

control gel. These observations suggested that WPC might distribute uniformly as the filler in the ordered network. However, surimi gel added with 3% WPC and CaCl_2 exhibited a finer compact structure with smaller voids, compared with that containing only WPC. This result indicated that CaCl_2 acted synergistically with WPC for gel improvement. Calcium ion therefore contributed to protein cross-linking during setting as evidenced by the stronger gel (Fig. 1). Modori gels without WPC addition had a structure containing aggregates of sparse pack spherical proteins and arranged in clusters. A large porous matrix with some cavities on the surface was also observed. When 3% WPC was added, more compact structure with smaller clusters of aggregated protein was formed as evidenced by the smaller voids. This suggested that WPC was effective in inhibiting protein degradation. Similar structures were found between modori gel containing 3% WPC without and with CaCl_2 addition.

CONCLUSION

The addition of WPC up to 3% (w/w) resulted in the increased breaking force and deformation with higher water-holding capacity of surimi from goatfish. Addition of CaCl_2 at 50 mmol/kg and 3% WPC effectively improved the gel strength of kamaboko gel but slightly lowered gel strength of modori gel from goatfish surimi. Thus, the use of WPC and CaCl_2 in goatfish surimi together with prior setting led to the improved gel-forming ability of goatfish surimi.

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Purification and biochemical properties of pepsins from the stomach of skipjack tuna (*Katsuwonus pelamis*)

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Abstract Pepsins 1 and 2 from the stomach of skipjack tuna (*Katsuwonus pelamis*) were purified to homogeneity by using a series of chromatographic purification involving DEAE-cellulose, Sephadex G-50 and Sephadex G-75 with increase in purity of 246-fold and 213-fold, respectively. Molecular weights of pepsins 1 and 2 were estimated by SDS–PAGE to be 33.9 and 33.7 kDa, respectively. The N-terminal amino acid sequences of the first 20 amino acids of both isoenzymes were YQDGTEPMTNDADLSYYGVI. The optimal pH and temperature for pepsin 1 were 2.5 and 50 °C, respectively, while pepsin 2 showed optimal activity at pH 2.0 and 45 °C. The activity of two pepsins was stable in the pH range of 2–5 and at temperatures up to 50 °C. The activity of purified pepsins was strongly inhibited by pepstatin A in a dose-dependent manner. SDS and cysteine showed inhibitory effects toward both pepsins. Activity of pepsin 2 was slightly activated by NaCl, but NaCl had no effect on pepsin 1. Pepsins 1 and 2 had high affinity and hydrolytic activity toward hemoglobin with K_m of 54 and 71 μ M, respectively. k_{cat} of pepsins 1 and 2 were 38.1 and 44.3 s^{-1} , respectively. Both pepsins effectively hydrolyzed bovine serum albumin, egg white, natural actomyosin from brownstripe red snapper muscle and acid-solubilized collagen

from arabesque greenling skin. Nevertheless, the hydrolytic activity was slightly less than that of pepsin from porcine stomach.

Keywords Skipjack tuna · Biochemical properties · Characterization · Pepsin · Purification · Hydrolysis

Introduction

Pepsin is the major digestive enzyme in stomach of animals and is secreted as pepsinogen from chief cells of oxyntic glands located in the stomach wall epithelium [1, 2]. In acidic environment, pepsinogen autocatalytically converts to an active form, pepsin [2–4]. Pepsin is composed of a single polypeptide chain of 321 amino acids and has a molecular weight of 35.5 kDa [5]. Its tertiary structure is stabilized in part by three disulfide bridges and a phosphate linkage. A phosphate group, attached to the hydroxyl group of a seryl residue, can be removed without loss of enzymatic activity [5]. The enzyme is quite stable in the pH range of 2–5 and rapidly loses activity at pH above 5, due to denaturation [1, 3, 5]. Pepsin has its primary specificity toward the amino acid residue that furnishes the NH group to the susceptible peptide bond, preferably phenylalanyl, tyrosyl, or tryptophanyl [5]. Pepsin and pepsinogen from stomach of various fish species such as polar cod [6], North Pacific bluefin tuna [7], Atlantic cod [8], sea bream [9], African coelacanth [4], pectoral rattail [10], smooth hound [11], Mandarin fish [12], European eel [13], and albacore tuna [3] have been purified and characterized.

Thailand is the world's largest producer and exporter of canned tuna. In 2008, Thai canned tuna was exported to several countries with a total volume of 484,894 metric tons and a value of 61,036 million baths [14]. The most

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commercially important tuna species for the Thai tuna industry is skipjack tuna, followed by yellow-fin tuna and tongol tuna, accounting for over 80% of Thai frozen tuna imports [14]. During canned tuna manufacturing, high amounts of processing wastes such as viscera, head, and bone are discarded. Tuna processing by-products are normally utilized as fishmeal or pet food, which have a lower market value. There is growing interest in obtaining higher value biochemicals and pharmaceuticals from fishery wastes, notably enzymes. Of these by-products, fish viscera can be used as a potential source of various enzymes, particularly proteases [15, 16]. Pepsin from the stomach of albacore tuna was isolated and partially purified by aqueous two-phase system (ATPS) [17]. Fish pepsins have been used for preparation of protein isolate [18], extraction of collagen [16, 19], and gelatin [20]. Due to a large amount of skipjack tuna stomach produced during manufacturing, it can serve as a promising source of pepsin for further applications, especially for the digestion of proteins to obtain the high-value products with bioactivity, e.g. nutraceutical properties or flavorant, etc. No information regarding the biochemical characteristics of pepsins from the stomach of skipjack tuna has been reported. Therefore, the objectives of this study were to purify pepsins from skipjack tuna stomach and to characterize some biochemical properties as well as hydrolytic ability toward different protein substrates.

Materials and methods

Chemicals

Bovine hemoglobin, β -mercaptoethanol (β ME), L-cysteine, adenosine 5'-triphosphate (ATP), molybdate, pepstatin A, ethylenediaminetetraacetic acid (EDTA), soybean trypsin inhibitor, 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Folin-Ciocalteu's phenol reagent was obtained from Merck (Darmstadt, Germany). Sephadex G-50 was procured from Pharmacia Biotech (Uppsala, Sweden). Diethylaminoethyl (DEAE)-cellulose was obtained from Whatman (Maidstone, UK). Sephadex G-75 was purchased from GE Healthcare UK Limited (Buckinghamshire, UK). 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).

Preparation of skipjack tuna stomach

Internal organs of skipjack tuna (*K. pelamis*) were obtained from Tropical Canning (Thailand) Public Co., Ltd.,

Songkhla, Thailand. The samples were packed in polyethylene bag, kept in ice with a solid/ice ratio of 1:2 (w/v), and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 30 min. Upon arrival, internal organs were excised, and the stomach was collected and placed in a polyethylene bag. The collected stomach was stored at -20°C until use.

Preparation of crude extract

Frozen stomach was thawed using running water ($26\text{--}28^{\circ}\text{C}$) until the core temperature reached -2 to 0°C . The sample was cut into pieces with a dimension of $0.5 \times 0.5\text{ cm}^2$. Sample was finely ground in liquid nitrogen using a National Model MX-T2GN blender (Taipei, Taiwan). Sample was then lyophilized using a SCANVAC CoolSafe™ freeze-dryer (CoolSafe 55, ScanLaf A/S, Lyngø, Denmark) and the dry powder was referred to as 'stomach powder'.

To prepare the crude extract, stomach powder was suspended in 20 mM sodium phosphate buffer (pH 7.0) containing 1 mM PMSF at a ratio of 1:9 (w/v) and stirred continuously at 4°C for 3 h. The suspension was centrifuged for 20 min at 4°C at $20,000\times g$ using a refrigerated centrifuge (H-2000B, Kokusan, Tokyo, Japan) to remove the tissue debris. The supernatant was collected and lyophilized using an EYELA FDU-1200 freeze-dryer (Tokyo Rikakikai Co., Ltd., Tokyo, Japan). Before use, the dry extract (10 g) was dissolved with 90 mL of 20 mM sodium phosphate buffer (pH 7.0), followed by centrifugation for 15 min at 4°C at $20,000\times g$. The supernatant was collected and referred to as "crude extract".

Purification of skipjack tuna pepsins

All steps of purification were carried out at 4°C . Crude extract was adjusted to pH 4 with 1 M HCl. The mixture was allowed to stand for 30 min. The acidified crude extract was then centrifuged at $20,000\times g$ for 15 min. To fully activate pepsin, the obtained supernatant was adjusted to pH 2 with 1 M HCl and gently stirred for 30 min. The solution was subjected to centrifugation as described previously prior to dialysis against 60 volumes of 20 mM sodium phosphate buffer (pH 5.0), referred to as 'starting buffer; SB'. Prepared solution was applied onto DEAE-cellulose column ($2.2 \times 35\text{ cm}$), previously equilibrated with SB. After the column was washed with SB until A_{280} was below 0.05, the elution was performed with a linear gradient of 0–0.5 M NaCl in SB at a flow rate of 0.5 mL/min. Fractions of 2.5 mL were collected and those with proteolytic activity were pooled. Two activity peaks (pepsins 1 and 2) were obtained, and pooled fractions from each peak were dialyzed with 60 volumes

of SB for 12 h and then concentrated by lyophilization. Both lyophilized fractions were further chromatographed on Sephadex G-50 column (3.9×70 cm) equilibrated with approximately two bed volumes of SB. Elution was carried out with the same buffer at the flow rate of 0.5 mL/min. Fractions (4.5 mL) were collected and those with proteolytic activity were pooled and lyophilized. Lyophilized Sephadex G-50 fraction was dissolved in distilled water and dialyzed against 60 volumes of SB for 12 h. The dialysate was then subjected to DEAE-cellulose column, and the enzyme was eluted with a linear gradient of 0–0.3 M NaCl. Fractions of 4.5 mL, with proteolytic activity, were pooled and dialyzed against 60 volumes of SB for 12 h prior to lyophilization. Lyophilized DEAE-cellulose fraction was dissolved in SB prior to loading onto Sephadex G-75 (3.2×56 cm) column equilibrated with approximately two bed volumes of SB. The proteases were eluted with SB at the flow rate of 0.5 mL/min and fractions of 4.5 mL were collected. Those fractions with proteolytic activity were pooled and lyophilized. The lyophilized fraction was dissolved in SB and re-chromatographed onto Sephadex G-75 column, followed by the elution with SB at the flow rate of 0.25 mL/min. Fractions (2.5 mL) were collected and those with proteolytic activity were pooled and lyophilized.

Pepsin from porcine stomach mucosa (EC 3.4.23.1) (750 units/mg dry matter, Sigma Chemical Co., St. Louis, MO, USA) was partially purified by 2 steps of purification using DEAE-cellulose column and Sephadex G-75 column as described previously.

Protein determination

Protein concentration was determined by measuring the absorbance at 280 nm or by the method of Lowry et al. [21] using bovine serum albumin as a standard.

Assay of proteolytic activity

Proteolytic activity was determined using hemoglobin as a substrate according to the method of Nalinanon et al. [16] with some modifications. To initiate the reaction, 200 μ L of enzyme solution suitably diluted were added into the assay mixture containing 200 μ L of 2% hemoglobin, 200 μ L of distilled water, and 600 μ L of reaction buffer. The reaction was conducted at pH 2.0 and 50 °C for 20 min. To terminate enzymatic reaction, 200 μ L of 50% (w/v) trichloroacetic acid (TCA) were added. Unhydrolyzed protein substrate was allowed to precipitate for 1 h at 4 °C, followed by centrifuging at $15,000 \times g$ for 10 min using a KUBOTA 3630 centrifuge (SiGMA Laborzentrifugen, Osterode am Harz, Germany). The oligopeptide content in the supernatant was measured at 280 nm. One unit of activity was defined as the

amount causing an increase of 1.0 in absorbance at 280 nm per min. A blank was run in the same manner, except that the enzyme was added into the reaction mixture after the addition of 50% (w/v) TCA.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and native-PAGE

SDS–PAGE was performed according to the method of Laemmli [22]. 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 ME) and boiled for 3 min. The samples (15 μ g protein) were loaded onto the gel made of 4% stacking and 12.5% or 12% or 7.5% separating gels and subjected to electrophoresis at a constant current of 15 mA per gel using a AE-6440 electrophoresis 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. Protein standards, including egg albumin (45.0 kDa), bovine pancreatic trypsinogen (24.0 kDa), bovine milk β -lactoglobulin (18.4 kDa), and egg white lysozyme (14.3 kDa) were used as molecular weight markers. Gels were imaged using a Canon image scanner (CanoScan LiDE 25, Canon Inc., Tokyo, Japan), and band intensities were quantified with the public domain digital analysis software, ImageJ (ImageJ 1.42q, National Institutes of Health, Bethesda, MD, USA). Native-PAGE was performed in the same manner, except SDS and β ME were omitted from the system.

Analysis of amino acid sequence

To analyze the N-terminal sequence, purified pepsins were electroblotted onto polyvinylidenedifluoride (PVDF) membrane (ProBlott®, Applied Biosystems, Foster City, CA, USA) after SDS–PAGE. The amino acid sequence of pepsins was analyzed using a protein sequencer, Procise 492 (Perkin Elmer, Foster City, CA, USA).

Biochemical properties

pH and temperature profile

To study pH profile, proteolytic activity of purified pepsins was measured at 50 °C using hemoglobin as a substrate at different pHs (pHs 1.0, 1.5, and 2.0 using 20 mM maleate buffer and pHs 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, and 8.0 using McIlvaine's buffer (0.2 M Na-phosphate and 0.1 M Na-citrate)). For temperature profile, the assay was performed at various temperatures (20, 30, 40, 45, 50, 55, 60, and 70 °C) at pH 2.0.

pH and thermal stability

For pH stability study, purified pepsin solutions were incubated at pHs 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 using McIlvaine's buffer at room temperature for 30 min. Residual activity was then determined using hemoglobin as a substrate at pH 2.0 and 50 °C for 20 min. For thermal stability, the purified pepsins were incubated at different temperatures (20, 30, 40, 50, 60, and 70 °C) for 30 min before rapid cooling using iced water. The residual activity was then assayed as previously described.

Effect of some protease inhibitors

Purified pepsin solutions (200 µL) were mixed with 200 µL of single protease inhibitor to obtain the final designated concentration (1 µM for pepstatin A, 0.1 mM for E-64, 0.1 mM for soybean trypsin inhibitor and 2 mM for EDTA). Mixtures were incubated at room temperature for 30 min. The residual activity was measured at pH 2.0 and 50 °C. Percent inhibition was then calculated. To study the inhibitory effect of pepstatin A toward pepsins, different molar ratios of pepstatin A to pepsin (0/1, 0.1/1, 0.25/1, 0.5/1, 1/1, 2/1, 4/1 and 10/1; mol/mol) were used. The mixtures of pepsin and pepstatin A were prepared, and the residual activity was determined at pH 2.0 and 50 °C.

Effect of some chemicals

Different chemicals (200 µL) were mixed with pepsin solution (200 µL) to obtain the concentration designated (0.5 mM for ATP and molybdate; 5 mM for NaCl and CaCl₂; 0.05% (w/v) for SDS and 50 mM for L-cysteine). The mixtures were kept at room temperature for 30 min. The remaining activity was determined at pH 2 and 50 °C, and the relative activity was calculated.

Kinetic studies

The activity was assayed with different final concentrations of hemoglobin ranging from 1 to 300 µM. The final pepsin concentration for the assay was 0.02 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 [23]. Values of turnover number (k_{cat}) were calculated from the following equation: $k_{\text{cat}} = V_{\max}/[E]$, where $[E]$ is the active enzyme concentration and $V_{\max}$ is the maximal velocity.

Hydrolysis of different protein substrates by pepsins

BSA, egg white, natural actomyosin (NAM) from brown-stripe red snapper (*Lutjanus vitta*) muscle and acid-solubi-

lized collagen (ASC) from arabesque greenling (*Pleurogrammus azonus*) skin were used as protein substrates. NAM and ASC were prepared following the methods of Benjakul et al. [24] and Nalinanon et al. [19], respectively. To hydrolyze the proteins, pepsins (1.6 units) were added to 2 mL of reaction mixture, containing 4 mg of different protein substrates in McIlvaine's buffer, pH 2.0, previously incubated at 50 °C for 10 min. The hydrolysis was conducted by incubating the mixture at 50 °C for 30 min. The control was performed by incubating the reaction mixture at 50 °C for 30 min without the addition of pepsin. At the designated time, 200 µL of reaction mixture was taken and mixed with 100 µL of 1 N NaOH containing 1% (w/v) SDS in order to terminate pepsin activity. The mixture was further mixed with 100 µL of SDS–PAGE sample buffer and boiled for 1 min. The reaction mixture was analyzed by SDS–PAGE using 12 or 7.5% separating gel and 4% stacking gel as previously described.

Statistical analysis

Experiments were run in triplicate. All data were subjected to analysis of variance (ANOVA), and the differences between means were evaluated by Duncan's multiple range test. For pair comparison, *T*-test was used. SPSS statistic program (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA) was used for data analysis.

Results and discussion

Purification of skipjack tuna pepsins

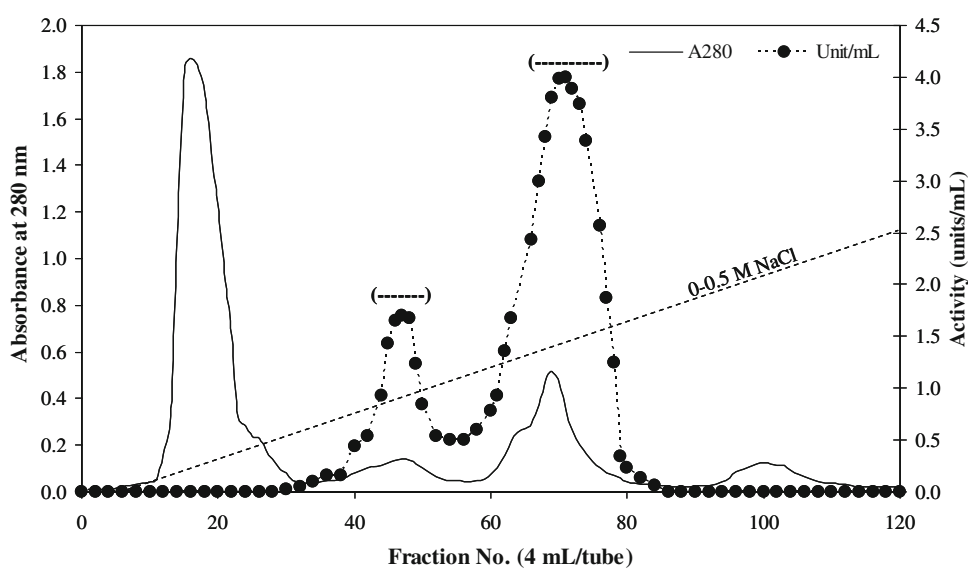
Purification of pepsins from the stomach of skipjack tuna is summarized in Table 1. The purification of pepsins was started by adjusting pH of the crude extract to pH 4 and 2, respectively. After acidification, an activity yield of approximately 111% was obtained, and 73.4% of proteins, mostly contaminating proteins, were removed. Acidification was used to remove other protein contaminants in stomach extract, leading to the higher purity of pepsin [10, 25]. Increase in purity of four-fold was obtained after acidification process. Acidified fraction was then subjected to ion-exchange chromatography using DEAE-cellulose. Two distinct peaks with proteolytic activity eluted by 0.07 and 0.15 M NaCl were designated as pepsins 1 and 2, respectively (Fig. 1). Purity of pepsins 1 and 2 was increased by 19- and 20-fold, respectively. Both DEAE-cellulose fractions were subsequently chromatographed onto Sephadex G-50 column. It was noted that 99.7% of protein, mainly contaminating proteins, were removed after gel filtration using Sephadex G-50 column.

Table 1 Purification of pepsins from the stomach of skipjack tuna

Purification step	Total protein (mg)	Total activity (units)*	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	6,250	2,383	0.381	1	100
Acidification	1,657	2,655	1.60	4	111
DEAE-cellulose (1st)					
Pepsin 1	44.1	316	7.17	19	13
Pepsin 2	57.4	438	7.63	20	18
Sephadex G-50					
Pepsin 1	7.77	155	19.9	52	6.5
Pepsin 2	9.67	170	16.0	42	7.2
DEAE-cellulose (2nd)					
Pepsin 1	2.17	121	55.7	146	5.1
Pepsin 2	2.94	153	52.2	137	6.4
Sephadex G-75 (1st)					
Pepsin 1	1.25	92.2	73.7	194	3.9
Pepsin 2	2.55	157	61.7	162	6.6
Sephadex G-75 (2nd)					
Pepsin 1	0.53	49.5	93.5	246	2.1
Pepsin 2	1.23	100	81.1	213	4.2

* One unit of activity was defined as the amount causing an increase of 1.0 in absorbance at 280 nm per min. Pepsin activity was assayed at pH 2.0, 50 °C for 20 min using hemoglobin as a substrate

Fig. 1 Elution profile of pepsins on DEAE-cellulose column. Acidified extract was dialyzed against SB and applied onto a DEAE-cellulose column. Elution was carried out with a linear gradient of 0–0.5 M NaCl in SB. (---): pooled fractions



The results showed that DEAE-cellulose and Sephadex G-50 chromatographies effectively removed the contaminants from the crude extract. However, some protein contaminants were still retained as examined by SDS-PAGE (data not shown). In order to purify pepsins to homogeneity, Sephadex G-50 fractions were concentrated and rechromatographed using DEAE-cellulose column, followed by Sephadex G-75 column twice. After the final purification step, purification fold of 246 and 213 with a yield of 2.1 and 4.2% were obtained for pepsins 1 and 2, respectively. Specific activity of 93.5 and 81.1 units/mg of protein were found for pepsins 1 and 2, respectively. The homogeneity

of purified pepsins was examined by SDS-PAGE. Pepsins 1 and 2 showed single band on SDS-PAGE under reducing conditions (Fig. 2). Molecular weights (MW) of pepsins 1 and 2 estimated from the mobilities in SDS-PAGE were 33.9 and 33.7 kDa, respectively. Based on native-PAGE, single band of each pepsin was observed (data not shown), confirming the purity of pepsin obtained. MWs of pepsins from the stomach of skipjack tuna were similar to those of pepsins or acid proteases from the stomach of Mandarin fish [12], sea bream [9], European eel [13], smooth hound [11], African coelacanth [4], albacore tuna [3], pectoral rattail [10], and orange roughy [26], which had

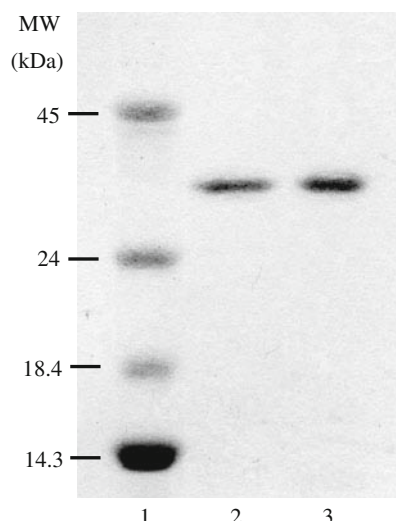


Fig. 2 SDS-PAGE of purified pepsins from the stomach of skipjack tuna. Lane 1: standard protein markers; lane 2: pepsin 1; lane 3: pepsin 2

MW ranging from 30 to 35 kDa. Although the MW of two pepsin isoforms from skipjack tuna are quite similar, some differences in enzymatic characteristics, especially their binding properties to ion-exchange column, DEAE-cellulose, were noticeable.

N-terminal amino acid sequence of pepsins

N-terminal amino acid sequence of pepsins from the stomach of skipjack tuna was aligned to compare with those of other pepsins as depicted in Fig. 3. The same N-terminal amino acid sequence of the first 20 amino acids, YQDGTPEMTNDADLSYYGVI, was found for pepsins 1 and 2. The result revealed that two pepsins from skipjack tuna stomach might share high identity. Based on the first 20 amino acids, pepsins from skipjack tuna showed high degree of sequence homology (95%), compared with pepsin 2 from North Pacific bluefin tuna [7]. The highly conserved amino acid sequence between pepsins from skipjack tuna and North Pacific bluefin tuna suggested that they

were genetically evolved from a common ancestor. According to the first 20 amino acids, pepsins from skipjack tuna showed the sequence homology of 50 and 45%, compared with pepsin from cod [27] and smooth hound [11], respectively. Relative low homology of skipjack tuna pepsins in comparison with pepsin B from porcine [28] and dog [29] and pepsin C from bullfrog [30] was observed.

pH and temperature profile

The effect of pH on the activity of pepsins from the stomach of skipjack tuna was determined over a pH range of 1.0–8.0 as shown in Fig. 4a. Pepsins 1 and 2 were highly active between pH 1.5 and 3.0. The optimal pH for hydrolysis of hemoglobin by pepsins 1 and 2 was 2.5 and 2.0, respectively. The optimum pH values of both pepsins were in accordance with those of pepsins obtained from the stomach of other fish species including bonito [31], polar cod [6], North Pacific bluefin tuna [7], African coelacanth [4], sea bream [9], pectoral rattail [10], smooth hound [11], skipjack tuna and tongol tuna [16], Mandarin fish [12], European eel [13], and albacore tuna [3], in which optimal pH ranged from 2.0 to 3.5. No pepsin activity was detected at the pH range of 6–8. Denaturation causing the loss in activity was very severe in neutral–alkaline pH ranges. This was possibly due to the conformational changes of enzyme under harsh condition, resulting in the lowered activity or complete loss in activity [3].

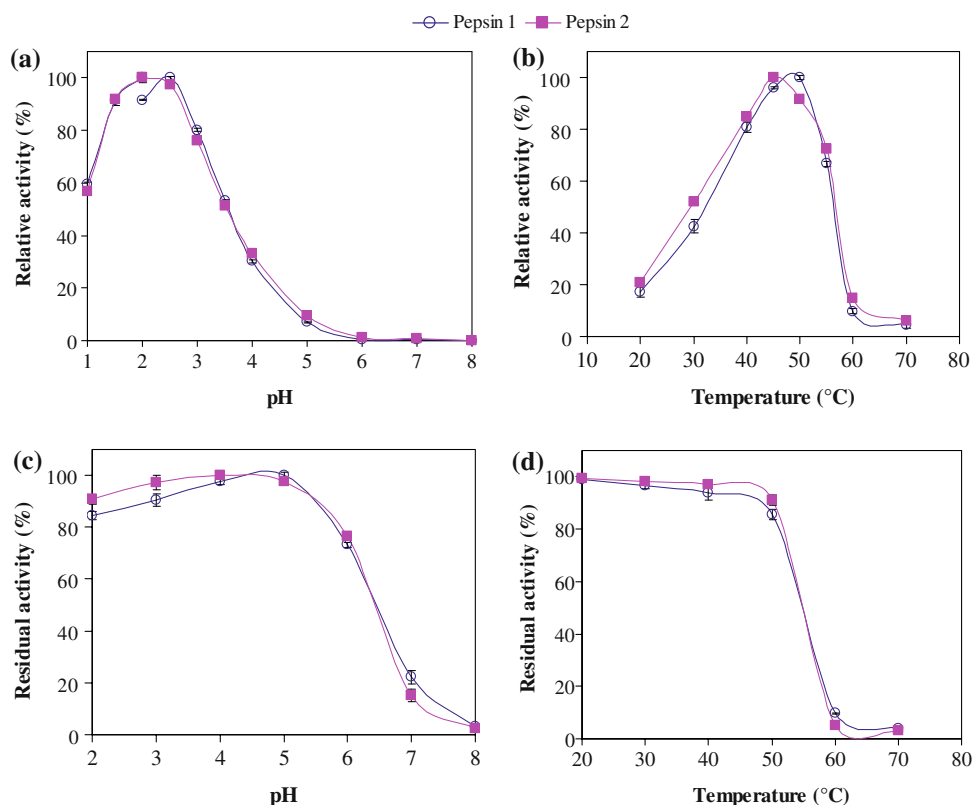
Temperature profiles of pepsins from the stomach of skipjack tuna are depicted in Fig. 4b. The optimal temperature of pepsins 1 and 2 was 50 and 45 °C, respectively, when hemoglobin was used as a substrate. The different temperature optima (40 and 55 °C) between two pepsins from sardine have also been reported [32]. The optimal temperature of pepsin 1 from skipjack tuna was in accordance with those of pepsins obtained from sea bream (P-II, P-III and P-IV) [9], tongol tuna [16], and albacore tuna [3]. The varying optimal temperatures of pepsin from other marine fish species which inhabit in different temperature zones such as tropical fish: sea bream (45–50 °C) [9],

	1	5	10	15	20
Skipjack tuna 1	-	Y Q D G T E P M T N D A D L S Y Y G V I - - -			
Skipjack tuna 2	-	Y Q D G T E P M T N D A D L S Y Y G V I - - -			
Bluefin tuna 2	-	Y Q D G T E P M T N D A D L S Y Y G V V S I G			
Bluefin tuna 3	-	A T A N Y M Y I N Q Y A D T I Y Y G A I S I G			
Smooth hound	-	- - - A T E P L S N Y L D S S Y F G D I S I G			
Cod	-	- - R T V E Q M K N E A D T E Y Y G V I S I G			
Bullfrog C	-	- A T A F E P L A N Y M D M S Y Y G E I S I G			
Porcine B	-	- A V A Y E P F T N Y L D S F Y F G E I S I G			
Dog B	N	D A V A Y E P F T N Y L D S S Y Y F G E I S I G			

Fig. 3 Comparison of N-terminal amino acid sequence of purified pepsins from the stomach of skipjack tuna with other pepsins: North Pacific bluefin tuna pepsin 2 and 3 [7]; smooth hound pepsin [11]; cod

pepsin [27]; bullfrog pepsin C [30]; porcine pepsin B [28], and dog pepsin B [29]. Amino acid residues different from skipjack tuna pepsin are boxed

Fig. 4 pH (a) and temperature (b) profiles and pH (c) and thermal (d) stabilities of purified pepsins from the stomach of skipjack tuna



subtropical fish: smooth hound (40 °C) [11], and cold water fish: polar cod (37 °C) [6] and pectoral rattail (45 °C) [10] have been reported. The optimal temperatures of pepsin from fresh water fish including Mandarin fish (40–45 °C) [12] and European eel (35–40 °C) [13] were also reported. In general, pepsins from colder-water fish had the lower temperature optima and were more heat-labile than those of animals from the warmer environment [6, 25, 33]. The differences in optimal temperature might be associated with the differences in enzyme conformation and bonds stabilizing the enzyme structure as governed by habitat, environment and genetics [1, 3, 16]. The sharp decrease in activity of both pepsins from skipjack tuna at temperature above 50 °C was observed, mainly due to thermal denaturation of pepsins.

pH and thermal stability

Pepsins 1 and 2 from the stomach of skipjack tuna were stable in the pH range of 2–5, in which the residual activity more than 80% was found (Fig. 4c). The decrease in activity of both pepsins was noticeable at pH 6, and pepsins became more susceptible to activity losses at neutral and alkaline pH. Similar result was reported for albacore tuna pepsin [3]. Orange roughy pepsins [26], Monterey acidic enzymes [34], and pectoral rattail pepsins [10] exhibited the stability at pH range of 2–6. Gildberg [1] reported that

pepsins from cold and temperate water fish were stable in the pH range of 2–5, whereas pepsins from warm water species such as bonito were quite stable even in neutral conditions [31]. The differences in pH stability indicated the different molecular properties including bondings stabilizing the structure as well as enzyme conformation among various species and anatomical location [10].

Thermal stability of pepsins 1 and 2 from the stomach of skipjack tuna is depicted in Fig. 4d. The activity of both pepsins was stable up to 50 °C with the residual activity more than 85%. After being heated at 60 °C for 30 min, activities of pepsins 1 and 2 were decreased to 9.7 and 4.7%, respectively. The result was in agreement with pepsin from dogfish stomach [35] and albacore tuna [3], which was stable at temperature below 50 °C. Disulfide linkages, the average hydrophobicity and the amount of intramolecular hydrogen bonds are considered to affect thermostability of enzymes [8].

Effect of protease inhibitors and some chemicals

The effects of various inhibitors and some chemicals on activity of pepsins 1 and 2 from the stomach of skipjack tuna were determined (Table 2). The proteolytic activity of both pepsins was strongly inhibited by pepstatin A, an aspartic proteinase inhibitor. Pepstatin A, a pentapeptide, is a very specific inhibitor that forms multiple, non-covalent

Table 2 Effect of various inhibitors and some chemicals on the activity of pepsins from the stomach of skipjack tuna

Inhibitors/chemicals	Concentration	Relative activity (%) ^a	
		Pepsin 1	Pepsin 2
Control		100	100
Pepstatin A	1 μ M	ND	ND
E-64	0.1 mM	97.6 $\pm$ 0.7	98.4 $\pm$ 0.8
EDTA	2 mM	101.3 $\pm$ 1.4	99.8 $\pm$ 1.7
Soybean trypsin inhibitor	0.1 mM	99.4 $\pm$ 1.9	99.1 $\pm$ 0.3
ATP	0.5 mM	97.3 $\pm$ 1.1	99.5 $\pm$ 0.8
Molybdate	0.5 mM	100.5 $\pm$ 0.6	99.9 $\pm$ 4.6
NaCl	5 mM	102.0 $\pm$ 1.6	107.4 $\pm$ 0.8
CaCl ₂	5 mM	99.8 $\pm$ 0.7	103.4 $\pm$ 1.0
SDS	0.05% (w/v)	59.9 $\pm$ 1.2	60.5 $\pm$ 0.4
Cysteine	50 mM	79.9 $\pm$ 5.1	88.3 $\pm$ 1.8

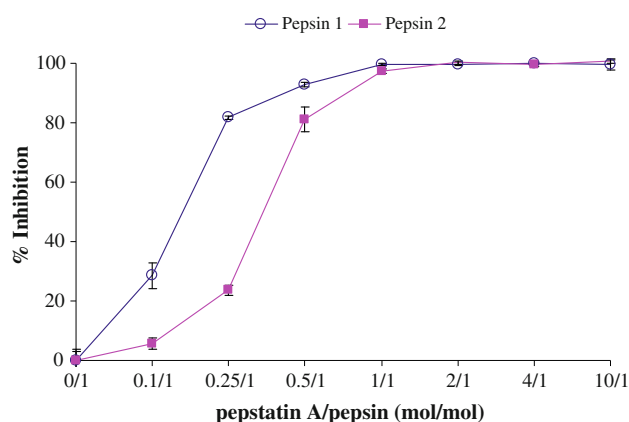
Enzyme solution was incubated with the same volume of inhibitors or chemicals at 25 °C for 30 min, and the residual activity was determined using hemoglobin as a substrate for 20 min at pH 2.0 and 50 °C

^a Mean $\pm$ SD from triplicate determinations

ND not detectable

interactions with the active site of aspartic proteinases and blocks the accessibility of a substrate to the active site cleft [36, 37]. On the other hand, E-64, specific inhibitor of cysteine proteinase, soybean trypsin inhibitor, a serine proteinase inhibitor and EDTA, a metalloproteinase inhibitor had negligible effect on the activity of purified pepsins (Table 2). Therefore, the inhibitory results confirmed that purified enzymes belonged to the aspartic proteinase.

Pepsin 1 from skipjack tuna was specifically bound to pepstatin A with a pepstatin A/pepsin molar ratio of 1:1, whereas pepstatin A completely inhibited pepsin 2 with a 2:1 stoichiometry (Fig. 5). The differences in susceptibility of both pepsins toward pepstatin A were also observed in

**Fig. 5** Effect of pepstatin A at different molar ratios on the inhibition of purified pepsins from the stomach of skipjack tuna

different isoforms of pepsins from the stomach of other fish species such as sea bream [9], European eel [13], and Mandarin fish [12]. Tanji et al. [4] reported that both pepsins from African coelacanth were nearly completely inhibited by an equimolar amount of pepstatin. Zhou et al. [12] found that pepsins III(a) and III(b) from Mandarin fish were strongly inhibited by pepstatin with a pepstatin/pepsin molar ratio of approximately 1:1. Nevertheless, pepsins I and II were not sensitive to pepstatin A and a 10-fold molar excess of pepstatin A was necessary for the complete inhibition of these two enzymes. Pepsins 1 and 2 from North Pacific bluefin tuna were strongly inhibited by pepstatin, whereas pepsin 3 was less sensitive to pepstatin. A 17-fold molar excess of pepstatin was required for complete inhibition of pepsin 3 [7].

Different chemicals were also used to elucidate their impact on activity of both pepsins (Table 2). ATP and molybdate, activator and inhibitor of cathepsin D [38], respectively, did not affect the activity of purified pepsins from skipjack tuna. Klomklao et al. [10] reported that ATP and molybdate had no impact on the activity of pepsins from pectoral rattail. Nalinanon et al. [3] also found that ATP and molybdate had no profound effect on the activity of albacore tuna pepsin. Cathepsin D is generally activated by ATP when bovine serum albumin, hemoglobin, parathyroid hormone, and a synthetic octapeptide were used as substrates, whereas pepsin, a homologous aspartic proteinase, was not activated [38]. NaCl (5 mM) slightly increased the activity of pepsin 2 from skipjack tuna by 7% ($p < 0.05$), but it had no impact on the activity of pepsin 1 ($p > 0.05$). CaCl₂ (5 mM) had no profound effect on activity of pepsin ($p > 0.05$). Sanchez-Chiang et al. [39] found that the activity of pepsin I from adult salmon (*Oncorhynchus keta*) toward hemoglobin was increased by approximately 20% in the presence of 5 mM NaCl, whereas the activity of pepsin II was not affected. The activity of both pepsins A and B from the stomach of pectoral rattail was increased in the presence of 5 mM CaCl₂, 5 mM MgCl₂, or 5 mM CoCl₂ [10]. However, the proteolytic activities of pepsins from pectoral rattail was continuously decreased as NaCl concentration increased (0–30%) [10]. Gildberg et al. [8] also reported that all pepsins from Atlantic cod were strongly depressed by the presence of NaCl at the concentrations of 5 and 10%. Salts possibly altered the structural conformation of either pepsin or the substrate used, in the way, which impeded or enhanced the binding ability between both enzyme and substrate [3]. The presence of 0.05% (w/v) SDS and 50 mM cysteine resulted in the loss in activity of both pepsins by approximately 40% and 12–20%, respectively. The same result was also reported for albacore tuna pepsin [3]. Nalinanon et al. [16] found that cysteine, a reducing agent, inhibited the pepsin activity of tuna stomach extracts in dose-dependent manner, mainly via

Table 3 Kinetic properties of purified pepsins from the stomach of skipjack tuna stomach for the hydrolysis of hemoglobin

Enzyme	K_m (μM)*	k_{cat} (s^{-1})*	k_{cat}/K_m ($\mu\text{M}^{-1} \text{s}^{-1}$)
Pepsin 1	$54 \pm 0.2\text{a}$	$38.1 \pm 0.2\text{a}$	0.71
Pepsin 2	$71 \pm 0.3\text{b}$	$44.3 \pm 0.2\text{b}$	0.63

K_m , k_{cat} values were determined using hemoglobin as a substrate at pH 2.0 and 50 °C

The final enzyme concentration for the assay was 0.02 mg/mL

Mean $\pm$ SD from triplicate determinations

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

breaking disulfide bonds of pepsin. Furthermore, SDS, an anionic surfactant, could act as a denaturant by disrupting non-covalent bonds that stabilized the pepsin structure [3].

Kinetic study

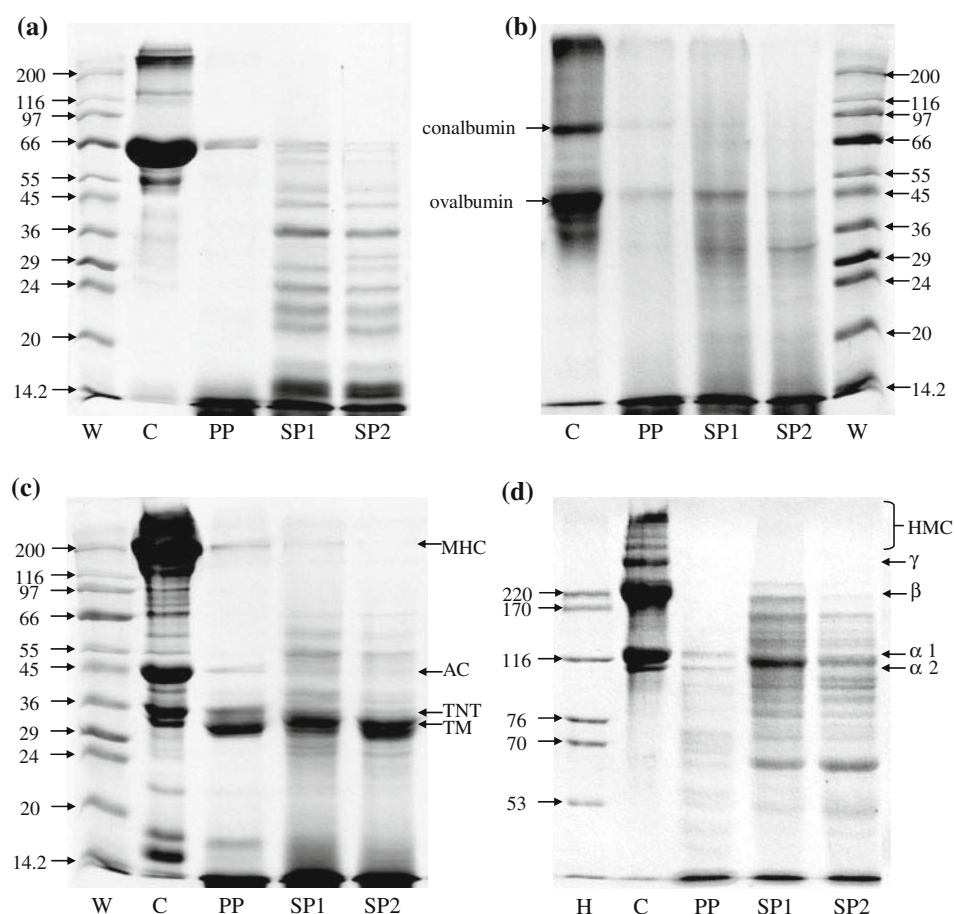
Kinetic constants, K_m and k_{cat} , for hydrolysis of hemoglobin of purified pepsins from the stomach of skipjack tuna were determined from Lineweaver–Burk plot (Table 3). The apparent K_m of pepsins 1 and 2 were estimated to be 54 and 71 μM , respectively. K_m of pepsin 1 was lower than

that of pepsin 2 ($p < 0.05$), suggesting that pepsin 1 had the higher affinity to hemoglobin, compared with pepsin 2. The turnover numbers (k_{cat}) of pepsins 1 and 2 were 38.1 and 44.3 s^{-1} , respectively. The relatively higher k_{cat} value obtained for pepsin 2 indicated that this isoenzyme could contribute to a higher rate of hemoglobin hydrolysis than pepsin 1. The specificity constants (k_{cat}/K_m) of pepsins 1 and 2 were 0.71 and 0.63 $\mu\text{M}^{-1} \text{s}^{-1}$, respectively, indicating that pepsin 1 had the greater catalytic efficiency for hemoglobin hydrolysis than pepsin 2.

Hydrolysis of different protein substrates by pepsins

The degradation patterns of different protein substrates including BSA, egg white, NAM, and ASC hydrolyzed by pepsin from porcine stomach and pepsins 1 and 2 from the stomach of skipjack tuna stomach at 50 °C for 30 min are shown in Fig. 6. All protein substrates used were susceptible to pepsin hydrolysis. The degree of hydrolysis of those protein substrates depended upon types of pepsin used. BSA was drastically hydrolyzed by pepsins (Fig. 6a). After hydrolysis of BSA by porcine pepsin, only a protein band (66 kDa) was retained, and approximately 89% of BSA was cleaved into small peptides with MW lower than 14.2 kDa. The deg-

Fig. 6 Hydrolysis of BSA (a), egg white (b), natural actomyosin (c), and acid-solubilized collagen (d) by porcine pepsin (PP) and purified pepsin 1 (SP1) and pepsin 2 (SP2) from skipjack tuna stomach at 50 °C for 30 min. W wide range MW protein marker, H high MW protein marker, C control (protein sample incubated without enzyme addition), MHC myosin heavy chain, AC actin, TNT troponin T, TM tropomyosin, HMC high molecular weight components. SDS–PAGEs were performed using 12% separating gel for all protein substrates (a, b, c) except 7.5% separating gel for acid-solubilized collagen (d)



radation patterns of BSA hydrolyzed by pepsins 1 and 2 were compared. The peptide fragments with MW of 40–66, 36, 21–29, and 15 kDa were produced when both pepsins were used. However, BSA was slightly more susceptible to hydrolysis by pepsin 2 than pepsin 1 as evidenced by the lower band intensity of proteins with MW of 40–66 kDa. Two major protein components in egg white, conalbumin, and ovalbumin were also hydrolyzed by pepsins (Fig. 6b). Conalbumin was completely hydrolyzed by both pepsins from skipjack tuna, whereas ovalbumin was more resistant to hydrolysis by those pepsins. Although porcine pepsin showed the greater ability to hydrolyze egg white proteins than pepsins 1 and 2, conalbumin and ovalbumin were still retained to some extents. After pepsin digestion of NAM from brownstripe red snapper muscle, a substantial decrease in myosin heavy chain (MHC) and actin with the concomitant formation of degradation products with MW range of 29–35 kDa as well as those having MW lower than 14.2 kDa was observed (Fig. 6c). MHC and actin are the main components of myofibrillar proteins in NAM, followed by troponin and tropomyosin, respectively. The result revealed that MHC and actin were more susceptible to pepsins 1 and 2 digestion, compared with porcine pepsin treatment as indicated by slightly higher remaining MHC and actin after hydrolysis by the latter. Troponin T and tropomyosin were also hydrolyzed to some extent. Degradation patterns of ASC from the skin of arabesque greenling digested by different pepsins are shown in Fig. 6d. After pre-heated at 50 °C for 10 min, ASC was readily hydrolyzed by all pepsins tested, particularly by porcine pepsin. No high molecular weight component (HMC), γ and β chains were retained in ASC after skipjack tuna pepsins digestion. The α -chains were also hydrolyzed to a high degree. Coincidentally, peptide fragment with MW of 62.6 kDa and other degradation peptides were produced. The α -components (α_1 and α_2) of ASC were almost completely hydrolyzed by porcine pepsin. These results revealed that cross-linked components (HMC, β -, and γ -components) as well as α -components of ASC could be hydrolyzed by pepsins from skipjack tuna stomach as well as porcine pepsin. However, the hydrolysis of all protein substrates by porcine pepsin was more pronounced than pepsins from skipjack tuna stomach. Additionally, pepsin 2 exhibited slightly higher effectiveness for hydrolysis of those protein substrates than pepsin 1. Therefore, skipjack tuna pepsins can be used for hydrolysis of proteins for further application, especially for the production of protein hydrolysates.

Conclusion

Pepsins 1 and 2 from the stomach of skipjack tuna were purified to homogeneity. Based on biochemical characteristics, they were consistent with those of pepsins from other

species. Skipjack tuna pepsins could be effectively used for hydrolysis purposes for food processing and other applications.

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Biochemical properties of pepsinogen and pepsin from the stomach of albacore tuna (*Thunnus alalunga*)

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ABSTRACT

Pepsinogen (PG) from the stomach of albacore tuna (*Thunnus alalunga*) was purified to homogeneity by using a series of chromatographies involving Sephacryl S-200HR, Sephadex G-50 and DEAE-cellulose with a 658-fold increase in purity. Based on the native-PAGE and zymography, PG showed a single band with pepsin activity. Molecular weights (MW) of PG and active pepsin were estimated to be 39.9 and 32.7 kDa as determined by SDS-PAGE, respectively. PG was converted to the corresponding pepsin through an intermediate form (MW $\approx$ 36.8 kDa) and the complete activation was observed after 30–60 min. The N-terminal amino acid sequence of the first 15 amino acids of activation segment of pepsinogen was FHKLPLIKGKTAREE. The optimal pH and temperature for pepsin activity were 2.0 and 50 °C, respectively. The activity was stable in the pH range of 2–5. Residual activity more than 85% was found after heating at temperatures up to 50 °C for 30 min. Pepsin activity was strongly inhibited by pepstatin A, whilst E-64, ethylenediaminetetraacetic acid (EDTA) and soybean trypsin inhibitor exhibited the negligible effect. SDS and cysteine also showed inhibitory effects, whilst ATP, molybdate, NaCl and CaCl₂ had no impact on pepsin activity.

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1. Introduction

Pepsin is the major digestive enzyme in stomach of animals, which is secreted as pepsinogen from chief cells of oxyntic glands located in the stomach wall epithelium (Kageyama, 2002). In acidic environment, pepsinogen rapidly converts to pepsin (Kageyama, 2002). During this activation reaction, both prosegment (activation segment) and the active enzyme undergo the conformational changes, and the proteolytic cleavage of the prosegment can occur in one or more steps by either an intra or intermolecular reaction (Richter, Tanaka, & Yada, 1998). Pepsin and pepsinogen from stomach of various fish species such as polar cod (Arunchalam & Haard, 1985), North Pacific bluefin tuna (Tanji, Kageyama, & Takahashi, 1988), Atlantic cod (Gildberg, Olsen, & Bjarnason, 1990), sea bream (Zhou, Fu, Zhang, Su, & Cao, 2007), African coelacanth (Tanji et al., 2007), pectoral rattail (Klomklao, Kishimura, Yabe, & Benjakul, 2007), smooth hound (Bougatef, Balti, Zaied, Souissi, & Nasri, 2008), Mandarin fish (Zhou et al., 2008) and European eel (Wu et al., 2009b) have been purified and characterised.

Albacore tuna (*Thunnus alalunga*), also known as longfin tuna, is recognised by its remarkably long and slender pectoral fins, about 30% of its fork length (Wu, Chiang, Chen, Hsu, & Yang, 2009a). It

has a high commercial value and has served as an important raw material for the production of canned tuna in Thailand. During canned tuna manufacturing, a high amount of viscera containing various proteases is generated. Stomach proteases, especially pepsin, can be recovered and used, mainly for hydrolysis purposes. Fish pepsin has been used for preparation of protein hydrolysate (Pavli-sko, Rial, De Vecchi, & Coppes, 1997) and for extraction of collagen (Nalinanon, Benjakul, Visessanguan, & Kishimura, 2007) and gelatin (Nalinanon, Benjakul, Visessanguan, & Kishimura, 2008a). Recently, stomach extract from albacore tuna has been used as the potential aid for collagen extraction from the skin of threadfin bream (Nalin-anon, Benjakul, Visessanguan, & Kishimura, 2008b). Therefore, alba-core stomach can be used as a promising source of pepsin for further applications. However, no information regarding the biochemical characteristics of pepsinogen and pepsin from the stomach of alba-core tuna has been reported. Therefore, the aims of this study were to purify pepsinogen from albacore tuna stomach and to characterise some biochemical properties of the corresponding pepsin.

2. Materials and methods

2.1. Chemicals

Bovine haemoglobin, β -mercaptoethanol (β ME), L-cysteine, adenosine 5'-triphosphate (ATP), molybdate, pepstatin A, ethylene-

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diaminetetraacetic acid (EDTA), soybean trypsin inhibitor, 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64) and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Folin–Ciocalteu's phenol reagent was obtained from Merck (Darmstadt, Germany). Sephacryl S-200HR and Sephadex G-50 were procured from Pharmacia Biotech (Uppsala, Sweden). Diethylaminoethyl (DEAE)-cellulose was obtained from Whatman (Maidstone, England). 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. Preparation of fish stomach

Internal organs of albacore tuna (*T. alalunga*) were obtained from Tropical Canning (Thailand) Public Co., Ltd., Songkhla, Thailand. The samples were packed in polyethylene bag, kept in ice with a solid/ice ratio of 1:2 (w/v) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon the arrival, internal organs were excised and only stomach was collected and placed in polyethylene bag. The collected stomach was stored at -20°C until use.

2.3. Preparation of crude extract

Frozen stomach was thawed using running water ($26\text{--}28^{\circ}\text{C}$) until the core temperature reached -2 to 0°C . The sample was cut into pieces with a dimension of $0.5 \times 0.5\text{ cm}^2$. Sample was finely ground in liquid nitrogen using a National Model MX-T2GN blender (Taipei, Taiwan). Sample was then lyophilised using an EYELA FDU-1200 freeze-dryer (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) and the dry powder was referred to as 'stomach powder'.

To prepare the crude extract, stomach powder was suspended in 20 mM sodium phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) at a ratio of 1:9 (w/v) and stirred continuously at 4°C for 3 h. The suspension was centrifuged for 20 min at 4°C at 20,000 g (H-2000B, Kokusan, Tokyo, Japan) to remove the tissue debris. The supernatant was collected and lyophilised. Before used, the dry extract powder (10 g) was dissolved with 60 mL of 20 mM sodium phosphate buffer (pH 7.0) referred to as 'starting buffer; SB', followed by centrifugation at 4°C for 10 min at 10,000 g. The supernatant was collected and referred to as "crude extract".

2.4. Purification of albacore tuna pepsinogen

All steps of purification were carried out at 4°C . Crude extract was applied onto Sephacryl S-200HR column ($3.9 \times 70\text{ cm}$), previously equilibrated with approximately two bed volumes of SB. Elution was carried out using the same buffer at a flow rate of 0.5 mL/min. Fractions of 4.5 mL were collected and those with proteolytic activity were pooled and lyophilised. Lyophilised Sephacryl S-200HR fraction was dissolved in SB prior to loading onto Sephadex G-50 (Pharmacia Biotech, Uppsala, Sweden) ($3.9 \times 70\text{ cm}$) column equilibrated with approximately two bed volumes of SB. The proteases were eluted with SB at the flow rate of 0.5 mL/min and fractions of 4.5 mL were collected. Those fractions with proteolytic activity were pooled and lyophilised. Lyophilised Sephadex G-50 fraction was dissolved in distilled water and dialysed against 60 volumes of SB for 12 h. The dialysate was then subjected to DEAE-cellulose (Whatman, Maidstone, England) column ($2.2 \times 35\text{ cm}$) equilibrated with SB. After the column was washed with SB until A_{280} was below 0.05, the elution was performed with a linear gradient of 0–0.75 M NaCl in SB at a flow rate of 0.5 mL/min. Fractions of 4.5 mL, with proteolytic activity were pooled and dialysed against 60 volumes of SB for 12 h prior to lyophilisation. The lyophilised

fraction was dissolved in SB and re-chromatographed onto Sephadex G-50 column, followed by the elution with SB at the flow rate of 0.5 mL/min. Fractions (2.5 mL) were collected and those with proteolytic activity were pooled and lyophilised. The lyophilised pooled fraction was dissolved and dialysed prior to loading onto DEAE-cellulose column ($2.2 \times 35\text{ cm}$) in the same manner described previously. The elution was performed with a linear gradient of 0.2–0.4 M NaCl in SB at a flow rate of 0.25 mL/min. Fractions (2 mL) were collected and those with proteolytic activity were combined and dialysed against 60 volumes of SB for 12 h. Dialysate obtained was lyophilised.

During purification, protein concentration was measured by monitoring the absorbance at 280 nm, and the proteolytic activity was measured using haemoglobin as a substrate at pH 2.0 and 50°C as per the method of Nalinanon et al. (2008b).

2.5. Protein determination

Protein concentration was determined by measuring the absorbance at 280 nm or by the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as a standard.

2.6. Assay of proteolytic activity

Proteolytic activity of purified enzyme was determined using haemoglobin as a substrate according to the method of Nalinanon et al. (2008b) with some modifications. To initiate the reaction, 200 μL of enzyme solution were added into the assay mixture containing 200 μL of 2% haemoglobin, 200 μL of distilled water and 625 μL of reaction buffer. Appropriate dilution was made to ensure that the amount of enzyme was not excessive for available substrate in the assay system. The reaction was conducted at pH 2.0 and 50°C for 20 min. To terminate enzymatic reaction, 200 μL of 50% (w/v) trichloroacetic acid (TCA) were added. Unhydrolysed protein substrate was allowed to precipitate for 1 h at 4°C , followed by centrifuging at 15,000 g for 10 min using a KUBOTA 3630 centrifuge (SiGMA Laborzentrifugen, Osterode am Harz, Germany). The oligopeptide content in the supernatant was measured at 280 nm. One unit of activity was defined as the amount causing an increase of 1.0 in absorbance at 280 nm per min. A blank was run in the same manner, except that the enzyme was added into the reaction mixture after the addition of 50% (w/v) TCA.

2.7. Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed according to the method of Laemmli (1970). Protein solutions were mixed at 1:1 (v/v) ratio with the SDS-PAGE sample buffer (0.125 M Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 10% βME) and boiled for 3 min. The samples (15 μg) were loaded onto the gel made of 4% stacking and 12.5% separating gels and subjected to electrophoresis at a constant current of 15 mA/gel using a AE-6440 electrophoresis 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 gel in a similar manner with SDS-PAGE, except that the sample was not heated and SDS and reducing agent were left out.

2.8. Enzyme zymography

Zymography of purified pepsinogen (PG) from the stomach of albacore tuna was performed as per the method of Diaz-Lopez, Moyano-Lopez, Alarcon-Lopez, Garcia-Carreno, and Navarrete del Toro (1998) and Nalinanon et al. (2008b) with a slight modification. Enzyme solution was applied onto native-PAGE in the same

manner as described previously. Electrophoresis was performed at constant current of 15 mA/gel at 4 °C. After electrophoresis, gels were removed and soaked in 0.1 M HCl to reduce the pH to 2.0 to activate the pepsin. After 15 min, the gels were soaked in haemoglobin solution (0.25% haemoglobin in 0.1 M glycine–HCl, pH 2.0) with constant agitation for 30 min at 4 °C to allow the substrate to penetrate into the gels. The gels were then soaked for 90 min in a fresh haemoglobin solution at 50 °C to develop the activity zone. After development of activity zone, gels were washed using distilled water and fixed for 15 min in 12% TCA solution prior to staining and destaining as described previously. Development of clear zones on blue background indicated proteolytic activity.

2.9. Analysis of amino acid sequence

To analyse the N-terminal sequence, the PG was electroblotted onto polyvinylidenedifluoride (PVDF) membrane (ProBlott®, Applied Biosystems, Foster City, CA, USA) after SDS–PAGE. The amino acid sequence of the enzyme was analysed using a protein sequencer, Procise 492 (Perkin–Elmer, Foster City, CA, USA).

2.10. Biochemical properties

2.10.1. Pepsinogen activation

The conversion reaction from pepsinogen (PG) to pepsin was monitored at various times. PG solution (25 µL) was mixed with 25 µL of 0.5 M glycine–HCl (pH 2.0). The mixture was incubated at 4 °C for different times (0, 0.25, 0.50, 1, 5, 10, 30 and 60 min). At the time designated, 50 µL of SDS–PAGE sample buffer were immediately added to terminate the conversion reaction. The reaction mixtures were then analysed by SDS–PAGE as previously described.

2.10.2. pH and temperature profile

To study pH profile, proteolytic activity of purified enzyme was measured at 50 °C using haemoglobin as a substrate at different pHs (pHs 1.0, 1.5 and 2.0 using 20 mM maleate buffer and pHs 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0 and 7.0 using Mcllvaine's buffer (0.2 M Na-phosphate and 0.1 M Na-citrate)). For temperature profile, the assay was performed at various temperatures (20, 30, 40, 45, 50, 55, 60 and 70 °C) at pH 2.0.

2.10.3. pH and thermal stability

For pH stability study, purified enzyme solution was incubated at pHs 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 using Mcllvaine's buffer at room temperature for 30 min. Residual activity was then determined. For thermal stability, the purified enzyme solution was incubated at different temperatures (20, 30, 40, 50, 60 and 70 °C) for 30 min before rapid cooling using iced water. The residual activity was then assayed using haemoglobin as a substrate at pH 2.0 and 50 °C for 20 min.

2.10.4. Effect of some protease inhibitors

Purified enzyme solution (200 µL) was mixed with 200 µL of single protease inhibitor to obtain the final designated concentration (0.1, 1 and 10 µM for pepstatin A, 0.1 mM for E-64, 0.1 mM for soybean trypsin inhibitor and 2 mM for EDTA). Mixtures were incubated at room temperature for 30 min. The residual activity was measured at pH 2.0 and 50 °C. Relative activity was then calculated.

2.10.5. Effect of some chemicals

Different chemicals (200 µL) were mixed with the enzyme solution (200 µL) to obtain the concentration designated (0.5 mM for ATP and molybdate; 5 mM for NaCl and CaCl₂; 0.05% (w/v) for SDS and 50 mM for L-cysteine). The mixtures were kept at room

temperature for 30 min. The remaining activity was determined at pH 2 and 50 °C and the relative activity was calculated.

2.11. Statistical analysis

All experiments were run in triplicate. Data were subjected to Analysis of Variance (ANOVA) and mean comparisons were carried out using Duncan's multiple range test. SPSS statistic programme (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA) was used for data analysis.

3. Results and discussion

3.1. Purification of pepsinogen

Purification of pepsinogen from the stomach of albacore tuna is summarised in Table 1. The purification of pepsinogen was started by separating the crude extract using Sephacryl S-200HR column. After separation, the fractions with pronounced proteolytic activity were pooled and subjected to further purification. Purity of 2-fold was observed in Sephacryl S-200HR fraction. A single activity peak was obtained for this fraction upon the subsequent chromatography on Sephadex G-50 (data not shown). The yield of 25% and the purity of 168-fold were observed after gel filtration using Sephadex G-50 column. The pooled activity fractions were then subjected to ion-exchange chromatography using DEAE-cellulose. Ion-exchange chromatography was used to remove contaminating proteins and to separate different pepsinogen and pepsin isoforms (Klomklao et al., 2007; Tanji et al., 1988; Zhou et al., 2007). Pooled fractions from single activity peak obtained after DEAE-cellulose chromatography showed high purity of 513-fold with 17% yield. The results showed that Sephadex G-50 and DEAE-cellulose chromatographies effectively removed the contaminants from the crude extract. However, some protein contaminants were still retained as examined by PAGE (data not shown). In order to completely remove those contaminants, the selected fractions were re-chromatographed onto Sephadex G-50 and DEAE-cellulose columns, respectively. After the final purification step, purification fold of 658 with a yield of 0.5% and a specific activity of 13.8 U/mg of protein were obtained.

3.2. Molecular weight of pepsinogen

Native-PAGE and zymography of purified PG are shown in Fig. 1a and b, respectively. PG showed a single protein band on

Table 1
Purification of pepsinogen from the stomach of albacore tuna.

Purification step	Total protein (mg)	Total activity (U)*	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	9989	1235	0.124	1	100
Step 1: Gel filtration Sephacryl S-200	1850	475	0.257	2	39
Step 2: Gel filtration Sephadex G-50	87	308	3.53	168	25
Step 3: Anion-exchange DEAE-cellulose	19	205	10.8	513	17
Step 4: Gel filtration Sephadex G-50	10	129	12.8	608	10
Step 5: Anion-exchange DEAE-cellulose	0.42	5.9	13.8	658	0.5

* One unit of activity was defined as the amount causing an increase of 1.0 in absorbance at 280 nm per min. Pepsin activity was assayed at pH 2.0, 50 °C for 20 min using haemoglobin as a substrate.

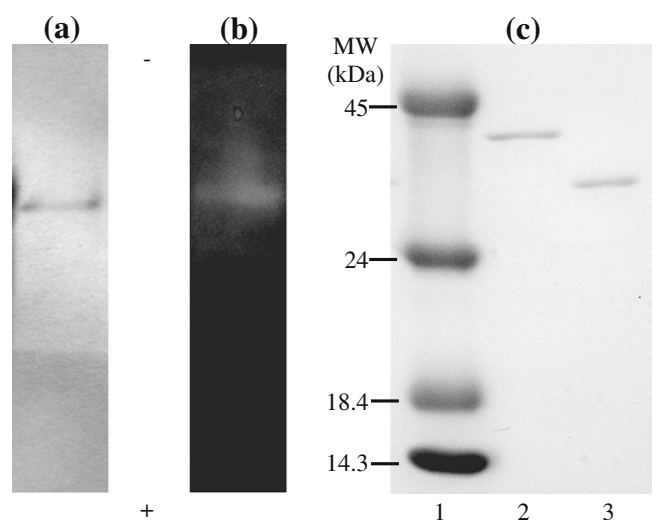


Fig. 1. Native-PAGE of purified pepsinogen (PG) from the stomach of albacore tuna (a), zymogram of albacore tuna pepsin (b) and SDS-PAGE of PG and pepsin (c). Lane 1: standard protein markers; lane 2: purified pepsinogen; lane 3: pepsin.

native-PAGE. Coincidentally, only one activity zone on the zymogram was noticeable, indicating the purity of the enzyme with pepsin activity developed under acidic condition used for activity staining. PG and its corresponding active form, pepsin, also exhibited a single band on SDS-PAGE under reducing condition (Fig. 1c). Molecular weights (MW) of PG and pepsin estimated from the mobilities in SDS-PAGE were 39.9 and 32.7 kDa, respectively. The results suggested that albacore pepsin is the monomeric protein. The MW of PG from albacore tuna was similar to those from polar cod (Arunchalam & Haard, 1985), North Pacific bluefin tuna (Tanji et al., 1988) and smooth hound (Bougatef et al., 2008). A slightly lower MWs of PGs from other fish species has been reported such as sea bream (Zhou et al., 2007), mandarin fish (Zhou et al., 2008), and European eel (Wu et al., 2009b), which had MW ranging from 32 to 38 kDa. After activation of PGs, the MWs of the corresponding pepsins from those fish species ranged from 29 to 32 kDa.

3.3. Activation of pepsinogen

Activation of PG was carried out at pH 2 and 4 °C and was monitored by the release of prosegment with the concomitant formation of pepsin (Fig. 2). Conversion of pepsinogens to pepsins proceeds autocatalytically at acidic pH by two different pathways, a one-step pathway to release the intact activation segment (prosegment) directly, and a stepwise pathway through a pseudopepsin(s) (Kageyama, 2002). PG from albacore tuna stomach was

converted to be active pepsin by a two-step process through an intermediate form (pseudopepsin), which had MW of 36.8 kDa. Tanji et al. (2007) found that the MWs of intermediate form of PG1 and PG2 from African coelacanth were 34.1 kDa. The generation of the intermediate form rapidly occurred in a few second during activation. The complete activation was observed after 30–60 min. Activation of PG by two-step process has been reported for PG2 from North Pacific bluefin tuna (Tanji et al., 1988), PG-I from sea bream (Zhou et al., 2007), PG1 and PG2 from African coelacanth (Tanji et al., 2007), PG-I and PG-II from Mandarin fish (Zhou et al., 2008), as well as PG-I and PG-III from European eel (Wu et al., 2009b). However, the direct conversion of pepsinogen to pepsin in one-step process was also reported for PG3 from North Pacific bluefin tuna (Tanji et al., 1988) and PG from smooth hound (Bougatef et al., 2008). In pepsinogen, the prosegment covers the active-site cleft, and at neutral or weakly alkaline conditions, the inactive zymogen is stabilised through electrostatic interactions between basic amino acid residues in the prosegment and acidic residues in the enzyme moiety of the molecule (Nielsen & Foltmann, 1993). In acidic condition, most carboxyls of pepsinogen are protonated, resulting in weakening of electrostatic interactions. The conformational changes also occur in the activation segment plus the first 13 residues of the pepsin moiety (Kageyama, 2002; Sielecki, Fujinaga, Read, & James, 1991). The active site is then exposed and the pepsinogen molecule cleaves off its own activation segment automatically (Kageyama, 2002).

3.4. N-terminal amino acid sequence of pepsinogen

The N-terminal amino acid sequence of PG from the stomach of albacore tuna was aligned to compare with those of others pepsinogens as depicted in Fig. 3. The N-terminal amino acid sequence of the first 15 amino acids of activation segment of PG was determined to be FHKLPLIKGKTAREE (Fig. 3). PG from albacore tuna had the similar N-terminal amino acid sequence to PG2 from North Pacific bluefin tuna (Tanji et al., 1988). The highly conserved amino acid sequence between PGs from albacore tuna and North Pacific bluefin tuna suggested that they were genetically evolved from a common ancestor. PG from albacore tuna showed the sequence homology of 60% and 40%, compared with PG2 from Mandarin fish (Zhou et al., 2008) and African coelacanth (Tanji et al., 2007), respectively. On the other hand, relatively low identities to PGs from smooth hound (Bougatef et al., 2008) and porcine (Nielsen & Foltmann, 1995; Ong & Perlmann, 1968) were observed. The first 15 amino acid of activation segment of PG from albacore tuna contained 5 basic amino acid residues including His2, Lys3, Lys8, Lys10 and Arg13. Basic amino acid residues at positions 3, 8, 10, 13 and 36 in the activation segment have been shown to be important in the stabilization of pepsin moiety at neutral pH (Bougatef et al., 2008; Richter et al., 1998). Therefore, the differences in those basic amino acids between various pepsinogens may govern a

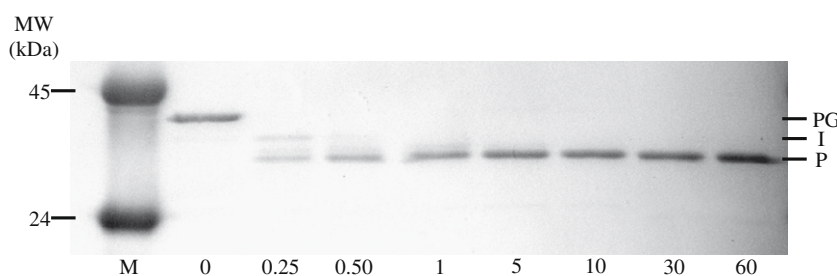


Fig. 2. Time courses of the activation of purified pepsinogen (PG) from the stomach of albacore tuna. The activation was carried out at pH 2.0 and 4 °C. The conversion reactions were terminated at the time designated and subjected to SDS-PAGE. M: standard protein markers; PG: pepsinogen; I: intermediate; P: pepsin. The numbers denote the time of activation (min).

	1				5	10				15					
Albacore tuna	F	H	K	L	P	L	I	K	G	K	T	A	R	E	E
Bluefin tuna 2	F	H	K	L	P	L	I	K	G	K	T	A	R	E	E
Coelacanth 1	I	A	K	V	P	L	I	K	E	X	P	L	R	A	I
Coelacanth 2	I	A	K	V	P	L	I	K	E	X	P	L	R	A	I
Coelacanth 3	L	I	S	V	P	L	K	R	F	X	S	I	R	E	S
Bluefin tuna 3	-	I	N	V	P	L	T	R	H	K	S	M	R	E	S
Smooth hound	L	L	R	V	P	L	R	K	G	K	S	T	L	D	V
Bluefin tuna 1	L	L	Q	V	P	L	E	K	G	Q	S	A	R	E	Y
Madarin PG-I	L	K	Q	V	P	L	E	K	G	K	T	A	-	-	-
Madarin PG-II	L	I	Q	V	P	L	E	K	G	K	T	A	R	E	L
Porcine A	L	V	K	V	P	L	V	R	K	K	S	L	R	Q	N
Porcine B	M	E	R	I	I	L	R	K	G	K	S	I	R	E	A

Fig. 3. Comparison of N-terminal amino acid sequence of purified pepsinogen (PG) from albacore tuna with other pepsinogens: North Pacific bluefin tuna 1, 2 and 3 (Tanji et al., 1988); coelacanth 1, 2 and 3 (Tanji et al., 2007); smooth hound (Bougatef et al., 2008); mandarin PG-I and PG-II (Zhou et al., 2008); porcine A (Ong and Perlmann, 1968); porcine B (Nielsen & Foltmann, 1995). Amino acid residues different from albacore tuna pepsinogen are boxed.

conformational change in the activation segment, resulting in the differences in stability and the activation mechanism of the enzyme.

3.5. pH and temperature profile

The effect of pH on the activity of pepsin from the stomach of albacore tuna was determined over a pH range of 1.0–7.0 as shown in Fig. 4a. The pepsin was highly active between pH 1.5–3.0 with a relative activity more than 80%. The optimal pH for hydrolysis of haemoglobin by pepsin was found at pH 2.0, which was in accordance with those of pepsins obtained from the stomach of bonito (Kubota & Ohnuma, 1970), polar cod (Arunchalam & Haard, 1985), African coelacanth (Tanji et al., 2007), smooth hound

(Bougatef et al., 2008), skipjack tuna and tongol tuna (Nalinanon et al., 2008b). Nevertheless, the higher optimal pHs (2.5–3.5) of pepsins from other fish species including North Pacific bluefin tuna (Tanji et al., 1988), sea bream (Zhou et al., 2007), pectoral rattail (Klomklao et al., 2007), Madarin fish (Zhou et al., 2008) and European eel (Wu et al., 2009b), have been reported. Activity of pepsin generally decreased at pHs higher and lower than the optimal pH. This was possibly due to the conformational changes of enzyme under harsh condition, resulting in the lowered activity (Benjakul, Visessanguan, & Tanaka, 2003; Nalinanon et al., 2008b).

Temperature profile of pepsin from the stomach of albacore tuna is depicted in Fig. 4b. The optimal temperature of pepsin was 50 °C when haemoglobin was used as a substrate. This optimal temperature was higher than those of pepsins obtained from

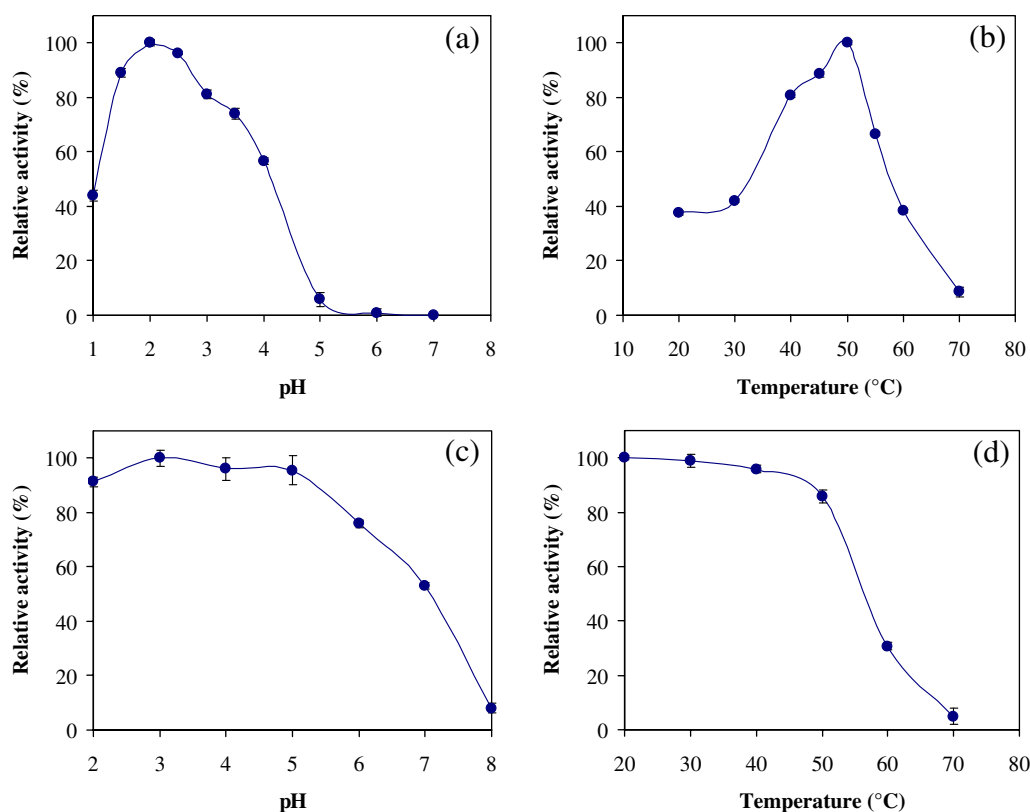


Fig. 4. pH (a) and temperature (b) profiles and pH (c) and thermal (d) stability of pepsin from the stomach of albacore tuna. Bars represent the standard deviation ($n = 3$).

marine subtropical fish, warm water fish and cold water fish, such as smooth hound (40 °C) (Bougatef et al., 2008), sea bream (45 °C for P-I) (Zhou et al., 2007) and polar cod (37 °C) (Arunchalam & Haard, 1985) and pectoral rattail (45 °C) (Klomklao et al., 2007), respectively. The varying optimal temperatures of pepsin from fresh water fish including Madarin fish (40–45 °C) (Zhou et al., 2008) and European eel (35–40 °C) (Wu et al., 2009b) have been reported. The differences in optimal temperature might be associated with the differences in enzyme conformation as governed by habitat, environment and genetics (Gildberg, 1988). A sharp decrease in activity of albacore pepsin at temperature above 50 °C was mostly likely owing to thermal denaturation.

3.6. pH and thermal stability

The pH stability of pepsin from the stomach of albacore tuna is shown in Fig. 4c. Albacore tuna pepsin was stable in the pH range of 2–5, in which the residual activity more than 85% was found. The marked decrease in activity was noticeable at pH above 5. Similar results were reported for orange roughy pepsins (Xu, Wong, Rogers, & Fletcher, 1996), Monterey acidic enzymes (Castillo-Yanez, Pacheco-Aguilar, Garcia-Carreno, & Toro, 2004) and pectoral rattail pepsins (Klomklao et al., 2007). Those enzymes were stable at pH range of 2–6 and became susceptible to activity loss at neutral and alkaline pH. Tanji et al. (2007) found that pepsin from African coelacanth at pH 4.5 had 30% of the maximal activity observed at pH 2. Pepsins from cold and temperate water fish are stable in the pH range of 2–5 (Gildberg, 1988), whereas pepsins from warm water species such as bonito are quite stable even under neutral conditions (Kubota & Ohnuma, 1970). The differences in pH stability indicated the different molecular properties including bondings stabilising the structure as well as enzyme conformation amongst various species and anatomical location (Klomklao et al., 2007).

Thermal stability of pepsin from the stomach of albacore tuna is shown in Fig. 4d. Albacore tuna pepsin was stable up to 50 °C with the residual activity more than 85%. Nevertheless, the sharp decrease in activity was noticeable at temperature above 50 °C. Relatively low activity was remained at 70 °C, suggesting the drastic loss in activity caused by thermal denaturation of pepsin. The result is in agreement with pepsin from dogfish stomach, which was stable at temperature below 50 °C (Guerard & Le Gal, 1987). At high temperatures, enzyme possibly underwent denaturation and lost its activity. Klomklao et al. (2007) reported that the activity of two pepsins purified from pectoral rattail was stable up to 40 °C and unstable at higher temperatures. Potential number of disulphide linkages, the average hydrophobicity and the amount of intramolecular hydrogen bonds are considered to affect thermostability of enzymes (Gildberg et al., 1990). Tello-Solis and Romero-Garcia (2001) observed the aggregation of porcine pepsin at temperature higher than 50 °C. Therefore, the irreversible loss in pepsin activity might be associated with thermal aggregation.

3.7. Effect of protease inhibitors and chemicals

Effects of various inhibitors and some chemicals on proteolytic activity of pepsin from the stomach of albacore tuna were determined as shown in Table 2. The proteolytic activity was strongly inhibited by pepstatin A with the concentration range of 0.1–10 µM. E-64, which is specific inhibitor to cysteine proteinase, EDTA, a metalloproteinase inhibitor and soybean trypsin inhibitor, the inhibitor specific to serine proteinase, had no effect on the activity. Pepstatin A, a pentapeptide, is very specific inhibitor, which is able to interact noncovalently with the active site of aspartic proteinases and blocks the accessibility of a substrate to the active-site cleft (Fusek & Větvíčka, 1995; Haard, 1994). Guerard and Le Gal (1987) reported that the activity of dogfish pepsin II was

Table 2

Effect of various inhibitors and some chemicals on the activity of pepsin from the stomach of albacore tuna.^a

Inhibitors/chemicals	Concentration	Relative activity (%) ^b
Control		100
Pepstatin A	0.1 µM	5.27 ± 0.2
	1 µM	ND
	10 µM	ND
E-64	0.1 mM	99.83 ± 0.8
EDTA	2 mM	96.26 ± 1.1
Soybean trypsin inhibitor	0.1 mM	100.0 ± 0.3
ATP	0.5 mM	100.7 ± 4.1
Molybdate	0.5 mM	100.0 ± 5.9
NaCl	5 mM	101.4 ± 4.3
CaCl ₂	5 mM	102.8 ± 4.1
SDS	0.05% (w/v)	ND
Cysteine	50 mM	23.98 ± 5.2

ND = not detectable.

^a Enzyme solution was incubated with the same volume of inhibitors or chemicals at 25 °C for 30 min and the residual activity was determined using haemoglobin as a substrate for 20 min at pH 2.0 and 50 °C.

^b Mean ± SD from triplicate determinations.

progressively reduced to about 50% of its initial value when pepstatin A at a level of 6×10^{-7} M was added, whilst a total inhibitory effect was obtained at 10^{-6} M. Pepsins from the stomach of skipjack tuna and tongol tuna were completely inhibited by 1 µM pepstatin A (Nalinanon et al., 2008b). Klomklao et al. (2007) reported that the activity of pepsins from the stomach of pectoral rattail was almost inhibited by 0.01 mM pepstatin A. Therefore, the inhibitory results confirmed that purified enzyme was aspartic proteinase, most likely pepsin.

ATP and molybdate, which are activator and inhibitor of cathepsin D, respectively (Pillai & Zull, 1985), had no profound effect on the activity (Table 2). The result was in accordance with Klomklao et al. (2007) who reported that ATP and molybdate had no impact on the activity of pepsin from pectoral rattail (Klomklao et al., 2007), skipjack tuna and tongol tuna (Nalinanon et al., 2008b). Pillai and Zull (1985) found that cathepsin D was generally activated by ATP when bovine serum albumin, haemoglobin, parathyroid hormone and a synthetic octapeptide were used as substrates, whereas pepsin, a homologous aspartic proteinase, was not activated. NaCl and CaCl₂ also had no impact on the activity of pepsin. Sanchez-Chiang, Cisternas, and Ponce (1987) found that the activity of pepsin I from adult salmon (*Oncorhynchus keta*) towards haemoglobin was increased by approximately 20% in the presence of 5 mM NaCl, whereas the activity of pepsin II was not affected. However, the activities of cod pepsin and porcine pepsin were strongly depressed in the presence of NaCl at the concentration of 5% and 10% (Gildberg et al., 1990). Klomklao et al. (2007) found that the activity of both pepsins A and B from pectoral rattail was increased in the presence of 5 mM CaCl₂, 5 mM MgCl₂ or 5 mM CoCl₂. Salts possibly altered the conformation of either pepsin or the substrate used in the way, which impeded or enhanced the binding ability between enzyme and substrate. Albacore pepsin was strongly inhibited by SDS and cysteine. Nalinanon et al. (2008b) found that cysteine, a reducing agent, inhibited the pepsin activity of tuna stomach extracts in a dose dependent manner via breaking disulphide bonds of pepsin. Furthermore, SDS, an anionic surfactant, could act as a denaturant by disrupting non-covalent bonds stabilizing the pepsin structure.

4. Conclusions

Pepsinogen from the stomach of albacore tuna was purified to homogeneity. On the basis of biochemical properties, pepsinogen was activated to pepsin at the acidic pH. Resultant pepsin exhibited

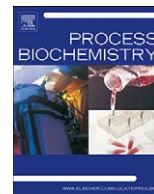
the proteolytic activity similar to other fish pepsins, particularly from tropical fish. Albacore pepsin could be used as a biotechnological alternative for food processing and other applications.

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Partitioning of protease from stomach of albacore tuna (*Thunnus alalunga*) by aqueous two-phase systems

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ABSTRACT

Partitioning of protease from stomach of albacore tuna using an aqueous two-phase system (ATPS) was investigated. The best ATPS conditions for protease partitioning from stomach extract (SE) and acidified counterpart (ASE) were 25% PEG1000–20% MgSO₄ and 15% PEG2000–15% MgSO₄, which increased the purity by 7.2-fold and 2.4-fold with the recovered activity of 85.7% and 89.1%, respectively. Electrophoretic study revealed that SE had a major protein with a molecular weight (MW) of 40.6 kDa, while protein with MW of 32.7 kDa was predominant in ASE and ATPS fractions. Pepsinogen in SE might be activated to pepsin by acidification and partitioning process. SE was quite stable at 0 and 4 °C up to 14 days. The loss in protease activity in ASE and selected ATPS fractions was more pronounced when storage time and temperature increased. Therefore, ATPS can be effectively used to recover and purify protease from albacore tuna stomach.

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1. 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]. Thus, high amounts of processing wastes such as viscera, head, and bone are discarded during canned tuna manufacturing. Fish viscera is a potential source for recovering enzymes such as proteinases that may have some unique properties for industrial applications, e.g. in the detergent, food, pharmaceutical, leather and silk industries [2]. Stomach protease, especially pepsin can be recovered and used, mainly for hydrolysis purposes. Fish pepsin has been used for preparation of protein isolate [3], extraction of collagen [1,4] 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. Partitioning in aqueous two-phase system (ATPS) has been shown to be powerful for separating and purifying the mixtures of proteins [6,7]. ATPS forms readily upon

mixing aqueous solutions of two hydrophilic polymers, or of a polymer and a salt, above a certain threshold concentration [8,9]. In general, ATPS yields a specific environment suitable for maintaining enzymes in their native structure and for concentration/purification by means of selective partitioning of the enzyme to one of the phases [7]. Coincidentally, ATPS can remove undesirable enzymes/proteins, unidentified polysaccharides and pigments that are present in the system [7,10]. ATPS can be scaled up and has the possibility for continuous steady state operation. Additionally, the inexpensive materials that form this system can be recycled [8]. Aqueous two-phase partitioning is a potentially useful clarification or partial purification technique for a number of reasons: (1) it is a gentle method, having little or no adverse effect on the biological activity of most proteins; (2) many of the polymers used exhibit protein-stabilizing properties; (3) the yield of protein recovered is generally high and (4) little technical difficulties arise during process scale-up [11]. ATPS has been applied for partitioning and recovery of various proteases such as trypsin [9,12], α -chymotrypsin [9], chymosin, pepsin [8], *Mucor bacilliformis* acid protease [13], *Bacillus subtilis* neutral protease [14] and tuna spleen proteinase [2]. Therefore, the objective of this study was to investigate the feasibility of utilizing ATPS for partitioning and recovery of protease from the stomach of albacore tuna.

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2. Methods

2.1. Chemicals

Polyethylene glycol (PEG) 1000 was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). PEG2000, PEG4000, trichloroacetic acid, tris (hydroxymethyl) aminomethane and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Hemoglobin, β -mercaptoethanol (β ME), L-tyrosine, wide range molecular weight markers and Coomassie Brilliant Blue G-250 were purchased from Sigma Chemical Co. (St. Louis, MO, USA.). Sodium dodecyl sulfate (SDS) and bovine serum albumin were obtained from Fluka (Buchs, Switzerland). *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). The salts and other chemicals with the analytical grade were procured from Merck (Darmstadt, Germany).

2.2. Fish stomach collection and preparation

Pooled internal organ of albacore tuna (*T. alalunga*) was obtained from Tropical Canning (Thailand) Public Co., Ltd., Songkhla, Thailand. The sample was packed in polyethylene bag, kept in ice with a solid/ice ratio of 1:2 (w/v) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon the arrival, internal organ was excised and only stomach was collected and placed in polyethylene bag. The stomach sample was stored at -20°C until use.

2.3. Preparation of stomach extract

Frozen stomach was thawed using running water ($26\text{--}28^{\circ}\text{C}$) until the core temperature reached -2 to 0°C . The sample was cut into pieces with a thickness of 1–1.5 cm. Sample was rapidly frozen in liquid nitrogen and then finely ground using a National Model MX-T2GN blender (Taipei, Taiwan) to a powder form. Stomach powder was suspended in deionized water at a ratio of 1:9 (w/v). The mixture was stirred continuously at 4°C for 30 min. The suspension was centrifuged for 30 min at 4°C at $8000 \times g$ using a Sorvall Model RC-B Plus refrigerated centrifuge (Newtown, CT, USA) to remove the tissue debris. The supernatant was collected and referred to as "stomach extract (SE)". To activate stomach protease, SE was adjusted to pH 2 with 1 M HCl and the mixture was allowed to stand at 4°C for 30 min [15]. The suspension was centrifuged for 30 min at 4°C at $10,000 \times g$ using a refrigerated centrifuge. The collected supernatant was referred to as "acidified stomach extract (ASE)".

2.4. Enzyme assay

Protease activity of tuna stomach extract was determined using hemoglobin as a substrate according to the method of An et al. [16] and Nalinanon et al. [4] with a slight modification. To initiate the reaction, 200 μl of stomach extract was added into the assay mixture containing 200 μl of 2% hemoglobin, 200 μl of distilled water and 625 μl of reaction buffer. Appropriate dilution was made to ensure that the amount of enzyme was not excessive for available substrate in the assay system. The reaction was conducted at pH 2.0 and 50°C for 20 min. To terminate enzymatic reaction, 200 μl of 50% (w/v) trichloroacetic acid (TCA) were added. Unhydrolyzed protein substrate was allowed to precipitate for 15 min at 4°C , followed by centrifuging at $4725 \times g$ for 10 min using a MIKRO 20 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). The oligopeptide content in the supernatant was determined by the Lowry method [17] using tyrosine as a standard. One unit of activity was defined as that releasing 1 μmol of tyrosine per min ($\mu\text{mol Tyr/min}$). A blank was run in the same manner, except that the stomach extract was added into the reaction mixture after the addition of 50% TCA (w/v). Protein concentration of stomach extract was measured by the method of Lowry et al. [17] using bovine serum albumin as a standard. Specific activity was calculated and expressed as units/mg protein.

2.5. Preparation of aqueous two-phase systems

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

2.5.1. Effect of salts on partitioning of protease in SE and ASE

To study the effect of salts on partitioning of the protease in SE and ASE using ATPS, different salts including $(\text{NH}_4)_2\text{SO}_4$, $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, MgSO_4 and K_2HPO_4 at different concentrations (15, 20 and 25%, w/w) were mixed with 20% PEG1000 in aqueous system. One ml of SE or ASE (1 mg protein/ml) was added into the 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 (Vortex-genie2, G-560E, USA). Phase separation was achieved by centrifugation for 5 min at $2000 \times g$. Top phase was carefully separated using a pasteur pipette and the interface of each tube was discarded. Volumes of the separated phases, top and bottom phases, were measured. Aliquots from each phase were taken for enzyme assay and protein determination.

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

$$\text{SA} = \frac{\text{protease activity}}{\text{protein concentration}}; \quad (\text{unit/mg protein}) \quad (1)$$

the purification factor as

$$\text{PF} = \frac{\text{SA}_e}{\text{SA}_i} \quad (2)$$

where SA_e is the SA of each phase and SA_i is the initial SA of SE or ASE.

The partition coefficient of protein concentration was defined as

$$K_p = \frac{C_T}{C_B} \quad (3)$$

where C_T and C_B are concentrations of protein in top and bottom phase, respectively. The volume ratio as

$$V_R = \frac{V_T}{V_B} \quad (4)$$

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

$$\text{Yield (\%)} = \frac{A_T}{A_i} \times 100 \quad (5)$$

where A_T is total protease activity in top phase and A_i is the initial protease activity of SE or ASE.

Based on purity and recovery yield, the appropriate salt in ATPS rendering the most effective partitioning was chosen for further study.

2.5.2. Effect of molecular weight and concentration of PEG on partitioning of protease in SE and ASE

To study the effect of the concentrations (15, 20 and 25%, w/w) of PEG1000, PEG2000 and PEG4000 on partitioning of protease in SE and ASE, 20% and 15% MgSO_4 were used in the system for SE and ASE partitioning, respectively. Partitioning was performed as described previously. Based on purity and recovery yield, the ATPS rendering the most effective partitioning was chosen for further study.

2.6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of SE, ASE and their selected ATPS fractions was performed according to the method of Laemmli [18]. 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 (0.75 μg protein) were loaded onto 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 Laboratories, Richmond, CA, USA). After electrophoresis, the gel was fixed with fixing solution, containing 40% (v/v) ethanol and 10% (v/v) acetic acid, for 60 min. The gel was then stained overnight with staining solution (0.1% (w/v) Coomassie Brilliant Blue G-250 in 2% (w/v) ortho-phosphoric acid and 10% (w/v) ammonium sulfate prepared at least 24 h prior to mixing with methanol at a ratio of 4:1 (v/v)). Protein patterns were visualized after washing the gel with 1% (v/v) acetic acid for several times to remove Coomassie particles until clear background was observed.

2.7. Storage stability of stomach protease

Protease activity of SE and ASE and their selected ATPS fractions (PSE and PASE, respectively) containing 0.1% (w/v) sodium azide was monitored daily for the first 4 days and thereafter every 2 days up to 2 weeks. The storage temperature studied included 0°C , 4°C and room temperature ($25\text{--}27^{\circ}\text{C}$).

2.8. Protein determination

Protein concentration was measured by the Bradford method [19] using bovine serum albumin as a standard.

2.9. Statistical analysis

All data were subjected to Analysis of Variance (ANOVA) and differences between means were evaluated by Duncan's Multiple Range Test [20]. SPSS statistic program (Version 10.0) (SPSS, 1.2, 1998) was used for data analysis.

3. Results and discussion

3.1. Effect of salts on partitioning of stomach protease in ATPS

Partitioning of SE and ASE from albacore tuna was carried out in several biphasic systems of 20% PEG1000 containing different salts, including $(\text{NH}_4)_2\text{SO}_4$, $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, MgSO_4 and K_2HPO_4 at various

Table 1Effect of phase composition in PEG1000–salt ATPS on partitioning of protease from the stomach of albacore tuna^a.

Phase composition (% w/w)	V_R	K_P	SA	PF	Yield (%)
20% PEG1000–15% (NH ₄) ₂ SO ₄	1.16 ± 0.02 ^{i**}	1.45 ± 0.03 ^d	18.7 ± 0.40 ^e	4.18 ± 0.08 ^e	81.6 ± 0.61 ^f
20% PEG1000–20% (NH ₄) ₂ SO ₄	0.68 ± 0.01 ^c	1.37 ± 0.06 ^d	18.1 ± 0.85 ^e	4.06 ± 0.19 ^e	67.7 ± 0.08 ^b
20% PEG1000–25% (NH ₄) ₂ SO ₄	0.58 ± 0.01 ^a	1.21 ± 0.03 ^c	16.9 ± 0.76 ^d	3.79 ± 0.18 ^d	60.3 ± 0.79 ^a
20% PEG1000–15% Na ₃ C ₆ H ₅ O ₇	ns	ns	ns	ns	ns
20% PEG1000–20% Na ₃ C ₆ H ₅ O ₇	0.85 ± 0.01 ^e	0.81 ± 0.02 ^a	6.12 ± 0.14 ^c	1.37 ± 0.03 ^c	79.4 ± 0.93 ^e
20% PEG1000–25% Na ₃ C ₆ H ₅ O ₇	0.75 ± 0.01 ^d	1.05 ± 0.09 ^b	5.17 ± 0.28 ^b	1.16 ± 0.06 ^b	76.8 ± 0.08 ^d
20% PEG1000–15% MgSO ₄	ns	ns	ns	ns	ns
20% PEG1000–20% MgSO ₄	1.26 ± 0.03 ^b	1.23 ± 0.02 ^c	27.7 ± 0.17 ^g	6.19 ± 0.04 ^g	87.9 ± 0.20 ^g
20% PEG1000–25% MgSO ₄	0.93 ± 0.01 ^f	1.10 ± 0.04 ^b	19.9 ± 0.39 ^f	4.46 ± 0.09 ^f	73.3 ± 1.13 ^c
20% PEG1000–15% K ₂ HPO ₄	1.00 ± 0.02 ^g	1.92 ± 0.04 ^e	4.35 ± 0.22 ^a	0.97 ± 0.05 ^a	80.2 ± 0.78 ^e
20% PEG1000–20% K ₂ HPO ₄	0.65 ± 0.01 ^c	2.17 ± 0.07 ^f	4.30 ± 0.48 ^a	0.96 ± 0.11 ^a	60.8 ± 0.64 ^a
20% PEG1000–25% K ₂ HPO ₄	0.61 ± 0.01 ^b	3.00 ± 0.10 ^g	4.10 ± 0.25 ^a	0.92 ± 0.06 ^a	60.6 ± 0.76 ^a

V_R : volume ratio (upper/lower); K_P : partition coefficient of protein in the upper phase; SA: specific activity (unit/mg protein); PF: purification factor; Yield: activity recovery yield. ns: no phase separation.

^a Means ± S.D. from triplicate determinations.

^{**} Different superscripts in the same column indicate the significant differences ($P < 0.05$).

concentrations (Tables 1 and 2, respectively). After phase separation, two phases were obtained, PEG-rich top phase and salt-rich lower phase. However, no phase separation was observed in the system containing 15% Na₃C₆H₅O₇ and 15% MgSO₄ for partitioning of SE. For ASE partitioning, activity in both phases from the system containing K₂HPO₄ was not detectable, while varying activity was observed in other systems. The result suggested that acidified proteases in ASE were completely denatured under the alkaline pH of PEG/K₂HPO₄ system (pH 8.8). The phase volume ratio (V_R) of all systems tested was generally decreased when salt concentration increased. 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 effects [21]. The formation of a water layer around the cation resulted in a more compact structure with a minor volume of PEG molecule.

For all ATPS studied, the proteases in both SE and ASE were partitioned predominantly in the PEG-rich top phase as evidenced by negligible or no protease activity in bottom phase (data not shown). In general, negatively charged proteins prefer the upper phase in PEG–salt systems, while positively charged proteins normally partition selectively to the bottom phase [2,22–24]. Above the isoelectric point (pI), enzyme/protein polyanions are accepted by PEG-rich phase in ATPS [7,25]. This contrasts with the rejection of inorganic polyanions and may be attributed to an inherent greater separation of point charges on amino acid side-chains over the surface of enzyme polyanions [7]. Such separation enhances the ability of the enzyme to interact with the PEG [7]. Reh et al. [26] reported that the transfer of both proteins, trypsin and α -

chymotrypsin to top phase was associated with negative enthalpic and entropic changes. Hence, stomach proteases, both non-activated and activated, partitioned in the top phase might be negatively charged. These results might be explained by acceptance of enzyme polyanions by PEG-rich phase in ATPS that occurred above pI. Since pepsin, a major acidic protease in stomach has pI less than 1 [27], the enzyme was negatively charged in all partitioning systems, which had pH ranging from 4.6 to 8.8. Xia et al. [28] studied the PEG-pepsin interaction at acidic pHs (1–4), postulating an electrostatic interaction between the protonated carboxyl groups of pepsin and the oxygen ether of PEG by hydrogen bond formation. Therefore, stomach proteases from albacore tuna might be partitioned towards the PEG-rich phase in all systems due to electrostatic interaction between enzyme surface and PEG. In PEG–salt systems, anions and cations distribute themselves differently between the phases depending on their affinity with water. Generally, two phases have to be electrically neutral and iso-osmotic. Thus, an interfacial potential is produced differently which may aid the partitioning of charged biomolecules [22]. SA, PF and activity recovery (% yield) of protease obtained from PEG1000–salt systems depended on types of salt used. The phase system containing MgSO₄ showed superior partitioning affinity for both SE and ASE to those containing other salts. For SE, a phase system containing 20% PEG1000 and 20% MgSO₄ gave the highest SA (27.7 units/mg protein), PF (6.2-fold) and yield (87.9%) ($P < 0.05$). The maximal yield of 81.1% from the system containing 20% PEG1000 and 15% MgSO₄ was obtained for partitioning of ASE ($P < 0.05$). Similar SA and PF were observed in system with other

Table 2Effect of phase composition in PEG1000–salt ATPS on partitioning of acidified protease from the stomach of albacore tuna^a.

Phase composition (% w/w)	V_R	K_P	SA	PF	Yield (%)
20% PEG1000–15% (NH ₄) ₂ SO ₄	0.98 ± 0.02 ^{h**}	1.74 ± 0.12 ^d	11.8 ± 0.49 ^e	1.63 ± 0.07 ^e	60.5 ± 1.49 ^e
20% PEG1000–20% (NH ₄) ₂ SO ₄	0.67 ± 0.01 ^c	1.96 ± 0.25 ^{de}	9.88 ± 0.12 ^c	1.36 ± 0.02 ^c	53.1 ± 0.47 ^c
20% PEG1000–25% (NH ₄) ₂ SO ₄	0.44 ± 0.02 ^a	1.12 ± 0.07 ^{bc}	11.8 ± 0.44 ^e	1.63 ± 0.06 ^e	38.0 ± 0.50 ^a
20% PEG1000–15% Na ₃ C ₆ H ₅ O ₇	1.37 ± 0.01 ^j	0.95 ± 0.03 ^{ab}	10.5 ± 0.15 ^d	1.45 ± 0.02 ^d	75.7 ± 0.39 ^g
20% PEG1000–20% Na ₃ C ₆ H ₅ O ₇	0.89 ± 0.03 ^e	0.77 ± 0.01 ^a	8.69 ± 0.03 ^b	1.20 ± 0.01 ^b	60.0 ± 0.19 ^{de}
20% PEG1000–25% Na ₃ C ₆ H ₅ O ₇	0.73 ± 0.01 ^d	0.76 ± 0.03 ^a	6.41 ± 0.05 ^a	0.88 ± 0.01 ^a	46.7 ± 0.42 ^b
20% PEG1000–15% MgSO ₄	2.52 ± 0.02 ^k	2.21 ± 0.09 ^e	15.9 ± 0.42 ^f	2.19 ± 0.01 ^f	81.1 ± 0.88 ^h
20% PEG1000–20% MgSO ₄	1.16 ± 0.01 ⁱ	2.05 ± 0.29 ^e	16.0 ± 0.16 ^f	2.20 ± 0.05 ^f	62.2 ± 0.21 ^f
20% PEG1000–25% MgSO ₄	0.95 ± 0.02 ^g	1.78 ± 0.27 ^d	16.0 ± 0.06 ^f	2.21 ± 0.01 ^f	59.0 ± 0.13 ^d
20% PEG1000–15% K ₂ HPO ₄	0.91 ± 0.01 ^f	1.73 ± 0.12 ^d	na	na	–
20% PEG1000–20% K ₂ HPO ₄	0.72 ± 0.01 ^d	1.33 ± 0.01 ^c	na	na	–
20% PEG1000–25% K ₂ HPO ₄	0.59 ± 0.01 ^b	0.84 ± 0.04 ^a	na	na	–

V_R : volume ratio (upper/lower); K_P : partition coefficient of protein in the upper phase; SA: specific activity (unit/mg protein); PF: purification factor; Yield: activity recovery yield. na: no protease activity.

^a Means ± S.D. from triplicate determinations.

^{**} Different superscripts in the same column indicate the significant differences ($P < 0.05$).

Table 3Effect of PEG molecular mass and concentration in a PEG–MgSO₄ ATPS on partitioning of protease from the stomach of albacore tuna^a.

Phase composition (% w/w)	V _R	K _P	SA	PF	Yield (%)
15% PEG1000–20% MgSO ₄	1.02 ± 0.01 ^{c**}	0.62 ± 0.02 ^e	22.7 ± 0.63 ^{de}	6.11 ± 0.07 ^d	84.2 ± 0.71 ^e
20% PEG1000–20% MgSO ₄	1.25 ± 0.01 ^e	1.24 ± 0.03 ^f	23.5 ± 0.91 ^e	6.34 ± 0.24 ^e	87.5 ± 0.52 ^{fg}
25% PEG1000–20% MgSO ₄	1.61 ± 0.01 ^h	1.30 ± 0.05 ^f	26.8 ± 0.60 ^f	7.23 ± 0.16 ^f	85.7 ± 0.56 ^{ef}
30% PEG1000–20% MgSO ₄	1.71 ± 0.01 ^j	1.61 ± 0.09 ^g	22.0 ± 0.97 ^d	5.92 ± 0.26 ^d	76.0 ± 1.37 ^a
15% PEG2000–20% MgSO ₄	0.78 ± 0.02 ^b	0.26 ± 0.01 ^{ab}	11.5 ± 0.42 ^b	3.10 ± 0.11 ^b	91.9 ± 1.41 ^{hi}
20% PEG2000–20% MgSO ₄	1.07 ± 0.01 ^d	0.35 ± 0.02 ^{cd}	11.4 ± 0.23 ^b	3.06 ± 0.06 ^b	98.6 ± 1.24 ^k
25% PEG2000–20% MgSO ₄	1.55 ± 0.01 ^g	0.36 ± 0.01 ^d	12.9 ± 0.26 ^c	3.49 ± 0.07 ^c	94.6 ± 0.34 ^j
30% PEG2000–20% MgSO ₄	1.74 ± 0.01 ^k	0.60 ± 0.05 ^e	3.26 ± 0.32 ^a	0.88 ± 0.09 ^a	33.3 ± 0.24 ^b
15% PEG4000–20% MgSO ₄	0.75 ± 0.01 ^a	0.25 ± 0.01 ^a	12.7 ± 0.20 ^c	3.41 ± 0.05 ^c	93.2 ± 0.24 ^{ij}
20% PEG4000–20% MgSO ₄	1.07 ± 0.01 ^d	0.28 ± 0.02 ^{abc}	13.2 ± 0.12 ^c	3.55 ± 0.03 ^c	89.9 ± 0.77 ^{gh}
25% PEG4000–20% MgSO ₄	1.47 ± 0.01 ^f	0.34 ± 0.05 ^{bcd}	3.32 ± 0.31 ^a	0.89 ± 0.08 ^a	50.6 ± 1.43 ^c
30% PEG4000–20% MgSO ₄	1.66 ± 0.01 ⁱ	0.38 ± 0.09 ^d	3.30 ± 0.43 ^a	0.89 ± 0.11 ^a	22.9 ± 3.95 ^a

V_R: volume ratio (upper/lower); K_P: partition coefficient of protein in the upper phase; SA: specific activity (unit/mg protein); PF: purification factor; Yield: activity recovery yield.^a Means ± S.D. from triplicate determinations.^{**} Different superscripts in the same column indicate the significant differences ($P < 0.05$).

MgSO₄ concentrations ($P > 0.05$). Therefore, the systems containing 20% or 15% MgSO₄ were appropriate for partitioning of protease in SE and ASE, respectively.

Partition coefficient (K) is used to characterize the proteins distribution in ATPS. K values for protease and protein partitioning are reported as K_E and K_P , respectively. Since protease was found only in upper phase, K_E of SE and ASE could not be calculated. Generally, the lowest K_P indicates a shift of contaminant proteins, nucleic acid and other undesirable components to the lower phase. When a single component must be extracted from a mixture, phase system compositions are often manipulated in such a manner that the component partitions into one of the phases, while the other components of the mixture partition into the other phase [29]. K_P values varied with types and concentrations of salt used for SE partitioning. Increasing salts concentration for ASE partitioning resulted in lower K_P . Salts might enhance the migration of the proteins to the lower phase by electrostatic repulsion effects [14]. Gündüz and Korkmaz [30] observed that an increase in phosphate concentration caused a decrease in the partition coefficient of negatively charged BSA at pH 7 ($pI = 4.82$). Therefore, the distribution of protein in ATPS of SE and ASE likely depended on the differences in surface charge of protease and others protein contaminants, which were governed by different salts. Han and Lee [14] mentioned that the surface charge of biological materials is one of the most significant factors affecting the separation of partitioning system. Furthermore, molecular weight, shape, hydrophobicity 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 [14]. Tables 1 and 2 show that increasing salt concentration resulted in a less activity recovery. This might be due to the denaturation of proteases caused by “salting out effect”. The same result was reported on partitioning of proteinase from tuna spleen [2]. Antov et al. [31] found that increasing concentration of ammonium sulfate resulted in less recovery yield of xylanase in the top phase.

3.2. Effect of PEG molecular weight on partitioning of stomach protease in ATPS

Partitioning of protease in SE and ASE using ATPS containing PEG with varying molecular weights and concentrations in the presence of 20% MgSO₄ or 15% MgSO₄ for SE and ASE, respectively, are shown in Tables 3 and 4. The results showed that all partitioning parameters in PEG/MgSO₄ systems were influenced by PEG molecular weight. At the same PEG concentration, K_P decreased with increasing PEG molecular weight. A preferential interaction between PEG molecule and protein domain decreased when the molecular weight of PEG increased due to its exclusion from the protein domain [2,29]. Tubio et al. [9] 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 [9]. PEG of the highest molecular weight (PEG8000) exclude the protein from the top phase driven by an entropically unfavorable term [9]. For systems formed by PEG of low molecular weight, the free energy changes adopt negative values (enthalpic changes overcomes entropic changes), while opposite behaviour ($\Delta G_{\text{transfer}} > 0$) occurs from ATPSs formed by PEGs of higher

Table 4Effect of PEG molecular mass and concentration in a PEG–MgSO₄ ATPS on partitioning of acidified protease from the stomach of albacore tuna^a.

Phase composition (% w/w)	V _R	K _P	SA	PF	Yield (%)
15% PEG1000–15% MgSO ₄	ns	ns	ns	ns	ns
20% PEG1000–15% MgSO ₄	2.49 ± 0.01 ^{h**}	2.18 ± 0.07 ^f	15.5 ± 0.98 ^f	2.22 ± 0.14 ^f	79.2 ± 2.31 ^b
25% PEG1000–15% MgSO ₄	2.78 ± 0.02 ^j	2.24 ± 0.05 ^{fg}	14.2 ± 0.11 ^e	2.03 ± 0.02 ^e	79.3 ± 0.13 ^b
30% PEG1000–15% MgSO ₄	2.92 ± 0.01 ^k	2.33 ± 0.03 ^g	11.2 ± 0.55 ^d	1.60 ± 0.08 ^d	74.7 ± 0.35 ^a
15% PEG2000–15% MgSO ₄	1.60 ± 0.01 ^c	0.86 ± 0.07 ^a	17.0 ± 0.69 ^g	2.43 ± 0.10 ^g	89.1 ± 3.03 ^e
20% PEG2000–15% MgSO ₄	1.86 ± 0.02 ^d	1.34 ± 0.05 ^c	15.8 ± 0.03 ^f	2.26 ± 0.01 ^f	96.3 ± 1.24 ^f
25% PEG2000–15% MgSO ₄	2.23 ± 0.01 ^f	2.04 ± 0.04 ^e	10.9 ± 0.23 ^d	1.56 ± 0.03 ^d	97.9 ± 1.01 ^f
30% PEG2000–15% MgSO ₄	2.52 ± 0.01 ⁱ	2.33 ± 0.04 ^g	8.46 ± 0.13 ^b	1.21 ± 0.02 ^b	86.3 ± 1.03 ^d
15% PEG4000–15% MgSO ₄	1.17 ± 0.01 ^a	1.11 ± 0.04 ^b	9.89 ± 0.13 ^c	1.42 ± 0.02 ^c	82.1 ± 1.19 ^c
20% PEG4000–15% MgSO ₄	1.55 ± 0.01 ^b	1.40 ± 0.04 ^c	10.1 ± 0.17 ^c	1.45 ± 0.02 ^c	87.7 ± 0.48 ^{de}
25% PEG4000–15% MgSO ₄	1.95 ± 0.01 ^e	1.91 ± 0.09 ^d	9.45 ± 0.11 ^c	1.35 ± 0.02 ^c	96.0 ± 0.76 ^f
30% PEG4000–15% MgSO ₄	2.45 ± 0.01 ^g	2.16 ± 0.03 ^f	7.19 ± 0.11 ^a	1.03 ± 0.02 ^a	96.8 ± 0.46 ^f

V_R: volume ratio (upper/lower); K_P: partition coefficient of protein in the upper phase; SA: specific activity (unit/mg protein); PF: purification factor; Yield: activity recovery yield. ns: no phase separation.^a Means ± S.D. from triplicate determinations.^{**} Different superscripts in the same column indicate the significant differences ($P < 0.05$).

molecular weights [9]. Furthermore, better partitioning of protein was achieved with lower molecular weight PEG, compared to that with higher molecular weight. This was possibly due to the fact that interfacial tension is lower when molecular weight of PEG is lower [32]. Therefore, PEG1000 was a suitable polymer for partitioning of protease in SE as indicated by the higher SA and PF than PEG with higher molecular weights. For partitioning of protease in ASE, PEG1000 showed high SA and PF. However, PEG2000 exhibited the higher activity recovery. It was speculated that ATPS system for protease purification might be governed by the state of enzyme, inactive (SE) or active (ASE), which possessed different physicochemical properties. ATPS of 25% PEG1000–20% MgSO_4 gave the highest PF (7.2-fold) with 85.7% yield for partitioning of protease in SE ($P < 0.05$). The 15% PEG2000–15% MgSO_4 afforded the greatest PF (2.4-fold) with 89.1% yield for ATPS in partitioning protease in ASE ($P < 0.05$). Those phase systems exhibiting the highest PF with appropriate recovery yield for SE or ASE were selected for further study and those fractions were referred to “PSE” and “PASE”, respectively.

To separate the protease in PEG-rich phase after two-phase separation, different methods can be used [33–35]. The target protease can be separated from PEG by back-extraction, in which a fresh salt phase is added to the collected PEG-rich phase containing target enzyme. By changing system conditions, target protease can be directed to the salt phase [33]. Thereafter, salt can be removed by dialysis or membrane filtration. Furthermore, ultrafiltration was used to separate PEG from the target enzyme [34,35]. Thus, further study on separation of protease from PEG is still needed.

3.3. Protein pattern of stomach protease partitioned with ATPS

The purity of protease from the stomach of albacore tuna after ATPS process was analyzed by SDS-PAGE (Fig. 1). SE contained a major band with a molecular weight (MW) of 40.6 kDa. Proteins with MWs of 25, 30, 56 and 62 kDa were also found. After acidification process, some of protein contaminants were removed and the major band with MW of 32.7 kDa was observed. Additionally, a large number of contaminating proteins were also removed after ATPS partitioning. A greater intensity of major protein band (32.7 kDa) was obtained in both PSE and PASE, in comparison with that found in SE and ASE. The major protease in stomach extract of albacore tuna has been reported to be aspartic proteinase, most likely pepsin [1]. Fish pepsin had MW of 30 and 35 kDa for sea bream [15] and smooth hound [36]. The migration of

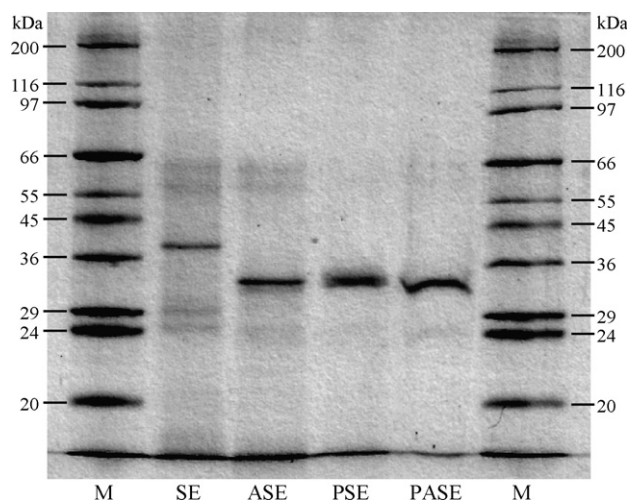


Fig. 1. SDS-PAGE patterns of stomach extract (SE) and acidified stomach extract (ASE), and their ATPS fractions (PSE and PASE). M denotes wide range MW protein markers.

major protein band of SE to lower molecular weight position after acidification or ATPS partitioning might be due to the autocatalytic activation of pepsinogen to pepsin under acidic condition and with two-phase systems, respectively. This finally removed 44 amino acid residues (the prosegment) from the N-terminal end of the zymogen peptide chain [37]. Nalinanon et al. [1] found that the activation of pepsin from tuna stomach, including albacore tuna, skipjack tuna and tongol tuna could be achieved at pH below 5.

3.4. Storage stability of stomach protease partitioned with ATPS

Fig. 2 shows the changes in protease activity of SE, ASE, PSE and PASE during storage at different temperatures up to 14 days. At 0 and 4 °C, SE was quite stable throughout 14 days of storage. However, the stability of PSE was lower as indicated by continuous decrease in

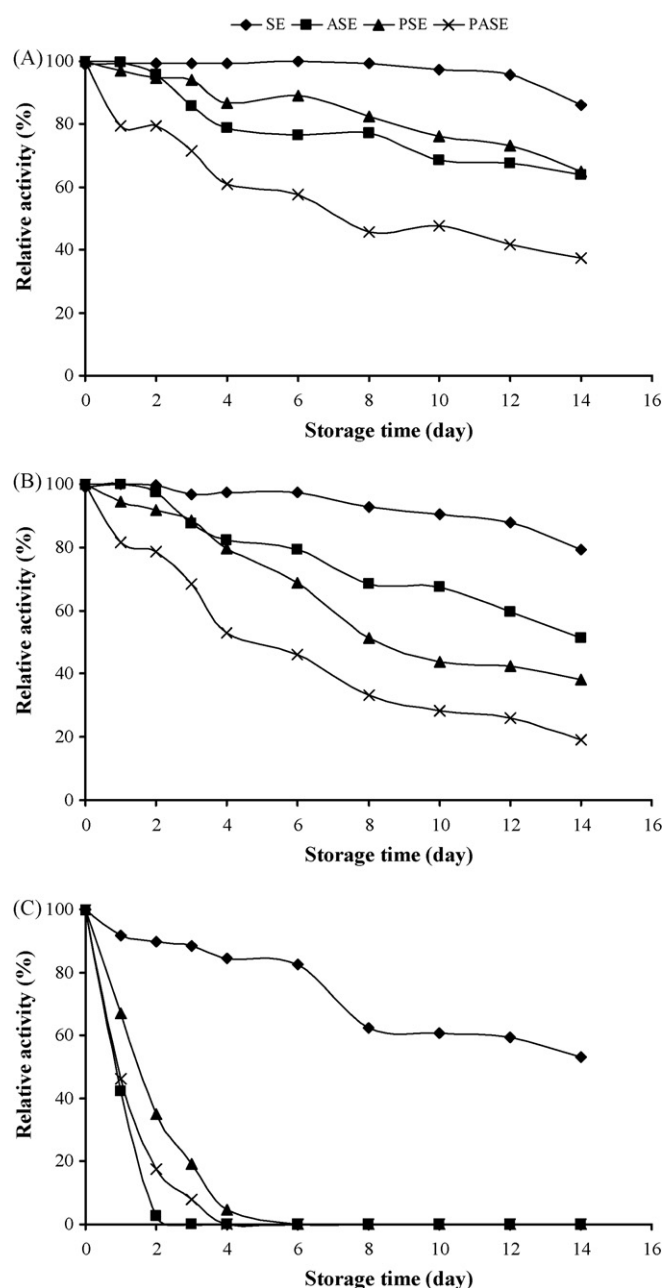


Fig. 2. Changes in protease activity of stomach extract (SE) and acidified stomach extract (ASE), and their ATPS fractions (PSE and PASE) during storage at 0 °C (A), 4 °C (B) and room temperature (C). Bars represent standard deviation from triplicate determinations.

activity with increasing storage time. Protease activity of ASE and PASE gradually decreased when storage time increased (Fig. 2A and B). Nevertheless, the greatest loss in protease activity was observed in PASE when compared to ASE and PSE. The greater decrease in protease activity was observed when stored at room temperature. For ASE, PSE and PASE, more than 80% of the initial activity was lost at day 3 of storage at room temperature (Fig. 2C). It was noted that the stability of stomach protease most likely depended on the form of enzyme and storage temperature. Additionally, PEG present in PSE and PASE had no stabilizing effect on protease. Protein-stabilizing ability of PEG has been reported [26,38]. Instability of stomach protease might be mainly due to autolysis process.

4. Conclusion

The optimum condition for partitioning of SE and ASE from the stomach of albacore tuna was 25% PEG1000–20% MgSO_4 and 15% PEG2000–15% MgSO_4 , respectively. The highest PF of 7.2-fold and 2.4-fold, with the yield of 85.7% and 89.1% was obtained for SE and ASE, respectively. After phase separation, protein with MW of 32.7 kDa became dominant. Protease in SE was stable at 0 and 4 °C throughout 14 days of storage, while ASE, PSE and PASE gradually lost their activity in time-dependent manner. Instability of protease was more pronounced in higher storage temperature. ATPS can be effectively used to recover and purify protease from albacore tuna stomach. However, changes in protease properties after partitioning should be considered.

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Tuna Pepsin: Characteristics and Its Use for Collagen Extraction from the Skin of Threadfin Bream (*Nemipterus* spp.)

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ABSTRACT: Pepsin from the stomach of albacore tuna, skipjack tuna, and tongol tuna was characterized. Pepsin from all tuna species showed maximal activity at pH 2.0 and 50 °C when hemoglobin was used as a substrate. Among the stomach extract of all species tested, that of albacore tuna showed the highest activity (40.55 units/g tissue) ($P < 0.05$). Substrate-Native-PAGE revealed that pepsin from albacore tuna and tongol tuna consisted of 2 isoforms, whereas pepsin from skipjack tuna had only 1 form. The activity was completely inhibited by pepstatin A, while EDTA (ethylenediaminetetraacetic acid), SBTI (soybean trypsin inhibitor), and E-64 (1-(*L*-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane) exhibited negligible effect. The activity was strongly inhibited by SDS (sodium dodecyl sulfate) (0.05% to 0.1%, w/v). Cysteine (5 to 50 mM) also showed an inhibitory effect in a concentration dependent manner. ATP, molybdate, NaCl, MgCl₂, and CaCl₂ had no impact on the activity. When tuna pepsin (10 units/g defatted skin) was used for collagen extraction from the skin of threadfin bream for 12 h, the yield of collagen increased by 1.84- to 2.32-fold and albacore pepsin showed the comparable extraction efficacy to porcine pepsin. The yield generally increased with increasing extraction time ($P < 0.05$). All collagen obtained with the aid of tuna pepsin showed similar protein patterns compared with those found in acid-solubilized collagen. Nevertheless, pepsin from skipjack tuna caused the degradation of α and β components. All collagens were classified as type I with large portion of β -chain. However, proteins with molecular weight (MW) greater than 200 kDa were abundant in acid-solubilized collagen.

Keywords: collagen, extraction, pepsin, threadfin bream, tuna

Introduction

Thailand is the top canned tuna producer and exporter in the world (DFT 2007). By the year 2006, Thai canned tuna was exported to several countries with the total volume of 416226 metric tons and a value of 40408 million bahts (The Customs Dept. 2007). Several tuna species, including albacore, skipjack, tongol, and yellowfin, are mainly used for the production of canned tuna in Thailand. During canned tuna manufacturing, high amounts of processing wastes such as viscera, head, and bone are discarded. Among those by-products, fish viscera can be used as a potential source of various enzymes, particularly proteases (Klomklao and others 2004). Pepsin is the major digestive enzyme in stomach of animals that are secreted as pepsinogen from chief cells of oxyntic glands located in the stomach wall epithelium. In acidic environment, pepsinogen rapidly converts to pepsin (Kageyama 2002). Pepsins and pepsinogens from stomach of various fish species such as Arctic fish capelin (Gildberg and Raa 1983), polar cod (Arunchalam and Haard 1985), North Pacific bluefin tuna (Tanji and others 1988), Atlantic cod (Gildberg and others 1990), sea bream (Zhou and others 2007), African coelacanth (Tanji and others 2007), and

pectoral rattail (Klomklao and others 2007) have been purified and characterized.

Apart from the tuna industry, the surimi industry has become important due to the increasing global market demand. Threadfin bream (*Nemipterus* spp.) is one of the important raw materials used for surimi production in Thailand in addition to bigeye snapper, lizardfish, croaker, and others (Benjakul and others 2005). During surimi production, a large quantity of by-products, especially skin and bone, are generated. Fish skin can serve as an alternative source for collagen since mammalian collagens are associated with several problems such as the outbreak of mad cow disease and the constraint for some religions, mainly Islam and Judaism. Therefore, skin collagen from several fish species such as sheep-head seabream (Ogawa and others 2003), Pacific whiting (Kim and Park 2004), Nile perch (Muyonga and others 2004), channel catfish (Liu and others 2007), and bigeye snapper (Nalinanon and others 2007) has been extracted and characterized. Generally, typical processing, especially acid solubilization, renders a low yield of collagen. To tackle the problem, pepsin has been applied since it is able to cleave peptides specifically in telopeptide region of collagen, leading to increased extraction efficiency (Nagai and others 2002; Nalinanon and others 2007). Use of commercial pepsin can be costly for collagen extraction. Tuna pepsin from stomach, a by-product, can be a promising enzyme due to its abundance and unique properties. Therefore, the objectives of this study were to characterize pepsin from the stomach of different tuna species, including albacore, skipjack, and tongol tuna, as well as to use tuna pepsin for collagen extraction from the skin of threadfin bream.

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Materials and Methods

Chemicals

Bovine hemoglobin, β -mercaptoethanol (β ME), *L*-tyrosine, *L*-cysteine, adenosine 5'-triphosphate (ATP), molybdate, bovine serum albumin, pepsin from porcine stomach mucosa (EC 3.4.23.1; powdered; 750 U/mg dry matter), and wide range protein markers were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Trichloroacetic acid, disodium hydrogen phosphate, sodium citrate, Folin-Ciocalteu's phenol reagent, acetic acid, and *p*-dimethylamino-benzaldehyde 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, Calif., U.S.A.). Type I collagen from calfskin was purchased from Elastin Products Co. Inc. (Owensville, Mo., U.S.A.).

Preparation of fish stomach and skin

Internal organs of skipjack tuna (*Katsuwonus pelamis*), albacore tuna (*Thunnus alalunga*), and tongol tuna (*Thunnus tonggol*) were obtained from Tropical Canning (Thailand) Public Co., Ltd., Songkhla, Thailand. Those samples were packed in polyethylene bag, kept in ice with a solid/ice ratio of 1:2 (w/v), and transported to the Dept. of Food Technology, Prince of Songkla Univ., Hat Yai within 1 h. Upon the arrival, pooled internal organs from each species were excised and only stomach was collected and placed in polyethylene bag. The stomach samples were stored at -20°C until use.

Skins of threadfin bream (*Nemipterus* spp.) obtained from Man A Frozen Foods Co. Ltd., Songkhla, Thailand, were kept in ice and transported to the Dept. of Food Technology within 1 h. The residual meat was removed manually from the skins. The cleaned skin was then washed with tap water, drained, and cut into small pieces (0.5×0.5 cm). The prepared skins were placed in polyethylene bags and stored at -20°C until use.

Preparation of stomach extract

Frozen stomach was thawed using running water (26 to 28°C) until the core temperature reached -2 to 0°C . The sample was cut into pieces with a thickness of 1 to 1.5 cm. Sample was rapidly frozen in liquid nitrogen and then finely ground using a Natl. Model MX-T2GN blender (Taipei, Taiwan) to a powder form. Stomach powder was suspended in 50 mM sodium phosphate buffer, pH 7.2, at a ratio of 1:9 (w/v) according to the method of Nalinanon and others (2007). The mixture was stirred continuously at 4°C for 30 min. The suspension was centrifuged for 30 min at 4°C at $7700 \times g$ using a Sorvall Model RC-B refrigerated centrifuge (Newtown, Ct., U.S.A.) to remove the tissue debris. The supernatant was collected and referred to as "stomach extract." The protein content in the extract was measured according to the method of Lowry and others (1951).

Assay of proteolytic activity

Proteolytic activity of tuna stomach extract was determined using hemoglobin as a substrate according to the method of An and others (1994) and Nalinanon and others (2007) with a slight modification. To initiate the reaction, 200 μL of stomach extract were added to the assay mixture containing 200 μL of 2% hemoglobin, 200 μL of distilled water and 625 μL of reaction buffer. Appropriate dilution was made to ensure that the amount of enzyme was not excessive for available substrate in the assay system. The reaction was conducted at pH 2.0 and 50°C for 20 min. To terminate enzymatic reaction, 200 μL of 50% (w/v) trichloroacetic acid

(TCA) were added. Unhydrolyzed protein substrate was allowed to precipitate for 15 min at 4°C , followed by centrifuging at $4725 \times g$ for 10 min using a MIKRO 20 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). The oligopeptide content in the supernatant was determined by the Lowry method (Lowry and others 1951) using tyrosine as a standard (An and others 1994). One unit of activity was defined as that releasing 1 μmole of tyrosine per minute ($\mu\text{mol Tyr/min}$). A blank was run in the same manner, except that the stomach extract was added into the reaction mixture after the addition of 50% TCA (w/v). Protein concentration of stomach extract was measured by the method of Lowry and others (1951) using bovine serum albumin as a standard. Specific activity was calculated and expressed as units per milligram protein.

pH and temperature profile

To study pH profile, proteolytic activity of stomach extract was measured at 50°C using hemoglobin as a substrate at different pHs (pHs 1 and 1.5 using 20 mM maleate buffer and pHs 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, and 7.0 using McIlvaine's buffer [0.2 M Na-phosphate and 0.1 M Na-citrate]). For temperature profile, the assay was performed at various temperatures (25, 30, 35, 40, 45, 50, 55, 60, 65, and 70°C) at pH 2.

Effect of some protease inhibitors

Stomach extract (200 μL) was mixed with 200 μL of single protease inhibitor to obtain the final designated concentration (0.1 mM E-64, 0.1 mM soybean trypsin inhibitor, 1 and 10 μM pepstatin A and 2 mM EDTA). The mixtures were incubated at room temperature (26 to 28°C) for 20 min. The residual activity was measured at pH 2 and 50°C . Relative activity was then calculated.

Effect of some chemicals

Different chemicals (200 μL) were mixed with stomach extract (200 μL) to obtain the concentration designated (0.1 and 0.5 mM for ATP and molybdate; 1 and 5 mM for NaCl, MgCl_2 and CaCl_2 ; 0.05 and 0.1% (w/v) for SDS, and 5, 25, and 50 mM for *L*-cysteine). The mixtures were kept at room temperature for 30 min. The remaining activity was determined at pH 2 and 50°C and the relative activity was calculated as described previously.

Activity staining of tuna pepsin

Activity staining of tuna pepsin was carried out as per the method of Diaz-Lopez and others (1998) with a slight modification. Stomach extract was mixed with sample buffer containing no SDS and β ME at a ratio of 1:1 (v/v). The samples (15 μg protein) were loaded onto the gels, consisting of 4% stacking gel and 12% separating gel, where SDS was excluded. Electrophoresis was performed at constant current of 15 mA per gel at 4°C . After electrophoresis, the gels were removed and soaked in 0.1 M HCl to reduce the pH to 2.0 to activate the pepsin. After 15 min, the gels were soaked in hemoglobin solution (0.25% hemoglobin in 0.1 M glycine-HCl at pH 2.0) with constant agitation for 30 min at 4°C to allow the substrate to penetrate into the gels. The gels were then soaked for 90 min in a fresh hemoglobin solution at 50°C to develop the activity zone. After development of activity zone, gels were washed using distilled water and fixed for 15 min in 12% TCA solution prior to staining with 0.125% Coomassie Blue R-250 in 45% methanol and 10% acetic acid and destained in 30% methanol and 10% acetic acid. Development of clear zones on blue background indicated proteolytic activity.

Use of tuna pepsin for the extraction of collagen from the skin of threadfin bream

Prior to collagen extraction, stomach extracts from different tuna species were adjusted to pH 2 with 1 M HCl and the mixture was allowed to stand at 4 °C for 10 min. The treated stomach extracts were centrifuged for 30 min at 4 °C at 5000 × g using a refrigerated centrifuge. The acidified supernatants were collected and used for collagen extraction.

The collagens from the skin of threadfin bream were extracted following the method of Nalinanon and others (2007) with a slight modification. All procedures were performed at 4 °C. To remove noncollagenous proteins, the skin was mixed with 0.1 N NaOH using a sample/alkaline solution ratio of 1:10 (w/v). The mixture was stirred continuously for 6 h. The alkaline solution was changed every 2 h. Then, the alkaline-treated skin was washed with cold water until neutral or faintly basic pHs of wash water were obtained. The treated skin was then defatted with 10% butyl alcohol with a solid/solvent ratio of 1:10 (w/v) for 18 h and the solvent was changed every 6 h. Defatted skin was washed with 10 volumes of cold water 3 times.

To extract the collagen, prepared skin was soaked in 0.5 M acetic acid with a solid/solvent ratio of 1:15 (w/v) in the absence and presence of acidified tuna stomach extract with the activity of 10 units/g defatted skin. Porcine pepsin at the same level was also used for the comparative study. The mixture was stirred at 4 °C for 6 and 12 h. At the designated time, the mixture was immediately mixed with pepstatin A to obtain a final concentration of 1 μM. The mixture was filtered with 2 layers of cheesecloth to remove unhydrolyzed tissues. The filtrates were subjected to the determination of hydroxyproline content and SDS-PAGE as described by Nalinanon and others (2007). The yield of collagen extracted was calculated based on hydroxyproline content in the extract in comparison with that of defatted skin.

Statistical analysis

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). An SPSS statistics program (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, U.S.A.) was used for data analysis.

Results and Discussion

pH and temperature profile of tuna pepsin

The effects of pH on the activity of pepsin from the stomach of albacore tuna, skipjack tuna, and tongol tuna are shown in Figure 1. The optimal pH for hydrolysis of hemoglobin of those pepsins was found at pH 2.0, which was in accordance with those of pepsins obtained from the stomach of polar cod (Arunchalam and Haard 1985), African coelacanth (Tanji and others 2007), and smooth hound (Bougatef and others 2008) when hemoglobin was used as a substrate. However, pepsins from North Pacific bluefin tuna (Tanji and others 1988) showed the maximal activity toward hemoglobin at pH 2.5. Bohak (1973) found that the conversion of pepsinogen to pepsin was observed in the pH range of 2 to 4. In this pH range, the zymogen undergoes a rapid conformational change to form an intermediate and the subsequent conversion of this intermediate to the active enzyme. At pH 2, stomach extract from albacore tuna exhibited the highest specific activity, followed by pepsin from skipjack tuna and tongol tuna, respectively. The activities of tuna pepsin decreased generally at very acidic pH and sharply decreased when the pH was above 3.0. At pHs higher and lower than the optimal pH, the enzyme possibly underwent the conformational changes under harsh condition, resulting in the lowered activity (Benjakul and others 2003). It was noted that no activity was observed at pH 5 or above. Therefore, the activation of tuna pepsin could be achieved at pH below 5.

