



## รายงานวิจัยฉบับสมบูรณ์

โครงการ การปรับปรุงคุณภาพผลิตภัณฑ์ประมงและการใช้ประโยชน์สูงสุดของผลพลอยได้จาก  
การแปรรูปสัตว์น้ำ: ระยะที่ 2

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สนับสนุนโดยสำนักงานคณะกรรมการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

## กิตติกรรมประกาศ

ขอขอบพระคุณสำนักงานกองทุนสนับสนุนการวิจัย และสำนักงานคณะกรรมการอุดมศึกษา สำหรับการสนับสนุนทุนส่งเสริมกลุ่มวิจัยเรื่อง “การปรับปรุงคุณภาพผลิตภัณฑ์ประมงและการใช้ประโยชน์สูงสุดของผลพลอยได้จากการแปรรูปสัตว์น้ำ: ระยะที่ 2” อันก่อให้เกิดองค์ความรู้ใหม่และเทคโนโลยีที่เป็นประโยชน์ต่อการพัฒนาอุตสาหกรรมสัตว์น้ำของไทย รวมทั้งได้สร้างกลุ่มวิจัยทางด้านการแปรรูปและการปรับปรุงคุณภาพสัตว์น้ำที่เข้มแข็งเพื่อการพัฒนาประเทศต่อไป

ศาสตราจารย์ ดร.สุทธีวัฒน์ เบญจกุล

## บทคัดย่อ

จากการศึกษาการใช้ประโยชน์จากผลพลอยได้จากการแปรรูปสัตว์น้ำเพื่อเพิ่มมูลค่าและการปรับปรุงคุณภาพของผลิตภัณฑ์จากสัตว์น้ำ โดยศึกษาการสกัดและจำแนกคุณลักษณะของคอลลาเจนจากหนังปลาและถุงลมปลา นอกจากนี้ได้ศึกษาการสกัดเจลาตินจากหนังปลาและหมึกกล้วย ซึ่งพบว่าสภาวะการสกัดมีผลต่อสมบัติของเจลาติน โดยความคงตัวต่อความร้อนของคอลลาเจน และโปรตีนเอสที่พบในหนังของปลาบางชนิดเป็นปัจจัยสำคัญที่มีผลต่อสมบัติของเจลาติน การฟอกขาวหนังหมึกกล้วยมีผลต่อสี อย่างไรก็ตามการฟอกขาวมีผลเสียต่อสมบัติของเจลาติน กระบวนการฟอสโฟไลเซชันเป็นกระบวนการที่สามารถเพิ่มสมบัติการเกิดเจลของเจลาติน โดยเฉพาะการใช้ร่วมกับแคทิออนชนิดไดวาเลนต์ เจลาตินจากหนังปลาและหมึกกล้วยสามารถใช้ในการเตรียมฟิล์ม และสมบัติของฟิล์มสามารถปรับปรุงโดยการเติมนาโนเคลย์ ซึ่งสมบัติการป้องกันการซึมผ่านไอน้ำของฟิล์มขึ้นกับชนิดและปริมาณของนาโนเคลย์ที่เติม สมบัติของแผ่นฟิล์มสามารถถูกดัดแปลงโดยการผสมกับโปรตีนหรือพอลิเมอร์อื่น แผ่นฟิล์มชนิดเอกทิฟสามารถเตรียมโดยการเติมน้ำมันหอมระเหย โดยส่งผลให้ฤทธิ์การต้านออกซิเดชันและฤทธิ์ในการยับยั้งจุลินทรีย์ของฟิล์มเพิ่มขึ้น เมื่อใช้แผ่นฟิล์มชนิดเอกทิฟในการห่อชิ้นปลาระหว่างการเก็บรักษาที่อุณหภูมิต่ำ พบว่าอายุการเก็บรักษานานขึ้นเมื่อเปรียบเทียบกับชุดควบคุมและชุดที่ห่อด้วยฟิล์มเจลาติน

การเก็บเกี่ยวโปรตีนเอสจากเครื่องในปลาหรือกุ้งสามารถกระทำได้โดยกระบวนการแยกที่พัฒนาขึ้น โดยเฉพาะอย่างยิ่ง aqueous two-phase และ aqueous three-phase โปรตีนเอสสามารถใช้ในการเตรียมโปรตีนไฮโดรไลเสตที่มีฤทธิ์ทางชีวภาพ เช่น ฤทธิ์ต้านออกซิเดชัน และฤทธิ์ยับยั้งเอนไซม์ ACE โดยมีการเตรียมโปรตีนไฮโดรไลเสตจากแหล่งต่างๆ เช่น ไข่ปลาทูน่า เจลาตินจากหนังปลา โปรตีนกล้ามเนื้อปลา และอื่นๆ นอกจากนี้มีการศึกษาการใช้เอนไซม์จากพืช เช่น glycosyl endopeptidase จากยางมะละกอ เมื่อเตรียมเจลาตินไฮโดรไลเสตที่มีระดับการย่อยสลายสูงขึ้น โปรตีนไฮโดรไลเสตสามารถทำงานเป็นอิมัลซิไฟเออร์ที่มีฤทธิ์เป็นสารต้านออกซิเดชัน โดยเฉพาะภายหลังการดัดแปลงด้วยกรดแทนนิก ดังนั้น โปรตีนไฮโดรไลเสตจากสัตว์น้ำจึงใช้ประโยชน์ได้มากขึ้น

เมื่อศึกษาการปรับปรุงสมบัติของเจลซูริมิจากปลาที่จับในภาคใต้ของประเทศไทยด้วยเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์ (MTGase) โดยศึกษาปัจจัยต่างๆ ที่มีผลต่อการเชื่อมประสานโปรตีนในซูริมิ นอกจากนี้โปรตีนไฮโดรไลเสตจากเมล็ดถั่วที่มีฤทธิ์ยับยั้งโปรตีนเอสที่ระดับที่เหมาะสมสามารถใช้เป็นโปรตีนเติมแต่งสำหรับปรับปรุงสมบัติเจลซูริมิโดยเฉพาะเมื่อใช้ร่วมกับ MTGase การใช้เกลือสังกะสีที่ระดับที่เหมาะสมสามารถเพิ่มความแข็งแรงของเจลซูริมิโดยกลไก “salt bridges”

จุลินทรีย์สายพันธุ์ใหม่หลายชนิดสามารถแยกและจำแนกจากผลิตภัณฑ์จากผลิตภัณฑ์สัตว์น้ำ เช่น *Idiomarina piscisalsi* sp. จากปลาร้า และมีการจำแนกจุลินทรีย์ที่ผลิตฮีสตามีน คือ *Tetragenococcus* จากผลิตภัณฑ์สัตว์น้ำหมัก นอกจากนี้มีการแยกแบคทีเรียสายพันธุ์ใหม่ที่สามารถผลิตเอนไซม์ทรานส์กลูตามิเนส คือ *Enterobacter siamensis* จากน้ำทิ้งจากการแปรรูปอาหารทะเลในประเทศไทย แบคทีเรียที่แยกได้ใหม่นี้ดังกล่าวสามารถใช้สำหรับผลิต MTGase

โดยภาพรวม งานวิจัยที่เกิดขึ้นจากโครงการสามารถให้ความเข้าใจที่ดีขึ้นสำหรับการปรับปรุงคุณภาพผลิตภัณฑ์สัตว์น้ำและการใช้ประโยชน์จากวัสดุเศษเหลือจากการแปรรูปสัตว์น้ำ การประยุกต์ใช้ความรู้และเทคโนโลยีที่ได้จึงเป็นประโยชน์ต่ออุตสาหกรรมแปรรูปสัตว์น้ำหรือหน่วยงานอื่นที่เกี่ยวข้อง

**คำหลัก:** ผลพลอยได้ ปลา หมึก คอลลาเจน เจลาติน โปรตีนเอส ฟิล์ม ซูริมิ เจล จุลินทรีย์ ฤทธิ์ทางชีวภาพ หนัง เครื่องใน

## Abstract

Utilization of fish processing byproducts was investigated to increase their value. The improvement of some fishery products was also performed. Collagen from fish skin as well as swim bladder was successfully extracted and characterized. Additionally, gelatins from fish and squid skin were extracted, in which the extraction conditions affected the properties of resulting gelatin. Thermal stability of mother collagen and indigenous heat-activated protease in skin of some fish species were the prime factors determining the properties of gelatin. Bleaching condition of squid skin was found to improve the color of resulting gelatin. However, it showed the negative effect on the property of gelatin gel. Phosphorylation was shown to enhance the gelling property of fish skin gelatin, particularly when subsequently induced by divalent cations. Gelatins from fish and squid skin were used for preparation of film. The properties of gelatin film were improved by nanoclay incorporation. The water vapor barrier property was improved when the nanoclay with the appropriate type and level was added. The property of gelatin film was also modified using the blend with other protein or polymer. The active films were also prepared via the incorporation of some selected essential oils. The antioxidant and antimicrobial activity of gelatin films increased. When the active based on gelatin film was applied to wrap the fish slices during the refrigerated storage, the shelf-life was extended in comparison with the control and those wrapped with gelatin film.

Proteases from fish and shrimp viscera were also recovered by the developed separation methods, especially aqueous two-phase and aqueous three-phase. The proteases were used for production of protein hydrolysates with bioactivities including antioxidative activity and ACE inhibitory activity. Several fish protein hydrolysate such as tuna roe protein hydrolysate, fish skin gelatin hydrolysate, fish muscle protein hydrolysate, etc. were produced. Furthermore, some plant proteases such as glycosyl endopeptidase from papaya latex were also used for production of gelatin hydrolysate with increased degree of hydrolysis. Fish protein hydrolysate could function as the antioxidative emulsifier, especially after modification with tannic acid. Therefore, fish protein hydrolysate could be better used.

Property improvement of gel from surimi from several fish species caught in the southern part of Thailand was investigated. Gel properties of surimi could be improved by using microbial transglutaminase (MTGase). Factors affecting the cross-linking of surimi protein by MTGase were also elucidated. Protein isolates from legume seeds with protease inhibitory activity at appropriate levels were shown to be the promising protein additive for improvement of surimi gel properties, especially when used in combination with MTGase. The uses of Zinc salts at the proper levels also increased the gel strength of surimi via 'salt bridge' mechanism.

Novel microorganisms isolated from fermented fishery products were identified. *Idiomarina piscisalsi* sp. Nov. was isolated from pla-ra. Histamine former, *Tetragenococcus*, were also isolated from Thai fermented fishery products. Various strains exhibited the different histamine forming ability. The novel transglutaminase-producing bacterium, *Enterobacter siamensis*, was isolated from seafood processing wastewater in Thailand. This newly isolated bacterium could be used as the MTGase producer.

Overall, the researches carried out can provide the better understanding on the improvement of fishery products and the utilization of fish processing byproducts. Further applications of knowledge or technologies can be of great benefit for fish processing industry or related association/organization.

Keywords: byproducts, fish, squid, quality, collagen, protease, gelatin, film, surimi, gel, microorganisms, bioactivity, skin, viscera

## Output ของโครงการวิจัย

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-เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

-ผลิตนักศึกษาระดับบัณฑิตศึกษา

ปริญญาเอก 12 คน

ปริญญาโท 3 คน

-การเข้าสู่ตำแหน่งวิชาการ

ผู้ช่วยศาสตราจารย์ 2 คน

รองศาสตราจารย์ 2 คน

ศาสตราจารย์ 1 คน

## 3. อื่น ๆ

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### 3.3 การเชื่อมโยงทางวิชาการกับนักวิชาการต่างประเทศจำนวน....4....ท่าน

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## Characteristics of myoglobin and haemoglobin-mediated lipid oxidation in washed mince from bighead carp (*Hypophthalmichthys nobilis*)

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### ABSTRACT

Myoglobin (Mb) and haemoglobin (Hb) accounted for 61% and 39% of the total haem-protein in bighead carp (*Hypophthalmichthys nobilis*) dark muscle, respectively. Molecular weight of Mb and monomeric-Hb was ~16 kDa. Haemin loss from metHb was more rapid, compared to metMb (pH 6.0, 4 °C). Pro-oxidative activities of oxyMb/Hb and metMb/Hb were examined in washed mince during 9 days of iced storage (pH 6.0). Soret measurements suggested the existence of holoMb throughout storage. For Hb, weakening of haem-globin linkage was observed, especially for metHb which had undetectable Soret after 3 days of storage. Loss of redness was more rapid and extensive in washed mince containing Hb, compared to Mb. During storage, Hb induced larger amounts of peroxides, thiobarbituric acid-reactive substances and hexanal than did Mb ( $p < 0.05$ ), especially for met-form. Thus, Hb had lower haemin affinity and was a stronger pro-oxidant than Mb.

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### 1. Introduction

Lipid oxidation in meat is accentuated during handling, processing, storage and cooking. This undesirable reaction lowers the quality and acceptability of muscle products. The haem proteins, both myoglobin (Mb) and haemoglobin (Hb), have been shown to promote lipid oxidation in model systems, especially at low pH (Grunwald & Richards, 2006; Maqsood & Benjakul, 2011; Thiansilakul, Benjakul, & Richards, 2011a).

Mb and Hb are monomeric and tetrameric haem proteins, respectively. They contain iron in a prosthetic haem group. Hb, which transports oxygen and other gases, is the main protein in blood and highly concentrated in the erythrocytes (Jensen, 2004). Blood capillaries can burst naturally and also during mechanical treatments such as filleting and mincing, leading to the distribution of Hb throughout the muscle. Mb, the oxygen-storage protein retained by the intracellular structure, is a major pigment in the dark muscle of fish (Chaijan, Benjakul, Visessanguan, & Faustman, 2004; Thiansilakul, Benjakul, & Richards, 2011b). Haem proteins contribute to the colour of muscle, depending upon their states and concentration (Faustman & Cassens, 1990). The content of Mb and Hb in fish depends on muscle types and fish species (Brown, 1962; Chaijan et al., 2004; Maqsood & Benjakul, 2011).

Immediately after death, Mb and Hb are mostly present in the reduced form or the ferrous state (Mb/Hb-Fe<sup>2+</sup>), which provides

the purple or red colour to the muscle, depending on the sixth ligand. With increasing storage time, the iron atom in the haem ring is further oxidised to the ferric state (Mb/Hb-Fe<sup>3+</sup>), resulting in the development of brown colour (Faustman & Cassens, 1990). H<sub>2</sub>O<sub>2</sub> formed upon oxidation of haem can further generate ferryl-haem protein (Mb/Hb-Fe<sup>4+</sup>), a strong pro-oxidant (Kanner & Harel, 1985). Autooxidation of haem proteins is responsible for the discoloration of meat and acceleration of lipid oxidation (Faustman & Cassens, 1990; Thiansilakul et al., 2011a). Lipid oxidation in washed Asian sea bass mince initiated by Mb brought about increased fishy and rancid off-odour, especially at low pH, in which metMb was the dominant form (Thiansilakul et al., 2011a). Pro-oxidative activity of Hb from different fish species, including Asian sea bass, tilapia and grouper varied, depending on the molecular properties of Hb (Maqsood & Benjakul, 2011). Richards, Dettmann, and Grunwald (2005) reported autooxidation and haemin loss of trout Mb and Hb associated with lipid oxidation in washed fish muscle at pH 6.3. Haemin loss rate was considered crucial in promoting lipid oxidation, compared to autooxidation rate. Trout Hb was prone to haemin loss and became a stronger promoter of lipid oxidation than trout Mb, while trout Mb had a more rapid autooxidation rate than trout Hb (Richards et al., 2005). Released haemin readily converts pre-formed lipid hydroperoxides to radicals that facilitate lipid oxidation (Van Der Zee, Barr, & Mason, 1996).

Dark-fleshed fish, containing substantial quantities of Mb and Hb in the dark muscle, are considered to be highly prone to discoloration and lipid oxidation. However, little has been reported on the relative efficacy of Mb and Hb to promote lipid oxidation. A

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better understanding of lipid oxidation initiated by these haem proteins would provide basic knowledge, leading to the development of preventive methods to inhibit lipid oxidation and improve overall quality of fish. The objective of this investigation was to compare the pro-oxidative activity of bighead carp Mb and Hb, both oxy- and met-forms, in washed bighead carp mince during iced storage.

## 2. Materials and methods

### 2.1. Chemicals

Triton X-100, sodium dodecyl sulphate (SDS),  $\beta$ -mercaptoethanol ( $\beta$ ME), sodium dithionite and ferrous chloride were procured from Fisher Scientific (Pittsburgh, PA). Streptomycin sulphate, ammonium thiocyanate, 2-thiobarbituric acid, cumene hydroperoxide and 1,1,3,3-tetramethoxypropane were purchased from Sigma (St. Louis, MO). Recombinant sperm whale Mb, apo-H64Y, was supplied by Dr. John Olson (Rice University, Houston, TX, USA). All chemicals were of analytical grade.

### 2.2. Collection of fish samples and blood

Blood from 55 juvenile bighead carp (*H. nobilis*) (7–8 cm in length) were obtained from the Columbia Environmental Research Center (Columbia, MO). Blood and fillets from adult bighead carp (50–60 cm in length) were obtained from Schafer Fisheries (Thomson, IL). Blood was removed from the caudal vein of newly-killed carp by severing the caudal peduncle and collecting blood in sodium heparin (30 U heparin per mL of blood drawn). The fish fillets and heparinised blood were placed in an insulated box containing frozen gel packs and transported to the Meat Science and Muscle Biology Laboratory, University of Wisconsin-Madison (Madison, WI) within 1 day of capture. Upon arrival, dark and light muscles were separated and cut into small pieces. Samples were ground using a KSM90WW household mixer (Kitchen Aid, Inc., St. Joseph, MI) until uniformity was obtained. Mince from dark and light muscle was used for preparation of Mb and washed mince, respectively, whereas heparinised blood was used for Hb preparation.

### 2.3. Preparation of haem proteins with oxy- and met-forms

#### 2.3.1. Preparation of Mb

Extraction and purification of Mb were performed according to the method of Thiansilakul et al. (2011b). Mince from dark muscle (100 g) was mixed with 300 mL of cold extracting buffer (10 mM Tris-HCl, pH 8.0 containing 1 mM EDTA and 25 g/L Triton X-100). The mixture was homogenised for 1 min at 13,000 rpm with a Polytron Type PT 10/35 probe (Brinkmann Instruments, Westbury, NY). Homogenate was subjected to centrifugation at 9600g for 10 min at 4 °C using a Beckman J-6B centrifuge (Beckman Instruments Inc., Palo Alto, CA). The supernatant obtained was filtered through a Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, UK). The filtrate, referred to as 'crude Mb extract', was subjected to ammonium sulphate (AS) fractionation (65–100% saturation). The precipitate obtained after centrifugation at 20,000g for 60 min was dissolved in a minimal volume of cold 5 mM Tris-HCl buffer, pH 8.5, which was referred to as 'starting buffer'. The mixture was then dialysed against 10 volumes of the same buffer with 10 changes at 4 °C for 20 h. The dialysate was immediately applied onto a Sephadex G-100 column (2.5 × 90 cm; Amersham Bioscience, Uppsala, Sweden), which was previously equilibrated with the starting buffer. The separation was conducted using the starting buffer at a flow rate of 0.5 mL/min. Fractions of 3 mL were collected and the absor-

bance at 280 and 540 nm was measured to monitor the proteins and haem proteins, respectively, using a UV-2401 spectrophotometer (Shimadzu Instruments, Inc., Columbia, MD). The fractions with high absorbance at 540 nm were pooled and referred to as 'Mb'. To locate Hb and Mb peaks, Hb from bighead carp blood (prepared as in Section 2.3.2) was loaded onto a Sephadex G-100 column and the retention time was used to differentiate between Hb and Mb. Pooled Sephadex G-100 fractions containing Mb and Hb were determined by measuring the absorbance at 525 nm to quantify the Mb and Hb contents in the dark muscle of bighead carp, respectively. The molar extinction coefficient of  $7.6 \times 10^{-3}$  was used for the calculation (Tang, Faustman, & Hoagland, 2004). Mb and Hb contents were expressed as mg/g dark mince.

#### 2.3.2. Preparation of Hb

Hb was prepared as per the method of Fyhn et al. (1973). Four volumes of cold 1.7% NaCl in 1 mM Tris buffer (pH 8.0) were added to heparinised blood. The mixture was centrifuged at 700g for 10 min at 4 °C. After the plasma was removed, the red blood cells were washed by suspending them three times in 10 volumes of the same buffer. Red blood cells were lysed in 3 volumes of 1 mM Tris (pH 8.0) for 1 h. One-tenth volume of 1 M NaCl was then added to aid in stromal removal before ultracentrifugation at 28,000g for 15 min at 4 °C using a Beckman L8-70 M ultracentrifuge (Beckman Instruments Inc.). Low molecular mass components (<10 kDa) were removed from 'Hb' using a prepacked PD-10 column (column volume: 13.5 mL, medium volume: 8.3 mL; Bio-Rad, Hercules, CA) eluted with 50 mM Tris buffer (pH 8.5) with gravity flow.

#### 2.3.3. Preparation of oxyMb/Hb and metMb/Hb

OxyMb/Hb and metMb/Hb were prepared according to the method of Tang et al. (2004) with some modifications. To obtain oxy-form, 1.5 mg of sodium dithionite was added to an aliquot of Mb or Hb solutions (3 mL; 0.1 mM haem). MetMb/Hb was prepared by adding 1.5 mg of potassium ferricyanide to 3 mL of Mb or Hb solutions (0.1 mM haem). To remove sodium dithionite and potassium ferricyanide, 3 mL of samples were applied onto a prepacked PD-10 columns and 20 mM phosphate buffer (pH 7.0) was used for elution with gravity flow.

#### 2.3.4. Characterisation of haem proteins

**2.3.4.1. SDS-PAGE.** Mb and Hb were subjected to SDS-PAGE according to the method of Laemmli (1970). Prestained protein marker (18–215 kDa) (Pierce, Rockford, IL) was used for MW estimation. Band intensity was determined by ImageJ program (Abramoff, Magalhaes, & Ram, 2004). Total intensity of all bands in each lane was defined as 100%.

**2.3.4.2. Mass spectrometry.** A C4 zip-tip (Millipore, Billerica, MA) was used to desalt Mb and Hb solutions. Desalted samples were analysed by electrospray ionisation mass spectrometry (ESI-MS) on an API365 triple quadrupole (Applied Biosystems, Foster City, CA). The ESI-MS raw spectra were obtained over the scan range from  $m/z$  600 to 1650 and determined as a true mass scale (Richards et al., 2005).

**2.3.4.3. Determination of Mb and Hb concentration.** The concentrations of Mb and Hb solutions, both oxy- and met-forms, were determined by measuring the absorbance at 525 nm. The molar extinction coefficient of  $7.6 \times 10^{-3}$  was used for the calculation and the concentration was expressed as  $\mu$ M haem protein.

**2.3.4.4. Haemin loss from metMb and metHb.** Haemin loss was determined as described by Grunwald and Richards (2006) using

the recombinant sperm whale Mb, apoH64Y, as a reagent to capture haemin from the met-forms of Mb and Hb. Haemin loss was monitored for metMb/Hb because haem is anchored in the globin of ferrous haem protein approximately 60 times stronger compared to met haem protein (Hargrove & Olson, 1996). ApoH64Y was added at a 4-fold molar excess to that of the haem proteins (10  $\mu$ M) on a monomer basis at pH 5.5 and 6.0. Absorbance measurements were taken at  $A_{600}$  and  $A_{700}$  over 4 days of 4 °C storage. Binding of released haemin from haem proteins to the apoH64Y resulted in an increased  $A_{600}$ . Rate of haemin loss was reported as absorbance difference at wavelength of 600 and 700 nm ( $A_{600}-A_{700}$ ).

#### 2.4. Effect of oxyMb, metMb, oxyHb and metHb on lipid oxidation in washed mince

##### 2.4.1. Preparation of washed mince

Washed mince of bighead carp was prepared following the method of Grunwald and Richards (2006) with a slight modification. The mince from light muscle was washed twice with cold distilled water with a mince/water ratio of 1:3 (w/v). The mixture was stirred continuously for 2 min with a glass rod and allowed to settle for 15 min. The mixture was filtered using 2 layers of cheesecloth. Thereafter, 3 volumes of cold 50 mM sodium phosphate buffer (pH 6.0) were mixed with washed mince, which was stirred and filtered twice as described previously. The retentate was mixed with cold 50 mM sodium phosphate buffer (pH 6.0) with a mince/buffer ratio of 1:1 (w/v) and homogenised for 3 min at a speed of 13,000 rpm. The pH of muscle slurry was checked and adjusted to the desirable pH (pH  $6.0 \pm 0.05$ ), using 0.5 M NaOH or 0.5 M HCl. After standing for 15 min, the mixture was centrifuged at 15,000g for 25 min. The pellet was collected and referred to as 'washed mince'. All processes were performed at 4 °C. Washed mince was measured for moisture content by AOAC method (AOAC, 2000), packaged in a polyethylene bag, vacuum-sealed and stored at  $-80$  °C.

##### 2.4.2. Preparation of washed mince containing different forms of Mb and Hb

Washed mince was thawed overnight at 4 °C and ground for three 3-s pulses using a Hamilton Beach Custom Grind Type CM04 coffee grinder (Hamilton Beach/Proctor Silex, Inc., Southern Pines, NC). The sample (20 g) was transferred to a 30-mL amber vial and combined with phosphate buffer (pH 6.0) to obtain a final moisture content of 90%. In order to inhibit the microbial growth, the streptomycin sulphate stock (2% w/v) was added to the washed mince with a final concentration of 200 ppm. The preliminary study showed that bighead carp dark muscle contained 85  $\mu$ mol Mb/kg mince and 13.3  $\mu$ mol Hb (53  $\mu$ mol haem basis)/kg mince. However, an appropriate amount of 24  $\mu$ mol haem/kg mince was able to initiate lipid oxidation in washed mince during the storage time (Richards et al., 2005). To study the impact of different haem proteins on initiation of lipid oxidation, the oxyMb, metMb, oxyHb or metHb stock was then added into washed mince to obtain a final concentration of 24  $\mu$ mol haem/kg mince. For the control sample, phosphate buffer was added instead of haem protein solution. The mixtures in vial were mixed thoroughly with a plastic spatula. All samples were stored on ice for 9 days. Melted ice was removed and the same amount of ice was replaced every day. Samples (3 g) were taken at Day 0, 1, 2, 3, 5, 7 and 9 of storage for analyses.

##### 2.4.3. Analyses

**2.4.3.1. Measurement of Soret peak in haem proteins.** To obtain the haem protein solution, washed mince samples with added haem proteins (1 g) were centrifuged at 14,000g for 10 min. The supernatants were then collected. Haem protein solutions were measured

for absorption spectra ranging from 380 to 450 nm, to monitor the Soret peak.

**2.4.3.2. Measurement of redness.** Colorimetric values of washed mince with different treatments were determined using a Minolta CR-300 Chroma Meter (Minolta Camera Co., Osaka, Japan). The aperture size was 1 cm. Illuminant C was used. A white calibration plate was used to calibrate the instrument. Washed mince samples were determined for the *a*-value (redness/greenness) of the *L*, *a* and *b*-system.

**2.4.3.3. Measurement of lipid peroxides.** Peroxide value (PV) was determined as described by Grunwald and Richards (2006). A standard curve was prepared using cumene hydroperoxide at concentrations ranging from 0 to 20  $\mu$ M. PV was calculated and expressed as  $\mu$ mol cumene hydroperoxide/kg sample.

**2.4.3.4. Measurement of thiobarbituric acid reactive substances (TBARS).** TBARS were determined following the method of Buege and Aust (1978). A standard curve was prepared using 1,1,3,3-tetramethoxypropane at concentrations ranging from 0 to 40  $\mu$ M. TBARS values were calculated and expressed as  $\mu$ mol malonaldehyde/kg sample.

**2.4.3.5. Measurement of hexanal content.** Hexanal content in washed mince with different treatments stored for 0, 3, 5 and 9 days was determined using a solid-phase microextraction (SPME) technique and gas chromatography (GC). The 10-mL vial (Agilent Technologies, Santa Clara, CA) with a metal cap and PTFE/silicone septum (MicroLiter Analytical Supplies Inc., Suwanee, GA) containing 1 g of sample was allowed to stand at room temperature for 15 min, followed by heating at 40 °C for 5 min. The SPME fibre (PDMS/DVB, fused silica,  $d_f$  65  $\mu$ m, needle size 23 ga) (Supelco, Bellefonte, PA) was conditioned at 260 °C for 30 min before use and then exposed to the headspace of the vial at 40 °C for 10 min.

GC analysis was performed using an Agilent 6890 gas chromatography equipped with a DB-5 capillary column (30 m length  $\times$  0.25 mm i.d.  $\times$  0.1  $\mu$ l film thickness; Agilent) and flame ionisation detector (FID). After injection of the SPME fibre into the GC/FID injection port, hexanal was desorbed from the fibre at 250 °C for 5 min. The flow rate of carrier gas (helium) was 1 mL/min. Inlet and detector temperatures were 250 and 270 °C, respectively. The oven temperature was programmed at 40 °C for 5 min with a 10 °C/min ramp rate until 90 °C. Identification of hexanal was based on its retention time. Quantification of hexanal in the sample was made using a standard curve of hexanal solution (0–0.25 ppm).

#### 2.5. Statistical analysis

Data from triplicate experiments were subjected to analysis of variance (ANOVA) with a confidence level of 95%. Comparison of means was carried out by Duncan's multiple range test (Steel & Torrie, 1980). For paired comparison, Student's *t*-test was used. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS 14.0 for Windows; SPSS Inc., Chicago, IL).

### 3. Results and discussion

#### 3.1. Characteristics of Mb and Hb from bighead carp

##### 3.1.1. Purification of Mb and Hb

Elution profile of haem proteins purified from bighead carp dark muscle using a Sephadex G-100 column is shown in Fig. 1A. After

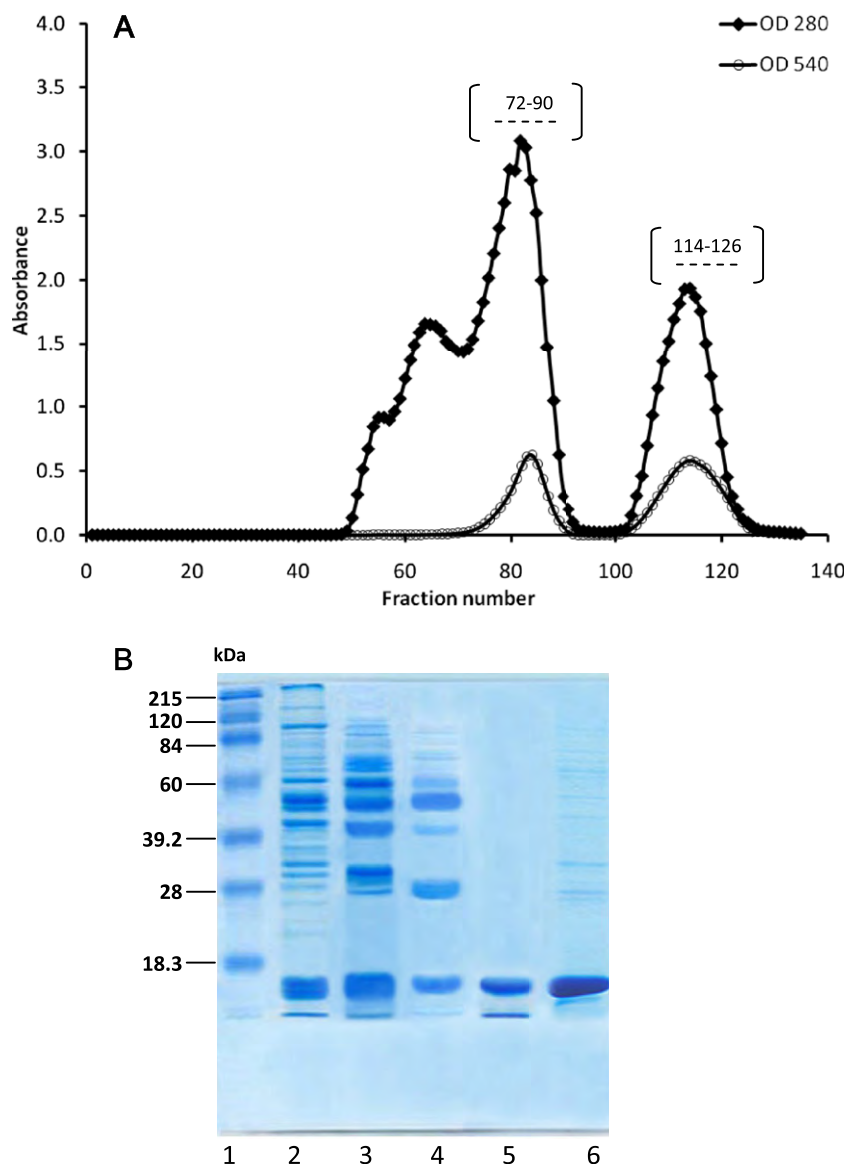


**Fig. 1.** Elution profile of Mb purified from bighead carp dark muscle on Sephadex G-100 (A) and SDS-PAGE pattern of crude extract and different fractions containing Mb from bighead carp dark muscle and Hb from bighead carp blood (B); lane 1, MW marker; lane 2, crude extract from bighead carp dark muscle; lane 3, AS fraction; lane 4, pooled Sephadex G-100 fraction I (No. 72–90); lane 5, pooled Sephadex G-100 fraction II (No. 114–126); lane 6, Hb from bighead carp blood.

being chromatographed, two major peaks at  $A_{540}$  were obtained. The two haem proteins, Mb and Hb, could be distinguished on the basis of different size. In general, Hb has 4-fold higher MW than Mb. Pooled fractions of the first peak having a larger size likely contained Hb as the major component, whereas pooled fractions of the second peak with a smaller size were presumed to contain Mb. An Hb peak in the bighead carp dark muscle extract was confirmed with elution profile of Hb from bighead carp blood on Sephadex G-100 column, in which the same retention time was observed. After purification, Mb and Hb contents in the dark muscle of bighead carp were determined to be 1.36 and 0.86 mg/g dark mince, respectively. Brown (1962) reported that dark muscle of yellowfin tuna (*Neothunnus macropterus*) contained Mb ranging from 5.3 to 24.4 mg/g, while Hb ranged from 0.5 to 3.8 mg/g.

Protein patterns of extract, AS fraction and Sephadex G-100 fraction from bighead carp dark muscle and Hb from bighead carp blood are shown in Fig. 1B. Crude extract contained proteins with MW ranging from 16 to 120 kDa (Fig. 1B, lane 2), while AS fraction consisted of proteins with MW range of 16–90 kDa (Fig. 1B, lane 3).

It was noted that protein with MW of ~16 kDa was dominant in both samples. The first peak obtained from Sephadex G-100 column (fraction No. 72–90) comprised several proteins and those with MW of 50, 28 and ~16 kDa were dominant (Fig. 1B, lane 4). The second peak (fraction No. 114–126) showed the predominant band with MW of ~16 kDa, which was probably Mb (Fig. 1B, lane 5). The densitometric analysis indicated that Mb band shown in Fig. 1B lane 5 represented 89% of total protein in pooled fractions of the second peak. This suggested an effective removal of contaminating proteins in crude extract from bighead carp dark muscle after being precipitated with AS (65–100% saturation) and chromatographed with Sephadex G-100 gel filtration. Protein pattern of Hb prepared from bighead carp blood exhibited the intense band at MW of ~16 kDa (Fig. 1B, lane 6) indicating that Hb was the main protein in red blood cells. Under the reducing condition of SDS-PAGE, a tetramer of Hb was dissociated into a monomer with MW of 16 kDa.

Molecular weight of Mb from the dark muscle of bighead carp and Hb from the blood were also determined by ESI-MS (Fig. 2).

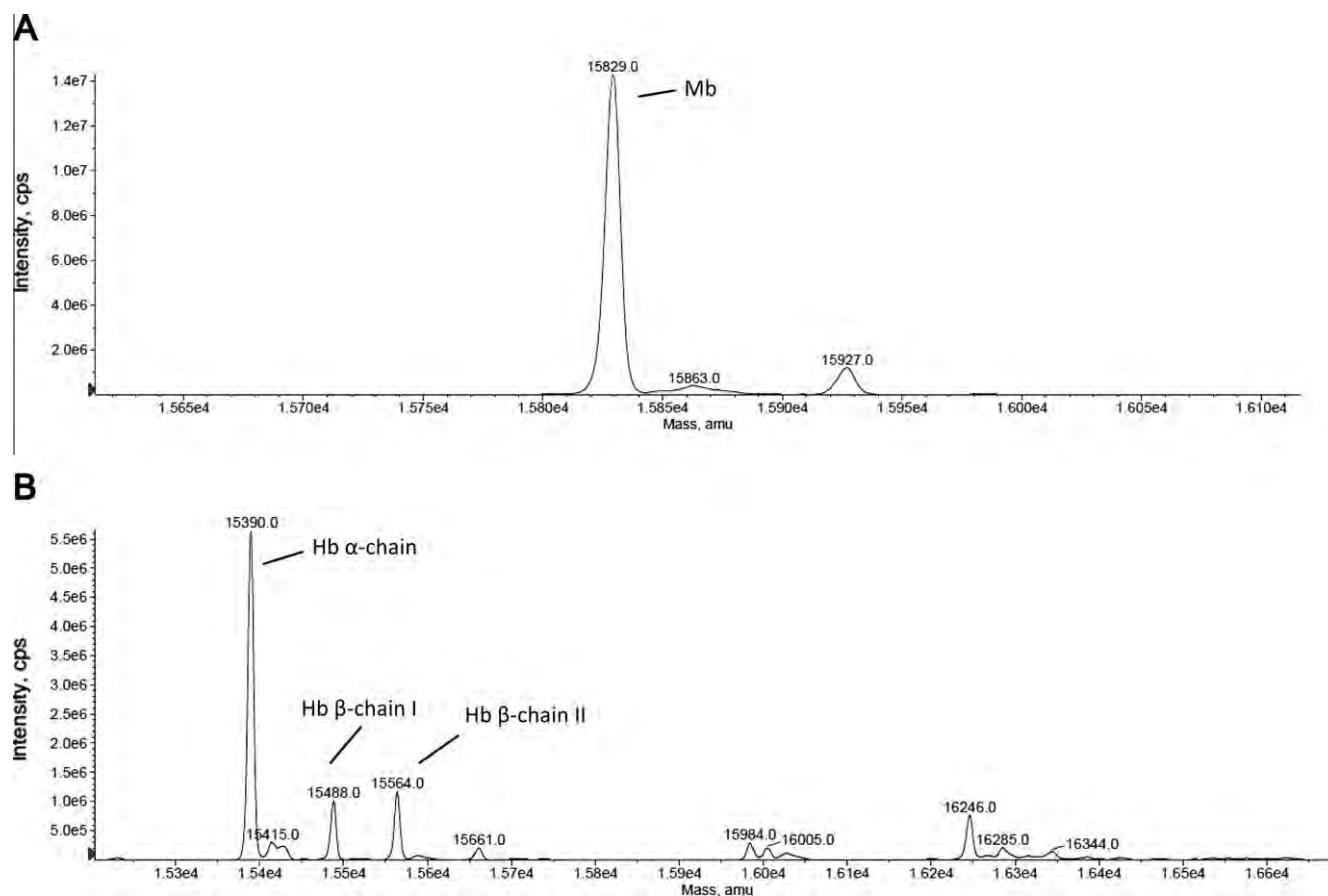


**Fig. 2.** ESI-MS of Mb from bighead carp dark muscle in pooled Sephadex G-100 fraction II (A) and Hb from adult bighead carp blood (B).

For Mb-enriched Sephadex G-100 fractions (the second peak), a major peak at 15,829 Da was obtained, most likely representing the globin of bighead carp Mb. Two minor peaks at 15,863 and 15,927 Da were also noticeable. Mb consists of a globin and the haem group with a MW of 616 Da. During ionisation, Mb is dissociated into the globin and haem moieties and they are detected as separate entities (Richards et al., 2005). For Hb extracted from the blood of adult bighead carp, a predominant peak at 15,390 Da was observed, representing  $\alpha$ -chain of Hb. Two additional peaks obtained at 15,488 and 15,564 Da represented two  $\beta$ -chains of Hb. Hb from adult and juvenile bighead carp were similar except that the juvenile Hb had no additional  $\beta$ -chain II of 15,564 Da (data not shown). Hilse and Braunitzer (1968) reported that Hb  $\alpha$ -chain of common carp (*Cyprinus carpio*) had 142 amino acids with MW of 15,248 Da.

### 3.1.2. Haemin loss from metMb and methb

Haemin loss from metMb and methb was examined at pH 6.0 during 4 °C storage (Fig. 3A). ApoH64Y with the tyrosine substitution has affinity for haemin (Hargrove et al., 1994). Haemin released from Mb or Hb was gathered by this apoglobin and a specific chromophore was generated (Grunwald & Richards, 2006; Hargrove et al., 1994). High value of  $A_{600}-A_{700}$  was found in methb sample, indicating that haemin loss from methb was rapid at pH 6.0. Conversely, little haemin loss from metMb was observed. Thus, methb had lower haemin affinity than metMb. For Mb, the highly conserved histidine at site FG3 and prevalent serine or threonine at F7 stabilise the haem moiety within the globin, while Hb contained leucine residues at these sites (Richards, 2010). Haemin loss from metMb increased rapidly when pH de-

creased from 6.0 to 5.5 (Fig. 3B). At acidic pH, an enhanced protonation of Mb induces conformational changes, as well as the unfolding of globin, which could reduce the stability of haem-globin linkage (Thiansilakul et al., 2011b). Sperm whale metMb had a 20-fold lower haemin affinity at pH 5.5, compared to pH 6.0, and 200-fold lower at pH 5.0, compared to pH 6.0 (Hargrove et al., 1994). Thiansilakul et al. (2011b) suggested a disruption of haem-globin complex of tuna oxy- and metMb at pH less than 5.0. Composition and conformation of globin greatly influenced haemin affinity in haem proteins.

### 3.2. Effect of oxyMb, metMb, oxyHb and methb on lipid oxidation in washed mince during iced storage

#### 3.2.1. Changes in Soret peak of the haem protein solutions after centrifugation of washed mince

Changes in wavelength of Soret peak of haem protein solutions after centrifugation of washed mince are shown in Fig. 4. At Day 0, oxyMb, oxyHb, metMb and methb samples had the Soret peak at wavelengths of 416, 412, 406 and 406 nm, respectively. Mb from eastern little tuna had the Soret peak at wavelength of 413 for oxy-form and 407 nm for met-form (Thiansilakul et al., 2011b). The wavelength of the Soret peak of the oxyMb sample gradually decreased to 410 nm when storage time increased up to Day 9. In oxyHb sample, the wavelength of the Soret peak sharply decreased to 404 nm at Day 2 of storage and increased to 406 nm at Day 5. Thereafter, the Soret peak remained constant at wavelength of 406 nm until Day 9 of storage. In washed mince containing metMb, the Soret peak decreased to 404 nm during 3–5 days of storage and then increased to 406 nm during 7–9 days of storage. It

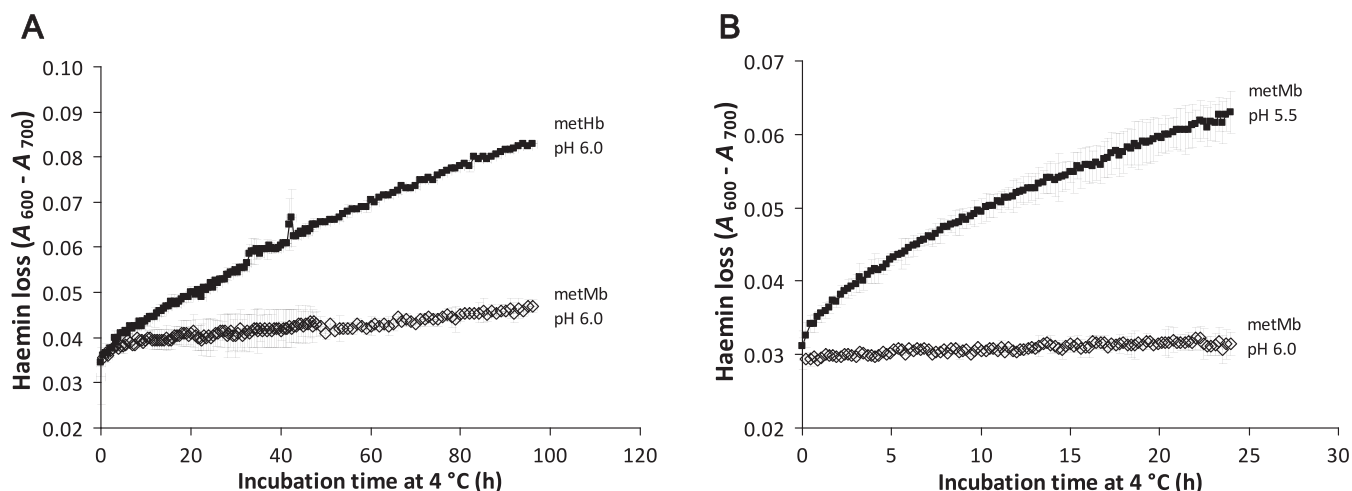


**Fig. 3.** Haemin loss ( $A_{600}-A_{700}$ ) from bighead carp metMb and metHb at pH 6.0 (A) and metMb at pH 5.5 and pH 6.0 (B) using apoH64Y as haemin loss reagent during storage at 4 °C for 96 and 24 h, respectively. Bars represent standard deviations ( $n = 3$ ).

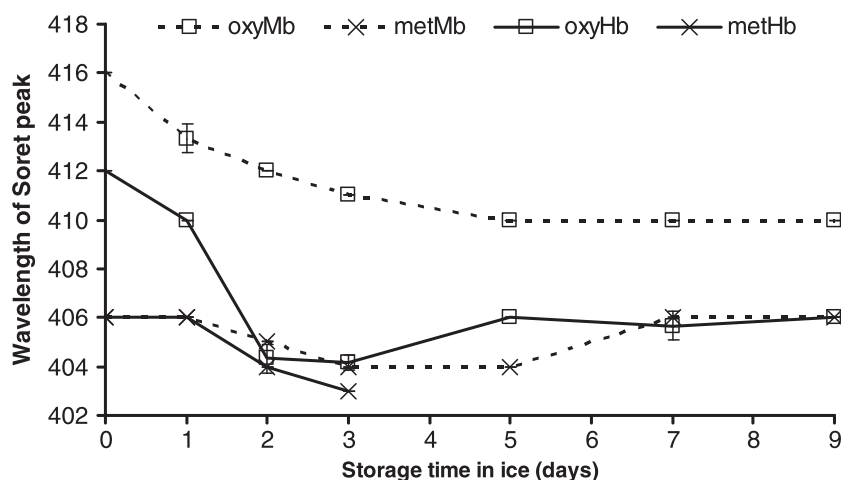


**Fig. 4.** Changes in wavelength of the Soret peak in supernatants of washed mince containing oxyMb, metMb, oxyHb and metHb from bighead carp during 9 days of iced storage, pH 6.0. Bars represent standard deviation ( $n = 3$ ).

was noted that the shift of the Soret wavelength of metMb sample was similar to that of oxyHb sample.

The Soret absorbance of metMb is reported to range from 406 to 410 nm (Smulevich et al., 2007; Swatland, 1989; Thiansilakul et al., 2011b). The reported Soret absorbance for metHb is 405 nm (Antonini & Brunori, 1971). FerrylMb is noted for having a Soret peak at 420 nm (Rao, Wilks, Hamberg, & Ortiz de Montellano, 1994) and crosslinking of Mb after ferrylMb formation is observed at 405 nm (Osawa & Williams, 1996). Changes in the Soret wavelength to a lower number (416 to 410 nm) of oxyMb sample suggested that oxyMb was gradually converted to metMb. Nearly all the oxyMb remained intact as holoMb during the 9 days of storage, since the peak in Soret region was still found (data not shown). For metMb sample, the shift of the Soret wavelength from 406 to 404 nm suggested the possible formation of ferrylMb and cross-linked Mb during storage. A cycling of ferrylMb and metMb can occur during storage, making it difficult to directly measure ferrylMb. Also, metMb tended to remain as holoMb during storage, based on Soret absorbance (data not shown).

For oxyHb, a sharp decrease in the Soret wavelength within the first 2 days of storage (412 to 404 nm) indicated the susceptibility of oxyHb to autooxidation. According to the reported Soret absor-

bance for crosslinking-haem protein at 405 nm (Osawa & Williams, 1996), the Soret wavelength of the oxyHb sample between 404 and 406 nm from Day 2 to 9 indicated the presence of metHb or a combination of metHb and a cross-linked Hb species. Approximately one-fourth of the oxyHb sample remained as holoHb during the 9 days of storage (data not shown). For the metHb sample, the wavelength of the Soret peak was observed at 406 nm at Day 1 and then it decreased to 403 nm after Day 3. Thereafter, no Soret peak was detectable after 3 days of storage, reflecting the disruption of haem-globin complex. A Soret band results mainly from the interaction of the haem moiety with globin. Hence it can be used to monitor the unfolding of haemoproteins (Thiansilakul et al., 2011b). Conformation changes of haem protein found in metHb sample was coincidental with rapid haemin loss from metHb at pH 6.0. A weak haem-globin linkage as well as rapid autooxidation of Hb revealed the lower stability of Hb than Mb.

### 3.2.2. Changes in redness

Redness ( $a$ -value) of washed mince without and with the addition of varying haem proteins during iced storage is shown in Fig. 5A. Washed mince without the addition of haem protein had low  $a$ -values throughout 9 days of storage. The result indicated

the efficiency of washing process in removal of water soluble pigments from bighead carp muscle. At Day 0, washed mince containing oxyMb exhibited the highest redness, followed by those containing added oxyHb, metMb, metHb and the control, respectively ( $p < 0.05$ ). Mb and Hb, the haem proteins, are the major pigments found in fish, especially in the dark muscle. Haem protein in the oxy-form is bright red, which is considered as fresh and attractive in meat. On the other hand, haem protein with the met-form is brown and rather unattractive (Faustman & Cassens, 1990). During

9 days of iced storage, the gradual decreases in redness of washed mince containing oxyMb and metMb were noticeable ( $p < 0.05$ ). For washed mince containing oxyHb and metHb, sharp decreases in redness were observed within the first 3 days of storage ( $p < 0.05$ ). Thereafter the redness tended to be stable and showed little difference between both samples. After 3 days of storage, washed mince with added oxyHb and metHb exhibited the lowest redness, compared with other samples, and maintained lowest redness until the end of storage ( $p < 0.05$ ).

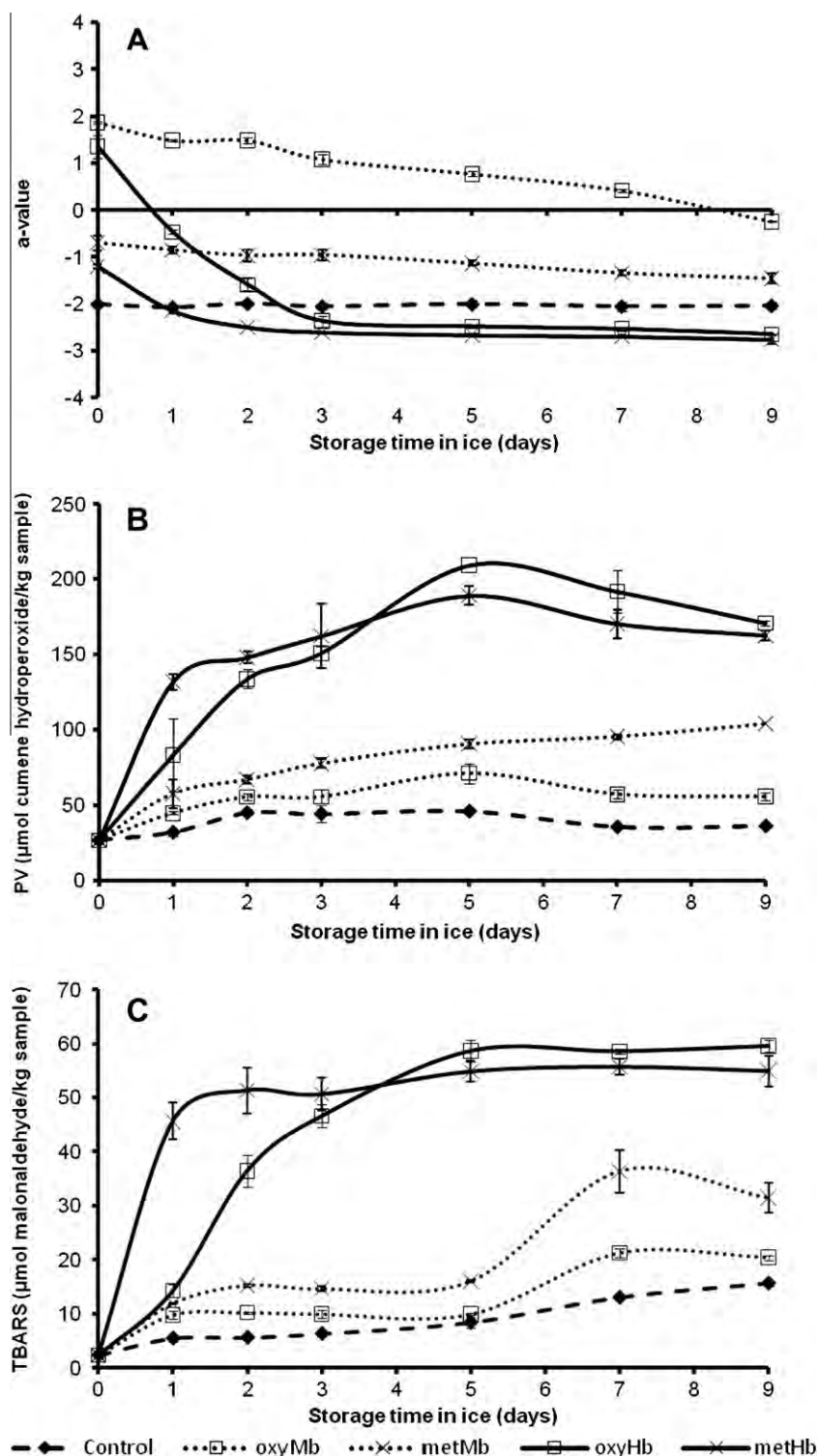


**Fig. 5.** Changes in redness (A), PV (B) and TBARS (C) values of washed mince added with oxyMb, metMb, oxyHb and metHb from bighead carp during 9 days of iced storage, pH 6.0. Bars represent standard deviation ( $n = 3$ ).

The relative amounts of the oxidation states of haem proteins in muscle contribute to the apparent colour (Faustman & Cassens, 1990; Tang et al., 2004). Mb or Hb in the oxy-form can be oxidised during storage, resulting in discoloration of washed mince. A rapid decrease in redness of washed mince containing oxyHb in the early stage of storage indicated the susceptibility of oxyHb to autooxidation, compared with oxyMb. Decrease in *a*-value was more rapid and extensive in washed mince containing metHb, compared to that with added metMb. This was likely to be due to the higher dissociation of haemin from metHb, while negligible haemin dissociation occurred in metMb (Fig. 3). Thus, changes in colour of washed mince containing Mb or Hb could be governed by the stability of haem proteins toward autooxidation as well as haem dissociation from globin.

### 3.2.3. Changes in lipid peroxides

Lipid peroxides (PV) in washed mince with and without the addition of oxyMb, metMb, oxyHb and metHb during 9 days of iced storage are depicted in Fig. 5B. Washed mince without haem proteins (the control) showed slight increase in PV as the storage time increased ( $p < 0.05$ ). The lowest PV was noticed in the control, compared with those containing haem proteins throughout 9 days of storage ( $p < 0.05$ ). Washed mince containing oxyMb and metMb had a gradual increase in PV as the storage time increased. When oxyHb and metHb were incorporated, washed mince exhibited rapid and extensive increases in PV up to 5 days of storage, followed by a slight decrease until the end of storage ( $p < 0.05$ ). The results indicated that Hb in both oxy- and met-forms were much more capable of promoting lipid peroxide formation than the Mb forms. One possible mechanism of rapid lipid oxidation induced by Hb was the greater haemin loss from Hb compared to Mb (Fig. 3). Haemin loss was also found to be more rapid in trout Hb, compared to trout Mb at pH 6.3 (Richards et al., 2005). A rapid haemin loss from metHb facilitated lipid oxidation in washed mince, whereas metMb with the retained porphyrin was less reactive for accelerating lipid oxidation. Recombinant haem proteins had higher ability to promote lipid oxidation in washed mince, compared to haem proteins that had higher haemin affinity (Grunwald & Richards, 2006).

Within the first 2 days of iced storage, washed mince containing metHb had higher PV than that added with oxyHb ( $p < 0.05$ ). Nevertheless, higher PV was observed in washed mince containing oxyHb during 3–9 days of storage. The potent pro-oxidative activity of metHb could be attributed to its rapid haemin loss (Richards et al., 2005). Essentially negligible rates of haemin loss were reported for oxyHb and deoxyHb, whereas extensive haemin release

was observed for metHb (Bunn & Jandl, 1966). Lipid oxidation in washed mince containing oxyHb was associated with superoxide radicals generated during autooxidation (Maqsood & Benjakul, 2011). Superoxide radicals can be converted to  $H_2O_2$ , which facilitates ferrylHb catalyst formation (Kanner & Harel, 1985) and increases the reactivity of dissociated haemin (Robinson, Dang, Dringen, & Bishop, 2009). OxyHb, on the other hand, is gradually oxidised to metHb, which is more prone to haemin loss. When comparing pro-oxidative activity between both forms of Mb, higher PV was found in washed mince containing metMb, compared with that having oxyMb throughout storage ( $p < 0.05$ ). MetMb was able to generate ferrylMb and cross-linked Mb, which could promote lipid oxidation (Vuletic, Osawa, & Aviram, 2000).

### 3.2.4. Changes in TBARS

Changes in TBARS of washed mince without and with the addition of varying haem proteins during iced storage are shown in Fig. 5C. In the presence of haem proteins, increases in TBARS were observed, suggesting the effective pro-oxidative activity of haem proteins in washed mince. For washed mince having oxyMb and metMb, a gradual increase in TBARS was noticed during the first 5 days of storage, followed by a sharper increase at Day 7 of storage ( $p < 0.05$ ). A rapid increase in TBARS was found in washed mince containing oxyHb within the first 5 days of storage and no change in TBARS was observed thereafter ( $p > 0.05$ ). For washed mince containing metHb, TBARS increased dramatically within the first 2 days of iced storage. No changes in TBARS were observed during 3–9 days of storage ( $p > 0.05$ ). Washed mince with the addition of oxyHb and metHb showed more rapid and extensive TBARS formation than those having oxyMb and metMb. A higher pro-oxidative activity has been reported for anodic Hb from trout blood when compared with Mb from trout cardiac tissue (Richards et al., 2005). Low haemin release from metMb should be considered as part of the mechanism by which lipid oxidation was accelerated. In addition,  $H_2O_2$  equivalents could be formed during Hb oxidation, which rapidly occurred compared to Mb oxidation. Thus, the formation of ferrylHb could be a part of the mechanism by which bighead carp Hb promoted lipid oxidation in washed mince. However, the strong pigment bleaching in washed mince containing oxy- or metHb during storage (Fig. 5A), which resulted from dissociation of haem–globin complex, indicated the more important role of haemin loss from Hb, compared to the role of ferrylHb catalyst. Monahan, Crackel, Gray, Buckley, and Morrissey (1993) reported that lipid oxidation was highest in washed pork muscle treated with Hb, followed by Mb and non-haem iron, respectively. However,

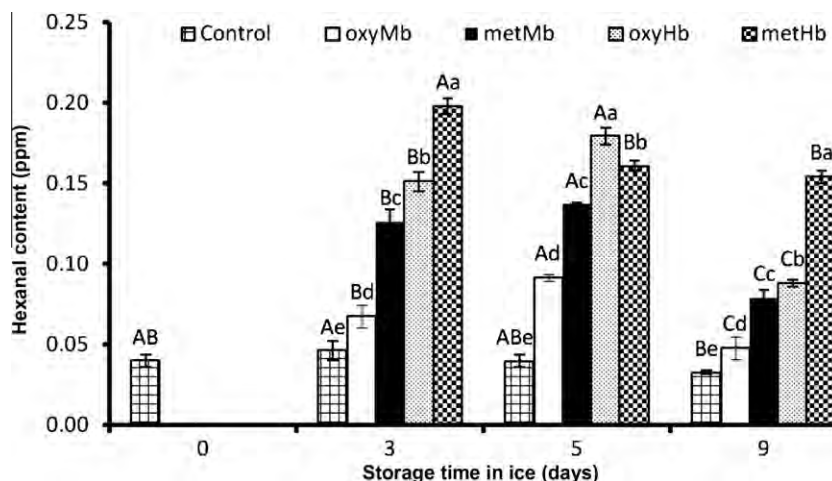


Fig. 6. Changes in hexanal content of washed mince added with oxyMb, metMb, oxyHb and metHb from bighead carp during 9 days of iced storage, pH 6.0. Bars represent standard deviation ( $n = 3$ ).

varying ratio of haem proteins, Mb to Hb, in different species might contribute to a different potential of haem proteins as pro-oxidants.

Based on TBARS in washed mince, metMb had a higher effect on acceleration of lipid oxidation in washed mince, when compared with oxyMb, throughout the storage ( $p < 0.05$ ). MetMb can react with  $H_2O_2$  or pre-formed lipid hydroperoxides to form ferrylMb catalyst that can stimulate lipid oxidation (Kanner & Harel, 1985).

### 3.2.5. Changes in hexanal content

Hexanal contents of washed mince added with and without haem proteins during 9 days of iced storage are shown in Fig. 6. Washed mince without added haem protein had the lowest hexanal content, in comparison with the other samples throughout 9 days of storage ( $p < 0.05$ ). During storage, the highest formation of hexanal was observed in washed mince incorporated with metHb, followed by oxyHb, metMb and oxyMb, respectively. However, at Day 5 of storage, higher hexanal content was observed in washed mince containing oxyHb than that having metHb ( $p < 0.05$ ). Hexanal contents in all samples gradually decreased at Day 9 of storage ( $p < 0.05$ ). Oxidation of lipids is a major cause of deterioration of fish and fish products, especially those containing high content of unsaturated fatty acids. Several derivatives of aldehyde, ketone and alcohol could be formed via lipid oxidation in washed silver carp induced by Hb (Fu, Xu, & Wang, 2009) and washed Asian sea bass with added Mb (Thiansilakul et al., 2011a). Hexanal generated during storage time would contribute to off-odour and fishy odour development in bighead carp muscle. The result also reconfirmed a higher impact of Hb than Mb on lipid oxidation, especially when met-form was present. Roles of dissociated haemin, ferryl-haem protein and cross-linked haem protein in lipid oxidation should be further examined to elucidate the specific mechanisms of haem-protein mediated lipid oxidation.

## 4. Conclusions

Mb and Hb were prepared from the dark muscle and blood of bighead carp, respectively. Mb and Hb monomer had MW of ~16 kDa. During 9 days of iced storage, Hb showed a higher negative impact on colour of washed mince in comparison with Mb. In the presence of haem proteins, lipid oxidation of washed mince occurred to a greater extent during storage time than in the control sample (washed mince without added haem proteins). Both Mb and Hb could accelerate lipid oxidation, in which primary and secondary oxidation products were formed. However, Hb was more pro-oxidative than Mb, compared on the basis of the same amount of haem. Decreasing the concentration of both haem proteins, especially Hb, could be an approach to retard lipid oxidation in fish and fish products so that quality can be maintained.

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## EFFECT OF KIAM WOOD EXTRACT AS INFLUENCED BY pH DURING OXYGENATION ON MACKEREL SURIMI GEL

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### ABSTRACT

*Impact of ethanolic kiam wood extract (EKWE) oxygenated at various pHs on the properties of gels from mackerel (*Rastrelliger kanagurta*) surimi was studied in comparison with commercial tannin (CT). When 0.3% CT and 0.15% EKWE oxygenated at alkaline pHs (8 and 9) were incorporated, surimi gel had the increases in breaking force by 123.4–135.7% and 136.9–157.5%, respectively, while the deformation was increased by 20.9–38.1% and 33.7–48.2%, respectively, compared with those of the control ( $P < 0.05$ ). Those increases were associated with the lowered expressible moisture content and the disappearance of myosin heavy chain band intensity. However, CT and EKWE oxygenated at pH 3 and 7 showed no pronounced effect. Gels added with 0.15% EKWE oxygenated at alkaline pH had the finer matrix with smaller strands and voids. Thus, the pH for oxygenation of phenolic compounds in EKWE had the impact on the gel strength of mackerel surimi.*

### PRACTICAL APPLICATIONS

Gel strength is a prime factor determining the quality and price of surimi. Several additives have been used to improve the gel strength. Recently the use of phenolic compounds in their oxidized form effectively enhanced gel strength of surimi. The extract from kiam wood is another natural additive exhibiting gel strengthening effect. Oxygenation of the extract at alkaline pH could increase the efficacy of the extract, in which the lower amount was required and no negative effect on the resulting gel was obtained.

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## INTRODUCTION

Surimi, a minced and washed fish flesh, has now become a very popular term. It has been used as a base material for the production of various imitation products such as crab stick, shrimp analogue, lobster analogue, etc. Lean fish with white meat containing low fat have been generally used to make surimi. Nowadays, over-exploitation of lean fish has resulted in their shortage. Dark flesh fish species currently make up 40–50% of the total fish catch in the world and could be a better alternative for lean fish for surimi production (Chaijan *et al.* 2004). Due to its high fat and myoglobin content, dark flesh fish commonly have low gel-forming ability and less whiteness. To overcome these problems, different washing methods have been developed (Shimizu 1965; Chen *et al.* 1997; Karayannakidis *et al.* 2007). A variety of additives have been used to improve the gel strength of surimi, including beef plasma protein (Benjakul *et al.* 2004), phosphate compounds (Julavittayanukul *et al.* 2006) and whey protein concentrate (Rawdkuen and Benjakul 2008).

Phenolic compounds have become increasingly important because of their dynamic properties as a cross-linker, anti-oxidant, antimicrobial agent, etc. (Nirmal and Benjakul 2009; Nuthong *et al.* 2009). Protein–phenolic interactions had impact on various food systems (Rawel *et al.* 2002; Strauss and Gibson 2004). Recently, Balange and Benjakul (2009a) reported the cross-linking activity of different oxidized phenolic compounds, i.e. tannic acid, catechin, caffeic acid and ferulic acid toward surimi proteins, which were associated with the increase in the gel strength of bigeye snapper (*Priacanthus tayenus*) surimi. Among different commercial phenolic compounds used, tannic acid in its oxidized form exhibited the highest gel strengthening effect, compared with others. The use of oxidized tannic acid in conjunction with the alkaline–saline washing process improved the properties of gel from mackerel surimi without the adverse effect on sensory properties (Balange and Benjakul 2009c). Oxidation of phenolic compounds results in the formation of quinone, which is highly reactive and is involved in the formation of strong covalent bonds with the proteins (Kroll *et al.* 2003). This oxidation can be achieved enzymatically or nonenzymatically. Enzymatic oxidation requires technical expertise and is costly. On the contrary, nonenzymatic oxidation is simple and not expensive (Prigent 2005). The covalent modification of proteins by phenolic oxidation products generated at alkaline pH has been studied extensively (Rawel *et al.* 2002; Kroll *et al.* 2003). Phenolic compounds could be oxidized nonenzymatically to quinone at pH 8 (Strauss and Gibson 2004; Balange and Benjakul 2009a). Nevertheless, protein modification with plant phenolic compounds at neutral or mildly acidic pH conditions, which are much more relevant to foods than alkaline pH, have not been intensively investigated.

Kiam (*Cotylelobium lanceotatum* craih) trees are very common in the southern part of Thailand. Pieces of wood from kiam tree have been traditionally submerged in sugar palm sap in Thailand to prevent or retard microbial fermentation (Chanthachum and Beuchat 1997). Kiam wood extract contained tannin as the major constituent and could be used as the natural protein cross-linker (Balange and Benjakul 2009b). To maximize the use of this wood extract as the processing aid, the preparation condition including pH adjustment prior to oxidation should be optimized. The objective of this research was to study the effect of different pHs on the oxidation of kiam wood phenolic compounds and to determine the properties of mackerel (*Rastrelliger kanagurta*) surimi gel added with kiam wood extracts oxidized at different pHs in comparison with commercial tannin (CT).

## MATERIALS AND METHODS

### Chemicals

Tannic acid (TA), sodium hydroxide (NaOH), hydrochloric acid, sodium carbonate,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , bovine serum albumin, Folin-Ciocalteu reagent and  $\beta$ -mercaptoethanol ( $\beta$ ME) were obtained from Sigma (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS), *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) and all chemicals for electrophoresis were procured from Bio-Rad Laboratories (Hercules, CA). Sodium chloride and ethanol were obtained from Merck (Darmstadt, Germany).

### Preparation of Kiam Wood Extracts

**Collection of Kiam Wood.** Kiam wood was obtained from a forest of the Phattalung province in Southern Thailand. The tree was about 15–20 years old and harvested in June 2008. The tree was cut by using a sawing machine; the leaves and branches were separated manually by cutting and the trunk was sun-dried for 3 months. The pieces of wood with an average thickness of 1.5 cm were dried in an oven at 70°C for 8 h. Prepared wood was then subjected to a portable grinding machine (Spong-90, Aylesbury, U.K.) with a sieve size of 6 mm. This coarse form was blended using a blender (National Model MK-K77, Tokyo, Japan) and finally sieved using a stainless steel sieve of 80 mesh size.

**Extraction of Phenolic Compounds from Kiam Wood Using Ethanol.** Ethanol extract from powdered kiam wood was prepared as per the method of Santoso *et al.* (2004) with slight modifications. Wood powder (10 g) was mixed with 150 mL of absolute ethanol. The mixture was homogenized

for 2 min at 15,000 rpm using a homogenizer (IKA Labortechnik, Selangor, Malaysia) and stirred at room temperature using a magnetic stirrer (IKA-Werke, Staufen, Germany) at room temperature (28–30C) for 3 h. The homogenate was then centrifuged at  $5,000 \times g$  for 10 min at 25C using a Sorvall Model RC-B plus centrifuge (Newtown, CT). The supernatant was filtered using Whatman filter paper No.1 (Whatman Schleicher & Schuell, Maidstone, England). The filtrate was then evaporated at 40C using a rotary evaporator model N-1000 (Eyela, Tokyo Rikakikai, Co. Ltd, Tokyo, Japan) until ethanol was almost removed. The extract was then dried in a forced-air oven at 70C for 4 h. Dried extract was powdered using a mortar and pestle. Extract powder referred to as ethanolic kiam wood extract (EKWE) was kept in a desiccator at 28–30C until used.

### **Preparation of Surimi**

Mackerel (*R. kanagurta*) with an average weight of 85–90 g were caught from the Songkhla coast along the Gulf of Thailand in September–October, 2008. Fish were stored in ice and offloaded approximately 36 h after capture. Upon the arrival to the dock in Songkhla, fish were iced with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. The fish were immediately washed and used for surimi preparation.

Surimi was prepared according to the method of Shimizu (1965) with a slight modification. After the flesh was removed manually and minced to uniformity using a mincer with a hole diameter of 5 mm, the mince was then suspended in four volumes of cold (5C) diluted alkaline salt solution (0.15% NaCl in 0.2%  $\text{NaHCO}_3$ , of which the ionic strength was 0.05 and the final pH was 7.0–7.1). The mixture was stirred gently for 20 min and the washed mince was filtered with a layer of nylon screen (Butterfly, Lao Hah Seng Lee Co., Ltd, Bangkok, Thailand). The washing process was repeated twice with cold water only. Finally, the washed mince was subjected to centrifugation using a Model CE 21K basket centrifuge (Grandiumpiant, Belluno, Italy) with a speed of  $700 \times g$  at 4C for 10 min. To the washed mince, 4% sucrose and 4% sorbitol were added and mixed well. The portion of mixture (500 g) was packaged in polyethylene bag and frozen using an air-blast freezer at –20C (Patkol, Patanakolkarn Co. Ltd., Bangkok, Thailand). Frozen surimi was stored at –20C for no longer than 2 weeks.

### **The Effect of pHs on the Oxidation of CT or EKWE Solutions and Its Subsequent Effect on the Gel Properties of Mackerel Surimi**

**Oxidation of CT and EKWE at Different pHs.** CT and EKWE were subjected to oxidation at different pHs as per the method of Strauss and

Gibson (2004) with slight modifications. The solutions (100 mL; 1% w/v) were adjusted to pHs 3, 7, 8 and 9, using 6 M NaOH or 6 M HCl. The prepared solutions were placed in a temperature-controlled water bath (40C) and subjected to oxygenation for 1 h by bubbling the solution with oxygen with the purity of 99.5–100% (TTS Gas Agency, Hat Yai, Songkhla, Thailand) to convert phenolic compounds to quinone, an oxidized form. After being oxygenated for 1 h, the solutions without and with neutralization were then determined for the content of remaining phenolic compound with the reduced form.

**Determination of Total Phenolic Content in CT or EKWE Oxidized at Different pHs.** The conversion of phenolic compounds in CT and EKWE to quinone, an oxidized form of CT and EKWE at different pHs was monitored indirectly by determining total phenolic content in CT or EKWE before and after oxidation according to the method of Slinkard and Singleton (1977) with slight modifications. CT or EKWE solutions (0.5 mL; 50 µg/mL) bubbled at different pHs were mixed with 0.5 mL of distilled water. To the mixture, 0.5 mL of Folin-Ciocalteu reagent (1:1 with water) and 2.5 mL of 2% sodium carbonate solution were added sequentially. The reaction mixture was mixed thoroughly and placed in dark for 40 min and the absorbance was read at 725 nm. The total phenolic content was calculated from the standard curve of tannins (100 µg/mL) and expressed as µg tannin/mL after blank subtraction. Blank for each extract was prepared in the same manner, except that distilled water was used instead of Folin-Ciocalteu reagent. The differences in the total phenolic content before and after oxidation indicated the formation of quinones.

**Surimi Gel Preparation.** Frozen surimi was tempered for 30 min in tap water (26–28C) until the core temperature reached 0–2C. The surimi was then cut into small pieces with an approximate thickness of 1 cm and placed in a mixer (National Model MK-K77, Tokyo, Japan). The moisture was adjusted to 80% and 2.5% salt was added. Neutralized CT and EKWE solutions oxygenated at different pHs (3, 7, 8 and 9) were added into the sols at levels of 0.30 and 0.15% (based on protein content), respectively (Balange and Benjakul 2009b). The mixtures were chopped for 4 min at 4C to obtain homogeneous sols. The sols were then stuffed into polyvinylidene casings with a diameter of 2.5 cm and both ends of the casings were sealed tightly. Sols were incubated at 40C for 30 min, followed by heating at 90C for 20 min (Benjakul *et al.* 2003). All gels were cooled in iced water and stored overnight at 4C prior to analyses.

## Measurement of Surimi Gel Properties

**Texture Analysis.** Texture analysis of gels was performed using a texture analyzer Model TA-XT2 (Stable Micro Systems, Surrey, U.K.). Gels were equilibrated and tested at room temperature. Five cylinder-shaped samples of 2.5 cm in length were prepared. The breaking force (gel strength) and deformation (elasticity/deformability) were measured using the texture analyzer equipped with a spherical plunger (5 mm diameter). The probe was pressed into the cut surface of a gel specimen perpendicularly at a constant plunger speed ( $60 \text{ mm min}^{-1}$ ) until the puncture occurred. The force in gram (g) required to puncture into the gel (breaking force) and the distance (mm) at which the spherical probe punctured into the gel (deformation) were recorded.

**Determination of Expressible Moisture Content.** Expressible moisture content was measured according to the method of Benjakul *et al.* (2001) with a slight modification. Gel samples were cut into a thickness of 5 mm, weighed ( $X$ ) and placed between three pieces of Whatman paper no. 4 at the bottom and two pieces on top of the sample. The standard weight (5 kg) was placed at the top and held for 2 min. The sample was then removed from the papers and weighed again ( $Y$ ). Expressible moisture content was calculated using the following equation:

$$\text{Expressible moisture content (\%)} = 100[(X - Y)/X]$$

**Determination of Whiteness.** Color of gels was determined using a JP7100F colorimeter (Juki Corporation, Tokyo, Japan).  $L^*$  (lightness),  $a^*$  (redness/greenness) and  $b^*$  (yellowness/blueness) were measured and whiteness was calculated as described by NFI (1991) as follows:

$$\text{Whiteness} = 100 - \left[ (100 - L^*)^2 + a^{*2} + b^{*2} \right]^{1/2}$$

**Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE).** Protein patterns of samples were analyzed under a reducing condition by SDS–PAGE according to the method of Laemmli (1970). To prepare the protein sample, 27 mL of 5% (w/v) SDS solution heated to 85°C was added to the sample (3 g). The mixture was then homogenized at a speed of 11,000 rpm for 2 min using a homogenizer (IKA Labortechnik, Selangor, Malaysia) for 2 min. The homogenate was incubated at 85°C for 1 h to dissolve total proteins. The samples were centrifuged at  $3,500 \times g$  for 20 min to remove undissolved debris. Protein concentration was determined by the Biuret

method (Robinson and Hodgen 1940) using bovine serum albumin as a standard. The sample was then mixed with sample buffer (4 mL of 10% SDS, 2 mL of glycerol, 1 mL of  $\beta$ -mercaptoethanol, 2.5 mL of 0.5 M Tris-HCl (pH 6.8) and 0.03 g Bromophenol blue) at 1:1 ratio (v/v). The samples (20  $\mu$ g protein) were loaded onto the polyacrylamide gel made of 10% running gel and 4% stacking gel, and subjected to electrophoresis at a constant current of 15 mA per gel, using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA). After separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid, and destained with 50% methanol (v/v) and 7.5% (v/v) acetic acid, followed by 5% methanol (v/v) and 7.5% (v/v) acetic acid.

**Determination of Solubility.** Solubility of protein in surimi gel added with EKWE oxygenated at different pHs was determined as described by Benjakul *et al.* (2001). Finely chopped gel sample (1 g) was solubilized with various solvents, including S1 (0.6 M KCl), S2 (20 mM Tris-HCl, pH 8.0), S3 (20 mM Tris-HCl, pH 8.0 containing 1% SDS), S4 (20 mM Tris-HCl, pH 8.0 containing 1% SDS and 8 M urea) and S5 (20 mM Tris-HCl, pH 8.0 containing 1% SDS, 2%  $\beta$ -mercaptoethanol and 8 M urea). The mixture was homogenized for 1 min, boiled for 2 min and stirred for 4 h at room temperature (28–30°C) using a magnetic stirrer (IKA-Werke, Staufen, Germany). The mixture was centrifuged at  $10,000 \times g$  for 30 min. Two mL of 50% (w/v) cold trichloroacetic acid (TCA) was added to 10 mL of supernatant. The mixture was kept at 4°C for 18 h prior to centrifugation at  $10,000 \times g$  for 20 min. The precipitate was washed with 10% (w/v) TCA, followed by solubilizing in 0.5 M NaOH. Protein concentration was determined by the Biuret method (Robinson and Hodgen 1940). Solubility of protein in surimi samples was expressed as the percentage of total protein in surimi gels solubilized directly in 0.5 M NaOH.

**Scanning Electron Microscopy (SEM).** The microstructure of gels was determined using SEM. Surimi gels added without and with EKWE (0.15%) solutions oxygenated at different pHs were fixed with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2). The samples were then rinsed for 1 h in distilled water before being dehydrated in ethanol with serial concentrations of 50, 70, 80, 90 and 100% (v/v). Dried samples were mounted on a bronze stub, and sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA). The specimens were observed with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 10 kV.

**Sensory Evaluation.** Mackerel surimi gels added with EKWE oxygenated at different pHs at a level of 0.15% were evaluated for color, appearance,

odor, taste, texture and overall liking by 30 nontrained panelists. A 9-point hedonic scale, in which a score of 1 = not like very much, 5 = neither like nor dislike and 9 = like extremely was used for evaluation (Meilgaard *et al.* 1990). The control gel (without addition of EKWE) was also assessed for likeness.

**Statistical Analysis.** All experiments were run in triplicate. For each run, chemical analyses were performed in triplicate. For physical analyses, e.g. expressible moisture, whiteness and textural properties, five determinations were conducted. Data were subjected to analysis of variance. Comparison of means was carried out by Duncan's multiple range tests (Steel and Torrie 1980). *t*-test was used for pair comparison. Analysis was performed using a SPSS package (SPSS 10.0 for Windows, SPSS Inc, Chicago, IL).

## RESULTS AND DISCUSSION

### Conversion of Phenolic Compounds in CT and EKWE to Oxidized Forms after Oxygenation at Different pHs

Oxidized form, mainly quinones, in CT and EKWE solutions oxygenated at different pHs were determined indirectly by using Folin-Ciocalteu reagent (Table 1). The amount of quinone formed in both CT and EKWE increased with increasing pHs of solutions ( $P < 0.05$ ). Nonsignificant increases in quinone content were found in CT and EKWE solutions when oxygenated at pH 3 and 7 ( $P > 0.05$ ). The amount of quinone in CT and EKWE solutions increased by 50.42% and 59.24%, respectively, when oxygenated at pH 8. The highest amount of quinone was formed in CT and EKWE solutions when oxygenated at pH 9, in which increases by 68.96% and 75.08% were obtained ( $P < 0.05$ ). Under the alkaline conditions, the hydroxyl groups are readily oxidized to hydroxyl radical (Huang and Chu 1992). Those radicals would react with the neutral molecules and cause the benzene ring to undergo hydrolyzation. Increasing pH induces the deprotonation of the phenolic hydroxyl group, eventually leading to the formation of quinones (Prigent 2005). Therefore, the conversion of phenolic compounds into quinones was more pronounced at alkaline conditions, compared with the acidic or neutral conditions. Furthermore, the content of quinone in CT and EKWE solutions oxygenated at different pHs, slightly decreased after neutralization ( $P > 0.05$ ). The quinones formed during oxidation are highly unstable and are prone to dimerization (Huang and Chu 1992). Additionally, the reduction of quinone formed could take place to some extent during neutralization. This resulted in the lower content of quinone detected in the solutions.

TABLE 1.  
TOTAL PHENOLIC CONTENT (MG/ML) OF COMMERCIAL TANNIN (CT) AND ETHANOLIC KIAM WOOD EXTRACT (EKWE)  
OXYGENATED AT DIFFERENT pHs

| Source of phenolic compounds | Treatments                           | pHs                       |                           |                            |                            |
|------------------------------|--------------------------------------|---------------------------|---------------------------|----------------------------|----------------------------|
|                              |                                      | 3                         | 7                         | 8                          | 9                          |
| CT                           | Without treatment                    | 50.00 ± 0.00a             | 50.00 ± 0.00a             | 50.00 ± 0.00a              | 50.00 ± 0.00a              |
|                              | After oxygenation                    | 47.5 ± 0.51aA<br>(5%)     | 46.87 ± 0.48aA<br>(6.26%) | 24.79 ± 0.61bB<br>(50.42%) | 15.52 ± 0.46bC<br>(68.96%) |
| EKWE                         | After oxygenation and neutralization | 47.15 ± 0.46aA<br>(5.7%)  | 46.87 ± 0.48aA<br>(6.26%) | 25.81 ± 0.25bB<br>(48.38%) | 16.93 ± 0.16bC<br>(66.14%) |
|                              | Without treatment                    | 50.00 ± 0.00a             | 50.00 ± 0.00a             | 50.00 ± 0.00a              | 50.00 ± 0.00a              |
|                              | After oxygenation                    | 49.48 ± 0.56aA<br>(1.04%) | 47.73 ± 0.16aA<br>(4.54%) | 20.38 ± 0.53bB<br>(59.24%) | 12.46 ± 0.26bC<br>(75.08%) |
|                              | After oxygenation and neutralization | 48.65 ± 0.31aA<br>(2.70%) | 47.73 ± 0.16aA<br>(4.54%) | 21.13 ± 0.26bB<br>(57.74%) | 13.14 ± 0.12bC<br>(73.72%) |

\* Different letters in the same column within the same source of phenolic compound indicate the significant differences ( $P < 0.05$ ). \*\* Different capital letters in the same row indicate the significant differences ( $P < 0.05$ ). Values in parenthesis indicate the % conversion of phenolic compounds to quinone. Values are mean ± standard deviation ( $n = 3$ ).  
CT, commercial tannin; EKWE, ethanolic kiam wood extract.

### Effect of CT or EKWE Oxygenated at Different pHs on Properties of Mackerel Surimi Gel

Breaking force and deformation of mackerel surimi gels added with 0.30% CT or 0.15% EKWE oxygenated at different pHs are shown in Fig. 1. Surimi gels added with CT or EKWE oxygenated at pH 3 or 7 had the increases in breaking force by 49.3% or 52.5% and in deformation by 20.9% or 38.2 %, respectively, compared with those of the control. For surimi gels added with 0.30% CT or 0.15% EKWE oxygenated at pH 8, the increases in breaking force by 123.4% or 135.7%, and in deformation, by 35.9 or 56.2%, were obtained, respectively, compared with that of the control ( $P < 0.05$ ). The highest breaking force was observed in the surimi gels added with 0.30% CT or 0.15% EKWE oxygenated at pH 9, in which increases by 136.9% and 157.5% were found, respectively. Similar deformation was noticeable in comparison with that found in gel added with either CT or EKWE oxygenated at pH 8 ( $P > 0.05$ ). The results revealed that the pH for the oxidation of phenolic compounds had a pronounced effect on gel properties of mackerel surimi. Breaking force and deformation of gels increased when both CT and EKWE oxygenated at alkaline pHs were incorporated ( $P < 0.05$ ). This might be due to the fact that the greater formation of quinones took place at alkaline pHs (Table 1). Quinones formed were electrophilic and could react with amino acids in a peptide chain via covalent bond. The covalent modification of proteins by phenolic oxidation products generated at alkaline pH has been studied extensively (Rawel *et al.* 2002). On the contrary, the deprotonation of phenolic hydroxyl groups occurred at a low extent at acidic or neutral pHs. As a consequence, hydroxyl groups were mostly present in the reduced forms. Those hydroxyl groups could interact with peptide chain, particularly carbonyl group of peptide backbone via hydrogen bond. Moreover, phenolic compounds could cross-link proteins via hydrophobic interaction (Prigent 2005). All surimi gels added with CT and EKWE oxygenated at all pHs had higher breaking force and deformation as compared with the control gels ( $P < 0.05$ ). It was noted that the breaking force of surimi gel could be much improved by addition of CT or EKWE oxygenated at pHs 8 or 9, most likely due to the formation of covalent bonds. The major chemical constituents in wood or bark from different trees were reported to be tannin, with a small amount of lignin (Yazaki and Collins 1994; Fradinho *et al.* 2002). At pH levels 8 and 9, EKWE exhibited the higher gel strengthening effect than did CT, even though the lower amount of the former was incorporated. Apart from tannin, ethanol might extract other phenolic compounds such as lignin from kiam wood. The presence of lignin and other phenolic compounds, along with tannin might have the synergistic effect on the improvement of gel strength of surimi. Recently,

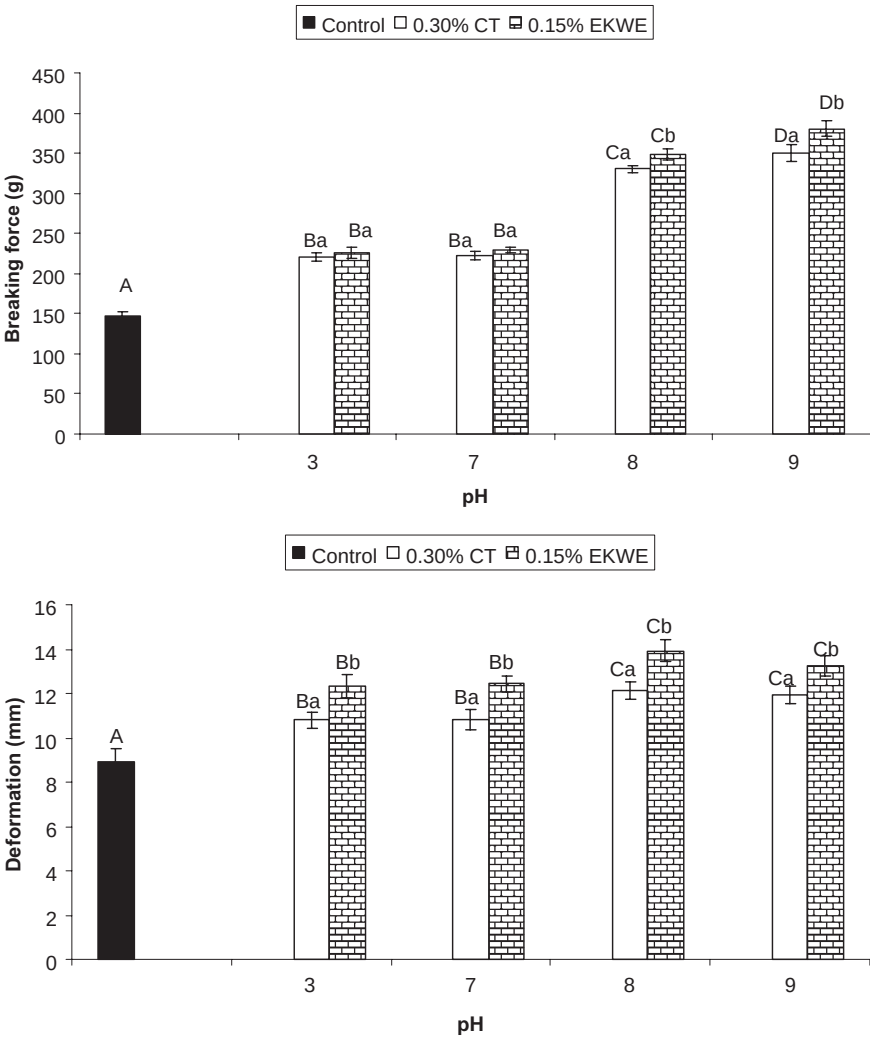


FIG. 1. BREAKING FORCE AND DEFORMATION OF GELS FROM MACKEREL SURIMI ADDED WITH 0.30% COMMERCIAL TANNIN (CT) OR 0.15% ETHANOLIC KIAM WOOD EXTRACT (EKWE) OXYGENATED AT DIFFERENT pHs. BARS REPRESENT THE STANDARD DEVIATION ( $n = 3$ ). DIFFERENT CAPITAL LETTERS ON THE BARS WITHIN THE SAME SOURCE OF PHENOLIC COMPOUNDS TOGETHER WITH THE CONTROL INDICATE THE SIGNIFICANT DIFFERENCES ( $P < 0.05$ ). THE DIFFERENT LETTERS ON THE BARS WITHIN THE SAME PH INDICATE THE SIGNIFICANT DIFFERENCES ( $P < 0.05$ ). BOTH CT AND EKWE WERE NEUTRALIZED AFTER OXYGENATION PRIOR TO THE ADDITION INTO SURIMI SOL.

Balange and Benjakul (2009b) reported that tannin was the major constituent in both water and ethanolic kiam wood extract.

### Effect of CT and EKWE Oxygenated at Different pHs on the Expressible Moisture Content of Mackerel Surimi Gel

When CT or EKWE oxygenated at different pHs was added into surimi gel, the expressible moisture content gradually decreased when pHs of both CT and EKWE subjected to oxygenation increased (Table 2). The decreases in expressible moisture contents were in accordance with the increased breaking force and deformation of resulting surimi gels (Fig. 1). During setting at 40C, proteins underwent some denaturation and aligned themselves gradually to form the network, which can imbibe water (Benjakul and Visessanguan 2003). In the presence of CT or EKWE, tannin and other components could interact with unfolded muscle proteins in which the network capable of holding water could be formed. It was suggested that quinones formed at alkaline pHs effectively cross-linked proteins or amino acids, resulting in the formation of stronger network with greater water-holding capacity. For gel incorporated with CT or EKWE oxygenated at pHs 3 or 7, the lower expressible moisture content was obtained compared with the control gel. Weak bonds stabilizing gel network including hydrogen bond or hydrophobic interaction might play a role in

TABLE 2.  
EXPRESSIBLE MOISTURE CONTENT AND WHITENESS OF GELS FROM MACKEREL SURIMI ADDED WITH COMMERCIAL TANNIN (CT) AND ETHANOLIC KIAM WOOD EXTRACT (EKWE) OXYGENATED AT DIFFERENT pHs

| Sources of phenolic compounds | Amount added (%) | pH of solution | Expressible moisture content (%) | Whiteness        |
|-------------------------------|------------------|----------------|----------------------------------|------------------|
| Control                       | 0                | —              | 11.18 ± 0.48a*                   | 69.25 ± 0.36a*   |
| CT                            | 0.30             | 3              | 8.46 ± 0.34bA**                  | 67.26 ± 0.34bA** |
|                               |                  | 7              | 8.56 ± 0.37bA                    | 67.06 ± 0.42bA   |
|                               |                  | 8              | 5.37 ± 0.21cA                    | 62.26 ± 0.42cA   |
|                               |                  | 9              | 4.45 ± 0.39dA                    | 58.22 ± 0.34dA   |
|                               |                  |                |                                  |                  |
| EKWE                          | 0.15             | 3              | 8.10 ± 0.23bA                    | 68.16 ± 0.46bB   |
|                               |                  | 7              | 8.02 ± 0.45bA                    | 68.76 ± 0.51bB   |
|                               |                  | 8              | 4.32 ± 0.33cB                    | 66.25 ± 0.55cB   |
|                               |                  | 9              | 3.12 ± 0.57dC                    | 64.17 ± 0.34dB   |

\* Different letters in the same column within the same source of phenolic compounds together with the control indicate the significant differences ( $P < 0.05$ ).

\*\* Different capital letters in the same column within the same pH used indicate the significant differences ( $P < 0.05$ ).

Values are mean ± standard deviation ( $n = 3$ ).

CT, commercial tannin; EKWE, ethanolic kiam wood extract.

building up the network that could imbibe the water. However, the efficacy in the water holding of gel added with CT or EKWE oxygenated at both pHs was lower than that of gel incorporated with CT or EKWE oxygenated at pH levels 8 and 9 ( $P < 0.05$ ). Thus, the pH of CT or EKWE during oxygenation had the impact on water holding capacity of gel. At the same pH used, EKWE yielded the gel with the lower expressible moisture content than did CT ( $P < 0.05$ ). This result was in agreement with the higher breaking force of gel added with EKWE, particularly those oxygenated at the alkaline pH range.

### **Effect of CT and EKWE Oxygenated at Different pHs on Whiteness of Mackerel Surimi Gel**

The decreases in whiteness of mackerel surimi gels were observed as the levels of pH for oxygenation of CT and EKWE increased up to pH 9 ( $P < 0.05$ ) (Table 2). These results were in agreement with O'Connell and Fox (2001), who reported that phenolic compounds were responsible for discoloration in the cheese products. The decrease in whiteness was more pronounced in the mackerel surimi gels with the addition of CT or EKWE oxygenated at pHs 8 and 9, compared with pHs 3 and 7 ( $P < 0.05$ ). The color of the solution becomes darker after being oxygenated in the alkaline pH range, probably due to the dimerization or polymerization of oxidized phenolic compounds (Huang and Chu 1992). Furthermore, the carbonyl of quinone might undergo Maillard reaction with amino group of surimi to a greater extent, compared with the reduced phenolic compounds in both CT and EKWE. Thus, it caused the increased darkening in surimi gel added with CT or EKWE oxygenated at alkaline pH, as indicated by the lowered whiteness. When CT and EKWE with the same pH for oxygenation were added, the resulting surimi gel added with the former had the lower whiteness than that added with the latter. This indicated that other components in EKWE might be less susceptible to darkening than tannin. As a consequence, the darkening was lower in surimi gel added with EKWE.

### **Effect of EKWE Oxygenated at Different pHs on the Protein Patterns of Mackerel Surimi Gels**

Due to the higher efficacy of EKWE in strengthening the surimi gel with the less adverse effect on whiteness, only gels incorporated with 0.15% EKWE oxygenated at different pHs were determined for protein patterns (Fig. 2). Surimi sol contained myosin heavy chain (MHC) and actin as the major proteins. Decrease in MHC band intensity was found in the control gel (without addition of EKWE), compared with that observed in sol. The result suggested that the formation of cross-linking stabilized by nondisulfide covalent bond mediated by indigenous transglutaminase took place, especially

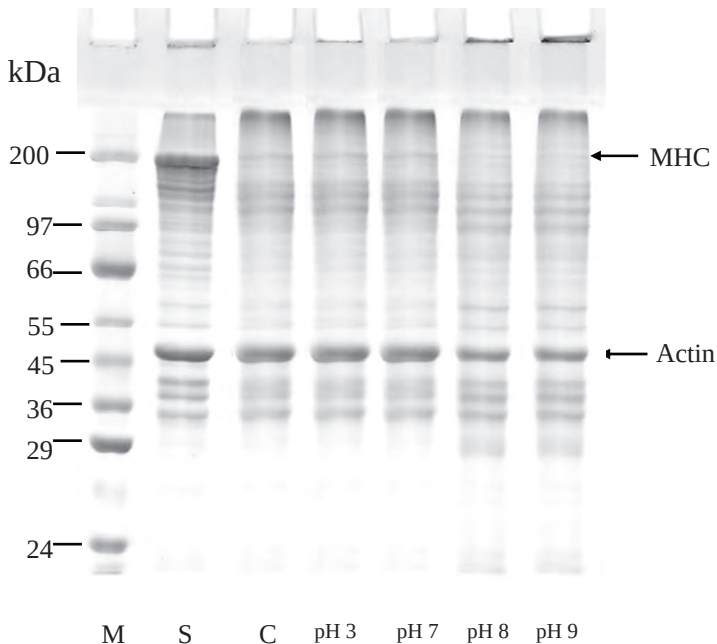


FIG. 2. SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS PATTERNS OF PROTEIN IN GELS FROM MACKEREL SURIMI ADDED WITH 0.15% ETHANOLIC KIAM WOOD EXTRACT (EKWE) OXYGENATED AT DIFFERENT pHs.

M, MARKER; S, SURIMI SOL; MHC, MYOSIN HEAVY CHAIN; C, THE CONTROL GEL.

during setting. MHC was most susceptible to cross-linking during setting (Benjakul and Visessanguan 2003). Benjakul and Visessanguan (2003) reported the decrease in MHC of surimi gel from bigeye snapper, particularly when the setting was implemented.

For surimi gels added with 0.15% EKWE oxygenated at alkaline pH, MHC band intensity decreased to a higher extent, compared with that found in the control gel. The results suggested that MHC underwent cross-linking induced by oxidized phenolic compounds in EKWE via nondisulfide covalent bonds effectively. For gels added with EKWE oxygenated at pH 3 and pH 7, MHC band was decreased slightly, compared with the control gels. However, MHC band disappeared almost completely when the gels were added with EKWE oxygenated at pHs 8 and 9. The coincidental formation of polymerized proteins was noticeable in the stacking gel. The results reconfirmed that enhanced cross-linking of proteins in surimi was related with the formation of quinones, which acted as the potential protein cross-linkers. The similar

protein pattern was observed in case of surimi gels added with CT oxygenated at alkaline pHs (data not shown). Phenols could be oxidized with ease, in an alkaline solution, to their corresponding quinones (Hurrell and Finot 1984). The quinone, a reactive electrophilic intermediate, can readily undergo attack by nucleophiles such as lysine, methionine, cysteine and tryptophan residues in a protein chain, forming strong covalent bonds (Hurrell and Finot 1984).

**Effect of EKWE Oxygenated at Different pHs on the Solubility of Mackerel Surimi Gels**

Solubility of surimi gels added with 0.15% EKWE oxygenated at different pHs in different solubilizing solutions is shown in Fig. 3. Solubility was found to be 28% and 25% in the control gels without EKWE when solubilized with 0.6 M KCl ( $S_1$ ) and 20 mM Tris-HCl (pH 8.0) ( $S_2$ ), respectively. Solubility was lower than 20% in the gels added with 0.15% EKWE oxygenated at pH 3 and pH 7 when solubilized with  $S_1$  and  $S_2$ . Nevertheless, the lowest solubility was observed in the gels added with 0.15% EKWE oxygenated at pH levels 8 and pH 9, when solubilized with  $S_1$  and  $S_2$ . Native myofibrillar

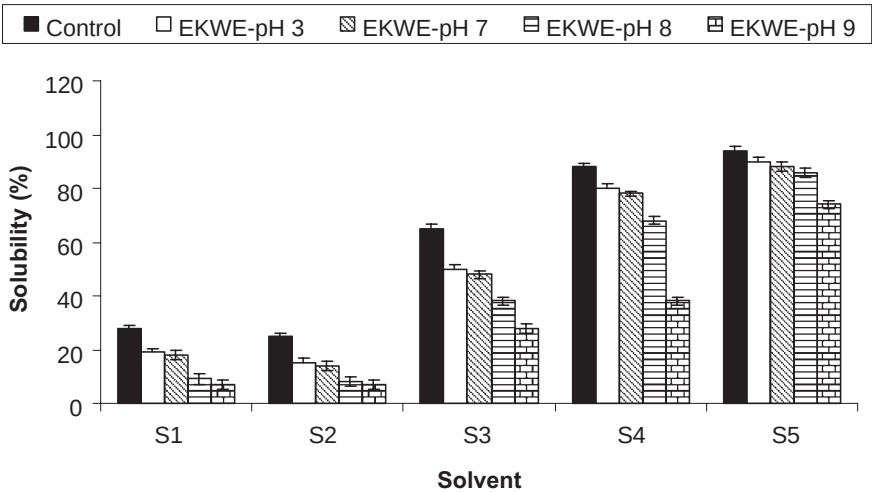
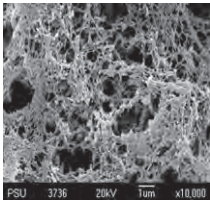


FIG. 3. SOLUBILITY OF MACKEREL SURIMI GELS ADDED WITH 0.15% ETHANOLIC KIAM WOOD EXTRACT (EKWE) OXYGENATED AT DIFFERENT pHs. SAMPLES WERE SOLUBILIZED IN DIFFERENT SOLVENTS AND SOLUBLE PROTEIN WAS DETERMINED BY THE BIURET ASSAY. 0.6 M KCl ( $S_1$ ); 20 mM TRIS-HCl, pH 8.0 ( $S_2$ ); 20 mM TRIS-HCl, pH 8.0, CONTAINING 1% SDS ( $S_3$ ); 20 mM TRIS-HCl, pH 8.0, CONTAINING 1% SDS AND 8 M UREA ( $S_4$ ) AND 20 mM TRIS-HCl, pH 8.0, CONTAINING 1% SDS, 8 M UREA AND 2%  $\beta$ -MERCAPTOETHANOL ( $S_5$ ). BARS REPRESENT THE STANDARD DEVIATION ( $n = 3$ ).

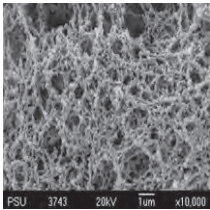
proteins are normally soluble in high ionic strength buffer (Suzuki 1981). The decrease in solubility of surimi gel suggested the formation of protein aggregates during setting and heating. When the gels were solubilized in 20 mM Tris-HCl (pH 8.0) containing 1% SDS ( $S_3$ ), the solubility was increased up to 65% in the control gel, while gels added with 0.15% EKWE oxygenated at pH 3, 7, 8 and 9 had 50, 48, 38 and 28% increases in solubility, respectively. SDS is capable of destroying hydrogen and some hydrophobic interactions (Hamada 1992). Further increases in solubility were observed in  $S_4$ , containing urea and SDS, indicating the presence of hydrophobic and hydrogen bonds in surimi gels. Hydrogen bonds might involve in the interactions between hydroxyl groups of phenolic compounds and the nitrogen or oxygen of lysine, arginine, histidine, asparagine, glutamine, serine, threonine, aspartic acid, glutamic acid, tyrosine, cysteine and tryptophan as hydrogen acceptor (Prigent 2005). Protonation of quinone could take place to some extent after neutralization (Table 1). As a consequence, hydroxyl groups could be regenerated partially. Hydrophobic interactions may occur between phenolic compounds and hydrophobic amino acids such as alanine, valine, isoleucine, leucine, methionine, phenylalanine, tyrosine, tryptophan, cysteine and glycine residues (Prigent 2005). Surimi gels added with EKWE oxygenated at pH values 8 and 9 had the lower solubility than those incorporated with EKWE oxygenated at pH levels 3 and 7 when  $S_4$  was used. This suggested that covalent bonds were formed in gels added with EKWE oxygenated at pH 8 and 9 to a high extent. When gel samples were solubilized in  $S_5$  containing urea, SDS and  $\beta$ ME, it was noticeable that a higher increase in solubility was observed in surimi gels added with EKWE oxygenated at pH 8 and 9 in comparison with that found in  $S_4$ . The result indicated the presence of disulfide bonds in the respective gels. When quinones attached to protein molecules, the conformational changes of protein possibly occurred in the way that favored the oxidation of sulfhydryl groups, leading to the increases in disulfide bond formation. Gels added with EKWE oxygenated at alkaline pHs, particularly at pH 9, had a lower solubility in  $S_5$ , compared with other gels ( $P < 0.05$ ). Thus, the nondisulfide covalent bonds induced by quinones, an oxidized phenolic compound, could play a partial role in gel strengthening. It was reported that quinone probably attached to amino groups of protein molecules in which the subsequent inter-molecular cross-linking could be formed (Rawel *et al.* 2002; Strauss and Gibson 2004). The solubility result reconfirmed the gel enhancing effect of oxidized phenolic compounds in EKWE via both disulfide and non-disulfide covalent bonds.

#### **Effect of EKWE Oxygenated at Different pH on the Microstructures of Gels from Mackerel Surimi**

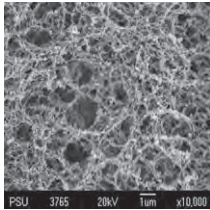
Microstructures of gels from mackerel surimi without (A) and with 0.15% EKWE oxygenated at different pHs (B) are illustrated in Fig. 4.



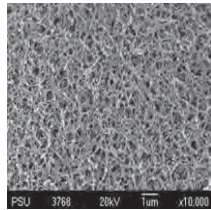
A) Control



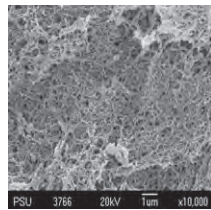
pH-3



pH-7



pH-8



pH-9

B) 0.15% EKWE

FIG. 4. ELECTRON MICROSCOPIC IMAGES OF GELS FROM MACKEREL SURIMI ADDED WITHOUT AND WITH 0.15% ETHANOLIC KIAM WOOD EXTRACT (EKWE) OXYGENATED AT DIFFERENT pHs (MAGNIFICATION: 10,000 $\times$ ). EKWE WERE NEUTRALIZED AFTER OXYGENATION PRIOR TO THE ADDITION INTO SURIMI SOL.

Surimi gels containing 0.15% EKWE oxygenated at pHs 3 and 7 had a finer gel network with less number of voids, compared with the control gel. This might be attributed to the formation of hydrophobic interaction and hydrogen bonds between phenolic compounds and muscle proteins in gel network. Surimi gels containing 0.15% EKWE oxygenated at pHs 8 and 9 had a finer gel and continuous matrix than other treatments. Nevertheless, the gel added with 0.15% EKWE oxygenated at pH 9 had very compact and denser network. This suggested that phenolic compounds oxidized at alkaline pH range effectively induced the cross-linking of proteins, in which interconnection between protein molecules could be formed to a higher extent. Those cross-links further underwent polymerization, leading to the formation of gel network with fibrillar structure. In addition to tannin, other polyphenolic compounds in EKWE might function synergistically in developing the gel matrix. The finer and more ordered structure of gels added with EKWE oxygenated at alkaline pHs correlated with the higher breaking force and deformation (Fig. 1) as well as the lower expressible moisture content of the resulting gels (Table 2).

### Effect of EKWE Oxygenated at Different pHs on the Sensory Properties of Mackerel Surimi Gels

Likeness scores of the control gels (without EKWE) and those containing 0.15% EKWE oxygenated at different pHs are depicted in Fig. 5. Addition of 0.15% EKWE oxygenated at pHs 3, 7 and 8 had no negative impact on the color, appearance, odor, taste, texture and overall likeness of resulting gels ( $P > 0.05$ ). However, the addition of 0.15% EKWE oxygenated at pH 9 resulted in the lower score for the appearance and color of resulting surimi gels, compared with other treatments including the control ( $P < 0.05$ ). The result was in accordance with the lowest whiteness of gel added with EKWE oxygenated at pH 9 (Table 2). Nevertheless, the addition of 0.15% EKWE oxygenated at pHs 8 and 9 yielded the gel with the higher texture score, compared with other gels ( $P < 0.05$ ). Higher texture score was also found in gels added with EKWE oxygenated at pHs 3 and 7, compared with that of the control gel ( $P < 0.05$ ). This was coincidental with the increased breaking force and deformation in the surimi gels added with EKWE oxygenated at pHs 8 and

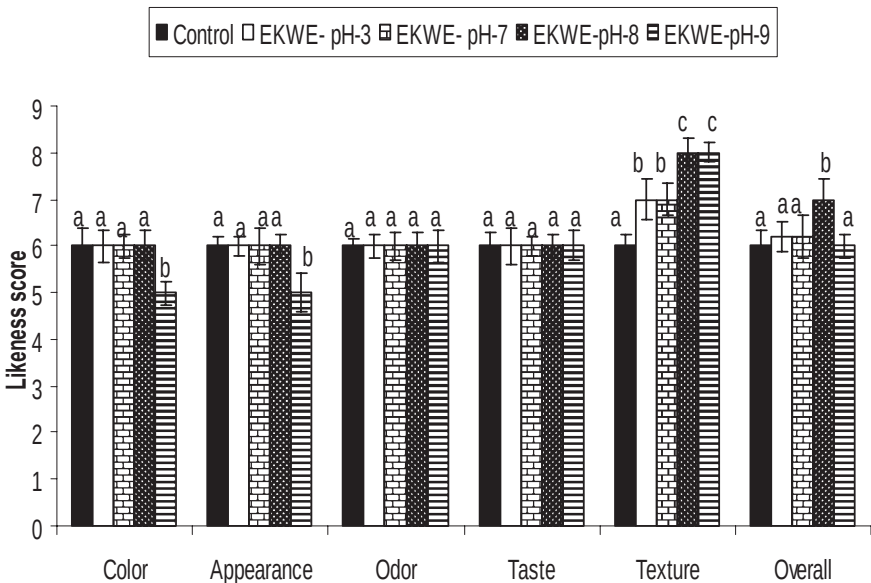


FIG. 5. LIKENESS SCORE OF SURIMI GELS FROM MACKEREL ADDED WITH 0.15% ETHANOLIC KIAM WOOD EXTRACT (EKWE) OXYGENATED AT DIFFERENT pHs. BARS REPRESENT STANDARD DEVIATION ( $n = 30$ ). DIFFERENT LETTERS ON THE BARS WITHIN THE SAME SENSORY ATTRIBUTE INDICATE SIGNIFICANT DIFFERENCES ( $P < 0.05$ ). EKWE WERE NEUTRALIZED AFTER OXYGENATION PRIOR TO THE ADDITION INTO SURIMI SOL.

pH 9 (Fig. 1). Phenolic compounds play a major role in the sensory attributes of many food products (O'Connell and Fox 2001). Tea extracts containing a high proportion of polyphenols were added to sherbet mixes, yoghurt and acidified milk drinks (Ioki and Suzuki 1992). Among all gel samples, those added with 0.15% EKWE oxygenated at pH 8 had the highest overall likeness score. Therefore, the addition of 0.15% EKWE oxygenated at pH 8 in this study had the positive impact on the overall sensory properties of mackerel surimi gels.

## CONCLUSIONS

The pH of CT and EKWE solution subjected to oxygenation was the key factor determining the oxidation of phenolic compounds, in which quinones were formed. Alkaline condition favored the formation of quinones which enhanced the cross-linking of proteins via both disulfide and non-disulfide covalent bonds. Therefore, incorporation of 0.15% EKWE oxygenated at pH 8 into the surimi yielded the gels with the improved mechanical and sensory properties.

## ACKNOWLEDGMENTS

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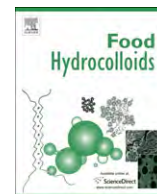
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# Effects of partial hydrolysis and plasticizer content on the properties of film from cuttlefish (*Sepia pharaonis*) skin gelatin

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## ABSTRACT

Properties of film from cuttlefish (*Sepia pharaonis*) ventral skin gelatin with different degree of hydrolysis (DH: 0.40, 0.80 and 1.20%) added with glycerol as plasticizer at various levels (10, 15 and 20%, based on protein) were investigated. Films prepared from gelatin with all DH had the lower tensile strength (TS) and elongation at break (EAB) but higher water vapor permeability (WVP), compared with the control film (without hydrolysis) ( $p < 0.05$ ). At the same glycerol content, both TS and EAB decreased, while WVP increased ( $p < 0.05$ ) with increasing %DH. At the same DH, TS generally decreased as glycerol content increased ( $p < 0.05$ ), however glycerol content had no effect on EAB when gelatins with 0.80 and 1.20% DH were used ( $p > 0.05$ ). DH and glycerol content had no marked impact on color and the difference in color ( $\Delta E^*$ ) of resulting films. Electrophoretic study revealed that degradation of gelatin and their corresponding films was more pronounced with increased %DH, resulting in the lower mechanical properties of films. Based on FTIR spectra, with the increasing %DH as well as glycerol content, higher amplitudes for amide-A and amide-B peaks were observed, compared with film from gelatin without hydrolysis (control film) due to the increased  $-\text{NH}_2$  group caused by hydrolysis and the lower interaction of  $-\text{NH}_2$  group in the presence of higher glycerol. Thermo-gravimetric analysis indicated that film prepared from gelatin with 1.20% DH exhibited the higher heat susceptibility and weight loss in the temperature range of 50–600 °C, compared with control film. Thus, both chain length of gelatin and glycerol content directly affected the properties of cuttlefish skin gelatin films.

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## 1. Introduction

Gelatin is a thermal denatured protein obtained from collagen by acidic or alkaline process (Arvanitoyannis, 2002; Gennadios, McHugh, Weller, & Krochta, 1994). Generally, gelatin has the wide range applications in food industries, cosmetics, biomedical, pharmaceutical applications, leather and encapsulation (Cho et al., 2004; Segtnan, Kvaal, Rukke, Schüller, & Isaksson, 2003; Slade & Levine, 1987). However, the occurrence of bovine spongiform encephalopathy (BSE), foot and mouth diseases have caused major concerns for human health (Cho, Gu, & Kim, 2005). Moreover, porcine gelatin can cause objections from some religions. As a consequence, an increasing interest has been paid to other gelatin sources, especially skin and bone from seafood processing by-products (Gómez-Guillén et al., 2002; Hoque, Benjakul, & Prodpran, 2010; Jongjareonrak, Benjakul, Vesessanguan, Prodpran, & Tanaka,

2006). Cuttlefish are one of the important fishery product in Thailand as well as other south-east Asian countries, and are exported worldwide. During processing of cuttlefish, skin is generated as a by-product with the low market value. To increase its profitability, cuttlefish skin has recently been used for gelatin extraction (Aewsiri, Benjakul, & Visessanguan, 2009; Hoque et al., 2010). Gelatin has been used as a material for preparing biodegradable films with an excellent gas barrier property (Gómez-Guillén et al., 2009; Jongjareonrak, Benjakul, Vesessanguan, Prodpran, et al., 2006). Biodegradable films made from renewable biopolymers can become an important environmental friendly packaging, thereby reducing plastic wastes (Hoque et al., 2010; Prodpran & Benjakul, 2005; Tharanathan, 2003).

Gelatin is a heterogeneous protein containing 20 different amino acids in their polypeptide chains (Arvanitoyannis, 2002). The physical and structural properties of gelatin are mainly influenced by the molecular weight distribution and amino acid composition that plays an important role in the rheological and barrier properties of the resulting films (Gómez-Guillén et al., 2009). The

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properties of films also depend on the type and concentration of plasticizer used (Gómez-Guillén et al., 2009). Carvalho et al. (2008) stated that the mechanical behaviors of gelatin films varied with molecular weight distributions of gelatin. Higher tensile strength and lower elongation values were reported for the film from high-molecular weight gelatin, compared with the films of lower molecular weight gelatins (Gómez-Guillén et al., 2009). Gelatin with the lower molecular mass caused by hydrolysis had the reduced thermal and mechanical properties (Langmaier, Kolomaznik, Sukop, & Mladek, 1999). Plasticizer type and concentration is another critical factor affecting the mechanical properties as well as water vapor barrier property of gelatin films (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2006; Sobral & Habitate, 2001). Due to hydrophilicity of glycerol, commonly used plasticizer in the protein based film, its use at the lower level could be a promising means to improve water barrier property. Along with the cleavages of peptide chains, the rigidity of films could be decreased and the lower amount of plasticizer could be required. However, there is no information about the impact of chain length of gelatin from cuttlefish skin, a by-product from cuttlefish processing, and the influence of glycerol content on the properties of resulting film exists. Thus, the objectives of the present study were to study the effects of gelatin from cuttlefish skin gelatin with different degree of hydrolysis (DH) and glycerol content on the properties of films.

## 2. Materials and methods

### 2.1. Chemicals

L-Leucine, 2,4,6-trinitrobenzenesulphonic acid (TNBS), bovine serum albumin and wide range molecular weight protein markers were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), glycerol, *p*-dimethylaminobenzaldehyde and tris(hydroxymethyl) aminomethane were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfates (SDS), Coomassie Blue R-250 and *N*, *N*, *N'*, *N'*-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Alcalase 2.4 L was provided by Novozymes (Bagsvaerd, Denmark). All chemicals were of analytical grade.

### 2.2. Collection and preparation of cuttlefish skin

Ventral skin of cuttlefish (*Sepia pharaonis*) was obtained from a dock in Songkhla, Thailand. Cuttlefish skin was stored in ice with a skin/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, cuttlefish skin was washed with tap water and cut into small pieces ( $1 \times 1 \text{ cm}^2$ ), placed in polyethylene bags and stored at  $-20^\circ\text{C}$  until use. Storage time was not longer than 2 months. Prior to gelatin extraction, the frozen skin was thawed using running water ( $25\text{--}26^\circ\text{C}$ ) until the core temperature reached  $0\text{--}2^\circ\text{C}$ .

### 2.3. Extraction of gelatin from cuttlefish skin

Gelatin was extracted from cuttlefish skin according to the method of Hoque et al. (2010). Skin was soaked in 0.05 M NaOH with a skin/solution ratio of 1:10 (w/v) with a gentle stirring at room temperature ( $26\text{--}28^\circ\text{C}$ ). The solution was changed every hour to remove non-collagenous proteins for totally 6 h. Alkali treated skin was then washed with distilled water until the neutral pH of wash water was obtained. The prepared skin was subjected to bleaching in 5%  $\text{H}_2\text{O}_2$ , using a sample/solution ratio of 1:10 (w/v) for 48 h at  $4^\circ\text{C}$ . The skin treated with  $\text{H}_2\text{O}_2$  was washed three times with 10 volumes of water. Gelatin was extracted from bleached skin

by using distilled water at  $60^\circ\text{C}$  for 12 h, using a sample/water ratio of 1:2 (w/v). During extraction, the mixture was stirred continuously using a paddle stirrer (RW20.n, IKA LABORTECHNIK, Staufen, Germany). The extract was centrifuged at  $8000 \times g$  for 30 min at room temperature using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Beckman Coulter, Inc., Palo Alto, CA, USA) to remove insoluble materials. The supernatant was collected and freeze-dried (Model Duratop™ IP/Dura Dry™ IP, FTS® System, Inc., Stone Ridge, NY, USA). The dry matter was referred to as 'gelatin powder' and subjected to hydrolysis. Gelatin and its hydrolysates were used for film preparation.

### 2.4. Preparation of gelatin hydrolysate

#### 2.4.1. Preparation of gelatin hydrolysate with different degree of hydrolysis (DH)

Gelatin was dissolved in 10 mM phosphate buffer (pH 8) to obtain the protein concentration of 3% (w/v). The pH of the mixture was readjusted to 8.0 using 2 M NaOH. The mixtures were incubated at  $50^\circ\text{C}$  for 10 min. The hydrolysis reaction was started by the addition of Alcalase at the amount calculated from the plot between log enzyme concentration and DH to obtain DH of 0.40, 0.80 and 1.20% as described by Benjakul and Morrissey (1997). After hydrolysis using different levels of Alcalase for 5 min, the resulting hydrolysates were subjected to heat treatment ( $90^\circ\text{C}$ ) in a temperature controlled water bath (Mettmert, GmbH+Co. KG, D-91126, Schwabach, Germany) for 15 min to fully inactivate the enzyme. Protein pattern of obtained hydrolysates was determined using SDS-PAGE made of 4% stacking gel and 12% separating gel. DH of hydrolysate was also determined.

#### 2.4.2. Determination of DH

DH of gelatin hydrolysate was determined according to the method of Benjakul and Morrissey (1997). Hydrolysate samples with appropriate dilution (125  $\mu\text{L}$ ) were added with 2.0 mL of 0.2 M phosphate buffer (pH 8.2) and 1.0 mL of 0.01% TNBS solution. The solution was mixed thoroughly and placed in a temperature controlled water bath at  $50^\circ\text{C}$  for 30 min in the dark. The reaction was terminated by adding 2.0 mL of 0.1 M sodium sulfite. The mixtures were cooled at room temperature for 15 min. The absorbance was read at 420 nm and  $\alpha$ -amino group was expressed in terms of L-leucine. The DH was defined as follows (Benjakul & Morrissey, 1997):

$$\text{DH} = [(L_s - L_0)/(L_{\max} - L_0)] \times 100$$

where  $L_s$  is the amount of  $\alpha$ -amino groups of gelatin hydrolysate sample.  $L_0$  is the amount of  $\alpha$ -amino groups in the original gelatin solution.  $L_{\max}$  is the total  $\alpha$ -amino groups in the original gelatin solution obtained after acid hydrolysis (6 N HCl at  $100^\circ\text{C}$  for 24 h).

### 2.5. Effect of partial hydrolysis and glycerol content on the properties of gelatin films

#### 2.5.1. Preparation of film from gelatin hydrolysates with different % DHs having various glycerol contents

Gelatin hydrolysate with different DHs was used for the preparation of film forming solution (FFS). Glycerol at different contents (10, 15 and 20%; based on protein) was used as a plasticizer. FFS ( $4 \pm 0.01 \text{ g}$ ) was cast onto a rimmed silicone resin plate ( $5 \times 5 \text{ cm}^2$ ), air-blown for 12 h at room temperature and dried with an environmental chamber (Binder, KBF 115 # 00-19735, D-78532, Tuttingen, Germany) at the temperature of  $25 \pm 0.5^\circ\text{C}$  and  $50 \pm 5\%$  relative humidity (RH) for 48 h. Dried films were manually peeled off and subjected to analyses.

### 2.5.2. Analyses

Prior to mechanical properties testing, films were conditioned for 48 h at  $50 \pm 5\%$  relative humidity (RH) at  $25 \pm 0.5^\circ\text{C}$ . For ATR-FTIR and TGA studies, films were conditioned in a desiccator containing dried silica gel for 3 weeks at room temperature ( $28\text{--}30^\circ\text{C}$ ) to obtain the most dehydrated films.

**2.5.2.1. Film thickness.** The thickness of film was measured using a micrometer (Mitutoyo, Model ID-C112PM, Serial No. 00320, Mitutoyo Corp, Kawasaki-shi, Japan). Ten random locations around each film sample were used for thickness determination.

**2.5.2.2. Mechanical properties.** Tensile strength (TS) and elongation at break (EAB) were determined as described by Iwata, Ishizaki, Handa, and Tanaka (2000) using the Universal Testing Machine (Lloyd Instrument, Hampshire, UK). Ten samples ( $2 \times 5\text{ cm}^2$ ) with the initial grip length of 3 cm were used for testing. The samples were clamped and deformed under tensile loading using a 100 N load cell with the cross-head speed of  $30\text{ mm min}^{-1}$  until the samples were broken. The maximum load and the final extension at break were used for calculation of TS and EAB, respectively.

**2.5.2.3. Water vapor permeability (WVP).** WVP was measured using a modified ASTM (American Society for Testing and Materials, 1989) method as described by Shiku, Yuca Hamaguchi, Benjakul, Visessanguan, and Tanaka (2004). The film was sealed on an aluminum permeation cup containing silica gel (0% RH) with silicone vacuum grease. The cup was placed at  $30^\circ\text{C}$  in a desiccator containing the distilled water. It was then weighed at 1 h intervals over an 8 h period. Six films were used for WVP testing. WVP of the film was calculated as follows (McHugh, Avena-Bustillos, & Krochta, 1993):

$$\text{WVP} \left( \text{g m}^{-1} \text{s}^{-1} \text{Pa}^{-1} \right) = w l A^{-1} t^{-1} (P_2 - P_1)^{-1}$$

where  $w$  is the weight gain of the cup (g);  $l$  is the film thickness (m);  $A$  is the exposed area of film ( $\text{m}^2$ );  $t$  is the time of gain (s);  $(P_2 - P_1)$  is the vapor pressure difference across the film (Pa).

**2.5.2.4. Color and transparency of the film.** Color of film was determined using a CIE colorimeter (Hunter associates laboratory, Inc., Reston, VA, USA). Color of the film was expressed as  $L^*$ ,  $a^*$  and  $b^*$ -values and total differences in color was expressed as  $\Delta E^*$ .  $\Delta E^*$  values were calculated according to the following equation (Gennadios, Weller, Hanna, & Froning, 1996)

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where  $\Delta L^*$ ,  $\Delta a^*$  and  $\Delta b^*$  are the differences between the color parameter of the samples and the color parameter of white standard ( $L^* = 93.61$ ,  $a^* = -0.93$  and  $b^* = 0.53$ ).

The transparency value of the film was calculated by the following equation (Han & Floros, 1997):

$$\text{Transparency value} = -\log T_{600}/x$$

where  $T_{600}$  is the fractional transmittance at 600 nm and  $x$  is the film thickness (mm). The greater transparency value represents the lower transparency.

**2.5.2.5. Electrophoretic analysis.** Protein patterns of gelatin film were analyzed under reducing condition using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Prior to analysis, the film samples were prepared according to the method of Jongjareonrak, Benjakul,

Vesessanguan, Prodpran, et al. (2006) with some modifications. Film samples (200 mg) were dissolved in 10 mL of 1% (w/v) SDS. The mixture was stirred continuously at room temperature for 12 h. Supernatants were obtained after centrifuging at  $3000 \times g$  for 5 min using Hettich Zentrifuge (MIKRO-20, D-78532, Tuttlingen, Germany). For gelatin hydrolysates, they were mixed with 1% (w/v) SDS at a ratio of 1:1 (v/v). Protein content of the prepared solutions of both gelatin hydrolysates and their corresponding film was determined according to the Biuret method (Robinson & Hodgen, 1940). The solutions were then mixed with sample buffer (0.5 M Tris-HCl, pH 6.8 containing 4% (w/v) SDS, 20% (v/v) glycerol with 10% (v/v)  $\beta$ -ME) at the ratio of 1:1 (v/v). Samples (30  $\mu\text{g}$  protein) were loaded onto the polyacrylamide gel made of 12% running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, gel was stained with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. Wide range molecular weight protein markers were used to estimate the molecular weight of proteins.

**2.5.2.6. Attenuated total reflectance-Fourier transforms infrared (ATR-FTIR) spectroscopy.** FTIR spectra of films prepared from gelatin without hydrolysis (0% DH) and that with 1.20% DH in the presence of glycerol at different levels were determined using a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) equipped with a horizontal ATR Trough plate crystal cell ( $45^\circ$  ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) at room temperature as described by Nuthong, Benjakul and Prodpran (2009). Films were placed onto the crystal cell and the cell was clamped into the mount of FTIR spectrometer. The spectra in the range of  $700\text{--}4000\text{ cm}^{-1}$  with automatic signal gain were collected in 32 scans at a resolution of  $4\text{ cm}^{-1}$  and were ratioed against a background spectrum recorded from the clean empty cell at  $25^\circ\text{C}$ .

**2.5.2.7. Thermo-gravimetric analysis (TGA).** Conditioned films were scanned using a thermo-gravimetric analyzer (TGA7, PerkinElmer, Norwalk, CT, USA) from 50 to  $600^\circ\text{C}$  at a rate of  $10^\circ\text{C/min}$  (Nuthong et al., 2009). Nitrogen was used as the purge gas at a flow rate of 20 mL/min.

### 2.6. Statistical analysis

Experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by Duncan's multiple range test (Steel & Torrie, 1980). Analysis was performed using the SPSS package (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

## 3. Results and discussion

Films from gelatin of cuttlefish skin with different DHs containing glycerol at varying levels exhibited the different properties and molecular characteristics.

### 3.1. Thickness

Thickness of films prepared from cuttlefish skin gelatin with different DHs containing various glycerol contents ranged from 0.036 to 0.040 mm (Table 1). At the same DH, glycerol content had no impact on thickness of the resulting films ( $p > 0.05$ ). Vanin, Sobral, Menegalli, Carvalho, and Habitante (2005) also observed the negligible differences in thickness of gelatin-based films with

**Table 1**

Mechanical properties, water vapor permeability and thickness of films from gelatin of cuttlefish skin without hydrolysis and with different DH containing glycerol at different levels.

| %DH/glycerol <sup>a</sup> | TS<br>(MPa)          | EAB (%)            | WVP<br>( $\times 10^{-10} \text{ g s}^{-1} \text{ m}^{-1} \text{ Pa}^{-1}$ ) | Thickness (mm)        |
|---------------------------|----------------------|--------------------|------------------------------------------------------------------------------|-----------------------|
| C/10                      | 45.63 $\pm$ 1.10 aA  | 3.91 $\pm$ 0.64 bA | 0.66 $\pm$ 0.11 cB                                                           | 0.037 $\pm$ 0.002 bA  |
| C/15                      | 40.39 $\pm$ 3.45 bA  | 4.29 $\pm$ 0.59 bA | 0.88 $\pm$ 0.03 bC                                                           | 0.039 $\pm$ 0.002 abA |
| C/20                      | 36.57 $\pm$ 2.56 cA  | 5.39 $\pm$ 0.16 aA | 1.06 $\pm$ 0.07 aB                                                           | 0.040 $\pm$ 0.002 aA  |
| 0.40/10                   | 23.55 $\pm$ 2.38 aB  | 2.98 $\pm$ 0.23 bB | 0.89 $\pm$ 0.10 bA                                                           | 0.038 $\pm$ 0.002 aA  |
| 0.40/15                   | 20.80 $\pm$ 2.44 abB | 3.23 $\pm$ 0.13 bB | 1.01 $\pm$ 0.06 aAB                                                          | 0.038 $\pm$ 0.001 aAB |
| 0.40/20                   | 18.96 $\pm$ 1.84 bB  | 3.65 $\pm$ 0.33 aB | 1.08 $\pm$ 0.07 aB                                                           | 0.040 $\pm$ 0.002 aA  |
| 0.80/10                   | 17.39 $\pm$ 2.17 aC  | 2.50 $\pm$ 0.34 aB | 0.96 $\pm$ 0.08 bA                                                           | 0.037 $\pm$ 0.002 aA  |
| 0.80/15                   | 15.84 $\pm$ 1.71 aC  | 2.65 $\pm$ 0.26 aC | 1.05 $\pm$ 0.07 aB                                                           | 0.038 $\pm$ 0.002 aAB |
| 0.80/20                   | 11.64 $\pm$ 1.70 bC  | 2.89 $\pm$ 0.45 aC | 1.11 $\pm$ 0.04 aAB                                                          | 0.039 $\pm$ 0.002 aAB |
| 1.20/10                   | 15.32 $\pm$ 1.77 aC  | 2.61 $\pm$ 0.69 aB | 1.01 $\pm$ 0.07 bA                                                           | 0.037 $\pm$ 0.001 aA  |
| 1.20/15                   | 12.83 $\pm$ 1.91 bD  | 2.66 $\pm$ 0.31 aC | 1.12 $\pm$ 0.06 aA                                                           | 0.036 $\pm$ 0.003 aB  |
| 1.20/20                   | 12.99 $\pm$ 1.18 bC  | 2.45 $\pm$ 0.29 aD | 1.18 $\pm$ 0.08 aA                                                           | 0.037 $\pm$ 0.002 aB  |

Values are given as mean  $\pm$  SD ( $n = 3$ ).

Different capital letters within the same glycerol content in the same column indicate significant differences ( $p < 0.05$ ).

Different letters within the same DH in the same column indicate significant differences ( $p < 0.05$ ).

<sup>a</sup> % Based on protein content.

the different levels of glycerol. Nevertheless, the film prepared from gelatin with higher DH (1.20%) showed a slightly lower thickness than that of the control film (without hydrolysis) ( $p < 0.05$ ). Gelatin with the shorter chain might align themselves to form the ordered network with the less protrusion. This more likely resulted in the lower thickness of obtained film.

### 3.2. Mechanical properties

Mechanical properties of films prepared from gelatin with different DHs in the presence of glycerol at varying contents are presented in Table 1. At the same level of glycerol used, film prepared from the control gelatin (without hydrolysis) showed the highest TS and EAB, as compared with the film obtained from gelatin with partial hydrolysis ( $p < 0.05$ ). TS and EAB decreased as % DH increased when the same level of glycerol was used ( $p < 0.05$ ). These results suggested that gelatin molecules with the shorter chain most likely established the weaker chain-to-chain interaction

or less junction zones mainly via hydrogen bond (Fig. 1). Furthermore, the increasing number of chain ends of gelatin with partial hydrolysis directly enhanced the mobility of chains. As a result, the weaker film network was formed. Hoque et al. (2010) reported that gelatin film mainly stabilized by the weak bond including hydrogen bond and hydrophobic interaction. The result indicated that integrity and chain length of gelatin molecules directly contributed to the formation of film network. A scheme illustrating the impact of chain length of gelatin on the matrix of resulting film is presented in Fig. 1.

At the same level of glycerol used, EAB decreased when gelatin with increasing DH was used for film preparation. The lower chain entanglement more likely took place when the shorter chains existed (Fig. 1). Therefore, the easier molecular slippage was presumed when the tensile force was applied. As a consequence, both TS and EAB of film with the shorter chains decreased. Since weak bonds were involved in gelatin film (Hoque et al., 2010), the interaction of gelatin molecules in the way which provided the

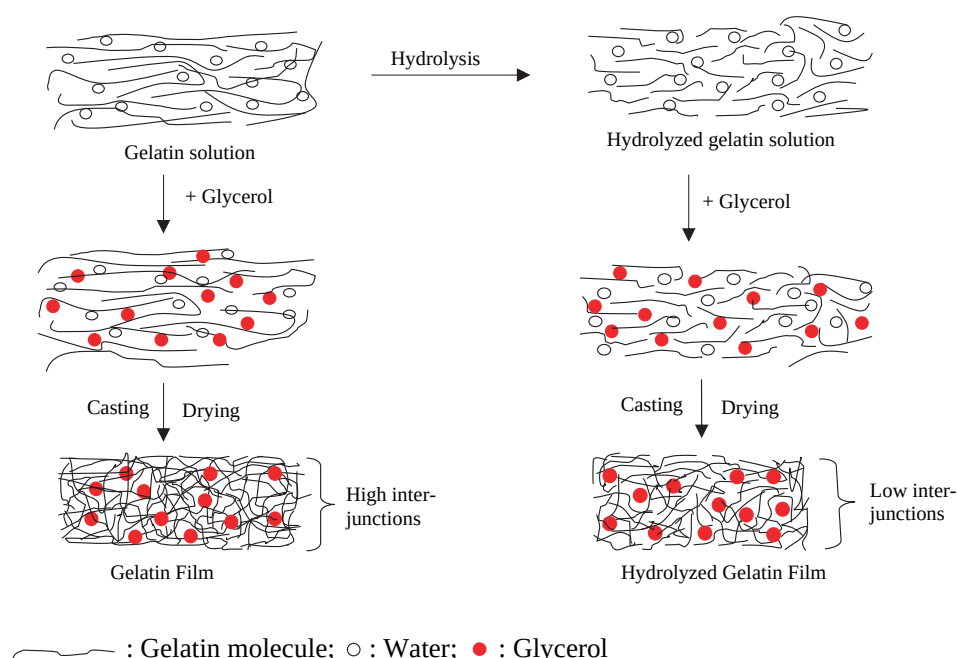


**Fig. 1.** A scheme illustrating the impact of chain length of gelatin on the matrix of resulting film.

longer chain could lower the rigidity of resulting films. Nevertheless, at the same level of glycerol, too short chains could not interact each other effectively to form the strong and elastic films. Jongjareonrak, Benjakul, Vesessanguan, Prodpran, et al. (2006) observed the lower TS of the films prepared from the bigeye snapper-skin gelatin than that of the brownstripe red snapper-skin gelatin films, where bigeye snapper-skin gelatin possessed the lower concentrations of high-molecular weight fractions with a concomitant increase in degradation peptides, compared with brownstripe red snapper-skin gelatin.

When glycerol at different levels was incorporated, film obtained from the control gelatin and those with different %DH showed the differences in both TS and EAB ( $p < 0.05$ ) (Table 1). Regardless of DH, TS decreased with increasing glycerol content ( $p < 0.05$ ). The increases in EAB were noticeable when glycerol contents increased ( $p < 0.05$ ). The increases in glycerol would lower the interaction between chains. However, EAB values obtained from film with 0.80 and 1.20% DH were not affected by the glycerol content used ( $p > 0.05$ ). The results suggested that the chain length was a crucial factor governing the TS and EAB of resulting film. Gontard, Guilbert, and Cuq (1993) reported that glycerol molecule could easily form hydrogen bond with protein chain, resulting in a reduction of intermolecular interaction. Also the mobility of macromolecules increased, leading to the decrease in TS and increase in EAB of films. Similar results was reported by Jongjareonrak, Benjakul, Vesessanguan, Prodpran, et al. (2006) who reported that decreased TS and increased EAB were obtained in gelatin film from bigeye snapper skin and brownstripe red snapper skin with increasing glycerol content (25–75%, based on protein). At higher DH (0.80 and 1.20% DH), shorter gelatin molecules with the higher mobility of chain ends might perform plasticizing effect by preventing protein–protein interaction but preferably formed H-bonds with water. Therefore, the mechanical properties of gelatin-based film were largely affected by the chain length of gelatin as well as glycerol content.

### 3.3. Water vapor permeability (WVP)

WVP of films prepared from gelatin without and with hydrolysis at different DH containing different glycerol levels is presented in Table 1. Film with the same glycerol level showed the increases in WVP as %DH increased ( $p < 0.05$ ). The highest WVP was found in film prepared from gelatin with 1.20% DH ( $p < 0.05$ ). These results suggested that the hydrolysis could expose more carboxylic group and amino group, which could form hydrogen bond with the water molecules. This resulted in the increased hydrophilicity of the

resulting films. Furthermore, the shorter gelatin molecules most likely yielded less dense and more permeable film matrix during film formation, leading to the higher WVP of the resulting film. Giménez, Gómez-Estaca, Alemán, Gómez-Guillén, and Montero (2009) observed that WVP of film significantly increased ( $p < 0.05$ ) when gelatin hydrolysates was incorporated in the films. Film from halibut skin gelatin with lower molecular weight also had the higher WVP, compared with that from the higher molecular weight (Carvalho et al., 2008).

When film with the same DH was tested, WVP increased with increasing glycerol content ( $p < 0.05$ ). However, no differences in WVP were observed between film with DH of 0.40–1.20% containing 15 and 20% glycerol content ( $p > 0.05$ ). These result suggested that hydroxyl group (–OH) of hygroscopic plasticizer, glycerol, which was able to interact with water, increased WVP of the film. Increased WVP was also found when the increasing glycerol content was used in fish skin gelatin film (Jongjareonrak, Benjakul, Vesessanguan, Prodpran, et al., 2006), pig skin gelatin film (Vanin et al., 2005) and fish muscle protein film (Paschoalick, Garcia, Sobral, & Habitante, 2003). Thus, degree of hydrolysis and glycerol level had the profound impact on WVP of film from cuttlefish skin gelatin.

### 3.4. Color of film

Table 2 shows the color of films prepared from cuttlefish skin gelatin with different DH and various glycerol levels. Higher  $L^*$ -value (lightness), lower  $b^*$ -value (yellowness) and  $\Delta E^*$  were obtained for film prepared from gelatin without hydrolysis, compared with those of films from gelatin with all DH ( $p < 0.05$ ). For  $a^*$ -value, it was noted that similar value was obtained for all films ( $p > 0.05$ ). However, films prepared from gelatin with 1.20% DH showed the lower  $a^*$ -value than film from gelatin without hydrolysis, when glycerol at 15 and 20% was used ( $p < 0.05$ ). Hydrolysis process rendered amino group (–NH<sub>2</sub>), which might undergo interaction with carbonyl group in gelatin via Maillard reaction, particularly during drying of film. Collagen and/or gelatin contains some amount of carbohydrates (glucose and galactose), which are attached to hydroxylysine residues of the peptide chain by O-glycosidic bonds. The presence of 2-O- $\alpha$ -D-glucosyl-O- $\beta$ -D-galactosyl-hydroxylysine and O- $\beta$ -D-galactosyl-hydroxylysine was reported in fish and invertebrate collagen (Burghagen, 1999). Petibois, Gouspillou, Wehbe, Delage, and Délérís (2006) also reported that the carbohydrate moieties are attached to the collagenous protein. Hoque et al. (2010) found the increased  $b^*$ -value due to heat treatment of FFS from the cuttlefish skin

**Table 2**  
Color of films from gelatin of cuttlefish skin without hydrolysis and with different DH containing glycerol at different levels.

| %DH/glycerol <sup>a</sup> | $L^*$                | $a^*$                | $b^*$              | $\Delta E^*$        |
|---------------------------|----------------------|----------------------|--------------------|---------------------|
| C/10                      | 91.13 $\pm$ 0.20 aA  | –1.29 $\pm$ 0.02 aA  | 2.55 $\pm$ 0.19 aB | 3.22 $\pm$ 0.23 aB  |
| C/15                      | 91.09 $\pm$ 0.13 aA  | –1.30 $\pm$ 0.04 aA  | 2.65 $\pm$ 0.11 aB | 3.32 $\pm$ 0.13 aB  |
| C/20                      | 91.10 $\pm$ 0.19 aA  | –1.28 $\pm$ 0.03 aA  | 2.62 $\pm$ 0.11 aB | 3.29 $\pm$ 0.14 aB  |
| 0.40/10                   | 90.89 $\pm$ 0.21 aB  | –1.33 $\pm$ 0.05 aA  | 2.85 $\pm$ 0.26 aA | 3.59 $\pm$ 0.13 aA  |
| 0.40/15                   | 90.78 $\pm$ 0.22 aBC | –1.34 $\pm$ 0.04 aAB | 2.95 $\pm$ 0.24 aA | 3.70 $\pm$ 0.33 aA  |
| 0.40/20                   | 90.83 $\pm$ 0.17 aB  | –1.30 $\pm$ 0.05 aAB | 2.82 $\pm$ 0.18 aA | 3.63 $\pm$ 0.27 aA  |
| 0.80/10                   | 90.88 $\pm$ 0.23 aB  | –1.32 $\pm$ 0.05 aA  | 2.92 $\pm$ 0.28 aA | 3.66 $\pm$ 0.26 aA  |
| 0.80/15                   | 90.75 $\pm$ 0.08 aB  | –1.33 $\pm$ 0.04 aAB | 2.86 $\pm$ 0.19 aA | 3.71 $\pm$ 0.13 aA  |
| 0.80/20                   | 90.69 $\pm$ 0.15 aB  | –1.31 $\pm$ 0.05 aAB | 2.80 $\pm$ 0.17 aA | 3.71 $\pm$ 0.20 aA  |
| 1.20/10                   | 90.84 $\pm$ 0.17 aB  | –1.32 $\pm$ 0.03 aA  | 2.81 $\pm$ 0.16 aA | 3.61 $\pm$ 0.17 bA  |
| 1.20/15                   | 90.56 $\pm$ 0.18 bC  | –1.37 $\pm$ 0.04 bB  | 2.92 $\pm$ 0.15 aA | 3.84 $\pm$ 0.10 aA  |
| 1.20/20                   | 90.66 $\pm$ 0.25 abB | –1.33 $\pm$ 0.05 abB | 2.87 $\pm$ 0.23 aA | 3.69 $\pm$ 0.22 abA |

Values are given as mean  $\pm$  SD ( $n = 3$ ).

Different capital letters within the same glycerol content in the same column indicate significant differences ( $p < 0.05$ ).

Different letters within the same DH in the same column indicate significant differences ( $p < 0.05$ ).

<sup>a</sup> % Based on protein content.

gelatin film. Therefore, hydrolysis of gelatin showed the impact on the color of resulting films. However, glycerol content did not show the marked effect on color. Gennadios et al. (1996) and Vanin et al. (2005) reported that neither plasticizer type (glycerol, polyethylene glycol, sorbitol) nor concentration had effect on the color difference of films from egg albumen and gelatin, respectively.

### 3.5. Light transmission and transparency

UV and visible light transmission at wavelength range of 200–800 nm of film from gelatin without hydrolysis and with different DH containing various glycerol contents are presented in Table 3. Light transmission in visible range (350–800 nm) of films was from 54.85 to 87.59%. The transmission of UV light at 280 nm was in the range of 5.83–9.67%. Very low transmission (0.01%) was found at 200 nm. Therefore, gelatin film effectively prevented the UV light, regardless of DH. Jongjareonrak, Benjakul, Vesessanguan, Prodpran, et al. (2006) also reported the higher UV light barrier capacity of gelatin film from bigeye snapper and brownstripe red snapper skin, compared with the synthetic film.

No differences in transparency value were observed between films from gelatin without hydrolysis and those with various DH, irrespective of glycerol content ( $p > 0.05$ ) (Table 3). Gelatin film has been considered as highly transparent film (Paschoalick et al., 2003). Highly transparent film was prepared from polyvinyl alcohol and gelatin blended film (Maria, de Carvalho, Sobral, Habitante, & Solorza-Feria, 2008). Both plasticizer type and concentration had no effect on transparency of film from pig skin gelatin (Vanin et al., 2005). Therefore, chain length of gelatin from cuttlefish skin had no impact on light transmission and transparency value of resulting films.

### 3.6. Electrophoretic protein patterns

The electrophoretic patterns of gelatin used for film preparation (without hydrolysis and with different %DH) and their corresponding films containing different glycerol contents are shown in Fig. 2A and B, respectively. Proteins with the molecular weight (MW) of  $\sim 105$  and  $\sim 97$  kDa were observed as two major bands in gelatin. After hydrolysis with Alcalase, those two major bands disappeared with coincidental occurrence of peptides with lower MW. For 0.40% DH sample, the small band with MW of  $\sim 90$  kDa and bands with MW lower than  $\sim 80$  kDa were found. For gelatin with 0.80 and 1.20% DH, band intensity of all proteins became lowered. Gelatin with 1.20% DH contained no proteins with MW

greater than  $\sim 80$  kDa. The results suggested that enzymatic hydrolysis caused the degradation of gelatin molecules, leading to the formation of shorter peptide chains. Those shorter chain molecules yielded the weaker film network with low TS and EAB (Fig. 1 and Table 1). Carvalho et al. (2008) reported the differences in molecular weight of gelatin from the skin of Atlantic halibut.

The similar protein patterns were observed between gelatin samples and their corresponding films (Fig. 2B). Additionally, two protein bands with MW of  $\sim 28$  and  $\sim 24$  kDa were observed in all films tested. No differences in protein pattern were observed between films containing different glycerol contents. The result suggested that film of all gelatin samples were most likely stabilized by weak bond, especially hydrogen bond. Limpisophon, Tanaka, Weng, Abe, and Osako (2009) found the no differences in the protein pattern of film with different glycerol and protein contents from under-utilized blue shark (*Prionace glauca*) skin gelatin. Hoque et al. (2010) also observed the similar protein pattern between FFS and film at different heating temperatures from cuttlefish skin gelatin. In the presence of SDS as well as  $\beta$ ME used for electrophoresis, hydrogen bond, hydrophobic interaction as well as disulfide bond in film network were destroyed. Gelatin contains negligible amount of cysteine. As a result, no disulfide bond was formed in the resulting film (Hoque et al., 2010). This was evidenced by the complete solubilization in water (data not shown). However, different protein components in gelatin with various MW directly influenced the properties of their resulting films, mainly stabilized by weak bonds.

### 3.7. FTIR spectroscopy

FTIR spectra of films prepared from gelatin without hydrolysis and gelatin with 1.20% DH in the presence of glycerol at different levels are shown in Fig. 3. The spectra of all films displayed the major bands at  $1631\text{ cm}^{-1}$  (amide-I, representing C=O stretching/hydrogen bonding coupled with COO),  $1538\text{ cm}^{-1}$  (amide-II, arising from bending vibration of N–H groups and stretching vibrations of C–N groups) and  $1235\text{ cm}^{-1}$  (amide-III, representing the vibrations in plane of C–N and N–H groups of bound amide or vibrations of  $\text{CH}_2$  groups of glycine) (Aewsiri et al., 2009; Muyonga, Cole, & Duodu, 2004). Yakimets et al. (2005) reported the similar result for bovine skin gelatin film where amide-I, amide-II and amide-III peaks were found at the wavenumbers of 1633, 1536 and  $1240\text{ cm}^{-1}$ , respectively. The peak situated around  $1033\text{ cm}^{-1}$  might be related to the interactions arising between plasticizer (OH group of glycerol) and film structure (Bergo & Sobral, 2007).

**Table 3**

Light transmittance (%) and transparency values of films from gelatin of cuttlefish skin without hydrolysis and with different DH containing glycerol at different levels.

| %DH/glycerol <sup>a</sup> | Wavelength (nm) |      |       |       |       |       |       |       | Transparency values |
|---------------------------|-----------------|------|-------|-------|-------|-------|-------|-------|---------------------|
|                           | 200             | 280  | 350   | 400   | 500   | 600   | 700   | 800   |                     |
| C/10                      | 0.01            | 8.08 | 62.44 | 74.83 | 82.06 | 84.84 | 86.50 | 87.59 | $1.40 \pm 0.03$ aA  |
| C/15                      | 0.01            | 7.03 | 59.47 | 72.91 | 80.47 | 83.33 | 85.10 | 86.27 | $1.38 \pm 0.04$ aA  |
| C/20                      | 0.01            | 8.55 | 65.69 | 77.73 | 83.96 | 86.04 | 87.30 | 88.11 | $1.37 \pm 0.02$ aA  |
| 0.40/10                   | 0.01            | 5.83 | 56.01 | 69.77 | 77.90 | 81.00 | 82.92 | 84.22 | $1.38 \pm 0.04$ aA  |
| 0.40/15                   | 0.01            | 6.97 | 58.46 | 71.41 | 79.22 | 82.24 | 84.13 | 85.42 | $1.36 \pm 0.07$ aA  |
| 0.40/20                   | 0.00            | 6.51 | 58.67 | 73.01 | 80.70 | 83.20 | 84.71 | 85.77 | $1.34 \pm 0.04$ aA  |
| 0.80/10                   | 0.01            | 7.01 | 54.85 | 67.93 | 76.69 | 80.51 | 82.95 | 84.64 | $1.38 \pm 0.03$ aA  |
| 0.80/15                   | 0.01            | 7.37 | 59.60 | 73.81 | 81.48 | 84.11 | 85.71 | 86.81 | $1.35 \pm 0.03$ aB  |
| 0.80/20                   | 0.01            | 8.26 | 63.49 | 75.96 | 82.44 | 84.59 | 85.92 | 86.83 | $1.33 \pm 0.04$ bA  |
| 1.20/10                   | 0.01            | 8.79 | 60.27 | 72.11 | 79.46 | 82.42 | 84.27 | 85.53 | $1.35 \pm 0.05$ aA  |
| 1.20/15                   | 0.01            | 9.67 | 61.34 | 74.63 | 81.85 | 84.27 | 85.74 | 86.76 | $1.33 \pm 0.05$ aA  |
| 1.20/20                   | 0.01            | 8.98 | 64.52 | 76.81 | 83.24 | 85.26 | 86.50 | 87.33 | $1.33 \pm 0.04$ aA  |

Values are given as mean  $\pm$  SD ( $n = 3$ ).

Different capital letters within the same glycerol content in the same column indicate significant differences ( $p < 0.05$ ).

Different letters within the same DH in the same column indicate significant differences ( $p < 0.05$ ).

<sup>a</sup> % Based on protein content.

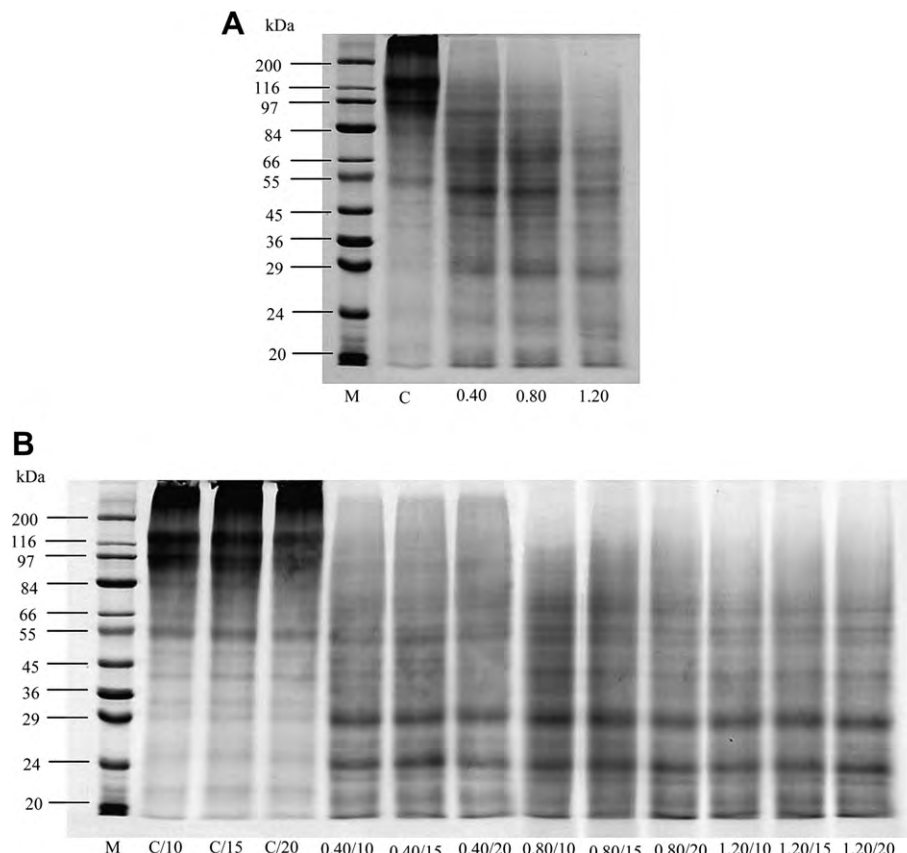


**Fig. 2.** Protein patterns of gelatin without hydrolysis and with different DH (A) and their corresponding films containing glycerol at different levels (B). M: protein marker; C: control (without hydrolysis); 0.40, 0.80 and 1.20 denote %DH; and 10, 15 and 20 represent glycerol level (%).

Generally, similar spectra of all samples were obtained in the range of  $1800\text{--}700\text{ cm}^{-1}$ , covering amide-I, II and III.

Furthermore, amide-A peak was found at  $3275\text{ cm}^{-1}$ , representing NH-stretching coupled with hydrogen bonding. Amide-B peak at  $2929\text{ cm}^{-1}$ , representing CH stretching and  $\text{--NH}_2$ , was also observed in the spectra. The amide-A and amide-B peaks at wavenumbers of  $3458$  and  $2926\text{ cm}^{-1}$ , respectively were formed in adult Nile perch collagen (Muyonga et al., 2004). At the same glycerol level, spectra of film prepared from gelatin with 1.20% DH showed the higher amplitudes for amide-A peak, compared with that of film prepared from gelatin without hydrolysis. Higher amount of  $\text{--NH}_2$  or  $\text{--NH}_3^+$  group obtained from hydrolysis process more likely resulted in the higher amplitude of amide-A and amide-B peaks obtained. For the film prepared from gelatin with the same DH containing different glycerol contents, it was noted that the higher amplitudes of amide-A and amide-B peaks were obtained as glycerol content increased. The result indicated that in the presence of glycerol at higher content, the interaction between gelatin molecules became less. As a result, the cross-linking of molecules, especially via H-bonds between amino group of either gelatin or gelatin with partial hydrolysis (1.20% DH) and H-acceptors was lowered. This was evidenced by the higher availability of amino group shown by the higher amplitudes of both amide peaks. The film in this present study contained different glycerol level as the plasticizer. As a consequence, some water might be within the film matrix, though it was equilibrated over the dry silica gel for 3 weeks. Water content affected FTIR spectra in the gelatin film without glycerol addition (Yakimets et al., 2005). This water might have the influence on FTIR spectra to some degree. Additionally, the physical ageing occurred after 3 weeks might suffer the film to some extent.

### 3.8. Thermo-gravimetric analysis (TGA)

TGA thermograms revealing thermal degradation behavior of films prepared from gelatin without hydrolysis and gelatin with 1.20% DH in the presence of glycerol at different levels are depicted in Fig. 4A and B. Their corresponding degradation temperatures ( $T_d$ ) and weight loss ( $\Delta w$ ) are shown in Table 4. All films from gelatin

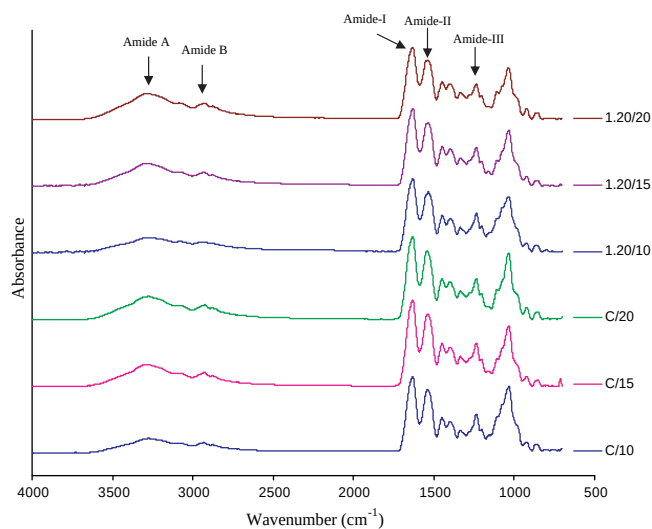


**Fig. 3.** FTIR spectra of films prepared from gelatin of cuttlefish skin without hydrolysis and 1.20% DH containing glycerol at different levels. C: control; 1.20 denotes %DH; and 10, 15 and 20 represent glycerol level (%).

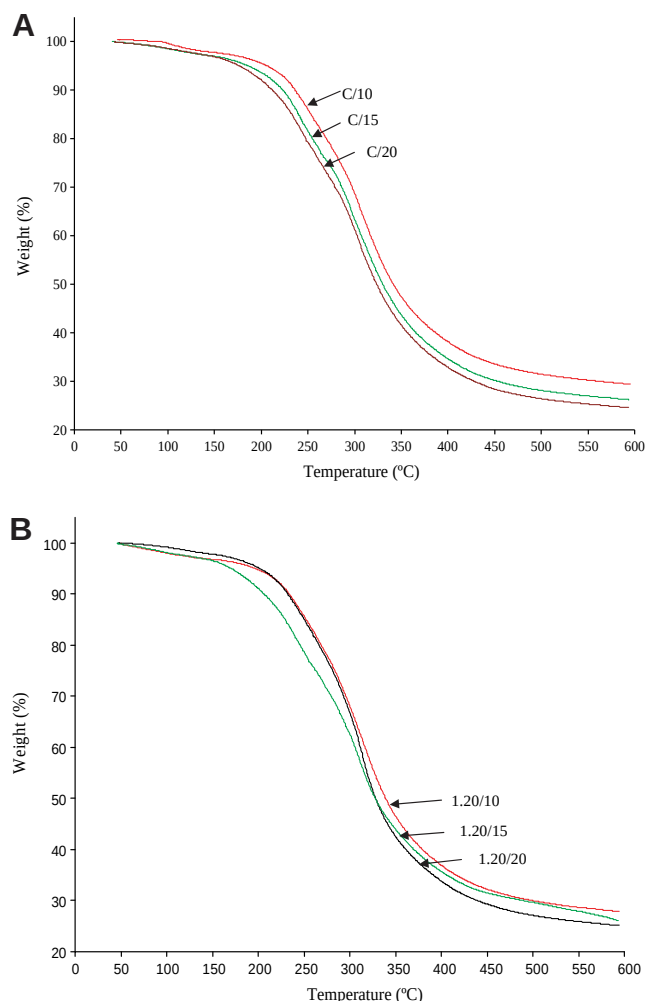


**Fig. 4.** Thermo-gravimetric curves of films prepared from gelatin of cuttlefish skin without hydrolysis (A) and 1.20% DH (B) containing glycerol at different levels. C: control; 1.20 denotes %DH; and 10, 15 and 20 represent glycerol level (%).

without hydrolysis and gelatin with 1.20% DH exhibited three main stages of weight loss. For all films, the first stage weight loss ( $\Delta w_1 = 2.10\text{--}3.70\%$ ) was observed approximately at temperature ( $T_{d1}$ ) of  $50.03\text{--}70.00^\circ\text{C}$ , possibly related to the loss of free water adsorbed in the film. The similar result was reported in collagen hydrolysate film plasticized with glycerol (GLY) and poly(ethylene glycols) at different molecular masses (Langmaier, Mokrejs, Kolomaznik, & Mladek, 2008) and porcine plasma protein film added with different cross-linking agents (Nuthong et al., 2009).

**Table 4**

Thermal degradation temperature ( $T_d$ ,  $^\circ\text{C}$ ) and weight loss ( $\Delta w$ , %) of films from cuttlefish skin gelatin without hydrolysis and 1.20% DH containing glycerol at different levels.

| %DH/glycerol <sup>a</sup> | $\Delta_1$             |              | $\Delta_2$             |              | $\Delta_3$             |              | Residue (%) |
|---------------------------|------------------------|--------------|------------------------|--------------|------------------------|--------------|-------------|
|                           | $T_{d1, \text{onset}}$ | $\Delta w_1$ | $T_{d2, \text{onset}}$ | $\Delta w_2$ | $T_{d3, \text{onset}}$ | $\Delta w_3$ |             |
| C/10                      | 70.00                  | 2.10         | 216.71                 | 14.94        | 290.18                 | 53.36        | 29.60       |
| C/15                      | 60.90                  | 2.54         | 209.68                 | 17.60        | 286.50                 | 55.25        | 24.61       |
| C/20                      | 59.51                  | 2.82         | 202.68                 | 18.81        | 271.40                 | 55.59        | 22.78       |
| 1.2/10                    | 49.22                  | 3.38         | 213.89                 | 16.46        | 255.80                 | 54.71        | 25.45       |
| 1.2/15                    | 50.03                  | 3.57         | 209.55                 | 19.08        | 253.32                 | 53.11        | 24.24       |
| 1.2/20                    | 50.18                  | 3.70         | 196.38                 | 21.70        | 249.47                 | 51.58        | 23.02       |

$\Delta_1$ ,  $\Delta_2$ , and  $\Delta_3$  denote the first, second and third stage weight loss, respectively, of film during heating scan.

<sup>a</sup> % Based on protein content.

The second stage weight loss ( $\Delta w_2 = 14.94\text{--}21.70\%$ ) appeared at  $T_{d2}$  ( $196.30\text{--}216.71^\circ\text{C}$ ) of all films, was most likely associated with the loss of glycerol compounds and structurally bound water. For the third stage weight loss,  $T_{d3}$  of  $271.40\text{--}290.18^\circ\text{C}$  were observed in films prepared from gelatin without hydrolysis, whereas  $T_{d3}$  of  $249.47\text{--}255.80^\circ\text{C}$  were found in those from gelatin with 1.20% DH. The lower  $T_d$  of the latter was more likely due to the shorter chains, which could undergo thermal degradation to a high extent, compared with the former.

The result revealed that at the same glycerol content, film prepared from gelatin with 1.20% DH showed the higher heat susceptibility, compared with film from gelatin without hydrolysis (control film). At any glycerol level, the control films showed the higher thermal degradation temperatures for  $T_{d1}$ ,  $T_{d2}$  and  $T_{d3}$  and the lower weight loss for  $\Delta w_1$  and  $\Delta w_2$ , compared with film from gelatin with 1.20% DH (Table 4). The result suggested that weak film network obtained from the gelatin with the shorter gelatin molecules, underwent easier thermal degradation and higher weight loss, compared with film from without hydrolysates gelatin. At the same DH, the lower  $T_d$  was obtained in the film containing the higher glycerol content. This confirmed the interfering effect of glycerol on gelatin interaction in the film network. Increased plasticizer contents were responsible for preventing the protein–protein interaction, resulting in the higher heat sensitivity of the films, in which the looser matrix of gelatin molecules was found. In general, higher residue from thermal degradation was obtained in film from gelatin without hydrolysis, compared with those from gelatin with 1.20% DH. The higher residue was also found in film with lower glycerol content. Thus, both chain length of gelatin molecules and glycerol content directly showed the pronounced impact on thermal stability of gelatin.

#### 4. Conclusion

Shorter gelatin molecules generated by hydrolysis yielded the film with the lower junction zone or shorter strands via weak bonds during film formation. This led to the lower mechanical properties and thermal stability of their resulting films. Increased amount of  $-\text{NH}_2$  and  $-\text{COOH}$  groups from hydrolysis process and  $-\text{OH}$  group of glycerol formed H-bond with water molecules resulted in the increased WVP. Therefore, the chain length of gelatin molecules and plasticizer concentration were crucial factors governing the properties of gelatin-based films from cuttlefish skin.

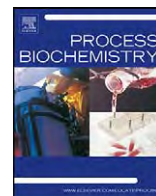
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# Impact of legume seed extracts on degradation and functional properties of gelatin from unicorn leatherjacket skin

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## ABSTRACT

Trypsin inhibitor was extracted from the seed flour of soybean (SB; *Glycine max*), mung bean (MB; *Vigna radiata*), cowpea bean (CP; *Vigna unguiculata*) and adzuki bean (AB; *Vigna angularis*) using 0.15 M NaCl, followed by heat precipitation at 70 °C. The extract from SB showed the highest specific trypsin inhibitory activity, followed by those from MB, CP and AB, respectively. Based on inhibitory activity staining, molecular weights (MWs) of trypsin inhibitor from SB, MB, CP and AB were 20.1, 14, 10 and 13 kDa, respectively. The SB extract powder (SBEP) containing trypsin inhibitor in the range of 10–100 TIU/g effectively prevented the degradation of  $\gamma$ -,  $\beta$ - and  $\alpha$ -chains of collagenolytic proteins of leatherjacket skin subjected to incubation at 50 °C for 30 min. The impact of SBEP on the extraction yield, physical and functional properties of gelatin from leatherjacket skin was investigated. The gelatin extracted in the presence of SBEP contained  $\alpha_1$  and  $\alpha_2$  chains as the predominant components with some degradation peptides. FTIR spectra indicated the significant loss of molecular order of triple helix and higher degradation was found in gelatin extracted in the absence of SBEP. Gelatin extracted in the presence of SBEP had the higher gel strength (232.8–268.5 g) than that extracted in the absence of SBEP (90.4 g). Higher foam stability (FS) but lower emulsion stability index (ESI) was observed in the former. Therefore, the addition of SBEP effectively prevented the degradation of gelatin from the skin of unicorn leatherjacket, thereby yielding the gelatin with improved gel strength and foam stability.

