รายงานวิจัยฉบับสมบูรณ์

โครงการ เอนไซม์โปรติเนสจากตับปลาทูน่าพันธุ์ครีบยาว (*Thunnus alalunga*): การทำบริสุทธิ์ การแยกส่วน และการประยุกต์ใช้ในการผลิตโปรตีนไฮโดรไลเสต

โดย รองศาสตราจารย์ ดร. สรรพสิทธิ์ กล่อมเกล้า

พฤษภาคม 2563

สัญญาเลขที่ RSA6080044

รายงานวิจัยฉบับสมบูรณ์

โครงการ เอนไซม์โปรติเนสจากตับปลาทูน่าพันธุ์ครีบยาว (*Thunnus alalunga*): การทำบริสุทธิ์ การแยกส่วน และการประยุกต์ใช้ในการผลิตโปรตีนไฮโดรไลเสต

รองศาสตราจารย์ ดร.สรรพสิทธิ์ กล่อมเกล้า มหาวิทยาลัยทักษิณ

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยทักษิณ
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. และมหาวิทยาลัยทักษิณ
ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

ขอขอบพระคุณสำนักงานกองทุนสนับสนุนการวิจัย และมหาวิทยาลัยทักษิณ สำหรับเงินอุดหนุนโครงการวิจัยเรื่อง “เอนไซม์โปรติเนสจากตับปลาทูน่าพันธุ์ครีบยาว (*Thunnus alalunga*): การทำบริสุทธิ์ การแยกส่วนและการประยุกต์ใช้ในการผลิตโปรตีนไฮโดรไลเสต” จำนวน 1,500,000 บาท ขอขอบพระคุณคณะอุตสาหกรรมเกษตรและชีวภาพ มหาวิทยาลัยทักษิณ ที่ให้การสนับสนุนด้านอุปกรณ์และเครื่องมือวิทยาศาสตร์สำหรับการวิจัยครั้งนี้

รองศาสตราจารย์ ดร.สรรพสิทธิ์ กล่อมเกล้า
หัวหน้าโครงการฯ

บทคัดย่อ

รหัสโครงการ: RSA6080044

ชื่อโครงการ: เอนไซม์โปรติเนสจากตับปลาทูน่าพันธุ์ครีบยาว (*Thunnus alalunga*): การทำบริสุทธิ์ การแยกส่วนและการประยุกต์ใช้ในการผลิตโปรตีนไฮโดรไลเสต

ชื่อนักวิจัย: รองศาสตราจารย์ ดร.สรรพสิทธิ์ กล่อมเกล้า มหาวิทยาลัยทักษิณ

E-mail Address: sappasith@tsu.ac.th

ระยะเวลาโครงการ: 30 พฤษภาคม 2560 ถึง 29 พฤษภาคม 2563

เอนไซม์ทริปซินสองไอโซฟอร์ม (เอ และ บี) จากตับปลาทูน่าพันธุ์ครีบยาว (*Thunnus alalunga*) สามารถทำบริสุทธิ์โดยการใช้ Sephacryl S-200 Sephadex G-50 และ DEAE-cellulose ตามลำดับ เอนไซม์ทริปซินทั้งสองปรากฏเป็นแถบโปรตีนเดี่ยวบนเจล native-PAGE เอนไซม์ทริปซิน เอ และ บี มีกิจกรรมสูงสุดที่อุณหภูมิ 60 และ 55 องศาเซลเซียส ตามลำดับ และมีพีเอชที่เหมาะสมที่พีเอช 8.5 เมื่อใช้ TAME เป็นสารตั้งต้น สารยับยั้งเอนไซม์ทริปซินจากถั่วเหลือง และ TLCK แสดงกิจกรรมการยับยั้งเอนไซม์อย่างมีประสิทธิภาพ ลำดับของกรดอะมิโนปลายสายด้านหมู่อะมิโนจำนวน 20 หน่วยย่อยของเอนไซม์ทริปซินทั้งสองไอโซฟอร์ม มีลำดับของกรดอะมิโนปลายสายด้านหมู่อะมิโนที่มีความคล้ายกันและคล้ายกับเอนไซม์ทริปซินจากสัตว์น้ำอื่น ๆ เมื่อศึกษาการใช้ส่วนสกัดเอนไซม์โปรติเนสจากตับปลาทูน่าพันธุ์ครีบยาวสำหรับการผลิตโปรตีนไฮโดรไลเสตจากกล้ามเนื้อปลาวัวพบว่าสภาวะที่เหมาะสมต่อการย่อยสลายโปรตีนในกล้ามเนื้อปลาวัว คือการใช้ส่วนสกัดจากตับปลาทูน่าพันธุ์ครีบยาวที่ระดับความเข้มข้นร้อยละ 5.5 โดยใช้ระยะเวลาในการย่อยสลาย 40 นาที และใช้อัตราส่วนระหว่างกล้ามเนื้อปลากับบัฟเฟอร์ เท่ากับ 1 : 3 (น้ำหนักต่อปริมาตร) นอกจากนี้จากการศึกษาคุณลักษณะของโปรตีนไฮโดรไลเสตที่ผ่านการทำแห้งแบบแช่เยือกแข็ง พบว่าโปรตีนไฮโดรไลเสตจากกล้ามเนื้อปลาวัวที่ผ่านการทำแห้งแบบแช่เยือกแข็งมีปริมาณโปรตีนสูง โปรตีนไฮโดรไลเสตที่ผลิตได้ประกอบด้วยกรดอะมิโนจำเป็นในปริมาณสูง (ร้อยละ 45.62) และมีสีเหลืองอ่อน จากการศึกษากิจกรรมการต้านออกซิเดชันและสมบัติเชิงหน้าที่ของโปรตีนไฮโดรไลเสตที่ผลิตจากกล้ามเนื้อปลาวัวโดยใช้เอนไซม์ทริปซินจากตับปลาทูน่าพันธุ์ครีบยาวที่มีระดับการย่อยสลายร้อยละ 60 พบว่า ค่ากิจกรรมการจับอนุมูลอิสระ DPPH ABTS FRAP และกิจกรรมการจับโลหะเพิ่มขึ้นเมื่อปริมาณเพิ่มขึ้นสำหรับสมบัติเชิงหน้าที่ พบว่า โปรตีนไฮโดรไลเสตที่ผ่านการย่อยสลายด้วยเอนไซม์ทริปซินเพิ่มความสามารถในการละลาย โปรตีนไฮโดรไลเสตแสดงคุณสมบัติระหว่างพื้นผิวซึ่งขึ้นกับระดับความเข้มข้น ดังนั้นผลการศึกษาแสดงให้เห็นว่ากล้ามเนื้อปลาวัวสามารถนำมาผลิตเป็นโปรตีนไฮโดรไลเสตและสามารถใช้เป็นส่วนผสมที่มีศักยภาพในอาหารฟังก์ชันรวมถึงใช้เป็นสารต้านอนุมูลอิสระธรรมชาติในระบบอาหารที่มีลิปิด

คำสำคัญ: โปรติเนส เอนไซม์ทริปซิน การทำบริสุทธิ์ โปรตีนไฮโดรไลเสต

Abstract

Project Code: RSA6080044

Project Title: Proteinases from albacore tuna (*Thunnus alalunga*) liver: Purification, partitioning and their application for protein hydrolysate production

Investigator: Assoc. Prof. Dr. Sappasith Klomklao Thaksin University

E-mail Address: sappasith@tsu.ac.th

Project Period: May 30, 2017 – May 29, 2020

Two trypsins (A and B) from the liver of albacore tuna (*Thunnus alalunga*) were purified to homogeneity using a series of column chromatographies including Sephacryl S-200, Sephadex G-50 and Diethylaminoethyl-cellulose. Both trypsins showed only one band on native-PAGE. Trypsin A and B exhibited the maximal activity at 60 $^{\circ}$C and 55 $^{\circ}$C, respectively, and had the same optimal pH at 8.5 using TAME as a substrate. The inhibition test demonstrated strong inhibition by soybean trypsin inhibitor and TLCK. The N-terminal amino acid sequence of 20 residues of two trypsin isoforms had high homology compared to those of other fish trypsin. Proteinases from liver extract from albacore tuna were used to produce protein hydrolysate from starry triggerfish muscle. Hydrolysis conditions for preparing protein hydrolysate from starry triggerfish muscle were optimized. Optimum conditions for triggerfish muscle hydrolysis were 5.5% liver extract, 40 min reaction time and fish muscle/buffer ratio of 1:3 (w/v). The freeze dried protein hydrolysate was characterized with respect to chemical composition, amino acid composition and color. The product contained high protein and exhibited high amount of essential amino acids (45.62%). It was light yellow in color. Protein hydrolysates from starry triggerfish muscle with a degree of hydrolysis (DH) of 60% were also prepared using trypsin from albacore tuna liver and investigated for antioxidant activity and functional properties. Antioxidant activities including DPPH, ABTS radical scavenging activity, FRAP and metal chelating activity of hydrolysate samples were dose dependent. For functional properties, hydrolysis by trypsin increased protein solubility. The hydrolysates possessed interfacial properties, which were governed by their concentrations. Therefore, the results of the present study suggest that starry triggerfish can effectively be converted to protein hydrolysate and could be a potential ingredient in functional food as well as natural antioxidants in lipid food systems.

Keywords: Proteinase, Trypsin, Purification, Protein hydrolysate

CONTENTS

Chapter 1

PROTEINASES FROM LIVER OF ALBACORE TUNA (*THUNNUS ALALUNGA*): OPTIMUM EXTRACTANT AND BIOCHEMICAL CHARACTERISTICS

Journal of Food Biochemistry

PAKTEERA SRIPOKAR[1], MANAT CHAIJAN[2], SOOTTAWAT BENJAKUL[3] AND SAPPASITH KLOMKLAO[4]

[1]*Biotechnology Program, Faculty of Technology and Community Development, Thaksin University, Phatthalung Campus, Phatthalung, 93210, Thailand*

[2]*Department of Food Technology, School of Agricultural Technology, Walailak University, Nakhon Si Thammarat 80160, Thailand*

[3]*Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla, Thailand*

[4]*Department of Food Science and Technology, Facculty of Technology and Community Development, Thaksin University, Phatthalung Campus, Phatthalung, 93210, Thailand*

ABSTRACT

Proteolytic activity of the extract from albacore tuna (*Thunnus alalunga*) liver was studied. Optimum pH and temperature for casein hydrolysis were 8.5 and 55C, respectively. The enzyme was stable to heat treatment up to 50C and in the pH range of 7.0–10.0 for 30–120 min. The proteolytic activity was strongly inhibited by soybean trypsin inhibitor, *N*-p-tosyl-L-lysine chloromethyl ketone and phenylmethylsulfonyl fluoride. Activities of the liver extract continuously decreased with increasing NaCl concentration (0–30%), while activities increased as CaCl2 concentration increased. Based on activity staining, the molecular weights of the proteinases in albacore tuna liver were 21, 24, 30 and 34 kDa. Optimum extraction medium for proteinase recovery from albacore tuna liver was also investigated. Extraction of the liver powder with 50 mM Na phosphate buffer (pH 7.0) containing 0.2% (v/v) Brij 35 rendered a higher recovery of proteinase activity than other extractants tested ($P < 0.05$). The results suggested that major proteinases in albacore tuna liver were heat-activated alkaline proteinases, most likely trypsin-like serine proteinases.

Keyword: Proteinase, Isolation, Viscera, Trypsin, Tuna

INTRODUCTION

The tuna processing industry, especially canning, has become increasingly important as an income generator for Thailand. Interms of volume, Thailand is the world's largest exporter of canned tuna, with over 20 million cans annually during the past 5 years. Large volumes of raw tuna go through the canning process, in which about two-thirds of whole fish are utilized (Nalinanon *et al*. 2010). Fish viscera is produced in large quantities and represents a waste disposal and potential pollution problem. However, these materials are a potential source of enzymes such as proteinases that may have some unique properties for industrial applications (Klomklao *et al*. 2006).

Proteinases have been known to degrade proteins through hydrolysis of peptide bonds (Klomklao *et al*. 2006). Proteinases play a vital role in biotechnology, food processing and other industries, as well as in a variety of physiological processes. Proteinases used in the industry are mainly derived from plant, animal and microbial sources, whereas their counterparts derived from marine and aquatic sources have not been extensively used (Simpson, 2000). Marine animals have adapted to different environmental conditions, and these adaptations, together with inter and intra species genetic variations, are associated with certain unique properties of their proteinase, compared with their counter past enzymes from animals, plants and microorganisms (Simpson, 2000). Some of there distinctive properties include higher catalytic activity/ stability at neutral to alkaline pH (Klomklao *et al.* 2007). From this, there is a great potential for the extraction and use of proteinases from fish sources, especially from the viscera.

Albacore tuna (*Thunnus alalunga*) is one of the important species commonly used for the production of canned tuna in Thailand (Nalinanon *et al*. 2009). Base on

our preliminary study, liver from albacore tuna contained high proteolytic activity. Understanding the properties of proteinases in albacore tuna liver may provide important information for industrial application. However, there is no information on molecular and the biochemical characteristics of proteinases in albacore tuna liver. Therefore, the objectives of this study were to characterize the proteinases and to study the effect of extractants on the proteinases isolation from albacore tuna liver.

MATERIALS AND METHODS

Chemicals

Sodium caseinate, bovine hemoglobin, 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, phenyl-methylsulfonyl fluoride (PMSF), Brij 35, Triton X-100, Tween 20, L-tyrosine and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). Trichloroacetic acid, sodium chloride, tris (hydroxymethyl) aminomethane, acetone and Folin-Ciocalteu's phenol reagent were purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), Coomassie Brilliant Blue R-250 and *N, N, N', N'*-tetramethyl ethylene diamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA). All other reagents were of analytical grade.

Fish Sample Preparation

Albacore tuna (*Thunnus alalunga*) internal organs were obtained from Tropical Canning (Thailand) Public Co. Ltd., Hat Yai, Songkhla. The samples were packed in polyethylene bags, kept in ice with a sample/ ice ratio of 1:3 (w/w) and

transported to the research laboratory within 2 h. Pooled internal organs were separated and only the liver was collected, immediately frozen and stored at -20°C until used.

Preparation of Liver Extract

Frozen livers were thawed using running water (26-28°C) until the core temperature reached -2 to 0°C. The samples were cut into pieces with a thickness of 1-1.5 cm and homogenized into powder in three volumes of acetone at -20°C for 30 min according to the method of Klomklao *et al*. (2007). The homogenate was filtrated in vacuum on Whatman No. 4 filter paper. The residue obtained was then homogenized in two volumes of acetone at -20°C for 30 min, and then the residue was air-dried at room temperature. Defatted liver powder obtained was stored at -20°C until used.

To prepare the liver extract, defatted liver powder was suspended in distilled water at a ratio of 1:9 (w/v) and stirred continuously at 4°C for 30 min. The suspension was centrifuged at 5,000×g for 30 min at 4°C using a Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA). The supernatant obtained was collected and referred to as “liver extract”.

Enzyme Assay

Proteolytic activity of liver extract from albacore tuna was measured using hemoglobin and casein as substrate (An *et al*. 1994). To initiate the reaction, 200 µL of liver extract was added into assay mixtures containing 200 µL of 2% (w/v) substrate, 200 µL of distilled water and 625 µL of reaction buffer. The enzymatic reaction was terminated by adding 200 µL of 50% (w/v) trichloroacetic acid (TCA).

The reaction mixture was centrifuged at 7,500×g for 10 min at room temperature. The oligopeptide content in the supernatant was determined by the Lowry assay (Lowry *et al*. 1951) using tyrosine as a standard. Activity was expressed as tyrosine equivalents in TCA-supernatant. One unit of activity was defined as that releasing 1 mmol of tyrosine per min (mmol/ Tyr/ min). A blank was run in the same manner, except the enzyme was added after 50% TCA (w/v) addition.

Protein Determination

Protein concentration was measured by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

Characterization of Proteinases from Liver of Albacore Tuna

pH and Temperature Profiles

Proteinase activity was measured using substrate-TCA-Lowry assay (An *et al*. 1994) at pH 2.0-5.0 (using hemoglobin) and pH 5.0-11.0 (using casein). Different buffers were used for different pH conditions: 0.2 M McIlvain's buffer (0.2 M sodium phosphate-0.1 M sodium citrate) for pH 2.0-7.0 and 0.1 M glycine-NaOH for pH 8.0-11.0 (Klomklao *et al.* 2006). The activity was assayed at 50°C for 15 min. For temperature profile study, the activity was assayed at different temperatures (20, 30, 40 ,50, 55, 60, 65, 70 and 80°C) for 15 min at pH 8.5.

pH and Thermal Stability

The effect of pH on enzyme stability was evaluated by measuring the residual activity after incubation at various pHs (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) for 30, 60 and 120 min at room temperature. To investigate thermal stability, the liver

extracts were incubated at different temperatures (0, 10, 20, 30, 40, 50, 60, 70 and 80°C) for 30, 60 and 120 min, followed by cooling in ice water. The residual activity was measured using casein as a substrate at pH 8.5 and 55°C for 15 min and reported as the relative activity (%) compared with the original activity.

Effect of NaCl

To study the effect of NaCl on proteinase activity, NaCl was added to the standard reaction assay to obtain the final concentration of 0, 5, 10, 15, 20, 25 and 30% (w/v). The residual activity was determined at 55°C and pH 8.5 for 15 min using casein as a substrate.

Effect of $CaCl_2$

The effect of $CaCl_2$ on proteinase activity was investigated. $CaCl_2$ was added to the standard reaction assay to obtain different final concentrations (0, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} and 10^{-2} M). The activity was determined at 55°C and pH 8.5 for 15 min using casein as a substrate.

Effect of Inhibitors

The effect of inhibitors on proteinase activity was determined according to the method of Klomklao *et al*. (2010a) by incubating the liver extract with an equal volume of proteinase inhibitor solution to obtain the final concentration designated (0.1 mM E-64, 1 mM *N*-ethylmaleimide, 1 g/L soybean trypsin inhibitor, 5 mM TLCK, 5 mM TPCK, 1 mM pepstatin A, 5 mM benzamidine, 1 mM PMSF and 2 mM EDTA). The mixture was allowed to stand at room temperature (26-28°C) for 30 min.

Thereafter, the remaining activity was measured by substrate-TCA-Lowry method (An *et al*. 1994). Percent inhibition was then calculated.

Activity Staining

Liver extract was separated on SDS polyacrylamide gel electrophoresis, followed by activity staining according to the method of Klomklao *et al*. (2010b). Liver extract was mixed with sample buffer (0.5 M Tris-HCl, pH 6.8 containing 20% (v/v) glycerol, 10% SDS and 0.3% bromophenol blue) with and without βME at a ratio of 1:1 (v/v). Two µg of proteins were loaded into the gel made of 4% stacking and 12% separating gels. The proteins were subjected to electrophoresis at a constant current of 15 mA per gel by a Mini-Protean II Cell apparatus. After electrophoresis, gels were immersed in 100 mL of 2% (w/v) casein in 50 mM Tris-HCl buffer, pH 8.5 for 1 h with constant agitation at 0°C to allow the substrate to penetrate into the gels. The gels were then transferred to 2% (w/v) casein in 0.1 M glycine-NaOH, pH. 8.5 and incubated at 55°C for 15 min with constant agitation to develop the activity zone. The gels were fixed and stained with 0.125% Coomassie blue R-250 in 45% ethanol and 10% acetic acid, and destained in 30% methanol and 10% acetic acid. Development of clear zones on blue background indicated proteolytic activity.

For the inhibitor study, liver extracts were incubated with an equal volume of proteinase inhibitor solutions to obtain the final concentration designated as described previously for 30 min at room temperature. After incubation, the mixtures were mixed with sample buffer at a ratio of 1:1 (v/v). The mixtures were loaded into the gel, and activity staining was performed as previously described.

Optimum Extractant for Recovery of Proteinases from Albacore Tuna Liver

Effect of Extractants on the Recovery of Proteinases from Albacore Tuna Liver

Different extraction media, including distilled water, 50 mM Na-phosphate buffer, pH 7.0 and 50 mM Tris-HCl, pH 7.0 were used to extract proteinases from liver of albacore tuna. The medium was added into the defatted liver powder with a ratio of 1:9 (w/v) and stirred continuously at 4°C for 30 min. The supernatant was recovered by centrifuging the slurry at 5,000× g for 30 min. The proteinase activity and protein content in the extracts were determined, and the yield and specific activity of the extracts obtained using different media were compared. The extractant rendering the highest yield was selected for further steps.

Effect of NaCl Concentration on the Recovery of Proteinases from Albacore Tuna Liver

Defatted liver powder was suspended in 50 mM Na-phosphate buffer, pH 7.0 containing different NaCl concentration (0, 0.25, 0.5, 0.75 or 1 M) at a ratio of 1:9 (w/v) and stirred continuously at 4°C for 30 min. The supernatant was recovered by centrifuging the slurry at 5,000× g for 30 min. The proteinase activity and protein content in the extracts were determined. The extraction yield and specific activity of the extracts were calculated. The extractant rendering the highest yield was chosen for further steps.

Effect of Surfactant on the Recovery of Proteinases from Albacore Tuna Liver

Defatted liver powder was suspended in 50 mM Na-phosphate buffer, pH 7.0 containing 0.2% (v/v) different surfactants (Brij 35, Tween 20, Triton X-100 and SDS) at a ratio of 1:9 (w/v) and stirred continuously at 4°C for 30 min. The

supernatant was recovered by centrifuging the slurry at 5,000× g for 30 min. The proteinase activity and protein content in the extracts were measured. The extraction yield and specific activity of the extracts were calculated.

Statistical Analysis

A completely randomized design was used throughout this study. Data were subjected to analysis of variance, and mean comparison was carried out using Duncan's Multiple Range Test (Steel and Torrie, 1980). Statistical analysis was performed using SPSS.

RESULTS AND DISCUSSION

Biochemical Properties of Proteinases from Liver of Albacore Tuna

pH and Temperature Profiles

The pH activity profile of proteinases from liver of albacore tuna is shown in Fig. 1a. The optimum pH for caseinolytic activity was found at pH 8.5. The relative activities at pH 8.0 and 9.0 were about 80.73% and 92.31%, respectively. Therefore, these proteinases are considered to be an alkaline proteinases. The activity of albacore tuna liver proteinases decreased in acidic and alkaline pH ranges. The effect of temperature on the proteinase activity was determined by assaying enzyme activity at different temperatures for 15 min at pH 8.5. Fig. 1b shows the enzyme activity of the liver extract as a function of temperature. The optimum temperature for the proteinase was 55°C. The enzyme activity gradually declined at temperatures beyond 55°C. Based on the optimum pH and temperature, the major proteinase from the liver of albacore tuna was characterized as heat-activated alkaline proteinases. Heat-activated alkaline proteinases have been found in digestive tracts of various fish. Alkaline

proteases from the red scorpionfish (*Scorpaena scrofa*) viscera exhibited optimal activity at pH 10.0 and 55ºC (Younes *et al*. 2014). The optimal pH and temperature of alkaline protease from viscera of bolti fish (*Tilapia nilotica*) were 8.0 and 45ºC, respectively with casein as a substrate (El-Beltagy *et al*. 2005). The optimum pH and temperature of serine protease from the viscera of sardinelle (*Sardinella aurita*) were around pH 8.0 and 60ºC, respectively (Ben *et al*. 2011). Klomklao *et al*. (2007) found that the optima pH and temperature of trypsin from pyloric ceca of bluefish (*Pomatomus saltatrix*) for the hydrolysis of benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) were 9.5 and 55 °C, respectively. Souza *et al*. (2007) reported that trypsin from the intestine and pyloric ceca of spotted goatfish (*Pseudopeneus maculates*) presented identical optima pH (9.0) and temperature (55ºC).

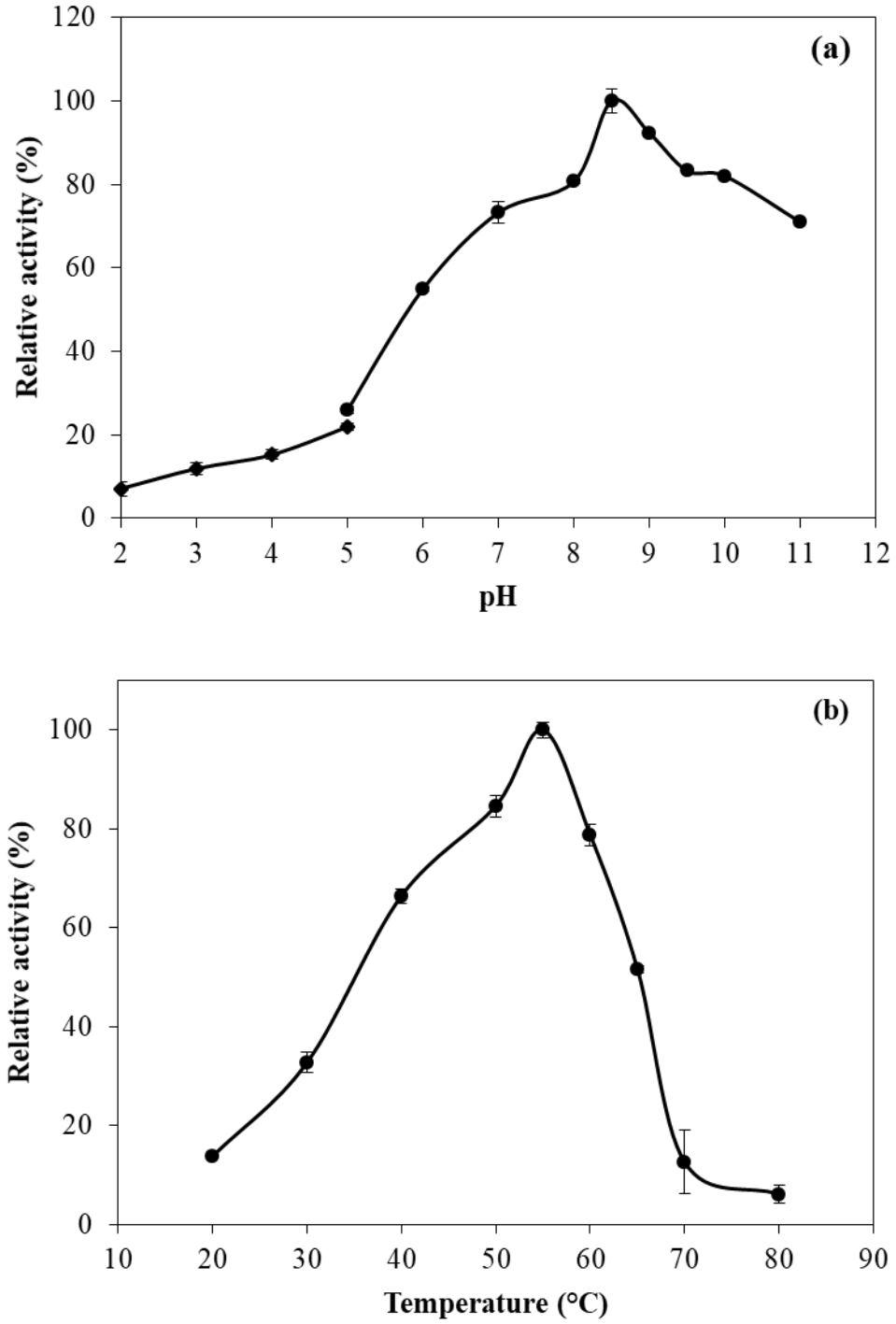

FIGURE 1. pH (A) AND TEMPERATURE (B) PROFILES OF PROTEINASES FROM LIVER OF ALBACORE TUNA.

Bars represent the standard deviation from triplicate determinations.

pH and Thermal stability

The pH stability profile showed that the albacore tuna liver proteinase is highly stable over a wide pH range, maintaining more than 80% of its original activity at pH values between 7.0-10.0 after incubation for 60 min at room temperature (Fig. 2a). With an extended incubation time, proteinase activity was lost to a greater extent. At pH below 7.0, the stability of the enzyme decreased sharply. The stability of the enzyme at a particular pH might be related to the net charge of the enzyme at that pH (Klomklao *et al*. 2010b). At acidic pHs, it is suggested that conformational changes in the enzyme affected the proper binding of the enzyme to the substrate (Klomklao *et al*. 2007). Alkaline proteases from the red scorpionfish (*Scorpaena scrofa*) viscera was extremely stable in the pH range of 5.0-12.0 (Younes *et al*. 2014). Serine protease from sardinelle (*Sardinella aurita*) viscera was stable in the pH range of 7.0-10.0 (Ben *et al*. 2011). Trypsin from intestine of Nile tilapia (*Oreochromis niloticus* L.) showed high stability at the pH range of 6.0-11.0 (Unajak *et al*. 2012). Trypsin from the intestine of Grey triggerfish (*Balistes capriscus*) was extremely stable in the pH range 7.0-12.0 (Jellouli *et al*. 2009). Trypsin from the hepatopancreas of the cuttlefish (*Sepia officinalis*) was stable in the pH range 6.0-10.0 (Balti *et al*. 2009).

Figure 2b showed the thermal stability of the liver proteinase from albacore tuna. The enzyme was stable when incubated at temperatures up to 50°C for 30-120 min. However, the enzyme was inactivated at temperatures above 50ºC. At high temperature, the enzymes possibly underwent denaturation and lost their activity (Klomklao *et al*. 2007). Generally, the stability of proteinases decreased when heating time was increased. Alkaline proteases from the red scorpionfish (*Scorpaena scrofa*) viscera were stable at temperatures ranging from 30 to 40°C for 30 min (Younes *et al*. 2014). Serine protease from sardinelle (*Sardinella aurita*) viscera is completely stable

at temperatures below 40°C (Ben *et al*. 2011). Trypsin from the pyloric ceca of Pacific saury (*Cololabis saira*) was stable in the temperature range of 20-50ºC (Klomklao *et al*. 2014). Trypsin from the hepatopancreas of the cuttlefish (*Sepia officinalis*) was stable at temperatures below 50ºC (Balti *et al*. 2009). Jellouli *et al*. (2009) found that trypsin from the intestine of Grey triggerfish (*Balistes capriscus*) was highly stable at temperature below 40ºC but was inactivated at high temperature.

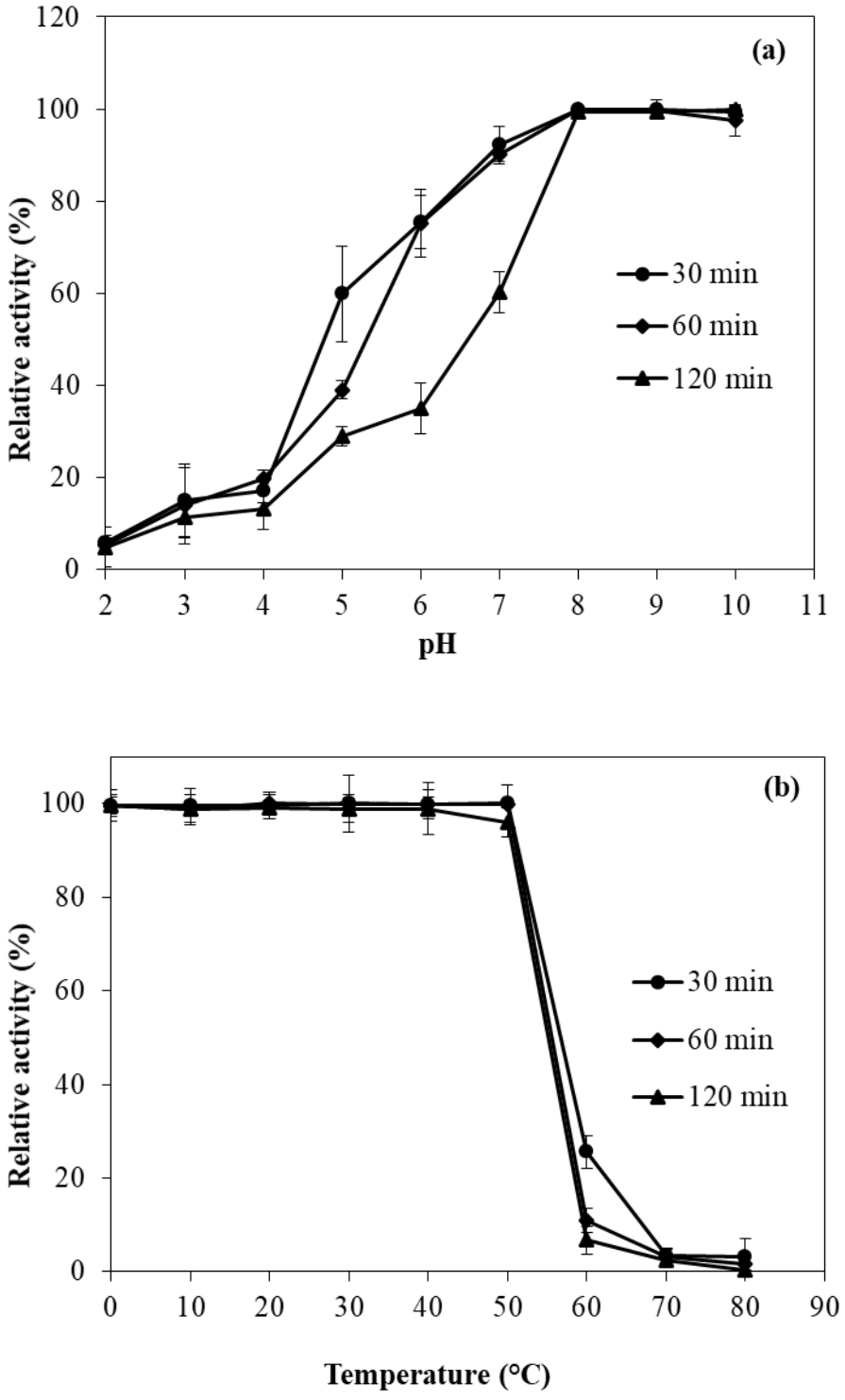

FIGURE 2. pH (A) AND THERMAL (B) STABILITY OF PROTEINASES FROM LIVER OF ALBACORE TUNA.

Bars represent the standard deviation from triplicate determinations.

Effect of NaCl

The effect of NaCl on the activity of proteinase from albacore tuna liver was studied at pH 8.5 and 55°C by the addition of NaCl to the reaction mixture. The activity of proteinases decreased gradually with increasing NaCl concentration (Fig. 3). Ben *et al*. (2011) reported that the relative activity of protease from viscera of sardinelle (*Sadinella aurita*) at 10% NaCl was approximately 20%. The activity of trypsin from hepatopancreas of the cuttlefish (*Sepia officinalis*) decreased with increasing NaCl concentration. The relative activity at 30% NaCl was approximately 39.4% (Balti *et al*. 2009). The activity of trypsin from the pyloric ceca of Pacific saury (*Cololabis saira*) decreased with increasing NaCl concentration up to 30% (Klomklao *et al*. 2014). Some losses in activity occurred as NaCl concentration increased, probably owing to the partial denaturation of proteinase caused by the "salting out" effect (Klomklao *et al*. 2011). The water molecule is drawn from the proteinase molecule by salt, leading to the aggregation of those enzymes (Klomklao *et al*. 2004). From the results, more than 30% of proteinase activity remained in the presence of a high concentration of NaCl (25%) Therefore, these proteinases from liver of albacore tuna may contribute to the protein hydrolysis in high salt fermented fish products such as fish sauce.

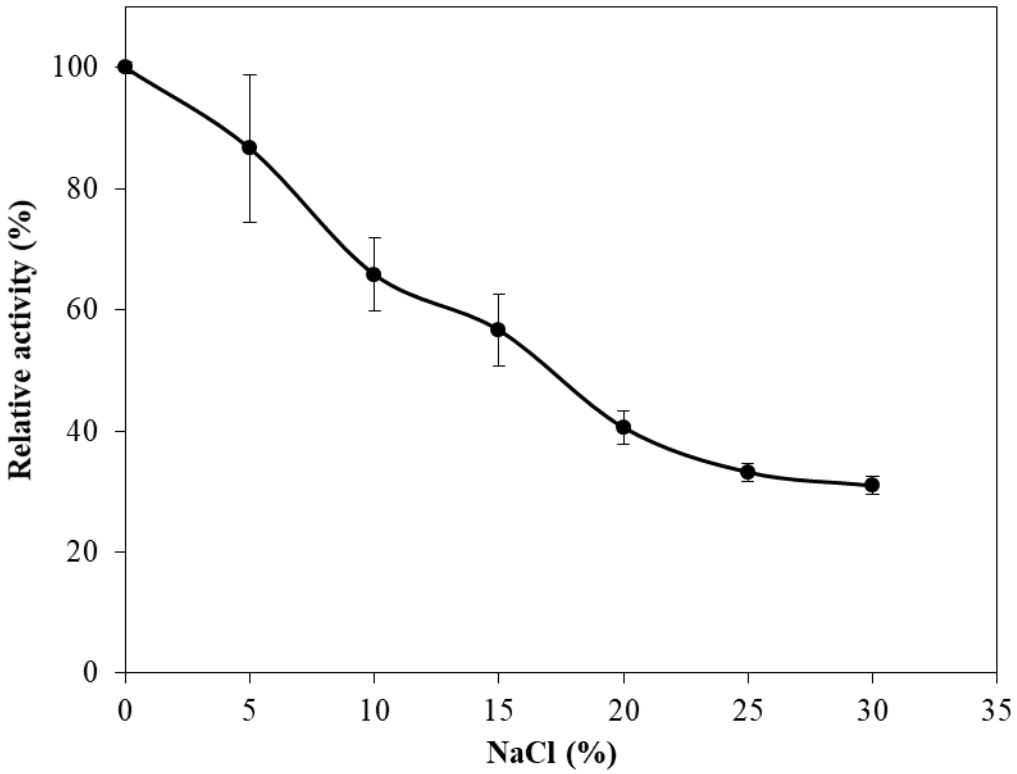

FIGURE 3. EFFECT OF NACL CONCENTRATION ON ACTIVITIES OF PROTEINASES FROM LIVER OF ALBACORE TUNA.

Bars represent the standard deviation from triplicate determinations.

Effect of $CaCl_2$

Proteinase activities of liver extract from albacore tuna increased with $CaCl_2$ addition (Fig. 4). When the concentration of $CaCl_2$ was increased from 0.1 µM to 0.1 mM, activity apparently increased. However, there was no further increase in the activity with $CaCl_2$ above 0.1 mM. Serine proteinases from the skeletal muscle of red sea bream (*Pagrus major*) was slightly activated by Ca^{2+} at the concentration of 5 mM (Wu *et al*. 2010). Younes *et al*. (2014) found that the addition 5 mM of $CaCl_2$ increased the activity of the crude protease extract from red scorpionfish (*Scorpaena scrofa*) viscera to 109%. It has been known that calcium ions promote the formation of active trypsin from trypsinogen and stabilize trypsin against autolysis (Klomklao *et al*. 2007). Two calcium-binding sites are present in trypsinogen (Kosslakoff *et al*. 1977). The primary site, with a higher affinity for calcium ions, is common in trypsinogen and trypsin, and the secondary site occurs only in the zymogen. The binding of calcium to trypsinogen induces a conformational change, which is associated with the formation of an active form. Therefore, calcium ions played an essential role in activation of proteinases from albacore tuna liver.

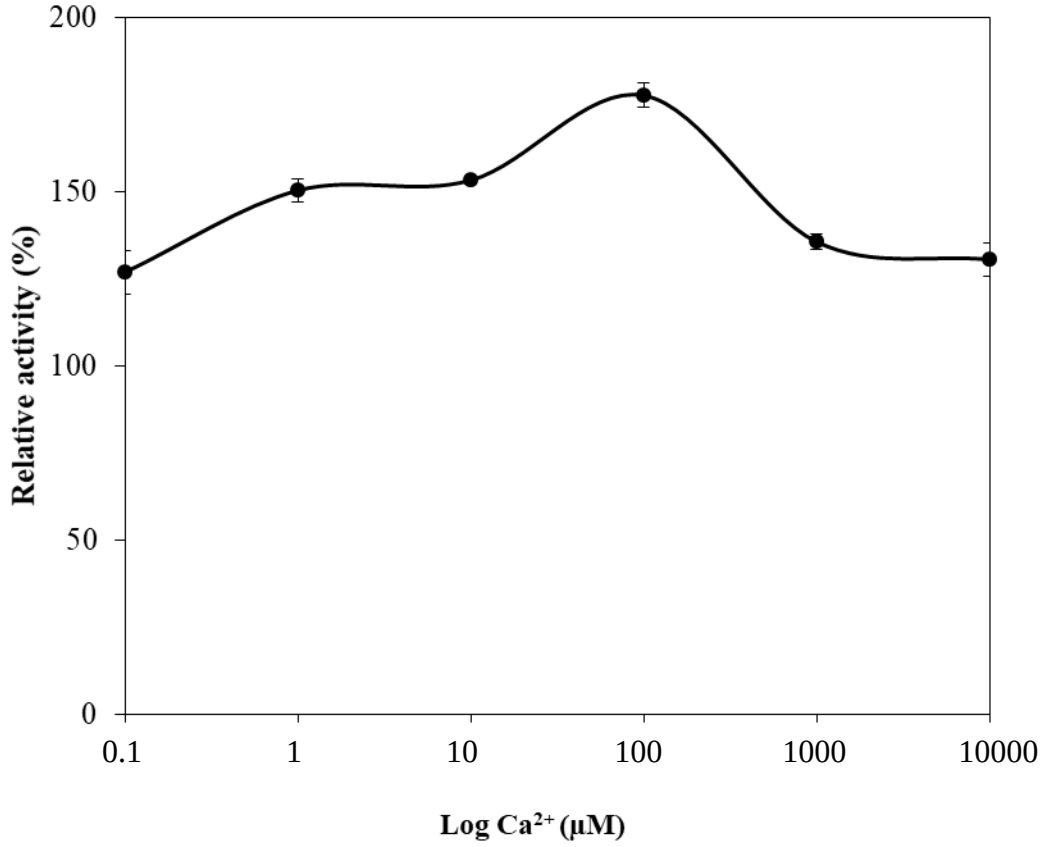

FIGURE 4. EFFECT OF $CACL_2$ ON ACTIVITIES OF PROTEINASES FROM LIVER OF ALBACORE TUNA.

Bars represent the standard deviation from triplicate determinations.

Effect of Inhibitors

Various proteinase inhibitors were assayed and evaluated in term of their effects on enzyme activity (Table 1). Strong inhibition by 1.0 g/L soybean trypsin inhibitor (78.70% inhibition), 5.0 mM TLCK (54.98% inhibition) and 1.0 mM PMSF (51.35% inhibition), was observed. The cysteine proteinase inhibitor (E-64, *N*-ethylmaleimide), metallo (EDTA), aspartic proteinases (pepstatin A) and chymotrypsin inhibitor (TPCK) showed the negligible inhibitory no effect on proteinase activity. These data indicated that the major proteinases from the liver of albacore tuna belonged to the serine proteinases, particulary trypsin or trypsin-like enzymes. Trypsin from zebra blenny (*Salaria basilisca*) viscera was completely inhibited by 1 mg/ml soybean trypsin inhibitor and partially inhibited by PMSF and a serine protease inhibitor (Ktari *et al*. 2012). Serine proteinase from the viscera of Sadinelle (*Sardinella aurita*) was strongly inhibited by PMSF, a serine protease inhibitor and soybean trypsin inhibitor (Ben *et al*. 2011). Protease from viscera of red scorpionfish (*Scorpaena scrofa*) were affected by 10 mM PMSF and a serine protease inhibitor (Younes *et al*. 2014).

TABLE 1. EFFECT OF VARIOUS INHIBITORS ON THE ACTIVITY OF PROTEINASES FROM LIVER OF ALBACORE TUNA*

Inhibitors	Concentration	Inhibition (%)**
Control		0a
E-64	0.1 mM	0.56±0.65a
N-ethylmaleimide	1.0 mM	0.07±0.33a
Soybean trypsin inhibitor	1.0 g/L	78.70±1.42d
TLCK	5.0 mM	54.98±1.54c
TPCK	5.0 mM	1.14±2.40a
Pepstatin A	1.0 mM	0.42±2.89a
Benzamidine	5.0 mM	20.80±4.51b
PMSF	1.0 mM	51.35±5.72c
EDTA	2.0 mM	0.50±3.82a

*Activity was analyzed using casein as a substrate for 15 min at pH 8.5 and 55°C.

**Mean ± S.D. from triplicate determinations.

The different letters in the same column denote the significant differences ($P<0.05$).

Activity Staining

The proteinase activity in albacore tuna liver extract was identified by separation on SDS-substrate polyacrylamide gels following by staining for proteolytic activity (Fig. 5). Under both reducing and non reducing conditions, a similar pattern of activity bands is depicted as clear zones on the dark background**.** Four activity bands were observed at the apparent molecular weight of 21, 24, 30 and 34 kDa. The molecular weight of the major activity band was estimated to be 21 kDa. Based on the molecular weight, the activity bands were tentatively identified as trypsin and/or trypsin-like serine proteinases. Trypsin consists of a single peptide chain with molecular weight typically of 24 kDa. However, the differences in trypsin maybe owing to the genetic variation among species (Klomklao *et al*. 2004). The molecular weight of trypsin from Greenland cod pyloric ceca was 23.5 kDa (Simpson and Haard. 1984). Klomklao *et al*. (2004) reported that the molecular weights of the major proteinase activity bands of tongol and yellowfin tuna were 21 kDa. The molecular weight of trypsin from viscera of hybrid catfish was 24 kDa by size exclusion chromatography and SDS-PAGE (Klomklao *et al*. 2011). Unajak *et al*. (2012) reported that the molecular weight of the trypsin of Nile tilapia (*Oreochromis niloticus* L.) intestine were 22.39 kDa by SDS-PAGE.

The effect of various proteinase inhibitors on the activity bands observed on SDS-substrate gel electrophoresis is depicted in Fig. 6. The activity bands were effectively inhibited when the liver extract was treated with soybean trypsin inhibitor and TLCK and partially inhibited by PMSF. Nevertheless, the activity bands were retained after mixing with E-64, *N*-ethylmaleimide, TPCK, pepstatin A, benzamidine and EDTA. The result confirmed that the major proteinases in liver of albacore tuna

are trypsin or trypsin-like enzyme. The major liver proteinase from albacore tuna will be further isolated, purified and characterized.

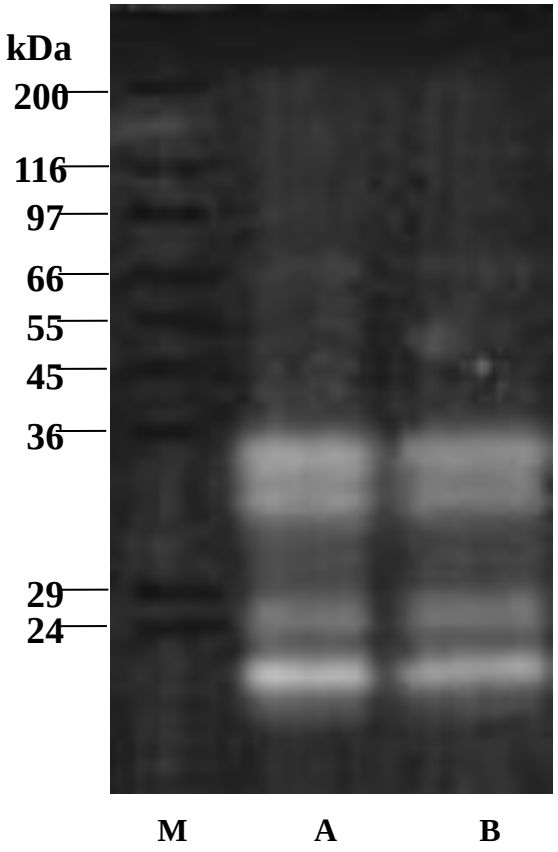

FIGURE 5. ACTIVITY STAINING OF LIVER PROTEINASES FROM ALBACORE TUNA UNDER NON REDUCING (A) AND REDUCING (B). M, molecular weight standard.

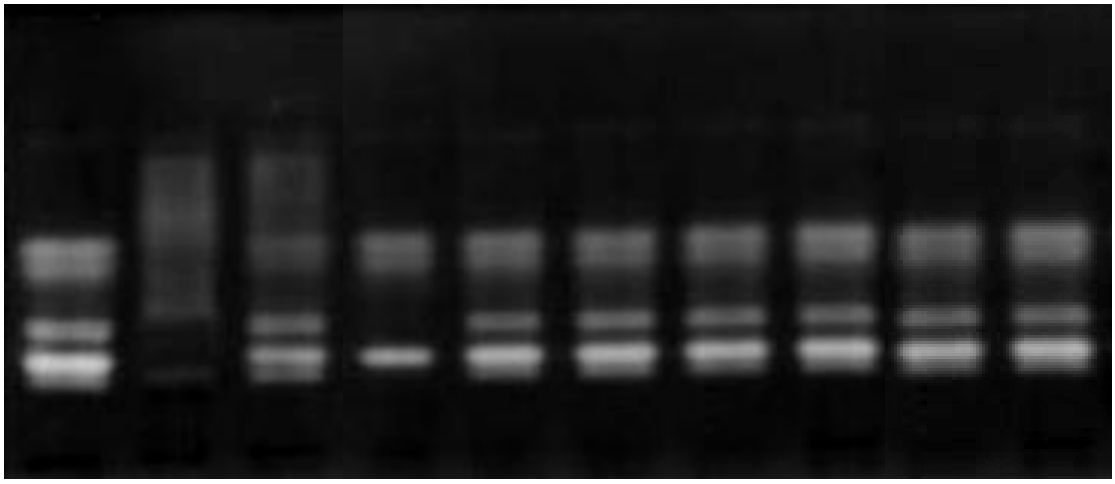

FIGURE 6. ACTIVITY STAINING OF LIVER PROTEINASES FROM ALBACORE TUNA WITH AND WITHOUT PROTEINASE INHIBITOR. A: control; B: soybean trypsin inhibitor; C: TLCK; D: PMSF; E: benzamidine; F: TPCK; G: E-64; H: EDTA; I: pepstatin A; J: *N*-ethylmaleimide.

Optimum Extractant for Recovery of Proteinases from Albacore Tuna Liver

Table 2 showed the effect of extractants on proteinase extraction from liver of albacore tuna. Liver extract using 50 mM Na-phosphate buffer, pH 7.0 showed the higher proteinase activity than those extracted with distilled water and 50 mM Tris-HCl, pH 7.0 when casein was used as substrate ($P < 0.05$). The results suggested that Na-phosphate buffer had a greater ability to extract proteinase than Tris-HCl and distilled water. Wu *et al*. (2010) used 25 mM sodium phosphate buffer, pH 7.5 for extracting the proteinases from the skeletal muscle of red sea bream (*Pagrus major*). 50 mM sodium phosphate buffer (pH 7.0) was used to extract pepsinogens and pepsins from the stomach of European eel (*Anguilla Anguilla*) (Wu *et al*. 2009). From the results, 50 mM Na-phosphate buffer, pH 7.0 was selected as the extraction medium for albacore tuna liver proteinase because the extract had the maximum proteinase activity.

Na-phosphate buffer containing different NaCl concentration were used to recover the proteinases from liver of albacore tuna (Table 3). When the concentration of NaCl was increased from 0 to 1.0 M, activity apparently decreased ($P < 0.05$). This probably due to the denaturation of proteinases from liver of albacore tuna at high salt concentration.

The effect of some surfactants on the recovery of proteinases from liver of albacore tuna is shown in Table 4. Addition of surfactants in 50 mM Na-phosphate buffer, pH 7 affected the yield of proteinase extracted at 4°C for 30 min. When the tuna liver powder was extracted with 50 mM Na-phosphate buffer, pH 7 containing 0.2% Brij 35, the highest yield or proteinase activity and specific activity were obtained ($P < 0.05$). The yield of proteinase extracted with the aid of Brij 35 was higher than that of proteinases extracted without Brij 35. The Brij 35 was added to

facilitate improved extraction of soluble cell material and to emulsify the small amount of lipid present in tuna liver extract to prevent lipid interference with the proteinase activity. Addition of 0.2% (v/v) of Brij 35 to the extract from Chinook salmon intestine resulted in a small increase in trypsin activity (Kurtovic *et al*. 2006). Klomklao *et al*. (2010) found that 50 mM Tris-HCl, pH 7.0 containing 0.5 M NaCl and 0.2% (v/v) Brij 35 showed the best of extraction medium for proteinases in hybrid catfish viscera.

TABLE 2. EFFECT OF EXTRACTION MEDIA ON THE RECOVERY OF PROTEINASES FROM LIVER OF ALBACORE TUNA*

Extraction media	Total activity (Units)	Total protein (mg)	Specific activity (Units/mg protein)**
Distilled water	196.71±3.75a	65.36±0.56a	3.01±0.08a
50 mM Tris-HCl, pH 7.0	247.13±1.58b	62.54±2.67a	3.96±0.18b
50 mM Na-phosphate buffer, pH 7.0	291.97±1.58c	64.43±0.71a	4.53±0.04c

*The defatted liver powder was extracted in different media at 4°C for 30 min and proteolytic activity was analyzed using casein as substrate for 15 min at pH 8.5 and 55°C.

**Mean ± S.D. from triplicate determinations.

The different letters in the same column denote the significant differences (P<0.05).

TABLE 3. EFFECT OF NACL CONCENTRATION ON THE RECOVERY OF PROTEINASES FROM LIVER OF ALBACORE TUNA*

NaCl concentration (M)	Total activity (Units)	Total protein (mg)	Specific activity (Units/mg protein)**
0	301.72±2.01d	66.08±0.77ab	4.57±0.07d
0.25	297.67±4.94cd	67.95±1.66bc	4.38±0.10d
0.50	287.28±3.31c	68.86±1.88c	4.17±0.08c
0.75	269.29±13.39b	71.18±0.34d	3.63±0.19a
1.00	245.99±4.58a	64.12±0.99a	3.83±0.71b

*The defatted liver powder was extracted in 50 mM Na-phosphate buffer, pH 7.0 in different NaCl concentration at 4°C for 30 min and proteolytic activity was analyzed using casein as substrate for 15 min at pH 8.5 and 55°C.

**Mean ± S.D. from triplicate determinations.

The different letters in the same column denote the significant differences (P<0.05).

TABLE 4. EFFECT OF SOME SURFACTANTS ON THE RECOVERY OF PROTEINASES FROM LIVER OF ALBACORE TUNA*

Surfactant	Total activity (Units)	Total protein (mg)	Specific activity (Units/mg protein)**
Control	299.31±1.16c	66.21±1.45b	4.52±0.09c
SDS	261.82±4.75b	66.21±2.29b	3.96±0.09a
Tween 20	306.28±3.95d	60.64±1.65a	5.05±0.17d
Triton X-100	249.15±1.58a	58.86±0.79a	4.23±0.04b
Brij 35	328.32±2.01e	59.45±1.85a	5.52±0.15e

*The defatted liver powder was extracted in 50 mM Na-phosphate buffer, pH 7.0 in different surfactants at 4°C for 30 min and proteolytic activity was analyzed using casein as substrate for 15 min at pH 8.5 and 55°C.

**Mean ± S.D. from triplicate determinations.

The different letters in the same column denote the significant differences (P<0.05).

CONCLUSION

Albacore tuna liver extract contained heat-activated alkaline serine proteinases as the predominant enzymes with optimum activity at pH 8.5 and 55ºC. The major proteinase from albacore tuna liver was most likely classified as trypsin or trypsin-like based on inhibitor study, effect of $CaCl_2$ and molecular mass and can be potential novel enzymes for further applications.

ACKNOWLEDGMENTS

This research was supported by the Thailand Research fund and Thaksin University. The TRF distinguished research professor grant was also acknowledged.

REFERENCES

AN, H., SEYMOUR, T.A., WU, J.W. and MORRISSEY, M.T. 1994. Assay systems and characterization of Pacific whiting (*Merluccius productus*) protease. J. Food Sci. 59, 277-289.

BALTI, R., BARKIA, A., BOUGATEF, A., KTARI, N. and NASRI, M. 2009. A heat-stable trypsin from the hepatopancreas of the cuttlefish (*Sepia officinalis*): Purification and characterization. Food Chem. 113, 146-154.

BEN, K.H., NASRI, R., BOUGATEF, A., GHORBEL, S. and NASRI, M. 2011. Low molecular weight serine protease from the viscera of sardinelle (*Sadinella aurita*) with collagenolytic activity: purification and characterization. Food Chem. 124, 788-794.

EL-BELTAGY, A.E., EL-ADAWY, T.A., RAHMA, E.H. and EL-BEDAWAY, A.A. 2005. Purification and characterization of an alkaline protease from the viscera of bolti fish (*Tilapia nilotica*). J. Food Biochem. 29, 445-458.

JELLOULI, K., BOUGATEF, A., DAASSI, D., BALTI, R., BARKIA, A. and NASRI, M. 2009. New alkaline trypsin from the intestine of Grey triggerfish (*Balistes capriscus*) with high activity at low temperature: Purification and characterization. Food Chem. 116, 644-650.

KLOMKLAO, S., BENJAKULl, S. and VISESSANGUAN, W. 2004. Comparative studies on proteolytic activity of spleen extracts from three tuna species commonly used in Thailand. J. Food Biochem. 28, 355-372.

KLOMKLAO, S., BENJAKUL, S., VISESSANGUAN, W., KISHIMURA, H. and SIMPSON, B.K. 2006. Purification and characterization of trypsin from the spleen of Tongol tuna (*Thunnus tonggol*). J. Agric. Food Chem. 54, 5617-5622.

KLOMKLAO, S., BENJAKUL, S., VISESSANGUAN, W., KISHIMURA, H. and SIMPSON, B.K. 2007. Purification and characterization of trypsins from the spleen of skipjack tuna (*Katsuwonus pelamis*). Food Chem. 100, 1580-1589.

KLOMKLAO, S., KISHIMURA, H. and BENJAKUL, S. 2008. Endogenous proteinases in true sardine (*Sardinops melanostictus*). Food Chem. 107, 213-220.

KLOMKLAO, S., BENJAKUL, S. and KISHIMURA, H. 2010a. Proteinases in hybrid catfish viscera: Characterization and effect of extraction media. J. Food Biochem. 34, 711-729.

KLOMKLAO, S., KISHIMURA, H., BENJAKUL, S., SIMPSON, B.K. and VISESSANGUAN, W. 2010b. Cationic trypsin: A predominant proteinase in Pacific saury (*Cololabis saira*) pyloric ceca. J. Food Biochem. 34, 1105-1123.

KLOMKLAO, S., BENJAKUL, S., KISHIMURA, H. and CHAIJAN, M. 2011. 24 kDa trypsin: A predominant protease purified from the viscera of hybrid

catfish (*Clarias macrocephalus* × *Clarias gariepinus*). Food Chem. 129, 739-746.

KLOMKLAO, S., KISHIMURA, H. and BENJAKUL, S. 2014. Anionic trypsin from the pyloric ceca of Pacific saury (*Cololabis saira*): Purification and biochemical characteristics. J. Aquat. Food Prod. Technol. 23, 186-200.

KOSSLAKOFF, A.A., CHAMBERS, J.L., KAY, L.M. and STROUD, R.M. 1977. Structure of bovine trypsinogen at 1.9 A resolution. Biochem. 16, 654-664.

KTARI, N., BKHAIRIA, I., JRIDI, M., HAMZA, I., RIADH, B.S. and NASRI, M. 2012. Digestive acid protease from zebra blenny (*Salaria basilisca*): Characteristics and application in gelatin extraction. Food Research Int. 57, 218-224.

KURTOVIC, I., MARSHALL, S.N. and SIMPSON, B.K. 2006. Isolation and characterization of a trypsin fraction from the pyloric ceca of Chinook salmon (*Oncorhynchus tshawytscha*). Comp. Biochem. Physiol. 143B, 432-440.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. AND RANDALL, R.J. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193, 256-275.

NALINANON, S., BENJAKUL, S., VISESSANGUAN, W. and KISHIMURA, H. 2009. Partitioning of protease from stomach of albacore tuna (*Thunnus alalunga*) by aqueous two-phase systems. Process Biochem. 44, 471-476

NALINANON, S., BENJAKUL, S. and KISHIMURA, H. 2010. Purification and biochemical properties of pepsins from the stomach of skipjack tuna (*Katsuwonus pelamis*). Eur. Food Res. Technol. 231, 259-269.

SIMPSON, B.K. and HAARD, H.F. 1984. Trypsin from Greenland cod (*Gadus ogac*). Isolation and comparative properties. Comp. Biochem. Physiol. 79B, 613-622.

SIMPSON, B.K. 2000. Digestive proteinases from marine animals. In: *Seafood Enzymes: Utilization and Influence on Postharvest Seafood Quality*, (N.M. Haard and B.K. Simpson, eds) pp. 531-540, Marcel Dekker, New York.

SOUZA, A.A.G., AMARAL, I.P.G., ESPIRITO SANTO, A.R., CARVALHO JR, L.B. and BEZERRA, R.S. 2007. Trypsin-like enzyme from intestine and pyloric caeca of spotted goatfish (*Pseudupeneus maculatus*). Food Chem. 100, 1429-1434.

STEEL, R.G.D. and TORRIE, J.H. 1980. Principles and Procedures of Statistics; A Biometrical Approach. 2nd Ed., McGraw-Hill, New York.

UNAJAK, S., MEESAWAT, P., PAEMANEE, A., AREECHON, N., ENGKAGUL, A., KOVITVADHI, U., KOVITVADHI, S., RUNGRUANGSAK-TORRISSEN, K. and CHOOWONGKOMON, K. 2012. Characterisation of thermostable trypsin and determination of trypsin isozymes from intestine of Nile tilapia (*Oreochromis niloticus* L.). Food Chem. 134, 1533-1542.

WU, T., SUN, L.C., DU, C.H., CAI, Q.F., ZHANG, Q.B., SU, W.J. and CAO, M.J. 2009. Identification of pepsinogens and pepsins from the stomach of European eel (*Anguilla anguilla*). Food Chem. 115, 137-142.

WU, G.P., CHEN, S.H., LIU, G.M., YOSHIDA, A., ZHANG, L.J., SU, W.J. and CAO, M.J. 2010. Purification and characterization of a collagenolytic serine proteinase from the skeletal muscle of red sea bream (*Pagrus major*). Comp. Biochem. Physiol. 155B, 281-287.

YOUNES, I., NASRI, R., BKHAIRIA, I., JELLOULI, K. and NASRI, M. 2014. New proteases extracted from red scorpionfish (*Scorpaena scrofa*) viscera: Characterization and application as a detergent additive and for shrimp waste deproteinization. Food Bioprod. Process. 94, 453-462.