Temperature profiles of pepsin from 3 tuna species are depicted in Figure 2. The optimal temperature of all pepsin was 50 °C when hemoglobin was used as a substrate. This optimal temperature was slightly higher than those of pepsin or acid proteinase obtained from other warm water fish species such as Monterey sardine (Castillo-Yañez and others 2004), and sea bream (Zhou and others 2007). The differences in optimal temperature might be associated with the differences in enzyme conformation as governed

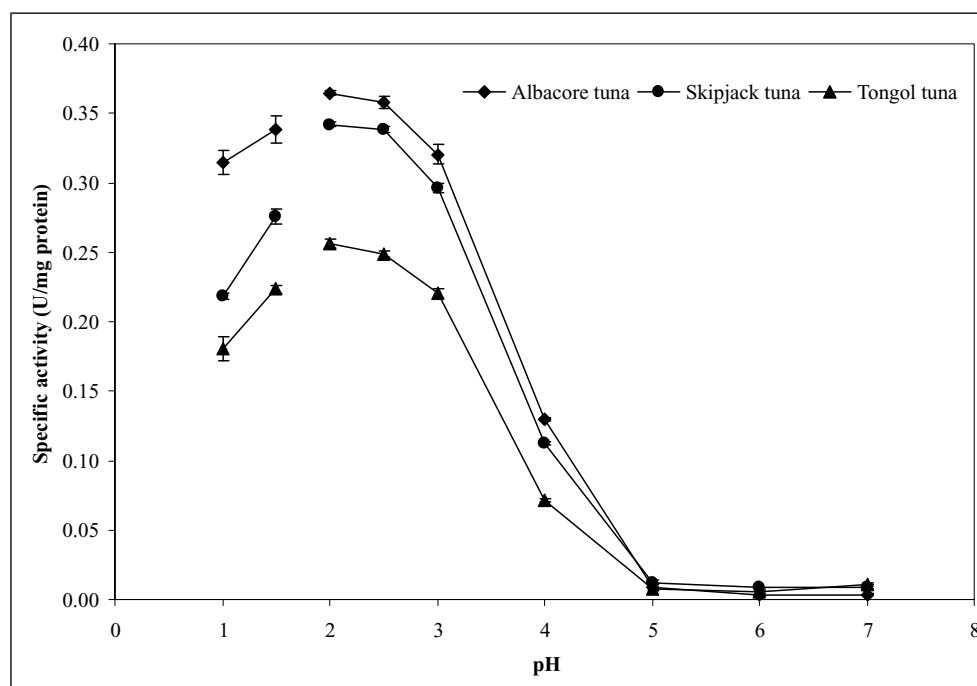


Figure 1 – pH profiles of pepsin from the stomach of different tuna species. Bars represent standard deviation from triplicate determinations.

by habitat, environment, and genetics (Gildberg 1988). A sharp decrease in activity was observed at temperatures above 55 °C, most likely due to thermal denaturation of enzyme. The result indicated that low activity was observed at 70 °C. At the optimal temperature, the highest specific activity was found in the stomach extract from albacore tuna, followed by those from skipjack tuna and tongol tuna.

Total activity of pepsin from stomach of different tuna species

Total pepsin activities in the stomach of 3 tuna species varied from 28.70 to 40.55 units/g. Under the optimal pH and temperature (Figure 1 and 2), albacore tuna stomach contained the highest activity (40.55 units/g) ($P < 0.05$), followed by tongol tuna stomach (37.94 units/g) and skipjack tuna stomach (28.70 units/g). The differences in pepsin content among species might be due to the differences in eating behavior, feed, or genetics. Klomklao and others (2004) also found varying amounts of trypsin in spleen among skipjack tuna, tongol tuna, and yellowfin tuna.

Activity staining of tuna pepsin

Tuna pepsins were separated on Native-PAGE, followed by staining for zymogram development (Figure 3). Pepsin from the stomach of albacore tuna and tongol tuna showed a similar pattern of activity band appeared as clear zones on the dark background. Two activity bands with different activity were found as indicated by differences in clear zone. The result indicated that pepsins in both albacore tuna and tongol tuna most likely consisted of 2 isoforms. For pepsin from skipjack tuna stomach, only 1 activity band was observed. In general, a major activity band of pepsin from all tuna species appeared at the similar position on zymogram, suggesting the similarity in size and charge distribution among pepsins. Different pepsin isoforms isolated from the stomach of different fish species have been reported. Acid proteases I and II from orange roughy (*Hoplostethus atlanticus*) stomach with molecular weight of 33.5 and 34.5 KDa, respectively, were purified (Xu and others 1996). Two isoforms of pepsins isolated from pectoral rattail (*Coryphaenoides pectoralis*) were classified as pepsin A and B (Klomklao and others 2007). Two major pepsinogens, PG1 and PG2,

and 1 minor pepsinogen, PG3, were purified from the gastric mucosa of African coelacanth, *Latimeria chalumnae* (Actinistia) (Tanji and others 2007). Four pepsinogens (PG-I, PG-II, PG-III, and PG-IV) from sea bream (*Sparus latus* Houuttuyn) stomach were purified and identified by Zhou and others (2007).

Effect of various protease inhibitors

The effect of various protease inhibitors on the activity of pepsin from different tuna species is shown in Table 1. The activity was completely inhibited by pepstatin A at concentrations of 1 and 10 μ M. Nevertheless, EDTA, soybean trypsin inhibitor, and E-64 at the concentrations used had no marked effect on the activity. Pepstatin A inhibits most aspartic proteinase (Umezawa 1976) with the exception of rennin and retroviral aspartic proteinases (Fusek and Větvicka 1995). Klomklao and others (2007) reported that activity of both pepsins A and B from pectoral rattail stomach was almost completely inhibited by pepstatin A. Tanji and others (2007) also reported that both pepsins from African coelacanth were nearly completely inhibited by an equimolar amount of pepstatin. E-64, which is specific inhibitor to cysteine proteinase, EDTA, a metalloproteinase inhibitor, and soybean trypsin inhibitor, the inhibitor specific to serine proteinase, had no impact on activity. Therefore, the major proteinase in tuna stomach extract was aspartic proteinase, most likely pepsin.

Effect of some chemicals

The effect of ATP, molybdate, NaCl, $MgCl_2$, $CaCl_2$, SDS, and cysteine on the activity of pepsin from the stomach of different tuna species is shown in Table 2. ATP and molybdate at concentrations of 0.1 and 0.5 mM had no profound effect on the activity. The result was in agreement with that of Klomklao and others (2007), who reported that ATP showed no influence on the activity of pepsins from pectoral rattail even though the concentration increased. Cathepsin D is generally activated by ATP; whereas pepsin is not activated (Pillai and Zull 1985; Klomklao and others 2007). Pillai and Zull (1985) found that half-maximal activation of cathepsin D occurs between 200 and 300 μ M ATP with all substrate used. Molybdate did not show inhibition on the activity of tuna stomach extract. Molybdate was reported to inhibit cathepsin

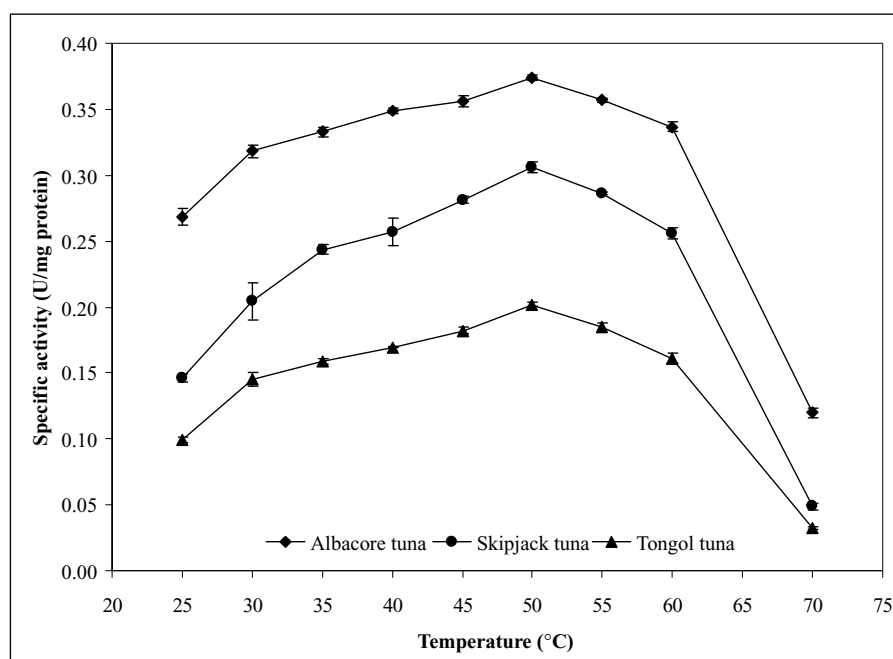


Figure 2—Temperature profiles of pepsin from the stomach of different tuna species. Bars represent standard deviation from triplicate determinations.

D (Pillai and Zull 1985). Furthermore, all salts tested, including NaCl, CaCl₂, and MgCl₂, also showed no marked influence on the activity of stomach extracts. Tuna pepsin was completely inhibited by SDS at concentrations of 0.05% and 0.1%, except for pepsin from skipjack tuna, which had 29.8% of activity remaining in the presence of 0.05% SDS. Cysteine inhibited the activity of stomach extracts in a dose-dependent manner. Pepsins contained 3 disulfide bonds and a phosphate linkage that stabilized their structure together with hydrogen bond, electrostatic, and hydrophobic interactions (Whitaker 1994). Cysteine acted as a reducing agent, which could destabilize enzyme structure by breaking disulfide bonds.

Use of tuna pepsin for collagen extraction

The yields of collagen extracted from threadfin bream skin using pepsin from the stomach of different tuna species for dif-

ferent times (6 and 12 h) in comparison with acid solubilization process (without pepsin) are shown in Table 3. The yields of collagen extracted with the aid of porcine pepsin at the same levels were also compared (Table 3). The highest yield (approximately 74.5% to 76%) was obtained when the skin was extracted using pepsin from albacore tuna or porcine pepsin at a level of 10 units/g of defatted skin for 12 h. At the same extraction time, the use of tuna pepsins and porcine pepsin resulted in the higher yield than the control (without pepsin). In general, longer reaction time afforded a higher yield, regardless of pepsin addition. The yield of collagen extracted by pepsin treatments for 12 h was 1.84- to 2.38-fold and 0.83- to 1.17-fold greater than that obtained from acid solubilization process with the extraction time of 12 and 48 h, respectively. It was suggested that hydrolysis at the telopeptide region was more pronounced with sufficient reaction time. Consequently, collagen could be solubilized

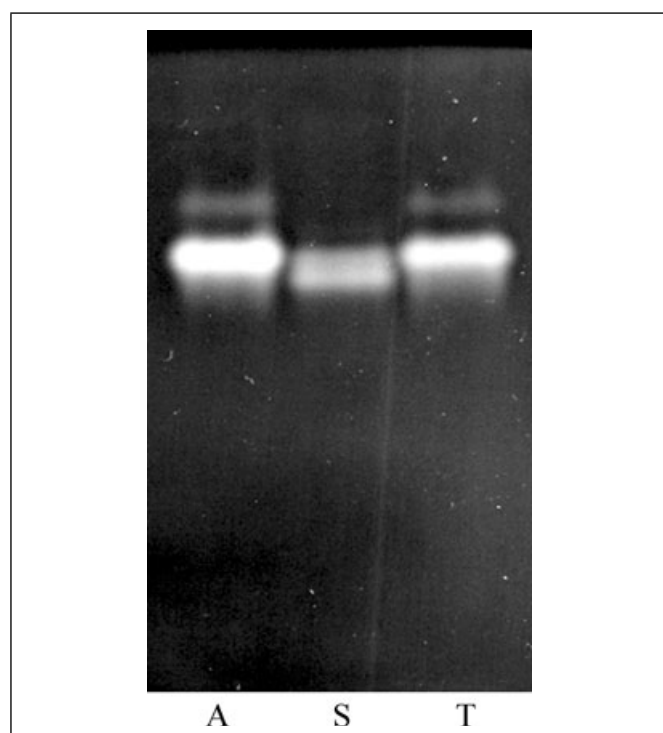


Figure 3—Zymogram of pepsin from the stomach of different tuna species. A = albacore tuna; S = skipjack tuna; T = tongol tuna.

Table 1—Effect of various protease inhibitors on the activity of pepsin from the stomach of different tuna species.^a

Inhibitors	Concentration	Relative activity (%) ^b		
		Albacore tuna	Skipjack tuna	Tongol tuna
Control		100	100	100
Pepstatin	1 μ M	ND	ND	ND
	10 μ M	ND	ND	ND
E-64	0.1 mM	100.3 $\pm$ 0.8	97.1 $\pm$ 1.9	98.1 $\pm$ 1.3
EDTA	0.2 mM	100.3 $\pm$ 0.9	97.5 $\pm$ 1.0	98.5 $\pm$ 0.8
Soybean trypsin inhibitor	0.1 mM	109.1 $\pm$ 0.2	105.6 $\pm$ 2.7	103.1 $\pm$ 0.9

^aEach enzyme solution was incubated with the same volume of inhibitor at room temperature (26 to 28 °C) for 20 min and the residual activity was determined using hemoglobin as a substrate for 20 min at pH 2.0 and 50 °C.

^bMean $\pm$ SD from triplicate determinations.

ND = not detectable.

Table 2—Effect of some chemicals on the activity of pepsin from the stomach of different tuna species.^a

Chemicals	Concentration	Relative activity (%) ^b		
		Albacore tuna	Skipjack tuna	Tongol tuna
Control		100	100	100
ATP	0.1 mM	103.8 $\pm$ 1.2	104.3 $\pm$ 0.7	104.8 $\pm$ 2.0
	0.5 mM	104.4 $\pm$ 0.5	106.5 $\pm$ 1.3	106.8 $\pm$ 1.3
Molybdate	0.1 mM	105.0 $\pm$ 0.2	102.7 $\pm$ 0.9	105.7 $\pm$ 0.6
	0.5 mM	105.0 $\pm$ 0.8	105.0 $\pm$ 0.7	105.4 $\pm$ 2.2
NaCl	1 mM	99.7 $\pm$ 0.6	105.9 $\pm$ 1.1	101.9 $\pm$ 0.8
	5 mM	103.7 $\pm$ 0.3	109.1 $\pm$ 2.7	106.5 $\pm$ 1.4
MgCl ₂	1 mM	100.4 $\pm$ 0.8	103.9 $\pm$ 1.0	103.2 $\pm$ 1.6
	5 mM	102.2 $\pm$ 0.4	103.6 $\pm$ 1.8	103.2 $\pm$ 0.3
CaCl ₂	1 mM	101.2 $\pm$ 0.4	102.6 $\pm$ 0.4	103.2 $\pm$ 1.5
	5 mM	105.6 $\pm$ 1.3	105.7 $\pm$ 1.0	106.1 $\pm$ 0.6
SDS	0.05% (w/v)	ND	29.8 $\pm$ 1.3	ND
	0.10% (w/v)	ND	ND	ND
Cysteine	5 mM	97.1 $\pm$ 0.6	97.2 $\pm$ 0.5	98.6 $\pm$ 0.4
	25 mM	94.4 $\pm$ 0.5	81.9 $\pm$ 1.4	76.4 $\pm$ 1.6
	50 mM	36.8 $\pm$ 5.4	33.5 $\pm$ 1.7	35.5 $\pm$ 3.4

^aEach enzyme solution was incubated with the same volume of chemical at room temperature (26 to 28 °C) for 30 min and the residual activity was determined using hemoglobin as a substrate for 20 min at pH 2.0 and 50 °C.

^bMean $\pm$ SD from triplicate determinations.

ND = not detectable.

Table 3—Total hydroxyproline (Hyp) and yield of collagen extracted from the skin of threadfin bream with the aid of pepsin from the stomach of different tuna species and porcine pepsin for different times.

Treatments ^A	Extraction time (h)	Total extracted Hyp ^B (mg/g defatted skin)	Yield ^C (%)
Control	6	2.19 $\pm$ 0.41 ^a	12.32 $\pm$ 2.29 ^a
(without pepsin)	12	4.00 $\pm$ 0.27 ^b	22.45 $\pm$ 1.50 ^b
	48	6.22 $\pm$ 0.15 ^c	34.90 $\pm$ 0.82 ^c
Albacore pepsin	6	10.55 $\pm$ 0.35 ^f	59.22 $\pm$ 1.95 ^f
	12	13.26 $\pm$ 0.24 ^{hi}	74.48 $\pm$ 1.35 ^{hi}
Skipjack pepsin	6	7.78 $\pm$ 0.22 ^d	43.68 $\pm$ 1.25 ^d
	12	11.36 $\pm$ 0.08 ^g	63.81 $\pm$ 0.44 ^g
Tongol pepsin	6	9.69 $\pm$ 0.21 ^e	54.41 $\pm$ 1.15 ^e
	12	12.81 $\pm$ 0.45 ^h	71.95 $\pm$ 2.53 ^h
Porcine pepsin	6	9.36 $\pm$ 0.53 ^e	52.56 $\pm$ 2.95 ^e
	12	13.52 $\pm$ 0.19 ^j	75.92 $\pm$ 1.05 ^j

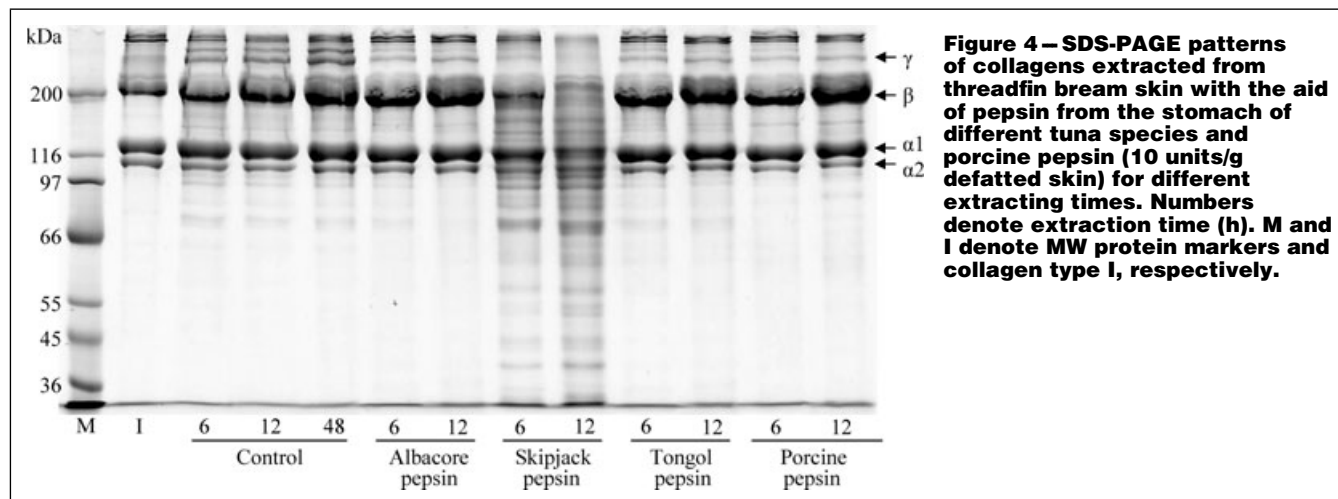
Means $\pm$ SD from triplicate determinations.

Different superscripts in the same column indicate the significant differences ($P < 0.05$).

^APepsin at a level of 10 units/g of defatted skin was used.

^BHydroxyproline content in the skin of threadfin bream was 17.81 mg/g defatted skin.

^CYield was calculated, based on hydroxyproline content in the collagen in comparison with that of defatted skin.



into the extracting solution in the presence of both tuna and porcine pepsins to a greater extent compared with the collagen extracted without pepsins. This result was in agreement with Nalinanon and others (2007) who found that the addition of bigeye snapper pepsin and porcine pepsin at a level of 20 units/g defatted skin resulted in the increased content of collagen extracted from bigeye snapper skin. Mammalian pepsin has been used for collagen extraction from fish skin, including ocellate puffer fish (Nagai and others 2002), black drum, sheephead seabream (Ogawa and others 2003), and channel catfish (Liu and others 2007). Albacore tuna pepsin exhibited higher efficiency in collagen extraction compared with skipjack tuna pepsin ($P < 0.05$), and showed similar effectiveness in increasing the yield to porcine pepsin and tongol pepsin. The differences in collagen yield were obtained when pepsins from different sources were used, suggesting the differences in the cleavage of telopeptide of collagen from threadfin bream skin. Due to the similar extraction efficiency, pepsin from albacore tuna could be used as a substitute for porcine pepsin.

Electrophoretic study of collagens extracted under different conditions revealed that β , $\alpha 1$, and $\alpha 2$ were the major components and collagen obtained was classified as type I (Figure 4). The result was in accordance with those found in skin collagen of black drum and sheephead seabream (Ogawa and others 2003), Pacific whiting (Kim and Park 2004), young and adult Nile perch (Muyonga and others 2004), channel catfish (Liu and others 2007), and bigeye snapper (Nalinanon and others 2007). Generally, type I collagen, a major collagen in fish skin, consists of 2 identical $\alpha 1$ chains and 1 $\alpha 2$ chain (Rochdi and others 2000; Yata and others 2001). No differences in proteins patterns were noticeable among collagens obtained from different extraction processes, except those treated with pepsin from skipjack tuna. Most protein bands were degraded with the concomitant formation of low molecular weight (MW) peptides or proteins when pepsin from skipjack tuna was used. It was suggested that pepsin from skipjack tuna hydrolyzed peptides not only at the telopeptide region but also within tropocollagen, as indicated by the decreased band intensity of β and α chains. Generally, the higher MW components, including γ -chain, were found at the higher extent in acid-solubilized collagen compared with those observed in pepsin-solubilized collagens. When reaction time increased, the intensity of β -chain of pepsin-solubilized collagen was noticeably increased, possibly due to the cleavage of higher MW components. Similar changes were also found in calfskin tropocollagen (Drake and others 1966), deep-sea redfish (Wang and others 2007), and bigeye snapper skin collagen (Nali-

nanon and others 2007) when pepsin was used for collagen extraction. Drake and others (1966) reported that native calfskin tropocollagen consisted of $\alpha:\beta:\gamma$ at a ratio of 32:65:3, whereas the components of pepsin-treated tropocollagen were changed to 72:23:3. Wang and others (2007) also found that the band intensity ratios of cross-linked chain (β and γ chains) to total monomer chain ($\alpha 1 + \alpha 2$) were lowered in pepsin-solubilized collagen extracted from deep-sea redfish when compared with acid-solubilized collagen. This result indicated that pepsin used in this study could cleave intermolecular bonds of collagen specifically. Zimmermann and others (1970) demonstrated that 2 types of intermolecular bonds, side-to-side and end-to-end bond, in calf collagen can easily be cleaved by pepsin, whereas head-to-tail bond is relatively pepsin resistant.

It was noted that similar MW of α chains was observed between acid solubilized collagen and tuna-pepsin solubilized collagen. However, Nalinanon and others (2007) reported that MW of α chains of collagen from bigeye snapper skin extracted with pepsin from the same fish species decreased slightly when compared with acid-solubilized collagen. Therefore, it was likely that the use of pepsin from the same fish species to extract the fish skin might cause more intensive cleavage at telopeptide region. Therefore, pepsins from both albacore and tongol could be used to extract collagen from threadfin bream skin effectively without the alteration of MW of components in resulting collagen.

Conclusions

Tuna pepsins showed optimal activity at pH 2 and 50 °C. The activity was strongly inhibited by pepstatin A, SDS, and cysteine. Among all tuna species, albacore tuna contains the highest pepsin activity ($P < 0.05$). Collagen extraction from threadfin bream skin could be achieved by soaking the prepared skin in 0.5 M acetic acid containing albacore pepsin at a level of 10 units/g defatted skin for 12 h. Albacore pepsin exhibited the comparable extraction efficiency to porcine pepsin and no detrimental effect on the integrity of resulting collagen was observed. Therefore, albacore pepsin could be used as the potential aid for collagen extraction from threadfin bream skin.

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Purification and characterization of trypsin from the pyloric caeca of brownstripe red snapper (*Lutjanus vitta*)

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ABSTRACT

Trypsin was purified from the pyloric caeca of brownstripe red snapper (*Lutjanus vitta*) by ammonium sulphate (40–60% saturation) precipitation, soybean trypsin inhibitor (SBTI)–Sephacrose 4B column and DEAE–Sephacel column chromatography. Purified trypsin showed a single band on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and native–PAGE. A yield of 4.9% with the purification-fold of 20 was obtained. Trypsin had an apparent molecular weight of 23 kDa. SBTI and *N*- ρ -tosyl-L-lysine-chloromethylketone (TLCK) showed a strong inhibitory effect on the purified trypsin, while other protease inhibitors exhibited negligible inhibition. Trypsin had maximal activity at pH 8.5 and 60 °C for the hydrolysis of α -*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA). It was stable within the temperature range of 25–55 °C and pH range of 7.0–10.0. Purified trypsin had a Michaelis–Menten constant (K_m) and catalytic constant (k_{cat}) of 0.507 mM and 4.71 s^{−1}, respectively, when BAPNA was used as the substrate. For the hydrolysis of α -*N*- ρ -tosyl-L-arginine methyl ester (TAME), K_m and k_{cat} were 0.328 mM and 112 s^{−1}, respectively.

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1. Introduction

Fish viscera generated during fishery processing can be used as an important source of digestive enzymes, including proteases. Proteases have been widely used for basic research and industrial applications (Shahidi & Kamil, 2001) and about 50% of the total sale of enzymes in industries consists of proteases. To obtain a variety of proteases, particularly those with unique properties, new sources of proteolytic enzymes have been studied, including proteases from fish, especially fish viscera (Souza, Amaral, Santo, Carvalho, & Bezerra, 2007).

Trypsin (EC 3.4.21.4) generally belongs to the group of serine proteinases. It specifically hydrolyses peptide bonds at the carboxyl terminus of lysine and arginine residues. This enzyme has been isolated from the pyloric caeca of various species of fish, including tambaqui (*Colossoma macropomum*) (Bezerra et al., 2001), Monterey sardine (*Sardinops sagax caerulea*) (Castillo-Yañez, Pacheco-Aguilar, García-Carreño, & Del Toro, 2005), chinook salmon (*Oncorhynchus tshawytscha*) (Kurtovic, Marshall, & Simpson, 2006), bigeye snapper (*Pricanthus macracanthus*) (Hau & Benjakul, 2006), arabesque greenling (*Pleuroprammus azonus*) (Kishimura, Hayashi, Miyashita, & Nonami, 2006a), spotted goatfish (*Pseudupeneus maculatus*) (Souza et al., 2007), walleye pollock (*Theragra chalcogramma*) (Kishimura, Klomklao, Benjakul, & Chun, 2008)

and mandarin fish (*Siniperca chuatsi*) (Lu et al., 2008). Trypsin can be used for the acceleration of protein hydrolysis. Klomklao, Benjakul, Visessanguan, Kishimura, and Simpson (2006a) reported that the addition of spleen from skipjack tuna containing trypsin could enhance the protein degradation of sardine muscle during fish sauce fermentation. Additionally, trypsin was used to produce a hydrolysate with antioxidative activity (Khantaphant & Benjakul, 2008).

Brownstripe red snapper (*Lutjanus vitta*) is one of the fish species used as raw materials for surimi production in Thailand (Jongjareonrak, Benjakul, Visessanguan, Nagai, & Tanaka, 2005). During processing, a large amount of viscera is generated. Thus, the use of viscera as the source of protease should maximise the utilisation of this by-product from this species. Among all viscera, pyloric caeca has been reported as an excellent source of trypsin (Hau & Benjakul, 2006). However, no information exists regarding the properties and characteristics of trypsins from pyloric caeca of brownstripe red snapper, which can be further used as a processing aid for various industries. Therefore, this study aimed to purify and characterise trypsin from pyloric caeca of brownstripe red snapper.

2. Materials and methods

2.1. Chemicals

Soybean trypsin inhibitor (SBTI), α -*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), *N*- ρ -tosyl-L-lysine-chloromethylketone

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(TLCK), ethylenediaminetetraacetic acid (EDTA), iodoacetic acid, 1-(L-trans-epoxysuccinyleucylamino)-4-guanidinobutane (E-64), pepstatin A, *N*-tosyl-L-phenylalanine chloromethylketone (TPCK), β -mercaptoethanol (β ME) and wide range molecular weight protein markers were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Coomassie Brilliant Blue G-250, sodium dodecyl sulphate (SDS) and *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Tris (hydroxymethyl) aminomethane (Tris-HCl) was procured from Merck (Darmstadt, Germany). Diethylaminoethyl (DEAE)-Sephacel and cyanobromide (CNBr)-activated Sepharose 4B were purchased from Amersham Biosciences (Uppsala, Sweden). All chemicals were of analytical grade.

2.2. Preparation of pyloric caeca extract

Brownstripe red snapper were purchased from a dock in Songkhla province, Thailand. Fish were kept in ice with fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, pyloric caeca from brownstripe red snapper were collected and pooled as the composite sample. Pyloric caeca were powdered in liquid nitrogen. Thereafter, the pyloric caeca extract was prepared according to the method of Khantaphant and Benjakul (2008) with a slight modification. Pyloric caeca powder was suspended in an extraction buffer (50 mM Tris-HCl buffer, pH 8.0, containing 10 mM CaCl_2) with a ratio of 1:10 (w/v). The mixture was homogenised for 2 min using a homogeniser (Model T25B, IKA labortechnik, Selangor, Malaysia) at 11,000 rpm. The homogenate was then continuously stirred for 30 min at 4 °C and centrifuged at 8000g for 30 min at 4 °C using a refrigerated centrifuge (Becman Coulter Model Avant J-E, Becman Coulter, Inc., CA, USA). The supernatant was filtered through a Whatman filter paper No. 1 (Schleicher & Schuell, Maidstone, England). The filtrate obtained was referred to as 'pyloric caeca extract, PCE'.

2.3. Preparation of ammonium sulphate fraction

PCE was subjected to 40–60% saturated ammonium sulphate precipitation, according to the method of Khantaphant and Benjakul (2008) with a slight modification. After the addition of ammonium sulphate, the mixture was stirred gradually at 4 °C for 30 min. Thereafter, the mixture was centrifuged at 8000g for 30 min at 4 °C and the pellet obtained was dissolved in the minimum volume of 50 mM Tris-HCl buffer, pH 8.0. The solution was dialysed against 20 volumes of the extraction buffer overnight at 4 °C with three changes of dialysis buffer. The dialysate was kept in ice and referred to as 'ammonium sulphate fraction, ASF'.

2.4. Purification of trypsin

ASF was purified using a series of chromatographies, including affinity column and ion-exchange column. The soybean trypsin inhibitor (SBTI)-Sepharose 4B column was prepared by following the instructions of the supplier. CNBr-activated Sepharose 4B was swollen and washed in 200 volumes of 1 mM HCl. The coupling between Sepharose gel and SBTI (1:0.027 (w/w)) was carried out at room temperature and the remaining active group on the medium was blocked by 0.1 M Tris-HCl, pH 8.0. ASF was applied onto the SBTI-Sepharose 4B column (1 × 10 cm) pre-equilibrated with 50 mM Tris-HCl, pH 7.8, containing 10 mM CaCl_2 and 0.5 M NaCl. The column was then washed with the same buffer containing no CaCl_2 with a flow rate of 0.5 ml/min until the absorbance of 280 nm was less than 0.005. The elution was carried out using 5 mM HCl with a flow rate of 1.0 ml/min. A fraction of 2 ml was collected and mixed rapidly with 0.5 ml of 100 mM Tris-HCl, pH

8.5, containing 10 mM CaCl_2 . The fractions were tested for trypsin activity, using BAPNA as a substrate. Active fractions were pooled and dialysed against 10 volumes of 10 mM Tris-HCl, pH 8.0, containing 1 mM CaCl_2 at 4 °C. Two changes of dialysis buffer were performed during dialysis.

The dialysate was then applied onto a DEAE-Sephacel column (2.5 × 15 cm) pre-equilibrated with 10 mM Tris-HCl, pH 8.0, containing 1 mM CaCl_2 . The column was washed with the same buffer until the absorbance of 280 nm was less than 0.005. The elution was then performed using a linear gradient of NaCl from 0 to 0.5 M in 10 mM Tris-HCl, pH 8.0, containing 1 mM CaCl_2 . Fractions (2 ml) were tested for trypsin activity. Active fractions were pooled and dialysed against 10 volumes of 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM CaCl_2 , with two changes of dialysis buffer. The dialysate was lyophilised prior to the storage at –20 °C.

2.5. Assay for trypsin activity

Trypsin activity was measured, using BAPNA as a substrate according to the method of Khantaphant and Benjakul (2008). A 200 μ l of sample was mixed with 200 μ l of distilled water and 1000 μ l of reaction buffer (50 mM Tris-HCl buffer, pH 8.0, containing 10 mM CaCl_2). The reaction was initiated by adding 200 μ l of 2 mg/ml BAPNA to the reaction mixture. After incubation for 20 min at 60 °C, 200 μ l of 30% acetic acid (v/v) were added to terminate the reaction. Production of *p*-nitroaniline was measured by monitoring the absorbance of reaction mixture at 410 nm (A_{410}). A blank was conducted in the same manner except that sample was added after addition of 30% acetic acid. Trypsin activity was calculated using the following equation:

$$\text{Trypsin activity (unit/ml)} = \frac{(A - A_0) \times \text{mixture volume (ml)} \times 1000}{8800 \times \text{reaction time (min)} \times 0.2}$$

where 8800 ($\text{cm}^{-1} \text{M}^{-1}$) is the extinction coefficient of *p*-nitroaniline; A and A_0 are A_{410} of sample and blank, respectively. One unit of activity was defined as that releasing 1 nmol of *p*-nitroaniline per min.

2.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and substrate-gel electrophoresis

SDS-PAGE was carried out according to the method of Laemmli (1970), using 12% separating gel and 4% stacking gel. Active fractions from the affinity or ion-exchange column were mixed with sample buffer (0.125 M Tris-HCl, pH 6.8, containing 4% SDS and 20% (v/v) glycerol) at a ratio of 2:1 (v/v). Proteins (4 and 0.8 μ g for affinity and ion-exchange fractions, respectively) were loaded onto the gel. The electrophoresis was run at a constant current of 15 mA per gel by a Mini-Protein II Cell apparatus (Bio-Rad, Hercules, CA, USA). Proteins from the affinity fraction were stained with 0.05% Coomassie Blue G-250 in 15% methanol and 5% acetic acid, while those from the ion-exchange fraction were stained with 0.2% silver nitrate dissolved in water and 0.076% formaldehyde. Native-PAGE was performed, using 12% separating gel in a similar manner, except that the sample was not heated and all solutions contained no SDS or any reducing agent. Destaining was carried out using 30% methanol and 10% acetic acid for Coomassie Blue-stained gel. Protein bands on silver-stained gel were developed using 6% sodium carbonate and 0.05% formaldehyde.

Substrate-gel electrophoresis was performed after electrophoresis. The gels were immersed in 100 ml of 50 mM Tris-HCl buffer, pH 7.5, containing 2% casein (w/v) for 1 h at 0 °C with a gentle agitation. Thereafter, the gels were transferred to 2% casein (w/v) in 50 mM Tris-HCl buffer with pH rendering the highest activity of trypsin. The mixture was incubated at 60 °C for 15 min with con-

tinuous agitation. The gels were then stained with 0.05% Coomassie Blue R-250 in 15% methanol and 5% acetic acid and destained in 30% methanol and 10% acetic acid. Proteolytic activity was indicated by the development of a clear zone on a blue background.

2.7. pH and temperature profile

The temperature profile of purified trypsin was determined at various temperatures (25, 35, 45, 50, 55, 60, 65 and 70 °C) at pH 8.0, using BAPNA as a substrate. For pH profile, the activity was studied over the pH range of 4.0–11.0, using the different buffers: 50 mM acetic acid–sodium acetate for pHs 4.0–6.0, 50 mM Tris–HCl buffer for pHs 7.0–9.0 and 50 mM glycine–NaOH buffer for pHs 10.0–11.0. The optimal temperature was used for the assay.

2.8. pH and thermal stability

The effects of pH and temperature on the stability of purified trypsin were evaluated by measuring the remaining trypsin activity after incubation at various pHs and temperatures. For thermal stability, purified trypsin, in 50 mM Tris–HCl containing 10 mM CaCl_2 (pH 8.5), was incubated at different temperatures (25, 35, 45, 50, 55, 60, 65 and 70 °C) for 30 min. Then the treated samples were rapidly cooled in iced water and the residual trypsin activity was assayed as previously described.

For pH stability, purified trypsin was mixed with an equal volume of different buffers containing 10 mM CaCl_2 with different pHs. The mixture was allowed to stand for 30 min at room temperature (25–28 °C) prior to assay at optimal pH and temperature, using BAPNA as a substrate, as described above.

2.9. Effect of inhibitors

Effects of various inhibitors on the activity of purified trypsin from brownstripe red snapper were determined according to the method of Khantaphant and Benjakul (2008). Purified trypsin in 50 mM Tris–HCl buffer (pH 8.5) containing 10 mM CaCl_2 was treated with an equal volume of protease inhibitor solutions to obtain the final concentrations designated (0.1 mM E-64, 1 mM iodoacetic acid, 1.0 mg/ml SBTI, 5 mM TLCK, 5 mM TPCK, 0.01 mM pepstatin A and 2 mM EDTA). The mixture was allowed to stand at room temperature (25–28 °C) for 15 min. The remaining activity was determined and the percentage inhibition was then calculated. The control was conducted in the same manner except that deionised water was used instead of inhibitors.

2.10. Kinetic studies

Kinetic studies of purified trypsin from brownstripe red snapper were carried out according to the method of Hau and Benjakul (2006) using BAPNA and TAME, ranging from 0.01 to 2.0 mM, as the substrates. The final enzyme concentration for the assay was 0.0055 mg protein/ml. The kinetic parameters, including the maximal velocity ($V_{\max}$) and Michaelis–Menten constant (K_m), were evaluated at pH 8.5 and 30 °C, using a Lineweaver–Burk double-reciprocal plot (Lineweaver & Burk, 1934). Catalytic constant (k_{cat}) was calculated from the following equation: $k_{\text{cat}} = V_{\max}/[E]$, where $[E]$ is molar concentration of enzyme calculated, based on the molecular weight determined by SDS–PAGE and protein concentration.

2.11. Determination of protein content

Protein concentration of all samples was measured by the method of Lowry, Rosebrough, Farr, and Randall (1951), except for the ion-exchange fraction, which was measured by the method

of Bradford (1976). Bovine serum albumin was used as a standard for both assays.

2.12. Statistical analysis

All data were subjected to analysis of variance (ANOVA) and differences between means were evaluated by Duncan's multiple range tests (Steel & Torrie, 1980). The SPSS Statistic Programme (Version 10.0) (SPSS Inc., Chicago, IL, USA) was used for data analysis.

3. Results and discussion

3.1. Purification of trypsin from pyloric caeca of brownstripe red snapper

Trypsin was purified from the pyloric caeca of brownstripe red snapper by a series of chromatographies after precipitation with ammonium sulphate (40–60% saturation) as summarised in Table 1. An 8-fold increase in purity was obtained by ammonium sulphate precipitation. Ammonium sulphate precipitation was a simple method and generally introduced as an initial step to remove other proteins from the crude extract. Hau and Benjakul (2006) found that ammonium sulphate (40–60% saturation) precipitation of trypsin from pyloric caeca of bigeye snapper resulted in a 4.53-fold increase in purity. ASF was further purified using SBTI-Sepharose 4B affinity column. This purification step resulted in an increase in purity of 13-fold, compared with that of PCE, and a yield of 16.0% was obtained. Two protein bands of the SBTI-affinity fraction were observed (Fig. 2A; lane A). SBTI-Sepharose 4B affinity chromatography was performed in the first step of purification of trypsin from Atlantic bonito (*Sarda sarda*) pyloric caeca with a yield 20.7% and 22.4-fold increase in purity and gel-filtration chromatography was further used to obtain a purity of 47.6-fold with 11.9% yield. (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2007a). However, trypsin was purified from the pyloric caeca of chinook salmon (Kurtovic et al., 2006), bigeye snapper (Hau & Benjakul, 2006) and New Zealand hoki (*Macruronus novaezealandiae*) (Shi, Marshall, & Simpson, 2007), using only SBTI-Sepharose 4B affinity chromatography, in which increases in purity of 6.5-, 34.0- and 44.6-fold, respectively, and the yields of 2.7%, 13% and 23.9% were obtained, respectively.

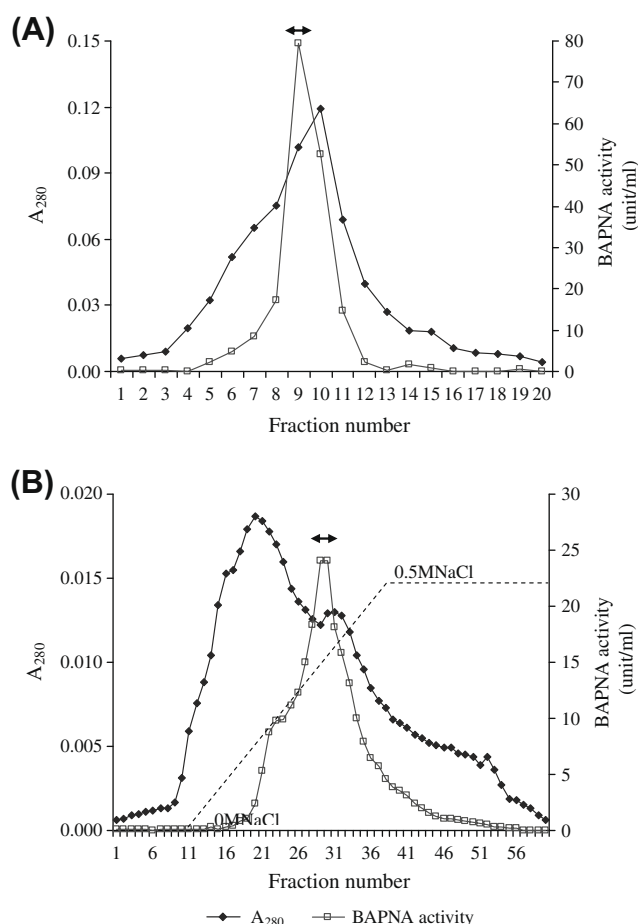
The SBTI-affinity fraction was subsequently subjected to a DEAE-Sepharcel column. After elution, using a linear gradient of 0–0.5 M NaCl, the active fractions were pooled (Fig. 1B). Only one protein band was found in the DEAE-Sepharcel fraction when determined using SDS–PAGE (Fig. 2A; lane 1). A purity of 20-fold was obtained for the DEAE-Sepharcel fraction. Ion-exchange chromatography, especially DEAE ion-exchange chromatography, was used to remove the contaminating proteins and to purify different trypsins from the pyloric caeca of starfish (*Asterina pectinifera*) (Kishimura & Hayashi, 2002) and from the spleen of yellowfin tuna (*Thunnus albacores*) (Klomklao et al., 2006b) and skipjack tuna (*Katsuwonus pelamis*) (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2007b). Trypsin from pyloric caeca of chum salmon (*Oncorhynchus keta*) was purified to homogeneity with 20.4-fold purity, using affinity chromatography, gel-filtration and DEAE ion-exchange chromatography (Sekizaki, Itoh, Murakami, Toyota, & Tanizawa, 2000). Ahsan and Watabe (2001) purified two trypsins from viscera of Japanese anchovy (*Engraulis japonicus*), using affinity chromatography, gel-filtration, and DEAE ion-exchange chromatography. Purities of 30- and 58-fold were achieved for trypsin I and trypsin II, respectively.

The DEAE-Sepharcel fraction showed a single band when determined using SDS–PAGE. The apparent molecular weight of the

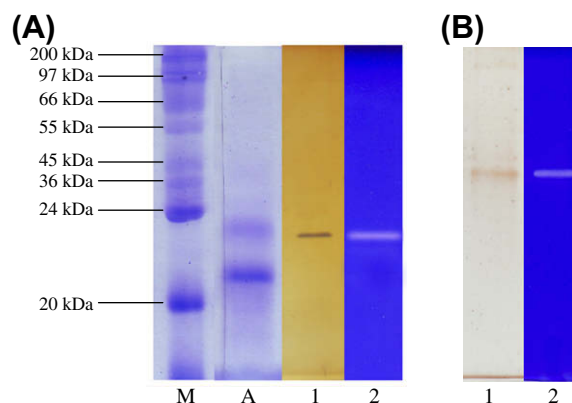
Table 1

Purification of trypsin from pyloric caeca of brownstripe red snapper.

Purification steps	Total protein (mg)	Total activity (unit ^a)	Specific activity (unit/mg)	Yield (%)	Purity (fold)
Pyloric caeca extract	271	5954	21.94	100	1
Ammonium sulphate fraction (40–60% saturation)	11.6	1918	164.9	32.2	8
SBTI-affinity chromatography	3.37	954.4	283.1	16.0	13
DEAE chromatography	0.665	289.2	434.7	4.9	20

^a One unit was defined as that releasing 1 nmol of *p*-nitroaniline per min.**Fig. 1.** Elution profile of ammonium sulphate (40–60% saturation) fraction on SBTI-Sepharose 4B column (A) and elution profile of SBTI-Sepharose 4B fraction on DEAE-Sepharcel column (B). Elution was performed using 5 mM HCl with a flow rate of 1.0 ml/min for the SBTI-Sepharose 4B column and using a gradient (0–0.5 M NaCl) with a flow rate of 0.5 ml/min for the DEAE-Sepharcel column. Fractions (2 ml) were determined for trypsin activity using BAPNA as a substrate. (↔): pooled fractions.

purified trypsin was estimated to be approximately 23 kDa (Fig. 2A). Based on native-PAGE (Fig. 2B), this fraction also showed a single protein band, reconfirming that the purified trypsin had only one isoform. Different molecular weights of trypsins from various fish were reported, such as 21 and 21.5 kDa for mandarin fish (Lu et al., 2008), 24 kDa for arabesque greenling and walleye pollock (Kishimura et al., 2006a, 2008), 25 kDa for Monterey sardine (Castillo-Yañez et al., 2005), 26 kDa for New Zealand hoki (Shi et al., 2007), 28 kDa for chinook salmon and bluefish (*Pomatomus saltatrix*) (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2007d; Kurtovic et al., 2006) and 38.5 kDa for tambaqui (Bezerra et al., 2001). The different molecular weights among trypsins from different sources may be due to genetic variations among

**Fig. 2.** SDS-PAGE (A) and native-PAGE (B) of purified trypsin from pyloric caeca of brownstripe red snapper. Lane A: Sepharose 4B fraction. Lanes 1 and 2: protein band and activity band of DEAE-Sepharcel fraction, respectively. M, molecular weight marker.

species. Generally, trypsins have been reported to have molecular weights between 20 and 30 kDa (Lu et al., 2008).

3.2. Effects of various protease inhibitors on the activity of trypsin from pyloric caeca of brownstripe red snapper

Protease inhibitors are very important tools for classifying the types of proteases (Kishimura, Tokuda, Klomklao, Benjakul, & Ando, 2006b). Table 2 shows the effects of various protease inhibitors on the activity of the purified trypsin. Trypsin from pyloric caeca of brownstripe red snapper was almost inhibited (99.2–99.6% inhibition) by soybean trypsin inhibitor (a serine proteinase inhibitor) and TLCK (a trypsin specific inhibitor). E-64, *N*-ethylmaleimide and iodoacetic acid (cysteine proteinase inhibitors), pepstatin A (an aspartic proteinase), EDTA (a metalloproteinase) and TPCK (a chymotrypsin specific inhibitor) had very small inhibitory effects on trypsin activities (0.2–1.4%). The result indicated that the protease purified from pyloric caeca of brownstripe red snapper was most likely a trypsin.

Table 2Effects of various protease inhibitors on inhibition of trypsin from brownstripe red snapper.^a

Inhibitors	Final concentration	% Inhibition
Control	–	–
E-64	0.1 mM	0.5
<i>N</i> -ethylmaleimide	1 mM	1.0
Iodoacetic acid	1 mM	0.3
SBTI	1 g/l	99.6
TLCK	5 mM	99.2
TPCK	5 mM	0.9
EDTA	2 mM	1.4
Pepstatin A	0.01 mM	0.2

^a The residual activities of trypsin after a 15 min incubation in the presence of inhibitors were assayed at 60 °C and pH 8.5 using BAPNA as substrate.

3.3. Effects of temperature and pH on the activity of trypsin from pyloric caeca of brownstripe red snapper

Temperature and pH profiles of purified trypsin from pyloric caeca of brownstripe red snapper are shown in Fig. 3a and b, respectively. The optimal temperature for BAPNA hydrolysis was 60 °C. However, the activity markedly decreased at temperatures above 60 °C, mainly due to thermal denaturation of trypsin (Khantaphant & Benjakul, 2008). The same optimal temperature (60 °C) of trypsin from menhaden (*Brevoortia* spp.) and mullet (*Mugil* spp.) (Pavlisko, Rial, & Coppes, 1999), Japanese anchovy (Ahsan & Watabe, 2001), chinook salmon (Kurtovic et al., 2006) and New Zealand hoki (Shi et al., 2007) was reported when BAPNA was used as a substrate. Trypsin from brownstripe red snapper had an optimal temperature higher than those reported for cold-water fish trypsins, which had optimal temperatures in the range of 40–45 °C (Simpson, 2000). The differences might be associated with different living temperatures of fish (Kishimura et al., 2008), as well as differences in substrates or assay conditions used (Klomklao et al., 2007a).

The maximal activity of trypsin from brownstripe red snapper was observed at pH 8.5. Marked loss in activity was found at pH 4.0. Furthermore, a decrease in activity was noticeable at very alkaline pHs. Trypsins from pyloric caeca of bigeye snapper and chinook salmon had optimal pHs of 8.0 (Hau & Benjakul, 2006; Kurtovic et al., 2006). Trypsin from spotted goatfish had an optimal pH of 9.0 (Souza et al., 2007); tambaqui and Japanese anchovy trypsins showed maximal activity at pH 9.5 (Ahsan & Watabe, 2001; Bezerra et al., 2001). Simpson (2000) reported that trypsins are generally more active at alkaline pH with optimal pH ranging from 7.5 to 10.5.

3.4. Thermal and pH stability of purified trypsin from pyloric caeca of brownstripe red snapper

Thermal and pH stabilities of purified trypsin from brownstripe red snapper are depicted in Fig. 3c and d, respectively. Trypsin was quite stable up to 55 °C with a residual activity of 95–99%. Decreases in activity were observed when heated at temperatures above 60 °C. At high temperatures, trypsin most likely underwent denaturation and lost its activity. Trypsin from brownstripe red snapper was more resistant to high temperature than were trypsins from chinook salmon, New Zealand hoki and bluefish, which lost their activity rapidly at temperatures above 40 °C (Klomklao et al., 2007d; Kurtovic et al., 2006; Shi et al., 2007). Furthermore, Bezerra et al. (2005) found that trypsin from Nile tilapia (*Oreochromis niloticus*) was stable up to 50 °C and similar thermal stability was reported for trypsins from Japanese anchovy (Ahsan & Watabe, 2001) and mullet (Pavlisko et al., 1999). In general, thermal stability of fish trypsin varies with species as well as with incubation conditions (Shahidi & Kamil, 2001).

For pH stability, trypsin from brownstripe red snapper showed high stability over neutral and alkaline pH ranges (pH 7.0–10.0). A sharp decrease in activity was more pronounced in the acidic pH range. This indicated the irreversible denaturation of trypsin at acidic pHs (Klomklao et al., 2007d). However, a slight decrease in activity was found at pH above 10.0. Trypsins from menhaden and mullet (Pavlisko et al., 1999), bigeye snapper (Hau & Benjakul, 2006), New Zealand hoki (Shi et al., 2007), bluefish (Klomklao et al., 2007d) and Atlantic bonito (Klomklao et al., 2007a) were stable in the neutral and alkaline pH ranges, in which 80–100% of activity was retained. Shahidi and Kamil (2001) concluded that fish trypsins were stable in the alkaline pH range.

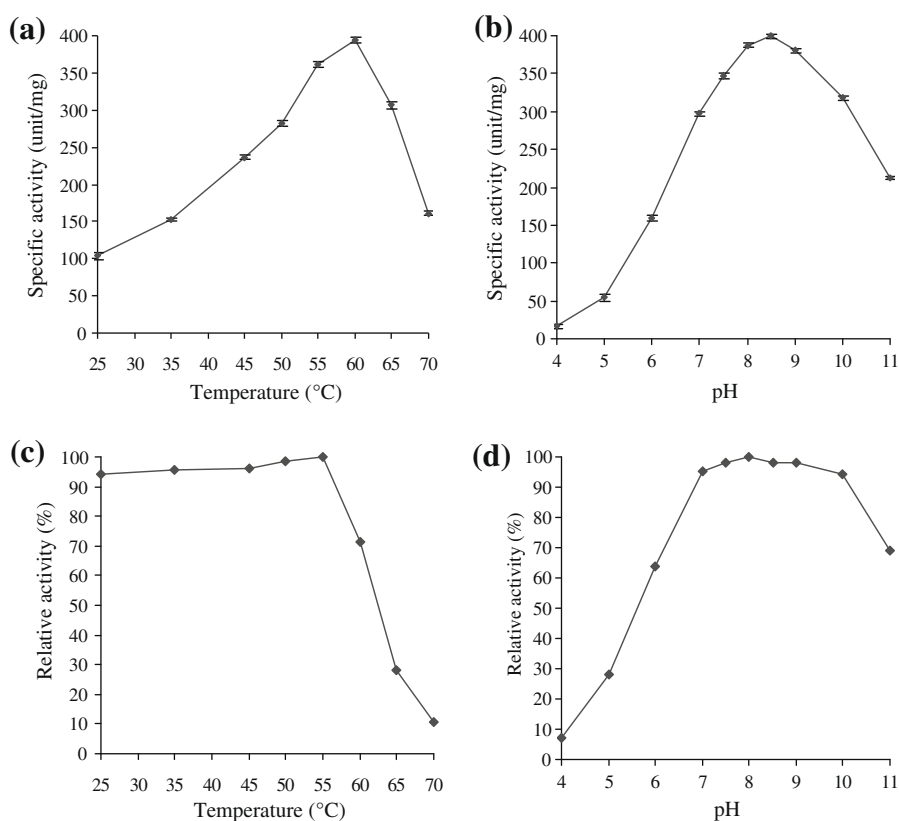


Fig. 3. Temperature (a) and pH (b) profiles and thermal (c) and pH (d) stability of purified trypsin from pyloric caeca of brownstripe red snapper. For temperature profile, the activity of trypsin was determined at pH 8.0 using BAPNA as substrate. pH profile was studied at 60 °C at various pHs. For thermal and pH stability, the remaining activity was assayed at 60 °C and pH 8.5 using BAPNA as substrate.

Table 3

Kinetic parameters of brownstripe red snapper trypsin and other fish trypsins.

Substrates	Trypsins	K_m ^A (mM)	k_{cat} ^A (s ⁻¹)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)
BAPNA	Brownstripe red snapper (<i>Lutjanus vitta</i>)	0.507	4.71	9.27
	New Zealand hoki (<i>Macruronus novaezealandiae</i>) ^a	0.06	0.33	5.5
	Chum salmon (<i>Oncorhynchus keta</i>) ^b	0.029	2.29	79.03
	Anchovy (<i>Engraulis japonica</i>) ^c	0.05	1.52	30.7
	Monterey sardine (<i>Sardinops sagax caerulea</i>) ^d	0.05	2.12	41.0
	Bigeye snapper (<i>Priacanthus macracanthus</i>) ^e	0.31	1.06	3.4
TAME	Brownstripe red snapper (<i>Lutjanus vitta</i>)	0.328	112	341
	New Zealand hoki (<i>Macruronus novaezealandiae</i>) ^a	2.08	19.00	9.1
	Chum salmon (<i>Oncorhynchus keta</i>) ^b	0.003	72.35	24117
	Anchovy (<i>Engraulis japonica</i>) ^c	0.84	39.67	47.2
	Tongol tuna (<i>Thunnus tonggol</i>) ^f	0.25	200	800

^A K_m and k_{cat} values of all trypsins were determined at 30 °C under optimal pH, except trypsins from New Zealand hoki and chum salmon, which were assayed at 25 °C.^a Shi et al. (2007).^b Sekizaki et al. (2000).^c Heu et al. (1995).^d Castillo-Yañez et al. (2005).^e Hau and Benjakul (2006).^f Klomklao et al. (2007c).

3.5. Kinetic study

Kinetic data for purified trypsin from brownstripe red snapper are summarised in Table 3. K_m and k_{cat} for the hydrolysis of BAPNA were 0.507 mM and 4.71 s⁻¹, respectively, and the corresponding values for the hydrolysis of TAME were 0.328 mM and 112 s⁻¹, respectively. The catalytic efficiencies (k_{cat}/K_m) for the hydrolysis of BAPNA and TAME were calculated to be 9.27 and 341 s⁻¹mM⁻¹, respectively. The result indicated that trypsin from brownstripe red snapper had a higher affinity for TAME than did BAPNA.

The kinetic parameters of trypsin from brownstripe red snapper were also compared with those of other fish species (Table 3). For the BAPNA hydrolysis reaction, trypsin from brownstripe red snapper had a higher K_m value, than had trypsins from New Zealand hoki (Shi et al., 2007), chum salmon (Sekizaki et al., 2000), anchovy (*Engraulis japonica*) (Heu, Kim, & Pyeun, 1995), Monterey sardine (Castillo-Yañez et al., 2005) and bigeye snapper (Hau & Benjakul, 2006). K_m is often associated with the affinity of the enzyme for substrate (Engel, 1977). Therefore, trypsin from brownstripe red snapper had a lower affinity for BAPNA than had those from other fish species. For TAME hydrolysis, a lower K_m was observed for trypsin from brownstripe red snapper, than those from New Zealand hoki (Shi et al., 2007) and anchovy (Heu et al., 1995). This indicated that trypsin from brownstripe red snapper had a higher affinity for TAME than had trypsins from New Zealand hoki and anchovy.

Trypsin showed a higher k_{cat} than did those from other fish species when BAPNA was used as a substrate. For the TAME hydrolysis reaction, trypsin from brownstripe red snapper also showed a higher k_{cat} than did those from New Zealand hoki (Shi et al., 2007), chum salmon (Sekizaki et al., 2000) and anchovy (Heu et al., 1995). However, k_{cat} of trypsin from tongol tuna (*Thunnus tonggol*) spleen (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2007c) was higher than that of trypsin from brownstripe red snapper. k_{cat} indicates the maximum number of enzymatic reactions catalysed per second (Fersht, 1977). Thus, the higher k_{cat} of trypsin suggests a higher rate of substrate hydrolysis.

Catalytic efficiency (k_{cat}/K_m) of trypsin from brownstripe red snapper was lower than those from chum salmon (Sekizaki et al., 2000), anchovy (Heu et al., 1995) and Monterey sardine (Castillo-Yañez et al., 2005) when BAPNA was used as a substrate. A lower k_{cat}/K_m was also observed with trypsin from chum salmon (Sekizaki et al., 2000) and from tongol tuna (Klomklao et al., 2007c) when TAME was used as a substrate. However, trypsin from brownstripe red snapper showed a higher catalytic efficiency than

did trypsins from New Zealand hoki and bigeye snapper when BAPNA was used as a substrate. A higher catalytic efficiency was noticeable, compared with those of New Zealand hoki and anchovy when TAME was used as a substrate. The higher catalytic efficiency, the more efficient the enzyme is in transforming the substrate to product (Fersht, 1977). This result suggests that trypsin from brownstripe red snapper was more effective in hydrolysis of BAPNA than were trypsins from New Zealand hoki and bigeye snapper and it also exhibited higher hydrolytic activity toward TAME in comparison with trypsin from New Zealand hoki and anchovy.

4. Conclusion

Based on the ability to hydrolyse specific synthetic substrates, molecular weight, optimal conditions of reaction and the response to a specific trypsin inhibitor, the purified protease from pyloric caeca of brownstripe red snapper was classified as trypsin. Trypsin exhibited the highest hydrolytic activity toward BAPNA and TAME at 60 °C and pH 8.5. Therefore, pyloric caeca may be an important source of trypsin, for further use.

Acknowledgements

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Trypsin from the Pyloric Ceca of Pectoral Rattail
(*Coryphaenoides pectoralis*): Purification and CharacterizationSAPPASITH KLOMKLAO,^{*,†} HIDEKI KISHIMURA,[‡] AND SOOTTAWAT BENJAKUL[§]

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Trypsin from the pyloric ceca of pectoral rattail (*Coryphaenoides pectoralis*) was purified and characterized. Purification was carried out by ammonium sulfate precipitation, followed by column chromatographies on Sephacryl S-200, DEAE-cellulose and Sephadex G-50. The enzyme was purified 89-fold with a yield of 2.2%. Purified trypsin had an apparent molecular weight of 24 kDa when analyzed using SDS–PAGE and size exclusion chromatography. Optimal profiles of pH and temperature of the enzyme were 8.5 and 45 °C, respectively, using *N*^ε-*p*-tosyl-L-arginine methyl ester hydrochloride as a substrate. It was stable in a wide pH range of 6–11 but unstable at a temperature greater than 40 °C. Trypsin was stabilized by calcium ion. The activity of purified trypsin was effectively inhibited by soybean trypsin inhibitor and TLCK and was partially inhibited by EDTA. Activity continuously decreased with increasing NaCl concentration (0–30%). The kinetic trypsin constants K_m and K_{cat} were 0.15 mM and 210 s⁻¹, respectively, while the catalytic efficiency (K_{cat}/K_m) was 1400 s⁻¹ mM⁻¹. The N-terminal amino acid sequence of trypsin was determined to be 12 residues (IVGGYECQEHSQ), and the sequence showed high homology to other fish trypsins.

KEYWORDS: Trypsin; purification; isolation; pyloric ceca; N-terminal amino acid sequence

INTRODUCTION

Cold-adapted organisms may possess enzymatic adaptabilities in different ways (1). The reduced enzymatic rates as a consequence of low temperatures may be compensated by increased enzyme production, change of the type of enzyme present in the system, or adaptability of preexisting enzymes (1). In the last case it would be expected that natural selection through evolution would favor enzymes with increased catalytic potential at low temperatures. The observed higher catalytic efficiency of cold-adapted enzymes was due to a more flexible tertiary structure, which enables the enzymes to lower the activation energy barrier during catalysis (1). Among this group of enzymes, the cold-adapted fish trypsins are of immense interest because they exhibit higher catalytic activity than their mammalian counterparts and preserve a high degree of activity at lower temperatures, making them more suitable for a number of biotechnological and food processing applications (2–4).

Trypsins (EC 3.4.21.4), a member of a large family of serine proteinases, specifically hydrolyze proteins and peptides at the carboxyl side of arginine and lysine residues and play major roles in biological process including digestion and activation of zymogens of chymotrypsin and other enzymes (5, 6). Trypsins have been isolated and characterized thoroughly on the basis of their physio-

chemical and enzymatic properties in a wide range of cold water as well as warm water fish such as crayfish (*Procambarus clarkii*) (7), mackerel (*Scomber japonicus*) (8), crawfish (*Procambarus clarkii*) (9), tambaqui (*Colossoma macropomum*) (10), and true sardine (*Sardinops melanostictus*) and arabesque greenling (*Pleuroprammus azonus*) (11). Recently, Klomklao et al. (5) purified and characterized three trypsin isoforms from skipjack tuna (*Katsuwonus pelamis*) spleen.

Pectoral rattail (*Coryphaenoides pectoralis*) is one of fish species adapted to temperatures below 4 °C and found in 600–1500 m depth around the northern parts of Pacific Ocean. Thus, this fish species may provide a source of specific cold adapted enzymes. On the basis of our preliminary study, the pyloric ceca from pectoral rattail showed high trypsin activity. However, the information regarding the molecular and biochemical characteristic of trypsin from the pyloric ceca of pectoral rattail has not been reported. The aims of this study were to purify and to characterize the biochemical properties of trypsin from the pyloric ceca of pectoral rattail.

MATERIALS AND METHODS

Chemicals. Ethylenediaminetetraacetic acid (EDTA), pepstatin A, soybean trypsin inhibitor, iodoacetic acid, *N*-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 1-(1-*trans*-epoxysuccinylleucylamino)-4-guanidinobutane (E-64), *N*-ethylmaleimide, β -mercaptoethanol (β ME), and bovine serum albumin were procured from Sigma Chemical Co. (St. Louis, MO). Sephacryl S-200 and

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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'*-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA).

Fish Sample Preparation. Pectoral rattail (*C. pectoralis*) was harvested along the Hakodate coast, Japan, stored in ice, and off-loaded approximately 24–26 h after catching. Fish were then transported in ice with a fish/ice ratio of 1:2 (w/w) to the Laboratory. Viscera were then excised and separated into individual organs. Only pyloric ceca was collected, immediately frozen, and stored at –20 °C until used.

Preparation of Crude Enzyme Extract. Frozen pyloric ceca 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 in three volumes of acetone at –20 °C for 30 min according to the method of Klomklao et al. (12). The homogenate was filtrated in vacuo 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 until dry and free of acetone odor.

To prepare the crude extract, the pyloric ceca powder was suspended in 10 mM Tris-HCl, pH 8.0, containing 1 mM CaCl₂ referred to as starting buffer (SB) at a ratio of 1:50 (w/v) and stirred continuously at 4 °C for 3 h. The suspension was centrifuged for 20 min at 4 °C at 20000g (H-200, Kokusan, Tokyo, Japan) to remove the tissue debris, and then the supernatant was lyophilized. Before use, the lyophilized sample (10 g) was dissolved with 50 mL of cold distilled water (4 °C) and referred to as “crude extract”.

Trypsin Purification. All purification steps were carried out at 4 °C. Crude extract was subjected to ammonium sulfate precipitation at 30–70% saturation and allowed to stand for 2 h. The precipitate was collected by centrifugation at 25000g for 20 min. The pellet obtained was dissolved in a minimal volume of SB and dialyzed with 10 volumes of SB with three changes overnight. The dialysate was then applied to a Sephacryl S-200 column (3.9 cm × 64 cm), which was equilibrated with SB. The separation was conducted using the SB at a flow rate of 0.5 mL/min, and 5 mL fractions were collected. The fractions containing trypsin were pooled. Pooled Sephacryl S-200 column fractions were dialyzed against SB for 10–12 h. The sample was then chromatographed on DEAE-cellulose (Whatman, England) column (2.2 cm × 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 below 0.05 and then eluted with a linear gradient of 0–0.25 M NaCl in SB at a flow rate of 0.5 mL/min. Fractions of 5 mL were collected, and those with TAME activity were pooled. Pooled fractions were dialyzed with SB with 10–12 h and then concentrated by lyophilization. After the first DEAE-cellulose chromatography, trypsin fractions were rechromatographed on the same column equilibrated with SB. The elution was performed with stepwise process using SB containing different NaCl concentrations: 0, 0.1, and 0.2 M, respectively, at a flow rate of 0.5 mL/min. Fractions of 5 mL were collected, and those with high trypsin activity were combined. Pooled fractions with TAME activity from second DEAE-cellulose column were dialyzed against SB for 10–12 h. The dialysate was lyophilized and then dissolved in distilled water prior to Sephadex G-50 column (3.9 cm × 64 cm) chromatography. Sample was loaded to the column and then eluted with the same buffer at a flow rate of 0.5 mL/min. Fractions of 5 mL were collected, and fractions with TAME activity were pooled and used for further study.

During purification, protein concentration was measured at 280 nm and trypsin activity was determined using TAME as a substrate.

Trypsin Activity Assay. Trypsin activity was measured by the method of Hummel (13) as modified by Klomklao et al. (14) 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*-tosylarginine 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.

pH and Temperature Profile. Trypsin activity was assayed over the pH range of 4.0–11.0 (50 mM acetate buffer for pH 4.0–7.0; 50 mM Tris-HCl buffer for pH 7.0–9.0, and 50 mM glycine–NaOH for pH 9.0–11.0) at 30 °C for 20 min. For 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.

pH and Thermal Stability. The effect of pH on enzyme stability was evaluated by measuring the residual activity after incubation at various pH values for 30 min at 30 °C. Different buffers used were mentioned above. 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.

Determination of Molecular Weight. The molecular weight of purified trypsin was determined using a size exclusion chromatography on Sephacryl S-200 column. The trypsin separated by size exclusion chromatography was estimated for its molecular weight by plotting the 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*₀) 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).

Effect of CaCl₂ on Thermal Stability. The effect of CaCl₂ on thermal stability was determined by heating the enzyme dissolved in 50 mM Tris-HCl, pH 8.0, in the presence of 2 mM EDTA or 2 mM CaCl₂ 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.

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.

Effect of Inhibitors. The effect of inhibitors on trypsin activity was determined according to the method of Klomklao et al. (15) 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.

Electrophoresis. 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 (0.125 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-Protein 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 manner similar to that for SDS–PAGE except that the sample was not heated and SDS and reducing agent were left out.

Determination of N-Terminal Amino Acid Sequence. The purified enzyme was 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) by injection on Spheri-5PTH column (220 mm × 2.1 mm) linked to an HPLC system. The system was calibrated using a PTH residue standard kit (Sigma-Aldrich, St. Louis, MO) containing all the 20 common amino acids.

Kinetic Studies. The activity was assayed with different final concentrations of TAME ranging from 0.01 to 0.10 mM. The final enzyme

Table 1. Purification of Trypsin from the Pyloric Ceca of Pectoral Rattail

purification steps	total activity (units) ^a	total protein (mg)	specific activity (units mg ⁻¹ protein)	purity (fold)	yield (%)
crude extract	402.0	3415.0	0.1	1	100
(NH ₄) ₂ SO ₄ (30–70%)	354.8	548.6	0.6	6	88.3
Sephacryl S-200	133.2	100.9	1.3	13	33.1
first DEAE-cellulose	41.2	8.1	5.1	51	10.2
second DEAE-cellulose	15.6	2.4	6.5	65	3.9
Sephadex G-50	8.9	1.0	8.9	89	2.2

^a One unit of activity was defined as the amount causing an increase of 1.0 in absorbance at 247 nm per min. Trypsin activity was assayed at pH 8.0, 30 °C for 20 min using TAME as a substrate.

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 (17). Values of turnover number (K_{cat}) were calculated from the following equation: $V_{\max}/[E] = K_{\text{cat}}$, where $[E]$ is the active enzyme concentration and $V_{\max}$ is the maximal velocity.

Protein Determination. Protein concentration was measured by the method of Lowry et al. (18) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Trypsin Purification. A summary of trypsin purification is presented in Table 1. An increase in purity of 6.0-fold was obtained when 30–70% ammonium sulfate was used. The ammonium sulfate precipitation process was able to fractionate the enzyme of interest. Moreover, ammonium sulfate precipitation was normally introduced as an initial step for removal of other proteins in trypsin purification (19). Castillo-Yanez et al. (20) reported that ammonium sulfate precipitation of trypsin from the pyloric ceca of Monterey sardine at 30–70% resulted in the increase in specific activity by 1.3-fold.

Ammonium sulfate fraction was further purified by gel filtration on Sephacryl S-200. Purification fold of 13.0 with a yield of 33.1% was observed after this step. Gel filtration was used to remove other proteins in true sardine viscera trypsin fraction, leading to the higher purity of trypsin (11).

Pooled active Sephacryl S-200 fractions were further purified using DEAE-cellulose column. After loading and washing, the column was eluted by using a 0–0.2 M NaCl linear gradient. Only one activity peak was found. A 51-fold increase in purity with a yield of 10.2% was observed. To refine the pooled fraction obtained from previous step, pooled active fractions were subjected to a second DEAE-cellulose column chromatography. After this step, a large amount of contaminated proteins was removed, resulting in a substantial increase in purification fold. Purity was increased by 65 with yields of 3.9%. Yoshinaka et al. (21) also chromatographed the trypsin from the eel viscera on an anion exchanger, DEAE-cellulose, twice, and two anionic trypsins were obtained with 22-fold purity. However, it was not completely separated from other contaminating proteins. Therefore, trypsin containing fractions were rechromatographed on Sephadex G-50.

When second DEAE-cellulose sample with trypsin activity was subjected to gel filtration on Sephadex G-50, a single peak was obtained. Purification fold of 89 with a yield of 2.2% was observed. Bezerra et al. (19) found that the use of gel filtration on Sephadex G-75 in the final step of the purification process of trypsin from the intestine of Nile tilapia led to an increase in trypsin activity by 21.68-fold.

Purity and Molecular Weight. Purity of the purified trypsin was examined via native gel electrophoresis. The enzyme was observed to migrate as a single band on native PAGE (Figure 1a), suggesting the homogeneity of the enzyme. For SDS–PAGE,

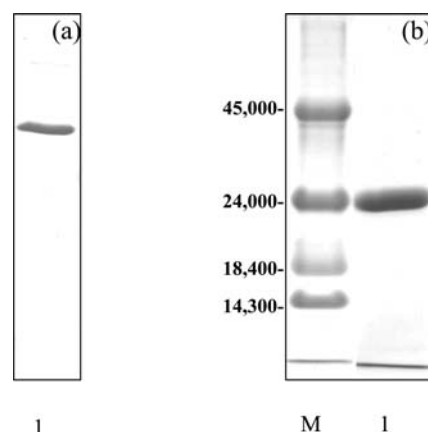


Figure 1. Protein pattern of purified trypsin from the pyloric ceca of pectoral rattail by native PAGE (a) and SDS–PAGE (b): M, molecular weight standards of egg albumin (45 kDa), bovine pancreatic trypsinogen (24 kDa), bovine-milk-betactoglobulin (18.4 kDa), and egg white lysozyme (14.3 kDa); lane 1, purified trypsin.

purified trypsin migrated as a single protein band at 24 kDa (Figure 1b). Also, the molecular mass of the enzyme was estimated to be 24 kDa by Sephacryl S-200 gel filtration (data not shown). These results demonstrated that purified trypsin from pectoral rattail pyloric ceca is a monomeric protein with the molecular mass of 24 kDa. Generally, fish trypsin has been reported to have a molecular weight in the range of 23–28 kDa. Purified alkaline protease from the viscera of boliti fish had a molecular weight of 23 kDa (22). The molecular mass of trypsin from the pyloric ceca of arabesque greenling was estimated to be approximately 24 kDa by SDS–PAGE (11). However, a slightly higher molecular weight (28 kDa) was reported for trypsin of starfish (23). Cao et al. (6) reported that the molecular weights of two trypsins (A and B) from carp hepatopancreas were estimated to be 28.5 and 28 kDa, respectively.

Optimal pH and Temperature. Trypsin from the pyloric ceca from pectoral rattail exhibited a maximal activity at pH 8.5 (Figure 2a). The activity of purified trypsin sharply decreased above optimum pH because of the denaturation of enzyme. The decrease in activity was also found in acidic pH ranges. Under acidic and alkaline pHs, the changes in enzyme conformation were possibly caused by charge repulsion. At extreme acidic or alkaline pH, protein or enzyme unfolds because of a decrease in electrostatic bonds (24). The optimum pH value of purified trypsin was similar to that of trypsin isolated from Monterey sardine pyloric ceca, which had an optimum of pH 8.0 when BAPNA was used as a substrate (20). Bezerra et al. (19) found that trypsin-like enzyme from Nile tilapia intestine had optimal pH of 8.0 when azocasein was used.

The effect of various temperatures on trypsin activity is depicted in Figure 2b. Purified trypsin showed the highest activity

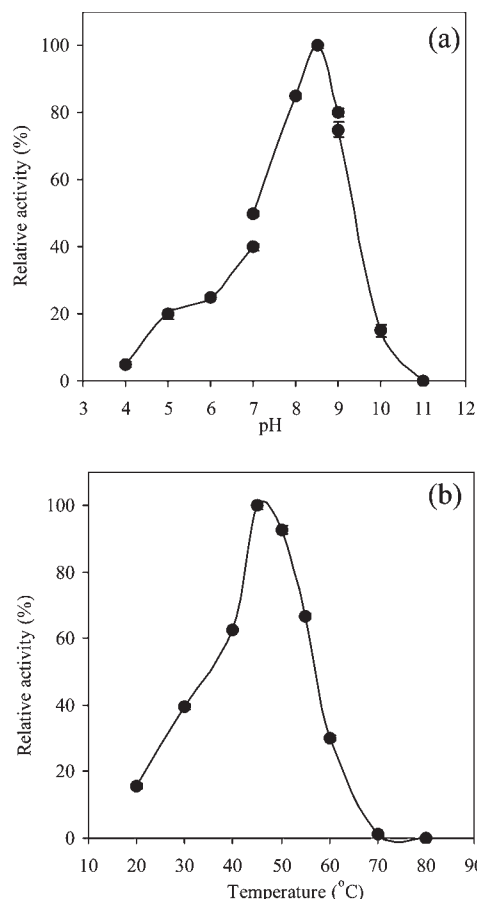


Figure 2. pH (a) and temperature (b) profiles of purified trypsin from the pyloric ceca of pectoral rattail. Bar indicates standard deviation from triplicate determination.

at 45 °C. A very sharp decrease in activity was observed above 50 °C. When assayed at 70 °C, no activity was found, presumably as a result of thermal inactivation. Enzymes were inactivated at high temperature, possibly because of the partial unfolding of the enzyme molecule. This result was similar to that of alkaline protease from the viscera of boliti fish, which had optimum temperatures of 45 °C (22). Heu et al. (25) also reported that trypsin from the viscera of anchovy had optimum temperature at 45 °C. However, the optimal temperature of the purified trypsin from the pyloric ceca of pectoral rattail is lower than that reported for trypsin from the pyloric ceca of bluefish, which had maximal activity at 55 °C (14). Bezerra et al. (10) reported that alkaline serine proteinase from tambaqui pyloric ceca exhibited optimal activity at 60 °C. The difference might be related to the difference in the temperatures of water where fish inhabited.

pH and Thermal Stability. The purified enzyme was stable over a wide pH range (6–11) (Figure 3a). However, at pH 5.0, the activity was slightly decreased and no activity was detected after incubation at pH 4.0. The stability of trypsin at a particular pH might be related to the net charge of the enzyme at that pH (20). Diminished stability at acidic pHs was found for trypsin from several fish species (4, 5, 11). From the result, the trypsin might undergo the denaturation under the acidic condition, where the conformational change took place and enzyme could not bind to the substrate properly.

The thermal stability of the purified trypsin was examined for 15 min in the temperature range of 20–80 °C (Figure 3b). The result showed that purified trypsin was stable up to 40 °C and slightly lost its stability at 50 °C. The purified trypsin was not

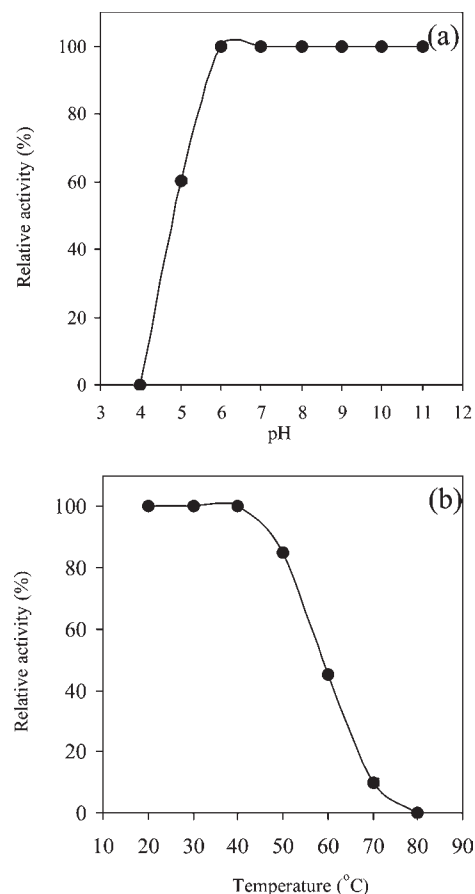


Figure 3. pH (a) and thermal (b) stability of purified trypsin from the pyloric ceca of pectoral rattail. Bar indicates standard deviation from triplicate determination.

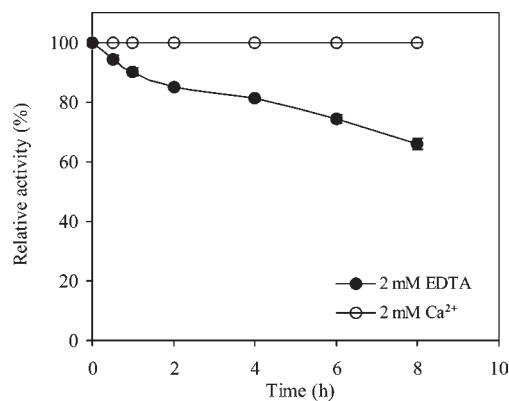


Figure 4. Effect of calcium ion and EDTA on the stability of purified trypsin from the pyloric ceca of pectoral rattail. The stability was tested at 40 °C for different times. Bar indicates standard deviation from triplicate determination.

stable at higher temperature. Approximately 45% of activity was retained after heating at 60 °C, but no trypsin activity was remained when heated to 80 °C. At high temperature, the enzyme possibly underwent denaturation and lost its activity. The result was in agreement with Castillo-Yanez et al. (20) who reported that trypsin from the pyloric ceca of Monterey sardine was stable up to 40 °C but unstable at temperatures higher than 40 °C. Trypsin from the viscera of anchovy was not stable at temperatures higher than 45 °C (25).

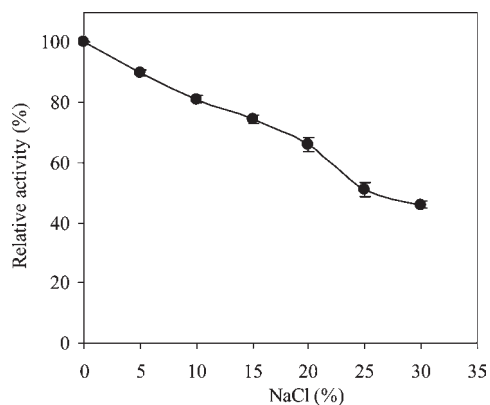


Figure 5. Effect of NaCl concentrations on the activity of purified trypsin from the pyloric ceca of pectoral rattail. Bar indicates standard deviation from triplicate determination.

Effect of Calcium Ions on Thermal Stability. The thermal stability of the purified trypsin from pectoral rattail pyloric ceca was highly dependent on the presence of calcium (Figure 4). Total activity was retained throughout 8 h of incubation at 40 °C in the presence of 2 mM calcium ion, while in the presence of 2 mM EDTA, the activity of purified trypsin decreased with increasing the time. These results indicated that pectoral rattail required calcium ion for its stability. Fish trypsin was stabilized by the calcium ion similarly to porcine pancreatic trypsin (11). Two calcium binding sites are presented in the bovine trypsinogen. The primary site, with a higher affinity for calcium ions, is common in trypsinogen and trypsin, and the secondary site is only found in the zymogen (4, 5, 11). The occupancy of the primary calcium-binding site stabilizes the protein toward thermal denaturation or autolysis (11). From the result, it can be concluded that purified trypsin from pyloric ceca of pectoral rattail possesses a primary calcium-binding site as reported in trypsin from tongol tuna (26), skipjack tuna (5), yellowfin tuna (4), and true sardine (11). Therefore, the stability of trypsin from pectoral rattail can be maintained by the addition of calcium ion.

Effect of NaCl. The activity of purified trypsin decreased with increasing NaCl concentration (0–30%) (Figure 5). Remaining activity of the enzyme at 30% NaCl was approximately 48%. The activity loss might be due to the denaturation of trypsin caused by the “salting out” effect (27). NaCl at higher concentration possibly competed with enzyme in water binding, resulting in a stronger protein–protein interaction, which was possibly associated with precipitation (24). This led to a loss in activity in the presence of high NaCl concentration. Ishida et al. (28) reported that thermostable proteinase in salted anchovy muscle was still active and able to degrade myofibrillar protein in commercial salted fillets containing 16–17% NaCl. From the result, the activity of 50% remained at 20% NaCl. Thus, trypsin from the pyloric ceca of pectoral rattail may have potential in accelerating the fish sauce fermentation.

Effect of Inhibitors. To confirm the identity of the purified trypsin from pectoral rattail pyloric ceca, the effect of various protease inhibitors on its activity was determined and is shown in Table 2. Among all proteinase inhibitors, soybean trypsin inhibitor and TLCK exhibited the highest inhibitor (91–100%). Inhibitors for chymotrypsin (TPCK), aspartic (pepstatin A), and cysteine (E-64, *N*-ethylmaleimide, iodoacetic acid) proteinases had no inhibitory effect on trypsin activity. The inhibition pattern of purified trypsin was similar to trypsins from other fish species, such as anchovy and Monterey sardine (20, 25). Bezerra et al. (10)

Table 2. Effect of Various Inhibitors on the Activity of Purified Trypsin from the Pyloric Ceca of Pectoral Rattail^a

inhibitors	concentration	inhibition (%) ^b
control		0
E-64	0.1 mM	0
<i>N</i> -ethylmaleimide	1 mM	0
iodoacetic acid	1 mM	0
soybean trypsin inhibitor	1.0 g/L	100
TLCK	5 mM	91.1 ± 0.34
TPCK	5 mM	0
pepstatin A	1 mM	0
EDTA	2 mM	10.1 ± 0.03

^a Each enzyme solution was incubated with the same volume of inhibitor at 25 °C for 15 min, and residual activity was analyzed using TAME as a substrate for 20 min at pH 8.0 and 30 °C. ^b Mean ± SD from triplicate determination.

Table 3. Kinetic Properties of Pectoral Rattail Pyloric Ceca Trypsin for the Hydrolysis of TAME^a

enzyme	K_m (mM)	K_{cat} (s ⁻¹)	K_{cat}/K_m (s ⁻¹ mM ⁻¹)
trypsin	0.15	210	1400

^a K_m and K_{cat} values were determined using TAME as a substrate at pH 8.0 and 30 °C. The final enzyme concentration for the assay was 0.1 mg/mL.

also reported that trypsin-like enzyme from tambaqui pyloric ceca was inhibited by some trypsin inhibitors, such as PMSF, benzamidine, and TLCK. From the result, it was noted that approximately 10% inhibition was found with 2 mM EDTA, indicating that the enzyme required metal ions for its activity. EDTA possibly affected the structure of enzyme or active site conformation, leading to the reduced activity. Moreover, EDTA could chelate the ion required for activity of enzymes. This might lead to the less availability of these ions. The inhibitory results confirmed that purified enzyme was trypsin, which possibly required metal ions for its activity.

Kinetic Study. The K_m and K_{cat} values were calculated using Lineweaver–Burk plots and are presented in Table 3. K_m and K_{cat} were 0.15 mM and 210 s⁻¹, respectively. The low K_m value obtained from pectoral rattail trypsin was close to those reported for trypsins from Atlantic croaker (29) and Greenland cod (3). In contrast, this K_m value was lower than those of tongol tuna (26) and yellowfin tuna (4). Therefore, purified trypsin exhibited higher affinity to TAME than those from tongol tuna and yellowfin tuna. The catalytic efficiency (K_{cat}/K_m) of pectoral rattail trypsin, 1400 s⁻¹ mM⁻¹, was close to those of trypsins from crayfish (7). Furthermore, the catalytic efficiency of purified enzyme was higher than those of mammalian trypsins reported (30).

N-Terminal Sequence. As shown in Figure 6, 12 residues of the N-terminal amino acid sequence of the purified trypsin were determined. The N-terminal amino acid sequences of purified trypsin were IVGGYECQEHSQ. N-Terminal amino acid sequences of purified enzyme were compared with those of skipjack tuna (5), true sardine, arabesque greenling (11), cod (31), salmon (32), dogfish (33), dog (34), porcine (35), and bovine (36). It was found that the sequence of the purified trypsin showed high homology to other trypsins, especially the sequence from the first to seventh residues. However, all fish trypsins had a charged Glu residue at position 6, while Thr is most common in mammalian pancreatic trypsin (Figure 6). The N-terminal sequence clearly demonstrated that purified trypsin from pectoral rattail pyloric ceca was most likely a member of the trypsin family.

Conclusion. The pectoral rattail enzyme purified in this research was shown to be true member of the trypsin family

	5	10
Trypsin	I V G G Y E C Q E H S Q	
Skipjack tuna	I V G G Y E C Q A H S Q	
True sardine	I V G G Y E C K A Y S Q	
Arabesque greenling	I V G G Y E C T P H T Q	
Cod	I V G G Y E C T K H S Q	
Salmon	I V G G Y E C K A Y S Q	
Dogfish	I V G G Y E C P K H A A	
Dog	I V G G Y T C E E N S V	
Porcine	I V G G Y T C A A N S V	
Bovine	I V G G Y T C G A N T V	

Figure 6. Comparison of N-terminal amino acid sequence of the purified trypsin from the pyloric ceca of pectoral rattail with other enzymes: skipjack tuna (5), true sardine, arabesque greenling (11), cod (31), salmon (32), dogfish (33), dog (34), porcine (35), and bovine (36).

of serine proteases. Confirmation included determination of molecular weight, substrate specificity, inhibitor study, and N-terminal sequencing. Enzymatic characteristics were essentially consistent with those of trypsin from other species.

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**CATIONIC TRYPSIN: A PREDOMINANT PROTEINASE IN
PACIFIC SAURY (*COLOLABIS SAIRA*) PYLORIC CECA**

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ABSTRACT

Cationic trypsin was purified from Pacific saury (Cololabis saira) pyloric ceca by the successive steps of ammonium sulfate precipitation, anion exchange chromatography and gel filtration chromatography. The purification and yield were 90-fold and 8.9%, respectively. The molecular weight was determined to be 24 kDa using Sephacryl S-200 and SDS-PAGE. Cationic trypsin was stable at pH 7–11 for 30 min at 30C, and its maximal activity against L-arginine methyl ester hydrochloride (TAME) was at pH 8.5. Thermostability for cationic trypsin was up to 50C for 15 min and its temperature

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optimum was 60°C. Cationic trypsin was stabilized by calcium ion. Activity decreased as NaCl concentration (0–30%) increased. Inhibitor susceptibility analysis revealed that the enzyme was inhibited effectively by soybean trypsin inhibitor and N-p-tosyl-L-lysine chloromethyl ketone. The K_m and K_{cat} of the enzyme were 0.17 mM and 200 s⁻¹, respectively. The N-terminal amino acid sequence of cationic trypsin was partially determined as IVGGYECQPH- and was very homologous to other trypsins.

PRACTICAL APPLICATIONS

Trypsin is a major member of the serine proteases, which constitute a large family of biologically important enzymes. Trypsins from various sources catalyze the hydrolysis of peptide bonds on the carboxyl sides of arginine and lysine. Hence, it is expected that like other trypsins, Pacific saury trypsin would also be used for processing aids in industry applications. Most trypsins are used for a variety of products in the food industry including baked foods, beer, wine, cereal, meat and fish products, and for production of protein hydrolysates and flavor extract. Trypsins are also used in mammalian cell culture to disaggregate adherent cells for research and the production of recombinant proteins, in diabetes diagnosis and therapy, in detergents and bating of leather.

INTRODUCTION

Enzymes are used as food-processing aids to improve product quality and stability. Approximately 60% of the enzymes used as industrial processing aids are proteinases (Haard 1992).

Fish digestive organs are one of the good sources for recovering proteolytic enzyme. They are a cheap source of proteinases whose utilization is one way of increasing the value of a resource. Also, proteinases from fish viscera may have some unique properties for industrial applications, e.g., laundry detergents, feed, leather bathing, silk degumming, cheese making, chill-proofing, meat tenderizing and fermented sauces (Simpson and Haard 1984; Haard 1992). Recently, the use of alkaline proteinases, especially trypsin, has increased remarkably because they are both stable and active under harsh conditions, such as at temperatures of 50–60°C, high pHs and in the presence of surfactants or oxidizing agents (Klomklao *et al.* 2005).

Trypsin, a member of a large family of serine proteases, specifically hydrolyzes proteins at the carboxyl side of arginine and lysine residues and

plays major roles in biological processes including digestion, activation of zymogens of chymotrypsin and other enzymes (Simpson 2000). Trypsins have been isolated and characterized thoroughly based on their physiochemical and enzymatic properties from several species of fish, e.g., crayfish (Kim *et al.* 1992, 1994), mackerel (Kim and Pyeun 1986), carp (Cao *et al.* 2000), crawfish (Jeong *et al.* 2000), capelin (Hjelmeland and Raa 1982), tambaqui (Bezerra *et al.* 2001), true sardine and arabesque greenling (Kishimura *et al.* 2006), skipjack tuna (Klomklao *et al.* 2007), yellowfin tuna (Klomklao *et al.* 2006a) and tongol tuna (Klomklao *et al.* 2006b).

Pacific saury (*Cololabis saira*) is one of the important fish species cultured in Japan. Also, it is used almost exclusively for food production. Based on our preliminary study, Pacific saury pyloric ceca has been reported to possess the high trypsin activity and two groups of trypsin including cationic trypsin (the major trypsin) and anionic trypsin were found. Understanding the biochemical characteristics of cationic trypsin which is the predominant enzyme in Pacific saury pyloric ceca may provide the important information for industrial application. Nevertheless, no information regarding the molecular and biochemical characteristics of cationic trypsin from the pyloric ceca of Pacific saury has been studied. This study aimed to purify and to characterize some molecular and enzymatic properties of major trypsin in pyloric ceca of Pacific saury.

MATERIALS AND METHODS

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 (β ME) and bovine serum albumin were procured from Sigma Chemical Co. (St. Louis, MO). Sephadex G-50 was 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).

Fish Sample Preparation

Fresh Pacific saury were purchased from the local fish market in Hakodate, Japan. The fish were transported in ice with a fish–ice ratio of 1:2 (w/w) to the Laboratory of Marine Products and Food Science, Research Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Japan within 30 min. Viscera were then excised carefully. Only pyloric ceca was collected, immediately frozen and stored at -20°C until used.

Preparation of Crude Extract

Frozen pyloric cecas were thawed using running water ($26\text{--}28^{\circ}\text{C}$) until the core temperature reached $-2\text{--}0^{\circ}\text{C}$. 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 vacuo* on ADVANTEC 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 until dry and free of acetone odor.

To prepare the extract, pyloric ceca powder was suspended in 10 mM Tris-HCl, pH 8.0 containing 1 mM CaCl_2 referred to as starting buffer (SB) at a ratio of 1:50 (w/v) and stirred continuously at 4°C for 3 h. The suspension was centrifuged for 10 min at 4°C at 10,000 g (H-200, Kokusan, Tokyo, Japan) to remove the tissue debris, and then the supernatant was lyophilized. Before use, the lyophilized sample was dissolved with distilled water and referred to as “crude extract.”

Cationic Trypsin Purification

All purification processes were carried out in a walk-in cold room (4°C). Purification of cationic trypsin from crude enzyme solution was undertaken by first employing ammonium sulfate precipitation with 30–70% saturation. The precipitate was collected and dissolved in a minimal volume of cold SB. The mixture was then dialyzed against SB overnight. The dialysate was immediately applied to a DEAE-cellulose (Whatman) column (2.2×18 cm) equilibrated with SB at a flow rate of 0.5 mL/min. The column was washed with SB until no absorbance at 280 nm was detected and then eluted with a linear gradient ranging from 0 to 0.5 M NaCl in SB at a flow rate of 0.5 mL/min. Fractions of 5 mL were collected and those with TAME activity were pooled. Trypsin activities were detected in both the unadsorbed and adsorbed fraction. However, only the unadsorbed fractions showed the main trypsin fractions were selected for further study. After DEAE-cellulose column chromatography, the flow-through fractions referred to as cationic trypsin were pooled,

dialyzed and then concentrated by lyophilization. Lyophilized cationic trypsin fractions were dissolved in cold distilled water and loaded onto a Sephadex G-50 column chromatography (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 5 mL were collected and the fractions with TAME activity were pooled and used for further study.

During purification, the fractions obtained were measured protein concentration at 280 nm, and the trypsin activity was measured using TAME as a substrate at pH 8.0 and 30C.

Trypsin Activity Assay

Trypsin activity was measured by the method of Hummel (1959) as modified by Klomklao *et al.* (2006a) 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 30C 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.

pH and Temperature Profile

Trypsin activity was assayed over the pH range of 4.0–11.0 (50 mM acetate buffer for pH 4.0–7.0, 50 mM Tris-HCl buffer for pH 7.0–9.0 and 50 mM glycine-NaOH for pH 9.0–11.0) at 30C for 20 min. For temperature profile study, the activity was assayed at different temperatures (20, 30, 40, 50, 55, 60, 65, 70 and 80C) for 20 min at pH 8.0.

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 30C. Different buffers used were mentioned above. For thermal stability, enzyme solution was diluted with 100 mM Tris-HCl, pH 8.0 at a ratio of 1:1 and incubated at different temperatures (20, 30, 40, 50, 60, 70 and 80C) for 15 min in a temperature-controlled water bath (Mettmert, Germany). Thereafter, treated samples were suddenly cooled in iced water. The residual activity was assayed using TAME as a substrate at pH 8.0 and 30C for 20 min.

Effect of Inhibitors

The effect of inhibitors on trypsin activity was determined according to the method of Klomklao *et al.* (2004) 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.

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.

Effect of CaCl₂ on Thermal Stability

The effect of CaCl₂ on thermal stability was determined by heating the enzyme dissolved in 50 mM Tris-HCl, pH 8.0 in the presence of 2 mM EDTA or 2 mM CaCl₂, at 40°C. After a specific heating time (0, 0.5, 1, 2, 4, 6 and 8 h), the samples were cooled in iced water and assayed for remaining activity.

SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (1970). Protein solutions were mixed at 1:1 (v/v) ratio with the SDS-PAGE sample buffer (0.125 M Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 10% 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.

Size Exclusion Chromatography

The molecular weight of purified trypsin 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 2000000). The standards used included aprotinin (M_r 6500), trypsinogen (M_r 24000), bovine serum albumin (M_r 66000) and catalase (M_r 232000).

Determination of N-Terminal Amino Acid Sequence

The purified enzyme was 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).

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 (Lineweaver and Burk 1934). Values of turnover number (K_{cat}) were calculated from the following equation: $V_{\max}/[E] = K_{\text{cat}}$, where $[E]$ is the active enzyme concentration and $V_{\max}$ is the maximal velocity.

Protein Determination

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

RESULTS AND DISCUSSION

Cationic Trypsin Purification

Table 1 summarizes the purification steps of cationic trypsin from the pyloric ceca of Pacific saury. The specific activity and purification fold were

TABLE 1.
PURIFICATION OF CATIONIC TRYPSIN FROM PACIFIC SAURY PYLORIC CECA

Purification steps	Total activity (U) [†]	Total protein (mg)	Specific activity (U/mg protein)	Purity (fold)	Yield (%)
Crude extract	486.0	3200	0.15	1.0	100.0
(NH ₄) ₂ SO ₄ (30–70%)	521.6	619.3	0.84	5.6	107.3
DEAE-cellulose	111.0	39.0	2.85	19.0	22.8
Sephadex G-50	43.2	3.2	13.5	90.0	8.9

[†] One unit of activity was defined as the amount causing an increase of 1.0 in absorbance at 247 nm per min. Trypsin activity was assayed at pH 8.0, 30°C for 20 min using L-arginine methyl ester hydrochloride (TAME) as a substrate.

0.84 U/mg protein and 5.6, respectively, when 30–70% ammonium sulfate was used. The total activity slightly increased after this step, suggesting that the enzyme became fully activated. The increase of total activity may be due to the activation of trypsinogen to the active trypsin. This finding is similar to the observation of Simpson and Haard (1984) in the purification of Greenland cod trypsin, indicating the presence of naturally occurring trypsin inhibitors that prevent autocatalytic activation of trypsinogen.

When the ammonium sulfate fractions were chromatographed on a DEAE-cellulose column, two trypsin activities were recovered as adsorbed and unadsorbed fractions. Major trypsin activity was found in unadsorbed fractions which were presumed to be cationic trypsin, while the minor trypsin activity was found after eluting with a linear gradient ranging from 0 to 0.5 M NaCl. The results suggested that two major groups of trypsin were present in the ammonium sulfate sample. However, only the major trypsin was further purified using Sephadex G-50 column chromatography. After DEAE-cellulose column, a large amount of contaminated proteins were removed, resulting in a substantial increase in purification fold. Purification fold of 19 with a yield of 22.8% was obtained by this step. Kishimura and Hayashi (2002) found that purification of cationic trypsin from the pyloric ceca of starfish by using anion exchanger, DEAE-cellulose was achieved and purification fold of 22 was obtained.

After Sephadex G-50 column chromatography, purity of the enzyme was increased 90-fold with an 8.9% recovery from the crude extract. Klomklao *et al.* (2006b) found that the use of the gel filtration on Sephadex G-50 in the final step of purification process of trypsin from tongol tuna spleen led to increase in trypsin activity by 401.7-fold.

SDS-PAGE

The molecular weight of purified cationic trypsin was estimated to be 24 kDa based on SDS-PAGE (Fig. 1), corresponding to that determined by Sephacryl S-200 gel filtration (data not shown). The results demonstrated that it is a monomeric protein with the molecular weight of 24 kDa. This molecular weight is similar to values reported for other trypsins, such as true sardine (Kishimura *et al.* 2006), anchovy (Kishimura *et al.* 2005), skipjack tuna (Klomklao *et al.* 2007), yellowfin tuna (Klomklao *et al.* 2006a) and tongol tuna (Klomklao *et al.* 2006b), etc., and is within the range of molecular weights for trypsins, from 20 to 25 kDa (Keil 1971).

pH Optimum and Stability

The pH dependence of cationic trypsin is depicted in Fig. 2a. The enzyme gave the maximal activity at pH 8.5. The enzyme displayed acidic lability

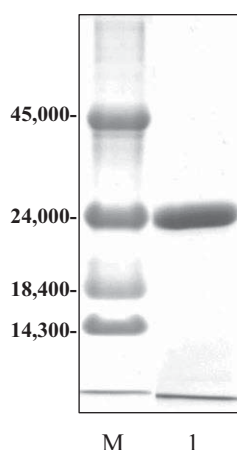


FIG. 1. SDS-PAGE OF PURIFIED CATIONIC TRYPSIN FROM PACIFIC SAURY PYLORIC CECA

M: molecular weight standard included egg albumin (45,000 Da), bovine pancreatic trypsinogen (24,000 Da), bovine milk β -lactoglobulin (18,400 Da) and egg white lysozyme (14,300 Da); lane 1: purified cationic trypsin.

below pH 6.0, probably because of the denaturation of enzyme. The decrease in activity was also found in the alkaline pH ranges of more than 9. Under acidic and alkaline pHs, the changes in enzyme conformation was possibly caused by charge repulsion, which is associated with a decrease in electrostatic bonds (Klomklao *et al.* 2005). The apparent pH-activity profiles of cationic trypsin from Pacific saury pyloric ceca was similar to that of trypsin from skipjack tuna (Klomklao *et al.* 2007), yellowfin tuna (Klomklao *et al.* 2006a) and true sardine (Kishimura *et al.* 2006).

Figure 2b shows the effect of pH on the stability of the enzyme. Purified cationic trypsin from pyloric ceca of Pacific saury was stable in the neutral and alkaline pH range (7–11). However, at pH values below 6, the stability of the enzyme decreased sharply. No activity was observed after incubation at pH 4, suggesting irreversible denaturation of trypsin at acidic pHs. Diminished stability at acidic pHs has been observed for trypsin from several fish species (Hjelmeland and Raa 1982; Kristjansson 1991; Kishimura *et al.* 2005; Klomklao *et al.* 2006a, 2007).

Temperature Optimum and Stability

Figure 3a illustrates the influence of different temperatures on the cationic trypsin activity. Trypsin showed the highest activity at 60°C. A sharp decrease in activity was found at temperature above 60°C, presumably as a

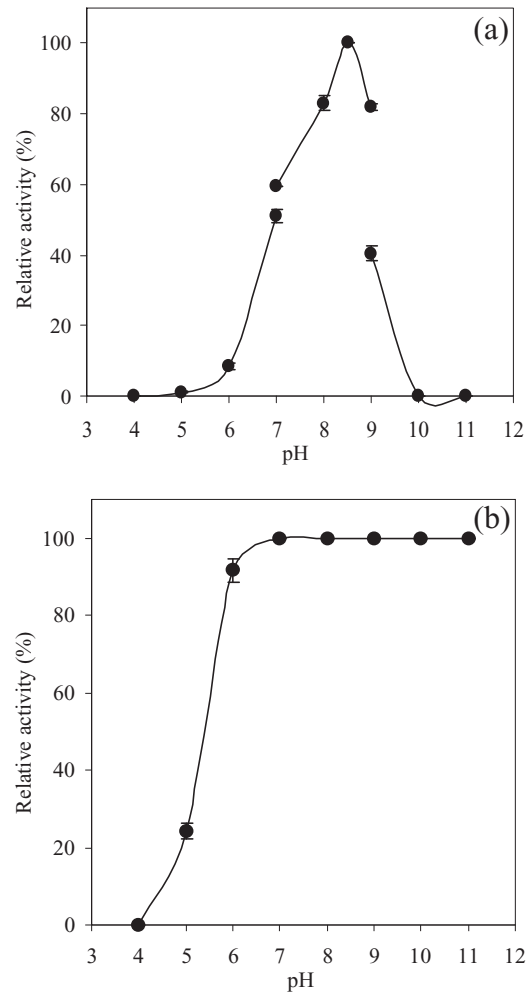


FIG. 2. PH PROFILE (a) AND PH STABILITY (b) OF PURIFIED CATIONIC TRYPSIN FROM PACIFIC SAURY PYLORIC CECA

result of thermal inactivation. This result was similar to that of trypsin from the hepatopancreas of crayfish with a temperature optimum of 60°C (Kim *et al.* 1994). Four trypsins from crayfish hepatopancreas had optimal temperatures in the range of 60–70°C (Jeong *et al.* 2000). Kristjansson (1991) also found that trypsin from the pyloric caeca of rainbow trout had an optimal temperature of 60°C. However, the optimal temperature of purified trypsin was higher than those of cod, capelin, anchovy and sardine, which ranged from 40 to 45°C

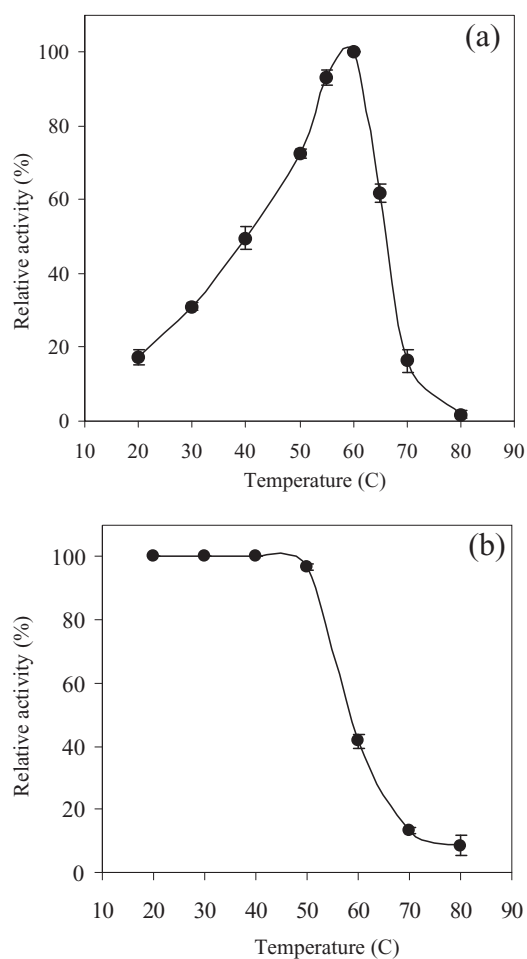


FIG. 3. TEMPERATURE PROFILE (a) AND THERMAL STABILITY (b) OF PURIFIED CATIONIC TRYPSIN FROM PACIFIC SAURY PYLORIC CECA

(Castillo-Yanez *et al.* 2005). The differences in optimal temperatures can be associated with the different living temperatures.

Thermostability of the cationic trypsin is shown in Fig. 3b. The enzyme was fully stable for 15 min at heat treatment up to 50°C, but at higher temperatures the stability decreased. The trypsin was almost completely inactivated at 80°C. Most fish trypsins are unstable at temperatures higher than 40–50°C (De Vecchi and Coppes 1996). Kishimura and Hayashi (2002) found that cationic trypsin activity of starfish pyloric ceca was stable up to 50°C and the activity

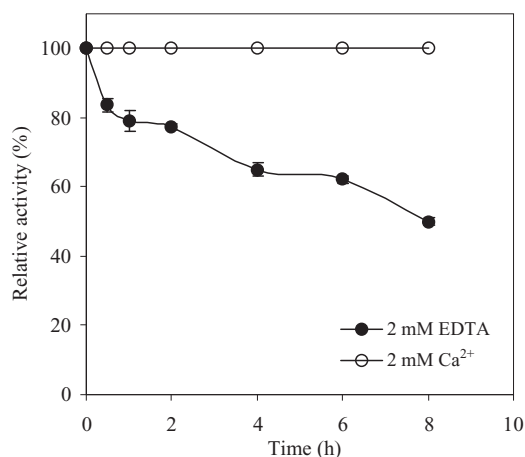


FIG. 4. EFFECT OF CALCIUM ION AND EDTA ON THE STABILITY OF PURIFIED CATIONIC TRYPSIN FROM PACIFIC SAURY PYLORIC CECA. THE STABILITY WAS TESTED AT 40°C FOR DIFFERENT TIMES

disappeared when heated above 70°C. Although the trypsin had a high maximal activity (60°C), the exposure to 60°C without TAME substrate induced some losses in activity. This result suggests that the surrounding environment such as the substrate may influence enzyme stability.

Effect of Calcium Ions on Thermal Stability

The thermal stability of the Pacific saury cationic trypsin was highly dependent on the presence of calcium (Fig. 4). The activity of the enzyme decreased with increasing the time in the presence of 2 mM EDTA, whereas, total activity was retained throughout 8 h at 40°C in the presence of 2 mM calcium ion. These results indicated that thermostability of this enzyme was affected by calcium ion. Similarly, trypsins of other fish species were activated and stabilized by calcium ions (Kristjansson 1991; Kishimura *et al.* 2005, 2006; Klomklao *et al.* 2006b, 2007). However, calcium ions did not show effect in enhancing the stability of trypsins from some fish species such as carp (Cao *et al.* 2000) and starfish (Kishimura and Hayashi 2002). These findings suggest that a notable difference in the structure of the primary calcium-binding site may exist in fish trypsin.

Effect of Inhibitors

The influence of various inhibitors on the purified cationic trypsin from the pyloric ceca of Pacific saury is shown in Table 2. Soybean trypsin inhibitor

TABLE 2.
EFFECT OF VARIOUS INHIBITORS ON THE ACTIVITY OF
PURIFIED CATIONIC TRYPSIN FROM PACIFIC SAURY
PYLORIC CECA[†]

Inhibitors	Concentration	% Inhibition [‡]
Control		0
E-64	0.1 mM	0
<i>N</i> -ethylmaleimide	1 mM	0
Iodoacetic acid	1 mM	0
Soybean trypsin inhibitor	1.0 g/L	96.36 ± 0.32
TLCK	5 mM	99.3 ± 0.17
TPCK	5 mM	0
Pepstatin A	0.01 mM	0
EDTA	2 mM	28.6 ± 0.11

[†] Each enzyme solution was incubated with the same volume of inhibitor at 25°C for 15 min and the residual activity was determined using L-arginine methyl ester hydrochloride (TAME) as a substrate for 20 min at pH 8.0 and 30°C.

[‡] Mean ± SD from triplicate determinations.

and TLCK were the most effective inhibitors, decreasing the activity by 96 and 99%, respectively, while specific inhibitors of chymotrypsin (TPCK), aspartyl proteinase (pepstatin A) as well as cysteine proteinase (E-64, *N*-ethylmaleimide and iodoacetic acid) had no effect at all on trypsin activity. TLCK deactivates only trypsin-like enzymes which have a similar specificity, as a result of alkylation of the active center histidine (Kim *et al.* 1992). The enzyme, in the presence of TLCK, is known to form a covalent bond with histidine in the catalytic site and to block the substrate-binding portion of the active center of the molecule (Kim *et al.* 1992; Klomklao *et al.* 2007). Inhibition by soybean trypsin inhibitor and TLCK suggests that the enzyme is a serine proteinase, mostly trypsin. Similar results were obtained for other fish trypsins (Hjelmeland and Raa 1982; Kristjansson 1991; Bezerra *et al.* 2001; Klomklao *et al.* 2004). However, EDTA, a specific for metalloproteinase (Klomklao *et al.* 2004), showed slightly inhibited activity. This result indicated that this enzyme require metal ions as cofactors for activity. EDTA possibly affected the structure of enzyme or active site conformation, leading to the reduced activity. From the results, it can conclude that the enzyme was trypsin, which possibly required metal ions for its activity.

Effect of NaCl

As shown in Fig. 5, the activity of cationic trypsin decreased with increasing NaCl concentrations (0–30%). The activity of the enzyme decreased by

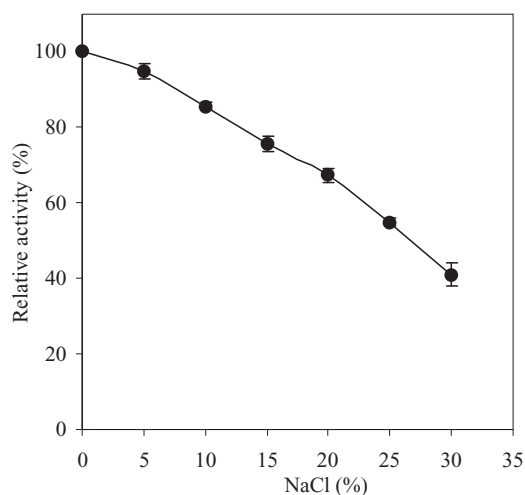


FIG. 5. EFFECT OF NACL CONCENTRATIONS ON ACTIVITY OF PURIFIED CATIONIC TRYPSIN FROM PACIFIC SAURY PYLORIC CECA

59% at 30% NaCl concentration. NaCl at high concentration possibly competed with enzyme in water binding, resulting in stronger protein–protein interaction, which was possibly associated with precipitation (Vojdani 1996; Klomklao *et al.* 2007). This might lead to the loss in activity in presence of high NaCl concentration. Similar effect was found in trypsin from skipjack tuna (Klomklao *et al.* 2007) and yellowfin tuna (Klomklao *et al.* 2006a). However, about 40% of activity was remained for the cationic trypsin in the presence of high salt concentration (30%). Therefore, this enzyme can be used for the improvement or acceleration in proteins hydrolysis of high salt fermented fish products such as fish sauce.

Kinetic Studies

The K_m and K_{cat} for hydrolysis of TAME by cationic trypsin from the pyloric ceca of Pacific saury were determined to be 0.17 mM and 200 s⁻¹, respectively, using Lineweaver-Burk plots (Table 3). K_m values for TAME with trypsin from Greenland cod (Simpson and Haard 1984) and mullet (Guizani *et al.* 1991) were determined to be 0.15–0.19 mM, which are in agreement with the present results. However, K_m of cationic trypsin from pyloric ceca of Pacific saury was lower than those of Atlantic cod (Simpson *et al.* 1989), skipjack tuna (Klomklao *et al.* 2007) and crayfish (Kim *et al.* 1994) (Table 3). This indicated that cationic trypsin had higher affinity to TAME than those from Atlantic cod trypsin, skipjack tuna as well as crayfish.

TABLE 3.
KINETIC CONSTANTS OF PURIFIED CATIONIC TRYPSIN
FROM PACIFIC SAURY PYLORIC CECA AND OTHER FISH
TRYPSINS FOR THE HYDROLYSIS OF TAME

Enzyme	K_m (mM)	K_{cat} (s^{-1})	K_{cat} / K_m ($s^{-1} \text{ mM}^{-1}$)
Pacific saury	0.17	200	1176.47
Crayfish†	0.33	200	590
Skipjack tuna‡	0.33	80	242.42
Greenland cod§	0.15	—	—
Mullet¶	0.19	—	—
Atlantic cod††	0.22	—	—

K_m , K_{cat} values were determined using L-arginine methyl ester hydrochloride (TAME) as a substrate at pH 8.0 and 30°C.

† Kim *et al.* (1994).

‡ Klomklao *et al.* (2007).

§ Simpson and Haard (1984).

¶ Guizani *et al.* (1991).

†† Simpson *et al.* (1989).

	5	10
Cationic trypsin	I V G G Y E C Q P H	
Skipjack tuna	I V G G Y E C Q A H	
True sardine	I V G G Y E C K A Y	
Arabesque greenling	I V G G Y E C T P H	
Japanese anchovy	I V G G Y E C Q A H	
Cod	I V G G Y E C T K H	
Salmon	I V G G Y E C K A Y	
Dogfish	I V G G Y E C P K H	
Dog	I V G G Y T C E E N	
Porcine	I V G G Y T C A A N	
Bovine	I V G G Y T C G A N	

FIG. 6. COMPARISON OF N-TERMINAL AMINO ACID SEQUENCE OF THE PURIFIED CATIONIC TRYPSIN FROM PACIFIC SAURY PYLORIC CECA WITH OTHER ENZYMES: SKIPJACK TUNA (KLOMKLAO *ET AL.* 2007), TRUE SARDINE, ARABESQUE GREENLING (KISHIMURA *ET AL.* 2006), JAPANESE ANCHOVY (KISHIMURA *ET AL.* 2005), COD (GUDMUNDSDOTTIR *ET AL.* 1993), SALMON (MALE *ET AL.* 1995), DOGFISH (TITANI *ET AL.* 1975), DOG (PINSKY *ET AL.* 1985), PORCINE (HERMODSON *ET AL.* 1973) AND BOVINE (WALSH 1970)

N-Terminal Amino Acid Sequence

The N-terminal amino acid sequence of the purified cationic trypsin was determined to be 10 amino acid residues. As shown in Fig. 6, the sequence was compared with those of other fish trypsins. The sequence (IVGGYECQPH) of purified cationic trypsin from Pacific saury pyloric ceca showed high homol-

ogy to fish trypsins. Generally, the N-terminal region of trypsin-like proteases, especially from first to seventh residues, demonstrates high homology (Cao *et al.* 2000). From the results, the N-terminal four amino acid sequence of cationic trypsin and other trypsins started with IVGG, which was a part of the proteolytic cleavage site in inactive trypsinogen. Therefore, this enzyme is truly a member of trypsin family.

CONCLUSION

The enzyme purified from the pyloric ceca of Pacific saury with maximal activity at pH 8.5 and 60°C was classified as trypsin based on molecular weight, substrate specificity, inhibitor study and N-terminal sequencing. Enzymatic characteristics were essentially consistent with those of trypsin from other species.

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Autolysis of goatfish (*Mulloidichthys martinicus*) mince: Characterisation and effect of washing and skin inclusion

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ABSTRACT

Autolysis of goatfish mince and washed mince incubated at different temperatures (30–70 °C) was investigated. The highest autolytic activity was generally observed in mince and washed mince at 60 °C as evidenced by the highest trichloroacetic acid (TCA) soluble peptide content and the greatest disappearance of myosin heavy chains (MHCs). Autolysis of both mince and washed mince was maximised at pH 4, and lower autolytic activity was observed at pH 7. *trans*-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E-64) showed the greatest inhibition of autolysis at pH 4, showing that at least one cysteine protease was active in goatfish muscle. Nevertheless, soybean trypsin inhibitor effectively inhibited the autolysis at neutral pH (pH 7), suggesting that goatfish muscle also contained at least one serine protease. Generally, autolysis of mince was more pronounced than that of washed mince, indicating that washing could lower the autolytic activity of mince. In the presence of skin, a higher autolysis was obtained with the goatfish mince. Therefore, both sarcoplasmic and myofibril-associated proteases in muscle as well as the contamination of skin likely contributed to the degradation of muscle proteins of goatfish.

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1. Introduction

Autolysis of fish muscle has generally been recognised as resulting from endogenous proteases and as being associated with the quality loss of fish flesh as well as the softening of gel products. Endogenous proteases, especially heat-activated and heat-stable proteases, have been known to play an essential role in surimi gel weakening (Benjakul, Visessanguan, & Leela-pong Wattana, 2003a). Disintegration of surimi gels is caused by severe degradation of myofibrillar proteins, particularly myosin at temperatures above 50 °C (Jiang, 2000). The degree of proteolysis varies with fish species, and is governed by the types of proteases. As a consequence, gel weakening of surimi or mince induced by proteases depends on species. A rapid breakdown of myofibrillar protein inhibits development of a three-dimensional gel network. Generally, cathepsin and heat-activated alkaline proteases mainly contribute to a decrease in gel strength, resulting in a brittle and non-elastic gel (An, Peters, & Seymour, 1996). Benjakul et al. (2003a) reported that heat-activated alka-

line proteases in bigeye snapper and lizardfish (*Saurida tumbil*) played an important role in degradation of muscle protein, especially at 60–65 °C. Based on their extractability, fish muscle proteases can be classified as sarcoplasmic and myofibril-associated proteases (Kinoshita, Toyohara, & Shimizu, 1990; Toyohara, Sakata, Yamashita, Kinoshita, & Shimizu, 1990). Sarcoplasmic proteases can be removed to some extent during the washing process, while myofibril-associated proteases still remain in surimi, resulting in gel weakening (modori).

Goatfish is an abundant species in the Gulf of Thailand and has low commercial values. So far, it has become more economically important as a raw material for surimi production. Nevertheless, goatfish surimi generally exhibited poor gel properties. Similar to other underutilised fishery resources, this common problem is due to the high endogenous proteolytic activity in the muscle (Greene & Babbitt, 1990). During the deboning process, skin can become a contaminant in the mince, especially from fish with soft and thin skin. Recently, fish skin was reported to contain proteases capable of degrading the β -, α_1 - and α_2 -chains of collagen (Nalinanon, Benjakul, Visessanguan, & Kishimura, 2008). Nevertheless, no information on proteolysis of goatfish has been reported. The objectives of this study were to characterise the predominant endogenous protease responsible for autolysis of goatfish muscle and to study the effect of washing and skin inclusion on autolysis.

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2. Materials and methods

2.1. Chemicals

Sodium chloride was obtained from Fisher Scientific (Pittsburgh, PA, USA). Folin-Ciocalteu's phenol reagent and trichloroacetic acid were purchased from Merck (Darmstadt, Germany). β -Mercaptoethanol (β -ME), ethylenediaminetetraacetic acid (EDTA), *trans*-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E-64), pepstatin A, soybean trypsin inhibitor (SBTI), and L-tyrosine were procured from Sigma Chemical Co. (St. Louis, MO, USA). Bovine serum albumin (BSA) was purchased from Fluka (Buchs, Switzerland). Sodium dodecyl sulphate (SDS), *N,N,N',N'*-tetramethylethylenediamine (TEMED) and Coomassie Brilliant Blue R-250 were obtained from Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Collection and preparation of goatfish

Goatfish (*Mulloidichthys martinicus*) with a size of 10–15 cm long were caught off the Songkhla-Pattani Coast in the Gulf of Thailand during May and July, 2007. Fish were immediately stored in ice after capture and off-loaded within 48 h, and were obtained from a dock in Songkhla, Thailand. Fish were transported in ice with the fish/ice ratio of 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University, Songkhla, Thailand within 2–3 h. They were headed, gutted and washed with water. The flesh was separated manually from skin and bone and kept on ice not longer than 2 h. Three different lots of fish were employed in this study.

2.3. Preparation of mince and washed mince

Flesh was minced using a motorised mincer with a plate diameter of 0.5 cm (Model 613, Chuang Zong Machine, San Chung, Taipei Hsien, Taiwan). The mince obtained was placed in polyethylene bags and was stored in ice not longer than 1–2 h before use. Washed mince was prepared according to the method of Toyohara et al. (1990) with a slight modification. Mince was homogenised with five volumes of 50 mM NaCl for 2 min using an IKA Labortechnik homogeniser (Selangor, Malaysia) at a speed of 11,000 rpm, followed by centrifuging at 9600g at 4 °C for 10 min using a Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA). The washing process was repeated twice. To minimise the denaturation, the preparation work was conducted at temperature below 4 °C.

2.4. Effect of temperature on autolysis of goatfish mince and washed mince

Mince and washed mince with the physiological pH of 6.9–7.1 (3 g) were incubated at different temperatures (30, 35, 40, 45, 50, 55, 60, 65 and 70 °C) in a temperature-controlled water bath (Mettmert, Schwabach, Germany) for 60 min. The autolytic reaction was terminated by addition of 27 ml of cold 5% trichloroacetic acid (w/v). The mixture was homogenised for 1 min at 11,000 rpm. The homogenate was centrifuged at 7500g for 10 min using a microcentrifuge (Model MIKRO20, Hettich ZENTRIFUGEN, Tuttlingen, Germany). TCA-soluble peptide content in the supernatant was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) using L-tyrosine as a standard and was expressed as μ mol tyrosine/g sample.

To determine the autolytic pattern of goatfish muscle, another 3 g of samples incubated at different temperatures for 60 min were mixed with 27 ml of 5% SDS (85 °C) to terminate the autolytic reaction. The mixture was then homogenised at 11,000 rpm for 1 min. The homogenate was then incubated at 85 °C for 60 min to solubi-

lise the sample. To remove undissolved debris, the mixture was centrifuged at 7500g for 10 min. The supernatant was subjected to SDS-PAGE analysis.

2.5. Effect of pH on autolysis of goatfish mince and washed mince

Mince and washed mince (3 g) were homogenised at 11,000 rpm for 1 min with 9 ml of different buffers having various pH. Those included 0.2 M McIlvaine's buffer (0.2 M Na-phosphate and 0.1 M Na-citrate) for pH 2.0–7.0 and phosphate-borate buffer (0.1 M Na_2HPO_4 –0.05 M $\text{Na}_2\text{B}_4\text{O}_7$) for pH 8.0–11.0. The homogenate was incubated at the optimal temperature. Autolysis was terminated after 60 min of incubation by the addition of 18 ml of cold 5% TCA or 5% SDS solution (85 °C) as previously described. TCA-soluble peptide content and SDS-PAGE protein patterns were determined, respectively.

2.6. Effect of various protease inhibitors on autolysis of mince and washed mince

Mince and washed mince (0.5 g) were homogenised (11,000 rpm for 1 min) with 1.5 ml of the buffer having the pH that shows the maximal activity. The homogenate was mixed with 2 ml of protease inhibitor solution to obtain the final designated concentration: 2 mM EDTA, 0.01 mM soybean trypsin inhibitor (SBTI), 0.01 mM E-64 and 1 μ M and 10 μ M pepstatin A (Benjakul, Visessanguan, & Turksuban, 2003b). The mixtures were allowed to stand in ice for 2 h, followed by incubation at the optimal temperature for 2 h. Autolysis was terminated by addition of 2 ml of either 10% TCA or 10% SDS solution (85 °C). TCA-soluble peptide content and SDS-PAGE protein patterns were then determined. The control used deionised water instead of protease inhibitor solution.

2.7. Effect of skin on autolysis of mince

To prepare mince with skin, fillets were directly minced without prior skin removal. Mince with and without skin (3 g) was incubated at the optimal temperature for different times (0, 10, 30, 60, 90 and 120 min). Autolysis was terminated by addition of 27 ml of either 5% TCA or 5% SDS solution (85 °C). TCA-soluble peptide contents and SDS-PAGE protein patterns were then determined.

2.8. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of mince and washed mince were obtained by SDS-PAGE according to the method of Leammli (1970). SDS-solubilised samples with 15 μ g of protein per channel were loaded onto the polyacrylamide gel made of a 10% running gel and a 4% stacking gel and were subjected to electrophoresis at a constant current of 15 mA per gel, using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid, and were destained with 50% methanol (v/v) and 7.5% (v/v) acetic acid, followed by 5% methanol (v/v) and 7.5% (v/v) acetic acid. Quantitative analysis of protein band intensity was performed using a Model GS-700 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA, USA) with Molecular Analyst Software version 1.4 (image analysis systems). The band intensity of myosin heavy chain (MHC) and actin in each sample was expressed as a percentage of that found in mince or washed mince.

2.9. Protein determination

Protein content was determined by the Biuret method (Robinson & Hodgen, 1940) using bovine serum albumin as the standard.

2.10. Statistical analysis

Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests (Steel & Torrie, 1980). Analysis was performed using a SPSS package (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Effect of temperature on autolysis of goatfish mince and washed mince

Autolysis of goatfish mince and washed mince incubated at various temperatures was monitored and expressed in terms of TCA-soluble peptide (Fig. 1A). The highest autolysis was observed at 60 °C. The activity decreased sharply at temperatures above 60 °C, most likely due to thermal denaturation of endogenous proteases. The results showed that temperature was a crucial factor affecting the autolysis. Proteolysis of fish muscle caused by endogenous proteases has been widely reported. Cathepsin L caused severe texture degradation in Pacific whiting (An, Seymour, Wu, & Morrissey, 1994), arrowtooth flounder (Visessanguan, Benjakul, & An, 2003) and chum salmon (Saeki, Iseya, Sugiura, & Seki, 1995). Serine proteases were found to be responsible for textural breakdown of threadfin bream (Toyohara & Shimizu, 1988) oval-filefish (Toyohara et al., 1990) and lizardfish (Suwansakornkul, Itoh, Hara, & Obatake, 1993). When comparing the autolytic activity between mince and washed mince, higher activity was found in mince. Fish mince contained both sarcoplasmic proteases and myofibril-asso-

ciated proteases. Washing the mince with 50 mM NaCl could remove sarcoplasmic proteases to some degree. Therefore, the use of a low molarity saline solution for mince washing was able to lower the autolysis of the resulting washed mince. Nevertheless, the protease associated with the myofibrils still remained as indicated by the protein degradation, which was still found in washed mince. Furthermore, washing not only removed the proteases, but also any co-factors for proteolytic activity. This might lead to the lowered proteolysis of washed mince. Generally, these enzymes contributed to degradation of proteins, leading to a poor gel network. Toyohara et al. (1990) reported that proteolytic degradation of fish gels could be classified into two types according to the localisation of the gel degradation inducing factor (GIF) in muscle cells, sarcoplasmic GIF, which could be easily washed out from the myofibrils and myofibrillar GIF, which was tightly associated with myofibrils. Both these types of proteases were responsible for gel softening (Kinoshita et al., 1990; Toyohara et al., 1990). Benjakul et al. (2003a) reported that the maximum autolysis of mince and washed mince from two species of bigeye snapper (*Priacanthus macracanthus* and *Priacanthus tayenus*) was found at 60 °C. Myofibril-bound serine proteases in carp (*Cyprinus carpio*) with an optimal temperature of 55 °C were responsible for the degradation of MHC and other myofibrillar proteins such as α -actinin, β -actin and tropomyosin (Jiang et al., 2006).

Autolysis patterns of goatfish mince and washed mince are shown in Fig. 2. Degradation was more pronounced at temperatures higher than 50 °C, as evidenced by the marked decrease in MHC band intensity. At 60 °C, the band intensity of MHC decreased by 80.1% and 62.4% for mince and washed mince, respectively, compared with that of the samples without incubation. For actin, no changes in band intensity were observed. Thus, it was noted that MHC was more susceptible to degradation than other easily observable muscle protein bands with SDS-PAGE. However, MHC with high band intensity was found in mince incubated at 70 °C, suggesting the heat inactivation of endogenous proteases. Cao et al. (1999) reported that myofibril-bound serine protease from lizardfish muscle hydrolyzed MHC at 55–60 °C effectively, whereas α -actinin and actin were not degraded. Benjakul et al. (2003a) reported that actin was regarded as a proteolytically resistant protein in comparison with MHC, β -tropomyosin and troponin T. The decrease in protein band intensity was in agreement with the increase in TCA-soluble peptide content (Fig. 1A). A larger decrease in the MHC band was found in mince than in washed mince, indicating the higher autolysis in the former. Thermal-activated proteases contributed to degradation of myofibrils in mince, whereas those proteases that are not removed by washing were mainly involved in degradation of MHC in washed mince. The results suggested that autolysis of goatfish mince could be minimised by thorough washing.

3.2. Effect of pH on autolysis of mince and washed mince

The pH profiles for the autolysis of mince and washed mince conducted at the optimal temperature (60 °C) for 60 min are shown in Fig. 1B. For both mince and washed mince, the major activity peak was found at pH 4 with a small activity peak at pH 7. Therefore, acidic proteases were the major proteases, while neutral proteases were present at low levels. Cathepsin, especially cathepsin D, had an optimal pH range of 3.0–4.5 (Makinodan, Akasaka, Toyohara, & Ikeda, 1982). Bonete, Manjon, Llorca, and Iborara (1984) found that cathepsin D from grey mullet had maximum activity at pH 4.0 and was stable up to 45 °C. Activity losses of 34.0% and 100% were found at 50 and 70 °C, respectively. Moreover, cathepsin L, a cysteine protease, was also presumed to exhibit its activity peak at acidic pH. Cathepsin L has several forms and a wide range of pH (3.0–6.5) for activity (Kang & Lanier, 2000).