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## 1. Introduction

Indigenous proteases associated with skin matrix degrade, destabilize and disintegrate the major components of native structure of collagen by disrupting the intra- and intermolecular cross-links of high molecular weight components like  $\gamma$ -,  $\beta$ -, and  $\alpha$ -chains during gelatin extraction at higher temperature, resulting in the substantial decrease in gel strength, foam ability, foam stability and viscosity of gelatins [1]. Gelatins which retained high molecular weight components including  $\gamma$ -,  $\beta$ -, and  $\alpha$ -chains have been known to possess the maximal functional properties, which can be applicable for food, medicine and pharmaceutical industries [2]. Collagenases are proteases capable of cleaving the peptide bonds in the native triple helical collagen molecules under physiological conditions [3]. These collagenases are classified into two major groups, metallo- and serine-collagenases. Collagenases are endopeptidases that have the unique ability to hydrolyse the major structural extracellular matrix proteins, especially the triple helical strand of type I and type II collagens at Gly<sup>775</sup>-Leu (Ile)<sup>776</sup>, giving rise to  $\frac{3}{4}$ - and

$\frac{1}{4}$ -cleavage peptide products [4]. Furthermore, non-collagenase proteases can cleave the collagen molecule in the telopeptide region and contribute to hydrolysis of the collagen molecule by disrupting the regions, in which intermolecular cross-links are formed [5]. Heat-activated serine proteases in unicorn leatherjacket skin (*Aluterus monoceros*) were involved in the drastic degradation of the  $\gamma$ -,  $\beta$ - and  $\alpha$ -chains of the gelatin extracted at 50 °C [1].

To produce the high-quality gelatin with negligible hydrolysis of peptides, the use of an appropriate protease inhibitor from natural sources could be a new approach and an effective means to suppress the indigenous protease-induced degradation of gelatin molecules due to its safety and lower price, in comparison with commercial protease inhibitors. The major protease inhibitors from seeds belonging to Gramineae, Leguminosae and Solanaceae families are the Kunitz and Bowman-Birk inhibitors [6]. Kunitz inhibitors are usually 8–22 kDa proteins, with two disulphide linkages and a single reactive site of trypsin, whereas Bowman-Birk inhibitors are usually 8–10 kDa proteins, with seven disulphide linkages and two reactive sites of trypsin and chymotrypsin [7]. The extracts from legume seeds were reported to prevent the autolysis of surimi, thereby improving the surimi gel properties [8].

Unicorn leatherjacket (*A. monoceros*) is one of the marine tropical fish, which is harvested in large quantities throughout the world. This species has been used for fillet production in Thailand, in

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which a large quantity of skin has been produced as by-product. Due to its thick skin, it can be a potential source of value-added products like collagen and gelatin. As a consequence, the increased revenue for processors can be achieved. Furthermore, the comprehensive utilization of leatherjacket skin is a promising means to bring about the biomaterial with environmental protective aspect. Nevertheless, there is no information regarding the suppression of indigenous protease-induced degradation in the skin of unicorn leatherjacket by the extracts of legume seed. Therefore, the objective of this investigation was to suppress the unwanted proteolysis of collagenolytic proteins in the skin of unicorn leatherjacket by legume seed extract and to elucidate the impact of extract on the functional properties of gelatin extracted by different processes.

## 2. Materials and methods

### 2.1. Materials

Soybean (*Glycine max*), mung bean (*Vigna radiata*), cowpea bean (*Vigna unguiculata*) and adzuki bean (*Vigna angularis*) were purchased from the local market, Hat Yai, Thailand. *N*- $\alpha$ -Benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), trypsin from bovine pancreas, casein from bovine milk, type I collagen from calf skin and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phosphoric acid was obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS), *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) and Coomassie blue R-250 were procured from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals were of analytical grade.

### 2.2. Fractionation of trypsin inhibitor from legume seeds

#### 2.2.1. Preparation of crude extracts

Legume seeds were finely ground using a blender (Model MX-T2GN, National, Taipei, Taiwan). Seed flour was defatted by mixing with hexane at a ratio of 1:5 (w/v). The mixture was shaken for 10 min and filtered through Whatman No. 1 filter paper (Whatman International, Ltd., Maidstone, England). The retentate was rinsed twice with hexane to remove the residual oil in the ground sample. Defatted sample was then air-dried at room temperature (28–30 °C) until dry and free of hexane odour. The dried defatted samples were extracted with 0.15 M NaCl at room temperature (26–28 °C) at the sample/medium ratio of 1:5 (w/v), with continuous stirring for 3 h, using IKA® Model Colour Squid [white] magnetic stirrer (BEC THAI, BKK, Thailand) [9]. The mixture was then subjected to centrifugation at 8000  $\times$  g at 25 °C for 30 min using a Beckman Model Avanti J-E centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA). The supernatant was referred to as 'crude extract'.

#### 2.2.2. Heat treatment of crude extract

Crude extracts were subjected to heat treatment at 70 °C for 15 min, followed by cooling with iced water. To remove coagulated debris, the extracts were centrifuged at 8000  $\times$  g for 5 min at room temperature. The supernatants were then dialysed extensively at 4 °C for 24 h against 20 volumes of distilled water with a change of solution every 6 h. The dialysates obtained were freeze-dried using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lyngø, Denmark). The powder obtained was referred to as 'seed extract powder'.

### 2.3. Characterisation of trypsin inhibitor in seed extracts

#### 2.3.1. Trypsin inhibitory activity assay

Trypsin inhibitory activity of all seed extract powders was measured by the method of Welham and Domoney [10] with a slight modification using BAPNA as substrate. A solution containing 200  $\mu$ l of inhibitor solution (2 mg/ml), 200  $\mu$ l of bovine pancreas trypsin (1 mg/ml) and 1000  $\mu$ l of 50 mM Tris-HCl, pH 7 containing 10 mM CaCl<sub>2</sub> was pre-incubated at 37 °C for 15 min. To initiate the reaction, 200  $\mu$ l of BAPNA (0.4 mg/ml in DMSO) (pre-warmed to 37 °C) were added and vortexed immediately to start the reaction. After incubating for 10 min, 200  $\mu$ l of 30% acetic acid (v/v) was added to terminate the reaction. The reaction mixture was centrifuged at 8000  $\times$  g for 5 min (Eppendorf Micro Centrifuge, MIK-RO20, Hettich Zentrifugan, Germany). Residual activity of trypsin was determined by measuring the absorbance at 410 nm due to *p*-nitroaniline released. One unit of proteolytic activity was defined as an increase of 0.01 absorbance unit ml<sup>-1</sup> min<sup>-1</sup> under the assay condition. One unit of trypsin inhibitory activity (TIU) was defined as the amount of inhibitor, which reduced trypsin activity by one unit.

#### 2.3.2. Protein determination

Protein concentration was determined by the Biuret method using BSA as a standard [11].

#### 2.3.3. Protein pattern and inhibitory activity staining

**2.3.3.1. Protein pattern.** SDS-PAGE was performed under non-reducing conditions, using 12% separating and 4% stacking gels according to the method of Laemmli [12].

The samples were mixed with the sample buffer (0.5 M Tris-HCl, pH 6.8, 20% (v/v) glycerol, 10% (w/v) SDS and 0.1% (w/v) bromophenol blue) at a ratio of 1:1 without heating. Samples (20  $\mu$ g protein) were loaded onto the gel and then subjected to electrophoresis at 15 mA/gel using a Mini Protean Tetra Cell units (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, one gel was stained with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with the mixture of 30% (v/v) methanol and 10% (v/v) acetic acid.

**2.3.3.2. Inhibitory activity staining.** After electrophoresis, another identical gel was washed in 2.5% (v/v) Triton X-100 for 15 min to remove SDS and renature the proteins. The gel was washed extensively with cold distilled water before soaking in 30 ml of a trypsin solution (1 mg/ml in 50 mM Tris-HCl, pH 7 containing 10 mM CaCl<sub>2</sub>) for 30 min at 4 °C to allow the trypsin to diffuse into the gel. The gel-trypsin solution was then incubated at 37 °C for 40 min and then rinsed with cold distilled water, fixed and stained with 0.05% (w/v) Coomassie brilliant blue R-250 to develop inhibitory zones. The apparent molecular weight (MW) of the trypsin inhibitor was estimated by comparing its R<sub>f</sub> with those of protein standards.

### 2.4. Effect of soybean extract powder (SBEP) towards autolysis of pretreated skin

#### 2.4.1. Collection and preparation of fish skin

The skin of unicorn leatherjacket (*A. monoceros*) was obtained from Sea Wealth Frozen Food Co., Ltd., Songkhla, Thailand. Upon arrival to the Department of Food Technology, Prince of Songkla University, Hat Yai, the skin was cleaned and washed with iced tap water (0–2 °C). Prepared skin was then cut into small pieces (0.5 cm  $\times$  0.5 cm), placed in polyethylene bags and stored at –20 °C until use. The storage time was less than 2 months.

#### 2.4.2. Pretreatment of unicorn leatherjacket skin

Prepared skin was pretreated following the method of Ahmad and Benjakul [13] with a slight modification. To remove non-collagenous proteins, the prepared skin was mixed with 0.1 M NaOH at a skin/alkali solution ratio of 1:20 (w/v). The mixture was stirred for 6 h at 4 °C using an overhead mechanical stirrer (W20.n, IKA-Werke GmbH & CO. KG, Stanfen, Germany) at a speed of 300 rpm. The alkali solution was changed every 2 h. The samples were then washed with iced tap water until neutral or faintly basic pH was obtained. Thereafter, the prepared skin was swollen by mixing the skins with 0.2 M phosphoric acid at a ratio of 1:10 (w/v). The mixture was stirred for 24 h at 4 °C. Finally, the swollen skin was washed thoroughly with iced tap water until neutral or faintly acidic pH of wash water was obtained. The pretreated skin was stored at –20 °C until use, but not longer than 2 months. Prior to study, the frozen pretreated skin was powdered in liquid nitrogen using a blender. The powder obtained was used for autolysis study.

#### 2.4.3. Autolysis inhibition study

Powdered pretreated skin (1 g) was homogenised with 3 ml of McIlvaine's buffer (0.2 M Na-phosphate and 0.1 M Na-citrate, pH 7) at a speed of 11,000 rpm at room temperature for 2 min using a homogeniser (Model T25 basic, IKA, Labortechnik, Selangor, Malaysia). The homogenate was mixed with SBEP at various levels (5–100 TIU/g pretreated skin). The mixtures were allowed to stand at 4 °C for 2 h to ensure the interaction between inhibitor and target serine proteases. To initiate the autolysis, the mixtures were incubated at 50 °C for 30 min in a temperature controlled water bath (Mettmert, Schwabach, Germany). The reaction was then terminated by addition of 5 ml of 10% (w/v) SDS (85 °C). The reaction mixture was further incubated at 85 °C in a water bath for 1 h, followed by centrifugation at 8000  $\times$  g for 10 min. After complete solubilisation, the autolytic pattern was determined by SDS-PAGE using 7.5% separating gel and 4% stacking gel. The control was performed in the same manner, except de-ionised water was added instead of SBEP.

### 2.5. Effects of SBEP on extraction and properties of gelatin from unicorn leatherjacket skin

To inhibit the indigenous serine proteases associated with pretreated skin, the mixtures of pretreated skin and distilled water at the ratio of 1:10 (w/v) were subjected to extraction using different processes as follows (Fig. 1):

- (1) *Process I.* Addition of SBEP at a level of 100 TIU/g pretreated skin, followed by pre-incubation at 4 °C for 12 h and extraction using distilled water at 50 °C for 12 h.
- (2) *Process II.* Same manner with process No. 1 except that SBEP was also added in distilled water at the level of 100 TIU/g used as the extracting medium.
- (3) *Process III.* Extraction using distilled water at 50 °C for 12 h in the presence of 100 TIU/g pretreated skin without pre-incubation with SBEP.

After extraction at 50 °C for 12 h, the mixtures from three processes were then filtered using two layers of cheesecloth. The filtrates were further filtered using a Whatman No. 4 filter paper with the aid of an electrical aspirator (Model VE-11, JEIO TECH, Seoul, Korea). The resultant filtrates were freeze-dried. Gelatins obtained from processes I, II and III were referred to as G1, G2 and G3, respectively. Gelatin extracted at 50 °C for 12 h without the addition of SBEP was used as the control and

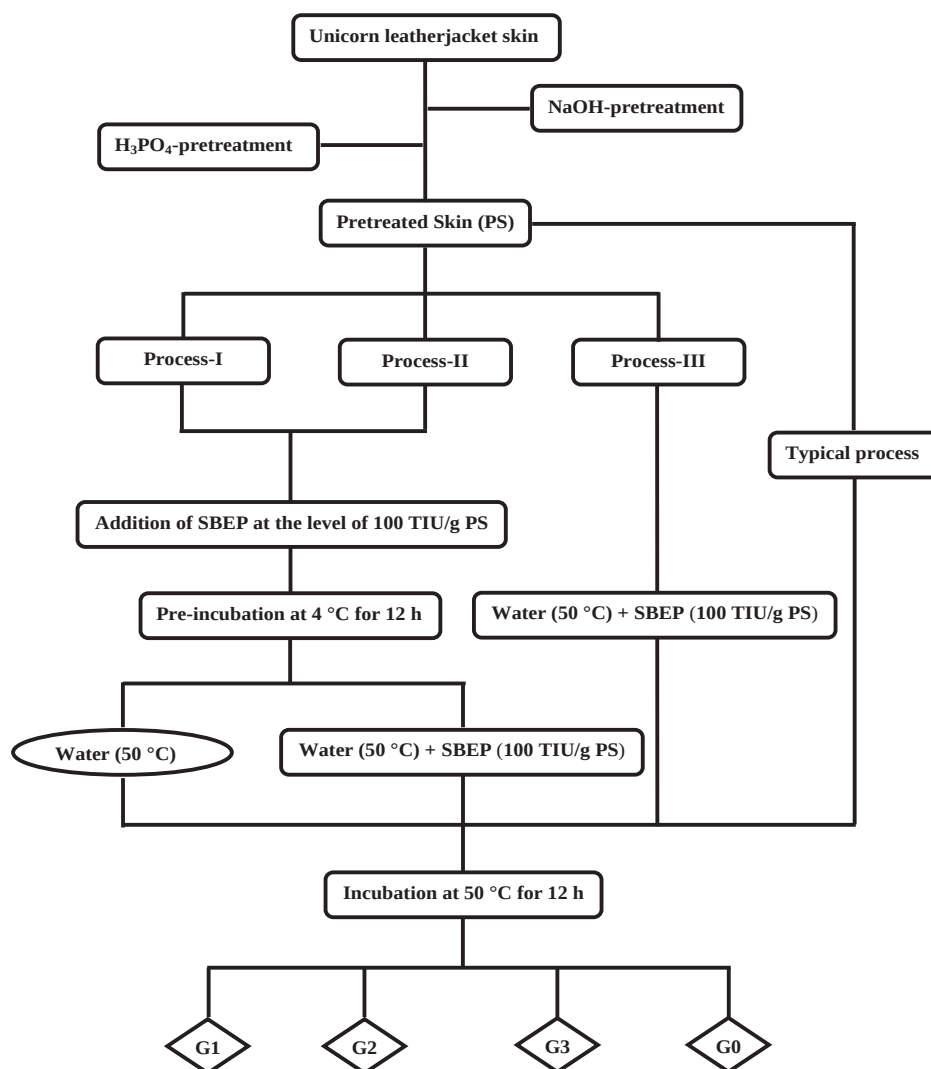


Fig. 1. Flow diagram of different gelatin extraction processes from the skin of unicorn leatherjacket using SBEP as the aid at 50 °C for 12 h.

referred to as 'G0'. All gelatin samples were subjected to SDS-PAGE as previously described and further analyses were also conducted as follows:

#### 2.5.1. Fourier transform infrared (FTIR) spectroscopy

Gelatin samples were subjected to FTIR analysis using Bruker Model EQUINOX 55 FTIR spectrometer (Bruker, Ettlingen, Germany) equipped with a deuterated L-alanine triglycine sulphate (DLATGS) detector was used. The horizontal attenuated total reflectance (HATR) accessory was mounted into the sample compartment. The internal reflection crystal (Pike Technologies, Madison, WI, USA), made of zinc selenide, had a 45° angle of incidence to the IR beam. Spectra were acquired at a resolution of 4 cm<sup>-1</sup> and the measurement range was 4000–500 cm<sup>-1</sup> (mid-IR region) at room temperature. Automatic signals were collected in 32 scans at a resolution of 4 cm<sup>-1</sup> and were ratioed against a background spectrum recorded from the clean and empty cell at 25 °C. Analysis of spectral data was carried out using the OPUS 3.0 data collection software programme (Bruker, Ettlingen, Germany). Prior to data analysis, the spectra were baseline corrected and normalised.

#### 2.5.2. Determination of colour

The colour of gelatin powder was measured by a Hunter lab colour meter (Colour Flex, Hunter Lab Inc., Reston, VA, USA) reported by the CIE system. *L*\*, *a*\* and *b*\* parameters indicate lightness/brightness, redness/greenness and yellowness/blueness, respectively. The colourimeter was warmed up for 10 min and calibrated with a white standard. Total difference in colour ( $\Delta E^*$ ) was calculated according to the following equation [14]:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where  $\Delta L^*$ ,  $\Delta a^*$  and  $\Delta b^*$  are the differences between the corresponding colour parameter of the sample and that of white standard ( $L^* = 93.63$ ,  $a^* = -0.95$  and  $b^* = 0.46$ ).

#### 2.5.3. Determination of gel strength

Gels of gelatin samples were prepared following the method of Fernandez-Daz et al. [15] with a slight modification. Gelatin samples were dissolved in distilled water at 60 °C to obtain the final concentration of 6.67% (w/v). The solution was stirred until the gelatin was solubilised completely, followed by incubating the samples in a refrigerator at 10 °C for 18 h for gel maturation. The dimensions of the sample were 3 cm in diameter and 2.5 cm in height. Gel strength of the gel samples prepared at 10 °C was determined using a Model TA-XT2 Texture Analyser (Stable Micro System, Surrey, UK) with a load cell of 5 kN and equipped with a 1.27 cm diameter flat-faced cylindrical Teflon plunger. The maximum force (in grams) was recorded when the penetration distance reached 4 mm. The speed of the plunger was 0.5 mm/s.

#### 2.5.4. Determination of emulsifying properties

Emulsion activity index (EAI) and emulsion stability index (ESI) of gelatin samples were determined according to the method of Aewsiri et al. [16]. Soybean oil (2 ml) and gelatin solution (1–3%, w/v, 6 ml) were homogenised using a homogeniser at a speed of 20,000 rpm for 1 min. Emulsions were pipetted out at 0 and 10 min and 100-fold diluted with 0.1% SDS. The mixture was mixed thoroughly for 10 s using a vortex mixer (Scientific Industries, Inc., Bohemia, NY, USA). *A*<sub>500</sub> of the resulting dispersion was measured using a double beam spectrophotometer (Model UV-1800, Shimadzu, Kyoto, Japan). EAI was calculated by the following equation:

$$EAI(m^2/g) = \frac{2 \times 2.303 \times A \times DF}{l\phi C}$$

where *A* = *A*<sub>500</sub>, *DF* = dilution factor (100), *l* = path length of cuvette (m),  $\phi$  = oil volume fraction and *C* = gelatin concentration in aqueous phase (g/m<sup>3</sup>).

$$ESI(min) = \frac{A_0}{A_0 - A_{10}} \times \Delta t$$

**Table 1**  
Trypsin inhibitory activity of different legume seed extract powders.

| Legume seeds                        | Trypsin inhibitor (units/g seed powder) | Total protein (mg/g seed powder) | Specific inhibitory activity (units/mg protein) |
|-------------------------------------|-----------------------------------------|----------------------------------|-------------------------------------------------|
| Soybean ( <i>G. max</i> )           | 70,231 ± 33a                            | 673.4 ± 3.2c                     | 104.3 ± 3.0a                                    |
| Mung bean ( <i>V. radiata</i> )     | 39,871 ± 21b                            | 700.2 ± 2.1a                     | 56.9 ± 2.3b                                     |
| Cowpea ( <i>V. unguiculata</i> )    | 11,631 ± 22c                            | 680.3 ± 2.9b                     | 17.1 ± 0.8c                                     |
| Adzuki bean ( <i>V. angularis</i> ) | 8,787 ± 56d                             | 660.7 ± 2.4d                     | 13.3 ± 0.9d                                     |

Mean ± SD (n = 3).

Different letters (a, b, c, d) within the same column indicate significant differences ( $P < 0.05$ ).

where  $A_0 = A_{500}$  at time of 0 min,  $A_{10} = A_{500}$  at time of 10 min and  $\Delta t = 10$  min.

#### 2.5.5. Determination of foaming properties

Foam expansion (FE) and foam stability (FS) of gelatin sample solutions were determined, as described by Shahidi et al. [17], with a slight modification. Gelatin solution (1–3%, w/v) was transferred into 100-ml cylinders. The solution was homogenised at a speed of 13,400 rpm for 1 min at room temperature. The sample was allowed to stand for 30, 60 and 90 min. FE and FS were then calculated using the following equations:

$$FE(\%) = \frac{V_T}{V_0} \times 100$$

$$FS(\%) = \frac{V_t}{V_0} \times 100$$

where  $V_T$  = total volume after whipping,  $V_0$  = the original volume before whipping and  $V_t$  = total volume after leaving at room temperature for different times (30, 60 and 90 min).

#### 2.6. Statistical analyses

All experiments were performed in triplicate and a completely randomised design (CRD) was used. Data were presented as means ± standard deviation and a probability value of  $<0.05$  was considered significant. Analysis of variance (ANOVA) was performed and the mean comparisons were done by *T*-test. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for Windows, SPSS Inc., Chicago, IL, USA).

### 3. Results and discussion

#### 3.1. Trypsin inhibitory activity of extracts from various legume seeds

Trypsin inhibitory activity, protein content and specific inhibitory activity of extract powders of soybean (SB), mung bean (MB), cowpea (CP) and adzuki bean (AB) are shown in Table 1. The highest trypsin inhibitory activity was observed in the extract powder from SB (70,231 TIU/g seed powder) ( $P < 0.05$ ), followed by the extract powders of MB (39,871 TIU/g seed powder), CP (11,631 TIU/g seed powder) and AB (8787 TIU/g seed powder). The results suggested that the legume seeds differed in the contents of trypsin inhibitor. For the specific activity, the extract powder from SB showed the highest value, followed by that from MB. Lower specific activity was found in the extract powder from CP and AB. The content of trypsin inhibitors in legume seeds can be varied, depending on the cultivars, climate, processing, etc. [18]. Therefore, the extract powder from SB was the potential source of trypsin inhibitors, which could be used to inhibit proteolysis of pretreated skin from unicorn leatherjacket. Soybean trypsin inhibitor usually forms a reversible stoichiometric complex with the serine protease active site [19].

#### 3.2. Protein pattern and inhibitory activity staining of various legume seed extracts

Protein patterns of extract powders from SB, MB, CP and AB under non-reducing condition are depicted in Fig. 2. Extract powders from different legume seeds contained a number of proteins with different MWs. The major bands of SB extract powder

appeared at apparent MWs of 200, 116, 95 and 21 kDa. Protein bands with MWs of 90, 40 and 14 kDa were predominant in MB extract powder. CP extract powder consisted of proteins with MWs of 117, 90, 40, 27, 22, and 10 kDa. For AB extract powder, protein bands with MWs of 86, 70, 62 and 13 kDa were observed. The result suggested that different legume seeds contained different proteins with varying MWs.

Inhibitory activity staining for trypsin inhibitor of various legume seed extract powders is depicted in Fig. 2. For SB extract powder, a single protein band with the MW of 20.1 kDa clearly appeared on the gel. The single protein bands with the MWs of 14, 10 and 13 kDa were observed for the extract powders of MB, CP and AB, respectively. Those protein bands retained after being subjected to hydrolysis by trypsin were most likely belonging to trypsin inhibitors. Garcia-Carreño et al. [20] reported that trypsin, chymotrypsin and papain inhibitors had MW ranging from 14.2 to 66 kDa. Benjakul et al. [21] also reported that the MW of trypsin inhibitors from bambara groundnuts was 13 kDa. Two trypsin inhibitory activity bands were observed for the extracts from cowpea (10 and 18 kDa) and pigeon pea (15 and 25 kDa) [21]. Generally, the Bowman–Birk inhibitor has a lower MW (8–10 kDa), compared with the Kunitz inhibitor ( $>20$  kDa). Two protease inhibitors, including trypsin–chymotrypsin inhibitor and trypsin inhibitor (Bowman–Birk type) with MWs of 15 and 10.5 kDa, respectively, were present in pigeon pea [22]. It was noted that no inhibitor bands were found when determined under reducing conditions, suggesting the loss in activity of inhibitor (data not shown). Damodaran [23] reported that proteins that require high structural stability to function as catalysts are usually stabilized by intra-molecular disulphide bonds and can be separated into lower MW proteins by the action of reducing agents. Therefore, trypsin inhibitors in all legume seed extract powders were more likely stabilised by disulphide bonds. Since soybean extract powder (SBEP) contained the

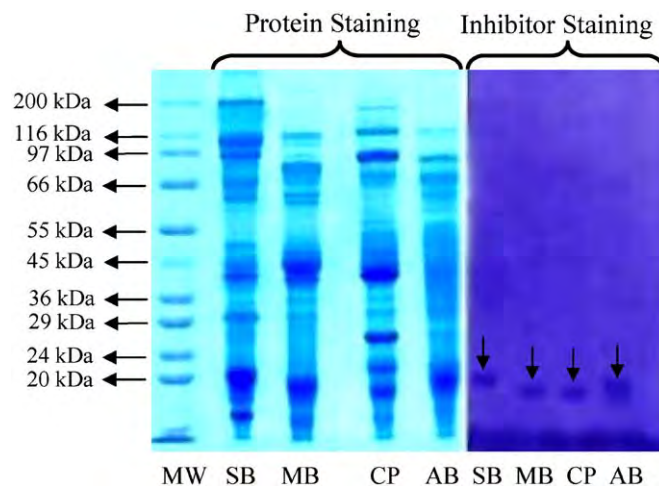


**Fig. 2.** Protein pattern and inhibitory activity staining of different legume seed extract powders. MW: wide range molecular weight marker; SB: soybean; MB: mung bean; CP: cowpea and AB: adzuki bean. Arrowheads indicate the trypsin inhibitor bands.

highest level of trypsin inhibitor, this extract powder was selected as the aid for gelatin extraction.

### 3.3. Effect of SBEP on the autolysis of pretreated skin from unicorn leatherjacket

The effect of SBEP at various concentrations on autolysis of unicorn leatherjacket skin is depicted in Fig. 3. The increasing inhibition was found as SBEP was added to obtain trypsin inhibitor up to 10 TIU/g pretreated skin. This was evidenced by the more retained  $\gamma$ -,  $\beta$ - and  $\alpha$ -chains with increasing SBEP levels added. Nevertheless, no differences in all protein bands were observed when SBEP containing trypsin inhibitor higher than 10 TIU/g pretreated skin was incorporated. In the absence of SBEP, the pronounced degradation was noticeable as indicated by the marked decrease in the band intensity of  $\gamma$ -,  $\beta$ - and  $\alpha$ -chains. Heat-activated serine protease in unicorn leatherjacket skin was involved in the drastic degradation of the  $\gamma$ -,  $\beta$ - and  $\alpha$ -chains of the gelatin extracted at 50 °C [1]. Furthermore, non-collagenolytic proteases might be involved in degradation of gelatin to some extent. Thus,  $\gamma$ -,  $\beta$ - and  $\alpha$ -chains of gelatin were totally retained when the level of trypsin inhibitor in SBEP was greater than 10 TIU/g pretreated skin. This confirmed the efficacy of SBEP in preventing the hydrolysis of gelatin molecules during extraction process. Intarasirisawat et al. [24] also reported that serine protease was the major enzyme causing the autolysis of skin from bigeye snapper. Thus, the addition of serine protease inhibitors, such as SBEP at appropriate concentration could suppress the proteolysis of  $\gamma$ -,  $\beta$ -,  $\alpha$ -chains of collagenolytic proteins in unicorn leatherjacket skin effectively.

### 3.4. Yields, characteristics and functional properties of gelatin from unicorn leatherjacket skin extracted in the presence of SBEP

#### 3.4.1. Yields

The yields of different gelatins including G0, G1, G2 and G3 extracted from unicorn leatherjacket skin at 50 °C for 12 h were  $13.2 \pm 0.5$ ,  $9.82 \pm 0.35$ ,  $9.9 \pm 0.43$  and  $11.14 \pm 0.36\%$  (wet weight basis), respectively. When gelatin was extracted in the presence of SBEP, the yield was generally decreased, compared with the typical process without SBEP incorporation (G0). Addition of SBEP during pre-incubation (G1) or both during pre-incubation and extraction, resulted in the lower yield, compared with the process without pre-incubation with SBEP (G3). Pre-incubation overnight might allow trypsin inhibitor in SBEP to penetrate into pretreated skin effectively. As a result, indigenous serine proteases associated with the skin matrix might be inhibited to a greater extent, thereby preventing the cleavage of peptide chains. This in turn retained the high

MW cross-linked components in the skin matrix, leading to a lower amount of extractable gelatin [24]. The degradation in pretreated skin without SBEP addition, particularly at temperature close to maximal autolytic activity (50 °C) used for gelatin extraction might result in the formation of shorter peptides, caused by the indigenous serine protease associated with skin matrix [1]. This possibly resulted in the ease of gelatin extraction. Higher temperature was also able to destroy the triple-helix of mother collagen, in which intra- and inter-molecular hydrogen bondings and covalent cross-links were cleaved, leading to helix-to-coil transition. In addition, some amide bonds in the elementary chains of collagen molecules also undergo hydrolysis [25]. Extraction process can influence the length of the polypeptide chains and the functional properties of the gelatin [26,27]. Therefore, the use of SBEP to prevent degradation lowered the extraction yield of gelatin from leatherjacket skin.

#### 3.4.2. Protein pattern

Based on SDS-PAGE pattern, the degradation of G0 was more pronounced as shown by almost complete loss of  $\gamma$ -,  $\beta$ - and  $\alpha$ -chains (Fig. 4) in comparison with that found in the pretreated skin. With the sufficient extraction time (12 h), the remaining collagenolytic proteases associated with skin matrix hydrolysed the collagen molecules extensively in G0. When SBEP was incorporated during pre-incubation or extraction, the degradation of both  $\beta$ - and  $\alpha$ -chains was inhibited (Fig. 4). However,  $\alpha$ -chains and cross-linked components of G1 and G2 were more retained, compared with those found in G3. The result suggested that the pre-incubation of pre-treated skin with SBEP at 4 °C for 12 h to ensure penetration of trypsin inhibitor of SBEP into skin was necessary. No marked differences in protein patterns between G1 and G2 were observed, suggesting that the further addition of SBEP during extraction of pretreated skin pre-incubated with SBEP had no profound impact on inhibition of degradation. However,  $\beta$ -chain was almost completely degraded in G3, whereas  $\alpha$ -chains were retained to some extent. This might be due to the proteolysis induced by the active indigenous serine proteases associated with collagen molecules. This was more likely a result of lower penetration of trypsin inhibitor of SBEP into skin matrix during gelatin extraction at 50 °C. In addition, proteins with MW lower than  $\alpha$ -chains (100 kDa) were also observed in G1, G2 and G3. It was suggested that the degradation of  $\alpha$ -,  $\beta$ - and  $\gamma$ -components during gelatin extraction still took place, mainly caused by thermal hydrolysis or the proteolysis mediated by the remaining indigenous proteases. The result was in agreement with Muyonga et al. [28] who reported

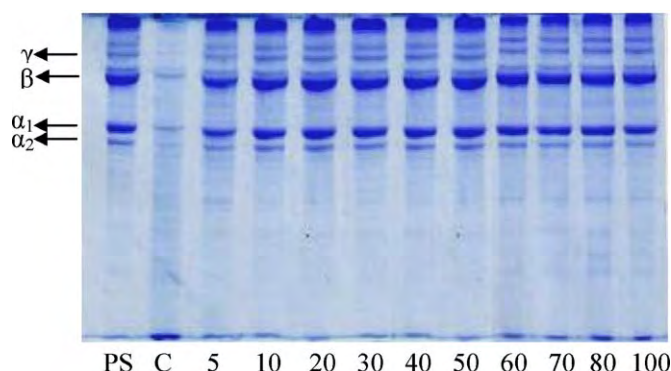


**Fig. 3.** SDS-PAGE pattern of pretreated skin from the unicorn leatherjacket incubated at 50 °C for 30 min in the presence of SBEP containing various concentrations of trypsin inhibitor. PS: pretreated skin and C: control (without SBEP). Numbers (5–100) denote the trypsin inhibitory units (TIU/g pretreated skin).

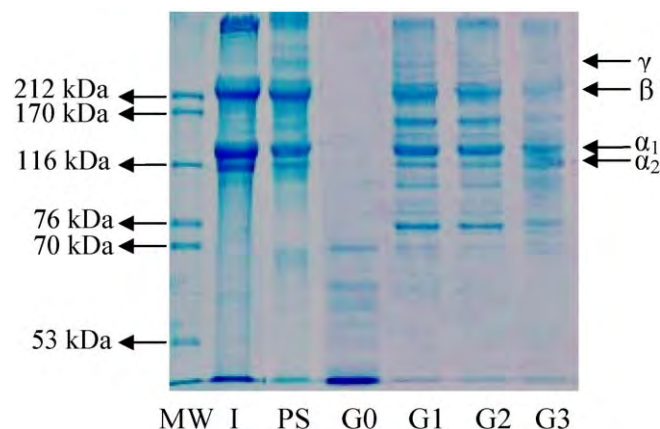


**Fig. 4.** SDS-PAGE pattern of gelatin extracted from the skin of unicorn leatherjacket by different processes. MW: high molecular weight marker; I: collagen type I from calf skin; and PS: pretreated skin. G0, G1, G2 and G3 denote the gelatin extracted by the typical process, processes I, II and III, respectively.

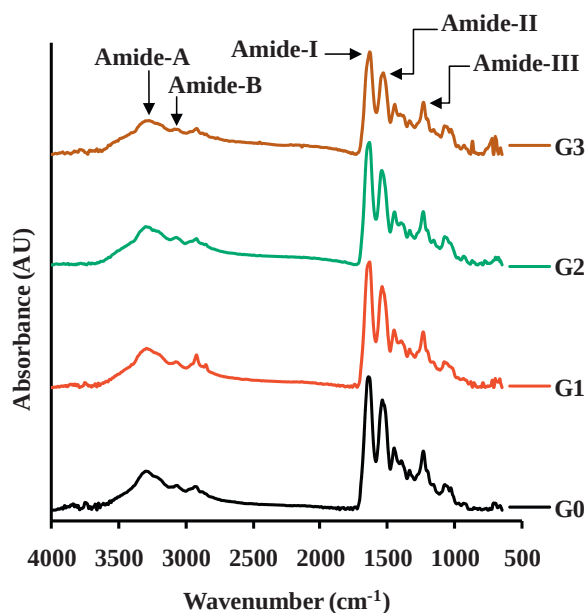


**Fig. 5.** Fourier transform infrared spectra of gelatin extracted from the skin of unicorn leatherjacket by different processes. G0, G1, G2 and G3 denote the gelatin extracted by the typical process, processes I, II and III, respectively.

that gelatin extracted at high temperature contained more peptides (MW less than  $\alpha$ -chains) and a lower proportion of high MW (greater than  $\beta$ -chain) material. The degradation of gelatin from bigeye snapper skin caused by some proteases during extraction process was reported by Jongjareonrak et al. [29]. Therefore, the use of SBEP containing trypsin inhibitor effectively prevented the degradation of  $\beta$ - and  $\alpha$ -chains in G1 and G2 and partially inhibited the degradation in G3 extracted from unicorn leatherjacket skin.

### 3.4.3. FTIR spectra

FTIR spectroscopy has been used to monitor the changes in the functional groups and secondary structure of all gelatin samples. FTIR spectra of G0, G1, G2 and G3 from unicorn leatherjacket skin are depicted in Fig. 5. All gelatin samples had the major peaks in amide region, but showed slight differences in the spectra. These spectra were in accordance with those reported by Muyonga et al. [28]. G0, G1, G2 and G3 displayed the amide-I bands at the wavenumbers of 1643.16, 1634.45, 1635.77 and 1633.18  $\text{cm}^{-1}$ , respectively. The spectral differences in amide-I, especially between G0 and other gelatins, were largely attributed to different conformation of polypeptide chains. The amide-I vibration mode is primarily a C=O stretching vibration coupled to contributions from the CN stretch, CCN deformation and in-plane NH bending modes [30]. The absorption peak at amide-I (1635.89  $\text{cm}^{-1}$ ) was a characteristic for the coil structure of gelatin [31]. Amide-I peak of G0 (1643.16  $\text{cm}^{-1}$ ) had the higher wavenumber, compared to that of G1 (1634.45  $\text{cm}^{-1}$ ), G2 (1635.77  $\text{cm}^{-1}$ ) and G3 (1633.18  $\text{cm}^{-1}$ ). This indicated the greater loss of triple helix of collagen structure of G0, due to the higher hydrolysis induced by heat-activated indigenous serine proteases associated with the skin matrix as well as thermal uncoupling of inter-molecular cross-links. In addition, G1, G2 and G3 showed the lower amplitude of amide-I peak, compared to G0, suggesting the higher content of intra-molecular cross-linking between  $\beta$ - or  $\alpha$ -chains in the formers. Lower amplitude was associated with the higher extent of molecular order due to interaction of C=O with adjacent chains via hydrogen bond. G1, G2 and G3 might retain cross-links during extraction in the presence of SBEP. However, triple helical structure normally held by intermolecular hydrogen bonds was extensively

destroyed caused by thermal uncoupling. The result was in agreement with the higher degradation of G0, in comparison with G1, G2 and G3 (Fig. 4). G0 contained a higher amount of unordered and low MW peptides, mainly mediated by indigenous proteases.

The characteristic absorption bands of G0, G1, G2 and G3 in amide-II region were noticeable at the wavenumbers of 1539.56, 1541.67, 1543.94 and 1532.65  $\text{cm}^{-1}$ , respectively. The amide-II vibration modes are attributed to an out-of-phase combination of CN stretch and inplane NH deformation modes of the peptide [30]. Lower amplitude was found in G1, G2 and G3, compared to G0, indicating that N-H was more involved in bonding with the adjacent molecules. In addition, amide-III was detected at the wavenumbers of 1235.03, 1236.11, 1235.68 and 1234.30  $\text{cm}^{-1}$  for G0, G1, G2 and G3, respectively. The amide-III represents the combination peaks between C-N stretching vibrations and N-H deformation from amide linkages as well as absorptions arising from wagging vibrations from  $\text{CH}_2$  groups from the glycine backbone and proline side-chains [32]. The results suggested that the disordered molecular structure of G0, G1, G2 and G3 was probably caused by the transformation of  $\alpha$ -helical to random coil structure during heating. Those changes were associated with the loss of triple helix state as a result of denaturation of collagen to gelatin [28]. In addition, absorption bands at the wavenumbers of 1075.23, 1079.60, 1075.95 and 1076.09  $\text{cm}^{-1}$  were found in G0, G1, G2 and G3, respectively. Those bands most likely arose from asymmetric stretching vibrations of phosphate groups of phosphorylated proteins coupled to  $-\text{CH}_2$  of the amino acid residues [1,32]. In the present study, phosphoric acid was used for pretreatment of the skin, in which some phosphates were attached to gelatin molecules [33].

Moreover, amide-A band, arising from the stretching vibrations of N-H group, appeared at wavenumbers of 3297.98, 3291.27, 3299.34 and 3271.46  $\text{cm}^{-1}$  for G0, G1, G2 and G3, respectively. Normally, a free N-H stretching vibration is found in the range of 3400–3440  $\text{cm}^{-1}$  [28]. When the N-H group of a peptide is involved in a hydrogen bond, the position is shifted to lower frequencies [34]. In amide-A region, the lower wavenumber was found in G3, compared to other gelatins. However, higher amplitude was found in G0, suggesting the presence of N-H group of shorter peptide fragments. The amide-B bands were observed at wavenumbers of 3064.66, 3074.45, 3070.87 and 3083.74  $\text{cm}^{-1}$  for G0, G1, G2 and G3, respectively, corresponding to asymmetric stretch vibration of  $=\text{C}-\text{H}$  as well as  $-\text{NH}_3^+$  [33]. G0 and G2 showed the lower wavenumber, compared to G1 and G3 at amide-B region, suggesting the interaction of  $-\text{NH}_3$  group between peptide chains. For G1, G2 and G3, the amplitude at the wavenumber of 2851.55, 2852.79 and 2849.51  $\text{cm}^{-1}$  (symmetrical) or 2921.10, 2921.43 and 2919.90  $\text{cm}^{-1}$  (asymmetrical) was higher, compared with that of G0, suggesting C-H stretching vibrations of the  $-\text{CH}_2$  groups which arose from the coupling of SBEP with indigenous serine protease associated with skin matrix [35,36]. Thus, it can be concluded that the secondary structure as well as molecular order of G1 and G2 was retained to high extent due to inhibition of indigenous serine proteases bound with unicorn leatherjacket skin.

### 3.4.4. Gel strength

Gel strength of G0, G1, G2 and G3 is shown in Table 2. G1 exhibited the highest gel strength ( $P < 0.05$ ), followed by G2, G3 and G0, respectively. The use of SBEP as a source of protease inhibitor for gelatin extraction markedly improved the gel strength of extracted gelatin ( $P < 0.05$ ). It was noted that gel strength of gelatin was dependent on the extraction process used. The higher gel strength was more likely due to the maintenance of chain length, which was prerequisite for better gelation. The difference in gel strength might be governed by MW distribution as well as the aggregation between gelatin molecules. The configuration of polypeptide chains and the way in which the inter-junction was developed to form the stronger

**Table 2**

Gel strength and colour of gelatin from the skin of unicorn leatherjacket extracted by different processes.

| Properties              | Bovine gelatin | G0            | G1             | G2             | G3             |
|-------------------------|----------------|---------------|----------------|----------------|----------------|
| Gel strength (g)        | 293.22 ± 5.71a | 90.42 ± 3.99e | 268.51 ± 3.07b | 244.57 ± 4.44c | 232.81 ± 4.87d |
| <i>L</i> <sup>*</sup>   | 35.85 ± 2.44a  | 32.28 ± 3.72a | 28.55 ± 3.33a  | 27.77 ± 4.20a  | 31.40 ± 3.19a  |
| <i>a</i> <sup>*</sup>   | −0.77 ± 0.14b  | 1.86 ± 0.38a  | 1.84 ± 0.44a   | 2.02 ± 0.32a   | 2.11 ± 0.22a   |
| <i>b</i> <sup>*</sup>   | 18.29 ± 1.96a  | 1.86 ± 0.59b  | 1.84 ± 0.38b   | 1.99 ± 0.54b   | 2.43 ± 0.61b   |
| Δ <i>E</i> <sup>*</sup> | 61.42 ± 0.54b  | 65.14 ± 0.06a | 65.93 ± 0.03a  | 62.33 ± 0.51b  | 60.46 ± 0.73b  |

G0, G1, G2 and G3 denote the gelatin extracted by the typical process, processes I, II and III, respectively.

Mean ± SD (*n* = 3).Different letters (a, b, c, d, e) in the same row indicate significant differences (*P* < 0.05).

network were crucial for gel formation [33]. Low MW peptides of G0 might not form the inter-junction zone effectively, leading to the lower aggregation of gelatin to form strong gel network. The hydrolysed β- and α-chains of G0 were not able to anneal correctly during gel maturation, thereby hindering the growth of the existing nucleation sites [37]. Additionally, gel strength is also governed by complex interactions determined by the amino acid composition and the ratio of α/β-chains present in the gelatin [38]. The quality of gelatin is generally determined by the gel strength or bloom value, including low (<150), medium (150–220) and high (220–300) [39]. Usually, the gel strength of commercial gelatins ranges from 100 to 300 g, but gelatins with gel strength of 250–260 g are the most desirable and suitable for a wide range of applications in the food industry, especially in the processing of jellies, canned meat, marshmallows and yoghurts [40]. In addition, acidic pretreatment could play the role in the determination of gel strength. Phosphate groups used for pretreatment might attach with some amino acids, leading to phosphorylation of all gelatins [33]. Phosphorylation may introduce the ionic interaction between phosphate groups and  $-\text{NH}_3^+$  of amino acids, thereby increasing the cross-links of proteins [41]. It was noted that G0, G1, G2, and G3 had the lower gel strength than commercial bovine gelatin (293.22 g) (*P* < 0.05). The gelling properties of fish gelatin were influenced by the source of raw materials, particularly in terms of amino acid composition [42]. The wide range of bloom values found in the various gelatins arises from differences in proline and hydroxyproline content in collagens of different species, and is also associated with the temperature of the habitat of animals. Some species of warm-water fish gelatins have been reported to exhibit relatively high gel strength, which is close to that of gelatin from bovine and porcine [43]. Gelatin with different gel strengths was reported for megrim (340 g) [44], red tilapia (128.1 g) [45], black tilapia (108.7 g) [45], bigeye snapper (105.7 g)

[46], brownstripe red snapper (218.6 g) [46], young and adult Nile perch (217 g and 240 g) [28].

### 3.4.5. Gel colour

Colour of gel from gelatins extracted with different processes is shown in Table 2. No difference in *L*<sup>\*</sup>-value was observed between all gelatin samples (*P* > 0.05). All gelatins, G0, G1, G2 and G3, had the higher redness (*a*<sup>\*</sup>-value), but lower yellowness (*b*<sup>\*</sup>-value) than those of bovine gelatin (*P* < 0.05). It indicated that bovine gelatin was more yellowish than gelatin from unicorn leatherjacket skin. G0 and G1 had higher Δ*E*<sup>\*</sup>-value than G2, G3 and bovine gelatin (*P* < 0.05). Thus, different extraction processes using SBEP as a source of trypsin inhibitor did not affect the colour of gelatin gel significantly. Gelatin manufacture generally has a good process to clarify the impurities from the gelatin solution, such as chemical clarification and filtration processes, in which the improved colour can be achieved.

### 3.4.6. Emulsifying properties

Emulsion activity index (EAI) and emulsion stability index (ESI) of gelatin from the skin of unicorn leatherjacket prepared by different processes are shown in Table 3. EAI of all gelatin samples decreased as the concentration of gelatin increased (*P* < 0.05). After 10 min, EAI of all samples decreased (*P* < 0.05). Among all samples, G0 showed the highest EAI, compared with others (*P* < 0.05), when the same concentration of gelatin was used. G0 with a larger amount of short chain peptides could migrate to the interface effectively and localised surrounding oil droplet at the faster rate than G1, G2 and G3 with the larger size. Additionally, G0 with higher degradation might have more charged groups, especially amino or carboxyl groups at the end of peptides, thereby having the ability to facilitate the stabilisation via electrostatic repulsion. High solubil-

**Table 3**

Emulsifying properties of gelatin from the skin of unicorn leatherjacket extracted by different processes.

| Samples | Gelatin (%) | Emulsion activity index (m <sup>2</sup> /g) |                             | Emulsion stability index (min) |
|---------|-------------|---------------------------------------------|-----------------------------|--------------------------------|
|         |             | 0 min                                       | 10 min                      |                                |
| G0      | 1           | 22.27 ± 0.37Aa <sup>x</sup>                 | 16.92 ± 0.64Aa <sup>y</sup> | 42.02 ± 5.11Ba                 |
|         | 2           | 12.07 ± 1.60Ba <sup>x</sup>                 | 8.42 ± 0.48Ba <sup>y</sup>  | 29.31 ± 5.17Bb                 |
|         | 3           | 8.22 ± 0.37Ca <sup>x</sup>                  | 6.94 ± 0.06Ca <sup>y</sup>  | 74.69 ± 8.46Aa                 |
| G1      | 1           | 17.38 ± 0.48Ab <sup>x</sup>                 | 13.87 ± 0.68Ab <sup>y</sup> | 54.45 ± 5.30Ba                 |
|         | 2           | 8.23 ± 0.06Bb <sup>x</sup>                  | 5.30 ± 0.21Cc <sup>y</sup>  | 28.19 ± 2.31Cb                 |
|         | 3           | 7.21 ± 0.14Cb <sup>x</sup>                  | 6.31 ± 0.03Bab <sup>y</sup> | 85.91 ± 3.30Aa                 |
| G2      | 1           | 13.35 ± 0.73Ac <sup>x</sup>                 | 11.21 ± 1.13Ac <sup>x</sup> | 43.65 ± 4.62Ba                 |
|         | 2           | 8.06 ± 0.13Bb <sup>x</sup>                  | 6.66 ± 0.20Bb <sup>y</sup>  | 58.49 ± 7.41Aa                 |
|         | 3           | 7.04 ± 0.16Cb <sup>x</sup>                  | 5.68 ± 0.22Cb <sup>y</sup>  | 48.01 ± 5.13ABb                |
| G3      | 1           | 14.58 ± 0.57Ac <sup>x</sup>                 | 11.40 ± 0.62Ac <sup>y</sup> | 47.40 ± 9.93Aa                 |
|         | 2           | 8.30 ± 0.23Bb <sup>x</sup>                  | 6.64 ± 0.34Bb <sup>y</sup>  | 50.30 ± 5.26Aa                 |
|         | 3           | 6.26 ± 0.08Cc <sup>x</sup>                  | 4.40 ± 0.69Cc <sup>y</sup>  | 47.46 ± 7.16Ab                 |

G0, G1, G2 and G3 denote the gelatin extracted by the typical process, processes I, II and III, respectively.

Mean ± SD (*n* = 3).Different letters (a, b, ab, c, d) in the same column within the same concentration indicate significant differences (*P* < 0.05).Different capital letters (A, B, AB, C, D) in the same column within the same gelatin sample indicate significant differences (*P* < 0.05).Different superscripts (x, y) in the same row indicate significant differences (*P* < 0.05).

**Table 4**  
Foaming properties of gelatin from the skin of unicorn leatherjacket extracted by different processes.

| Samples | Gelatin (%) | Foam expansion (%)           | Foam stability (%)           |                               |                               |
|---------|-------------|------------------------------|------------------------------|-------------------------------|-------------------------------|
|         |             |                              | 30 min                       | 60 min                        | 90 min                        |
| G0      | 1           | 226.66 ± 2.88Ca <sup>w</sup> | 195.0 ± 2.88Ba <sup>x</sup>  | 131.66 ± 2.88Bc <sup>y</sup>  | 101.66 ± 2.88Bc <sup>z</sup>  |
|         | 2           | 243.33 ± 5.77Ba <sup>w</sup> | 201.66 ± 5.0Ba <sup>x</sup>  | 138.33 ± 2.88ABc <sup>y</sup> | 111.66 ± 5.77Ad <sup>z</sup>  |
|         | 3           | 255.83 ± 5.20Aa <sup>w</sup> | 225.0 ± 5.0Ab <sup>x</sup>   | 145.0 ± 5.0Ac <sup>y</sup>    | 110.0 ± 5.0ABc <sup>z</sup>   |
| G1      | 1           | 130.83 ± 5.20Cd <sup>w</sup> | 116.66 ± 2.88Cd <sup>x</sup> | 110.0 ± 5.0Cd <sup>x</sup>    | 110.0 ± 5.0Bb <sup>x</sup>    |
|         | 2           | 145.66 ± 5.13Bd <sup>w</sup> | 140.0 ± 5.0Bc <sup>w</sup>   | 138.33 ± 2.88Bc <sup>w</sup>  | 138.33 ± 2.88Ac <sup>w</sup>  |
|         | 3           | 165.50 ± 5.76Ac <sup>w</sup> | 150.0 ± 5.0Ad <sup>x</sup>   | 150.0 ± 5.0Ac <sup>x</sup>    | 145.0 ± 5.0Ab <sup>x</sup>    |
| G2      | 1           | 148.33 ± 2.88Cc <sup>w</sup> | 147.50 ± 2.50Cc <sup>w</sup> | 146.66 ± 2.88Cb <sup>w</sup>  | 140.0 ± 5.0Ca <sup>x</sup>    |
|         | 2           | 215.91 ± 3.71Bb <sup>w</sup> | 204.16 ± 3.81Ba <sup>x</sup> | 205.0 ± 5.0Ba <sup>x</sup>    | 190.0 ± 5.0Ba <sup>y</sup>    |
|         | 3           | 241.66 ± 2.88Ab <sup>w</sup> | 241.66 ± 2.88Aa <sup>w</sup> | 228.33 ± 2.88Aa <sup>x</sup>  | 232.50 ± 6.61Aa <sup>x</sup>  |
| G3      | 1           | 168.33 ± 2.88Bb <sup>w</sup> | 161.66 ± 2.88Bb <sup>x</sup> | 156.66 ± 2.88Aa <sup>x</sup>  | 141.66 ± 2.88Ba <sup>y</sup>  |
|         | 2           | 186.66 ± 7.63Ac <sup>w</sup> | 173.33 ± 2.88Ab <sup>x</sup> | 165.0 ± 5.0Ab <sup>x</sup>    | 150.83 ± 3.81ABb <sup>y</sup> |
|         | 3           | 153.16 ± 4.64Cd <sup>w</sup> | 163.33 ± 5.77Bc <sup>w</sup> | 164.16 ± 6.29Ab <sup>w</sup>  | 152.50 ± 6.61Ab <sup>w</sup>  |

G0, G1, G2 and G3 denote the gelatin extracted by the typical process, processes I, II and III, respectively.

Mean ± SD (*n* = 3).

Different letters (a, b, c, d) in the same column within the same concentration indicate significant differences (*P* < 0.05).

Different capital letters in the same column (A, B, AB, C, D) within the same gelatin sample indicate significant differences (*P* < 0.05).

Different superscripts in the same row (w, x, y, z) indicate significant differences (*P* < 0.05).

ity of the protein in the dispersed phase increases the emulsifying efficiency, because the protein molecules should be able to migrate to the surface of the fat droplets rapidly [47]. When comparing EAI between G1, G2 and G3, it was found that G1 showed the highest EAI (*P* < 0.05). Although G1 had the higher  $\alpha$ - or  $\beta$ -chains than G3, the molecular properties including amphiphilic property as well as the chain length could favor G1 molecules to localise at interface more properly. This was associated with the increased stability of emulsions to coalescence during emulsification. Apart from chain length and hydrophilic–lipophilic balance (HLB) of peptide, concentration and flexibility of protein, pH and ionic strength of food system and pretreatment of protein before emulsification are also important factors for emulsifying properties [48]. Differences in the intrinsic properties, composition and conformation of gelatins determined the functional properties of gelatin from skin of two species of big-eye snapper [49]. Therefore, the extraction process using SBEP as the aid directly affected the emulsifying properties of gelatin from the skin of unicorn leatherjacket.

#### 3.4.7. Foaming properties

Foam expansion (FE) and foam stability (FS) of all gelatin samples at various levels are shown in Table 4. FE and FS of all gelatin samples mostly increased with increasing gelatin concentrations (*P* < 0.05). However, for G3 sample, FE decreased when the concentration was higher than 2% (*P* < 0.05). Foams with higher concentration of proteins were denser and more stable owing to an increase in the thickness of interfacial films [50]. Among all samples, G0 had the highest FE and FS (*P* < 0.05). G0 with lower chain length might migrate to air–water interface at the fastest rate. In general, proteins, which rapidly adsorb at the newly created air–liquid interface during bubbling, unfolding and molecular rearrangement at the interface, exhibit better foaming ability than proteins that adsorb slowly and resist unfolding at the interface [51]. G1 and G2 with the larger proportion of  $\beta$ - and  $\alpha$ -chains could not migrate to the air–water interface effectively as G0. Nevertheless, G2 with 3% concentration had the FE comparable to that of G0 (*P* > 0.05). FS increased with protein concentrations, depending on the protein surface properties. During 30–90 min, foams of G1 were quite stable at all concentrations used. FS is directly affected by protein concentration, which influences the thickness, mechanical strength and cohesiveness of the film [50]. For G2 and G3, the lower FS was observed with increasing time, compared with G1. Among all samples, FS of G0 decreased more drastically, compared with other samples during 30–90 min. Low MW peptides of G0 could

not form well-ordered film at the interface, resulting in poor FS. Foam stability of G1, G2 and G3 might be due to flexible proteins, which enhanced viscosity of the aqueous phase and strong films could be formed [52]. The stability of foams depends on various parameters, such as the rate of attaining equilibrium surface tension, bulk and surface viscosities, steric stabilization, and electrical repulsion between the two sides of the foam lamella [53]. Foam stability of gelatins extracted with the aid of SBEP (G1, G2 and G3) can be explained by the combined effect of cohesive forces between the molecules in the foam lamella and the prevention of drainage in the lamella due to longer peptide length of  $\alpha$ - and  $\beta$ -chains. Foam stability of protein solutions is generally positively correlated with MW of peptides [54]. Gravitational drainage of liquid from the lamella and disproportionation of gas bubbles via inter bubble gas diffusion contribute to instability of foams [16]. Coalescence of bubbles occurs because of liquid drainage from the lamella film as two gas bubbles approach each other, leading to film thinning and rupture [55]. Thus, SBEP incorporated during pre-incubation and extraction had the impact on interfacial properties of gelatin from the skin of unicorn leatherjacket.

#### 4. Conclusion

SBEP containing trypsin inhibitor in the range of 10–100 TIU/g effectively prevented the hydrolysis of  $\gamma$ -,  $\beta$ - and  $\alpha$ -chains of collagenolytic proteins of leatherjacket skin subjected to incubation at 50 °C for 30 min. SBEP used in gelatin extraction process affected the molecular and functional properties of resulting gelatins. The addition of SBEP, especially with pre-incubation for 12 h, prior to extraction could retain the  $\beta$ - and  $\alpha$ -chains in resulting gelatin via inhibition of indigenous serine proteases, in which the gel strength increased but foam expansion became poorer. However, extraction yield was lowered when SBEP was used as the aid for gelatin extraction. Thus, the process I, in which pre-incubation of pretreated skin with SBEP at 100 TIU/g was implemented, can be an alternative extraction method to obtain the gelatin with the improved gel property.

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## Isolation and characterisation of collagen extracted from the skin of striped catfish (*Pangasianodon hypophthalmus*)

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### ABSTRACT

Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) from the skin of striped catfish (*Pangasianodon hypophthalmus*) were isolated and characterised. The yields of ASC and PSC were 5.1% and 7.7%, based on the wet weight of skin, respectively, with the accumulated yield of 12.8%. Both ASC and PSC comprising two different  $\alpha$ -chains ( $\alpha_1$  and  $\alpha_2$ ) were characterised as type I and contained imino acid of 206 and 211 imino acid residues/1000 residues, respectively. Peptide maps of ASC and PSC hydrolysed by either lysyl endopeptidase or V8 protease were slightly different and totally differed from those of type I calf skin collagen, suggesting some differences in amino acid sequences and collagen structure. Fourier transform infrared (FTIR) spectra of both ASC and PSC were almost similar and pepsin hydrolysis had no marked effect on the triple-helical structure of collagen. Both ASC and PSC had the highest solubility at acidic pH. A loss in solubility was observed at a pH greater than 4 or when NaCl concentration was higher than 2% (w/v).  $T_{max}$  of ASC and PSC were 39.3 and 39.6 °C, respectively, and shifted to a lower temperature when rehydrated with 0.05 M acetic acid. Zeta potential studies indicated that ASC and PSC exhibited a net zero charge at pH 4.72 and 5.43, respectively. Thus, ASC and PSC were slightly different in terms of composition and structure, leading to somewhat different properties.

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### 1. Introduction

Collagen is the most abundant protein in vertebrates and constitutes about 30% of the total proteins. Collagen, a right-handed triple superhelical rod, is unique in its ability to form insoluble fibres that have high tensile strength (Gelse, Poschl, & Aigner, 2003). There are at least 27 different types of collagen, named type I–XXVII (Birk & Bruckner, 2005). Type I collagen is commonly found in connective tissues, including tendons, bones and skins (Muyonga, Cole, & Duodu, 2004). All members of the collagen family are characterised by domains with repetitions of the proline-rich tripeptides, Gly-X-Y, involved in the formation of the triple helix (Muyonga et al., 2004). Collagen has a wide range of applications in the leather and film industries, in cosmetic and biomedical materials, and as food (Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005). In pharmaceutical applications, collagen can be used for production of wound dressings, vitreous implants and as carriers for drug delivery. In addition, collagen has been used to produce edible casings for the meat processing industries (sausages/salami/snack sticks).

Due to the outbreak of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and the foot-and-mouth disease (FMD) crisis, the uses of collagen and collagen-derived products of land animal origin have become of more concern (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2005). In addition, the collagens extracted from bovine sources are prohibited for Sikhs and Hindus, whilst porcine collagen cannot be consumed by Muslims and Jews, both of whom require bovine to be religiously prepared. As a consequence, the alternative sources of collagen, especially from aquatic animals including freshwater and marine fish and mollusks have received increasing attention (Shen, Kurihara, & Takahashi, 2007).

*Pangasianodon hypophthalmus* or Pla Sawai (in Thai), a large freshwater catfish, belongs to the order Siluriformes and is a member of Pangasidae family. It is one of the most important aquaculture species in Thailand (Froese & Pauly, 2007), especially in the northeast part of Thailand. This fish is also known as Siamese shark or sutchi catfish and is native to the Chao Phraya river in Thailand and the Mekong in Vietnam. It has become an important fish for many countries like Indonesia, Malaysia and China (Roberts & Vidthayanon, 1991). This freshwater fish normally lives in a tropical climate and prefers water with a pH of 6.5–7.5 and a temperature range of 22–26 °C. Adults reach up to 130 cm (4 ft) in length and can weigh up to a maximum of 44.0 kg (97 lb) (Roberts &

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Vidthayanon, 1991). Its meat has been popular among the consumers worldwide. During processing and filleting, a huge amount of skin from this fish is generated as a byproduct, which can be used as a potential source for collagen extraction. The skin from this fish is thick and tough, which may be associated with the collagen cross-links, especially cross-linking caused by hydroxylysine. However, no information on composition and molecular properties of collagen from the skin of this species has been reported. Thus, the objective of the present study was to extract and characterise the collagen, both acid soluble collagen (ASC) and pepsin soluble collagen (PSC), from the skin of striped catfish.

## 2. Materials and methods

### 2.1. Fish skin preparation

Whole fresh farmed striped catfish (*P. hypophthalmus*) (approximately 2 years old) weighing  $1 \pm 0.5$  kg/fish stored on ice were procured from the fish market of Hat Yai, Songkhla, Thailand. Fish were stored in ice with a fish/ice ratio of 1:2 (w/w) and transported within 1 h to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand. Upon arrival, fish were washed using tap water and deskinning. The skin was washed with cold water ( $5\text{--}8^\circ\text{C}$ ) and cut into small pieces ( $0.5 \times 0.5\text{ cm}^2$ ). The prepared skin samples were packed in polyethylene bags and kept at  $-20^\circ\text{C}$  until used. The storage time was not longer than 1 month.

### 2.2. Chemicals

$\beta$ -Mercaptoethanol ( $\beta$ -ME), V8 protease from *Staphylococcus aureus* (EC3.4.21.19, 800 U/mg powder); pepsin from porcine stomach mucosa (EC3.4.23.1; powdered; 750 U/mg dry matter) and type I collagen from calf skin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). High molecular weight markers were obtained from GE Healthcare UK (Buckinghamshire, UK). Sodium dodecyl sulphate (SDS), trichloroacetic acid, Folin-Ciocalteu's phenol reagent, acetic acid and tris(hydroxymethyl) aminomethane were obtained from Merck (Darmstadt, Germany). Lysyl endopeptidase from *Achromobacter lyticus* (EC3.4.21.50; 4.5 AU/mg protein) were procured from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

### 2.3. Pretreatment of skin

To remove non-collagenous proteins, the prepared fish skin was mixed with 0.1 M NaOH at a skin/alkali solution ratio of 1:10 (w/v). The mixture was continuously stirred for 6 h at  $4^\circ\text{C}$  and the alkali solution was changed every 2 h. The treated skin was then washed with cold water until a neutral or faintly basic pH of wash water was reached. The pH of wash water was determined using a digital pH meter (Sartorius North America, Edgewood, NY, USA).

### 2.4. Extraction of acid soluble collagen (ASC)

ASC was extracted as per the method of Nagai and Suzuki (2000) with slight modification. All processes were carried out at  $4^\circ\text{C}$  with continuous stirring. The pretreated skins were defatted with 10% butyl alcohol with a solid/solvent ratio of 1:10 (w/v) for 48 h and the solvent was changed every 8 h. Defatted skin was washed with cold water ( $5\text{--}8^\circ\text{C}$ ), followed by soaking in 0.5 M acetic acid with a solid/solvent ratio of 1:15 (w/v) for 24 h. The mixture was filtered through two layers of cheese cloth and the residue was re-extracted under the same conditions. Both filtrates were combined. The collagen was precipitated by adding

NaCl (powder) to a final concentration of 2.6 M in the presence of 0.05 M tris(hydroxymethyl) aminomethane, pH 7.0. The resultant precipitate was collected by centrifuging at 20,000g for 60 min, using a refrigerated centrifuge Avanti® J-E (Beckman Coulter, Inc., Palo Alto, CA, USA). The pellet was dissolved in a minimum volume of 0.5 M acetic acid, and dialysed against 50 volumes of 0.1 M acetic acid for 24 h, followed by the dialysis in the same volume of distilled water for another 24 h. The dialysate was freeze dried and was referred to as "acid soluble collagen; ASC". The yield of ASC was calculated from the percentage of dry weight of collagen extracted in comparison with the wet weight of the initial skin used.

### 2.5. Extraction of pepsin soluble collagen (PSC)

The undissolved residue obtained after ASC extraction was used for PSC extraction. The residue was soaked in 0.5 M acetic acid with a solid/solvent ratio of 1:15 (w/v) and porcine pepsin (20 U/g residue) was added. The mixtures were continuously stirred at  $4^\circ\text{C}$  for 48 h, followed by filtration with two layers of cheesecloth. The filtrate was subjected to precipitation and the pellet was dialysed in the same manner as those for ASC previously described. The dialysate was freeze dried and was referred to as "pepsin soluble collagen; PSC". The yield of PSC was also calculated in the same manner as for ASC. Additionally, the accumulated yield of collagen was calculated from the yields of both ASC and PSC.

### 2.6. Analyses

Both ASC and PSC were subjected to the following analyses.

#### 2.6.1. Amino acid analysis

The samples were hydrolysed under reduced pressure in 4 M methane sulphonic acid containing 0.2% (v/v) 3-(2-aminoethyl) indole at  $115^\circ\text{C}$  for 24 h. The hydrolysates were neutralised with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2) (Kittipattananabawon, Benjakul, Visessanguan, Kishimura, & Shahidi, 2010). An aliquot of 0.4 ml was applied to an amino acid analyser (MLC-703; Atto Co., Tokyo, Japan).

#### 2.6.2. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed following the method of Laemmli (1970). The samples were dissolved in 5% SDS and the mixtures were incubated at  $85^\circ\text{C}$  for 1 h in temperature controlled water bath (Memmert, Schwabach, Germany). The mixture was centrifuged at 4000g for 5 min using a microcentrifuge (MIKRO20, Hettich Zentrifugen, Germany) at room temperature to remove undissolved debris. Solubilised samples were mixed at a ratio of 1:1 (v/v) with the sample buffer (0.5 M Tris HCl, pH 6.8, containing 4% SDS and 20% glycerol) containing 10%  $\beta$ -ME. The mixtures were kept in boiling water for 2 min. Samples (15  $\mu\text{g}$  protein) were loaded onto polyacrylamide gels comprising a 7.5% running gel and a 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel for 1 h and 30 min using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, the gel was stained with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. High molecular weight markers were used to estimate the molecular weight of proteins. The markers used included myosin (200 kDa),  $\alpha_2$ -macroglobulin (170 kDa),  $\beta$ -galactosidase (116 kDa), transferrin (76 kDa) and glutamate dehydrogenase (53 kDa). Type I calf skin collagen was used as a standard. Quantitative analysis of protein band intensity was performed using a Model GS-700 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA, USA) with Molecular Analyst Software version 1.4 (image analysis system).

### 2.6.3. Peptide mapping of collagen

Peptide mapping of ASC and PSC was performed according to the method of Kittiphattanabawon et al. (2005) with a slight modification. The samples (6 mg) were dissolved in 1 ml of 0.1 M sodium phosphate, pH 7.2 containing 0.5% (w/v) SDS. Then, the mixtures were preheated at 45 °C for 3 h and 300 µl of the prepared mixtures were transferred to test tubes for digestion. To initiate the digestion, 20 µl of each enzyme solution, either *S. aureus* V8 protease or lysyl endopeptidase from *A. lyticus*, with concentrations of 5 and 50 µl/ml, respectively, were added to the mixtures. The reaction mixtures were then incubated at 37 °C for 1 h. The reactions were terminated by subjecting the reaction mixture to boiling water for 3 min. Peptides generated by the protease digestion were separated by SDS–PAGE using a 7.5% running gel and a 4% stacking gel, followed by staining and destaining as previously described. Peptide mapping of calf skin collagen type I was also conducted in the same manner and the peptide patterns were compared.

### 2.6.4. ATR-FTIR analysis

Both ASC and PSC were subjected to attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR). FTIR spectrometer (Model Equinox 55, Bruker, Ettlingen, Germany) equipped with a horizontal ATR trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) was used. For spectra analysis, the collagen samples were placed onto the crystal cell and the cell was clamped into the mount of the FTIR spectrometer. The spectra in the range of 400–4000 cm<sup>-1</sup> with automatic signal gain were collected in 32 scans at a resolution of 4 cm<sup>-1</sup> and were ratioed against a background spectrum recorded from the clean empty cell at 25 °C.

### 2.6.5. Differential scanning calorimetry

Differential scanning calorimetry (DSC) of ASC and PSC was run following the method of Rochdi, Foucat, and Renou (2000) with a slight modification. The samples were rehydrated by adding deionised water or 0.05 M acetic acid to dried samples at a solid/solution ratio of 1:40 (w/v). The mixtures were allowed to stand for 2 days at 4 °C. DSC was performed using a differential scanning calorimeter (Perkin Elmer, Model DSC7, Norwalk, CA, USA). Temperature calibration was run using the Indium standard. The samples were accurately weighed into aluminium pans and sealed. The samples were scanned at 1 °C/min over the range of 20–50 °C using iced water as the cooling medium. An empty pan was used as the reference. Total denaturation enthalpy ( $\Delta H$ ) was estimated by measuring the area of the DSC thermogram. The maximum transition temperature ( $T_{max}$ ) was estimated from the thermogram.

### 2.6.6. Zeta potential analysis

ASC and PSC were dissolved in 0.5 M acetic acid to obtain a final concentration of 0.05% (w/v). The mixtures were continuously stirred at 4 °C using a magnetic stirrer model BIG SQUID (IKA®-Werke GmbH & CO.KG, Stanfen, Germany) until the samples were completely solubilised.

The zeta ( $\zeta$ ) potential of collagen solutions was measured with a zeta potential analyser (model ZetaPALS; Brookhaven Instruments Co., Holtsville, NY, USA). Solutions (20 ml) were transferred to an autotitrator (model BI-ZTU, Brookhaven Instruments Co.), in which the pH of the solutions were adjusted to 2–6 using either 1.0 M nitric acid or 1.0 M KOH. The zeta potential for each solution was recorded.

### 2.6.7. Solubility

The solubility of ASC and PSC was determined by the method of Montero, Jimenez-Colmenero, and Borderias (1991) with a slight modification. Collagens were dissolved in 0.5 M acetic acid to ob-

tain a final concentration of 3 mg/ml and the mixture was stirred at 4 °C for 24 h. Thereafter, the mixture was centrifuged at 5000g for 15 min at 4 °C. The supernatant was used for solubility study.

**2.6.7.1. Effect of pH on solubility.** Collagen solution (3 mg/ml; 8 ml) was transferred to a 50 ml centrifuge tube and the pH was adjusted with either 6 N NaOH or 6 N HCl to obtain the final pH ranging from 1 to 10. The volume of solution was made up to 10 ml by deionised water (Hydrochem group-Harlte pool, Cleveland, UK) previously adjusted to the same pH as the collagen solution. The solution was centrifuged at 20,000g for 30 min at 4 °C. Protein content in the supernatant was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) using bovine serum albumin as a standard. Relative solubility was calculated in comparison with that obtained at the pH giving the highest solubility.

**2.6.7.2. Effect of NaCl on solubility.** Collagen solution (6 mg/ml; 5 ml) was mixed with 5 ml of NaCl in 0.5 M acetic acid at various concentrations to give the final concentrations of 0%, 1%, 2%, 3%, 4%, 5% and 6%. The mixture was stirred continuously at 4 °C for 30 min, followed by centrifuging at 20,000g for 30 min at 4 °C. Protein content in the supernatant was measured and the relative solubility was calculated as previously described.

### 2.6.8. Statistical analyses

All experiments were performed in triplicate and a completely randomised design (CRD) was used. Analysis of variance (ANOVA) was performed and means comparison were done by Duncan's multiple range tests. For comparison, the T-test was used (Steel & Torrie, 1980). Analysis was performed using a SPSS statistical package (SPSS 11.0 for Windows, SPSS Inc, Chicago, IL, USA).

## 3. Results and discussion

### 3.1. Yield of ASC and PSC from the skin of striped catfish

ASC and PSC were isolated from the skin of striped catfish with yields of 5.1% and 7.7% (based on the wet weight of skin), respectively. The skin was not completely solubilised by 0.5 M acetic acid, as shown by the low yield of ASC. This result was in agreement with Jongjareonrak, Benjakul, Visessanguan, and Tanaka (2005) who reported the incomplete solubilisation of bigeye snapper skin in 0.5 M acetic acid. The present result was explained by the fact that collagen molecules in striped catfish skin were most likely cross-linked by covalent bonds through the condensation of aldehyde groups at the telopeptide region as well as the inter-molecular cross-linking, leading to a decrease in the solubility of collagen (Foegeding, Lanier, & Hultin, 1996; Zhang et al., 2007). The yields of ASC and PSC from brownbanded bamboo shark (*Chiloscyllium punctatum*) were 9.38% and 8.86% (wet weight basis), respectively (Kittiphattanabawon et al., 2010) and those from bigeye snapper skin were 6.4% and 1.1% (wet weight basis), respectively (Jongjareonrak, Benjakul, Visessanguan, Nagai, & Tanaka, 2005), whereas for brownstripe red snapper skin, the yields of ASC and PSC were 9% and 4.7% (wet weight basis), respectively (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2005). With further limited pepsin digestion, the cross-linked molecules at the telopeptide region were cleaved, resulting in further extraction. Pepsin was able to cleave specifically at the telopeptide region of collagen from the skin of bigeye snapper (Nalinanon, Benjakul, Visessanguan, & Kishimura, 2007). The combined yield of ASC and PSC was 12.8%. Thus pepsin could be used as an aid for increasing the extraction yield of collagen from the skin of striped catfish.

### 3.2. Amino acid composition

ASC and PSC from the skin of striped catfish showed similar amino acid compositions as shown in Table 1. Both collagens had (309–317 glycine residues/1000 residues) as the major amino acid, followed by proline (120–126 residues/1000 residues), alanine (116–114 residues/1000 residues) and hydroxyproline (86–91 residues/1000 residues). Generally, glycine in collagen represents nearly one third of the total residues and occurs as every third residue in collagen except for the first 14 amino acid residues from the N-terminus and the first 10 residues from the C-terminus (Foe-geding et al., 1996). The imino acid content (proline + hydroxyproline) of ASC and PSC was 206 and 217/1000 residues, which was higher than those of most fish collagens such as grass carp skin collagen (186 residues/1000 residues) and bigeye snapper skin collagen (193 residues/1000 residues) (Kittiphattanabawon et al., 2005; Zhang et al., 2007). The variation in imino acid content amongst different species is mostly due to different living environments of habitat, particularly temperature. Additionally, the imino acid content was reported to have a major influence on the thermal stability of collagen (Muyonga et al., 2004). Pro + Hyp rich zones of collagen molecules are most likely involved in the formation of junction zones that are stabilised by hydrogen bonding (Johnston-Banks, 1990). The pyrrolidine rings of proline and hydroxyproline impose restrictions on the conformation of the polypeptide chain and help to strengthen the triple helix (Wong, 1989). Thus, collagens from striped catfish are expected to be more stable than those from the other fish species. Hydroxylysine (5–6 residues/1000 residues) was found in both ASC and PSC from striped catfish skin. Hydroxylysine might undergo cross-linking, leading to the compact structure of collagen from striped catfish skin (Balian & Bowes, 1977). When comparing the imino acid content between ASC and PSC, it was found that the former contained a slightly lower content than the latter. It was suggested that the teleopeptides removed by pepsin digestion contained fewer imino acids. As a result, the PSC obtained had a higher proportion of imino acids. No cysteine and tryptophan were found in either ASC or PSC. Generally, type I collagen has low amounts of cysteine (~0.2%) and methionine (~1.24–1.33%) (Owusu-Apenten, 2002).

The amino acid composition of collagen from striped catfish skin was found to be almost similar to those of collagen from other

freshwater fish including common carp, channel catfish and silver carp (Duan, Zhang, Du, Yao, & Konno, 2009).

### 3.3. Gel electrophoresis

The electrophoretic patterns of ASC and PSC from the skin of striped catfish are shown in Fig. 1. Both ASC and PSC consisted of  $\alpha$ 1- and  $\alpha$ 2-chains at a ratio of approximately 2:1. Therefore both collagens should most likely be classified as type I collagen. Similar electrophoretic patterns of type I collagen from the skin of brown stripe red snapper were reported by Jongjareonrak, Benjakul, Visessanguan, Nagai, et al. (2005). Apart from  $\alpha$ -chains, both ASC and PSC also contained high molecular weight (MW) components, including  $\beta$ - and  $\gamma$ -components as well as their cross-linked molecules. Generally, starving fish have more cross-linked collagen than those fed well (Love, Yamaguchi, Creach, & Lavety, 1976). However, the cross-linking of collagen in fish skins is extremely low and the highly cross-linked molecules are rarely found (Cohen-Solal, Louis, Allian, & Meunier, 1981). When comparing the proportion of high MW components between ASC and PSC, the former contained the higher band intensity of  $\beta$ - and  $\gamma$ -chains as well as more cross-linked components than the latter. The result suggested that the intra- and inter-molecular cross-links of collagens were richer in ASC than in PSC. After digestion by pepsin, some  $\beta$ - and  $\gamma$ -components of ASC might be cleaved into  $\alpha$ -components, as evidenced by the increased band intensity of the  $\alpha$ -chains. Similar electrophoretic protein patterns were found in ASC and PSC from the skin of large fin long barbel catfish (*Mystus macropterus*) (Zhang, Liu, & Li, 2009). Pepsin cleaves the cross-link containing the telopeptide, and the  $\beta$ -chain is concomitantly converted to two  $\alpha$ -chains (Sato et al., 2000). Type I collagen was found in the skins of hake (Montero, Borderias, Turnay, & Leyzarbe, 1990), trout (Montero et al., 1990), Nile perch (Muyonga et al., 2004) and bigeye snapper (Kittiphattanabawon et al., 2005).

### 3.4. Peptide mapping

The peptide maps of ASC and PSC from striped catfish skin digested by lysyl endopeptidase and V8 protease with type 1 calf skin collagen as a control are shown in Fig. 2. For peptide maps of all collagens digested with lysyl endopeptidase, all components

**Table 1**

Amino acid composition of acid soluble collagen and pepsin soluble collagen from the skin of striped catfish (*Pangasianodon hypophthalmus*) (expressed as residues per 1000 total amino acid residues).

| Amino acids              | ASC  | PSC  |
|--------------------------|------|------|
| Alanine                  | 116  | 114  |
| Arginine                 | 54   | 54   |
| Aspartic acid/asparagine | 46   | 44   |
| Cysteine                 | 0    | 0    |
| Glutamic acid/glutamine  | 80   | 77   |
| Glycine                  | 309  | 317  |
| Histidine                | 4    | 4    |
| Isoleucine               | 14   | 12   |
| Leucine                  | 26   | 24   |
| Lysine                   | 27   | 26   |
| Hydroxylysine            | 5    | 6    |
| Methionine               | 10   | 10   |
| Phenylalanine            | 13   | 12   |
| Hydroxylproline          | 86   | 91   |
| Proline                  | 120  | 126  |
| Serine                   | 37   | 34   |
| Threonine                | 24   | 24   |
| Tyrosine                 | 5    | 3    |
| Tryptophan               | 0    | 0    |
| Valine                   | 23   | 22   |
| Total                    | 1000 | 1000 |
| Imino acid               | 206  | 211  |

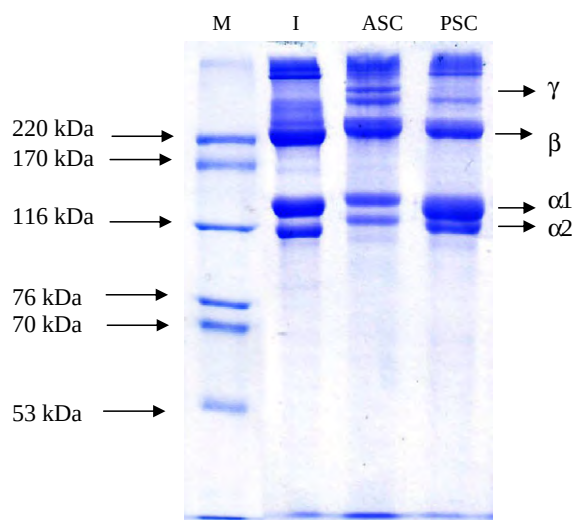


**Fig. 1.** SDS-PAGE of acid soluble collagen (ASC) and pepsin soluble collagen (PSC) from the skin of striped catfish. M, high molecular weight markers; I: type I calf skin collagen.

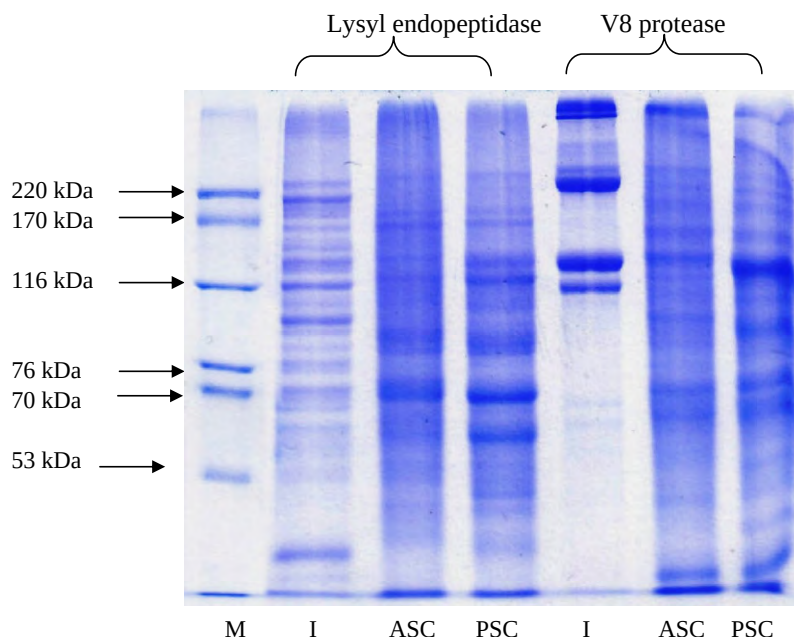


**Fig. 2.** Peptide maps of ASC and PSC from the skin of striped catfish digested by lysyl endopeptidase and V8 protease. M, high MW markers; I, type I calf skin collagen.

including  $\alpha$ -1,  $\alpha$ -2,  $\beta$ - and  $\gamma$ -chains were markedly hydrolysed and varying degradation peptides with different molecular weights were obtained for all collagens. Peptides with MW of 178, 162, 138, 123, 87, 76, 66, 65 and 54 kDa were the dominant products of ASC and PSC after being hydrolysed with lysyl endopeptidase. When comparing between ASC and PSC, the former was more susceptible to hydrolysis by lysyl endopeptidase than the latter.  $\alpha$ -Chains were more retained in the latter. For ASC and PSC digested with V8 protease,  $\beta$ - and  $\gamma$ -components were almost completely hydrolysed. V8 protease shows a high specific preference for glutamic acid and aspartic acid residues of proteins (Vercaigne-Marko, Kosciarz, Nedjar-Arroume, & Guillochon, 2000). Due to the slightly lower contents of glutamic acid and aspartic acid (75 and 45 residues/1000 residues) in calf skin collagen (Herbage, Bouillet, & Bernengo, 1977), ASC (80 and 46 residues/1000 residues) and PSC (77 and 44 residues/1000 residues) might be more susceptible to hydrolysis by V8 protease. After hydrolysis with V8 protease, peptides with MW of 95.4, 66 and 25.7 kDa were obtained for ASC. For PSC, peptides with MW of 71.1, 61.6 and 26.3 kDa were observed. However, type I calf skin collagen was resistant to the hydrolysis by V8 protease. When comparing the peptide maps between ASC and PSC hydrolysed by the V8 protease, PSC was more resistant to hydrolysis than ASC, as indicated by a greater band intensity of the  $\alpha$ -1 chain.

It was found that all collagens were more susceptible to hydrolysis by lysyl endopeptidase than by V8 protease. The differences in the peptide maps between the different collagens generated by lysyl endopeptidase and V8 protease digestion suggested some differences in their primary structure (Nagai & Suzuki, 2002). For PSC, some portions of the telopeptides were presumably removed by pepsin. As a result, chain lengths as well as amino acids at both the C- and N-termini were different. This might determine the accessibility of collagen molecules to proteinases, leading to varying degrees of hydrolysis between ASC and PSC. Peptide maps of collagens were reported to differ amongst sources and species (Mizuta, Yamasa, Miyagi, & Yoshinaka, 1999). Thus, ASC and PSC from the skin of striped catfish might be different in terms of domain or cross-links and totally different from type I calf skin collagen in term of sequence and composition of amino acids.

### 3.5. ATR-FTIR spectra

ATR-FTIR spectra of both ASC and PSC from the skin of striped catfish are depicted in the Fig. 3. FTIR spectra for both ASC and PSC were similar to those of collagens from other fish species (Muyonga et al., 2004). The amide A band of ASC and PSC was found at a wavenumber of  $3321\text{ cm}^{-1}$ . According to Doyle, Bendit, and Blout (1975), a free N-H stretching vibration occurs in the range of  $3400\text{--}3440\text{ cm}^{-1}$  and when the NH group of a peptide is involved in a hydrogen bond, the position is shifted to lower frequencies, usually around  $3300\text{ cm}^{-1}$ . The result indicated that the NH groups of this collagen were involved in hydrogen bonding, probably with a carbonyl group of the peptide chain. The amide B band positions of ASC and PSC were found at wavenumbers of  $2926$  and  $2928\text{ cm}^{-1}$ , respectively, representing the asymmetrical stretch of  $\text{CH}_2$  (Muyonga et al., 2004). The amide I band of ASC and PSC were found at wavenumbers of  $1651$  and  $1649\text{ cm}^{-1}$ , respectively. Due to the greater non-helical portion of the telopeptides in ASC, intramolecular H-bond between C=O of the peptide backbone and the adjacent hydrogen donor should be lower in ASC, in comparison with PSC. The amide I band with characteristic frequencies in the range from  $1600$  to  $1700\text{ cm}^{-1}$  was mainly associated with the stretching vibrations of the carbonyl group (C=O bond) along the polypeptide backbone (Payne & Veis, 1988), and was a sensitive marker of the peptide secondary structure (Surewicz & Mantsch, 1988). The amide II band of ASC and PSC was situated at a wavenumber of  $1551\text{ cm}^{-1}$ , whilst the amide III band of ASC and PSC was located at wavenumbers of  $1242$  and  $1244\text{ cm}^{-1}$ , respectively. The amide II and amide III bands represent N-H bending vibrations and C-H stretching, respectively (Payne & Veis, 1988). Furthermore, a strong C-H stretching also occurred at wavenumber of  $1747\text{ cm}^{-1}$  for both ASC and PSC, which was in agreement with the findings of Duan et al. (2009) who found a similar stretching pattern of collagen from the skin of common carp, a freshwater fish.

The IR ratios between the amide III and  $1454\text{ cm}^{-1}$  of ASC and PSC were 1.17 and 1.16, respectively. An IR ratio of approximately 1 indicates the presence of helical structure (Plepis, Goissis, & Das-Gupta, 1996). Due to the similarity of the IR ratio between

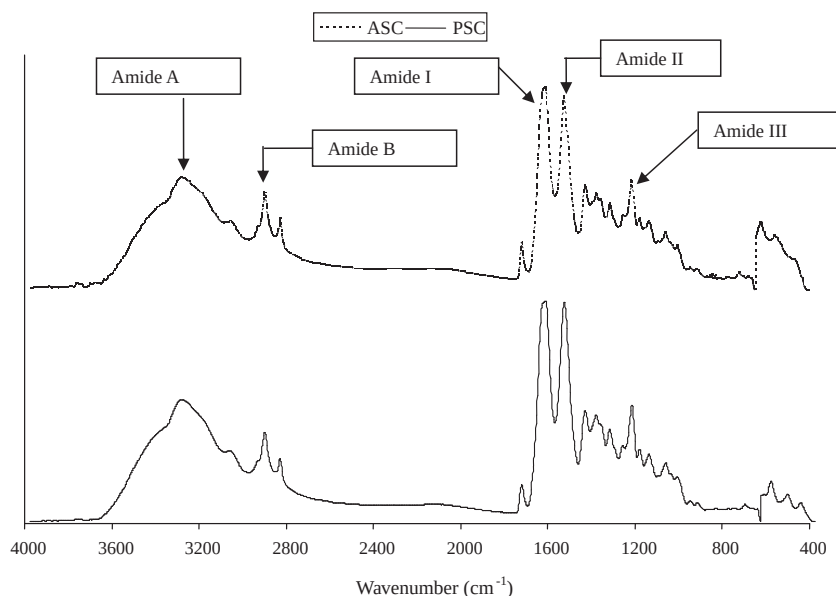


Fig. 3. Fourier transform infrared spectra of ASC and PSC from the skin of striped catfish.

ASC and PSC, pepsin hydrolysis apparently had no pronounced effect on the triple-helical structure of PSC. However, there might be the slight differences between ASC and PSC, especially at the telopeptide region, which was cleaved by pepsin. Nagai, Suzuki, and Nagashima (2008) reported that there were some differences between the secondary structural components such as  $\alpha$ -helix,  $\beta$ -sheet,  $\beta$ -turn and other random coils between ASC and PSC from the skin of the common mink whale (*Balaenoptera acutorostrata*).

### 3.6. Thermal stability

DSC thermograms of ASC and PSC from the skin of striped catfish rehydrated in 0.05 M acetic acid and deionised water are shown in Fig. 4. The endothermic peaks, with maximum temperatures ( $T_{\max}$ ) of 39.66 and 39.31 °C, were observed for ASC and PSC rehydrated in deionised water, respectively. When ASC and PSC were rehydrated in acetic acid, it was noted that  $T_{\max}$  shifted to lower temperatures, 35.35 and 35.38 °C for ASC and PSC, respectively. The result suggested that the intramolecular hydrogen bonds stabilising the triple helix structure of collagen might be disrupted to some levels in the presence of acetic acid, mainly due to the repulsion of collagen molecules in acidic solution (Ahmad & Benjakul, 2010). Furthermore, a higher cross-linkage of striped catfish collagen more likely contributed to the higher  $T_{\max}$  of both ASC and PSC.  $T_{\max}$  of ASC was similar to that of PSC in both media, suggesting no differences in the denaturation temperature between both collagens. The triple helix structure was predominant in PSC (Hickman et al., 2000).  $T_{\max}$  of collagen from the skin of striped catfish was slightly higher than that of pig skin collagen (37 °C) (Ikoma, Kobayashi, Tanaka, Walsh, & Mann, 2003) but was close to that of calf skin collagen (40.8 °C) (Komsa-Penkova, Koyonava, Kostov, & Tenchov, 1999). In contrast,  $T_{\max}$  of collagen from striped catfish was much higher than that of collagen from cold-water fish skin including cod skin (15 °C) and that of other tropical fish such as brownstripe red snapper (31.5 °C) and bigeye snapper (30.4 °C) (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2005). The difference in  $T_{\max}$  amongst collagens from different species was correlated with the different imino acid contents (proline and hydroxyproline), body temperature and environmental temperature (Kittiphattanabawon et al., 2005; Nagai et al., 2008). The water temperature from where the striped catfish were caught ranged from 27 to 32 °C. The higher content of imino acids is associated with increasing thermal denaturation

(Wong, 1989). The denaturation temperature in fish species is also correlated with the environment, in which they are living. The higher imino acid content of ASC and PSC (206 and 217 residues/1000 residues) was noticeable, compared with those of collagen from the skin of cod (154 residues/1000 residues) (Duan et al., 2009), bigeye snapper (193 residues/1000 residues) (Kittiphattanabawon et al., 2005), carp (190 residues/1000 residues) (Duan et al., 2009) and brown banded bamboo shark (207/1000 residues) (Kittiphattanabawon et al., 2010).

When the transition enthalpy ( $\Delta H$ ) of both ASC and PSC was determined, PSC had the higher  $\Delta H$  than ASC when rehydrated in the same medium. The higher  $\Delta H$  of PSC might be associated with the higher imino acid content (Table 1) after the removal of telopeptides. The lower  $\Delta H$  was found when acetic acid was used for rehydration, in comparison with that found in collagen rehydrated with deionised water. Acetic acid can cleave hydrogen bonds, which stabilise the triple-helical structure of collagen (Xiong, 1997). Thus, the collagen structure was destabilised, leading to decreased thermal stability of collagens, as shown by the lowered  $T_{\max}$  and enthalpy.

### 3.7. Zeta potentials

The zeta potentials of ASC and PSC solutions at different pHs are shown in Fig. 5. The zero surface net charge of ASC and PSC was observed at pH 4.72 and 5.43, respectively. Vojdani (1996) reported that a protein in an aqueous system has a zero net charge at its isoelectric point (pI), when the positive charges are balanced out by the negative charges (Bonner, 2007). Difference in surface charge of ASC and PSC might be ascribed to the difference in acidic and basic amino acid residues, which was more likely governed by the removal of telopeptides by pepsin. ASC and PSC from the skin of brown banded bamboo shark had a net charge of zero at pH 6.21 and 6.56, respectively (Kittiphattanabawon et al., 2010).

### 3.8. Collagen solubility

#### 3.8.1. Effect of pH

The effect of pH on the solubility of ASC and PSC from the skin of striped catfish is shown in Fig. 6a. Both ASC and PSC showed a maximum solubility at pH 2 ( $P < 0.05$ ). In general, both collagens were solubilised in the acidic pH range (1–4) (Jongjareonrak,

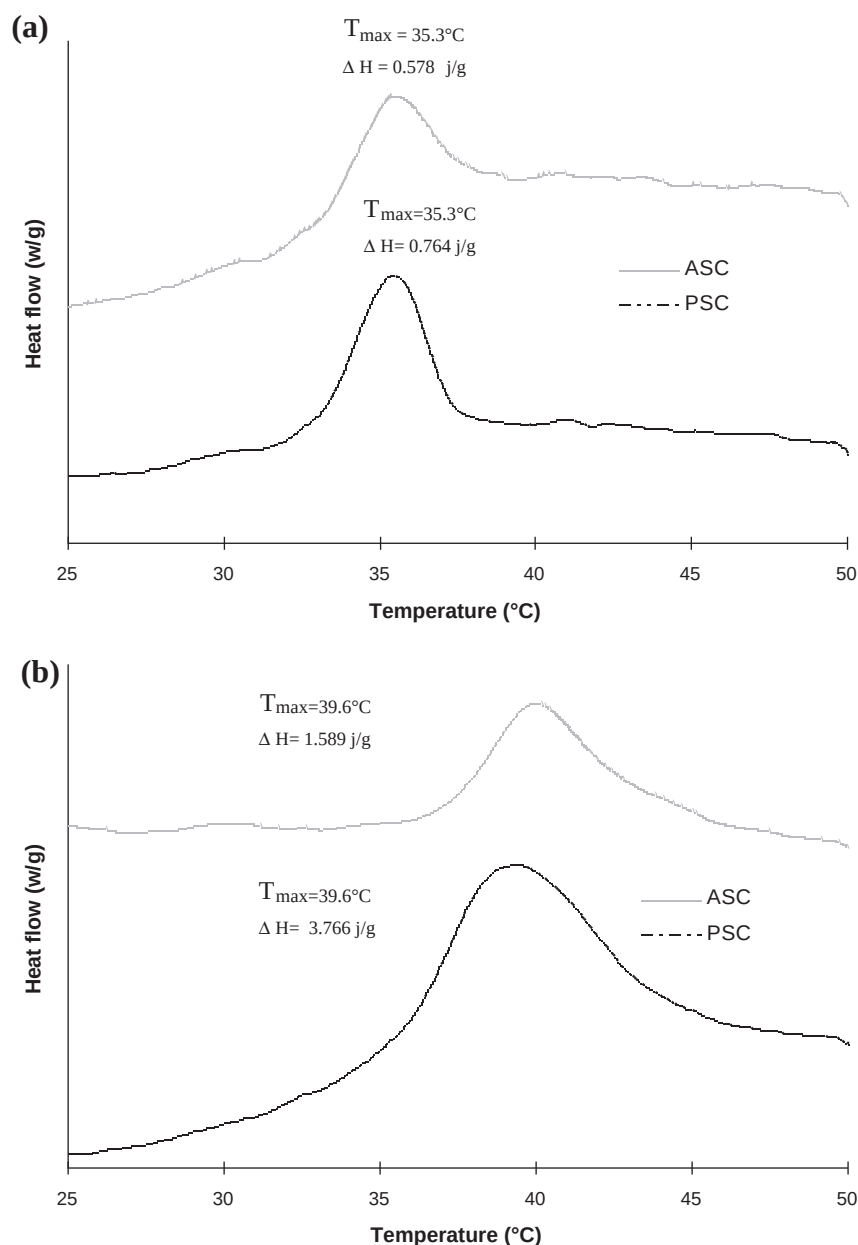


Fig. 4. DSC thermogram of ASC and PSC from the skin of striped catfish dispersed in 0.05 M acetic acid (a) and in deionised water (b).

Benjakul, Visessanguan, Nagai, et al., 2005b). There was a sharp decrease in solubility of both ASC and PSC at a pH higher than 4

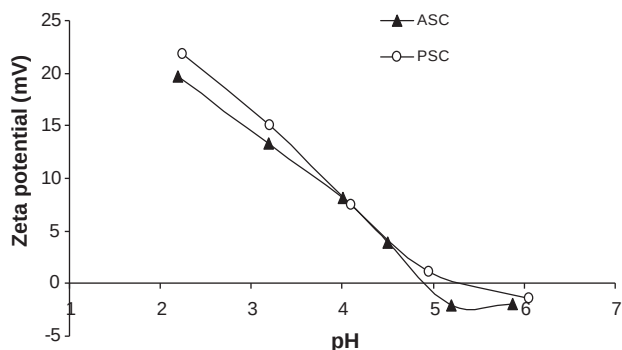


Fig. 5. Zeta ( $\zeta$ ) potential of ASC and PSC from the skin of striped catfish at different pH.

( $P < 0.05$ ). Marked decreases in solubility were observed in the neutral and alkaline pH ranges. When the pH is lower or higher than pI, the net charge of protein molecules are greater and the solubility is increased by the repulsion forces between chains (Vojdani, 1996). In contrast, when the total net charges of protein molecules are zero, the hydrophobic–hydrophobic interaction increases, thereby leading to the precipitation and aggregation at the pI. It has been reported that collagen has isoelectric points ranging from pH 6 to 9 (Foegeding et al., 1996). The lowest solubility of ASC and PSC was obtained at around pH 7. This result was in accordance with the solubility of collagen from trout muscle and skin, which was lowest at pH 7 (Montero et al., 1991). From the result, ASC had a solubility profile similar to PSC.

### 3.8.2. Effect of salt concentration

The effect of NaCl on the solubility of ASC and PSC is shown in Fig. 6b. Solubility of ASC and PSC in 0.5 M acetic acid remained constant in the presence of NaCl at concentrations of 0–2% ( $P > 0.05$ ).

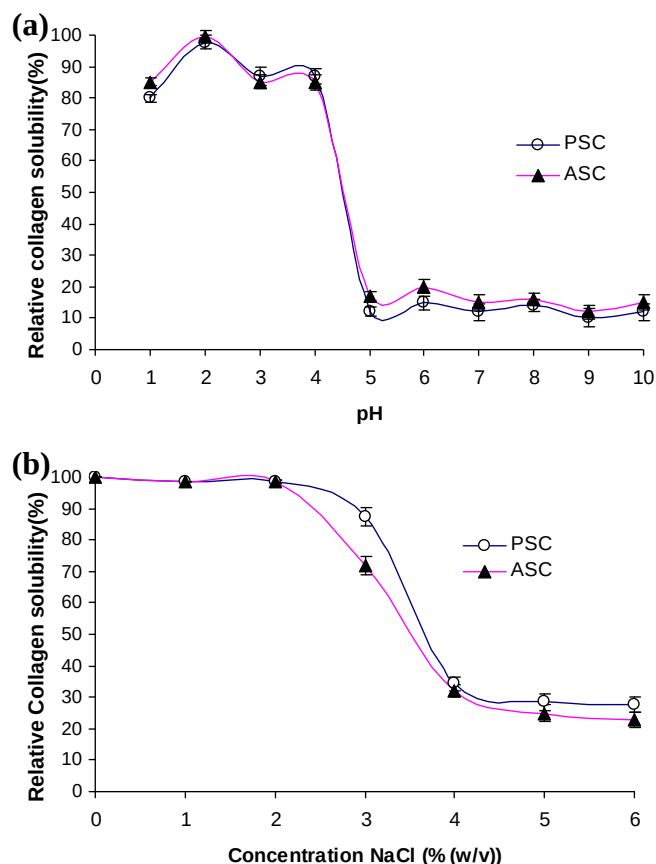


Fig. 6. Relative solubility (%) of ASC and PSC from the skin of striped catfish as affected by different pH (a) and NaCl (b) concentrations.

However a slight decrease in solubility was observed at 3% NaCl. A drastic decrease was noticeable when the concentration was increased to 4% and above ( $P < 0.05$ ). The solubility of collagens from the skin of trout, hake, bigeye snapper (*Priacanthus tayenus*), and bigeye snapper (*Priacanthus marcracanthus*) in acetic acid solution generally decreased with increasing NaCl concentration (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2005a; Kittiphattananabawon et al., 2005; Montero et al., 1991). The decrease in solubility of collagens could be described as being due to a 'salting out' effect, which occurred at relatively high NaCl concentrations (Asghar & Henrickson, 1982). An increase in ionic strength causes a reduction in protein solubility by enhancing hydrophobic-hydrophobic interactions between protein chains, and increasing the competition for water with the ionic salts, leading to the induced protein precipitation (Vojdani, 1996). Similar behaviours were found for ASC and PSC. However, PSC showed a greater solubility than ASC at NaCl concentrations greater than 2%. The result was in accordance with that of Jongjareonrak, Benjakul, Visessanguan, Nagai, et al. (2005). A greater solubility of PSC could be due to the partial hydrolysis of high MW cross-linked molecules by pepsin. In addition, the differences in amino acid compositions and structure between ASC and PSC might result in such differences.

#### 4. Conclusion

Both ASC and PSC were successfully isolated from the skin of striped catfish and classified as type I collagen. Telopeptides of ASC were cleaved by pepsin, resulting in the increased yield of PSC. Both ASC and PSC had a much higher  $T_{max}$ , as compared to those from temperate and cold-water fish. ASC and PSC with a pI of 6.21 and 6.56, respectively, showed high solubility in the acidic

pH ranges. However, the solubility decreased in the presence of NaCl at concentrations above 2%. Pepsin aided extraction can serve as a tool for obtaining the greater yield without a marked effect on the triple-helical structure. Therefore, the skin of striped catfish could be an alternative source of collagen for further applications.

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# Isolation, characterisation and stability of myoglobin from Eastern little tuna (*Euthynnus affinis*) dark muscle

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## ABSTRACT

This study aimed to purify and characterise the myoglobin from the dark muscle of Eastern little tuna (*Euthynnus affinis*). Myoglobin purified by ammonium sulphate precipitation (65–100% saturation), followed by Sephadex G-75 chromatography had a molecular weight of 15,680 Da. The isoelectric point (pI) of both oxymyoglobin (OxyMb) and metmyoglobin (MetMb) was estimated to be 5.25, as determined by zeta potential analysis. Transition temperatures were 61 and 60 °C, for OxyMb and MetMb, respectively. The colour values ( $L^*$ ,  $a^*$  and  $b^*$ ) and absorption spectra of the myoglobin solutions differed significantly, depending upon the form of myoglobin. OxyMb and MetMb had the Soret bands at 413 and 407 nm, respectively. The pH and thermal stability of myoglobin were tested under a pH range of 3–11 and a temperature range of 4–70 °C, respectively. Loss of haem–globin complex and autoxidation were dominant at pH 3, as evidenced by the disappearance of the Soret band and the formation of MetMb. Heating at temperature above 60 °C had a great impact on myoglobin denaturation. With increasing temperature and incubation time, OxyMb was susceptible to oxidation and conformational changes, whilst MetMb tended to be more stable. Thus, the form of myoglobin governed its properties and stability.

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## 1. Introduction

Myoglobin has been known to be a major contributor to the colour of muscle, depending on its redox states (DeoMb, OxyMb and MetMb), and concentration (Faustman & Cassens, 1990). Furthermore, myoglobin has a close relationship with lipid oxidation, which could influence the deterioration of foods. The lipid oxidation in fish muscle was promoted by the oxidation of myoglobin, which played a considerable role in the generation of hydroperoxides (Sohn et al., 2005).

Myoglobin is made up of a single polypeptide chain, globin and a prosthetic haem group, an iron (II) protoporphyrin-IX complex (Pegg & Shahidi, 1997). The molecular weight of myoglobin is determined to be 14–18 kDa; fish myoglobin is generally smaller than the mammalian counterpart (Chaijan, Benjakul, Visessanguan, & Faustman, 2007; Fosmire & Brown, 1976; Satterlee & Zachariah, 1972; Ueki & Ochiai, 2004; Yamaguchi, Takeda, Ogawa, & Hashimoto, 1979). The stability of myoglobin varies with species, due to the different amino acid sequences and secondary structures of globin. Chaijan et al. (2007) demonstrated that sardine myoglobin was prone to oxidation and denaturation at temperature above

40 °C, and at very acidic or alkaline pHs. Moreover, the rate of myoglobin autoxidation was related to oxygen concentration (Faustman & Cassens, 1990). The stability of myoglobin, which affects colour, as well as the lipid oxidation of meat, is associated with its autoxidation rate. Tuna, has been known to provide one of the most remarkable red muscles, in which myoglobin is found at high concentrations (Chow, Ochiai, & Watabe, 2004; Ueki & Ochiai, 2004). To obtain fish with high quality during storage, the suppression of myoglobin oxidation is required.

Tuna and tuna products are widely consumed in many parts of the world because they are rich in proteins. Tuna viscera were used as the raw materials for fish sauce production (Dissaraphong, Benjakul, Visessanguan, & Kishimura, 2006). Additionally, the tuna head has been used as a source of fish oil, as it contains a high amount of  $\omega$ -3 fatty acids (Chantachum, Benjakul, & Sriwirat, 2000). Eastern little tuna (*Euthynnus affinis*) is a species available in the Gulf of Thailand and the Indian Ocean, and provides a high global economic value as a low-mercury tuna for canning and sashimi (Fisheries Foreign Affairs Division, 2007; Hajeb et al., 2009). However, no information regarding the myoglobin of the Eastern little tuna has been reported. The objective of this investigation was to purify and characterise the myoglobin from Eastern little tuna dark muscle, and to study the properties and stability of myoglobin over a wide range of pHs and temperatures.

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## 2. Materials and methods

### 2.1. Chemicals

Sodium dodecyl sulphate (SDS),  $\beta$ -mercaptoethanol ( $\beta$ ME), Triton X-100, pyridine, bathophenanthroline disulphonic acid and wide range protein markers were purchased from Sigma (St. Louis, MO, USA). Acrylamide,  $N,N,N',N'$ -tetramethylethylenediamine (TEMED) and bis-acrylamide were purchased from Fluka (Buchs, Switzerland). Sodium dithionite was obtained from Riedel (Seeize, Germany). Sodium chloride, trichloroacetic acid, sodium nitrite and iron standard solution were procured from Merck (Damstadt, Germany).

### 2.2. Fish samples

Five Eastern little tuna (*E. affinis*) with the average weight of 0.5–0.55 kg were obtained from the dock in Songkhla province, Thailand. The fish were placed in ice with a fish/ice ratio of 1:2 (w/w), and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla. The fish were immediately washed, filleted and manually excised. The dark muscles were collected, pooled as the composite sample, and minced until uniformity was obtained. The mince was kept in ice for 1 h prior to myoglobin extraction.

### 2.3. Purification of myoglobin from Eastern little tuna

Extraction and purification of myoglobin was performed according to the method of Trout and Gutzke (1996) with a slight modification. Eastern little tuna mince (100 g) was mixed with 300 ml of cold extracting medium (10 mM Tris–HCl, pH 8.0, containing 1 mM EDTA and 25 g/l Triton X-100). The mixture was homogenised for 1 min at a speed of 13,000 rpm using an IKA Labortechnik homogeniser (Selangor, Malaysia). After centrifugation at 9600g for 10 min at 4 °C using an Avanti J-E centrifuge (Beckman Coulter, Palo Alto, CA, USA), the supernatant was filtered through a Whatman No. 4 filter paper. The filtrate referred to as “crude myoglobin extract” was subjected to ammonium sulphate fractionation (65–100% saturation). The precipitate obtained was dissolved in a minimal volume of cold 5 mM Tris–HCl buffer, pH 8.5, which was referred to as the “starting buffer”. The mixture was then dialysed against 10 volumes of the same buffer, with 20 changes, at 4 °C. The dialysate was immediately applied onto a Sephadex G-75 column (2.6 × 70 cm; Amersham Bioscience, Uppsala, Sweden), which was equilibrated with the starting buffer. The separation was conducted using the starting buffer at a flow rate of 0.5 ml/min. Fractions of 3 ml were collected and measured at 280 and 540 nm using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). The fractions with the higher absorbance at 540 nm were pooled and subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

#### 2.3.1. SDS–PAGE

The purity and molecular weight (MW) of myoglobin were determined by SDS–PAGE, according to the method of Laemmli (1970). Protein samples were mixed at a 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol and 10%  $\beta$ ME). Samples were loaded onto polyacrylamide gels comprising a 4% stacking gel and a 17.5% running gel, and were subjected to electrophoresis at a constant current of 15 mA/gel, using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, the gel was stained with 0.02% (w/v) Coomassie Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid, and was destained with 25% (v/v) methanol and 10%

(v/v) acetic acid. A wide range of markers [myosin (205 kDa),  $\beta$ -galactosidase (116 kDa), phosphorylase (97 kDa), fructose-6-phosphate kinase (84 kDa), albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa),  $\alpha$ -lactalbumin (14.2 kDa) and aprotinin (6.5 kDa)], were used for MW estimation.

#### 2.3.2. Preparation of OxyMb and MetMb

OxyMb and MetMb were prepared according to the method of Tang, Faustman, and Hoagland (2004) with some modifications. An aliquot (5 ml; 2 mg protein/ml) of purified myoglobin was converted to OxyMb by the addition of 50 mg of sodium dithionite. MetMb was prepared by adding 50 mg of potassium ferricyanide to 5 ml of the purified myoglobin solution. The sodium dithionite and potassium ferricyanide were removed by dialysis of the sample against 10 volumes of cold 50 mM phosphate buffer, pH 7.0; the dialysis buffer was changed 20 times. The protein concentrations of the dialysates, representing OxyMb and MetMb, were adjusted to 0.2 mg protein/ml.

#### 2.3.3. Determination of protein concentration

The protein concentrations of the myoglobin solutions were determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). Bovine serum albumin was used as the protein standard.

### 2.4. Characterisation of Eastern little tuna myoglobin

#### 2.4.1. Colour

The colours of OxyMb and MetMb solutions (0.2 mg protein/ml) were measured and expressed as  $L^*$ ,  $a^*$  and  $b^*$  values using a colourimeter (Juki Corp, Tokyo, Japan).  $L^*$ ,  $a^*$  and  $b^*$  values represented lightness, redness/greenness and yellowness/blueness, respectively.

#### 2.4.2. Absorption spectra

The absorption spectra of OxyMb and MetMb solutions were taken using a spectrophotometer. The spectra were recorded from 350 to 750 nm at a scanning rate of 1000 nm/min using 50 mM phosphate buffer, pH 7.0, as blank.

#### 2.4.3. Proportion of myoglobin forms

The proportions of the three myoglobin forms; deoxymyoglobin (DeoMb), OxyMb and MetMb were calculated by a modified Krzywicki's equation (Tang et al., 2004) as follows:

$$[\text{DeoMb}] = -0.543R_1 + 1.594R_2 + 0.552R_3 - 1.329$$

$$[\text{OxyMb}] = 0.722R_1 - 1.432R_2 - 1.659R_3 + 2.599$$

$$[\text{MetMb}] = -0.159R_1 - 0.085R_2 + 1.262R_3 - 0.520$$

where  $R_1 = A_{582}/A_{525}$ ,  $R_2 = A_{557}/A_{525}$  and  $R_3 = A_{503}/A_{525}$ .

#### 2.4.4. Differential scanning calorimetry (DSC)

DSC thermograms of OxyMb and MetMb solutions were determined according to the method of Chaijan et al. (2007) using a Perkin Elmer Differential Scanning Calorimeter (DSC) (Model DSC-7, Norwalk, CT, USA). Temperature calibration was performed using the Indium thermogram. The specimen (about 12 mg) was accurately weighed, placed into an aluminium pan, and sealed. Scanning was performed at a heating rate of 2 °C/min over a temperature range of 20–100 °C. The iced water was used as a cooling medium and the system was equilibrated at 20 °C for 5 min prior to the scan. The empty aluminium pan was used as a reference. The temperature at each peak maximum was recorded

as the maximum transition temperature ( $T_m$ ) and the total denaturation enthalpy ( $\Delta H$ ).

#### 2.4.5. Zeta potential analysis

Zeta ( $\zeta$ ) potential of OxyMb and MetMb solutions was measured by a Zeta potential analyser model ZetaPALS (Brookhaven Instruments Co., Holtsville, NY, USA). Solutions (20 ml) were transferred to an autotitrator model, BI-ZTU (Brookhaven Instruments Co., Holtsville, NY, USA), in which the pHs of solutions were adjusted to 2–12, using either 1.0 M nitric acid or 1.0 M KOH. The obtained zeta potential of each solution at all pHs was recorded.

#### 2.5. Effect of pH and temperature on the properties and stability of Eastern little tuna myoglobin

The solutions of OxyMb and MetMb were dialysed against 10 volumes of cold 50 mM phosphate buffers, with various pH values (pHs 3, 5, 7, 9 and 11), for 15 h, according to the method of [Chaijan et al. \(2007\)](#). The protein concentration of myoglobin solutions was adjusted to 0.2 mg/ml with the corresponding buffer. All procedures were carried out at 4 °C.

The thermal stability of OxyMb and MetMb was also studied. Three millilitre of OxyMb and MetMb solution were pipetted into a test tube and incubated at 4, 20, 30, 40, 50, 60 and 70 °C in an ice bath, or a temperature controlled water bath (Mettler, Schwabach, Germany), for 10, 30 and 60 min, according to the method of [Chaijan et al. \(2007\)](#). At the designated time, the samples were taken for analyses.

##### 2.5.1. Haem protein degradation and myoglobin oxidation

The haem protein degradation of the treated solutions was determined by the changes in absorption spectra and Soret band ([Swatland, 1989](#)). To determine the oxidation of myoglobin, the proportions of three myoglobin forms; DeoMb, OxyMb and MetMb were calculated following a modified Krzywicki's equations ([Tang et al., 2004](#)).

##### 2.5.2. Myoglobin aggregation ratio

The treated myoglobin solutions were filtered through a Millipore filter (pore size, 0.45  $\mu$ m) (Millipore, Bedford, MA, USA). The absorbance of the filtrate was determined at 280 nm. The percentage of the absorbance decrement to the untreated original absorbance was calculated as the myoglobin aggregation ratio ([Chow et al., 2004](#)).

##### 2.5.3. Tryptophan fluorescence

To monitor the changes in protein structure, tryptophan fluorescence of the treated myoglobin solutions was measured with a RF-1501 fluorometer (Shimadzu, Kyoto, Japan) at an excitation wavelength of 280 nm and an emission wavelength of 325 nm according to the method of [Chanthai, Ogawa, Tamiya, and Tsuchiya \(1996\)](#).

##### 2.5.4. Non-haem iron content

The non-haem iron content of treated myoglobin solutions was determined according to the method of [Schricker, Miller, and Stouffer \(1982\)](#) with a slight modification. One millilitre of OxyMb and MetMb was pipetted into a screw cap test tube, and 50  $\mu$ l of 0.39% (w/v) sodium nitrite were added. Four millilitre of a mixture of 40% trichloroacetic acid and 6 M HCl (ratio of 1:1 (v/v), freshly prepared) were added. The tightly capped tubes were incubated at 65 °C for 22 h, and then cooled at room temperature for 2 h. The supernatant (400  $\mu$ l) was mixed with 2 ml of the non-haem iron colour reagent, a mixture of bathophenanthroline disulfonic acid, double-deionised water and saturated sodium acetate solution, at a ratio of 1:20:20 (w/v/v), freshly prepared. After vortexing

and standing for 10 min, the absorbance was measured at 540 nm. The non-haem iron content was calculated from an iron standard curve. Iron standard solutions [ $\text{Fe}(\text{NO}_3)_3$  in  $\text{HNO}_3$ ], with concentrations ranging from 0 to 2 ppm, were used.

#### 2.6. Statistical analysis

Experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by the Duncan's multiple range test. For pair comparison, *t*-test was used ([Steel & Torrie, 1980](#)). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

### 3. Results and discussion

#### 3.1. Purification of myoglobin from Eastern little tuna dark muscle

Crude myoglobin extract from Eastern little tuna dark muscle was subjected to ammonium sulphate precipitation, followed by gel filtration using the Sephadex G-75 column. After being chromatographical using the Sephadex G-75 column, two major peaks exhibiting  $A_{280}$  were obtained ([Fig. 1A](#)). The second peak had a brown colour, thus it most likely contained myoglobin as the major component.

The crude myoglobin extract and its fractions, i.e., the 65–100% ammonium sulphate and Sephadex G-75 fractions, were analysed

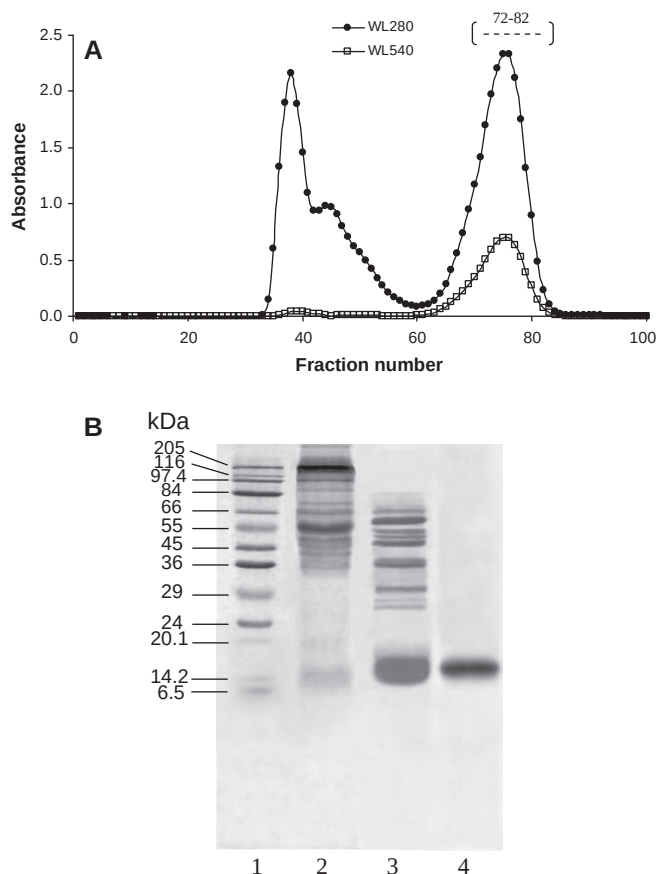


**Fig. 1.** Elution profile of myoglobin from Eastern little tuna dark muscle on Sephadex G-75 (A), and SDS-PAGE pattern of the crude myoglobin extract and its fractions (B). Lane 1, wide range markers; lane 2, crude myoglobin extract; lane 3, ammonium sulphate precipitated fraction; lane 4, Sephadex G-75 fraction (fraction Nos. 72–82).

for their protein patterns using SDS–PAGE as shown in Fig 1B. Crude myoglobin extract contained a large number of proteins with MW greater than 36 kDa (Fig. 1B, lane 2). After ammonium sulphate precipitation (65–100% saturation), proteins with MW higher than 84 kDa were discarded. Coincidentally, the band intensity of the proteins with MW lower than 36 kDa, especially with MW of 15 kDa, became more intense (Fig. 1B, lane 3). The contaminating proteins with MW higher than 15 kDa could be further removed by Sephadex G-75 gel filtration chromatography. Pooled fractions (fraction Nos. 72–82) showed a single band on the SDS–PAGE, suggesting the homogeneity of the purified protein. The MW of Eastern little tuna myoglobin was estimated to be 15,680 Da. The obtained MW was similar to that of other tuna species, including bigeye tuna (15,540 Da) and yellowfin tuna (16,000 Da), but was larger than that of mackerel (14,900 Da) and sardine (14,600 Da) (Fosmire & Brown, 1976; Ueki & Ochiai, 2004; Yamaguchi et al., 1979). The MW of myoglobin from porcine, ovine and sperm whale were 17,700, 17,100, and 17,000 Da, respectively. The MW of fish myoglobin is generally smaller than mammalian myoglobin (Satterlee & Zachariah, 1972).

### 3.2. Colour and absorption spectra of OxyMb and MetMb

The colours of myoglobin from Eastern little tuna with a concentration of 0.2 mg protein/ml, were expressed as  $L^*$  (lightness),  $a^*$  (redness) and  $b^*$  (yellowness) values; these were 79.12, 11.11 and 33.30 for the OxyMb solution, and 82.72, 4.22 and 38.85 for the MetMb solution, respectively. A higher  $a^*$  value and a lower  $b^*$  value for the OxyMb solution in comparison with the MetMb solution ( $P < 0.05$ ) indicated that OxyMb was more reddish than MetMb. Myoglobin autoxidation was found to cause an increase in the  $L$  value and a decrease in the  $a$  value (Chen & Chow, 2001). Therefore, a higher  $L^*$  value with a coincidentally lower  $a^*$  value for the MetMb solution suggested the autoxidation of myoglobin. The colour of fresh meat is governed by the relative amounts of three forms of myoglobin; DeoMb, OxyMb and MetMb (Lindahl, Lundstrom, & Tornberg, 2001). DeoMb is the purple pigment of deep muscles. Upon exposure to air, myoglobin combines with oxygen to form the bright red OxyMb, which is considered attractive by the consumer. However, the contact of myoglobin with oxygen also leads to the formation of the oxidised form, MetMb, which is brown or grey, and rather unattractive (Faustman & Cassens, 1990).

The absorption spectra of OxyMb and MetMb solutions from dark muscle of Eastern little tuna are depicted in Fig. 2A and B. The intense peaks in the blue region (350–450 nm) corresponding to the Soret bands, were found at 413 and 407 nm for OxyMb and MetMb solutions, respectively. The Soret bands of OxyMb and MetMb purified from tuna (*Thunnus albacares*) were noticeable at 414 and 406 nm (Smulevich et al., 2007); the bands of OxyMb and MetMb from meat were noticeable at 416 and 410 nm, respectively (Swatland, 1989). A Soret band results mainly from the interaction of the haem moiety with apomyoglobin, hence it can be used to monitor the unfolding of hemoproteins (Chen & Chow, 2001). In the region of 450–750 nm, the peaks were found at wavelengths of 542 and 577 nm for the OxyMb solution and 502 and 630 nm for the MetMb solution. Similar spectra patterns were reported for the equine heart myoglobin, which had peak wavelengths at 544 and 582 nm for OxyMb, and 503 and 632 nm for MetMb (Tang et al., 2004). Tang et al. (2004) demonstrated that three redox forms of myoglobin resulted in individually different absorption spectra, which had an isobestic point at 525 nm. The maximum wavelengths at 503, 557 and 582 nm representing MetMb, DeoMb and OxyMb, respectively, have been used for determining the relative proportions of the myoglobin redox forms in aqueous solution (Tang et al., 2004). The OxyMb solution had the highest

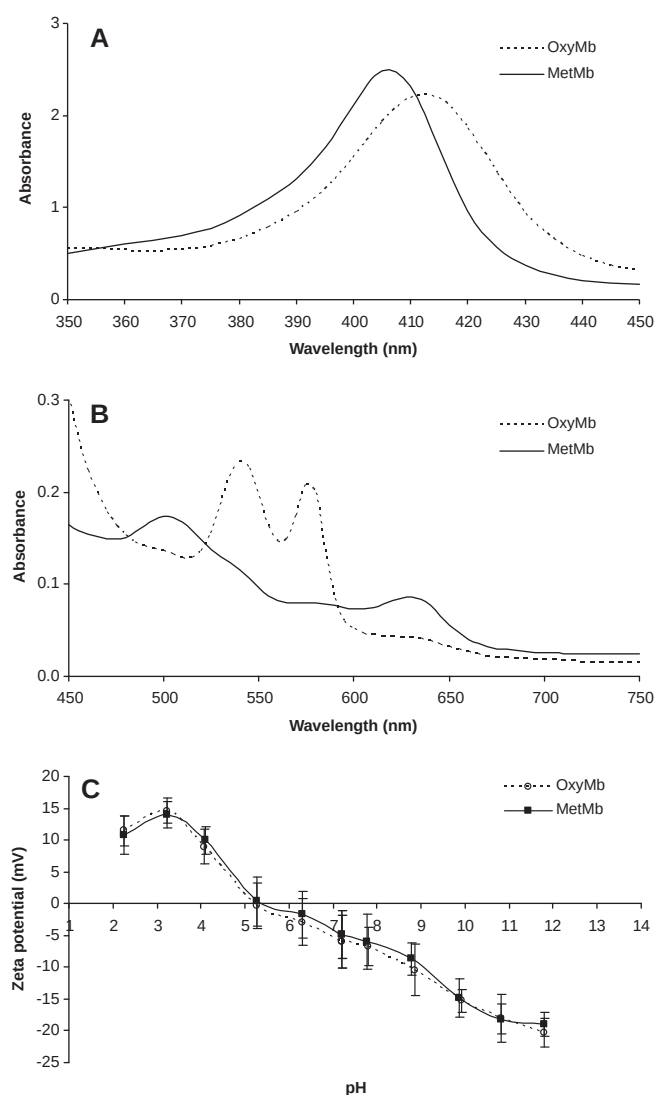


Fig. 2. The absorption spectra in the region of 350–450 nm (A), 450–750 nm (B), and the zeta potential (C) of OxyMb and MetMb from Eastern little tuna dark muscle as a function of pH. Bars represent standard deviation ( $n = 3$ ).

proportions of OxyMb (61.88%), followed by MetMb (30.45%) and DeoMb (7.67%). This indicated that the oxidation of OxyMb to MetMb more likely occurred during the preparation of OxyMb solution. For the MetMb solution, MetMb was dominant (91.47%); DeoMb (4.53%) and OxyMb (4.00%) were present at lower levels.

### 3.3. Differential scanning calorimetry (DSC) of OxyMb and MetMb

Transition temperatures ( $T_m$ ) and total denaturation enthalpy ( $\Delta H$ ) of OxyMb and MetMb were analysed by DSC. A  $T_m$  value of 61 and 60 °C and a  $\Delta H$  value of 0.105 and 0.519 J/g were found for OxyMb and MetMb, respectively. Chen, Lin, and Chen (2004) reported that the  $T_m$  of myoglobin from migratory, demersal, aquaculture and elasmobranch fish species was in the range of 46.4–71.8 °C. The thermal stability of myoglobin varies with fish species due to the different amino acid sequence and the secondary structure of globin (Chen et al., 2004; Colonna, Irace, Bismuto, Servillo, & Balestrieri, 1983). The low conformational stability of fish myoglobin in comparison with mammalian myoglobin has been reported (Chanthai et al., 1996; Colonna et al., 1983). A slight difference in  $T_m$  was observed between OxyMb, the ferrous form, and MetMb, the ferric form. For bovine myoglobin,  $T_m$  was 74.0 °C for OxyMb,

73.7 °C for DeoMb and 73.3 °C for MetMb (Sepe et al., 2005). For sardine myoglobin, MetMb was most easily denatured by heat when compared with DeoMb and OxyMb (Chaijan et al., 2007). Thus, oxygen or water molecules, which could bind with iron within the porphyrin ring, might contribute to the conformational stability of all forms of myoglobin.

### 3.4. Zeta potential of OxyMb and MetMb

The zeta potential of OxyMb and MetMb from Eastern little tuna as a function of pH, is shown in Fig 2C. A similar surface charge was observed for both OxyMb and MetMb over the whole pH range (pH 2–12) ( $P > 0.05$ ), most likely due to the same amino acid composition of globin. Both forms of myoglobin exhibited positive charges at acidic pH, whilst negative charges were obtained at pH greater than 5.25. The differences in the surface net charge at different pH were governed by the protonation or deprotonation of amino acids, particularly those localised on the surface of molecules. When pH is close to the isoelectric point (pI), the net charge of protein is zero (Copeland, 1994; Pace, Trevino, Prabhakaran, & Scholtz, 2004). A zero net charge of both forms of myoglobin was observed at pH 5.25, which presumably corresponded to the pI of the myoglobin from this species. Mackerel and sardine myoglobins have pI of 5.8–5.9, whilst horse myoglobin have pI of 8.1 (Shiraki, Kudou, Fujiwara, Imanaka, & Takagi, 2002; Yamaguchi et al., 1979).

### 3.5. Effect of pH on properties and stability of OxyMb and MetMb

The absorption spectra of OxyMb and MetMb from Eastern little tuna at varying pH values, are displayed in Fig. 3. In the region of 350–450 nm, the Soret bands disappeared at pH 3, for the OxyMb and MetMb, indicating a weak haem–globin complex at extremely acidic pH. Low pH could reduce the stability of the haem–globin linkage and increase the autoxidation rate (Chaijan et al., 2007; Renerre, Anton, & Gatellier, 1992). At pH 3, the proportions of the MetMb form were the highest for both the OxyMb solution (67.87%) and the MetMb solution (96.14%). This result confirmed the autoxidation, as indicated by MetMb formation at acidic pH. The Soret bands were noticed over a pH range of 5–11 for both forms of myoglobin. However, the peaks of both forms decreased in area and shifted to the higher wavelength, as pH increased. These pointed out a degradation of the haem structure and a change of myoglobin into a reduced form under alkaline condition. On the other hand, the neutral pH yielded a compact and unique

spatial structure of myoglobin (Chen et al., 2004). When pH increased from 5 to 11, the proportions of OxyMb increased from 25.31% to 57.69% for OxyMb, and from 4.32% to 25.86% for MetMb. Phillips, Teodoro, Li, Smith, and Olson (1999) reported the occurrence of hydroxyl-MetMb at high pH, which appears to be similar to OxyMb. In a pH range of 5–9, OxyMb exhibited a greater change in the absorption spectra than did MetMb. This indicated that OxyMb was less stable than MetMb, in those pH ranges.

Based on the absorption spectra of both myoglobin forms in the region of 450–750 nm, the OxyMb peak was dominant at pH  $\geq 7$ , indicating the stability of OxyMb at neutral to alkaline pHs (Fig. 3B). For the MetMb solution, the typical absorption spectra of MetMb were obtained at pH 5 and 7, suggesting the stability of MetMb at neutral to slightly acidic pH (Fig. 3D). According to the Bohr's effect, the proton-catalysed displacement process is mainly responsible for promoting the autoxidation of myoglobin at acidic pHs, whilst the oxygenation of myoglobin might occur at alkaline pHs (Renerre et al., 1992).

The tryptophan fluorescent intensity of OxyMb and MetMb from Eastern little tuna, in a pH range of 3–11, is shown in Fig. 4A. Tryptophan is involved in forming favourable and specific tertiary interactions in the native apomyoglobin structure (Ivana, Simona, Pier Luigi, Rita, & Gaetano, 2000). The fluorescence properties of the tryptophan residues can be used to determine the polarity of its local environment and the unfolding of proteins (Copeland, 1994). For both OxyMb and MetMb, the highest intensity in tryptophan fluorescence was noticeable at pH 3 ( $P < 0.05$ ). At acidic pH, an enhanced protonation of myoglobin might induce conformational changes, as well as the unfolding of apomyoglobin, as evidenced by an increase in fluorescent intensity. Acid-denatured myoglobin is associated with protein unfolding and a loss of the haem group (Chaijan et al., 2007). At pH 5, which was close to the pI of myoglobin from Eastern little tuna, the haem–globin complex was compact and stable. However, the tryptophan fluorescent intensity decreased as pH increased up to pH 11 ( $P < 0.05$ ), indicating the stability of both myoglobin forms at very alkaline pH. This result was in agreement with the highest peak amplitude in absorption spectra (Fig. 3). Pace et al. (2004) reported that hydrophobic clusters and other native-like structures of protein could exist even under strongly denaturing conditions. A similar behaviour was previously reported in sardine myoglobin, which exhibited the highest fluorescent intensity at very acidic pH, whereas the lowest intensity was found at neutral to alkaline pH (Chaijan et al., 2007). The tryptophan fluorescent intensity of

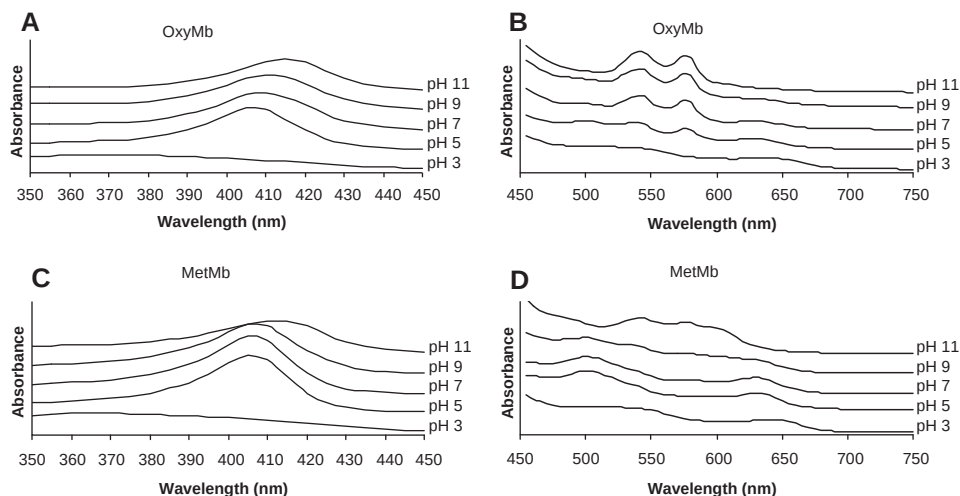


**Fig. 3.** Effect of pH on the absorption spectra at 4 °C for OxyMb in the region of 350–450 (A), and 450–750 nm (B), and for MetMb in the region of 350–450 (C) and 450–750 nm (D) from Eastern little tuna dark muscle.

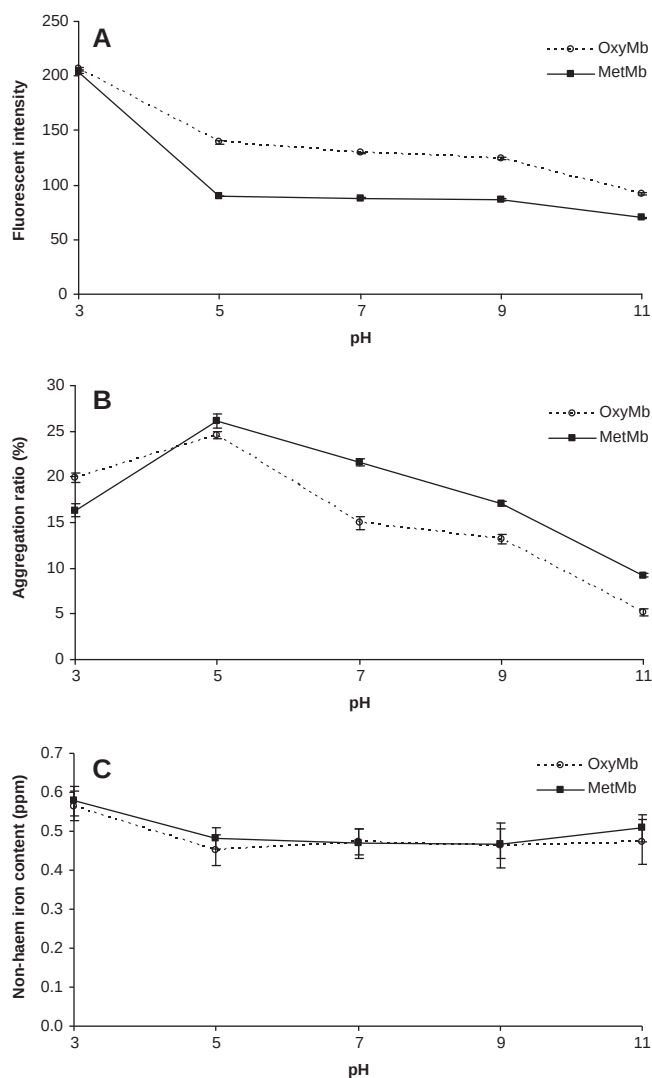


**Fig. 4.** Effect of pH on tryptophan fluorescent intensity (A), aggregation ratio (B) and non-haem iron content (C), at 4 °C of OxyMb and MetMb from Eastern little tuna dark muscle. Bars represent standard deviation ( $n = 3$ ).

OxyMb was higher than that of MetMb when tested at a pH range of 5–11 ( $P < 0.05$ ). The result suggested that OxyMb, the ferrous form, was more susceptible to unfolding or conformational changes than was MetMb, the ferric form.

The aggregation ratio of OxyMb and MetMb at varying pH is depicted in Fig. 4B. The highest aggregation ratio for both myoglobin solutions was observed at pH 5, which was 24.60% and 26.14% for OxyMb and MetMb, respectively ( $P < 0.05$ ). This indicated that the highest compactness of the haem–globin complex was at a pH close to the pI (5.25) of the purified myoglobin from Eastern little tuna. At the pI, the charges on the protein surface are generally arranged so that there are more attractive than repulsive interactions (Pace et al., 2004). The aggregation ratio for both myoglobin solutions decreased when the pH was further from pH 5, and showed the lowest values at pH 11 (5.20% for OxyMb and 9.19% for MetMb) ( $P < 0.05$ ). The charges of the proteins in a pH significantly different from the pI contribute favourably to the repulsive interactions, which destabilise the native states of the proteins (Pace et al., 2004). At pH above 5, a higher aggregation ratio was found in the MetMb solution ( $P < 0.05$ ). The native state of myoglobin is characterised by a tightly folded conformation with a haem group bound to the protein in a hydrophobic pocket (Pegg & Shahidi, 1997). The conformational changes of apomyoglobin, induced by hydrogen ions or hydroxyl ions, could lead a susceptible haem group to destruction or autoxidation (Chaijan et al., 2007; Renner et al., 1992).

The non-haem iron content of OxyMb and MetMb in a pH range of 3–11 is depicted in Fig. 4C. No differences in non-haem iron content were found between the OxyMb and the MetMb solutions ( $P > 0.05$ ), suggesting that the myoglobin forms had no effect on iron releasing from the haem structure, at all pHs tested. For both forms of myoglobin, no differences in the non-haem iron content were observed in a pH range of 5–11 ( $P > 0.05$ ), whilst the highest non-haem iron content was observed at pH 3 (0.56 ppm for OxyMb and 0.58 ppm for MetMb) ( $P < 0.05$ ). These results suggested that the disruption of the porphyrin ring probably occurred at very acidic pH, leading to the release of free iron, named “non-haem iron”. Normally, three edges of the porphyrin are buried in the protein interior and are stabilised by hydrophobic interactions with the apolar side chains that line the haem pocket. The fourth edge contains the solvent-exposed haem propionates, which interact electrostatically with the polar amino acid side chains located on the surface of the protein (Liong, Dou, Scott, Olson, & Phillips,

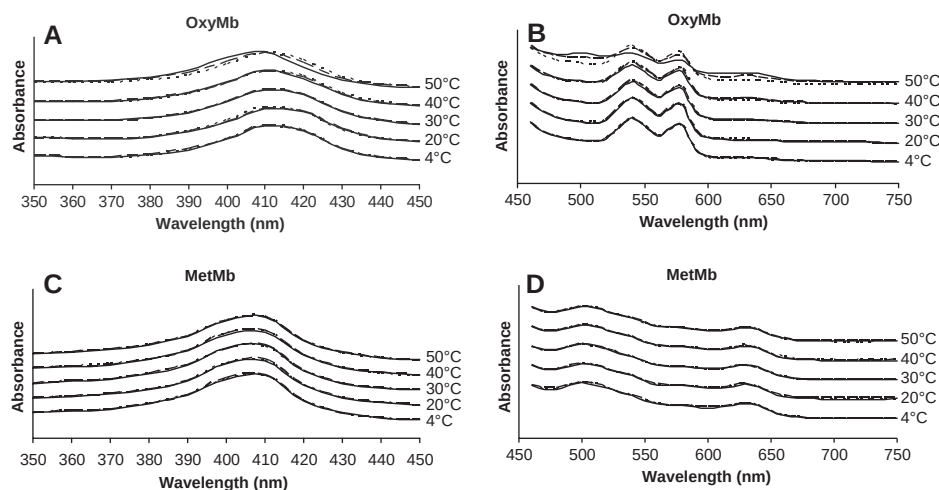


**Fig. 5.** Effect of heating temperatures and time on the absorption spectra at pH 7 for OxyMb in the region of 350–450 (A), and 450–750 nm (B), and for MetMb in the region of 350–450 (C) and 450–750 nm (D), from Eastern little tuna dark muscle: 10 min (.....), 30 min (- - - -) and 60 min (—).

2001). In accordance with the result of the Soret band, the destabilised haem–globin linkage and the haem destruction, might promote the release of iron from haem for both forms of myoglobin at pH 3. It was most likely that the porphyrin ring in the hydrophobic pocket of myoglobin was stable under neutral and alkaline condition. Tang, Kalsbeck, Olson, and Bocian (1998) reported that the unfolding of apoprotein and the hydration of the haem pocket were the key factors in the cleavage of the iron–histidine bond in myoglobin.

### 3.6. Effect of temperature on properties and stability of OxyMb and MetMb

The absorption spectra of OxyMb and MetMb from Eastern little tuna, incubated at 4–70 °C, are shown in Fig. 5. Both forms of myoglobin were precipitated when the temperature reached 60 °C. This coincided with the result of DSC, in which the transition temperature of myoglobin from Eastern little tuna was 60–61 °C. Below the denaturation temperature, the denaturation proceeded slowly, but it was very fast at temperatures above the denaturation one (Chen et al., 2004). A negligible change in the absorption spectra was observed for the MetMb solution when it was heated up to 50 °C, for both the 350–450 nm and the 450–750 nm regions, regardless of heating time, indicating the high stability of MetMb. For the OxyMb solution, the change in the absorption spectra was noticeable. With elevated temperatures, the Soret bands of OxyMb were slightly decreased in intensity and shifted to a lower wavelength, suggesting that OxyMb changed into an oxidised form. Moreover, the disappearance of OxyMb spectra in the region of 450–750 nm was found when OxyMb was heated at 50 °C for 60 min. With increasing temperature and incubation time, the proportions of the MetMb form in the OxyMb solutions increased by 34.64% whereas the OxyMb form decreased by 39.64% ( $P < 0.05$ ). The higher the temperature and time, the higher was the autoxidation rate of myoglobin (Chaijan et al., 2007).

The tryptophan fluorescent intensity of the OxyMb and MetMb solutions heated at varying temperatures and times, is depicted in Fig. 6A. For the OxyMb solution, the fluorescent intensities increased with increasing temperature up to 60 °C ( $P < 0.05$ ), whilst no changes were noticeable in the MetMb solution ( $P > 0.05$ ). Both myoglobin solutions showed a sharp increase in fluorescent intensities when the heating temperature was 70 °C ( $P < 0.05$ ). Additionally, the longer the incubation time, the greater was the increase in the fluorescent intensity ( $P < 0.05$ ). At higher temperature and longer time, unfolding of globin might have occurred, causing the exposure of tryptophan and tyrosine residues. From the result, the OxyMb solution had a greater intensity of tryptophan fluorescence than did the MetMb solution over the heating temperatures used ( $P < 0.05$ ). Heating brings about a modification of the tryptophan environment in the molecule. The change in the protein structure exposes the haem to the surroundings, thereby increasing the pro-oxidative activity of the heated myoglobin (Kristensen & Andersen, 1997).

The aggregation ratio of the OxyMb and MetMb solutions, subjected to heating at varying temperatures, is shown in Fig. 6B. For the OxyMb solution, a negligible change in the aggregation ratio was observed at 4 and 20 °C ( $P > 0.05$ ). At temperatures above 20 °C, the aggregation ratios increased with increasing temperature and incubation time (4.34–82.53% for OxyMb, and 1.97–55.75% for MetMb) ( $P < 0.05$ ). No marked changes in the aggregation ratios were noticeable in the MetMb solution heated at a temperature range of 4–60 °C. The sharpest increase in the aggregation ratios was found at 70 °C ( $P < 0.05$ ). The native state of a protein in solution is characterised by a highly specific and tightly folded conformation. With increasing temperature and longer times, the subsequent aggregation of globin might be en-

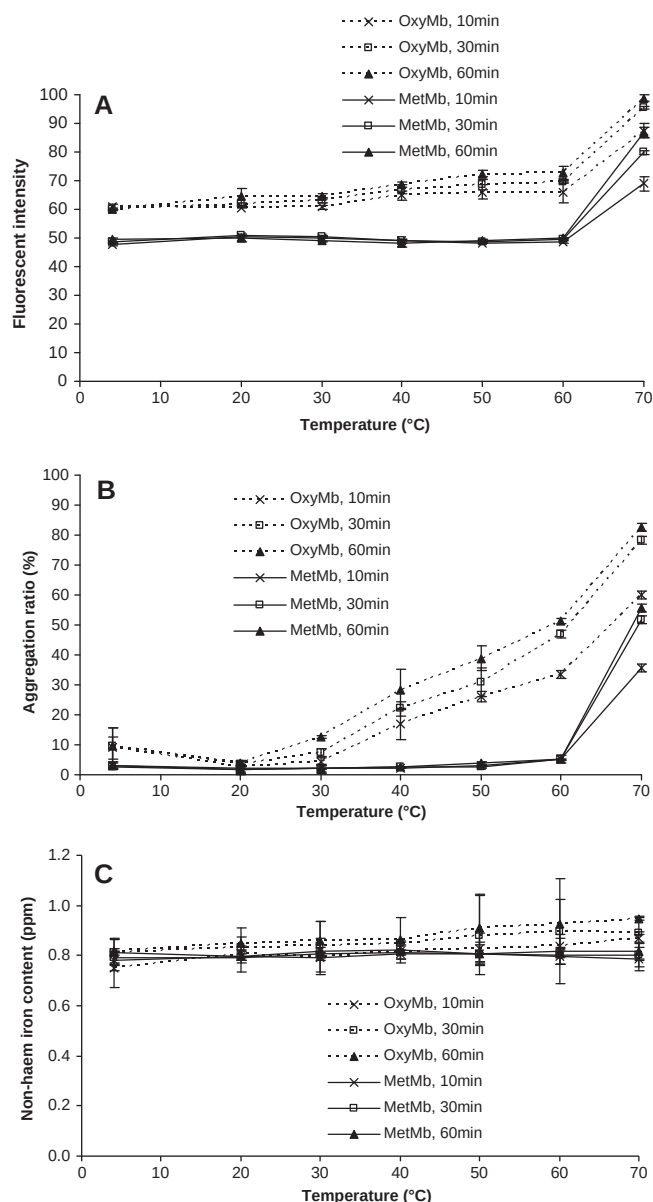


Fig. 6. Effect of heating temperatures on the tryptophan fluorescent intensity (A), aggregation ratio (B), and non-haem iron content (C), at pH 7 of OxyMb, and MetMb from Eastern little tuna dark muscle. Bars represent standard deviation ( $n = 3$ ).

hanced via hydrophobic interaction of unfolded molecules. Cope-land (1994) reported that elevated temperatures could significantly increase the rate of collisions between the unfolded protein molecules. The MetMb solution required a higher temperature for aggregation, indicating a smaller conformational change for MetMb, in comparison with OxyMb, over a wide range of temperatures.

The release of the non-haem iron content from OxyMb and MetMb at varying temperatures and times is shown in Fig. 6C. When temperature increased up to 70 °C, no increase in the non-haem iron content for MetMb solution was observed ( $P > 0.05$ ), whereas an increase was found in the OxyMb solution, with increasing temperature and time (0.75–0.95 ppm) ( $P < 0.05$ ). Kristensen and Andersen (1997) reported a slight increase in the free iron (0.46–0.50 ppm) from horse heart MetMb after heating at temperatures between 25 and 90 °C. The result suggested that increasing temperature up to 70 °C for 60 min had no effect on the haem degradation and iron released from the MetMb solution, whilst the

conformational changes of apomyoglobin in OxyMb solution might have weakened a porphyrin ring, leading to iron released. The three important functions of the globin portion of myoglobin are to sequester haem, enhance coordination with the proximal histidine, and inhibit oxidation of the iron atom (Liong et al., 2001). The released iron might act as a pro-oxidant and has been associated with enhanced lipid oxidation. However, Kristensen and Andersen (1997) revealed that heating haem iron had a more significant effect on pro-oxidative activity than did heating free iron. Therefore, a suppression of any conformational changes would be required to maintain the haem structure, and consecutively decrease the pro-oxidative activity of myoglobin.

#### 4. Conclusions

Myoglobin isolated from dark muscle of Eastern little tuna with MW of 15,680 Da had different colour and absorption spectra, depending on its forms, OxyMb and MetMb. pH and heating could induce the destabilisation of myoglobin. The tuna myoglobin was prone to oxidation and denaturation at pH 3 and temperatures above 60 °C. The MetMb form was more stable than OxyMb. Processing of tuna muscle at mild conditions could reduce the changes of myoglobin, in which colour changes and pro-oxidative activity can be retarded. By this way, the quality of tuna meat with a high market value could be maintained during handling or distribution.

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## Low molecular weight trypsin from hepatopancreas of freshwater prawn (*Macrobrachium rosenbergii*): Characteristics and biochemical properties

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### ABSTRACT

Trypsin was purified to homogeneity from hepatopancreas of freshwater prawn (HFWP) (*Macrobrachium rosenbergii*) using a series of chromatographies including Q-Sepharose, Superdex 75 and MonoQ columns. HFWP trypsin was purified 525-fold with a yield of 10.6%. Based on native-PAGE, the purified trypsin showed a single band. Trypsin had a molecular weight of 17 kDa as estimated by SDS-PAGE. The optimal pH and temperature for Boc-Val-Pro-Arg-MCA hydrolysis were 8.0 and 55 °C, respectively. Trypsin was stable to heat treatment up to 40 °C, and over a pH range of 7.0–11.0. Trypsin activity was strongly inhibited by soybean trypsin inhibitor, N-p-tosyl-L-lysine chloromethyl ketone (TLCK) and Pefabloc SC and was partially inhibited by ethylenediaminetetraacetic acid (EDTA). Apparent  $K_m$  value of trypsin was 0.24  $\mu$ M and  $K_{cat}$  value was 607.56 s<sup>-1</sup> for Boc-Val-Pro-Arg-MCA. The N-terminal amino acid sequence of 20 residues of HFWP trypsin was IVGGDEAAPGEFPHQISMVQV, which was highly homologous with those from other species of prawn. HFWP trypsin also showed high collagenolytic activity toward prawn, shrimp and fish collagens, suggesting its possible role in muscle softening of freshwater prawn during extended storage.

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### 1. Introduction

Freshwater prawn (FWP) is widely consumed in Thailand due to its delicacy. However, this species is not successfully marketable due to the loss in textural acceptability. During postmortem handling and storage, prawn meat becomes soft and mushy, probably as a result of indigenous proteases. Softening of meat was developed within 4 days of iced storage and was most pronounced in the first segment of abdomen (Sriket, Benjakul, & Visessanguan, 2010). Sriket, Benjakul, and Visessanguan (2011a) reported that proteases from FWP muscle had very low proteolytic activity and no collagenolytic activity, whilst protease from hepatopancreas showed high hydrolytic activity on natural actomyosin (NAM) and pepsin soluble collagen (PSC) extracted from FWP muscle.

Shrimp hepatopancreas has been reported to have high proteolytic activities (Oh, Kim, Kim, & Kim, 2000) and most digestive enzymes in crustaceans were classified to be serine protease and metalloprotease (Garcia-Carreño, Patricia Hernandez-Cortes, & Haard, 1994). Amongst proteolytic enzymes, serine collagenase has an impact on the softening of muscle (Ezquerro Brauer, Salazar Leyva,

Bringas Alvarado, & Rouzaud Sandez, 2003). Hepatopancreas from white shrimp, *Penaeus vannamei* (Ezquerro, Garcia-Carreño, & Haard, 1997) and Northern shrimp, *Pandalus eous* (Aoki, Ahsan, Matsuo, Hagiwara, & Watabe, 2003) possessed collagenolytic/gelatinolytic activities. Recently, serine protease, mainly trypsin-like protease released from hepatopancreas, was reported to cause the softening of FWP muscle during extended iced storage (Sriket, Benjakul, Visessanguan, & Kishimura, 2011b). Therefore, better understanding of trypsin from FWP, especially from the digestive organs, could pave the way to prevent or lower the activity of those proteases, thereby retarding the quality loss of FWP. Nevertheless, no information regarding the characteristic and biochemical properties of purified trypsin from hepatopancreas of freshwater prawn (HFWP) has been reported. Therefore, this study aimed to purify and characterise trypsin responsible for the muscle degradation from HFWP.

### 2. Materials and methods

#### 2.1. Chemicals

Q-Sepharose, Superdex 75 and MonoQ were obtained from Amersham Pharmacia Biotech AB (Uppsala, Sweden). t-Butyloxy-carbonyl-Val-Pro-Arg-4-methyl-coumaryl-7-amide (Boc-Val-Pro-Arg-MCA) and other synthetic fluorogenic peptide substrates

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(MCA-substrates) were purchased from Peptide Institute (Osaka, Japan). Pefabloc SC was obtained from Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA), pepstatin A, soybean trypsin inhibitor, N-p-tosyl-L-lysine chloromethyl ketone (TLCK), N-tosyl-L-phenyl-alanine chloromethyl ketone (TPCK), 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), N-ethylmaleimide,  $\beta$ -mercaptoethanol ( $\beta$ ME), bovine gelatin and bovine serum albumin were procured from Sigma Chemical Co. (St. Louis, MO, USA.). Protein molecular weight markers were obtained from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals used were of analytical grade.

## 2.2. Sample preparation

Live FWP (*M. rosenbergii*) with a size of 30–35 prawn/kg were obtained from a farm in Phatthalung province, Thailand. The samples were ice-shocked with iced-water (0 °C). The hepatopancreas was removed manually and immediately mixed with liquid nitrogen. The frozen sample was placed in a polyethylene bag, sealed and stored at –40 °C until use.

## 2.3. Preparation of hepatopancreas extract

Hepatopancreas were homogenised with three volumes of acetone at –20 °C for 3 min using an IKA homogeniser (Model T25, Selangor, Malaysia) according to the method of Klomklao, Benjakul, Kishimura, Osako, and Tanaka (2010). The homogenate was filtered *in vacuo* through Whatman No. 4 filter paper. The residue obtained was then homogenised in two volumes of acetone at –20 °C for 3 min, and then the residue was air-dried in a vacuum desiccator until the sample was dry and free of acetone odour. Defatted hepatopancreas powder obtained was stored at –40 °C until use.

To prepare the hepatopancreas extract, the powder was suspended in 50 mM Tris–HCl, pH 7.5 containing 5 mM CaCl<sub>2</sub> referred to as “starting buffer; SB” at a ratio of 1:10 (w/v) and stirred continuously at 4 °C for 3 h. The suspension was centrifuged at 12,000g for 10 min at 4 °C using a refrigerated centrifuge (Kubota, Model 7780II, Tokyo, Japan) to remove the tissue debris, and the supernatant obtained was referred to as “crude extract”.

## 2.4. Purification of trypsin from FWP hepatopancreas

All purification processes were carried out at 4 °C in the laboratory of Faculty of Fisheries, Nagasaki University, Japan. Fractions obtained from all purification steps were subjected to the measurement of protein content and trypsin activity. Protein concentration during purification steps was determined by monitoring the absorbance of sample solution at 280 nm.

After filtration through a 0.2- $\mu$ m filter, the sample was then applied to a Q-Sepharose anion exchange column (3.0  $\times$  45 cm), which was equilibrated with approximately two-bed volumes of SB. The sample was loaded onto the column at a flow rate of 0.5 ml/min. The column was washed with SB until A<sub>280</sub> was less than 0.05 and then eluted with a linear gradient of 0.0–0.6 M NaCl in SB at a flow rate of 0.5 ml/min. Fractions of 5 ml were collected and the fractions with trypsin activity were pooled and concentrated by ultrafiltration with YM-10 membrane (Millipore, Bedford, MA, USA).

The concentrated Q-Sepharose fractions were filtered with a 0.2- $\mu$ m filter and further subjected to a Superdex 75 column (2.6  $\times$  60 cm) previously equilibrated with approximately two-bed volumes of SB. The elution was performed with the same buffer at a flow rate of 0.5 ml/min. Fractions of 6 ml were collected and those with trypsin activity were pooled and further purified by a MonoQ anion exchange column previously equilibrated with SB. The sample was loaded onto the column at a flow rate of

0.5 ml/min. The column was washed with SB until A<sub>280</sub> was less than 0.05 and then eluted with a linear gradient of 0.0–0.6 M NaCl in SB at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected and the fractions with trypsin activity were pooled, dialysed with SB for 12 h and used for further study.

## 2.5. Trypsin activity assay

Trypsin activity was measured by the method of Yoshida et al. (2009) using Boc-Val-Pro-Arg-MCA as a substrate. Appropriately diluted enzyme (100  $\mu$ l) was added to 800  $\mu$ l of 50 mM Tris–HCl buffer (pH 8.0). The reaction was immediately initiated by the addition of 100  $\mu$ l of 50  $\mu$ M substrate and incubated at 55 °C for 10 min. To stop the reaction, 1.5 ml of the stopping agent (methyl alcohol:n-butyl alcohol:distilled water = 35:30:35, v/v/v) was added. The fluorescence intensity of liberated 7-amino-4-methylcoumarin (AMC) was measured by a spectrofluorometer (Model RF-1500, Shimadzu Co., Kyoto, Japan) at the excitation wavelength of 380 nm and the emission wavelength of 450 nm. One unit of enzyme activity was defined as the amount of the enzyme, which released 1  $\mu$ mol AMC per min.

## 2.6. pH and temperature profile

Trypsin activity was assayed over the pH range of 4.0–11.0 (50 mM acetate buffer for pHs 4.0–7.0; 50 mM Tris–HCl buffer for pHs 7.0–9.0 and 50 mM glycine–NaOH for pHs 9.0–11.0) at 55 °C for 10 min. For the temperature profile study, the activity was assayed at different temperatures (20, 30, 40, 50, 60, 70 and 80 °C) for 10 min at pH 8.0.

## 2.7. pH and thermal stability

The effect of pH on enzyme stability was evaluated by measuring the residual activity after incubation at various pHs for 30 min at 30 °C. Enzyme solution was mixed with different buffers mentioned above at a ratio of 1:1 (v/v). For thermal stability, the enzyme solution was mixed with 100 mM Tris–HCl, pH 8.0 at a ratio of 1:1 (v/v) and incubated at different temperatures (20, 30, 40, 50, 60, 70 and 80 °C) for 15 min in a temperature controlled water bath (Thermo Minder Mini-80, Taiyo, Tokyo, Japan). Thereafter, the treated samples were suddenly cooled in iced water. The residual activity was assayed using Boc-Val-Pro-Arg-MCA as a substrate at pH 8.0 and 55 °C for 10 min.

## 2.8. Effect of inhibitors

The effect of inhibitors on trypsin activity was determined according to the method of Klomklao, Benjakul, Visessanguan, Kishimura, and Simpson (2007). Enzyme solution was mixed with an equal volume of protease inhibitor solution to obtain the final concentration designated (0.1 mM E-64, 1.0 g/l soybean trypsin inhibitor, 5 mM pefabloc SC, 5 mM TLCK, 5 mM TPCK, 1 mM pepstatin A and 10 mM EDTA). The mixture was allowed to stand at room temperature (26–28 °C) for 15 min. Thereafter, the remaining activity was measured and percent inhibition was calculated.

## 2.9. Determination of protein concentration

Protein concentration was determined by monitoring the absorbance of sample solution at 280 nm.

## 2.10. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed according to the method of Laemmli (1970). Protein solutions were mixed at 1:1 (v/v) ratio with the

SDS–PAGE sample buffer (0.125 M Tris–HCl, pH 6.8; 4% SDS; 20% glycerol; 10%  $\beta$ -mercaptoethanol) and boiled for 3 min. The samples (10  $\mu$ g) were loaded onto the gel made of 4% stacking and 15% separating gels and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Protean II Cell apparatus (Atto Co., Tokyo, Japan). After electrophoresis, the gels were stained with 0.1% Coomassie brilliant blue R-250 in 50% methanol and 7% acetic acid and destained with 7% acetic acid.

Native-PAGE was performed using 15% separating gels in a similar manner, except that the sample was not heated and SDS and reducing agent were left out.

### 2.11. Activity staining

Activity staining of purified trypsin towards bovine gelatin was performed according to the method described by Lødemel and Olsen (2003) with a slight modification. Purified trypsin was mixed with sample buffer (0.125 M Tris–HCl, pH 6.8; 4% SDS; 20% glycerol) at a ratio of 1:1 (v/v). The mixture containing 1  $\mu$ g protein was loaded onto the gel made of 4% stacking and 15% separating gels containing 0.1% bovine gelatin. The electrophoresis was conducted at 4 °C. After electrophoresis, the gel was washed twice in 2.5% Triton X-100 for 15 min. The remaining Triton X-100 was removed by washing the gel (three times) with 50 mM Tris–HCl buffer pH 7.0. Gel was incubated in 50 mM Tris–HCl, pH 8.0 containing 5 mM  $\text{CaCl}_2$  and 0.02%  $\text{NaN}_3$  at 37 °C for 6 h. The gel was rinsed using cold distilled water prior to staining and destaining, as described above. Development of clear zones on a blue background indicated gelatinolytic activity.

### 2.12. Determination of N-terminal amino acid sequence

Purified trypsin was subjected to SDS–PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was briefly stained by Coomassie brilliant blue (CBB) and the protein bands were excised. The N-terminal amino acid sequence of trypsin was determined using a protein sequencer (Shimadzu, Model PPSQ-33A, Kyoto, Japan).

### 2.13. Substrate specificity

To study the substrate specificity of the trypsin, various synthetic fluorogenic MCA-substrates were used and the activity of trypsin toward different substrates was determined as previously described.

### 2.14. Kinetic studies

The activity was assayed with different final concentrations of Boc-Val-Pro-Arg-MCA and Boc-Leu-Lys-Arg-MCA. The final enzyme concentration for the assay was 0.015  $\mu$ g/ml. The determinations were repeated twice and the respective kinetic parameters, including  $V_{\text{max}}$  and  $K_m$ , were evaluated by plotting the data on a Lineweaver–Burk double-reciprocal graph (Lineweaver & Burk, 1934). Values of turnover number ( $K_{\text{cat}}$ ) were calculated from the following equation:  $V_{\text{max}}/[E] = K_{\text{cat}}$ , where  $[E]$  was the active enzyme concentration.

### 2.15. Hydrolysis of various collagens by purified trypsin

Purified trypsin (100  $\mu$ l) with the activity of 0.01 unit determined by Boc-Val-Pro-Arg-MCA assay was added to the reaction mixture containing 4 mg collagen and 800  $\mu$ l of 50 mM Tris–HCl, pH 7.0, containing 5 mM  $\text{CaCl}_2$ . Pepsin soluble collagen (PSC) from freshwater prawn, PSC from white shrimp and acid soluble collagen (ASC) from yellowtail meats were isolated according to the method

of Sriket, Benjakul, Visessanguan, and Kijroongrojana (2007). The hydrolysis was conducted by incubating the mixture at 25 °C for 0, 5, 10 and 15 min. The control was performed in the same manner for 15 min, except distilled water was added instead of trypsin. The reaction was terminated by adding the preheated solution containing 2% SDS, 8 M urea and 2%  $\beta$ ME (85 °C). The mixture was further incubated at 85 °C for 60 min to solubilise total proteins. The solution was centrifuged at 5000g for 10 min at room temperature to remove the debris. The supernatant was then subjected to SDS–PAGE using 7.5% separating gel and 4% stacking gel.

### 2.16. Statistical analysis

The experiments were run in triplicate, except for the kinetic study, which was carried out in duplicate. Data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's multiple range test (DMRT) (Steel & Torrie, 1980). Statistical analyses were performed using the Statistical Package for Social Science (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA).

## 3. Results and discussion

### 3.1. Purification of HFWP trypsin

Purification steps of HFWP trypsin are summarised in Table 1. The purification of trypsin was started by separating the crude extract using a Q-Sepharose anion exchange column. After separation, the fractions with pronounced trypsin activity were pooled and a purity of 14.3-fold was observed. A single activity peak was obtained for the pooled Q-Sepharose fractions upon the subsequent chromatography on a Superdex 75 column (Fig. 1). The yield of 23.8% and the purity of 27.7-fold were observed after this gel filtration. The pooled fractions with trypsin activity were then subjected to MonoQ anion exchange column. Ion-exchange chromatography was used to remove contaminating proteins and to separate different trypsin isoforms (Klomklao et al., 2006b). Pooled fractions from single activity peak obtained after MonoQ anion exchange column showed high purity of 525-fold with 10.6% yield. The results showed that Q-Sepharose anion exchange, Superdex 75 and MonoQ anion exchange chromatographies effectively removed the contaminants from the crude extract. As a consequence, the purified trypsin was obtained.

### 3.2. Purity and molecular weight

Based on native gel-electrophoresis, there was only a single protein band (Fig. 2a), indicating the high purity of trypsin. HFWP trypsin also showed a single band on SDS–PAGE (Fig. 2b). Molecular weight of HFWP trypsin was estimated to be 17 kDa. This value was in accordance with that determined by Superdex 75 column (data not shown). The result suggested that HFWP trypsin was monomeric, however, the molecular weight of trypsin was quite

**Table 1**  
Purification of trypsin from hepatopancreas of freshwater prawn.

| Purification step | Total activity (units) | Total protein (mg) | Specific activity (units/mg protein) | Purity (fold) | Yield (%) |
|-------------------|------------------------|--------------------|--------------------------------------|---------------|-----------|
| Crude enzyme      | 1418.59                | 5827               | 0.24                                 | 1.0           | 100       |
| Q Sepharose       | 1037.45                | 301.9              | 3.44                                 | 14.3          | 73.1      |
| Superdex 75       | 337.27                 | 50.76              | 6.64                                 | 27.7          | 23.8      |
| MonoQ             | 150.09                 | 1.19               | 126                                  | 525           | 10.6      |

Trypsin activity was assayed at pH 8 and 55 °C for 10 min using Boc-Val-Pro-Arg-MCA as substrate.