Chapter 2

Aqueous two-phase partitioning of liver proteinase from albacore tuna (*Thunnus alalunga*): Application to starry triggerfish (*Abalistes stellaris*) muscle hydrolysis

International Journal of Food Propertites

Pakteera Sripokar[1], Manat Chaijan[2], Soottawat Benjakul[3], Asami Yoshida[4]

and Sappasith Klomklao[5,*]

[1]*Biotechnology Program, Faculty of Technology and Community Development, Thaksin University, Phatthalung Campus, Phatthalung, 93210, Thailand*

[2]*Department of Food Technology, School of Agricultural Technology, Walailak University, Nakhon Si Thammarat, 80160, Thailand*

[3]*Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla, Thailand*

[4]*Graduate School of Fisheries Science and Environmental Studies, Nagasaki University, 1-14 Bunkyo, Nagasaki 852-8521, Japan*

[5]*Department of Food Science and Technology, Faculty of Technology and Community Development, Thaksin University, Phatthalung Campus, Phatthalung, 93210, Thailand*

ABSTRACT

The potential of aqueous two-phase system (ATPS) for the purification and recovery of proteinase from albacore tuna (*Thunnus alalunga*) liver was explored. Influence of phase compositions such as type of phase forming salts, PEG molecular weight, concentration of salt and PEG, pH of the system and NaCl addition on partitioning of proteinase was investigated. ATPS comprising PEG1000 (25%, w/w) and NaH_2PO_4 (20%, w/w) at pH 7.0 provided the best condition for the maximum partitioning of proteinase into the top phase and gave the highest purification factor (5.58-fold) and specific activity (20.65 unit/mg protein). The yield of 89.99% was obtained. The addition of NaCl up to a final concentration of 6% (w/w) decreased the degree of purification and enzyme recovery of proteinase. Based on electrophoresis and activity staining, the fractionated proteinases had the MW 21, 24, 30 and 34 kDa. The effect of fractionated proteinases on starry triggerfish (*Abalistes stellaris*) muscle hydrolysis was also studied. Fractionated proteinases were able to hydrolyze triggerfish muscle in a dose-dependent manner. Overall, results demonstrated the feasibility of ATPS for the recovery and purification of proteinase without the need for multiple steps and the obtained proteinase can be further in preparation of protein hydrolysate.

Keywords: Aqueous two-phase system, Proteinase, Partitioning, Viscera, Hydrolysis

INTRODUCTION

Albacore tuna (*Thunnus alalunga*) is an important raw material used for the production of canned tuna in Thailand.[1] Large volumes of raw tuna go through the canning process, by which about two-thirds of whole fish is utilized.[2] During canned tuna manufacturing, high amounts of viscera are generated.

Fish viscera is known to be a rich source of proteinases that have high activity over a wide range of pH and temperature conditions[3] and exhibit high catalytic activity at relatively low concentration.[3] Proteinases have been widely used in food, medical-phamaceutical, cosmetic and other industries. Fish proteinases has been used for preparation of protein isolate[4] extraction of collagen and gelatin.[5] Nowadays, the efficient and economical downstream processes for the partitioning and purification of biomolecules that give high yield and high purity of the product have been demanded by industries.

The powerful and versatile aqueous two-phase system (ATPS) has been employed as an efficient tool in several biotechnology processes for the partitioning of biomolecules like proteins, enzymes, nucleic acids, animal, plant and microbial cells.[6] ATPS forms readily upon mixing aqueous solution of two hydrophobic polymers, or of a polymer and salt, above a certain threshold concentration.[6] Proteins are partitioned between the two phases with a partition coefficient that can be modified by changing the experimental conditions of the medium such as pH, salts and ionic strength, among others.[7] The basis of separation is the selective distribution of a given biomolecule between the phases, depending on the characteristics of the phase system, properties of the biomolecule and the interaction between them.[6] ATPS has several advantages such as low processing time and energy consumption, high capacity and yield, biocompatibility, easy to scale up and non-toxic.[6] ATPS has

been successfully used for partitioning and recovery of various proteinases such as trypsin[8], tuna spleen proteinase[2], tuna stomach protease[9], bromelain[10] and protease from *Caltropis procera* latex.[11] Therefore, the objective of this study was to determine the optimal conditions for partitioning and separating proteinase from albacore tuna liver by ATPS. The other aims of this were to apply the fractionated enzyme for starry triggerfish muscle protein hydrolysis.

MATERIALS AND METHODS

Chemicals

Polyethylene glycol (PEG) 1000 and 4000 were obtained from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). Sodium caseinate, β-mercaptoethanol (βME), L-tyrosine, high-molecular-weight markers, low-molecular-weight markers and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA). Trichloroacetic acid, tris (hydroxymethyl) aminomethane and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250 and *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). The salts and other chemicals with the analytical grade were procured from Merck (Darmstadt, Germany).

Preparation of crude proteinase extract

Internal organs from albacore tuna (*Thunnus alalunga*) were obtained from Tropical Canning (Thailand) Public Co. Ltd., Hat Yai, Songkhla. Pooled internal organs were then excised and separated into individual organs. Only the liver was collected, immediately frozen and stored at -20°C until used. Frozen livers were

thawed using running water (26-28°C) until the core temperature reached -2 to 0°C. The samples were cut into pieces with a thickness of 1-1.5 cm and homogenized into powder in three volumes of acetone at -20°C for 30 min according to the method of Klomklao et al.[12]

To prepare the liver extract, the liver powder was suspended in 50 mM Na-phosphate buffer, pH 7.0 containing 0.2% Brij 35 at a ratio of 1:9 (w/v) and stirred continuously at 4°C for 30 min. The suspension was centrifuged for 30 min at 4°C at 5,000× g to remove the tissue debris. The supernatant was collected and referred to as "liver extract".

Preparation of aqueous two-phase system

ATPS was prepared in 10-ml centrifuge tubes by adding the different amounts of PEG and salts together with liver extract according to the method of Klomklao et al.[2]

Effect of salts on proteinases partitioning: To study the effect of salts on partitioning of the proteinases from liver extract using ATPS, different salts including NaH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$, K_2HPO_4, $Na_3C_6H_5O_7$ and Na_2SO_4 at different concentrations (15, 20 and 25%, w/w) were mixed with 20% PEG1000 in aqueous system. Distilled water was used to adjust the system to obtain the final weight of 5 g. The mixtures were mixed continuously for 3 min using a Vortex mixer (Vortexgenic 2, G-560E, USA). Phase separation was achieved by centrifugation for 5 min at 5,000× g. Top phase was carefully separated using a pasteur pipette and the interface of each tube was discarded. Volumes of the separated phases were measured. Aliquots from each phase were taken for enzyme assay and protein determination.

The specific activity of stomach proteinase in the aqueous two-phase system was defined as

$$SA = \frac{\text{proteinase activity}}{\text{protein concentration}} ; \text{(Unit/mg protein)}$$

The purification factor as

$$PF = \frac{SA_e}{SA_i}$$

where SA_e is the SA of each phase and SA_i is the initial SA of crude extract.

The partition coefficient of protein concentration was defined as:

$$K_P = \frac{C_T}{C_B}$$

where C_T and C_B are concentrations of protein in top and bottom phase, respectively.

The partition coefficient of proteinase activity was defined as:

$$K_E = \frac{A_T}{A_B}$$

where A_T and A_B are proteinase activity in top and bottom phase, respectively.

The volume ratio as:

$$V_R = \frac{V_T}{V_B}$$

where V_T and V_B are top and bottom phase volume, respectively and the protease activity recovery yield was defined as:

$$\text{Yield (\%)} = \frac{A_T \times 100}{A_i}$$

where A_T is total proteinase activity in top phase and A_i is the initial proteinase activity of crude extract. Based on purity and recovery yield, the appropriate salt in ATPS rendering the most effective partitioning was selected for further study.

Effect of molecular weight and concentration of PEG on proteinases partitioning: To study the effect of the concentrations (10%, 15%, 20%, 25% and 30%, w/w) of PEG1000 and PEG4000 on partitioning of proteinase in tuna liver extract, NaH_2PO_4 at a level of 20% was used in the system.[2] Partitioning was performed as previously described. All experiments were run in duplicate. The ATPS rendering the most effective partitioning was chosen. Based on purity and recovery yield, the ATPS rendering the most effective partitioning was chosen for further study.

Effect of pH on the proteinases partitioning: ATPS containing PEG1000 (25%, w/w) and NaH_2PO_4 (20%, w/w) was used for study on the effect of pH on liver extract proteinase partitioning. The original pH of the system was measured and then adjusted to 3.0, 5.0, 7.0, 9.0 and 11.0 with 1 M HCl or 1 M NaOH. Partitioning was performed as previously deseribed. The system pH showing the highest purity and recovery yield was selected for further study.

Effect of NaCl on the proteinases partitioning: The phase system containing 25% PEG1000 and 20% NaH_2PO_4 at pH 7.0 was chosen for study on the effect of NaCl on proteinase partitioning. Adjustment of salt content in the system was made by addition of NaCl (solid form) into the system to obtain concentrations of 0%, 2%, 4%, and 6% (w/w). Partitioning was performed as previously deseribed. The ATPS

rendering the most effective partitioning was chosen. Phase with high specific activity was dialyzed against 10 volumes of 50 mM Tris-HCl (pH 7.5) for 18 h with three changes of buffer in the first 3 h and five changes in the last 15 h. ATPS fraction with highest purity and yield was used for hydrolysis study.

Enzyme assay and protein determination: Proteinase activity of liver extract was measured using casein-TCA-Lowry assay.[13, 14] To initiate the reaction, 200 µL of liver extract was added into assay mixtures containing 200 µL of 2% (w/v) casein, 200 µL of distilled water and 625 µL of assay buffer (0.1 M glycine-NaOH, pH 8.5). The mixture was incubated at 55°C for precisely 15 min. The enzymatic reaction was terminated by adding 200 µL of 50% (w/v) trichloroacetic acid (TCA). The reaction mixture was centrifuged at 7,500×g for 10 min at room temperature. The oligopeptide content in the supernatant was determined by the Lowry assay[14] using tyrosine as a standard. One unit of activity was defined as that releasing 1 mmol of tyrosine per min (mmol Tyr/ min). A blank was run in the same manner, except the enzyme was added after 50% TCA (w/v) addition. Protein concentration was measured by the method of Bradford[15] using bovine serum albumin as a standard.

Characterization of recovered proteinase

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and activity Staining: SDS-PAGE was performed according to the method of Laemmli.[16] Protein solutions were mixed at 1:1 (v/v) ratio with the SDS-PAGE sample buffer (0.5 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol and 10% βME) and boiled for 3 min. The samples (20 µg) were loaded on the gel made of 4% stacking and 12% separating gels and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-

Protean II cell apparatus (Bio-Rad, Hercules, CA, USA). After electrophoresis, the gels were stained with 0.2% Coomassie Brilliant Blue R-250 in 45% methanol and 10% acetic acid and destained with 30% methanol and 10% acetic acid.

Liver extract and selected phase with high SA and yield obtained from ATPS were separated on SDS-PAGE, followed by activity staining according to the method of Klomklao et al.[2] The samples were mixed with sample buffer (0.5 M Tris-HCl, pH 6.8 containing 4% SDS, 20% (v/v) glycerol and 10% βME) at a ratio of 1:1 (v/v). Two µg of proteins were loaded into the gel made of 4% stacking and 12% separating gels. The proteins were subjected to electrophoresis at a constant current of 15 mA per gel by a Mini-Protean II Cell apparatus (Bio-Rad, Hercules, CA, USA). After electrophoresis, gels were immersed in 100 mL of 2% (w/v) casein in 50 mM Tris-HCl buffer, pH 7.5 for 1 h with constant agitation at 0ºC to allow the substrate to penetrate into the gels. The gels were then transferred to 2% (w/v) casein in 0.1 M glycine-NaOH, pH. 8.5 and incubated at 55ºC for 15 min with constant agitation to develop the activity zone. The gels were fixed and stained with 0.125% Coomassie blue R-250 in 45% ethanol and 10% acetic acid and destained in 30% methanol and 10% acetic acid. Development of clear zones on blue background indicated proteolytic activity.

Hydrolysis of starry triggerfish muscle by fractionated proteinase: Starry triggerfish (*Abalistes stellaris*) with the length of 10 cm were purchased from the dock in Trang, Thailand. The fish, off-loaded approximately 18-24 h after capture, were placed on ice at a fish/ ice ratio of 1:2 (w/w) and transported to the Department of Food Science and Technology, Thaksin University, Phatthalung, within 2 h. Upon arrival, the fish were filleted and the ordinary muscle was collected and ground to

uniformity. A portion of mince (500 g) was placed in a polyethylene bag and stored at -20°C until used.

Fractionated proteinase (0, 25, 50, 75 and 100 unit) was added to the reaction mixture containing 3 g starry triggerfish mince and 6 ml of 0.1 M glycine-NaOH, pH 8.5. The hydrolysis was conducted by incubating the mixture at 55°C for 0, 15, 30, 60 and 120 min. The control was performed by incubating the reaction mixture at 55°C for 120 min without the addition of fractionated enzyme. At hydrolysis time designated, 1 ml of sample was taken and mixed with 1 ml of 1% SDS solution (85°C) before placing in a water bath at 85°C for 15 min to inactivate proteinase. The degree of hydrolysis of protein hydrolysate was analyzed according to the method of Benjakul and Morrissey.[17]

To monitor the protein pattern, another lot of sample was added with hot 5% SDS solution (85°C) to terminate the reaction and solubilize total protein. All samples was subjected to SDS-PAGE.[16]

The degree of hydrolysis (DH) of protein hydrolysate was analyzed according to the method of Benjakul and Morrissey.[17] The samples (125 µl) were mixed throughly with 2.0 mL of 0.2125 M phosphate buffer, pH 8.2 and 1.0 mL of 0.01% TNBS solution. The mixtures were then placed in a 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 sulfite. The mixtures were cooled at ambient temperature for 15 min. The absorbance was measured at 420 nm and α-amino acid content was calculated and 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 α-amino acid released at time t. L_0 is the amount of α-amino acid in original starry triggerfish muscle. L_{max} is the total amount of α-amino acid in

original starry triggerfish muscle obtained after acid hydrolysis with 6 N HCl at 100°C for 24h.

Statistical analysis

A completely randomized design was used throughout this study. Data were subjected to analysis of variance (ANOVA), and mean comparison was carried out using Duncan's Multiple Range Test.[18] Statistical analysis was performed using the Statistical Pacleage for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Use of ATPS for partitioning of proteinase

Effect of salts on the proteinase partitioning in ATPS: Table 1 shows the effects of type and concentration of salts on the partitioning and recovery of proteinase from liver of albacore tuna. The proteinase partitioning was assayed in several biphasis systems of 20% PEG1000 containing different salts, including NaH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$, K_2HPO_4, $Na_3C_6H_5O_7$ and Na_2SO_4 at various concentrations. After phase separation, two phases were obtained. The upper phase becomes PEG-rich and the lower phase becomes salt-rich. However, no phase separation was observed in the system containing 20% PEG1000-15% NaH_2PO_4. Raghavarao et al.[19] 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). The addition of salts to the aqueous PEG solution led to an arrangement of ordered water molecules around PEG molecule due to their water structure breaking effect.[12] For all ATPS studied, the proteinase was partitioned predominantly in the PEG-rich top phase, principally those with hydrophobic characteristics.[20] In general, negatively charged proteins

prefer the upper phase in PEG-salt systems, while positively charged proteins normally partition selectively to the bottom phase.[20] Hence, liver proteinase partitioned in the top phase might be negatively charged. SA, PF and %yield of proteinase obtained from PEG1000-salt systems depended on types of salt used. System of composition 20% PEG1000 and 20% NaH_2PO_4 showed the highest SA (8.56 units/µg protein), PF (2.31-fold) and yield (68.75%) indicating that this system has the best capacity of separating proteinase from liver of albacore tuna. Therefore, the system containing 20% NaH_2PO_4 was selected for further study on the effect of PEG concentration on proteinase partitioning and recovery. Senphan and Benjakul[21] reported that ATPS comprising PEG1000 (15% w/w) and $MgSO_4$ (25% w/w) provided the best condition for the maximal partitioning of proteases from hepatopancreas of Pacific white shrimp into the top phase and gave the highest PF (8.6-fold) and yield (65.5%). Protease from *Calotropis procera* latex was separated in the top PEG-rich phase in ATPS composed of 12% PEG4000 and 17% $MgSO_4$.[22] Ketnawa et al.[23] found that the best ATPS condition for protease partitioning from viscera extract of Giant catfish (*Pangasianodon gigas*) was 15% PEG2000-15% $Na_3C_6H_5O_7$ with 1% (w/w) NaCl, which increased the purity by 3.33-fold and recovery yield (64.18%).

The distribution of the proteins in ATPS is characterized by partition coefficient *K*. *K* values for proteinase and protein partitioning are reported as K_E and K_P, respectively. From the results, the lowest K_P (0.53) and the highest K_E (7.56) were found in the system of 20% PEG1000-25% NaH_2PO_4. Generally, the lowest K_P indicates a shift of contaminant proteins, nucleic acid and other undersirable components to the lower phase. For K_E, high K_E value indicates that only proteinase from crude extract was partitioned more to the top phase. Hence, the extraction

conditions employed, resulted in the enrichment of specific proteinase activity, which was due to the differential partitioning of the desired proteinase and contaminating enzymes and proteins to the opposite phases. Johansson[24] reported that the partition of a protein is influenced by the presence of salts. This effect increases with the net charge of protein. There has been found that the efficiency of the salts in promoting phase separation reflects the lyotropic series (a classification of ions based upon salting-out or salting-in ability).[22] Their effectiveness is mainly determined by the nature of the anion. Multi-charged anions being the most effective are ordered of SO_4^{2-} > HPO_4^{2-} > acetate > Cl^-, whereas the order of cations is usually given as $(NH_4)^+$ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+} [22].

Table 1 also showed that increasing salt concentration resulted in less activity recovery. Loss in activity might be due to the denaturation of proteinases causes by "salting out" effect.[25] Isable and Otero[26] found that the presence of high concentrations of salt in the reaction medium greatly decreased both the yield and the selectivity towards the trisaccharide from lactose. Pan and Li[27] also reported that increasing NaH_2PO_4 concentration resulted in less activity recovery as well as poorer specific activity. Therefore, the type of salt and concentration used were critical for albacore tuna liver proteinase recovery or partitioning in ATPS.

TABLE 1 Effect of phase composition in PEG1000-salt ATPS on partitioning of liver proteinase from albacore tuna.

Phase composition (%, w/w)	V_R	K_P	K_E	SA	PF	Yield
20% PEG1000-15% NaH_2PO_4	-	-	-	-	-	-
20% PEG1000-20% NaH_2PO_4	1.83	0.53	7.01	8.56	2.31	68.75
20% PEG1000-25% NaH_2PO_4	0.83	1.14	7.56	3.00	0.81	41.84
20% PEG1000-15% $(NH_4)_2SO_4$	1.07	1.02	5.70	3.72	1.00	46.49
20% PEG1000-20% $(NH_4)_2SO_4$	0.90	1.00	3.47	3.03	0.82	37.74
20% PEG1000-25% $(NH_4)_2SO_4$	0.64	1.08	4.97	3.12	0.84	39.83
20% PEG1000-15% $MgSO_4$	2.45	0.65	0.37	3.61	0.98	41.32
20% PEG1000-20% $MgSO_4$	1.36	0.79	0.44	2.67	0.72	31.65
20% PEG1000-25% $MgSO_4$	0.92	0.90	0.53	1.39	0.37	16.50
20% PEG1000-15% K_2HPO_4	0.97	1.09	1.98	4.10	1.11	64.12
20% PEG1000-20% K_2HPO_4	0.57	1.04	2.10	3.23	0.87	52.30
20% PEG1000-25% K_2HPO_4	0.67	1.08	4.25	3.09	0.83	50.97
20% PEG1000-15% $Na_3C_6H_5O_7$	1.09	0.75	3.27	3.68	0.99	48.21
20% PEG1000-20% $Na_3C_6H_5O_7$	0.86	0.96	3.34	2.49	0.67	38.52
20% PEG1000-25% $Na_3C_6H_5O_7$	0.72	0.86	1.13	1.62	0.44	22.53
20% PEG1000-15% Na_2SO_4	1.08	1.47	1.08	3.74	1.01	47.85
20% PEG1000-20% Na_2SO_4	0.85	1.31	0.81	2.41	0.65	35.87
20% PEG1000-25% Na_2SO_4	0.67	1.42	0.87	1.74	0.47	30.10

(-) No phase separation.

V_R: volume ratio (upper/lower); K_P: partition coefficient of protein; K_E: partition coefficient of proteinases; SA: specific activity (U/(g protein) in the upper phase; PF: purification factor in the upper phase; Yield: recovery yield in the upper phase.

Effect of PEG molecular weight on the proteinase partitioning in ATPS: Proteinase partitioning using ATPS with varying concentration of PEG and 20% NaH_2PO_4 is depicted in Table 2. No phase separation was observed in the 10% PEG1000 with 20% NaH_2PO_4. The V_R of the system ranged from 0.53-1.51. An increase in the PEG molecular mass reduces free volume by increasing the chain length of the PEG polymer, resulting in partitioning of the biomolecules to the bottom phase. The increase in polymer weight causes the reduction of free volume of the top phase, so the partition of biomolecules in the salt-rich bottom phase decreases the partitioning coefficient.[7] With PEG1000 and 4000, all proteinases partitioned into the top phase (K_E >1). However use of the higher molecular weight PEG gave a higher K_E, compared with the lower molecular weight. Thus, K_E values depended on the PEG molecular weight. Tubio et al.[6] suggested that for ATPS formed by PEG of low molecular weight (600-3350 kDa), the protein transfer to the top phase is enthalpically driven mainly due to a strong interaction between PEG and the protein. PEG of the highest molecular weight (PEG8000) exclude the protein from the top phase driven by an entropically unfavorable term.[7] For K_P value, when PEG with higher molecular weight was used, the K_P increased. The lowest K_P (0.44) was observed in ATPS composed of 25% PEG1000 and 20% NaH_2PO_4. High in K_P values indicating most of proteins were more partitioning to the top phase, while the high in K_E implying only the target enzyme was partitioned to the top phase.[22] The highest SA (17.03 units/mg protein) and PF (4.60-fold) of proteinase was obtained in 25% PEG1000 and 20% NaH_2PO_4 systems. Therefore, PEG1000 was a suitable polymer for partitioning of proteinase in albacore tuna liver as indicated by the higher SA and PF than PEG with higher molecular weight. This was possibly due to the fact that interfacial tension is lower when molecular weight of PEG is lower.[25] A preferential

interaction between PEG molecule and protein domain decreased when the molecular weight of PEG increased because of its exclusion from the protein domain.[7] Moreover, the surface charge of biological materials is one of the most significant factors affecting the separation by using partitioning. Molecular weight, shape and specific binding sites of biological materials also affect the partition profiles. Electrical interaction and repulsion between charged aqueous phase systems and the proteins affect the partitioning of system.[2] Among all ATPS tested, system comprising 25% PEG1000 and 20% NaH_2PO_4 partitioned the proteinase to the top PEG-rich phase and undesired protein to the bottom salt phase most effectively.

Effect of pH on the proteinase partitioning in ATPS: The influence of pH on the partitioning of proteinase from liver of albacore tuna was investigated using the ATPS composition of 25% PEG1000-20% NaH_2PO_4 which provided the highest proteinase recovery. The pH of the ATPS was adjusted to 3.0, 5.0, 7.0, 9.0 and 11.0 in comparison to the control system (without pH adjustment). In general, the pH had influenced on protein partitioning, either by changing the charge of the solute or by altering the ratio of the charged species present. Negatively charged proteins prefer the upper PEG-rich phase and positively charged proteins partition to the lower salt phase. Therefore, as the pH increases above the isoelectric point (pI) of a protein, it becomes negatively charged, its interaction with PEG becomes stronger and the partition coefficient increases.[22] A higher PF was found when the system pH increased up to 7.0 (Fig. 1). The highest PF (5.58-fold) and recovery yield (89.99%) were obtained in the system pH of 7.0. However, the decrease in the recovery and PF was observed when the pH of system of 9 and 11 was used. Most of the biomolecules, especially proteins and enzymes, are stable at neutral pH that is favorable condition to

conduct the ATPS partitioning. Enzyme stability slightly reduced in the acidic area, but it was dramatically lost at pH above 9.0.[22]

Effect of NaCl on the proteinase partitioning in ATPS: Partitioning of proteinase from liver of albacore tuna in the presence of NaCl was also studied in the system of 25% PEG1000-20% NaH_2PO_4 at pH 7.0 (Fig. 2). In general, addition of NaCl to the ATPS results in an increase in the hydrophobic difference due to generation of an electrical potential difference between two phases. The result showed that purification factor (PF) and yield decreased with increasing NaCl concentration (Fig. 2). The PF of proteinase decreased from 5.25 (no NaCl addition) to 1.76 (6%, w/w). Higher concentration of NaCl showed a significantly negative effect on partitioning and yield of the enzyme, probably due to protein denaturation and precipitation at high concentration of this salt.[28] These results were in agreement with Ketnawa et al.[23] who studied the PF of alkaline protease from fish viscera in 15% PEG2000-15% $Na_3C_6H_5O_7$ ATPS, using NaCl at different concentrations, ranging from 1% (w/w) to 7% (w/w). The PF of alkaline protease were significantly decreased from 15.34 at 1% (w/w) NaCl to 10.67 at 7% (w/w) NaCl.

TABLE 2 Effect of PEG molecular mass and concentration in a PEG-NaH_2PO_4 ATPS on partitioning of liver proteinase from albacore tuna.

Phase composition (%, w/w)	V_R	K_P	K_E	SA	PF	Yield
10% PEG1000-20% NaH_2PO_4	-	-	-	-	-	-
15% PEG1000-20% NaH_2PO_4	1.04	0.87	1.97	7.45	2.01	70.88
20% PEG1000-20% NaH_2PO_4	1.05	0.77	3.93	9.34	2.52	66.62
25% PEG1000-20% NaH_2PO_4	1.18	0.44	8.88	17.03	4.60	73.70
30% PEG1000-20% NaH_2PO_4	1.51	0.45	2.27	12.21	3.30	53.28
10% PEG4000-20% NaH_2PO_4	0.53	0.73	1.05	4.32	1.17	16.52
15% PEG4000-20% NaH_2PO_4	0.77	0.86	1.14	6.75	1.82	28.30
20% PEG4000-20% NaH_2PO_4	0.87	0.98	1.64	4.91	1.33	21.15
25% PEG4000-20% NaH_2PO_4	1.25	1.34	1.66	5.07	1.37	25.95
30% PEG4000-20% NaH_2PO_4	1.40	1.67	1.01	3.30	0.46	20.13

(-) No phase separation.

V_R: volume ratio (upper/lower); K_P: partition coefficient of protein; K_E: partition coefficient of proteinases; SA: specific activity (U/(g protein) in the upper phase; PF: purification factor in the upper phase; Yield: recovery yield in the upper phase.

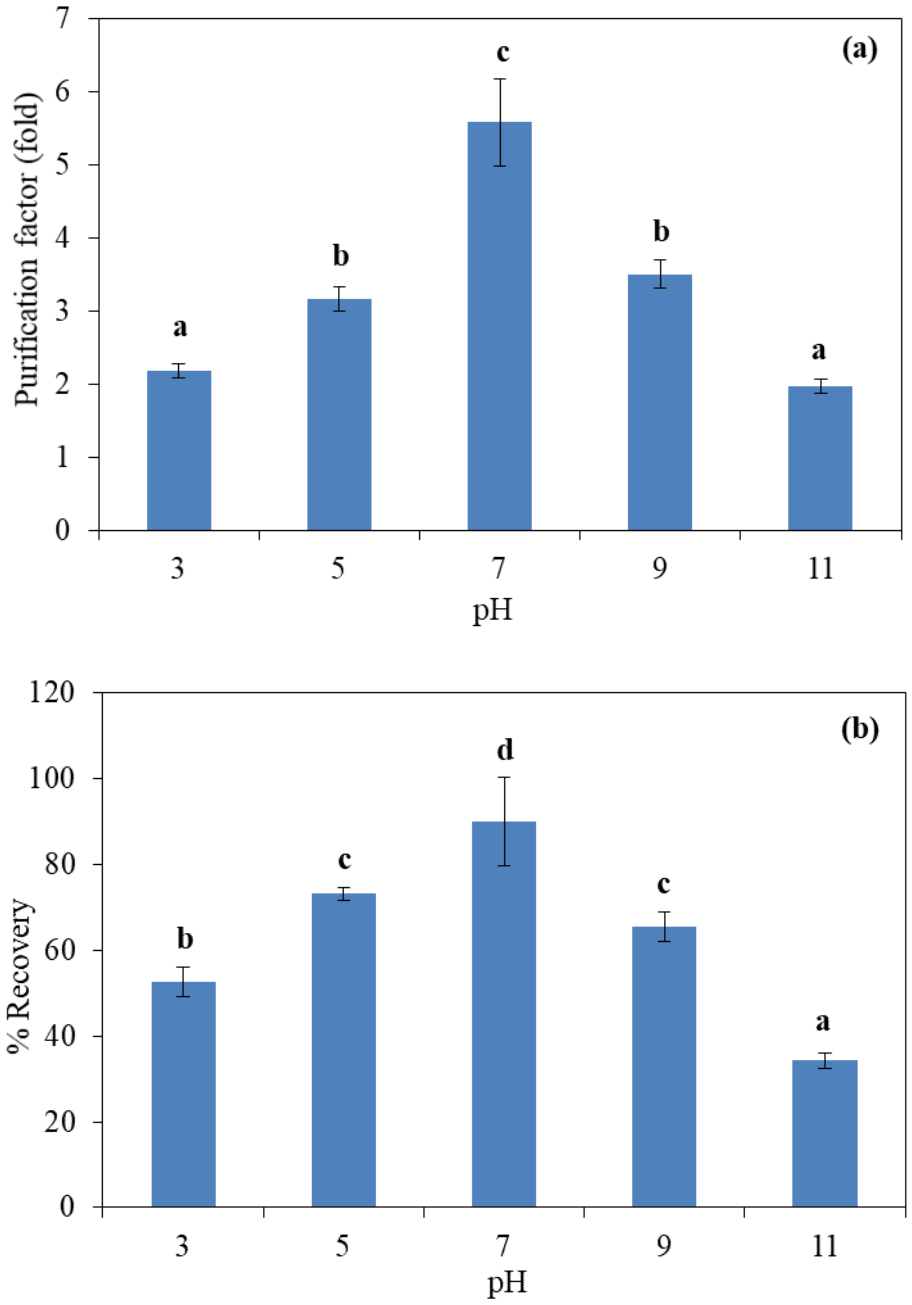

FIGURE 1 Effect of system pH on the purification factor (a) and proteinase recovery (b) of liver proteinase partitioning in 25% PEG1000-20% NaH_2PO_4 ATPS. Bars represented the standard deviation from triplicate determinations. Different letters within the same parameter indicate the significant differences (P<0.05).

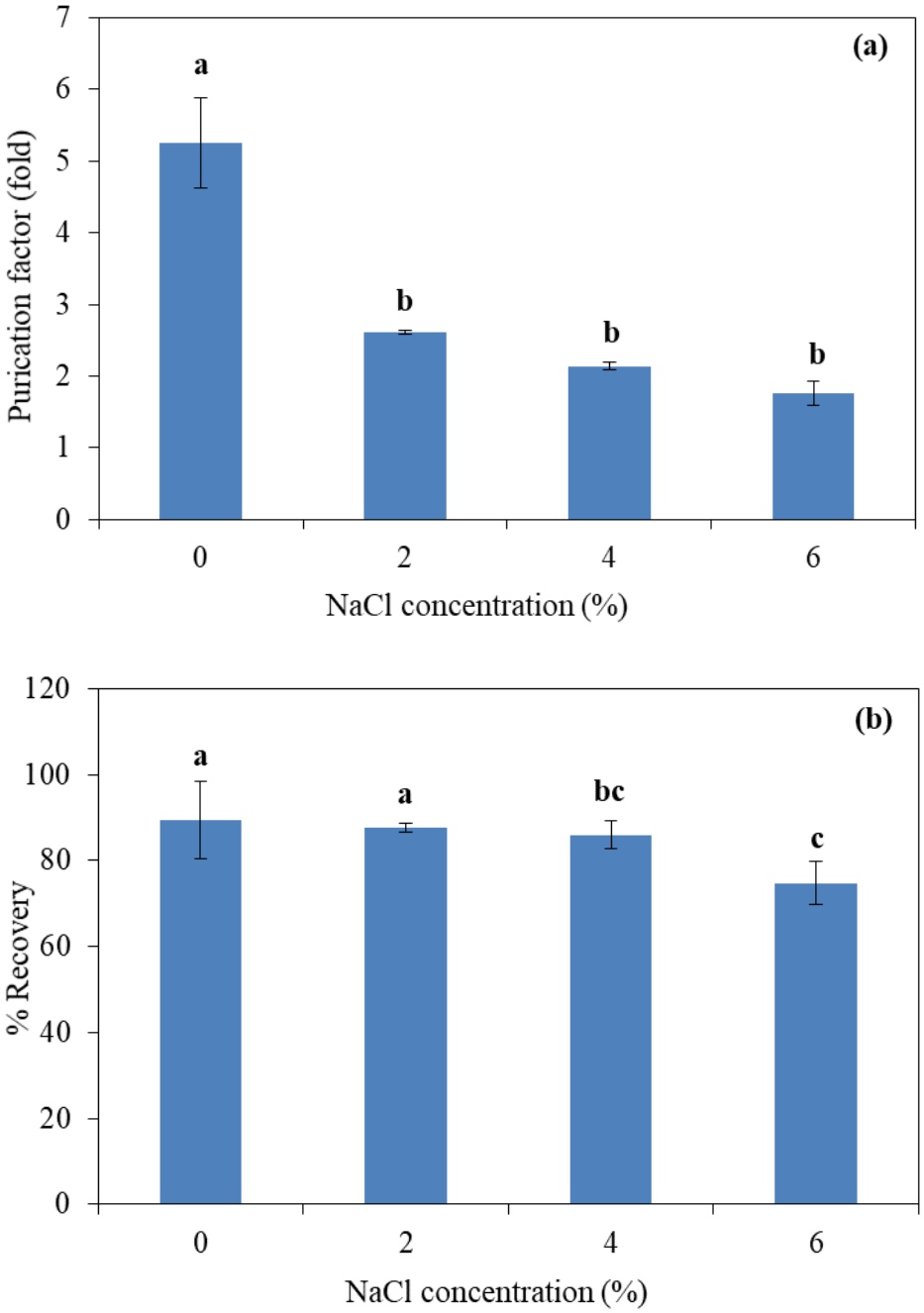

FIGURE 2 Effect of NaCl concentration on the purification factor (a) and proteinase recovery (b) of liver proteinase partitioning in 25% PEG1000-20% NaH_2PO_4 ATPS at pH 7.0. Bars represented the standard deviation from triplicate determinations. Different letters within the same parameter indicate the significant differences (P<0.05).

Characterization of recovered proteinase

Protein pattern and activity staining of proteinase from albacore tuna liver partitioned with ATPS: SDS-PAGE pattern of proteinase obtained from the partial purification using the ATPS process is shown in Fig. 3. Crude liver extract contained a variety of proteins of different molecular weight. However, a large number of contaminating proteins were removed after partitioning with ATPS, particularly proteins with higher or lower molecular weight. As a result, a higher purity of interested proteinase was obtained. When the proteins or enzymes to be separated differ significantly in their structural properties from others, partitioning can be carried out successfully.[29]

Activity staining of proteinase in liver extract and various fractions obtained from ATPS process were analyzed on SDS-substrate gel (Fig. 4). The presence of the clear zone suggested that it is the proteinase that can be hydrolyzed casein in the gel. The band intensity with apparent MW of 21, 24, 30 and 34 kDa slightly increased after the ATPS process, suggesting the higher specific activity of proteinase loaded into the gel. Molecular weights of trypsin-like enzymes from pyloric ceca brownstripe red snapper were 20, 24-29, 45 and 97 kDa; bigeye snapper were 17, 20, 22, 45 and 97 kDa; and threadfin bream were 20, 22, 36 and 45 kDa.[30]

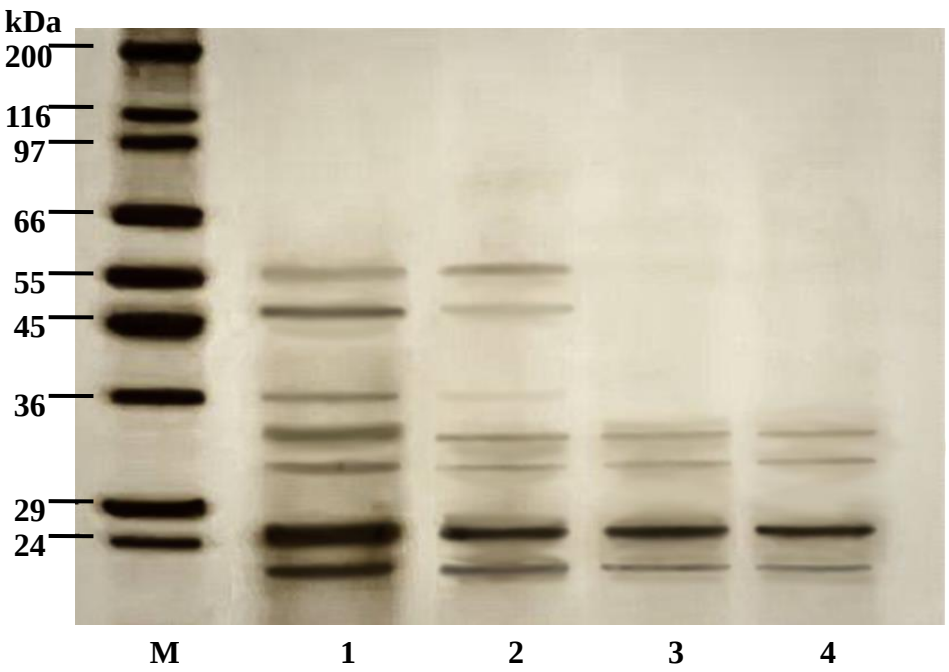

FIGURE 3 SDS-PAGE of liver extract and ATPS fraction from albacore tuna. M, molecular weight standard; lane 1, liver extract; lane 2, 20% PEG1000-20% NaH_2PO_4 ATPS fraction; lane 3, 25% PEG1000-20% NaH_2PO_4 ATPS fraction; lane 4, 25% PEG1000-20% NaH_2PO_4, pH 7.0 ATPS fraction.

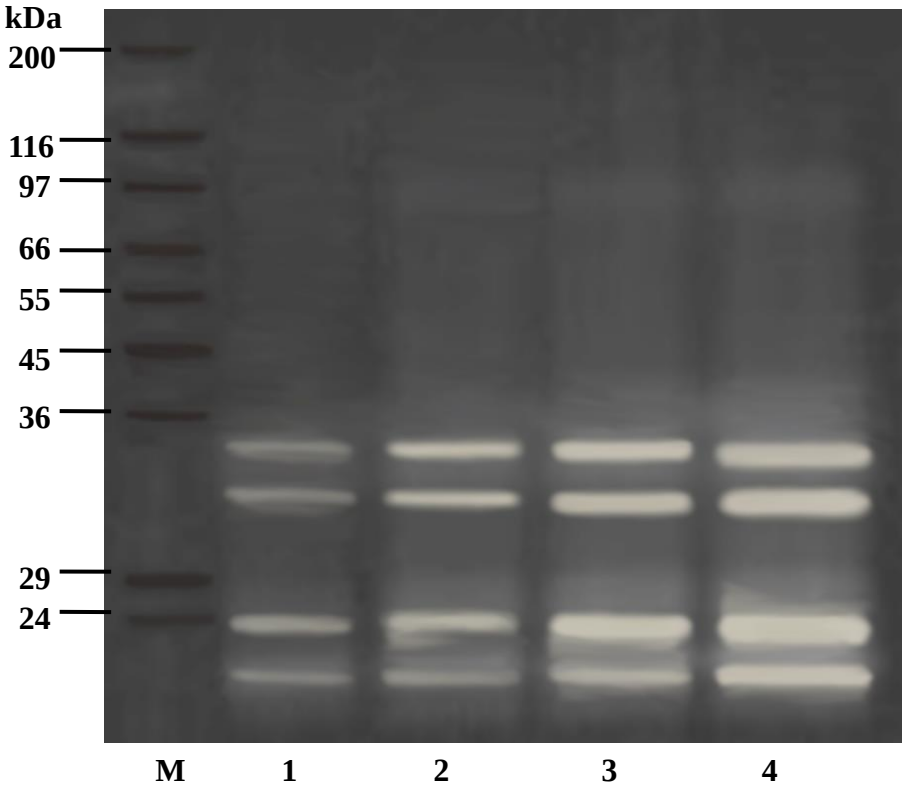

FIGURE 4 Activity staining of liver extract and ATPS fraction from albacore tuna. M, molecular weight standard; lane 1, liver extract; lane 2, 20% PEG1000-20% NaH_2PO_4 ATPS fraction; lane 3, 25% PEG1000-20% NaH_2PO_4 ATPS fraction; lane 4, 25% PEG1000-20% NaH_2PO_4, pH 7.0 ATPS fraction.

Hydrolysis of starry triggerfish muscle using fractionated proteinases: When starry triggerfish muscle was hydrolyzed using fractionated proteinase (0, 25, 50 75 and 100 unit), a rapid hydrolysis was found within the first 15-30 min, followed by a slower hydrolysis rate up to 120 min (Fig. 5). The fast hydrolysis in the initial phase indicated that a large number of peptide bonds were hydrolyzed.[31] Thereafter, the hydrolysis rate was decreased, mainly due to a decrease in available hydrolysis sites, enzyme autodigestion and/or product inhibition.[30] At the same time of hydrolysis, higher DH was observed with higher activity level of fractionated proteinase used. The results of this study revealed that, the degree of hydrolysis increased with increasing hydrolysis time. Also, the degradation of protein increased by increasing the enzyme concentration. Naveena et al.[31] reported that when using protease from *Cucumis trigonus* in buffalo meat samples, the increase in proteolysis can be correlated with significantly higher protein solubility. Senphan and Benjakul[21] found that proteinases recovered from hepatopancreases using the combined partitioning systems could be used for gelatin hydrolysis.

The proteolytic degradation pattern of starry triggerfish muscle protein analyzed by SDS-PAGE, revealed that among all proteins, myosin heavy chain (MHC) was susceptible to hydrolysis, followed by actin (Fig. 6). The band intensity of MHC decreased with increasing fractionated liver proteinase concentration up to 50 unit. Total disappearance of MHC was observed when enzyme at concentration of 75 and 100 unit was used. For actin, no hydrolytic degradation was observed when starry triggerfish muscle was incubated in the presence of 25 and 50 unit fractionated proteinases from albacore tuna liver. However, the degradation increased as the enzyme concentration increased. Also, the degradation rate was lower than that of MHC. Therefore, the rate of hydrolysis was dependent on the amount of enzyme

added. From the result, it was noted that autolysis of sample (without fractionated proteinase addition) occurred to some extent during incubation at 55°C. From the results, proteinases recovered from liver of albacore tuna using ATPS could be an alternative potential aid for production of fish protein hydrolysate, in which the cost of enzyme could be reduced.

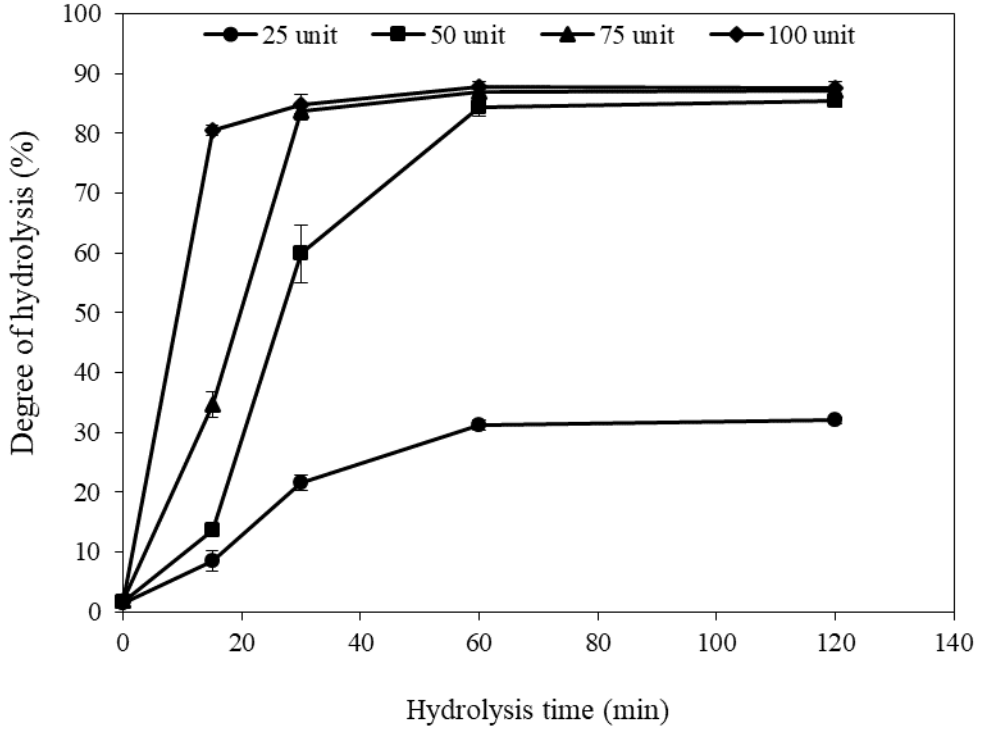

FIGURE 5 Degree of hydrolysis (DH) of starry triggerfish muscle during hydrolysis with liver enzyme from ATPS fraction (top phase of system 25% PEG1000-20% NaH_2PO_4, pH 7.0). The hydrolytic reaction was performed at pH 8.5, 55°C. Bars represented the standard deviation from triplicate determinations.

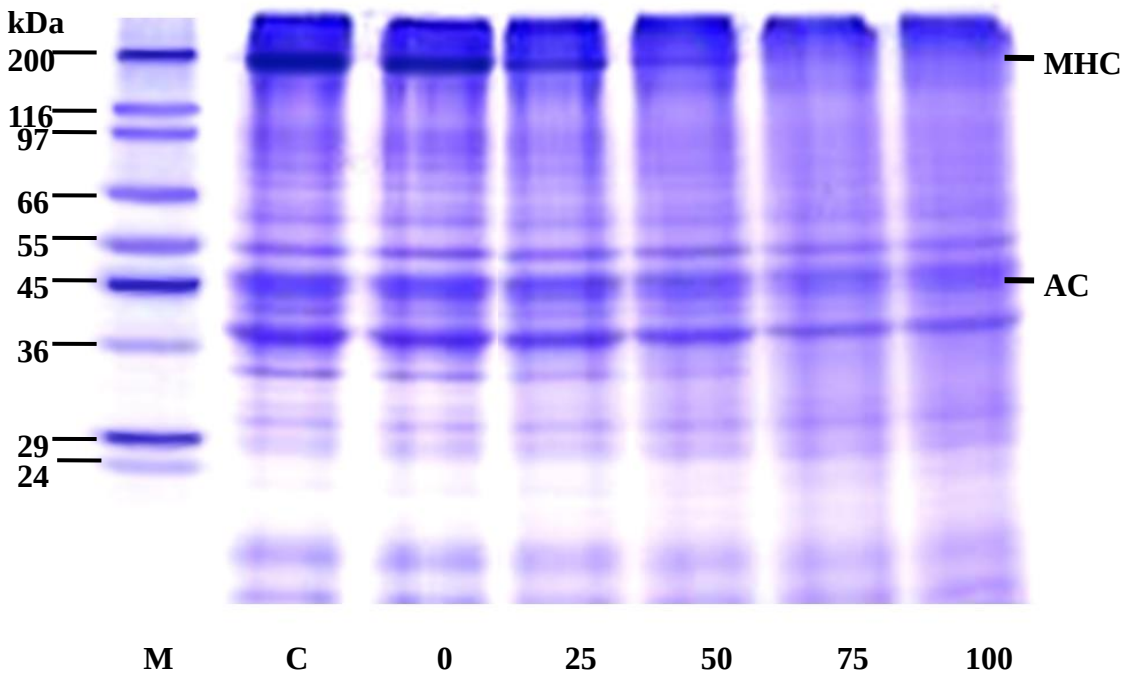

FIGURE 6 SDS-PAGE patterns of starry triggerfish muscle with liver enzyme from ATPS fraction (top phase of system 25% PEG1000-20% NaH_2PO_4, pH 7.0). The hydrolytic reaction was performed at pH 8.5, 55°C. Numbers indicated enzyme activity (unit) the fraction used. MHC: myosin heavy chain; AC: actin.

CONCLUSION

ATPS can be effectively used to recover and purify proteinase from albacore tuna liver. ATPS with 25% PEG1000-20% NaH_2PO_4, pH 7.0 provided the best enzyme recovery and purity. NaCl concentration had no effect on partitioning of the target enzyme. Based on the protein degradation of starry triggerfish muscle, it is suggested that the fractionated proteinase from albacore tuna liver is suitable for protein hydrolysate.

FUNDING

This research was supported by the Thailand Research fund and Thaksin University for Project No. RSA6080044. The TRF distinguished research professor grant was also acknowledged.

REFERENCES

1. Nalinanon, S.; Benjakul, S.; Visessanguan, W.; Kishimura, H. Tuna pepsin: Characteristics and its use for collagen extraction from the skin of threadfin bream (*Nemipterus* spp.). Journal of Food Science **2008**, *73*, 413-419.
2. Klomklao, S.; Benjakul, S.; Visessanguan, W.; Simpson, B.K.; Kishimura, H. Partitioning and recovery of proteinase from tuna spleen by aqueous two-phase systems. Process Biochemistry **2005**, *40*, 3061-3067.
3. El Hadj Ali, N.; Hmidet, N.; Zouari-Fakhfakh, N.; Ben Khaled, H.; Nasri, M. Alkaline chymotrypsin from striped sea bream (*Lithognathus mormyrus*) viscera: Purification and characterization. Journal of Agricultural and Food Chemistry **2010**, *58*, 9787- 9792.

4. Devi, B.G.; Latha, K.P.J.H. Isolation, partial purification and characterization of alkaline
protease from seeds of *Cucumis melo var agrestis*. International Journal of Research in
Engineering and Technology **2014**, *3*, 88-97.
5. Ketnawa, S.; Martinez-Alvarez, O.; Benjakul, S.; Rawdkuen, S. Gelatin hydrolysates from farmed Giant catfish skin using alkaline proteases and its antioxidative function of simulated gastro-intestinaldigestion. Food Chemistry **2016**, *192*, 34-42.
6. Tubio, G.; Nerli, B.; Pico, G. Partitioning features of bovine trypsin and α-chymotrypsin in polyethyleneglycol-sodium citrate aqueous two-phase systems. Journal of Chromatography B **2007**, *852*, 244-249.
7. Goja, A.M.; Yang, H.; Cui, M.; Li, C. Aqueous two-phase extraction advances for bioseparation. *Journal* of *Bioprocessing* and *Biotechniques* **2013**, *4*, 1-8.
8. Oliveira, L.A.; Sarubbo, L.A.; Porto, A.L.F.; Campos-Takaki, G.M.; Tambourgi, E.B. Partition of trypsin in aqueous two-phase systems of poly (ethylene glycol) and cashew-nut tree gum. Process Biochemistry **2002**, *38*, 693-699.
9. Nalinanoon, S.; Benjakul, S.; Visessanguan, W.; Kishimura, H. Partitioning of protease from stomach of albacore tuna (*Thunnus alalunga*) by aqueous two-phase systems. Process Biochemistry **2009**, *44*, 471-476.
10. Ketnava, S.; Rawdkuen, S.; Chaiwat, P. Two phase partitioning and collagen hydrolysis of bromelain from pineapple peel *Nang Lae* cultivar. Biochemical Engineering Journal **2010**, *52*, 205-211.
11. Rawdkuen, S.; Pintathong, P.; Chaiwut, P.; Benjakul, S. The partitioning of protease from *Calotropis procera* latex by aqueous two-phase systems and its

hydrolytic pattern on muscle proteins. Food and Bioproducts Processing **2011**, *89*, 73-80.

12. Klomklao, S.; Kishimura, H.; Yabe, M.; Benjakul, S. Purification and characterization of two pepsins from the stomach of pectoral rattail (*Coryphaenoides pectoralis*). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology **2007**, *147*, 682-689.
13. An, H.; Seymour, T.A.; Wu, J.W.; Morrisey, M.T. Assay systems and characterization of Pacific whiting (*Merluccius productus*) protease. Journal of Food Science **1994**, *59*, 277-289.
14. Lowry, O.H.; Rosebrough, N.J.; Fan, A.L.; Randall, R.J. Protein measurement with Folin phenol reagent. The Journal of Biological Chemistry **1951**, *193*, 256-275.
15. Bradford, M.A. Rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Analytical Biochemistry **1976**, *72*, 248-254.
16. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **1970**, *227*, 680-685.
17. Benjakul, S.; Morrissey, M.T. Protein hydrolysates from Pacific whiting solid wastes. Journal of Agricultural and Food Chemistry **1997**, *45*, 3423-3430.
18. Steel, R.G.D.; Torrie, J.H. *Principles and Procedures of Statistics*; McGraw-Hill, New York, 1980; 481 pp.
19. Raghavarao, K.S.M.S.; Ruinn, M.R.; Todd, P. Recent developments in aqueous two phase extraction in bioprocessing. Separation and Purification Method **1998**, *27*, 1-49.

20. Yang, S.; Huang, Z.; Jiang, Z.; Li, L. Partition and purification of a thermostable xylanase produced by *Paecilomyces thermophila* in solid-state fermentation using aqueous two-phase systems. Process Biochemistry **2008**, *43*, 56-61.
21. Senphan, T.; Benjakul, S. Use of the combined phase partitioning systems for recovery of proteases from hepatopancreas of Pacific white shrimp. Separation and Purification Technology **2014**, *129*, 57-63.
22. Chaiwat, P.; Rawdkuen, S.; Benjakul, S. Extraction of protease from *Calotropis procera* latex by polyethylene glycol-salts biphasic system. Process Biochemistry **2010**, *45*, 1148-1155.
23. Ketnawa, S.; Benjakul, S.; Ling, T.C.; Martinez-Alvarez, O.; Rawdkuen, S. Enhanced recovery of alkaline protease from fish viscera by phase partitioning and its application. Chemistry Central Journal **2013**, *7*, 1-9.
24. Johansson, G. Affinity partitioning of proteins using aqueous two-phase systems. In *Protein Purification: Principles, High-Resolution Methods, and Applications*; Janson, J.C., Ed.; Wiley-VCH: London, 1998; 443-460.
25. Klomklao, S.; Benjakul, S.; Visessanguan, W. Comparative studies on proteolytic activity of spleen extracts from three tuna species commonly used in Thailand. Journal of Food Biochemistry **2004**, *28*, 355-372.
26. Isable, D.V.M.; Otero, C. Biphasic aqueous media containing polyethylene glycol for the enzymatic synthesis of oligosaccharides from lactose. Enzyme and Microbial Technology **2003**, *33*, 118-126.
27. Pan, I.H.; Li, Y.K. Rapid process for purification of an extracellular β-xylosidase by aqueous two-phase extraction. Journal of Chromatography B **2001**, *754*, 179-184.

28. Han, J.H.; Lee, C.H. Effects of salts and poly(ethylene glycol)-palmitate on the partitioning of proteins and *Bacillus subtilis* neutral protease in aqueous two-phase systems. Colloids and Surfaces B: Biointerfaces **1997**, *9*, 109-116.
29. Rosa, P.A.J.; Azevedo, A.M.; Sommerfeld, S.; Mutter, A.; Aires-Barros, M.R. Application of aqueous two-phase systems to antibody purification: A multi stage approach. Journal of Biotechnology **2009**, *139*, 306-313.
30. Khantaphant, S.; Benjakul, S. Comparative study on the proteases from fish pyloric caeca and the use for production of gelatin hydrolysate with antioxidative activity. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology **2008**, *151*, 410-419.
31. Naveena, B.M.; Mendiratta, S.K.; Anjaneyulu, A.S.R. Tenderizaiton of buffalo meat using plant proteases from *Cucumis trigounus* Roxb (Kachri) and Zingiber officinale roscoe (*Ginger rhizome*). Meat Science **2004**, *68*, 363-369.

Chapter 3

Two trypsin isoforms from albacore tuna (*Thunnus alalunga*) liver: Purification and physicochemical and biochemical characterization

International Journal of Biological Macromolecules

Sappasith Klomklao[a,*], Soottawat Benjakul[b]

[a] Department of Food Science and Technology, Faculty of Technology and Community Development, Thaksin University, Phatthalung Campus, Phatthalung, 93210, Thailand

[b] Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand

ABSTRACT

Two trypsins (A and B) from the liver of albacore tuna (*Thunnus alalunga*) were purified to homogeneity using a series of column chromatographies including Sephacryl S-200, Sephadex G-50 and Diethylaminoethyl-cellulose. Purity was increased to 80.35- and 101.23-fold with approximately 3.1 and 19.2% yield for trypsins A and B, respectively. The molecular weights of trypsins A and B were estimated to be 21 and 24 kDa, respectively, by SDS-PAGE and size exclusion chromatography. Both trypsins showed only one band on native-PAGE. Trypsins A and B exhibited the maximal activity at 60 °C and 55 °C, respectively, and had the same optimal pH at 8.5 using N^{α}-*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME) as a substrate. Stabilities of both trypsins were well maintained at a temperature up to 50°C and in the pH range of 7.0 to 11.0 and were highly dependent on the presence of calcium ion. The inhibition test demonstrated strong inhibition by soybean trypsin inhibitor and TLCK. Activity of both trypsins continuously decreased with increasing NaCl concentration (0-30%). The N-terminal amino acid sequence of 20 residues of the two trypsin isoforms had homology when compared to those of other fish trypsins.

Keywords: Trypsin · Tuna · Isolation · Viscera · N-terminal amino acid sequence

1. Introduction

Seafood waste constitutes at present a serious environmental problem; that waste needs appropriate management. Fish viscera constitute approximately 20% of the fish biomass and are a rich source of digestive proteinases. Hence, the proteinase recovery from fishery waste would be of great importance because it would not only alleviate the serious concerns related to the visceral waste management but also would help produce novel low-cost proteinases for industrial application [1].

Trypsin (EC 3.4.21.4) is one of the main digestive proteinases found in fish viscera, especially pyloric ceca and intestine [2]. It is a serine proteinase, which is produced as an inactive precursor. It has a function in the hydrolysis of target proteins at the amino acids arginine and lysine [3]. Trypsin has various industrial applications, especially in food industries, due to its high stability and activity under harsh conditions, such as in the presence of surfactants and oxidative agents [4]. Trypsins have been extracted and characterized thoroughly based on their biochemical properties from several species of fish, e.g. the viscera of striped sebream (*Lithognathus mormyrus*) [5], Goby (*Zosterisessor ophiocephalus*) [6], the intestine of Grey triggerfish (*Balistes capriscus*) [7], the spleen of skipjack tuna (*Katsuwonus pelamis*) [8], yellowfin tuna (*Thunnus albacores*) [9] and the pyloric ceca of Chinook salmon (*Oncorhynchus tshawytscha*) [10]. Recently, Sripokar et al. [11] reported that albacore tuna liver contained high proteolytic activity and the major proteinases were heat-activated alkaline proteinases, most likely trypsin-like serine proteinases. However, the molecular and the biochemical characteristics of trypsin or trypsin-like enzymes in albacore tuna liver still remain unknown. Therefore, in the present study, we attempted to isolate and characterize trypsins from the liver of albacore tuna and obtain basic information about their biochemical and kinetic properties.

2. Materials and methods

2.1 Chemicals

Ethylenediaminetetraacetic acid (EDTA), pepstatin A, soybean trypsin inhibitor, iodoacetic acid, *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, β-mercaptoethanol (βME) and bovine serum albumin were procured from Sigma Chemical Co. (St. Louis, MO, USA.). Sephacryl S-200 and Sephadex G-50 were purchased from Pharmacia Biotech (Uppsala, Sweden). Diethylaminoethyl (DEAE)-cellulose was obtained from Whatman (Maidstone, England). N^{α}-*p*-tosyl-L-arginine methyl ester hydrochloride (TAME) was purchased from Wako Pure Chemicals (Osaka, Japan). Sodium chloride, tris (hydroxymethyl) aminomethane and Folin-Ciocalteu's phenol reagent 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).

2.2 Liver extract preparation

Albacore tuna (*Thunnus alalunga*) internal organs were obtained from Tropical Canning (Thailand) Public Co. Ltd., Hat Yai, Songkhla. Pooled internal organs were then excised and separated into individual organs. Only the liver was collected, immediately frozen and stored at -20°C until used. Frozen livers were thawed using running water (26-28°C) until the core temperature reached -2 to 0°C. The samples were cut into pieces with a thickness of 1-1.5 cm and homogenized into powder in three volumes of acetone at -20°C for 30 min according to the method of Klomklao et al. [8].

To prepare the liver extract, the liver powder was suspended in 50 mM Na-phosphate buffer, pH 7.0 containing 1 mM $CaCl_2$ referred to as starting buffer (SB) at a ratio of 1:9 (w/v) and stirred continuously at 4°C for 30 min. The suspension was centrifuged for 30 min at 4°C at 5,000×g to remove the tissue debris. The supernatant was collected and referred to as "liver extract".

2.3 Trypsin purification from albacore tuna liver

All purification processes were carried out in a walk-in cold room (4°C). Fractions obtained from all purification steps were subjected to the measurement of protein content and trypsin activity.

2.3.1 Sephacryl S-200 column chromatography

Liver extract (15 ml) was chromatographed on Sephacryl S-200 column (3.9×64 cm), which was equilibrated with approximately two bed volumes of SB. Sample was loaded onto column and then eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions of 5 ml were collected and those with TAME activity were pooled, lyopholized and further purified by Sephadex G-50 column.

2.3.2 Sephadex G-50 column chromatography

Lyopholized fractions with TAME activity after Sephacryl S-200 column chromatography were dissolved in distilled water and loaded onto a Sephadex G-50 column (3.9×64 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 3 ml were collected and those with TAME activity were pooled and further purified by anion exchanger DEAE-cellulose chromatography.

2.3.3 DEAE-cellulose chromatography

Pooled fractions with TAME activity from Sephadex G-50 column chromatography were collected and dialyzed against SB for 12 h. After that, the sample was chromatographed on DEAE-cellulose (Whatman, England) column (2.2×18 cm) 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_{280} was less than 0.05 and then eluted with a linear gradient of 0.05-0.45 M NaCl in SB at a flow rate of 0.5 ml/min. Fractions of 5 ml were collected and the fractions with TAME activity were pooled. Two activity peaks (trypsin A and B) were obtained and pooled fractions from each peak were dialyzed with SB for 12 h and then concentrated by lyophilization and used for further study.