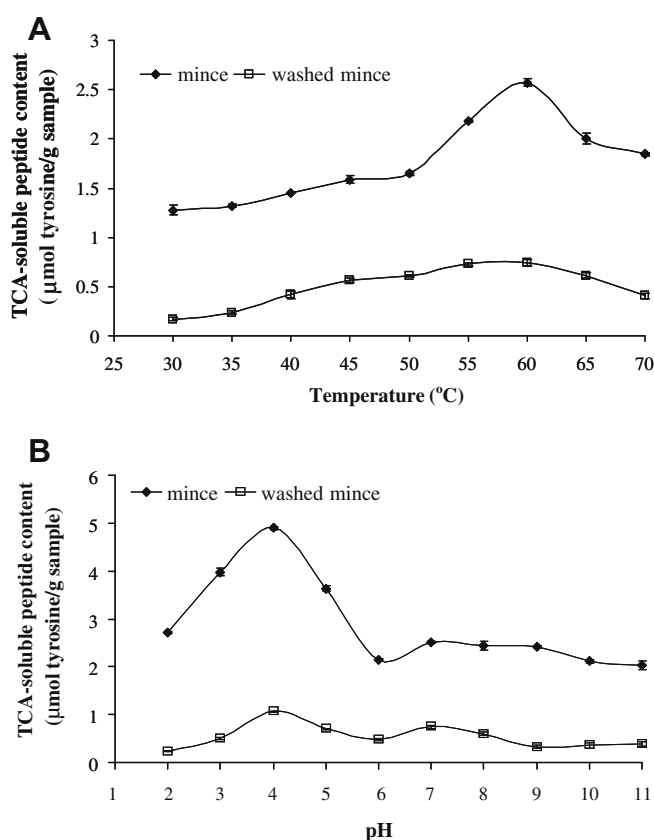


Fig. 1. TCA-soluble peptide content of goatfish mince and washed mince incubated at different temperatures (A) and pH (B). Autolysis was conducted for 60 min. The temperature profile was determined at physiological pH (~7). For the pH profile study, the autolysis was performed at 60 °C. Bars represent the standard deviation of triplicate determinations.

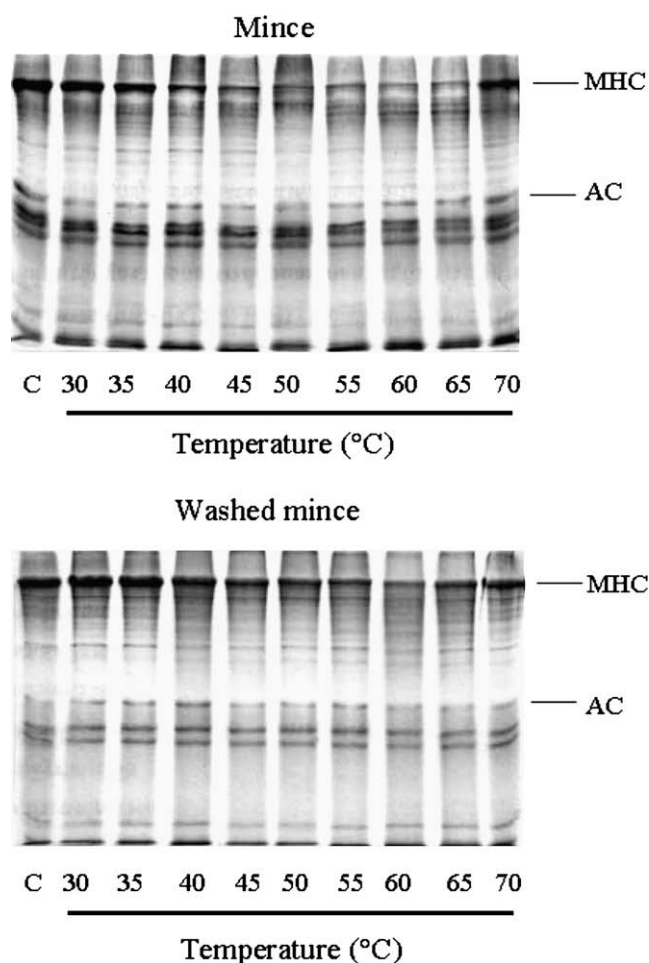


Fig. 2. Autolytic SDS–PAGE pattern of goatfish mince and washed mince incubated at different temperatures for 60 min. Numbers designate the incubation temperatures (°C). MHC, myosin heavy chain; AC, actin; C, control (no incubation).

Visessanguan et al. (2003) reported that a predominant heat-activated protease in arrowtooth flounder displayed optimal activity at pH 5.0–5.5. Since calpain was not stable at temperatures above 40 °C (Wang, Ma, Su, Chen, & Jiang, 1993), other proteases might play a role in the degradation at neutral and slightly alkaline condition. Among all proteases (cathepsin D, neutral protease, calpain and alkaline protease), only alkaline protease could show activity at pH 6.8 and 60 °C in meat paste (Makinodan, Toyohara, & Niwa, 1985). Autolysis of goatfish muscle was most likely mediated by heat-activated acidic proteases at pH lower than 6. At the autolysis peak (pH 4), lower activity was found in washed mince, compared with mince. This reconfirmed that washing could remove some proteases in muscle, resulting in lower amounts of proteases.

3.3. Effect of various protease inhibitors on autolysis of mince and washed mince

The effect of various protease inhibitors on autolysis of mince and washed mince at pH 4 and 7 is shown in Table 1. For autolysis at pH 4, E-64, a specific cysteine protease inhibitor, showed the highest inhibitory activity towards autolysis in goatfish mince (63.3%) and washed mince (54.0%). Inhibitors tested including 10 μ M pepstatin A (an aspartic protease inhibitor) and SBTI (a serine protease inhibitor) also showed inhibition of autolysis at this pH. However, EDTA and metallo-protease inhibitor had little effect on inhibition of both samples. The results suggested that different

types of proteases are involved in autolysis of goatfish muscle at acidic pH. Autolysis of lizardfish was inhibited by E-64 (Benjakul et al., 2003b). Klomklao, Kishimura, and Benjakul (2008) reported that aspartic protease was the major protease in true sardine, when tested at pH 3. For autolysis at pH 7, SBTI was the most effective protease inhibitor with 49.7% and 75.4% inhibition for mince and washed mince, respectively. Since the highest activity was found at acidic pH and autolysis was most strongly inhibited by E-64, cysteine proteases including cathepsin could be the main proteases in mince and washed mince. Additionally, serine proteases were another group of proteases present in goatfish muscle but to a lesser extent. Myofibril-bound protease in lizardfish (*Saurida wanieso*) was classified as a trypsin-like protease, which showed optimal activity at 50–60 °C and pH 7–8, and it was also inhibited by SBTI, leupeptin and *N*-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (Cao, Hara, & Ishihara, 2000). Other inhibitors also showed inhibition towards autolysis, suggesting that goatfish contained a variety of proteases, which had different characteristics.

Protein patterns of mince and washed mince autolyzed at pH 4 and 7 in the presence of different protease inhibitors are shown in Fig. 3. Degradation of MHC was more pronounced in mince than in washed mince. For the same sample, higher degradation was found at pH 4 than pH 7. The results were in agreement with the higher TCA-soluble peptide content at pH 4 (Fig. 1B). The lowest band intensity of MHC was observed in the control (without protease inhibitor). MHCs of both mince and washed mince were most retained in the presence of E-64 when tested at pH 4, while SBTI was the most effective inhibitor towards the degradation of MHC at pH 7. For mince and washed mince with added E-64 and incubated at 60 °C and pH 4, the MHC band intensity was 70.8% and 80.2%, respectively, compared with that of a corresponding sample without incubation. When incubated at pH 7 and 60 °C, the MHC band intensity of mince and washed mince added with SBTI was 71.9% and 85.4%, respectively, compared with that of a corresponding sample without incubation. Greater retention of the MHC band in samples with inhibitors gives a higher % inhibition (Table 1). Pepstatin A and EDTA had no impact on autolysis. The results reconfirmed that cysteine proteases and serine proteases were most likely responsible for protein degradation of this species. However, serine proteases most likely contribute more to the autolysis of surimi or mince products, which have a neutral pH. Suwansakornkul et al. (1993) reported that both cysteine and serine proteases were responsible for MHC degradation of washed lizardfish mince and varied with species.

3.4. Effect of skin on autolysis of muscle

The effect of skin on the autolysis of goatfish mince incubated at 60 °C for different times is shown in Fig. 4. At the same incubation times, TCA-soluble peptide content of mince with skin (4.63 μ mol tyrosine/g sample) was greater than that of mince without skin (3.75 μ mol tyrosine/g sample) ($P < 0.05$). This result indicated that skin might contain proteases, which were able to hydrolyze muscle proteins. Endogenous skin proteases of bigeye snapper (*P. macracanthus*) were classified as serine proteases, which were strongly inhibited by SBTI (Intarasirisawat et al., 2007). Nalinanon et al. (2008) found that skin of bigeye snapper (*P. taylori*) contained proteases capable of degrading β -, β_1 - and α_2 -chains of gelatin. Longer incubation times resulted in higher TCA-soluble peptide content for both mince with and without skin ($P < 0.05$), suggesting that the proteins or peptides underwent autolysis caused by thermal-activated endogenous proteases localised either in the muscle or associated with the skin to a larger extent. Therefore, contamination of mince with skin during surimi production may play a role in enhanced degradation of myofibrils in goatfish surimi or mince products.

Table 1
Effect of various protease inhibitors on autolysis of goatfish mince and washed mince

Inhibitor	Concentration	% Inhibition			
		Mince		Washed mince	
		pH 4	pH 7	pH 4	pH 7
Control		0 ^{eA,a,b,c}	0 ^{fA}	0 ^{dA}	0 ^{dA}
SBTI	0.01 mM	29.9 ± 1.06 ^{bc}	49.7 ± 0.14 ^{aB}	29.8 ± 2.98 ^{bcC}	75.4 ± 5.32 ^{aA}
E-64	0.01 mM	63.3 ± 0.38 ^{aA}	18.2 ± 2.43 ^{cD}	54.0 ± 1.12 ^{aB}	31.0 ± 5.02 ^{bcC}
Pepstatin A	1 µM	13.9 ± 1.01 ^{cB}	12.2 ± 0.032 ^d	26.1 ± 0.67 ^{cA}	22.8 ± 3.46 ^{cA}
Pepstatin A	10 µM	28.6 ± 2.88 ^{bA}	19.6 ± 0.99 ^{bB}	30.6 ± 3.86 ^{bA}	37.4 ± 11.2 ^{bA}
EDTA	2 mM	8.02 ± 3.44 ^{dB}	8.5 ± 0.35 ^{eB}	1.38 ± 0.042 ^{dC}	22.3 ± 0.51 ^{cA}

^a Different superscripts in the same column indicate significant differences ($P < 0.05$).

^b Different capital superscripts in the same row indicate significant differences ($P < 0.05$).

^c Values are given as mean ± SD from triplicate determinations.

The protein patterns of goatfish mince with and without skin autolyzed at 60 °C for different times are shown in Fig. 5. MHC band intensity decreased when the incubation time increased with the concomitant occurrence of degradation products. This result was in agreement with the increase in TCA-soluble peptide content of both mince with and without skin as incubation time increased. Actin was degraded to a much lower extent compared to MHC. Within 30 min, a slightly lower intensity of MHC band was observed in mince with skin than mince without skin. The MHC band intensity of mince without and with skin decreased by 52.3% and 60.0% after 10 min of incubation and by 68.7% and 75.3% after 30 min of incubation, respectively, compared with that of a corresponding sample without incubation. However, no differences in protein patterns were found with increasing incubation time. It was suggested that mince with skin contained higher amount of

proteases, resulting in greater degradation of muscle. Therefore, endogenous skin proteases, especially heat-activated proteases or collagenolytic enzymes, might be involved in the degradation of muscle proteins in surimi contaminated with skins. Deskinning prior to deboning or appropriate refining could be a means of reducing degradation induced by skin proteases.

4. Conclusion

Autolysis of goatfish mince took place at high temperature with the activity peak at 60 °C. MHC was the most susceptible to hydrolysis. At physiological pH, serine proteases were most likely a major contributor to protein degradation. However, cysteine proteases were dominant at acidic pH. Inclusion of skin also resulted in increased autolysis. Therefore, degradation of myofibrillar

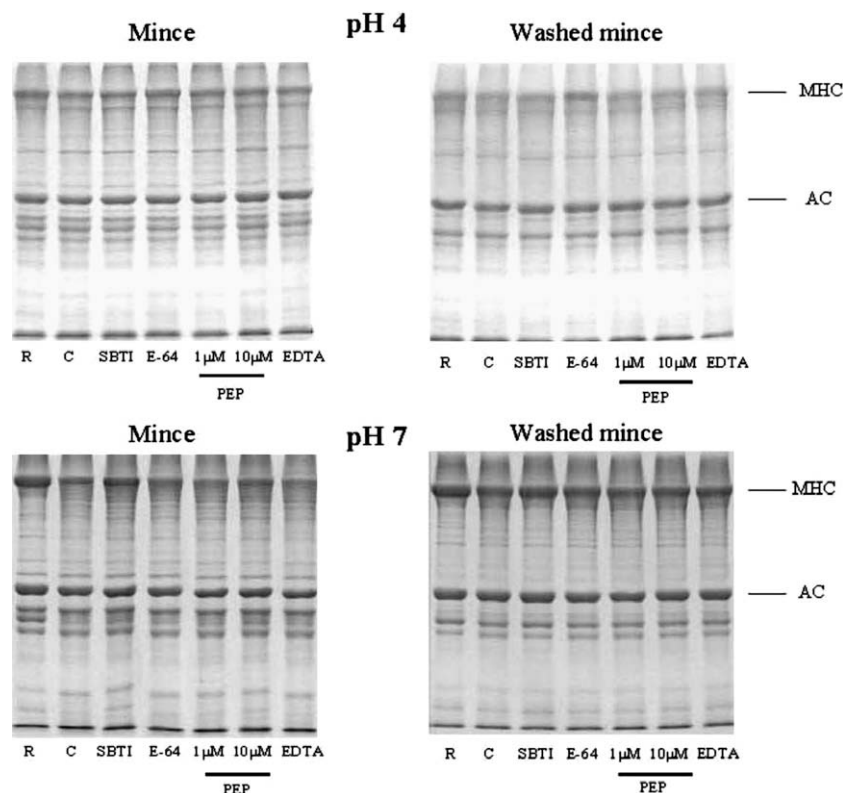


Fig. 3. Autolytic SDS-PAGE pattern of goatfish mince and washed mince with addition of various protease inhibitors at pH 4 and 7. Autolysis was conducted at 60 °C for 120 min. MHC, myosin heavy chain; AC, actin; R, raw material (without inhibitor and no incubation); SBTI, soybean trypsin inhibitor; E-64, *trans*-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane; PEP, pepstatin A; EDTA, ethylenediaminetetraacetic acid.

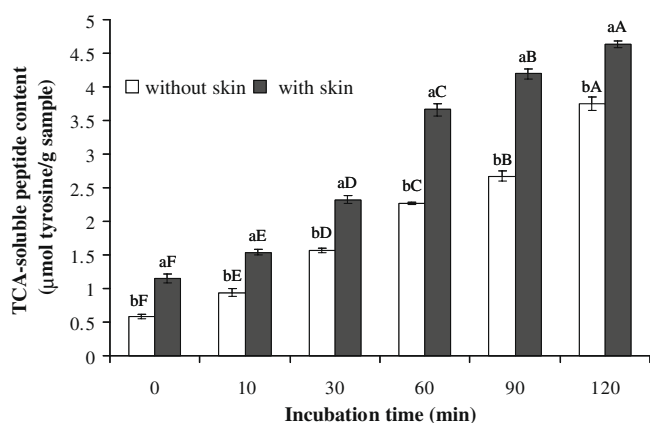


Fig. 4. TCA-soluble peptide content of goatfish mince with and without skin incubated at 60 °C for different times. Bars represent the standard deviation of triplicate determinations. Different letters in the same incubation time indicate significant differences ($P < 0.05$). Different capital letters in the same sample indicate significant differences ($P < 0.05$).

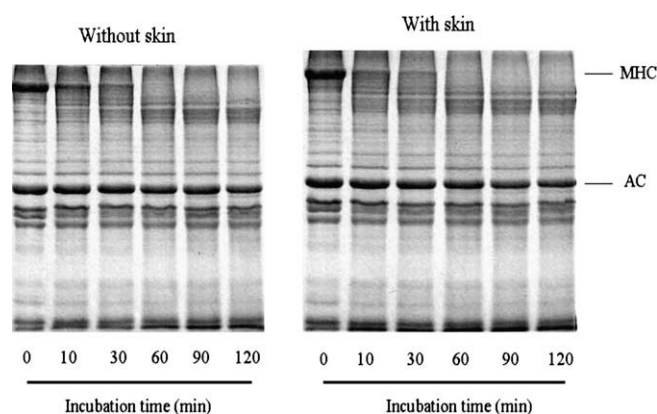


Fig. 5. Autolytic pattern of goatfish mince without and with skin incubated at 60 °C for different times. Numbers designate the incubation times (min). MHC, myosin heavy chain; AC, actin.

proteins of goatfish muscle was governed by endogenous proteases as well as contamination with skin. This was most likely associated with gel weakening of the surimi obtained from this species.

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Original article

Autolysis and biochemical properties of endogenous proteinases in Japanese sandfish (*Arctoscopus japonicus*)Sappasith Klomklao,^{1*} Hideki Kishimura,² Soottawat Benjakul³ & Benjamin K. Simpson⁴

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Summary Autolysis of Japanese sandfish (*Arctoscopus japonicus*) was studied. The highest autolysis was observed at 60 °C. Optimal pH for autolysis was found at both acidic and alkaline pH values. Autolytic activity decreased with increasing NaCl concentration. Further assay of the extract from Japanese sandfish exhibited the maximum activity at 55 °C and pH 9.5. The Japanese sandfish proteinases were markedly inhibited by pepstatin A at pH 3.5, while soybean trypsin inhibitor, PMSF and TLCK inhibited activities of proteinases at pH 9.5, suggesting Japanese sandfish proteinases were both aspartic and serine proteinases. The extracts were stable to heat treatment up to 50 °C for 15 min and still remained high relative activity after incubated at 50 °C for up to 8 h. Maximum stability of the extracts was observed between pH 6 and 11 and no changes of activity were found after incubation at pH 9.5 for up to 8 h. Activity of Japanese sandfish extract continuously decreased as NaCl concentration increased (0–30%), while activity increased as CaCl₂ concentration increased. The results suggest that major proteinases in Japanese sandfish were trypsin-like serine proteinases.

Keywords Autolysis, degradation, fish sauce, protease, proteolysis.

Introduction

Fish sauce is the liquid product developed during fermentation of heavily salted fish material in closed tanks at tropical temperatures (Klomklao *et al.*, 2006a). Generally, fish sauce is produced by adding the salt to the fish with the ratios of fish to salt of 2:1 or 3:1 and fermenting at ambient temperature for 6–12 months or longer (Dissaraphong *et al.*, 2006). A great variety of raw materials can be used for fish sauce manufacture and the proteolytic enzymes must be sufficient for tissue or protein hydrolysis. Fish sauce is mainly produced from anchovies, sardine, mackerel and herring (Siringan *et al.*, 2006). During fermentation, protein hydrolysis is induced by endogenous proteinases in fish muscle and digestive tract as well as proteinases produced by halophilic bacteria (Siringan *et al.*, 2006). The volatile compounds contributing to flavour of fish sauce are

produced by non-enzymatic reactions of various components and enzymatic reactions by endogenous enzymes of fish origin and those of microorganisms surviving during fermentation (Dissaraphong *et al.*, 2006). Thus, understanding properties of endogenous proteinases is important in the degradation of muscle protein and may support an information for the production of fish sauce.

The most important proteolytic enzymes from fish viscera and muscle are the aspartic and serine proteinases (Klomklao *et al.*, 2004). Proteolytic activity in digestive organs and muscles of various fish species used for fish sauce production has been studied. Alkaline proteinase was predominant in fish muscle and brine during the ripening of salted anchovy (Hernandez-Herrero *et al.*, 1999). Siringan *et al.* (2006) reported that both acid and alkaline proteinases have been found in Indian anchovy. Acid, neutral and alkaline proteinases have been found in Monterey sardine (*Sardinops sagax caerulea*) muscle (Lugo-Sanchez *et al.*, 1997). Ishida *et al.* (1995) reported that neutral serine

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proteinase from muscle anchovy displayed optimum catalytic activity at pH 7.5. Two acid proteinases were purified of sardine (*Sardina pilchardus*) stomach (Noda & Murakami, 1981). Acid proteinase purified from Monterey sardine (*Sardinops sagas caerulea*) viscera had an optimal pH of 2.5 (Castillo-Yañez *et al.*, 2004). Castillo-Yañez *et al.* (2005) isolated trypsin from pyloric ceca of Monterey sardine (*S. s. caerulea*) and found that the enzyme had an optimal activity at pH 8.0 and 50 °C.

Japanese sandfish (*Arctoscopus japonicus*) is one of the preferable species commonly used for fish sauce production in Japan due to its high endogenous proteinase activity and abundance. Based on our preliminary study, many different proteinases were found in Japanese sandfish. However, no information on proteolysis of Japanese sandfish, which might contribute to protein hydrolysis during high salt fermentation, has been reported. Therefore, the objective of this investigation was to study the autolysis and to characterise biochemical properties of endogenous proteinases in Japanese sandfish.

Materials and methods

Chemicals

Sodium caseinate, bovine haemoglobin, 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, phenylmethanesulfonyl fluoride (PMSF), L-tyrosine and bovine serum albumin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Trichloroacetic acid (TCA), sodium chloride, Tris(hydroxymethyl)aminomethane and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany).

Fish sample

Fresh Japanese sandfish (*A. japonicus*), with an average body weight of 55–60 g, was purchased from a local fish market in Hakodate, Japan. The fish were transported in ice with a fish/ice ratio of 1:2 (w/w) to the Laboratory of Marine Products and Food Science, Research Faculty of Fisheries Sciences, Hokkaido University, Japan. Defatted Japanese sandfish powder was then prepared according to the method of Klomklao *et al.* (2007a). The defatted powder was stored at –20 °C until used.

Protein determination

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

Autolytic activity assay

Autolytic activity was measured according to the method of Visessanguan *et al.* (2001) with a slight modification. Whole Japanese sandfish was ground using a meat grinder with a 5 mm plate. Three grams of ground Japanese sandfish were incubated for 40 min in a water bath at various temperatures (30–80 °C), and the autolytic reaction was stopped by adding 27 mL of ice-cold 5% (w/v) TCA. The concentration of the soluble peptides released was measured according to the Lowry method (Lowry *et al.*, 1951).

To construct a pH profile, 3 g of ground fish was added with 12 mL of buffer (McIlvain's buffer consisting of 0.2 M sodium phosphate and 0.1 M sodium citrate for the pH range of 2.0–7.0, and 0.1 M glycine–NaOH for the pH range of 8.0–11.0) and incubated for 40 min at 60 °C in a water bath. The autolytic reaction was terminated by addition of 15 mL of ice-cold 9% (w/v) TCA. Soluble peptides were determined as describe above.

The effect of NaCl on autolytic activity was also studied. NaCl was added into ground sample to obtain the final concentrations (0%, 5%, 10%, 15%, 20%, 25% and 30% (w/w)). After incubated the mixture at 60 °C for 40 min, 27 mL of ice-cold 5% (w/w) TCA was added and TCA-soluble peptides were determined. Blanks were prepared by adding TCA before incubation at 60 °C. Autolytic activity was expressed as nmol of tyrosine released min^{–1} g^{–1} mince.

Preparation of Japanese sandfish extract

Defatted Japanese sandfish powder was suspended in 20 mM phosphate buffer, pH 7.0 at a ratio of 1:20 (w/v) and stirred continuously at 4 °C for 3 h. The suspension was centrifuged for 15 min at 4 °C and 20 000 × *g* (H-200; Kokusan, Tokyo, Japan) to remove the tissue debris, and then the supernatant was lyophilised. Before used, the lyophilised sample (10 g) was dissolved in 30 mL of cold distilled water (4 °C) and referred to as 'Japanese sandfish extract'.

Enzyme assay

Proteinase activity of Japanese sandfish extract was assayed using haemoglobin and casein as substrates according to the method of Klomklao *et al.* (2004). Activity was determined according to the TCA-Lowry assay. Fish extract (200 µL) was added into assay mixtures containing 200 µL of 2% (w/v) substrate solution, 200 µL of distilled water and 625 µL of reaction buffer. The mixture was incubated at the test pH and temperature for precisely 20 min. Enzymatic reaction was terminated by adding 200 µL of 50% (w/v) TCA. Unhydrolysed protein substrate was allowed to precipitate for 1 h at 4 °C, followed by centrifuging at

10 000 $\times$ g for 10 min. The oligopeptide content in the supernatant was determined by the Lowry assay (Lowry *et al.*, 1951) using tyrosine as a standard. One unit of activity was defined as that releasing 1 nmol of tyrosine per min (nmol Tyr⁻¹ min⁻¹). A blank was run in the same manner, except the enzyme was added after addition of 50% (w/v) TCA.

pH and temperature profile

Proteolytic activity was assayed over the pH range of 2.0–11.0 (McIlvaine's buffer for pH 2.0–7.5 and 0.1 M glycine–NaOH for pH 8.0–11.0) at 60 °C for 20 min. For 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 3.5 and 9.5.

pH and thermal stability

The effect of pH on enzyme stability was evaluated by measuring the residual activity after incubation at various pHs at room temperature for 30 min. Different buffers used were mentioned above. The 200 μ L of Japanese sandfish extract was also incubated with McIlvaine's buffer, pH 9.5 at a ratio of 1:1 (v/v) at room temperature for various times (0, 10, 20, 30, 60, 120, 240, 360 and 540 min). The remaining activity was determined using casein as a substrate at pH 9.5 and 55 °C for 20 min.

For thermal stability, enzyme solution was subjected to heating at different temperatures (20–80 °C) for 15 min in a temperature-controlled water bath (Mettler, Schwabach, Germany). Thereafter, the treated samples were suddenly cooled in iced, and then the activity was determined. Japanese sandfish extract was also incubated at 50 °C for up to 8 h. Heated-treated samples were immediately cooled in iced water and tested for the remaining activity.

Effect of inhibitors on proteinase activity

The effect of inhibitors on proteinase activity was determined according to the method of Klomklao *et al.* (2006b) 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 mM PMSF, 1.0 g L⁻¹ 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 30 min. Thereafter, the remaining activity was measured and per cent inhibition was calculated.

Effect of NaCl

Effect of NaCl on proteinase activity in Japanese sandfish extract 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 55 °C and pH 9.5 for 20 min using casein as a substrate.

Effect of CaCl₂ on proteinase activity

To study the effect of CaCl₂ on proteinase activity, CaCl₂ was added into the standard reaction assay to obtain the 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 9.5 for 20 min using casein as a substrate.

Statistical analysis

A completely randomised design was used throughout this study and the experiments were done in triplicate. Data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's multiple range test (Steel & Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for Windows; SPSS Inc., Chicago, IL, USA).

Results and discussion

Autolysis in Japanese sandfish

Effects of temperature and pH on autolytic activity are shown in Fig. 1a and b, respectively. Highest TCA-soluble peptides in Japanese sandfish were observed at 60 °C (Fig. 1a). This result indicated that heat-activated proteinase is primarily responsible for the autolysis of Japanese sandfish proteins. The pH profile of autolytic activity analysed at 60 °C showed two peak at pH 3.5 and 9.0 (Fig. 1b), suggesting that different groups of proteinases were presumed to be present in Japanese sandfish. Siringan *et al.* (2006) reported that maximum autolytic activity of Indian anchovy was found at 60 °C. Autolysis of arrowtooth flounder muscle is due to a heat-activated proteinase active at both acidic and alkaline pH values (Visessanguan *et al.*, 2001). Serine proteinase isolated from salted anchovy muscle exhibited maximal activity at 55 °C (Ishida *et al.*, 1994). On the basis of the temperature and pH optima, autolytic profile of Japanese sandfish indicated the involvement of heat-activated proteinases active at both acidic and alkaline pH values.

Effect of NaCl on autolysis

The continuous decrease in TCA-soluble oligopeptides was observed as NaCl concentration increased (Fig. 2). However, at 25% (w/v) NaCl, the autolytic activity was still remained. The results suggested that endogenous proteinase in Japanese sandfish could hydrolyse muscle

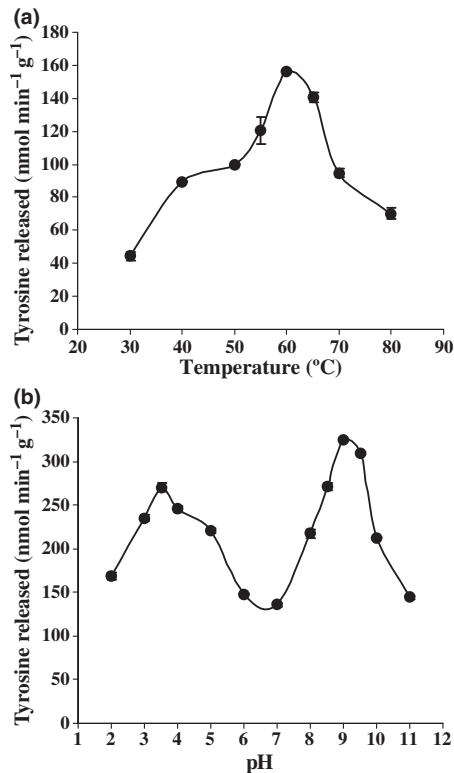


Figure 1 Temperature (a) and pH (b) profiles of Japanese sandfish autolytic activity. Bars represent the standard deviation from triplicate determination.

proteins, even at high salt concentration, but to a lesser extent than in the absence of salt. Ishida *et al.* (1994) reported that thermostable proteinase in salted anchovy muscle may be still active and able to degrade myofibrillar protein in commercial salted fillets containing 16–17% NaCl. Trypsin-like proteinase activity was observed during the first month of anchovy fermentation in 22–28% NaCl (Orejana & Liston, 1982). Siringan *et al.* (2006) also reported that the autolytic activity in Indian anchovy decreased with increasing NaCl concentration. From the result, incubation at optimum temperature (60 °C) and salt reduction, from 25% (w/v) to 10–20% (w/v), might accelerate autolysis in Japanese sandfish.

pH and temperature profiles of Japanese sandfish proteinase activity

The effect of pH on the proteinase activity is shown in Fig. 3a. Optimal pH values of Japanese sandfish extract were found at pH 3.5 and 9.5. The results indicated that both acid and alkaline proteinases were predominant in Japanese sandfish. When activity was assayed at pH 3.5 and 9.5 at different temperatures, activity peak was observed at 55 °C (Fig. 3b). A sharp decrease in activity was observed at temperature above 55 °C, due to

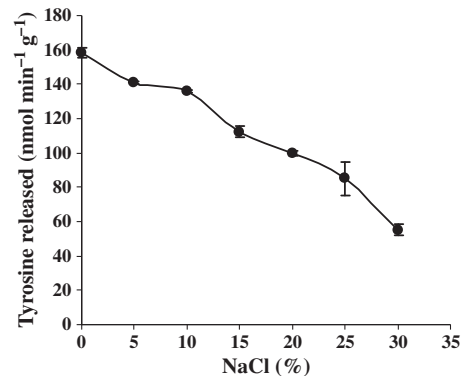


Figure 2 Effect of NaCl on autolytic activity of Japanese sandfish. Bars represent the standard deviation from triplicate determination.

thermal denaturation. At the same test temperature, the activity assayed at pH 9.5 was higher than that observed at pH 3.5 (Fig. 3b). The high activity at pH 9.5 and 55 °C is postulated to be due to heat-stable alkaline proteinase. This result was in agreement with the higher rate of autolysis at alkaline pH value. Heat stable alkaline proteinase has often been reported to be responsible for fish protein degradation. Alkaline proteinase with the optimum pH of 8 and temperature of 62 °C was found in herring muscle (Stoknes *et al.*, 1993). At pH 3.5, it was observed at 55 °C. This was presumed to be due to Japanese sandfish containing aspartic proteinases such as cathepsin D, pepsin, etc. Jiang *et al.* (1993) reported that cathepsin D from mackerel and milkfish muscle had an optimum pH of approximately 3 with optimum temperatures of 45 and 50 °C, respectively. Pepsin enzymes with an optimum pH of 3–3.5 were found in stomach of pectoral rattail (Klomklao *et al.*, 2007b). From the results, the activity was higher at pH 9.5 than at other pH values (Fig. 3a and b). Therefore, the major proteinases in Japanese sandfish were possibly heat-stable alkaline proteinase. Generally, fish sauce fermentation is produced at temperatures of 30–35 °C and pH 5.3–5.8, which were far different from the optimum values of endogenous proteinases. This would partly explain the limited rate of protein hydrolysis during fish sauce fermentation.

Effect of inhibitors on Japanese sandfish proteinase activity

All four types of inhibitors, that is, serine, cysteine, aspartic, and metalloproteinase, were tested with the extract from Japanese sandfish to determine the class of active proteinase at pH 3.5 and 9.5 (Table 1). Proteinase activity was mainly inhibited by pepstatin A (89%) when tested at pH 3.5, whereas activity of 72–93% was inhibited in the presence of serine proteinase inhibitors including soybean trypsin inhibitor, PMSF and TLCK when assayed at pH 9.5 (Table 1). High inhibition by

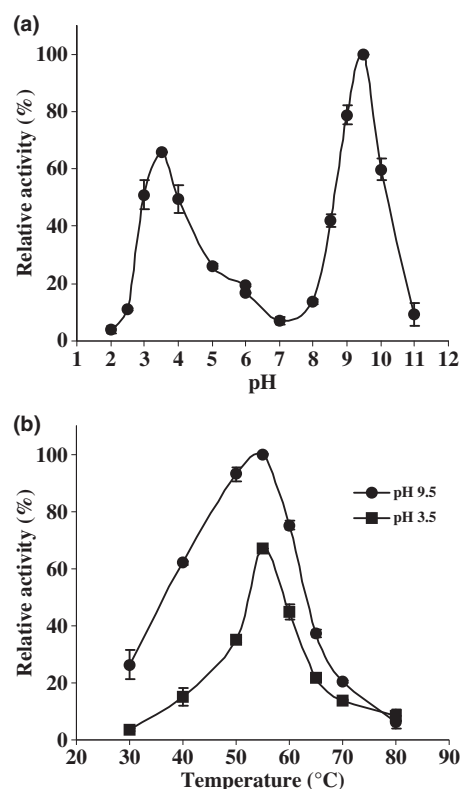


Figure 3 pH (a) and temperature (b) profiles of proteinases from Japanese sandfish. Bars represent the standard deviation from triplicate determination.

pepstatin A at pH 3.5 and soybean trypsin inhibitor, PMSF and TLCK at pH 9.5 indicated the presence of aspartic and serine proteinase, especially trypsin, respectively. Siringan *et al.* (2006) reported that general serine proteinase inhibitors (PMSF, leupeptin) and specific trypsin inhibitors (soybean trypsin inhibitor and TLCK) effectively inhibited crude proteinases from Indian anchovy. Specific inhibitors of cysteine (E-64, *N*-ethylmaleimide, iodoacetic acid) showed negligible (<14%) inhibition of the activity. When EDTA, specific for metalloproteinase (Klomklao *et al.*, 2004), was used, it reduced the activity of fish enzyme at both pH with different degrees varying from 33% to 50%, indicating the presence of proteinases which required metal ions for their activity. Those enzymes include calpain, which needs calcium ion for activation, and metalloproteinases. The high inhibition of TPCK towards the endogenous proteinases in Japanese sandfish indicated the presence of chymotrypsin. From the inhibitory studies it can be inferred that various proteinases were present in Japanese sandfish. However, trypsin-like serine proteinases were the major endogenous proteinase in Japanese sandfish and aspartic proteinase was presented as the minor one.

Table 1 Effect of various inhibitors on Japanese sandfish proteinases activity^a

Inhibitors	Concentration	Inhibition ^b	
		pH 3.5	pH 9.5
Control		0	0
E-64	0.1 mM	10.23 ± 1.09	5.78 ± 2.12
<i>N</i> -ethylmaleimide	1 mM	13.11 ± 0.98	11.19 ± 3.10
Iodoacetic acid	1 mM	7.87 ± 0.45	12.62 ± 0.18
PMSF	1 mM	27.62 ± 0.18	71.77 ± 1.90
Soybean trypsin inhibitor	1.0 g L ⁻¹	24.50 ± 5.30	81.99 ± 0.38
TLCK	5 mM	30.62 ± 1.94	92.66 ± 4.94
TPCK	5 mM	25.25 ± 0.35	61.18 ± 3.18
Pepstatin A	0.01 mM	88.75 ± 3.18	36.45 ± 3.80
EDTA	2 mM	32.62 ± 0.88	49.14 ± 6.08

^aJapanese sandfish extract (200 µL) was mixed with 200 µL of single protease inhibitor to obtain the final concentration shown. The residual proteolytic activity was measured using casein and haemoglobin as substrate.

^bValues are mean ± standard deviation from triplicate determinations.

pH and thermal stability of Japanese sandfish proteinase activity

Endogenous proteinases from Japanese sandfish were stable over a broad pH range (pHs 6–11) (Fig. 4). However, it was unstable at pH below 6. No activity was found after incubated at pH 2 and 3, suggesting irreversible denaturation of enzymes at acidic pHs. Inactivation of enzyme activity at acidic pH was also reported for the trypsin from true sardine (Kishimura *et al.*, 2006) and skipjack tuna (Klomklao *et al.*, 2007a). When the fish enzymes were incubated with 0.1 M glycine–NaOH, pH 9.5 for various times (0–540 min), no changes in relative activity of the enzyme were observed up to 8 h (data not shown). The results suggested that the enzyme was very stable at alkaline pH value, especially at pH 9.5.

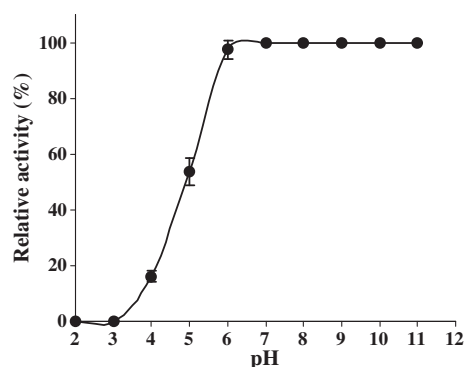


Figure 4 Effect of pH on the stability of proteinase from Japanese sandfish. Bars represent the standard deviation from triplicate determination.

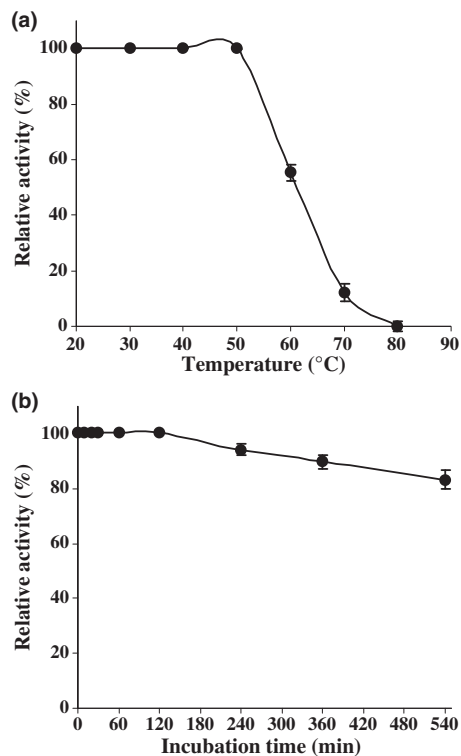


Figure 5 Effect of heating temperature (a) and heating time (b) on the stability of proteinase from Japanese sandfish. The enzymes were heated at different temperatures for 15 min. To study the effect of heating time, the enzyme was heated at 50 °C for different times. Residual activity was determined using casein as a substrate. Bars represent the standard deviation from triplicate determination.

Japanese sandfish proteinases were stable for 15 min when heated up to 50 °C (Fig. 5a). After heat treatment at 60 °C, activities of approximately 50% were retained. No activities were remained after heat treatment at 80 °C. At high temperature, enzyme possibly underwent denaturation and lost their activity. When Japanese sandfish enzyme was subjected to heating at 55 °C for various times (0–540 min) (Fig. 5b), no marked changes in relative activity were found after heating up to 2 h. However, the relative activity of proteinase enzyme slightly decreased when increased the time. Approximately 85% activity remained after heating for 8 h. The results suggested that the Japanese sandfish enzyme was very heat stable up to 8 h at 55 °C. High thermal stability of endogenous proteinases at 55 °C would be advantageous for acceleration of fish sauce production. From the results, incubation the salted fish mixture at optimal condition would maximise autolytic activity without lessening the enzyme activity and might accelerate the protein hydrolysis during fermentation.

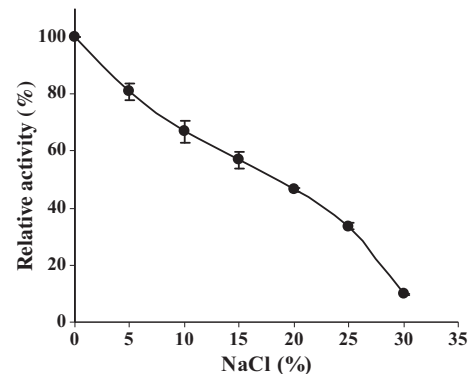


Figure 6 Effect of NaCl on proteinase activity of the extracts from Japanese sandfish. Bars represent the standard deviation from triplicate determination.

Effect of NaCl on Japanese sandfish proteinase activity

The activity decreased with increasing NaCl concentration (Fig. 6). The loss in activity about 70% was noticeable in the presence of 25% (w/v) NaCl. The decrease in activity might be due to the denaturation of enzymes. The 'salting out' effect was postulated to cause the enzyme denaturation. The water molecule is drawn from the enzyme molecule by salt, leading to the aggregation of those enzymes (Klomklao *et al.*, 2004, 2006c). At 10–20% (w/v) NaCl, more than 50% of activity was remained. Thus, lowering salt content would be a means to increase the rate of protein hydrolysis.

Effect of CaCl₂ on Japanese sandfish proteinase activity

Proteinase activity of Japanese sandfish increased with the addition of calcium chloride (Fig. 7). At a concentration lower than 1 µM, calcium chloride had no effect on the endogenous proteinase activity of Japanese sandfish. When the concentration of calcium was

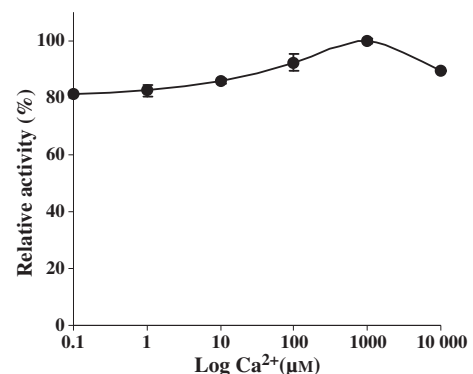


Figure 7 Effect of CaCl₂ on proteinase activity of the extracts from Japanese sandfish. Bars represent the standard deviation from triplicate determination.

increased from 1 μ M to 1 mM, activity apparently increased. However, there was no increase in the activity with calcium chloride above 1 mM. The result was in agreement with Klomklao *et al.* (2004) who found that calcium below 1 μ M had no influence on trypsin activity from tuna spleen, however when the concentration of calcium was increased from 1 μ M to 1 mM, the activity increased. Calcium ions promote the formation of active trypsin from trypsinogen and stabilised trypsin against autolysis (Klomklao *et al.*, 2004). Calcium ions not only protected trypsin against self-digestion, but it also slightly increased its activity (Klomklao *et al.*, 2007a). Kishimura *et al.* (2006) reported the existence of a specific calcium binding site on trypsinogen. These results reconfirmed that predominant endogenous proteinase in Japanese sandfish was trypsin-like enzyme. Therefore, the addition of CaCl_2 into the salted fish mixture during fermentation would be an effective way for acceleration of fish sauce production.

Conclusion

Both acid and alkaline proteinases were found in Japanese sandfish. Trypsin-like enzyme with optimal activity at pH 9.5 and 55 °C was the predominant in fish extract based on inhibitor study, optimal pH and effect of calcium ion. Activity decreased with increasing NaCl concentration. Therefore, the reduction of salt content combined with incubation at optimal condition and adding calcium ion could be the effective way for acceleration of fish sauce production.

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Use of pyloric caeca extract from bigeye snapper (*Priacanthus macracanthus*) for the production of gelatin hydrolysate with antioxidative activity

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ABSTRACT

Proteolytic activity of pyloric caeca extract (PCE) from bigeye snapper (*Priacanthus macracanthus*) was studied. The highest activity was observed at 55 °C and pH 8.0 when casein, *N*^ε-Benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) and *N*^ε-*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME) were used as the substrates. The activity was inhibited markedly by 1 mg/ml soybean trypsin inhibitor, whereas E-64, pepstatin A and EDTA exhibited a negligible effect on activity. The results suggested that a trypsin-like enzyme was most likely the major proteinase in PCE. As determined by activity staining, two proteolytic activity bands with apparent molecular weights of 55 and 24 kDa were found. Gelatin hydrolysate from bigeye snapper skin prepared using PCE exhibited the increases in 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2, 2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activities and ferric reducing antioxidative power (FRAP) as degrees of hydrolysis (DHs) increased ($P < 0.05$). Hydrolysates derived from gelatin using Alcalase combined with PCE showed the highest ABTS radical scavenging activity ($P < 0.05$). Gelatin hydrolysate prepared using Alcalase in combination with Neutrase or PCE at 500 and 1000 ppm, respectively, retarded the oxidation in both linoleic acid oxidation and lecithin liposome oxidation systems. The antioxidative peptide of gelatin hydrolysate had a molecular weight of 1.7 kDa.

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1. Introduction

Free radical-mediated lipid peroxidation, oxidative stress and antioxidants have been widely studied. Under normal conditions, reactive oxygen species (ROS) and free radicals are effectively eliminated in organisms by the antioxidant defense systems such as antioxidant enzymes and non-enzymatic factors (Qian, Jung, & Kim, 2008). A shift in the balance between ROS generation and destruction leads to overproduction or decreased detoxification, which is associated with chronic diseases such as cardiovascular diseases, neurodegenerative disorders, diabetes and certain types of cancer (Ferreira, Baptista, Vilas-Boas, & Barros, 2007). Thus, antioxidants may function to prevent the formation of radicals or to detoxify free radicals. Protein hydrolysates or peptides have been reported to exhibit antioxidative activity. Gelatin hydrolysates from fish skin including Alaska pollack (Kim et al., 2001) and jumbo squid (*Dosidicus gigas*) (Mendis, Rajapakse, & Kim, 2005) showed antioxidative activity via radical scavenging and by retarding lipid peroxidation.

Bigeye snapper (*Priacanthus macracanthus*) is one of the preferable species for surimi production in Thailand. During surimi processing, skin and viscera are generally produced and become by-products, which are commonly used as low-value animal feeds (Hau & Benjakul, 2006). Fish skin can be used as a new material for gelatin production. Nevertheless, utilisation of fish gelatin is limited since it mostly has a lower gel strength and melting temperature, compared to mammalian gelatin (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2006). Therefore, the production of gelatin hydrolysates containing bioactive peptides that may act as potential physiological modulators of metabolism may be an alternative use of fish gelatin (Kim, Je, & Kim, 2007).

Fish viscera generated during processing is a potential source of enzymes such as proteinases that may have some unique properties for industrial applications (Klomklao, Benjakul, Visessanguan, Simpson, & Kishimura, 2005). Trypsin (EC 3.4.21.4) is one of the serine proteases in fish viscera and is commonly synthesized as a proenzyme by pancreatic acinar cells (Kishimura & Hayashi, 2002). Trypsin and trypsin-like enzymes have been isolated and characterised from the intestine of Nile tilapia (*Oreochromis niloticus*) (Bezerra et al., 2001), pyloric caeca of Monterey sardine (*Sardinops sagax caerulea*) (Castillo-Yañez, Pacheco-Aguilar, Garcia-Carreño, &

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Toro, 2005) and pyloric caeca of bluefish (*Pomatomus saltatrix*) (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2007). To maximise the utilisation of fish processing wastes, the extraction of gelatin from skin, which can be used for the production of hydrolysate with antioxidative activity using various proteinases, is promising. The aims of this study were to characterise the proteolytic activity of pyloric caeca extract (PCE) from bigeye snapper and to produce gelatin hydrolysate from bigeye snapper skin possessing antioxidative activity using PCE or PCE in combination with other commercial proteinases.

2. Materials and methods

2.1. Chemicals

Ethylenediaminetetraacetic acid (EDTA), pepstatin A, soybean trypsin inhibitor (SBTI), *trans*-Epoxy succinyl-L-leucyl-amino (4-guanidino) butane (E-64), L-tyrosine, bovine serum albumin (BSA), β -mercaptoethanol, *N* α -Benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), *N* α -*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME), 2,4,6-trinitrobenzene sulphonic acid solution (TNBS), 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), L-leucine, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), malondialdehyde and thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid (TCA), calcium chloride (CaCl₂), Tris (hydroxymethyl) aminomethane and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS), Coomassie Brilliant Blue R-250, G-250 and *N,N,N',N'*-tetramethyl ethylene diamine were procured from Bio-Rad laboratories (Hercules, CA, USA). 2,4,6-Tripyridyl-s-riazine (TPTZ), ferric chloride hexahydrate and potassium persulfate were purchased from Fluka Chemical Co. (Buchs, Switzerland).

2.2. Collection of bigeye snapper pyloric caeca and skin

Fresh bigeye snapper (*P. macracanthus*) with an average weight of 120–150 g were obtained from a dock in Songkhla province, Thailand. Whole fish were stored in ice and off-loaded approximately 36–48 h after capture. After purchasing, fish were placed in ice with the fish/ice ratio of approximately 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 2 h. Upon arrival, fish were washed with tap water (26–27 °C) and eviscerated manually. Viscera were excised and only pyloric caeca was collected. Pyloric caeca was frozen in liquid nitrogen and then ground into powder in liquid nitrogen using a National Model MX-T2GN blender (Taipei, Taiwan) according to the method of García-Carreño, Dimes, and Haard (1993). The powder was placed in polyethylene bags and stored at –20 °C for not longer than 2 months.

Skins were collected and residual meat was removed manually. Cleaned skin was washed with tap water, cut with scissors into small pieces (0.5 × 0.5 cm) and placed in polyethylene bags. Skins were stored at –20 °C until use. The samples were stored not longer than 3 months.

2.3. Preparation of pyloric caeca extract (PCE) from bigeye snapper

2.3.1. Preparation of crude extract

Crude extract was prepared according to the method of Hau and Benjakul (2006) with a slight modification. Pyloric caeca powder (10 g) was homogenised with 100 ml of extracting buffer (50 mM Tris-HCl, pH 8.0 containing 10 mM CaCl₂) at a speed of 11,200 rpm for 2 min using a homogeniser (Model T25B, IKA Labortechnik, Selangor, Malaysia). The homogenate was then stirred continuously

for 30 min at 4 °C and centrifuged in a refrigerated centrifuge (Sorvall, Model RC-B Plus, Newtown, CT, USA) at 11,200 × g for 30 min at 4 °C. The supernatant was filtered using one piece of Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, England) to remove the fat layer. The filtrate was referred to as the “crude extract”.

2.3.2. Ammonium sulphate precipitation

The crude extract was subjected to ammonium sulphate precipitation between 40 and 60% saturation according to the method of Hau and Benjakul (2006). The pellet obtained after centrifugation at 11,200 × g for 30 min at 4 °C was dissolved in 5 ml of 50 mM Tris-HCl, pH 8.0. The solution was then dialysed against 15 volumes of extraction buffer for 12 h at 4 °C with three changes of dialysis buffer. Dialysis bag (Viskase Companies, Inc, Tokyo, Japan) with a nominal molecular weight cut-off of 14,000 Da was boiled in 20 volumes of distilled water for 5 min before use. The dialysate was kept at 4 °C and defined as “pyloric caeca extract; PCE”.

2.4. Assays for proteolytic activity

2.4.1. Proteinase activity

Proteinase activity of PCE was assayed using casein as a substrate according to the method of Klomklao, Benjakul, and Visessanguan (2004). PCE (200 μ l) was added into the assay mixtures containing 2 mg of casein, 200 μ l of distilled water and 625 μ l of reaction buffer. The mixture was incubated for 20 min. The enzymatic reactions were then terminated by adding 200 μ l of 50% (w/v) TCA. Unhydrolysed protein substrate was allowed to precipitate for 1 h at 4 °C, followed by centrifuging at 6500 × g for 10 min. The oligopeptide content in the supernatant was determined by the Lowry assay (Lowry, Rosebrough, Fan, & Randall, 1951) using tyrosine (99% purity) as a standard. One unit of activity was defined as that releasing 1 μ mole of tyrosine per min (μ mol/Tyr/min). A blank was run in the same manner, except that the enzyme was added after the addition of 50% TCA (w/v).

2.4.2. Trypsin activity

Trypsin activity was measured using BAPNA and TAME as substrates according to the methods of Hau and Benjakul (2006) and Kishimura and Hayashi (2002), respectively. When BAPNA was used as a substrate, the sample (200 μ l) with an appropriate dilution was added to 200 μ l of distilled water and 1000 μ l of 50 mM Tris-HCl at the designated pH, containing 10 mM CaCl₂. To initiate the reaction, 200 μ l of BAPNA (2 mg/ml) was added and mixed thoroughly. After incubation at the designated temperature for 10 min, 200 μ l of 30% acetic acid (v/v) was added to terminate the reaction. The absorbance of the reaction mixture was read at 410 nm. Trypsin amidase activity was then calculated using the following formula:

$$\text{Activity (U/ml)} = \frac{(A - A_0) \times \text{Final volume of the mixture (ml)} \times 1000}{8800 \times \text{Time of the reaction (min)} \times 0.2 \text{ (ml)}}$$

where 8800 cm^{–1}M^{–1} is the extinction coefficient of *p*-nitroaniline; A and A₀ are the A₄₁₀ of the sample and the blank, respectively.

When TAME was used as a substrate, the enzyme solution with an appropriate dilution (20 μ l) was mixed with 3.0 ml of 1 mM TAME in 10 mM Tris-HCl buffer at the designated pH and temperature for 20 min. Production of *p*-tosyl-arginine was measured by monitoring the increase in absorbance at 247 nm. One unit of enzyme activity was defined as the amount of enzyme that hydrolysed 1 mM of TAME in 1 min.

2.5. pH and temperature profiles

Activity of PCE was determined over the pH ranges of 6–11 using the different buffers containing 10 mM CaCl_2 . Buffers used included 50 mM sodium acetate for pH 6.0; 50 mM Tris-HCl for pH 7–9; and 50 mM glycine-NaOH for pH 10–11. The activity was assayed at 55 °C using the different substrates as previously described. For the temperature profile study, the activity was determined at various temperatures (25, 30, 40, 50, 55, 60, 65 and 70 °C) at optimum pH using the different substrates.

2.6. Effect of inhibitors

Effect of various inhibitors on proteolytic activity was determined using casein, BAPNA and TAME as substrates. PCE previously dialysed against 15 volumes of 50 mM Tris-HCl, pH 8.0 overnight at 4 °C was added with an equal volume of inhibitor solutions to obtain the final concentrations designated (0.1 mM E-64, 0.1 mM and 0.001 mM pepstatin A, 2 mM EDTA, and 1 mg/ml SBTI). The residual activity was measured at the optimal pH and temperature. Percentage inhibition was then calculated.

2.7. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and substrate gel electrophoresis

2.7.1. SDS-PAGE

SDS-PAGE was done using the method of Laemmli (1970). PCE was mixed with sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol and 10% β -mercaptoethanol) at a ratio of 1:1 (v/v). The mixture (10 μg of protein) was loaded onto the gel having a 4% stacking gel and a 10% separating gel. The electrophoresis was run at a constant current of 15 mA per gel using a Mini-Protein II cell apparatus (Bio-Rad Laboratories Inc., Richmond, CA, USA). After electrophoresis, the gel was stained with 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 7.5% acetic acid for 3 h and destained using a solution containing 50% methanol and 7.5% acetic acid for 3 h. The molecular weights of the protein bands were estimated by comparing with a wide range molecular weight standard (Sigma Chemical Co.) including aprotinin (6.5 kDa), α -lactalbumin (14.2 kDa), trypsin inhibitor (20 kDa), trypsinogen (24 kDa), carbonic anhydrase, bovine erythrocytes (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), ovalbumin (45 kDa), glutamic dehydrogenase (55 kDa), albumin (66 kDa), phosphorylase B (97 kDa), β -galactosidase (116 kDa) and myosin (200 kDa).

2.7.2. Substrate gel electrophoresis

Substrate gel electrophoresis was performed as described by García-Carreño et al. (1993). PCE was mixed with sample buffer at a ratio of 1:1 (v/v). Sample (10 μg of protein) was loaded onto the polyacrylamide gel and subjected to electrophoresis at a constant current of 15 mA per gel using the Mini-Protein II cell apparatus. After electrophoresis, gels were immersed in 100 ml of 2% (w/v) casein in 50 mM Tris-HCl buffer, pH 7, for 1 h with constant agitation at 0 °C to allow the casein to penetrate into the gels. Gels were then transferred to 100 ml of 2% (w/v) casein in 50 mM Tris-HCl, pH 8.5 and incubated at 55 °C for 15 min to develop activity zones. The gels were then stained and destained as described previously. Development of clear zones on the blue background indicated proteolytic activity.

2.8. Preparation of skin gelatin hydrolysate

2.8.1. Extraction of gelatin from fish skin

Gelatin was extracted from bigeye snapper skin according to the method of Jongjareonrak et al. (2006) with a slight modification.

Skins were soaked in 0.1 M NaOH with a skin/solution ratio of 1:10 (w/v) at room temperature (25 °C) with a gentle stirring. The solution was changed every 30 min three times to remove non-collagenous proteins and pigments. Alkaline-treated skins were then washed with tap water until the neutral or faintly basic pH wash waters were obtained as determined by a pH meter. The skins were then soaked in 0.05 M acetic acid with a skin/solution ratio of 1:10 (w/v) for 3 h at room temperature with a gentle stirring to swell the collagenous material in the fish skin matrix. Acid-treated skins were washed as previously described. The swollen fish skins were soaked in distilled water with a skin/water ratio of 1:10 (w/v) at 45 °C for 12 h with continuous stirring to extract the gelatin from the skins. The mixture was then filtered using two layers of cheesecloth. The resultant filtrate was freeze-dried. The dry sample was blended until the fine power was obtained using a blender (Moulinex Type AY46R4, Lyon, France). Gelatin powder contained $8.46 \pm 0.36\%$ moisture, $89.8 \pm 1.61\%$ protein, $2.9 \pm 0.15\%$ ash content and $0.46 \pm 0.04\%$ fat as determined by methods of the AOAC (2000) with the method numbers of 950.46, 928.08, 920.153 and 960.39, respectively.

2.8.2. Uses of various single proteinases

Hydrolysates from bigeye snapper skin gelatin were prepared by dissolving gelatin in Mcllvaine buffer (0.2 M Na phosphate and 0.1 M Na citrate) at the desired pH to obtain a protein concentration of 2%. The pH of gelatin solutions was adjusted to pH 8.0 for hydrolysis using PCE and Alcalase and to 7.0 for hydrolysis using Neutrase. The hydrolysis reaction was started by the addition of the selected enzyme at the amount calculated from the plot between log (enzyme concentration) and DH to obtain DH of 5, 10 and 15% for PCE and Neutrase and DHs of 5, 10, 15, 20 and 25% for Alcalase as described by Benjakul and Morrissey (1997). DH was determined by the 2,4,6-trinitrobenzene sulphonic acid (TNBS) technique as described by Adler-Nissen and Olsen (1979). After 1 h of hydrolysis at optimal temperature (55, 55 and 60 °C for PCE, Alcalase and Neutrase, respectively), the enzymes were inactivated by heating at 90 °C for 15 min in a water bath (model W350, Memmert, Schwabach, Germany). The mixture was then centrifuged at $2000 \times g$ at 4 °C for 10 min. The supernatant referred to as a “gelatin hydrolysate” was tested for antioxidative activities.

2.8.3. Uses of various combined proteinases

Two steps for the hydrolysis using different proteinases were used in some cases. First, a gelatin hydrolysate was prepared using PCE, Alcalase or Neutrase to obtain a DH of 15, 25 and 15%, respectively, under optimal conditions for each enzyme as previously described. Further hydrolysis was conducted using another proteinase under its optimal condition for 1 h. Thereafter, enzymes were inactivated by heating at 90 °C for 15 min in a water bath. The mixture was then centrifuged at $2000 \times g$ at 4 °C for 10 min and the supernatant was collected and referred to as a gelatin hydrolysate. Hydrolysates obtained were subjected to analysis for antioxidative activity.

2.9. Determination of antioxidant activity

2.9.1. DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by Binsan et al. (2008). To the sample (1.5 ml), 1.5 ml of 0.15 mM DPPH in 50% ethanol was added. The mixture was mixed well using a Vortex mixer (Scientific Industries, Inc, Bohemia, NY, USA) and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was measured at 517 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The blank was prepared in the same manner, except that distilled water was

used instead of the sample. A standard curve was prepared using Trolox in the range of 10–60 μM . The activity was expressed as μmol Trolox equivalents (TE)/mg protein of gelatin hydrolysate.

2.9.2. ABTS radical scavenging activity

ABTS radical scavenging activity was determined 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 dark. The solution was then diluted by mixing 1 ml of ABTS solution with 50 ml of methanol to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using the UV-1601 spectrophotometer. Fresh ABTS solution was prepared daily. Sample (150 μl) was mixed with 2850 μl of ABTS solution and the mixture was left at room temperature for 2 h in dark. The absorbance was then measured at 734 nm. A standard curve of Trolox ranging from 50 to 600 μM was prepared. The activity was expressed as μmol Trolox equivalents (TE)/mg protein of gelatin hydrolysate.

2.9.3. Ferric reducing antioxidant power (FRAP)

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 $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 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 $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 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 tripyridyltriazine complex (colored product) was measured by reading the absorbance at 593 nm. The standard curve was prepared using Trolox ranging from 50 to 600 μM . The activity was expressed as μmol Trolox equivalents (TE)/mg protein of gelatin hydrolysate.

2.9.4. Linoleic oxidation system

The antioxidant activity of the gelatin hydrolysates in a linoleic acid model system was determined according to the method of Sakanaka, Tachibana, Ishihara, and Juneja (2004). The hydrolysate samples were dissolved in 1.5 ml of 0.1 M phosphate buffer (pH 7.0) to obtain concentrations of 100, 500 and 1000 ppm. Each solution (0.5 ml) was mixed with 1 ml of 50 mM linoleic acid in 99.5% ethanol, and the mixtures were stored at 40 °C in the dark. At regular intervals, aliquots of the mixture were taken for determination of oxidation using the ferric thiocyanate method according to the method of Thiansilakul, Benjakul, and Shahidi (2007). To 50 ml of the mixture, 2.35 ml of 75% ethanol, 50 ml of 30% ammonium thiocyanate and 50 ml of 20 mM ferrous chloride solution in 3.5% HCl were added and mixed thoroughly. After 3 min, the absorbance of the colored solution was read at 500 nm. Mixtures containing BHT at 25 or 100 ppm were used for comparative purposes. The control was prepared in the same manner except that no protein hydrolysate or antioxidants were added.

2.9.5. Lecithin liposome system

The antioxidative activity of gelatin hydrolysates in a lecithin liposome system was determined as described by Thiansilakul et al. (2007). Lecithin was suspended in deionised water at a concentration of 8 mg/ml by stirring with a glass rod, followed by sonicating for 30 min in a sonicating bath (model Transsonic 460/H, Elma, Singen, Germany). A hydrolysate sample at a concentration of 100, 500 and 1000 ppm (3 ml) was added to the lecithin liposome system (15 ml). The liposome suspension was then sonicated for 2 min. To initiate the assay, 20 μl of cupric acetate (0.15 M) was added. The mixture was shaken at 120 rpm using a shaker (Unimax

1010, Heidolph, Schwabach, Germany) at 37 °C in the dark. The control and systems containing 25 or 100 ppm BHT were also prepared. At regular intervals, oxidation of the liposome was monitored by determining the thiobarbituric acid reactive substances (TBARS) (Lee & Hendricks, 1997) and conjugated dienes (Frankel, Huang, & Aeschbach, 1997).

2.9.5.1. Determination of thiobarbituric acid reactive substances (TBARS). TRARS value was determined according to the method of Lee and Hendricks (1997). Liposome sample (1 ml) was mixed with 20 μl of 0.2% butylated hydroxytoluene and 2 ml of TBA solution (15% TCA/0.375% TBA/0.025 N HCl). The mixtures were mixed well and placed in the boiling water to develop the pink color. The mixture was cooled using the running tap water, followed by centrifugation at $5000 \times g$ for 20 min. The absorbance of supernatant was read at 532 nm using a UV-1601 spectrophotometer. The standard curve was prepared in the same manner using malondialdehyde (MDA) as a standard. TBARS was expressed as MDA/ml liposome.

2.9.5.2. Determination of conjugated diene. Conjugated diene content was measured as per the method of Frankel et al. (1997). Liposome sample (0.1 ml) was dissolved in methanol (5.0 ml). The absorbance of the solution obtained was read at 234 nm using the UV-1601 spectrophotometer.

2.10. Fractionation of antioxidative peptides from gelatin hydrolysate

Gelatin hydrolysates prepared using Alcalase + Neutrase or Alcalase + PCE were subjected to gel filtration chromatography. The hydrolysate was loaded onto a Sephadex G-25 column (2.5×50 cm; Bio-Rad Laboratories, Hercules, CA, USA). The elution was done with 50 mM sodium phosphate buffer (pH 7.0) at a flow rate of 1 ml/min. The 3-ml fractions were collected and their absorbance was read at 220 and 280 nm. Gramicidin (1880 Da), vitamin B12 (1355 Da), flavin adenine dinucleotide (829.5 Da) and Gly-Tyr (238.25 Da) were used as the molecular weight standards. The fractions were subjected to analysis for ABTS radical scavenging activity.

2.11. Statistical analysis

All data were subjected to analysis of variance (ANOVA) and the differences between means were evaluated by Duncan's Multiple Range Test (Steel & Torrie, 1980). Analysis was performed using an SPSS package (SPSS 10.0 for Windows, SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. pH and temperature profile of PCE

The effects of pH on the proteolytic activities of PCE using different substrates are shown in Fig. 1(A). The optimal pH of PCE for casein, BAPNA and TAME hydrolysis was 8.0. Proteolytic activity of PCE decreased at acidic and very alkaline pH, probably due to the denaturation of the enzymes. Pyloric caeca have been reported as a source of proteolytic enzyme, particularly trypsin or trypsin-like enzymes. Alkaline proteolytic activity from tambaqui (*Colossoma macropomum*) had an optimum pH ranging from 7.0 to 9.0 when azocasein was used as a substrate (Bezerrar, Santos, Lino, Vieira, & Carvalho, 2000). Trypsin obtained from Monterey sardine pyloric caeca had an optimal pH of 8.0 for BAPNA hydrolysis (Castillo-Yañez et al., 2005). Trypsin from pyloric caeca of the starfish (*Asterina pectinifera*) showed an optimal pH of 8.0 when TAME was used as

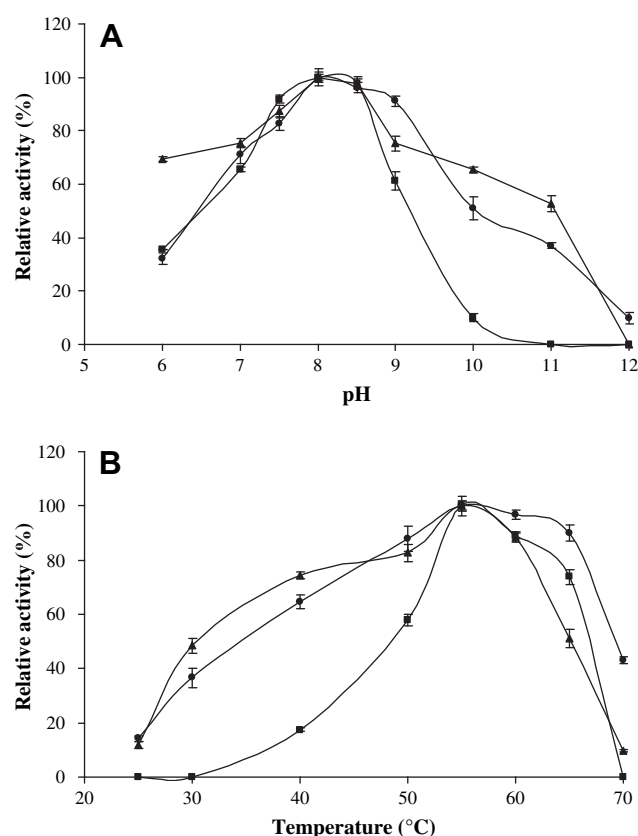


Fig. 1. Effect of pH (A) and temperature (B) on the proteolytic activity of PCE from bigeye snapper using casein, *N*^ε-Benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), and *N*^ε-*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME) as substrates. Bars represent standard deviation from triplicate determinations. (●) casein; (■) BAPNA; (▲) TAME.

a substrate (Kishimura & Hayashi, 2002). In addition, Heu, Kim, and Pyeun (1995) reported that trypsin and chymotrypsin from anchovy viscera had an optimum activity at pH 9.0 and 8.0 when casein and synthetic substrates (BAPNA and BTEE) were used as substrates, respectively. The results suggested that alkaline proteinases, most likely trypsin or trypsin-like enzyme, were present in the pyloric caeca of bigeye snapper.

The optimal temperature of proteinases in PCE from bigeye snapper was 55 °C (Fig. 1(B)) when casein, BAPNA and TAME were used as substrates. The activity decreased when the temperature was increased up to 70 °C. The loss in activity was presumably caused by thermal denaturation of the enzymes (Hau & Benjakul, 2006). The optimal temperature of proteinases in PCE was similar to those of other fish trypsins reported previously. Hau and Benjakul (2006) reported that the optimum temperature of trypsin from the pyloric caeca of bigeye snapper (*P. macracanthus*) was 55 °C when BAPNA was used as a substrate. Trypsin from the pyloric caeca of bluefish (*P. saltatrix*) had the optimal temperature for the hydrolysis of BAPNA at 55 °C (Klomklao et al., 2007). Two isozymes of trypsin purified from the viscera of Japanese anchovy (*Engraulis japonica*) had maximal activities at around 60 °C for hydrolysis of TAME (Kishimura, Hayashi, Miyashita, & Nonami, 2005). Alkaline serine proteinase from pyloric caeca of tambaqui (*C. macropomum*) exhibited the maximal activity at 60 °C when azocasein was used as a substrate (Bezerra et al., 2001). Furthermore, Klomklao et al. (2004) reported that tuna splenic extract contained heat-activated alkaline serine proteinases as the major enzymes. These had an optimum temperature of 55 °C when casein

Table 1

Effect of various inhibitors on the proteolytic activity of PCE of bigeye snapper using different substrates.

Inhibitors	Concentration	Inhibition (%) ^a		
		Casein	BAPNA	TAME
E-64	0.1 mM	0	11.1 ± 1.6	2.4 ± 0.3
Pepstatin A	0.1 and 0.001 mM	12.1 ± 1.2	0	6.2 ± 1.2
EDTA	2 mM	6.4 ± 0.7	3.1 ± 0.5	9.1 ± 1.3
SBTI	1 mg/ml	72.2 ± 1.3	98.3 ± 1.1	76.2 ± 2.7

All assays were carried out at pH 8.0 and 55 °C.

^a Mean ± SD from triplicate determinations.

was used as a substrate. The results indicated that PCE contained proteinases, which were active at high temperature.

3.2. Effect of inhibitors

The effect of various inhibitors on PCE proteinases is shown in Table 1. When casein was used as a substrate, PCE proteinase activity was highly inhibited by 1.0 mg/ml soybean trypsin inhibitor (72.2%). Specific inhibitors of aspartic proteinases (pepstatin A) showed partial inhibition (12.1%). EDTA, specific for metalloproteinases (Castillo-Yañez et al., 2005) had a slight inhibitory effect on proteolytic activity with approximately 6.4% inhibition. A specific inhibitor of cysteine proteinases (E-64) had no inhibitory effect on proteinase activity of PCE. The results indicated that the major proteinases of PCE were serine proteinases. Furthermore, when BAPNA and TAME were used as substrates, the activities of PCE were strongly inhibited by SBTI (98.3 and 76.2%, respectively), whereas E-64 and EDTA showed slight inhibitory activity towards PCE. EDTA possibly affected the structure of the enzyme or active site conformation, leading to the reduced activity (Klomklao et al., 2007). Since BAPNA and TAME are synthetic substrates specific for trypsin, the considerable inhibition by SBTI confirmed that trypsin was the major proteinase in PCE. This finding was in agreement with that of trypsin from pyloric caeca of the same species of bigeye snapper (*P. macracanthus*), in which 0.1 mM SBTI could inhibit proteinase effectively when BAPNA was used as the substrate (Hau & Benjakul, 2006).

3.3. SDS-PAGE and substrate gel electrophoresis

PCE contained a protein with an apparent molecular weight (MW) of 24 kDa as the major band. Additionally, the proteins with the apparent MW of 55 and 200 kDa were also found in PCE (Fig. 2A). When comparing protein patterns under reducing and non-reducing conditions, it was noted that the band intensity of proteins with MW of 55 and 24 kDa slightly decreased under the reducing condition. However, no marked changes were found with the protein having a MW of 200 kDa. The results suggested that proteins in PCE were partially stabilised by disulphide bonds. For activity staining, two proteolytic activity bands of PCE with MW of 24 and 55 kDa were noticeable (Fig. 2B). The activity band with a MW of 24 kDa was much larger than that of the protein with a MW of 55 kDa. Generally, fish trypsin has been reported to have a molecular weight in the range of 23–28 kDa, e.g., Japanese anchovy trypsin (24 kDa) (Kishimura et al., 2005) and bigeye snapper trypsin (23.8 kDa) (Hau & Benjakul, 2006). The activity band decreased slightly under reducing conditions. Thus, the enzyme configuration might be stabilised by disulphide bonds to some extent. Serine proteases including trypsin and chymotrypsin contain three conserved disulphide bonds, which connect the loops around the substrate binding pocket (Várallyay, Lengyel, Gráf, & Szilágyi, 1997).

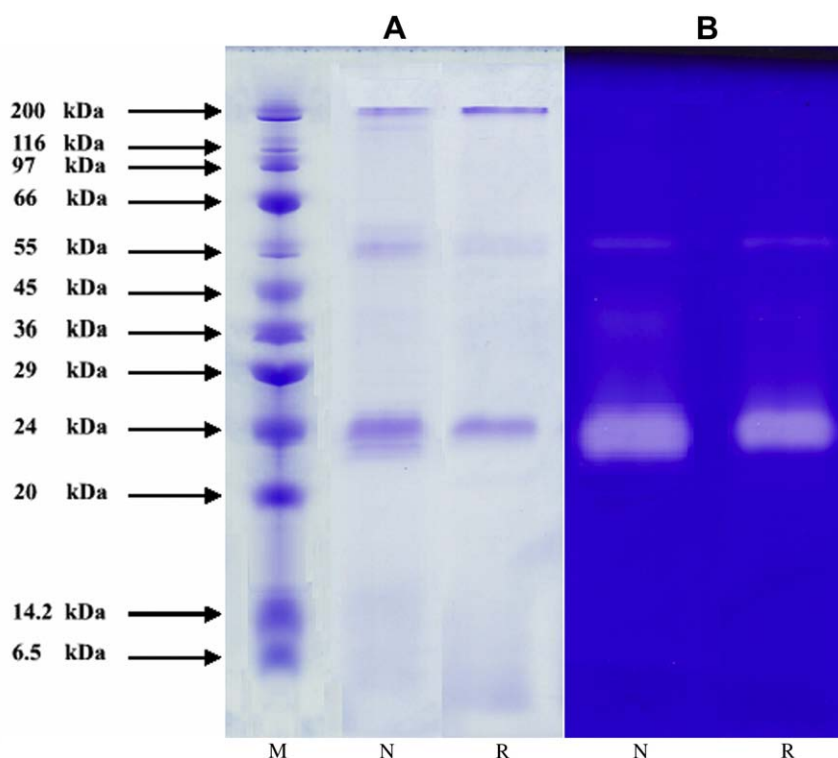


Fig. 2. SDS-polyacrylamide gel electrophoresis (A) and substrate gel electrophoresis (B) of PCE from bigeye snapper. M: marker; N: non-reducing condition; R: reducing condition.

3.4. Antioxidative activity of gelatin hydrolysates from bigeye snapper skin prepared using various single proteases

3.4.1. DPPH radical scavenging activity

All gelatin hydrolysates prepared using various enzymes had increased DPPH radical scavenging activity as DH increased ($P < 0.05$) (Fig. 3(A)). At 15% DH, hydrolysates prepared using Neutrase showed the highest activity ($P < 0.05$), followed by those prepared using Alcalase and PCE, respectively. At DH of 5 and 10%, hydrolysates prepared using Alcalase had higher activity than the others. Very high amounts of Neutrase and PCE were required for production of gelatin hydrolysates with DH greater than 15%. Thiansilakul et al. (2007) reported that DPPH radical scavenging activity of round scad muscle protein hydrolysates prepared using Flavourzyme and Alcalase increased with increasing DH up to 60 and 40%, respectively. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH is often used to evaluate the antioxidant activity (Qian et al., 2008). The results show that the gelatin hydrolysates from bigeye snapper skin possibly contained substances that were hydrogen donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction.

3.4.2. ABTS radical scavenging activity

With increasing DH, all gelatin hydrolysates showed increased ABTS $\cdot$ radical scavenging activity ($P < 0.05$) (Fig. 3(B)). At the same DH, hydrolysates prepared using PCE showed the highest activity ($P < 0.05$), followed by those prepared using Alcalase and Neutrase, respectively. The ability of hydrolysates to scavenge DPPH and ABTS radicals varied with the enzymes used. Hydrolysates prepared using PCE had the highest ABTS radical scavenging activity but showed the lowest DPPH radical scavenging activity ($P < 0.05$), compared to hydrolysates prepared using the other enzymes. The

results suggested that those hydrolysates might scavenge two radicals, ABTS and DPPH, differently. It is well known that the radical system used for antioxidant evaluation may influence the experimental results, and two or more radical systems are required to investigate the radical scavenging capacities of a selected antioxidant (Yu et al., 2002). The pre-formed radical monocation of ABTS $\cdot^+$ is generated by oxidation of ABTS with potassium persulphate and is reduced in the presence of hydrogen-donating antioxidants and of chain breaking antioxidants (Binsan et al., 2008). Therefore, PCE was appropriate to produce gelatin hydrolysates that had the ability to scavenge free radicals, thereby preventing lipid oxidation via a chain breaking reaction.

3.4.3. Ferric reducing antioxidant power (FRAP)

FRAP of gelatin hydrolysates prepared using different proteinases increased as DH increased ($P < 0.05$) (Fig. 3(C)). At 15% DH, the hydrolysates prepared using Neutrase showed the highest FRAP, compared with the other hydrolysates ($P < 0.05$). The hydrolysates prepared using Neutrase with 15% DH and the hydrolysates prepared using Alcalase with 25% DH had similar FRAP. The reducing power of protein hydrolysates from mackerel by autolysis and with Protease N increased gradually with increasing hydrolysis time (Wu, Chen, & Shiau, 2003). The antioxidant potential of gelatin hydrolysates was estimated from their ability to reduce the TPTZ–Fe (III) complex to the 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 (Ferreira et al., 2007). From these results, gelatin hydrolysates prepared with Neutrase had the pronounced effect of donating electrons that led to the retardation of propagation of lipid oxidation (Binsan et al., 2008).

Differences in DPPH, ABTS radical scavenging activity and FRAP between gelatin hydrolysates from bigeye snapper skin prepared using different enzymes possibly resulted from existing differences

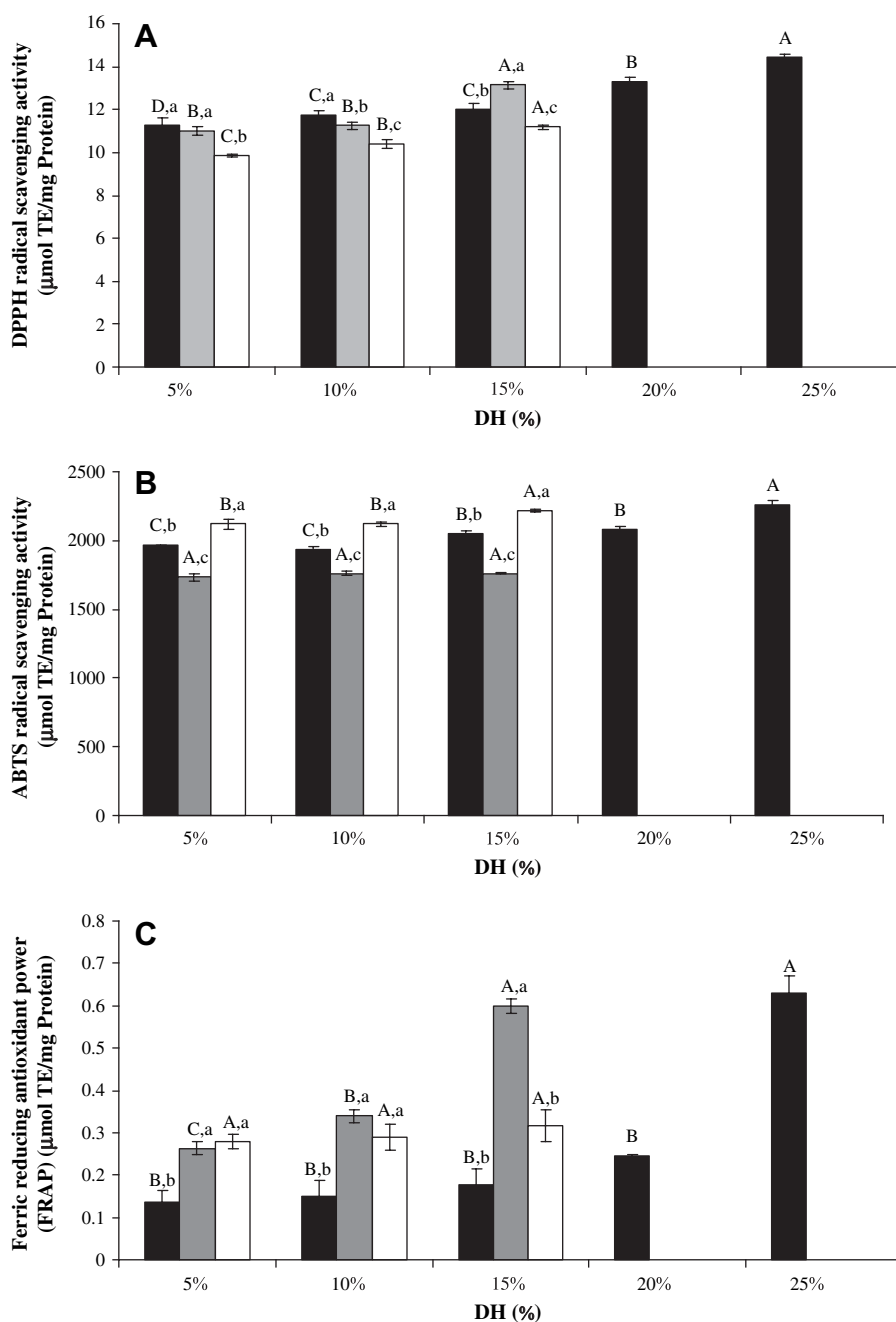


Fig. 3. DPPH radical scavenging activity (A), ABTS radical scavenging activity (B) and ferric-reducing antioxidant power (C) of skin gelatin hydrolysates prepared using Alcalase, Neutrase or PCE with different DHs. Bars represent standard deviation from triplicate determinations. Different capital letters indicate significant differences ($P < 0.05$) with the same enzyme. Different lowercase letters indicate significant differences ($P < 0.05$) with the same DH. ■: Alcalase; ■: Neutrase; □: PCE.

in the enzyme specificity toward protein substrates, in which a wide variety of peptides with different modes of actions for inhibiting lipid oxidation were generated during hydrolysis. Changes in size, amount, the exposure of the terminal amino groups of the products obtained and the composition of free amino acids or small peptides affect the antioxidative activity (Thiansilakul et al., 2007; Wu et al., 2003). Low molecular weight peptides generally showed higher antioxidant activity (Qian et al., 2008). Thus, gelatin hydrolysates from bigeye snapper skin with high DPPH radical scavenging activity and FRAP were prepared by using Neutrase or Alcalase to obtain DH of 15 and 25%, respectively. To produce gelatin hydrolysates with high ABTS radical scavenging

activity, PCE or Alcalase could be used to obtain DH of 15 and 25%, respectively. Those hydrolysates were prepared and another hydrolysis using a second enzyme was then conducted to increase the antioxidative activity of the resulting hydrolysates.

3.5. Antioxidative activity of gelatin hydrolysates from bigeye snapper skin prepared using a two-step hydrolysis

Gelatin hydrolysates from bigeye snapper skin were prepared by a two-step hydrolysis using two different enzymes including Alcalase + Neutrase, Alcalase + PCE, Neutrase + Alcalase, Neutrase + PCE, PCE + Alcalase and PCE + Neutrase. The first step of the

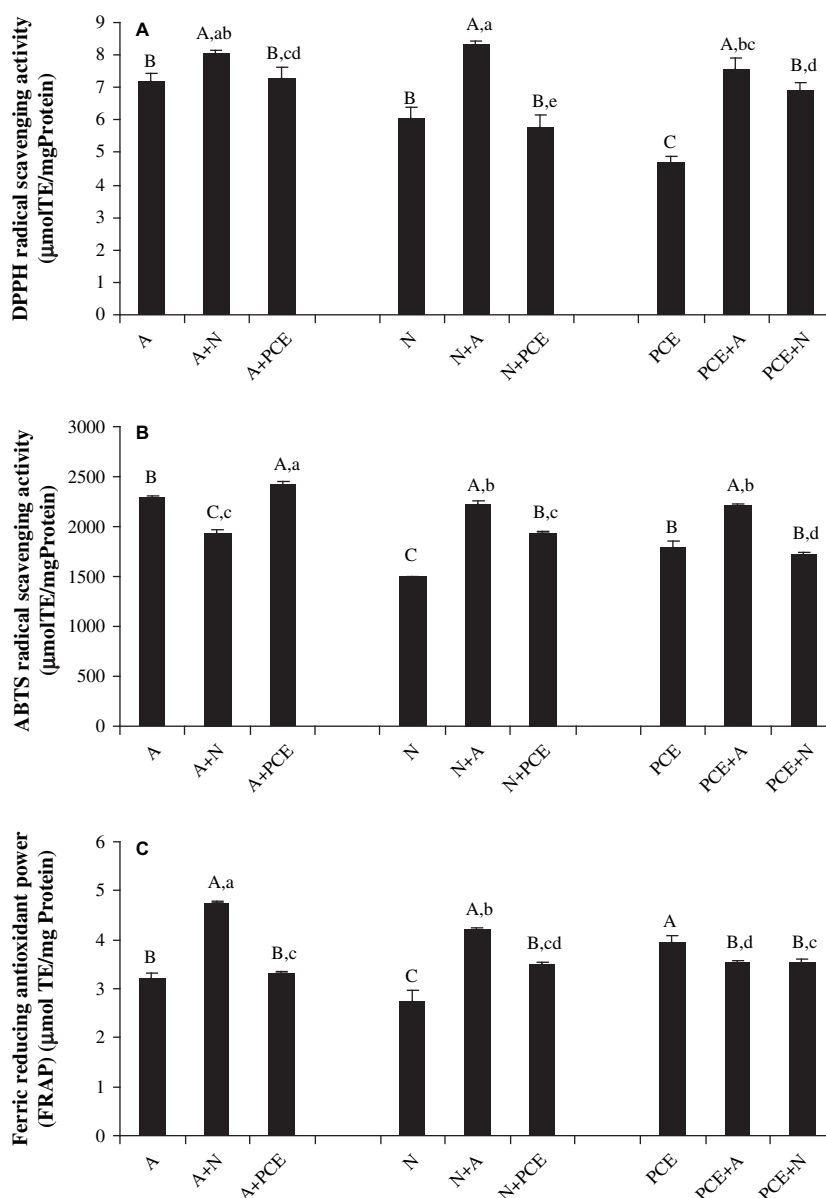


Fig. 4. DPPH radical scavenging activity (A), ABTS radical scavenging activity (B) and ferric-reducing antioxidant power (C) of skin gelatin hydrolysates prepared using a two-step hydrolysis with different enzymes. A: Alcalase; N: Neutrase; PCE: pyloric caeca extract. Bars represent standard deviation from triplicate determinations. Different capital letters indicate significant differences ($P < 0.05$) with the same first-step enzyme. Different lowercase letters indicate significant differences ($P < 0.05$) of the hydrolysates with a two-step hydrolysis.

hydrolysis was done under the optimum conditions for that enzyme with a selected DH (Alcalase = 25%, Neutrase = 15% and PCE = 15%). The second step was conducted with a different enzyme using the optimal conditions for that enzyme.

3.5.1. DPPH radical scavenging activity

The DPPH radical scavenging activity of gelatin hydrolysates from bigeye snapper skin using the combination of two enzymes is shown in Fig. 4(A). Gelatin hydrolysates prepared using combination of two enzymes showed increased DPPH radical scavenging activities except for hydrolysates prepared using Alcalase + PCE and Neutrase + PCE, which showed no increase in activity ($P > 0.05$), compared with hydrolysates prepared using Alcalase and Neutrase alone. Hydrolysates produced by the combination of Alcalase and Neutrase (N+A and A+N) showed the highest DPPH radical scavenging activity ($P < 0.05$). The results suggested that peptides

generated from the combined hydrolysis using two different enzymes had a greater ability to scavenge DPPH radicals or to donate hydrogen. Wu et al. (2003) reported that the DPPH radical scavenging activities of mackerel (*Scomber australasicus*) protein hydrolysates obtained by both autolysis and Protease N increased gradually with increasing hydrolysis time.

3.5.2. ABTS radical scavenging activity

ABTS radical scavenging activity of gelatin hydrolysates prepared by combined enzymes is shown in Fig. 4(B). The hydrolysates exhibited ABTS radical scavenging activity differently, depending on the enzymes used. For hydrolysates prepared using Alcalase for the first hydrolysis, the use of Neutrase for the second step of hydrolysis resulted in lower activity, whereas the use of PCE in combination yielded hydrolysates with increased activity ($P < 0.05$). For hydrolysates using Neutrase for the first step of the

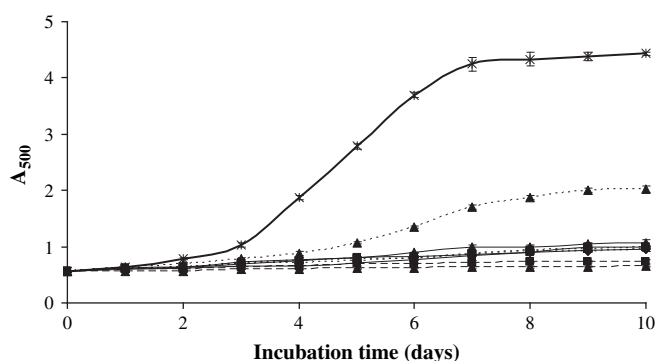


Fig. 5. Effect of gelatin hydrolysates prepared using Alcalase + Neutrase or Alcalase + PCE at various amounts on the A_{500} of the linoleic acid system in comparison with the control, 25 and 100 ppm BHT. Bars represent the SD from triplicate determinations. (—●—) control; (---□---) 25 ppm BHT; (---△---) 100 ppm BHT; (---▲---) 100 ppm Alcalase + Neutrase; (---■---) 500 ppm Alcalase + Neutrase; (---◆---) 1000 ppm Alcalase + Neutrase; (---▲---) 100 ppm Alcalase + PCE; (---■---) 500 ppm Alcalase + PCE; (---◆---) 1000 ppm Alcalase + PCE.

hydrolysis, the use of both Alcalase and PCE as the second step could enhance the activity of the resulting hydrolysates. The use of Alcalase to further hydrolyse the hydrolysates first prepared using PCE resulted in increased activity, whereas the use of Neutrase had no impact ($P > 0.05$). Thus, peptides produced during hydrolysis by different enzymes could be varied in term of ABTS radical scavenging activity.

3.5.3. Ferric reducing antioxidant power (FRAP)

The FRAP of gelatin hydrolysates prepared using different combinations of enzymes is shown in Fig. 4(C). Hydrolysates prepared with Alcalase in the first hydrolysis, followed by Neutrase showed the highest FRAP ($P < 0.05$) and FRAP was greater than that of hydrolysates prepared using only Alcalase ($P < 0.05$). However, PCE had no effect on the FRAP of hydrolysates when it was used for the second step of hydrolysis. For hydrolysate prepared using Neutrase, the uses of Alcalase or PCE for the second hydrolysis resulted in increased FRAP, but the former yielded hydrolysates with higher FRAP than the latter. On the other hand, the use of Alcalase and Neutrase for the second hydrolysis of hydrolysates first prepared using PCE led to decreased FRAP ($P < 0.05$). The differences in FRAP might be governed by peptides in the hydrolysates. A number of studies have already shown that the antioxidant activity of hydrolysates was dependent on their molecular weight distribution (Wu et al., 2003). Kim et al. (2001) prepared hydrolysates with antioxidative activity from Alaska pollack skin gelatin using serial digestions yielding antioxidant activity in the descending order of Alcalase, Pronase E, and collagenase.

3.5.4. Antioxidative activity of gelatin hydrolysates in a linoleic oxidation system

Both gelatin hydrolysates prepared using Alcalase + Neutrase or Alcalase + PCE showed antioxidative activity in the linoleic oxidation system. Linoleic acid, an unsaturated fatty acid, is usually used as a model compound in lipid oxidation and antioxidation-

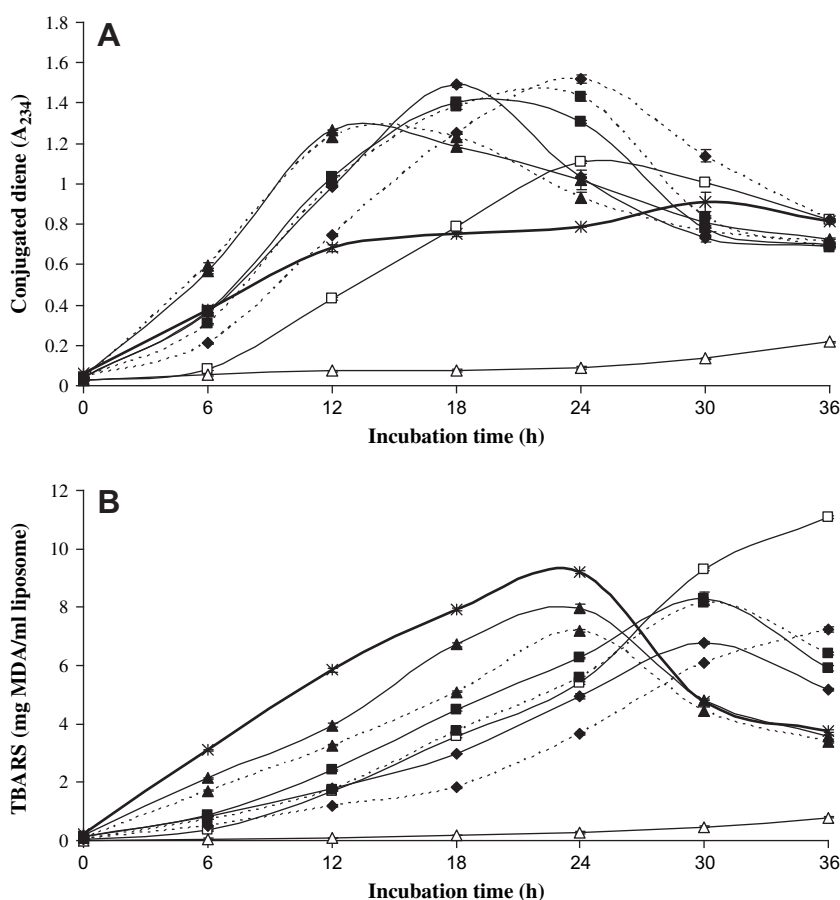


Fig. 6. Effect of gelatin hydrolysates prepared using Alcalase + Neutrase or Alcalase + PCE at various amounts on the formation of conjugated dienes (A) and thiobarbituric acid reactive substances (TBARS) (B) in the lecithin liposome system in comparison with the control, and systems containing 25 or 100 ppm BHT. Bars represent the SD from triplicate determinations. (—●—) control; (---□---) 25 ppm BHT; (---△---) 100 ppm BHT; (---▲---) 100 ppm Alcalase + Neutrase; (---■---) 500 ppm Alcalase + Neutrase; (---◆---) 1000 ppm Alcalase + Neutrase; (---▲---) 100 ppm Alcalase + PCE; (---■---) 500 ppm Alcalase + PCE; (---◆---) 1000 ppm Alcalase + PCE.

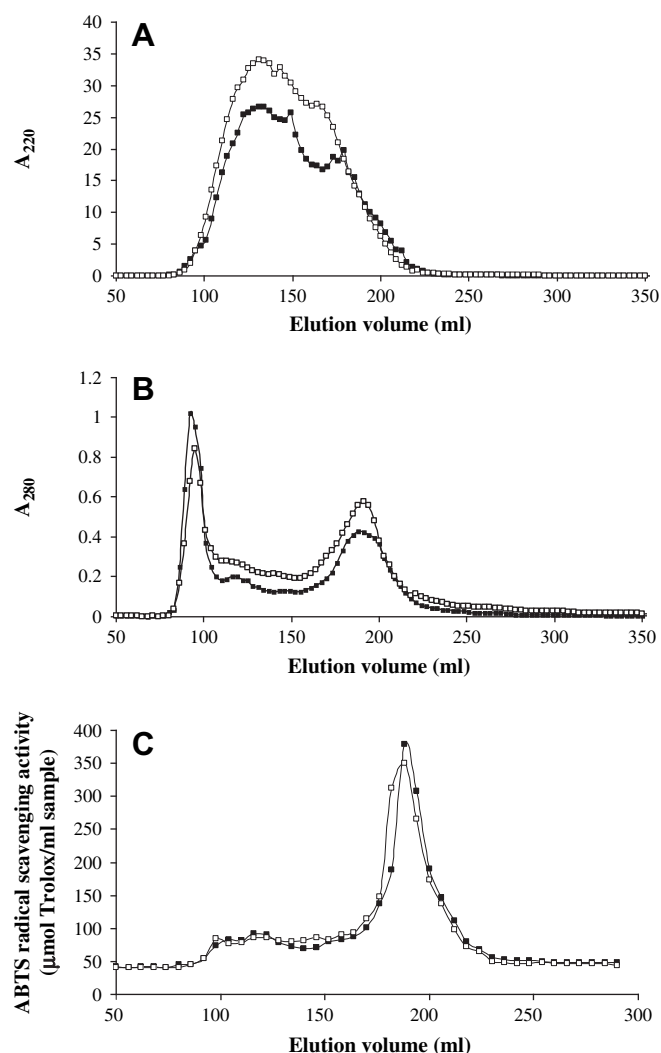


Fig. 7. Separation of antioxidative peptide fractions from gelatin hydrolysates prepared using Alcalase + Neutrase or Alcalase + PCE by Sephadex G-25. (A): A_{220} ; (B): A_{280} ; (C): ABTS radical scavenging activity, (■) Alcalase + PCE; (□) Alcalase + Neutrase.

related assays of emulsion system, in which carbon-centered, peroxy radicals and hydroperoxides are involved in the oxidation process (Burton & Ingold, 1986). The antioxidant activity of gelatin hydrolysates was determined using the thiocyanate method, with BHT for comparison. As shown in Fig. 5, during incubation, the system containing gelatin hydrolysates prepared using Alcalase + PCE or Alcalase + Neutrase at all concentrations lowered the oxidation, compared with that of the control ($P < 0.05$). Additionally, gelatin hydrolysates exhibited antioxidant activity in a dose-dependent manner. However, the system containing 25 and 100 ppm BHT had the lowest oxidation, in comparison with those of the gelatin hydrolysates. Gelatin hydrolysates prepared using Alcalase + Neutrase at 100 ppm showed lower inhibitory activity, compared to 500 and 1000 ppm. At a concentration of 100 ppm, the hydrolysates prepared using Alcalase + PCE showed higher antioxidative activity than that using Alcalase + Neutrase as evidenced by the smaller increase in A_{500} . From the results, the antioxidative activity of gelatin hydrolysates was possibly attributed to the ability of peptides to interfere with the propagation cycle of lipid peroxidation, thereby slowing radical-mediated linoleic acid oxidation (Thiansilakul et al., 2007). Antioxidative peptides with 5–16 amino acid residues could inhibit auto-

oxidation of linoleic acid (Chen, Muramoto, & Yamauchi, 1995). Additionally, peptides from Alaska pollack skin (Kim et al., 2001), jumbo squid (*Dosidicus gigas*) skin gelatin (Mendis et al., 2005) and bullfrog skin (Qian et al., 2008) also showed inhibitory activity in the linoleic acid model system. In the free radical-mediated lipid peroxidation system, antioxidative activity of peptides or proteins is dependent on molecular size and properties such as hydrophobicity and electron transferring ability of the amino acid residues in the sequence (Qian et al., 2008). Therefore, it is suggested that the peptides released from gelatin hydrolysates of bigeye snapper skin by enzymatic hydrolysis had the antioxidant properties. Many antioxidative peptides have hydrophobic amino acid residues such as Val, or Leu at the N-terminus of the peptides, which are more likely able to interact with the fatty acids (Chen et al., 1995; Kim et al., 2001).

3.5.5. Antioxidative activity in a lecithin liposome system

Gelatin hydrolysates prepared using Alcalase + Neutrase or Alcalase + PCE with different enzyme concentrations affected the oxidation of the lecithin liposome system differently as indicated by the different conjugated dienes and TBARS values (Fig. 6). Only a slight increase in conjugated dienes and TBARS values were observed throughout the 36 h incubation period for the liposome system containing 100 ppm BHT. The concentration of conjugated dienes in all samples significantly increased, followed by a decrease. The rate of increase varied with the samples and concentrations used. The formation of conjugated dienes occurs at the early stages of lipid oxidation (Frankel et al., 1997) and hydroperoxides are expected to decompose to create secondary products. The decrease or reaching of a stagnant level in conjugated dienes was generally accompanied by an increase in TBARS (Peña-Ramos & Xiong, 2002). Both gelatin hydrolysates could inhibit the early stages of lipid oxidation (formation of conjugated dienes or hydroperoxides) as well as retard propagation of the oxidation process (degradation of hydroperoxide to TBARS) (Klompong, Benjakul, Kantachote, Hayes, & Shahidi, 2008). Gelatin hydrolysates prepared using Alcalase + Neutrase were generally more effective in inhibiting the lipid oxidation in the lecithin liposome system than were gelatin hydrolysates prepared using Alcalase + PCE as shown by the lower conjugated diene formation throughout the incubation. Additionally, an increase in TBARS values of the liposome system containing both hydrolysates was lower than the control and the efficiency in retarding lipid oxidation was dependent on the concentration used. For the control and the system with 100 ppm of added hydrolysates, the decrease in TBARS was found after 24 h of incubation, while systems containing hydrolysates at 500 ppm or hydrolysates prepared from Alcalase + PCE at 1000 ppm had decreases in TBARS only after 30 h. In general, liposomes may be appropriate lipid models to evaluate antioxidants for both food and lipoprotein particles containing phospholipids (Frankel et al., 1997). Polar portions of peptides might interact with the liposomes of phospholipids, where they function effectively as antioxidant at the interface.

3.6. Fractionation of antioxidative peptides from bigeye snapper skin gelatin hydrolysates

Gelatin hydrolysates prepared using Alcalase + Neutrase or Alcalase + PCE were fractionated using Sephadex-G25 gel filtration chromatography and A_{220} (Fig. 7(A)) to indicate the peptide bond, and A_{280} (Fig. 7(B)) representing peptides, proteins or amino acids with aromatic rings (Amarowicz & Shahidi, 1996) were monitored. Both hydrolysates had two peaks of A_{280} , reflecting the presence of high and low MW peptides or proteins. A peak for A_{220} was found in both hydrolysates, showing the presence of peptide bonds in the

hydrolysates. However, the peak height varied with the enzyme used for the hydrolysate preparation. The results suggested that there was a difference in the amount and size of proteins or peptides generated by the different hydrolysis processes.

ABTS radical scavenging activity of fractions from gelatin hydrolysates prepared using Alcalase + Neutrase and Alcalase + PCE was investigated. As shown in Fig. 7(C), the fraction containing peptides with a MW of 1.7 kDa of both hydrolysates showed the highest antioxidative activity. The fraction from the hydrolysates using Alcalase + PCE exhibited slightly higher antioxidative activity than its Alcalase + Neutrase counterpart. The activity peak of hydrolysates was in accordance with the A_{220} peak, indicating that the peptides contributed to the antioxidative activity. The antioxidative activity of the peptides isolated from gelatin hydrolysates of Alaska pollack skin depended on their amino acid sequences. Kim et al. (2001) reported that peptides from gelatin hydrolysates of Alaska pollack skin ranged from 1.5 to 4.5 kDa. Yang, Ho, Chu, and Chow (2008) reported that gelatin hydrolysates from cobia (*Rachycentron canadum*) skin containing peptides having a MW below 700 Da showed antioxidant activity. From the results, gelatin hydrolysates from bigeye snapper skin most likely contained certain peptides that were electron donors or could scavenge free radicals to terminate the radical chain reaction.

4. Conclusion

Pyloric caeca of bigeye snapper contained trypsin-like enzymes classified based on MW, substrate specificity and the inhibition study. The extract of pyloric caeca could be used to produce gelatin hydrolysates with antioxidative activity, in which ABTS radical scavenging activity was the highest, compared with hydrolysates prepared using Alcalase or Neutrase. The use of a two-step hydrolysis using PCE in combination with Alcalase or Neutrase, regardless of hydrolysis order, could increase the antioxidative activity of the resulting hydrolysates. Gelatin hydrolysates prepared using Alcalase in combination with Neutrase or PCE exhibited antioxidative activity in the linoleic acid oxidation and the lecithin liposome systems. The peptides with a MW of 1.7 kDa seemed to be the peptide mainly involved in the antioxidant properties of hydrolysates. Thus, PCE can be used to produce the hydrolysates from fish skin gelatin with antioxidative activity, which could be a potential source for a natural antioxidant.

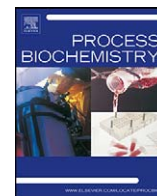
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Antioxidative and ACE inhibitory activities of protein hydrolysates from the muscle of brownstripe red snapper prepared using pyloric caeca and commercial proteases

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ABSTRACT

Protein hydrolysates from the muscle of brownstripe red snapper (*Lutjanus vitta*) prepared using Alcalase or Flavourzyme as the first step with 40% degree of hydrolysis (DH), followed by hydrolysis with pyloric caeca protease (PCP) as the second step for 2 (HAP) and 1 h (HFP), respectively, were prepared and determined for their antioxidative and angiotensin I-converting enzyme (ACE) inhibitory activities. HAP had the higher DPPH and ABTS radical scavenging activity and ferric reducing antioxidant power (FRAP), while HFP showed the higher ferrous chelating activity and ACE inhibitory activity ($p < 0.05$). Both HAP and HFP were able to retard lipid oxidations in lecithin–liposome and β -carotene–linoleic acid oxidation model systems in dose-dependent manner. HAP and HFP contained 87.36 and 86.55% protein (wet basis), respectively with glutamic acid/glutamine as the major amino acids, followed by aspartic acid/asparagine, lysine, alanine and leucine, respectively. HFP showed a slightly greater efficiency in prevention of lipid oxidation in all systems tested. Antioxidative activities, except DPPH radical scavenging activity, of both HAP and HFP after being subjected to gastrointestinal tract model system (GIMs) increased, suggesting the enhancement of antioxidative activities of both hydrolysates after ingestion.

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1. Introduction

Lipid oxidation is one of the major deteriorative processes in many types of foods, leading to the changes in food quality and nutritional value. Additionally, potentially toxic reaction products can be produced. Lipid oxidations have been known to be the major causes of many serious human diseases, such as cardiovascular disease, cancer, and neurological disorders as well as the aging process [1,2]. To prevent oxidative deterioration of foods and to provide protection against serious diseases, such as cancer and atherosclerosis [3,4], it is important to inhibit the oxidation of lipids and the formation of free radicals occurring in the foodstuff and living body. To tackle the problem, antioxidants, both natural and synthetic ones, have been used widely. Nevertheless, synthetic antioxidants have been suspected of being responsible for toxicity in the long term and their use in foodstuffs is restricted or prohibited in some countries [5–7]. Therefore, there is a growing interest on natural antioxidants, especially peptides derived from hydrolyzed food proteins. Protein hydrolysates from several fish species including round scad (*Decapterus maruadsi*) [8], yellow stripe trevally

(*Selaroides leptolepis*) [9], Pacific hake (*Merluccius productus*) [10], tilapia (*Oreochromis niloticus*) [11], silver carp (*Hypophthalmichthys molitrix*) [12] and smooth hound (*Mustelus mustelus*) [13] have been reported to possess antioxidative activities.

Hypertension has been considered as the most common serious chronic health problem [14]. Since angiotensin I-converting enzyme (ACE) (EC 3.4.15.1) is physiologically important in raising blood pressure, the inhibition of ACE activity can lead to an overall antihypertensive effect. The synthetic ACE inhibitors are now widely used as pro-drugs but these synthetic ACE inhibitors can cause many significant undesirable side effects [14,15]. Therefore, the natural safe compounds are desirable for prevention of hypertension instead of the synthetic counterpart. Among those, food protein derived peptides are promising natural products exhibiting ACE inhibitory activities. Protein hydrolysates with antihypertensive activity have also produced from sardinelle (*Sardina pilchardus*) by-products [16], tuna cooking juice [17], salmon protein [18] and tilapia [19].

In Thailand, brownstripe red snapper (*Lutjanus vitta*) is one of the raw materials for surimi production [20]. Besides being produced into surimi, this species and its viscera, especially pyloric caeca, can be used as raw material for production of protein hydrolysate and as the source of proteases, respectively. Production of fish protein hydrolysates with bioactivity can pave the way

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for full utilization of these species. Many factors affect the bioactivity of protein hydrolysates, e.g. type of proteases [9], steps of hydrolysis [21], etc.

This work aims to study antioxidative and ACE inhibitory activities of protein hydrolysate from brownstripe red snapper muscle prepared using its pyloric caeca protease in combination with commercial proteases via 2-step hydrolysis and to investigate the bioactivities of selected hydrolysate after digestion in gastrointestinal tract model system.

2. Materials and methods

2.1. Enzymes and chemicals

Alcalase 2.4L (E.C. 3.4.21.62) (2.4 AU/g) and Flavourzyme 500L (E.C. 3.4.21.77) (500 LPU/g) were provided by Novozyme (Bagsvaerd, Denmark). 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-trinitrobenzenesulfonic acid (TNBS), 1,1,3,3-tetramethoxypropane, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid (ferrozine), 2,4,6-tripyridyl-triazine (TPTZ), L- α -phosphatidylcholine (lecithin) and linoleic acid were purchased from Sigma (St. Louis, MO, USA). Thiobarbituric acid (TBA), potassium persulfate, β -carotene and Tween 40 were obtained from Fluka (Buchs, Switzerland). Tris (hydroxymethyl) aminomethane (Tris-HCl) was procured from Merck (Darmstadt, Germany) and sodium sulfite was obtained from Riedel-deHaën (Seelze, Germany). All chemicals were of analytical grade.

2.2. Preparation of pretreated fish mince

Brownstripe red snapper, stored in ice and off-loaded approximately 24–36 h after capture, were purchased from a dock in Songkhla province, Thailand. Fish were transported in ice with the fish/ice ratio of 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, whole fish were washed and only flesh was separated manually. Flesh was minced to uniformity using Moulinex AY46 blender (Group SEB, Lyon, France) and phospholipid membrane was removed by further homogenizing with nine volumes of cold 8 mmol L⁻¹ CaCl₂ solution containing 5 mmol L⁻¹ citric acid [22] using an IKA Labortechnik homogenizer (Selangor, Malaysia) at a speed of 11000 rpm for 2 min. After continuous stirring for 60 min at 4 °C, the sample was centrifuged at 4000 $\times$ g for 15 min at 4 °C using a Beckman Coulter centrifuge Model Avant J-E (Beckman Coulter, Inc., Fullerton, CA, USA). Thereafter, the pellet was washed by homogenizing with five volumes of cold distilled water using a homogenizer at a speed of 11000 rpm for 2 min, followed by stirring at 4 °C for 15 min prior to centrifuging at 9600 $\times$ g for 10 min at 4 °C. The washing process was repeated twice. Pretreated mince obtained was kept in polyethylene bags and placed in ice until use, but not longer than 2 h.

2.3. Preparation of proteases from pyloric caeca

Pyloric caeca from brownstripe red snapper was collected and powdered in liquid nitrogen. Thereafter, the pyloric caeca extract was prepared according to the method of Khantaphant and Benjakul [20]. Pyloric caeca powder was suspended in ten volumes of extraction buffer (50 mmol L⁻¹ Tris-HCl buffer, pH 8.0, containing 10 mmol L⁻¹ CaCl₂). The mixture was homogenized at 11000 rpm for 2 min. The homogenate was continuously stirred for 30 min at 4 °C and centrifuged at 8000 $\times$ g for 30 min at 4 °C. The supernatant was filtered through a Whatman filter paper No. 1 (Schleicher & Schuell, Maidstone, England). The filtrate obtained was further subjected to 40–60% saturation ammonium sulfate precipitation. After stirring at 4 °C for 30 min, the mixture was centrifuged at 8000 $\times$ g for 30 min at 4 °C and the pellet obtained was dissolved in 50 mmol L⁻¹ Tris-HCl buffer, pH 8.0 followed by dialysis against 20 volumes of the extraction buffer overnight at 4 °C with three changes of dialysis buffer. The dialysate was kept in ice and referred to as 'pyloric caeca protease; PCP'. The proteolytic activity of PCP was determined using casein as a substrate under optimal condition (60 °C and pH 8.5)

2.4. Preparation of protein hydrolysate from brownstripe red snapper

2.4.1. One-step hydrolysis using different single proteases

Pretreated brownstripe red snapper mince with a protein content of 95.42% protein (dry basis) determined by Kjeldahl method [23] was mixed with 50 mmol L⁻¹ Tris-HCl buffer with pH of 7.0, 8.0 and 8.5 for hydrolysis by Flavourzyme, Alcalase and PCP, respectively, to obtain a final protein concentration of 20 g protein L⁻¹. The mixtures were homogenized at a speed of 11,000 rpm for 1 min and the pH was rechecked and readjusted using 1 mol L⁻¹ NaOH or 1 mol L⁻¹ HCl. The homogenates were pre-incubated for 10 min at 50 °C for Alcalase and Flavourzyme and at 60 °C for PCP [20]. To start the hydrolysis, the different levels of enzymes were added into the mixture to obtain the desirable degree of hydrolysis (DH) of 20, 30 and 40% following the method of Benjakul and Morrissey [24]. After 2 h of hydrolysis, the reactions were inactivated by placing the mixture in boiling water for 10 min. Thereafter, the mixture was centrifuged at 2000 $\times$ g at 4 °C for 10 min. The super-

natant was collected and referred to as protein hydrolysate prepared using Alcalase (HA), Flavourzyme (HF) or PCP (HP). All protein hydrolysates were determined for antioxidative activities.

2.4.2. Two-step hydrolysis using different proteases

After 2 h of the first hydrolysis, the mixtures with DH of 40%, which had the highest antioxidative activities, were heated for 10 min in boiling water and adjusted to the desirable pH using 1 mol L⁻¹ NaOH or 1 mol L⁻¹ HCl for proteases used for the second step. Those proteases included Alcalase, Flavourzyme and PCP. The same amount of proteases used in the first step was added into the pre-incubated mixture with optimal temperature of each protease. Reaction was conducted for 1, 2, 3 and 5 h. At the time designated, the reaction was terminated by submerging the mixture in boiling water for 10 min. The mixture was then centrifuged at 2000 $\times$ g at 4 °C for 10 min and the supernatant was collected and adjusted to neutral pH. The neutral solution was referred to as 'protein hydrolysates' and lyophilized to obtain hydrolysate powder. The hydrolysates obtained from HP with further hydrolysis using Alcalase (HPA) or Flavourzyme (HPF), hydrolysate from HA using PCP or Flavourzyme for the second step (HAP and HAF) and hydrolysate from HF using PCP or Alcalase for the second step (HFP and HFA) were determined for their antioxidative and ACE inhibitory activities.

2.5. Determination of antioxidative activities

2.5.1. DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to the method of Blois [25] with a slight modification. Sample solution (1.5 mL) was added with 1.5 mL of 0.1 mmol L⁻¹ DPPH in 950 mL L⁻¹ ethanol. The mixture was allowed to stand for 30 min in dark at room temperature. The resulting solution was measured at 517 nm. The control was prepared in the same manner except that distilled water was used instead of the sample. The DPPH radical scavenging activity was calculated from Trolox standard curve (0–60 μ mol L⁻¹) and expressed as μ mol Trolox equivalents (TE) g⁻¹ protein.

2.5.2. ABTS radical scavenging activity

ABTS radical scavenging activity was determined as described by Re et al. [26]. ABTS radical (ABTS^{•+}) was produced by reacting ABTS stock solution (7.4 mmol L⁻¹ ABTS) with 2.6 mmol L⁻¹ potassium persulfate at the ratio of 1:1 (v/v). The mixture was allowed to react in the dark for 12 h at room temperature. Prior to assay, the ABTS^{•+} solution was diluted with methanol to obtain an absorbance of 1.1 ($\pm$ 0.02) at 734 nm. To initiate the reaction, 150 μ L of sample was mixed with 2.85 mL of ABTS^{•+} solution. The mixture was incubated at room temperature for 2 h in dark. The absorbance was then read at 734 nm. A Trolox standard curve (0–200 μ mol L⁻¹) was prepared. Distilled water was used instead of the sample and prepared in the same manner to obtain the control. ABTS radical scavenging activity was expressed as μ mol TE g⁻¹ protein.

2.5.3. Ferric reducing antioxidant power (FRAP)

The ability of samples to reduce ferric ion (Fe³⁺) was evaluated by the method of Benzie and Strain [27]. FRAP reagent (a freshly prepared mixture of 10 mmol L⁻¹ TPTZ solution in 40 mmol L⁻¹ HCl, 20 mmol L⁻¹ FeCl₃·6H₂O solution and 300 mmol L⁻¹ acetate buffer, pH 3.6 (1:1:10, v/v/v)) (2.85 mL) was incubated at 37 °C for 30 min prior to mixing with 150 μ L of sample. The reaction mixture was allowed to stand in dark for 30 min at room temperature. Absorbance at 593 nm was read and FRAP was calculated from the Trolox standard curve (0–60 μ mol L⁻¹) and expressed as μ mol TE g⁻¹ protein. The control was prepared in the same manner except that distilled water was used instead of the sample.

2.5.4. Ferrous chelating activity

Chelating activity of samples towards ferrous ion (Fe²⁺) was measured by the method of Benjakul et al. [28] with a slight modification. Sample (200 μ L) was mixed with 800 μ L of distilled water. Thereafter, 0.1 mL of 2 mmol L⁻¹ FeCl₂ and 0.2 mL of 5 mmol L⁻¹ ferrozine were added. The mixture was allowed to react for 20 min at room temperature. The absorbance was then read at 562 nm. The standard curve of EDTA (0–1.0 mmol L⁻¹) was prepared. The control was prepared in the same manner except that distilled water was used instead of the sample. Ferrous chelating activity was expressed as μ mol EDTA equivalents g⁻¹ protein.

2.6. Determination of ACE inhibitory activity

The angiotensin I-converting enzyme inhibitory activity was determined as described by Hayakari et al. [29] with slight modifications. Sample (0.3 mL) was incubated with 725 unit ACE L⁻¹ (50 μ L) at 37 °C for 5 min. Thereafter, the incubated mixture was added into the assay mixture (0.2 mL) which was 100 mmol L⁻¹ K₂HPO₄ buffer (pH 8.3) containing 600 mmol L⁻¹ NaCl and 3 mmol L⁻¹ hippuryl-L-histidyl-L-leucine (HHL). The mixture was incubated at 37 °C for 15 min. To terminate the reaction, 1.5 mL of 30 g L⁻¹ 2,4,6-trichloro-1,3,5-triazine (dissolved in dioxane) and 3 mL of 0.2 mol L⁻¹ K₂HPO₄ buffer (pH 8.3) were added and mixed thoroughly. The mixture was left for 10 min until the solution was clear and the absorbance at 382 nm was determined. Sample blank was prepared in the same manner except that HHL was added after the reaction mixture was terminated. The control was prepared

by using distilled water instead of the sample, whereas the control blank was done in the same manner with the control but HHL was added after the termination. % inhibition of ACE was determined using the following equation:

$$\% \text{ Inhibition} = \frac{A_c - A_s}{A_c} \times 100$$

where $A_c = A_{\text{control}} - A_{\text{control blank}}$ and $A_s = A_{\text{sample}} - A_{\text{sample blank}}$.

2.7. Determination of antioxidative activity in different model systems

2.7.1. β -Carotene linoleic acid emulsion model system

The antioxidative activity of the sample in the β -carotene linoleic acid emulsion model system was determined as described by Binsan et al. [30]. β -Carotene (1 mg) was dissolved in 10 mL of chloroform. Thereafter, the solution (3 mL) was added to 20 mg linoleic acid and 200 mg Tween 40. Chloroform was then removed by purging with nitrogen. Fifty milliliters of oxygenated distilled water were added to the β -carotene emulsion and mixed well. Hydrolysate (200 μ L) was then mixed with 3 mL of oxygenated β -carotene emulsion to obtain the final concentrations of 500 and 1000 ppm. The oxidation of β -carotene emulsion was monitored spectrophotometrically at 470 nm after 0, 10, 20, 30, 40, 60, 90 and 120 min of incubation at 50 °C in dark. BHT at levels of 100 ppm was also used. The control was prepared by using distilled water instead of hydrolysate in the assay system.

2.7.2. Lecithin liposome model system

The antioxidative activity of protein hydrolysates in a lecithin liposome system was determined according to the method of Frankel et al. [31] slightly modified by Thiansilakul et al. [8]. Lecithin liposome system was prepared by suspending lecithin in deionized water at a concentration of 8 g L⁻¹. The mixture was stirred with a glass rod followed by sonification for 30 min in a sonicating bath (Elma Model S30H, Singen, Germany). Protein hydrolysate (3 mL) was added to the lecithin liposome system (15 mL) to obtain a final concentration of 1000 ppm. The mixture was sonicated for 2 min. To initiate the reaction, 20 mL of 0.15 mol L⁻¹ cupric acetate were added. The mixture was shaken in the dark at 120 rpm using a shaker (Heidolph Model Unimax 1010, Schwabach, Germany) at 37 °C. The systems containing 25 or 100 ppm BHT were also prepared. The control was prepared in the same manner, except that distilled water was used instead of sample. Oxidation in lecithin liposome systems was monitored at 6 h intervals by determining the formation of TBARS and conjugated dienes.

2.8. Determination of thiobarbituric acid reactive substances (TBARS)

Thiobarbituric acid reactive substances (TBARS) were determined as described by Buege and Aust [32] with a slight modification. Sample was homogenized with TBARS solution (3.75 g L⁻¹ TBA, 150 g L⁻¹ TCA and 0.25 mol L⁻¹ HCl) with a ratio of 1:4 (w/v). The mixture was heated in boiling water for 10 min to develop the pink color. Then the mixture was cooled with running water and centrifuged at 5000 $\times$ g for 10 min at room temperature using Hettich centrifuge (Hettich Model MIKRO-20, Tuttlingen, Germany). The supernatant was collected and measured at 532 nm using a UV-1800 Spectrophotometer (Shimadzu, Kyoto, Japan). TBARS was calculated from a standard curve of malonaldehyde (MDA) (0–10 mg L⁻¹) and expressed as mg MDA kg⁻¹ sample.

2.9. Determination of conjugated diene

Conjugated diene formed in the sample was measured according to the method of Frankel et al. [31]. Sample (0.1 mL) was dissolved in methanol (5.0 mL) and conjugated dienes were measured as the increase in absorbance at 234 nm.

2.10. Preparation of gastrointestinal tract model system (GIMs)

Gastrointestinal tract model system was prepared according to the method of Lo et al. [33] with slight modification. Hydrolysate powder was dissolved in distilled water to obtain a concentration of 50 g protein L⁻¹. The solution was adjusted to pH 2.0 with 1 mol L⁻¹ HCl and pepsin dissolved in 0.1 mol L⁻¹ HCl was added to obtain the final concentration of 40 g pepsin kg⁻¹ protein. The mixture was incubated at 37 °C for 1 h with continuous shaking (Memmert Model SV 1422, Schwabach, Germany). Thereafter, the pH of reaction mixture was raised to 5.3 with 1 mol L⁻¹ NaOH before adding 20 g pancreatin kg⁻¹ protein and the pH of mixture was adjusted to 7.5 with 1 mol L⁻¹ NaOH. The mixture was incubated at 37 °C for 3 h with continuous shaking. The digestion was terminated by submerging the mixture in boiling water for 10 min. During digestion, the mixture was randomly taken and determined for antioxidative activities at 0, 20, 40, 60, 80, 100, 120, 150, 180, 210 and 240 min.

2.11. Proximate analysis

HAP and HFP were determined for protein, fat, ash and moisture contents according to the methods of AOAC [23] with the analytical number of 992.15, 991.36, 942.05 and 950.46, respectively.

2.12. Amino acid analysis

HAP and HFP were hydrolyzed under reduced pressure in 4.0 M methanesulfonic acid containing 0.2% (v/v) 3-(2-aminoethyl) indole at 115 °C for 24 h. The hydrolysates were neutralized with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.4 mL was applied to an amino acid analyzer (MLC-703; Atto Co., Tokyo, Japan).

2.13. Determination of protein concentration

Protein concentration was measured by the method of Lowry et al. [34] using bovine serum albumin as a standard.

2.14. Statistical analysis

Experiments were run in triplicate. All data were subjected to analysis of variance (ANOVA) and differences between means were evaluated by Duncan's Multiple Range Test. For pair comparison, T-test was used [35]. SPSS Statistic Program (Version 10.0) (SPSS Inc, Chicago, IL, USA) was used for data analysis.

3. Results and discussion

3.1. Antioxidative activities of brownstripe red snapper protein hydrolysates prepared with single step of hydrolysis using different proteases

Protein hydrolysates from brownstripe red snapper muscle prepared using protease from pyloric caeca of brownstripe red snapper, Alcalase and Flavourzyme referred to as HP, HA and HF, respectively, with different DH (20, 30 and 40% DH) showed varying antioxidative activities (Fig. 1).

3.1.1. DPPH radical scavenging activity

The DPPH radical scavenging activity of HA increased with increasing DH ($p < 0.05$) (Fig. 1(a)). HF showed the similar activity at all DH used ($p > 0.05$), whereas HP showed no further increase in activity when DH was higher than 30% ($p > 0.05$). At all designated DH, HA and HF showed the higher activity than did HP ($p < 0.05$). The result suggested that the peptides in different hydrolysates might be different in term of chain length and amino acid sequence, which contributed to varying capabilities of scavenging DPPH radicals. The increase in DPPH radical scavenging activity of HA was in agreement with Thiansilakul et al. [8] who reported the increases in DPPH radical scavenging activity as the DH of the hydrolysate from round scad muscle protein prepared using Flavourzyme and Alcalase increased. On the other hand, Klompong et al. [9] found that DPPH radical scavenging activity of protein hydrolysate prepared from the muscle of yellow stripe trevally using Flavourzyme and Alcalase decreased when DH increased. Invert correlation between DH and DPPH radical scavenging activity was obtained for protein hydrolysates prepared from alkaline-aided channel catfish protein isolates using Protamex [36]. You et al. [37] reported that loach protein hydrolysate showed the greater DPPH radical scavenging activity when DH increased. DPPH is a stable free radical and can be scavenged with a proton-donating substance, such as an antioxidant [25]. Therefore, protein hydrolysates from brownstripe red snapper muscle more likely contained peptides acting as hydrogen donors, thereby scavenging free radicals by converting them into more stable products.

3.1.2. ABTS radical scavenging activity

In general, ABTS radical scavenging activities of protein hydrolysates increased as DH increased ($p < 0.05$) (Fig. 1(b)). The highest activity was observed in HP and HA with 40% DH ($p < 0.05$). However, no difference in activity was found in HF with all DH used ($p > 0.05$). Among all hydrolysates, HA had the lowest activity for all DH tested ($p < 0.05$). At DH of 20%, HF showed higher ABTS radical scavenging activity than did HP ($p < 0.05$). Conversely, HP

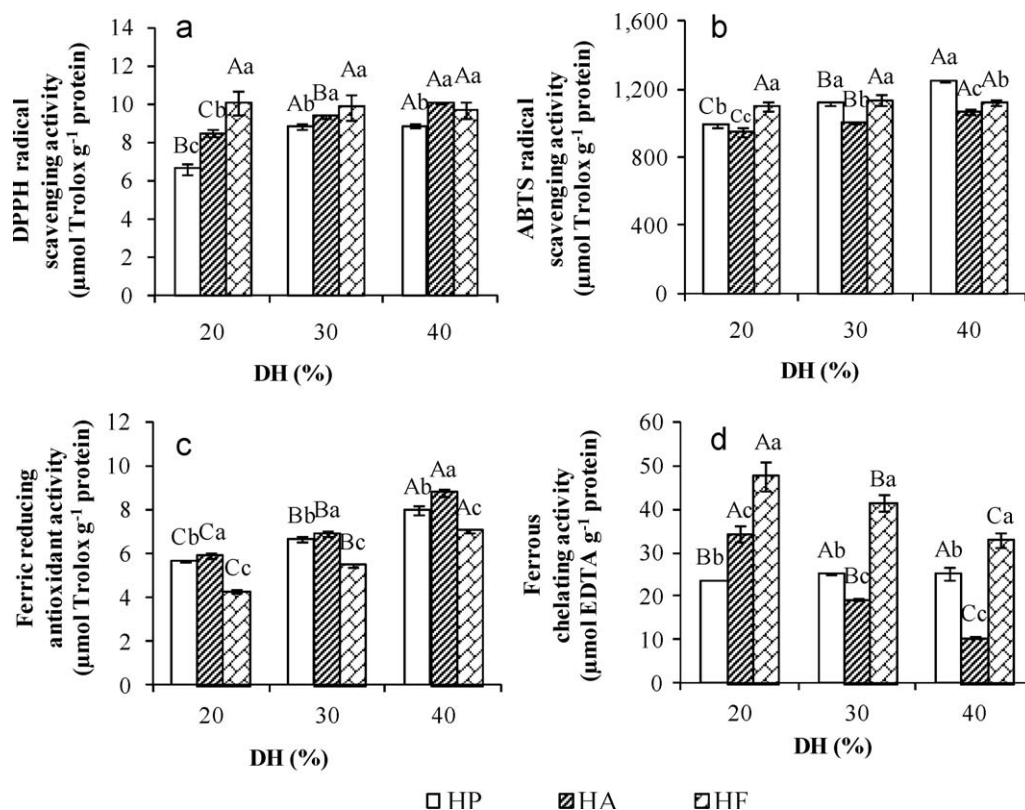


Fig. 1. Antioxidative activities of protein hydrolysate from brownstripe red snapper muscle prepared using pyloric caeca protease (PCP) from brownstripe red snapper (HP), Alcalase (HA) and Flavourzyme (HF) with different DHs. Bars represent the standard deviation ($n=3$). Different capital letters within the same enzyme used indicate significant differences ($p < 0.05$). Different letters within the same DH indicate significant differences ($p < 0.05$).

had the highest activity when DH of 40% was used ($p < 0.05$). Protein hydrolysate from alkali-solubilized tilapia protein prepared using various proteases showed a sharp increase in ABTS radical scavenging activity when DH increased from 18 to 23% [11]. Loach protein hydrolysate showed a similar result, in which ABTS radical scavenging activity increased with increasing DH [37].

For both DPPH and ABTS assays, HF showed no differences in activities with all DH tested ($p > 0.05$). ABTS radical assay is used for determining the antioxidative activity, in which the radical is quenched to form ABTS-radical complex [26]. Generally, all hydrolysates contained peptides, which were able to scavenge ABTS radicals, leading to the termination of radical chain reaction.

3.1.3. Ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant power (FRAP) measures the reducing ability against ferric ion (Fe^{3+}), indicating the ability of hydrolysates to transfer an electron to the free radical [27]. FRAP of different hydrolysates with varying DH is depicted in Fig. 1(c). An increase in FRAP was observed in all hydrolysates when DH increased ($p < 0.05$). FRAP of HA was generally higher than those of HP and HF at all DHs tested ($p < 0.05$). The result suggested that HA had the greater reducing power than did others, leading to the greater efficacy in prevention and retardation of propagation in lipid oxidation. However, protein hydrolysates prepared from alkaline-aided channel catfish protein isolates showed the decrease in reducing power with increasing DH [36]. Raghavan et al. [11] reported that alkaline-solubilized tilapia protein hydrolysate prepared using Flavourzyme showed the increase in reducing ferric ion when DH increased. The hydrolysis most likely increased the reducing power, especially when the cleavage of peptides increased

as indicated by the increase in DH. The result indicated that peptides generated from the hydrolysis by different proteases had the different capacities of providing electron to the radicals.