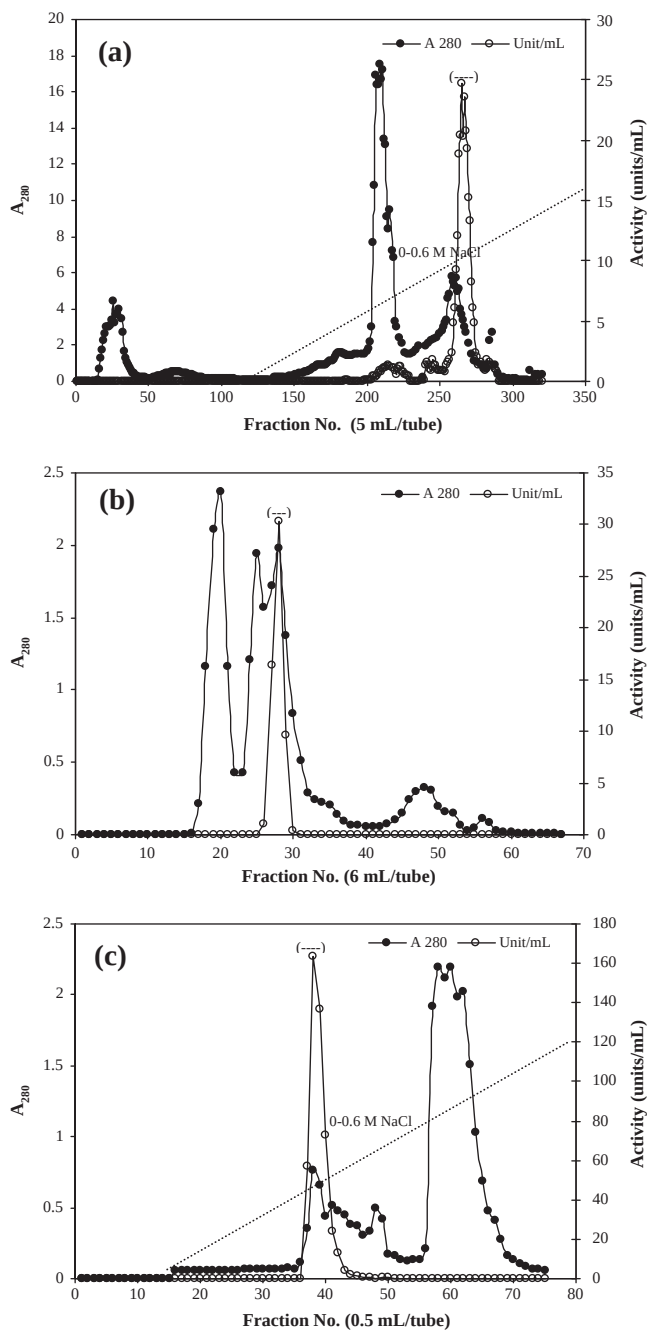


**Fig. 1.** Purification of trypsin from hepatopancreas of freshwater prawn. (a) Elution profile of trypsin on Q-Sepharose anion exchange column; (b) Superdex 75 gel filtration column and (c) MonoQ anion exchange column. Fractions under the bars were pooled.

different from those of mammalian, fish and shrimp trypsins. Trypsins purified from black tiger prawn (27–29 kDa) (Lu, Liu, & Tsai, 1990), white shrimp (*Penaeus setiferus*) (24 kDa) (Gates, 1969) and crayfish (*Procambarus clarkia*) (23.8, 27.9, 24.8 and 31.4 kDa) (Kim, Meyers, & Godber, 1992) have been reported. The activity band was confirmed by substrate gel electrophoresis, in which a clear band with the same molecular weight appeared on a blue background (Fig. 2c). The result indicated that purified HFWP trypsin was able to hydrolyse gelatin. Aoki et al. (2003) reported that four gelatinolytic proteases were found in hepatopancreas of Northern shrimp (*Pandalus eous*). Generally, trypsins have molecular weights between 20 and 25 kDa (Klomklao, 2008). The

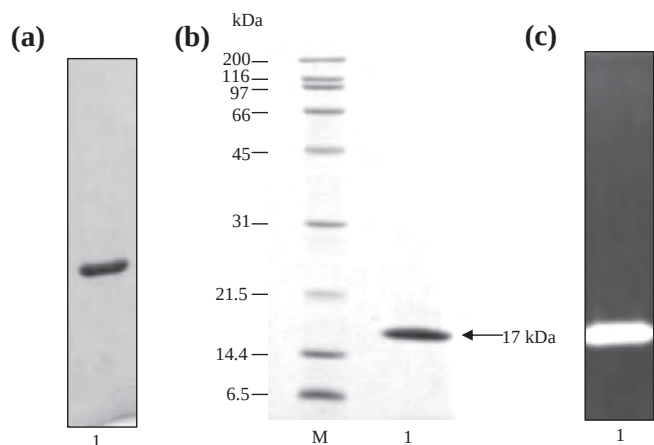


**Fig. 2.** Protein pattern from native-PAGE (a), SDS-PAGE (b) and activity staining (c) of purified trypsin from hepatopancreas of freshwater prawn. M, molecular weight standard; lane 1, trypsin.

molecular weight of trypsin-like enzymes from crustacea varies widely. This present study therefore firstly reported the trypsin with low molecular weight (17 kDa) in crustacean and fish. Nevertheless, the possibility that autolytic degradation causing such a low molecular weight should not be excluded (Lu et al., 2008).

### 3.3. pH and temperature profile

Purified HFWP trypsin had the increases in activity as the pH increased and reached the maximum at pH 8.0 ( $P < 0.05$ ) (Fig. 3a). The optimal pH of purified HFWP trypsin was in accordance with those of trypsins from other fish and shellfish (Castillo-Yanez, Pacheco-Aguilar, Garcia-Carreno, & Navarrete-Del Toro, 2005; Hjelmeland & Raa, 1982; Jiang, Moody, & Chen, 1991). Activity of trypsin decreased when pHs were higher than the optimal pH. The lowered activity at very acidic and alkaline pH was plausibly due to the conformational changes of enzyme under harsh conditions (Klomklao, Kishimura, Benjakul, Simpson, & Visessanguan, 2010).

The temperature profile of HFWP trypsin is depicted in Fig. 3b. The optimal temperature of trypsin was 55 °C ( $P < 0.05$ ) when Boc-Val-Pro-Arg-MCA was used as a substrate. The optimal temperature of purified HFWP trypsin was similar to that of trypsin from other crustacean, which ranges from 50 to 60 °C (Jiang et al., 1991; Maeda-Martinez, Obregon-Barboza, Navarrete-Del Toro, Obregon-Barboza, & Garcia-Carreno, 2000). However, the optimal temperature of purified HFWP trypsin was higher than those of trypsins obtained from hepatopancreas from other fish and shellfish such as Japanese sea bass (*Lateolabrax japonicus*) (40–45 °C) (Cai et al., 2011), carp (40–45 °C) (Cao et al., 2000) and crab (45 °C) (Galgani & Nagayama, 1987). The differences in optimal temperature might be governed by habitat, environment and genetic factors (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2006a). A sharp decrease in activity of HFWP trypsin at temperatures above 60 °C was most likely due to thermal denaturation.

### 3.4. pH and thermal stability

The pH stability of HFWP trypsin is shown in Fig. 3c. HFWP trypsin was stable in pH values ranging from 7.0 to 11.0. The inactivation was more pronounced at pH values below 7. A complete loss in activity was observed after incubating trypsin at pH 4. Osnes and Mohr (1985) reported that trypsins from krill, *Euphausia superba*, were inactivated at pH 4.5, and fish trypsins are stable only in alkaline pH (Simpson, 2000). The stability of trypsin at a

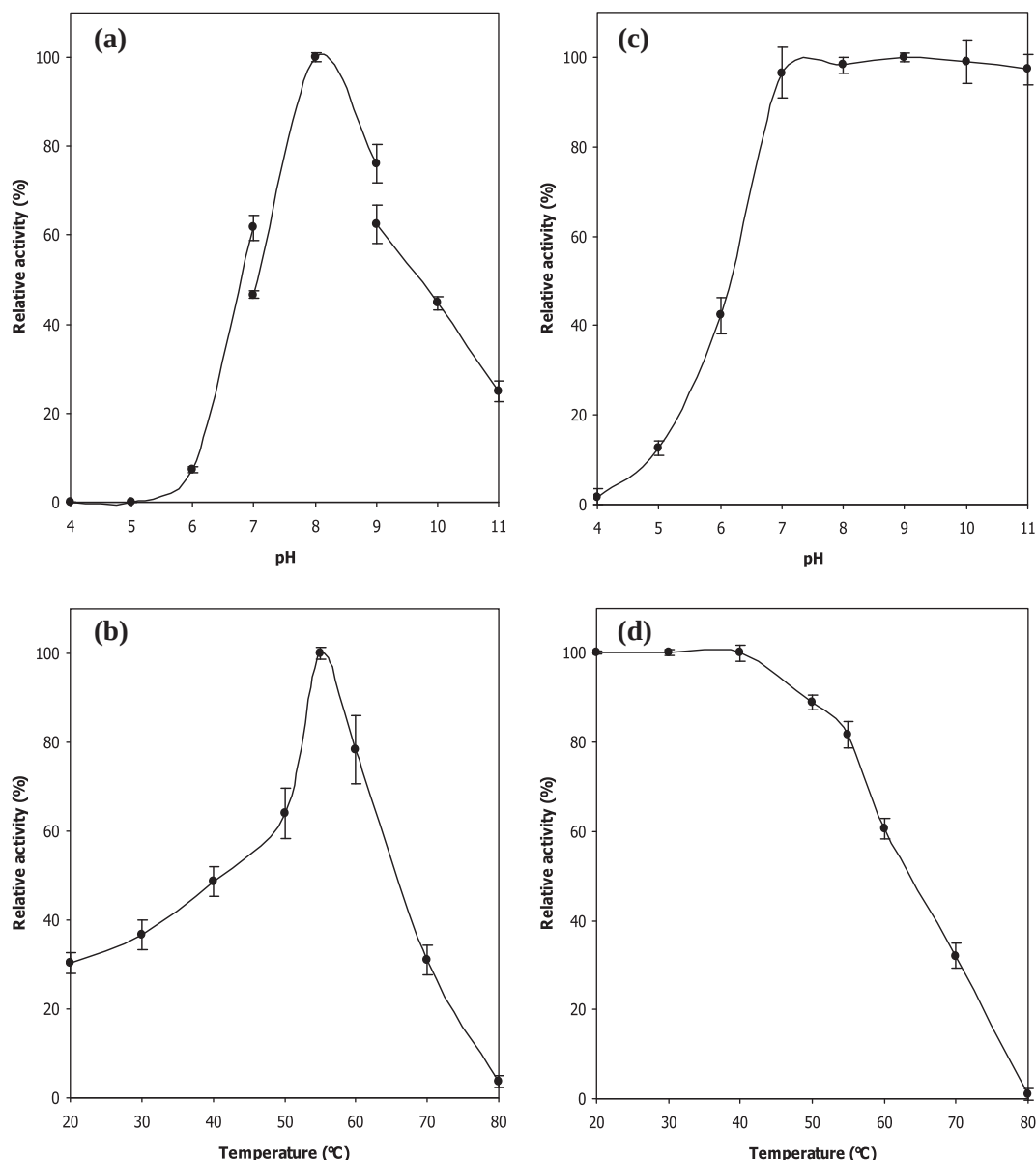


**Fig. 3.** pH and temperature profiles (a and b) and pH and thermal stabilities (c and d) of purified trypsin from hepatopancreas of freshwater prawn.

particular pH might be related to the balance of charge of the enzyme (Castillo-Yanez et al., 2005). A similar pH effect on activity has been reported for trypsin from several fish species (Cai et al., 2011; Castillo-Yanez et al., 2005; Klomklao, Kishimura, Nonami, & Benjakul, 2009). Thus, HFWP trypsin might undergo denaturation under the acidic conditions, where the conformational change took place and enzyme could not bind to the substrate properly. Inactivation at acidic pH is a common phenomenon for anionic tryptins (Kim et al., 1992).

Thermal stability of HFWP trypsin is depicted in Fig. 3d. HFWP trypsin was stable for 15 min when heated up to 40 °C (Fig. 3d). The loss in activity was noticeable after incubation at temperature above 40 °C for 15 min. When the enzyme was heated at sufficiently high temperature, the configuration could be altered. As a consequence, the lowered activity could be attained. In the presence of a substrate during the activity assay, the enzyme might be stabilised to some extent. On the other hand, the enzyme was directly subjected to heat treatment in the absence of a substrate for the thermal stability study. No trypsin activities remained after heat treatment at 80 °C. After heat treatment at 60 °C, activities of

approximately 60% were retained. At high temperatures, the enzyme more likely underwent denaturation and lost its activity. The results were in agreement with Kim et al. (1992) who reported that trypsin activity of crayfish hepatopancreas was stable up to 40 °C and activity almost disappeared when heated at temperatures higher than 60 °C. Trypsin from the hepatopancreas of Atlantic blue crab was stable at temperatures ranging from 30 to 50 °C for 30 min, but the activity was rapidly lost at temperatures above 50 °C (Dendinger & O'Connor, 1990). Brun and Wojtowicz (1976) reported that trypsin activity of rock crab hepatopancreas was reduced by 60% after incubation at temperatures >50 °C for 20 min. Enzymes are inactivated at high temperature due to the partial unfolding of their molecules. The mechanism for increasing thermal stability of proteins appears to be due to a strengthening of hydrophobic interactions and disulphide bonds in the interior of the protein molecule (Kim, Meyers, Pyeun, & Godber, 1994). Numerous disulphide linkages, as well as stronger hydrophobic interactions in the interior of the protein contribute to protein thermal stability. Disulphide bonds may stabilise a folded conformation of trypsin (Klomklao et al., 2009). Fish and shellfish tryptins had a lower

thermal stability than those from mammals, possibly caused by the lower number of intramolecular disulphide bonds in fish and shellfish trypsin, compared with the mammal counterpart (Klomklao et al., 2009; Lu et al., 1990). When the internal temperature during cooking was not sufficiently high, trypsin still retained and caused substantial softening of shrimp meat during storage or distribution.

### 3.5. Effect of inhibitors

The effect of various inhibitors on the activity of HFWP trypsin was determined as shown in Table 2. Trypsin was markedly inhibited by trypsin inhibitors, such as soybean trypsin inhibitor (99.23% inhibition), TLCK (97.94% inhibition) and pefabloc SC (99.35% inhibition). No differences in % inhibition were found among those three inhibitors ( $P > 0.05$ ). Soybean trypsin inhibitor is a single polypeptide that forms a stable stoichiometric enzymically inactive complex with trypsin, thereby reducing the availability of trypsin. TLCK is a competitive inhibitor with trypsin. When the enzyme binds to TLCK at active site, the substrate cannot be hydrolysed. Pefabloc SC or 4-(2-Aminoethyl)-benzenesulfonylfluoride hydrochloride (AEBSF) is an irreversible proteinase inhibitor with broad specificity for serine proteinases. TPCK is a competitive inhibitor of chymotrypsin. Inhibitors for cysteine and aspartic proteases involving E-64 and pepstatin A did not show inhibitory effects towards trypsin activity. No inhibition was also observed when TPCK, a specific inhibitor for chymotrypsin, was used. The result confirms that this purified enzyme was serine proteases, most likely trypsin. Kim et al. (1992) reported that the activities of trypsin from hepatopancreas of crayfish were inhibited by soybean trypsin inhibitors and TLCK. The enzymatic activities of two trypsin from the hepatopancreas of Japanese sea bass were also effectively inhibited by pefabloc SC (Cai et al., 2011). EDTA, which chelates the metal ions required for the enzyme, and partially lowered trypsin activity (9.71% inhibition). Removal of calcium ions might affect trypsin structure, resulting in some losses in activity. The result suggests that trypsin most likely required metal ions as cofactors for activity. It has been reported that calcium ions are required for fish and crustacean trypsin (Klomklao et al., 2009; Zwilling, Pfeleiderer, Sonneborn, Kraft, & Stucky, 1969). However, it was noted that soybean trypsin inhibitor effectively inhibited trypsin from hepatopancreas of FWP. Thus, the extracts from soybean or other legumes containing trypsin inhibitor could be used to inhibit the proteolytic activity from hepatopancreas, which was more likely released to the flesh and caused the softening of this species.

### 3.6. Substrate specificity

Among all fluorescent MCA-substrates tested, Boc-Val-Pro-Arg-MCA and Boc-Leu-Lys-Arg-MCA were hydrolysed by purified

HFWP trypsin more effectively than other specific synthetic substrates. The result suggested that the substrates containing arginine (Boc-Phe-Ser-Arg-MCA) or lysine (Boc-Glu-Lys-Lys-MCA and Boc-Val-Leu-Lys-MCA) residues at the P1 site were more preferable for trypsin (Table 3). Nevertheless, the substrate containing arginine residue was hydrolysed more than that with lysine residue (Table 3). A similar result was obtained for other fish trypsin (Cai et al., 2011), however, chymotrypsin substrates (Suc-Leu-Leu-Val-Tyr-MCA and Suc-Ala-Ala-Pro-Phe-MCA), cathepsin L substrate (Z-Phe-Arg-MCA) and aminopeptidase substrate (Arg-MCA) were not hydrolysed by HFWP trypsin. These results strongly indicated that the purified HFWP was trypsin.

Since HFWP trypsin could cleave arginine and lysine residues, this enzyme might be responsible for muscle softening of this species during extended iced storage. FWP meat was found to have a high content of arginine (34.91 mg/100 g) and lysine (27.11 mg/100 g) (Sriket et al., 2011c).

**Table 3**

Substrate specificity of purified trypsin from hepatopancreas of freshwater prawn on synthetic fluorogenic substrates.

| Substrates (10 $\mu$ M) | Relative activity (%)        |
|-------------------------|------------------------------|
| Boc-Val-Pro-Arg-MCA     | 100 $\pm$ 3.06 <sup>b</sup>  |
| Boc-Phe-Ser-Arg-MCA     | 72.6 $\pm$ 4.45 <sup>c</sup> |
| Boc-Leu-Lys-Arg-MCA     | 120 $\pm$ 2.88 <sup>a</sup>  |
| Boc-Glu-Lys-Lys-MCA     | 38.1 $\pm$ 5.43 <sup>d</sup> |
| Boc-Val-Leu-Lys-MCA     | 17.4 $\pm$ 3.55 <sup>e</sup> |
| Arg-MCA                 | 0                            |
| Z-Phe-Arg-MCA           | 0                            |
| Suc-Leu-Leu-Val-Tyr-MCA | 0                            |
| Suc-Ala-Ala-Pro-Phe-MCA | 0                            |

Trypsin activity was assayed at pH 8 and 55 °C for 10 min using various MCA-substrates. Different superscripts indicate the significant differences ( $p < 0.05$ ).

<sup>a</sup> Means  $\pm$  SD ( $n = 3$ ).

**Table 4**

Kinetic parameters of purified trypsin from hepatopancreas of freshwater prawn and other fish trypsin.

| Substrates          | Trypsins                         | $K_m$<br>( $\mu$ M) <sup>a</sup> | $K_{cat}$<br>( $S^{-1}$ ) <sup>a</sup> | $K_{cat}/K_m$<br>( $S^{-1} \mu M^{-1}$ ) |
|---------------------|----------------------------------|----------------------------------|----------------------------------------|------------------------------------------|
| Boc-Val-Pro-Arg-MCA | Freshwater prawn                 | 0.24                             | 607.56                                 | 2531.51                                  |
|                     | Sea bass trypsin A <sup>**</sup> | 0.58                             | 5.51                                   | 9.45                                     |
|                     | Sea bass trypsin B <sup>**</sup> | 0.59                             | 25.75                                  | 43.58                                    |
| Boc-Leu-Lys-Arg-MCA | Freshwater prawn                 | 0.56                             | 26.16                                  | 46.72                                    |
|                     | Sea bass trypsin A <sup>**</sup> | 0.39                             | 5.42                                   | 14.07                                    |
|                     | Sea bass trypsin B <sup>**</sup> | 0.82                             | 12.19                                  | 14.86                                    |

<sup>a</sup>  $K_m$  and  $k_{cat}$  values of all trypsin were determined at 37 °C under optimal pH.

<sup>\*\*</sup> Cai et al. (2011).

**Table 2**

Effect of various inhibitors on the activity of purified trypsin from hepatopancreas of freshwater prawn.

| Inhibitors  | Concentration | % Inhibition                  |
|-------------|---------------|-------------------------------|
| Control     | –             | 0                             |
| Pestain A   | 1 mM          | 0                             |
| E-64        | 0.1 mM        | 0                             |
| SBTI        | 1 g/L         | 99.23 $\pm$ 0.02 <sup>a</sup> |
| TLCK        | 5 mM          | 97.94 $\pm$ 0.01 <sup>a</sup> |
| Pefabloc SC | 5 mM          | 99.35 $\pm$ 0.03 <sup>a</sup> |
| TPCK        | 5 mM          | 0                             |
| EDTA        | 10 mM         | 9.71 $\pm$ 0.17 <sup>b</sup>  |

Enzyme solution was incubated with the same volume of inhibitor at 25 °C for 15 min and the residual activity was determined using Boc-Val-Pro-Arg-MCA as substrate for 10 min at pH 8 and 55 °C. Different superscripts indicate the significant differences ( $p < 0.05$ ).

<sup>a</sup> Means  $\pm$  SD ( $n = 3$ ).

|                     | 5       | 10              | 15                | 20            |
|---------------------|---------|-----------------|-------------------|---------------|
| Freshwater prawn    | I V G G | D E A A         | P G E F P         | H Q I S M Q V |
| Black Tiger prawn   | I V G G | T A V T         | P G E F P         | Y Q L S F Q D |
| Sea bass            | I V G G | Y E C T P       | Y S Q P H         | Q V S L N S   |
| Yellowfin tuna      | I V G G | Y E C Q A H S   | Q P H Q V S L N A |               |
| Skipjack tuna       | I V G G | Y E C Q A H S   | Q P H Q V S L N S |               |
| Arabesque greenling | I V G G | Y E C T P H T   | Q A H Q V S L D S |               |
| Dog                 | I V G G | Y T C E E N S   | V P V Q V S L N A |               |
| Porcine             | I V G G | Y T C E A A N S | V P Y Q V S L N S |               |
| Bovine              | I V G G | Y T C G A N T   | V P Y Q V S L N S |               |

**Fig. 4.** Comparison of N-terminal amino acid sequence of the purified trypsin from hepatopancreas of freshwater prawn with other trypsin. Black tiger prawn (Lu et al., 1990), sea bass (Cai et al., 2011), yellowfin tuna (Klomklao et al., 2006b), skipjack tuna (Klomklao et al., 2009), arabesque greenling (Kishimura, Hayashi, Miyashita, & Nonami, 2006), dog (Pinsky, LaForge, & Scheele, 1985), porcine (Hermanson, Ericsson, Neurath, & Walsh, 1973) and bovine (Walsh, 1970). Identical amino acid residues were boxed.

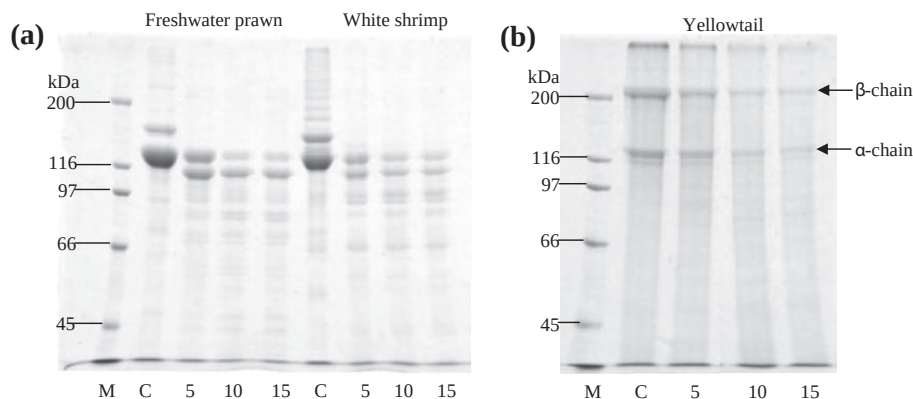


**Fig. 5.** Protein patterns of pepsin soluble collagen from freshwater prawn and white shrimp (a) and acid soluble collagen from yellowtail (b) incubated with trypsin from hepatopancreas of freshwater prawn at 25 °C for different times. M: molecular weight marker, C: collagen incubated without trypsin for 15 min. Numbers denote the incubation time (min) at 25 °C.

### 3.7. Kinetic study

Kinetic data for purified HFWP trypsin are summarised in Table 4.  $K_m$  and  $k_{cat}$  for the hydrolysis of Boc-Val-Pro-Arg-MCA were 0.24  $\mu\text{M}$  and 607.5  $\text{s}^{-1}$ , respectively, and the corresponding values for the hydrolysis of Boc-Leu-Lys-Arg-MCA were 0.56  $\mu\text{M}$  and 26.1  $\text{s}^{-1}$ , respectively. The catalytic efficiencies ( $k_{cat}/K_m$ ) for the hydrolysis of Boc-Val-Pro-Arg-MCA and Boc-Leu-Lys-Arg-MCA were calculated to be 2531.5 and 46.72  $\mu\text{M}^{-1}$ , respectively.

When comparing  $K_m$  of purified HFWP trypsin with two trypsins from Japanese sea bass hepatopancreas, it was found that HFWP trypsin had lower  $K_m$  values than trypsins from Japanese sea bass (Table 4). The result indicated that HFWP trypsin had a higher affinity for Boc-Val-Pro-Arg-MCA than did sea bass trypsins. For the  $K_{cat}$  value, HFWP trypsin also showed higher  $K_{cat}$  value on both Boc-Val-Pro-Arg-MCA and Boc-Leu-Lys-Arg-MCA than did trypsins from hepatopancreas of Japanese sea bass. HFWP trypsin also had higher catalytic efficiencies on both Boc-Val-Pro-Arg-MCA and Boc-Leu-Lys-Arg-MCA than did trypsin from Japanese sea bass. Thus, hepatopancreas trypsin might have higher hydrolytic activity on muscle proteins than did trypsins from hepatopancreas of Japanese sea bass.

### 3.8. N-terminal sequencing

The N-terminal amino acid sequence of HFWP trypsin was aligned to compare with those of other trypsins as depicted in Fig. 4. The N-terminal amino acid sequence of the first 20 amino acids of purified trypsin was determined to be IVGGDEAAPGEFPH-QISMVQV (Fig. 4). When N-terminal amino acid sequences of purified trypsin were compared with those of other shrimp, fish and three mammals, it was found that the sequence of the purified HFWP trypsin displayed high homology to that of black tiger prawn trypsin, especially the sequence from the first to fourth (IVGG) and ninth to thirteenth (PGEFP) residues. However, HFWP and all fish trypsins had a charged Glu residue at position 6, whereas Thr is most common in mammalian pancreatic trypsin (Fig. 4). Moreover, the sequences of HFWP trypsin and other trypsins started with IVGG after the proteolytic cleavage of inactive trypsinogen. The result confirmed that HFWP trypsin was most likely a member of the trypsin family.

### 3.9. Collagen hydrolysis by purified HFWP trypsin

Protein patterns of PSC extracted from FWP and white shrimp muscle incubated with purified HFWP trypsin at 25 °C for 0–

15 min are shown in (Fig. 5a). Marked degradation of the  $\alpha$ -chain was found after 5 min of hydrolysis, with concomitant occurrence of degradation products having a lower molecular weight (Fig. 5a). The protein pattern of ASC type I extracted from yellowtail muscle incubated with purified HFWP trypsin is depicted in Fig. 5b. The degradations of  $\beta$  and  $\alpha$ -chains were observed after 5 min of incubation. The decrease in band intensity of  $\beta$  and  $\alpha$ -chains became more pronounced with increasing incubation time.

These results suggested that HFWP trypsin had high collagenolytic activity toward prawn and fish collagens. This result was in agreement with Aoki et al. (2003) who found that proteases from Northern shrimp (*Pandalus eous*) were able to hydrolyse collagen. In general, collagens in fish and shellfish muscle contain lower cross-links than those from warm-blooded vertebrates. Collagens from fish and shellfish are more heat labile than land animal collagen (Hernandez-Herrero, Duflos, Malle, & Bouquelet, 2003). Therefore, trypsin released from FWP hepatopancreas may play a role in postmortem degradation of prawn muscle during extended iced storage.

## 4. Conclusion

Low molecular weight trypsin from hepatopancreas of freshwater prawn showed high hydrolytic activity at pH 8.0 and 55 °C. Based on substrate specificity, the inhibitor study and N-terminal sequencing, the enzyme purified was found to belong to trypsin family. Trypsin from FWP also showed high hydrolytic activity toward prawn collagen. Therefore, trypsin released from hepatopancreas more than likely plays a role in muscle softening of this species during extended iced storage.

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## Properties and antioxidant activity of fish skin gelatin film incorporated with citrus essential oils

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### ABSTRACT

Properties of protein-based film from fish skin gelatin incorporated with different citrus essential oils, including bergamot, kaffir lime, lemon and lime (50% based on protein) in the presence of 20% and 30% glycerol were investigated. Films containing 20% glycerol had higher tensile strength (TS) but lower elongation at break (EAB), compared with those prepared with 30% glycerol, regardless of essential oils incorporated ( $p < 0.05$ ). Films incorporated with essential oils, especially from lime, at both glycerol levels showed the lower TS but higher EAB than the control films (without incorporated essential oil) ( $p < 0.05$ ). Water vapour permeability (WVP) of films containing essential oils was lower than that of control films for both glycerol levels ( $p < 0.05$ ). Films with essential oils had varying  $\Delta E^*$  (total colour difference), where the highest value was observed in that added with bergamot essential oil ( $p < 0.05$ ). Higher glycerol content increased EAB and WVP but decreased TS of films. Fourier transforms infrared (FTIR) spectra indicated that films added with essential oils exhibited higher hydrophobicity with higher amplitude at wavenumber of  $2874\text{--}2926\text{ cm}^{-1}$  and  $1731\text{--}1742\text{ cm}^{-1}$  than control film. Film incorporated with essential oils exhibited slightly lower thermal degradation resistance, compared to the control film. Varying effect of essential oil on thermal degradation temperature and weight loss was noticeable, but all films prepared using 20% glycerol had higher thermal degradation temperature with lower weight loss, compared with those containing 30% glycerol. Films added with all types of essential oils had rough cross-section, compared with control films, irrespective of glycerol levels. However, smooth surface was observed in all film samples. Film incorporated with lemon essential oil showed the highest ABTS radical scavenging activity and ferric reducing antioxidant power (FRAP) ( $p < 0.05$ ), while the other films had lower activity. Thus, the incorporation of different essential oils and glycerol levels directly affected the properties of gelatin-based film from fish skin.

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### 1. Introduction

Development of biodegradable packaging materials is an effective alternative to synthetic packaging material from petrochemical products, which are non-biodegradable and have the negative impact on environment. Moreover, biodegradable materials are eco-friendly, non-toxic and shown to have many desirable physico-chemical characteristics over synthetic ones. The biodegradable film can be made from neutral biopolymers, including polysaccharides, proteins and lipids or the combination of these materials (Huber, Embuscado, Pavlath, & Orts, 2009; Tharanathan, 2003). Among these biopolymers, proteins have been extensively used for the development of biodegradable films due to their abundance and good film-forming ability. Proteins are heteropolymers containing a variety of amino acids, which can undergo a wide

range of interactions and chemical reactions (Stevens, 1999). The protein-based films have excellent oxygen, carbon dioxide and volatile compounds barrier properties, in comparison with synthetic films under low relative humidity condition (Limpan, Prodpran, Benjakul, & Prasarnpran, 2010).

Gelatin is a natural biopolymer, which can be derived from collagen. It has gained more attention as material for edible films (Arvanitoyannis, 2002; Jongjareonrak, Benjakul, Visessanguan, Prodpran, & Tanaka, 2006a). Gelatin edible films prepared from bovine and porcine skin with high puncture strength, low puncture deformation and high water vapour permeability were reported (Sobral, Menegalli, Hubinger, & Roques, 2001). Fish skin gelatin had poor gelling property, however, can be used as film forming material to widen its field of application. Generally, properties of film vary depending on the source of gelatin, plasticiser and other factors (Gomez-Guillen et al., 2009). Mechanical properties of film from brownstripe red snapper skin gelatin were superior to those of bigeye snapper skin gelatin film at any protein and plasticiser concentrations tested (Jongjareonrak, Benjakul, Visessanguan, &

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Tanaka, 2006b). However, gelatin films have poor water barrier vapour property, thereby limiting its use as potential packaging (Gomez-Guillen et al., 2009; Krochta, 1997). This is due to its hydrophilicity in nature (Gennaidios, Brandenburg, Weller, & Testin, 1993). To tackle this problem, the incorporation of hydrophobic substances such as lipid, fatty acid and wax has been implemented to improve water barrier property (Limpisophon, Tanaka, & Osako, 2010; Prodpran, Benjakul, & Artharn, 2007; Soazo, Rubiol, & Verdini, 2011).

Essential oils from various plants have been known to exhibit antioxidant property, which can extend shelf-life by lowering lipid oxidation in foods (Oussalah, Caillet, Salmieri, Saucier, & Lacroix, 2004). Therefore the incorporation of essential oils, especially from various citrus, into the films could be an approach to lower water vapour permeability. Simultaneously, smart edible film with antioxidant activity can be gained. However, rare information regarding the improvement of water barrier property of fish gelatin-based film, using citrus essential oils has been reported. This study aimed to elucidate the impact of different citrus essential oils and glycerol levels on properties of fish skin gelatin film.

## 2. Materials and methods

### 2.1. Fish gelatin and chemicals

Fish skin gelatin from tilapia (~240 bloom) was purchased from Lapi Gelatine S.p.A (Empoli, Italy). Essential oils from bergamot, kaffir lime, lemon and lime were purchased from Botanicessence (Bangkok, Thailand).

2,2-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picryl hydrazyl (DPPH), 2,4,6-triphenyl-1,3,5-triazine (TPTZ), glycerol and Tween 20 were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 2.2. Preparation of film from gelatin incorporated with different essential oils

To prepare film forming solution (FFS), gelatin powder was mixed with distilled water and heated at 70 °C for 30 min to obtain the protein concentration of 3.5% (w/v). Glycerol at concentrations of 20% and 30% (w/w) of protein content was used as a plasticiser. Different essential oils previously mixed with Tween 20 at 25% (w/w, based on essential oil) were added into FFS at a level of 50% (w/w, based on protein content). FFS was homogenised at 22,000 rpm for 3 min using a homogeniser (IKA Labortechnik homogeniser, Selangor, Malaysia). The dissolved air in the FFS was removed by a vacuum pump (Diaphragm vacuum pump, Wertheim Germany) for 30 min at room temperature.

To prepare the film, FFS (4 g) was cast onto a rimmed silicone resin plate (50 × 50 mm<sup>2</sup>) and air-blown for 12 h at room temperature prior to further drying at 25 °C and 50 ± 5% RH for 24 h in an environmental chamber (WTB Binder, Tuttlingen, Germany). The resulting films were manually peeled off and subjected to analyses. Control films were prepared from FFS without essential oils added.

### 2.3. Determination of film properties

#### 2.3.1. Film thickness

The thickness of film was measured using a micrometer (Mitutoyo, Model ID-C112PM, Serial No. 00320, Mitutoyo Corp., Kawasaki-shi, Japan). Five random locations around each film of 10 film samples were used for average thickness determination.

#### 2.3.2. Mechanical properties

Prior to testing the mechanical properties, films were conditioned for 48 h at 25 °C and 50 ± 5% RH. Tensile strength (TS) and elongation at break (EAB) were determined as described by Iwata, Ishizaki, Handa, and Tanaka (2000), with a slight modification, using the Universal Testing Machine (Lloyd Instrument, Hampshire, UK) equipped with tensile load cell of 100 N. Ten samples (2 × 5 cm<sup>2</sup>) with initial grip length of 3 cm were used for testing. Cross-head speed was set at 30 mm/min.

#### 2.3.3. Water vapour permeability (WVP)

WVP was measured using a modified ASTM method (American Society for Testing & Materials, 1989) as described by Shiku, Hamaguchi, Benjakul, Visessanguan, and Tanaka (2004). The film was sealed on an aluminium permeation cup containing dried silica gel (0% RH) with silicone vacuum grease and a rubber gasket to hold the film in place. The cups were placed in a desiccator containing the distilled water at 30 °C. The cups were weighed at 1 h intervals over a 10 h period. WVP of the film was calculated as follows:

$$\text{WVP (g m}^{-1} \text{ s}^{-1} \text{ Pa}^{-1}) = w l A^{-1} t^{-1} (P_2 - P_1)^{-1}$$

where  $w$  is the weight gain of the cup (g);  $l$  is the film thickness (m);  $A$  is the exposed area of film (m<sup>2</sup>);  $t$  is the time of gain (s);  $P_2 - P_1$  is the vapour pressure difference across the film (Pa).

#### 2.3.4. Colour differences

Colour differences of film samples were determined using a CIE colourimeter (Hunter associates laboratory, Inc., VA, USA).  $D_{65}$  (day light) and a measure cell with opening of 30 mm was used. Total difference of colour ( $\Delta E^*$ ) was calculated as follows (Gennaidios, Weller, Hanna, & Froning, 1996):

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where  $\Delta L^*$ ,  $\Delta a^*$  and  $\Delta b^*$  are the differences between the colour parameter of the samples and the colour parameter of the white standard ( $L^* = 93.45$ ,  $a^* = -0.81$ ,  $b^* = 0.33$ ).

#### 2.3.5. Light transmittance and transparency value

The light transmittance of films was measured at the ultraviolet and visible range (200–800 nm) using a UV-vis spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) as described by Shiku et al. (2004). The transparency value of film was calculated using the following equation, (Han & Floros, 1997):

$$\text{Transparency value} = -\log T_{600}/x$$

where  $T_{600}$  is the fractional transmittance at 600 nm and  $x$  is the film thickness (mm). The greater transparency value represents the lower transparency of film.

#### 2.3.6. Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy

Prior to analysis, films were conditioned in a desiccator containing  $P_2O_5$  for 2 weeks at room temperature to obtain the most dehydrated films (Sobral et al., 2001). Films were scanned with a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) equipped with a horizontal ATR Trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) at 25 °C as described by Nuthong, Benjakul, and Prodpran (2009). Films were placed onto the crystal cell and the cell was clamped into the mount of FTIR spectrometer. The spectra in the range of 650–4000 cm<sup>-1</sup> with automatic signal gain were collected in 32 scans at a resolution of 4 cm<sup>-1</sup> and were ratioed against a background spectrum recorded from the clean empty cell at 25 °C.

### 2.3.7. Thermo-gravimetric analysis (TGA)

Prior to testing, films were conditioned in a desiccator containing  $P_2O_5$  for 2 weeks at room temperature. Conditioned films were scanned using a thermo-gravimetric analyzer (TGA7, PerkinElmer, Norwalk, CT, USA) from 50 to 600 °C at a rate of 10 °C/min (Nuthong et al., 2009). Nitrogen was used as the purge gas at a flow rate of 20 ml/min.

### 2.3.8. Scanning electron microscopy (SEM)

Morphology of surface and cross-section of film samples were visualised using a scanning electron microscope (SEM) (Quanta 400, FEI, Eindhoven, the Netherlands). For cross-section, samples were fractured under liquid nitrogen prior to visualisation. Then, the samples were mounted on bronze stub and sputtered with gold (Sputter coater SPI-Module, PA, USA) in order to make the sample conductive. The photographs were taken at an acceleration voltage of 15 kV.

### 2.4. Determination of antioxidant activity of fish skin gelatin films incorporated with essential oils

Films were solidified using liquid nitrogen in mortar and ground with a pestle. Ground film (0.25 g) was mixed with 5 ml of methanol and stirred vigorously for 3 h. The mixture was centrifuged at 2700g for 10 min using a centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Beckman Coulter, Inc., Palo Alto, CA, USA). The supernatant obtained was analysed for DPPH radical scavenging activity, ABTS radical scavenging activity and ferric reducing antioxidant power.

#### 2.4.1. DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by Wu, Chen, and Shiau (2003) with a slight modification. Sample (1.5 ml) was added with 1.5 ml of 0.15 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) in 95% ethanol. The mixture was mixed vigorously and allowed to stand at room temperature in dark for 30 min. The absorbance of the resulting solution was measured at 517 nm using a spectrophotometer. Sample blank was prepared in the same manner except that 95% methanol was used instead of DPPH solution. A standard curve was prepared using Trolox in the range of 10–60  $\mu$ M. The activity was calculated after the sample blank subtraction and expressed as  $\mu$ mol Trolox equivalents (TE)/g dried film.

#### 2.4.2. ABTS radical scavenging activity

ABTS radical scavenging activity was assayed as per the method of Arnao, Cano, and Acosta (2001) with a slight modification. The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in dark. The solution was then diluted by mixing 1 ml of ABTS solution with 50 ml of methanol in order to obtain an absorbance of  $1.1 \pm 0.02$  units at 734 nm using a spectrophotometer. Fresh ABTS solution was prepared for each assay. Sample (150  $\mu$ l) was mixed with 2850  $\mu$ l of ABTS solution and the mixture was left at room temperature for 2 h in dark. The absorbance was then measured at 734 nm using a spectrophotometer. Sample blank was prepared in the same manner except that methanol was used instead of ABTS solution. A standard curve of Trolox ranging from 50 to 600  $\mu$ M was prepared. The activity was calculated after sample blank subtraction and was expressed as  $\mu$ mol Trolox equivalents (TE)/g dried film.

#### 2.4.3. Ferric reducing antioxidant power (FRAP)

FRAP was assayed according to the method of Benzie and Strain (1996). Stock solutions included 30 mM acetate buffer (pH 3.6),

10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM  $FeCl_3 \cdot 6H_2O$  solution. A working solution was prepared freshly by mixing 25 ml of acetate buffer, 2.5 ml of TPTZ solution and 2.5 ml of  $FeCl_3 \cdot 6H_2O$  solution. The mixed solution was incubated at 37 °C for 30 min in a water bath (Memmert, D-91126, Schwabach, Germany) and was referred to as FRAP solution. A sample (150  $\mu$ l) was mixed with 2850  $\mu$ l of FRAP solution and kept for 30 min in dark at room temperature. The ferrous tripyridyltriazine complex (coloured product) was measured by reading the absorbance at 593 nm. Sample blank was prepared by omitting  $FeCl_3$  from FRAP solution and distilled water was used instead. The standard curve was prepared using Trolox ranging from 50 to 600  $\mu$ M. The activity was calculated after sample blank subtraction and was expressed as  $\mu$ mol Trolox equivalents (TE)/g dried film.

### 2.5. Statistical analysis

All experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by Duncan's multiple range test. For pair comparison, T-test was used (Steel & Torrie, 1980). Analysis was performed using the SPSS package (SPSS for windows, SPSS Inc., Chicago, IL, USA).

## 3. Results and discussion

### 3.1. Mechanical properties and thickness

Mechanical properties and thickness of fish skin gelatin films incorporated with different citrus essential oils (bergamot, kaffir lime, lemon and lime) along with 20% or 30% glycerol are shown in Table 1. The thickness of fish skin gelatin films incorporated with essential oils was higher than that of the control film (without incorporated essential oils), regardless of types of essential oil incorporated ( $p < 0.05$ ) (Table 1). When the same type of essential oil was used, there were no differences in thickness between films containing 20% and 30% glycerol ( $p > 0.05$ ). Negligible differences in thickness of gelatin-based films with the different levels of glycerol were reported (Vanin, Sobral, Menegalli, Carvalho, & Habitante, 2005).

At the same level of glycerol used, the control film showed higher TS, but lower EAB, compared with those incorporated with essential oils ( $p < 0.05$ ). At the same level of glycerol, film containing bergamot essential oil had the highest TS with coincidentally lowest EAB, compared with those incorporated with other essential oils ( $p < 0.05$ ). On the other hand, that added with lime essential oil exhibited the lowest TS but highest EAB ( $p < 0.05$ ). Films prepared using 20% glycerol had higher TS, but lower EAB than those with 30% glycerol ( $p < 0.05$ ). Similar results have been reported for gelatin film from cuttlefish skin, where increasing glycerol content enhanced the flexibility of film (Hoque, Benjakul, & Prodpran, 2011). Addition of essential oils at 50% possibly resulted in the lowered interaction between gelatin molecules. As a consequence, the decrease in rigidity with the concomitant increase in extensibility/elasticity of film was gained. Addition of lipid or oil in protein-based or polysaccharide-based films may hinder polymer chain-to-chain interactions and provide flexible domains within the film (Limpisophon et al., 2010). Hoque, Benjakul, and Prodpran (2010) reported that gelatin film was mainly stabilised by the weak bond including hydrogen bond and hydrophobic interaction. Interactions between polar polymer molecules are much stronger than those between non-polar molecules (lipid) and between polar polymer and non-polar molecules (Yang & Paulson, 2000). Among all essential oils tested, lime essential oil showed the highest plasticising effect than others. This was most likely governed by the different components in different essential oils.

**Table 1**

Properties of films from fish skin gelatin containing 20% and 30% glycerol in the presence of different citrus essential oils at 50% of protein.

| Glycerol (%) | Essential oils | TS (MPa)                       | EAB (%)                        | WVP ( $\times 10^{-11} \text{ gm}^{-1} \text{ s}^{-1} \text{ Pa}^{-1}$ ) | Thickness (mm)                 |
|--------------|----------------|--------------------------------|--------------------------------|--------------------------------------------------------------------------|--------------------------------|
| 20           | Control        | 49.09 $\pm$ 2.34 <sup>aA</sup> | 9.61 $\pm$ 2.06 <sup>aA</sup>  | 3.62 $\pm$ 0.26 <sup>aB</sup>                                            | 0.040 $\pm$ 0.001 <sup>b</sup> |
|              | Bergamot       | 42.42 $\pm$ 2.03 <sup>bA</sup> | 15.29 $\pm$ 2.92 <sup>bB</sup> | 3.15 $\pm$ 0.31 <sup>bA</sup>                                            | 0.047 $\pm$ 0.002 <sup>a</sup> |
|              | Kaffir Lime    | 36.87 $\pm$ 1.33 <sup>cA</sup> | 31.43 $\pm$ 4.29 <sup>cA</sup> | 2.95 $\pm$ 0.23 <sup>bA</sup>                                            | 0.048 $\pm$ 0.002 <sup>a</sup> |
|              | Lemon          | 32.82 $\pm$ 1.17 <sup>dA</sup> | 39.06 $\pm$ 5.10 <sup>bB</sup> | 2.81 $\pm$ 0.04 <sup>bA</sup>                                            | 0.048 $\pm$ 0.001 <sup>a</sup> |
|              | Lime           | 27.32 $\pm$ 1.75 <sup>eA</sup> | 52.21 $\pm$ 5.62 <sup>aB</sup> | 2.91 $\pm$ 0.11 <sup>bB</sup>                                            | 0.049 $\pm$ 0.003 <sup>a</sup> |
| 30           | Control        | 42.86 $\pm$ 4.97 <sup>aB</sup> | 13.25 $\pm$ 3.99 <sup>dA</sup> | 4.07 $\pm$ 0.17 <sup>aA</sup>                                            | 0.041 $\pm$ 0.001 <sup>b</sup> |
|              | Bergamot       | 36.52 $\pm$ 1.75 <sup>bB</sup> | 19.96 $\pm$ 3.72 <sup>dA</sup> | 3.22 $\pm$ 0.11 <sup>bA</sup>                                            | 0.048 $\pm$ 0.002 <sup>a</sup> |
|              | Kaffir Lime    | 34.22 $\pm$ 0.89 <sup>bB</sup> | 30.93 $\pm$ 5.46 <sup>cA</sup> | 3.38 $\pm$ 0.23 <sup>bA</sup>                                            | 0.047 $\pm$ 0.001 <sup>a</sup> |
|              | Lemon          | 31.06 $\pm$ 2.31 <sup>cB</sup> | 52.66 $\pm$ 6.14 <sup>bA</sup> | 2.85 $\pm$ 0.15 <sup>cA</sup>                                            | 0.048 $\pm$ 0.003 <sup>a</sup> |
|              | Lime           | 25.87 $\pm$ 4.48 <sup>dA</sup> | 69.79 $\pm$ 7.39 <sup>aA</sup> | 3.37 $\pm$ 0.08 <sup>bA</sup>                                            | 0.047 $\pm$ 0.002 <sup>a</sup> |

Different superscript lowercase letters in the same column under the same glycerol content indicate significant differences ( $p < 0.05$ ). Different superscript uppercase letters in the same column under the same essential oil indicate significant differences ( $p < 0.05$ ).

<sup>a</sup> Mean  $\pm$  SD ( $n = 3$ ).

However, strengthening effects of essential oil were reported in various protein-based film. Atarés, De Jesús, Talens, and Chiralt (2010) reported that the incorporation of cinnamon essential oil into soybean protein isolate (SPI) increased the tensile strength of film. Essential oil might induce rearrangement of protein network in the way which strengthened the films. Some compounds in essential oil might be able to cross-link gelatin chain, thereby increasing the rigidity of film. Essential oils consist of various compounds and high concentrations of phenolic compounds such as carvacrol, eugenol and thymol were reported (Burt, 2004). The phenolic compounds were able to react with more than one protein site and led to protein cross-links (Haslam, 1989). Therefore, different compounds had varying properties, thereby affecting the film matrix differently.

### 3.2. Water vapour permeability (WVP)

WVP of fish skin gelatin films prepared using 20% and 30 % glycerol and incorporated with different essential oils is shown in Table 1. When comparing the effect of glycerol on WVP of control films, that containing 20% glycerol had lower WVP than did that with 30% glycerol ( $p < 0.05$ ). Hydrophilic glycerol could increase the hydrophilicity of films. When all essential oils were incorporated, the decrease in WVP was obtained, compared with the control ( $p < 0.05$ ), regardless of glycerol content. There were no differences in WVP amongst films incorporated with different essential oils when 20% glycerol was used ( $p > 0.05$ ). For films containing 30% glycerol, the lowest WVP was observed in that incorporated with lemon essential oil ( $p < 0.05$ ). It was noted that no difference in WVP between films added with the same essential oil when different glycerol contents were used, except for films added with lemon essential oil. Therefore, the incorporation of essential oils into gelatin film directly affected WVP of resulting films. Monoterpene hydrocarbon was generally found in essential oils (Ikeda, Stanley, Vannier, & Spitler, 1962) and contributed to increased hydrophobicity of film. The presence of a hydrophobic disperse phase, even at small ratio, limits water vapour transfer since it introduces the interfere in hydrophilic phase and increases the tortuosity factor of mass transfer (Atarés et al., 2010). Furthermore, water vapour transfer normally occurs through the hydrophilic portion of film network and depends on hydrophilic/hydrophobic ratio of film constituent (Hernandez, 1994). Similar results were observed for thyme essential oil incorporated hake protein-based film, in which the decrease of WVP was obtained with increasing levels of essential oil (Pires et al., 2011).

### 3.3. Colour differences, light transmittance and film transparency

Total colour differences ( $\Delta E^*$ ) of films containing 20% and 30% glycerol, which were incorporated with bergamot, kaffir lime,

lemon and lime essential oils are shown in Table 2. Among all samples, that incorporated with bergamot essential oil had the highest  $\Delta E^*$  ( $p < 0.05$ ). Higher  $\Delta E^*$  in agreement with the higher  $b^*$ -value and lower  $a^*$ -value of films (data not shown). This might be due to the yellow colour of bergamot essential oil. However,  $\Delta E^*$  of film added with kaffir lime essential oil was lower than that of control film ( $p < 0.05$ ). Therefore, incorporation of different essential oils had an impact on the colour of resulting gelatin film.

Light transmission at selected wavelengths from 200 to 800 nm in UV and visible ranges and transparency value of films from fish skin gelatin containing glycerol (20% and 30%) and different essential oils (Bergamot, Kaffir Lime, Lemon and Lime) are shown in Table 2. Decreases in light transmission of films incorporated with essential oils for both glycerol levels (20% and 30%) were observed at all wavelengths, compared with control film. The result indicated that essential oils were able to impede the light transmission through the film. This decreased light transmission was possibly due to the light scattering at the interface of essential oil droplets imbedded in the film matrix. All films from fish skin gelatin had a good barrier property in the UV-ranges (200–280 nm), especially for film incorporated with all types of essential oils. The higher UV light barrier capacity was reported for gelatin films from bigeye snapper and brownstripe red snapper skin (Jongjareonrak et al., 2006a) and cuttlefish skin (Hoque et al., 2011). For visible range, films added with essential oil, especially that containing lemon essential oil, showed the better barrier property for light transmission. This was more likely due to the increase in opaqueness of films containing essential oils, which were distributed throughout the films.

Based on transparency value (Table 2), films incorporated with all types of essential oils had the lower transparency than control film containing both glycerol levels (20% and 30%) as evidenced by the higher transparency value. Higher transparency value indicated that the film had the lower transparency. Essential oil droplets localised in the film matrix lowered the transparency of gelatin film, more likely due to the light scattering effect. Film incorporated with bergamot essential oil was more transparent than those added with other essential oils ( $p < 0.05$ ). Therefore, the incorporation of essential oil had the impact on appearance and light barrier properties of gelatin film.

### 3.4. Fourier-transform infrared (FTIR) spectroscopy

FTIR spectra of films from fish skin gelatin containing 20% and 30% glycerol in the presence of different essential oils are illustrated in Fig. 1A and B, respectively. Generally, films containing both glycerol levels, with and without the addition of different essential oils showed the similar major peaks but the amplitudes of peaks varied. The band situated at the wavenumber of 1033–1039  $\text{cm}^{-1}$  was found in all film samples, corresponding to the

**Table 2**

Colour difference, light transmittance and transparency value of films from fish skin gelatin containing 20% and 30% glycerol in the presence of different citrus essential oils at 50% of protein.

| Glycerol (%) | Essential oils | Light transmittance (%) at different wavelength (nm) |       |       |       |       |       |       |       | Transparency value    | $\Delta E^*$      |
|--------------|----------------|------------------------------------------------------|-------|-------|-------|-------|-------|-------|-------|-----------------------|-------------------|
|              |                | 200                                                  | 280   | 350   | 400   | 500   | 600   | 700   | 800   |                       |                   |
| 20           | Control        | 0.00                                                 | 55.70 | 79.94 | 82.41 | 85.12 | 86.66 | 87.69 | 88.43 | $2.14 \pm 0.21^{dA}$  | $2.31 \pm 0.08^c$ |
|              | Bergamot       | 0.00                                                 | 15.17 | 40.34 | 61.12 | 66.98 | 69.51 | 71.23 | 72.76 | $4.28 \pm 0.01^{cA}$  | $2.92 \pm 0.07^a$ |
|              | Kaffir Lime    | 0.00                                                 | 23.51 | 47.57 | 53.35 | 59.52 | 62.98 | 65.70 | 68.32 | $5.48 \pm 0.01^{bA}$  | $2.08 \pm 0.11^d$ |
|              | Lemon          | 0.00                                                 | 16.45 | 42.35 | 48.79 | 55.46 | 59.52 | 62.54 | 65.14 | $5.46 \pm 0.01^{bA}$  | $2.45 \pm 0.10^b$ |
|              | Lime           | 0.00                                                 | 26.01 | 49.25 | 54.90 | 60.95 | 64.26 | 66.78 | 69.19 | $5.66 \pm 0.10^{aA}$  | $2.30 \pm 0.02^c$ |
| 30           | Control        | 0.00                                                 | 53.67 | 81.33 | 83.67 | 85.89 | 86.99 | 87.72 | 88.14 | $2.15 \pm 0.01^{dA}$  | $2.39 \pm 0.05^c$ |
|              | Bergamot       | 0.00                                                 | 19.34 | 40.74 | 63.47 | 69.01 | 71.32 | 73.04 | 74.71 | $4.45 \pm 0.23^{cA}$  | $3.19 \pm 0.04^a$ |
|              | Kaffir Lime    | 0.01                                                 | 18.61 | 47.52 | 53.40 | 59.52 | 62.87 | 65.54 | 68.13 | $5.65 \pm 0.19^{aA}$  | $2.09 \pm 0.08^d$ |
|              | Lemon          | 0.01                                                 | 22.26 | 46.31 | 51.91 | 57.61 | 61.21 | 64.06 | 66.58 | $5.31 \pm 0.06^{bB}$  | $2.45 \pm 0.05^b$ |
|              | Lime           | 0.01                                                 | 20.78 | 50.14 | 55.89 | 61.83 | 64.93 | 67.20 | 69.37 | $5.46 \pm 0.07^{abA}$ | $2.25 \pm 0.04^c$ |

Different superscript lowercase letters in the same column under the same glycerol content indicate significant differences ( $p < 0.05$ ). Different superscript uppercase letters in the same column under the same essential oil indicate significant differences ( $p < 0.05$ ).

\* Mean  $\pm$  SD ( $n = 3$ ).

glycerol (OH group) added as a plasticiser (Bergo & Sobral, 2007). All films had the major bands at  $1630\text{ cm}^{-1}$  (amide-I, illustrating C=O stretching/hydrogen bonding coupled with COO),  $1538\text{ cm}^{-1}$  (amide-II, presenting the bending vibrations of N-H groups and stretching vibrations of C-N groups) and  $1237\text{ cm}^{-1}$  (amide-III, illustrating the vibrations in-plane of C-N and N-H groups of bound amide or vibrations of  $\text{CH}_2$  groups of glycine) (Aewsiri, Benjakul, & Visessanguan, 2009; Muyonga, Cole, & Duodu, 2004a). Hoque et al. (2010) also found the similar spectra for cuttlefish skin gelatin film, where amide-I, II and III bands were found at the wavenumbers of  $1630$ ,  $1539$  and  $1235\text{ cm}^{-1}$ , respectively. An amide-A band was observed at the wavenumber of  $3272$ – $3271\text{ cm}^{-1}$  for all film samples. Amide-B band was also found at  $2923$ – $2925\text{ cm}^{-1}$  for all film samples. The amide-A band represented the NH-stretching coupled with hydrogen bonding and amide-B band represented the CH stretching and  $-\text{NH}_3^+$  at wavenumber of  $2928\text{ cm}^{-1}$  (Muyonga, Cole, & Duodu, 2004b).

Amplitude of peaks at wavenumbers  $2853.62\text{ cm}^{-1}$  and  $2923.58\text{ cm}^{-1}$  increased when the films were incorporated with essential oils. Those peaks represent the methylene asymmetrical and symmetrical stretching vibration of the aliphatic C-H in  $\text{CH}_2$  and  $\text{CH}_3$  groups, respectively (Guillén & Cabo, 1997, 2004). Both the methylene asymmetrical stretching bands at approximately  $2853\text{ cm}^{-1}$  and methylene symmetrical stretching band near  $2924\text{ cm}^{-1}$  were obviously present in most lipids (Guillén & Cabo, 2004). The spectra of control films (without incorporated essential oil) for both 20% and 30% glycerol levels showed the highest amplitude of amide-A followed by films incorporated with kaffir lime, bergamot, lime and lemon essential oils, respectively. Film incorporated with lemon essential oil exhibited the highest amplitude for amide-B peak and peak with wavenumber of  $2877\text{ cm}^{-1}$ , followed by film added with lime, bergamot, kaffir lime essential oil, respectively, irrespective of glycerol content. As a result, films incorporated with essential oils generally had the increases in amplitude of peaks at wavenumbers of  $2854\text{ cm}^{-1}$  and  $2924\text{ cm}^{-1}$ . This was in agreement with the decreases in amplitude of peaks at wavenumbers of  $3284\text{ cm}^{-1}$  (amide-A). Among all types of essential oils, film incorporated with lemon essential oil more likely had the highest hydrophobicity of film, as evidenced by the highest amplitude of peaks at wavenumbers of  $2854\text{ cm}^{-1}$  and  $2924\text{ cm}^{-1}$ , corresponding to CH of  $\text{CH}_2$ ,  $\text{CH}_3$  hydrophobic moieties.

Furthermore, the strong peak was observed at about  $1742\text{ cm}^{-1}$  for film incorporated with lime essential oils, regardless of glycerol levels, while the tiny peaks at wavenumbers of  $1731$ – $1734\text{ cm}^{-1}$  were found in films incorporated with bergamot, kaffir lime and lime essential oils. Those peaks more likely represented the C=O stretching vibration of aldehyde or ester carbonyl groups (Guillén & Cabo, 1997, 2004). The difference in peak position depended

on the varying compounds constituted in different essential oils. Nevertheless, there was no peak at around  $1742$ – $1734\text{ cm}^{-1}$  found in control film. Addition of essential oils in gelatin film could increase the hydrophobicity of film, as evidenced by existing peak at wavenumber of  $2854\text{ cm}^{-1}$ ,  $2924\text{ cm}^{-1}$  and  $1745\text{ cm}^{-1}$ , especially for film added with lemon essential oil. This was supported by the lowest WVP of film incorporated with lemon essential oil for both glycerol levels (Table 1). Therefore, the incorporation of essential oils into gelatin film could decrease WVP of fish skin gelatin film.

### 3.5. Thermo-gravimetric analysis (TGA)

Degradation temperatures ( $T_d$ ), weight loss ( $\Delta w$ ) and residue of films containing 20% and 30% glycerol in the presence of different essential oils are presented in Table 3. Both control films with 20% and 30% glycerol exhibited three main stages of weight loss. However, four main stages of weight loss were found in films incorporated with all types of essential oils, regardless of glycerol levels. For all films, the first stage weight loss at  $3.91$ – $7.81\%$  was observed with degradation temperature ( $T_{d1}$ ) of  $67.50$ – $87.50^\circ\text{C}$ . The weight loss at this temperature range was possibly due to the loss of free water, as well as other volatile compounds absorbed in the film. It was noted that lower weight loss was found in film incorporated with essential oil, suggesting lower water absorbed in the film matrix, due to higher hydrophobicity of those films, compared to the control film. The similar result was observed in cuttlefish skin gelatin film (Hoque et al., 2011) and porcine-plasma protein film (Nuthong et al., 2009). The second stage of weight loss ( $\Delta w_2 = 14.28$ – $28.37\%$ ) appeared at  $T_{d2}$  of  $200.75$ – $239.92^\circ\text{C}$ . This transition revealed the degradation of film matrix. The result was in agreement with Hoque et al. (2011) who reported that the degradation temperature in the range of  $196.30$ – $216.71^\circ\text{C}$  found in cuttlefish skin gelatin film was mostly associated with the loss of glycerol compound (plasticiser) and smaller size protein fraction, as well as structurally bound water. For the third stage of weight loss,  $\Delta w_3$  of  $36.46$ – $49.39\%$  and  $T_{d3}$  of  $305.48$ – $322.69^\circ\text{C}$  were obtained for all film samples, mostly associated with the degradation of the larger-size or associated protein fraction. For the fourth stage of weight loss,  $\Delta w_4$  of  $13.13$ – $16.95\%$  and  $13.45$ – $17.88\%$  with  $T_{d4}$  of  $401.92$ – $417.64^\circ\text{C}$  and  $385.60$ – $413.47^\circ\text{C}$ , respectively, were obtained for films incorporated with different essential oils and added with 20% and 30% glycerol, respectively. Nevertheless, for both control films, the fourth stage of weight loss ( $\Delta w_4$ ) disappeared. It was noted that the fourth stage of weight loss might be associated with the loss of thermally stable components constituted in essential oils incorporated in film matrix.

Additionally, lower  $T_{d3}$  was found in films incorporated with all essential oils, compared with the control films for both glycerol levels used. The result was coincidental with the higher weight loss. This might be owing to the plasticising effect in which incorporated essential oils might impede interaction between gelatin molecules in the film network. This result was in agreement with the lowered TS and higher EAB of films incorporated with essential oils (Table 1). Film prepared using 20% glycerol, with and without incorporated essential oils, showed the higher heat resistance than those prepared using 30% glycerol, as evidenced by the higher  $T_{d2}$ ,  $T_{d3}$  and  $T_{d4}$  and lower weight loss for  $\Delta w_2$ ,  $\Delta w_3$  and  $\Delta w_4$ . This reconfirmed that glycerol at higher content interfered protein interaction in film network. In general, lower residue (or char content) from thermal degradation was found in films incorporated with essential oils, compared with the control film, regardless of glycerol levels. Thus, both glycerol contents and essential oils had the marked impact on thermal stability of gelatin film.

### 3.6. Film morphology

SEM micrographs of the surface and freeze-fractured cross-section of films from fish skin gelatin containing glycerol at 20%

and 30% and different essential oils are illustrated in Fig. 2. The control film (without incorporated essential oils) had the smooth and continuous surface for both glycerol levels. Smooth surface was also observed in the films incorporated with essential oils, regardless of glycerol content. This result indicated that FFS had the stable emulsion system and no collapse of emulsion occurred during FFS dehydration. For cross-section, the control films prepared at 20% and 30% glycerol had smooth and compact structure. Nevertheless, the control film containing 20% glycerol had some discontinuous zone, compared with others. With the addition of essential oils, the cross-section of film became rougher, compared with the control film when the same glycerol level was used. The disruption of protein–protein interaction in the film matrix arose from droplets of essential oils would enhance roughness of film cross-section. Those oil droplets were more likely localised inside the film network as observed in cross-section, where no dispersion of oil droplet on the surface of film was noticeable. The film micro-structure might be associated with the reduction in tensile strength and lower WVP of films incorporated with essential oils, compared with control film (without incorporated essential oil). Oil droplet could obstruct the transfer of water molecules through the film network (Karbowiak, Debeaufort, & Voilley, 2007). As a

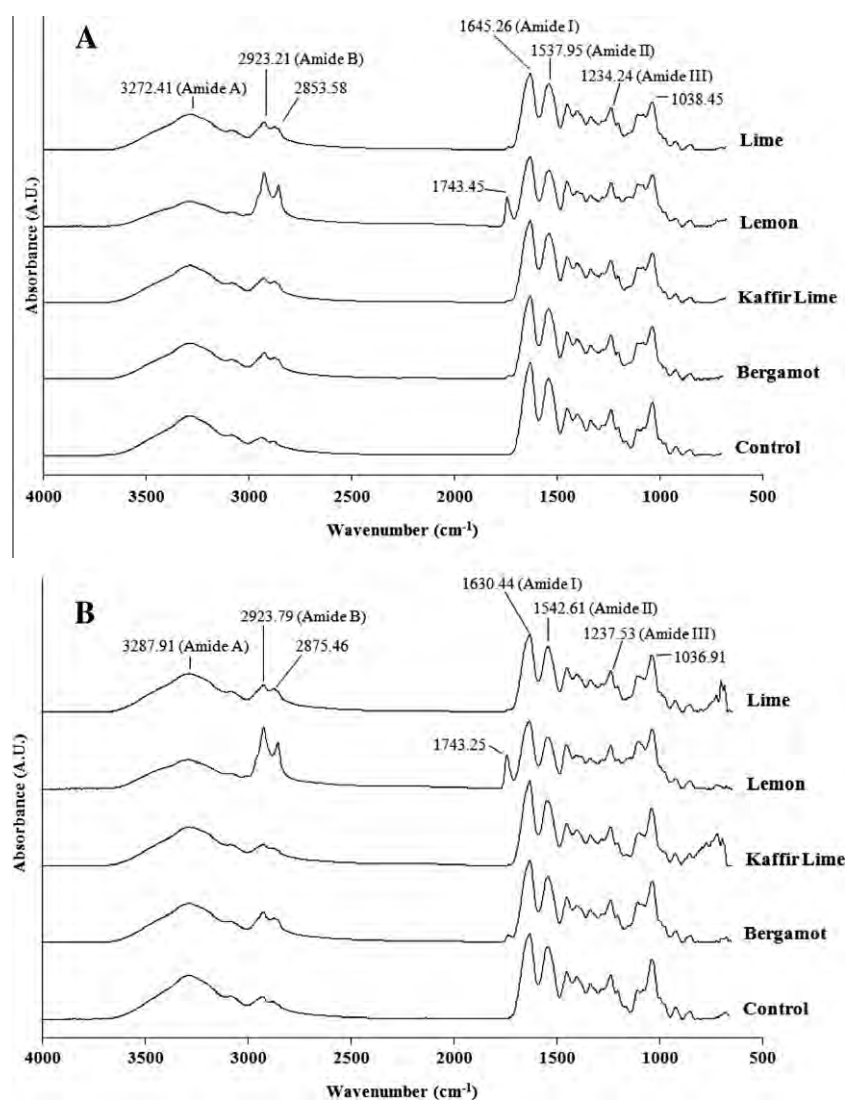


Fig. 1. ATR-FTIR spectra of films from fish skin gelatin containing 20% (A) and 30% (B) glycerol in the presence of different citrus essential oils at 50% of protein.

**Table 3**

Thermal degradation temperatures ( $T_d$ , °C) and weight loss ( $\Delta w$ , %) of films from fish skin gelatin containing 20 and 30% glycerol in the presence of different citrus essential oils at 50% of protein.

| Glycerol (%) | Essential oils | $\Delta_1$     |              | $\Delta_2$     |              | $\Delta_3$     |              | $\Delta_4$     |              | Residue (%) |
|--------------|----------------|----------------|--------------|----------------|--------------|----------------|--------------|----------------|--------------|-------------|
|              |                | $T_{d1,onset}$ | $\Delta w_1$ | $T_{d2,onset}$ | $\Delta w_2$ | $T_{d3,onset}$ | $\Delta w_3$ | $T_{d4,onset}$ | $\Delta w_4$ |             |
| 20           | Control        | 75.05          | 7.81         | 229.81         | 20.05        | 322.69         | 49.23        | –              | –            | 22.01       |
|              | Bergamot       | 84.21          | 3.91         | 239.92         | 14.28        | 318.79         | 48.84        | 417.64         | 13.13        | 19.13       |
|              | Kaffir Lime    | 70.00          | 6.25         | 211.49         | 15.03        | 319.28         | 47.11        | 401.92         | 14.83        | 16.79       |
|              | Lemon          | 77.50          | 5.76         | 221.68         | 14.52        | 310.24         | 49.39        | 405.01         | 13.35        | 15.62       |
|              | Lime           | 87.50          | 4.21         | 233.59         | 16.50        | 313.53         | 44.97        | 411.87         | 14.33        | 17.37       |
| 30           | Control        | 77.50          | 7.12         | 215.65         | 28.37        | 318.89         | 42.79        | –              | –            | 21.72       |
|              | Bergamot       | 73.75          | 6.51         | 209.37         | 18.73        | 315.57         | 45.89        | 413.47         | 13.45        | 15.43       |
|              | Kaffir Lime    | 67.50          | 6.17         | 200.75         | 21.32        | 314.91         | 39.98        | 395.07         | 17.88        | 14.65       |
|              | Lemon          | 80.00          | 6.37         | 220.54         | 24.84        | 309.37         | 36.46        | 409.07         | 16.16        | 16.17       |
|              | Lime           | 72.50          | 5.69         | 218.14         | 26.46        | 305.48         | 36.75        | 385.6          | 16.69        | 14.41       |

$\Delta_1$ ,  $\Delta_2$ ,  $\Delta_3$  and  $\Delta_4$  denote the first, second, third and fourth stage weight loss, respectively, of film during heating scan.

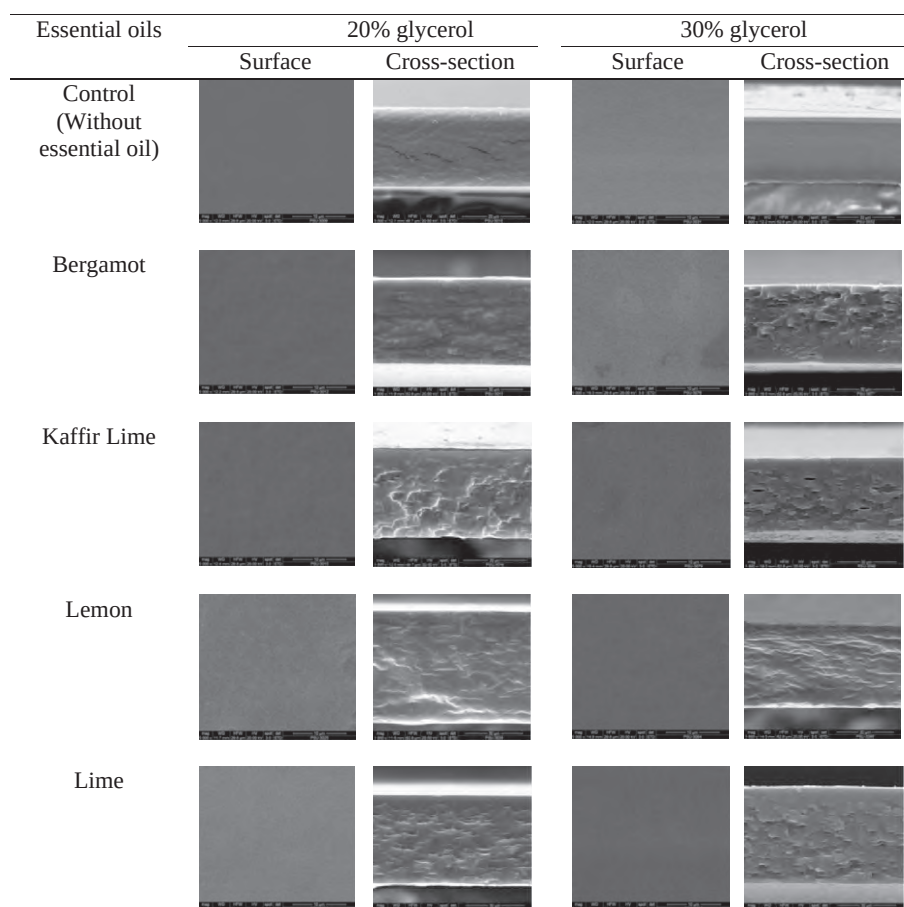


**Fig. 2.** SEM micrographs of surface (magnification: 5000 $\times$ ) and cross-section (magnification: 1800 $\times$ ) of films from fish skin gelatin containing 20% and 30% glycerol in the presence of different citrus essential oils at 50% of protein.

result, a homogeneous dispersion of oil droplets would give a lower WVP in the emulsion film.

### 3.7. Antioxidative activity of fish skin gelatin films incorporated with essential oils

Antioxidative activity expressed as DPPH radical scavenging activity, ABTS radical scavenging activity and ferric reducing antioxidant power (FRAP) of films from fish skin gelatin containing 20% and 30% glycerol in the presence of different essential oils is shown in Table 4. Control film without essential oils had no DPPH radical scavenging activity and possessed very low FRAP and ABTS

radical scavenging activity. Nevertheless, Gómez-Guillén, Ihl, Bifani, Silva, and Montero (2007) reported that films prepared from tuna skin gelatin showed antioxidative activity as determined by FRAP and ABTS assays. Control film containing 30% glycerol had slightly higher FRAP and ABTS radical scavenging activity, compared with that having 20% glycerol.

Generally, essential oils have been reported as the excellent source of antioxidant (Wu, Lee, Ho, & Chang, 1982). Film incorporated with all types of essential oils at both glycerol levels showed an antioxidant activity. ABTS radical-scavenging activity and FRAP of film incorporated with lemon essential oil was highest, followed by film added with bergamot essential oil. For DPPH radical-

**Table 4**

Antioxidant activity of films from fish skin gelatin containing 20% and 30% glycerol in the presence of different citrus essential oils at 50% of protein.

| Glycerol (%) | Essential oils | Antioxidant activity ( $\mu\text{mol}$ Trolox equivalents (TE)/g dried film) |                                |                                |
|--------------|----------------|------------------------------------------------------------------------------|--------------------------------|--------------------------------|
|              |                | DPPH                                                                         | FRAP                           | ABTS                           |
| 20           | Control        | 0.00 <sup>dA</sup>                                                           | 0.04 $\pm$ 0.01 <sup>dB</sup>  | 0.14 $\pm$ 0.30 <sup>dB</sup>  |
|              | Bergamot       | 0.25 $\pm$ 0.01 <sup>aB</sup>                                                | 1.17 $\pm$ 0.14 <sup>bB</sup>  | 4.33 $\pm$ 0.59 <sup>bB</sup>  |
|              | Kaffir Lime    | 0.04 $\pm$ 0.02 <sup>cdB</sup>                                               | 0.36 $\pm$ 0.11 <sup>cB</sup>  | 1.98 $\pm$ 0.33 <sup>cB</sup>  |
|              | Lemon          | 0.15 $\pm$ 0.01 <sup>bB</sup>                                                | 2.76 $\pm$ 0.08 <sup>aB</sup>  | 28.01 $\pm$ 2.07 <sup>aA</sup> |
|              | Lime           | 0.02 $\pm$ 0.02 <sup>cdB</sup>                                               | 0.30 $\pm$ 0.08 <sup>cdA</sup> | 2.54 $\pm$ 0.33 <sup>cB</sup>  |
| 30           | Control        | 0.00 <sup>dA</sup>                                                           | 0.18 $\pm$ 0.02 <sup>dA</sup>  | 0.64 $\pm$ 0.11 <sup>cA</sup>  |
|              | Bergamot       | 0.42 $\pm$ 0.06 <sup>aA</sup>                                                | 2.15 $\pm$ 0.08 <sup>bA</sup>  | 6.25 $\pm$ 0.47 <sup>bA</sup>  |
|              | Kaffir Lime    | 0.13 $\pm$ 0.01 <sup>cA</sup>                                                | 0.68 $\pm$ 0.05 <sup>cA</sup>  | 3.99 $\pm$ 0.64 <sup>bA</sup>  |
|              | Lemon          | 0.27 $\pm$ 0.01 <sup>bA</sup>                                                | 3.42 $\pm$ 0.08 <sup>aA</sup>  | 31.18 $\pm$ 2.08 <sup>aA</sup> |
|              | Lime           | 0.09 $\pm$ 0.02 <sup>cA</sup>                                                | 0.46 $\pm$ 0.02 <sup>dA</sup>  | 4.24 $\pm$ 0.28 <sup>bA</sup>  |

Different superscript lowercase letters in the same column under the same glycerol content indicate significant differences ( $p < 0.05$ ). Different superscript uppercase letters in the same column under the same essential oil indicate significant differences ( $p < 0.05$ ).

<sup>a</sup> Mean  $\pm$  SD ( $n = 3$ ).

scavenging activity, film incorporated with bergamot essential oil showed the highest activity ( $p < 0.05$ ), followed by film added with lemon essential oil. Films incorporated with different essential oils containing 30% glycerol mostly had the higher antioxidant activity analysed by all assays than those with 20% glycerol ( $p < 0.05$ ). This result suggested that more loosen structure of film network found in film containing 30% glycerol favoured the release of essential oils, which had antioxidative activity. Furthermore, the interaction of antioxidant compounds in different essential oils might be different. As a consequence, the binding of those compounds with film matrix could be different. Thus, the release of free antioxidant compounds could be varied.

#### 4. Conclusion

The incorporation of citrus essential oils into fish skin gelatin film directly affected the mechanical properties, water vapour permeability, colour and transparency of the film. The tested essential oils incorporated in fish skin gelatin film at 50% of protein exhibited plasticising effect, mostly owing to the decreased gelatin–gelatin interaction. Water vapour permeability was lowered when all types of essential oils were incorporated. Antioxidative activity of films incorporated with essential oils varied, depending on types of essential oils. Lemon essential oil at a level of 50% of protein could be potentially used to enhance flexibility and water vapour barrier property of fish skin gelatin film and rendered the film with high antioxidative activity.

#### Acknowledgements

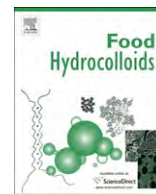
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## Properties of film from cuttlefish (*Sepia pharaonis*) skin gelatin incorporated with cinnamon, clove and star anise extracts

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### ABSTRACT

Properties of film from cuttlefish (*Sepia pharaonis*) ventral skin gelatin without and with partial hydrolysis (1.2% degree of hydrolysis) incorporated with 1% ethanolic extract of cinnamon (CME), clove (CLE) and star anise (SAE) were determined. Films with different herb extracts (without and with oxidation) had higher tensile strength (TS) but lower elongation at break (EAB), compared with the control film (without addition of herb extracts) ( $p < 0.05$ ). Lower water vapor permeability (WVP) and  $L^*$ -value but higher  $b^*$ - and  $\Delta E^*$ -values were observed when the extracts were incorporated ( $p < 0.05$ ). Electrophoretic study revealed that cross-linking was pronounced in films containing different herb extracts. Oxidized extracts yielded films with higher TS and WVP than those without oxidized herb extracts ( $p < 0.05$ ). Generally, similar properties were noticeable for films from gelatin with and without partial hydrolysis. Nevertheless, higher mechanical properties were obtained for the latter. FTIR spectra indicated that protein–polyphenol interactions were involved in the film. Thermo-gravimetric analysis revealed that films incorporated with SAE or SAE with oxidation (OSAE) exhibited lower heat susceptibility and weight loss in the temperature range of 50–600 °C, compared with control film. Films with SAE and OSAE had smoother surface for gelatin without hydrolysis; however, coarser surface was observed in film from gelatin with partial hydrolysis. Thus, the incorporation of different herb extracts directly affected the properties of film from cuttlefish skin gelatin with and without hydrolysis.

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### 1. Introduction

Biodegradable films made from renewable biopolymers have become an important environmental friendly packaging, thereby reducing plastic wastes (Hoque, Benjakul, & Prodpran, 2010a; Prodpran & Benjakul, 2005; Tharanathan, 2003). Most synthetic films are non-biodegradable, leading to environmental pollution and serious ecological problems (Tharanathan, 2003). Gelatin is a natural biopolymer obtained by thermal degradation of collagen (Arvanitoyannis, 2002). Gelatin has been used as a material for preparing biodegradable films with an excellent gas barrier property (Jongiareonrak, Benjakul, Vesessanguan, Prodpran, & Tanaka, 2006; Tharanathan, 2003). Protein-based films have good barrier characteristics against gas, organic vapor and oil as compared to synthetic films, which can maintain the quality of food products (Jiang, Tang, Wen, Li, & Yang, 2007). However, the poor mechanical properties and the high water vapor permeability are the main

drawbacks of protein films for their application as a packaging material (McHugh & Krochta, 1994).

To modify the properties of protein film, different chemical cross-linking agents and enzyme including gossypol, formaldehyde, glutaraldehyde (de Carvalho & Grosso, 2004), glyoxal (Nuthong, Benjakul, & Prodpran, 2009a) and transglutaminase (Mariniello et al., 2003) have been used. However, the toxicity and high cost of some cross-linking agents have led to their limitation for the application in films (Cao, Fu, & He, 2007). Polyphenols are known to react with side chain amino groups of peptides, leading to the formation of protein cross-links (Strauss & Gibson, 2004).

Different plant or herb extracts have been incorporated in gelatin films to enhance antioxidant and/or antimicrobial properties such as murta ecotypes leave extracts incorporated in tuna-skin gelatin film (Gómez-Guillén, Ihl, Bifani, Silva, & Montero, 2007), oregano or rosemary aqueous extracts in bovine-hide and tuna-skin gelatin film (Gómez-Estaca, Bravo, Gómez-Guillén, Alemán, & Montero, 2009) and borage extract in sole skin or commercial fish skin gelatin (Gómez-Estaca, Giménez, Montero, & Gómez-Guillén, 2009). Additionally, tuna-skin gelatin film incorporated with oregano and rosemary extracts had altered properties including higher glass

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transition temperature, decreased deformability and increased water solubility (Gómez-Estaca, Montero, Fernández-Martín, Alemán, & Gómez-Guillén, 2009) and improved light barrier properties (Gómez-Estaca, Bravo, et al., 2009). Rattaya, Benjakul, and Prodpran (2009) found that fish skin gelatin films incorporated with oxygenated seaweed (*Turbinaria ornata*) extract exhibited higher EAB, and lower WVP and film solubility than the control film ( $p < 0.05$ ). Recently, Hoque, Benjakul, and Prodpran (2010b) found that gelatin with shorter chain length produces film with poorer properties. Incorporation of selected herb extracts may improve the properties of those films. However, there is no information about the effect of incorporation of herb (cinnamon, clove and star anise) extracts on the properties of gelatin-based film. Thus, the objectives of the present study were to study the effects of three different herb (cinnamon, clove and star anise) extracts with and without oxidation on the properties of films from cuttlefish skin gelatin with and without partial hydrolysis.

## 2. Materials and methods

### 2.1. Chemicals

L-Leucine, 2,4,6-trinitrobenzenesulphonic acid (TNBS), bovine serum albumin and wide range molecular weight protein markers were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide ( $H_2O_2$ ), glycerol, *p*-dimethylaminobenzaldehyde and tris(hydroxymethyl) aminomethane were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250 and *N*, *N*, *N'*, *N'*-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Folin–Ciocalteu's reagent and gallic acid were obtained from Fluka (Buchs, Switzerland). Alcalase 2.4 L was provided by Novozymes (Bagsvaerd, Denmark). All chemicals were of analytical grade.

### 2.2. Preparation of herb extracts

#### 2.2.1. Collection and preparation of herb

Three different herbs namely cinnamon (*Cinnamomum zeylanicum*), clove (*Syzygium aromaticum*) and star anise (*Illicium verum*) were obtained from local market in Hat Yai, Thailand. Herbs were dried using a hot-air oven at 35 °C until the final moisture content of 10–12% was obtained. Dried herbs were then ground and sieved to obtain uniformity using a screen with mesh 35 with an aperture size of 500 µm, ASTM E11, serial number 5666533 (FRITSCH GMBH, Laborgerätebau, Industriestrasse 8, D-55743 Idar-Oberstein, Germany).

#### 2.2.2. Preparation of herb extracts

Herb extracts were prepared according to the method of Santoso, Yoshie-Stark, and Suzuki (2004) with some modifications. To prepare herb extracts, the herb powder (25 g) was mixed with 80% ethanol using a powder/solvent ratio of 1:20 (w/v). The mixtures were then stirred continuously at room temperature for 3 h. The mixtures were centrifuged at 5000×g for 10 min at room temperature using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Beckman Coulter, Inc., Palo Alto, CA, USA) to remove undissolved debris. The supernatant was collected and filtered through a Whatman filter paper No. 1 (Schleicher & Schuell, Maidstone, England). The solvent in the extract was removed by a rotary evaporator (EYELA, Rotary Vacuum Evaporator, N-1000 series, Tokyo Rikakikai Co., Ltd., Chuo-ku, Tokyo, Japan) at 40 °C to obtain the concentrated herb extract. To remove the residual ethanol, the extract was purged with nitrogen gas. The extract was further dried using a freeze dryer to obtain the dry extract. Dried

extract was ground into a powder using a mortar and pestle. Extract powder was referred to as cinnamon extract (CME), clove extract (CLE) and star anise extract (SAE). CME, CLE and SAE powder was placed in amber bottles, closed tightly and stored in a dessicator until use.

#### 2.2.3. Determination of total phenolic content of herb extracts

Total phenolic content of herb extracts was determined with Folin–Ciocalteu reagent according to the method of Singleton, Orthofer, and Lamuela-Raventos (1998) using gallic acid as a standard. Extract was dissolved in 25% ethanol to obtain the concentration of 0.5% (w/v). The solution (0.5 mL) was added to 100 µL of Folin–Ciocalteu reagent (two-fold diluted with de-ionized water) and mixed thoroughly. After 3 min, 1.5 mL of 2%  $Na_2CO_3$  was added. The mixture was allowed to stand at room temperature for 30 min. The absorbance was measured at 760 nm using a spectrophotometer (UV-160, Shimadzu, Kyoto, Japan). The concentration of total phenolic compounds in the extract was calculated from a standard curve generated with gallic acid (0.01–0.1 mg/mL) and expressed as mg of gallic acid equivalent (GAE)/g powder.

#### 2.2.4. Preparation of oxidized herb extracts

Oxidized herb extracts were prepared as per the method of Strauss and Gibson (2004) with some modifications. Herb extracts (CME, CLE and SAE) were dissolved using 25% ethanol to obtain a concentration of 0.5% (w/v). The solutions were then adjusted to pH 12 by adding 6 M NaOH. The prepared solutions were placed in a temperature controlled water bath (Mettler, GmbH+Co. KG, D-91126, Schwabach, Germany) at 40 °C and subjected to oxygenation by bubbling the solution with oxygen gas with a purity of 99.8% (TTS Gas Agency, Hat Yai, Songkhla, Thailand) for 30 min to convert the phenolic compounds to quinone. After being oxygenated for 30 min, the solution was then neutralized using 2 M HCl and the final volume of the solution was adjusted to 25 mL by using distilled water and was referred to as “oxidized herb extract” named oxidized cinnamon extract (OCME), oxidized clove extract (OCLE) and oxidized star anise extract (OSAE). All oxidized extracts were analyzed for quinone formation using Folin–Ciocalteu reagent according to the methods of Balange and Benjakul (2010) with a slight modification. The conversion of phenolic compounds in different herb extracts to quinone was monitored indirectly by determining total phenolic content in different herb extracts before and after oxidation. The differences in the total phenolic content before and after oxidation indicated the formation of quinone and were expressed in percentages.

### 2.3. Collection and preparation of cuttlefish skin

Ventral skin of cuttlefish (*Sepia pharaonis*) was obtained from a dock in Songkhla, Thailand. Cuttlefish skin was stored in ice with a skin/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, cuttlefish skin was washed with tap water and cut into small pieces (1 × 1 cm<sup>2</sup>), placed in polyethylene bags and stored at –20 °C until use. Storage time was not longer than 2 months. Prior to gelatin extraction, the frozen skin was thawed using running water (25–26 °C) until the core temperature reached 0–2 °C.

### 2.4. Preparation of gelatin and partially hydrolyzed gelatin from cuttlefish skin

Gelatin was extracted from cuttlefish skin according to the method of Hoque et al. (2010a). Skin was soaked in 0.05 M NaOH with a skin/solution ratio of 1:10 (w/v) with gentle stirring at room

temperature (26–28 °C). The solution was changed every hour to remove non-collagenous proteins for totally 6 h. Alkali treated skin was then washed with distilled water until a neutral pH of wash water was obtained. The prepared skin was subjected to bleaching in 5% H<sub>2</sub>O<sub>2</sub>, using a sample/solution ratio of 1:10 (w/v) for 48 h at 4 °C. The skin treated with H<sub>2</sub>O<sub>2</sub> was washed three times with 10 volumes of distilled water. Gelatin was extracted from bleached skin by using distilled water at 60 °C for 12 h, using a sample/water ratio of 1:2 (w/v). During extraction, the mixture was stirred continuously using a paddle stirrer (RW20.n, IKA LABORTECHNIK, Staufen, Germany). The extract was centrifuged at 8000×g for 30 min at room temperature using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Beckman Coulter, Inc., Palo Alto, CA, USA) to remove insoluble materials. The supernatant was collected and freeze-dried (Model Duratop™ IP/Dura Dry™ IP, FTS® System, Inc., Stone Ridge, NY, USA). The dry matter was referred to as 'gelatin powder'.

To prepare gelatin with partial hydrolysis, gelatin was dissolved in distilled water to obtain a final protein concentration of 3% (w/v) and the pH of the mixture was adjusted to 8.0 using 6 M NaOH. The mixtures were incubated at 50 °C for 10 min. The hydrolysis reaction was started by the addition of Alcalase at the amount calculated from the plot between log enzyme concentration and degree of hydrolysis (DH) to obtain DH of 1.2% as described by Benjakul and Morrissey (1997). After hydrolysis by using Alcalase for 5 min, the resulting hydrolysates were subjected to heat treatment (90 °C) in a temperature controlled water bath (Mettler, GmbH+Co. KG, D-91126, Schwabach, Germany) for 15 min to fully inactivate the enzyme. Resulting hydrolysate was neutralized using 6 M HCl and used for film preparation.

## 2.5. Effect of different herb extracts on the properties of gelatin films

### 2.5.1. Preparation of film from gelatin incorporated with different herb extracts

Prior to film preparation, gelatin powder was dissolved in distilled water and heated at 70 °C for 30 min (Hoque et al., 2010a). Glycerol at a level of 20% (based on protein content) was added to solutions of gelatin and gelatin with 1.2% DH both containing 3% protein and mixed thoroughly. The mixtures obtained were referred to as 'film-forming solution; FFS'.

Different herb extracts (CME, CLE and SAE) and those subjected to oxidation (OCME, OCLE and OSAE) were incorporated into FFS at a level of 1% (based on protein content). Control films were prepared by omitting herb extracts from FFS. The mixtures were stirred at room temperature for 1 h. FFS (4 ± 0.01 g) was cast onto a rimmed silicone resin plate (5 × 5 cm<sup>2</sup>), air-blown for 12 h at room temperature and dried with an environmental chamber (Binder, KBF 115 # 00-19735, D-78532, Tuttlingen, Germany) at a temperature of 25 ± 0.5 °C and 50 ± 5% relative humidity (RH) for 48 h. Dried films were manually peeled off and subjected to analyses.

## 2.6. Analyses

Prior to mechanical properties testing, films were conditioned for 48 h at 50 ± 5% relative humidity (RH) at 25 ± 0.5 °C. For SEM, ATR-FTIR and TGA studies, films were conditioned in a dessicator containing dried silica gel for 3 weeks at room temperature (28–30 °C) to obtain the most dehydrated films.

### 2.6.1. Film thickness

The thickness of film was measured using a digital micrometer (Mitutoyo, Model ID-C112PM, Serial No. 00320, Mitutoyo Corp.,

Kawasaki-shi, Japan). Ten random locations around each film sample were used for thickness determination.

### 2.6.2. Mechanical properties

Tensile strength (TS) and elongation at break (EAB) were determined as described by Iwata, Ishizaki, Handa, and Tanaka (2000) using the Universal Testing Machine (Lloyd Instrument, Hampshire, UK). Ten samples (2 × 5 cm<sup>2</sup>) with initial grip length of 3 cm were used for testing. The samples were clamped and deformed under tensile loading using a 100 N load cell with cross-head speed of 30 mm/min until the samples were broken. The maximum load and the final extension at break were used for calculation of TS and EAB, respectively.

### 2.6.3. Water vapor permeability (WVP)

WVP was measured using a modified ASTM (American Society for Testing and Materials, 1989) method as described by Shiku, Yuca Hamaguchi, Benjakul, Visessanguan, and Tanaka (2004). The film was sealed on an aluminum permeation cup containing silica gel (0% RH) with silicone vacuum grease. The cup was placed at 30 °C in a dessicator containing distilled water. It was then weighed at 1 h intervals for up to 8 h. Five films were used for WVP testing. WVP of the film was calculated as follows:

$$\text{WVP} \left( \text{g m}^{-1} \text{s}^{-1} \text{Pa}^{-1} \right) = w l A^{-1} t^{-1} (P_2 - P_1)^{-1}$$

where  $w$  is the weight gain of the cup (g);  $l$  is the film thickness (m);  $A$  is the exposed area of film (m<sup>2</sup>);  $t$  is the time of gain (s); ( $P_2 - P_1$ ) is the vapor pressure difference across the film (Pa).

### 2.6.4. Color, light transmission and transparency of the film

Color of film was determined using a CIE colorimeter (Hunter associates laboratory, Inc., Reston, VA, USA). Color of the film was expressed as  $L^*$ ,  $a^*$  and  $b^*$ -values. Total difference in color ( $\Delta E^*$ ) was calculated according to the following equation (Gennadios, Weller, Hanna, & Froning, 1996):

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where  $\Delta L^*$ ,  $\Delta a^*$  and  $\Delta b^*$  are the differences between the corresponding color parameter of the sample and that of white standard ( $L^* = 93.63$ ,  $a^* = -0.95$  and  $b^* = 0.46$ ).

Light transmission of films against ultraviolet (UV) and visible light was measured at selected wavelengths between 200 and 800 nm, using a UV–Visible spectrophotometer (model UV-160, Shimadzu Co., Kyoto, Japan) according to the method of Jongiareonrak et al. (2006).

The transparency value of the film was calculated by the following equation (Han & Floros, 1997):

$$\text{Transparency value} = -\log T_{600}/x$$

where  $T_{600}$  is the fractional transmittance at 600 nm and  $x$  is the film thickness (mm). The greater transparency value represents lower transparency of the films.

### 2.6.5. Film solubility

Film solubility in water was determined according to the method of Gennadios, Handa, Froning, Weller, and Hanna (1998) with a slight modification. The conditioned film sample (3 × 2 cm<sup>2</sup>) was weighed and placed in 50 mL centrifuge tubes containing 10 mL of distilled water with 0.1% (w/v) sodium azide. The mixture was shaken continuously at room temperature for 24 h using a shaker (Heidolph UNIMAX 1010, Schwabach, Germany). Undissolved debris film matter was determined after centrifugation

at 3000×g for 10 min at 25 °C and drying them at 105 °C for 24 h to obtain the dry unsolubilized film matter. The weight of solubilized dry matter was calculated by subtracting the weight of unsolubilized dry matter from the initial weight of dry matter and expressed as the percentage of total weight.

#### 2.6.6. Electrophoretic analysis

Protein patterns of all film samples were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Prior to analysis, the film samples were prepared according to the method of Jongiareonrak et al. (2006) with some modifications. Film samples (100 mg) were dissolved in 10 mL of 1% (w/v) SDS. The mixture was stirred continuously at room temperature for 12 h. Supernatants were obtained after centrifugation at 3000×g for 5 min using Hettich Zentrifuge (MIKRO-20, D-78532, Tuttlingen, Germany). Proteins in supernatants were determined using the Biuret method (Robinson & Hodgen, 1940). The supernatants were then mixed with sample buffer (0.5 M Tris–HCl, pH 6.8 containing 4% (w/v) SDS, 20% (v/v) glycerol with 10% (v/v) β-ME) at a ratio of 1:1 (v/v). Samples (30 mg protein) were loaded onto the polyacrylamide gel made of 7.5% (for films from gelatin without hydrolysis) and 12% (for films from gelatin with 1.2% DH) running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, gel was stained with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. Wide range molecular weight protein markers were used to estimate the molecular weight of proteins.

Quantitative analysis of protein band intensity was performed using a Model GS-700 Imaging Densitometer (Bio-Red Laboratories, Hercules, CA, USA) with Molecular Analyst Software version 1.4 (image analysis systems).

#### 2.7. Characterization of gelatin film incorporated with selected herb extract

Films prepared from gelatin and partially hydrolyzed gelatin incorporated without and with star anise extracts (without and with oxidation) was characterized.

##### 2.7.1. Attenuated total reflectance-Fourier transforms infrared (ATR-FTIR) spectroscopy

FTIR spectra of films without and with addition of SAE and OSAE were collected using a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) equipped with a horizontal ATR Trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) at 25 °C as described by Nuthong et al. (2009a). Sample was placed onto the

crystal cell and the cell was clamped into the mount of FTIR spectrometer. The spectra in the range of 700–4000 cm<sup>-1</sup> with automatic signal gain were collected in 32 scans at a resolution of 4 cm<sup>-1</sup> and were ratioed against a background spectrum recorded from the clean empty cell at 25 °C.

##### 2.7.2. Thermo-gravimetric analysis (TGA)

Conditioned films were scanned using a thermo-gravimetric analyzer (TGA7, PerkinElmer, Norwalk, CT, USA) from 50 to 600 °C at a rate of 10 °C/min (Nuthong et al., 2009a). Nitrogen was used as the purge gas at a flow rate of 20 mL/min.

##### 2.7.3. Microstructure

Microstructure of upper surface and cross-section of the film samples were visualized using a scanning electron microscope (SEM) (JSM-5800LV, JEOL, Tokyo, Japan) at an accelerating voltage of 10 kV. Prior to visualization, the film samples were mounted on brass stub and sputtered with gold in order to make the sample conductive, and photographs were taken at 8000× magnification. For cross-section, freeze-fractured films were mounted around stubs using double sided adhesive tape, coated with gold and observed at the 3000× magnification.

#### 2.8. Statistical analysis

Experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by Duncan's multiple range test (Steel & Torrie, 1980). Analysis was performed using the SPSS package (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

### 3. Results and discussion

#### 3.1. Characteristics of herb extracts

Extraction yield and total phenolic content of three different herb extract powders are shown in Table 1. The highest yield was obtained for CLE (23.2%), followed by CME (19.4%) and SAE (16.0%), respectively. Total phenolic contents of CME, CLE and SAE expressed as gallic acid equivalent, were 138.1, 125.0 and 34.4 mg/g powder, respectively. CME generally contained the highest content of phenolic compounds, though it had the lower yield, when compared with CLE. Su et al. (2007) found the phenolic content in cinnamon methanolic extract at a level of 148 mg GAE/g powder. Among all extracts, SAE showed the lowest phenolic content and yield. Thus, different herbs rendered the extracts with different extractable phenolic compounds. Kim and Lee (2004) also reported significant differences in total phenolic content from six different herbs due to genotypic and environmental differences namely, climate, location, temperature, fertility, diseases and pest exposure

**Table 1**  
Total phenolic content and color of three different herb extracts.

| Herb      | Extracted dry powder <sup>a</sup> | Total phenolic content <sup>b</sup> | Total phenolic content <sup>c</sup> | Color of extract solution <sup>d</sup> |     |     |     |      |      |      |      |
|-----------|-----------------------------------|-------------------------------------|-------------------------------------|----------------------------------------|-----|-----|-----|------|------|------|------|
|           |                                   |                                     |                                     | L*                                     |     | a*  |     | b*   |      | ΔE*  |      |
|           |                                   |                                     |                                     | w/o                                    | w   | w/o | w   | w/o  | w    | w/o  | w    |
| Cinnamon  | 19.4                              | 138.1                               | 49.5                                | 3.2                                    | 1.4 | 0.6 | 0.5 | −2.0 | 0.0  | 90.5 | 92.3 |
| Clove     | 23.2                              | 125.0                               | 52.2                                | 1.5                                    | 1.1 | 1.1 | 0.8 | −1.0 | −0.5 | 92.2 | 92.8 |
| Star anis | 16.0                              | 34.8                                | 11.9                                | 2.5                                    | 1.4 | 0.9 | 0.5 | −1.9 | −0.7 | 91.3 | 92.3 |

w/o: Without oxidation; w: with oxidation.

<sup>a</sup> Yield of extracts was calculated as percentage (w/w) of initial herbs' weight.

<sup>b</sup> Total phenolic content (before oxidation) was expressed as mg gallic acid equivalents/g powder (mg GAE/g of powder).

<sup>c</sup> Total phenolic content (after oxidation) was expressed as mg gallic acid equivalents/g powder (mg GAE/g of powder).

<sup>d</sup> 4 mL of 0.5% (w/v) extract solution were used for color determination.

within species, choice of parts tested, time of taking samples and determination methods.

Different  $L^*$ -,  $a^*$ -,  $b^*$ -values and  $\Delta E^*$  were observed among different extract solutions (0.5%, w/v) (Table 1). The differences in color of different extract solutions might be attributed to the variation in pigments, composition and response to the polarity of the solvent (Tian et al., 2009).

The formation of quinones, an oxidized form of phenolic compounds, in different herb extracts varied (Table 1). After oxidation process, phenolic compounds were converted to quinone by 64.2, 58.2 and 65.8% for CME, CLE and SAE, respectively. The greatest extent of quinone formation occurred with SAE. The results suggest possible formation of quinone. Quinones react with amino or sulfhydryl side chains of polypeptides to form C–N or C–S covalent bonds (Strauss & Gibson, 2004). Additionally, changes in color were observed for oxidized herb extracts. Oxidized herb extract solutions had lower  $L^*$ -values and higher  $b^*$ - and  $\Delta E^*$ -values, as compared with those of herb extracts without oxidation. The changes in color of extracts upon oxidation were more likely related with the formation of quinone. Quinones are brown or dark in color. Discolorations due to conversion of phenol to quinone were reported by Pospíšila, Nešpurek, Zweifel, and Kuthanc (2002).

### 3.2. Effects of incorporation of different herb extracts on the properties of film from cuttlefish skin gelatin

Films from both gelatin and partially hydrolyzed gelatin incorporated with three different herb extracts with and without oxidation exhibited different properties and molecular characteristics.

#### 3.2.1. Thickness

Thickness of films prepared from cuttlefish skin gelatin incorporated with different herb extracts is shown in Table 2. For films from both gelatin and partially hydrolyzed gelatin, no differences in thickness were found between films incorporated without and with different herb extracts ( $p > 0.05$ ). Rattaya et al. (2009) found similar thickness of gelatin film incorporated without and with oxygenated seaweed extracts. Films from both gelatin and partially hydrolyzed gelatin had similar thickness, regardless of herb extract incorporated.

#### 3.2.2. Mechanical properties

Mechanical properties of films prepared from gelatin and partially hydrolyzed gelatin incorporated with different herb extracts without and with oxidation is presented in Table 2. Films obtained from gelatin incorporated with CME, CLE and SAE showed higher TS, but lower EAB, as compared with the control film (without addition of herb extracts) ( $p < 0.05$ ). TS of films were increased by 19.3, 19.1 and 16.2% when CME, CLE and SAE at a level of 1% were incorporated, respectively. For the films prepared from partially hydrolyzed gelatin, those with SAE had increase in TS by 33.1%, compared with that of the control film. However, there were no changes in TS of films containing other non-oxidized extracts ( $p > 0.05$ ). No changes in EAB of resulting films were noticeable when different herb extracts were incorporated ( $p > 0.05$ ). The results suggested that interactions between proteins and phenolic compounds in herb extracts were determined by the chain length of gelatin. Gelatin without hydrolysis, which possessed the higher chain length, more likely provided more reactive groups within one chain for interaction with phenolic compounds via hydrogen bonds and hydrophobic interactions, leading to film strengthening. As a result, inter-connection between gelatin molecules was more pronounced. Polyphenol contained hydrophobic groups which entered into hydrophobic region of protein by hydrophobic interaction (Shi & Di, 2000). Furthermore, hydroxyl group of polyphenol was able to combine with hydrogen acceptor in gelatin molecules by hydrogen bonds. A scheme illustrating the impact of herb extracts without and with oxidation on the cross-linking of gelatin molecules via several bondings in the film matrix is presented in Fig. 1. Rattaya et al. (2009) also reported increased mechanical properties of gelatin film incorporated with seaweed extracts through protein–polyphenol hydrophobic interactions and hydrogen bonds. Protein–polyphenol interactions thus altered the properties of the gelatin films incorporated with different plant extracts including oregano and rosemary extracts (Gómez-Estaca, Montero, et al., 2009) and borage extract (Gómez-Estaca, Giménez, et al., 2009). Increases in TS were reported in ferulic acid treated gelatin films at pH 7 and tannic acid treated films at pH 9 (Cao et al., 2007), tannic acid, caffeic acid and ferulic acid treated porcine-plasma protein films at pH 7–10 (Nuthong, Benjakul, & Prodpran, 2009b) and ferulic acid treated soy protein isolate films (Ou, Wang, Tang, Huang, & Jackson, 2005).

When oxidized herb extracts were incorporated, greater increases in TS were obtained, in comparison with those found with

**Table 2**

Mechanical properties, water vapor permeability and thickness of films from gelatin and partially hydrolyzed gelatin from cuttlefish skin incorporated without and with herb extracts without and with oxidation.

| Source of materials          | Herb extracts | TS (MPa)            | EAB (%)           | WVP ( $\times 10^{-10}$ g s <sup>-1</sup> m <sup>-1</sup> Pa) | Thickness (mm)      |
|------------------------------|---------------|---------------------|-------------------|---------------------------------------------------------------|---------------------|
| Gelatin                      | C             | 32.78 $\pm$ 3.10 d  | 5.92 $\pm$ 0.70a  | 0.96 $\pm$ 0.03 a                                             | 0.032 $\pm$ 0.003 a |
|                              | CME           | 39.11 $\pm$ 1.73 c  | 4.31 $\pm$ 0.41 b | 0.80 $\pm$ 0.06 c                                             | 0.030 $\pm$ 0.003 a |
|                              | CLE           | 39.04 $\pm$ 1.43 c  | 4.68 $\pm$ 0.39 b | 0.77 $\pm$ 0.07 c                                             | 0.030 $\pm$ 0.002 a |
|                              | SAE           | 38.08 $\pm$ 1.90 c  | 4.73 $\pm$ 0.28 b | 0.79 $\pm$ 0.06 c                                             | 0.031 $\pm$ 0.003 a |
|                              | OCME          | 44.06 $\pm$ 1.96 b  | 4.82 $\pm$ 0.22 b | 0.88 $\pm$ 0.05 b                                             | 0.030 $\pm$ 0.002 a |
|                              | OCLE          | 43.13 $\pm$ 1.82 b  | 4.69 $\pm$ 0.26 b | 0.88 $\pm$ 0.05 b                                             | 0.029 $\pm$ 0.003 a |
|                              | OSAE          | 46.96 $\pm$ 2.03 a  | 4.28 $\pm$ 0.30 b | 0.90 $\pm$ 0.02 b                                             | 0.029 $\pm$ 0.002 a |
| Partially hydrolyzed gelatin | C             | 11.10 $\pm$ 2.06 c  | 2.0 $\pm$ 0.41 a  | 1.05 $\pm$ 0.05 a                                             | 0.031 $\pm$ 0.002 a |
|                              | CME           | 13.33 $\pm$ 1.27 bc | 1.68 $\pm$ 0.67 a | 0.85 $\pm$ 0.06 c                                             | 0.030 $\pm$ 0.002 a |
|                              | CLE           | 13.01 $\pm$ 1.06 bc | 1.74 $\pm$ 0.41 a | 0.82 $\pm$ 0.04 c                                             | 0.029 $\pm$ 0.002 a |
|                              | SAE           | 14.77 $\pm$ 2.10 b  | 1.83 $\pm$ 0.15 a | 0.83 $\pm$ 0.05 c                                             | 0.029 $\pm$ 0.002 a |
|                              | OCME          | 19.74 $\pm$ 1.83 a  | 1.76 $\pm$ 0.55 a | 0.95 $\pm$ 0.04 b                                             | 0.030 $\pm$ 0.001 a |
|                              | OCLE          | 18.34 $\pm$ 1.54 a  | 1.88 $\pm$ 0.22 a | 0.96 $\pm$ 0.07 b                                             | 0.029 $\pm$ 0.003 a |
|                              | OSAE          | 20.31 $\pm$ 1.76 a  | 1.68 $\pm$ 0.31 a | 0.94 $\pm$ 0.05 b                                             | 0.030 $\pm$ 0.002 a |

Values are given as mean  $\pm$  SD (n = 3).

Different letters in the same column within the same source of gelatin indicate the significant differences ( $p < 0.05$ ).

C: control (without herb extracts); CME, CLE, SAE: cinnamon, clove and star anise extracts, respectively.

OCME, OCLE, OSAE: the oxidized cinnamon, clove and star anise extracts, respectively.

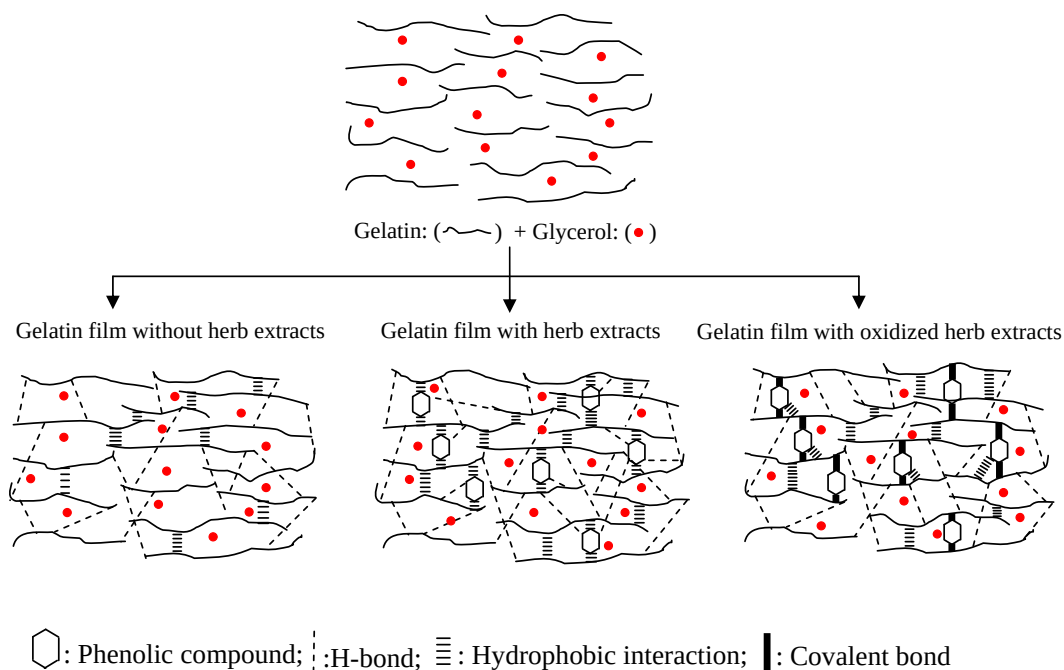


Fig. 1. A scheme illustrating the impact of herb extracts without and with oxidation on the cross-linking of gelatin molecules in the film matrix.

the addition of extracts (without oxidation). With the addition of oxidized herb extracts, TS of gelatin film were increased by 34.4, 31.5 and 43.2% for OCME, OCLE and OSAE incorporated film, respectively, as compared with the control film. For the films from partially hydrolyzed gelatin, the addition of OCME, OCLE and OSAE increased TS of resulting films by 77.8, 65.2 and 82.9%, respectively, as compared with the control. The result suggested that under alkaline pH and in the presence of oxygen, phenolic compounds were converted to quinone. The quinone as a protein-crosslinker could interact with nucleophilic amino group of gelatin (Strauss & Gibson, 2004). Quinones react with amino or sulfhydryl side chains of polypeptides to form covalent C–N or C–S bonds (Strauss & Gibson, 2004). Oxidized phenolic compounds in different herb extracts might contribute to the formation of non-disulfide covalent bond. Thus, the incorporation of oxidized herb extracts effectively improved TS of resulting film. With the addition of OSAE, film prepared from partially hydrolyzed gelatin had higher percentage increase (82.9%) in TS than the film prepared from gelatin (43.2%). The result suggested that the hydrolysis could expose more amino groups at N-termini. Those amino groups could serve as the nucleophilic domain for the attack of oxidized polyphenols, in which subsequent cross-links were formed. Therefore, the mechanical properties of gelatin-based film were largely affected by the addition of herb extracts and chain length of gelatin. Additionally, the oxidized herb extracts exhibited greater strengthening effects on resulting films.

### 3.2.3. Water vapor permeability (WVP)

WVP of films prepared from gelatin containing different herb extracts without and with oxidation is presented in Table 2. Decreases in water vapor permeability (WVP) of film were observed, compared with the control film for both gelatin and partially hydrolyzed gelatin, when different herb extracts were incorporated. Increasing amount of cross-links via hydrogen and hydrophobic interaction might form film network with decreased free volume of the polymeric matrix, resulting in the lower WVP of the resulting films. Gelatin film incorporated with seaweed extract

had significantly lower WVP, compared with the control (without addition of seaweed extract) (Rattaya et al., 2009). Gómez-Guillén et al. (2007) also found decreased WVP of tuna-fish (*Thunnus thynnus*) gelatin-based edible films incorporated with murta (*Ugni molinae* Turcz.) leaves (Soloyo Chico) extracts. A decrease in WVP of soy protein isolate films cross-linked with ferulic acid was also reported (Ou et al., 2005). No differences in WVP were observed among films incorporated with the three non-oxidized herb extracts ( $p > 0.05$ ). When oxidized herb extract was incorporated, the resulting films showed higher WVP, compared with their non-oxidized counterpart ( $p < 0.05$ ). Nevertheless, no differences in WVP were found among films incorporated with oxidized extract from different herbs ( $p > 0.05$ ). These results might be due to the presence of higher number of polar groups of oxidized phenolic compounds in the extracts dispersed in the films. The barrier characteristics of film were affected by the chemical nature of the macromolecule, structural/morphological characteristics of the polymeric matrix, chemical nature of the additives and degree of cross-linking (McHugh & Krochta, 1994). Thus, incorporation of cinnamon, clove and star anise extract had the profound impact on WVP of film from cuttlefish skin gelatin. The extract and oxidized extracts had an impact on WVP of film from cuttlefish skin gelatin.

### 3.2.4. Color of film

Table 3 shows the color of films prepared from cuttlefish skin gelatin containing different herb extracts without and with oxidation. Lower  $L^*$ -value (lightness), higher  $b^*$ -value (yellowness) and  $\Delta E^*$  (color difference) were obtained for films containing all herb extracts, compared with the control (without addition of herb extracts) ( $p < 0.05$ ). When comparing the color of control films from gelatin and partially hydrolyzed gelatin, it was found that the former had the higher  $L^*$ -value but lower  $b^*$ - and  $\Delta E^*$ -values ( $p < 0.05$ ). Moderate changes in  $L^*$ -,  $a^*$ - and  $\Delta E^*$ -values were observed in the films incorporated with CLE and SAE (both without and with oxidation). The changes in color were more pronounced in the films containing CME (both without and with oxidation), as evidenced by the lowest  $L^*$ -value, and the highest  $b^*$ - and

**Table 3**

Color of films from gelatin and partially hydrolyzed gelatin from cuttlefish skin incorporated without and with herb extracts without and with oxidation.

| Source of materials          | Herb extracts | Color           |                 |                |                |
|------------------------------|---------------|-----------------|-----------------|----------------|----------------|
|                              |               | L*              | a*              | b*             | ΔE*            |
| Gelatin                      | C             | 91.29 ± 0.06 a  | −1.31 ± 0.06 d  | 2.71 ± 0.14 e  | 3.27 ± 0.12 e  |
|                              | CME           | 89.77 ± 0.08 e  | −0.95 ± 0.07 b  | 4.61 ± 0.30 c  | 5.67 ± 0.22 b  |
|                              | CLE           | 90.52 ± 0.27 c  | −1.68 ± 0.08 e  | 4.56 ± 0.14 c  | 5.38 ± 0.09 c  |
|                              | SAE           | 90.83 ± 0.09 b  | −1.20 ± 0.04 c  | 3.77 ± 0.14 d  | 4.35 ± 0.13 d  |
|                              | OCME          | 88.13 ± 0.11 f  | −0.21 ± 0.04 a  | 7.61 ± 0.22 a  | 9.06 ± 0.23 a  |
|                              | OCLE          | 90.23 ± 0.12 d  | −1.72 ± 0.06 e  | 5.00 ± 0.41 b  | 5.57 ± 0.09 b  |
|                              | OSAE          | 90.15 ± 0.10 d  | −1.26 ± 0.13 cd | 4.82 ± 0.14 bc | 5.59 ± 0.08 b  |
| Partially hydrolyzed gelatin | C             | 90.65 ± 0.28 ab | −1.32 ± 0.05 c  | 2.92 ± 0.19 d  | 3.70 ± 0.19 d  |
|                              | CME           | 89.89 ± 0.15 c  | −0.90 ± 0.08 a  | 4.54 ± 0.36 b  | 5.54 ± 0.30 b  |
|                              | CLE           | 90.82 ± 0.65 a  | −1.47 ± 0.11 d  | 3.52 ± 0.38 c  | 4.20 ± 0.71 cd |
|                              | SAE           | 90.43 ± 0.20 ab | −1.21 ± 0.05 c  | 3.51 ± 0.29 c  | 4.44 ± 0.18 c  |
|                              | OCME          | 88.15 ± 0.10 d  | −0.92 ± 0.08 b  | 6.92 ± 0.29 a  | 8.47 ± 0.28 a  |
|                              | OCLE          | 90.29 ± 0.34 bc | −1.65 ± 0.09 e  | 4.26 ± 0.45 b  | 5.12 ± 0.51 b  |
|                              | OSAE          | 90.19 ± 0.09 bc | −1.33 ± 0.11 c  | 4.53 ± 0.31 b  | 5.35 ± 0.29 b  |

Values are given as mean ± SD (n = 3).

Different letters in the same column within the same source of gelatin indicate the significant differences (p &lt; 0.05).

C: control (without herb extracts); CME, CLE, SAE: cinnamon, clove and star anise extracts, respectively.

OCME, OCLE, OSAE: the oxidized cinnamon, clove and star anise extracts, respectively.

ΔE\*-values, compared with those of control films and films incorporated with other herb extracts (p < 0.05). For all films, the incorporation of oxidized herb extracts resulted in the greater changes in color of resulting films than the extract without oxidation. This was evidenced by the lower L\*-value and the increases in b\*- and ΔE\*-values. These results suggested that the changes in color of resulting films were most likely attributed to the color components in the different herbs extracts (Table 1). Changes in color of film were also observed in gelatin film incorporated with seaweed extracts due to pigments in the seaweed extracts (Rattaya et al., 2009). Porcine-plasma protein-based film incorporated with oxidized caffeic acid showed increase in b\*-value (Nuthong et al., 2009b). Therefore, incorporation of different herb extracts had an impact on the color of resulting films from gelatin.

### 3.2.5. Light transmission and transparency

Transmission of UV and visible light at wavelength range of 200–800 nm of films from gelatin and partially hydrolyzed gelatin containing herb extracts without and with oxidation are shown in Table 4. The transmission of UV light was found very low at 200 nm

and at 280 nm for all films. Therefore, gelatin films effectively prevented the transmission of UV light at these wavelengths, regardless of herb extract incorporation and degree of hydrolysis of gelatin used for film preparation. Jongiareonrak et al. (2006) also reported higher UV light barrier capacity of gelatin film from bigeye snapper and brownstripe red snapper skin, compared with the synthetic film. The films obtained from bigeye snapper (*Priacanthus tayenus*) surimi proteins (Chinabhark, Benjakul, & Prodpran, 2007) also showed excellent UV protection. In general, light transmission in visible range (350–800 nm) for all films was in the range of 52.12–89.96%. With the addition of different herb extracts, moderate variations in light transmission of resulting films were observed in the visible range. In general, the incorporation of herb extracts showed no marked effects on transmission of visible light of the resulting films. On the other hand, increases in transparency value of gelatin films incorporated with herb extracts were observed, compared with the control (p < 0.05). However, no differences in transparency value were observed among all films treated with herb extract with and without oxidation. The increased transparency value indicated the lowered transparency

**Table 4**

Light transmittance (%) and transparency values of films from gelatin and partially hydrolyzed gelatin from cuttlefish skin incorporated without and with herb extracts without and with oxidation.

| Source of materials          | Herb extracts | Wavelength (nm) |       |       |       |       |       |       |       | Transparency values |
|------------------------------|---------------|-----------------|-------|-------|-------|-------|-------|-------|-------|---------------------|
|                              |               | 200             | 280   | 350   | 400   | 500   | 600   | 700   | 800   |                     |
| Gelatin                      | C             | 0.00            | 8.19  | 61.40 | 74.65 | 82.23 | 84.66 | 86.02 | 86.85 | 3.42 ± 0.03 b       |
|                              | CME           | 0.01            | 10.31 | 61.47 | 72.12 | 80.39 | 83.64 | 85.42 | 87.42 | 3.47 ± 0.04 a       |
|                              | CLE           | 0.00            | 9.48  | 58.02 | 76.18 | 86.58 | 88.47 | 89.39 | 89.96 | 3.49 ± 0.04 a       |
|                              | SAE           | 0.01            | 13.92 | 65.84 | 78.41 | 85.09 | 86.88 | 87.81 | 88.40 | 3.48 ± 0.02 a       |
|                              | OCME          | 0.00            | 6.53  | 52.12 | 67.79 | 78.51 | 83.31 | 85.74 | 87.75 | 3.48 ± 0.04 a       |
|                              | OCLE          | 0.01            | 10.63 | 56.24 | 73.11 | 84.10 | 86.29 | 87.47 | 88.80 | 3.48 ± 0.02 a       |
|                              | OSAE          | 0.00            | 9.03  | 62.77 | 76.61 | 85.60 | 87.94 | 89.01 | 89.65 | 3.47 ± 0.02 a       |
| Partially hydrolyzed gelatin | C             | 0.00            | 8.47  | 65.47 | 75.54 | 83.28 | 85.76 | 86.87 | 87.75 | 3.49 ± 0.04 b       |
|                              | CME           | 0.01            | 10.43 | 62.78 | 76.80 | 83.61 | 85.69 | 86.77 | 87.82 | 3.55 ± 0.05 a       |
|                              | CLE           | 0.00            | 7.78  | 54.22 | 70.67 | 82.37 | 84.61 | 85.81 | 87.90 | 3.57 ± 0.04 a       |
|                              | SAE           | 0.00            | 8.60  | 65.72 | 76.43 | 83.64 | 85.94 | 86.89 | 88.05 | 3.53 ± 0.04 ab      |
|                              | OCME          | 0.00            | 5.72  | 52.73 | 70.33 | 81.82 | 85.46 | 87.92 | 88.45 | 3.56 ± 0.02 a       |
|                              | OCLE          | 0.01            | 13.60 | 67.62 | 77.97 | 86.31 | 88.03 | 88.89 | 89.25 | 3.57 ± 0.06 a       |
|                              | OSAE          | 0.01            | 11.29 | 66.61 | 77.43 | 83.76 | 85.94 | 86.88 | 88.65 | 3.54 ± 0.05 ab      |

Values are given as mean ± SD (n = 3).

Different letters in the same column within the same source of gelatin indicate the significant differences (p &lt; 0.05).

C: control (without herb extracts); CME, CLE, SAE: cinnamon, clove and star anise extracts, respectively.

OCME, OCLE, OSAE: the oxidized cinnamon, clove and star anise extracts, respectively.

of films. The variation in light transmission and transparency of films incorporated with different herb extracts might be due to the pigments which might affect the color, transparency as well as overall appearance of resulting films differently. Therefore, the addition of different herb extracts either non-oxidized or oxidized form had no profound impact on light transmission and transparency value of resulting films.

### 3.2.6. Film solubility

Solubility of films from gelatin and partially hydrolyzed gelatin containing herb extracts without and with oxidation is presented in Table 5. With the addition of different herb extracts, solubility of non-hydrolyzed gelatin film was lowered compared with that of the control film ( $p < 0.05$ ), except for the films containing CME, where no differences were observed ( $p > 0.05$ ). Control film showed the highest film solubility (96.02%), while films incorporated with the oxidized herb extracts exhibited lower film solubility. Among all films incorporated with oxidized herb extracts, that treated with OSAE had the lowest film solubility ( $p < 0.05$ ). The lower solubility of film incorporated with oxidized herb extract might result from the stronger structure of film network through higher extent of protein–polyphenol interaction as evidenced by the higher TS of film. However, all films obtained from partially hydrolyzed gelatin were completely solubilized, irrespective of addition of different herbs extracts, either non-oxidized or oxidized forms. Shorter gelatin molecules could not be able to form strong film network to resist the water during solubilization process for 24 h. Furthermore, the shorter chains were able to be leached out with ease. Film solubility can be viewed as a measure of the water resistance and integrity of a film (Rhim, Gennadios, Handa, Weller, & Hanna, 2000). Therefore, addition of different oxidized herb extracts could enhance the interaction between gelatin molecules by non-disulfide covalent bond, leading to the lower solubility (Fig. 1). It was noted that gelatin contained negligible content of cysteine. Thus, disulfide bond formation may not have taken place in the gelatin films.

### 3.2.7. Electrophoretic protein patterns

Protein patterns of films prepared from gelatin and partially hydrolyzed gelatin incorporated with different herb extracts (both without and with oxidation) are shown in Fig. 2A and B,

**Table 5**

Solubility of films from gelatin and partially hydrolyzed gelatin from cuttlefish skin incorporated without and with herb extracts without and with oxidation.

| Source of materials          | Herb extracts | Film solubility (%) |
|------------------------------|---------------|---------------------|
| Gelatin                      | C             | 96.02 ± 0.61 a      |
|                              | CME           | 95.04 ± 0.74 ab     |
|                              | CLE           | 94.45 ± 0.42 b      |
|                              | SAE           | 94.61 ± 0.98 b      |
|                              | OCME          | 92.91 ± 1.19 c      |
|                              | OCLE          | 92.86 ± 0.41 c      |
|                              | OSAE          | 91.67 ± 0.78 d      |
| Partially hydrolyzed gelatin | C             | 100                 |
|                              | CME           | 100                 |
|                              | CLE           | 100                 |
|                              | SAE           | 100                 |
|                              | OCME          | 100                 |
|                              | OCLE          | 100                 |
|                              | OSAE          | 100                 |

Values are given as mean ± SD (n = 3).

Different letters in the same column within the same source of gelatin indicate the significant differences ( $p < 0.05$ ).

C: control (without herb extracts); CME, CLE, SAE: cinnamon, clove and star anise extracts, respectively.

OCME, OCLE, OSAE: the oxidized cinnamon, clove and star anise extracts, respectively.

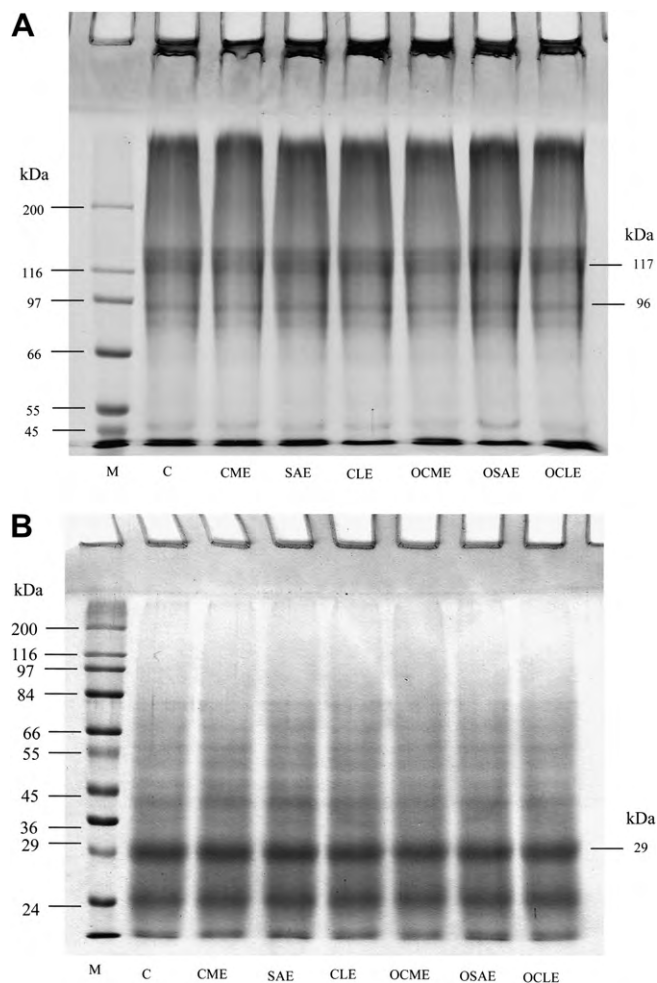


**Fig. 2.** Protein patterns of films from cuttlefish skin gelatin (A) and partially hydrolyzed gelatin (B) containing different herb extracts. M: protein marker; C: control (without addition of herb extracts); CME: cinnamon extract; SAE: star anise extract; CLE: clove extract; OCME: oxidized cinnamon extract; OSAE: oxidized star anise extract; OCLE: oxidized clove extract.

respectively. Proteins with molecular weight (MW) of ~117 and ~95 kDa were found as the major proteins in film from gelatin. Aewsiri, Benjakul, and Visessanguan (2009) and Hoque et al. (2010a) also reported that protein with molecular weight of 118 and 97 kDa was the dominant component in gelatin extracted from ventral skin of cuttlefish. Phenolic compounds in the extracts might contribute to protein–polyphenol cross-linking. Band intensities of those two major protein bands of gelatin films incorporated with all extracts (without oxidation) were similar to those of control film (without addition of herb extracts). For films with herb extracts, weak bonds stabilizing film network may have been destroyed by all denaturants, leading to the dissociation of cross-links. However, the slight decreases in those protein bands were observed when oxidized extracts were incorporated. Band intensity of protein with MW of 97 kDa in film added with OCME, OSAE and OCLE was decreased by 24.62, 26.8 and 25.2%, respectively. This might be due to the formation of large aggregate as evidenced by the appearance of protein band on the stacking gel. These indicated that non-disulfide covalent bonds induced by oxidized extracts were formed to some extent. For films from partially hydrolyzed gelatin, similar results were obtained. However, it was noted that no proteins with MW of 118 and 97 kDa were present in the films, regardless of addition of herb extracts. Band intensity of protein with MW of

29 kDa of film incorporated with OCME, OSAE and OCLE was decreased by 12.05, 24.8 and 17.5%, respectively. This change was concomitant with the formation of high MW protein. Greater contents of cross-links formed were related with the increases in TS (Table 2).

### 3.3. Characteristics of film incorporated with star anise extracts

Films incorporated with star anise extracts, both without and with oxidation, showed higher mechanical properties and lower  $b^*$ -value as compared with the film incorporated with other herb extracts. Therefore, the films incorporated with star anise extracts were further characterized.

#### 3.3.1. FTIR spectroscopy

FTIR spectra of films prepared from gelatin and partially hydrolyzed gelatin incorporated with star anise extracts (without and with oxidation) are shown in Fig. 3. The spectra of all films exhibited the major bands at  $1631\text{ cm}^{-1}$  (amide-I, representing C=O stretching/hydrogen bonding coupled with COO),  $1536\text{ cm}^{-1}$  (amide-II, arising from bending vibration of N–H groups and stretching vibrations of C–N groups) and  $1234\text{ cm}^{-1}$  (amide-III, representing the vibrations in plane of C–N and N–H groups of bound amide or vibrations of  $\text{CH}_2$  groups of glycine) (Aewsiri et al., 2009; Muyonga, Cole, & Duodu, 2004). Pranoto, Lee, and Park (2007) reported a similar result for fish gelatin film, where amide-I, amide-II and amide-III peaks were found at the wavenumbers of 1656, 1550 and  $1240\text{ cm}^{-1}$ , respectively. The peak situated around  $1033\text{ cm}^{-1}$  might be related to the interactions arising between plasticizer (OH group of glycerol) and film structure (Bergo & Sobral, 2007). Generally, similar spectra were obtained for films without and with SAE or OSAE in the range of  $1800\text{--}700\text{ cm}^{-1}$ , covering amide-I, amide-II and amide-III observed at similar wavenumber for both films from gelatin and partially hydrolyzed gelatin.

Furthermore, amide-A peak was found at  $3275\text{ cm}^{-1}$ , representing NH-stretching coupled with hydrogen bonding. Amide-B peak at  $2928\text{ cm}^{-1}$ , representing CH stretching and  $\text{NH}_3^+$ , was also observed in the spectra (Muyonga et al., 2004). For film from gelatin, the wavenumber of amide-A peak shifted from  $3275\text{ cm}^{-1}$  for the control to  $3277$  and  $3287\text{ cm}^{-1}$  for films incorporated with SAE and OSAE, respectively. For film from partially hydrolyzed gelatin with SAE and OSAE, wavenumber of amide-A peak shifted from  $3276\text{ cm}^{-1}$  for the control to  $3278$  and  $3290\text{ cm}^{-1}$ , respectively. The amplitude of amide-A peak of the control gelatin film

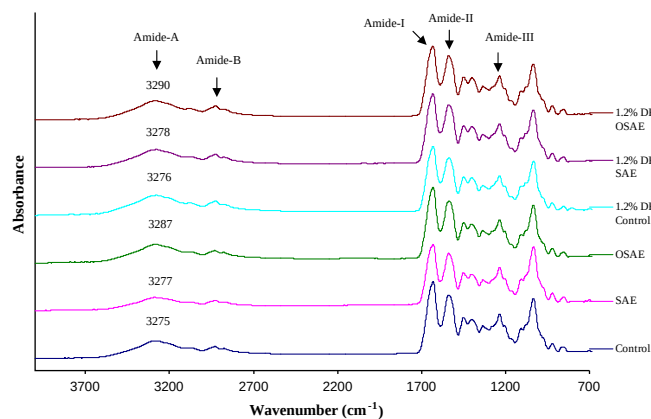


**Fig. 3.** FTIR spectra of films prepared from gelatin and partially hydrolyzed (1.2% DH) gelatin from cuttlefish skin containing star anise extracts without and with oxidation. Control: film without addition of the extract; SAE: film added with star anise extract; OSAE: film added with oxidized star anise extract.

(Abs = 0.15) decreased markedly in film incorporated with SAE (both without and with oxidation) (Abs = 0.11–0.13). For film prepared from partially hydrolyzed gelatin, the amplitude of amide-A peak decreased from 0.17 to 0.14–0.15 when the SAE without and with oxidation was incorporated. In general, film with OSAE had slightly higher amplitudes, in comparison with film with SAE. Similar result was observed for amide-B peak. These results suggested that SAE and OSAE induced interaction between phenolic compounds and  $\text{NH}_2$  group of gelatin. This led to cross-linking of gelatin. Protein cross-linking led to higher diffraction of film, which affects the spectra obtained using ATR techniques. Nuthong et al. (2009a) also observed similar results in porcine-

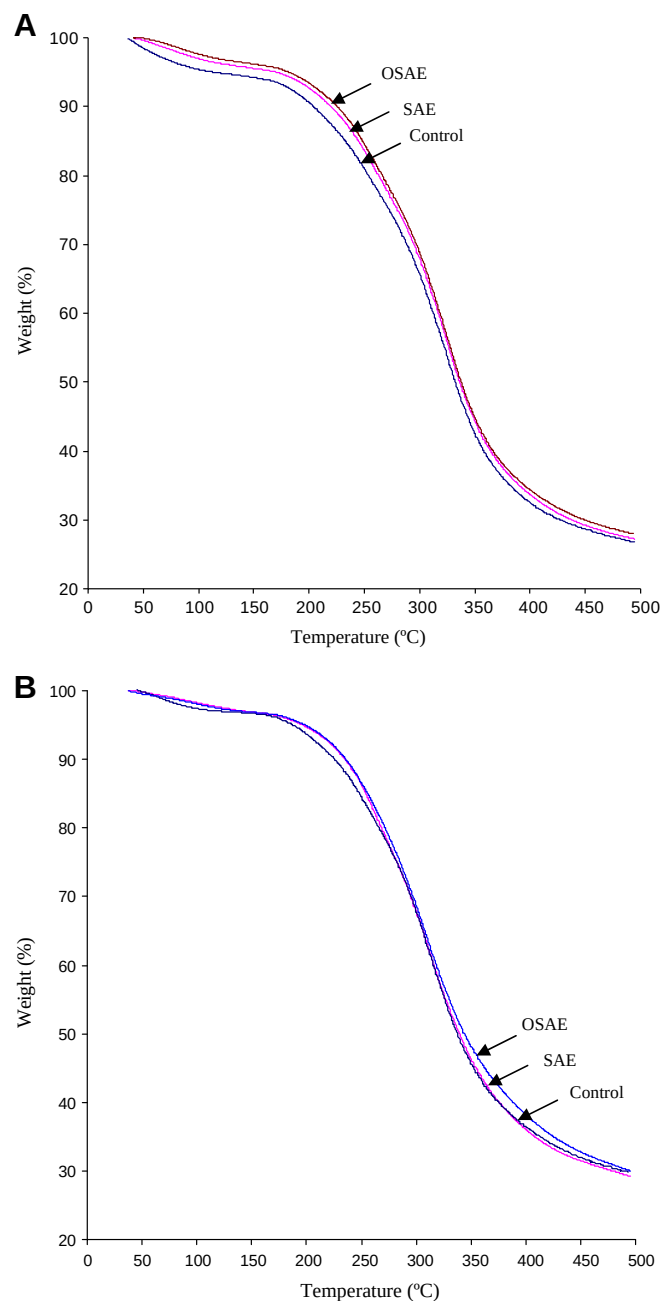


**Fig. 4.** Thermo-gravimetric curves of films prepared from cuttlefish skin gelatin (A) and partially hydrolyzed gelatin (B) containing star anise extract without and with oxidation. Control: film without addition of the extract; SAE: film added with star anise extract; OSAE: film added with oxidized star anise extract.

**Table 6**  
Thermal degradation temperature ( $T_d$ , °C) and weight loss ( $\Delta w$ , %) of films from gelatin and partially hydrolyzed gelatin from cuttlefish skin incorporated without and with star anise extracts without and with oxidation.

| Source of materials          | Herb extracts | $\Delta_1$     |              | $\Delta_2$     |              | $\Delta_3$     |              | Residue (%) |
|------------------------------|---------------|----------------|--------------|----------------|--------------|----------------|--------------|-------------|
|                              |               | $T_{d1,onset}$ | $\Delta w_1$ | $T_{d2,onset}$ | $\Delta w_2$ | $T_{d3,onset}$ | $\Delta w_3$ |             |
| Gelatin                      | C             | 53.30          | 3.53         | 245.90         | 21.10        | 270.18         | 61.10        | 25.10       |
|                              | SAE           | 58.82          | 2.92         | 250.40         | 18.56        | 278.45         | 60.45        | 29.61       |
|                              | OSAE          | 61.31          | 2.13         | 252.39         | 17.25        | 282.10         | 59.50        | 31.78       |
| Partially hydrolyzed gelatin | C             | 46.24          | 4.07         | 242.38         | 22.60        | 251.80         | 58.92        | 21.45       |
|                              | SAE           | 48.21          | 3.65         | 244.15         | 18.78        | 256.25         | 55.23        | 23.20       |
|                              | OSAE          | 50.48          | 3.40         | 245.98         | 18.20        | 258.90         | 53.57        | 24.92       |

$\Delta_1$ ,  $\Delta_2$ , and  $\Delta_3$  denote the first, second and third stage weight loss, respectively, of film.  
SAE and OSAE: star anise extracts without and with oxidation, respectively.

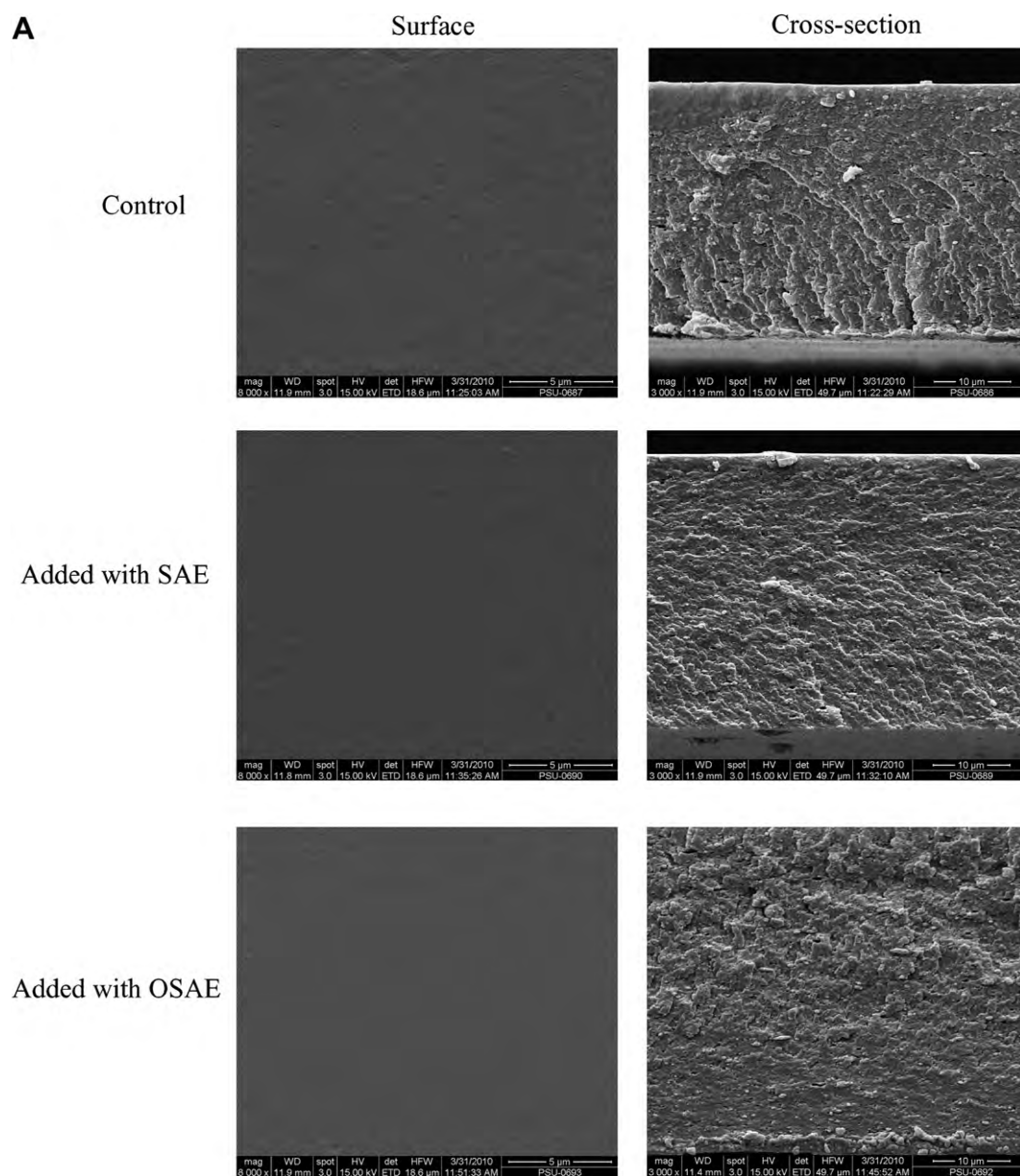


**Fig. 5.** SEM micrographs of surface and cross-section of films from cuttlefish skin gelatin (A) and partially hydrolyzed gelatin (B) containing star anise extract without and with oxidation. Control: film without addition of the extract; SAE: film added with star anise extract; OSAE: film added with oxidized star anise extract.

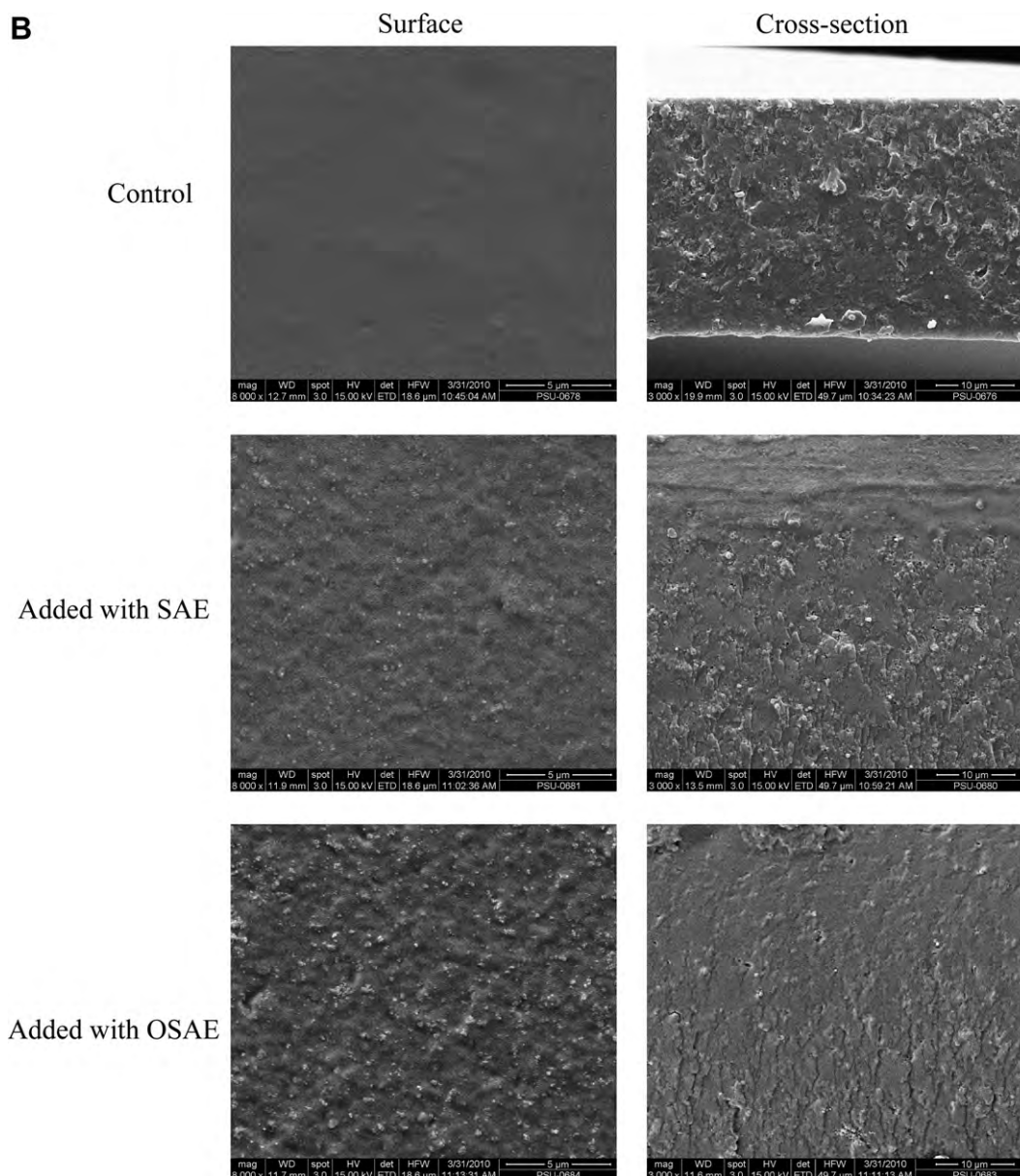


Fig. 5. (continued).

plasma protein films incorporated with 2% glyoxal and 3% caffeic acid with or without oxygenation. However, it was also noted that higher amplitudes of amide-A and amide-B peaks were observed in film from partially hydrolyzed than film from gelatin. The result reconfirmed the changes of peptides in partially hydrolyzed gelatin. Higher amount of  $-NH_2$  or  $-NH_3^+$  group obtained from hydrolysis process more likely resulted in the higher amplitude of amide-A and amide-B peaks obtained. Protein cross-linking was prominent in film network with the addition of SAE and OSAE as indicated by the changes in functional group and conformation of proteins elucidated by the changes in FTIR spectra (Ahmad & Benjakul, 2011).

### 3.3.2. Thermo-gravimetric analysis (TGA)

TGA thermograms revealing thermal degradation behavior of films prepared from gelatin and partially hydrolyzed gelatin in the presence of star anise extract (without and with oxidation) are depicted in Fig. 4A and B. Their corresponding degradation

temperatures ( $T_d$ ) and weight loss ( $\Delta w$ ) are shown in Table 6. Three main stages of weight loss were observed for all films from both gelatin and partially hydrolyzed gelatin. For all films, the first stage of weight loss ( $\Delta w_1 = 2.13$ – $4.07\%$ ) was observed approximately at temperature ( $T_{d1}$ ) of  $46.24$ – $61.31$  °C, possibly associated with the loss of free water adsorbed on the film. A similar result was found in porcine-plasma protein film containing different cross-linking agents (Nuthong et al., 2009a) and in collagen hydrolysate film plasticized with glycerol and poly(ethylene glycols) (Langmaier, Mokrejs, Kolomaznik, & Mladek, 2008). The second stage of weight loss ( $\Delta w_2 = 17.25$ – $22.60\%$ ) appeared in the region of  $T_{d2}$  ( $242.38$ – $252.39$  °C) for all films. This was most likely associated with the loss of lower molecular weight protein fractions, glycerol compounds and also structurally bound water. For the third stage of weight loss,  $T_{d3}$  of  $270.18$ – $282.10$  °C were observed in films prepared from gelatin, whereas  $T_{d3}$  of  $251.80$ – $258.90$  °C were found in those from partially hydrolyzed gelatin. These changes mainly resulted from the degradation of the gelatin with the larger

size. The lower Td of the latter was more likely due to the shorter chains, which could undergo thermal degradation to a higher extent, compared with the former.

The result suggested that the film with SAE showed higher heat resistance than the control films (without addition of star anise extracts) from both gelatin and partially hydrolyzed gelatin. For both gelatin materials, the control films showed lower thermal degradation temperatures for Td<sub>1</sub>, Td<sub>2</sub> and Td<sub>3</sub> and higher weight loss for  $\Delta w_1$ ,  $\Delta w_2$  and  $\Delta w_3$ , as compared to films with SAE and OSAE (Table 6). The result suggested that higher amount of bonding between phenolic compounds of star anise extracts and gelatin molecules yielded stronger film network, leading to the higher heat resistance of the resulting films. For the same materials, films with OSAE had higher Td<sub>1</sub>, Td<sub>2</sub> and Td<sub>3</sub> with lower  $\Delta w$ . Thus, OSAE most likely increased the thermal stability of resulting film. This was due to the increased stronger bonds induced by OSAE in the film matrix. Non-disulfide covalent bonds may be predominant in films incorporated with OSAE, compared to those with SAE. However, at the same condition, film from partially hydrolyzed gelatin added with star anise extract showed higher heat susceptibility, compared with film from gelatin. The result suggested that film network obtained from the gelatin with the shorter gelatin molecules underwent rapid thermal degradation and had higher weight loss, compared with film from gelatin. Also, herb extracts showed the pronounced impact on thermal stability of gelatin film via protein–polyphenol cross-linking.

### 3.3.3. Microstructure

SEM micrographs of the surface and cross-section of films from gelatin and partially hydrolyzed gelatin incorporated with SAE and OSAE are illustrated in Fig. 5A and B, respectively. For the control film, smooth surface was observed for both films from gelatin and partially hydrolyzed gelatin. Smooth surface was also obtained in the film prepared from gelatin incorporated with SAE and OSAE. However, a rough surface was obtained in the film from partially hydrolyzed gelatin when SAE and OSAE were added. The rough surface was more obvious in film with OSAE, compared to that with SAE. For cross-section, film obtained from gelatin incorporated with SAE showed a more compact-structure as compared with the control. Increased number of non-covalent protein–polyphenol interaction might contribute to the formation of compact film structure. However, OSAE addition resulted in coarser cross-sectional structure of their resulting films. The roughness might be governed by conversion of phenol to quinone, which induced the large aggregation in the film matrix via non-disulfide bonds. Nuthong et al. (2009a, 2009b) also found rougher cross-section of film with oxygenated caffeic acid than that with non-oxygenated caffeic acid.

Coarser surfaces observed in films prepared from partially hydrolyzed gelatin after the incorporation with SAE and OSAE was probably caused by the protruding of polymerized chains. However, the long chain could form the junction zone throughout the film network, leading to the smoother surface irrespective of SAE and OSAE incorporation. For the cross-section, the smaller micro-voids with the denser internal structure were noticed in the film treated with SAE, compared with the control film.

## 4. Conclusion

Incorporation of herb extracts including cinnamon, clove and star anise extracts into gelatin and partially hydrolyzed gelatin increased the TS and decreased WVP of resulting films. However, those extracts could affect the color of resulting films to some degree. Star anise extract was the most effective in improving the mechanical properties and water barrier property of the gelatin

films. Extracts with oxidation showed the greater efficiency in increasing the strength of films than non-oxidized counterpart. However, the molecular weight or chain length of gelatin played a role in cross-linking mediated by phenolic compounds in the extracts and also determined the microstructure of resulting film. Therefore, herb extracts can be used as the natural protein cross-linkers, which is able to modify the properties of film from gelatin or other proteins.

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# Quality changes of sea bass slices wrapped with gelatin film incorporated with lemongrass essential oil

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## ABSTRACT

Microbiological, chemical and physical changes of sea bass slices wrapped with gelatin film incorporated with 25% (w/w) lemongrass essential oil (LEO) during storage of 12 days at 4 °C were investigated. Sea bass slices wrapped with LEO film had the retarded growth of lactic acid bacteria (LAB), psychrophilic bacteria and spoilage microorganisms including H<sub>2</sub>S-producing bacteria and Enterobacteriaceae throughout storage of 12 days in comparison with the control and those wrapped with gelatin film without LEO (G film) ( $P < 0.05$ ). Lowered changes of colour, K value, total volatile base nitrogen (TVB) and TBARS value were also found in LEO film wrapped samples, compared with those wrapped with G film and control, respectively. Therefore, the incorporation of LEO into gelatin film could enhance the antimicrobial and antioxidative properties of the film, thereby maintaining the qualities and extending the shelf-life of the sea bass slices stored at refrigerated temperature.

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## 1. Introduction

Increasing demand for high quality ready-to-cook fish with the extended shelf-life has initiated the development of several innovative techniques to keep quality attributes as long as possible and yield the safe products (Maftoonazad and Badii, 2009). To satisfy these requirements, the uses of conventional chemical additives in food formulation have been decreased. The alternative use of natural products has been receiving more and more attention, mainly due to additional functional properties of these natural products (Sánchez-González et al., 2011). Essential oils (EOs) fulfilled the part of increasing demand for natural additives, since they possess antibacterial, antiviral and antimycotic properties (Holley and Patel, 2005). Among EOs, oregano and thyme EOs have been frequently used for food preservation due to their antimicrobial and antioxidant properties (Chouliara et al., 2007). EO contains phenolic compounds such as thymol, carvacrol, *p*-cymene and  $\gamma$ -terpinene and has been known to inhibit both Gram negative and Gram-positive microorganisms (Sivropoulou et al., 1996).

Active packaging systems, based on the incorporation of natural antimicrobial or antioxidative substances in food packaging materials, provide the safety benefits, control undesirable changes of food qualities and extend shelf-life of meat and meat based products (Suppakul et al., 2003). Films incorporated with cinnamon EO have been reported to extend the shelf-life of rainbow trout during storage at

4 °C for 16 days, mainly by retarding the microbial growth and lowering the lipid oxidation (Ojagh et al., 2010). Generally fish muscle slices are known for their high nutritional value, due to high contents of protein, phospholipids, polyunsaturated fatty acids (PUFA) and essential minerals (Simopoulos, 2002). Nevertheless, sea bass slices are perishable, associated with microbial growth (Masniyom et al., 2002). Lipid oxidation also took place in sea bass slices during the refrigerated storage (Masniyom et al., 2002). The use of protein films, especially those based on fish gelatin with antimicrobial and antioxidative activities could be a means to prolong the shelf-life of sea bass slices during refrigerated storage. Therefore, the objective of this investigation was to study the impact of gelatin-based film incorporated with lemongrass EO on the shelf-life extension of sea bass slices stored at 4 °C.

## 2. Materials and methods

### 2.1. Chemicals

Lemongrass (*Cymbopogon citratus*) essential oil (LEO) was purchased from Botanicessence essential oils (Suanlung, BKK, Thailand). Malonaldehyde bis (dimethyl acetal), thiobarbituric acid (TBA) and anion-exchange resin-AG (R) 1-X<sup>4</sup>, 400 mesh Cl (chloride)-form were purchased from Bio-Rad (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Trichloroacetic acid (TCA), perchloric acid (PCA), hydrochloric acid, potassium hydroxide, ammonium hydroxide, sodium chloride, ammonium sulfate, MRS culture media, standard plate count agar, triple sugar iron agar (IA), and eosin methylene blue

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agar (EMB) were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

## 2.2. Preparation of skin from unicorn leatherjacket and gelatin extraction

The skin of unicorn leatherjacket (*Aluterus monoceros*) was obtained from Sea Wealth Frozen Food Co., Ltd., Songkhla, Thailand. Upon arrival at the Department of Food Technology, Prince of Songkla University, Hat Yai, the skin was washed with iced tap water (0–2 °C). The prepared skin was then cut into small pieces (0.5 × 0.5 cm<sup>2</sup>), placed in polyethylene bags and stored at –20 °C until use.

Prepared skin was subjected to pretreatment and subsequent gelatin extraction following the method of [Ahmad and Benjakul \(2011\)](#). Gelatin powder obtained contained 85.61% protein as determined by the Kjeldhal method ([AOAC, 1999](#)).

## 2.3. Formulation and preparation of gelatin films

For the preparation of film forming solution (FFS), fish gelatin powder was mixed with distilled water to obtain the final protein concentration of 3% (w/v). The mixture was stirred at room temperature for 30 min, followed by heating at 60 °C for 15 min. After complete solubilisation, LEO as an antimicrobial agent at the concentration of 25% (w/w) of total protein was then added into FFS. To stabilise emulsion, tween-20 was also added at 15% (w/w) based on LEO content. Control films were also performed in the same way except that glycerol (25%, w/w protein) was added instead of LEO. The mixture was homogenised at a speed of 11,000 rpm for 2 min using a homogeniser (Model T25 basic, IKA®18, 19 Labortechnik, Selangor, Malaysia) and allowed to stand for 5 min at room temperature (28–30 °C) to reduce the air bubbles formed during homogenisation. The filmogenic solution was then subjected to film casting. FFS (4 ± 0.01 g) was cast onto a rimmed silicone resin plate (5 × 5 cm<sup>2</sup>), air-blown for 12 h at room temperature, followed by drying in an environmental chamber (Binder, KBF 115 # 00-19735, D-78532, Tuttlingen, Germany) at 25 ± 0.5 °C and 50 ± 5% relative humidity (RH) for 24 h. Dried film samples were aseptically peeled off and used for wrapping sea bass slices.

## 2.4. Effect of gelatin film incorporated with LEO on quality changes of sea bass slices

Fresh sea bass (*Lates calcarifer*) were purchased from a local market in Hat Yai, Songkhla, Thailand and transported in ice with fish/ice ratio of 1:2 (w/w) to the laboratory at 4 °C within 30 min. Sea bass were filleted and sliced to a thickness of 1.5 cm. For each slice, films (5 × 5 cm<sup>2</sup>) were placed on both sides. Films used in this study included gelatin film incorporated without (G film) and with LEO (LEO film). LEO film did not show any smell associated with essential oil incorporated. Subsequently, the samples were placed in polystyrene-trays (9 × 7 cm<sup>2</sup>), wrapped with M WRAP extensible polypropylene film (Thickness# 11 µm, MMP Corporation Ltd., Bangkok, Thailand) prior to storage at 4 °C for 12 days. Control samples were performed in the same manner except that the slices were not covered with any film. During storage, the samples were randomly taken every 2 days for analyses.

## 2.5. Microbiological analyses

Microbiological analyses were performed by the spread plate method ([Sallam, 2007](#)). Samples were collected aseptically and used as the composite sample. The sample (25 g) was placed in a Stomacher bag containing 225 ml of 0.85% saline solution. After mixing for 1 min in a Stomacher blender (Stomacher M400, Seward Ltd., Worthington, England), further serial dilutions were prepared from

this homogenate using 0.85% saline solution as the diluent. Appropriate dilutions were used for microbiological analyses.

### 2.5.1. Total viable count and psychrophilic bacterial count

Total viable count and psychrophilic bacterial count were determined by spreading 0.1 ml of an appropriately diluted homogenate on plate count agar containing 1% NaCl, followed by incubation at 35 °C for 3 days and 4 °C for 10 days, respectively ([Sallam, 2007](#)).

### 2.5.2. Hydrogen sulphide (H<sub>2</sub>S) producing bacterial count

H<sub>2</sub>S-producing bacteria were grown on triple sugar iron agar by the spread plate method using 0.1 ml of an appropriately diluted homogenate. Plates were incubated at 25 °C for 3 days. Black colonies, due to the precipitation of ferrous sulphide on this medium, were counted ([Sallam, 2007](#)).

### 2.5.3. Enterobacteriaceae count

For the determination of enterobacterial count, 0.1 ml of an appropriately diluted homogenate was spread on Eosin methylene blue agar (EMB) and incubated at 37 °C for 24 h.

### 2.5.4. Lactic acid bacteria count

Lactic acid bacterial count was determined by inoculating 0.1 ml of an appropriately diluted homogenate on double-layered plates of MRS Agar (Oxoid). A spread plate method was used. Then the plates were incubated at 37 °C for 72 h ([Asahara et al., 2001](#)).

## 2.6. Chemical analyses

### 2.6.1. pH measurement

pH measurement was performed by the method described by [Lopez-Caballero et al. \(2007\)](#) with a slight modification. Sample (2 g) was homogenised with 10 volumes of deionised water for 1 min. The homogenate was kept at room temperature for 5 min. The pH was determined using a pH-meter (Sartorius North America, Edgewood, NY, USA).

### 2.6.2. Determination of total volatile base (TVB) content

TVB contents were determined following the method of [Conway and Byrne \(1936\)](#). Sample (5 g) was homogenised with 4% of trichloroacetic acid at a ratio of 1: 2 (w/v). The homogenate was filtered through Whatman No 1 paper (Schleicher & Schuell, Maidstone, England). The filtrate (1 ml) was placed in the outer ring. The inner ring solution (1% boric acid containing the Conway indicator) was pipetted into the inner ring. To initiate the reaction, K<sub>2</sub>CO<sub>3</sub> (1 ml) was mixed with the filtrate. The Conway unit was closed and incubated at 37 °C for 60 min. The inner ring solution was then titrated with 0.02 N HCl until the green colour turned to pink.

### 2.6.3. Determination of thiobarbituric acid reactive substances (TBARS)

TBARS were determined as described by [Buege and Aust \(1978\)](#). The ground sample (0.5 g) was homogenised with 2.5 ml of solution containing 0.375% thiobarbituric acid (w/v), 15% trichloroacetic acid (w/v) and 0.25 M HCl. The mixture was heated in a boiling water bath (95–100 °C) for 10 min to develop a pink colour, cooled with running tap water and centrifuged at 3600 g at 25 °C for 20 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane at the concentrations ranging from 0 to 6 ppm. TBARS were calculated and expressed as mg malonaldehyde/kg sample.

### 2.6.4. Determination of K value

K value was determined using anion-exchange column chromatography ([Uchiyama and Kakuda, 1984](#)). A ground sample (1 g) was subjected to a serial extraction using 10% PCA and 5% PCA. The final extract (2 ml) was adjusted to pH 9.4 using 0.5 N NH<sub>3</sub>(aq). The

prepared extract (pH 9.4) was loaded onto an anion-exchange column ( $1 \times 10 \text{ cm}^2$ ) containing Resin-AG (R) 1-X<sup>4</sup>, 400 mesh Cl-form. The column was rinsed using deionised water (20 ml). The elution was performed using 45 ml of solution A (0.001 N HCl). The eluate was collected, and the volume was made up to 50 ml using solution A. Thereafter, the column was eluted with 45 ml of solution B (0.01 N HCl containing 0.6 M NaCl). The resulting eluate was made up to 50 ml using solution B. Both eluates were read at 250 nm using the corresponding eluent (solution A or B) as the blank. *K* value was calculated as follows:

$$K\text{-value}(\%) = \frac{A}{A+B} \times 100$$

where *A* is *A*<sub>250</sub> of eluate A representing the amount of inosine (HxR) and hypoxanthine (Hx), and *B* is *A*<sub>250</sub> of eluate B representing the amount of ATP, ADP, AMP, and IMP.

## 2.7. Physical analyses

The colour of samples was measured by a Hunter lab colour meter (Colour Flex, Hunter Lab Inc., Reston, VA, USA) and was reported by the CIE system. *L*<sup>\*</sup>, *a*<sup>\*</sup> and *b*<sup>\*</sup> parameters indicate lightness/brightness, redness/greenness and yellowness/blueness, respectively. The colourimeter was calibrated with a white standard.

## 2.8. Statistical analyses

Experiments were performed in triplicate (*n* = 3) and a completely randomised design (CRD) was used. Data were presented as means  $\pm$  standard deviation and a probability value of  $<0.05$  was considered significant. Analysis of variance (ANOVA) was performed and the mean comparisons were done by Duncan's multiple range tests. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for Windows, SPSS Inc., Chicago, IL, USA).