2.4 Trypsin activity assay

Trypsin activity was measured by the method of Hummel [12] as modified by Klomklao et al. [8] using TAME as a substrate. Enzyme solution with an appropriate dilution (20 µl) was mixed with 3.0 ml of 1 mM TAME in 10 mM Tris-HCl buffer, pH 8.0 and incubated at 30°C for 20 min. Production of *p*-tosyl-arginine was measured by monitoring the increment in absorbance at 247 nm. One unit of activity was defined as the amount causing an increase of 1.0 in absorbance at 247 nm per min.

2.5 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 30°C for 20 min. For the temperature profile study, the

activity was assayed at different temperatures (20, 30, 40, 50, 55, 60, 65, 70 and 80°C) for 20 min at pH 8.0.

2.6 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. Different buffers were used from the above mentioned experiment. For thermal stability, enzyme solution was diluted 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 (Memmert, Germany). Thereafter, the treated samples were suddenly cooled in iced water. The residual activity was assayed using TAME as a substrate at pH 8.0 and 30°C for 20 min. The effect of $CaCl_2$ on thermal stability was also determined by heating the enzyme dissolved in 50 mM Tris-HCl, pH 8.0 in the presence of 2 mM EDTA or 2 mM $CaCl_2$, at 40°C for different times (0, 0.5, 1, 2, 4, 6 and 8 h). At the time designated, the samples were cooled in iced water and assayed for remaining activity.

2.7 Determination of molecular weight

The molecular weight of purified trypsins was determined using size exclusion chromatography on Sephacryl S-200 column. The trypsin separated on size exclusion chromatography was estimated for its molecular weight by plotting available partition coefficient (K_{av}) against the logarithm of molecular weight of the protein standards. The elution volume (V_e) was measured for each protein standard and the trypsins. Void volume (V_o) was estimated by the elution volume of blue dextran (M_r

2,000,000). The standards used included aprotinin (M_r 6,500), trypsinogen (M_r 24,000), bovine serum albumin (M_r 66,000) and catalase (M_r 232,000).

2.8 Effect of NaCl

Effect of NaCl on trypsin activity was studied. NaCl was added into the standard reaction assay to obtain the final concentrations of 0, 5, 10, 15, 20, 25 and 30% (w/v). The residual activity was determined at 30°C and pH 8.0 for 20 min using TAME as a substrate.

2.9 Effect of inhibitors

The effect of inhibitors on trypsin activity was determined according to the method of Klomklao et al. [13] by incubating enzyme solution with an equal volume of proteinase inhibitor solution to obtain the final concentration designated (0.1 mM E-64, 1 mM N-ethylmaleimide, 1 mM iodoacetic acid, 1.0 g/l soybean trypsin inhibitor, 5 mM TLCK, 5 mM TPCK, 1 mM pepstatin A and 2 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.

2.10 Polyacrylamide gel electrophoresis

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 (0.125M Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 10% β-mercaptoethanol) and boiled for 3 min. The samples (15 µg) were loaded on the gel made of 4% stacking and 12.5% 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 12.5% separating gels in a similar manner, except that the sample was not heated and SDS and reducing agent were left out.

2.11 Determination of N-terminal amino acid sequence

The purified enzymes were subjected to SDS-PAGE under reducing conditions and electrophoretically transferred to polyvinylidenedifluoride (PVDF) membrane. After the membrane was briefly stained by Coomassie brilliant blue, the band of protein was applied to a protein sequencer, Procise 492 (Perkin-Elmer, Foster, CA, USA).

2.12 Kinetic studies

The activity was assayed with different final concentrations of TAME ranging from 0.01 to 0.10 mM. The final enzyme concentration for the assay was 0.1 mg/ml. The determinations were repeated twice and the respective kinetic parameters including V_{max} and K_m were evaluated by plotting the data on a Lineweaver-Burk double-reciprocal graph [14]. Values of turnover number (K_{cat}) were calculated from the following equation: $V_{max}/[E] = K_{cat}$, where [E] is the active enzyme concentration.

2.13 Protein determination

Protein concentration was measured by the method of Lowry et al. [15] using bovine serum albumin as a standard.

3. Results and discussion

3.1 Trypsin purification from albacore tuna liver

Trypsins from the liver of albacore tuna were isolated and purified successively by the three-step procedure described in the materials and methods section. The results of the purification procedure are summarized in Table 1. In the first step, purity of 2.25-fold was obtained with Sephacryl S-200 chromatography. Pooled active fractions obtained from the first step were loaded to Sephadex G-50 column chromatography. After this step, a purification fold of 4.63 with a yield of 45.42% was observed. Subsequently, fractions showing trypsin activity were chromatographed on DEAE-cellulose anion-exchange chromatography column. Two peaks showing trypsin activity were observed after elution with a linear gradient of NaCl (0.05-0.45 M) (Fig. 1). Based on the elution order, these enzymes were assigned as trypsins A and B. Purity was increased to 80.35- and 101.23-fold with a recovery of 3.1 and 19.2% for trypsins A and B, respectively. Klomklao et al. [16] purified two trypsins from the skipjack tuna intestine by using Sephacryl S-200, Sephadex G-50 and DEAE-cellulose, and purification fold of 177 and 257 were obtained. Trypsin from hybrid catfish viscera was purified by ammonium sulphate fractionation and a series of chromatographies with a 47.6-fold increase in specific activity and 12.7% yield [22].

The purity of the enzymes was determined by using native-PAGE. As depicted in Fig. 2a, trypsins A and B migrated as a single protein band and displayed the different mobilities in native-PAGE, indicating the homogeneity of both enzymes.

For SDS-PAGE, each purified trypsin gave a single band and the apparent molecular weights of trypsins A and B were estimated to be 21 and 24 kDa, respectively, corresponding to that measured by gel filtration using Sephacryl S-200

(data not shown). The results confirm that trypsins A and B are monomeric proteins. The molecular weight of both trypsins was similar to those of mammalian and fish trypsins. Generally, fish trypsins were found to have molecular masses in the range of 20 and 28 kDa [16]. Trypsin from zebra blenny viscera had an apparent molecular weight of 27 kDa as estimated by SDS-PAGE and gel filtration [17]. Nasri et al. [6] reported that the molecular weight of an alkaline calcium dependent trypsin from the viscera of Goby was approximately 23.2 kDa using SDS-PAGE and gel filtration. The apparent molecular weight of trypsin A and B from yellowfin tuna spleen was estimated to be 24 kDa by size exclusion chromatography and SDS-PAGE [9].

Table 1

Purification of trypsins from the liver of albacore tuna

Purification steps	Total activity (units)*	Total protein (mg)	Specific activity (units/mg protein)	Purity (fold)	Yield (%)
Crude extract	589.47	897	0.65	1	100
Sephacryl S-200	310.57	213	1.46	2.25	52.67
Sephadex G-50	267.77	89	3.01	4.63	45.42
DEAE-Cellulose					
Trypsin A	18.28	0.35	52.23	80.35	3.10
Trypsin B	113.18	1.72	65.80	101.23	19.20

*Trypsin activity was assayed at pH 8.0, 30°C for 20 min using TAME as a substrate.

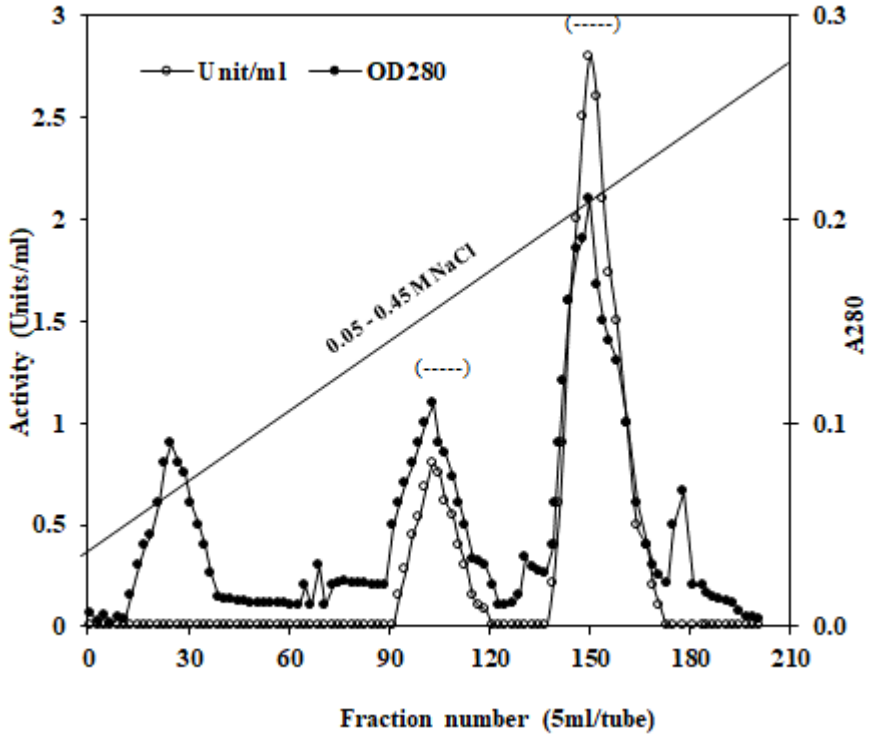

Fig.1. Elution profile of trypsins from albacore tuna liver on the DEAE-cellulose column. Elution was carried out with a linear gradient of 0.05-0.45 M NaCl in SB.

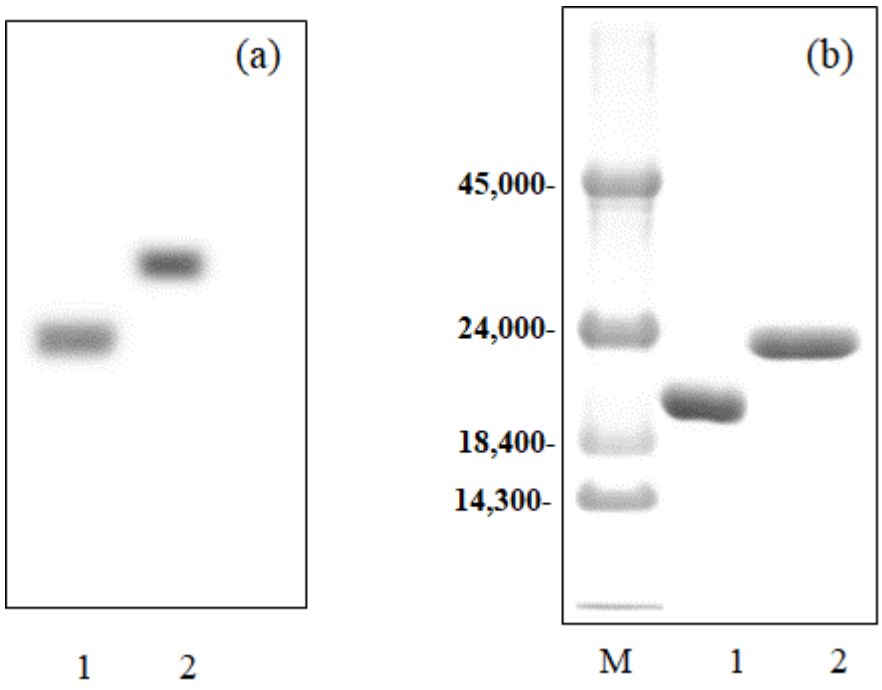

Fig. 2. Protein pattern from native-PAGE (a) and SDS-PAGE (b) of purified trypsins A and B from albacore tuna liver. M, molecular weight standard; lane 1, trypsin A; lane 2, trypsin B.

3.2 Optimal pH and temperature

The pH curves of trypsins A and B from albacore tuna liver are illustrated in Fig. 3a. Both trypsins were active between pH 7.0 and 9.0 and showed the maximal activity toward TAME at pH 8.5. Loss of activity was found at very acidic and alkaline pHs. No activity was observed at pH 11.0. The sharp decrease in TAME hydrolysis by both trypsins at low and high pH might be attributed to denaturation [9].

Temperature activity profiles of trypsins A and B are shown in Fig. 3b. Trypsins A and B had optimal temperatures of 60 and 55°C, respectively. At temperatures above 70°C, an appreciable decrease in activity of both trypsins was observed, presumably due to thermal inactivation. Inactivation of the enzymes at high temperature was possibly due to unfolding of the molecules. Trypsin from zebra blenny viscera had a temperature optimum of 60°C [17]. Klomklao et al. [9] reported that trypsin A and B from the spleen of yellowfin tuna had the maximal activity at 55 and 60°C, respectively. The optimum temperature for trypsin activity from golden grey mullet viscera was 50°C [18]. Nevertheless, these optimal temperatures were higher than those reported for trypsin from Grey triggerfish [7], vermiculated sailfin catfish [19] and carp [20], which had the optimum temperatures in the range of 30-40°C. The difference might be related to the different temperatures of water where the fish inhabited.

3.3 pH and thermal stability

Fig. 4a shows the pH stability profile of purified trypsins A and B from albacore tuna liver. Both trypsins were highly stable over a wide pH range, maintaining 100% of their original activities between pH 7.0-11.0. Nevertheless, some losses in trypsin activities were found at slightly acidic pH for both trypsins. No

activities were observed for trypsins A and B after incubation at pH 4.0. The trypsins stability at particular pH may be relevant to the net charge of the enzyme at that pH [9]. At extreme pH, strong intramolecular electrostatic repulsion caused by high net charge results in swelling and unfolding of the protein molecules [20-21]. Enzyme activity inactivation at acidic pH was also reported for trypsin from Grey triggerfish [7], hybrid catfish [22], zebra blenny [17], Goby [6] and Tunisian barbel [23]. From these results, the liver of albacore tuna would be a potential source of trypsins for certain food processing operations that require high alkaline conditions.

For thermal stability, both trypsins A and B purified from albacore tuna liver were highly stable at temperatures below 50°C (Fig. 4b). However, both enzymes were inactivated at higher temperatures (Fig. 4b). The relative activities at 80°C for trypsin A and B were about 5.12% and 0.25%, respectively, of their initial activity. At high temperatures, enzyme possibly underwent denaturation and lost their activity.

The thermal stability of the purified trypsin A and B was also investigated by incubating the enzymes in the presence of 2 mM EDTA or 2 mM $CaCl_2$, at 40°C for different times (0, 0.5, 1, 2, 4, 6 and 8 h). As depicted in Fig. 5, in the presence of 2 mM calcium ion, approximately 85% of the initial activities remained after 8 h of incubation at 40°C. However, the trypsin activity of both enzymes decreased with increasing time in the presence of 2 mM EDTA. Purified trypsin B was rather stable in the presence of 2 mM EDTA than purified trypsin A, especially when the incubation time increased. These results indicated that trypsin A and B from the liver of albacore tuna were most likely stabilized by calcium ion. The presence of calcium ions activates trypsinogen to trypsin and increases the thermal stability of the enzyme. This stabilizing effect is accomplished by a conformational change in the molecule of trypsin, resulting in a more compact structure [13]. Klomklao et al. [22] reported that

trypsin purified from the viscera of hybrid catfish was stabilized by calcium ion. El Hadj Ali et al. [5] also found that the stability of trypsin from striped seabream viscera was enhanced by the addition of $CaCl_2$. On the other hand, trypsin from Nile tilapia intestine was not stabilized by calcium ion [24]. These finding suggest a difference in the structure of the primary calcium binding site among different marine fish trypsins.

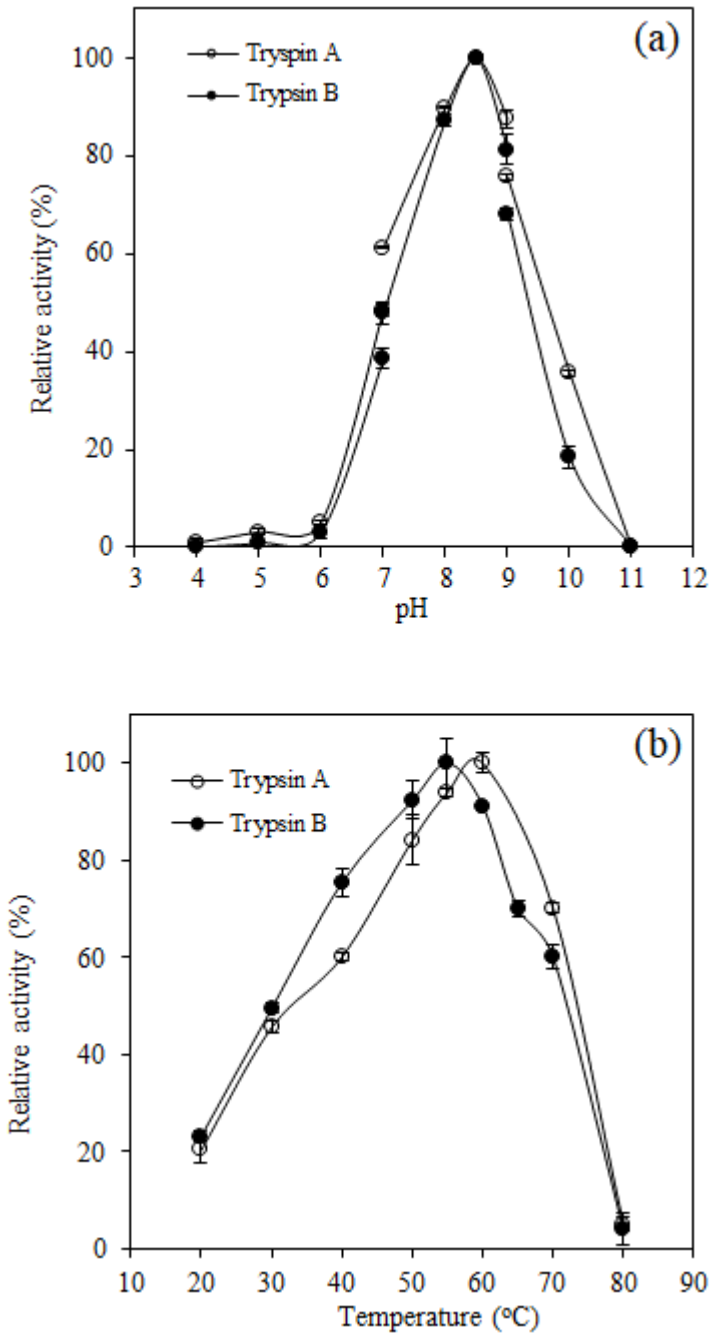

Fig. 3. pH (a) and temperature (b) profiles of purified trypsin A, and B from albacore tuna liver.

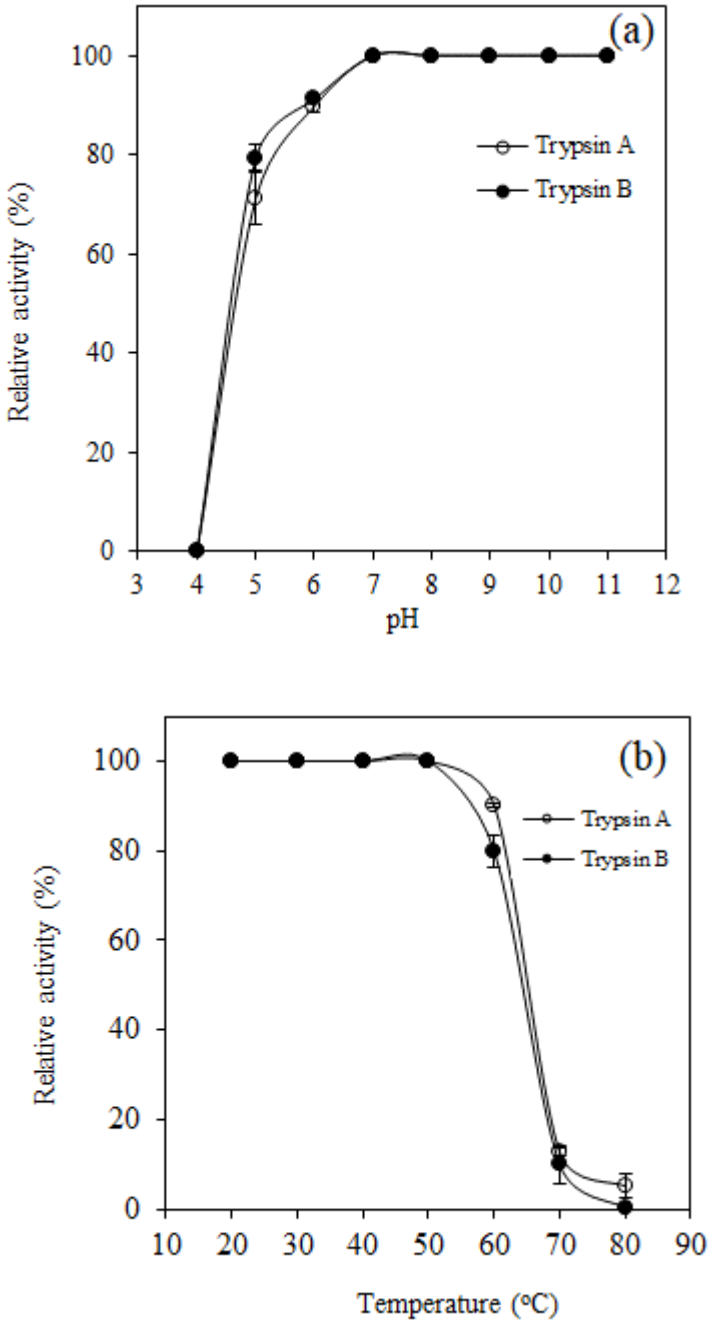

Fig. 4. pH (a) and thermal (b) stability of purified trypsin A and B from albacore tuna liver.

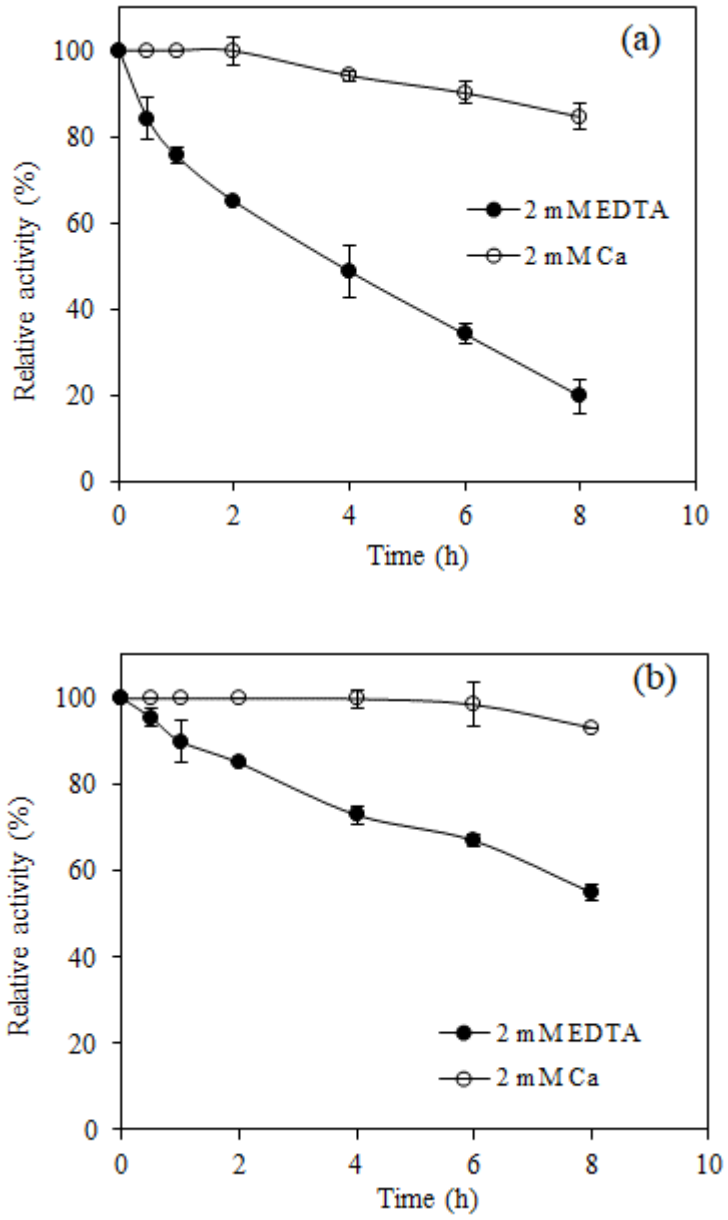

Fig. 5. Effect of calcium ion and EDTA on the stability of purified trypsin A (a) and B (b) from albacore tuna liver. The stability was tested at 40°C for different times.

3.4 Effect of NaCl

As shown in Fig. 6, the trypsin activities of both enzymes decreased gradually with increasing NaCl concentrations. In the presence of NaCl ranging from 5% to 30%, trypsin B exhibited slightly higher trypsin activity than trypsin A, indicating that trypsin B was more tolerant to NaCl than trypsin A. At 30% NaCl, remaining trypsin activities were approximately 50% and 54% for trypsins A and B, respectively. The decrease in activity might be due to the enzyme denaturation. The 'salting out' effect was postulated to cause the denaturation of enzymes. The water molecule is drawn from the molecule of trypsin by salt, leading to the aggregation of those enzymes [9]. Based on these results, more than 50% of activity remained for both trypsins A and B at high salt concentration (30%). Therefore, these trypsins from albacore tuna liver can be involved in protein hydrolysis in high salt fermented fish products such as fish sauce.

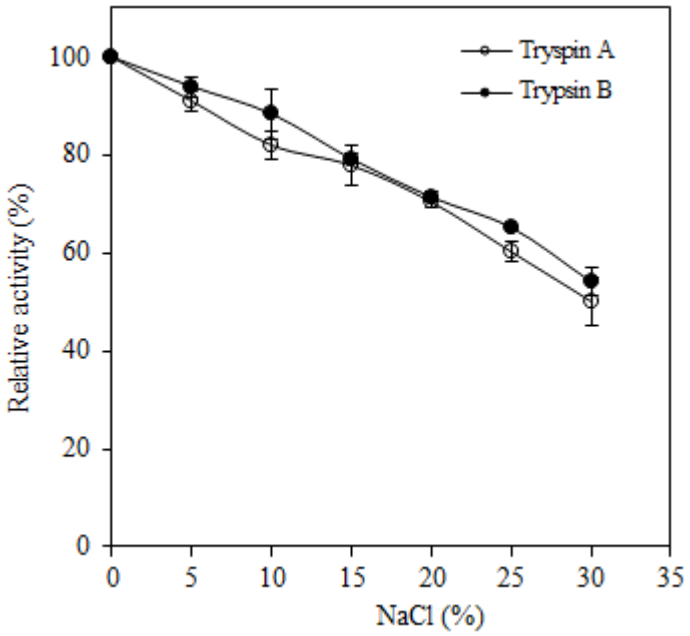

Fig. 6. Effect of NaCl concentrations on activities of purified trypsin A and B from albacore tuna liver.

3.5 Effect of inhibitors

Proteinases can be classified by their sensitivity to various inhibitors. The effect of different proteinase inhibitors, such as specific group reagents and chelating agents on the trypsin activity were studied (Table 2). The trypsin activities of both enzymes were highly affected by soybean trypsin inhibitor, known as a trypsin inhibitor and TLCK, a trypsin specific inhibitor. Further, the metalloproteinase inhibitor (2 mM EDTA), displayed partial inhibition towards both trypsins A and B with different degrees. Trypsin A was inhibited by EDTA to a greater extent, compared with trypsin B. However, cysteine and aspartic proteinases inhibitors and a chymotrypsin specific inhibitor (TPCK) showed no inhibitory effects on the trypsin activity of both enzymes. The results confirmed that both enzymes are serine proteinases, which possibly require metal ions for their activities. Purified trypsin from carnivorous catfish was strongly inhibited using soybean trypsin inhibitor and PMSF [25]. Ktari et al. [17] reported that soybean trypsin inhibitor and PMSF showed a strong inhibitory effect on the purified trypsin from zebra blenny viscera. Klomklao et al. [22] also reported that the trypsin activity from the viscera of hybrid catfish was effectively inhibited by soybean trypsin inhibitor and TLCK and partially inhibited by EDTA.

Table 2

Effect of various inhibitors on the activity of purified trypins from albacore tuna liver*

Inhibitors	Concentration	% Inhibition	
		Trypsin A	Trypsin B
Control		0	0
E-64	0.1 mM	0	0
N-ethylmaleimide	1 mM	0	0
Iodoacetic acid	1 mM	0	0
Soybean trypsin inhibitor	1.0 g/l	87.50±0.68	91.68±0.28
TLCK	5 mM	98.75±1.12	99.91±0.17
TPCK	5 mM	0	0
Pepstatin A	0.01 mM	0	0
EDTA	2 mM	18.54±0.40	8.18±0.73

*Each enzyme solution was incubated with the same volume of inhibitor at 25°C for 15 min and the residual activity was determined using TAME as a substrate for 20 min at pH 8.0 and 30°C.

3.6 Kinetic study

Table 3 shows the kinetic constants K_m and K_{cat} of the purified trypsins A and B from the liver of albacore tuna for TAME hydrolysis measured using Lineweaver-Burk plots. K_m values of trypsins A and B were 0.23 and 0.32 mM, respectively. K_{cat} values of trypsins A and B were 67.77 and 85.58 S^{-1}, respectively. The Km and Kcat of the purified trypsins from albacore tuna liver were close to those reported for trypsins from yellowfin tuna [9] and Japanese sea bass [26]. The K_m value of trypsin A was lower than that of trypsin B. This result suggests that trypsin A has higher affinity to TAME, compared with trypsin B. For K_{cat} (turnover number), trypsin B had a higher value than trypsin A. Nevertheless, the catalytic efficiency value for trypsin A was higher than that of trypsin B. This result suggests that trypsin A would be more efficient in transforming the substrate to product. Furthermore, the catalytic efficiency of trypsins from albacore tuna liver was higher than those reported for mammalian trypsins [27].

Table 3

Kinetic properties of albacore tuna liver trypsins for the hydrolysis of TAME

Enzyme	K_m (mM)	K_{cat} (S^{-1})	K_{cat} / K_m (S^{-1} mM^{-1})
Trypsin A	0.23±0.02	67.77±0.01	294.65
Trypsin B	0.32±0.01	85.58±0.03	267.44

K_m, K_{cat} values were determined using TAME as a substrate at pH 8.0 and 30°C.

3.7 N-terminal sequencing

Generally, trypsins are regarded to have arisen from a common ancestor by divergent evolution as they share similarities not only in biological functions, but also in active sites, primary and even three-dimensional structures. The N-terminal (20 residues) amino acid sequences of trypsins A and B were IVGGYECQAHSQPWQVSLNA and IVGGYECQAHTQPHQVSLNA (Fig. 7), indicating that the N terminus of the enzymes was unblocked. The N-terminal amino acid sequences of the trypsins A and B from albacore tuna liver were aligned with those of other animal trypsins (Fig. 7). Being similar to other fish trypsins, both trypsins had a charged Glu residue at position 6, where Thr is most common in mammalian pancreatic trypsins. The N-terminal sequences of trypsins A and B from albacore tuna liver clearly showed that they are closely-related members of the trypsin family.

	5 10 15 20
Trypsin A	I V G G Y E C Q A H S Q P W Q V S L N A
Trypsin B	I V G G Y E C Q A H T 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
True sardine	I V G G Y E C K A Y S Q P W 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
Japanese anchovy	I V G G Y E C Q A H S Q P H T V S L N S
Cod	I V G G Y E C T K H S Q A H Q V S L N S
Salmon	I V G G Y E C K A Y S Q T H Q V S L N S
Dogfish	I V G G Y E C P K H A A P W T V S L N V
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 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. 7. Comparison of N-terminal amino acid sequences of the purified trypsin A and B from albacore tuna liver with other enzymes: skipjack tuna [16], true sardine, arabesque greenling [28], Japanese anchovy [29], cod [30], salmon [31], dogfish [32], dog [33], porcine [34], and bovine [35].

4. Conclusion

The proteases were purified from the liver of albacore tuna. After purification, two enzymes were obtained. The characterization, with specific substrate, inhibitors and the N-terminal sequence, demonstrated that these proteases are trypsin. Furthermore, it showed interesting features, such as high activity and stability over a large alkaline pH range and high activity at elevated salt concentrations. These properties have confirmed that fish viscera may be used as a source of trypsin with potential for industrial applications.

Acknowledgments

This research was supported by the Thailand Research fund and Thaksin University for Project No. RSA6080044. The TRF distinguished research professor grant was also acknowledged.

References

[1] A. Bougatef**,** Trypsins from fish processing waste: Characteristics and biotechnological applications e comprehensive review, J. Clean Prod. 57 (2009) 257-265.

[2] P. Sripokar, T. Poonsin, M. Chaijan, S. Benjakul, S. Klomklao, Proteinases from the liver of albacore tuna (*Thunnus Alalunga*): Optimum extractant and biochemical characteristics, J. Food Biochem. 40 (2016) 10-19.

[3] S. Unajak, P. Meesawat, A. Paemanee, N. Areechon, A. Engkagul, U. Kovitvadhi, S. Kovitvadhi, K. Rungruangsak-Torrissen, K. Choowongkomon, Characterisation of thermostable trypsin and determination of trypsin

isozymes from intestine of Nile tilapia (*Oreochromis niloticus* L.), Food Chem. 134 (2012) 1533-1541.

[4] S. Klomklao, S. Benjakul, W. Visessanguan, B.K. Simpson, H. Kishimura, Partitioning and recovery of proteinase from tuna spleen by aqueous two-phase systems, Process Biochem. 40 (2005) 3061-3067.

[5] N. El Hadj Ali, N. Hmidet, A. Bougatef, R. Nasri, M. Nasri, A laundry detergent-stable alkaline trypsin from striped seabream (*Lithognathus mormyrus*) viscera: Purification and characterization, J. Agric. Food Chem. 57 (2009) 10943-10950.

[6] R. Nasri, A. Sila, N. Ktari, I. Lassoued, A. Bougatef, M. Karra-Chaabouni, M. Nasri, Calcium dependent, alkaline detergent-stable trypsin from the viscera of Goby (*Zosterisessor ophiocephalus*): Purification and characterization, Process Biochem. 47 (2012) 1957-1964.

[7] K. Jellouli, A. Bougatef, D. Daassi, R. Balti, A. Barkia, M. Nasri, New alkaline trypsin from the intestine of Grey triggerfish (*Balistes capriscus*) with high activity at low temperature: Purification and characterization, Food Chem. 116 (2009) 644-650.

[8] S. Klomklao, S. Benjakul, W. Visessanguan, H. Kishimura, B.K. Simpson, Purification and characterisation of trypsins from the spleen of skipjack tuna (*Katsuwonus pelamis*), Food Chem. 100 (2007) 1580-1589.

[9] S. Klomklao, S. Benjakul, W. Visessanguan, H. Kishimura, B.K. Simpson, H. Saeki, Trypsins from yellowfin tuna (*Thunnus albacores*) spleen: Purification and characterization, Comp. Biochem. Physiol. B. 144 (2006) 47-56.

[10] I. Kurtovic, S.N. Marshall, B.K. Simpson, Isolation and characterization of a trypsin fraction from the pyloric ceca of chinook salmon (*Oncorhynchus tshawytscha*), Comp. Biochem. Physiol. B. 143 (2006) 432-440.

[11] P. Sripokar, M. Chaijan, S. Benjakul, H. Kishimura, S. Klomklao, Enzymatic hydrolysis of starry triggerfish (*Abalistes stellaris*) muscle using liver proteinase from albacore tuna (*Thunnus alalunga*), J. Food Sci. Technol. (2016) doi: 10.1007/s13197-015-2138-z

[12] B.C.W. Hummel, A modified spectrophotometric determination of chymotrypsin, trypsin, and thrombin, Can. J. Biochem. Phys. 37 (1959) 1393-1399.

[13] S. Klomklao, S. Benjakul, W. Visessanguan, W, Comparative studies on proteolytic activity of spleen extracts from three tuna species commonly used in Thailand, J. Food Biochem. 28 (2004) 355-372.

[14] H. Lineweaver, D. Burk, The determination of enzyme dissociation constants, J. Am. Chem. Soc. 56 (1934) 665-666.

[15] O.H. Lowry, N.J. Rosebrough, A.L. Fan, R.J. Randall, 1951. Protein measurement with Folin phenol reagent, J. Biol. Chem. 193 (1951) 256-275.

[16] S. Klomklao, H. Kishimura, Y. Nonami, S. Benjakul, Biochemical properties of two isoforms of trypsin purified from the intestine of skipjack tuna (*Katsuwonus pelamis*), Food Chem. 115 (2009) 155-162.

[17] N. Ktari, H.B. Khaled, R. Nasri, K. Jellouli, S. Ghorbel, M. Nasri, Trypsin from zebra blenny (*Salaria basilisca*) viscera: Purification, characterisation and potential application as a detergent additive, Food Chem. 130 (2012) 467-474.

[18] I. Bkhairia, H.B. Khaled, N. Ktari, N. Miled, M. Nasri, S. Ghorbel, Biochemical and molecular characterisation of a new alkaline trypsin from *Liza aurata*:

Structural features explaining thermal stability, Food Chem. 196 (2016) 1346-1354.

[19] A.G. Villalba-Villalba, J.C. Ramírez-Suárez, E.M. Valenzuela-Soto, G.G. Sánchez, G.C. Ruiz, R. Pacheco-Aguilar, Trypsin from viscera of vermiculated sailfin catfish, *Pterygoplichthys disjunctivus*, Weber, 1991: Its purification and characterization, Food Chem. 141 (2013) 940-945.

[20] B.K. Khangembam, R. Chakrabarti, Trypsin from the digestive system of carp Cirrhinus mrigala: Purification, characterization and its potential application, Food Chem. 175 (2015) 386-394.

[20] S. Damodaran, Amino acids, peptides, and proteins, in: O.R. Fennema (Ed.), Food Chemistry Quality, Marcel Dekker, New York, 1996, pp. 322-429.

[21] S. Benjakul, W. Visessanguan, P. Thummaratwasik, P, Isolation and characterization of trypsin inhibitors from some Thai legume seeds, J. Food Biochem. 24 (2000) 107-127.

[22] S. Klomklao, S. Benjakul, H. Kishimura, M. Chaijan, 24 kDa Trypsin: A predominant protease purified from the viscera of hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*), Food Chem. 129 (2011) 739-746.

[23] A. Sila, R. Nasri, M. Jridi, R. Balti, M. Nasri, A. Bougatef, Characterisation of trypsin purified from the viscera of Tunisian barbel (*Barbus callensis*) and its application for recovery of carotenoproteins from shrimp wastes, Food Chem. 132 (2012) 1287-1295.

[24] R.S. Bezerra, E.J.F. Lins, R.B. Alencar, P.M.G. Paiva, M.E.C. Chaves, L.C.B.B. Coelho, J.L.B. Carvalho, Alkaline proteinase from intestine of Nile tilapia (*Oreochromis niloticus*), Process Biochem. 40 (2005) 1829-1834.

[25] C.W.V Santos, M.E.C. Marques, H.A. Tenório, E.C. Miranda, H.J.V. Pereira, Purification and characterization of trypsin from *Luphiosilurus alexandri* pyloric cecum, Biochem. Biophys. Rep. 8 (2016) 29-33.

[26] Q.F. Cai, Y.K. Jiang, L.G. Zhou, L.C. Sun, G.M. Liu, K. Osatomi, M.J. Cao, Biochemical characterization of trypsins from the hepatopancreas of Japanese sea bass (*Lateolabrax japonicus*)

[27] B.K. Simpson, Digestive proteinases from marine animals, in: N.M. Haard, B.K. Simpson (Eds.), Seafood Enzymes: Utilization and Influence on Postharvest Seafood Quality, Marcel Dekker, New York, 2000, pp. 531-540.

[28] H. Kishimura, K. Hayashi, Y. Miyashita, Y. Nonami, Y, Characteristics of trypsins from the viscera of true sardine (*Sardinops melanostictus*) and the pyloric caeca of arabesque greenling (*Pleuroprammus azonus*), Food Chem. 97 (2006) 65-70.

[29] H. Kishimura, K. Hayashi, Y. Miyashita, Y., Nonami, Characteristics of two trypsin isozymes from the viscera of Japanese anchovy (*Engraulis japonica*), J. Food Biochem. 29 (2005) 459-469.

[30] A. Gudmundsdottir, E. Gudmundsdottir, S. Oskarsson, J.B. Bjanrnason, A.K. Eakin, C.S. Craik, Isolation and characterization of cDNAs from Atlantic cod encoding two different forms of trypsinogen, Eur. J. Biochem. 217 (1993) 1091-1097.

[31] R. Male, L.B. Lorens, A.O. Smalas, K.R. Torrissen, Molecular cloning and characterization of anionic and cationic variants of trypsin from Atlantic salmon, Eur. J. Biochem. 232 (1995) 677-685.

[32] K. Titani, L.H. Ericsson, H. Neurath, K.A. Walsh, Amino acid sequence of dogfish trypsin, Biochemistry. 14 (1975) 1358-1366.

[33] S.D. Pinsky, K.S. Laforge, G. Scheele, Differential regulation of trypsinogen mRNA translation: Full-length mRNA sequences encoding two oppositely charged trypsinogen isoenzymes in the dog pancreas, Mol. Cell. Biol. 5 (1985) 2669-2676.

[34] M.A. Hermodson, L.H. Ericsson, H. Neurath, K.A. Walsh, Determination of the amino sequence of porcine trypsin by sequenator analysis, Biochemistry. 12 (1973) 3146-3153.

[35] K.A. Walsch, Trypsinogens and trypsins of various species, Method. Enzymol. 19 (1970) 41-63.

Chapter 4

Enzymatic hydrolysis of starry triggerfish (*Abalistes stellaris*) muscle using liver proteinase from albacore tuna (*Thunnus alalunga*)

Journal of Food Science and Technology

P Sripokar[1] · M Chaijan[2] · S Benjakul[3] · H Kishimura[4] · S Klomklao[5*]

[1]Biotechnology Program, Faculty of Technology and Community Development, Thaksin University, Phatthalung Campus, Phatthalung, 93210, Thailand

[2]Department of Food Technology, School of Agricultural Technology, Walailak University, Nakhon Si Thammarat 80160, Thailand

[3]Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand

[4]Laboratory of Marine Products and Food Science, Research Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido, 041-8611, Japan.

[5]Department of Food Science and Technology, Faculty of Technology and Community Development, Thaksin University, Phatthalung Campus, Phatthalung, 93210, Thailand

Abstract

Proteinases from liver extract from albacore tuna (*Thunnus alalunga*) were used to produce protein hydrolysate from starry triggerfish (*Abalistes stellaris*) muscle. Hydrolysis conditions for preparing protein hydrolysate from starry triggerfish muscle were optimized. Enzyme level, reaction time and fish muscle/buffer ratio significantly affected the hydrolysis ($p<0.05$). Optimum conditions for triggerfish muscle hydrolysis were 5.5% liver extract, 40 min reaction time and fish muscle/buffer ratio of 1:3 (w/v). The freeze dried protein hydrolysate was characterized with respect to chemical composition, amino acid composition and color. The product contained 91.73% protein, 2.04% lipid and 6.48% ash. The protein hydrolysate exhibited high amount of essential amino acids (45.62%). It was light yellow in color (L^* = 82.94, a^* = 0.84, b^* = 22.83). The results indicate that the extract from liver of albacore tuna could be used to produce fish protein hydrolysate and protein hydrolysate from starry triggerfish muscle may potentially serve as a good source of desirable peptide and amino acids.

Keywords Protein hydrolysate · Proteinases · Tuna · Industrial application

Introduction

Proteins from fish processing byproducts can be modified to improve their quality and functional characteristics by enzymatic hydrolysis (Shahidi 1994). Utilizing proteolytic enzymes, fish protein hydrolysate (FPH) can be prepared with the peptides having new and/or improved properties. Generally, protein can be hydrolyzed by chemical process or protease enzymes. However, FPH obtained from these two methods are different in quality (Wisuthiphaet and Kongruang 2015). Enzymatic hydrolysis of food proteins is an efficient way to recover potent bioactive peptides. Fish protein hydrolysates have been shown to have potential for nutritional or pharmaceutical applications (Thiansilakul et al. 2007; Wu et al. 2003). Protein hydrolysis decreases the peptide size, and thereby making hydrolysates the most available amino acid source for various physiological functions of human body. Protein hydrolysates are used as readily available sources of protein for humans and animals due to their good functional properties (Neklyudov et al. 2000).

The starry triggerfish (*Abalistes stellaris*), or flat-tailed triggerfish belongs to order Tetraodontiformes and is a member of the Balistidae family. Generally, it is widely used as fish meal. In Thailand, starry triggerfish has not been used for human consumption due to its tough skin. To increase the value of this fish species, the production of new value-added products such as protein hydrolysates, with nutritive value and bioactivity, can pave the way for its full utilization. Many factors affect the bioactivity of protein hydrolysates, e.g. type and concentration of proteinases (Benjakul and Morrissey 1997), steps of hydrolysis (Thiansilakul et al. 2007), etc. Protein hydrolysates have been produced and characterized from several sources, e.g. toothed ponyfish (*Gazza minuta*) (Klomklao et al. 2013), Catla (*Catla catla*) (Bhaskar

and Mahendrakar 2008), yellowfin tuna (*Thunnus albacores*) (Guerard 2001) and bigeye snapper (*Priacanthus macracanthus*) (Phanturat et al. 2010).

Fish viscera generated during processing contain a variety of enzymes including proteinases. Proteinases are potential enzymes for industrial applications and could produce the new bioactive molecules (Klomklao et al. 2005). Albacore tuana (*Thunnus alalunga*) is the potential raw materials for canned production in Thailand (Nalinanon et al. 2008). Processing wastes generated during canned tuna processing, especially viscera can be used as the essential source of proteinases. Sripokar et al. (2015) reported that the liver of albacore tuna contained high proteolytic activity and major proteinases were heat-activate alkaline proteinases, most likely trypsin-like serine proteinases.

Recovery and use of proteinases from fish viscera for fish protein hydrolysate is a promising approach to minimize the economics and ecologicals problem of this processing waste. Also, the production of protein hydrolysate from fish muscle using albacore tuna liver extract has not been reported. Hence, the purpose of this work was to study the production and characterization of hydrolysate from starry triggerfish muscle using albacore tuna liver extract.

Materials and methods

Chemical

β-Mercaptoethanol (βME), 2,4,6-trinitrobenzenesulfonic acid (TNBS), *L*-leucine and bovine serum albumin were procured from Sigma Chemical Co. (St. Louis, MO, USA). Tris (hydroxymethyl) aminomethane (Tris-HCl), sodium sulphite and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS) and Coomassie Brilliant Blue R-250 were

procured from Bio-Rad Laboratories (Hercules, CA, USA). All of chemicals used were of analytical grade.

Fish sample preparation

Albacore tuna (*Thunnus alalunga*) internal organs were obtained from Tropical Canning (Thailand) Public Co. Ltd., Hat Yai, Songkhla. The samples were packed in polyethylene bags, kept in ice with a sample/ ice ratio of 1:3 (w/w) and transported to the research laboratory within 2 h. Pooled internal organs were separated and only the liver was collected, immediately frozen and stored at -20°C until used.

Starry triggerfish (*Abalistes stellaris*) with the length of 30-35 cm were purchased from the dock in Trang, Thailand. The fish, off-loaded approximately 18-24 h after capture, were placed on ice at a fish/ ice ratio of 1:2 (w/w) and transported to the Department of Food Science and Technology, Thaksin University, Phatthalung, within 2 h. Upon arrival, the fish were filleted and the ordinary muscle was collected and ground to uniformity. A portion of mince (500 g) was placed in a polyethylene bag and stored at -20°C until used.

Preparation of Liver Extract

Frozen livers were thawed using running water (26-28°C) until the core temperature reached -2 to 0°C. The samples were cut into pieces with a thickness of 1-1.5 cm and homogenized into powder in three volumes of acetone at -20°C for 30 min according to the method of Klomklao et al. (2007). The homogenate was filtrated in vacuum on Whatman No. 4 filter paper. The residue obtained was then

homogenized in two volumes of acetone at -20°C for 30 min. The residue was left at room temperature until dried and free of acetone odor.

To prepare the liver extract, defatted liver powder was suspended in 50 mM Na-phosphate, pH 7.0 at a ratio of 1:9 (w/v) and stirred continuously at 4°C for 30 min. The suspension was centrifuged at 5,000× g for 30 min at 4°C using a Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA). The supernatant obtained was collected and referred to as "liver extract".

Study on the optimum condition for production of starry triggerfish protein hydrolystate using tuna liver extract

Effect of extract concentration on DH

Effect of liver extract concentrations (0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5%, 5%, 5.5%, 6%, 6.5%, 7%, 7.5%, 10%, 15% and 20% (w/w)) on degree of hydrolysis (DH) was studied. Different amounts of liver extract were added to the suspension of starry triggerfish mince in 0.1 M glycine-NaOH buffer, pH 8.5 (1:2 ratio, w/v) and the reaction was carried out at 55ºC, for 30 min (Sripokar et al. 2015). The termination of hydrolytic reaction was done by heating the reaction mixture at 90ºC for 15 min in a water bath. The supernatant was obtained by centrifuging at 5000g for 10 min. DH was then determined. The extract concentration, which was able to hydrolyze fish mince with the highest DH, was selected for further study.

Effect of reaction time on DH

To study the effect of reaction time on DH, starry triggerfish mince was added with 0.1 M glycine-NaOH buffer (pH 8.5) at the ratio of 1:2 (w/v). The reaction was initiated by adding 5.5% liver extract. The mixtures were shaken at 55°C for 5, 10,

15, 20, 30, 40, 60, 80 and 100 min. At designated time, the reaction was stopped and the supernatant was prepared in the same manner as previously described. DH was then determined. The reaction time rendering the highest DH was chosen for further steps.

Effect of starry triggerfish muscle and buffer ratio on DH

Starry triggerfish mince was mixed with 0.1 M glycine-NaOH buffer, pH 8.5 at a ratio of 1:0.5, 1:1, 1:2, 1:3, 1:4 and 1:5 (w/v). Liver extract at a level of 5.5% was added and the reaction was maintained for 40 min at 55°C. The hydrolysate obtained was determined for DH. The buffer ratio used for mixing the mince showing the highest DH was selected for further study.

Determination of α-amino acids and DH

The α-amino acid content was determined according to the method of Klomklao et al. (2013). Properly diluted hydrolysate samples (125 μl) 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 (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 ambient temperature for 15 min. The absorbance was measured at 420 nm and α-amino acid was expressed in term 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 α-amino acid released at time t. L_0 is the amount of α-amino acid in the original starry triggerfish muscle homogenate. L_{max} is total α-amino acid in the original starry triggerfish muscle homogenate obtained after acid hydrolysis with 6 N HCl at 100°C for 24 h.

Production of starry triggerfish protein hydrolysate

Starry triggerfish mince (20 g) was mixed with 0.1 M glycine-NaOH buffer, pH 8.5 at a ratio of 1:3 (w/v) and pre-incubated at 55°C for 10 min (Klomklao et al. 2013; Sripokar et al. 2015). The enzyme hydrolysis was started by adding albacore tuna liver extract at a level of 5.5% (w/w). The reaction was conducted at pH 8.5 and 55°C for 40 min. After 40 min of hydrolysis, the enzyme was inactivated by heating at 90°C for 15 min in a water bath. The mixture was then centrifuged at 5000g at 4°C for 10 min and the supernatant was collected. Starry triggerfish protein hydrolysate was freeze-dried using a Dura-Top™µp freeze-dryer (FTS systems Inc., Stone Ridge, NY, USA). The freeze-dried starry triggerfish protein hydrolysate obtained was subjected to analyses.

Proximate analysis

Moisture, protein, fat and ash contents were determined according to the method of AOAC (2005).

Amino acid analysis

Amino acid compositions of freeze-dried hydrolysate were determined according to the method of Cohen and Michaud (1993). Hydrolysate (10 µg) was dissolved in 10 mM HCl (10 µl) and treated with 0.2 M borate buffer pH 9.3 (30 µl).

The sample solution was reacted with 10 mM 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (10 µl) to form stable unsymmetric urea derivatives. Amino acids were separated by reverse phase HPLC (AccQ Tag column, Waters, Milford, MA, USA) at 37°C, using gradient mobile phase: deionized water, acetonitrile and eluent A (sodium acetate, phosphoric acid, triethylamine) and detected by a UV detector (Waters 486, Milford, MA, USA) at 254 nm and a fluorescence detector (Jasco FP-920, Great Dunmow, Essex, UK) with excitation wavelength at 250 nm and emission wavelength at 395 nm. The amount of amino acids was calculated, based on the peak area in comparison with that of standard. The amino acid content was expressed as a percentage of total amino acids in the sample.

Color measurement

The color of freeze-dried hydrolysate was measured by the Hunter lab colorimeter and reported by the CIE system. L^*, a^* and b^* parameters, indicating lightness, redness and yellowness, respectively.

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 proximate composition, the independence t-test was used for pair comparison. (Steel and Torrie 1980). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for Windows; SPSS Inc., Chicago, IL, USA).

Results and discussion

Optimum condition for starry triggerfish protein hydrolystate production using albacore tuna liver extract

Enzymatic hydrolysis is influenced by several factors like temperature, pH, enzyme to substrate level and time that cooperatively influence the enzyme activity thereby making the process more controllable (Liaset et al. 2000). Based on my previous study, liver proteinase from albacore tuna had optimum pH and temperature at 8.5 and 55°C, respectively. An appreciable decrease in enzyme activity was observed at temperature above 55°C, due to thermal denaturation (Sripokar et al. 2015). The optimal pH and temperature were 9.0 and 50°C, respectively, when toothed ponyfish (*Gazza minuta*) muscle was used as a substrate (Klomklao et al. 2013). Alcalase had maximal activity at pH 8.5 and 55°C for hydrolysis of Persian sturgeon viscera (Ovissipour et al. 2009). Benjakul and Morrissey (1997) found that Alcalase and Neutrase showed optimum activity against Pacific whiting solid wastes at pH 9.5, 60°C and pH 7.0, 55°C, respectively. Therefore, pH 8.5 and temperature 55°C was chosen as the optimal condition for protein hydrolysate production from starry triggerfish using liver proteinase from albacore tuna.

Effect of liver extract concentration on DH

Degree of hydrolysis (DH), which indicates the percentage of peptide bonds cleaved (Adler-Nissen 1986), is essential because several properties of protein hydrolysates are closely related to DH (Nielsen 1997). The DH of starry triggerfish muscle treated with tuna liver extract increased when the liver extract level was increased (Fig. 1). Significant changes in DH occurred with the liver extract treatment at levels ranging from 0.5 to 5.5% ($p<0.05$). However, no increase for DH was found with treatment of liver extract at level above 5.5%. The value of DH in this study was

similar to that obtained in toothed ponyfish hydrolysate treated with catfish viscera extract (Klomklao et al. 2013). Shahidi et al. (1995) reported that considerable soluble protein was released during initial phase and no increase in soluble hydrolysate was observed when additional enzyme was added during the stationary phase of hydrolysis. Morr et al. (1985) suggested that, the overall reaction involved at least two steps. In the first step, the enzyme molecules become associated with and bound to the fish particles. Subsequently, hydrolysis took place, resulting in the release of soluble peptides and amino acids.

When $\log_{10}$ (liver extract concentration) was plotted against DH, a linear relationship and the regressive equations were obtained (Fig. 2). Klomklao et al. (2013) found a similar linear relationship between %DH and log of enzyme concentration, when hydrolyzing toothed ponyfish muscle with catfish viscera extract. The relationship is further supported by Benjakul and Morissey (1997) for Pacific white shrimp protein hydrolysate derived with Alcalase and Neutrase and Thiansilakul et al. (2007) for round scad derived with Flavourzyme. From the regression, amount of liver extract required to prepare starry triggerfish muscle protein hydrolysate with required DH could be calculated.

Effect of reaction time on DH

Fig. 3. shows the effect of hydrolysis time on DH of starry triggerfish muscle using albacore tuna liver extract. A high rate of hydrolysis was obtained during the initial stage (10-40 min), owing to a large number of peptide bonds available. After 40 min of hydrolysis, the enzymatic reaction more likely reached the steady-state phase. The stability in the hydrolysis rate, observed in the later stage, was mainly due to a decrease in available substrate, enzyme auto-digestion and product inhibition (Khantaphant et al. 2011). Generally, the enzyme absorbs rapidly onto the insoluble

protein particles and the polypeptide chains that are loosely bound to the surface are then cleaved. The more compacted core proteins are hydrolyzed more slowly. The rate of enzymic cleavage of peptide bond controls the overall rate of hydrolysis (Benjakul and Morrissey 1997). Fish gelatin was hydrolyzed using proteases from hepatopancreas of Pacific white shrimp, a rapid hydrolysis was found within the first 30-40 min, followed by a slower hydrolysis rate up to 120 min (Senphan and Benjakul, 2014). Ovissipour et al. (2009) produced protein hydrolysates from Persian sturgeon (*Acipensor persicus*) viscera by optimizing the hydrolysis time. The highest DH%, 46.13 was achieved at 55°C after 205 min. Klomklao et al. (2013) investigated the enzymatic hydrolysis of toothed ponyfish (*Gazza minuta*) muscle with viscera extract from hybrid catfish (*Clarias macrocephalus*×*Clarias gariepinus*). A rapid reaction rate of both DH and NR was obtained in the first 15 min. From the results, the reaction time of 40 min was found to be optimal for starry triggerfish protein hydrolysate production.

Effect of fish muscle/buffer ratio on DH

The effect of substrate/buffer ratio on hydrolysis of starry triggerfish muscle using albacore tuna liver extract is shown in Fig. 4. In general, an increase in starry triggerfish mince/buffer resulted in an increase in DH. The ratio above 1:3 did not cause a significant increase in DH (p>0.05). Sufficient buffer gave buffering capacity for the reaction, worked as media for enzyme dispersion and was considered as an important factor for starry triggerfish muscle hydrolysis. From the results, fish muscle/buffer ratio of 1:3 (w/v) was sufficient for enzymatic reaction. Increased water or buffer added to substrate enhanced enzyme homogeneity, promoted tissue swelling, and reduced the localized concentration of hydrolysis products (Surowka and Fix 1994). Klomklao et al. (2013) used catfish viscera extract to recover the proteinases

substances from toothed ponyfish muscle and found that an increase in the ratio of added buffer resulted in an increase in DH. Benjakul and Morrissey (1997) reported that an increase in Pacific whiting solid waste/buffer ratio resulted in an increase in α-amino acid concentration as well as NR.

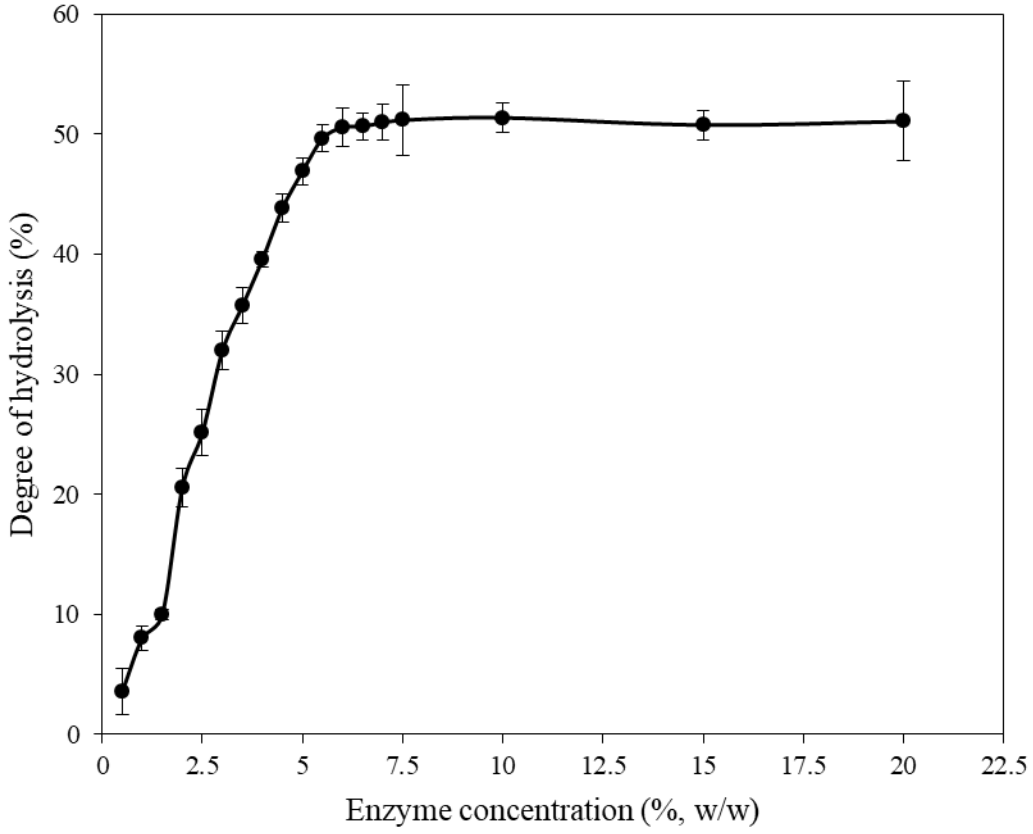

Fig. 1 Effect of albacore tuna liver extract concentration on DH of starry triggerfish muscle. Bars represent the standard deviation from triplicate determinations.

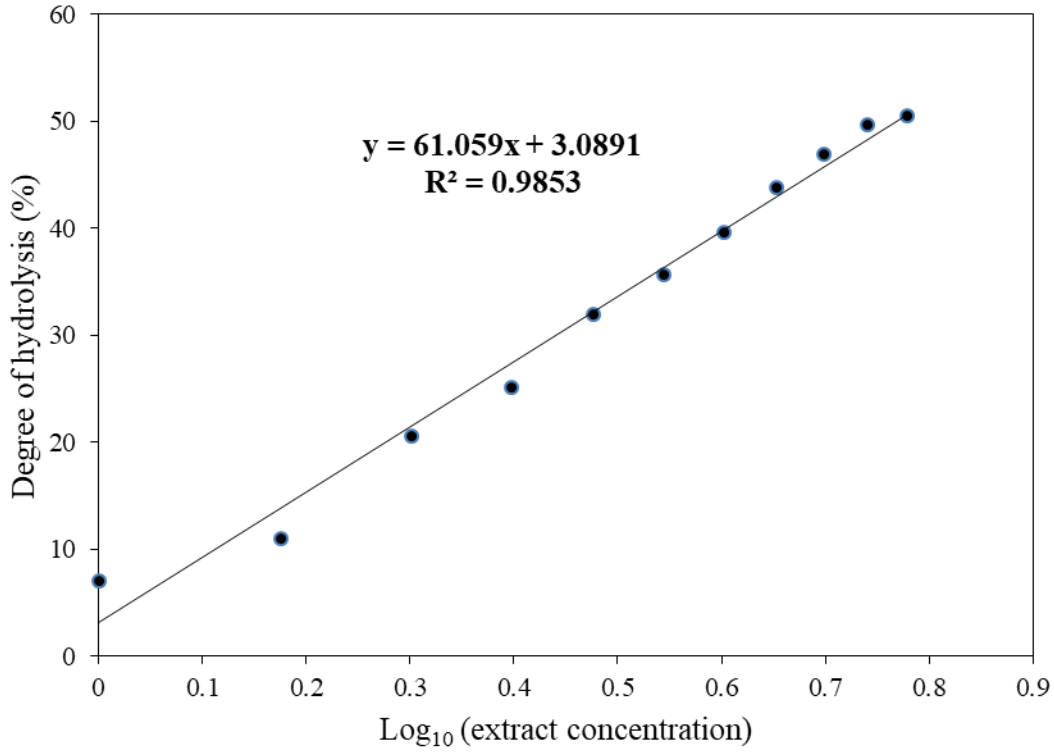

Fig. 2 Relation between $\log_{10}$ (extract concentration) and DH for starry triggerfish muscle treated with albacore tuna liver extract.