3.1.4. Ferrous chelating activity

Ferrous chelating activities of hydrolysates prepared using different proteases with different DHs are shown in Fig. 1(d). Chelating activity against Fe^{2+} of HP slightly increased when DH increased up to 30% ($p < 0.05$). For HA and HF, the decreases in ferrous chelating activities were found as DH increased ($p < 0.05$). Ferrous ion (Fe^{2+}) is the most powerful pro-oxidant among metal ions [38], leading to the initiation and acceleration of lipid oxidation by interaction with hydrogen peroxide in a Fenton reaction to produce the reactive oxygen species, hydroxyl free radical ($\text{OH}^{\bullet}$) [39]. Therefore chelation of metal ions by peptides in hydrolysates could retard the oxidative reaction. The result indicated that a higher DH rendered HA and HF with lower metal-chelating activities. The shorter chain of peptides might lose their ability to form the complex with Fe^{2+} . At DH of 20%, HF showed the highest chelating activity ($p < 0.05$), followed by HA and HP, respectively. Peptides in HF could effectively chelate the Fe^{2+} , leading to the retardation of initiation stage. The result indicated that the limited hydrolysis of muscle protein resulted in the enhanced ferrous chelating activity, compared with the excessive hydrolysis. The higher chelating activity of HP was coincidental with the higher DPPH and ABTS radical scavenging activity and FRAP, as the DH increased. Fe^{2+} chelating activity of round scad protein hydrolysate prepared using Alcalase showed the increase in chelating activity with increasing DH, but those treated with Flavourzyme showed no difference in activity at all DH tested [8]. With the same enzymes used, chelating activity of protein hydrolysate prepared from the muscle of yellow stripe trevally

using Flavourzyme and Alcalase increased with increasing DH [9]. Higher ferrous chelating activity was reported for hydrolysate of silver carp using Alcalase and Flavourzyme when DH increased [12]. Apart from Fe, other transition metals, such as Cu and Co can affect the rate of lipid oxidation and decomposition of hydroperoxide. Theodore et al. [36] reported that Cu^{2+} chelating activity of catfish protein hydrolysate increased with increasing DH.

Some proteins and peptides can chelate metal ions like Fe^{2+} due to the presence of carboxyl and amino groups in the side chains of acidic and basic amino acids [10,40]. Alcalase is endopeptidase capable of hydrolyzing proteins with broad specificity for peptide bonds and prefers for the uncharged residue, whereas Flavourzyme is a mixture of endo- and exopeptidase enzyme, which can produce both amino acids and peptides [41]. Hydrolysates showing different antioxidative activities might be attributed to the differences in the exposed side chains of peptides as governed by the specificity of different proteases towards peptide bonds in the proteins [42]. DH also greatly influenced the peptide chain length. The higher DH, the more cleavage of peptide chains took place. Peptides with various sizes and compositions had different capacities of scavenging or quenching free radicals [8,9]. PCP, Alcalase and Flavourzyme more likely cleaved the peptide bonds in brownstripe red snapper muscle at different positions, resulting in the different products with varying antioxidative activities. With 40% DH, all hydrolysates functioned more effectively as primary antioxidant, whereas the secondary antioxidative activity, chelating ability, was lowered. To enhance the antioxidative activity of peptides, especially as the primary antioxidant, the hydrolysate with 40% DH was prepared for the first step of hydrolysis.

3.2. Antioxidative and ACE inhibitory activities of brownstripe red snapper protein hydrolysate prepared with two-step hydrolysis using different proteases

3.2.1. DPPH radical scavenging activity

DPPH radical scavenging activity of protein hydrolysates with various hydrolysis times in the second step of hydrolysis using another protease is shown in Fig. 2(a). For HAF, HAP, HFA and HFP, the activities increased with increasing time up to 2 h ($p < 0.05$). Thereafter, the gradual decrease was observed when hydrolysis times were 3 and 5 h ($p < 0.05$). No changes in DPPH radical scavenging activity were observed for HPF, while HPA showed the maximal activity at 3 h of hydrolysis ($p < 0.05$). The results suggested that scavenging activity against DPPH radical was enhanced by additional hydrolysis using another enzyme with an appropriate time. Generally, hydrolysates prepared by further hydrolyzing the hydrolysate obtained from the first step of hydrolysis with another protease for 2 h had a greater scavenging ability against DPPH radical.

3.2.2. ABTS radical scavenging activity

ABTS radical scavenging activity of protein hydrolysates prepared by two-step hydrolysis is shown in Fig. 2(b). Only HAF and HAP showed the increases in ABTS radical scavenging activity, compared with their parent counterparts, HA. HAP with the hydrolysis time of 2 h for the second step had the highest activity ($p < 0.05$). This suggested the enhancement of ABTS radical scavenging activity when PCP was used for further hydrolysis of HA. However, the further hydrolysis of HF with another protease had no impact on the increases in activity ($p > 0.05$). For HP, the decrease in activities was obtained when the second step of hydrolysis was performed ($p < 0.05$). The results suggested that the second step of hydrolysis could slightly increase ABTS radical scavenging activity, depending on the first hydrolysate as well as the types of protease used for the second step. It was noted that the abilities of scavenging ABTS and DPPH radicals by hydrolysates were different. This might be due to

the differences in ability of scavenging the different radicals, ABTS and DPPH, by the same peptide.

3.2.3. Ferric reducing antioxidant power (FRAP)

Fig. 2(c) shows FRAP of protein hydrolysates prepared using two-step hydrolysis. The increases in FRAP were observed for all hydrolysates, when the second step of hydrolysis was applied. For HF and HP, when Alcalase was used for the second step of hydrolysis, the continuous increases in FRAP were observed ($p < 0.05$). For other hydrolysates, the hydrolysis time was the factor affecting the FRAP of resulting hydrolysate, depending on the first hydrolysate and the types of protease used for the second step of hydrolysis. The increase in FRAP with increasing hydrolysis time was coincidental with the increase in DPPH radical scavenging activity (Fig. 2(a)). For HAF, HAP and HPF, the activities were increased with increasing hydrolysis time up to 2 h ($p < 0.05$). For HFP, the hydrolysis time more than 1 h had the negative effect on FRAP ($p < 0.05$). Generally, further hydrolysis with another protease led to the increase in FRAP, but the activities of resulting hydrolysate were governed by hydrolysis time.

3.2.4. Ferrous chelating activity

Ferrous chelating activities of hydrolysates prepared by two-step hydrolysis are shown in Fig. 2(d). HFA and HPA showed the marked decrease in chelating activities when the second hydrolysis was conducted ($p < 0.05$). Alcalase used in the second step might generate peptides with the lower ability in Fe^{2+} chelating. The abilities of HAF, HAP, HFP and HPF in chelating Fe^{2+} ion were more pronounced, compared with their parent hydrolysate counterparts. The increases were observed when a certain time of the second hydrolysis was used. Among all protein hydrolysates, HFP showed the highest ferrous chelating activity, especially when the second step hydrolysis time of 1 h was used ($p < 0.05$).

3.3. ACE inhibitory activity

The inhibitory effect of all hydrolysates prepared using two-step hydrolysis against angiotensin I-converting enzyme (ACE) was determined as shown in Fig. 2(e). For the first hydrolysate prepared using the different single proteases (0 h), HF showed the highest ACE inhibition (11.44%) ($p < 0.05$). Flavourzyme might produce the peptides with ACE inhibitory activity. Raghavan and Kristinsson [19] used Cryotin and Flavourzyme for hydrolysis of tilapia protein and found the higher ACE inhibition by hydrolysate prepared using Flavourzyme. When the second step of hydrolysis using another protease was performed, HFA and HFP showed the higher ACE inhibition when hydrolysis time of 2 and 3 h was used, respectively. HAP also showed high ACE inhibitory activity. Wu et al. [43] reported that shark meat treated with enzyme showed the higher ACE inhibition than that of untreated one. ACE inhibition by tilapia protein hydrolysate was reported, especially with increasing DH [19]. Furthermore, smaller peptides are the better ACE inhibitors than the larger counterpart [19]. The hydrolysis might release ACE inhibitory peptides in hydrolysate [43]. Hydrolysates from muscle of different fish have been reported to possess ACE inhibitory activity, e.g. hydrolysates from tilapia protein [19], freshwater clam muscle [44], shark meat [43], Atlantic salmon, Coho salmon, Alaska pollack and southern blue whiting muscle [45].

From the results, the use of Alcalase for the first hydrolysis and the use of PCP in the second hydrolysis for 2 h (HAP) yielded the resulting hydrolysate with the higher DPPH and ABTS radical scavenging activities and FRAP, compared to other hydrolysates ($p < 0.05$). However HF hydrolyzed with PCP in the second hydrolysis step for 1 h (HFP) showed much higher chelating activity, compared with others ($p < 0.05$). Therefore, HFP and HAP prepared with 1 and 2 h for the second step of hydrolysis, respectively, were

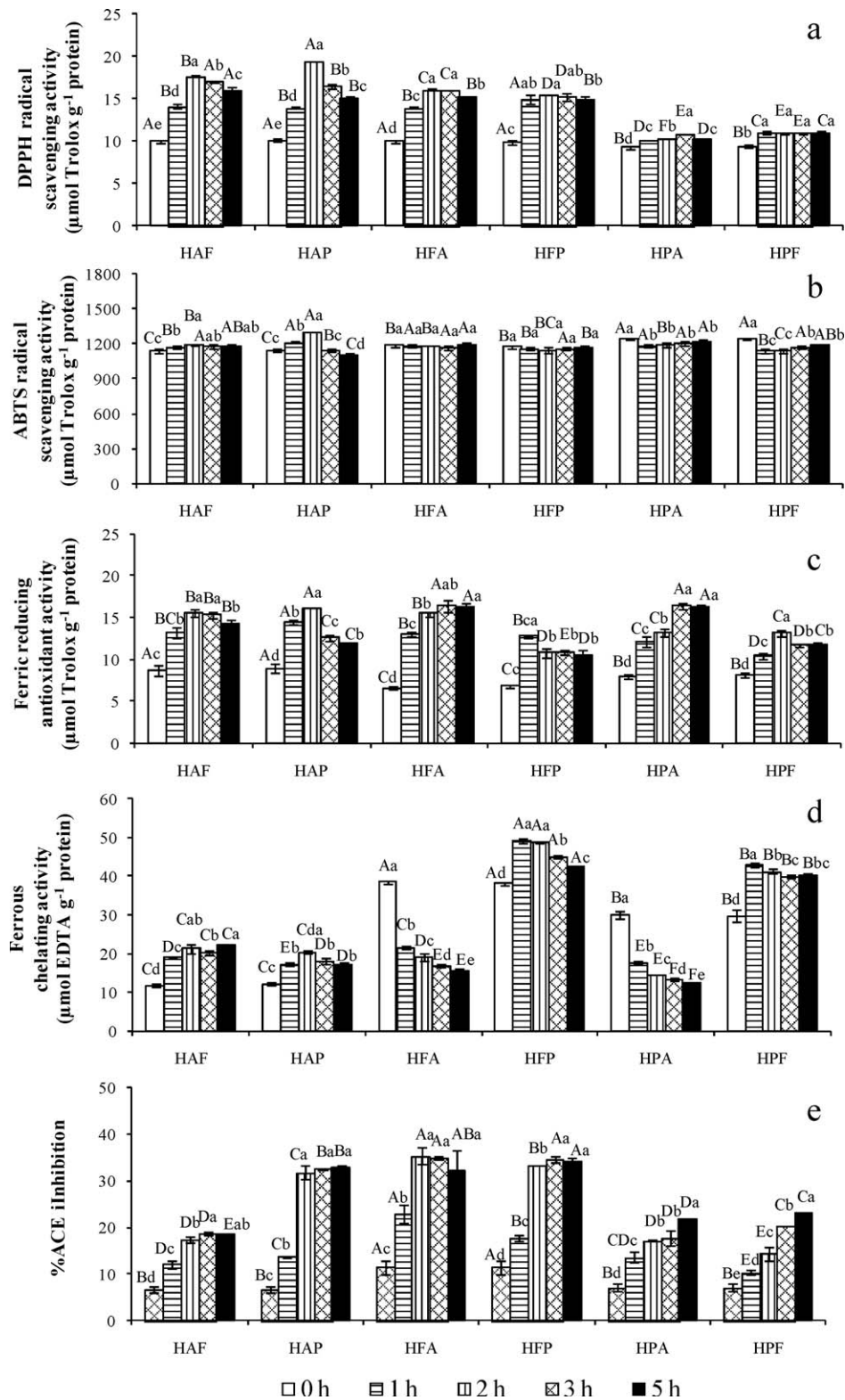


Fig. 2. Antioxidative activities (a–d) and ACE inhibitory activity (e) of protein hydrolysate from brownstripe red snapper muscle prepared by two-step hydrolysis process with different hydrolysis times in the second step of hydrolysis. HAF and HAP: HA hydrolyzed with Flavourzyme and PCP, respectively. HFA and HFP: HF hydrolyzed with Alcalase and PCP, respectively. HPA and HPF: HP hydrolyzed with Alcalase and Flavourzyme, respectively. Protein concentration used for ACE inhibitory assay was 5 g L^{-1} . Bars represent the standard deviation ($n=3$). Different capital letters within the same hydrolysis time indicate significant differences ($p < 0.05$). Different letters within the same treatment indicate significant differences ($p < 0.05$).

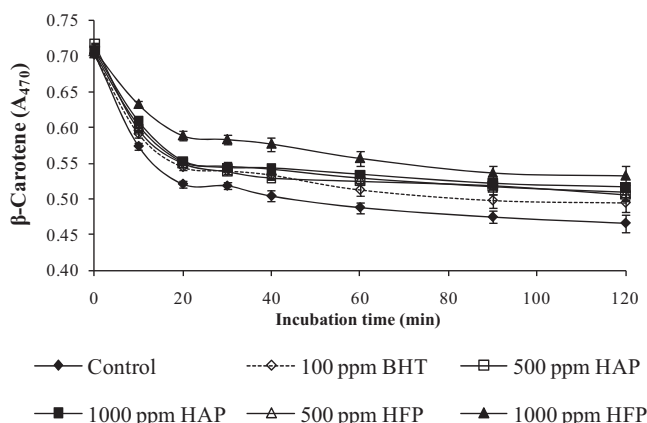


Fig. 3. Changes in A_{470} of β -carotene linoleic acid system in the absence or the presence of protein hydrolysate from brownstripe red snapper muscle at different levels. HAP: HA hydrolyzed with PCP. HFP: HF hydrolyzed with PCP. BHT is butylated hydroxytoluene. Bars represent the standard deviation ($n = 3$).

selected for further study. DH of selected protein hydrolysates, HAP and HFP, were 62.7 and 61.8%, respectively.

3.4. Antioxidative activities of brownstripe red snapper protein hydrolysate in different oxidation model systems

3.4.1. β -Carotene linoleic acid emulsion model system

The antioxidative activity of selected protein hydrolysates, HAP and HFP, were studied in β -carotene linoleic acid oxidation model system as shown in Fig. 3. The decrease in A_{470} indicates the oxidation of β -carotene in the system caused by the oxidation process. When the oxidation of linoleic acid occurs, free radicals formed are able to attack the highly unsaturated β -carotene molecules. As a result, β -carotene is oxidized, leading to the losses in chromophore and characteristic orange color of β -carotene [30]. The presence of antioxidant in linoleic acid emulsion system hinders β -carotene bleaching, due to the chain-breaking inhibition of lipid peroxidation by neutralizing the linoleic free radical formed. Systems containing HAP and HFP at levels of 500 and 1000 ppm retarded the decrease in A_{470} up to 120 min ($p < 0.05$). However, no difference in A_{470} was observed between the system containing 100 ppm BHT and those with 500 and 1000 ppm HAP and 500 ppm HFP within the first 40 min ($p > 0.05$). This indicated that the preventive effect of hydrolysates (500 and 1000 ppm) was equivalent to that of 100 ppm BHT. Thereafter, the lower β -carotene bleaching in the systems containing these hydrolysates was observed, compared with the system added with 100 ppm BHT up to 120 min. The result indicated that in emulsion system, HAP and HFP could act as the better chain breaker than BHT, especially with increasing reaction time. Among the hydrolysates, 1000 ppm HFP showed the highest inhibitory activity against β -carotene bleaching throughout the reaction time of 120 min ($p < 0.05$). The ability of hydrolysates to prevent the bleaching of β -carotene was more likely governed by their amphiphilic properties of amino acids compositions. Localization/orientation at the interface of hydrophobic part of peptides to oil phase and of hydrophilic portion to aqueous phase at the interface is the major principle for emulsion stabilization [30]. The result suggested that protein hydrolysates tested contained peptides with both hydrophilic and hydrophobic portions, in which the peptide segments with hydrophobicity in nature could adsorb at the oil droplet, while the hydrophilic domains preferably suspended in the aqueous phase. The localization of peptides with antioxidative activity at the oil-water interface could favor their antioxidative activity for oil droplet. As a result, the antioxidative activity could be maximized at the interface of emulsion.

3.4.2. Lecithin liposome model system

Different concentrations of HAP and HFP, 500 and 1000 ppm, affected the oxidation of the lecithin liposome system differently as indicated by different conjugated dienes (CD) (Fig. 4(A)) and TBARS values (Fig. 4(B)). All systems containing hydrolysate samples, except for that added with 100 ppm BHT, showed the similar pattern of CD formation, in which the increase was found within the first 12–18 h of reaction. Thereafter, the decrease in CD was observed. However, the rate of changes varied with the hydrolysates added and concentration used. In the early incubation time (0–6 h), the delay in CD formation was observed. System containing 1000 ppm HFP showed the longer delay time, compared with systems containing other hydrolysates, indicating the higher resistance to oxidation of the system [8]. After 6 h, the remarkable increases in CD were observed, especially within the first 18 h ($p < 0.05$). Within the first 6–12 h of incubation, the increasing rate of CD formation in the system varied with the hydrolysates and the concentrations used. At the same concentration used, the system with HFP showed a lower CD formation as evidenced by lower A_{234} and the systems containing higher concentration of hydrolysates showed a lower CD formation ($p < 0.05$). When comparing with the control, liposome system added with 500 and 1000 ppm HFP and 1000 ppm HAP had the lower CD than the control ($p < 0.05$), indicating that these hydrolysates had the ability to retard the formation of CD [41]. However, after 18 h, CD of all samples added with hydrolysates was higher than that of the control and gradually decreased up to 36 h. Subsequently, CD was quite stable up to 60 h of incubation. The decrease in CD was probably due to the decomposition of CD into secondary products. However, no changes in CD of liposome system added with 100 ppm BHT was observed throughout 60 h of incubation ($p > 0.05$). For the system added with 25 ppm BHT, the increase in CD was found after 12 h of incubation and reached the plateau at h 36.

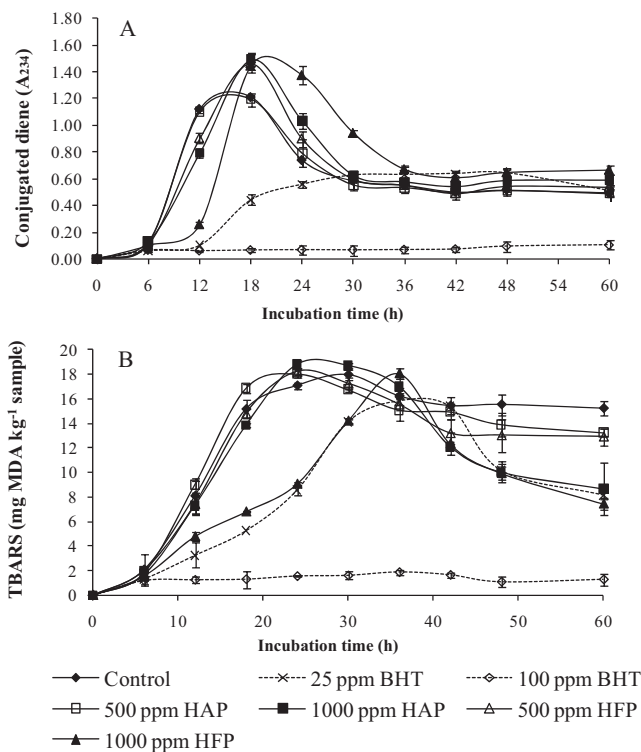


Fig. 4. Changes in conjugated diene (A) and thiobarbituric acid reactive substances (TBARS) value (B) in the lecithin liposome system in the absence or the presence of protein hydrolysate from brownstripe red snapper muscle at different levels. HAP: HA hydrolyzed with PCP. HFP: HF hydrolyzed with PCP. BHT is butylated hydroxytoluene. Bars represent the standard deviation ($n = 3$).

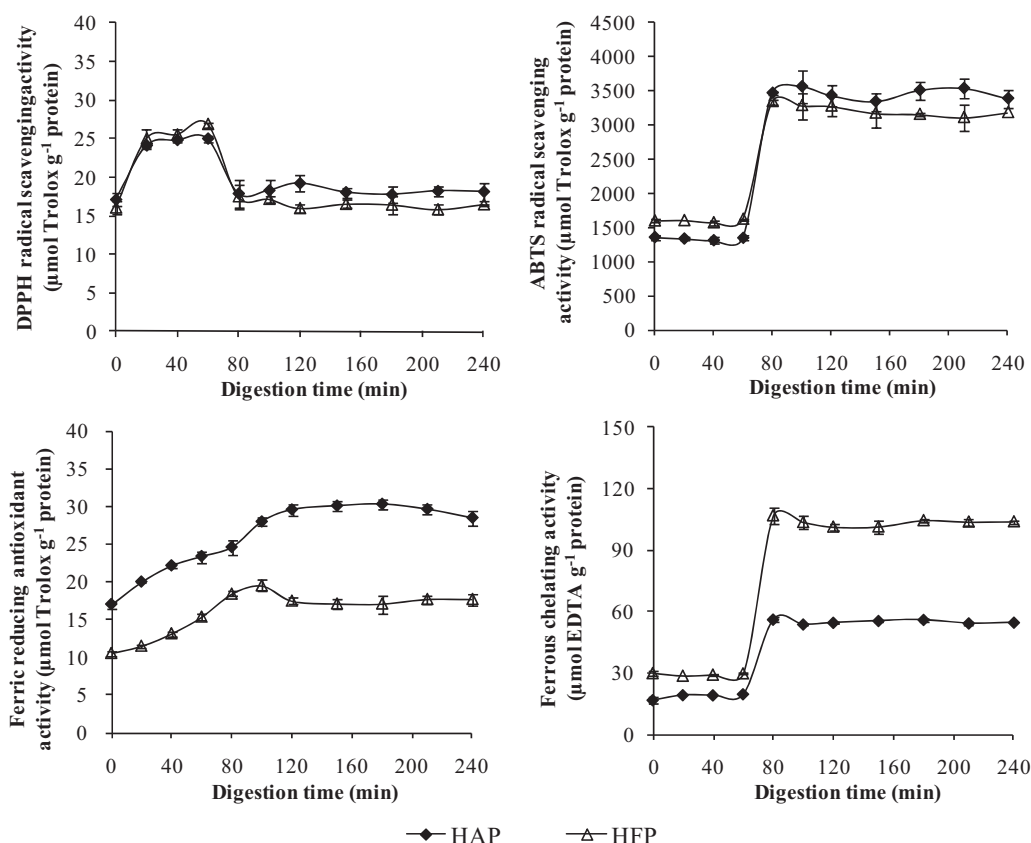


Fig. 5. Antioxidative activities of protein hydrolysate from brownstripe red snapper muscle prepared by two-step hydrolysis process in gastrointestinal tract model system. HAP: HA hydrolyzed with PCP. HFP: HF hydrolyzed with PCP. Bars represent standard deviation ($n = 3$).

The formation of secondary lipid oxidation products was evaluated by TBARS. The sharp increases in TBARS were observed within the first 24 h of incubation when 500 and 1000 ppm HAP and 500 ppm HFP were added to liposome system ($p < 0.05$). However, the system added with 1000 ppm HFP showed the lower TBARS than those added with other hydrolysates ($p < 0.05$). It was noted that system added with 1000 ppm HFP had the similar TBARS, compared with that containing 25 ppm BHT. The early formation of TBARS indicated the decomposition of the primary products to the secondary products. After the particular incubation time, the gradual decreases in TBARS were observed, possibly due to the loss of volatile compounds produced in those systems. HFP, especially at 1000 ppm, showed the noticeable antioxidant activity in liposome system throughout the 60 h of incubation time as evidenced by the lower TBARS formation ($p < 0.05$). The result suggested the greater ability of 1000 ppm HFP in retardation of the formation of secondary oxidation products. Liposome system added with 100 ppm BHT showed no changes in TBARS throughout 60 h of incubation ($p > 0.05$).

Liposomes are appropriate lipid models to evaluate antioxidative activity in lipid food or lipoprotein particles containing phospholipids [31]. Polar portions of peptides in hydrolysate might interact with the polar environment of lecithin liposomes, resulting in the better protection against lipid oxidation at the interface. It could be concluded that protein hydrolysates, especially 1000 ppm HFP, could retard lipid oxidation via their radical scavenging activity, reducing power and metal chelating activity.

In food-related systems, antioxidant activity is mainly due to the scavenging of free radical and the chain-breaking inhibition of lipid oxidation [46]. Therefore, the hydrolysate from brownstripe red snapper could retard lipid oxidation *in vitro* assay systems and

oxidation model systems. The results suggested that hydrolysate from brownstripe red snapper protein could function as a natural antioxidant in food or functional food.

3.5. Changes in antioxidative activity in gastrointestinal tract model system (GIMs)

GIMs was used to simulate the ingestion system of human body and the remaining antioxidative activities of HAP and HFP were monitored (Fig. 5). When protein hydrolysate was oral administered, their bioactive peptides should be resistant to hydrolysis by digestive proteases in order to be absorbed and reach the target organ to function as antioxidant [47]. Both HAP and HFP showed the similar activities in GIMs. Both hydrolysates showed the increase in DPPH radical scavenging activity within the first hour of digestion. Thereafter, during intestinal simulated system, DPPH radical scavenging activities were similar to that of original hydrolysate and remained constant up to 240 min. On the other hand, the increase in FRAP was found for both HAP and HFP during the first hour of digestion ($p < 0.05$). The further increases were also found in the intestinal simulated system up to 120 and 100 min for HAP and HFP, respectively. Thereafter, no changes in FRAP were found up to 240 min for HAP, whereas the slight decrease up to 240 min was observed for HFP ($p > 0.05$). Nevertheless, neither ABTS radical scavenging nor ferrous chelating activities were changed within the first hour (stomach simulated system) ($p > 0.05$). With further hydrolysis in intestinal simulated system, the sharp increases in both ABTS radical scavenging and ferrous chelating activities were obtained but no changes in activity were found with increasing digestion time up to 240 min ($p > 0.05$).

Table 1

Proximate composition of protein hydrolysate from the muscle of brownstripe red snapper prepared by 2-step hydrolysis process using Alcalase/pyloric caeca protease (PCP) (HAP) and Flavourzyme/PCP (HFP).

Compositions (% wet weight)	HAP	HFP
Protein	87.36 ± 0.12 ^{†,†a}	86.55 ± 0.19 ^b
Moisture	11.16 ± 0.39 ^a	11.96 ± 0.52 ^a
Fat	0.61 ± 0.06 ^a	0.64 ± 0.04 ^a
Ash	1.76 ± 0.73 ^a	1.78 ± 0.32 ^a

[†] Mean ± SD (n = 3).

[‡] Different superscripts in the same row indicate the significant differences (p < 0.05).

Pepsin might hydrolyze HAP and HFP to some degrees, yielding the new peptides with DPPH radical scavenging activity and reducing power. It was noted that HAP showed the higher FRAP than HFP during the first hour of digestion. Further digestion with pancreatin (intestinal simulated system) caused the dramatic increase in ABTS radical scavenging activity, FRAP and ferrous chelating activity, but with different rates. During pancreatin digestion, HAP showed the higher DPPH, ABTS radical scavenging activities and FRAP, but lower ferrous chelating activity, compared with HFP. The result suggested that pancreatin might cleave the peptides to some degrees, leading to the release of new antioxidative peptides. This could enhance the antioxidative activities of hydrolysates. Generally, GIMs may actually lead to generation of more potent bioactive peptides [48]. The antioxidative activities of protein hydrolysates after incubation in GIMs was dependent on peptides in hydrolysate, in term of size, amino acid composition and sequence, which could be targeted by digestive proteases [48]. The results showed that the antioxidative activity of both HAP and HFP could be preserved after treatment with these gastrointestinal enzymes in GIMs (p < 0.05). Thus, both the antioxidative activities of HAP and HFP were more likely preserved after digestion in the real gastrointestinal tract of human body.

3.6. Proximate compositions of protein hydrolysates

Chemical compositions of HAP and HFP are shown in Table 1. HAP and HFP had high protein content (87.36 and 86.55% for HAP and HFP, respectively). This suggested that both HAP and HFP were the essential source of proteins. During hydrolysis, protein was solubilized and the insoluble non-protein matter was removed, resulting in the high protein content in the resulting hydrolysate [24]. Lipid content in raw material also affected the protein content in resulting hydrolysate. The high amount of lipids resulted in the low amount of solubilized protein [49]. In the present study, the pretreatment with CaCl₂ and citric acid was also performed to remove phospholipid membrane prior to hydrolysis. As a consequence, both HAP and HFP contained very low fat content. Ash content of 1.76–1.78% in both hydrolysates represented the salt formed during pH adjustment using acid or alkaline.

3.7. Amino acid composition of protein hydrolysates

Amino acid compositions of HAP and HFP are shown in Table 2. Both HAP and HFP contained glutamic acid/glutamine as the major amino acids and were also rich in aspartic acid/asparagine, alanine, lysine and leucine. However, both hydrolysates contained low levels of cysteine and tryptophan. Based on total amino acids, essential amino acids made up 38.01 and 37.87% of HAP and HFP, respectively. Therefore, they could serve as the excellent source of useful nutrients. Generally, the differences in amino acid composition between HAP and HFP depended on the existing differences in enzyme specificity and hydrolysis conditions [41]. The differ-

Table 2

Amino acid composition of protein hydrolysate from the muscle of brownstripe red snapper prepared by 2-step hydrolysis process using Alcalase/pyloric caeca protease (PCP) (HAP) and Flavourzyme/PCP (HFP).

Amino acids (%)	HAP	HFP
Alanine	9.38	9.56
Arginine	5.00	5.06
Aspartic acid/asparagine	10.65	10.73
Cysteine	0.12	0.14
Glutamic acid/glutamine	16.23	16.92
Glycine	7.48	7.21
Histidine	1.71	1.60
Isoleucine ^a	4.14	4.07
Leucine ^a	8.54	8.62
Lysine ^a	9.51	9.76
Methionine ^a	2.93	2.92
Phenylalanine ^a	2.65	2.45
Proline	3.66	3.46
Serine	5.15	5.02
Threonine ^a	5.10	4.91
Tyrosine	2.33	2.14
Tryptophane	0.28	0.29
Valine ^a	5.15	5.15

^a Essential amino acids.

ences in amino acid composition probably determined the varying antioxidative activity between both hydrolysates.

4. Conclusion

Protein hydrolysates prepared from the muscle of brownstripe red snapper using protease from its pyloric caeca in combination with commercial enzymes could provide the peptides with antioxidative and ACE inhibitory activities. Protein hydrolysates prepared using Alcalase and Flavourzyme at the first step, followed by further hydrolysis using protease from its pyloric caeca showed the highest antioxidative activities, both *in vitro* and oxidation model systems. Antioxidative activities of protein hydrolysates remained constant or increased after digestion in gastrointestinal tract model system. Furthermore, protein hydrolysates contained high protein content with glutamic acid/glutamine as the major amino acids. Therefore, hydrolysate from brownstripe red snapper muscle by two-step hydrolysis could serve as a natural antioxidant for food preservation or as the functional foods.

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Functionalities and antioxidant properties of protein hydrolysates from the muscle of ornate threadfin bream treated with pepsin from skipjack tuna

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ABSTRACT

Functional properties and antioxidant activities of protein hydrolysates prepared from ornate threadfin bream (*Nemipterus hexodon*) muscle, using skipjack tuna pepsin, with different degrees of hydrolysis (DH: 10%, 20% and 30%), were evaluated. Emulsifying and foaming properties of hydrolysates were governed by their DH and concentrations used. Hydrolysates with 20% DH had the highest scavenging activities for ABTS and DPPH radicals. However, chelating activity of hydrolysates for ferrous ion increased as DH increased. Size exclusion chromatography of the hydrolysate with 20% DH using Sephadex G-25 revealed that antioxidative peptides with molecular weight of approximately 1.3 kDa exhibited the highest ABTS radical-scavenging activity. *In vitro* simulated gastrointestinal digestion indicated that ABTS radical-scavenging activity of the antioxidative peptides was not affected by pepsin hydrolysis, whilst further digestion by pancreatin enhanced the activity. Therefore, protein hydrolysate from the muscle of ornate threadfin bream produced by skipjack tuna pepsin can be used as a promising source of functional peptides with antioxidant properties.

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1. Introduction

The use of enzyme technologies for protein recovery and modification has led to the production of a broad spectrum of food ingredients and industrial products (Kristinsson & Rasco, 2000). Proteases from different sources are commonly employed to obtain a more selective hydrolysis due to their specificity for peptide bonds adjacent to certain amino acid residues (Khantaphant & Benjakul, 2008). Enzymatic hydrolysis of food proteins is an efficient way to recover potent bioactive peptides (Thiansilakul, Benjakul, & Shahidi, 2007a). Fish protein hydrolysates have been shown to have potential for nutritional or pharmaceutical applications (Thiansilakul et al., 2007a; Wu, Chen, & Shiau, 2003). Numerous peptides derived from hydrolysed food proteins have been shown to have antioxidant activity. Fish protein hydrolysates such as skin gelatin hydrolysate from brownstripe red snapper (Khantaphant & Benjakul, 2008) or meat protein hydrolysates from yellow travelled (Klompong, Benjakul, Kantachote, & Shahidi, 2007; Klompong et al., 2009), round scad (Thiansilakul et al., 2007a; Thiansilakul, Benjakul, & Shahidi, 2007b), mackerel (Wu et al., 2003) and loach (You, Zhao, Regenstien, & Ren, 2010) have been reported to exhibit antioxidant activity. Fish protein hydrolysates can be used in food

systems, comparable to other pertinent protein hydrolysates (Kristinsson & Rasco, 2000).

Thailand is the world's largest producer and exporter of canned tuna. The most commercially important tuna species for the Thai tuna industry is skipjack tuna, followed by yellow-fin tuna and tongol tuna, accounting for over 80% of Thai frozen tuna imports (Nalinanon, Benjakul, & Kishimura, 2010). Due to a large amount of tuna viscera discarded during manufacturing, it can serve as a promising source of pepsin for further application. Recovery of pepsin from tuna stomach is an approach to minimise the economic and ecological problems of this processing discard. Additionally, fish protein hydrolysate with bioactivity prepared with the aid of fish proteases can be obtained as a new value-added product with high market value (Khantaphant & Benjakul, 2008). Skipjack tuna pepsins were recently purified and characterised by Nalinanon et al. (2010). Pepsins from skipjack tuna effectively hydrolysed several protein substrates, including bovine serum albumin, egg white, natural actomyosin from brownstripe red snapper muscle and acid-solubilised collagen from arabesque greenling skin.

Ornate threadfin bream (*Nemipterus hexodon*) belongs to the order Perciformes and is a member of the Nemipteridae family, found in tropical and sub-tropical Indo-West Pacific. Ornate threadfin bream is a potential raw material and widely used for surimi production in Thailand. However, the price of surimi fluctuates, depending on the world market. To increase the value of meat from

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this fish species, the production of protein hydrolysates with nutritive value as well as good functional properties can be an alternative way for its full utilisation. The use of fish pepsin recovered from stomach of tuna, a by-product from canned tuna processing, for hydrolysate production, could lower the cost of commercial proteases. Furthermore, the production of protein hydrolysate from fish muscle using tuna pepsin has also not been reported. Therefore, the objectives of this investigation were to study the functionalities and antioxidant properties of the protein hydrolysate prepared from ornate threadfin bream (*N. hexodon*) muscle using skipjack tuna pepsin and to determine the molecular weight, pH and thermal stabilities, as well as the activity after *in vitro* simulated gastrointestinal digestion, of the antioxidant peptides in the resulting hydrolysate.

2. Materials and methods

2.1. Chemicals

Bovine haemoglobin, bovine serum albumin (BSA), pepsin from porcine gastric mucosa (EC 3.4.23.1) (P7000, 422 units/mg solid), L-leucine, 2,4,6-trinitrobenzenesulphonic acid (TNBS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulphonic acid sodium salt (ferrozine) were purchased from Sigma Chemical Co. (St. Louis, MO). Pancreatin was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Folin–Ciocalteu's phenol reagent, trichloroacetic acid (TCA), potassium persulphate, sodium sulphite and ferrous chloride were obtained from Merck (Darmstadt, Germany). Sephadex G-25 was purchased from GE Healthcare UK Limited (Little Chalfont, Buckinghamshire, UK). Sodium dodecyl sulphate (SDS), Coomassie Blue R-250, and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA). All chemicals used were of analytical grade.

2.2. Preparation of skipjack tuna stomach

Internal organs of skipjack tuna (*Katsuwonus pelamis*) were obtained from Tropical Canning (Thailand) Public Co., Ltd., Songkhla, Thailand. The samples were packed in polyethylene bags, kept in ice with a solid:ice ratio of 1:2 (w/v) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, within 30 min. Upon arrival, internal organs were excised and the stomach was collected and cut into 0.5 cm × 0.5 cm pieces. Samples were frozen in liquid nitrogen and finely ground using a National Model MX-T2GN blender (Taipei, Taiwan). Samples were then lyophilised using a SCANVAC CoolSafe™ freeze-dryer (Cool-Safe 55, ScanLaf A/S, Lyngø, Denmark). The dry powder referred to as 'stomach powder' was stored at –20 °C until use.

Ornate threadfin bream (*N. hexodon*) was purchased from the dock in Songkhla, Thailand. The fish, off-loaded approximately 18–24 h after capture, were placed in ice at a fish-to-ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, within 1 h. Upon arrival, fish were filleted and the ordinary muscle was collected. Size reduction of muscle was carried out by chopping manually into small pieces. The prepared sample was kept in ice until use.

2.3. Preparation of pepsin from skipjack tuna stomach and activity assay

To prepare the skipjack tuna pepsin extract, the stomach powder was suspended in 20 mM sodium phosphate buffer (pH 7.0)

containing 1 mM PMSF at a ratio of 1:19 (w/v) and stirred continuously at 4 °C for 3 h. The suspension was centrifuged for 20 min at 4 °C at 20,000g (Avanti® J-E, Beckman Coulter, Inc., Palo Alto, CA) to remove the tissue debris. The supernatant was collected and then adjusted to pH 4 with 1 M HCl. The mixture was allowed to stand at 4 °C for 30 min. The prepared extract was then centrifuged at 20,000g for 15 min at 4 °C. To fully activate pepsin, the pH of acidified supernatant was adjusted to pH 2 with 1 M HCl and gently stirred for 30 min at 4 °C. The enzyme solution was subjected to centrifugation and the supernatant was collected in the same manner as described previously and referred to as "skipjack tuna pepsin".

Proteolytic activity of skipjack tuna pepsin was determined using haemoglobin as a substrate, as described by Nalinanon et al. (2010). The reaction was conducted at pH 2.0 and 50 °C for 20 min. One unit of activity was defined as the amount causing an increase of 1.0 in absorbance at 280 nm per min.

2.4. Preparation of protein substrate from ornate threadfin bream muscle

Protein substrate from ornate threadfin bream muscle was prepared by an acid solubilisation process, according to the method of Pérez-Mateos and Lanier (2006), with some modifications. Chopped fish muscle was homogenised with eight volumes of deionised water (0–6 °C) for 2 min in a blender at maximum speed (Type AY46, Moulinex, Groupe SEB Thailand Ltd., Bangkok, Thailand). To solubilise proteins, the pH of the mixture was adjusted to 2.0 using 1 M HCl. The samples were centrifuged at 5000g for 10 min at 4 °C. After centrifugation, the upper layer (containing neutral lipids) and the bottom sediment layer (containing connective tissue and insoluble lipid membranes) were discarded. The middle layer, containing solubilised proteins, was filtered through four layers of cheesecloth. The filtrate was then adjusted to pH 5.5 using 1 M NaOH with gradual stirring prior to centrifugation at 5000g for 10 min at 4 °C. The pellet was dispersed in four volumes of deionised water (0–6 °C) and then the pH of the protein suspension was adjusted to 2.0. Acid-solubilised protein fraction, with a high proportion of dissociated proteins which were more likely susceptible to hydrolysis, was used as a protein substrate for preparation of hydrolysate.

2.5. Production of protein hydrolysate from ornate threadfin bream muscle

Acid-solubilised protein fraction from the muscle of ornate threadfin bream was mixed with McIlvaine's buffer (0.2 M disodium hydrogen phosphate and 0.1 M sodium citrate) (pH 2.0) at a ratio of 1:1 (w/v). The mixture was pre-incubated at 50 °C for 15 min. The enzymatic hydrolysis was started by adding skipjack tuna pepsin with the amount (140–650 µl) required to gain 10%, 20% and 30% DH into 250 ml of mixture, as described by Benjakul and Morrissey (1997). After 1 h of hydrolysis, the enzyme was inactivated by adjusting the pH of the reaction mixture to 7.0 prior to heating at 90 °C for 15 min. The mixture was then centrifuged at 2000g for 10 min. The supernatant was then collected and lyophilised using a freeze-dryer. Lyophilised protein hydrolysate obtained was subjected to analyses.

2.6. Determination of α -amino acid content and DH

The α -amino acid content was determined according to the method of Benjakul and Morrissey (1997). To properly diluted hydrolysate samples (125 µl), 2.0 ml of 0.2 M phosphate buffer (pH 8.2) and 1.0 ml of 0.01% TNBS solution were added. The solution was mixed thoroughly and placed in a temperature controlled

water bath (Model W350, Memmert, Schwabach, Germany) at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulphite. The mixtures were cooled at room temperature for 15 min. The absorbance was read at 420 nm and α -amino acid was expressed in terms of L-leucine. DH was calculated as follows:

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

where L_t is the amount of α -amino acid released at time t . L_0 is the amount of α -amino acid in the original acid-solubilised protein substrate. L_{max} is total α -amino acid in the original acid-solubilised protein substrate obtained after acid hydrolysis (6 M HCl at 100 °C for 24 h).

2.7. Functional properties of protein hydrolysate

2.7.1. Solubility

To determine protein solubility, hydrolysate samples (10 mg) were dispersed in 8 ml of deionised water and pH of the mixture was adjusted to 3, 5, 7 and 9 with either 1 M HCl or 1 M NaOH. The mixture was stirred at room temperature for 30 min. The volume of solutions was made up to 10 ml by distilled water, previously adjusted to the same pH as the sample solution, prior to centrifugation at 5000g for 15 min. Protein content in the supernatant was determined using the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951), using bovine serum albumin as a standard. Total protein content in the sample was determined after solubilisation of the sample in 0.5 M NaOH. Protein solubility was calculated as follows:

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

2.7.2. Emulsifying properties

The emulsifying activity index (EAI) and the emulsion stability index (ESI) were determined according to the method of Pearce and Kinsella (1978), with slight modification. Soybean oil (2 ml) and protein hydrolysate solutions (0.10%, 0.25% and 0.50% protein, 6 ml) were homogenised (Model T25 basic; IKA Labortechnik, Selangor, Malaysia) at a speed of 20,000 rpm for 1 min. An aliquot of the emulsion (50 μ l) was pipetted from the middle portion of the container at 0 and 10 min after homogenisation and subsequently diluted 100-fold using 0.1% sodium dodecyl sulphate (SDS) solution. The mixture was mixed thoroughly for 10 s using a vortex mixer (G-560E, Vortex-Genie 2, Scientific Industries, Inc., Bohemia, NY). A_{500} of the resulting dispersion was measured using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). EAI and ESI were calculated by the following formulae:

$$EAI(m^2/g) = (2 \times 2.303 \times A \times DF)/l\Phi C$$

where $A = A_{500}$, DF = dilution factor (100), l = path length of cuvette (m), Φ = oil volume fraction and C = protein concentration in aqueous phase (g/m^3);

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

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

2.7.3. Foaming properties

Foam expansion (FE) and foam stability (FS) of hydrolysate solutions were determined as described by Shahidi, Han, and Synowiecki (1995) with slight modification. Hydrolysate solutions (20 ml) with 0.10%, 0.25% and 0.50% protein concentrations were transferred into a 100-ml cylinder. The solutions were homogenised at 13,400 rpm for 1 min at room temperature. The samples

were allowed to stand for 0 and 60 min. FE and FS were then calculated using the following equations:

$$FE(\%) = (V_T/V_0) \times 100$$

$$FS(\%) = (V_t/V_0) \times 100$$

where V_T is total volume after whipping, V_0 is the original volume before whipping, and V_t is total volume after leaving at room temperature for 60 min.

2.8. Determination of antioxidant activity

2.8.1. DPPH radical-scavenging activity

DPPH radical-scavenging activity was determined by DPPH assay, as described by Binsan et al. (2008). To the sample (1.5 ml), 1.5 ml of 0.15 mM DPPH in 95% ethanol were added. The mixture was mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was measured at 517 nm using a UV-1800 spectrophotometer (Shimadzu). The blank was prepared in the same manner, except that distilled water was used instead of the sample. A standard curve was prepared using Trolox in the range of 10–60 μ M. The activity was expressed as μ mol Trolox equivalents (TE)/g protein of hydrolysate.

2.8.2. ABTS radical-scavenging activity

ABTS radical-scavenging activity was determined by ABTS assay, as described by Binsan et al. (2008). The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 50 ml methanol, in order to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using a spectrophotometer. Fresh ABTS solution was prepared for each assay. Sample (150 μ l) was mixed with 2850 μ l of ABTS solution and the mixture was left at room temperature for 2 h in the dark. The absorbance was then measured at 734 nm using a spectrophotometer. The blank was prepared in the same manner, except that distilled water was used instead of the sample. A standard curve of Trolox ranging from 50 to 600 μ M was prepared. The activity was expressed as μ mol TE/g protein of hydrolysate.

2.8.3. Chelating activity on Fe^{2+}

The chelation of Fe^{2+} was measured using the method of Boyer and McCleary (1987), with slight modification. Diluted sample (4.7 ml) was mixed with 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine. The reaction mixture was allowed to stand for 20 min at room temperature. The absorbance was then read at 562 nm. The blank was prepared by using the distilled water instead of the sample. The chelating activity was calculated as follows:

$$\text{Chelating activity}(\%) = [(B - A)/B] \times 100$$

where A is A_{562} of sample and B is A_{562} of the blank.

2.9. Fractionation and molecular weight distribution of antioxidant peptides from protein hydrolysate

The hydrolysate (20% DH) rendering the highest antioxidant activity was subjected to gel filtration chromatography. The hydrolysate was loaded onto a Sephadex G-25 column (1.6×70 cm). The elution was carried out with 20 mM sodium phosphate buffer (pH 7.0) at a flow rate of 1 ml/min. The 2-ml fractions were collected and their absorbance was read at 220 and 280 nm. The fractions were subjected to analysis for ABTS radical-scavenging activity.

The fractions with the maximum ABTS radical-scavenging activities were pooled, referred to as “antioxidative peptide fraction”, and used for further studies. The molecular weight distribution of antioxidative peptides was determined from the retention time of selected fraction, in comparison with those of protein standards including gramicidin (1880 Da), vitamin B₁₂ (1355 Da), flavin adenine dinucleotide (829.5 Da) and Gly-Tyr (238.25 Da).

2.10. pH and thermal stability of antioxidant peptides

For the pH stability study, the antioxidant peptides fraction was adjusted to different pHs (1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11) using 1 M HCl and 1 M NaOH and incubated at room temperature for 30 min. Thereafter, the pH of the samples was adjusted to 7.0. The residual antioxidant activities were determined using the ABTS assay.

To determine thermal stability, 5 ml of antioxidative peptide fraction (pH 7.0) were transferred into a screw-capped test tube. The tube was capped tightly and placed in a boiling water bath (100 °C) for 0, 15, 30, 60, 90, 120 and 180 min. The treated samples were suddenly cooled in iced water. The sample without incubation (25 °C) was used as the control. The residual antioxidant activities were determined by ABTS assay.

2.11. *In vitro* pepsin–pancreatin simulated gastrointestinal (GI) digestion of antioxidant peptides

Simulated GI digestion using an *in vitro* pepsin–pancreatin hydrolysis was carried out according to the method of Cinq-Mars, Hu, Kitts, and Li-Chan (2008) and You et al. (2010), with slight modification. The pH of the antioxidant peptide fraction containing 3 mg protein (15 ml) was adjusted to 2.0 with 6 M HCl. Pepsin was then added (E/S 1:35 w/w), and the mixture was incubated with continuous shaking (W350, Memmert, Schwabach, Germany) for 1 h at 37 °C. The pH was then adjusted to 5.3 with 0.9 M NaHCO₃ solution and further to pH 7.5 with 6 M NaOH. Pancreatin was added (E/S 1:25 w/w), and the mixture was further incubated with continuous shaking for 3 h at 37 °C. To terminate the digestion, the solution was submerged in boiling water for 10 min. Then, the GI digest was cooled to room temperature and centrifuged at 5000g for 15 min. The supernatant was used for analysis.

To investigate the changes in ABTS radical-scavenging activity of peptide digested during the simulated GI digestion, aliquots of GI digests were removed every 30 min for 3 h during the *in vitro* digestion.

2.12. Protein determination

Protein concentration was determined using the Lowry method (Lowry et al., 1951). Bovine serum albumin was used as a standard.

2.13. Statistical analysis

All experiments were performed in triplicate. The data were subjected to analysis of variance (ANOVA) and the differences between means were evaluated by Duncan's multiple range test. SPSS statistic programme (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL.) was used for data analysis.

3. Results and discussion

3.1. Solubility of protein hydrolysates with different DH

The solubilities of hydrolysates from the muscle of ornate threadfin bream with different DHs at various pHs are shown in

Table 1

Solubility of hydrolysates from ornate threadfin bream muscle prepared using pepsin from skipjack tuna with different DHs at various pHs.

pH	DH (%)		
	10	20	30
3	97.8 ± 2.0b [†] B [‡]	92.8 ± 2.5aA	91.4 ± 1.2aA
5	71.2 ± 1.1aA	93.1 ± 0.6aB	92.4 ± 0.5aB
7	97.0 ± 0.5bA	97.5 ± 1.9bA	98.2 ± 1.8bA
9	96.6 ± 0.7bA	99.3 ± 1.0bB	98.1 ± 1.8bAB

Means ± SD (n = 3).

[†] Different small letters in the same column indicate significant differences (*p* < 0.05).

[‡] Different capital letters in the same row indicate significant differences (*p* < 0.05).

Table 1. All hydrolysates were soluble over a wide pH range, in which more than 70% solubility was obtained. The hydrolysates were generally soluble in alkaline pH to a greater extent, compared with acidic pH, except at pH 3 for hydrolysate with 10% DH. Protein hydrolysates from yellow stripe trevally (*Selaroides leptolepis*) meat, hydrolysed by Alcalase and Flavourzyme with different DHs (5–25%), also showed high solubility (>85%) in the pH range of 2–12 (Klompong et al., 2007). The lowest solubility was found in the hydrolysate with 10% DH at pH 5 (*p* < 0.05). The result suggested that proteins or peptides with high molecular weight (MW) remaining after hydrolysis were precipitated at this pH, which was close to the isoelectric point (pI) of myofibrillar proteins. In general, the degradation of proteins to smaller peptides leads to more soluble products (Klompong et al., 2007; Gbogouri, Linder, Fanni, & Parmentier, 2004). The smaller peptides from myofibrillar proteins are expected to have proportionally more polar residues, with the ability to form hydrogen bonds with water and augment solubility (Gbogouri et al., 2004). At pH 5 and 9, the solubilities of hydrolysates were increased with increasing DH from 10% to 20% DH (*p* < 0.05). No differences in the solubility of hydrolysate with different DH were observed at pH 7 (*p* > 0.05). Enzymatic hydrolysis potentially affects the molecular size and hydrophobicity, as well as polar and ionisable groups of protein hydrolysates (Mutilangi, Panyam, & Kilara, 1996). The balance of hydrophilic and hydrophobic forces of peptides is another crucial influence on the solubility of protein hydrolysate (Klompong et al., 2007; Gbogouri et al., 2004). Therefore, the differences in solubility of hydrolysates with different DH might be determined by the size of peptides, the hydrophobic–hydrophilic balance, as well as the charge group of the peptides produced during the hydrolysis process.

3.2. Emulsifying properties of protein hydrolysates with different DH

Emulsion activity index (EAI) and emulsion stability index (ESI) of hydrolysates from ornate threadfin bream muscle with different DH at various concentrations (0.10%, 0.25%, and 0.50%) are shown in Table 2. Generally, both EAI and ESI of hydrolysates with different DH varied, particularly when the protein concentration increased (*p* < 0.05). At 0.10% protein, hydrolysate with 10% DH showed the highest EAI and ESI, when compared with those having higher DHs (*p* < 0.05). However, no differences in EAI were found with increasing DH, when hydrolysates at 0.25% and 0.50% were tested (*p* > 0.05). Mutilangi et al. (1996) postulated that higher contents of higher MW peptides or more hydrophobic peptides contribute to the stability of the emulsion. On the other hand, excessive hydrolysis brings about the loss of emulsifying properties (Kristinsson & Rasco, 2000; Klompong et al., 2007; Gbogouri et al., 2004). Klompong et al. (2007) reported that both EAI and ESI of protein hydrolysates from yellow stripe trevally meat decreased with increasing DH. The peptides with low MW may not be amphiphilic enough to exhibit good emulsifying properties

Table 2
Emulsifying and foaming properties of hydrolysates from ornate threadfin bream muscle prepared using pepsin from skipjack tuna with different DHs at various protein concentrations.

Hydrolysate concentrations (% protein)	DH (%)	EAI (m ² /g)	ESI (min)	FE (%)	FS (%)
0.10	10	116 ± 2.6c [‡]	25.8 ± 2.2bC	128 ± 3.1bA	69 ± 5.3bA
	20	109 ± 2.3bC	15.2 ± 0.6aA	126 ± 2.4bA	57 ± 2.5aA
	30	102 ± 2.9aC	16.1 ± 0.4aA	120 ± 2.3aA	55 ± 2.9aA
0.25	10	58.6 ± 1.5bB	18.4 ± 2.3bB	128 ± 3.0bA	75 ± 3.2bAB
	20	54.8 ± 1.0aB	18.5 ± 1.0bB	127 ± 2.1bA	65 ± 5.2abAB
	30	55.0 ± 0.2aB	17.5 ± 0.7bB	118 ± 3.3aA	60 ± 6.1aA
0.50	10	29.9 ± 2.3aA	14.1 ± 0.5aA	131 ± 4.5bA	78 ± 2.5cB
	20	30.3 ± 0.3aA	18.6 ± 2.5bB	126 ± 4.5abA	70 ± 4.8bB
	30	30.1 ± 1.1aA	17.8 ± 0.8bB	122 ± 4.0aA	61 ± 2.3aA

Means ± SD (n = 3).

[†] Different small letters in the same column within the same hydrolysate concentration indicate significant differences ($p < 0.05$).

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

(Chobert, Bertrand-Harb, & Nicolas, 1988). Lin and Chen (2006) proposed that the emulsification process includes two steps: (1) deformation and disruption of droplets which increase the specific surface area of emulsion and (2) stabilisation of this newly-formed interface by emulsifier or surfactant. The hydrolysate with 10% DH could assemble at the interface and stabilise the oil droplet to a higher extent. With the same DH, EAI of hydrolysates decreased with increasing concentrations ($p < 0.05$). With 20% and 30% DH, no differences in ESI were noticeable as the hydrolysate concentrations were above 0.25% ($p > 0.05$). At low protein concentrations, protein adsorption at the oil–water interface is diffusion controlled. At high protein concentration, the activation energy barrier does not allow protein migration to take place in a diffusion-dependent manner, leading to the accumulation of proteins in the aqueous phase (Lawal, 2004; Thiansilakul et al., 2007a). Thus, proteins or peptides were most likely localised in the aqueous phase and a lower amount of proteins or peptides migrated to the interface (Thiansilakul et al., 2007a). The increase in protein–protein interaction resulted in a lower protein concentration at the interface, in which a thinner film stabilising the oil droplet is formed. Therefore, emulsifying properties of hydrolysate were governed by the molecular properties, particularly the size of peptides and the concentration employed.

3.3. Foaming properties of protein hydrolysates with different DH

Foaming properties are physicochemical characteristics of proteins to form and stabilise foams (Thiansilakul et al., 2007a). Foam expansion (FE) and foam stability (FS) of hydrolysates from ornate threadfin bream muscle with different DH at various concentrations (0.10%, 0.25%, and 0.50%) are shown in Table 2. At the same concentration of hydrolysate used, slight decreases in FE were observed when DH of hydrolysate increased ($p < 0.05$). This is possibly due to a lesser alignment of small peptides at the air–water interface (Nalinanon, Benjakul, Visessanguan, & Kishimura, 2008). With the same DH, protein concentrations had no impact on FE ($p > 0.05$). Foam formation is governed by three factors, including transportation, penetration and reorganisation of molecules at the air–water interface (Klompong et al., 2007). In general, proteins, which rapidly adsorb at the newly-created air–liquid interface during bubbling and undergo unfolding and molecular rearrangement at the interface, exhibit better foaming ability than proteins that adsorb slowly and resist unfolding at the interface (Damodaran, 1997). FE after whipping for 60 min was monitored to indicate the FS of protein hydrolysates. FS was slightly decreased with increasing DH of hydrolysate. A similar result was also reported in protein hydrolysates from yellow stripe trevally meat prepared by Alcalase and Flavourzyme with 5–25% DH (Klompong et al., 2007). It was suggested that low MW peptides could not

maintain well-ordered orientation of the molecule at the interface. However, at the same DH, FS of hydrolysate was improved by increasing concentration ($p < 0.05$). Lawal (2004) postulated that an increase in foam stability with increasing concentration was a result of formation of stiffer foams. FS is enhanced by flexible protein domains, which enhance viscosity of the aqueous phase, protein concentration and film thickness (Phillips, Whitehead, & Kinsella, 1994).

3.4. Antioxidant activities of protein hydrolysates with different DHs

Antioxidant activities, as determined by ABTS, DPPH and Fe²⁺ chelating assays of hydrolysates with different DH values are shown in Table 3. The differences in activities determined by both assays were observed amongst protein hydrolysates with different DHs. The highest ABTS and DPPH radical-scavenging activities were found in hydrolysates with 20% DH (159 and 8.58 μmol TE/g protein, respectively; $p < 0.05$). ABTS and DPPH radical-scavenging activities of protein hydrolysates with 10% DH were 151 and 3.15 μmol TE/g protein, respectively, whereas that of 30% DH had activities of 151 and 6.21 μmol TE/g protein, respectively. Thus peptides in hydrolysates with various DH might differently scavenge two different radicals, ABTS and DPPH radicals. However, Klompong et al. (2007) found that no differences in DPPH radical-scavenging activities were observed for yellow stripe trevally protein hydrolysate prepared by Flavourzyme with different DHs, ranging from 5% to 25%. Generally, hydrolysates contained peptides or proteins, which were hydrogen donors and could react with radicals to convert them to more stable products, thereby terminating the radical chain reaction (Khantaphant & Benjakul, 2008). ABTS assay is an excellent tool for determining the antioxidant activity of hydrogen-donating compounds (scavengers of aqueous phase radicals) and of chain-breaking antioxidants (scavenger of lipid peroxy radicals) (Binsan et al., 2008). The ABTS radical is relatively stable and is readily reduced by antioxidants (Klompong et al., 2009; Miller, Rice-Evans, Davies, Gopinathan, &

Table 3
Antioxidant activities of hydrolysates from ornate threadfin bream muscle prepared using pepsin from skipjack tuna with different DHs as determined by various assays.

Activities tested	DH (%)		
	10	20	30
ABTS (μmol TE/g protein)	150 ± 0.82a [†]	159 ± 1.01b	151 ± 0.74a
DPPH (μmol TE/g protein)	3.15 ± 0.35a	8.58 ± 0.24c	6.21 ± 0.12b
Chelating activity (%)	45.9 ± 1.72a	51.3 ± 0.50b	58.1 ± 0.59c

TE, Trolox equivalents.

Mean ± SD (n = 3).

[†] Different letters in the same row indicate significant differences ($p < 0.05$).

Milner, 1993). With high ABTS radical-scavenging activity, it was postulated that antioxidative compounds were most likely hydrophilic. DPPH radical-scavenging activity is generally used to determine hydrogen-donating ability of protein hydrolysates (Wu et al., 2003; Thiansilakul et al., 2007b). The stable DPPH radical displays a maximum absorbance at 517 nm in ethanol. When DPPH encounters a hydrogen-donating substance, the radical would be scavenged, as visualised by changing its colour from purple to yellow, and the absorbance is reduced (Shimada, Fujikawa, Yahara, & Nakamura, 1992). The results so obtained suggest that the hydrolysates contained amino acids or peptides that were electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction.

As the DH increased, chelating activity on Fe^{2+} increased ($p < 0.05$). Hydrolysates with 30% DH exhibited the highest Fe^{2+} chelating activity (58.1%). The result indicated that metal ion-binding capacity of protein hydrolysates was enhanced by pepsin hydrolysis. The result was in accordance with Klompong et al. (2007), who reported that chelating activity on Fe^{2+} of yellow stripe trevally protein hydrolysates, prepared by both Alcalase and Flavourzyme, increased with increasing DH. The chelation of Fe^{2+} was used to determine the ability of hydrolysates in metal chelating. Ferrozine quantitatively forms complexes with Fe^{2+}

ion. In the presence of chelating agents, the complex formation is disrupted, resulting in the decrease in colour formation (Thiansilakul et al., 2007b). The results indicated that hydrolysates had a potential chelating ability towards iron.

It was noted that the protein hydrolysate from ornate threadfin bream with 20% DH showed the highest radical-scavenging activities, whilst the hydrolysate with 30% DH exhibited the highest Fe^{2+} chelating activity. It was speculated that different peptides with varying size as well as amino acid sequence might have different modes of action. Therefore, hydrolysates with appropriate DH could be prepared, to maximise their functions as radical scavengers and metal chelators.

3.5. Fractionation and molecular weight distribution of antioxidant peptides from protein hydrolysate

Hydrolysates from the muscle of ornate threadfin bream with 20% DH prepared using the pepsin from skipjack tuna, which showed the highest radical-scavenging activity, were fractionated using Sephadex G-25 gel filtration chromatography. A_{220} was used to monitor peptide bonds, whereas A_{280} was the parameter representing peptides, proteins or amino acids with aromatic rings (Fig. 1A). A distinct peak of A_{280} was observed in the hydrolysate,

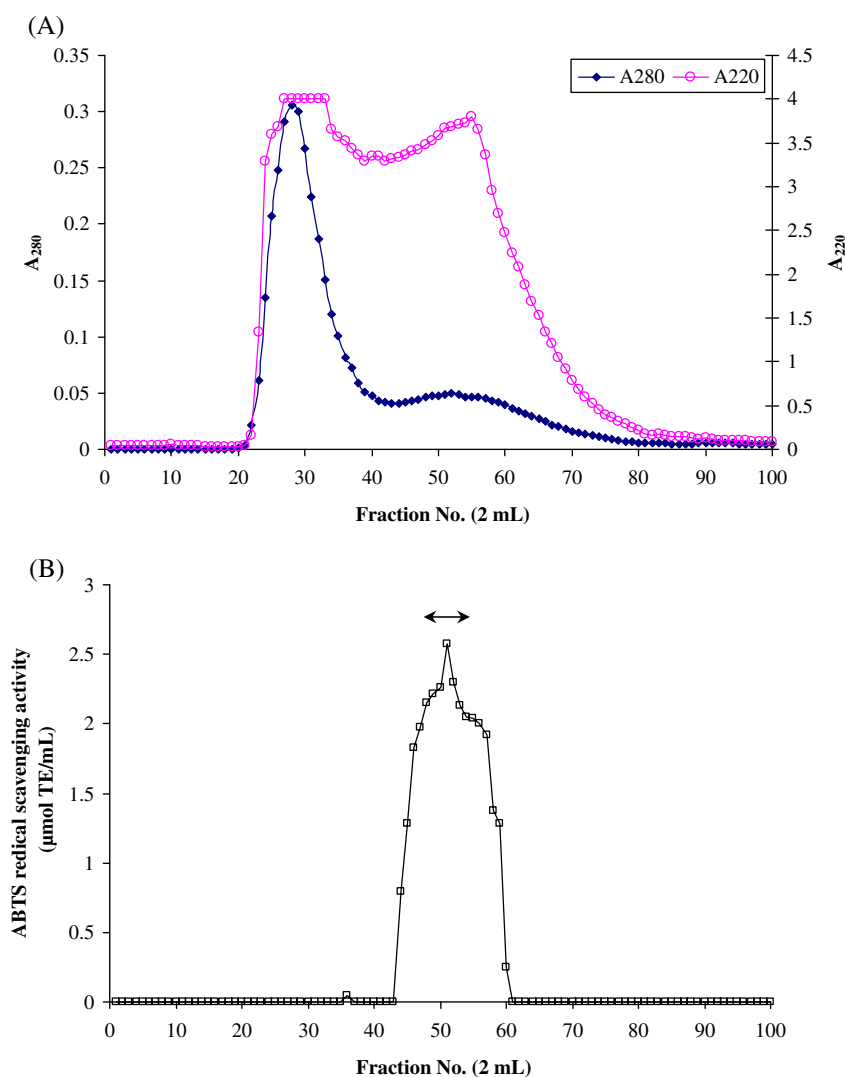


Fig. 1. Separation of antioxidant peptides from hydrolysate of ornate threadfin bream muscle prepared using pepsin from skipjack tuna. (A) A_{220} and A_{280} ; (B) ABTS radical-scavenging activity. ($\leftrightarrow$): pooled fractions.

indicating the presence of proteins or peptides mainly containing aromatic amino acids, with high and low MW. The hydrolysate had two peaks of A_{220} , indicating the presence of peptide bonds in the hydrolysate. The first peak of A_{220} was found in the same fractions as the A_{280} peak. Low A_{280} was detected in the second A_{220} peak, indicating a high proportion of low MW peptides in the hydrolysate.

As shown in Fig. 1B, the fraction containing peptides with a MW of 1.3 kDa of hydrolysate prepared using skipjack tuna pepsin showed the highest ABTS radical-scavenging activity. The activity peak was located at the same position of the second A_{220} peak, indicating that low MW peptides contributed to the antioxidant activity. The result suggested that the hydrolysate tested most likely contained certain peptides that were electron donors or could scavenge free radicals to terminate the radical chain reaction. Fractions 49–53, showing the highest ABTS radical-scavenging activity, were pooled and used as a source of antioxidant peptides.

3.6. pH and thermal stability of antioxidant peptides

The influences of pH on the stability of antioxidant peptides fractionated by Sephadex G-25 column chromatography are depicted in Fig. 2A. ABTS radical-scavenging activity of the antioxidant peptide fraction remained constant over the pH range of 1–

10. At pH 11, ABTS radical-scavenging activity slightly decreased ($p < 0.05$). The results suggested that antioxidant peptides exhibiting ABTS radical-scavenging activity might lose their activity to some extent at high pH. Due to the stability over a wide pH range, antioxidant peptides from the muscle of ornate threadfin bream have potential for application in any food system at extreme pH.

Thermal stability of antioxidant peptide fraction as monitored by ABTS radical-scavenging activities is shown in Fig. 2B. ABTS radical-scavenging activities of antioxidant peptide fraction were quite stable when heated at 100 °C up to 180 min, where activities of more than 97% were retained. A small loss in ABTS radical scavenging activity might be due to either degradation or aggregation of some antioxidant peptides, caused by heat treatment. In general, proteins were vulnerable to heat treatment, leading to the aggregation of protein and the exposure of hydrophobic domain (Sikorski & Naczek, 1981). Nevertheless, peptides with smaller sizes were more stable to aggregation at high temperature (Zayas, 1997). Peptides derived from many protein sources with increased hydrophobicity have been reported to correlate with antioxidant properties (Chen, Muramoto, Yamauchi, & Nokihara, 1996; Faithong, Benjakul, Phatcharat, & Binsan, 2010). The result indicated that peptides with low MW were most likely stable following heat treatment. Therefore, the antioxidant peptides can be incorporated in cooked food systems without significant loss of their antioxidant activities.

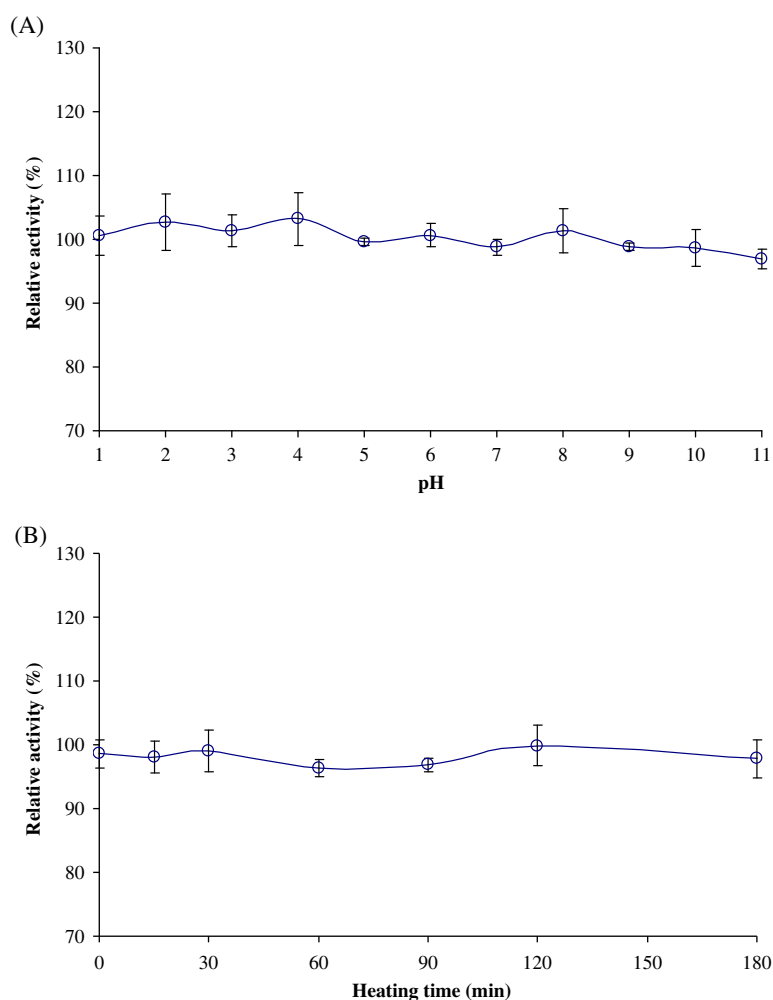


Fig. 2. pH (A) and thermal (B) stabilities of antioxidant peptides from hydrolysate of ornate threadfin bream using pepsin from skipjack tuna fractionated by Sephadex G-25 column chromatography. Bars represent the standard deviation ($n = 3$).

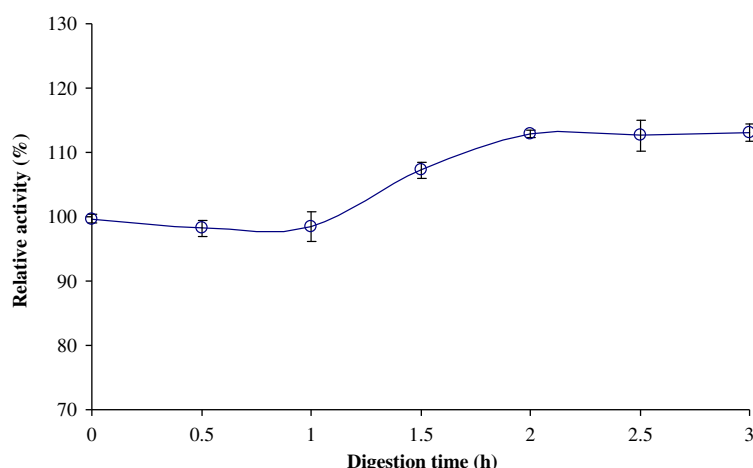


Fig. 3. Changes in ABTS radical-scavenging activity during sequential *in vitro* digestion of antioxidant peptides from hydrolysate of ornate threadfin bream using pepsin from skipjack tuna fractionated by Sephadex G-25 column chromatography. The first stage: from 0 to 1.0 h, digested by pepsin; the second stage: from 1.0 to 3.0 h, digested by pancreatin. Bars represent the standard deviation ($n = 3$).

3.7. *In vitro* pepsin–pancreatin simulated GI digestion of antioxidant peptides

An *in vitro* pepsin–pancreatin hydrolysis simulating GI digestion was used to illustrate its impact on ABTS radical-scavenging activity of antioxidant peptides (Sephadex G-25 fraction) from protein hydrolysate of ornate threadfin bream prepared using skipjack tuna pepsin (Fig. 3). Pepsin digestion for 1 h did not alter ABTS radical-scavenging activity ($p > 0.05$). This might be due to the cleavage limitation of pepsin on antioxidant peptides, which were previously digested by skipjack tuna pepsin. As a result, no degradation or generation of new antioxidant peptides took place. After further digestion by pancreatin for 30–60 min, ABTS radical-scavenging activity was increased by about 7–12%. It was suggested that antioxidative peptides were modified by pancreatin digestion to enhance their ABTS radical-scavenging activity. In addition, the increase of hydrophilic property of GI digests after pancreatin treatment has been reported to favour trapping of ABTS radical of loach protein hydrolysate (You et al., 2010). Additionally, You et al. (2010) reported that GI digests had increased reducing power, chelating ability of Cu^{2+} and hydroxyl radical-scavenging activities by 77%, 12% and 12%, respectively. Therefore, antioxidant peptides were most likely stable in real digestion system after ingestion in both stomach and intestine, which have high proteolytic activity under acidic and alkaline pH, respectively.

4. Conclusions

Production of protein hydrolysates from ornate threadfin bream muscle, exerting good functionalities and antioxidant activities, could be achieved by skipjack tuna pepsin hydrolysis. Based on pH and thermal stability as well as GI digestion, antioxidant peptides in protein hydrolysate can be incorporated as a multifunctional ingredient into foods.

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The effects of pretreatments on antioxidative activities of protein hydrolysate from the muscle of brownstripe red snapper (*Lutjanus vitta*)

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ABSTRACT

Different pretreatments of mince from brownstripe red snapper (*Lutjanus vitta*) including 1) washing; 2) membrane separation; 3) washing followed by membrane separation and 4) membrane separation followed by washing were conducted prior to hydrolysis. Among the resulting minces, that subjected to membrane separation with subsequent washing (MS/W) contained the lowest remaining myoglobin content, phospholipid content, heme iron and non-heme iron contents ($p < 0.05$) and showed the lowest TBARS values throughout 9 days of storage at 4 °C in the presence and absence of 0.15 mol L⁻¹ cupric acetate ($p < 0.05$). When hydrolysates from 1) mince, 2) MS/W and 3) protein isolate from MS/W (PI) with different degree of hydrolysis (DH) (20, 30 and 40%) were prepared using proteases from pyloric caeca of brownstripe red snapper, antioxidative activities determined by DPPH, ABTS radical scavenging activities, ferric reducing antioxidant power and metal chelating activity varied with hydrolysates and DH. Antioxidative activities increased with increasing DH up to 40% ($p < 0.05$). At all DH tested, hydrolysate prepared from MS/W exhibited the highest antioxidative activities determined by all assays, compared to those from mince and PI ($p < 0.05$). Hydrolysate from MS/W with 40% DH had the molecular weight lower than 6.5 kDa as determined by SDS-PAGE. In liposome oxidation system, the addition of hydrolysate from MS/W resulted in the lower TBARS, compared with the control throughout the incubation period of 48 h at room temperature (25–28 °C). Therefore, fish mince with membrane separation followed by washing was the most appropriate source for production of hydrolysate possessing antioxidative activity with the lowered amount of lipids and pro-oxidants.

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1. Introduction

Numerous fish protein hydrolysates have been shown to have antioxidative activities such as gelatin hydrolysate from the skin of Alaska pollack (Kim et al., 2001), protein hydrolysate from the frame of yellowfin sole (Jun, Park, Jung, & Kim, 2004), protein hydrolysate from the muscle of round scad (Thiansilakul, Benjakul, & Shahidi, 2007a) and yellow stripe trevally (Klompong, Benjakul, Kantachote, & Shahidi, 2007). One critical problem of hydrolysate preparation from these proteinaceous sources is the presence of pro-oxidants such as heme proteins and unstable lipid substrates (Raghavan & Kristinsson, 2008). As a consequence, the desirable antioxidative activity can be lowered and the hydrolysate may exhibit the pro-oxidative activity to some extent.

Fish muscle contains myoglobin and other heme proteins, which in turn become the major pro-oxidants in muscle (Chaijan, Benjakul, Visessanguan, & Faustman, 2005; Thanonkaew, Benjakul, Visessanguan, & Decker, 2006). Furthermore, phospholipids, major components of cell membranes, are believed to readily prone to oxidative deterioration due to their highly unsaturated fatty acid composition (Borst, Visser, Kouptsova, & Visser, 2000). In fish, especially lean fish, phospholipids make up most of the lipids of the cell (Liang & Hultin, 2005a). The presence of these pro-oxidants and lipids could decrease the stability of protein hydrolysates and may limit their use in food systems (Raghavan & Kristinsson, 2008). Removal of these compounds could therefore alleviate such problems occurring in hydrolysate from fish muscle.

Brownstripe red snapper (*Lutjanus vitta*), a lean fish, is one of the main raw materials for surimi production in Thailand (Khantaphant & Benjakul, 2008). Apart from being processed into surimi, its flesh can be used as the raw material for production of protein hydrolysate with bioactivities. To produce protein hydrolysate from brownstripe red snapper with high antioxidative activity and negligible pro-

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oxidant or lipids susceptible to oxidation, the appropriate pretreatment of muscle by washing and/or membrane separation before hydrolysis should therefore taken into consideration.

Pyloric caeca of various fish has been reported to be the important source of trypsin such as arabesque greenling (Kishimura, Hayashi, Miyashita, & Nonami, 2006), New Zealand hoki (Shi, Marshall, & Simpson, 2007) and bluefish (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2007). Recently, protease from pyloric caeca of brownstripe red snapper has been shown to hydrolyze fish skin gelatin effectively (Khantaphant & Benjakul, 2008). Along with the pretreatment, proteases from pyloric caeca of brownstripe red snapper could be used as the potential aid for the preparation of protein hydrolysate with antioxidative activity, in which the new value-added product of health benefit could be produced. The objectives of this study were to elucidate the effect of washing and membrane separation on the removal of pro-oxidant and membranes and to produce protein hydrolysates from pretreated mince of brownstripe red snapper with the lowered pro-oxidative activity using proteases from its pyloric caeca.

2. Materials and methods

2.1. Chemicals

2,4,6-trinitrobenzenesulfonic acid (TNBS), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2, 4, 6-tripyridyl-triazine (TPTZ), 1,1,3,3-tetramethoxypropane, bathophenanthroline disulfonic acid and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid (ferrozine) were purchased from Sigma (St. Louis, MO, USA). Thiobarbituric acid (TBA) and potassium persulfate were obtained from Fluka (Buchs, Switzerland). Trichloroacetic acid (TCA), iron standard solution and sodium nitrite were procured from Merck (Darmstadt, Germany). Sodium sulfite and ammoniumthiocyanate were obtained from Riedel-deHaën (Seelze, Germany). All chemicals were of analytical grade.

2.2. Preparation of mince with different pretreatments

2.2.1. Mince preparation

Brownstripe red snapper, stored in ice and off-loaded approximately 3 days after capture, were purchased from a dock in Songkhla province, Thailand. Fish were transported in ice with the fish/ice ratio of 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, whole fish were washed and only flesh was separated manually. Flesh was minced to uniformity using a Moulinex AY46 blender (Group SEB, Lyon, France). The mince obtained was placed in polyethylene bags and kept in ice not longer than 2 h before use.

2.2.2. Preparation of washed mince

Mince was homogenized with five volumes of cold distilled water using an IKA Labortechnik homogenizer (Selangor, Malaysia) at a speed of 11,000 rpm for 2 min, followed by stirring at 4 °C for 15 min prior to centrifuging at $9600 \times g$ for 10 min at 4 °C using a Beckman Coulter centrifuge Model Avant J-E (Beckman Coulter, Inc., Fullerton, CA, USA). The washing process was repeated twice. The sample obtained was referred to as 'washed mince; W'.

2.2.3. Preparation of mince with membrane separation

Membrane was removed from mince by treatment with Ca^{2+} and citric acid according to the method of Liang and Hultin (2005b) with a slight modification. Mince was homogenized with nine volumes of cold $8 \text{ mmol L}^{-1} \text{ CaCl}_2$ solution in the presence of 5 mmol L^{-1} citric acid using a homogenizer at a speed of 11,000 rpm for 2 min. After continuous stirring for 60 min at 4 °C, the sample

was centrifuged at $4000 \times g$ for 15 min at 4 °C and the mince obtained was referred to as 'membrane separated mince; MS'.

2.2.4. Preparation of membrane separated/washed mince and washed/membrane separated mince

Minces subjected to washing prior to membrane separation (W/MS) and to membrane separation followed by washing (MS/W) were also prepared. All samples were subjected to analyses.

2.3. Analyses

2.3.1. Determination of myoglobin content

Myoglobin content was determined by direct spectrophotometric method (Chaijan et al., 2005). Sample (2.0 g) was mixed with 20 mL of cold 40 mmol L^{-1} phosphate buffer (pH 6.8), followed by homogenization at 13,500 rpm for 10 s. The mixture was centrifuged at $3000 \times g$ for 30 min at 4 °C and the supernatant was filtered through Whatman filter paper No. 1 (Schleicher & Schuell, Maidstone, England). The absorbance of supernatant was read at 525 nm. Myoglobin content was calculated from the molar extinction coefficient of 7.6×10^{-3} and a molecular weight of 16,110 (Gomez-Basauri & Regenstein, 1992). The myoglobin content was expressed as mg/100 g dry sample.

2.3.2. Determination of heme iron and non-heme iron contents

Heme iron content was calculated based on the content of myoglobin, which contains iron at a level of 0.35 g/100 g (Gomez-Basauri & Regenstein, 1992). Heme iron content was expressed as mg/100 g dry sample.

Non-heme iron content was determined according to the method of Chaijan et al. (2005). Sample (1.0 g) was weighed and transferred into a screw cap test tube and 50 mL of 3.9 g L^{-1} sodium nitrite and 4 mL of a mixture of 400 g L^{-1} TCA and 6 mol L^{-1} HCl (1:1 (v/v) ratio, freshly prepared) were added. The tightly capped tubes were placed in a temperature controlled water bath (Model W350, Memmert, Schwabach, Germany) for 22 h at 65 °C followed by cooling to room temperature (25–28 °C) for 2 h. The supernatant (400 μL) was mixed with 2 mL of the non-heme iron color reagent, a freshly prepared mixture of bathophenanthroline disulfonic acid, double-deionized water and saturated sodium acetate solution at a ratio of 1:20:20 (w/v/v). After thorough mixing, the mixture was allowed to stand for 10 min and the absorbance was measured at 540 nm. The non-heme iron content was calculated from the iron standard curve using iron standard solution (0–2 mg L^{-1}). The non-heme iron content was expressed as mg/100 g dry sample.

2.3.3. Determination of phospholipid content

Phospholipid content was determined based on the direct spectrophotometric measurement of complex formation between phospholipids and ammonium ferrothiocyanate as described by Stewart (1980). Lipids were extracted from the sample by the method of Bligh and Dyer (1959). Thereafter, lipids (20 μL) were dissolved in chloroform to a final volume of 2 mL. One mL of thiocyanate reagent (a mixture of 0.10 mol L^{-1} ferric chloride hexahydrate and 0.40 mol L^{-1} ammoniumthiocyanate) was added. After thorough mixing for 1 min, the lower layer was removed and the absorbance at 488 nm was measured. A standard curve was prepared with phosphatidylcholine (0–0.1 g L^{-1}). The phospholipid content was expressed as mg/100 g dry sample.

2.4. Occurrence of lipid oxidation in mince with different pretreatments

Mince with different pretreatments was mixed with sodium azide as an antimicrobial agent to obtain a final concentration of

0.1 g kg⁻¹. The mixture (50 g) was added with 20 µL of 0.15 mol L⁻¹ cupric acetate and the samples were stored at 4 °C and taken every 3 days for determination of lipid oxidation.

2.4.1. Determination of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) were determined as described by Benjakul, Visessanguan, Phongkanpai, and Tanaka (2005) with a slight modification. Sample was homogenized with TBARS solution (3.75 g L⁻¹ TBA, 150 g L⁻¹ TCA and 0.25 mol L⁻¹ HCl) with a ratio of 1:4 (w/v). The mixture was heated in boiling water for 10 min to develop the pink color. Then the mixture was cooled with running water and centrifuged at 5000 × g for 10 min at room temperature using Hettich centrifuge (Model MIKRO-20, Hettich, Tuttlingen, Germany). The supernatant was collected and measured at 532 nm using a UV-1601 Spectrophotometer (Shimadzu, Kyoto, Japan). TBARS was calculated from a standard curve of malonaldehyde (MDA) (0–10 mg L⁻¹) and expressed as mg MDA/kg sample.

2.4.2. Determination of conjugated diene

Conjugated diene formed in liposome oxidation system was measured according to the method of Frankel, Huang, and Aeschbach (1997). Sample (0.1 mL) was dissolved in methanol (5.0 mL) and conjugated dienes were measured as the increase in absorbance at 234 nm.

2.5. Dissociation of actomyosin complex in mince with different pretreatments

Mince with different pretreatments were determined for ATPase activity. Prior to assay, natural actomyosin (NAM) was prepared as described by Benjakul, Seymour, Morrissey, and An (1997) with a slight modification. Pretreated mince was homogenized in cold 0.6 mol L⁻¹ KCl (pH 7.0) at a ratio of 1:10 (w/v) using a homogenizer with a speed of 11,000 rpm for 20 s, followed by a 20 s rest interval to a total extraction time of 4 min. Thereafter, the homogenate was centrifuged at 5000 × g for 30 min at 4 °C. Three volumes of cold distilled water were added to precipitate NAM prior to centrifugation at 5000 × g for 20 min at 4 °C. NAM pellet was dissolved in a minimal volume of cold 50 mmol L⁻¹ potassium phosphate buffer (pH 7.0) containing 0.6 mol L⁻¹ KCl. NAM solution was kept in ice and used within 1 h.

Mg²⁺-ATPase activities of NAM were assayed according to the method of Benjakul et al. (1997). NAM solution (0.5 mL) was added into 0.3 mL of 0.5 mol L⁻¹ Tris-maleate buffer (pH 7.0) and 2 mmol L⁻¹ MgCl₂ was added to a mixture to a final volume of 9.5 mL. Thereafter, 0.5 mL of 20 mmol L⁻¹ ATP was added to initiate the reaction. The reaction was conducted at 25 °C for exactly 10 min and 5 mL of cold 150 g L⁻¹ TCA were added to terminate the reaction. The reaction mixture was centrifuged at 3500 × g for 5 min and the inorganic phosphate liberated in the supernatant was measured as described by Fiske and Subbarow (1925). Specific activity was expressed as µmol inorganic phosphate released/mg protein/min. Blank was performed by adding the cold TCA prior to the addition of ATP. The decrease in Mg²⁺-ATPase indicated the dissociation of actomyosin complex, in which actin was not stable in the presence of high salt concentration (Torikai & Konno, 1996).

2.6. Preparation of proteases from pyloric caeca

Pyloric caeca from brownstripe red snapper was collected and powered in liquid nitrogen. Thereafter, the pyloric caeca extract was prepared according to the method of Khantaphant and Benjakul (2008) with a slight modification. Pyloric caeca powder was suspended in ten volumes of extraction buffer (50 mmol L⁻¹ Tris–HCl

buffer, pH 8.0 containing 10 mmol L⁻¹ CaCl₂). The mixture was homogenized using a homogenizer at 11,000 rpm for 2 min. The homogenate was continuously stirred for 30 min at 4 °C and centrifuged at 8000 × g for 30 min at 4 °C. The supernatant was filtered through a Whatman filter paper No. 1. The filtrate obtained was further subjected to 40–60% saturation ammonium sulfate precipitation. After stirring at 4 °C for 30 min, the mixture was centrifuged at 8000 × g for 30 min at 4 °C and the pellet obtained was dissolved in 50 mmol L⁻¹ Tris–HCl buffer, pH 8.0 followed by dialysis against 20 volumes of the extraction buffer overnight at 4 °C with three changes of dialysis buffer. The dialysate was kept in ice and referred to as 'pyloric caeca extract; PCE'. PCE was determined for proteolytic activity using casein as a substrate under optimal condition (60 °C and pH 8.5) (Khintaphant & Benjakul, 2008).

2.7. Preparation of brownstripe red snapper protein hydrolysates

Mince, mince subjected to membrane separation and washing (MS/W) and protein isolate (PI) were used for preparation of protein hydrolysate. PI was prepared as described by Rawdkuen, Sai-Ut, Khamsorn, Chaijan, and Benjakul (2009) with a slight modification. MS/W mince was homogenized with nine volumes of cold distilled water at a speed of 11,000 rpm for 1 min. The homogenate was adjusted to pH 11.2 using 2 mol L⁻¹ NaOH. Homogenate was then centrifuged at 5000 × g for 10 min at 4 °C. The alkaline-soluble fraction was collected and adjusted to pH 5.5 (the isoelectric point of muscle proteins) using 2 mol L⁻¹ HCl, followed by centrifuging at 5000 × g for 10 min at 4 °C. The pellet was mixed with distilled water at a ratio of 1:2 (w/v) and the final pH was adjusted to 7.0 with 2 mol L⁻¹ NaOH. The sample obtained was referred to as 'protein isolate; PI'.

To prepare protein hydrolysate, the samples were mixed with 50 mmol L⁻¹ Tris–HCl buffer, pH 8.5 to obtain the protein concentration of 20 g L⁻¹. The mixtures were homogenized at a speed of 11,000 rpm for 1 min and the homogenates were pre-incubated at 60 °C for 10 min. Hydrolytic reaction was started by adding PCE with different levels to gain the desirable degree of hydrolysis of 20, 30 and 40% following the method of Benjakul and Morrissey (1997). After 2 h of hydrolysis, the reactions were terminated by heating the mixture in boiling water for 10 min. The mixtures were centrifuged at 2000 × g at 4 °C for 10 min and the supernatants were collected and referred to as 'protein hydrolysates'. All protein hydrolysates were determined for antioxidative activities.

2.8. Determination of antioxidative activities

2.8.1. DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to the method of Khantaphant and Benjakul (2008). Sample solution (1.5 mL) was added with 1.5 mL of 0.1 mmol L⁻¹ 1,1-diphenyl-2-picrylhydrazyl (DPPH) in 950 mL L⁻¹ ethanol. The mixture was allowed to stand for 30 min in dark at room temperature. The resulting solution was measured at 517 nm. The blank was prepared in the same manner except that distilled water was used instead of the sample. The DPPH radical scavenging activity was calculated from Trolox standard curve (0–60 µmol L⁻¹) and expressed as µmol Trolox equivalents (TE)/g protein.

2.8.2. ABTS radical scavenging activity

ABTS radical scavenging activity was determined as described by Re et al. (1999) and modified slightly by Khantaphant and Benjakul (2008). ABTS radical (ABTS^{•+}) was produced by reacting ABTS stock solution (7.4 mmol L⁻¹ 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)) with 2.6 mmol L⁻¹ potassium persulfate at the ratio of 1:1 (v/v). The mixture was allowed to react in dark for 12 h at

room temperature. Prior to assay, ABTS^{•+} solution was diluted with methanol to obtain an absorbance of 1.1 (± 0.02) at 734 nm. To initiate the reaction, 150 μ L of sample was mixed with 2.85 mL of ABTS^{•+} solution. The absorbance was then read at 734 nm after 2 h dark incubation at room temperature. ABTS radical scavenging activity was expressed as μ mol TE/g protein.

2.8.3. Ferric reducing antioxidant power (FRAP)

The ability of samples to reduce ferric ion (Fe^{3+}) was evaluated by the method of Benzie and Strain (1996). FRAP reagent (a freshly prepared mixture of 10 mmol L⁻¹ TPTZ solution in 40 mmol L⁻¹ HCl, 20 mmol L⁻¹ $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution and 300 mmol L⁻¹ acetate buffer, pH 3.6 (1:1:10 v/v/v)) (2.85 mL) was incubated at 37 °C for 30 min prior to mixing with 150 μ L of sample. The reaction mixture was allowed to stand in dark for 30 min at room temperature. Absorbance at 593 nm was read and FRAP was expressed as μ mol TE/g protein.

2.8.4. Ferrous chelating activity

Chelating activity of protein hydrolysate towards ferrous ion (Fe^{2+}) was measured by the method of Benjakul et al. (2005) with a slight modification. Sample (200 μ L) was mixed with 800 μ L of distilled water. Thereafter, 0.1 mL of 2 mmol L⁻¹ FeCl_2 and 0.2 mL of 5 mmol L⁻¹ ferrozine were added. The mixture was allowed to react for 20 min at room temperature. The absorbance was then read at 562 nm. The standard curve of EDTA (0–2 mmol L⁻¹) was prepared and ferrous chelating activity was expressed as μ mol EDTA equivalents/g protein.

2.9. Determination of antioxidative activity in a lecithin liposome system

The antioxidative activity of protein hydrolysates in a lecithin liposome system was determined according to the method of Frankel et al. (1997) slightly modified by Thiansilakul et al. (2007a). Lecithin liposome system was prepared by suspending lecithin in deionized water at a concentration of 8 g L⁻¹. The mixture was stirred with a glass rod followed by sonicating for 30 min in a sonicating bath (Model S30H, Elma, Singen, Germany). Protein hydrolysate (3 mL) was added to the lecithin liposome system (15 mL) to a final concentration of 1000 mg L⁻¹ followed by sonicating the liposome suspension for 2 min. To initiate the reaction, 20 mL of 0.15 mol L⁻¹ cupric acetate were added. The mixture was shaken at 120 rpm using a shaker (Heidolph Model Unimax 1010, Schwabach, Germany) at 37 °C in dark. The control and systems containing 25 or 100 mg L⁻¹ BHT were also prepared. Oxidation in lecithin liposome systems was monitored at 6 h intervals by determining the formation of TBARS and conjugated dienes.

2.10. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were determined for protein pattern using SDS-PAGE (Laemmli, 1970) with 40 g L⁻¹ stacking gel and 100 or 200 g L⁻¹ separating gel for mince without and with hydrolysis, respectively. Samples were mixed with sample buffer (0.125 mol L⁻¹ Tris–HCl, pH 6.8 containing 40 g L⁻¹ SDS and 200 mL L⁻¹ (v/v) glycerol at a ratio of 1:1 (v/v)). Proteins (15 μ g) were loaded onto the gel. The electrophoresis was run at a constant current of 15 mA per gel by a Mini-Protean II Cell apparatus (Bio-Rad, Hercules, CA, USA). The gels were fixed and stained with 0.5 g L⁻¹ Coomassie Brilliant Blue R-250 in 150 mL L⁻¹ methanol and 50 mL L⁻¹ acetic acid and destained in 300 mL L⁻¹ methanol and 100 mL L⁻¹ acetic acid. Wide range molecular weight marker was used to estimate the molecular weight.

2.11. Determination of protein concentration

Protein concentration was measured by the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as a standard.

2.12. Statistical analysis

Experiments were run in triplicate. All data were subjected to analysis of variance (ANOVA) and differences between means were evaluated by Duncan's multiple range test (Steel & Torrie, 1980). SPSS Statistic Program (Version 10.0) (SPSS Inc, Chicago, IL, USA) was used for data analysis.

3. Results and discussion

3.1. Effect of different pretreatment on composition of mince

Mince from brownstripe red snapper with different pretreatments including wash mince (W), membrane separated mince (MS), washed/membrane separated mince (W/MS) and membrane separated/washed mince (MS/W) had the different compositions (Fig. 1). All treatments resulted in the decreases in myoglobin, phospholipid, heme iron and non-heme iron contents, compared with the control (C) ($p < 0.05$). However, washing showed no profound effect on non-heme iron content ($p > 0.05$) (Fig. 1(d)). In general, mince subjected to both washing and membrane separation (W/MS and MS/W) showed the higher decreases in myoglobin, phospholipid and heme iron, compared with those pretreated with only one process (W and MS) ($p < 0.05$). The result suggested that pretreatment by washing together with membrane separation was able to remove heme pigment as well as phospholipids from mince. Phospholipids are the major lipids in cell membranes (Borst et al., 2000).

Washing is the process used for the removal of sarcoplasmic protein and pro-oxidative aqueous components naturally present in fish muscle (Chen, Chiu, & Huang, 1997; Sannaveerappa, Sandberg, & Undeland, 2007). Myoglobin in ordinary muscle of sardine and mackerel mince was removed by 23 and 75% by washing process (Chaijan, Benjakul, Visessanguan, & Faustman, 2004). Thus, washing could lower the myoglobin and heme iron effectively. Baxter and Skonberg (2008) reported that washing not only removed sarcoplasmic proteins, but also concentrated the myofibrillar proteins. After washing, mince had the same muscle structure with the reduced content of aqueous pro-oxidants and endogenous triacylglycerols (Larsson, Almgren, & Undeland, 2007). In addition, washing could lower the phospholipid content in the resulting mince. Due to the polar head of phospholipid associated with cell membrane, it was more likely leached out during washing to some extent. After washing, mince had the decreases in myoglobin, phospholipid, heme iron and non-heme iron by 49.56, 42.55, 49.56 and 3.24%, compared with those found in the mince, respectively. When the mince was subjected to membrane separation, the lower content of phospholipid was obtained ($p < 0.05$).

In muscle tissue, phospholipid membranes are connected with cytoskeletal proteins through electrostatic attraction between the acidic phospholipids of membranes and the basic amino acid residues of the cytoskeletal proteins (Haleva, Ben-Tal, & Diamant, 2004). When mince was subjected to membrane separation using the solution containing Ca^{2+} ion and citric acid, the interaction between Ca^{2+} with the polar head of phospholipids as well as the competition between citric acid and the acidic phospholipids of membranes bound to the basic amino acid residues of cytoskeletal proteins more likely took place. As a result, the release of phospholipid membrane from attached cytoskeletal proteins occurred. Liang and Hultin (2005a) reported that polycarboxylic groups of citric acid might

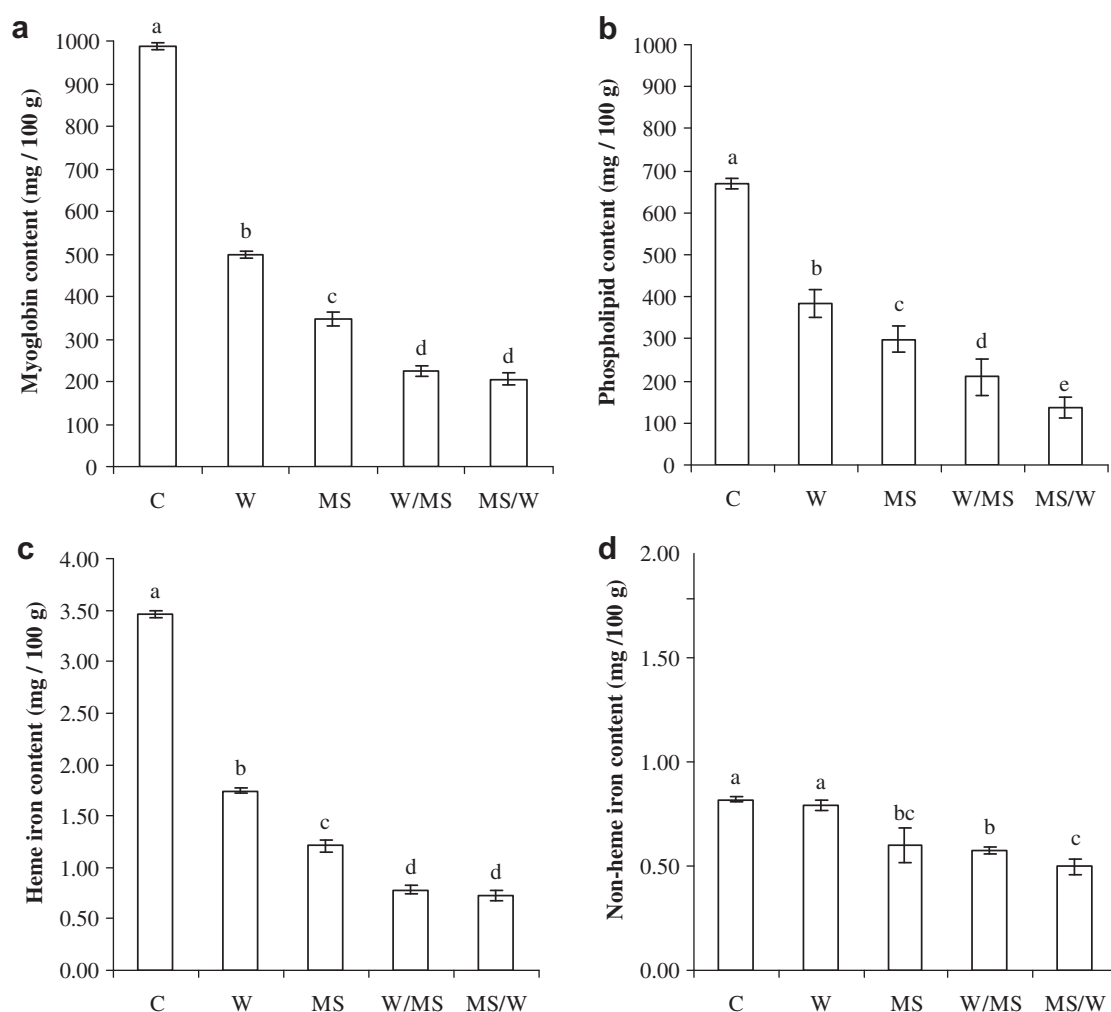


Fig. 1. Myoglobin (a), phospholipid (b), heme iron (c) and non-heme iron (d) contents of brownstripe red snapper mince with different pretreatments; W: washed mince, MS: membrane separated mince, W/MS: washed/membrane separated mince and MS/W: membrane separated/washed mince. Bars represent the standard deviation from triplicate determinations. Different letters indicate significant differences ($p < 0.05$).

compete with acidic phospholipids to bind with the basic amino acid residues of the cytoskeletal proteins, while Ca^{2+} induced the formation of calcium–phospholipid complex. Apart from the removal of phospholipids, the membrane separation process used also resulted in the lowering of myoglobin, heme iron and non-heme iron. Due to the polar nature of the medium used, myoglobin as well as iron could be soluble in the medium. Furthermore, [Chen et al. \(1997\)](#) reported that washing with cold water for 15 min eliminated 34% oil, compared with unwashed mince and 83% oil elimination was obtained when washing time of 135 min was used.

After membrane separation process, myoglobin, phospholipid, heme and non-heme irons were removed by 65.04, 55.33, 65.04 and 26.97%, compared to those found in mince. When both washing and membrane separation processes were applied, all components tested decreased to a higher extent, compared to those found in mince with only one pretreatment or mince without pretreatment. Therefore, both processes exhibited the synergistic effect on removal of pro-oxidant and phospholipid membrane. It was noted that the sample with membrane separation, followed by washing had the lower phospholipid and non-heme iron contents than those with prior washing and membrane separation ($p < 0.05$). However, no differences in myoglobin and heme iron contents were noticeable between both samples ($p > 0.05$). Therefore, membrane separation/washing process was the appropriate pretreatment as

evidenced by the lowest contents of pro-oxidants and phospholipids in prepared mince.

3.2. Lipid oxidation in mince with different pretreatments

The remaining pro-oxidants and phospholipid membrane in mince might accelerate the oxidative reaction during handling or storage. During 9 days of storage at 4 °C, TBARS of mince with different pretreatments in the presence and absence of Cu^{2+} are depicted in [Fig. 2](#). In the absence of Cu^{2+} , TBARS in all pretreated samples increased as the storage time increased up to 6 days ($p < 0.05$). Nevertheless, TBARS of the control sample had the continuous increase in TBARS throughout the storage time of 9 days ($p < 0.05$). At day 9, TBARS of the control was almost 3-fold higher than that of pretreated mince. At the same storage time, the control showed the higher TBARS, compared with other samples with pretreatment ($p < 0.05$). The pronounced formation of TBARS in mince indicated the susceptibility of mince, which might be associated with the high content of phospholipid membrane with high PUFA as well as pro-oxidants. Among different pretreated mince, MS/W showed the lowest TBARS value, compared with other samples during 3–9 days of storage ($p < 0.05$). The lowest TBARS of MS/W was in agreement with the lowest remaining phospholipid and other pro-oxidative components ([Fig. 1](#)).

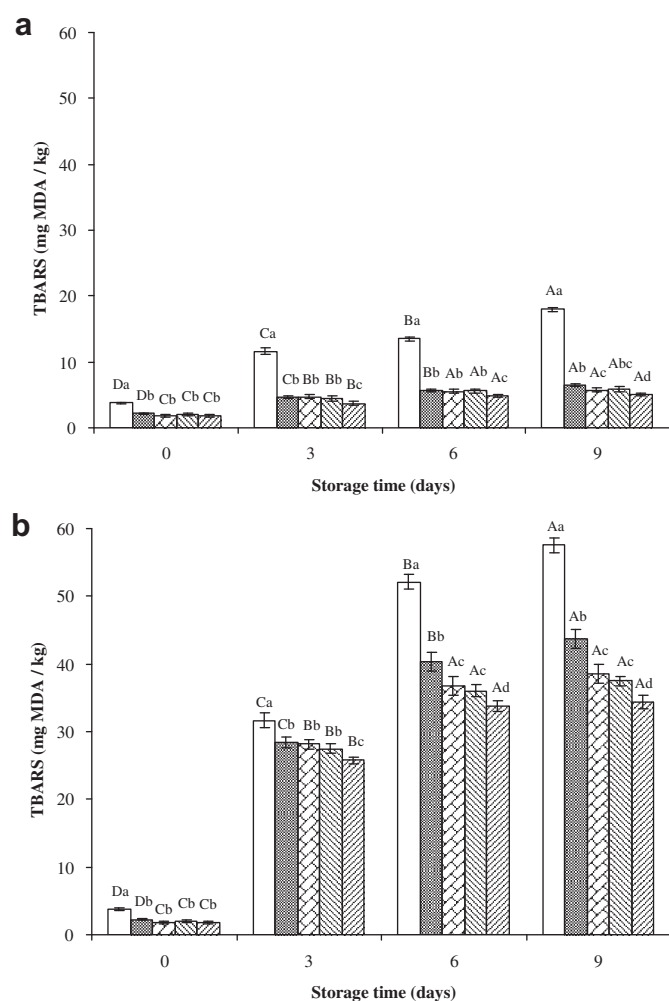


Fig. 2. TBARS value of brownstripe red snapper mince with different pretreatments in the absence (a) and presence (b) of 0.15 mol L^{-1} cupric acetate, during storage at 4°C for 9 days. C: control mince ($\square$), W: washed mince ($\▤$), MS: membrane separated mince ($\▨$), W/MS: washed/membrane separated mince ($\▩$) and MS/W: membrane separated/washed mince ($\▧$). Bars represent the standard deviation from triplicate determinations. Different capital letters within the same pretreatment indicate significant differences ($p < 0.05$) and different letters within the same day of storage indicate significant differences ($p < 0.05$).

In the presence of Cu^{2+} , all samples showed the higher TBARS, in comparison with those without Cu^{2+} . Cu^{2+} has been used as the catalyst for lipid oxidation in different systems (Decker, Crum, & Calvert, 1992; Thanonkaew et al., 2006). The oxidation rates of all samples added with Cu^{2+} were enhanced as shown by higher TBARS. Thanonkaew et al. (2006) studied the effects of metal ions (Fe^{2+} and Cu^{2+}) addition to cuttlefish paste and found that the metal ions could accelerate lipid oxidation. Moreover, the remaining pro-oxidants in mince might accelerate the oxidative deterioration of lipids, particularly phospholipids with high PUFA content. The similar results were obtained, compared to those without Cu^{2+} , in which MS/W containing the lowest phospholipids had the lowest TBARS throughout the storage time, compared with other samples ($p < 0.05$).

3.3. Hydrolysis of mince using protease from pyloric caeca of brownstripe red snapper

MS/W, PI and mince were used to produce protein hydrolysates using protease from pyloric caeca of brownstripe red snapper. It

was noted that mince contained myosin heavy chain (MHC) and actin as the major proteins. For pretreated sample (MS/W) and PI, proteins with molecular weight of 26.9, 32.5 and 43.2 kDa disappeared. During washing or membrane separation, some sarco-plasmic proteins and some cytoskeletal proteins interacted with phospholipid membrane were removed. Similar protein patterns were observed between MS/W and PI. For all samples, the typical initial rapid hydrolysis rate was observed within the first 30 min as evidenced by the sharp increase in DH, followed by a slower hydrolysis rate (Fig. 3). Firstly, a large number of peptide bonds were hydrolyzed (Shahidi, Xiao-Qing, & Synowiecki, 1995). Thereafter, proteases might have the lower activity towards the proteins or peptides with the shorter chains. At the same hydrolysis time, the higher DH was obtained in hydrolysate prepared with the

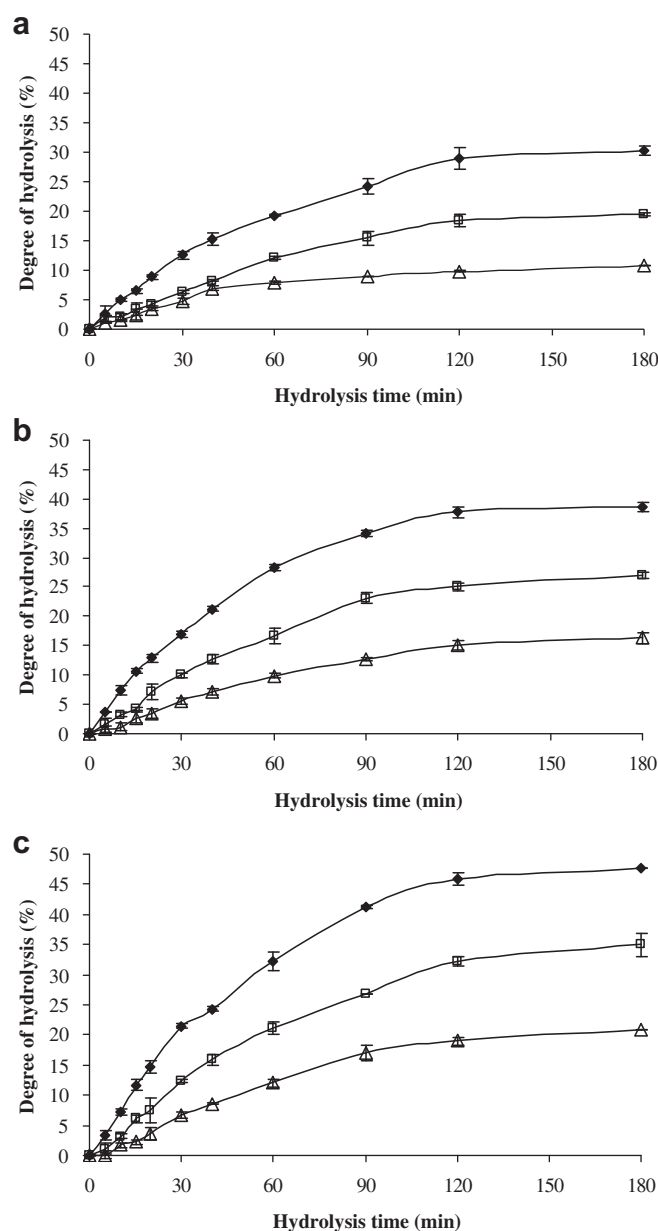


Fig. 3. Changes in degree of hydrolysis (DH) of control mince (a), membrane separated/washed mince (MS/W) (b) and protein isolate (PI) (c), using protease from pyloric caeca of brownstripe red snapper at various levels: ($\triangle$): 1 unit/g, ($\square$): 2 units/g and ($\blacklozenge$): 4 units/g. The reactions were performed at 60°C and pH 8.5. Bars represent the standard deviation from triplicate determinations.

higher activity of PCE ($p < 0.05$). The result suggested that higher amount of proteases used, the more peptide bonds were cleaved. At the same protease level used, PI was found to be the most susceptible to cleavage as indicated by the highest DH ($p < 0.05$).

Elimination of other proteins as well as lipids, which functioned as the barrier for hydrolysis of myofibrillar proteins by proteases, also enhanced the hydrolysis induced by proteases. Moreover, membrane separation process might cause the structural alternation of proteins by interruption of electrostatic linkage between proteins by Ca^{2+} and citric acid. Furthermore, the changes in muscle protein configuration of pretreated mince were also supported by the decreases in Mg^{2+} -ATPase activity. A decrease in Mg^{2+} -ATPase activity by 13.75%, compared with that found in mince (data not shown), was found in natural actomyosin extracted from MS/W, indicating the disassociation of actomyosin molecule. Mg^{2+} -ATPase activity is used as an indicator of conformational changes of actomyosin complex (Benjakul et al., 1997). The decrease in Mg^{2+} -ATPase activity confirmed the dissociation of actin from myosin (Torikai & Konno, 1996). Therefore, the dissociation of actomyosin might lead to the increased susceptibility of actin and myosin to be hydrolyzed by proteases used for hydrolysis. The loosen protein structures were more prone to be cleaved by proteases. When PI was used as substrate for hydrolysis, the greater hydrolysis took place. During alkaline solubilisation, the repulsion between protein molecules resulted in the dissociation of actomyosin complex. This was indicated by the marked decrease in Mg^{2+} -ATPase activity by 36.27%, in comparison with mince (data not shown). As a consequence, the individual or looser complex could be cleaved with ease as evidenced by the higher DH. Thus, the configuration of protein was another important factor governing the rate of hydrolysis.

3.4. Antioxidative activities of protein hydrolysate prepared from mince with different pretreatments

3.4.1. DPPH radical scavenging activity

DPPH radical scavenging activities of H-C, H-MS/W and H-PI with different DHs are shown in Fig. 4a. As DH increased from 20 to 30%, H-MS/W showed the increase in DPPH radical scavenging activity but no differences were found between the samples with 30 and 40% DH ($p > 0.05$). No differences in DPPH radical scavenging activity were observed for H-C and H-PI at all DHs ($p > 0.05$). DPPH radical scavenging activity indicated the hydrogen-donating ability of antioxidant (Khantaphant & Benjakul, 2008). The result suggested that H-MS/W with 30 and 40% DH could act as hydrogen donors more effectively than other hydrolysates. Protein hydrolysates from mackerel (*Scomber austriasicus*) (Wu, Chen, & Shiau, 2003) and round scad (*Decapterus maruadsi*) (Thiansilakul et al., 2007a) prepared using enzymatic hydrolysis were reported to possess DPPH radical scavenging activity.

3.4.2. ABTS radical scavenging activity

H-C, H-MS/W and H-PI showed the increase in ABTS radical scavenging activities with increasing DH ($p < 0.05$) (Fig. 4(b)). The highest activity was observed in all hydrolysates with 40% DH ($p < 0.05$). However, no difference in activity was found between H-PI with 20 and 30% DH ($p > 0.05$). Among the hydrolysates, H-C showed the lowest ABTS radical scavenging activity, whereas hydrolysate from MS/W exhibited the highest activity for all DH tested ($p < 0.05$). At 40% DH, H-MS/W had the highest ABTS radical scavenging activity, compared with other hydrolysates ($p < 0.05$). ABTS radical assay determine the antioxidative activity, in which the radical is quenched to form ABTS-radical complex (Khantaphant & Benjakul, 2008). Protein hydrolysates derived from loach (*Misgurnus anguillicaudatus*) (You, Zhao, Cui, Zhao, & Yang, 2009), yellow stripe trevally (*Selaroides leptolepis*) (Klompok, Benjakul,

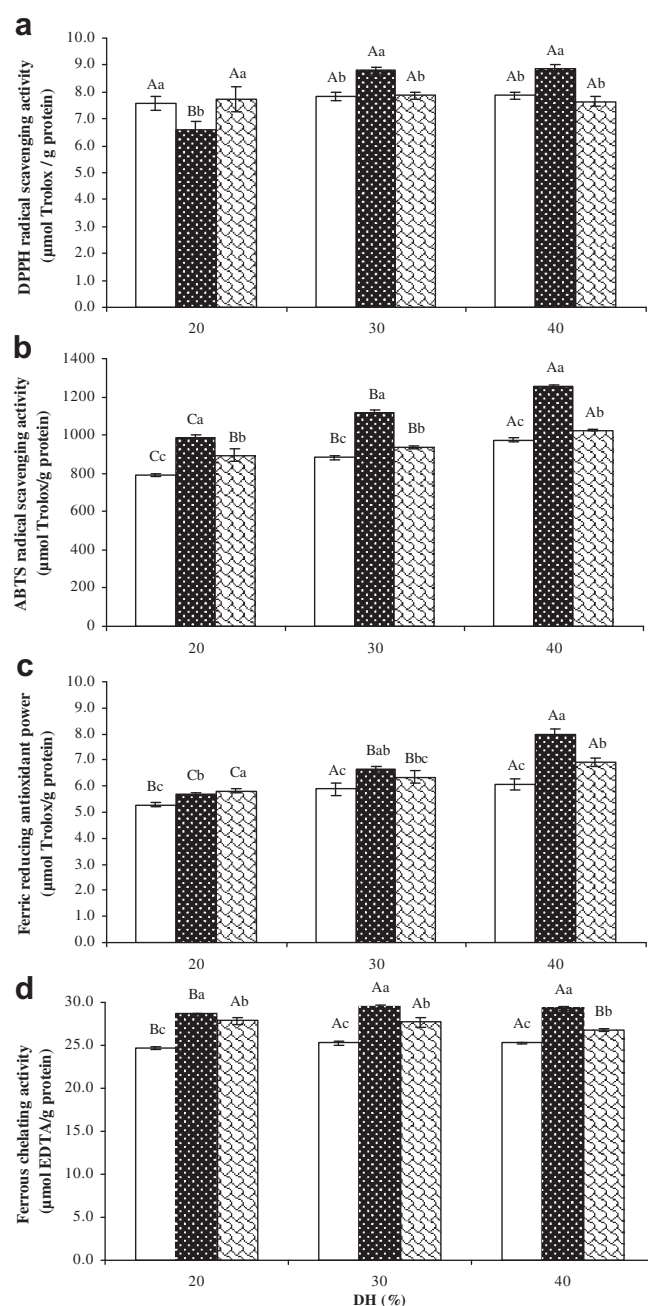


Fig. 4. DPPH radical scavenging activity (a), ABTS radical scavenging activity (b), ferric reducing antioxidant power (c) and metal chelating activity (d) of hydrolysates prepared from control mince (□), MS/W: membrane separated/washed mince (▨) and PI: protein isolate (▩) using protease from pyloric caeca with various degrees of hydrolysis (DHs). Bars represent the standard deviation from triplicate determinations. Different capital letters within the same pretreatment indicate significant differences ($p < 0.05$) and different letters within the same DH indicate significant differences ($p < 0.05$).

Kantachote, Hayes, & Shahidi, 2008) and alkali-solubilized tilapia (*Oreochromis niloticus*) (Raghavan, Kristinsson, & Leeuwenburgh, 2008) have been reported to have ABTS radical scavenging activities.

3.4.3. Ferric reducing antioxidant power (FRAP)

FRAP of different hydrolysates with different DH (20, 30 and 40%) is depicted in Fig. 4(c). All hydrolysates had the increases in FRAP when DH increased ($p < 0.05$) except for H-C, in which no difference was observed in samples with 30 and 40% DH ($p > 0.05$).

FRAP of H-MS/W and H-PI was generally higher than that of H-C at all DHs used ($p < 0.05$). At the same DH, H-MS/W showed the highest activity, in comparison with others ($p < 0.05$). FRAP generally measures the reducing ability against ferric ion (Fe^{3+}). This ability indicates the ability of hydrolysates to donate the electron to the free radical (Khantaphant & Benjakul, 2008). Therefore, it could be concluded that H-MS/W, especially at high DH, had the reducing power, leading to the prevention and retardation of propagation of lipid oxidation. Hydrolysates from yellow stripe trevally (Klompong et al., 2008) and alkali-solubilized tilapia (Raghavan et al., 2008) have been reported to possess FRAP.

3.4.4. Ferrous chelating activity

Ferrous chelating activities of hydrolysates prepared from mince, MS/W and PI with different DHs are shown in Fig. 4(d). Chelating activity against Fe^{2+} of H-C and H-MS/W slightly increased when DH increased up to 30% ($p < 0.05$). On the other hand, slight decrease in ferrous chelating activities was found in H-PI as DH was greater than 30% ($p < 0.05$). Among all hydrolysates, H-MS/W showed the highest chelating activity for all DH ($p < 0.05$). Ferrous ion (Fe^{2+}) is the most powerful pro-oxidant among metal ions. This ion can interact with hydrogen peroxide in a Fenton reaction to produce the reactive oxygen species and hydroxyl free radical (OH), leading to the initiation and/or acceleration of lipid oxidation (Stohs & Bagchi, 1995). Therefore, chelation of metal ions by peptides in hydrolysates would retard the oxidative reaction (Klompong et al., 2008). The result indicated that H-MS/W was able to chelate pro-oxidative metals, resulting in the lowered oxidation. Furthermore, the higher chelating activity of H-MS/W was coincidental with the higher DPPH and ABTS radical scavenging activity and FRAP. Ferrous chelating activity has been reported for hydrolysate of silver carp (*Hypophthalmichthys molitrix*) (Dong et al., 2008), round scad (Thiansilakul et al., 2007a) and yellow stripe trevally (Klompong et al., 2008). Therefore, all hydrolysates could function as both primary and secondary antioxidants via scavenging free radical and chelating the metal ions, respectively. It could be observed that when mince was subjected to different pretreatments, the peptides generated showed varying activity, though the same DH was used. Therefore, different antioxidative peptides were produced from the samples with different pretreatments. Pretreatment might affect the structure of proteins, in which hydrolysis could take place at

different positions in peptide chains. Thiansilakul et al. (2007a) and Klompong et al. (2008) reported that the differences in antioxidative activities among hydrolysates might result from the differences in the structure and size of the peptides of the resulting hydrolysate. Since the hydrolysis was performed using the same protease, the different antioxidative activities of resulting hydrolysate was governed by pretreatments. DH also greatly influenced the peptide chain length as well as the exposure of terminal amino groups of products obtained (Thiansilakul, Benjakul, & Shahidi, 2007b). The higher DH, the more cleavage of peptide chains took place. Peptides with various sizes and compositions had various capacities of scavenging or quenching free radicals (Klompong et al., 2007).

3.5. Protein pattern of protein hydrolysate prepared from mince with different pretreatments

Protein patterns of hydrolysates from mince without and with pretreatment having 40% DH are shown in Fig. 5(b). For mince with different pretreatments, myosin heavy chain (MHC) and actin were the major components. Slight decrease in MHC band intensity was noticeable in MS/W and PI, probably due to slight degradation of MHC during the process. After hydrolysis, no MHC and actin were detectable. H-C showed two major protein bands with molecular weight (MW) of 5.8 and 10.4 kDa. H-MS/W and H-PI showed smear bands with MW lower than 6.5 kDa. However, the smear band of H-MS/W was found with MW range of 5.1–6.5 kDa and protein bands with MW of 4.6–10.4 kDa were found in H-PI. The result indicated that the types and sizes of peptides in hydrolysate from mince with different pretreatments varied. In general, the hydrolysates prepared at 40% DH showed the highest antioxidative activities. Therefore, hydrolysates with 40% DH were selected to study for their ability in inhibition of lipid oxidation in lecithin-liposome oxidation model system, in comparison with commercial antioxidants.

3.6. Antioxidative activity of hydrolysate from mince with different pretreatments in a lecithin liposome system

Protein hydrolysates prepared from mince with different pretreatments affected the oxidation of the lecithin liposome system differently as indicated by different conjugated dienes and TBARS values (Fig. 6 (a) and (b)), respectively. No changes in conjugated

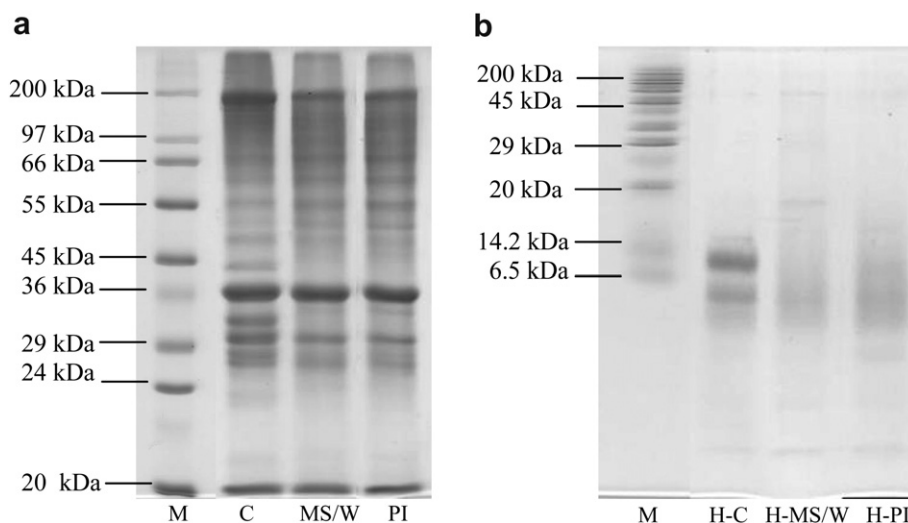


Fig. 5. Protein pattern of brownstripe red snapper mince with different pretreatments before (a) and after (b) hydrolysis with protease from pyloric caeca to obtain 40% DH. M: molecular weight marker; C: control mince; MS/W: membrane separated/washed mince; PI: protein isolate; H-C: hydrolysate from C; H-MS/W: hydrolysate from MS/W and H-PI: hydrolysate from PI.

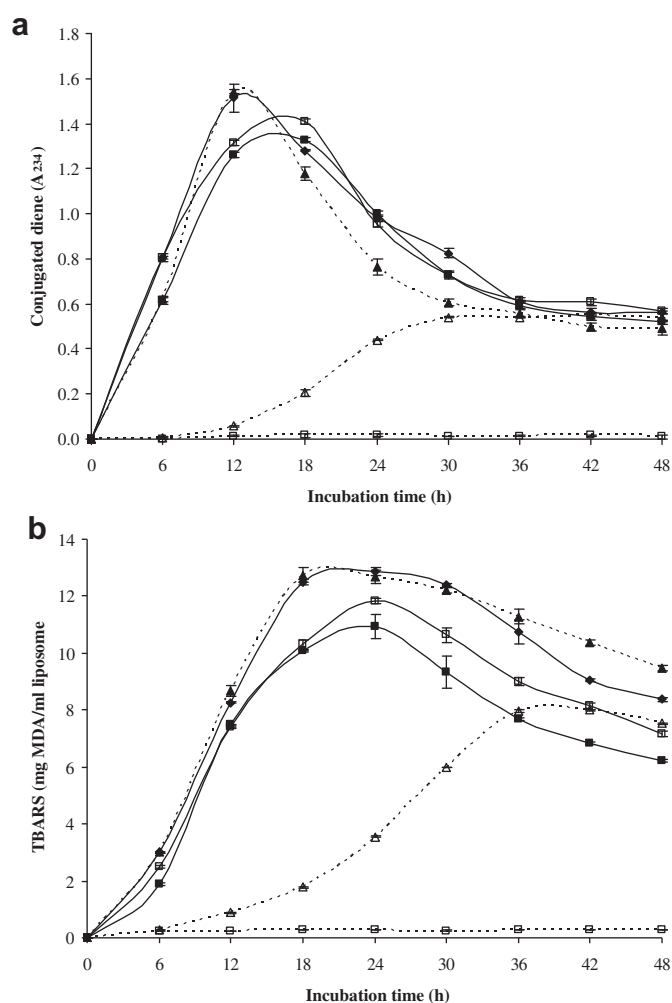


Fig. 6. The formation of conjugated diene (a) and thiobarbituric acid reactive substances (TBARS) (b) in lecithin-liposome model system in the presence of different hydrolysates from brownstripe red snapper mince with different pretreatments having 40% DH. (---▲---): control, (---△---): 25 mg L⁻¹ BHT, (---□---): 100 mg L⁻¹ BHT, (---◆---): 1000 mg L⁻¹ H-C, (---■---): 1000 mg L⁻¹ H-MS/W and (---□---): 1000 mg L⁻¹ H-PI. Bars represent the standard deviation from triplicate determinations.

dienes of liposome system added with 100 mg L⁻¹ BHT were observed throughout 48 h of incubation ($p > 0.05$). For the system added with 25 mg L⁻¹ BHT, the increase in conjugated dienes was found after 12 h of incubation. An increase in conjugated dienes followed by a sharp decrease was observed from the control system and all systems containing all hydrolysates at a level of 1000 mg L⁻¹ but the rate of changes varied with the hydrolysate added (Fig. 6 (a)). Within the first 12 h of incubation, liposome system added with H-C and the control system showed the higher conjugated diene than those added with H-MS/W, H-PI and BHT ($p < 0.05$). The results indicated that H-MS/W and H-PI in liposome system could retard the formation of conjugated diene. The higher conjugated diene in the system added with H-C might be due to the remaining pro-oxidants in minces, which could induce the oxidation within the first period of incubation. However, after 12 h, the sharp decreases in conjugated diene were observed from the system added with H-C and the control. Conjugated diene is generated during the lipid oxidative reaction and hydroperoxides are expected to decompose to the secondary products which can be determined by TBARS value (Frankel et al., 1997).

Changes in TBARS of liposome system during incubation of 48 h are shown in Fig. 6 (b). The increase in TBARS indicated the

formation of secondary lipid oxidation products. The sharp increases in TBARS were observed in the first 18 h of incubation ($p < 0.05$), when H-C was added to liposome system. The systems added with H-MS/W and H-PI showed the increase in TBARS within the first 24 h ($p < 0.05$). Thereafter, the gradual decreases in TBARS were observed ($p < 0.05$). However, the value of TBARS obtained from the former system was lower than that of the latter ($p < 0.05$). A gradual decrease in TBARS was found during 18–48 h for the control and the system added with H-C and during 24–48 h for the systems added with H-MS/W and H-PI (Fig. 6 (b)). The result suggested the greater ability of H-MS/W and H-PI in retardation of the formation of secondary oxidation products determined by TBARS. The first formation of TBARS indicated that the primary products were decomposed to the secondary products, especially aldehydes. The decrease in TBARS when the storage time increased was probably due to the loss in those volatile secondary products. In general, the increases in conjugated diene and TBARS values of the liposome systems containing H-MS/W and H-PI were lower than that of the control and the system containing H-C. Thus, H-MS/W and H-PI could retard the early stages of lipid oxidation as well as the advanced stage of oxidation via their radical scavenging activity, reducing power and metal chelating activity. Liposomes are appropriate lipid models to evaluate antioxidants for both food and lipoprotein particles containing phospholipids (Frankel et al., 1997). Polar portions of peptides in hydrolysate might interact with the liposomes of phospholipids, where they functioned effectively as antioxidant at the interface.

4. Conclusion

Pretreatment of mince played a role in lowering pro-oxidants and phospholipid membrane in mince. Cleavage of protein was affected by pretreatment and antioxidative activity of protein hydrolysate was also governed by pretreatment. Fish mince subjected to membrane separation, followed by washing was appropriate for production of hydrolysate possessing antioxidative activity with the lesser amount of pro-oxidants and phospholipid membrane. The hydrolysate obtained can be further used to retard lipid oxidation in food system with the negligible pro-oxidative activity.

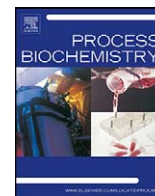
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Extraction of protease from *Calotropis procera* latex by polyethylene glycol–salts biphasic system

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ABSTRACT

The protease from the latex of *Calotropis procera* was isolated by an aqueous two-phase system (ATPS). The systems consist of polyethylene glycol (PEG 4000, 6000 and 8000) at concentrations of 9, 12 and 15% (w/w) with salts (Na-citrate, MgSO₄, K₂HPO₄, and (NH₄)₂SO₄) at concentrations of 11, 14 and 17% (w/w) were investigated. The highest protease recovery was found in the PEG-rich phase of the system, comprising of 12% PEG 4000–17% MgSO₄. For optimization of the system to obtain the higher yield of protease, the system pH (4, 7 and 10) or NaCl addition (2, 4 and 6%, w/w) was studied. At acidic (pH 4.0) and alkaline (9.0) conditions of the systems the reduction of K_E and protease recovery was clearly observed compared to that of the neutral pH (7.0). The addition of NaCl up to a final concentration of 6% (w/w) significantly increased the yield to 107% of the control. Molecular weight distribution and activity staining showed that the isolated protease had the molecular weight of ~38 kDa. However, the isolated protease had no activity under reducing condition (β ME). Under cathodic electrophoresis, protease from *C. procera* showed the same protein pattern to purified papain.

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1. Introduction

Proteases from plant sources have received special attention from the pharmaceutical industry and by food biotechnology because of their properties and functions [1,2]. Among them, those from plant latex were introduced for meat tenderization for several years [3,4]. The presence of proteolytic enzymes in the latex of plants is believed to be a defense mechanism function. *Calotropis procera* is a plant found in tropical and sub-tropical regions. It is well known for its great capacity of producing latex which exudates from the green damaged parts. Scientific reports have mentioned various medicinal activities of *C. procera* latex, such as insecticidal [5], anti-fungal [6] and wound healing [7]. Some biochemical properties of the enzyme containing in the latex of *C. procera* have been documented and named as procerain [8]. It has been reported that *C. procera* latex is a potential material for enzyme purification [9]. However, the enzyme extraction involved several steps such as precipitation and chromatography. Nitsawang et al. [10] purified papain from *Carica papaya* latex by using two-step salt precipitation. Ammonium sulfate precipitation and CM-Sepharose column were used for procerain purification from the latex of *C. procera* [8]. These

processes are time and cost consuming, inducing loss of enzyme activity.