## 3. Results and discussion

### 3.1. Changes in microbial loads

Total viable count (TVC) of sea bass slices wrapped without and with films (G film or LEO film) during storage at 4 °C for 12 days is shown in Fig. 1a. TVC of all samples at day 0 was 4.5 log CFU/g, indicating the presence of some microorganisms, possibly contaminated during slice preparation, packaging, etc. TVC of the control sample (without wrapping) increased significantly during the first 4 days of storage, with an approximate value of 7.2 log CFU/g at day 4 ( $P < 0.05$ ). This load became higher and reached 7.9 log CFU/g at day 12, suggesting the increase in microorganisms acquired during storage. For LEO-film wrapped samples, TVC increased gradually and did not exceed the value of 5.6 log CFU/g at day 12. Thus, LEO film had a profound effect on the inhibition of microbial growth. For G-film wrapped samples, a similar TVC was observed, compared with that of control samples within the first 6 days of storage, but slightly lower TVC was found during 6–12 days ( $P < 0.05$ ). The incorporation of the LEO in gelatin film resulted in a delay in microbial spoilage of sea bass slices. Films incorporated with chitosan or other essential oils could retard the microbial growth in beef (Oussalah et al., 2004), bologna slices (Zivanovic et al., 2005) or cold-smoked sliced sardine (Gómez-Estaca et al., 2007). LEO-film wrapped samples had the lower TVC by approximately 2 log CFU/g, as compared to G-film or control samples, after 12 days of storage. Generally, the total number of microorganisms varies enormously and ranges from  $10^2$  to  $10^7$  CFU/cm<sup>2</sup> in fish (Liston, 1980). Initial bacteriological count was  $<10^2$  CFU/g in the sea bass (*L. calcarifer*) flesh (Harpaz et al., 2003). Microbial inhibition of LEO film could be attributed to the

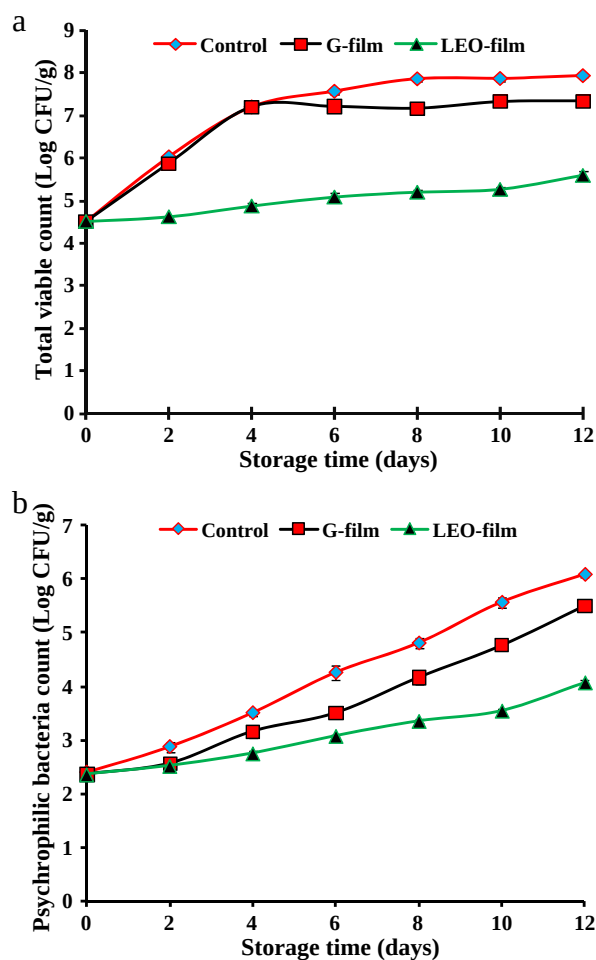


**Fig. 1.** Total viable bacterial count (a) and psychrophilic bacterial count (b) of sea bass slices wrapped without and with films (LEO film or G film) during storage at 4 °C for 12 days. Control: unwrapped samples, G film: samples wrapped with gelatin film, LEO film: samples wrapped with gelatin film incorporated with lemongrass essential oil. Bars represent the standard deviation (*n* = 3).

hydrophobic nature that enables LEO and its components to partition in the lipids of bacterial cell membrane, thereby disturbing the structures and rendering it more permeable (Sikkema et al., 1994).

The continuous increases in psychrophilic bacterial count in all samples were noticeable with increasing storage time up to day 12 ( $P < 0.05$ ). During the storage, samples wrapped with LEO film had the lowest psychrophilic bacterial count, in comparison with the control and G-film wrapped samples ( $P < 0.05$ ) (Fig. 1b). During the storage, lower psychrophilic bacterial count was obtained in the G-film wrapped samples, as compared to that found in the control. At the end of storage (day 12), psychrophilic bacterial count of the control, those wrapped with G film and LEO film was 6.0, 5.5 and 4.0 log CFU/g, respectively. The result indicated the antimicrobial activity of LEO toward psychrophilic bacteria in sea bass slices during storage at 4 °C for 12 days.

Lactic acid bacteria (LAB) count was 4.0 log CFU/g in sea bass slices at day 0. LAB count gradually increased when storage time increased ( $P < 0.05$ ). LAB increased to the levels of 7.2, 6.7 and 5.9 log CFU/g at the end of storage for the control, G-film wrapped samples and LEO-film wrapped samples, respectively (Fig. 2a). LABs are facultative anaerobes, which are able to grow under high concentrations of CO<sub>2</sub> and constitute a substantial part of the natural microflora of stored food products under anaerobic conditions (Mastromatteo et al., 2009). The use of LEO film resulted in a reduction in LAB counts by 1.4 log CFU/g in the sea bass slices ( $P < 0.05$ ), respectively. Zaika et

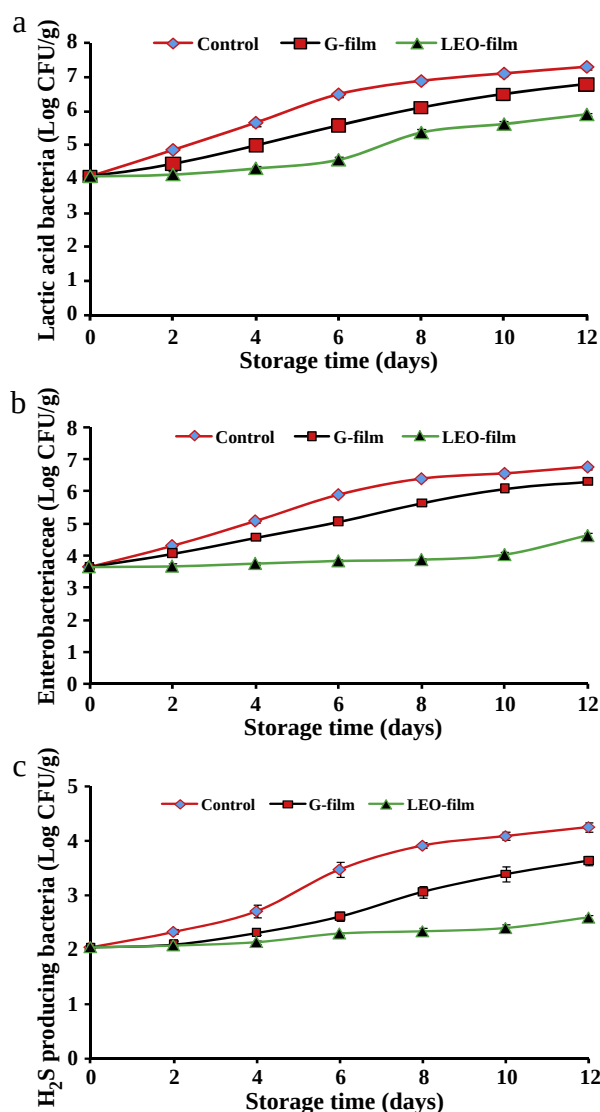


Fig. 2. Lactic acid bacteria (a), Enterobacteriaceae (b) and H<sub>2</sub>S-producing bacteria (c) counts of sea bass slices wrapped without and with films (LEO film or G film) during storage at 4 °C for 12 days. Key: see the caption for Fig. 1. Bars represent the standard deviation (n=3).

al. (1983) reported a reduction of 4 log CFU/g in LAB populations in pure culture after the addition of 4 g/l (0.4%) of oregano oil. When LAB were present in fish slices, other components more likely acted protectively on LAB, as compared to pure cultures. Due to controlled release of active compounds from gelatin film matrix, the concentration of LEO released was usually low. Hence, the inhibitory activity of LEO in film might be lower than free LEO. LAB were also found to be part of the natural microflora of sea bass fillets (*Dicentrarchus labrax*), irrespective of packaging treatment (Kostaki et al., 2009). The use of MAP (60% CO<sub>2</sub>/30% N<sub>2</sub>/10% O<sub>2</sub>) in combination with 0.2% (v/w) thyme oil could delay the growth of LAB on sea bass fillets (*D. labrax*) and increased only 4 log CFU/g during the first 16 days of storage (Kostaki et al., 2009). Although LAB are the most resistant among the Gram-positive bacteria towards the antimicrobial action of essential oils, thyme oil along with MAP was able to retard their growth (Kostaki et al., 2009). It was noted that gelatin film also showed the antimicrobial activity. Gelatin film might contain the antimicrobial peptides which could be released to the surface of slice samples. It was reported that gelatin peptides showed antimicrobial activity toward several microorganisms (Gómez-Guillén et al., 2010).

For Enterobacteriaceae (Fig. 2b), considered as a hygiene indicator, the initial count at day 0 was 3.6 log CFU/g, but reached 6.7 log CFU/g in the control samples at day 12. Nevertheless, LEO-film wrapped samples had the lower counts by 2.2 log CFU/g, as compared to controls, at day 12 of storage. Lower count observed in LEO-film wrapped samples was more likely associated with protective action of LEO against the spoilage bacteria ( $P < 0.05$ ). G film showed a little impact on the inhibition of Enterobacteriaceae and the count of 6.3 log CFU/g was obtained at the end of storage. Aerobic microorganisms might have the retarded growth when the surface of slice samples was covered by gelatin film. Gelatin film was reported to have excellent oxygen barrier property (Chiou et al., 2008).

Changes in H<sub>2</sub>S-producing bacterial count of sea bass slices during 12 days of storage are depicted in Fig. 2c. H<sub>2</sub>S-producing bacterial count was lowest in samples wrapped with LEO film as compared to others during 4–12 days of storage ( $P < 0.05$ ). Therefore, LEO incorporated into gelatin film was shown to retard the growth of spoilage bacteria, which were able to produce H<sub>2</sub>S. At the end of storage, the control, those wrapped with G film and LEO film had H<sub>2</sub>S-producing bacterial counts of 4.2, 3.6 and 2.5 log CFU/g, respectively. Specific spoilage organisms such as H<sub>2</sub>S-producing bacteria and Enterobacteriaceae are mostly predominant in the spoilage of fish and fish products, causing off-flavours and rejection (Liston, 1992). Similar inhibition of H<sub>2</sub>S producing bacteria growth by gelatin film was also observed. It was suggested that H<sub>2</sub>S-producing bacteria might proliferate at lower level when the surface of the sample had the lower oxygen level. For the sea bass slices (*D. labrax*), the combined use of thyme oil and MAP inhibited the growth of H<sub>2</sub>S-producing bacteria during 21 days of refrigerated storage and their population remained at 3.5 log CFU/g (Kostaki et al., 2009). H<sub>2</sub>S-producing bacteria including *Shewanella putrefaciens* at 4.6 log CFU/g were found in ice-stored sea bass (*D. labrax*) at the time of spoilage (day 15) (Kyrana and Lougvois, 2002).

### 3.2. Changes in chemical compositions

#### 3.2.1. Total volatile base content

TVB content of sea bass slices wrapped without and with films (G film or LEO film) during storage at 4 °C for 12 days is shown in Fig. 3. At day 0, TVB content of sea bass slices was 12.47 mg N/100 g. As the storage time increased, a continuous increase in TVB content was observed in all samples ( $P < 0.05$ ), but the increasing rate varied among samples. A gradual increase in TVB content was observed throughout the storage of 12 days for G-film and LEO-film wrapped samples, reaching a value of 18.34 and 15.84 mg N/100 g

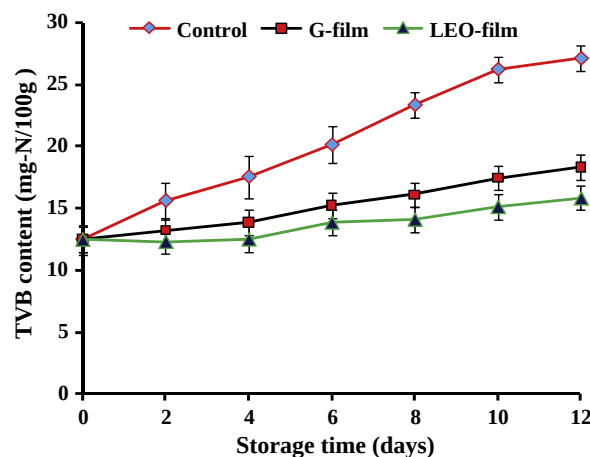


Fig. 3. TVB content of sea bass slices wrapped without and with films (LEO film or G film) during storage at 4 °C for 12 days. Key: see the caption for Fig. 1. Bars represent the standard deviation (n=3).

at day 12, respectively. However, a rapid increase in TVB content was noticed in the control, which reached a value of 27.11 mg N/100 g muscle at day 12. At the end of the storage period (14 days), TVB content of gutted sea bass (*D. labrax*) was 48 mg N/100 g (Cakli et al., 2007). Hozbor et al. (2006) reported the increase in TVB content in sea salmon during aerobic storage in ice at 0 °C for 10 days. TVB content in freshly caught fish is typically between 5 and 20 mg N/100 g, and TVB content of 30–35 mg N/100 g is usually regarded as the limit of acceptability for fish (Huss, 1988). It has been suggested that the TVB content is affected by species, season, harvesting area, age and sex of fish (Kilinc and Cakli, 2005). TVB represents the sum of ammonia, dimethylamine (DMA), trimethylamine (TMA) and other basic volatile nitrogenous compounds. DMA and TMA were the degradation products of trimethylamine oxide (TMAO), a fish typical molecule which has an important role in osmoregulation. DMA is mostly produced by endogenous enzymes and TMA is generated by bacterial enzymes (Zeisel et al., 1985). All samples had TVB content below the maximum value of 35 mg N/100 g muscle, specified by the EC guidelines (Commission Decision 95/149/EC, 1995). TVB content generally correlated well with microbial load (Fig. 1a) in the corresponding samples. Thus, the inhibitory effect of LEO film against microbial growth could retard or lower the production of microbial degradation products.

### 3.2.2. pH

pH of sea bass slices wrapped without and with films (G film or LEO film) during storage at 4 °C for 12 days is shown in Fig. 4. pH of the control increased from 5.97 to 7.42 after storage for 12 days. Generally, pH of all fish samples slightly increased, when the storage time increased ( $P < 0.05$ ). The increase in pH value of fish samples during the storage period was associated with the accumulation of alkaline compounds such as ammonia and TMA, etc. generated from microbial enzymatic actions (Lopez-Caballero et al., 2007). Herland et al. (2007) reported an increase in pH of wild cod during storage in ice and pH of 7.2 was found at day 15. The lower increase in the pH of sea bass slices wrapped with LEO film was observed, when compared with other samples ( $P < 0.05$ ). The result was in accordance with the lower microbial count in LEO film wrapped samples. For sea bass slices wrapped with G film, pH of slices was similar to that of samples wrapped with LEO film. Postmortem pH can vary from 6.0 to 7.1, depending on season, species and other factors (Simeonidou et al., 1998). The pH of air-stored sea bass muscle was found to be 7.1 after 9 days of storage, presumably due to the production of basic amines (Pastoriza et al., 1996).

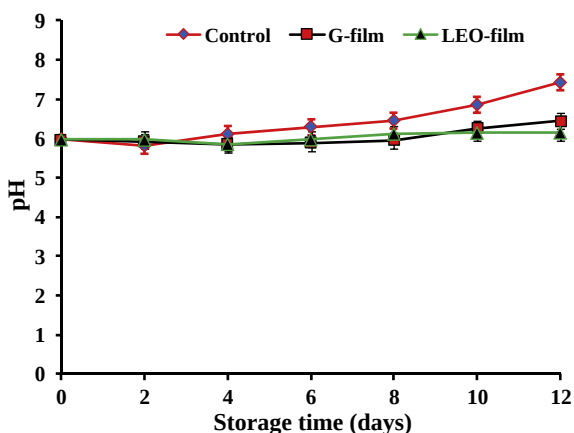


Fig. 4. pH of sea bass slices wrapped without and with films (LEO film or G film) during storage at 4 °C for 12 days. Key: see the caption for Fig. 1. Bars represent the standard deviation (n = 3).

### 3.2.3. K value

K value of sea bass slices wrapped without and with different films during storage at 4 °C for 12 days is shown in Fig. 5. K value has been used as the freshness index in fish and fish products. At day 0, K value of sea bass slices was 16.44%, and the value increased to 66.78% in the control samples at the end of the storage period. K values of 47.38% and 37.67% were detected at day 12 for G-film and LEO-film wrapped samples, respectively. The increase of K value primarily resulted from the decomposition of adenosine triphosphate (ATP) and derivatives in the sea bass slices during the storage. Ozogul et al. (2004) showed that K value was strongly affected by the fast depletion of inosine monophosphate (IMP). The pattern and rate of nucleotide degradation differed between and within fish species, type of muscle, and stress of fish during capture and storage temperature (Shoji and Saeki, 1989). During postmortem handling and storage, indigenous enzymes hydrolyse ATP to different derivatives such as adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (HxR), and finally hypoxanthine (Hx) (Yamagata and Low, 1995). However, the enzymes produced by microorganisms might involve in decomposition of ATP and its derivatives to some degree. This was reflected by the higher K value in the control, in which the highest microbial load was found (Surette et al., 1990). The K value of sea bass (*Lateolabrax japonicus*) increased during the refrigerated storage and reached a maximum of 85% on day 10 (Chang et al., 1998). The K value of 50% was considered as the quality limit or rejection of sea bass stored at refrigerated temperature (Chang et al., 1998). In general, a K value of 20% or lower indicates very good quality fish, while 60% has been realized as the rejection limit (Nirmal and Benjakul, 2009). The control samples were considered unacceptable based on the K value (66%). Nevertheless, the K value for LEO-film wrapped sea bass slices did not reach such a value even at the end of the storage (day 12). The lower K value in LEO-film wrapped samples was due to the protective effect of active film against bacteria, which secretes enzymes involved in the degradation of ATP and related compounds (namely dephosphorylases) during storage (Shoji and Saeki, 1989). Ozogul et al. (2004) detected a K value of 80% for vacuum-packed sardine stored at 4 °C on the rejection day (day 9), while a lower K value of 39% was determined on the day of rejection (day 17) for sea bream stored at  $2 \pm 2$  °C (Alasalvar et al., 2001).

### 3.3. Changes in physical property

$L^*$ —(lightness/brightness),  $a^*$ —(redness/greenness) and  $b^*$ —(yellowness/blueness) of sea bass slices wrapped without and with

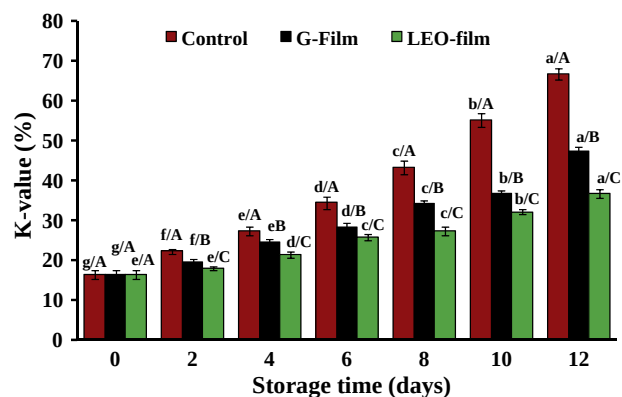


Fig. 5. K value of sea bass slices wrapped without and with films (LEO film or G film) during storage at 4 °C for 12 days. Key: see the caption for Fig. 1. Bars represent the standard deviation (n = 3). Different lowercase letters on the bars within the same sample denote significant difference ( $P < 0.05$ ). Different uppercase letters on the bars within the same storage time denote significant difference ( $P < 0.05$ ).

**Table 1**

Colour of sea bass slices wrapped without and with films (LEO film or G film) during storage at 4 °C for 12 days.

| Storage time (days) | L*              |                 |                 | a*             |                 |                 | b*             |                |                |
|---------------------|-----------------|-----------------|-----------------|----------------|-----------------|-----------------|----------------|----------------|----------------|
|                     | C               | G film          | LEO film        | C              | G film          | LEO film        | C              | G film         | LEO film       |
| 0                   | 44.16 ± 1.43abA | 44.16 ± 1.43abA | 44.16 ± 1.43abA | −0.44 ± 1.13cA | −0.44 ± 1.13bA  | −0.44 ± 1.13cA  | 2.46 ± 1.93bA  | 2.46 ± 1.93bA  | 2.46 ± 1.93bcA |
| 2                   | 44.24 ± 3.15abA | 46.17 ± 1.23aA  | 43.18 ± 0.34abA | −0.50 ± 0.11cB | 0.14 ± 0.10abA  | −0.36 ± 0.21bcB | 2.24 ± 0.68bAB | 2.57 ± 0.13bA  | 1.61 ± 0.26cB  |
| 4                   | 44.88 ± 1.69aA  | 43.91 ± 0.23bA  | 43.36 ± 1.51abA | 1.69 ± 0.28bA  | −0.11 ± 0.06abC | 0.31 ± 0.15abcB | 4.95 ± 0.75aA  | 2.72 ± 0.27bB  | 2.22 ± 0.07cB  |
| 6                   | 42.38 ± 0.17abA | 42.76 ± 0.92bcA | 44.11 ± 0.45aA  | 2.19 ± 0.84abA | 0.16 ± 0.08abB  | 0.43 ± 0.09abB  | 5.53 ± 0.57aA  | 3.03 ± 0.64abB | 2.47 ± 0.42bcB |
| 8                   | 42.12 ± 1.10bA  | 41.17 ± 2.08cdA | 43.23 ± 1.51abA | 2.44 ± 0.62abA | 0.43 ± 1.19abB  | 0.68 ± 0.59aB   | 5.68 ± 0.14aA  | 3.80 ± 1.14abB | 3.71 ± 0.98abB |
| 10                  | 39.37 ± 1.30cA  | 39.20 ± 1.98deA | 41.60 ± 0.99bA  | 2.69 ± 0.28abA | 0.61 ± 0.81abB  | 0.96 ± 0.48aB   | 5.76 ± 1.21aA  | 4.30 ± 0.91aAB | 3.83 ± 0.78abB |
| 12                  | 36.12 ± 0.86dC  | 38.74 ± 1.13eB  | 41.98 ± 0.66bA  | 3.19 ± 0.56aA  | 0.90 ± 0.76aB   | 1.04 ± 0.35aB   | 6.26 ± 0.40aA  | 4.55 ± 0.64aB  | 4.08 ± 0.52aB  |

Values are given as mean ± standard deviation (n = 3).

Different lowercase letters in the same column indicate significant differences ( $P < 0.05$ ).Different uppercase letters in the same row within the same colour parameter indicate significant differences ( $P < 0.05$ ).

C: control samples, G film: samples wrapped with gelatin film, LEO film: samples wrapped with gelatin film incorporated with lemongrass essential oil.

films (G film or LEO film) during storage at 4 °C for 12 days are shown in Table 1. Generally, all sea bass slices showed the decrease in  $L^*$ -values with increasing storage time ( $P < 0.05$ ). However, it was noted that the samples wrapped with LEO film had lower changes in  $L^*$ -values, followed by those wrapped with G film ( $P < 0.05$ ). Myoglobin presented in postmortem muscle slices is a major contributor to the colour of sea bass slices (*L. calcarifer*), which has an impact on consumers' acceptability (Thiansilakul et al., 2011). Immediately after death, myoglobin present in sea bass muscles slices is mostly present in the reduced form ( $\text{MbFe}^{2+}$ ), which provides the desirable red pigmentation to the muscle. With increasing storage time, the iron atom in the heme ring is further oxidised to the ferric state ( $\text{MbFe}^{3+}$ ), resulting in the development of brown colour (Baron and Andersen, 2002). The converting process of ferrous myoglobin into ferric metmyoglobin is termed "autoxidation," which is responsible for the discolouration of meat and acceleration of lipid oxidation (Baron and Andersen, 2002). Thiansilakul et al. (2011) reported that metmyoglobin accumulation and lipid oxidation of refrigerated sea bass slices (*L. calcarifer*), took place with increasing storage time up to day 6. Thus, the decrease in  $L^*$ -value might be due to the oxidation of red oxymyoglobin to metmyoglobin, yielding sea bass slices with an unattractive brown colour. The increases in  $a^*$ -values in all samples were observed throughout the storage time. The highest change was found in control samples, while G-film and LEO-film wrapped sample showed a slight increase in  $a^*$ -values, compared to control samples. Thus the application of gelatin film either without or with LEO, on sea bass slices surface could increase the stability of the red meat colour to some degree. In general, both G film and LEO film could provide a barrier of moisture migration and gas permeability. Samples wrapped with LEO film showed the lower changes in colour values, probably due to the antioxidant and antimicrobial properties of LEO. LEO has been reported to inhibit the growth of various pathogenic bacteria, yeasts and molds as well as lactic acid bacteria (Coma et al., 2002). Metabolite products generated by some microorganisms such as hydrogen peroxide have been known to be associated with discolouration of meat and meat products (Cayre et al., 2005). The  $a^*$ -value of fresh beef steaks whose surface was sprayed with a rosemary and ascorbic acid solution was maintained during refrigeration (Djenane et al., 2003). All samples had increases in  $b^*$ -value, indicating the increase in yellowness of samples. Similar to  $a^*$ -value,  $b^*$ -value increased to the highest extent in the control. The increase in  $b^*$ -value was plausibly related with the increase in TBARS value (Fig. 6). Lipid oxidation products, especially aldehydes, could serve as the source of carbonyl compounds for non-enzymatic browning reaction, namely Maillard reaction (Wu et al., 2011). Thus, gelatin film and LEO could retard the colour changes of sea bass slices to some extent during the storage.

### 3.3.1. TBARS value

During the storage, a significant increase in TBARS was observed in control samples as compared to the samples wrapped with G film or LEO film, respectively, ( $P < 0.05$ ) (Fig. 6). The result suggested that lipid oxidation in sea bass slices could be retarded when LEO film was applied, probably due to the antioxidant activity of LEO as well as its low oxygen permeability characteristics of LEO film (Xu et al., 2005). Thus, incorporation of LEO into gelatin film enhanced the antioxidant property of the resulting film, as evidenced by lower TBARS of samples wrapped with LEO film, compared to G film. Sacchetti et al. (2005) reported LEOs as effective free radical-scavengers and metal chelating agents. The antioxidant activities of the essential oils have been attributed to assorted mechanisms, including prevention of radical chain initiation, binding of transition metal ion catalysts, decomposition of peroxides and interaction with the free radicals (Perumalla and Hettiarachchy, 2011). The increase in TBARS was lowered in samples wrapped with LEO film in comparison with the control samples ( $P < 0.05$ ). Samples wrapped with G film, regardless of LEO incorporated, also showed the lower TBARS than the control ( $P < 0.05$ ). G film might function as a barrier to oxygen permeability and only a small amount of oxygen could therefore contact with samples. As a result, the lower oxidation rate was obtained. Psychrotrophic bacteria, mainly *Pseudomonas* species, produce lipase and phospholipase, causing an increase in free fatty acids (Koka and Weimer, 2001). These free fatty acids are highly susceptible to oxidation and form unstable lipid hydroperoxide. This hydroperoxide is readily decomposed to shorter chain products such as

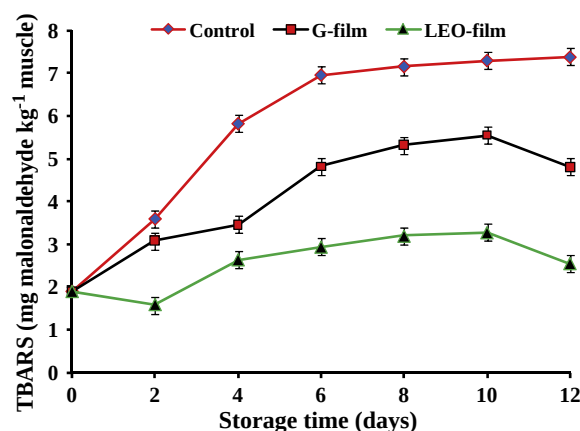


Fig. 6. TBARS value of sea bass slices wrapped without and with films (LEO film or G film) during storage at 4 °C for 12 days. Key: see the caption for Fig. 1. Bars represent the standard deviation (n = 3).

aldehydes, which can be detected as TBARS (Benjakul et al., 2005). Lowered lipid oxidation was in accordance with the lower microbial growth of sea bass slices wrapped with LEO film (Fig. 1a).

#### 4. Conclusion

An active gelatin-based film could be achieved by incorporation with LEO. LEO film could retard lipid oxidation and microbial growth more effectively than G film, indicating the higher antimicrobial and antioxidant properties of the LEO film. Thus, LEO film could be used as an active packaging to maintain quality and extend shelf-life of the sea bass slices at 4 °C.

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# The effect of Fenton's reactants and aldehydes on the changes of myoglobin from Eastern little tuna (*Euthynnus affinis*) dark muscle

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**Abstract** The influences of Fenton's reactants ( $\text{H}_2\text{O}_2$  and  $\text{FeCl}_2$ ) and aldehydes (hexanal and hexenal) on changes of oxymyoglobin and metmyoglobin from Eastern little tuna (*Euthynnus affinis*) dark muscle were studied. In the presence of  $\text{H}_2\text{O}_2$ , both oxymyoglobin and metmyoglobin were rapidly oxidized into ferrylmyoglobin based on spectra patterns. In the presence of  $\text{Fe}^{2+}$  and/or  $\text{H}_2\text{O}_2$ , the changes in fluorescent intensity of myoglobin were noticeable, but there were no changes in aggregation ratio. Release of non-heme iron from myoglobin was mainly governed by  $\text{H}_2\text{O}_2$ . When aldehydes were incorporated, the oxidation of oxymyoglobin and conformational changes of globin were more pronounced. No release of non-heme iron was noticeable, suggesting the stability of heme moiety toward aldehydes. Hexenal had a great impact on cross-linking of oxymyoglobin and metmyoglobin via covalent modification. Alteration of myoglobin redox state might be enhanced by conformational changes of globin induced by both Fenton's reactants and aldehydes.

**Keywords** Myoglobin · Ferrylmyoglobin ·  $\text{H}_2\text{O}_2$  · Cross-linking · Hexanal · Hexenal

## Introduction

Myoglobin, a predominant pigment protein found in dark fleshed-fish species, has been suggested for its relationship with lipid oxidation [1, 2]. Lipid oxidation is a major cause of deterioration of food and food products, especially those containing high content of unsaturated fatty acids. Oxidation of myoglobin is associated with the accelerated lipid oxidation [1]. The role of heme dissociation in the ability of different heme proteins to promote lipid oxidation processes was reported by Richards et al. [2].

Deoxymyoglobin and oxymyoglobin are in the ferrous state ( $\text{Fe}^{2+}$ ), whereas metmyoglobin is in the ferric state ( $\text{Fe}^{3+}$ ). Autoxidation of oxymyoglobin results in the formation of metmyoglobin and superoxide ( $\text{•OOH/O}_2^{\text{•-}}$ ), which rapidly dismutate to  $\text{H}_2\text{O}_2$  and  $\text{O}_2$  [3]. The interaction of  $\text{H}_2\text{O}_2$  with metmyoglobin led very rapidly to generation of active species, perferrylmyoglobin ( $\text{•MbFe(IV) = O}$ ) and ferrylmyoglobin species ( $\text{MbFe(IV) = O}$ ), which could initiate lipid peroxidation [3, 4]. The ferrous myoglobin species, deoxymyoglobin and oxymyoglobin, can likewise react with  $\text{H}_2\text{O}_2$ , resulting in the formation of ferrylmyoglobin by direct two-electron oxidation [5]. Additionally,  $\text{O}_2^{\text{•-}}$  can also reduce dissociated hemein to heme that further reacts with  $\text{H}_2\text{O}_2$  to form either hydroxyl radical or a non-protein bound ferryl-heme [6]. These products are able to abstract a hydrogen atom from a polyunsaturated fatty acid and initiates lipid oxidation. Chan et al. [3] revealed that  $\text{H}_2\text{O}_2$ , presumably generated during oxymyoglobin oxidation, played an important role in mediating oxymyoglobin and lipid oxidation in liposome system. The oxidation of phosphatidylcholine by ferrylmyoglobin was found to be approximately sevenfold greater than that observed for native myoglobin [7]. The potential of ferrylmyoglobin to oxidize lipids is dependent on  $\text{H}_2\text{O}_2$ , lipid hydroperoxide

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production, the concentration of reducing agents, and their compartmentalization in the muscle cells [8].

Lipid oxidation could generate a variety of secondary products, predominantly *n*-alkanals, *trans*-2-alkenals, 4-hydroxy-*trans*-2-alkenals, and malondialdehyde [9]. The aldehyde products are more water-soluble than their parent compounds and could potentially interact with myoglobin [9, 10]. Hexanal, hexenal, and 4-hydroxynonenal were reported to enhance the oxidation of tuna oxymyoglobin [11]. Porcine metmyoglobin formation was greater in the presence of 4-hydroxynonenal [12]. Lynch and Faustman [10] also determined the effect of aldehyde lipid oxidation products on oxymyoglobin oxidation, metmyoglobin reduction, and the catalytic activity of metmyoglobin as a lipid prooxidant in vitro. Aldehydic lipid oxidation products covalently attached to myoglobin and subsequently cause structural alterations, which would make the protein more susceptible to oxidation [9].

The process of ferrous myoglobin converting into ferric metmyoglobin is responsible for discoloration of meat and acceleration of lipid oxidation [8, 11]. However, less information regarding the influence of the oxidation products of myoglobin and lipid on stability of fish myoglobin has been reported. A better understanding of this subject would provide promising approaches for inhibition of lipid oxidation as well as improving overall quality of fish or meat. Eastern little tuna (*Euthynnus affinis*) is a species available in the Gulf of Thailand and the Indian Ocean. It provides the high global economic value for canning and sashimi [13]. This species contains red muscle with a high amount of myoglobins, which may undergo oxidation with ease, especially in the presence of prooxidative compounds, such as Fenton's reactants and aldehydic lipid oxidation products. Therefore, the objective of this investigation was to study the stability of myoglobin from Eastern little tuna dark muscle as influenced by Fenton's reactants and selected aldehydes.

## Materials and methods

### Chemicals

$\beta$ -Mercaptoethanol ( $\beta$ ME), Triton X-100, pyridine, hexanal, *trans*-2-hexen-1-al, low-range protein markers, and bathophenanthroline disulfonic acid were purchased from Sigma (St. Louis, MO, USA). Sodium dithionite and ferrous chloride were obtained from Riedel (Seeize, Germany). Hydrogen peroxide, trichloroacetic acid, sodium nitrite, and iron standard solution were procured from Merck (Damstadt, Germany).

### Fish collection and preparation

Twenty Eastern little tuna (*Euthynnus affinis*) with the average weight of 0.5–0.55 kg were obtained from the

dock in Songkhla province, Thailand. The fish were off-loaded 24 h after capture, placed in ice with a fish/ice ratio of 1:2 (w/w), and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla within 1 h. Upon arrival, fish were washed with cold water (5 °C), beheaded, and eviscerated. Thereafter, the fish were filleted, and the dark muscle was manually excised and collected within the same batch. The composite sample was minced until the uniformity was obtained.

### Purification of myoglobin from Eastern little tuna

Extraction and purification of myoglobin were performed according to the method of Thiansilakul et al. [14]. Eastern little tuna mince (100 g) was mixed with 300 mL of cold extracting medium (10 mM Tris-HCl, pH 8.0 containing 1 mM EDTA, and 25 g/l Triton X-100). The mixture was homogenized for 1 min at a speed of 13,000 rpm using an IKA Labortechnik homogenizer (Selangor, Malaysia). After centrifugation at 9,600  $\times g$  for 10 min at 4 °C using an Avanti J-E centrifuge (Beckman Coulter, Palo Alto, CA, USA), the supernatant was filtered through a Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, UK). The filtrate referred to as “crude myoglobin extract” was subjected to ammonium sulfate fractionation (65–100% saturation). The precipitate obtained after centrifugation at 20,000  $\times g$  for 60 min was dissolved in a minimal volume of cold 5 mM Tris-HCl buffer, pH 8.5, which was referred to as “starting buffer”. The mixture was then dialyzed against 10 volumes of the same buffer with 20 changes at 4 °C. The dialysate was immediately applied onto a Sephadex G-75 column (2.6  $\times$  70 cm; Amersham Bioscience, Uppsala, Sweden), which was previously equilibrated with the starting buffer. The separation was conducted using the starting buffer at a flow rate of 0.5 mL/min. Fractions of 3 mL were collected and measured at 280 and 540 nm using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). The fractions with the high absorbance at 540 nm were pooled and used as “tuna myoglobin”.

### Preparation of oxymyoglobin and metmyoglobin

Oxymyoglobin and metmyoglobin were prepared according to the method of Tang et al. [15] with some modifications. An aliquot (5 mL and 0.25 mM) of tuna myoglobin was converted to oxymyoglobin by the addition of 0.1 g of sodium dithionite. Metmyoglobin was prepared by adding 0.1 g of potassium ferricyanide into 5 mL of the tuna myoglobin solution. The sodium dithionite and potassium ferricyanide were then removed by dialysis of the sample against 10 volumes of cold 50 mM phosphate

buffer, pH 7.0 with 20 changes of dialysis buffer. The concentrations of oxymyoglobin and metmyoglobin were adjusted to 0.2 mM.

#### Determination of myoglobin concentration

The concentration of myoglobin (mM) was determined by measuring the absorbance at 525 nm. The molar extinction coefficient of  $7.6 \times 10^{-3}$  was used for calculation [15].

#### Effect of Fenton's reactants on the changes of Eastern little tuna myoglobin

The changes in oxymyoglobin and metmyoglobin as affected by Fenton's reactants were monitored according to the method of O'Grady et al. [1] with a slight modification. In a final volume of 4 mL, the solutions of oxymyoglobin and metmyoglobin (0.2 mM) were incubated with  $\text{FeCl}_2$  (0.2 mM) and/or  $\text{H}_2\text{O}_2$  (0.1 mM). The different procedures for addition of  $\text{FeCl}_2$  and/or  $\text{H}_2\text{O}_2$  into myoglobin solutions were used. Those included myoglobin +  $\text{FeCl}_2$  (MF), myoglobin +  $\text{H}_2\text{O}_2$  (MH), myoglobin +  $\text{FeCl}_2$  +  $\text{H}_2\text{O}_2$  (MFH), myoglobin +  $\text{H}_2\text{O}_2$  +  $\text{FeCl}_2$  (MHF), myoglobin + the mixture of  $\text{FeCl}_2$  and  $\text{H}_2\text{O}_2$  previously mixed for 1, 5, and 10 min (MM1, MM5, and MM10). After the thorough mixing, all samples were stored at 4 °C for 1 h and analyzed for the changes of myoglobin.

#### Effect of aldehydes on the changes of Eastern little tuna myoglobin

To study the effect of hexanal and hexenal on the changes of oxymyoglobin and metmyoglobin, the solutions of oxymyoglobin and metmyoglobin (0.15 mM) were added with hexanal or hexenal (1 mM) at a ratio of 1:1 to obtain a final concentration of 0.5 mM aldehyde. Control was aldehyde-free and was prepared in the same manner, except an equivalent volume of ethanol was used instead. The treated samples were collected at 0, 1, 2, 4, 6, and 8 days of incubation at 4 °C for analysis. To determine the cross-linking effect of aldehydes, the mixture containing hexanal and hexenal at a final concentration of 12.5 mM was also prepared and incubated at 4 °C for 1 day prior to SDS-PAGE analysis.

#### Analyses of heme protein degradation and myoglobin oxidation

Heme protein degradation was determined by the changes in absorption spectra and Soret band [16]. The proportions of three myoglobin forms, deoxymyoglobin, oxymyoglobin, and metmyoglobin, were calculated following the modified Krzywicki's equations [15]. To determine the oxidation of

myoglobin by the effect of Fenton's reactants, the ratio of absorbance at 580 and 525 nm ( $A_{580}/A_{525}$ ) was calculated [11]. A high  $A_{580}/A_{525}$  value indicates a high proportion of oxymyoglobin.

#### Tryptophan fluorescent intensity

Tryptophan fluorescent intensity of treated myoglobin solutions was measured using an RF-1501 fluorometer (Shimadzu, Kyoto, Japan) at an excitation wavelength of 280 nm and an emission wavelength of 325 nm according to the method of Chanthai et al. [17].

#### Myoglobin aggregation ratio

Myoglobin solutions were filtered through a Millipore filter (pore size, 0.45  $\mu\text{m}$ ) (Millipore, Bedford, MA, USA). The filtrate was determined for the protein content [15]. The percentage of the decrease in protein content relative to that of the initial sample (time = 0) was calculated as myoglobin aggregation ratio [18].

#### Non-heme iron content

Non-heme iron content of myoglobin solutions was determined according to the method of Schricker et al. [19] with a slight modification. One milliliter of oxymyoglobin and metmyoglobin solutions was pipetted into a screw cap test tube, and 50  $\mu\text{L}$  of 0.39% (w/v) sodium nitrite was added. Four milliliters of a mixture of 40% trichloroacetic acid and 6 M HCl (ratio of 1: 1 (v/v), prepared freshly) were added. The tightly capped tubes were incubated at 65 °C for 22 h and then cooled at room temperature for 2 h. The supernatant (400  $\mu\text{L}$ ) was mixed with 2 mL of the non-heme iron color reagent, a mixture of bathophenanthroline disulfonic acid, double-deionized water, and saturated sodium acetate solution at a ratio of 1:20:20 (w/v/v), prepared freshly. After vortexing and standing for 10 min, the absorbance was measured at 540 nm. The non-heme iron content was calculated from iron standard curve. The iron standard solutions ( $\text{Fe}(\text{NO}_3)_3$  in  $\text{HNO}_3$ ), with the concentrations ranging from 0 to 2 ppm, were used.

#### SDS-polyacrylamide gel electrophoresis

To determine the protein pattern of myoglobins, the treated myoglobin solutions were subjected to SDS-PAGE according to the method of Laemmli [20]. Samples (20  $\mu\text{g}$ ) with reducing and non-reducing conditions were loaded onto polyacrylamide gels comprising 4% stacking gel and 17.5% running gel and subjected to electrophoresis using a Mini Protean II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). The low-molecular-weight-protein

standards including bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa),  $\alpha$ -lactalbumin (14.2 kDa), and aprotinin (6.5 kDa) were used for molecular weight estimation.

### Statistical analysis

Experiments were run in triplicate using three different batches of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test. For pair comparison, *t*-test was used [21]. Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA).

## Results and discussion

### Effect of Fenton's reactants on the changes of Eastern little tuna myoglobin

#### *Changes in absorption spectra*

Oxymyoglobin solution contained oxymyoglobin as the predominant form (68.91%), followed by metmyoglobin (23.64%) and deoxymyoglobin (7.45), respectively. For metmyoglobin solution, a complete metmyoglobin formation (100%) was found. The strong absorption of myoglobin was located in a wavelength region of 350–450 nm or Soret band, in which the intense peaks at 413 and 406 nm were observed for the control oxymyoglobin and metmyoglobin, respectively (Fig. 1). The typical spectrum in a region of 450–750 nm representing the redox state of myoglobin had the peaks at wavelengths of 542 and 577 nm for oxymyoglobin and of 502 and 630 nm for metmyoglobin.

When  $\text{Fe}^{2+}$  and/or  $\text{H}_2\text{O}_2$  were added, myoglobin redox state was altered as evidenced by the changes in absorption characteristics of oxymyoglobin and metmyoglobin solutions. Absorbance spectra of MF for oxymyoglobin and metmyoglobin solution were similar to that of the control oxymyoglobin solution, suggesting the reducing capability of  $\text{Fe}^{2+}$ . The highest oxymyoglobin content was obtained in the control and MF of oxymyoglobin solution ( $p < 0.05$ ) (Table 1). In the presence of  $\text{H}_2\text{O}_2$  with or without  $\text{Fe}^{2+}$  added, the great changes in absorption spectra were observed in both oxymyoglobin and metmyoglobin solutions. MH, MFH, and MHF from oxymyoglobin and metmyoglobin solutions presenting a Soret peak shifted to a higher wavelength ( $\sim 419$  nm). Additionally, they had the increased absorbance in the region of 500–600 nm along with the unsharpened peak at wavelength of  $\sim 545$  nm.

The similar spectra patterns were evidenced for ferrylmyoglobin of sperm whale and horse heart [22, 23]. Prasad et al. [24] reported the similar wavelength of absorption peaks between oxymyoglobin and ferrylmyoglobin from horse heart by which the latter had the drastic decrease in peak height. Therefore, both oxymyoglobin and metmyoglobin could be rapidly oxidized by  $\text{H}_2\text{O}_2$ , leading to the formation of ferrylmyoglobin via one or two-electron oxidation [1, 5]. For both oxymyoglobin and metmyoglobin solutions, MM1 more likely exhibited the typical spectrum of ferrylmyoglobin. Conversely, MM5 and MM10 tended to possess the different absorption spectra. Increasing time for Fenton's reaction ( $\text{Fe}^{2+} + \text{H}_2\text{O}_2$ ) might decrease the  $\text{H}_2\text{O}_2$  content and concurrently increased the amount of hydroxyl radicals that exhibited the prooxidative effect on myoglobin. During the oxidation of oxymyoglobin solutions as accelerated by  $\text{H}_2\text{O}_2$  or hydroxyl radical, the decreases in oxymyoglobin contents were noticeable, and the lowest content was obtained in MM10 for both oxymyoglobin and metmyoglobin solutions ( $p < 0.05$ ) (Table 1). In the presence of  $\text{H}_2\text{O}_2$  or Fenton's reactants,  $A_{580}/A_{525}$ , which indicated the proportion of oxymyoglobin form, was greater in MH, compared with the control for metmyoglobin solution ( $p < 0.05$ ). This possibly resulted from a presence of ferrylmyoglobin, an oxygen-containing myoglobin ( $\text{MbFe(IV)} = \text{O}$ ), which had a typical spectrum related to that of oxymyoglobin form [24]. O'Grady et al. [1] revealed that the oxidation of oxymyoglobin in the presence of both Fenton's reactants may not involve hydroxyl radical production but proceeds via a direct interaction between  $\text{H}_2\text{O}_2$  and oxymyoglobin. Thus,  $\text{H}_2\text{O}_2$  was a necessary reactant in the conversion of oxymyoglobin or metmyoglobin to a hypervalent ferrylmyoglobin, which is known to be an effective prooxidant [1, 8].

#### *Changes in tryptophan fluorescent intensity*

Changes in tryptophan fluorescent intensity of oxymyoglobin and metmyoglobin solutions from Eastern little tuna as affected by  $\text{Fe}^{2+}$  and/or  $\text{H}_2\text{O}_2$  are depicted in Fig. 2a. The highest tryptophan fluorescent intensity was found in MH of both myoglobin solutions ( $p < 0.05$ ).  $\text{H}_2\text{O}_2$  was not only capable of oxidizing oxymyoglobin and metmyoglobin into ferrylmyoglobin, but also enhanced conformational changes of apomyoglobin as evidenced by a marked increase in fluorescent intensity. DeGray et al. [25] reported that the reaction between  $\text{H}_2\text{O}_2$  and recombinant sperm whale myoglobin resulted in the formation of globin-centered radicals, especially at tyrosine and tryptophan residues. Decreases in fluorescent intensity were observed in MF, MFH, MHF, MM1, MM5, and MM10 of both myoglobin solutions, when compared with the corresponding controls ( $p < 0.05$ ). Heavy metal salt such as  $\text{FeCl}_2$  added into

**Fig. 1** Effect of  $\text{Fe}^{2+}$  and/or  $\text{H}_2\text{O}_2$  on the absorption spectra in a region of 350–450 and 450–750 nm of oxymyoglobin (a and b) and metmyoglobin (c and d) solutions from Eastern little tuna dark muscle

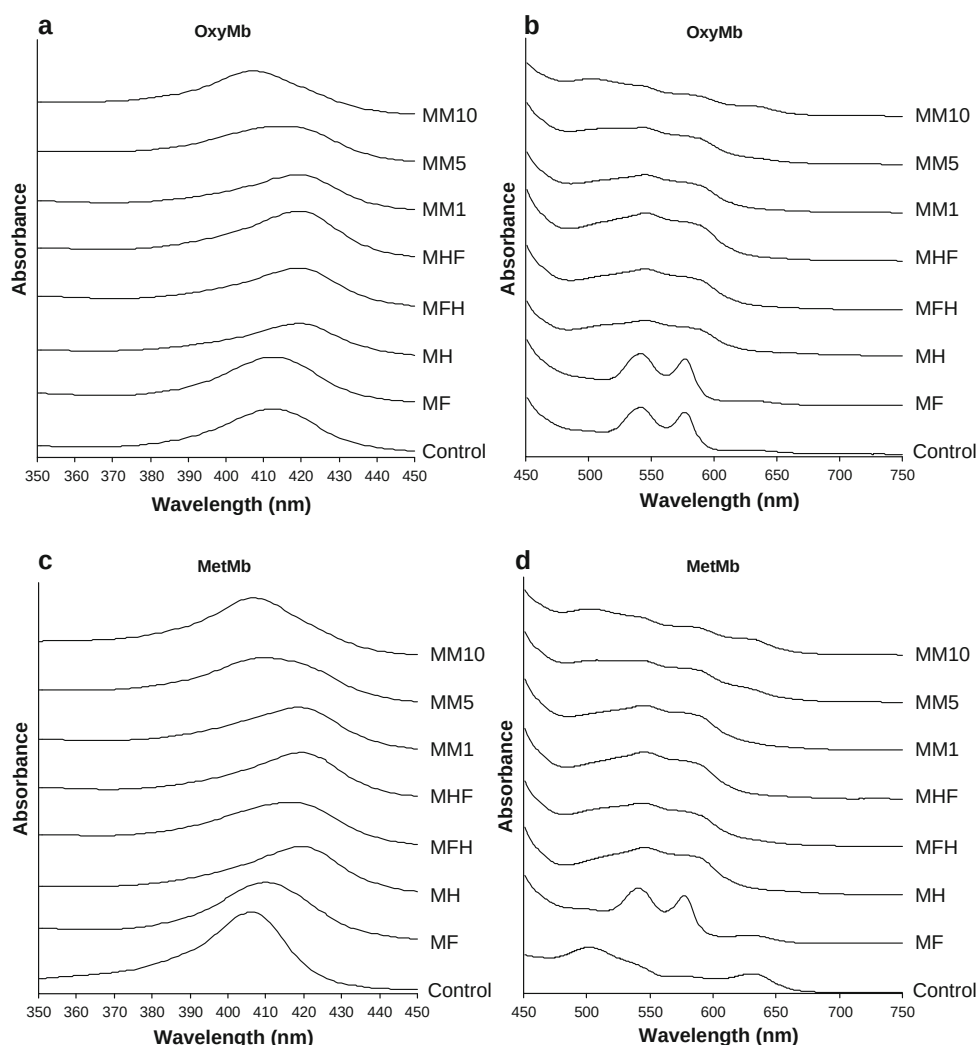


**Table 1**  $A_{580}/A_{525}$  value of oxymyoglobin and metmyoglobin solutions from Eastern little tuna dark muscle as affected by  $\text{Fe}^{2+}$  and/or  $\text{H}_2\text{O}_2$

| Treatments | $A_{580}/A_{525}^\dagger$ |                       |
|------------|---------------------------|-----------------------|
|            | OxyMb                     | MetMb                 |
| Control    | $1.24 \pm 0.08^{aA*}$     | $0.48 \pm 0.00^{dB}$  |
| MF         | $1.22 \pm 0.07^{aA}$      | $1.13 \pm 0.06^{aA}$  |
| MH         | $0.89 \pm 0.01^{bB}$      | $0.95 \pm 0.00^{bA}$  |
| MFH        | $0.89 \pm 0.01^{bA}$      | $0.87 \pm 0.01^{deA}$ |
| MHF        | $0.91 \pm 0.01^{bA}$      | $0.91 \pm 0.01^{cdA}$ |
| MM1        | $0.87 \pm 0.01^{bB}$      | $0.91 \pm 0.00^{bcA}$ |
| MM5        | $0.79 \pm 0.01^{cB}$      | $0.83 \pm 0.00^{cA}$  |
| MM10       | $0.67 \pm 0.00^{dB}$      | $0.71 \pm 0.00^{fA}$  |

$^\dagger$  A high  $A_{580}/A_{525}$  value indicates a high proportion of oxymyoglobin

\* Different superscripts in the same column indicate significant differences ( $p < 0.05$ ). Different capital superscripts in the same row indicate significant differences ( $p < 0.05$ )

myoglobin solutions might act as the protein denaturant by disruption of electrostatic interaction, resulting in an irreversible conformational change or denaturation of protein [26]. Hydroxyl radicals produced from Fenton's reaction could oxidize the proteins, contributing to either protein cross-linking or fragmentation [27, 28]. Moreover, the modified protein might bury tryptophan or aromatic domain inside, as evidenced by the decrease in fluorescent intensity. However, slight differences in fluorescent intensity among MFH, MHF, MM1, MM5, and MM10 of both myoglobin solutions could be observed ( $p < 0.05$ ). This might be related with the different prooxidant effect of hydroxyl radicals toward apomyoglobin. When comparing the impact of Fenton's reaction time on fluorescent intensity, it was found that reaction time had no influence on fluorescent intensity of metmyoglobin. Nevertheless, the longer reaction caused the gradual decrease in fluorescent intensity of oxymyoglobin solution. Furthermore, the order of addition of reactant ( $\text{H}_2\text{O}_2$  and  $\text{FeCl}_2$ ) only had the effect on

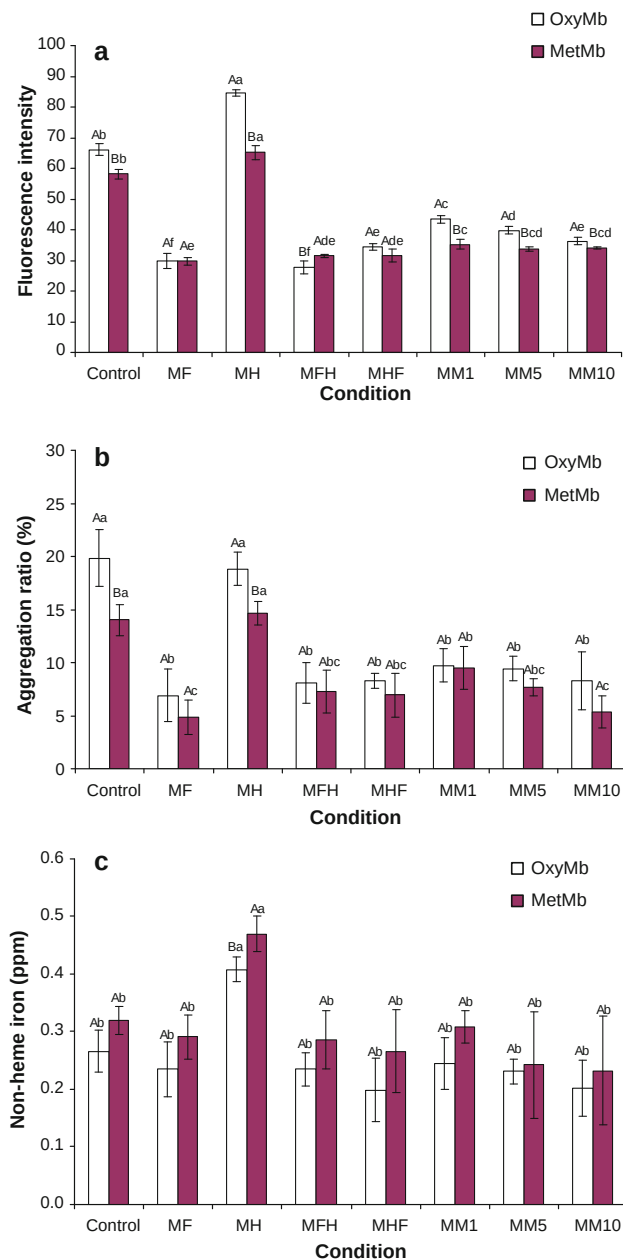


**Fig. 2** Effect of  $\text{Fe}^{2+}$  and/or  $\text{H}_2\text{O}_2$  on tryptophan fluorescent intensity (a), aggregation ratio (b), and non-heme iron content (c) of oxymyoglobin and metmyoglobin solutions from Eastern little tuna dark muscle. Bars represent the standard deviation ( $n = 3$ ). Different letters within the same myoglobin form indicate significant differences ( $p < 0.05$ ). Different capital letters within the same treatment indicate significant differences ( $p < 0.05$ )

fluorescent intensity of oxymyoglobin ( $p < 0.05$ ). Thus, the globin configuration was affected by Fenton's reactants, but the degree of changes depended on the form of myoglobin.

#### Changes in aggregation ratio

Aggregation ratio of oxymyoglobin and metmyoglobin from Eastern little tuna in the presence of  $\text{Fe}^{2+}$  and/or

$\text{H}_2\text{O}_2$  is shown in Fig. 2b. Following 1 h of incubation at 4 °C, aggregation ratio of the control oxymyoglobin and metmyoglobin was 19.84 and 14.00%, respectively. For both myoglobin solutions, no differences in aggregation ratio were observed for MH and its control ( $p > 0.05$ ). The lower ratios were noticeable in the presence of  $\text{Fe}^{2+}$ , regardless of  $\text{H}_2\text{O}_2$  added ( $p < 0.05$ ). Decrease in stabilization of the native structure of protein is presumed to be the key step for protein aggregation [29].  $\text{H}_2\text{O}_2$  showed the insignificant effect on myoglobin aggregation, while  $\text{Fe}^{2+}$  and Fenton's reactants could suppress the aggregation of myoglobin. Oxymyoglobin and metmyoglobin solutions, which had the low tryptophan fluorescent intensity (Fig. 2a), might have hydrophilic amino residues existing at exterior portion along with charge shielding from iron salt ( $\text{FeCl}_2$ ). Therefore, aggregation of globin enhanced via hydrophobic interaction could be reduced [29]. The control and MH of oxymyoglobin solution exhibited the higher aggregation ratios than those of metmyoglobin solution ( $p < 0.05$ ). This suggested a more conformational change of oxymyoglobin in comparison with metmyoglobin, regardless of  $\text{H}_2\text{O}_2$  incorporation. However, no differences in aggregation ratio were observed between oxymyoglobin and metmyoglobin solutions when  $\text{Fe}^{2+}$  was incorporated ( $p > 0.05$ ). No difference in protein patterns among all samples was noticeable (data not shown), indicating negligible change in the molecular weight of myoglobin as influenced by Fenton's reactants.

#### Changes in non-heme iron content

The released of non-heme iron content from oxymyoglobin and metmyoglobin solutions as affected by  $\text{Fe}^{2+}$  and/or  $\text{H}_2\text{O}_2$  is shown in Fig. 2c. Non-heme iron content increased, when  $\text{H}_2\text{O}_2$  was added into oxymyoglobin and metmyoglobin solution ( $p < 0.05$ ). Normally, heme group is surrounded in a hydrophobic pocket-like structure of globin, in which iron has occupied four sites with nitrogen of porphyrin ring and the other one site with histidine residue of globin [30].  $\text{H}_2\text{O}_2$  with oxidizing power might induce the change of myoglobin redox state as well as structure of globin, thereby enhancing iron released from porphyrin ring. In the presence of  $\text{Fe}^{2+}$  with or without  $\text{H}_2\text{O}_2$  addition, no changes in iron released from both myoglobin solutions were observed, compared with the control myoglobin ( $p > 0.05$ ). It suggested that porphyrin ring of heme in hydrophobic pocket of myoglobin was probably stable in the presence of  $\text{Fe}^{2+}$  or Fenton's reactants. For the same treatment, no difference in non-heme iron content was noticeable between oxymyoglobin and metmyoglobin except for MH that  $\text{H}_2\text{O}_2$  might cause the release of free iron from metmyoglobin at a higher extent.

## Effect of aldehyde on the changes of Eastern little tuna myoglobin

### Changes in absorption spectra

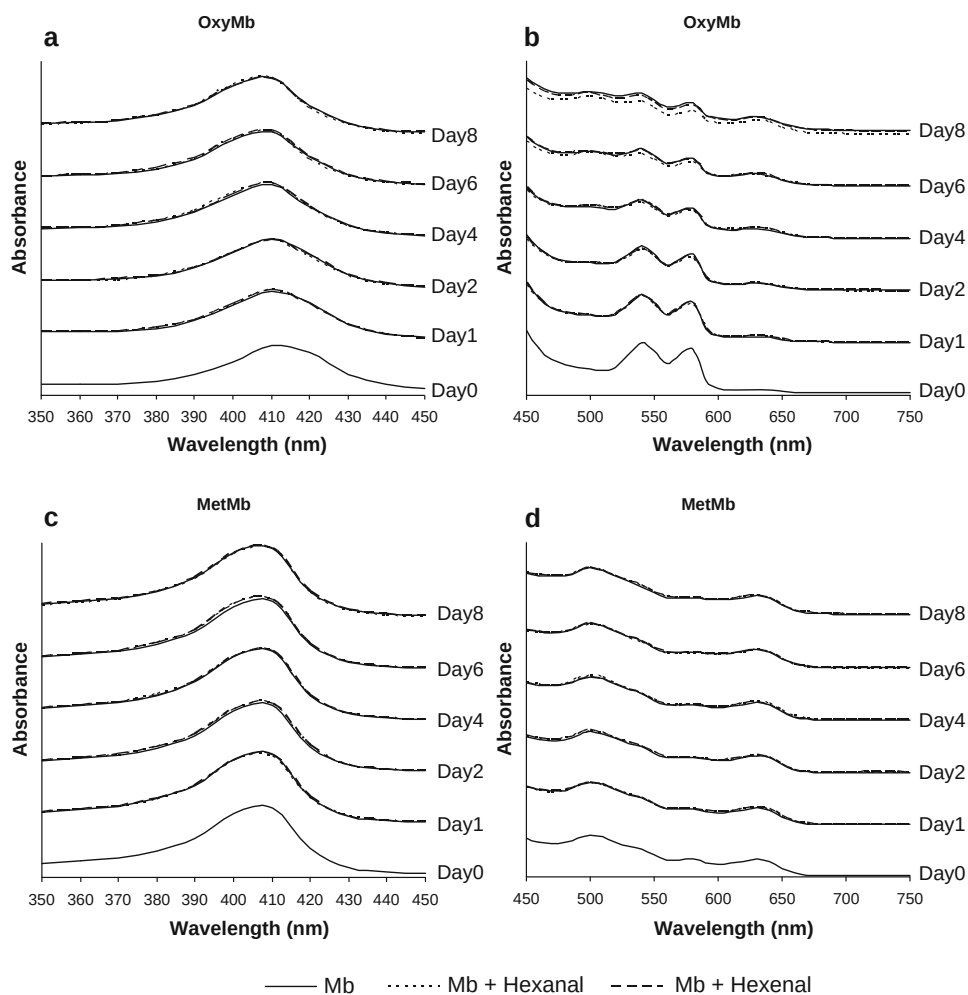
The effect of hexanal and hexenal on changes in absorption spectra of oxymyoglobin and metmyoglobin solutions from Eastern little tuna is presented in Fig. 3. For oxymyoglobin solution, as the incubation time increased up to 8 days, the peak of Soret bands was slightly increased in intensity and shifted to a lower wavelength. Concurrently, the typical spectra of oxymyoglobin in a region of 450–750 nm gradually disappeared. These implied the alteration of oxymyoglobin into an oxidized form induced by aldehyde, especially with the sufficient incubation time. After incubation for 1 day, the proportion of metmyoglobin form in the control and oxymyoglobin solution incorporated with hexanal and hexenal increased to 31.03, 34.48, and 34.90%, respectively ( $p < 0.05$ ). With increasing incubation time up to 8 days, the highest formation of metmyoglobin was found in oxymyoglobin solution added with

hexenal (76.47%), followed by hexanal (71.07%) and the control (68.73%), respectively ( $p < 0.05$ ). The result suggested that the oxidation of oxymyoglobin was induced by aldehydes, in which hexenal had a greater impact than its saturated counterpart, hexanal. Faustman et al. [9] and Lynch and Faustman [10] reported that metmyoglobin formation was greater in the presence of  $\alpha$ - $\beta$ -unsaturated aldehydes than their saturated counterparts having equivalent carbon chain length. A negligible change in the absorption spectra was observed for metmyoglobin solutions in both 350–450 and 450–750 nm regions, regardless of incubation time, indicating that aldehydes had no effect on the stability of metmyoglobin.

### Changes in tryptophan fluorescent intensity

Changes in tryptophan fluorescent intensity of oxymyoglobin and metmyoglobin solutions in the presence of hexanal and hexenal during the incubation period of up to 8 days are presented in Fig. 4a. Within the first day of incubation, an increase in fluorescent intensity was

**Fig. 3** Effect of hexanal and hexenal on the absorption spectra in a region of 350–450 and 450–750 nm of oxymyoglobin (a and b) and metmyoglobin (c and d) solutions from Eastern little tuna dark muscle as the function of incubation time



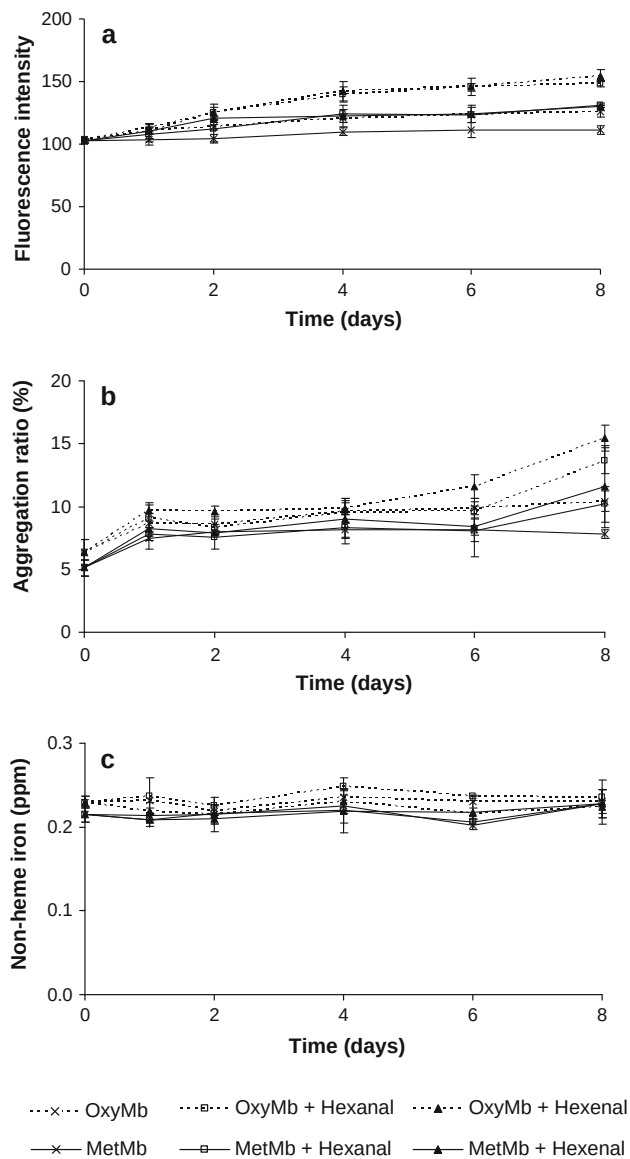


**Fig. 4** Effect of hexanal and hexenal on tryptophan fluorescent intensity (a), aggregation ratio (b), and non-heme iron content (c) of oxymyoglobin and metmyoglobin solutions from Eastern little tuna dark muscle as the function of incubation time. Bars represent the standard deviation ( $n = 3$ )

observed for both myoglobin solutions added with hexenal ( $p < 0.05$ ), whereas negligible changes were found in the controls and solutions containing hexanal ( $p > 0.05$ ). Thereafter, fluorescent intensities of all samples gradually increased as the incubation time increased ( $p < 0.05$ ). Hexanal and hexenal had a great impact on conformational changes of oxymyoglobin and metmyoglobin as indicated by the higher fluorescent intensity after incubation for 2 days ( $p < 0.05$ ). In the presence of aldehyde, unfolding of globin might be enhanced, causing the considerable exposure of tryptophan residues. A more susceptibility to structural changes was found in oxymyoglobin, compared

with metmyoglobin. Maheswarappa et al. [31] reported that alteration of myoglobin native form in adduction with aldehyde reduced the redox stability of oxymyoglobin. Therefore, the conformational changes of globin induced by hexanal or hexenal were more pronounced in ferrous myoglobin that was sensitive to oxidation.

#### Changes in aggregation ratio

Aggregation ratio of oxymyoglobin and metmyoglobin solutions added with hexanal and hexenal as a function of time is shown in Fig. 4b. The increase in aggregation ratio of all samples was observed with increasing incubation time up to 8 days ( $p < 0.05$ ), except for the control metmyoglobin that had a constant value after 1 day of incubation time ( $p > 0.05$ ). For both myoglobin solutions, no difference in aggregation ratio was found between myoglobin without and with the addition of aldehydes during 6 days of incubation ( $p > 0.05$ ). At day 8, both myoglobin solutions incorporated with hexanal or hexenal had a higher aggregation ratio, compared with the control ( $p < 0.05$ ). The result implied the profound impact of hexanal and hexenal on conformational changes of myoglobin with extended time. In addition, aggregation ratio of oxymyoglobin was generally greater than metmyoglobin counterpart ( $p < 0.05$ ). Libondi et al. [32] reported that the occurrence of intermolecular cross-linking in protein was induced by malondialdehyde, accompanied by the change in the secondary structure. Therefore, aggregation of myoglobin was governed by the presence of aldehyde as well as the reaction time.

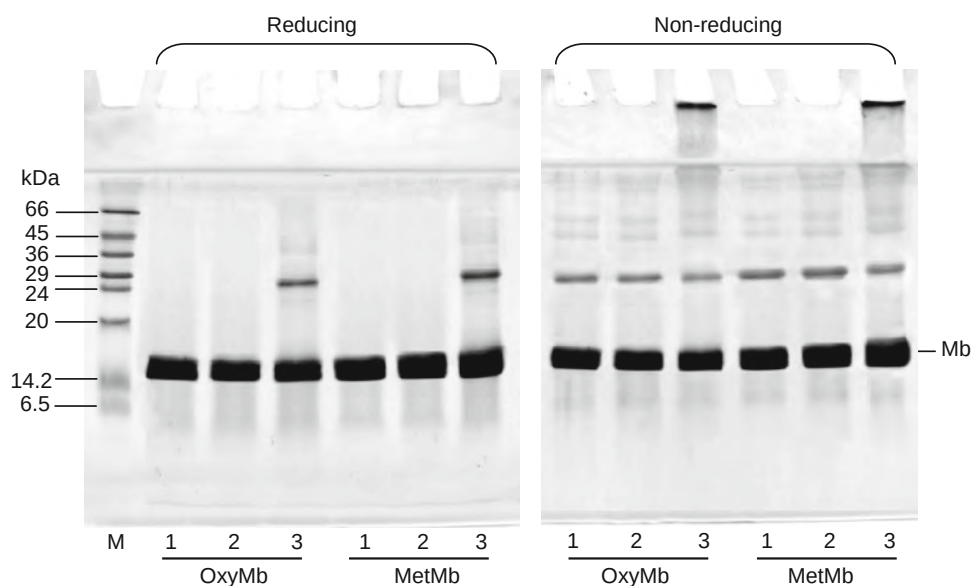
#### Changes in non-heme iron content

The effect of hexanal and hexenal on changes in non-heme iron content of oxymyoglobin and metmyoglobin solutions at varying times is presented in Fig. 4c. As the incubation time increased, no remarkable changes in non-heme iron content were obtained in all myoglobins, irrespective of aldehydes ( $p > 0.05$ ). The result demonstrated that aldehydes, both hexanal and hexenal, had no effect on iron released from heme complex. Heme iron is normally cleft within porphyrin ring of heme pocket and is localized far from the surface of myoglobin molecule [30]. Although the conformational changes of globin occurred to some extent, it had less impact on iron release from porphyrin ring of myoglobin.

#### Changes in protein pattern

Oxymyoglobin and metmyoglobin from Eastern little tuna dark muscle containing hexanal or hexenal at a concentration of 12.5 mM were determined for protein patterns

**Fig. 5** SDS-PAGE pattern of oxymyoglobin and metmyoglobin solutions from Eastern little tuna dark muscle containing 12.5 mM hexanal or hexenal at 4 °C for 1 day under-reducing and non-reducing conditions. *M* low-molecular-weight markers; *1* control myoglobin; *2* myoglobin added with hexanal; *3* myoglobin added with hexenal



using SDS-PAGE as shown in Fig. 5. The obvious band appeared at molecular weight around 15 kDa was presumed to be a myoglobin. Additionally, a band with molecular weight of 30 kDa was observed under the non-reducing condition in the control oxymyoglobin and metmyoglobin (without addition of aldehydes). This protein was most likely stabilized by the disulfide bond and might be a dimer of myoglobin. Hexenal had no effect on the changes in protein pattern of both myoglobins. This might be owing to the negligible cross-linking effect of hexenal at level used in this study. Proteins with the molecular weight of 30 kDa and the higher molecular weight components were observed in oxymyoglobin and metmyoglobin solutions added with hexenal (25 mM) under the reducing and non-reducing conditions. This result suggested that hexenal could induce the cross-linking of myoglobin via non-disulfide covalent bonds. On the other hand, no changes in protein pattern were observed when aldehydes were incorporated at a low concentration (0.5 mM) (data not shown). Lee et al. [11] reported a mono-adduct of 4-hydroxynonenal, a reactive aldehyde, to tuna myoglobin by covalent modification that was expected to accelerate the oxidation of oxymyoglobin.

## Conclusions

The oxidation of oxymyoglobin or metmyoglobin from the dark muscle of Eastern little tuna mediated by  $H_2O_2$  resulted in the generation of ferrylmyoglobin.  $Fe^{2+}$  and/or  $H_2O_2$  could induce the conformational changes of globin without aggregation of myoglobin.  $H_2O_2$  might weaken the porphyrin ring, leading to release of non-heme iron from myoglobin. Hexanal and hexenal caused structural changes

of globin but had no effect on the release of non-heme iron. Hexenal had a greater impact on the formation of metmyoglobin, compared with hexanal, and induced the formation of covalent cross-links of myoglobin. Fenton's reactants and aldehyde could therefore accelerate the changes of fish myoglobin; however, it depended on its forms.

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## The effect of heating conditions on polyphenol oxidase, proteases and melanosis in pre-cooked Pacific white shrimp during refrigerated storage

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### ABSTRACT

Pre-cooked Pacific white shrimp (*Litopenaeus vannamei*) is an important shrimp product. However melanosis, especially in the cephalothorax including carapace and internal organs, is more likely caused by the remaining polyphenol oxidase (PPO) after pre-cooking. Thus, PPO from carapace and proteases from hepatopancreas of Pacific white shrimp were characterised and the remaining activities of both enzymes were monitored in pre-cooked shrimp during storage at 4 °C. Based on activity staining using L-β-(3,4 dihydroxyphenyl) alanine as a substrate, PPO consisted of two isoforms with apparent molecular weight of 210 and 220 kDa. No difference in activity band was observed when analysed under reducing and non-reducing condition. Proteases from hepatopancreas were able to activate PPO to some degree. For the *in vitro* study, both enzymes were quite stable when heated at temperature up to 70 °C but the loss in activities increased with increasing heating time (0–120 s). When Pacific white shrimp were pre-cooked to obtain different core temperatures (50–90 °C), different PPO and protease activities were retained. Higher core temperatures were associated with lower PPO and protease activities, but higher cooking loss. When the shrimp were pre-cooked at 80 °C, the residual PPO and protease activities were 3.9% and 5.4%, respectively and cooking yield of 95.6% was obtained. The resulting pre-cooked shrimp possessed lower melanosis score during 7 days of storage at 4 °C. Thus, pre-cooking of shrimp to obtain a core temperature of 80 °C, with a holding time of 30 s, could prevent the severe cooking loss and lower melanosis during subsequent storage.

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### 1. Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is nowadays the most important shrimp for aquaculture, replacing *Penaeus monodon* and *Penaeus chinensis* (FAO, 2009). After capture, fresh shrimp are easily perishable, leading to quality loss. The loss in quality is associated with microbial spoilage, chemical changes and melanosis (blackening). Melanosis is a natural post-mortem biochemical process in crustacean caused by polyphenol oxidase (PPO). PPO induces the oxidation of phenolic substrates to quinones, which undergo autooxidation and polymerisation to form high-molecular-weight dark pigments, melanin (Kim, Marshall, & Wei, 2000). PPO from some crustacean has been known to be activated by proteases (Martínez-Álvarez, López-Caballero, Montero, & Gómez-Guillén, 2005).

Nowadays, a variety of shrimp and shrimp products have been available in the market. Among all products, pre-cooked shrimp have become popular due to its reddish colour, which is desirable

for consumers (Martínez-Álvarez, López-Caballero, Gómez-Guillén, & Montero, 2009). Apart from increasing the colour attraction of shrimp products, pre-cooking is used to extend the shelf-life. Pre-cooking can inactivate some enzymes associated with quality loss and destroy microorganisms, thereby providing stable products. Pre-cooking of shrimp at boiling temperature for 2 min was enough to achieve deactivation of PPO (Martínez-Álvarez et al., 2009). Additionally, Martínez-Álvarez et al. (2009) reported that shrimp packed under vacuum before cooking had lower melanosis during the chilled storage. For shrimp pre-cooked using steam-oven or traditional cooking, negligible melanosis was obtained when shrimp were previously sprayed with 4-hexylresorcinol based formula. Generally, pre-cooked shrimp products are frozen and distributed worldwide. However, pre-cooked shrimp are still facing melanosis, more likely due to the remaining PPO, which can be the major cause of this problem. Melanosis in pre-cooked shrimp takes place during subsequent handling and storage under the refrigerated condition in retail shops. Melanosis initially occurs at carapace and spreads to other portions. This discolouration leads to quality losses of pre-cooked shrimp. Pre-cooking at sufficiently high temperature might be the means to prevent melanosis by full

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inactivation of PPO. Also sufficient heating can inactivate proteases, mainly localised in the cephalothorax, which can induce PPO activity. Nevertheless, such a harsh condition can lower the yield of pre-cooked shrimp. So far, a little amount of information regarding melanosis in pre-cooked shrimp and the impact of pre-cooking condition on melanosis and yield has been reported. Thus, the aims of this investigation were to study the effect of heating conditions on PPO, proteases and yield, as well as to monitor melanosis in pre-cooked Pacific white shrimp during refrigerated storage.

## 2. Materials and methods

### 2.1. Chemicals

L- $\beta$ -(3,4 dihydroxyphenyl) alanine (L-DOPA), L-tyrosine, Brij-35 and casein were purchased from Sigma–Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA) was obtained from Riedel–de-Haen (Seelze, Germany). Ammonium sulphate,  $\beta$ -mercaptoethanol ( $\beta$ ME), Tris (hydroxymethyl) aminomethane, trichloroacetic acid and sodium chloride were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS), *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) and all chemicals for electrophoresis were procured from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals used were of analytical grade.

### 2.2. Sample collection and preparation

Pacific white shrimp (*L. vannamei*) with the size of 55–60 shrimp/kg were purchased from the dock in Songkhla province, Thailand. The shrimp were kept in ice with a shrimp/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon arrival, shrimp were washed with tap water and stored in ice until used (not more than 2 h).

### 2.3. Preparation of crude PPO and crude protease extracts of Pacific white shrimp

Cephalothoraxes of white shrimp, the source of PPO and proteases, were manually separated from the whole shrimp. Additionally, carapace and hepatopancreas were also collected separately. Whole cephalothoraxes, carapace and hepatopancreas were powdered with liquid nitrogen. The powder obtained was used for extraction of PPO and proteases.

To extract PPO, powder either from whole cephalothoraxes or carapace (50 g) was mixed with 150 ml of 0.05 M sodium phosphate buffer, pH 7.2 containing 1.0 M NaCl and 0.2% Brij 35 according to the method of Simpson, Marshall, and Otwell (1987) with a slight modification. The mixture was stirred continuously at 4 °C for 30 min, followed by centrifugation at 8000g at 4 °C for 30 min using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). Solid ammonium sulphate was added into the supernatant to obtain 40% saturation. The mixture was allowed to stand at 4 °C for 30 min and then centrifuged at a speed of 12,500g at 4 °C for 30 min. The obtained pellet was collected and dispersed in a minimum volume of 0.05 M sodium phosphate buffer, pH 7.2. The pellet solution was dialysed against 15 volumes of the same buffer at 4 °C with three changes overnight. After dialysis, insoluble materials in the dialysate were removed by centrifugation at 3000g at 4 °C for 30 min and the supernatant was named 'crude PPO extract'.

For protease extraction, the powder either from whole cephalothoraxes or hepatopancreas (20 g) was mixed with two volumes of 0.01 M sodium phosphate buffer (pH 7.6) (Brauer, Leyva, Alvarado,

& Sández, 2003). The mixture was homogenised for 5 min using a homogeniser (IKA labortechnik, Selangor, Malaysia) at a speed of 11,000 rpm. The homogenate was stirred for 30 min at 4 °C, followed by centrifugation at 10,000g for 30 min at 4 °C using a refrigerated centrifuge. The supernatant was named 'crude protease extract' (CPE).

### 2.4. Determination of PPO and protease activities

The PPO activity was determined by monitoring the rate of dopachrome formation using L-DOPA as a substrate according to the method of Nirmal and Benjakul (2010) with a slight modification. Crude PPO extract (100  $\mu$ l) was mixed with 100  $\mu$ l of distilled water and 400  $\mu$ l of 0.05 M sodium phosphate buffer (pH 6.0). The assay mixture was added to 600  $\mu$ l of 15 mM L-DOPA, and the solution incubated at 45 °C to initiate the reaction. The reaction was run for 3 min at 45 °C and the formation of dopachrome was monitored by measuring the absorbance at 475 nm ( $A_{475}$ ) using a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). One unit of PPO activity was defined as the enzyme causing an increase in the absorbance by 0.001 per min. Enzyme and substrate blanks were prepared by excluding the substrate and enzyme, respectively, from the reaction mixture, and distilled water was used instead. Activity was expressed as units/g powder.

The proteolytic activity of CPE was determined using casein as substrate according to the method of An, Seymour, Wu, and Morrissey (1994). To the preincubated reaction mixture containing 2 mg of casein, 200  $\mu$ l of distilled water and 625  $\mu$ l of reaction buffer (0.05 M Tris–HCl buffer, pH 8.0), CPE (200  $\mu$ l) was added to initiate the reaction. The mixture was incubated at 70 °C. After 10 min, the reaction was terminated by adding 200  $\mu$ l of 50% (w/v) trichloroacetic acid (TCA). Unhydrolysed protein substrate was allowed to precipitate for 15 min at 4 °C and centrifuged at 8500g for 10 min. The oligopeptide content in the supernatant was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) using tyrosine as a standard. One unit of activity was defined as that releasing 1 mmol of tyrosine/min/ml. A blank was run in the same manner, except that the enzyme was added after addition of 50% TCA (w/v). The activity was expressed as units/g powder.

### 2.5. Characterisation of PPO and proteases

#### 2.5.1. Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE) and activity staining of PPO crude extract

SDS–PAGE of PPO crude extract from carapace was carried out as per the Laemmli method (Laemmli, 1970). The extract was mixed with the sample buffer containing 1.5 M  $\beta$ ME at a ratio of 1:1 (v/v) without heating. The sample (15  $\mu$ l) was loaded onto the polyacrylamide gel made of 7.5% running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, one of the two identical gels was immersed in a sodium phosphate buffer (pH 6.0) containing 15 mM L-DOPA for 25 min at 25 °C. The PPO activity zone appeared as the dark band. Another gel was stained by 0.125% Coomassie Brilliant Blue R-250 and de-stained in 25% methanol and 10% acetic acid. Protein standards (Sigma Chemical Co., St. Louis, MO, USA) were used for the estimation of molecular weight of proteins.

#### 2.5.2. Effect of proteases on the activity of PPO

To the PPO crude extract from carapace (100  $\mu$ l), 100  $\mu$ l of CPE from hepatopancreas were added. The mixture was incubated at room temperature (25 °C) for 30 min before PPO activity assay. For the control, the assay was run in the same manner, except that deionised water was used instead of CPE.

### 2.5.3. Thermal stability of PPO from carapace and protease from hepatopancreas

The PPO crude extract from carapace and the CPE from hepatopancreas were subjected to heating at different temperatures (40, 50, 60, 70, 80 and 90 °C) for 0, 15, 30, 60 and 120 s. The extracts were cooled rapidly using iced water. The residual activities of PPO and protease in the extracts were then determined and expressed as the relative activity, compared to that found in sample without heating.

### 2.6. Effect of pre-cooking temperatures on melanosis, enzyme activity, cooking loss and cooking yield

Whole Pacific white shrimp (head-on) was pre-cooked by submerging the samples in boiling water (100 °C) until the core temperature of the second segment of shrimp reached 50, 55, 60, 65, 70, 75, 80, 85 and 90 °C. To measure the core temperature, the thermo-couple (Union, Kowloon, Hong Kong) was inserted into the middle of the second segment of abdomen. The samples were cooled rapidly in iced water for 1 min and then the shrimp samples were drained for 5 min at 4 °C. The samples were referred to as 'pre-cooked shrimp'. All samples were subjected to measurement of PPO and protease activities, melanosis assessment, cooking yield and cooking loss.

#### 2.6.1. Determination of cooking yield and cooking loss

Cooking yield and cooking loss were measured by weighing the shrimp before and after pre-cooking. Shrimp were pre-cooked to obtain the designated core temperatures, immediately cooled in iced water for 1 min and drained at 4 °C for 5 min. The 'pre-cooked shrimp' were weighed. Cooking yield and cooking loss were calculated using the following equations:

$$\text{Cooking yield (\%)} = (B/A) \times 100$$

$$\text{Cooking loss (\%)} = ((A-B)/A) \times 100$$

where A is the weight before pre-cooking and B is the weight after pre-cooking, followed by cooling in iced water.

#### 2.6.2. Determination of melanosis

To monitor melanosis in the samples, 14 shrimp were placed on a polystyrene tray (8 × 8 in<sup>2</sup>.) and covered with shrink film. Thereafter, samples were stored at 4 °C for 7 days. The samples were taken every day for melanosis assessment. Melanosis or blackening of pre-cooked shrimp was evaluated through visual inspection by ten trained panelists using 10-point scoring test following the method of Montero, Lopez-Caballero, and Perez-Mateos (2001) with some modifications. Shrimp stored at 4 °C for 0, 2, 4, 6, 8 and 10 days were cooked to obtain the core temperature of 60 °C for 1 min. Those samples were used as the references with melanosis score of 0, 2, 4, 6, 8 and 10, respectively. Training was conducted for two sessions. Panelists were asked to give the melanosis score (0–10), where 0 = complete absent of black spots; 2 = slight (up to 20% of shrimp's surface affected); 4 = moderate (20–40% of shrimp's surface affected); 6 = notable (40–60% of shrimp's surface affected); 8 = severe (60–80% of shrimp's surface affected); 10 = extremely heavy (80–100% of shrimp's surface affected).

### 2.7. Statistical analysis

The experiments were run in triplicate with three different lots of samples. Data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's multiple range test (DMRT) (Steel & Torrie, 1980). Analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

## 3. Results and discussion

### 3.1. Activity staining and the effect of protease from hepatopancreas on the activity of PPO from carapace

Activity staining of PPO from the carapace of Pacific white shrimp in the absence or presence of proteases from hepatopancreas determined under non-reducing and reducing conditions is shown in Fig. 1a. PPO from carapace consisted of two isoforms with the apparent molecular weight (MW) of 210 and 220 kDa (Fig. 1a). No differences in activity bands were observed under non-reducing and reducing conditions ( $p > 0.05$ ). The result suggested that the disulphide bond was not involved in stabilisation of PPO from Pacific white shrimp. When the activity band of PPO from whole cephalothorax was determined, only one activity band with MW of 210 kDa was found. Therefore, PPO localised in carapace and cephalothorax was different in terms of the isoforms presented. The PPO isoform, with a MW of 220 kDa, might be dominated by an isoform having a MW of 210 kDa, which was predominant in cephalothorax. Higher proportion of cephalothorax to carapace was associated with a dilution of the PPO isoform, with a MW of 220 kDa, by proteinaceous substances in cephalothorax. PPO was able to induce the oxidation of DOPA to DOPA-quinone and the intermediate products, which subsequently underwent polymerisation to melanin (Nirmal & Benjakul, 2010). Nirmal and Benjakul (2009, 2010) also found that the MW of PPO from the Pacific white

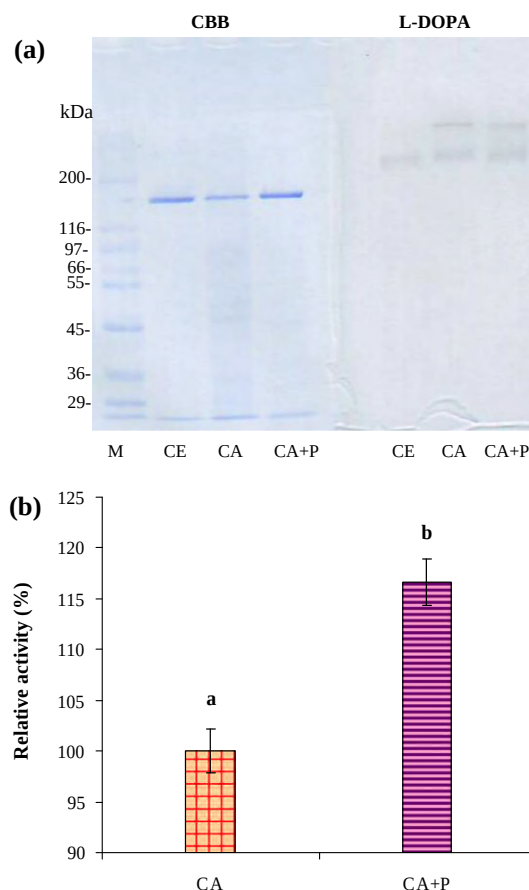


**Fig. 1.** Protein pattern and activity staining of PPO from Pacific white shrimp (a) and PPO activity as affected by proteases from hepatopancreas (b). M: Molecular-weight markers; CBB: gel stained with Coomassie Brilliant Blue; L-DOPA: gel stained with 15 mM L-DOPA in sodium phosphate buffer (pH 6.0). CE: Crude extracts of cephalothorax; CA: crude extracts of carapace; CA + P: crude extracts of carapace mixed with proteases from hepatopancreas (1:1).

shrimp cephalothorax was 210 kDa. The active PPO from deep water pink shrimp (*Parapenaeus longirostris*) had MW of 212 kDa (Zamorano, Martínez-Álvarez, Montero, & Gómez-Guillén, 2009). Benjakul, Visessanguan, and Tanaka (2005) reported that the PPO from the kuruma prawn cephalothoraxes had MW of 160 kDa. From the protein pattern of the crude extract from both carapace and whole cephalothorax, it was found that a protein with a MW of 180 kDa was predominant. Smaller MW of proteins/peptides were also observed in crude extracts. However, the major protein did not exhibit PPO activity.

Proteases from hepatopancreas were found to activate PPO as evidenced by increased PPO activity when proteases from hepatopancreas were incorporated (Fig. 1b). In the presence of proteases from hepatopancreas, the relative activity of 116% was obtained in comparison with the control (without proteases from hepatopancreas). Trypsin, which was the dominant protease in hepatopancreas, was able to activate the inactive form of PPO (pro-PPO) (Díaz-Tenorio, García-Carreño, & Pacheco-Aguilar, 2007).

### 3.2. Thermal stability of PPO from carapace and proteases from hepatopancreas

Thermal stability of PPO from carapace and proteases from hepatopancreas of Pacific white shrimp is shown in Fig. 2. PPO from carapace (Fig. 2a) was more likely stable up to 80 °C and the pronounced loss in activity was obtained at 90 °C. With increasing heating time, the greater loss in activity was found in the sample heated at 80 and 90 °C ( $p < 0.05$ ). When heating temperatures of 50–70 °C were used, only slight decrease in PPO activity was obtained. On the other hand, a slight increase in the PPO activity was observed when heated at 40 °C for a longer time. This

was possibly due to the enhanced PPO activity by proteases, which were active at that temperature. The result was in agreement with Giménez, Martínez-Alvarez, Montero, and Gómez-Guillén (2010) who found that PPO from carapace had the residual activity of 40% at high temperature (55–65 °C). Also, PPO still showed high activity at temperatures around 60–70 °C (Williams, Davidson, & Mamo, 2003; Zamorano et al., 2009). Approximately 12.8% of activity was retained after heating at 90 °C for 120 s. The remaining PPO in the shrimp after pre-cooking gave rise to the formation of a black spot (Williams et al., 2003). Therefore, PPO was quite heat stable and more likely played a role in melanosis during subsequent storage.

The thermal stability of crude proteases from the hepatopancreas of Pacific white shrimp is depicted in Fig. 2b. The activity of proteases from hepatopancreas decreased as heating temperatures increased ( $p < 0.05$ ). At temperature above 70 °C, a sharp decrease in activity was noticeable. This was mainly due to the thermal denaturation of proteases. In general, the loss in activity was slightly increased when heating time increased. However, a sharp decrease in proteolytic activity was obtained within 15 s when heated at 80 and 90 °C. Muhlia-Almazán and García-Carreño (2002) reported that trypsin and chymotrypsin are the most abundant proteolytic enzymes in the midgut gland of shrimp. Fish trypsin was stable at high temperature (Hau & Benjakul, 2006). Thus, trypsin in hepatopancreas that was retained after heating could be involved in the activation of PPO to some degree.

### 3.3. Effect of pre-cooking temperature on PPO, proteases, melanosis and property of pre-cooked shrimp

#### 3.3.1. Effect on PPO and protease activities

The effect of pre-cooking temperatures on PPO and protease activities of Pacific white shrimp cephalothoraxes is shown in Fig. 3. The activity of PPO in cephalothoraxes (Fig. 3a) sharply decreased after heating at 50 °C. The higher decrease in PPO activity was obtained as the pre-cooking temperature increased ( $p < 0.05$ ). It was noted that no PPO activity was detectable after being heated at 90 °C. However, the activity of PPO was still detectable in an *in vitro* study at 90 °C (Fig. 2). PPO from pre-cooked shrimp might not be extracted completely since some PPO were trapped with coagulated proteins, leading to lowered extractability. The result suggested that PPO more likely lost their activity due to the thermal denaturation. Unfolding of the native structure to a random coiled structure of enzyme leads to the loss in catalytic activity (Kim et al., 2000). The PPO activity in shrimp was dependent on different factors, such as species, method and season of capture, molting cycle, sex and proteases (Yan & Taylor, 1991). The protease activity of pre-cooked Pacific white shrimp is depicted in Fig. 3b. The proteolytic activity in cephalothoraxes continuously decreased with increasing pre-cooking temperatures. At a core temperature of less than 70 °C, proteases were moderately remained. At temperature above 70 °C, a marked decrease in proteolytic activity was observed. However, the remaining proteases might be able to activate PPO to some degree, thereby inducing melanosis in pre-cooked shrimp during storage. Arthropods PPO are present as zymogens (pro-PPOs) that are activated by a serine protease (Zamorano et al., 2009). The accessibility of the proteolytic enzymes to pro-PPOs is an important factor affecting melanosis in the shrimp tissue.

#### 3.3.2. Effect of pre-cooking temperature on melanosis during refrigerated storage

The melanosis score of pre-cooked Pacific white shrimp with different core temperatures during 7 days of refrigerated storage is shown in Fig. 4. At day 0, no melanosis was observed in all samples (score = 0). When the storage time increased, the melanosis

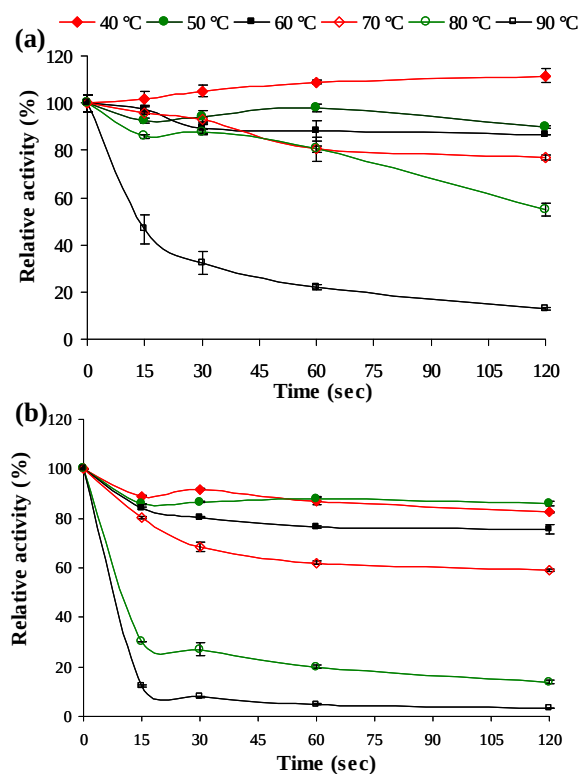


**Fig. 2.** Thermal stability of crude PPO from carapace (a) and crude protease (b) from hepatopancreas of Pacific white shrimp. For PPO, the residual activity was determined at 45 °C, pH 6.0 for 3 min using L-DOPA as a substrate. The residual activity of proteases was determined at 70 °C, pH 8.0 for 10 min using casein as a substrate. Bars represent the standard deviation ( $n = 3$ ).

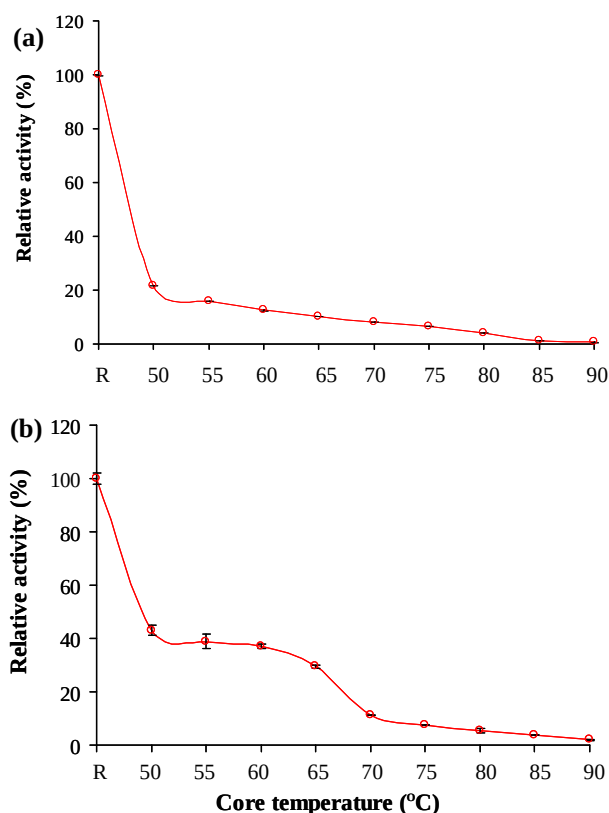


**Fig. 3.** Relative activities of PPO (a) and protease (b) in cephalothoraxes obtained from pre-cooked Pacific white shrimp with different core temperatures. R: Raw sample without pre-cooking. Bars represent the standard deviation ( $n = 3$ ).

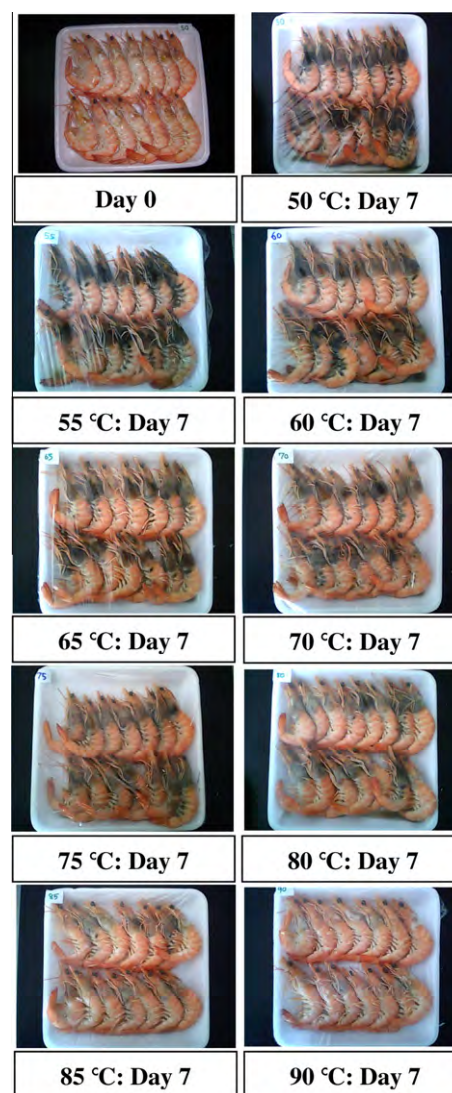


**Fig. 5.** Pre-cooked Pacific white shrimp with different core temperatures at day 0 and 7 of refrigerated storage.

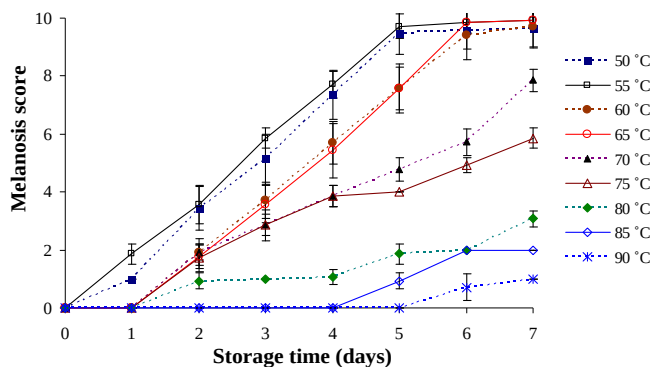


**Fig. 4.** Melanosis score of pre-cooked Pacific white shrimp with different core temperatures during 7 days of refrigerated storage. Bars represent the standard deviation ( $n = 3$ ).

score of all samples increased ( $p < 0.05$ ). However, no melanosis was noticeable in the shrimp with the core temperatures of 85 and 90 °C within the first 4 and 5 days of storage, respectively. Therefore, the melanosis was more retarded when the core temperatures of pre-cooked shrimp increased ( $p < 0.05$ ). The result suggested that PPO was likely inactivated at high temperature, due to thermal denaturation. Pre-cooked Pacific white shrimp with different core temperatures (50–90 °C) at day 0 and 7 of refrigerated storage are illustrated in Fig. 5. At day 0, no melanosis was found in all the samples, regardless of the core temperatures. After 7 days of storage, melanosis was developed in cephalothoraxes as well as pleopods. This phenomenon was caused by endogenous

PPO (Diaz-Tenorio et al., 2007). The highest melanosis was found in pre-cooked shrimp with the lowest core temperature, whilst the lower melanosis was detectable in shrimp pre-cooked at higher temperature. No melanosis took place in the sample with a core temperature of 90 °C. PPO, which played a role in melanosis, could be inactivated at high temperature, 90 °C.

### 3.3.3. Effect of pre-cooking temperature on cooking yield and cooking loss

The cooking yield and cooking loss of pre-cooked Pacific white shrimp with different core temperatures are shown in Fig. 6a and b, respectively. No differences in the cooking yield and cooking loss were found in the samples having core temperatures of 50–65 °C ( $p > 0.05$ ). The cooking loss of pre-cooked Pacific white shrimp increased when the core temperatures of heating increased ( $p < 0.05$ ). With increasing core temperatures, the amount of water retained in the shrimp meat decreased with a coincidental increase in the fat and protein contents (Benjakul, Visessanguan, Kijroongrojana, & Sriket, 2008). The water was mainly lost as a result of heat-induced denaturation of proteins during cooking of meat. This led to less water being entrapped within the protein structures (Aaslyng, Bejerholm, Ertbjerg, Bertram, & Anderson, 2003).

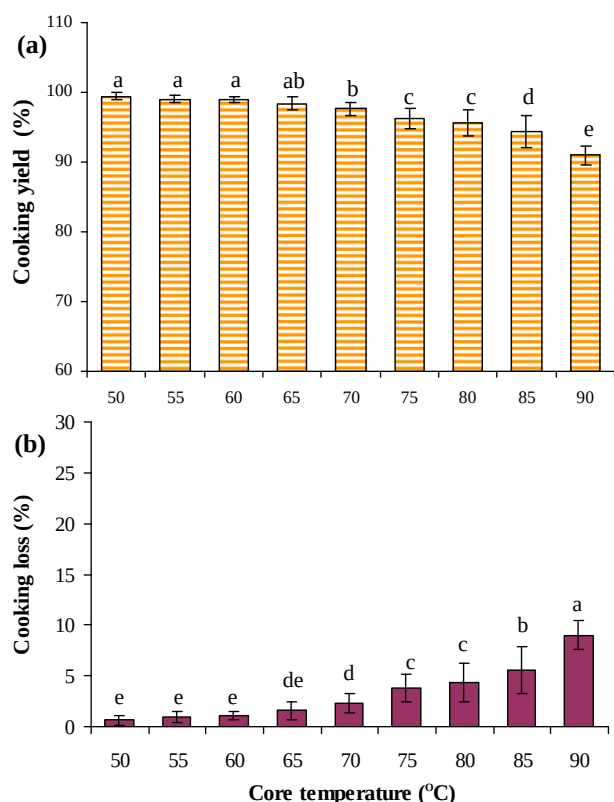


**Fig. 6.** Cooking yield (a) and cooking loss (b) of pre-cooked Pacific white shrimp with different core temperatures. Bars represent standard deviation ( $n = 3$ ). Different letters on the bars indicate significant differences ( $p < 0.05$ ).

Pre-cooking at 85 and 90 °C resulted in a higher cooking loss with a lower cooking yield ( $p < 0.05$ ). Therefore, the pre-cooking temperature directly affected the cooking loss and cooking yield of Pacific white shrimp. However, Martínez-Álvarez et al. (2009) reported that the weight loss of several cooked shrimp was not affected by the cooking method or by melanosis-inhibiting treatment. Even though high temperature could prevent melanosis effectively, it caused a detrimental effect on the cooking yield. Pre-cooking at 80 °C with a holding time of 30 s did not cause a severe cooking loss and it also retarded the melanosis during refrigerated storage more effectively than pre-cooking at 75 °C ( $p < 0.05$ ). This pre-cooking condition would be a promising means to lower melanosis and result in satisfactory cooking yield. In order to reduce the activity of PPO retained in pre-cooked shrimp, the use of antimelanosis agent in combination with pre-cooking should be further studied.

#### 4. Conclusions

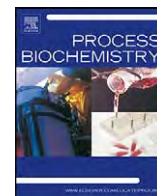
PPO, proteases and melanosis of pre-cooked Pacific white shrimp were affected by core temperatures after pre-cooking. PPO and proteases were quite stable when heated at temperature lower than 80 °C. The activities of both enzymes decreased with increasing pre-cooking time. Pre-cooked shrimp had increased cooking loss but lowered melanosis formation after 7 days of refrigerated storage when core temperature increased. Therefore, the appropriate pre-cooking condition should be implemented along with the use of antimelanosis agent to control melanosis in pre-cooked shrimp, while minimising the cooking loss.

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# Antioxidative and ACE inhibitory activities of protein hydrolysates from the muscle of brownstripe red snapper prepared using pyloric caeca and commercial proteases

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## ABSTRACT

Protein hydrolysates from the muscle of brownstripe red snapper (*Lutjanus vitta*) prepared using Alcalase or Flavourzyme as the first step with 40% degree of hydrolysis (DH), followed by hydrolysis with pyloric caeca protease (PCP) as the second step for 2 (HAP) and 1 h (HFP), respectively, were prepared and determined for their antioxidative and angiotensin I-converting enzyme (ACE) inhibitory activities. HAP had the higher DPPH and ABTS radical scavenging activity and ferric reducing antioxidant power (FRAP), while HFP showed the higher ferrous chelating activity and ACE inhibitory activity ( $p < 0.05$ ). Both HAP and HFP were able to retard lipid oxidations in lecithin–liposome and  $\beta$ -carotene–linoleic acid oxidation model systems in dose-dependent manner. HAP and HFP contained 87.36 and 86.55% protein (wet basis), respectively with glutamic acid/glutamine as the major amino acids, followed by aspartic acid/asparagine, lysine, alanine and leucine, respectively. HFP showed a slightly greater efficiency in prevention of lipid oxidation in all systems tested. Antioxidative activities, except DPPH radical scavenging activity, of both HAP and HFP after being subjected to gastrointestinal tract model system (GIMs) increased, suggesting the enhancement of antioxidative activities of both hydrolysates after ingestion.

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## 1. Introduction

Lipid oxidation is one of the major deteriorative processes in many types of foods, leading to the changes in food quality and nutritional value. Additionally, potentially toxic reaction products can be produced. Lipid oxidations have been known to be the major causes of many serious human diseases, such as cardiovascular disease, cancer, and neurological disorders as well as the aging process [1,2]. To prevent oxidative deterioration of foods and to provide protection against serious diseases, such as cancer and atherosclerosis [3,4], it is important to inhibit the oxidation of lipids and the formation of free radicals occurring in the foodstuff and living body. To tackle the problem, antioxidants, both natural and synthetic ones, have been used widely. Nevertheless, synthetic antioxidants have been suspected of being responsible for toxicity in the long term and their use in foodstuffs is restricted or prohibited in some countries [5–7]. Therefore, there is a growing interest on natural antioxidants, especially peptides derived from hydrolyzed food proteins. Protein hydrolysates from several fish species including round scad (*Decapterus maruadsi*) [8], yellow stripe trevally

(*Selaroides leptolepis*) [9], Pacific hake (*Merluccius productus*) [10], tilapia (*Oreochromis niloticus*) [11], silver carp (*Hypophthalmichthys molitrix*) [12] and smooth hound (*Mustelus mustelus*) [13] have been reported to possess antioxidative activities.

Hypertension has been considered as the most common serious chronic health problem [14]. Since angiotensin I-converting enzyme (ACE) (EC 3.4.15.1) is physiologically important in raising blood pressure, the inhibition of ACE activity can lead to an overall antihypertensive effect. The synthetic ACE inhibitors are now widely used as pro-drugs but these synthetic ACE inhibitors can cause many significant undesirable side effects [14,15]. Therefore, the natural safe compounds are desirable for prevention of hypertension instead of the synthetic counterpart. Among those, food protein derived peptides are promising natural products exhibiting ACE inhibitory activities. Protein hydrolysates with antihypertensive activity have also produced from sardinelle (*Sardina pilchardus*) by-products [16], tuna cooking juice [17], salmon protein [18] and tilapia [19].

In Thailand, brownstripe red snapper (*Lutjanus vitta*) is one of the raw materials for surimi production [20]. Besides being produced into surimi, this species and its viscera, especially pyloric caeca, can be used as raw material for production of protein hydrolysate and as the source of proteases, respectively. Production of fish protein hydrolysates with bioactivity can pave the way

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for full utilization of these species. Many factors affect the bioactivity of protein hydrolysates, e.g. type of proteases [9], steps of hydrolysis [21], etc.

This work aims to study antioxidative and ACE inhibitory activities of protein hydrolysate from brownstripe red snapper muscle prepared using its pyloric caeca protease in combination with commercial proteases via 2-step hydrolysis and to investigate the bioactivities of selected hydrolysate after digestion in gastrointestinal tract model system.

## 2. Materials and methods

### 2.1. Enzymes and chemicals

Alcalase 2.4 L (E.C. 3.4.21.62) (2.4 AU/g) and Flavourzyme 500 L (E.C. 3.4.21.77) (500 LPU/g) were provided by Novozyme (Bagsvaerd, Denmark). 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-trinitrobenzenesulfonic acid (TNBS), 1,1,3,3-tetramethoxypropane, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid (ferrozine), 2,4,6-triphenyl-1,3,5-triazine (TPTZ), L- $\alpha$ -phosphatidylcholine (lecithin) and linoleic acid were purchased from Sigma (St. Louis, MO, USA). Thiobarbituric acid (TBA), potassium persulfate,  $\beta$ -carotene and Tween 40 were obtained from Fluka (Buchs, Switzerland). Tris (hydroxymethyl) aminomethane (Tris-HCl) was procured from Merck (Darmstadt, Germany) and sodium sulfite was obtained from Riedel-deHaën (Seelze, Germany). All chemicals were of analytical grade.

### 2.2. Preparation of pretreated fish mince

Brownstripe red snapper, stored in ice and off-loaded approximately 24–36 h after capture, were purchased from a dock in Songkhla province, Thailand. Fish were transported in ice with the fish/ice ratio of 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, whole fish were washed and only flesh was separated manually. Flesh was minced to uniformity using Moulinex AY46 blender (Group SEB, Lyon, France) and phospholipid membrane was removed by further homogenizing with nine volumes of cold 8 mmol L<sup>-1</sup> CaCl<sub>2</sub> solution containing 5 mmol L<sup>-1</sup> citric acid [22] using an IKA Labortechnik homogenizer (Selangor, Malaysia) at a speed of 11000 rpm for 2 min. After continuous stirring for 60 min at 4 °C, the sample was centrifuged at 4000  $\times$  g for 15 min at 4 °C using a Beckman Coulter centrifuge Model Avant J-E (Beckman Coulter, Inc., Fullerton, CA, USA). Thereafter, the pellet was washed by homogenizing with five volumes of cold distilled water using a homogenizer at a speed of 11000 rpm for 2 min, followed by stirring at 4 °C for 15 min prior to centrifuging at 9600  $\times$  g for 10 min at 4 °C. The washing process was repeated twice. Pretreated mince obtained was kept in polyethylene bags and placed in ice until use, but not longer than 2 h.

### 2.3. Preparation of proteases from pyloric caeca

Pyloric caeca from brownstripe red snapper was collected and powdered in liquid nitrogen. Thereafter, the pyloric caeca extract was prepared according to the method of Khantaphant and Benjakul [20]. Pyloric caeca powder was suspended in ten volumes of extraction buffer (50 mmol L<sup>-1</sup> Tris-HCl buffer, pH 8.0, containing 10 mmol L<sup>-1</sup> CaCl<sub>2</sub>). The mixture was homogenized at 11000 rpm for 2 min. The homogenate was continuously stirred for 30 min at 4 °C and centrifuged at 8000  $\times$  g for 30 min at 4 °C. The supernatant was filtered through a Whatman filter paper No. 1 (Schleicher & Schuell, Maidstone, England). The filtrate obtained was further subjected to 40–60% saturation ammonium sulfate precipitation. After stirring at 4 °C for 30 min, the mixture was centrifuged at 8000  $\times$  g for 30 min at 4 °C and the pellet obtained was dissolved in 50 mmol L<sup>-1</sup> Tris-HCl buffer, pH 8.0 followed by dialysis against 20 volumes of the extraction buffer overnight at 4 °C with three changes of dialysis buffer. The dialysate was kept in ice and referred to as 'pyloric caeca protease; PCP'. The proteolytic activity of PCP was determined using casein as a substrate under optimal condition (60 °C and pH 8.5)

### 2.4. Preparation of protein hydrolysate from brownstripe red snapper

#### 2.4.1. One-step hydrolysis using different single proteases

Pretreated brownstripe red snapper mince with a protein content of 95.42% protein (dry basis) determined by Kjeldahl method [23] was mixed with 50 mmol L<sup>-1</sup> Tris-HCl buffer with pH of 7.0, 8.0 and 8.5 for hydrolysis by Flavourzyme, Alcalase and PCP, respectively, to obtain a final protein concentration of 20 g protein L<sup>-1</sup>. The mixtures were homogenized at a speed of 11,000 rpm for 1 min and the pH was rechecked and readjusted using 1 mol L<sup>-1</sup> NaOH or 1 mol L<sup>-1</sup> HCl. The homogenates were pre-incubated for 10 min at 50 °C for Alcalase and Flavourzyme and at 60 °C for PCP [20]. To start the hydrolysis, the different levels of enzymes were added into the mixture to obtain the desirable degree of hydrolysis (DH) of 20, 30 and 40% following the method of Benjakul and Morrissey [24]. After 2 h of hydrolysis, the reactions were inactivated by placing the mixture in boiling water for 10 min. Thereafter, the mixture was centrifuged at 2000  $\times$  g at 4 °C for 10 min. The super-

natant was collected and referred to as protein hydrolysate prepared using Alcalase (HA), Flavourzyme (HF) or PCP (HP). All protein hydrolysates were determined for antioxidative activities.

#### 2.4.2. Two-step hydrolysis using different proteases

After 2 h of the first hydrolysis, the mixtures with DH of 40%, which had the highest antioxidative activities, were heated for 10 min in boiling water and adjusted to the desirable pH using 1 mol L<sup>-1</sup> NaOH or 1 mol L<sup>-1</sup> HCl for proteases used for the second step. Those proteases included Alcalase, Flavourzyme and PCP. The same amount of proteases used in the first step was added into the pre-incubated mixture with optimal temperature of each protease. Reaction was conducted for 1, 2, 3 and 5 h. At the time designated, the reaction was terminated by submerging the mixture in boiling water for 10 min. The mixture was then centrifuged at 2000  $\times$  g at 4 °C for 10 min and the supernatant was collected and adjusted to neutral pH. The neutral solution was referred to as 'protein hydrolysates' and lyophilized to obtain hydrolysate powder. The hydrolysates obtained from HP with further hydrolysis using Alcalase (HPA) or Flavourzyme (HPF), hydrolysate from HA using PCP or Flavourzyme for the second step (HAP and HAF) and hydrolysate from HF using PCP or Alcalase for the second step (HFP and HFA) were determined for their antioxidative and ACE inhibitory activities.

### 2.5. Determination of antioxidative activities

#### 2.5.1. DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to the method of Blois [25] with a slight modification. Sample solution (1.5 mL) was added with 1.5 mL of 0.1 mmol L<sup>-1</sup> DPPH in 950 mL L<sup>-1</sup> ethanol. The mixture was allowed to stand for 30 min in dark at room temperature. The resulting solution was measured at 517 nm. The control was prepared in the same manner except that distilled water was used instead of the sample. The DPPH radical scavenging activity was calculated from Trolox standard curve (0–60  $\mu$ mol L<sup>-1</sup>) and expressed as  $\mu$ mol Trolox equivalents (TE) g<sup>-1</sup> protein.

#### 2.5.2. ABTS radical scavenging activity

ABTS radical scavenging activity was determined as described by Re et al. [26]. ABTS radical (ABTS\*) was produced by reacting ABTS stock solution (7.4 mmol L<sup>-1</sup> ABTS) with 2.6 mmol L<sup>-1</sup> potassium persulfate at the ratio of 1:1 (v/v). The mixture was allowed to react in the dark for 12 h at room temperature. Prior to assay, the ABTS\* solution was diluted with methanol to obtain an absorbance of 1.1 ( $\pm$ 0.02) at 734 nm. To initiate the reaction, 150  $\mu$ L of sample was mixed with 2.85 mL of ABTS\* solution. The mixture was incubated at room temperature for 2 h in dark. The absorbance was then read at 734 nm. A Trolox standard curve (0–200  $\mu$ mol L<sup>-1</sup>) was prepared. Distilled water was used instead of the sample and prepared in the same manner to obtain the control. ABTS radical scavenging activity was expressed as  $\mu$ mol TE g<sup>-1</sup> protein.

#### 2.5.3. Ferric reducing antioxidant power (FRAP)

The ability of samples to reduce ferric ion (Fe<sup>3+</sup>) was evaluated by the method of Benzie and Strain [27]. FRAP reagent (a freshly prepared mixture of 10 mmol L<sup>-1</sup> TPTZ solution in 40 mmol L<sup>-1</sup> HCl, 20 mmol L<sup>-1</sup> FeCl<sub>3</sub>.6H<sub>2</sub>O solution and 300 mmol L<sup>-1</sup> acetate buffer, pH 3.6 (1:1:10, v/v/v)) (2.85 mL) was incubated at 37 °C for 30 min prior to mixing with 150  $\mu$ L of sample. The reaction mixture was allowed to stand in dark for 30 min at room temperature. Absorbance at 593 nm was read and FRAP was calculated from the Trolox standard curve (0–60  $\mu$ mol L<sup>-1</sup>) and expressed as  $\mu$ mol TE g<sup>-1</sup> protein. The control was prepared in the same manner except that distilled water was used instead of the sample.

#### 2.5.4. Ferrous chelating activity

Chelating activity of samples towards ferrous ion (Fe<sup>2+</sup>) was measured by the method of Benjakul et al. [28] with a slight modification. Sample (200  $\mu$ L) was mixed with 800  $\mu$ L of distilled water. Thereafter, 0.1 mL of 2 mmol L<sup>-1</sup> FeCl<sub>2</sub> and 0.2 mL of 5 mmol L<sup>-1</sup> ferrozine were added. The mixture was allowed to react for 20 min at room temperature. The absorbance was then read at 562 nm. The standard curve of EDTA (0–1.0 mmol L<sup>-1</sup>) was prepared. The control was prepared in the same manner except that distilled water was used instead of the sample. Ferrous chelating activity was expressed as  $\mu$ mol EDTA equivalents g<sup>-1</sup> protein.

### 2.6. Determination of ACE inhibitory activity

The angiotensin I-converting enzyme inhibitory activity was determined as described by Hayakari et al. [29] with slight modifications. Sample (0.3 mL) was incubated with 725 unit ACE L<sup>-1</sup> (50  $\mu$ L) at 37 °C for 5 min. Thereafter, the incubated mixture was added into the assay mixture (0.2 mL) which was 100 mmol L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub> buffer (pH 8.3) containing 600 mmol L<sup>-1</sup> NaCl and 3 mmol L<sup>-1</sup> hippuryl-L-histidyl-L-leucine (HHL). The mixture was incubated at 37 °C for 15 min. To terminate the reaction, 1.5 mL of 30 g L<sup>-1</sup> 2,4,6-trichloro-1,3,5-triazine (dissolved in dioxane) and 3 mL of 0.2 mol L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub> buffer (pH 8.3) were added and mixed thoroughly. The mixture was left for 10 min until the solution was clear and the absorbance at 382 nm was determined. Sample blank was prepared in the same manner except that HHL was added after the reaction mixture was terminated. The control was prepared

by using distilled water instead of the sample, whereas the control blank was done in the same manner with the control but HHL was added after the termination. % inhibition of ACE was determined using the following equation:

$$\% \text{ Inhibition} = \frac{A_c - A_s}{A_c} \times 100$$

where  $A_c = A_{\text{control}} - A_{\text{control blank}}$  and  $A_s = A_{\text{sample}} - A_{\text{sample blank}}$ .

## 2.7. Determination of antioxidative activity in different model systems

### 2.7.1. $\beta$ -Carotene linoleic acid emulsion model system

The antioxidative activity of the sample in the  $\beta$ -carotene linoleic acid emulsion model system was determined as described by Binsan et al. [30].  $\beta$ -Carotene (1 mg) was dissolved in 10 mL of chloroform. Thereafter, the solution (3 mL) was added to 20 mg linoleic acid and 200 mg Tween 40. Chloroform was then removed by purging with nitrogen. Fifty milliliters of oxygenated distilled water were added to the  $\beta$ -carotene emulsion and mixed well. Hydrolysate (200  $\mu$ L) was then mixed with 3 mL of oxygenated  $\beta$ -carotene emulsion to obtain the final concentrations of 500 and 1000 ppm. The oxidation of  $\beta$ -carotene emulsion was monitored spectrophotometrically at 470 nm after 0, 10, 20, 30, 40, 60, 90 and 120 min of incubation at 50 °C in dark. BHT at levels of 100 ppm was also used. The control was prepared by using distilled water instead of hydrolysate in the assay system.

### 2.7.2. Lecithin liposome model system

The antioxidative activity of protein hydrolysates in a lecithin liposome system was determined according to the method of Frankel et al. [31] slightly modified by Thiansilakul et al. [8]. Lecithin liposome system was prepared by suspending lecithin in deionized water at a concentration of 8 g L<sup>-1</sup>. The mixture was stirred with a glass rod followed by sonification for 30 min in a sonicating bath (Elma Model S30H, Singen, Germany). Protein hydrolysate (3 mL) was added to the lecithin liposome system (15 mL) to obtain a final concentration of 1000 ppm. The mixture was sonicated for 2 min. To initiate the reaction, 20 mL of 0.15 mol L<sup>-1</sup> cupric acetate were added. The mixture was shaken in the dark at 120 rpm using a shaker (Heidolph Model Unimax 1010, Schwabach, Germany) at 37 °C. The systems containing 25 or 100 ppm BHT were also prepared. The control was prepared in the same manner, except that distilled water was used instead of sample. Oxidation in lecithin liposome systems was monitored at 6 h intervals by determining the formation of TBARS and conjugated dienes.

## 2.8. Determination of thiobarbituric acid reactive substances (TBARS)

Thiobarbituric acid reactive substances (TBARS) were determined as described by Buege and Aust [32] with a slight modification. Sample was homogenized with TBARS solution (3.75 g L<sup>-1</sup> TBA, 150 g L<sup>-1</sup> TCA and 0.25 mol L<sup>-1</sup> HCl) with a ratio of 1:4 (w/v). The mixture was heated in boiling water for 10 min to develop the pink color. Then the mixture was cooled with running water and centrifuged at 5000  $\times$  g for 10 min at room temperature using Hettich centrifuge (Hettich Model MIKRO-20, Tuttlingen, Germany). The supernatant was collected and measured at 532 nm using a UV-1800 Spectrophotometer (Shimadzu, Kyoto, Japan). TBARS was calculated from a standard curve of malonaldehyde (MDA) (0–10 mg L<sup>-1</sup>) and expressed as mg MDA kg<sup>-1</sup> sample.

## 2.9. Determination of conjugated diene

Conjugated diene formed in the sample was measured according to the method of Frankel et al. [31]. Sample (0.1 mL) was dissolved in methanol (5.0 mL) and conjugated dienes were measured as the increase in absorbance at 234 nm.

## 2.10. Preparation of gastrointestinal tract model system (GIMs)

Gastrointestinal tract model system was prepared according to the method of Lo et al. [33] with slight modification. Hydrolysate powder was dissolved in distilled water to obtain a concentration of 50 g protein L<sup>-1</sup>. The solution was adjusted to pH 2.0 with 1 mol L<sup>-1</sup> HCl and pepsin dissolved in 0.1 mol L<sup>-1</sup> HCl was added to obtain the final concentration of 40 g pepsin kg<sup>-1</sup> protein. The mixture was incubated at 37 °C for 1 h with continuous shaking (Memmert Model SV 1422, Schwabach, Germany). Thereafter, the pH of reaction mixture was raised to 5.3 with 1 mol L<sup>-1</sup> NaOH before adding 20 g pancreatin kg<sup>-1</sup> protein and the pH of mixture was adjusted to 7.5 with 1 mol L<sup>-1</sup> NaOH. The mixture was incubated at 37 °C for 3 h with continuous shaking. The digestion was terminated by submerging the mixture in boiling water for 10 min. During digestion, the mixture was randomly taken and determined for antioxidative activities at 0, 20, 40, 60, 80, 100, 120, 150, 180, 210 and 240 min.

### 2.11. Proximate analysis

HAP and HFP were determined for protein, fat, ash and moisture contents according to the methods of AOAC [23] with the analytical number of 992.15, 991.36, 942.05 and 950.46, respectively.

## 2.12. Amino acid analysis

HAP and HFP were hydrolyzed under reduced pressure in 4.0 M methanesulfonic acid containing 0.2% (v/v) 3-(2-aminoethyl) indole at 115 °C for 24 h. The hydrolysates were neutralized with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.4 mL was applied to an amino acid analyzer (MLC-703; Atto Co., Tokyo, Japan).

## 2.13. Determination of protein concentration

Protein concentration was measured by the method of Lowry et al. [34] using bovine serum albumin as a standard.

## 2.14. Statistical analysis

Experiments were run in triplicate. All data were subjected to analysis of variance (ANOVA) and differences between means were evaluated by Duncan's Multiple Range Test. For pair comparison, T-test was used [35]. SPSS Statistic Program (Version 10.0) (SPSS Inc, Chicago, IL, USA) was used for data analysis.

# 3. Results and discussion

## 3.1. Antioxidative activities of brownstripe red snapper protein hydrolysates prepared with single step of hydrolysis using different proteases

Protein hydrolysates from brownstripe red snapper muscle prepared using protease from pyloric caeca of brownstripe red snapper, Alcalase and Flavourzyme referred to as HP, HA and HF, respectively, with different DH (20, 30 and 40% DH) showed varying antioxidative activities (Fig. 1).

### 3.1.1. DPPH radical scavenging activity

The DPPH radical scavenging activity of HA increased with increasing DH ( $p < 0.05$ ) (Fig. 1(a)). HF showed the similar activity at all DH used ( $p > 0.05$ ), whereas HP showed no further increase in activity when DH was higher than 30% ( $p > 0.05$ ). At all designated DH, HA and HF showed the higher activity than did HP ( $p < 0.05$ ). The result suggested that the peptides in different hydrolysates might be different in term of chain length and amino acid sequence, which contributed to varying capabilities of scavenging DPPH radicals. The increase in DPPH radical scavenging activity of HA was in agreement with Thiansilakul et al. [8] who reported the increases in DPPH radical scavenging activity as the DH of the hydrolysate from round scad muscle protein prepared using Flavourzyme and Alcalase increased. On the other hand, Klompong et al. [9] found that DPPH radical scavenging activity of protein hydrolysate prepared from the muscle of yellow stripe trevally using Flavourzyme and Alcalase decreased when DH increased. Invert correlation between DH and DPPH radical scavenging activity was obtained for protein hydrolysates prepared from alkaline-aided channel catfish protein isolates using Protamex [36]. You et al. [37] reported that loach protein hydrolysate showed the greater DPPH radical scavenging activity when DH increased. DPPH is a stable free radical and can be scavenged with a proton-donating substance, such as an antioxidant [25]. Therefore, protein hydrolysates from brownstripe red snapper muscle more likely contained peptides acting as hydrogen donors, thereby scavenging free radicals by converting them into more stable products.

### 3.1.2. ABTS radical scavenging activity

In general, ABTS radical scavenging activities of protein hydrolysates increased as DH increased ( $p < 0.05$ ) (Fig. 1(b)). The highest activity was observed in HP and HA with 40% DH ( $p < 0.05$ ). However, no difference in activity was found in HF with all DH used ( $p > 0.05$ ). Among all hydrolysates, HA had the lowest activity for all DH tested ( $p < 0.05$ ). At DH of 20%, HF showed higher ABTS radical scavenging activity than did HP ( $p < 0.05$ ). Conversely, HP

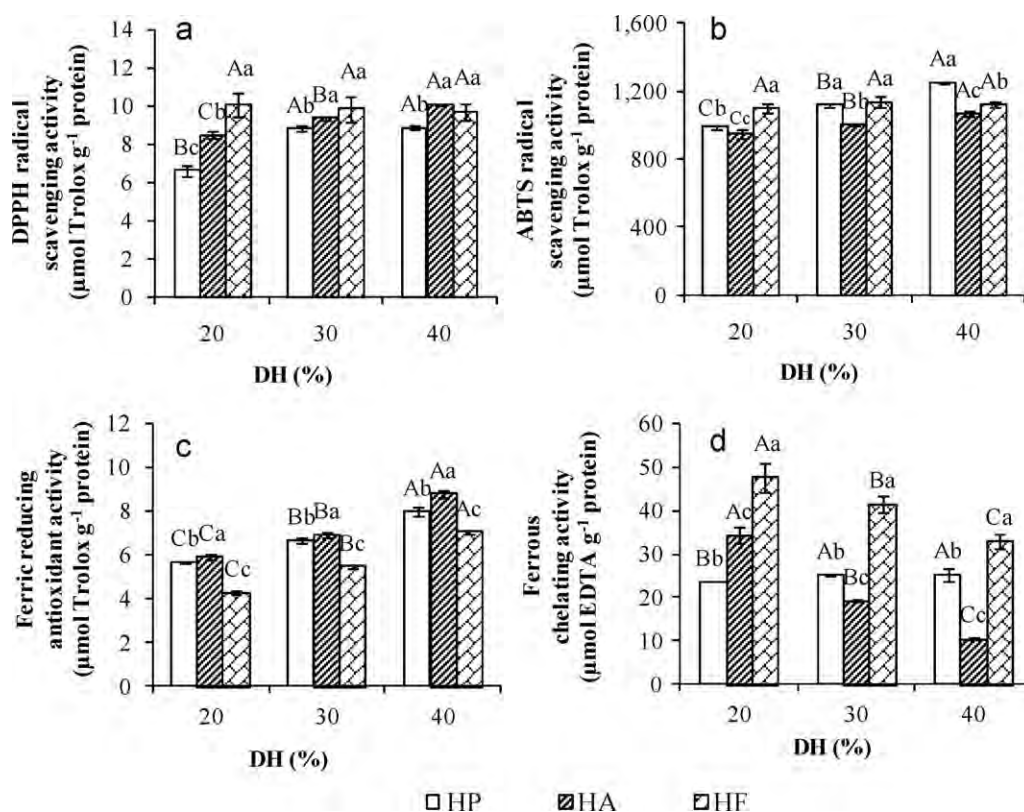


**Fig. 1.** Antioxidative activities of protein hydrolysate from brownstripe red snapper muscle prepared using pyloric caeca protease (PCP) from brownstripe red snapper (HP), Alcalase (HA) and Flavourzyme (HF) with different DHs. Bars represent the standard deviation ( $n=3$ ). Different capital letters within the same enzyme used indicate significant differences ( $p < 0.05$ ). Different letters within the same DH indicate significant differences ( $p < 0.05$ ).

had the highest activity when DH of 40% was used ( $p < 0.05$ ). Protein hydrolysate from alkali-solubilized tilapia protein prepared using various proteases showed a sharp increase in ABTS radical scavenging activity when DH increased from 18 to 23% [11]. Loach protein hydrolysate showed a similar result, in which ABTS radical scavenging activity increased with increasing DH [37].

For both DPPH and ABTS assays, HF showed no differences in activities with all DH tested ( $p > 0.05$ ). ABTS radical assay is used for determining the antioxidative activity, in which the radical is quenched to form ABTS-radical complex [26]. Generally, all hydrolysates contained peptides, which were able to scavenge ABTS radicals, leading to the termination of radical chain reaction.

### 3.1.3. Ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant power (FRAP) measures the reducing ability against ferric ion ( $\text{Fe}^{3+}$ ), indicating the ability of hydrolysates to transfer an electron to the free radical [27]. FRAP of different hydrolysates with varying DH is depicted in Fig. 1(c). An increase in FRAP was observed in all hydrolysates when DH increased ( $p < 0.05$ ). FRAP of HA was generally higher than those of HP and HF at all DHs tested ( $p < 0.05$ ). The result suggested that HA had the greater reducing power than did others, leading to the greater efficacy in prevention and retardation of propagation in lipid oxidation. However, protein hydrolysates prepared from alkaline-aided channel catfish protein isolates showed the decrease in reducing power with increasing DH [36]. Raghavan et al. [11] reported that alkaline-solubilized tilapia protein hydrolysate prepared using Flavourzyme showed the increase in reducing ferric ion when DH increased. The hydrolysis most likely increased the reducing power, especially when the cleavage of peptides increased

as indicated by the increase in DH. The result indicated that peptides generated from the hydrolysis by different proteases had the different capacities of providing electron to the radicals.

### 3.1.4. Ferrous chelating activity

Ferrous chelating activities of hydrolysates prepared using different proteases with different DHs are shown in Fig. 1(d). Chelating activity against  $\text{Fe}^{2+}$  of HP slightly increased when DH increased up to 30% ( $p < 0.05$ ). For HA and HF, the decreases in ferrous chelating activities were found as DH increased ( $p < 0.05$ ). Ferrous ion ( $\text{Fe}^{2+}$ ) is the most powerful pro-oxidant among metal ions [38], leading to the initiation and acceleration of lipid oxidation by interaction with hydrogen peroxide in a Fenton reaction to produce the reactive oxygen species, hydroxyl free radical ( $\text{OH}^\bullet$ ) [39]. Therefore chelation of metal ions by peptides in hydrolysates could retard the oxidative reaction. The result indicated that a higher DH rendered HA and HF with lower metal-chelating activities. The shorter chain of peptides might lose their ability to form the complex with  $\text{Fe}^{2+}$ . At DH of 20%, HF showed the highest chelating activity ( $p < 0.05$ ), followed by HA and HP, respectively. Peptides in HF could effectively chelate the  $\text{Fe}^{2+}$ , leading to the retardation of initiation stage. The result indicated that the limited hydrolysis of muscle protein resulted in the enhanced ferrous chelating activity, compared with the excessive hydrolysis. The higher chelating activity of HP was coincidental with the higher DPPH and ABTS radical scavenging activity and FRAP, as the DH increased.  $\text{Fe}^{2+}$  chelating activity of round scad protein hydrolysate prepared using Alcalase showed the increase in chelating activity with increasing DH, but those treated with Flavourzyme showed no difference in activity at all DH tested [8]. With the same enzymes used, chelating activity of protein hydrolysate prepared from the muscle of yellow stripe trevally

using Flavourzyme and Alcalase increased with increasing DH [9]. Higher ferrous chelating activity was reported for hydrolysate of silver carp using Alcalase and Flavourzyme when DH increased [12]. Apart from Fe, other transition metals, such as Cu and Co can affect the rate of lipid oxidation and decomposition of hydroperoxide. Theodore et al. [36] reported that  $\text{Cu}^{2+}$  chelating activity of catfish protein hydrolysate increased with increasing DH.

Some proteins and peptides can chelate metal ions like  $\text{Fe}^{2+}$  due to the presence of carboxyl and amino groups in the side chains of acidic and basic amino acids [10,40]. Alcalase is endopeptidase capable of hydrolyzing proteins with broad specificity for peptide bonds and prefers for the uncharged residue, whereas Flavourzyme is a mixture of endo- and exopeptidase enzyme, which can produce both amino acids and peptides [41]. Hydrolysates showing different antioxidative activities might be attributed to the differences in the exposed side chains of peptides as governed by the specificity of different proteases towards peptide bonds in the proteins [42]. DH also greatly influenced the peptide chain length. The higher DH, the more cleavage of peptide chains took place. Peptides with various sizes and compositions had different capacities of scavenging or quenching free radicals [8,9]. PCP, Alcalase and Flavourzyme more likely cleaved the peptide bonds in brownstripe red snapper muscle at different positions, resulting in the different products with varying antioxidative activities. With 40% DH, all hydrolysates functioned more effectively as primary antioxidant, whereas the secondary antioxidative activity, chelating ability, was lowered. To enhance the antioxidative activity of peptides, especially as the primary antioxidant, the hydrolysate with 40% DH was prepared for the first step of hydrolysis.

### 3.2. Antioxidative and ACE inhibitory activities of brownstripe red snapper protein hydrolysate prepared with two-step hydrolysis using different proteases

#### 3.2.1. DPPH radical scavenging activity

DPPH radical scavenging activity of protein hydrolysates with various hydrolysis times in the second step of hydrolysis using another protease is shown in Fig. 2(a). For HAF, HAP, HFA and HFP, the activities increased with increasing time up to 2 h ( $p < 0.05$ ). Thereafter, the gradual decrease was observed when hydrolysis times were 3 and 5 h ( $p < 0.05$ ). No changes in DPPH radical scavenging activity were observed for HPF, while HPA showed the maximal activity at 3 h of hydrolysis ( $p < 0.05$ ). The results suggested that scavenging activity against DPPH radical was enhanced by additional hydrolysis using another enzyme with an appropriate time. Generally, hydrolysates prepared by further hydrolyzing the hydrolysate obtained from the first step of hydrolysis with another protease for 2 h had a greater scavenging ability against DPPH radical.

#### 3.2.2. ABTS radical scavenging activity

ABTS radical scavenging activity of protein hydrolysates prepared by two-step hydrolysis is shown in Fig. 2(b). Only HAF and HAP showed the increases in ABTS radical scavenging activity, compared with their parent counterparts, HA. HAP with the hydrolysis time of 2 h for the second step had the highest activity ( $p < 0.05$ ). This suggested the enhancement of ABTS radical scavenging activity when PCP was used for further hydrolysis of HA. However, the further hydrolysis of HF with another protease had no impact on the increases in activity ( $p > 0.05$ ). For HP, the decrease in activities was obtained when the second step of hydrolysis was performed ( $p < 0.05$ ). The results suggested that the second step of hydrolysis could slightly increase ABTS radical scavenging activity, depending on the first hydrolysate as well as the types of protease used for the second step. It was noted that the abilities of scavenging ABTS and DPPH radicals by hydrolysates were different. This might be due to

the differences in ability of scavenging the different radicals, ABTS and DPPH, by the same peptide.

#### 3.2.3. Ferric reducing antioxidant power (FRAP)

Fig. 2(c) shows FRAP of protein hydrolysates prepared using two-step hydrolysis. The increases in FRAP were observed for all hydrolysates, when the second step of hydrolysis was applied. For HF and HP, when Alcalase was used for the second step of hydrolysis, the continuous increases in FRAP were observed ( $p < 0.05$ ). For other hydrolysates, the hydrolysis time was the factor affecting the FRAP of resulting hydrolysate, depending on the first hydrolysate and the types of protease used for the second step of hydrolysis. The increase in FRAP with increasing hydrolysis time was coincidental with the increase in DPPH radical scavenging activity (Fig. 2(a)). For HAF, HAP and HPF, the activities were increased with increasing hydrolysis time up to 2 h ( $p < 0.05$ ). For HFP, the hydrolysis time more than 1 h had the negative effect on FRAP ( $p < 0.05$ ). Generally, further hydrolysis with another protease led to the increase in FRAP, but the activities of resulting hydrolysate were governed by hydrolysis time.

#### 3.2.4. Ferrous chelating activity

Ferrous chelating activities of hydrolysates prepared by two-step hydrolysis are shown in Fig. 2(d). HFA and HPA showed the marked decrease in chelating activities when the second hydrolysis was conducted ( $p < 0.05$ ). Alcalase used in the second step might generate peptides with the lower ability in  $\text{Fe}^{2+}$  chelating. The abilities of HAF, HAP, HFP and HPF in chelating  $\text{Fe}^{2+}$  ion were more pronounced, compared with their parent hydrolysate counterparts. The increases were observed when a certain time of the second hydrolysis was used. Among all protein hydrolysates, HFP showed the highest ferrous chelating activity, especially when the second step hydrolysis time of 1 h was used ( $p < 0.05$ ).

#### 3.3. ACE inhibitory activity

The inhibitory effect of all hydrolysates prepared using two-step hydrolysis against angiotensin I-converting enzyme (ACE) was determined as shown in Fig. 2(e). For the first hydrolysate prepared using the different single proteases (0 h), HF showed the highest ACE inhibition (11.44%) ( $p < 0.05$ ). Flavourzyme might produce the peptides with ACE inhibitory activity. Raghavan and Kristinsson [19] used Cryotin and Flavourzyme for hydrolysis of tilapia protein and found the higher ACE inhibition by hydrolysate prepared using Flavourzyme. When the second step of hydrolysis using another protease was performed, HFA and HFP showed the higher ACE inhibition when hydrolysis time of 2 and 3 h was used, respectively. HAP also showed high ACE inhibitory activity. Wu et al. [43] reported that shark meat treated with enzyme showed the higher ACE inhibition than that of untreated one. ACE inhibition by tilapia protein hydrolysate was reported, especially with increasing DH [19]. Furthermore, smaller peptides are the better ACE inhibitors than the larger counterpart [19]. The hydrolysis might release ACE inhibitory peptides in hydrolysate [43]. Hydrolysates from muscle of different fish have been reported to possess ACE inhibitory activity, e.g. hydrolysates from tilapia protein [19], freshwater clam muscle [44], shark meat [43], Atlantic salmon, Coho salmon, Alaska pollack and southern blue whiting muscle [45].

From the results, the use of Alcalase for the first hydrolysis and the use of PCP in the second hydrolysis for 2 h (HAP) yielded the resulting hydrolysate with the higher DPPH and ABTS radical scavenging activities and FRAP, compared to other hydrolysates ( $p < 0.05$ ). However HF hydrolyzed with PCP in the second hydrolysis step for 1 h (HFP) showed much higher chelating activity, compared with others ( $p < 0.05$ ). Therefore, HFP and HAP prepared with 1 and 2 h for the second step of hydrolysis, respectively, were

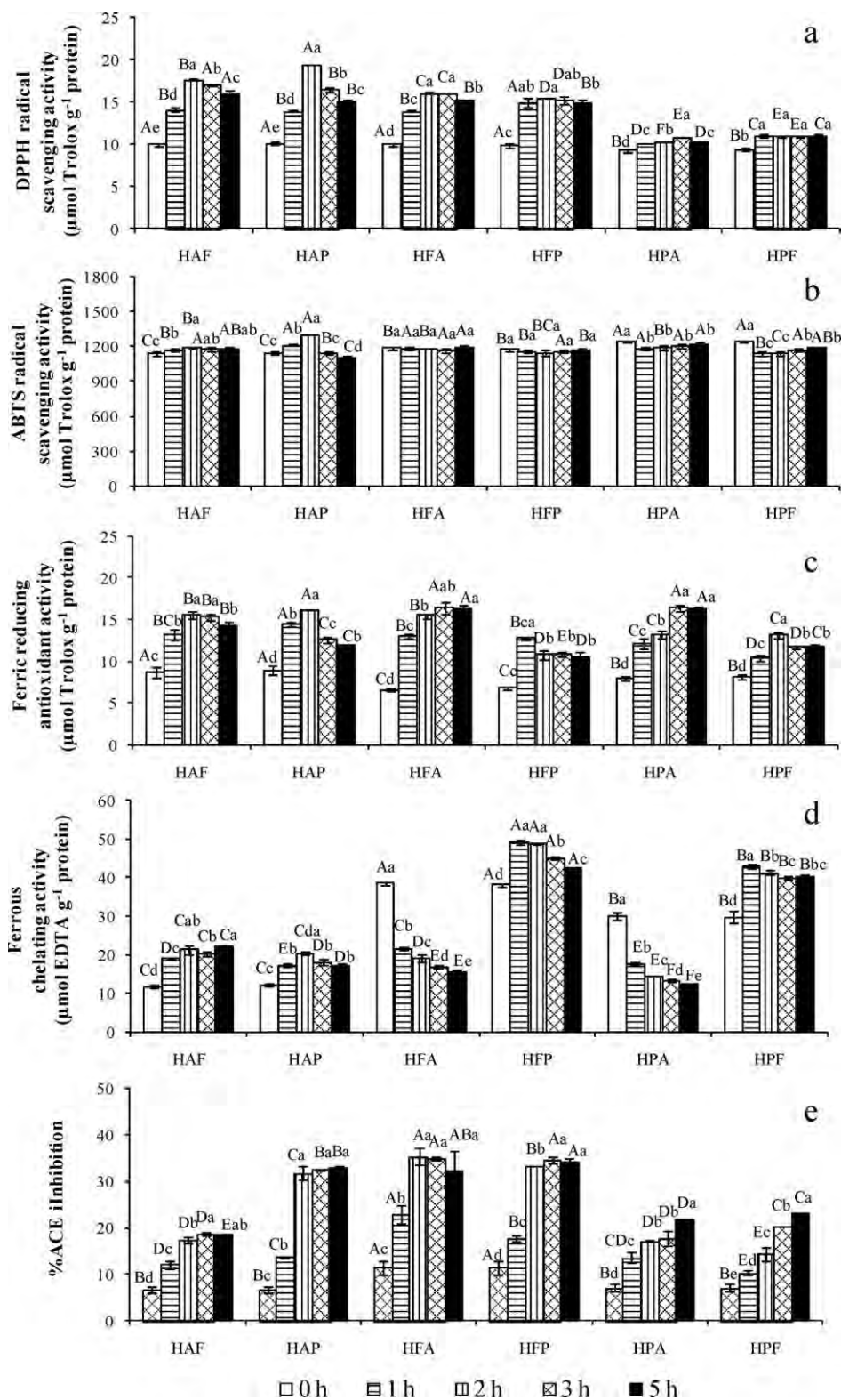


**Fig. 2.** Antioxidative activities (a–d) and ACE inhibitory activity (e) of protein hydrolysate from brownstripe red snapper muscle prepared by two-step hydrolysis process with different hydrolysis times in the second step of hydrolysis. HAF and HAP: HA hydrolyzed with Flavourzyme and PCP, respectively. HFA and HFP: HF hydrolyzed with Alcalase and PCP, respectively. HPA and HPF: HP hydrolyzed with Alcalase and Flavourzyme, respectively. Protein concentration used for ACE inhibitory assay was  $5 \text{ g L}^{-1}$ . Bars represent the standard deviation ( $n = 3$ ). Different capital letters within the same hydrolysis time indicate significant differences ( $p < 0.05$ ). Different letters within the same treatment indicate significant differences ( $p < 0.05$ ).

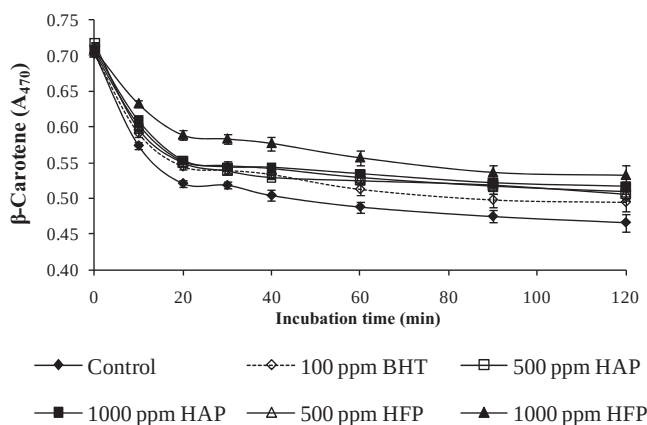


**Fig. 3.** Changes in  $A_{470}$  of  $\beta$ -carotene linoleic acid system in the absence or the presence of protein hydrolysate from brownstripe red snapper muscle at different levels. HAP: HA hydrolyzed with PCP. HFP: HF hydrolyzed with PCP. BHT is butylated hydroxytoluene. Bars represent the standard deviation ( $n=3$ ).

selected for further study. DH of selected protein hydrolysates, HAP and HFP, were 62.7 and 61.8%, respectively.

#### 3.4. Antioxidative activities of brownstripe red snapper protein hydrolysate in different oxidation model systems

##### 3.4.1. $\beta$ -Carotene linoleic acid emulsion model system

The antioxidative activity of selected protein hydrolysates, HAP and HFP, were studied in  $\beta$ -carotene linoleic acid oxidation model system as shown in Fig. 3. The decrease in  $A_{470}$  indicates the oxidation of  $\beta$ -carotene in the system caused by the oxidation process. When the oxidation of linoleic acid occurs, free radicals formed are able to attack the highly unsaturated  $\beta$ -carotene molecules. As a result,  $\beta$ -carotene is oxidized, leading to the losses in chromophore and characteristic orange color of  $\beta$ -carotene [30]. The presence of antioxidant in linoleic acid emulsion system hinders  $\beta$ -carotene bleaching, due to the chain-breaking inhibition of lipid peroxidation by neutralizing the linoleic free radical formed. Systems containing HAP and HFP at levels of 500 and 1000 ppm retarded the decrease in  $A_{470}$  up to 120 min ( $p < 0.05$ ). However, no difference in  $A_{470}$  was observed between the system containing 100 ppm BHT and those with 500 and 1000 ppm HAP and 500 ppm HFP within the first 40 min ( $p > 0.05$ ). This indicated that the preventive effect of hydrolysates (500 and 1000 ppm) was equivalent to that of 100 ppm BHT. Thereafter, the lower  $\beta$ -carotene bleaching in the systems containing these hydrolysates was observed, compared with the system added with 100 ppm BHT up to 120 min. The result indicated that in emulsion system, HAP and HFP could act as the better chain breaker than BHT, especially with increasing reaction time. Among the hydrolysates, 1000 ppm HFP showed the highest inhibitory activity against  $\beta$ -carotene bleaching throughout the reaction time of 120 min ( $p < 0.05$ ). The ability of hydrolysates to prevent the bleaching of  $\beta$ -carotene was more likely governed by their amphiphilic properties of amino acids compositions. Localization/orientation at the interface of hydrophobic part of peptides to oil phase and of hydrophilic portion to aqueous phase at the interface is the major principle for emulsion stabilization [30]. The result suggested that protein hydrolysates tested contained peptides with both hydrophilic and hydrophobic portions, in which the peptide segments with hydrophobicity in nature could adsorb at the oil droplet, while the hydrophilic domains preferably suspended in the aqueous phase. The localization of peptides with antioxidative activity at the oil-water interface could favor their antioxidative activity for oil droplet. As a result, the antioxidative activity could be maximized at the interface of emulsion.

##### 3.4.2. Lecithin liposome model system

Different concentrations of HAP and HFP, 500 and 1000 ppm, affected the oxidation of the lecithin liposome system differently as indicated by different conjugated dienes (CD) (Fig. 4(A)) and TBARS values (Fig. 4(B)). All systems containing hydrolysate samples, except for that added with 100 ppm BHT, showed the similar pattern of CD formation, in which the increase was found within the first 12–18 h of reaction. Thereafter, the decrease in CD was observed. However, the rate of changes varied with the hydrolysates added and concentration used. In the early incubation time (0–6 h), the delay in CD formation was observed. System containing 1000 ppm HFP showed the longer delay time, compared with systems containing other hydrolysates, indicating the higher resistance to oxidation of the system [8]. After 6 h, the remarkable increases in CD were observed, especially within the first 18 h ( $p < 0.05$ ). Within the first 6–12 h of incubation, the increasing rate of CD formation in the system varied with the hydrolysates and the concentrations used. At the same concentration used, the system with HFP showed a lower CD formation as evidenced by lower  $A_{234}$  and the systems containing higher concentration of hydrolysates showed a lower CD formation ( $p < 0.05$ ). When comparing with the control, liposome system added with 500 and 1000 ppm HFP and 1000 ppm HAP had the lower CD than the control ( $p < 0.05$ ), indicating that these hydrolysates had the ability to retard the formation of CD [41]. However, after 18 h, CD of all samples added with hydrolysates was higher than that of the control and gradually decreased up to 36 h. Subsequently, CD was quite stable up to 60 h of incubation. The decrease in CD was probably due to the decomposition of CD into secondary products. However, no changes in CD of liposome system added with 100 ppm BHT was observed throughout 60 h of incubation ( $p > 0.05$ ). For the system added with 25 ppm BHT, the increase in CD was found after 12 h of incubation and reached the plateau at h 36.

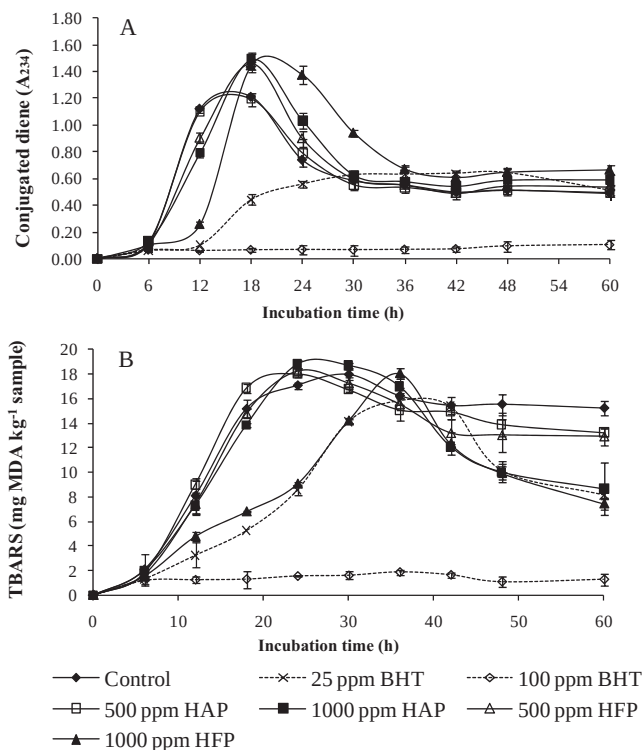


**Fig. 4.** Changes in conjugated diene (A) and thiobarbituric acid reactive substances (TBARS) value (B) in the lecithin liposome system in the absence or the presence of protein hydrolysate from brownstripe red snapper muscle at different levels. HAP: HA hydrolyzed with PCP. HFP: HF hydrolyzed with PCP. BHT is butylated hydroxytoluene. Bars represent the standard deviation ( $n=3$ ).

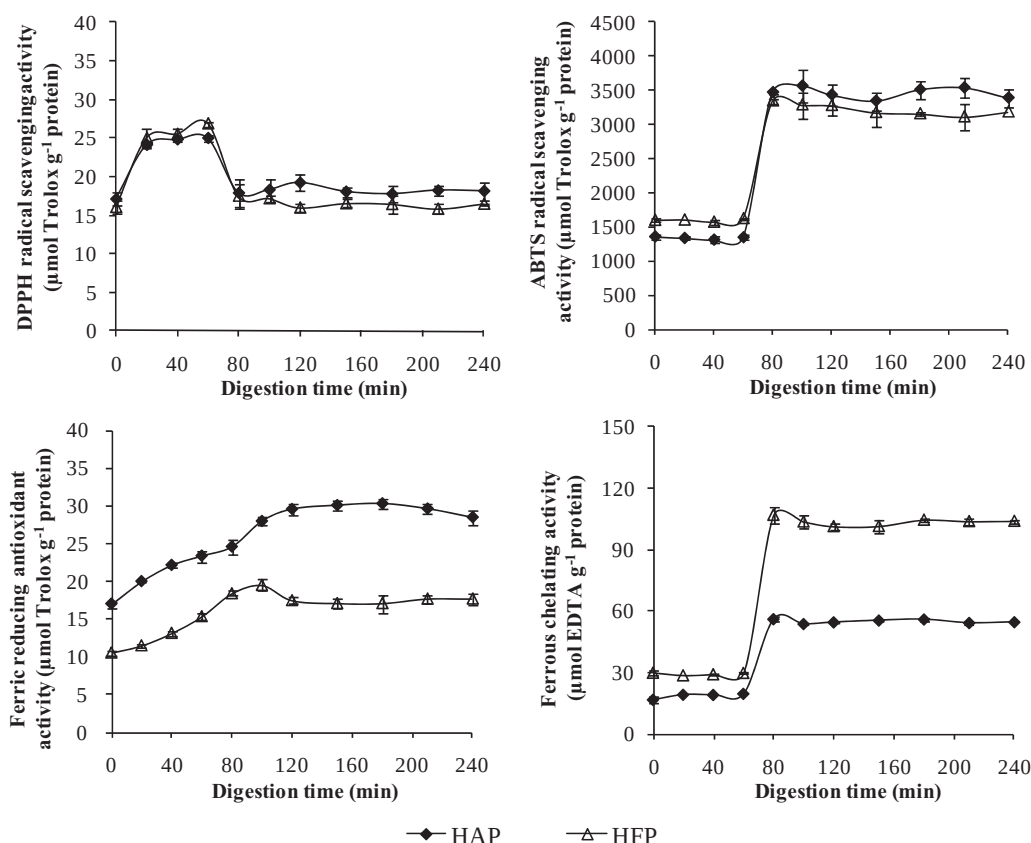


Fig. 5. Antioxidative activities of protein hydrolysate from brownstripe red snapper muscle prepared by two-step hydrolysis process in gastrointestinal tract model system. HAP: HA hydrolyzed with PCP. HFP: HF hydrolyzed with PCP. Bars represent standard deviation ( $n=3$ ).

The formation of secondary lipid oxidation products was evaluated by TBARS. The sharp increases in TBARS were observed within the first 24 h of incubation when 500 and 1000 ppm HAP and 500 ppm HFP were added to liposome system ( $p < 0.05$ ). However, the system added with 1000 ppm HFP showed the lower TBARS than those added with other hydrolysates ( $p < 0.05$ ). It was noted that system added with 1000 ppm HFP had the similar TBARS, compared with that containing 25 ppm BHT. The early formation of TBARS indicated the decomposition of the primary products to the secondary products. After the particular incubation time, the gradual decreases in TBARS were observed, possibly due to the loss of volatile compounds produced in those systems. HFP, especially at 1000 ppm, showed the noticeable antioxidative activity in liposome system throughout the 60 h of incubation time as evidenced by the lower TBARS formation ( $p < 0.05$ ). The result suggested the greater ability of 1000 ppm HFP in retardation of the formation of secondary oxidation products. Liposome system added with 100 ppm BHT showed no changes in TBARS throughout 60 h of incubation ( $p > 0.05$ ).

Liposomes are appropriate lipid models to evaluate antioxidative activity in lipid food or lipoprotein particles containing phospholipids [31]. Polar portions of peptides in hydrolysate might interact with the polar environment of lecithin liposomes, resulting in the better protection against lipid oxidation at the interface. It could be concluded that protein hydrolysates, especially 1000 ppm HFP, could retard lipid oxidation via their radical scavenging activity, reducing power and metal chelating activity.

In food-related systems, antioxidant activity is mainly due to the scavenging of free radical and the chain-breaking inhibition of lipid oxidation [46]. Therefore, the hydrolysate from brownstripe red snapper could retard lipid oxidation *in vitro* assay systems and

oxidation model systems. The results suggested that hydrolysate from brownstripe red snapper protein could function as a natural antioxidant in food or functional food.

### 3.5. Changes in antioxidative activity in gastrointestinal tract model system (GIMs)

GIMs was used to simulate the ingestion system of human body and the remaining antioxidative activities of HAP and HFP were monitored (Fig. 5). When protein hydrolysate was oral administered, their bioactive peptides should be resistant to hydrolysis by digestive proteases in order to be absorbed and reach the target organ to function as antioxidant [47]. Both HAP and HFP showed the similar activities in GIMs. Both hydrolysates showed the increase in DPPH radical scavenging activity within the first hour of digestion. Thereafter, during intestinal simulated system, DPPH radical scavenging activities were similar to that of original hydrolysate and remained constant up to 240 min. On the other hand, the increase in FRAP was found for both HAP and HFP during the first hour of digestion ( $p < 0.05$ ). The further increases were also found in the intestinal simulated system up to 120 and 100 min for HAP and HFP, respectively. Thereafter, no changes in FRAP were found up to 240 min for HAP, whereas the slight decrease up to 240 min was observed for HFP ( $p > 0.05$ ). Nevertheless, neither ABTS radical scavenging nor ferrous chelating activities were changed within the first hour (stomach simulated system) ( $p > 0.05$ ). With further hydrolysis in intestinal simulated system, the sharp increases in both ABTS radical scavenging and ferrous chelating activities were obtained but no changes in activity were found with increasing digestion time up to 240 min ( $p > 0.05$ ).

**Table 1**

Proximate composition of protein hydrolysate from the muscle of brownstripe red snapper prepared by 2-step hydrolysis process using Alcalase/pyloric caeca protease (PCP) (HAP) and Flavourzyme/PCP (HFP).

| Compositions (% wet weight) | HAP                          | HFP                       |
|-----------------------------|------------------------------|---------------------------|
| Protein                     | 87.36 ± 0.12 <sup>†,‡a</sup> | 86.55 ± 0.19 <sup>b</sup> |
| Moisture                    | 11.16 ± 0.39 <sup>a</sup>    | 11.96 ± 0.52 <sup>a</sup> |
| Fat                         | 0.61 ± 0.06 <sup>a</sup>     | 0.64 ± 0.04 <sup>a</sup>  |
| Ash                         | 1.76 ± 0.73 <sup>a</sup>     | 1.78 ± 0.32 <sup>a</sup>  |

<sup>†</sup> Mean ± SD (n = 3).

<sup>‡</sup> Different superscripts in the same row indicate the significant differences (p < 0.05).

Pepsin might hydrolyze HAP and HFP to some degrees, yielding the new peptides with DPPH radical scavenging activity and reducing power. It was noted that HAP showed the higher FRAP than HFP during the first hour of digestion. Further digestion with pancreatin (intestinal simulated system) caused the dramatic increase in ABTS radical scavenging activity, FRAP and ferrous chelating activity, but with different rates. During pancreatin digestion, HAP showed the higher DPPH, ABTS radical scavenging activities and FRAP, but lower ferrous chelating activity, compared with HFP. The result suggested that pancreatin might cleave the peptides to some degrees, leading to the release of new antioxidative peptides. This could enhance the antioxidative activities of hydrolysates. Generally, GIMs may actually lead to generation of more potent bioactive peptides [48]. The antioxidative activities of protein hydrolysates after incubation in GIMs was dependent on peptides in hydrolysate, in term of size, amino acid composition and sequence, which could be targeted by digestive proteases [48]. The results showed that the antioxidative activity of both HAP and HFP could be preserved after treatment with these gastrointestinal enzymes in GIMs (p < 0.05). Thus, both the antioxidative activities of HAP and HFP were more likely preserved after digestion in the real gastrointestinal tract of human body.

### 3.6. Proximate compositions of protein hydrolysates

Chemical compositions of HAP and HFP are shown in Table 1. HAP and HFP had high protein content (87.36 and 86.55% for HAP and HFP, respectively). This suggested that both HAP and HFP were the essential source of proteins. During hydrolysis, protein was solubilized and the insoluble non-protein matter was removed, resulting in the high protein content in the resulting hydrolysate [24]. Lipid content in raw material also affected the protein content in resulting hydrolysate. The high amount of lipids resulted in the low amount of solubilized protein [49]. In the present study, the pretreatment with CaCl<sub>2</sub> and citric acid was also performed to remove phospholipid membrane prior to hydrolysis. As a consequence, both HAP and HFP contained very low fat content. Ash content of 1.76–1.78% in both hydrolysates represented the salt formed during pH adjustment using acid or alkaline.

### 3.7. Amino acid composition of protein hydrolysates

Amino acid compositions of HAP and HFP are shown in Table 2. Both HAP and HFP contained glutamic acid/glutamine as the major amino acids and were also rich in aspartic acid/asparagine, alanine, lysine and leucine. However, both hydrolysates contained low levels of cysteine and tryptophan. Based on total amino acids, essential amino acids made up 38.01 and 37.87% of HAP and HFP, respectively. Therefore, they could serve as the excellent source of useful nutrients. Generally, the differences in amino acid composition between HAP and HFP depended on the existing differences in enzyme specificity and hydrolysis conditions [41]. The differ-

**Table 2**

Amino acid composition of protein hydrolysate from the muscle of brownstripe red snapper prepared by 2-step hydrolysis process using Alcalase/pyloric caeca protease (PCP) (HAP) and Flavourzyme/PCP (HFP).

| Amino acids (%)            | HAP   | HFP   |
|----------------------------|-------|-------|
| Alanine                    | 9.38  | 9.56  |
| Arginine                   | 5.00  | 5.06  |
| Aspartic acid/asparagine   | 10.65 | 10.73 |
| Cysteine                   | 0.12  | 0.14  |
| Glutamic acid/glutamine    | 16.23 | 16.92 |
| Glycine                    | 7.48  | 7.21  |
| Histidine                  | 1.71  | 1.60  |
| Isoleucine <sup>a</sup>    | 4.14  | 4.07  |
| Leucine <sup>a</sup>       | 8.54  | 8.62  |
| Lysine <sup>a</sup>        | 9.51  | 9.76  |
| Methionine <sup>a</sup>    | 2.93  | 2.92  |
| Phenylalanine <sup>a</sup> | 2.65  | 2.45  |
| Proline                    | 3.66  | 3.46  |
| Serine                     | 5.15  | 5.02  |
| Threonine <sup>a</sup>     | 5.10  | 4.91  |
| Tyrosine                   | 2.33  | 2.14  |
| Tryptophane                | 0.28  | 0.29  |
| Valine <sup>a</sup>        | 5.15  | 5.15  |

<sup>a</sup> Essential amino acids.

ences in amino acid composition probably determined the varying antioxidative activity between both hydrolysates.

## 4. Conclusion

Protein hydrolysates prepared from the muscle of brownstripe red snapper using protease from its pyloric caeca in combination with commercial enzymes could provide the peptides with antioxidative and ACE inhibitory activities. Protein hydrolysates prepared using Alcalase and Flavourzyme at the first step, followed by further hydrolysis using protease from its pyloric caeca showed the highest antioxidative activities, both *in vitro* and oxidation model systems. Antioxidative activities of protein hydrolysates remained constant or increased after digestion in gastrointestinal tract model system. Furthermore, protein hydrolysates contained high protein content with glutamic acid/glutamine as the major amino acids. Therefore, hydrolysate from brownstripe red snapper muscle by two-step hydrolysis could serve as a natural antioxidant for food preservation or as the functional foods.