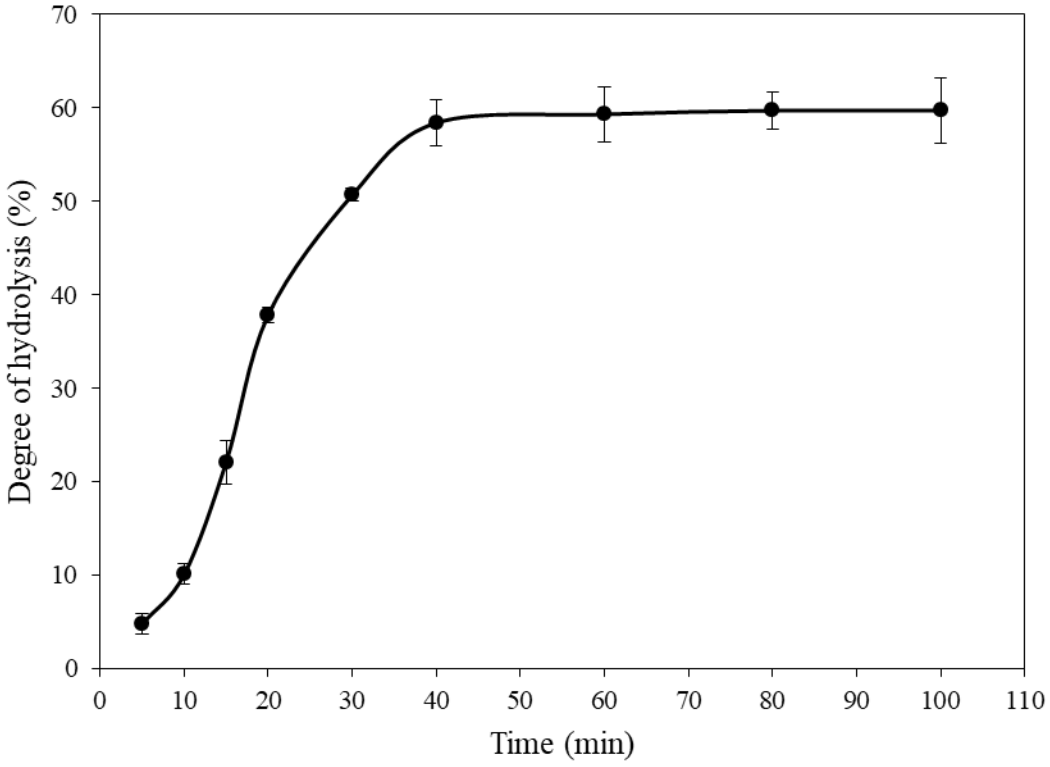

Fig. 3 Effect of hydrolysis time on DH for starry triggerfish muscle with albacore tuna liver extract. Bars represent the standard deviation from triplicate determinations.

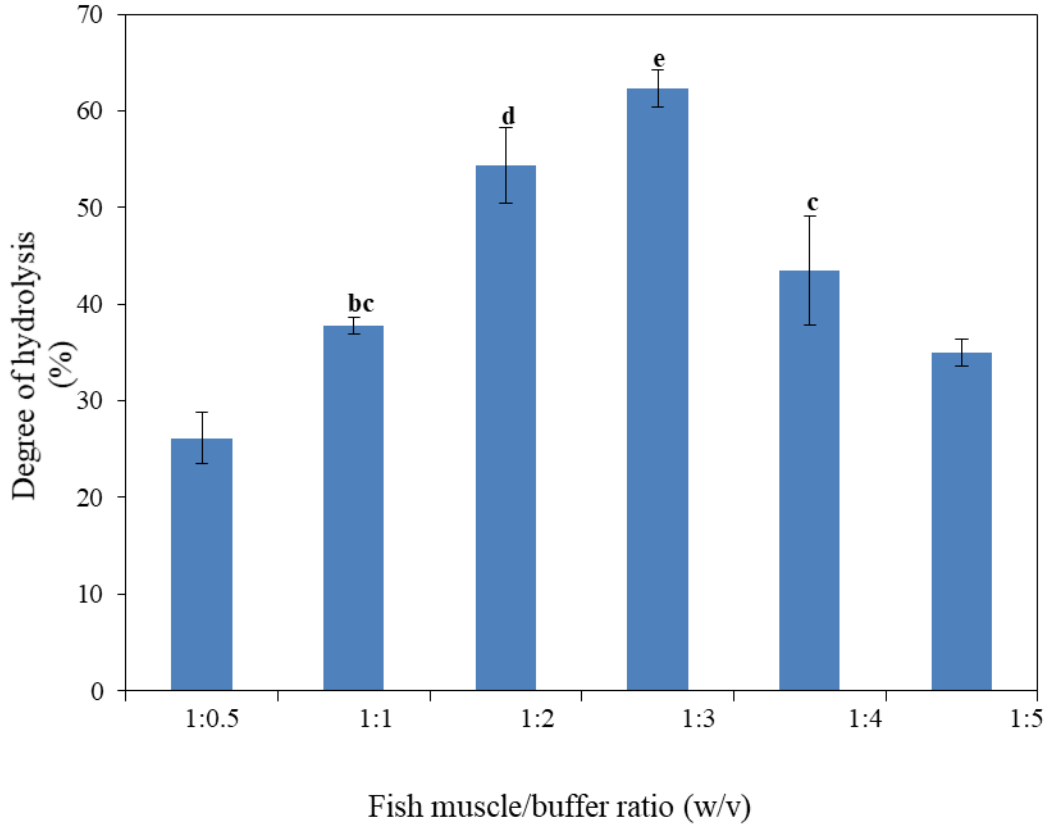

Fig. 4 Effect of fish muscle/buffer ratio on DH for starry triggerfish muscle with albacore tuna liver extract. Different letters indicate significant differences (p<0.05). Bars represent the standard deviation from triplicate determinations.

Proximate composition of protein hydrolysate

The proximate composition of starry triggerfish muscle and freeze-dried starry triggerfish protein hydrolysate are depicted in Table 1. The freeze-dried hydrolysate and starry triggerfish muscle contained 10.86% and 78.27% moisture, respectively. On dry weight basis, freeze-dried hydrolysate contained higher protein content but lower lipid content than starry triggerfish muscle. The high protein content reported for fish protein hydrolysates is due to the solubilization of proteins during hydrolysis and the removal of insoluble solid matter by centrifugation (Chalamaiah et al. 2010). High protein content of fish protein hydrolysates demonstrates its potential use as protein supplements for human nutrition (Chalamaiah et al. 2012). The low fat content of fish protein hydrolysates is because of removal of lipids with insoluble protein fractions by centrifugation (Chalamaiah et al. 2012). Decreasing lipid content in the protein hydrolysates might significantly increase stability of the material towards lipid oxidation, which may also enhance the product stability (Kristinsson and Rasco 2000). During the hydrolysis process, the muscle cell membranes tend to round up and form insoluble vesicles, leading to the removal of membrane structured lipid (Shahidi et al. 1995). The freeze-dried protein hydrolysate had a higher ash content (6.48%) than did starry triggerfish mince (3.71%). The relatively high ash content of fish protein hydrolysates is due to the use of glycine-NaOH buffer during the enzymatic reaction and usage of added acid or base for adjustment of pH of medium (Choi et al. 2009).

Table 1. Proximate composition of freeze-dried hydrolysate produced from starry triggerfish muscle and starry triggerfish muscle.*

Compositions	Freeze-dried hydrolysate	Starry triggerfish muscle
Protein**	91.73±1.16b***	84.66±2.04a
Lipid**	2.04±1.07a	11.89±4.79b
Ash**	6.48±1.55b	2.69±1.52a
Moisture	10.86±2.65a	78.27±0.32b

*Mean±SD from triplicate determination.
**Dry weight basis.
***The different letters in the same row denote the significant differences (p<0.05).

Amino acid composition

Table 2 shows the amino acid compositions of the freeze-dried starry triggerfish protein hydrolysate. Protein hydrolysate was rich in glycine, lysine and leucine, which accounted for 15.33%, 11.67% and 10.27% of the total amino acids, respectively. The nutritive value of any ingredient depends on the proteins capacity to fulfill the needs of organisms with respect to essential amino acids. From the result, the protein hydrolysate had an essential amino acid/non-essential amino acid ratio of 0.84. Fish and shellfish have been reported to contain the high essential amino acid/non-essential amino acid ratio (Twasaki and Harada 1985). Protein hydrolysate from toothed ponyfish (*Gazza minuta*) muscle had an essential amino acid/non-essential amino acid ratio of 0.93 (Klomklao et al. 2013). Thiansilakul et al. (2007) reported that the round scad protein hydrolysate had an essential amino acid/non-essential amino acid ratio of 0.92. The ratio of essential amino acid/non-essential amino acid of protein hydrolysate from yellowfin tuna (*Thunnus albacores*) head using Alcalase and Protemax was 1.04 and 1.43, respectively (Ovissipour et al. 2010). Starry triggerfish protein hydrolysate contained a low level of proline (3.07%). The presence of proline residues in the centre of the peptides generally contributes to the bitterness. Therefore the peptidase, which can cleave the hydrophobic amino acids and proline, is capable of debittering protein hydrolysate (Capiralla et al. 2002). Hevia and Olcott (1977) reported that bitter peptide from ficin-treated fish concentrate contained glycine, isoleucine, phenyalanine and valine. Thiansilakul et al. (2007) reported that bitter peptide from round scad protein hydrolysate contained leucine, valine, phenylalanine and isoleucine. Since starry triggerfish protein hydrolysate had a high protein content and high essential amino acid/non-essential amino acid ratio, it could be used as a source of protein ingredient for industrial applications.

Color

Color influences the overall acceptability of food products. The freeze-dried protein hydrolysate was light yellow in color (L^* = 82.94, a^* = 0.84, b^* = 22.83) (Table 3). The result was in agreement with Sathivel et al. (2003) who reported that protein hydrolysate from whole herring (*Clupea harengus*) was light yellow in color (L^* = 89.4, a^* = 3.3, b^* = 8.0). However, Thiansilakul et al. (2007) found that round scad protein hydrolysate was brownish yellow in color (L^* = 58.00, a^* = 8.38, b^* = 28.32). The dark color of fish protein hydrolysate was probably from the oxidation of myoglobin and the melanin pigment of the raw materials (Benjakul and Morrissey, 1997). Therefore, the varying color of fish protein hydrolysate depended on the composition of the raw material and the hydrolysis condition.

Table 2. Amino acid composition of freeze-dried hydrolysate produced from starry triggerfish muscle.

Amino acids	Content (%)
Essential amino acids	
Isoleucine	3.72
Leucine	10.27
Lysine	11.67
Methionine	4.85
Phenylalanine	3.47
Threonine	5.77
Valine	5.87
Non-essential amino acids	
Alanine	9.11
Arginine	3.77
Aspartic acid/Asparagine	2.97
Cysteine	0.6
Glutamic acid/Glutamine	8.2
Glycine	15.33
Histidine	2.89
Proline	3.07
Serine	4.52
Tyrosine	3.93
Total	100.01

Table 3. L^*, a^* and b^*-values of freeze-dried hydrolysate produced from starry triggerfish muscle.

Color characteristics	Freeze-dried hydrolysate
L^*	82.94±2.45
a^*	0.82±1.87
b^*	22.83±0.64

Values are mean ± SD from triplicate determination.

Conclusion

The results of this research clearly revealed that low valued marine fish had a potential to be utilized as protein source for producing protein hydrolysates. Optimum conditions for triggerfish muscle hydrolysis were, 5.5% liver extract from albacore tuna, 40 min reaction time and fish muscle/buffer ratio of 1:3 (w/v). Hydrolysate from triggerfish muscle may potentially serve as a good source of desirable peptides and amino acids.

Acknowledgements

This research was supported by the Thailand Research fund and Thaksin University. Authors would like to thank the Office of the Higher Education Commission for financial support. The TRF distinguished research professor grant was also acknowledged.

Reference

AOAC (2005) Official methods of analysis. Association of official analytical chemists international, 18th edn. AOAC International, Gaithersberg

Adler-Nissen J (1986) Enzymatic hydrolysis of food proteins. Elsevier Spplied Science, London

Benjakul S, Morrissey MT (1997) Protein hydrolysates from Pacific whiting solid wastes. J Agric Food Chem 45:3423-3430

Bhaskar N, Mahendrakar NS (2008) Protein hydrolysate from visceral waste proteins of Catla (*Catla catla*): Optimization of hydrolysis condition for a commercial neutral protease. Biores Technol 99:4105-4111

Capiralla H, Hiroi T, Hirokawa T, Maeda S (2002) Purification and characterization of a hydrophobic amino acid-specific endopeptidase from *Halobacterium halobium* S9 with potential application in debittering of protein hydrolysates. Process Biochem 38:571-579

Chalamaiah M, Narsing Rao G, Rao DG, Jyothirmayi T (2010) Protein hydrolysates from meriga (*Cirrhinus mrigala*) egg and evaluation of their functional properties. Food Chem 120:652-657

Chalamaiah M, Dinesh kumar B, Hemalatha R, Jyothirmayi T (2012) Fish protein hydrolysates: Proximate composition, amino acid composition, antioxidant activities and applications: A review. Food Chem 135:3020-3038

Choi YJ, Hur S, Choi BD, Konno K, Park JW (2009) Enzymatic hydrolysis of recovered protein from frozen small croaker and functional properties of its hydrolysates. J Food Sci 74:C17-C24

Cohen SA, Michaud DP (1993) Synthesis of a fluorescent derivatizing reagent, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, and its application for the analysis of hydrolysate amino acids via high-performance liquid chromatography. Anal Biochem 211:279-287

Guerard F, Dufosse L, De La Broise D, Binet A (2001) Enzymatic hydrolysis of proteins from yellowfin tuna (*Thunnus albacores*) wastes using Alcalase. J Molec Cata B 11:1051-1059

Hevia P, Olcott HS (1977) Flavor of enzyme-solubilized fish protein concentrate fractions. J Agric Food Chem 25:772-775

Khantaphant S, Benjakula S, Kishimura H (2011) Antioxidative and ACE inhibitory activities of protein hydrolysates from the muscle of brownstripe red snapper prepared using pyloric caeca and commercial proteases. Process Biochem 46:318-327

Klomklao S, Benjakul S, Visessanguan W, Simpson BK, Kishimura H (2005) Partitioning and recovery of proteinase from tuna spleen by aqueous two-phase systems. Process Biochem 40:3061-3067

Klomklao S, Kishimura H, Yabe M, Benjakul S (2007) Purification and characterization of two pepsins from the stomach of pectoral rattail (*Coryphaenoides pectoralis*). Comp Biochem Physiol 147B:682-689

Klomklao S, Kishimura H, Benjakul S (2013) Use of viscera extract from hybrid catfish (*Clarias macracephalus*×*Clarias gariepinus*) for the production of protein hydrolysate from toothed ponyfish (*Gazza minuta*) muscle. Food Chem 136:1006-1012

Kristinsson HG, Rasco BA (2000) Fish protein hydrolysates: Production, biochemical, and functional properties. Crit Rev Food Sci Nutr 40:43-81

Liaset B, Lied E, Espe M (2000) Enzymatic hydrolysis of by-products from the fish-filleting industry; chemical characterization and nutritional evaluation. J Sci Food Agric 80:581-589

Morr V, German B, Kinsella JE, Regenstein JM, Van Buren JP, Kilara A, Lewis BA, Mangino ME. (1985) A collaborative study to develop a standardized food protein solubility procedure. J Food Sci 50:1715-1718

Nalinanon S, Benjakul S, Visessanguan W, Kishimura H (2008) Partitioning of protease from stomach of albacore tuna (*Thunnus alalunga*) by aqueous two-phase systems. Process Biochem 44: 471-476

Neklyudov AD, Ivankin AN, Berdutina AV (2000) Properties and uses of protein hydrolysates. Applied Biochem Microbiol 36:452–459

Nielsen PM (1997) Functionality of protein hydrolysates. In: Damodaran S, Paraf A (eds) Food proteins and their applications. Marcel Dekker, New York

Ovissipour M, Abedian A, Motamedzadegan A, Rasco B, Safari R, Shahidi H (2009) The effect of enzymatic hydrolysis time and temperature on the properties of protein hydrolysates from Persian sturgeon (*Acipenser persicus*) viscera. Food Chem 115: 238-242

Ovissipour M, Benjakul S, Safari R, Motamedzagan A (2010) Fish protein hydrolysates production from yellowfin tuna *Thunnus albacores* head using Alcalase and Protamex. Int Aquat Res 2: 87-95

Phanturat P, Benjakul S, Visessanguan W, Roytrakul S (2010) Use of pyloric caeca extract from bigeye snapper (*Priacanthus macracanthus*) for the production of gelatin hydrolysate with antioxidative activity. LWT-Food Sci Technol 43:86-97

Senphan T, Benjakul S (2014) Use of the combined phase partitioning systems for recovery of proteases from hepatopancreas of Pacific white shrimp. Sep Purif Technol 129:57-63

Shahidi F (1994) Proteins from seafood processing discards. In: Sikorski ZE,Pan BS, Shahidi F (Eds) Seafood proteins. Chapman & Hall, New York

Shahidi F, Xiao-Qing H, Synowiecki J (1995) Production and characteristics of protein hydrolysates from capelin (*Mallotus villosus*). Food Chem 53:285-293

Sripokar P, Poonsin T, Chaijan M, Benjakul S, Klomklao S (2015) Proteinases from liver of albacore tuna (*Thunnus alalunga*): Optimum extractant and biochemical characteristics. J Food Biochem. Accepted

Steel RGD, Torrie JH (1980) Principles and procedures of statistics: A biometrical approach. McGraw-Hill, New York

Surowka K, Fik M (1994) Studied on the recovery of proteinaceous substrates from chicken heads: II-Application of pepsin to the production of protein hydrolysate. J Sci Food Agric 65:289-286.

Thiansilakul Y, Benjakul S, Shahidi F (2007) Antioxidant activity of protein hydrolysate from round scad muscle using Alcalase. J Food Biochem 31:266-287

Twasaki M, Harada R (1985) Proximate and amino acid composition of the roe and muscle of selected marine species. J Food Sci 50:1585-1587

Wisuthipheat N, Kongruang S (2015) Production of fish protein hydrolysates by acid and enzymatic hydrolysis. J Med Bioeng 4:466-470

Wu HC, Chen HM, Shiau CY (2003) Free amino acid and peptide as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). Food Res Int 36:949-957

Chapter 5

Antioxidant and functional properties of protein hydrolysates obtained from starry triggerfish muscle using trypsin from albacore tuna liver

Biocatal. Agric. Biotechnol.

Pakteera Sripokar[1], Soottawat Benjakul[2], Sappasith Klomklao[3*]

[1]Biotechnology Program, Faculty of Agro- and Bio-Industry, Thaksin University, Phatthalung Campus, Phatthalung, 93210, Thailand

[2]Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla, Thailand

[3]Department of Food Science and Technology, Facculty of Agro- and Bio-Industry, Thaksin University, Phatthalung Campus, Phatthalung, 93210, Thailand

Abstract

Protein hydrolysates from starry triggerfish (*Abalistes stellaris*) muscle with a degree of hydrolysis (DH) of 60% were prepared using trypsin from albacore tuna (*Thunnus alalunga*) liver. The hydrolysates were investigated for antioxidant activity and functional properties. Antioxidant activities including DPPH (2, 2-diphynyl-1-picrylhydrazyl) radical savenging activity, ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activity, ferric reducing antioxidant power (FRAP) and metal chelating activity of hydrolysate samples were dose dependent. After being subjected to gastrointestinal model systems, the DPPH radical scavenging activity and metal chelating activity of the hydrolysates increased, especially in the duodenal condition, suggesting the enhancement of those activities of hydrolysate after ingestion. For functional properties, hydrolysis by trypsin increased protein solubility to above 72.81% over a wide pH range. The hydrolysates possessed interfacial properties, which were governed by their concentrations. An increase in concentration of up to 2.0% (w/v) favored the emulsifying activity index (EAI) and emulsion stability index (ESI), while a further increase to 3.0% (w/v) diminished emulsifying properties. Foam expansion and foam stability increased as the protein concentration increased. Therefore, the results of the present study suggest that starry triggerfish can effectively be converted to protein hydrolysate and could be a potential ingredient in functional food as well as natural antioxidants in lipid food systems.

Keywords : Protein hydrolysate, Starry triggerfish, Antioxidant, Functional Properties

Introduction

Protein demand is growing; driven by a rising population, changing food preferences and a growing recognition of the importance of protein as a key ingredient for health and nutrition. The supplementation of dietary protein has been becoming more popular, especially for people on restrictive diets, athletes and the elderly (Egerton et al. 2018). Worldwide, fish protein hydrolysate (FPH) has been one of the most researched fish products of the last decade. The greater attention emerging towards fish protein hydrolysate is due to their bioactive characteristics and growing global market. Previous studies of FPH have shown that, when added to food, they can contribute to water holding, emulsification and texture properties (Halim et al. 2016). Increased solubility is a frequently reported and valued property of FPH (Benjakul and Yarnpakdee 2014). Bioactive characteristics such as antioxidation, antihypertension, antibacterial and antiprolifiration have also been reported (Halim et al. 2018; Khositanon et al. 2018; Singh and Vij 2018; Song et al. 2016; Senphan and Benjakul 2014; Khantaphant et al. 2011). Antioxadant characteristics can be an important feature for food preservation and providing potential health benefits (Phanturat et al. 2010). Numerous peptides derived from hydrolyzed fish protein have been shown to have antioxidant activities such as protein hydrolysate from the muscle of ornate threadfin bream (Nalinanon et al. 2011), brownstripe red snapper (Khantaphant et al. 2011), toothed ponyfish (Klomklao et al. 2013), seabass (Senphan and Benjakul 2014), whole tilapia waste (Tejpal et al. 2017), blue whiting (Egerton et al. 2018) and eel (Halim et al. 2018). Fish protein hydrolysates can be used in food systems, comparable to other pertinent protein hydrolysates (Kristinsson and Rasco 2000). The use of enzyme technologies for the recovery and modification of protein has led to the production of a broad spectrum of

food ingredients and industrial products (Kristinsson and Rasco 2000). Enzymatic hydrolysis of food proteins is an efficient way to recover potent bioactive peptides (Thiansilakul et al. 2007). Proteases from different sources are commonly employed to obtain a more selective hydrolysis due to their specity for peptide bonds adjacent to certain amino acid residues (Nalinanon et al. 2011: Klomklao et al. 2010).

Thailand is the world's largest producer and exporter of canned tuna. Albacore tuna is one of the most commercially important tuna species for the Thai tuna industry (Klomklao et al. 2018). Because of a large amount of tuna viscera, especially liver, discarded during processing, it can serve as a promising source of trypsin for further use. Trypsin recovery from tuna liver is an approach to minimize the economic and ecological problems of the manufacturing discard. Furthermore, 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 and Benjakul 2008). Trypsin from albacore tuna liver were recently partitioned, purified and characterized (Sripokar et al. 2015; Klomklao et al. 2018).

The starry triggerfish (*Abalistes stellaris*), or flat-tailed triggerfish, belongs to the order Tetraodontiformes and is a member of the Balistidae family (Sripokar et al. 2015b). This fish is used primarily for fish meal and are not considered palatable for direct human consumption in Thailand. To increase the value of this fish species, the production of new value-added products such as protein hydrolysates, with nutritive value and bioactivity, can pave the way for its full utilization. The use of trypsin from albacore tuna liver, for hydrolysate production, could lower the cost of commercial proteases. Based on our previous study, optimum conditions for starry triggerfish muscle hydrolysis using trypsin from albacore tuna liver were 5.5 % trypsin, a 40 min reaction time and a fish muscle/buffer ratio of 1:3 (w/v). However, no information

regarding the antioxidative activity and biochemical properties of starry triggerfish hydrolysate treated with trypsin from albacore tuna liver has been reported. Therefore, the objectives of the present investigation were to study the functionalities and antioxidant properties of protein hydrolysate prepared from starry triggerfish muscle using trypsin from albacore tuna liver.

Materials and Methods

Chemicals

L-leucine, 2,4,6-trinitrobenzenesulfonic acid (TNBS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 3-(2-pyridyl)-5-6-diphenyl-1,2,4-triazine-4',4''-disulphonic acid sodium salt (ferrozine) and bovine serum albumin were procured from Sigma Chemical Co. (St. Louis, MO, USA.). Tris (hydroxymethyl) aminomethane (Tris-HCl), sodium sulfite, potassium persulphate, ferrous chloride and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS) were procured from Bio-Rad Laboratories (Hercules, CA, USA). All of the chemicals used were of analytical grade.

Fish sample preparation

Liver of albacore tuna (*Thunnus alalunga*) were obtained from Tropical Canning (Thailand) Public Co. Ltd., Hat Yai, Songkhla. The samples were packed in polyethylene bags, kept in ice with a sample/ice ratio of 1:3 (w/w) and transported to the research laboratory within 2 h. Pooled internal organs were separated and only the liver was collected. Liver was cut and homogenized into powder in three volumes of acetone at -20 °C for 30 min according to the method of Klomklao et al. (2007). The

homogenate was filtrated in vacuo on Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, UK). The residue obtained was then homogenized in two volumes of acetone at -20 °C for 30 min. The residue was left at room temperature until dried and free of acetone odor.

Starry triggerfish (*Abalistes stellaris*) with a length of 30-35 cm were purchased from a dock in Trang, Thailand. The fish, off-loaded approximately 18-24 h after capture, were placed on ice at a fish/ice ratio of 1:2 (w/w) and transported to the research laboratory within 2 h. Upon arrival, the fish were filleted and the ordinary muscle was collected and ground to uniformity. A portion of mince (500 g) was placed in a polyethylene bag and stored at -20 °C until needed.

Trypsin preparation and activity assay

To prepare the albacore tuna trypsin extract, liver powder was suspended in 50 mM Tris-HCl, pH 8.5, containing 0.2% Brij 35 at a ratio of 1:9 (w/v) and stirred continuously at 4 °C for 30 min. The suspension was centrifuged at 5000×g for 30 min at 4 °C using a Sorvall Modell RC-B Plus centrifuge (Newtown, CT, USA). The supernatant was collected and referred to as “albacore tuna trypsin”.

Albacore tuna trypsin was determined for trypsin activity using TAME as a substrate, as described by Klomklao and Benjakul (2018). Enzyme solution with an appropriate dilution (20 µl) was mixed with 3.0 ml of 1 mM TAME in 10 mM Tris-HCl buffer, pH 8.0 and incubated at 30 °C for 20 min. Production of *p*-tosyl-arginine was measured by monitoring the increment in absorbance at 247 nm. One unit of activity was defined as the amount causing an increase of 1.0 in absorbance at 247 nm per min.

Production of protein hydrolysate from starry triggerfish muscle

Starry triggerfish mince (20 g) was mixed with 0.1 M glycine-NaOH buffer, pH 8.5 at a ratio of 1:3 (w/v) and pre-incubated at 55 °C for 10 min (Klomklao et al. 2013; Sripokar et al. 2015b). The enzyme hydrolysis was started by adding albacore tuna trypsin at a level of 5.5% (w/w). The reaction was conducted at pH 8.5 and 55 °C for 40 min. After 40 min of hydrolysis, the enzyme was inactivated by heating at 90 °C for 15 min in a water bath (Memmert, Schwabach, Germany). The mixture was then centrifuged at 5,000×g at 4 °C for 10 min. The supernatant was then collected and lyophilized using a Dura-TopTM lp freeze-dryer (FTS systems Inc., Stone Ridge, NY, USA). The freeze-dried protein hydrolysate obtained was subjected to analyses.

Determination of α-amino acids and DH

The α-amino acid content and DH was measured according to the method of Klomklao et al. (2013).

Determination of antioxidative activity

The starry triggerfish protein hydrolysate with 60% DH was dissolved in distilled water at obtained concentrations of 5, 10, 20, 30 and 40 mg protein/ml. Antioxidant activity was determined as follows.

DPPH radical-scavenging activity

DPPH radical scavenging activity was determined as described by Nalinanon et al. (2011) and Laywisakul et al. (2017) with a slight modification. For the sample (1.5 ml), 1.5 ml of 0.15 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) in 95% ethanol were added. The mixture was then 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 UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). The blank was prepared in the same manner, except that the distilled water was used instead of the sample. DPPH radical scavenging activity was calculated according to the following equation (Yen and Wu 1999):

$$\text{DPPH radical scavenging activity} = \left(1 - \frac{A_{517} \text{ of sample}}{A_{517} \text{ of control}}\right) \times 100$$

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±0.02 units at 734 nm using a spectrophotometer. Fresh ABTS solution was prepared for each assay. The sample (150µl) was mixed with 2,850 µl 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. ABTS radical scavenging activity was calculated according to the following equation:

$$\text{ABTS radical scavenging activity} = \left(1 - \frac{A_{734} \text{ of sample}}{A_{734} \text{ of control}}\right) \times 100$$

Ferric reducing antioxidant power (FRAP) assay

FRAP was assayed according to the method of Benzie and Strain (1996). Stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3 6H_2O$ solution. A working solution was prepared fresh by mixing 25 ml of acetate buffer, 2.5 ml of TPTZ solution and 2.5 ml of $FeCl_3 6H_2O$ solution. The mixed solution was incubated at 37 °C for 30 min and was referred to as the FRAP solution. A sample (150 μl) was mixed with 2,850 μl of FRAP solution and kept for 30 min in the dark. The ferrous tripyridyltriazine complex (colored product) was measured by reading the absorbance at 593 nm. Increased absorbance of the reaction mixture indicates the increasing ferric reducing antioxidant power.

Metal chelating activity

The chelating activity on Fe^{2+} was determined using the method of Boyer and McCleary (1987) with a slight modification. The 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 measured at 562 nm. The blank was conducted in the same manner but distilled water was used instead of the sample. The chelating activity was calculated as follows:

$$\text{Chelating activity} = \frac{(1 - A_{562} \text{ of sample})}{A_{562} \text{ of control}} \times 100$$

pH and thermal stability of antioxidant peptides

Starry triggerfish protein hydrolysate was dissolved in distilled water to obtain the concentration of 40 mg protein/ml. The 5 ml of sample solutions were adjusted to

pHs 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 with 1 or 6 M HCl and 1 or 6 M NaOH and incubated at room temperature for 30 min. The pHs of sample solutions were then adjusted to 7.0 with 1 M phosphate buffer. The final volume of all solutions was brought up to 20 ml using distilled water. The residual antioxidant activities were determined using DPPH and metal chelating assay.

To determine thermal stability, starry triggerfish protein hydrolysate at a concentration of 40 mg protein/ml was prepared using distilled water as a medium. The 5 ml of sample solutions 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, 45, 60, 90, 120, 150 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 using DPPH and metal chelating assay.

Stability in gastrointestinal tract model system

Gastrointestinal tract model system was prepared according to the method of Lo et al. (2006) with a slight modification. The hydrolysate was dissolved in distilled water to obtain a concentration of 0.5 g/ml. The solution was adjusted to pH 2.0 with 1 M HCl and pepsin dissolved in 0.1 M HCl was added to obtain the final concentration of 40 g pepsin/kg hydrolysate. The mixture was incubated at 37 °C for 1 h with a continuous shaking (Memmert Model SV 1422, Schwabach, Germany). Thereafter, the pH of the reaction mixture was raised to 5.3 with 1 M NaOH before the addition of 20 g pancreatin/kg hydrolysate. Subsequently, the pH of the mixture was adjusted to 7.5 with 1 M NaOH. The mixture was incubated at 37 °C for 3 h with a continuous shaking. The digestion was terminated by submerging the mixture in boiling water for 10 min. During digestion, the mixture was randomly taken at 0, 15,

30, 60, 90, 120, 150, 180 and 210 min for determination of DPPH radical scavenging activity and metal chelating activity.

Functional properties of protein hydrolysate

Solubility

To determine protein solubility, starry triggerfish hydrolysate samples (200 mg) were dispersed in 20 ml of deionized water and the 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 25 ml by distilled water, previously adjusted to the same pH as the sample solution, prior to centrifugation at 5,000×g for 15 min. Protein content in the supernatant was determined using the Lowry method (Lowry et al. 1951), with bovine serum albumin as a standard. Total protein content in the same was determined after solubilization of the sample in 0.5 M NaOH. Protein solubility was calculated as follows:

$$\text{Solubility (\%)} = \frac{\text{protein content in supernatant}}{\text{total protein content in sample}} \times 100$$

Emulsifying properties

The emulsifying activity index (EAI) and the emulsion stability index (ESI) were used to measure the emulsifying properties of protein hydrolysate. EAI and ESI were determined according to the method of Pearce and Kinsella (1978) with a slight modification. Soybean oil (2 ml) and protein hydrolysate solutions (0.5%, 1.0%, 2.0% and 3.0%, 6 ml) were homoginized (Model T25 basic; IKA Labortecnik, Selangor, Malaysia) at a speed of 20,000 rpm for 1 min. An aliquot of the emulsion (50 μl) was

pipette from the middle portion of the container at 0 and 10 min after homogenization 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), ϕ = oil volume fraction and C = protein concentration in aqueous phase (g/m3);

ESI (min) = $A_0 \times \Delta t/\Delta A$

where $\Delta A = A_0 - A_{10}$ and $\Delta t = 10$ min.

Foaming properties

Foam expansion (FE) and foam stability (FS) of hydrolysate solutions were determined according to the method of Shahidi et al. (1995) with a slight modification. Hydrolysate solutions (20 ml) with 0.5%, 1.0%, 2.0% and 3.0% protein concentrations were transferred into a 100-ml cylinder. The solutions were homogenized at 13,400 rpm for 1 min at room temperature. The samples were allowed to stand for 0, 30 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 different times (30 and 60 min).

Protein determination

Protein concentration was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Statistical analysis

Experiments were run in triplicate using three different lots of samples. All data were subjected to analysis of variance (ANOVA) and differences between means were evaluated by Duncan's multiple range test (Steel and Torrie 1980). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA).

Results and discussion

Antioxidative activities

DPPH radical-scavenging activity

The relatively stable DPPH radical has been widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and thus to evaluate the antioxidant activity (Jao and Ko 2002). The DPPH radical scavenging activity of protein hydrolysate from starry triggerfish muscle prepared using albacore tuna trypsin with the DH of 60% was measured at different concentrations (0, 5, 10, 20, 30 and 40 mg/ml) and the results are shown in Fig. 1. The DPPH radical scavenging activity increased as the concentration of protein hydrolysate increased up to 20 mg/ml ($p<0.05$). However, no differences in activity were observed as concentration increased from 30 to 40 mg/ml. DPPH is a stable free radical that exhibits a maximal absorbance at 517 nm in ethanol. When DPPH encounters a proton-donating substance, such as an antioxidant, the radical is scavenged. The color is changed from

purple to yellow and the absorbance is decreased (Khantaphant and Benjakul 2008). Hence, starry triggerfish hydrolysate obtained could donate hydrogen atom to free radicals and become more stable diamagnetic molecule, leading to the termination of the radical chain reaction (Khantaphant and Benjakul 2008). Nevertheless, the efficiency in hydrogen donation of peptides produced was governed by their concentration.

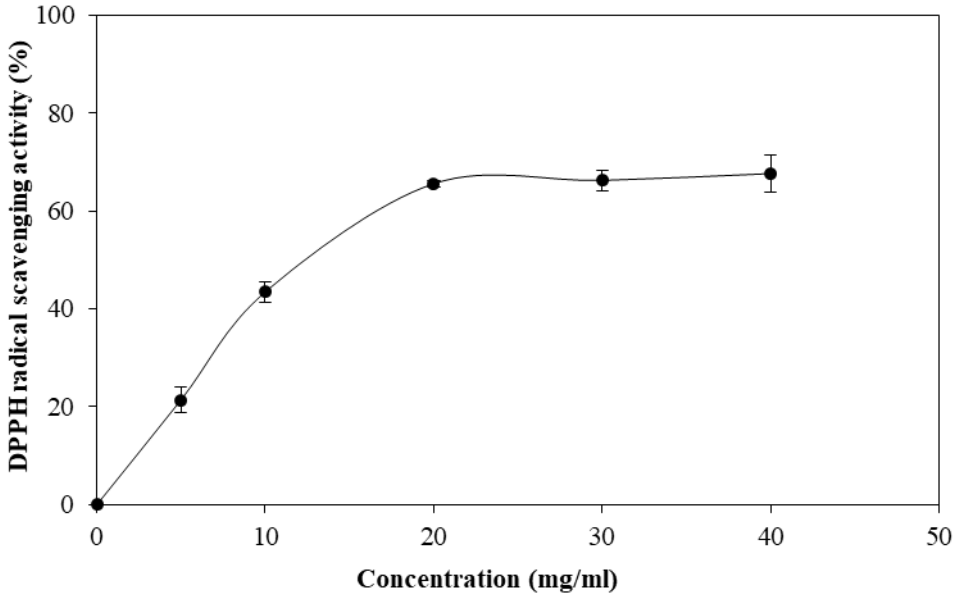

Fig. 1 DPPH radical scavenging activity of starry triggerfish protein hydrolysate at different concentrations. Bars represent the standard deviation from triplicate determinations.

ABTS radical-scavenging activity

ABTS radical assay determine the antioxidative activity, in which the radical is quenched to form ABTS-radical complex (Khantaphant and Benjakul 2008). With increasing protein concentration, protein hydrolysate showed increased ABTS radical scavenging activity ($p<0.05$) (Fig. 2). The highest ABTS radical scavenging activity was found in hydrolysate with 40 mg/ml (47%). 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 amino acid sequence in peptides might affect the antioxidant activity. The ABTS radical is relatively stable and is readily decreased by antioxidants (Klomklao et al. 2013). With high ABTS radical scavenging activity, it was postulated that antioxidative compounds were most likely hydrophilic.

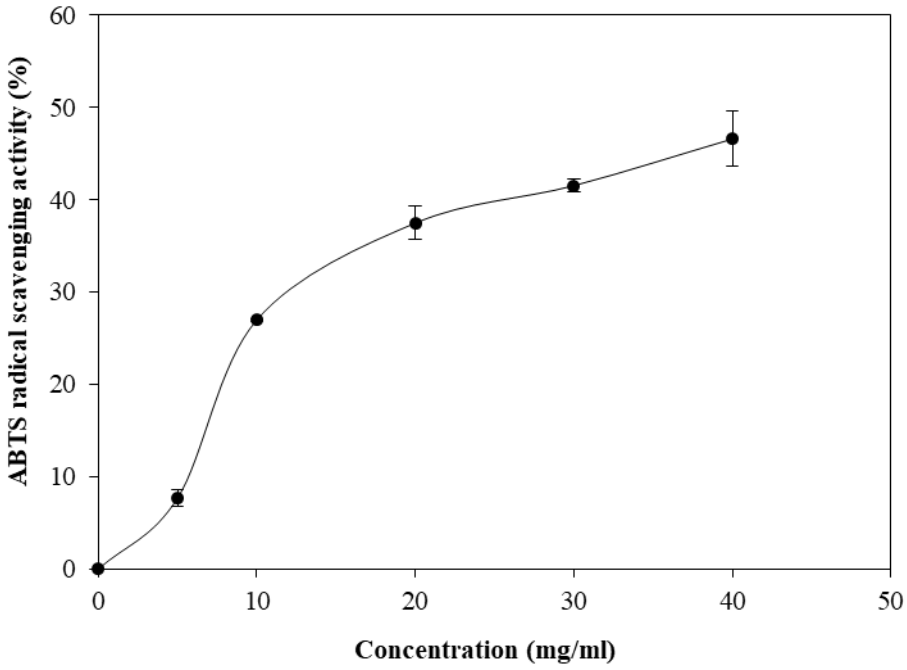

Fig. 2 ABTS radical scavenging activity of starry triggerfish protein hydrolysate at different concentrations. Bars represent the standard deviation from triplicate determinations.

Ferric reducing antioxidant power (FRAP)

FRAP of starry triggerfish protein hydrolysate were investigated at different concentrations ranging from 0 to 40 mg/ml and the results are illustrated in Fig 3. FRAP increased significantly with the increase in the concentration of hydrolysate. The greater reducing power indicated that hydrolysates could donate an electron to free radicals, leading to the prevention or retardation of propagation (Klomklao et al. 2013). Results obtained were in agreement with the published reports (Intarasirisawat et al. 2012; Tejpal et al. 2017). FRAP is generally used to measure the capacity of a substance in reducing TPTZ-Fe(III) complex to TPTZ-Fe(II) complex (Binsan et al. 2008). In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each compound (Thiansilakul et al. 2007). The results suggested that antioxidative compounds in protein hydrolysate tested showed higher FRAP when higher concentrations were used. The differences in FRAP might be governed by peptides in the hydrolysates. The reducing power of starry triggerfish protein hydrolysate could be used to reduce DNA damage, mutagenesis, careinogenesis and inhibition of pathogenic bacterial growth (Gulcin et al. 2010).

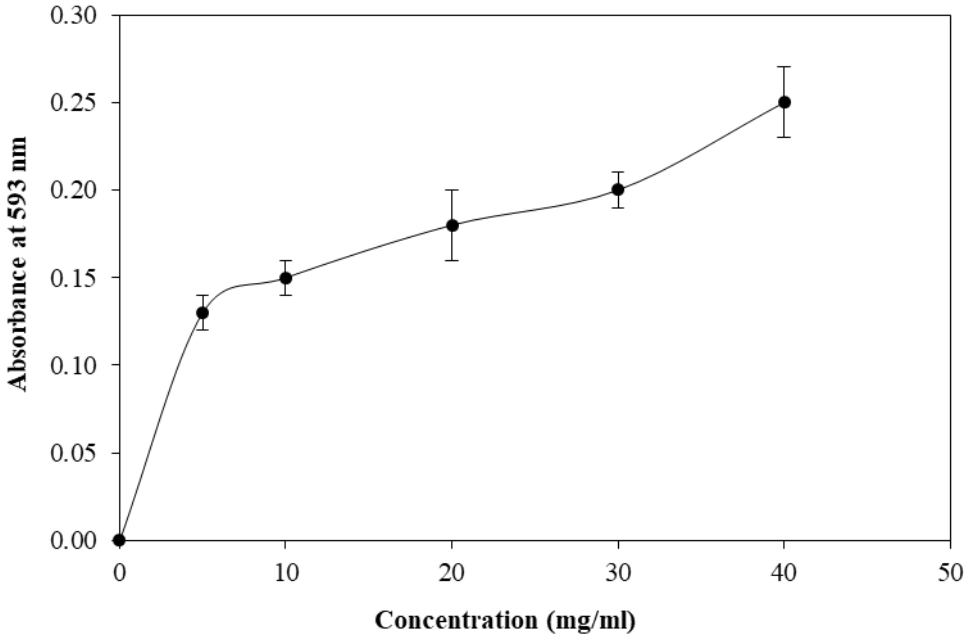

Fig. 3 Ferric reducing antioxidant power of starry triggerfish protein hydrolysate at different concentrations. Bars represent the standard deviation from triplicate determinations.

Metal chelating activity

As the concentration increased up to 20 mg/ml, chelating activity on Fe^{2+} of starry triggerfish protein hydrolysate increased ($p<0.05$) (Fig. 4). However, no changes of metal chelating activity of hydrolysates with a concentration of more than 20 mg/ml was observed. Peptides in hydrolysates could chelate the prooxidants, leading to decreased lipid oxidation (Klomklao et al. 2013). The chelation of Fe^{2+} represents the ability of hydrolysates in metal chelating (Nalinanon et al. 2011). Ferrozine quantitatively forms complexes with Fe^{2+} ion. In the presence of chelating agents the complex formation is disrupted, affecting the decrease in color formation (Thiansilakul et al. 2007). The chelation of transition metal ions by an antioxidant or antioxidative peptide would reduce the availability of pro-oxidative metal ions, and could also by this means reduce lipid oxidation (Thammarat et al. 2015). From the results, hydrolysates from starry triggerfish muscle treated with trypsin from albacore tuna liver had a potential chelating ability toward iron and the metal chelating activity of starry triggerfish protein hydrolysate were dose dependent.

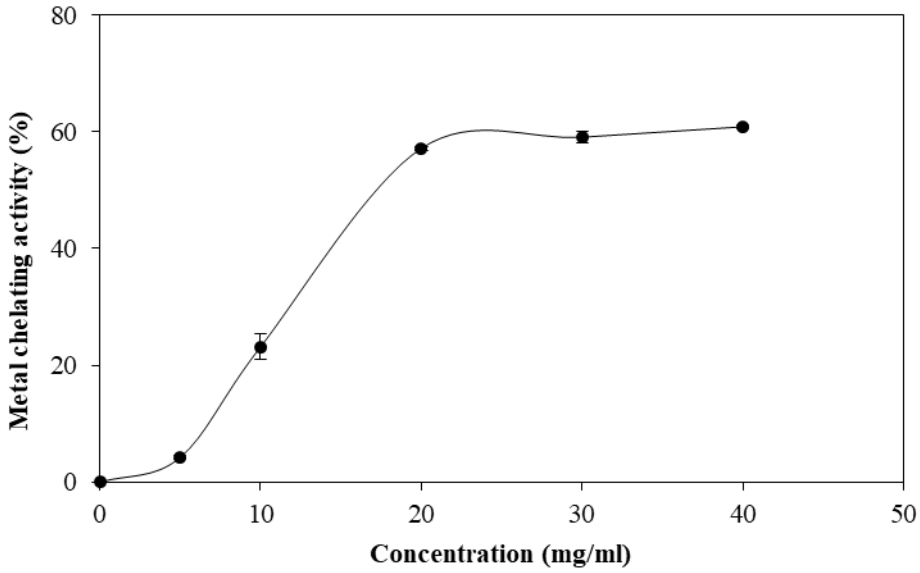

Fig. 4 Metal chelating activity of starry triggerfish protein hydrolysate at different concentrations. Bars represent the standard deviation from triplicate determinations.

Functional properties of protein hydrolysate

Protein solubility

The protein solubilities of the lyophilized protein hydrolysates from starry triggerfish muscle obtained from trypsin from albacore tuna liver was determined at pH 3.0, 5.0, 7.0 and 9.0 and the results are shown in Fig 5. All hydrolysates were soluble over a wide pH range, in which more than 72% solubility was obtained. The minimum solubility values were presented at pH 5.0 (72.81% solubility) and maximum solubility values at pH 3.0 (93.97% solubility) were observed ($p<0.05$). The change in solubility can be attributed to the net load of the amino acid residues after the hydrolysis process, which increases as the pH moves away from the isoelectric point, promoting the aggregation of hydrophobic interaction (Taheri et al. 2013). The result suggested that proteins or peptides remaining after hydrolysis were precipitated at pH 5.0, which was close to the isoelectric point (pI) of myofibrillar proteins (Klomklao et al. 2013). The solubilities of protein hydrolysates were quite low at pH 4 as reported in salmon by products (Gbogouri et al. 2004) and yellow stripe (Klompong et al. 2007). The pH affects the charge on the weakly acidic and basic side chain groups and hydrolysates generally show low solubility at their isoelectric points (Naqash and Nazeer 2013). Solubility is one of the most important functional properties of a protein and can be increased by the hydrolysis process (Klompong et al. 2007). Good solubility of proteins is required in many functional applications, especially for emulsions, foams and gels. Soluble proteins provide a homogeneous dispersibility of the molecules in colloidal systems and enhance the interfacial properties (Zayas 1997). Enzymatic hydrolysis potentially affects the molecular size and hydrophobicity, as well as polar and ionizable groups of protein

hydrolysates (Klompong et al. 2007). The balance of hydrophilic and hydrophobic forces of peptides is another crucial influence on solubility (Gbogouri et al. 2004).

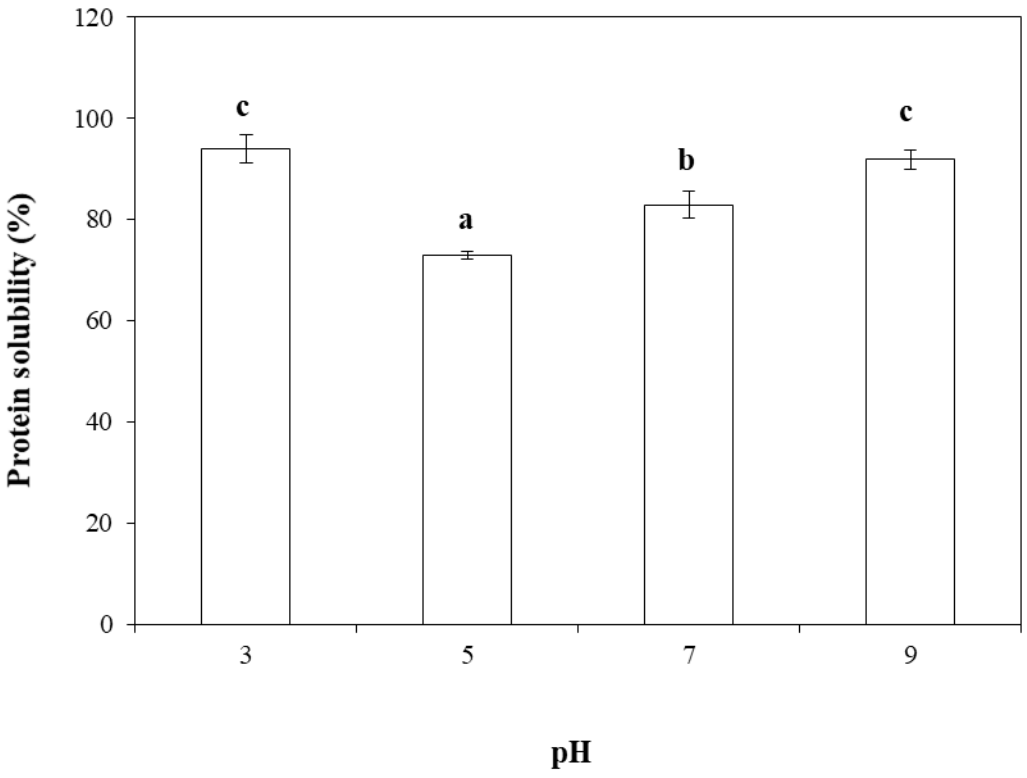

Fig. 5 Solubility of starry triggerfish protein hydrolysates at various pHs. Bars represent the standard deviation from triplicate determinations.

Emulsifying properties

Emulsifying activity index (EAI) and emulsion stability index (ESI) of hydrolysates from starry triggerfish muscle with different concentrations (0.5%, 1.0%, 2.0% and 3.0% w/v) are shown in Fig 6. Both EAI and ESI were concentration-dependent (Fig 6a, 6b). An increase in concentration favoured emulsifying activity up to 2.0% (w/v) concentration, while a further increase in concentration at 3.0% (w/v) reduced emulsifying activity (Fig 6a). Similarly, the initial increase in protein concentration favoured increase emulsion stability up to 2.0%, after which a decline was observed with a further increase in concentration (Fig 6b). The initial increase in protein concentration facilitated enhanced interaction between the oil phase and the aqueous phase. However, as the concentration increased, a point was reached where a further increase in protein concentration led to an accumulation of proteins in the aqueous phase. This development resulted in a decrease of emulsifying activity (Lawal 2004). Emulsion stability diminished after 2.0%, presumably due to an increase in protein-protein interaction at the expense of protein-oil interaction. 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) stabilization of this newly-formed interface by emulsifier or surfactant. Protein hydrolysates are surface-active materials and promote an oil-in-water emulsion due to their hydrophilic and hydrophobic groups and their charge (Kristinsson and Rasco 2000; Klomklao et al. 2013). From the results, emulsifying characteristics of hydrolysate from starry triggerfish muscle with 60% DH were governed by concentration employed.

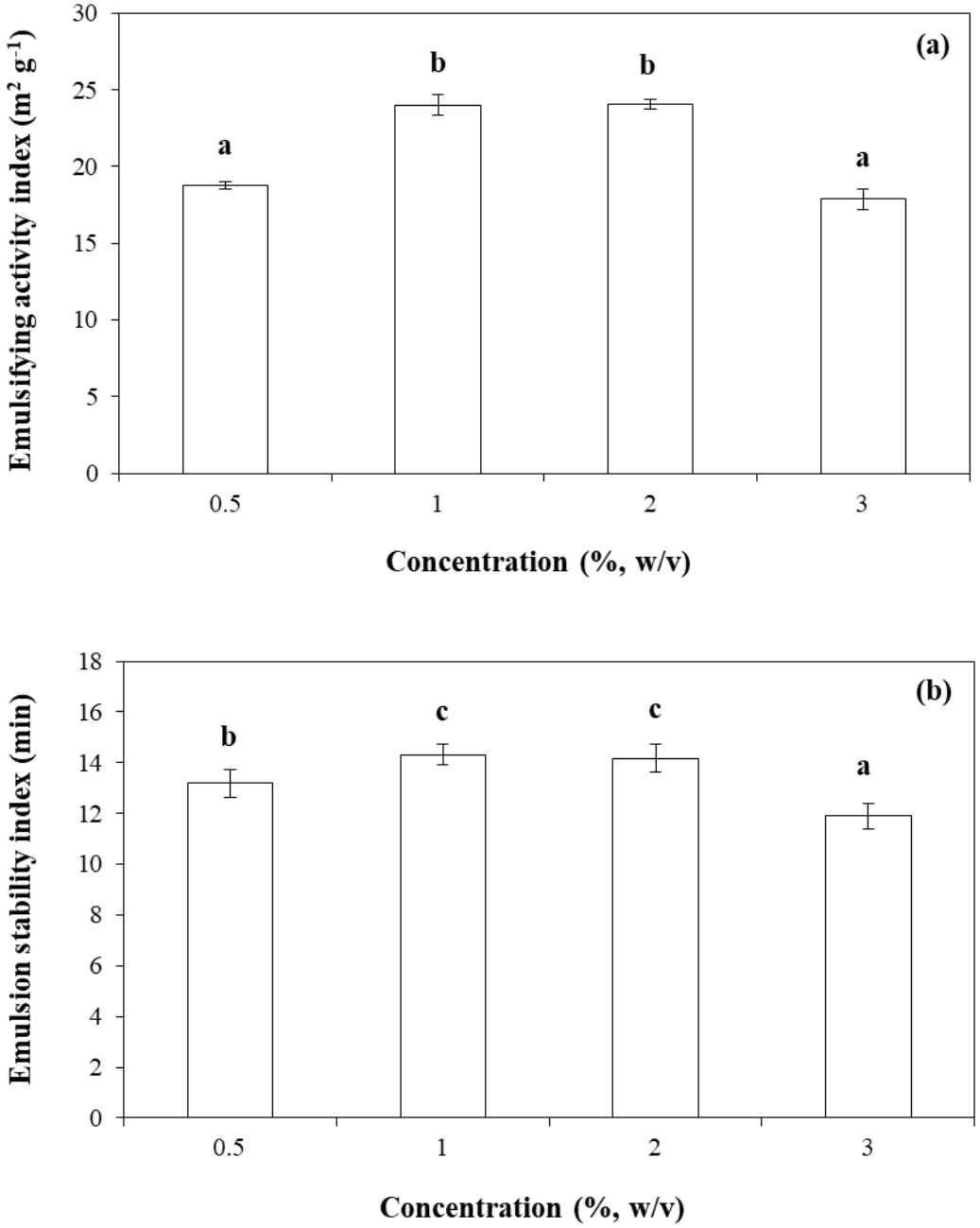

Fig. 6 Effect of concentration of starry triggerfish protein hydrolysate on emulsifying activity index (a) and emulsion stability index (b). Bars represent the standard deviation from triplicate determinations.

Foaming properties

Foam expansion and foam stability of starry triggerfish protein hydrolysates at various concentrations (0.5%, 1.0%, 2.0% and 3.0%) are depicted in Table 1. Foam expansion at 0 min after whipping indicated the foam abilities of protein hydrolysates, which increased from 128.89% to 208.89% when hydrolysate concentrations increased from 0.5% to 3.0% ($p<0.05$). Sanchez and Panito (2005) reported that an increase in protein concentration resulted in a higher rate of diffusion. Foam expansion after whipping was monitored for 30 and 60 min to indicate the foam stability of protein hydrolysates. Formation of foam is governed by three factors, including transportation, penetration and reorganization of molecules at the air-water interface (Klomklao et al. 2013). Foam expansion after whipping for 30 and 60 min was monitored to indicate the foam stability of starry triggerfish protein hydrolysate. Foam stability was increased with increasing concentration of the hydrolysates. The results suggest that foam stability of the hydrolysates from starry triggerfish muscle was improved by increasing concentration. A similar result was also reported in protein hydrolysates from toothed ponyfish muscle produced with viscera extract from hybrid catfish (Klomklao et al. 2013). Foam stability depends on the nature of the film and reflects the extant of protein-protein interaction within the matrix (Mutilangi et al. 1996). Foam stability is enhanced by flexible protein domains, which enhance viscosity of the aqueous phase, protein concentration and film thickness (Phillips et al. 1994).

Table 1 Foaming properties of starry triggerfish protein hydrolysate at varying concentrations

Hydrolysate concentrations (%, w/v)	FE* (%)	FS* (%)	
		30 min	60 min
0.5	128.89±1.92a**	118.89±1.92a	97.78±3.85a
1.0	147.78±1.94b	131.48±1.70b	115.56±5.09b
2.0	187.78±1.92c	154.44±1.92c	122.20±8.39bc
3.0	208.89±10.18d	170.00±8.82d	130.00±3.33c

*Mean ± SD from triplicate determinations.

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

pH and thermal stability of antioxidant peptides

The stability of bioactive peptides to pH is an important criteria for GI stability, because food encounters different pH at different digestion stages. Gastric pH is considered among one of the important factors affecting the survival of bioactive molecules during their passage through the stomach (Singh and Vij 2018). The pH in the human stomach ranges from 2 to 5, and food will take at least 2 h to pass through stomach after ingestion (Plessas et al. 2017). On the other hand, bile in the large intestine maintains an almost neutral pH (Plessas et al. 2017). The influences of pH on the stability of antioxidant peptides are depicted in Fig. 7a. DPPH radical-scavenging activity and metal chelating activity of the antioxidant peptide remained constant over the pH range of 1-10. At pH 11, DPPH radical-scavenging activity and metal chelating activity slightly decreased ($p<0.05$). The results suggested that antioxidant peptides exhibiting DPPH radical-scavenging activity and metal chelating activity might lose their activity to some extent at high pH. The loss of activities at this pH may be because of alkaline hydrolysis which affects the amount, size, structure, amino acid composition and hydrophobic of peptides.

Thermal stability of bioactive peptides are important because food products undergo several heat treatments before they reach the market. Generally, thermal treatment can cause protein denaturation, association, and aggregation (Singh and Vij 2018). Thermal stability of antioxidant activity of the starry triggerfish protein hydrolysates with 60% DH as monitored by DPPH radical-scavenging activity and metal chelating activity assay is shown in Fig. 7b. DPPH radical-scavenging activity and metal chelating activity of hydrolysate were stable when heated at 100 °C up to 180 min, where activities of 100% were retained. In general, proteins are heat sensitive, which can lead to their aggregation. However, it has been reported that low

molecular weight peptides are heat-stable (Nalinanon et al. 2011). Smaller size peptides were more stable to aggregation at high temperatures (Zayas 1997). These results indicate that starry triggerfish protein hydrolysates with 60% DH could be incorporated in cooked food systems without a significant loss of their antioxidant activities.

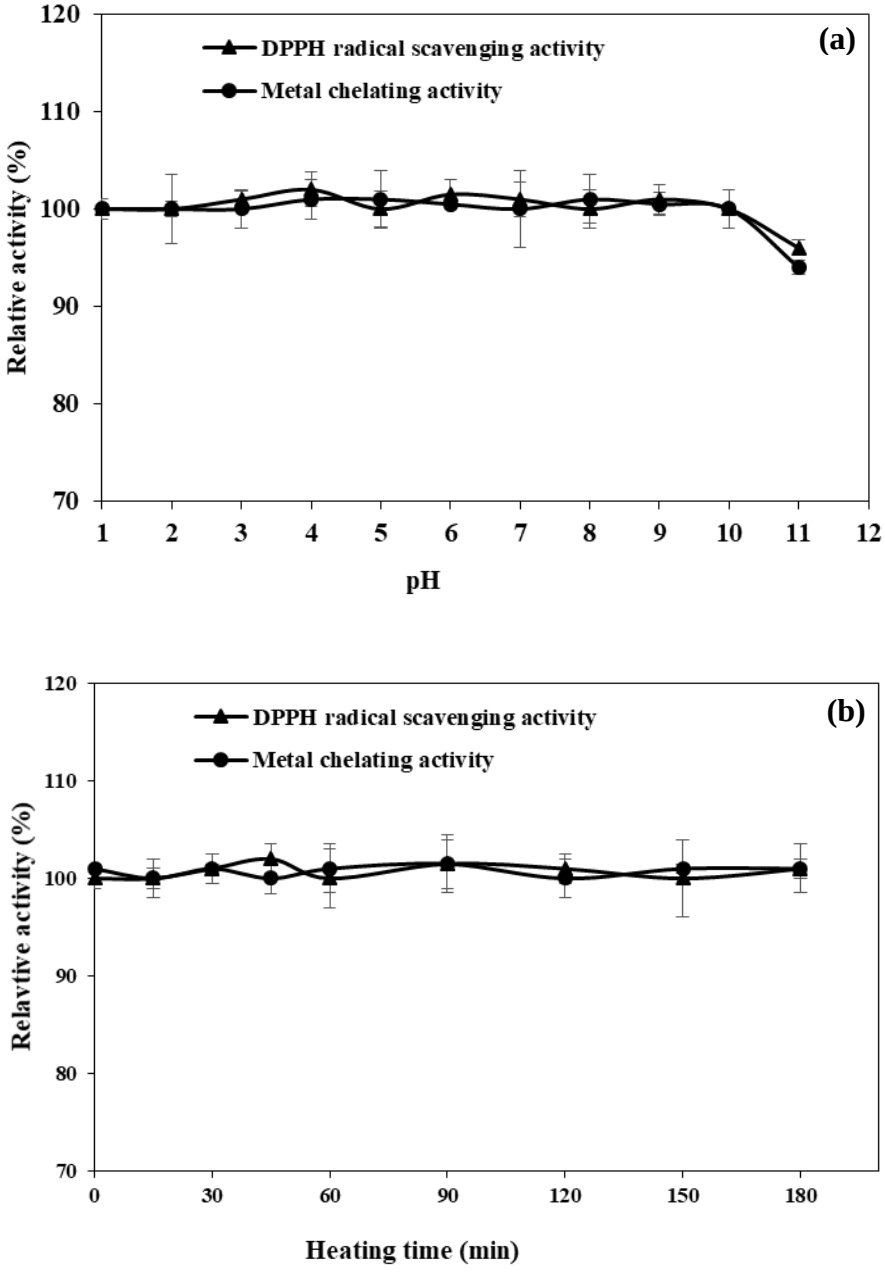

Fig. 7 Effect of pH (a) and heating time (b) on DPPH radical scavenging activity and metal chelating activity of starry triggerfish protein hydrolysate. Bars represent the standard deviation from triplicate determinations.