In this respect, partitioning in an aqueous two-phase system (ATPS) has shown to be powerful for separating and purifying the mixtures of proteins [11]. It could be used to combine several features of the early processing steps in only one or two partitioning operations. It offers many advantages: a biocompatible environment, low interfacial tension, low energy, ease of scale-up, and continuous operations [12]. Coincidentally, ATPS can remove undesirable proteins, unidentified polysaccharides and pigments that are present in the system [8]. Together with the removal of insoluble and major classes of contaminants this technique has been developed as a primary purification step in the overall recovery [11]. The basis for separation by ATPS is the selective distribution of biomolecules between the phases quantified by the partition coefficient. It is important to maximize the differences in partition coefficient between target protein and impurities. The coefficient value depends on various parameters from biomolecule (size, concentration and surface property), phase compositions (type, concentration and molecular weight of polymers or salts), pH and temperature of the system [12]. The pH value and the presence of electrolytes in the system have a pronounced effect on the partitioning of proteins between the two phases [13]. The phase behavior is also influenced by the presence of salts, the effect depending on both their type and concentration [14]. A favorable interaction between polyethylene glycol (PEG) and pro-

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tein molecule decreased when the molecular weight (MW) of PEG increased due to its exclusion from the protein molecule [15,16]. ATPS has been applied for recovery of various proteases including, bromelain [17], papain [10], trypsin [15] and pepsin [16]. Nonetheless, extraction of the protease from *C. procera* latex by ATPS had not been reported.

Hence, in view of this potential application and scale-up, it would be interesting to look into extraction of proteolytic enzyme from *C. procera* latex by using ATPS. The present paper will also report the optimization of the ATPS and the verification of the extracted protein by gel electrophoresis.

2. Materials and methods

2.1. Chemicals and raw materials

Polyethylene glycol (PEG), sodium dodecyl sulfate (SDS), bovine serum albumin (BSA), casein and L-cysteine were obtained from Fluka (Buchs, Switzerland). Beta-mercaptoethanol (β ME) and Coomassie Brilliant Blue G-250 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ammonium sulfate $((\text{NH}_4)_2\text{SO}_4)$, magnesium sulfate (MgSO_4), potassium phosphate (K_2HPO_4), trichloroacetic acid (TCA) and other chemicals with analytical grade were obtained from Merck (Darmstadt, Germany). Latex of *C. procera* was collected from Nayong, Trang Province, Thailand.

2.2. Latex preparation

Latex was collected in a clean tube by breaking the *C. procera* stems. The obtained latex was diluted with distilled water (1:1, v/v). It was mixed well and then centrifuged at $15,000 \times g$ at 4°C for 10 min. The supernatant was filtered through a Whatman paper no. 1. This sample was referred to as the “crude enzyme extract” and was used for further study.

2.3. Caseinolytic activity assay

An enzyme sample of 0.10 ml was mixed with 1.10 ml of 1% (w/v) casein in 0.10 M Tris–HCl, pH 8.0 containing 12 mM cysteine. The reaction was started by incubating the mixture at 37°C for 20 min. The reaction was stopped by adding 1.8 ml of 5% TCA. After centrifugation at $3000 \times g$ for 15 min, the absorption of the soluble peptide in supernatant was measured at 280 nm. One of caseinolytic activity unit is defined as the amount of enzyme needed to produce an increment of 0.01 absorbance unit per minute at the assayed condition [18].

2.4. Protein determination

The protein concentration in the sample was measured by the Bradford method [19] using BSA as a protein standard.

2.5. Aqueous two-phase partitioning

The ATPS was prepared in 50-ml centrifuge tubes according to the method in Klomklao et al. [15] with a modification. Various amounts and molecular weights of PEG (PEG 4000, 6000 and 8000) as well as salts (Na-citrate, MgSO_4 , K_2HPO_4 , and $(\text{NH}_4)_2\text{SO}_4$) were added to the 30% (w/w) of crude extract from *C. procera* to generate the biphasic system.

2.5.1. Effect of PEG on the partitioning of protease from *C. procera* latex

To study the effect of PEG on the protease partitioning from *C. procera* latex using ATPS, different concentrations (9, 12 and 15%, w/w) and MW (4000, 6000 and 8000) of PEG were mixed with 14% w/w MgSO_4 in an aqueous system. The distilled water was used to adjust the system obtaining the total weight of 15 g. The mixtures were gently mixed for 15 min before pH measurement. Phase separation was achieved by centrifuging the mixture at $7000 \times g$ at 4°C for 30 min. The top and bottom phases were carefully separated by using Pasteur pipette. Aliquot from each phase was taken for enzyme activity assay and protein determination. The phase composition giving the highest proteolytic yield was chosen for further study.

2.5.2. Effect of salts on the partitioning of protease from *C. procera* latex

To study the effect of salts on the protease partitioning from *C. procera* latex using ATPS, different salts (Na-citrate, MgSO_4 , K_2HPO_4 , and $(\text{NH}_4)_2\text{SO}_4$) with different concentrations (11, 14 and 17%, w/w) were mixed with the constant 12% PEG 4000. Two-phase separation was performed as previously described.

2.5.3. Effect of pH on the partitioning of protease from *C. procera* latex

Based on the protease recovery, the ATPS containing 12% PEG 4000–17% MgSO_4 and 30% (w/w) crude latex was chosen for optimization of the protease partitioning. The original pH of the system was measured and then adjusted to 4.0, 7.0 and 9.0 with 1 M HCl or 1 M NaOH.

2.5.4. Effect of NaCl on the partitioning of protease from *C. procera* latex

The selected system of 12% PEG 4000–17% MgSO_4 and 30% (w/w) crude latex was also chosen for study the effect of NaCl on protease 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). Complete phase separation was achieved by centrifugation after the whole system was filled up to 15 g.

2.5.5. ATPS extraction parameters

ATPS parameters were calculated as follows:

The volume ratio (V_R) was defined as:

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

where V_T and V_B are top and the bottom phase volume, respectively.

The partition coefficient of protein (K_P) was defined as:

$$K_P = \frac{P_T}{P_B}$$

where P_T and P_B are concentrations of protein in the top and the bottom phase, respectively.

The partition coefficient of enzyme (K_E) was defined as:

$$K_E = \frac{E_T}{E_B}$$

where E_T and E_B are concentrations of enzyme in the top and the bottom phase, respectively.

The specific activity (SA) of extracted protease in each phase of the ATPS was defined as:

$$SA = \frac{\text{Total protease activity}}{\text{Total protein content}}; \text{ unit/mg protein}$$

The purification factor (PF) was defined as:

$$PF = \frac{SA_E}{SA_I}$$

where SA_E is the SA of each phase and SA_I is the SA of initial phase (crude latex before partitioning).

The protease activity recovery (Yield) was defined as:

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

where A_T is total protease activity in top phase and A_I is the initial protease activity (crude latex before partitioning).

From the protease recovery, the protease obtained from the ATPS fraction that rendered the maximal yield was chosen for further study.

2.6. Electrophoresis

2.6.1. Cathodic electrophoresis

The experiment was carried out according to the method of Nitsawang et al. [10] using a polyacrylamide gel consisting of 15% (w/v) separating gel (pH 4.3) and 7.5% (w/v) stacking gel (pH 6.7). The electrode buffer consisted of 0.36 M beta-alanine–0.14 M acetic acid, pH 4.3. The protein sample was diluted (1:1, v/v) with stacking buffer containing 10% sucrose and 0.002% basic fuchsin (used as a tracking dye) prior to loading onto the gel. Electrophoresis was run at a constant current of 40 mA/gel using a Mini Protean Tetra Cell unit (Bio-Rad Laboratories, Richmond, CA, USA). The protein samples migrated toward the cathode during electrophoresis. After electrophoresis, the gel was stained overnight with staining solution (0.02%, w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with a solution of methanol–acetic acid.

2.6.2. SDS-PAGE

SDS-PAGE of *C. procera* crude extract and the protease fractions extracted from ATPS fractions were performed according to the method of Laemmli [20]. Protein solutions were mixed at a 1:1 (v/v) ratio with the sample buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol) with or without β ME. The samples (10 and 2 μ g protein for protein and activity staining, respectively) were loaded onto the gel made of 4% stacking and 15% separating gels. They were subjected to an electrophoresis set at a constant current of 20 mA/gel. After electrophoresis, the gel was stained overnight with staining solutions (0.02%, w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol, and 7.5% (v/v) acetic acid and destained as described above.

2.6.3. Protease activity staining

The protease separated on the gel was verified by using activity staining as the method of Garcia-Carreño et al. [21]. The gel was immersed in 50 ml of 2% (w/v) casein in 50 mM Tris–HCl buffer, pH 8.0 containing 12 mM cysteine with constant agitation at 4°C for 30 min. The reaction was generated by incubating the gel at 37°C for 15 min. The treated gel was then stained and destained as described above.

Table 1Effect of phase composition in PEG–14% MgSO₄ on partitioning of protease from *Calotropis procera* latex.

Phase composition (% w/w)	V _R	K _P	K _E	SA	PF	Yield (%)
9% PEG 4000–14% MgSO ₄	ns	ns	ns	ns	ns	ns
12% PEG 4000–14% MgSO ₄	1.73	0.02	0.07	883.6 ± 32	4.92 ± 0.74	3.88 ± 0.39
15% PEG 4000–14% MgSO ₄	0.89	0.01	0.001	51.9 ± 33	0.29 ± 0.18	0.14 ± 0.03
9% PEG 6000–14% MgSO ₄	1.16	0.03	0.04	257.9 ± 41	1.44 ± 0.23	1.69 ± 0.21
12% PEG 6000–14% MgSO ₄	0.71	0.01	0.01	119.1 ± 7	0.66 ± 0.32	0.31 ± 0.18
15% PEG 6000–14% MgSO ₄	1.79	0.02	0.001	50.1 ± 17	0.28 ± 0.09	0.45 ± 0.10
9% PEG 8000–14% MgSO ₄	1.13	0.03	0.02	180.5 ± 35	1.01 ± 0.19	1.01 ± 0.15
12% PEG 8000–14% MgSO ₄	0.59	0.03	0.01	27.2 ± 8	0.15 ± 0.05	0.16 ± 0.07
15% PEG 8000–14% MgSO ₄	1.25	0.03	0.01	40.1 ± 5	0.22 ± 0.03	0.60 ± 0.10

V_R: volume ratio (top/bottom); K_P: partition coefficient of protein in the top phase; K_E: partition coefficient of enzyme in the top phase; SA: specific activity (unit/mg protein); PF: purification factor; Yield: activity recovery; ns: no phase separation.

The development of a clear zone on the dark background indicated the caseinolytic activity of protease from *C. procera* latex.

3. Results and discussion

3.1. Effect of PEG on the partitioning of protease from *C. procera* latex

Influences of PEG molecular weight (4000–8000) and concentration (9–15%, w/w) on differential partitioning of *C. procera* protease activity (K_E) and total protein (K_P) were studied. As shown in Table 1, partitioning of protease from *C. procera* latex was strongly dependent on the MW and the concentration of PEG. No phase separation was observed in the system of 14% MgSO₄ with 9% PEG 4000. In general, either PEG 4000 or salts alone, no phase separation was found. This indicates that the combination of both PEG and salt was necessary for partitioning process. Phase separation will occur at the concentration of polymer or salt were above a certain critical value of binodal curve. Raghavarao et al. [12] reported that two phases are formed when the polymer concentration is in the range of 8–16% (w/w) and salt concentration must be as high as 10% (w/w). In addition, the lower MW, the higher the concentration of the polymer is required for phase formation. The result showed that the V_R of the system ranged from 0.59 to 1.79 (Table 1). The distribution of the protein and protease in ATPS are reported by K_P and K_E, respectively. 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. The K_E decreased with an increase in PEG concentration and MW (Table 1). This was a result from reduction of protease partitioning to the PEG-rich top phase. It has been stated that an increase in polymer concentration or MW resulted in the partition of biomolecule to the bottom phase [17,22]. The increase in concentration of enzyme in the bottom phase (decrease in K_E) is due to more dominant effect of volume exclusion over salting out [17]. Similarly, the decrease in SA and PF with increases of PEG molecular weight and concentration were possibly due to more favorable of *C. procera* protease to the bottom phase when compared to those of other proteins. Babu et al. [17] reported that PEG concentration is more influent to the higher MW polyphenol oxidase (104 kDa) than the lower MW of bromelain (23 kDa). It has been found that the system consisted of PEG 1000 using for penicillin acylase isolation gave the high yield compared with that of PEG 3350 and 8000 [23]. Similarly, the lower MW of PEG 4000 produced the higher yield of alkaline protease than that of PEG 6000 and 9000 [24].

From the result of PEG influence on partitioning of *C. procera* protease, 12% PEG 4000 was the most effective for partitioning the enzyme to the PEG-rich top phase with the highest PF of 4.92-folds. Moreover, this system also showed the highest proteolytic yield, K_E and SA. Therefore, the PEG 4000 at 12% (w/w) was chosen for study the effect of salts on protease partitioning.

3.2. Effect of salts on the partitioning of protease from *C. procera* latex

The effect of salts on the partitioning of protease from *C. procera* latex was studied in several biphasis compositions of 12% PEG 4000 with different types of salts (Na-citrate, MgSO₄, K₂HPO₄ and (NH₄)₂SO₄) and their concentrations (11, 14 and 17%, w/w). Salts are frequently used in ATPS to improve partitioning of the target molecules between the phases [25]. As shown in Table 2, the system consists of 11% Na-citrate and 11% MgSO₄ resulted in no phase separation. It is probably due to the amount of both salts were not sufficient to generate the two-phase formation in this partitioning of protease from *C. procera* latex. The mechanism of biphasic generation in PEG–salt systems is dependent on balancing of enthalpic and entropic forces involved in the aqueous hydration of the solutes [11]. The partitioning of proteolytic enzyme is also dependent on “volume exclusion effect” of the polymer and “salting-out effect” of the salts.

The effect of salts on the partitioning factors is shown in Table 2. It can be found that the partitioning *C. procera* protease was strongly dependent on the types and amounts of salts; Na-citrate, MgSO₄, K₂HPO₄ and (NH₄)₂SO₄. The increase in salt concentration from 11 to 17% resulted in the decreases in most of partition parameters. Enhancement of salt quantity provided the higher proportion of salt-rich bottom phase leading to practically reduced of V_R. Johansson [13] reported that the partitioning of a protein is influenced by the presence of salts and this effect is enhanced with the net charge of the protein increase. The SA and PF were decreased when increase of salt concentration. It can be found that increase in salt quantity provided the salting-out effect [17]. Consequently, both of target and non-target proteins were more partitioned to the PEG-rich top phase and resulted in decreases of SA and PF of the top phase (Table 2). The shift of protease as well as the non-desired proteins in *C. procera* latex to the top phase when raising the salt concentration is probably due to similar hydrophobic properties on their surface. Rabelo et al. [22] reported that decreased of PF when phosphate salt and copolymer concentration are increased probably due to the copolymer molecules interact with enzyme molecules, mainly the hydrophobic interactions. Raghavarao et al. [12] also reported that molecular weight, shape, surface hydrophobicity and specific binding sites of biological materials also affect the partition profiles.

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) [11]. Their effectiveness is mainly determined by the nature of the anion. Multi-charged anions being the most effective are ordered of SO₄^{2−} > HPO₄^{2−} > acetate > Cl[−], whereas the order of cations is usually given as (NH₄)⁺ > K⁺ > Na⁺ > Li⁺ > Mg²⁺ > Ca²⁺ [26]. It can be observed that, at the high salt concentration, partitioning of *C. procera* protease was affected by anion species rather than cations. For example, at the ATPS of 17% salts, the SO₄^{2−} provided the

Table 2Effect of phase composition in 12% PEG 4000–salts on partitioning of protease from *Calotropis procera* latex.

Phase composition (% w/w)	V_R	K_P	K_E	SA	PF	Yield (%)
12% PEG 4000–11% Na-citrate	ns	ns	ns	ns	ns	ns
12% PEG 4000–14% Na-citrate	0.67	0.17	0.17	366.5 ± 64	2.04 ± 0.36	7.97 ± 1.17
12% PEG 4000–17% Na-citrate	0.21	0.09	0.09	241.4 ± 10	1.34 ± 0.06	1.79 ± 0.15
12% PEG 4000–11% $MgSO_4$	ns	ns	ns	ns	ns	ns
12% PEG 4000–14% $MgSO_4$	1.73	0.02	0.07	883.6 ± 17	4.92 ± 0.70	3.88 ± 0.41
12% PEG 4000–17% $MgSO_4$	1.13	0.20	0.20	273.9 ± 7	1.53 ± 0.04	23.58 ± 0.36
12% PEG 4000–11% K_2HPO_4	1.27	0.13	0.13	315.4 ± 14	1.76 ± 0.08	12.84 ± 0.07
12% PEG 4000–14% K_2HPO_4	1.08	0.07	0.07	152.8 ± 18	0.85 ± 0.10	6.06 ± 0.93
12% PEG 4000–17% K_2HPO_4	0.49	0.08	0.08	158.1 ± 3	0.88 ± 0.02	3.13 ± 0.28
12% PEG 4000–11% $(NH_4)_2SO_4$	1.17	0.22	0.22	250.1 ± 22	1.39 ± 0.13	19.21 ± 1.41
12% PEG 4000–14% $(NH_4)_2SO_4$	0.94	0.10	0.10	248.6 ± 22	1.38 ± 0.13	8.36 ± 0.70
12% PEG 4000–17% $(NH_4)_2SO_4$	0.42	0.10	0.10	237.5 ± 9	1.32 ± 0.05	3.92 ± 0.65

V_R : volume ratio (top/bottom); K_P : partition coefficient of protein in the top phase; K_E : partition coefficient of enzyme in the top phase; SA: specific activity (unit/mg protein); PF: purification factor; Yield: activity recovery; ns: no phase separation.

highest proteolytic yield than those of HPO_4^{2-} and citrate, respectively (Table 2). This was the same as the lyotropic series. However, at the system of 14% salts, the protease recovery was seemed to be affected by cation. It can be found that the yields of 8.36, 7.97, 6.06 and 3.88% were obtained from the cation series of $(NH_4)^+ > Na^+ > K^+ > Mg^{2+}$, respectively. This was quite comparable to the lyotropic series.

The maximal proteolytic yield of 23.58% was obtained from the system containing 12% PEG 4000–17% $MgSO_4$. Klomklao et al. [27]

reported that the highest SA and PF in partitioning of protease from tuna spleen was also obtained by using 20% PEG 1000 and 20% $MgSO_4$. Based on the highest proteolytic yield obtained, the protease from the fraction of 12% PEG 4000–17% $MgSO_4$ was chosen for study the protein pattern on the gel electrophoresis.

Protein patterns of crude enzyme extract and extracted protease partitioned from 12% PEG 4000–14 and 17% $MgSO_4$ are shown in Fig. 1. Under non-reducing condition, the migration of protein composition was quite different among the crude, the top and the

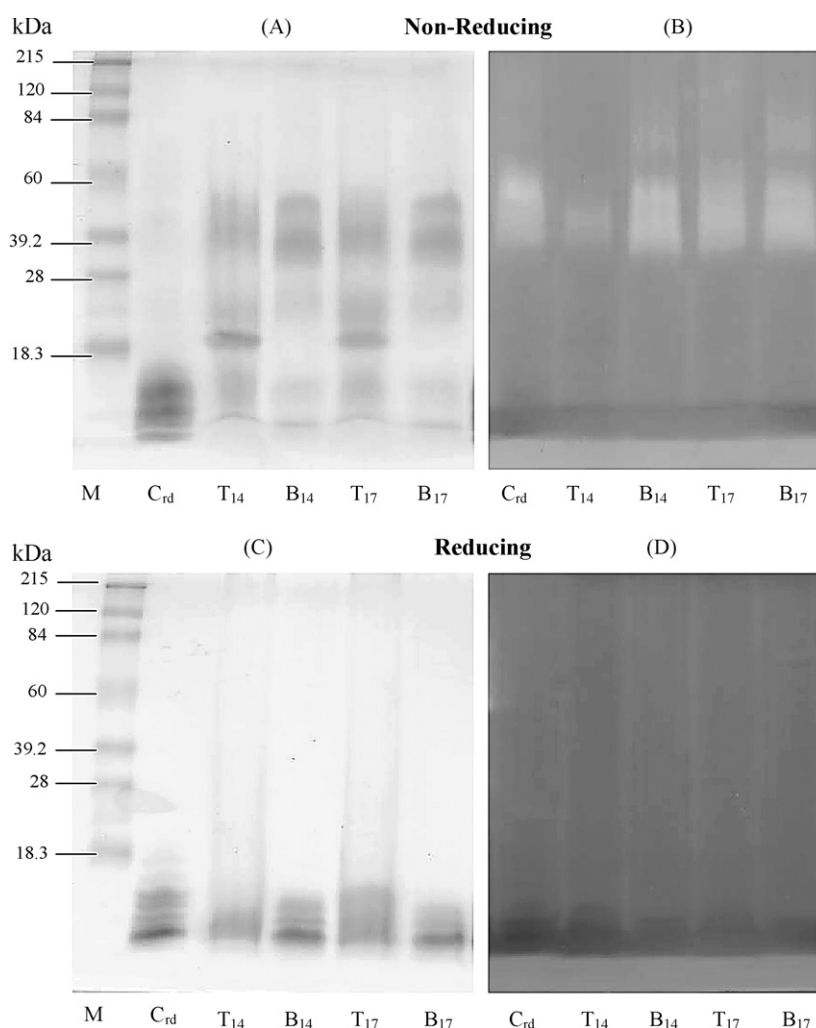


Fig. 1. SDS-PAGE patterns (A, C) and activity staining (B, D) of *Calotropis procera* latex extract and their ATPS fractions under non-reducing and reducing conditions. M: molecular weight marker; C_{rd} : crude enzyme extract; T and B: top phase and bottom phase of their ATPS fractions consisting of 12% PEG 4000–14% $MgSO_4$ (T_{14} and B_{14}) and 12% PEG 4000–17% $MgSO_4$ (T_{17} and B_{17}).

bottom phase. The major protein bands of crude *C. procera* latex were observed with the MW around 11 and 38 kDa (Fig. 1A). There has been another report that the major bands on SDS-PAGE of laticifer protein (LP) from *C. procera* appeared to be around 74, 43, 28, and 13 kDa and that belonging of 28 kDa was the maximal detectable proteolytic activity [28]. Using ATPS condition of 12% PEG 4000–14% MgSO_4 , the proteins were effectively separated to the PEG-rich top phase (T_{14} in Fig. 1A). When MgSO_4 amount was increased from 14 to 17% (w/w), more target protease was partitioned to the top phase as shown by higher band intensity of protein at the position around 38 kDa. However, the non-desired protein with MW around 20 kDa was still contaminated in the top phase. To verify the band of proteolytic enzyme, activity staining was performed by using substrate (casein) gel electrophoresis. The clear zone generated by proteolytic activity was found at the protein band around 38 kDa. The previous reports revealed that the maximum proteolytic activity of *C. procera* protein band was found with that possessed around 28.8 [8] and 30 kDa [28]. Increase in MgSO_4 from 14 to 17% resulted in more partitioning of protease to the PEG-rich top phase as seen by higher intensity of the clear zone (Fig. 1B, T_{17}). This result substantiated to the increase in proteolytic yield from 3.88 to 23.58% in the Table 2.

Under reducing condition, degradation of the protein component in all fractions including crude latex was observed. This result suggested that autolysis was occurred when presence of reducing agent. The proteolytic enzyme consisting in the *C. procera* belongs to cysteine protease class like the most other plant proteases [8,28]. This enzyme type was stabilized with disulfide bond and its activity was activated by reducing agent especially the thiol specific activator such as dithiothreitol, L-cysteine, reduced glutathione and βME [8]. In this experiment of reducing condition, the βME probably activated the enzyme activity to promote the autolysis as well as reduced the disulfide bonds in their molecule. From this consideration, the protein components in *C. procera* latex were digested into the small peptides and could not be detected proteolytic activity as shown in Fig. 1C and D, respectively.

3.3. Effect of pH on the partitioning of protease from *C. procera* latex

The influence of pH on the partitioning of protease from *C. procera* was investigated by using the ATPS composition of 12% PEG 4000–17% MgSO_4 which provided the highest protease recovery. The pH of the ATPS was adjusted to 4.0, 7.0 and 9.0 in comparison to the pH 5.7 of the control system (without pH adjustment). It was found that increasing the pH to 7.0 and 9.0 markedly increased in the K_E and proteolytic recovery (Fig. 2A). The highest protease recovery (85.6%) and K_E (0.76) were obtained in the system pH of 7.0. At alkaline condition (pH 9.0), decrease of the recovery (44.31%) was found when compared to that of neutral pH. In general, the pH had influence 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 [29]. At low pH, the target protease is positively charged and prefers the salt-rich phase. This explains the reduction of both K_E and enzyme recovery in the experiment at pH 4.0. However, some results showed that the major increase in partition coefficient is observed when the protein charge changes from positive to neutral [23]. When proteins are in solution at the pH of their pI , they will have no net molecular charge. Electrostatic repulsion between protein molecules will then be at a minimum and interactions via hydrophobic groups on the surface of the protein are more likely to occur. From this reason, the protein

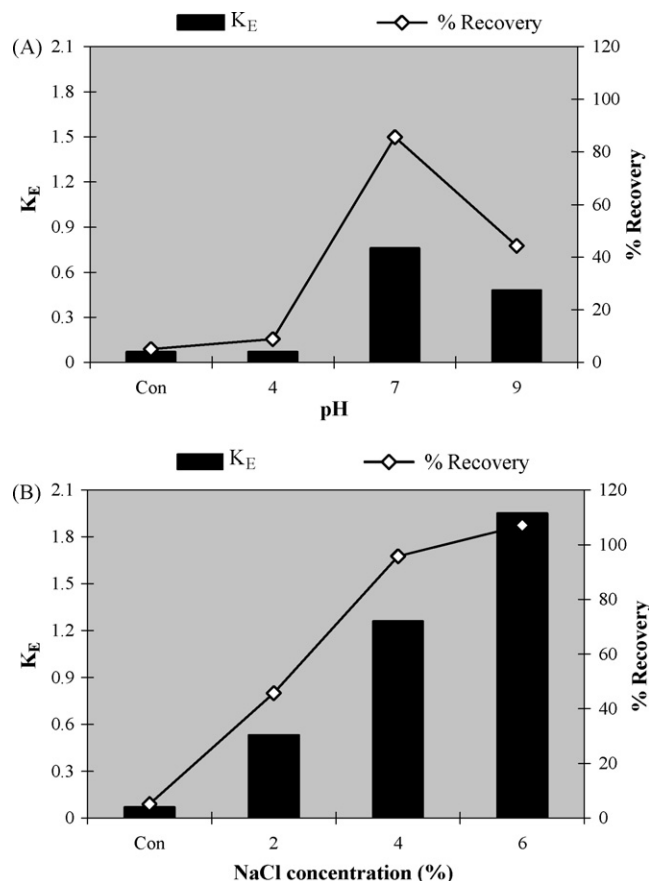


Fig. 2. Effect of system pH (A) and NaCl concentration (B) upon the distribution of recovered protease from *Calotropis procera* latex in 12% PEG 4000–17% MgSO_4 system.

was more favor to the PEG-rich top phase. It might be concluded that increase in the K_E and protease recovery at pH 7.0 and 9.0 was the result from the protease become closely to its neutral charge. At high pH (9.0), the yield and partition coefficient are lowered compared to neutral pH (7.0). This is probably due to inappropriate state of the charge in enzyme active site at the alkaline condition.

3.4. Effect of NaCl on the partitioning of protease from *C. procera* latex

Partitioning of protease from *C. procera* latex in the presence of NaCl was also studied in the system of 12% PEG 4000–17% MgSO_4 . The result showed that partition coefficient of protease (K_E) increase with the addition of NaCl (Fig. 2B). NaCl addition increased the partition coefficient higher than 1 when the level of NaCl addition was higher than 2% (w/w). Moreover, the protease recovery of 95 and 107% were obtained from the addition of NaCl at 4 and 6% (w/w), respectively. Asenjo et al. [29] reported that the resolution of the ATPS is increased with NaCl concentration. There have some reports of NaCl addition to PEG–salt systems that the salt increased the difference in hydrophobicity of the phases, promoting the partition of the more hydrophobic proteins to the upper phase [29,30]. This means that the difference between partition coefficient of two proteins increases with NaCl concentration. Marcos et al. [23] revealed that addition of NaCl to PEG 3350–base ATPS significantly increases the partition of the penicillin acylase from *Escherichia coli*. Oliveira et al. [31] reported that partition coefficients can be dramatically increased when high concentrations of NaCl were added to aqueous two-phase systems. The NaCl presence at increasing concentrations induces a transfer of total proteins,

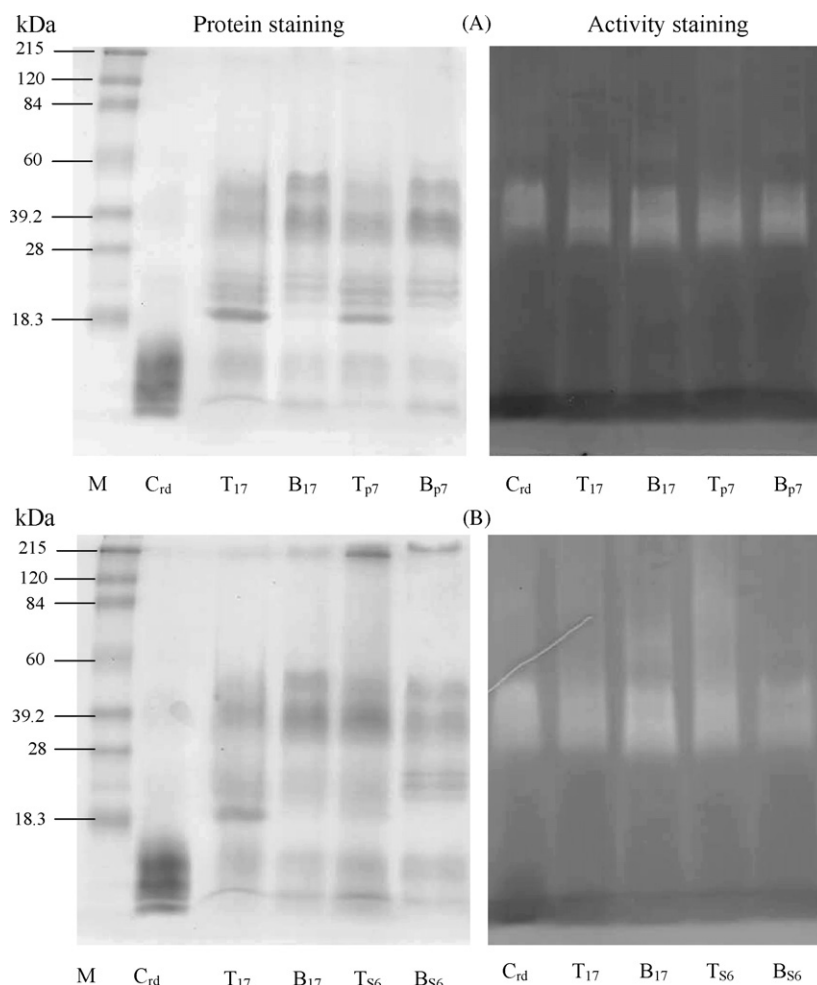


Fig. 3. Effect of system pH (A) and NaCl addition (B) on protein and activity patterns of *Calotropis procera* latex extract and their ATPS fractions. M: molecular weight marker; C_{rd}: crude enzyme extract; T and B: top phase and bottom phase of their ATPS fractions consisting of 12% PEG 4000–17% MgSO₄ (T₁₇ and B₁₇), adjusting to pH 7 (T_{p7} and B_{p7}) and salt content addition to 6% (w/w) (T_{s6} and B_{s6}).

including trypsin, to the top phase, increasing thereby the recovery values from 57 to 92% [32]. Salt is expected to have two different influences on the partitioning of proteins. It alters the behavior of the detergent as evidenced by changes in the cloud point and another may also interact with the protein. The intrinsic charges of the protein are increasingly shielded by higher salt concentrations and hydrophobicity increases [33]. Moreover, hydrophobic characteristic of the enzymes is also prominent; enzyme with higher hydrophobic surface tends to be more partitioning to the PEG phase. From the result, it can be presumable that the hydrophobicity of *C. procera* protease was higher than those of other proteins composed in the latex. As a consequent, the protease was more transferred to the top phase of PEG.

3.5. Protein pattern of the optimized condition for ATPS

The SDS-PAGE pattern of ATPS fractions obtained from the system of 12% PEG 4000–17% MgSO₄, pH 7 is shown in Fig. 3A. The crude extract and original ATPS fractions before pH adjustment were also used as the references in both SDS-PAGE and activity staining. The top phase contained the major protein bands with MW of around 38 and 20 kDa, while the MW of 45 and 38 kDa were still remained in the bottom phase. For activity staining, increase of clear zone was markedly observed in the top phase of the pH adjusted fraction when comparing with the original top phase fraction. However, the bottom phase of this fraction still exhibited high

protease activity as shown by the high hydrolysis zone on the substrate gel.

The protein pattern and activity staining of the fraction obtained from NaCl addition are shown in Fig. 3B. The fractions of the system added with 6% (w/w) NaCl showed slightly different compared with the original system (12% PEG 4000–17% MgSO₄ without salt addition). There were a number of protein bands in the top phase. High intensity of the protein band with MW of 38 kDa was observed. This means that the protease from *C. procera* latex was more partitioned to the top phase and this result can be comparable to that of Fig. 2B. The activity staining substantiated the previous result in which the proteolytic activity on the substrate gel was markedly increased.

Native cathodic polyacrylamide gel electrophoresis (PAGE) was used to study the protein component in each phase after separation from ATPS. The cathodic PAGE has been reported to be useful to investigate the mixture of similar MW proteins which are very difficult to separate them by SDS-PAGE [34]. It has recently been used to identify the 23 kDa cysteine proteases; papain, chymopain, glycyl endopeptidase and caricain comprised in *Carica papaya* latex [10,35].

The result in Fig. 4A shows that the major protease presented in *C. procera* latex possessed its mobility as far as that of papain marker (lane Pp). Based on pH 4.3 of electrode buffer used, the protein band with the same distance mobility of papain should have *pI* closely to 8.75 [35]. There were other major proteins that greatly moved toward anode faster than the papain. They were conse-

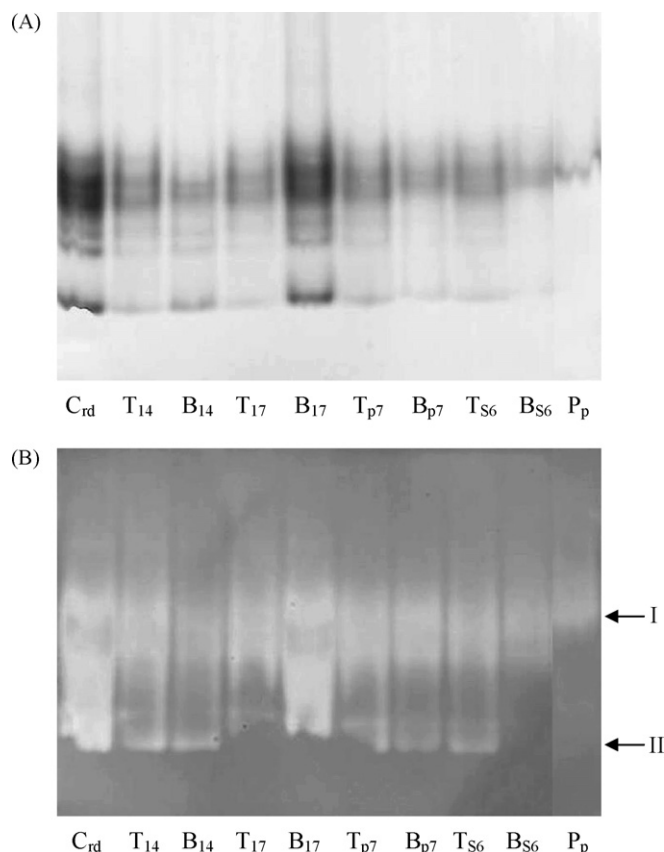


Fig. 4. Cathodic gel electrophoresis (A) and activity staining (B) of *Calotropis procera* latex extracts and their ATPS fractions compared to purified papain (P_p). Cr_d: crude enzymes extract; T and B: top phase and bottom phase of their ATPS fractions consisting of 12% PEG 4000–14% MgSO₄ (T₁₄ and B₁₄), 12% PEG 4000–17% MgSO₄ (T₁₇ and B₁₇), adjusting to pH 7 (T_{p7} and B_{p7}) and salt content addition to 6% (w/w) (T_{s6} and B_{s6}).

quently located below the papain in the gel (Fig. 4A). These proteins possibly had their *pI* higher than 8.75. This is due to they possessed more positive net charge than the papain at pH 4.3 of this electrophoresis condition [35]. It is notice that an isoelectrofocusing method might be more disclosing the exact *pI* of protein. Nevertheless, the purpose of this experiment was just comparison the protein components in each separated phase from ATPS.

Verification of proteolytic activities of protein bands is shown in Fig. 4B. It was observed that the latex from *C. procera* contained at least two proteases (proteases I and II). However, it has been reported that there were at least four proteases composing in *C. procera* latex [8]. It should be noted that the protease II was highly basic *pI* because it had the most positive net charge in this electrophoresis condition used. Although the result of activity staining on the gel was not sufficient for quantitative analysis of enzyme activity, it suggested different patterns of protease bands obtained from the latex of *C. procera* and was useful to compare the protease partitioning in each ATPS composition.

4. Conclusion

The protease from *C. procera* latex was extracted by ATPS and provided the highest recovery in the system comprised of 12% PEG 4000–17% MgSO₄. It showed the molecular weight of ~38 kDa with high activity on casein substrate gel electrophoresis. The pH and NaCl concentration had high significantly influenced on partitioning of the *C. procera* protease. Higher protease recovery was obtained when the system pH was adjusted to 7.0 or at the pres-

ence of 6% (w/w) NaCl in the ATPS. Protein profile on the gel electrophoresis indicated that the *C. procera* latex contains at least two proteases belonging to MW ~38 kDa, highly basic *pI*, and partitioned to the PEG-rich top phase.

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The partitioning of protease from *Calotropis procera* latex by aqueous two-phase systems and its hydrolytic pattern on muscle proteins

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ABSTRACT

The protease from the latex of *Calotropis procera* was isolated by an aqueous two-phase system (ATPS). Polyethylene glycol (PEG 1000, 2000 and 3000) at a concentration of 12, 15, and 18% (w/w) with salts ((NH₄)₂SO₄, K₂HPO₄ and MgSO₄) at a concentration of 14, 17, and 20% (w/w) were investigated. The highest protease recovery (74.6%) was found in the PEG-rich phase of the system ($p < 0.05$), comprising of 18% PEG 1000 and 14% MgSO₄. Protein patterns and activity staining showed that the isolated protease had a molecular weight of ~31 kDa without the oligosaccharide attached to the molecule. Degradation of muscle proteins in beef, farmed giant catfish, and squid was observed by the electrophoresis of sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE). The degradation of myofibrillar proteins (myosin heavy chain: MHC and actin: AC) of farmed giant catfish was higher than that of beef and squid muscles as indicated by the degradation proteins with lower molecular weight.

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Keywords: Aqueous two phase; *Calotropis procera*; Latex; Protease; Muscle protein

1. Introduction

Proteases have been exploited commercially in the food industry in processes such as papain for meat tenderizing (to separate partially connective tissues), ficin and bromelain for brewing (to solubilize grain proteins and stabilize beer), and α -amylase for cookies (to improve crispiness) (Walsh, 2002). Proteases from plant sources have received special attention from the pharmaceutical industry, cosmetic, and by food biotechnology because their activity over wide ranges of temperature and acidity. Bromelain from pineapple was used as a tenderizing agent in beef (Lizuka and Aishima, 1999), squid (Melendo et al., 1997) and in coarse dry sausage (Melendo et al., 1996). Protease from ginger rhizome is a new interesting hydrolytic enzyme that has also been increasingly used for meat tenderization (Naveena et al., 2004). Proteases from plant latex were also widely used to reduce meat toughness for a long time (Kang and Warner, 1974; Ashie et al., 2002; Remezani

et al., 2003). These proteases include papain from *Carica papaya* (Nitsawang et al., 2006), ficin from *Ficus carica* (Huang et al., 2008), and cysteine protease from *Funastrum clausum* (Morcelle et al., 2004). In general, protease in plant latex plays a role in the defense mechanism of plants. *Calotropis procera* is a plant found in tropical and sub-tropical regions. It is well-known for its great capacity to produce latex, which is exuded from damaged parts. Various parts of *C. procera* including latex, have been used in traditional medicine (Chitme et al., 2004). However, the biochemical properties of enzymes contained in the latex of *C. procera* is limited.

Nowadays, industries demand for efficient and economical downstream processes for the partitioning and purification of biomolecules that give high yield and purity. Partitioning in an aqueous two-phase system (ATPS) has shown to be powerful for separating and purifying the mixtures of proteins (Huddleston et al., 1991). It offers many advantages: a bio-compatible environment, low interfacial tension, low energy,

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ease of scale-up, and continuous operations (Raghavarao et al., 1998). ATPS forms readily upon mixing aqueous solutions of two hydrophilic polymers, or of a polymer and a salt, above a certain threshold concentration (Tubio et al., 2007). It could be used to combine several features of the early processing steps in only one or two partitioning operations. Coincidentally, ATPS can remove undesirable enzymes/proteins, unidentified polysaccharides and pigments that are present in the system (Dubey and Jagannadham, 2003; Morcelle et al., 2004). Together with the removal of insoluble and major classes of contaminants, this technique has been developed as a primary purification step in the overall recovery (Huddleston et al., 1991). ATPS has been applied for the partitioning and recovery of various proteases such as bromelain (Babu et al., 2008), papain (Nitsawang et al., 2006), trypsin (Klomklao et al., 2005), chymosin and pepsin (Spelzini et al., 2005; Nalinanon et al., 2009), amyloglucosidase (Tanuja et al., 1997) and ricin B (Zhang et al., 2005). Dubey and Jagannadham (2003) reported that the latex of *C. procera* contained 4 stable cysteine proteases. However, extraction of the protease from *C. procera* latex by ATPS has not been reported. Therefore, the objective of this research was to isolate the proteolytic enzyme from *C. procera* latex by using an aqueous two-phase system. The other aims of this work were to characterize and apply the isolated enzyme in muscle proteins.

2. Materials and methods

2.1. Chemicals and raw materials

Polyethylene glycol (PEG), sodium dodecyl sulfate (SDS) and bovine serum albumin (BSA) were obtained from Fluka (Buchs, Switzerland). Beta-mercaptoethanol (β ME) and Coomassie Brilliant Blue G-250 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$), magnesium sulfate (MgSO_4), potassium phosphate (K_2HPO_4), trichloroacetic acid (TCA), hydrochloric acid, tris-(hydroxymethyl)-aminomethane and other chemicals with analytical grade were obtained from Merck (Darmstadt, Germany).

Latex of *C. procera* was collected from Nayong, Trang Province, Thailand. Beef and squid muscles were purchased from Bando Market, Chiang Rai Province, Thailand. Farmed giant catfish was obtained from a farm in Phan, Chiang Rai Province, Thailand.

2.2. Latex preparation

Latex was collected in a clean tube by breaking the *C. procera* stems. The collected latex was diluted with distilled water (1:1, v/v). It was mixed well and then centrifuged at $15,000 \times g$ at 4°C for 10 min. The obtained supernatant was filtered through a Whatman paper No. 1. This sample was referred to as the “crude extract” and was used for further study.

2.3. Aqueous two phase partitioning

The ATPS was prepared in 10-ml centrifuge tubes according to the method in Nalinanon et al. (2009). Various amounts and molecular weights of PEG (1000, 2000 and 3000 Da) as well as salts ($(\text{NH}_4)_2\text{SO}_4$, K_2HPO_4 , and MgSO_4) were added to the

crude extract from *C. procera* to generate the biphasic system.

2.3.1. Effect of salts on the partitioning of protease from *C. procera* latex

To study the effect of salts on the partitioning of protease from *C. procera* latex using ATPS, different salts ($(\text{NH}_4)_2\text{SO}_4$, K_2HPO_4 , and MgSO_4 at different concentrations (14, 17 and 20%, w/w) were mixed with 18% PEG 1000 in an aqueous system (the concentration of PEG 1000 was fixed to make sure that a two-phase formation would be obtained). Thirty percentages of the crude extract (w/w) were added into the system. The remaining distilled water was used to adjust the system to obtain the final weight of 15 g. The mixtures were mixed continuously for 3 min using a Vortex mixer (Vortex-genie2, G-560E, USA), and then they were gently mixed for 15 min. Phase separation was achieved by centrifuging the mixture at $9,000 \times g$ for 30 min. The top phase was carefully separated using a Pasteur pipette. Volumes of the separated top and bottom phases were measured and recorded. Aliquots from each phase were taken for enzyme activity assay and protein determination. The phase composition giving the highest proteolytic yield was chosen for further study.

2.3.2. Effect of PEG on the partitioning of protease from *C. procera* latex

Fourteen percentages of MgSO_4 were used in the system to study the effect of the concentrations (12, 15 and 18%, w/w) and molecular weights (1000, 2000 and 3000 Da) of PEG on the partitioning of protease from *C. procera* latex. A two-phase separation was performed as previously described. ATPS parameters were calculated as follows:

The volume ratio (V_R) is as:

$$V_R = \frac{V_T}{V_B} \quad (1)$$

where V_T and V_B are top and bottom phase volume, respectively.

The partition coefficient of protein (K_P) was defined as:

$$K_P = \frac{P_T}{P_B} \quad (2)$$

where P_T and P_B are concentrations of protein in top and bottom phase, respectively.

The partition coefficient of enzyme (K_E) was defined as:

$$K_E = \frac{E_T}{E_B} \quad (3)$$

where E_T and E_B are concentrations of enzymes (unit/ml) in top and bottom phase, respectively.

The specific activity (SA) of extracted protease in each phase of the ATPS was defined as:

$$SA = \frac{\text{total protease activity}}{\text{total protein content}}; \text{ unit mg protein}^{-1} \quad (4)$$

The purification factor (PF) as:

$$PF = \frac{SA_E}{SA_I} \quad (5)$$

where SA_E is the SA of each phase and SA_I is the SA of the initial phase (crude latex before partitioning).

The protease activity recovery (yield) was defined as:

$$\text{yield (\%)} = \frac{A_T}{A_I} \times 100 \quad (6)$$

where A_T is the total protease activity in the top phase and A_I is the initial protease activity (crude latex before partitioning).

From the protease recovery, the protease from the ATPS fraction that rendered the maximal yield was chosen for further study.

2.4. Caseinolytic activity assay

An enzyme sample of 0.1 ml was mixed with 1.1 ml of 1% (w/v) casein in 0.1 M Tris–HCl, pH 8.0 containing 12 mM of cysteine. The mixture was incubated at 37 °C for 20 min. After that, the reaction was stopped by adding 1.8 ml of 5% TCA. After centrifugation at $3,000 \times g$ for 15 min, the absorbance of the supernatant was measured at 280 nm. One caseinolytic unit is defined as the amount of enzymes needed to produce an increment of 0.01 absorbance unit per minute at the assayed condition (Vallés et al., 2007).

2.5. Protein determination

The protein concentration in the sample was measured by the Bradford method (Bradford, 1976) using BSA as a protein standard.

2.6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

2.6.1. Protein staining

SDS-PAGE of crude extract and the extracted protease from ATPS fractions was performed according to the Laemmli method (Laemmli, 1970). Protein solutions were mixed at a 1:1 (v/v) ratio with the sample buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol). The samples (10 and 2 µg protein for protein and activity staining, respectively) were loaded onto the gel made of 4% stacking and 15% separating gels. They were subjected to an electrophoresis set at a constant current of 15 mA per gel using a Mini Protean Tetra Cell unit (Bio-Rad Laboratories, Richmond, CA, USA). After electrophoresis, the gel was stained overnight with staining solution (0.02% (w/v) Coomassie Brilliant Blue R-250) in 50% (v/v) methanol, and 7.5% (v/v) acetic acid. Protein patterns were then visualized after destaining the gel until a clear background was achieved.

2.6.2. Protease activity staining

The protease separated on the gel was verified by using activity staining as the method described in Garcia-Carreno et al. (1993). The gel was immersed in 50 mL of 2% (w/v) casein in 50 mM Tris–HCl buffer, pH 8.0 containing 12 mM cysteine for 45 min, and agitated constantly at 4 °C. The gel was then incubated at 37 °C for 30 min with constant agitation to generate the reaction. The treated gel was then stained and destained as described above. The appearance of a clear zone on the dark background indicated the *C. procera* latex protease activity.

2.6.3. Glycoprotein staining

Glycoprotein staining was conducted by using a GelCode® Glycoprotein Staining Kit (PIERCE Biotechnology, IL, USA). The protein separated by electrophoresis was fixed by immersing the gel in 30 ml of 50% (v/v) methanol for 30 min, followed by

washing the gel by gently agitating it with 3% (v/v) acetic acid for 10 min (repeated twice). The gel was transferred to 25 ml of oxidizing solution and then washed with 3% (v/v) acetic acid for 10 min before treating it with 15 ml of GelCode® Glycoprotein Stain for 15 min. The gel was incubated with 25 ml of reducing solution for 10 min and then washed by 3% (v/v) acetic acid. The magenta band could be observed in the presence of glycoprotein.

2.7. Effect of selected ATPS fraction on hydrolytic pattern of muscle proteins

Two grams of muscles (beef, giant catfish and squid) were marinated with 0, 3, 5, 10, 20, 30, and 50 units of isolated protease from the top phase of the ATPS (18% PEG 1000–14% MgSO₄) and then incubated at a refrigerated temperature (~4 °C) for 60 min. Twenty units of the crude extract were also used as the control. Three grams of a marinated sample were added with 27 ml of 5% SDS (85 °C). The mixtures were homogenized at a speed of 11,000 rpm for 1 min using an IKA Labortechnik homogenizer (Selangor, Malaysia). The homogenate was incubated at 85 °C for 60 min, followed by centrifugation at $8,000 \times g$ for 5 min at room temperature using a Mikro 20 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). The supernatants were mixed at a 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8 containing 4% SDS, 20% glycerol and 10% βME) and boiled for 3 min. The samples (15 µg protein) were loaded into the polyacrylamide gel (10% running and 4% stacking gel) and subjected to electrophoresis as previously described.

2.8. Statistical analysis

All experiments were conducted and analyzed in triplicate. Means and standard deviations were calculated and compared using the Duncan's multiple range test (DMRT). Analysis was performed using a SPSS package (SPSS 10.0 for windows, SPSS, Inc. Chicago, IL).

3. Results and discussion

3.1. Effect of salts on partitioning of protease from *C. procera* latex

The partitioning was carried out in several biphasis compositions of 18% PEG 1000 with different salts ((NH₄)₂SO₄, MgSO₄ and K₂HPO₄) and their concentrations (14, 17 and 20%, w/w). Salts are frequently used in ATPS to improve partitioning target molecules between the phases. In general, no phase separation was found in either PEG 1000 or salts on their own. This indicates that the combination of both PEG and salt was necessary for the partitioning process. Phase separation will occur if the concentrations of polymers or salts are above a certain critical value of the binodal curve. Raghavarao et al. (1998) reported that two phases are formed when the polymer concentration is in the range of 8–16% (w/w), and salt concentration must be as high as 10% (w/w). In addition, the lower the molecular weight, the higher the polymer concentration is required for phase formation. From the result, it was shown that the two-phase separations of PEG-rich top phase and salt-rich bottom phase were obtained in all systems comprising of 18% PEG and 14–20% salts. The mechanism of phase separation in PEG-salt systems is dependent on balancing enthalpic and entropic forces involved in the aqueous hydration of the solutes (Huddleston et al., 1991).

Table 1 – Effect of phase composition in PEG 1000-salts ATPS on partitioning of protease from *Calotropis procera* latex.

Phase composition (% w/w)	V _R	K _P	K _E	SA	PF	Yield (%)
18%PEG 1000–14% (NH ₄) ₂ SO ₄	2.96 ± 0.16f	0.13 ± 0.00a	0.57 ± 0.02e	293 ± 7.80e	5.82 ± 0.16e	65.7 ± 2.71g
18%PEG 1000–17% (NH ₄) ₂ SO ₄	1.94 ± 0.11cd	0.18 ± 0.01a	0.52 ± 0.02d	266 ± 7.71d	5.29 ± 0.15d	62.2 ± 1.51f
18%PEG 1000–20% (NH ₄) ₂ SO ₄	0.93 ± 0.03a	0.16 ± 0.01a	0.34 ± 0.01c	308 ± 1.73e	6.12 ± 0.03e	11.9 ± 0.69c
18%PEG 1000–14% K ₂ HPO ₄	2.31 ± 0.06de	0.33 ± 0.01ab	1.06 ± 0.02f	163 ± 0.60b	3.23 ± 0.01b	68.0 ± 3.39g
18%PEG 1000–17% K ₂ HPO ₄	1.62 ± 0.02bc	0.60 ± 0.03b	1.49 ± 0.01g	265 ± 6.46d	5.27 ± 0.13d	56.6 ± 1.90e
18%PEG 1000–20% K ₂ HPO ₄	1.22 ± 0.15ab	4.00 ± 0.57c	2.59 ± 0.02h	144 ± 0.57b	2.87 ± 0.01b	15.7 ± 1.28d
18%PEG 1000–14% MgSO ₄	7.41 ± 0.76g	0.16 ± 0.01a	0.53 ± 0.01d	205 ± 9.92c	4.08 ± 0.20c	74.6 ± 1.00h
18%PEG 1000–17% MgSO ₄	2.48 ± 0.25ef	0.03 ± 0.01a	0.05 ± 0.01b	154 ± 32.6b	3.01 ± 0.65b	6.93 ± 1.63b
18%PEG 1000–20% MgSO ₄	1.77 ± 0.21c	0.03 ± 0.00a	0.03 ± 0.00a	79.8 ± 1.97a	1.58 ± 0.04a	2.61 ± 0.08a

V_R: volume ratio (top/bottom); K_P: partition coefficient of protein in the top phase; K_E: partition coefficient of enzyme in the top phase SA: specific activity (unit/mg protein) of the top phase; PF: purification factor; yield: activity recovery. Different letters in the same column indicate significant differences (*p* < 0.05).

The basis for separation by ATPS is the selective distribution of biomolecules between the phases, quantified by the partition coefficient (Raghavarao et al., 1998). The protease was partitioned prominently in the polymer phase, principally those with hydrophobic characteristics the enzyme. Dubey and Jagannadham (2003) reported that most amino acids in procerain, a cysteine protease in *C. procera* latex, were hydrophobic amino acids, with especially high amounts of tyrosine and tryptophan. Glycine and valine were predominant non-polar amino acids present in *C. gigantean*. In PEG-salt systems, proteolytic enzyme partitioning depends on a “volume exclusion effect” of the polymer and a “salting-out effect” of the salts. The effect of salts and their concentrations on the partitioning (V_R, K_P, SA, PF and yield) are shown in Table 1. The V_R of the assayed systems generally decreased when salt concentration increased. The addition of salts to the aqueous PEG solution led to an arrangement of ordered water molecules around the PEG molecules. This was due to their water structure breaking effects (Farruggia et al., 2004). The formation of a water layer around the cation resulted in a more compact structure with a minor volume of PEG molecules. This led to the decrease in volume of the PEG-rich top phase.

The distribution of the protein and proteases in ATPS are reported by K_P and K_E, respectively. High K_P values indicate that most of the proteins from the crude extract were partitioning more to the top phase, while the high K_E was the only protease to favor the top phase. From the results, the highest K_P (4.00) and the highest K_E (2.59) were found in the system of 18% PEG 1000–20% K₂HPO₄. In contrast, the phase composition of 18% PEG 1000–20% MgSO₄ showed the lowest K_P (0.03), indi-

cating that both desired proteases and contaminant proteins would shift to the lower phase.

The phase containing 18% PEG 1000 and 14% (NH₄)₂SO₄ gave the highest SA (293 unit/mg protein⁻¹) and PF (5.82) (*p* < 0.05), whereas the maximal proteolytic yield of 74.6% was obtained from the system containing 18% PEG 1000 and 14% MgSO₄. Babu et al. (2008) reported that the system of 18% PEG 1500 and 20% K₂HPO₄ provided the highest activity recovery of bromelain from pineapple. It was reported that using 20% PEG 1000 and 20% MgSO₄ gave the highest SA and PF in the partitioning of protease from tuna spleen (Klomklao et al., 2007). The same result was also found in the extraction of protease from the stomach of an albacore tuna (Nalinanon et al., 2009). Johansson (1998) reported that the partition of a protein is influenced by the presence of salts. This effect increases with the net charge of the protein. The presence of salt may affect the partitioning in two ways: (1) the weakening or strengthening of the interactions, or (2) the interaction between ionized groups with the opposite net charge of proteins. The effectiveness of the salt is mainly determined by the nature of the anion. Multi-charged anions are the most effective in the order of SO₄²⁻ > HPO₄²⁻ > CH₃COO⁻ > Cl⁻. The order of cations is usually given as NH₄⁺ > K⁺ > Na⁺ > Li⁺ > Mg²⁺ > Ca²⁺ (Roe, 2000). Huddleston et al. (1991) concluded that the effectiveness of various salts in promoting phase separation reflects the lyotropic series (a classification of ions based upon salting-out ability).

Based on the highest proteolytic yield obtained, the 14% MgSO₄ was chosen for further experiment on the effect of PEG on the partitioning of protease from *C. procera* latex.

Table 2 – Effect of phase composition in PEG–14% MgSO₄ ATPS on partitioning of protease from *Calotropis procera* latex.

Phase composition (% w/w)	V _R	K _P	K _E	SA	PF	Yield (%)
12%PEG 1000–14% MgSO ₄	ns	ns	ns	ns	ns	ns
15%PEG 1000–14% MgSO ₄	ns	ns	ns	ns	ns	ns
18%PEG 1000–14% MgSO ₄	7.41 ± 0.8c	0.16 ± 0.01ab	0.53 ± 0.01f	205 ± 9.92a	4.08 ± 0.2a	74.6 ± 1.00f
12%PEG 2000–14% MgSO ₄	ns	ns	ns	ns	ns	ns
15%PEG 2000–14% MgSO ₄	2.84 ± 0.39ab	0.01 ± 0.01a	0.18 ± 0.01d	1279 ± 213b	25.6 ± 4.2b	45.80 ± 0.16d
18%PEG 2000–14% MgSO ₄	3.32 ± 0.44b	na	0.02 ± 0.00b	na	na	5.71 ± 0.36b
12%PEG 3000–14% MgSO ₄	2.62 ± 0.15ab	0.26 ± 0.03b	0.32 ± 0.00e	361 ± 2a	7.17 ± 0.05a	65.2 ± 1.53e
15%PEG 3000–14% MgSO ₄	2.24 ± 0.20a	0.03 ± 0.01a	0.05 ± 0.01c	208 ± 10a	4.12 ± 0.20a	10.5 ± 1.67c
18%PEG 3000–14% MgSO ₄	2.65 ± 0.04ab	0.02 ± 0.01a	0.01 ± 0.00a	24.0 ± 8a	0.47 ± 0.17a	1.10 ± 0.02a

V_R: volume ratio (top/bottom); K_P: partition coefficient of protein in the top phase; K_E: partition coefficient of enzyme in the top phase SA: specific activity (unit/mg protein); PF: purification factor; yield: activity recovery. ns: no phase separation; na: no protein content. Different letters in the same column indicate significant differences (*p* < 0.05).

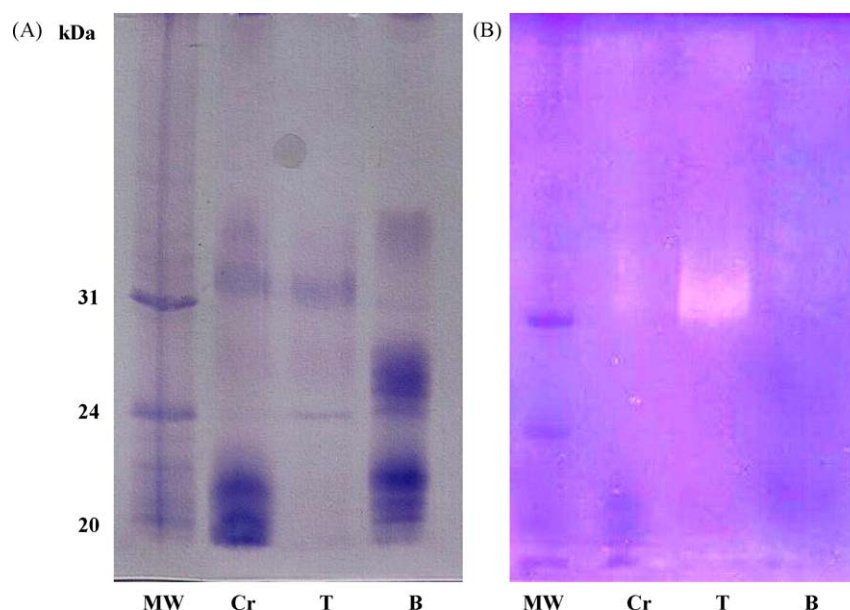


Fig. 1 – SDS-PAGE patterns (A) and activity staining (B) of *Calotropis procera* latex extract and its ATPS fractions of 18% PEG 1000–14% MgSO_4 ; MW: molecular weight marker; Cr: crude extract; T: top phase and B: bottom phase.

3.2. Effect of PEG on the partitioning of protease from *C. procera* latex

This study examined the partitioning of protease from *C. procera* latex in ATPS with different molecular weights (MW) and concentrations of PEG at 14% MgSO_4 . As shown in Table 2, the protease partitioning was strongly dependent on the MW and the PEG concentration. No phase separation was observed in the 14% MgSO_4 system with 12% PEG 1000, 15% PEG 1000, and 12% PEG 2000. As mentioned before, two-phase formation requires high concentrations of low MW polymer, or requires low concentrations with high MW polymer. The V_R of the system ranged from 2.24 to 7.41. Most of the systems produced a value of V_R higher than 1.0, indicating that the volume fraction was distributed in the top phase rather than in the bottom phase. The K_P obtained from all systems was lower than 1, indicating that most of the protein components from *C. procera* latex preferably partitioned to the bottom phase of the ATPS system. A favorable interaction between PEG molecules and protein domain decreased when the MW of PEG increased because of its' exclusion from the protein domain (Reh et al., 2002; Klomklao et al., 2005; Nalinanon et al., 2009). Tubio et al. (2009) reported that the best ATPS separation of trypsin from bovine pancreas was found in the system with a molecular weight of PEG 3350 compared with 600, 1000, 1450, and 8000. However, Hotha and Banik (1997) found that for the production of alkaline protease by ATPS, the lower MW of PEG 4000 produced a higher yield than PEG 6000 and 9000. Marcos et al. (1999) also reported that a system consisting of PEG 1000 for penicillin acylase production achieved a high yield compared with that of PEG 3350 and 8000.

Among all ATPS tested, systems comprising of 18% PEG 1000 and 14% MgSO_4 effectively partitioned the protease from *C. procera* latex to the PEG-rich top phase. Under this condition, the highest proteolytic yield of 74.6% was recovered in the top phase, providing PF of 4.08 fold. Though the highest PF was observed in the system of 15% PEG 2000 and 14% MgSO_4 , it provided only 45.80% yield. Therefore, the fraction of the ATPS system containing 18% PEG 1000–14% MgSO_4 with the

highest yield was selected for biochemical properties characterization.

3.3. Protein pattern and activity staining of protease from *C. procera* latex and its ATPS fractions

Fig. 1A shows the protein patterns of the crude extract and extracted protease fractions from 18% PEG 1000–14% MgSO_4 . For the migration of protein composition in the crude extract, the top phase and bottom phase of their fractions were quite different. The three major protein bands of crude *C. procera* latex were observed with a MW of around 20 kDa, ~31 kDa and above 31 kDa. This corresponds with the investigation of Freitas et al. (2007), but the protein band at around 31 kDa of that study was the main component instead of that below 20 kDa in the present research. Using ATPS with 18% PEG 1000–14% MgSO_4 , the effective protease separation was obtained (as shown in the Fig. 1A) by most of the proteins partitioning to the salt-rich bottom phase. Only the protein band with the MW of around 31 kDa was separated to the PEG-rich top phase. It was reported that the purified protease from *C. procera* appeared as a single band at around 29 kDa in a reducing condition of SDS-PAGE (Dubey and Jagannadham, 2003).

To verify the band of proteolytic enzymes, activity staining was performed by using substrate (casein) gel electrophoresis. The clear zone by proteolytic activity on the dark background resulted only at the protein band around 31 kDa (Fig. 1B). This clear zone was more apparent in the band of the top phase of ATPS than that of the crude latex. There is previous report that the maximum proteolytic activity of *C. procera* protein found a protein band possessing around 30 kDa (Freitas et al., 2007). Low detectable clear zones were observed in both the crude extract and in the bottom phase of their ATPS fractions. This is probably due to a rather low specific activity of protease content in both fractions. In contrast, the top phase fraction is mainly composed of activated protease, which results in detectable activity of clearer zones. For this result, the protein component of *C. procera*

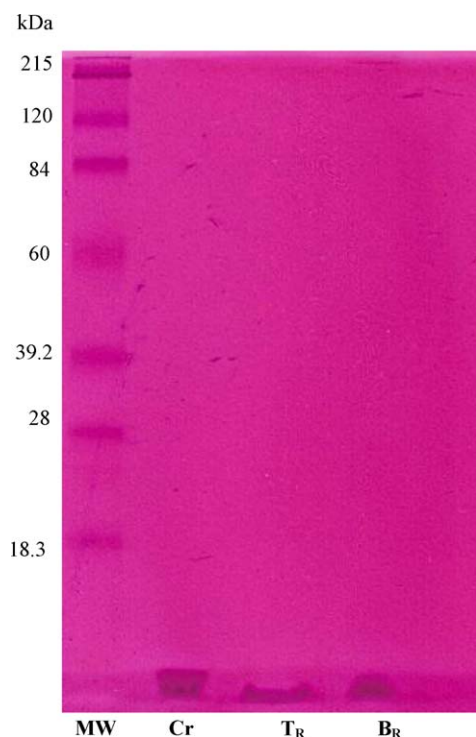


Fig. 2 – Glycoprotein staining of *C. procera* latex extract and its ATPS fractions 18% PEG 1000–14% MgSO_4 ; MW: molecular weight marker; Cr: crude extract; T: top phase and B: bottom phase.

latex with the MW of ~ 31 kDa was the predominant protease.

3.4. Glycoprotein staining of protease from *C. procera* latex and its ATPS fractions

The presence of carbohydrates in the protease from the *C. procera* latex was investigated. The crude extract and its 18% PEG 1000–14% MgSO_4 ATPS fractions were subjected to glycoprotein staining. This staining method was reported to detect high glycosylated proteoglycans (protein glycosaminoglycans) or glycoproteins (protein oligosaccharides) (Møller and Poulsen, 2002) in which polypeptides covalently bonded to a carbohydrate moiety (Mathews and Holde, 1990). The result (as depicted in Fig. 2) revealed that all of the latex major proteins of *C. procera* latex were not contained in their car-

bohydrate molecules. Similar results were obtained by Dubey and Jagannadham (2003) in which protease from *C. procera* was devoid of carbohydrate moiety in procera.

3.5. Effect of the protease fraction on the hydrolytic pattern of muscle proteins

Proteolytic patterns of the muscle samples treated with ATPS isolated *C. procera* latex protease are depicted in Fig. 3. The PEG-rich phase of 18% PEG 1000–14% MgSO_4 system was used as the isolated protease fraction in this study. Different protease activity units (0–50 units) were added to three kinds of muscle samples; beef, farmed giant catfish, and squid muscles. The treated samples were incubated at a refrigerated temperature (4°C) for 60 min. Increasing protease activity of isolated protease fractions on the treated muscle samples were observed, evidenced by gradually decreasing numbers of protein bands as well as the protein band intensity as compared with the control (without the fraction addition). The degradation of protein increased by increasing the enzyme concentration. Naveena et al. (2004) reported that when using protease from *Cucumis trigonus* in buffalo meat samples, the increase in proteolysis can be correlated with significantly higher protein solubility. Bacterial proteolytic enzyme treatment of muscle protein showed a reduction in the level of higher MW fractions due to the degradation of myosin, thus increasing the meat tenderness (Jorgova et al., 1989).

From the Fig. 3, it is also evident that protein breakdown in all of the enzyme treated samples was generated. High amounts of protein decomposition were more visible in the squid and giant catfish muscles than that of the beef sample. Not only was the myosin heavy chain (MHC) degraded by the ATPS enzyme fraction, but also by the actin (AC), especially in the giant catfish muscle. The squid and giant catfish muscles, MHC band was extensively degraded into lower MW when the concentration of the enzyme fraction was increased. Furthermore, high breakdowns of protein were markedly clearer in the giant catfish than in the beef for both MHC and AC bands. This is probably due to the co-hydrolysis from the endogenous enzymes contained in the giant catfish. The result also showed that there was a slight change in degradation of the AC band in squid and beef muscles, even with increased enzyme fraction concentrations when compared to those of the giant catfish muscle. It can be explained by the difference in protein structure and composition among these three muscles.

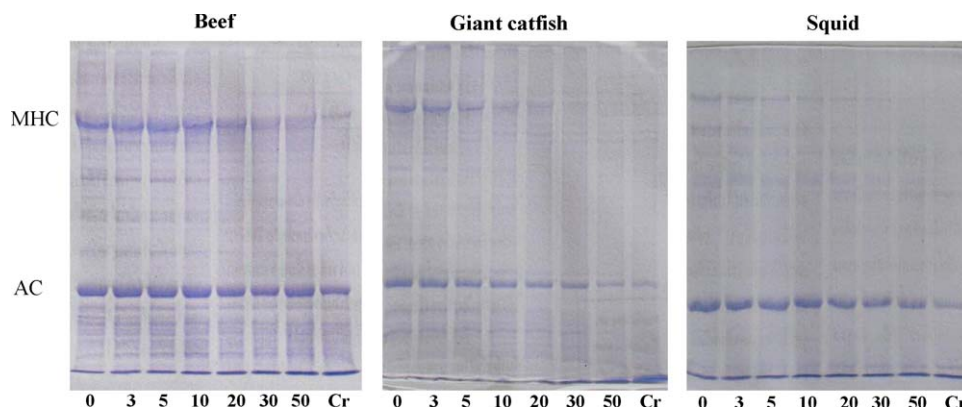


Fig. 3 – SDS-PAGE patterns of muscle samples incubated with enzyme from ATPS fraction (top phase of system 18% PEG 1000–14% MgSO_4) at refrigerated temperature. Numbers indicated enzyme activity (unit) the fraction used. MHC: myosin heavy chains; AC: actin; Cr: crude enzymes extract (20 units).

The degradation of muscle protein plays a major role in determining the tenderness and water holding capacity of meat during postmortem storage (Melody et al., 2004). Myosin was degraded very intensely throughout the meat, and totally in the presence of high concentrations of protease fractions. AC, on the other hand, was only slightly hydrolyzed unless a high amount of enzymes were added. Nonetheless, differences between the controls and the treated meat with lower enzyme concentrations were unclear in all samples.

In conclusion, the protease from *C. procera* latex was extracted by the aqueous two-phase system. The top phase from 18% PEG 1000–14% MgSO₄ provided the highest protease recovery (74.6%). By using casein substrate gel electrophoresis, the protease from the phase partitioning showed the MW of ~31 kDa with high specificity. The protease obtained had no oligosaccharide in the molecule as determined by the glycoprotein staining. Based on the protein degradation of beef, giant catfish and squid muscles, it is suggested that the protease fraction from *C. procera* latex is suitable for meat tenderization.

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Short communication

Three-phase partitioning of protease from *Calotropis procera* latexSaroat Rawdkuen^{a,*}, Phanuphong Chaiwut^b, Punyawatt Pintathong^b, Soottawat Benjakul^c^a Food Technology Program, School of Agro-Industry, Mae Fah Luang University, Chiang Rai 57100, Thailand^b School of Cosmetic Science, Mae Fah Luang University, Muang, Chiang Rai 57100, Thailand^c Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

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ABSTRACT

Three-phase partitioning (TPP) was used to partially purify protease from the latex of *Calotropis procera* (*C. procera*). To optimize the TPP for protease isolation a ratio of crude extract to *t*-butanol, percent saturation of $(\text{NH}_4)_2\text{SO}_4$, and the cycle of TPP was required. The highest proteolytic recovery (first cycle) of 182% with a purification of 0.95 folds was obtained at the interphase of the system comprising the ratio of the crude extract to *t*-butanol of 1.0:0.5 with the presence of 50% $(\text{NH}_4)_2\text{SO}_4$. The second cycle of TPP was prepared by adding of $(\text{NH}_4)_2\text{SO}_4$ up to 65% (w/v) to the bottom phase obtained from 30% $(\text{NH}_4)_2\text{SO}_4$ –1.0:0.5 system of the first TPP. A purification of 6.92-fold was achieved with about 132% activity recovery. SDS-PAGE and zymography profiles revealed the substantial isolation of protease from *C. procera* latex by the TPP. The molecular weight of major protease was found to be around 28 kDa. The present study shows high interesting outcomes and could be used as a primary purification process in comparison with existing literature's values.

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1. Introduction

Three-phase partitioning (TPP) is a relatively recent bioseparation technique, which employs collective operation of principles involved in numerous techniques for protein precipitation. The TPP has widely been used for the extraction and purification of various proteins [1–5]. It uses $(\text{NH}_4)_2\text{SO}_4$ with certain saturation to precipitate the protein, and *t*-butanol was added to make three-phase layers and to remove some small molecular weight compounds such as lipids, phenolics and some detergents [6]. In general, biomolecules are recovered in a purified form at the inter-phase, while the contaminants mostly partition in *t*-butanol (top phase) and aqueous phase (bottom phase) [3]. This method was scalable and could be used directly with the crude suspensions [4].

Proteases from plant sources have received special attention from the pharmaceutical industry and by food biotechnology because their properties of activity over wide range of temperature and pHs. They have been exploited commercially in the food industry such as papain for meat tenderizing and ficin and bromelain for brewing [7]. Although recently enzymes from microorganism have been widely commercially produced, consumer still aware for used it as an edible ingredients into their meals. Therefore, natural sources from both plant and animal tissues still desired.

Calotropis procera is a plant found in tropical and sub-tropical regions. It is well-known for its great capacity of producing latex which exudates from the green damaged parts. Scientific reports have mentioned various medicinal activities of *C. procera* latex, such as insecticidal [8], anti-fungal [9] and wound healing [10]. Some biochemical properties of the enzyme containing in the latex of *C. procera* have been documented and named as procerain [11]. It has been reported that *C. procera* latex is a potential material for enzyme purification [12,13]. Consequently, scientific researches of protease extraction from *C. procera* have recently been reported. Protease activity recovery of 74.6% with 4.08-fold of purification was obtained from *C. procera* latex by using aqueous two-phase system [13]. However, there is no report of using TPP as the single method to separate the protease from this potential source. Therefore, the aim of this study was to optimize the separation process of protease from the latex of *C. procera* by using TPP technique.

2. Materials and methods

2.1. Chemicals and raw materials

Polyethylene glycol (PEG), sodium dodecyl sulfate (SDS), bovine serum albumin (BSA), casein and L-cysteine were obtained from Fluka (Buchs, Switzerland). Beta-mercaptoethanol (β -ME) and Coomassie Brilliant Blue G-250 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ammonium sulfate $((\text{NH}_4)_2\text{SO}_4)$,

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magnesium sulfate (MgSO_4), trichloroacetic acid (TCA), tert-butanol (*t*-butanol) and other chemicals with analytical grade were obtained from Merck (Darmstadt, Germany). Latex of *C. procera* was collected from Nayong, Trang Province, Thailand.

2.2. Latex preparation

Latex was collected in a clean tube by breaking the *C. procera* stems and then diluted with distilled water (1:1, v/v). After mixing, it was centrifuged at $8000 \times g$ at 4°C for 10 min. The obtained supernatant was referred to as the “crude extract” [protein content: 3.49 mg/ml, activity: 1138 unit/ml, specific activity: 326 unit/mg protein] and used for further study.

2.3. Three-phase partitioning

2.3.1. Effect of crude extract to *t*-butanol ratio on protease partitioning

The TPP was carried out as described by Roy and Gupta [3]. The effect of the ratio of crude extract to *t*-butanol was studied. Firstly, *t*-butanol was added to the crude extract at the ratios of crude extract to *t*-butanol of 1.0:0.5, 1.0:1.0, 1.0:1.5, and 1.0:2.0 (v/v) with a constant $(\text{NH}_4)_2\text{SO}_4$ saturation of 30%. The mixture was mixed thoroughly and then allowed to stand for 60 min before subjecting to centrifuge at $5000 \times g$ for 10 min to facilitate the separation of phases. The lower aqueous layer and the interfacial precipitate were collected and dialyzed against water overnight at 4°C . After dialysis, the samples were analyzed for protease activity and total protein content. The ratio employing the highest enzyme recovery was chosen for further study.

2.3.2. Effect of ammonium sulfate saturation on protease partitioning

The $(\text{NH}_4)_2\text{SO}_4$ saturations at 20, 30, 40, and 50% affecting the partitioning of protease was also investigated by using the ratio of crude extract to *t*-butanol (with the highest enzyme recovery) obtained from 2.3.1. The lower aqueous layer and the interfacial precipitate were collected and dialyzed against water overnight at 4°C . After dialysis, the samples were analyzed for protease activity and total protein content. The system providing the highest protease recovery was chosen for further study.

2.3.3. Optimization of TPP for proteases recovery

The phase obtained from the first TPP that gave the highest activity recovery was chosen as the starting material for optimization in the second TPP. The selected phase (without dialysis) was mixed with the *t*-butanol in ratio of 1.0:0.5 and $(\text{NH}_4)_2\text{SO}_4$ was added to the mixture to obtain the final saturations of 50, 55, 60, and 65%. After the complete phase separation, the phases were collected as previously mentioned. The dialyzed phases from the second TPP were subjected to protease activity and total protein content analysis.

2.4. Caseinolytic activity assay

An enzyme sample of 0.10 ml was mixed with 1.10 ml of 1% (w/v) casein in 0.10 M Tris–HCl (pH 8.0) containing 12 mM cysteine. The reaction was started by incubation the mixture at 37°C for 20 min. The reaction was stopped by adding 1.8 ml of 5% TCA. After centrifugation at $3000 \times g$ for 15 min, the absorption of the soluble peptides in supernatant was measured at 280 nm. One of caseinolytic activity units is defined as the amount of enzymes needed to produce an increment of 0.01 absorbance unit per minute at the assayed condition [13].

2.5. Protein determination

Protein concentrations were measured by Bradford method using BSA as a protein standard [14].

2.6. Electrophoresis

2.6.1. SDS-PAGE

SDS-PAGE of the samples was performed according to the method of Laemmli [15] with slight modification. Protein solutions were mixed at a 1:1 (v/v) ratio with the sample buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol). The samples (10 and $2\mu\text{g}$ protein for protein and activity staining, respectively) were loaded onto the gel made of 4% stacking and 15% separating gels. They were subjected to an electrophoresis set at a constant current of 20 mA/gel. For protein staining, the gel obtained after electrophoresis was stained overnight with a solution of 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol, and 7.5% (v/v) acetic acid. Gels were destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid for 40 min, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid for 20 min. Pierce blue prestained protein molecular weight marker mix consists of (kDa) myosin (215), phosphorylase B (120), BSA (84), ovalbumin (60), carbonic anhydrase (39.2), trypsin inhibitor (28) and lysozyme (18.3) was used.

2.6.2. Protease activity staining

The protease separated on the gel was verified by using activity staining as done in Garcia-Carreno et al. [16]. The gel was immersed in 50 ml of 2% (w/v) casein in 50 mM Tris–HCl buffer, pH 8.0 containing 12 mM cysteine for 30 min with constant agitation at 4°C . The reaction was generated by incubation the gel at 37°C for 15 min. The treated gel was then stained and destained as described above. The development of a clear band on the dark background indicated the caseinolytic activity of protease from *C. procera* latex.

3. Results and discussion

3.1. Effect of the ratio of crude extract to *t*-butanol on protease partitioning

The effect of crude extract to *t*-butanol ratio for protease partitioning in the first TPP was firstly investigated. Different TPP experiments with various extract to *t*-butanol ratios (1.0:0.5, 1.0:1.0, 1.0:1.5 and 1.0:2.0) at 30% (w/v) ammonium sulfate saturation were performed. The highest protease recovery (99.3%) and purification fold (1.13-fold) were obtained from the bottom phase of a TPP system with the ratio of 1.0:0.5. An increase in *t*-butanol volume resulted in a decrease in activity recovery and purification fold values of the bottom phase. This may be attributed to the synergistic effects of the increase in concentration of *t*-butanol and decrease in saturation of ammonium sulfate [5]. Dennison and Lovrien [6] reported that only 0.2–0.5 ml of *t*-butanol usually is required per milliliter of beginning aqueous sample to precipitate out protein. The best results were obtained when the ratio was 1.0:1.0 used for partitioning of α -galactosidase from fermented media of *Aspergillus oryzae* with 60% $(\text{NH}_4)_2\text{SO}_4$ saturation [4]. The ratio of sample to *t*-butanol content is higher than one, the denaturation of the protein is more likely [4]. The partitioning of protease in the interphase was contrary with the bottom phase. However, the yield obtained was still lower than 30% in all ratios tested.

As the results, most protease was still remained in the aqueous phase rather than in the interphase. Therefore, the ratio of crude extract to *t*-butanol of 1.0:0.5 which provided the highest recovery

was selected for investigating the effect of $(\text{NH}_4)_2\text{SO}_4$ saturation on partitioning of *C. procera* protease.

3.2. Effect of ammonium sulfate saturation on protease partitioning

The study was performed by maintaining the ratio of crude extract to *t*-butanol at 1.0:0.5 and varying the $(\text{NH}_4)_2\text{SO}_4$ saturation in the ranges of 20, 30, 40, and 50%. Generally, one starts with a minimum salt saturation of 20% and optimizes this so as to obtain the maximum amount of the desired protein in the interfacial precipitate [1,2]. It was found that the protease recovery of 182% yield with 0.95 folds of purification was obtained in the interphase of the system contained 50% $(\text{NH}_4)_2\text{SO}_4$. Increase of $(\text{NH}_4)_2\text{SO}_4$ saturation resulted in increase of protease recovery in the interphase. However, the purity of enzyme seemed to be reduced when $(\text{NH}_4)_2\text{SO}_4$ saturation was raised. Narayan et al. [5] reported that the increasing in concentration of $(\text{NH}_4)_2\text{SO}_4$, the degree of purification decreased significantly. As in conventional salting out with $(\text{NH}_4)_2\text{SO}_4$, the extent of protein precipitation in TPP is a function of the $(\text{NH}_4)_2\text{SO}_4$ concentration [17]. The “salting out effect” plays a major role in the system contained high salt concentration. The principle of sulfate ion for salting out protein has been viewed in five different ways namely, ionic strength effects, kosmotropy, cavity surface tension and enhancement osmotic stressor (dehydration), exclusion crowding agent, and binding of sulfate ion to cationic sites of protein [5].

As the result obtained, the system consisted of 50% $(\text{NH}_4)_2\text{SO}_4$ was seemed to be the optimum condition for protease partitioning due to its highest protease yield at the interphase. However, this fraction gave low enzyme purity. While the highest purity was found in the bottom phase of the system contained 30% $(\text{NH}_4)_2\text{SO}_4$. This system was also belonged to the highest yield among all of the bottom phase. Pike and Dennison [18] also reported that 30% (w/v) $(\text{NH}_4)_2\text{SO}_4$ is the best salt concentration for carrying out TPP efficient.

3.3. Optimization of TPP for recovery of protease from *C. procera* latex

The system condition from the first TPP (1.0:0.5 extract: *t*-butanol (v/v), 30% $(\text{NH}_4)_2\text{SO}_4$ saturation) providing the highest protease recovery in the aqueous phase was used as the starting

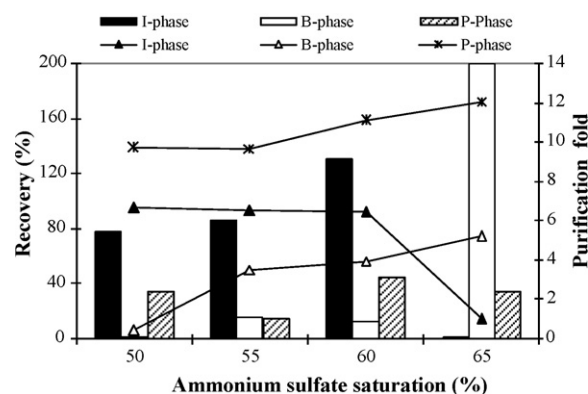


Fig. 1. Optimization of $(\text{NH}_4)_2\text{SO}_4$ saturation for protease partitioning by the second step three-phase partitioning. The bottom phase of the system of 1.0:0.5 extract: *t*-butanol in the presence 30% $(\text{NH}_4)_2\text{SO}_4$ was used as the starting material for the second TPP. Bar [■] and line [—] represent the protease recovery and purification fold, respectively. I-phase: interphase, B-phase: bottom phase and P-phase: precipitate.

material for optimization of enzyme partitioning in the second TPP. The reason for carrying out the second cycle of TPP was that sometimes the first TPP cycle could not remove the contaminant proteins efficiently. It was expected that the target protease will be more partitioned to the interphase with the highest yield and purity. Saxena et al. [19] reported that the aqueous phase containing most of the desired protein when subjected to a second cycle of TPP results in considerable purification of the target protein. Therefore, in this study, $(\text{NH}_4)_2\text{SO}_4$ was added to the aqueous phase of the first TPP fraction to obtain the final saturation of 50, 55, 60, and 65%. Interestingly, it was found that the precipitated phase was also observed as the fourth phase. This can be explained by increase of salt addition provided over salting out effect and protein subsequently precipitated as indicated by the fourth phase occurred. As shown in Fig. 1, increasing of purification fold in the precipitate as well as in the bottom phase was found when the salt content increased. At the salt content of 65%, most protease was recovered in the bottom phase (202.7%) with a little remaining in the interphase (1.1%). However, residue protease was precipitated (35.2%) with the highest purification of 12.79 folds. Kiss et al. [20] concluded that the amount of protein precipitated in the interphase was delineated as a function of the composition of the partitioning system as well as the initial protein concentration.

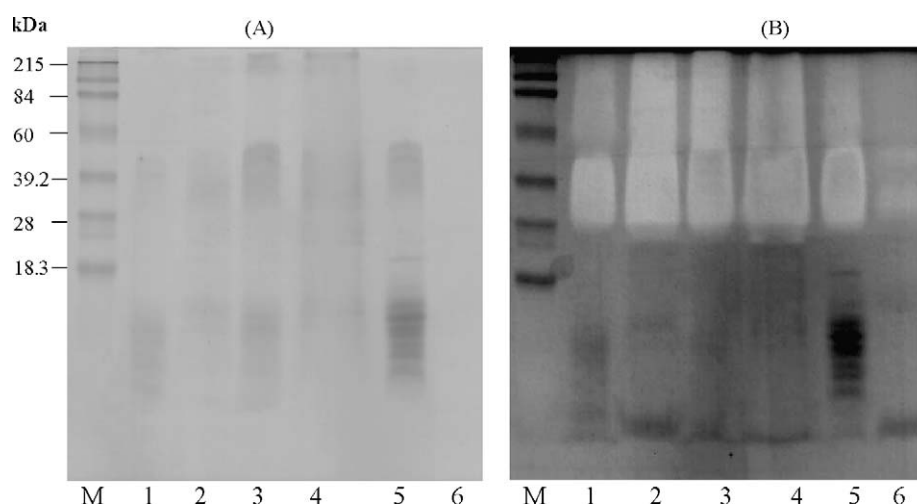


Fig. 2. Protein pattern (A, 10 μg protein) and activity staining (B, 2 μg protein) of *Calotropis procera* latex and their fractions from three-phase partitioning. M: molecular weight marker, 1: crude latex, 2: interphase of 1.0:0.5 *t*-butanol–50% $(\text{NH}_4)_2\text{SO}_4$, 3: interphase of 60% $(\text{NH}_4)_2\text{SO}_4$, 4: precipitate of 60% $(\text{NH}_4)_2\text{SO}_4$, 5: bottom phase of 1.0:0.5 *t*-butanol–50% $(\text{NH}_4)_2\text{SO}_4$, and 6: bottom phase of 60% $(\text{NH}_4)_2\text{SO}_4$.

Table 1Recovery of protease from *Calotropis procera* latex in the interphase (I) of TPP.

Sample	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude latex	5,633 ± 107	18.48 ± 1.34	304.82 ± 20.54	1.00 ± 0.00	100 ± 0.0
I-1st TPP-50% AS ^a	10,252 ± 244	35.40 ± 0.32	289.58 ± 4.33	0.95 ± 0.03	182 ± 2.1
I-2nd TPP-60% AS ^b	7,445 ± 505	3.53 ± 0.05	2,110.00 ± 110.00	6.92 ± 0.34	132 ± 8.9

^a The system consists of the ratio of crude extract to *t*-butanol of 1.0:0.5–50% (NH₄)₂SO₄.^b The system consists of the ratio of bottom phase of the first TPP (1.0:0.5 *t*-butanol, 30%, saturation of (NH₄)₂SO₄) with the addition of (NH₄)₂SO₄ to 60%. I: interphase, AS: (NH₄)₂SO₄.

Increasing activity recovery was found in the interphase of the system containing (NH₄)₂SO₄ less than 60% (Fig. 1). As expected, the highest yield (132%) and high enzyme purity (6.92 folds) was found in the interphase of the system added with 60% (NH₄)₂SO₄. In view of interest, the second TPP system containing the crude extract to *t*-butanol ratio of 1.0:0.5 with the addition of (NH₄)₂SO₄ up to 60% was efficient to partial purified the protease from *C. procera*. Narayan et al. [5] reported that the optimum condition for extraction peroxidase from the leave of *Ipomoea palmate* consisted of the crude extract to *t*-butanol of 1.0:1.0 and 30% (NH₄)₂SO₄. By using this condition resulted in about 160% activity recover and 2-fold of purification in the aqueous phase of the first TPP.

Protein patterns of the crude extract and their fractions from the first TPP and second TPP are shown in Fig. 2A. The major protein bands with the molecular weight (MW) of around 39–50 and less than 18.3 kDa were found as the major component in the crude extract (lane 1A). It was observed that the protein pattern of the precipitate fraction (lane 4A) was present as higher band intensity than those of the crude extract and other fractions. For the first TPP containing the crude extract to *t*-butanol at the ratio of 1.0:0.5 with 50% (NH₄)₂SO₄, a numbers of protein band mostly presented in the bottom phase (lane 5A) than in the interphase (lane 2A). There are previous reports that the maximum proteolytic activity of *C. procera* protein found a protein band possessing around 30 kDa [20,21]. It was reported that the purified protease from *C. procera* appeared as a single band at around 29 kDa in a reducing condition of SDS-PAGE [11].

Fig. 2B shows the activity staining (zymography) of the crude extract and the fractions from TPP optimization. Clear zone on the dark background was remarked as the activity of protease against casein substrate on the gel. A protein band with the molecular weight of ~30 kDa was clearly observed in all fractions. Depending on the intensity of the clear zone, the fraction of the interphase provided interesting protease activity (lanes 2B and 3B) as expected. The results revealed that TPP was able to partition the proteases into the interphase of system in which the salt and *t*-butanol content was optimized.

3.4. Overall partial purification of protease from *C. procera* latex by TPP

The isolation of protease from *C. procera* latex (by the first TPP with 50% (w/v) (NH₄)₂SO₄ and 1.0:0.5 ratio of the crude extract to *t*-butanol) gave the highest protease recovery (182%) in the interphase (Table 1). However, for further optimization of enzyme partitioning in the second TPP, the bottom phase of the system containing 30% saturated (NH₄)₂SO₄ was used as it provided the highest yield among the bottom phases of all conditions tested. Performing the second TPP with 60% saturated (NH₄)₂SO₄ resulted in 132% recovery in the interphase with higher purity (6.92 folds). High protease recovery obtained may probably due to the TPP led to simultaneous activation of the enzyme which results of such an apparently observed value more than 100%. Singh et al. [22] reported that the tertiary structure of serin protease (proteinase K) was altered by TPP, reflecting remarkable enhancement of the enzyme activity. Dennison and Lovrein [6] reported that *Bacillus*

subtilis protease, *Saccharomyces cerevisiae* invertase, *Candida cylindracea* lipase gave a yield of 300, 100 and 900%, respectively, after TPP. These unusual percentage yields were believed to be due to adopting a number of amino acid residues on the enzyme molecule. Sharma and Gupta [1,2] also reported that enzyme activation frequently observed during TPP may be a result of increased flexibility in the enzyme molecule.

From the second TPP, the overall result of protease activity recovery, as well as of final purity, is considerably better than the first TPP. Roy and Gupta [3] reported that using TPP for biological activity recovery provided the best results with high recovery activity (90–98%) and high purification fold (65–100-fold). Increasing of purification fold of 18 times with 81% activity recovery was achieved with the second TPP. Saxena et al. [19] also found that by using the second TPP can increase the purification fold of amylase inhibitor and trypsin inhibitor from 8.9 and 8.65-fold to 20.1 and 16-fold purification, respectively.

To compare between the first and second TPP in terms of recovery and purity of enzyme from *C. procera*, the first TPP possessed higher protease yield of 182% than that of the second TPP (132%). In addition, purity of enzyme was increased from 0.95 folds in the first step to 6.92 folds in the second step. This indicates that purification of enzyme was greatly obtained during operation of the second TPP. It can be pointed that only first step was sufficient when consideration of enzyme recovery. However, when regarding to the purity of the enzyme, the second step should be needed. Thus procedures utilizing the two cycles of TPP constitute a fairly simple and efficient isolation process for protease and they also yield a partial purified protein. The availability of this simple partial purification strategy should be employed in production of protease with a more efficient and economic way.

4. Conclusion

The TPP was a useful technique for partial purification of protease from *C. procera*. It is a simple, quick and economical technique and scaling up is convenient. The TPP system consists of the ratio of crude extract to *t*-butanol of 1.0:0.5 and 50% (w/v) (NH₄)₂SO₄ gave the highest yield but rather low purity in the interphase. Increasing the (NH₄)₂SO₄ saturation of the bottom phase to the first TPP up to 60% could improve the purity of the enzyme up to 6.92 folds.

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Trypsin Inhibitor from 3 Legume Seeds: Fractionation and Proteolytic Inhibition Study

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ABSTRACT: The trypsin inhibitor from navy beans (*Phaseolus vulgaris*), red kidney beans (*Phaseolus vulgaris* L.), and adzuki beans (*Vigna angularis*) provided by the Royal Project Foundation in Thailand was isolated by heat and ammonium sulfate (AS) precipitation. Incubation at 70 °C for 10 min produced the highest trypsin inhibitor recovery for all legumes. The AS precipitation with 60% to 80% saturation (precipitate IV) resulted in 41-, 88-, and 34-fold of the purity and (–)26%, 126%, and (–)47% of percentage of activity increase for navy beans, red kidney beans, and adzuki beans, respectively. The trypsin inhibitors had a molecular weight of 132 kDa for navy beans, 118 kDa for red kidney beans, and 13 kDa for adzuki beans under nonreducing conditions. The obtained precipitate IV fraction from each legume effectively prevented the degradation of the tilapia muscle with concentration dependent. The myosin heavy chain increased as the concentration of the inhibitor fraction increased, especially at the highest level of addition. The result indicated that the precipitate IV from these legumes have potential for use as a protease inhibitor in fishery related products.

Keywords: adzuki beans, legume, navy beans, proteolytic, red kidney beans, trypsin inhibitor

Introduction

Since Kunitz isolated the soybean trypsin inhibitor in 1945, inhibitors of proteolytic enzymes have been shown to generally occur in nature. Recently, the number of isolated, purified, characterized and utilized trypsin inhibitors from legumes has increased substantially. Protease inhibitors contained in legumes that are specific for serine protease were categorized into 2 families, the Kunitz trypsin inhibitor with a molecular weight (MW) of about 20 to 24 kDa and the Bowman–Birk inhibitor (BBI) with a MW of approximately 7 to 8 kDa (Birk 2003). Varieties of legume seeds are used as a natural source for trypsin inhibitor extraction and characterization. Trypsin inhibitor has been isolated from *Swartzia pickellii* (Cavalcanti and others 2002), *Crotalaria paulina* (Pando and others 2008), mungbean (Kansal and others 2008), cowpea (Samour 2005), *Cassia obtusifolia* (Liao and others 2007). However, soybean trypsin inhibitor is the most widely used and commercially produced. Thermal precipitation successfully isolated some protease inhibitors such as BBI from Jobs' tears seeds (Ary and others 1988); Thermal precipitation has also been found to isolate trypsin inhibitor from cowpea, pigeon pea, and bambara groundnut (Benjakul and others 1999a). In addition, trypsin inhibitor from *Cassia obtusifolia* (Liao and others 2007), mung bean (Wang and others 2006) and *Albizia kalkora* (Zhou and others 2008) was successfully fractionated using ammonium sulfate saturations of 50%, 60%, and 80% (w/v).

Proteolysis may be an unwanted process in some food processing. Martinez and Gildberg (1988) reported that the breakdown of connective tissue and myofibrillar protein in fish may cause the stomach and intestine to burst, leading to leakage of

proteolytic enzymes from the digestive tract into the surrounding tissues. Endogenous serine proteases were found to cause continual degradation of myofibrillar proteins in the muscle model system (Gómez-Guillén and others 2002; Benjakul and others 2003; Ohkubo and others 2008; Yarnpakdee and others 2009). Using proteinase inhibitors to control proteolysis in muscle model systems has been extensively reported. Ramírez and others (2002) reported that crude extracts from kidney beans, peas, chickpeas, lentils, and soybeans were able to inhibit proteolytic activities in Mexican flounder and Atlantic croaker. Proteinase inhibitor derived from black cowpea and soybean seeds showed inhibitory activity against viscera proteinases of threadfin bream, manifested by a reduction in modori-inducing proteinase activity (Benjakul and others 1999b). Alarcón and others (2001) also reported 10 µL of pigeon pea, guamuchil, ironwood, palo blanco, mesquite, soybean, chickpea, or green pea extract inhibited digestive proteases of yellow snapper and dog snapper by 40% to 80%; however, there is less information about using protease inhibitor fractions obtained from all of these legumes (navy bean, red kidney bean and adzuki bean) to inhibit unwanted proteolysis in fresh water fish muscle. Our objective was to isolate and evaluate the effect of trypsin inhibitor fractions from three legumes from The Royal Project Foundation for proteolytic inhibition of the tilapia muscle.

Materials and Methods

Chemicals and raw materials

N-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA); trypsin from the bovine pancreas; β-mercaptoethanol (βME); casein from bovine milk; bovine serum albumin (BSA); coomassie brilliant Blue R-250 and *N*, *N*', *N*', *N*'-tetramethyl ethylene diamine (TEMED) were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Sodium dodecyl sulfate (SDS) was procured from Fluka Chemical Co. (Ronkonkoma, N.Y., U.S.A.). Navy beans (*Phaseolus vulgaris*), red kidney beans (*Phaseolus vulgaris* L.), and adzuki beans (*Vigna angularis*) were obtained from The Royal Project Foundation,

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Chiang Mai, Thailand. Nile tilapia (*Tilapia nilotica*) was obtained from Lotus supermarket, Chiang Rai, Thailand.

Fractionation of trypsin inhibitor from legume seeds

Crude extract preparation. Whole legume seeds were ground to a particle size of 20 meshes using a coffee mill. Seed flour was defatted by mixing it with hexane for 10 min at a ratio of 1 : 5 (w/v). The mixture was filtered through Whatman nr 1 filter paper and the sediment was rinsed 3 times with hexane to remove the residual oil in the ground sample. The defatted sample was air-dried at room temperature (approximately 28 °C) until dry and free of hexane odor. The defatted samples were extracted with 0.02 M NaOH for the navy beans and red kidney beans and with water for the adzuki beans at the ratio 1 : 5 (w/v) and then shaken at 180 rpm for 2 h at room temperature, which gives the highest trypsin inhibitor recovery according to the previous studies (Wati and others 2009). The supernatant was recovered by centrifuging at $8000 \times g$ for 30 min. This supernatant was referred to as “a crude extract” and used for further fractionation processes.

Heat treatment precipitation. The crude extracts were heated between 50 and 70 °C for 10 min and then cooled with iced water (Benjakul and others 1999a). To remove coagulated debris, the extracts were centrifuged at $8000 \times g$ for 5 min at room temperature. The total inhibitory activity and the specific inhibitory activity of the trypsin inhibitor in the supernatant were determined. The highest specific activity of the heat treatment fraction was selected for the study.

Ammonium sulfate (AS) fractionation. Heat treated fraction was added to solid AS to reach a concentration of 20% (w/v) saturation. The mixture was stirred at 4 °C for 2 h and centrifuged at $7000 \times g$ for 15 min at 4 °C. The precipitated protein was collected and referred to as “precipitate I: 0% to 20% AS.” The 20% saturated AS supernatant was treated with solid AS to reach 40% saturation and the protein precipitate was referred to as “precipitate II: 20% to 40% AS.” The proteins in the supernatant were further fractionated with 60% and 80% (w/v) saturation and the pellets were referred to as “precipitates III: 40% to 60% AS” and “precipitate IV: 60% to 80% AS,” respectively. The supernatant obtained after adding 80% (w/v) saturation AS was referred to as “supernatant IV: > 80% AS.” All precipitates were dissolved in a 10 mM phosphate buffer (pH 7.4), containing 0.9 mM CaCl_2 and 0.05 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ at ratio of 1 : 9 (w/v). All fractions were dialyzed against the same buffer overnight at 4 °C with the dialysis buffer being changed 4 times to remove residual AS. It was stored at 4 °C until used.

Trypsin inhibitory activity assay

The trypsin inhibitor activity in all of the bean fractions were measured by using BAPNA as a substrate according to the method used by Benjakul and others (1999a). A solution containing 100 μL of the sample (0.5 to 0.6 mg protein), 200 μL (20 $\mu\text{g}/\text{mL}$) trypsin, and 100 μL of distilled water was preincubated at 37 °C for 10 min. Then, 500 μL (0.4 mg/mL) of BAPNA was added to start the reaction. After incubating at 37 °C for 10 min, 100 μL 30% (v/v) acetic acid were added to terminate the reaction; it was then subjected to centrifugation at $2000 \times g$ for 10 min. The trypsin activity was measured by absorbance at 410 nm due to the released of *p*-nitroaniline. One unit of trypsin inhibitor was measured at a decrease of 0.01 in absorbance at 410 nm under assay conditions compared with the control (without inhibitor) sample.

Protein determination

Protein concentration was determined by the Biuret method (Gornall and others 1948), using BSA as a standard.

Electrophoresis

Protein pattern. SDS-PAGE was carried out by the Laemmli (1970) method, using 15% (v/v) separating and 4% (v/v) stacking gels. The samples were mixed with the sample buffer (0.5 M Tris HCl, pH 6.8, glycerol, 10% (w/v) SDS, and bromophenol blue) without a reducing agent. Using Mini Protean Tetra Cell unit (Bio-Rad Laboratories, Inc., Richmond, Calif., U.S.A.), 20 μg of protein were loaded and then separated at 15 mA/gel. After separation, the protein was stained with Coomassie Brilliant Blue R-250 and destained with a methanol-acetic solution.

Inhibitory activity staining. For inhibitory activity staining, the protein separated by electrophoresis was subjected to incubation with 50 mL of a mixture of 0.2 mg/mL trypsin solution in Tris-HCl pH 8.3 for 60 min at 4 °C to allow the trypsin to diffuse into the gel. It was then washed with distilled water. The gel was incubated at 37 °C for 90 min with 1% (w/v) casein in 0.1 M phosphate buffer (pH 6), and then it was rinsed with distilled water, fixed, and stained with Coomassie Brilliant Blue R-250 to display inhibitory zones. The apparent molecular weight of the trypsin inhibitor was estimated by comparing the R_f with those of the protein standard marker.

Effect of isolated trypsin inhibitor in the muscle model system

Preparation of crude trypsin. Crude trypsin was prepared from tilapia viscera according to the method of Bezerra and others (2004). Viscera (10 g) was collected and homogenized with

Table 1 – Effect of heat treatment on recovery of trypsin inhibitor from 3 legume seeds.

Sample	Treatment ^A	Total protein (mg)	Total inhibitory activity ($\times 10^3$ units)	Specific inhibitory activity (u/mg)	Increasing activity ^B (%)
Navy beans	Crude	1785 $\pm$ 38b ^C	564 $\pm$ 21a	316 $\pm$ 7.6a	-
	50 °C	1738 $\pm$ 44b	756 $\pm$ 72a	435 $\pm$ 51.9b	34.7 $\pm$ 17.2a
	70 °C	1616 $\pm$ 47a	1446 $\pm$ 93b	895 $\pm$ 74.0c	156.8 $\pm$ 23.0b
Red kidney beans	Crude	2352 $\pm$ 19b	481 $\pm$ 30a	205 $\pm$ 10.3a	-
	50 °C	2243 $\pm$ 8a	678 $\pm$ 80a	302 $\pm$ 34.2b	41.9 $\pm$ 24.4a
	70 °C	2152 $\pm$ 57a	1690 $\pm$ 99b	785 $\pm$ 60.4c	252.8 $\pm$ 41b
Adzuki beans	Crude	1076 $\pm$ 76b	595 $\pm$ 42a	553 $\pm$ 78.2a	-
	50 °C	823 $\pm$ 40b	614 $\pm$ 135a	746 $\pm$ 196.0a	15.4 $\pm$ 9.9a
	70 °C	390 $\pm$ 7a	1214 $\pm$ 107b	3113 $\pm$ 109.2b	104.3 $\pm$ 18.0b

^ACrude extracts with the best condition were heated at 70 °C for 10 min and then cooled with ice water. The heated extracts were centrifuged at $8000 \times g$ for 15 min to remove the heat coagulated debris.

^BIncreasing activity = [(total activity of the treatment – total activity of the starting material)/total activity of the starting material] $\times$ 100.

^CDifferent letters in the same legume indicate significant differences ($P < 0.05$).

Trypsin inhibitor from 3 legume seeds . . .

250 mL of 0.9% (w/v) NaCl using a homogenizer. The homogenate was centrifuged at $10000 \times g$ for 10 min at 4 °C to remove cell debris and nuclei. The supernatant obtained was incubated at 45 °C for 30 min and centrifuged at $10000 \times g$ for 10 min at room temperature. The supernatant was used as “a crude trypsin” for further study. One unit (U) of trypsin activity was defined as the amount of enzyme capable of hydrolyzing casein to produce a 0.001 change in absorbance per minute.

Minced fish preparation. The fish was skinned and gutted to obtain a fillet. The fillet was washed with tap water to remove loose scales, blood or remaining viscera and then minced in a meat mincer. The sample obtained was referred to as “minced tilapia.”

Proteolysis study of minced tilapia. The minced tilapia (2 g) was weighed precisely and mixed with 55 units of crude trypsin (obtained from the previous step to 2000 μ L and the ratio of the meat to enzyme was 1 : 1). It was then incubated at 50 °C at different time intervals (10, 20, 30, 60, 90, 120, and 180 min). Total of 18 mL of 5% SDS solution (85 °C) was added before being homogenized at 10000 rpm for 1 min. The homogenate was then incubated at 85 °C in a water bath for 1 h to solubilize the protein. To remove undissolved debris, the mixture was centrifuged at $5000 \times g$ for 10 min. The supernatant was subjected to protein pattern analysis by the SDS-PAGE method as previously mentioned.

Proteolytic inhibition of minced tilapia by precipitate IV fraction. The proteolytic inhibition of minced tilapia by precipitate IV fraction from each legume was measured according to the method of Rawdkuen and others (2007). The inhibitor fraction of navy beans, red kidney beans and adzuki beans (100, 300, 500, 700, 1000, 2000 μ L) was added to 2 g of minced fish. The bean and fish sample was mixed and incubated in a water bath at 50 °C for 2 h. Proteolysis was terminated by adding 18 mL of 5% SDS solution (85 °C). The proteolytic inhibition pattern of the inhibitor fraction was measured by subjecting the solubilized protein to SDS-PAGE.

Statistical analysis

All experiments were conducted and analyzed in triplicate. Means and standard deviations were calculated using Duncan's test. Analysis was performed using an SPSS package (SPSS 10.0 for Windows, SPSS Inc., Chicago, Ill., U.S.A.).

Results and Discussion

Fractionation of trypsin inhibitor from legume seeds

Heat treatment. The seed extracts were subjected to heat treatment at 50 and 70 °C for 10 min. The inhibitory activity increased when the temperature increased to 70 °C. Extraction at 70 °C from

Table 2 – Ammonium sulfate fractionation of trypsin inhibitor from 3 legume seeds.

Sample	Fraction ^A	Total protein (mg)	Total inhibitory activity ($\times 10^3$ units)	Specific inhibitory activity (u/mg)	Increasing activity ^B (%)	Purification (fold) ^C
Navy beans	Heat treated	1616 $\pm$ 47e	1446 $\pm$ 93b	895 $\pm$ 74a ^D	-	1.0 $\pm$ 0.0a
	Precipitate I	440 $\pm$ 6c	4411 $\pm$ 229d	10025 $\pm$ 563b	205 $\pm$ 19c	11.2 $\pm$ 0.2b
	Precipitate II	393 $\pm$ 6c	4673 $\pm$ 343d	11890 $\pm$ 838ab	223 $\pm$ 17c	13.3 $\pm$ 0.4bc
	Precipitate III	240 $\pm$ 67b	2404 $\pm$ 121c	10016 $\pm$ 3230ab	66 $\pm$ 10b	11.2 $\pm$ 3.3bc
	Precipitate IV	32 $\pm$ 2a	417 $\pm$ 26a	13031 $\pm$ 612c	(-)71 $\pm$ 2a	14.6 $\pm$ 0.5c
	Supernatant IV	656 $\pm$ 20d	2180 $\pm$ 394c	3323 $\pm$ 491a	50 $\pm$ 22b	3.7 $\pm$ 0.4a
Red kidney beans	Heat treated	2152 $\pm$ 57e	1690 $\pm$ 99b	785 $\pm$ 60a	-	1.0 $\pm$ 0.0a
	Precipitate I	657 $\pm$ 19d	4469 $\pm$ 5d	6802 $\pm$ 263c	165 $\pm$ 16c	8.7 $\pm$ 0.5b
	Precipitate II	518 $\pm$ 3c	6196 $\pm$ 70e	11961 $\pm$ 355d	267 $\pm$ 19d	15.2 $\pm$ 1.8c
	Precipitate III	138 $\pm$ 0.5b	1,015 $\pm$ 3a	7,355 $\pm$ 96b	(-)40 $\pm$ 3a	9.4 $\pm$ 0.7b
	Precipitate IV	83 $\pm$ 3a	1086 $\pm$ 7a	13084 $\pm$ 1235e	(-)36 $\pm$ 4a	16.7 $\pm$ 0.8d
	Supernatant IV	671 $\pm$ 14a	3205 $\pm$ 125c	4776 $\pm$ 349bc	90 $\pm$ 7b	6.1 $\pm$ 1.2b
Adzuki beans	Heat treated	390 $\pm$ 7d	1214 $\pm$ 107d	3113 $\pm$ 109b	-	1.0 $\pm$ 0.0b
	Precipitate I	-	-	-	-	-
	Precipitate II	34 $\pm$ 0.4b	127 $\pm$ 6a	3735 $\pm$ 139b	(-)89 $\pm$ 0.9a	1.2 $\pm$ 0.0b
	Precipitate III	144 $\pm$ 2c	597 $\pm$ 33c	4145 $\pm$ 218b	(-)51 $\pm$ 4c	1.3 $\pm$ 0.0b
	Precipitate IV	17 $\pm$ 1a	316 $\pm$ 17b	18588 $\pm$ 1534c	(-)74 $\pm$ 2b	6.0 $\pm$ 0.2c
	Supernatant IV	460 $\pm$ 4e	292 $\pm$ 24b	635 $\pm$ 156a	(-)76 $\pm$ 4b	0.2 $\pm$ 0.0a

^AI-IV: fraction obtained by ammonium sulfate (AS) fractionation: precipitate I (0-20), precipitate II (20-40), precipitate III (40-60), precipitate IV (60-80), and 60-80 supernatant.

^BIncreasing activity = [(total activity of the treatment – total activity of the starting material)/total activity of the starting material] $\times$ 100.

^CPurification (fold) = [specific activity of treatment/specific activity of starting material].

^DDifferent letters in the same legume indicate significant differences ($P < 0.05$).

Table 3 – Fractionation of trypsin inhibitor from 3 legume seeds.

Sample	Step	Total protein (mg)	Total inhibitory activity ($\times 10^3$ units)	Specific inhibitory activity (u/mg)	Increasing activity ^A (%)	Purification (fold) ^B
Navy beans	Crude	1785 $\pm$ 38c	564 $\pm$ 21b	316 $\pm$ 8a ^E	-	1.0 $\pm$ 0.0a
	^C Heat treatment	1616 $\pm$ 47b	1446 $\pm$ 93c	895 $\pm$ 74b	156.8 $\pm$ 23b	2.8 $\pm$ 0.2a
	^D AS precipitation	32 $\pm$ 2a	417 $\pm$ 26a	13031 $\pm$ 612c	(-)25.9 $\pm$ 7a	41.2 $\pm$ 2.5b
Red kidney beans	Crude	2352 $\pm$ 19c	481 $\pm$ 30a	205 $\pm$ 10a	-	1.0 $\pm$ 0.0a
	^C Heat treatment	2152 $\pm$ 57b	1690 $\pm$ 99c	785 $\pm$ 60b	252.8 $\pm$ 41b	3.8 $\pm$ 0.4a
	^D AS precipitation	83 $\pm$ 3a	1086 $\pm$ 7b	18084 $\pm$ 1235c	126.2 $\pm$ 14a	88.2 $\pm$ 9.7b
Adzuki beans	Crude	1076 $\pm$ 76c	595 $\pm$ 42b	553 $\pm$ 78a	-	1.0 $\pm$ 0.0a
	^C Heat treatment	390 $\pm$ 7b	1214 $\pm$ 107c	3113 $\pm$ 109b	104.3 $\pm$ 18b	5.6 $\pm$ 0.9a
	^D AS precipitation	17 $\pm$ 1a	316 $\pm$ 17a	18588 $\pm$ 1534c	(-)46.6 $\pm$ 6a	33.6 $\pm$ 7.4b

^AIncreasing activity = [(total activity of the treatment – total activity of the starting material)/total activity of the starting material] $\times$ 100.

^BPurification (fold) = [specific activity of treatment/specific activity of starting material].

^CCrude extracts with the best condition were heated at 70 °C for 10 min and then cooled with ice water. The heated extracts were centrifuged at $8,000 \times g$ for 15 min to remove the heat coagulated debris.

^DFraction obtained by ammonium sulfate (AS) fractionation (precipitate IV; 60-80).

^EDifferent letters in the same legume indicate significant differences ($P < 0.05$).

Trypsin inhibitor from 3 legume seeds . . .

the present study produced the highest specific inhibitory activity compared to 895 and 785 as well as 3113 for navy beans, red

kidney beans, and adzuki beans, respectively (Table 1). The highest and lowest percent increase activity was found in red kidney beans (253%) and adzuki beans (15%), respectively. However, the heat might have caused the trypsin inhibitor to loosen its compact structure, which is normally stabilized by a number of disulfide bonds (Godbole and others 1994). Benjakul and others (1999a) reported that the trypsin inhibitory activity of some legumes increased slightly after heat treatment at 60 °C, but decreased when extracts were heated at 100 °C. Other studies reported that heat treatment leads to the conformational changes in many proteins (Lin and others 1991; Baptista and others 2003). Prakash and Narasinga Rao (1988) also reported noncovalent interaction, especially for hydrophobic interaction, can be disrupted by heating above 50 °C, causing a release of some target proteins. Dissociation of high molecular size protein also depends on pH, ionic strength, protein concentration, and temperature.

Ammonium sulfate (AS) fractionation. The heat treated sample (70 °C for 10 min) with the highest specific activity was subjected to AS precipitation. The highest purification fold was found in precipitate IV (60% to 80% AS saturation) for all legume seeds (Table 2); however, high purity resulted in lower increasing activity (%) compared to the starting material. The highest % activity increase was found in precipitate II for navy beans and red kidney beans, while precipitate III gave the highest percentage of activity increase for adzuki beans. From this result, it can be stated that the trypsin inhibitor from the 3 legumes is more concentrated in precipitate IV (60% to 80% AS saturation).

Thermal treatment and AS fractionation (heating at 70 °C for 10 min) with AS precipitation at 60% to 80% saturation were used for trypsin inhibitor fractionation. The fraction results are shown in Table 3. Purification folds of 41, 88, and 34 with the increasing activity of (–)26%, 126%, and (–)47% were obtained for navy beans, red kidney beans, and adzuki beans, respectively. Of the 3 legumes, red kidney bean showed the highest recovery as indicated by their highest percent activity increase, followed by navy beans and adzuki beans. Nevertheless, the highest specific inhibitory activity was obtained from adzuki beans. Different protein compositions in each legume resulted in different specific precipitations when salt was added. In general, water molecules are removed from the

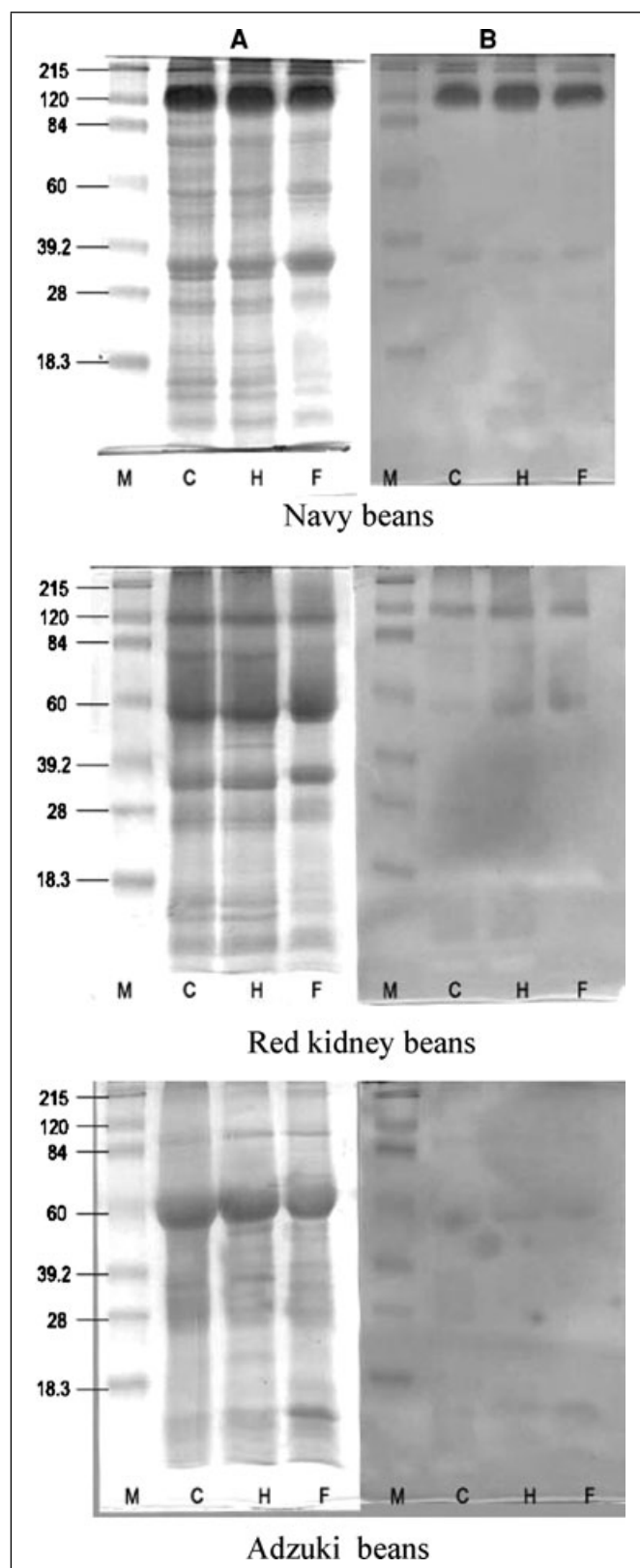


Figure 1—Protein pattern (A) and inhibitory activity staining (B) of crude and protein fractions of 3 legume seeds. (M) molecular weight marker; (C) crude extract; (H) heat treatment fraction (70 °C for 10 min); (F) precipitate IV (60% to 80% AS saturation).

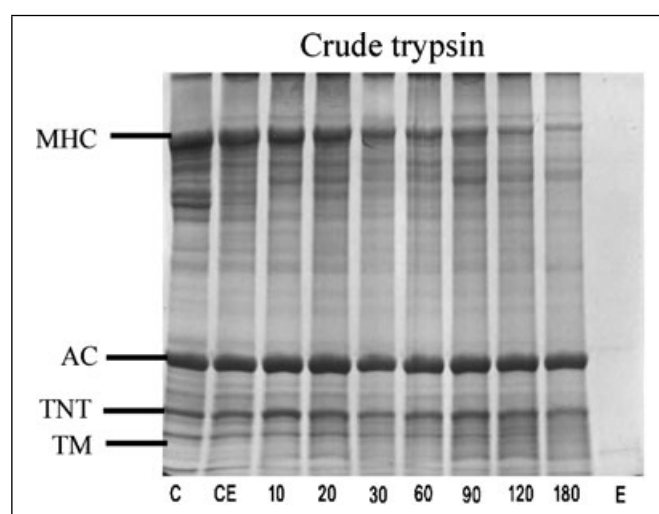


Figure 2—Proteolysis pattern of tilapia muscle treated with crude trypsin at 50 °C for different incubation times. (C) control without incubation; (CE) control with enzyme at 0 min; (E) crude trypsin. Numbers (10 to 180) denote the incubation time (min). (MHC) myosin heavy chain; (AC) actin; (TNT) troponin-T; (TM) tropomyosin.

protein's surface, and hydrophobic patches are exposed by surface-located residues such as leucine, valine, phenylalanine, and so on. These then interact with each other, causing the aggregation of the protein present. Salting out by ammonium sulfate precipitation results in separation, with the more hydrophobic proteins precipitating first, and the least hydrophobic proteins precipitating last (Scopes 1996).

Protein and inhibitory pattern of fractionated trypsin inhibitor

Protein pattern. SDS-PAGE patterns of the fraction from navy beans, red kidney beans and adzuki beans are shown in Figure 1A. For navy beans the precipitate IV (60% to 80% AS, lane F) consisted of the major protein components with MW of 215, 132, and 34 kDa. Many minor protein bands (MW < 28 kDa) disappeared in the precipitate IV fraction (lane F) when compared with the crude extract (lane C) or heat precipitate fraction (lane H). AS precipitation could remove the small protein molecule of the red kidney beans, leaving only the 3 major bands with MWs of 118, 59, and 32 kDa. The protein pattern from extracted adzuki beans also showed the same result at the crude extract. However, the numbers of bands were removed by fractionation. Major protein bands with MW of 62 kDa were found in the precipitate IV and in the crude extract. Meanwhile, the minor protein band with the MW of 13 kDa appeared after the heat treated fraction was fractionated using 60% to 80% AS saturation (lane F).

Inhibitory activity staining. For the inhibitor activity stained gel, trypsin inhibitors with different apparent molecular sizes were found in different legume extracts (Figure 1B). For navy beans, a protein band with the MW of 132 kDa was an inhibitor that remained after being treated with the gel with trypsin. A single band of inhibitor with apparent MW of 118 kDa was observed in the red kidney beans, whereas a band with apparent MW of 13 kDa was found in the adzuki beans extract. The bands that still retained inhibitory activity staining were proteins that could not be hydrolyzed by trypsin in the assay conditions.

Proteolysis of minced tilapia

Figure 2 shows the proteolysis of tilapia muscle in the presence of "crude trypsin" incubated at 50 °C with various incubation times (10 to 180 min). Myosin heavy chain (MHC) was found to be the major protein band, followed by actin (AC), troponin (TNT), and tropomyosin (TM). The crude trypsin from tilapia viscera showed hydrolytic activity with decreasing MHC intensity as the incubation time increased. Within 30 min of incubation, a slightly decreased in MHC band intensity was observed in tilapia muscle, indicating that the degradation of protein had started. The changes in intensity of the actin band were not observed despite prolonged incubation time. There were no changes in the MHC band intensity when incubated for 120 and 180 min. However, at 180 min of incubation, the lowest numbers of protein bands were observed. Yongsawatdigul and others (2000) reported that the proteolysis of surimi made from tropical tilapia was well inhibited by soybean and leupeptin, implying that serine protease might be the major protease involved in proteolysis of the tilapia muscle. From this result, an incubation time of 120 min at 50 °C in the presence of crude viscera trypsin was chosen for further study.

Effect of precipitate IV fraction from legumes in minced tilapia

Figure 3 shows the proteolytic inhibition of tilapia muscle by precipitate IV fraction from navy beans, red kidney beans, and

adzuki beans. Different concentrations of inhibitor fraction from 3 legumes (100 to 2000 μ L) were applied into minced tilapia.

The intensity of the MHC band clearly decreased when crude trypsin (lane CE) was added to the sample. This indicates that crude trypsin isolated from tilapia viscera results in high hydrolytic activity on minced tilapia. However, markedly increased MHC band intensity was observed when the volume of the fraction increased. Addition of trypsin inhibitor fraction from the 3 legumes revealed the inhibition activity against the proteolysis of muscle tilapia. The fraction from navy beans effectively inhibited the proteolysis of tilapia muscle when the fraction concentration was 500 μ L,

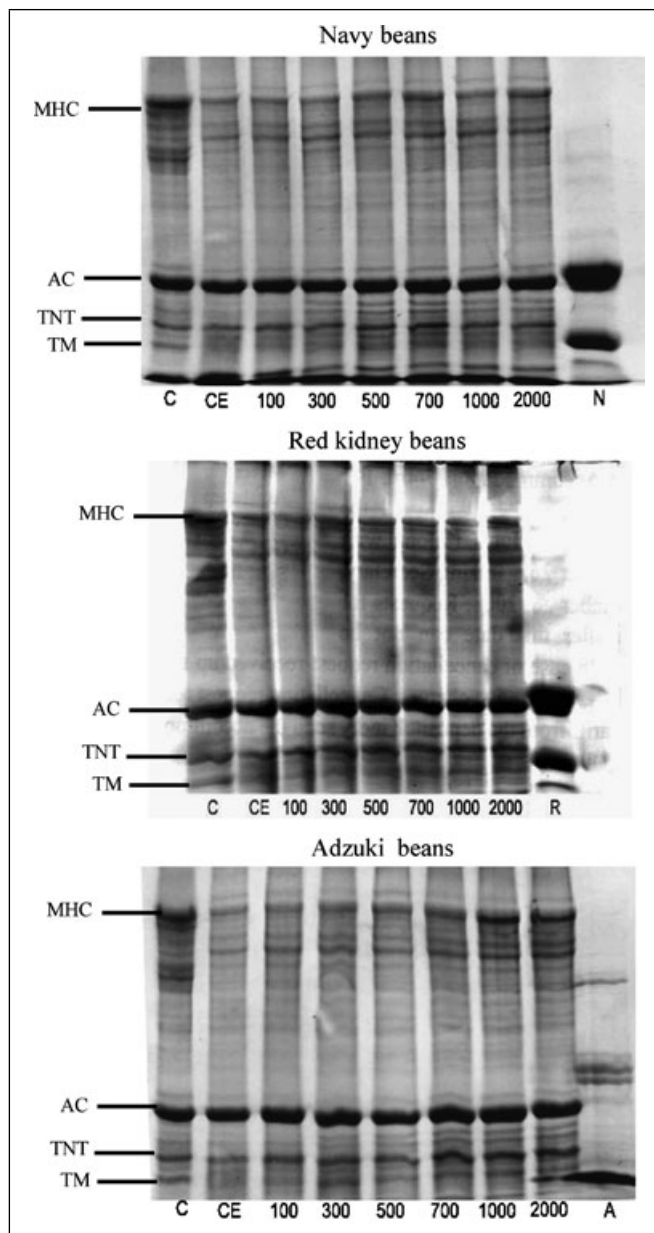


Figure 3—Effect of trypsin inhibitor fraction on proteolytic inhibition of minced tilapia. (C) minced tilapia; (CE) minced with crude trypsin; (N) precipitate IV of navy beans; (R) precipitate IV of red kidney beans; (A) precipitate IV of adzuki beans. Numbers (100 to 2000) denote the volume (μ L) of the precipitate IV from each legume added to the muscle. (MHC) myosin heavy chain; (AC) actin; (TNT) troponin-T; (TM) tropomyosin. Minced tilapia was incubated with inhibitor fraction and crude trypsin at 50 °C for 2 h.

likewise in red kidney beans when the fraction was around 300 μ L, and again in adzuki at a level of 100 μ L. Proteolysis of tilapia muscle was almost inhibited when the adzuki fraction concentration was 2000 μ L. The addition of soybean trypsin inhibitor completely inhibited the proteolysis of tilapia muscle, while E-64 only inhibited 36% of proteolysis (Yongsawatdigul and others 2000). Our results also showed that the amount of precipitate IV needed from each legume to inhibit proteolysis in the tilapia muscle depended on the amount of inhibitor in the fraction. Adzuki beans required the least amount, followed by red kidney beans and then navy beans.

Conclusions

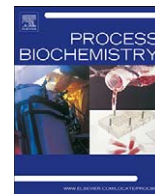
Trypsin inhibitor was fractionated from navy beans, red kidney beans, and adzuki beans by heat treatment (70 °C for 10 min) and ammonium sulfate precipitation (precipitate IV: 60% to 80% saturation). The purity of the inhibitor was 34- to 88-fold with the percent activity increase range of (–)47% to 253%. The trypsin inhibitor fraction from these legumes effectively inhibited protease activity of the tilapia muscle. The adzuki bean fraction showed the highest inhibitory activity against proteolysis of tilapia muscle.

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Three-phase partitioning of trypsin inhibitor from legume seeds

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ABSTRACT

Three-phase partitioning (TPP) was used to isolate trypsin inhibitors from navy bean (NB), red kidney bean (RK) and adzuki bean (AZ) from the Royal Project Foundation in Thailand. The method was to mix the crude extract with solid ammonium sulfate (30% saturation, w/v) and *tert*-butanol (*t*-butanol) in order to obtain the three phases. The trypsin inhibitor was purified to 5-, 14- and 7-fold with 315%, 441% and 228% recovery for NB, RK and AZ, respectively. The SDS-PAGE showed the major inhibitor band with the molecular weights (MWs) of 132, 118 and 13 kDa for NB, RK and AZ, respectively. The fractions from NB and AZ showed higher pH stability compared to that of the RK, and they had the optimum pH ranges of 7–9. The highest relative inhibitory activity of the fractions of NB and RK were found at 50 °C, and all fractions were quite stable at 90 °C for 60 min of incubation. Increasing the concentration of salt (up to 3%, w/v) did not significantly decrease the inhibitory activity of all fractions ($p > 0.05$). The trypsin inhibitors from the three legumes were unable to inhibit the autolysis of Pacific whiting and arrowtooth flounder muscles.

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1. Introduction

Plant seeds contain several biologically active proteins that play various specialized functions. The most representative molecules are hydrolytic enzymes, inhibitors, lectin and the ribosome inactivating proteins [1]. Some proteinase inhibitors have been found and isolated from plant storage organs such as seeds, tubers and endosperm. The serine protease inhibitors that were isolated from leguminous seeds were classified into three families: the Bowman–Birk type inhibitors (BBIs), the Kunitz type inhibitors, and the potato inhibitor I families [2]. The Kunitz type inhibitors have molecular weight (MW) approximately more than 20 kDa, with low cysteine content and a single reactive site. Meanwhile, the Bowman–Birk type has MW of ~8–10 kDa with high cysteine content and two reactive sites [3]. Soybean trypsin inhibitor, the most well-studied plant inhibitor, significantly inhibited the proteolysis of tilapia surimi [4], tuna viscera [5], Bigeye snapper [6] and Crucian carp [7]. Ramírez et al. [8] stated that the crude extract from kidney bean, pea, chickpea, lentil and soybean effectively decrease the *modori phenomenon* from several fish species by inhibiting the responsible enzymes. Since the application of plant seed protease inhibitors is expanding, especially in seafood based product, it is important to find new sources of

protease inhibitors with novel properties and to understand the characteristics of these protease inhibitors for their efficient and effective usage.

The grain of food legumes is second in importance only to cereals as a source of human and animal food. It is estimated that less than 4% of global legume production is currently consumed as human food. There are several varieties of legume seeds planted in the Royal Project Foundation of Thailand, including soybeans, bush beans, navy beans, adzuki beans, red kidney beans, black, red and green beans, and also garden peas. These legumes were grown at experimental stations in the hilly areas of Chiang Mai, Thailand (450–500 m above sea level) [9].

Recently, the separation and purification of enzymes have been developed to be more efficient, economical and scalable in order to isolate target proteins [10]. Three-phase partitioning (TPP) is an emerging technique that has been used for the extraction and purification of biomolecules. The TPP uses ammonium sulfate with certain saturation to precipitate the protein, and *t*-butanol was added to make three-phase layers and to remove some small MW compounds such as lipids, phenolics and some detergents [11]. The upper *t*-butanol phase containing non-polar compounds was separated from the lower aqueous phase with polar compounds by an interfacial protein precipitation. TPP involved techniques such as salting out, isoionic precipitation, co-solvent precipitation, osmolytic and kosmotropic precipitation of proteins [11]. This method was scalable and could be used directly with the crude suspensions. The partitioning process was affected by the

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hydrophilicity and MW of the protein and the physical conditions (temperature and pH) of the assay [12]. TPP was developed as an 'upstream' technique, but frequently it is also useful for the downstream of isolation on a milliliter volume scale. The method is also commonly used as a one-step purification protocol [13,14]. The single step of TPP successfully purified a wheat germ bifunctional protease/α-amylase inhibitor with 25-fold purification and an activity recovery of 85% [15]. A combination of TPP and hydrophobic interaction chromatography was also used to purify alkaline phosphatase from chicken intestine with the recovery and final purity of 61% and 80, respectively [16].