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## Antioxidative and functional properties of protein hydrolysate from defatted skipjack (*Katsuwonus pelamis*) roe

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### ABSTRACT

Antioxidative and functional properties of protein hydrolysate from defatted skipjack (*Katsuwonus pelamis*) roe, hydrolysed by Alcalase 2.4 L (RPH) with different degrees of hydrolysis (DH) at various concentrations were examined. As DH increased, the reduction of DPPH, ABTS radicals scavenging activities and reducing power were noticeable ( $p < 0.05$ ). The increases in metal chelating activity and superoxide scavenging activity were attained with increasing DH ( $p < 0.05$ ). However, chelating activity gradually decreased at DH above 30%. All activities except superoxide anion radical scavenging activity increased as the concentration of hydrolysate increased ( $p < 0.05$ ). Hydrolysis using Alcalase could increase protein solubility to above 80% over a wide pH range (2–10). The highest emulsion ability index (EAI) and foam stability (FS) of hydrolysates were observed at low DH (5%) ( $p < 0.05$ ). Concentrations of hydrolysates determined interfacial properties differently, depending on DH. The molecular weight distribution of RPH with 5% DH (RPH5) was determined using Sephadex G-75 column. Two major peaks with the molecular weight of 57.8 and 5.5 kDa were obtained. Fraction with MW of 5.5 had the strongest metal chelating activity and ABTS radical scavenging activity. The results reveal that protein hydrolysates from defatted skipjack roe could be used as food additives possessing both antioxidant activity and functional properties.

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### 1. Introduction

Enzymatic hydrolysis has been implemented to prepare protein hydrolysate in order to improve its functional properties, particularly solubility, and to enhance bioactivity. Hydrolysis provides the shorter chain peptides with excellent solubility over a wide pH range. However, a very high degree of hydrolysis can have negative effects on the functional properties, especially the interfacial properties (Klompong, Benjakul, Kantachote, & Shahidi, 2007). Various commercial proteases such as Alcalase (EC 3.4.21.62), Flavourzyme (EC 3.4.11.1), Protamex (EC 3.4.21.62/3.4.24.28), Neutrase (EC 3.4.24.28), porcine pepsin (EC 3.4.23.1), etc. have been used to produce hydrolysate with varying properties. Alcalase has been reported for its efficacy in preparation of fish protein hydrolysate from many sources (Chalamaiah, Rao, Rao, & Jyothirmayi, 2009; Klompong et al., 2007; Thiansilakul, Benjakul, & Shahidi, 2007) and it showed higher proteolytic activity, compared with acid and neutral enzymes (Balaswamy, 2011; Klompong et al., 2007). Generally, the obtained peptides have been found to possess

bioactivity e.g. radical scavenging activity, calcium binding capacity, chelating (Klompong et al., 2007), antihypertensive (Bougatef et al., 2008), antiproliferative (Picot et al., 2006), immunomodulating (Kitts & Weiler, 2003) and prolyl endopeptidase inhibitory activities (Sørensen, Kildal, Stepaniak, Pripp, & Sørhaug, 2004).

Fish roe is well known as a nutritive source for human consumption, particularly polyunsaturated fatty acids (PUFAs) (Intarasirisawat, Benjakul, & Visessanguan, 2011) and functional proteins such as vitellogenin and vitellogenin derivatives (lipovitellin and phosvitin). Those proteins are mainly located in egg yolk and are naturally present as granule form of lipovitellin–phosvitin complex with low solubility (Castellani, Belhomme, David-Briand, Guérin-Dubiard, & Anton, 2006). Hydrolysate from herring (*Clupea harengus*) gonad prepared using Alcalase with good functional properties was reported by Sathivel et al. (2003). Chalamaiah et al. (2009) reported that protein hydrolysates from underutilised meriga (*Cirrhinus mrigala*) fish egg prepared by Alcalase and papain exhibited high protein solubility over wide pH range (2–12) with good fat adsorption capacity, foam capacity and emulsifying capacity.

The tuna processing industry has been economically important for Thailand as the essential income generator. Among all tuna, skipjack has served as the most common species with the largest

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amount for canned tuna production in Thailand. During the butchering process, a large amount of roe has been collected and generally used as the animal feed with the low market value. To maximise the use of those byproducts, the hydrolysis can be implemented to enhance the functional properties and bioactivities, especially antioxidative activity, etc. The hydrolysate from tuna roe might possess the different characteristics and properties as determined by the unique proteins and lipoprotein, etc. Nevertheless, there is a little information regarding protein hydrolysates from the roe of tuna and their antioxidative activities and functional properties as affected by degree of hydrolysis (DH) and concentration used. Therefore, the aim of this investigation was to study the antioxidative activities and functional properties of protein hydrolysate from roe of skipjack tuna, a most commonly species used for canned tuna production in Thailand.

## 2. Materials and methods

### 2.1. Chemicals

Alcalase 2.4 L (2.4 units/ml) was obtained from Novo Nordisk (Bagsvaerd, Denmark). Nitro-blue tetrazolium salt (NBT), phenazine methosulphate (PMS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), nicotinamide adenine dinucleotide (NADH) and 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulphonic acid monosodium salt hydrate (ferrozine), 2,4,6-trinitrobenzenesulphonic acid (TNBS), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma Chemical Co. (St. Louis, MO). Sephadex G-75 was obtained from GE Healthcare UK Limited (Buckinghamshire, UK). All reagents were of analytical grade.

### 2.2. Preparation of defatted roe

Roe of frozen skipjack tuna with the size of 55–60 g/roe was obtained from Songkla Canning Public Co., Ltd., Songkhla, Thailand in November and December, 2011. Frozen whole skipjack tuna, stored at  $-20^{\circ}\text{C}$  for not longer than 1–2 months after capture, were thawed using the running water. After being thawed, skipjack tuna were subjected to butchering and the roe was collected, and placed in polyethylene bag and stored at  $-20^{\circ}\text{C}$  for not longer than 3 months. To prepare the defatted roe, the Bligh and Dyer method (Bligh & Dyer, 1959) was used as previously described by Intarasirisawat et al. (2011).

### 2.3. Preparation of roe protein hydrolysate

Roe protein hydrolysate (RPH) was prepared from defatted skipjack tuna roe using Alcalase. The defatted sample (2.5 g dry matter) was suspended in 95 ml of distilled water to obtain the protein concentration of 20 mg/ml. The mixture was preincubated at  $50^{\circ}\text{C}$  for 20 min prior to enzymatic hydrolysis. The hydrolysis reaction was initiated by the addition of Alcalase at levels of 1% and 5% (w/w solid matter). The reaction was conducted at pH 8 and  $50^{\circ}\text{C}$  for up to 5 h. The mixture (1 ml) was taken at designated times (0, 5, 10, 20, 30, 40, 60, 90, 120, 150, 180, 240 and 300 min), mixed with 1 ml of 1% SDS solution ( $85^{\circ}\text{C}$ ) and then placed into a water bath at  $85^{\circ}\text{C}$  for 15 min to terminate the enzymatic reaction and solubilise the sample. The hydrolysate obtained was subjected to the determination of degree of hydrolysis (DH). Hydrolysis time providing high DH was selected for further study.

The effect of Alcalase concentrations on DH of RPH was studied. Different amounts of Alcalase (1%, 2%, 5%, 6% and 10%, w/w solid matter) were added into the defatted roe suspension (2.5%, w/v) adjusted to pH 8.0 using 2 M NaOH and pre-incubated at  $50^{\circ}\text{C}$

for 20 min. The mixtures were mixed thoroughly. The hydrolysis was preceded for the selected time (1 h) and the enzyme was inactivated as previously described. RPHs were determined for DH.  $\text{Log}_{10}$  (enzyme concentration) vs. DH was plotted. From the regression equation, the enzyme concentrations required to hydrolyse defatted tuna roe powder to obtain the desired DHs (5%, 10%, 20%, 30%, 40% and 50%) were calculated. RPH samples with different DHs were freeze-dried using a SCANVAC CoolSafe™ freeze-dryer (CoolSafe 55, ScanLaf A/S, Lyngø, Denmark). Samples were placed in polyethylene bag and kept at  $-40^{\circ}\text{C}$  until analyses.

### 2.4. Determination of DH

DH of RPH was determined according to the method of Benjakul and Morrissey (1997). RPH samples with appropriate dilution ( $125^{\circ}\mu\text{l}$ ) were added with 2.0 ml of 0.2 M phosphate buffer (pH 8.2) and 1.0 ml of 0.01% TNBS solution. The solution was mixed thoroughly and placed in a temperature controlled water bath (Mettmert, Bavaria, Germany) at  $50^{\circ}\text{C}$  for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulphite. The mixtures were cooled at room temperature for 15 min. The absorbance was read at 420 nm and  $\alpha$ -amino group was expressed in terms of L-leucine. The DH was defined as follows:

$$\text{DH} = [(L_s - L_0)/(L_{\text{max}} - L_0)] \times 100$$

where  $L_s$  is the amount of  $\alpha$ -amino groups of hydrolysate sample.  $L_0$  is the amount of  $\alpha$ -amino groups in the original defatted roe.  $L_{\text{max}}$  is the total  $\alpha$ -amino groups in the defatted roe obtained after acid hydrolysis (6 N HCl at  $100^{\circ}\text{C}$  for 24 h).

### 2.5. Determination of antioxidative activities

#### 2.5.1. DPPH radical scavenging activity

The DPPH radical scavenging activity of RPH was determined as described by Wu, Chen, and Shiau (2003) with a slight modification. To 1.5 ml of sample solution with varying protein concentrations (5, 10 and 20 mg/ml), 1.5 ml of 0.15 mM DPPH in 50% ethanol were added. The mixture was mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was measured at 517 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The blank was prepared in the same manner, except that distilled water was used instead of the sample. A standard curve was prepared using Trolox in the range of 10–60  $\mu\text{M}$ . The activity was expressed as  $\mu\text{mol}$  Trolox equivalents (TE)/ml.

#### 2.5.2. ABTS radical scavenging activity

The ABTS radical scavenging activity of RPH was determined as described by Thiansilakul et al. (2007). The stock solutions included 14.8 mM ABTS solution and 5.2 mM potassium persulphate solution. The working solution was prepared by mixing two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in dark. The solution was then diluted by mixing 1 ml of ABTS solution with 50 ml of methanol in order to obtain an absorbance of  $1.1 \pm 0.02$  units at 734 nm using a spectrophotometer. Fresh ABTS solution was prepared daily. A sample (150  $\mu\text{l}$ ) with different protein concentrations (5, 10 and 20 mg/ml) was mixed with 2850  $\mu\text{l}$  of ABTS solution and the mixture was left at room temperature for 2 h in dark. The absorbance was then measured at 734 nm using a spectrophotometer. A standard curve of Trolox ranging from 50 to 600  $\mu\text{M}$  was prepared. The activity was expressed as  $\mu\text{mol}$  Trolox equivalents (TE)/ml.

#### 2.5.3. Superoxide radical scavenging activity

The superoxide radical scavenging activity was measured by the reduction of NBT according to the method of Hazra, Biswas, and

Mandal (2008) with a slight modification. The superoxide radicals, generated by non-enzymatic phenazine methosulphate–nicotinamide adenine dinucleotide (PMS/NADH), reduce nitro blue tetrazolium (NBT) to a purple formazan. One millilitre of reaction mixture contained phosphate buffer (20 mM, pH 7.4), NADH (73  $\mu$ M), NBT (50  $\mu$ M), PMS (15  $\mu$ M) and samples at various concentrations (0–20 mg/ml). After incubation for 2 min at room temperature (28–30 °C), the absorbance at 562 nm was measured against a blank to determine the quantity of formazan generated. Trolox with the range of 0–600  $\mu$ M was used as standard. The superoxide radical scavenging activity was expressed as  $\mu$ mol Trolox equivalents (TE)/ml.

#### 2.5.4. Reducing power

The reducing power was determined by the method of Oyaizu (1986). The sample solution (0.5 ml) with different protein concentrations (5, 10 and 20 mg/ml) was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. An aliquot (2.5 ml) of 10% trichloroacetic acid was added to the mixture, followed by centrifugation at 3,000g (Hettich mikro 20, Tuttlingen, Germany) for 10 min. The upper layer of solution (2.5 ml) was mixed with 2.5 ml of distilled water and 2.5 ml of 0.1% ferric chloride and the absorbance was read at 700 nm. Trolox with the concentration ranging from 0 to 600  $\mu$ M was used as standard. The reducing power value was expressed as  $\mu$ mol Trolox equivalents (TE)/ml.

#### 2.5.5. Metal chelating activity

The chelating activity on  $\text{Fe}^{2+}$  was determined using the method of Decker and Welch (1990). One millilitre of sample solution (5, 10 and 20 mg protein/ml) was mixed with 3.7 ml of distilled water. The mixture was then reacted with 0.1 ml of 2 mM  $\text{FeCl}_2$  and 0.2 ml of 5 mM ferrozine for 20 min at room temperature. The absorbance was read at 562 nm. The blank was prepared in the same manner except that distilled water was used instead of the sample. EDTA with the concentration range of 0–3  $\mu$ M was used as standard. The chelating activity was then calculated and expressed as  $\mu$ mol EDTA equivalent/ml.

### 2.6. Determination of functional properties

#### 2.6.1. Solubility

The solubility was tested at different pHs following the method of Castellani, Martinet, David-Briand, Guérin-Dubiard, and Anton (2003) with a slight modification. Sample was dispersed in distilled water and the pH was adjusted to different pHs (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0) using either 6 N HCl or 6 N NaOH. The volume of the mixture was adjusted to desired volume to obtain the final concentration of 10 mg protein/ml using distilled water with the corresponding pH. After stirring for 90 min, the mixtures were centrifuged at 1,800g for 30 min using a Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA) at room temperature. The supernatant was collected and subjected to the determination of the protein content using the Lowry's method (Lowry, Rosebrough, Farr, & Randall, 1951).

#### 2.6.2. Foaming properties

The foaming ability and foam stability of samples were determined according to the method of Shahidi, Han, and Synowiecki (1995). The samples were dissolved in distilled water (pH 7.0) to obtain designated protein concentrations (5, 10 and 20 mg/ml). Twenty millilitres of solutions were homogenised in a 50 ml-cylinder at a speed of 16,000 rpm to incorporate the air for 1 min. The total volume was measured at 0 and 30 min after whipping. The foaming ability was expressed as foam expansion at 0 min, while foam stability was expressed as foam expansion after 30 min of

whipping. The foam expansion was calculated according to the following equation (Sathe & Salunkhe, 1981):

$$\text{Foam expansion}(\%) = [(A - B)/B] \times 100$$

$$\text{Foam stability}(\%) = [(A_{30\text{min}} - B)/(A_{0\text{min}} - B)] \times 100$$

where  $A$  = volume after whipping (ml) and  $B$  = volume before whipping (ml).

#### 2.6.3. Emulsifying properties

The emulsion activity index (EAI) and emulsion stability index (ESI) were determined according to the method of Pearce and Kinsella (1978) with a slight modification. Samples at different protein concentrations (5, 10 and 20 mg/ml) were prepared using distilled water. Sample solution (6 ml) and soybean oil (2 ml) were homogenised using a homogeniser (model T25 basic, IKA LABORTECHNIK, Selangor, Malaysia) at a speed of 20,000 rpm for 1 min. The emulsions were pipetted out at 0 and 10 min and 100-fold diluted with 0.1% SDS. The mixture was mixed thoroughly for 10 s using a vortex mixer. The absorbance measured immediately ( $A_0$ ) and after 10 min ( $A_{10}$ ) of emulsification using a spectrophotometer were used to calculate EAI and ESI as follows (Pearce & Kinsella, 1978):

$$\text{EAI} (\text{m}^2/\text{g}) = (2 \times 2.303A)DF/l\phi C$$

$$\text{ESI} (\text{min}) = A_0 \times \Delta t / (A_0 - A_{10})$$

where  $A$ ,  $A_{500}$ ;  $l$ , path length of cuvette (m);  $DF$ , the dilution factor (100);  $\phi$ , oil volume fraction;  $C$ , protein concentration in aqueous phase ( $\text{g}/\text{m}^3$ ) and  $\Delta t$ , 10 min. The control was prepared by mixing distilled water and oil in the absence of sample.

### 2.7. Molecular weight distribution

RPH with 5%DH (RPH5), which exhibited the highest emulsifying properties and some antioxidative properties, was chosen to determine the molecular weight distribution using gel filtration chromatography. RPH5 was dissolved in distilled water (mobile phase) to yield a protein concentration of 50 mg/ml. The mixture was filtered through 0.45  $\mu$ m Nylon filter membrane (25 mm, id.) (Xiboshi®, Tianjin, China). The filtrate (2 ml) was chromatographed on a Sephadex G-75 column (1.5  $\times$  67 cm), equilibrated with approximately two bed volumes of elution buffer. A sample was loaded onto the column and then eluted with same buffer at a flow rate of 0.5 ml/min. Fractions of 3 ml were collected and their absorbance was monitored at 280 nm. The obtained fractions were subjected to the determination of metal chelating activity and scavenging activity toward ABTS and superoxide anion radicals as previously described.

The molecular weight of RPH5 was estimated by plotting the available partition coefficient ( $K_{av}$ ) against the logarithm of the molecular weight of the protein standards. The elution volume ( $V_e$ ) was measured for each protein standard and RPH5. The void volume ( $V_0$ ) was estimated by the elution volume of blue dextran ( $M_r = 2000000$ ). The low molecular weight markers included aprotinin ( $M_r = 6500$ ), lysozyme ( $M_r = 14,600$ ), soy bean trypsin inhibitor ( $M_r = 29000$ ), ovalbumin ( $M_r = 43000$ ) and bovine serum albumin ( $M_r = 66000$ ).

#### 2.7.1. Statistic analysis

All experiments were run in triplicate. All analyses were conducted in triplicate, except for the emulsion properties, which were performed in five replicates. Statistical analysis was performed using one-way analysis of variance (ANOVA). Mean comparison was carried out using Duncan's multiple range test (Steel & Torrie, 1980).

### 3. Results and discussion

#### 3.1. Effect of Alcalase concentration and hydrolysis time on protein hydrolysis

Alcalase was the only protease, which was able to hydrolyse defatted skipjack roe, whilst Flavourzyme and Protamex could not hydrolyse roe proteins (data not shown). Chalamaiah et al. (2009) found that meriga fish egg hydrolysate prepared using Alcalase had higher DH (62% DH) than that using papain (17.1%). Alcalase has been reported to preferably hydrolyse hydrophobic amino acid residues (Chalamaiah et al., 2009; Klompong et al., 2007). Fig. 1a displays DHs of hydrolysate from defatted skipjack roe (RPH) as affected by hydrolysis time. The DHs of RPH gradually increased with increasing the hydrolysis time ( $p < 0.05$ ). Rapid hydrolysis was observed within the first 1 h. Thereafter, a slower rate of hydrolysis was found. A reduction in the hydrolysis rate might be due to the limitation of the substrates for hydrolysis. The short chain peptides formed might not be cleaved by Alcalase. A decrease in hydrolysis rate could also be attributed to a decrease in peptide bonds available for hydrolysis, enzyme inhibition and deactivation (Dong et al., 2008). An initial rapid phase was observed when a large number of peptide linkages were cleaved per unit time, rendering a large proportion of soluble protein.

The most compact core proteins were hydrolysed more slowly. At the same time, the available substrate decreased as the reaction time increased. The reduction of hydrolysis rate might be hypothetically attributed to a decrease in the concentration of peptide bonds available for hydrolysis, the decrease in enzyme activity or a product inhibition (Safari et al., 2009). For the same hydrolysis time, the higher amount of enzyme used resulted in higher DH ( $p < 0.05$ ). In the present study, the hydrolysis time (1 h), whose initial velocity was obtained, was selected for further study.

Different amounts of Alcalase were added to hydrolyse defatted skipjack roe for 1 h. When  $\log_{10}$  (enzyme concentration) and DH were plotted, a linear relationship was observed (Fig. 1b). From this regression, the exact concentration of Alcalase required to hydrolyse defatted skipjack roe to obtain designated DH could be calculated. Alcalase is an alkaline microbial protease produced from *Bacillus licheniformis* (Chalamaiah et al., 2009). Alcalase has been successfully used to hydrolyse a variety of fish proteins such as underutilised meriga fish egg (Chalamaiah et al., 2009), herring byproducts (Sathivel et al., 2003), black scabbardfish (*Aphanopus carbo*) by-products and tuna fish by-product (Guerard, Guimas, & Binet, 2002). At a level of 0.5% (w/w) Alcalase, meriga fish egg (6.5% substrate) reached 62% DH (Chalamaiah et al., 2009), whilst 10.1% DH was found for herring gonad (50% substrate) (Sathivel et al., 2003). Difference in DH among different proteinaceous

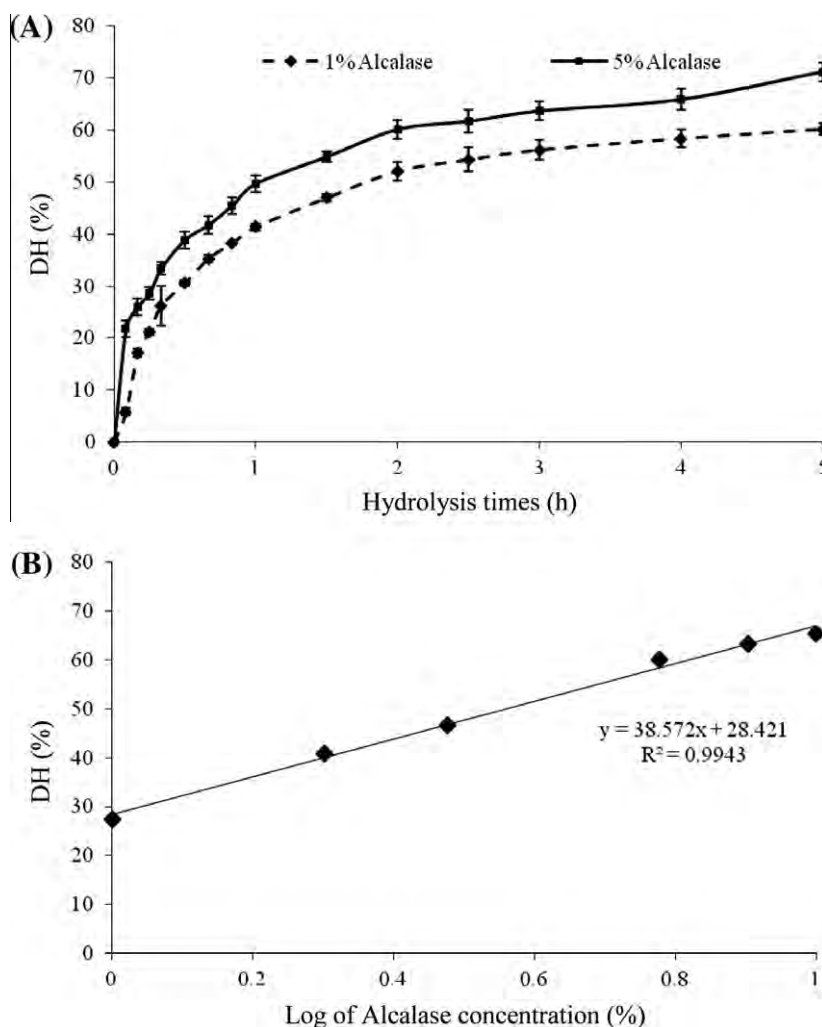


**Fig. 1.** Degree of hydrolysis of defatted skipjack roe (2%, dry basis) hydrolysed by 1% and 5% of Alcalase for various times (0–5 h) (A), and relationship between DH and log concentration of Alcalase for hydrolysis of defatted skipjack roe (B). The reaction was run for 1 h at pH 8.0, 50 °C.

materials used for hydrolysis was more likely determined by the protein substrate and amino acid composition (Chalamaiah et al., 2009; Sathivel et al., 2003).

### 3.2. Antioxidative properties

The antioxidative properties of RPH having different DHs (5–50%) at various concentrations (5–20 mg protein/ml) were evaluated using different assays (DPPH, ABTS, superoxide anion radical scavenging activities, reducing power and chelating capacity).

#### 3.2.1. DPPH radical scavenging activity

DPPH radical scavenging assay has been widely used to evaluate antioxidative properties of compounds as free radical scavengers or hydrogen donors (Klompong et al., 2007). RPH (5–20 mg/ml) showed a scavenging effect on DPPH radical in a dose dependent manner ranging from 35 to 339  $\mu\text{mol TE/ml}$  (Fig. 2A). DPPH scavenging activity was also reported in protein hydrolysate prepared from yellow stripe trevally meat (Klompong et al., 2007) and egg yolk (Sakanaka & Tachibana, 2006) prepared using Alcalase. Increasing amount of active peptides could react with free radicals to convert them to stable products more effectively (Batista,

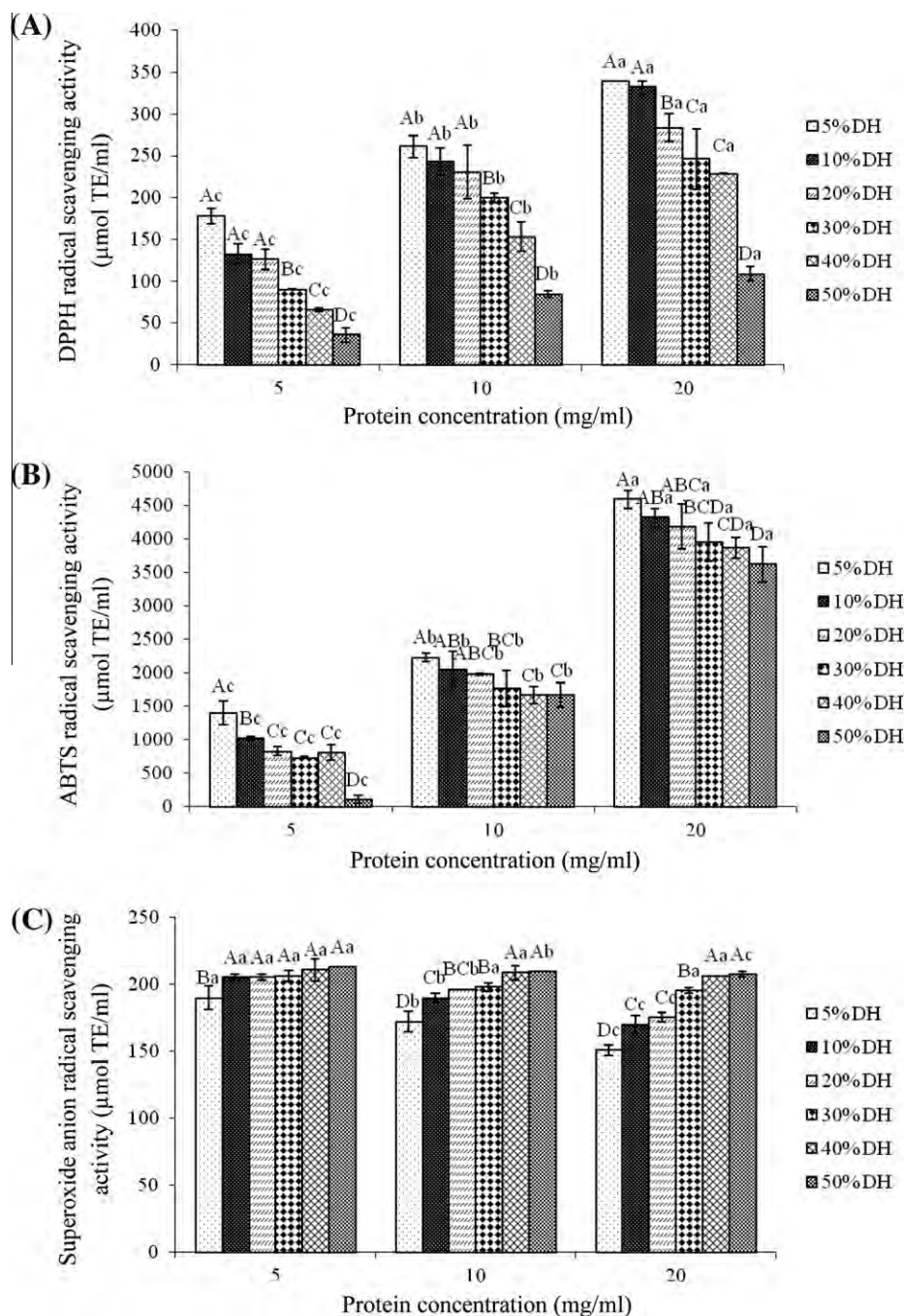


**Fig. 2.** DPPH radical (A), ABTS radical (B), Superoxide anion radical (C) scavenging activities of hydrolysates from defatted skipjack roe with different DHs at varying concentrations. Bars represent standard deviations ( $n = 3$ ). Different uppercase letters within the same concentration indicate significant differences ( $p < 0.05$ ). Different lowercase letters within the same DH indicate significant differences ( $p < 0.05$ ).

Ramos, Coutinho, Bandarra, & Nunes, 2010). At the same concentration used, the highest activity was found with RPH having 5%DH (339  $\mu\text{mol TE/ml}$ ). As the DH increased, a decrease in activity was observed ( $p < 0.05$ ). This might be governed by a difference in chain length, amino acid composition, amino acid side chain (Klompong et al., 2007) and hydrophobicity (Cheng, Xiong, & Chen, 2010). Higher enzymatic cleavage resulted in the formation of shorter chain peptides possessing more hydrophilicity. Those peptides had less efficacy in scavenging DPPH $^{\bullet}$  radical, an oil-soluble free radical. Peptides from hydrolysate with higher DH could not interact properly with hydrophobic peroxy radicals, due to the lack of hydrophobic domain of peptides (Cheng et al., 2010). The antioxidant activity of protein hydrolysates also depends on the protease and hydrolysis conditions employed (Batista et al., 2010). The results indicated that RPH contained peptides acting as electron donors and free radical scavengers, thereby terminating the chain reaction.

### 3.2.2. ABTS radical scavenging activity

The assay of ABTS $^+$  radical scavenging activity can be applied to both lipophilic and hydrophilic compounds, and has been widely used as an antioxidant activity assay (You, Zhao, Cui, Zhao, & Yang, 2009). As shown in Fig. 2B, RPH samples at all DHs were able to sequester ABTS radical in a dose dependent manner ( $p < 0.05$ ). At the same level, RPH with 5%DH showed the highest activity ( $p < 0.05$ ). The peptides in the hydrolysate with higher DH might lose scavenging activity toward ABTS $^+$ . Nalinanon, Benjakul, Kishimura, and Shahidi (2011) also reported that hydrolysates from ornate threadfin bream muscle having above 20% DH showed the decreased ability in scavenging ABTS radical. The amino acid sequence in peptides might affect the antioxidant activity. Also, the free amino acids produced determined the scavenging activity. Based on ABTS $^+$  scavenging assay, Cys was the most active amino acid, followed by Trp, Tyr and His (Gómez-Ruiz, López-Expósito, Pihlanto, Ramos, & Recio, 2008). Peptides containing these amino acids might be responsible for higher ABTS radical scavenging activity. In general, similar results were obtained in comparison with those of DPPH radical scavenging activity (Fig. 2A). Thus, the peptides in RPH, particularly with 5%DH, were able to scavenge the radicals, thereby retarding the propagation stage. As a result, the lipid oxidation could be impeded in the presence of those hydrolysates.

### 3.2.3. Superoxide radical scavenging activity

The superoxide anion radical ( $\text{O}_2^-$ ) scavenging capacity assay has been used to monitor the ability of hydrophilic antioxidants to directly react with this physiologically relevant radical (Moure, Dominguez, & Parajo, 2006). A higher superoxide radical scavenging activity was observed with increasing DH. However, it was noted that there was difference in the superoxide anion radical scavenging activity among RPH having DHs from 10 to 50 at a low protein concentration (5 mg/ml). When the concentration of RPH increased, the superoxide anion radical generally decreased. This was possibly due to the self – aggregation of peptides induced by  $\text{O}_2^-$ , thereby lowering functional group to quench the tested radicals. Furthermore, this result indicated that the smaller peptides apparently showed higher superoxide anion radical scavenging activity than those having long chain length (Klompong et al., 2007). Huang, Zhou, Ma, Cai, and Li (2010) reported that the purified peptide from egg shell protein hydrolysate with MW of 618.86 Da showed superoxide anion scavenging activity with  $\text{IC}_{50}$  at 0.10 mg/ml. Superoxide anion radical is an important reactive oxygen species (ROS) involving the formation of highly reactive species such as hydroxyl radical and  $\text{H}_2\text{O}_2$ , which is associated with cellular damage and is implicated in the ageing process, and numerous age-related degenerative diseases such as cancer, heart

disease, macular degeneration, Parkinson's disease and chronic diseases (Li, Jiang, Zhang, Mu, & Liu, 2008). The results suggested that some peptides, especially those with the shorter chain length, exhibited the ability in scavenging or trapping the superoxide anion. As a consequence, the rate of lipid oxidation could be lowered in the presence of RPH with the appropriate DH. The result suggested that peptides in RPH exhibited different scavenging mechanisms toward ABTS, DPPH and superoxide anion radicals.

### 3.2.4. Metal chelating activity

The metal chelating activity of RPH as affected by DH and concentration is depicted in Fig. 3A. At concentrations of 5 and 10 mg/ml, no differences in the metal chelating activity were found when DH increased ( $p > 0.05$ ). Nevertheless, the chelating activity increased with increasing DH up to 40% ( $p < 0.05$ ), when a concentration of 20 mg/ml was used. Furthermore, the marked decrease in the metal chelating activity was found when DH was 50% ( $p < 0.05$ ). Peptides with smaller size were not able to form the complex with metals. Xu, Katayama, and Mine (2007) reported that phosvitin oligophosphopeptides, produced by partial dephosphorylation, followed by trypsin hydrolysis, had lower chelating activity than did phosvitin. Therefore, the peptide chain length was more likely essential for the chelating activity of RPH. Steric hindrance found in RPH with a longer chain might lower the ability to migrate and chelate the target metal ion. It was noted that chelating activity of all RPHs was in a dose dependent manner. Phosvitin is a highly phosphorylated protein, which possesses a strong metal chelating activity (Xu et al., 2007). The metal chelating activity of phosvitin and derivatives is mainly related with the phosphorus content and amino acid composition (Xu et al., 2007). Additionally, their unique conformation, blocks of phosphoserines in singular way, carrying up to 15 consecutive residues, is also reported as an ideal binding site for transition metals such as iron, copper, cobalt and essential minerals such as calcium and zinc (Castellani, Guérin-Dubiard, David-Briand, & Anton, 2004). Transition metals in food could accelerate the rate of autoxidation and breakdown of hydroperoxide to volatile compounds. Transition metal ions react very quickly with peroxides by acting as one-electron donors to form alkoxyl radical (Klompong et al., 2007). Therefore, the chelation of transition metal ions by antioxidant or antioxidative peptide resulted in a lower availability of prooxidative metal ions. As a consequence, the lipid oxidation could be retarded.

### 3.2.5. Reducing power

The reducing power assay is often used to evaluate the ability of an antioxidant to donate an electron or hydrogen (Klompong et al., 2007). Antioxidative peptides in protein hydrolysate were able to reduce the  $\text{Fe}^{3+}$ /ferric cyanide complex to the ferrous form.  $\text{Fe}^{2+}$  complex can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. The ability of RPH with various DHs to reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  was determined and expressed as reducing power in comparison with trolox (Fig. 3B). All RPH samples containing peptides showed reducing power in the range of 1307–5999  $\mu\text{mol TE/ml}$ . The highest reducing power was obtained in RPH having 5%DH ( $p < 0.05$ ). The reducing power of all samples increased with increasing concentrations ( $p < 0.05$ ). A little difference in reducing power among RPHs with different DHs could be noticeable at a protein concentration higher than 10 mg/ml. The results suggested that the RPH with excessive hydrolysis generally had a lower reducing power. The reducing power of RPH was also in accordance with DPPH – (Fig. 2A) and ABTS-scavenging activities (Fig. 2B). Therefore, limited hydrolysis rendered RPH with better antioxidative properties. The hydrolysate from yellow stripe trevally prepared using Alcalase had the decreased reducing power when DH increased (Klompong et al., 2007). On the other hand, hydrolysates

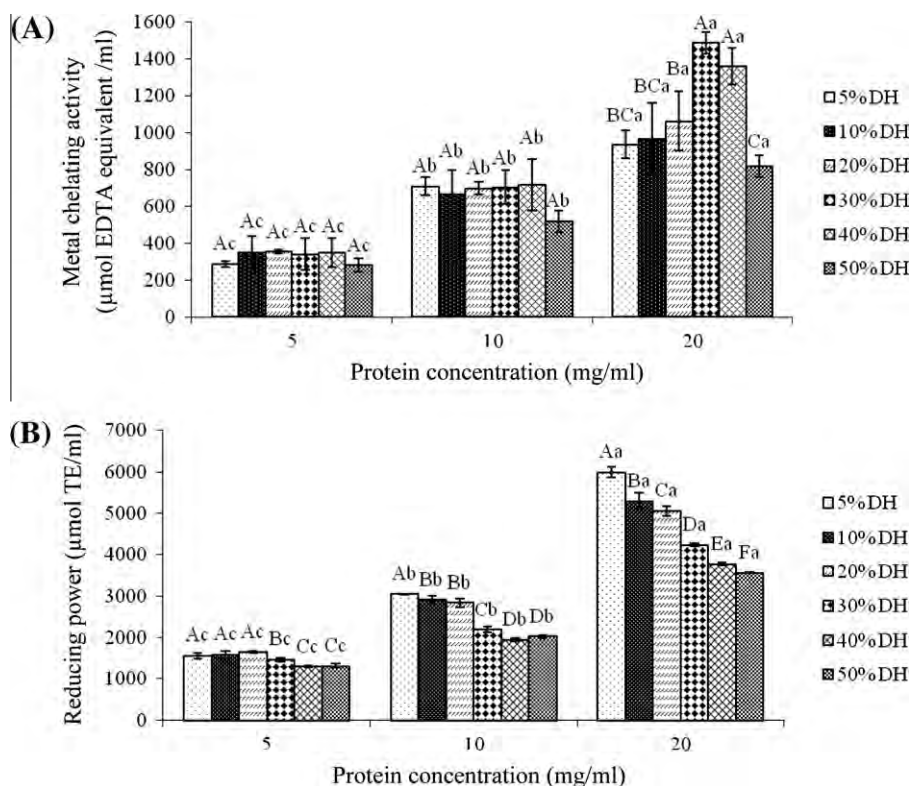


**Fig. 3.** Metal chelating activities (A) and reducing power (B) of hydrolysates from defatted skipjack roe with different DHs at varying concentrations. Bars represent standard deviations ( $n = 3$ ). Different uppercase letters within the same concentration indicate significant differences ( $p < 0.05$ ). Different lowercase letters within the same DH indicate significant differences ( $p < 0.05$ ).

prepared from several sources using varying enzymes such as black scabbard fish by-product (Batista et al., 2010), loach (*Misgurnus anguillicaudatus*) meat (You et al., 2009) and round scad (*Decapterus maruadsi*) protein hydrolysate (Thiansilakul et al., 2007) had higher reducing activity with increasing DH. The difference in reducing power might be caused by different amino acid sequence and length of the obtained peptides (Thiansilakul et al., 2007). DH greatly influenced the peptide chain length as well as the exposure of the terminal amino groups of products (Thiansilakul et al., 2007).

Therefore, RPH possessed different modes of actions and DH was a major factor determining antioxidant activity. Additionally, the concentration was another factor influencing antioxidative activity.

### 3.3. Functional properties

#### 3.3.1. Protein solubility

Solubility is one of the most important functional properties of a protein and can be increased by hydrolysis process (Klompong et al., 2007). RPH at various DHs (5–50%) was soluble over wide pH range with solubility of 82–99% (data not shown). The solubility of RPH was higher than that of meriga egg hydrolysate (72–90%) (Chalamaiah et al., 2009). The lowest solubility of all samples was noticeable at pH 3, whilst the hydrolysate from meriga fish egg prepared with the aid of Alcalase showed the lowest solubility at pH 4. The lowest solubility at pH 3 was plausibly owing to the lowest repulsion of proteins at their pI. Phosvitin, a protein in fish roe, has a pI of 4.0 (Castellani et al., 2006). The enzymatic hydrolysis potentially affects the molecular size and hydrophobicity, as well as polar and ionisable groups of protein hydrolysates (Klompong et al., 2007). The balance of hydrophilic and hydrophobic forces

of peptides is another crucial influence on solubility (Gbogouri, Linder, Fanni, & Parmentier, 2004; Klompong et al., 2007). Generally, RPH with higher DH tended to show a higher solubility. The smaller peptides are expected to have proportionally more polar residues with the ability to form hydrogen bonds with water and augment solubility (Gbogouri et al., 2004). As a consequence, hydrolysates with smaller peptides were more soluble. RPHs with high solubility over a wide pH range can be therefore applied widely in formulated food systems.

#### 3.3.2. Emulsifying properties

The EAI and ESI of RPH with different DHs at varying protein concentrations are depicted in Fig. 4. The EAI and ESI of RPH were in the range of 5.1–25.16  $\text{m}^2/\text{g}$  and 14.2–24.3 min, respectively. RPH with 5% DH showed the highest EAI ( $p < 0.05$ ). No difference in EAI was found when DH was in the range of 10–50% ( $p > 0.05$ ). EAI increased when the protein concentration increased, regardless of DH ( $p < 0.05$ ). Extensive hydrolysis commonly yielded the small peptides. Small peptides were more likely hydrophilic in nature. As a result, those peptides could not adsorb at the interface. They were more likely localised in the aqueous phase. Thus, a weaker EAI was generally observed as DH increased. As the RPH concentration increased, the EAI of RPHs having DH of 10–50% increased, whilst the decrease in EAI was observed for RPH having 5% DH ( $p < 0.05$ ). Peptides in RPH with 5% DH more likely had an amphiphilic characteristic, which favoured the migration of peptides to oil–water interface. The difference in the EAI of RPHs with different DHs might be associated with the varying chain length of their peptides. The excessive amount of protein hydrolysate might favour the self aggregation of long chain peptides. As a result, the lower amount of peptides could localise at oil–water interface. Apart from the peptide size, the amphiphilicity of peptides is

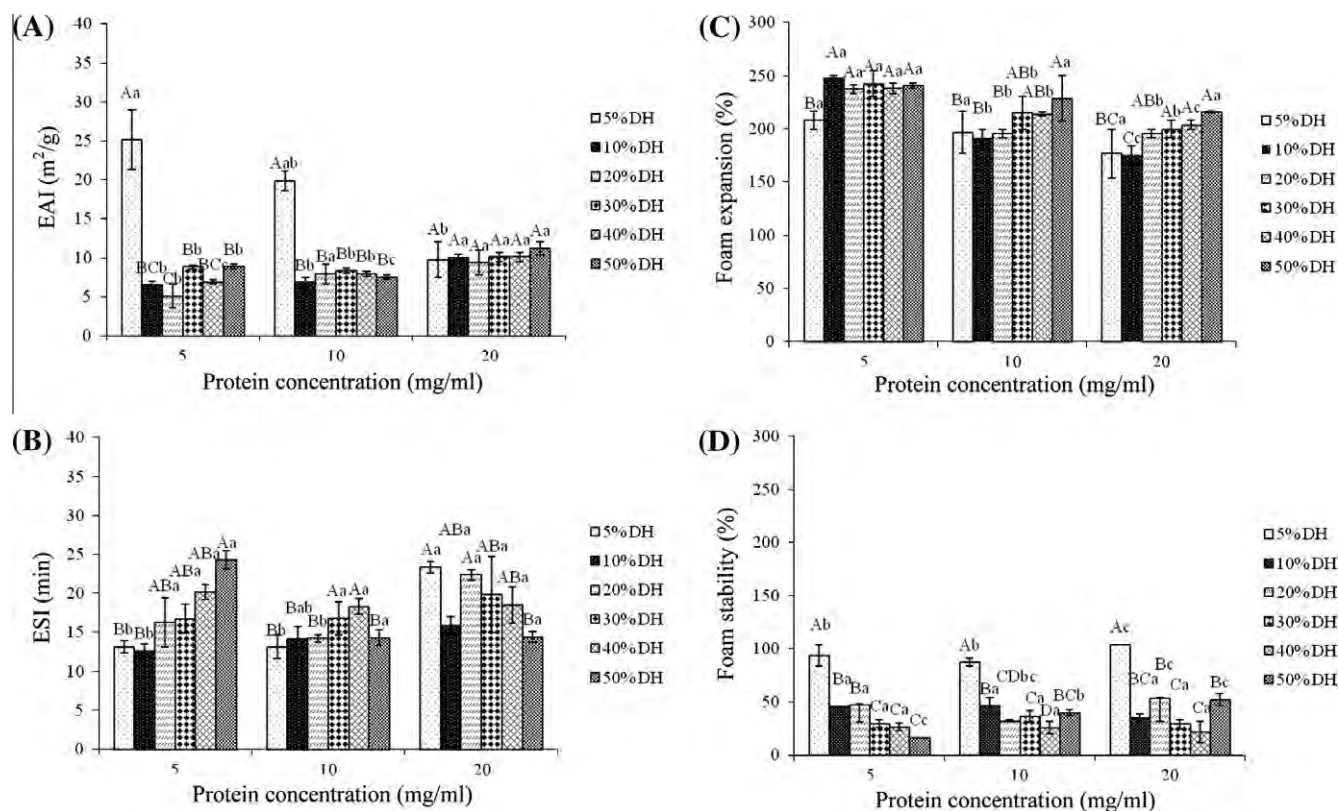


**Fig. 4.** Emulsion ability index (A), emulsion stability index (B), foam ability (C), foam stability (D) of hydrolysate from defatted skipjack roe with different DHs at varying concentrations. Bars represent standard deviations ( $n = 3$ ). Different uppercase letters within the same concentration indicate significant differences ( $p < 0.05$ ). Different lowercase letters within the same DH indicate significant differences ( $p < 0.05$ ).

important for interfacial properties. When comparing with a random structural phosphorylated protein such as sodium caseinate ( $EAI = 10.3\text{--}39.8 \text{ m}^2/\text{g}$ ;  $ESI = 20\text{--}99 \text{ min}$ ), it was found that all RPH samples had lower emulsifying capacity and stability. Poorer emulsifying properties of RPH sample might be due to the loss of tertiary structure of protein (Sathivel et al., 2003) and a steric effect (Klompong et al., 2007). A globular protein tends to form a highly elastic adsorbed layer, viscous solution and steric effect, contributing to higher emulsion stability (Sathivel et al., 2004). Egg proteins characterised by emulsifying properties are phosvitin and lipoprotein, which could stabilise o/w emulsion mainly from electrostatic repulsion force (Castellani et al., 2006).

RPH with 50% DH showed the highest ESI when determined at 5 mg/ml ( $p < 0.05$ ). Similar ESI was observed between RPH with different DHs when tested at higher concentrations. However, the ESI of RPH possessing 50%DH was lower than that having 5%DH at a concentration of 20 mg/ml ( $p < 0.05$ ). The hydrolysate might form the strong and thick film surrounding oil droplet. Nevertheless, the high charge density of phosvitin might result in a negligible adsorption capacity at o/w interface (Castellani et al., 2006). It was noted that the ESI varied with DH and concentration, suggesting that molecular property, chain length and amount of RPH at the interface determined the ESI of different RPH.

### 3.3.3. Foaming properties

The foam expansion ability (FA) and foam stability (FS) after standing at room temperature for 30 min of RPH with various DHs (5–50%) are shown in Fig. 4. As the DH increased, the RPH had higher FA ( $p < 0.05$ ). The lowest FA was found with the RPH having 5%DH when tested at 5 mg/ml ( $p < 0.05$ ). When DH increased, FA increased, depending on the concentrations used. The hydrolysate with high DH more likely had the smaller peptides,

which could migrate to the air–water interface faster. With higher amount of peptides in RPH, a lower FA was attained. The higher concentration of RPH might result in the self aggregation and lowered the migration of peptides to the air–water interface. This was evidenced by the lower EAI with increasing concentration.

For foam stability, RPH with 5%DH had the highest FS at all concentrations tested ( $p < 0.05$ ). For all samples, the FS remained unchanged when the concentration increased, except for RPH with 50%DH where FS increased with increasing concentration. It was found that RPH with a lower DH containing longer chain length yielded the foam with higher stability. This was plausibly due to the fact that peptides with the longer chain could form the thicker and stronger film surrounding air bubbles. Foam formation is governed by three factors, including transportation, penetration and reorganisation of molecules at the air–water interface (Klompong et al., 2007). A protein is required to migrate rapidly to the air–water interface, unfolding and rearranging at the interface. Aewsiri et al. (2011) suggested that the foaming capacity of gelatin can be improved by incorporating fatty acid ester, yielding more hydrophobic residues, which increased the capacity of decreasing surface tension of foam.

### 3.4. Molecular weight distribution

Since RPH having 5%DH (RPH5) displayed the highest antioxidative and functional properties when compared with others, it was determined for molecular weight distribution. After separation on Sephadex G-75, two main peaks of  $A_{280}$  with molecular weight (MW) of 57.8 (S1) and 5.5 kDa (S2) were obtained (Fig. 5A).  $A_{280}$  indicates the presence of peptide, proteins, amino acids and more likely revealed the presence of tyrosine, tryptophan and other aromatic compounds in the peptides (Thiansilakul et al., 2007). The

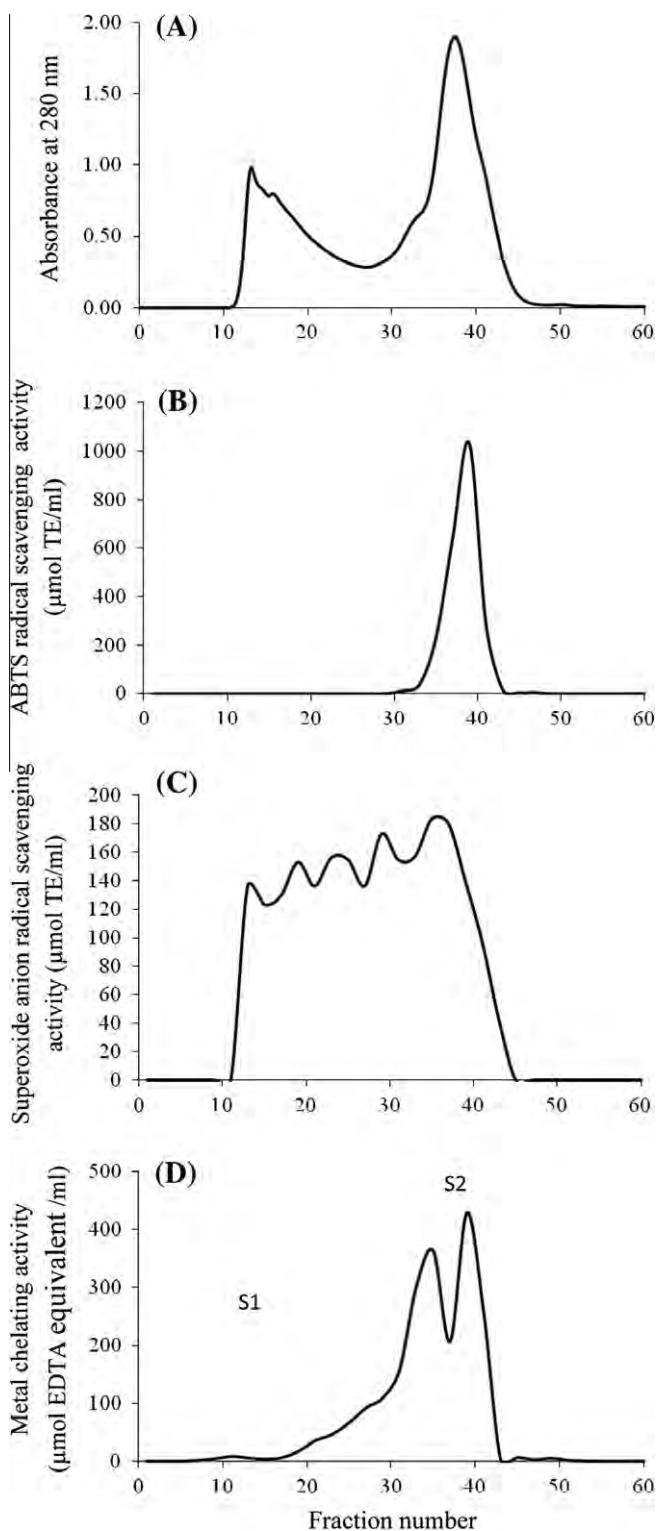


Fig. 5. Molecular weight distribution of RPH5 determined by Sephadex G-75 column.

peak of S2 was larger than that of S1, suggesting the high proportion of low MW peptides in the RPH5.

When the obtained fractions were measured for ABTS radical scavenging activity, superoxide anion radical and metal chelating activity, those fractions exhibited varying antioxidative activities. For metal chelating activity (Fig. 5D) and ABTS radical scavenging activity (Fig. 5B), peptides in S2 (F31–47) notably showed a

stronger activity than S1 ( $p < 0.05$ ). Peptides in selected fractions (F13–F43) could retard the generation of superoxide anion radical (Fig. 5C). Low MW peptides possessing metal chelating activity and ABTS radical scavenging activity were generated by enzymatic hydrolysis. The enhanced exposure of the functional groups of proteins/peptides, such as phosphoserine group, could favour their antioxidative activities. Balaswamy (2011) reported that roe protein hydrolysate from catla catla prepared using Alcalase yielded a high proportion of shorter peptides below 14 kDa. The peptides in the protein hydrolysates from meriga egg had an average MW of >10 kDa (Chalamaiah et al., 2009). A hydrolysate from mackerel mince with MW of 1400 Da had stronger antioxidative properties than that of small peptides (MW of 900 and 200 Da). Klompong et al. (2009) reported that hydrolysates from yellow stripe trevally muscle with MW of 2.4 kDa exhibited higher antioxidative properties than those with MW of 35, 0.47 and 57 kDa, respectively.

#### 4. Conclusion

RPH prepared by Alcalase had antioxidant activity, both radical scavenging activity and metal chelating activity. The obtained hydrolysates had high solubility and interfacial properties. RPH5, which exhibited the highest antioxidative and functional properties, contained two major peptides with MW of 57.8 and 5.5 kDa. Thus, the RPH powder from skipjack could be alternatively served as an ingredient, which can serve as the emulsifier in a food emulsion and can simultaneously act as an antioxidant to prevent the lipid oxidation. Thus, this safe nutritive additive can be used in food industry.

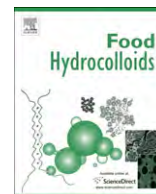
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# Characteristics of gelatin from the skin of unicorn leatherjacket (*Aluterus monoceros*) as influenced by acid pretreatment and extraction time

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## ABSTRACT

Gelatins from the skin of unicorn leatherjacket (*Aluterus monoceros*) pretreated with different acids (0.2 M acetic acid or 0.2 M phosphoric acid) and extracted with distilled water at 45 °C for various times (4 and 8 h) were characterized. Yields of 5.23–9.18 or 6.12–11.54% (wet weight basis) were obtained for gelatins extracted from the skin pretreated with 0.2 M acetic acid or 0.2 M phosphoric acid, respectively. Extracted gelatins contained  $\alpha_1$  and  $\alpha_2$  chains as the predominant components and some degradation peptides. The absorption bands of gelatins in FTIR spectra were mainly situated in the amide band region (amide I, amide II and amide III) and showed the significant loss of molecular order of triple helix. Gelatin samples had a relative solubility greater than 90% in the wide pH ranges (1–10). The gel strength of gelatin from skin pretreated with phosphoric acid (GPA) was higher than that of gelatin from skin pretreated with acetic acid (GAA). Both GPA and GAA had the lower gel strength than that of commercial bovine gelatin ( $P < 0.05$ ). Net charge of GAA and GPA became zero at pHs of 6.64–7.15 and 6.78–7.26, respectively, as determined by zeta potential titration. Emulsifying and foaming properties of GAA and GPA increased with increasing concentrations (1–3%, w/v). Those properties were governed by pretreatments and extraction time. Thus gelatin can be successfully extracted from unicorn leatherjacket skin using the appropriate acid pretreatment and extraction time.

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## 1. Introduction

Gelatin is a denatured fibrous protein derived from collagen by partial thermal hydrolysis. It is an important functional biopolymer that has a very broad application for food, material, pharmacy and photography industries (Hao et al., 2009). Under specific conditions of temperature, solvent and pH, gelatin macromolecules can exhibit flexibility sufficient to achieve a wide variety of conformations. Nevertheless, the characteristics and functionalities are dependent on its molecular structure. Due to its amphoteric nature, specific triple-stranded helical structure (not observed in synthetic polymers) and its interaction with water, which is different from synthetic hydrophilic polymers, gelatin has become indispensable (Kozlov & Burdygina, 1983).

Different types of gelatins have varying thermal and rheological properties such as melting and gelling temperatures and bloom strength (Norziah, Al-Hassan, Khairulnizam, Mordi, & Norita, 2009).

Gelatin extraction from fish skin is generally achieved by pretreatment with acid or alkali to give the desired properties (Arnesen & Gildberg, 2006). Efficiency of gelatin extraction depends on the method, in which collagens are pretreated. There are two types of gelatin with different characteristics including type-A, acid-treated collagen (isoelectric point at pH 6–9) and type-B, an alkaline treated (isoelectric point at pH 5) (Benjakul, Oungbho, Visessanguan, Thiansilakul, & Roytrakul, 2009). Acid treatment is suitable for less fully cross-linked collagens commonly found in pig or fish skins, whereas alkaline treatment is appropriate for the more complex collagens found in bovine hides (Gómez-Guillén et al., 2002).

Generally, the traditional sources of gelatin are bovine and porcine skins and bones. The global demand for gelatin has been increasing over the years. Recent reports indicate that the annual world production of gelatin is nearly 326,000 tons, with pig skin derived gelatin accounting for the highest (44%) output, followed by bovine hides (28%), bones (27%), and other sources (1%) (GME, 2009). Gelatin from aquatic sources has been recognized to be free of viral or other infection or the risk of contamination with bovine spongiform encephalopathy (BSE) (Sadowska, Kolodziejka,

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& Niecikowska, 2003). Additionally, it can be used to replace porcine gelatin, which is prohibited for Muslims or Judaism. It is estimated that fish-processing waste after filleting accounts for approximately 75% of the total fish weight (Shahidi, 1994) and 30% of the waste is in the form of bones and skins (Gómez-Guillén et al., 2002). Those by-products can be served as the promising raw material for gelatin extraction. As a consequence, fish gelatin can be used widely, especially for food and pharmaceutical applications. To maximize the yield and the functionalities, the extraction process should be optimized to obtain the gelatin with desired properties.

Unicorn leatherjacket (*Aluterus monoceros*) belongs to the order Tetraodontiformes and is a member of the Monacanthidae family. This species has been used for fillet production in Thailand, especially for the export as frozen fillets. As a consequence, a large amount of skin has been produced as by-product. Due to its thick skin, it can be a potential source of gelatin. The objective of this study was to extract and characterize the gelatin from the skin of unicorn leatherjacket pretreated with different acids and extracted for various times.

## 2. Materials and methods

### 2.1. Chemicals

$\beta$ -Mercaptoethanol ( $\beta$ -ME) and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). High molecular weight markers were purchased from GE Healthcare UK (Buckinghamshire, UK). Folin–Ciocalteu's phenol reagent, acetic acid and phosphoric acid were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250 and *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). Food grade bovine gelatin was obtained from Halagel Co., Ltd. (Bangkok, Thailand).

### 2.2. Fish skin preparation

The skin of unicorn leatherjacket (*A. monoceros*) was obtained from Sea Wealth Frozen Food Co., Ltd., Songkhla, Thailand. Upon arrival to the Department of Food Technology, Prince of Songkla University, Hat Yai, the skin was cleaned and washed with iced tap water (0–2 °C). Prepared skin was then cut into small pieces (0.5 × 0.5 cm<sup>2</sup>), placed in polyethylene bags and stored at –20 °C until use. The storage time was less than 2 months.

### 2.3. Extraction of gelatin from the skin of unicorn leatherjacket

Before gelatin extraction, skin was soaked in 0.1 M NaOH with a skin/solution ratio of 1:10 (w/v). The mixture was stirred continuously for 2 h at 4 °C at a speed of 150 rpm using an overhead stirrer equipped with propeller (RW 20.n, IKA®-Werke GmbH & CO.KG, Staufen, Germany). The alkaline solution was changed every 1 h to remove non collagenous proteins and pigments. Alkaline-treated skin was then washed with tap water until neutral or faintly basic pH of wash water was obtained. The skin was then soaked in 0.2 M acetic acid or 0.2 M phosphoric acid with a skin/solution ratio of 1:10 (w/v) for 24 h with gentle stirring at 4 °C. The acidic solution was changed every 12 h to swell the collagenous material in the fish skin matrix. Acid-pretreated skin was washed thoroughly with tap water until wash water became neutral or faintly basic. To extract gelatin, the swollen skin was soaked in distilled water (45 °C) with a skin/water ratio of 1:10 (w/v) in a temperature-controlled water bath (W350, Memmert, Schwabach, Germany) for 4 and 8 h with a continuous stirring at a speed of 150 rpm. The mixture was then filtered using

two layers of cheesecloth. The filtrate was further filtered using a Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England) with the aid of JEIO Model VE-11 electric aspirator (JEIO TECH, Korea). The resultant filtrate was freeze-dried using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lyngby, Denmark). The dry matter extracted from skin pretreated with acetic acid and extracted for 4 and 8 h was referred to as 'GAA4' and 'GAA8', respectively. That extracted from skin pretreated with phosphoric acid and extracted for 4 and 8 h was referred to as 'GPA4' and 'GPA8', respectively. All gelatin samples were weighed, calculated for extraction yield and were also subjected to analyses.

### 2.4. Analyses

#### 2.4.1. Determination of ash content

Ash content was determined following the method of AOAC (2000) with the analytical number of 920.153. Ash content was expressed as % (dry weight basis).

#### 2.4.2. Electrophoretic analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970). Gelatin samples were dissolved in 5% SDS and the mixtures were incubated at 85 °C for 1 h. The mixtures were centrifuged at 5000 g for 5 min at room temperature using a microcentrifuge (MIK-RO20, Hettich Zentrifugan, Germany) to remove undissolved debris. Gelatin samples were mixed at a 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS and 20% glycerol). Samples (15 µg protein) were loaded onto polyacrylamide gels comprising a 7.5% running gel and a 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, the gel was stained with 0.05% (w/v) Coomassie Blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. High molecular weight markers (GE Healthcare UK, Buckinghamshire, UK) were used to estimate the molecular weight of the proteins. Food grade bovine gelatin was used as a standard gelatin.

#### 2.4.3. Fourier transform infrared (FTIR) spectra analysis

FTIR spectra of freeze-dried gelatin samples were recorded using a horizontal ATR Trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) equipped with a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) at room temperature. For spectra analysis, freeze-dried gelatin samples were placed on the crystal cell and the cell was clamped into the mount of the FTIR spectrometer. The spectra in the range of 400–4000 cm<sup>–1</sup> were ratioed and automatic signals gained were collected in 32 scans at a resolution of 4 cm<sup>–1</sup> against a background spectrum recorded from the clean empty cell at 25 °C.

#### 2.4.4. Measurement of $\zeta$ -potential

Gelatin samples were dissolved in distilled water at a concentration of 0.5 mg/ml. The mixture was stirred at room temperature for 6 h. The  $\zeta$ -potential of each sample (20 ml) was measured using a zeta potential analyzer (ZetaPALS, Brookhaven Instruments Co., Holtsville, NY, USA).  $\zeta$ -Potential of samples adjusted to different pHs with 1.0 M nitric acid or 1.0 M KOH using an autotitrator (BI-ZTU, Brookhaven Instruments Co., Holtsville, New York, USA) was determined. The pl was estimated from pH rendering  $\zeta$ -potential of zero.

#### 2.4.5. Determination of gel strength

Gels of gelatin were prepared by the method of Fernández-Daz, Montero, and Gómez-Guillén (2001) with a slight modification.

Gelatin samples were dissolved in distilled water at 60 °C to obtain the final concentration of 6.67% (w/v). The solution was stirred until the gelatin was solubilized completely and cooled in a refrigerator at 10 °C for 16–18 h for gel maturation. The dimensions of the sample were 3 cm in diameter and 2.5 cm in height. The gel strength of the sample gels prepared at 10 °C was determined using a Model TA-XT2 Texture Analyzer (Stable Micro System, Surrey, UK) with a load cell of 5 kN and equipped with a 1.27 cm diameter flat-faced cylindrical Teflon® plunger. The maximum force (in grams) was recorded when the penetration distance reached 4 mm. The speed of the plunger was 0.5 mm/s.

#### 2.4.6. Color measurement

The color of gelatin gels (6.67% w/v) was measured by a Hunter lab color meter (Color Flex, Hunter Lab Inc., Reston, VA, USA) reported by the CIE system.  $L^*$ ,  $a^*$  and  $b^*$  parameters indicate lightness/brightness, redness/greenness and yellowness/blueness, respectively. The colorimeter was warmed up for 10 min and calibrated with a white standard.

#### 2.4.7. Determination of solubility

Solubility of gelatin was determined at different pH following the method of [Montero, Jimenez-Colmenero, and Borderias \(1991\)](#) with a slight modification. The gelatin samples were dissolved in distilled water at 60 °C to obtain a final concentration of 2% (w/v) and the mixture was stirred at room temperature until the gelatins were completely solubilized. The gelatin solutions were adjusted to different pH (1–10) with either 6 N NaOH or 6 N HCl. The volume of solutions was made up to 10 ml with distilled water, previously adjusted to the same pH of gelatin solution. The solutions were centrifuged at 8500 g at room temperature for 10 min. Protein content in the supernatant was determined by the Lowry method ([Lowry, Rosebrough, Farr, & Randall, 1951](#)) using bovine serum albumin as a standard. Relative solubility was calculated in comparison with that obtained at the pH giving the highest solubility.

#### 2.4.8. Determination of emulsifying properties

Emulsion activity index (EAI) and emulsion stability index (ESI) of gelatin samples were determined according to the method of [Pearce and Kinsella \(1978\)](#) with a slight modification. Soy bean oil (2 ml) and gelatin solution (1, 2 or 3% gelatin, 6 ml) were homogenized using a homogenizer (Model T25 basic; IKA Labortechnik, Selangor, Malaysia) at a speed of 20,000 rpm for 1 min. Emulsions were pipetted out at 0 and 10 min and 100-fold diluted with 0.1% SDS. The mixture was mixed thoroughly for 10 s using a vortex mixer (Scientific Industries, Inc., Bohemia, NY, USA).  $A_{500}$  of the resulting dispersion was measured using a spectrophotometer (UV-160, Shimadzu, Kyoto, Japan). EAI and ESI were calculated by the following formulae ([Aewsiri, Benjakul, & Visessanguan, 2009](#)):

$$\text{EAI} \left( \text{m}^2/\text{g} \right) = (2 \times 2.303 \times A \times \text{DF}) / l \theta C$$

where  $A = A_{500}$ , DF = dilution factor (100),  $l$  = path length of cuvette (m),  $\theta$  = oil volume fraction and  $C$  = protein concentration in aqueous phase ( $\text{g}/\text{m}^3$ )

$$\text{ESI} (\text{min}) = A_0 / (A_0 - A_{10}) \times \Delta t$$

where  $A_0 = A_{500}$  at time of 0 min;  $A_{10} = A_{500}$  at time of 10 min and  $\Delta t = 10$  min.

#### 2.4.9. Determination of foaming properties

Foam expansion (FE) and foam stability (FS) of gelatin sample solutions were determined, as described by [Shahidi, Xiao-Qing, and Synowiecki \(1995\)](#), with a slight modification. Gelatin solution (1, 2

or 3%) was transferred into 100 ml cylinders. The solution was homogenized at a speed of 13,400 rpm for 1 min at room temperature. The sample was allowed to stand for 0, 20, 40 and 60 min. FE and FS were then calculated using the following equations:

$$\text{FE} (\%) = (V_T / V_0) \times 100$$

$$\text{FS} (\%) = (V_t / V_0) \times 100$$

where  $V_T$  = total volume after whipping;  $V_0$  = the original volume before whipping and  $V_t$  = total volume after leaving at room temperature for different times (20, 40 and 60 min).

### 2.5. Statistical analyses

All experiments were performed in triplicate and a completely randomized design (CRD) was used. Data were presented as means  $\pm$  standard deviation and the probability value of  $<0.05$  was considered significant. Analysis of variance (ANOVA) was performed and mean comparisons were done by Duncan's multiple range tests ([Steel & Torrie, 1980](#)). Analysis was performed using a SPSS package (SPSS 11.0 for windows, SPSS Inc, Chicago, IL, USA).

## 3. Results and discussion

### 3.1. Extraction yield and ash content

Gelatin extracted from skin of unicorn leatherjacket pretreated with 0.2 M acetic acid (GAA) or 0.2 M phosphoric acid (GPA) showed the different yields when different extraction times (4 and 8 h) were used. During heating at 45 °C, hydrogen bonds stabilizing triple helix of mother collagen were destroyed, leading to helix-to-coil transition. This resulted in the conversion of collagen to soluble gelatin ([Benjakul et al., 2009](#)). Additionally, some peptide bonds were cleaved. Yield was increased as the extraction times increased from 4 to 8 h ( $P < 0.05$ ). Yields of GAA4, GAA8, GPA4 and GPA8 were 5.23, 9.18, 6.12 and 11.54% (wet weight basis), respectively. The higher yields of GAA8 and GPA8 were attributed to the sufficient time in disrupting the bonds of collagen. The results were in accordance with [Arnesen and Gildberg \(2007\)](#) who reported that increasing extraction times were associated with the increase in yield of gelatin from Atlantic salmon skin. At the same extraction time, GPA showed the higher yield than did GAA. Even at the same concentration (molarity), phosphoric acid with higher ionic strength and lower pH could facilitate the swelling process caused by more repulsive force among the collagen molecules compared with acetic acid. With the loosen structure of swollen collagen, warm water could penetrate into the skin matrix effectively ([Stainsby, 1987](#)). Phosphoric acid pretreatment more likely provided a greater swelling power for destabilization of acid labile cross-links at the telopeptide region and amide bonds of the triple helical structure of collagen as well as non-covalent intra- and inter-molecular bonds, compared with acetic acid ([Gómez-Guillén & Montero, 2001](#)). Though the swelling took place, gelatin could not be completely extracted due to a large number of cross-links at the telopeptide region as well as inter-molecular cross-links, leading to the compact structure ([Ahmad, Benjakul, & Nalinanon, 2010](#)). Thus, swelling process affecting the extraction efficiency was dependent on the types of acid used.

Ash contents of gelatin, both GPA than GAA, were quite high when compared with the recommended value (2.5%) ([Jones, 1977](#)). Ash contents were 8.10 and 12.3% (dry weight basis) for GAA4 and GAA8, respectively. GPA4 and GPA8 had the ash content of 12.4 and 15.45%, respectively. High ash content in all gelatin samples indicated the presence of inorganic salt, which might be generated

during the pretreatment with either alkali or acid. Phosphates incorporated into the gelatin pretreated with phosphoric acid more likely contributed to the higher ash content in the resulting gelatin.

### 3.2. Protein patterns

Protein patterns of gelatins extracted from the skin of unicorn leatherjacket with different acid pretreatments for different extraction times are shown in Fig. 1. Different protein patterns were noticeable when different extraction times were used. With increasing extraction times, the degradation of major components with the concomitant formation of low molecular weight peptides was observed. For GAA, high intensity of  $\gamma$ -,  $\beta$ - and  $\alpha$ -chains was still found when extraction times of up to 4 h were used. Nevertheless, those components were hydrolyzed to higher extent as the extraction time increased. The  $\alpha$ -chain was still noticed in GAA extracted with extraction time up to 12 h. Gelatins with high content of  $\alpha$ -chain were reported to possess better functional properties (Gómez-Guillén et al., 2002). For GPA, the greater amount of peptides with molecular weight lower than  $\alpha$ -chains was generated as the extraction time increased. At all extraction times used, GPA contained the lower band intensity for all major bands including  $\alpha$ -,  $\beta$ -, and  $\gamma$ -chains, while the proteins with low molecular weight were dominant. In general, the formation of peptide fragments is associated with the low viscosity, low melting point, low setting point, high setting time, as well as decreased

bloom strength of gelatin (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2006). For both GAA and GPA,  $\beta$ -chain and  $\alpha$ -chain were found as the major components, especially when extraction time was less than 4 h. The protein patterns were different from those of commercial bovine gelatin, in which proteins with molecular weight of 145,000 and 141,000 Da were the major components. This was probably due to the different pretreatment and extraction conditions. In gelatin manufacture, the conversion of collagen to gelatin yields the molecules of varying mass, due to the cleavage of inter-chain cross-links and some unfavorable breakages of inter-chain peptide linkage (Zhou, Mulvaney, & Regenstein, 2006). From the result, GAA and GPA extracted for the extended times were more prone to degradation, in which shorter chains were obtained. Acid used for pretreatment played a role in protein components of resulting gelatin. When phosphoric acid was used, the highly loosen structure might be more susceptible to hydrolysis during extraction at high temperature. Thus extraction time and pretreatment had the influence on the components and the length of the polypeptide chains. Since the excessive extraction time yielded the gelatin with the remarkable degradation, GAA and GPA extracted for 4 and 8 h were prepared and used for further characterization.

### 3.3. ATR-FTIR analysis

FTIR spectra of gelatin extracted from the skin of unicorn leatherjacket with different acid pretreatments and extraction times are depicted in Fig. 2. Spectra of GAA4, GAA8, GPA4 and GPA8 displayed the major peaks in amide region. These spectra were in accordance with those reported by Muyonga, Cole, and Duodu (2004). FTIR spectroscopy has been used to monitor the changes in the functional groups and secondary structure of gelatin samples (Muyonga et al., 2004). GAA4, GAA8, GPA4 and GPA8 exhibited the amide I bands at the wavenumber of 1634.73, 1649.11, 1642.59, and 1646.51  $\text{cm}^{-1}$ , respectively. The amide I vibration mode is primarily a C=O stretching vibration coupled to contributions from the CN stretch, CCN deformation and in-plane NH bending modes (Bandekar, 1992). The absorption peak at amide I (1634.73  $\text{cm}^{-1}$ ) was characteristic for the coil structure of gelatin (Yakimets et al., 2005). The spectral differences in amide I of different gelatin samples were largely attributed to different conformation of polypeptide chains. Amide I peaks of GAA8 and GPA8 shifted to higher

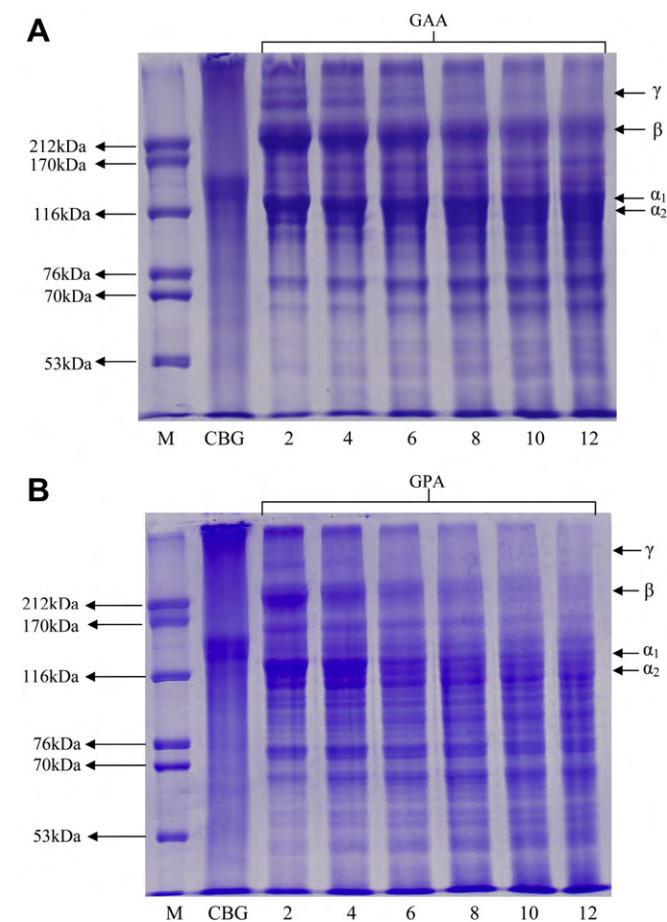


**Fig. 1.** SDS-PAGE pattern of gelatin extracted from the skin of unicorn leatherjacket with different acid pretreatment using 0.2 M of acetic acid (A) or phosphoric acid (B) and extraction times. M: high molecular weight markers; CBG: commercial bovine gelatin. Numbers denote the extraction times (h).

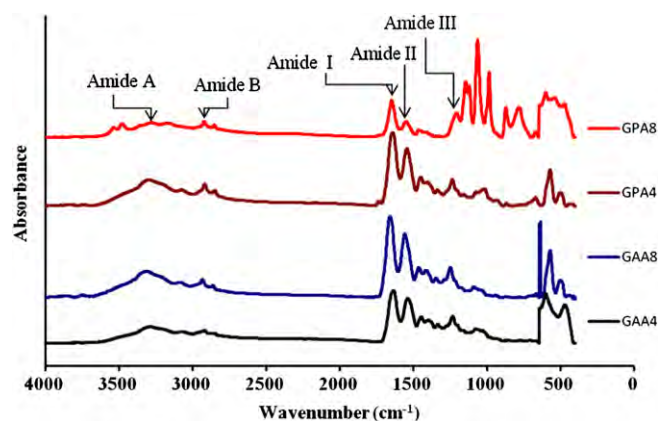


**Fig. 2.** Fourier transform infrared spectra of gelatin from the skin of unicorn leatherjacket with different acid pretreatment and extraction times. GAA4 and GAA8: gelatin extracted from the skin of unicorn leatherjacket pretreated with 0.2 M acetic acid and extracted for 4 and 8 h, respectively. GPA4 and GPA8: gelatin extracted from the skin of unicorn leatherjacket pretreated with 0.2 M phosphoric acid and extracted for 4 and 8 h, respectively.

frequency, compared to those of GAA4 and GPA4. This indicated the greater loss of molecular order due to thermal uncoupling of inter-molecular cross-links as the longer extraction was used. In addition, GAA4 showed the lower amplitude compared to GAA8, indicating the greater disruption of intra-molecular bonding and more exposure of C=O of GAA8. Lower amplitude was associated with the higher extent of molecular order due to interaction of C=O with adjacent chains via hydrogen bond. Nevertheless, GPA4 showed the higher amplitude than did GPA8. GPA8 most likely lost the secondary structure during extraction, while aggregation might occur to some extent during freeze-drying. The decrease in peak intensity and narrowing of peak area might reflect the association of peptide fragments (Prystupa & Donald, 1996). Since, GAA8 and GPA8 contained a higher amount of unordered and low molecular weight peptides due to longer extraction time, the reactive groups (C=O) were more exposed and became more reactive between  $\alpha$ -chains, especially during freeze-drying.

The characteristic absorption bands of GAA4, GAA8, GPA4 and GPA8 in amide II region were noticeable at the wavenumbers of 1539.69, 1547.49, 1545.77 and 1549.87  $\text{cm}^{-1}$ , respectively. The amide II vibration modes are attributed to an out-of-phase combination of CN stretch and in-plane NH deformation modes of the peptide group (Bandekar, 1992; Lavalie, Adams, & Levin, 1982). Lower amplitude was found in GPA8, compared to GAA8, GPA4 and GAA4, indicating that N–H was more involved in bonding with the adjacent  $\alpha$ -chains. In addition, amide III was detected around the wavenumber of 1234.81, 1237.74, 1236.99, 1206.03  $\text{cm}^{-1}$  for GAA4, GAA8, GPA4 and GPA8, respectively. The amide III represented the combination peaks between C–N stretching vibrations and N–H deformation from amide linkages as well as absorptions arising from wagging vibrations from  $\text{CH}_2$  groups from the glycine backbone and proline side-chains (Jackson, Choo, Watson, Halliday, & Mantsch, 1995). GAA8 had the lower amplitude at amide III region than GAA4. However, GPA8 exhibited the higher amplitude of amide III band than GPA4. The greater disorder of molecular structure due to transformation of an  $\alpha$ -helical to a random coil structure occurred during heating and these changes were associated with loss of triple-helix state as a result of denaturation of collagen to gelatin (Muyonga et al., 2004). GPA8 also had the high intensity peaks at wavenumbers of 1144.04, 1119.62, 1063.38 and 985.59  $\text{cm}^{-1}$ . These bands were associated with the C–O stretching vibrations of the short peptide chains (Jackson et al., 1995). The occurrence of those peaks was coincidental with more degradation of peptide chains in GPA8 sample (Fig. 1). In addition, the prominent absorption bands in GPA4 and GPA8 around the wavenumber of 1074.06 and 1063.38  $\text{cm}^{-1}$  respectively, most likely arose from asymmetric stretching vibrations of phosphate groups of phosphorylated proteins coupled to  $-\text{CH}_2$  of the amino acid residues (Jackson et al., 1995).

Moreover, the amide A band, arising from the stretching vibrations of N–H group, appeared at 3293.18, 3300.66, 3298.23 and 3275.53  $\text{cm}^{-1}$  for GAA4, GAA8, GPA4 and GPA8, respectively. Normally, a free N–H stretching vibration occurs in the range of 3400–3440  $\text{cm}^{-1}$ . When the N–H group of a peptide is involved in a hydrogen bond, the position is shifted to lower frequencies (Doyle, Blout, & Bendit, 1975). At amide A region, the lower amplitude as well as the lower wavenumber were found in GPA8, compared to GPA4, indicating that N–H group of shorter peptide fragments in GPA8 was involved in hydrogen bonding. GPA8 might also form new covalent inter-molecular cross-links during freeze-drying. The amide A also tends to join with the  $\text{CH}_2$  stretch peak when carboxylic acid groups exist in a dimeric inter-molecular interaction (Kemp, 1987). The amide B was observed at 3073.13, 3075.22, 3076.61 and 3165.97  $\text{cm}^{-1}$  for GAA4, GAA8, GPA4 and GPA8, respectively, corresponding to asymmetric stretch vibration

of  $=\text{C}-\text{H}$  as well as  $-\text{NH}_3^+$ . Among all samples, GPA8 showed the lowest wavenumber of amide B peak, suggesting the interaction of  $-\text{NH}_3$  group between peptide chains. Thus, it can be concluded that the secondary structure of gelatins obtained from the skin of unicorn leatherjacket was affected by acid pretreatment and extraction time. The low molecular weight peptides formed during the extraction for long time were more likely able to form covalent cross-links during freeze-drying process.

### 3.4. Measurement of $\zeta$ -potential

The  $\zeta$ -potential values of GAA and GPA were measured as a function of pH as shown in Fig. 3. Net charge of zero was obtained at pH 7.26 and 6.78 for GPA4 and GPA8, respectively. Net charge of GAA4 and GAA8 became zero at pH of 7.15 and 6.64, respectively. At the pH near the isoelectric point, both GAA and GPA had the decreased repulsion with increased hydrophobic interaction among gelatin molecules. At pl, the positive charges are balanced out by the negative charges (Bonner, 2007). The isoelectric points of GPA8 and GAA8 were in acidic range. With increasing extraction time, deamidation might take place, in which glutamic acid and aspartic acid could be formed. This led to the increase in acidic amino acids associated with the decrease in pl. Furthermore, the difference in the pl between GAA and GPA might be due to the differences in reactive groups, which could accept or donate proton differently at various pH. The result also suggested that different acid pretreatments also had the impact on pl of resulting gelatin to some degree.

### 3.5. Solubility

High solubility of both GAA and GPA was observed in the wide pH range (1–10). The relative solubility of both gelatins was greater than 90% at all pH tested. Both GAA and GPA showed the lower relative solubility at pH 8.0 ranging from 90.4 to 91.1% and 91.8 to 92.4%, respectively. At pH close to pl, hydrophobic interaction among gelatin molecules increased, and the total net charge of protein molecules became zero, which was associated with the decreased repulsion. The higher solubility at pH far away from pl can be attributed to increased electrostatic repulsion of the protonated or deprotonated gelatin molecules. Normally, gelatin type-A or acid-processed gelatin has isoelectric points varying from 6.5 to 9.0 (Foegeding, Lanier, & Hultin, 1996). During acid pretreatment of skin, some glutamine and asparagine can be converted to their acidic forms, i.e. glutamic acid and aspartic acid, respectively (Jamilah & Harvinder, 2002). Bovine bone gelatin had the lowest solubility at pH 5 (90.6%). Bovine gelatin has been identified as type-B gelatin with pl of approximately 4.88 (Zhang, Li,

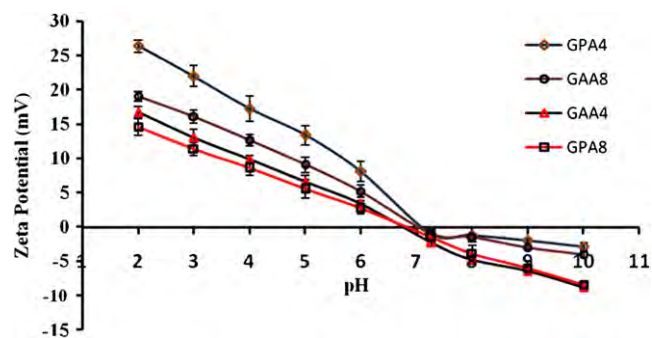


Fig. 3. Zeta potential of gelatin from the skin of unicorn leatherjacket with different acid pretreatment and extraction times. Key: see the caption for Fig. 2.

**Table 1**

Gel strength and color of gelatin from the skin of unicorn leatherjacket with different acid pretreatment and extraction times.

| Properties       | Commercial bovine gelatin | GPA4           | GPA8           | GAA4          | GAA8          |
|------------------|---------------------------|----------------|----------------|---------------|---------------|
| Gel strength (g) | 293.22 ± 5.71A            | 149.77 ± 1.22B | 121.92 ± 3.76C | 56.90 ± 3.23D | 51.73 ± 0.77E |
| Color            |                           |                |                |               |               |
| $L^*$            | 35.85 ± 2.44B             | 34.35 ± 1.12B  | 45.89 ± 2.19A  | 30.67 ± 2.34D | 18.26 ± 0.97C |
| $a^*$            | −0.77 ± 0.14C             | 0.37 ± 0.05B   | 3.33 ± 1.21A   | 0.54 ± 0.41B  | −0.69 ± 0.11C |
| $b^*$            | 18.29 ± 1.96A             | 2.16 ± 0.19C   | 5.47 ± 1.01B   | 0.83 ± 1.23D  | −4.60 ± 1.23E |

Mean ± SD ( $n = 3$ ). Key: see the caption for Fig. 2.Different capital letters in the same row indicate significant differences ( $P < 0.05$ ).

& Shi, 2006). The difference in solubility of different gelatins might result from the differences in molecular weight and the content of polar and non-polar groups in amino acids (Zayas, 1997). Due to high solubility of all gelatin samples at pH 1–10, they can be used widely and effectively since solubility is a prerequisite for most functionality of food proteins.

### 3.6. Gel strength

GAA and GPA extracted with different times had the varying gel strength (Table 1). Gel strength of gelatin extracted decreased as the extraction times increased, regardless of acid pretreatments ( $P < 0.05$ ). At the same extraction time, GPA exhibited the higher gel strength than did GAA ( $P < 0.05$ ). The gel strength varied considerably with the acid treatments used for swelling process from the skin of dover sole (Giménez, Turnay, Lizarbe, Montero, & Gómez-Guillén, 2005). The difference in bloom values might also be governed by molecular weight distribution, as well as by complex interactions determined by the amino acids composition and the ratio of  $\alpha/\beta$ -chains present in the gelatin. For gelatin from the skin of unicorn leatherjacket, it was found that the content of major components including  $\beta$ - and  $\alpha$ -chains was not the major factor governing the gel formation of gelatin. The configuration of protein and the way the inter-junction was developed to form the stronger network were crucial for gel formation. Location of imino acids in the peptide chain also contributed to gel formation. Limited hydrolysis, in which the free  $\alpha$ -chains and some oligomers could be released, might favor the aggregation or cross-links, mainly via hydrogen bond. In addition, during acidic pretreatment, some phosphate groups might attach with some amino acids, leading to phosphorylation of GPA4 and GPA8. Phosphorylation may introduce the ionic interaction between phosphate groups and  $-\text{NH}_3^+$  of amino acids, increasing the cross-links of proteins (Guo, Li, Zhu, & Zhao, 2005). Since, GPA8 had the shorter chain length, the network formed might not be strong, and even some ionic interaction could be enhanced. However, both GAA (GAA4 and GAA8) and GPA (GPA4 and GPA8) had the lower gel strength than did commercial bovine gelatin (293.22 g) ( $P < 0.05$ ) (Table 1). This was most likely governed by the differences in the imino acid content. Mammalian gelatins generally have the high amount of hydroxyproline and proline (Benjakul et al., 2009). Fish gelatins have lower hydroxyproline contents (about 7–10% of the total amino acids) compared with that of bovine gelatin (14%) (Nalinanon, Benjakul, Visessanguan, & Kishimura, 2008). The gelling properties of gelatin were also influenced by the source of raw materials, which vary in proline and hydroxyproline contents (Jongjareonrak et al., 2006). Hydroxyl groups of amino acids, especially hydroxyproline, play a part in inter-chain hydrogen bonding via a bridging water molecule as well as direct hydrogen bonding to the carbonyl group (Wong, 1989).

### 3.7. Color of gel

Gel color of gelatin extracted from the skin of unicorn leatherjacket with different acid pretreatments and various extraction

times is shown in Table 1. Higher  $L^*$ -value of GPA8 gel was observed, compared with GPA4 ( $P < 0.05$ ) (Table 1). On the other hand,  $L^*$ -value decreased as GAA was extracted for a longer time ( $P < 0.05$ ). Marked increase in  $L^*$ -value of GPA8 was most likely associated with the greater aggregation via  $-\text{NH}_3^+$  group during freeze-drying. The larger aggregate exhibited the greater light scattering effect. However, GAA8 with lower chain length showed the low  $L^*$ -value, suggesting the clearer solution of gelatin before gelation. Gelatin manufacture generally has a good process to clarify the impurities from the gelatin solution, such as chemical clarification and filtration processes. For redness ( $a^*$ -value) and yellowness ( $b^*$ -value), GPA8 showed the higher  $a^*$ - and  $b^*$ -values than did GPA4 ( $P < 0.05$ ). Higher degradation of GPA, especially when extraction time increased, might provide more  $-\text{NH}_3^+$  group, which underwent Maillard reaction to a higher extent. Conversely, the decreases in  $a^*$ - and  $b^*$ -values were found in GAA as the extraction time increased. With acetic acid pretreatment, pigments in the skin were more likely removed to a greater extent, compared with that pretreated with phosphoric acid. Color did not affect functional properties of gelatin (Ockerman & Hansen, 1988).

### 3.8. Emulsifying properties of gelatin

Emulsion activity index (EAI) and emulsion stability index (ESI) of GAA and GPA from the skin of unicorn leatherjacket are presented in Table 2. EAI and ESI of GPA (GPA4 and GPA8) were higher than GAA (GAA4 and GAA8). For the same gelatin, EAI decreased when the extraction time increased ( $P < 0.05$ ). With increasing degradation, peptides with the shorter chain, which had more hydrophilicity were preferably localized in aqueous phase. As a result, the lower portion of gelatin migrated to oil–water

**Table 2**

Emulsifying properties of gelatin from the skin of unicorn leatherjacket with different acid pretreatment and extraction times.

| Samples | Concentration of gelatin (%) | Emulsion activity index ( $\text{m}^2/\text{g}$ ) | Emulsion stability index (min) |
|---------|------------------------------|---------------------------------------------------|--------------------------------|
| GPA4    | 1                            | 39.17 ± 0.33aA                                    | 8.58 ± 0.25bC                  |
|         | 2                            | 20.34 ± 0.09aB                                    | 10.35 ± 0.12aB                 |
|         | 3                            | 15.16 ± 0.19bC                                    | 39.63 ± 0.17aA                 |
| GPA8    | 1                            | 35.21 ± 0.78cA                                    | 12.58 ± 0.64aB                 |
|         | 2                            | 18.87 ± 0.60bB                                    | 10.59 ± 0.45aC                 |
|         | 3                            | 16.86 ± 0.43aC                                    | 35.81 ± 0.55bA                 |
| GAA4    | 1                            | 37.48 ± 1.38bA                                    | 9.98 ± 1.21bB                  |
|         | 2                            | 19.72 ± 0.67abB                                   | 10.28 ± 0.21aB                 |
|         | 3                            | 13.49 ± 0.09cC                                    | 35.74 ± 0.24bA                 |
| GAA8    | 1                            | 33.63 ± 0.10dA                                    | 9.19 ± 0.45bB                  |
|         | 2                            | 16.33 ± 0.41cB                                    | 10.38 ± 0.85aB                 |
|         | 3                            | 16.82 ± 0.11aB                                    | 13.10 ± 0.65cA                 |

Mean ± SD ( $n = 3$ ). Key: see the caption for Fig. 2.Different letters in the same column within the same concentration indicate significant differences ( $P < 0.05$ ).Different capital letters in the same column within the same gelatin sample indicate significant differences ( $P < 0.05$ ).

**Table 3**

Foaming properties of gelatin from the skin of unicorn leatherjacket with different acid pretreatment and extraction times.

| Samples | Concentration of gelatin (%) | Foam expansion (%)            | Foam stability (%)            |                               |                              |
|---------|------------------------------|-------------------------------|-------------------------------|-------------------------------|------------------------------|
|         |                              |                               | 20 min                        | 40 min                        | 60 min                       |
| GPA4    | 1                            | 116.35 ± 0.23bC <sup>W</sup>  | 111.11 ± 0.12bC <sup>X</sup>  | 110.0 ± 0.32bC <sup>Y</sup>   | 108.00 ± 0.51bC <sup>Z</sup> |
|         | 2                            | 133.05 ± 0.34bB <sup>W</sup>  | 130.52 ± 0.25bB <sup>X</sup>  | 115.15 ± 0.43bB <sup>Y</sup>  | 111.00 ± 0.54bB <sup>Z</sup> |
|         | 3                            | 150.33 ± 0.16aA <sup>W</sup>  | 132.97 ± 0.24cA <sup>X</sup>  | 121.05 ± 0.53cA <sup>Y</sup>  | 119.58 ± 0.67bA <sup>Z</sup> |
| GPA8    | 1                            | 126.32 ± 2.23aB <sup>W</sup>  | 121.11 ± 3.45aC <sup>Wx</sup> | 118.34 ± 3.40aC <sup>xy</sup> | 115.56 ± 2.34aC <sup>X</sup> |
|         | 2                            | 147.54 ± 2.12aA <sup>W</sup>  | 141.13 ± 2.34aB <sup>X</sup>  | 134.23 ± 2.17aB <sup>Y</sup>  | 128.98 ± 2.12aB <sup>Z</sup> |
|         | 3                            | 144.65 ± 3.45bA <sup>Wx</sup> | 147.35 ± 0.34aA <sup>W</sup>  | 143.67 ± 2.34aA <sup>Wx</sup> | 139.46 ± 3.78aA <sup>X</sup> |
| GAA4    | 1                            | 110.34 ± 3.23cC <sup>W</sup>  | 106.34 ± 2.47cC <sup>Wx</sup> | 102.34 ± 2.13cC <sup>xy</sup> | 98.86 ± 2.23cC <sup>Y</sup>  |
|         | 2                            | 129.34 ± 2.33cB <sup>W</sup>  | 115.34 ± 2.34dB <sup>X</sup>  | 109.34 ± 2.89cB <sup>Y</sup>  | 107.34 ± 4.57cB <sup>Y</sup> |
|         | 3                            | 139.34 ± 4.32bA <sup>W</sup>  | 124.54 ± 5.62dA <sup>X</sup>  | 119.45 ± 2.87cA <sup>xy</sup> | 115.45 ± 3.45bA <sup>Y</sup> |
| GAA8    | 1                            | 118.15 ± 0.22bC <sup>W</sup>  | 115.07 ± 0.43bC <sup>X</sup>  | 113.09 ± 0.65bC <sup>Y</sup>  | 111.05 ± 0.32bC <sup>Z</sup> |
|         | 2                            | 128.15 ± 0.29cB <sup>W</sup>  | 126.04 ± 0.55cB <sup>X</sup>  | 118.55 ± 0.55bB <sup>Y</sup>  | 115.09 ± 0.54bB <sup>Z</sup> |
|         | 3                            | 152.63 ± 0.31aA <sup>W</sup>  | 141.41 ± 0.32bA <sup>X</sup>  | 138.04 ± 0.77bA <sup>Y</sup>  | 135.06 ± 0.39aA <sup>Z</sup> |

Mean ± SD (*n* = 3). Key: see the caption for Fig. 2.Different letters in the same column within the same concentration indicate significant differences (*P* < 0.05).Different capital letters in the same column within the same gelatin sample indicate significant differences (*P* < 0.05).Different superscripts in the same row indicate significant differences (*P* < 0.05).

interface. At a level of 3%, ESI of gelatin extracted for a longer time was lower than those of gelatin with shorter extraction time (*P* < 0.05). The highest ESI of GPA4, GPA8, GAA4 and GAA8 was found at the level of 3% (*P* < 0.05). In case of GAA8, the aggregated proteins might be rigid and could not unfold rapidly at the interface and form a film around an oil droplet effectively. At a level of 3%, emulsions containing GPA4 were more stable than those of the GAA4 (*P* < 0.05). Additionally, GPA8 also exhibited the higher ESI than GAA8 at the level of 3% (*P* < 0.05). However, no difference in ESI was found between GPA8 and GAA8 at the level of 2% (*P* > 0.05). Phosphate groups incorporated into gelatin could stabilize the protein film at the interface via ionic interaction more effectively. GPA4 and GPA8 possesses surface activity at the oil–water interface, thereby having the ability to facilitate the formation and stabilization of fine droplets during and after emulsification. The stabilization of emulsion against coalescence/flocculation is greatly dependent on the force of electrostatic repulsions between the adsorbed proteins on the interfacial protein film. Surh, Decker, and McClements (2006) found that the oil-in-water emulsion prepared with high molecular weight fish gelatin (120 kDa) was more stable than that prepared with low molecular weight fish gelatin (50 kDa). Thickness of an adsorbed gelatin membrane increased with increasing molecular weight. This was associated with the increased stability of emulsions to coalescence during homogenization (Lobo & Svereika, 2003). EAI and ESI of bovine bone gelatin were higher than those of all fish skin gelatin samples (Aewsiri et al., 2009). This possibly resulted from the differences in the intrinsic properties, composition and conformation of gelatins from both sources (Benjakul et al., 2009).

### 3.9. Foaming properties of gelatin

Foam expansion (FE) and foam stability (FS) of GAA (GAA4 and GAA8) and GPA (GPA4 and GPA8) at different concentrations are shown in Table 3. FE and FS of both gelatins mostly increased with increasing gelatin concentrations (*P* < 0.05). However, for GPA8, no difference in FE was observed between 2 and 3% (*P* > 0.05). Foams with higher concentration of proteins were denser and more stable because of an increase in the thickness of interfacial films (Zayas, 1997). At the concentration lower than 2%, GAA had the lower FE than GPA (*P* < 0.05). GPA with the shorter chains could migrate to the air–water interface more effectively than GAA, having the larger proportion of β- and α-chains. FS increased with protein

concentrations, depending on the protein surface properties. The foaming ability of proteins is related to their film-forming ability at the air–water interface. In general, proteins, which rapidly adsorb at the newly created air–liquid interface during bubbling, unfolding and molecular rearrangement at the interface, exhibit better foaming ability than proteins that adsorb slowly and resist unfolding at the interface (Damodaran, 1997). The stability of foams depends on various parameters, such as the rate of attaining equilibrium surface tension, bulk and surface viscosities, steric stabilization, and electrical repulsion between the two sides of the foam lamella (Liu, Xu, Yuan, & Jiang, 2003). Foam stability of GAA and GPA can be explained by the combined effect of cohesive forces between the molecules in the foam lamella, due to the hydrophobic groups, and the prevention of drainage in the lamella, which was achieved only by the gelatin molecules present in the lamella and anchored into the interface by the long hydrophobic chains. Gravitational drainage of liquid from the lamella and disproportionation of gas bubbles via inter bubble gas diffusion contribute to instability of foams (Aewsiri et al., 2009). Coalescence of bubbles occurs because of liquid drainage from the lamella film as two gas bubbles approach each other, leading to film thinning and rupture (Damodaran, 2005). Thus, acid pretreatments and extraction times also had impact on interfacial properties of gelatin from skin of unicorn leatherjacket.

### 4. Conclusion

GAA and GPA with different characteristics could be extracted with warm water after acid pretreatment. Phosphoric acid used in swelling process was found to be efficient disrupter of skin tissue, compared to acetic acid. However, the extraction for a longer time could shorten the gelatin chain. Additionally, type of acid for pretreatment also had the impact on functional properties of resulting gelatin. The gel strength of GPA was higher than that of GAA and interfacial properties of both gelatins were somehow affected by extraction time. Therefore, the skin of unicorn leatherjacket could serve as raw material for gelatin extraction after the appropriate acid pretreatment and with proper extraction time.

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# Comparative study on protein cross-linking and gel enhancing effect of microbial transglutaminase on surimi from different fish

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## Abstract

**BACKGROUND:** Microbial transglutaminase (MTGase) has been used to increase the gel strength of surimi. Nevertheless, its effectiveness varies with fish species. The aim of this study was to elucidate the effect of MTGase at different levels on protein cross-linking and gel property of surimi from threadfin bream, Indian mackerel and sardine in the presence and absence of endogenous transglutaminase.

**RESULT:** Breaking force of all surimi gels increased as MTGase levels ( $0\text{--}0.6\text{ U g}^{-1}$ ) increased except for threadfin bream surimi gel, where the breaking force decreased at  $0.6\text{ U g}^{-1}$  ( $P < 0.05$ ). In the presence of EDTA, the gel strengthening effect was lower, suggesting the combined effect of endogenous transglutaminase with MTGase. With the addition of MTGase, the gel with the highest increase in breaking force showed highest decrease in myosin heavy chain. When cross-linking activity of MTGase on natural actomyosin (NAM) was determined, the highest decreasing rate in  $\epsilon$ -amino group content with the concomitant increased formation of cross-linked proteins was found in NAM from threadfin bream. The reactivity of muscle proteins toward MTGase-induced cross-linking was in agreement with surimi gel strengthening.

**CONCLUSION:** The composition and properties of muscle proteins of varying fish species more likely determined protein cross-linking induced by MTGase, thereby affecting their gel properties.

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**Keywords:** microbial transglutaminase; surimi; protein cross-linking; gel forming ability

## INTRODUCTION

Transglutaminase (TGase, EC 2.3.2.13) is a transferase which is able to catalyse protein cross-linking by an acyl transfer reaction.<sup>1</sup> The  $\gamma$ -carboxamide group of glutamine residue in protein serves as an acyl donor, while the amino group of primary amines or  $\epsilon$ -amino group of lysine residues acts as an acyl acceptor. The reaction results in the formation of  $\epsilon$ -( $\gamma$ -glutamyl) lysine isopeptide, providing the cross-linked proteins or peptides.<sup>2</sup> The enzyme can be found in various living tissues, such as found in microorganisms, vertebrates, invertebrates and plants.<sup>3</sup> TGase-catalysed reaction can be used to modify the functional properties of food proteins. TGase plays an important role in cross-linking of fish muscle proteins pre-incubated at  $25\text{--}40^\circ\text{C}$ , resulting in the improved gel quality.<sup>4</sup> Seki *et al.*<sup>5</sup> reported that endogenous TGase contributes to the polymerisation of myosin heavy chain of pollack surimi. Increases in  $\epsilon$ -( $\gamma$ -glutamyl) lysine bond, which is a product of TGase reaction, in salted fish meat paste during setting have been reported.<sup>6,7</sup>

Surimi is concentrated myofibrillar protein obtained from mechanically deboned fish flesh, which is washed with cold water. Theoretically, any fish can be used to produce surimi but properties of surimi gel vary, depending on fish species. Both intrinsic and extrinsic factors including species, freshness, endogenous enzyme, processing parameters, protein concentration, pH, ionic strength, and temperature determine gelling properties of surimi.<sup>4,8,9</sup> In general, lean fish have been used for surimi production in Thailand.

Threadfin bream has been widely used for surimi production because of its white colour, good flavour and strong gel-forming ability. Its myofibrillar proteins are also highly stable in frozen storage.<sup>10</sup> Owing to insufficient amounts of these lean fish as raw material, dark-fleshed pelagic fish such as sardine and mackerel have gained attention for surimi production. Although they are cheaper, their high content of dark muscle associated with the high content of lipid and myoglobin makes them difficult to yield high-quality surimi as evidenced by poor gel-forming ability of those species.<sup>11</sup> Since about 40% of the total fish catch in the world is dark-muscle pelagic species, there is great interest in developing methods that make use of those species for human consumption.<sup>12</sup> To improve the setting of surimi, microbial transglutaminase (MTGase) has been widely used to induce the polymerisation of proteins, thereby increasing the gel strength of surimi.<sup>13,14</sup> Asagami *et al.*<sup>15</sup> found that the amount of MTGase

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added highly depended on fish species and some other factors such as freshness, protein quality, and harvesting season. The gel strengthening effect of MTGase was lower when surimi was produced from lizardfish that was not fresh.<sup>16</sup> This might be a drawback for the surimi industry to achieve gel improvement of surimi by MTGase. A better understanding of the gel enhancing effect of MTGase toward surimi from different fish species could lead to a maximised improvement of surimi gel quality. Thus, the objective of this study was to investigate the impact of MTGase on protein cross-linking and gel forming ability of surimi from different fish species, including threadfin bream, Indian mackerel, and sardine.

## MATERIAL AND METHODS

### Chemicals and enzyme

All chemicals for gel preparation and analyses were of analytical grade. 2,4,6-Trinitrobenzenesulfonic acid (TNBS), *N*-Carbobenzoxy(CBZ)-L-glutaminylglycine, sodium dodecyl sulfate (SDS),  $\beta$ -mercaptoethanol, glycerol, high-molecular-weight marker, and glutaraldehyde were purchased from Sigma (St Louis, MO, USA). *N,N,N',N'*-Tetramethyl ethylene diamine (TEMED), acrylamide, and bisacrylamide were procured from Fluka (Buchs, Switzerland). Sodium hydrogen carbonate was obtained from Merck (Darmstadt, Germany). Microbial transglutaminase (MTGase) from *Streptovorticillum mobaraense* (TG-K) containing 10 g kg<sup>-1</sup> of pure enzyme was supplied by Ajinomoto (Thailand) Co., Ltd. (Bangkok, Thailand).

### Fish samples

Fresh threadfin bream (*Nemipterus furcosus*), Indian mackerel (*Rastrelliger kanagurta*), and sardine (*Sardinella gibbosa*) with the sizes of 100–120, 140–160 and 50–60 g per fish, respectively, were purchased from a dock in Songkhla province, Thailand. The fish, off-loaded approximately 36–48 h after capture, were transported in ice with a fish/ice ratio of 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand, within 3 h. The fish were immediately washed and drained before using for the preparation of surimi.

### Preparation of surimi

Fish were beheaded and eviscerated, and then washed. Fish skin and bone were removed manually and the flesh was minced to uniformity using a mincer with a hole diameter of 5 mm. The mince was then washed with cold water (5–8 °C), using a mince/water ratio of 1:3 (w/w). The mixture was stirred gently for 3 min and washed mince was filtered with a layer of nylon screen. The washing process was carried out three times. Finally, the washed mince was subjected to centrifugation using a Model CE 21 K basket centrifuge (Grandiumpiant, Belluno, Italy) with a speed of 700  $\times g$  for 15 min. Washed mince was mixed thoroughly with 40 g kg<sup>-1</sup> sorbitol and 40 g kg<sup>-1</sup> sucrose. The mixture (500 g) was packed in a polyethylene bag, kept at –18 °C and was referred to as 'frozen surimi'. The frozen surimi was used within 2 weeks of frozen storage.

### Preparation of crude microbial transglutaminase

Crude MTGase was prepared according to the method of Visessanguan *et al.*<sup>17</sup> with a slight modification. Commercial enzyme powder (4 g) was dissolved with 20 mL of 20 mmol kg<sup>-1</sup> Tris-HCl, pH 7.0 at 4 °C. The mixture was stirred gradually for 20 min,

followed by centrifugation at 17 500  $\times g$  at 4 °C for 30 min using a refrigerated centrifuge (Avanti J-E Centrifuge; Beckman Coulter, Fullerton, CA, USA). Supernatant was filtered through a Whatman filter paper (Whatman Ltd, Maidstone, UK) and then through a 0.20 mm nylon syringe filter. The filtrate was dialysed against 15 volumes of 20 mmol kg<sup>-1</sup> Tris-HCl, pH 7.0, at 4 °C for 36 h, with four changes of dialysis buffer. The dialysate was centrifuged at 17 500  $\times g$  for 30 min at 4 °C. The resulting supernatant was analysed for TGase activity.

### Determination of microbial transglutaminase activity

MTGase activity was measured by the hydroxamate method.<sup>18</sup> Freshly prepared substrate mixture containing 350  $\mu$ L of 0.1 mol L<sup>-1</sup> Tris-acetate, pH 6.0, 25  $\mu$ L of 2.0 mol L<sup>-1</sup> hydroxylamine, 75  $\mu$ L of 0.1 mol L<sup>-1</sup> *N*-Carbobenzoxy(CBZ)-L-glutaminylglycine and 25  $\mu$ L of deionised water was used. To initiate the reaction, 25  $\mu$ L of crude extract were added and reaction was performed for 10 min at 37 °C. The reaction was terminated by adding 500  $\mu$ L of 150 g kg<sup>-1</sup> trichloroacetic acid containing 50 g kg<sup>-1</sup> ferric chloride. The resulting suspension was centrifuged at 9000  $\times g$  using a refrigerated centrifuge for 5 min and the absorbance was measured at 525 nm using a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). The calibration was performed using L-glutamic acid- $\gamma$ -monohydroxamic acid as standard. One unit of MTGase was defined as the amount of enzyme required to catalyse the formation of 1  $\mu$ mol hydroxamic acid min<sup>-1</sup> at pH 6.0 and 37 °C.

### Effect of microbial transglutaminase on gel properties of surimi from different fish species

Surimi was added with 25 g kg<sup>-1</sup> NaCl and the moisture content was adjusted to 80 g kg<sup>-1</sup>. After grinding for 3 min, various amounts of MTGase (0, 0.2, 0.4 and 0.6 U g<sup>-1</sup> surimi) were added to the paste. The mixture was chopped for another 2 min at 4 °C to obtain the homogenous paste. The paste was then stuffed into polyvinylidene casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. Two-step heated gels were prepared by setting the paste at 40 °C for 30 min, followed by heating at 90 °C for 20 min. The gels were then cooled in iced water and stored for 24 h at 4 °C prior to analysis. To study the role of only MTGase (without endogenous TGase) on gel properties, 10 mmol kg<sup>-1</sup> EDTA was added into the surimi paste to inactivate endogenous TGase.<sup>19,20</sup> The gels were then prepared in the same manner. All gels were analysed.

### Texture analysis

Texture analysis of surimi gels was carried out using a Model TA-XT2 texture analyser (Stable Micro Systems, Guildford, UK). Gels were equilibrated at room temperature (25–30 °C) before analysis. Five cylindrical samples (2.5 cm in length) were prepared and tested. Breaking force (strength) and deformation (cohesiveness/elasticity) were measured by the texture analyser equipped with a spherical plunger (5 mm diameter; depression speed 60 mm min<sup>-1</sup>).

### Determination of colour and whiteness

Gel samples from each treatment were subjected to whiteness measurement using a colourFlex (HunterLab, Reston, VA, USA). Illuminant C was used as the light source of measurement. CIE  $L^*$ ,  $a^*$ , and  $b^*$  values were measured. Whiteness was calculated using the equation:<sup>21</sup> whiteness =  $100 - [100 - (L^{*2} + a^{*2} + b^{*2})]^{1/2}$

### Determination of expressible moisture content

Expressible moisture content was measured according to the method of Benjakul *et al.*<sup>4</sup> Cylindrical gel samples were cut into a thickness of 5 mm, weighed (*X*), and placed between two pieces of Whatman paper No. 1 at the bottom and one piece of paper on the top. A standard weight (5 kg) was placed on the top of the sample for 2 min, and then the sample was removed from the papers and weighed again (*Y*). Expressible moisture content was calculated and expressed as percentage of sample weight as follows: expressible moisture content (%) =  $[(X - Y)/X] \times 100$ .

### Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Protein patterns of surimi gels were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli.<sup>22</sup> To prepare the protein sample, 27 mL of 50 g L<sup>-1</sup> SDS solution heated to 85 °C were added to the sample (3 g). The mixture was then homogenised using a homogeniser (IKA Labortechnik, Selangor, Malaysia) at a speed of 11 000 rpm for 2 min. The homogenate was incubated at 85 °C for 1 h to dissolve total proteins. The samples were centrifuged at 3500 × *g* for 20 min to remove undissolved debris. Supernatant was determined for protein content using the Biuret method.<sup>23</sup> The samples (10 µg protein) were loaded onto the polyacrylamide gel made of 10% running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel, using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.2 g L<sup>-1</sup> Coomassie brilliant blue R-250 in 500 mL L<sup>-1</sup> methanol and 75 mL L<sup>-1</sup> acetic acid and destained with 500 g L<sup>-1</sup> methanol and 75 mL L<sup>-1</sup> acetic acid, followed by 50 mL L<sup>-1</sup> methanol and 75 mL L<sup>-1</sup> acetic acid.

### Effect of microbial transglutaminase on cross-linking of natural actomyosin from different fish species

#### Preparation of natural actomyosin

Natural actomyosin (NAM) was prepared according to the method of Benjakul *et al.*<sup>24</sup> Fish mince (10 g) was homogenised in 100 mL of chilled 0.6 mol L<sup>-1</sup> KCl, pH 7.0 for 4 min at a speed of 11 000 rpm using a homogeniser. Over-heating during extraction was avoided by keeping the sample in an iced container and each 20 s of homogenisation was followed by a 20 s rest interval. The homogenate was centrifuged at 5000 × *g* for 30 min at 4 °C. Three volumes of chilled water (0–2 °C) were added to precipitate NAM, which was then collected by centrifuging at 5000 × *g* for 20 min at 4 °C using a refrigerated centrifuge. The pellets were then dissolved by gradually stirring in an equal volume of chilled 0.6 mol L<sup>-1</sup> KCl, pH 7.0 for 30 min at 4 °C.

#### Study on cross-linking of natural actomyosin by microbial transglutaminase

For cross-linking reaction, NAM solution (5 mg mL<sup>-1</sup>) was pre-heated at 40 °C for 5 min and MTGase (0, 10 and 20 U g<sup>-1</sup> protein) was added. The reaction was performed for different times (0, 1, 3, 5, 10, 20 and 30 min). To terminate the reaction, the assay mixtures were heated at 100 °C for 5 min. All samples were subjected to analyses of  $\epsilon$ -amino group content and protein pattern.

#### Determination of the $\epsilon$ -amino group content

The  $\epsilon$ -amino group content was measured according to the method of Bubnis and Ofner<sup>25</sup> with a slight modification. Sample (1 mL) was placed in 50 mL screw cap test tube. One millilitre of

4 g L<sup>-1</sup> sodium hydrogen carbonate and 1 mL of 5 g L<sup>-1</sup> TNBS were added. The reaction mixture was heated at 40 °C for 4 h with continuous shaking (SV1422; Memmert, Schwabach, Germany). Three millilitres of 6 mol L<sup>-1</sup> hydrochloric acid was added and the mixture was autoclaved at 120 °C for 1 h using an autoclave (SX-500; Tomy, Tokyo, Japan). The hydrolysate was then diluted with 5 mL of deionised water and extracted with three volumes of ethyl ether three times. The aqueous phase was removed and heated for 15 min in a temperature controlled water bath (W350; Memmert) to evaporate the residual ethyl ether. The aliquot was diluted with three volumes of deionised water and the absorbance was measured at 346 nm using a spectrophotometer.

### Statistical analysis

The experiments were run in triplicate with three different lots of samples. Data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's multiple range tests. A *T*-test was used for pair comparison.<sup>26</sup> Analysis was performed using the Statistical Package for Social Science Package (SPSS 11.0 for windows; SPSS Inc., Chicago, IL, USA).

## RESULTS AND DISCUSSION

### Effect of microbial transglutaminase on the gel properties of different surimi in the presence and absence of EDTA

#### Breaking force and deformation

Breaking force and deformation of gels from threadfin bream, Indian mackerel, and sardine added with various levels of MTGase in the presence and absence of EDTA at a level of 10 mmol kg<sup>-1</sup> are shown in Fig. 1. Without EDTA, the breaking force of gel from all surimi increased as MTGase levels increased up to the particular level (*P* < 0.05). For gel from threadfin bream surimi, the addition of MTGase at 0.2 U g<sup>-1</sup> surimi yielded the highest breaking force, which was 2.3-fold higher than that of the control. When MTGase higher than 0.4 U g<sup>-1</sup> was added, the decrease in breaking force was observed (*P* < 0.05). For surimi from Indian mackerel and sardine, breaking force of gel added with MTGase increased with increasing MTGase levels (*P* < 0.05). At the same level of MTGase added, gels from threadfin bream surimi had the highest breaking force, followed by Indian mackerel and sardine respectively. Breaking force of surimi from threadfin bream, Indian mackerel and sardine increased by 133.28, 351.9 and 48.9% when MTGase at level of 0.2, 0.6 and 0.6 U g<sup>-1</sup> was incorporated, respectively. It was noted that the increase in breaking force was lowest in sardine surimi gel when MTGase at all levels was added (*P* < 0.05). This indicated that the strong gel development was not achieved by addition of MTGase. The extent of setting response depends on not only TGase activity level but also the conformation of actomyosin, which varies among fish species.<sup>27</sup> Similar results were obtained for deformation. Deformation of threadfin bream surimi added with MTGase more than 0.2 U g<sup>-1</sup> decreased continuously as MTGase level increased (*P* < 0.05), while there was no change in deformation for surimi from other two species (*P* > 0.05). A higher amount of MTGase added might induce the formation of non-disulfide covalent bond to a greater extent. As a result, the strength of gel matrix was enhanced.<sup>16</sup> Nevertheless, the breaking force and deformation of surimi from threadfin bream decreased as the excessive amount of MTGase was added. This was possibly due to the self-aggregation of MTGase, thereby losing its protein cross-linking activity. This phenomenon was also observed in several previous studies.<sup>15,28–30</sup> The decrease in breaking force in threadfin bream surimi, one of

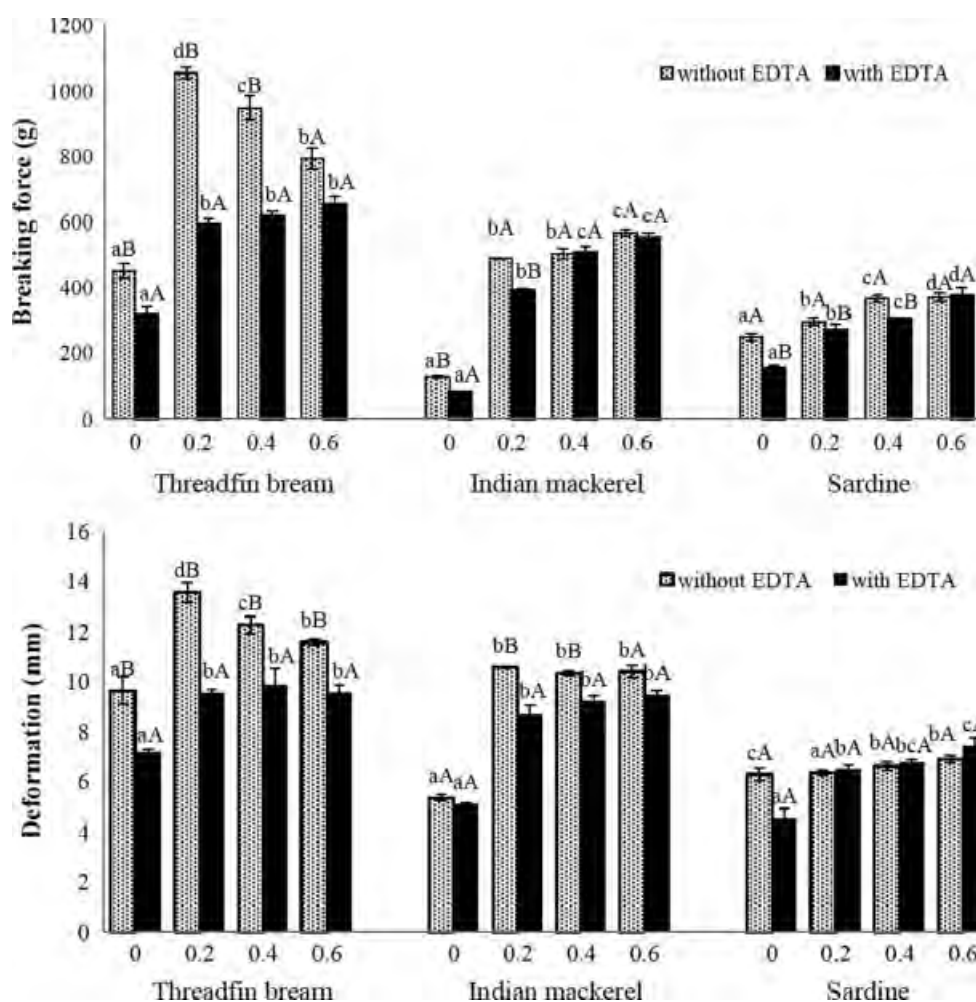


**Figure 1.** Breaking force and deformation of gels from threadfin bream, Indian mackerel, and sardine added with various levels of MTGase (0–0.6 U g<sup>-1</sup> surimi) in the absence and presence of EDTA. Bars represent the standard deviation ( $n = 3$ ). Different letters within the same level of EDTA and the same fish species indicate significant differences ( $P < 0.05$ ). Different capital letters within the same level of MTGase and the same fish species indicate significant differences ( $P < 0.05$ ). Numbers designate the level of MTGase added (U g<sup>-1</sup> surimi).

the good gel formers,<sup>10</sup> might be also due to the excessive and rapid aggregation of proteins caused by both endogenous TGase and MTGase, particularly at the high level. The gel with the random aggregate generally shows the poorer mechanical property.<sup>31,32</sup> In the presence of EDTA, endogenous TGase was inhibited and only MTGase played a role in protein cross-linking. Therefore, the level of MTGase alone might not cause the rapid and excessive aggregation. For surimi from sardine and Indian mackerel, which had the lower gel forming ability, the pronounced aggregation could not take place, even in the high level of MTGase.

In the presence of EDTA, all surimi gels also had the increases in breaking force and deformation with increasing MTGase levels up to the particular level ( $P < 0.05$ ). In the presence of EDTA, the lower breaking force and deformation were found in all surimi gels ( $P < 0.05$ ). The result indicated that endogenous TGase played a role in setting, in which non-disulfide covalent bonds were formed. The higher breaking force and deformation were observed when EDTA was excluded. No differences in breaking force were found in surimi from Indian mackerel and sardine when MTGase greater than 0.2 and 0.4 U g<sup>-1</sup> was added, respectively. Also, there was no difference in deformation between sardine surimi with and without EDTA when MTGase at levels of 0.2–0.6 U g<sup>-1</sup> was added ( $P > 0.05$ ).

Thus, MTGase showed its efficacy in protein cross-linking, even when endogenous TGase was omitted. EDTA was able to chelate Ca<sup>2+</sup>, which is required for activation of calcium-dependent endogenous TGase. The combined effect of endogenous TGase with MTGase added in surimi on gel strengthening was therefore obtained, but varied with species. Gel formation of Alaska pollack surimi was totally inhibited in the presence of 5 mmol kg<sup>-1</sup> EDTA.<sup>7</sup> Van Phu *et al.*<sup>20</sup> reported that the addition of 10 mmol kg<sup>-1</sup> EDTA in surimi from white croaker resulted in the decrease in gel strength approximately 2.5-fold compared with that of the gels without EDTA. Seki *et al.*<sup>5</sup> found that the endogenous TGase is essentially involved in the setting of surimi and plays a major role in the strengthening surimi gel. Both endogenous TGase and exogenous MTGase enable to improve the functionality of surimi by increasing protein cross-linking.<sup>13</sup> Asagami *et al.*<sup>15</sup> reported that the amount of MTGase added for strengthening surimi gel highly depended on fish species and other factors such as freshness, protein quality, and harvesting season.

#### Expressible moisture content

The expressible moisture content of surimi gel added with MTGase at various levels without and with EDTA is shown in Table 1. The

**Table 1.** Expressible moisture content and whiteness of gels from threadfin bream, Indian mackerel and sardine surimi added with different levels of MTGase (0–0.6 units g<sup>-1</sup> surimi) in the absence and presence of EDTA (10 mmol kg<sup>-1</sup>)

| Parameter                        | MTGase levels | Threadfin bream             |                            | Indian mackerel            |                            | Sardine                     |                            |
|----------------------------------|---------------|-----------------------------|----------------------------|----------------------------|----------------------------|-----------------------------|----------------------------|
|                                  |               | Without EDTA                | With EDTA                  | Without EDTA               | With EDTA                  | Without EDTA                | With EDTA                  |
| Expressible moisture content (%) | 0             | 2.98 ± 0.08 <sup>bA**</sup> | 3.43 ± 0.10 <sup>aB</sup>  | 5.51 ± 0.28 <sup>cA</sup>  | 5.68 ± 0.06 <sup>bA</sup>  | 4.83 ± 0.13 <sup>bA</sup>   | 4.90 ± 0.05 <sup>bB</sup>  |
|                                  | 0.2           | 2.89 ± 0.53 <sup>bA</sup>   | 3.40 ± 0.33 <sup>aB</sup>  | 4.83 ± 0.18 <sup>bA</sup>  | 5.55 ± 0.26 <sup>abB</sup> | 4.76 ± 0.17 <sup>bA</sup>   | 4.81 ± 0.56 <sup>bA</sup>  |
|                                  | 0.4           | 2.66 ± 0.22 <sup>abA</sup>  | 3.30 ± 0.51 <sup>aB</sup>  | 4.79 ± 0.26 <sup>aA</sup>  | 5.44 ± 0.22 <sup>abB</sup> | 4.67 ± 0.29 <sup>bA</sup>   | 4.76 ± 0.30 <sup>abA</sup> |
|                                  | 0.6           | 2.35 ± 0.33 <sup>aA</sup>   | 3.24 ± 0.41 <sup>aB</sup>  | 4.60 ± 0.47 <sup>aA</sup>  | 5.24 ± 0.10 <sup>aB</sup>  | 4.40 ± 0.31 <sup>aA</sup>   | 4.58 ± 0.26 <sup>aA</sup>  |
| Whiteness                        | 0             | 79.59 ± 0.53 <sup>abA</sup> | 83.82 ± 0.31 <sup>aB</sup> | 67.38 ± 0.36 <sup>aA</sup> | 70.37 ± 0.41 <sup>aB</sup> | 71.72 ± 0.32 <sup>aA</sup>  | 75.55 ± 0.44 <sup>aB</sup> |
|                                  | 0.2           | 79.25 ± 0.14 <sup>aA</sup>  | 83.88 ± 0.52 <sup>aB</sup> | 68.33 ± 0.36 <sup>bA</sup> | 70.74 ± 0.47 <sup>aB</sup> | 72.39 ± 0.79 <sup>abA</sup> | 75.88 ± 0.75 <sup>aB</sup> |
|                                  | 0.4           | 79.59 ± 0.61 <sup>bA</sup>  | 83.96 ± 0.25 <sup>aB</sup> | 68.89 ± 0.11 <sup>cA</sup> | 70.80 ± 0.08 <sup>aB</sup> | 72.62 ± 0.07 <sup>bA</sup>  | 75.98 ± 0.15 <sup>aB</sup> |
|                                  | 0.6           | 80.13 ± 0.26 <sup>bA</sup>  | 83.98 ± 0.26 <sup>aB</sup> | 68.30 ± 0.23 <sup>bA</sup> | 70.79 ± 0.50 <sup>aB</sup> | 72.74 ± 0.32 <sup>bA</sup>  | 76.22 ± 0.26 <sup>aB</sup> |

Values are given as mean ± SD (*n* = 3).

<sup>a,b,c,\*</sup> Different lowercase superscripts within the same column under the same parameter tested indicate the significant differences (*P* < 0.05).

<sup>A,B,\*\*</sup> Different uppercase superscripts within the same row under the same fish species indicate the significant differences (*P* < 0.05).

decreases in expressible moisture content were observed with increasing MTGase levels for all surimi gels, regardless of EDTA addition. Addition of MTGase induced some increase in WHC of the samples and also a more porous microstructure.<sup>33,34</sup> At the same levels of MTGase, the lowest expressible moisture content was observed in surimi gels from threadfin bream, a lean fish, indicating that a higher water amount was retained in the gel matrix. Different expressible moisture content between surimi gels from different species suggested the differences in water holding capacity of gel network. Higher fat content in surimi from Indian mackerel and sardine, dark-fleshed fish, might impede the aggregation of proteins in the gel matrix. It has been reported that fatty and medium fatty fish do not respond to MTGase as fast as lean fish, because fat has an adverse effect on the binding properties of the fish.<sup>35</sup> Also, hydrophobicity of those lipids might lower the water binding capacity of gel. During setting at 40 °C, proteins underwent some denaturation and aligned themselves gradually to form the network, which can imbibe water.<sup>4</sup> When EDTA was added into gel, the increase in expressible moisture content was noticed. In the presence of EDTA, less calcium ion was available for TGase activation. As a consequence, a lower TGase activity was assumed, leading to a lower cross-linking of protein. The weakened gel matrix might have the lower water holding capacity. In general, lower expressible moisture content of gels suggested more water retained in the gel network.<sup>8</sup> The decrease in expressible moisture content was in agreement with the higher breaking force and deformation for surimi gel from threadfin bream (Fig. 1). However, this phenomenon was not found in surimi gel from Indian mackerel and sardine. There are several factors involving in water holding capacity of gels. Indian mackerel and sardine are pelagic dark-fleshed fish containing a higher amount of fats in comparison with threadfin bream. The fat remained in surimi might lower or have the influence on water binding of surimi gel. Additionally, the amino composition in different surimi varied, thereby affecting the water holding capacity differently. When MTGase was added especially in the range of 0.4–0.6 U g<sup>-1</sup> surimi the cross-linking of proteins could be enhanced, resulting in the formation of stronger network with the greater water-holding capacity.

#### Whiteness

Slight increases in whiteness were found in surimi gels added with MTGase at higher levels in the absence of EDTA (*P* < 0.05).

However, there was no difference in whiteness of surimi gels added with increasing levels of MTGase in the presence of EDTA (*P* > 0.05). Among surimi gels from three fish species, that from Indian mackerel had the lowest whiteness, followed by those from sardine and threadfin bream. Dark-fleshed fish such as mackerel and sardine has high content of dark muscle associated with high content of myoglobin, resulting in natural dark colour of surimi.<sup>11,36</sup> The whiteness of surimi gels increased when EDTA was incorporated. The increase in whiteness might be associated with the light scattering effect from released water of gel matrix, which was higher in gel added with EDTA. Thus, the addition of MTGase had generally no profound impact on the whiteness of resulting surimi gel.

#### Protein patterns

Protein patterns of surimi gels added with different levels of MTGase in the absence and presence of EDTA are depicted in Fig. 2. Surimi paste contained myosin heavy chain (MHC) and actin as the major proteins. Nevertheless, MHC band intensity varied with species. The paste from sardine surimi contained the lower MHC band intensity than those from threadfin bream and Indian mackerel. Decrease in MHC band intensity was observed in the gel without MTGase addition, compared to that found in paste. The result suggested the formation of cross-links stabilised by non-disulfide covalent bond mediated by endogenous TGase, especially during setting. Among all samples, no MHC band was retained in surimi gel from sardine which possibly resulted from endogenous TGase. A lower initial amount of MHC might undergo cross-linking almost completely during setting, resulting in the negligible MHC retained. Montero and Gomez-Guillen<sup>37</sup> found that kamaboko gel of sardine (preset at 35 °C and then cooked at 90 °C) had no MHC remaining as a consequence of setting, in which more covalent bonds could be formed. Furthermore, proteolysis of MHC still occurred to some extent during setting.<sup>37</sup> The highest MHC band intensity in gel without MTGase addition was observed in surimi from Indian mackerel, suggesting the poorest setting phenomenon in this species. This was in accordance with the lowest breaking force and deformation of this species (Fig. 1). MHC was most susceptible to cross-linking during setting.<sup>4</sup> MHC band disappeared when MTGase at 0.2 U g<sup>-1</sup> surimi was added in all gel samples, mainly via non-disulfide covalent bond.<sup>28</sup> No marked changes in actin were obtained in the presence and

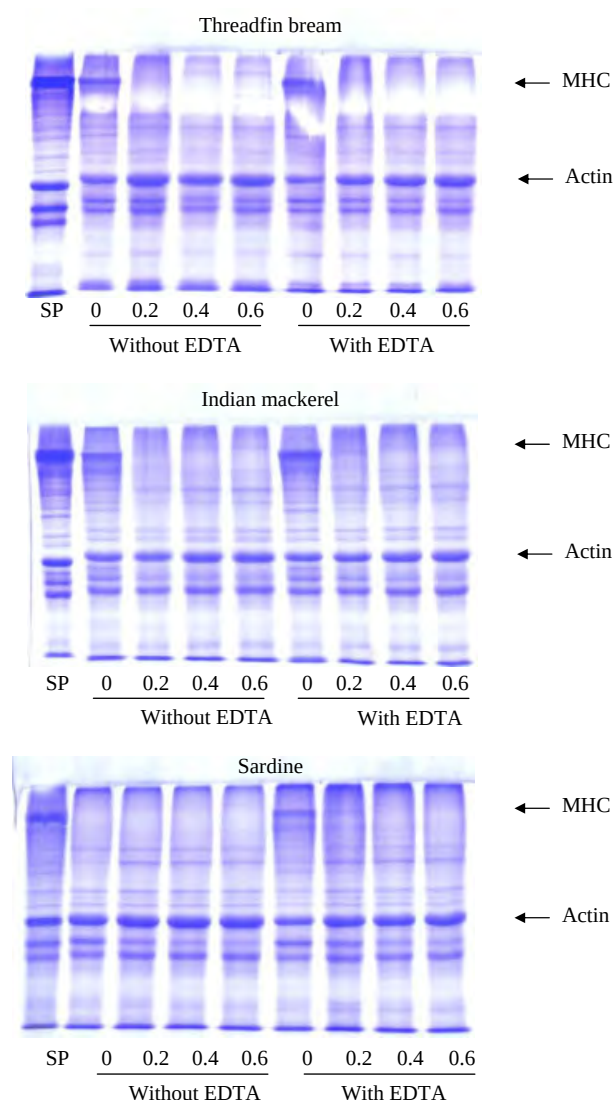


**Figure 2.** Protein pattern of surimi gels from threadfin bream, Indian mackerel, and sardine added with various levels of MTGase (0–0.6 U g<sup>-1</sup> surimi) in the absence and presence of EDTA. SP, surimi paste; MHC, myosin heavy chain. Numbers designate the level of MTGase added (U g<sup>-1</sup> surimi).

absence of MTGase. MHC appeared to be a preferable substrate for cross-linking induced by either endogenous TGase or MTGase. Nevertheless, actin could not serve as a substrate for TGase. Nakahara *et al.*<sup>38</sup> reported that MTGase and carp TGase could not cross-link actin molecules. The decrease in MHC band intensity was concomitant with the increased breaking force and deformation (Fig. 1). Thus, MTGase together with endogenous TGase effectively induced polymerisation of MHC, a protein contributing to the gel network formation. As a result, the stronger gel network was formed. The result was in accordance with Jiang *et al.*<sup>39</sup> who reported that cross-linking of MHC occurred rapidly in mackerel surimi added with MTGase.

In the presence of EDTA, MHC band was more retained in all samples, especially when MTGase at level of 0 and 0.2 U g<sup>-1</sup> was added. In the presence of EDTA, less calcium ion was available for endogenous TGase activation. As a consequence, a lower TGase activity was assumed, leading to a lower cross-linking of protein, especially MHC. Although there was no MHC retained in all surimi

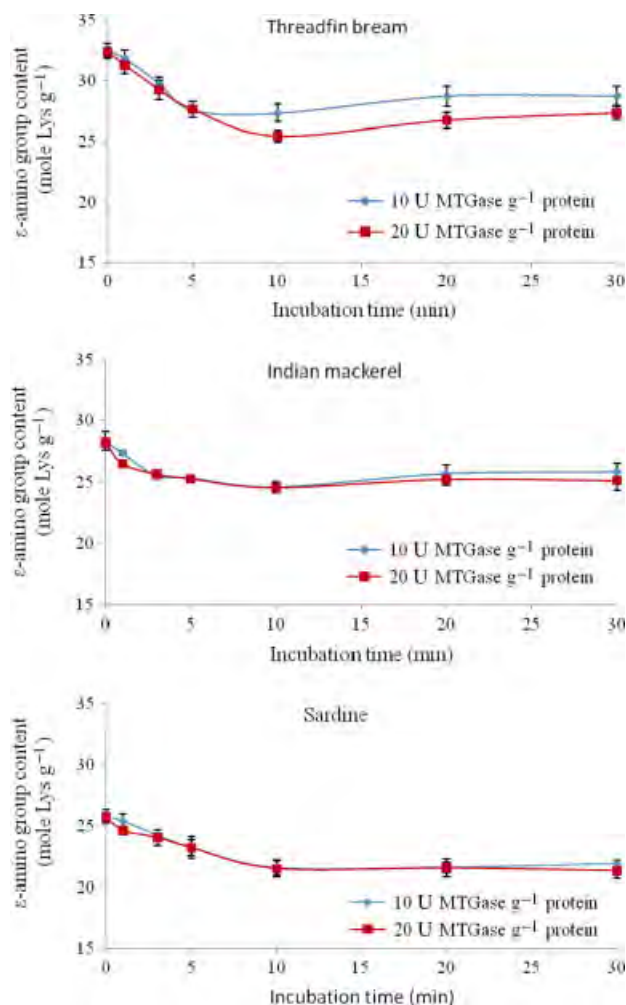


**Figure 3.** Changes in  $\epsilon$ -amino group contents of natural actomyosin from threadfin bream, Indian mackerel, and sardine added with MTGase (10 and 20 U g<sup>-1</sup> protein) as a function of incubation time at 40 °C. Bars represent the standard deviation ( $n = 3$ ).

when MTGase particularly at levels higher than 0.2 U g<sup>-1</sup> was added, gel property was different with species. Therefore, not only degree of cross-linking, but also configuration of gel matrix as well as other intrinsic factors determined gel properties of surimi.

### Effect of microbial transglutaminase on protein cross-linking of natural actomyosin from different fish species

#### $\epsilon$ -Amino group content

Changes in  $\epsilon$ -amino group contents of NAM from threadfin bream, Indian mackerel, and sardine added with two levels of MTGase are demonstrated in Fig. 3. Addition of MTGase into NAM resulted in the decrease in  $\epsilon$ -amino group content, especially within the first 10 min ( $P < 0.05$ ). During setting at 40 °C for 10 min, MTGase catalysed an acyl transfer reaction between the  $\gamma$ -carboxamide group of a peptide-bound glutamyl residue (acyl donors) and  $\epsilon$ -amino group of a peptide-bound lysyl residue. Peptide chains are covalently connected through  $\epsilon$ -( $\gamma$ -glutamyl)lysine bonds. This resulted in the reduction of  $\epsilon$ -amino group content in NAM. NAM extracted from threadfin bream with the highest amount of  $\epsilon$ -amino group content ( $32.46 \pm 0.42$  mole Lys g<sup>-1</sup>) had the highest reduction rate, followed by NAM from sardine and Indian mackerel, respectively. After 10 min of incubation, threadfin bream NAM

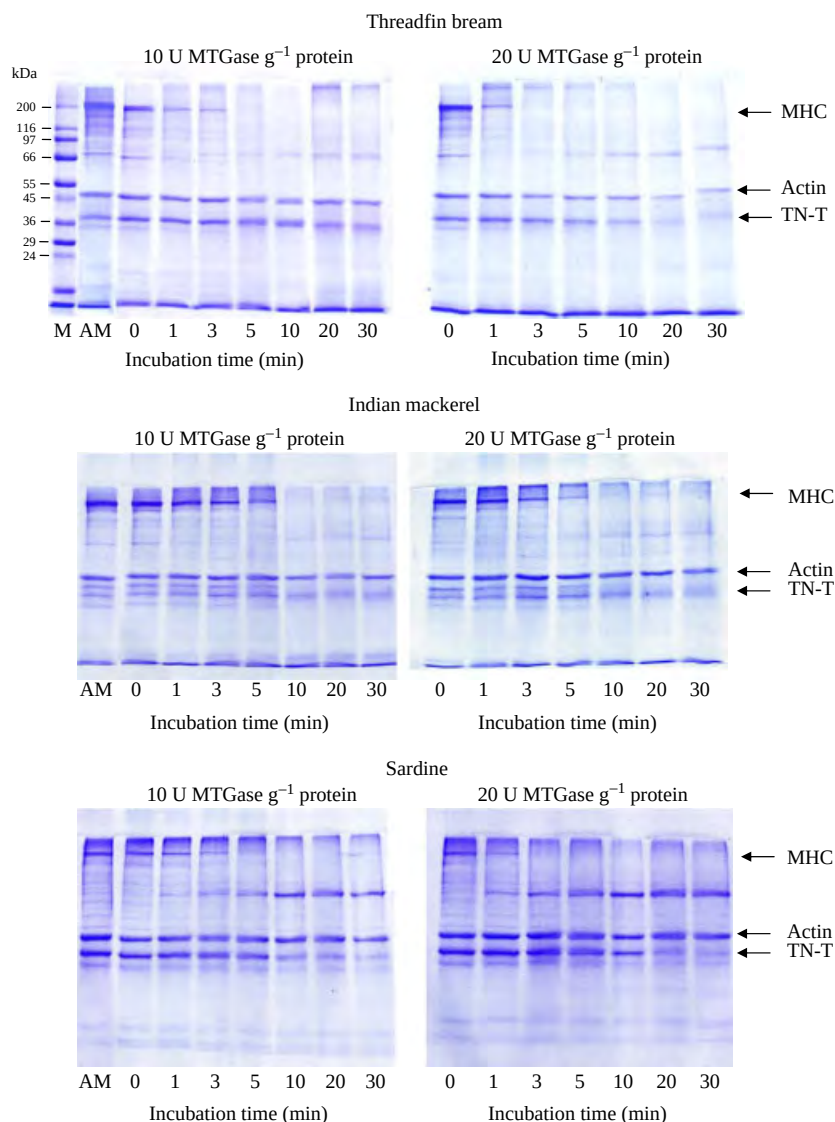


**Figure 4.** Protein patterns of natural actomyosin from threadfin bream, Indian mackerel, and sardine added with MTGase at 10 and 20 U g<sup>-1</sup> protein as a function of incubation time at 40 °C. AM, actomyosin; MHC, myosin heavy chain; TN-T, troponin-T. Numbers designate the incubation time (min).

had the lower  $\epsilon$ -amino group content, especially as the amount of MTGase increased. On the other hand, no further changes in  $\epsilon$ -amino group content were obtained after 10 min. Thus, cross-linking of proteins mainly occurred within the first 10 min.  $\epsilon$ -amino group contents of threadfin bream NAM at 10 min of incubation added with 10 or 20 U g<sup>-1</sup> protein decreased by 15.8% or 21.4%, respectively, compared with those of initial sample. At the same incubation time (10 min),  $\epsilon$ -amino group content of NAM from Indian mackerel and sardine decreased by 13.1% and 16.4%, compared with that observed at time 0 min. Jiang *et al.*<sup>28</sup> found that MTGase-containing hairtail actomyosin incubated at 45 °C decreased greatly at the first 5 min of incubation and cross-linking of MHC also became prominent within a short period. MTGase acts as an intermediate factor in coupling glutamyl and lysine residues in one strong complex by affinity relation through covalent bonds between 3-glutamyl and 5-lysine. The lowest amount of  $\epsilon$ -amino group content in sardine was in accordance with the lowest improvement of gel strength by MTGase (Fig. 1). The result suggested that the limited  $\epsilon$ -amino group content

also affected protein cross-linking by MTGase. Ahmmed *et al.*<sup>40</sup> suggested that there are remarkable differences in the myofibrillar protein within the same muscle type in different species. Although TGase is known to catalyse the cross-linking between glutamine and lysine residues in proteins, structure of substrate containing glutamine and lysine residues had the direct impact on cross-linking induced by TGase. The compact globular structures of the 11S and 7S soybean proteins, for instance, make them rather poor substrates for TGase, despite their relatively high glutamine contents.<sup>41</sup> Thus, the decreasing rate of  $\epsilon$ -amino group content might be another factor affecting the cross-linking as well as gel-forming ability of proteins in surimi.

#### Protein patterns

Natural actomyosin from threadfin bream, Indian mackerel and sardine were incubated with two levels of MTGase at 40 °C for up to 30 min and their protein patterns were analysed by SDS-PAGE (Fig. 4). The intensity of MHC band decreased as the incubation time increased. The disappearance of MHC indicated the formation

of MHC cross-links mainly via the formation the  $\varepsilon$ -( $\gamma$ -glutamyl) lysine isopeptide induced by MTGase. Cross-links were not be dissociated by the mixture of SDS and mercaptoethanol used for electrophoresis.<sup>39</sup> When MTGase at a level of 10 U g<sup>-1</sup> was used, MHC band intensity of threadfin bream and sardine NAM rapidly decreased within the first 5–10 min of incubation, which was correlated with the reduction of  $\varepsilon$ -amino group contents (Fig. 3). It was noted that MHC proportion in NAM varied with species. Threadfin bream NAM showed the highest MHC band intensity, followed by Indian mackerel and sardine respectively. MHC has been known to serve as the major contributor for gel formation and determine gel property of fish protein.<sup>38</sup> With the higher content of preferable substrate, MHC, MTGase could induce the cross-linking of protein substrate to a higher extent. Jiang *et al.*<sup>13</sup> reported that polymerisation of MHC caused by MTGase in pollack surimi was much faster in golden threadfin–bream surimi. At the same incubation time, the less intense MHC band was retained as the higher amount of MTGase (20 U g<sup>-1</sup>) was added. Although MHC band intensity decreased markedly, actin band was rarely changed in NAM from sardine and Indian mackerel. However, actin band intensity of NAM from threadfin bream slightly decreased with increasing incubation time, especially at higher MTGase concentration. Araki and Seki<sup>27</sup> reported that the rates of changes in MHC from various fish actomyosin incubated with carp TGase at 25 °C were significantly different and concluded that the rate of TGase-mediated cross-linking depends upon the substrate specificity. For NAM from Indian mackerel and sardine, it was found that a protein with MW of 35 kDa, which was likely to be troponin-T, decreased in band intensity when the incubation time was longer than 5 min. That protein was decreased in threadfin bream NAM as the incubation time was longer than 10–20 min. Thus, troponin-T also served as substrate for cross-linking reaction induced by MTGase, but reactivity for reaction was governed by species. Coincidentally, the protein with MW of 100 kDa was found in all samples especially for NAM from sardine. This might be the dimer of tropomyosin induced by MTGase. Nakahara *et al.*<sup>38</sup> found that MTGase preferentially cross-linked connectin, followed by MHC, troponin-T and actin, respectively. Thus, the cross-linking of NAM from different species varied and substrate specificity was also different.

## CONCLUSION

The addition of MTGase enhanced the gel-forming ability of surimi from three fish species, but the strengthening effect varied. Gels from threadfin bream which had the highest  $\varepsilon$ -amino group content showed the highest gel strength, when MTGase (0.2–0.6 U g<sup>-1</sup>) was added, followed by Indian mackerel and sardine, respectively. The reactivity of muscle proteins toward MTGase-induced cross-linking and MHC proportion were the key factors determining the gel strength of surimi or fish muscle proteins. Thus, the composition and property of muscle proteins of varying fish species, which more likely determined the ability of MTGase in protein cross-linking, should be further investigated.

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## Cryoprotective effect of gelatin hydrolysate from blacktip shark skin on surimi subjected to different freeze-thaw cycles

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### ABSTRACT

The cryoprotective effects of gelatin hydrolysate from blacktip shark (*Carcharhinus limbatus*) skin with different degrees of hydrolysis (DHs: 5, 10, 20 and 30%) and commercial cryoprotectant (sucrose/sorbitol blend, 3:1) on surimi from threadfin bream (*Nemipterus* spp.) subjected to 3 and 6 freeze-thaw cycles were investigated.  $\text{Ca}^{2+}$ -ATPase, total sulfhydryl group content and solubility of all natural actomyosin (NAM) extracted from surimi decreased with increasing freeze-thaw cycles, while surface hydrophobicity and disulfide bond content increased ( $P < 0.05$ ). Among all samples, the highest  $\text{Ca}^{2+}$ -ATPase activity with the coincidental lowest surface hydrophobicity was observed in surimi added with gelatin hydrolysate having 10%DH after repeated freeze-thawing ( $P < 0.05$ ). On the other hand, surimi without cryoprotectant showed the lowest  $\text{Ca}^{2+}$ -ATPase and the highest surface hydrophobicity ( $P < 0.05$ ). The similar solubility was obtained between samples added with commercial cryoprotectant and gelatin hydrolysate (10%DH) ( $P > 0.05$ ). The results suggest that gelatin hydrolysate with 10%DH was able to prevent the denaturation of surimi protein comparably to commercial cryoprotectant. However, it exhibited the higher protective effect on denaturation of myosin heavy chain, especially globular head domain. Therefore, gelatin hydrolysate can be an alternative cryoprotectant with the lower sweetness in surimi products.

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### 1. Introduction

Freezing or frozen storage is an important process to prolong the shelf-life of surimi and surimi products. However, temperature fluctuation and abuse during transportation, storage or consumption lead to the deterioration of surimi quality. The native protein structure is generally stabilized by many forces, including hydrogen bonds, dipole–dipole interactions, electrostatic interactions and disulfide linkages (Benjakul & Bauer, 2000). Such bondings and interactions can be destabilized by temperature fluctuation or during extended frozen storage (Benjakul & Sutthipan, 2009; Benjakul, Visessanguan, Thongkaew, & Tanaka, 2003). To alleviate or retard protein denaturation in muscle food, cryoprotectants have been widely used, especially in surimi.

A cryoprotectant is a substance which protects biological tissue from freezing damage by slowing the rate of ice crystal growth

(Alvarez, Fernández, & Canet, 2010). The most commonly used cryoprotectants in the surimi industry are low molecular weight sugars such as sorbitol and sucrose, among others (Jin et al., 2010). Cryoprotectants are known to lower the denaturation and/or aggregation of myofibrillar protein during frozen storage, thereby maintaining functional properties, such as gel-forming ability, water holding capacity and solubility, of proteins. However, the common cryoprotectants, particularly sucrose, impart a sweet taste to surimi products which is unacceptable to consumers. Thus, alternative cryoprotectants with reduced sweetness have gained increasing attention. Ruttanapornwareesakul et al. (2006) reported that protein hydrolysate from shrimp head containing short chain peptides could inhibit denaturation of fish myofibrillar protein. Moreover, protein hydrolysates from various sources were also used as a cryoprotectant in fish products (Cheung, Liceaga, & Li-Chan, 2009; Hossain et al., 2004; Somjit, Ruttanapornwareesakul, Hara, & Nozaki, 2005; Yamashita, Zhang, & Nozaki, 2003; Zhang, Yamashita, & Nozaki, 2002).

Recently, gelatin from blacktip shark skin has been extracted successfully and it had the similar bloom strength and setting

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behavior to bovine bone gelatin (Kittiphattanabawon, Benjakul, Visessanguan, & Shahidi, 2010). To maximize the use of gelatin from shark skin, the production of gelatin hydrolysate can be of choice to widen its application, especially as the cryoprotectant with no sweetness. However, no information regarding the use of gelatin hydrolysate as a cryoprotectant in surimi, especially those subjected to repeated freeze-thawing, has been reported. Therefore, the objective of this work was to study the cryoprotective effect of gelatin hydrolysate from black shark (*Carcharhinus limbatus*) skin on surimi with multiple freeze-thaw cycles in comparison with commercial cryoprotectants.

## 2. Materials and methods

### 2.1. Chemicals and gelatin

All chemicals were of analytical grade. Sucrose, sorbitol and trichloroacetic acid (TCA) were obtained from Merck (Darmstadt, Germany). Tris-maleate, 2,4,6-trinitrobenzene sulfonic acid (TNBS), adenosine triphosphate (ATP), 1-anilinonaphthalene-8-sulfonic acid (ANS), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and 2-nitro-5-thiosulfolbenzoate (NTSB) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA).

### 2.2. Preparation of gelatin hydrolysate from blacktip shark skin

#### 2.2.1. Shark skin preparation

Blacktip shark (*C. limbatus*) skin was obtained from Blue Ocean Food Products Co., Ltd. in Samutsakhon province, Thailand. The shark skin was prepared according to the method of Kittiphattanabawon et al. (2010).

#### 2.2.2. Extraction of gelatin

Gelatin from shark skin was extracted following the method of Kittiphattanabawon et al. (2010). Prior to extraction, the prepared skin was pretreated by mixing with  $0.1 \text{ mol L}^{-1}$  NaOH using a sample to solution ratio of 1:10 (g:mL) for 2 h at  $20^\circ\text{C}$ . The pretreated skin was subsequently demineralized using  $1 \text{ mol L}^{-1}$  HCl at a sample to solution ratio of 1:10 (g:mL) for 1 h at  $20^\circ\text{C}$ . Thereafter, the demineralized skin was swollen by mixing the skin with  $0.2 \text{ mol L}^{-1}$  acetic acid at a sample to solution ratio of 1:10 (g:mL) for 15 min at room temperature ( $26\text{--}28^\circ\text{C}$ ). For all pretreatment steps, the mixtures were continuously stirred using a stirrer (W20.n, IKA®-Werke GmbH & CO.KG, Stanfen, Germany). For each pretreatment step, the resulting skin was washed thoroughly with tap water until wash water became neutral or mildly acidic or basic in pH.

To extract gelatin, the swollen skin was mixed with distilled water at  $45^\circ\text{C}$  using a sample to water ratio of 1:2 (g:mL). The mixtures were continuously stirred for 6 h. The mixtures were then filtered using a Buchner funnel with Whatman No.4 filter paper (Whatman International, Ltd., Maidstone, England). Then, the filtrates were freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lyngø, Denmark). The resulting gelatin was used for preparation of gelatin hydrolysate.

#### 2.2.3. Preparation of crude enzyme from papaya (*Carica papaya*) latex

Fresh latex was collected from locally grown papaya in Thailand according to the method of Nitsawang, Hatti-Kaul, and Kanasawud (2006). Four to six longitudinal incisions were made on the unripen papaya fruit using a stainless steel knife. The exuded latex was collected using a receiving container. The latex was then transferred to a beaker and stored at temperature below  $10^\circ\text{C}$  and used within 3 h.

To prepare crude enzyme, the latex was mixed with cold distilled water ( $\leq 4^\circ\text{C}$ ) at the latex to water ratio of 1:1 (g:mL). The mixture was gently stirred at  $4^\circ\text{C}$  for 1 h. Then, the mixture was centrifuged at  $9000\times g$  at  $4^\circ\text{C}$  for 20 min using a refrigerated centrifuge (Avanti® J-E, Beckman Coulter, Palo Alto, CA, USA). The supernatant was filtered using a Whatman No.1 filter paper, followed by freeze-drying. The resulting powder referred to as “crude enzyme from papaya latex” was kept at  $-40^\circ\text{C}$  until use.

### 2.2.4. Production of gelatin hydrolysate with different DHs

Gelatin hydrolysates with different DHs from shark skin were prepared. Gelatin (3 g) was dissolved in 80 mL of distilled water. The pH of mixture was adjusted to 7.5 with  $1 \text{ mol L}^{-1}$  NaOH. The volume of solution was made up to 100 mL with distilled water previously adjusted to pH 7.5 to obtain a protein concentration of  $30 \text{ g L}^{-1}$ . The hydrolysis reaction was started by the addition of the crude enzyme from papaya latex at the amount calculated from the plot between log (enzyme concentration) and DH to obtain DH of 5, 10, 20 and 30% as described by Benjakul and Morrissey (1997). DH was determined by the 2,4,6-trinitrobenzene sulfonic acid (TNBS) method as described by Adler-Nissen (1979). After 1 h of hydrolysis at optimal temperature ( $40^\circ\text{C}$ ), the enzyme was inactivated by heating at  $90^\circ\text{C}$  for 15 min in a water bath (model W350, Memmert, Schwabach, Germany). The mixture was then centrifuged at  $5000\times g$  at room temperature for 10 min. The supernatant was freeze-dried and kept at  $4^\circ\text{C}$  until use. The obtained powder was referred to as “gelatin hydrolysate” and was stored at  $-20^\circ\text{C}$  until use.

### 2.3. Preparation of surimi from threadfin bream (*Nemipterus spp.*)

Threadfin bream was purchased from a dock, Songkhla province, Thailand. The fish were placed in ice with a fish to ice ratio of 1:2 (g:g) in a polystyrene box. The sample was transported to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, the fish was deheaded and filleted. The flesh was minced using a mincer with the hole diameter of 5 mm. The mince was suspended in 3 volumes of cold water ( $4^\circ\text{C}$ ). The mixture was stirred gently for 5 min and the washed mince was filtered with two layer of cheese cloth. Washing was repeated three times. Finally, the washed mince was centrifuged at  $700\times g$  for 15 min using a basket centrifuge (model CE 21 K, Grandiumpiant, Belluno, Italy). The washed mince was mixed with gelatin hydrolysate having different DHs (5, 10, 20 and 30%). Sample added with commercial cryoprotectant (sucrose/sorbitol blend, 3:1 (g:g)) was used as the control. The amount of gelatin hydrolysate and commercial cryoprotectant was  $80 \text{ g kg}^{-1}$  washed mince. All mixtures were referred to as “surimi”.

### 2.4. Freeze-thawing of surimi

The surimi (50 g) without and with gelatin hydrolysates or commercial cryoprotectant (4 g) were packed in polyethylene bag and frozen at  $-20^\circ\text{C}$  using an air-blast freezer for 24 h. The frozen samples were thawed with running tap water within approximately 30 min until the core temperature of surimi reached  $0\text{--}2^\circ\text{C}$ . The freeze-thawing was performed for 0, 3 and 6 cycles. Thawed samples were kept in ice until use for analysis.

### 2.5. Preparation of natural actomyosin (NAM)

NAM was prepared according to the method of Benjakul, Seymour, Morrissey, and An (1997) with a slight modification. To avoid any interfering effect from gelatin hydrolysate or cryoprotectant on the measurement of physicochemical properties of

NAM, gelatin hydrolysate or cryoprotectant was removed before NAM extraction. Surimi (4 g) was homogenized in 40 mL of chilled distilled water (2 °C) using a homogenizer (model T25 basic, IKA Labortechnik, Selangor, Malaysia) at a speed of 11,000 rpm for 1 min. The homogenate was centrifuged at  $10,000\times g$  for 10 min at 2 °C.

To extract NAM, washed surimi was further homogenized with 40 mL of 1.2 mol L<sup>-1</sup> KCl, pH 7 for 4 min. The beaker containing the sample was placed in ice and each 20 s of blending was followed by a 20 s rest interval to avoid overheating during extraction. The extract was centrifuged at  $5000\times g$  for 30 min at 2 °C. Three volumes of chilled deionized water were added to the supernatant to precipitate NAM. NAM was then collected by centrifuging at  $5000\times g$  for 20 min at 2 °C, and the pellet was dissolved in an appropriate volume of chilled 1.2 mol L<sup>-1</sup> KCl, pH 7.0 by stirring for 30 min at 2 °C. The extracted NAM was kept at 4 °C and used within 12 h.

## 2.6. Determination of physico-chemical properties

### 2.6.1. Ca<sup>2+</sup>-ATPase activity

Ca<sup>2+</sup>-ATPase activity was determined using the method of Benjakul et al. (1997). The prepared NAM with the protein concentration of 4 g L<sup>-1</sup> (250 µL) was mixed with 150 µL of 0.5 mol L<sup>-1</sup> Tris-maleate (pH 7), 250 µL of 0.1 mol L<sup>-1</sup> CaCl<sub>2</sub> and 1725 µL of distilled water. The mixture was vigorously mixed using a vortex mixer (model G-560E, Scientific Industries, Inc., Bohemia, NY, USA). To initiate the reaction, 20 mmol L<sup>-1</sup> ATP (125 µL) was added into the mixture and mixed thoroughly. The mixture was incubated at room temperature for 10 min and the reaction was terminated by adding 1250 µL of chilled 100 g L<sup>-1</sup> trichloroacetic acid (TCA, 4 °C). The reaction mixture was centrifuged at  $5000\times g$  for 5 min at room temperature using a centrifuge (model Allegra™ 25R, Beckman Coulter, Inc., Kerkfeld, Germany). The inorganic phosphate liberated in the supernatant was measured by the method of Fiske and Subbarow (1925). Specific activity was expressed as µmol inorganic phosphate released mg<sup>-1</sup>protein min<sup>-1</sup>. A blank solution was prepared by adding chilled TCA prior to addition of ATP. The Ca<sup>2+</sup>-ATPase activity of NAM extracted from samples after freeze-thawing at 3 and 6 cycles was reported as residual Ca<sup>2+</sup>-ATPase activity (%), which was calculated in comparison with that obtained from the sample without freeze-thawing.

### 2.6.2. Protein solubility

Prior to determination of protein solubility, gelatin hydrolysate or cryoprotectant was removed by thorough washing as previously described. The washed surimi was homogenized with 10 volume of 1.2 mol L<sup>-1</sup> KCl (pH 7) for 4 min. The beaker containing the sample was placed in ice and each 20 s of homogenization was followed by a 20 s rest interval to avoid overheating during extraction. The extract was centrifuged at  $5000\times g$  for 30 min at 2 °C. The protein content in the obtained supernatant was determined by the Biuret method (Robinson & Hodgen, 1940) using bovine serum albumin as a standard. The protein solubility (%) was calculated in comparison with that obtained from the sample without freeze-thawing.

### 2.6.3. Surface hydrophobicity

Protein surface hydrophobicity was determined by the method of Benjakul et al. (1997). NAM solution was diluted to 0.125, 0.25, 0.5 and 1 g L<sup>-1</sup> using 10 mmol L<sup>-1</sup> phosphate buffer, pH 6.0 containing 0.6 mol L<sup>-1</sup> NaCl. The diluted NAM solutions (4 mL) were mixed well with 20 µL of 8 mmol L<sup>-1</sup> 1-anilinonaphthalene-8-sulfonic acid (ANS) in 0.1 mol L<sup>-1</sup> phosphate buffer, pH 7.0. The

relative fluorescence intensity of ANS-protein conjugates was measured using a spectrofluorometer (RF-15001, Shimadzu, Kyoto, Japan) at the excitation wavelength of 374 nm and the emission wavelength of 485 nm. Protein surface hydrophobicity was calculated from initial slopes of plots of relative fluorescence intensity versus protein concentration (g L<sup>-1</sup>) using a linear regression analysis. The initial slope was referred to as "SoANS".

### 2.6.4. Total sulfhydryl group content

Total sulfhydryl group content was determined, using 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) according to the method of Ellman (1959) as modified by Benjakul et al. (1997) with a slight modification. To 1 mL of NAM solution (4 g L<sup>-1</sup>), 9 mL of 0.2 mol L<sup>-1</sup> Tris-HCl buffer, pH 8, containing 8 mol L<sup>-1</sup> urea, 20 g L<sup>-1</sup> SDS and 10 mmol L<sup>-1</sup> EDTA, were added. To 3 mL of the mixture, 0.3 mL of 1 g L<sup>-1</sup> DTNB, dissolved in 0.2 mol L<sup>-1</sup> Tris-HCl (pH 8.0) was added and the mixture was incubated at 40 °C for 25 min. The absorbance at 412 nm was measured using a double beam spectrophotometer (model UV-1800, Shimadzu, Kyoto, Japan). A blank was conducted by replacing the sample with 0.6 mol L<sup>-1</sup> KCl. Sulfhydryl group content was calculated, using the extinction coefficient of 13,600 L mol<sup>-1</sup> cm<sup>-1</sup> and was expressed as mol 10<sup>5</sup> g protein<sup>-1</sup>.

### 2.6.5. Disulfide bond content

Disulfide bond content in NAM was determined using 2-nitro-5-thiosulfolbenzoate (NTSB) assay, according to the method of Thannhauser, Konishi, and Scheraga (1987). To 0.5 mL of NAM sample (1 g L<sup>-1</sup>), 3 mL of freshly prepared NTSB assay solution were added. The mixture was mixed thoroughly and incubated in the dark at room temperature for 25 min. Absorbance at 412 nm was measured using a spectrophotometer. A blank was conducted by replacing the sample with 0.6 mol L<sup>-1</sup> KCl. Disulfide bond content was calculated using the extinction coefficient of 13,900 L mol<sup>-1</sup> cm<sup>-1</sup> and was expressed as mol 10<sup>6</sup> g protein<sup>-1</sup>.

## 2.7. Statistical analysis

All experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by using Duncan's multiple range test (Steel & Torrie, 1980). For pair comparison, *T*-test was used. Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA).

## 3. Results and discussion

### 3.1. Ca<sup>2+</sup>-ATPase activity

Residual Ca<sup>2+</sup>-ATPase activity of NAM from surimi added without and with gelatin hydrolysate or commercial cryoprotectant as influenced by multiple freeze-thaw cycles is shown in Fig. 1A. Loss in Ca<sup>2+</sup>-ATPase activity in all samples was more pronounced as freeze-thaw cycle increased (*P* < 0.05). Surimi without cryoprotectant had the highest decrease in Ca<sup>2+</sup>-ATPase activity after 6 freeze-thaw cycles, in which 22.9% activity was retained. Ruttanapornvareesakul et al. (2006) reported that surimi from lizardfish without cryoprotectant was particularly susceptible to denaturation and aggregation. The decrease in ATPase activity is a good indicator for monitoring the denaturation of myofibrillar proteins during iced and frozen storage (Benjakul et al., 1997). Generation of ice crystals and the increase in ionic strength of the system induced myosin denaturation and the disruption of the actin-myosin complex during frozen or freeze-thaw process (Benjakul & Sutthiphan, 2009). Generally, aggregation and denaturation take place in myosin, especially globular heads responsible for

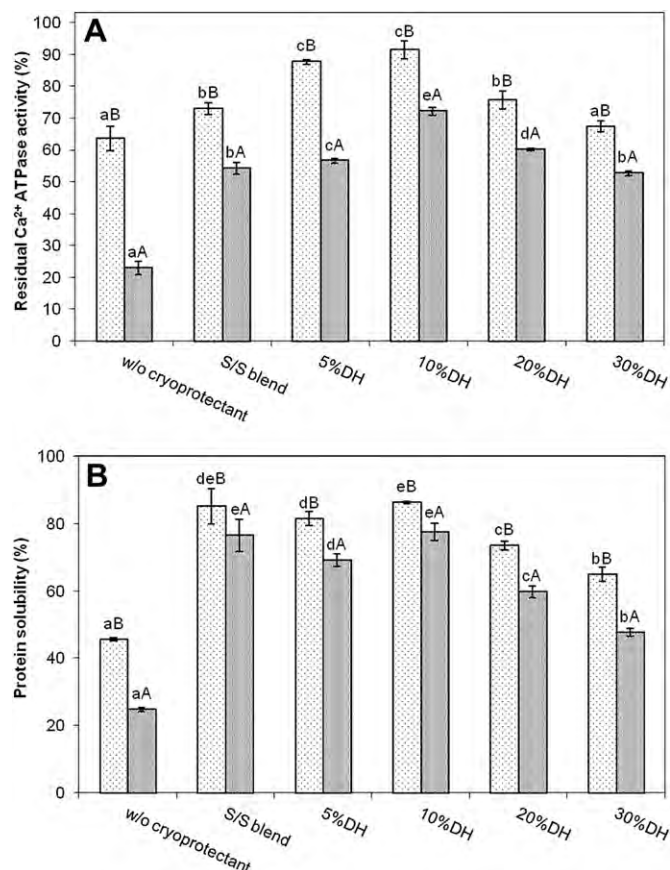


**Fig. 1.**  $\text{Ca}^{2+}$ -ATPase activity of natural actomyosin (A) and protein solubility (B) of surimi added with gelatin hydrolysate with different DHs and subjected to 3 and 6 freeze-thaw cycles. Different letters and capital letters on the bars within the same freeze-thaw cycle and the same cryoprotectant, respectively, indicate significant differences ( $P < 0.05$ ). □ 3 freeze-thaw cycles, ■ 6 freeze-thaw cycles.