Changes in antioxidative activity in gastrointestinal tract model systems (GIMs)

GIMs was used to simulate the ingestion system of human body and the remaining antioxidative activities of starry triggerfish protein hydrolysate were monitored (Fig. 8). When protein hydrolysate was orally administrated, their bioactive peptides should be resistant to hydrolysis by digestive proteases in order to be adsorbed and reach the target organ to function as an antioxidant (Burkitt 2001). Starry triggerfish protein hydrolysates showed a slight increase in DPPH radical scavenging activity and metal chelating activity during pepsin digestion ($p<0.05$). Pepsin might hydrolyze starry triggerfish protein hydrolysate to some degrees, yielding the new peptides with DPPH radical scavenging activity and metal chelating activity. With further hydrolysis in the intestinal simulated system, mark increases in DPPH radical scavenging and metal chelating activity were obtained within 90 min under duodenal conditions. Thereafter, no changes in DPPH radical scavenging activity and metal chelating were found during 150 and 210 min of incubation ($p>0.05$). 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 protein hydrolysates. Generally, gastrointestinal tract actually leads to the generation of more potent bioactive peptides (Megías et al. 2009). Khantaphant et al. (2011) found increased antioxidative activity of protein hydrolysate from the muscle of brownstripe red snapper using flavourzyme after being ingested in the simulated model system. Senphan and Benjakul (2014) also reported that when the hydrolysates from seabass skin prepared using ammonium sulphate precipitated fraction from Pacific white shrimp hepatopancreas with 40% DH was subjected to GIMs, ABTS radical scavenging activity and chelating activity increased, especially in the duodenal condition. The antioxidative activities of protein

hydrolysates after incubation in GIMs was dependent on peptides in the hydrolysate, in terms of size, amino acid composition and sequence, which could be targeted by digestive proteases (Megías et al. 2009). From the results, the antioxidative activity of starry triggerfish hydrolysate could be preserved after treatment with these gastrointestinal enzymes in GIMs. Therefore, the antioxidative activities of starry triggerfish hydrolysate were more likely preserved after digestion in the real gastrointestinal tract of the human body.

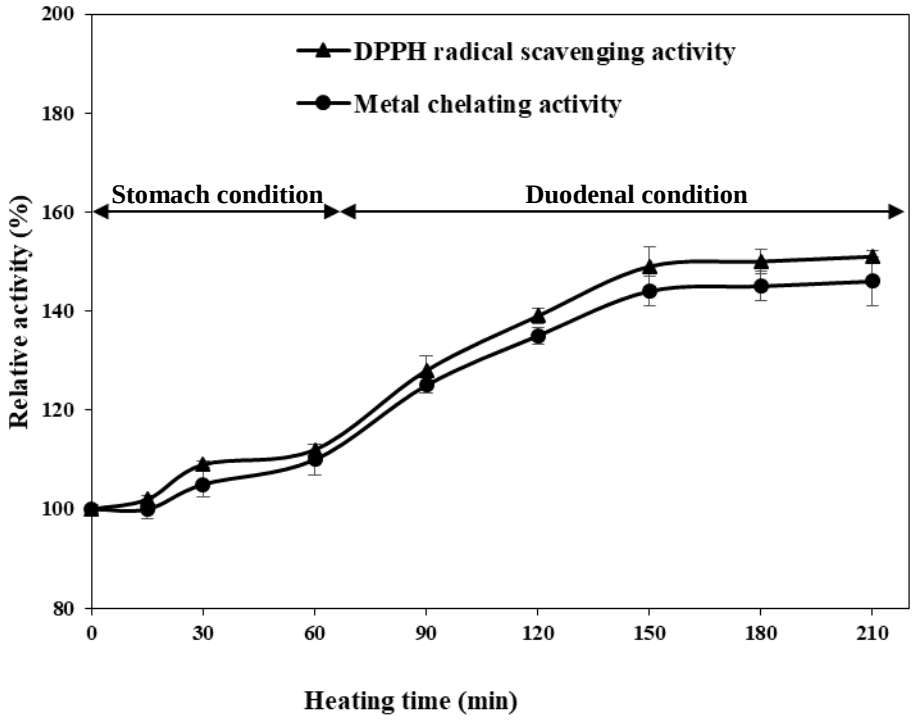

Fig. 8 Antioxidative activities of hydrolysate from starry triggerfish protein hydrolysate in gastrointestinal tract model system. Bars represent the standard deviation from triplicate determinations.

Conclusion

The protein hydrolysates derived from starry triggerfish using trypsin from albacore tuna liver appear to be good sources of desirable peptides. The protein

hydrolysate could be used as an emulsifier and as a foaming agent with antioxidant activities. Hence, starry triggerfish protein hydrolysate can be used in food systems as a natural additive possessing antioxidative properties. Furthermore, 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.

Acknowledgments

This research was supported by the Thailand Research Fund and Thaksin University for Project No. RSA6080044 to Dr. Sappasith Klomklao.

References

Adler-Nissen J. 1986. Enzymatic hydrolysis of food proteins. London: Elsevier Supplied Science.

Benjakul S, Yarnpakdee S, Senphan T, Halldorsdottir SM, Kristinsson HG. 2014. Fish protein hydrolysates: Antioxidants and Functional Components in Aquatic Foods.

Benzie IFF, Strain JJ. 1996. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power the FRAP assay. Anal Biochem. 239:70-76.

Binsan W, Benjakul S, Visessanguan W, Roytrakul S, Tanaka M, Kishimura H. 2008. Antioxidative activity of Mungoong, an extract paste, from the caphalothorax of white shrimp (*Litopenaeus vannamei*). Food Chem 106:185-193.

Boyer RF, McCleary CJ. 1987. Superoxide ion as a primary reductant in ascorbate-mediated ferritin iron release. Free Rad Biol Med. 3:389-395.

Burkitt M. 2001. A critical overview of the chemistry of copper-dependent low density lipoprotein oxidation: roles of lipid hydroperoxides, α-tocopherol, thiol, and ceruloplasmin. Arch Biochem Biophys 394:117-135.

Damodaran S. 1997. Protein-stabilized foams and emulsions. In: Damodaran S, Paraf A, Eds. Food protein and their applications. New York: Marcel Dekker, 57-110.

Dickinson E. 1989. Surface and emulsifying properties of caseins. J Dairy Res 56:471-477.

Egerton S, Culloty S, Whooley J, Stanton C, Ross RP. 2018. Characterization of protein hydrolysates from blue whiting (*Micromesistius poutassou*) and their application in beverage fortification. Food Chem. 245:698-706.

Gbogouri GA, Linder M, Fanni J, Parmentier M. 2004. Influence of hydrolysis degree on the functional properties of salmon byproducts hydrolysates. J Food Sci. 69:C615-622.

Gomez-Ruiz JA, Lopez-Exposito I, Pihlanto A, Ramos M, Recio I. 2008. Antioxidant activity of ovine casein hydrolysates: identification of active peptides by HPLC-MS/MS. Eur Food Res Technol. 227:1061-1067.

Halim NRA, Yusof HM, Sarbon NM. 2016. Functional and bioactive properties of fish protein hydolysates and peptides: A comprehensive review. Trends Food Sci Technol. 51:24-33.

Halim NRA, Azlan A, Yusof HM, Sarbon NM. 2018. Antioxidant and anticancer activities of enzymatic eel (*monopterus* sp) protein hydrolysate as influenced by different molecular weight. Biocatal Agric Biotechnol. 16:10-16.

Intarasirisawat R, Benjakul S, Visessanguan W, Wu J. 2012. Antioxidative and functional properties of protein hydrolysate from defatted skipjack (*Katsuwonous pelamis*) roe. Food Chem. 135:3039-3048.

Jao CL, Ko WC. 2002. 1,1-dipheny1-2-picrylhydrazyl (DPPH) radical scavenging by protein hydrolyzates from Tuna cooking juice. Fisheries Sci. 68:430-435

Khantaphant S, Benjakul S. 2008. Comparative study on the proteases from fish pyloric caeca and the use for production of gelatin hydrolysate with antioxidative activity. Comp Biochem Physiol B. 151:410-419.

Khantaphant S, Benjakul S, Kishimura H. 2011. Antioxidative and ACE inhibitory activities of protein hydrolysates from the muscle of brownstripe red snapper prepared using pyloric caeca and commercial proteases. Process Biochem. 46:318-327.

Khositanon P, Inpratom D, Somwang T, Lawsipo P, Roytrakul S, Choksawangkarn W. 2018. Antibacterial and anticancer activities of protein hydrolysate from fish sauce byproduct. The 6th 1 International Conference on Biochemistry and Molecular Biology.

Klomklao S, Benjakul S, Visessanguan W, Simpson BK, Kishimura H. 2005. Partitioning and recovery of proteinase from tuna spleen by aqueous two-phase systems. Process Biochem. 40:3061-3067.

Klomklao S, Benjakul S, Visessanguan W, Kishimura H, Simpson BK. 2007. Purification and characterization of trypsins from the spleen of skipjack tuna (*Katsuwonus pelamis*). Food Chem. 100:1580-1589.

Klomklao S, Benjakul S, Kishimura H, Osaka K, Tanaka M. 2010. Effect of salts and polyethylene glycol on the partitioning and recovery of trypsin from hybrid catfish viscera in aqueous two phase systems. J Food Biochem. 34:730-747.

Klomklao S, Benjakul S, Kishimura H. 2013. Functional properties and antioxidative activity of protein hydrolysates from toothed ponyfish muscle treated with viscera extract from hybrid catfish. Int J Food Sci Technol. 48:1483-1489.

Klomklao S, Benjakul S. 2018. Two trypsin isoforms from albacore tuna (*Thunnus alalunga*) liver: Purification and physicochemical and biochemical characterization. Int J Biol Macromol. 107:1864-1870.

Klompong V, Benjakul S, Kantachote D, Shahidi F. 2007. Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. Food Chem. 102:1317-1327.

Klompong V, Benjakul S, Kantachote D, Hayes KD, Shahidi F. 2008. Comparative study on antioxidative activity of yellow stripe trevally protein hydrolysate produced from Allcalase and Flavourzyme. Int J Food Sci Technol. 43:1019-1026.

Kristinsson HG, Rasco BA. 2000. Fish protein hydrolysates: production, biochemical and functional properties. Crit Rev Food Sci Nutr. 40:43-81.

Lawal OS. 2004. Functionality of African locust bean (*Parkia biglobosa*) protein isolate: effects of pH, ionic strength and various protein concentrations. Food Chem. 86:345-355.

Laywisadkul S, Weerawatanakorn M, Maneerattanarungrojn C, Sujipuli K. 2017. Investigating the antioxidant and preventing DNA-damage properties of various honeys in Phitsanulok province. Thaksin U J. 5:93-103.

Li Y, Jiang B, Zhang T, Mu Y, Liu J. 2008. Antioxidant and free radical-scavenging activities of chickpea protein hydrolysate (CPH). Food Chem. 106:444-450.

Lin LH, Chen KM. 2006. Preparation and surface activity of gelatin derivative surfactants. Coll Surf. 272:8-14.

Lo WMY, Farnworth ER, Li-Chan ECY. 2006. Angiotensin I-converting enzyme inhibitory activity of soy protein digests in a dynamic model system simulating the upper gastrointestinal tract. J Food Sci. 71:231-237.

Lowry OH, Rosebrough NJ, Fan AL, Randall RJ. 1951. Protein measurement with Folin phenol reagent. J Biol Chem. 193:256-275.

Megías C, Pedroche J, Yust MDM, Alaiz M, Girón-Calle J, Millán F. 2009. Stability of sunflower protein hydrolysates in simulated gastric and intestinal fluids and Cao-2 cell extracts. LWT-Food Sci Technol. 42:1496-1500.

Mutilangi WAM, Panyam D, Kilara A. 1996. Functional properties of hydrolysates from proteolysis of heat-denatured whey protein isolate. J Food Sci. 61:270-275.

Nalinanon S, Benjakul S, Kishimura H, Shahidi F. 2011. Functionalities and antioxidant properties of protein hydrolysates from the muscle of ornate threadfin bream treated with pepsin from skipjack tuna. Food Chem. 124:1354-1362.

Naqash SY, Nazeer RA. 2013. Antioxidant and functional properties of protein hydrolysates from pink perch (*Nemipterus japonicas*) muscle. J Food Sci Technol. 50:972-978.

Pearce KN, Kinsella JE. 1978. Emulsifying properties of proteins: evaluation of a turbidimetric technique. J Agric Food Chem. 26:716-723.

Phanturat P, Benjakul S, Visessanguan W, Roytrakul S. 2010. Use of pyloric caeca extract from bigeye snapper (*Priacanthus macracanthus*) for the production of

gelatin hydrolysate with antioxidative activity. LWT-Food Sci Technol. 43:86-97.

Phillips LG, Whitehead DM, Kinsella JE. 1994. Protein stabilized foams. In: Phillips LG, Whitehead DM, Kinsella JE, eds. Structure-function of food proteins. New York, USA: Academic Press, 131-152.

Plessas S, Nouska G, Karapetsas A, Kazakos S, Alexopoulos A, Mantzourani L. 2017. Isolation, characterization and evaluation of the probiotic potential of a novel Lactobacillus strain isolated from Feta-type cheese. Food Chem. 226:102-108.

Sanchez CC, Panito JMR. 2005. Interfacial, foaming and emulsifying characteristics of sodium caseinate as influenced by protein concentration in solution. Food Hydro. 19:407-416.

Senphan T, Benjakul S. 2014. Antioxidative activities of hydrolysates from seabass skin prepared using protease from hepatopancreas of Pacific white shrimp. J Func Food. 6:147-156.

Shahidi F, Han XQ, Synowiecki J. 1995. Production and characteristics of protein hydrolysates from capelin (*Mallotus villosus*). Food Chem. 53:285-293.

Singh BP, Vij S. 2018. In *vitro* stability of bioactive peptides derived from fermented soy milk against heat treatment, pH and gastrointestinal enzymes. LWT-Food Sci Technol. 91:303-307.

Song R, Yang P, Wei R, Ruan G. 2016. Antioxidative, antibacterial, and food functional oroperties of the half-fin anchovy hydrolysates-glucose conjugates formed via maillard reaction. Molecules. 21:795-809.

Sripokar P, Poonsin T, Chaijan M, Benjakul S, Klomklao S. 2015a. Proteinases from liver of albacore tuna (*Thunnus alalunga*): optimum extractant and biochemical characteristics. J Food Biochem. 40:10-19.

Sripokar P, Chaijan M, Benjakul S, Kishimura H, Klomklao S. 2015b. Enzymatic hydrolysis of starry triggerfish (*Abalistes stellaris*) muscle using liver proteinase from albacore tuna (*Thunnus alalunga*). J Food Sci Technol. 53:1047-1054.

Steel RGD, Torrie JH. 1980. Principle and Procedure of Statistics; A biometrical approach. New York: McGraw-Hill.

Taheri A, Anvar SAA, Ahari H, Fogliano V. 2013. Comparison the functional properties of protein hydrolysates from poultry by-products and rainbow trout (*Onchorhynchus mykiss*) viscera. Iranian J Fish Sci. 12:154-169.

Tejpal, CS, Vijayagopal P, Elavarasan K, Prabu DL, Lekshmi RGK, Asha KK, Anandan R, Chatterjee NS, Mathew S. 2017. Antioxidant, functional properties and amino acid composition of pepsin-derived protein hydrolysates from whole tilapia waste as influenced by pre-processing ice storage. J Food Sci Technol. 54:4257-4267.

Thammarat K, Leena N, Punnanee S, Soottawat B. 2015. Functional and antioxidative properties of Bambara groundnut (*Voandzeia subterranean*) protein hydrolysates. Int Food Res J. 22:1584-1595.

Thiansilakul Y, Benjakul S, Shahidi F. 2007. Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scad (*Decapterus maruadsi*). Food Chem. 103:1385-1394.

Yen G, Wu J. 1999. Antioxidant and radical scavenging properties of extract from *Ganoderma tsugae*. Food Chem. 65:375-379.

You L, Zhao M, Regenstein JM, Ren J. 2010. Changes in the antioxidant activity of loach (*Misgurnus anguillicaudatus*) protein hydrolysates during a simulated gastrointestinal digestion. Food Chem. 120:810-816.

Waniska RD, Kinsella JE. 1979. Foaming properties of proteins: Evaluation of a column aeration apparatus using ovalbumin. J Food Sci. 44:1398-1411.

Wu HC, Chen HM, Shiau CY. 2003. Free amino acid and peptide as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriaicus*). Food Res Int. 36:949-957.

Zayas JF. 1997. Solubility of proteins. In Functionality of proteins in food (pp. 6-22). Berlin: Springer-Verlag.

OUTPUT

Publication

1. Sripokar, P., Poonsin, T., Chaijan, M., Benjakul, S. and **Klomklao, S.** 2016. Proteinases from liver of albacore tuna (*Thunnus alalunga*): Optimum extractant and biochemical characteristics. J. Food Biochem. 40: 10-19.

2. Sripokar, P., Chaijan, M., Benjakul, S., Yoshida, A. and **Klomklao, S.** 2017. Aqueous two-phase partitioning of liver proteinase from albacore tuna (*Thunnus alalunga*): Application to starry triggerfish (*Abalistes stellaris*) muscle hydrolysis. Int. J. Food Prop. 20, No S2: 1600-1612.

3. **Klomklao, S.** and Benjakul, S. 2018. Two trypsin isoforms from albacore tuna (*Thunnus alalunga*) liver: Purification and physicochemical and biochemical characterization. Int. J. Biol. Macromol. 107:1864-1870.

4. Sripokar, P., Chaijan, M., Benjakul, S., Kishimura, H. and **Klomklao, S.** 2016. Enzymatic hydrolysis of starry triggerfish (*Abalistes stellaris*) muscle using liver proteinase from albacore tuna (*Thunnus alalunga*). J. Food Sci. Technol. 53: 1047-1054.

5. Sripokar, P., Benjakul, S. and **Klomklao, S.** 2019. Antioxidant and functional properties of protein hydrolysates obtained from starry triggerfish muscle using trypsin from albacore tuna liver. Biocatal. Agric. Biotechnol. 17: 447-454.

Proceeding

1. Sripokar, P., **Klomklao, S.** and Chaijan, M. 2014. Extraction and biochemical properties of proteinases from liver of albacore tuna (*Thunnus alalunga*). Food Innovation ASIA Conference 2014, June 12-13, 2014. BITEC, Bangkok, Thailand.

2. Sripokar, P., Chaijan, M. and **Klomklao, S.** 2015. Biochemical properties of proteinase from liver of albacore tuna (*Thunnus alalunga*) partitioned by aqueous two-phase system. The 25th Thaksin University National Conference 2015, June 10-12, 2015. Thaksin University, Songkhla, Thailand.

3. Sripokar, P., **Klomklao, S.** and Chaijan, M. 2015. Use of aqueous two-phase system for partitioning and recovery of proteinases from liver of albacore tuna (*Thunnus alalunga*) . Food Innovation ASIA Conference 2015, June 18-19, 2015. BITEC, Bangkok, Thailand.

4. **Klomklao, S.,** Benjakul, S. and Kishimura, H. 2016. Purification and characterization of trypsins from the liver of albacore tuna (*Thunnus alalunga*). Tokyo 8th International Conference on Engineering & Technology, Computer, Basic & Applied Sciences, September 27-28, 2016. Shinjuku New City Hotel, Tokyo, Japan.

5. Sripokar, P., Chaijan, M. and **Klomklao, S.** 2016. Use of liver extract from albacore tuna (*Thunnus alalunga*) for the production of protein hydrolysate from starry triggerfish (*Abalistes stellaris*) muscle. International Conference on Food and Applied Bioscience, February 4-5, 2016. The Empress Hotel, Chiang Mai, Thailand.

6. Sripokar, P. and **Klomklao, S.** 2018. Antioxidant and functional properties of protein hydrolysate from starry triggerfish (*Abalistes stellaris*) muscle treated with albacore tuna liver extract (*Thunnus alalunga*). The 20th Food Innovation ASIA Conference 2018, June 14-16, 2018. BITEC, Bangkok, Thailand.

APPENDIX

Journal of
Food Biochemistry

Journal of Food Biochemistry ISSN 1745–4514

PROTEINASES FROM THE LIVER OF ALBACORE TUNA (*THUNNUS ALALUNGA*): OPTIMUM EXTRACTANT AND BIOCHEMICAL CHARACTERISTICS

PAKTEERA SRIPOKAR[1], TANCHANOK POONSIN[1], MANAT CHAIJAN[2], SOOTTAWAT BENJAKUL[3] and SAPPASITH KLOMKLAO[4,5]

[1]Biotechnology Program, Department of Food Science and Technology, Faculty of Technology and Community Development, Thaksin University, Phatthalung Campus, Phatthalung 93210, Thailand
[2]Department of Food Technology, School of Agricultural Technology, Walailak University, Nakhon Si Thammarat, Thailand
[3]Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla, Thailand
[4]Department of Food Science and Technology, Faculty of Technology and Community Development, Thaksin University, Phatthalung Campus, Phatthalung 93210, Thailand

[5]Corresponding author.
TEL: 66-7469-3996;
FAX: 66-7469-3996;
EMAIL: sappasith@tsu.ac.th

Received for Publication March 26, 2015
Accepted for Publication June 23, 2015

doi:10.1111/jfbc.12174

ABSTRACT

Proteolytic activity of the extract from albacore tuna (*Thunnus alalunga*) liver was studied. Optimum pH and temperature for casein hydrolysis were 8.5 and 55C, respectively. The enzyme was stable to heat treatment up to 50C and in the pH range of 7.0–10.0 for 30–120 min. The proteolytic activity was strongly inhibited by soybean trypsin inhibitor, *N*-p-tosyl-L-lysine chloromethyl ketone and phenylmethylsulfonyl fluoride. Activities of the liver extract continuously decreased with increasing NaCl concentration (0–30%), while activities increased as $CaCl_2$ concentration increased. Based on activity staining, the molecular weights of the proteinases in albacore tuna liver were 21, 24, 30 and 34 kDa. Optimum extraction medium for proteinase recovery from albacore tuna liver was also investigated. Extraction of the liver powder with 50 mM Na phosphate buffer (pH 7.0) containing 0.2% (v/v) Brij 35 rendered a higher recovery of proteinase activity than other extractants tested ($P < 0.05$). The results suggested that major proteinases in albacore tuna liver were heat-activated alkaline proteinases, most likely trypsin-like serine proteinases.

PRACTICAL APPLICATIONS

High amount of albacore tuna liver–containing proteinases is generated during canned tuna manufacturing. Major proteinases found in albacore tuna liver were trypsin-like serine proteinase that can be recovered and used mainly for hydrolysis purposes. Therefore, it is expected that albacore tuna liver can be used as a promising source of proteinase for further applications.

INTRODUCTION

The tuna processing industry, especially canning, has become increasingly important as an income generator for Thailand. In terms of volume, Thailand is the world's largest exporter of canned tuna, with over 20 million cans annually during the past five years. Large volumes of raw tuna go through the canning process, in which about two-thirds of whole fish are utilized (Nalinanon *et al.* 2010). Fish viscera are produced in large quantities and represent a waste disposal and potential pollution problem. However, this material is a potential source of enzymes such as proteinases that may have some unique properties for industrial applications (Klomklao *et al.* 2006).

Proteinases have been known to degrade proteins through hydrolysis of peptide bonds (Klomklao *et al.* 2006). Proteinases play a vital role in biotechnology, food processing and other industries, as well as in a variety of physiological processes. Proteinases used in the industry are mainly derived from plant, animal and microbial sources,

whereas their counterparts derived from marine and aquatic sources have not been extensively used (Simpson 2000). Marine animals have adapted to different environmental conditions, and these adaptations, together with inter- and intraspecies genetic variations, are associated with certain unique properties of their proteinase, compared with their counterpart enzymes from animals, plants and microorganisms (Simpson 2000). Some of these distinctive properties include higher catalytic activity/stability at neutral to alkaline pH (Klomklao *et al.* 2007). From this, there is a great potential for the extraction and use of proteinases from fish sources, especially from the viscera.

Albacore tuna (*Thunnus alalunga*) is one of the important species commonly used for the production of canned tuna in Thailand (Nalinanon *et al.* 2009). Based on our preliminary study, liver from albacore tuna contained high proteolytic activity. Understanding the properties of proteinases in albacore tuna liver may provide important information for industrial application. However, there is no information on molecular and the biochemical characteristics of proteinases in albacore tuna liver. Therefore, the objectives of this study were to characterize the proteinases and to study the effect of extractants on the proteinase isolation from albacore tuna liver.

MATERIALS AND METHODS

Chemicals

Sodium caseinate, bovine hemoglobin, ethylenediaminetetraacetic acid (EDTA), pepstatin A, soybean trypsin inhibitor, *N*-p-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), *N*-ethylmaleimide, phenylmethylsulfonyl fluoride (PMSF), Brij 35, Triton X-100, Tween 20, L-tyrosine and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). Trichloroacetic acid (TCA), sodium chloride, tris (hydroxymethyl) aminomethane, acetone and Folin–Ciocalteu phenol reagent were purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), Coomassie Brilliant Blue R-250 and *N,N,N′,N′*-tetramethyl ethylene diamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA). All other reagents were of analytical grade.

Fish Sample Preparation

The internal organs of albacore tuna (*T. alalunga*) were obtained from Tropical Canning (Thailand) Public Co., Ltd., Hat Yai, Songkhla, Thailand. The samples were packed in polyethylene bags, kept in ice with a sample/ice ratio of 1:3 (w/w) and transported to the research laboratory within 2 h. Pooled internal organs were separated and only the liver was collected, immediately frozen and stored at −20C until use.

Preparation of Liver Extract

Frozen livers were thawed using running water (26–28C) until the core temperature reached −2–0C. The samples were cut into pieces with a thickness of 1–1.5 cm and homogenized into powder in 3 volumes of acetone at −20C for 30 min according to the method of Klomklao *et al.* (2007). The homogenate was filtrated in vacuum on Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England). The residue obtained was then homogenized in 2 volumes of acetone at −20C for 30 min and then the residue was air-dried at room temperature. Defatted liver powder obtained was stored at −20C until use.

To prepare the liver extract, defatted liver powder was suspended in distilled water at a ratio of 1:9 (w/v) and stirred continuously at 4C for 30 min. The suspension was centrifuged at 5,000 × g for 30 min at 4C using a Sorvall Model RC-B Plus centrifuge (Newtown, CT). The supernatant obtained was collected and referred to as "liver extract."

Enzyme Assay

Proteolytic activity of the liver extract from albacore tuna was measured using hemoglobin and casein as substrate (An *et al.* 1994). To initiate the reaction, 200 μL of the liver extract was added into assay mixtures containing 200 μL of 2% (w/v) substrate, 200 μL of distilled water and 625 μL of reaction buffer. The enzymatic reaction was terminated by adding 200 μL of 50% (w/v) TCA. The reaction mixture was centrifuged at 7,500 × g for 10 min at room temperature. The oligopeptide content in the supernatant was determined by the Lowry assay (Lowry *et al.* 1951) using tyrosine as a standard. Activity was expressed as tyrosine equivalents in the TCA supernatant. One unit of activity was defined as that releasing 1 mmol of tyrosine per minute (mmol/Tyr/min). A blank was run in the same manner, except that the enzyme was added after 50% TCA (w/v) addition.

Protein Determination

Protein concentration was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Characterization of Proteinases from the Liver of Albacore Tuna

pH and Temperature Profiles. Proteinase activity was measured using substrate-TCA-Lowry assay (An *et al.* 1994) at pH 2.0–5.0 (using hemoglobin) and pH 5.0–11.0 (using

casein). Different buffers were used for different pH conditions: 0.2 M McIlvain's buffer (0.2 M sodium phosphate–0.1 M sodium citrate) for pH 2.0–7.0 and 0.1 M glycine–NaOH for pH 8.0–11.0 (Klomklao *et al.* 2006). The activity was assayed at 50C for 15 min. For temperature profile study, the activity was assayed at different temperatures (20, 30, 40, 50, 55, 60, 65, 70 and 80C) for 15 min at pH 8.5.

pH and Thermal Stability. The effect of pH on enzyme stability was evaluated by measuring the residual activity after incubation at various pH (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) for 30, 60 and 120 min at room temperature. To investigate thermal stability, the liver extracts were incubated at different temperatures (0, 10, 20, 30, 40, 50, 60, 70 and 80C) for 30, 60 and 120 min, followed by cooling in ice water. The residual activity was measured using casein as a substrate at pH 8.5 and 55C for 15 min and reported as the relative activity (%) compared with the original activity.

Effect of NaCl. To study the effect of NaCl on proteinase activity, NaCl was added to the standard reaction assay to obtain the final concentrations of 0, 5, 10, 15, 20, 25 and 30% (w/v). The residual activity was determined at 55C and pH 8.5 for 15 min using casein as a substrate.

Effect of $CaCl_2$. The effect of $CaCl_2$ on the proteinase activity was investigated. $CaCl_2$ was added to the standard reaction assay to obtain different final concentrations (0, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} and 10^{-2} M). The activity was determined at 55C and pH 8.5 for 15 min using casein as a substrate.

Effect of Inhibitors. The effect of inhibitors on the proteinase activity was determined according to the method of Klomklao *et al.* (2010a) by incubating the liver extract with an equal volume of proteinase inhibitor solution to obtain the final concentration designated (0.1 mM E-64, 1 mM *N*-ethylmaleimide, 1 g/L soybean trypsin inhibitor, 5 mM TLCK, 5 mM TPCK, 1 mM pepstatin A, 5 mM benzamidine, 1 mM PMSF and 2 mM EDTA). The mixture was allowed to stand at room temperature (26–28C) for 30 min. Thereafter, the remaining activity was measured by substrate-TCA-Lowry method (An *et al.* 1994). Percentage inhibition was then calculated.

Activity Staining. Liver extract was separated on SDS–polyacrylamide gel electrophoresis (SDS-PAGE), followed by activity staining according to the method of Klomklao *et al.* (2010b). Liver extract was mixed with sample buffer (0.125 M Tris–HCl [pH 6.8] containing 20% [v/v] glycerol, 10% SDS and 0.3% bromophenol blue) with and without βME at a ratio of 1:1 (v/v). Two micrograms of proteins were loaded into the gel made of 4% stacking and 12% separating gels. The proteins were subjected to electrophoresis at a constant current of 15 mA per gel by a Mini-Protean II Cell apparatus (Atto Co., Tokyo, Japan). After electrophoresis, gels were washed with distilled water before soaking in 100 mL of 2% (w/v) casein in 50 mM Tris–HCl buffer (pH 8.5) for 1 h with constant agitation at 0C to allow the substrate to penetrate into the gels. The gels were then transferred to 2% (w/v) casein in 0.1 M glycine–NaOH (pH 8.5) and incubated at 55C for 15 min with constant agitation to develop the activity zone. The gels were fixed and stained with 0.125% Coomassie Blue R-250 in 45% ethanol and 10% acetic acid, and destained in 30% methanol and 10% acetic acid. Development of clear zones on blue background indicated proteolytic activity.

For the inhibitor study, liver extracts were incubated with an equal volume of proteinase inhibitor solutions to obtain the final concentration designated as described previously for 30 min at room temperature. After incubation, the mixtures were mixed with sample buffer at a ratio of 1:1 (v/v). The mixtures were loaded into the gel, and activity staining was performed as previously described.

Optimum Extractant for Recovery of Proteinases from Albacore Tuna Liver

Effect of Extractants on the Recovery of Proteinases from Albacore Tuna Liver. Different extraction media, including distilled water, 50 mM Na phosphate buffer (pH 7.0) and 50 mM Tris–HCl (pH 7.0) were used to extract proteinases from the liver of albacore tuna. The medium was added into the defatted liver powder with a ratio of 1:9 (w/v) and stirred continuously at 4C for 30 min. The supernatant was recovered by centrifuging the slurry at 5,000 × g for 30 min. The proteinase activity and protein content in the extracts were determined, and the yield and specific activity of the extracts obtained using different media were compared. The extractant rendering the highest yield was selected for further steps.

Effect of NaCl Concentration on the Recovery of Proteinases from Albacore Tuna Liver. Defatted liver powder was suspended in 50 mM Na phosphate buffer (pH 7.0) containing different NaCl concentrations (0, 0.25, 0.5, 0.75 or 1 M) at a ratio of 1:9 (w/v) and stirred continuously at 4C for 30 min. The supernatant was recovered by centrifuging the slurry at 5,000 × g for 30 min. The proteinase activity and protein content in the extracts were determined. The extraction yield and specific activity of the

extracts were calculated. The extractant rendering the highest yield was chosen for further steps.

Effect of Surfactant on the Recovery of Proteinases from Albacore Tuna Liver

Defatted liver powder was suspended in 50 mM Na phosphate buffer (pH 7.0) containing 0.2% (v/v) different surfactants (Brij 35, Tween 20, Triton X-100 and SDS) at a ratio of 1:9 (w/v) and stirred continuously at 4C for 30 min. The supernatant was recovered by centrifuging the slurry at 5,000 × g for 30 min. The proteinase activity and protein content in the extracts were measured. The extraction yield and specific activity of the extracts were calculated.

Statistical Analysis

A completely randomized design was used throughout this study. Data were subjected to analysis of variance, and mean comparison was carried out using Duncan's multiple range test (Steel and Torrie 1980). Statistical analysis was performed using SPSS (SPSS, Inc., Chicago, IL).

RESULTS AND DISCUSSION

Biochemical Properties of Proteinases from the Liver of Albacore Tuna

pH and Temperature Profiles. The pH activity profile of proteinases from the liver of albacore tuna is shown in Fig. 1a. The optimum pH for caseinolytic activity was found at pH 8.5. The relative activities at pH 8.0 and 9.0 were about 80.73 and 92.31%, respectively. Therefore, these proteinases are considered to be an alkaline proteinase. The activity of albacore tuna liver proteinases decreased in acidic and alkaline pH ranges. The effect of temperature on the proteinase activity was determined by assaying enzyme activity at different temperatures for 15 min at pH 8.5. Figure 1b shows the enzyme activity of the liver extract as a function of temperature. The optimum temperature for the proteinase was 55C. The enzyme activity gradually declined at temperatures beyond 55C. Based on the optimum pH and temperature, the major proteinase from the liver of albacore tuna was characterized as heat-activated alkaline proteinases. Heat-activated alkaline proteinases have been found in the digestive tracts of various fish. Alkaline proteases from the red scorpionfish (*Scorpaena scrofa*) viscera exhibited optimal activity at pH 10.0 and 55C (Younes *et al.* 2014). The optimal pH and temperature of alkaline protease from the viscera of bolti fish (*Tilapia nilotica*) were 8.0 and 45C, respectively, with casein as a substrate (El-Beltagy *et al.* 2005). The optimum pH and temperature of serine protease from the viscera of sardinelle (*Sardinella aurita*) were around pH 8.0 and 60C, respectively (Ben *et al.* 2011). Klomklao *et al.* (2007) found that the optimal pH and temperature of trypsin from pyloric ceca of bluefish (*Pomatomus saltatrix*) for the hydrolysis of benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) were 9.5 and 55C, respectively. Souza *et al.* (2007) reported that trypsin from the intestine and pyloric ceca of spotted goatfish (*Pseudupeneus maculatus*) presented identical optimal pH (9.0) and temperature (55C).

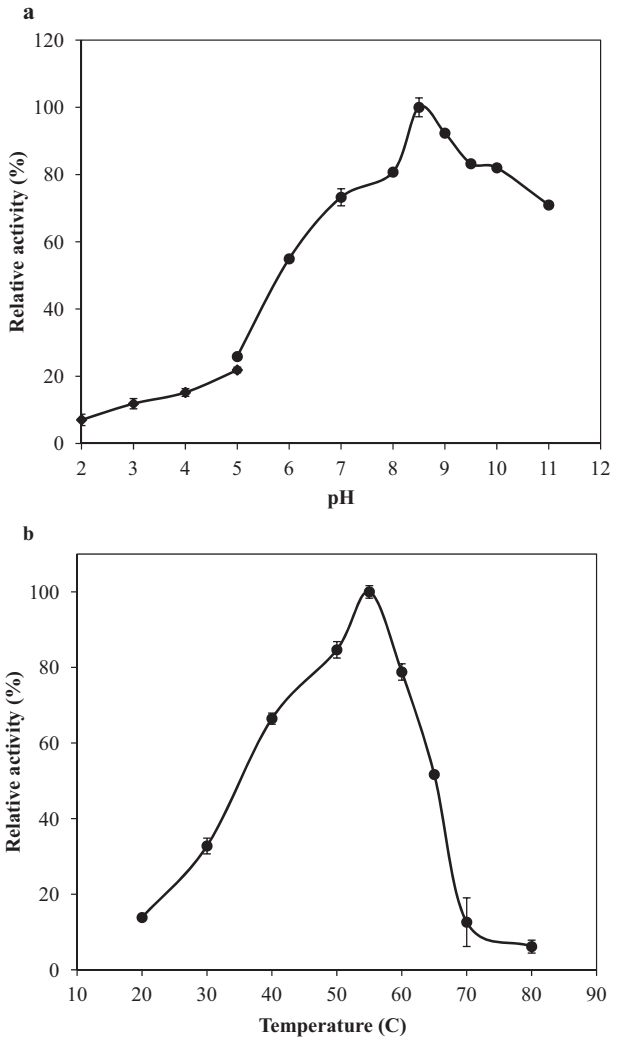

FIG. 1. pH (A) AND TEMPERATURE (B) PROFILES OF PROTEINASES FROM THE LIVER OF ALBACORE TUNA
Bars represent the standard deviation from triplicate determinations.

pH and Thermal Stability. The pH stability profile showed that the albacore tuna liver proteinase is highly stable over a wide pH range, maintaining more than 80% of its original activity at pH values between 7.0 and 10.0 after incubation for 60 min at room temperature (Fig. 2a). With

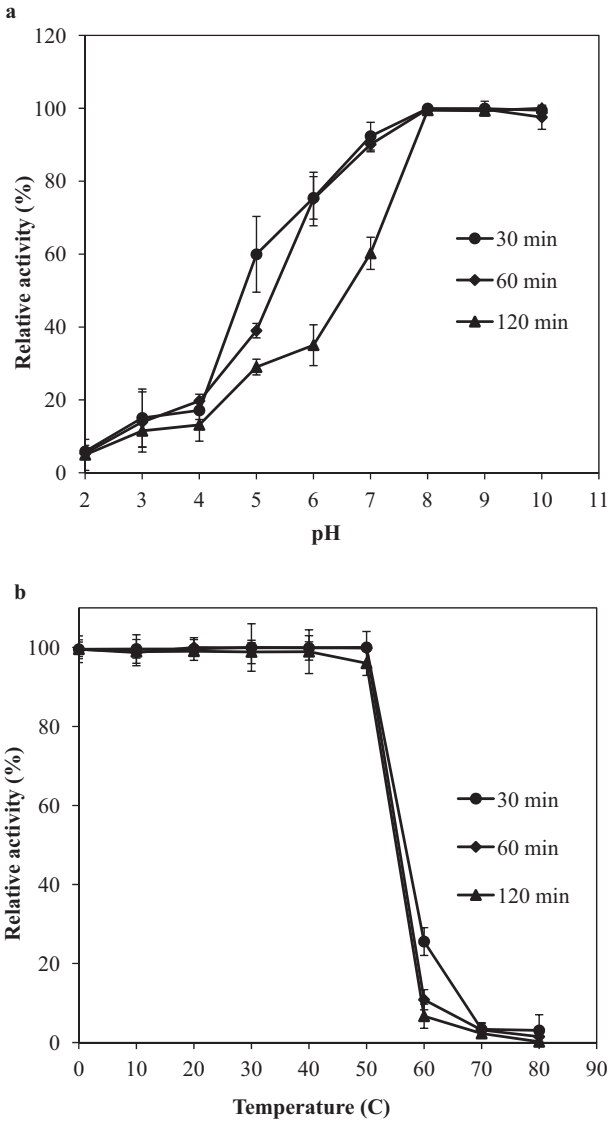

FIG. 2. pH (A) AND THERMAL (B) STABILITY OF PROTEINASES FROM THE LIVER OF ALBACORE TUNA
Bars represent the standard deviation from triplicate determinations.

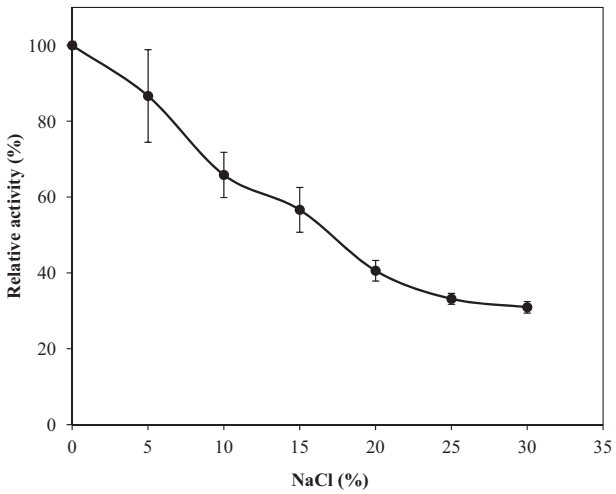

FIG. 3. EFFECT OF NaCl CONCENTRATION ON THE ACTIVITIES OF PROTEINASES FROM THE LIVER OF ALBACORE TUNA
Bars represent the standard deviation from triplicate determinations.

an extended incubation time, proteinase activity was lost to a greater extent. At pH below 7.0, the stability of the enzyme decreased sharply. The stability of the enzyme at a particular pH might be related to the net charge of the enzyme at that pH (Klomklao *et al.* 2010b). At acidic pH, it is suggested that conformational changes in the enzyme affected the proper binding of the enzyme to the substrate (Klomklao *et al.* 2007). Alkaline proteases from the red scorpionfish (*Sc. scrofa*) viscera were extremely stable in the pH range of 5.0–12.0 (Younes *et al.* 2014). Serine protease from sardinelle (*Sa. aurita*) viscera was stable in the pH range of 7.0–10.0 (Ben *et al.* 2011). Trypsin from intestine of Nile tilapia (*Oreochromis niloticus* L.) showed high stability at the pH range of 6.0–11.0 (Unajak *et al.* 2012). Trypsin from the intestine of grey triggerfish (*Balistes capriscus*) was extremely stable in the pH range of 7.0–12.0 (Jellouli *et al.* 2009). Trypsin from the hepatopancreas of the cuttlefish (*Sepia officinalis*) was stable in the pH range of 6.0–10.0 (Balti *et al.* 2009).

Figure 2b shows the thermal stability of the liver proteinase from albacore tuna. The enzyme was stable when incubated at temperatures up to 50C for 30–120 min. However, the enzyme was inactivated at temperatures above 50C. At high temperature, the enzymes possibly underwent denaturation and lost their activity (Klomklao *et al.* 2007). Generally, the stability of proteinases decreased when heating time was increased. Alkaline proteases from the red scorpionfish (*Sc. scrofa*) viscera were stable at temperatures ranging from 30 to 40C for 30 min (Younes *et al.* 2014). Serine protease from sardinelle (*Sa. aurita*) viscera is completely stable at temperatures below 40C (Ben *et al.* 2011). Trypsin from the pyloric ceca of Pacific saury (*Cololabis saira*) was stable in the temperature range of 20–50C (Klomklao *et al.* 2014). Trypsin from the hepatopancreas of the cuttlefish (*Se. officinalis*) was stable at temperatures below 50C (Balti *et al.* 2009). Jellouli *et al.* (2009) found that trypsin from the intestine of grey triggerfish (*B. capriscus*) was highly stable at temperature below 40C but was inactivated at high temperature.

Effect of NaCl. The effect of NaCl on the activity of proteinase from albacore tuna liver was studied at pH 8.5 and 55C by the addition of NaCl to the reaction mixture. The activity of proteinases decreased gradually with increasing NaCl concentration (Fig. 3). Ben *et al.* (2011) reported that

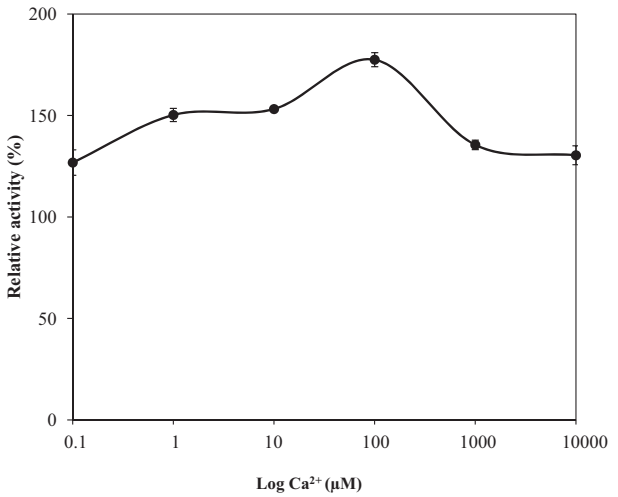

FIG. 4. EFFECT OF $CaCl_2$ ON THE ACTIVITIES OF PROTEINASES FROM THE LIVER OF ALBACORE TUNA
Bars represent the standard deviation from triplicate determinations.

TABLE 1. EFFECT OF VARIOUS INHIBITORS ON THE ACTIVITY OF PROTEINASES FROM THE LIVER OF ALBACORE TUNA*

Inhibitors	Concentration	Inhibition (%)†
Control		0[a]
E-64	0.1 mM	0.56 ± 0.65[a]
N-ethylmaleimide	1.0 mM	0.07 ± 0.33[a]
Soybean trypsin inhibitor	1.0 g/L	78.70 ± 1.42[d]
TLCK	5.0 mM	54.98 ± 1.54[c]
TPCK	5.0 mM	1.14 ± 2.40[a]
Pepstatin A	1.0 mM	0.42 ± 2.89[a]
Benzamidine	5.0 mM	20.80 ± 4.51[b]
PMSF	1.0 mM	51.35 ± 5.72[c]
EDTA	2.0 mM	0.50 ± 3.82[a]

The different letters in the same column denote significant differences ($P < 0.05$).
* Activity was analyzed using casein as a substrate for 15 min at pH 8.5 and 55C.
† Mean ± SD from triplicate determinations.
E-64, 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; TLCK, *N*-p-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

the relative activity of protease from the viscera of sardinelle (*Sa. aurita*) at 10% NaCl was approximately 20%. The activity of trypsin from the hepatopancreas of the cuttlefish (*Se. officinalis*) decreased with increasing NaCl concentration. The relative activity at 30% NaCl was approximately 39.4% (Balti *et al.* 2009). The activity of trypsin from the pyloric ceca of Pacific saury (*C. saira*) decreased with increasing NaCl concentration up to 30% (Klomklao *et al.* 2014). Some losses in activity occurred as NaCl concentration increased, probably owing to the partial denaturation of proteinase caused by the "salting out" effect (Klomklao *et al.* 2011). The water molecule is drawn from the proteinase molecule by salt, leading to the aggregation of those enzymes (Klomklao *et al.* 2004). From the results, more than 30% of proteinase activity remained in the presence of a high concentration of NaCl (25%). Therefore, these proteinases from the liver of albacore tuna may contribute to the protein hydrolysis in high salt–fermented fish products such as fish sauce.

Effect of $CaCl_2$. Proteinase activities of the liver extract from albacore tuna increased with $CaCl_2$ addition (Fig. 4). When the concentration of $CaCl_2$ was increased from 0.1 μM to 0.1 mM, the activity apparently increased. However, there was no further increase in the activity with $CaCl_2$ above 0.1 mM. Serine proteinases from the skeletal muscle of red sea bream (*Pagrus major*) were slightly activated by Ca^{2+} at the concentration of 5 mM (Wu *et al.* 2010). Younes *et al.* (2014) found that the addition of 5 mM of $CaCl_2$ increased the activity of the crude protease extract from red scorpionfish (*Sc. scrofa*) viscera to 109%. It has been known that calcium ions promote the formation of active trypsin from trypsinogen and stabilize trypsin against autolysis (Klomklao *et al.* 2007). Two calcium-binding sites are present in trypsinogen (Kosslakoff *et al.* 1977). The primary site, with a higher affinity for calcium ions, is common in trypsinogen and trypsin, and the secondary site occurs only in the zymogen. The binding of calcium to trypsinogen induces a conformational change, which is associated with the formation of an active form. Therefore, calcium ions played an essential role in the activation of proteinases from albacore tuna liver.

Effect of Inhibitors. Various proteinase inhibitors were assayed and evaluated in terms of their effects on the enzyme activity (Table 1). Strong inhibition by 1.0 g/L soybean trypsin inhibitor (78.70% inhibition), 5.0 mM TLCK (54.98% inhibition) and 1.0 mM PMSF (51.35% inhibition) was observed. The cysteine proteinase inhibitor (E-64, *N*-ethylmaleimide), metallo (EDTA), aspartic proteinases (pepstatin A) and chymotrypsin inhibitor (TPCK) showed the negligible inhibitory no effect on proteinase activity. These data indicated that the major proteinases from the liver of albacore tuna belonged to the serine proteinases, particularly trypsin or trypsin-like enzymes. Trypsin from zebra blenny (*Salaria basilisca*) viscera was completely inhibited by 1 mg/mL soybean trypsin inhibitor and partially inhibited by PMSF and a serine protease inhibitor (Ktari *et al.* 2012). Serine proteinase from the viscera of sardinelle (*Sa. aurita*) was strongly inhibited by PMSF, a serine protease inhibitor and soybean trypsin

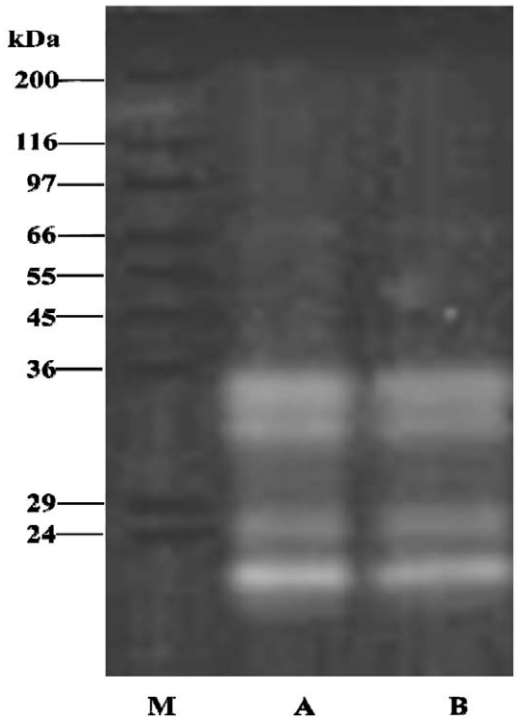

FIG. 5. ACTIVITY STAINING OF LIVER PROTEINASES FROM ALBACORE TUNA UNDER NONREDUCING (A) AND REDUCING (B) CONDITIONS
M, molecular weight standard.

inhibitor (Ben *et al.* 2011). Protease from the viscera of red scorpionfish (*Sc. scrofa*) was affected by 10 mM PMSF and a serine protease inhibitor (Younes *et al.* 2014).

Activity Staining. The proteinase activity in the albacore tuna liver extract was identified by separation on SDS–substrate polyacrylamide gels followed by staining for proteolytic activity (Fig. 5). Under both reducing and nonreducing conditions, a similar pattern of activity bands is depicted as clear zones on the dark background. Four activity bands were observed at the apparent molecular weights of 21, 24, 30 and 34 kDa. The molecular weight of the major activity band was estimated to be 21 kDa. Based on the molecular weight, the activity bands were tentatively identified as trypsin and/or trypsin-like serine proteinases. Trypsin consists of a single peptide chain with molecular weight typically of 24 kDa. However, the differences in trypsin may be owed to the genetic variation among species (Klomklao *et al.* 2004). The molecular weight of trypsin from Greenland cod pyloric ceca was 23.5 kDa (Simpson and Haard 1984). Klomklao *et al.* (2004) reported that the molecular weights of the major proteinase activity bands of tongol and yellowfin tuna were 21 kDa. The molecular weight of trypsin from the viscera of hybrid catfish was 24 kDa by size exclusion chromatography and SDS-PAGE (Klomklao *et al.* 2011). Unajak *et al.* (2012) reported that the molecular weight of the trypsin of Nile tilapia (*O. niloticus* L.) intestine was 22.39 kDa by SDS-PAGE.

The effect of various proteinase inhibitors on the activity bands observed on SDS-substrate gel electrophoresis is depicted in Fig. 6. The activity bands were effectively inhibited when the liver extract was treated with soybean trypsin inhibitor and TLCK and partially inhibited by PMSF. Nevertheless, the activity bands were retained after mixing with E-64, *N*-ethylmaleimide, TPCK, pepstatin A, benzamidine and EDTA. The result confirmed that the major proteinases in the liver of albacore tuna are trypsin or trypsin-like enzyme. The major liver proteinase from albacore tuna will be further isolated, purified and characterized.

Optimum Extractant for Recovery of Proteinases from Albacore Tuna Liver

Table 2 shows the effect of extractants on proteinase extraction from the liver of albacore tuna. Liver extract using 50 mM Na phosphate buffer (pH 7.0) showed higher proteinase activity than those extracted with distilled water and 50 mM Tris–HCl (pH 7.0) when casein was used as

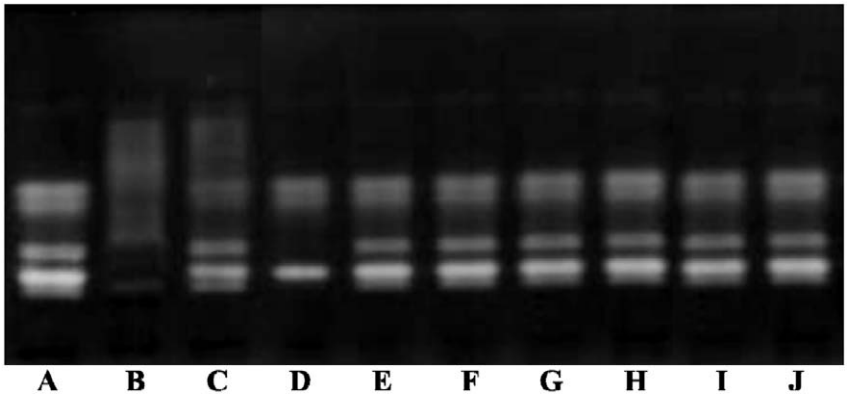

FIG. 6. ACTIVITY STAINING OF LIVER PROTEINASES FROM ALBACORE TUNA WITH AND WITHOUT PROTEINASE INHIBITOR
A: control; B: soybean trypsin inhibitor; C: TLCK (*N*-p-tosyl-L-lysine chloromethyl ketone); D: PMSF (phenylmethylsulfonyl fluoride); E: benzamidine; F: TPCK (*N*-tosyl-L-phenylalanine chloromethyl ketone); G: E-64; H: EDTA (ethylenediaminetetraacetic acid); I: pepstatin A; J: *N*-ethylmaleimide.

TABLE 2. EFFECT OF EXTRACTION MEDIA ON THE RECOVERY OF PROTEINASES FROM THE LIVER OF ALBACORE TUNA*

Extraction media	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)†
Distilled water	196.71 ± 3.75[a]	65.36 ± 0.56[a]	3.01 ± 0.08[a]
50 mM Tris–HCl, pH 7.0	247.13 ± 1.58[b]	62.54 ± 2.67[a]	3.96 ± 0.18[b]
50 mM Na phosphate buffer, pH 7.0	291.97 ± 1.58[c]	64.43 ± 0.71[a]	4.53 ± 0.04[c]

The different letters in the same column denote significant differences ($P < 0.05$).
* The defatted liver powder was extracted in different media at 4C for 30 min and proteolytic activity was analyzed using casein as substrate for 15 min at pH 8.5 and 55C.
† Mean ± SD from triplicate determinations.

substrate ($P < 0.05$). The results suggested that Na phosphate buffer had a greater ability to extract proteinase than Tris–HCl and distilled water. Wu *et al.* (2010) used 25 mM sodium phosphate buffer (pH 7.5) for extracting the proteinases from the skeletal muscle of red sea bream (*P. major*). Further, 50 mM sodium phosphate buffer (pH 7.0) was used to extract pepsinogens and pepsins from the stomach of European eel (*Anguilla anguilla*) (Wu *et al.* 2009). From the results, 50 mM Na phosphate buffer (pH 7.0) was selected as the extraction medium for albacore tuna liver proteinase because the extract had the maximum proteinase activity.

Na phosphate buffer containing different NaCl concentrations was used to recover the proteinases from the liver of albacore tuna (Table 3). When the concentration of NaCl was increased from 0 to 1.0 M, the activity apparently decreased ($P < 0.05$). This is probably due to the denaturation of proteinases from the liver of albacore tuna at high salt concentration.

The effect of some surfactants on the recovery of proteinases from the liver of albacore tuna is shown in Table 4. The addition of surfactants in 50 mM Na phosphate buffer (pH 7) affected the yield of proteinase extracted at 4C for 30 min. When the tuna liver powder was extracted with 50 mM Na phosphate buffer (pH 7) containing 0.2% Brij 35, the highest yield or proteinase activity and specific activity were obtained ($P < 0.05$). The yield of proteinase extracted with the aid of Brij 35 was higher than that of proteinases extracted without Brij 35. Brij 35 was added to facilitate improved extraction of soluble cell material and to

TABLE 3. EFFECT OF NaCl CONCENTRATION ON THE RECOVERY OF PROTEINASES FROM THE LIVER OF ALBACORE TUNA*

NaCl concentration (M)	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)†
0	301.72 ± 2.01[a]	66.08 ± 0.77[ab]	4.57 ± 0.07[a]
0.25	297.67 ± 4.94[ab]	67.95 ± 1.66[bc]	4.38 ± 0.10[a]
0.50	287.28 ± 3.31[b]	68.86 ± 1.88[c]	4.17 ± 0.08[b]
0.75	269.29 ± 13.39[c]	71.18 ± 0.34[d]	3.63 ± 0.19[d]
1.00	245.99 ± 4.58[d]	64.12 ± 0.99[a]	3.83 ± 0.71[c]

The different letters in the same column denote significant differences ($P < 0.05$).
* The defatted liver powder was extracted in 50 mM Na phosphate buffer (pH 7.0) in different NaCl concentration at 4C for 30 min and proteolytic activity was analyzed using casein as substrate for 15 min at pH 8.5 and 55C.
† Mean ± SD from triplicate determinations.

TABLE 4. EFFECT OF SOME SURFACTANTS ON THE RECOVERY OF PROTEINASES FROM THE LIVER OF ALBACORE TUNA*

Surfactant	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)†
Control	299.31 ± 1.16[c]	66.21 ± 1.45[a]	4.52 ± 0.09[c]
SDS	261.82 ± 4.75[b]	66.21 ± 2.29[a]	3.96 ± 0.09[a]
Tween 20	306.28 ± 3.95[d]	60.64 ± 1.65[b]	5.05 ± 0.17[d]
Triton X-100	249.15 ± 1.58[a]	58.86 ± 0.79[b]	4.23 ± 0.04[b]
Brij 35	328.32 ± 2.01[e]	59.45 ± 1.85[b]	5.52 ± 0.15[e]

The different letters in the same column denote significant differences ($P < 0.05$).
* The defatted liver powder was extracted in 50 mM Na phosphate buffer (pH 7.0) in different surfactants at 4C for 30 min and proteolytic activity was analyzed using casein as substrate for 15 min at pH 8.5 and 55C.
† Mean ± SD from triplicate determinations.
SDS, sodium dodecyl sulfate.

emulsify the small amount of lipid present in tuna liver extract to prevent lipid interference with the proteinase activity. The addition of 0.2% (v/v) of Brij 35 to the extract from Chinook salmon intestine resulted in a small increase in trypsin activity (Kurtovic *et al.* 2006). Klomklao *et al.* (2010a) found that 50 mM Tris–HCl (pH 7.0) containing 0.5 M NaCl and 0.2% (v/v) Brij 35 showed the best of extraction medium for proteinases in hybrid catfish viscera.

CONCLUSION

Albacore tuna liver extract contained heat-activated alkaline serine proteinases as the predominant enzymes with optimum activity at pH 8.5 and 55C. The major proteinase from albacore tuna liver was most likely classified as trypsin or trypsin-like based on inhibitor study, effect of $CaCl_2$ and molecular mass and can be potential novel enzymes for further applications.

ACKNOWLEDGMENTS

This research was supported by the Thailand Research Fund and Thaksin University. The TRF distinguished research professor grant was also acknowledged.

CONFLICT OF INTEREST

There were no conflicts of interest related to this study.

REFERENCES

AN, H., SEYMOUR, T.A., WU, J.W. and MORRISSEY, M.T. 1994. Assay systems and characterization of Pacific whiting (*Merluccius productus*) protease. J. Food Sci. *59*, 277–289.

BALTI, R., BARKIA, A., BOUGATEF, A., KTARI, N. and NASRI, M. 2009. A heat-stable trypsin from the hepatopancreas of the cuttlefish (*Sepia officinalis*): Purification and characterization. Food Chem. *113*, 146–154.

BEN, K.H., NASRI, R., BOUGATEF, A., GHORBEL, S. and NASRI, M. 2011. Low molecular weight serine protease from the viscera of sardinelle (*Sardinella aurita*) with collagenolytic activity: Purification and characterization. Food Chem. *124*, 788–794.

EL-BELTAGY, A.E., EL-ADAWY, T.A., RAHMA, E.H. and EL-BEDAWAY, A.A. 2005. Purification and characterization of an alkaline protease from the viscera of bolti fish (*Tilapia nilotica*). J. Food Biochem. *29*, 445–458.

JELLOULI, K., BOUGATEF, A., DAASSI, D., BALTI, R., BARKIA, A. and NASRI, M. 2009. New alkaline trypsin from the intestine of Grey triggerfish (*Balistes capriscus*) with high activity at low temperature: Purification and characterization. Food Chem. *116*, 644–650.

KLOMKLAO, S., BENJAKUL, S. and VISESSANGUAN, W. 2004. Comparative studies on proteolytic activity of spleen extracts from three tuna species commonly used in Thailand. J. Food Biochem. *28*, 355–372.