However there is less information about TPP as the method to isolate protease inhibitors from legumes, especially from the Royal Project Foundation of Thailand. Therefore, this study was conducted to isolate and characterize trypsin inhibitors from three legumes. The effect of isolated inhibitor fractions on the autolysis inhibition of Pacific whiting and arrowtooth flounder muscle was also investigated.

2. Material and methods

2.1. Chemicals and raw materials

N-α-Benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), trypsin from bovine pancreas, β-mercaptoethanol (βME) and *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) were purchased from Sigma Chemical Co. (St. Louis, MO). Casein from bovine milk, bovine serum albumin (BSA) and Coomassie Brilliant Blue R-250 were procured from Fluka Chemica-Biochemika (Buchs, Switzerland). *tert*-Butanol (*t*-butanol) and sodium dodecyl sulfate (SDS) were purchased from Ajax Finechem Pty. Ltd. (Auckland, NZ). The GelCode[®] Glycoprotein Staining Kit was purchased from Pierce Biotechnology Perbio (Rockford, IL). Navy beans (*Phaseolus vulgaris*), red kidney beans (*P. vulgaris* L.) and adzuki beans (*Vigna angularis*) were obtained from the Royal Project Foundation, Chiang Mai, Thailand. Frozen Pacific whiting and arrowtooth flounder filets were obtained from Pacific Seafood (OR, USA) and grocery store in Raleigh (NC, USA).

2.2. Fractionation of trypsin inhibitor from legume seeds

2.2.1. Preparation of crude extracts

Legume seeds were ground using a coffee mill to a particle size of 20 meshes. Seed flour was defatted by mixing it with hexane for 10 min at a ratio of 1:5 (w/v). The mixture was filtered through Whatman No. 1 filter paper and the sediment was rinsed with hexane thrice to remove the residual oil in the ground sample. The defatted sample was air-dried at an ambient temperature (28–30 °C) until dry, and until free of hexane odor. The defatted samples were extracted with 0.02 M NaOH for NB and RK, and water for AZ at the ratio 5:1 (v/w), and then shaken at 180 rpm for 2 h, which gave the highest trypsin inhibitor recovery according to the previous studies [17]. The supernatant was recovered by centrifuging at 8000 × *g* for 30 min. This supernatant was referred to as 'a crude extract' and used for further fractionation processes.

2.2.2. Heat treatment precipitation

Crude extracts were heated at 70 °C for 10 min (the highest recovery of trypsin inhibitors from previous work) and then cooled with ice water. To remove coagulated debris, the extracts were centrifuged at 8000 × *g* for 5 min at room temperature. The activity and specific activity of trypsin inhibitor in the supernatant were determined. The supernatant obtained was referred to as 'heat extract' and used for the next extraction process.

2.2.3. Three-phase partitioning (TPP)

TPP was carried out as described by Roy and Gupta [18]. The heat extract (Section 2.2.2) was added with solid ammonium sulfate to 30% (w/v) saturation and vortexed gently to dissolve the salt, followed by the addition of *t*-butanol with the ratio of 1:1 (v/v). The mixture was allowed to stand for 1 h at room temperature and then subjected to centrifuge at 5000 × *g* for 10 min to facilitate the separation of phases (Fig. 1). The lower aqueous layer (bottom phase) and the interfacial precipitate (middle phase) were collected. The latter was dissolved in 0.05 M Tris-HCl pH 8.2 (containing 0.02 M CaCl₂) with the ratio of 1:1 (w/v). Both phases were dialyzed against water at 4 °C to remove ammonium sulfate. The specific activity of each phase was determined. The phases that gave the highest specific activity were chosen for characterization.

2.3. Characterization of trypsin inhibitor from legume seeds

2.3.1. Trypsin inhibitory activity assay

Trypsin inhibitory activity was measured according to the method of Benjakul et al. [19] by using BAPNA as a substrate. A solution containing 100 μl of the sample, 200 μl (20 μg/mL) of trypsin and 100 μl of distilled water was pre-incubated at 37 °C for 10 min. Then 500 μl (0.4 mg/mL) of BAPNA (pre-warmed to 37 °C) was added to start the reaction. After incubation at 37 °C for 10 min, 100 μl of 30% (v/v) acetic acid was added to terminate the reaction; it was then subjected to centrifugation at 2000 × *g* for 10 min. The activity of the trypsin was measured by absorbance at 410 nm due to *p*-nitroaniline released. One unit of trypsin inhibitor was determined and defined as 0.01 decrease in absorbance at 410 nm under assay conditions compared to the control (without inhibitor) sample.

2.3.2. Protein determination

Protein concentration was determined by the Biuret method [20] by using BSA as a standard.

2.3.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

2.3.3.1. Protein pattern. SDS-PAGE was carried out by the method of Laemmli [21] using 15% separating and 4% stacking gels. The samples were mixed with the sample buffer (0.5 M Tris-HCl, pH 6.8, glycerol, 10% SDS and bromophenol blue) at a ratio of 1:1 in the absence of βME and unheated. Twenty micrograms of protein were loaded and then subjected to separate at 15 mA/gel using Mini Protean Tetra Cell units (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the protein was stained with Coomassie Brilliant Blue R-250 and destained with a methanol-acetic acid solution.

2.3.3.2. Inhibitory activity staining. For inhibitory activity staining, the protein separated by electrophoresis was subjected to incubation with 50 ml of a mixture of 0.2 mg/mL trypsin solution in Tris-HCl pH 8.3 for 60 min at 4 °C to allow the trypsin to diffuse into the gel, and then it was washed with distilled water. The gel was incubated at 37 °C for 90 min with 1% (w/v) casein in a 0.1 M phosphate buffer, pH 6.0 and then rinsed with distilled water, fixed and stained with Coomassie Brilliant Blue R-250 to develop detectable inhibitory zones. The apparent MW of the trypsin inhibitor was estimated by comparing the *R_f* with those of the protein standard marker.

2.3.3.3. Glycoprotein staining. Glycoprotein staining was conducted using the GelCode[®] Glycoprotein Staining Kit. The protein separated by electrophoresis was fixed by immersing the gel in 30 ml of 50% (v/v) methanol for 30 min and followed by washing the gel by gently agitating it with 3% (v/v) acetic acid for 10 min (repeated twice). The gel was transferred to 25 ml of oxidizing solution and then washed with 3% (v/v) acetic acid for 10 min before staining with 15 ml of GelCode[®] Glycoprotein Stain for 15 min. The gel was incubated for 10 min with 25 ml of reducing solution and washed by 3% (v/v) acetic acid. The glycoprotein appears as magenta bands.

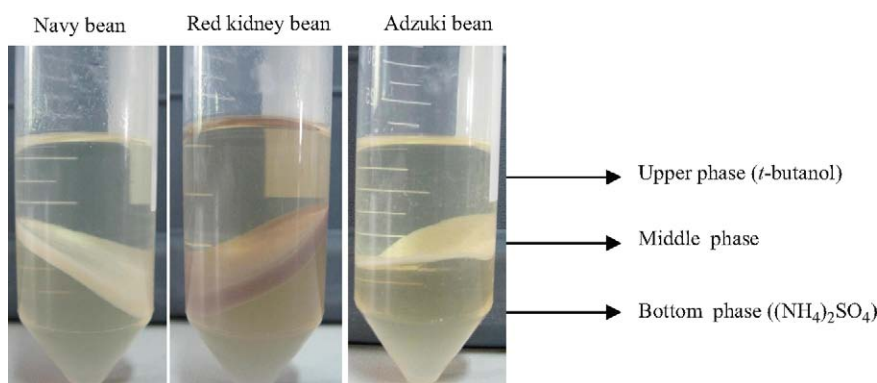


Fig. 1. Three-phase partitioning of three legume seeds. The extract was saturated with 30% ammonium sulfate and *t*-butanol with the ratio of 1:1 (v/v).

2.3.4. Thermal profile and stability

The phase that gave the highest trypsin inhibitor recovery (bottom phase of all legume seeds) was then subjected to assay the trypsin inhibitory activity at different temperatures (40, 50, 60, 70, 80, 90 and 100 °C). The inhibitory activity against trypsin was expressed as the relative inhibitory activity, compared with that of the untreated sample.

For thermal stability, the fractions were incubated at 90 °C for various time periods of 10, 20, 30, 40, 50, 60 and 90 min. The heat treated samples were immediately cooled in an ice water and tested for the remaining inhibitory activity.

2.3.5. pH profile and stability

The pH profile was tested by assaying the trypsin activity in the presence of the inhibitor fraction at different pHs (3–12) of the assay conditions. Glycine (pH 3), sodium acetate (pHs 4–5), sodium phosphate buffer (pHs 6–7), Tris–HCl buffer (pHs 8–9) and sodium carbonate (pHs 11–12) were used. The residual trypsin activity was measured and expressed as the relative inhibitory activity.

The pH stability was tested by incubating the trypsin inhibitor fractions in the buffer solution at different pHs (3–12) for 10 min at room temperature. The residual trypsin activity was determined and reported as the relative inhibitory activity as previously mentioned.

2.3.6. Salt stability

The trypsin inhibitor fractions were incubated at room temperature for 10 min in the presence of NaCl ranging from 0% to 3% (w/v) at a ratio of 1:1. The mixture was analyzed for its inhibitory activity against trypsin. The residual activity was reported as the relative inhibitory activity, compared with the untreated sample.

2.4. Effect of trypsin inhibitor on autolysis inhibition of muscle model system

2.4.1. Minced fish preparation

Frozen Pacific whiting and arrowtooth flounder fillets were thawed using running water and then minced with a meat mincer. The obtained samples were referred to as “minced fish” and used as the muscle model system for the autolysis inhibition study.

2.4.2. Autolysis inhibition study

The autolysis inhibition of minced fish by isolated trypsin inhibitors was measured according to the method of Rawdkuen et al. [22]. The trypsin inhibitor fractions of NB, RK and AZ (100, 300, 500, 700, 1000 and 2000 µl) were added to 2 g of minced fish. The mixture was then incubated in a water bath at 55 °C for 1 h and 60 °C for 30 min for Pacific whiting and arrowtooth flounder, respectively. The addition of 18 ml of 5% SDS solution (85 °C) terminated the autolysis. The mixture was homogenized by using homogenizer (IKA Ultra Turrax, Germany) for 1 min at 10,000 rpm and then incubated at 85 °C in a water bath for 1 h and centrifuged at 5000 × g for 10 min. The protein content in the supernatant was determined by using the Biuret method. Autolytic patterns of minced fish were determined using SDS PAGE as previously described.

2.5. Statistical analysis

All experiments were conducted and analyzed in triplicate. Means and standard deviations were calculated and compared using the Duncan's test. Analysis was performed using a SPSS package (SPSS 16.0 for windows, SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Three-phase partitioning of trypsin inhibitor from legume seeds

TPP with ammonium sulfate and *t*-butanol was used to fractionate the trypsin inhibitor from three legume seeds. The heat extract of the three legumes was added with solid ammonium sulfate (30% saturation, w/v) followed by the addition of 1:1 *t*-butanol. After 1 h, the three phases were formed and collected separately. The partitioning of the trypsin inhibitor by TPP is summarized in Table 1. Specific inhibitory activity (unit/mg protein) was achieved by the bottom phase for NB (2172) and RK (2094). Meanwhile, the middle phase of AZ (15,545) was given the highest specific inhibitory activity. The salting out mechanism as the part of TPP was used to separate proteins by using the kosmotrope ability of the sulfate ion [23]. Small precipitates obtained for AZ might be due to the higher hydrophilic site exposed in this protein. Meanwhile, for NB and RK, the bulk water associated with the salt ions, which made less water available to partake in the salvation layer around the protein. This leads to hydrophobic interactions. The increasing of the total units might be because of the increased flexibility of the molecule, which then probably leads to enhanced activity of the enzymes [14,15]. Singh et al. [24] reported the remarkable increase in the catalytic activity of proteinase K obtained from TPP treatment could be attributed to the presence of an acetate ion at the active site and the excited state of the proteinase K as indicated by an overall higher temperature factor. The difference between conventional salting out and TPP were the additions of *t*-butanol that binds to the protein, and also the increases in buoyancy against gravity that makes it float between the aqueous and the organic layer [25]. Sulfate that has kosmotrope and anionic “water structure promoter” generates a synergistic effect with *t*-butanol in water with a powerful structure promoter; therefore, the sulfate ion concentration used is much lower than normally required for salting out the protein [15]. The best concentration of solid ammonium sulfate used in TPP is generally around 30% saturation (w/v) [12,15,26,27]. The purity of the trypsin inhibitor increased as the number of the isolation steps increased. Therefore, heat treatment was used as the pre-treatment to remove some heat labile proteins in the sample. The TPP gave the yield of 315,441 and 228 with purification folds of 5.65, 14.15 and 7.3 for the bottom phase of NB, RK and AZ, respectively. The highest trypsin inhibitor recovery was obtained in RK, followed by NB and AZ. The saturation of 30% ammonium sulfate was sufficient enough to remove other proteins that precipitate in the middle phase and

Table 1
Three-phase partitioning of trypsin inhibitor from three legume seeds.

Sample	Step	Volume (ml)	Total protein (mg)	Total inhibitory activity (units)	Specific inhibitory activity (unit/mg)	Yield (%)	Purification (fold)
Navy bean	Crude	13	391	150,423	384 ± 61a [*]	100 ± 0a	1 ± 0a
	Heat treatment ^a	10	305	246,570	808 ± 9b	163 ± 18b	2.1 ± 0.02b
	Bottom phase ^b	19.23	218	475,000	2,172 ± 27d	315 ± 47c	5.6 ± 0.05c
	Middle phase ^b	9.77	130	241,045	1,852 ± 244c	160 ± 79b	4.8 ± 0.47d
Red kidney bean	Crude	15	499	73,740	148 ± 21a	100 ± 0a	1 ± 0a
	Heat treatment ^a	10	317	223,960	705 ± 6b	303 ± 1b	4.7 ± 0.13b
	Bottom phase ^b	14.5	155	325,496	2,094 ± 72d	441 ± 39c	14.1 ± 0.42c
	Middle phase ^b	13.12	209	294,045	1,406 ± 92c	398 ± 15c	9.5 ± 0.02d
Adzuki bean	Crude	15	194	130,185	671 ± 59a	100 ± 0a	1 ± 0a
	Heat treatment ^a	10	95	225,880	2,370 ± 79b	173 ± 25c	3.5 ± 0.15b
	Bottom phase ^b	13	60	298,051	4,920 ± 1,734c	228 ± 30d	7.3 ± 0.09c
	Middle phase ^b	1.63	2	37,154	15,545 ± 3,992d	28 ± 4b	23.2 ± 0.20d

^a Crude extracts were heated at 70 °C for 10 min and then centrifuged at 8000 × g for 15 min to remove the heat coagulated debris.

^b Fraction obtained by three-phase partitioning (TPP) with 30% saturation of ammonium sulfate.

^{*} Different letters in the same legume indicate significant differences ($p < 0.05$).

leave the trypsin inhibitor in the bottom phase of the RK fraction. However, the highest specific inhibitory activity and purification factor were achieved by AZ. The amount of protein precipitated in the middle layer of AZ was lower than that of NB and RK. It means that 30% saturation can only remove small amounts of protein but still have inhibitory activity. This phenomenon leads to higher specific activity of the trypsin inhibitor. Due to the high recovery of the isolated trypsin inhibitor, this method is potential of low cost mass production trypsin inhibitor. Soybean trypsin inhibitor was successfully purified by the modified metal-TPP with 72% recovery and 13-fold purification [18]. Sharma and Gupta [15] used the TPP as a single step process to purify protease/amylase inhibitors from wheat germ. It obtained 25-fold purity and 85% activity recovery. This result was similar to the aqueous two-phase systems (ATPS) where the target proteins usually were recovered from the bottom

phase or ammonium sulfate phase. The contaminants were contained in the top phase in ATPS (PEG phase) and TPP (butanol phase). Platis et al. [28] recovered the target protein monoclonal antibodies from transgenic tobacco plants by using ATPS as the initial purification step to remove polyphenols followed by chromatography techniques.

3.2. Protein pattern, inhibitory activity and glycoprotein staining

3.2.1. Protein pattern

Protein patterns of the crude extract and fractions of NB, RK and AZ are shown in Fig. 2. For NB, there is no significant change of protein pattern from the crude extract (lane C) and the heat treatment (lane H). The major protein bands for NB were 215, 132 and 77 kDa. Even though no changes of protein pattern were

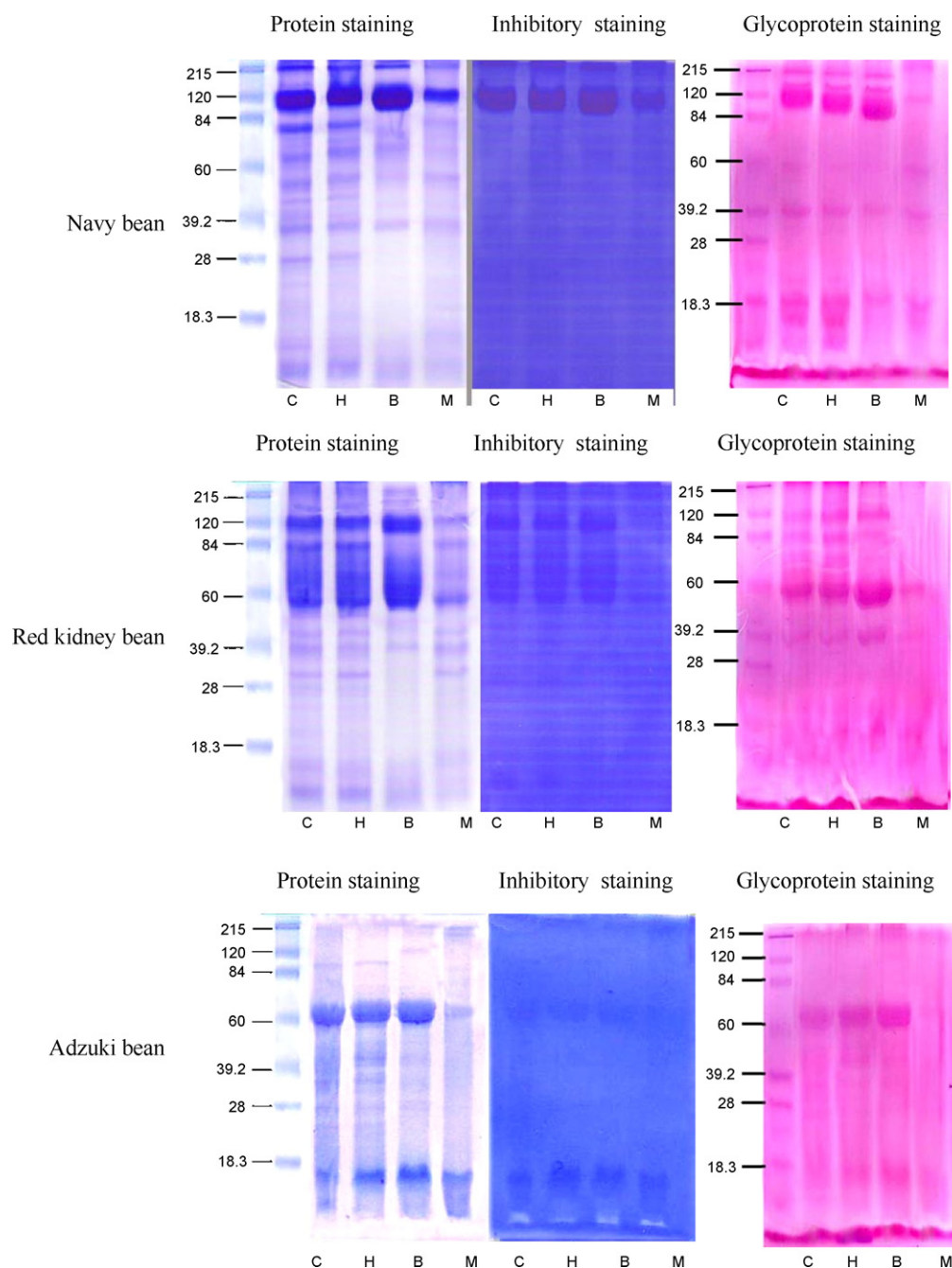


Fig. 2. Protein, inhibitor and glycoprotein staining of the samples from three legume seeds. C, crude; H, heat treatment; B, bottom phase; M, middle phase.

observed for the heat treatment sample, increasing inhibitory activity recovery was obtained (data not shown). Heat treatment leads to the conformational changes in many proteins resulted in the release of some reactive proteins. The TPP process could remove the small MW of proteins from the sample as shown in lanes B and M. The protein band with the MW of 77 kDa disappeared, and the protein band with the MWs of 215 and 132 kDa still remained with higher intensity in the bottom phase compared to that of the middle phase. The protein patterns of the bottom and middle phase of NB were identical; however, a decreased intensity was observed in lane M.

Protein bands of the crude and heat treatment samples with the MWs of 118, 59, 38 and 32 kDa were observed in RK. After TPP, the bottom phase (lane B) showed only three major bands with the MWs of 118, 59 and 38 kDa. Nevertheless, the middle phase (lane M) had the same pattern as shown in lane H with lower band intensity. The AZ presented two major protein bands with the MWs of 62 and 13 kDa after the TPP process. The minor protein bands with the MWs of 39 and 35 kDa were completely removed by the TPP. The low MW of the protein might have been removed in the upper layer (*t*-butanol phase), because the *t*-butanol could remove only low MW proteins by denaturation [29]. In the TPP process, the lipids dissolved in the upper *t*-butanol layer, while most proteins (nucleic acids and insoluble aggregates formed into macromolecules) were in the middle phase. The remaining macromolecules were solubilized in the salt phase [30]. From our results, it can be stated that the proteins have a tendency to concentrate in the bottom phase of the mixture. However, it is totally dependent on the structure of the protein molecule inside those legumes.

3.2.2. Inhibitory activity staining

Fig. 2 shows the inhibitory activity staining for the three legumes seeds. For NB, a single protein band with the MW of 132 kDa still clearly appeared on the gel. The presence of this band in the inhibitory activity staining condition suggested that it is the trypsin inhibitor. The single protein band with the MWs of 118 and 13 kDa was observed for RK and AZ, respectively. Our results revealed that TPP could partition the trypsin inhibitor into the salt phase for NB and RK, while the trypsin inhibitor of AZ was effectively isolated in the interfacial phase of the system.

3.2.3. Glycoprotein staining

It has been reported that glycoprotein was found in legume seeds with an estimated MW range of 45–55 kDa. In this study, the crude extract and isolated protein was stained for the glycoprotein content, as depicted in Fig. 2. This staining was used to detect high glycosylated proteoglycans (protein glycosaminoglycans) or glycoproteins (protein oligosaccharides) [31]. Structural glycoprotein consists of polypeptide covalently bonded to a carbohydrate moiety [32]. The glycoprotein staining revealed that the entire major protein bands contained oligosaccharide. For NB, the protein bands with the MWs of 215, 132 and 39 kDa appeared as a magenta band. A group of protein bands with the MWs of 113, 59 and 38 kDa and 62 and 13 kDa were observed for RK and AZ, respectively. These bands were identified as the glycoprotein. In addition, the protein band with the MWs of 132, 113 and 13 kDa for NB, RK and AZ also appeared at the inhibitory activity staining. This evidence confirmed that the trypsin inhibitor from NB, RK and AZ was glycoprotein.

Based on the recovery of the trypsin inhibitor obtained by using TPP, the bottom phase for all legumes was chosen for further study.

3.3. pH profile and stability

The trypsin inhibitor fraction was incubated with various pHs (3–12) in the assay condition. The highest residual activity was

observed at pHs 7, 8 and 9 for AZ, RK and NB, respectively. However, decreased relative inhibitory activity of the trypsin inhibitor fraction from all kinds of legumes was observed at strong acidic and alkaline conditions (data not shown). The trypsin inhibitor isolated from most legumes had the optimum pH of around 8 [33]. Rai et al. [34] reported that the pH 8 as the optimum for inhibitory activity of the serine Kunitz trypsin inhibitor isolation from leaves of *Terminalia arjuna*.

The pH stability of the trypsin inhibitor isolation from NB, RK and AZ was also investigated. There was no significant decrease in relative inhibitory activity of the trypsin inhibitor isolation from NB and AZ after incubation at various pHs (3–12) for 10 min ($p > 0.05$). The markedly decrease of relative inhibitory activity was observed for RK when the pH ranges were 9–12 (data not shown). The remaining residual trypsin activity of 50% was observed when the RK fraction has incubated with the basic condition (pH 12). Benjakul et al. [19] reported that the stability of trypsin inhibitor isolation from cowpea, pigeon pea and bambara groundnuts was over a wide pH ranges (3–9). Inhibitor isolated from horse gram (*Dolichos biflorus*) was stable between the pHs of 3–11 [35]. Our results revealed that, the trypsin inhibitor fractions from NB and AZ were more stable in the various pH ranges compared to that of RK. A decrease in activity was observed in the acidic ranges (pHs 3–6). When protein was exposed in the extreme pH values, the denaturation (unfolding) of the protein molecule occurred because of the strong electrostatic repulsion of ionized groups leading to losses of their function [36].

3.4. Temperature profile and stability

For thermal profile, the trypsin inhibitor of NB, RK and AZ was determined at a temperature ranging from 40 to 100 °C. The highest inhibitory activity of each fraction from each legume was found at 50 °C for RK and 60 °C for NB and AZ (Fig. 3). The relative inhibitory activity was 98%, 102% and 110% for NB, RK and AZ at each optimum temperature. As temperature used for incubation was increased, the relative inhibitory activity decreased constantly and reached the lowest point at 100 °C. Rekha et al. [37] reported that the optimum temperature for trypsin inhibitor isolation from mungbean seeds was 50 °C. As the temperature increases, more molecules have enough kinetic energy to undergo the reaction. If the temperature is raised above the optimum point, the kinetic energy of the enzyme and water molecules is so great that the structure of the enzyme molecule starts to disrupt [38]. Therefore, a decrease in activity was detected when the temperature increased.

For thermal stability, the trypsin inhibitory activity of NB, RK and AZ was determined after the fraction was incubated at 90 °C and ranging in incubation times from 10 to 90 min (Fig. 4). There is

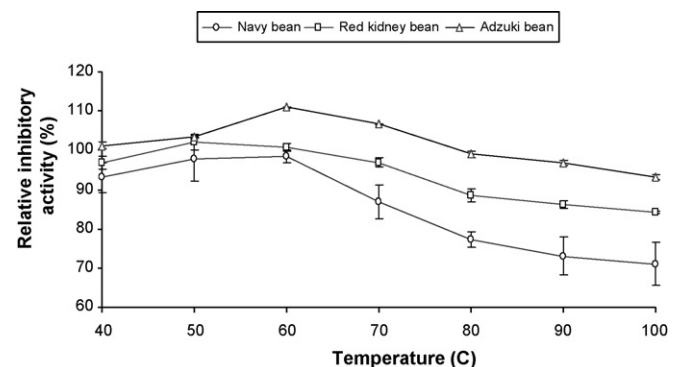


Fig. 3. Thermal profiles of isolated trypsin inhibitor from three legume seeds. Fractions were assayed at various temperatures (40–100 °C). Bars represent standard deviations from triplicate determinations.

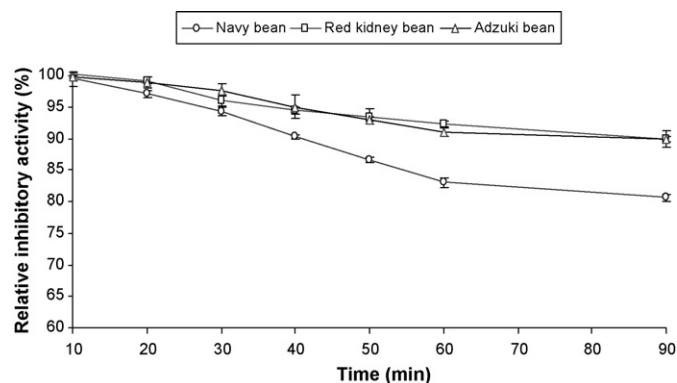


Fig. 4. Thermal stability of isolated trypsin inhibitor from three legume seeds. Fractions were incubated at 90 °C for various times (10–90 min). Bars represent standard deviations from triplicate determinations.

a significant decrease in the relative inhibitory activity when the NB, RK and AZ fractions were incubated for 20 min ($p > 0.05$). The level of thermal stability causes the changes in the tertiary structure, leading to a less ordered arrangement of the polypeptide chain that can decrease the enzyme activity [39]. The resistance of

trypsin inhibitors to thermal denaturation was shown by the retention of inhibitory activity of up to 80% after incubation at 90 °C for 90 min. The thermal stability of proteinase inhibitor depends on its amino acid composition and structural conformation. A tightly coiled conformation with multiple disulfide bonds is considered to be responsible for the resistance of thermal denaturation [40]. The isolated trypsin inhibitor from NB, RK and AZ seemed to be more stable than that of the trypsin inhibitor from cowpea, pigeon pea and bambara groundnut, which were heated steadily for up to 30 min at 90 °C [19]. From three legumes, NB showed the lowest thermal stability when compared with others. The trypsin inhibitor isolated from *Calliandra selloi* Macbride was found to be quite stable at 80 °C for 30 min [41]. Richardson [42] reported that the Bowman–Birk inhibitor was more stable compared to the Kunitz type trypsin inhibitors, which displayed significant heat stability. This stability is probably due to the fact that the Bowman–Birk inhibitor consists of cysteine-rich polypeptide chain bridged by seven conserved disulfide bonds.

3.5. Salt stability

The effect of NaCl on the inhibitory activity of the trypsin inhibitor isolated from three legume seeds was studied. Slightly

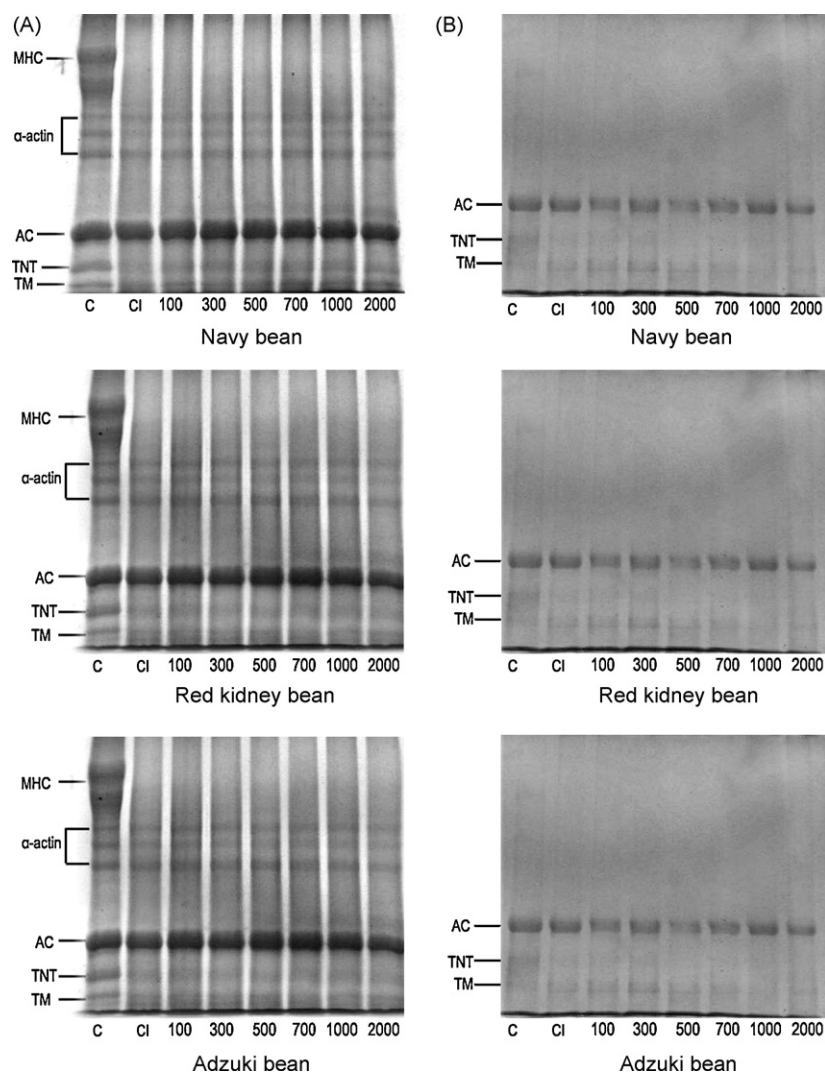


Fig. 5. Effect of isolated trypsin inhibitor fraction from three legume seeds on autolysis inhibition of Pacific whiting (A) and arrowtooth flounder (B) minced. C, minced; CI, minced with incubation. Numbers (100–2000) designate the fraction volume (μl) used; MHC, myosin heavy chain; α-actin, alpha-actin; AC, actin; TNT, troponin-T; TM, tropomyosin.

decreased inhibitory activity was observed for NB and AZ, yet meanwhile the relative inhibitory activity of RK reached the lowest point when the salt concentration increased ($p > 0.05$) (data not shown). The relative inhibitory activity of each legume reached 99%, 95% and 97% for NB, RK and AZ when the salt concentration addition was up to 3% (w/v). The trypsin inhibitor from the three legumes was fairly stable with salt concentrations of up to 3% (w/v), demonstrated by the high relative inhibitory activity (>95%). The effect of salt is considered to involve ion–dipole association or hydrogen bonding of a decrease in the double-bond character and the consequent loss of polypeptide-chain rigidity [43]. However, a low level of salt concentration could increase the solubility of the protein inside the legume via the “salting process” which results in more inhibitors released into the system.

3.6. Effect of isolated trypsin inhibitor in muscle model system

The autolysis inhibition study of minced Pacific whiting is shown in Fig. 5A. The major bands of Pacific whiting muscle were myosin heavy chain (MHC), alpha-actinin (α -actin), actin (AC), troponin-T (TNT) and tropomyosin (TM). When the muscle was incubated at 55 °C for 60 min, the MHC completely disappeared and decreased in intensity of α -actin (lane CI). However, the actin band did not undergo hydrolysis by this incubation condition. This phenomenon was caused by high protease activity of the Pacific whiting muscle. An et al. [44] reported that high proteolytic activity of Pacific whiting was caused by the most active protease that was identified as cathepsin L. The protease was also found as the predominant heat-activated proteinase in the muscle extract of arrowtooth flounder, which was responsible for high proteolytic activity [45]. When the Pacific whiting muscle was incubated with the trypsin inhibitor fractions from NB, RK and AZ, there were no changes in the protein pattern between the control (lane CI) and the muscle that was incubated with the trypsin inhibitor fraction (100–2000 μ l). It showed that the MHC was completely hydrolyzed by the protease contained in the muscle. The addition of the trypsin inhibitor fraction (at all levels) from all kinds of legumes could not inhibit the autolysis of the Pacific whiting muscle. Increasing intensities of α -actin were observed in the muscle incubated with NB and RK. Meanwhile, adzuki beans only showed autolytic inhibition on α -actin.

The incapability of the trypsin inhibitor to isolate from three legume seeds might correlate with the different target protease responsible for the proteolytic activity of the Pacific whiting muscle, cathepsin L (cysteine proteinase) [44]. Therefore, the trypsin inhibitor isolation from three legumes was unable to inhibit the proteolytic activity in the Pacific whiting muscle. It may also be stated that the inhibitor fraction from these legumes contains only the trypsin inhibitor.

The autolysis inhibition study of arrowtooth flounder by the trypsin inhibitor fractions from NB, RK and AZS is depicted in Fig. 5B. For minced arrowtooth flounder, the same pattern of proteolysis was observed. However, high hydrolytic activity was found as indicated by the disappearance of MHC and other protein bands. Only the actin band was observed in the incubated minced fish at the optimum condition of protease activity presented in this fish. Different concentrations of trypsin inhibitor fractions of NB, RK and AZ (100–2000 μ l) were applied into the minced arrowtooth flounder. The protein bands of MHC, and α -actin were still not observed for all inhibitor fractions added. This result indicated that the trypsin inhibitor fraction from all legume seeds could not inhibit the autolysis of this fish muscle as well as of Pacific whiting.

The presence of heat stable cysteine proteinase (cathepsin L) in arrowtooth flounder was presumed as the major cause of the high hydrolytic activity in the muscle, especially at elevated tempera-

tures [46]. Visessanguan et al. [45] reported partial or complete inhibition of cathepsin L isolation from minced arrowtooth flounder that was obtained by E-64 and iodoacetate containing thiol-blocking agents. These results clearly confirmed that the inhibitor fraction from the TPP of the three legumes contained only the trypsin inhibitor.

4. Conclusion

Trypsin inhibitor was partially purified from NB, RK and AZ by heat treatment (70 °C for 10 min) and by three-phase partitioning (30% saturation of solid ammonium sulfate, w/v and *t*-butanol). The purification folds of 5–14 with the recovery of 228–441% from the bottom phase of three legumes were obtained. The trypsin inhibitors from NB and AZ were quite stable at a wide range of pH, and meanwhile the RK loosened its activity by up to 50% in an alkaline condition. High temperature and salt concentration did not affect the inhibitory activity of the inhibitor fraction from the three legumes. The autolysis of Pacific whiting and arrowtooth flounder was not inhibited by the addition of trypsin inhibitor fraction from all the legumes.

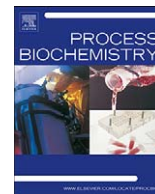
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Three-phase partitioning of trypsin inhibitor from legume seeds

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ABSTRACT

Three-phase partitioning (TPP) was used to isolate trypsin inhibitors from navy bean (NB), red kidney bean (RK) and adzuki bean (AZ) from the Royal Project Foundation in Thailand. The method was to mix the crude extract with solid ammonium sulfate (30% saturation, w/v) and *tert*-butanol (*t*-butanol) in order to obtain the three phases. The trypsin inhibitor was purified to 5-, 14- and 7-fold with 315%, 441% and 228% recovery for NB, RK and AZ, respectively. The SDS-PAGE showed the major inhibitor band with the molecular weights (MWs) of 132, 118 and 13 kDa for NB, RK and AZ, respectively. The fractions from NB and AZ showed higher pH stability compared to that of the RK, and they had the optimum pH ranges of 7–9. The highest relative inhibitory activity of the fractions of NB and RK were found at 50 °C, and all fractions were quite stable at 90 °C for 60 min of incubation. Increasing the concentration of salt (up to 3%, w/v) did not significantly decrease the inhibitory activity of all fractions ($p > 0.05$). The trypsin inhibitors from the three legumes were unable to inhibit the autolysis of Pacific whiting and arrowtooth flounder muscles.

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1. Introduction

Plant seeds contain several biologically active proteins that play various specialized functions. The most representative molecules are hydrolytic enzymes, inhibitors, lectin and the ribosome inactivating proteins [1]. Some proteinase inhibitors have been found and isolated from plant storage organs such as seeds, tubers and endosperm. The serine protease inhibitors that were isolated from leguminous seeds were classified into three families: the Bowman–Birk type inhibitors (BBIs), the Kunitz type inhibitors, and the potato inhibitor I families [2]. The Kunitz type inhibitors have molecular weight (MW) approximately more than 20 kDa, with low cysteine content and a single reactive site. Meanwhile, the Bowman–Birk type has MW of ~8–10 kDa with high cysteine content and two reactive sites [3]. Soybean trypsin inhibitor, the most well-studied plant inhibitor, significantly inhibited the proteolysis of tilapia surimi [4], tuna viscera [5], Bigeye snapper [6] and Crucian carp [7]. Ramírez et al. [8] stated that the crude extract from kidney bean, pea, chickpea, lentil and soybean effectively decrease the *modori phenomenon* from several fish species by inhibiting the responsible enzymes. Since the application of plant seed protease inhibitors is expanding, especially in seafood based product, it is important to find new sources of

protease inhibitors with novel properties and to understand the characteristics of these protease inhibitors for their efficient and effective usage.

The grain of food legumes is second in importance only to cereals as a source of human and animal food. It is estimated that less than 4% of global legume production is currently consumed as human food. There are several varieties of legume seeds planted in the Royal Project Foundation of Thailand, including soybeans, bush beans, navy beans, adzuki beans, red kidney beans, black, red and green beans, and also garden peas. These legumes were grown at experimental stations in the hilly areas of Chiang Mai, Thailand (450–500 m above sea level) [9].

Recently, the separation and purification of enzymes have been developed to be more efficient, economical and scalable in order to isolate target proteins [10]. Three-phase partitioning (TPP) is an emerging technique that has been used for the extraction and purification of biomolecules. The TPP uses ammonium sulfate with certain saturation to precipitate the protein, and *t*-butanol was added to make three-phase layers and to remove some small MW compounds such as lipids, phenolics and some detergents [11]. The upper *t*-butanol phase containing non-polar compounds was separated from the lower aqueous phase with polar compounds by an interfacial protein precipitation. TPP involved techniques such as salting out, isoionic precipitation, co-solvent precipitation, osmolytic and kosmotropic precipitation of proteins [11]. This method was scalable and could be used directly with the crude suspensions. The partitioning process was affected by the

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hydrophilicity and MW of the protein and the physical conditions (temperature and pH) of the assay [12]. TPP was developed as an 'upstream' technique, but frequently it is also useful for the downstream of isolation on a milliliter volume scale. The method is also commonly used as a one-step purification protocol [13,14]. The single step of TPP successfully purified a wheat germ bifunctional protease/α-amylase inhibitor with 25-fold purification and an activity recovery of 85% [15]. A combination of TPP and hydrophobic interaction chromatography was also used to purify alkaline phosphatase from chicken intestine with the recovery and final purity of 61% and 80, respectively [16].

However there is less information about TPP as the method to isolate protease inhibitors from legumes, especially from the Royal Project Foundation of Thailand. Therefore, this study was conducted to isolate and characterize trypsin inhibitors from three legumes. The effect of isolated inhibitor fractions on the autolysis inhibition of Pacific whiting and arrowtooth flounder muscle was also investigated.

2. Material and methods

2.1. Chemicals and raw materials

N-α-Benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), trypsin from bovine pancreas, β-mercaptoethanol (βME) and *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) were purchased from Sigma Chemical Co. (St. Louis, MO). Casein from bovine milk, bovine serum albumin (BSA) and Coomassie Brilliant Blue R-250 were procured from Fluka Chemica-Biochemika (Buchs, Switzerland). *tert*-Butanol (*t*-butanol) and sodium dodecyl sulfate (SDS) were purchased from Ajax Finechem Pty. Ltd. (Auckland, NZ). The GelCode[®] Glycoprotein Staining Kit was purchased from Pierce Biotechnology Perbio (Rockford, IL). Navy beans (*Phaseolus vulgaris*), red kidney beans (*P. vulgaris* L.) and adzuki beans (*Vigna angularis*) were obtained from the Royal Project Foundation, Chiang Mai, Thailand. Frozen Pacific whiting and arrowtooth flounder filets were obtained from Pacific Seafood (OR, USA) and grocery store in Raleigh (NC, USA).

2.2. Fractionation of trypsin inhibitor from legume seeds

2.2.1. Preparation of crude extracts

Legume seeds were ground using a coffee mill to a particle size of 20 meshes. Seed flour was defatted by mixing it with hexane for 10 min at a ratio of 1:5 (w/v). The mixture was filtered through Whatman No. 1 filter paper and the sediment was rinsed with hexane thrice to remove the residual oil in the ground sample. The defatted sample was air-dried at an ambient temperature (28–30 °C) until dry, and until free of hexane odor. The defatted samples were extracted with 0.02 M NaOH for NB and RK, and water for AZ at the ratio 5:1 (v/w), and then shaken at 180 rpm for 2 h, which gave the highest trypsin inhibitor recovery according to the previous studies [17]. The supernatant was recovered by centrifuging at 8000 × *g* for 30 min. This supernatant was referred to as 'a crude extract' and used for further fractionation processes.

2.2.2. Heat treatment precipitation

Crude extracts were heated at 70 °C for 10 min (the highest recovery of trypsin inhibitors from previous work) and then cooled with ice water. To remove coagulated debris, the extracts were centrifuged at 8000 × *g* for 5 min at room temperature. The activity and specific activity of trypsin inhibitor in the supernatant were determined. The supernatant obtained was referred to as 'heat extract' and used for the next extraction process.

2.2.3. Three-phase partitioning (TPP)

TPP was carried out as described by Roy and Gupta [18]. The heat extract (Section 2.2.2) was added with solid ammonium sulfate to 30% (w/v) saturation and vortexed gently to dissolve the salt, followed by the addition of *t*-butanol with the ratio of 1:1 (v/v). The mixture was allowed to stand for 1 h at room temperature and then subjected to centrifuge at 5000 × *g* for 10 min to facilitate the separation of phases (Fig. 1). The lower aqueous layer (bottom phase) and the interfacial precipitate (middle phase) were collected. The latter was dissolved in 0.05 M Tris-HCl pH 8.2 (containing 0.02 M CaCl₂) with the ratio of 1:1 (w/v). Both phases were dialyzed against water at 4 °C to remove ammonium sulfate. The specific activity of each phase was determined. The phases that gave the highest specific activity were chosen for characterization.

2.3. Characterization of trypsin inhibitor from legume seeds

2.3.1. Trypsin inhibitory activity assay

Trypsin inhibitory activity was measured according to the method of Benjakul et al. [19] by using BAPNA as a substrate. A solution containing 100 μl of the sample, 200 μl (20 μg/mL) of trypsin and 100 μl of distilled water was pre-incubated at 37 °C for 10 min. Then 500 μl (0.4 mg/mL) of BAPNA (pre-warmed to 37 °C) was added to start the reaction. After incubation at 37 °C for 10 min, 100 μl of 30% (v/v) acetic acid was added to terminate the reaction; it was then subjected to centrifugation at 2000 × *g* for 10 min. The activity of the trypsin was measured by absorbance at 410 nm due to *p*-nitroaniline released. One unit of trypsin inhibitor was determined and defined as 0.01 decrease in absorbance at 410 nm under assay conditions compared to the control (without inhibitor) sample.

2.3.2. Protein determination

Protein concentration was determined by the Biuret method [20] by using BSA as a standard.

2.3.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

2.3.3.1. Protein pattern. SDS-PAGE was carried out by the method of Laemmli [21] using 15% separating and 4% stacking gels. The samples were mixed with the sample buffer (0.5 M Tris-HCl, pH 6.8, glycerol, 10% SDS and bromophenol blue) at a ratio of 1:1 in the absence of βME and unheated. Twenty micrograms of protein were loaded and then subjected to separate at 15 mA/gel using Mini Protean Tetra Cell units (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the protein was stained with Coomassie Brilliant Blue R-250 and destained with a methanol-acetic acid solution.

2.3.3.2. Inhibitory activity staining. For inhibitory activity staining, the protein separated by electrophoresis was subjected to incubation with 50 ml of a mixture of 0.2 mg/mL trypsin solution in Tris-HCl pH 8.3 for 60 min at 4 °C to allow the trypsin to diffuse into the gel, and then it was washed with distilled water. The gel was incubated at 37 °C for 90 min with 1% (w/v) casein in a 0.1 M phosphate buffer, pH 6.0 and then rinsed with distilled water, fixed and stained with Coomassie Brilliant Blue R-250 to develop detectable inhibitory zones. The apparent MW of the trypsin inhibitor was estimated by comparing the R_f with those of the protein standard marker.

2.3.3.3. Glycoprotein staining. Glycoprotein staining was conducted using the GelCode[®] Glycoprotein Staining Kit. The protein separated by electrophoresis was fixed by immersing the gel in 30 ml of 50% (v/v) methanol for 30 min and followed by washing the gel by gently agitating it with 3% (v/v) acetic acid for 10 min (repeated twice). The gel was transferred to 25 ml of oxidizing solution and then washed with 3% (v/v) acetic acid for 10 min before staining with 15 ml of GelCode[®] Glycoprotein Stain for 15 min. The gel was incubated for 10 min with 25 ml of reducing solution and washed by 3% (v/v) acetic acid. The glycoprotein appears as magenta bands.

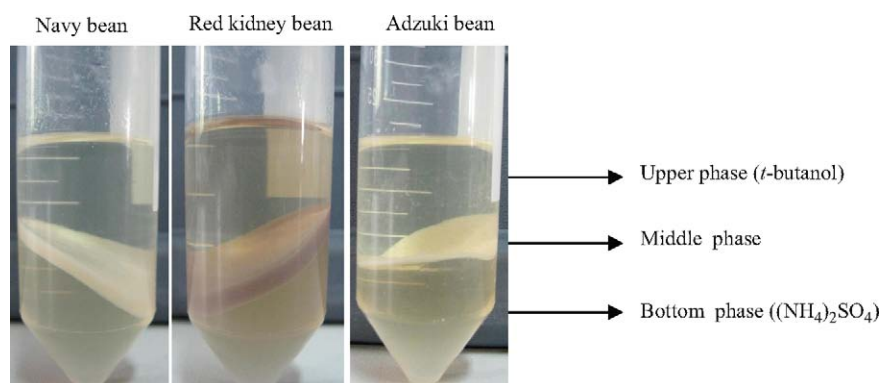


Fig. 1. Three-phase partitioning of three legume seeds. The extract was saturated with 30% ammonium sulfate and *t*-butanol with the ratio of 1:1 (v/v).

2.3.4. Thermal profile and stability

The phase that gave the highest trypsin inhibitor recovery (bottom phase of all legume seeds) was then subjected to assay the trypsin inhibitory activity at different temperatures (40, 50, 60, 70, 80, 90 and 100 °C). The inhibitory activity against trypsin was expressed as the relative inhibitory activity, compared with that of the untreated sample.

For thermal stability, the fractions were incubated at 90 °C for various time periods of 10, 20, 30, 40, 50, 60 and 90 min. The heat treated samples were immediately cooled in an ice water and tested for the remaining inhibitory activity.

2.3.5. pH profile and stability

The pH profile was tested by assaying the trypsin activity in the presence of the inhibitor fraction at different pHs (3–12) of the assay conditions. Glycine (pH 3), sodium acetate (pHs 4–5), sodium phosphate buffer (pHs 6–7), Tris–HCl buffer (pHs 8–9) and sodium carbonate (pHs 11–12) were used. The residual trypsin activity was measured and expressed as the relative inhibitory activity.

The pH stability was tested by incubating the trypsin inhibitor fractions in the buffer solution at different pHs (3–12) for 10 min at room temperature. The residual trypsin activity was determined and reported as the relative inhibitory activity as previously mentioned.

2.3.6. Salt stability

The trypsin inhibitor fractions were incubated at room temperature for 10 min in the presence of NaCl ranging from 0% to 3% (w/v) at a ratio of 1:1. The mixture was analyzed for its inhibitory activity against trypsin. The residual activity was reported as the relative inhibitory activity, compared with the untreated sample.

2.4. Effect of trypsin inhibitor on autolysis inhibition of muscle model system

2.4.1. Minced fish preparation

Frozen Pacific whiting and arrowtooth flounder fillets were thawed using running water and then minced with a meat mincer. The obtained samples were referred to as “minced fish” and used as the muscle model system for the autolysis inhibition study.

2.4.2. Autolysis inhibition study

The autolysis inhibition of minced fish by isolated trypsin inhibitors was measured according to the method of Rawdkuen et al. [22]. The trypsin inhibitor fractions of NB, RK and AZ (100, 300, 500, 700, 1000 and 2000 µl) were added to 2 g of minced fish. The mixture was then incubated in a water bath at 55 °C for 1 h and 60 °C for 30 min for Pacific whiting and arrowtooth flounder, respectively. The addition of 18 ml of 5% SDS solution (85 °C) terminated the autolysis. The mixture was homogenized by using homogenizer (IKA Ultra Turrax, Germany) for 1 min at 10,000 rpm and then incubated at 85 °C in a water bath for 1 h and centrifuged at 5000 × g for 10 min. The protein content in the supernatant was determined by using the Biuret method. Autolytic patterns of minced fish were determined using SDS PAGE as previously described.

2.5. Statistical analysis

All experiments were conducted and analyzed in triplicate. Means and standard deviations were calculated and compared using the Duncan's test. Analysis was performed using a SPSS package (SPSS 16.0 for windows, SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Three-phase partitioning of trypsin inhibitor from legume seeds

TPP with ammonium sulfate and *t*-butanol was used to fractionate the trypsin inhibitor from three legume seeds. The heat extract of the three legumes was added with solid ammonium sulfate (30% saturation, w/v) followed by the addition of 1:1 *t*-butanol. After 1 h, the three phases were formed and collected separately. The partitioning of the trypsin inhibitor by TPP is summarized in Table 1. Specific inhibitory activity (unit/mg protein) was achieved by the bottom phase for NB (2172) and RK (2094). Meanwhile, the middle phase of AZ (15,545) was given the highest specific inhibitory activity. The salting out mechanism as the part of TPP was used to separate proteins by using the kosmotrope ability of the sulfate ion [23]. Small precipitates obtained for AZ might be due to the higher hydrophilic site exposed in this protein. Meanwhile, for NB and RK, the bulk water associated with the salt ions, which made less water available to partake in the salvation layer around the protein. This leads to hydrophobic interactions. The increasing of the total units might be because of the increased flexibility of the molecule, which then probably leads to enhanced activity of the enzymes [14,15]. Singh et al. [24] reported the remarkable increase in the catalytic activity of proteinase K obtained from TPP treatment could be attributed to the presence of an acetate ion at the active site and the excited state of the proteinase K as indicated by an overall higher temperature factor. The difference between conventional salting out and TPP were the additions of *t*-butanol that binds to the protein, and also the increases in buoyancy against gravity that makes it float between the aqueous and the organic layer [25]. Sulfate that has kosmotrope and anionic “water structure promoter” generates a synergistic effect with *t*-butanol in water with a powerful structure promoter; therefore, the sulfate ion concentration used is much lower than normally required for salting out the protein [15]. The best concentration of solid ammonium sulfate used in TPP is generally around 30% saturation (w/v) [12,15,26,27]. The purity of the trypsin inhibitor increased as the number of the isolation steps increased. Therefore, heat treatment was used as the pre-treatment to remove some heat labile proteins in the sample. The TPP gave the yield of 315,441 and 228 with purification folds of 5.65, 14.15 and 7.3 for the bottom phase of NB, RK and AZ, respectively. The highest trypsin inhibitor recovery was obtained in RK, followed by NB and AZ. The saturation of 30% ammonium sulfate was sufficient enough to remove other proteins that precipitate in the middle phase and

Table 1
Three-phase partitioning of trypsin inhibitor from three legume seeds.

Sample	Step	Volume (ml)	Total protein (mg)	Total inhibitory activity (units)	Specific inhibitory activity (unit/mg)	Yield (%)	Purification (fold)
Navy bean	Crude	13	391	150,423	384 ± 61a [*]	100 ± 0a	1 ± 0a
	Heat treatment ^a	10	305	246,570	808 ± 9b	163 ± 18b	2.1 ± 0.02b
	Bottom phase ^b	19.23	218	475,000	2,172 ± 27d	315 ± 47c	5.6 ± 0.05c
	Middle phase ^b	9.77	130	241,045	1,852 ± 244c	160 ± 79b	4.8 ± 0.47d
Red kidney bean	Crude	15	499	73,740	148 ± 21a	100 ± 0a	1 ± 0a
	Heat treatment ^a	10	317	223,960	705 ± 6b	303 ± 1b	4.7 ± 0.13b
	Bottom phase ^b	14.5	155	325,496	2,094 ± 72d	441 ± 39c	14.1 ± 0.42c
	Middle phase ^b	13.12	209	294,045	1,406 ± 92c	398 ± 15c	9.5 ± 0.02d
Adzuki bean	Crude	15	194	130,185	671 ± 59a	100 ± 0a	1 ± 0a
	Heat treatment ^a	10	95	225,880	2,370 ± 79b	173 ± 25c	3.5 ± 0.15b
	Bottom phase ^b	13	60	298,051	4,920 ± 1,734c	228 ± 30d	7.3 ± 0.09c
	Middle phase ^b	1.63	2	37,154	15,545 ± 3,992d	28 ± 4b	23.2 ± 0.20d

^a Crude extracts were heated at 70 °C for 10 min and then centrifuged at 8000 × g for 15 min to remove the heat coagulated debris.

^b Fraction obtained by three-phase partitioning (TPP) with 30% saturation of ammonium sulfate.

^{*} Different letters in the same legume indicate significant differences ($p < 0.05$).

leave the trypsin inhibitor in the bottom phase of the RK fraction. However, the highest specific inhibitory activity and purification factor were achieved by AZ. The amount of protein precipitated in the middle layer of AZ was lower than that of NB and RK. It means that 30% saturation can only remove small amounts of protein but still have inhibitory activity. This phenomenon leads to higher specific activity of the trypsin inhibitor. Due to the high recovery of the isolated trypsin inhibitor, this method is potential of low cost mass production trypsin inhibitor. Soybean trypsin inhibitor was successfully purified by the modified metal-TPP with 72% recovery and 13-fold purification [18]. Sharma and Gupta [15] used the TPP as a single step process to purify protease/amylase inhibitors from wheat germ. It obtained 25-fold purity and 85% activity recovery. This result was similar to the aqueous two-phase systems (ATPS) where the target proteins usually were recovered from the bottom

phase or ammonium sulfate phase. The contaminants were contained in the top phase in ATPS (PEG phase) and TPP (butanol phase). Platis et al. [28] recovered the target protein monoclonal antibodies from transgenic tobacco plants by using ATPS as the initial purification step to remove polyphenols followed by chromatography techniques.

3.2. Protein pattern, inhibitory activity and glycoprotein staining

3.2.1. Protein pattern

Protein patterns of the crude extract and fractions of NB, RK and AZ are shown in Fig. 2. For NB, there is no significant change of protein pattern from the crude extract (lane C) and the heat treatment (lane H). The major protein bands for NB were 215, 132 and 77 kDa. Even though no changes of protein pattern were

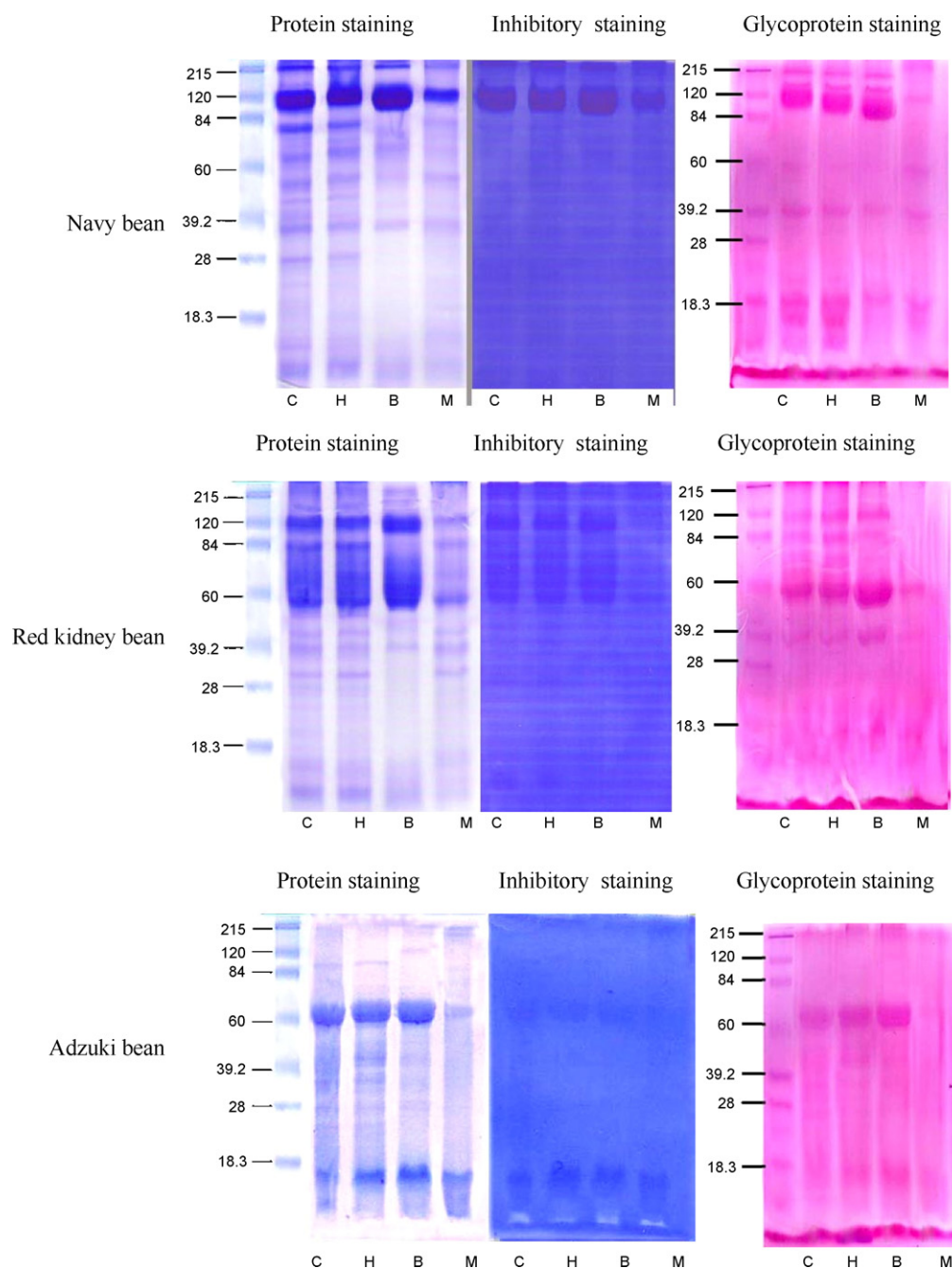


Fig. 2. Protein, inhibitor and glycoprotein staining of the samples from three legume seeds. C, crude; H, heat treatment; B, bottom phase; M, middle phase.

observed for the heat treatment sample, increasing inhibitory activity recovery was obtained (data not shown). Heat treatment leads to the conformational changes in many proteins resulted in the release of some reactive proteins. The TPP process could remove the small MW of proteins from the sample as shown in lanes B and M. The protein band with the MW of 77 kDa disappeared, and the protein band with the MWs of 215 and 132 kDa still remained with higher intensity in the bottom phase compared to that of the middle phase. The protein patterns of the bottom and middle phase of NB were identical; however, a decreased intensity was observed in lane M.

Protein bands of the crude and heat treatment samples with the MWs of 118, 59, 38 and 32 kDa were observed in RK. After TPP, the bottom phase (lane B) showed only three major bands with the MWs of 118, 59 and 38 kDa. Nevertheless, the middle phase (lane M) had the same pattern as shown in lane H with lower band intensity. The AZ presented two major protein bands with the MWs of 62 and 13 kDa after the TPP process. The minor protein bands with the MWs of 39 and 35 kDa were completely removed by the TPP. The low MW of the protein might have been removed in the upper layer (*t*-butanol phase), because the *t*-butanol could remove only low MW proteins by denaturation [29]. In the TPP process, the lipids dissolved in the upper *t*-butanol layer, while most proteins (nucleic acids and insoluble aggregates formed into macromolecules) were in the middle phase. The remaining macromolecules were solubilized in the salt phase [30]. From our results, it can be stated that the proteins have a tendency to concentrate in the bottom phase of the mixture. However, it is totally dependent on the structure of the protein molecule inside those legumes.

3.2.2. Inhibitory activity staining

Fig. 2 shows the inhibitory activity staining for the three legumes seeds. For NB, a single protein band with the MW of 132 kDa still clearly appeared on the gel. The presence of this band in the inhibitory activity staining condition suggested that it is the trypsin inhibitor. The single protein band with the MWs of 118 and 13 kDa was observed for RK and AZ, respectively. Our results revealed that TPP could partition the trypsin inhibitor into the salt phase for NB and RK, while the trypsin inhibitor of AZ was effectively isolated in the interfacial phase of the system.

3.2.3. Glycoprotein staining

It has been reported that glycoprotein was found in legume seeds with an estimated MW range of 45–55 kDa. In this study, the crude extract and isolated protein was stained for the glycoprotein content, as depicted in Fig. 2. This staining was used to detect high glycosylated proteoglycans (protein glycosaminoglycans) or glycoproteins (protein oligosaccharides) [31]. Structural glycoprotein consists of polypeptide covalently bonded to a carbohydrate moiety [32]. The glycoprotein staining revealed that the entire major protein bands contained oligosaccharide. For NB, the protein bands with the MWs of 215, 132 and 39 kDa appeared as a magenta band. A group of protein bands with the MWs of 113, 59 and 38 kDa and 62 and 13 kDa were observed for RK and AZ, respectively. These bands were identified as the glycoprotein. In addition, the protein band with the MWs of 132, 113 and 13 kDa for NB, RK and AZ also appeared at the inhibitory activity staining. This evidence confirmed that the trypsin inhibitor from NB, RK and AZ was glycoprotein.

Based on the recovery of the trypsin inhibitor obtained by using TPP, the bottom phase for all legumes was chosen for further study.

3.3. pH profile and stability

The trypsin inhibitor fraction was incubated with various pHs (3–12) in the assay condition. The highest residual activity was

observed at pHs 7, 8 and 9 for AZ, RK and NB, respectively. However, decreased relative inhibitory activity of the trypsin inhibitor fraction from all kinds of legumes was observed at strong acidic and alkaline conditions (data not shown). The trypsin inhibitor isolated from most legumes had the optimum pH of around 8 [33]. Rai et al. [34] reported that the pH 8 as the optimum for inhibitory activity of the serine Kunitz trypsin inhibitor isolation from leaves of *Terminalia arjuna*.

The pH stability of the trypsin inhibitor isolation from NB, RK and AZ was also investigated. There was no significant decrease in relative inhibitory activity of the trypsin inhibitor isolation from NB and AZ after incubation at various pHs (3–12) for 10 min ($p > 0.05$). The markedly decrease of relative inhibitory activity was observed for RK when the pH ranges were 9–12 (data not shown). The remaining residual trypsin activity of 50% was observed when the RK fraction has incubated with the basic condition (pH 12). Benjakul et al. [19] reported that the stability of trypsin inhibitor isolation from cowpea, pigeon pea and bambara groundnuts was over a wide pH ranges (3–9). Inhibitor isolated from horse gram (*Dolichos biflorus*) was stable between the pHs of 3–11 [35]. Our results revealed that, the trypsin inhibitor fractions from NB and AZ were more stable in the various pH ranges compared to that of RK. A decrease in activity was observed in the acidic ranges (pHs 3–6). When protein was exposed in the extreme pH values, the denaturation (unfolding) of the protein molecule occurred because of the strong electrostatic repulsion of ionized groups leading to losses of their function [36].

3.4. Temperature profile and stability

For thermal profile, the trypsin inhibitor of NB, RK and AZ was determined at a temperature ranging from 40 to 100 °C. The highest inhibitory activity of each fraction from each legume was found at 50 °C for RK and 60 °C for NB and AZ (Fig. 3). The relative inhibitory activity was 98%, 102% and 110% for NB, RK and AZ at each optimum temperature. As temperature used for incubation was increased, the relative inhibitory activity decreased constantly and reached the lowest point at 100 °C. Rekha et al. [37] reported that the optimum temperature for trypsin inhibitor isolation from mungbean seeds was 50 °C. As the temperature increases, more molecules have enough kinetic energy to undergo the reaction. If the temperature is raised above the optimum point, the kinetic energy of the enzyme and water molecules is so great that the structure of the enzyme molecule starts to disrupt [38]. Therefore, a decrease in activity was detected when the temperature increased.

For thermal stability, the trypsin inhibitory activity of NB, RK and AZ was determined after the fraction was incubated at 90 °C and ranging in incubation times from 10 to 90 min (Fig. 4). There is

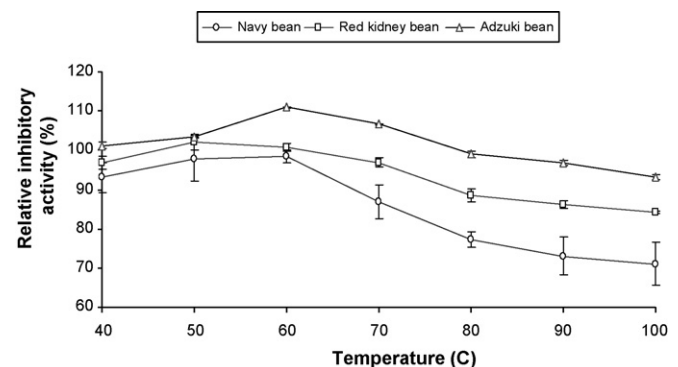


Fig. 3. Thermal profiles of isolated trypsin inhibitor from three legume seeds. Fractions were assayed at various temperatures (40–100 °C). Bars represent standard deviations from triplicate determinations.

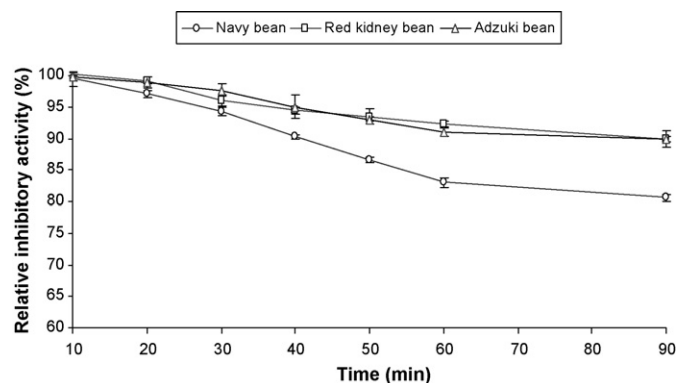


Fig. 4. Thermal stability of isolated trypsin inhibitor from three legume seeds. Fractions were incubated at 90 °C for various times (10–90 min). Bars represent standard deviations from triplicate determinations.

a significant decrease in the relative inhibitory activity when the NB, RK and AZ fractions were incubated for 20 min ($p > 0.05$). The level of thermal stability causes the changes in the tertiary structure, leading to a less ordered arrangement of the polypeptide chain that can decrease the enzyme activity [39]. The resistance of

trypsin inhibitors to thermal denaturation was shown by the retention of inhibitory activity of up to 80% after incubation at 90 °C for 90 min. The thermal stability of proteinase inhibitor depends on its amino acid composition and structural conformation. A tightly coiled conformation with multiple disulfide bonds is considered to be responsible for the resistance of thermal denaturation [40]. The isolated trypsin inhibitor from NB, RK and AZ seemed to be more stable than that of the trypsin inhibitor from cowpea, pigeon pea and bambara groundnut, which were heated steadily for up to 30 min at 90 °C [19]. From three legumes, NB showed the lowest thermal stability when compared with others. The trypsin inhibitor isolated from *Calliandra selloi* Macbride was found to be quite stable at 80 °C for 30 min [41]. Richardson [42] reported that the Bowman–Birk inhibitor was more stable compared to the Kunitz type trypsin inhibitors, which displayed significant heat stability. This stability is probably due to the fact that the Bowman–Birk inhibitor consists of cysteine-rich polypeptide chain bridged by seven conserved disulfide bonds.

3.5. Salt stability

The effect of NaCl on the inhibitory activity of the trypsin inhibitor isolated from three legume seeds was studied. Slightly

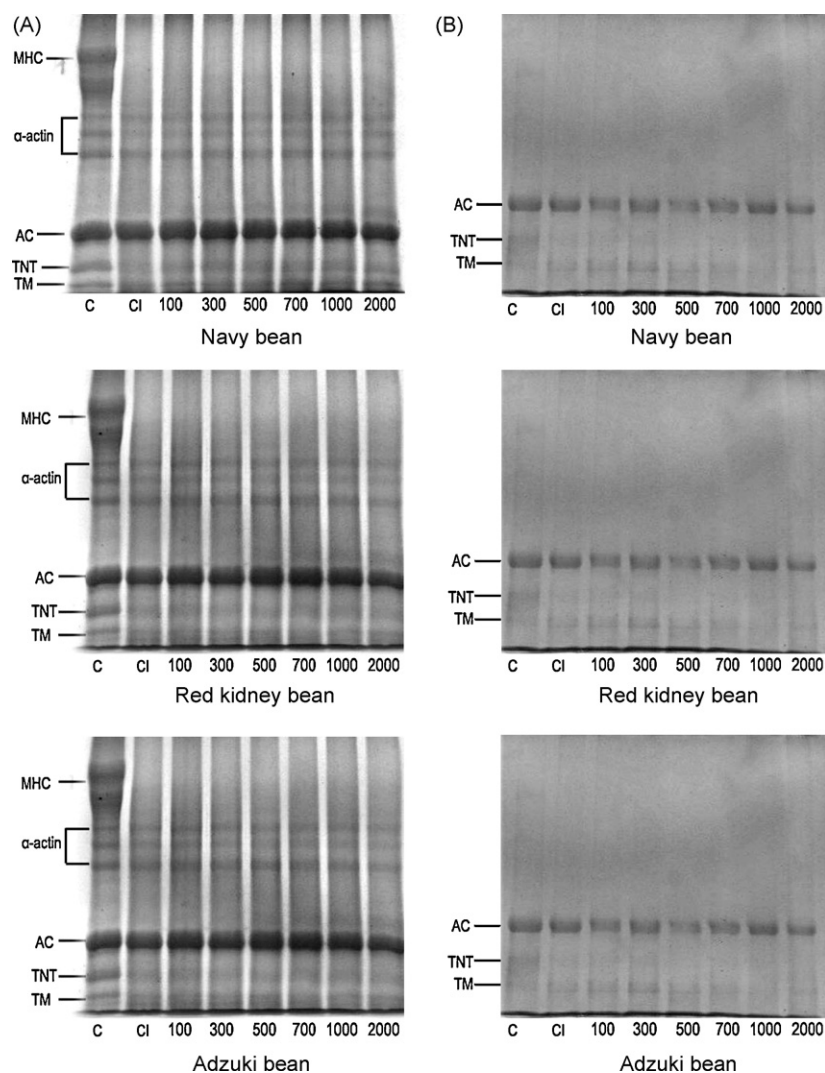


Fig. 5. Effect of isolated trypsin inhibitor fraction from three legume seeds on autolysis inhibition of Pacific whiting (A) and arrowtooth flounder (B) minced. C, minced; CI, minced with incubation. Numbers (100–2000) designate the fraction volume (µl) used; MHC, myosin heavy chain; α-actin, alpha-actin; AC, actin; TNT, troponin-T; TM, tropomyosin.

decreased inhibitory activity was observed for NB and AZ, yet meanwhile the relative inhibitory activity of RK reached the lowest point when the salt concentration increased ($p > 0.05$) (data not shown). The relative inhibitory activity of each legume reached 99%, 95% and 97% for NB, RK and AZ when the salt concentration addition was up to 3% (w/v). The trypsin inhibitor from the three legumes was fairly stable with salt concentrations of up to 3% (w/v), demonstrated by the high relative inhibitory activity ($>95\%$). The effect of salt is considered to involve ion–dipole association or hydrogen bonding of a decrease in the double-bond character and the consequent loss of polypeptide-chain rigidity [43]. However, a low level of salt concentration could increase the solubility of the protein inside the legume via the “salting process” which results in more inhibitors released into the system.

3.6. Effect of isolated trypsin inhibitor in muscle model system

The autolysis inhibition study of minced Pacific whiting is shown in Fig. 5A. The major bands of Pacific whiting muscle were myosin heavy chain (MHC), α -actinin (α -actin), actin (AC), troponin-T (TNT) and tropomyosin (TM). When the muscle was incubated at 55 °C for 60 min, the MHC completely disappeared and decreased in intensity of α -actin (lane CI). However, the actin band did not undergo hydrolysis by this incubation condition. This phenomenon was caused by high protease activity of the Pacific whiting muscle. An et al. [44] reported that high proteolytic activity of Pacific whiting was caused by the most active protease that was identified as cathepsin L. The protease was also found as the predominant heat-activated proteinase in the muscle extract of arrowtooth flounder, which was responsible for high proteolytic activity [45]. When the Pacific whiting muscle was incubated with the trypsin inhibitor fractions from NB, RK and AZ, there were no changes in the protein pattern between the control (lane CI) and the muscle that was incubated with the trypsin inhibitor fraction (100–2000 μ l). It showed that the MHC was completely hydrolyzed by the protease contained in the muscle. The addition of the trypsin inhibitor fraction (at all levels) from all kinds of legumes could not inhibit the autolysis of the Pacific whiting muscle. Increasing intensities of α -actin were observed in the muscle incubated with NB and RK. Meanwhile, adzuki beans only showed autolytic inhibition on α -actin.

The incapability of the trypsin inhibitor to isolate from three legume seeds might correlate with the different target protease responsible for the proteolytic activity of the Pacific whiting muscle, cathepsin L (cysteine proteinase) [44]. Therefore, the trypsin inhibitor isolation from three legumes was unable to inhibit the proteolytic activity in the Pacific whiting muscle. It may also be stated that the inhibitor fraction from these legumes contains only the trypsin inhibitor.

The autolysis inhibition study of arrowtooth flounder by the trypsin inhibitor fractions from NB, RK and AZS is depicted in Fig. 5B. For minced arrowtooth flounder, the same pattern of proteolysis was observed. However, high hydrolytic activity was found as indicated by the disappearance of MHC and other protein bands. Only the actin band was observed in the incubated minced fish at the optimum condition of protease activity presented in this fish. Different concentrations of trypsin inhibitor fractions of NB, RK and AZ (100–2000 μ l) were applied into the minced arrowtooth flounder. The protein bands of MHC, and α -actin were still not observed for all inhibitor fractions added. This result indicated that the trypsin inhibitor fraction from all legume seeds could not inhibit the autolysis of this fish muscle as well as of Pacific whiting.

The presence of heat stable cysteine proteinase (cathepsin L) in arrowtooth flounder was presumed as the major cause of the high hydrolytic activity in the muscle, especially at elevated tempera-

tures [46]. Visessanguan et al. [45] reported partial or complete inhibition of cathepsin L isolation from minced arrowtooth flounder that was obtained by E-64 and iodoacetate containing thiol-blocking agents. These results clearly confirmed that the inhibitor fraction from the TPP of the three legumes contained only the trypsin inhibitor.

4. Conclusion

Trypsin inhibitor was partially purified from NB, RK and AZ by heat treatment (70 °C for 10 min) and by three-phase partitioning (30% saturation of solid ammonium sulfate, w/v and *t*-butanol). The purification folds of 5–14 with the recovery of 228–441% from the bottom phase of three legumes were obtained. The trypsin inhibitors from NB and AZ were quite stable at a wide range of pH, and meanwhile the RK loosened its activity by up to 50% in an alkaline condition. High temperature and salt concentration did not affect the inhibitory activity of the inhibitor fraction from the three legumes. The autolysis of Pacific whiting and arrowtooth flounder was not inhibited by the addition of trypsin inhibitor fraction from all the legumes.

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Type I collagen from the skin of ornate threadfin bream (*Nemipterus hexodon*): Characteristics and effect of pepsin hydrolysis

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ABSTRACT

Type I collagen from the skin of ornate threadfin bream (*Nemipterus hexodon*) was purified and characterised. Purified type I collagen contained $[\alpha 1(I)]_2\alpha 2(I)$ as the dominant component with the co-presence of $\alpha 1(I)\alpha 2(I)\alpha 3(I)$. It was rich in glycine and alanine with high content of imino acids (188 residues/1000 residues). The maximum transition temperature (T_m) and the total denaturation enthalpy (ΔH) of purified type I collagen was 33.35 °C and 0.819 J/g, respectively. The isoelectric point (pI) of purified type I collagen was estimated to be 6.40. After hydrolysis of purified type I collagen using pepsin, the band intensity ratios of $\alpha 1/\alpha 2$ -chains were increased ($P < 0.05$). The cross-linked components were effectively hydrolysed by pepsin 1 and 2 from skipjack tuna stomach and porcine pepsin at 4 °C without the cleavage of β - and α -chains. At 50 °C, they were more susceptible to porcine pepsin hydrolysis, followed by pepsin 2 and 1, respectively.

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1. Introduction

Collagen is the fibrous protein that contributes to the unique physiological functions of connective tissues in skins, tendons, bones and cartilages, and is associated with the toughness of mammalian muscle (Foegeding, Lanier, & Hultin, 1996; Kittiphattanabawon, Benjakul, Visessanguan, Kishimura, & Shahidi, 2010; Muyonga, Cole, & Duodu, 2004; Ogawa et al., 2003; Yan et al., 2008). The structural unit of collagen is tropocollagen, a rod-shaped protein consisting of three polypeptides unit (called α -chains) intertwined to form a triple-helical structure (Wong, 1989). Each polypeptide chain forms a left-handed helix and consists of repeating triplets, $(\text{Gly-X-Y})_n$, where X and Y are, with a high possibility, proline or hydroxyproline (Ogawa et al., 2003). The repetitive occurrence of glycine is absent in the first 14 amino acid residues from the N-terminus and the first 10 amino acids from the C-terminus. These end portions are termed “telopeptides” (Foegeding et al., 1996). The covalent cross-links at the telopeptide regions between the collagen molecules are formed by the condensation of aldehyde groups, as well as the formation of a Schiff's base when the aldehyde reacts with an amino group (Foegeding et al., 1996; Jongjareonrak, Benjakul, Visessanguan, Nagai, & Tanaka, 2005). These cross-linking molecules generally cause the decrease in the solubility of collagen (Belitz, Grosch, & Schieberle,

2004; Foegeding et al., 1996). At least 19 variants of collagen (types I–XIX) have been reported (Bailey, Paul, & Knott, 1998). Type I collagen, $[\alpha 1(I)]_2\alpha 2$, is the major collagen found predominantly in the connective tissues, such as skin, bone, and tendon, and contains two identical $\alpha 1(I)$ chains and one $\alpha 2$ -chain (Trelstad, Catanese, & Rubin, 1976). Chromatographic and electrophoretic analyses revealed that most of the teleost skin collagens also contained a unique subunit, $\alpha 3$ (Kimura, Ohno, Miyauchi, & Uchida, 1987). The existence of $\alpha 1\alpha 2\alpha 3$ and $(\alpha 1)_2\alpha 2$ heterotrimer of type I collagen from the skin of some teleostean and chondrosteian fish species has been reported (Kimura, 1992; Kimura & Ohno, 1987; Kimura et al., 1987).

Currently, the increasing attention of alternative sources for replacement of mammalian collagen has been paid, especially from seafood processing by-products. Fish skin, a by-product from fish processing, is one of the alternative sources for collagen preparation. Collagens from several fish species, such as arabesque greenling (Nalinanon, Benjakul, & Kishimura, 2010a), brownbanded bamboo shark (Kittiphattanabawon et al., 2010), cod (Duan, Zhang, Du, Yao, & Konno, 2009), eagle ray (Bae et al., 2008), walleye pollock (Yan et al., 2008), deep-sea red fish (Wang, An, Xin, Zhao, & Hu, 2007), bigeye snapper (Benjakul et al., 2010; Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005; Nalinanon, Benjakul, Visessanguan, & Kishimura, 2007), channel catfish (Liu, Li, & Guo, 2007), Pacific whiting (Kim & Park, 2004), black drum (Ogawa et al., 2003), sheephead seabream (Ogawa et al., 2003), ocellate puffer fish (Nagai, Araki, & Suzuki, 2002) and Japanese

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seabass, chub mackerel and bullhead shark (Nagai & Suzuki, 2000), have been isolated and characterised. Pepsin, mainly from porcine stomach, was used to improve the extraction efficiency of collagen for a decade. Recently, pepsins from the stomach of several fish species, such as bigeye snapper, albacore tuna, tongol tuna and skipjack tuna, have also been used as the aid for collagen extraction (Benjakul et al., 2010; Nalinanon et al., 2010a; Nalinanon et al., 2007; Nalinanon, Benjakul, Visessanguan, & Kishimura, 2008). Nalinanon et al. (2008) found that albacore tuna pepsin exhibited the comparable extraction efficiency of collagen from the skin of threadfin bream to porcine pepsin, and no detrimental effect on the integrity of resulting collagen was observed. Nevertheless, pepsin from skipjack tuna caused the degradation of the α and β components. The differences in the hydrolysis of collagen matrix by pepsin might be dependent on several factors, such as the source and type of collagen, and the type of pepsin and hydrolysis condition. Therefore, the objective of this study was to isolate and characterise the major collagen from the skin of ornate threadfin bream (*Nemipterus hexodon*), one of the fish species commonly used for surimi production in Thailand, as well as to demonstrate the hydrolysis of type I collagen by different pepsins, including porcine pepsin and skipjack tuna pepsin 1 and 2.

2. Materials and methods

2.1. Chemicals

Bovine haemoglobin, β -mercaptoethanol (β ME), type I collagen from calf skin and pepsin from porcine stomach mucosa (EC 3.4.23.1), (750 U/mg dry matter) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid, disodium hydrogen phosphate, sodium citrate, and acetic acid were obtained from Merck (Darmstadt, Germany). Coomassie Blue R-250 and *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). Sodium dodecyl sulphate (SDS) and bovine serum albumin were obtained from Fluka (Buchs, Switzerland). High-molecular-weight protein markers, DEAE-sephacel and Sephadex G-75 were purchased from GE Healthcare UK Limited (Buckinghamshire, UK). TOYOPEARL® CM-650M was purchased from Tosoh Corporation (Tokyo, Japan).

2.2. Preparation of fish stomach and skin

Stomach of skipjack tuna (*Katsuwonus pelamis*) was obtained from Tropical Canning (Thailand) Public Co., Ltd., Songkhla, Thailand. The sample was packed in a polyethylene bag, kept in ice with a solid/ice ratio of 1:2 (w/v) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand, within 1 h. Upon arrival, the stomach was cleaned with cold water and placed in a polyethylene bag. The stomach sample was stored at -20°C until use.

Ornate threadfin bream (*N. hexodon*) was obtained after being off-loaded for 6 h from the dock in Songkhla, Thailand. The fish were kept in ice with a solid/ice ratio of 1:2 (w/v) and were transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand, within 30 min. Upon arrival, the skin was removed and washed with cold water, drained and cut into small pieces ($0.5 \times 0.5 \text{ cm}^2$). The skin sample was kept in a polyethylene bag and stored at -20°C until use.

2.3. Preparation of pepsins

2.3.1. Isolation of pepsins

The frozen stomach was thawed using running water ($26\text{--}28^{\circ}\text{C}$) until the core temperature reached $-2\text{--}0^{\circ}\text{C}$. The sample

was cut into pieces with dimensions of $0.5 \times 0.5 \text{ cm}^2$. Sample was frozen in liquid nitrogen and finely ground using a National Model MX-T2GN blender (Taipei, Taiwan). The sample was then lyophilised using a SCANVAC CoolSafe™ freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark).

To prepare the skipjack pepsin extract, the stomach powder was suspended in 20 mM sodium phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) at a ratio of 1:19 (w/v), and was stirred continuously at 4°C for 3 h. The suspension was centrifuged for 20 min at 4°C at 20,000g (Avanti® J-E, Beckman Coulter, Inc., Palo Alto, CA, USA) to remove the tissue debris. The supernatant was collected and then adjusted to pH 4 with 1 M HCl. The mixture was allowed to stand at 4°C for 30 min. The treated stomach extract was then centrifuged at 20,000g for 15 min at 4°C . To fully activate pepsin, the pH of the acidified supernatant was adjusted to pH 2 with 1 M HCl, and was gently stirred for 30 min at 4°C . The enzyme solution was collected in the same manner as described previously prior to dialysis against 60 volumes of 20 mM sodium phosphate buffer (pH 5.0), referred to as 'starting buffer, SB' for 12 h at 4°C . The dialysate was collected, and was referred to as "skipjack pepsin extract".

To prepare the porcine pepsin solution, the enzyme powder (1.0 g) was dissolved with 120 ml of SB, followed by centrifugation at 4°C for 30 min at 20,000g. The supernatant was collected and used for further study.

2.3.2. Partial purification of pepsins

All the steps of purification were carried out at 4°C . Skipjack pepsin extract was applied onto DEAE-sephacel column ($1.6 \times 18 \text{ cm}$), previously equilibrated with SB. After the column was washed with SB until the A_{280} was below 0.05, the elution was performed with a linear gradient of 0–0.5 M NaCl in SB, at a flow rate of 0.5 ml/min. Fractions of 3 ml were collected and those with proteolytic activity were pooled. Two activity peaks (skipjack tuna pepsin 1 and 2, referred to as SP1 and SP2, respectively) were obtained and the pooled fractions from each peak were dialysed with 60 volumes of SB for 12 h. The dialysates were then subjected to lyophilisation. Lyophilised fractions were dissolved in SB and chromatographed onto Sephadex G-75 ($1.6 \times 70 \text{ cm}$) column, followed by elution with SB at a flow rate of 0.5 ml/min. Fractions of 3 ml were collected and the fractions with proteolytic activity were combined and used for further study. The porcine pepsin solution was purified in the same manner. The purified porcine pepsin fraction was collected and referred to as "PP". During purification, the protein concentration was measured by monitoring the absorbance at 280 nm, and the proteolytic activity was determined using haemoglobin as a substrate at pH 2.0, and 50°C .

2.4. Enzyme assay

The proteolytic activity of the pepsin extract was determined using haemoglobin as a substrate according to the method of Nalinanon, Benjakul, and Kishimura (2010b). To initiate the reaction, 200 μl of enzyme solution were added into the assay mixture containing 200 μl of 2% haemoglobin, 200 μl of distilled water and 625 μl of reaction buffer. An appropriate dilution was made to ensure that the amount of enzyme was not excessive for available substrate in the assay system. The reaction was conducted at pH 2.0 and 50°C for 10 min. To terminate enzymatic reaction, 200 μl of 50% (w/v) trichloroacetic acid (TCA) were added. Unhydrolysed protein substrate was allowed to precipitate for 1 h at 4°C , followed by centrifuging at 4250g for 10 min using a MIKRO 20 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). The oligopeptide content in the supernatant was measured at 280 nm. One unit (U) of activity was defined as the amount causing an increase of 1.0 in absorbance at 280 nm per min. A blank was

run in the same manner, except that the enzyme was added into the reaction mixture after the addition of 50% (w/v) TCA.

2.5. Preparation of type I collagen from the skin of ornate threadfin bream

2.5.1. Isolation of skin collagen

Acid solubilised collagen from the skin of ornate threadfin bream was isolated following the method of Nalinanon et al. (2007) with some modifications. All procedures were performed at 4 °C. To remove non-collagenous proteins, the skin was mixed with 0.1 M NaOH using a sample/alkaline solution ratio of 1:10 (w/v). The mixture was stirred continuously for 6 h. The alkaline solution was changed every 2 h. Then, the alkaline treated skin was washed with cold water until neutral or faintly basic pHs of wash water were obtained. The treated skin was then defatted with 10% (v/v) butyl alcohol with a solid/solvent ratio of 1:10 (w/v) for 18 h and the solvent was changed every 6 h. Defatted skin was washed with 10 volumes of cold water for three times prior to lyophilisation.

To extract the collagen, the lyophilised skin was soaked in 0.5 M acetic acid with a sample/solution ratio of 1:100 (w/v) for 48 h, with gentle stirring. The mixture was then centrifuged at 20,000g for 1 h at 4 °C. The supernatant was referred to as 'acid solubilised collagen, ASC'.

2.5.2. Fractionation of type I collagen

Type I collagen was fractionated according to the method of Yata, Yoshida, Fujisawa, Mizuta, and Yoshinaka (2001) with a slight modification. ASC was salted out by adding NaCl to a final concentration of 2.0 M, followed by centrifugation at 10,000g for 20 min. The precipitate was further extracted with 0.5 M acetic acid containing 11% (w/v) ammonium sulphate for 24 h. The mixture was then centrifuged at 10,000g for 20 min. This procedure was repeated three times. The resultant precipitate was referred to as "type I collagen".

2.5.3. Purification of type I collagen

Fractionated type I collagen was dissolved in 0.5 M acetic acid and dialysed against 60 volumes of the starting buffer (50 mM sodium acetate, pH 4.8) for 48 h; the dialysis solution was changed every 6 h. The dialysate was applied onto a TOYOPEARL® CM-650M column (1.8 × 20 cm), previously equilibrated with 10 volumes of the starting buffer, at a flow rate of 60 ml/h. After loading, the unbound proteins were washed by the same buffer until A_{230} was less than 0.05. Elution was achieved with a linear gradient of 0–0.5 M NaCl at a flow rate of 60 ml/h with a total volume of 270 ml. The fractions (3 ml) were collected and measured for the absorbance at 230 nm by a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). Appropriate fractions were pooled and dialysed against distilled water, followed by lyophilisation. The lyophilised collagen was referred to as "purified type I collagen".

2.6. Characterisation of type I collagen

2.6.1. Hydroxyproline content and conversion factor for collagen

The purified type I collagen was dehydrated with acetone prior to the determination of hydroxyproline content, as described by Nalinanon et al. (2007). The hydroxyproline content was calculated and expressed as mg/g of dried purified type I collagen. The conversion factor of hydroxyproline content to collagen content was calculated as:

$$\text{Conversion factor} = \frac{100}{\text{Hydroxyproline content (\% dry wt.)}}$$

2.6.2. UV absorption measurement

The purified type I collagen was dissolved in 0.5 M acetic acid to obtain a concentration of 1 mg/ml. The solution was placed into a quartz cell with a path length of 1 cm. The UV absorption spectrum of collagen was measured using a spectrophotometer. Prior to the measurement, the base line was set with 0.5 M acetic acid. The spectrum was obtained by scanning the wavelength in the range of 190–350 nm with a scan speed of 50 nm/min at room temperature.

2.6.3. Amino acid analysis

The purified type I collagen was hydrolysed under reduced pressure in 4.0 M methanesulfonic acid containing 0.2% (v/v) 3-(2-aminoethyl)indole at 115 °C for 24 h. The hydrolysates were neutralised with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.4 ml was applied to an amino acid analyser (MLC-703; Atto Co., Tokyo, Japan).

2.6.4. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed by the method of Laemmli (1970). The samples were mixed with 5% (w/v) SDS and heated at 85 °C for 1 h. The mixtures were then centrifuged at 8500g for 5 min to remove undissolved debris. Solubilised samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% (w/v) SDS, 20% (v/v) glycerol) in the presence or absence of 10% (v/v) βME. Samples (15 µg protein) were loaded onto a polyacrylamide gel made of 7.5% separating gel and 4% stacking gel, and subjected to electrophoresis at a constant current of 15 mA/gel, using a Mini-PROTEAN II unit (Bio-Rad Laboratories Inc., Richmond, CA, USA). After electrophoresis, the gels were fixed with a mixture of 50% (v/v) methanol and 10% (v/v) acetic acid for 45 min, followed by staining with 0.05% (w/v) Coomassie Blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid for 3 h. Finally, they were destained with the mixture of 30% (v/v) methanol and 10% (v/v) acetic acid for 45 min. High-molecular-weight protein markers (GE Healthcare UK Limited, Buckinghamshire, UK) were used to estimate the molecular weight of proteins. Type I collagen from calf skin was used as the standard. The gels were imaged using a Canon image scanner (CanoScan LiDE25, Canon Inc., Tokyo, Japan) and the band intensities were quantified with the public domain digital analysis software, ImageJ (ImageJ 1.42q, National Institutes of Health, Bethesda, MD, USA).

2.6.5. Determination of subunit compositions of type I collagen

The analysis of the subunit compositions of purified type I collagen was performed according to the method of Kittiphattanabawon et al. (2010) and Nalinanon et al. (2010a) with a slight modification. Purified type I collagen (20 mg) were dissolved in 10 ml of starting buffer (50 mM sodium acetate buffer, pH 4.8 containing 2 M urea) and incubated at 60 °C for 30 min. The mixtures were centrifuged at 20,000g at room temperature (25–26 °C) for 30 min. The supernatant was applied onto a TOYOPEARL® CM-650M column (1.8 × 20 cm) previously equilibrated with 10 volumes of the starting buffer at a flow rate of 60 ml/h. After loading, the unbound proteins were washed by the same buffer until the A_{230} was less than 0.05. Elution was achieved with a linear gradient of 0–0.2 M NaCl in the same buffer, at a flow rate of 60 ml/h, with a total volume of 270 ml. The eluant was monitored at 230 nm and fractions (3 ml) were collected. The selected fractions were subjected to SDS–PAGE using 7.5% separating gel and 4% stacking gel, as previously described.

2.6.6. Differential scanning calorimetry (DSC)

DSC analysis of the collagen samples was carried out following the method of Nalinanon et al. (2007) with a slight modification. The samples were rehydrated by adding 0.05 M acetic acid to dried

samples at a solid/solution ratio of 1:40 (w/v). The mixture was allowed to stand for 2 days at 4 °C. DSC analysis was performed using a differential scanning calorimeter (Model DSC 7, Norwalk, CT, USA). Temperature calibration was done using the indium thermogram. The collagen solutions (5–10 mg) were accurately weighed into aluminium pans and sealed. The samples were scanned at 1 °C/min over the range of 20–50 °C using iced water as the cooling medium. An empty pan was used as the reference. The maximum transition temperature (T_m) was estimated from the DSC thermogram. Total denaturation enthalpy (ΔH) was estimated by measuring the area in the DSC thermogram.

2.6.7. Measurement of zeta (ζ) potential

Purified type I collagen was dissolved in 0.5 M acetic acid at a concentration of 0.5 mg/ml. The mixture was stirred at 4 °C for 12 h. The ζ -potential of the sample (20 ml) was measured using a Zeta potential analyser (ZetaPALS, Brookhaven Instruments Co., Holtsville, NY, USA). The pH of the samples was adjusted to different values with 1.0 M Nitric acid or 1.0 M KOH using an Autotitrator (BI-ZTU, Brookhaven Instruments Co., Holtsville, NY, USA). The isoelectric point (pI) was estimated from pH rendering zero ζ -potential.

2.7. Hydrolysis of type I collagen by pepsin

PP, SP1 or SP2 (4 U) was added to 2 ml of a reaction mixture, containing 8 mg of purified type I collagen in 0.5 M acetic acid, pH 2.0. The hydrolysis was conducted by incubating the mixture at 4 °C for 24 h or at 50 °C for 0, 0.5, 1, 2 and 3 h. The control was performed by incubating the reaction mixture at 4 °C for 24 h or 50 °C for 3 h without the addition of pepsins. At the designated time, 200 μ l of reaction mixture was taken and mixed with 100 μ l of 1 M NaOH containing 1% (w/v) SDS in order to terminate the pepsin activity. The mixture was further mixed with 100 μ l of SDS–PAGE sample buffer and boiled for 1 min. The reaction mixture was analysed by SDS–PAGE using 7.5% separating gel and 4% stacking gel, as previously described.

2.8. Statistical analysis

All data were subjected to Analysis of Variance (ANOVA), and the differences between means were evaluated by Duncan's multiple

range test. The SPSS statistic programme (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA) was used for data analysis.

3. Results and discussion

3.1. Extraction of ASC and purification of type I collagen

ASC from the skin of ornate threadfin bream was extracted with the yield of $24.9 \pm 1.1\%$ (dry wt.). The hydroxyproline content in the skin and ASC was 49.5 ± 0.4 and 72.3 ± 0.5 mg/g dry wt., respectively. The hydroxyproline content of ASC increased by approximately 1.46-fold, compared with that found in the skin. The result suggested that non-collagenous matters could be removed effectively from the skin prior to collagen extraction. To separate type I collagen, ASC was fractionated by NaCl precipitation. The precipitate was further extracted with 0.5 M acetic acid containing 11% (w/v) ammonium sulphate. Yata et al. (2001) isolated pepsin-solubilised collagen from the skin of common horse mackerel, yellow sea bream and tiger puffer fish with the same method, and found that type I collagen was a major collagen in the precipitate, whereas the supernatant contained type V collagen. After salt fractionation, the supernatant was further precipitated by 20% (w/v) ammonium sulphate. No collagen was recovered, suggesting that very low or no type V collagen in ASC from the skin of ornate threadfin bream was solubilised by acetic acid (data not shown). The collected precipitate was subjected to further purification using TOYOPEARL® CM-650M ion-exchange column chromatography (Fig. 1). The peak (fractions 50–62) showed the typical SDS–PAGE pattern of type I collagen with two different α bands, $\alpha_1(I)$ and $\alpha_2(I)$ (see inset). It also contained β and γ components as well as other higher molecular weight cross-linked components. The band intensity of α_1 was approximately 2-fold higher than that of α_2 , indicating the existence of [$\alpha_1(I)$] $_2$ $\alpha_2(I)$ in collagen triple helix. Fractions No. 50–62 containing collagen components were pooled and used for further study. Based on the precipitation by ammonium sulphate at acidic pH and SDS–PAGE pattern, the collagen fraction obtained could be classified as type I collagen. Purified type I collagen had the higher hydroxyproline content (78.6 ± 0.3 mg/g dry wt.) in comparison with that of ASC ($P < 0.05$), indicating a greater purity in the former. When the absorbance spectrum was determined for purified type I collagen, the distinct absorption peak was observed at 230 nm. This was mainly attributed to the absorption of the peptide bonds in the

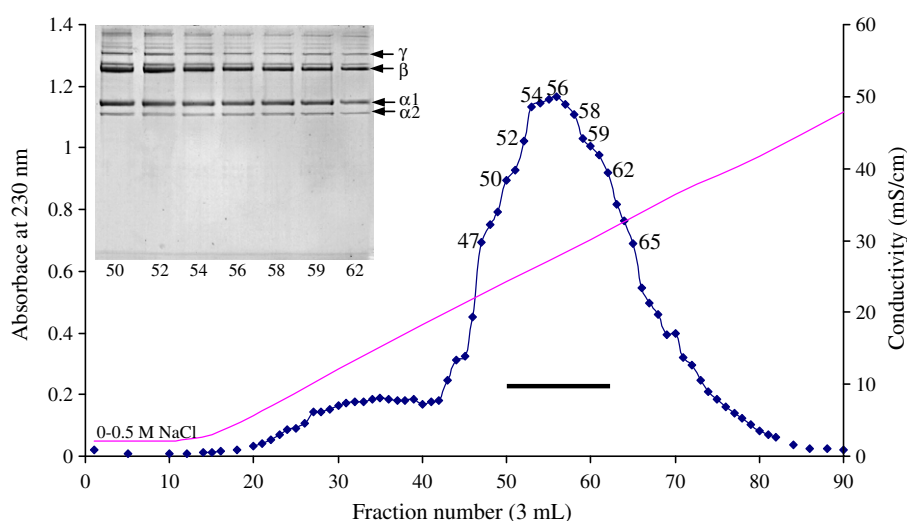


Fig. 1. TOYOPEARL® CM-650M ion-exchange column chromatography of type I collagen isolated from the skin of ornate threadfin bream. The column (1.8 × 20 cm) was equilibrated with 50 mM sodium acetate, pH 4.8. Adsorbed proteins were eluted with a linear gradient of 0–0.5 M NaCl over a total volume of 270 ml, at a flow rate of 60 ml/h. The inset shows the SDS–PAGE pattern (7.5% separating gel and 4% stacking gel) of the fractions indicated by the numbers.

polypeptides chains of collagen. No absorption peak was detected at 280 nm (data not shown). Thus, type I collagen from the skin of ornate threadfin bream was obtained with negligible contamination of other proteins.

With reference to hydroxyproline content, the conversion factor of hydroxyproline to collagen from the skin of ornate threadfin bream was calculated to be 12.7. The conversion factor of collagen from other sources, such as Baltic cod backbone (15.7) (Żelec-howska, Sadowska, & Turk, 2010) and skin (14.7) (Sadowska, Kołodziejka, & Niecikowska, 2003) has been reported. This factor might be useful for the estimation of collagen content in raw material, and the yield of extraction, as well as the purity assessment of the resultant collagen.

3.2. Subunit composition of purified type I collagen

The elution profiles of the purified type I collagen on the TOYOPEARL® CM-650M column, after being denatured by heat treatment in the presence of 2 M urea, are shown in Fig. 2. Three peaks containing different collagen components were obtained. The chromatographic fractions indicated by numbers were subjected to SDS–PAGE analysis as shown in the inset. The first peaks (fraction No. 37) contained only a band of α -chain. The second peak (fraction No. 41) consisted of two distinct components, β 11 (dimer of α 1) and α -chains. High MW component, γ -chain and β 12 (dimer of α 1 and α 2) were concentrated in fraction No. 47–51 of the third peak, whilst α 2 was observed in fraction No. 49–51. Since the α -chains found in fraction No. 37 and 41 were eluted by different NaCl concentrations but had similar molecular mobility, both α 1- and α 3-chains were most likely present in purified collagen. Kimura et al. (1987) and Matsui, Ishida, and Kimura (1991) reported that the electrophoretic mobility in SDS–PAGE of α 3 from chum salmon, as well as other teleosts, was almost identical to that of α 1. Therefore, the purified collagen from the skin of ornate threadfin bream not only comprised $[\alpha 1(I)]_2\alpha 2(I)$ but also $\alpha 1(I)\alpha 2(I)\alpha 3(I)$. The existence of two molecular forms, $[\alpha 1(I)]_2\alpha 2(I)$ and $\alpha 1(I)\alpha 2(I)\alpha 3(I)$, were reported in fish type I collagen (Kimura et al., 1987). The α 3-chain was reported as the intermediate between α 1 and α 2 (Matsui et al., 1991).

3.3. Amino acid composition of purified type I collagen

The amino acid composition of purified type I collagen from the skin of ornate threadfin bream, expressed as residues per 1000 total amino acid residues, is shown in Table 1. The purified type I collagen was rich in glycine, alanine and proline. It had relatively low contents of tyrosine and histidine and it contained no cysteine and tryptophan. The results were in accordance with those of type I collagens from the skin of other fish species, such as common horse mackerel, yellow sea bream, tiger puffer fish (Yata et al., 2001), black drum, sheephead seabream (Ogawa et al., 2003), brownstripe red snapper (Jongjareonrak et al., 2005) and arabesque greenling (Nalinanon et al., 2010a). The imino acid (proline and hydroxyproline) content of purified type I collagen was 188 residues/1000 residues, which was slightly lower than that of type I collagen from calf skin (215 residues/1000 residues) (Ogawa et al., 2003), young and adult Nile perch (193–200 residues/1000 residues) (Muyonga et al., 2004) and bigeye snapper (193 residues/1000 residues) (Kittiphattanabawon et al., 2005). However, the imino acid content of purified collagen was relatively higher than those reported for collagen from the skin of cold water fish species, such as arabesque greenling (Nalinanon et al., 2010a), cod (Duan et al., 2009), deep-sea red fish (Wang et al., 2007) and Pacific whiting (Kim & Park, 2004), which had imino acid content ranging from 154 to 165 residues/1000 residues. The imino acids contribute to the stability of helical structure of collagen (Ikoma, Kobayashi, Tanaka, Walsh, & Mann, 2003; Jongjareonrak et al., 2005). The stability of collagen was proportional to the total content of imino acids (Kittiphattanabawon et al., 2010). Pyrrolidine rings of proline and hydroxyproline impose restrictions on the conformation of the polypeptide chain and help to strengthen the triple helix (Bae et al., 2008; Wong, 1989). The difference in the imino acid content amongst the animals was associated with the different living environments (Foegeding et al., 1996; Kittiphattanabawon et al., 2010; Nalinanon et al., 2010a; Regenstein & Zhou, 2007). Hydroxylysine (8 residues/1000 residues) was found in purified type I collagen, suggesting the partial cross-linking of collagen via covalent bonds (Mechanic, Katz, Henmi, Noyes, & Yamauchi, 1987; Wong, 1989).

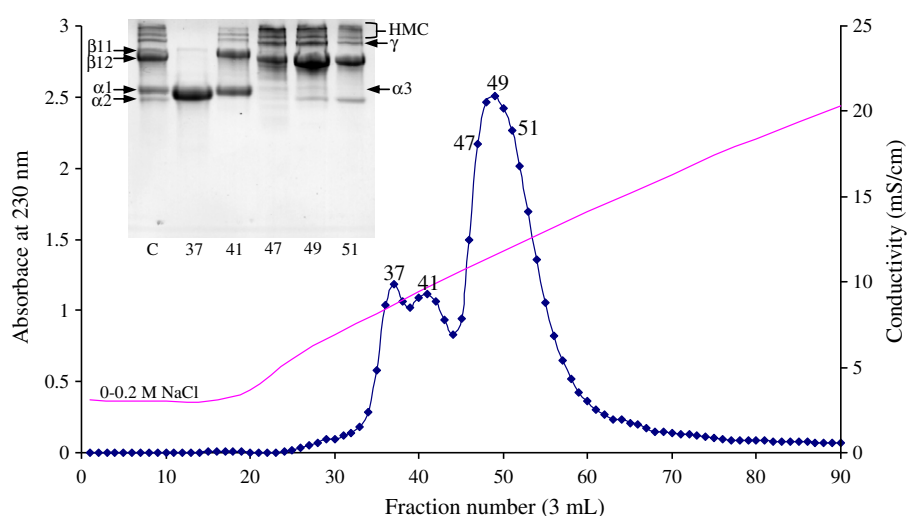


Fig. 2. TOYOPEARL® CM-650M ion-exchange column chromatography of denatured type I collagen from the skin of ornate threadfin bream. The column (1.8 × 20 cm) was equilibrated with 50 mM sodium acetate, pH 4.8, containing 2 M urea. Adsorbed proteins were eluted with a linear gradient of 0–0.2 M NaCl over a total volume of 270 ml at a flow rate of 60 ml/h. The inset shows the SDS–PAGE pattern (7.5% separating gel and 4% stacking gel) of the fractions indicated by the numbers. C and HMC denote collagen and high MW cross-linked components, respectively.

Table 1

Amino acid composition of purified type I collagen from the skin of ornate threadfin bream (residues/1000 residues).

Amino acid	Purified type I collagen
Alanine	134
Arginine	53
Aspartic acid/asparagine	49
Cysteine	0
Glutamic acid/glutamine	72
Glycine	321
Histidine	5
Isoleucine	9
Leucine	23
Lysine	27
Hydroxylysine	8
Methionine	14
Phenylalanine	14
Hydroxyproline	84
Proline	104
Serine	35
Threonine	22
Tyrosine	4
Tryptophan	0
Valine	21
Total	1000
Imino acids ^a	188

^a Imino acids include proline and hydroxyproline.

3.4. Thermal stability of purified type I collagen

DSC analysis of purified type I collagen from the skin of ornate threadfin bream and type I collagen from calf skin rehydrated in 0.05 M acetic acid was performed. The maximum transition temperature (T_m) and total denaturation enthalpy (ΔH) of purified type I collagen was 33.35 °C and 0.819 J/g, respectively (data not shown), which were lower than that of type I collagen from calf skin (T_m = 39.27 °C; ΔH = 1.204 J/g). This might be due to the lower content of imino acids (hydroxyproline and proline) of collagen from the skin of ornate threadfin bream. The thermal stability of collagen is associated with the restriction of the secondary structure of the polypeptide chain governed by the pyrrolidine rings of proline and hydroxyproline, and partially by hydrogen bonding through the hydroxyl group of the hydroxyproline (Benjakul et al., 2010; Piez & Gross, 1960). The T_m of purified type I collagens from the skin of ornate threadfin bream was similar to that of collagen from subtropical and tropical species, such as bigeye snapper (32.5 °C) (Nalinanon et al., 2007) and channel catfish (32.5 °C) (Liu et al., 2007), black drum (34.2 °C) (Ogawa et al., 2003), sheep-head seabream (34 °C) (Ogawa et al., 2003), eagle ray (34.1 °C) (Bae et al., 2008), and brownbanded bamboo shark (Kittiphattanabawon et al., 2010). Lower denaturation temperatures of skin collagen from several cold and temperate water fishes including cod (15 °C) (Duan et al., 2009), arabesque greenling (15.7 °C) (Nalinanon et al., 2010a), deep-sea redfish (16.1 °C) (Wang et al., 2007), Pacific whiting (21.7 °C) (Kim & Park, 2004), walleye pollock (24.6 °C) (Yan et al., 2008), Japanese seabass (26.5 °C), chub mackerel (25.6 °C), bullhead shark (25 °C) (Nagai & Suzuki, 2000) and ocellate puffer fish (28 °C) (Nagai et al., 2002), have been reported. The differences in denaturation temperature amongst the collagens from different fish species were correlated with the imino acid content, body temperature and environmental temperature (Kittiphattanabawon et al., 2010; Nagai et al., 2002; Rigby, 1968).

3.5. ζ -Potential of purified type I collagen

The ζ -potential, representing the surface charge of purified type I collagen from the skin of ornate threadfin bream at different pHs,

was determined. The surface net charge of purified type I collagen decreased as the pH increased and it became zero at pH 6.40 (data not shown). The pH where the ζ -potential is zero corresponds to the pI of the protein (Ma et al., 2009; Nalinanon et al., 2010b). Thus, the pI of purified type I collagen was estimated to be 6.40. Purified type I collagen had a net positive or a negative charge when pH values were below and above their pI, respectively. The pI of purified type I collagen was found in a slight acidic pH, which was in accordance with those found in the collagen from the skin of arabesque greenling (Nalinanon et al., 2010b) and brownbanded bamboo shark (Kittiphattanabawon et al., 2010). This might be due to a higher content of acidic amino acids, aspartic acid and glutamic acid, compared to basic amino acids, including histidine, lysine and arginine (Nalinanon et al., 2010b).

3.6. Hydrolysis of purified type I collagen by different pepsins

The degradation patterns of purified type I collagen from the skin of ornate threadfin bream (C-OTB) digested by porcine pepsin (C-PP), skipjack pepsin 1 (C-SP1) and 2 (C-SP2) at a level of 0.5 U/mg collagen, for 24 h at 4 °C, are shown in Fig. 3. This condition was carried out to simulate the hydrolysis of type I collagen during the extraction of pepsin-solubilised collagen. The result revealed that the major components including β , $\alpha 1$, and $\alpha 2$, were fully retained (Fig. 3a). However, some degradation bands were observed in C-PP, C-SP1 and C-SP2, as evidenced by the increase in band intensity at the dye front. The degradation was more pronounced in C-SP2, in which degradation products with molecular weight (MW) of 67 and 71 kDa were observed. The small peaks of those bands were noticeable in the densitogram (Fig. 3a and b). Pepsin treatment of C-OTB had an impact on the proportion of collagen components. In general, β - and γ -chains (dimer and trimer, respectively) as well as very high (VMC) and high MW components (HMC) in collagens decreased after treatment with all pepsins. Cleavage of the telopeptide region more likely resulted in the conversion of cross-links, such as β - and γ -chains, to the α -chain (Fig. 3b). C-OTB contained the higher proportion of cross-linked chains (β , γ and VMC) than that of pepsin treated collagen. VMC in C-OTB disappeared when treated with pepsin, regardless of the type of pepsin used. This VMC was degraded to smaller components, such as β , $\alpha 1$ or $\alpha 2$, by pepsin. Substantial decreases in VMC, HMC, β - and γ -chains, with the concomitant formation of $\alpha 1$, were observed. The band intensity ratios of $\alpha 1/\alpha 2$ -chains were significantly increased from 2.18 (C-OTB) to 2.37, 3.00 and 3.45 for C-PP, C-SP1 and C-SP2, respectively (Table 2). The result showed that amongst the pepsin used, SP2 had the greatest ability to cleave intermolecular cross-links in C-OTB and to produce a monomeric chain. SP1 exhibited less detrimental effect on the collagen structure, as evidenced by the higher ratio of collagen components (β , $\alpha 1$ and $\alpha 2$) to that of lower MW components produced from pepsin hydrolysis. The conversion of cross-linked components to monomer chains, by removing the cross-link containing telopeptide, was reported for pepsin-solubilised collagens from the skin of deep-sea redfish (Wang et al., 2007), black drum, and sheephead seabream (Ogawa et al., 2003). There were no differences in the relative mobilities of $\alpha 1$ and $\alpha 2$ -chains between C-OTB and C-PP. Slight differences in relative mobilities between C-OTB and collagen with skipjack pepsin treatments (C-SP1 and C-SP2) were observed. The MWs of $\alpha 1$ and $\alpha 2$ -chains estimated to be 128 and 116 kDa, respectively, were observed in both C-OTB and C-PP. For C-SP1 and C-SP2, the MWs of the $\alpha 1$ -chains were estimated to be 126 and 122 kDa, respectively, whereas those of $\alpha 2$ -chains were 115 and 112 kDa, respectively. Short peptides with MWs of 2–6 kDa and 1–4 kDa from the telopeptides of $\alpha 1$ and $\alpha 2$, respectively, were produced by the action of pepsin. These results suggested that both pepsins from skipjack tuna partially hydrolysed C-OTB at either telopeptide regions

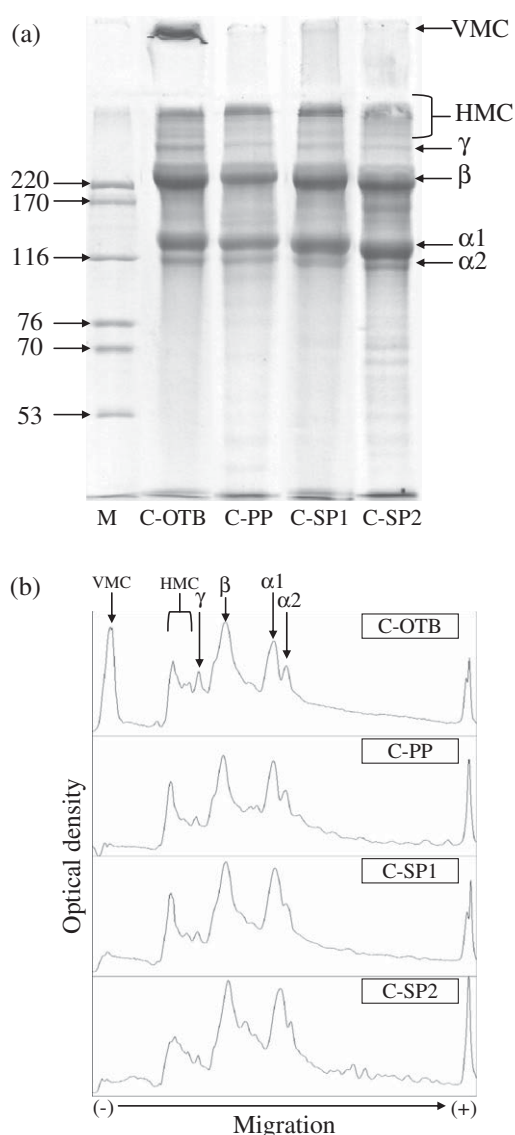


Fig. 3. Protein patterns (a) and densitograms (b) of purified type I collagen (C-OTB) and purified type I collagen treated with porcine pepsin (C-PP), skipjack pepsin 1 (C-SP1) and skipjack pepsin 2 (C-SP2), at a level of 0.5 unit/mg collagen for 24 h at 4 °C. VMC and HMC denote very high and high MW cross-linked components of collagen, respectively. M denotes standard protein marker.

Table 2

Band intensity ratios of $\alpha 1$ -chain to $\alpha 2$ -chain and cross-linked chains to the total monomer chains of purified type I collagen from the skin of ornate threadfin bream, subjected to hydrolysis by different pepsins.

Components	Band intensity ratio ^A			
	C-OTB	C-PP	C-SP1	C-SP2
$\alpha 1/\alpha 2$	$2.18 \pm 0.10a^B$	$2.37 \pm 0.05b$	$3.00 \pm 0.12c$	$3.45 \pm 0.09d$
$\beta/(\alpha 1 + \alpha 2)$	$1.39 \pm 0.06c$	$1.18 \pm 0.04b$	$1.21 \pm 0.05b$	$0.97 \pm 0.04a$
$\gamma/(\alpha 1 + \alpha 2)$	$0.33 \pm 0.01c$	$0.18 \pm 0.01b$	$0.16 \pm 0.02b$	$0.11 \pm 0.04a$
HMC/ $(\alpha 1 + \alpha 2)$	$0.62 \pm 0.04b$	$0.59 \pm 0.03ab$	$0.55 \pm 0.02a$	$0.52 \pm 0.03a$
VMC/ $(\alpha 1 + \alpha 2)$	$0.61 \pm 0.01b$	$0.02 \pm 0.01a$	$0.04 \pm 0.02a$	$0.03 \pm 0.02a$

^A Mean $\pm$ standard deviation ($n = 3$).

^B Different letters in the same row indicate the significant differences ($P < 0.05$). C-OTB denotes purified type I collagen. C-PP, C-SP1 and C-SP2 denote purified type I collagens treated with porcine pepsin, skipjack pepsin 1 and skipjack pepsin 2, respectively.

or the end of helical region. A slight decrease in MW, by about 1–2 kDa, has been reported for pepsin-solubilised collagens from the skin of bigeye snapper, in comparison with that of the acid

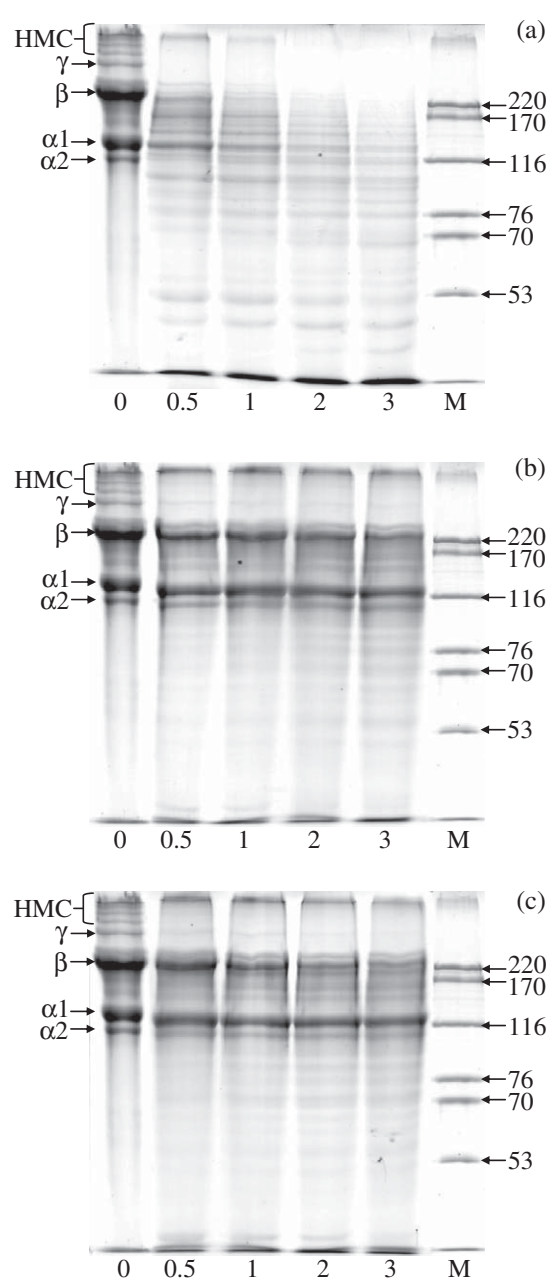


Fig. 4. Degradation patterns of purified type I collagen treated with PP (a), SP1 (b) and SP2 (c) at a level of 0.5 unit/mg collagen, at 50 °C, for different times. Number of each lane denotes pepsin hydrolysis time (h). HMC and M denote high MW cross-linked components of collagen and standard protein marker, respectively.

solubilised collagen (Nalinanon et al., 2007). Although no changes in the relative mobilities of α -chains in C-PP were observed, a partial cleavage of collagen at the telopeptide regions also took place when porcine pepsin was used, as evidenced by the increase in the $\alpha 1/\alpha 2$ proportion. Therefore, different pepsins were able to cleave collagen cross-links at different positions, particularly at the telopeptide region.

The degradation patterns of the purified type I collagen from the skin of ornate threadfin bream treated with PP, SP1 and SP2, at a level of 0.5 U/mg collagen and at 50 °C for different times, are shown in Fig. 4. Hydrolysis was more pronounced with increasing digestion time. The HMC and the γ -chain almost disappeared after 30 min of pepsin digestion. The degradation was much higher in purified type I collagen treated with porcine pepsin (Fig. 4a),

compared with that found in the collagen treated with skipjack tuna pepsin. The major components, including γ -, β - and α -chains, were drastically hydrolysed by porcine pepsin after 30 min, with the concomitant formation of degradation bands with MWs of 47, 52 and 70–101 kDa. Those major components were completely hydrolysed after 2 h of digestion. Furthermore, the $\alpha 2$ -chain was more susceptible to porcine pepsin hydrolysis than the $\alpha 1$ -chain. Purified type I collagen was therefore more resistant to skipjack tuna pepsin hydrolysis (Fig. 4b and c). The major components were retained after 3 h of skipjack tuna pepsin hydrolysis. The results revealed that SP2 had a slightly greater hydrolysis activity on purified type I collagen than did SP1. The lower band intensity of HMC, β - and α -chain of purified type I collagen treated with SP2 was observed, compared with that of SP1 hydrolysis. The similar result was reported by Nalinanon, Benjakul, and Kishimura (2010c) who found that purified pepsin 2 from the stomach of skipjack tuna exhibited slightly higher effectiveness for hydrolysis of ASC from the skin of arabesque greenling, compared with purified pepsin 1. Therefore, fish pepsin, especially from skipjack tuna, could be used to increase the extraction efficacy of collagen with the negligible cleavage of the major components of collagen. This revealed the greater advantage of fish pepsin as the aid for the extraction of pepsin-solubilised collagen from fish skin.

4. Conclusions

Acid solubilised collagen (ASC) from the skin of ornate threadfin bream contained type I collagen with $[\alpha 1(I)]_2\alpha 2(I)$ as the major components; $\alpha 1(I)\alpha 2(I)\alpha 3(I)$ was also found as minor component. Type I collagen showed a varied susceptibility to hydrolysis by pepsin, depending on hydrolysis temperature and type of pepsin used. Pepsin from the stomach of skipjack tuna showed the greater ability to cleave the cross-linked components without the cleavage of major components of collagen, whereas porcine pepsin more likely hydrolysed all components, especially at high temperature. Therefore, pepsin from skipjack tuna can be applied to effectively recover the residual collagen in acid-treated skin, from which the higher yield can be obtained. Additionally, skipjack tuna pepsin can be used as the potential alternative for bovine or porcine pepsins, which may have the limitation for application in some religions.

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Extraction and characterisation of pepsin-solubilised collagen from the skin of unicorn leatherjacket (*Aluterus monoceros*)

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ABSTRACT

Pepsin-solubilised collagen (PSC) was extracted from the skin of unicorn leatherjacket (*Aluterus monoceros*), using 0.5 M acetic acid in the presence of pepsin from albacore tuna, yellowfin tuna or porcine pepsin at a level of 20 units/g of defatted skin. Yields of $8.48 \pm 0.3\%$, $8.40 \pm 0.3\%$ and $7.56 \pm 0.4\%$ (wet weight basis) were obtained for PSC extracted with the aid of albacore tuna pepsin (APSC), yellowfin tuna pepsin (YPSC) and porcine pepsin (PPSC), respectively. All PSCs were classified as Type I collagen containing two α_1 -chains and one α_2 -chain with no disulphide bond. The peptide maps of different PSCs hydrolysed by V8 protease and lysyl endopeptidase were different. ATR-FTIR spectra analysis revealed that PSC molecules had the compact triple helical structure stabilised mainly by the hydrogen bond. $T_{\max}$ of all PSCs (31.68 – 31.98 °C) shifted to lower values (29.33 – 29.40 °C) when dispersed in 0.05 M acetic acid, indicating the conformational changes in the collagen structure induced by acid. Relative viscosity of 0.03% PSC in 0.1 M acetic acid solution decreased progressively as the temperature increased from 4 to 52 °C, indicating thermal destabilisation or denaturation of PSC molecules. All PSCs were soluble in the pH range of 1–6 and sharply decreased at neutral pH. Decreases in solubility were observed in the presence of NaCl, especially at concentrations above 2% (w/v). Therefore, the skin of unicorn leatherjacket could serve as a potential source of collagen.

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1. Introduction

Collagen is the predominant protein of connective tissue in animals, making up about 30% of the total protein content (Birk & Bruckner, 2005). It possesses a unique protein sequence and is able to form insoluble fibres with a high tensile strength (Gelse, Poschl, & Aigner, 2003). Collagen has a wide range of applications in various branches of industry. Its pharmaceutical and biomedical uses include tissue engineering for implants in humans, inhibition of angiogenic diseases, treatment of hypertension, urinary incontinence and osteoarthritis (Lee, Singla, & Lee, 2001). It is also a very attractive substrate for fragrance and cosmetic applications (Tzaphlidou, 2004). Its denatured form (gelatin) has been widely used in the food industry (Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005). All members of the collagen family are characterised by domains with repetitions of the proline-rich tripeptide Gly-X-Y, involved in the formation of trimeric collagen triple helix (Muyonga, Cole, & Duodu, 2004; Ramachandran, 1988).

In general, the traditional sources of collagen are bovine and porcine skins and bones. Porcine collagen is unacceptable for some religions, for example Judaism and Islam, while those from bovine

sources are at risk of contamination with bovine spongiform encephalopathy (BSE) (Fernandez-Diaz, Montero, & Gomez-Guillen, 2001).

With the rapid development of seafood processing industries, huge quantities of by-products have been discarded, which may cause pollution and emit offensive odours. Hence, the comprehensive utilisation of these by-products, especially the production of value-added products, is a promising means to increase revenue for the producers and accelerate the development of the seafood processing industry. Fish skin is an important source for collagen, which can be used as a replacement for mammalian sources. In general, a low yield of collagen is obtained with traditional processes. Since pepsin has been reported to cleave peptides in the telopeptide region, the extraction of collagen partially cleaved by pepsin resulted in a higher yield (Jongjareonrak, Benjakul, Visessanguan, Nagai, & Tanaka, 2005; Nalinanon, Benjakul, Visessanguan, & Kishimura, 2007a). Due to the enormous amount of fish viscera, especially stomach, pepsin of fish origin can be recovered and used to increase the extraction efficiency of collagen from fish skin (Nalinanon, Benjakul, Visessanguan, & Kishimura, 2007b).

Unicorn leatherjacket (*Aluterus monoceros*) belongs to the order Tetraodontiformes and is a member of the Monacanthidae family. This species has been used for fillet production, in which a large amount of skin has been produced as by-product. Due to

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its thick skin, it is therefore a potential source of collagen, especially when fish pepsin is implemented as the extraction aid. The objective of this study was to isolate and characterise the pepsin-solubilised collagen (PSC) from the skin of unicorn leatherjacket (*A. monoceros*), with the aid of different pepsins including tuna and porcine pepsins.

2. Materials and methods

2.1. Chemicals

Bovine haemoglobin, β -mercaptoethanol (β -ME), V8 protease from *Staphylococcus aureus* (EC3.4.21.19), L-tyrosine, bovine serum albumin, pepsin from porcine stomach mucosa (EC3.4.23.1; powdered, 750 U/mg dry matter), and Type I collagen from calf skin were purchased from Sigma Chemical Co. (St. Louis, MO). High molecular weight markers were purchased from GE Healthcare UK (Aylesbury, UK). Sodium dodecyl sulphate (SDS), trichloroacetic acid, Folin-Ciocalteu's phenol reagent, acetic acid and tris(hydroxymethyl)aminomethane were obtained from Merck (Darmstadt, Germany). Lysyl endopeptidase from *Achromobacter lyticus* (EC3.4.21.50) was procured from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

2.2. Fish skin and stomach preparation

The skin of unicorn leatherjacket (*A. monoceros*) was obtained from Sea Wealth Frozen Food Co., Ltd., Songkhla, Thailand. Stomachs of albacore tuna (*Thunnus alalunga*) and yellowfin tuna (*Thunnus albacares*) were obtained from Tropical Canning Public Co., Ltd., Songkhla, Thailand. The samples were packed in polythene bags and kept in ice using a solid/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. The skin was removed manually and the cleaned skin was washed with iced tap water (0–2 °C). Prepared skin was then cut into small pieces (0.5 × 0.5 cm²). Both prepared skin and stomachs were placed in polyethylene bags and stored at –20 °C until use. The storage time was less than 2 months.

2.3. Preparation of stomach extract

The frozen stomachs of both types of tuna were thawed using running water (26–28 °C) until the core temperature reached –2 to 0 °C. The sample was cut into pieces (1 × 1 cm²) and finely ground in liquid nitrogen using a National Model MX-T2GN blender (Taipei, Taiwan), according to the method of Klomklao, Benjakul, and Visessanguan (2004). Stomach powder was suspended in 50 mM sodium phosphate buffer (pH 7.2) at a ratio of 1:10 (w/v). The mixture was stirred continuously at 4 °C for 30 min. The suspension was centrifuged at 7700g for 30 min at 4 °C using a Beckman Model Avanti J-E centrifuge (Beckman Coulter, Inc., Fullerton, CA) to remove the tissue debris. The supernatant was collected and referred to as 'stomach extract'. The protein content in the stomach extract was measured as per the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as a standard.

2.4. Assay of proteolytic activity

Prior to assay, the stomach extracts from albacore tuna or yellowfin tuna or porcine pepsin were adjusted to pH 2 with 1 M HCl and the mixtures were allowed to stand at 4 °C for 10 min, as described by Nalinanon et al. (2007a). The treated stomach extracts were centrifuged at 5000g for 10 min at 4 °C, using a refrigerated centrifuge. The acidified supernatants were collected and used as the sources of activated pepsin.

Proteolytic activity of acidified stomach extract from both tuna and pepsin powder from porcine stomach mucosa was determined using haemoglobin as a substrate, as per the method of Nalinanon et al. (2007a), with a slight modification. To initiate the reaction, 200 μ l of stomach extract were added into the assay mixture containing 200 μ l of 2% haemoglobin, 200 μ l of distilled water and 625 μ l of McIlvaine's buffer (0.2 M Na-phosphate and 0.1 M Na citrate, pH 2.0). Appropriate dilution was made to ensure that the amount of enzyme was not excessive for the available substrate in the assay system. The reaction was conducted at 50 °C for 20 min. To terminate the enzymatic reaction, 200 μ l of 50% (w/v) trichloroacetic acid (TCA) were added. Unhydrolysed protein substrate was allowed to precipitate for 15 min at 4 °C, followed by centrifuging at 4725g for 10 min at room temperature (26–28 °C) using a MIKRO 20 centrifuge (Hettich Zentrifugen Tuttlingen, Germany). The oligopeptide content in the supernatant was measured by the Lowry method (Lowry et al., 1951), using tyrosine as a standard. One unit of activity was defined as the amount releasing 1 μ mol of tyrosine per min. A blank was performed in the same manner, except that the acidified stomach extract was added into the reaction mixture after the addition of 50% TCA (w/v).