ATPase activity, during freeze-thawing process (Sultanbawa & Li-Chan, 2001). In the presence of gelatin hydrolysate or commercial cryoprotectant, residual  $\text{Ca}^{2+}$ -ATPase activity higher than 50% was obtained after 6 freeze-thaw cycles. The results suggest that gelatin hydrolysate more likely exhibited cryoprotective effect on myosin in surimi subjected to multiple freeze-thawing. Peptides in hydrolysate with high proportion of hydrophilic amino acids could bind water, thereby lowering the migration of water to form ice crystals. Squid protein hydrolysate prevented denaturation of lizardfish myofibrillar protein during freezing. Squid protein hydrolysate was composed of hydrophilic amino acids, which probably played a vital role in preventing ice crystal formation, leading to structural stabilization of myofibrillar protein during freezing (Hossain et al., 2004).

When comparing the cryoprotective effect of gelatin hydrolysate with different DHs, hydrolysate with 10%DH showed the highest cryoprotective effect as evidenced by the high  $\text{Ca}^{2+}$ -ATPase activity retained, particularly after 6 freeze-thaw cycles. After 6 freeze-thaw cycles, samples added with gelatin hydrolysate with 10% DH had the higher  $\text{Ca}^{2+}$ -ATPase activity than others ( $P < 0.05$ ), while similar activity was observed between sample containing commercial cryoprotectant and gelatin hydrolysate with 30%DH ( $P > 0.05$ ). Khan et al. (2003) reported that addition of fish protein hydrolysates from five fish species suppressed the loss of  $\text{Ca}^{2+}$ -ATPase activity in lizardfish surimi, whereas the surimi without addition of fish protein hydrolysate had the marked loss in  $\text{Ca}^{2+}$ -ATPase activity. Short-chain peptides may be important in

protection against freezing by inhibiting ice crystal growth (Damodaran, 2007). The results indicate that gelatin hydrolysate with 10%DH exhibited the preventive effect against the denaturation of surimi protein induced by freeze-thaw process.

### 3.2. Protein solubility

Protein solubility in 0.6 mol  $\text{L}^{-1}$  KCl of surimi added without and with gelatin hydrolysate or commercial cryoprotectant after being freeze-thawed for 3 and 6 cycles is shown in Fig. 1B. The solubility decreased with increasing freeze-thaw cycles in all samples ( $P < 0.05$ ). Nevertheless, the highest decrease in protein solubility was obtained in the sample without cryoprotectants ( $P < 0.05$ ). The decrease in protein solubility indicated the denaturation of proteins as induced by freeze-thaw process. The result was in agreement with Benjakul and Bauer (2000) who reported that solubility of cod muscle protein decreased when the freeze-thaw cycles increased. The decrease in solubility might be caused by protein aggregation during frozen storage or freeze-thaw process (Cheung et al., 2009; Sriket, Benjakul, Visessanguan, & Kijroongrojana, 2007). The decrease in protein solubility was coincidental with the decrease in  $\text{Ca}^{2+}$ -ATPase activity (Fig. 1A). White shrimp and black tiger shrimp had the decrease in solubility as the freeze-thaw cycles increased (Sriket et al., 2007). Sultanbawa and Li-Chan (2001) reported that surimi without cryoprotectant lost more water due to freeze denaturation. The decrease in protein solubility is a primary criterion of protein denaturation during frozen storage, resulting from hydrophobic interaction, disulfide bond and ionic interaction (Sultanbawa & Li-Chan, 1998).

The higher solubility was observed in surimi added with gelatin hydrolysate, compared with that of the sample without any cryoprotectant ( $P < 0.05$ ). It was noted that DH of gelatin hydrolysate affected their ability in prevention of loss in protein solubility. Gelatin hydrolysate with 10%DH showed a similar effect with commercial cryoprotectant ( $P > 0.05$ ). Gelatin hydrolysate with higher DH (20 and 30%) had the less ability in preventing solubility losses of surimi subjected to freeze-thawing. Peptides with smaller chain length might have the lower impact on impeding the aggregation of surimi proteins during freeze-thawing since they were preferably localized in the aqueous phase. Damodaran (2007) reported that the  $-\text{Gly}-\text{Pro}-\text{X}-$  and  $-\text{Gly}-\text{Z}-\text{X}-$  tripeptide repeat sequences in gelatin hydrolysate might play a role in their ice crystal growth inhibitory property. Thus, gelatin hydrolysate with appropriate DH was able to prevent denaturation of surimi protein by lowering the protein aggregation during freezing or thawing process.

### 3.3. Surface hydrophobicity

The changes of surface hydrophobicity (SoANS) in NAM extracted from surimi added without and with gelatin hydrolysate or commercial cryoprotectant after freeze-thawing with different cycles are depicted in Fig. 2. For surimi without freeze-thawing, the slight decrease in SoANS was observed when gelatin hydrolysate was added. The decrease was more intense as DH increased ( $P < 0.05$ ). It was most likely due to the fact that gelatin hydrolysate with high hydrophilicity in nature could interact with NAM via hydrophilic–hydrophilic interaction. As a result, hydrophilic domains of NAM could be more exposed as indicated by the lowered SoANS. For all samples, SoANS increased when the number of freeze-thaw cycles increased ( $P < 0.05$ ). However, the increase in SoANS was lowered in surimi added with gelatin hydrolysate with 5 and 10%DH after 3 freeze-thaw cycles, compared with others ( $P < 0.05$ ). Among all samples, the lowest SoANS was observed in surimi added with gelatin hydrolysate

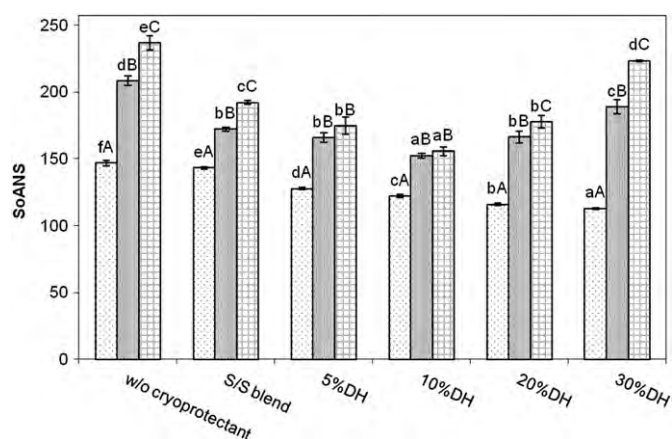


**Fig. 2.** Surface hydrophobicity of natural actomyosin from surimi added with gelatin hydrolysate with different DHs and subjected to 3 and 6 freeze-thaw cycles. Different letters and capital letters on the bars within the same freeze-thaw cycle and the same cryoprotectant, respectively, indicate significant differences ( $P < 0.05$ ). □ fresh surimi, ▨ 3 freeze-thaw cycles, ■ 6 freeze-thaw cycles.

having 10%DH ( $P < 0.05$ ), whereas surimi without cryoprotectant had the highest SoANS ( $P < 0.05$ ). The increase in SoANS indicated the conformational changes of NAM, in which hydrophobic domain was exposed. The increase in SoANS of all samples was generally in agreement with the decrease in  $\text{Ca}^{2+}$ -ATPase (Fig. 1A). An alternation of myosin head, when hydrophobic interior protein was exposed, reflected protein denaturation or degradation (Mutilangi, Panyam, & Kilara, 1996). The formation of ice crystals and the destruction of the hydrate layers surrounding polar residues followed by hydrophobic interactions are considered to be the prime causes of freeze-induced denaturation of fish protein (Hanafusa, 1973; Némethy & Scheraga, 1962). When gelatin hydrolysate was added, the increase in SoANS was retarded. From the result, gelatin hydrolysate having 10%DH could suppress the denaturation of fish protein as indicated by the lowest increase in surface hydrophobicity. Hossain et al. (2004) reported that addition of squid protein hydrolysate in lizardfish myofibrillar protein resulted in the increased amount of unfrozen water due to the interaction between hydrophilic side chains of peptides and hydrate water in the hydration sphere of myofibrillar proteins. However, gelatin hydrolysate with higher DH (20 and 30%) with higher hydrophilicity had the lower ability in prevention of protein denaturation in surimi subjected to multiple freeze-thawing process.

### 3.4. Total sulfhydryl group content and disulfide bond content

The changes of total sulfhydryl group content in NAM extracted from surimi added without and with gelatin hydrolysate or commercial cryoprotectant after multiple freeze-thaw process are depicted in Fig. 3A. Total sulfhydryl group content generally decreased with increasing number of freeze-thaw cycle ( $P < 0.05$ ). The total sulfhydryl group content decreased by 9.44–23.93% of initial value with 3 freeze-thaw cycles. Slight decrease was obtained when surimi was subjected to freeze-thawing with 6 cycles. Myosin contains 42 sulfhydryl group (Benjakul & Bauer, 2000; Buttkeus, 1970). These reactive sulfhydryl groups are susceptible to oxidation, especially during frozen storage (Benjakul & Bauer, 2000; Buttkeus, 1970; Sultanbawa & Li-Chan, 2001). Among all samples, total sulfhydryl group content in surimi added with commercial cryoprotectant and gelatin hydrolysate with 10%DH decreased to a lower extent after 6 freeze-thaw cycles, in which the content decreased by 12.88 and 14.24% of the initial value,

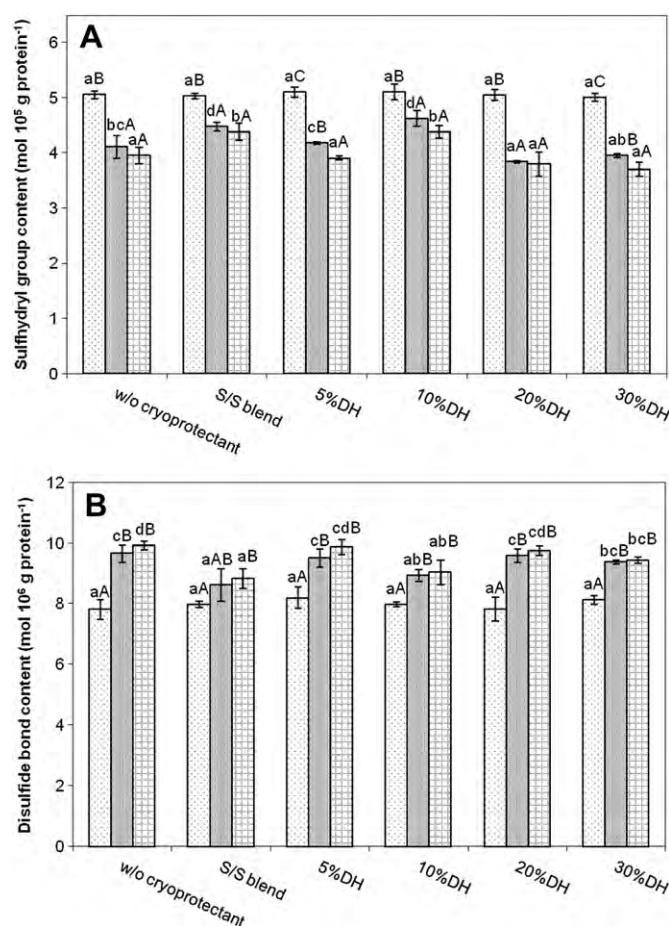


**Fig. 3.** Total sulfhydryl group content (A) and disulfide bond content (B) of natural actomyosin from surimi added with gelatin hydrolysate with different DHs and subjected to 3 and 6 freeze-thaw cycles. Different letters and capital letters on the bars within the same freeze-thaw cycle and the same cryoprotectant, respectively, indicate significant differences ( $P < 0.05$ ). □ fresh surimi, ▨ 3 freeze-thaw cycles, ■ 6 freeze-thaw cycles.

respectively. Therefore, gelatin hydrolysate with 10%DH could retard the loss of total sulfhydryl group content in surimi during freeze-thaw process comparable to that of the commercial cryoprotectant.

The changes of disulfide bond content after 3 and 6 cycles of freeze-thawing are shown in Fig. 3B. Disulfide bond content in all samples generally increased when the number of freeze-thaw cycles increased ( $P < 0.05$ ). Nevertheless, a similar disulfide bond content was observed in NAM extracted from surimi subjected to 3 and 6 freeze-thaw cycles ( $P > 0.05$ ). No difference in disulfide bond content between surimi added with commercial cryoprotectant and gelatin hydrolysate having 10%DH was observed after freeze-thaw process, either 3 or 6 cycles ( $P > 0.05$ ). Surimi without cryoprotectant showed the highest disulfide bond content after repeated freeze-thawing ( $P < 0.05$ ). The increase in disulfide bond content was generally coincidental with the decrease in  $\text{Ca}^{2+}$ -ATPase activity (Fig. 1A) and total sulfhydryl group content (Fig. 3A). The result suggests that freeze-thaw process possibly induced the exposure of sulfhydryl groups, which were further oxidized to disulfide bonds. Benjakul et al. (1997) reported that the oxidation of sulfhydryl groups, especially in the head region, caused the decrease in  $\text{Ca}^{2+}$ -ATPase activity. The result indicated that the oxidation of sulfhydryl group and the decrease in  $\text{Ca}^{2+}$ -ATPase activity could be retarded by the addition of gelatin hydrolysate having 10%DH.

#### 4. Conclusion

Gelatin hydrolysate with 10%DH could prevent the denaturation of surimi protein comparable to that of commercial cryoprotectant (6 g 100 g<sup>-1</sup> sucrose and 2 g 100 g<sup>-1</sup> sorbitol) but exhibited a higher protective effect on denaturation of myosin heavy chain. Therefore, hydrolysate with appropriate DH could serve as an alternative cryoprotectant to reduce the sweetness in surimi products. However, the effect of gelatin hydrolysate (10%DH) on physical properties, particularly gel forming ability, of surimi as well as sensory characteristics needs to be further studied.

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# EFFECTS OF BAMBARA GROUNDNUT PROTEIN ISOLATE ON PROTEIN DEGRADATION AND GEL PROPERTIES OF SURIMI FROM SARDINE (*SARDINELLA ALBELLA*)

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## ABSTRACT

Effects of protein isolate from bambara groundnut (BGPI) at different levels (0–1.5%) on autolysis and gel property of surimi from sardine (*Sardinella albella*) were investigated. Higher inhibitory activity against autolysis for kamaboko (40/90C) and modori (65/90C) gels was observed when BGPI level increased up to 1%. BGPI effectively prevented the degradation of myosin heavy chain in a concentration-dependent manner in both gels. This was associated with the increased breaking force and deformation of gels added with BGPI ( $P < 0.05$ ). Water-holding capacity of gels was improved with increasing level of BGPI incorporated ( $P < 0.05$ ). However, whiteness of kamaboko and modori gels slightly decreased with increasing BGPI levels ( $P < 0.05$ ). Microstructure of kamaboko and modori gels added with 1% BGPI had the denser and more ordered fibrillar structure, indicating a preventive effect of BGPI on hydrolysis of myofibrillar proteins.

## PRACTICAL APPLICATIONS

Surimi from dark-fleshed fish is generally prone to protein degradation caused by heat-activated proteases. Proteolytic disintegration of myofibrillar proteins leads to gel weakening of surimi. Gel-forming ability of myofibrillar proteins is the most important functional requirement to provide the superior quality of surimi-based products. Protein isolate from bambara groundnut (BGPI) containing protease inhibitors could be used to lower autolysis of sardine surimi caused by endogenous proteases. In addition, BGPI might serve as a co-gelling agent or binder in surimi gels. Thus, BGPI could be used as the promising protein additive for surimi industry.

## INTRODUCTION

Gel-forming ability of myofibrillar proteins is the most important functional requirement to provide the superior quality of surimi-based products, and it depends on the both intrinsic and extrinsic factors. The rapid and severe breakdown of myofibrillar proteins, particularly myosin, at higher temperature inhibits the development of three-dimensional gel network. This leads to gel weakening of surimi-based products (Morrissey *et al.* 1993). Gel softening, so-called modori, is induced by endogenous heat-activated proteases, which are able to degrade myofibrillar proteins (Sangorin *et al.* 2000; Liu *et al.* 2008; Hu *et al.* 2010). Gel weakening varies with species but is generally

caused by two types of proteases, serine-type and cysteine-type proteases (Rawdkuen and Benjakul 2008; Van Phu *et al.* 2010).

Because of the overexploitation of lean fish, which have been used for surimi production, pelagic dark-fleshed fish have been paid increasing attention as alternative source of raw material for surimi production. However, surimi from dark-fleshed fish has poor gel-forming ability. This is associated with high proteolytic activity, sarcoplasmic protein, myoglobin and fat contents (Haard *et al.* 1994; Chaijan *et al.* 2004). Chaijan *et al.* (2004) stated that myoglobin and sarcoplasmic protein from dark muscle can be removed to some extent with washing, particularly with increasing NaCl and washing cycle. However, myofibril-associated proteases

remained in surimi contribute to gel weakening (Cao *et al.* 1999; Benjakul *et al.* 2003a). Shimizu *et al.* (1992) proposed that poor gel-forming properties of muscle from dark-fleshed species was caused by the presence of heat-stable proteases, which are active in degrading myosin during the heating at temperature range of 50–70°C. Tsukamasa and Shimizu (1989) and Rawdkuen *et al.* (2007) reported that the maximized modori phenomenon of surimi from *Sardinop melanosticta* and *Sardinella gibbosa* occurred when the gels were incubated at temperatures 60 and 70°C, respectively.

To alleviate the soft texture problem caused by endogenous proteases in surimi-based foods, some additives containing protease inhibitors have been used such as beef plasma protein, chicken plasma protein (CPP), porcine plasma protein, egg white and whey protein concentrate (WPC) (Benjakul *et al.* 2004a, 2010; Rawdkuen *et al.* 2007; Rawdkuen and Benjakul 2008). Sodium caseinate, wheat gluten and soy protein isolate (SPI) have been used to improve the gel-forming capacity of sardine surimi (Gomez-Guillen and Montero 1996; Gomez-Guillen *et al.* 1996, 1997). However, some additives have been prohibited due to their association with disadvantages such as bovine spongiform encephalopathy, outbreak of avian influenza, off odor, off-color and off-favor in resulting surimi-based products (Rawdkuen and Benjakul 2008).

Legume seeds are considerably rich in Kunitz and Bowman-Birk type protease inhibitors (Guillamon *et al.* 2008). Bambara groundnut is an indigenous legume in the southern part of Thailand. Benjakul *et al.* (2000a) reported that partially purified protease inhibitors from bambara ground nuts were quite stable at 90°C for 30 min at pH 7.0. Apart from inhibitory action, protein isolate from bambara groundnut (BGPI) exhibited gelling property, high water-holding capacity, high protein solubility at either side of isoelectric point and bulking density for successful utilization in various food products (Eltayeb *et al.* 2011). Therefore, the addition of BGPI possessing the protease inhibitory activity should pave the way for gel improvement of sardine surimi. The objective of this study was to investigate the effects of BGPI on autolysis and gel-forming ability of sardine (*Sardinella albella*) surimi.

## MATERIALS AND METHODS

### Chemicals and Surimi

Trichloroacetic acid (TCA), sodium chloride and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Bovine serum albumin, L-tyrosine, *N*- $\alpha$ -Benzoyl-DL-arginine-*p*-nitroanilide (BAPNA),  $\beta$ -mercaptoethanol ( $\beta$ -ME), trypsin from bovine pancreas (*N*-Benzoyl-DL-arginine ethyl ester [BAEE] 10,200 units/

mg) and wide-range molecular weight (MW) protein markers were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium dodecyl sulphate, Coomassie Blue R-250, and *N*, *N*, *N'*, *N'*-tetramethyl ethylene diamine were procured from Bio-Rad Laboratories (Hercules, CA). All chemicals were of analytical grade.

Frozen surimi grade A from sardine (*Sardinella albella*) was obtained from Man A Frozen Foods Co., Ltd. (Songkhla, Thailand) and kept at –20°C until use but not longer than 2 months.

### Preparation of BGPI

Bambara groundnut (*Vigna subterranean*) was purchased from local market, Hat Yai, Songkhla, Thailand. The sample was dehulled and ground using a blender (Moulinex, Type AY46, Shenzhen, Guangdong, China) to obtain the fine powder. The powder was sieved using a mesh with an aperture size of 500  $\mu$ m (35, ASTM E11, serial number 5666533, FRITSCH GmbH, Idar-Oberstein, Germany).

BGPI was prepared according to the methods of Hoque *et al.* (2011) with a slight modification. Bambara groundnut powder was suspended in 10 volumes of 0.2% NaOH solution (pH 12). The mixture was stirred continuously for 2 h at room temperature (28–30°C), followed by centrifugation at 8,000  $\times$  g for 30 min at 25°C (Avanti J-E centrifuge, Beckman Coulter, Inc., Fullerton, CA). The supernatant was collected, and pH was adjusted to 4.5 using 6 N HCl. The precipitate formed was recovered by centrifugation at 8,000  $\times$  g for 30 min. The pellet was washed twice with 10 volumes of distilled water adjusted to pH 4.5 using 6 N HCl, followed by centrifugation at 8,000  $\times$  g for 30 min. The resulting pellet was freeze-dried. Dried powder obtained was referred to as “bambara groundnut protein isolate, BGPI.” BGPI was placed in polyethylene bag and stored at –40°C until use. BGPI contained 86.2% protein, 6.04% fat, 3.11% ash and 3.16% moisture, as determined by the method of the Association of Official Analytical Chemists (AOAC) (AOAC 2000).

### Trypsin Inhibitory Activity Assay

Trypsin inhibitory activity of BGPI was measured by the method of Benjakul *et al.* (2000a), with a slight modification using BAPNA as substrate. Trypsin inhibitors from BGPI were extracted by continuous stirring BGPI (5 mg/mL) in 25 mL of 0.2 M Tris-HCl, pH 8.5 at a speed of 300 rpm at room temperature (26–28°C) for 90 min using magnetic stirrer (IKA Model Color Squid white, BEC THAI, Bangkok, Thailand). The mixtures were then subjected to centrifugation at 12,000  $\times$  g at 25°C for 25 min. The supernatant was used for determination of trypsin inhibitors.

To assay for trypsin inhibitor, the solution containing 200  $\mu$ L of inhibitor solution (protein isolate supernatant), 200  $\mu$ L of bovine pancreas trypsin (0.1 mg/mL) and 1,000  $\mu$ L of 50 mM Tris-HCl, pH 8.5 containing 10 mM  $\text{CaCl}_2$  was preincubated at 37C for 15 min. To initiate the reaction, 200  $\mu$ L of BAPNA (0.4 mg/mL in dimethyl sulfoxide, prewarmed to 37C) were added and vortexed immediately to start the reaction. After incubating for 10 min, 200  $\mu$ L of 30% acetic acid (v/v) was added to terminate the reaction. For the control, distilled water was used instead of inhibitor solution. The reaction mixture was centrifuged at  $8,000 \times g$  for 5 min at room temperature. The activity of trypsin in the absence and presence of inhibitor solution was determined by measuring the absorbance at 410 nm because of *p*-nitroaniline released. One unit of proteolytic activity was defined as an increase of 0.01 absorbance unit mL/min under the assay condition. One unit of trypsin inhibitory activity was defined as the amount of inhibitor, which reduced trypsin activity by one unit.

### Autolysis Study of Sardine Surimi

Autolysis of sardine surimi was determined as per the method of Yarnpakdee *et al.* (2009). Three grams of defrosted surimi (pH 7) were placed as a thin layer in a beaker, followed by incubating at different temperatures (45, 50, 55, 60, 65, 70, 75 and 80C) in a temperature-controlled water bath (Mettmert, Schwabach, Germany) for 60 min. The autolytic reaction was terminated by addition of 27 mL of cold 5% TCA (w/v). The mixture was homogenized for 2 min at 11,000 rpm (IKA homogenizer, Labortechnik, Selangor, Malaysia). The homogenate was centrifuged at  $8,000 \times g$  for 10 min using a microcentrifuge (Model MIKRO20, Hettich-Zentrifugen, Tuttlingen, Germany). TCA-soluble peptide content in the supernatant was determined by the Lowry method (Lowry *et al.* 1951) using L-tyrosine as a standard and was expressed as  $\mu$ mol tyrosine/g sample.

To determine the autolytic pattern of sardine surimi, another three grams of sample incubated at different temperatures for 60 min were mixed with 27 mL of hot 5% sodium dodecyl sulfate (SDS) (85C) to terminate the autolytic reaction. The mixture was then homogenized at a speed of 11,000 rpm for 1 min. The homogenate was then incubated at 85C for 60 min to solubilize the sample. To remove undissolved debris, the mixture was centrifuged at  $8,000 \times g$  for 10 min. The supernatant was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) analysis.

### Effect of BGPI on Gel Properties of Sardine Surimi

Frozen surimi was partially thawed at 4C for 2–3 h, cut into small pieces with an approximate thickness of 1 cm. Pre-

pared surimi was placed in the mixer (National Model MKK77, Tokyo, Japan) and subsequently chopped for 1 min. BGPI, containing trypsin inhibitor at a level of  $11,098 \pm 64$  units/g, was added into surimi to obtain the different concentrations (0, 0.25, 0.5, 1 and 1.5% w/w). The final moisture content of samples was adjusted to 80% (w/w) by adding iced water. Thereafter, salt (2.5%, w/w) was added. The mixture was chopped for additionally 3 min, and the temperature was maintained at below 7C during chopping. The sol was stuffed into a polyvinylidene chloride casing with a diameter of 2.5 cm, and both ends were sealed tightly. Kamaboko and modori gels were prepared by incubating the sol at 40 and 65C for 30 min, respectively, followed by heating at 90C for 20 min. All gels were cooled in iced water for 30 min and stored at 4C overnight prior to analyses.

## Analyses

**Textural Analysis.** Textural analysis of surimi gels was carried out using a texture analyzer (Model TA-XT2, Stable Micro Systems, Surrey, UK). Gels kept at 4C were equilibrated at room temperature (28–30C) before analysis. Six cylindrical samples (2.5 cm in diameter; 2.5-cm length) were prepared. Breaking force and deformation were measured by the texture analyzer according to the method of Benjakul *et al.* (2010). A spherical probe with a diameter of 5 mm was pressed into the cut surface of a gel specimen perpendicularly at a constant depression speed (60 mm/min) until puncture occurred. The force (breaking force) and the distance at which the ball probe punctured into the surimi (breaking distance or deformation) were both recorded.

**Determination of Whiteness.** Gel samples from each treatment were subjected to whiteness measurement using a colorimeter (HunterLab, ColorFlex, Hunter Associates Laboratory, Reston, VA). White standard plate was used for calibration prior to the measurement. Illuminant C was used as the light source of measurement.  $L^*$ ,  $a^*$  and  $b^*$  values were measured. Whiteness was calculated using the following equation (Park 1994):

$$\text{Whiteness} = 100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{1/2}$$

**Determination of Expressible Moisture Content.** Expressible moisture content was measured according to the method of Rawdkuen and Benjakul (2008). Gel samples were cut to a thickness of 5 mm, weighed ( $X$ ) and placed between three pieces of filter papers (Whatman No.1, Whatman International, Ltd., Maidstone, England) at the

bottom and two pieces of paper on the top. A standard weight (5 kg) was placed on the top of the sample for 2 min, and then the sample was removed from the papers and weighed again (*Y*). Expressible moisture content was calculated with the following equation and expressed as the percentage of sample weight:

$$\text{Expressible moisture content (\%)} = [X - Y/X] \times 100$$

#### Determination of TCA-soluble Peptide Content.

TCA-soluble peptide content was determined according to the method of Benjakul *et al.* (2010). Three grams of gel samples were homogenized with 27 mL of cold 5% (w/v) TCA for 2 min at 12,000 rpm. The homogenate was allowed to stand in ice for 60 min and centrifuged at  $8,000 \times g$  for 10 min. TCA-soluble peptide content in the supernatant was measured as previously described and expressed as  $\mu\text{mol}$  tyrosine/g sample.

**SDS-PAGE.** Protein patterns of surimi and gels were analyzed by SDS-PAGE according to the method of Laemmli (1970). To prepare the protein sample, 27 mL of 5% (w/v) hot SDS (85C) solution was added to the sample (3 g). The mixture was then homogenized for 2 min at speed of 12,000 rpm. The homogenate was incubated at 85C for 1 h to dissolve total proteins. The samples were then centrifuged at  $8,000 \times g$  for 20 min to remove undissolved debris. Protein concentration in the supernatants was determined as per the method of Lowry *et al.* (1951). Solubilized samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris-HCl, pH 6.8, containing 4% SDS, 20% glycerol and 10%  $\beta$ -ME) and boiled for 3 min. The samples (15  $\mu\text{g}$  protein) were loaded onto the polyacrylamide gel made of 10% running gel and 4% stacking gel, and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini Protean III unit (Bio-Rad Laboratories, Inc., Richmond, CA). After separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid, and destained with 50% methanol (v/v) and 7.5% (v/v) acetic acid, followed by 5% methanol (v/v) and 7.5% (v/v) acetic acid.

**Scanning Electron Microscopy.** Microstructure of surimi gels was determined using scanning electron microscopy. Kamaboko and modori gels (thickness of 2–3 mm) without and with 1% (w/w) BGPI addition were fixed with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2). The samples were then rinsed for 1 h in distilled water before being dehydrated in ethanol with a serial concentration of 50, 70, 80, 90 and 100% (v/v). Dried samples were

mounted on a bronze stub and sputter-coated with gold (Sputter coater, SPI-Module, West-Chester, PA). The specimens were observed with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 20 kV.

**Statistical Analysis.** All experiments were run in triplicate. Data were subjected to analysis of variance. Comparison of means was carried out by Duncan's multiple range tests (Steel and Torrie 1980). Analysis was performed using a SPSS package (SPSS 17.0 for Windows, SPSS Inc., Chicago, IL).

## RESULTS AND DISCUSSION

### Autolysis of Sardine Surimi as Affected by Different Temperatures

Autolysis of sardine surimi at different temperatures that was monitored and expressed in terms of TCA-soluble peptides is shown in Fig. 1A. TCA-soluble peptide content of sardine surimi increased as the temperature increased, and the highest value was obtained at 65C ( $P < 0.05$ ). At temperature above 65C, the marked decrease in TCA-soluble peptide content was noticed most likely because of thermal denaturation of endogenous proteases at high temperature. The result suggested the presence of heat-activated proteases, which were able to hydrolyze the muscle proteins in surimi at high temperature, especially during thermal gelation process. Mince from goatfish and bigeye snapper showed the highest autolysis at 60 and 65C, respectively (Rawdkuen and Benjakul 2008; Yarnpakdee *et al.* 2009). Rawdkuen *et al.* (2007) reported that the highest autolysis of surimi from *Sardinella gibbosa* was found at 70C. The differences in autolysis profile of surimi from different species were more likely due to the different types of proteases present in surimi. The myofibril-associated proteases play a profound role in weakening the gel because they are still retained after the washing process (Cao *et al.* 1999; Benjakul *et al.* 2003a). As a result, those proteases were able to hydrolyze proteins in surimi, particularly at elevated temperature.

Autolysis patterns of sardine surimi incubated at different temperatures are shown in Fig. 1B. Degradation was more prominent at temperatures higher than 50C, as evidenced by the marked disappearance in myosin heavy chain (MHC) and actin bands. The coincidental formation of protein or peptide with MW of 108 kDa was found. This band might represent the degradation product of MHC induced by endogenous proteases. At temperature higher than 70C, MHC and actin were more retained mainly due to heat inactivation of endogenous proteases. In general, the

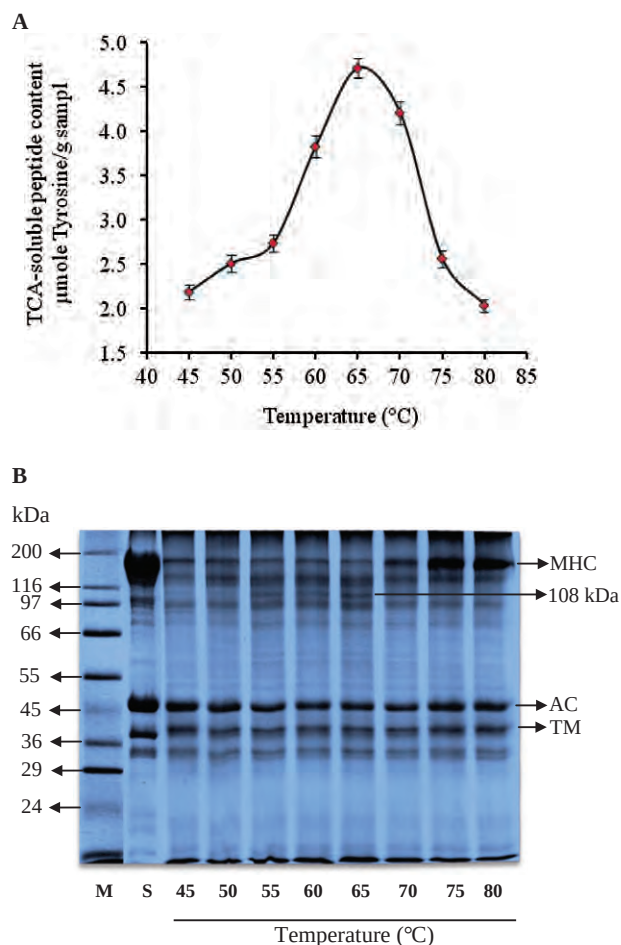


**FIG. 1.** TRICHLOROACETIC ACID (TCA)-SOLUBLE PEPTIDE CONTENTS (A) AND PROTEIN PATTERN (B) OF SARDINE SURIMI INCUBATED AT DIFFERENT TEMPERATURES

Autolysis was conducted for 60 min. MHC, myosin heavy chain; AC, actin; TM, tropomyosin; M, standard molecular markers; S, surimi sol. Numbers designate the incubation temperatures (°C). Bars represent the standard deviation ( $n = 3$ ).

decrease in protein band intensity was in agreement with the increase in TCA-soluble peptide content (Fig. 1A). Therefore, the degradation of MHC was maximized at 65°C, and it was optimal temperature for gel weakening in sardine surimi.

### Effect of BGPI on Textural Properties of Sardine Surimi Gels

Breaking force and deformation of both kamaboko and modori gels containing BGPI with trypsin inhibitory activity (13,100 units/g) at different levels are shown in Fig. 2. Breaking force and deformation of both kamaboko and modori gels containing BGPI increased with increasing

BGPI level up to 1% ( $P < 0.05$ ). Nevertheless, the highest breaking force was found in kamaboko gel added with 1.5% BGPI compared with that containing 1% BGPI ( $P < 0.05$ ). Breaking force and deformation of kamaboko gels from sardine surimi added with 1.5% BGPI increased by 44.9 and 21.4%, respectively. For modori gel added with 1% BGPI, breaking force and deformation increased by 125.5 and 50.3%, respectively, compared with those of the control gel (without BGPI). Comparatively, breaking force and deformation of kamaboko gels were higher than those of modori gels. This was probably due to the greater cross-linking of proteins induced by endogenous transglutaminase during setting at 40°C of kamaboko gel. As consequence, high covalent bondings, especially  $\epsilon$ -( $\gamma$ -glutamyl) lysine linkages, were formed. These cross-links were plausibly resistant to

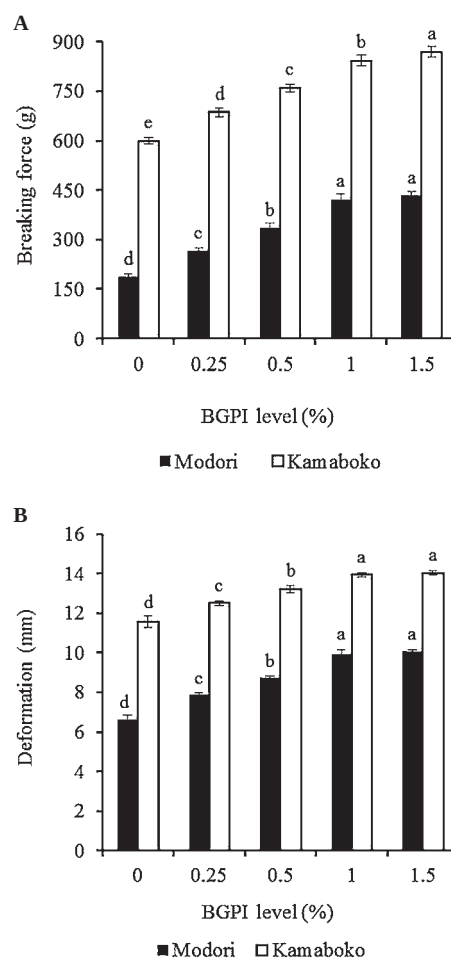


**FIG. 2.** BREAKING FORCE (A) AND DEFORMATION (B) OF MODORI AND KAMABOKO GELS FROM SARDINE SURIMI ADDED WITH BAMBARA GROUNDNUT PROTEIN ISOLATE (BGPI) AT DIFFERENT LEVELS

Bars represent the standard deviation ( $n = 06$ ). Different letters on the bars within the same gel indicate significant differences ( $P < 0.05$ ).

**TABLE 1.** WHITENESS AND EXPRESSIBLE MOISTURE CONTENT OF MODORI AND KAMABOKO GELS FROM SARDINE SURIMI ADDED WITH BGPI AT DIFFERENT LEVELS

| BGPI level (%) | Whiteness                  |                           | Expressible moisture (%)  |                           |
|----------------|----------------------------|---------------------------|---------------------------|---------------------------|
|                | Modori                     | Kamaboko                  | Modori                    | Kamaboko                  |
| 0              | 70.42 ± 0.22 <sup>a</sup>  | 69.61 ± 0.15 <sup>a</sup> | 11.96 ± 0.40 <sup>a</sup> | 7.66 ± 0.36 <sup>a</sup>  |
| 0.25           | 70.18 ± 0.43 <sup>a</sup>  | 69.28 ± 0.20 <sup>a</sup> | 8.73 ± 0.43 <sup>b</sup>  | 7.26 ± 0.37 <sup>a</sup>  |
| 0.50           | 69.58 ± 0.25 <sup>b</sup>  | 68.62 ± 0.13 <sup>b</sup> | 7.81 ± 0.46 <sup>c</sup>  | 6.64 ± 0.48 <sup>b</sup>  |
| 1.0            | 69.41 ± 0.09 <sup>bc</sup> | 68.18 ± 0.29 <sup>c</sup> | 6.45 ± 0.54 <sup>d</sup>  | 6.06 ± 0.38 <sup>bc</sup> |
| 1.5            | 69.03 ± 0.43 <sup>c</sup>  | 67.82 ± 0.40 <sup>c</sup> | 5.80 ± 0.37 <sup>d</sup>  | 5.71 ± 0.38 <sup>c</sup>  |

Mean ± S.D. ( $n = 3$ ). Different superscripts in the same column indicate significant differences ( $P < 0.05$ ).

hydrolysis induced by proteases. Although these bondings might be formed to lower extent in modori gel, the degradation still took place in this gel. In the presence of BGPI with sufficient proteolytic inhibitory activity, the degradation of surimi muscle proteins could be impeded. Luo *et al.* (2004) reported that breaking force of surimi from Alaska pollock incubated at 50C for 60 min increased with addition of 10% SPI. The results suggested that BGPI at level of 1.5% played a crucial role in inhibiting endogenous heat-activated proteases in surimi from sardine. Benjakul *et al.* (1999) found that the extract from bambara groundnut effectively inhibited modori-inducing proteases in threadfin bream modori gel. Furthermore, some proteins in protein isolate might serve as gelling agents in surimi gelation. Adebowale *et al.* (2011) reported that 7S vicilin, a major storage protein in BGPI, possessed high gelling property that might act as co-gelling agents/binder in surimi gel. Those proteins from BGPI at the appropriate levels therefore possibly induced the formation of strong gel network of surimi. Tang (2008) reported that vicilin-rich protein isolates from various Phaseolus legumes exhibited irreversible gelation property via the formation of new disulfide bonds. Therefore, those legume proteins might form network along with muscle proteins during gelation. As a result, muscle protein substrate for protease action might be lowered. Furthermore, the proteins in BGPI had high water-holding capacity (Eltayeb *et al.* 2011). This might contribute to the increased water retention in gel network (Table 1). The result suggested that the addition of BGPI in surimi could strengthen gel via protease inhibition and its binding or co-gelling effects.

### Effect of BGPI on Whiteness of Sardine Surimi Gel

The whiteness of modori and kamaboko gels added with BGPI at different levels is shown in Table 1. The slight decrease in whiteness was noticed in both gels as BGPI

levels increased ( $P < 0.05$ ). This result was accordance with Luo *et al.* (2004) who reported a linear decrease in whiteness of surimi gels from Alaska pollock and common carp when SPI levels added increased. Additionally, Benjakul *et al.* (2000b) also reported that when partially purified proteinase inhibitors from cowpea, pigeon pea and bambara groundnut seeds were incorporated, slight decreases in whiteness of surimi gel from threadfin bream were observed. Decrease in whiteness was most probably caused by indigenous pigments in BGPI. The whiteness of kamaboko and modori gels were decreased by 2.6 and 2.0%, respectively, with the addition of 1.5% BGPI ( $P < 0.05$ ). Generally, modori gels had a slightly higher whiteness compared with kamaboko gel regardless of BGPI levels used. Nevertheless, no changes in whiteness were observed in either kamaboko or modori gels when added with 0.25% BGPI. Thus, BGPI could be used as a protein additive in surimi to improve the gel strength without causing marked changes in whiteness.

### Effect of BGPI on Expressible Moisture Content of Sardine Surimi Gel

Expressible moisture contents of kamaboko and modori gels added with BGPI at various levels are shown in Table 1. The expressible moisture content decreased as the level of BGPI added increased in both gels ( $P < 0.05$ ). This indicated that water-holding capacity of both gels was improved with increasing level of BGPI resulting in a greater amount of water retained in the gel matrix. Lowered expressible moisture contents of kamaboko gels from *Sardinella gibbosa* and some tropical fish surimi were observed when CPP and WPC were added (Rawdkuen *et al.* 2007; Rawdkuen and Benjakul 2008). Protein additives with high water-holding capacity might absorb water, especially when they underwent gelation. The lowest expressible moisture contents were found in both kamaboko and modori gels containing 1.5% added BGPI in which the decreases by 25.5 and 51.5%, respectively, were observed. The decrease in expressible moisture content was in accordance with the increased breaking force and deformation of kamaboko and modori gels (Fig. 2). The expressible moisture content of the control kamaboko gel was lower than that of the control modori gel. The higher expressible moisture content of modori gel was associated with higher whiteness (Table 1). This was attributed to the light-scattering effect of released water in modori gel. This result suggested that kamaboko gel had higher water-holding capacity than the modori gel. This might be associated by less degradation of muscle protein in surimi in kamaboko gels, especially when BGPI was added. Therefore, the addition of BGPI increased water-holding capacity of gel matrix.

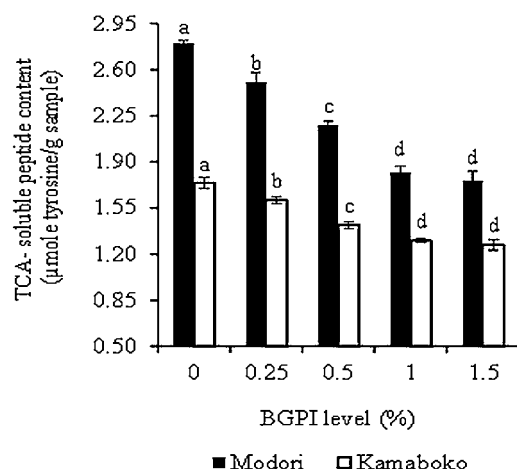


**FIG. 3.** TRICHLOROACETIC ACID (TCA)-SOLUBLE PEPTIDE CONTENT OF MODORI AND KAMABOKO GELS FROM SARDINE SURIMI ADDED WITH BAMBARA GROUNDNUT PROTEIN ISOLATE (BGPI) AT DIFFERENT LEVELS

Bars represent the standard deviation ( $n = 3$ ). Different letters on the bars within the same gel indicate significant differences ( $P < 0.05$ ).

### Effect of BGPI on the Degradation of Sardine Surimi Gel

**TCA-Soluble Peptide Content of Surimi Gel.** Degradation of proteins in kamaboko and modori gel without and with BGPI addition at different levels expressed as TCA-soluble peptide content is shown in Fig. 3. The highest TCA-soluble peptide content was obtained in both gels without BGPI addition, indicating the pronounced degradation caused by endogenous proteases at both heating conditions (40/90 and 65/90C). The highest TCA-soluble peptide content was in accordance with the lowest breaking force and deformation in the control kamaboko and modori gels (without BGPI) (Fig. 2). TCA-soluble peptide content of modori and kamaboko gel decreased as the levels of BGPI increased up to 1% ( $P < 0.05$ ). However, no further decreases in TCA-soluble peptide content in both gels were observed with addition of 1.5% BGPI ( $P > 0.05$ ). Thus, 1% (w/w) BGPI addition might be enough to inhibit modori-inducing proteases in sardine surimi gels. Improved gel strength of kamaboko gel added with 1.5% BGPI might be attributed to gel-enhancing ability of proteins in legume protein isolate. Generally, TCA-soluble peptide contents in the modori gels were greater than those of the kamaboko gels, indicating that greater degradation of surimi proteins occurred in modori gels compared with kamaboko gels. Incubation at 65C for 30 min during modori gel preparation could favor the degradation of surimi proteins induced by endogenous proteases, which had the optimal temperature of 65C (Fig. 1). An *et al.* (1996) and Benjakul *et al.* (2003b) proposed that degradation occurred during heat-

induced gelation is considered to result from the action of heat-activated endogenous proteases. Accordingly, the decrease in TCA-soluble peptide content in gels, especially modori gel by additions of BGPI, reflected the ability of BGPI in inhibiting proteolysis mediated by endogenous proteases. Inhibition of proteolysis should be an effective means for controlling the undesirable gel weakening of surimi from dark-fleshed fish during setting at high temperatures. Thus, protein degradation could be retarded by addition of BGPI at sufficient amount.

**Protein Patterns of Surimi Gel.** Protein patterns of kamaboko and modori gels added with BGPI at different levels (0–1.5%) are shown in Fig. 4. The lowest band

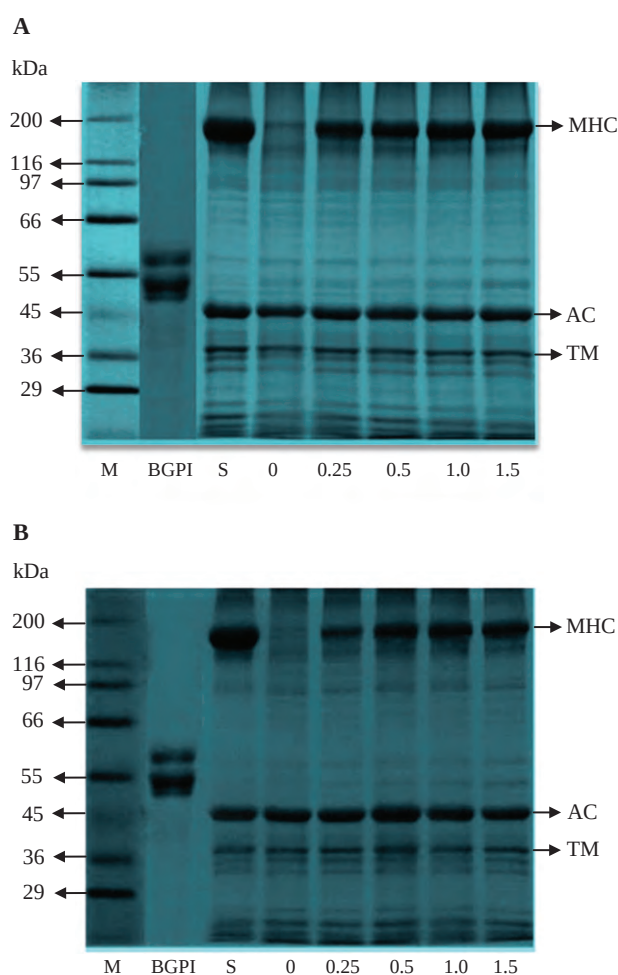


**FIG. 4.** SODIUM DODECYL SULFATE – POLYACRYLAMIDE GEL ELECTROPHORESIS PATTERN OF MODORI (A) AND KAMABOKO (B) GELS FROM SARDINE SURIMI ADDED WITH BGPI AT DIFFERENT LEVELS

MHC, myosin heavy chain; AC, actin; TM, tropomyosin; M, standard molecular weight markers; S, surimi sol; BGPI, bambara groundnut protein isolate. Numbers designate the level of BGPI added (% w/w).

intensity of MHC was found in kamaboko and modori gels containing no BGPI, indicating that the highest polymerization and degradation of MHC caused by endogenous transglutaminase and proteases, respectively. Both actin and tropomyosin were more retained in kamaboko gel compared with modori gel. This indicated that actin and tropomyosin in modori gel underwent degradation to some degree. In general, MHC is more liable to proteolysis caused by endogenous proteases (Rawdkuen and Benjakul 2008; Yarnpakdee *et al.* 2009; Benjakul *et al.* 2010). The intensity of MHC band increased as the level of BGPI increased (Fig. 4, lane 4–8). This result was coincidental with the

decrease in TCA-soluble peptide content (Fig. 3) and the increases in breaking force and deformation in both kamaboko and modori gels (Fig. 2). At the same level of BGPI, higher band intensity of MHC was found in modori gel compared with kamaboko gel. This result suggested that the MHC was less degraded by endogenous proteases mainly because of the presence of inhibitors in BGPI. For kamaboko gel, the retained MHC more likely underwent cross-linking mediated by endogenous transglutaminase more effectively, especially during setting at 40°C. Therefore, the lower MHC band was found in kamaboko gel. Benjakul and Visessanguan (2003) reported the increased formation

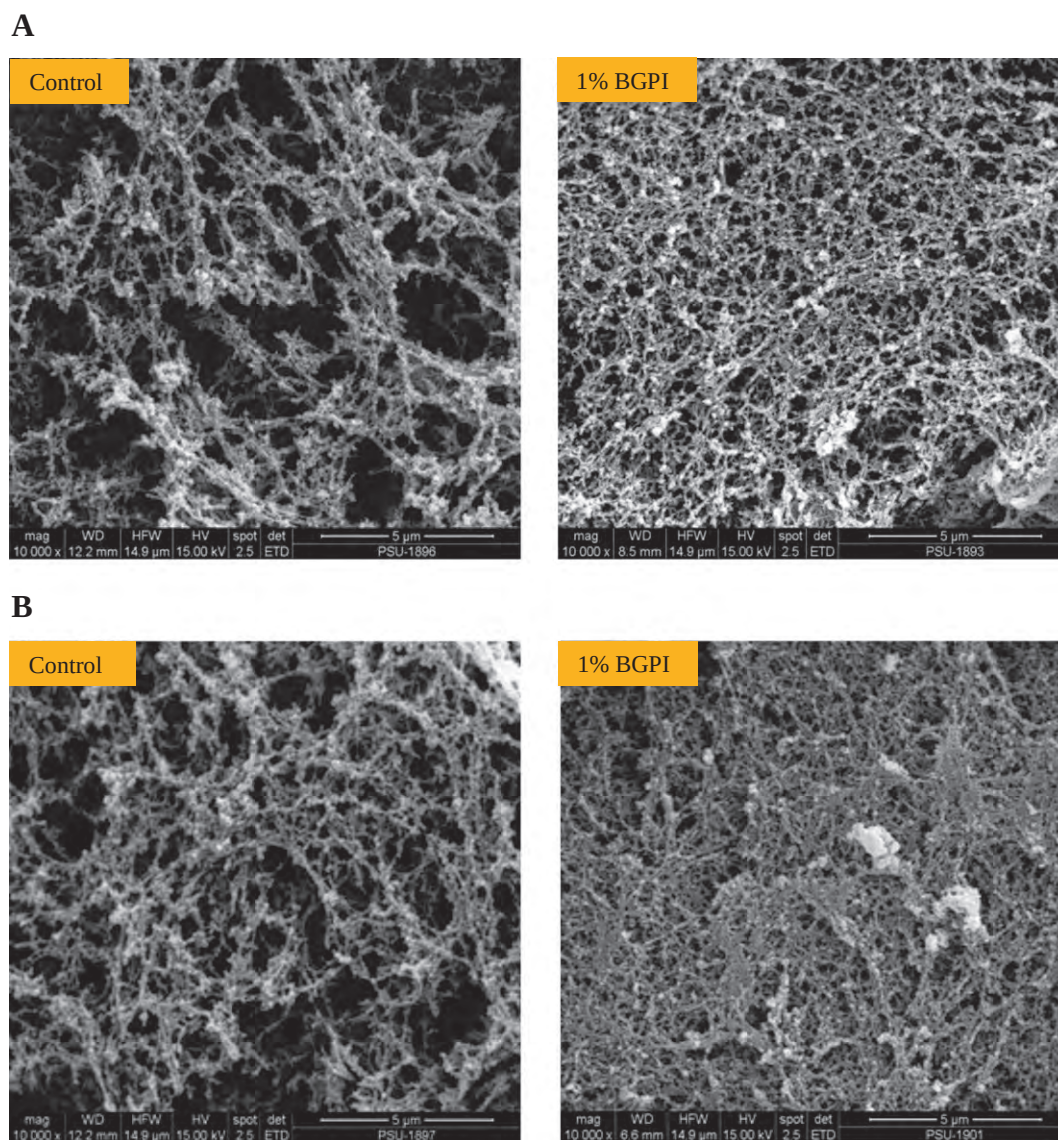


**FIG. 5.** ELECTRON MICROSCOPICAL IMAGE OF MODORI (A) AND KAMABOKO (B) GELS FROM SARDINE SURIMI CONTAINING 1% BAMBARA GROUNDNUT PROTEIN ISOLATE (BGPI) AND THE CONTROL (WITHOUT BGPI)  
Magnification:  $\times 10,000$ .

of nondisulfide covalent bonds, which coincided with increased gel strength and the decrease in MHC band intensity in two species of bigeye snapper induced by endogenous transglutaminase. The increased gel strength of surimi was associated with the increases in cross-linking of MHC and  $\epsilon$ -( $\gamma$ -glutamyl) lysine isopeptide formed (Kumazawa *et al.* 1995). Moreover, at setting temperature (40C), proteolysis still occurred, but at a lower extent, compared with 65C, as evidenced by low TCA-soluble peptide content in kamaboko gel (Fig. 3). Benjakul *et al.* (2004b) reported the proteolysis in surimi from some tropical fish during setting. It can be confirmed that the addition of BGPI could prevent the degradation of proteins in sardine surimi, which in turn favored cross-linking mediated by transglutaminase. As consequence, a strong three-dimensional gel network was formed.

### Effect of BGPI on Microstructure of Sardine Surimi Gel

Microstructure study revealed that kamaboko gel added with 1% BGPI had well-structured matrix with a highly interconnected network of strands than that of control gel (without BGPI) (Fig. 5B). These observations suggested that BGPI might distribute uniformly as the filler in the ordered network and simultaneously prevented the degradation of muscle proteins mediated by endogenous proteases. Rawdkuen and Benjakul (2008) reported that kamaboko gel from bigeye snapper surimi added with 3% WPC was more compact with smaller clusters of aggregated protein than that of the control gel (without WPC). For modori gels (Fig. 5A), that without BGPI addition had the looser structure with larger number of voids compared with kamaboko control gel (Fig. 5B). This was most likely due to high degradation of myofibrillar proteins, particularly during heating at 65C for 30 min. When 1% BGPI was added in modori gel, more compact and finer three-dimensional protein network with smaller voids was observed compared with that of modori gel without BGPI (control) (Fig. 5A). This reconfirmed that BGPI was effective in inhibiting protein degradation. This result was coincidental with the higher breaking force and deformation of modori gel added with 1% BGPI compared with the control gels (Fig. 2). Thus, the addition of 1% BGPI resulted in the improved gel network formation with increased gel strength and water-holding capacity.

### CONCLUSIONS

Autolysis of sardine surimi caused by heat-activated proteases could be partially inhibited by BGPI. The addition of BGPI at concentrations up to 1.5% (w/w) yielded the gel with increased breaking force and deformation along with

higher water-holding capacity. However, addition of BGPI at higher amount caused slight decrease in whiteness. Thus, BGPI at an appropriate level could be an alternative food-grade inhibitor to improve gel properties of surimi from sardine.

### ACKNOWLEDGMENTS

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# Functionalities and antioxidant properties of protein hydrolysates from the muscle of ornate threadfin bream treated with pepsin from skipjack tuna

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## ABSTRACT

Functional properties and antioxidant activities of protein hydrolysates prepared from ornate threadfin bream (*Nemipterus hexodon*) muscle, using skipjack tuna pepsin, with different degrees of hydrolysis (DH: 10%, 20% and 30%), were evaluated. Emulsifying and foaming properties of hydrolysates were governed by their DH and concentrations used. Hydrolysates with 20% DH had the highest scavenging activities for ABTS and DPPH radicals. However, chelating activity of hydrolysates for ferrous ion increased as DH increased. Size exclusion chromatography of the hydrolysate with 20% DH using Sephadex G-25 revealed that antioxidative peptides with molecular weight of approximately 1.3 kDa exhibited the highest ABTS radical-scavenging activity. *In vitro* simulated gastrointestinal digestion indicated that ABTS radical-scavenging activity of the antioxidative peptides was not affected by pepsin hydrolysis, whilst further digestion by pancreatin enhanced the activity. Therefore, protein hydrolysate from the muscle of ornate threadfin bream produced by skipjack tuna pepsin can be used as a promising source of functional peptides with antioxidant properties.

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## 1. Introduction

The use of enzyme technologies for protein recovery and modification has led to the production of a broad spectrum of food ingredients and industrial products (Kristinsson & Rasco, 2000). Proteases from different sources are commonly employed to obtain a more selective hydrolysis due to their specificity for peptide bonds adjacent to certain amino acid residues (Khantaphant & Benjakul, 2008). Enzymatic hydrolysis of food proteins is an efficient way to recover potent bioactive peptides (Thiansilakul, Benjakul, & Shahidi, 2007a). Fish protein hydrolysates have been shown to have potential for nutritional or pharmaceutical applications (Thiansilakul et al., 2007a; Wu, Chen, & Shiau, 2003). Numerous peptides derived from hydrolysed food proteins have been shown to have antioxidant activity. Fish protein hydrolysates such as skin gelatin hydrolysate from brownstripe red snapper (Khantaphant & Benjakul, 2008) or meat protein hydrolysates from yellow travelly (Klompong, Benjakul, Kantachote, & Shahidi, 2007; Klompong et al., 2009), round scad (Thiansilakul et al., 2007a; Thiansilakul, Benjakul, & Shahidi, 2007b), mackerel (Wu et al., 2003) and loach (You, Zhao, Regenstien, & Ren, 2010) have been reported to exhibit antioxidant activity. Fish protein hydrolysates can be used in food

systems, comparable to other pertinent protein hydrolysates (Kristinsson & Rasco, 2000).

Thailand is the world's largest producer and exporter of canned tuna. The most commercially important tuna species for the Thai tuna industry is skipjack tuna, followed by yellow-fin tuna and tongol tuna, accounting for over 80% of Thai frozen tuna imports (Nalinanon, Benjakul, & Kishimura, 2010). Due to a large amount of tuna viscera discarded during manufacturing, it can serve as a promising source of pepsin for further application. Recovery of pepsin from tuna stomach is an approach to minimise the economic and ecological problems of this processing discard. Additionally, fish protein hydrolysate with bioactivity prepared with the aid of fish proteases can be obtained as a new value-added product with high market value (Khantaphant & Benjakul, 2008). Skipjack tuna pepsins were recently purified and characterised by Nalinanon et al. (2010). Pepsins from skipjack tuna effectively hydrolysed several protein substrates, including bovine serum albumin, egg white, natural actomyosin from brownstripe red snapper muscle and acid-solubilised collagen from arabesque greenling skin.

Ornate threadfin bream (*Nemipterus hexodon*) belongs to the order Perciformes and is a member of the Nemipteridae family, found in tropical and sub-tropical Indo-West Pacific. Ornate threadfin bream is a potential raw material and widely used for surimi production in Thailand. However, the price of surimi fluctuates, depending on the world market. To increase the value of meat from

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this fish species, the production of protein hydrolysates with nutritive value as well as good functional properties can be an alternative way for its full utilisation. The use of fish pepsin recovered from stomach of tuna, a by-product from canned tuna processing, for hydrolysate production, could lower the cost of commercial proteases. Furthermore, the production of protein hydrolysate from fish muscle using tuna pepsin has also not been reported. Therefore, the objectives of this investigation were to study the functionalities and antioxidant properties of the protein hydrolysate prepared from ornate threadfin bream (*N. hexodon*) muscle using skipjack tuna pepsin and to determine the molecular weight, pH and thermal stabilities, as well as the activity after *in vitro* simulated gastrointestinal digestion, of the antioxidant peptides in the resulting hydrolysate.

## 2. Materials and methods

### 2.1. Chemicals

Bovine haemoglobin, bovine serum albumin (BSA), pepsin from porcine gastric mucosa (EC 3.4.23.1) (P7000, 422 units/mg solid), L-leucine, 2,4,6-trinitrobenzenesulphonic acid (TNBS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulphonic acid sodium salt (ferrozine) were purchased from Sigma Chemical Co. (St. Louis, MO). Pancreatin was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Folin–Ciocalteu's phenol reagent, trichloroacetic acid (TCA), potassium persulphate, sodium sulphite and ferrous chloride were obtained from Merck (Darmstadt, Germany). Sephadex G-25 was purchased from GE Healthcare UK Limited (Little Chalfont, Buckinghamshire, UK). Sodium dodecyl sulphate (SDS), Coomassie Blue R-250, and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA). All chemicals used were of analytical grade.

### 2.2. Preparation of skipjack tuna stomach

Internal organs of skipjack tuna (*Katsuwonus pelamis*) were obtained from Tropical Canning (Thailand) Public Co., Ltd., Songkhla, Thailand. The samples were packed in polyethylene bags, kept in ice with a solid:ice ratio of 1:2 (w/v) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, within 30 min. Upon arrival, internal organs were excised and the stomach was collected and cut into 0.5 cm × 0.5 cm pieces. Samples were frozen in liquid nitrogen and finely ground using a National Model MX-T2GN blender (Taipei, Taiwan). Samples were then lyophilised using a SCANVAC CoolSafe™ freeze-dryer (Cool-Safe 55, ScanLaf A/S, Lynge, Denmark). The dry powder referred to as 'stomach powder' was stored at −20 °C until use.

Ornate threadfin bream (*N. hexodon*) was purchased from the dock in Songkhla, Thailand. The fish, off-loaded approximately 18–24 h after capture, were placed in ice at a fish-to-ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, within 1 h. Upon arrival, fish were filleted and the ordinary muscle was collected. Size reduction of muscle was carried out by chopping manually into small pieces. The prepared sample was kept in ice until use.

### 2.3. Preparation of pepsin from skipjack tuna stomach and activity assay

To prepare the skipjack tuna pepsin extract, the stomach powder was suspended in 20 mM sodium phosphate buffer (pH 7.0)

containing 1 mM PMSF at a ratio of 1:19 (w/v) and stirred continuously at 4 °C for 3 h. The suspension was centrifuged for 20 min at 4 °C at 20,000g (Avanti® J-E, Beckman Coulter, Inc., Palo Alto, CA) to remove the tissue debris. The supernatant was collected and then adjusted to pH 4 with 1 M HCl. The mixture was allowed to stand at 4 °C for 30 min. The prepared extract was then centrifuged at 20,000g for 15 min at 4 °C. To fully activate pepsin, the pH of acidified supernatant was adjusted to pH 2 with 1 M HCl and gently stirred for 30 min at 4 °C. The enzyme solution was subjected to centrifugation and the supernatant was collected in the same manner as described previously and referred to as "skipjack tuna pepsin".

Proteolytic activity of skipjack tuna pepsin was determined using haemoglobin as a substrate, as described by Nalinanon et al. (2010). The reaction was conducted at pH 2.0 and 50 °C for 20 min. One unit of activity was defined as the amount causing an increase of 1.0 in absorbance at 280 nm per min.

### 2.4. Preparation of protein substrate from ornate threadfin bream muscle

Protein substrate from ornate threadfin bream muscle was prepared by an acid solubilisation process, according to the method of Pérez-Mateos and Lanier (2006), with some modifications. Chopped fish muscle was homogenised with eight volumes of deionised water (0–6 °C) for 2 min in a blender at maximum speed (Type AY46, Moulinex, Groupe SEB Thailand Ltd., Bangkok, Thailand). To solubilise proteins, the pH of the mixture was adjusted to 2.0 using 1 M HCl. The samples were centrifuged at 5000g for 10 min at 4 °C. After centrifugation, the upper layer (containing neutral lipids) and the bottom sediment layer (containing connective tissue and insoluble lipid membranes) were discarded. The middle layer, containing solubilised proteins, was filtered through four layers of cheesecloth. The filtrate was then adjusted to pH 5.5 using 1 M NaOH with gradual stirring prior to centrifugation at 5000g for 10 min at 4 °C. The pellet was dispersed in four volumes of deionised water (0–6 °C) and then the pH of the protein suspension was adjusted to 2.0. Acid-solubilised protein fraction, with a high proportion of dissociated proteins which were more likely susceptible to hydrolysis, was used as a protein substrate for preparation of hydrolysate.

### 2.5. Production of protein hydrolysate from ornate threadfin bream muscle

Acid-solubilised protein fraction from the muscle of ornate threadfin bream was mixed with McIlvaine's buffer (0.2 M disodium hydrogen phosphate and 0.1 M sodium citrate) (pH 2.0) at a ratio of 1:1 (w/v). The mixture was pre-incubated at 50 °C for 15 min. The enzymatic hydrolysis was started by adding skipjack tuna pepsin with the amount (140–650 µl) required to gain 10%, 20% and 30% DH into 250 ml of mixture, as described by Benjakul and Morrissey (1997). After 1 h of hydrolysis, the enzyme was inactivated by adjusting the pH of the reaction mixture to 7.0 prior to heating at 90 °C for 15 min. The mixture was then centrifuged at 2000g for 10 min. The supernatant was then collected and lyophilised using a freeze-dryer. Lyophilised protein hydrolysate obtained was subjected to analyses.

### 2.6. Determination of $\alpha$ -amino acid content and DH

The  $\alpha$ -amino acid content was determined according to the method of Benjakul and Morrissey (1997). To properly diluted hydrolysate samples (125 µl), 2.0 ml of 0.2 M phosphate buffer (pH 8.2) and 1.0 ml of 0.01% TNBS solution were added. The solution was mixed thoroughly and placed in a temperature controlled

water bath (Model W350, Memmert, Schwabach, Germany) at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulphite. The mixtures were cooled at room temperature for 15 min. The absorbance was read at 420 nm and  $\alpha$ -amino acid was expressed in terms of L-leucine. DH was calculated as follows:

$$DH = [(L_t - L_0)/(L_{max} - L_0)] \times 100$$

where  $L_t$  is the amount of  $\alpha$ -amino acid released at time  $t$ .  $L_0$  is the amount of  $\alpha$ -amino acid in the original acid-solubilised protein substrate.  $L_{max}$  is total  $\alpha$ -amino acid in the original acid-solubilised protein substrate obtained after acid hydrolysis (6 M HCl at 100 °C for 24 h).

## 2.7. Functional properties of protein hydrolysate

### 2.7.1. Solubility

To determine protein solubility, hydrolysate samples (10 mg) were dispersed in 8 ml of deionised water and pH of the mixture was adjusted to 3, 5, 7 and 9 with either 1 M HCl or 1 M NaOH. The mixture was stirred at room temperature for 30 min. The volume of solutions was made up to 10 ml by distilled water, previously adjusted to the same pH as the sample solution, prior to centrifugation at 5000g for 15 min. Protein content in the supernatant was determined using the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951), using bovine serum albumin as a standard. Total protein content in the sample was determined after solubilisation of the sample in 0.5 M NaOH. Protein solubility was calculated as follows:

$$\text{Solubility}(\%) = \frac{\text{protein content in supernatant}}{\text{total protein content in sample}} \times 100$$

### 2.7.2. Emulsifying properties

The emulsifying activity index (EAI) and the emulsion stability index (ESI) were determined according to the method of Pearce and Kinsella (1978), with slight modification. Soybean oil (2 ml) and protein hydrolysate solutions (0.10%, 0.25% and 0.50% protein, 6 ml) were homogenised (Model T25 basic; IKA Labortechnik, Selangor, Malaysia) at a speed of 20,000 rpm for 1 min. An aliquot of the emulsion (50  $\mu$ l) was pipetted from the middle portion of the container at 0 and 10 min after homogenisation and subsequently diluted 100-fold using 0.1% sodium dodecyl sulphate (SDS) solution. The mixture was mixed thoroughly for 10 s using a vortex mixer (G-560E, Vortex-Genie 2, Scientific Industries, Inc., Bohemia, NY).  $A_{500}$  of the resulting dispersion was measured using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). EAI and ESI were calculated by the following formulae:

$$EAI(m^2/g) = (2 \times 2.303 \times A \times DF)/l\Phi C$$

where  $A = A_{500}$ ,  $DF$  = dilution factor (100),  $l$  = path length of cuvette (m),  $\Phi$  = oil volume fraction and  $C$  = protein concentration in aqueous phase ( $g/m^3$ );

$$ESI(\text{min}) = A_0 \times \Delta t / \Delta A$$

where  $\Delta A = A_0 - A_{10}$  and  $\Delta t = 10$  min.

### 2.7.3. Foaming properties

Foam expansion (FE) and foam stability (FS) of hydrolysate solutions were determined as described by Shahidi, Han, and Synowiecki (1995) with slight modification. Hydrolysate solutions (20 ml) with 0.10%, 0.25% and 0.50% protein concentrations were transferred into a 100-ml cylinder. The solutions were homogenised at 13,400 rpm for 1 min at room temperature. The samples

were allowed to stand for 0 and 60 min. FE and FS were then calculated using the following equations:

$$FE(\%) = (V_T/V_0) \times 100$$

$$FS(\%) = (V_t/V_0) \times 100$$

where  $V_T$  is total volume after whipping,  $V_0$  is the original volume before whipping, and  $V_t$  is total volume after leaving at room temperature for 60 min.

## 2.8. Determination of antioxidant activity

### 2.8.1. DPPH radical-scavenging activity

DPPH radical-scavenging activity was determined by DPPH assay, as described by Binsan et al. (2008). To the sample (1.5 ml), 1.5 ml of 0.15 mM DPPH in 95% ethanol were added. The mixture was mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was measured at 517 nm using a UV-1800 spectrophotometer (Shimadzu). The blank was prepared in the same manner, except that distilled water was used instead of the sample. A standard curve was prepared using Trolox in the range of 10–60  $\mu$ M. The activity was expressed as  $\mu$ mol Trolox equivalents (TE)/g protein of hydrolysate.

### 2.8.2. ABTS radical-scavenging activity

ABTS radical-scavenging activity was determined by ABTS assay, as described by Binsan et al. (2008). The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 50 ml methanol, in order to obtain an absorbance of  $1.1 \pm 0.02$  units at 734 nm using a spectrophotometer. Fresh ABTS solution was prepared for each assay. Sample (150  $\mu$ l) was mixed with 2850  $\mu$ l of ABTS solution and the mixture was left at room temperature for 2 h in the dark. The absorbance was then measured at 734 nm using a spectrophotometer. The blank was prepared in the same manner, except that distilled water was used instead of the sample. A standard curve of Trolox ranging from 50 to 600  $\mu$ M was prepared. The activity was expressed as  $\mu$ mol TE/g protein of hydrolysate.

### 2.8.3. Chelating activity on $Fe^{2+}$

The chelation of  $Fe^{2+}$  was measured using the method of Boyer and McCleary (1987), with slight modification. Diluted sample (4.7 ml) was mixed with 0.1 ml of 2 mM  $FeCl_2$  and 0.2 ml of 5 mM ferrozine. The reaction mixture was allowed to stand for 20 min at room temperature. The absorbance was then read at 562 nm. The blank was prepared by using the distilled water instead of the sample. The chelating activity was calculated as follows:

$$\text{Chelating activity}(\%) = [(B - A)/B] \times 100$$

where  $A$  is  $A_{562}$  of sample and  $B$  is  $A_{562}$  of the blank.

## 2.9. Fractionation and molecular weight distribution of antioxidant peptides from protein hydrolysate

The hydrolysate (20% DH) rendering the highest antioxidant activity was subjected to gel filtration chromatography. The hydrolysate was loaded onto a Sephadex G-25 column ( $1.6 \times 70$  cm). The elution was carried out with 20 mM sodium phosphate buffer (pH 7.0) at a flow rate of 1 ml/min. The 2-ml fractions were collected and their absorbance was read at 220 and 280 nm. The fractions were subjected to analysis for ABTS radical-scavenging activity.

The fractions with the maximum ABTS radical-scavenging activities were pooled, referred to as “antioxidative peptide fraction”, and used for further studies. The molecular weight distribution of antioxidative peptides was determined from the retention time of selected fraction, in comparison with those of protein standards including gramicidin (1880 Da), vitamin B<sub>12</sub> (1355 Da), flavin adenine dinucleotide (829.5 Da) and Gly-Tyr (238.25 Da).

### 2.10. pH and thermal stability of antioxidant peptides

For the pH stability study, the antioxidant peptides fraction was adjusted to different pHs (1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11) using 1 M HCl and 1 M NaOH and incubated at room temperature for 30 min. Thereafter, the pH of the samples was adjusted to 7.0. The residual antioxidant activities were determined using the ABTS assay.

To determine thermal stability, 5 ml of antioxidative peptide fraction (pH 7.0) were transferred into a screw-capped test tube. The tube was capped tightly and placed in a boiling water bath (100 °C) for 0, 15, 30, 60, 90, 120 and 180 min. The treated samples were suddenly cooled in iced water. The sample without incubation (25 °C) was used as the control. The residual antioxidant activities were determined by ABTS assay.

### 2.11. *In vitro* pepsin–pancreatin simulated gastrointestinal (GI) digestion of antioxidant peptides

Simulated GI digestion using an *in vitro* pepsin–pancreatin hydrolysis was carried out according to the method of Cinq-Mars, Hu, Kitts, and Li-Chan (2008) and You et al. (2010), with slight modification. The pH of the antioxidant peptide fraction containing 3 mg protein (15 ml) was adjusted to 2.0 with 6 M HCl. Pepsin was then added (E/S 1:35 w/w), and the mixture was incubated with continuous shaking (W350, Memmert, Schwabach, Germany) for 1 h at 37 °C. The pH was then adjusted to 5.3 with 0.9 M NaHCO<sub>3</sub> solution and further to pH 7.5 with 6 M NaOH. Pancreatin was added (E/S 1:25 w/w), and the mixture was further incubated with continuous shaking for 3 h at 37 °C. To terminate the digestion, the solution was submerged in boiling water for 10 min. Then, the GI digest was cooled to room temperature and centrifuged at 5000g for 15 min. The supernatant was used for analysis.

To investigate the changes in ABTS radical-scavenging activity of peptide digested during the simulated GI digestion, aliquots of GI digests were removed every 30 min for 3 h during the *in vitro* digestion.

### 2.12. Protein determination

Protein concentration was determined using the Lowry method (Lowry et al., 1951). Bovine serum albumin was used as a standard.

### 2.13. Statistical analysis

All experiments were performed in triplicate. The data were subjected to analysis of variance (ANOVA) and the differences between means were evaluated by Duncan's multiple range test. SPSS statistic programme (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL,) was used for data analysis.

## 3. Results and discussion

### 3.1. Solubility of protein hydrolysates with different DH

The solubilities of hydrolysates from the muscle of ornate threadfin bream with different DHs at various pHs are shown in

**Table 1**

Solubility of hydrolysates from ornate threadfin bream muscle prepared using pepsin from skipjack tuna with different DHs at various pHs.

| pH | DH (%)                                  |              |               |
|----|-----------------------------------------|--------------|---------------|
|    | 10                                      | 20           | 30            |
| 3  | 97.8 ± 2.0b <sup>†</sup> B <sup>‡</sup> | 92.8 ± 2.5aA | 91.4 ± 1.2aA  |
| 5  | 71.2 ± 1.1aA                            | 93.1 ± 0.6aB | 92.4 ± 0.5aB  |
| 7  | 97.0 ± 0.5bA                            | 97.5 ± 1.9bA | 98.2 ± 1.8bA  |
| 9  | 96.6 ± 0.7bA                            | 99.3 ± 1.0bB | 98.1 ± 1.8bAB |

Means ± SD (n = 3).

<sup>†</sup> Different small letters in the same column indicate significant differences ( $p < 0.05$ ).

<sup>‡</sup> Different capital letters in the same row indicate significant differences ( $p < 0.05$ ).

**Table 1.** All hydrolysates were soluble over a wide pH range, in which more than 70% solubility was obtained. The hydrolysates were generally soluble in alkaline pH to a greater extent, compared with acidic pH, except at pH 3 for hydrolysate with 10% DH. Protein hydrolysates from yellow stripe trevally (*Selaroides leptolepis*) meat, hydrolysed by Alcalase and Flavourzyme with different DHs (5–25%), also showed high solubility (>85%) in the pH range of 2–12 (Klompong et al., 2007). The lowest solubility was found in the hydrolysate with 10% DH at pH 5 ( $p < 0.05$ ). The result suggested that proteins or peptides with high molecular weight (MW) remaining after hydrolysis were precipitated at this pH, which was close to the isoelectric point (pI) of myofibrillar proteins. In general, the degradation of proteins to smaller peptides leads to more soluble products (Klompong et al., 2007; Gbogouri, Linder, Fanni, & Parmentier, 2004). The smaller peptides from myofibrillar proteins are expected to have proportionally more polar residues, with the ability to form hydrogen bonds with water and augment solubility (Gbogouri et al., 2004). At pH 5 and 9, the solubilities of hydrolysates were increased with increasing DH from 10% to 20% DH ( $p < 0.05$ ). No differences in the solubility of hydrolysate with different DH were observed at pH 7 ( $p > 0.05$ ). Enzymatic hydrolysis potentially affects the molecular size and hydrophobicity, as well as polar and ionisable groups of protein hydrolysates (Mutilangi, Panyam, & Kilara, 1996). The balance of hydrophilic and hydrophobic forces of peptides is another crucial influence on the solubility of protein hydrolysate (Klompong et al., 2007; Gbogouri et al., 2004). Therefore, the differences in solubility of hydrolysates with different DH might be determined by the size of peptides, the hydrophobic–hydrophilic balance, as well as the charge group of the peptides produced during the hydrolysis process.

### 3.2. Emulsifying properties of protein hydrolysates with different DH

Emulsion activity index (EAI) and emulsion stability index (ESI) of hydrolysates from ornate threadfin bream muscle with different DH at various concentrations (0.10%, 0.25%, and 0.50%) are shown in Table 2. Generally, both EAI and ESI of hydrolysates with different DH varied, particularly when the protein concentration increased ( $p < 0.05$ ). At 0.10% protein, hydrolysate with 10% DH showed the highest EAI and ESI, when compared with those having higher DHs ( $p < 0.05$ ). However, no differences in EAI were found with increasing DH, when hydrolysates at 0.25% and 0.50% were tested ( $p > 0.05$ ). Mutilangi et al. (1996) postulated that higher contents of higher MW peptides or more hydrophobic peptides contribute to the stability of the emulsion. On the other hand, excessive hydrolysis brings about the loss of emulsifying properties (Kristinsson & Rasco, 2000; Klompong et al., 2007; Gbogouri et al., 2004). Klompong et al. (2007) reported that both EAI and ESI of protein hydrolysates from yellow stripe trevally meat decreased with increasing DH. The peptides with low MW may not be amphiphilic enough to exhibit good emulsifying properties

**Table 2**  
Emulsifying and foaming properties of hydrolysates from ornate threadfin bream muscle prepared using pepsin from skipjack tuna with different DHs at various protein concentrations.

| Hydrolysate concentrations (% protein) | DH (%) | EAI (m <sup>2</sup> /g) | ESI (min)    | FE (%)       | FS (%)       |
|----------------------------------------|--------|-------------------------|--------------|--------------|--------------|
| 0.10                                   | 10     | 116 ± 2.6c <sup>†</sup> | 25.8 ± 2.2bC | 128 ± 3.1bA  | 69 ± 5.3bA   |
|                                        | 20     | 109 ± 2.3bC             | 15.2 ± 0.6aA | 126 ± 2.4bA  | 57 ± 2.5aA   |
|                                        | 30     | 102 ± 2.9aC             | 16.1 ± 0.4aA | 120 ± 2.3aA  | 55 ± 2.9aA   |
| 0.25                                   | 10     | 58.6 ± 1.5bB            | 18.4 ± 2.3bB | 128 ± 3.0bA  | 75 ± 3.2bAB  |
|                                        | 20     | 54.8 ± 1.0aB            | 18.5 ± 1.0bB | 127 ± 2.1bA  | 65 ± 5.2abAB |
|                                        | 30     | 55.0 ± 0.2aB            | 17.5 ± 0.7bB | 118 ± 3.3aA  | 60 ± 6.1aA   |
| 0.50                                   | 10     | 29.9 ± 2.3aA            | 14.1 ± 0.5aA | 131 ± 4.5bA  | 78 ± 2.5cB   |
|                                        | 20     | 30.3 ± 0.3aA            | 18.6 ± 2.5bB | 126 ± 4.5abA | 70 ± 4.8bB   |
|                                        | 30     | 30.1 ± 1.1aA            | 17.8 ± 0.8bB | 122 ± 4.0aA  | 61 ± 2.3aA   |

Means ± SD (n = 3).

<sup>†</sup> Different small letters in the same column within the same hydrolysate concentration indicate significant differences ( $p < 0.05$ ).

<sup>‡</sup> Different capital letters in the same column within the same DH indicate significant differences ( $p < 0.05$ ).

(Chobert, Bertrand-Harb, & Nicolas, 1988). Lin and Chen (2006) proposed that the emulsification process includes two steps: (1) deformation and disruption of droplets which increase the specific surface area of emulsion and (2) stabilisation of this newly-formed interface by emulsifier or surfactant. The hydrolysate with 10% DH could assemble at the interface and stabilise the oil droplet to a higher extent. With the same DH, EAI of hydrolysates decreased with increasing concentrations ( $p < 0.05$ ). With 20% and 30% DH, no differences in ESI were noticeable as the hydrolysate concentrations were above 0.25% ( $p > 0.05$ ). At low protein concentrations, protein adsorption at the oil–water interface is diffusion controlled. At high protein concentration, the activation energy barrier does not allow protein migration to take place in a diffusion-dependent manner, leading to the accumulation of proteins in the aqueous phase (Lawal, 2004; Thiansilakul et al., 2007a). Thus, proteins or peptides were most likely localised in the aqueous phase and a lower amount of proteins or peptides migrated to the interface (Thiansilakul et al., 2007a). The increase in protein–protein interaction resulted in a lower protein concentration at the interface, in which a thinner film stabilising the oil droplet is formed. Therefore, emulsifying properties of hydrolysate were governed by the molecular properties, particularly the size of peptides and the concentration employed.

### 3.3. Foaming properties of protein hydrolysates with different DH

Foaming properties are physicochemical characteristics of proteins to form and stabilise foams (Thiansilakul et al., 2007a). Foam expansion (FE) and foam stability (FS) of hydrolysates from ornate threadfin bream muscle with different DH at various concentrations (0.10%, 0.25%, and 0.50%) are shown in Table 2. At the same concentration of hydrolysate used, slight decreases in FE were observed when DH of hydrolysate increased ( $p < 0.05$ ). This is possibly due to a lesser alignment of small peptides at the air–water interface (Nalinanon, Benjakul, Visessanguan, & Kishimura, 2008). With the same DH, protein concentrations had no impact on FE ( $p > 0.05$ ). Foam formation is governed by three factors, including transportation, penetration and reorganisation of molecules at the air–water interface (Klompong et al., 2007). In general, proteins, which rapidly adsorb at the newly-created air–liquid interface during bubbling and undergo unfolding and molecular rearrangement at the interface, exhibit better foaming ability than proteins that adsorb slowly and resist unfolding at the interface (Damodaran, 1997). FE after whipping for 60 min was monitored to indicate the FS of protein hydrolysates. FS was slightly decreased with increasing DH of hydrolysate. A similar result was also reported in protein hydrolysates from yellow stripe trevally meat prepared by Alcalase and Flavourzyme with 5–25% DH (Klompong et al., 2007). It was suggested that low MW peptides could not

maintain well-ordered orientation of the molecule at the interface. However, at the same DH, FS of hydrolysate was improved by increasing concentration ( $p < 0.05$ ). Lawal (2004) postulated that an increase in foam stability with increasing concentration was a result of formation of stiffer foams. FS is enhanced by flexible protein domains, which enhance viscosity of the aqueous phase, protein concentration and film thickness (Phillips, Whitehead, & Kinsella, 1994).

### 3.4. Antioxidant activities of protein hydrolysates with different DHs

Antioxidant activities, as determined by ABTS, DPPH and Fe<sup>2+</sup> chelating assays of hydrolysates with different DH values are shown in Table 3. The differences in activities determined by both assays were observed amongst protein hydrolysates with different DHs. The highest ABTS and DPPH radical-scavenging activities were found in hydrolysates with 20% DH (159 and 8.58 μmol TE/g protein, respectively;  $p < 0.05$ ). ABTS and DPPH radical-scavenging activities of protein hydrolysates with 10% DH were 151 and 3.15 μmol TE/g protein, respectively, whereas that of 30% DH had activities of 151 and 6.21 μmol TE/g protein, respectively. Thus peptides in hydrolysates with various DH might differently scavenge two different radicals, ABTS and DPPH radicals. However, Klompong et al. (2007) found that no differences in DPPH radical-scavenging activities were observed for yellow stripe trevally protein hydrolysate prepared by Flavourzyme with different DHs, ranging from 5% to 25%. Generally, hydrolysates contained peptides or proteins, which were hydrogen donors and could react with radicals to convert them to more stable products, thereby terminating the radical chain reaction (Khantaphant & Benjakul, 2008). ABTS assay is an excellent tool for determining the antioxidant activity of hydrogen-donating compounds (scavengers of aqueous phase radicals) and of chain-breaking antioxidants (scavenger of lipid peroxyl radicals) (Binsan et al., 2008). The ABTS radical is relatively stable and is readily reduced by antioxidants (Klompong et al., 2009; Miller, Rice-Evans, Davies, Gopinathan, &

**Table 3**  
Antioxidant activities of hydrolysates from ornate threadfin bream muscle prepared using pepsin from skipjack tuna with different DHs as determined by various assays.

| Activities tested        | DH (%)                   |              |              |
|--------------------------|--------------------------|--------------|--------------|
|                          | 10                       | 20           | 30           |
| ABTS (μmol TE/g protein) | 150 ± 0.82a <sup>†</sup> | 159 ± 1.01b  | 151 ± 0.74a  |
| DPPH (μmol TE/g protein) | 3.15 ± 0.35a             | 8.58 ± 0.24c | 6.21 ± 0.12b |
| Chelating activity (%)   | 45.9 ± 1.72a             | 51.3 ± 0.50b | 58.1 ± 0.59c |

TE, Trolox equivalents.

Mean ± SD (n = 3).

<sup>†</sup> Different letters in the same row indicate significant differences ( $p < 0.05$ ).

Milner, 1993). With high ABTS radical-scavenging activity, it was postulated that antioxidative compounds were most likely hydrophilic. DPPH radical-scavenging activity is generally used to determine hydrogen-donating ability of protein hydrolysates (Wu et al., 2003; Thiansilakul et al., 2007b). The stable DPPH radical displays a maximum absorbance at 517 nm in ethanol. When DPPH encounters a hydrogen-donating substance, the radical would be scavenged, as visualised by changing its colour from purple to yellow, and the absorbance is reduced (Shimada, Fujikawa, Yahara, & Nakamura, 1992). The results so obtained suggest that the hydrolysates contained amino acids or peptides that were electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction.

As the DH increased, chelating activity on  $\text{Fe}^{2+}$  increased ( $p < 0.05$ ). Hydrolysates with 30% DH exhibited the highest  $\text{Fe}^{2+}$  chelating activity (58.1%). The result indicated that metal ion-binding capacity of protein hydrolysates was enhanced by pepsin hydrolysis. The result was in accordance with Klompong et al. (2007), who reported that chelating activity on  $\text{Fe}^{2+}$  of yellow stripe trevally protein hydrolysates, prepared by both Alcalase and Flavourzyme, increased with increasing DH. The chelation of  $\text{Fe}^{2+}$  was used to determine the ability of hydrolysates in metal chelating. Ferrozine quantitatively forms complexes with  $\text{Fe}^{2+}$

ion. In the presence of chelating agents, the complex formation is disrupted, resulting in the decrease in colour formation (Thiansilakul et al., 2007b). The results indicated that hydrolysates had a potential chelating ability towards iron.

It was noted that the protein hydrolysate from ornate threadfin bream with 20% DH showed the highest radical-scavenging activities, whilst the hydrolysate with 30% DH exhibited the highest  $\text{Fe}^{2+}$  chelating activity. It was speculated that different peptides with varying size as well as amino acid sequence might have different modes of action. Therefore, hydrolysates with appropriate DH could be prepared, to maximise their functions as radical scavengers and metal chelators.

### 3.5. Fractionation and molecular weight distribution of antioxidant peptides from protein hydrolysate

Hydrolysates from the muscle of ornate threadfin bream with 20% DH prepared using the pepsin from skipjack tuna, which showed the highest radical-scavenging activity, were fractionated using Sephadex G-25 gel filtration chromatography.  $A_{220}$  was used to monitor peptide bonds, whereas  $A_{280}$  was the parameter representing peptides, proteins or amino acids with aromatic rings (Fig. 1A). A distinct peak of  $A_{280}$  was observed in the hydrolysate,

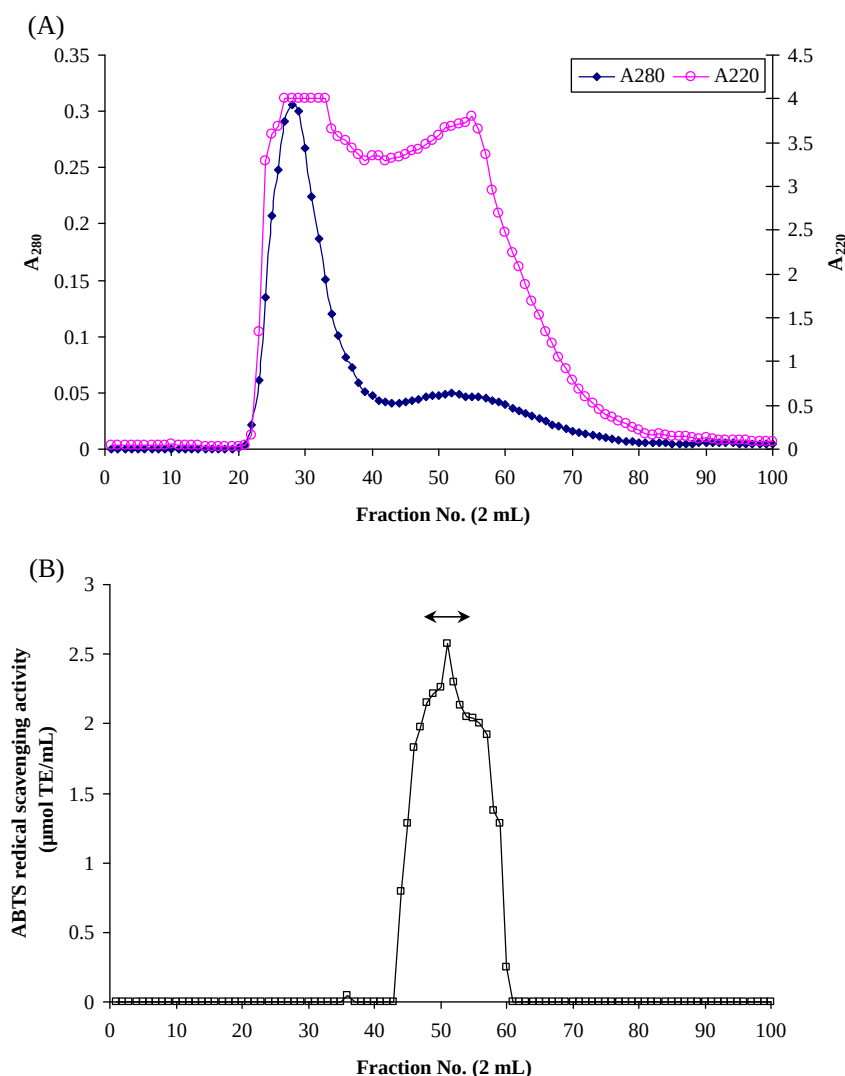


**Fig. 1.** Separation of antioxidant peptides from hydrolysate of ornate threadfin bream muscle prepared using pepsin from skipjack tuna. (A)  $A_{220}$  and  $A_{280}$ ; (B) ABTS radical-scavenging activity. ( $\leftrightarrow$ ): pooled fractions.

indicating the presence of proteins or peptides mainly containing aromatic amino acids, with high and low MW. The hydrolysate had two peaks of  $A_{220}$ , indicating the presence of peptide bonds in the hydrolysate. The first peak of  $A_{220}$  was found in the same fractions as the  $A_{280}$  peak. Low  $A_{280}$  was detected in the second  $A_{220}$  peak, indicating a high proportion of low MW peptides in the hydrolysate.

As shown in Fig. 1B, the fraction containing peptides with a MW of 1.3 kDa of hydrolysate prepared using skipjack tuna pepsin showed the highest ABTS radical-scavenging activity. The activity peak was located at the same position of the second  $A_{220}$  peak, indicating that low MW peptides contributed to the antioxidant activity. The result suggested that the hydrolysate tested most likely contained certain peptides that were electron donors or could scavenge free radicals to terminate the radical chain reaction. Fractions 49–53, showing the highest ABTS radical-scavenging activity, were pooled and used as a source of antioxidant peptides.

### 3.6. pH and thermal stability of antioxidant peptides

The influences of pH on the stability of antioxidant peptides fractionated by Sephadex G-25 column chromatography are depicted in Fig. 2A. ABTS radical-scavenging activity of the antioxidant peptide fraction remained constant over the pH range of 1–

10. At pH 11, ABTS radical-scavenging activity slightly decreased ( $p < 0.05$ ). The results suggested that antioxidant peptides exhibiting ABTS radical-scavenging activity might lose their activity to some extent at high pH. Due to the stability over a wide pH range, antioxidant peptides from the muscle of ornate threadfin bream have potential for application in any food system at extreme pH.

Thermal stability of antioxidant peptide fraction as monitored by ABTS radical-scavenging activities is shown in Fig. 2B. ABTS radical-scavenging activities of antioxidant peptide fraction were quite stable when heated at 100 °C up to 180 min, where activities of more than 97% were retained. A small loss in ABTS radical scavenging activity might be due to either degradation or aggregation of some antioxidant peptides, caused by heat treatment. In general, proteins were vulnerable to heat treatment, leading to the aggregation of protein and the exposure of hydrophobic domain (Sikorski & Naczki, 1981). Nevertheless, peptides with smaller sizes were more stable to aggregation at high temperature (Zayas, 1997). Peptides derived from many protein sources with increased hydrophobicity have been reported to correlate with antioxidant properties (Chen, Muramoto, Yamauchi, & Nokihara, 1996; Faithong, Benjakul, Phatcharat, & Binsan, 2010). The result indicated that peptides with low MW were most likely stable following heat treatment. Therefore, the antioxidant peptides can be incorporated in cooked food systems without significant loss of their antioxidant activities.

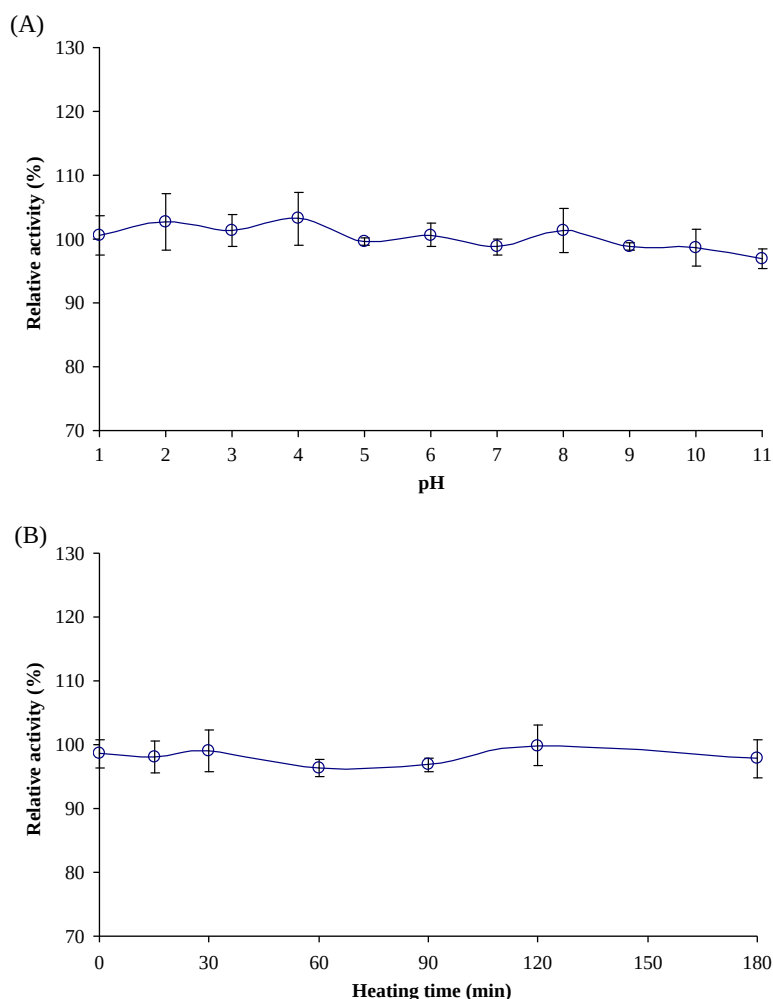


Fig. 2. pH (A) and thermal (B) stabilities of antioxidant peptides from hydrolysate of ornate threadfin bream using pepsin from skipjack tuna fractionated by Sephadex G-25 column chromatography. Bars represent the standard deviation ( $n = 3$ ).

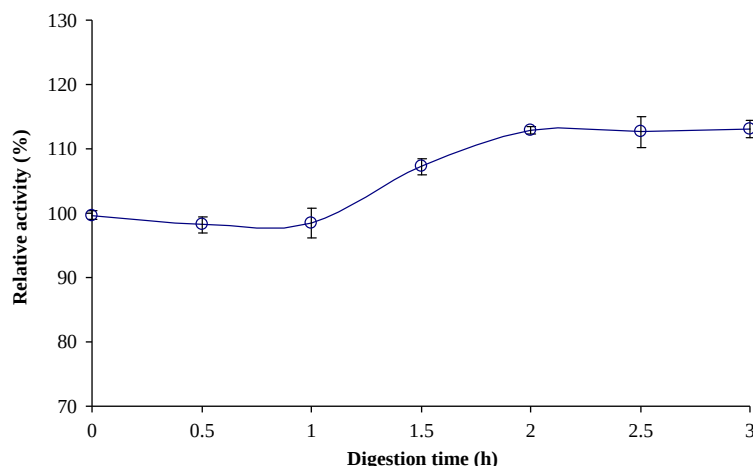


**Fig. 3.** Changes in ABTS radical-scavenging activity during sequential *in vitro* digestion of antioxidant peptides from hydrolysate of ornate threadfin bream using pepsin from skipjack tuna fractionated by Sephadex G-25 column chromatography. The first stage: from 0 to 1.0 h, digested by pepsin; the second stage: from 1.0 to 3.0 h, digested by pancreatin. Bars represent the standard deviation ( $n = 3$ ).

### 3.7. *In vitro* pepsin–pancreatin simulated GI digestion of antioxidant peptides

An *in vitro* pepsin–pancreatin hydrolysis simulating GI digestion was used to illustrate its impact on ABTS radical-scavenging activity of antioxidant peptides (Sephadex G-25 fraction) from protein hydrolysate of ornate threadfin bream prepared using skipjack tuna pepsin (Fig. 3). Pepsin digestion for 1 h did not alter ABTS radical-scavenging activity ( $p > 0.05$ ). This might be due to the cleavage limitation of pepsin on antioxidant peptides, which were previously digested by skipjack tuna pepsin. As a result, no degradation or generation of new antioxidant peptides took place. After further digestion by pancreatin for 30–60 min, ABTS radical-scavenging activity was increased by about 7–12%. It was suggested that antioxidative peptides were modified by pancreatin digestion to enhance their ABTS radical-scavenging activity. In addition, the increase of hydrophilic property of GI digests after pancreatin treatment has been reported to favour trapping of ABTS radical of loach protein hydrolysate (You et al., 2010). Additionally, You et al. (2010) reported that GI digests had increased reducing power, chelating ability of  $\text{Cu}^{2+}$  and hydroxyl radical-scavenging activities by 77%, 12% and 12%, respectively. Therefore, antioxidant peptides were most likely stable in real digestion system after ingestion in both stomach and intestine, which have high proteolytic activity under acidic and alkaline pH, respectively.

## 4. Conclusions

Production of protein hydrolysates from ornate threadfin bream muscle, exerting good functionalities and antioxidant activities, could be achieved by skipjack tuna pepsin hydrolysis. Based on pH and thermal stability as well as GI digestion, antioxidant peptides in protein hydrolysate can be incorporated as a multifunctional ingredient into foods.

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# Gelatin hydrolysate from blacktip shark skin prepared using papaya latex enzyme: Antioxidant activity and its potential in model systems

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## ABSTRACT

Antioxidant activities of gelatin hydrolysates from blacktip shark skin prepared using papaya latex enzyme with different degrees of hydrolysis (DH: 10%, 20%, 30% and 40%) were evaluated. All antioxidant activity indices of hydrolysates increased with increasing DH ( $P < 0.05$ ). When gelatin hydrolysate with 40% DH was determined for its pH and thermal stability, ORAC and chelating activity remained constant or slightly increased in a wide pH range (1–9) and during heating (100 °C) for 240 min. It was also stable in simulated gastrointestinal tract model system. Moreover, gelatin hydrolysate at a level of 500 and 1000 ppm could inhibit lipid oxidation in both  $\beta$ -carotene linoleate and cooked comminuted pork model systems. Therefore, gelatin hydrolysate from blacktip shark skin (40% DH) can potentially be used as an alternative source of natural antioxidants.

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## 1. Introduction

Blacktip shark (*Carcharhinus limbatus*) has been used for shark fin and fillet production in Thailand. Shark skin is one of the potential sources for gelatin (Kittiphattanabawon, Benjakul, Visessanguan, & Shahidi, 2010). Recently, gelatin hydrolysate from bigeye snapper skin, prepared using fish pyloric caeca, has been reported to possess antioxidant activity including DPPH and ABTS radical scavenging activity and ferric reducing antioxidant power (Khantaphant & Benjakul, 2008; Phanturat, Benjakul, Visessanguan, & Roytrakul, 2010). Mendis, Rajapakse, and Kim (2005) reported that Gly and Pro in peptide from gelatin hydrolysate of hoki skin prepared with the aid of trypsin determined the radical scavenging activity. In addition, a peptide isolated from gelatin hydrolysate of Alaska pollack skin prepared using two-step hydrolysis with Alcalase and Pronase E, respectively, which had Gly-Glu-Hyp at N-terminus, showed high antioxidant activity (Kim et al., 2001). Nevertheless, gelatin hydrolysate generally has a low degree of hydrolysis (DH), possibly due to the molecular constraint associated with its amino acid sequence/composition. Gelatin with a glycine content of around 33% might not be a preferable substrate for proteases. Glycyl endopeptidase is one of the four papaya

cysteine proteinases, including papain, glycyl endopeptidase, chymopapain and caricain. It is a major component which constitutes almost 30% of total protein in the latex of *Carica papaya* and has high specificity to cleave only peptide bonds with Gly at P<sub>1</sub> (Buttle, 1994). Thus, papaya latex, which is cheap and available in tropical countries, can serve as a potential protease for production of gelatin hydrolysate with high DH. Nevertheless, no information regarding gelatin hydrolysate from blacktip shark skin with high DH and their antioxidant activity has been reported. Therefore, this investigation aimed to produce gelatin hydrolysate with different DHs and study their antioxidant activity and stability. The use of gelatin hydrolysate in different model systems was also studied.

## 2. Materials and methods

### 2.1. Chemicals/enzymes

All chemicals were of analytical grade. 2,4,6-Trinitrobenzenesulphonic acid (TNBS), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tripyridyltriazine (TPTZ), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulphonic acid sodium salt (ferrozine), ethylenediaminetetraacetic acid (EDTA), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), 5,5-dimethyl-1-pyrroline N-oxide (DMPO), N,N-dimethyl p-nitroaniline (DPN), histidine, sodium hypochlorite (NaOCl), taurine,

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potassium iodide (KI), fluorescein, 2,2'-azobis(2-methylpropionamidine) (AAPH), trolox, pepsin from porcine gastric mucosa (EC 3.4.23.1), pancreatin from porcine pancreas, trypsin from bovine pancreas (EC 3.4.21.4), bile extract porcine,  $\beta$ -carotene and Tween® 40 were purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada). Linoleic acid was purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA).

## 2.2. Preparation of gelatin hydrolysate from blacktip shark skin

### 2.2.1. Shark skin preparation

Blacktip shark (*C. limbatus*) skin was obtained from Blue Ocean Food Products Co., Ltd. in Samutsakhon province, Thailand. The shark skin was prepared according to the method of Kittiphattanabawon et al. (2010).

### 2.2.2. Extraction of gelatin

Gelatin from shark skin was extracted with distilled water at 45 °C for 6 h following the method of Kittiphattanabawon et al. (2010). The resulting gelatin was used for the preparation of gelatin hydrolysate.

### 2.2.3. Preparation of crude enzyme from papaya (*Carica papaya*) latex

Fresh latex was collected from locally grown papaya in Hat Yai, Thailand. Four to six longitudinal incisions were made on the unripen papaya fruit using a stainless steel knife. The exuded latex was collected using a receiving container. The latex was then transferred to a beaker and stored below 10 °C and used within 3 h.

To prepare crude enzyme, the latex was mixed with cold distilled water ( $\leq 4$  °C) at a latex to water ratio of 1:1 (w/v). The mixture was gently stirred at 4 °C for 1 h. Then, the mixture was centrifuged at 9,000g at 4 °C for 20 min using a refrigerated centrifuge (Avanti® J-E, Beckman Coulter, Palo Alto, CA, USA). The supernatant was filtered using a Whatman No.1 filter paper, followed by freeze-drying. The resulting powder referred to as “crude enzyme from papaya latex” was kept at –40 °C until use. It contained 0.92 g protein/g as determined by the method of Lowry, Rosebrough, Farr, and Randall (1951).

The proteolytic activity of prepared crude enzyme was 6.03 U/mg protein. The activity was assayed using casein as a substrate at pH 7.5 and 40 °C according to the method of Khantaphant and Benjakul (2008). After termination of enzymatic reaction, the oligopeptide content in the supernatant was measured as per the Lowry method (Lowry et al., 1951) using tyrosine as a standard. One unit of activity was defined as that releasing 1  $\mu$ mol of tyrosine per min ( $\mu$ mol Tyr/min).

### 2.2.4. Production of gelatin hydrolysate with different degrees of hydrolysis (DH)

Gelatin hydrolysates with different DHs from shark skin were prepared. Gelatin (3 g) was dissolved in 80 ml of distilled water. The pH of mixture was adjusted to 7.5 with 1 M NaOH. The volume of solution was made up to 100 ml by distilled water previously adjusted to pH 7.5 to obtain a protein concentration of 3% (w/v). The hydrolysis reaction was started by the addition of the crude enzyme from papaya latex at the amounts of 0.46, 1.62, 5.18 and 15.23 g/100 g protein, which were calculated from the plot between log (enzyme concentration) and DH to obtain DH of 10%, 20%, 30% and 40%, respectively (Benjakul & Morrissey, 1997). After 1 h of hydrolysis at 40 °C, the enzyme was inactivated by heating at 90 °C for 15 min in a temperature controlled water bath (model W350, Memmert, Schwabach, Germany). The mixture was then centrifuged at 5000g at room temperature for 10 min. The supernatant was freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The obtained powder referred to as “gelatin

hydrolysate” was placed in polyethylene bag and stored at –20 °C until use.

## 2.3. Determination of antioxidant activities

### 2.3.1. DPPH radical scavenging activity

The DPPH radical scavenging activity was determined as described by Binsan et al. (2008). A standard curve was prepared using trolox in the range of 0–50  $\mu$ M. The activity was expressed as  $\mu$ mol trolox equivalents (TE)/g sample.

### 2.3.2. ABTS radical scavenging activity

The ABTS radical scavenging activity was determined as described by Binsan et al. (2008). A standard curve of trolox ranging from 0 to 500  $\mu$ M was prepared. The activity was expressed as  $\mu$ mol trolox equivalents (TE)/g sample.

### 2.3.3. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was determined using the electron paramagnetic resonance (EPR) spectrometric method as described by Madhujith and Shahidi (2008) with slight modifications. The samples were dissolved in deionised water to obtain final concentration of 10 mg/ml. The sample (100  $\mu$ l) was mixed with 10 mM H<sub>2</sub>O<sub>2</sub> (300  $\mu$ l) and 17.6 mM DMPO (200  $\mu$ l). The reaction was initiated by adding 450  $\mu$ l of 100  $\mu$ M FeSO<sub>4</sub>. The mixture was left at room temperature for 1 min. The mixture was injected to the sample cavity of a Bruker E-scan (Bruker Biospin Co., Billerica, MA, USA) and then the spectrum was recorded by the software of the equipment using the following parameters:  $5.02 \times 10^2$  receiver gain, 1.86 G modulation amplitude, 2.621 s sweep time, 8 scans, 100.00 G sweep width, 3495.258 G centre field, 5.12 ms time constant, 9.795 GHz microwave frequency, 86.00 kHz modulation frequency, and 1.86 G modulation amplitude. Deionised water was used as the control. The EPR spectra were plotted between the EPR signals and the magnetic field. The amplitude of the second peak of the spectrum represented the amount of DMPO-OH adducts, which related with the amount of hydroxyl radicals scavenged. Trolox (0–50  $\mu$ M) was used as the standard. The hydroxyl radical scavenging activity was expressed as  $\mu$ mol trolox equivalents (TE)/g sample.

### 2.3.4. Oxygen radical absorbance capacity

Oxygen radical absorbance capacity (ORAC) was determined using the method of Madhujith and Shahidi (2007) with slight modifications. The samples were dissolved in 75 mM phosphate buffer (pH 7.0) to obtain a final concentration of 0.1 mg/ml. The prepared sample (20  $\mu$ l) was loaded onto black polystyrene, non-treated 96-well microplate (Costar Corning Inc., Corning, NY, USA). Only the internal wells of the microplate were used. The loaded microplate was inserted to a FLUOstar OPTIMA microplate reader (BMG Labtechnologies GmbH, Offenberg, Germany) equipped with FLUOstar OPTIMA evaluation software version 1.30-0. The samples were equilibrated at 37 °C for 10 min. Then 200  $\mu$ l of 0.11  $\mu$ M fluorescein dissolved in 75 mM phosphate buffer (pH 7.0) were automatically injected to the sample at the first cycle. The reaction was started at the second cycle by automatic injection of 75  $\mu$ l of 60 mM AAPH. The reaction was performed at 37 °C. The fluorescence intensity was measured every 150 s for 62 cycles with excitation and emission filters of 485 and 520 nm, respectively. A gain adjustment was performed by pipetting 200  $\mu$ l of fluorescein (0.11  $\mu$ M) onto a designated well before starting the program to optimise signal amplification. The control was prepared in the same manner, except that 75 mM phosphate buffer (pH 7.0) was used instead of the sample. The kinetic curve (AUC) of the samples was plotted between fluorescence intensity and the number of cycles. Area below the curve directly relates

with the ORAC of sample. Trolox (0–50  $\mu\text{M}$ ) was used as the standard. The ORAC was expressed as  $\mu\text{mol}$  trolox equivalents (TE)/g sample.

#### 2.3.5. Ferric reducing antioxidant power (FRAP)

FRAP was determined according to the method of Benzie and Strain (1996). The standard curve was prepared using trolox ranging from 0 to 500  $\mu\text{M}$ . The activity was expressed as  $\mu\text{mol}$  trolox equivalents (TE)/g sample.

#### 2.3.6. Chelating activity of ferrous ion

Ferrous ion chelating activity was measured by the method of Thiansilakul, Benjakul, and Shahidi (2007b). EDTA with the concentration range of 0–50  $\mu\text{M}$  was used as the standard. The  $\text{Fe}^{2+}$  chelating activity was expressed as  $\mu\text{mol}$  EDTA equivalents (EE)/g sample.

#### 2.3.7. Singlet oxygen scavenging activity

The capacity of samples to scavenge singlet oxygen was measured as described by Chandrasekara and Shahidi (2011) with slight modifications. The samples and all reagents were dissolved in 45 mM sodium phosphate buffer (pH 7.4). The sample solution (10 mg/ml; 200  $\mu\text{l}$ ) was mixed with 700  $\mu\text{l}$  of the mixture including 200  $\mu\text{l}$  of 0.2 mM DPN, 100  $\mu\text{l}$  of 100 mM histidine, 100  $\mu\text{l}$  of 200 mM sodium hypochlorite (NaOCl) and 250  $\mu\text{l}$  of 45 mM phosphate buffer (pH 7.4). The reaction was initiated by adding 100  $\mu\text{l}$  of 200 mM  $\text{H}_2\text{O}_2$ . The reaction mixtures were then incubated at room temperature (23–25  $^{\circ}\text{C}$ ) for 40 min. The absorbance was read at 440 nm using a spectrophotometer. The sample blanks were run for each sample, replacing DPN, histidine and NaOCl with the buffer for background correction. The blank was prepared in the same manner except that the buffer was used instead of the sample. A standard curve of trolox (0–10 mM) was prepared. The singlet oxygen scavenging activity was expressed as  $\mu\text{mol}$  trolox equivalents (TE)/g sample.

#### 2.3.8. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was assayed according to the method of Wettasinghe and Shahidi (2000). Trolox (0–10 mM) was used as the standard. The hydrogen peroxide scavenging activity was expressed as  $\mu\text{mol}$  trolox equivalents (TE)/g sample.

#### 2.3.9. Hypochlorous acid scavenging capacity

Hypochlorous acid (HOCl) scavenging capacity was determined using the method of Chandrasekara and Shahidi (2011) with slight modifications. Samples were prepared by dissolution in distilled water. HOCl was freshly prepared by adjusting the pH of a 1% (v/v) of NaOCl to 6.2 with 1% (v/v) sulphuric acid. The concentration of HOCl was determined by reading the absorbance at 235 nm and using the molar extinction coefficient of  $100 \text{ M}^{-1} \text{ cm}^{-1}$ . The samples (1 mg/ml; 100  $\mu\text{l}$ ) were consecutively mixed with 100  $\mu\text{l}$  of 150 mM taurine, 100  $\mu\text{l}$  of 0.5 mM HOCl and 700  $\mu\text{l}$  of 50 mM phosphate buffer containing 0.15 M NaCl (pH 7.4). The mixtures were incubated at room temperature for 10 min. The reaction was initiated by adding 10  $\mu\text{l}$  of 2 M KI. The absorbance was read at 350 nm using a spectrophotometer. The sample blanks were run for each sample, replacing taurine, HOCl and phosphate buffer with distilled water for background correction. The blank was prepared in the same manner except that distilled water was used instead of the sample. Trolox (0–500  $\mu\text{M}$ ) was used as the standard. The hypochlorous acid scavenging activity was expressed as  $\mu\text{mol}$  trolox equivalents (TE)/g sample.

#### 2.4. Stability studies of selected gelatin hydrolysate

The gelatin hydrolysate with a DH of 40% which showed the highest antioxidant activity was used for stability studies.

##### 2.4.1. pH stability

The gelatin hydrolysate was dissolved in distilled water to obtain a final concentration of 50 mg solid/ml. The pH of gelatin hydrolysate solution (10 ml) was adjusted to different pHs (1, 3, 5, 7 and 9) using 1 M HCl or 1 M NaOH. The volume of solution was made up to 25 ml by distilled water previously adjusted to the same pH as the solution. The mixtures were incubated at room temperature for 1 h. Thereafter, the pH of the mixtures was adjusted to 7.0 and their volumes were made up to 50 ml with distilled water. The residual antioxidant activities were determined using the chelating activity and ORAC assays and expressed as the activity (%) relative to that obtained without pH adjustment.

##### 2.4.2. Thermal stability

The gelatin hydrolysate was dissolved in distilled water to obtain a final concentration of 50 mg solid/ml. The pH of gelatin hydrolysate solution (10 ml) was adjusted to 7 and the volume of solution was made up to 50 ml with distilled water. Ten millilitres of the solution were transferred to screw-capped test tube (PYREX<sup>®</sup>, New York, NY, USA). The tube was capped tightly and placed in a boiling water bath (100  $^{\circ}\text{C}$ ) for 0, 15, 30, 60, 120, 180 and 240 min. After designated heating times, treated samples were immediately cooled in iced water. The residual antioxidant activities were determined using the chelating activity and ORAC assays and expressed as the activity (%) relative to those without heat treatment.

##### 2.4.3. Stability in gastrointestinal tract model systems

To simulate the *in vivo* resistance to digestion, the sequential reaction, which mimics the conditions of the digestive organ, was used as described by Enari, Takahashi, Kawarasaki, Tada, and Tatsuta (2008) with slight modifications. Gelatin hydrolysate solution (10 mg/ml; 100 ml) was mixed with 10 ml of 10 mM phosphate buffer (pH 6.8). The mixture was incubated for 2 min at 37  $^{\circ}\text{C}$  (oral condition). Then 0.5 ml of 1 M HCl–KCl buffer (pH 1.5) was added to produce an acidic condition (pH 1.5), followed by adding 32 U/ml of pepsin solution in 1 M HCl–KCl buffer (pH 1.5) (5 ml) and incubating for 60 min at 37  $^{\circ}\text{C}$  (stomach condition). Thereafter, the pH was adjusted to 6.8 with 1 M  $\text{NaHCO}_3$  (1 ml), and the enzyme mixture of bile and pancreatic juice (1 ml) that contained pancreatin (10 mg/ml), trypsin (14,600 U/ml) and bile extract (13.5 mg/ml) in 10 mM phosphate buffer (pH 8.2), was added to the solution, followed by incubation at 37  $^{\circ}\text{C}$  for 3 h (duodenal condition). To investigate the changes in chelating activity and ORAC of the gelatin hydrolysate during the simulated gastrointestinal digestion, aliquots of gastrointestinal digests were randomly taken at 0, 0.5, 1, 2, 3 and 4 h during the *in vitro* digestion. After sampling, the sample was immediately placed in a boiling water bath for 10 min. The residual antioxidant activities were determined using the chelating activity and ORAC assays and expressed as the activity (%) relative to that without any treatment.

#### 2.5. Antioxidant activity in model systems

##### 2.5.1. $\beta$ -Carotene-linoleate model system

The  $\beta$ -carotene-linoleate model system represents an oil-in-water emulsion system. The antioxidant activity in  $\beta$ -carotene-linoleate model system was determined according to the method of Chandrasekara and Shahidi (2010) with slight modifications. To prepare  $\beta$ -carotene oil-in-water emulsion, 1 mg of  $\beta$ -carotene was dissolved in 10 ml of chloroform. The  $\beta$ -carotene solution

(0.2 ml) was mixed with linoleic acid (20 mg) and Tween® 40 (200 mg). Chloroform was removed under a nitrogen stream. Oxygenated distilled water (50 ml) was added to the mixture which was then homogenised at a speed of 11000 rpm for 1 min using a Polytron PT 3000 (Brinkmann Instruments, Rexdale, ON, Canada). The prepared emulsion was kept in the dark at room temperature. Gelatin hydrolysate dissolved in distilled water (30 µl) with different concentrations (1,000, 5000 and 10,000 ppm) was manually pipetted into sample wells of a Costar flat-bottom 96-well microplate. The samples were equilibrated at 45 °C for 10 min. To initiate the reaction, the emulsion (270 µl) was automatically injected to the sample and the final concentrations of gelatin hydrolysate in the system were 100, 500 and 1000 ppm. The reaction was performed at 45 °C. The absorbance at 450 nm was monitored every 5 min up to 120 min. The microplate reader was programmed to perform additional shaking of the contents in wells before each reading was taken. The negative and positive controls were prepared in the same manner, except that distilled water and 100 ppm BHA, respectively, were used instead of the sample. The retardation of decrease in the absorbance at 450 nm indicated the ability to prevent oxidation of the system.

### 2.5.2. Cook comminuted pork model system

The cook comminuted pork model system was prepared according to the method of [Shahidi and Alexander \(1998\)](#). Fresh comminuted pork was purchased from a local market (St. John's, NL, Canada). The comminuted pork (80 g) was mixed with 20 g of distilled water containing gelatin hydrolysate to obtain the different final concentrations of 100, 500 and 1000 ppm. The meat mixture was homogenised at 12,000 rpm for 1 min using a homogeniser. The homogenate was cooked at 80 °C in a controlled temperature water bath for 40 min with gentle stirring every 5 min. The cooked meat was cooled to room temperature. The cooled meat was transferred to polyethylene bag and kept at 4 °C. After designated time (0, 3, 5, 7, 10 and 14 days), the samples were randomly taken for TBARS determination according to the method of [Wijeratne, Amarowicz, and Shahidi \(2006\)](#). The TBARS values were then calculated using the standard curve of malondialdehyde (MDA) and expressed as mg MDA equivalents/kg sample.

### 2.6. Statistical analysis

All experiments were carried out in triplicate. Data were subjected to the analysis of variance (ANOVA) and mean comparisons were performed by using Duncan's multiple range test ([Steel & Torrie, 1980](#)). Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA).

## 3. Results and discussion

### 3.1. Antioxidant activity of gelatin and gelatin hydrolysates with different DHs

DPPH and ABTS radical scavenging activities of gelatin hydrolysates with different DHs are shown in Figs. 1A and 1B, respectively. In general, the increases in radical scavenging activity were found in hydrolysate, compared with gelatin. The results indicate that antioxidative peptides were produced during the hydrolysis. DPPH radical scavenging activity gradually increased as %DH increased from 0 to 30 ( $P < 0.05$ ) (Fig. 1A). The sharp increase in DPPH radical scavenging activity was noticeable at 40%DH. [Thiansilakul, Benjakul, and Shahidi \(2007a\)](#) reported that the DPPH radical scavenging activity of round scad muscle protein hydrolysates prepared using Flavourzyme and Alcalase increased with increasing DH up to 60%

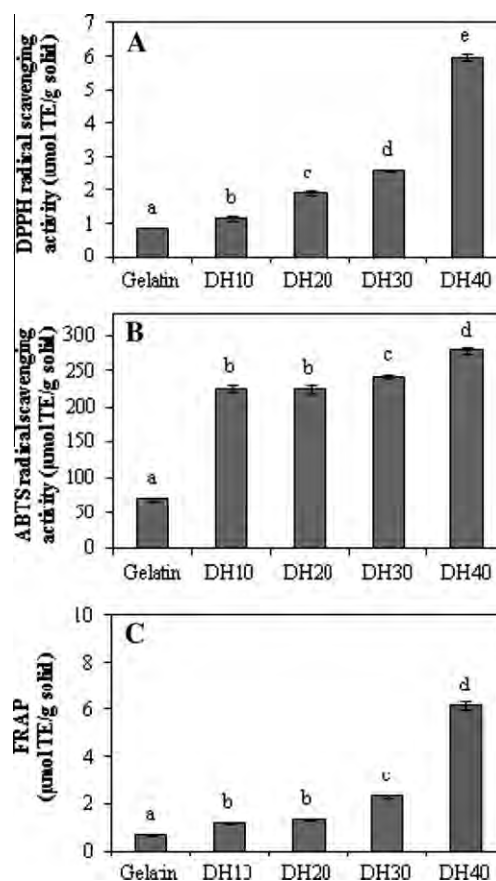


**Fig. 1.** DPPH radical scavenging activity (A), ABTS radical scavenging activity (B), and ferric reducing antioxidant power (FRAP) (C) of gelatin and gelatin hydrolysates with different DHs. Bars represent standard deviation ( $n = 3$ ). Different letters on the bars denote significant differences ( $P < 0.05$ ).

and 40%, respectively. The DPPH radical scavenging activity of peanut protein hydrolysate increased as DH increased from 10% to 20% but no difference in activity was observed as DH increased from 20% to 40% ([Jamdar et al., 2010](#)). For ABTS radical scavenging activity (Fig. 1B), the activity increased with increasing DH ( $P < 0.05$ ). However, the ABTS radical scavenging activity of gelatin hydrolysate with 10%DH and 20%DH was not different ( $P > 0.05$ ). Gelatin hydrolysates from bigeye snapper skin with DH ranging from 5% to 25% prepared using Alcalase had increased ABTS scavenging activity with increasing DH ([Phanturat et al., 2010](#)). The DPPH and ABTS radical scavenging activity is based on the ability of antioxidants to donate a hydrogen atom or an electron to stabilise radicals, by converting it to the non-radical species ([Binsan et al., 2008](#); [Chandrasekara & Shahidi, 2011](#)). The results suggest that gelatin hydrolysates, especially at 40%DH, had the ability to scavenge free radicals, thereby preventing lipid oxidation via a chain breaking reaction.

Hydroxyl radical scavenging activity of gelatin hydrolysate with %DH of 10, 20, 30 and 40 was 5.23, 5.59, 5.69 and 5.93 µmol TE/g sample, whilst no activity was detectable for gelatin. Hydroxyl radicals generated by Fenton's reaction were spin trapped with DMPO to give DMPO-OH adducts. The radical adducts were detected using an EPR spectrometer. The obtained EPR spectra had a 1:2:2:1 quartet pattern ([Harbour, Chow, & Bolton, 1974](#)), as shown in Fig. 2. The EPR spectrum of DMPO-OH adduct alone (control) (Fig. 2A) had a much higher intensity than that of gelatin hydrolysates with 40%DH (Fig. 2E). The amplitude of the spectra decreased when hydrolysate with higher DH was incorporated (Fig. 2B–E). Therefore, the hydroxyl radical scavenging activity was enhanced

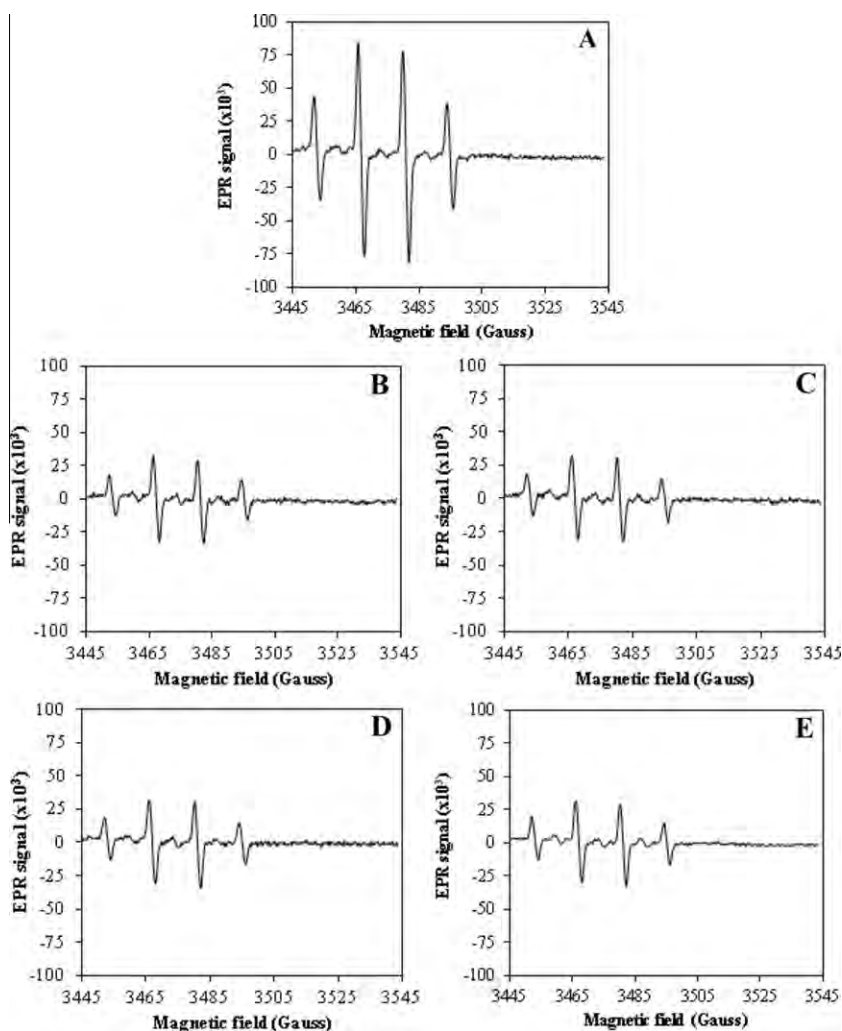


**Fig. 2.** Electron paramagnetic resonance (EPR) signals of DMPO-OH adduct alone (A), DMPO-OH adduct in the presence of gelatin hydrolysates with 10%DH (B), 20%DH (C), 30%DH (D) and 40%DH (E) at a concentration of 10 mg/ml.

when gelatin was hydrolysed with papaya latex enzyme. Hydroxyl radical is an extremely reactive oxygen-centred radical, which generates other radicals by reacting with the biomolecules (proteins, DNA and lipids) and it is a major reactive oxygen species causing lipid peroxidation (Chandrasekara & Shahidi, 2011). Therefore, it was implied that gelatin hydrolysates might be able to inhibit lipid peroxidation via scavenging of hydroxyl radicals.

The oxygen radical absorbance capacity (ORAC) of gelatin hydrolysate with different DHs ranged from 268.16 to 709.42  $\mu\text{mol TE/g}$  sample, whereas the ORAC of 158.92  $\mu\text{mol TE/g}$  sample was found for gelatin. Higher DH generally resulted in an increase in ORAC of gelatin hydrolysates ( $P < 0.05$ ). ORAC is the assay, which determines the antioxidant activity of compounds by scavenging peroxyl radical, which is thermally generated through the decomposition of the azo compound, AAPH. Fluorescein, which has been used as a fluorescence probe, reacts with peroxyl radical and hence causing fluorescence decay. The fluorescence decay was inhibited in the presence of antioxidants (Chandrasekara & Shahidi, 2011). The intensity can be measured at 485 nm excitation and 525 nm emission wavelengths. The noticeable fluorescence decay was observed in the presence of gelatin hydrolysate. Amongst all samples, gelatin hydrolysate with 40%DH showed the highest inhibition toward the decay ( $P < 0.05$ ) (Fig. 3). Peroxyl radicals, which are formed by direct reaction of triplet oxygen with alkyl radicals during the

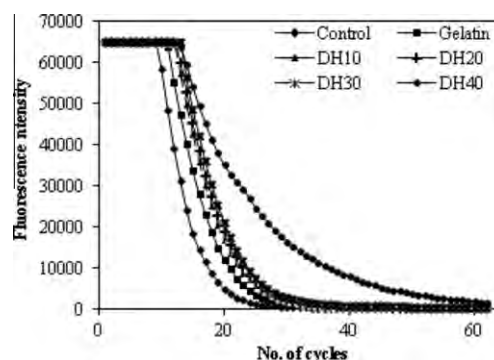


**Fig. 3.** Fluorescence decay curves of fluorescein in the presence of gelatin and gelatin hydrolysates with different DHs. Hydrolysate at a concentration of 0.1 mg/ml was used for oxygen radical absorbance capacity (ORAC) assay.

propagation step in fatty acid oxidation, produce hydroperoxides by abstracting hydrogen from other molecules. In the presence of heat, UV light or transition metals, hydroperoxides can be easily decomposed to alkoxy radicals by the homolytic cleavage of the oxygen and oxygen bond, which has a low dissociation energy of 44 kcal/mol than the 90 kcal/mol for the bond between oxygen and hydrogen of the hydroperoxide. The alkoxy radical is cleaved by homolytic  $\beta$ -scission of the C–C bond and produces oxo

compound, aldehydes, acids, alcohols, and short-chain hydrocarbons, which are the main causes of rancid off-flavour in foods (Choe & Min, 2005). The result indicates that gelatin hydrolysate with 40%DH was the most powerful antioxidant amongst all samples examined for donating a hydrogen atom to the peroxy radical.

The ferric reducing antioxidant power (FRAP) of gelatin and gelatin hydrolysates with %DH of 10, 20, 30 and 40 was 0.69, 1.17, 1.30, 2.29 and 6.18  $\mu\text{mol TE/g sample}$  (Fig. 1C). Gelatin hydrolysate with 40%DH had the highest activity ( $P < 0.05$ ) that was 8.95 and 5.28 fold higher than that of gelatin and gelatin hydrolysate with 10%DH, respectively. Increases in reducing power of hydrolysate with increasing DH have been reported in round scad protein hydrolysate prepared using Flavourzyme and gelatin hydrolysate from bigeye snapper prepared using Alcalase, Neutrase and pepsin extract (Phanturat et al., 2010; Thiansilakul et al., 2007a). FRAP is generally used to measure the capacity of a substance in reducing TPTZ–Fe(III) complex to TPTZ–Fe(II) complex (Benzie & Strain, 1996; Binsan et al., 2008). The result suggests that gelatin hydrolysate with 40%DH possibly contained higher amounts of peptides which donated electrons to free radicals, thereby terminating the chain reaction.

The ferrous ( $\text{Fe}^{2+}$ ) chelating activity of gelatin hydrolysate increased when DH increased ( $P < 0.05$ ) (Fig. 4A). Similar results were observed in the protein hydrolysate from round scad muscle (Thiansilakul et al., 2007a), peanut (Jamdar et al., 2010) and porcine plasma (Liu, Kong, Xiong, & Xia, 2010). The DH greatly influenced the peptide chain length as well as the increase in termini. Liu et al. (2010) reported that the cleavage of peptides led to an enhanced metal ion binding due to increased concentration of carboxylic groups and amino groups of the peptides. Therefore, gelatin hydrolysate with higher DH showed a higher chelating activity. In addition, the chelating activity of gelatin hydrolysate was much higher than that of gelatin, approximately by 7.23–25.08 folds. The presence of transition metals in foods is a major factor in the promotion of lipid oxidation (Klompang, Benjakul, Kantachote, & Shahidi, 2007; McClements & Decker, 2000). Therefore, gelatin hydrolysate, especially that with 40%DH, could act as a chelating agent, which was able to retard lipid oxidation in foods.

Singlet oxygen, which is a highly reactive, electrophilic and non-radical molecule, can be formed by the reaction between photosensitizers and triple oxygen in the presence of light. The reaction rate of singlet oxygen with foods is much greater than that

of triplet oxygen due to the low activation energy (Min & Boff, 2002). Singlet oxygen can directly react with electron-rich double bonds of unsaturated fatty acids without the formation of free-radical intermediates (Choe & Min, 2005). The singlet oxygen scavenging activity of gelatin (352.63  $\mu\text{mol TE/g sample}$ ) was lower than that of gelatin hydrolysate (648.68–1362.72  $\mu\text{mol TE/g sample}$ ) ( $P < 0.05$ ) (Fig. 4B). The singlet oxygen scavenging activity of gelatin hydrolysate increased when DH increased ( $P < 0.05$ ). Peptides with the shorter chain length might be able to trap or bind with singlet oxygen to a higher extent. As a result the lower available singlet oxygen could have a lower ability in accelerating the lipid oxidation process. Therefore, gelatin hydrolysate, especially 40%DH, can act as a singlet oxygen scavenger.

Hydrogen peroxide, which is a weak oxidising agent, is not directly involved in the initiation of lipid oxidation because its reduction potential is lower than that of unsaturated fatty acids (Choe & Min, 2005). However, hydrogen peroxide can be implicated indirectly in lipid oxidation. Hydrogen peroxide is the precursor for the generation of hydroxyl radical, which is a strong initiator of lipid oxidation (Choe & Min, 2005). Hydrogen peroxide scavenging activity of gelatin hydrolysate increased slightly as DH increased ( $P < 0.05$ ) (Fig. 4C). However, the difference in hydrogen peroxide scavenging activity was not observed between gelatin hydrolysate with 10%DH and 20%DH ( $P > 0.05$ ) and between samples with 30%DH and 40%DH ( $P > 0.05$ ). These results suggest that the hydrogen peroxide scavenging activity of gelatin was slightly increased after hydrolysis.

Hypochlorous acid (HOCl), which is a potent chlorinating and oxidising agent, is generated by myeloperoxidase in the presence of  $\text{H}_2\text{O}_2$  and  $\text{Cl}^-$  (Erickson, 1997). Myeloperoxidase localised in blood can be contaminated into muscle. Although the blood is washed off, residual concentrations of myeloperoxidase may be sufficient to accelerate oxidation in stored muscle foods (Erickson, 1997). HOCl scavenging activity of gelatin hydrolysate increased as DH increased up to 30%DH ( $P < 0.05$ ). HOCl scavenging activity of gelatin and gelatin hydrolysate with 10%DH was not different ( $P > 0.05$ ) and there was no difference in the HOCl scavenging activity between gelatin hydrolysate with 30%DH and 40%DH ( $P > 0.05$ ). HOCl inhibition of gelatin hydrolysate at a concentration of 1 mg/ml ranged from 28.18% to 32.21% (data not shown), whilst that of plant extract from *Spondias pinnata* at a concentration of 0.1 mg/ml was about 50% (Hazra, Biswas, & Mandal, 2008). Thus,

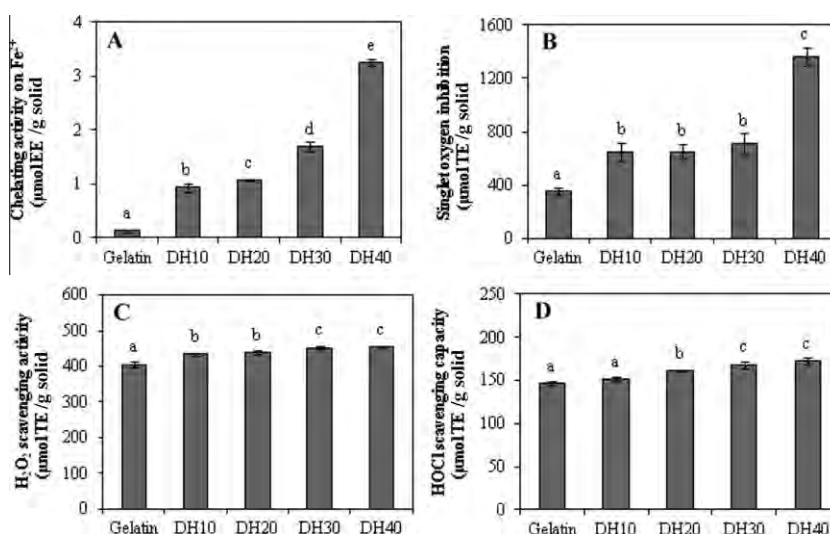


**Fig. 4.** Chelating activity of  $\text{Fe}^{2+}$  (A), singlet oxygen inhibition (B), hydrogenperoxide scavenging activity (C) and hypochlorous acid scavenging capacity (D) by gelatin hydrolysates with different DHs. Bars represent standard deviation ( $n = 3$ ). Different letters on the bars denote significant differences ( $P < 0.05$ ).

gelatin hydrolysates from blacktip shark skin were able to scavenge HOCl, thereby lowering lipid oxidation in meat and meat products.

From the above measurements of antioxidant activity using different assays, antioxidant activity of gelatin was enhanced upon hydrolysis using enzymes from papaya latex. Nevertheless, the  $H_2O_2$  and HOCl scavenging activities were slightly increased after hydrolysis. In addition, gelatin hydrolysate with 40%DH showed the highest antioxidant activity via not only chain breaking, but also preventive mechanisms. Changes in size, amount, exposure of the terminal amino groups of the products obtained, and composition of free amino acids or small peptides affect the antioxidant activity (Thiansilakul et al., 2007a; Wu, Chen, & Shiau, 2003). The small peptides generally showed a higher antioxidant activity (Qian, Jung, & Kim, 2008). Therefore, gelatin hydrolysate with 40%DH was selected for further studies. Furthermore, gelatin hydrolysate with the highest DH (~40%DH) could be prepared by enzyme from papaya latex (data not shown).

### 3.2. Stability of selected gelatin hydrolysate

The ORAC and chelating activity assays were used for stability study. Peroxyl radical can abstract hydrogen atom from unsaturated fatty acids to form hydroperoxides, which are decomposed to aldehydes, acids, alcohols and short-chain hydrocarbons. These decomposition products are the main cause of rancid off-flavour in foods (Choe & Min, 2005). In addition,  $Fe^{2+}$  can stimulate lipid peroxidation by the Fenton reaction and can also accelerate lipid peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals (Hazra et al., 2008).

#### 3.2.1. pH stability

The effect of pH on ORAC and chelating activity of gelatin hydrolysate with 40%DH is shown in Fig. 5A. The ORAC of gelatin hydrolysate was quite stable over the pH range of 1–7 ( $P > 0.05$ ). At pH 9, the activity increased by approximately 121%, compared with that found in the sample without pH adjustment. Meanwhile, the chelating activity of gelatin hydrolysate increased by 768–891% after pH adjustment, possibly due to the changes of charges in peptides, particularly at N- and C-termini, mediated by pH adjustment. Dong et al. (2008) reported that increasing concentration of carboxylic groups and amino groups in branches of the acidic and basic amino acids led to an enhanced  $Fe^{2+}$  binding. Thus, it suggests that gelatin hydrolysate with 40%DH had a potential for application in any food system over pH range of 1–9 without any loss in activity.

#### 3.2.2. Thermal stability

Thermal stability of antioxidant activity of gelatin hydrolysate with 40%DH monitored by ORAC and chelating activity is shown in Fig. 5B. No changes in ORAC were observed at all heating times (0–240 min) ( $P > 0.05$ ). It was noted that chelating activity slightly increased when heating time increased up to 15 min ( $P < 0.05$ ). Thereafter, the activity was stable up to 240 min of heating ( $P > 0.05$ ). Zayas (1997) reported that smaller size peptides were more stable to aggregation at high temperatures. Furthermore, gelatin hydrolysate was hydrophilic in nature with a lesser proportion of hydrophobic domain. This result indicates that gelatin hydrolysate with 40%DH could be incorporated in any food subjected to thermal processed at 100 °C for up to 240 min.

#### 3.2.3. Stability in gastrointestinal tract model system

The gastrointestinal tract model system was used to mimic the digestive system containing several proteases. The remaining antioxidant activity of the gelatin hydrolysate with 40%DH, as monitor by ORAC and chelating activity, after different digestion times is

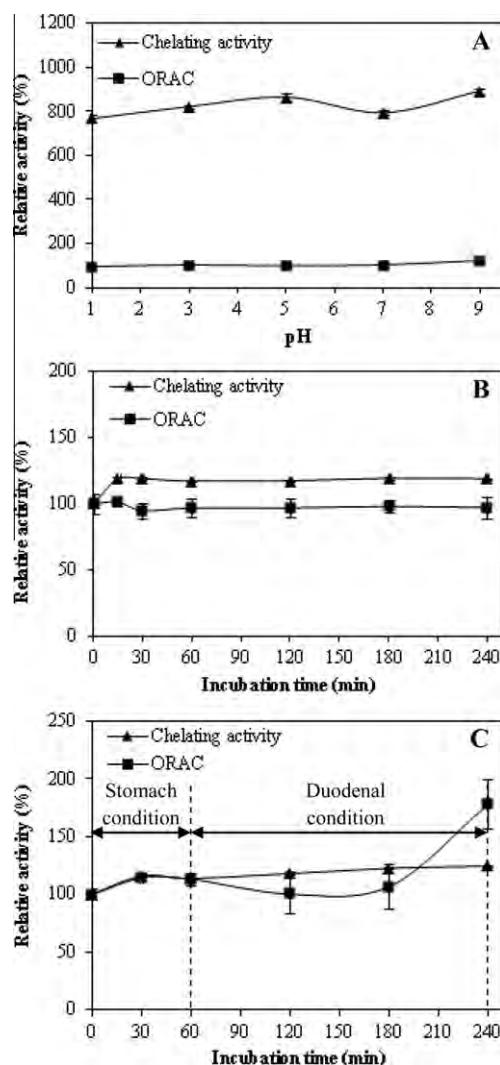


Fig. 5. pH (A), thermal (B) and digestive (C) stabilities of gelatin hydrolysate with 40%DH as monitored by the oxygen radical absorbance capacity (ORAC) and chelating activity assays. Bars represent standard deviation ( $n = 3$ ).

presented in Fig. 5C. The ORAC slightly increased after pepsin digestion ( $P < 0.05$ ). Under duodenal condition, ORAC remained constant during 60 and 180 min ( $P > 0.05$ ), but increased at the end of digestion (time = 240 min) ( $P < 0.05$ ). After digestion in duodenum, some new peptides with low MW were probably produced. These peptides might have high ORAC and hence the increased ORAC was noticeable. Nalinanon, Benjakul, Kishimura, and Shahidi (2011) also found increased antioxidant activity of protein hydrolysate from ornate threadfin bream prepared after being ingested in the simulated model system. On the other hand, only slight increase in chelating activity was observed over digestion time of 0–240 min ( $P < 0.05$ ). The results show that the antioxidant activity of gelatin hydrolysate with 40%DH was most likely stable in the real human digestion system.

### 3.3. Antioxidant activity of selected gelatin hydrolysate in model systems

#### 3.3.1. $\beta$ -Carotene-linoleate model system

The antioxidant activity of gelatin hydrolysate with 40%DH in a  $\beta$ -carotene-linoleate model system in comparison with BHA is shown in Fig. 6A. The decrease in  $A_{450}$  indicates the oxidation of  $\beta$ -carotene in the system caused by free radicals from oxidation

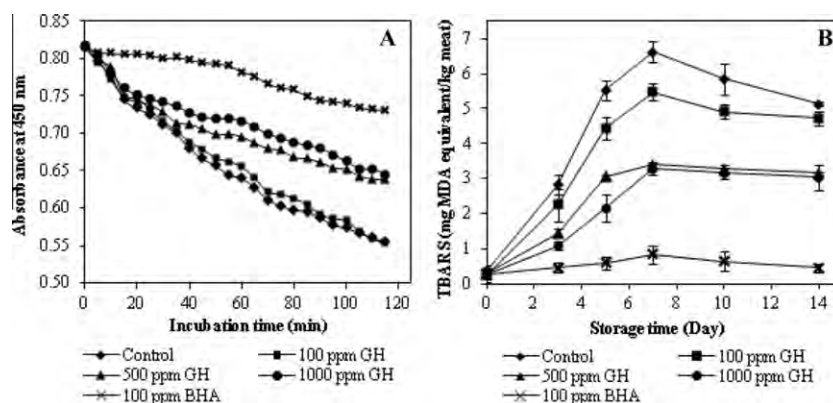


**Fig. 6.** Changes in lipid oxidation of  $\beta$ -carotene-linoleate model system (A) and cooked comminuted pork model system (B) added with gelatin hydrolysate with 40%DH at different levels. GH and BHA denote gelatin hydrolysate and butylated hydroxyanisole, respectively. Bars represent standard deviation ( $n = 3$ ).

of linoleic acid (Chandrasekara & Shahidi, 2010). In the presence of antioxidants,  $\beta$ -carotene bleaching was retarded mainly due to the elimination of free radicals. No difference in  $A_{450}$  between the systems without antioxidant (control) and with 100 ppm gelatin hydrolysate was noticeable up to 120 min of incubation time ( $P > 0.05$ ). Systems containing gelatin hydrolysate at the levels of 500 and 1000 ppm effectively retarded the decrease in  $A_{450}$ . However, their antioxidant activity was lower than BHA at a level of 100 ppm. The difference in antioxidant activity between gelatin hydrolysate and BHA might be caused by the difference in their polarity. The hydrophobic antioxidants have higher efficiency than hydrophilic antioxidants in preventing oxidation in oil-in-water emulsion systems by preferably orienting at the oil–water interface (Wijeratne et al., 2006). Gelatin hydrolysate at levels of 500 and 1000 ppm was therefore able to retard lipid oxidation in oil-in-water emulsions.

### 3.3.2. Cooked comminuted pork model system

Lipid oxidation in cooked comminuted pork containing gelatin hydrolysate of 40%DH at levels of 100, 500 and 1000 ppm and BHA (100 ppm) was monitored during storage at 4 °C for 14 days, using TBARS, as shown in Fig. 6B. Generally, the TBARS of cook comminuted pork with and without antioxidants increased as the storage time increased up to 7 days of storage ( $P < 0.05$ ). Thereafter, the decrease in TBARS was observed until the end of storage ( $P < 0.05$ ). This was probably due to the loss of oxidation products formed, particularly low MW volatile compounds. Malondialdehyde and other short-chain products of lipid oxidation are not stable and are decomposed to alcohols and acids, which are not determined by the TBA test (Fernández, Pérez-Álvarez, & Fernández-López, 1997). The meat system containing higher levels of gelatin hydrolysate had lower TBARS during the storage period ( $P < 0.05$ ). After 7 days of storage, no difference in TBARS between the meat system containing gelatin hydrolysate at levels of 500 and 1000 ppm was noticeable ( $P > 0.05$ ). The inhibition of TBARS production in meat system added with gelatin hydrolysate was most likely due to its radical scavenging activity as well as chelating activity toward  $Fe^{2+}$ , a prooxidant in meat system. However, BHA (100 ppm) showed a better preventive effect on lipid oxidation than gelatin hydrolysate as evidenced by the lowest TBARS values during the storage period. This result was in accordance with that observed in  $\beta$ -carotene-linoleate model system (Fig. 6A). Lipophilicity of antioxidants appeared to be positively associated with their activity in cooked muscle foods and in oil-in-water emulsions. Zhong and Shahidi (2012) reported that lipophilised-epigallocatechin-gallate (EGCG), which were more

lipophilic than their parent EGCG molecule, showed a higher antioxidant activity.

## 4. Conclusions

The antioxidant activity of gelatin hydrolysates from blacktip shark skin prepared using papaya latex enzymes varied with DH. Gelatin hydrolysate with 40%DH could act as an antioxidant in oil-in-water emulsion and cooked comminuted pork system. In addition, its antioxidant activity remained unchanged or increased in a wide range of pH (1–9) and during heating (100 °C) for 240 min. The activity was also stable after digestion in a gastrointestinal tract model system. Therefore, it might serve as a potential source of natural antioxidant to prevent lipid oxidation in various foods.

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# Impact of zinc salts on heat-induced aggregation of natural actomyosin from yellow stripe trevally

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## ABSTRACT

Impact of zinc sulphate ( $\text{ZnSO}_4$ ) and zinc chloride ( $\text{ZnCl}_2$ ) on heat-induced aggregation of natural actomyosin (NAM) extracted from yellow stripe trevally (*Selaroides leptolepis*) was investigated. In the presence of  $\text{ZnSO}_4$  or  $\text{ZnCl}_2$ , the transition temperature ( $T_{\text{max}}$ ) of myosin shifted from  $47.83 \pm 0.30$  °C to  $46.05 \pm 0.36$  and  $46.49 \pm 0.49$  °C, with the coincidental decreases in  $\Delta H$  from  $1.07 \pm 0.03$  J/g to  $0.63 \pm 0.02$  and  $0.67 \pm 0.04$  J/g, respectively ( $P < 0.05$ ). Additionally,  $\text{Ca}^{2+}$ -ATPase activity of NAM decreased with increasing the concentrations of  $\text{ZnSO}_4$  or  $\text{ZnCl}_2$  during heating up to 40 °C. During heating from 20 to 75 °C, higher turbidity, surface hydrophobicity and disulphide bond formation were obtained in NAM added with  $\text{ZnSO}_4$  or  $\text{ZnCl}_2$  at temperatures ranging from 40 to 75 °C, compared with the control. Nevertheless, a higher aggregation was found in NAM added with  $\text{ZnSO}_4$ , compared with  $\text{ZnCl}_2$ . Zeta potential ( $\zeta$ ) analysis suggested that the surface of NAM added with  $\text{ZnSO}_4$  became less negatively charged, compared with that of  $\text{ZnCl}_2$  counterpart. Transmission electron microscopy showed that the structure of NAM was highly interconnected, finer and denser when zinc salts, especially  $\text{ZnSO}_4$  were incorporated. Therefore,  $\text{ZnSO}_4$  could be used to induce aggregation of fish muscle proteins, thereby improving gelling property of fish mince or surimi.

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## 1. Introduction

Surimi is the concentrated myofibrillar proteins, prepared from fish mince subjected to thorough washing, in which undesirable components including sarcoplasmic proteins, lipids, and haem pigments, are removed. Lean fish have been widely used as the raw material for surimi production owing to the good gel forming ability and whiteness. Due to the limited fish resources, especially lean fish, pelagic dark fleshed fish have been investigated more as a potential alternative raw material for surimi production (Chaijan, Benjakul, Visessanguan, & Faustman, 2004). However, a poor gel forming ability of dark fleshed fish is generally observed thereby limiting the utilisation of this species. To improve the properties of surimi gel from dark fleshed fish, novel and cheap additives capable of improving gel quality of mince or surimi have gained increasing attention.

Divalent cations like calcium, magnesium and zinc are known to alter the functionality of proteins during gelation (Mathew, Shamasundar, Kumar, & Prakash, 2009; Morales, Ramirez, Vivanco, & Vazquez, 2001). Sorgentini, Wagner, and Anon (1995) reported that aqueous dispersions of native soy isolate had the highest turbidity when  $\text{MgCl}_2$  at a level of 40 mM was present. The role of zinc salts in combination with sucrose on the cryoprotection and aggre-

gation of fish actomyosin was studied by MacDonald, Lanier, and Giesbrecht (1996). MacDonald et al. (1996) reported that increasing  $\text{ZnSO}_4$  concentration above 0.1 mM decreased  $\text{Ca}^{2+}$ -ATPase activity by inducing denaturation and consequent aggregation in these samples. Haque and Aryana (2002) found that gels with  $\text{CuSO}_4$ ,  $\text{FeSO}_4$ , and  $\text{ZnSO}_4$  had aggregates that appeared clustered and more fused together than the control. At pH values sufficiently far from the isoelectric point of proteins, divalent ions such as  $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mg}^{2+}$  induce protein cross-linking via the salt bridges between negatively charged carboxyl groups (Hongprabhas & Barbut, 1997). Mathew et al. (2009) reported that the binding of zinc salts to actomyosin from oil sardine induced conformational changes with the exposure of functional groups such as sulphhydryl groups, which subsequently undergo oxidation to disulphide bond.

Yellow stripe trevally (*Selaroides leptolepis*) is one of the abundant dark fleshed fish species in Southern Thailand. Surimi from this species had low gel forming ability, compared with surimi from lean fish (Arfat & Benjakul, in press). The addition of divalent cations, especially zinc at an appropriate concentration could be a promising means to improve the gel property by enhancing the aggregation of proteins via salt bridges. From our previous study, the addition of  $\text{ZnSO}_4$  (1–5 mmol/kg) into surimi from threadfin bream surimi was able to increase the breaking force and deformation (unpublished data). However, the role of zinc salts on the physicochemical changes and heat-induced aggregation of fish myofibrillar proteins, which play an essential role in gelation, has

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not yet been elucidated. The present study was undertaken to elucidate the impact of two different zinc salts on the thermal stability, physicochemical changes and heat-induced aggregation of natural actomyosin from yellow stripe trevally (*S. leptolepis*).

## 2. Materials and methods

### 2.1. Chemicals

Adenosine-5'-triphosphate (ATP), 8-anilino-1-naphthalenesulphonic acid (ANS), guanidine thiocyanate, sodium hydrogen sulphite,  $\beta$ -mercaptoethanol ( $\beta$ -ME) and Tris-maleate were obtained from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Zinc sulphate ( $\text{ZnSO}_4$ ) and zinc chloride ( $\text{ZnCl}_2$ ) were purchased from Ajax Finechem. Pty., Ltd., (Taren Point, NSW, Australia). Potassium chloride, sodium chloride, calcium chloride, trichloroacetic acid, potassium dihydrogen phosphate and ammonium molybdate were procured from Merck (Darmstadt, Germany). 5,5-Dithiobis (2-nitrobenzoic acid) (DTNB) was purchased from Wako Pure Chemical Industries (Tokyo, Japan). Bovine serum albumin (BSA) was obtained from Fluka (Buchs, Switzerland).

### 2.2. Collection and preparation of fish

Yellow stripe trevally (*S. leptolepis*) with an average weight of 65–75 g were caught from Songkhla coast along the Gulf of Thailand during January and February, 2012. The fish, off-loaded approximately 12 h after capture, were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 45 min. The fish were headed, gutted and washed with water. The flesh was separated manually from skin and bone and kept on ice prior to natural actomyosin (NAM) preparation. The storage time was not longer than 2 h.

### 2.3. Preparation of natural actomyosin (NAM)

NAM was prepared according to the method of Balange and Benjakul (2010) with a slight modification. Yellow stripe trevally muscle was homogenised in chilled 0.6 M KCl, pH 7.0 at a ratio of 1:10 (w/v) using a homogeniser (IKA, Labortechnik, Selangor, Malaysia). To avoid overheating, the sample was placed in ice and homogenised for 20 s, followed by a 20 s rest interval for a total extraction time of 4 min. The extract was centrifuged at 5000g for 30 min at 4 °C using a refrigerated centrifuge (Avanti J-E Centrifuge; Beckman Coulter, Fullerton, CA, USA). Three volumes of chilled deionised water (0–2 °C) were added to precipitate NAM. The NAM was collected by centrifuging at 5000g for 20 min at 4 °C. The NAM pellet was dissolved in chilled 0.6 M KCl, pH 7.0 for 30 min at 4 °C and then centrifuged at 5000g for 20 min at 4 °C. The supernatant was collected and used as NAM.

### 2.4. Study on the effect of $\text{ZnSO}_4$ and $\text{ZnCl}_2$ at different concentrations on heat-induced aggregation of NAM

NAM extracted from yellow stripe trevally was diluted to 1 mg/ml with chilled 0.6 M KCl (pH 7.0). NAM solutions containing  $\text{ZnSO}_4$  or  $\text{ZnCl}_2$  at different concentrations (25, 50, 100  $\mu\text{M}$ ) were heated at a heating rate of 0.65 °C/min from 20 to 75 °C using a digital thermoregulator (TH5/150, Ratek, Boronia, Victoria, Australia). NAM without the addition of zinc salt was used as the control. The samples were taken every 5 °C of temperature increment. At the designated temperature, the samples were cooled immediately with iced water. The samples obtained were subjected to analyses.

#### 2.4.1. Determination of thermal protein aggregation

Different NAM solutions (1 mg protein/ml) were placed in cuvette (light path length of 1 cm). The degree of protein aggregation was estimated by measuring the turbidity of NAM solutions after being heated to the designated temperatures at a wavelength of 660 nm (Benjakul, Visessanguan, Ishizaki, & Tanaka, 2001) using a UV–visible spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan).

#### 2.4.2. Determination of surface hydrophobicity

Surface hydrophobicity was measured as per the method of Li-Chan, Nakai, and Wood (1985) as modified by Benjakul et al. (2001) using 8-anilino-1-naphthalenesulphonic acid (ANS) as a probe. Heated NAM solutions were diluted to 0.125, 0.25, 0.5 and 1 mg/ml using the same buffer. To 2.0 ml of diluted NAM solution, 10  $\mu\text{l}$  of 10 mM ANS dissolved in 50 mM potassium phosphate buffer (pH 7.0) were added and the mixtures were mixed thoroughly. Sample blanks of each protein concentration were prepared in the same manner, except the same volume of 50 mM potassium phosphate buffer (pH 7.0) was used instead of ANS solution. Fluorescence intensity was measured using a RF-1501 spectrofluorometer (Shimadzu, Kyoto, Japan) at the excitation and emission wavelength of 374 and 485 nm, respectively. Surface hydrophobicity was calculated from initial slope of plot of fluorescence intensity against protein concentration using a linear regression analysis. The initial slope was referred to as  $S_0\text{ANS}$ .

#### 2.4.3. Determination of total sulphydryl groups and disulphide bond contents

Determination of total sulphydryl group content was done using 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB) following the method of Ellman (1959) as modified by Benjakul et al. (2001). To 1.0 ml of sample solutions, 9 ml of 0.2 M Tris–HCl buffer, pH 8.2, containing 8 M urea, 2% SDS and 10 mM ethylenediaminetetraacetic acid (EDTA) were added. To 4 ml of the mixture, 0.4 ml of 0.1% DTNB was added and incubated at 40 °C for 25 min. The absorbance at 412 nm was then measured. A sample blank of each sample was conducted in the same manner except that distilled water was used instead of DTNB. The sulphydryl group content was calculated using the extinction coefficient of 13,600  $\text{M}^{-1} \text{cm}^{-1}$ . The determination of disulphide bond content was carried out by using 2-nitro-5-thiosulphobenzoate (NTSB) (Thannhauser, Konishi, & Scheraga, 1987). To 0.5 ml of sample solution, 3.0 ml of freshly prepared NTSB assay solution were added. The mixture was incubated in the dark at room temperature (26–28 °C) for 25 min. A sample blank of each sample was prepared in the same manner but the distilled water was used instead of NTSB assay solution. The absorbance at 412 nm was measured. The disulphide bond content was calculated using the extinction coefficient of 13,900  $\text{M}^{-1} \text{cm}^{-1}$ .

#### 2.4.4. Determination of $\text{Ca}^{2+}$ -ATPase activity

The  $\text{Ca}^{2+}$ -ATPase activity of NAM samples was determined according to the method of Jiang, Hwang, and Chen (1988) as modified by Benjakul, Seymour, Morrissey, and An (1997). To 1 ml of different NAM solutions (1 mg protein/ml) incubated at different temperatures (20, 30, 40, 50 and 60 °C), 0.6 ml of 0.5 M Tris-maleate, pH 7.0, was added. The mixture was added with  $\text{CaCl}_2$  solution to obtain a final concentration of 10 mM  $\text{CaCl}_2$  with the total volume of 9.5 ml. To initiate the reaction, 0.5 ml of 20 mM ATP was added. The reaction was conducted for exactly 10 min at 25 °C and was terminated by adding 5 ml of chilled 15% (w/v) trichloroacetic acid. The reaction mixture was centrifuged at 3500g for 5 min and the inorganic phosphate liberated in the supernatant was measured by the method of Fiske and Subbarow (1925).  $\text{Ca}^{2+}$ -ATPase activity was expressed as  $\mu\text{moles}$  of inorganic phosphate released per mg protein per min. A blank solution was

prepared by adding chilled trichloroacetic acid prior to addition of ATP.

## 2.5. Study on the effect of $\text{ZnSO}_4$ and $\text{ZnCl}_2$ on thermal transition, charge, size and microstructure of NAM

### 2.5.1. Differential scanning calorimetry

The thermal transition of NAM from yellow stripe trevally was determined using the differential scanning calorimetry (DSC) (Perkin-Elmer, Model DSCM, Norwalk, CT, USA). NAM pellet samples (15–20 mg wet weight) containing  $\text{ZnSO}_4$  or  $\text{ZnCl}_2$  with a final concentration of 100  $\mu\text{M}$  were placed in the DSC hermetic pans, assuring a good contact between the sample and the pan bottom. Sample without addition of zinc salt was used as the control. An empty hermetic pan was used as a reference. The samples were scanned at 10  $^{\circ}\text{C}/\text{min}$  over the range of 20–100  $^{\circ}\text{C}$ . The maximum transition temperature ( $T_{\text{max}}$ ) was measured and the enthalpy ( $\Delta H$ ) was estimated by measuring the area under the DSC transition curve. The system was calibrated using indium.

### 2.5.2. Zeta potential and particle size

NAM solutions (1 mg protein/ml) with different treatments heated to reach the final temperature of 75  $^{\circ}\text{C}$  were stirred gently for 6 h in ice. Thereafter, the mixtures were allowed to stand at 4  $^{\circ}\text{C}$  for 30 min prior to analysis. The zeta ( $\zeta$ ) potential and particle size of NAM solutions were measured using a ZetaPlus zeta potential analyser (Brookhaven Instruments Corporation, Holtsville, NY) at room temperature.

### 2.5.3. Transmission electron microscopy

NAM solutions (1 mg protein/ml) with different treatments heated to reach the final temperature of 75  $^{\circ}\text{C}$ , followed by cooling, were diluted to 0.2 mg/ml with 50 mM potassium phosphate buffer containing 0.6 M KCl (pH 7). A drop of sample was fixed for 5 min on a carbon-coated grid, negatively stained with 4% uranyl acetate for 5 min and washed with distilled water until the grid was cleaned. The specimens were visualized using a JEOL JEM-2010 transmission electron microscope (JEOL Ltd., Tokyo, Japan) (60,000 $\times$ ) at an accelerating voltage of 160 kV.

## 2.6. Protein determination

The protein content was determined by the Biuret method (Robinson & Hodgen, 1940) using bovine serum albumin as the standard.

## 2.7. Statistical analysis

The experiments were run in triplicate with three lots of sample. Analysis of variance (ANOVA) was performed and the mean comparisons were carried out by the Duncan's multiple range tests (Steel & Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS 17.0 for windows: SPSS Inc., Chicago, IL, USA).

## 3. Results and discussion

### 3.1. Effect of $\text{ZnSO}_4$ and $\text{ZnCl}_2$ at different concentrations on heat-induced aggregation of NAM

#### 3.1.1. Thermal protein aggregation

Changes in turbidity of NAM solutions in the absence and in the presence of  $\text{ZnSO}_4$  or  $\text{ZnCl}_2$  at different concentrations during heat-

ing from 20 to 75  $^{\circ}\text{C}$  were monitored (Fig. 1). Turbidity represents a rough estimate of the degree of aggregation and is affected by environmental conditions (pH and/or ionic strength) (Hermansson, 1986). A marked increase in the turbidity of all NAM samples was observed above 35  $^{\circ}\text{C}$  ( $P < 0.05$ ). The higher rate in turbidity development was found with increasing concentrations of zinc salts ( $P < 0.05$ ). At the same concentration used,  $\text{ZnSO}_4$  resulted in the higher turbidity at all temperatures, compared with  $\text{ZnCl}_2$  and the control, respectively. Nevertheless, no difference in turbidity was found between NAM containing  $\text{ZnSO}_4$  and  $\text{ZnCl}_2$  at a level of 25  $\mu\text{M}$  ( $P > 0.05$ ). The increased turbidity of NAM was therefore induced by  $\text{ZnSO}_4$  and  $\text{ZnCl}_2$ . This was more likely governed by a 'salt bridge' between proteins.  $\text{Zn}^{2+}$  could act as the bridge between negatively charged residues, particularly  $\text{COO}^-$ . As a result, a large aggregate could be formed. Furthermore, protein molecules also underwent aggregation via hydrophobic interaction, electrostatic and hydrogen bonds, Van der Waals interaction and disulphide bond during a heat induced gelation process (Chawla, Venugopal, & Nair, 1996; Xiong, 1997). Divalent metal ions might cause changes in protein conformation and interact with negative charges on the polypeptide chains of proteins, thereby inducing cross-linking via salt bridges (Ding et al., 2011).