KLOMKLAO, S., BENJAKUL, S., VISESSANGUAN, W., KISHIMURA, H. and SIMPSON, B.K. 2006. Purification and characterization of trypsin from the spleen of Tongol tuna (*Thunnus tonggol*). J. Agric. Food Chem. *54*, 5617–5622.

KLOMKLAO, S., BENJAKUL, S., VISESSANGUAN, W., KISHIMURA, H. and SIMPSON, B.K. 2007. Purification and characterization of trypsins from the spleen of skipjack tuna (*Katsuwonus pelamis*). Food Chem. *100*, 1580–1589.

KLOMKLAO, S., BENJAKUL, S. and KISHIMURA, H. 2010a. Proteinases in hybrid catfish viscera: Characterization and effect of extraction media. J. Food Biochem. *34*, 711–729.

KLOMKLAO, S., KISHIMURA, H., BENJAKUL, S., SIMPSON, B.K. and VISESSANGUAN, W. 2010b. Cationic trypsin: A predominant proteinase in Pacific saury (*Cololabis saira*) pyloric ceca. J. Food Biochem. *34*, 1105–1123.

KLOMKLAO, S., BENJAKUL, S., KISHIMURA, H. and CHAIJAN, M. 2011. 24 kDa trypsin: A predominant protease purified from the viscera of hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*). Food Chem. *129*, 739–746.

KLOMKLAO, S., KISHIMURA, H. and BENJAKUL, S. 2014. Anionic trypsin from the pyloric ceca of Pacific saury (*Cololabis saira*): Purification and biochemical characteristics. J. Aquat. Food Prod. Technol. *23*, 186–200.

KOSSLAKOFF, A.A., CHAMBERS, J.L., KAY, L.M. and STROUD, R.M. 1977. Structure of bovine trypsinogen at 1.9 A resolution. Biochemistry *16*, 654–664.

KTARI, N., BKHAIRIA, I., JRIDI, M., HAMZA, I., RIADH, B.S. and NASRI, M. 2012. Digestive acid protease from zebra blenny (*Salaria basilisca*): Characteristics and application in gelatin extraction. Food Res. Int. *57*, 218–224.

KURTOVIC, I., MARSHALL, S.N. and SIMPSON, B.K. 2006. Isolation and characterization of a trypsin fraction from the pyloric ceca of Chinook salmon (*Oncorhynchus tshawytscha*). Comp. Biochem. Physiol. B Biochem. Mol. Biol. *143B*, 432–440.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. *193*, 256–275.

NALINANON, S., BENJAKUL, S., VISESSANGUAN, W. and KISHIMURA, H. 2009. Partitioning of protease from stomach of albacore tuna (*Thunnus alalunga*) by aqueous two-phase systems. Process Biochem. *44*, 471–476.

NALINANON, S., BENJAKUL, S. and KISHIMURA, H. 2010. Purification and biochemical properties of pepsins from the stomach of skipjack tuna (*Katsuwonus pelamis*). Eur. Food Res. Technol. *231*, 259–269.

SIMPSON, B.K. 2000. Digestive proteinases from marine animals. In *Seafood Enzymes: Utilization and Influence on Postharvest Seafood Quality* (N.M. Haard and B.K. Simpson, eds) pp. 531–540, Marcel Dekker, New York, NY.

SIMPSON, B.K. and HAARD, H.F. 1984. Trypsin from Greenland cod (*Gadus ogac*). Isolation and comparative properties. Comp. Biochem. Physiol. B Biochem. Mol. Biol. *79B*, 613–622.

SOUZA, A.A.G., AMARAL, I.P.G., ESPIRITO SANTO, A.R., CARVALHO JR, L.B. and BEZERRA, R.S. 2007. Trypsin-like enzyme from intestine and pyloric caeca of spotted goatfish (*Pseudupeneus maculatus*). Food Chem. *100*, 1429–1434.

STEEL, R.G.D. and TORRIE, J.H. 1980. *Principles and Procedures of Statistics: A Biometrical Approach*, 2nd Ed., McGraw-Hill, New York, NY.

UNAJAK, S., MEESAWAT, P., PAEMANEE, A., AREECHON, N., ENGKAGUL, A., KOVITVADHI, U., KOVITVADHI, S., RUNGRUANGSAK-TORRISSEN, K. and CHOOWONGKOMON, K. 2012. Characterisation of thermostable trypsin and determination of trypsin isozymes from intestine of Nile tilapia (*Oreochromis niloticus* L.). Food Chem. *134*, 1533–1542.

WU, G.P., CHEN, S.H., LIU, G.M., YOSHIDA, A., ZHANG, L.J., SU, W.J. and CAO, M.J. 2010. Purification and characterization of a collagenolytic serine proteinase from the skeletal muscle of red sea bream (*Pagrus major*). Comp. Biochem. Physiol. B Biochem. Mol. Biol. *155B*, 281–287.

WU, T., SUN, L.C., DU, C.H., CAI, Q.F., ZHANG, Q.B., SU, W.J. and CAO, M.J. 2009. Identification of pepsinogens and pepsins from the stomach of European eel (*Anguilla anguilla*). Food Chem. *115*, 137–142.

YOUNES, I., NASRI, R., BKHAIRIA, I., JELLOULI, K. and NASRI, M. 2014. New proteases extracted from red scorpionfish (*Scorpaena scrofa*) viscera: Characterization and application as a detergent additive and for shrimp waste deproteinization. Food Bioprod. Process. *94*, 453–462.

International Journal of Food Properties

ISSN: 1094-2912 (Print) 1532-2386 (Online) Journal homepage: http://www.tandfonline.com/loi/ljfp20

Aqueous two-phase partitioning of liver proteinase from albacore tuna (Thunnus alalunga): Application to starry triggerfish (Abalistes stellaris) muscle hydrolysis

Pakteera Sripokar, Manat Chaijan, Soottawat Benjakul, Asami Yoshida & Sappasith Klomklao

To cite this article: Pakteera Sripokar, Manat Chaijan, Soottawat Benjakul, Asami Yoshida & Sappasith Klomklao (2017) Aqueous two-phase partitioning of liver proteinase from albacore tuna (Thunnus alalunga): Application to starry triggerfish (Abalistes stellaris) muscle hydrolysis, International Journal of Food Properties, 20:sup2, 1600-1612, DOI: 10.1080/10942912.2017.1350705

To link to this article: https://doi.org/10.1080/10942912.2017.1350705

Accepted author version posted online: 20 Jul 2017.
Published online: 21 Dec 2017.

INTERNATIONAL JOURNAL OF FOOD PROPERTIES
2017, VOL. 20, NO. S2, S1600–S1612
https://doi.org/10.1080/10942912.2017.1350705

Aqueous two-phase partitioning of liver proteinase from albacore tuna (*Thunnus alalunga*): Application to starry triggerfish (*Abalistes stellaris*) muscle hydrolysis

Pakteera Sripokar[a], Manat Chaijan[b], Soottawat Benjakul[c], Asami Yoshida[d], and Sappasith Klomklao[e]

[a]Biotechnology Program, Faculty of Technology and Community Development, Thaksin University, Phatthalung Campus, Phatthalung, Thailand; [b]Department of Food Technology, School of Agricultural Technology, Walailak University, Nakhon Si Thammarat, Thailand; [c]Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla, Thailand; [d]Graduate School of Fisheries Science and Environmental Studies, Nagasaki University, Nagasaki, Japan; [e]Department of Food Science and Technology, Faculty of Technology and Community Development, Thaksin University, Phatthalung Campus, Phatthalung, Thailand

ABSTRACT

The potential of aqueous two-phase system (ATPS) for the purification and recovery of proteinase from albacore tuna (*Thunnus alalunga*) liver was explored. Influence of phase compositions such as type of phase forming salts, PEG molecular weight, concentration of salt and PEG, pH of the system, and NaCl addition on partitioning of proteinase was investigated. ATPS comprising PEG1000 (25%, w/w) and NaH_2PO_4 (20%, w/w) at pH 7.0 provided the best condition for the maximum partitioning of proteinase into the top phase and gave the highest purification factor (5.58-fold) and specific activity (20.65 unit/mg protein). The yield of 89.99% was obtained. The addition of NaCl up to a final concentration of 6% (w/w) decreased the degree of purification and enzyme recovery of proteinase. Based on electrophoresis and activity staining, the fractionated proteinases had the MW 21, 24, 30, and 34 kDa. The effect of fractionated proteinases on starry triggerfish (*Abalistes stellaris*) muscle hydrolysis was also studied. Fractionated proteinases were able to hydrolyze triggerfish muscle in a dose-dependent manner. Overall, results demonstrated the feasibility of ATPS for the recovery and purification of proteinase without the need for multiple steps, and the obtained proteinase can be further in preparation of protein hydrolysate.

ARTICLE HISTORY
Received 7 March 2017
Accepted 30 June 2017

KEYWORDS
Aqueous two-phase system; Proteinase; Partitioning; Viscera; Hydrolysis

Introduction

Albacore tuna (*Thunnus alalunga*) is an important raw material used for the production of canned tuna in Thailand.[1] Large volumes of raw tuna go through the canning process, by which about two-thirds of whole fish is utilized.[2] During canned tuna manufacturing, high amounts of viscera are generated.

Fish viscera is known to be a rich source of proteinases that have high activity over a wide range of pH and temperature conditions[3] and exhibit high catalytic activity at relatively low concentration.[3] Proteinases have been widely used in food, medical-phamaceutical, cosmetic, and other industries. Fish proteinases have been used for preparation of protein isolate[4] extraction of collagen and gelatin.[5] Nowadays, the efficient and economical downstream processes for the

CONTACT Sappasith Klomklao sappasith@tsu.ac.th Department of Food Science and Technology, Faculty of Technology and Community Department, Thaksin University, Phatthalung Campus, Phatthalung, 93210, Thailand.

Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/ljfp.

partitioning and purification of biomolecules that give high yield and high purity of the product have been demanded by industries.

The powerful and versatile aqueous two-phase system (ATPS) has been employed as an efficient tool in several biotechnology processes for the partitioning of biomolecules like proteins, enzymes, nucleic acids, animal, plant, and microbial cells.[6] ATPS forms readily upon mixing aqueous solution of two hydrophobic polymers, or of a polymer and salt, above a certain threshold concentration.[6] Proteins are partitioned between the two phases with a partition coefficient that can be modified by changing the experimental conditions of the medium such as pH, salts, and ionic strength, among others.[7] The basis of separation is the selective distribution of a given biomolecule between the phases, depending on the characteristics of the phase system, properties of the biomolecule, and the interaction between them.[6] ATPS has several advantages such as low processing time and energy consumption, high capacity and yield, biocompatibility, easy to scale up, and non-toxicity.[6] ATPS has been successfully used for partitioning and recovery of various proteinases such as trypsin,[8] tuna spleen proteinase,[2] tuna stomach protease,[9] bromelain,[10] and protease from *Caltropis procera* latex.[11] Therefore, the objective of this study was to determine the optimal conditions for partitioning and separating proteinase from albacore tuna liver by ATPS. The other aims of this were to apply the fractionated enzyme for starry triggerfish muscle protein hydrolysis.

Materials and methods

Chemicals

Polyethylene glycol (PEG) 1000 and 4000 were obtained from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). Sodium caseinate, β-mercaptoethanol (βME), L-tyrosine, wide range molecular weight markers, and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA). Trichloroacetic acid, tris (hydroxymethyl) aminomethane, and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250, and *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). The salts and other chemicals with the analytical grade were procured from Merck (Darmstadt, Germany).

Preparation of crude proteinase extract

Internal organs from albacore tuna (*Thunnus alalunga*) were obtained from Tropical Canning (Thailand) Public Co. Ltd., Hat Yai, Songkhla. Pooled internal organs were then excised and separated into individual organs. Only the liver was collected, immediately frozen and stored at -20°C until used. Frozen livers were thawed using running water (26–28°C) until the core temperature reached -2 to 0°C. The samples were cut into pieces with a thickness of 1–1.5 cm and homogenized into powder in three volumes of acetone at -20°C for 30 min according to the method of Klomklao et al.[12]

To prepare the liver extract, the liver powder was suspended in 50 mM Na-phosphate buffer, pH 7.0 containing 0.2% Brij 35 at a ratio of 1:9 (w/v) and stirred continuously at 4°C for 30 min. The suspension was centrifuged for 30 min at 4°C at 5,000×g to remove the tissue debris. The supernatant was collected and referred to as "liver extract."

Preparation of aqueous two-phase system

ATPS was prepared in 10-ml centrifuge tubes by adding the different amounts of PEG and salts together with liver extract according to the method of Klomklao et al.[2]

Effect of salts on proteinases partitioning

To study the effect of salts on partitioning of the proteinases from liver extract using ATPS, different salts including NaH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$, K_2HPO_4, $Na_3C_6H_5O_7$, and Na_2SO_4 at different concentrations (15, 20, and 25%, w/w) were mixed with 20% PEG1000 in aqueous system. Distilled water was used to adjust the system to obtain the final weight of 5 g. The mixtures were mixed continuously for 3 min using a Vortex mixer (Vortexgenic 2, G-560E, USA). Phase separation was achieved by centrifugation for 5 min at 5,000×g. Top phase was carefully separated using a pasteur pipette, and the interface of each tube was discarded. Volumes of the separated phases were measured. Aliquots from each phase were taken for enzyme assay and protein determination.

The specific activity of stomach proteinase in the aqueous two-phase system was defined as

$$\mathrm{SA} = \frac{\text{proteinase activity}}{\text{protein concentration}}\ (\text{Unit/mg protein}); \tag{1}$$

The purification factor as

$$\mathrm{PF} = \frac{\mathrm{SA_e}}{\mathrm{SA_i}} \tag{2}$$

where SA_e is the SA of each phase, and SA_i is the initial SA of crude extract. The partition coefficient of protein concentration was defined as:

$$\mathrm{K_P} = \frac{\mathrm{C_T}}{\mathrm{C_B}} \tag{3}$$

where C_T and C_B are concentrations of protein in top and bottom phase, respectively. The partition coefficient of proteinase activity was defined as:

$$\mathrm{K_E} = \frac{\mathrm{E_T}}{\mathrm{E_B}} \tag{4}$$

where E_T and E_B are proteinase activity in top and bottom phase, respectively.

The volume ratio is:

$$\mathrm{V_R} = \frac{\mathrm{V_T}}{\mathrm{V_B}} \tag{5}$$

where V_T and V_B are top and bottom phase volume, respectively, and the protease activity recovery yield was defined as:

$$\mathrm{Yield}(\%) = \frac{\mathrm{A_T}}{\mathrm{A_i}} \times 100 \tag{6}$$

where A_T is total proteinase activity in top phase, and A_i is the initial proteinase activity of crude extract. Based on purity and recovery yield, the appropriate salt in ATPS rendering the most effective partitioning was selected for further study.

Effect of molecular weight and concentration of PEG on proteinases partitioning

To study the effect of the concentrations (10%, 15%, 20%, 25%, and 30%, w/w) of PEG1000 and PEG4000 on partitioning of proteinase in tuna liver extract, NaH_2PO_4 at a level of 20% was used in the system.[2] Partitioning was performed as previously described. All experiments were run in duplicate. Based on purity and recovery yield, the ATPS rendering the most effective partitioning was chosen for further study.

Effect of pH on the proteinases partitioning

ATPS containing PEG1000 (25%, w/w) and NaH_2PO_4 (20%, w/w) was used for study on the effect of pH on liver extract proteinase partitioning. The original pH of the system was measured and then

adjusted to 3.0, 5.0, 7.0, 9.0, and 11.0 with 1 M HCl or 1 M NaOH. Partitioning was performed as previously described. The system pH showing the highest purity and recovery yield was selected for further study.

Effect of NaCl on the proteinases partitioning

The phase system containing 25% PEG1000 and 20% NaH_2PO_4 at pH 7.0 was chosen for study on the effect of NaCl on proteinase partitioning. Adjustment of salt content in the system was made by addition of NaCl (solid form) into the system to obtain concentrations of 0%, 2%, 4%, and 6% (w/w). Partitioning was performed as previously described. The ATPS rendering the most effective partitioning was chosen. Phase with high specific activity was dialyzed against 10 volumes of 50 mM Tris-HCl (pH 7.5) for 18 h with three changes of buffer in the first 3 h and five changes in the last 15 h. ATPS fraction with highest purity and yield was used for hydrolysis study.

Enzyme assay and protein determination

Proteinase activity of liver extract was measured using casein-TCA-Lowry assay.[13,14] To initiate the reaction, 200 µL of liver extract was added into assay mixtures containing 200 µL of 2% (w/v) casein, 200 µL of distilled water, and 625 µL of assay buffer (0.1 M glycine-NaOH, pH 8.5). The mixture was incubated at 55°C for precisely 15 min. The enzymatic reaction was terminated by adding 200 µL of 50% (w/v) trichloroacetic acid (TCA). The reaction mixture was centrifuged at 7,500×g for 10 min at room temperature. The oligopeptide content in the supernatant was determined by the Lowry assay[14] using tyrosine as a standard. One unit of activity was defined as that releasing 1 mmol of tyrosine per min (mmol Tyr/min). A blank was run in the same manner, except the enzyme was added after 50% TCA (w/v) addition. Protein concentration was measured by the method of Bradford[15] using bovine serum albumin as a standard.

Characterization of recovered proteinase

SDS-polyacrylamide gel electrophoresis and activity staining

SDS-PAGE was performed according to the method of Laemmli.[16] Protein solutions were mixed at 1:1 (v/v) ratio with the SDS-PAGE sample buffer (0.5 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 10% βME) and boiled for 3 min. The samples (20 µg) were loaded on the gel made of 4% stacking and 12% separating gels and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Protean II cell apparatus (Bio-Rad, Hercules, CA, USA). After electrophoresis, the gels were stained with 0.2% Coomassie Brilliant Blue R-250 in 45% methanol and 10% acetic acid and destained with 30% methanol and 10% acetic acid.

Liver extract and selected phase with high SA and yield obtained from ATPS were separated on SDS-PAGE, followed by activity staining according to the method of Klomklao et al.[2] The samples were mixed with sample buffer (0.5 M Tris-HCl, pH 6.8 containing 4% SDS, 20% (v/v) glycerol, and 10% βME) at a ratio of 1:1 (v/v). Two µg of proteins were loaded into the gel made of 4% stacking and 12% separating gels. The proteins were subjected to electrophoresis at a constant current of 15 mA per gel by a Mini-Protean II Cell apparatus (Bio-Rad, Hercules, CA, USA). After electrophoresis, gels were immersed in 100 mL of 2% (w/v) casein in 50 mM Tris-HCl buffer, pH 7.5 for 1 h with constant agitation at 0°C to allow the substrate to penetrate into the gels. The gels were then transferred to 2% (w/v) casein in 0.1 M glycine-NaOH, pH. 8.5, and incubated at 55°C for 15 min with constant agitation to develop the activity zone. The gels were fixed and stained with 0.125% Coomassie blue R-250 in 45% ethanol and 10% acetic acid and destained in 30% methanol and 10% acetic acid. Development of clear zones on blue background indicated proteolytic activity.

Hydrolysis of starry triggerfish muscle by fractionated proteinase

Starry triggerfish (*Abalistes stellaris*) with the length of 10 cm were purchased from the dock in Trang, Thailand. The fish, off-loaded approximately 18–24 h after capture, were placed on ice at a

fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Science and Technology, Thaksin University, Phatthalung, within 2 h. Upon arrival, the fish were filleted, and the ordinary muscle was collected and ground to uniformity. A portion of mince (500 g) was placed in a polyethylene bag and stored at -20°C until used.

Fractionated proteinase (0, 25, 50, 75, and 100 unit) was added to the reaction mixture containing 3 g starry triggerfish mince and 6 ml of 0.1 M glycine-NaOH, pH 8.5. The hydrolysis was conducted by incubating the mixture at 55°C for 0, 15, 30, 60, and 120 min. The control was performed by incubating the reaction mixture at 55°C for 120 min without the addition of fractionated enzyme. At hydrolysis time designated, 1 ml of sample was taken and mixed with 1 ml of 1% SDS solution (85° C) before placing in a water bath at 85°C for 15 min to inactivate proteinase. The degree of hydrolysis of protein hydrolysate was analyzed according to the method of Benjakul and Morrissey.[17]

To monitor the protein pattern, another lot of sample was added with hot 5% SDS solution (85°C) to terminate the reaction and solubilize total protein. All samples was subjected to SDS-PAGE.[16] The degree of hydrolysis (DH) of protein hydrolysate was analyzed according to the method of Benjakul and Morrissey.[17] The samples (125 µl) were mixed throughly with 2.0 mL of 0.2125 M phosphate buffer, pH 8.2, and 1.0 mL of 0.01% TNBS solution. The mixtures were then placed in a 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 sulfite. The mixtures were cooled at ambient temperature for 15 min. The absorbance was measured at 420 nm, and α-amino acid content was calculated and expressed in terms of *L*-leucine. DH was calculated as follows:

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

where L_t is the amount of α-amino acid released at time t. L_0 is the amount of α-amino acid in original starry triggerfish muscle. L_{max} is the total amount of α-amino acid in original starry triggerfish muscle obtained after acid hydrolysis with 6 N HCl at 100°C for 24 h.

Statistical analysis

A completely randomized design was used throughout this study. Data were subjected to analysis of variance (ANOVA), and and mean comparison was carried out using Duncan's Multiple Range Test.[18] Statistical analysis was performed using the Statistical Pacleage for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

Results and discussion

Use of ATPS for partitioning of proteinase

Effect of salts on the proteinase partitioning in ATPS

Table 1 shows the effects of type and concentration of salts on the partitioning and recovery of proteinase from liver of albacore tuna. The proteinase partitioning was assayed in several biphasis systems of 20% PEG1000 containing different salts, including NaH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$, K_2HPO_4, $Na_3C_6H_5O_7$, and Na_2SO_4 at various concentrations. After phase separation, two phases were obtained. The upper phase becomes PEG rich, and the lower phase becomes salt rich. However, no phase separation was observed in the system containing 20% PEG1000-15% NaH_2PO_4. Raghavarao et al.[19] 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). The addition of salts to the aqueous PEG solution led to an arrangement of ordered water molecules around PEG molecule due to their water structure breaking effect.[12] For all ATPS studied, the proteinase was partitioned predominantly in the PEG-rich top phase, principally those with hydrophobic characteristics.[20] In general, negatively charged proteins prefer the upper phase in PEG-salt systems, while positively charged proteins normally partition selectively to the bottom phase.[20] Hence,

Table 1. Effect of phase composition in PEG1000-salt ATPS on partitioning of liver proteinase from albacore tuna.

Phase composition (%, w/w)	V_R	K_P	K_E	SA	PF	Yield
20% PEG1000-15% NaH_2PO_4	–	–	–	–	–	–
20% PEG1000-20% NaH_2PO_4	1.83	0.53	7.01	8.56	2.31	68.75
20% PEG1000-25% NaH_2PO_4	0.83	1.14	7.56	3.00	0.81	41.84
20% PEG1000-15% $(NH_4)_2SO_4$	1.07	1.02	5.70	3.72	1.00	46.49
20% PEG1000-20% $(NH_4)_2SO_4$	0.90	1.00	3.47	3.03	0.82	37.74
20% PEG1000-25% $(NH_4)_2SO_4$	0.64	1.08	4.97	3.12	0.84	39.83
20% PEG1000-15% $MgSO_4$	2.45	0.65	0.37	3.61	0.98	41.32
20% PEG1000-20% $MgSO_4$	1.36	0.79	0.44	2.67	0.72	31.65
20% PEG1000-25% $MgSO_4$	0.92	0.90	0.53	1.39	0.37	16.50
20% PEG1000-15% K_2HPO_4	0.97	1.09	1.98	4.10	1.11	64.12
20% PEG1000-20% K_2HPO_4	0.57	1.04	2.10	3.23	0.87	52.30
20% PEG1000-25% K_2HPO_4	0.67	1.08	4.25	3.09	0.83	50.97
20% PEG1000-15% $Na_3C_6H_5O_7$	1.09	0.75	3.27	3.68	0.99	48.21
20% PEG1000-20% $Na_3C_6H_5O_7$	0.86	0.96	3.34	2.49	0.67	38.52
20% PEG1000-25% $Na_3C_6H_5O_7$	0.72	0.86	1.13	1.62	0.44	22.53
20% PEG1000-15% Na_2SO_4	1.08	1.47	1.08	3.74	1.01	47.85
20% PEG1000-20% Na_2SO_4	0.85	1.31	0.81	2.41	0.65	35.87
20% PEG1000-25% Na_2SO_4	0.67	1.42	0.87	1.74	0.47	30.10

(-) No phase separation.

V_R: volume ratio (upper/lower); K_P: partition coefficient of protein; K_E: partition coefficient of proteinases; SA: specific activity (U/(g protein) in the upper phase; PF: purification factor in the upper phase; Yield: recovery yield in the upper phase.

liver proteinase partitioned in the top phase might be negatively charged. SA, PF, and % yield of proteinase obtained from PEG1000-salt systems depended on types of salt used. System of composition 20% PEG1000 and 20% NaH_2PO_4 showed the highest SA (8.56 units/μg protein), PF (2.31-fold) and yield (68.75%) indicating that this system has the best capacity of separating proteinase from liver of albacore tuna. Therefore, the system containing 20% NaH_2PO_4 was selected for further study on the effect of PEG concentration on proteinase partitioning and recovery. Senphan and Benjakul[21] reported that ATPS comprising PEG1000 (15% w/w) and $MgSO_4$ (25% w/w) provided the best condition for the maximal partitioning of proteases from hepatopancreas of Pacific white shrimp into the top phase and gave the highest PF (8.6-fold) and yield (65.5%). Protease from *Calotropis procera* latex was separated in the top PEG-rich phase in ATPS composed of 12% PEG4000 and 17% $MgSO_4$.[22] Ketnawa et al.[23] found that the best ATPS condition for protease partitioning from viscera extract of Giant catfish (*Pangasianodon gigas*) was 15% PEG2000-15% $Na_3C_6H_5O_7$ with 1% (w/w) NaCl, which increased the purity by 3.33-fold and recovery yield (64.18%).

The distribution of the proteins in ATPS is characterized by partition coefficient *K*. *K* values for proteinase and protein partitioning are reported as K_E and K_P, respectively. From the results, the lowest K_P (0.53) and the highest K_E (7.56) were found in the system of 20% PEG1000 - 20% NaH_2PO_4 and 20% PEG1000 - 25% NaH_2PO_4, respectively. Generally, the lowest K_P indicates a shift of contaminant proteins, nucleic acid, and other undersirable components to the lower phase. For K_E, high K_E value indicates that only proteinase from crude extract was partitioned more to the top phase. Hence, the extraction conditions employed resulted in the enrichment of specific proteinase activity, which was due to the differential partitioning of the desired proteinase and contaminating enzymes and proteins to the opposite phases. Johansson[24] reported that the partition of a protein is influenced by the presence of salts. This effect increases with the net charge of protein. It has been found that the efficiency of the salts in promoting phase separation reflects the lyotropic series (a classification of ions based upon salting-out or salting-in ability).[22] Their effectiveness is mainly determined by the nature of the anion. Multicharged anions being the most effective are ordered of SO_4^{2-} > HPO_4^{2-} > acetate > Cl^-, whereas the order of cations is usually given as $(NH_4)^+$ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+} [22].

Table 1 also showed that increasing salt concentration resulted in less activity recovery. Loss in activity might be due to the denaturation of proteinases causes by "salting out" effect.[25] Isable and

Otero[26] found that the presence of high concentrations of salt in the reaction medium greatly decreased both the yield and the selectivity toward the trisaccharide from lactose. Pan and Li[27] also reported that increasing NaH_2PO_4 concentration resulted in less activity recovery as well as poorer specific activity. Therefore, the type of salt and concentration used were critical for albacore tuna liver proteinase recovery or partitioning in ATPS.

Effect of PEG molecular weight on the proteinase partitioning in ATPS

Proteinase partitioning using ATPS with varying concentration of PEG and 20% NaH_2PO_4 is depicted in Table 2. No phase separation was observed in the 10% PEG1000 with 20% NaH_2PO_4. The V_R of the system ranged from 0.53 to 1.51. Generally, an increase in the PEG molecular mass reduces free volume by increasing the chain length of the PEG polymer, resulting in partitioning of the biomolecules to the bottom phase. The increase in polymer weight causes the reduction of free volume of the top phase, so the partition of biomolecules in the salt-rich bottom phase decreases the partitioning coefficient.[7] With PEG1000 and 4000, all proteinases partitioned into the top phase (K_E >1). However, use of the lower molecular weight PEG gave a higher K_E, compared with the higher molecular weight. Thus, K_E values depended on the PEG molecular weight. Tubio et al.[6] suggested that for ATPS formed by PEG of low molecular weight (600–3350 kDa), the protein transfer to the top phase is enthalpically driven mainly due to a strong interaction between PEG and the protein. PEG of the highest molecular weight (PEG8000) excludes the protein from the top phase driven by an entropically unfavorable term.[7] For K_P value, when PEG with higher molecular weight was used, the K_P increased. The lowest K_P (0.44) was observed in ATPS composed of 25% PEG1000 and 20% NaH_2PO_4. High in K_P values indicating most of proteins were more partitioning to the top phase, while the high in K_E implying only the target enzyme was partitioned to the top phase.[22] The highest SA (17.03 units/mg protein) and PF (4.60-fold) of proteinase was obtained in 25% PEG1000 and 20% NaH_2PO_4 systems. Therefore, PEG1000 was a suitable polymer for partitioning of proteinase in albacore tuna liver as indicated by the higher SA and PF than PEG with higher molecular weight. This was possibly due to the fact that interfacial tension is lower when molecular weight of PEG is lower.[25] A preferential interaction between PEG molecule and protein domain decreased when the molecular weight of PEG increased because of its exclusion from the protein domain.[7] Moreover, the surface charge of biological materials is one of the most significant factors affecting the separation by using partitioning. Molecular weight, shape, and specific binding sites of biological materials also affect the partition profiles. Electrical interaction and repulsion between charged aqueous phase systems and the proteins affect the partitioning of system.[2] Among all ATPS tested, system comprising 25% PEG1000 and 20% NaH_2PO_4 partitioned the proteinase to the top PEG-rich phase and undesired protein to the bottom salt phase most effectively.

Table 2. Effect of PEG molecular mass and concentration in a PEG-NaH_2PO_4 ATPS on partitioning of liver proteinase from albacore tuna.

Phase composition (%, w/w)	V_R	K_P	K_E	SA	PF	Yield
10% PEG1000-20% NaH_2PO_4	–	–	–	–	–	–
15% PEG1000-20% NaH_2PO_4	1.04	0.87	1.97	7.45	2.01	70.88
20% PEG1000-20% NaH_2PO_4	1.05	0.77	3.93	9.34	2.52	66.62
25% PEG1000-20% NaH_2PO_4	1.18	0.44	8.88	17.03	4.60	73.70
30% PEG1000-20% NaH_2PO_4	1.51	0.45	2.27	12.21	3.30	53.28
10% PEG4000-20% NaH_2PO_4	0.53	0.73	1.05	4.32	1.17	16.52
15% PEG4000-20% NaH_2PO_4	0.77	0.89	1.14	6.75	1.82	28.30
20% PEG4000-20% NaH_2PO_4	0.87	0.98	1.64	4.91	1.33	21.15
25% PEG4000-20% NaH_2PO_4	1.25	1.34	1.66	5.07	1.37	25.95
30% PEG4000-20% NaH_2PO_4	1.40	1.67	1.01	3.30	0.46	20.13

(-) No phase separation.

V_R: volume ratio (upper/lower); K_P: partition coefficient of protein; K_E: partition coefficient of proteinases; SA: specific activity (U/(g protein) in the upper phase; PF: purification factor in the upper phase; Yield: recovery yield in the upper phase.

Effect of pH on the proteinase partitioning in ATPS

The influence of pH on the partitioning of proteinase from liver of albacore tuna was investigated using the ATPS composition of 25% PEG1000-20% NaH_2PO_4 that provided the highest proteinase recovery. The pH of the ATPS was adjusted to 3.0, 5.0, 7.0, 9.0, and 11.0 in comparison to the control system (without pH adjustment). In general, the pH had influenced on protein partitioning, either by changing the charge of the solute or by altering the ratio of the charged species present. Negatively charged proteins prefer the upper PEG-rich phase, and positively charged proteins partition to the lower salt phase. Therefore, as the pH increases above the isoelectric point (pI) of a protein, it becomes negatively charged, its interaction with PEG becomes stronger, and the partition coefficient increases.[22] A higher PF was found when the system pH increased up to 7.0 (Fig. 1). The highest PF (5.58-fold) and recovery yield (89.99%) were obtained in the system pH of 7.0. However, the decrease in the recovery and PF was observed when the pH of system of 9 and 11 was used. Most of the biomolecules, especially proteins and enzymes, are stable at neutral pH that is favorable condition to conduct the ATPS partitioning. Enzyme stability slightly reduced in the acidic area, but it was dramatically lost at pH above 9.0.[22]

Effect of NaCl on the proteinase partitioning in ATPS

Partitioning of proteinase from liver of albacore tuna in the presence of NaCl was also studied in the system of 25% PEG1000-20% NaH_2PO_4 at pH 7.0 (Fig. 2). In general, addition of NaCl to the ATPS

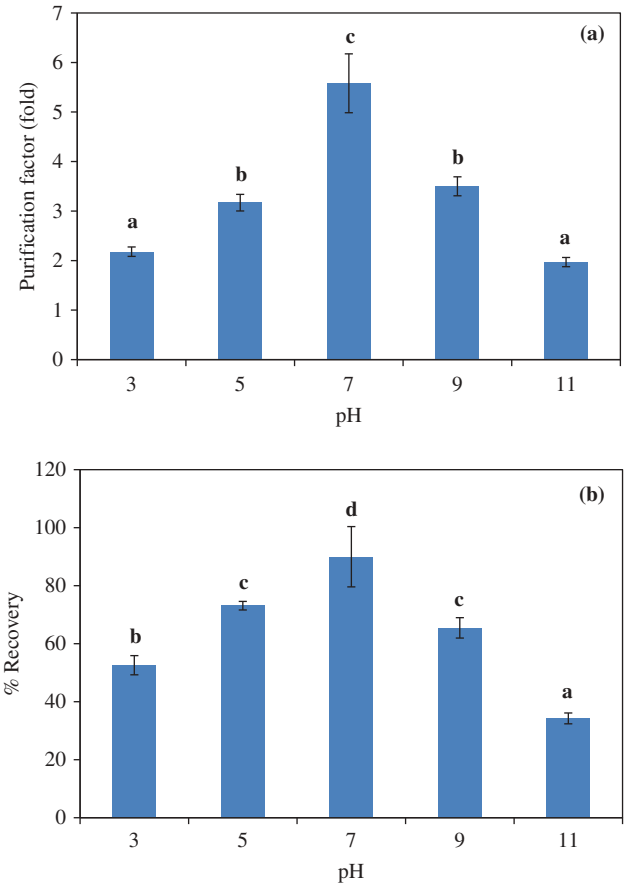

Figure 1. Effect of system pH on the purification factor (a) and proteinase recovery (b) of liver proteinase partitioning in 25% PEG1000-20% NaH_2PO_4 ATPS. Bars represented the standard deviation from triplicate determinations. Different letters within the same parameter indicate the significant differences ($P<0.05$).

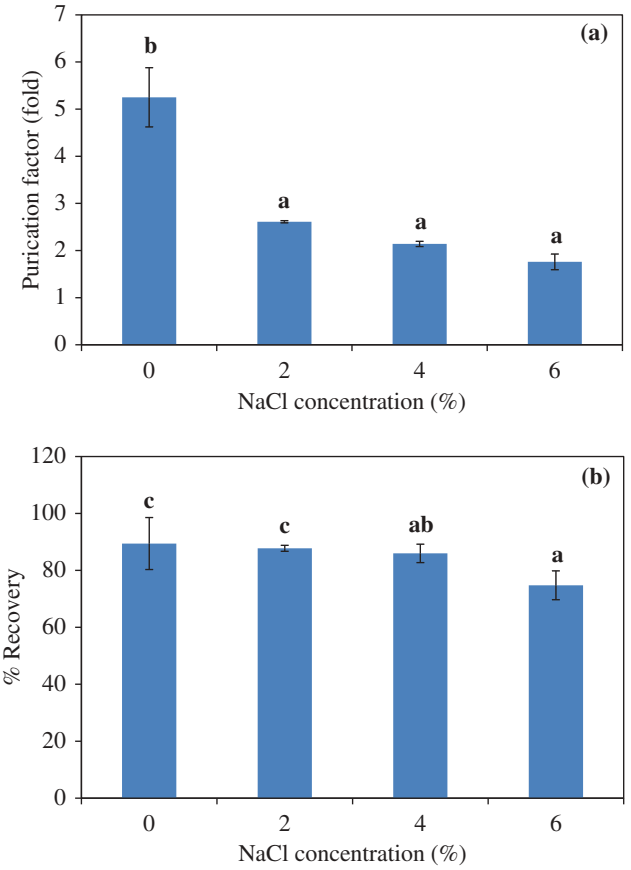

Figure 2. Effect of NaCl concentration on the purification factor (a) and proteinase recovery (b) of liver proteinase partitioning in 25% PEG1000-20% NaH_2PO_4 ATPS at pH 7.0. Bars represented the standard deviation from triplicate determinations. Different letters within the same parameter indicate the significant differences ($P<0.05$).

results in an increase in the hydrophobic difference due to generation of an electrical potential difference between two phases. The result showed that purification factor (PF) and yield decreased with increasing NaCl concentration (Fig. 2). The PF of proteinase decreased from 5.25 (no NaCl addition) to 1.76 (6%, w/w). Higher concentration of NaCl showed a significantly negative effect on partitioning and yield of the enzyme, probably due to protein denaturation and precipitation at high concentration of this salt.[28] These results were in agreement with Ketnawa et al.[23] who studied the PF of alkaline protease from fish viscera in 15% PEG2000-15% $Na_3C_6H_5O_7$ ATPS, using NaCl at different concentrations, ranging from 1% (w/w) to 7% (w/w). The PF of alkaline protease was significantly decreased from 15.34 at 1% (w/w) NaCl to 10.67 at 7% (w/w) NaCl.

Characterization of recovered proteinase

Protein pattern and activity staining of proteinase from albacore tuna liver partitioned with ATPS

SDS-PAGE pattern of proteinase obtained from the partial purification using the ATPS process is shown in Fig. 3. Crude liver extract contained a variety of proteins of different molecular weight. However, a large number of contaminating proteins were removed after partitioning with ATPS, particularly proteins with higher or lower molecular weight. As a result, a higher purity of interested

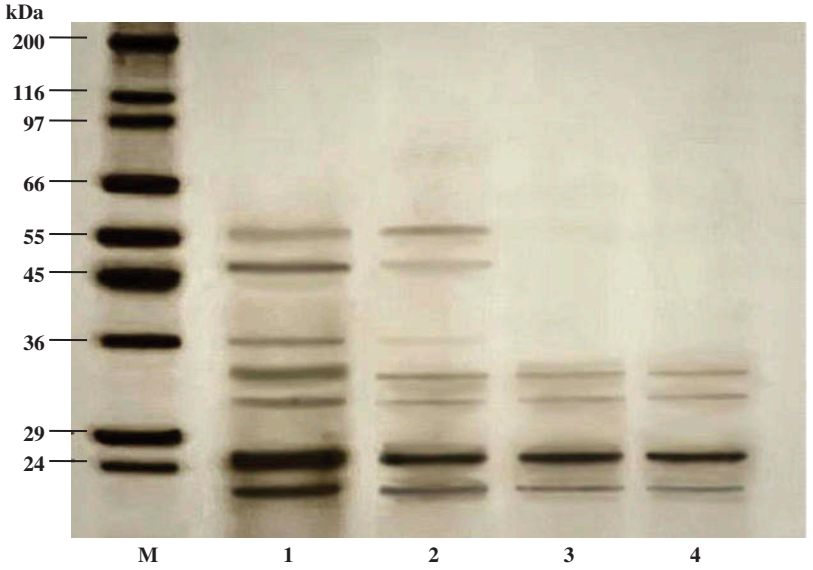

Figure 3. SDS-PAGE of liver extract and ATPS fraction from albacore tuna. M, molecular weight standard; lane 1, liver extract; lane 2, 20% PEG1000-20% NaH_2PO_4 ATPS fraction; lane 3, 25% PEG1000-20% NaH_2PO_4 ATPS fraction; lane 4, 25% PEG1000-20% NaH_2PO_4, pH 7.0 ATPS fraction.

proteinase was obtained. When the proteins or enzymes to be separated differ significantly in their structural properties from others, partitioning can be carried out successfully.[29]

Activity staining of proteinase in liver extract and various fractions obtained from ATPS process were analyzed on SDS-substrate gel (Fig. 4). The presence of the clear zone suggested that it is the proteinase that can be hydrolyzed casein in the gel. The band intensity with apparent MW of 21, 24, 30, and 34 kDa slightly increased after the ATPS process, suggesting the higher specific activity of proteinase loaded into the gel. Molecular weights of trypsin-like enzymes from pyloric ceca brown-stripe red snapper were 20, 24–29, 45, and 97 kDa; bigeye snapper were 17, 20, 22, 45, and 97 kDa; and threadfin bream were 20, 22, 36, and 45 kDa.[30]

Hydrolysis of starry triggerfish muscle using fractionated proteinases

When starry triggerfish muscle was hydrolyzed using fractionated proteinase (0, 25, 50 75, and 100 unit), a rapid hydrolysis was found within the first 15–30 min, followed by a slower hydrolysis rate up to 120 min (Fig. 5). The fast hydrolysis in the initial phase indicated that a large number of peptide bonds were hydrolyzed.[31] Thereafter, the hydrolysis rate was decreased, mainly due to a decrease in available hydrolysis sites, enzyme autodigestion and/or product inhibition.[30] At the same time of hydrolysis, higher DH was observed with higher activity level of fractionated proteinase used. The results of this study revealed that the degree of hydrolysis increased with increasing hydrolysis time. Also, the degradation of protein increased by increasing the enzyme concentration. Naveena et al.[31] reported that when using protease from *Cucumis trigonus* in buffalo meat samples, the increase in proteolysis can be correlated with significantly higher protein solubility. Senphan and Benjakul[21] found that proteinases recovered from hepatopancreases using the combined partitioning systems could be used for gelatin hydrolysis.

The proteolytic degradation pattern of starry triggerfish muscle protein analyzed by SDS-PAGE revealed that among all proteins, myosin heavy chain (MHC) was susceptible to hydrolysis, followed by actin (Fig. 6). The band intensity of MHC decreased with increasing fractionated liver proteinase concentration up to 50 unit. Total disappearance of MHC was observed when enzyme at concentration of 75 and 100 unit was used. For actin, no hydrolytic degradation was observed when starry triggerfish

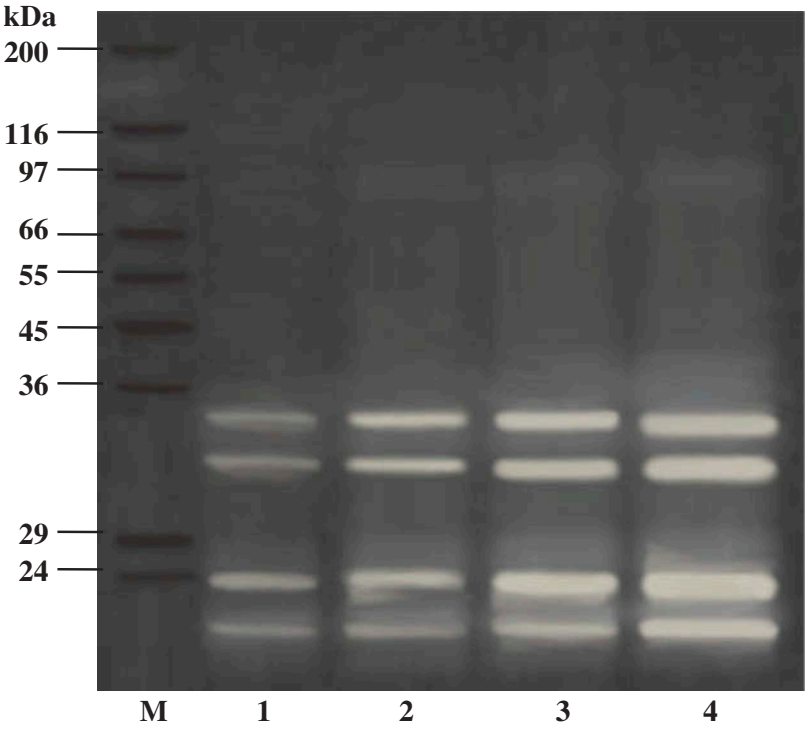

Figure 4. Activity staining of liver extract and ATPS fraction from albacore tuna. M, molecular weight standard; lane 1, liver extract; lane 2, 20% PEG1000-20% NaH_2PO_4 ATPS fraction; lane 3, 25% PEG1000-20% NaH_2PO_4 ATPS fraction; lane 4, 25% PEG1000-20% NaH_2PO_4, pH 7.0 ATPS fraction.

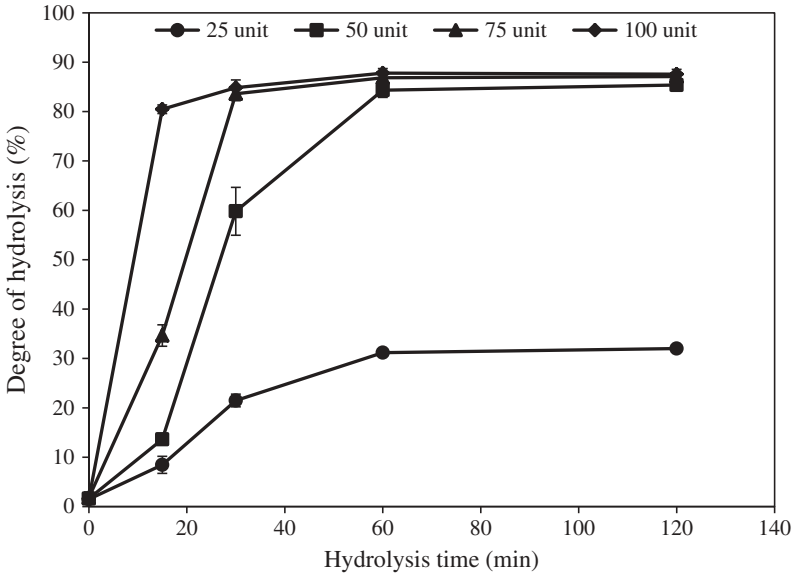

Figure 5. Degree of hydrolysis (DH) of starry triggerfish muscle during hydrolysis with liver enzyme from ATPS fraction (top phase of system 25% PEG1000-20% NaH_2PO_4, pH 7.0). The hydrolytic reaction was performed at pH 8.5, 55°C. Bars represented the standard deviation from triplicate determinations.

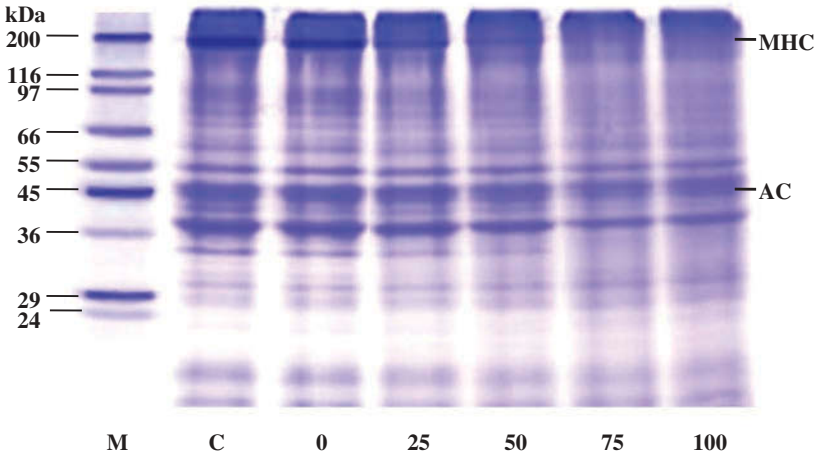

Figure 6. SDS-PAGE patterns of starry triggerfish muscle with liver enzyme from ATPS fraction (top phase of system 25% PEG1000-20% NaH_2PO_4, pH 7.0). The hydrolytic reaction was performed at pH 8.5, 55°C. Numbers designate the enzyme level (unit). M: molecular weight standard; C: control MHC: myosin heavy chain; AC: actin.

muscle was incubated in the presence of 25 and 50 unit fractionated proteinases from albacore tuna liver. However, the degradation increased as the enzyme concentration increased. Also, the degradation rate was lower than that of MHC. Therefore, the rate of hydrolysis was dependent on the amount of enzyme added. From the result, it was noted that autolysis of sample (without fractionated proteinase addition) occurred to some extent during incubation at 55°C. From the results, proteinases recovered from liver of albacore tuna using ATPS could be an alternative potential aid for production of fish protein hydrolysate, in which the cost of enzyme could be reduced.

Conclusion

ATPS can be effectively used to recover and purify proteinase from albacore tuna liver. ATPS with 25% PEG1000-20% NaH_2PO_4, pH 7.0 provided the best enzyme recovery and purity. NaCl concentration had no effect on partitioning of the target enzyme. Based on the protein degradation of starry triggerfish muscle, it is suggested that the fractionated proteinase from albacore tuna liver could be used for protein hydrolysate production.

Funding

This research was supported by the Thailand Research fund and Thaksin University for Project No. RSA6080044. The TRF distinguished research professor grant was also acknowledged.

References

1. Nalinanon, S.; Benjakul, S.; Visessanguan, W.; Kishimura, H. Tuna Pepsin: Characteristics and its use for Collagen Extraction from the Skin of Threadfin Bream (*Nemipterus* spp.). Journal of Food Science **2008**, *73*, 413–419.
2. Klomklao, S.; Benjakul, S.; Visessanguan, W.; Simpson, B.K.; Kishimura, H. Partitioning and Recovery of Proteinase from Tuna Spleen by Aqueous Two-phase Systems. Process Biochemistry **2005**, *40*, 3061–3067.
3. El Hadj Ali, N.; Hmidet, N.; Zouari-Fakhfakh, N.; Ben Khaled, H.; Nasri, M. Alkaline Chymotrypsin from Striped Sea Bream (*Lithognathus Mormyrus*) Viscera: Purification and Characterization. Journal of Agricultural and Food Chemistry **2010**, *58*, 9787–9792.
4. Devi, B.G.; Latha, K.P.J.H. Isolation, Partial Purification and Characterization of Alkaline Protease from Seeds of *Cucumis Melo Var Agrestis*. International Journal of Research in Engineering and Technology **2014**, *3*, 88–97.

5. Ketnawa, S.; Martinez-Alvarez, O.; Benjakul, S.; Rawdkuen, S. Gelatin Hydrolysates from Farmed Giant Catfish Skin using Alkaline Proteases and its Antioxidative Function of Simulated Gastro-Intestinaldigestion. Food Chemistry **2016**, *192*, 34–42.
6. Tubio, G.; Nerli, B.; Pico, G. Partitioning Features of Bovine Trypsin and α-Chymotrypsin in Polyethyleneglycol-sodium Citrate Aqueous Two-Phase Systems. Journal of Chromatography B **2007**, *852*, 244–249.
7. Goja, A.M.; Yang, H.; Cui, M.; Li, C. Aqueous Two-phase Extraction Advances for Bioseparation. Journal of Bioprocessing and Biotechniques **2013**, *4*, 1–8.
8. Oliveira, L.A.; Sarubbo, L.A.; Porto, A.L.F.; Campos-Takaki, G.M.; Tambourgi, E.B. Partition of Trypsin in Aqueous Two-Phase Systems of Poly (Ethylene Glycol) and Cashew-nut Tree Gum. Process Biochemistry **2002**, *38*, 693–699.
9. Nalinanoon, S.; Benjakul, S.; Visessanguan, W.; Kishimura, H. Partitioning of Protease from Stomach of Albacore Tuna (*Thunnus alalunga*) by Aqueous Two-phase Systems. Process Biochemistry **2009**, *44*, 471–476.
10. Ketnava, S.; Rawdkuen, S.; Chaiwat, P. Two Phase Partitioning and Collagen Hydrolysis of Bromelain from Pineapple Peel *Nang Lae* Cultivar. Biochemical Engineering Journal **2010**, *52*, 205–211.
11. Rawdkuen, S.; Pintathong, P.; Chaiwut, P.; Benjakul, S. The Partitioning of Protease from *Calotropis Procera* Latex by Aqueous Two-Phase Systems and its Hydrolytic Pattern on Muscle Proteins. Food and Bioproducts Processing **2011**, *89*, 73–80.
12. Klomklao, S.; Kishimura, H.; Yabe, M.; Benjakul, S. Purification and Characterization of Two Pepsins from the Stomach of Pectoral Rattail (*Coryphaenoides pectoralis*). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology **2007**, *147*, 682–689.
13. An, H.; Seymour, T.A.; Wu, J.W.; Morrisey, M.T. Assay Systems and Characterization of Pacific Whiting (*Merluccius Productus*) Protease. Journal of Food Science **1994**, *59*, 277–289.
14. Lowry, O.H.; Rosebrough, N.J.; Fan, A.L.; Randall, R.J. Protein Measurement with Folin Phenol Reagent. The Journal of Biological Chemistry **1951**, *193*, 256–275.
15. Bradford, M.A. Rapid and Sensitive Method for the Quantification of Microgram Quantities of Protein Utilizing the Principle of Protein Dye Binding. Analytical Biochemistry **1976**, *72*, 248–254.
16. Laemmli, U.K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature **1970**, *227*, 680–685.
17. Benjakul, S.; Morrissey, M.T. Protein Hydrolysates from Pacific Whiting Solid Wastes. Journal of Agricultural and Food Chemistry **1997**, *45*, 3423–3430.
18. Steel, R.G.D.; Torrie, J.H. *Principles and Procedures of Statistics*; McGraw-Hill: New York, **1980**; 481 pp.
19. Raghavarao, K.S.M.S.; Ruinn, M.R.; Todd, P. Recent Developments in Aqueous Two Phase Extraction in Bioprocessing. Separation and Purification Method **1998**, *27*, 1–49.
20. Yang, S.; Huang, Z.; Jiang, Z.; Li, L. Partition and Purification of a Thermostable Xylanase Produced by *Paecilomyces Thermophila* in Solid-State Fermentation using Aqueous Two-Phase Systems. Process Biochemistry **2008**, *43*, 56–61.
21. Senphan, T.; Benjakul, S. Use of the Combined Phase Partitioning Systems for Recovery of Proteases from Hepatopancreas of Pacific White Shrimp. Separation and Purification Technology **2014**, *129*, 57–63.
22. Chaiwat, P.; Rawdkuen, S.; Benjakul, S. Extraction of Protease from *Calotropis Procera* Latex by Polyethylene Glycol-Salts Biphasic System. Process Biochemistry **2010**, *45*, 1148–1155.
23. Ketnawa, S.; Benjakul, S.; Ling, T.C.; Martinez-Alvarez, O.; Rawdkuen, S. Enhanced Recovery of Alkaline Protease from fish Viscera by Phase Partitioning and its Application. Chemistry Central Journal **2013**, *7*, 1–9.
24. Johansson, G. Affinity Partitioning of Proteins using Aqueous Two-Phase Systems. In *Protein Purification: Principles, High-Resolution Methods, and Applications*; Janson, J.C.; Ed.; Wiley-VCH: London, **1998**; 443–460.
25. Klomklao, S.; Benjakul, S.; Visessanguan, W. Comparative Studies on Proteolytic Activity of Spleen Extracts from Three Tuna Species Commonly used in Thailand. Journal of Food Biochemistry **2004**, *28*, 355–372.
26. Isable, D.V.M.; Otero, C. Biphasic Aqueous Media Containing Polyethylene Glycol for the Enzymatic Synthesis of Oligosaccharides from Lactose. Enzyme and Microbial Technology **2003**, *33*, 118–126.
27. Pan, I.H.; Li, Y.K. Rapid Process for Purification of an Extracellular β-xylosidase by Aqueous Two-phase Extraction. Journal of Chromatography B **2001**, *754*, 179–184.
28. Han, J.H.; Lee, C.H. Effects of Salts and Poly(Ethylene Glycol)-Palmitate On the Partitioning of Proteins and *Bacillus Subtilis* Neutral Protease in Aqueous Two-Phase Systems. Colloids and Surfaces B: Biointerfaces **1997**, *9*, 109–116.
29. Rosa, P.A.J.; Azevedo, A.M.; Sommerfeld, S.; Mutter, A.; Aires-Barros, M.R. Application of Aqueous Two-Phase Systems to Antibody Purification: A Multi Stage Approach. Journal of Biotechnology **2009**, *139*, 306–313.
30. Khantaphant, S.; Benjakul, S. Comparative Study on the Proteases from Fish Pyloric Caeca and the Use for Production of Gelatin Hydrolysate with Antioxidative Activity. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology **2008**, *151*, 410–419.
31. Naveena, B.M.; Mendiratta, S.K.; Anjaneyulu, A.S.R. Tenderizaiton of Buffalo Meat using Plant Proteases from *Cucumis Trigounus* Roxb (Kachri) and Zingiber Officinale Roscoe (*Ginger rhizome*). Meat Science **2004**, *68*, 363–369.

International Journal of Biological Macromolecules 107 (2018) 1864–1870

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Two trypsin isoforms from albacore tuna (*Thunnus alalunga*) liver: Purification and physicochemical and biochemical characterization

Sappasith Klomklao [a,*], Soottawat Benjakul [b]

[a] Department of Food Science and Technology, Faculty of Technology and Community Development, Thaksin University, Phatthalung Campus, Phatthalung, 93210, Thailand
[b] Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand

ARTICLE INFO

Article history:
Received 22 May 2017
Received in revised form 10 October 2017
Accepted 10 October 2017
Available online 12 October 2017

Keywords:
Trypsin
Tuna
Isolation
Viscera
N-terminal amino acid sequence

ABSTRACT

Two trypsins (A and B) from the liver of albacore tuna (*Thunnus alalunga*) were purified to homogeneity using a series of column chromatographies including Sephacryl S-200, Sephadex G-50 and Diethylaminoethyl-cellulose. Purity was increased to 80.35- and 101.23-fold with approximately 3.1 and 19.2% yield for trypsins A and B, respectively. The molecular weights of trypsins A and B were estimated to be 21 and 24 kDa, respectively, by SDS-PAGE and size exclusion chromatography. Both trypsins showed only one band on native-PAGE. Trypsins A and B exhibited the maximal activity at 60 °C and 55 °C, respectively, and had the same optimal pH at 8.5 using N^{α}-*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME) as a substrate. Stabilities of both trypsins were well maintained at a temperature up to 50 °C and in the pH range of 7.0–11.0 and were highly dependent on the presence of calcium ion. The inhibition test demonstrated strong inhibition by soybean trypsin inhibitor and TLCK. Activity of both trypsins continuously decreased with increasing NaCl concentration (0–30%). The N-terminal amino acid sequence of 20 residues of the two trypsin isoforms had homology when compared to those of other fish trypsins.

1. Introduction

Seafood waste constitutes at present a serious environmental problem; that waste needs appropriate management. Fish viscera constitute approximately 20% of the fish biomass and are a rich source of digestive proteinases. Hence, the proteinase recovery from fishery waste would be of great importance because it would not only alleviate the serious concerns related to the visceral waste management but also would help produce novel low-cost proteinases for industrial application [1].

Trypsin (EC 3.4.21.4) is one of the main digestive proteinases found in fish viscera, especially pyloric ceca and intestine [2]. It is a serine proteinase, which is produced as an inactive precursor. It has a function in the hydrolysis of target proteins at the amino acids arginine and lysine [3]. Trypsin has various industrial applications, especially in food industries, due to its high stability and activity under harsh conditions, such as in the presence of surfactants and oxidative agents [4]. Trypsins have been extracted and characterized thoroughly based on their biochemical properties from several species of fish, e.g. the viscera of striped sebream (*Lithognathus mormyrus*) [5], Goby (*Zosterisessor ophiocephalus*) [6], the intestine of Grey triggerfish (*Balistes capriscus*) [7], the spleen of skipjack tuna (*Katsuwonus pelamis*) [8], yellowfin tuna (*Thunnus albacores*) [9] and the pyloric ceca of Chinook salmon (*Oncorhynchus tshawytscha*) [10]. Recently, Sripokar et al. [11] reported that albacore tuna liver contained high proteolytic activity and the major proteinases were heat-activated alkaline proteinases, most likely trypsin-like serine proteinases. However, the molecular and the biochemical characteristics of trypsin or trypsin-like enzymes in albacore tuna liver still remain unknown. Therefore, in the present study, we attempted to isolate and characterize trypsins from the liver of albacore tuna and obtain basic information about their biochemical and kinetic properties.

2. Materials and methods

2.1. Chemicals

Ethylenediaminetetraacetic acid (EDTA), pepstatin A, soybean trypsin inhibitor, iodoacetic acid, *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, β-mercaptoethanol (βME) and bovine serum albumin were procured from Sigma

* Corresponding author.
E-mail address: sappasith@tsu.ac.th (S. Klomklao).

https://doi.org/10.1016/j.ijbiomac.2017.10.059
0141-8130/

Chemical Co. (St. Louis, MO, USA.). Sephacryl S-200 and Sephadex G-50 were purchased from Pharmacia Biotech (Uppsala, Sweden). Diethylaminoethyl (DEAE)-cellulose was obtained from Whatman (Maidstone, England). N^{α}-*p*-tosyl-L-arginine methyl ester hydrochloride (TAME) was purchased from Wako Pure Chemicals (Osaka, Japan). Sodium chloride, tris (hydroxymethyl) aminomethane and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS), Coomassie Blue R-250 and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Liver extract preparation

Albacore tuna (*Thunnus alalunga*) internal organs were obtained from Tropical Canning (Thailand) Public Co. Ltd., Hat Yai, Songkhla. Pooled internal organs were then excised and separated into individual organs. Only the liver was collected, immediately frozen and stored at −20 °C until used. Frozen livers were thawed using running water (26–28 °C) until the core temperature reached −2 to 0 °C. The samples were cut into pieces with a thickness of 1–1.5 cm and homogenized into powder in three volumes of acetone at −20 °C for 30 min according to the method of Klomklao et al. [8].

To prepare the liver extract, the liver powder was suspended in 50 mM Na-phosphate buffer, pH 7.0 containing 1 mM $CaCl_2$ referred to as starting buffer (SB) at a ratio of 1:9 (w/v) and stirred continuously at 4 °C for 30 min. The suspension was centrifuged for 30 min at 4 °C at 5000 × g to remove the tissue debris. The supernatant was collected and referred to as "liver extract".

2.3. Trypsin purification from albacore tuna liver

All purification processes were carried out in a walk-in cold room (4 °C). Fractions obtained from all purification steps were subjected to the measurement of protein content and trypsin activity.