2.5. Extraction of pepsin-solubilised collagen (PSC)

All procedures were carried out at 4 °C with gentle stirring. The collagen from the skin of unicorn leatherjacket was extracted following the method of Nalinanon et al. (2007b) with slight modification. Skin was soaked in 0.1 M NaOH with a skin/solution ratio of 1:20 (w/v), with continuous stirring to remove non-collagenous proteins for 6 h. The solution was changed every 2 h. The alkaline-treated skin was then washed with cold water until the pH of the wash water became neutral or faintly basic. Residual fat in the skin was removed using 10% (v/v) butyl alcohol with a sample/solution ratio of 1:10 for 18 h with a change of solution every 6 h. Defatted skin was thoroughly washed with 15 volumes of cold water (4–5 °C).

To extract PSC, the prepared skin was soaked in 0.5 M acetic acid with a solid/solution ratio of 1:15 (w/v) for 48 h in the presence of acidified stomach extracts or acidified porcine pepsin at a level of 20 units/g of defatted skin. The mixture was filtered with two layers of cheesecloth to remove undissolved debris. The filtrate was added with NaCl to obtain a final concentration of 2.6 M in the presence of 0.05 M tris(hydroxymethyl)aminomethane (pH 7.0). The resultant precipitate was collected by centrifuging at 20,000g for 60 min at 4 °C. The pellets were dissolved in 10 volumes of 0.5 M acetic acid and dialysed against 9 volumes of 0.1 M acetic acid and distilled water, respectively. The dialysate was finally freeze-dried using a Model Coolsafe 55, Scanvac, (Coolsafe, Lyngby, Denmark). The yield of PSC was calculated and expressed as dry matter/wet weight of skin. PSC was then subjected to analysis.

2.6. Amino acid analysis

PSC samples were hydrolysed under reduced pressure in 4 M methanesulfonic acid containing 0.2% (v/v) 3-(2-aminoethyl)indole at 115 °C for 24 h. The hydrolysates were neutralised with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.4 ml was applied to an amino acid analyser (MLC-703; Atto Co., Tokyo, Japan).

2.7. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed by the method of Laemmli (1970). The PSC samples were dissolved in 5% SDS and the mixtures were incubated at 85 °C for 1 h. The mixture was centrifuged at 4000g

for 5 min at room temperature to remove undissolved debris. Solubilised samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS and 20% glycerol) in the presence or absence of 10% β -ME. The mixtures were boiled in boiling water for 2 min. Samples (15 μ g protein) were loaded onto polyacrylamide gels comprising a 7.5% running gel and a 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel, using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Hercules, CA). After electrophoresis, gel was stained with 0.05% (w/v) Coomassie Blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. High molecular weight markers were used to estimate the molecular weight of proteins. Type I calf skin collagen was used as a standard.

2.8. Peptide mapping of collagen

Peptide mapping of PSC was performed according to the method of Saito, Kunisaki, Urano, and Kimura (2002) with slight modification. The sample (0.2 mg) were dissolved in 0.1 ml of 0.1 M sodium phosphate (pH 7.2) containing 0.5% (w/v) SDS. After the addition of 10 μ l of the sample buffer containing 5 μ g of V8 protease from *S. aureus* or 0.05 μ g of lysyl endopeptidase from *A. lyticus* to collagen solutions, the reaction mixture was incubated at 37 °C for 25 and 5 min for V8 protease and lysyl endopeptidase, respectively. The reaction was terminated by submerging the reaction mixture in boiling water for 3 min. Peptides generated by the protease digestion were determined by SDS–PAGE using 7.5% separating gel and 4% stacking gel. High molecular weight markers (GE Healthcare UK) were used to estimate the molecular weight of peptides.

2.9. ATR-FTIR analysis

PSC samples from the skin of unicorn leatherjacket were subjected to attenuate total reflectance/Fourier-transform infrared spectroscopy (ATR/FTIR). FTIR spectrometer (Model Equinox 55, Bruker, Ettlingen, Germany) equipped with a horizontal ATR trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technologies Inc., Madison, WI) was used. For spectral analysis, the PSC samples were placed onto the crystal cell and the cell was clamped into the mount of the FTIR spectrometer. The spectra in the range of 600–4000 cm^{-1} with automatic signal gain were collected in 32 scans at a resolution of 4 cm^{-1} and were compared against a background spectrum recorded from the clean empty cell at 25 °C.

2.10. Differential scanning calorimetry

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

2.11. Viscosity of collagen solution

Collagen was dissolved in 0.1 M acetic acid to obtain a concentration of 0.03% (w/v). The solution (500 ml) was subjected to viscosity measurement using a Brookfield Synchroelectric viscometer (model DVII+, Brookfield Eng. Labs Inc., Stoughton, MA) with spindle No. 1 and speed of 100 rpm. Collagen solution was heated from 4 to 52 °C with a heating rate of 4 °C/min. At the designated temperature, the solution was held for 30 min prior to viscosity determination. The relative viscosity was calculated in comparison to that obtained at 4 °C.

2.12. Collagen solubility

The collagen solubility was determined by the method of Montero, Jimenez-Colmenero, and Borderias (1991) with slight modification. Collagen was dissolved in 0.5 M acetic acid to obtain a final concentration of 3 mg/ml and the mixture was stirred at 4 °C until collagen was completely solubilised.

2.12.1. Effect of pH on collagen solubility

Collagen solution (8 ml) was transferred to a centrifuge tube and the pH was adjusted with either 6 N NaOH or 6 N HCl to obtain a final pH ranging from 1 to 10. The volume of solutions was made up to 10 ml by deionised water previously adjusted to the same pH as the collagen solution. The solution was centrifuged at 20,000g for 30 min at 4 °C. Protein content in the supernatant was measured by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard. Relative solubility was calculated in comparison to that obtained at the pH rendering the highest solubility.

2.12.2. Effect of NaCl on collagen solubility

Collagen (6 mg/ml) was dissolved in 0.5 M acetic acid. Solution (5 ml) was added with 5 ml of NaCl in 0.5 M acetic acid at various concentrations (0%, 2%, 4%, 6%, 8%, 10% and 12% (w/v)), in which the final concentrations of 0%, 1%, 2%, 3%, 4%, 5% and 6% were obtained. The mixture was stirred continuously at 4 °C for 30 min, followed by centrifuging at 20,000g for 30 min at 4 °C. Protein content in the supernatant was measured and the relative solubility was calculated as previously described.

2.13. Statistical analyses

All experiments were performed in triplicate and a completely randomised design (CRD) was used. Data were presented as means $\pm$ standard deviation and a probability value of <0.05 was considered significant. Analysis of variance (ANOVA) was performed and mean comparisons were done by Duncan's multiple range tests. Analysis was performed using SPSS 11.0 for Windows (SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Yields of PSC from the skin of unicorn leatherjacket

The yields of APSC and YPSC extracted with the aid of albacore tuna and yellowfin tuna stomach extract were $8.48 \pm 0.3\%$ and $8.40 \pm 0.3\%$ (wet weight basis), respectively. A lower yield ($7.56 \pm 0.4\%$) was obtained for PPSC extracted with the aid of porcine pepsin. Without pepsin, much lower yield ($4.19 \pm 0.4\%$) was obtained. The skin was not completely solubilised in 0.5 M acetic acid. The dense covalent cross-linking through the condensation of aldehyde groups at the telopeptide regions of collagen chains and the inter-molecular cross-linked collagen were not readily solubilised by acetic acid (Jongjareonrak et al., 2005). The results sug-

gested that skin of unicorn leatherjacket contained a large amount of cross-links, leading to a low solubility of collagen in acid (Foege-ding, Lanier, & Hultin, 1996; Zhang et al., 2007). With the further limited hydrolysis by pepsin, the cross-links at the telopeptide region were most likely cleaved as shown by the higher extractability. Collagen could be solubilised into the extracting solution in the presence of both tuna stomach extracts as well as porcine pepsin to a greater extent, compared with that extracted without pepsin. The results are in agreement with Nalinanon et al. (2007b) who reported the increased efficiency of collagen extraction with the aid of pepsin from bigeye snapper stomach or porcine pepsin. The differences in the collagen yield were governed by the sources of pepsin. Different pepsins might cleave telopeptide regions of collagens from skin of unicorn leatherjacket to different degrees. Due to the superior extraction efficiency of pepsins from both albacore tuna or yellowfin tuna, they could be used as promising replacers for porcine pepsin.

3.2. Amino acid composition of PSC from the skin of unicorn leatherjacket

Amino acid composition of PSC extracted from the skin of unicorn leatherjacket is presented in Table 1. For all PSCs extracted with the aid of pepsins from different sources including APSC, YPSC and PPSC, glycine was the most predominant amino acid in all collagens. Generally, glycine represents nearly one-third of the total residues and occurs at every third residue in collagen except for the first 14 amino acid residues from the N-terminus and the first 10 amino acids from the C-terminus (Burghagen, 1999). Alanine was found as the second most abundant amino acid in all collagens. All collagens contained no cysteine and negligible tryptophan content. The imino acid content (hydroxyproline and proline) of APSC, YPSC and PPSC was 187, 169, 192 residues/1000 residues, respectively. Thus, APSC and PPSC contained the higher imino acid content, compared with YPSC. Calf skin collagen contained 215 residues/1000 residues (Giraud-Guille, Besseau, Chopin, Durand, & Herbage, 2000). The degree of hydroxylation of proline was calculated to be around 40% in PSC from the skin of unicorn leatherjacket. The degree of hydroxylation of proline residues plays an

important role in stabilising the triple helix of collagen (Ramachandran, 1988). Hydroxylation of proline in APSC and PPSC was slightly higher than that in YPSC, suggesting a slightly more complex structure of both PSCs than that from YPSC as shown by the higher degree of hydroxylation. APSC and PPSC generally showed the similar amino acid composition, but had some differences in comparison with YPSC. APSC and PPSC had the higher contents of glycine and alanine than did YPSC. On the other hand, the former possessed lower contents of aspartic acid, asparagine, glutamic acid, glutamine, valine, leucine and lysine than the latter. The result suggested that pepsin used for collagen extraction had an influence on the amino acid composition of resulting collagen.

3.3. Protein pattern of PSC from the skin of unicorn leatherjacket

Protein patterns of PSC from the skin of unicorn leatherjacket determined under reducing and non-reducing conditions are illustrated in Fig. 1. All PSCs consisted of two α_1 -chains and one α_2 -chain. High molecular weight components, including β -, γ -components as well as the cross-linked constituents were also observed in all PSCs. PSCs isolated from the skin of unicorn leatherjacket were most likely classified as Type I collagen, which consisted of a heterotrimer of two identical α_1 -chains and one α_2 -chain (Muyonga et al., 2004). A slight difference in the protein pattern was noticeable in YPSC, compared with APSC and PPSC. Slight degradation of major proteins with the concomitant formation of low molecular weight peptides was noticeable when pepsin from yellowfin tuna stomach was used. It was suggested that pepsin from yellowfin tuna stomach was able to hydrolyse tropocollagen, as indicated by the decreased band intensity of β - and α -chains. It was noted that molecular weights of α -chains of all collagens were slightly lower than that of ASC. The results are in accordance with Nalinanon et al. (2007a) who reported that molecular weights of α -chains of collagen from bigeye snapper skin extracted with the aid of pepsin from the same fish species decreased slightly when compared with ASC. Hence, it can be inferred that pepsin most likely cleaved some peptides in the telopeptide region. As a consequence, a slight decrease in molecular weight of PSC was noticeable, in comparison with that of ASC. Generally, the higher molecular weight components including γ -chain were found at a lower content in PSC, compared with those of ASC. This result indicated that pepsin used in this study could cleave inter-molecular cross-links

Table 1

Amino acid composition of the pepsin-solubilised collagen from the skin of unicorn leatherjacket (expressed as residues/1000 residues).

Amino acid	APSC	YPSC	PPSC
Aspartic acid/asparagine	49	55	47
Threonine	27	30	27
Serine	38	39	36
Glutamic acid/glutamine	74	86	72
Glycine	320	290	325
Alanine	140	134	141
Cysteine	0	0	0
Valine	22	26	21
Methionine	12	13	12
Isoleucine	8	12	7
Leucine	18	27	16
Tyrosine	2	5	2
Phenylalanine	12	15	12
Hydroxylysine	4	4	4
Lysine	28	33	27
Histidine	6	8	6
Arginine	53	53	53
Tryptophan	0	1	0
Hydroxyproline	81	72	83
Proline	106	97	109
Imino acid	187	169	192

APSC: pepsin solubilised collagen using albacore tuna pepsin, YPSC: pepsin solubilised collagen using yellowfin pepsin and PPSC: pepsin solubilised collagen using porcine pepsin.

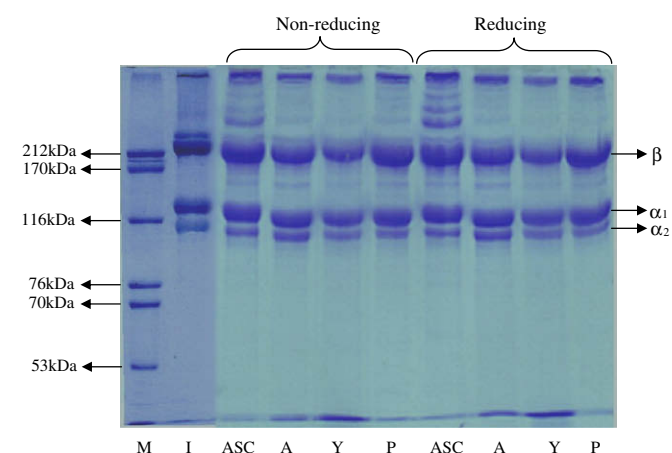


Fig. 1. SDS-PAGE pattern of pepsin-solubilised collagen from the skin of unicorn leatherjacket under reducing and non-reducing conditions. M: high molecular weight markers, I: Type I calf skin collagen, ASC: acid-solubilised collagen, A: pepsin-solubilised collagen using albacore tuna pepsin, Y: pepsin-solubilised collagen using yellowfin tuna pepsin and P: pepsin-solubilised collagen using porcine pepsin.

of α -chains. It was demonstrated that two types of inter-molecular bonds, side-to-side and end-to-end bond, in calf collagen can easily be cleaved by pepsin, whereas head-to-tail bond is relatively pepsin resistant (Zimmermann, Pikkarainen, Fietzek, & Kuhn, 1970). Drake, Davison, Bump, and Schmitt (1966) reported that most of intra- and inter-molecular cross-links found in collagen occur through the telopeptide region.

No differences in the electrophoretic patterns of PSC were noticeable in the presence and absence of β -ME. This indicated that there was no disulphide bond in PSCs. Some of the telopeptides of calf skin tropocollagen are vulnerable to pepsin actions, since intra-molecular cross-links are broken on pepsin digestion and fragments comprising a small fraction of the molecule (approximately 1%) become dialysable (Drake et al., 1966).

3.4. Peptide map of PSC from the skin of unicorn leatherjacket

Lysyl endopeptidases from *A. lyticus* and *S. aureus* V8 protease have been employed for peptide mapping of collagens (Kittiphattanabawon et al., 2005). The peptide maps of PSC digested by both enzymes are shown in Fig. 2. When V8 protease was used, the band intensities of β - and α -chains decreased with the concomitant increase in lower molecular weight peptides. Among all PSCs, YPSC was more susceptible to hydrolysis by V8 protease. This was evidenced by the almost total disappearance of all major components such as α -, β -chains, etc. When comparing between APSC and PPSC, the former was more prone to hydrolysis by V8 protease than was the latter. V8 protease shows a high specific preference for glutamic acid and aspartic acid residues of proteins (Kittiphattanabawon et al., 2005). Due to higher content of aspartic acid and glutamic acid (Table 1), YPSC was more prone to hydrolysis by V8 protease than APSC and PPSC. When lysyl endopeptidase was used, similar results were observed. YPSC underwent hydrolysis to the highest extent. PPSC was more resistant to hydrolysis, compared with APSC. However, different peptide maps were noticeable when two different enzymes were used for digestion.

The differences in peptide maps between the different PSCs generated by lysyl endopeptidase or V8 protease digestion suggested that there might be some differences in their primary structures, especially the α -helix strand, in terms of sequence as well as amino acid composition (Jongjareonrak et al., 2005). Thus the degree of hydrolysis by protease might be different, due to variation

in the collagen structure and composition. Peptide maps of collagens were reported to differ among sources and species (Mizuta, Yamasa, Miyagi, & Yoshinaka, 1999). Thus, PSCs prepared with the aid of different pepsins might be different in terms of domain or cross-links, as shown by the difference in peptide maps generated by limited digestion under suitable conditions for each enzyme.

3.5. Thermal denaturation of PSC from the skin of unicorn leatherjacket

Thermal transitions of PSC from the skin of unicorn leatherjacket dispersed in 0.05 M acetic acid or deionised water are depicted in Fig. 3. Endothermic peaks with maximum peak temperatures (T_{max}) of 29.36 ± 0.7 , 29.34 ± 0.5 and 29.33 ± 0.5 °C were found in APSC, YPSC and PPSC dispersed in 0.05 M acetic acid, respectively. When PSC were suspended in deionised water, T_{max} values of 31.73 ± 0.8 , 31.68 ± 0.7 and 31.98 ± 0.8 °C were observed, respectively. It was noted that T_{max} shifted to a lower temperature ($p < 0.05$), when PSC was dispersed in acetic acid. The results suggested that intra-molecular hydrogen bonds stabilising the triple helix structure of collagen might be disrupted at some levels, in the presence of acetic acid, mainly due to the repulsion of collagen molecules in acidic solution. This alteration resulted in the decrease in T_{max} . For the same medium used for suspending PSC, similar T_{max} was noticeable among all PSC samples. Nevertheless, differences in ΔH were noticeable among all three samples. In acetic acid, YPSC showed the lowest ΔH , while PPSC had the highest

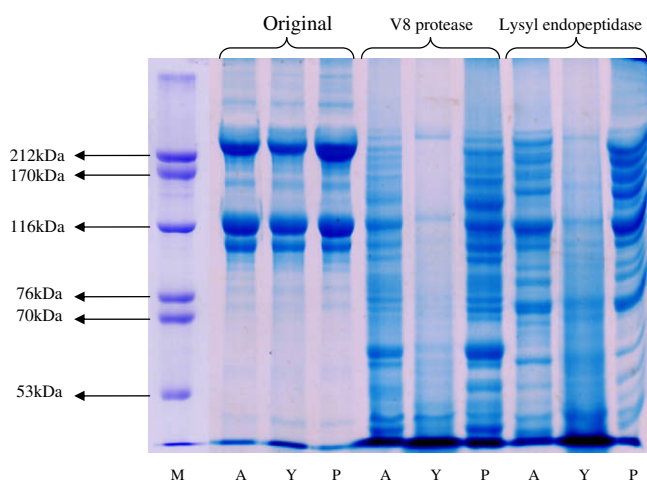


Fig. 2. Peptide maps of pepsin-solubilised collagens from the skin of unicorn leatherjacket digested by V8 protease and lysyl endopeptidase. M: high molecular weight markers, A: pepsin-solubilised collagen using albacore tuna pepsin, Y: pepsin-solubilised collagen using yellowfin pepsin and P: pepsin-solubilised collagen using porcine pepsin.

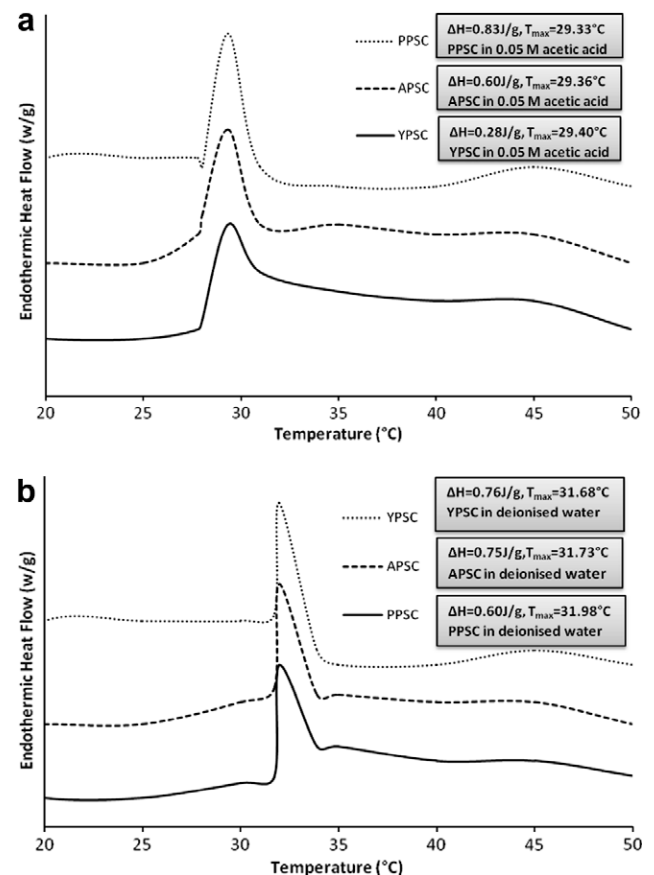


Fig. 3. DSC thermogram of pepsin-solubilised collagen from the skin of unicorn leatherjacket dispersed in 0.05 M acetic acid (a) and in deionised water (b). APSC: pepsin-solubilised collagen using albacore tuna pepsin, YPSC: pepsin-solubilised collagen using yellowfin pepsin and PPSC: pepsin-solubilised collagen using porcine pepsin.

ΔH ($p < 0.05$). For PSC dispersed in deionised water, the lowest ΔH was found in PPSC, compared with APSC and YPSC ($p < 0.05$). The differences in $T_{\max}$ and ΔH among all PSC samples might be governed by the composition and sequence of amino acid as well as the tertiary structure of collagen. $T_{\max}$ values of PSC samples from the skin of unicorn leatherjacket were much lower than that of pig skin collagen (37 °C) (Ikoma, Kobayashi, Tanaka, Walsh, & Mann, 2003) and that of calf skin collagen (40.8 °C) (Komsa-Penkova, Koyonava, Kostov, & Tenchov, 1999). It was found that PSC from the skin of unicorn leatherjacket showed higher $T_{\max}$ values than collagen from other fish species, such as bigeye snapper (28.68 °C), ocellate puffer fish (28.0 °C) and grass carp (24.6 °C) (Kittiphattanabawon et al., 2005; Nagai, Araki, & Suzuki, 2002; Zhang et al., 2007).

Thermal stability of collagen is influenced by the imino acid content. The higher the imino acid content, the more stability is obtained. The molecular structure of collagen is maintained mainly by restrictions on changes in the secondary structure of the polypeptide chain, imposed by the pyrrolidine rings of proline and hydroxyproline, and also partially stabilised by the hydrogen bond through the hydroxyl group of hydroxyproline (Zhang et al., 2007). Factors influencing the change of collagen include the content of imino acid, degree of hydroxylation of proline and the content of the Gly-Pro-Hyp sequence (Burjanadze, 1999). The thermal stability of the collagen triple helix is attributed to the hydrogen-bonded networks, mediated by water molecules, which connect the hydroxyl group of hydroxyproline in one strand to the main chain amide or carboxyl groups of another chain (Babu & Ganesh, 2001). Therefore, the differences in hydroxyproline content might determine the thermal transition temperatures of collagens from different fish species.

3.6. ATR-FTIR of PSC from the skin of unicorn leatherjacket

FTIR spectra of PSCs from the skin of unicorn leatherjacket are depicted in Fig. 4. The absorption bands in the spectra of all PSC samples were situated in the amide band region such as amide I (1600–1700 cm^{-1}), amide II (1500–1600 cm^{-1}) and amide III (1200–1300 cm^{-1}) as previously reported by Muyonga et al. (2004). For the spectrum of APSC, the characteristic absorption bands at wave numbers of 3293.06, 3079.61, 1631.76, 1546.71 and 1235.36 cm^{-1} were observed, whereas YPSC showed main absorption bands at wave numbers of 3294.37, 3079.74, 1639.71,

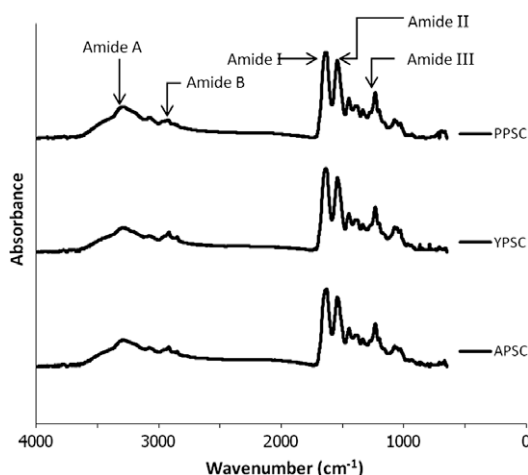


Fig. 4. Fourier-transform infrared spectra of pepsin-solubilised collagen from the skin of unicorn leatherjacket. APSC: pepsin-solubilised collagen using albacore tuna pepsin, YPSC: pepsin-solubilised collagen using yellowfin pepsin and PPSC: pepsin-solubilised collagen using porcine pepsin.

1545.34 and 1235.14 cm^{-1} . The characteristic absorption bands of PPSC were noticeable at wave numbers of 3294.37, 3085.53, 1634.77, 1546.30 and 1235.67 cm^{-1} . The amide I bands are originated from C=O stretching vibrations coupled to N–H bending vibrations, CN stretch and CCN deformation (Bandekar, 1992). For amide I band, APSC had the lowest wave number, indicating the interaction of C=O with the adjacent chains, while YPSC had the highest wave number. The amide II bands of all PSCs were obtained at similar wave number, representing N–H bending vibrations coupled to C–N stretching vibrations. Generally, the shift to the lower wave number showed the existence of hydrogen bonds in each collagen (Li, Liu, Gao, & Chen, 2004). The amide III represented the combination peaks between N–H deformation and C–N stretching vibrations and was involved with the triple helical structure of collagen (Muyonga et al., 2004). The triple helical structure of collagens extracted with the aid of pepsin was confirmed from the IR ratio between amide III and 1450 cm^{-1} which was approximately 1. The other bands, arising from the stretching vibrations of N–H group, of a medium to weak intensity, appeared at 3293.06, 3294.37 and 3294.37 cm^{-1} , corresponding to amide A, which occurs commonly in the range of 3280–3300 cm^{-1} . The amide B with similar wave number (3079.61 cm^{-1} , 3079.74 cm^{-1}) was observed for APSC and YPSC. Higher wave number of 3085.53 cm^{-1} was noticeable for PPSC. Amide B corresponds to asymmetric stretch vibration of =C–H as well as $-\text{NH}_3^+$.

Fourier-transform infrared study indicated that PSC from skin of unicorn leatherjacket had some differences in functional groups and inter- and intra-molecular interaction. Thus, the pepsin used might affect the resulting PSC to some degree.

3.7. Viscosity of PSC from the skin of unicorn leatherjacket

Relative viscosities of PSCs in 0.1 M acetic acid subjected to heat-treatment at different temperatures are depicted in Fig. 5. Collagen solutions are highly structured systems associated with the high viscosity. The collagen is irreversibly destroyed as a result of thermal denaturation. Viscosity of APSC and YPSC decreased continuously when heated up to 32 °C. Rate of decrease was retarded in the temperature range of 36–52 °C. The relative decrease of viscosity up to 32 °C might be due to continuous disruption of hydrogen bonds, which stabilised the structure of collagen. Nevertheless, PPSC had the marked decrease at temperature above 32 °C.

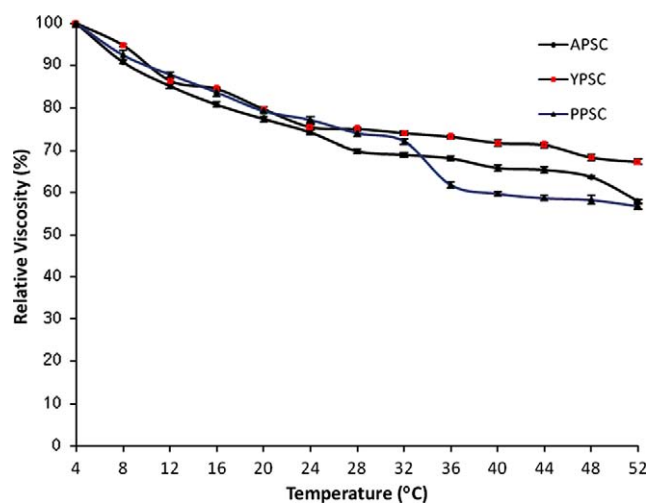


Fig. 5. Relative viscosity of the solution of pepsin-solubilised collagen from the skin of unicorn leatherjacket at different temperatures. APSC: pepsin-solubilised collagen using albacore tuna pepsin, YPSC: pepsin-solubilised collagen using yellowfin pepsin and PPSC: pepsin-solubilised collagen using porcine pepsin.

The high viscosity can be attributed to the high proportion of β - and γ -chains, which favoured the entanglement of molecules (Ogawa et al., 2004). Collagen denatures completely at a temperature above 40 °C to a mixture of random coils of single, double and triple strands (Wong, 1989). Denaturation of collagen structure due to heat-treatment is associated with changes in viscosity. Among all PSCs, APSC showed the highest viscosity (22.8 cP at 4 °C), followed by YPSC (21.1 cP at 4 °C) and PPSC (19.6 cP at 4 °C), respectively. APSC might contain a higher proportion of inter- and intra-molecular cross-links stabilising the structure of collagen.

3.8. Effects of pH and NaCl on collagen solubility

Fig. 6 shows the effect of pH and NaCl on the solubility of PSC from the skin of unicorn leatherjacket. All PSCs were solubilised in the pH range of 1–6, with the highest solubility at pH 2 ($p < 0.05$). The decrease in solubility was observed in the pH range of 7–8 ($p < 0.05$). The dissolved protein was found to precipitate in this pH range. The observation of precipitation and aggregation at a particular pH can be attributed to hydrophobic interaction among

collagen molecules, which increases at pI, and the total net charge of protein molecules becomes zero (Jongjareonrak et al., 2005). Slight increase in solubility was noticeable at pH 9 and 10. This was probably due to the repulsive effect of collagen molecules at pH above pI. The differences in the pH determining the solubility of collagens were caused by the differences in the molecular properties and conformations of collagens (Kittiphattanabawon et al., 2005). All PSCs had a slight decrease in solubility at NaCl concentrations of 0–2%. Drastic decrease was observed at NaCl concentration higher than 2% (Fig. 6b). At NaCl concentration of 4%, APSC showed the highest solubility ($p < 0.05$), followed by YPSC and PPSC, respectively. At high NaCl concentration, salting-out effect might occur. As a result, salt might bind with water and collagen molecules might more likely undergo precipitation. The differences in amino acid composition and structure might have an impact on solubility, especially at high NaCl concentration. Thus, the collagen extracted by different sources of pepsin might have different molecular properties, leading to the varied characteristics of collagens.

4. Conclusion

Collagens (PSC) could be extracted from the skin of unicorn leatherjacket with the aid of pepsin from tuna stomach. The collagens extracted with the aid of albacore tuna pepsin were characterised as Type I collagen, exhibiting similar properties to the PSC extracted by using porcine pepsin. PSC were solubilised in acidic pH range, while NaCl at higher concentration could lower their solubility. Thus, the applications should be maximised based on their characteristics.

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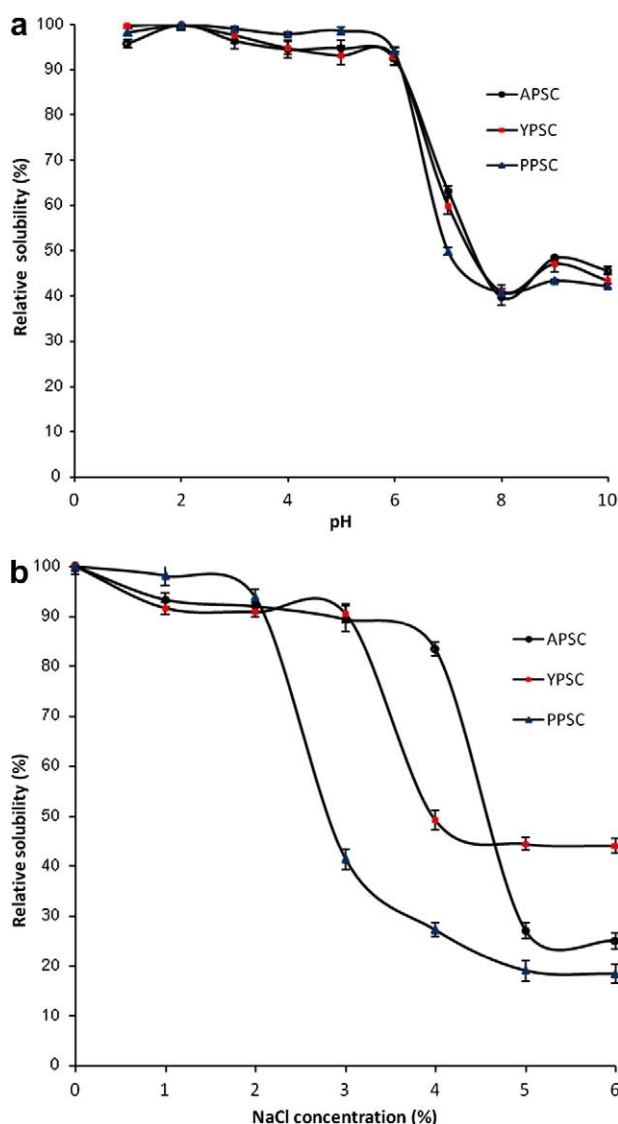
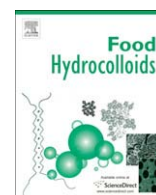


Fig. 6. Relative solubility (%) of pepsin-solubilised collagen from the skin of unicorn leatherjacket as affected by different pH (a) and NaCl (b) concentrations. APSC: pepsin-solubilised collagen using albacore tuna pepsin, YPSC: pepsin-solubilised collagen using yellowfin pepsin and PPSC: pepsin-solubilised collagen using porcine pepsin.

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Compositional and physicochemical characteristics of acid solubilized collagen extracted from the skin of unicorn leatherjacket (*Aluterus monoceros*)

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ABSTRACT

Acid solubilized collagen (ASC) was extracted from the skin of unicorn leatherjacket (*Aluterus monoceros*) using 0.5 M acetic acid, followed by precipitation with 2.6 M NaCl. ASC with the yield of 4.19% (wet weight basis) was identified as type I collagen, which was composed of two α_1 chains and one α_2 chain. Different peptide maps were observed between ASC hydrolyzed by V8 protease and lysyl endopeptidase. The maps were also different from those of type I collagen from calf skin, suggesting the differences in amino acid sequences between both collagens. Glycine was the most predominant amino acid. ASC contained the relatively higher content of alanine, but lower contents of proline and hydroxyproline, compared with calf skin collagen. FTIR analysis showed that ASC was in triple helix structure. T_{max} of ASC dispersed in 0.05 M acetic acid and deionized water were 27.7 and 35.8 °C, respectively. Relative viscosity of 0.03% (w/v) ASC dissolved in 0.1 M acetic acid decreased continuously as the temperature increased from 4 to 52 °C, indicating thermal destabilization or denaturation of ASC molecules. ASC had the solubility greater than 90% in very acidic pH range (pH 1–4) and the solubility decreased continuously with increasing NaCl concentrations (0–6%). Net charge of ASC and calf skin collagen became zero at pHs of 5.58 and 5.68, respectively as determined by zeta potential titration. Therefore, skin of unicorn leatherjacket can be used as an alternative collagenous source.

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1. Introduction

Fish processing industry is an important source of income for Thailand. A large amount of by-products (60–70%) including skins, bones, scales, fins, head, guts and frame is generated and is usually used as animal feed with low market value. These underutilized resources have attracted the increasing attention as the raw material for marketable value-added product such as collagen, gelatin, hydrolysate, etc (Benjakul & Morrissey, 1997; Jongjareonrak, Benjakul, Visessanguan, Nagai, & Tanaka, 2005).

Collagen is one of most abundant biological macromolecule of extracellular matrix where it provides the major structural and mechanical support to tissues. It constitutes about 30% of total proteins found in multicellular organisms and contains at least 27 different types of collagen, named from type I to type XXVII, which varies considerably in their complexity and diversity of their structure (Birk & Bruckner, 2005). All members of collagen family are characterized by containing domains with repetitions of the glycine-rich tripeptides Gly-X-Y involved in the formation of

primary structure of trimeric collagen triple helix. Collagen triple helix is stabilized mainly by inter- and intra-chain water mediated hydrogen bonding as well as direct inter-chain hydrogen bonding (Brodsky & Persikov, 2005). Collagen contents vary considerably with fish species, age and season (Nagai, Araki, & Suzuki, 2002). Nevertheless, collagen obtained from different species and habitats might be different in terms of molecular compositions and properties (Foegeding, Lanier, & Hultin, 1996).

Collagen is one of the best biomaterials, which has wide range of applications in food industry, pharmaceuticals, biomedical, leather industry, cosmetics and tissue engineering due to its excellent biocompatibility and biodegradability (Zhang, Li, & Shi, 2006). Due to its amphoteric nature, the interaction of collagen with other molecules has the desired effect on its rheological, chemical and biochemical properties. Collagen is readily available and non-toxic and can serve as an excellent basis for biomaterials (Sionkowska, Skopinska-Wisniewska, & Wisniewski, 2009).

Unicorn leatherjacket (*Aluterus monoceros*) belongs to the order Tetraodontiformes and is a member of the Monacanthidae family, found around the 'Gulf of Thailand' in subtropical oceans between latitudes 43°N and 35°S, at the depths down to 50 m. Its length varies from 30 to 76 cm. Although fish collagen has been extracted from different marine or fresh water fish with varying inhabiting

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environment, the information regarding the collagen from the skin of unicorn leatherjacket, which is one of the species used for fillet production in Thailand, especially for export, is scarce. The aim of this study was to isolate and characterize acid solubilized collagen (ASC) from skin of unicorn leatherjacket in comparison with commercial calf skin collagen.

2. Materials and methods

2.1. Chemicals

β -mercaptoethanol (β -ME), V8 protease from *Staphylococcus aureus* (EC 3.4.21.19), bovine serum albumin, calf skin type I collagen and lysyl endopeptidase from *Achromobacter lyticus* (EC 3.4.21.50) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). High molecular weight protein marker was purchased from GE Healthcare UK (Buckinghamshire, UK). Sodium dodecyl sulfate (SDS), Folin-Ciocalteu's phenol reagent, acetic acid and tris (hydroxymethyl) aminomethane were obtained from Merck (Darmstadt, Germany).

2.2. Fish skin preparation

The skin of unicorn leatherjacket (*A. monoceros*) was obtained from Sea Wealth Frozen Food Co., Ltd., Songkhla, Thailand. The skin was kept in ice using a skin/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. The remaining flesh was removed manually from the skin. The cleaned skin was washed with iced tap water (0–2 °C). Prepared skin was then cut into small pieces (0.5 × 0.5 cm²) using the scissor, placed in polyethylene bags and stored at –20 °C until use. The storage time was less than 2 months.

2.3. Extraction of acid solubilized collagen (ASC)

All the procedures were carried out at 4 °C. ASC from the skin of unicorn leatherjacket was extracted following the method of Nalinanon, Benjakul, Visessanguan, and Kishimura (2007) with a slight modification. Skin was soaked in 0.1 M NaOH with a skin/solution ratio of 1:20 (w/v) with the continuous stirring to remove non-collagenous proteins for 6 h using an overhead stirrer model W20.n (IKA®-Werke GmbH & CO.KG, Stanfen, Germany). The solution was changed every 2 h. The alkaline treated skin was then washed with cold water until the pH of the wash water became neutral or faintly basic. Residual fat in the skin was removed using 10% (v/v) butyl alcohol with a sample/solution ratio of 1:10 (w/v) for 18 h with a change of solution every 6 h. Defatted skin was thoroughly washed with 15 volumes of cold water (4–5 °C).

To extract ASC, the prepared skin was mixed with 0.5 M acetic acid with a skin/solution ratio of 1:15 (w/v). The mixture was stirred continuously for 48 h. The mixture was filtered with two layers of cheesecloth to remove undissolved debris. The undissolved debris was re-extracted under the same conditions. Both filtrates were combined and precipitated using 2.6 M NaCl in the presence of 0.05 M tris(hydroxymethyl) aminomethane (pH 7.0). The resultant precipitate was collected by centrifuging at 20,000g using a Beckman model Avanti J-E centrifuge (Beckman coulter, Inc., Palo Alto, CA, USA) for 60 min at 4 °C. The pellet was dissolved in 10 volumes of 0.5 M acetic acid and dialyzed against 20 volumes of 0.1 M acetic acid and distilled water, respectively. The dialyze was finally freeze-dried using a Scanvac Model Coolsafe 55 freeze drier (Coolsafe, Lyngø, Denmark). The yield of ASC was calculated and expressed as dry matter/wet weight of skin. Additionally, the yield of ASC was calculated based on the original collagen content in the skin. Hydroxyproline content in the skin was determined by

spectrophotometric method (Bergman & Loxley, 1963). The factor of 10.25 obtained from amino acid composition of ASC was used to convert the hydroxyproline content to collagen. ASC was then subjected to analyses.

2.4. Amino acid analysis

Collagen samples were hydrolyzed under reduced pressure in 4 M methane sulfonic acid containing 0.2% (v/v) 3-(2-aminoethyl) indole at 115 °C for 24 h. The hydrolysates were neutralized with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.4 ml was applied to an amino acid analyzer (MLC-703; Atto Co., Tokyo, Japan). The degree of hydroxylation of proline was calculated as the percentage of hydroxyproline from total imino acids.

2.5. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method of Laemmli (1970). Collagen samples were suspended in 5% (w/v) SDS prior to incubation at 85 °C for 1 h. The mixture was centrifuged at 4,000g for 5 min at room temperature to remove undissolved debris. Solubilized samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% (w/v) SDS, 20% (v/v) glycerol and bromophenol blue) in the presence or absence of 10% (v/v) β -ME. The mixtures were boiled in boiling water for 2 min. Samples (15 μ g protein) were loaded onto polyacrylamide gels comprising 7.5% separating gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel using a Mini-PROTEIN II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, gel was stained with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with the mixture of 30% (v/v) methanol and 10% (v/v) acetic acid. High molecular weight protein markers were used to estimate the molecular weight of proteins. Calf skin type I collagen was used as a standard.

2.6. Peptide mapping of collagen

Peptide mapping of ASC and calf skin collagen was performed according to the method of Saito, Kunisaki, Urano, and Kimura (2002) with a slight modification. The samples (0.2 mg) were dissolved in 0.1 ml of 0.1 M sodium phosphate (pH 7.2) containing 0.5% (w/v) SDS. After the addition of 10 μ l of the reaction buffer (0.1 M sodium phosphate, pH 7.2) containing 5 μ g of V8 protease from *S. aureus* or 0.05 μ g of lysyl endopeptidase from *A. lyticus* to collagen solutions, the reaction mixture was incubated at 37 °C for 25 and 50 min for V8 protease and lysyl endopeptidase hydrolysis, respectively. The reaction was terminated by submerging the reaction mixture in the boiling water for 3 min. Peptides generated by the protease digestion were determined using SDS-PAGE using 7.5% separating gel and 4% stacking gel. High molecular weight protein markers were used to estimate the molecular weight of peptides.

2.7. ATR-FTIR analysis

ASC from the skin of unicorn leatherjacket and calf skin collagen were subjected to Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR). FTIR spectrometer (Model Equinox 55, Bruker, Ettlingen, Germany) equipped with a horizontal ATR trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) was used. Prior to analysis, calf skin type I collagen (Sigma chemical Co.) was dissolved in 10 volumes of 0.5 M acetic acid. The mixture was stirred at 4 °C for 12 h and then dialyzed against 20

volumes of 0.1 M acetic acid for 24 h, followed by dialysis using 20 volumes of distilled water for 24 h. The dialyzed sample was freeze-dried.

For spectra analysis, the freeze-dried samples were placed onto the crystal cell and the cell was clamped into the mount of FTIR spectrometer. The spectra in the range of 400–4000 cm^{-1} with automatic signal gain were collected in 32 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean empty cell at 25 °C.

2.8. Differential scanning calorimetry

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

2.9. Viscosity of collagen solution

ASC was dissolved in 0.1 M acetic acid to obtain a concentration of 0.03% (w/v). The solution (500 ml) was subjected to viscosity measurement using a Brookfield synchroelectric viscometer (model DVII+, Brookfield Eng Labs Inc., Stoughton, MA, USA) with the spindle No.1 and speed of 100 rpm. ASC solution was heated from 4 to 50 °C with a heating rate of 4 °C/min (Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005). At the designated temperature, the solution was held for 30 min prior to viscosity determination. The relative viscosity was calculated in comparison with that obtained at 4 °C. T_d was defined as the temperature causing 50% decrease in the relative viscosity of collagen solution.

2.10. Collagen solubility

ASC solubility at different pH and NaCl concentration was determined by the method of Kittiphattanabawon et al. (2005).

2.10.1. Effect of pH on collagen solubility

ASC was dissolved in 0.5 M acetic acid to obtain a final concentration of 3 mg/ml and the mixture was stirred at 4 °C until collagen was completely solubilized. The pH of ASC solution (8 ml) was adjusted with either 6 N NaOH or 6 N HCl to obtain the final pH ranging from 1 to 10. The volume of solutions was made up to 10 ml by deionized water previously adjusted to the same pH as the collagen solution. The solution was centrifuged at 20,000g for 30 min at 4 °C. The supernatants were 10-fold diluted using distilled water. Protein content in the diluted supernatants was determined by the Lowry method using bovine serum albumin as a standard (Lowry, Rosebrough, Farr, & Randall, 1951). Relative solubility was calculated in comparison with that obtained at the pH rendering the highest solubility.

2.10.2. Effect of NaCl on collagen solubility

ASC (6 mg/ml) was dissolved in 0.5 M acetic acid. ASC solution (5 ml) was added with 5 ml of NaCl in 0.5 M acetic acid at various concentrations (0–12%; w/v), in which the final concentrations of

0–6% (w/v) were obtained. The mixture was stirred continuously at 4 °C for 30 min, followed by centrifuging at 20,000g for 30 min at 4 °C. Protein content in the supernatant was measured and the relative solubility was calculated.

2.11. Measurement of ζ -potential

ASC and calf skin type I collagen were dissolved in 0.5 M acetic acid at a concentration of 0.5 mg/ml. The mixture was stirred at 4 °C for 12 h. The ζ -potential of each sample (20 ml) was measured using a zeta potential analyzer (ZetaPALS, Brookhaven Instruments Co., Holtsville, NY, USA). ζ -potential of samples adjusted to different pHs with 1.0 M nitric acid or 1.0 M KOH using an autotitrator (BI-ZTU, Brookhaven Instruments co., Holtsville, NY, USA) was determined. pH rendering zero ζ -potential was obtained from the titration curve.

2.12. Statistical analysis

All experiments were performed in triplicate and a completely randomized design (CRD) was used. Analysis of variance (ANOVA) was used and mean comparison was performed using Duncan's multiple range tests (Steel & Torrie, 1980). Analysis was performed using an SPSS package (SPSS 11.0 for windows, SPSS Inc, Chicago, IL, USA).

3. Results and discussion

3.1. Yield

Acid solubilized collagen (ASC) was extracted from the skin of unicorn leatherjacket with the yield of $4.19 \pm 0.4\%$ (based on wet weight of skin). Skin contained the collagen at the level of 22.67 g/100 g. Therefore, the yield based on the initial collagen presented in the skin or % recovery was 18.47%. The skin was not completely solubilized with 0.5 M acetic acid even with two repetitions of extraction. This result suggested a high amount of cross-links at the telopeptide region as well as other inter-molecular cross-links, leading to low solubility of collagen in acid (Zhang et al., 2007). The presence of cross-links was more likely associated with the tough and elastic skin of this species (Ahmad & Benjakul, 2010).

3.2. Amino acid compositions

Amino acid compositions of ASC from the skin of unicorn leatherjacket and calf skin collagen are presented in Table 1. Glycine is the major amino acid in both collagens. Generally, glycine represents nearly one-third of the total residues and occurs at every third residue in collagen except for the first 14 amino acid residues from the N-terminus and the first 10 residues from the C-terminus (Burghagen, 1999). Alanine was found as the second abundant amino acid. However ASC from unicorn leatherjacket skin had the higher content of alanine than that of calf skin collagen. No cysteine was found in both collagens. Tryptophan was observed in only calf skin collagen, but was not found in ASC. The imino acid content (hydroxyproline and proline) of ASC was 190 residues/1000 residues, while calf skin collagen contained 215 residues/1000 residues. Therefore, ASC had the lower imino acid content than calf skin collagens, which was in accordance with the report of Jongjareonrak et al. (2005). The degree of hydroxylation of proline was calculated to be 42.6% in ASC. Ramachandran (1988) reported that the degree of hydroxylation of proline residues plays an important role in stabilizing the triple helix of collagen. The results suggested that ASC might have the lower thermal stability than mammalian collagen. ASC contained the higher content of

Table 1

Amino acid composition of ASC from skin of unicorn leatherjacket and calf skin collagen (expressed as residues/1000 residues).

Amino acids	Unicorn leatherjacket skin collagen	Calf skin collagen
Aspartic acid/Asparagine	45	45
Threonine	27	18
Serine	33	33
Glutamic acid/Glutamine	74	75
Glycine	321	330
Alanine	141	119
Cysteine	0	0
Valine	21	21
Methionine	13	6
Isoleucine	9	11
Leucine	17	23
Tyrosine	4	3
Phenylalanine	13	3
Hydroxylysine	5	7
Lysine	27	26
Histidine	6	5
Arginine	53	50
Tryptophan	0	3
Hydroxyproline	81	94
Proline	109	121
Imino acid	190	215

threonine, methionine and phenylalanine but lower leucine than did calf skin collagen. Thus, ASC from unicorn leatherjacket skin was different from calf skin collagen in terms of amino acid compositions, which might determine the differences in properties between both collagens.

3.3. SDS-polyacrylamide gel electrophoresis

Protein patterns of ASC from the skin of unicorn leatherjacket under reducing and non-reducing conditions are shown in Fig. 1. ASC consisted of two α -chains (α_1 and α_2). The band intensity of α_1 -chain was approximately 2-fold higher than that of α_2 . High molecular weight components, particularly β -components, as well as other cross-linked molecules with higher molecular weight were also observed in ASC. Protein pattern of ASC generally was similar to those of the type I collagen from calf skin, indicating that ASC from the skin of unicorn leatherjacket was most likely type I collagen. No differences in the electrophoretic patterns of ASC were observed, when analyzed in the presence and absence of β -ME,

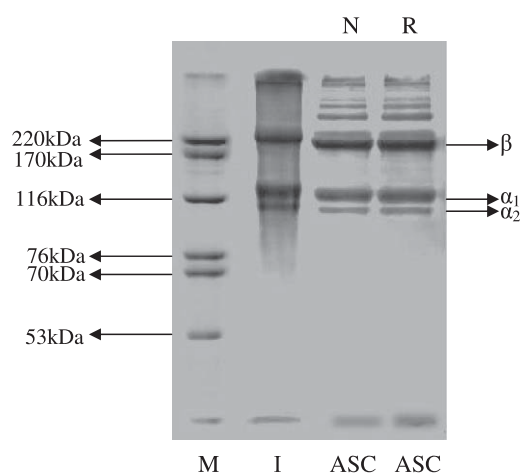


Fig. 1. SDS-PAGE pattern of ASC from the skin of unicorn leatherjacket under reducing (R) and non-reducing (N) conditions. M: high molecular weight markers; I: calf skin type I collagen and ASC: acid solubilized collagen.

indicating that there was no disulfide bond in ASC. This result was in accordance with the absence of cysteine in both collagens (Table 1). It was noted that ASC from the skin of unicorn leatherjacket contained a high amount of cross-linked proteins. This might reflect the high content of cross-links in their skin, which resulted in the difficulty of acid solubilization of collagen from the skin of this species. Cross-linking of α -chains was also observed in collagens from other fish species such as brownbanded bamboo shark (Kittiphattanabawon, Benjakul, Visessanguan, Kishimura, & Shahidi, 2009).

3.4. Peptide mapping of collagen

Lysyl endopeptidases from *A. lyticus* and V8 protease from *S. aureus* have been employed for peptide mapping of collagens (Kittiphattanabawon et al., 2005). The peptide maps of ASC digested by both enzymes are shown in Fig. 2. When V8 protease was used, the band intensities of γ - and β -chains disappeared. Band intensity of α -chains decreased with the coincidental increase in lower molecular weight peptides. However, calf skin collagen was resistant to hydrolysis by V8 protease. Thus, ASC was more susceptible to hydrolysis, compared to calf skin collagen by V8 protease. The result suggested that the primary structure of ASC from skin of unicorn leatherjacket was different from that of calf skin collagen, particularly in terms of sequence of amino acids as well as amino acid composition (Table 1). V8 protease shows a high specific preference for glutamic acid and aspartic acid residues of proteins (Jongjareonrak et al., 2005).

For peptide map of ASC digested by lysyl endopeptidase, all major components including α -, β - and γ -chains as well as high MW cross-links were completely hydrolyzed. It was noted that calf skin collagen was digested by lysyl endopeptidase, whereas it was resistant to V8 protease. Also it was found that the patterns of peptides were different between peptide map of ASC digested by V8 and lysyl endopeptidase. The result reconfirmed that ASC from unicorn leatherjacket skin had the different sequence and composition of amino acids as well as the degree of cross-linking, in comparison with calf skin type I collagen.

3.5. Differential scanning calorimetry

DSC thermograms of ASC from the skin of unicorn leatherjacket dispersed in 0.05 M acetic acid or deionized water are depicted in

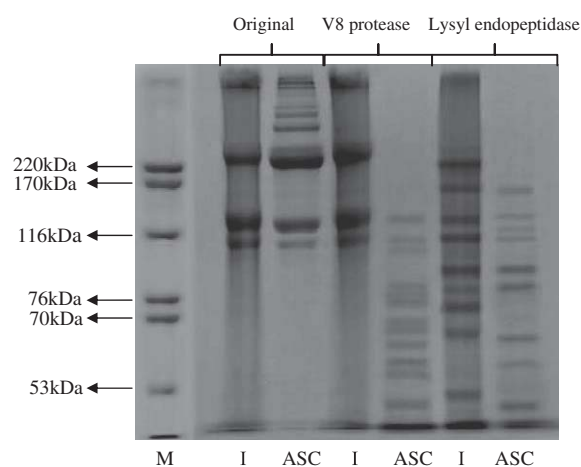


Fig. 2. Peptide maps of ASC from the skin of unicorn leatherjacket and calf skin collagen digested by V8 protease and lysyl endopeptidase. M: high molecular weight markers; I: calf skin type I collagen and ASC: acid solubilized collagen from the skin of unicorn leatherjacket.

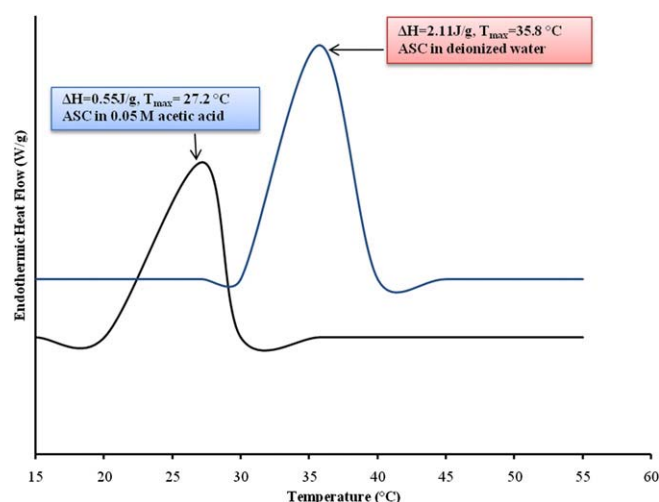


Fig. 3. DSC thermogram of ASC from the skin of unicorn leatherjacket dispersed in 0.05 M acetic acid and in deionized water.

Fig. 3. Endothermic peaks with the maximum transition temperatures ($T_{\max}$) at 27.2 and 35.8 °C were observed for ASC rehydrated in 0.05 M acetic acid and deionized water, respectively. For ASC rehydrated in acetic acid, $T_{\max}$ shifted to a lower temperature (27.2 °C), compared with that found in the sample rehydrated with deionized water. The result suggested that the hydrogen bonds which stabilize the secondary structure of collagen were partially disrupted by acetic acid. The thermal stability of the collagen triple helix is attributed to the hydrogen-bonded networks, mediated by water molecules, which connect the hydroxyl group of hydroxyproline in one strand to the main chain amide or carboxyl groups of another chain (Babu & Ganesh, 2001). In the presence of acetic acid, the hydrogen bonding might be disrupted to some extent due to electrostatic repulsion of ASC molecules and the breakage of hydrogen bond stabilizing the collagen molecules, resulting in the decrease in $T_{\max}$ in acidic solution. $T_{\max}$ of ASC was much lower than that of calf skin collagen (40.8 °C) (Kittiphattanabawon et al., 2005). The lower $T_{\max}$ of ASC from the skin of unicorn leatherjacket might be related to the lower content of imino acids than that of the calf skin collagen. The higher the imino acid content, the more thermal stability was obtained (Zhang et al., 2007). In addition, a higher enthalpy ($\Delta H = 2.11$ J/g) was also observed for ASC rehydrated in deionized water, compared with that of ASC dispersed in 0.05 M acetic acid ($\Delta H = 0.55$ J/g). The differences in ΔH among ASC rehydrated with two different media might be governed by hydrogen bonding in collagen structure.

3.6. ATR-FTIR analysis

FTIR spectra of ASC from the skin of unicorn leatherjacket and calf skin collagen are shown in Fig. 4. For ASC of unicorn leatherjacket, five characteristic amide bands representing amide A, amide B, amide I, amide II and amide III were found at the wavenumbers of 3294.86, 2920.87, 1646.24, 1549.08, and 1236.76 cm^{-1} , respectively. For calf skin collagen, the corresponding amide bands were observed at the wavenumbers of 3295.61, 2933.94, 1635.76, 1545.29 and 1235.57 cm^{-1} . The absorption spectrum of ASC from Nile perch skin was situated in the amide region including amide I (1651–1662 cm^{-1}), amide II (1540–1560 cm^{-1}) and amide III (1230–1242 cm^{-1}) (Muyonga, Cole, & Duodu, 2004). The amide I band is originated from C=O stretching vibrations coupled to N–H bending vibrations, CN stretch and CCN deformation (Bandekar, 1992). Dialyzed calf skin

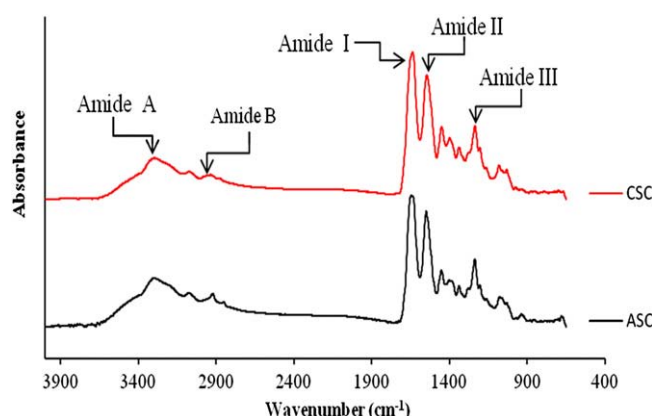


Fig. 4. Fourier transform infrared spectra of ASC from the skin of unicorn leatherjacket and calf skin collagen (CSC).

collagen had the lower wavenumber at amide I region, as compared to ASC, suggesting the more interaction of C=O with adjacent chains via hydrogen bond of the former. Generally, the lower wavenumber of bands corresponds to higher hydrogen bonding potential (Li, Liu, Gao, & Chen, 2004). It was presumed that intra-molecular interaction between α -chains in calf skin tropocollagen was more pronounced than ASC. Previous research suggested the following secondary structures in the amide I region: β -turn, 1660–1700 cm^{-1} ; α -helix, 1645–1659 cm^{-1} ; irregular structure, 1640–1644 cm^{-1} ; and β -sheet or extended structure, 1620–1640 cm^{-1} (Farrell, Wickham, Unruh, Qi, & Hoagland, 2001). For undialyzed calf skin collagen, an additional peak at amide I region with the wavenumber of 1728.90 cm^{-1} was found (data not shown). After the excessive dialysis, acetic acid or other acids used for collagen solubilization were completely removed. This was confirmed by the disappearance of peak at 1728.90 cm^{-1} , which more likely represented C=O stretching of the carboxyl group from acetic acid or other acids (Kaminska & Sionkowska, 1996).

The lower wavenumber of amide II peak was also found in calf skin collagen as compared to ASC, indicating that N–H of calf skin collagen was more involved in bonding with the adjacent α -chains. The amide III represented the combination peaks between C–N stretching vibrations and N–H deformation from amide linkages as well as absorptions arising from wagging vibrations from CH_2 groups from the glycine backbone and proline side-chains (Jackson, Choo, Watson, Halliday, & Mantsch, 1995). This amide III was detected around the wavenumber of 1236.76 and 1235.57 cm^{-1} for ASC and calf skin collagen, respectively. The ratio of amide III and 1451.30 cm^{-1} was 0.86 and 0.85 for ASC and calf skin collagen, respectively. In general, the ratio of 1 indicated the triple helical structure (Plepis, Goisis, & Das, 1996). Therefore, there was similarity in helical structure between both samples. Nevertheless, calf skin collagen might have the higher intra- and inter-molecular cross-links, especially the higher hydrogen bonding in stabilizing the triple helix, as evidenced by the higher $T_{\max}$ of calf skin collagen, compared with that of ASC. The amide A band, arising from the stretching vibrations of N–H group, appeared at 3294.86 and 3295.61 cm^{-1} in ASC and calf skin collagen, respectively. Doyle, Blout, and Bendit (1975) reported that a free N–H stretching vibration commonly occurs in the range of 3400–3440 cm^{-1} . The amide B was observed at 2920.87 and 2933.94 cm^{-1} for ASC and calf skin collagen, respectively, corresponding to asymmetric stretch vibration of =C–H as well as $-\text{NH}_2^+$. The shift of amide B of calf skin collagen to higher wavenumber was probably associated with free NH_2^+ group of lysine residues or of N-termini. Thus, FTIR spectrum of ASC from the skin of unicorn leatherjacket had slight

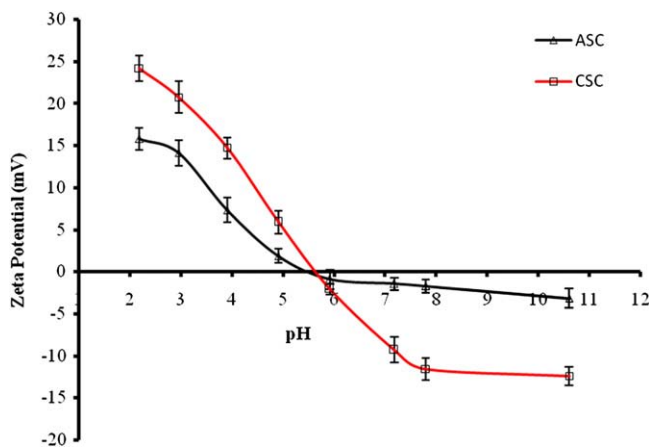


Fig. 5. Zeta potential of ASC from the skin of unicorn leatherjacket and calf skin collagen (CSC) at different pHs. Bars represent the standard deviation ($n = 3$).

differences in functional groups and inter- and intra-molecular interaction, compared with calf skin collagen type I.

3.7. Measurement of ζ -potential

The ζ -potential values of ASC and calf skin collagen type I measured as a function of pH are shown in Fig. 5. The surface net charge of ASC and calf skin collagen became zero at pH of 5.58 and 5.68, respectively. Protein molecules in an aqueous system have zero net charge at their isoelectric points (pI), in which the positive charges are balanced out by the negative charges (Bonner, 2007). Thus, pIs of ASC and calf skin collagen were estimated to be 5.58 and 5.68, respectively. The pIs of ASC and calf skin collagen were in acidic range, possibly due to the higher density of carboxyl groups. It was noted that the molecular charge of ASC remained very low in the alkaline region. This might be associated with the low solubility of ASC in the alkaline pH range, in which the repulsion force between the molecules with the negative charge was not sufficient to cause the complete solubilization. The slight difference in the pI of ASC and calf skin collagen might be caused by the differences in amino acid composition and configuration between collagens.

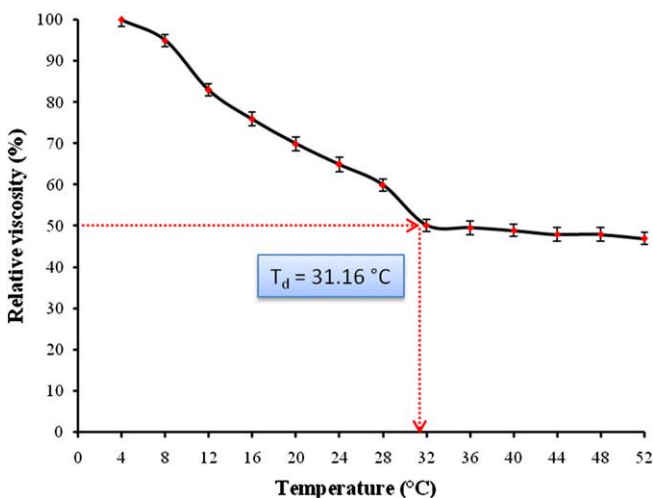


Fig. 6. Relative viscosity of ASC from the skin of unicorn leatherjacket at different temperatures. Bars represent the standard deviation ($n = 3$).

3.8. Viscosity of collagen solution

One of the physicochemical characteristics of collagen is its high viscosity. Collagen solutions are highly structured systems which even at low concentrations are characterized by a high degree of viscosity due to stronger electrostatic repulsion among the collagen molecular chains in solution (Zhang, Liu, & Li, 2009). Collagen is irreversibly destroyed as a result of the thermal denaturation. The triple helix structure of collagen stabilized by hydrogen bonds was converted into the random coil configuration by the process of thermal depolymerization, which was accompanied by a change in physical properties, such as viscosity, sedimentation, diffusion, light scattering and optical activity (Gurdak, Booth, Roberts, Rouxhet, & Dupont-Gillain, 2006). The continuous decreases in relative viscosity of ASC in 0.1 M acetic acid were observed when the temperature increased up to 52 °C ($P < 0.05$) (Fig. 6). At higher temperature, the rearrangement of all chains, α , β or γ chains, might take place and the less compact configuration was obtained, leading to the decrease in viscosity of solution. From the result, T_d of ASC was estimated to be 31.16 °C. The high viscosity can be attributed to the high proportion of β and γ chains with a higher

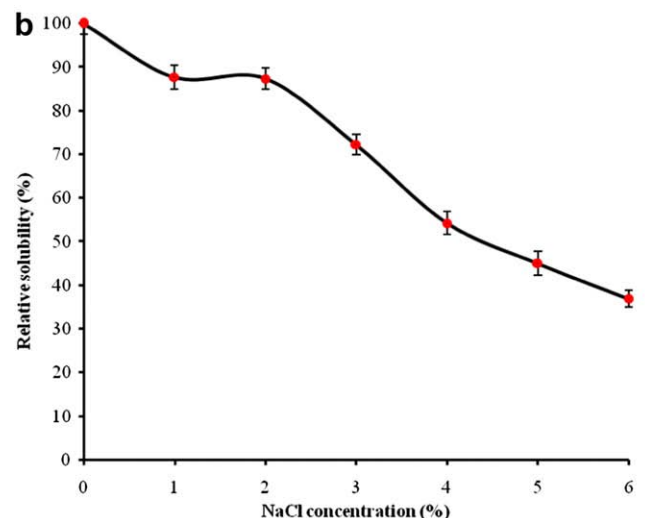
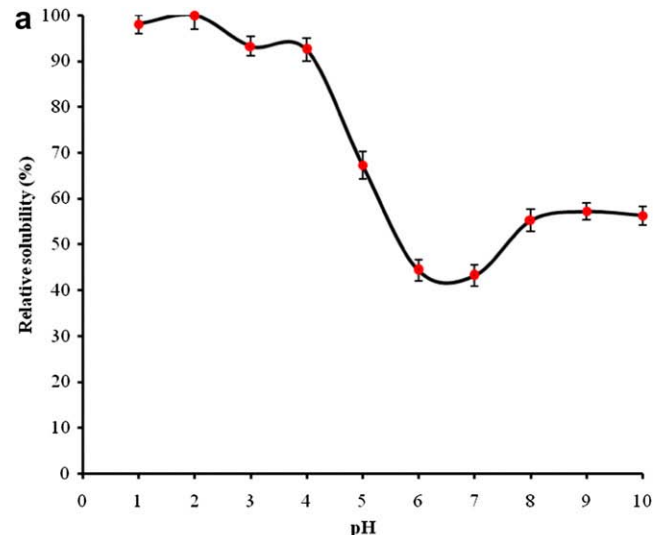


Fig. 7. Relative solubility (%) of ASC from the skin of unicorn leatherjacket as affected by different pHs (a) and NaCl concentration (b). Bars represent the standard deviation ($n = 3$).

average molecular weight (Ogawa et al., 2004). Heat-treatment at high temperature can break down the hydrogen bonds, which stabilize collagen structure (Wong, 1989), leading to the dissociation of collagen molecules.

3.9. Effects of pH and NaCl on collagen solubility

Fig. 7(a) shows the effect of pH on the solubility of ASC from skin of unicorn leatherjacket. ASC was soluble in the pH ranging from 1 to 4 and the highest solubility was observed at pH 2 ($P < 0.05$). The lowest solubility was observed at pH 6–7 ($P < 0.05$), which was in agreement with the pH rendering the net charge of zero. At the pH near the isoelectric point, ASC and calf skin collagen molecules are unstable and tend to coagulate or flocculate due to increased hydrophobic interaction among collagen molecules (Jongjareonrak et al., 2005). The higher solubility at lower pH could arise from the increased repulsive force among collagen molecules. At alkaline pH, the slight increase in solubility was also observed ($P < 0.05$). ASC had the decrease in solubility in the presence of salt. The slight decrease in solubility was observed in the presence of 1–2% and the sharp decrease was found as the salt concentration increased up to 6% ($P < 0.05$). At 6% NaCl, the solubility of 36.91% was obtained. The lowered solubility of ASC was mainly due to the 'salting out' effect.

4. Conclusion

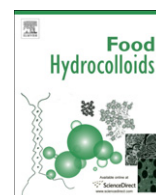
ASC could be extracted from unicorn leatherjacket skin. The collagen consisted of two α -chains (α_1 and α_2) and were characterized as type I collagen. ASC was in triple helical structure and was solubilized in acidic pH range and lost its solubility as the concentration of salt increased. The applications of ASC should be maximized based on its characteristics. Due to the low yield of ASC from the skin of unicorn leatherjacket, the improvement of extraction method should be further studied.

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Characteristics of gelatin from the skin of unicorn leatherjacket (*Aluterus monoceros*) as influenced by acid pretreatment and extraction time

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Extraction

ABSTRACT

Gelatins from the skin of unicorn leatherjacket (*Aluterus monoceros*) pretreated with different acids (0.2 M acetic acid or 0.2 M phosphoric acid) and extracted with distilled water at 45 °C for various times (4 and 8 h) were characterized. Yields of 5.23–9.18 or 6.12–11.54% (wet weight basis) were obtained for gelatins extracted from the skin pretreated with 0.2 M acetic acid or 0.2 M phosphoric acid, respectively. Extracted gelatins contained α_1 and α_2 chains as the predominant components and some degradation peptides. The absorption bands of gelatins in FTIR spectra were mainly situated in the amide band region (amide I, amide II and amide III) and showed the significant loss of molecular order of triple helix. Gelatin samples had a relative solubility greater than 90% in the wide pH ranges (1–10). The gel strength of gelatin from skin pretreated with phosphoric acid (GPA) was higher than that of gelatin from skin pretreated with acetic acid (GAA). Both GPA and GAA had the lower gel strength than that of commercial bovine gelatin ($P < 0.05$). Net charge of GAA and GPA became zero at pHs of 6.64–7.15 and 6.78–7.26, respectively, as determined by zeta potential titration. Emulsifying and foaming properties of GAA and GPA increased with increasing concentrations (1–3%, w/v). Those properties were governed by pretreatments and extraction time. Thus gelatin can be successfully extracted from unicorn leatherjacket skin using the appropriate acid pretreatment and extraction time.

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1. Introduction

Gelatin is a denatured fibrous protein derived from collagen by partial thermal hydrolysis. It is an important functional biopolymer that has a very broad application for food, material, pharmacy and photography industries (Hao et al., 2009). Under specific conditions of temperature, solvent and pH, gelatin macromolecules can exhibit flexibility sufficient to achieve a wide variety of conformations. Nevertheless, the characteristics and functionalities are dependent on its molecular structure. Due to its amphoteric nature, specific triple-stranded helical structure (not observed in synthetic polymers) and its interaction with water, which is different from synthetic hydrophilic polymers, gelatin has become indispensable (Kozlov & Burdygina, 1983).

Different types of gelatins have varying thermal and rheological properties such as melting and gelling temperatures and bloom strength (Norziah, Al-Hassan, Khairulnizam, Mordi, & Norita, 2009).

Gelatin extraction from fish skin is generally achieved by pretreatment with acid or alkali to give the desired properties (Arnesen & Gildberg, 2006). Efficiency of gelatin extraction depends on the method, in which collagens are pretreated. There are two types of gelatin with different characteristics including type-A, acid-treated collagen (isoelectric point at pH 6–9) and type-B, an alkaline treated (isoelectric point at pH 5) (Benjakul, Oungbho, Visessanguan, Thiansilakul, & Roytrakul, 2009). Acid treatment is suitable for less fully cross-linked collagens commonly found in pig or fish skins, whereas alkaline treatment is appropriate for the more complex collagens found in bovine hides (Gómez-Guillén et al., 2002).

Generally, the traditional sources of gelatin are bovine and porcine skins and bones. The global demand for gelatin has been increasing over the years. Recent reports indicate that the annual world production of gelatin is nearly 326,000 tons, with pig skin derived gelatin accounting for the highest (44%) output, followed by bovine hides (28%), bones (27%), and other sources (1%) (GME, 2009). Gelatin from aquatic sources has been recognized to be free of viral or other infection or the risk of contamination with bovine spongiform encephalopathy (BSE) (Sadowska, Kolodziejka,

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& Niecikowska, 2003). Additionally, it can be used to replace porcine gelatin, which is prohibited for Muslims or Judaism. It is estimated that fish-processing waste after filleting accounts for approximately 75% of the total fish weight (Shahidi, 1994) and 30% of the waste is in the form of bones and skins (Gómez-Guillén et al., 2002). Those by-products can be served as the promising raw material for gelatin extraction. As a consequence, fish gelatin can be used widely, especially for food and pharmaceutical applications. To maximize the yield and the functionalities, the extraction process should be optimized to obtain the gelatin with desired properties.

Unicorn leatherjacket (*Aluterus monoceros*) belongs to the order Tetraodontiformes and is a member of the Monacanthidae family. This species has been used for fillet production in Thailand, especially for the export as frozen fillets. As a consequence, a large amount of skin has been produced as by-product. Due to its thick skin, it can be a potential source of gelatin. The objective of this study was to extract and characterize the gelatin from the skin of unicorn leatherjacket pretreated with different acids and extracted for various times.

2. Materials and methods

2.1. Chemicals

β -Mercaptoethanol (β -ME) and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). High molecular weight markers were purchased from GE Healthcare UK (Buckinghamshire, UK). Folin–Ciocalteu's phenol reagent, acetic acid and phosphoric acid were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250 and *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). Food grade bovine gelatin was obtained from Halagel Co., Ltd. (Bangkok, Thailand).

2.2. Fish skin preparation

The skin of unicorn leatherjacket (*A. monoceros*) was obtained from Sea Wealth Frozen Food Co., Ltd., Songkhla, Thailand. Upon arrival to the Department of Food Technology, Prince of Songkla University, Hat Yai, the skin was cleaned and washed with iced tap water (0–2 °C). Prepared skin was then cut into small pieces (0.5 × 0.5 cm²), placed in polyethylene bags and stored at –20 °C until use. The storage time was less than 2 months.

2.3. Extraction of gelatin from the skin of unicorn leatherjacket

Before gelatin extraction, skin was soaked in 0.1 M NaOH with a skin/solution ratio of 1:10 (w/v). The mixture was stirred continuously for 2 h at 4 °C at a speed of 150 rpm using an overhead stirrer equipped with propeller (RW 20.n, IKA®-Werke GmbH & CO.KG, Staufen, Germany). The alkaline solution was changed every 1 h to remove non collagenous proteins and pigments. Alkaline-treated skin was then washed with tap water until neutral or faintly basic pH of wash water was obtained. The skin was then soaked in 0.2 M acetic acid or 0.2 M phosphoric acid with a skin/solution ratio of 1:10 (w/v) for 24 h with gentle stirring at 4 °C. The acidic solution was changed every 12 h to swell the collagenous material in the fish skin matrix. Acid-pretreated skin was washed thoroughly with tap water until wash water became neutral or faintly basic. To extract gelatin, the swollen skin was soaked in distilled water (45 °C) with a skin/water ratio of 1:10 (w/v) in a temperature-controlled water bath (W350, Memmert, Schwabach, Germany) for 4 and 8 h with a continuous stirring at a speed of 150 rpm. The mixture was then filtered using

two layers of cheesecloth. The filtrate was further filtered using a Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England) with the aid of JEIO Model VE-11 electric aspirator (JEIO TECH, Korea). The resultant filtrate was freeze-dried using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark). The dry matter extracted from skin pretreated with acetic acid and extracted for 4 and 8 h was referred to as 'GAA4' and 'GAA8', respectively. That extracted from skin pretreated with phosphoric acid and extracted for 4 and 8 h was referred to as 'GPA4' and 'GPA8', respectively. All gelatin samples were weighed, calculated for extraction yield and were also subjected to analyses.

2.4. Analyses

2.4.1. Determination of ash content

Ash content was determined following the method of AOAC (2000) with the analytical number of 920.153. Ash content was expressed as % (dry weight basis).

2.4.2. Electrophoretic analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970). Gelatin samples were dissolved in 5% SDS and the mixtures were incubated at 85 °C for 1 h. The mixtures were centrifuged at 5000 g for 5 min at room temperature using a microcentrifuge (MIK-RO20, Hettich Zentrifugan, Germany) to remove undissolved debris. Gelatin samples were mixed at a 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS and 20% glycerol). Samples (15 µg protein) were loaded onto polyacrylamide gels comprising a 7.5% running gel and a 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, the gel was stained with 0.05% (w/v) Coomassie Blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. High molecular weight markers (GE Healthcare UK, Buckinghamshire, UK) were used to estimate the molecular weight of the proteins. Food grade bovine gelatin was used as a standard gelatin.

2.4.3. Fourier transform infrared (FTIR) spectra analysis

FTIR spectra of freeze-dried gelatin samples were recorded using a horizontal ATR Trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) equipped with a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) at room temperature. For spectra analysis, freeze-dried gelatin samples were placed on the crystal cell and the cell was clamped into the mount of the FTIR spectrometer. The spectra in the range of 400–4000 cm^{–1} were ratioed and automatic signals gained were collected in 32 scans at a resolution of 4 cm^{–1} against a background spectrum recorded from the clean empty cell at 25 °C.

2.4.4. Measurement of ζ -potential

Gelatin samples were dissolved in distilled water at a concentration of 0.5 mg/ml. The mixture was stirred at room temperature for 6 h. The ζ -potential of each sample (20 ml) was measured using a zeta potential analyzer (ZetaPALS, Brookhaven Instruments Co., Holtsville, NY, USA). ζ -Potential of samples adjusted to different pHs with 1.0 M nitric acid or 1.0 M KOH using an autotitrator (BI-ZTU, Brookhaven Instruments Co., Holtsville, New York, USA) was determined. The pI was estimated from pH rendering ζ -potential of zero.

2.4.5. Determination of gel strength

Gels of gelatin were prepared by the method of Fernández-Daz, Montero, and Gómez-Guillén (2001) with a slight modification.

Gelatin samples were dissolved in distilled water at 60 °C to obtain the final concentration of 6.67% (w/v). The solution was stirred until the gelatin was solubilized completely and cooled in a refrigerator at 10 °C for 16–18 h for gel maturation. The dimensions of the sample were 3 cm in diameter and 2.5 cm in height. The gel strength of the sample gels prepared at 10 °C was determined using a Model TA-XT2 Texture Analyzer (Stable Micro System, Surrey, UK) with a load cell of 5 kN and equipped with a 1.27 cm diameter flat-faced cylindrical Teflon® plunger. The maximum force (in grams) was recorded when the penetration distance reached 4 mm. The speed of the plunger was 0.5 mm/s.

2.4.6. Color measurement

The color of gelatin gels (6.67% w/v) was measured by a Hunter lab color meter (Color Flex, Hunter Lab Inc., Reston, VA, USA) reported by the CIE system. L^* , a^* and b^* parameters indicate lightness/brightness, redness/greenness and yellowness/blueness, respectively. The colorimeter was warmed up for 10 min and calibrated with a white standard.

2.4.7. Determination of solubility

Solubility of gelatin was determined at different pH following the method of [Montero, Jimenez-Colmenero, and Borderias \(1991\)](#) with a slight modification. The gelatin samples were dissolved in distilled water at 60 °C to obtain a final concentration of 2% (w/v) and the mixture was stirred at room temperature until the gelatins were completely solubilized. The gelatin solutions were adjusted to different pH (1–10) with either 6 N NaOH or 6 N HCl. The volume of solutions was made up to 10 ml with distilled water, previously adjusted to the same pH of gelatin solution. The solutions were centrifuged at 8500 g at room temperature for 10 min. Protein content in the supernatant was determined by the Lowry method ([Lowry, Rosebrough, Farr, & Randall, 1951](#)) using bovine serum albumin as a standard. Relative solubility was calculated in comparison with that obtained at the pH giving the highest solubility.

2.4.8. Determination of emulsifying properties

Emulsion activity index (EAI) and emulsion stability index (ESI) of gelatin samples were determined according to the method of [Pearce and Kinsella \(1978\)](#) with a slight modification. Soy bean oil (2 ml) and gelatin solution (1, 2 or 3% gelatin, 6 ml) were homogenized using a homogenizer (Model T25 basic; IKA Labortechnik, Selangor, Malaysia) at a speed of 20,000 rpm for 1 min. Emulsions were pipetted out at 0 and 10 min and 100-fold diluted with 0.1% SDS. The mixture was mixed thoroughly for 10 s using a vortex mixer (Scientific Industries, Inc., Bohemia, NY, USA). A_{500} of the resulting dispersion was measured using a spectrophotometer (UV-160, Shimadzu, Kyoto, Japan). EAI and ESI were calculated by the following formulae ([Aewsiri, Benjakul, & Visessanguan, 2009](#)):

$$\text{EAI} \left(\text{m}^2/\text{g} \right) = (2 \times 2.303 \times A \times \text{DF}) / l \theta C$$

where $A = A_{500}$, DF = dilution factor (100), l = path length of cuvette (m), θ = oil volume fraction and C = protein concentration in aqueous phase (g/m^3)

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

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

2.4.9. Determination of foaming properties

Foam expansion (FE) and foam stability (FS) of gelatin sample solutions were determined, as described by [Shahidi, Xiao-Qing, and Synowiecki \(1995\)](#), with a slight modification. Gelatin solution (1, 2

or 3%) was transferred into 100 ml cylinders. The solution was homogenized at a speed of 13,400 rpm for 1 min at room temperature. The sample was allowed to stand for 0, 20, 40 and 60 min. FE and FS were then calculated using the following equations:

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

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

where V_T = total volume after whipping; V_0 = the original volume before whipping and V_t = total volume after leaving at room temperature for different times (20, 40 and 60 min).

2.5. Statistical analyses

All experiments were performed in triplicate and a completely randomized design (CRD) was used. Data were presented as means $\pm$ standard deviation and the probability value of <0.05 was considered significant. Analysis of variance (ANOVA) was performed and mean comparisons were done by Duncan's multiple range tests ([Steel & Torrie, 1980](#)). Analysis was performed using a SPSS package (SPSS 11.0 for windows, SPSS Inc, Chicago, IL, USA).

3. Results and discussion

3.1. Extraction yield and ash content

Gelatin extracted from skin of unicorn leatherjacket pretreated with 0.2 M acetic acid (GAA) or 0.2 M phosphoric acid (GPA) showed the different yields when different extraction times (4 and 8 h) were used. During heating at 45 °C, hydrogen bonds stabilizing triple helix of mother collagen were destroyed, leading to helix-to-coil transition. This resulted in the conversion of collagen to soluble gelatin ([Benjakul et al., 2009](#)). Additionally, some peptide bonds were cleaved. Yield was increased as the extraction times increased from 4 to 8 h ($P < 0.05$). Yields of GAA4, GAA8, GPA4 and GPA8 were 5.23, 9.18, 6.12 and 11.54% (wet weight basis), respectively. The higher yields of GAA8 and GPA8 were attributed to the sufficient time in disrupting the bonds of collagen. The results were in accordance with [Arnesen and Gildberg \(2007\)](#) who reported that increasing extraction times were associated with the increase in yield of gelatin from Atlantic salmon skin. At the same extraction time, GPA showed the higher yield than did GAA. Even at the same concentration (molarity), phosphoric acid with higher ionic strength and lower pH could facilitate the swelling process caused by more repulsive force among the collagen molecules compared with acetic acid. With the loosen structure of swollen collagen, warm water could penetrate into the skin matrix effectively ([Stainsby, 1987](#)). Phosphoric acid pretreatment more likely provided a greater swelling power for destabilization of acid labile cross-links at the telopeptide region and amide bonds of the triple helical structure of collagen as well as non-covalent intra- and inter-molecular bonds, compared with acetic acid ([Gómez-Guillén & Montero, 2001](#)). Though the swelling took place, gelatin could not be completely extracted due to a large number of cross-links at the telopeptide region as well as inter-molecular cross-links, leading to the compact structure ([Ahmad, Benjakul, & Nalinanon, 2010](#)). Thus, swelling process affecting the extraction efficiency was dependent on the types of acid used.

Ash contents of gelatin, both GPA than GAA, were quite high when compared with the recommended value (2.5%) ([Jones, 1977](#)). Ash contents were 8.10 and 12.3% (dry weight basis) for GAA4 and GAA8, respectively. GPA4 and GPA8 had the ash content of 12.4 and 15.45%, respectively. High ash content in all gelatin samples indicated the presence of inorganic salt, which might be generated

during the pretreatment with either alkali or acid. Phosphates incorporated into the gelatin pretreated with phosphoric acid more likely contributed to the higher ash content in the resulting gelatin.

3.2. Protein patterns

Protein patterns of gelatins extracted from the skin of unicorn leatherjacket with different acid pretreatments for different extraction times are shown in Fig. 1. Different protein patterns were noticeable when different extraction times were used. With increasing extraction times, the degradation of major components with the concomitant formation of low molecular weight peptides was observed. For GAA, high intensity of γ -, β - and α -chains was still found when extraction times of up to 4 h were used. Nevertheless, those components were hydrolyzed to higher extent as the extraction time increased. The α -chain was still noticed in GAA extracted with extraction time up to 12 h. Gelatins with high content of α -chain were reported to possess better functional properties (Gómez-Guillén et al., 2002). For GPA, the greater amount of peptides with molecular weight lower than α -chains was generated as the extraction time increased. At all extraction times used, GPA contained the lower band intensity for all major bands including α -, β -, and γ -chains, while the proteins with low molecular weight were dominant. In general, the formation of peptide fragments is associated with the low viscosity, low melting point, low setting point, high setting time, as well as decreased

bloom strength of gelatin (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2006). For both GAA and GPA, β -chain and α -chain were found as the major components, especially when extraction time was less than 4 h. The protein patterns were different from those of commercial bovine gelatin, in which proteins with molecular weight of 145,000 and 141,000 Da were the major components. This was probably due to the different pretreatment and extraction conditions. In gelatin manufacture, the conversion of collagen to gelatin yields the molecules of varying mass, due to the cleavage of inter-chain cross-links and some unfavorable breakages of inter-chain peptide linkage (Zhou, Mulvaney, & Regensteinst, 2006). From the result, GAA and GPA extracted for the extended times were more prone to degradation, in which shorter chains were obtained. Acid used for pretreatment played a role in protein components of resulting gelatin. When phosphoric acid was used, the highly loosen structure might be more susceptible to hydrolysis during extraction at high temperature. Thus extraction time and pretreatment had the influence on the components and the length of the polypeptide chains. Since the excessive extraction time yielded the gelatin with the remarkable degradation, GAA and GPA extracted for 4 and 8 h were prepared and used for further characterization.

3.3. ATR-FTIR analysis

FTIR spectra of gelatin extracted from the skin of unicorn leatherjacket with different acid pretreatments and extraction times are depicted in Fig. 2. Spectra of GAA4, GAA8, GPA4 and GPA8 displayed the major peaks in amide region. These spectra were in accordance with those reported by Muyonga, Cole, and Duodu (2004). FTIR spectroscopy has been used to monitor the changes in the functional groups and secondary structure of gelatin samples (Muyonga et al., 2004). GAA4, GAA8, GPA4 and GPA8 exhibited the amide I bands at the wavenumber of 1634.73, 1649.11, 1642.59, and 1646.51 cm^{-1} , respectively. The amide I vibration mode is primarily a C=O stretching vibration coupled to contributions from the CN stretch, CCN deformation and in-plane NH bending modes (Bandekar, 1992). The absorption peak at amide I (1634.73 cm^{-1}) was characteristic for the coil structure of gelatin (Yakimets et al., 2005). The spectral differences in amide I of different gelatin samples were largely attributed to different conformation of polypeptide chains. Amide I peaks of GAA8 and GPA8 shifted to higher

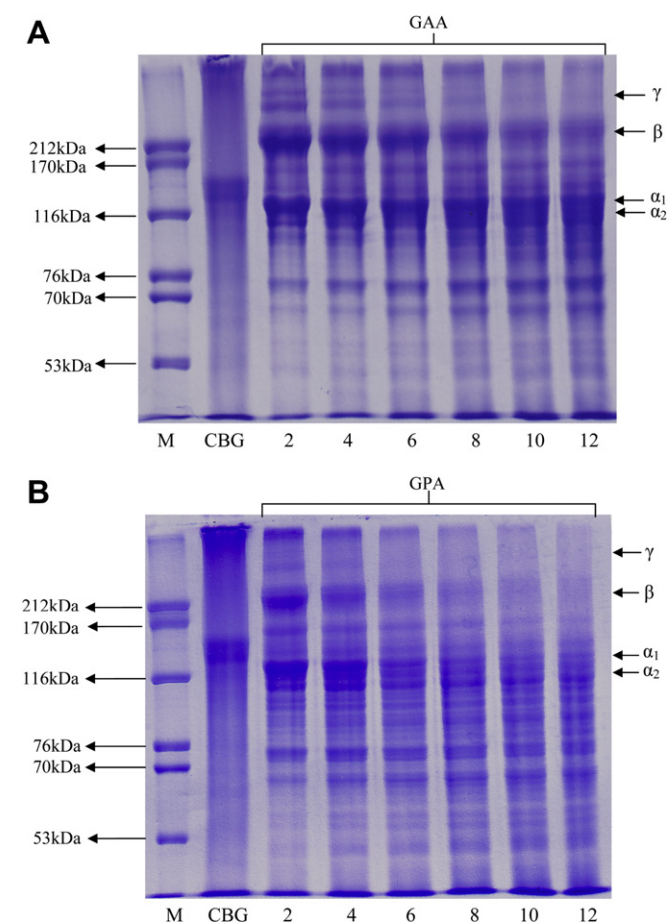


Fig. 1. SDS-PAGE pattern of gelatin extracted from the skin of unicorn leatherjacket with different acid pretreatment using 0.2 M of acetic acid (A) or phosphoric acid (B) and extraction times. M: high molecular weight markers; CBG: commercial bovine gelatin. Numbers denote the extraction times (h).

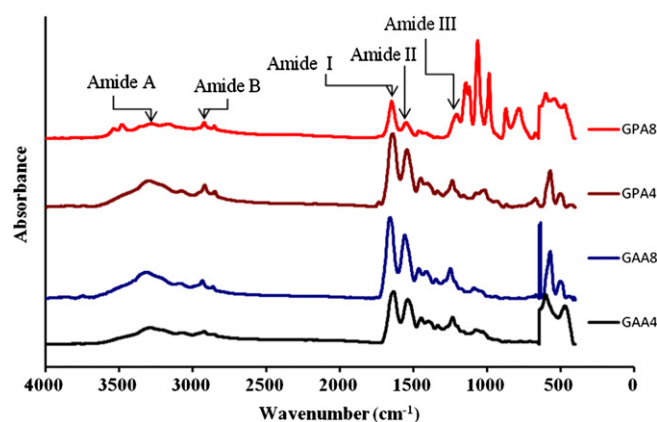


Fig. 2. Fourier transform infrared spectra of gelatin from the skin of unicorn leatherjacket with different acid pretreatment and extraction times. GAA4 and GAA8: gelatin extracted from the skin of unicorn leatherjacket pretreated with 0.2 M acetic acid and extracted for 4 and 8 h, respectively. GPA4 and GPA8: gelatin extracted from the skin of unicorn leatherjacket pretreated with 0.2 M phosphoric acid and extracted for 4 and 8 h, respectively.

frequency, compared to those of GAA4 and GPA4. This indicated the greater loss of molecular order due to thermal uncoupling of inter-molecular cross-links as the longer extraction was used. In addition, GAA4 showed the lower amplitude compared to GAA8, indicating the greater disruption of intra-molecular bonding and more exposure of C=O of GAA8. Lower amplitude was associated with the higher extent of molecular order due to interaction of C=O with adjacent chains via hydrogen bond. Nevertheless, GPA4 showed the higher amplitude than did GPA8. GPA8 most likely lost the secondary structure during extraction, while aggregation might occur to some extent during freeze-drying. The decrease in peak intensity and narrowing of peak area might reflect the association of peptide fragments (Prystupa & Donald, 1996). Since, GAA8 and GPA8 contained a higher amount of unordered and low molecular weight peptides due to longer extraction time, the reactive groups (C=O) were more exposed and became more reactive between α -chains, especially during freeze-drying.

The characteristic absorption bands of GAA4, GAA8, GPA4 and GPA8 in amide II region were noticeable at the wavenumbers of 1539.69, 1547.49, 1545.77 and 1549.87 cm^{-1} , respectively. The amide II vibration modes are attributed to an out-of-phase combination of CN stretch and in-plane NH deformation modes of the peptide group (Bandeekar, 1992; Lavalie, Adams, & Levin, 1982). Lower amplitude was found in GPA8, compared to GAA8, GPA4 and GAA4, indicating that N–H was more involved in bonding with the adjacent α -chains. In addition, amide III was detected around the wavenumber of 1234.81, 1237.74, 1236.99, 1206.03 cm^{-1} for GAA4, GAA8, GPA4 and GPA8, respectively. The amide III represented the combination peaks between C–N stretching vibrations and N–H deformation from amide linkages as well as absorptions arising from wagging vibrations from CH_2 groups from the glycine backbone and proline side-chains (Jackson, Choo, Watson, Halliday, & Mantsch, 1995). GAA8 had the lower amplitude at amide III region than GAA4. However, GPA8 exhibited the higher amplitude of amide III band than GPA4. The greater disorder of molecular structure due to transformation of an α -helical to a random coil structure occurred during heating and these changes were associated with loss of triple-helix state as a result of denaturation of collagen to gelatin (Muyonga et al., 2004). GPA8 also had the high intensity peaks at wavenumbers of 1144.04, 1119.62, 1063.38 and 985.59 cm^{-1} . These bands were associated with the C–O stretching vibrations of the short peptide chains (Jackson et al., 1995). The occurrence of those peaks was coincidental with more degradation of peptide chains in GPA8 sample (Fig. 1). In addition, the prominent absorption bands in GPA4 and GPA8 around the wavenumber of 1074.06 and 1063.38 cm^{-1} respectively, most likely arose from asymmetric stretching vibrations of phosphate groups of phosphorylated proteins coupled to $-\text{CH}_2$ of the amino acid residues (Jackson et al., 1995).

Moreover, the amide A band, arising from the stretching vibrations of N–H group, appeared at 3293.18, 3300.66, 3298.23 and 3275.53 cm^{-1} for GAA4, GAA8, GPA4 and GPA8, respectively. Normally, a free N–H stretching vibration occurs in the range of 3400–3440 cm^{-1} . When the N–H group of a peptide is involved in a hydrogen bond, the position is shifted to lower frequencies (Doyle, Blout, & Bendit, 1975). At amide A region, the lower amplitude as well as the lower wavenumber were found in GPA8, compared to GPA4, indicating that N–H group of shorter peptide fragments in GPA8 was involved in hydrogen bonding. GPA8 might also form new covalent inter-molecular cross-links during freeze-drying. The amide A also tends to join with the CH_2 stretch peak when carboxylic acid groups exist in a dimeric inter-molecular interaction (Kemp, 1987). The amide B was observed at 3073.13, 3075.22, 3076.61 and 3165.97 cm^{-1} for GAA4, GAA8, GPA4 and GPA8, respectively, corresponding to asymmetric stretch vibration

of $=\text{C}-\text{H}$ as well as $-\text{NH}_3^+$. Among all samples, GPA8 showed the lowest wavenumber of amide B peak, suggesting the interaction of $-\text{NH}_3$ group between peptide chains. Thus, it can be concluded that the secondary structure of gelatins obtained from the skin of unicorn leatherjacket was affected by acid pretreatment and extraction time. The low molecular weight peptides formed during the extraction for long time were more likely able to form covalent cross-links during freeze-drying process.

3.4. Measurement of ζ -potential

The ζ -potential values of GAA and GPA were measured as a function of pH as shown in Fig. 3. Net charge of zero was obtained at pH 7.26 and 6.78 for GPA4 and GPA8, respectively. Net charge of GAA4 and GAA8 became zero at pH of 7.15 and 6.64, respectively. At the pH near the isoelectric point, both GAA and GPA had the decreased repulsion with increased hydrophobic interaction among gelatin molecules. At pI, the positive charges are balanced out by the negative charges (Bonner, 2007). The isoelectric points of GPA8 and GAA8 were in acidic range. With increasing extraction time, deamidation might take place, in which glutamic acid and aspartic acid could be formed. This led to the increase in acidic amino acids associated with the decrease in pI. Furthermore, the difference in the pI between GAA and GPA might be due to the differences in reactive groups, which could accept or donate proton differently at various pH. The result also suggested that different acid pretreatments also had the impact on pI of resulting gelatin to some degree.

3.5. Solubility

High solubility of both GAA and GPA was observed in the wide pH range (1–10). The relative solubility of both gelatins was greater than 90% at all pH tested. Both GAA and GPA showed the lower relative solubility at pH 8.0 ranging from 90.4 to 91.1% and 91.8 to 92.4%, respectively. At pH close to pI, hydrophobic interaction among gelatin molecules increased, and the total net charge of protein molecules became zero, which was associated with the decreased repulsion. The higher solubility at pH far away from pI can be attributed to increased electrostatic repulsion of the protonated or deprotonated gelatin molecules. Normally, gelatin type-A or acid-processed gelatin has isoelectric points varying from 6.5 to 9.0 (Foegeding, Lanier, & Hultin, 1996). During acid pretreatment of skin, some glutamine and asparagine can be converted to their acidic forms, i.e. glutamic acid and aspartic acid, respectively (Jamilah & Harvinder, 2002). Bovine bone gelatin had the lowest solubility at pH 5 (90.6%). Bovine gelatin has been identified as type-B gelatin with pI of approximately 4.88 (Zhang, Li,

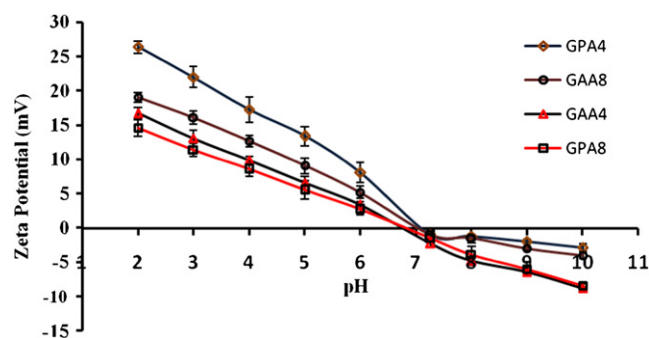


Fig. 3. Zeta potential of gelatin from the skin of unicorn leatherjacket with different acid pretreatment and extraction times. Key: see the caption for Fig. 2.

Table 1

Gel strength and color of gelatin from the skin of unicorn leatherjacket with different acid pretreatment and extraction times.

Properties	Commercial bovine gelatin	GPA4	GPA8	GAA4	GAA8
Gel strength (g)	293.22 ± 5.71A	149.77 ± 1.22B	121.92 ± 3.76C	56.90 ± 3.23D	51.73 ± 0.77E
Color					
L^*	35.85 ± 2.44B	34.35 ± 1.12B	45.89 ± 2.19A	30.67 ± 2.34D	18.26 ± 0.97C
a^*	−0.77 ± 0.14C	0.37 ± 0.05B	3.33 ± 1.21A	0.54 ± 0.41B	−0.69 ± 0.11C
b^*	18.29 ± 1.96A	2.16 ± 0.19C	5.47 ± 1.01B	0.83 ± 1.23D	−4.60 ± 1.23E

Mean ± SD ($n = 3$). Key: see the caption for Fig. 2.Different capital letters in the same row indicate significant differences ($P < 0.05$).

& Shi, 2006). The difference in solubility of different gelatins might result from the differences in molecular weight and the content of polar and non-polar groups in amino acids (Zayas, 1997). Due to high solubility of all gelatin samples at pH 1–10, they can be used widely and effectively since solubility is a prerequisite for most functionality of food proteins.

3.6. Gel strength

GAA and GPA extracted with different times had the varying gel strength (Table 1). Gel strength of gelatin extracted decreased as the extraction times increased, regardless of acid pretreatments ($P < 0.05$). At the same extraction time, GPA exhibited the higher gel strength than did GAA ($P < 0.05$). The gel strength varied considerably with the acid treatments used for swelling process from the skin of dover sole (Giménez, Turnay, Lizarbe, Montero, & Gómez-Guillén, 2005). The difference in bloom values might also be governed by molecular weight distribution, as well as by complex interactions determined by the amino acids composition and the ratio of α/β -chains present in the gelatin. For gelatin from the skin of unicorn leatherjacket, it was found that the content of major components including β - and α -chains was not the major factor governing the gel formation of gelatin. The configuration of protein and the way the inter-junction was developed to form the stronger network were crucial for gel formation. Location of imino acids in the peptide chain also contributed to gel formation. Limited hydrolysis, in which the free α -chains and some oligomers could be released, might favor the aggregation or cross-links, mainly via hydrogen bond. In addition, during acidic pretreatment, some phosphate groups might attach with some amino acids, leading to phosphorylation of GPA4 and GPA8. Phosphorylation may introduce the ionic interaction between phosphate groups and $-\text{NH}_3^+$ of amino acids, increasing the cross-links of proteins (Guo, Li, Zhu, & Zhao, 2005). Since, GPA8 had the shorter chain length, the network formed might not be strong, and even some ionic interaction could be enhanced. However, both GAA (GAA4 and GAA8) and GPA (GPA4 and GPA8) had the lower gel strength than did commercial bovine gelatin (293.22 g) ($P < 0.05$) (Table 1). This was most likely governed by the differences in the imino acid content. Mammalian gelatins generally have the high amount of hydroxyproline and proline (Benjakul et al., 2009). Fish gelatins have lower hydroxyproline contents (about 7–10% of the total amino acids) compared with that of bovine gelatin (14%) (Nalinanon, Benjakul, Visessanguan, & Kishimura, 2008). The gelling properties of gelatin were also influenced by the source of raw materials, which vary in proline and hydroxyproline contents (Jongjareonrak et al., 2006). Hydroxyl groups of amino acids, especially hydroxyproline, play a part in inter-chain hydrogen bonding via a bridging water molecule as well as direct hydrogen bonding to the carbonyl group (Wong, 1989).

3.7. Color of gel

Gel color of gelatin extracted from the skin of unicorn leatherjacket with different acid pretreatments and various extraction

times is shown in Table 1. Higher L^* -value of GPA8 gel was observed, compared with GPA4 ($P < 0.05$) (Table 1). On the other hand, L^* -value decreased as GAA was extracted for a longer time ($P < 0.05$). Marked increase in L^* -value of GPA8 was most likely associated with the greater aggregation via $-\text{NH}_3^+$ group during freeze-drying. The larger aggregate exhibited the greater light scattering effect. However, GAA8 with lower chain length showed the low L^* -value, suggesting the clearer solution of gelatin before gelation. Gelatin manufacture generally has a good process to clarify the impurities from the gelatin solution, such as chemical clarification and filtration processes. For redness (a^* -value) and yellowness (b^* -value), GPA8 showed the higher a^* - and b^* -values than did GPA4 ($P < 0.05$). Higher degradation of GPA, especially when extraction time increased, might provide more $-\text{NH}_3^+$ group, which underwent Maillard reaction to a higher extent. Conversely, the decreases in a^* - and b^* -values were found in GAA as the extraction time increased. With acetic acid pretreatment, pigments in the skin were more likely removed to a greater extent, compared with that pretreated with phosphoric acid. Color did not affect functional properties of gelatin (Ockerman & Hansen, 1988).

3.8. Emulsifying properties of gelatin

Emulsion activity index (EAI) and emulsion stability index (ESI) of GAA and GPA from the skin of unicorn leatherjacket are presented in Table 2. EAI and ESI of GPA (GPA4 and GPA8) were higher than GAA (GAA4 and GAA8). For the same gelatin, EAI decreased when the extraction time increased ($P < 0.05$). With increasing degradation, peptides with the shorter chain, which had more hydrophilicity were preferably localized in aqueous phase. As a result, the lower portion of gelatin migrated to oil–water

Table 2

Emulsifying properties of gelatin from the skin of unicorn leatherjacket with different acid pretreatment and extraction times.

Samples	Concentration of gelatin (%)	Emulsion activity index (m^2/g)	Emulsion stability index (min)
GPA4	1	39.17 ± 0.33aA	8.58 ± 0.25bC
	2	20.34 ± 0.09aB	10.35 ± 0.12aB
	3	15.16 ± 0.19bC	39.63 ± 0.17aA
GPA8	1	35.21 ± 0.78cA	12.58 ± 0.64aB
	2	18.87 ± 0.60bB	10.59 ± 0.45aC
	3	16.86 ± 0.43aC	35.81 ± 0.55bA
GAA4	1	37.48 ± 1.38bA	9.98 ± 1.21bB
	2	19.72 ± 0.67abB	10.28 ± 0.21aB
	3	13.49 ± 0.09cC	35.74 ± 0.24bA
GAA8	1	33.63 ± 0.10dA	9.19 ± 0.45bB
	2	16.33 ± 0.41cB	10.38 ± 0.85aB
	3	16.82 ± 0.11aB	13.10 ± 0.65cA

Mean ± SD ($n = 3$). Key: see the caption for Fig. 2.Different letters in the same column within the same concentration indicate significant differences ($P < 0.05$).Different capital letters in the same column within the same gelatin sample indicate significant differences ($P < 0.05$).

Table 3

Foaming properties of gelatin from the skin of unicorn leatherjacket with different acid pretreatment and extraction times.

Samples	Concentration of gelatin (%)	Foam expansion (%)	Foam stability (%)		
			20 min	40 min	60 min
GPA4	1	116.35 ± 0.23bC ^W	111.11 ± 0.12bC ^X	110.0 ± 0.32bC ^Y	108.00 ± 0.51bC ^Z
	2	133.05 ± 0.34bB ^W	130.52 ± 0.25bB ^X	115.15 ± 0.43bB ^Y	111.00 ± 0.54bB ^Z
	3	150.33 ± 0.16aA ^W	132.97 ± 0.24cA ^X	121.05 ± 0.53cA ^Y	119.58 ± 0.67bA ^Z
GPA8	1	126.32 ± 2.23aB ^W	121.11 ± 3.45aC ^{Wx}	118.34 ± 3.40aC ^{Xy}	115.56 ± 2.34aC ^X
	2	147.54 ± 2.12aA ^W	141.13 ± 2.34aB ^X	134.23 ± 2.17aB ^Y	128.98 ± 2.12aB ^Z
	3	144.65 ± 3.45bA ^{Wx}	147.35 ± 0.34aA ^W	143.67 ± 2.34aA ^{Wx}	139.46 ± 3.78aA ^X
GAA4	1	110.34 ± 3.23cC ^W	106.34 ± 2.47cC ^{Wx}	102.34 ± 2.13cC ^{Xy}	98.86 ± 2.23cC ^Y
	2	129.34 ± 2.33cB ^W	115.34 ± 2.34dB ^X	109.34 ± 2.89cB ^Y	107.34 ± 4.57cB ^Y
	3	139.34 ± 4.32bA ^W	124.54 ± 5.62dA ^X	119.45 ± 2.87cA ^{Xy}	115.45 ± 3.45bA ^Y
GAA8	1	118.15 ± 0.22bC ^W	115.07 ± 0.43bC ^X	113.09 ± 0.65bC ^Y	111.05 ± 0.32bC ^Z
	2	128.15 ± 0.29cB ^W	126.04 ± 0.55cB ^X	118.55 ± 0.55bB ^Y	115.09 ± 0.54bB ^Z
	3	152.63 ± 0.31aA ^W	141.41 ± 0.32bA ^X	138.04 ± 0.77bA ^Y	135.06 ± 0.39aA ^Z

Mean ± SD ($n = 3$). Key: see the caption for Fig. 2.Different letters in the same column within the same concentration indicate significant differences ($P < 0.05$).Different capital letters in the same column within the same gelatin sample indicate significant differences ($P < 0.05$).Different superscripts in the same row indicate significant differences ($P < 0.05$).

interface. At a level of 3%, ESI of gelatin extracted for a longer time was lower than those of gelatin with shorter extraction time ($P < 0.05$). The highest ESI of GPA4, GPA8, GAA4 and GAA8 was found at the level of 3% ($P < 0.05$). In case of GAA8, the aggregated proteins might be rigid and could not unfold rapidly at the interface and form a film around an oil droplet effectively. At a level of 3%, emulsions containing GPA4 were more stable than those of the GAA4 ($P < 0.05$). Additionally, GPA8 also exhibited the higher ESI than GAA8 at the level of 3% ($P < 0.05$). However, no difference in ESI was found between GPA8 and GAA8 at the level of 2% ($P > 0.05$). Phosphate groups incorporated into gelatin could stabilize the protein film at the interface via ionic interaction more effectively. GPA4 and GPA8 possesses surface activity at the oil–water interface, thereby having the ability to facilitate the formation and stabilization of fine droplets during and after emulsification. The stabilization of emulsion against coalescence/flocculation is greatly dependent on the force of electrostatic repulsions between the adsorbed proteins on the interfacial protein film. Surh, Decker, and McClements (2006) found that the oil-in-water emulsion prepared with high molecular weight fish gelatin (120 kDa) was more stable than that prepared with low molecular weight fish gelatin (50 kDa). Thickness of an adsorbed gelatin membrane increased with increasing molecular weight. This was associated with the increased stability of emulsions to coalescence during homogenization (Lobo & Svereika, 2003). EAI and ESI of bovine bone gelatin were higher than those of all fish skin gelatin samples (Aewsiri et al., 2009). This possibly resulted from the differences in the intrinsic properties, composition and conformation of gelatins from both sources (Benjakul et al., 2009).

3.9. Foaming properties of gelatin

Foam expansion (FE) and foam stability (FS) of GAA (GAA4 and GAA8) and GPA (GPA4 and GPA8) at different concentrations are shown in Table 3. FE and FS of both gelatins mostly increased with increasing gelatin concentrations ($P < 0.05$). However, for GPA8, no difference in FE was observed between 2 and 3% ($P > 0.05$). Foams with higher concentration of proteins were denser and more stable because of an increase in the thickness of interfacial films (Zayas, 1997). At the concentration lower than 2%, GAA had the lower FE than GPA ($P < 0.05$). GPA with the shorter chains could migrate to the air–water interface more effectively than GAA, having the larger proportion of β - and α -chains. FS increased with protein

concentrations, depending on the protein surface properties. The foaming ability of proteins is related to their film-forming ability at the air–water interface. In general, proteins, which rapidly adsorb at the newly created air–liquid interface during bubbling, unfolding and molecular rearrangement at the interface, exhibit better foaming ability than proteins that adsorb slowly and resist unfolding at the interface (Damodaran, 1997). The stability of foams depends on various parameters, such as the rate of attaining equilibrium surface tension, bulk and surface viscosities, steric stabilization, and electrical repulsion between the two sides of the foam lamella (Liu, Xu, Yuan, & Jiang, 2003). Foam stability of GAA and GPA can be explained by the combined effect of cohesive forces between the molecules in the foam lamella, due to the hydrophobic groups, and the prevention of drainage in the lamella, which was achieved only by the gelatin molecules present in the lamella and anchored into the interface by the long hydrophobic chains. Gravitational drainage of liquid from the lamella and disproportionation of gas bubbles via inter bubble gas diffusion contribute to instability of foams (Aewsiri et al., 2009). Coalescence of bubbles occurs because of liquid drainage from the lamella film as two gas bubbles approach each other, leading to film thinning and rupture (Damodaran, 2005). Thus, acid pretreatments and extraction times also had impact on interfacial properties of gelatin from skin of unicorn leatherjacket.

4. Conclusion

GAA and GPA with different characteristics could be extracted with warm water after acid pretreatment. Phosphoric acid used in swelling process was found to be efficient disrupter of skin tissue, compared to acetic acid. However, the extraction for a longer time could shorten the gelatin chain. Additionally, type of acid for pretreatment also had the impact on functional properties of resulting gelatin. The gel strength of GPA was higher than that of GAA and interfacial properties of both gelatins were somehow affected by extraction time. Therefore, the skin of unicorn leatherjacket could serve as raw material for gelatin extraction after the appropriate acid pretreatment and with proper extraction time.

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Indigenous proteases in the skin of unicorn leatherjacket (*Aluterus monoceros*) and their influence on characteristic and functional properties of gelatin

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ABSTRACT

Indigenous proteases in the skin of unicorn leatherjacket (*Aluterus monoceros*) were characterised using autolytic study. Maximised autolysis was found at pH 7 and 50 °C. Autolysis was markedly inhibited by 0.04 mM soybean trypsin inhibitor (SBTI), suggesting that heat activated serine protease was predominant in the skin. The impact of indigenous proteases on the properties of gelatin extracted from unicorn leatherjacket skin was investigated. Gelatin was extracted from unicorn leatherjacket skin using distilled water at 50 °C for 12 h in the presence and absence of 0.04 mM SBTI. In the presence of SBTI, the degradation was markedly inhibited, but a lower gelatin extraction yield was obtained ($P < 0.05$). Extracted gelatins contained α_1 and α_2 chains as the predominant components with some degradation peptides. FTIR spectra indicated a greater loss of molecular order of the triple helix and a higher degradation was found in gelatin extracted in the absence of 0.04 mM SBTI. The net charge of gelatin samples extracted with and without 0.04 mM SBTI became zero at pHs of 8.45 and 7.31, respectively, as determined by ζ -potential titration. Higher gel strength (320.68 ± 3.02 g) was obtained in gelatin extracted with SBTI, compared with that of gelatin extracted without SBTI (288.63 ± 1.44 g). High emulsifying activity index but lower emulsifying stability index was observed in the former. Therefore, heat-activated serine protease was involved in the degradation of gelatin molecules, thereby affecting the yield, proteinaceous components and properties of gelatin from unicorn leatherjacket skin.

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1. Introduction

Proteolysis induced by heat-activated and heat-stable indigenous proteases associated with skin matrix can contribute to the destabilisation as well as disintegration of collagen structure by disrupting the intra- and intermolecular cross-links (Wu et al., 2008). Collagenases have the unique ability to degrade the major structural extracellular matrix proteins, especially the triple helical strand of type I and type II collagens at Gly⁷⁷⁵-Leu (Ile)⁷⁷⁶. After these initial and specific cleavages, $\frac{3}{4}$ - and $\frac{1}{4}$ -cleavage products are formed, and collagen is spontaneously denatured (helix-to-coil transition) to gelatin (Miller et al., 1976). Collagenases are classified into two major groups, metallocollagenases and serine collagenases (Aoki, Ahsan, Matsuo, Hagiwara, & Watabe, 2003). Furthermore, non-collagenase proteinases can cleave the collagen molecule in the telopeptide region and contribute to hydrolysis of the collagen molecule by disrupting the regions, in which intermolecular cross-links are formed (Bornstein & Traus, 1979).

Heat-activated serine protease in bigeye snapper skin was involved in the drastic degradation of the β - and α -chains of the gelatin extracted at 60 °C (Intarasirisawat et al., 2007). These enzymes are bound with matrix components such as collagens (Woessner, 1991). The proteolytic degradation of high molecular weight components caused by indigenous proteases during extraction of gelatin at high temperature resulted in adverse effects on gel-forming properties of resulting gelatin (Intarasirisawat et al., 2007). The proteolytic breakdown of collagen structure is most likely related to the disintegration of connective tissues, which has been implicated in quality deterioration of products. Gelatin structures with large amount of high molecular weight components, like γ -, β , and α -chains, have been known to possess the maximal functional properties including gelation (Kittiphattanabawon, Benjakul, Visessanguan, & Shahidi, 2010), film forming ability (Hoque, Benjakul, & Prodpran, 2010), etc. Therefore, it was necessary to produce the gelatin with negligible hydrolysis of peptides for further applications in pharmacy, medicine and food industry. Hence, the use of an appropriate protease inhibitor can be an effective means to obtain the gelatin with limited or negligible hydrolysis of peptides. Nevertheless, no information regarding the characteristics of indigenous proteases in the skin of unicorn

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leatherjacket (*Alutherus monoceros*) and their role in properties of gelatin extracted has been reported. Unicorn leatherjacket is an economically important species commonly used for frozen fillet production. Thick skin of this species, generated during deskinning process, can serve as the promising raw material for gelatin extraction. Therefore, the objectives of this investigation were to study the autolysis of skin from unicorn leatherjacket and to elucidate their impact on gelatin extraction and characteristics of resulting gelatin.

2. Materials and methods

2.1. Chemicals

2,4,6-Trinitrobenzenesulfonic acid (TNBS), sodium sulphite, L-leucine, bovine serum albumin, ethylenediaminetetraacetic acid (EDTA), 1,10-phenanthroline monohydrate, ethylene-bis (oxyethylenitrilo) tetraacetic acid (EGTA), *trans*-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E-64), iodoacetic acid, pepstatin A, phenylmethanesulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), β -mercaptoethanol (β -ME) and type I collagen from calf skin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Folin–Ciocalteu's phenol reagent and phosphoric acid were purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS), *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) and Coomassie blue R-250 were procured from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals were of analytical grade.

2.2. Collection and preparation of fish skin

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

2.3. Pretreatment of unicorn leatherjacket skin

Prepared skin was pretreated following the method of Ahmad and Benjakul (2010a), with a slight modification. To remove non-collagenous proteins, the prepared skin was mixed with 0.1 M NaOH at a skin/alkali solution ratio of 1:20 (w/v). The mixture was stirred for 6 h at 4 °C using an overhead mechanical stirrer (W20.n, IKA®-Werke GmbH & CO.KG, Stanfen, Germany) at a speed of 300 rpm. The alkali solution was changed every 2 h. The samples were then washed with iced tap water until neutral or faintly basic pH was obtained. Thereafter, the prepared skin was swollen by mixing the skins with 0.2 M phosphoric acid at a ratio of 1:10 (w/v). The mixture was stirred for 24 h at 4 °C. Finally, the swollen skin was washed thoroughly with iced tap water until neutral or faintly acidic pH of wash water was obtained. The pretreated skin was stored at –20 °C until use.

2.4. Autolysis study of pretreated unicorn leatherjacket skin

Prior to study, the frozen pretreated skin was powderised in liquid nitrogen using a blender (Model MX-T2GN, National, Taipei, Taiwan). The powder obtained was used for autolysis study.

2.4.1. Temperature profile

Autolytic activity was assayed according to the method of Morrissey, Wu, Lin, and An (1993), with a slight modification. Pretreated skin powder (3 g) was added to 9 ml of McIlvaine's buffer

(0.2 M Na-phosphate and 0.1 M Na-citrate), pH 7.0. The mixture was homogenised for 2 min at a speed of 11,000 rpm using a homogeniser (Model T25 basic, IKA, Labortechnik, Selangor, Malaysia). The resulting homogenate was incubated at different temperatures (40, 45, 50, 55, 60, 70, 80 and 90 °C) for 30 min in a temperature-controlled water bath (Mettler, Schwabach, Germany). Autolytic reaction was terminated by addition of 18 ml of 10% SDS (85 °C). The reaction mixture was further incubated at 85 °C for 1 h in a temperature-controlled water bath. The supernatant was obtained by centrifuging at 8500g for 15 min using a microcentrifuge (Model MIKRO20, Hettich Zentrifugen, Germany) and was subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis. Samples were also determined for free amino group content.

2.4.2. pH profile

Autolytic activity of pretreated skin was determined at various pHs using different buffers at 50 °C for 30 min. Buffers used included 50 mM maleate buffer for pH 1; McIlvaine's buffer (0.2 M Na-phosphate and 0.1 M Na-citrate) for pHs 2–6, 50 mM Tris–HCl buffer for pHs 7–9 and 0.1 M Na_2HPO_4 – $\text{Na}_2\text{B}_4\text{O}_7$ buffer for pH 10. After the incubation at 50 °C for 30 min, the autolysis was terminated and was monitored in the same manner as the temperature profile study.

2.4.3. Inhibitor study

Pretreated skin powder (0.5 g) was homogenised with 1.5 ml of McIlvaine's buffer (pH 7) at a speed of 11,000 rpm for 2 min using a homogeniser. The homogenate was mixed with 2 ml of various protease inhibitors to obtain the different final concentrations. Those inhibitors included EDTA (10 mM), EGTA (10 mM), iodoacetic acid (10 mM), E-64 (1 mM), pepstatin A (1 mM) and 1,10-phenanthroline (10 mM), PMSF (1 mM) and SBTI (0.04 mM). The mixtures were allowed to stand in ice for 2 h, followed by incubation at optimal temperature (50 °C) for 30 min. The reaction was terminated by addition of 2 ml of 10% (w/v) SDS (85 °C). After complete solubilisation, the autolytic pattern was then analysed by using SDS–PAGE. The control was performed in the same manner, but deionised water was added instead of protease inhibitor solution.

2.4.4. Determination of free amino group content

Free amino group content was determined following the method of Benjakul and Morrissey (1997). Properly diluted samples (125 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 at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulphite. The mixtures were cooled down at room temperature for 15 min. The absorbance was measured at 420 nm using a double beam spectrophotometer (Model UV-1800, Shimadzu, Kyoto, Japan) and free amino group content was expressed in terms of L-leucine.

2.4.5. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein patterns were determined using SDS–PAGE according to the method of Laemmli (1970) using a 4% stacking gel and a 7.5% separating gel. Samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol). Samples (15 μg) were loaded onto the gel. After electrophoresis using 15 mA/gel (Mini Protein II, Bio-Rad, Hercules, CA, USA), the gel was stained with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. Type I collagen was used as the standard.

2.5. Study on the impact of indigenous proteases on extraction and characteristics of gelatin from unicorn leatherjacket skin

To inhibit indigenous serine proteases, the pretreated skin was mixed with 0.04 mM SBTI solution at a pretreated skin/SBTI solution ratio of 1:5 (w/v). The mixture was stirred for 24 h at 4 °C using an overhead mechanical stirrer at a speed of 150 rpm. Then, the mixtures in the absence and presence of 0.04 mM SBTI were incubated at 50 °C in a temperature-controlled water bath for 12 h with continuous stirring to extract the gelatin. The mixtures were then filtered using two layers of cheesecloth. The filtrates were further filtered using a Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England) with the aid of an electric aspirator, (Model VE-11, JEIO TECH, Korea). The resultant filtrate was freeze-dried using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark). Gelatins extracted in the absence and presence of 0.04 mM SBTI were referred to as GAI and GPI, respectively. Both gelatin samples were subjected to SDS-PAGE as previously described and further analyses were also conducted as follows:

2.5.1. Fourier transform infrared spectroscopy (FTIR)

FTIR analysis was performed as per the method of Ahmad, Benjakul, and Nalinanon (2010). A Bruker Model EQUINOX 55 FTIR spectrometer (Bruker, Ettlingen, Germany) equipped with a deuterated L-alanine triglycine sulphate (DLATGS) detector was used. The horizontal attenuated total reflectance (HATR) accessory was mounted into the sample compartment. The internal reflection crystal (Pike Technologies, Madison, WI, USA), made of zinc selenide, had a 45° angle of incidence to the IR beam. Spectra were acquired at a resolution of 4 cm⁻¹ and the measurement range was 4000–650 cm⁻¹ (mid-IR region) at room temperature. Automatic signals were collected in 32 scans at a resolution of 4 cm⁻¹ and were normalised against a background spectrum recorded from the clean, empty cell at 25 °C. Analysis of spectral data was carried out using the OPUS 3.0 data collection software programme (Bruker, Ettlingen, Germany).

2.5.2. ζ-Potential analysis

Gelatin samples were dissolved in distilled water at a concentration of 0.5 mg/ml. The mixtures were stirred at room temperature for 6 h. The ζ-potential of each sample (20 ml) was measured using a ζ-potential analyser (ZetaPALS, Brookhaven Instruments Co., Holtsville, NY, USA). ζ-Potential of samples, adjusted to different pHs with 1.0 M nitric acid or 1.0 M KOH using an autotitrator (BI-ZTU, Brookhaven Instruments Co., Holtsville, New York, USA), was determined. The pI was estimated from pH rendering ζ-potential of zero.

2.5.3. Determination of gel strength

Gels of gelatin were prepared by the method of Fernandez-Daz, Montero, and Gómez-Guillén (2001), with a slight modification. Gelatin samples were dissolved in distilled water (60 °C) to obtain the final concentration of 6.67% (w/v). The solution was stirred until the gelatin was solubilised completely, followed by incubating the samples in a refrigerator at 10 °C for 18 h for gel maturation. The dimensions of the sample were 3 cm in diameter and 2.5 cm in height. The gel strength of the samples prepared at 10 °C was determined using a Model TA-XT2 Texture Analyser (Stable Micro System, Surrey, UK) with a load cell of 5 kN and equipped with a 1.27 cm diameter flat-faced cylindrical Teflon® plunger. The maximum force (in grams) was recorded when the penetration distance reached 4 mm. The speed of the plunger was 0.5 mm/s.

2.5.4. Determination of gel microstructure

Microstructures of gelatin gels were visualised using a scanning electron microscope (SEM). Gelatin gels having a thickness of 2–3 mm were fixed with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 12 h. The samples were then rinsed with distilled water for 1 h and dehydrated in ethanol with a serial concentration of 50%, 70%, 80%, 90% and 100% (v/v). Dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA). The specimens were observed with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 15 kV.

2.5.5. Determination of emulsifying properties

Emulsion activity index (EAI) and emulsion stability index (ESI) of gelatin samples were determined according to the method of Pearce and Kinsella (1978) as modified by Aewsiri, Benjakul, and Visessanguan (2009). Soy bean oil (2 ml) and gelatin solution (1% gelatin, 6 ml) were homogenised using a homogeniser at a speed of 20,000 rpm for 1 min. Emulsions were pipetted out at 0 and 10 min and 100-fold diluted with 0.1% SDS. The mixture was mixed thoroughly for 10 s using a vortex mixer (Scientific Industries, Inc., Bohemia, NY, USA). A_{500} of the resulting dispersion was measured using a spectrophotometer. EAI and ESI were calculated by the following equation:

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

where $A = A_{500}$, DF = dilution factor (100), l = path length of cuvette (m), θ = oil volume fraction and C = protein concentration in aqueous phase (g/m³)

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

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

2.6. Statistical analyses

All experiments were performed in triplicate and a completely randomised design (CRD) was used. Data were presented as means ± standard deviation and a probability value <0.05 was considered significant. Analysis of variance (ANOVA) was performed and the mean comparisons were done by *T*-test. Analysis was performed using SPSS 11.0 for Windows (SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Effect of temperature and pH on autolysis of pretreated skin

The autolytic degradation of pretreated skin at different temperatures, as monitored by free amino group content, is shown in Fig. 1a. The maximal autolytic activity of pretreated skin was observed at 50 °C as indicated by the highest free amino group content ($P < 0.05$). However, a decrease in autolytic activity was found when the temperature was above 50 °C, suggesting the thermal denaturation of the indigenous proteases. When the protein patterns of pretreated skin, autolysed at different temperatures, were compared, the highest degradation of β-chain and α-chains was obtained at 50 °C (Fig. 1c). This was concomitant with the formation of degradation products with low molecular weight appearing at the dye front. Furthermore, the high molecular weight cross-linked proteins were also degraded at 50–70 °C. β- and α-chains constituted as the major proteins in the collagen from unicorn leatherjacket skin (Ahmad & Benjakul, 2010a). The results suggested that the proteases still remained within the skin after pretreatment with alkali and acid for non-collagenous protein removal and swelling processes, respectively. Those heat-activated

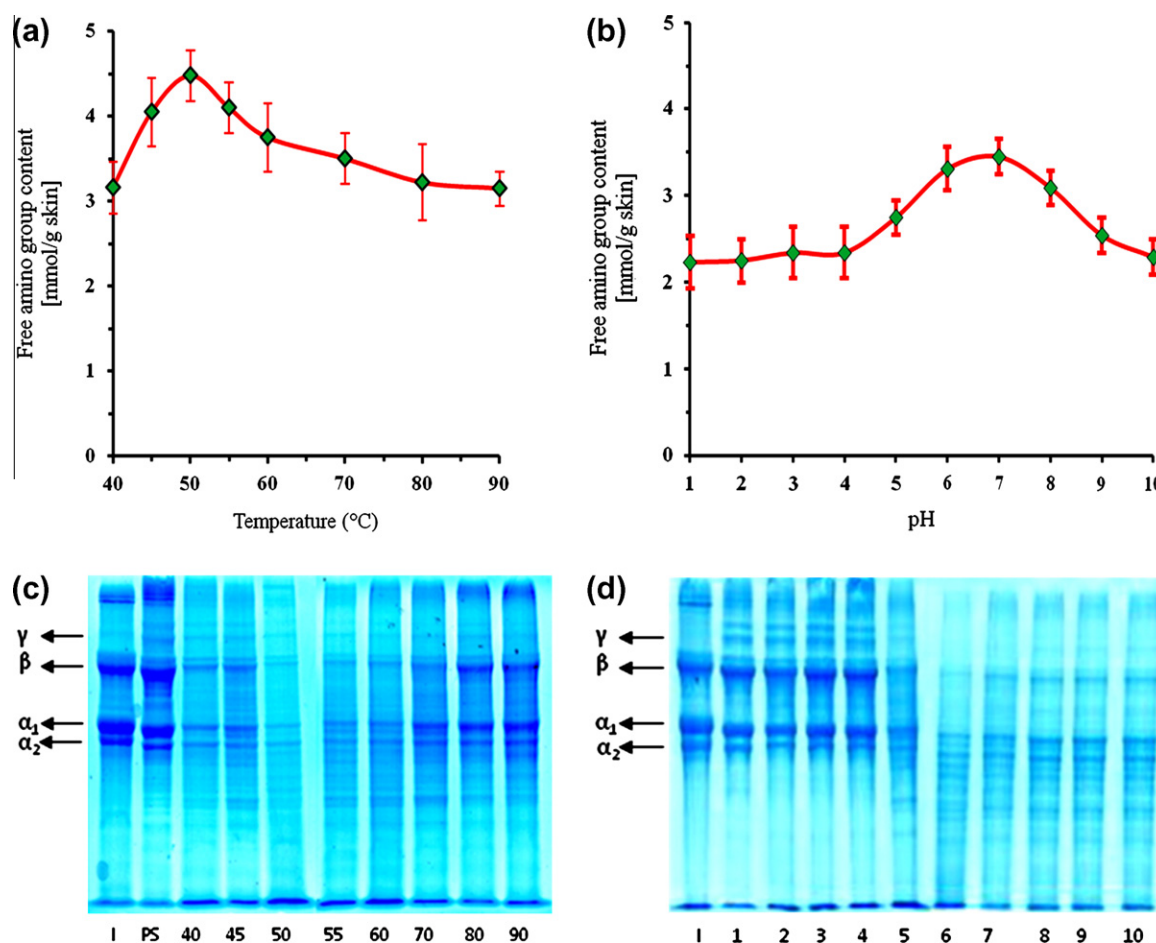


Fig. 1. pH and temperature profiles for autolysis of pretreated skin from unicorn leatherjacket. For the temperature profile, autolysis was performed at pH 7 for 30 min at different temperatures. For the pH profile, autolysis was conducted at 50 °C for 30 min at different pHs. For Fig. 1c and 1d; I: type I collagen from calf skin. For Fig. 1c; PS: pretreated skin. Numbers denote the temperatures (°C). For Fig. 1d; Numbers denote the pHs. Bars represent the standard deviation ($n = 3$).

proteases were able to cleave γ -, β - and α -chains most effectively at 50 °C as shown in Fig. 1c. Thus, those proteases more likely contributed to the disintegration of gelatin molecules during the extraction process, which is normally conducted at high temperature. Pretreatment removed not only the proteases to some extent, but also some metallic ions or co-factors required for proteolytic activity of bigeye snapper skin (Intarasirisawat et al., 2007). In the present study, pretreatment could lower the autolysis to some degree, compared with the skin without pretreatment (data not shown). Nevertheless, the remaining proteases were shown to be involved in hydrolysis of collagenous components in the skin.