At temperature above 35  $^{\circ}\text{C}$ , unfolding of some protein molecules occurred, more likely due to the instability of hydrogen bonds. The  $T_{\text{onset}}$  of myosin in all samples was around 40  $^{\circ}\text{C}$  (data not shown), where the alteration of protein molecules more likely take place. As a consequence, the greater numbers of reactive groups and hydrophobic portions were exposed (Niwa, 1992). In the presence of  $\text{Zn}^{2+}$ , this divalent cation might induce cross-linking of protein molecules via the exposed carboxyl group. As a result, the higher aggregation via salt bridge could be enhanced, along with other bondings including hydrophobic interaction and disulphide bond. When higher levels of zinc salt were present, a higher aggregation was noticeable. Thus, the cross-linking activity was determined by the amount of zinc ions. When comparing the cross-linking activity between  $\text{ZnSO}_4$  and  $\text{ZnCl}_2$  towards NAM,  $\text{ZnSO}_4$  exhibited higher capacity of forming the protein aggregate than  $\text{ZnCl}_2$ . The greater screening effect of  $\text{ZnSO}_4$  on the negatively charged carboxyl groups of proteins was reported in comparison with  $\text{ZnCl}_2$  (Twomey, Keogh, Mehra, & O'Kennedy, 1997). The formation of large aggregates is presumably a prerequisite to the formation of a good elastic gel (Chan, Gill, & Paulson, 1992a). Chan et al. (1992a) reported that the poorer aggregating ability of hering actomyosin reflected the inferior gelling properties of surimi. Therefore, the aggregation of NAM from yellow stripe trevally was enhanced by the addition of zinc salt, especially  $\text{ZnSO}_4$ .

#### 3.1.2. Surface hydrophobicity

Surface hydrophobicity ( $S_0\text{ANS}$ ) of different NAM solutions in the absence and presence of zinc salts ( $\text{ZnSO}_4$  or  $\text{ZnCl}_2$ ) at different

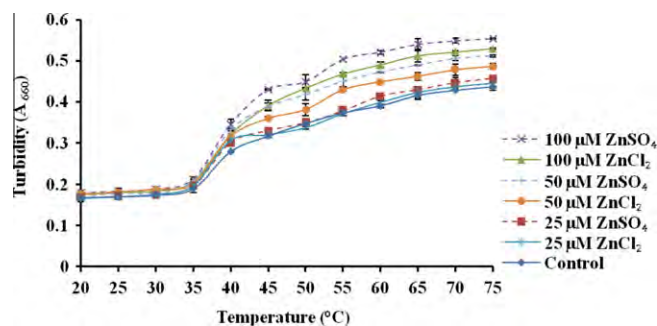


Fig. 1. Turbidity of NAM solutions (1 mg protein/ml) containing  $\text{ZnSO}_4$  or  $\text{ZnCl}_2$  at different concentrations during heating from 20 to 75  $^{\circ}\text{C}$ . Heating rate was 0.65  $^{\circ}\text{C}/\text{min}$ . Bars represent the standard deviation ( $n = 3$ ).

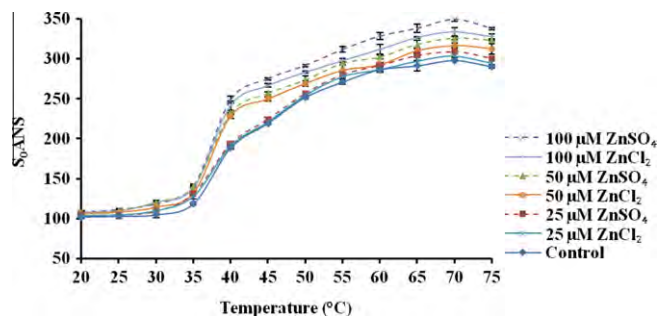


**Fig. 2.** Surface hydrophobicity of NAM solutions (1 mg protein/ml) containing  $\text{ZnSO}_4$  or  $\text{ZnCl}_2$  at different concentrations during heating from 20 to 75 °C. Heating rate was 0.65 °C/min. Bars represent the standard deviation ( $n = 3$ ).

concentrations heated from 20 to 75 °C is illustrated in Fig. 2.  $S_0\text{ANS}$  of control NAM increased continuously after heating at a temperature above 35 °C up to 70 °C ( $P < 0.05$ ). The increase in  $S_0\text{ANS}$  indicated the exposure of hydrophobic domains of NAM during heating. Addition of either  $\text{ZnSO}_4$  or  $\text{ZnCl}_2$  at 50 and 100 μM further increased  $S_0\text{ANS}$ , particularly when the temperatures were above 35 °C ( $P < 0.05$ ). This confirmed that  $\text{Zn}^{2+}$  in solution could induce the conformational change of NAM to a higher extent, particularly when more exposure of reactive group was favoured at higher temperatures. At temperature above 70 °C, slight decreases in  $S_0\text{ANS}$  were observed for all samples, suggesting that an hydrophobic interaction between proteins molecules most likely took place. ANS, a fluorescence probe, has been found to bind to hydrophobic amino acids containing an aromatic ring, such as phenylalanine and tryptophan, and can be used to indicate the conformational changes occurring in the proteins (Benjakul et al., 1997). The increase in ANS binding could be due to either the presence of exposed hydrophobic sites on unfolded, not aggregated proteins, or unfolded and aggregated proteins. The exposure of hydrophobic domains has been suggested as a prerequisite for the formation of large myosin aggregates via hydrophobic interaction (Chan, Gill, & Paulson, 1992b). Upon heating, the aromatic hydrophobic amino acid residues could be exposed to a greater extent (Visessanguan, Ogawa, Nakai, & An, 2000). In the presence of zinc salts at higher concentration, the attachment of  $\text{Zn}^{2+}$  with protein molecules could be augmented. As a result, the alteration of protein conformation could be increased as indicated by the higher  $S_0\text{ANS}$  ( $P < 0.05$ ). The rate of the increase in  $S_0\text{ANS}$  of NAM containing  $\text{ZnSO}_4$  was higher than those containing  $\text{ZnCl}_2$ , when the same concentration was used. This might be due to the higher reactivity of  $\text{ZnSO}_4$  on binding or interacting with proteins, in which the exposure of hydrophobic domain was favoured. The effect of other divalent cations such as  $\text{Ca}^{2+}$  on the surface hydrophobicity was also reported in other proteins. The addition of 1–15 mM  $\text{CaCl}_2$  induced structural changes in  $\beta$ -lactoglobulin, resulting in an increased hydrophobicity (Jeyarajah & Allen, 1994). Thus, the aggregation of NAM by both ionic and hydrophobic interaction could be more induced when  $\text{ZnSO}_4$  or  $\text{ZnCl}_2$  was incorporated. The result was in agreement with a higher turbidity of NAM when zinc salts were added (Fig. 1). The result suggested that Zn divalent was able to improve the gel strength of surimi via enhancing hydrophobic interaction, apart from ionic interaction or salt bridges.

### 3.1.3. Total sulphydryl group and disulphide bond contents

The total sulphydryl (SH) group content and disulphide bond formation of NAM with or without the addition of  $\text{ZnSO}_4$  or  $\text{ZnCl}_2$  at various concentrations during heating from 20 to 75 °C is shown in Fig. 3. The total SH content of control NAM decreased continu-

ously after heating at a temperature above 35 °C. In the presence of  $\text{ZnSO}_4$  or  $\text{ZnCl}_2$  at all concentrations, a higher decrease in the total SH content was observed, especially at temperatures above 35 °C ( $P < 0.05$ ). However, the rate of decrease in total SH content was higher in NAM with  $\text{ZnSO}_4$ , compared with those with  $\text{ZnCl}_2$ , when the same concentration was used. The total SH content of the NAM solutions decreased with a concomitant increase in the disulphide bond formation, particularly at temperatures above 40 °C (Fig. 3B). However, no differences in the total SH group content and disulphide bond content were found in NAM added with either  $\text{ZnSO}_4$  or  $\text{ZnCl}_2$  at a level of 25 μM, compared with those of the control ( $P > 0.05$ ). The elevated temperature most likely resulted in the enhanced oxidation of sulphydryl groups with the accompanied disulphide bond formation. Disulphide bond formation of fish actomyosin and myosin occurred at 40 and 45 °C, respectively (Niwa, 1992). Yarnpakdee, Benjakul, Visessanguan, and Kijroongrojana (2009) reported that the disulphide bond formation in goatfish actomyosin required a temperature above 40 °C.

Zinc salts might induce the conformational change of NAM, in which sulphydryl groups might be exposed to a higher extent and favoured the oxidation process. This was evidenced by the decrease in the total SH group content with a concomitant increase in the disulphide bond formation of NAM as the concentration of zinc salts increased ( $P < 0.05$ ). Mathew et al. (2009) reported that the addition of zinc led to a decrease in the free SH group content of actomyosin. A decrease in the total SH group content was reported to be due to the formation of disulphide bonds through the oxidation of SH group or disulphide interchanges (Hayakawa & Nakai, 1985). An inter-molecular disulphide bond is formed by the oxidation of two cysteine molecules on adjacent protein chains (Lanier, 2000). At the same concentration, the presence of  $\text{ZnSO}_4$  resulted in a higher formation of disulphide bond of NAM. The result was in accordance with a higher decrease in the SH group content. The formation of disulphide bond correlated well with the turbidity of the NAM solution (Fig. 1). The result suggested that a stronger gel of surimi could be obtained via an increased formation of disulphide bond in the presence of zinc divalent. The change of

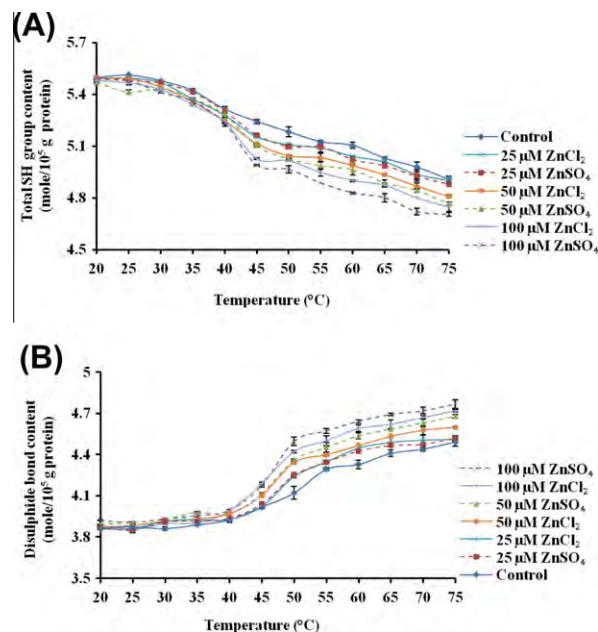


**Fig. 3.** Total sulphydryl group content (A) and disulphide bond content (B) of NAM solutions (1 mg protein/ml) containing  $\text{ZnSO}_4$  or  $\text{ZnCl}_2$  at different concentrations during heating from 20 to 75 °C. Heating rate was 0.65 °C/min. Bars represent standard deviation ( $n = 3$ ).

**Table 1**Ca<sup>2+</sup>-ATPase activity of NAM solutions (1 mg protein/ml) containing ZnSO<sub>4</sub> or ZnCl<sub>2</sub> at different concentrations after heating from 20 to 75 °C.

| Temperature (°C) | Control                    | ZnSO <sub>4</sub> (μM) |                |               | ZnCl <sub>2</sub> (μM) |                |               |
|------------------|----------------------------|------------------------|----------------|---------------|------------------------|----------------|---------------|
|                  |                            | 25                     | 50             | 100           | 25                     | 50             | 100           |
| 20               | 0.19 ± 0.02aA <sup>*</sup> | 0.18 ± 0.01aAB         | 0.15 ± 0.02aBC | 0.13 ± 0.01aC | 0.18 ± 0.02aAB         | 0.15 ± 0.02aBC | 0.12 ± 0.01aC |
| 30               | 0.13 ± 0.03bA              | 0.12 ± 0.02bA          | 0.10 ± 0.03bAB | 0.07 ± 0.01bC | 0.12 ± 0.02bA          | 0.10 ± 0.01bAB | 0.08 ± 0.03bC |
| 40               | 0.06 ± 0.02cA              | 0.05 ± 0.01cAB         | 0.04 ± 0.01cB  | 0.01 ± 0.01cC | 0.05 ± 0.02cAB         | 0.04 ± 0.01cB  | 0.01 ± 0.03cC |
| 50               | ND <sup>†</sup>            | ND                     | ND             | ND            | ND                     | ND             | ND            |
| 60               | ND                         | ND                     | ND             | ND            | ND                     | ND             | ND            |
| 70               | ND                         | ND                     | ND             | ND            | ND                     | ND             | ND            |

Different lowercase letters in the same column indicate significant differences ( $P < 0.05$ ).Different uppercase letters in the same row indicate significant differences ( $P < 0.05$ ).<sup>\*</sup> Values are given as mean ± SD ( $n = 3$ ).<sup>†</sup> ND: Not detectable.

actomyosin conformation correlates with the exposure of functional groups such as sulphhydryl groups. Subsequently, those groups most likely undergo disulphide bond formation (Benjakul et al., 2001).

### 3.1.4. Ca<sup>2+</sup>-ATPase Activity

The effect of ZnSO<sub>4</sub> and ZnCl<sub>2</sub> at various concentrations on the Ca<sup>2+</sup>-ATPase activity of NAM as a function of heating temperature is shown in Table 1. No Ca<sup>2+</sup>-ATPase activity of NAM was detected when heated at temperatures above 40 °C. The Ca<sup>2+</sup>-ATPase activity of NAM decreased continuously as the temperature increased from 20 to 40 °C ( $P < 0.05$ ). In the presence of ZnSO<sub>4</sub> or ZnCl<sub>2</sub>, the decrease in Ca<sup>2+</sup>-ATPase activity was more pronounced with increasing concentrations of ZnSO<sub>4</sub> or ZnCl<sub>2</sub>. At 50 and 100 μM, both ZnSO<sub>4</sub> and ZnCl<sub>2</sub> caused a marked loss in the Ca<sup>2+</sup>-ATPase activity. The inhibitory effect of 100 μM zinc salts was more profound when the temperatures were 30 and 40 °C ( $P < 0.05$ ). Foege-ding, Lanier, and Hultin (1996) reported that Ca<sup>2+</sup>-ATPase activity is a sensitive indicator of myosin denaturation. Ca<sup>2+</sup>-ATPase is also used as a good indicator of the integrity of myosin molecule (Benjakul, Visessanguan, Aewsiri, Tanaka, & Nikoo, 2011; Benjakul et al., 1997). Mathew et al. (2009) reported the complete inhibition of ATPase activity of actomyosin by zinc salt at a concentration of 1 mM. Prevalent Zn<sup>2+</sup> might induce the denaturation of Ca<sup>2+</sup>-ATPase by inducing the conformational change of myosin, especially at head portion. The SH groups located in the head portion (SH1 and SH2) play an essential role in the Ca<sup>2+</sup>-ATPase activity (Kielley & Bradley, 1956). The result indicated that zinc salts at higher concentrations were effective in altering the conformation of myosin. Conformational changes and aggregation between myosin, particularly at head portions, induced by Zn<sup>2+</sup> was postulated to cause a decrease in the Ca<sup>2+</sup>-ATPase activity. Therefore, zinc in conjunction with increasing temperature could induce the denaturation of NAM and the denatured proteins could further undergo aggregation.

## 3.2. Effect of ZnSO<sub>4</sub> and ZnCl<sub>2</sub> on thermal transition, charge, size and microstructure of NAM

### 3.2.1. Thermal transition

Transition temperature ( $T_{max}$ ) and enthalpy ( $\Delta H$ ) of NAM from yellow stripe trevally in the presence of ZnSO<sub>4</sub> or ZnCl<sub>2</sub> at a concentration of 100 μM and the control NAM are shown in Table 2. The control NAM showed two major endothermic peaks with  $T_{max}$  of 47.83 and 64.15 °C, respectively. The first and second peaks were postulated to be the transitions of myosin and actin, respectively (Hastings, Rodger, Park, Matthews, & Anderson, 1985). The observed  $T_{max}$  of myosin and actin were in the temperature range reported for various fish species. Yarnpakdee et al. (2009) reported

that NAM from goatfish had two endothermic transitions, with the  $T_{max}$  of myosin and actin found at 47.4 and 63.5 °C, respectively. Fresh bigeye snapper (*Priacanthus tayenus*) muscle proteins had two major endothermic peaks, with the  $T_{max}$  at 47.7 and 70.6 °C, and  $\Delta H$  values of 1.42 and 0.69 J/g, respectively (Benjakul, Visessanguan, & Leelapongwattana, 2002). Myosin and actin of Japanese stingfish had  $T_{max}$  of 40.9 and 61.1 °C, respectively (Nagai et al. 1999). Whole cod muscle showed two maximal transitions on the DSC thermogram, with the  $T_{max}$  at about 45 and 75 °C (Hastings et al., 1985), and whole muscle of fresh hake also showed two endothermic transitions with the  $T_{max}$  values of 46 and 75 °C (Beas, Wagner, Crupkin, & Anon 1990). In the presence of ZnSO<sub>4</sub> or ZnCl<sub>2</sub>, the  $T_{max}$  of myosin shifted to the lower temperatures of 46.05 and 46.49 °C, respectively. The coincidental decreases in  $\Delta H$  were observed for both peaks ( $P < 0.05$ ). Lower  $T_{max}$  and  $\Delta H$  indicated that myosin became less thermally stable in the presence of ZnSO<sub>4</sub> or ZnCl<sub>2</sub>. However, the  $T_{max}$  and  $\Delta H$  of actin were not affected by both ZnSO<sub>4</sub> and ZnCl<sub>2</sub> ( $P > 0.05$ ). The result suggested that myosin might undergo a conformational change when ZnSO<sub>4</sub> or ZnCl<sub>2</sub> was incorporated, while those salts have no impact on the actin structure. Generally, myosin was unstable and easily irreversibly denatured during both handling and processing. Actin, on the other hand, was more stable and its denaturation was largely reversible (Hastings et al. 1985). Zn<sup>2+</sup> more likely interacted with the negatively charged residues of myosin molecules. As a consequence, the configurational change could be induced. This resulted in an ease in the thermal denaturation of myosin with a subsequent increased aggregation. It was presumed that myosin with less thermal stability could undergo the denaturation, followed by aggregation with ease. As a consequence, the protein network could be formed more effectively, especially in the presence of Zn divalent. Thermodynamic stability, charge distribution and hydrophobic characteristics are important to the structure function relationship of proteins (Damodaran, 1994). Different conformational changes and protein–protein interactions of myofibrillar proteins occurred during gelation (Chawla et al., 1996).

### 3.2.2. Zeta potential and particle size

The Zeta potential ( $\zeta$ ) values of the surface charge of NAM containing ZnSO<sub>4</sub> or ZnCl<sub>2</sub> at a level of 100 μM after heating to 75 °C, and of the control NAM were determined (Table 3). The highest negative charge was found in the control ( $P < 0.05$ ). The NAM solutions became less negatively charged when ZnSO<sub>4</sub> or ZnCl<sub>2</sub> at 100 μM were incorporated ( $P < 0.05$ ). The negative charge of the proteins was governed by acidic amino acids like aspartic acid and glutamic acid in the NAM at a neutral pH. When NAM was heated, the proteins were more unfolded, exposing the charged amino acids. Zn<sup>2+</sup> ion might neutralise the negative charge of the protein during heating, as indicated by the lowered negative charge, as ZnSO<sub>4</sub> or ZnCl<sub>2</sub> was added (Table 3). This behaviour

**Table 2**

Thermal transition ( $T_{\max}$ ) and enthalpy ( $\Delta H$ ) of NAM containing 100  $\mu\text{M}$   $\text{ZnSO}_4$  or 100  $\mu\text{M}$   $\text{ZnCl}_2$ .

| Treatment | Peak I                        |                              | Peak II                       |                              |
|-----------|-------------------------------|------------------------------|-------------------------------|------------------------------|
|           | $T_{\max} (^{\circ}\text{C})$ | $\Delta H$ (J/g protein)     | $T_{\max} (^{\circ}\text{C})$ | $\Delta H$ (J/g protein)     |
| Control   | 47.83 $\pm$ 0.30 <sup>a</sup> | 1.07 $\pm$ 0.03 <sup>a</sup> | 64.15 $\pm$ 0.36 <sup>a</sup> | 0.23 $\pm$ 0.02 <sup>a</sup> |
| ZC        | 46.49 $\pm$ 0.49 <sup>b</sup> | 0.67 $\pm$ 0.04 <sup>b</sup> | 63.71 $\pm$ 0.44 <sup>a</sup> | 0.19 $\pm$ 0.03 <sup>a</sup> |
| ZS        | 46.05 $\pm$ 0.36 <sup>b</sup> | 0.63 $\pm$ 0.02 <sup>b</sup> | 63.60 $\pm$ 0.35 <sup>a</sup> | 0.20 $\pm$ 0.04 <sup>a</sup> |

Different lowercase letters in the same column indicate significant differences ( $p < 0.05$ ).

<sup>a</sup> Values are given as mean  $\pm$  SD ( $n = 3$ ).

**Table 3**

Zeta potential and particle size of NAM solutions (1 mg protein/ml) containing 100  $\mu\text{M}$   $\text{ZnSO}_4$  or 100  $\mu\text{M}$   $\text{ZnCl}_2$  after heating to 75  $^{\circ}\text{C}$ .

| Treatment | Zeta potential (mV)            | Particle size (nm)               |
|-----------|--------------------------------|----------------------------------|
| Control   | −14.41 $\pm$ 0.64 <sup>c</sup> | 710.78 $\pm$ 25.68 <sup>c</sup>  |
| ZC        | −2.67 $\pm$ 0.32 <sup>b</sup>  | 1242.37 $\pm$ 34.78 <sup>b</sup> |
| ZS        | −0.92 $\pm$ 0.47 <sup>a</sup>  | 1558.89 $\pm$ 33.62 <sup>a</sup> |

Different lowercase letters in the same column indicate significant differences ( $P < 0.05$ ).

<sup>a</sup> Values are given as mean  $\pm$  SD ( $n = 3$ ).

could be attributed to ionic interactions between protein molecules and  $\text{Zn}^{2+}$  ions. Ding et al. (2011) reported that divalent metal ions might cause changes in protein conformation and interact with negative charges on the polypeptide chains of proteins and induce cross-linking by the formation of salt bridges. However, the higher decrease in negative charge was observed in NAM added with  $\text{ZnSO}_4$ , compared with  $\text{ZnCl}_2$  ( $P < 0.05$ ). The higher drop in negative charge by  $\text{ZnSO}_4$  was likely due to the greater screening effect of  $\text{ZnSO}_4$  on the negatively charged carboxyl groups of proteins (Twomey, Keogh, Mehra, & O'Kennedy, 1997). With the addition of zinc salts, progressive decreases in the electronegativity of protein molecules with a lowered electrostatic repulsion between protein molecules could occur. This resulted in the induced protein aggregation as evidenced by the increased turbidity of NAM incorporated with both zinc salts (Fig. 1).

The average particle sizes of control NAM solution, and the NAM solution containing  $\text{ZnSO}_4$  or  $\text{ZnCl}_2$  at a level of 100  $\mu\text{M}$  after heating to 75  $^{\circ}\text{C}$ , are shown in Table 3. The particle size of NAM increased from 710.78 nm for the control to 1242.37 and 1558.89 nm in the presence of  $\text{ZnCl}_2$  and  $\text{ZnSO}_4$ , respectively ( $P < 0.05$ ). The increase in particle sizes of NAM was in accordance with the increase in turbidity (Fig. 1). The aggregation of the heated protein molecules not only affected the size of particulates formed, but also had an impact on the surface charge of the aggregates. These results suggested that  $\text{Zn}^{2+}$  played a role in the modification of protein charge. This more likely determined the physicochemical changes of protein molecules, in which the aggregation of proteins was enhanced. With sufficient heat, proteins underwent unfolding. Simultaneously,  $\text{Zn}^{2+}$  was able to bind with protein molecules. As a consequence, the aggregation was enhanced as indicated by the increase in the particle size of the protein aggregate.

### 3.2.3. Microstructure

The microstructure of control NAM solution, the NAM solutions containing  $\text{ZnSO}_4$  or  $\text{ZnCl}_2$  at 100  $\mu\text{M}$  after heating to 75  $^{\circ}\text{C}$ , revealed that the aggregation of NAM occurred differently, as depicted in Fig. 4. A highly interconnected and denser network was developed in NAM with  $\text{ZnSO}_4$ , followed by NAM with  $\text{ZnCl}_2$  and the control, respectively. This was concomitant with the turbidity development, the increase in hydrophobicity and in disulphide

bond formation. It was noted that a looser protein network was formed in the control (Fig. 4C). Van den Berg, Rosenberg, VanBoekel, Rosenberg, and Van de Velde (2009) reported that aggregated protein constituents were engaged in network formation. The alignment of myofibrillar proteins as well as the aggregation pattern induced by  $\text{ZnSO}_4$  was different from those mediated by  $\text{ZnCl}_2$ . This might be due to the different reactivity of NAM towards both zinc salts. Gelation is the result of protein denaturation, followed by the aggregation via inter-molecular covalent bonds and noncovalent interactions, including disulphide bonds and hydrophobic interactions (Lee & Lanier, 1995). The heat treatment might provide sufficient energy for the unfolding of protein molecules, which allowed  $\text{Zn}^{2+}$  to interact easily and large aggregates were formed among actomyosin molecules by various bonds.  $\text{Zn}^{2+}$  also served

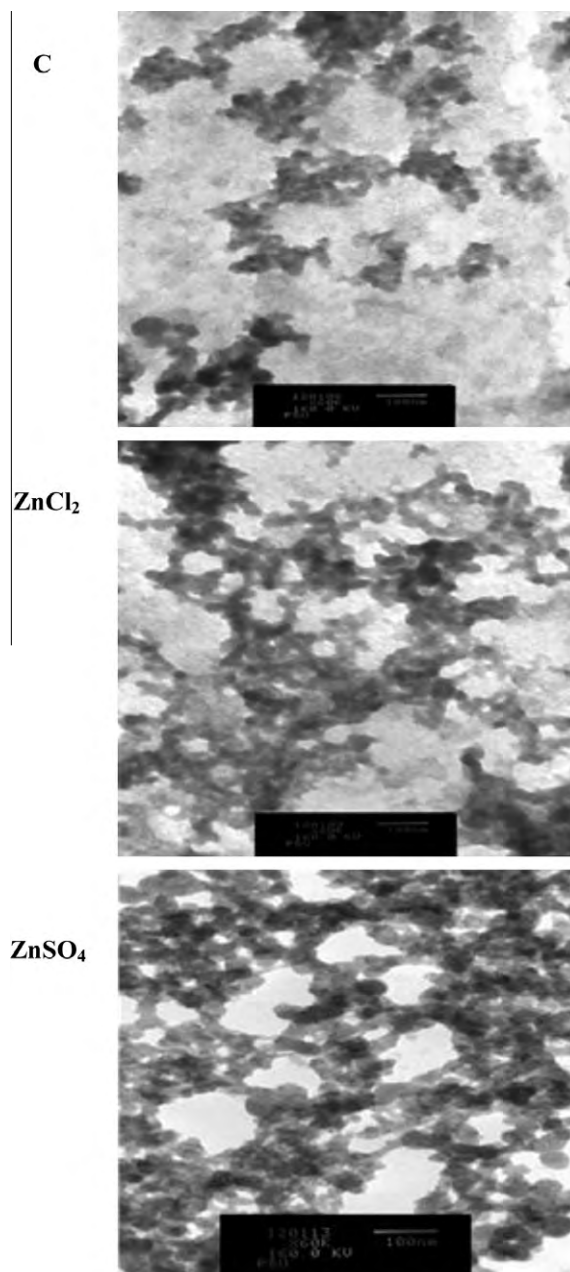


**Fig. 4.** Transmission electron micrograph of NAM solutions containing 100  $\mu\text{M}$   $\text{ZnSO}_4$  or 100  $\mu\text{M}$   $\text{ZnCl}_2$  after heating at 0.65  $^{\circ}\text{C}/\text{min}$  to 75  $^{\circ}\text{C}$ . C: control NAM solution (without zinc salt). Heated NAM was cooled rapidly in iced water prior to TEM analysis. Magnification: 60,000 $\times$ .

as the contributor in bridging between negatively charged groups on adjacent unfolded protein molecules. The result indicated that  $\text{Zn}^{2+}$  was able to induce the aggregation of NAM, but the degree of aggregation varied, depending on the zinc salt.

#### 4. Conclusion

Heat-induced aggregation of NAM solution was governed by the heating temperature, type and concentration of zinc salts. The presence of  $\text{ZnSO}_4$  was associated with the higher aggregation of NAM, than with  $\text{ZnCl}_2$ , mainly through the induction of higher hydrophobic interactions, and disulphide bond and ionic interactions. Salts bridge, mediated by  $\text{Zn}^{2+}$  plays a role in the aggregation of NAM. Therefore, zinc salts, particularly  $\text{ZnSO}_4$ , can serve as a promising additive to improve the aggregation of muscle proteins, thereby strengthening surimi gels.

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# Indigenous proteases in the skin of unicorn leatherjacket (*Aluterus monoceros*) and their influence on characteristic and functional properties of gelatin

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## ABSTRACT

Indigenous proteases in the skin of unicorn leatherjacket (*Aluterus monoceros*) were characterised using autolytic study. Maximised autolysis was found at pH 7 and 50 °C. Autolysis was markedly inhibited by 0.04 mM soybean trypsin inhibitor (SBTI), suggesting that heat activated serine protease was predominant in the skin. The impact of indigenous proteases on the properties of gelatin extracted from unicorn leatherjacket skin was investigated. Gelatin was extracted from unicorn leatherjacket skin using distilled water at 50 °C for 12 h in the presence and absence of 0.04 mM SBTI. In the presence of SBTI, the degradation was markedly inhibited, but a lower gelatin extraction yield was obtained ( $P < 0.05$ ). Extracted gelatins contained  $\alpha_1$  and  $\alpha_2$  chains as the predominant components with some degradation peptides. FTIR spectra indicated a greater loss of molecular order of the triple helix and a higher degradation was found in gelatin extracted in the absence of 0.04 mM SBTI. The net charge of gelatin samples extracted with and without 0.04 mM SBTI became zero at pHs of 8.45 and 7.31, respectively, as determined by  $\zeta$ -potential titration. Higher gel strength ( $320.68 \pm 3.02$  g) was obtained in gelatin extracted with SBTI, compared with that of gelatin extracted without SBTI ( $288.63 \pm 1.44$  g). High emulsifying activity index but lower emulsifying stability index was observed in the former. Therefore, heat-activated serine protease was involved in the degradation of gelatin molecules, thereby affecting the yield, proteinaceous components and properties of gelatin from unicorn leatherjacket skin.

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## 1. Introduction

Proteolysis induced by heat-activated and heat-stable indigenous proteases associated with skin matrix can contribute to the destabilisation as well as disintegration of collagen structure by disrupting the intra- and intermolecular cross-links (Wu et al., 2008). Collagenases have the unique ability to degrade the major structural extracellular matrix proteins, especially the triple helical strand of type I and type II collagens at Gly<sup>775</sup>-Leu (Ile)<sup>776</sup>. After these initial and specific cleavages,  $\frac{3}{4}$ - and  $\frac{1}{4}$ -cleavage products are formed, and collagen is spontaneously denatured (helix-to-coil transition) to gelatin (Miller et al., 1976). Collagenases are classified into two major groups, metallocollagenases and serine collagenases (Aoki, Ahsan, Matsuo, Hagiwara, & Watabe, 2003). Furthermore, non-collagenase proteinases can cleave the collagen molecule in the telopeptide region and contribute to hydrolysis of the collagen molecule by disrupting the regions, in which intermolecular cross-links are formed (Bornstein & Traus, 1979).

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Heat-activated serine protease in bigeye snapper skin was involved in the drastic degradation of the  $\beta$ - and  $\alpha$ -chains of the gelatin extracted at 60 °C (Intarasirisawat et al., 2007). These enzymes are bound with matrix components such as collagens (Woessner, 1991). The proteolytic degradation of high molecular weight components caused by indigenous proteases during extraction of gelatin at high temperature resulted in adverse effects on gel-forming properties of resulting gelatin (Intarasirisawat et al., 2007). The proteolytic breakdown of collagen structure is most likely related to the disintegration of connective tissues, which has been implicated in quality deterioration of products. Gelatin structures with large amount of high molecular weight components, like  $\gamma$ -,  $\beta$ , and  $\alpha$ -chains, have been known to possess the maximal functional properties including gelation (Kittiphattanabawon, Benjakul, Visessanguan, & Shahidi, 2010), film forming ability (Hoque, Benjakul, & Prodpran, 2010), etc. Therefore, it was necessary to produce the gelatin with negligible hydrolysis of peptides for further applications in pharmacy, medicine and food industry. Hence, the use of an appropriate protease inhibitor can be an effective means to obtain the gelatin with limited or negligible hydrolysis of peptides. Nevertheless, no information regarding the characteristics of indigenous proteases in the skin of unicorn

leatherjacket (*Alutherus monoceros*) and their role in properties of gelatin extracted has been reported. Unicorn leatherjacket is an economically important species commonly used for frozen fillet production. Thick skin of this species, generated during deskinning process, can serve as the promising raw material for gelatin extraction. Therefore, the objectives of this investigation were to study the autolysis of skin from unicorn leatherjacket and to elucidate their impact on gelatin extraction and characteristics of resulting gelatin.

## 2. Materials and methods

### 2.1. Chemicals

2,4,6-Trinitrobenzenesulfonic acid (TNBS), sodium sulphite, L-leucine, bovine serum albumin, ethylenediaminetetraacetic acid (EDTA), 1,10-phenanthroline monohydrate, ethylene-bis (oxyethylenitrilo) tetraacetic acid (EGTA), *trans*-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E-64), iodoacetic acid, pepstatin A, phenylmethanesulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI),  $\beta$ -mercaptoethanol ( $\beta$ -ME) and type I collagen from calf skin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Folin–Ciocalteu's phenol reagent and phosphoric acid were purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS), *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) and Coomassie blue R-250 were procured from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals were of analytical grade.

### 2.2. Collection and preparation of fish skin

The skin of unicorn leatherjacket (*A. monoceros*) was obtained from Sea Wealth Frozen Food Co., Ltd., Songkhla, Thailand. Upon arrival to the Department of Food Technology, Prince of Songkla University, Hat Yai, the skin was cleaned and washed with iced tap water (0–2 °C). Prepared skin was then cut into small pieces (0.5 × 0.5 cm<sup>2</sup>), placed in polyethylene bags and stored at –20 °C until use. The storage time was less than 2 months.

### 2.3. Pretreatment of unicorn leatherjacket skin

Prepared skin was pretreated following the method of Ahmad and Benjakul (2010a), with a slight modification. To remove non-collagenous proteins, the prepared skin was mixed with 0.1 M NaOH at a skin/alkali solution ratio of 1:20 (w/v). The mixture was stirred for 6 h at 4 °C using an overhead mechanical stirrer (W20.n, IKA®-Werke GmbH & CO.KG, Stanfen, Germany) at a speed of 300 rpm. The alkali solution was changed every 2 h. The samples were then washed with iced tap water until neutral or faintly basic pH was obtained. Thereafter, the prepared skin was swollen by mixing the skins with 0.2 M phosphoric acid at a ratio of 1:10 (w/v). The mixture was stirred for 24 h at 4 °C. Finally, the swollen skin was washed thoroughly with iced tap water until neutral or faintly acidic pH of wash water was obtained. The pretreated skin was stored at –20 °C until use.

### 2.4. Autolysis study of pretreated unicorn leatherjacket skin

Prior to study, the frozen pretreated skin was powderised in liquid nitrogen using a blender (Model MX-T2GN, National, Taipei, Taiwan). The powder obtained was used for autolysis study.

#### 2.4.1. Temperature profile

Autolytic activity was assayed according to the method of Morrissey, Wu, Lin, and An (1993), with a slight modification. Pretreated skin powder (3 g) was added to 9 ml of McIlvaine's buffer

(0.2 M Na-phosphate and 0.1 M Na-citrate), pH 7.0. The mixture was homogenised for 2 min at a speed of 11,000 rpm using a homogeniser (Model T25 basic, IKA, Labortechnik, Selangor, Malaysia). The resulting homogenate was incubated at different temperatures (40, 45, 50, 55, 60, 70, 80 and 90 °C) for 30 min in a temperature-controlled water bath (Mettmert, Schwabach, Germany). Autolytic reaction was terminated by addition of 18 ml of 10% SDS (85 °C). The reaction mixture was further incubated at 85 °C for 1 h in a temperature-controlled water bath. The supernatant was obtained by centrifuging at 8500g for 15 min using a microcentrifuge (Model MIKRO20, Hettich Zentrifugen, Germany) and was subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis. Samples were also determined for free amino group content.

#### 2.4.2. pH profile

Autolytic activity of pretreated skin was determined at various pHs using different buffers at 50 °C for 30 min. Buffers used included 50 mM maleate buffer for pH 1; McIlvaine's buffer (0.2 M Na-phosphate and 0.1 M Na-citrate) for pHs 2–6, 50 mM Tris–HCl buffer for pHs 7–9 and 0.1 M Na<sub>2</sub>HPO<sub>4</sub>–Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> buffer for pH 10. After the incubation at 50 °C for 30 min, the autolysis was terminated and was monitored in the same manner as the temperature profile study.

#### 2.4.3. Inhibitor study

Pretreated skin powder (0.5 g) was homogenised with 1.5 ml of McIlvaine's buffer (pH 7) at a speed of 11,000 rpm for 2 min using a homogeniser. The homogenate was mixed with 2 ml of various protease inhibitors to obtain the different final concentrations. Those inhibitors included EDTA (10 mM), EGTA (10 mM), iodoacetic acid (10 mM), E-64 (1 mM), pepstatin A (1 mM) and 1,10-phenanthroline (10 mM), PMSF (1 mM) and SBTI (0.04 mM). The mixtures were allowed to stand in ice for 2 h, followed by incubation at optimal temperature (50 °C) for 30 min. The reaction was terminated by addition of 2 ml of 10% (w/v) SDS (85 °C). After complete solubilisation, the autolytic pattern was then analysed by using SDS–PAGE. The control was performed in the same manner, but deionised water was added instead of protease inhibitor solution.

#### 2.4.4. Determination of free amino group content

Free amino group content was determined following the method of Benjakul and Morrissey (1997). Properly diluted samples (125  $\mu$ l) were mixed thoroughly with 2.0 ml of 0.2125 M phosphate buffer, pH 8.2, followed by the addition of 1.0 ml of 0.01% TNBS solution. The mixtures were then placed in a temperature controlled water bath at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulphite. The mixtures were cooled down at room temperature for 15 min. The absorbance was measured at 420 nm using a double beam spectrophotometer (Model UV-1800, Shimadzu, Kyoto, Japan) and free amino group content was expressed in terms of L-leucine.

#### 2.4.5. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein patterns were determined using SDS–PAGE according to the method of Laemmli (1970) using a 4% stacking gel and a 7.5% separating gel. Samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol). Samples (15  $\mu$ g) were loaded onto the gel. After electrophoresis using 15 mA/gel (Mini Protein II, Bio-Rad, Hercules, CA, USA), the gel was stained with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. Type I collagen was used as the standard.

### 2.5. Study on the impact of indigenous proteases on extraction and characteristics of gelatin from unicorn leatherjacket skin

To inhibit indigenous serine proteases, the pretreated skin was mixed with 0.04 mM SBTI solution at a pretreated skin/SBTI solution ratio of 1:5 (w/v). The mixture was stirred for 24 h at 4 °C using an overhead mechanical stirrer at a speed of 150 rpm. Then, the mixtures in the absence and presence of 0.04 mM SBTI were incubated at 50 °C in a temperature-controlled water bath for 12 h with continuous stirring to extract the gelatin. The mixtures were then filtered using two layers of cheesecloth. The filtrates were further filtered using a Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England) with the aid of an electric aspirator, (Model VE-11, JEIO TECH, Korea). The resultant filtrate was freeze-dried using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lyngø, Denmark). Gelatins extracted in the absence and presence of 0.04 mM SBTI were referred to as GAI and GPI, respectively. Both gelatin samples were subjected to SDS-PAGE as previously described and further analyses were also conducted as follows:

#### 2.5.1. Fourier transform infrared spectroscopy (FTIR)

FTIR analysis was performed as per the method of Ahmad, Benjakul, and Nalinanon (2010). A Bruker Model EQUINOX 55 FTIR spectrometer (Bruker, Ettlingen, Germany) equipped with a deuterated L-alanine triglycine sulphate (DLATGS) detector was used. The horizontal attenuated total reflectance (HATR) accessory was mounted into the sample compartment. The internal reflection crystal (Pike Technologies, Madison, WI, USA), made of zinc selenide, had a 45° angle of incidence to the IR beam. Spectra were acquired at a resolution of 4 cm<sup>-1</sup> and the measurement range was 4000–650 cm<sup>-1</sup> (mid-IR region) at room temperature. Automatic signals were collected in 32 scans at a resolution of 4 cm<sup>-1</sup> and were normalised against a background spectrum recorded from the clean, empty cell at 25 °C. Analysis of spectral data was carried out using the OPUS 3.0 data collection software programme (Bruker, Ettlingen, Germany).

#### 2.5.2. ζ-Potential analysis

Gelatin samples were dissolved in distilled water at a concentration of 0.5 mg/ml. The mixtures were stirred at room temperature for 6 h. The ζ-potential of each sample (20 ml) was measured using a ζ-potential analyser (ZetaPALS, Brookhaven Instruments Co., Holtsville, NY, USA). ζ-Potential of samples, adjusted to different pHs with 1.0 M nitric acid or 1.0 M KOH using an autotitrator (BI-ZTU, Brookhaven Instruments Co., Holtsville, New York, USA), was determined. The pI was estimated from pH rendering ζ-potential of zero.

#### 2.5.3. Determination of gel strength

Gels of gelatin were prepared by the method of Fernandez-Daz, Montero, and Gómez-Guillén (2001), with a slight modification. Gelatin samples were dissolved in distilled water (60 °C) to obtain the final concentration of 6.67% (w/v). The solution was stirred until the gelatin was solubilised completely, followed by incubating the samples in a refrigerator at 10 °C for 18 h for gel maturation. The dimensions of the sample were 3 cm in diameter and 2.5 cm in height. The gel strength of the samples prepared at 10 °C was determined using a Model TA-XT2 Texture Analyser (Stable Micro System, Surrey, UK) with a load cell of 5 kN and equipped with a 1.27 cm diameter flat-faced cylindrical Teflon® plunger. The maximum force (in grams) was recorded when the penetration distance reached 4 mm. The speed of the plunger was 0.5 mm/s.

#### 2.5.4. Determination of gel microstructure

Microstructures of gelatin gels were visualised using a scanning electron microscope (SEM). Gelatin gels having a thickness of 2–3 mm were fixed with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 12 h. The samples were then rinsed with distilled water for 1 h and dehydrated in ethanol with a serial concentration of 50%, 70%, 80%, 90% and 100% (v/v). Dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA). The specimens were observed with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 15 kV.

#### 2.5.5. Determination of emulsifying properties

Emulsion activity index (EAI) and emulsion stability index (ESI) of gelatin samples were determined according to the method of Pearce and Kinsella (1978) as modified by Aewsiri, Benjakul, and Visessanguan (2009). Soy bean oil (2 ml) and gelatin solution (1% gelatin, 6 ml) were homogenised using a homogeniser at a speed of 20,000 rpm for 1 min. Emulsions were pipetted out at 0 and 10 min and 100-fold diluted with 0.1% SDS. The mixture was mixed thoroughly for 10 s using a vortex mixer (Scientific Industries, Inc., Bohemia, NY, USA).  $A_{500}$  of the resulting dispersion was measured using a spectrophotometer. EAI and ESI were calculated by the following equation:

$$\text{EAI (m}^2/\text{g)} = (2 \times 2.303 \times A \times \text{DF}) / l \phi C$$

where  $A = A_{500}$ , DF = dilution factor (100),  $l$  = path length of cuvette (m),  $\phi$  = oil volume fraction and  $C$  = protein concentration in aqueous phase (g/m<sup>3</sup>)

$$\text{ESI (min)} = A_0 / (A_0 - A_{10}) \times \Delta t$$

where  $A_0 = A_{500}$  at time of 0 min;  $A_{10} = A_{500}$  at time of 10 min and  $\Delta t = 10$  min.

### 2.6. Statistical analyses

All experiments were performed in triplicate and a completely randomised design (CRD) was used. Data were presented as means ± standard deviation and a probability value <0.05 was considered significant. Analysis of variance (ANOVA) was performed and the mean comparisons were done by *T*-test. Analysis was performed using SPSS 11.0 for Windows (SPSS Inc., Chicago, IL).

## 3. Results and discussion

### 3.1. Effect of temperature and pH on autolysis of pretreated skin

The autolytic degradation of pretreated skin at different temperatures, as monitored by free amino group content, is shown in Fig. 1a. The maximal autolytic activity of pretreated skin was observed at 50 °C as indicated by the highest free amino group content ( $P < 0.05$ ). However, a decrease in autolytic activity was found when the temperature was above 50 °C, suggesting the thermal denaturation of the indigenous proteases. When the protein patterns of pretreated skin, autolysed at different temperatures, were compared, the highest degradation of β-chain and α-chains was obtained at 50 °C (Fig. 1c). This was concomitant with the formation of degradation products with low molecular weight appearing at the dye front. Furthermore, the high molecular weight cross-linked proteins were also degraded at 50–70 °C. β- and α-chains constituted as the major proteins in the collagen from unicorn leatherjacket skin (Ahmad & Benjakul, 2010a). The results suggested that the proteases still remained within the skin after pretreatment with alkali and acid for non-collagenous protein removal and swelling processes, respectively. Those heat-activated

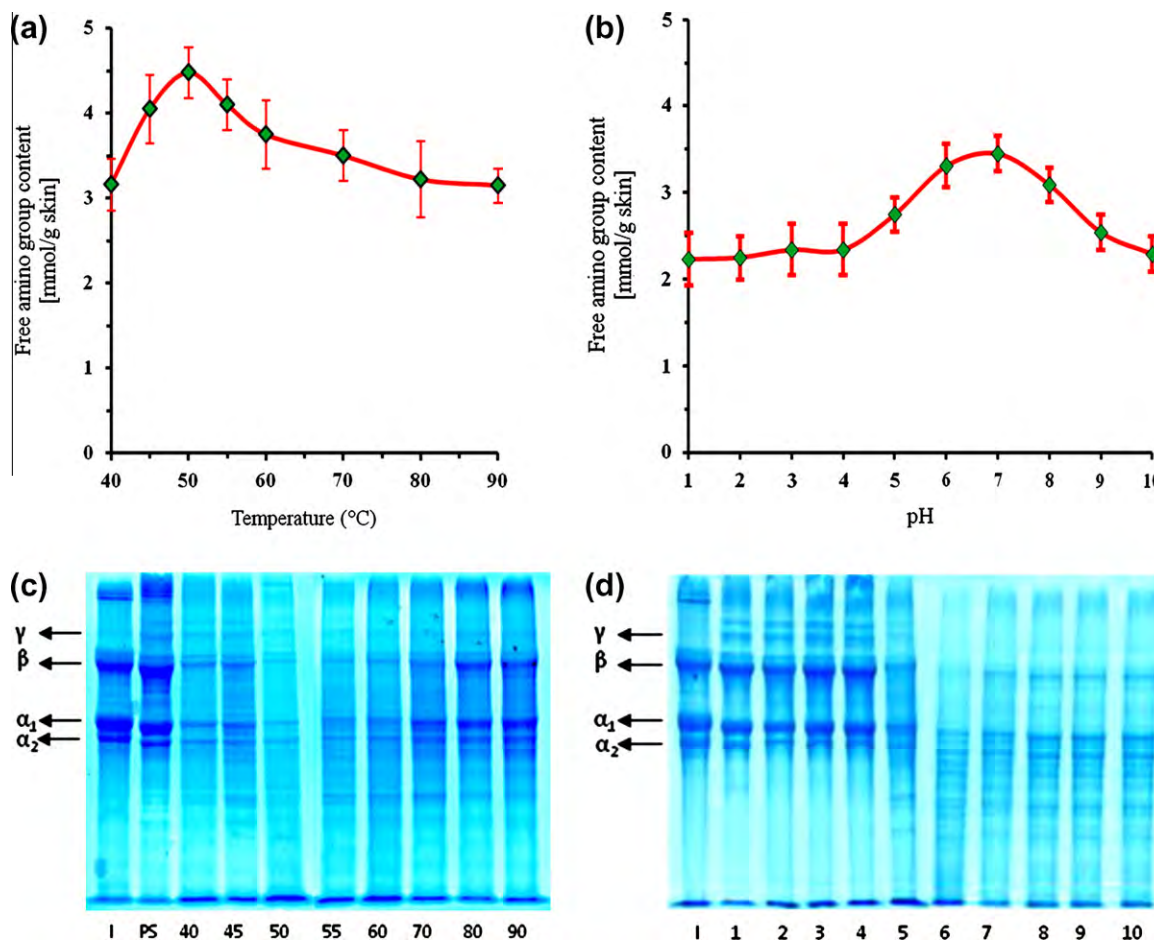


**Fig. 1.** pH and temperature profiles for autolysis of pretreated skin from unicorn leatherjacket. For the temperature profile, autolysis was performed at pH 7 for 30 min at different temperatures. For the pH profile, autolysis was conducted at 50 °C for 30 min at different pHs. For Fig. 1c and 1d; I: type I collagen from calf skin. For Fig. 1c; PS: pretreated skin. Numbers denote the temperatures (°C). For Fig. 1d; Numbers denote the pHs. Bars represent the standard deviation ( $n = 3$ ).

proteases were able to cleave  $\gamma$ -,  $\beta$ - and  $\alpha$ -chains most effectively at 50 °C as shown in Fig. 1c. Thus, those proteases more likely contributed to the disintegration of gelatin molecules during the extraction process, which is normally conducted at high temperature. Pretreatment removed not only the proteases to some degree, compared with the skin without pretreatment (data not shown). Nevertheless, the remaining proteases were shown to be involved in hydrolysis of collagenous components in the skin.

The pH profile for pretreated skin autolysis at 50 °C is shown in Fig. 1b. The highest free amino group content was obtained at pH 7.  $\gamma$ -,  $\beta$ - and  $\alpha$ -chains were almost degraded at pHs 6–7 with a concomitant formation of low-molecular-weight peptides (Fig. 1d). The results indicated that the neutral proteases were dominant in the pretreated unicorn leatherjacket skin, causing the hydrolysis of collagen molecules. At very acidic or alkaline pHs, the degradation was markedly decreased as evidenced by the low free amino group content and the greater band intensity of  $\beta$ - and  $\alpha$ -chains retained. This might be due to the inactivation of the indigenous protease under these conditions due to the conformational changes or ionisation of R-groups of amino acids, especially at the active site of proteases. Although collagen could be solubilised in acidic pH range (Ahmad & Benjakul, 2010a), the degradation was negligible in such a range, especially pH 1–4. Despite the presence of soluble substrates, no hydrolysis was noticeable. This reconfirmed the

inactivation of indigenous protease in an acidic pH range. On the other hand, the pronounced degradation was noticeable at neutral pH, which is commonly used for gelatin extraction.

### 3.2. Inhibitor study towards autolysis of pretreated skin

The effect of various inhibitors on autolysis of unicorn leatherjacket pretreated skin is depicted in Fig. 2. Among all protease inhibitors tested, 0.04 mM SBTI and 1 mM PMSF showed the highest inhibition towards autolysis when tested at pH 7, whilst other inhibitors, including EDTA, EGTA, E-64, iodoacetic acid, pepstatin A and 1,10-phenanthroline, had no inhibitory effect on autolysis.  $\gamma$ -,  $\beta$ - and  $\alpha$ -chains of pretreated skin were mostly retained in the presence of PMSF, whilst SBTI was found to be the most effective inhibitor towards the degradation of those components. PMSF irreversibly inhibits serine proteases by sulphonylation of the serine residue in the active site of the protease (James, 1978). The active site in each serine protease includes a serine residue, a histidine residue, and an aspartate residue (Johnson, Roderick, & Cook, 2005). Therefore, it can be inferred that a serine protease was the major protease associated with unicorn leatherjacket pretreated skin. In the absence of inhibitors, the pronounced hydrolysis was noticeable as evidenced by the marked decrease in the band intensity of  $\gamma$ -,  $\beta$ - and  $\alpha$ -chains. Intarasirisawat et al. (2007) reported that serine protease was the major enzyme causing the autolysis of skin from bigeye snapper.

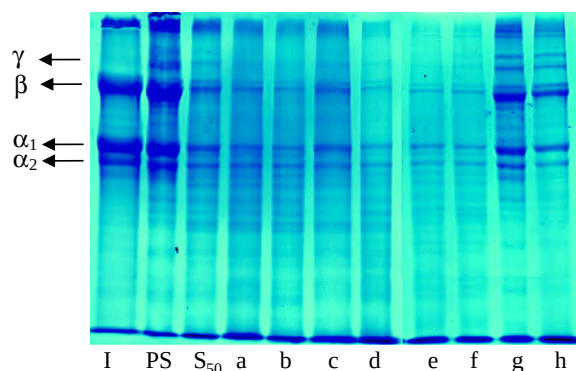


**Fig. 2.** SDS–PAGE pattern of pretreated skin from the unicorn leatherjacket incubated in the presence of various inhibitors: (a) 10 mM EDTA; (b) 10 mM EGTA; (c) 1 mM E-64; (d) 10 mM iodoacetic acid; (e) 10 mM 1,10-phenanthroline; (f) 1 mM pepstatin A; (g) 1 mM PMSF; (h) 0.04 mM SBTI; I: collagen type I from calf skin. PS: pretreated skin and  $S_{50}$ : control (without protease inhibitors). The autolysis was performed at pH 7.0 and 50 °C for 30 min.

### 3.3. Yield and characteristics of gelatin from pretreated skin as affected by protease inhibitor

#### 3.3.1. Yield

Yields of gelatin extracted from pretreated skin in the absence (GAI) and in the presence of 0.04 mM SBTI (GPI) at 50 °C for 12 h are 7.34% and 5.29% (wet weight basis), respectively. When SBTI was incorporated during the extraction, a decrease in the yield was obtained. SBTI most likely inhibited the activity of indigenous serine proteases associated with the skin matrix, thereby preventing the cleavage of peptide chains. This in turn retained the high molecular weight components in the skin matrix, leading to a lower amount of extractable gelatin. The degradation in pretreated skin without SBTI addition, particularly at temperature close to the optimal temperature (50 °C), might result in the formation of shorter peptides as well as loosening of the skin matrix. This possibly resulted in the ease of gelatin extraction. During heating at 50 °C, hydrogen bonds stabilising triple-helix of mother collagen were destroyed, leading to helix-to-coil transition (Wong, 1989). This resulted in the conversion of collagen to soluble gelatin. Therefore, the addition of protease inhibitor was shown to lower the extraction yield of gelatin from unicorn leatherjacket skin.

#### 3.3.2. Protein pattern

Based on protein pattern, GAI contained the lower band intensity of  $\beta$ - and  $\alpha$ -chains, in comparison with that found in the pretreated skin and GPI (Fig. 3). The  $\beta$ -chain was almost completely degraded in GAI, whereas,  $\alpha$ -chains were retained to some extent. The result suggested that  $\alpha$ -chains and cross-linked components of GPI were retained more when SBTI was incorporated during extraction at high temperature. With the extended extraction time (12 h), the remaining collagenolytic protease associated with skin might hydrolyse the collagen molecules progressively in GAI, especially at the optimal temperature (50 °C). In addition, proteins with molecular weights lower than  $\alpha$ -chains (100 kDa) were also observed in both GAI and GPI, suggesting the degradation of  $\alpha$ ,  $\beta$ - and  $\gamma$ -components during gelatin extraction caused by thermal hydrolysis or the proteolysis mediated by the remaining indigenous proteases. The result was in agreement with Muyonga, Cole, and Duodu (2004) who reported that gelatin extracted at high temperature contained more peptides with a molecular weight less than the  $\alpha$ -chains and a lower proportion of component having a molecular weight greater than the  $\beta$ -chain. The degradation of gelatin from bigeye snapper skin caused by some proteases during extraction process was also reported (Jongjareonrak, Benjakul,

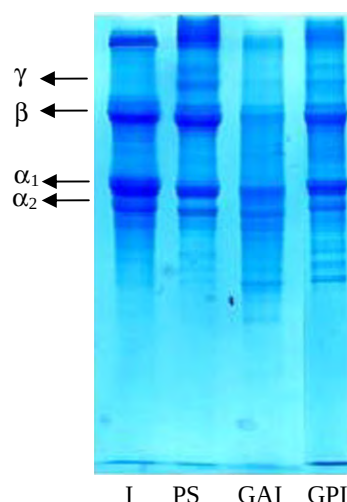


**Fig. 3.** SDS–PAGE pattern of gelatin extracted from the skin of unicorn leatherjacket in the absence (GAI) and in the presence of 0.04 mM of SBTI (GPI). I: collagen type I from calf skin. PS: pretreated skin.

Visessanguan, Prodpran, & Tanaka, 2006). The formation of degradation peptides is associated with the low viscosity, low melting point, low setting point, high setting time, as well as decreased bloom strength of gelatin (Muyonga et al., 2004). Therefore, the addition of SBTI effectively prevented the degradation of  $\beta$ - and  $\alpha$ -chains in gelatin from unicorn leatherjacket skin.

#### 3.3.3. FTIR spectra

FTIR spectra of GAI and GPI from the skin of unicorn leatherjacket are depicted in Fig. 4a. Spectra of both gelatins had the major peaks in the amide region, but showed slight differences in the spectra. These spectra were in accordance with those reported by Muyonga et al. (2004). GAI and GPI displayed the amide-I bands at the wavenumbers of 1635.89 and 1630.82  $\text{cm}^{-1}$ , respectively. The amide-I vibration mode resulted primarily from a C=O stretching vibration coupled to contributions from the CN stretch, CCN deformation and in-plane NH bending modes (Bandekar, 1992). The absorption peak at amide-I was characteristic for the coil structure of gelatin (Yakimets et al., 2005). The amide-I peak of GAI appeared at a higher wavenumber, compared to that of GPI. This indicated the greater loss of triple helix and enhanced hydrolysis of collagen caused by heat-activated indigenous proteases in the skin matrix. In addition, GPI showed the shift to a lower wavenumber and lower amplitude for the amide-I peak, compared to GAI. This was probably associated with the higher extent of molecular order due to interaction of C=O with adjacent chains via hydrogen bonds. This indicated that GPI retained more intermolecular cross-links during extraction at high temperature in the presence of SBTI. In the absence of SBTI, the triple helical structure, held by intermolecular hydrogen bonds, was destroyed to the higher extent. The result was in agreement with the higher degradation of GAI, in comparison with GPI (Fig. 3).

The characteristic absorption bands of GAI and GPI in the amide-II region were noticeable at the wavenumbers of 1545.82 and 1533.77  $\text{cm}^{-1}$ , respectively. The amide-II vibration modes are attributed to an out-of-plane combination of CN stretch and in-plane N–H deformation modes of the peptide group (Bandekar, 1992). A shift to a lower wavenumber and lower amplitude were found in GPI, compared to GAI, indicating that N–H was more involved in bonding with the adjacent molecules probably via H-bonding. In addition, amide-III was detected around the wavenumbers of 1236.53 and 1233.74  $\text{cm}^{-1}$  for GAI and GPI, respectively.

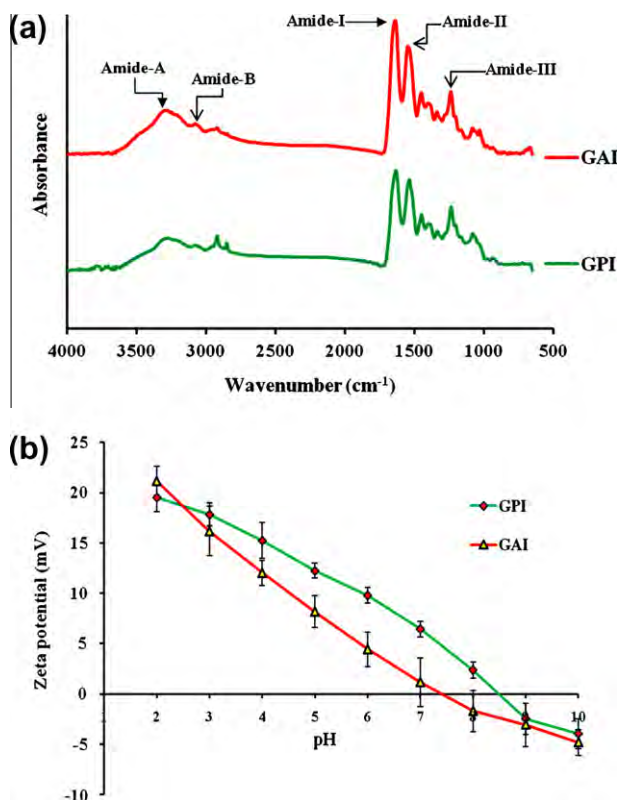


**Fig. 4.** Fourier transform infrared spectra (a) and  $\zeta$ -potential (b) at different pH of gelatin extracted from the skin of unicorn leatherjacket in the absence (GAI) and presence of 0.04 mM of SBTI (GPI). For Fig. 4b: Bars represent standard deviation ( $n = 3$ ).

The amide-III represented combination peaks between C–N stretching vibrations and N–H deformation from amide linkages, as well as absorptions arising from wagging vibrations from  $\text{CH}_2$  groups of the glycine backbone and proline side-chains (Jackson, Choo, Watson, Halliday, & Mantsch, 1995). The results suggested a disordered molecular structure for both GAI and GPI, probably due to the transformation of  $\alpha$ -helical to random coil structure during heating. Those changes were associated with the loss of the triple helix state as a result of denaturation of collagen to gelatin (Muyonga et al., 2004). In addition, absorption bands in GAI and GPI around the wavenumbers of 1080.57 and 1079.13  $\text{cm}^{-1}$ , respectively, most likely arose from asymmetric stretching vibrations of phosphate groups of phosphorylated proteins coupled to  $-\text{CH}_2$  of the amino acid residues (Jackson et al., 1995). In the present study, phosphoric acid was used for pretreatment of the skin, in which some phosphates were attached to gelatin molecules (Ahmad & Benjakul, 2010b).

The amide-A band, arising from the stretching vibrations of the N–H group, appeared at 3275.77 and 3276.24  $\text{cm}^{-1}$  for GAI and GPI, respectively. Normally, a free N–H stretching vibration is found in the range of 3400–3440  $\text{cm}^{-1}$  (Muyonga et al., 2004). When the N–H group of a peptide is involved in a hydrogen bond, the position is shifted to lower frequencies (Doyle, Blout, & Bendit, 1975). At the amide-A region, a slightly higher wavenumber was found in GPI, compared to GAI. However, a higher amplitude was found in GAI at the amide-A region, indicating the presence of N–H group of shorter peptide fragments. The amide-B was observed at 3075.29 and 3074.28  $\text{cm}^{-1}$  for GAI and GPI, respectively, corresponding to an asymmetric stretch vibration of  $=\text{C}-\text{H}$  and  $-\text{NH}_3^+$  (Ahmad & Benjakul, 2010b). GPI showed the lower wavenumber of amide-B peak, suggesting the interaction of  $-\text{NH}_3$  group between peptide chains. Peaks at the wavenumber of 2849.24

(symmetrical) and 2918.31  $\text{cm}^{-1}$  (asymmetrical), representing C–H stretching vibrations of the  $-\text{CH}_2$  groups, were found in both GAI and GPI spectra. However, the difference in the spectra between both samples in the region was probably due to the coupling of SBTI with gelatin (Chen et al., 2008; D'Souza, Devi, Shridhar, & Naik, 2008). Thus, it can be concluded that the secondary structure of gelatins obtained from the skin of unicorn leatherjacket was affected by the addition of SBTI during extraction process.

### 3.3.4. $\zeta$ -Potential

Zeta-potential ( $\zeta$ ) representing the surface charge of GAI and GPI at different pH is shown in Fig. 4b. A net charge of zero was obtained at pH 7.31 and 8.45, for GAI and GPI, respectively. At a pH near the isoelectric point, both GAI and GPI had decreased repulsion with increased interaction among gelatin molecules. Protein molecules in an aqueous system have zero net charge at their isoelectric points (pI), in which the positive charges are balanced out by the negative charges (Bonner, 2007). It was found that the  $\zeta$ -potential was higher for GPI in the pH range of 4–8. The differences in  $\zeta$ -potential, as well as pI between both gelatins, were most likely due to the differences in the amino acid compositions. Indigenous proteases present in the skin might enhance the extraction efficiency, in which a larger content of several peptides could be released. On the other hand, only a small amount of peptides, mainly those with higher MW, was extracted in the presence of SBTI. The result suggested that indigenous proteases involved in gelatin extraction had an impact on pI of the resulting gelatin to some degree.

### 3.3.5. Gel strength

The gel strength of GAI and GPI is shown in Table 1. GPI had a higher gel strength than GAI ( $P < 0.05$ ). The difference in gel strength might be governed by molecular weight distribution, as well as the aggregation between gelatin molecules. Low molecular weight peptides of GAI might not form the inter-junction zone effectively, leading to a lower aggregation of gelatin needed to form strong gel network. The quality of gelatin is generally determined by the gel strength or bloom value, including low (<150), medium (150–220) and high bloom (220–300) (Johnston-Bank, 1983). The configuration of polypeptide chains and the way in which the inter-junction was developed to form a stronger network were both crucial for gel formation (Ahmad & Benjakul, 2010b). The gelation of gelatin is considered to be due to a partial recovery of the triple helical structure in gelatin, in which regions rich in Gly-Pro-Hyp are of crucial importance for the stabilisation of the triple helices. In addition, acidic pretreatment also played a role in the determination of gel strength. Phosphate groups used for pretreatment might attach with some amino acids, leading to phosphorylation of both GAI and GPI (Ahmad & Benjakul, 2010b). Phosphorylation may introduce the ionic interaction between phosphate groups and  $-\text{NH}_3^+$  of amino acids, thereby increasing the aggregation (Guo, Li, Zhu, & Zhao, 2005). It was noted that

**Table 1**

Gel strength, colour and emulsifying properties of gelatin extracted from the skin of unicorn leatherjacket in the absence (GAI) and presence of 0.04 mM of SBTI (GPI).

|                               | GAI                | GPI                |
|-------------------------------|--------------------|--------------------|
| Gel strength (g)              | 288.63 $\pm$ 1.44b | 320.68 $\pm$ 3.02a |
| EAI ( $\text{m}^2/\text{g}$ ) | 30.21 $\pm$ 0.86b  | 34.32 $\pm$ 0.12a  |
| ESI (min)                     | 22.62 $\pm$ 0.38b  | 18.91 $\pm$ 1.10b  |
| $L^*$                         | 28.92 $\pm$ 1.28a  | 32.54 $\pm$ 2.20a  |
| $a^*$                         | 0.83 $\pm$ 0.02b   | 1.43 $\pm$ 0.11a   |
| $b^*$                         | −0.20 $\pm$ 0.04b  | 0.81 $\pm$ 0.15a   |

Mean  $\pm$  SD ( $n = 3$ ).

Different letters in the same row indicate the significant differences ( $p < 0.05$ ).

GPI had a higher gel strength than commercial bovine gelatin (293.22 g) ( $P < 0.05$ ) (Ahmad & Benjakul, 2010b). In general, fish gelatins have lower hydroxyproline content (about 7–10% of the total amino acids), compared with that of bovine gelatin (14%) (Nalinanon, Benjakul, Visessanguan, & Kishimura, 2008). The gelling properties of gelatin were also influenced by the source of raw materials, which vary in proline and hydroxyproline contents (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2006). Therefore, the use of SBTI, as a protease inhibitor during the gelatin extraction, markedly improved the gel strength of extracted gelatin. This was mainly due to the maintenance of chain length, which was a prerequisite for better gelation.

### 3.3.6. Gel colour

The colour of gel from both GAI and GPI is shown in Table 1. A higher  $L^*$ -value of GPI gel was observed, compared with GAI ( $P < 0.05$ ). The higher  $L^*$ -value of GPI was most likely associated with the greater interaction of higher molecular weight peptides,

in which the larger aggregates were formed. A larger aggregate more likely exhibited a greater light scattering effect. However, GPI had a higher  $b^*$ -value (yellowness) and  $a^*$ -value (redness) than those of GAI. Gelatin manufactures generally have a good method to clarify the impurities from the gelatin solution, such as chemical clarification and filtration processes. Generally, the colour did not affect functional properties of gelatin (Ockerman & Hansen, 1988).

### 3.3.7. Microstructure of gel

The microstructures of GAI and GPI gels are illustrated in Fig. 5. Both GAI and GPI gels were sponge or coral-like in structure. GPI gel had a denser and more irregular network of interconnected fibrous structure with smaller voids, compared with GAI gel. Larger voids were found in GAI and were possibly correlated with low aggregation of peptide chains during gelation. Generally, the arrangement and association of protein molecules in the gel matrix directly contributed to the gel strength of the gelatin (Benjakul, Oungbho, Visessanguan, Thiansilakul, & Roytrakul, 2009). The network of GAI gel with fewer inter-junctions might be disrupted easily by an applied force, resulting in lower gel strength. Therefore, the use of SBTI had an impact on the network of gelatin gel, in which chain length of peptide molecules were retained, and interaction or aggregation could take place more effectively.

### 3.3.8. Emulsifying properties

Emulsion activity index (EAI) and emulsion stability index (ESI) of GAI and GPI are presented in Table 1. At a gelatin concentration of 1%, EAI of GPI was higher than GAI ( $P < 0.05$ ). Conversely, ESI of GAI was higher than GPI ( $P < 0.05$ ). This indicated that there was a difference in emulsifying properties between GAI and GPI. GAI contained a higher content of low molecular weight peptides, which had more hydrophilicity and preferably localised in the aqueous phase. As a result, the lower portion of gelatin migrated to the oil–water interface, leading to the low emulsifying property. Nevertheless, emulsion containing GPI was less stable than that with GAI ( $P < 0.05$ ). Oil-in-water emulsion, prepared with high molecular weight fish gelatin (120 kDa) was more stable than that prepared with low molecular weight fish gelatin (50 kDa). The thickness of adsorbed gelatin film increased with increasing molecular weight (Ahmad & Benjakul, 2010b). This was associated with the increased stability of emulsions to coalescence during homogenisation (Lobo & Svereika, 2003). In the present study, bulky  $\alpha$ -,  $\beta$ - and  $\gamma$ -chains prevalent in GPI could not align themselves properly, where a strong film was formed effectively around an oil droplet. Additionally, GAI with higher degradation might have more charged groups, especially amino or carboxyl groups at the end of peptides, thereby having the ability to facilitate the stabilisation of emulsion via electrostatic repulsion. The stabilisation of emulsion against coalescence/flocculation is greatly dependent on the force of electrostatic repulsions between the adsorbed proteins on the interfacial protein film (Aewsiri et al., 2009). Differences in the intrinsic properties, composition and conformation of gelatins determined the functional properties of gelatin from the skin of two species of bigeye snapper (Benjakul et al., 2009).

## 4. Conclusion

Serine protease was the major enzyme in pretreated skin from unicorn leatherjacket and was involved in the drastic degradation of collagen/gelatin at high temperature, used for gelatin extraction. The addition of 0.04 mM SBTI during extraction could retain the  $\beta$ - and  $\alpha$ -chains in resulting gelatin via inhibition of indigenous proteases, in which the gel strength and emulsifying activity were increased. However, it is important to note that the extraction yield was lowered when SBTI was incorporated.

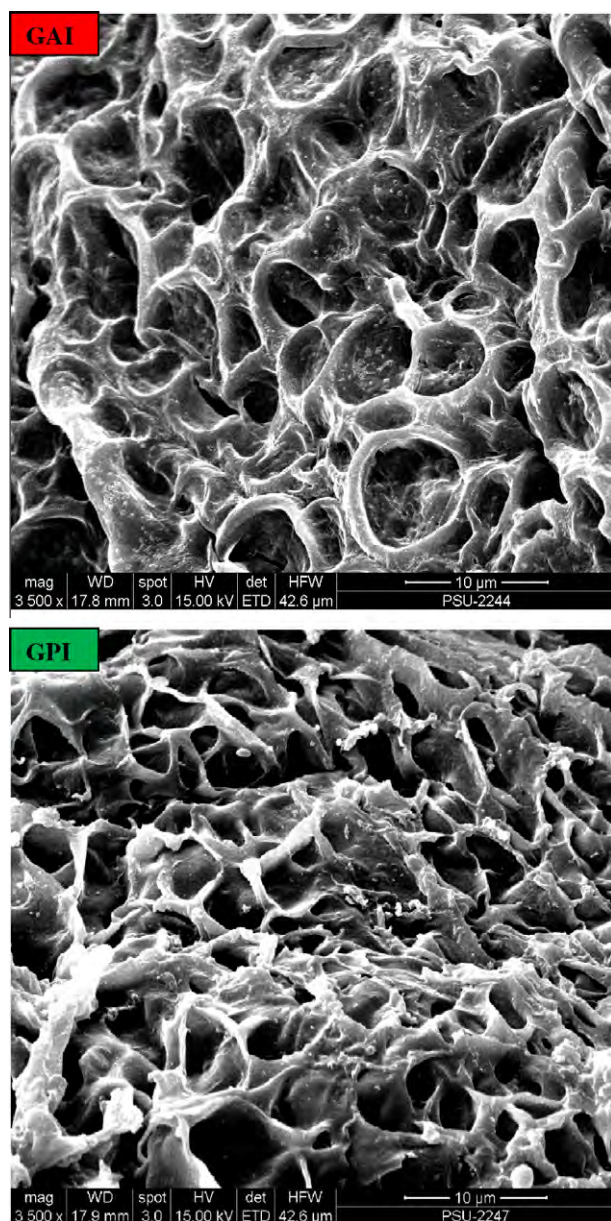


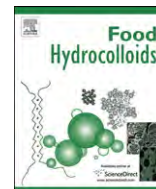
Fig. 5. SEM micrographs of gel from gelatin extracted from the skin of unicorn leatherjacket in the absence (GAI) and presence of 0.04 mM of SBTI (GPI). Magnification: 3500 $\times$ .

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# Physico-mechanical and antimicrobial properties of gelatin film from the skin of unicorn leatherjacket incorporated with essential oils

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## ABSTRACT

Gelatin films incorporated with bergamot (BO) and lemongrass oil (LO) at various concentrations as glycerol substitute were prepared and characterised. Incorporation of BO and LO at 5–25% (w/w protein) resulted in the decreases in both tensile strength (TS) and elongation at break (EAB) of the films. Water vapour permeability (WVP) were decreased in LO incorporated films, while it was increased in film added with BO at level higher than 5% ( $P < 0.05$ ). Film solubility and transparency values decreased, and the films had the lowered light transmission in the visible range when BO and LO were incorporated. Films incorporated with LO showed inhibitory effect in a concentration dependent manner against *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella typhimurium*, but BO added film inhibited only *L. monocytogenes* and *S. aureus*. Films containing both BO and LO did not inhibit *Pseudomonas aeruginosa*. Significant change of molecular organisation and higher intermolecular interactions among gelatin molecules were found in the film structure as determined by FTIR. Thermogravimetric analysis (TGA) demonstrated that films added with BO and LO exhibited enhanced heat stability with higher degradation temperature, compared with control film. Scanning electron microscopic (SEM) images revealed the presence of micro-pores in the essential oil incorporated films, which contributed to physical properties of the resulting films. Thus, gelatin films incorporated with BO and LO can be used as active packaging, but the properties could be modified, depending on essential oil added.

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## 1. Introduction

Nowadays, eco-friendly materials from natural polymers have gained increasing attention for the development of packaging to reduce environmental pollution and ecological related problems caused by non-biodegradable plastic packaging (Petersen et al., 1999). Packaging is widely used for the protection of food quality, thereby ensuring hygiene and extending the shelf life of perishable items, especially those susceptible to oxidative and microbiological deterioration (Coma, 2008). Proteins, lipids and polysaccharides are employed for the preparation of bio-based packaging for food industry (Bao, Xu, & Wang, 2009). Gelatin is one of the versatile biomaterial obtained from collagen by the controlled hydrolysis of the fibrous insoluble collagen present in the bones and skin generated as waste during animal slaughtering and fish processing (Ahmad, Benjakul, Ovissipour, & Prodpran, 2011). It has been known for the excellent film forming ability (Hoque, Benjakul, & Prodpran, 2010). Gelatin-based film used for coating or packaging

could maintain the quality of foods during storage, due to its barrier to oxygen, light and prevention of dehydration and lipid oxidation (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2011). Several properties of gelatin film, such as mechanical properties, permeability, light absorption, transparency, antimicrobial activity and antioxidant ability, are influenced by the addition of active substances (Pires et al., 2009). Essential oils derived from natural sources have been added to the film as antimicrobial agent (Pereda, Ponce, Marcovich, Ruseckaite, & Martucci, 2011).

Essential oils categorised as GRAS (generally recognised as safe) by U.S. Food and Drug Administration (Persico et al., 2009) can be considered as the potential alternatives to synthetic additives (Valentao et al., 2002). Essential oils especially from bergamot and lemongrass appear as interesting natural compounds with great potential use in foodstuffs preservation. *In vitro* studies have revealed significant antimicrobial effects of these essential oils (Lv, Liang, Yuan, & Li, 2011; Naik, Fomda, Jaykumar, & Bhat, 2010). Thus the incorporation of bergamot essential oil and lemongrass essential oil into gelatin films offers the possibility not only of imparting antimicrobial activity, but also improving the physico-chemical properties. In general, essential oils, the odorous volatile

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products of an aromatic plant's secondary metabolism, are well-known as antimicrobial agents that could be used to control food spoilage and food-borne pathogenic bacteria (Kalemba & Kunicka, 2003). Essential oil-based antimicrobial packaging has high potential for food applications (Emiroglu, Yemis, Coskun, & Candogan, 2010). The antimicrobial activity of essential oils is assigned to a number of small terpenoids and phenolic compounds (thymol, carvacrol, eugenol) (Oussalah, Caillet, Saucier, & Lacroix, 2007). Terpenes have the ability to disrupt and penetrate into lipid structure of the cell wall of bacteria, leading to denaturation of proteins and destruction of cell membrane, cytoplasmic leakage, cell lysis and eventually cell death (Emiroglu et al., 2010). The decrease in pH that occurs due to cell membrane disruption resulted in a loss of control of cellular processes such as DNA transcription, protein synthesis and enzyme activity (Oussalah et al., 2007). Essential oils also penetrate into mitochondrial membrane, leading to the greater permeability of organelle and the  $K^+$  ion leakage process (Oussalah et al., 2007).

Unicorn leatherjacket (*Aluterus monoceros*) is one of the marine tropical fish, which is harvested in large quantities throughout the world. This species has been used for fillet production in Thailand, in which a large quantity of skin has been produced as by-product (Ahmad & Benjakul, 2010). Due to its thick skin, it can be a potential source for gelatin production (Ahmad & Benjakul, 2011). As a consequence, the increased revenue for processors can be achieved. Furthermore, gelatin from unicorn leatherjacket skin can be used for smart film/packaging with antimicrobial activity. Selected natural antimicrobial agents, especially essential oils can be incorporated into films to provide their preventive role. Therefore, the objectives of this investigation were to prepare and characterise the gelatin film from unicorn leatherjacket skin and to assess the antimicrobial property of film added with bergamot and lemongrass essential oils.

## 2. Materials and methods

### 2.1. Chemicals

Essential oils of bergamot (BO, *Citrus bergamia*) and lemongrass (LO, *Cymbopogon citratus*) were purchased from Botanicessence essential oils (Suanlung, Bangkok, Thailand). Bovine serum albumin (BSA) and high molecular weight protein markers were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Glycerol, phosphoric acid, *p*-dimethylaminobenzaldehyde and tris(hydroxymethyl) aminomethane were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS), *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) and Coomassie blue R-250 were procured from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals were of analytical grade.

### 2.2. Microorganisms

*Escherichia coli* (ATCC 25922), *Listeria monocytogenes* (ATCC 7644), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923) and *Salmonella typhimurium* (ATCC 14028) were obtained from the Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand.

### 2.3. Collection and preparation of the skin from unicorn leatherjacket

The skin of unicorn leatherjacket (*A. monoceros*) was obtained from Sea Wealth Frozen Food Co., Ltd., Songkhla, Thailand. Upon arrival to the Department of Food Technology, Prince of Songkla University, Hat Yai, the skin was washed with iced tap water

(0–2 °C). Prepared skin was then cut into small pieces ( $0.5 \times 0.5 \text{ cm}^2$ ), placed in polyethylene bags and stored at –20 °C until use. The storage time was less than 2 months.

### 2.4. Extraction of gelatin from the skin of unicorn leatherjacket

Prepared skin was pretreated following the method of Ahmad and Benjakul (2011) with a slight modification. To remove non-collagenous proteins, the prepared skin was mixed with 0.1 M NaOH using a skin/alkali solution ratio of 1:20 (w/v). The mixture was stirred for 6 h at 4 °C using an overhead mechanical stirrer (W20.n, IKA®-Werke GmbH & CO.KG, Stanfen, Germany) at a speed of 300 rpm. The alkali solution was changed every 2 h. The samples were then washed with iced tap water until neutral or faintly basic pH was obtained. Thereafter, the prepared skin was swollen by mixing the skins with 0.2 M phosphoric acid at a ratio of 1:10 (w/v). The mixture was stirred for 12 h at 4 °C. The swollen skin was washed thoroughly with iced tap water until neutral or faintly acidic pH of wash water was obtained. To the swollen skin, distilled water was added at the ratio of 1:10 (w/v). Soybean seed extract containing trypsin inhibitor was subsequently added to obtain the final concentration of 100 TIU/g of swollen skin, to inhibit the indigenous serine proteases associated with skin matrix. The mixture was pre-incubated at 4 °C for 12 h, followed by extraction at 50 °C for 12 h. After extraction, the mixture was then filtered using two layers of cheesecloth. The filtrate was further filtered using a Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England) with the aid of an electrical aspirator, (Model VE-11, JEIO TECH, Seoul, Korea). The resultant filtrate was freeze-dried using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lyngø, Denmark). Gelatin powder obtained contained 85.61% protein as determined by the Kjeldhal method (AOAC, 1999).

### 2.5. Formulation and preparation of gelatin films

Film forming solution (FFS) was prepared by mixing the gelatin powder with distilled water to obtain the protein concentration of 3% (w/v). After dissolution, glycerol and essential oils either from bergamot (BO) or lemongrass (LO) were added into FFS as plasticiser and antimicrobial agent, respectively. Based on the protein, the mixture between glycerol and essential oil at various ratios (25:0, 20:5, 15:10, 10:15, 5:20 and 0:25% w/w) was added into FFS to obtain the final concentration of 25% of protein. To stabilise emulsion, tween-20 was added as an emulsifier at 15% based on the added essential oil. The mixture was homogenised at a speed of 11,000 rpm for 2 min using a homogeniser (Model T25 basic, IKA®18, 19 Labortechnik, Selangor, Malaysia) and allowed to stand for 5 min at room temperature (28–30 °C) before being subjected to film casting. FFS ( $4 \pm 0.01 \text{ g}$ ) was cast onto a rimmed silicone resin plate ( $5 \times 5 \text{ cm}^2$ ), air-blown for 12 h at room temperature, followed by drying in an environmental chamber (Binder, KBF 115 # 00-19735, D-78532, Tuttlingen, Germany) at  $25 \pm 0.5 \text{ °C}$  and  $50 \pm 5\%$  relative humidity (RH) for 24 h. Dried film samples were manually peeled off and subjected to following analyses.

### 2.6. Analyses

Prior to testing, film samples were conditioned for 48 h at  $50 \pm 5\%$  relative humidity (RH) and  $25 \pm 0.5 \text{ °C}$ . For ATR-FTIR, SEM and TGA studies, films were conditioned in a desiccator containing dried silica gel for 1 week to minimise the plasticising effect of water, followed by drying in a desiccator containing  $\text{P}_2\text{O}_5$  gel for 2 weeks at room temperature (28–30 °C) to obtain the most dehydrated films.

### 2.6.1. Film thickness

The thickness of film samples was measured using a digital micrometer (Mitutoyo, Model ID-C112PM, Serial No. 00320, Mitutoyo Corp., Kawasaki-shi, Japan). Ten random locations around each film sample were used for determination of thickness.

### 2.6.2. Mechanical properties

Tensile strength (TS) and elongation at break (EAB) of film samples were determined as described by Iwata, Ishizaki, Handa and Tanaka (2000) using the Universal Testing Machine (Lloyd Instrument, Hampshire, UK). The test was performed in the controlled room at 25 °C and  $\sim 50 \pm 5\%$  RH. Ten film samples ( $2 \times 5 \text{ cm}^2$ ) with the initial grip length of 3 cm were used for testing. The film samples were clamped and deformed under tensile loading using a 100 N load cell with the cross-head speed of 30 mm/min until the samples were broken. The maximum load and the final extension at break were used for calculation of TS and EAB, respectively.

### 2.6.3. Water vapour permeability (WVP)

WVP was measured using a modified ASTM (American Society for Testing and Materials) method as described by Shiku, Hamaguchi, Benjakul, Visessanguan, and Tanaka (2004). The film samples were sealed on an aluminium permeation cup containing dried silica gel (0% RH) with silicone vacuum grease and rubber gasket. The cups were placed at 30 °C in a desiccator containing the distilled water, followed by weighing after every 1 h intervals for up to 8 h. Five film samples were used for WVP testing. WVP of the film was calculated as follows:

$$\text{WVP (g m}^{-1} \text{ s}^{-1} \text{ Pa}^{-1}) = w/A^{-1} t^{-1} (P_2 - P_1)^{-1}$$

where  $w$  is the weight gain of the cup (g);  $l$  is the film thickness (m);  $A$  is the exposed area of film ( $\text{m}^2$ );  $t$  is the time of gain (s);  $(P_2 - P_1)$  is the vapour pressure difference across the film (4244.9 Pa at 30 °C).

### 2.6.4. Film solubility

Solubility of film samples in water was determined according to the method of Gennadios, Handa, Froning, Weller, and Hanna (1998) with a slight modification. The conditioned film samples ( $3 \times 2 \text{ cm}^2$ ) were weighed and placed in 50 ml-centrifuge tube containing 10 ml of distilled water with 0.1% (w/v) sodium azide. The mixtures were shaken continuously at room temperature for 24 h using a shaker (Heidolph UNIMAX 1010, Schwabach, Germany). Undissolved debris was obtained after centrifugation at  $3000 \times g$  for 10 min at 25 °C using a centrifuge (Allegra 25R Centrifuge, Beckman Coulter, Krefeld, Germany). The pellets were dried at 105 °C for 24 h to obtain the dry unsolubilised film matter. The weight of solubilised dry matter was calculated by subtracting the weight of unsolubilised dry matter from the initial weight of dry matter and expressed as the percentage of total weight.

### 2.6.5. Colour, light transmission and transparency

Colour of film samples was determined using a CIE colourimeter (Hunter associates laboratory, Inc., Reston, VA, USA). Colour of the film was expressed as  $L^*$  – (lightness/brightness),  $a^*$  – (redness/greenness) and  $b^*$  – (yellowness/blueness) values. Total difference in colour ( $\Delta E^*$ ) was calculated according to the following equation (Gennadios, Weller, Hanna, & Froning, 1996).

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where  $\Delta L^*$ ,  $\Delta a^*$  and  $\Delta b^*$  are the differences between the corresponding colour parameter of the sample and that of white standard ( $L^* = 93.63$ ,  $a^* = -0.95$  and  $b^* = 0.46$ ).

Light transmission of films against ultraviolet (UV) and visible light was measured at selected wavelengths between 200 and 600 nm, using a UV–Visible spectrophotometer (model UV-160, Shimadzu, Kyoto, Japan) according to the method of Jongjareonrak et al. (2011). The transparency value of film was calculated by the following equation (Han & Floros, 1997):

$$\text{Transparency value} = (-\log T_{600})/x$$

where  $T_{600}$  is the fractional transmittance at 600 nm and  $x$  is the film thickness (mm). The higher transparency value represents the lower transparency of films.

### 2.6.6. Electrophoretic analysis

Protein patterns of film samples were analysed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Gelatin or film samples (200 mg) were mixed with 10 ml of 5% (w/v) SDS. The mixtures were stirred continuously at room temperature for 12 h. Supernatants were obtained after centrifugation at  $5000 \times g$  for 5 min at room temperature. Protein content in the supernatant of all samples was determined using the Biuret method (Robinson & Hogden, 1940). Supernatants were then mixed with sample buffer (0.5 M Tris–HCl, pH 6.8 containing 4% (w/v) SDS and 20% (v/v) glycerol at the ratio of 1:1 (v/v)). Samples (15  $\mu\text{g}$  protein) were loaded onto the polyacrylamide gel made of 7.5% (w/v) separating gel and 4% (w/v) stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini Protein II unit. High molecular weight markers (GE Healthcare UK, Buckinghamshire, UK) were used to estimate the molecular weight of the proteins.

### 2.6.7. Antimicrobial properties

Antimicrobial activity of film samples was assessed using the agar diffusion method according to Ponce, Roura, del Valle, and Moreira (2008). The zone of inhibition on solid media was used for determining the antimicrobial effects of films against two typical food pathogens including Gram-negative bacteria, (*E. coli*, *S. typhimurium*, *P. aeruginosa*) and Gram-positive bacteria (*L. monocytogenes*, *S. aureus*). Film samples incorporated with BO and LO were cut into a disc shape of 10 mm diameter using a circular knife and then placed on Mueller Hinton (Merck, Darmstadt, Germany) agar plates, which had been previously smeared with 100  $\mu\text{l}$  of inoculums containing approximately  $10^6$ – $10^7$  CFU/ml of tested bacteria. The plates were then incubated at 37 °C for 24 h and the diameter of inhibition zone was measured. The total area was used to evaluate the antimicrobial potential of film samples. Antimicrobial index of film samples was then measured based on the total area using the following equation:

$$\text{Antimicrobial index} = \frac{\text{Area of inhibition zone} - \text{Area of film}}{\text{Area of film}}$$

## 2.7. Characterisation of selected films

Gelatin films incorporated without and with BO and LO at selected essential oil: glycerol ratios (15:10 and 25:0), representing medium and high essential oil levels, respectively, were further subjected for characterisation.

### 2.7.1. Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR)

FTIR spectra of film samples were determined using a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) equipped with a horizontal ATR Trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) at 25 °C as described by [Nuthong, Benjakul, and Prodpran \(2009\)](#). Samples were placed onto the crystal cells and the cells were clamped into the mount of FTIR spectrometer. The spectra in the range of 650–4000 cm<sup>-1</sup> with automatic signal gain were collected in 32 scans at a resolution of 4 cm<sup>-1</sup> and were ratioed against a background spectrum recorded from the clean empty cell at 25 °C. Analysis of spectral data was carried out using the OPUS 3.0 data collection software programme (Bruker, Ettlingen, Germany). Prior to data analysis, the spectra were baseline corrected and normalised.

### 2.7.2. Thermo-gravimetric analysis (TGA)

Dried film samples were scanned using a thermogravimetric analyser (TGA-7, Perkin Elmer, Norwalk, CT, USA) from 40 to 600 °C at a rate of 10 °C/min ([Nuthong et al., 2009](#)). Nitrogen was used as the purge gas at a flow rate of 20 ml/min.

### 2.7.3. Microstructure

Microstructure of upper surface and cryo-fractured cross-section of the film samples was visualised using a scanning electron microscope (SEM) (JEOL JSM-5800 LV, Tokyo, Japan) at an accelerating voltage of 10 kV. The film samples were cryo-fractured by immersion in liquid nitrogen. Prior to visualisation, the film samples were mounted on brass stub and sputtered with gold in order to make the sample conductive, and photographs were taken at 8000× magnification for surface. For cross-section, cryo-fractured films were mounted around stubs perpendicularly using double sided adhesive tape, coated with gold and observed at the 5000× magnification.

## 2.8. Statistical analyses

Experiments were performed in triplicate ( $n = 3$ ) with three different lots of film samples and a completely randomised design (CRD) was used. Data were presented as means  $\pm$  standard deviation and a probability value of  $<0.05$  was considered significant. Analysis of variance (ANOVA) was performed and the mean comparisons were done by Duncan's multiple range tests. Statistical analysis was performed using the Statistical package for social sciences (SPSS for Windows, SPSS Inc., Chicago, IL, USA).

## 3. Results and discussion

### 3.1. Thickness

Thickness of gelatin films incorporated with bergamot (BO) and lemongrass oil (LO) at various concentrations is shown in [Table 1](#). Thickness of films incorporated with BO (5–25%) increased in comparison with the control ( $P < 0.05$ ). It ranged from 0.045 to 0.048 mm. However, no significant difference in thickness was observed between films incorporated with BO in the range of 10–25% ( $P > 0.05$ ). The results suggested that peptide chains of gelatin could not form the compact film network in the presence of BO. LO incorporated films had similar thickness, regardless of LO concentrations used ( $P < 0.05$ ). [Altio, Altio, and Tihminlioglu \(2010\)](#) reported that the incorporation of thyme oil had no effect on thickness of chitosan films. Protruded structure formed during film formation might be induced by the compounds in BO, which could interact with gelatin to form agglomerates or particulates. BO and LO incorporated films might be lost to different degrees, leading to different final solid content of films. According to [Han and Krochta \(1999\)](#), film thickness is influenced by the solid content of the film forming solution.

Thickness of BO incorporated films increased ( $P < 0.05$ ). It was plausibly due to the protruded structures mediated by the different chemical components present in BO. Thus, BO might contribute to the formation of the loose film matrix with the increased thickness. Apparently, LO was more compatible with the gelatin matrix than BO, resulting in formation of compact structure without significant increase in the thickness. Furthermore, different components in BO ( $\alpha$ -pinene, myrcene, limonene,  $\alpha$ -bergaptene,  $\beta$ -bisabolene, etc) and LO (myrcene, citronellal, geraniol, neral, limonene, citral, etc) might interact with gelatin to different degrees. In addition, the compatibility of BO and LO with gelatin matrix and the inter-junction between gelatin developed in the presence of those compounds in different ways might determine the compactness of film matrix, thereby resulting in varying film thickness. However, [Hoque, Benjakul, and Prodpran \(2011\)](#) did not find any difference in the thickness of film incorporated with various extract of cinnamon, clove and star anise. [Rattaya, Benjakul, and Prodpran \(2009\)](#) also found similar thickness of gelatin film incorporated without and with oxygenated seaweed extracts.

### 3.2. Mechanical properties

Tensile strength (TS) and elongation at break (EAB) of gelatin films incorporated with BO and LO at various concentrations are

**Table 1**  
Tensile strength (TS), elongation at break (EAB), water vapour permeability (WVP) and thickness of gelatin films from the skin of unicorn leatherjacket incorporated with bergamot (BO) and lemongrass essential oil (LO) at various concentrations.

| Film samples | TS (MPa)            | EAB (%)           | WVP $\times (10^{-10} \text{ gs}^{-1} \text{ m}^{-1} \text{ Pa}^{-1})$ | Thickness (mm)      |
|--------------|---------------------|-------------------|------------------------------------------------------------------------|---------------------|
| Control      | 33.35 $\pm$ 5.35abC | 6.99 $\pm$ 3.51aA | 1.21 $\pm$ 0.065bA                                                     | 0.042 $\pm$ 0.001cA |
| 5% BO        | 36.34 $\pm$ 3.14a   | 8.76 $\pm$ 3.45a  | 1.26 $\pm$ 0.028b                                                      | 0.048 $\pm$ 0.002a  |
| 10% BO       | 30.80 $\pm$ 6.41b   | 7.33 $\pm$ 2.12a  | 1.88 $\pm$ 0.032a                                                      | 0.045 $\pm$ 0.001b  |
| 15% BO       | 27.94 $\pm$ 3.34bc  | 7.18 $\pm$ 4.60a  | 1.94 $\pm$ 0.057a                                                      | 0.046 $\pm$ 0.001b  |
| 20% BO       | 27.96 $\pm$ 7.24bc  | 3.70 $\pm$ 2.04b  | 1.87 $\pm$ 0.105a                                                      | 0.045 $\pm$ 0.001b  |
| 25% BO       | 23.75 $\pm$ 6.85c   | 3.06 $\pm$ 2.00b  | 1.84 $\pm$ 0.132a                                                      | 0.046 $\pm$ 0.001b  |
| 5% LO        | 43.82 $\pm$ 6.56A   | 3.48 $\pm$ 0.92C  | 1.21 $\pm$ 0.040A                                                      | 0.045 $\pm$ 0.002A  |
| 10% LO       | 39.05 $\pm$ 5.96B   | 4.13 $\pm$ 2.14BC | 1.03 $\pm$ 0.066BC                                                     | 0.046 $\pm$ 0.001A  |
| 15% LO       | 34.07 $\pm$ 3.61C   | 4.80 $\pm$ 1.04BC | 1.00 $\pm$ 0.060C                                                      | 0.046 $\pm$ 0.001A  |
| 20% LO       | 25.84 $\pm$ 2.36D   | 5.90 $\pm$ 1.66AB | 1.12 $\pm$ 0.082AB                                                     | 0.047 $\pm$ 0.001A  |
| 25% LO       | 21.21 $\pm$ 3.36E   | 5.66 $\pm$ 2.34AB | 1.07 $\pm$ 0.041BC                                                     | 0.046 $\pm$ 0.001A  |

Values are given as mean  $\pm$  SD ( $n = 3$ ).

Different letters or different capital letters in the same column within the film containing the same essential oil together with the control indicate significant differences ( $P < 0.05$ ).

shown in Table 1. TS of resulting films was affected by the addition of BO and LO. The presence of BO in film structure caused the decreases in both TS and EAB, when BO at levels above 5 and 20%, respectively, was incorporated. For film added with LO, the decrease in TS was found as the level of LO was more than 10% ( $P < 0.05$ ). On the other hand, the lowered EAB was obtained with the addition of LO at levels of 5–15% ( $P < 0.05$ ), while 20–25% LO had no effect on EAB ( $P > 0.05$ ). The results suggest that the incorporation of essential oils or other extra-polymeric components at an excessive amount more likely resulted in the development of a heterogeneous film structure, featuring discontinuities or irregularities. Nevertheless, the essential oil at an appropriate level could strengthen the film matrix via enhancing the interaction between protein chains. Cagri, Ustunol, and Ryser (2001) concluded that incorporation of additives other than cross-linking agents generally lowers TS. In the present study, TS and EAB were decreased with increasing essential oil levels. This was concomitant with the lowered glycerol content in the films. Glycerol has been used widely as plasticiser in protein films (Hoque et al., 2011). As a result, the excessive interaction between protein molecules was gained, leading to the increased rigidity with less extensibility. Essential oil is usually liquid at room temperature and its presence in the film structure in the form of oil droplets can easily be deformed, enhancing the film flexibility (Fabra, Talens, & Chiralt, 2008). The results were consistent with Altioek et al. (2010) who reported that increasing concentration of thyme oil decreased TS of chitosan film. It is suggested that BO and LO contained different compounds, thereby affecting protein interaction differently. Therefore, the incorporation of either BO or LO as the substitute of glycerol resulted in the lower plasticising effect. As a consequence, the film had less stretchability. Nevertheless, it was noted that TS was also decreased. The excessive rigidity of brittle film caused the ease of breaking when only low tensile force was applied. Those effects were governed by the compounds in both BO and LO.

### 3.3. Water vapour permeability (WVP)

WVP of gelatin films incorporated with BO and LO at various concentrations is presented in Table 1. WVP of BO incorporated films was slightly increased from 1.21 to 1.84–1.94 as the concentrations of BO were higher than 5% ( $P < 0.05$ ). BO in film might distribute irregularly, leading to the heterogeneous film structure. Nevertheless, LO at levels higher than 5% caused the decreases in WVP of resulting film ( $P < 0.05$ ). This was coincidental with the lower glycerol presented in the film. Generally, plasticiser e.g.,

glycerol located between adjacent chains of gelatin molecules decreased the intermolecular forces, increased the free volume of the system and favoured the mobility of polypeptide chains in the film matrix. The increased mobility results in greater free volume and segmental motions, which facilitates the migration of water vapour molecules through the film (Rodriguez, Oses, Ziani, & Mate, 2006). The water vapour transfer process in films also depends on the hydrophilic–hydrophobic ratio of the film constituents. Pranoto, Salokhe, and Rakshit (2005) reported that WVP of alginate-based films tended to increase as higher amounts of garlic oil were incorporated (0.3–0.4%). It was noted that BO and LO incorporation affected WVP of film differently. BO incorporation at higher level caused the increase in WVP but LO more likely led to the lower WVP of films. This might be due to the differences in the hygroscopic nature of the oils used, which caused the different ability to attract water to the film network. Interactions of oil components with some hydrophilic protein domains could promote the decrease in the hydrophobic character of film matrix (Perez-Gago & Krochta, 2001). Zivanovic, Chi, and Draughon (2005) found that the WVP of chitosan films enriched with oregano oil decreased due to hydrophobicity of the oils. Thus, types of essential oil not only affected the mechanical property, but also water vapour permeability.

### 3.4. Film solubility

Solubility of gelatin films incorporated with BO and LO at various concentrations is shown in Table 2. Control film showed the highest film solubility (97.80%), while films incorporated with BO and LO exhibited lower film solubility ( $P < 0.05$ ). Solubility of both BO and LO incorporated films decreased as the concentration of essential oil increased from 5 to 25% ( $P < 0.05$ ). However, no significant difference was noted in BO and LO incorporated films, when the essential oil incorporated increased from 5 to 15% ( $P < 0.05$ ). The incorporation of BO or LO might be associated with the hydrophobic nature of oil. Non-polar components of oil favourably interacted with hydrophobic domain of gelatin, leading to the increase in hydrophobicity of the resulting film. As a result, the solubility of film became lowered. Film solubility can be viewed as a measure of the water resistance and integrity of a film (Rhim, Gennadios, Handa, Weller, & Hanna, 2000). Additionally, the higher degree of substitution of glycerol by essential oil could lower hydrophilicity of film, thereby lowering the soluble substance of films. Therefore, the addition of BO and LO could decrease solubility of gelatin films. Films with high solubility have the potential for developing edible packaging

**Table 2**

Colour and film solubility of gelatin films prepared from the skin of unicorn leatherjacket incorporated with bergamot (BO) and lemongrass essential oil (LO) at various concentrations.

| Film samples | Colour          |               |               |                 | Film solubility (%) |
|--------------|-----------------|---------------|---------------|-----------------|---------------------|
|              | $L^*$           | $a^*$         | $b^*$         | $\Delta E^*$    |                     |
| Control      | 80.41 ± 1.53bcB | 1.98 ± 0.29aA | 5.85 ± 0.46bA | 14.60 ± 1.62abA | 97.80 ± 0.78aA      |
| 5% BO        | 81.02 ± 2.19bc  | 1.91 ± 0.39a  | 5.67 ± 0.59bc | 13.96 ± 2.29 ab | 93.37 ± 0.57b       |
| 10% BO       | 83.20 ± 0.44a   | 1.32 ± 0.07b  | 5.26 ± 0.25c  | 11.71 ± 0.51c   | 93.14 ± 0.37b       |
| 15% BO       | 80.53 ± 0.46bc  | 1.80 ± 0.03a  | 6.62 ± 0.07a  | 14.74 ± 0.44 ab | 93.07 ± 0.45b       |
| 20% BO       | 79.59 ± 0.38c   | 2.10 ± 0.08a  | 6.07 ± 0.04b  | 15.44 ± 0.36a   | 90.04 ± 0.46c       |
| 25% BO       | 81.67 ± 0.12ab  | 0.55 ± 0.07c  | 5.64 ± 0.16bc | 13.31 ± 0.07bc  | 89.82 ± 0.96c       |
| 5% LO        | 86.72 ± 0.75A   | 0.29 ± 0.14B  | 4.16 ± 0.38A  | 7.91 ± 0.85B    | 93.54 ± 0.66B       |
| 10% LO       | 85.84 ± 0.05A   | 0.44 ± 0.09B  | 4.60 ± 0.20A  | 8.90 ± 0.14B    | 92.30 ± 0.65B       |
| 15% LO       | 85.24 ± 1.22A   | 0.59 ± 0.26B  | 5.02 ± 0.91A  | 9.64 ± 1.53B    | 92.04 ± 0.57B       |
| 20% LO       | 83.51 ± 4.60A   | 0.83 ± 0.91B  | 5.79 ± 2.80A  | 11.54 ± 5.45AB  | 89.81 ± 0.50C       |
| 25% LO       | 84.24 ± 0.26A   | 0.55 ± 0.07B  | 5.38 ± 0.12A  | 10.68 ± 0.28B   | 89.16 ± 0.65C       |

Values are given as mean ± SD ( $n = 3$ ).

Different letters or different capital letters in the same column within the film containing the same essential oil together with the control indicate significant differences ( $P < 0.05$ ).

material intended for easy solubility. The gradual release of an antimicrobial from a packaging film to the food surface may have an advantage over dipping and spraying. Direct application of antimicrobial agents onto food surfaces by spraying, dipping or coating has proven to be less effective as there is loss of activity because of leaching onto the food, enzymatic activity, and reaction with other food components (Ouattara, Simard, Piette, Bégin, & Holley, 2000). Additionally, the dissolution of film, an edible packaging, provides the advantage for application since the removal of film is not required before cooking or consumption.

### 3.5. Colour

Colour parameters of gelatin films incorporated with BO and LO at various concentrations are shown in Table 2. Colour of films was affected by the type and levels of essential oil incorporated. Increased  $L^*$ -values with the coincidental decreases in  $a^*$ - and  $b^*$ -values as well as  $\Delta E^*$ -values were found in films incorporated with 10 and 25% BO, compared to the control films ( $P < 0.05$ ). For films incorporated with LO at all concentrations tested ( $P > 0.05$ ),  $L^*$ -values increased, while  $a^*$ - and  $b^*$ -values decreased, regardless of concentrations used ( $P < 0.05$ ).  $\Delta E^*$ -values of film added with LO decreased as LO was added at all levels, except for that added with 20% LO, where no changes in  $\Delta E^*$  were observed ( $P > 0.05$ ). The increase in  $L^*$ -values in the films incorporated with LO could be due to light scattering effect of essential oil droplets in film matrix. However, there was no obvious difference in all colour parameters in films incorporated with BO ( $P > 0.05$ ). These results suggested that incorporation of essential oil had influence on colour of film, but changes were mediated by the type of essential oil. The incorporation of cinnamon oil in chitosan-based films considerably increased its total colour difference, whereas such pronounced effect was not found with thyme and clove oil (Hosseini, Razavi, & Mousavi, 2009). Considerable effects of the incorporation of cinnamon oil on the colour of caseinate-based and soy protein-based edible films were found (Atarés, Bonilla, & Chiralt, 2010; Atarés, De Jesús, Talens, & Chiralt, 2010). However, no marked effect on the colour parameters of those films was obtained, when ginger oil was added. It was suggested that essential oil also influenced the colour of gelatin film, depending on the type and concentrations of essential oil incorporated.

### 3.6. Light transmission and transparency

Transmission of UV and visible light at wavelength range of 200–800 nm of gelatin films incorporated with BO and LO at

various concentrations is shown in Table 3. The transmission of UV light was very low at 200 and 280 nm for all films, regardless of types and concentrations of essential oils used. Films incorporated with essential oils at higher levels had lower transmission in UV range at 280 nm. The result suggested that both essential oil effectively prevented the transmission of UV and visible light of gelatin films. Light transmission in visible range (490–600 nm) for control films was in the range of 44.58–56.48%. In visible range, control films showed higher light transmission, compared with those incorporated with BO and LO. Optical properties of films are an important attribute which influences its appearance, marketability, and their suitability for various applications. Clear edible films are typically desirable with higher applicability and acceptability in food packaging systems. Lowered transmission was observed when the concentration of BO incorporated increased. The result suggested that BO, especially at higher amount, with high light transmission barrier more likely contributed to limited light transmission of films at visible ranges. LO also showed varying effects on transmission of visible light, depending on the levels of LO incorporated. Light transmission more likely depended on the distribution of essential oil in the film matrix as well the interaction between essential oil and gelatin. This led to the differences in film matrix morphology with different light transmission.

When either BO or LO was incorporated, films were more transparent, compared with the control film. Essential oil, which is clear and transparent in nature, might contribute to transparency of film to some extent. BO incorporated films showed the lower transparency values as the BO level increased, indicating that films became more transparent ( $P < 0.05$ ). For films incorporated with LO, the slight decreases in transparency value were obtained as the concentration increased ( $P < 0.05$ ), but the degree of decrease was lower than that found in BO added films. In transparent material, non-uniformities in the composition of the material can cause significant changes in optical properties (Rauwendaal, 2001, pp 90). Nevertheless, Prodpran, Benjakul, and Artharn (2007) reported that transparency of films was not significantly affected by the addition of palm oil. Thus, incorporation of essential oil in films directly had influence on the light transmission and transparency of resulting films.

### 3.7. Protein pattern

Protein patterns of gelatin films incorporated with BO and LO at various concentrations are shown in Fig. 1. High molecular weight (MW) components, including  $\alpha_1$ -,  $\alpha_2$ -, and  $\beta$ -components with MW

**Table 3**  
Light transmittance (%) and transparency values of gelatin films from the skin of unicorn leatherjacket incorporated with bergamot (BO) and lemongrass essential oil (LO) at various concentrations.

| Film samples | Wavelength (nm) |       |       |       |       |       |       |       | Transparency values |
|--------------|-----------------|-------|-------|-------|-------|-------|-------|-------|---------------------|
|              | 200             | 280   | 350   | 400   | 500   | 600   | 700   | 800   |                     |
| Control      | 0.01            | 18.38 | 44.58 | 47.93 | 50.70 | 53.25 | 55.30 | 56.48 | 3.08 ± 0.00 aA      |
| 5% BO        | 0.00            | 9.32  | 43.96 | 41.19 | 45.61 | 48.26 | 50.68 | 53.60 | 2.99 ± 0.02b        |
| 10% BO       | 0.00            | 7.66  | 28.99 | 40.59 | 44.58 | 46.52 | 48.18 | 49.99 | 2.87 ± 0.03c        |
| 15% BO       | 0.00            | 4.68  | 23.18 | 34.37 | 39.46 | 43.65 | 47.05 | 50.32 | 2.72 ± 0.02d        |
| 20% BO       | 0.00            | 3.22  | 17.32 | 28.37 | 32.64 | 36.67 | 39.33 | 41.37 | 2.73 ± 0.06d        |
| 25% BO       | 0.00            | 4.42  | 18.95 | 29.36 | 33.40 | 36.45 | 39.06 | 40.83 | 2.76 ± 0.00d        |
| 5% LO        | 0.00            | 8.99  | 23.01 | 27.21 | 30.70 | 33.17 | 36.52 | 38.52 | 2.92 ± 0.04C        |
| 10% LO       | 0.00            | 9.26  | 23.00 | 26.80 | 31.15 | 33.60 | 37.37 | 39.78 | 2.91 ± 0.04C        |
| 15% LO       | 0.00            | 1.46  | 15.69 | 22.69 | 28.33 | 31.99 | 35.29 | 38.17 | 2.94 ± 0.01C        |
| 20% LO       | 0.00            | 21.77 | 40.18 | 44.37 | 47.28 | 49.69 | 51.38 | 52.67 | 2.69 ± 0.00D        |
| 25% LO       | 0.00            | 1.85  | 20.81 | 30.55 | 37.92 | 44.06 | 48.59 | 52.24 | 3.03 ± 0.01B        |

Values are given as mean ± SD ( $n = 3$ ).

Different letters or different capital letters in the same column within the film containing the same essential oil together with the control indicate significant differences ( $P < 0.05$ ).

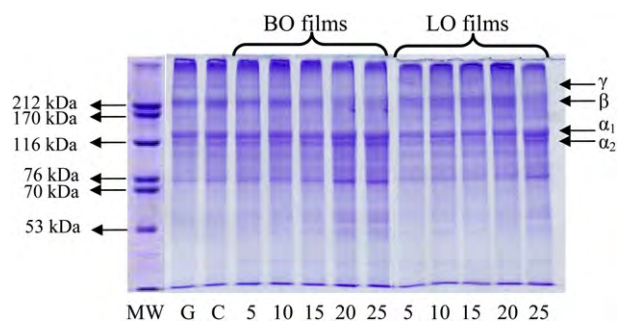


**Fig. 1.** Protein patterns of gelatin films from the skin of unicorn leatherjacket incorporated with bergamot (BO) and lemongrass essential oil (LO) at various concentrations. MW: High molecular weight marker; G: Gelatin; C: Control film; Numbers (5–25) denote the concentrations of BO and LO incorporated (%).

of 120, 116 and 212 kDa, respectively, as well as the cross-linked constituents were also observed in gelatin.  $\beta$ -chain of BO and LO incorporated films disappeared as the concentration increased from 0 to 25%, in comparison with that found in the gelatin (G) and the control films (C). This was coincidental with the increase in band intensity of  $\alpha$ -chain and protein with MW of 76 kDa. Small bands with MW of 74–75 kDa were predominant in films incorporated with 20–25% BO and LO. BO and LO might induce the formation of cross-links via covalent bonds. Also, it was suggested that  $\beta$ -chain was more susceptible to cross-linking induced by both essential oils. The cross-linking in film matrix was more pronounced with increasing essential oil. This was in accordance with the continuous decrease in EAB of the film (Table 1). Thus the addition of essential oils had the impact on protein patterns of films, more likely owing to the cross-linking activity of active compounds in essential oils.

### 3.8. Antimicrobial properties

Antimicrobial properties of gelatin films incorporated with BO and LO at various concentrations are presented in Table 4. BO showed the inhibition towards *S. aureus* and *L. monocytogenes*, but could not inhibit *E. coli*, *S. typhimurium* and *P. aeruginosa*. Inhibition zones increased significantly with increasing BO concentration. For LO, it was able to inhibit all microorganism tested except *P. aeruginosa*. It was noted that *E. coli*, *S. typhimurium* and *P. aeruginosa* are Gram-negative, in which cell wall lipopolysaccharides of Gram-negative bacteria might prevent active components from reaching the cytoplasmic membrane (Ouattara, Simard, Holley, Piette, & Bégin, 1997). The higher inhibition for LO

incorporated films was observed for the Gram-positive bacteria (*S. aureus* and *L. monocytogenes*), while the lower inhibition was found for the Gram-negative bacteria (*S. typhimurium* and *E. coli*). Essential oils have been employed as food additive for the preservation of a wide range of foods, including vegetables, rice, fruit, dairy products, fish, meat and poultry (Burt, 2004). The action of essential oils against food spoilage organisms and food-borne pathogens is higher against Gram-positive than Gram-negative bacteria (Rasooli, 2007). This might be due to the presence of an additional external membrane surrounding the cell wall in Gram-negative bacteria, which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering (Sharma, Jain, Yadav, & Sharma, 2010). The mechanism of antimicrobial activity of essential oils is related with the attack on the phospholipid present in cell membranes, which causes increased permeability and leakage of cytoplasm, or in their interaction with enzymes located on the cell wall (Emiroglu et al., 2010). Thus, the resistance of Gram-negative bacteria to the essential oils likely lies in the protective role of their cell wall lipopolysaccharides or outer membrane proteins. Dadalioglu and Evrendilek (2004) reported the powerful inhibitory effect of organo essential oil (OEO) on the growth of *E. coli* O157:H7, *L. monocytogenes*, *S. typhimurium*, and *S. aureus*. Antimicrobial activity of OEO was due to two components: the phenolic compound carvacrol and monoterpene *p*-cymene. Burt (2004) described the action of carvacrol as the disintegration of the external membrane of Gram-negative bacteria followed by the release of the lipopolysaccharides present, resulting in increased permeability of the cytoplasm membrane to ATP. In another study, the incorporation of 1% OEO to whey protein isolated (WPI)-based film was effective against *E. coli* O157:H7 and *Pseudomonas* species on the surface of beef (Oussalah, Caillet, Salmiéri, Saucier, & Lacroix, 2004). OEO (2%) was required for WPI films to reach the minimum inhibitory level against *S. aureus*, *S. enteritidis*, *L. monocytogenes*, *Listeria plantarum*, and *E. coli* O157:H7 (Pelissari, Grossmann, Yamashita, & Pineda, 2009). In general, BO had lower inhibitory effect towards all microorganisms tested than LO. Inhibition was increased with increasing concentration of essential oil. However, inhibition of film towards some microorganisms reached the maximum at some particular concentration. This indicated that the ability of compounds in essential oil to inhibit microorganisms was dependent on the concentration of essential oil in the films.

### 3.9. FTIR spectra

FTIR spectra of gelatin films incorporated with BO and LO at selected concentrations are depicted in Fig. 2. FTIR spectra have

**Table 4**

Antimicrobial index of gelatin films prepared from the skin of unicorn leatherjacket incorporated with bergamot (BO) and lemongrass essential oil (LO) at various concentrations.

| Film samples | <i>E. coli</i> | <i>S. typhimurium</i> | <i>P. aeruginosa</i> | <i>S. aureus</i> | <i>L. monocytogenes</i> |
|--------------|----------------|-----------------------|----------------------|------------------|-------------------------|
| Control      | 0 ± 0E         | 0 ± 0F                | 0 ± 0                | 0 ± 0fC          | 0 ± 0eE                 |
| 5% BO        | 0 ± 0          | 0 ± 0                 | 0 ± 0                | 967 ± 0e         | 0 ± 0e                  |
| 10% BO       | 0 ± 0          | 0 ± 0                 | 0 ± 0                | 1089 ± 1d        | 405 ± 2d                |
| 15% BO       | 0 ± 0          | 0 ± 0                 | 0 ± 0                | 1217 ± 1c        | 442 ± 2c                |
| 20% BO       | 0 ± 0          | 0 ± 0                 | 0 ± 0                | 1351 ± 1b        | 563 ± 1b                |
| 25% BO       | 0 ± 0          | 0 ± 0                 | 0 ± 0                | 2355 ± 1a        | 820 ± 5a                |
| 5% LO        | 0 ± 0E         | 206 ± 2E              | 0 ± 0                | 2104 ± 1B        | 1163 ± 1D               |
| 10% LO       | 831 ± 3D       | 314 ± 2D              | 0 ± 0                | 5155 ± 2A        | 1210 ± 2C               |
| 15% LO       | 964 ± 3C       | 767 ± 1C              | 0 ± 0                | 5155 ± 1A        | 3044 ± 1B               |
| 20% LO       | 990 ± 2B       | 1591 ± 2B             | 0 ± 0                | 5155 ± 1A        | 5155 ± 2A               |
| 25% LO       | 1677 ± 1A      | 1662 ± 1A             | 0 ± 0                | 5155 ± 1A        | 5156 ± 2A               |

Values are given as mean ± SD ( $n = 3$ ).

Different letters or different capital letters in the same column within the film containing the same essential oil together with the control indicate significant differences ( $P < 0.05$ ).

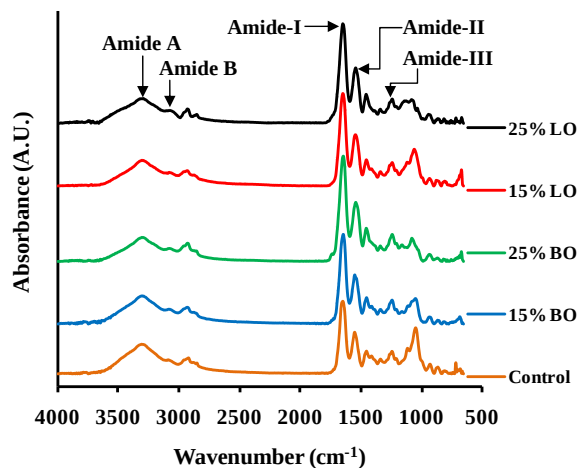


Fig. 2. Fourier transform infrared spectra of gelatin films from the skin of unicorn leatherjacket incorporated with bergamot (BO) and lemongrass essential oil (LO) at various concentrations.

been used to monitor the functional groups and structural changes of film samples at molecular level through a detailed spectral analysis. All gelatin film samples had major peaks in amide region, which showed slight differences in the spectra. These spectra were in accordance with those reported by *Muyonga, Cole, and Duodu (2004)*. Control film, 15% BO, 25% BO, 15% LO and 25% LO incorporated gelatin films displayed the amide-I bands at the wavenumbers of 1646.96, 1642.96, 1642.30, 1646.47 and 1646.92  $\text{cm}^{-1}$ , respectively. The amide-I vibration mode is primarily a C=O stretching vibration coupled with the CN stretch, CCN deformation and in plane NH bending modes (*Bandekar, 1992*). The spectral differences between different film samples in amide-I region were largely attributed to different conformation and orientation of polypeptide chains as affected by incorporation of BO. The shifts of amide-I peak to lower wavenumber of BO added films were associated with a decrease in molecular order due to conformational change (*Altiok et al., 2010*). The extent of changes in molecular order was affected by the concentration of BO and LO incorporated and intermolecular interaction formed during film drying process. These results are correlated with the decrease in tensile performance as the concentration of BO and LO incorporated in the films increased. Essential oil incorporated at higher concentration replaced the glycerol, suggesting the higher content of intra-molecular cross-linking between chains, especially  $\beta$ -chains in the gelatin molecules. Lower amplitude of amide-I peak in control films was associated with the higher extent of intermolecular interaction, more likely between C=O and adjacent gelatin chains via hydrogen bond. The characteristic absorption bands of control film, 15% BO, 25% BO, 15% LO and 25% LO incorporated films in amide-II region were noticeable at the wavenumbers of 1549.11, 1548.38, 1539.94, 1542.77 and 1540.47  $\text{cm}^{-1}$ , respectively. The amide-II vibration modes are attributed to out-of-plane combination of the NH in plane bend and the CN stretching vibration with smaller contributions from the CO in plane bend and the CC and NC stretching vibrations (*Jackson, Choo, Watson, Halliday, & Mantsch, 1995*). The shift of amide-II to lower wavenumber of essential oil incorporated films was noticed, suggesting the presence of intermolecular interaction between gelatin molecules and essential oil. *Yamane, Williams, and Barry (1995)* suggested terpene–protein interaction since terpenes could partition into the intracellular protein domains. Lower amplitude at amide-II region was also found in control film, compared to BO and LO incorporated films, indicating that N–H was more involved in bonding with the

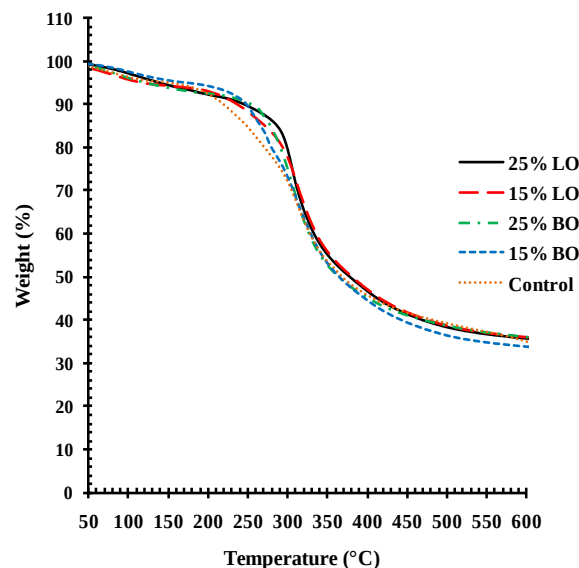


Fig. 3. Thermo-gravimetric curves of gelatin films from the skin of unicorn leatherjacket incorporated with bergamot (BO) and lemongrass essential oil (LO) at various concentrations.

adjacent molecules due to lesser amount of plasticiser. In addition, amide-III peak was detected at the wavenumbers of 1240.55, 1240.01, 1238.77, 1239.14 and 1239.08  $\text{cm}^{-1}$  for the control film, 15% BO, 25% BO, 15% LO and 25% LO incorporated films, respectively. The amide-III represents the combination peaks between C–N stretching vibrations and N–H deformation from amide linkages as well as absorptions arising from wagging vibrations from  $\text{CH}_2$  groups from the glycine backbone and proline side-chains of gelatin molecules (*Jackson et al., 1995*). The results suggested the lower molecular order of BO and LO incorporated films, compared to the control film (*Bandekar, 1992*). In addition, absorption bands at the wavenumbers of 1045.37, 1046.68 and 1056.90  $\text{cm}^{-1}$  were found in control film, 15% BO and 15% LO incorporated films, respectively. Those bands most likely arose from asymmetric stretching vibrations of –OH groups of glycerol (plasticiser) coupled to  $-\text{CH}_2$  of the amino acid residues of gelatin molecules (*Bergo & Sobral, 2007*). Lower amplitude was found at the wavenumbers of 1076.29, and 1079.18  $\text{cm}^{-1}$  due to replacement of glycerol by 25% of BO and LO, respectively (*Bergo & Sobral, 2007*). These results reconfirm that the lower glycerol was used in the films. This mainly resulted in the loss in film elasticity or flexibility.

Moreover, amide-A band, arising from the stretching vibration of N–H group, appeared at wavenumbers of 3308.81, 3307.28, 3305.92, 3307.13 and 3293.83  $\text{cm}^{-1}$  for the control film, 15% BO, 25% BO, 15% LO and 25% LO incorporated film, respectively. When the

Table 5

Thermal degradation temperature ( $T_d$ , °C) and weight loss ( $\Delta w$ , %) of gelatin films from the skin of unicorn leatherjacket incorporated with bergamot (BO) and lemongrass essential oil (LO) at selected concentrations.

| Film samples | $\Delta_1$             |              | $\Delta_2$             |              | Residue (%) |
|--------------|------------------------|--------------|------------------------|--------------|-------------|
|              | $T_{d1, \text{onset}}$ | $\Delta w_1$ | $T_{d2, \text{onset}}$ | $\Delta w_2$ |             |
| Control      | 31.67                  | 4.64         | 157.41                 | 61.23        | 34.14       |
| 15% BO       | 49.73                  | 5.16         | 244.52                 | 61.16        | 33.68       |
| 25% BO       | 44.18                  | 7.33         | 257.19                 | 56.97        | 35.68       |
| 15% LO       | 40.89                  | 5.79         | 292.46                 | 57.19        | 37.02       |
| 25% LO       | 54.22                  | 8.09         | 302.14                 | 56.44        | 35.47       |

$\Delta_1$  and  $\Delta_2$  denote the first and second stage weight loss, respectively, of film during TGA heating scan.

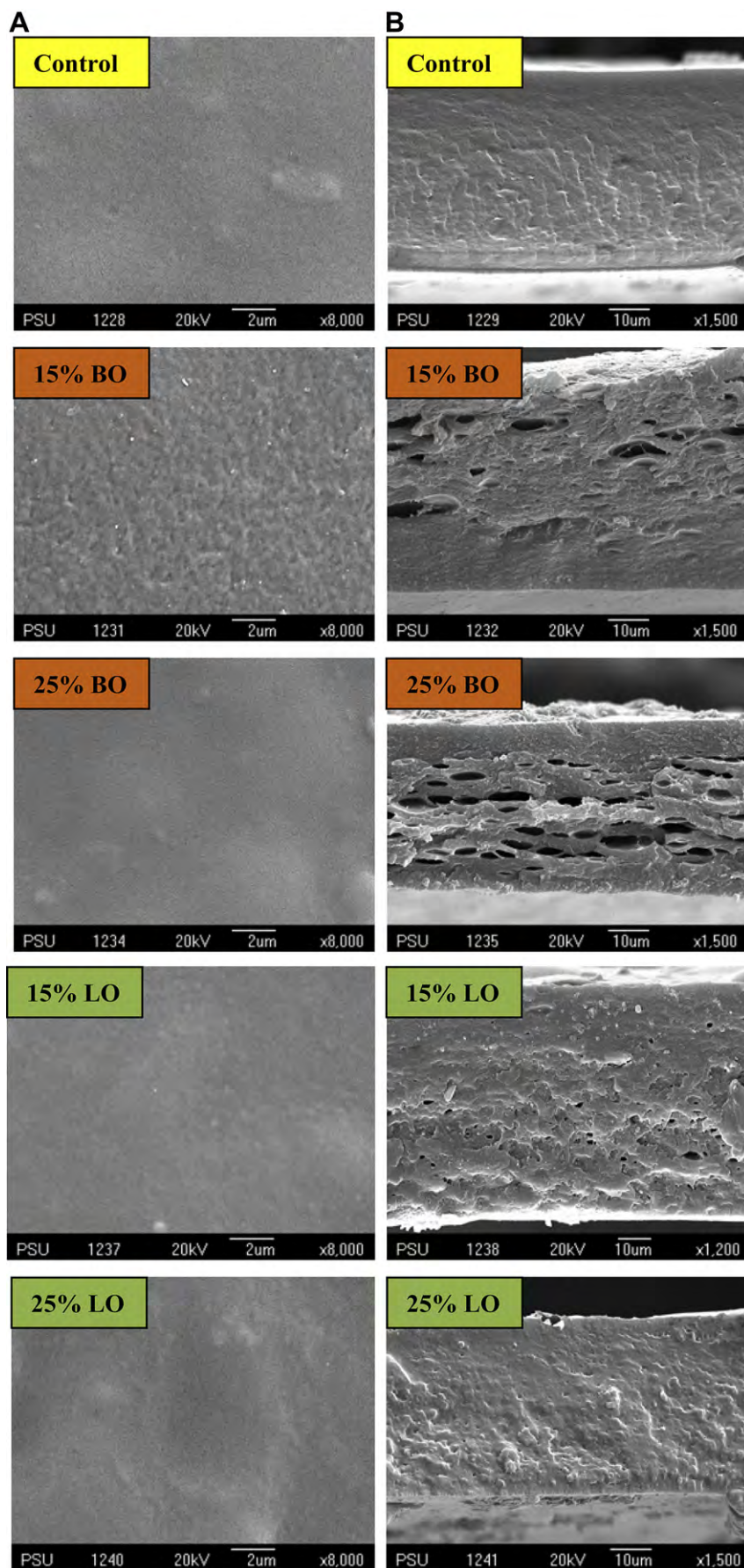


**Fig. 4.** SEM micrographs of surface (A) and cryo-fractured cross-section (B) of gelatin films from the skin of unicorn leatherjacket incorporated with bergamot (BO) and lemongrass essential oil (LO) at various concentrations.

N–H group of a peptide is involved in a hydrogen bond, the position shifted to lower frequencies (Doyle, Blout, & Bendit, 1975). In amide-A region, the lower wavenumber was found in 25% LO added film, compared to other films. The amide-B bands were observed at wavenumbers of 3089.03, 3084.76, 3078.58, 3080.97 and 3072.37  $\text{cm}^{-1}$  for the control film, 15% BO, 25% BO, 15% LO and 25% LO incorporated films, respectively, corresponding to asymmetric stretch vibration of  $\text{C}=\text{H}$  as well as  $-\text{NH}_2^+$  of peptide fragments of gelatin molecules (Ahmad & Benjakul, 2011). BO and LO incorporated films showed the lower wavenumber at amide-B region, compared to control film, suggesting the interaction of  $-\text{NH}_2$  group between peptide chains. In addition, the hydrocarbon chains of essential oils give asymmetric and symmetric  $\text{CH}_2$  vibrations at 2920–2932 and 2850–2853  $\text{cm}^{-1}$ , respectively (Lewis & McElhaney, 1996). The heights and areas of these two peaks are proportional to the amount of the essential oils present in films. Therefore, incorporation of essential oils (BO and LO) altered the molecular organisation and intermolecular interaction in the film matrix.

### 3.10. Thermo-gravimetric analysis (TGA)

TGA thermograms revealing thermal degradation behaviour of gelatin films incorporated without and with BO and LO at selected concentrations are shown in Fig. 3. Their degradation temperatures ( $T_d$ ) and weight loss ( $\Delta w$ ) are presented in Table 5. Two main stages of weight loss were observed in all films. The first stage weight loss ( $\Delta w_1 = 4.64\text{--}8.09\%$ ) was observed over the onset temperature ( $T_{d1}$ ) ranging from 41.67 to 54.22  $^{\circ}\text{C}$ , mostly associated with the loss of free water adsorbed in the film, along with the evaporation of essential oils. The second stage weight loss ( $\Delta w_2 = 61.23\%$ ) for control film appeared at the onset temperature of 157.41  $^{\circ}\text{C}$  ( $T_{d2}$ ), while films incorporated with BO and LO at 15 and 25% showed weight loss ( $\Delta w_2 = 56.44\text{--}61.16\%$ ) over the temperature ( $T_{d2}$ ) ranging from 244.52 to 302.14  $^{\circ}\text{C}$ , depending on the film samples. This was most likely due to the degradation or decomposition of protein components, glycerol and bound essential oil compounds. In general, films incorporated with essential oils had increased thermal stability compared to the control film, as evidenced by the higher  $T_{d2}$ . With the increase of essential oil content from 15 to 25% in films, the degradation temperature increased but the weight loss decreased. The similar result was found in thyme oil incorporated chitosan films (Altiok et al., 2010) and starch–chitosan films incorporated with oregano essential oil (Pelissari et al., 2009). An enhanced thermal stability of gelatin films incorporated with essential oil was attributed to the interaction between gelatin and essential oils, yielding the stronger film network, thus leading to the higher heat resistance of the resulting film, compared with control film. Hoque et al. (2011) also observed high heat stability of gelatin film incorporated with star anise extract, where higher amount of bonding between phenolic compounds of star anise extracts and gelatin molecules yielded stronger film network with increased heat resistance. Additionally, all films had residual mass (representing char content) at 600  $^{\circ}\text{C}$  in the range of 33.68–37.02%. Slight difference in char content was most likely due to different formulation of films and compositions of essential oils obtained from two different sources. Therefore, TGA curves showed clearly that essential oil incorporated contributed to a substantial improvement in the thermal stability of the gelatin film.

### 3.11. Microstructure

SEM micrographs of the surface (A) and cryo-fractured cross-section (B) of gelatin films incorporated with BO and LO at selected concentrations are illustrated in Fig. 4. The control film had

compact, smooth and homogeneous surface without grainy and porous structure, indicating that an ordered matrix was formed. Films incorporated with BO and LO at a level of 25% had relatively smoother surfaces, however pores and cavities were found in the films. BO and LO incorporated at higher concentration in the film forming solution might be dispersed more evenly. Essential oil might be evaporated during drying, leading to the formation of micro-pores throughout the films. However, the rough surface and looser texture with sponge-like structure were obtained in films incorporated with 15% BO and 15% LO. Generally, roughness of surface structure was more pronounced in films incorporated with essential oils than that found in control film. This was possibly due to the arrangements of protein molecules during film formation that might take place in different ways between two different types of films. This might be also associated with the coexisting of protruded film structure as indicated by the increased thickness of resulting films (Table 1). The existence of holes or pores might be related to the volatility of the essential oil. In fact, these spaces were filled by essential oil that had evaporated from the film surface (Sanchez-Gonzalez, Pastor, Vargas, Chiralt, & Gonzalez-Martinez, 2011). The porosity of film was different between films added with BO and LO at the same level used. The larger pore and a higher number of spaces of film added with BO was in agreement with higher WVP of this film, compared with LO incorporated films. Thus, film structure as well as the distribution of oil droplets might be associated with the properties of film, particularly water vapour permeability of resulting film.

## 4. Conclusion

The properties of gelatin films were affected by the addition of BO and LO. The BO and LO incorporated films with porous structure showed the decreased tensile performance (i.e., tensile strength and elongation at break). Both BO and LO affected the film differently due to the different modes of action determined by compounds presented in essential oils. Films incorporated with LO exhibited more antibacterial activity than BO added films against microorganisms studied. The films were generally more effective against Gram-positive bacteria (*S. aureus* and *L. monocytogenes*) than Gram-negative bacteria (*E. coli* and *S. typhimurium*), but they could not inhibit *P. aeruginosa*. Therefore, physical, mechanical and antibacterial properties of gelatin films can be modified by selected essential oil.

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## The effects of pretreatments on antioxidative activities of protein hydrolysate from the muscle of brownstripe red snapper (*Lutjanus vitta*)

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### ABSTRACT

Different pretreatments of mince from brownstripe red snapper (*Lutjanus vitta*) including 1) washing; 2) membrane separation; 3) washing followed by membrane separation and 4) membrane separation followed by washing were conducted prior to hydrolysis. Among the resulting minces, that subjected to membrane separation with subsequent washing (MS/W) contained the lowest remaining myoglobin content, phospholipid content, heme iron and non-heme iron contents ( $p < 0.05$ ) and showed the lowest TBARS values throughout 9 days of storage at 4 °C in the presence and absence of 0.15 mol L<sup>-1</sup> cupric acetate ( $p < 0.05$ ). When hydrolysates from 1) mince, 2) MS/W and 3) protein isolate from MS/W (PI) with different degree of hydrolysis (DH) (20, 30 and 40%) were prepared using proteases from pyloric caeca of brownstripe red snapper, antioxidative activities determined by DPPH, ABTS radical scavenging activities, ferric reducing antioxidant power and metal chelating activity varied with hydrolysates and DH. Antioxidative activities increased with increasing DH up to 40% ( $p < 0.05$ ). At all DH tested, hydrolysate prepared from MS/W exhibited the highest antioxidative activities determined by all assays, compared to those from mince and PI ( $p < 0.05$ ). Hydrolysate from MS/W with 40% DH had the molecular weight lower than 6.5 kDa as determined by SDS-PAGE. In liposome oxidation system, the addition of hydrolysate from MS/W resulted in the lower TBARS, compared with the control throughout the incubation period of 48 h at room temperature (25–28 °C). Therefore, fish mince with membrane separation followed by washing was the most appropriate source for production of hydrolysate possessing antioxidative activity with the lowered amount of lipids and pro-oxidants.

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### 1. Introduction

Numerous fish protein hydrolysates have been shown to have antioxidative activities such as gelatin hydrolysate from the skin of Alaska pollack (Kim et al., 2001), protein hydrolysate from the frame of yellowfin sole (Jun, Park, Jung, & Kim, 2004), protein hydrolysate from the muscle of round scad (Thiansilakul, Benjakul, & Shahidi, 2007a) and yellow stripe trevally (Klompong, Benjakul, Kantachote, & Shahidi, 2007). One critical problem of hydrolysate preparation from these proteinaceous sources is the presence of pro-oxidants such as heme proteins and unstable lipid substrates (Raghavan & Kristinsson, 2008). As a consequence, the desirable antioxidative activity can be lowered and the hydrolysate may exhibit the pro-oxidative activity to some extent.

Fish muscle contains myoglobin and other heme proteins, which in turn become the major pro-oxidants in muscle (Chaijan, Benjakul, Visessanguan, & Faustman, 2005; Thanonkaew, Benjakul, Visessanguan, & Decker, 2006). Furthermore, phospholipids, major components of cell membranes, are believed to readily prone to oxidative deterioration due to their highly unsaturated fatty acid composition (Borst, Visser, Kouptsova, & Visser, 2000). In fish, especially lean fish, phospholipids make up most of the lipids of the cell (Liang & Hultin, 2005a). The presence of these pro-oxidants and lipids could decrease the stability of protein hydrolysates and may limit their use in food systems (Raghavan & Kristinsson, 2008). Removal of these compounds could therefore alleviate such problems occurring in hydrolysate from fish muscle.

Brownstripe red snapper (*Lutjanus vitta*), a lean fish, is one of the main raw materials for surimi production in Thailand (Khantaphant & Benjakul, 2008). Apart from being processed into surimi, its flesh can be used as the raw material for production of protein hydrolysate with bioactivities. To produce protein hydrolysate from brownstripe red snapper with high antioxidative activity and negligible pro-

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oxidant or lipids susceptible to oxidation, the appropriate pretreatment of muscle by washing and/or membrane separation before hydrolysis should therefore taken into consideration.

Pyloric caeca of various fish has been reported to be the important source of trypsin such as arabesque greenling (Kishimura, Hayashi, Miyashita, & Nonami, 2006), New Zealand hoki (Shi, Marshall, & Simpson, 2007) and bluefish (Klomkiao, Benjakul, Visessanguan, Kishimura, & Simpson, 2007). Recently, protease from pyloric caeca of brownstripe red snapper has been shown to hydrolyze fish skin gelatin effectively (Khantaphant & Benjakul, 2008). Along with the pretreatment, proteases from pyloric caeca of brownstripe red snapper could be used as the potential aid for the preparation of protein hydrolysate with antioxidative activity, in which the new value-added product of health benefit could be produced. The objectives of this study were to elucidate the effect of washing and membrane separation on the removal of pro-oxidant and membranes and to produce protein hydrolysates from pretreated mince of brownstripe red snapper with the lowered pro-oxidative activity using proteases from its pyloric caeca.

## 2. Materials and methods

### 2.1. Chemicals

2,4,6-trinitrobenzenesulfonic acid (TNBS), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2, 4, 6-tripyridyl-triazine (TPTZ), 1,1,3,3-tetramethoxypropane, bathophenanthroline disulfonic acid and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid (ferrozine) were purchased from Sigma (St. Louis, MO, USA). Thiobarbituric acid (TBA) and potassium persulfate were obtained from Fluka (Buchs, Switzerland). Trichloroacetic acid (TCA), iron standard solution and sodium nitrite were procured from Merck (Darmstadt, Germany). Sodium sulfite and ammoniumthiocyanate were obtained from Riedel-deHaën (Seelze, Germany). All chemicals were of analytical grade.

### 2.2. Preparation of mince with different pretreatments

#### 2.2.1. Mince preparation

Brownstripe red snapper, stored in ice and off-loaded approximately 3 days after capture, were purchased from a dock in Songkhla province, Thailand. Fish were transported in ice with the fish/ice ratio of 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, whole fish were washed and only flesh was separated manually. Flesh was minced to uniformity using a Moulinex AY46 blender (Group SEB, Lyon, France). The mince obtained was placed in polyethylene bags and kept in ice not longer than 2 h before use.

#### 2.2.2. Preparation of washed mince

Mince was homogenized with five volumes of cold distilled water using an IKA Labortechnik homogenizer (Selangor, Malaysia) at a speed of 11,000 rpm for 2 min, followed by stirring at 4 °C for 15 min prior to centrifuging at  $9600 \times g$  for 10 min at 4 °C using a Beckman Coulter centrifuge Model Avant J-E (Beckman Coulter, Inc., Fullerton, CA, USA). The washing process was repeated twice. The sample obtained was referred to as 'washed mince; W'.

#### 2.2.3. Preparation of mince with membrane separation

Membrane was removed from mince by treatment with  $\text{Ca}^{2+}$  and citric acid according to the method of Liang and Hultin (2005b) with a slight modification. Mince was homogenized with nine volumes of cold  $8 \text{ mmol L}^{-1}$   $\text{CaCl}_2$  solution in the presence of  $5 \text{ mmol L}^{-1}$  citric acid using a homogenizer at a speed of 11,000 rpm for 2 min. After continuous stirring for 60 min at 4 °C, the sample

was centrifuged at  $4000 \times g$  for 15 min at 4 °C and the mince obtained was referred to as 'membrane separated mince; MS'.

#### 2.2.4. Preparation of membrane separated/washed mince and washed/membrane separated mince

Minces subjected to washing prior to membrane separation (W/MS) and to membrane separation followed by washing (MS/W) were also prepared. All samples were subjected to analyses.

### 2.3. Analyses

#### 2.3.1. Determination of myoglobin content

Myoglobin content was determined by direct spectrophotometric method (Chaijan et al., 2005). Sample (2.0 g) was mixed with 20 mL of cold  $40 \text{ mmol L}^{-1}$  phosphate buffer (pH 6.8), followed by homogenization at 13,500 rpm for 10 s. The mixture was centrifuged at  $3000 \times g$  for 30 min at 4 °C and the supernatant was filtered through Whatman filter paper No. 1 (Schleicher & Schuell, Maidstone, England). The absorbance of supernatant was read at 525 nm. Myoglobin content was calculated from the molar extinction coefficient of  $7.6 \times 10^{-3}$  and a molecular weight of 16,110 (Gomez-Basauri & Regenstein, 1992). The myoglobin content was expressed as mg/100 g dry sample.

#### 2.3.2. Determination of heme iron and non-heme iron contents

Heme iron content was calculated based on the content of myoglobin, which contains iron at a level of 0.35 g/100 g (Gomez-Basauri & Regenstein, 1992). Heme iron content was expressed as mg/100 g dry sample.

Non-heme iron content was determined according to the method of Chaijan et al. (2005). Sample (1.0 g) was weighed and transferred into a screw cap test tube and 50 mL of  $3.9 \text{ g L}^{-1}$  sodium nitrite and 4 mL of a mixture of  $400 \text{ g L}^{-1}$  TCA and  $6 \text{ mol L}^{-1}$  HCl (1:1 (v/v) ratio, freshly prepared) were added. The tightly capped tubes were placed in a temperature controlled water bath (Model W350, Memmert, Schwabach, Germany) for 22 h at 65 °C followed by cooling to room temperature (25–28 °C) for 2 h. The supernatant (400  $\mu\text{L}$ ) was mixed with 2 mL of the non-heme iron color reagent, a freshly prepared mixture of bathophenanthroline disulfonic acid, double-deionized water and saturated sodium acetate solution at a ratio of 1:20:20 (w/v/v). After thorough mixing, the mixture was allowed to stand for 10 min and the absorbance was measured at 540 nm. The non-heme iron content was calculated from the iron standard curve using iron standard solution (0–2  $\text{mg L}^{-1}$ ). The non-heme iron content was expressed as mg/100 g dry sample.

#### 2.3.3. Determination of phospholipid content

Phospholipid content was determined based on the direct spectrophotometric measurement of complex formation between phospholipids and ammonium ferrothiocyanate as described by Stewart (1980). Lipids were extracted from the sample by the method of Bligh and Dyer (1959). Thereafter, lipids (20  $\mu\text{L}$ ) were dissolved in chloroform to a final volume of 2 mL. One mL of thiocyanate reagent (a mixture of  $0.10 \text{ mol L}^{-1}$  ferric chloride hexahydrate and  $0.40 \text{ mol L}^{-1}$  ammoniumthiocyanate) was added. After thorough mixing for 1 min, the lower layer was removed and the absorbance at 488 nm was measured. A standard curve was prepared with phosphatidylcholine (0–0.1  $\text{g L}^{-1}$ ). The phospholipid content was expressed as mg/100 g dry sample.

### 2.4. Occurrence of lipid oxidation in mince with different pretreatments

Mince with different pretreatments was mixed with sodium azide as an antimicrobial agent to obtain a final concentration of

0.1 g kg<sup>-1</sup>. The mixture (50 g) was added with 20 µL of 0.15 mol L<sup>-1</sup> cupric acetate and the samples were stored at 4 °C and taken every 3 days for determination of lipid oxidation.

#### 2.4.1. Determination of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) were determined as described by Benjakul, Visessanguan, Phongkanpai, and Tanaka (2005) with a slight modification. Sample was homogenized with TBARS solution (3.75 g L<sup>-1</sup> TBA, 150 g L<sup>-1</sup> TCA and 0.25 mol L<sup>-1</sup> HCl) with a ratio of 1:4 (w/v). The mixture was heated in boiling water for 10 min to develop the pink color. Then the mixture was cooled with running water and centrifuged at 5000 × g for 10 min at room temperature using Hettich centrifuge (Model MIKRO-20, Hettich, Tuttlingen, Germany). The supernatant was collected and measured at 532 nm using a UV-1601 Spectrophotometer (Shimadzu, Kyoto, Japan). TBARS was calculated from a standard curve of malonaldehyde (MDA) (0–10 mg L<sup>-1</sup>) and expressed as mg MDA/kg sample.

#### 2.4.2. Determination of conjugated diene

Conjugated diene formed in liposome oxidation system was measured according to the method of Frankel, Huang, and Aeschbach (1997). Sample (0.1 mL) was dissolved in methanol (5.0 mL) and conjugated dienes were measured as the increase in absorbance at 234 nm.

#### 2.5. Dissociation of actomyosin complex in mince with different pretreatments

Mince with different pretreatments were determined for ATPase activity. Prior to assay, natural actomyosin (NAM) was prepared as described by Benjakul, Seymour, Morrissey, and An (1997) with a slight modification. Pretreated mince was homogenized in cold 0.6 mol L<sup>-1</sup> KCl (pH 7.0) at a ratio of 1:10 (w/v) using a homogenizer with a speed of 11,000 rpm for 20 s, followed by a 20 s rest interval to a total extraction time of 4 min. Thereafter, the homogenate was centrifuged at 5000 × g for 30 min at 4 °C. Three volumes of cold distilled water were added to precipitate NAM prior to centrifugation at 5000 × g for 20 min at 4 °C. NAM pellet was dissolved in a minimal volume of cold 50 mmol L<sup>-1</sup> potassium phosphate buffer (pH 7.0) containing 0.6 mol L<sup>-1</sup> KCl. NAM solution was kept in ice and used within 1 h.

Mg<sup>2+</sup>-ATPase activities of NAM were assayed according to the method of Benjakul et al. (1997). NAM solution (0.5 mL) was added into 0.3 mL of 0.5 mol L<sup>-1</sup> Tris-maleate buffer (pH 7.0) and 2 mmol L<sup>-1</sup> MgCl<sub>2</sub> was added to a mixture to a final volume of 9.5 mL. Thereafter, 0.5 mL of 20 mmol L<sup>-1</sup> ATP was added to initiate the reaction. The reaction was conducted at 25 °C for exactly 10 min and 5 mL of cold 150 g L<sup>-1</sup> TCA were added to terminate the reaction. The reaction mixture was centrifuged at 3500 × g for 5 min and the inorganic phosphate liberated in the supernatant was measured as described by Fiske and Subbarow (1925). Specific activity was expressed as µmol inorganic phosphate released/mg protein/min. Blank was performed by adding the cold TCA prior to the addition of ATP. The decrease in Mg<sup>2+</sup>-ATPase indicated the dissociation of actomyosin complex, in which actin was not stable in the presence of high salt concentration (Torikai & Konno, 1996).

#### 2.6. Preparation of proteases from pyloric caeca

Pyloric caeca from brownstripe red snapper was collected and powered in liquid nitrogen. Thereafter, the pyloric caeca extract was prepared according to the method of Khantaphant and Benjakul (2008) with a slight modification. Pyloric caeca powder was suspended in ten volumes of extraction buffer (50 mmol L<sup>-1</sup> Tris–HCl

buffer, pH 8.0 containing 10 mmol L<sup>-1</sup> CaCl<sub>2</sub>). The mixture was homogenized using a homogenizer at 11,000 rpm for 2 min. The homogenate was continuously stirred for 30 min at 4 °C and centrifuged at 8000 × g for 30 min at 4 °C. The supernatant was filtered through a Whatman filter paper No. 1. The filtrate obtained was further subjected to 40–60% saturation ammonium sulfate precipitation. After stirring at 4 °C for 30 min, the mixture was centrifuged at 8000 × g for 30 min at 4 °C and the pellet obtained was dissolved in 50 mmol L<sup>-1</sup> Tris–HCl buffer, pH 8.0 followed by dialysis against 20 volumes of the extraction buffer overnight at 4 °C with three changes of dialysis buffer. The dialysate was kept in ice and referred to as 'pyloric caeca extract; PCE'. PCE was determined for proteolytic activity using casein as a substrate under optimal condition (60 °C and pH 8.5) (Khantaphant & Benjakul, 2008).

#### 2.7. Preparation of brownstripe red snapper protein hydrolysates

Mince, mince subjected to membrane separation and washing (MS/W) and protein isolate (PI) were used for preparation of protein hydrolysate. PI was prepared as described by Rawdkuen, Sai-Ut, Khamsorn, Chaijan, and Benjakul (2009) with a slight modification. MS/W mince was homogenized with nine volumes of cold distilled water at a speed of 11,000 rpm for 1 min. The homogenate was adjusted to pH 11.2 using 2 mol L<sup>-1</sup> NaOH. Homogenate was then centrifuged at 5000 × g for 10 min at 4 °C. The alkaline-soluble fraction was collected and adjusted to pH 5.5 (the isoelectric point of muscle proteins) using 2 mol L<sup>-1</sup> HCl, followed by centrifuging at 5000 × g for 10 min at 4 °C. The pellet was mixed with distilled water at a ratio of 1:2 (w/v) and the final pH was adjusted to 7.0 with 2 mol L<sup>-1</sup> NaOH. The sample obtained was referred to as 'protein isolate; PI'.

To prepare protein hydrolysate, the samples were mixed with 50 mmol L<sup>-1</sup> Tris–HCl buffer, pH 8.5 to obtain the protein concentration of 20 g L<sup>-1</sup>. The mixtures were homogenized at a speed of 11,000 rpm for 1 min and the homogenates were pre-incubated at 60 °C for 10 min. Hydrolytic reaction was started by adding PCE with different levels to gain the desirable degree of hydrolysis of 20, 30 and 40% following the method of Benjakul and Morrissey (1997). After 2 h of hydrolysis, the reactions were terminated by heating the mixture in boiling water for 10 min. The mixtures were centrifuged at 2000 × g at 4 °C for 10 min and the supernatants were collected and referred to as 'protein hydrolysates'. All protein hydrolysates were determined for antioxidative activities.

#### 2.8. Determination of antioxidative activities

##### 2.8.1. DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to the method of Khantaphant and Benjakul (2008). Sample solution (1.5 mL) was added with 1.5 mL of 0.1 mmol L<sup>-1</sup> 1, 1-diphenyl-2-picrylhydrazyl (DPPH) in 950 mL L<sup>-1</sup> ethanol. The mixture was allowed to stand for 30 min in dark at room temperature. The resulting solution was measured at 517 nm. The blank was prepared in the same manner except that distilled water was used instead of the sample. The DPPH radical scavenging activity was calculated from Trolox standard curve (0–60 µmol L<sup>-1</sup>) and expressed as µmol Trolox equivalents (TE)/g protein.

##### 2.8.2. ABTS radical scavenging activity

ABTS radical scavenging activity was determined as described by Re et al. (1999) and modified slightly by Khantaphant and Benjakul (2008). ABTS radical (ABTS<sup>•+</sup>) was produced by reacting ABTS stock solution (7.4 mmol L<sup>-1</sup> 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)) with 2.6 mmol L<sup>-1</sup> potassium persulfate at the ratio of 1:1 (v/v). The mixture was allowed to react in dark for 12 h at

room temperature. Prior to assay, ABTS<sup>•+</sup> solution was diluted with methanol to obtain an absorbance of 1.1 ( $\pm 0.02$ ) at 734 nm. To initiate the reaction, 150  $\mu$ L of sample was mixed with 2.85 mL of ABTS<sup>•+</sup> solution. The absorbance was then read at 734 nm after 2 h dark incubation at room temperature. ABTS radical scavenging activity was expressed as  $\mu$ mol TE/g protein.

### 2.8.3. Ferric reducing antioxidant power (FRAP)

The ability of samples to reduce ferric ion ( $\text{Fe}^{3+}$ ) was evaluated by the method of Benzie and Strain (1996). FRAP reagent (a freshly prepared mixture of 10 mmol L<sup>-1</sup> TPTZ solution in 40 mmol L<sup>-1</sup> HCl, 20 mmol L<sup>-1</sup>  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  solution and 300 mmol L<sup>-1</sup> acetate buffer, pH 3.6 (1:1:10 v/v/v)) (2.85 mL) was incubated at 37 °C for 30 min prior to mixing with 150  $\mu$ L of sample. The reaction mixture was allowed to stand in dark for 30 min at room temperature. Absorbance at 593 nm was read and FRAP was expressed as  $\mu$ mol TE/g protein.

### 2.8.4. Ferrous chelating activity

Chelating activity of protein hydrolysate towards ferrous ion ( $\text{Fe}^{2+}$ ) was measured by the method of Benjakul et al. (2005) with a slight modification. Sample (200  $\mu$ L) was mixed with 800  $\mu$ L of distilled water. Thereafter, 0.1 mL of 2 mmol L<sup>-1</sup>  $\text{FeCl}_2$  and 0.2 mL of 5 mmol L<sup>-1</sup> ferrozine were added. The mixture was allowed to react for 20 min at room temperature. The absorbance was then read at 562 nm. The standard curve of EDTA (0–2 mmol L<sup>-1</sup>) was prepared and ferrous chelating activity was expressed as  $\mu$ mol EDTA equivalents/g protein.

### 2.9. Determination of antioxidative activity in a lecithin liposome system

The antioxidative activity of protein hydrolysates in a lecithin liposome system was determined according to the method of Frankel et al. (1997) slightly modified by Thiansilakul et al. (2007a). Lecithin liposome system was prepared by suspending lecithin in deionized water at a concentration of 8 g L<sup>-1</sup>. The mixture was stirred with a glass rod followed by sonicating for 30 min in a sonicating bath (Model S30H, Elma, Singen, Germany). Protein hydrolysate (3 mL) was added to the lecithin liposome system (15 mL) to a final concentration of 1000 mg L<sup>-1</sup> followed by sonicating the liposome suspension for 2 min. To initiate the reaction, 20 mL of 0.15 mol L<sup>-1</sup> cupric acetate were added. The mixture was shaken at 120 rpm using a shaker (Heidolph Model Unimax 1010, Schwabach, Germany) at 37 °C in dark. The control and systems containing 25 or 100 mg L<sup>-1</sup> BHT were also prepared. Oxidation in lecithin liposome systems was monitored at 6 h intervals by determining the formation of TBARS and conjugated dienes.

### 2.10. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were determined for protein pattern using SDS-PAGE (Laemmli, 1970) with 40 g L<sup>-1</sup> stacking gel and 100 or 200 g L<sup>-1</sup> separating gel for mince without and with hydrolysis, respectively. Samples were mixed with sample buffer (0.125 mol L<sup>-1</sup> Tris–HCl, pH 6.8 containing 40 g L<sup>-1</sup> SDS and 200 mL L<sup>-1</sup> (v/v) glycerol at a ratio of 1:1 (v/v)). Proteins (15  $\mu$ g) were loaded onto the gel. The electrophoresis was run at a constant current of 15 mA per gel by a Mini-Protean II Cell apparatus (Bio-Rad, Hercules, CA, USA). The gels were fixed and stained with 0.5 g L<sup>-1</sup> Coomassie Brilliant Blue R-250 in 150 mL L<sup>-1</sup> methanol and 50 mL L<sup>-1</sup> acetic acid and destained in 300 mL L<sup>-1</sup> methanol and 100 mL L<sup>-1</sup> acetic acid. Wide range molecular weight marker was used to estimate the molecular weight.

### 2.11. Determination of protein concentration

Protein concentration was measured by the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as a standard.

### 2.12. Statistical analysis

Experiments were run in triplicate. All data were subjected to analysis of variance (ANOVA) and differences between means were evaluated by Duncan's multiple range test (Steel & Torrie, 1980). SPSS Statistic Program (Version 10.0) (SPSS Inc, Chicago, IL, USA) was used for data analysis.

## 3. Results and discussion

### 3.1. Effect of different pretreatment on composition of mince

Mince from brownstripe red snapper with different pretreatments including wash mince (W), membrane separated mince (MS), washed/membrane separated mince (W/MS) and membrane separated/washed mince (MS/W) had the different compositions (Fig. 1). All treatments resulted in the decreases in myoglobin, phospholipid, heme iron and non-heme iron contents, compared with the control (C) ( $p < 0.05$ ). However, washing showed no profound effect on non-heme iron content ( $p > 0.05$ ) (Fig. 1(d)). In general, mince subjected to both washing and membrane separation (W/MS and MS/W) showed the higher decreases in myoglobin, phospholipid and heme iron, compared with those pretreated with only one process (W and MS) ( $p < 0.05$ ). The result suggested that pretreatment by washing together with membrane separation was able to remove heme pigment as well as phospholipids from mince. Phospholipids are the major lipids in cell membranes (Borst et al., 2000).

Washing is the process used for the removal of sarcoplasmic protein and pro-oxidative aqueous components naturally present in fish muscle (Chen, Chiu, & Huang, 1997; Sannaveerappa, Sandberg, & Undeland, 2007). Myoglobin in ordinary muscle of sardine and mackerel mince was removed by 23 and 75% by washing process (Chaijan, Benjakul, Visessanguan, & Faustman, 2004). Thus, washing could lower the myoglobin and heme iron effectively. Baxter and Skonberg (2008) reported that washing not only removed sarcoplasmic proteins, but also concentrated the myofibrillar proteins. After washing, mince had the same muscle structure with the reduced content of aqueous pro-oxidants and endogenous triacylglycerols (Larsson, Almgren, & Undeland, 2007). In addition, washing could lower the phospholipid content in the resulting mince. Due to the polar head of phospholipid associated with cell membrane, it was more likely leached out during washing to some extent. After washing, mince had the decreases in myoglobin, phospholipid, heme iron and non-heme iron by 49.56, 42.55, 49.56 and 3.24%, compared with those found in the mince, respectively. When the mince was subjected to membrane separation, the lower content of phospholipid was obtained ( $p < 0.05$ ).

In muscle tissue, phospholipid membranes are connected with cytoskeletal proteins through electrostatic attraction between the acidic phospholipids of membranes and the basic amino acid residues of the cytoskeletal proteins (Haleva, Ben-Tal, & Diamant, 2004). When mince was subjected to membrane separation using the solution containing  $\text{Ca}^{2+}$  ion and citric acid, the interaction between  $\text{Ca}^{2+}$  with the polar head of phospholipids as well as the competition between citric acid and the acidic phospholipids of membranes bound to the basic amino acid residues of cytoskeletal proteins more likely took place. As a result, the release of phospholipid membrane from attached cytoskeletal proteins occurred. Liang and Hultin (2005a) reported that polycarboxylic groups of citric acid might

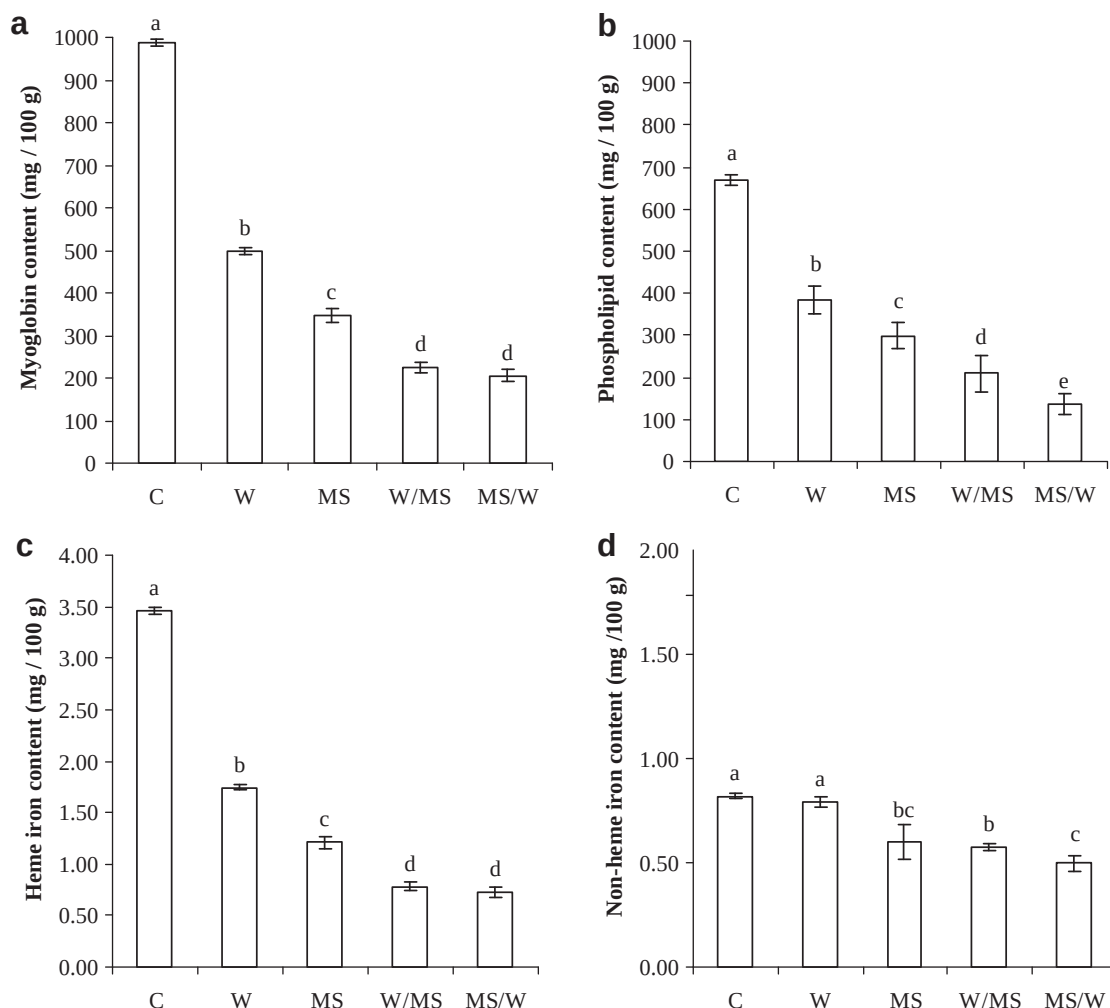


**Fig. 1.** Myoglobin (a), phospholipid (b), heme iron (c) and non-heme iron (d) contents of brownstripe red snapper mince with different pretreatments; W: washed mince, MS: membrane separated mince, W/MS: washed/membrane separated mince and MS/W: membrane separated/washed mince. Bars represent the standard deviation from triplicate determinations. Different letters indicate significant differences ( $p < 0.05$ ).

compete with acidic phospholipids to bind with the basic amino acid residues of the cytoskeletal proteins, while  $\text{Ca}^{2+}$  induced the formation of calcium–phospholipid complex. Apart from the removal of phospholipids, the membrane separation process used also resulted in the lowering of myoglobin, heme iron and non-heme iron. Due to the polar nature of the medium used, myoglobin as well as iron could be soluble in the medium. Furthermore, [Chen et al. \(1997\)](#) reported that washing with cold water for 15 min eliminated 34% oil, compared with unwashed mince and 83% oil elimination was obtained when washing time of 135 min was used.

After membrane separation process, myoglobin, phospholipid, heme and non-heme irons were removed by 65.04, 55.33, 65.04 and 26.97%, compared to those found in mince. When both washing and membrane separation processes were applied, all components tested decreased to a higher extent, compared to those found in mince with only one pretreatment or mince without pretreatment. Therefore, both processes exhibited the synergistic effect on removal of pro-oxidant and phospholipid membrane. It was noted that the sample with membrane separation, followed by washing had the lower phospholipid and non-heme iron contents than those with prior washing and membrane separation ( $p < 0.05$ ). However, no differences in myoglobin and heme iron contents were noticeable between both samples ( $p > 0.05$ ). Therefore, membrane separation/washing process was the appropriate pretreatment as

evidenced by the lowest contents of pro-oxidants and phospholipids in prepared mince.

### 3.2. Lipid oxidation in mince with different pretreatments

The remaining pro-oxidants and phospholipid membrane in mince might accelerate the oxidative reaction during handling or storage. During 9 days of storage at 4 °C, TBARS of mince with different pretreatments in the presence and absence of  $\text{Cu}^{2+}$  are depicted in [Fig. 2](#). In the absence of  $\text{Cu}^{2+}$ , TBARS in all pretreated samples increased as the storage time increased up to 6 days ( $p < 0.05$ ). Nevertheless, TBARS of the control sample had the continuous increase in TBARS throughout the storage time of 9 days ( $p < 0.05$ ). At day 9, TBARS of the control was almost 3-fold higher than that of pretreated mince. At the same storage time, the control showed the higher TBARS, compared with other samples with pretreatment ( $p < 0.05$ ). The pronounced formation of TBARS in mince indicated the susceptibility of mince, which might be associated with the high content of phospholipid membrane with high PUFA as well as pro-oxidants. Among different pretreated mince, MS/W showed the lowest TBARS value, compared with other samples during 3–9 days of storage ( $p < 0.05$ ). The lowest TBARS of MS/W was in agreement with the lowest remaining phospholipid and other pro-oxidative components ([Fig. 1](#)).