2.3.1. Sephacryl S-200 column chromatography

Liver extract was chromatographed on Sephacryl S-200 column (3.9 × 64 cm), which was equilibrated with approximately two bed volumes of SB. Sample was loaded onto column and then eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions of 5 ml were collected and those with TAME activity were pooled, lyopholized and further purified by Sephadex G-50 column.

2.3.2. Sephadex G-50 column chromatography

Lyopholized fractions with TAME activity after Sephacryl S-200 column chromatography were dissolved in distilled water and loaded onto a Sephadex G-50 column (3.9 × 64 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 3 ml were collected and those with TAME activity were pooled and further purified by anion exchanger DEAE-cellulose chromatography.

2.3.3. DEAE-cellulose chromatography

Pooled fractions with TAME activity from Sephadex G-50 column chromatography were collected and dialyzed against SB for 12 h. After that, the sample was chromatographed on DEAE-cellulose (Whatman, England) column (2.2 × 18 cm) 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_{280} was less than 0.05 and then eluted with a linear gradient of 0.05-0.45 M NaCl in SB at a flow rate of 0.5 ml/min. Fractions of 5 ml were collected and the fractions with TAME activity were pooled. Two activity peaks (trypsins A and B) were obtained and pooled fractions from each peak were dialyzed with SB for 12 h and then concentrated by lyophilization and used for further study.

2.4. Trypsin activity assay

Trypsin activity was measured by the method of Hummel [12] as modified by Klomklao et al. [8] using TAME as a substrate. Enzyme solution with an appropriate dilution (20 μl) was mixed with 3.0 ml of 1 mM TAME in 10 mM Tris-HCl buffer, pH 8.0 and incubated at 30 °C for 20 min. Production of *p*-tosyl-arginine was measured by monitoring the increment in absorbance at 247 nm. One unit of activity was defined as the amount causing an increase of 1.0 in absorbance at 247 nm per min.

2.5. 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 30 °C for 20 min. For the temperature profile study, the activity was assayed at different temperatures (20, 30, 40, 50, 55, 60, 65, 70 and 80 °C) for 20 min at pH 8.0.

2.6. 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. Different buffers were used from the above mentioned experiment. For thermal stability, enzyme solution was diluted 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 (Memmert, Germany). Thereafter, the treated samples were suddenly cooled in iced water. The residual activity was assayed using TAME as a substrate at pH 8.0 and 30 °C for 20 min. The effect of $CaCl_2$ on thermal stability was also determined by heating the enzyme dissolved in 50 mM Tris-HCl, pH 8.0 in the presence of 2 mM EDTA or 2 mM $CaCl_2$, at 40 °C for different times (0, 0.5, 1, 2, 4, 6 and 8 h). At the time designated, the samples were cooled in iced water and assayed for remaining activity.

2.7. Determination of molecular weight

The molecular weight of purified trypsins was determined using size exclusion chromatography on Sephacryl S-200 column. The trypsin separated on size exclusion chromatography was estimated for its molecular weight by plotting available partition coefficient (K_{av}) against the logarithm of molecular weight of the protein standards. The elution volume (V_e) was measured for each protein standard and the trypsins. Void volume (V_o) was estimated by the elution volume of blue dextran (M_r 2,000,000). The standards used included aprotinin (M_r 6500), trypsinogen (M_r 24,000), bovine serum albumin (M_r 66,000) and catalase (M_r 232,000).

2.8. Effect of NaCl

Effect of NaCl on trypsin activity was studied. NaCl was added into the standard reaction assay to obtain the final concentrations of 0, 5, 10, 15, 20, 25 and 30% (w/v). The residual activity was determined at 30 °C and pH 8.0 for 20 min using TAME as a substrate.

2.9. Effect of inhibitors

The effect of inhibitors on trypsin activity was determined according to the method of Klomklao et al. [13] by incubating enzyme solution with an equal volume of proteinase inhibitor

solution to obtain the final concentration designated (0.1 mM E-64, 1 mM *N*-ethylmaleimide, 1 mM iodoacetic acid, 1.0 g/l soybean trypsin inhibitor, 5 mM TLCK, 5 mM TPCK, 1 mM pepstatin A and 2 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.

2.10. Polyacrylamide gel electrophoresis

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% β-mercaptoethanol) and boiled for 3 min. The samples (15 μg) were loaded on the gel made of 4% stacking and 12.5% 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 12.5% separating gels in a similar manner, except that the sample was not heated and SDS and reducing agent were left out.

2.10. Determination of N-terminal amino acid sequence

The purified enzymes were subjected to SDS-PAGE under reducing conditions and electrophoretically transferred to polyvinylidenedifluoride (PVDF) membrane. After the membrane was briefly stained by Coomassie brilliant blue, the band of protein was applied to a protein sequencer, Procise 492 (Perkin-Elmer, Foster, CA, USA).

2.12. Kinetic studies

The activity was assayed with different final concentrations of TAME ranging from 0.01 to 0.10 mM. The final enzyme concentration for the assay was 0.1 mg/ml. The determinations were repeated twice and the respective kinetic parameters including V_{max} and K_m were evaluated by plotting the data on a Lineweaver-Burk double-reciprocal graph [14]. Values of turnover number (K_{cat}) were calculated from the following equation: $V_{max}/[E] = K_{cat}$, where [E] is the active enzyme concentration.

2.13. Protein determination

Protein concentration was measured by the method of Lowry et al. [15] using bovine serum albumin as a standard.

3. Results and discussion

3.1. Trypsin purification from albacore tuna liver

Trypsins from the liver of albacore tuna were isolated and purified successively by the three-step procedure described in the materials and methods section. The results of the purification procedure are summarized in Table 1. In the first step, purity of 2.25-fold was obtained with Sephacryl S-200 chromatography. Pooled active fractions obtained from the first step were loaded to Sephadex G-50 column chromatography. After this step, a purification fold of 4.63 with a yield of 45.42% was observed. Subsequently, fractions showing trypsin activity were chromatographed on DEAE-cellulose anion-exchange chromatography column. Two peaks showing trypsin activity were observed after elution with a linear gradient of NaCl (0.05–0.45 M) (Fig. 1). Based on the elution order, these enzymes were assigned as trypsins A and B. Purity was increased to 80.35- and 101.23-fold with a recovery of 3.1 and 19.2% for trypsins A and B, respectively. Klomklao et al. [16] purified two

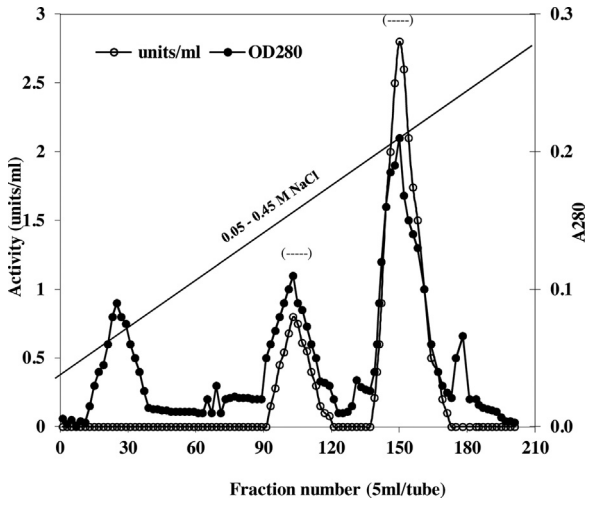

Fig. 1. Elution profile of trypsins from albacore tuna liver on the DEAE-cellulose column. Elution was carried out with a linear gradient of 0.05-0.45 M NaCl in SB.

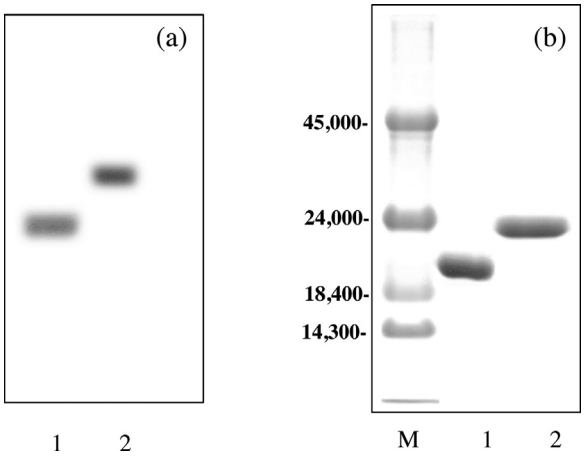

Fig. 2. Protein pattern from native-PAGE (a) and SDS-PAGE (b) of purified trypsins A and B from albacore tuna liver. M, molecular weight standard; lane 1, trypsin A; lane 2, trypsin B.

trypsins from the skipjack tuna intestine by using Sephacryl S-200, Sephadex G-50 and DEAE-cellulose, and purification fold of 177 and 257 were obtained. Trypsin from hybrid catfish viscera was purified by ammonium sulphate fractionation and a series of chromatographies with a 47.6-fold increase in specific activity and 12.7% yield [22].

The purity of the enzymes was determined by using native-PAGE. As depicted in Fig. 2a, trypsins A and B migrated as a single protein band and displayed the different mobilities in native-PAGE, indicating the homogeneity of both enzymes.

For SDS-PAGE (Fig. 2b), each purified trypsin gave a single band and the apparent molecular weights of trypsins A and B were estimated to be 21 and 24 kDa, respectively, corresponding to that measured by gel filtration using Sephacryl S-200 (data not shown). The results confirm that trypsins A and B are monomeric proteins. The molecular weight of both trypsins was similar to those of mammalian and fish trypsins. Generally, fish trypsins were found to have molecular masses in the range of 20 and 28 kDa [16]. Trypsin from zebra blenny viscera had an apparent molecular weight of 27 kDa as estimated by SDS-PAGE and gel filtration [17]. Nasri et al. [6] reported that the molecular weight of an alkaline calcium dependent trypsin from the viscera of Goby was approximately 23.2 kDa using SDS-PAGE and gel filtration. The apparent molecular weight

Table 1
Purification of trypsins from the liver of albacore tuna.

Purification steps	Total activity (units)[a]	Total protein (mg)	Specific activity (units/mg protein)	Purity (fold)	Yield (%)
Crude extract	589.47	897	0.65	1	100
Sephacryl S-200	310.57	213	1.46	2.25	52.69
Sephadex G-50	267.77	89	3.01	4.63	45.42
DEAE-Cellulose					
Trypsin A	18.28	0.35	52.23	80.35	3.10
Trypsin B	113.18	1.72	65.80	101.23	19.20

[a] Trypsin activity was assayed at pH 8.0, 30 °C for 20 min using TAME as a substrate.

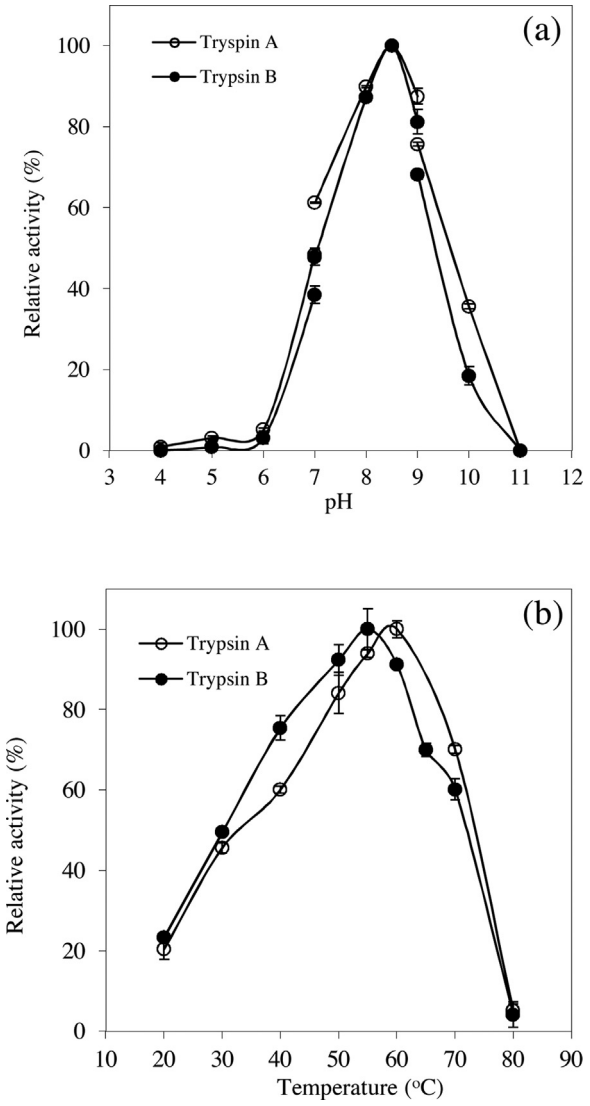

Fig. 3. pH (a) and temperature (b) profiles of purified trypsins A, and B from albacore tuna liver.

of trypsin A and B from yellowfin tuna spleen was estimated to be 24 kDa by size exclusion chromatography and SDS-PAGE [9].

3.2. Optimal pH and temperature

The pH curves of trypsins A and B from albacore tuna liver are illustrated in Fig. 3a. Both trypsins were active between pH 7.0 and 9.0 and showed the maximal activity toward TAME at pH 8.5. Loss of activity was found at very acidic and alkaline pHs. No activity was observed at pH 11.0. The sharp decrease in TAME hydrolysis by both trypsins at low and high pH might be attributed to denaturation [9].

Temperature activity profiles of trypsins A and B are shown in Fig. 3b. Trypsins A and B had optimal temperatures of 60 and 55 °C, respectively. At temperatures above 70 °C, an appreciable decrease in activity of both trypsins was observed, presumably due to thermal inactivation. Inactivation of the enzymes at high temperature was possibly due to unfolding of the molecules. Trypsin from zebra blenny viscera had a temperature optimum of 60 °C [17]. Klomklao et al. [9] reported that trypsin A and B from the spleen of yellowfin tuna had the maximal activity at 55 and 60 °C, respectively. The optimum temperature for trypsin activity from golden grey mullet viscera was 50 °C [18]. Nevertheless, these optimal temperatures were higher than those reported for trypsin from Grey triggerfish [7], vermiculated sailfin catfish [19] and carp [20], which had the optimum temperatures in the range of 30–40 °C. The difference might be related to the different temperatures of water where the fish inhabited.

3.3. pH and thermal stability

Fig. 4a shows the pH stability profile of purified trypsins A and B from albacore tuna liver. Both trypsins were highly stable over a wide pH range, maintaining 100% of their original activities between pH 7.0–11.0. Nevertheless, some losses in trypsin activities were found at slightly acidic pH for both trypsins. No activities were observed for trypsins A and B after incubation at pH 4.0. The trypsins stability at particular pH may be relevant to the net charge of the enzyme at that pH [9]. At extreme pH, strong intramolecular electrostatic repulsion caused by high net charge results in swelling and unfolding of the protein molecules [20,21]. Enzyme activity inactivation at acidic pH was also reported for trypsin from Grey triggerfish [7], hybrid catfish [22], zebra blenny [17], Goby [6] and Tunisian barbel [23]. From these results, the liver of albacore tuna would be a potential source of trypsins for certain food processing operations that require high alkaline conditions.

For thermal stability, both trypsins A and B purified from albacore tuna liver were highly stable at temperatures below 50 °C (Fig. 4b). However, both enzymes were inactivated at higher temperatures (Fig. 4b). The relative activities at 80 °C for trypsin A and B were about 5.12% and 0.25%, respectively, of their initial activity. At high temperatures, enzyme possibly underwent denaturation and lost their activity.

The thermal stability of the purified trypsins A and B was also investigated by incubating the enzymes in the presence of 2 mM EDTA or 2 mM $CaCl_2$, at 40 °C for different times (0, 0.5, 1, 2, 4, 6 and 8 h). As depicted in Fig. 5, in the presence of 2 mM calcium ion, approximately 85% of the initial activities remained after 8 h of incubation at 40 °C. However, the trypsin activity of both enzymes decreased with increasing time in the presence of 2 mM EDTA. Purified trypsin B was rather stable in the presence of 2 mM EDTA than purified trypsin A, especially when the incubation time increased. These results indicated that trypsins A and B from the liver of albacore tuna were most likely stabilized by calcium ion. The presence of calcium ions activates trypsinogen to trypsin and increases the thermal stability of the enzyme. This stabilizing effect is accom-

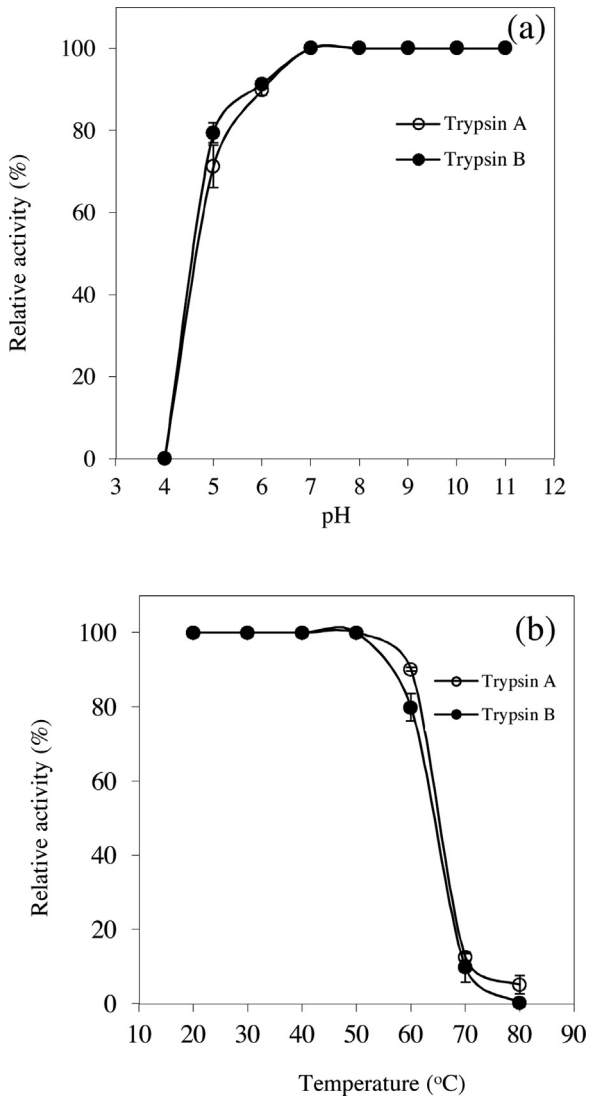

Fig. 4. pH (a) and thermal (b) stability of purified trypsin A and B from albacore tuna liver.

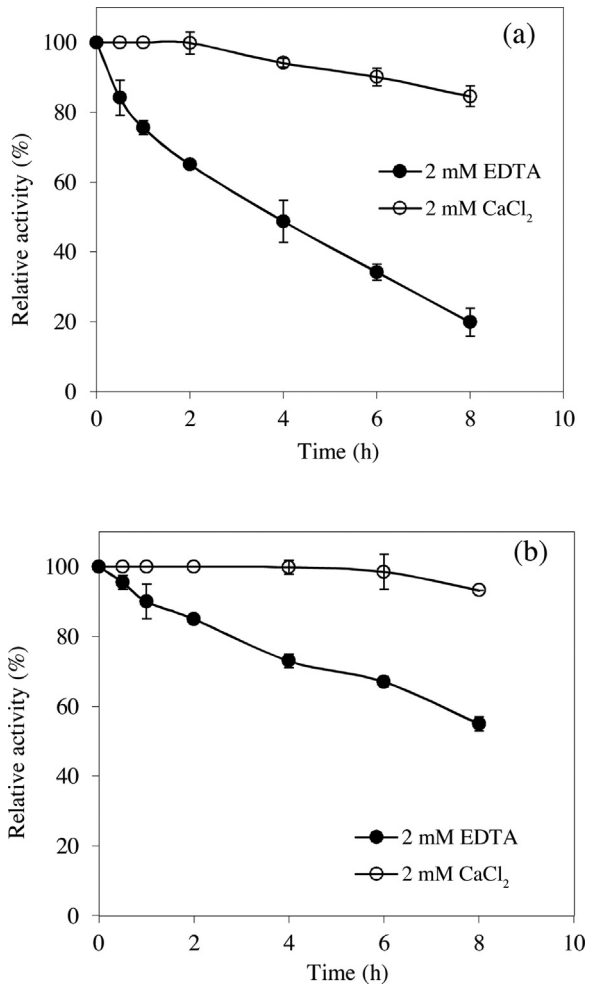

Fig. 5. Effect of calcium ion and EDTA on the stability of purified trypsins A (a) and B (b) from albacore tuna liver. The stability was tested at 40 °C for different times.

plished by a conformational change in the molecule of trypsin, resulting in a more compact structure [13]. Klomklao et al. [22] reported that trypsin purified from the viscera of hybrid catfish was stabilized by calcium ion. El Hadj Ali et al. [5] also found that the stability of trypsin from striped seabream viscera was enhanced by the addition of $CaCl_2$. On the other hand, trypsin from Nile tilapia intestine was not stabilized by calcium ion [24]. These findings suggest a difference in the structure of the primary calcium binding site among different marine fish trypsins.

3.4. Effect of NaCl

As shown in Fig. 6, the trypsin activities of both enzymes decreased gradually with increasing NaCl concentrations. In the presence of NaCl ranging from 5% to 30%, trypsin B exhibited slightly higher trypsin activity than trypsin A, indicating that trypsin B was more tolerant to NaCl than trypsin A. At 30% NaCl, remaining trypsin activities were approximately 50% and 54% for trypsins A and B, respectively. The decrease in activity might be due to the enzyme denaturation. The 'salting out' effect was postulated to cause the denaturation of enzymes. The water molecule is drawn from the molecule of trypsin by salt, leading to the aggregation of those enzymes [9]. Based on these results, more than 50% of activity

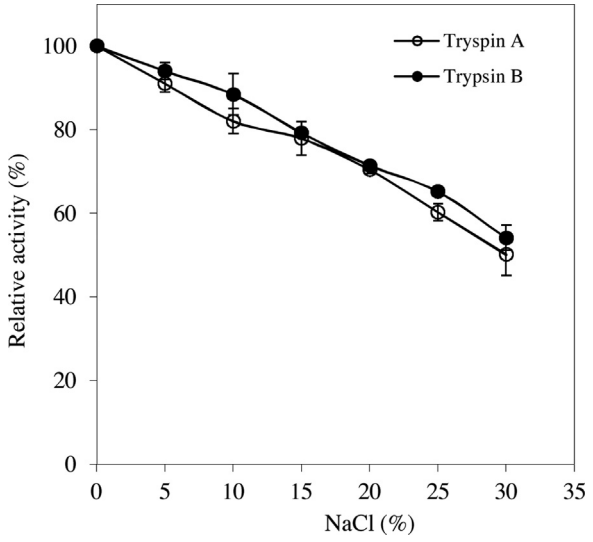

Fig. 6. Effect of NaCl concentrations on activities of purified trypsins A and B from albacore tuna liver.

Table 2
Effect of various inhibitors on the activity of purified trypsins from albacore tuna liver[a].

Inhibitors	Concentration	% Inhibition	
		Trypsin A	Trypsin B
Control		0	0
E-64	0.1 mM	0	0
N-ethylmaleimide	1 mM	0	0
Iodoacetic acid	1 mM	0	0
Soybean trypsin inhibitor	1.0 g/l	87.50 ± 0.68	91.68 ± 0.28
TLCK	5 mM	98.75 ± 1.12	99.91 ± 0.17
TPCK	5 mM	0	0
Pepstatin A	0.01 mM	0	0
EDTA	2 mM	18.54 ± 0.40	8.18 ± 0.73

[a] Each enzyme solution was incubated with the same volume of inhibitor at 25 °C for 15 min and the residual activity was determined using TAME as a substrate for 20 min at pH 8.0 and 30 °C.

Table 3
Kinetic properties of albacore tuna liver trypsins for the hydrolysis of TAME.

Enzyme	K_m (mM)	K_{cat} (S^{-1})	K_{cat}/K_m (S^{-1} mM^{-1})
Trypsin A	0.23 ± 0.02	67.77 ± 0.01	294.65
Trypsin B	0.32 ± 0.01	85.58 ± 0.03	267.44

K_m, K_{cat} values were determined using TAME as a substrate at pH 8.0 and 30 °C.

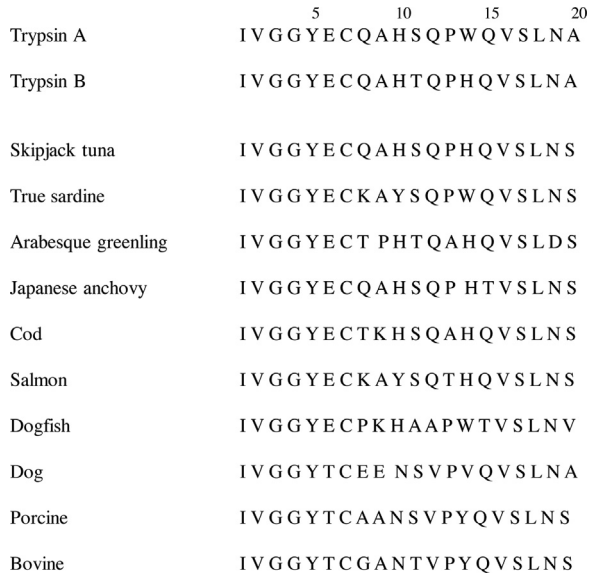

Fig. 7. Comparison of N-terminal amino acid sequences of the purified trypsins A and B from albacore tuna liver with other enzymes: skipjack tuna [16], true sardine, arabesque greenling [28], Japanese anchovy [29], cod [30], salmon [31], dogfish [32], dog [33], porcine [34], and bovine [35].

remained for both trypsins A and B at high salt concentration (30%). Therefore, these trypsins from albacore tuna liver can be involved in protein hydrolysis in high salt fermented fish products such as fish sauce.

3.5. Effect of inhibitors

Proteinases can be classified by their sensitivity to various inhibitors. The effect of different proteinase inhibitors, such as specific group reagents and chelating agents on the trypsin activity were studied (Table 2). The trypsin activities of both enzymes were highly affected by soybean trypsin inhibitor, known as a trypsin inhibitor and TLCK, a trypsin specific inhibitor. Further, the metalloproteinase inhibitor (2 mM EDTA), displayed partial inhibition towards both trypsins A and B with different degrees. Trypsin A was inhibited by EDTA to a greater extent, compared with trypsin B. However, cysteine and aspartic proteinases inhibitors and a chymotrypsin specific inhibitor (TPCK) showed no inhibitory effects on the trypsin activity of both enzymes. The results confirmed that both enzymes are serine proteinases, which possibly require metal ions for their activities. Purified trypsin from carnivorous catfish was strongly inhibited using soybean trypsin inhibitor and PMSF [25]. Ktari et al. [17] reported that soybean trypsin inhibitor and PMSF showed a strong inhibitory effect on the purified trypsin from zebra blenny viscera. Klomklao et al. [22] also reported that the trypsin activity from the viscera of hybrid catfish was effectively inhibited by soybean trypsin inhibitor and TLCK and partially inhibited by EDTA.

3.6. Kinetic study

Table 3 shows the kinetic constants K_m and K_{cat} of the purified trypsins A and B from the liver of albacore tuna for TAME hydrolysis measured using Lineweaver-Burk plots. K_m values of trypsins A and B were 0.23 and 0.32 mM, respectively. K_{cat} values of trypsins A and B were 67.77 and 85.58 S^{-1}, respectively. The K_m and K_{cat} of the purified trypsins from albacore tuna liver were close to those reported for trypsins from yellowfin tuna [9] and Japanese sea bass [26]. The K_m value of trypsin A was lower than that of trypsin B. This result suggests that trypsin A has higher affinity to TAME, compared with trypsin B. For K_{cat} (turnover number), trypsin B had a higher value than trypsin A. Nevertheless, the catalytic efficiency value for trypsin A was higher than that of trypsin B. This result suggests that trypsin A would be more efficient in transforming the substrate to product. Furthermore, the catalytic efficiency of trypsins from albacore tuna liver was higher than those reported for mammalian trypsins [27].

3.7. N-terminal sequencing

Generally, trypsins are regarded to have arisen from a common ancestor by divergent evolution as they share similarities not only in biological functions, but also in active sites, primary and even three-dimensional structures. The N-terminal (20 residues) amino acid sequences of trypsins A and B were IVGGYECQAHSQPWQVSLNA and IVGGYECQAHTQPHQVSLNA (Fig. 7), indicating that the N terminus of the enzymes was unblocked. The N-terminal amino acid sequences of the trypsins A and B from albacore tuna liver were aligned with those of other animal trypsins (Fig. 7). Being similar to other fish trypsins, both trypsins had a charged Glu residue at position 6, where Thr is most common in mammalian pancreatic trypsins. The N-terminal sequences of trypsins A and B from albacore tuna liver clearly showed that they are closely-related members of the trypsin family.

4. Conclusion

The proteases were purified from the liver of albacore tuna. After purification, two enzymes were obtained. The characterization, with specific substrate, inhibitors and the N-terminal sequence, demonstrated that these proteases are trypsin. Furthermore, it showed interesting features, such as high activity and stability over a large alkaline pH range and high activity at elevated salt concentrations. These properties have confirmed that fish viscera may be used as a source of trypsin with potential for industrial applications.

Acknowledgments

This research was supported by the Thailand Research Fund and Thaksin University for Project No. RSA6080044. The TRF distinguished research professor grant was also acknowledged.

References

[1] A. Bougatef, Trypsins from fish processing waste: characteristics and biotechnological applications - comprehensive review, J.Clean Prod. 57 (2009) 257–265.
[2] P. Sripokar, T. Poonsin, M. Chaijan, S. Benjakul, S. Klomklao, Proteinases from the liver of albacore tuna *(Thunnus Alalunga)*: Optimum extractant and biochemical characteristics, J. Food Biochem. 40 (2016) 10–19.
[3] S. Unajak, P. Meesawat, A. Paemanee, N. Areechon, A. Engkagul, U. Kovitvadhi, S. Kovitvadhi, K. Rungruangsak-Torrissen, K. Choowongkomon, Characterisation of thermostable trypsin and determination of trypsin isozymes from intestine of Nile tilapia (*Oreochromis niloticus* L.), Food Chem. 134 (2012) 1533–1541.
[4] S. Klomklao, S. Benjakul, W. Visessanguan, B.K. Simpson, H. Kishimura, Partitioning and recovery of proteinase from tuna spleen by aqueous two-phase systems, Process Biochem. 40 (2005) 3061–3067.
[5] N. El Hadj, N. Ali, A. Hmidet, R. Bougatef, M. Nasri, A laundry detergent-stable alkaline trypsin from striped seabream (*Lithognathus mormyrus*) viscera: purification and characterization, J. Agric. Food Chem. 57 (2009) 10943–10950.
[6] R. Nasri, A. Sila, N. Ktari, I. Lassoued, A. Bougatef, M. Karra-Chaabouni, M. Nasri, Calcium dependent, alkaline detergent-stable trypsin from the viscera of Goby (*Zosterisessor ophiocephalus*): purification and characterization, Process Biochem. 47 (2012) 1957–1964.
[7] K. Jellouli, A. Bougatef, D. Daassi, R. Balti, A. Barkia, M. Nasri, New alkaline trypsin from the intestine of Grey triggerfish (*Balistes capriscus*) with high activity at low temperature: purification and characterization, Food Chem. 116 (2009) 644–650.
[8] S. Klomklao, S. Benjakul, W. Visessanguan, H. Kishimura, B.K. Simpson, Purification and characterisation of trypsins from the spleen of skipjack tuna (*Katsuwonus pelamis*), Food Chem. 100 (2007) 1580–1589.
[9] S. Klomklao, S. Benjakul, W. Visessanguan, H. Kishimura, B.K. Simpson, H. Saeki, Trypsins from yellowfin tuna (*Thunnus albacores*) spleen: purification and characterization, Comp. Biochem. Physiol. B 144 (2006) 47–56.
[10] I. Kurtovic, S.N. Marshall, B.K. Simpson, Isolation and characterization of a trypsin fraction from the pyloric ceca of chinook salmon (*Oncorhynchus tshawytscha*), Comp. Biochem. Physiol. B. 143 (2006) 432–440.
[11] P. Sripokar, M. Chaijan, S. Benjakul, H. Kishimura, S. Klomklao, Enzymatic hydrolysis of starry triggerfish (*Abalistes stellaris*) muscle using liver proteinase from albacore tuna (*Thunnus alalunga*), J. Food Sci. Technol. 53 (2016) 1047–1054.
[12] B.C.W. Hummel, A modified spectrophotometric determination of chymotrypsin, trypsin, and thrombin, Can. J. Biochem. Phys. 37 (1959) 1393–1399.
[13] S. Klomklao, S. Benjakul, W. Visessanguan, Comparative studies on proteolytic activity of spleen extracts from three tuna species commonly used in Thailand, J. Food Biochem. 28 (2004) 355–372.
[14] H. Lineweaver, D. Burk, The determination of enzyme dissociation constants, J. Am. Chem. Soc. 56 (1934) 665–666.
[15] O.H. Lowry, N.J. Rosebrough, A.L. Fan, R.J. Randall, 1951. Protein measurement with Folin phenol reagent, J. Biol. Chem. 193 (1951) 256–275.
[16] S. Klomklao, H. Kishimura, Y. Nonami, S. Benjakul, Biochemical properties of two isoforms of trypsin purified from the intestine of skipjack tuna (*Katsuwonus pelamis*), Food Chem. 115 (2009) 155–162.
[17] N. Ktari, H.B. Khaled, R. Nasri, K. Jellouli, S. Ghorbel, M. Nasri, Trypsin from zebra blenny (*Salaria basilisca*) viscera: purification, characterisation and potential application as a detergent additive, Food Chem. 130 (2012) 467–474.
[18] I. Bkhairia, H.B. Khaled, N. Ktari, N. Miled, M. Nasri, S. Ghorbel, Biochemical and molecular characterisation of a new alkaline trypsin from *Liza aurata*: Structural features explaining thermal stability, Food Chem. 196 (2016) 1346–1354.
[19] A.G. Villalba-Villalba, J.C. Ramírez-Suárez, E.M. Valenzuela-Soto, G.G. Sánchez, G.C. Ruiz, R. Pacheco-Aguilar, Trypsin from viscera of vermiculated sailfin catfish, *Pterygoplichthys disjunctivus*, Weber, 1991: its purification and characterization, Food Chem. 141 (2013) 940–945.
[20] B.K. Khangembam, R. Chakrabarti, Trypsin from the digestive system of carp *Cirrhinus mrigala*: purification, characterization and its potential application, Food Chem. 175 (2015) 386–394.
[21] S. Benjakul, W. Visessanguan, P. Thummaratwasik, Isolation and characterization of trypsin inhibitors from some Thai legume seeds, J. Food Biochem. 24 (2000) 107–127.
[22] S. Klomklao, S. Benjakul, H. Kishimura, M. Chaijan, 24 kDa Trypsin: a predominant protease purified from the viscera of hybrid catfish (*Clarias macrocephalus x Clarias gariepinus*), Food Chem. 129 (2011) 739–746.
[23] A. Sila, R. Nasri, M. Jridi, R. Balti, M. Nasri, A. Bougatef, Characterisation of trypsin purified from the viscera of Tunisian barbel (*Barbus callensis*) and its application for recovery of carotenoproteins from shrimp wastes, Food Chem. 132 (2012) 1287–1295.
[24] R.S. Bezerra, E.J.F. Lins, R.B. Alencar, P.M.G. Paiva, M.E.C. Chaves, L.C.B.B. Coelho, J.L.B. Carvalho, Alkaline proteinase from intestine of Nile tilapia (*Oreochromis niloticus*), Process Biochem. 40 (2005) 1829–1834.
[25] C.W.V. Santos, M.E.C. Marques, H.A. Tenório, E.C. Miranda, H.J.V. Pereira, Purification and characterization of trypsin from *Luphiosilurus alexandri* pyloric cecum, Biochem. Biophys. Rep. 8 (2016) 29–33.
[26] Q.F. Cai, Y.K. Jiang, L.G. Zhou, L.C. Sun, G.M. Liu, K. Osatomi, M.J. Cao, Biochemical characterization of trypsins from the hepatopancreas of Japanese sea bass (*Lateolabrax japonicus*), Comp. Biochem. Physiol B. 159 (2011) 183–189.
[27] B.K. Simpson, Digestive proteinases from marine animals, in: N.M. Haard, B.K. Simpson (Eds.), Seafood Enzymes: Utilization and Influence on Postharvest Seafood Quality, Marcel Dekker, New York, 2000, pp. 531–540.
[28] H. Kishimura, K. Hayashi, Y. Miyashita, Y. Nonami, Characteristics of trypsins from the viscera of true sardine (*Sardinops melanostictus*) and the pyloric caeca of arabesque greenling (*Pleuroprammus azonus*), Food Chem. 97 (2006) 65–70.
[29] H. Kishimura, K. Hayashi, Y. Miyashita, Y. Nonami, Characteristics of two trypsin isozymes from the viscera of Japanese anchovy (*Engraulis japonica*), J. Food Biochem. 29 (2005) 459–469.
[30] A. Gudmundsdottir, E. Gudmundsdottir, S. Oskarsson, J.B. Bjanrnason, A.K. Eakin, C.S. Craik, Isolation and characterization of cDNAs from Atlantic cod encoding two different forms of trypsinogen, Eur. J. Biochem. 217 (1993) 1091–1097.
[31] L.B. Male, A.O. Lorens, Molecular cloning and characterization of anionic and cationic variants of trypsin from Atlantic salmon, Eur. J. Biochem. 232 (1995) 677–685.
[32] L.H. Titani, H. Ericsson, Amino acid sequence of. dogfish trypsin, Biochemistry 14 (1975) 1358–1366.
[33] K.S. Pinsky, Differential regulation of trypsinogen. mRNA translation Full-length mRNA sequences encoding two oppositely charged trypsinogen isoenzymes in the dog pancreas, Mol. Cell. Biol. 5 (1985) 2669–2676.
[34] M.A. Hermodson, L.H. Ericsson, H. Neurath, K.A. Walsh, Determination of the amino sequence of porcine trypsin by sequenator analysis, Biochemistry 12 (1973) 3146–3153.
[35] K.A. Walsch, Trypsinogens and trypsins of various species, Method Enzymol. 19 (1) (1970) 41–63.

J Food Sci Technol (February 2016) 53(2):1047–1054
DOI 10.1007/s13197-015-2138-z

ORIGINAL ARTICLE

Enzymatic hydrolysis of starry triggerfish (*Abalistes stellaris*) muscle using liver proteinase from albacore tuna (*Thunnus alalunga*)

P. Sripokar[1] · M. Chaijan[2] · S. Benjakul[3] · H. Kishimura[4] · S. Klomklao[5]

Revised: 29 November 2015 / Accepted: 11 December 2015 / Published online: 23 December 2015

Abstract Proteinases from liver extract from albacore tuna (*Thunnus alalunga*) were used to produce protein hydrolysate from starry triggerfish (*Abalistes stellaris*) muscle. Hydrolysis conditions for preparing protein hydrolysate from starry triggerfish muscle were optimized. Enzyme level, reaction time and fish muscle/buffer ratio significantly affected the hydrolysis ($p < 0.05$). Optimum conditions for triggerfish muscle hydrolysis were 5.5 % liver extract, 40 min reaction time and fish muscle/buffer ratio of 1:3 (*w*/*v*). The freeze-dried protein hydrolysate was characterized with respect to chemical composition, amino acid composition and color. The product contained 91.73 % protein, 2.04 % lipid and 6.48 % ash. The protein hydrolysate exhibited high amount of essential amino acids (45.62 %). It was light yellow in color (L^* = 82.94, a^* = 0.84, b^* = 22.83). The results indicate that the extract from liver of albacore tuna could be used to produce fish protein hydrolysate and protein hydrolysate from starry triggerfish muscle may potentially serve as a good source of desirable peptide and amino acids.

Keywords Protein hydrolysate · Proteinases · Tuna · Industrial application

✉ S. Klomklao
sappasith@tsu.ac.th

[1] Biotechnology Program, Faculty of Technology and Community Development, Phatthalung Campus, Thaksin University, Phatthalung 93210, Thailand

[2] Department of Food Technology, School of Agricultural Technology, Walailak University, Nakhon Si Thammarat 80160, Thailand

[3] Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

[4] Laboratory of Marine Products and Food Science, Research Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

[5] Department of Food Science and Technology, Faculty of Technology and Community Development, Phatthalung Campus, Thaksin University, Phatthalung 93210, Thailand

Introduction

Proteins from fish processing byproducts can be modified to improve their quality and functional characteristics by enzymatic hydrolysis (Shahidi 1994). Utilizing proteolytic enzymes, fish protein hydrolysate (FPH) can be prepared with the peptides having new and/or improved properties. Generally, protein can be hydrolyzed by chemical process or protease enzymes. However, FPH obtained from these two methods are different in quality (Wisuthipheat and Kongruang 2015). Enzymatic hydrolysis of food proteins is an efficient way to recover potent bioactive peptides. Fish protein hydrolysates have been shown to have potential for nutritional or pharmaceutical applications (Thiansilakul et al. 2007; Wu et al. 2003). Protein hydrolysis decreases the peptide size, and thereby making hydrolysates the most available amino acid source for various physiological functions of human body. Protein hydrolysates are used as readily available sources of protein for humans and animals due to their good functional properties (Neklyudov et al. 2000).

The starry triggerfish (*Abalistes stellaris*), or flat-tailed triggerfish belongs to order Tetraodontiformes and is a member of the Balistidae family. Generally, it is widely used as fish meal. In Thailand, starry triggerfish has not been used for human consumption due to its tough skin. To increase the value of this fish species, the production of new value-added products such as protein hydrolysates, with nutritive value and bioactivity, can pave the way for its full utilization. Many factors

affect the bioactivity of protein hydrolysates, e.g. type and concentration of proteinases (Benjakul and Morrissey 1997), steps of hydrolysis (Thiansilakul et al. 2007), etc. Protein hydrolysates have been produced and characterized from several sources, e.g. toothed ponyfish (*Gazza minuta*) (Klomklao et al. 2013), Catla (*Catla catla*) (Bhaskar and Mahendrakar 2008), yellowfin tuna (*Thunnus albacores*) (Guerard et al. 2001) and bigeye snapper (*Priacanthus macracanthus*) (Phanturat et al. 2010).

Fish viscera generated during processing contain a variety of enzymes including proteinases. Proteinases are potential enzymes for industrial applications and could produce the new bioactive molecules (Klomklao et al. 2005). Albacore tuana (*Thunnus alalunga*) is the potential raw materials for canned production in Thailand (Nalinanon et al. 2008). Processing wastes generated during canned tuna processing, especially viscera can be used as the essential source of proteinases. Sripokar et al. (2015) reported that the liver of albacore tuna contained high proteolytic activity and major proteinases were heat-activated alkaline proteinases, most likely trypsin-like serine proteinases.

Recovery and use of proteinases from fish viscera for fish protein hydrolysate are a promising approach to minimize the economics and ecologicals problem of this processing waste. Also, the production of protein hydrolysate from fish muscle using albacore tuna liver extract has not been reported. Hence, the purpose of this work was to study the production and characterization of hydrolysate from starry triggerfish muscle using albacore tuna liver extract.

Materials and methods

Chemical

β-Mercaptoethanol (βME), 2,4,6-trinitrobenzenesulfonic acid (TNBS), *L*-leucine and bovine serum albumin were procured from Sigma Chemical Co. (St. Louis, MO, USA). Tris (hydroxymethyl) aminomethane (Tris-HCl), sodium sulphite and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS) and Coomassie Brilliant Blue R-250 were procured from Bio-Rad Laboratories (Hercules, CA, USA). All of chemicals used were of analytical grade.

Fish sample preparation

Albacore tuna (*Thunnus alalunga*) internal organs were obtained from Tropical Canning (Thailand) Public Co. Ltd., Hat Yai, Songkhla. The samples were packed in polyethylene bags, kept in ice with a sample/ice ratio of 1:3 (*w*/*w*) and transported to the research laboratory within 2 h. Pooled internal organs were separated and only the liver was collected, immediately frozen and stored at −20 °C until used.

Starry triggerfish (*Abalistes stellaris*) with the length of 30–35 cm were purchased from the dock in Trang, Thailand. The fish, off-loaded approximately 18–24 h after capture, were placed on ice at a fish/ice ratio of 1:2 (*w*/*w*) and transported to the Department of Food Science and Technology, Thaksin University, Phatthalung, within 2 h. Upon arrival, the fish were filleted and the ordinary muscle was collected and ground to uniformity. A portion of mince (500 g) was placed in a polyethylene bag and stored at −20 °C until used.

Preparation of liver extract

Frozen livers were thawed using running water (26–28 °C) until the core temperature reached −2 to 0 °C. The samples were cut into pieces with a thickness of 1–1.5 cm and homogenized into powder in three volumes of acetone at −20 °C for 30 min according to the method of Klomklao et al. (2007). The homogenate was filtrated in vacuum on Whatman No. 4 filter paper. The residue obtained was then homogenized in two volumes of acetone at -20 °C for 30 min. The residue was left at room temperature until dried and free of acetone odor.

To prepare the liver extract, defatted liver powder was suspended in 50 mM Na-phosphate, pH 7.0 at a ratio of 1:9 (*w*/*v*) and stirred continuously at 4 °C for 30 min. The suspension was centrifuged at 5000×g for 30 min at 4 °C using a Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA). The supernatant obtained was collected and referred to as "liver extract".

Study on the optimum condition for production of starry triggerfish protein hydrolysate using tuna liver extract

Effect of extract concentration on DH

Effect of liver extract concentrations (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 10, 15 and 20 % (*w*/*w*)) on degree of hydrolysis (DH) was studied. Different amounts of liver extract were added to the suspension of starry triggerfish mince in 0.1 M glycine-NaOH buffer, pH 8.5 (1:2 ratio, *w*/*v*) and the reaction was carried out at 55 °C, for 30 min (Sripokar et al. 2015). The termination of hydrolytic reaction was done by heating the reaction mixture at 90 °C for 15 min in a water bath. The supernatant was obtained by centrifuging at 5000 g for 10 min. DH was then determined. The extract concentration, which was able to hydrolyze fish mince with the highest DH, was selected for further study.

Effect of reaction time on DH

To study the effect of reaction time on DH, starry triggerfish mince was added with 0.1 M glycine-NaOH buffer (pH 8.5) at

the ratio of 1:2 (*w*/*v*). The reaction was initiated by adding 5.5 % liver extract. The mixtures were shaken at 55 °C for 5, 10, 15, 20, 30, 40, 60, 80 and 100 min. At designated time, the reaction was stopped and the supernatant was prepared in the same manner as previously described. DH was then determined. The reaction time rendering the highest DH was chosen for further steps.

Effect of starry triggerfish muscle and buffer ratio on DH

Starry triggerfish mince was mixed with 0.1 M glycine-NaOH buffer, pH 8.5 at a ratio of 1:0.5, 1:1, 1:2, 1:3, 1:4 and 1:5 (*w*/*v*). Liver extract at a level of 5.5 % was added and the reaction was maintained for 40 min at 55 °C. The hydrolysate obtained was determined for DH. The buffer ratio used for mixing the mince showing the highest DH was selected for further study.

Determination of α-amino acids and DH

The α-amino acid content was determined according to the method of Klomklao et al. (2013). Properly diluted hydrolysate samples (125 μl) 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 (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 ambient temperature for 15 min. The absorbance was measured at 420 nm and α-amino acid was expressed in term of *L*-leucine. DH was calculated as follows:

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

where L_t is the amount of α-amino acid released at time t. L_0 is the amount of α-amino acid in the original starry triggerfish muscle homogenate. L_{max} is total α-amino acid in the original starry triggerfish muscle homogenate obtained after acid hydrolysis with 6 N HCl at 100 °C for 24 h.

Production of starry triggerfish protein hydrolysate

Starry triggerfish mince (20 g) was mixed with 0.1 M glycine-NaOH buffer, pH 8.5 at a ratio of 1:3 (*w*/*v*) and pre-incubated at 55 °C for 10 min (Klomklao et al. 2013; Sripokar et al. 2015). The enzyme hydrolysis was started by adding albacore tuna liver extract at a level of 5.5 % (*w*/*w*). The reaction was conducted at pH 8.5 and 55 °C for 40 min. After 40 min of hydrolysis, the enzyme was inactivated by heating at 90 °C for 15 min in a water bath. The mixture was then centrifuged at 5000 g at 4 °C for 10 min and the supernatant was collected. Starry triggerfish protein hydrolysate was freeze-dried using a Dura-Top™μp freeze-dryer (FTS systems Inc., Stone Ridge, NY, USA). The freeze-dried starry triggerfish protein hydrolysate obtained was subjected to analyses.

Proximate analysis

Moisture, protein, fat and ash contents were determined according to the method of AOAC (2005).

Amino acid analysis

Amino acid compositions of freeze-dried hydrolysate were determined according to the method of Cohen and Michaud (1993). Hydrolysate (10 μg) was dissolved in 10 mM HCl (10 μl) and treated with 0.2 M borate buffer pH 9.3 (30 μl). The sample solution was reacted with 10 mM 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (10 μl) to form stable unsymmetric urea derivatives. Amino acids were separated by reverse phase HPLC (AccQ Tag column, Waters, Milford, MA, USA) at 37 °C, using gradient mobile phase: deionized water, acetonitrile and eluent A (sodium acetate, phosphoric acid, triethylamine) and detected by a UV detector (Waters 486, Milford, MA, USA) at 254 nm and a fluorescence detector (Jasco FP-920, Great Dunmow, Essex, UK) with excitation wavelength at 250 nm and emission wavelength at 395 nm. The amount of amino acids was calculated, based on the peak area in comparison with that of standard. The amino acid content was expressed as a percentage of total amino acids in the sample.

Color measurement

The color of freeze-dried hydrolysate was measured by the Hunter lab colorimeter and reported by the CIE system. L^*, a^* and b^* parameters, indicating lightness, redness and yellowness, respectively.

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 proximate composition, the independence t-test was used for pair comparison (Steel and Torrie 1980). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for Windows; SPSS Inc., Chicago, IL, USA).

Results and discussion

Optimum condition for starry triggerfish protein hydrolysate production using albacore tuna liver extract

Enzymatic hydrolysis is influenced by several factors like temperature, pH, enzyme to substrate level and time that cooperatively influence the enzyme activity thereby making the process more controllable (Liaset et al. 2000). Based on my previous study, liver proteinase from albacore tuna had optimum pH and temperature at 8.5 and 55 °C, respectively. An appreciable decrease in enzyme activity was observed at temperature above 55 °C, due to thermal denaturation (Sripokar et al. 2015). The optimal pH and temperature were 9.0 and 50 °C, respectively, when toothed ponyfish (*Gazza minuta*) muscle was used as a substrate (Klomklao et al. 2013). Alcalase had maximal activity at pH 8.5 and 55 °C for hydrolysis of Persian sturgeon viscera (Ovissipour et al. 2009). Benjakul and Morrissey (1997) found that Alcalase and Neutrase showed optimum activity against Pacific whiting solid wastes at pH 9.5, 60 °C and pH 7.0, 55 °C, respectively. Therefore, pH 8.5 and temperature 55 °C was chosen as the optimal condition for protein hydrolysate production from starry triggerfish using liver proteinase from albacore tuna.

Effect of liver extract concentration on DH

Degree of hydrolysis (DH), which indicates the percentage of peptide bonds cleaved (Adler-Nissen 1986), is essential because several properties of protein hydrolysates are closely related to DH (Nielsen 1997). The DH of starry triggerfish muscle treated with tuna liver extract increased when the liver extract level was increased (Fig. 1). Significant changes in DH occurred with the liver extract treatment at levels ranging from 0.5 to 5.5 % ($p < 0.05$). However, no increase for DH was found with treatment of liver extract at level above 5.5% (Fig. 1). The value of DH in this study was similar to that obtained in toothed ponyfish hydrolysate treated with catfish viscera extract (Klomklao et al. 2013). Shahidi et al. (1995) reported that considerable soluble protein was released during initial phase and no increase in soluble hydrolysate was observed when additional enzyme was added during the stationary phase of hydrolysis. Morr et al. (1985) suggested that, the overall reaction involved at least two steps. In the first step, the enzyme molecules become associated with and bound to the fish particles. Subsequently, hydrolysis took place, resulting in the release of soluble peptides and amino acids.

When $\log_{10}$ (liver extract concentration) was plotted against DH, a linear relationship and the regressive equations were obtained (Fig. 2). Klomklao et al. (2013) found a similar linear relationship between %DH and log of enzyme concentration, when hydrolyzing toothed ponyfish muscle with catfish viscera extract. The relationship is further supported by Benjakul and Morrissey (1997) for Pacific white shrimp protein hydrolysate derived with Alcalase and Neutrase and Thiansilakul et al. (2007) for round scad derived with Flavourzyme. From the regression, amount of liver extract required to prepare starry triggerfish muscle protein hydrolysate with required DH could be calculated.

Effect of reaction time on DH

Figure 3 shows the effect of hydrolysis time on DH of starry triggerfish muscle using albacore tuna liver extract. A high rate of hydrolysis was obtained during the initial stage (10–40 min), owing to a large number of peptide bonds available. After 40 min of hydrolysis, the enzymatic reaction more likely reached the steady-state phase. The stability in the hydrolysis rate, observed in the later stage, was mainly due to a decrease in available substrate, enzyme auto-digestion and product inhibition (Khantaphant et al. 2011). Generally, the enzyme absorbs rapidly onto the insoluble protein particles and the polypeptide chains that are loosely bound to the surface are then cleaved. The more compacted core proteins are hydrolyzed more slowly. The rate of enzymic cleavage of peptide bond controls the overall rate of hydrolysis (Benjakul and Morrissey 1997). Fish gelatin was hydrolyzed using proteases from hepatopancreas of Pacific white shrimp, a rapid hydrolysis was found within the first 30–40 min, followed by a slower hydrolysis rate up to 120 min (Senphan and Benjakul 2014). Ovissipour et al. (2009) produced protein hydrolysates from Persian sturgeon (*Acipensor persicus*) viscera by optimizing the hydrolysis time. The highest DH%, 46.13 was achieved at 55 °C after 205 min. Klomklao et al. (2013) investigated the enzymatic hydrolysis of toothed ponyfish (*Gazza minuta*) muscle with viscera extract from hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*). A rapid reaction rate of both DH and NR was obtained in the first 15 min. From the results, the reaction time of 40 min was found to be optimal for starry triggerfish protein hydrolysate production.

Effect of fish muscle/buffer ratio on DH

The effect of substrate/buffer ratio on hydrolysis of starry triggerfish muscle using albacore tuna liver extract is shown in Fig. 4. In general, an increase in starry triggerfish mince/buffer resulted in an increase in DH. The ratio above 1:3 did not cause a significant increase in DH ($p > 0.05$). Sufficient buffer gave buffering capacity for the reaction, worked as media for enzyme dispersion and was considered as an important factor for starry triggerfish muscle hydrolysis. From the results, fish muscle/buffer ratio of 1:3 (*w*/*v*) was sufficient for enzymatic reaction. Increased water or buffer added to

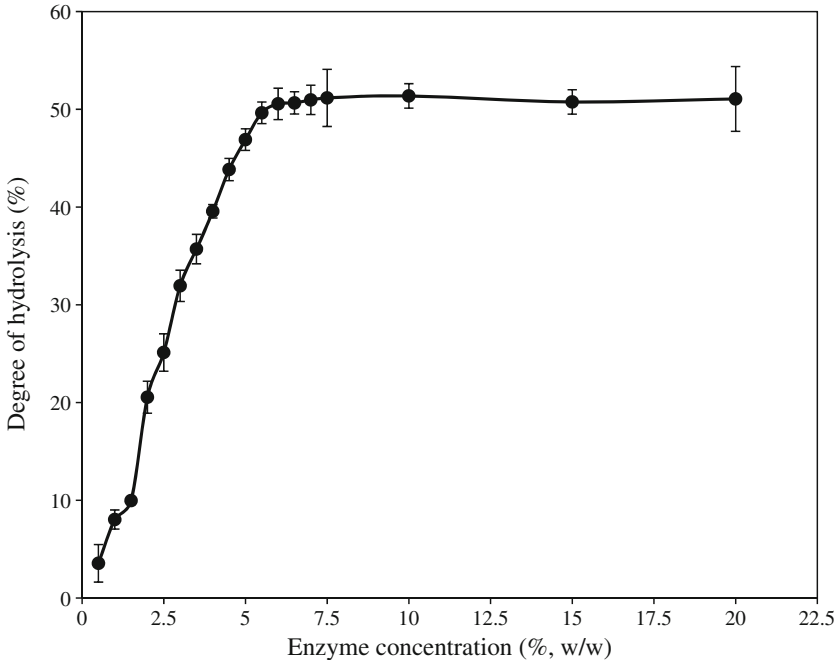

Fig. 1 Effect of albacore tuna liver extract concentration on DH of starry triggerfish muscle. Bars represent the standard deviation from triplicate determinations

substrate enhanced enzyme homogeneity, promoted tissue swelling, and reduced the localized concentration of hydrolysis products (Surowka and Fik 1994). Klomklao et al. (2013) used catfish viscera extract to recover the proteinases substances from toothed ponyfish muscle and found that an increase in the ratio of added buffer resulted in an increase in DH. Benjakul and Morrissey (1997) reported that an increase in Pacific whiting solid waste/buffer ratio resulted in an increase in α-amino acid concentration as well as NR.

Proximate composition of protein hydrolysate

The proximate composition of starry triggerfish muscle and freeze-dried starry triggerfish protein hydrolysate are depicted in Table 1. The freeze-dried hydrolysate and starry triggerfish muscle contained 10.86 % and 78.27 % moisture, respectively. On dry weight basis, freeze-dried hydrolysate contained higher protein content but lower lipid content than starry triggerfish muscle. The high protein content reported for fish protein hydrolysates is due to the solubilization of proteins

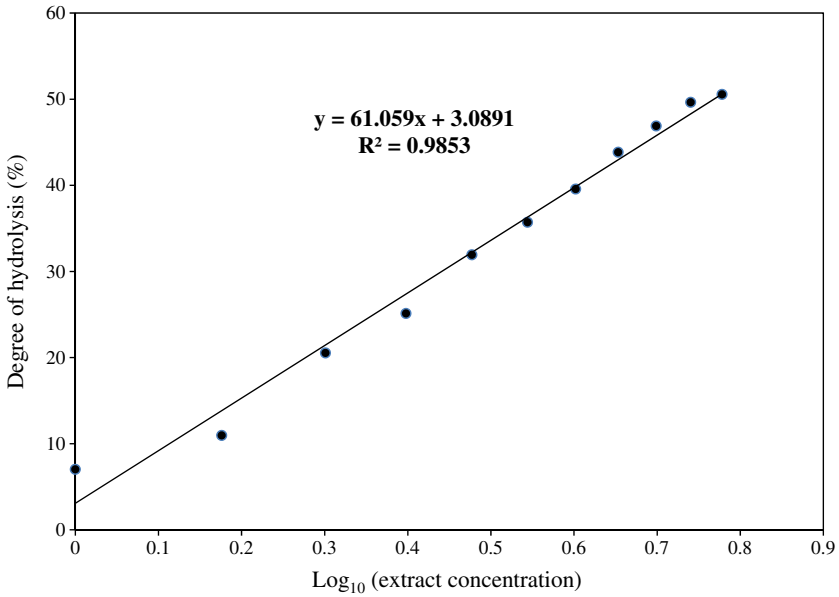

Fig. 2 Relation between $\log_{10}$ (extract concentration) and DH for starry triggerfish muscle treated with albacore tuna liver extract

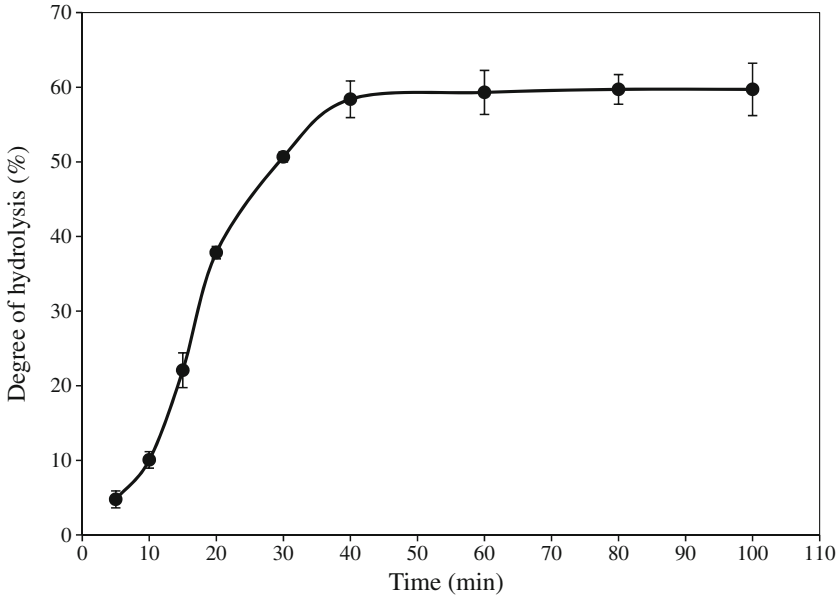

Fig. 3 Effect of hydrolysis time on DH for starry triggerfish muscle with albacore tuna liver extract. Bars represent the standard deviation from triplicate determinations

during hydrolysis and the removal of insoluble solid matter by centrifugation (Chalamaiah et al. 2010). High protein content of fish protein hydrolysates demonstrates its potential use as protein supplements for human nutrition (Chalamaiah et al. 2012). The low fat content of fish protein hydrolysates is because of removal of lipids with insoluble protein fractions by centrifugation (Chalamaiah et al. 2012). Decreasing lipid content in the protein hydrolysates might significantly increase stability of the material towards lipid oxidation, which may also enhance the product stability (Kristinsson and Rasco 2000). During the hydrolysis process, the muscle cell membranes tend to round up and form insoluble vesicles, leading to the removal of membrane structured lipid (Shahidi et al. 1995). The freeze-dried protein hydrolysate had a higher ash content (6.48 %) than did starry triggerfish mince (3.71 %). The relatively high ash content of fish protein hydrolysates is due to the use of glycine-NaOH buffer during the enzymatic reaction and usage of added acid or base for adjustment of pH of medium (Choi et al. 2009).

Amino acid composition

Table 2 shows the amino acid compositions of the freeze-dried starry triggerfish protein hydrolysate. Protein hydrolysate was rich in glycine, lysine and leucine, which accounted for 15.33, 11.67 and 10.27 % of the total amino acids, respectively. The

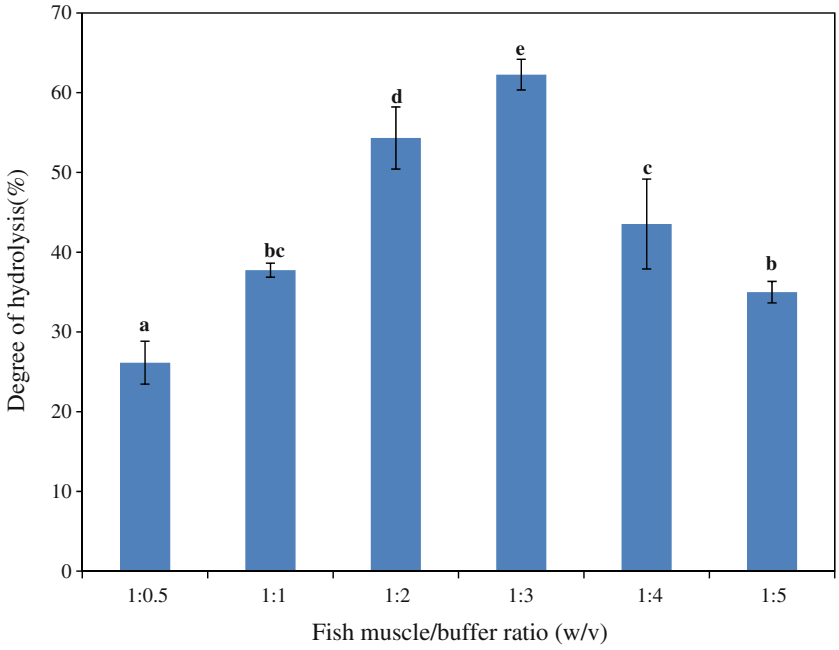

Fig. 4 Effect of fish muscle/buffer ratio on DH for starry triggerfish muscle with albacore tuna liver extract. Different letters indicate significant differences ($p < 0.05$). Bars represent the standard deviation from triplicate determinations

Table 1 Proximate composition of freeze-dried hydrolysate produced from starry triggerfish muscle and starry triggerfish muscle

Compositions	Freeze-dried hydrolysate	Starry triggerfish muscle
Protein[a]	91.73 ± 1.16b[***]	84.66 ± 2.04a
Lipid[a]	2.04 ± 1.07a	11.89 ± 4.79b
Ash[a]	6.48 ± 1.55b	2.69 ± 1.52a
Moisture	10.86 ± 2.65a	78.27 ± 0.32b

Mean ± SD from triplicate determination

[a] Dry weight basis

[***] The different letters in the same row denote the significant differences ($p < 0.05$)

nutritive value of any ingredient depends on the proteins capacity to fulfill the needs of organisms with respect to essential amino acids. From the result, the protein hydrolysate had an essential amino acid/non-essential amino acid ratio of 0.84. Fish and shellfish have been reported to contain the high essential amino acid/non-essential amino acid ratio (Twasaki and Harada 1985). Protein hydrolysate from toothed ponyfish (*Gazza minuta*) muscle had an essential amino acid/non-essential amino acid ratio of 0.93 (Klomklao et al. 2013). Thiansilakul et al. (2007) reported that the round scad protein hydrolysate had an essential amino acid/non-essential amino acid ratio of 0.92. The ratio of essential amino acid/non-essential amino acid of protein hydrolysate from yellowfin tuna (*Thunnus albacores*) head using Alcalase and Protemax was 1.04 and 1.43, respectively (Ovissipour et al. 2010). Starry triggerfish protein hydrolysate contained a low level of proline (3.07 %). The presence of proline residues in the centre of the peptides generally contributes to the bitterness. Therefore the peptidase, which can cleave the hydrophobic amino acids and proline, is capable of debittering protein hydrolysate (Capiralla et al. 2002). Hevia and Olcott (1977) reported that bitter peptide from ficin-treated fish concentrate contained glycine, isoleucine, phenyalanine and valine. Thiansilakul et al. (2007) reported that bitter peptide from round scad protein hydrolysate contained leucine, valine, phenylalanine and isoleucine. Since starry triggerfish protein hydrolysate had a high protein content and high essential amino acid/non-essential amino acid ratio, it could be used as a source of protein ingredient for industrial applications.

Table 2 Amino acid composition of freeze-dried hydrolysate produced from starry triggerfish muscle

Amino acids	Content (%)
Essential amino acids	
Isoleucine	3.72
Leucine	10.27
Lysine	11.67
Methionine	4.85
Phenylalanine	3.47
Threonine	5.77
Valine	5.87
Non-essential amino acids	
Alanine	9.11
Arginine	3.77
Aspartic acid/Asparagine	2.97
Cysteine	0.6
Glutamic acid/Glutamine	8.2
Glycine	15.33
Histidine	2.89
Proline	3.07
Serine	4.52
Tyrosine	3.93

Color

Color influences the overall acceptability of food products. The freeze-dried protein hydrolysate was light yellow in color ($L^* = 82.94$, $a^* = 0.84$, $b^* = 22.83$) (Table 3). The result was in agreement with Sathivel et al. (2003) who reported that protein hydrolysate from whole herring (*Clupea harengus*) was light yellow in color ($L^* = 89.4$, $a^* = 3.3$, $b^* = 8.0$). However, Thiansilakul et al. (2007) found that round scad protein hydrolysate was brownish yellow in color ($L^* = 58.00$, $a^* = 8.38$, $b^* = 28.32$). The dark color of fish protein hydrolysate was probably from the oxidation of myoglobin and the melanin pigment of the raw materials (Benjakul and Morrissey 1997). Therefore, the varying color of fish protein hydrolysate depended on the composition of the raw material and the hydrolysis condition.

Conclusion

The results of this research clearly revealed that low valued marine fish had a potential to be utilized as protein source for producing protein hydrolysates. Optimum conditions for triggerfish muscle hydrolysis were, 5.5 % liver extract from albacore tuna, 40 min reaction time and fish muscle/buffer ratio of 1:3 (w/v). Hydrolysate from triggerfish muscle may

Table 3 L^*, a^* and b^*-values of freeze-dried hydrolysate produced from starry triggerfish muscle

Color characteristics	Freeze-dried hydrolysate
L^*	82.94 ± 2.45
a^*	0.84 ± 1.87
b^*	22.83 ± 0.64

Values are mean ± SD from triplicate determination

potentially serve as a good source of desirable peptides and amino acids.

Acknowledgments This research was supported by the Thailand Research Fund and Thaksin University. Authors would like to thank the Office of the Higher Education Commission for financial support. The TRF distinguished research professor grant was also acknowledged.

References

Adler-Nissen J (1986) Enzymatic hydrolysis of food proteins. Elsevier Spplied Science, London

AOAC (2005) Official methods of analysis. Association of official analytical chemists international, 18th edn. AOAC International, Gaithersberg

Benjakul S, Morrissey MT (1997) Protein hydrolysates from Pacific whiting solid wastes. J Agric Food Chem 45:3423–3430

Bhaskar N, Mahendrakar NS (2008) Protein hydrolysate from visceral waste proteins of Catla (*Catla catla*): optimization of hydrolysis condition for a commercial neutral protease. Bioresour Technol 99:4105–4111

Capiralla H, Hiroi T, Hirokawa T, Maeda S (2002) Purification and characterization of a hydrophobic amino acid-specific endopeptidase from *Halobacterium halobium* S9 with potential application in debittering of protein hydrolysates. Process Biochem 38:571–579

Chalamaiah M, Narsing Rao G, Rao DG, Jyothirmayi T (2010) Protein hydrolysates from meriga (*cirrhinus mrigala*) egg and evaluation of their functional properties. Food Chem 120:652–657

Chalamaiah M, Dinesh Kumar B, Hemalatha R, Jyothirmayi T (2012) Fish protein hydrolysates: proximate composition, amino acid composition, antioxidant activities and applications: a review. Food Chem 135:3020–3038

Choi YJ, Hur S, Choi BD, Konno K, Park JW (2009) Enzymatic hydrolysis of recovered protein from frozen small croaker and functional properties of its hydrolysates. J Food Sci 74:C17–C24

Cohen SA, Michaud DP (1993) Synthesis of a fluorescent derivatizing reagent, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, and its application for the analysis of hydrolysate amino acids via high-performance liquid chromatography. Anal Biochem 211:279–287

Guerard F, Dufosse L, De La Broise D, Binet A (2001) Enzymatic hydrolysis of proteins from yellowfin tuna (*Thunnus albacores*) wastes using Alcalase. J Mol Catal B 11:1051–1059

Hevia P, Olcott HS (1977) Flavor of enzyme-solubilized fish protein concentrate fractions. J Agric Food Chem 25:772–775

Khantaphant S, Benjakula S, Kishimura H (2011) Antioxidative and ACE inhibitory activities of protein hydrolysates from the muscle of brownstripe red snapper prepared using pyloric caeca and commercial proteases. Process Biochem 46:318–327

Klomklao S, Benjakul S, Visessanguan W, Simpson BK, Kishimura H (2005) Partitioning and recovery of proteinase from tuna spleen by aqueous two-phase systems. Process Biochem 40:3061–3067

Klomklao S, Kishimura H, Yabe M, Benjakul S (2007) Purification and characterization of two pepsins from the stomach of pectoral rattail (*Coryphaenoides pectoralis*). Comp Biochem Physiol 147B:682–689

Klomklao S, Kishimura H, Benjakul S (2013) Use of viscera extract from hybrid catfish (*Clarias macracephalus* × *Clarias gariepinus*) for the production of protein hydrolysate from toothed ponyfish (*Gazza minuta*) muscle. Food Chem 136:1006–1012

Kristinsson HG, Rasco BA (2000) Fish protein hydrolysates: production, biochemical, and functional properties. Crit Rev Food Sci Nutr 40: 43–81

Liaset B, Lied E, Espe M (2000) Enzymatic hydrolysis of by-products from the fish-filleting industry; chemical characterization and nutritional evaluation. J Sci Food Agric 80:581–589

Morr V, German B, Kinsella JE, Regenstein JM, Van Buren JP, Kilara A, Lewis BA, Mangino ME (1985) A collaborative study to develop a standardized food protein solubility procedure. J Food Sci 50:1715–1718

Nalinanon S, Benjakul S, Visessanguan W, Kishimura H (2008) Partitioning of protease from stomach of albacore tuna (*Thunnus alalunga*) by aqueous two-phase systems. Process Biochem 44: 471–476

Neklyudov AD, Ivankin AN, Berdutina AV (2000) Properties and uses of protein hydrolysates. Appl Biochem Microbiol 36:452–459

Nielsen PM (1997) Functionality of protein hydrolysates. In: Damodaran S, Paraf A (eds) Food proteins and their applications. Marcel Dekker, New York

Ovissipour M, Abedian A, Motamedzadegan A, Rasco B, Safari R, Shahidi H (2009) The effect of enzymatic hydrolysis time and temperature on the properties of protein hydrolysates from Persian sturgeon (*Acipenser persicus*) viscera. Food Chem 115:238–242

Ovissipour M, Benjakul S, Safari R, Motamedzagan A (2010) Fish protein hydrolysates production from yellowfin tuna *Thunnus albacores* head using Alcalase and Protamex. Int Aquat Res 2:87–95

Phanturat P, Benjakul S, Visessanguan W, Roytrakul S (2010) Use of pyloric caeca extract from bigeye snapper (*Priacanthus macracanthus*) for the production of gelatin hydrolysate with antioxidative activity. LWT Food Sci Technol 43:86–97

Sathivel S, Bechtel PJ, Babbitt J, Smiley S, Crapo C, Reppond KD, Prinyawiwatkul W (2003) Biochemical and functional properties of herring (*Clupea harengus*) byproduct hydrolysate. J Food Sci 68:2196–2200

Senphan T, Benjakul S (2014) Use of the combined phase partitioning systems for recovery of proteases from hepatopancreas of Pacific white shrimp. Sep Purif Technol 129:57–63

Shahidi F (1994) Proteins from seafood processing discards. In: Sikorski ZE, Pan BS, Shahidi F (eds) Seafood proteins. Chapman & Hall, New York

Shahidi F, Xiao-Qing H, Synowiecki J (1995) Production and characteristics of protein hydrolysates from capelin (*Mallotus villosus*). Food Chem 53:285–293

Sripokar P, Poonsin T, Chaijan M, Benjakul S, Klomklao S (2015) Proteinases from liver of albacore tuna (*Thunnus alalunga*): optimum extractant and biochemical characteristics. J Food Biochem. Accepted

Steel RGD, Torrie JH (1980) Principles and procedures of statistics: a biometrical approach. McGraw-Hill, New York

Surowka K, Fik M (1994) Studied on the recovery of proteinaceous substrates from chicken heads: II-application of pepsin to the production of protein hydrolysate. J Sci Food Agric 65:289–286

Thiansilakul Y, Benjakul S, Shahidi F (2007) Antioxidant activity of protein hydrolysate from round scad muscle using Alcalase. J Food Biochem 31:266–287

Twasaki M, Harada R (1985) Proximate and amino acid composition of the roe and muscle of selected marine species. J Food Sci 50:1585–1587

Wisuthipheat N, Kongruang S (2015) Production of fish protein hydrolysates by acid and enzymatic hydrolysis. J Med Bioeng 4:466–470

Wu HC, Chen HM, Shiau CY (2003) Free amino acid and peptide as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). Food Res Int 36:949–957

Biocatalysis and Agricultural Biotechnology 17 (2019) 447–454

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Antioxidant and functional properties of protein hydrolysates obtained from starry triggerfish muscle using trypsin from albacore tuna liver

Pakteera Sripokar[a], Soottawat Benjakul[b], Sappasith Klomklao[c,*]

[a] Biotechnology Program, Faculty of Agro- and Bio-Industry, Thaksin University, Phatthalung Campus, Phatthalung 93210, Thailand
[b] Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
[c] Department of Food Science and Technology, Faculty of Agro- and Bio-Industry, Thaksin University, Phatthalung Campus, Phatthalung 93210, Thailand

ARTICLE INFO

Keywords:
Protein hydrolysates
Starry triggerfish
Antioxidant
Functional properties
Proteolysis
Trypsin

ABSTRACT

Protein hydrolysates from starry triggerfish (*Abalistes stellaris*) muscle with a degree of hydrolysis (DH) of 60% were prepared using trypsin from albacore tuna (*Thunnus alalunga*) liver. The hydrolysates were investigated for antioxidant activity and functional properties. Antioxidant activities including DPPH (2,2-diphynyl-1-picrylhydrazyl) radical scavenging activity, ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activity, ferric reducing antioxidant power (FRAP) and metal chelating activity of hydrolysate samples were dose dependent. After being subjected to gastrointestinal model systems, the DPPH radical scavenging activity and metal chelating activity of the hydrolysates increased, especially in the duodenal condition, suggesting the enhancement of those activities of hydrolysates after ingestion. For functional properties, hydrolysis by the trypsin increased the solubility of hydrolysates to above 72.8% over a wide pH range. The hydrolysates possessed interfacial properties, which were governed by their concentrations. An increase in concentration of up to 2.0% (w/v) favoured the emulsifying activity index (EAI) and emulsion stability index (ESI), while a further increase to 3.0% (w/v) diminished emulsifying properties. Foam expansion and foam stability increased as the protein concentration increased. Therefore, the results of the present study suggest that starry triggerfish muscle can effectively be converted to protein hydrolysates, and the hydrolysates could be a potential ingredient in functional food as well as natural antioxidants in lipid food systems.

1. Introduction

Protein demand is growing; driven by a rising population, changing food preferences and a growing recognition of the importance of protein as a key ingredient for health and nutrition. The supplementation of dietary protein has been becoming more popular, especially for people on restrictive diets, athletes and the elderly (Egerton et al., 2018). Worldwide, fish protein hydrolysates (FPH) has been one of the most researched fish products of the last decade. The greater attention emerging towards FPH is due to their bioactive characteristics and growing global market. Previous studies of FPH have shown that, when added to food, they can contribute to water holding, emulsification and texture properties (Halim et al., 2016). Increased solubility is a frequently reported and valued property of FPH (Benjakul et al., 2014). Bioactive characteristics such as antioxidation, antihypertension, antibacterial and antiprolifiration have also been reported (Halim et al., 2018; Khositanon et al., 2018; Singh and Vij, 2018; Song et al., 2016; Senphan and Benjakul, 2014; Khantaphant et al., 2011). Antioxidant characteristics can be an important feature for food preservation and providing potential health benefits (Phanturat et al., 2010). Numerous peptides derived from hydrolyzed fish protein have been shown to have antioxidant activities such as protein hydrolysates from the muscle of ornate threadfin bream (Nalinanon et al., 2011), brownstripe red snapper (Khantaphant et al., 2011), toothed ponyfish (Klomklao et al., 2013), seabass (Senphan and Benjakul, 2014), whole tilapia waste (Tejpal et al., 2017), blue whiting (Egerton et al., 2018) and eel (Halim et al., 2018). FPH can be used in food systems, comparable to other pertinent protein hydrolysates (Kristinsson and Rasco, 2000). The use of enzyme technologies for the recovery and modification of protein has led to the production of a broad spectrum of food ingredients and industrial products (Kristinsson and Rasco, 2000). Enzymatic hydrolysis of food proteins is an efficient way to recover potent bioactive peptides (Thiansilakul et al., 2007). 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 (Nalinanon et al., 2011; Klomklao et al., 2010).

* Corresponding author.
E-mail address: sappasith@tsu.ac.th (S. Klomklao).

https://doi.org/10.1016/j.bcab.2018.12.013
Received 9 October 2018; Received in revised form 5 December 2018; Accepted 11 December 2018
Available online 19 December 2018
1878-8181/

Thailand is the world's largest producer and exporter of canned tuna. Albacore tuna is one of the most commercially important tuna species for the Thai tuna industry (Klomklao and Benjakul, 2018). Because of a large amount of tuna viscera, especially liver, discarded during processing, it can serve as a promising source of trypsin for further use. Trypsin (EC 3.4.21.4) recovery from tuna liver is an approach to minimize the economic and ecological problems of the manufacturing discard. Furthermore, FPH with bioactivity prepared with the aid of fish proteases can be obtained as a new value-added product with high market value (Khantaphant and Benjakul, 2008). Trypsin from albacore tuna liver was recently partitioned, purified and characterized (Sripokar et al., 2016a; Klomklao and Benjakul, 2018).

The starry triggerfish (*Abalistes stellaris*), or flat-tailed triggerfish, belongs to the order Tetraodontiformes and is a member of the Balistidae family (Sripokar et al., 2016b). This fish is used primarily for fish meal and are not considered palatable for direct human consumption in Thailand. To increase the value of this fish species, the production of new value-added products such as protein hydrolysates, with nutritive value and bioactivity, can pave the way for its full utilization. The use of trypsin from albacore tuna liver, for hydrolysate production, could lower the cost of commercial proteases. Based on our previous study, optimum conditions for starry triggerfish muscle hydrolysis using trypsin from albacore tuna liver were 5.5% trypsin, at 55 °C for 40 min reaction time and a fish muscle/buffer ratio of 1:3 (w/v). However, no information regarding the antioxidative activity and biochemical properties of starry triggerfish hydrolysates treated with the trypsin from albacore tuna liver has been reported. Therefore, the objectives of the present investigation were to study the functionalities and antioxidant properties of protein hydrolysates prepared from starry triggerfish muscle using the trypsin from albacore tuna liver.

2. Materials and methods

2.1. Chemicals

L-leucine, 2,4,6-trinitrobenzenesulfonic acid (TNBS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 3-(2-pyridyl)-5–6-diphenyl-1,2,4-triazine-4′,4″-disulphonic acid sodium salt (ferrozine) and bovine serum albumin were procured from Sigma Chemical Co. (St. Louis, MO, USA.). Tris (hydroxymethyl) aminomethane (Tris), sodium sulfite, potassium persulphate, ferrous chloride and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS) were procured from Bio-Rad Laboratories (Hercules, CA, USA). Pepsin from porcine gastric mucosa (EC 3.4.23.1) and pancreatin from porcine pancreas were procured from Sigma–Aldrich, Inc. (St. Louis, MO, USA). All of the chemicals used were of analytical grade.

2.2. Fish sample preparation

Liver of albacore tuna (*Thunnus alalunga*) were obtained from Tropical Canning (Thailand) Public Co. Ltd., Hat Yai, Songkhla. The samples were packed in polyethylene bags, kept in ice with a sample/ice ratio of 1:3 (w/w) and transported to the research laboratory within 2 h. Pooled internal organs were separated and only the liver was collected. Liver was cut and homogenized into powder in three volumes of acetone at −20 °C for 30 min according to the method of Klomklao et al. (2007). The homogenate was filtrated in vacuo on Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, UK). The residue obtained was then homogenized in two volumes of acetone at −20 °C for 30 min. The residue was left at room temperature until dried and free of acetone odor.

Starry triggerfish (*Abalistes stellaris*) with a length of 30–35 cm were purchased from a dock in Trang, Thailand. The fish, off-loaded approximately 18–24 h after capture, were placed on ice at a fish/ice ratio of 1:2 (w/w) and transported to the research laboratory within 2 h. Upon arrival, the fish were filleted and the ordinary muscle was collected and ground to uniformity. A portion of mince (500 g) was placed in a polyethylene bag and stored at −20 °C until needed.

2.3. Trypsin preparation and activity assay

To prepare the albacore tuna trypsin extract, liver powder was suspended in 50 mM Tris-HCl buffer, pH 8.5, containing 0.2% Brij 35 at a ratio of 1:9 (w/v) and stirred continuously at 4 °C for 30 min. The suspension was centrifuged at 5000 × *g* at 4 °C for 30 min using a Sorvall Modell RC-B Plus centrifuge (Newtown, CT, USA). The supernatant was collected and referred to as “albacore tuna trypsin”.

Albacore tuna trypsin was determined for trypsin activity using N^{α}-p-Tosyl-L-arginine methyl ester hydrochloride (TAME) as a substrate, as described by Klomklao and Benjakul (2018). Enzyme solution with an appropriate dilution (20 µl) was mixed with 3.0 ml of 1 mM TAME in 10 mM Tris-HCl buffer, pH 8.0 and incubated at 30 °C for 20 min. Production of *p*-tosyl-arginine was measured by monitoring the increment in absorbance at 247 nm. One unit of activity was defined as the amount causing an increase of 1.0 in absorbance at 247 nm per min.

2.4. Production of protein hydrolysates from starry triggerfish muscle

Starry triggerfish mince (20 g) was mixed with 0.1 M glycine-NaOH buffer, pH 8.5 at a ratio of 1:3 (w/v) and pre-incubated at 55 °C for 10 min (Klomklao et al., 2013; Sripokar et al., 2016b). The enzyme hydrolysis was started by adding albacore tuna trypsin at a level of 5.5% (w/w). The reaction was conducted at pH 8.5 and 55 °C for 40 min. After 40 min of hydrolysis, the enzyme was inactivated by heating at 90 °C for 15 min in a water bath (Memmert, Schwabach, Germany). The mixture was then centrifuged at 5000 × *g* at 4 °C for 10 min. The supernatant was then collected and lyophilized using a Dura-TopTM lp freeze-dryer (FTS systems Inc., Stone Ridge, NY, USA). The freeze-dried protein hydrolysates obtained were subjected to analyses.

2.5. Determination of α-amino acids and DH

The α-amino acid content and DH was measured according to the method of Klomklao et al. (2013). 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 sulfite. The mixtures were cooled at room temperature for 15 min. The absorbance was read at 420 nm and α-amino acid was expressed in term of L-leucine. DH was calculated as follows:

$$\text{DH}\ (\%) = \left[\frac{L_t - L_0}{L_{max} - L_0}\right] \times 100$$

where L_t is the amount of α-amino acid released at time *t*. L_0 is the amount of α-amino acid in the original starry triggerfish muscle homogenate. L_{max} is total α-amino acid in the original starry triggerfish muscle homogenate obtained after acid hydrolysis with 6 N HCl at 100 °C for 24 h.

2.6. Determination of antioxidative activity

The starry triggerfish protein hydrolysates with 60% DH was dissolved in distilled water at obtained concentrations of 5, 10, 20, 30 and 40 mg hydrolysates/ml. Antioxidant activity was determined as follows.

2.6.1. DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by

Nalinanon et al. (2011) and Laywisadkul et al. (2017) with a slight modification. For the sample (1.5 ml), 1.5 ml of 0.15 mM DPPH in 95% ethanol were added. The mixture was then 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 UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). The blank was prepared in the same manner, except that the distilled water was used instead of the sample. DPPH radical scavenging activity was calculated according to the following equation (Yen and Wu, 1999):

$$\text{DPPH radical scavenging activity (\%)} = \left[1-\left(\frac{A_{517}\text{ of sample}}{A_{517}\text{ of control}}\right)\right]\times 100$$

2.6.2. 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 ± 0.02 units at 734 nm using a spectrophotometer. Fresh ABTS solution was prepared for each assay. The sample (150 µl) was mixed with 2850 µl 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. ABTS radical scavenging activity was calculated according to the following equation:

$$\text{ABTS radical scavenging activity (\%)} = \left[1-\left(\frac{A_{734}\text{ of sample}}{A_{734}\text{ of control}}\right)\right]\times 100$$

2.6.3. Ferric reducing antioxidant power (FRAP) assay

FRAP was assayed according to the method of Benzie and Strain (1996). Stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and 20 mM $FeCl_36H_2O$ solution. A working solution was prepared fresh by mixing 25 ml of acetate buffer, 2.5 ml of TPTZ solution and 2.5 ml of $FeCl_36H_2O$ solution. The mixed solution was incubated at 37 °C for 30 min and was referred to as the FRAP solution. A sample (150 µl) was mixed with 2850 µl of FRAP solution and kept for 30 min in the dark. The ferrous TPTZ complex (colored product) was measured by reading the absorbance at 593 nm. Increased absorbance of the reaction mixture indicates the increasing ferric reducing antioxidant power.

2.6.4. Metal chelating activity

The chelating activity on Fe^{2+} was determined using the method of Boyer and McCleary (1987) with a slight modification. The 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 at room temperature for 20 min. The absorbance was then measured at 562 nm. The blank was conducted in the same manner but distilled water was used instead of the sample. The chelating activity was calculated as follows:

$$\text{Chelating activity (\%)} = \left[1-\left(\frac{A_{562}\text{ of sample}}{A_{562}\text{ of control}}\right)\right]\times 100$$

2.7. pH and thermal stability of antioxidant peptides

Starry triggerfish protein hydrolysates were dissolved in distilled water to obtain the concentration of 40 mg hydrolysates/ml. The 5 ml of sample solutions were adjusted to pHs 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 with 1 or 6 M HCl and 1 or 6 M NaOH and incubated at room temperature for 30 min. The pHs of sample solutions were then adjusted to 7.0 with 1 M phosphate buffer. The final volume of all solutions was brought up to 20 ml using distilled water. The residual antioxidant activities were determined using DPPH and metal chelating assay.

To determine thermal stability, starry triggerfish protein hydrolysate at a concentration of 40 mg hydrolysates/ml were prepared using distilled water as a medium. The 5 ml of sample solutions 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, 45, 60, 90, 120, 150 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 using DPPH and metal chelating assay.

2.8. Stability in gastrointestinal tract model system

Gastrointestinal tract model system was prepared according to the method of Lo et al. (2006) with a slight modification. The hydrolysate was dissolved in distilled water to obtain a concentration of 0.5 g/ml. The solution was adjusted to pH 2.0 with 1 M HCl and pepsin dissolved in 0.1 M HCl was added to obtain the final concentration of 40 g pepsin/kg hydrolysate. The mixture was incubated at 37 °C for 1 h with a continuous shaking (Memmert Model SV 1422, Schwabach, Germany). Thereafter, the pH of the reaction mixture was raised to 5.3 with 1 M NaOH before the addition of 20 g pancreatin/kg hydrolysates. Subsequently, the pH of the mixture was adjusted to 7.5 with 1 M NaOH. The mixture was incubated at 37 °C for 3 h with a continuous shaking. The digestion was terminated by submerging the mixture in boiling water for 10 min. During digestion, the mixture was randomly taken at 0, 15, 30, 60, 90, 120, 150, 180 and 210 min for determination of DPPH radical scavenging activity and metal chelating activity.

2.9. Functional properties of protein hydrolysates

2.9.1. Solubility

To determine solubility of the hydrolysates, starry triggerfish hydrolysate samples (200 mg) were dispersed in 20 ml of deionized water and the 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 25 ml by distilled water, previously adjusted to the same pH as the sample solution, prior to centrifugation at 5000 × *g* for 15 min. Protein content in the supernatant was determined using the Lowry method (Lowry et al., 1951), with bovine serum albumin as a standard. Total protein content in the same was determined after solubilization of the sample in 0.5 M NaOH. Solubility of the hydrolysates was calculated as follows:

$$\text{Solubility (\%)} = \left(\frac{\text{protein content in supernatant}}{\text{total protein content in sample}}\right)\times 100$$

2.9.2. Emulsifying properties

The emulsifying activity index (EAI) and the emulsion stability index (ESI) were used to measure the emulsifying properties of protein hydrolysate. EAI and ESI were determined according to the method of Pearce and Kinsella (1978) with a slight modification. Soybean oil (2 ml) and protein hydrolysate solutions (0.5%, 1.0%, 2.0% and 3.0%, 6 ml) were homogenized (Model T25 basic; IKA Labortecnik, Selangor, Malaysia) at a speed of 20,000 rpm for 1 min. An aliquot of the emulsion (50 µl) was pipette from the middle portion of the container at 0 and 10 min after homogenization and subsequently diluted 100-fold using 0.1% SDS solution. The mixture was mixed thoroughly for 10 s using a vortex mixer (G-560E, Vortex-Genie 2, Scientific Industries, Inc., Bohemia, NY). Absorbance at 500 nm of the resulting dispersion was measured using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). EAI and ESI were calculated by the following formulae:

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

where A is A_{500}, DF is dilution factor (100), l is path length of cuvette (m), ϕ is oil volume fraction and C is concentration of hydrolysates in aqueous phase (g/m^3);

$$ESI\ (min) = \left(\frac{A_0 \times \Delta t}{\Delta A}\right)$$

where ΔA is $A_0 - A_{10}$ and Δt is 10 min.

2.9.3. Foaming properties

Foam expansion (FE) and foam stability (FS) of hydrolysate solutions were determined according to the method of Shahidi et al. (1995) with a slight modification. Hydrolysate solutions (20 ml) with 0.5%, 1.0%, 2.0% and 3.0% concentrations were transferred into a 100-ml cylinder. The solutions were homogenized at 13,400 rpm for 1 min at room temperature. The samples were allowed to stand for 0, 30 and 60 min. FE and FS were then calculated using the following equations:

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

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

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 different times (30 and 60 min).

2.10. Statistical analysis

Experiments were run in triplicate using three different lots of samples. All data were subjected to analysis of variance (ANOVA) and differences between means were evaluated by Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Antioxidative activities

3.1.1. DPPH radical scavenging activity

The relatively stable DPPH radical has been widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and thus to evaluate the antioxidant activity (Jao and Ko, 2002). The DPPH radical scavenging activity of protein hydrolysates from starry triggerfish muscle prepared using albacore tuna trypsin with the DH of 60% was measured at different concentrations (0, 5, 10, 20, 30 and 40 mg/ml) and the results are shown in Fig. 1a. The DPPH radical scavenging activity increased as the concentration of protein hydrolysates increased up to 20 mg/ml ($p < 0.05$). However, no differences in activity were observed as concentration increased from 30 to 40 mg/ml. DPPH is a stable free radical that exhibits a maximal absorbance at 517 nm in ethanol. When DPPH encounters a proton-donating substance, such as an antioxidant, the radical is scavenged. The color is changed from purple to yellow and the absorbance is decreased (Khantaphant and Benjakul, 2008). Hence, starry triggerfish hydrolysates obtained could donate hydrogen atom to free radicals and become more stable diamagnetic molecule, leading to the termination of the radical chain reaction (Khantaphant and Benjakul, 2008). Nevertheless, the efficiency in hydrogen donation of peptides produced was governed by their concentration.

3.1.2. ABTS radical scavenging activity

ABTS radical assay determine the antioxidative activity, in which the radical is quenched to form ABTS-radical complex (Khantaphant and Benjakul, 2008). With increasing concentration, protein hydrolysate showed increased ABTS radical scavenging activity ($p < 0.05$) (Fig. 1b). The highest ABTS radical scavenging activity was found in hydrolysate with 40 mg/ml (47%). 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 amino acid sequence in peptides might affect the antioxidant activity. The ABTS radical is relatively stable and is readily decreased by antioxidants (Klomklao et al., 2013). With high ABTS radical scavenging activity, it was postulated that antioxidative compounds were most likely hydrophilic.

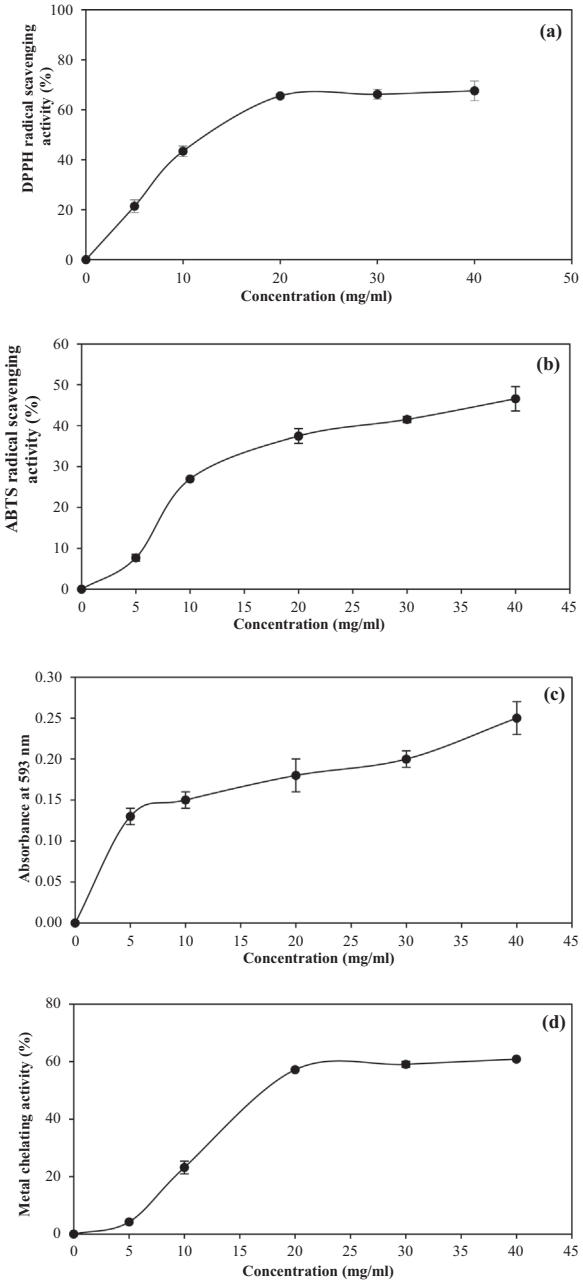

Fig. 1. DPPH radical (a) and ABTS radical (b) scavenging activity, ferric reducing antioxidant power (c) and metal chelating activity (d) of starry triggerfish protein hydrolysate at different concentrations. Bars represent the standard deviation from triplicate determinations.

3.1.3. FRAP

FRAP of starry triggerfish protein hydrolysates was investigated at different concentrations ranging from 0 to 40 mg/ml and the results are illustrated in Fig. 1c. FRAP increased significantly with the increase in the concentration of hydrolysates. The greater reducing power indicated that hydrolysates could donate an electron to free radicals, leading to the prevention or retardation of propagation (Klomklao et al., 2013). Results obtained were in agreement with the published reports (Intarasirisawat et al., 2012; Tejpal et al., 2017). FRAP is generally used to measure the capacity of a substance in reducing TPTZ-Fe (III) complex to TPTZ-Fe(II) complex (Binsan et al., 2008). In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each compound (Thiansilakul et al., 2007). The results suggested that antioxidative compounds in protein hydrolysates tested showed higher FRAP when higher concentrations were used. The differences in FRAP might be governed by peptides in the hydrolysates. The reducing power of starry triggerfish protein hydrolysate could be used to reduce DNA damage, mutagenesis, carcinogenesis and inhibition of pathogenic bacterial growth (Gulcin et al., 2010).

3.1.4. Metal chelating activity

As the concentration increased up to 20 mg/ml, chelating activity on Fe^{2+} of starry triggerfish protein hydrolysates increased ($p < 0.05$) (Fig. 1d). However, no changes of metal chelating activity of hydrolysates with a concentration of more than 20 mg/ml was observed. Peptides in hydrolysates could chelate the prooxidants, leading to decreased lipid oxidation (Klomklao et al., 2013). The chelation of Fe^{2+} represents the ability of hydrolysates in metal chelating (Nalinanon et al., 2011). Ferrozine quantitatively forms complexes with Fe^{2+} ion. In the presence of chelating agents the complex formation is disrupted, affecting the decrease in color formation (Thiansilakul et al., 2007). The chelation of transition metal ions by an antioxidant or antioxidative peptide would reduce the availability of pro-oxidative metal ions, and could also by this means reduce lipid oxidation (Thammarat et al., 2015). From the results, hydrolysates from starry triggerfish muscle treated with trypsin from albacore tuna liver had a potential chelating ability toward iron and the metal chelating activity of starry triggerfish protein hydrolysates was dose dependent.

3.2. Functional properties of protein hydrolysates

3.2.1. Solubility of the hydrolysates

The solubility of the hydrolysates of the lyophilized protein hydrolysates from starry triggerfish muscle obtained from trypsin from albacore tuna liver was determined at pH 3.0, 5.0, 7.0 and 9.0 and the results are shown in Fig. 2. All hydrolysates were soluble over a wide pH range, in which more than 72% solubility was obtained. The minimum solubility values were presented at pH 5.0 (72.8% solubility) and maximum solubility values at pH 3.0 (94.0% solubility) were observed ($p < 0.05$). The change in solubility can be attributed to the net charge of the amino acid residues after the hydrolysis process, which increases as the pH moves away from the isoelectric point, promoting the aggregation of hydrophobic interaction (Taheri et al., 2013). The solubilities of protein hydrolysates were quite low at pH 4 as reported in salmon by products (Gbogouri et al., 2004) and yellow stripe (Klompong et al., 2007). The same result was also reported in silver carp (Dong et al., 2008) which showed decrease in solubility at the pH 4–5 and drastically increased with increase in pH. The result suggested that near pH 4–5 proteins or peptides remaining after hydrolysis were precipitated at this pH, which was close to the isoelectric point (pI). The pH affects the charge on the weakly acidic and basic side chain groups and hydrolysates generally show low solubility at their isoelectric points (Naqash and Nazeer, 2013). Solubility is one of the most important functional properties of a protein and can be increased by the hydrolysis process (Klompong et al., 2007). Good solubility of proteins is required in many functional applications, especially for emulsions, foams and gels. Soluble proteins provide a homogeneous dispersibility of the molecules in colloidal systems and enhance the interfacial properties (Zayas, 1997). Enzymatic hydrolysis potentially affects the molecular size and hydrophobicity, as well as polar and ionizable groups of protein hydrolysates (Klompong et al., 2007). The balance of hydrophilic and hydrophobic forces of peptides is another crucial influence on solubility (Gbogouri et al., 2004).

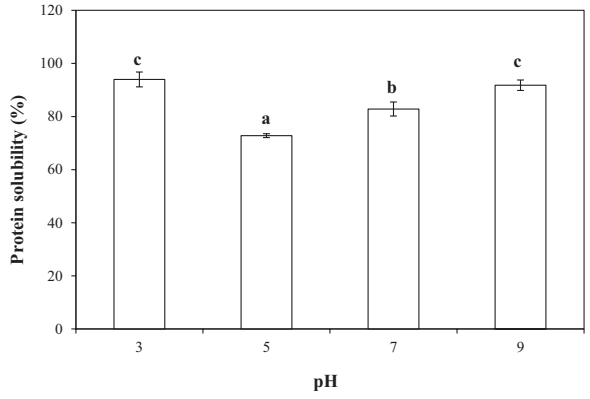

Fig. 2. Solubility of starry triggerfish protein hydrolysates at various pHs. Bars represent the standard deviation from triplicate determinations.

3.2.2. Emulsifying properties

Emulsifying activity index (EAI) and emulsion stability index (ESI) of hydrolysates from starry triggerfish muscle with different concentrations (0.5%, 1.0%, 2.0% and 3.0% w/v) are shown in Fig. 3. Both EAI and ESI were concentration-dependent (Fig. 3a, 3b). An increase in concentration favoured emulsifying activity up to 2.0% (w/v) concentration, while a further increase in concentration at 3.0% (w/v) reduced emulsifying activity (Fig. 3a). Similarly, the initial increase in concentration favoured increase emulsion stability up to 2.0%, after

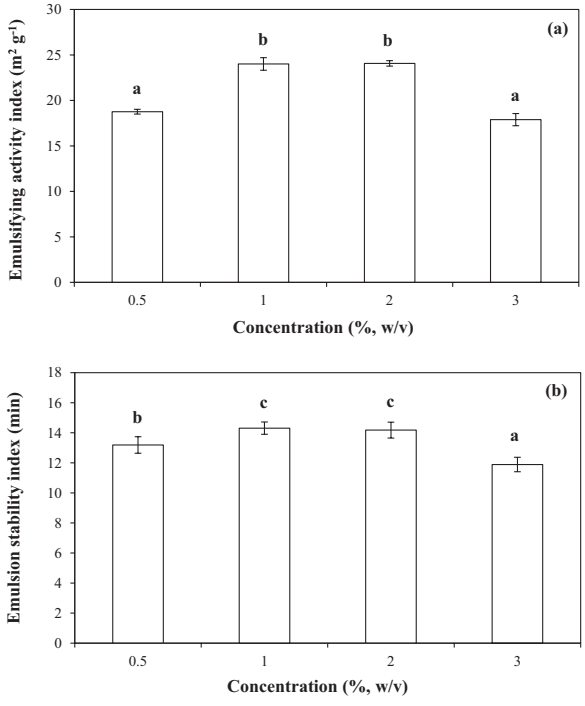

Fig. 3. Effect of concentration of starry triggerfish protein hydrolysate on emulsifying activity index (a) and emulsion stability index (b). Bars represent the standard deviation from triplicate determinations.

which a decline was observed with a further increase in concentration (Fig. 3b). The initial increase in concentration of hydrolysates facilitated enhanced interaction between the oil phase and the aqueous phase. However, as the concentration increased, a point was reached where a further increase in concentration led to an accumulation of proteins in the aqueous phase. This development resulted in a decrease of emulsifying activity (Lawal, 2004). Emulsion stability diminished after 2.0%, presumably due to an increase in protein-protein interaction at the expense of protein and peptide-oil interaction. 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) stabilization of this newly-formed interface by emulsifier or surfactant. Protein hydrolysates are surface-active materials and promote an oil-in-water emulsion due to their hydrophilic and hydrophobic groups and their charge (Kristinsson and Rasco, 2000; Klomklao et al., 2013). From the results, emulsifying characteristics of hydrolysates from starry triggerfish muscle with 60% DH were governed by concentration employed.

3.2.3. Foaming properties

Foam expansion and foam stability of starry triggerfish protein hydrolysates at various concentrations (0.5%, 1.0%, 2.0% and 3.0%) are depicted in Table 1. Foam expansion at 0 min after whipping indicated the foam abilities of protein hydrolysates, which increased from 128.89% to 208.89% when hydrolysate concentrations increased from 0.5% to 3.0% ($p < 0.05$). Sanchez and Panito (2005) reported that an increase in protein concentration resulted in a higher rate of diffusion. Foam expansion after whipping was monitored for 30 and 60 min to indicate the foam stability of protein hydrolysates. Formation of foam is governed by three factors, including transportation, penetration and reorganization of molecules at the air-water interface (Klomklao et al., 2013). Foam expansion after whipping for 30 and 60 min was monitored to indicate the foam stability of starry triggerfish protein hydrolysates. Foam stability was increased with increasing concentration of the hydrolysates. The results suggest that foam stability of the hydrolysates from starry triggerfish muscle was improved by increasing concentration. A similar result was also reported in protein hydrolysates from toothed ponyfish muscle produced with viscera extract from hybrid catfish (Klomklao et al., 2013). Foam stability depends on the nature of the film and reflects the extent of protein-protein interaction within the matrix (Mutilangi et al., 1996). Foam stability is enhanced by flexible protein domains, which enhance viscosity of the aqueous phase, protein concentration and film thickness (Phillips et al., 1994).

3.3. pH and thermal stability of antioxidant peptides

The stability of bioactive peptides to pH is an important criteria for gastrointestinal (GI) stability, because food encounters different pH at different digestion stages. Gastric pH is considered among one of the important factors affecting the survival of bioactive molecules during their passage through the stomach (Singh and Vij, 2018). The pH in the human stomach ranges from 2 to 5, and food will take at least 2 h to pass through stomach after ingestion (Plessas et al., 2017). On the other hand, bile in the large intestine maintains an almost neutral pH (Plessas et al., 2017). The influences of pH on the stability of antioxidant peptides are depicted in Fig. 4a. DPPH radical scavenging activity and metal chelating activity of the antioxidant peptide remained constant over the pH range of 1–10. At pH 11, DPPH radical scavenging activity and metal chelating activity slightly decreased ($p < 0.05$). The results suggested that antioxidant peptides exhibiting DPPH radical scavenging activity and metal chelating 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 starry triggerfish have potential for application in any food system at extreme pH.

Thermal stability of bioactive peptides are important because food products undergo several heat treatments before they reach the market. Generally, thermal treatment can cause protein denaturation, association, and aggregation (Singh and Vij, 2018). Thermal stability of antioxidant activity of the starry triggerfish protein hydrolysates with 60% DH as monitored by DPPH radical scavenging activity and metal chelating activity assay is shown in Fig. 4b. DPPH radical scavenging activity and metal chelating activity of hydrolysates were stable when heated at 100 °C up to 180 min, where activities of 100% were retained. In general, proteins are heat sensitive, which can lead to their aggregation. However, it has been reported that low molecular weight peptides are heat-stable (Nalinanon et al., 2011). Smaller size peptides were more stable to aggregation at high temperatures (Zayas, 1997). These results indicate that starry triggerfish protein hydrolysates with 60% DH could be incorporated in cooked food systems without a significant loss of their antioxidant activities.

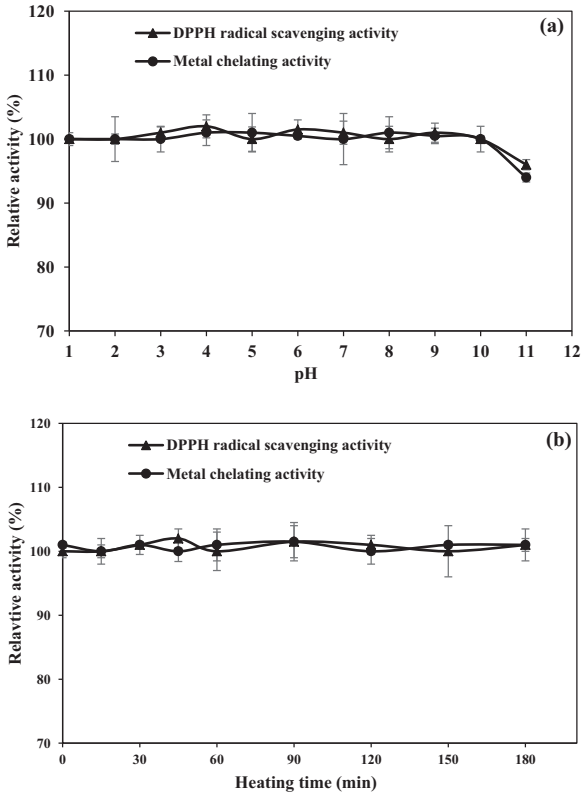

Fig. 4. Effect of pH (a) and heating time (b) on DPPH radical scavenging activity and metal chelating activity of starry triggerfish protein hydrolysate. Bars represent the standard deviation from triplicate determinations.

Table 1
Foaming properties of starry triggerfish protein hydrolysate at varying concentrations.

Hydrolysate concentrations (%, w/v)	FE* (%)	FS* (%)	
		30 min	60 min
0.5	128.89 ± 1.92a**	118.89 ± 1.92a	97.78 ± 3.85a
1.0	147.78 ± 1.94b	131.48 ± 1.70b	115.56 ± 5.09b
2.0	187.78 ± 1.92c	154.44 ± 1.92c	122.20 ± 8.39bc
3.0	208.89 ± 10.18d	170.00 ± 8.82d	130.00 ± 3.33c

* Mean ± SD from triplicate determinations.

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

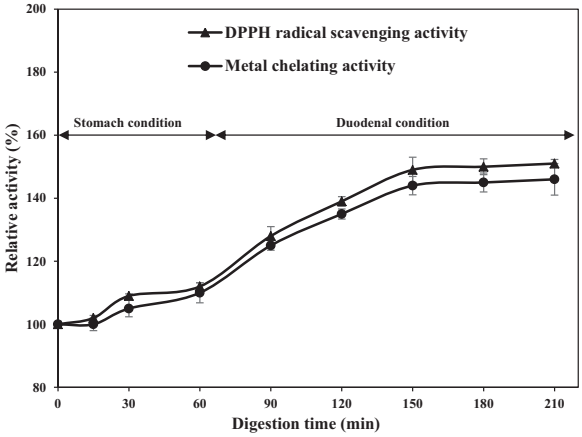

Fig. 5. Antioxidative activities of hydrolysate from starry triggerfish protein hydrolysate in gastrointestinal tract model system. Bars represent the standard deviation from triplicate determinations.

3.4. Changes in antioxidative activity in gastrointestinal tract model systems (GIMs)

GIMs was used to simulate the ingestion system of human body and the remaining antioxidative activities of starry triggerfish protein hydrolysates were monitored (Fig. 5). When protein hydrolysates were orally administrated, their bioactive peptides should be resistant to hydrolysis by digestive proteases in order to be adsorbed and reach the target organ to function as an antioxidant (Burkitt, 2001). Starry triggerfish protein hydrolysates showed a slight increase in DPPH radical scavenging activity and metal chelating activity during pepsin digestion ($p < 0.05$). Pepsin might hydrolyze starry triggerfish protein hydrolysates to some degrees, yielding the new peptides with DPPH radical scavenging activity and metal chelating activity. The further increases in DPPH radical scavenging and metal chelating activities were also found in the intestinal simulated system up to 150 min. Thereafter, no changes in DPPH radical scavenging and metal chelating activities were found during 150 and 210 min of incubation ($p > 0.05$). 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 protein hydrolysates. Generally, gastrointestinal tract actually leads to the generation of more potent bioactive peptides (Megías et al., 2009). Khantaphant et al. (2011) found increased antioxidative activity of protein hydrolysates from the muscle of brownstripe red snapper using flavourzyme after being ingested in the simulated model system. Senphan and Benjakul (2014) also reported that when the hydrolysates from seabass skin prepared using ammonium sulfate precipitated fraction from Pacific white shrimp hepatopancreas with 40% DH was subjected to GIMs, ABTS radical scavenging activity and chelating activity increased, especially in the duodenal condition. The antioxidative activities of protein hydrolysates after incubation in GIMs were dependent on peptides in the hydrolysates, in terms of size, amino acid composition and sequence, which could be targeted by digestive proteases (Megías et al., 2009). From the results, the antioxidative activity of starry triggerfish hydrolysates could be preserved after treatment with these gastrointestinal enzymes in GIMs. Therefore, the antioxidative activities of starry triggerfish hydrolysates were more likely preserved after digestion in the real gastrointestinal tract of the human body.

4. Conclusion

The protein hydrolysates derived from starry triggerfish using trypsin from albacore tuna liver appear to be good sources of desirable peptides. The protein hydrolysates could be used as an emulsifier and as a foaming agent with antioxidant activities. Hence, starry triggerfish protein hydrolysates can be used in food systems as a natural additive possessing antioxidative properties. Furthermore, based on pH and thermal stabilities as well as GI digestion, antioxidant peptides in protein hydrolysates can be incorporated as a multifunctional ingredient into foods.

Acknowledgments

This research was supported by the Thailand Research Fund and Thaksin University, Thailand, for Project No. RSA6080044 to Dr. Sappasith Klomklao.

References

Benjakul, S., Yarnpakdee, S., Senphan, T., Halldorsdottir, S.M., Kristinsson, H.G., 2014. Fish Protein Hydrolysates: Antioxidants and Functional Components in Aquatic Foods. Wiley, Newyork.
Benzie, I.F.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power the FRAP assay. Anal. Biochem. 239, 70–76.
Binsan, W., Benjakul, S., Visessanguan, W., Roytrakul, S., Tanaka, M., Kishimura, H., 2008. Antioxidative activity of Mungoong, an extract paste, from the caphalothorax of white shrimp (*Litopenaeus vannamei*). Food Chem. 106, 185–193.
Boyer, R.F., McCleary, C.J., 1987. Superoxide ion as a primary reductant in ascorbate-mediated ferritin iron release. Free Rad. Biol. Med. 3, 389–395.
Burkitt, M., 2001. A critical overview of the chemistry of copper-dependent low density lipoprotein oxidation: roles of lipid hydroperoxides, α-tocopherol, thiol, and ceruloplasmin. Arch. Biochem. Biophys. 394, 117–135.
Dong, S., Dongfeng, M.Z., Liu, Z., Zhao, Y., Yang, H., 2008. Antioxidant and biochemical properties of protein hydrolysates prepared from Silver carp (*Hypophyhalmichthys molitrix*). Food Chem. 107, 1485–1493.
Egerton, S., Culloty, S., Whooley, J., Stanton, C., Ross, R.P., 2018. Characterization of protein hydrolysates from blue whiting (*Micromesistius poutassou*) and their application in beverage fortification. Food Chem. 245, 698–706.
Gbogouri, G.A., Linder, M., Fanni, J., Parmentier, M., 2004. Influence of hydrolysis degree on the functional properties of salmon byproducts hydrolysates. J. Food Sci. 69, C615–C622.
Gulcin, I., Huyut, Z., Elmastas, M., Aboul-Enein, H.Y., 2010. Radical scavenging and antioxidant activity of tannic acid. Arab. J. Chem. 3, 43–45.
Halim, N.R.A., Yusof, H.M., Sarbon, N.M., 2016. Functional and bioactive properties of fish protein hydolysates and peptides: a comprehensive review. Trends Food Sci. Technol. 51, 24–33.
Halim, N.R.A., Azlan, A., Yusof, H.M., Sarbon, N.M., 2018. Antioxidant and anticancer activities of enzymatic eel (*monopterus* sp) protein hydrolysate as influenced by different molecular weight. Biocatal. Agric. Biotechnol. 16, 10–16.
Intarasirisawat, R., Benjakul, S., Visessanguan, W., Wu, J., 2012. Antioxidative and functional properties of protein hydrolysate from defatted skipjack (*Katsuwonous pelamis*) roe. Food Chem. 135, 3039–3048.
Jao, C.L., Ko, W.C., 2002. 1,1-dipheny1-2-picrylhydrazyl (DPPH) radical scavenging by protein hydrolyzates from Tuna cooking juice. Fish. Sci. 68, 430–435.
Khantaphant, S., Benjakul, S., 2008. Comparative study on the proteases from fish pyloric caeca and the use for production of gelatin hydrolysate with antioxidative activity. Comp. Biochem. Physiol. 151, 410–419.
Khantaphant, S., Benjakul, S., Kishimura, H., 2011. Antioxidative and ACE inhibitory activities of protein hydrolysates from the muscle of brownstripe red snapper prepared using pyloric caeca and commercial proteases. Process Biochem. 46, 318–327.
Khositanon, P., Inpratom, D., Somwang, T., Lawsipo, P., Roytrakul, S., Choksawangkarn, W., 2018. Antibacterial and anticancer activities of protein hydrolysate from fish sauce byproduct. In: Proceedings of the 6th International Conference on Biochemistry and Molecular Biology.
Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., Simpson, B.K., 2007. Purification and characterization of trypsins from the spleen of skipjack tuna (*Katsuwonus pelamis*). Food Chem. 100, 1580–1589.
Klomklao, S., Benjakul, S., Kishimura, H., Osaka, K., Tanaka, M., 2010. Effect of salts and polyethylene glycol on the partitioning and recovery of trypsin from hybrid catfish viscera in aqueous two phase systems. J. Food Biochem. 34, 730–747.
Klomklao, S., Benjakul, S., Kishimura, H., 2013. Functional properties and antioxidative activity of protein hydrolysates from toothed ponyfish muscle treated with viscera extract from hybrid catfish. Int. J. Food Sci. Technol. 48, 1483–1489.
Klomklao, S., Benjakul, S., 2018. Two trypsin isoforms from albacore tuna (*Thunnus alalunga*) liver: purification and physicochemical and biochemical characterization. Int. J. Biol. Macromol. 107, 1864–1870.
Klompong, V., Benjakul, S., Kantachote, D., Shahidi, F., 2007. Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. Food Chem. 102, 1317–1327.
Kristinsson, H.G., Rasco, B.A., 2000. Fish protein hydrolysates: production, biochemical and functional properties. Crit. Rev. Food Sci. Nutr. 40, 43–81.
Lawal, O.S., 2004. Functionality of African locust bean (*Parkia biglobosa*) protein isolate: effects of pH, ionic strength and various protein concentrations. Food Chem. 86, 345–355.

Laywisadkul, S., Weerawatanakorn, M., Maneerattanarungrojn, C., Sujipuli, K., 2017. Investigating the antioxidant and preventing DNA-damage properties of various honeys in Phitsanulok province. Thaksin Univ. J. 5, 93–103.
Lin, L.H., Chen, K.M., 2006. Preparation and surface activity of gelatin derivative surfactants. Coll. Surf. 272, 8–14.
Lo, W.M.Y., Farnworth, E.R., Li-Chan, E.C.Y., 2006. Angiotensin I-converting enzyme inhibitory activity of soy protein digests in a dynamic model system simulating the upper gastrointestinal tract. J. Food Sci. 71, 231–237.
Lowry, O.H., Rosebrough, N.J., Fan, A.L., Randall, R.J., 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193, 256–275.
Megías, C., Pedroche, J., Yust, M.D.M., Alaiz, M., Girón-Calle, J., Millán, F., 2009. Stability of sunflower protein hydrolysates in simulated gastric and intestinal fluids and Cao-2 cell extracts. LWT-Food Sci. Technol. 42, 1496–1500.
Mutilangi, W.A.M., Panyam, D., Kilara, A., 1996. Functional properties of hydrolysates from proteolysis of heat-denatured whey protein isolate. J. Food Sci. 61, 270–275.
Nalinanon, S., Benjakul, S., Kishimura, H., Shahidi, F., 2011. Functionalities and antioxidant properties of protein hydrolysates from the muscle of ornate threadfin bream treated with pepsin from skipjack tuna. Food Chem. 124, 1354–1362.
Naqash, S.Y., Nazeer, R.A., 2013. Antioxidant and functional properties of protein hydrolysates from pink perch (*Nemipterus japonicas*) muscle. J. Food Sci. Technol. 50, 972–978.
Pearce, K.N., Kinsella, J.E., 1978. Emulsifying properties of proteins: evaluation of a turbidimetric technique. J. Agric. Food Chem. 26, 716–723.
Phanturat, P., Benjakul, S., Visessanguan, W., Roytrakul, S., 2010. Use of pyloric caeca extract from bigeye snapper (*Priacanthus macracanthus*) for the production of gelatin hydrolysate with antioxidative activity. LWT-Food Sci. Technol. 43, 86–97.
Phillips, L.G., Whitehead, D.M., Kinsella, J.E., 1994. Protein stabilized foams. In: Phillips, L.G., Whitehead, D.M., Kinsella, J.E. (Eds.), Handbook Structure-Function of Food Proteins. Academic Press, New York, pp. 131–152.
Plessas, S., Nouska, G., Karapetsas, A., Kazakos, S., Alexopoulos, A., Mantzourani, L., 2017. Isolation, characterization and evaluation of the probiotic potential of a novel Lactobacillus strain isolated from Feta-type cheese. Food Chem. 226, 102–108.
Sanchez, C.C., Panito, J.M.R., 2005. Interfacial, foaming and emulsifying characteristics of sodium caseinate as influenced by protein concentration in solution. Food Hydrocolliods 19, 407–416.
Senphan, T., Benjakul, S., 2014. Antioxidative activities of hydrolysates from seabass skin prepared using protease from hepatopancreas of Pacific white shrimp. J. Func. Food 6, 147–156.
Shahidi, F., Han, X.Q., Synowiecki, J., 1995. Production and characteristics of protein hydrolysates from capelin (*Mallotus villosus*). Food Chem. 53, 285–293.
Singh, B.P., Vij, S., 2018. *In vitro* stability of bioactive peptides derived from fermented soy milk against heat treatment, pH and gastrointestinal enzymes. LWT-Food Sci. Technol. 91, 303–307.
Song, R., Yang, P., Wei, R., Ruan, G., 2016. Antioxidative, antibacterial, and food functional oroperties of the half-fin anchovy hydrolysates-glucose conjugates formed via maillard reaction. Molecules 21, 795–809.
Sripokar, P., Poonsin, T., Chaijan, M., Benjakul, S., Klomklao, S., 2016a. Proteinases from liver of albacore tuna (*Thunnus alalunga*): optimum extractant and biochemical characteristics. J. Food Biochem. 40, 10–19.
Sripokar, P., Chaijan, M., Benjakul, S., Kishimura, H., Klomklao, S., 2016b. Enzymatic hydrolysis of starry triggerfish (*Abalistes stellaris*) muscle using liver proteinase from albacore tuna (*Thunnus alalunga*). J. Food Sci. Technol. 53, 1047–1054.
Steel, R.G.D., Torrie, J.H., 1980. Principle and Procedure of Statistics; A Biometrical Approach. McGraw-Hill, New York.
Taheri, A., Anvar, S.A.A., Ahari, H., Fogliano, V., 2013. Comparison the functional properties of protein hydrolysates from poultry by-products and rainbow trout (*Onchorhynchus mykiss*) viscera. Iran. J. Fish. Sci. 12, 154–169.
Tejpal, C.S., Vijayagopal, P., Elavarasan, K., Prabu, D.L., Lekshmi, R.G.K., Asha, K.K., Anandan, R., Chatterjee, N.S., Mathew, S., 2017. Antioxidant, functional properties and amino acid composition of pepsin-derived protein hydrolysates from whole tilapia waste as influenced by pre-processing ice storage. J. Food Sci. Technol. 54, 4257–4267.
Thammarat, K., Leena, N., Punnanee, S., Soottawat, B., 2015. Functional and antioxidative properties of Bambara groundnut (*Voandzeia subterranean*) protein hydrolysates. Int. Food Res. J. 22, 1584–1595.
Thiansilakul, Y., Benjakul, S., Shahidi, F., 2007. Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scad (*Decapterus maruadsi*). Food Chem. 103, 1385–1394.
Yen, G., Wu, J., 1999. Antioxidant and radical scavenging properties of extract from *Ganoderma tsugae*. Food Chem. 65, 375–379.
Zayas, J.F., 1997. Solubility of proteins. In: Zayas, J.F. (Ed.), Handbook of Functionality of Proteins in Food. Springer-Verlag, Berlin, pp. 6–22.