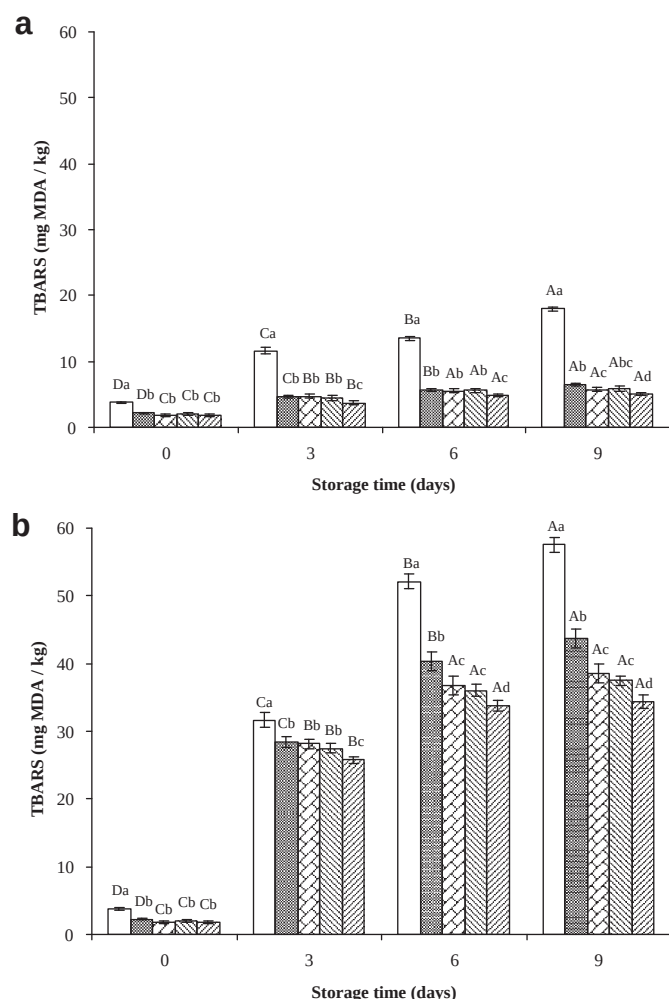


**Fig. 2.** TBARS value of brownstripe red snapper mince with different pretreatments in the absence (a) and presence (b) of  $0.15 \text{ mol L}^{-1}$  cupric acetate, during storage at  $4^\circ\text{C}$  for 9 days. C: control mince ( $\square$ ), W: washed mince ( $\▤$ ), MS: membrane separated mince ( $\▨$ ), W/MS: washed/membrane separated mince ( $\▩$ ) and MS/W: membrane separated/washed mince ( $\▧$ ). Bars represent the standard deviation from triplicate determinations. Different capital letters within the same pretreatment indicate significant differences ( $p < 0.05$ ) and different letters within the same day of storage indicate significant differences ( $p < 0.05$ ).

In the presence of  $\text{Cu}^{2+}$ , all samples showed the higher TBARS, in comparison with those without  $\text{Cu}^{2+}$ .  $\text{Cu}^{2+}$  has been used as the catalyst for lipid oxidation in different systems (Decker, Crum, & Calvert, 1992; Thanonkaew et al., 2006). The oxidation rates of all samples added with  $\text{Cu}^{2+}$  were enhanced as shown by higher TBARS. Thanonkaew et al. (2006) studied the effects of metal ions ( $\text{Fe}^{2+}$  and  $\text{Cu}^{2+}$ ) addition to cuttlefish paste and found that the metal ions could accelerate lipid oxidation. Moreover, the remaining pro-oxidants in mince might accelerate the oxidative deterioration of lipids, particularly phospholipids with high PUFA content. The similar results were obtained, compared to those without  $\text{Cu}^{2+}$ , in which MS/W containing the lowest phospholipids had the lowest TBARS throughout the storage time, compared with other samples ( $p < 0.05$ ).

### 3.3. Hydrolysis of mince using protease from pyloric caeca of brownstripe red snapper

MS/W, PI and mince were used to produce protein hydrolysates using protease from pyloric caeca of brownstripe red snapper. It

was noted that mince contained myosin heavy chain (MHC) and actin as the major proteins. For pretreated sample (MS/W) and PI, proteins with molecular weight of 26.9, 32.5 and 43.2 kDa disappeared. During washing or membrane separation, some sarco-plasmic proteins and some cytoskeletal proteins interacted with phospholipid membrane were removed. Similar protein patterns were observed between MS/W and PI. For all samples, the typical initial rapid hydrolysis rate was observed within the first 30 min as evidenced by the sharp increase in DH, followed by a slower hydrolysis rate (Fig. 3). Firstly, a large number of peptide bonds were hydrolyzed (Shahidi, Xiao-Qing, & Synowiecki, 1995). Thereafter, proteases might have the lower activity towards the proteins or peptides with the shorter chains. At the same hydrolysis time, the higher DH was obtained in hydrolysate prepared with the

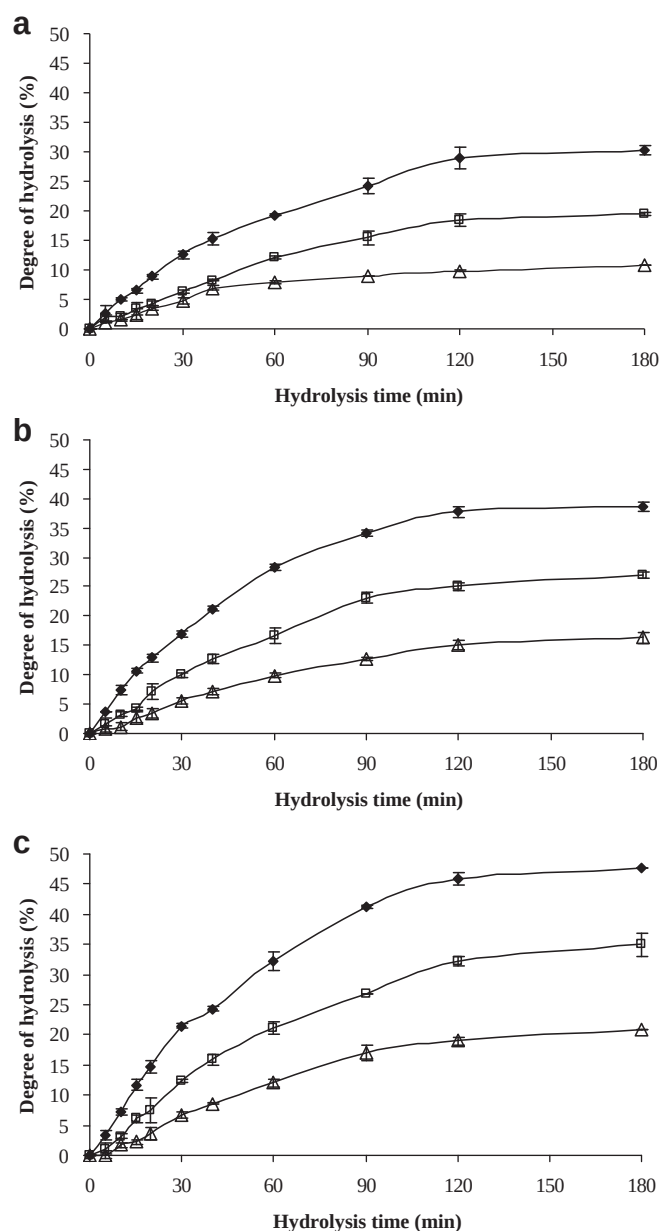


**Fig. 3.** Changes in degree of hydrolysis (DH) of control mince (a), membrane separated/washed mince (MS/W) (b) and protein isolate (PI) (c), using protease from pyloric caeca of brownstripe red snapper at various levels: ( $\triangle$ ): 1 unit/g, ( $\square$ ): 2 units/g and ( $\blacklozenge$ ): 4 units/g. The reactions were performed at  $60^\circ\text{C}$  and pH 8.5. Bars represent the standard deviation from triplicate determinations.

higher activity of PCE ( $p < 0.05$ ). The result suggested that higher amount of proteases used, the more peptide bonds were cleaved. At the same protease level used, PI was found to be the most susceptible to cleavage as indicated by the highest DH ( $p < 0.05$ ).

Elimination of other proteins as well as lipids, which functioned as the barrier for hydrolysis of myofibrillar proteins by proteases, also enhanced the hydrolysis induced by proteases. Moreover, membrane separation process might cause the structural alternation of proteins by interruption of electrostatic linkage between proteins by  $\text{Ca}^{2+}$  and citric acid. Furthermore, the changes in muscle protein configuration of pretreated mince were also supported by the decreases in  $\text{Mg}^{2+}$ -ATPase activity. A decrease in  $\text{Mg}^{2+}$ -ATPase activity by 13.75%, compared with that found in mince (data not shown), was found in natural actomyosin extracted from MS/W, indicating the disassociation of actomyosin molecule.  $\text{Mg}^{2+}$ -ATPase activity is used as an indicator of conformational changes of actomyosin complex (Benjakul et al., 1997). The decrease in  $\text{Mg}^{2+}$ -ATPase activity confirmed the dissociation of actin from myosin (Torigai & Konno, 1996). Therefore, the dissociation of actomyosin might lead to the increased susceptibility of actin and myosin to be hydrolyzed by proteases used for hydrolysis. The loosen protein structures were more prone to be cleaved by proteases. When PI was used as substrate for hydrolysis, the greater hydrolysis took place. During alkaline solubilisation, the repulsion between protein molecules resulted in the dissociation of actomyosin complex. This was indicated by the marked decrease in  $\text{Mg}^{2+}$ -ATPase activity by 36.27%, in comparison with mince (data not shown). As a consequence, the individual or looser complex could be cleaved with ease as evidenced by the higher DH. Thus, the configuration of protein was another important factor governing the rate of hydrolysis.

### 3.4. Antioxidative activities of protein hydrolysate prepared from mince with different pretreatments

#### 3.4.1. DPPH radical scavenging activity

DPPH radical scavenging activities of H-C, H-MS/W and H-PI with different DHs are shown in Fig. 4a. As DH increased from 20 to 30%, H-MS/W showed the increase in DPPH radical scavenging activity but no differences were found between the samples with 30 and 40% DH ( $p > 0.05$ ). No differences in DPPH radical scavenging activity were observed for H-C and H-PI at all DHs ( $p > 0.05$ ). DPPH radical scavenging activity indicated the hydrogen-donating ability of antioxidant (Khantaphant & Benjakul, 2008). The result suggested that H-MS/W with 30 and 40% DH could act as hydrogen donors more effectively than other hydrolysates. Protein hydrolysates from mackerel (*Scomber austriasicus*) (Wu, Chen, & Shiau, 2003) and round scad (*Decapterus maruadsi*) (Thiansilakul et al., 2007a) prepared using enzymatic hydrolysis were reported to possess DPPH radical scavenging activity.

#### 3.4.2. ABTS radical scavenging activity

H-C, H-MS/W and H-PI showed the increase in ABTS radical scavenging activities with increasing DH ( $p < 0.05$ ) (Fig. 4(b)). The highest activity was observed in all hydrolysates with 40% DH ( $p < 0.05$ ). However, no difference in activity was found between H-PI with 20 and 30% DH ( $p > 0.05$ ). Among the hydrolysates, H-C showed the lowest ABTS radical scavenging activity, whereas hydrolysate from MS/W exhibited the highest activity for all DH tested ( $p < 0.05$ ). At 40% DH, H-MS/W had the highest ABTS radical scavenging activity, compared with other hydrolysates ( $p < 0.05$ ). ABTS radical assay determine the antioxidative activity, in which the radical is quenched to form ABTS-radical complex (Khantaphant & Benjakul, 2008). Protein hydrolysates derived from loach (*Misgurnus anguillicaudatus*) (You, Zhao, Cui, Zhao, & Yang, 2009), yellow stripe trevally (*Selaroides leptolepis*) (Klompang, Benjakul,

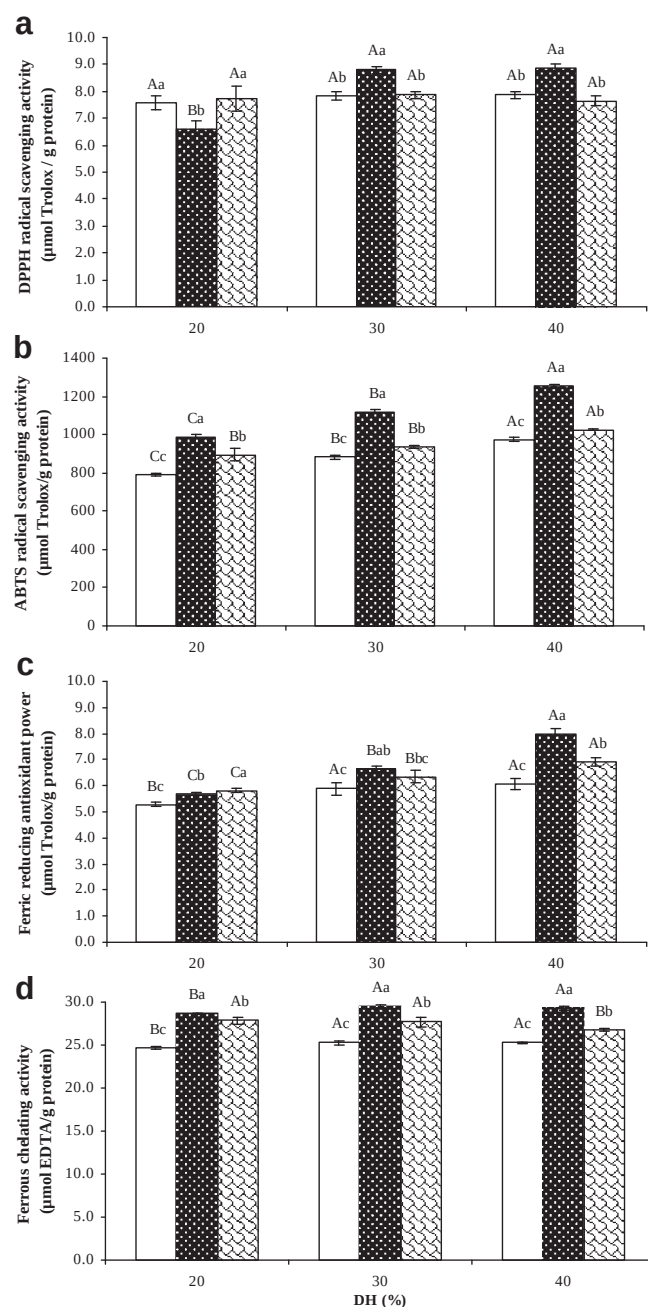


**Fig. 4.** DPPH radical scavenging activity (a), ABTS radical scavenging activity (b), ferric reducing antioxidant power (c) and metal chelating activity (d) of hydrolysates prepared from control mince (□), MS/W: membrane separated/washed mince (▨) and PI: protein isolate (▩) using protease from pyloric caeca with various degrees of hydrolysis (DHs). Bars represent the standard deviation from triplicate determinations. Different capital letters within the same pretreatment indicate significant differences ( $p < 0.05$ ) and different letters within the same DH indicate significant differences ( $p < 0.05$ ).

Kantachote, Hayes, & Shahidi, 2008) and alkali-solubilized tilapia (*Oreochromis niloticus*) (Raghavan, Kristinsson, & Leeuwenburgh, 2008) have been reported to have ABTS radical scavenging activities.

#### 3.4.3. Ferric reducing antioxidant power (FRAP)

FRAP of different hydrolysates with different DH (20, 30 and 40%) is depicted in Fig. 4(c). All hydrolysates had the increases in FRAP when DH increased ( $p < 0.05$ ) except for H-C, in which no difference was observed in samples with 30 and 40% DH ( $p > 0.05$ ).

FRAP of H-MS/W and H-PI was generally higher than that of H-C at all DHs used ( $p < 0.05$ ). At the same DH, H-MS/W showed the highest activity, in comparison with others ( $p < 0.05$ ). FRAP generally measures the reducing ability against ferric ion ( $\text{Fe}^{3+}$ ). This ability indicates the ability of hydrolysates to donate the electron to the free radical (Khantaphant & Benjakul, 2008). Therefore, it could be concluded that H-MS/W, especially at high DH, had the reducing power, leading to the prevention and retardation of propagation of lipid oxidation. Hydrolysates from yellow stripe trevally (Klompong et al., 2008) and alkali-solubilized tilapia (Raghavan et al., 2008) have been reported to possess FRAP.

#### 3.4.4. Ferrous chelating activity

Ferrous chelating activities of hydrolysates prepared from mince, MS/W and PI with different DHs are shown in Fig. 4(d). Chelating activity against  $\text{Fe}^{2+}$  of H-C and H-MS/W slightly increased when DH increased up to 30% ( $p < 0.05$ ). On the other hand, slight decrease in ferrous chelating activities was found in H-PI as DH was greater than 30% ( $p < 0.05$ ). Among all hydrolysates, H-MS/W showed the highest chelating activity for all DH ( $p < 0.05$ ). Ferrous ion ( $\text{Fe}^{2+}$ ) is the most powerful pro-oxidant among metal ions. This ion can interact with hydrogen peroxide in a Fenton reaction to produce the reactive oxygen species and hydroxyl free radical (OH), leading to the initiation and/or acceleration of lipid oxidation (Stojs & Bagchi, 1995). Therefore, chelation of metal ions by peptides in hydrolysates would retard the oxidative reaction (Klompong et al., 2008). The result indicated that H-MS/W was able to chelate pro-oxidative metals, resulting in the lowered oxidation. Furthermore, the higher chelating activity of H-MS/W was coincidental with the higher DPPH and ABTS radical scavenging activity and FRAP. Ferrous chelating activity has been reported for hydrolysate of silver carp (*Hypophthalmichthys molitrix*) (Dong et al., 2008), round scad (Thiansilakul et al., 2007a) and yellow stripe trevally (Klompong et al., 2008). Therefore, all hydrolysates could function as both primary and secondary antioxidants via scavenging free radical and chelating the metal ions, respectively. It could be observed that when mince was subjected to different pretreatments, the peptides generated showed varying activity, though the same DH was used. Therefore, different antioxidative peptides were produced from the samples with different pretreatments. Pretreatment might affect the structure of proteins, in which hydrolysis could take place at

different positions in peptide chains. Thiansilakul et al. (2007a) and Klompong et al. (2008) reported that the differences in antioxidative activities among hydrolysates might result from the differences in the structure and size of the peptides of the resulting hydrolysate. Since the hydrolysis was performed using the same protease, the different antioxidative activities of resulting hydrolysate was governed by pretreatments. DH also greatly influenced the peptide chain length as well as the exposure of terminal amino groups of products obtained (Thiansilakul, Benjakul, & Shahidi, 2007b). The higher DH, the more cleavage of peptide chains took place. Peptides with various sizes and compositions had various capacities of scavenging or quenching free radicals (Klompong et al., 2007).

#### 3.5. Protein pattern of protein hydrolysate prepared from mince with different pretreatments

Protein patterns of hydrolysates from mince without and with pretreatment having 40% DH are shown in Fig. 5(b). For mince with different pretreatments, myosin heavy chain (MHC) and actin were the major components. Slight decrease in MHC band intensity was noticeable in MS/W and PI, probably due to slight degradation of MHC during the process. After hydrolysis, no MHC and actin were detectable. H-C showed two major protein bands with molecular weight (MW) of 5.8 and 10.4 kDa. H-MS/W and H-PI showed smear bands with MW lower than 6.5 kDa. However, the smear band of H-MS/W was found with MW range of 5.1–6.5 kDa and protein bands with MW of 4.6–10.4 kDa were found in H-PI. The result indicated that the types and sizes of peptides in hydrolysate from mince with different pretreatments varied. In general, the hydrolysates prepared at 40% DH showed the highest antioxidative activities. Therefore, hydrolysates with 40% DH were selected to study for their ability in inhibition of lipid oxidation in lecithin-liposome oxidation model system, in comparison with commercial antioxidants.

#### 3.6. Antioxidative activity of hydrolysate from mince with different pretreatments in a lecithin liposome system

Protein hydrolysates prepared from mince with different pretreatments affected the oxidation of the lecithin liposome system differently as indicated by different conjugated dienes and TBARS values (Fig. 6 (a) and (b)), respectively. No changes in conjugated

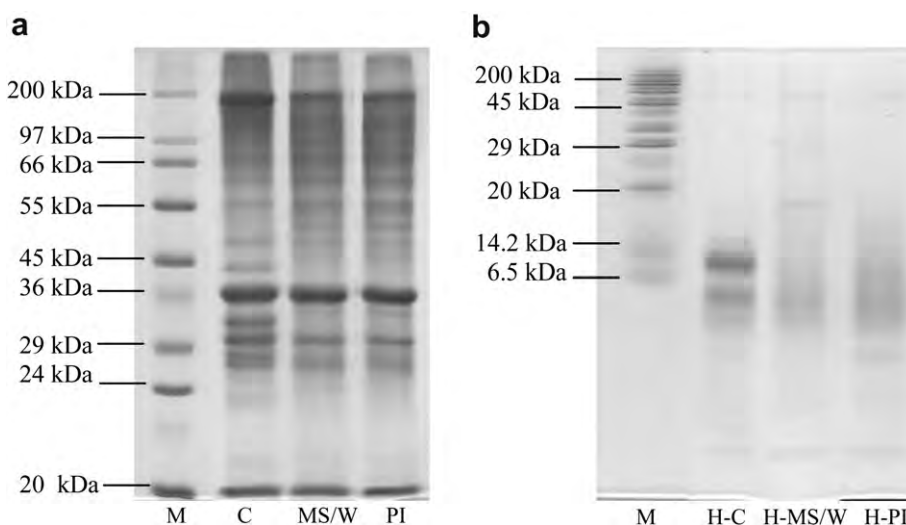


**Fig. 5.** Protein pattern of brownstripe red snapper mince with different pretreatments before (a) and after (b) hydrolysis with protease from pyloric caeca to obtain 40% DH. M: molecular weight marker; C: control mince; MS/W: membrane separated/washed mince; PI: protein isolate, H-C hydrolysate from C, H-MS/W: hydrolysate from MS/W and H-PI: hydrolysate from PI.

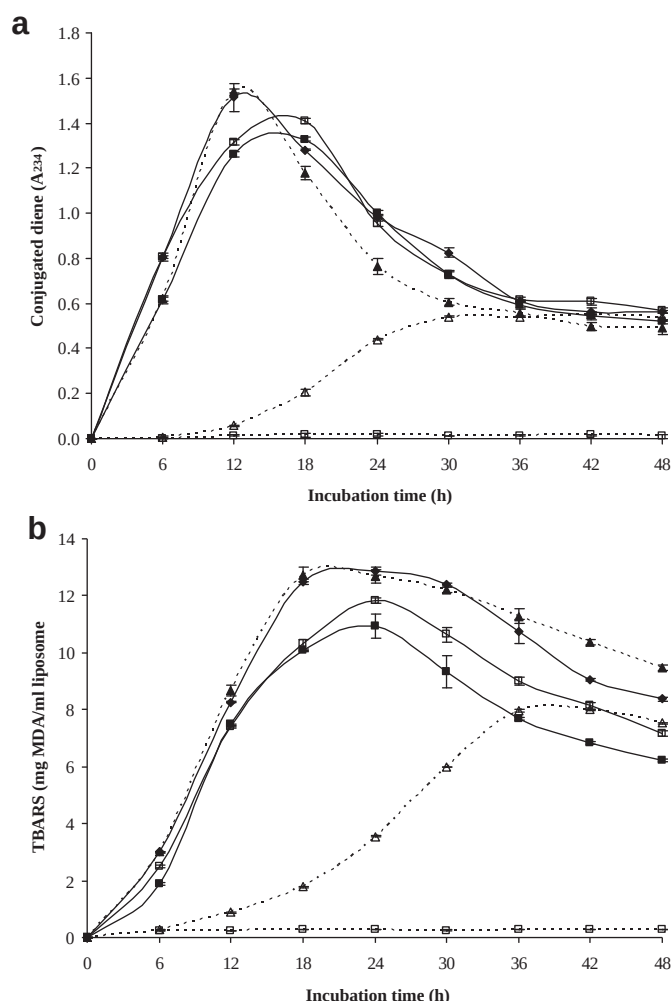


**Fig. 6.** The formation of conjugated diene (a) and thiobarbituric acid reactive substances (TBARS) (b) in lecithin-liposome model system in the presence of different hydrolysates from brownstripe red snapper mince with different pretreatments having 40% DH. (---▲---): control, (---△---): 25 mg L<sup>-1</sup> BHT, (---□---): 100 mg L<sup>-1</sup> BHT, (---◆---): 1000 mg L<sup>-1</sup> H-C, (---■---): 1000 mg L<sup>-1</sup> H-MS/W and (---□---): 1000 mg L<sup>-1</sup> H-PI. Bars represent the standard deviation from triplicate determinations.

dienes of liposome system added with 100 mg L<sup>-1</sup> BHT were observed throughout 48 h of incubation ( $p > 0.05$ ). For the system added with 25 mg L<sup>-1</sup> BHT, the increase in conjugated dienes was found after 12 h of incubation. An increase in conjugated dienes followed by a sharp decrease was observed from the control system and all systems containing all hydrolysates at a level of 1000 mg L<sup>-1</sup> but the rate of changes varied with the hydrolysate added (Fig. 6 (a)). Within the first 12 h of incubation, liposome system added with H-C and the control system showed the higher conjugated diene than those added with H-MS/W, H-PI and BHT ( $p < 0.05$ ). The results indicated that H-MS/W and H-PI in liposome system could retard the formation of conjugated diene. The higher conjugated diene in the system added with H-C might be due to the remaining pro-oxidants in minces, which could induce the oxidation within the first period of incubation. However, after 12 h, the sharp decreases in conjugated diene were observed from the system added with H-C and the control. Conjugated diene is generated during the lipid oxidative reaction and hydroperoxides are expected to decompose to the secondary products which can be determined by TBARS value (Frankel et al., 1997).

Changes in TBARS of liposome system during incubation of 48 h are shown in Fig. 6 (b). The increase in TBARS indicated the

formation of secondary lipid oxidation products. The sharp increases in TBARS were observed in the first 18 h of incubation ( $p < 0.05$ ), when H-C was added to liposome system. The systems added with H-MS/W and H-PI showed the increase in TBARS within the first 24 h ( $p < 0.05$ ). Thereafter, the gradual decreases in TBARS were observed ( $p < 0.05$ ). However, the value of TBARS obtained from the former system was lower than that of the latter ( $p < 0.05$ ). A gradual decrease in TBARS was found during 18–48 h for the control and the system added with H-C and during 24–48 h for the systems added with H-MS/W and H-PI (Fig. 6 (b)). The result suggested the greater ability of H-MS/W and H-PI in retardation of the formation of secondary oxidation products determined by TBARS. The first formation of TBARS indicated that the primary products were decomposed to the secondary products, especially aldehydes. The decrease in TBARS when the storage time increased was probably due to the loss in those volatile secondary products. In general, the increases in conjugated diene and TBARS values of the liposome systems containing H-MS/W and H-PI were lower than that of the control and the system containing H-C. Thus, H-MS/W and H-PI could retard the early stages of lipid oxidation as well as the advanced stage of oxidation via their radical scavenging activity, reducing power and metal chelating activity. Liposomes are appropriate lipid models to evaluate antioxidants for both food and lipoprotein particles containing phospholipids (Frankel et al., 1997). Polar portions of peptides in hydrolysate might interact with the liposomes of phospholipids, where they functioned effectively as antioxidant at the interface.

#### 4. Conclusion

Pretreatment of mince played a role in lowering pro-oxidants and phospholipid membrane in mince. Cleavage of protein was affected by pretreatment and antioxidative activity of protein hydrolysate was also governed by pretreatment. Fish mince subjected to membrane separation, followed by washing was appropriate for production of hydrolysate possessing antioxidative activity with the lesser amount of pro-oxidants and phospholipid membrane. The hydrolysate obtained can be further used to retard lipid oxidation in food system with the negligible pro-oxidative activity.

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# The partitioning of protease from *Calotropis procera* latex by aqueous two-phase systems and its hydrolytic pattern on muscle proteins

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## ABSTRACT

The protease from the latex of *Calotropis procera* was isolated by an aqueous two-phase system (ATPS). Polyethylene glycol (PEG 1000, 2000 and 3000) at a concentration of 12, 15, and 18% (w/w) with salts ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, K<sub>2</sub>HPO<sub>4</sub> and MgSO<sub>4</sub>) at a concentration of 14, 17, and 20% (w/w) were investigated. The highest protease recovery (74.6%) was found in the PEG-rich phase of the system ( $p < 0.05$ ), comprising of 18% PEG 1000 and 14% MgSO<sub>4</sub>. Protein patterns and activity staining showed that the isolated protease had a molecular weight of ~31 kDa without the oligosaccharide attached to the molecule. Degradation of muscle proteins in beef, farmed giant catfish, and squid was observed by the electrophoresis of sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE). The degradation of myofibrillar proteins (myosin heavy chain: MHC and actin: AC) of farmed giant catfish was higher than that of beef and squid muscles as indicated by the degradation proteins with lower molecular weight.

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**Keywords:** Aqueous two phase; *Calotropis procera*; Latex; Protease; Muscle protein

## 1. Introduction

Proteases have been exploited commercially in the food industry in processes such as papain for meat tenderizing (to separate partially connective tissues), ficin and bromelain for brewing (to solubilize grain proteins and stabilize beer), and  $\alpha$ -amylase for cookies (to improve crispiness) (Walsh, 2002). Proteases from plant sources have received special attention from the pharmaceutical industry, cosmetic, and by food biotechnology because their activity over wide ranges of temperature and acidity. Bromelain from pineapple was used as a tenderizing agent in beef (Lizuka and Aishima, 1999), squid (Melendo et al., 1997) and in coarse dry sausage (Melendo et al., 1996). Protease from ginger rhizome is a new interesting hydrolytic enzyme that has also been increasingly used for meat tenderization (Naveena et al., 2004). Proteases from plant latex were also widely used to reduce meat toughness for a long time (Kang and Warner, 1974; Ashie et al., 2002; Remezan

et al., 2003). These proteases include papain from *Carica papaya* (Nitsawang et al., 2006), ficin from *Ficus carica* (Huang et al., 2008), and cysteine protease from *Funastrum clausum* (Morcelle et al., 2004). In general, protease in plant latex plays a role in the defense mechanism of plants. *Calotropis procera* is a plant found in tropical and sub-tropical regions. It is well-known for its great capacity to produce latex, which is exuded from damaged parts. Various parts of *C. procera* including latex, have been used in traditional medicine (Chitme et al., 2004). However, the biochemical properties of enzymes contained in the latex of *C. procera* is limited.

Nowadays, industries demand for efficient and economical downstream processes for the partitioning and purification of biomolecules that give high yield and purity. Partitioning in an aqueous two-phase system (ATPS) has shown to be powerful for separating and purifying the mixtures of proteins (Huddleston et al., 1991). It offers many advantages: a bio-compatible environment, low interfacial tension, low energy,

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ease of scale-up, and continuous operations (Raghavarao et al., 1998). ATPS forms readily upon mixing aqueous solutions of two hydrophilic polymers, or of a polymer and a salt, above a certain threshold concentration (Tubio et al., 2007). It could be used to combine several features of the early processing steps in only one or two partitioning operations. Coincidentally, ATPS can remove undesirable enzymes/proteins, unidentified polysaccharides and pigments that are present in the system (Dubey and Jagannadham, 2003; Morcelle et al., 2004). Together with the removal of insoluble and major classes of contaminants, this technique has been developed as a primary purification step in the overall recovery (Huddleston et al., 1991). ATPS has been applied for the partitioning and recovery of various proteases such as bromelain (Babu et al., 2008), papain (Nitsawang et al., 2006), trypsin (Klomklao et al., 2005), chymosin and pepsin (Spelzini et al., 2005; Nalinanon et al., 2009), amyloglucosidase (Tanuja et al., 1997) and ricin B (Zhang et al., 2005). Dubey and Jagannadham (2003) reported that the latex of *C. procera* contained 4 stable cysteine proteases. However, extraction of the protease from *C. procera* latex by ATPS has not been reported. Therefore, the objective of this research was to isolate the proteolytic enzyme from *C. procera* latex by using an aqueous two-phase system. The other aims of this work were to characterize and apply the isolated enzyme in muscle proteins.

## 2. Materials and methods

### 2.1. Chemicals and raw materials

Polyethylene glycol (PEG), sodium dodecyl sulfate (SDS) and bovine serum albumin (BSA) were obtained from Fluka (Buchs, Switzerland). Beta-mercaptoethanol ( $\beta$ ME) and Coomassie Brilliant Blue G-250 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Ammonium sulfate ( $(\text{NH}_4)_2\text{SO}_4$ ), magnesium sulfate ( $\text{MgSO}_4$ ), potassium phosphate ( $\text{K}_2\text{HPO}_4$ ), trichloroacetic acid (TCA), hydrochloric acid, tris-(hydroxymethyl)-aminomethane and other chemicals with analytical grade were obtained from Merck (Darmstadt, Germany).

Latex of *C. procera* was collected from Nayong, Trang Province, Thailand. Beef and squid muscles were purchased from Bando Market, Chiang Rai Province, Thailand. Farmed giant catfish was obtained from a farm in Phan, Chiang Rai Province, Thailand.

### 2.2. Latex preparation

Latex was collected in a clean tube by breaking the *C. procera* stems. The collected latex was diluted with distilled water (1:1, v/v). It was mixed well and then centrifuged at  $15,000 \times g$  at  $4^\circ\text{C}$  for 10 min. The obtained supernatant was filtered through a Whatman paper No. 1. This sample was referred to as the “crude extract” and was used for further study.

### 2.3. Aqueous two phase partitioning

The ATPS was prepared in 10-ml centrifuge tubes according to the method in Nalinanon et al. (2009). Various amounts and molecular weights of PEG (1000, 2000 and 3000 Da) as well as salts ( $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{K}_2\text{HPO}_4$ , and  $\text{MgSO}_4$ ) were added to the

crude extract from *C. procera* to generate the biphasic system.

#### 2.3.1. Effect of salts on the partitioning of protease from *C. procera* latex

To study the effect of salts on the partitioning of protease from *C. procera* latex using ATPS, different salts ( $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{K}_2\text{HPO}_4$ , and  $\text{MgSO}_4$  at different concentrations (14, 17 and 20%, w/w) were mixed with 18% PEG 1000 in an aqueous system (the concentration of PEG 1000 was fixed to make sure that a two-phase formation would be obtained). Thirty percentages of the crude extract (w/w) were added into the system. The remaining distilled water was used to adjust the system to obtain the final weight of 15 g. The mixtures were mixed continuously for 3 min using a Vortex mixer (Vortex-genie2, G-560E, USA), and then they were gently mixed for 15 min. Phase separation was achieved by centrifuging the mixture at  $9,000 \times g$  for 30 min. The top phase was carefully separated using a Pasteur pipette. Volumes of the separated top and bottom phases were measured and recorded. Aliquots from each phase were taken for enzyme activity assay and protein determination. The phase composition giving the highest proteolytic yield was chosen for further study.

#### 2.3.2. Effect of PEG on the partitioning of protease from *C. procera* latex

Fourteen percentages of  $\text{MgSO}_4$  were used in the system to study the effect of the concentrations (12, 15 and 18%, w/w) and molecular weights (1000, 2000 and 3000 Da) of PEG on the partitioning of protease from *C. procera* latex. A two-phase separation was performed as previously described. ATPS parameters were calculated as follows:

The volume ratio ( $V_R$ ) is as:

$$V_R = \frac{V_T}{V_B} \quad (1)$$

where  $V_T$  and  $V_B$  are top and bottom phase volume, respectively.

The partition coefficient of protein ( $K_P$ ) was defined as:

$$K_P = \frac{P_T}{P_B} \quad (2)$$

where  $P_T$  and  $P_B$  are concentrations of protein in top and bottom phase, respectively.

The partition coefficient of enzyme ( $K_E$ ) was defined as:

$$K_E = \frac{E_T}{E_B} \quad (3)$$

where  $E_T$  and  $E_B$  are concentrations of enzymes (unit/ml) in top and bottom phase, respectively.

The specific activity (SA) of extracted protease in each phase of the ATPS was defined as:

$$SA = \frac{\text{total protease activity}}{\text{total protein content}}; \text{ unit mg protein}^{-1} \quad (4)$$

The purification factor (PF) as:

$$PF = \frac{SA_E}{SA_I} \quad (5)$$

where  $SA_E$  is the SA of each phase and  $SA_I$  is the SA of the initial phase (crude latex before partitioning).

The protease activity recovery (yield) was defined as:

$$\text{yield (\%)} = \frac{A_T}{A_I} \times 100 \quad (6)$$

where  $A_T$  is the total protease activity in the top phase and  $A_I$  is the initial protease activity (crude latex before partitioning).

From the protease recovery, the protease from the ATPS fraction that rendered the maximal yield was chosen for further study.

#### 2.4. Caseinolytic activity assay

An enzyme sample of 0.1 ml was mixed with 1.1 ml of 1% (w/v) casein in 0.1 M Tris–HCl, pH 8.0 containing 12 mM of cysteine. The mixture was incubated at 37 °C for 20 min. After that, the reaction was stopped by adding 1.8 ml of 5% TCA. After centrifugation at  $3,000 \times g$  for 15 min, the absorbance of the supernatant was measured at 280 nm. One caseinolytic unit is defined as the amount of enzymes needed to produce an increment of 0.01 absorbance unit per minute at the assayed condition (Vallés et al., 2007).

#### 2.5. Protein determination

The protein concentration in the sample was measured by the Bradford method (Bradford, 1976) using BSA as a protein standard.

#### 2.6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

##### 2.6.1. Protein staining

SDS-PAGE of crude extract and the extracted protease from ATPS fractions was performed according to the Laemmli method (Laemmli, 1970). Protein solutions were mixed at a 1:1 (v/v) ratio with the sample buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol). The samples (10 and 2 µg protein for protein and activity staining, respectively) were loaded onto the gel made of 4% stacking and 15% separating gels. They were subjected to an electrophoresis set at a constant current of 15 mA per gel using a Mini Protean Tetra Cell unit (Bio-Rad Laboratories, Richmond, CA, USA). After electrophoresis, the gel was stained overnight with staining solution (0.02% (w/v) Coomassie Brilliant Blue R-250) in 50% (v/v) methanol, and 7.5% (v/v) acetic acid. Protein patterns were then visualized after destaining the gel until a clear background was achieved.

##### 2.6.2. Protease activity staining

The protease separated on the gel was verified by using activity staining as the method described in Garcia-Carreno et al. (1993). The gel was immersed in 50 mL of 2% (w/v) casein in 50 mM Tris–HCl buffer, pH 8.0 containing 12 mM cysteine for 45 min, and agitated constantly at 4 °C. The gel was then incubated at 37 °C for 30 min with constant agitation to generate the reaction. The treated gel was then stained and destained as described above. The appearance of a clear zone on the dark background indicated the *C. procera* latex protease activity.

##### 2.6.3. Glycoprotein staining

Glycoprotein staining was conducted by using a GelCode® Glycoprotein Staining Kit (PIERCE Biotechnology, IL, USA). The protein separated by electrophoresis was fixed by immersing the gel in 30 ml of 50% (v/v) methanol for 30 min, followed by

washing the gel by gently agitating it with 3% (v/v) acetic acid for 10 min (repeated twice). The gel was transferred to 25 ml of oxidizing solution and then washed with 3% (v/v) acetic acid for 10 min before treating it with 15 ml of GelCode® Glycoprotein Stain for 15 min. The gel was incubated with 25 ml of reducing solution for 10 min and then washed by 3% (v/v) acetic acid. The magenta band could be observed in the presence of glycoprotein.

#### 2.7. Effect of selected ATPS fraction on hydrolytic pattern of muscle proteins

Two grams of muscles (beef, giant catfish and squid) were marinated with 0, 3, 5, 10, 20, 30, and 50 units of isolated protease from the top phase of the ATPS (18% PEG 1000–14% MgSO<sub>4</sub>) and then incubated at a refrigerated temperature (~4 °C) for 60 min. Twenty units of the crude extract were also used as the control. Three grams of a marinated sample were added with 27 ml of 5% SDS (85 °C). The mixtures were homogenized at a speed of 11,000 rpm for 1 min using an IKA Labortechnik homogenizer (Selangor, Malaysia). The homogenate was incubated at 85 °C for 60 min, followed by centrifugation at  $8,000 \times g$  for 5 min at room temperature using a Mikro 20 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). The supernatants were mixed at a 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8 containing 4% SDS, 20% glycerol and 10% βME) and boiled for 3 min. The samples (15 µg protein) were loaded into the polyacrylamide gel (10% running and 4% stacking gel) and subjected to electrophoresis as previously described.

#### 2.8. Statistical analysis

All experiments were conducted and analyzed in triplicate. Means and standard deviations were calculated and compared using the Duncan's multiple range test (DMRT). Analysis was performed using a SPSS package (SPSS 10.0 for windows, SPSS, Inc. Chicago, IL).

### 3. Results and discussion

#### 3.1. Effect of salts on partitioning of protease from *C. procera* latex

The partitioning was carried out in several biphasis compositions of 18% PEG 1000 with different salts ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, MgSO<sub>4</sub> and K<sub>2</sub>HPO<sub>4</sub>) and their concentrations (14, 17 and 20%, w/w). Salts are frequently used in ATPS to improve partitioning target molecules between the phases. In general, no phase separation was found in either PEG 1000 or salts on their own. This indicates that the combination of both PEG and salt was necessary for the partitioning process. Phase separation will occur if the concentrations of polymers or salts are above a certain critical value of the binodal curve. Raghavarao et al. (1998) reported that two phases are formed when the polymer concentration is in the range of 8–16% (w/w), and salt concentration must be as high as 10% (w/w). In addition, the lower the molecular weight, the higher the polymer concentration is required for phase formation. From the result, it was shown that the two-phase separations of PEG-rich top phase and salt-rich bottom phase were obtained in all systems comprising of 18% PEG and 14–20% salts. The mechanism of phase separation in PEG-salt systems is dependent on balancing enthalpic and entropic forces involved in the aqueous hydration of the solutes (Huddleston et al., 1991).

**Table 1 – Effect of phase composition in PEG 1000-salts ATPS on partitioning of protease from *Calotropis procera* latex.**

| Phase composition (% w/w)                                       | V <sub>R</sub> | K <sub>P</sub> | K <sub>E</sub> | SA           | PF           | Yield (%)    |
|-----------------------------------------------------------------|----------------|----------------|----------------|--------------|--------------|--------------|
| 18%PEG 1000–14% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> | 2.96 ± 0.16f   | 0.13 ± 0.00a   | 0.57 ± 0.02e   | 293 ± 7.80e  | 5.82 ± 0.16e | 65.7 ± 2.71g |
| 18%PEG 1000–17% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> | 1.94 ± 0.11cd  | 0.18 ± 0.01a   | 0.52 ± 0.02d   | 266 ± 7.71d  | 5.29 ± 0.15d | 62.2 ± 1.51f |
| 18%PEG 1000–20% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> | 0.93 ± 0.03a   | 0.16 ± 0.01a   | 0.34 ± 0.01c   | 308 ± 1.73e  | 6.12 ± 0.03e | 11.9 ± 0.69c |
| 18%PEG 1000–14% K <sub>2</sub> HPO <sub>4</sub>                 | 2.31 ± 0.06de  | 0.33 ± 0.01ab  | 1.06 ± 0.02f   | 163 ± 0.60b  | 3.23 ± 0.01b | 68.0 ± 3.39g |
| 18%PEG 1000–17% K <sub>2</sub> HPO <sub>4</sub>                 | 1.62 ± 0.02bc  | 0.60 ± 0.03b   | 1.49 ± 0.01g   | 265 ± 6.46d  | 5.27 ± 0.13d | 56.6 ± 1.90e |
| 18%PEG 1000–20% K <sub>2</sub> HPO <sub>4</sub>                 | 1.22 ± 0.15ab  | 4.00 ± 0.57c   | 2.59 ± 0.02h   | 144 ± 0.57b  | 2.87 ± 0.01b | 15.7 ± 1.28d |
| 18%PEG 1000–14% MgSO <sub>4</sub>                               | 7.41 ± 0.76g   | 0.16 ± 0.01a   | 0.53 ± 0.01d   | 205 ± 9.92c  | 4.08 ± 0.20c | 74.6 ± 1.00h |
| 18%PEG 1000–17% MgSO <sub>4</sub>                               | 2.48 ± 0.25ef  | 0.03 ± 0.01a   | 0.05 ± 0.01b   | 154 ± 32.6b  | 3.01 ± 0.65b | 6.93 ± 1.63b |
| 18%PEG 1000–20% MgSO <sub>4</sub>                               | 1.77 ± 0.21c   | 0.03 ± 0.00a   | 0.03 ± 0.00a   | 79.8 ± 1.97a | 1.58 ± 0.04a | 2.61 ± 0.08a |

V<sub>R</sub>: volume ratio (top/bottom); K<sub>P</sub>: partition coefficient of protein in the top phase; K<sub>E</sub>: partition coefficient of enzyme in the top phase SA: specific activity (unit/mg protein) of the top phase; PF: purification factor; yield: activity recovery. Different letters in the same column indicate significant differences (*p* < 0.05).

The basis for separation by ATPS is the selective distribution of biomolecules between the phases, quantified by the partition coefficient (Raghavarao et al., 1998). The protease was partitioned prominently in the polymer phase, principally those with hydrophobic characteristics the enzyme. Dubey and Jagannadham (2003) reported that most amino acids in procerain, a cysteine protease in *C. procera* latex, were hydrophobic amino acids, with especially high amounts of tyrosine and tryptophan. Glycine and valine were predominant non-polar amino acids present in *C. gigantean*. In PEG-salt systems, proteolytic enzyme partitioning depends on a “volume exclusion effect” of the polymer and a “salting-out effect” of the salts. The effect of salts and their concentrations on the partitioning (V<sub>R</sub>, K<sub>P</sub>, SA, PF and yield) are shown in Table 1. The V<sub>R</sub> of the assayed systems generally decreased when salt concentration increased. The addition of salts to the aqueous PEG solution led to an arrangement of ordered water molecules around the PEG molecules. This was due to their water structure breaking effects (Farruggia et al., 2004). The formation of a water layer around the cation resulted in a more compact structure with a minor volume of PEG molecules. This led to the decrease in volume of the PEG-rich top phase.

The distribution of the protein and proteases in ATPS are reported by K<sub>P</sub> and K<sub>E</sub>, respectively. High K<sub>P</sub> values indicate that most of the proteins from the crude extract were partitioning more to the top phase, while the high K<sub>E</sub> was the only protease to favor the top phase. From the results, the highest K<sub>P</sub> (4.00) and the highest K<sub>E</sub> (2.59) were found in the system of 18% PEG 1000–20% K<sub>2</sub>HPO<sub>4</sub>. In contrast, the phase composition of 18% PEG 1000–20% MgSO<sub>4</sub> showed the lowest K<sub>P</sub> (0.03), indi-

cating that both desired proteases and contaminant proteins would shift to the lower phase.

The phase containing 18% PEG 1000 and 14% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> gave the highest SA (293 unit mg protein<sup>-1</sup>) and PF (5.82) (*p* < 0.05), whereas the maximal proteolytic yield of 74.6% was obtained from the system containing 18% PEG 1000 and 14% MgSO<sub>4</sub>. Babu et al. (2008) reported that the system of 18% PEG 1500 and 20% K<sub>2</sub>HPO<sub>4</sub> provided the highest activity recovery of bromelain from pineapple. It was reported that using 20% PEG 1000 and 20% MgSO<sub>4</sub> gave the highest SA and PF in the partitioning of protease from tuna spleen (Klomklao et al., 2007). The same result was also found in the extraction of protease from the stomach of an albacore tuna (Nalinanon et al., 2009). Johansson (1998) reported that the partition of a protein is influenced by the presence of salts. This effect increases with the net charge of the protein. The presence of salt may affect the partitioning in two ways: (1) the weakening or strengthening of the interactions, or (2) the interaction between ionized groups with the opposite net charge of proteins. The effectiveness of the salt is mainly determined by the nature of the anion. Multi-charged anions are the most effective in the order of SO<sub>4</sub><sup>2-</sup> > HPO<sub>4</sub><sup>2-</sup> > CH<sub>3</sub>COO<sup>-</sup> > Cl<sup>-</sup>. The order of cations is usually given as NH<sub>4</sub><sup>+</sup> > K<sup>+</sup> > Na<sup>+</sup> > Li<sup>+</sup> > Mg<sup>2+</sup> > Ca<sup>2+</sup> (Roe, 2000). Huddleston et al. (1991) concluded that the effectiveness of various salts in promoting phase separation reflects the lyotropic series (a classification of ions based upon salting-out ability).

Based on the highest proteolytic yield obtained, the 14% MgSO<sub>4</sub> was chosen for further experiment on the effect of PEG on the partitioning of protease from *C. procera* latex.

**Table 2 – Effect of phase composition in PEG–14% MgSO<sub>4</sub> ATPS on partitioning of protease from *Calotropis procera* latex.**

| Phase composition (% w/w)         | V <sub>R</sub> | K <sub>P</sub> | K <sub>E</sub> | SA          | PF           | Yield (%)     |
|-----------------------------------|----------------|----------------|----------------|-------------|--------------|---------------|
| 12%PEG 1000–14% MgSO <sub>4</sub> | ns             | ns             | ns             | ns          | ns           | ns            |
| 15%PEG 1000–14% MgSO <sub>4</sub> | ns             | ns             | ns             | ns          | ns           | ns            |
| 18%PEG 1000–14% MgSO <sub>4</sub> | 7.41 ± 0.8c    | 0.16 ± 0.01ab  | 0.53 ± 0.01f   | 205 ± 9.92a | 4.08 ± 0.2a  | 74.6 ± 1.00f  |
| 12%PEG 2000–14% MgSO <sub>4</sub> | ns             | ns             | ns             | ns          | ns           | ns            |
| 15%PEG 2000–14% MgSO <sub>4</sub> | 2.84 ± 0.39ab  | 0.01 ± 0.01a   | 0.18 ± 0.01d   | 1279 ± 213b | 25.6 ± 4.2b  | 45.80 ± 0.16d |
| 18%PEG 2000–14% MgSO <sub>4</sub> | 3.32 ± 0.44b   | na             | 0.02 ± 0.00b   | na          | na           | 5.71 ± 0.36b  |
| 12%PEG 3000–14% MgSO <sub>4</sub> | 2.62 ± 0.15ab  | 0.26 ± 0.03b   | 0.32 ± 0.00e   | 361 ± 2a    | 7.17 ± 0.05a | 65.2 ± 1.53e  |
| 15%PEG 3000–14% MgSO <sub>4</sub> | 2.24 ± 0.20a   | 0.03 ± 0.01a   | 0.05 ± 0.01c   | 208 ± 10a   | 4.12 ± 0.20a | 10.5 ± 1.67c  |
| 18%PEG 3000–14% MgSO <sub>4</sub> | 2.65 ± 0.04ab  | 0.02 ± 0.01a   | 0.01 ± 0.00a   | 24.0 ± 8a   | 0.47 ± 0.17a | 1.10 ± 0.02a  |

V<sub>R</sub>: volume ratio (top/bottom); K<sub>P</sub>: partition coefficient of protein in the top phase; K<sub>E</sub>: partition coefficient of enzyme in the top phase SA: specific activity (unit/mg protein); PF: purification factor; yield: activity recovery. ns: no phase separation; na: no protein content. Different letters in the same column indicate significant differences (*p* < 0.05).

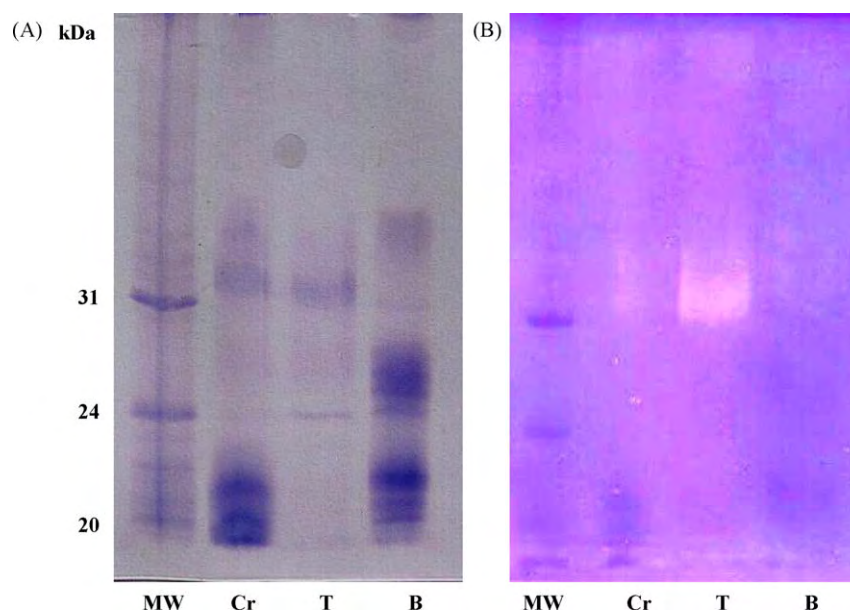


**Fig. 1 – SDS-PAGE patterns (A) and activity staining (B) of *Calotropis procera* latex extract and its ATPS fractions of 18% PEG 1000–14%  $\text{MgSO}_4$ ; MW: molecular weight marker; Cr: crude extract; T: top phase and B: bottom phase.**

### 3.2. Effect of PEG on the partitioning of protease from *C. procera* latex

This study examined the partitioning of protease from *C. procera* latex in ATPS with different molecular weights (MW) and concentrations of PEG at 14%  $\text{MgSO}_4$ . As shown in Table 2, the protease partitioning was strongly dependent on the MW and the PEG concentration. No phase separation was observed in the 14%  $\text{MgSO}_4$  system with 12% PEG 1000, 15% PEG 1000, and 12% PEG 2000. As mentioned before, two-phase formation requires high concentrations of low MW polymer, or requires low concentrations with high MW polymer. The  $V_R$  of the system ranged from 2.24 to 7.41. Most of the systems produced a value of  $V_R$  higher than 1.0, indicating that the volume fraction was distributed in the top phase rather than in the bottom phase. The  $K_P$  obtained from all systems was lower than 1, indicating that most of the protein components from *C. procera* latex preferably partitioned to the bottom phase of the ATPS system. A favorable interaction between PEG molecules and protein domain decreased when the MW of PEG increased because of its' exclusion from the protein domain (Reh et al., 2002; Klomklao et al., 2005; Nalinanon et al., 2009). Tubio et al. (2009) reported that the best ATPS separation of trypsin from bovine pancreas was found in the system with a molecular weight of PEG 3350 compared with 600, 1000, 1450, and 8000. However, Hotha and Banik (1997) found that for the production of alkaline protease by ATPS, the lower MW of PEG 4000 produced a higher yield than PEG 6000 and 9000. Marcos et al. (1999) also reported that a system consisting of PEG 1000 for penicillin acylase production achieved a high yield compared with that of PEG 3350 and 8000.

Among all ATPS tested, systems comprising of 18% PEG 1000 and 14%  $\text{MgSO}_4$  effectively partitioned the protease from *C. procera* latex to the PEG-rich top phase. Under this condition, the highest proteolytic yield of 74.6% was recovered in the top phase, providing PF of 4.08 fold. Though the highest PF was observed in the system of 15% PEG 2000 and 14%  $\text{MgSO}_4$ , it provided only 45.80% yield. Therefore, the fraction of the ATPS system containing 18% PEG 1000–14%  $\text{MgSO}_4$  with the

highest yield was selected for biochemical properties characterization.

### 3.3. Protein pattern and activity staining of protease from *C. procera* latex and its ATPS fractions

Fig. 1A shows the protein patterns of the crude extract and extracted protease fractions from 18% PEG 1000–14%  $\text{MgSO}_4$ . For the migration of protein composition in the crude extract, the top phase and bottom phase of their fractions were quite different. The three major protein bands of crude *C. procera* latex were observed with a MW of around 20 kDa, ~31 kDa and above 31 kDa. This corresponds with the investigation of Freitas et al. (2007), but the protein band at around 31 kDa of that study was the main component instead of that below 20 kDa in the present research. Using ATPS with 18% PEG 1000–14%  $\text{MgSO}_4$ , the effective protease separation was obtained (as shown in the Fig. 1A) by most of the proteins partitioning to the salt-rich bottom phase. Only the protein band with the MW of around 31 kDa was separated to the PEG-rich top phase. It was reported that the purified protease from *C. procera* appeared as a single band at around 29 kDa in a reducing condition of SDS-PAGE (Dubey and Jagannadham, 2003).

To verify the band of proteolytic enzymes, activity staining was performed by using substrate (casein) gel electrophoresis. The clear zone by proteolytic activity on the dark background resulted only at the protein band around 31 kDa (Fig. 1B). This clear zone was more apparent in the band of the top phase of ATPS than that of the crude latex. There is previous report that the maximum proteolytic activity of *C. procera* protein found a protein band possessing around 30 kDa (Freitas et al., 2007). Low detectable clear zones were observed in both the crude extract and in the bottom phase of their ATPS fractions. This is probably due to a rather low specific activity of protease content in both fractions. In contrast, the top phase fraction is mainly composed of activated protease, which results in detectable activity of clearer zones. For this result, the protein component of *C. procera*

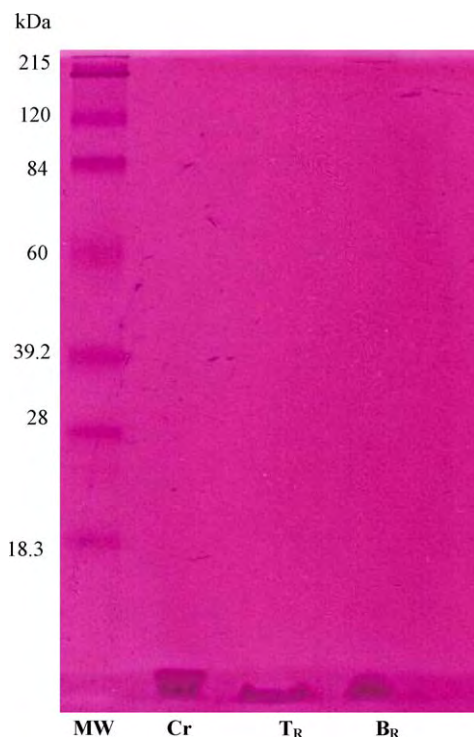


**Fig. 2 – Glycoprotein staining of *C. procera* latex extract and its ATPS fractions 18% PEG 1000–14%  $\text{MgSO}_4$ ; MW: molecular weight marker; Cr: crude extract; T: top phase and B: bottom phase.**

latex with the MW of  $\sim 31$  kDa was the predominant protease.

### 3.4. Glycoprotein staining of protease from *C. procera* latex and its ATPS fractions

The presence of carbohydrates in the protease from the *C. procera* latex was investigated. The crude extract and its 18% PEG 1000–14%  $\text{MgSO}_4$  ATPS fractions were subjected to glycoprotein staining. This staining method was reported to detect high glycosylated proteoglycans (protein glycosaminoglycans) or glycoproteins (protein oligosaccharides) (Møller and Poulsen, 2002) in which polypeptides covalently bonded to a carbohydrate moiety (Mathews and Holde, 1990). The result (as depicted in Fig. 2) revealed that all of the latex major proteins of *C. procera* latex were not contained in their car-

bohydrate molecules. Similar results were obtained by Dubey and Jagannadham (2003) in which protease from *C. procera* was devoid of carbohydrate moiety in procerain.

### 3.5. Effect of the protease fraction on the hydrolytic pattern of muscle proteins

Proteolytic patterns of the muscle samples treated with ATPS isolated *C. procera* latex protease are depicted in Fig. 3. The PEG-rich phase of 18% PEG 1000–14%  $\text{MgSO}_4$  system was used as the isolated protease fraction in this study. Different protease activity units (0–50 units) were added to three kinds of muscle samples; beef, farmed giant catfish, and squid muscles. The treated samples were incubated at a refrigerated temperature ( $4^\circ\text{C}$ ) for 60 min. Increasing protease activity of isolated protease fractions on the treated muscle samples were observed, evidenced by gradually decreasing numbers of protein bands as well as the protein band intensity as compared with the control (without the fraction addition). The degradation of protein increased by increasing the enzyme concentration. Naveena et al. (2004) reported that when using protease from *Cucumis trigonus* in buffalo meat samples, the increase in proteolysis can be correlated with significantly higher protein solubility. Bacterial proteolytic enzyme treatment of muscle protein showed a reduction in the level of higher MW fractions due to the degradation of myosin, thus increasing the meat tenderness (Jorgova et al., 1989).

From the Fig. 3, it is also evident that protein breakdown in all of the enzyme treated samples was generated. High amounts of protein decomposition were more visible in the squid and giant catfish muscles than that of the beef sample. Not only was the myosin heavy chain (MHC) degraded by the ATPS enzyme fraction, but also by the actin (AC), especially in the giant catfish muscle. The squid and giant catfish muscles, MHC band was extensively degraded into lower MW when the concentration of the enzyme fraction was increased. Furthermore, high breakdowns of protein were markedly clearer in the giant catfish than in the beef for both MHC and AC bands. This is probably due to the co-hydrolysis from the endogenous enzymes contained in the giant catfish. The result also showed that there was a slight change in degradation of the AC band in squid and beef muscles, even with increased enzyme fraction concentrations when compared to those of the giant catfish muscle. It can be explained by the difference in protein structure and composition among these three muscles.

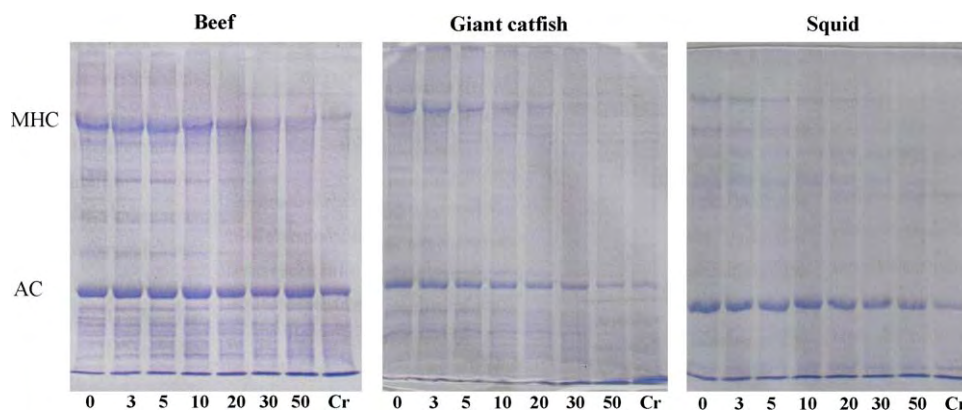


**Fig. 3 – SDS-PAGE patterns of muscle samples incubated with enzyme from ATPS fraction (top phase of system 18% PEG 1000–14%  $\text{MgSO}_4$ ) at refrigerated temperature. Numbers indicated enzyme activity (unit) the fraction used. MHC: myosin heavy chains; AC: actin; Cr: crude enzymes extract (20 units).**

The degradation of muscle protein plays a major role in determining the tenderness and water holding capacity of meat during postmortem storage (Melody et al., 2004). Myosin was degraded very intensely throughout the meat, and totally in the presence of high concentrations of protease fractions. AC, on the other hand, was only slightly hydrolyzed unless a high amount of enzymes were added. Nonetheless, differences between the controls and the treated meat with lower enzyme concentrations were unclear in all samples.

In conclusion, the protease from *C. procera* latex was extracted by the aqueous two-phase system. The top phase from 18% PEG 1000–14% MgSO<sub>4</sub> provided the highest protease recovery (74.6%). By using casein substrate gel electrophoresis, the protease from the phase partitioning showed the MW of ~31 kDa with high specificity. The protease obtained had no oligosaccharide in the molecule as determined by the glycoprotein staining. Based on the protein degradation of beef, giant catfish and squid muscles, it is suggested that the protease fraction from *C. procera* latex is suitable for meat tenderization.

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## Type I collagen from the skin of ornate threadfin bream (*Nemipterus hexodon*): Characteristics and effect of pepsin hydrolysis

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### ABSTRACT

Type I collagen from the skin of ornate threadfin bream (*Nemipterus hexodon*) was purified and characterised. Purified type I collagen contained  $[\alpha 1(I)]_2\alpha 2(I)$  as the dominant component with the co-presence of  $\alpha 1(I)\alpha 2(I)\alpha 3(I)$ . It was rich in glycine and alanine with high content of imino acids (188 residues/1000 residues). The maximum transition temperature ( $T_m$ ) and the total denaturation enthalpy ( $\Delta H$ ) of purified type I collagen was 33.35 °C and 0.819 J/g, respectively. The isoelectric point (pI) of purified type I collagen was estimated to be 6.40. After hydrolysis of purified type I collagen using pepsin, the band intensity ratios of  $\alpha 1/\alpha 2$ -chains were increased ( $P < 0.05$ ). The cross-linked components were effectively hydrolysed by pepsin 1 and 2 from skipjack tuna stomach and porcine pepsin at 4 °C without the cleavage of  $\beta$ - and  $\alpha$ -chains. At 50 °C, they were more susceptible to porcine pepsin hydrolysis, followed by pepsin 2 and 1, respectively.

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### 1. Introduction

Collagen is the fibrous protein that contributes to the unique physiological functions of connective tissues in skins, tendons, bones and cartilages, and is associated with the toughness of mammalian muscle (Foegeding, Lanier, & Hultin, 1996; Kittiphattanabawon, Benjakul, Visessanguan, Kishimura, & Shahidi, 2010; Muyonga, Cole, & Duodu, 2004; Ogawa et al., 2003; Yan et al., 2008). The structural unit of collagen is tropocollagen, a rod-shaped protein consisting of three polypeptides unit (called  $\alpha$ -chains) intertwined to form a triple-helical structure (Wong, 1989). Each polypeptide chain forms a left-handed helix and consists of repeating triplets,  $(\text{Gly-X-Y})_n$ , where X and Y are, with a high possibility, proline or hydroxyproline (Ogawa et al., 2003). The repetitive occurrence of glycine is absent in the first 14 amino acid residues from the N-terminus and the first 10 amino acids from the C-terminus. These end portions are termed “telopeptides” (Foegeding et al., 1996). The covalent cross-links at the telopeptide regions between the collagen molecules are formed by the condensation of aldehyde groups, as well as the formation of a Schiff's base when the aldehyde reacts with an amino group (Foegeding et al., 1996; Jongjareonrak, Benjakul, Visessanguan, Nagai, & Tanaka, 2005). These cross-linking molecules generally cause the decrease in the solubility of collagen (Belitz, Grosch, & Schieberle,

2004; Foegeding et al., 1996). At least 19 variants of collagen (types I–XIX) have been reported (Bailey, Paul, & Knott, 1998). Type I collagen,  $[\alpha 1(I)]_2\alpha 2$ , is the major collagen found predominantly in the connective tissues, such as skin, bone, and tendon, and contains two identical  $\alpha 1(I)$  chains and one  $\alpha 2$ -chain (Trelstad, Catanese, & Rubin, 1976). Chromatographic and electrophoretic analyses revealed that most of the teleost skin collagens also contained a unique subunit,  $\alpha 3$  (Kimura, Ohno, Miyauchi, & Uchida, 1987). The existence of  $\alpha 1\alpha 2\alpha 3$  and  $(\alpha 1)_2\alpha 2$  heterotrimer of type I collagen from the skin of some teleostean and chondrosteian fish species has been reported (Kimura, 1992; Kimura & Ohno, 1987; Kimura et al., 1987).

Currently, the increasing attention of alternative sources for replacement of mammalian collagen has been paid, especially from seafood processing by-products. Fish skin, a by-product from fish processing, is one of the alternative sources for collagen preparation. Collagens from several fish species, such as arabesque greenling (Nalinanon, Benjakul, & Kishimura, 2010a), brownbanded bamboo shark (Kittiphattanabawon et al., 2010), cod (Duan, Zhang, Du, Yao, & Konno, 2009), eagle ray (Bae et al., 2008), walleye pollock (Yan et al., 2008), deep-sea red fish (Wang, An, Xin, Zhao, & Hu, 2007), bigeye snapper (Benjakul et al., 2010; Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005; Nalinanon, Benjakul, Visessanguan, & Kishimura, 2007), channel catfish (Liu, Li, & Guo, 2007), Pacific whiting (Kim & Park, 2004), black drum (Ogawa et al., 2003), sheephead seabream (Ogawa et al., 2003), ocellate puffer fish (Nagai, Araki, & Suzuki, 2002) and Japanese

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seabass, chub mackerel and bullhead shark (Nagai & Suzuki, 2000), have been isolated and characterised. Pepsin, mainly from porcine stomach, was used to improve the extraction efficiency of collagen for a decade. Recently, pepsins from the stomach of several fish species, such as bigeye snapper, albacore tuna, tongol tuna and skipjack tuna, have also been used as the aid for collagen extraction (Benjakul et al., 2010; Nalinanon et al., 2010a; Nalinanon et al., 2007; Nalinanon, Benjakul, Visessanguan, & Kishimura, 2008). Nalinanon et al. (2008) found that albacore tuna pepsin exhibited the comparable extraction efficiency of collagen from the skin of threadfin bream to porcine pepsin, and no detrimental effect on the integrity of resulting collagen was observed. Nevertheless, pepsin from skipjack tuna caused the degradation of the  $\alpha$  and  $\beta$  components. The differences in the hydrolysis of collagen matrix by pepsin might be dependent on several factors, such as the source and type of collagen, and the type of pepsin and hydrolysis condition. Therefore, the objective of this study was to isolate and characterise the major collagen from the skin of ornate threadfin bream (*Nemipterus hexodon*), one of the fish species commonly used for surimi production in Thailand, as well as to demonstrate the hydrolysis of type I collagen by different pepsins, including porcine pepsin and skipjack tuna pepsin 1 and 2.

## 2. Materials and methods

### 2.1. Chemicals

Bovine haemoglobin,  $\beta$ -mercaptoethanol ( $\beta$ ME), type I collagen from calf skin and pepsin from porcine stomach mucosa (EC 3.4.23.1), (750 U/mg dry matter) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid, disodium hydrogen phosphate, sodium citrate, and acetic acid were obtained from Merck (Darmstadt, Germany). Coomassie Blue R-250 and *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). Sodium dodecyl sulphate (SDS) and bovine serum albumin were obtained from Fluka (Buchs, Switzerland). High-molecular-weight protein markers, DEAE-sephacel and Sephadex G-75 were purchased from GE Healthcare UK Limited (Buckinghamshire, UK). TOYOPEARL® CM-650M was purchased from Tosoh Corporation (Tokyo, Japan).

### 2.2. Preparation of fish stomach and skin

Stomach of skipjack tuna (*Katsuwonus pelamis*) was obtained from Tropical Canning (Thailand) Public Co., Ltd., Songkhla, Thailand. The sample was packed in a polyethylene bag, kept in ice with a solid/ice ratio of 1:2 (w/v) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand, within 1 h. Upon arrival, the stomach was cleaned with cold water and placed in a polyethylene bag. The stomach sample was stored at  $-20^{\circ}\text{C}$  until use.

Ornate threadfin bream (*N. hexodon*) was obtained after being off-loaded for 6 h from the dock in Songkhla, Thailand. The fish were kept in ice with a solid/ice ratio of 1:2 (w/v) and were transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand, within 30 min. Upon arrival, the skin was removed and washed with cold water, drained and cut into small pieces ( $0.5 \times 0.5 \text{ cm}^2$ ). The skin sample was kept in a polyethylene bag and stored at  $-20^{\circ}\text{C}$  until use.

### 2.3. Preparation of pepsins

#### 2.3.1. Isolation of pepsins

The frozen stomach was thawed using running water ( $26\text{--}28^{\circ}\text{C}$ ) until the core temperature reached  $-2\text{--}0^{\circ}\text{C}$ . The sample

was cut into pieces with dimensions of  $0.5 \times 0.5 \text{ cm}^2$ . Sample was frozen in liquid nitrogen and finely ground using a National Model MX-T2GN blender (Taipei, Taiwan). The sample was then lyophilised using a SCANVAC CoolSafe™ freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark).

To prepare the skipjack pepsin extract, the stomach powder was suspended in 20 mM sodium phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) at a ratio of 1:19 (w/v), and was stirred continuously at  $4^{\circ}\text{C}$  for 3 h. The suspension was centrifuged for 20 min at  $4^{\circ}\text{C}$  at 20,000g (Avanti® J-E, Beckman Coulter, Inc., Palo Alto, CA, USA) to remove the tissue debris. The supernatant was collected and then adjusted to pH 4 with 1 M HCl. The mixture was allowed to stand at  $4^{\circ}\text{C}$  for 30 min. The treated stomach extract was then centrifuged at 20,000g for 15 min at  $4^{\circ}\text{C}$ . To fully activate pepsin, the pH of the acidified supernatant was adjusted to pH 2 with 1 M HCl, and was gently stirred for 30 min at  $4^{\circ}\text{C}$ . The enzyme solution was collected in the same manner as described previously prior to dialysis against 60 volumes of 20 mM sodium phosphate buffer (pH 5.0), referred to as 'starting buffer, SB' for 12 h at  $4^{\circ}\text{C}$ . The dialysate was collected, and was referred to as "skipjack pepsin extract".

To prepare the porcine pepsin solution, the enzyme powder (1.0 g) was dissolved with 120 ml of SB, followed by centrifugation at  $4^{\circ}\text{C}$  for 30 min at 20,000g. The supernatant was collected and used for further study.

#### 2.3.2. Partial purification of pepsins

All the steps of purification were carried out at  $4^{\circ}\text{C}$ . Skipjack pepsin extract was applied onto DEAE-sephacel column ( $1.6 \times 18 \text{ cm}$ ), previously equilibrated with SB. After the column was washed with SB until the  $A_{280}$  was below 0.05, the elution was performed with a linear gradient of 0–0.5 M NaCl in SB, at a flow rate of 0.5 ml/min. Fractions of 3 ml were collected and those with proteolytic activity were pooled. Two activity peaks (skipjack tuna pepsin 1 and 2, referred to as SP1 and SP2, respectively) were obtained and the pooled fractions from each peak were dialysed with 60 volumes of SB for 12 h. The dialysates were then subjected to lyophilisation. Lyophilised fractions were dissolved in SB and chromatographed onto Sephadex G-75 ( $1.6 \times 70 \text{ cm}$ ) column, followed by elution with SB at a flow rate of 0.5 ml/min. Fractions of 3 ml were collected and the fractions with proteolytic activity were combined and used for further study. The porcine pepsin solution was purified in the same manner. The purified porcine pepsin fraction was collected and referred to as "PP". During purification, the protein concentration was measured by monitoring the absorbance at 280 nm, and the proteolytic activity was determined using haemoglobin as a substrate at pH 2.0, and  $50^{\circ}\text{C}$ .

### 2.4. Enzyme assay

The proteolytic activity of the pepsin extract was determined using haemoglobin as a substrate according to the method of Nalinanon, Benjakul, and Kishimura (2010b). To initiate the reaction, 200  $\mu\text{l}$  of enzyme solution were added into the assay mixture containing 200  $\mu\text{l}$  of 2% haemoglobin, 200  $\mu\text{l}$  of distilled water and 625  $\mu\text{l}$  of reaction buffer. An appropriate dilution was made to ensure that the amount of enzyme was not excessive for available substrate in the assay system. The reaction was conducted at pH 2.0 and  $50^{\circ}\text{C}$  for 10 min. To terminate enzymatic reaction, 200  $\mu\text{l}$  of 50% (w/v) trichloroacetic acid (TCA) were added. Unhydrolysed protein substrate was allowed to precipitate for 1 h at  $4^{\circ}\text{C}$ , followed by centrifuging at 4250g for 10 min using a MIKRO 20 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). The oligopeptide content in the supernatant was measured at 280 nm. One unit (U) of activity was defined as the amount causing an increase of 1.0 in absorbance at 280 nm per min. A blank was

run in the same manner, except that the enzyme was added into the reaction mixture after the addition of 50% (w/v) TCA.

## 2.5. Preparation of type I collagen from the skin of ornate threadfin bream

### 2.5.1. Isolation of skin collagen

Acid solubilised collagen from the skin of ornate threadfin bream was isolated following the method of Nalinanon et al. (2007) with some modifications. All procedures were performed at 4 °C. To remove non-collagenous proteins, the skin was mixed with 0.1 M NaOH using a sample/alkaline solution ratio of 1:10 (w/v). The mixture was stirred continuously for 6 h. The alkaline solution was changed every 2 h. Then, the alkaline treated skin was washed with cold water until neutral or faintly basic pHs of wash water were obtained. The treated skin was then defatted with 10% (v/v) butyl alcohol with a solid/solvent ratio of 1:10 (w/v) for 18 h and the solvent was changed every 6 h. Defatted skin was washed with 10 volumes of cold water for three times prior to lyophilisation.

To extract the collagen, the lyophilised skin was soaked in 0.5 M acetic acid with a sample/solution ratio of 1:100 (w/v) for 48 h, with gentle stirring. The mixture was then centrifuged at 20,000g for 1 h at 4 °C. The supernatant was referred to as 'acid solubilised collagen, ASC'.

### 2.5.2. Fractionation of type I collagen

Type I collagen was fractionated according to the method of Yata, Yoshida, Fujisawa, Mizuta, and Yoshinaka (2001) with a slight modification. ASC was salted out by adding NaCl to a final concentration of 2.0 M, followed by centrifugation at 10,000g for 20 min. The precipitate was further extracted with 0.5 M acetic acid containing 11% (w/v) ammonium sulphate for 24 h. The mixture was then centrifuged at 10,000g for 20 min. This procedure was repeated three times. The resultant precipitate was referred to as "type I collagen".

### 2.5.3. Purification of type I collagen

Fractionated type I collagen was dissolved in 0.5 M acetic acid and dialysed against 60 volumes of the starting buffer (50 mM sodium acetate, pH 4.8) for 48 h; the dialysis solution was changed every 6 h. The dialysate was applied onto a TOYOPEARL® CM-650M column (1.8 × 20 cm), previously equilibrated with 10 volumes of the starting buffer, at a flow rate of 60 ml/h. After loading, the unbound proteins were washed by the same buffer until  $A_{230}$  was less than 0.05. Elution was achieved with a linear gradient of 0–0.5 M NaCl at a flow rate of 60 ml/h with a total volume of 270 ml. The fractions (3 ml) were collected and measured for the absorbance at 230 nm by a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). Appropriate fractions were pooled and dialysed against distilled water, followed by lyophilisation. The lyophilised collagen was referred to as "purified type I collagen".

## 2.6. Characterisation of type I collagen

### 2.6.1. Hydroxyproline content and conversion factor for collagen

The purified type I collagen was dehydrated with acetone prior to the determination of hydroxyproline content, as described by Nalinanon et al. (2007). The hydroxyproline content was calculated and expressed as mg/g of dried purified type I collagen. The conversion factor of hydroxyproline content to collagen content was calculated as:

$$\text{Conversion factor} = \frac{100}{\text{Hydroxyproline content (\% dry wt.)}}$$

### 2.6.2. UV absorption measurement

The purified type I collagen was dissolved in 0.5 M acetic acid to obtain a concentration of 1 mg/ml. The solution was placed into a quartz cell with a path length of 1 cm. The UV absorption spectrum of collagen was measured using a spectrophotometer. Prior to the measurement, the base line was set with 0.5 M acetic acid. The spectrum was obtained by scanning the wavelength in the range of 190–350 nm with a scan speed of 50 nm/min at room temperature.

### 2.6.3. Amino acid analysis

The purified type I collagen was hydrolysed under reduced pressure in 4.0 M methanesulfonic acid containing 0.2% (v/v) 3-(2-aminoethyl)indole at 115 °C for 24 h. The hydrolysates were neutralised with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.4 ml was applied to an amino acid analyser (MLC-703; Atto Co., Tokyo, Japan).

### 2.6.4. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed by the method of Laemmli (1970). The samples were mixed with 5% (w/v) SDS and heated at 85 °C for 1 h. The mixtures were then centrifuged at 8500g for 5 min to remove undissolved debris. Solubilised samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% (w/v) SDS, 20% (v/v) glycerol) in the presence or absence of 10% (v/v) βME. Samples (15 µg protein) were loaded onto a polyacrylamide gel made of 7.5% separating gel and 4% stacking gel, and subjected to electrophoresis at a constant current of 15 mA/gel, using a Mini-PROTEAN II unit (Bio-Rad Laboratories Inc., Richmond, CA, USA). After electrophoresis, the gels were fixed with a mixture of 50% (v/v) methanol and 10% (v/v) acetic acid for 45 min, followed by staining with 0.05% (w/v) Coomassie Blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid for 3 h. Finally, they were destained with the mixture of 30% (v/v) methanol and 10% (v/v) acetic acid for 45 min. High-molecular-weight protein markers (GE Healthcare UK Limited, Buckinghamshire, UK) were used to estimate the molecular weight of proteins. Type I collagen from calf skin was used as the standard. The gels were imaged using a Canon image scanner (CanoScan LiDE25, Canon Inc., Tokyo, Japan) and the band intensities were quantified with the public domain digital analysis software, ImageJ (ImageJ 1.42q, National Institutes of Health, Bethesda, MD, USA).

### 2.6.5. Determination of subunit compositions of type I collagen

The analysis of the subunit compositions of purified type I collagen was performed according to the method of Kittiphattanabawon et al. (2010) and Nalinanon et al. (2010a) with a slight modification. Purified type I collagen (20 mg) were dissolved in 10 ml of starting buffer (50 mM sodium acetate buffer, pH 4.8 containing 2 M urea) and incubated at 60 °C for 30 min. The mixtures were centrifuged at 20,000g at room temperature (25–26 °C) for 30 min. The supernatant was applied onto a TOYOPEARL® CM-650M column (1.8 × 20 cm) previously equilibrated with 10 volumes of the starting buffer at a flow rate of 60 ml/h. After loading, the unbound proteins were washed by the same buffer until the  $A_{230}$  was less than 0.05. Elution was achieved with a linear gradient of 0–0.2 M NaCl in the same buffer, at a flow rate of 60 ml/h, with a total volume of 270 ml. The eluant was monitored at 230 nm and fractions (3 ml) were collected. The selected fractions were subjected to SDS–PAGE using 7.5% separating gel and 4% stacking gel, as previously described.

### 2.6.6. Differential scanning calorimetry (DSC)

DSC analysis of the collagen samples was carried out following the method of Nalinanon et al. (2007) with a slight modification. The samples were rehydrated by adding 0.05 M acetic acid to dried

samples at a solid/solution ratio of 1:40 (w/v). The mixture was allowed to stand for 2 days at 4 °C. DSC analysis was performed using a differential scanning calorimeter (Model DSC 7, Norwalk, CT, USA). Temperature calibration was done using the indium thermogram. The collagen solutions (5–10 mg) were accurately weighed into aluminium pans and sealed. The samples were scanned at 1 °C/min over the range of 20–50 °C using iced water as the cooling medium. An empty pan was used as the reference. The maximum transition temperature ( $T_m$ ) was estimated from the DSC thermogram. Total denaturation enthalpy ( $\Delta H$ ) was estimated by measuring the area in the DSC thermogram.

#### 2.6.7. Measurement of zeta ( $\zeta$ ) potential

Purified type I collagen was dissolved in 0.5 M acetic acid at a concentration of 0.5 mg/ml. The mixture was stirred at 4 °C for 12 h. The  $\zeta$ -potential of the sample (20 ml) was measured using a Zeta potential analyser (ZetaPALS, Brookhaven Instruments Co., Holtsville, NY, USA). The pH of the samples was adjusted to different values with 1.0 M Nitric acid or 1.0 M KOH using an Autotitrator (BI-ZTU, Brookhaven Instruments Co., Holtsville, NY, USA). The isoelectric point (pI) was estimated from pH rendering zero  $\zeta$ -potential.

#### 2.7. Hydrolysis of type I collagen by pepsin

PP, SP1 or SP2 (4 U) was added to 2 ml of a reaction mixture, containing 8 mg of purified type I collagen in 0.5 M acetic acid, pH 2.0. The hydrolysis was conducted by incubating the mixture at 4 °C for 24 h or at 50 °C for 0, 0.5, 1, 2 and 3 h. The control was performed by incubating the reaction mixture at 4 °C for 24 h or 50 °C for 3 h without the addition of pepsins. At the designated time, 200  $\mu$ l of reaction mixture was taken and mixed with 100  $\mu$ l of 1 M NaOH containing 1% (w/v) SDS in order to terminate the pepsin activity. The mixture was further mixed with 100  $\mu$ l of SDS–PAGE sample buffer and boiled for 1 min. The reaction mixture was analysed by SDS–PAGE using 7.5% separating gel and 4% stacking gel, as previously described.

#### 2.8. Statistical analysis

All data were subjected to Analysis of Variance (ANOVA), and the differences between means were evaluated by Duncan's multiple

range test. The SPSS statistic programme (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA) was used for data analysis.

### 3. Results and discussion

#### 3.1. Extraction of ASC and purification of type I collagen

ASC from the skin of ornate threadfin bream was extracted with the yield of  $24.9 \pm 1.1\%$  (dry wt.). The hydroxyproline content in the skin and ASC was  $49.5 \pm 0.4$  and  $72.3 \pm 0.5$  mg/g dry wt., respectively. The hydroxyproline content of ASC increased by approximately 1.46-fold, compared with that found in the skin. The result suggested that non-collagenous matters could be removed effectively from the skin prior to collagen extraction. To separate type I collagen, ASC was fractionated by NaCl precipitation. The precipitate was further extracted with 0.5 M acetic acid containing 11% (w/v) ammonium sulphate. Yata et al. (2001) isolated pepsin-solubilised collagen from the skin of common horse mackerel, yellow sea bream and tiger puffer fish with the same method, and found that type I collagen was a major collagen in the precipitate, whereas the supernatant contained type V collagen. After salt fractionation, the supernatant was further precipitated by 20% (w/v) ammonium sulphate. No collagen was recovered, suggesting that very low or no type V collagen in ASC from the skin of ornate threadfin bream was solubilised by acetic acid (data not shown). The collected precipitate was subjected to further purification using TOYOPEARL® CM-650M ion-exchange column chromatography (Fig. 1). The peak (fractions 50–62) showed the typical SDS–PAGE pattern of type I collagen with two different  $\alpha$  bands,  $\alpha_1(I)$  and  $\alpha_2(I)$  (see inset). It also contained  $\beta$  and  $\gamma$  components as well as other higher molecular weight cross-linked components. The band intensity of  $\alpha_1$  was approximately 2-fold higher than that of  $\alpha_2$ , indicating the existence of  $[\alpha_1(I)]_2\alpha_2(I)$  in collagen triple helix. Fractions No. 50–62 containing collagen components were pooled and used for further study. Based on the precipitation by ammonium sulphate at acidic pH and SDS–PAGE pattern, the collagen fraction obtained could be classified as type I collagen. Purified type I collagen had the higher hydroxyproline content ( $78.6 \pm 0.3$  mg/g dry wt.) in comparison with that of ASC ( $P < 0.05$ ), indicating a greater purity in the former. When the absorbance spectrum was determined for purified type I collagen, the distinct absorption peak was observed at 230 nm. This was mainly attributed to the absorption of the peptide bonds in the

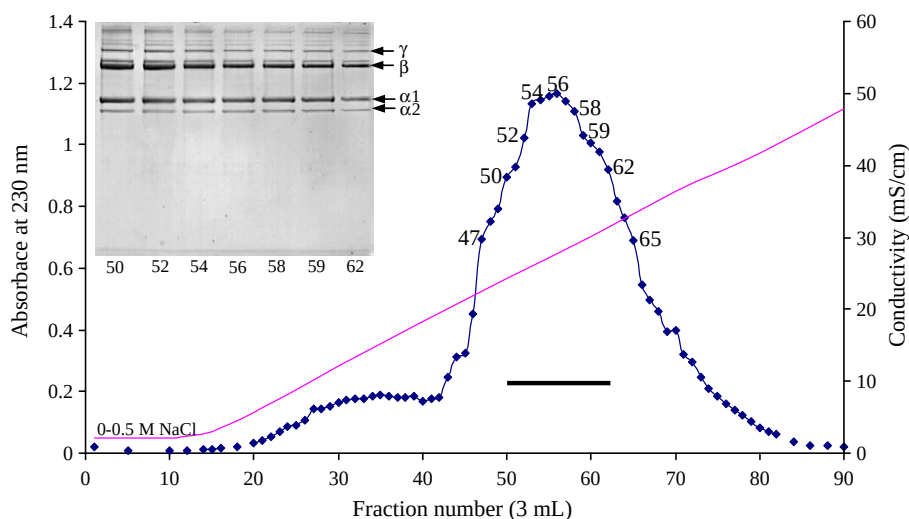


**Fig. 1.** TOYOPEARL® CM-650M ion-exchange column chromatography of type I collagen isolated from the skin of ornate threadfin bream. The column (1.8 × 20 cm) was equilibrated with 50 mM sodium acetate, pH 4.8. Adsorbed proteins were eluted with a linear gradient of 0–0.5 M NaCl over a total volume of 270 ml, at a flow rate of 60 ml/h. The inset shows the SDS–PAGE pattern (7.5% separating gel and 4% stacking gel) of the fractions indicated by the numbers.

polypeptides chains of collagen. No absorption peak was detected at 280 nm (data not shown). Thus, type I collagen from the skin of ornate threadfin bream was obtained with negligible contamination of other proteins.

With reference to hydroxyproline content, the conversion factor of hydroxyproline to collagen from the skin of ornate threadfin bream was calculated to be 12.7. The conversion factor of collagen from other sources, such as Baltic cod backbone (15.7) (Żelec-howska, Sadowska, & Turk, 2010) and skin (14.7) (Sadowska, Kołodziejka, & Niecikowska, 2003) has been reported. This factor might be useful for the estimation of collagen content in raw material, and the yield of extraction, as well as the purity assessment of the resultant collagen.

### 3.2. Subunit composition of purified type I collagen

The elution profiles of the purified type I collagen on the TOYOPEARL® CM-650M column, after being denatured by heat treatment in the presence of 2 M urea, are shown in Fig. 2. Three peaks containing different collagen components were obtained. The chromatographic fractions indicated by numbers were subjected to SDS–PAGE analysis as shown in the inset. The first peaks (fraction No. 37) contained only a band of  $\alpha$ -chain. The second peak (fraction No. 41) consisted of two distinct components,  $\beta$ 11 (dimer of  $\alpha$ 1) and  $\alpha$ -chains. High MW component,  $\gamma$ -chain and  $\beta$ 12 (dimer of  $\alpha$ 1 and  $\alpha$ 2) were concentrated in fraction No. 47–51 of the third peak, whilst  $\alpha$ 2 was observed in fraction No. 49–51. Since the  $\alpha$ -chains found in fraction No. 37 and 41 were eluted by different NaCl concentrations but had similar molecular mobility, both  $\alpha$ 1- and  $\alpha$ 3-chains were most likely present in purified collagen. Kimura et al. (1987) and Matsui, Ishida, and Kimura (1991) reported that the electrophoretic mobility in SDS–PAGE of  $\alpha$ 3 from chum salmon, as well as other teleosts, was almost identical to that of  $\alpha$ 1. Therefore, the purified collagen from the skin of ornate threadfin bream not only comprised [ $\alpha$ 1(I)]<sub>2</sub> $\alpha$ 2(I) but also  $\alpha$ 1(I) $\alpha$ 2(I) $\alpha$ 3(I). The existence of two molecular forms, [ $\alpha$ 1(I)]<sub>2</sub> $\alpha$ 2(I) and  $\alpha$ 1(I) $\alpha$ 2(I) $\alpha$ 3(I), were reported in fish type I collagen (Kimura et al., 1987). The  $\alpha$ 3-chain was reported as the intermediate between  $\alpha$ 1 and  $\alpha$ 2 (Matsui et al., 1991).

### 3.3. Amino acid composition of purified type I collagen

The amino acid composition of purified type I collagen from the skin of ornate threadfin bream, expressed as residues per 1000 total amino acid residues, is shown in Table 1. The purified type I collagen was rich in glycine, alanine and proline. It had relatively low contents of tyrosine and histidine and it contained no cysteine and tryptophan. The results were in accordance with those of type I collagens from the skin of other fish species, such as common horse mackerel, yellow sea bream, tiger puffer fish (Yata et al., 2001), black drum, sheephead seabream (Ogawa et al., 2003), brownstripe red snapper (Jongjareonrak et al., 2005) and arabesque greenling (Nalinanon et al., 2010a). The imino acid (proline and hydroxyproline) content of purified type I collagen was 188 residues/1000 residues, which was slightly lower than that of type I collagen from calf skin (215 residues/1000 residues) (Ogawa et al., 2003), young and adult Nile perch (193–200 residues/1000 residues) (Muyonga et al., 2004) and bigeye snapper (193 residues/1000 residues) (Kittiphattanabawon et al., 2005). However, the imino acid content of purified collagen was relatively higher than those reported for collagen from the skin of cold water fish species, such as arabesque greenling (Nalinanon et al., 2010a), cod (Duan et al., 2009), deep-sea red fish (Wang et al., 2007) and Pacific whiting (Kim & Park, 2004), which had imino acid content ranging from 154 to 165 residues/1000 residues. The imino acids contribute to the stability of helical structure of collagen (Ikoma, Kobayashi, Tanaka, Walsh, & Mann, 2003; Jongjareonrak et al., 2005). The stability of collagen was proportional to the total content of imino acids (Kittiphattanabawon et al., 2010). Pyrrolidine rings of proline and hydroxyproline impose restrictions on the conformation of the polypeptide chain and help to strengthen the triple helix (Bae et al., 2008; Wong, 1989). The difference in the imino acid content amongst the animals was associated with the different living environments (Foegeding et al., 1996; Kittiphattanabawon et al., 2010; Nalinanon et al., 2010a; Regenstein & Zhou, 2007). Hydroxylysine (8 residues/1000 residues) was found in purified type I collagen, suggesting the partial cross-linking of collagen via covalent bonds (Mechanic, Katz, Henmi, Noyes, & Yamauchi, 1987; Wong, 1989).

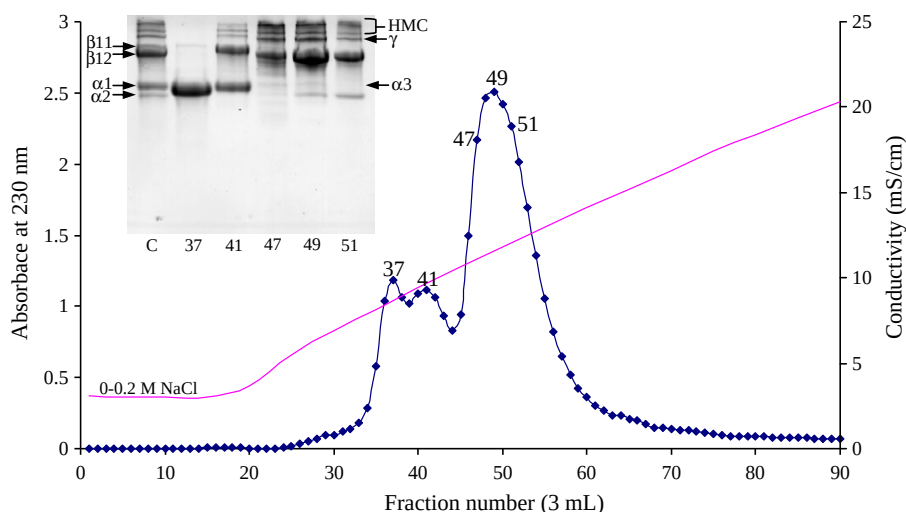


**Fig. 2.** TOYOPEARL® CM-650M ion-exchange column chromatography of denatured type I collagen from the skin of ornate threadfin bream. The column (1.8 × 20 cm) was equilibrated with 50 mM sodium acetate, pH 4.8, containing 2 M urea. Adsorbed proteins were eluted with a linear gradient of 0–0.2 M NaCl over a total volume of 270 ml at a flow rate of 60 ml/h. The inset shows the SDS–PAGE pattern (7.5% separating gel and 4% stacking gel) of the fractions indicated by the numbers. C and HMC denote collagen and high MW cross-linked components, respectively.

**Table 1**

Amino acid composition of purified type I collagen from the skin of ornate threadfin bream (residues/1000 residues).

| Amino acid               | Purified type I collagen |
|--------------------------|--------------------------|
| Alanine                  | 134                      |
| Arginine                 | 53                       |
| Aspartic acid/asparagine | 49                       |
| Cysteine                 | 0                        |
| Glutamic acid/glutamine  | 72                       |
| Glycine                  | 321                      |
| Histidine                | 5                        |
| Isoleucine               | 9                        |
| Leucine                  | 23                       |
| Lysine                   | 27                       |
| Hydroxylysine            | 8                        |
| Methionine               | 14                       |
| Phenylalanine            | 14                       |
| Hydroxyproline           | 84                       |
| Proline                  | 104                      |
| Serine                   | 35                       |
| Threonine                | 22                       |
| Tyrosine                 | 4                        |
| Tryptophan               | 0                        |
| Valine                   | 21                       |
| Total                    | 1000                     |
| Imino acids <sup>a</sup> | 188                      |

<sup>a</sup> Imino acids include proline and hydroxyproline.

### 3.4. Thermal stability of purified type I collagen

DSC analysis of purified type I collagen from the skin of ornate threadfin bream and type I collagen from calf skin rehydrated in 0.05 M acetic acid was performed. The maximum transition temperature ( $T_m$ ) and total denaturation enthalpy ( $\Delta H$ ) of purified type I collagen was 33.35 °C and 0.819 J/g, respectively (data not shown), which were lower than that of type I collagen from calf skin ( $T_m = 39.27$  °C;  $\Delta H = 1.204$  J/g). This might be due to the lower content of imino acids (hydroxyproline and proline) of collagen from the skin of ornate threadfin bream. The thermal stability of collagen is associated with the restriction of the secondary structure of the polypeptide chain governed by the pyrrolidine rings of proline and hydroxyproline, and partially by hydrogen bonding through the hydroxyl group of the hydroxyproline (Benjakul et al., 2010; Piez & Gross, 1960). The  $T_m$  of purified type I collagens from the skin of ornate threadfin bream was similar to that of collagen from subtropical and tropical species, such as bigeye snapper (32.5 °C) (Nalinanon et al., 2007) and channel catfish (32.5 °C) (Liu et al., 2007), black drum (34.2 °C) (Ogawa et al., 2003), sheephead seabream (34 °C) (Ogawa et al., 2003), eagle ray (34.1 °C) (Bae et al., 2008), and brownbanded bamboo shark (Kittiphattanabawon et al., 2010). Lower denaturation temperatures of skin collagen from several cold and temperate water fishes including cod (15 °C) (Duan et al., 2009), arabesque greenling (15.7 °C) (Nalinanon et al., 2010a), deep-sea redfish (16.1 °C) (Wang et al., 2007), Pacific whiting (21.7 °C) (Kim & Park, 2004), walleye pollock (24.6 °C) (Yan et al., 2008), Japanese seabass (26.5 °C), chub mackerel (25.6 °C), bullhead shark (25 °C) (Nagai & Suzuki, 2000) and ocellate puffer fish (28 °C) (Nagai et al., 2002), have been reported. The differences in denaturation temperature amongst the collagens from different fish species were correlated with the imino acid content, body temperature and environmental temperature (Kittiphattanabawon et al., 2010; Nagai et al., 2002; Rigby, 1968).

### 3.5. $\zeta$ -Potential of purified type I collagen

The  $\zeta$ -potential, representing the surface charge of purified type I collagen from the skin of ornate threadfin bream at different pHs,

was determined. The surface net charge of purified type I collagen decreased as the pH increased and it became zero at pH 6.40 (data not shown). The pH where the  $\zeta$ -potential is zero corresponds to the pI of the protein (Ma et al., 2009; Nalinanon et al., 2010b). Thus, the pI of purified type I collagen was estimated to be 6.40. Purified type I collagen had a net positive or a negative charge when pH values were below and above their pI, respectively. The pI of purified type I collagen was found in a slight acidic pH, which was in accordance with those found in the collagen from the skin of arabesque greenling (Nalinanon et al., 2010b) and brownbanded bamboo shark (Kittiphattanabawon et al., 2010). This might be due to a higher content of acidic amino acids, aspartic acid and glutamic acid, compared to basic amino acids, including histidine, lysine and arginine (Nalinanon et al., 2010b).

### 3.6. Hydrolysis of purified type I collagen by different pepsins

The degradation patterns of purified type I collagen from the skin of ornate threadfin bream (C-OTB) digested by porcine pepsin (C-PP), skipjack pepsin 1 (C-SP1) and 2 (C-SP2) at a level of 0.5 U/mg collagen, for 24 h at 4 °C, are shown in Fig. 3. This condition was carried out to simulate the hydrolysis of type I collagen during the extraction of pepsin-solubilised collagen. The result revealed that the major components including  $\beta$ ,  $\alpha_1$ , and  $\alpha_2$ , were fully retained (Fig. 3a). However, some degradation bands were observed in C-PP, C-SP1 and C-SP2, as evidenced by the increase in band intensity at the dye front. The degradation was more pronounced in C-SP2, in which degradation products with molecular weight (MW) of 67 and 71 kDa were observed. The small peaks of those bands were noticeable in the densitogram (Fig. 3a and b). Pepsin treatment of C-OTB had an impact on the proportion of collagen components. In general,  $\beta$ - and  $\gamma$ -chains (dimer and trimer, respectively) as well as very high (VMC) and high MW components (HMC) in collagens decreased after treatment with all pepsins. Cleavage of the telopeptide region more likely resulted in the conversion of cross-links, such as  $\beta$ - and  $\gamma$ -chains, to the  $\alpha$ -chain (Fig. 3b). C-OTB contained the higher proportion of cross-linked chains ( $\beta$ ,  $\gamma$  and VMC) than that of pepsin treated collagen. VMC in C-OTB disappeared when treated with pepsin, regardless of the type of pepsin used. This VMC was degraded to smaller components, such as  $\beta$ ,  $\alpha_1$  or  $\alpha_2$ , by pepsin. Substantial decreases in VMC, HMC,  $\beta$ - and  $\gamma$ -chains, with the concomitant formation of  $\alpha_1$ , were observed. The band intensity ratios of  $\alpha_1/\alpha_2$ -chains were significantly increased from 2.18 (C-OTB) to 2.37, 3.00 and 3.45 for C-PP, C-SP1 and C-SP2, respectively (Table 2). The result showed that amongst the pepsin used, SP2 had the greatest ability to cleave intermolecular cross-links in C-OTB and to produce a monomeric chain. SP1 exhibited less detrimental effect on the collagen structure, as evidenced by the higher ratio of collagen components ( $\beta$ ,  $\alpha_1$  and  $\alpha_2$ ) to that of lower MW components produced from pepsin hydrolysis. The conversion of cross-linked components to monomer chains, by removing the cross-link containing telopeptide, was reported for pepsin-solubilised collagens from the skin of deep-sea redfish (Wang et al., 2007), black drum, and sheephead seabream (Ogawa et al., 2003). There were no differences in the relative mobilities of  $\alpha_1$  and  $\alpha_2$ -chains between C-OTB and C-PP. Slight differences in relative mobilities between C-OTB and collagen with skipjack pepsin treatments (C-SP1 and C-SP2) were observed. The MWs of  $\alpha_1$  and  $\alpha_2$ -chains estimated to be 128 and 116 kDa, respectively, were observed in both C-OTB and C-PP. For C-SP1 and C-SP2, the MWs of the  $\alpha_1$ -chains were estimated to be 126 and 122 kDa, respectively, whereas those of  $\alpha_2$ -chains were 115 and 112 kDa, respectively. Short peptides with MWs of 2–6 kDa and 1–4 kDa from the telopeptides of  $\alpha_1$  and  $\alpha_2$ , respectively, were produced by the action of pepsin. These results suggested that both pepsins from skipjack tuna partially hydrolysed C-OTB at either telopeptide regions

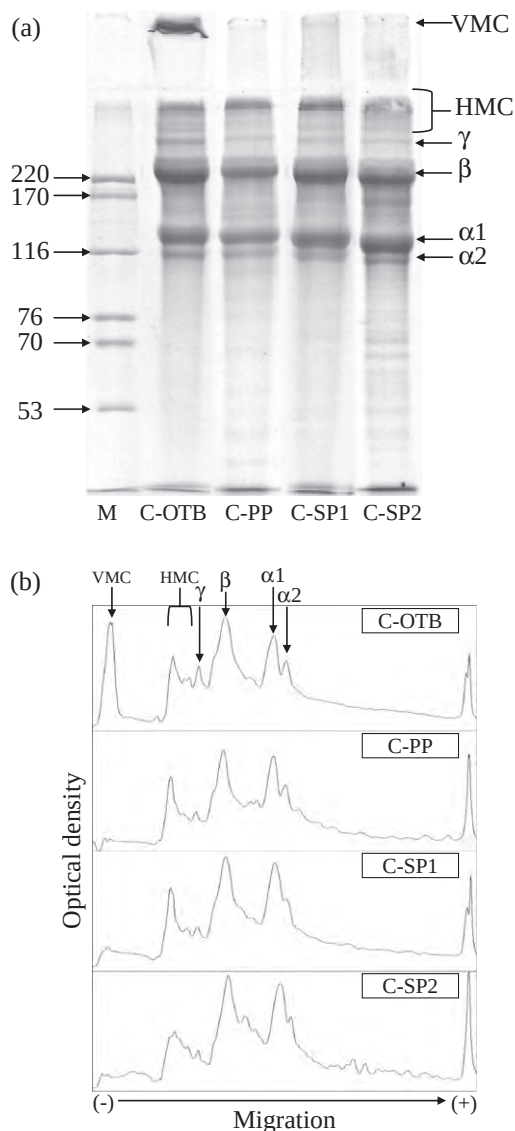


**Fig. 3.** Protein patterns (a) and densitograms (b) of purified type I collagen (C-OTB) and purified type I collagen treated with porcine pepsin (C-PP), skipjack pepsin 1 (C-SP1) and skipjack pepsin 2 (C-SP2), at a level of 0.5 unit/mg collagen for 24 h at 4 °C. VMC and HMC denote very high and high MW cross-linked components of collagen, respectively. M denotes standard protein marker.

**Table 2**

Band intensity ratios of  $\alpha 1$ -chain to  $\alpha 2$ -chain and cross-linked chains to the total monomer chains of purified type I collagen from the skin of ornate threadfin bream, subjected to hydrolysis by different pepsins.

| Components                     | Band intensity ratio <sup>A</sup> |                   |                  |                  |
|--------------------------------|-----------------------------------|-------------------|------------------|------------------|
|                                | C-OTB                             | C-PP              | C-SP1            | C-SP2            |
| $\alpha 1/\alpha 2$            | $2.18 \pm 0.10a^B$                | $2.37 \pm 0.05b$  | $3.00 \pm 0.12c$ | $3.45 \pm 0.09d$ |
| $\beta/(\alpha 1 + \alpha 2)$  | $1.39 \pm 0.06c$                  | $1.18 \pm 0.04b$  | $1.21 \pm 0.05b$ | $0.97 \pm 0.04a$ |
| $\gamma/(\alpha 1 + \alpha 2)$ | $0.33 \pm 0.01c$                  | $0.18 \pm 0.01b$  | $0.16 \pm 0.02b$ | $0.11 \pm 0.04a$ |
| HMC/ $(\alpha 1 + \alpha 2)$   | $0.62 \pm 0.04b$                  | $0.59 \pm 0.03ab$ | $0.55 \pm 0.02a$ | $0.52 \pm 0.03a$ |
| VMC/ $(\alpha 1 + \alpha 2)$   | $0.61 \pm 0.01b$                  | $0.02 \pm 0.01a$  | $0.04 \pm 0.02a$ | $0.03 \pm 0.02a$ |

<sup>A</sup> Mean  $\pm$  standard deviation ( $n = 3$ ).

<sup>B</sup> Different letters in the same row indicate the significant differences ( $P < 0.05$ ). C-OTB denotes purified type I collagen. C-PP, C-SP1 and C-SP2 denote purified type I collagens treated with porcine pepsin, skipjack pepsin 1 and skipjack pepsin 2, respectively.

or the end of helical region. A slight decrease in MW, by about 1–2 kDa, has been reported for pepsin-solubilised collagens from the skin of bigeye snapper, in comparison with that of the acid

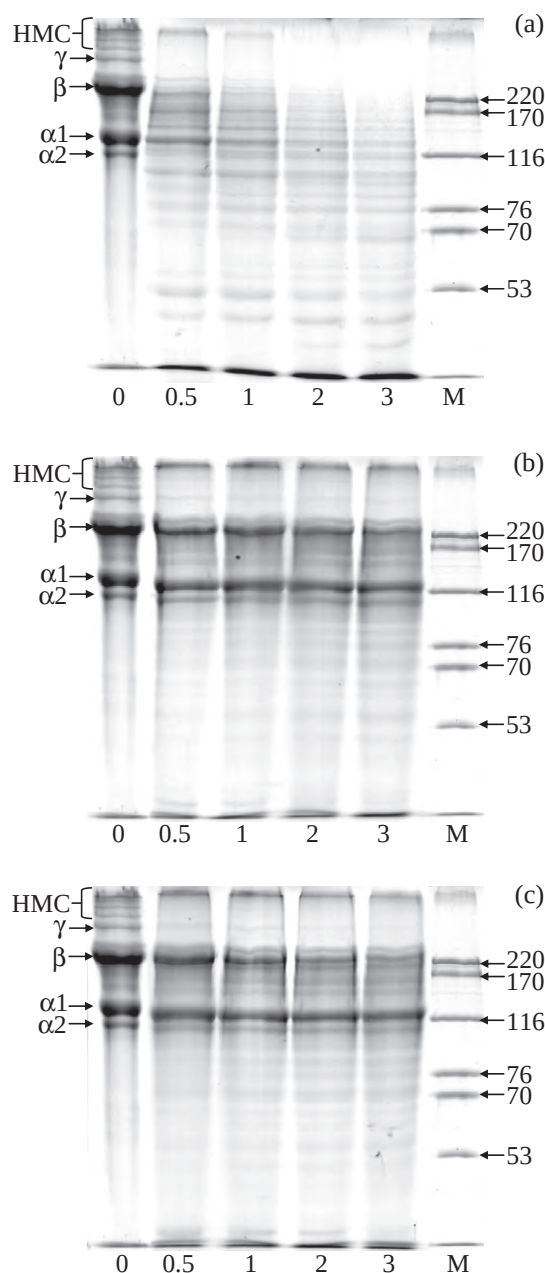


**Fig. 4.** Degradation patterns of purified type I collagen treated with PP (a), SP1 (b) and SP2 (c) at a level of 0.5 unit/mg collagen, at 50 °C, for different times. Number of each lane denotes pepsin hydrolysis time (h). HMC and M denote high MW cross-linked components of collagen and standard protein marker, respectively.

solubilised collagen (Nalinanon et al., 2007). Although no changes in the relative mobilities of  $\alpha$ -chains in C-PP were observed, a partial cleavage of collagen at the telopeptide regions also took place when porcine pepsin was used, as evidenced by the increase in the  $\alpha 1/\alpha 2$  proportion. Therefore, different pepsins were able to cleave collagen cross-links at different positions, particularly at the telopeptide region.

The degradation patterns of the purified type I collagen from the skin of ornate threadfin bream treated with PP, SP1 and SP2, at a level of 0.5 U/mg collagen and at 50 °C for different times, are shown in Fig. 4. Hydrolysis was more pronounced with increasing digestion time. The HMC and the  $\gamma$ -chain almost disappeared after 30 min of pepsin digestion. The degradation was much higher in purified type I collagen treated with porcine pepsin (Fig. 4a),

compared with that found in the collagen treated with skipjack tuna pepsin. The major components, including  $\gamma$ -,  $\beta$ - and  $\alpha$ -chains, were drastically hydrolysed by porcine pepsin after 30 min, with the concomitant formation of degradation bands with MWs of 47, 52 and 70–101 kDa. Those major components were completely hydrolysed after 2 h of digestion. Furthermore, the  $\alpha 2$ -chain was more susceptible to porcine pepsin hydrolysis than the  $\alpha 1$ -chain. Purified type I collagen was therefore more resistant to skipjack tuna pepsin hydrolysis (Fig. 4b and c). The major components were retained after 3 h of skipjack tuna pepsin hydrolysis. The results revealed that SP2 had a slightly greater hydrolysis activity on purified type I collagen than did SP1. The lower band intensity of HMC,  $\beta$ - and  $\alpha$ -chain of purified type I collagen treated with SP2 was observed, compared with that of SP1 hydrolysis. The similar result was reported by Nalinanon, Benjakul, and Kishimura (2010c) who found that purified pepsin 2 from the stomach of skipjack tuna exhibited slightly higher effectiveness for hydrolysis of ASC from the skin of arabesque greenling, compared with purified pepsin 1. Therefore, fish pepsin, especially from skipjack tuna, could be used to increase the extraction efficacy of collagen with the negligible cleavage of the major components of collagen. This revealed the greater advantage of fish pepsin as the aid for the extraction of pepsin-solubilised collagen from fish skin.

#### 4. Conclusions

Acid solubilised collagen (ASC) from the skin of ornate threadfin bream contained type I collagen with  $[\alpha 1(I)]_2\alpha 2(I)$  as the major components;  $\alpha 1(I)\alpha 2(I)\alpha 3(I)$  was also found as minor component. Type I collagen showed a varied susceptibility to hydrolysis by pepsin, depending on hydrolysis temperature and type of pepsin used. Pepsin from the stomach of skipjack tuna showed the greater ability to cleave the cross-linked components without the cleavage of major components of collagen, whereas porcine pepsin more likely hydrolysed all components, especially at high temperature. Therefore, pepsin from skipjack tuna can be applied to effectively recover the residual collagen in acid-treated skin, from which the higher yield can be obtained. Additionally, skipjack tuna pepsin can be used as the potential alternative for bovine or porcine pepsins, which may have the limitation for application in some religions.

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# Use of Protein Hydrolysate from Yellow Stripe Trevally (*Selaroides leptolepsis*) as Microbial Media

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**Abstract** The objective of this study was to investigate the potential use of protein hydrolysate from yellow stripe trevally as a nitrogen source for the growth of different microorganisms. Protein hydrolysates from yellow stripe trevally with different degrees of hydrolysis (5, 15 and 25%) prepared using Alcalase (HA) or Flavourzyme (HF) were determined in comparison with commercial Bacto Peptone. For bacteria, *Staphylococcus aureus* and *Escherichia coli*, HF with 25% DH (HF<sub>25</sub>) yielded the highest cell density and specific growth rate ( $\mu_{\max}$ ) and the lowest generation time ( $t_d$ ) ( $p<0.05$ ). For yeasts, *Saccharomyces cerevisiae* and *Candida albicans*, Bacto Peptone yielded the higher growth rate than did HA and HF ( $p<0.05$ ), whereas no differences in  $\mu_{\max}$  and  $t_d$  were observed for fungus, *Aspergillus oryzae* ( $p>0.05$ ). The pH of culture broth containing HF<sub>25</sub> decreased markedly during the first 8 hours of cultivation of *S. aureus* and *E. coli* ( $p<0.05$ ). This directly lowered the colony size of *S. aureus* ( $p<0.05$ ). However, buffered culture broth containing HF<sub>25</sub> rendered the similar growth and colony size of *S. aureus* ( $p>0.05$ ), compared with that containing Bacto Peptone. Scanning

electron microscopic study revealed no differences in size and shape of microorganisms cultured in HF<sub>25</sub> and Bacto Peptone ( $p>0.05$ ).

**Keywords** Protein hydrolysate · Yellow stripe trevally · Microbial growth · Peptone · Alcalase · Flavourzyme

## Introduction

Fish processing by-products and under-utilized species are commonly recognized as low-value resources with negligible market value. In addition, their inappropriate disposal is a major cause for environmental pollution. To tackle these problems, hydrolysis processes have been developed to convert such resources into marketable and acceptable forms. Protein hydrolysates from different fish species such as tuna, cod, salmon and unspecified fish have been used as nitrogenous sources for microbial growth (Dufosse et al. 2001). Soluble fish protein hydrolysate from extensive hydrolysis comprises free amino acids and low-molecular-weight peptides, which can be used as an excellent nitrogen source for microbial growth and thus can be used as microbial peptone. The peptides with the molecular weight of 6,500 Da, di-peptides and amino acids from tuna treated with Alcalase served as a suitable nitrogenous source in microbial media (Guerard et al. 2001). Recently, protein hydrolysates from yellow stripe trevally have been produced successfully using Alcalase and Flavourzyme (Klompong et al. 2007a, b). Degree of hydrolysis (DH) can affect the functional properties and antioxidative activity of resulting protein hydrolysate (Klompong et al. 2007a). DH influencing peptide chain length may have the impact on the ease of utilization by microorganisms. In addition, different peptides produced by different proteases

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can be used by microorganisms at varying degrees. Therefore, the objective of this study was to investigate the potential use of protein hydrolysate from yellow stripe trevally produced by Alcalase or Flavourzyme as a nitrogen source for the growth of different microorganisms including bacteria, yeasts and mold.

## Materials and Methods

### Enzymes and Cultivation Media

Alcalase and Flavourzyme were obtained from Novo Nordisk (Bagsvaerd, Denmark) and East Asiatic Company Ltd. (Bangkok, Thailand), respectively. Bacto Peptone was purchased from Difco Laboratories (Sparks, MD, USA). Nutrient Broth (NB) was procured from Merck (Darmstadt, Germany), and Potato Dextrose Broth (PDB) was obtained from Himedia Laboratories (Mumbai, Maharashtra, India).

### Fish Sample Collection and Preparation

Yellow stripe trevally (*Selaroides leptolepis*) caught along the coast of the Andaman Sea with the size of 65 g per fish, off-loaded approximately 24–36 hours after capture, were obtained from the fishing port in Satul province, Thailand. Fish were washed, and the meat was separated manually. The meat was minced using a mincer with 0.4-cm-diameter holes. The mince was stored in ice until use.

### Production of Protein Hydrolysates from Yellow Stripe Trevally

Mince (60 g) was suspended in 240 ml of distilled water. The mixture was homogenized using a homogenizer (IKA Labortechnik, Selangor, Malaysia) at a speed of 11,000 rpm for 1 minute. The homogenate was adjusted to pHs 8.5 and 7.0 and preincubated at 60 or 50 °C for 20 minutes prior to enzymatic hydrolysis using Alcalase and Flavourzyme, respectively. The hydrolytic reaction was started by the addition of 0.0076, 0.05 and 0.33% Alcalase (w/w) or 0.26, 1.58, 9.77% Flavourzyme (w/w) based on the protein content of mince (Klompong et al. 2007a). The reaction was conducted as per the pH-stat method (Adler-Nissen 1986) for 20 minutes to obtain the DH of 5, 15, 25% as described by Klompong et al. (2007a).

### Media Formulas

Different media including Nutrient Broth (NB) and agar (NA), yeast–malt broth (YMB) and agar (YMA) and Malt Extract Broth (MEB) and agar (MEA) were prepared for culturing bacteria, yeasts and mold, respec-

tively. To study the use of HA or HF as the replacer for commercial peptone, Bacto Peptone was substituted by HA with DHs of 5% (HA<sub>5</sub>), 15% (HA<sub>15</sub>) and 25% (HA<sub>25</sub>) or HF with DHs of 5% (HF<sub>5</sub>), 15% (HF<sub>15</sub>) and 25% (HF<sub>25</sub>) at the amount yielding an equivalent level of nitrogen content to Bacto Peptone determined by Kjeldahl method (AOAC 2000).

### Preparation of Microorganisms and Inocula

*Staphylococcus aureus* TISTR 118, *Escherichia coli* TISTR 780 and *Saccharomyces cerevisiae* TISTR 5017 were obtained from the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand. *Candida albicans* PSSCMI 7010 and *Aspergillus oryzae* PSSCMI 3004 were gifts from the Department of Microbiology, Prince of Songkla University, Hat Yai, Thailand. Bacteria were kept on NA slants, whereas yeasts and mold were kept on PDA slants at 4 °C until use. To activate microorganisms before culturing, the inocula were prepared. The inocula were adjusted to obtain A<sub>660</sub> of 0.02 and then subjected to centrifugation at 3,000×g (Hettich, Mikro 20, Zentrifugen, Germany) for 20 minutes at 25 °C. Supernatants were discarded, and the cells were washed twice with normal saline (0.85% NaCl solution). The fresh culture media were added to the cell pellet to obtain A<sub>660</sub> of 0.02. The obtained cell suspension was used as late log phase inocula. For mold, *A. oryzae* was sub-cultured onto PDA slant and incubated at 25 °C for 48 hours. This process was conducted twice to produce spores. Spore suspension with the concentration of 10<sup>6</sup> spores/ml measured by direct count using hemacytometer was prepared.

### Determination of Growth Kinetic of Microorganisms

Growth rates were determined by submerged cultivation. To 50 ml of tested broths containing HA or HF at different DHs or standard broths containing Bacto Peptone, 1.5 ml of inocula with A<sub>660</sub> of 0.02 or 10<sup>6</sup> spores/ml was added. NB, YMB and MEB were used for culturing bacteria, yeasts and mold, respectively. Incubation was carried out at 37 °C for 24 hours for bacteria and at 25 °C for 48 hours for yeasts with continuous shaking at 120 rpm. The growth of bacteria and yeasts were monitored by turbidity (A<sub>660</sub>) measurements every 4 hours. For mold, dry cell weight (DCW) measurement was used to monitor the growth every 4 hours up to 48 hours. Briefly, 2 ml of liquid culture was centrifuged at 3,000×g for 20 minutes at 25 °C (Hettich, Mikro 20, Zentrifugen, Germany). The cell pellet was resuspended in 2 ml normal saline and centrifuged under the same conditions. Washed cells were dried at 105 °C until the constant dry weight was obtained.

Growth curves of each microorganism cultured in different media containing HA or HF (5, 15 and 25% DH) or commercial peptone (Bacto Peptone) were obtained by plotting  $A_{660}$  for bacteria and yeasts or DCW for mold against incubation time. Specific growth rate ( $\mu_{\max}$ ) and generation time ( $t_d$ ) were then calculated (Lynch and Poole 1979) as follows:

$$\mu_{\max} = \frac{\ln x_t - \ln x_0}{t_t - t_0}$$

$$t_d = 0.693/\mu$$

where  $x_0$  is initial population;  $x_t$  is population at instant  $t$ ;  $t_0$  is initial time and  $t_t$  is instant time.

Protein hydrolysate with the DH rendering the highest growth rate of all microorganisms was selected for further study.

#### Measurement of Growth and Colony Size of Microorganisms Cultured in Medium Containing Protein Hydrolysate

To study the effect of HF<sub>25</sub> on qualitative microbial growths, the colony size was determined. One loopful of each bacterium cultured in NA slants for 15 hours and yeast cultured in YMA slant for 24 hours was sub-cultured on NA and YMA containing Bacto Peptone or HF<sub>25</sub> at equivalent total nitrogen (HF<sub>25tn</sub>) or total solid (HF<sub>25ts</sub>) contents to Bacto Peptone. The culture was incubated at 37 °C for 15 hours for bacteria and at 25 °C for 24 hours for yeasts. Thereafter, bacteria and yeasts were inoculated into NB and YMB containing HF<sub>25tn</sub>, HF<sub>25ts</sub> or Bacto Peptone for bacteria and yeasts, respectively, and then incubated at 37 °C for 15 hours for bacteria and at 25 °C for 24 hours for yeasts with continuous shaking at 120 rpm. Culture broths obtained were adjusted with corresponding culture media to obtain  $A_{660}$  of 0.5. For mold, *A. oryzae* was sub-cultured onto MEA slant containing HF<sub>25tn</sub>, HF<sub>25ts</sub> or Bacto Peptone and incubated at 25 °C for 48 hours twice to produce spores. Spore suspension was prepared as previously described and adjusted to  $10^6$  spores/ml using sterile normal saline. Serial dilutions of bacteria, yeasts and mold were prepared. Thereafter, aliquots of 0.1 ml dilution were pipetted onto corresponding agar plates containing HF<sub>25tn</sub>, HF<sub>25ts</sub> or Bacto Peptone and then spread using a sterile spreader. The plates were incubated at 37 °C for 24 hours for bacteria and incubated at 25 °C for 48 hours for yeasts and mold before counting and measuring colony diameter. The colony counts were recorded, and the diameters of 20 colonies were measured per plate.

#### Measurement of Growth and Colony Size of *S. aureus* Cultured in Medium Containing Protein Hydrolysate Supplemented with Buffer

To study the effect of buffer to improve the colony size of *S. aureus*, all NB and NA used for culturing *S. aureus* were supplemented with 0.2% dipotassium hydrogen phosphate ( $K_2HPO_4$ ) and the pH was adjusted to  $6.8 \pm 0.2$  using 1 M NaOH or HCl. One loopful of *S. aureus* cultured in NA slants for 15 hours was sub-cultured on NA, which was supplemented with 0.2%  $K_2HPO_4$  and contained HF<sub>25</sub> at equivalent total nitrogen (HF<sub>25tn</sub>) and total solid (HF<sub>25ts</sub>) contents to Bacto Peptone or NA containing Bacto Peptone and 0.2%  $K_2HPO_4$  (pH 6.8). The culture was incubated at 37 °C for 15 hours. Thereafter, *S. aureus* was inoculated into NB, which was supplemented with 0.2%  $K_2HPO_4$  and contained HF<sub>25tn</sub>, HF<sub>25ts</sub> or Bacto Peptone and then incubated at 37 °C with continuous shaking at 120 rpm. Culture broths were taken after cultivation for 4, 8 and 12 hours, representing the log phase of *S. aureus*. Culture broths obtained were adjusted with NB to obtain  $A_{660}$  of 0.5. Serial dilutions were prepared with sterile normal saline. Thereafter, aliquots of 0.1 ml dilution were pipetted onto NA, which was supplemented with 0.2%  $K_2HPO_4$  and contained HF<sub>25tn</sub>, HF<sub>25ts</sub> or Bacto Peptone and then spread using a sterile spreader. The plates were incubated at 37 °C for 24 hours before counting and measuring colony diameter. The colony counts were recorded, and the diameters of 20 colonies were measured per plate.

#### Determination of Microbial Morphology

Morphology of the late log phase of bacteria and yeasts and the stationary phase of mold cultured in NA and YMA and MEA containing HF<sub>25ts</sub> or Bacto Peptone was analyzed by a scanning electron microscopy (JEOL JSM-5800LV, Tokyo, Japan).

#### Statistical Analysis

One-way analysis of variance (ANOVA) was used, and mean comparison was performed by Duncan's multiple range tests (Steel and Torrie 1980). Statistical analysis was carried out using SPSS statistic program (Version 11.0) for Windows (SPSS Inc. Chicago, IL, USA).

## Results and Discussion

#### Effect of Protein Hydrolysate from Yellow Stripe Trevally Prepared Using Alcalase or Flavourzyme with Different DHs on Microbial Growth

Growth curves of bacteria, yeasts and mold cultured in the media containing HA or HF, in comparison with those

cultured in media with Bacto Peptone, are depicted in Figs. 1, 2 and 3, respectively. As the cultivation time increased, cell densities of all microorganisms increased, regardless of media used, and reached the plateau differently, depending on the types of microorganism ( $p < 0.05$ ). pHs of media were changed differently and varied with microorganisms.  $\mu_{\max}$  and  $t_d$  also varied with media as well as types of hydrolysate and DHs used (Table 1).

#### Bacteria

For *S. aureus*, no changes in cell density were found within the first 4 hours of cultivation period. Thereafter, the log phase started and reached stationary phase at hour 16 (Fig. 1a). The media containing HA or HF with 25% DH

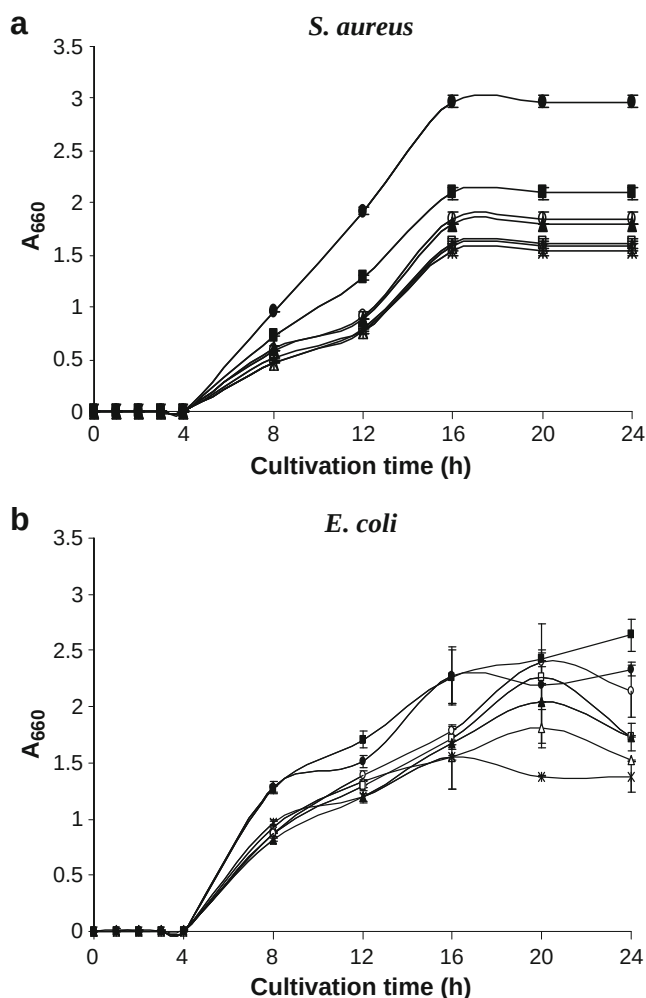


**Fig. 1** Growth curve of *S. aureus* (a) and *E. coli* (b) during cultivation in NB containing protein hydrolysate from yellow stripe trevally prepared using Alcalase (HA) and Flavourzyme (HF) with different DHs: HA with DH of 5% ( $\Delta$ ), 15% ( $\square$ ), 25% ( $\circ$ ); HF with DH of 5% ( $\blacktriangle$ ), 15% ( $\blacksquare$ ), 25% ( $\bullet$ ), Bacto Peptone (\*). Bars represent standard deviations from triplicate determinations

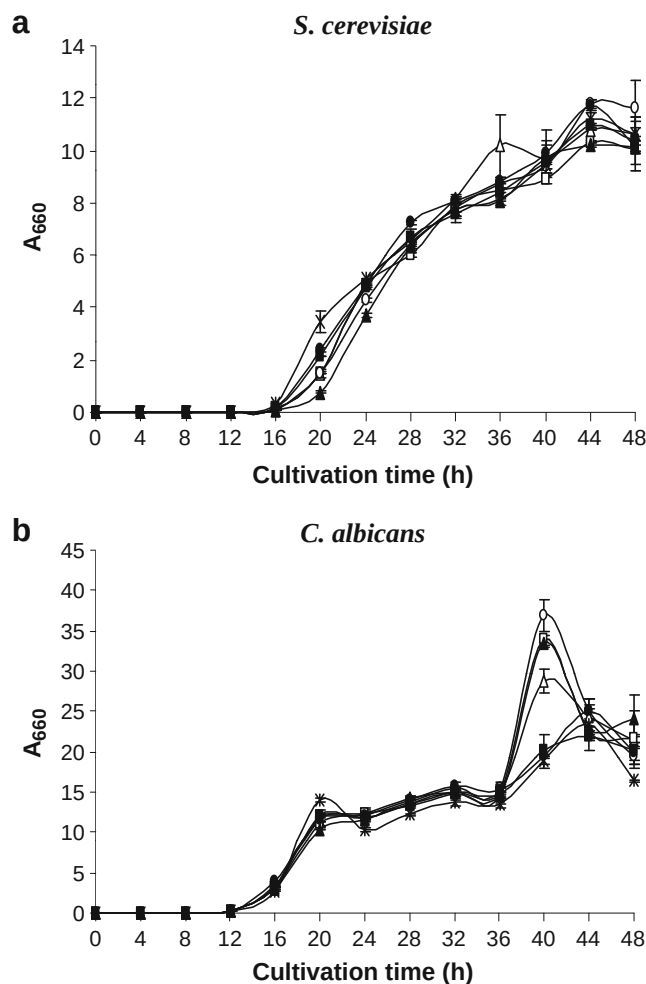


**Fig. 2** Growth curve of *S. cerevisiae* (a) and *C. albicans* (b) during cultivation in YMB containing protein hydrolysate from yellow stripe trevally prepared using Alcalase (HA) and Flavourzyme (HF) with different DHs: HA with DH of 5% ( $\Delta$ ), 15% ( $\square$ ), 25% ( $\circ$ ); HF with DH of 5% ( $\blacktriangle$ ), 15% ( $\blacksquare$ ), 25% ( $\bullet$ ), Bacto Peptone (\*). Bars represent standard deviations from triplicate determinations

(HA<sub>25</sub> and HF<sub>25</sub>) showed the higher cell density and  $\mu_{\max}$  with a lower  $t_d$  than did those with HA or HF at lower DHs ( $p < 0.05$ ). At the same DH tested, HF yielded the greater cell density and  $\mu_{\max}$  and lower  $t_d$  than did HA, manifesting that the types of enzyme used for hydrolysate preparation affected the growth of *S. aureus*. All media containing HA or HF exhibited the higher cell density and  $\mu_{\max}$  with lower  $t_d$  than did that containing Bacto Peptone except for HA<sub>5</sub>. The pHs of media containing HF started to drop at hour 4. This was coincidental with the beginning of log phase. The decrease in pH was more intense at hour 8, followed by the gradual increase (data not shown). The medium containing HF<sub>25</sub> had the lower pH than did others. This was concomitant with the higher cell density. The result suggested that the extraordinary growth of *S. aureus* occurred in the medium containing HF<sub>25</sub>.

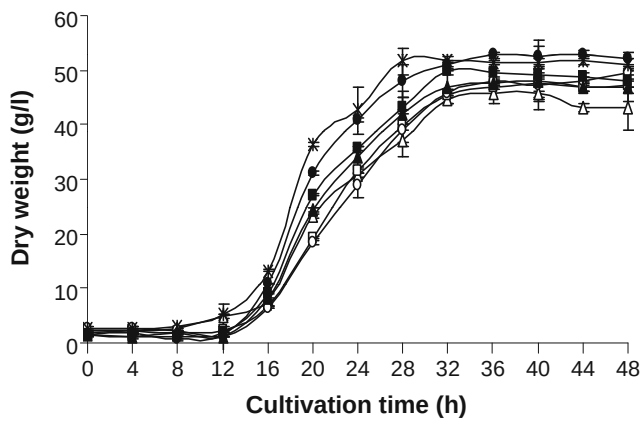


**Fig. 3** Growth curve of *A. oryzae* during cultivation in MEB containing protein hydrolysate from yellow stripe trevally prepared using Alcalase (HA) and Flavourzyme (HF) with different DHs: HA with DH of 5% ( $\Delta$ ), 15% ( $\square$ ), 25% ( $\circ$ ); HF with DH of 5% ( $\blacktriangle$ ), 15% ( $\blacksquare$ ), 25% ( $\bullet$ ), Bacto Peptone (\*). Bars represent standard deviations from triplicate determinations

*Escherichia coli* maintained the lag phase up to 4 hours of cultivation period, followed by the log phase and reached the stationary phase at hours 16–24 (Fig. 1b). *E. coli* cultured in media containing HA or HF with various DHs or Bacto Peptone started the log phase at the same time but reached stationary phase and death phase at different times. Although the media containing HF<sub>25</sub> or HF<sub>15</sub> showed the greatest cell density, that containing HF<sub>25</sub> possessed the highest  $\mu_{\max}$  and the lowest  $t_d$  ( $p < 0.05$ ). Generally, the media containing HA or HF with higher DHs exhibited the higher cell density and  $\mu_{\max}$  with lower  $t_d$ . The media containing HF yielded the higher cell density and  $\mu_{\max}$  with the lower  $t_d$  than did those containing HA with the same DH. Thus, the types of enzyme used to produce HA and HF also affected the growth of *E. coli*. All media containing HA or HF exhibited the higher cell density and  $\mu_{\max}$  with the lower  $t_d$  than did media containing Bacto Peptone except HA<sub>5</sub>. The pHs of all media containing HA or HF with all DHs except HF<sub>25</sub> increased gradually with increasing cultivation time (data not shown). For the medium with HF<sub>25</sub>, the pH decreased within the first 8 hours of cultivation. The pH drop of medium containing HF<sub>25</sub> was much more intense than was other media. This was concomitant with the highest cell density and  $\mu_{\max}$  and the lowest  $t_d$  ( $p < 0.05$ ). However, the continuous increase in pH was observed during 8–24 hours of cultivation. The increase in pH was associated mostly with the increase in populations.

The differences between cell density,  $\mu_{\max}$ ,  $t_d$  and pH changes of *S. aureus* and *E. coli* cultured in media containing various protein hydrolysates (HA<sub>5</sub>, HA<sub>15</sub>, HA<sub>25</sub>, HF<sub>5</sub>, HF<sub>15</sub>, HF<sub>25</sub>) or Bacto Peptone might be due to the differences in size of peptide. HA and HF with different DHs contained peptides with different sizes, in

**Table 1** Specific growth rate ( $\mu_{\max}$ ,  $h^{-1}$ ) and generation time ( $t_d$ , min) of microorganisms cultured in media containing protein hydrolysate from yellow stripe trevally prepared using Alcalase (HA) and Flavourzyme (HF) with different DHs compared with Bacto Peptone

| Peptones      | DH (%) | <i>S. aureus</i>        |                       | <i>E. coli</i>          |                       | <i>S. cerevisiae</i>   |                       | <i>C. albicans</i>      |                        | <i>A. oryzae</i>        |                         |
|---------------|--------|-------------------------|-----------------------|-------------------------|-----------------------|------------------------|-----------------------|-------------------------|------------------------|-------------------------|-------------------------|
|               |        | $\mu_{\max}$            | $t_d$                 | $\mu_{\max}$            | $t_d$                 | $\mu_{\max}$           | $t_d$                 | $\mu_{\max}$            | $t_d$                  | $\mu_{\max}$            | $t_d$                   |
| HA            | 5      | 0.95±0.01 <sup>c</sup>  | 44±0.26 <sup>c</sup>  | 1.38±0.05 <sup>b</sup>  | 30±1.01 <sup>b</sup>  | 0.30±0.02 <sup>c</sup> | 139±7.00 <sup>c</sup> | 0.33±0.01 <sup>b</sup>  | 127±4.43 <sup>bc</sup> | 0.26±0.00 <sup>ns</sup> | 158±1.17 <sup>ns</sup>  |
|               | 15     | 0.98±0.01 <sup>d</sup>  | 42±0.43 <sup>cd</sup> | 1.57±0.10 <sup>ab</sup> | 26±1.58 <sup>ab</sup> | 0.30±0.00 <sup>c</sup> | 138±2.12 <sup>c</sup> | 0.31±0.00 <sup>c</sup>  | 134±1.93 <sup>c</sup>  | 0.26±0.03 <sup>ns</sup> | 158±11.66 <sup>ns</sup> |
|               | 25     | 1.02±0.02 <sup>c</sup>  | 41±0.64 <sup>c</sup>  | 1.52±0.12 <sup>ab</sup> | 27±2.22 <sup>ab</sup> | 0.26±0.01 <sup>d</sup> | 161±5.24 <sup>d</sup> | 0.31±0.02 <sup>bc</sup> | 132±7.27 <sup>bc</sup> | 0.26±0.01 <sup>ns</sup> | 159±14.85 <sup>ns</sup> |
| HF            | 5      | 1.05±0.02 <sup>bc</sup> | 40±0.63 <sup>bc</sup> | 1.43±0.12 <sup>b</sup>  | 29±2.37 <sup>b</sup>  | 0.39±0.03 <sup>b</sup> | 106±6.91 <sup>b</sup> | 0.32±0.01 <sup>bc</sup> | 131±2.46 <sup>c</sup>  | 0.27±0.00 <sup>ns</sup> | 151±2.37 <sup>ns</sup>  |
|               | 15     | 1.07±0.01 <sup>b</sup>  | 39±0.38 <sup>b</sup>  | 1.46±0.14 <sup>b</sup>  | 28±2.78 <sup>ab</sup> | 0.20±0.00 <sup>e</sup> | 205±0.47 <sup>e</sup> | 0.33±0.01 <sup>b</sup>  | 126±2.19 <sup>b</sup>  | 0.27±0.01 <sup>ns</sup> | 153±6.78 <sup>ns</sup>  |
|               | 25     | 1.19±0.00 <sup>a</sup>  | 35±0.02 <sup>a</sup>  | 1.72±0.09 <sup>a</sup>  | 24±1.23 <sup>a</sup>  | 0.17±0.00 <sup>f</sup> | 238±1.33 <sup>f</sup> | 0.27±0.01 <sup>d</sup>  | 152±8.06 <sup>d</sup>  | 0.27±0.02 <sup>ns</sup> | 157±9.40 <sup>ns</sup>  |
| Bacto Peptone |        | 0.97±0.01 <sup>de</sup> | 43±0.60 <sup>d</sup>  | 1.40±0.07 <sup>b</sup>  | 30±1.56 <sup>b</sup>  | 0.61±0.03 <sup>a</sup> | 68±3.26 <sup>a</sup>  | 0.41±0.01 <sup>a</sup>  | 102±1.46 <sup>a</sup>  | 0.28±0.02 <sup>ns</sup> | 149±9.27 <sup>ns</sup>  |

Means ± standard deviations from triplicate determinations.

Different superscripts in the same column indicate significant differences ( $p < 0.05$ )

ns non-significant difference ( $p > 0.05$ ).

which molecular weights lower than 204, 70 and 7 kDa were found with DHs of 5, 15 and 25%, respectively (Klompong et al. 2007b). This could contribute to a different biomass production of bacteria. Reductions of the molecular size of hydrolysate appeared when the DH increased (Klompong et al. 2007b). The results suggested that the DH of hydrolysates correlated with the maximum cell density,  $\mu_{\max}$ ,  $t_d$  and pH changes in both cultures. Dufosse et al. (2001) also found a correlation between the peptone hydrolysis rate and the biomass production of *E. coli* and *Lactobacillus casei*. Peptides with the molecular weight of 6,500 Da, di-peptides and amino acids from tuna treated with Alcalase were a suitable nitrogenous source in microbial media (Guerard et al. 2001). HF<sub>25</sub> was prepared by hydrolyzing yellow stripe trevally with Flavourzyme and had MW lower than 7 kDa (Klompong et al. 2007b). Thus, it was a suitable nitrogenous source for culturing bacteria. It could be concluded that HF<sub>25</sub> with MW lower than 7 kDa might provide the valuable nutrients, which were readily available for growths of both *S. aureus* and *E. coli*.

Differences in maximum cell densities and growth rates of *S. aureus* and *E. coli* were noticeable when different hydrolysates and DHs were used ( $p < 0.05$ ). For all DHs, HA showed the inferior growth stimulation to HF. The result suggested the certain differences in hydrolysate composition such as size of peptides, amino acid composition of peptides, vitamins, fatty acids and inhibitory peptides. At the same DH, HA and HF might contain different peptides due to the cleavage of peptides at different positions or amino acids, generating various amino acids and peptides. Owing to exopeptidase activity, HF might contain more free amino acids that were readily available for bacterial growth than did HA. Aspmo et al. (2005) found that proteolytic enzyme clearly affected the growth performance of microorganisms cultured in media containing protein hydrolysate. The results suggested that HA and HF showed the better efficacy for bacterial growth than did commercial peptone (Bacto Peptone) ( $p < 0.05$ ).

In fish muscle, glycogen is the main carbohydrate. After capture, postmortem degradation of glycogen to glucose-6-phosphate and glucose occurs via phosphorylytic pathway and via hydrolytic or amylolytic pathway (Eskin 1990). This sugar could be consumed by bacteria as a carbon source (Boyd and Hoerl 1991). After sugar fermentation, organic acids were produced, resulting in the dramatic drop of pH of the medium containing HF<sub>25</sub> used for culturing *S. aureus* and *E. coli*. The result suggested that medium containing HF<sub>25</sub> might have the low buffering capacity. pH increases or decreases in culturing system depended on media and buffering capacity (Fernandez-Lopez et al. 2008). Protein and free amino acid are major contributors to pH buffering in the

biological system. After 8 hours of cultivation, pH of media culturing *S. aureus* and *E. coli* increased gradually. When protein and amino acid were degraded during microbial growth, ammonia, non-protein nitrogen and amino acid catabolism products were accumulated (Nychas et al. 1998; Parente et al. 1994).

## Yeasts

The log phase of *S. cerevisiae* started after cultivation for 16 hours (Fig. 2a). *S. cerevisiae* cultured in media containing HA or HF with various DHs or Bacto Peptone reached the plateau after 44 hours. Similar growth curves were obtained for *S. cerevisiae* cultured in all media. The medium containing HF<sub>5</sub> showed the greater  $\mu_{\max}$  and the lowest  $t_d$  than did those added with HA or HF having other DHs. Nevertheless, the medium containing Bacto Peptone possessed the highest  $\mu_{\max}$  and the lowest  $t_d$  ( $p < 0.05$ ) and had the greatest cell density during 16–24 hours of cultivation. Thereafter, no differences in cell densities in all media were distinguishably observed. Basically, the media containing HA or HF with higher DHs exhibited the lower  $\mu_{\max}$  and higher  $t_d$  ( $p < 0.05$ ). The pH of all media for culturing *S. cerevisiae* decreased after 12 hours, corresponding to the beginning of log phase at hour 16 (data not shown). As the cell density increased, the pHs slightly decreased in all media. However, a slight increase in pH was noticeable after 24 hours. The lower pH of the medium containing Bacto Peptone was more distinguished than other media, likely associated with the highest cell density and  $\mu_{\max}$  and the lowest  $t_d$ , particularly during 16–24 hours of cultivation ( $p < 0.05$ ).

The log phase of *C. albicans* began after cultivation for 12 hours and reached stationary phase at hour 20 (Fig. 2b). *C. albicans* cultured in all media began log phase synchronously. Nevertheless, the system containing Bacto Peptone possessed the highest  $\mu_{\max}$  and the lowest  $t_d$  ( $p < 0.05$ ) and showed the greatest cell density during 12–20 hours of cultivation. Thereafter, no differences in cell density among the media containing HA or HF were observed ( $p > 0.05$ ). Generally, the media added with HA or HF having the higher DHs exhibited the lower  $\mu_{\max}$  and higher  $t_d$  ( $p < 0.05$ ). The pHs of all media decreased at hour 12. This was associated with the multiplying of cells in the late lag phase found at hour 12 (data not shown). In general, the pH gradually increased after 24 hours of cultivation. The pH of the medium containing Bacto Peptone was lower than those of other media, particularly during 12–20 hours ( $p < 0.05$ ). *C. albicans* reached stationary phase within 4 hours after the beginning of log phase. After the easily fermentable sugar (dextrose in YMB) was exhausted, *C. albicans* used slow fermenting nutrients (Fernandez-Lopez et al. 2008). *C. albicans* expressed the

**Table 2** Growth ( $A_{660}=0.5$ ) and colony size of microorganisms cultured in media containing protein hydrolysate from yellow stripe trevally prepared using Flavourzyme with 25% DH based on totalnitrogen (HF<sub>25tn</sub>) and total solid contents (HF<sub>25ts</sub>) of Bacto Peptone, compared with Bacto Peptone

| Microorganisms       | Number (log CFU/ml)     |                         |                         | Colony diameter (cm)    |                         |                         |
|----------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
|                      | HF <sub>25tn</sub>      | HF <sub>25ts</sub>      | Bacto Peptone           | HF <sub>25tn</sub>      | HF <sub>25ts</sub>      | Bacto Peptone           |
| <i>S. aureus</i>     | 6.57±0.08 <sup>a</sup>  | 6.40±0.08 <sup>b</sup>  | 6.39±0.02 <sup>b</sup>  | 0.11±0.02 <sup>b</sup>  | 0.22±0.03 <sup>a</sup>  | 0.19±0.05 <sup>a</sup>  |
| <i>E. coli</i>       | 7.15±0.05 <sup>b</sup>  | 7.26±0.03 <sup>a</sup>  | 7.09±0.06 <sup>b</sup>  | 0.27±0.07 <sup>ns</sup> | 0.31±0.05 <sup>ns</sup> | 0.24±0.04 <sup>ns</sup> |
| <i>S. cerevisiae</i> | 5.48±0.02 <sup>b</sup>  | 5.21±0.03 <sup>c</sup>  | 5.80±0.02 <sup>a</sup>  | 0.20±0.02 <sup>ns</sup> | 0.20±0.01 <sup>ns</sup> | 0.21±0.03 <sup>ns</sup> |
| <i>C. albicans</i>   | 5.80±0.01 <sup>ns</sup> | 5.78±0.15 <sup>ns</sup> | 5.99±0.19 <sup>ns</sup> | 0.16±0.04 <sup>ns</sup> | 0.19±0.02 <sup>ns</sup> | 0.23±0.04 <sup>ns</sup> |
| <i>A. oryzae</i>     | 3.77±0.07 <sup>ns</sup> | 3.78±0.01 <sup>ns</sup> | 3.81±0.05 <sup>ns</sup> | 0.62±0.12 <sup>ns</sup> | 0.64±0.12 <sup>ns</sup> | 0.70±0.10 <sup>ns</sup> |

Means ± standard deviations from triplicate determinations for numbers and from 20 determinations for colony diameter

Different superscripts in the same row within the same parameters indicate significant differences ( $p<0.05$ ); *ns* non-significant difference ( $p>0.05$ ).

Diauxie at hours 40–44. Diauxie was observed at low sugar concentration, independent of nitrogen supplementation and the types of sugar (Batistote et al. 2006). When the primary carbon sources are absent or present at concentrations low enough to limit growth, others nitrogen source such as amides, amino acids and peptides can be used as a carbon source. Batistote et al. (2006) reported that the growth of *S. cerevisiae* in medium containing maltose and glucose supplemented with different nitrogen sources exhibited diauxie. *C. albicans* reached the death phase at the period of 40–44 hours.

## Mold

*Aspergillus oryzae* was in the lag phase up to 12 hours of cultivation period before the log phase was started (Fig. 3). *A. oryzae* reached stationary phase synchronously for all media at hour 32 except for the medium containing Bacto Peptone, which reached plateau at hour 28. That might be due to the greater biomass in the medium containing Bacto Peptone during 12–20 hours of cultivation ( $p<0.05$ ), leading to the faster beginning of stationary phase. However, no significant differences of  $\mu_{\max}$  and  $t_d$  were found among the

media containing HA, HF and Bacto Peptone (Table 1). DHs and enzyme types had no impact on  $\mu_{\max}$  and  $t_d$  of *A. oryzae* cultured in media containing HA or HF that might be due to the ability of *A. oryzae* in producing extracellular enzymes. The pHs of all media began to drop at hour 8 and decreased continuously up to 48 hours of cultivation (data not shown). As the dry biomass increased, the pHs decreased in all systems. The medium containing Bacto Peptone exhibited the lowest pH ( $p<0.05$ ), which was concomitant with the greatest growth in this system. The decreased pH observed after growth of *A. Oryzae* is likely to be caused by assimilation of sugars and a metabolic capability (Pitt and Hocking 1999). For all bacteria tested, growth rates and maximum cell densities were higher when HF<sub>25</sub> was used as a replacer of Bacto Peptone. However, HF<sub>25</sub> was inferior to Bacto Peptone for yeast cultivation. In addition, no difference was observed between HF<sub>25</sub> and Bacto Peptone for mold culturing. Each peptone has its own biologic characteristics and could not meet the requirements of all microorganisms during cultivation (Kurbanoglu and Algur 2002). Performances of peptones for production of cell biomass also exhibited large differences, depending on the strain tested (Boyd and Hoerl 1991).

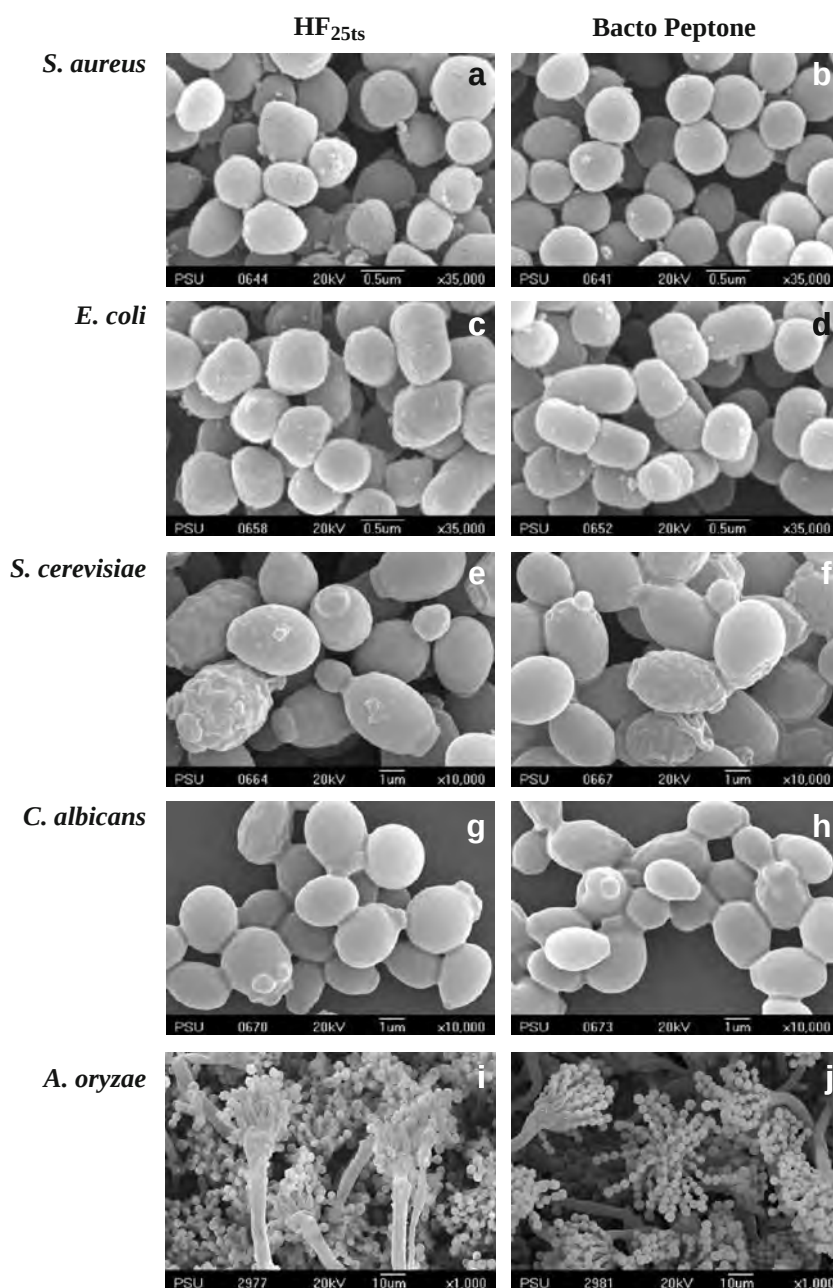
**Table 3** Growth and colony size of *S. aureus* cultured in NA and NB containing buffer and protein hydrolysate from yellow stripe trevally prepared using Flavourzyme with 25% DH based on total nitrogen(HF<sub>25tn</sub>) and total solid contents (HF<sub>25ts</sub>) of Bacto Peptone, compared with Bacto Peptone during log phase

| Duration (hours)   | Number (log CFU/ml)    |                        |                        | Colony diameter (cm)    |                         |                         |
|--------------------|------------------------|------------------------|------------------------|-------------------------|-------------------------|-------------------------|
|                    | 4                      | 8                      | 12                     | 4                       | 8                       | 12                      |
| HF <sub>25tn</sub> | 7.67±0.03 <sup>a</sup> | 7.80±0.04 <sup>a</sup> | 7.99±0.02 <sup>a</sup> | 0.20±0.03 <sup>ns</sup> | 0.21±0.03 <sup>ns</sup> | 0.19±0.02 <sup>ns</sup> |
| HF <sub>25ts</sub> | 7.33±0.08 <sup>b</sup> | 7.54±0.01 <sup>b</sup> | 7.96±0.03 <sup>a</sup> | 0.21±0.05 <sup>ns</sup> | 0.20±0.02 <sup>ns</sup> | 0.18±0.02 <sup>ns</sup> |
| Bacto Peptone      | 7.17±0.07 <sup>c</sup> | 7.45±0.09 <sup>b</sup> | 7.75±0.07 <sup>b</sup> | 0.19±0.02 <sup>ns</sup> | 0.19±0.01 <sup>ns</sup> | 0.18±0.02 <sup>ns</sup> |

Means ± standard deviations from triplicate determinations for numbers and from 20 determinations for colony diameter

Different superscripts in the same column indicate the significant differences ( $p<0.05$ ); *ns* non-significant difference ( $p>0.05$ )

**Fig. 4** Scanning electron microscopic photographs of *S. aureus*, *E. coli*, *S. cerevisiae*, *C. albicans* and *A. oryzae* cultured on media containing protein hydrolysate from yellow stripe trevally prepared using Flavourzyme with 25% DH based on total solid content of Bacto Peptone (HF<sub>25ts</sub>) and those cultured on Bacto Peptone



#### Effect of Protein Hydrolysate from Yellow Stripe Trevally Prepared Using Flavourzyme on Microbial Growth and Colony Size

Growth and colony size of microorganisms cultured in agar medium containing HF<sub>25</sub> as a replacer of Bacto Peptone (based on total nitrogen and solid contents) are shown in Table 2.

#### *Staphylococcus aureus*

Although the cell density was adjusted to 0.5 of A<sub>660</sub> to obtain a similar cell density, total viable count of *S. aureus* cultured

on NB containing HF<sub>25tn</sub> was greater than that observed in NB with HF<sub>25ts</sub> or Bacto Peptone ( $p < 0.05$ ). As a consequence, *S. aureus* cultured on NA comprising HF<sub>25tn</sub> exhibited the smaller colony than that found on NA containing Bacto Peptone and HF<sub>25ts</sub> ( $p < 0.05$ ). The smaller size of colony cultured on medium comprising HF<sub>25tn</sub> might be affected by the decrease in pH during culturing.

#### *Escherichia coli*

*Escherichia coli* cultured in NB comprising HF<sub>25ts</sub> had the greater number than that found in NB containing HF<sub>25tn</sub> or Bacto Peptone ( $p < 0.05$ ). NB containing HF<sub>25ts</sub> possibly

**Table 4** Size of microorganisms cultured on media containing protein hydrolysate from yellow stripe trevally prepared using Flavourzyme with 25% DH based on total solid contents of Bacto Peptone (HF<sub>25ts</sub>), compared with Bacto Peptone

| Microorganisms       |                | Cell size (μm)          |                         |
|----------------------|----------------|-------------------------|-------------------------|
|                      |                | HF <sub>25ts</sub>      | Bacto Peptone           |
| <i>S. aureus</i>     | Diameter       | 0.67±0.09 <sup>ns</sup> | 0.66±0.05 <sup>ns</sup> |
| <i>E. coli</i>       | Length         | 0.84±0.12 <sup>ns</sup> | 0.79±0.10 <sup>ns</sup> |
|                      | Width          | 0.58±0.04 <sup>ns</sup> | 0.54±0.03 <sup>ns</sup> |
| <i>S. cerevisiae</i> | Length         | 3.68±0.70 <sup>ns</sup> | 3.50±0.57 <sup>ns</sup> |
|                      | Width          | 2.73±0.32 <sup>ns</sup> | 2.70±0.22 <sup>ns</sup> |
| <i>C. albicans</i>   | Length         | 3.01±0.39 <sup>ns</sup> | 2.81±0.33 <sup>ns</sup> |
|                      | Width          | 2.34±0.31 <sup>ns</sup> | 2.26±0.29 <sup>ns</sup> |
| <i>A. oryzae</i>     | Spore diameter | 6.30±0.48 <sup>ns</sup> | 6.01±0.43 <sup>ns</sup> |

Means ± standard deviations from 20 determinations based on SEM photomicrograph

ns non-significant difference ( $p>0.05$ )

contained a suitable amount of nitrogen source for the growth of *E. coli* than did NB containing HF<sub>25tn</sub>. However, no significant difference in colony size of *E. coli* cultured on NA containing HF<sub>25tn</sub>, HF<sub>25ts</sub> and Bacto Peptone was observed ( $p>0.05$ ).

An excessive amount of nitrogen did not increase the microbial growth (Lynch and Poole 1979). The lower growth of *E. coli* was found in the medium containing HF<sub>25tn</sub> than that comprising HF<sub>25ts</sub>. The inhibition might be due to the differences in nutrient composition, unsuitable carbon and nitrogen ratio (C/N ratio) and some toxic materials (Dufosse et al. 2001). Kurbanoglu and Algur (2002) found that ram horn hydrolysates at a level of 4% were found to be optimal for bacterial growth and applications higher than 4% had an inhibitory effect on growth.

#### *Saccharomyces cerevisiae*

A great number of *S. cerevisiae* cultured in YMB comprising Bacto Peptone was found, when compared with that cultured in YMB containing HF<sub>25tn</sub> or HF<sub>25ts</sub> ( $p<0.05$ ). YMB comprising Bacto Peptone might contain a suitable peptide size for the growth of *S. cerevisiae* under the condition used. However, a similar colony size was observed among *S. cerevisiae* cultured on YMA containing HF<sub>25tn</sub>, HF<sub>25ts</sub> and Bacto Peptone ( $p>0.05$ ).

#### *Candida albicans*

No differences in the number and colony size of *C. albicans* cultured on YMA containing HF<sub>25tn</sub>, HF<sub>25ts</sub> and Bacto Peptone were observed ( $p>0.05$ ).

#### *Aspergillus oryzae*

Similar number and colony size of *A. oryzae* cultured in MEB containing HF<sub>25tn</sub>, HF<sub>25ts</sub> or Bacto Peptone were obtained ( $p>0.05$ ). It might be due to the fact that all protein hydrolysates were available substrates as a nitrogen source for *A. oryzae*.

The media containing different amounts of nitrogen sources from yellow stripe trevally protein hydrolysate (HF<sub>25tn</sub>, HF<sub>25ts</sub>) showed no effect on the quantitative and qualitative growth of *C. albicans* and *A. oryzae*. Differences in growth of *S. aureus*, *E. coli* and *S. cerevisiae* observed when culturing in HF<sub>25</sub> and Bacto Peptone could be related to the differences in the amino acid composition of peptides in protein hydrolysates. In addition, the amount of hydrolysate used could exhibit either stimulating or inhibitory effects on microbial growth, depending upon the types of microorganisms. The amounts of nitrogen sources affected the growth of *S. aureus* markedly for both positive and negative aspects. The growth of *S. aureus* in this system was most likely associated with the decrease in pH. Thus, the use of buffer to maintain the pH of medium could be a promising means to solve the problem about colony size.

#### Effect of Buffer on Growth and Colony Size of *S. aureus*

When 0.2% K<sub>2</sub>HPO<sub>4</sub> was supplemented in all media to maintain the pH during microbial cultivation, total viable count of *S. aureus* cultured in the medium containing HF<sub>25tn</sub> was higher than that found in media containing HF<sub>25ts</sub> or Bacto Peptone ( $p<0.05$ ) (Table 3). When the cultivation time increased, the growth of *S. aureus* in all media increased throughout the log phase (4–12 hours) (data not shown). *S. aureus* cultured in NB containing HF<sub>25tn</sub> exhibited the higher cell density than did NB containing HF<sub>25ts</sub> or Bacto Peptone, respectively, up to 24 hours of cultivation time ( $p<0.05$ ). *S. aureus* started the log phase at hour 3, and the stationary phase was observed at hour 16. However, the pH of the medium containing HF<sub>25tn</sub> showed the greater decrease than did the media containing HF<sub>25ts</sub> or Bacto Peptone ( $p<0.05$ ). The more decrease in pH was related with the higher cell density of *S. aureus*. Nevertheless, the decrease in pH of NB supplemented with buffer was not as much as that of NB without buffer (data not shown). In addition, no difference in colony size of *S. aureus* was observed among the media containing HF<sub>25tn</sub>, HF<sub>25ts</sub> and Bacto Peptone supplemented with 0.2 % K<sub>2</sub>HPO<sub>4</sub> ( $p>0.05$ ). K<sub>2</sub>HPO<sub>4</sub> acts as a potential buffer in culturing system. The result reconfirmed that acidity might affect colony size as previously mentioned (Table 2). In the presence of buffer, *S. aureus* in the medium containing HF<sub>25tn</sub> showed the higher growth than that cultured in the

medium containing HF<sub>25ts</sub> ( $p < 0.05$ ). This might be associated with the higher amount of nitrogen source for the growth. However, in the late log phase at hour 12 of cultivation, no difference between the number of *S. aureus* culturing in media containing HF<sub>25tn</sub> and HF<sub>25ts</sub> was observed ( $p > 0.05$ ). Normally used as inoculants, cells in the late log phase are important. Thus, the medium containing HF<sub>25ts</sub> was selected for further study.

#### Effect of Protein Hydrolysate from Yellow Stripe Trevally on Microbial Morphology

Microstructures and size of microbial cells are shown in Fig. 4 and Table 4. Sizes of cells of all microorganisms cultured on NA containing HF<sub>25ts</sub> were similar to those of microorganisms cultured on NA with Bacto Peptone ( $p > 0.05$ ). Cells of *S. aureus* were round. Nevertheless, the cells cultured on NA comprising Bacto Peptone had a more regular spherical shape and finer surface than those cultured on NA containing HF<sub>25ts</sub> (Fig. 4a, b). *E. coli* was short rod in shape. The cells cultured on NA containing HF<sub>25ts</sub> were similar in length and width to those cultured on NA containing Bacto Peptone ( $p > 0.05$ ) (Fig. 4c, d). However, the cells cultured on Bacto Peptone had a more regular shape and smoother surface than that cultured in NA containing HF<sub>25ts</sub>. Microscopic features of *S. cerevisiae* were unicellular and ellipsoidal in shape and showed the doubling by budding (Fig. 4e, f). *C. albicans* were single ellipsoidal cells (Fig. 4g, h). Both *S. cerevisiae* and *C. albicans* cultured on YMA containing HF<sub>25ts</sub> had the similar size and shape, in comparison with those cultured on YMA containing Bacto Peptone. For mold, *A. oryzae* cultured on MEA containing HF<sub>25ts</sub> or Bacto Peptone produced upright conidiophores (Fig. 4i, j), which are simple and terminate in a globose or clavate swelling (Tortora et al. 1997). No difference in size and shape of the conidia, which were I-celled and globose in shape, were observed on MEA containing HF<sub>25ts</sub> and Bacto Peptone ( $p > 0.05$ ). However, *A. oryzae* started producing spore at hour 38 and hour 40 when cultured on MEA containing HF<sub>25ts</sub> and Bacto Peptone, respectively (data not shown). The differences in starting production of conidiospores of *A. oryzae* culturing on MEA containing HF<sub>25ts</sub> and Bacto Peptone might be governed by peptide composition.

#### Conclusions

Efficacy of protein hydrolysate from yellow stripe trevally as microbial nutrient varied with DH, enzyme and amount of hydrolysate used. Microbial kinetics of bacteria, yeasts and mold cultured in media containing protein hydrolysate

from yellow stripe trevally produced by Flavourzyme with 25% DH were comparable to those of commercial Bacto Peptone. Therefore, production of fish protein hydrolysate for upgrading low market value species as a high-value nitrogenous substrate for microbial growth can be achieved.

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