The pH profile for pretreated skin autolysis at 50 °C is shown in Fig. 1b. The highest free amino group content was obtained at pH 7. γ -, β - and α -chains were almost degraded at pHs 6–7 with a concomitant formation of low-molecular-weight peptides (Fig. 1d). The results indicated that the neutral proteases were dominant in the pretreated unicorn leatherjacket skin, causing the hydrolysis of collagen molecules. At very acidic or alkaline pHs, the degradation was markedly decreased as evidenced by the low free amino group content and the greater band intensity of β - and α -chains retained. This might be due to the inactivation of the indigenous protease under these conditions due to the conformational changes or ionisation of R-groups of amino acids, especially at the active site of proteases. Although collagen could be solubilised in acidic pH range (Ahmad & Benjakul, 2010a), the degradation was negligible in such a range, especially pH 1–4. Despite the presence of soluble substrates, no hydrolysis was noticeable. This reconfirmed the

inactivation of indigenous protease in an acidic pH range. On the other hand, the pronounced degradation was noticeable at neutral pH, which is commonly used for gelatin extraction.

3.2. Inhibitor study towards autolysis of pretreated skin

The effect of various inhibitors on autolysis of unicorn leatherjacket pretreated skin is depicted in Fig. 2. Among all protease inhibitors tested, 0.04 mM SBTI and 1 mM PMSF showed the highest inhibition towards autolysis when tested at pH 7, whilst other inhibitors, including EDTA, EGTA, E-64, iodoacetic acid, pepstatin A and 1,10-phenanthroline, had no inhibitory effect on autolysis. γ -, β - and α -chains of pretreated skin were mostly retained in the presence of PMSF, whilst SBTI was found to be the most effective inhibitor towards the degradation of those components. PMSF irreversibly inhibits serine proteases by sulphonylation of the serine residue in the active site of the protease (James, 1978). The active site in each serine protease includes a serine residue, a histidine residue, and an aspartate residue (Johnson, Roderick, & Cook, 2005). Therefore, it can be inferred that a serine protease was the major protease associated with unicorn leatherjacket pretreated skin. In the absence of inhibitors, the pronounced hydrolysis was noticeable as evidenced by the marked decrease in the band intensity of γ -, β - and α -chains. Intarasirisawat et al. (2007) reported that serine protease was the major enzyme causing the autolysis of skin from bigeye snapper.

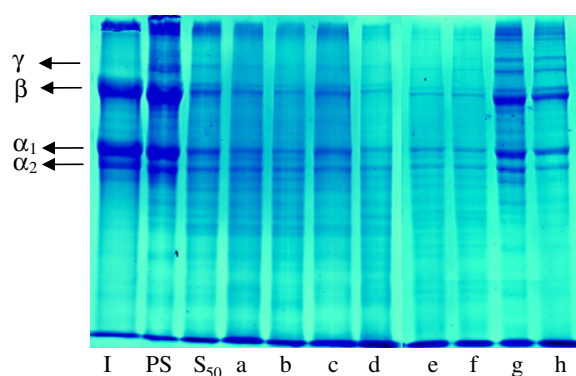


Fig. 2. SDS–PAGE pattern of pretreated skin from the unicorn leatherjacket incubated in the presence of various inhibitors: (a) 10 mM EDTA; (b) 10 mM EGTA; (c) 1 mM E-64; (d) 10 mM iodoacetic acid; (e) 10 mM 1,10-phenanthroline; (f) 1 mM pepstatin A; (g) 1 mM PMSF; (h) 0.04 mM SBTI ; I: collagen type I from calf skin. PS: pretreated skin and S_{50} : control (without protease inhibitors). The autolysis was performed at pH 7.0 and 50 °C for 30 min.

3.3. Yield and characteristics of gelatin from pretreated skin as affected by protease inhibitor

3.3.1. Yield

Yields of gelatin extracted from pretreated skin in the absence (GAI) and in the presence of 0.04 mM SBTI (GPI) at 50 °C for 12 h are 7.34% and 5.29% (wet weight basis), respectively. When SBTI was incorporated during the extraction, a decrease in the yield was obtained. SBTI most likely inhibited the activity of indigenous serine proteases associated with the skin matrix, thereby preventing the cleavage of peptide chains. This in turn retained the high molecular weight components in the skin matrix, leading to a lower amount of extractable gelatin. The degradation in pretreated skin without SBTI addition, particularly at temperature close to the optimal temperature (50 °C), might result in the formation of shorter peptides as well as loosening of the skin matrix. This possibly resulted in the ease of gelatin extraction. During heating at 50 °C, hydrogen bonds stabilising triple-helix of mother collagen were destroyed, leading to helix-to-coil transition (Wong, 1989). This resulted in the conversion of collagen to soluble gelatin. Therefore, the addition of protease inhibitor was shown to lower the extraction yield of gelatin from unicorn leatherjacket skin.

3.3.2. Protein pattern

Based on protein pattern, GAI contained the lower band intensity of β - and α -chains, in comparison with that found in the pretreated skin and GPI (Fig. 3). The β -chain was almost completely degraded in GAI, whereas, α -chains were retained to some extent. The result suggested that α -chains and cross-linked components of GPI were retained more when SBTI was incorporated during extraction at high temperature. With the extended extraction time (12 h), the remaining collagenolytic protease associated with skin might hydrolyse the collagen molecules progressively in GAI, especially at the optimal temperature (50 °C). In addition, proteins with molecular weights lower than α -chains (100 kDa) were also observed in both GAI and GPI, suggesting the degradation of α , β - and γ -components during gelatin extraction caused by thermal hydrolysis or the proteolysis mediated by the remaining indigenous proteases. The result was in agreement with Muyonga, Cole, and Duodu (2004) who reported that gelatin extracted at high temperature contained more peptides with a molecular weight less than the α -chains and a lower proportion of component having a molecular weight greater than the β -chain. The degradation of gelatin from bigeye snapper skin caused by some proteases during extraction process was also reported (Jongjareonrak, Benjakul,

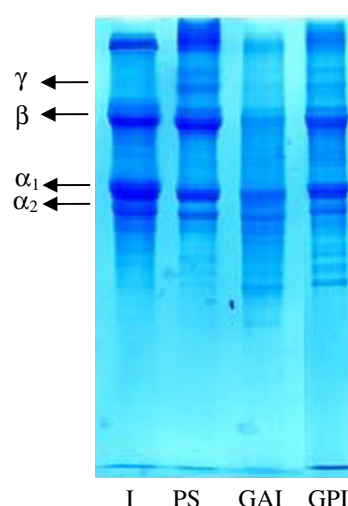


Fig. 3. SDS–PAGE pattern of gelatin extracted from the skin of unicorn leatherjacket in the absence (GAI) and in the presence of 0.04 mM of SBTI (GPI). I: collagen type I from calf skin. PS: pretreated skin.

Visessanguan, Prodpran, & Tanaka, 2006). The formation of degradation peptides is associated with the low viscosity, low melting point, low setting point, high setting time, as well as decreased bloom strength of gelatin (Muyonga et al., 2004). Therefore, the addition of SBTI effectively prevented the degradation of β - and α -chains in gelatin from unicorn leatherjacket skin.

3.3.3. FTIR spectra

FTIR spectra of GAI and GPI from the skin of unicorn leatherjacket are depicted in Fig. 4a. Spectra of both gelatins had the major peaks in the amide region, but showed slight differences in the spectra. These spectra were in accordance with those reported by Muyonga et al. (2004). GAI and GPI displayed the amide-I bands at the wavenumbers of 1635.89 and 1630.82 cm^{-1} , respectively. The amide-I vibration mode resulted primarily from a C=O stretching vibration coupled to contributions from the CN stretch, CCN deformation and in-plane NH bending modes (Bandeekar, 1992). The absorption peak at amide-I was characteristic for the coil structure of gelatin (Yakimets et al., 2005). The amide-I peak of GAI appeared at a higher wavenumber, compared to that of GPI. This indicated the greater loss of triple helix and enhanced hydrolysis of collagen caused by heat-activated indigenous proteases in the skin matrix. In addition, GPI showed the shift to a lower wavenumber and lower amplitude for the amide-I peak, compared to GAI. This was probably associated with the higher extent of molecular order due to interaction of C=O with adjacent chains via hydrogen bonds. This indicated that GPI retained more intermolecular cross-links during extraction at high temperature in the presence of SBTI. In the absence of SBTI, the triple helical structure, held by intermolecular hydrogen bonds, was destroyed to the higher extent. The result was in agreement with the higher degradation of GAI, in comparison with GPI (Fig. 3).

The characteristic absorption bands of GAI and GPI in the amide-II region were noticeable at the wavenumbers of 1545.82 and 1533.77 cm^{-1} , respectively. The amide-II vibration modes are attributed to an out-of-plane combination of CN stretch and in-plane N–H deformation modes of the peptide group (Bandeekar, 1992). A shift to a lower wavenumber and lower amplitude were found in GPI, compared to GAI, indicating that N–H was more involved in bonding with the adjacent molecules probably via H-bonding. In addition, amide-III was detected around the wavenumbers of 1236.53 and 1233.74 cm^{-1} for GAI and GPI, respectively.

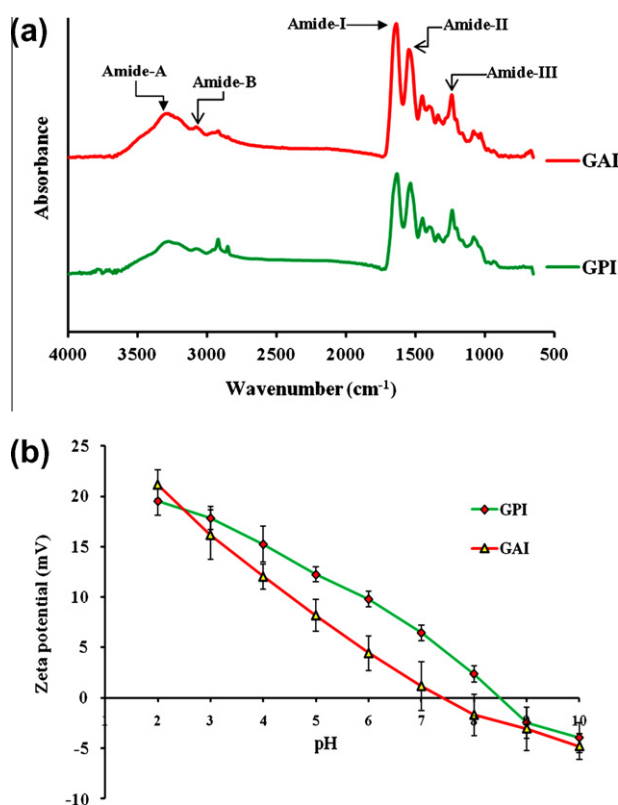


Fig. 4. Fourier transform infrared spectra (a) and ζ -potential (b) at different pH of gelatin extracted from the skin of unicorn leatherjacket in the absence (GAI) and presence of 0.04 mM of SBTI (GPI). For Fig. 4b: Bars represent standard deviation ($n = 3$).

The amide-III represented combination peaks between C–N stretching vibrations and N–H deformation from amide linkages, as well as absorptions arising from wagging vibrations from CH_2 groups of the glycine backbone and proline side-chains (Jackson, Choo, Watson, Halliday, & Mantsch, 1995). The results suggested a disordered molecular structure for both GAI and GPI, probably due to the transformation of α -helical to random coil structure during heating. Those changes were associated with the loss of the triple helix state as a result of denaturation of collagen to gelatin (Muyonga et al., 2004). In addition, absorption bands in GAI and GPI around the wavenumbers of 1080.57 and 1079.13 cm^{-1} , respectively, most likely arose from asymmetric stretching vibrations of phosphate groups of phosphorylated proteins coupled to $-\text{CH}_2$ of the amino acid residues (Jackson et al., 1995). In the present study, phosphoric acid was used for pretreatment of the skin, in which some phosphates were attached to gelatin molecules (Ahmad & Benjakul, 2010b).

The amide-A band, arising from the stretching vibrations of the N–H group, appeared at 3275.77 and 3276.24 cm^{-1} for GAI and GPI, respectively. Normally, a free N–H stretching vibration is found in the range of 3400–3440 cm^{-1} (Muyonga et al., 2004). When the N–H group of a peptide is involved in a hydrogen bond, the position is shifted to lower frequencies (Doyle, Blout, & Bendit, 1975). At the amide-A region, a slightly higher wavenumber was found in GPI, compared to GAI. However, a higher amplitude was found in GAI at the amide-A region, indicating the presence of N–H group of shorter peptide fragments. The amide-B was observed at 3075.29 and 3074.28 cm^{-1} for GAI and GPI, respectively, corresponding to an asymmetric stretch vibration of $=\text{C}-\text{H}$ and $-\text{NH}_3^+$ (Ahmad & Benjakul, 2010b). GPI showed the lower wavenumber of amide-B peak, suggesting the interaction of $-\text{NH}_3$ group between peptide chains. Peaks at the wavenumber of 2849.24

(symmetrical) and 2918.31 cm^{-1} (asymmetrical), representing C–H stretching vibrations of the $-\text{CH}_2$ groups, were found in both GAI and GPI spectra. However, the difference in the spectra between both samples in the region was probably due to the coupling of SBTI with gelatin (Chen et al., 2008; D'Souza, Devi, Shridhar, & Naik, 2008). Thus, it can be concluded that the secondary structure of gelatins obtained from the skin of unicorn leatherjacket was affected by the addition of SBTI during extraction process.

3.3.4. ζ -Potential

Zeta-potential (ζ) representing the surface charge of GAI and GPI at different pH is shown in Fig. 4b. A net charge of zero was obtained at pH 7.31 and 8.45, for GAI and GPI, respectively. At a pH near the isoelectric point, both GAI and GPI had decreased repulsion with increased interaction among gelatin molecules. Protein molecules in an aqueous system have zero net charge at their isoelectric points (pI), in which the positive charges are balanced out by the negative charges (Bonner, 2007). It was found that the ζ -potential was higher for GPI in the pH range of 4–8. The differences in ζ -potential, as well as pI between both gelatins, were most likely due to the differences in the amino acid compositions. Indigenous proteases present in the skin might enhance the extraction efficiency, in which a larger content of several peptides could be released. On the other hand, only a small amount of peptides, mainly those with higher MW, was extracted in the presence of SBTI. The result suggested that indigenous proteases involved in gelatin extraction had an impact on pI of the resulting gelatin to some degree.

3.3.5. Gel strength

The gel strength of GAI and GPI is shown in Table 1. GPI had a higher gel strength than GAI ($P < 0.05$). The difference in gel strength might be governed by molecular weight distribution, as well as the aggregation between gelatin molecules. Low molecular weight peptides of GAI might not form the inter-junction zone effectively, leading to a lower aggregation of gelatin needed to form strong gel network. The quality of gelatin is generally determined by the gel strength or bloom value, including low (<150), medium (150–220) and high bloom (220–300) (Johnston-Bank, 1983). The configuration of polypeptide chains and the way in which the inter-junction was developed to form a stronger network were both crucial for gel formation (Ahmad & Benjakul, 2010b). The gelation of gelatin is considered to be due to a partial recovery of the triple helical structure in gelatin, in which regions rich in Gly-Pro-Hyp are of crucial importance for the stabilisation of the triple helices. In addition, acidic pretreatment also played a role in the determination of gel strength. Phosphate groups used for pretreatment might attach with some amino acids, leading to phosphorylation of both GAI and GPI (Ahmad & Benjakul, 2010b). Phosphorylation may introduce the ionic interaction between phosphate groups and $-\text{NH}_3^+$ of amino acids, thereby increasing the aggregation (Guo, Li, Zhu, & Zhao, 2005). It was noted that

Table 1

Gel strength, colour and emulsifying properties of gelatin extracted from the skin of unicorn leatherjacket in the absence (GAI) and presence of 0.04 mM of SBTI (GPI).

	GAI	GPI
Gel strength (g)	288.63 $\pm$ 1.44b	320.68 $\pm$ 3.02a
EAI (m^2/g)	30.21 $\pm$ 0.86b	34.32 $\pm$ 0.12a
ESI (min)	22.62 $\pm$ 0.38b	18.91 $\pm$ 1.10b
L^*	28.92 $\pm$ 1.28a	32.54 $\pm$ 2.20a
a^*	0.83 $\pm$ 0.02b	1.43 $\pm$ 0.11a
b^*	−0.20 $\pm$ 0.04b	0.81 $\pm$ 0.15a

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

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

GPI had a higher gel strength than commercial bovine gelatin (293.22 g) ($P < 0.05$) (Ahmad & Benjakul, 2010b). In general, fish gelatins have lower hydroxyproline content (about 7–10% of the total amino acids), compared with that of bovine gelatin (14%) (Nalinanon, Benjakul, Visessanguan, & Kishimura, 2008). The gelling properties of gelatin were also influenced by the source of raw materials, which vary in proline and hydroxyproline contents (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2006). Therefore, the use of SBTI, as a protease inhibitor during the gelatin extraction, markedly improved the gel strength of extracted gelatin. This was mainly due to the maintenance of chain length, which was a prerequisite for better gelation.

3.3.6. Gel colour

The colour of gel from both GAI and GPI is shown in Table 1. A higher L^* -value of GPI gel was observed, compared with GAI ($P < 0.05$). The higher L^* -value of GPI was most likely associated with the greater interaction of higher molecular weight peptides,

in which the larger aggregates were formed. A larger aggregate more likely exhibited a greater light scattering effect. However, GPI had a higher b^* -value (yellowness) and a^* -value (redness) than those of GAI. Gelatin manufactures generally have a good method to clarify the impurities from the gelatin solution, such as chemical clarification and filtration processes. Generally, the colour did not affect functional properties of gelatin (Ockerman & Hansen, 1988).

3.3.7. Microstructure of gel

The microstructures of GAI and GPI gels are illustrated in Fig. 5. Both GAI and GPI gels were sponge or coral-like in structure. GPI gel had a denser and more irregular network of interconnected fibrous structure with smaller voids, compared with GAI gel. Larger voids were found in GAI and were possibly correlated with low aggregation of peptide chains during gelation. Generally, the arrangement and association of protein molecules in the gel matrix directly contributed to the gel strength of the gelatin (Benjakul, Oungbho, Visessanguan, Thiansilakul, & Roytrakul, 2009). The network of GAI gel with fewer inter-junctions might be disrupted easily by an applied force, resulting in lower gel strength. Therefore, the use of SBTI had an impact on the network of gelatin gel, in which chain length of peptide molecules were retained, and interaction or aggregation could take place more effectively.

3.3.8. Emulsifying properties

Emulsion activity index (EAI) and emulsion stability index (ESI) of GAI and GPI are presented in Table 1. At a gelatin concentration of 1%, EAI of GPI was higher than GAI ($P < 0.05$). Conversely, ESI of GAI was higher than GPI ($P < 0.05$). This indicated that there was a difference in emulsifying properties between GAI and GPI. GAI contained a higher content of low molecular weight peptides, which had more hydrophilicity and preferably localised in the aqueous phase. As a result, the lower portion of gelatin migrated to the oil–water interface, leading to the low emulsifying property. Nevertheless, emulsion containing GPI was less stable than that with GAI ($P < 0.05$). Oil-in-water emulsion, prepared with high molecular weight fish gelatin (120 kDa) was more stable than that prepared with low molecular weight fish gelatin (50 kDa). The thickness of adsorbed gelatin film increased with increasing molecular weight (Ahmad & Benjakul, 2010b). This was associated with the increased stability of emulsions to coalescence during homogenisation (Lobo & Svereika, 2003). In the present study, bulky α -, β - and γ -chains prevalent in GPI could not align themselves properly, where a strong film was formed effectively around an oil droplet. Additionally, GAI with higher degradation might have more charged groups, especially amino or carboxyl groups at the end of peptides, thereby having the ability to facilitate the stabilisation of emulsion via electrostatic repulsion. The stabilisation of emulsion against coalescence/flocculation is greatly dependent on the force of electrostatic repulsions between the adsorbed proteins on the interfacial protein film (Aewsiri et al., 2009). Differences in the intrinsic properties, composition and conformation of gelatins determined the functional properties of gelatin from the skin of two species of bigeye snapper (Benjakul et al., 2009).

4. Conclusion

Serine protease was the major enzyme in pretreated skin from unicorn leatherjacket and was involved in the drastic degradation of collagen/gelatin at high temperature, used for gelatin extraction. The addition of 0.04 mM SBTI during extraction could retain the β - and α -chains in resulting gelatin via inhibition of indigenous proteases, in which the gel strength and emulsifying activity were increased. However, it is important to note that the extraction yield was lowered when SBTI was incorporated.

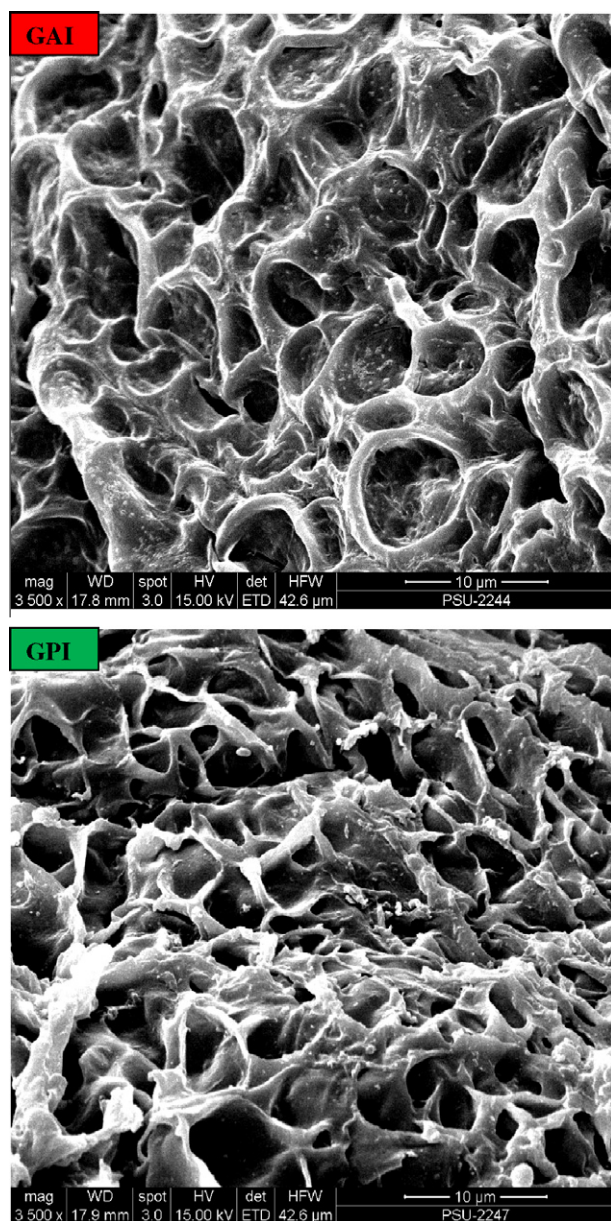


Fig. 5. SEM micrographs of gel from gelatin extracted from the skin of unicorn leatherjacket in the absence (GAI) and presence of 0.04 mM of SBTI (GPI). Magnification: 3500 $\times$.

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Chemical compositions and characterisation of skin gelatin from farmed giant catfish (*Pangasianodon gigas*)

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ABSTRACT

Gelatin was extracted from the skin of farmed giant catfish (*Pangasianodon gigas*) with a yield of 20.1 g/100 g skin sample on the basis of wet weight. The chemical composition and properties of gelatin were characterised. The gelatin had high protein (89.1 g/100 g) but low fat (0.75 g/100 g) content and contained a high number of imino acids (proline and hydroxyproline) (211 residues per 1000 residues). Giant catfish skin gelatin had a slightly different amino acid composition than calf skin gelatin. The bloom strength of the gelatin gel from giant catfish skin gelatin (153 g) was greater than that of calf skin gelatin (135 g) ($P < 0.05$). Viscosity, foam capacity and foam stability of gelatin from giant catfish skins were in general greater than those of the gelatin from calf skin tested. SDS-PAGE of giant catfish skin gelatin showed a high band intensity for the major protein components, especially, α -, β - and γ -components and was similar to that of standard calf skin collagen type I.

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1. Introduction

Gelatin, the denatured form of collagen, has been extensively applied in the food industry as an ingredient to improve the elasticity, consistency and stability of foods. Its parent form, collagen, constitutes approximately 30 g/100 g of total animal protein. Skin, bones, the vascular system, tendons and the connective tissue sheaths surrounding muscle are the major sources of collagen. In general, gelatin is manufactured from the waste generated during animal slaughter and processing, i.e., skin and bone (Patil, Dalev, Mark, Vassileva, & Fakirov, 2000). There are two different processes to obtain gelatin from skins and bones of animals, an acid process (gelatin A with an isoelectric point at pH 6–9) and an alkaline process (gelatin B with an isoelectric point at pH 5) (Stainsby, 1987). The physicochemical properties of gelatins are generally influenced by several factors, such as the method of preparation and the intrinsic properties, including the chemical composition, the amino acid sequence as well as the chain length of the protein

components (Badii & Howell, 2006). In addition, functional properties and the length of the polypeptides of gelatin could be influenced by the chemicals used for pretreatment and the conditions used, such as temperature and time during extraction (Kolodziejaska, Kaczorowski, Piotrowsia, & Sadowska, 2004). The source and types of collagen in the raw material, which vary in proline and hydroxyproline contents, will affect the gelling properties, thermal properties and functional properties of gelatin. Collagen from varying species is associated with the temperature of the animal's living habitat (Norland, 1990).

Gelatin from alternative sources has received attention in recent years due to the outbreak of bovine spongiform encephalopathy (BSE) and the foot-and-mouth disease crisis (Helcke, 2000). In addition, gelatin produced from bovine and porcine skins cannot be used for some foods due to esthetic and religious objections (Sadowska, Kolodziejaska, & Niecikowska, 2003). Gelatins from alternative sources have been produced from various fish species such as cod and hake skin (Fernández-Díaz, Montero, & Gómez-Guillén, 2001), Nile perch skin and bone (Muyonga, Cole, & Duodu, 2004), shark cartilage (Cho et al., 2004), skate skin (Cho, Jahncke, Chin, & Eun, 2006), bigeye snapper and brownstripe red snapper

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skin (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2006), Alaska Pollock (Zhou, Mulvaney, & Regenstein, 2006), Atlantic salmon skin (Arnesen & Gildberg, 2007), sin croaker and shortfin scad (Cheow, Norizah, Kyaw, & Howell, 2007). However, little information regarding the characteristics of gelatin from freshwater fish (Zhang, Wang, Herring, & Oh, 2007), especially from commercially important species involving those used for domestic consumption in Thailand, such as farmed giant catfish, has been reported. Therefore, the aims of this investigation were to extract the gelatin from the skin of farmed giant catfish and to study its physicochemical characteristics as well as its functional properties.

2. Materials and methods

2.1. Chemicals

Sodium dodecyl sulfate (SDS), acetic acid, and sodium hydroxide were obtained from Merck (Darmstadt, Germany). Methanesulfonic acid, citrate buffer, calf skin gelatin (bloom strength 120–150 g) and calf skin acid-soluble type I collagen were from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). All chemicals for electrophoresis were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Fish skin preparation

Farmed giant catfish (*Pangasianodon gigas*) with an average total length of 75–80 cm were obtained from a farm in Chiang Rai. The fish were sacrificed by iced-shock and then were cooled in ice during transportation to the School of Agro-Industry, Mae Fah Luang University, Chiang Rai with the ratio of fish per ice of 1:2 (wt/wt). Fish were washed with tap water. The skin was then removed and scrapped to remove the adhering fat and foreign matter and cut into small pieces (0.5 × 0.5 cm). Prepared skin was kept on ice prior to gelatin extraction.

2.3. Extraction of fish skin gelatin

Gelatin was extracted from washed giant catfish skin as described in Jongjareonrak, Benjakul, Visessanguan, and Tanaka (2006). To remove non-collagenous proteins and pigments, washed skin was soaked in 0.2 mol/l NaOH with a skin to solution ratio of 1:10 (w/v) at 4 ± 1 °C with a continuous gentle stirring. The solution was changed every 30 min for 3 times. Alkaline-treated skin was then washed with tap water until neutral or faintly basic pH (pH < 7.5) of wash water was obtained. To swell the collagenous material in the fish skin matrix, the alkaline-treated skin was soaked in 0.05 mol/l acetic acid with a skin to solution ratio of 1:10 (w/v) for 3 h at room temperature (25 ± 1 °C) with a continuous gentle stirring. Acid-treated skin was washed as previously described. The swollen fish skin was soaked in distilled water with a skin/water ratio of 1:10 (w/v) at 45 ± 1 °C for 12 h with continuous stirring to extract the gelatin. The mixture was then filtered using two layers of cheesecloth. The resultant filtrate was freeze-dried and the dry matter from freeze-dried process was ground and referred to as “gelatin powder”.

2.4. Proximate analysis of gelatins

The moisture, ash and fat contents of the gelatin powder were determined according to the AOAC methods number 927.05, 942.05 and 920.39 B, respectively (AOAC, 2000). Protein content was measured by the Kjeldahl method according to the AOAC method number 984.13 (AOAC, 2000) and a nitrogen conversion factor of 5.4 was used for calculation of protein content of gelatin.

2.5. Determination of amino acid composition

Gelatin samples were hydrolysed under reduced pressure in 4.0 mol/l methanesulfonic acid containing 0.2 ml/100 ml 3-(2-aminoethyl)indole at 115 °C for 24 h, and the hydrolysates were neutralised with 3.5 mol/l NaOH and diluted with 0.2 mol/l citrate buffer (pH 2.2). An aliquot of 0.4 ml was applied to an amino acid analyser (MLC-703; Atto Co., Tokyo, Japan).

2.6. Determination of foam capacity and foam stability

Foaming capacity and the stability of gelatin sample were determined according to the method of Cho et al. (2004) with modifications. The gelatin solution (2 g/100 ml) was prepared by dissolving the gelatin dry powder in distilled water and incubating in a water bath at 60 °C for 30 min. The gelatin sample solution was then left at room temperature for 20 min followed by homogenisation at a speed of 11,000 rpm, using a homogeniser (IKA Labor-technik, Selangor, Malaysia) to incorporate air for 5 min at room temperature. The whipped sample was immediately transferred into a 25 ml cylinder and the total volume was read after 30 s. The foaming capacity was calculated according to the following equation:

$$\text{Foaming capacity (\%)} = [(A - B)/B] \times 100$$

where *A* is the total volume after whipping (ml), *B* is the total volume before whipping (ml).

The whipped sample was allowed to stand at ambient temperature for 3 min and the volume of whipped sample was then recorded. Foam stability was calculated as follows:

$$\text{Foaming stability (\%)} = [(A - B)/B] \times 100$$

where *A* is the total volume after standing (ml), *B* is the total volume before standing (ml).

2.7. Determination of viscosity

Viscosity of gelatin sample was determined according to the method of Cho et al. (2006). Gelatin solutions (10 g/100 ml) were prepared by dissolving the dry powder in distilled water and heating at 60 °C. Viscosity was determined using a computerised Brookfield digital viscometer (Model DV-II, Brookfield Engineering, MA, USA) equipped with a No. 1 spindle (Model RVT) at 60 rpm at 40 ± 1 °C. The viscosity was read and reported in term of cP.

2.8. Gelatin gel preparation

Gelatin gels were prepared according to the British Standard 757: 1975 method (BSI, 1975) with a slight modification. Gelatin powder was mixed with distilled water to obtain a final concentration of 6.67 g/100 ml. The mixture was stirred thoroughly and left at room temperature for 30 min to allow the gelatin to absorb water and swell. The mixture (30 ml) was then transferred to a 50 ml-beaker (PYREX®, USA) and incubated at 42 °C for 30 min in a temperature-controlled water bath with occasional stirring. The beakers were then kept in a temperature-controlled chamber at 10 °C and allowed to stand for 16–18 h before determination of bloom gel strength.

2.9. Determination of gelatin gel colour

The colour of gelatin gel samples was measured after the gels were incubated at 10 °C for 16–18 h and reported in the L^* , a^* , b^*

mode of CIE using a colorimeter (Model ColorFlex, HunterLab, Reston, VA, USA).

2.10. Determination of bloom gel strength

Bloom gel strength was determined according to the British Standard 757: 1975 method (BSI, 1975) with a slight modification. Bloom gel strength at 10 °C was determined using a Model TA-XT2 Texture analyser (Stable Micro System, Surrey, UK) using a load cell of 5 kg equipped with a 1.27 cm diameter flat-faced cylindrical Teflon® plunger. The dimensions of the sample were 3.8 cm in diameter and 2.7 cm in height. The maximum force (in grams), taken when the penetration distance of 4 mm was obtained, was recorded. The speed of the plunger was 0.5 mm/s. The measurement was performed in triplicate.

2.11. Electrophoretic analysis

Protein patterns of gelatin samples were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). The samples (1 g) were dissolved in 10 ml of 5 g/100 ml SDS solution. The mixtures were heated at 85 °C for 1 h in a water bath to dissolve the proteins. Supernatants were collected after centrifuging by using a Mikro 20 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany) at 3000×g for 3 min. The supernatants were then mixed with sample buffer (0.5 mol/l Tris-HCl, pH 6.8 containing 4 g/100 ml SDS, 20 ml/100 ml glycerol) with and without 10 ml/100 ml beta-mercaptoethanol (β ME) at the ratio of 1:1 (v/v) and boiled in boiling water for 3 min. Samples (20 μ g protein) were loaded into the polyacrylamide gel made with a 7.5 g/100 ml running gel and a 4 g/100 ml stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini Protean II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, the gel was stained with 0.05 g/100 ml Coomassie blue R-250 in 15 ml/100 ml methanol and 5 ml/100 ml acetic acid and destained with 30 ml/100 ml methanol and 10 ml/100 ml acetic acid. Calf skin acid-soluble type I collagen (Sigma Chemical Co., St. Louis, MO, USA) was used as a reference.

2.12. Statistical analysis

Data were subjected to analysis as mean $\pm$ SD from triplicate determinations.

3. Results and discussion

3.1. Composition of fish skin gelatin

Gelatin was extracted from farmed giant catfish skin with a yield of 20.1 g/100 g skin sample on the basis of wet weight. The gelatin yield recoveries have been reported to vary among fish species, mainly due to the differences in collagen content, the compositions of the skin as well as the skin matrix. The yields of gelatin obtained from fish skin have been reported for sin croaker (14.3 g/100 g) and shortfin scad (7.25 g/100 g) (Cheow et al., 2007), bigeye snapper (6.5 g/100 g) and brownstripe red snapper (9.4 g/100 g) (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2006), Atlantic salmon (15.3 g/100 g) and cod (11.8 g/100 g) by a 2 step extraction (Arnesen & Gildberg, 2007). The higher yield of gelatin was due to the higher collagen content in the skin of giant catfish than other species. Furthermore, the high degree of cross-linking via covalent bonds caused the decrease in solubility of collagen and might lead to a lower amount of extractable gelatin (Foegeding, Lanier, & Hultin, 1996). The extraction conditions, including solvent, temperature

and extraction time, might affected the yield of the extracted gelatin.

The proximate composition of farmed giant catfish skin gelatin is shown in Table 1. Generally, skin gelatin of giant catfish had high protein and low moisture, fat and ash contents, suggesting the efficient removal of fat and minerals from the skin material. Gelatin from giant catfish skin was lower in ash content (0.33 g/100 g), compared with those from bigeye snapper (3.2 g/100 g), brownstripe red snapper (1.9 g/100 g) (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2006), skate (1.42 g/100 g) (Cho et al., 2006), sin croaker (5.43 g/100 g) and shortfin scad (5.9 g/100 g).

The amino acid composition of farmed giant catfish and calf skin gelatin expressed as residues per 1000 total amino acid residues is shown in Table 2. Giant catfish skin gelatin was rich in glycine (35.9 g/100 g), alanine (10.6 g/100 g), and proline (12.4 g/100 g). Hydroxyproline accounted for 87 residues per 1000 residues. The numbers of imino acids, proline and hydroxyproline, in giant catfish skin gelatins were 211 residues per 1000 residues. The imino acid content in skin gelatin from farmed giant catfish was higher than that reported in bigeye snapper and brownstripe red snapper (195 and 197 residues per 1000 residues, respectively) (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2006), Atlantic salmon and cod (166 and 154 residues per 1000 residues, respectively) but lower than that in calf skin gelatin (219 residues per 1000 residues). The stability of the triple helical structure in renatured gelatins was associated with the total content of pyrrolidine imino acids (Ledward, 1986). Hydroxyproline plays an essential role in the stabilisation of the triple-helix strands of collagen via its hydrogen bonding ability through its -OH group (Ledward, 1986). Gelatin with higher content of hydroxyproline is believed to have higher viscoelastic properties and its ability to develop triple helix structures, which are important for stabilising the gelatin gel structure (Gómez-Guillén et al., 2002). However, the size of the protein chains also determines the gelatin properties. From the result, cysteine, tryptophan, asparagine and glutamine were not found in gelatin from both sources. Cysteine and tryptophan are not commonly present in gelatin (Foegeding et al., 1996). In addition, asparagine and glutamine were oxidized to aspartic acid and glutamic acid, respectively, during sample preparation by acid hydrolysis method.

3.2. Foam capacity and foam stability of gelatin

Foam capacity and foam stability of gelatin are shown in Table 3. The foam capacity of gelatin from farmed giant catfish (130%) was higher than that from calf skin (110%), indicating the difference in foaming ability between the two gelatins. Foam formation is generally controlled by transportation, penetration and reorganisation of protein molecules at the air–water interface. A protein must be capable of migrating rapidly to the air–water interface, unfolding and rearranging at the interface to express good foaming ability (Halling, 1981). The positive correlation between hydrophobicity of unfolded proteins and foaming characteristics has been reported (Townsend & Nakai, 1983). The foaming capacity of

Table 1
Proximate compositions of gelatins from farmed giant catfish skin.

Compositions	Contents (g/100 g wet weight)
Protein	89.1 $\pm$ 0.5
Moisture	9.3 $\pm$ 0.3
Ash	0.3 $\pm$ 0.1
Fat	0.7 $\pm$ 0.1

Values are given as mean $\pm$ SD from triplicate determinations.

Table 2

Amino acid compositions of gelatins from calf and farmed giant catfish skin (residues per 1000 total amino acid residues).

Amino acids	Source of skin gelatin	
	Giant catfish	Calf
Hydroxyproline	87	93
Aspartic acid	15	30
Threonine	24	16
Serine	36	34
Glutamic acid	62	63
Proline	124	126
Glycine	359	356
Alanine	106	104
Valine	22	21
Methionine	10	5
Isoleucine	13	13
Leucine	23	25
Tyrosine	3	3
Phenylalanine	13	13
Lysine	32	32
Hydroxylysine	5	7
Histidine	4	3
Arginine	63	57
Total	1001	1001

protein was improved by making it more flexible, by exposing more hydrophobic residues and by increasing its capacity to decrease surface tension (Mutilangi, Panyam, & Kilara, 1996). For the adsorption at the air–water interface, molecules should contain hydrophobic regions (Mutilangi et al., 1996). The additional hydrophobic residues could form a large hydrophobic sphere on the surface of the polypeptide, so the foaming capacity could be improved. From the results, the difference in foam capacity between both skin gelatins could possibly be due to the higher content of hydrophobic amino acid contents (alanine, valine, isoleucine, leucine, proline, methionine, phenylalanine, tyrosine and tryptophan) of giant catfish skin gelatin (315 residues per 1000 residues) than calf skin gelatin (308 residues per 1000 residues) tested. For foam stability, it was noticeable that gelatin from giant catfish skin exhibited the greater value of foam stability compared with that of calf skin. Foam stability of protein solutions is generally positively correlated with molecular weight of peptides (van der Ven, Gruppen, de Bont, & Voragen, 2002). In addition, foam stability depends on the nature of the film and indicates the extent of protein–protein interaction within the matrix (Mutilangi et al., 1996).

3.3. Viscosity of gelatin

The viscosity of gelatin sample solutions (10 g/100 ml) is shown in Table 3. The viscosity of farmed giant catfish skin gelatin (112.5 cP) was almost 4 times greater than that of calf skin gelatin (31.3 cP). Viscosity is the second most important commercial physical property of a gelatin (Johnston-Bank, 1983). The viscosity of gelatin solutions varies with sources in terms of molecular weight and molecular size distribution of proteins (Sperling, 1985),

Table 3

The viscosity, foam capacity, foam stability and bloom strength of gelatin from farmed giant catfish and calf skin.

Properties	Source of skin gelatin	
	Giant catfish	Calf
Viscosity (cP)	112.5 ± 0.2	31.3 ± 0.2
Foam capacity (%)	130.0 ± 10.0	110.0 ± 10.0
Foam stability (%)	35.0 ± 3.5	16.7 ± 2.8
Bloom strength (g)	153.0 ± 2.9	134.8 ± 1.5

Values are given as mean ± SD from triplicate determinations.

concentration, pH (Cho et al., 2006) as well as temperature (Arnesen & Gildberg, 2007) of the gelatin solutions. From the results, at the same condition tested the difference in viscosity between gelatin from giant catfish skin and calf skin could be due mainly to the molecular weight distribution of protein components in gelatins.

3.4. Colour of gelatin gels

Colour values of gelatin gels from giant catfish and calf skin expressed as L^* , a^* and b^* are shown in Table 4. In general, L^* values of gelatin gels from giant catfish skin (20.43) were higher than that of calf skin (15.45). However, gelatin gels from calf skin gave higher $-a^*$ and b^* values than did giant catfish skin. These results suggested that gelatin gels from giant catfish skin had a lighter colour but lower yellowness and greenness than that of calf skin. The colour of gelatin is generally dependant on the raw materials extracted and whether it is the first, second or later extraction.

3.5. Bloom strength of gelatin gel

Bloom strength of the gelatin gels is shown in Table 3. Giant catfish skin gelatin gel exhibited higher bloom strength (153.0 g) than that of calf skin gelatin (134.8 g) tested, suggesting the difference in gel forming ability of the two specific gelatins used. The quality of gelatin is generally determined by the gel strength or bloom value, including low (<150), medium (150–220) and high bloom (220–300) (Johnston-Bank, 1983). Gelatins with different bloom strength were reported for Atlantic salmon (108 g), cod (71 g) (Arnesen & Gildberg, 2007), sin croaker (125 g), shortfin scad (177 g) (Cheow et al., 2007), bigeye snapper (105.7 g), brownstripe red snapper (218.6 g) (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2006), young and adult Nile perch (217 g and 240 g, respectively) (Muyonga et al., 2004). The difference in bloom strength among species was possibly due to the different composition, particularly in terms of amino acid composition and size of protein chains (Muyonga et al., 2004) as well as their living habitat (Norland, 1990). Protein degradation during gelatin extraction had been reported in bigeye snapper (Jongjareonrak, Benjakul, Visessanguan, Prodpran, & Tanaka, 2006). Protein degradation fragments, if present, may reduce the ability of α -chains to anneal correctly by hindering the growth of the existing nucleation sites (Ledward, 1986). As a result, functional properties such as bloom strength, foam ability, foam stability and viscosity of gelatins might be decreased.

3.6. SDS-PAGE patterns of gelatin gels

Protein patterns of gelatin from giant catfish skin gelatin under non-reducing and reducing condition (in the absence and presence of β ME) are shown in Fig. 1. In general, no differences were seen in protein patterns of gelatin under non-reducing and reducing conditions, suggesting no disulfide bond stabilising the protein structures in the gelatin obtained from giant catfish skin. α_1 - and α_2 -chains were found as the major components and similar to that of standard collagen type I. β - and γ -components were also observed in the gelatin from giant catfish skin. In addition, proteins

Table 4

Colour values of gelatin gel from farmed giant catfish and calf skin.

Sources of skin gelatin	L^*	a^*	b^*
Giant catfish	20.43 ± 0.06	−0.61 ± 0.13	1.36 ± 0.16
Calf	15.45 ± 0.23	−1.86 ± 0.15	3.72 ± 0.19

Values are given as mean ± SD from triplicate determinations.

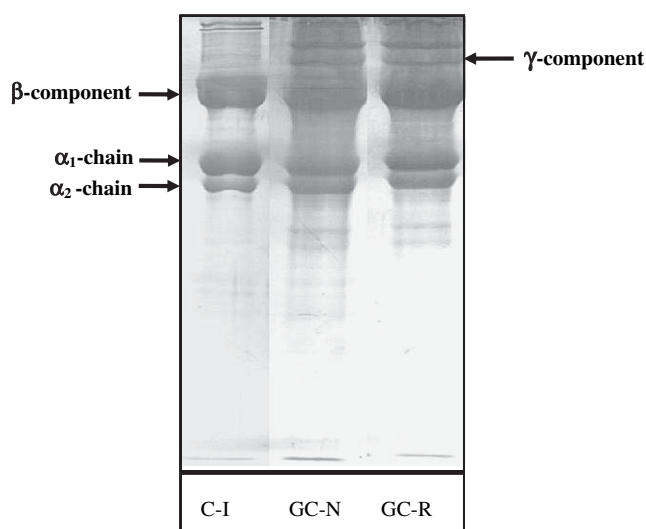


Fig. 1. Protein patterns of gelatin from farmed giant catfish skin under reducing and non-reducing conditions. C-I, GC-N and GC-R denote standard collagen type I, giant catfish skin gelatin under non-reducing condition and giant catfish skin gelatin under reducing condition, respectively.

with molecular weights lower than α -chains (~ 100 kDa) were also observed in the giant catfish skin gelatin sample. This might be due to the degradation of α , β - and/or γ -components during gelatin extraction or preparation. Gelatin with high proportion of α and β chains has also been extracted from Alaska Pollock (Zhou et al., 2006). Similar results have been reported in Nile perch skin gelatins (Muyonga et al., 2004). The extraction at higher temperature led to lower molecular weight peptides. Heat-induced breakage of protein components in gelatin occurred during the extraction process of Nile perch skin gelatin and was enhanced with higher temperature of extraction (Muyonga et al., 2004). Furthermore, the degradation of gelatin from bigeye snapper skin caused by some proteinases during extraction process had been reported (Jongjareonrak, Benjakul, Visessanguan, Prodpran, et al., 2006). The formation of degradation peptides is associated with the low viscosity, low melting point, low setting point, high setting time, as well as decreased bloom strength of gelatin (Ledward, 1986; Muyonga et al., 2004; Tavernier, 1989).

4. Conclusion

Gelatin was extracted as a high yield from the skin of farmed giant catfish. Giant catfish skin gelatin had a slightly different amino acid composition compared with those of calf skin gelatin. The functional properties, including viscosity, foam capacity, foam stability and bloom strength of gelatin from giant catfish skin were greater than that of the calf skin tested and were categorised as medium bloom value gelatin. Gelatin from farmed giant catfish skin contained α -, β - and γ -components.

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Properties of gelatin films from giant catfish skin and bovine bone: a comparative study

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Abstract Edible films were prepared from the gelatin of farmed giant catfish skin (GC) (*Pangasianodon gigas*), bovine bone gelatin (BB) and their combination. The physical, mechanical, thermal and chemical properties of the resulting films were characterized and compared. The molecular weight distributions of the giant catfish skin gelatin-containing samples had high quantities of α -chains, β -components and high molecular weight cross-links. The bovine bone gelatin-containing samples showed low contents of major bands with high degradation band components. The GC films had higher water activity (0.45) and mechanical properties [tensile strength: TS 41 MPa and elongation at break: EAB 34 (%)] but lower thickness (38 μ m) and water vapor permeability than those of BB films. The lowest light transmission (200–800 nm) and film transparency (3.34) were found in the GC films. However, the color attribute (L , a and b) of BB films was closer to the low-density polyethylene commercial film (ΔE 1.2). The highest value of film and protein solubility (100%) was found in BB film, while the lowest value (41–56%) was found in GC film. The GC films showed the most compact, smooth and continuous surface without porous structures, which corresponds to the greater properties of films.

Keywords Gelatin film · Giant catfish · Fish skin gelatin · Bovine bone gelatin · Edible film

Introduction

The deterioration of food during storage and transportation could be retarded or prolonged using better packaging. In general, packaging is needed for the protection of food from the outside environmental air and/or from post-contamination by microorganisms. Traditional non-biodegradable plastic films are extensively used for all industries over the world. The destruction of these plastics by burning is considered to be one of the contributors to global warming. To reduce the problem, edible or biodegradable films/materials are being applied in various kinds of packaging and are partly substituting for plastic containers [1]. In addition, edible or biodegradable films could make mass transfer barriers for moisture, oxygen, carbon dioxide, lipids, flavors and aromas between food products and the atmosphere [2, 3]. Edible films prepared from different types or sources of biomaterials such as proteins, polysaccharide, lipids or their blends exhibit different film properties. Among all materials, proteins are considered to provide desirable mechanical, gas barrier and transparency properties, as well as high nutritional value [4]. In recent years, various kinds of protein have gained the interested researchers to develop edible films. Gelatin is an important protein widely used for producing edible films/packaging. This is due to the uniqueness of gelatin among various hydrocolloids to form thermo-reversible properties with a melting point close to human body temperature. This is particularly significant in edible and pharmaceutical applications [5].

Gelatins are usually obtained by partial degradation or denaturation of collagen from bones and hides of bovine or porcine. However, gelatin from alternative sources has received more attention due to the outbreak of bovine spongiform encephalopathy (BSE) and the foot-and-mouth

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disease crisis [6]. Additionally, gelatin from bovine and porcine sources cannot be used for some foods due to esthetic and religious objections [7]. Gelatins from alternative sources have been produced from various fish by-products such as skin, bones, fins and scales [8]. Recently, gelatin from the skin of farmed giant catfish, which is one of the commercially important fresh water fish species, was successfully extracted and characterized [8]. One of the possible applications of giant catfish skin gelatin is the production of edible films. Therefore, in this work, the giant catfish skin gelatin was used to form edible film, and the properties of the film were also characterized and compared with the films from bovine bone gelatin (one of the most used gelatins) and their gelatin blends.

Materials and methods

Chemicals

Trichloroacetic acid, Folin-Ciocalteu's phenol reagent, glycerol and other analytical grade reagents were obtained from Merck (Darmstadt, Germany). Beta-mercaptoethanol (β ME), L-tyrosine, bovine serum albumin (BSA) and protein markers were purchased from Sigma Chemical Co. (St. Louis, MO, USA.). Electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Food grade bovine bone gelatin (bloom strength ~ 245) was obtained from Halagel (Thailand) Co., Ltd. (Bangkok, Thailand).

Fish skin preparation

Farmed giant catfish (*Pangasianodon gigas*) with a range of total length of 75–80 cm were obtained from a farm in Chiang Rai and transported to the School of Agro-Industry, Mae Fah Luang University, Chiang Rai. The fish was washed with tap water. The skin was then removed and scraped to remove fat and foreign matter, and it was then cut into small pieces (0.5×0.5 cm). The prepared skin was kept on ice prior to the gelatin extraction.

Extraction of fish skin gelatin

Gelatin was extracted from the washed giant catfish skin by the method described in Jongjareonrak et al. [9]. To remove non-collagenous proteins and pigments, the washed skin was soaked in 0.2 M NaOH with a skin to solution ratio of 1:10 (w/v) at 4 ± 1 °C with continuous gentle stirring. The solution was changed 3 times every 30 min. The alkaline-treated skin was then washed with tap water until it was neutral or faintly basic ($\text{pH} < 7.5$). To swell the collagenous material in the fish skin matrix, the

alkaline-treated skin was soaked in 0.05 mol/L acetic acid with a skin to solution ratio of 1:10 (w/v) for 3 h at room temperature (25 ± 1 °C) with continuous gentle stirring. Acid-treated skin was washed as previously described. The swollen fish skin was soaked in distilled water with a skin/water ratio of 1:10 (w/v) at 45 ± 1 °C for 12 h with continuous stirring to extract the gelatin. The mixture was then filtered using two layers of cheesecloth. The resultant filtrate was freeze-dried. The dry matter from freeze-dried process was ground and then referred to as “gelatin powder”.

Preparation of gelatin films

Gelatin powder was mixed with distilled water to obtain the film-forming solution (FFS) with a protein concentration of 4% (w/v). Glycerol was used as a plasticizer at a concentration of 25/100 g protein. The solution was incubated at 60 °C for 30 min in a water bath occasionally stirred. De-aerated film-forming solution (4 ± 0.01 g) was cast onto a rimmed silicone resin plate (50×50 mm) and dried with a ventilated oven environmental chamber (model H110 K-30DM; Seiwa Riko Co., Tokyo, Japan) at 25 ± 0.5 °C and $50 \pm 5\%$ relative humidity (RH) for 24 h. The dried films obtained were manually peeled.

Analyses of gelatin film properties

Film thickness

The film thickness was measured using a micrometer (Gotech, Model GT-313-A, Gotech Testing Machines Inc, Taiwan). Nine random locations around each of the ten film samples were used for thickness determination.

Water activity

The water activity (A_w) of the gelatin film samples was determined with a Novasina (Novasina AG, Pfäffikon, Switzerland) at a constant temperature of 25 ± 1 °C. Three samples in each treatment were used for testing.

Mechanical properties

Prior to testing the mechanical properties, the films were conditioned for 48 h at $50 \pm 5\%$ RH at 25 °C. The tensile strength (TS) and elongation at break (EAB) were determined with the modification of ASTM method D882–97 (ASTM., 1998) [10] using a Universal Testing Machine (Lloyd Instrument, Hampshire, UK). Five samples (2×5 cm) with an initial grip length of 3 cm were used for testing. The cross-head speed was set at 30 mm/min.

Water vapor permeability

Water vapor permeability (WVP) of the film was measured using the ASTM E96-95 standard method (ASTM., 1999), a modified ASTM method [11]. The films were sealed onto a permeation cup containing silica gel (0% RH) with silicone vacuum grease and an O-ring to hold the film in place. The cups were then placed in a desiccator saturated with water vapor at 30 °C. The cups were weighed at 1-h interval over an 8-h period, and the WVP of films was calculated as follows:

$$\text{WVP} = wxA^{-1}t^{-1}(P_2 - P_1)^{-1}$$

where w is the weight gain of the cup (g), x is the film thickness (m), A is the area of exposed film (m^2), t is the time of gain (s), and $(P_2 - P_1)^{-1}$ is the vapor pressure differential across the film (Pa). The WVP was expressed as $\text{g m}^{-1} \text{s}^{-1} \text{Pa}^{-1}$. A total of five samples were determined for each film.

Color, light transmission and film transparency

The color of the film was determined using a CIE colorimeter (Hunter associates laboratory, Inc., VA, USA) and expressed as L , a and b . From the Hunter color values, total color difference (ΔE) was calculated as:

$$\Delta E = \left\{ (L - L')^2 + (a - a')^2 + (b - b')^2 \right\}^{1/2}$$

where L' , a' and b' are values of standard low-density polyethylene (LDPE) film ($L' = 90.492$, $a' = -1.014$ and $b' = 0.642$).

The ultraviolet (UV) and visible light barrier properties of the films were measured at selected wavelengths between 200 and 800 nm, using UV-16001 spectrophotometer (Shimadzu, Kyoto, Japan). The film transparency was calculated by the following equation [12]:

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

where T_{600} is the fractional transmittance at 600 nm and x is the film thickness (mm).

Fourier transform infrared (FTIR) spectra analysis

Prior to analysis, films were conditioned in a desiccator containing silica gel for 7 days at room temperature to obtain the most dehydrated films possible. The FTIR spectra of the films were recorded using a horizontal ATR Trough plate crystal cell (451ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI) equipped with a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) at room temperature. For the spectra analysis, the film sample was

placed onto the crystal cell and it was clamped into the mount of the FTIR spectrometer. A spectrum in the range of $400\text{--}4,000 \text{ cm}^{-1}$ with automatic signal gain was collected over 16 scans at a resolution of 4 cm^{-1} . They were rationed against a background spectrum recorded from the clean empty cell at 25 °C. The spectra obtained were used to determine possible interactions of functional groups between gelatin molecules.

Differential scanning calorimetry

Prior to analysis, the films were conditioned in a desiccator containing silica gel for 1 week at room temperature to obtain the most dehydrated films possible. The films' thermal properties were determined using Perkin Elmer Differential Scanning Calorimetry (DSC) (Model DSC-7, Norwalk, CT, USA). Temperature calibration was performed using the Indium thermogram. The film samples (5–10 mg) were accurately weighed into aluminum pans, sealed and scanned over a range of $20\text{--}150 \text{ }^\circ\text{C}$ with a heating rate of $5 \text{ }^\circ\text{C}/\text{min}$. Ice water was used as a cooling medium, and the system was equilibrated at $20 \text{ }^\circ\text{C}$ for 5 min prior to the scan. The empty aluminum pan was used as a reference. The transition temperature and the transition enthalpy were estimated from the maximum of endothermic peak and the area under the DSC thermogram.

Film solubility and protein solubility

The film solubility was determined according to the method of Gennadios et al. [13]. The conditioned film samples were weighed and placed in a 50-mL centrifuge tube containing 10 mL of distilled water. The mixture was shaken at a speed of 250 rpm using a shaker (Heidolph Inkubator 10,000, Schwabach, Germany) for 24 h. The undissolved debris was then removed by centrifugation at $3,000 \text{ g}$ for 20 min. The pellet was dried at $105 \text{ }^\circ\text{C}$ for 24 h and weighed. The weight of the solubilized dry matter was calculated by subtracting it from the initial weight of the dry matter. It was then expressed as a percentage of the total weight.

To determine the protein solubility, the protein concentration in the supernatant was determined using the Lowry method [14]. Protein solubility was expressed as the percentage of the total protein in the film, which was solubilized with 0.5 M NaOH for 24 h.

Electrophoretic analysis

The protein patterns of gelatin, film-forming solution and the gelatin films were analyzed by SDS-PAGE according to the method of Laemmli [15]. To solubilize the films, the samples were mixed with a solubilizing solution (1% SDS).

The mixtures were heated at 85 °C for 1 h in a water bath to dissolve the proteins. The solubilized samples were then mixed with a sample buffer (0.5 M Tris–HCl, pH 6.8 containing 4% (w/v) SDS, and 20% (v/v) glycerol) and 10% (v/v) β ME at a ratio of 1:1 (v/v). The samples (16 μ g protein) were loaded into the polyacrylamide gel made of 7.5% running gel and of 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini Protean II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, the gel was stained with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and then destained with 30% (v/v) methanol and 10% (v/v) acetic acid.

Surface morphology and gelatin film microstructure

The surface morphology of the gelatin films was examined using a Canon Powershot A95 digital camera (Canon Marketing (Thailand) Co., Ltd., Bangkok, Thailand). The gelatin films' microstructure was observed using a scanning electron microscope (SEM) (JEOL JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 10 kV and magnification at 10,000 $\times$.

Statistical analysis

The data were subjected to analysis of variance (ANOVA). A comparison of means was carried out by Duncan's multiple range test. The analysis was performed using a SPSS package (SPSS 10.0 for window, SPSS Inc, Chicago, IL).

Results and discussion

Thickness of gelatin film

The thickness of gelatin-based films from giant catfish skin (GC), bovine bone (BB) and their combination (CG) is shown in Table 1. The average thickness of all the films was in a range of 38–40 μ m, and the highest film thickness was observed in the CG film. The thickness of all the films

was similar to those values of bigeye snapper (35.32 μ m) and brown stripe red snapper (37.18 μ m) skin gelatin films (3% protein) as found in Jongjareonrak et al. [9]. Thickness generally affects film properties such as mechanical properties, water vapor permeability, light transmission and film transparency.

Mechanical properties of gelatin film

The mechanical properties of gelatin-based film were expressed in terms of tensile strength (TS) and elongation at break (EAB) (Table 1). The TS of gelatin films varied with sources of gelatin (GC, BB and CG). Among all gelatin, the GC skin gelatin rendered the film with the highest TS (40.74 MPa) and EAB (34.14%) ($p < 0.05$). The films from BB and CG showed no differences in TS ($p > 0.05$). The result suggested the difference in film-forming characteristics of gelatin between two sources. Different protein constituents, as well as other components, in different gelatin sources, might govern the film formation. Film formation generally takes place by the development of a three-dimensional network of protein molecules by ionic, hydrophobic and hydrogen bonds [16]. From the results, EAB of GC film was greater than BB film (24.63%). When BB was combined with GC in a ratio of 50:50, the EAB was improved ($p < 0.05$). The increasing TS and EAB of film is attributed to the occurrence of advanced cross-linking molecules within film structures or by the formation of high molecular weight biopolymers.

Water activity and water vapor permeability of gelatin film (A_w , WVP)

Film cast from GC, BB and CG had water activity in the range of 0.42–0.45 ($p > 0.05$) (Table 1). Water activity affects the growth and multiplication of microorganisms. When there is $A_w < 0.9$, growth of most bacteria is inhibited. Microorganisms cease growth at $A_w < 0.6$. The interactions between water and food components mutually change the properties of food products, especially their stability, texture, taste, and lead to spoilage [17].

Table 1 Physical and mechanical properties of gelatin film

Sample	Thickness* (μ m)	Tensile strength (MPa)	Elongation (%)	Water activity (A_w)	WVP ($\times 10^{-10}$ gm $^{-1}$ s $^{-1}$ Pa $^{-1}$)
BB	38.71 $\pm$ 1.99 ^{ab}	32.56 $\pm$ 6.72 ^a	24.63 $\pm$ 10.96 ^a	0.419 $\pm$ 0.003 ^a	0.81 $\pm$ 0.04 ^a
GC	38.06 $\pm$ 2.09 ^a	40.74 $\pm$ 5.18 ^b	34.14 $\pm$ 6.07 ^b	0.448 $\pm$ 0.037 ^a	0.91 $\pm$ 0.06 ^b
CG	39.97 $\pm$ 0.76 ^b	34.91 $\pm$ 3.74 ^a	31.33 $\pm$ 5.41 ^{ab}	0.423 $\pm$ 0.005 ^a	1.14 $\pm$ 0.09 ^c

Different superscripts in the same column indicate significant differences ($p < 0.05$)

BB bovine bone gelatin film, GC giant catfish skin gelatin film, CG: combination of bovine bone: giant catfish gelatin film (50:50)

* Values are given as mean $\pm$ SD from 10 determinations

The WVP of film from GC, BB and CG was shown in Table 1. The differences in WVP were observed among films prepared from different sources of gelatins ($p < 0.05$). The CG film showed the highest WVP, while the lowest one was observed in the BB film. Fish gelatins are known to contain lower proline and hydroxyproline; thus, their hydrophobicity increases when compared with gelatin from mammal sources [18]. Constituents with lower polarity could absorb less water from the surrounding atmosphere. Water vapor permeation through a hydrophilic film depends on both diffusivity and solubility of moisture in the film matrix [19]. Additionally, more pronounced protein cross-linking of the film along with more rigid and denser structures might retard water diffusion through the films. The CG film's water vapor transmissibility rates were higher than GC and BB films, suggesting a decreased value of the film as a barrier to moisture. Film with higher thickness (CG) could adsorb more water from the environment. Water vapor permeability is dependent on the relative polarity of the polymers used. If the films are cationic and strongly hydrophilic, water interacts with the polymer matrix and the water vapor permeability increases.

Light transmission and transparency of gelatin film

Light transmission in UV (200–280 nm) and visible ranges (350–800 nm), as well as the transparency of all films, is shown in Table 2. Generally, all films exhibited lower light transmission in the UV range than in the visible range. The light transmission in the UV range was from 0 to 52.37%, while the transmission in the visible range was from 70 to 90%. The highest transmission in all the selected wavelengths was found in the BB film, while the lowest was found in the GC film. This result suggested that gelatin-based films could prevent UV transmission and therefore retard lipid oxidation induced by UV light in a food system. The result was consistent with Jongjareonrak et al. [9] who observed low transmissions of light in the UV range of fish gelatin-based films. Aromatic amino acids (tyrosine and phenylalanine) are well known to be sensitive chromophores in absorbing light at a wavelength below 300 nm

[20]. The increase in light transmission at selected wavelengths of films was in the order of GC, CG and BB. Light transmission values of GC, BB and CG were in a similar range as bigeye snapper and brownstripe red snapper skin gelatin [9].

The highest transparency was obtained in BB film, but no significant difference was observed in CG and GC ($p > 0.05$) (Table 2). The higher transparency value indicated that the film was less transparent. From the result, it was suggested that GC and CG films were more transparent than BB films. This indicated that the transparency properties of BB films could be improved by combining them with GC. Giant catfish skin gelatin film was transparent and clear enough for use as seen by the packaging.

Color of gelatin film

The Hunter L (Lightness), a (redness/greenness) and b (yellowness/blueness) color values of gelatin-based films are presented in Table 3. The GC films showed significantly lower values of L and a but highest in b value compared to the others ($p < 0.05$). When compared to the low-density polyethylene film, GC and BB films showed the highest and the lowest of total color differences, respectively. The total color difference (ΔE) value of BB films (1.20) was lower than CG (1.42) and GC (1.56) films, respectively. This indicated that the BB film had color properties closer to the low-density polyethylene film. Among all films, BB film had a lighter color and more yellowness than other films where the highest redness was observed in GC films.

Protein pattern of gelatin film

Protein patterns of gelatin, film-forming solution and gelatin-based film from GC, BB and CG are shown in Fig. 1. The electrophoretic analysis of gelatin in all film-forming solutions showed similar molecular weight distribution to the GC. The predominant bands including α -1, α -2 chains and their dimers (β -components) were observed. Marked differences in protein patterns were observed between BB

Table 2 Light transmission and transparency of gelatin film

Sample	Wavelength (nm)								Transparency*
	200	280	350	400	500	600	700	800	
BB	0.13	52.37	81.03	86.73	89.33	90.07	90.50	90.70	3.39 ± 0.029^b
GC	0.13	34.10	70.90	74.73	78.00	79.30	80.13	80.73	3.34 ± 0.006^a
CG	0.00	39.50	74.20	79.40	82.90	84.23	85.03	85.60	3.34 ± 0.020^a

Different superscripts in the same column indicate significant differences ($p < 0.05$)

BB bovine bone gelatin film, GC giant catfish skin gelatin film, CG combination of bovine bone: giant catfish gelatin film (50:50)

* Values are given as mean $\pm$ SD from triplicate determinations

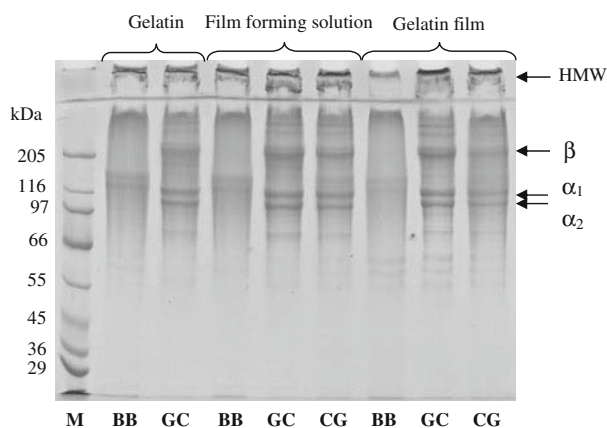
Table 3 The Hunter color value (L , a and b) and total color difference (ΔE) of gelatin film

Sample	L^*	a	b	ΔE
BB	90.45 ± 0.08^b	-1.23 ± 0.07^{ab}	1.81 ± 0.09^a	1.195 ± 0.082^a
GC	90.18 ± 0.08^a	-1.32 ± 0.12^a	2.14 ± 0.04^b	1.563 ± 0.044^c
CG	90.41 ± 0.13^b	-1.15 ± 0.07^b	2.05 ± 0.10^b	1.419 ± 0.105^b

Different superscripts in the same column indicate significant differences ($p < 0.05$)

BB bovine bone gelatin film, GC giant catfish skin gelatin film, CG combination of bovine bone: giant catfish gelatin film (50:50)

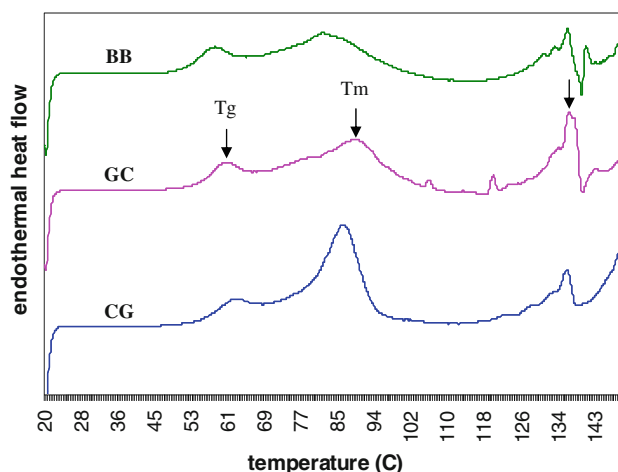
* Values are given as mean $\pm$ SD from triplicate determinations

**Fig. 1** SDS-PAGE pattern of gelatin, film-forming solution and films. *M* protein markers, *BB* bovine bone gelatin, *GC* giant catfish skin gelatin, *CG* combination of bovine bone: giant catfish skin gelatin (50:50)

and GC. The major components of gelatin (α -1, α -2 and β) were clearly observed in giant catfish skin gelatin, while these components were not found in BB. In addition, a high intensity of high molecular weight (HMW) bands (β - and γ -components) was observed in all GC-containing samples, especially in film-forming solutions and films. From the results, no β - and γ -component and slight α -1- and α -2-chains band intensity was observed in BB, BB film-forming solutions and BB films. The concomitant degradation bands (MW < 100 kDa) were also found in all BB-containing samples. The lower content of high MW cross-links and α -chains might result in the lowered mechanical properties of the BB films, unlike the GC and CG films that have higher mechanical properties.

Thermal properties

The transition temperature (T_i) and transition enthalpy of GC, BB and CG films are shown in Fig. 2. Generally, the transition temperature and transition enthalpy of gelatin films from all samples showed no significant differences, as observed from the maximum of the endothermic transition temperature. Figure 2 presents a typical curve for gelatin showing a glass transition (first small peak: T_g), melting (big endothermic peak: T_m) and deterioration peaks. The

**Fig. 2** DSC thermograms of gelatin films. *BB* bovine bone gelatin film, *GC* giant catfish skin gelatin film, *CG* combination of bovine bone: giant catfish skin gelatin (50:50) film

glass transition, melting and deterioration peaks of the films prepared from GC, BB and CG were around 55–60 °C, 83–89 °C and 134–140 °C, respectively.

In addition, there is a relatively small endothermic peak (between T_m and deterioration peaks) in GC film, while it is not found in the others. In gelatin, the T_g reported has been explained by the block copolymer model for amino acid content of gelatin. T_g of gelatin occurs at ~ 60 °C and is associated with the glass transition of an amino acid blocks in the peptide chain. Native fish gelatin film had T_m of 76.5 °C, which is higher than those films made from bovine skin gelatin that had the T_m of 65.06 °C as found by de Carvalho & Grosso [21]. On the other hand, the dry film made from pigskin gelatin had T_m of 91 °C [22]. This difference of the thermal property is reasonable due to the different specification of gelatin source used. However, Bigi et al. [23] reported that the first transition is more likely related to the volatilization of absorbed water in the gelatin sample, while the higher temperature transition is related to the relative amount of triple helix in gelatin.

The transition temperature indicated the precise temperature that caused a disruption of the protein interaction formed during film preparation. T_i from GC (89.50 °C) was higher than that of BB (88.42 °C) and CG (83.36 °C). The

higher T_g might be due to the greater imino acid amount (proline and hydroxyproline) of GC (211 residues/1,000 residues) [8]. The amount of imino acids showed the direct positive correlation with the thermal stability of protein via a hydrogen bond [24]. In addition, the integrity and molecular weight of protein chains might contribute to the network structure of the films obtained [25]. The film from BB comprised of proteins with lower molecular weight and greater content of degradation peptides, compared with those of GC (Fig. 1). This might be associated with the weaker network interaction of BB films. As a consequence, melting at lower temperature was found as indicated by the lower T_g in the films from BB (88.42 °C) and CG (83.36 °C). However, CG films had the lowest T_g value (83.36 °C). According to Jongjareonrak et al. [9], the transition peak observed in the DSC curves of gelatin films indicated the presence of the triple-helical structure. Thus, thermal properties of gelatin films were affected by the gelatin source.

The typical DSC thermograms of the gelatin film are shown in Fig. 2. During the heating scan, two endothermic peaks were observed. First, a step change in heat flow was assigned to a glass-to-rubber transition relating to the amorphous regions of a biopolymer. They were in a glassy state at the water contents of interest at room temperature [26, 27]. The glass transition was followed by an endotherm associated with the melting and dissociation of the ordered regions. The resulting thermograms indicated that before the heating scan was taken, gelatin films from BB, GC and their combination (CG) had a partial crystalline structure. These results are also consistent with previous reports [28, 29].

FTIR spectroscopy

FTIR spectra of GC, BB and CG films are depicted in Fig. 3. The spectrum of all gelatin films showed a similar pattern, which indicates that there were no major changes in the functional groups of the gelatin samples used. Gelatin films (GC and CG) revealed absorption band at $3,273\text{ cm}^{-1}$, (NH-stretching), while BB film showed the major absorption band at $3,293\text{ cm}^{-1}$. An increased absorption at $\sim 3,293$ of BB film compared with others was probably due to the stretching bands of OH-absorbed water molecules. In addition, the exact shape of the difference bands may be influenced by changes in the amide A band of the protein, whose maximum was found at $3,340\text{ cm}^{-1}$. Absorption bands in the spectra of all films were situated in the amide band region. The amide I and amide II bands of all films were observed at $1,630$ and $1,536\text{ cm}^{-1}$, respectively. These were similar to the amide I ($1,630\text{ cm}^{-1}$) and amide II ($1,545\text{ cm}^{-1}$) of the gelatin films from both bigeye snapper and brownstripe red snapper skin gelatin [9]. Muyonga et al. [30] also reported the amide I and amide II to be at

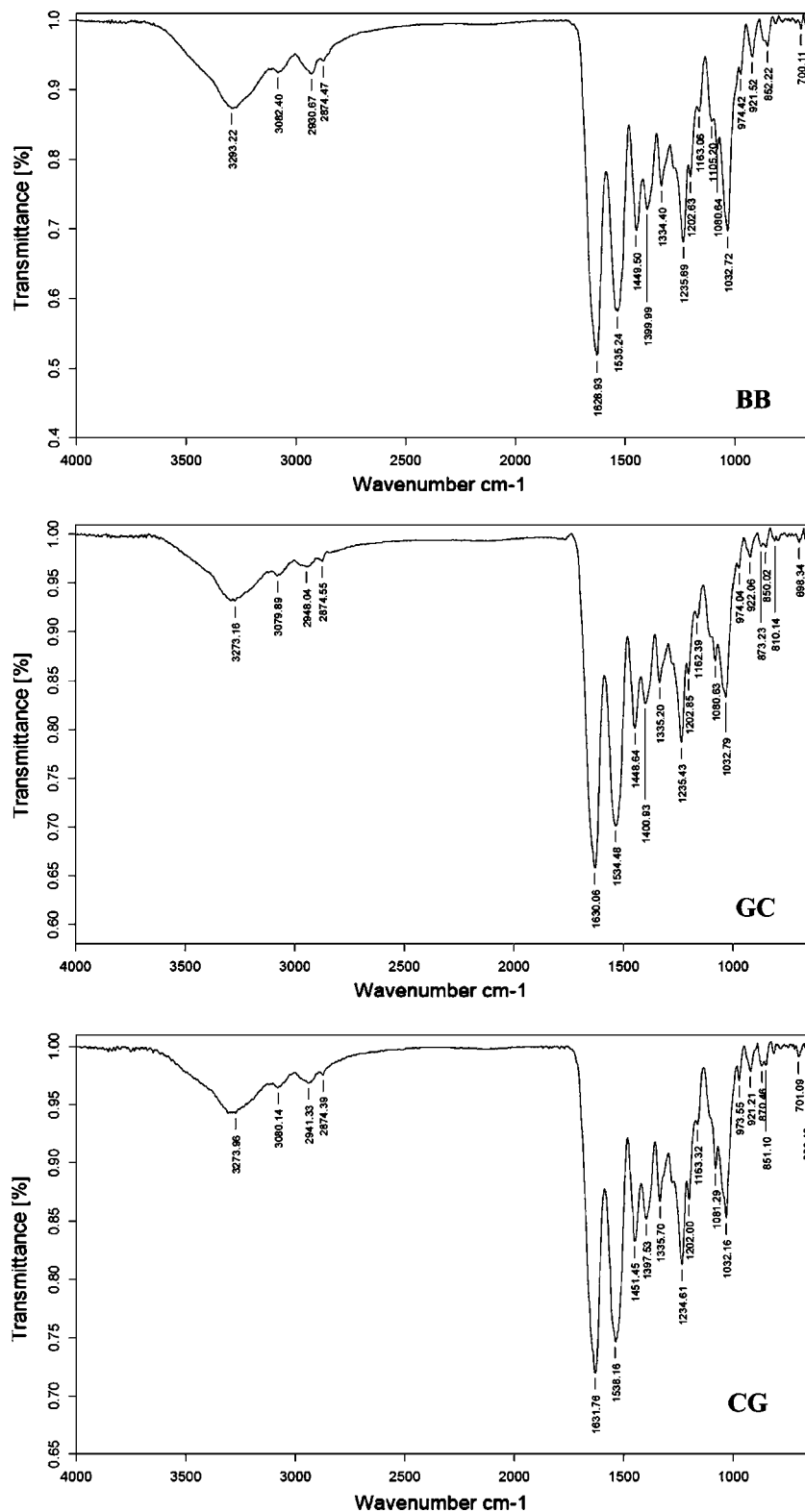
$1,700$ – $1,600$ and $1,560$ – $1,500\text{ cm}^{-1}$, respectively. The amide I vibration mode is primarily a C = O stretching vibration coupled with contributions from the CN stretch, CCN deformation and in-plane NH bending modes. The amide II vibration mode is attributed to an out-of-phase combination of CN stretch and in-plane NH deformation modes of the peptide group [31]. The amide II peak is considered to be much more sensitive to hydration than to secondary structure change [32]. In this study, FTIR spectra analysis was determined in a dry state, and the changes in the amide II band might be related to possible alterations in protein secondary structure. The infrared spectroscopic analysis technique is generally used to determine the secondary structure of protein via the absorption in the amide I and amide II region. From the result, the peaks at amide I and amide II were more intense when GC and BB were combined (CG films). The change in amide I and amide II band intensity for the CG film compared with the GC film suggested that the combination of BB and GC might affect the helix structure and/or alteration in the protein secondary structure of their resulting film (CG). From the result, the change in the films' FTIR spectra when BB was combined with GC revealed that there might be some interaction between both gelatins. These interactions and/or structural alterations might be associated with the mechanical properties of the resulting films.

Film solubility and protein solubility

The film solubility and protein solubility of GC, BB and CG films are shown in Table 4. From the results, 100% film solubility and protein solubility were observed in the BB films, where only 41.40% film solubility and 56.20% protein solubility were observed in GC. However, when BB was combined with GC, the resulting films (CG) had an intermediate solubility. The lower film and protein solubility observed in the film from GC suggested that the polypeptides in GC underwent more aggregation, leading to more cross-linking and higher molecular weights resulting in lower solubility. The combination of BB and GC might inhibit some inter-chain interactions between GC protein molecules and therefore increase the film and protein solubility of CG film.

The SDS-PAGE results of insoluble and soluble fractions of all films are depicted in Fig. 4. The protein patterns of the insoluble fractions of both GC and CG films were similar to their gelatin patterns. For soluble fractions, all films contained only low molecular weight peptides. These results suggested that high molecular weight peptides including α_1 -, α_2 -, β - and γ -components were the major compositions involved in the formation of film network structures, which underwent cross-linking during film formation, so the films became less soluble.

Fig. 3 FTIR spectra of gelatin films. *BB* bovine bone gelatin film, *GC* giant catfish skin gelatin film, *CG* combination of bovine bone: giant catfish skin gelatin (50:50) film



Surface morphology and gelatin film microstructure

The surface morphology of gelatin film from bovine bone, giant catfish skin and their combination was similar to

each other with uniform transparency (Fig. 5). When the film sheet covered the black background, the color of the background remained clearly observable. However, there was more turbidity of the gelatin film present in the dark

Table 4 Film solubility and protein solubility of gelatin film

Sample	Film solubility* (%)	Protein solubility* (%)
BB	100 ± 0.00 ^c	100 ± 0.00 ^c
GC	41.40 ± 1.84 ^a	56.20 ± 1.75 ^a
CG	48.68 ± 3.20 ^b	60.64 ± 2.50 ^b

Different superscripts in the same column indicate significant differences ($p < 0.05$)

BB bovine bone gelatin film, GC giant catfish skin gelatin film, CG combination of bovine bone: giant catfish gelatin film (50:50)

* Values are given as mean ± SD from triplicate determinations

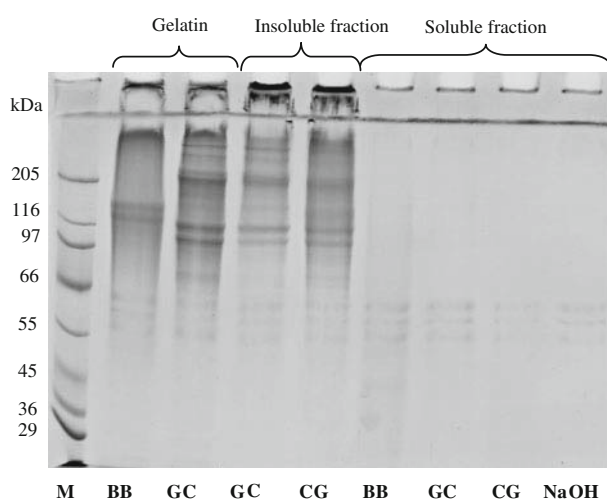


Fig. 4 Protein patterns of insoluble and soluble fractions of gelatin film. M protein markers, BB bovine bone gelatin, GC giant catfish skin gelatin, CG combination of bovine bone: giant catfish skin gelatin (50:50), NaOH solubilized film with 0.5 M NaOH

background compared to the green one, especially for the film containing giant catfish skin gelatins (data not shown). These results were similar to the light transmission and transparency of the gelatin film as mentioned above. Based on the film surface morphology property, applications of edible gelatin film were interesting, especially in food products. When the customers see the product packed inside the package, it is easy decide to buy that product.

SEM micrographs of gelatin films from GC, BB and CG are also observed (data not shown). The GC film had a smooth and continuous surface without grainy and porous structures. This indicated that a film with ordered matrix was formed. For BB films, porous surface with cracks was observed throughout the film surface. However, when GC was combined, the surface of the film became smoother with less cracks and pores. These results suggested that the molecular weight or chain length of polypeptide in gelatin samples might be responsible for the surface structure and properties, especially in the WVP of the resulting films.

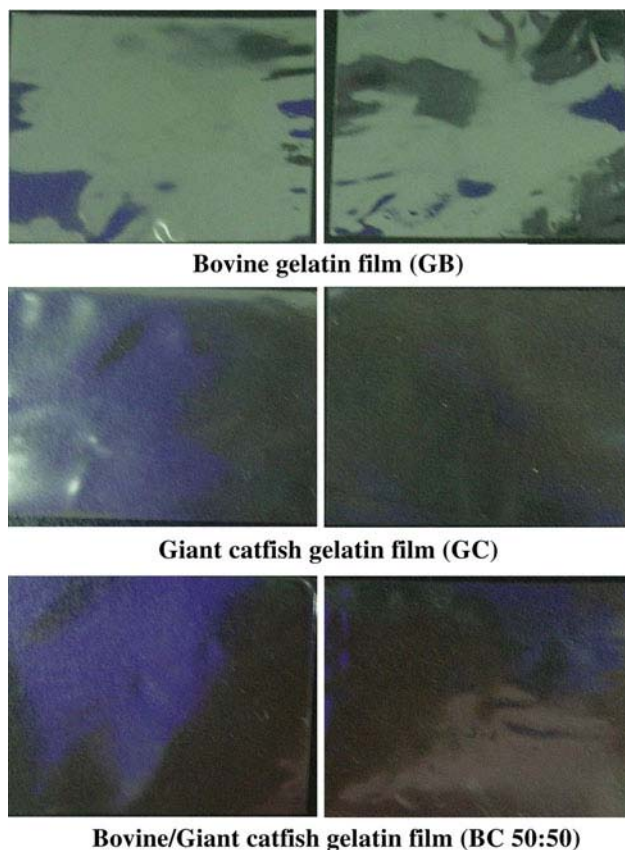


Fig. 5 Surface morphology of gelatin films from bovine bone gelatin, giant catfish skin gelatin and their combination of bovine bone: giant catfish skin gelatin (50:50)

Conclusion

The results demonstrated significant differences in both physical and chemical properties between GC and BB films. GC films had lower light transmission and film transparency, but they were higher in WVP and mechanical properties compared to BB film. The combination of BB and GC could improve the properties of BB film. Specific characteristic of GC-based films can benefit some food packaging applications.

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Natrinema gari sp. nov., a halophilic archaeon isolated from fish sauce in Thailand

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Two Gram-negative, rod-shaped, halophilic archaea, designated strains HIS40-3^T and HDS3-1, were isolated from anchovy fish sauce (nam-pla) collected from two different locations in Thailand. The two strains were able to grow at 20–60 °C (optimum 37–40 °C), at 1.7–5.1 M NaCl (optimum 2.6–3.4 M NaCl) and at pH 5.5–8.5 (optimum pH 6.0–6.5). Hypotonic treatment with less than 1.7 M NaCl caused cell lysis. The major polar lipids of the isolates were C₂₀C₂₀ and C₂₀C₂₅ derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate, two glycolipids and one unidentified lipid. The DNA G + C contents were 64.0–65.4 mol%. In addition to phenotypic and chemotaxonomic characteristics, phylogenetic analysis based on 16S rRNA gene sequence similarities showed that strains HIS40-3^T and HDS3-1 were related most closely to species of the genus *Natrinema*. Levels of 16S rRNA gene sequence similarity between strains HIS40-3^T and HDS3-1 and the type strains of recognized *Natrinema* species were 99.1–96.6%. The two novel strains could be distinguished from recognized *Natrinema* species on the basis of low levels of DNA–DNA relatedness and differences in whole-cell protein patterns and phenotypic properties. Levels of 16S rRNA gene sequence similarity and DNA–DNA relatedness between the two strains were 99.7 and 77.7%, respectively, suggesting that they should be classified as representing a single species. Based on these taxonomic data, strains HIS40-3^T and HDS3-1 are considered to represent a novel species of the genus *Natrinema*, for which the name *Natrinema gari* sp. nov. is proposed. The type strain is HIS40-3^T (=BCC 24370^T =JCM 14663^T =PCU 303^T).

Fish sauce (nam-pla) is a traditional fermented fish product commonly used as a condiment in South-East Asia. Apart from its unique and pleasant flavour, it provides an important supplementary source of nitrogen

in the diet of people in this region. Fish sauce contains nitrogen at 20 g l⁻¹, of which 16% is present as amino acids (Phithakpol *et al.*, 1995). Fish sauce contains a high concentration of NaCl, allowing various halophilic microorganisms to thrive (Lopetcharat *et al.*, 2001; Tanasupawat & Komagata, 2001). Nevertheless, just a few types of bacteria have been isolated from fish sauce samples and subjected to taxonomic study. In Thailand, *Halobacterium salinarum* and *Halococcus thailandensis* have been isolated from fish sauce samples: these are extremely halophilic archaea that grow optimally at 20–25% NaCl (Thongthai *et al.*, 1992; Namwong *et al.*, 2007). Other bacteria isolated from fish sauce are moderately halophilic micro-organisms

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains HIS40-3^T and HDS3-1 are AB289741 and AB289743, respectively.

Two-dimensional TLC of polar lipids extracted from strains HIS40-3^T and HDS3-1, maximum-parsimony and maximum-likelihood phylogenetic trees based on 16S rRNA gene sequences and patterns of whole-cell proteins are available as supplementary material with the online version of this paper.

that grow optimally at 3–15 % NaCl, such as *Tetragenococcus halophilus*, *Tetragenococcus muriaticus*, *Halobacillus thailandensis* and *Lentibacillus juripiscarius* (Thongsanit *et al.*, 2002; Chaayanan *et al.*, 1999; Namwong *et al.*, 2005). At the time of writing, the genus *Natrinema* comprises five recognized species. *Natrinema pellirubrum* and *Natrinema pallidum* were created by McGenity *et al.* (1998) as a result of reclassification of strains of *Halobacterium salinarum* and *Halobacterium halobium*, respectively. On the basis of 16S rRNA gene sequence analysis, phenotypic properties and polar lipid composition, *Natrinema versiforme* (Xin *et al.*, 2000) and *Natrinema altunense* (Xu *et al.*, 2005) were also included within the genus. *Natrinema ejinorensis* has been described more recently (Castillo *et al.*, 2006). Here, we describe the taxonomic properties of two extremely halophilic archaea, strains HIS40-3^T and HDS3-1, isolated from fermented fish sauce, and these are suggested to represent a novel species of the genus *Natrinema*.

Strains HIS40-3^T and HDS3-1 were isolated from anchovy fish sauce fermented for 40 days collected from Samut Prakarn Province and an anchovy fish sauce sample fermented for 3 months collected from Samut Songkram Province, central Thailand, respectively. Samples were plated on agar plates of halophilic medium [per litre: 250 g NaCl, 5 g Casamino acids, 5 g yeast extract, 1 g sodium glutamate, 2 g KCl, 3 g trisodium citrate, 20 g MgSO₄·7H₂O, 0.036 g FeCl₄·4H₂O, 0.36 mg MnCl₂·4H₂O, 20 g agar (pH 7.2)] and incubated at 37 °C for 1–2 weeks. A pure culture was obtained by repeated transfers of separate colonies on agar plates of the same medium. *Nnm. pallidum* JCM 8980^T, *Nnm. pellirubrum* JCM 10476^T and *Nnm. altunense* JCM 12890^T were used as reference strains in all tests of phenotypic characteristics. Unless otherwise stated, strains were grown in liquid medium (with shaking at 200 r.p.m.) or on agar plates of the halophilic medium and cultivated at 37 °C for 1–2 weeks.

Phenotypic characterization was carried out in accordance with the recommended minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997). Colony and cell morphology were examined for cells grown on agar plates at 37 °C for 14 days. Catalase and oxidase activities and hydrolysis of gelatin, casein, starch and Tween 80 were determined according to the methods of Barrow & Feltham (1993). Casamino acids were omitted from the test medium for determination of the hydrolysis of gelatin and casein. Utilization of sugars, alcohols, amino acids and organic acids and acid production were determined in modified Leifson medium supplemented with 0.01 % (w/v) yeast extract and 4.3 M NaCl, but with casitone and Tris/HCl omitted (Leifson, 1963). Growth at various temperatures (20–60 °C) was examined. NaCl requirement was determined in medium containing various NaCl concentrations (0–5.1 M). Similarly, the requirement of the strains for Mg²⁺ was tested in halophilic medium without MgSO₄·7H₂O but supplemented with 0–1.0 M MgCl₂. Growth was determined by measuring culture turbidity at

600 nm. Anaerobic growth was tested on agar plates in the presence of nitrate (1 g l⁻¹), L-arginine (1 g l⁻¹) or DMSO (10 g l⁻¹). Production of indole and reduction of nitrate and nitrite were also tested. Determination of the antibiotic susceptibility of the strains was tested according to the methods described by Stan-Lotter *et al.* (2002). Menaquinones were analysed as described by Komagata & Suzuki (1987). Polar lipids were determined according to the method of Minnikin *et al.* (1984).

DNA was isolated and purified according to the method of Saito & Miura (1963). The G + C content was determined by the method of Tamaoka & Komagata (1984) by using reversed-phase HPLC. DNA–DNA hybridization was determined as reported by Ezaki *et al.* (1989) and levels of relatedness were determined by the colorimetric method as reported by Tanasupawat *et al.* (2000). The 16S rRNA gene sequences of strains HIS40-3^T and HDS3-1, comprising 1405 and 1353 bp, respectively, were amplified by PCR with primers D30F (5'-ATTCCGGTTGATCCTGC-3', positions 6–12 according to the *Escherichia coli* numbering system) and D56R (5'-CTTGTTACGACTT-3', positions 1492–1509). The amplified DNA fragment was separated by agarose gel electrophoresis and was recovered by using a GenElute Minus EtBr Spin Column (Sigma). The sequence was determined by using a BigDye Terminator Cycle Sequencing Ready Reaction kit (v. 3.0; Applied Biosystems) in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) with the following primers: D30F, D33R (5'-TCGCGCCTGCGCCCCGT-3', positions 344–360), D34R (5'-GGTCTCGCTCGTTGCCTG-3', positions 1096–1113) and D56R. The sequence was compared with reference 16S rRNA gene sequences available in the GenBank and EMBL databases obtained from the National Center for Biotechnology Information database using BLAST searches. The alignment was subjected to phylogenetic analysis with the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood and maximum-parsimony methods, by using programs in the CLUSTAL_X and MEGA 4 packages (Thompson *et al.*, 1997; Tamura *et al.*, 2007). Confidence in the branching pattern was assessed by analysis of 1000 bootstrap replicates (Felsenstein, 1985).

Cells of strains HIS40-3^T and HDS3-1 were motile, Gram-negative rods (0.5–0.8 × 2.0–3.0 µm) (Fig. 1). Colonies formed on agar plates were circular (1–2 mm in diameter), smooth, translucent and pale-orange pigmented. The two strains were able to grow over a wide range of NaCl concentrations, from 1.7 M (approximately 10 %) to 5.1 M (approximately 30 %). Hypotonic treatment with less than 1.7 M NaCl caused cell lysis. Strains HIS40-3^T and HDS3-1 grew optimally in the presence of 2.6–3.4 M (15–20 %) NaCl, similar to most halophilic archaea (Grant *et al.*, 2001). The strains grew over a wide range of MgCl₂ concentrations from 0 to 1.0 M and grew optimally at around 0.1–0.2 M. The two strains grew at 20–60 °C (optimum 37–40 °C) and at pH 5.5–8.5 (optimum pH 6.0–6.5). The two strains were positive for catalase and oxidase. Indole production from tryptophan was

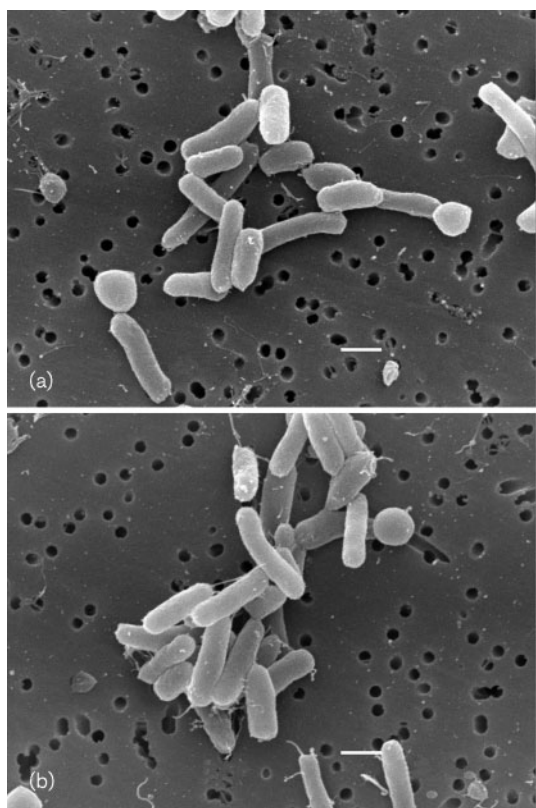


Fig. 1. Scanning electron micrographs of cells of strain HIS40-3^T (a) and strain HDS3-1 (b) grown on halophilic medium at 37 °C. Bars, 1 µm.

negative. Casein, starch and Tween 80 were not hydrolysed. Gelatin was liquefied. The strains showed anaerobic growth in the presence of DMSO but not with nitrate or arginine. Nitrate was not reduced and gas formation was not observed. The strains were sensitive to rifampicin, bacitracin and novobiocin. Strains HIS40-3^T and HDS3-1 utilized several carbohydrates. Among them, strong acid formation was observed only from glycerol for both strains and from arabinose for strain HIS40-3^T. There were some differences in the utilization of carbon sources between strains HIS40-3^T and HDS3-1. Some characteristics that distinguish strains HIS40-3^T and HDS3-1 from other members of the genus *Natrinema* are summarized in Table 1.

Strains HIS40-3^T and HDS3-1 possessed two menaquinones, MK-8 (76.3 and 74.6 %, respectively) and MK-8(H₂) (23.7 and 25.5 %), which are commonly detected in species of the genus *Natrinema* (McGenity *et al.*, 1998; Xin *et al.*, 2000). Two-dimensional TLC revealed that the two novel strains possessed glycerol diether analogues of phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP), phosphatidylglycerol sulfate (PGS), pigment, two glycolipids and one unidentified lipid (see Supplementary Fig. S1, available in IJSEM Online). The core lipids were C₂₀C₂₀ and C₂₀C₂₅ diether, as shown

from the two PG and PGP spots. The two strains showed two glycolipid spots, tentatively designated GL1 and 2, which might correspond to molecules containing C₂₀C₂₀ and C₂₀C₂₅ diether moieties. These two glycolipids were also found in varying amounts in the reference strains *Nnm. pallidum* JCM 8980^T, *Nnm. pellirubrum* JCM 10476^T and *Nnm. altunense* JCM 12890^T (McGenity *et al.*, 1998; Xu *et al.*, 2005). The DNA G + C contents of strains HIS40-3^T and HDS3-1 were 65.4 and 64.0 mol%, respectively.

The neighbour-joining phylogenetic tree constructed on the basis of 16S rRNA gene sequence data for the two new isolates and other representative *Natrinema* species is shown in Fig. 2. Strains HIS40-3^T and HDS3-1 formed a distinct cluster that fell within the genus *Natrinema*. Trees constructed according to the maximum-likelihood and maximum-parsimony methods are shown in Supplementary Fig. S2. 16S rRNA gene sequence similarity between the two novel strains was 99.7 %, suggesting that they should be classified as representing a single species or as members of very closely related species. This is in accordance with the morphological and chemotaxonomic similarities detailed above. The nearest neighbours of strains HIS40-3^T and HDS3-1 were *Nnm. pallidum* JCM 8980^T (99.1 and 99.1 % 16S rRNA gene sequence similarity, respectively), *Nnm. pellirubrum* JCM 10476^T (98.7 and 98.7 %), *Nnm. altunense* JCM 12890^T (98.5 and 98.5 %), *Nnm. versiforme* JCM 10478^T (98.4 and 98.4 %) and *Nnm. ejinorensis* JCM 13890^T (96.6 and 96.7 %).

The DNA–DNA hybridization study revealed that strains HIS40-3^T and HDS3-1 were closely related, exhibiting levels of relatedness of 73.5–77.7 % to each other; however, strains HIS40-3^T and HDS3-1 showed only low levels of DNA–DNA relatedness to *Nnm. pallidum* JCM 8980^T (40.5 and 42.5 %, respectively), *Nnm. pellirubrum* JCM 10476^T (18.7 and 22.0 %), *Nnm. altunense* JCM 12890^T (13.2 and 16.0 %) and *Nnm. versiforme* JCM 10478^T (19.5 and 18.2 %), indicating that these two novel strains are not members of any of these *Natrinema* species. Each of the values was obtained from two independent determinations. Moreover, the protein pattern of the novel strains was markedly different from those of the *Natrinema* species representatives analysed herein (Supplementary Fig. S3).

In conclusion, on the basis of growth requirements, poor utilization of carbohydrates, antibiotic susceptibility, menaquinone content, overall phospholipid composition, DNA G + C contents and 16S rRNA gene sequence analysis, strains HIS40-3^T and HDS3-1 are considered to represent a single species of the genus *Natrinema*. However, they could be differentiated from recognized *Natrinema* species based on levels of DNA–DNA relatedness and differences in whole-cell protein patterns. The results of the present study thus suggest that strains HIS40-3^T and HDS3-1 represent a novel species of the genus *Natrinema*, for which the name *Natrinema gari* sp. nov. is proposed.

Table 1. Differential characteristics between strains HIS40-3^T and HDS3-1 and recognized *Natrinema* species

Strains: 1, strain HIS40-3^T; 2, strain HDS3-1; 3, *Nnm. pallidum* JCM 8980^T; 4, *Nnm. pellirubrum* JCM 10476^T; 5, *Nnm. altunense* JCM 12890^T (data in columns 1–5 from this study unless indicated); 6, *Nnm. versiforme* JCM 10478^T (data from Xin *et al.*, 2000); 7, *Nnm. ejinorensis* JCM 13890^T (Castillo *et al.*, 2006). +, Positive; –, negative; +/-, doubtful; ND, no data available.

Characteristic	1	2	3	4	5	6	7
Cell morphology	Rods	Rods	Rods	Rods	Rods	Pleomorphic	Pleomorphic
Motility	+	+	+	+	+	–	–
Pigmentation	Pale orange	Pale orange	Pale orange	Pale orange	Pale orange	Light red	Light red
NaCl concentration (M) required to prevent cell lysis	>1.7	>1.7	>1.7	>1.7	>2.1	>1.0	>1.5
NaCl requirement (M)	≥1.7	≥1.7	≥1.7	≥1.7	≥2.1	≥1.5	≥1.8
NaCl optimum (M)	2.6–3.4	2.6–3.4	3.4–4.3	2.6–3.4	2.6–3.4	3.4–4.3	3.4
MgCl ₂ optimum (M)	0.1–0.2	0.1–0.2	0.1–0.2	0.1–0.2	0.1–0.2	0.15	ND
Temperature for growth (°C)							
Range	20–60	20–60	25–60	20–60	20–60	20–53	25–50
Optimum	37–40	37–40	30–37	30–37	37–40	37–46	37
pH for growth							
Range	5.5–8.5	5.5–8.5	5.5–8.5	5.5–8.5	6.0–8.5	6.0–8.0	6.0–8.5
Optimum	6.0–6.5	6.0–6.5	7.0–7.5	7.0–7.5	6.5–7.0	6.5–7.0	7.0
Anaerobic growth in the presence of nitrate	–	–	+	–	+	+	–
Reduction of nitrate to nitrite	–	–	+	+	+	+	–
Gas formation from nitrate	–	–	–	–	–	+	+
Oxidase activity	+	+	+	–	–	+	+
Indole formation	–	–	–	–	–	+	–
Hydrolysis of:							
Gelatin	+	+	+	–	+	–	+
Starch	–	–	–	–	–	+/-	+
Tween 80	–	–	+	–	+	+	+
DNA G + C content (mol%)	65.4	64.0	63.9	62.9	65.6*	64.2	64.7

*Data from Xu *et al.* (2005).

Description of *Natrinema gari* sp. nov.

Natrinema gari (ga'ri. L. gen. n. *gari* of a fish sauce, pertaining to the isolation of strains from fermented fish sauce).

Cells are motile, Gram-negative rods, 0.5–0.8 × 2.0–3.0 µm in size. Colonies are pale orange, smooth, circular and elevated. Growth is chemo-organotrophic. Requires at least 1.7 M NaCl for growth (optimum 2.6–3.4 M NaCl).

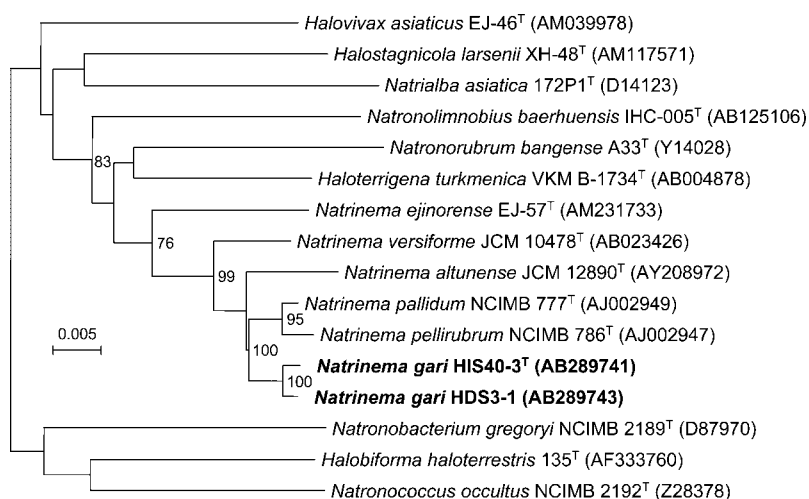


Fig. 2. Phylogenetic tree showing the relationships between strains HIS40-3^T, HDS3-1 and related archaeal species based on 16S rRNA gene sequences. The branching pattern was generated according to the neighbour-joining method. Bootstrap values above 70 %, based on 1000 replications, are shown at nodes. Bar, 0.5 substitutions per 100 nucleotide positions.

Growth occurs at 0–1.0 M MgCl₂ (optimum 0.1–0.2 M MgCl₂). The pH range for growth is 5.5–8.5 (optimum pH 6.0–6.5). The temperature range for growth is 20–60 °C (optimum 37–40 °C). Grows anaerobically in the presence of DMSO but not nitrate. Catalase- and oxidase-positive. Nitrate and nitrite are not reduced. Negative for production of indole. Casein, starch and Tween 80 are not hydrolysed. Gelatin is hydrolysed. L-Arabinose, D-glucose and glycerol are utilized for growth. Does not utilize inulin, lactose, maltose, D-mannitol, D-mannose, melibiose, rhamnose, D-ribose, sorbitol, sucrose, D-xylose or citrate. Acid is produced from L-arabinose and glycerol. Susceptible to bacitracin (10 U), novobiocin (5 µg) and rifampicin (30 µg), but resistant to ampicillin (10 µg), chloramphenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), neomycin (30 µg), streptomycin (10 µg) and tetracycline (30 µg). The predominant menaquinone is MK-8. Cells contain C₂₀C₂₀ and C₂₀C₂₅ derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate, two glycolipids and one unidentified lipid. The DNA G+C content of the type strain is 65.4 mol%.

The type strain, HIS40-3^T (=BCC 24370^T =JCM 14663^T =PCU 303^T), was isolated from fermented fish sauce in Thailand.

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Halobacterium piscisalsi sp. nov., from fermented fish (*pla-ra*) in Thailand

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A Gram-negative, motile, rod-shaped, extremely halophilic archaeon, designated strain HPC1-2^T, was isolated from *pla-ra*, a salt-fermented fish product of Thailand. Strain HPC1-2^T was able to grow at 20–60 °C (optimum at 37–40 °C), at 2.6–5.1 M NaCl (optimum at 3.4–4.3 M NaCl) and at pH 5.0–8.0 (optimum at pH 7.0–7.5). Hypotonic treatment with less than 1.7 M NaCl caused cell lysis. The major polar lipids of the isolate were C₂₀C₂₀ derivatives of phosphatidylglycerol, methylated phosphatidylglycerol phosphate, phosphatidylglycerol sulfate, triglycosyl diether, sulfated triglycosyl diether and sulfated tetraglycosyl diether. The G+C content of the DNA was 65.5 mol%. 16S rRNA gene sequence analysis indicated that the isolate represented a member of the genus *Halobacterium* in the family *Halobacteriaceae*. Based on 16S rRNA gene sequence similarity, strain HPC1-2^T was related most closely to *Halobacterium salinarum* DSM 3754^T (99.2 %) and *Halobacterium jilantaiense* JCM 13558^T (97.8 %). However, low levels of DNA–DNA relatedness suggested that strain HPC1-2^T was genotypically different from these closely related type strains. Strain HPC1-2^T could also be differentiated based on physiological and biochemical characteristics. Therefore, strain HPC1-2^T is considered to represent a novel species of the genus *Halobacterium*, for which the name *Halobacterium piscisalsi* sp. nov. is proposed. The type strain is HPC1-2^T (=BCC 24372^T=JCM 14661^T=PCU 302^T).

Pla-ra is a fermented fish product from Thailand made by mixing freshwater fish with salt and roasted rice and leaving this mixture to ferment at room temperature for 6–12 months (Phithakpol *et al.*, 1995). The product is rich in various nutrients, particularly amino acids and peptides, and contains a high concentration of salt (about 1.2–3.1 M NaCl), conditions that are suitable for the growth of

halophilic bacteria (Saisithi *et al.*, 1966; Phithakpol *et al.*, 1995; Tanasupawat & Komagata, 2001; Chamroensaksri *et al.*, 2008; Tanasupawat *et al.*, 2007). Nevertheless, no extremely halophilic bacteria have previously been isolated from *pla-ra*, and in particular none of the genus *Halobacterium*, family *Halobacteriaceae*, which are reported to inhabit highly saline environments (Elazari-Volcani, 1957; Thongthai *et al.*, 1992; Yang *et al.*, 2006). At the time of writing, the genus *Halobacterium* comprises fifteen species with validly published names. Of these, all but three species, namely *Halobacterium salinarum* (Ventosa & Oren, 1996), *Halobacterium noricense* (Gruber *et al.*, 2004) and *Halobacterium jilantaiense* (Yang *et al.*, 2006), have subsequently been transferred to other genera or classed as synonyms of already described species. Here, the taxonomic properties of a novel, extremely halophilic archaeon, strain HPC1-2^T, isolated from *pla-ra* are described.

Abbreviations: PG, phosphatidylglycerol; PGP-Me, methylated phosphatidylglycerol phosphate; PGS, phosphatidylglycerol sulfate; S-TeGD, sulfated tetraglycosyl diether; S-TGD, sulfated triglycosyl diether; TGD, triglycosyl diether.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HPC1-2^T is AB285020.

Maximum-parsimony and maximum-likelihood phylogenetic trees showing the relationships between strain HPC1-2^T and related archaeal species based on 16S rRNA gene sequences are available with the online version of this paper.

Strain HPC1-2^T was isolated from *pla-ra* samples collected from local markets in Thailand. Samples were plated on agar plates of halophilic medium [comprising (per litre distilled water): 250 g NaCl, 5 g Casamino acids, 5 g yeast extract, 1 g sodium glutamate, 2 g KCl, 3 g trisodium citrate, 20 g MgSO₄·7H₂O, 0.036 g FeCl₄·4H₂O, 0.00036 g MnCl₂·4H₂O, 20 g agar (pH 7.2)] and incubated at 37 °C for 1–2 weeks. A pure culture was obtained by repeated transfers of separate colonies on agar plates of the same medium. *Hbt. salinarum* JCM 8978^T and *Hbt. jilantaiense* JCM 13558^T(=NG4^T) were used as reference strains in all tests except DNA base composition. Unless otherwise stated, strains were grown in liquid (with shaking at 200 r.p.m.) or on agar plates of the halophilic medium and cultivated at 37 °C for 1–2 weeks.

Phenotypic tests were performed in accordance with the proposed minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997). Growth at various temperatures (20–60 °C) was examined. NaCl requirement was determined in the above medium containing various NaCl concentrations (0–5.1 M). Similarly, the requirement of the strains for Mg²⁺ was tested in halophilic medium lacking MgSO₄·7H₂O but supplemented with 0–1.0 M MgCl₂. Growth was determined by measuring culture turbidity at 600 nm. Cell motility and morphology were examined by phase contrast and transmission electron microscopy of liquid cultures grown for 7 days. Gram staining was carried out as described by Dussault (1955). Colony morphology was observed by growth on agar plates of the halophilic medium after incubation at 37 °C for 7 days. Anaerobic growth was tested on agar plates in the presence of nitrate (1 g l⁻¹), L-arginine (1 g l⁻¹) or DMSO (10 g l⁻¹). Catalase and oxidase activities and the hydrolysis of casein, gelatin, starch and Tween 80 were tested according to the methods of Barrow & Feltham (1993). Casamino acids were omitted from the test medium for determination of hydrolysis of casein and gelatin. Additional enzyme activities were determined by using API test kits (API ZYM and API 20E) at 37 °C as recommended by the manufacturer (bioMérieux). Utilization of sugars, alcohols, amino acids and organic acids, and acid production from various substrates were determined in modified Leifson medium supplemented with 0.01 % (w/v) yeast extract and 4.3 M NaCl, but lacking casitone and Tris-HCl (Leifson, 1963). Nitrate reduction, H₂S formation and indole production were tested as described by Oren *et al.* (1997). Tests for DNase, urease, methyl red, the Voges–Proskauer reaction, lysine and ornithine decarboxylases were performed as described by Gerhardt *et al.* (1981). Determination of antibiotic susceptibility was tested according to the methods of Stan-Lotter *et al.* (2002). Menaquinones were analysed as described by Komagata & Suzuki (1987). Polar lipids were determined according to the method of Minnikin *et al.* (1984).

DNA was isolated and purified according to the method of Saito & Miura (1963). The G + C content was determined

with the method of Tamaoka & Komagata (1984) by using reversed-phase HPLC. DNA–DNA hybridization was performed as reported by Ezaki *et al.* (1989) and levels of relatedness were determined by using the colorimetric method given by Tanasupawat *et al.* (2000). The 16S rRNA gene sequence of strain HPC1-2^T, comprising 1375 bp, was PCR-amplified with primers D30F (5'-ATTCCGG-TTGATCCTGC-3'; positions 6–12 according to the *Escherichia coli* numbering system) and D56R (5'-CTTGTTACGACTT-3'; positions 1492–1509). The amplified DNA fragment was separated by agarose gel electrophoresis and was recovered by using a GenElute Minus EtBr Spin Column (Sigma). The sequence was determined by using the BigDye Terminator Cycle Sequencing Ready Reaction kit version 3.0 (Applied Biosystems) in an ABI PRISM 310 genetic analyser (Applied Biosystems) with the following primers: D30F, D33R (5'-TCGCGCCTGCGCCCCGT-3'; positions 344–360), D34R (5'-GGTCTCGCTCGTTGCCTG-3'; positions 1096–1113) and D56R. The sequence was compared with reference 16S rRNA gene sequences available in the GenBank and EMBL databases obtained from the National Center for Biotechnology Information database by using BLAST searches. The alignment was subjected to phylogenetic analysis with the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood and maximum-parsimony methods by using programs in the CLUSTAL_X and MEGA 4 packages (Thompson *et al.*, 1997; Tamura *et al.*, 2007). Confidence in the branching pattern was assessed by analysis of 1000 bootstrap replicates.

Cells of strain HPC1-2^T were motile rods (0.5–1.0 × 1.0–2.0 µm) that possessed peritrichous flagella (Fig. 1). On halophilic agar medium, colonies of strain HPC1-2^T were circular, smooth, translucent and red. Cell lysis occurred in 1.7 M NaCl solution. Cells stained Gram-negative. Strain HPC1-2^T was capable of growing over a wide concentration

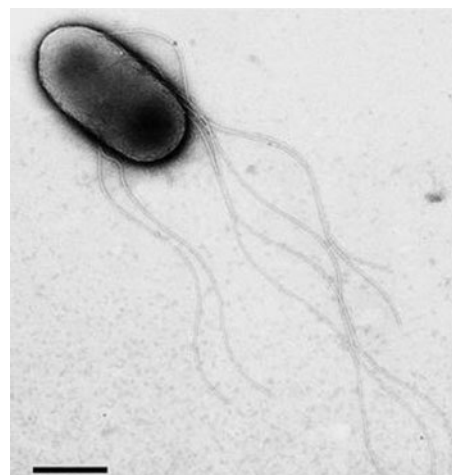


Fig. 1. Transmission electron micrograph of cells of strain HPC1-2^T grown in halophilic medium at 37 °C. Bar, 1 µm.

Table 1. Differential characteristics between strain HPC1-2^T and recognized *Halobacterium* species

Taxa: 1, strain HPC1-2^T; 2, *Hbt. salinarum* DSM 3754^T; 3, *Hbt. jilantaiense* JCM 13558^T; 4, *Hbt. noricense* A1^T (data from Gruber *et al.*, 2004). +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4
Cell morphology	Rods	Small rods	Rods	Irregular rods
Pigmentation	Red	Red	Red	Light red
pH range for growth	4.5–8.5	5.0–8.5	5.0–8.0	5.2–7.0
Temperature range for growth (°C)	20–60	20–60	20–60	28–50
Optimum NaCl concentration for growth (M)	3.4–4.3	3.4–4.3	3.4–4.3	2.5–3.0
Optimum MgCl ₂ concentration for growth (M)	0.5–0.6	0.5–0.6	0.5–0.6	0.6–0.9
NaCl concentration (M) required to prevent cell lysis	>1.7	>1.7	>2.1	ND
Oxidase	+	+	+	–
Hydrolysis of:				
Casein	+	+	+	ND
Gelatin	+	+	+	–
Tween 80	+	–	–	ND
Anaerobic growth with DMSO	+	+	+	–
Acid production from glycerol	+	+	–	+
Utilization of carbon sources:				
Citrate	+	+	–	ND
Inulin	–	+	+	ND
Lactose	+	–	–	ND
L-Lysine	–	–	+	ND
Maltose	–	+	–	–
D-Mannose	–	–	+	ND
Melezitose	+	–	–	ND
D-Xylose	–	+	–	–
Enzyme assay (API ZYM)				
Acid phosphatase	+	+	+	–
Alkaline phosphatase	+	+	+	–
Cystine arylamidase	–	–	–	+
Esterase (C4)	+	–	+	+
Esterase lipase (C8)	+	–	–	ND
Leucine arylamidase	–	–	–	ND
Lipase (C14)	+	–	–	ND
Naphthol-AS-BI-phosphohydrolase	+	+	+	–
Valine arylamidase	–	–	–	+
DNA G + C content (mol%)	65.5	67.1–71.2*	64.2†	54.5

*Data from Grant (2001).

†Data from Yang *et al.* (2006).

range of NaCl from 2.6 to 5.1 M. It grew optimally in the presence of 3.4–4.3 M NaCl, similar to most extremely halophilic Archaea (Grant, 2001). It also grew over a wide range of MgCl₂ concentrations from 0 to 1 M, with optimum growth at about 0.5–0.6 M. Strain HPC1-2^T grew in the temperature range 20–60 °C (optimum 37–40 °C) and pH range 5.0–8.0 (optimum pH 7.0–7.5). Catalase and oxidase activity tests were positive. No indole formation was observed in the presence of tryptophan. Cells hydrolysed gelatin, casein and Tween 80, but not starch. Strain HPC1-2^T showed anaerobic growth in the presence of DMSO, nitrate and L-arginine. Nitrate was not reduced and no gas formation was observed.

Two-dimensional TLC revealed that strain HPC1-2^T possessed C₂₀C₂₀ derivatives of phosphatidylglycerol (PG), methylated phosphatidylglycerol phosphate (PGP-Me), phosphatidylglycerol sulfate (PGS), triglycosyl diether (TGD), sulfated triglycosyl diether (S-TGD) and sulfated tetraglycosyl diether (S-TeGD) and pigments. The overall polar lipid pattern was similar to those of *Hbt. salinarum* DSM 3754^T (Ventosa & Oren, 1996) and *Hbt. jilantaiense* JCM 13558^T (Yang *et al.*, 2006). The presence of S-TGD can be used to distinguish the above three taxa from *Hbt. noricense* (Gruber *et al.*, 2004). Strain HPC1-2^T had menaquinones MK-8 (98 %) and MK-8 (H₂) (2 %). The DNA G + C content of strain HPC1-2^T was 65.5 mol%, which was similar to that of

Hbt. salinarum DSM 3754^T (Ventosa & Oren, 1996) and *Hbt. jilantaiense* JCM 13558^T (Yang *et al.*, 2006) but not to that of *Hbt. noricense* A1^T (Gruber *et al.*, 2004). The range of G + C contents of recognized species of the genus *Halobacterium* are 57–60 mol% for the minor DNA component and 67.1–71.2 mol% for the major DNA component (Grant, 2001). Characteristics that distinguish strain HPC1-2^T from recognized members of the genus *Halobacterium* are summarized in Table 1.

Fig. 2 shows the phylogenetic tree constructed by the neighbour-joining method in which strain HPC1-2^T clustered with the type strains of species of the genus *Halobacterium*. Trees constructed with the maximum-likelihood and maximum-parsimony methods are shown in Supplementary Figs S1 and S2 (available in IJSEM Online). Levels of 16S rRNA gene sequence similarity between strain HPC1-2^T and *Hbt. salinarum* DSM 3754^T, *Hbt. jilantaiense* JCM 13558^T and *Hbt. noricense* A1^T were 99.2, 97.8 and 96.6 %, respectively. However, strain HPC1-2^T showed levels of DNA–DNA relatedness of only 36.5 ± 2.5 % with *Hbt. salinarum* DSM 3754^T and 19.3 ± 2.1 % with *Hbt. jilantaiense* JCM 13558^T. These values are the mean $\pm$ SD obtained from three independent determinations. The results presented herein indicate that strain HPC1-2^T represents a novel species of the genus *Halobacterium*, for which the name *Halobacterium piscisalsi* sp. nov. is proposed.

Description of *Halobacterium piscisalsi* sp. nov.

Halobacterium piscisalsi (pis.ci.sal'si. L. n. *piscis* fish; L. adj. *salsus* salted, salt; N.L. gen. n. *piscisalsi* of salted fish).

Cells are rod-shaped ($0.5\text{--}1.0 \times 1.0\text{--}2.0$ μm) and motile by means of peritrichous flagella. Cells stain Gram-negative and are chemo-organotrophic. Colonies on plates of halophilic agar medium are red, smooth, circular and elevated. Requires at least 2.6 M NaCl for growth; optimal growth occurs at 3.4–4.3 M NaCl. Cell lysis occurs upon hypotonic treatment with less than 1.7 M NaCl. Growth occurs at 0–1 M MgCl₂, optimally at around 0.5–0.6 M MgCl₂. The temperature and pH ranges for growth are 20–60 °C (optimum at 37–40 °C) and 5.0–8.0 (optimum at pH 7.0–7.5). Grows anaerobically in the presence of nitrate, L-arginine and DMSO. Catalase- and oxidase-positive. Nitrate and nitrite are not reduced. Methyl red, Voges–Proskauer reaction and indole production are also negative. Casein, gelatin and Tween 80 are hydrolysed, but starch is not. The following substrates are utilized for growth: lactose, melezitose, glycerol and citrate. Acid is produced from glycerol. Does not utilize L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, *myo*-inositol, inulin, maltose, D-mannitol, D-mannose, melibiose, raffinose, rhamnose, D-ribose, salicin, sorbitol, sucrose, trehalose, D-xylose, acetate, L-glycine, L-alanine, L-arginine, L-aspartate, L-glutamate or L-lysine. Produces alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), acid phosphatase and naphthol-AS-BI-phosphohydrolase, but not leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase, lysine decarboxylase, ornithine decarboxylase, urease or DNase. Sensitive to bacitracin (10 U), neomycin (30 μg)

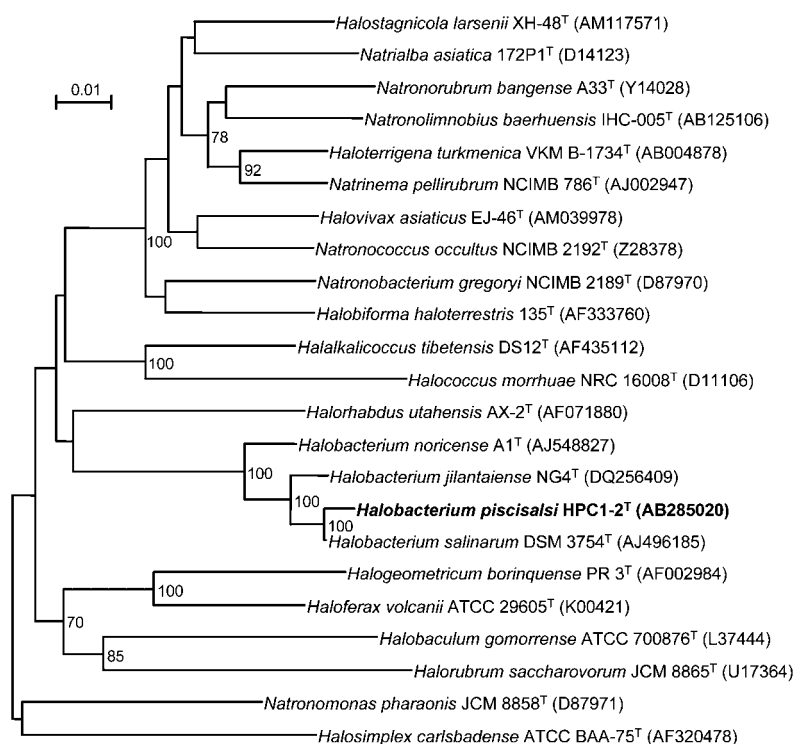


Fig. 2. Phylogenetic tree showing the relationships between strain HPC1-2^T and related archaeal species based on 16S rRNA gene sequences. The branching pattern was generated according to the neighbour-joining method. Bootstrap percentages above 70 %, based on 1000 replications, are shown at the nodes. Bar, 0.01 substitutions per 100 nucleotide positions.

and novobiocin (5 µg), but not to ampicillin (10 µg), chloramphenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), rifampicin (30 µg), streptomycin (10 µg) or tetracycline (30 µg). The major polar lipids are C₂₀C₂₀ derivatives of PG, PGP-Me, PGS, TGD, S-TGD and S-TeGD. The predominant menaquinone is MK-8. The G + C content of the DNA is 65.5 mol%.

The type strain, HPC1-2^T (=BCC 24372^T=JCM 14661^T=PCU 302^T), was isolated from *pla-ra* (salt-fermented fish product) in Thailand.

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Degradation of histamine by extremely halophilic archaea isolated from high salt-fermented fishery products

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ABSTRACT

The presence of high level of histamine is detrimental to the quality and safety of fish sauce. Therefore, this study aimed to study the ability of extremely halophilic archaea to reduce histamine under high salt condition and to examine the enzyme activity potentially involved. Of 156 extremely halophilic archaea isolated from various salt-fermented fishery products, HDS3-1 from an anchovy fish sauce sample fermented for 3 months, exhibited the highest histamine degradation activity when cultured in halophilic medium containing 5 mM histamine (free-base), followed by HDS1-1, HPC1-2, and HIS40-3, respectively. HDS3-1 was classified as *Natrinema gari* based on 16S rRNA gene sequence similarities and did not exhibit decarboxylase activity toward all tested amino acids. Based on *in vitro* cytotoxicity assay, the treatment with whole cell extract of HDS3-1 to all cell lines tested resulted in dose-dependent inhibitions of the cell growth with the IC₅₀ values higher than 250 µg ml⁻¹. Histamine-degrading activity of HDS3-1 was located in the intracellular fraction and required 1-methoxy-5-methylphenazinium methyl-sulfate (PMS) as an electron carrier. The optimal pH, salt concentration, and temperature for histamine degradation were pH 6.5–8, 3.5–5 M NaCl, and 40–55 °C, respectively. The activity was fully retained at pH 6.5–9, in the presence of NaCl above 2.5 M, and at temperature lower than 50 °C. The results suggested that histamine-degrading activity of HDS3-1 was most likely associated with salt-tolerant and thermo-neutrophilic histamine dehydrogenase.

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1. Introduction

The presence of high levels of histamine is detrimental to the quality and safety of foods, particularly fish sauce and other fermented fishery products from scombroid species. Histamine in foods is mainly induced by histamine decarboxylase activity from several kinds of bacteria. The presence of histamine in fermented foods does not usually represent any health hazard to individuals unless large amounts are ingested. Typical symptoms may be observed in certain individuals, and include nausea, sweating, headache, and hyper- or hypotension [1]. The Food and Drug Administration (FDA) established an advisory level of 500 ppm to be toxic to human health [2]. Histamine is heat stable and is not detectable through organoleptic analysis by even trained panelists. Except for the gamma irradiation, no other food processing

methods are available for histamine degradation [3]. Therefore histamine, if present, is difficult to destroy and posts a risk of food intoxication.

The presence of histamine-degrading enzymes either histamine oxidases or histamine dehydrogenases has been reported in various higher organisms [4–6] as well as in microorganisms [7–10]. Therefore, the application of starter strains possessing histamine-degrading activity might be a way for decreasing the amount of histamine produced *in situ* [5,7]. Nevertheless, the applications of these microorganisms and enzymes have been restricted by unfavorable physiological conditions for growth and enzyme activity such as low oxygen concentration, low pH value, undesirable temperature, and especially in the high salinity. The extremely halophilic archaea, in particular, are well adapted to saturated NaCl concentrations (grow optimally above 3.4–5.1 mol l⁻¹ or 20–30% NaCl). They have a number of novel molecular characteristics, especially for their enzymes that function in high salt concentration (3–4 M NaCl), such as lipase [11], protease [12], and glucose dehydrogenase [13]. Therefore, in this present study, extremely

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halophilic archaea isolated from several salt-fermented products were screened for the ability to degrade histamine in high salt condition.

2. Materials and methods

2.1. Chemicals

Histamine dihydrochloride, dimethyl sulfoxide (DMSO) and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) were purchased from Sigma Chemical Company (St. Louis, MO). The other chemicals were of analytical grade.

2.2. Isolation and culture conditions

Extremely halophilic archaea were isolated from various salt-fermented fishery products made in Thailand. Fish sauce samples were collected during early, middle, and late stages of the fermentation from many factories, whereas other fermented fish samples were obtained from local markets. The samples were spread onto agar plates of halophilic medium [per litre: 250 g NaCl, 5 g Casamino acids, 5 g yeast extract, 1 g sodium glutamate, 2 g KCl, 3 g trisodium citrate, 20 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.036 g $\text{FeCl}_3 \cdot 4\text{H}_2\text{O}$, 0.36 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 20 g agar (pH 7.2)]. The plates were incubated at 37 °C for 7 days. Several colonies with different morphological characteristics were picked. A pure culture was obtained by repeated transfers of separated colonies on agar plates of the same medium. The single colony was inoculated into the halophilic broth and incubated at 37 °C in a shaker incubator (Sartorius, Certomat® BS-1, Goettingen, Germany) at 200 rpm for 7 days. Cells were harvested by centrifugation of cultured broth at $10,000 \times g$ at 4 °C for 15 min. The pellet was washed twice with halophilic liquid medium. The obtained pellet was suspended in halophilic liquid medium containing 20% (v/v) glycerol and placed in a 2 ml cryotube. The stock cultures were kept in the –20 °C freezer until used.

2.3. Screening of histamine-degrading halophilic archaea

The inoculum was prepared by inoculating 250 μl of stock culture into 5 ml of halophilic medium and incubated at 37 °C in a shaker incubator at 200 rpm for 7 days. Inoculum was subsequently added at 5% (v/v) into halophilic liquid medium containing 5 mM histamine (free-base) and incubated at 37 °C in a shaker incubator at 200 rpm for 7 days. The supernatant was obtained by centrifugation of cultured broth at $10,000 \times g$ at 4 °C for 15 min. The histamine concentration of supernatant was measured following the AOAC method [14]. The histamine-degrading activity was expressed as the percentage degradation of histamine in the supernatant.

2.4. Identification of selected strains by 16S rRNA gene sequencing

DNA was isolated and purified according to the method of Saito and Miura [15]. The 16S rRNA gene sequences consisting of more than 1200 bp were PCR amplified with primers D30F (5'-ATTCGGTTCATCCTGC-3', positions 6–22 according to the *Escherichia coli* numbering system) and D56R (5'-GYTACCTGTGTACGACTT-3', positions 1492–1509). The amplified DNA fragment was separated by agarose gel electrophoresis and recovered by using GenElute Minus EtBr Spin Column (Sigma). The sequence was determined using BigDye Terminator Cycle Sequencing Ready Reaction kit (ver. 3.0: Applied Biosystems) in the ABI PRISM 310 Genetic analyzer (Applied Biosystems) with the following primers: D30F, D33R (5'-TCGCGCTGCGCCCGT-3', positions 344–360), D34R (5'-GGTCTCGCTGCTGCCTG-3', positions 1096–1113), and D56R. The sequence was compared with reference 16S rRNA gene sequences available in the GenBank and EMBL databases obtained from the National Center for Biotechnology Information database using BLAST searches. The alignment was subjected to phylogenetic analysis with the neighbour-joining [16] by using programs in the CLUSTALX [17] and MEGA4 packages [18]. Confidence in the branching pattern was assessed by analysis of 1000 bootstrap replicates [19].

2.5. Test for amino acid decarboxylation

The inoculum of the selected strains was inoculated to halophilic liquid medium supplemented with histidine, tryptophan, lysine, ornithine, arginine, tyrosine, and phenylalanine at 1% (w/v) at the same time. The cultures were incubated at 37 °C in a shaker incubator at 200 rpm for 7 and 14 days. The supernatant was collected by centrifugation of cultured broth at $10,000 \times g$ at 4 °C for 15 min. Biogenic amines in supernatant were extracted and derivitized according to the procedure of Eerola et al. [20] with a slight modification. In brief, the supernatant (480 μl) was added with 20 μl of 2 mg ml^{-1} 1,7-diaminoheptane as the internal standard and 500 μl of 0.4 M perchloric acid. The mixture was mixed thoroughly by a vortex mixer for 5 min and then centrifuged at a speed of $15,000 \times g$ at 25 °C for 5 min. The obtained supernatant (300 μl) was mixed with 60 μl of 2N NaOH and 90 μl of saturated sodium bicarbonate. A 600 μl of 10 mg ml^{-1} dansyl chloride was added to each sample, mixed, and then incubated for 45 min at 40 °C. Residual dansyl chloride was removed by adding 30 μl of 25% (v/v) ammonia and centrifuged at $3500 \times g$ for 5 min. The supernatant was collected and filtered through a 0.45 μm membrane filter prior to analysis by a high performance liquid chromatography (HPLC). A Waters Separation Module 2690

was operated to give a flow rate of 1.5 ml min^{-1} of the mobile phase including 0.1% (v/v) acetic acid (solvent A) and acetonitrile containing 0.1% (v/v) acetic acid (solvent B). Elution program was carried out as follows: a linear gradient from 45% to 60% B in 15 min, a linear gradient from 60% to 90% B in 10 min, an isocratic elution at 90% B for 5 min, a linear decrease from 90% to 45% B in 2 min, followed by an isocratic elution at 45% B for 5 min. Separation was achieved using a column of Hypersil BDS C18 (300 mm $\times$ 7.8 mm i.d.) set at 40 °C. Samples (20 μl) were injected and a photo diode array (Model Waters 996), set at the wavelength of 254 nm, was used as the detector. Data were processed and analyzed using Millennium 32 software (Waters, Milford, MA, USA).

2.6. Cytotoxicity testing

The cytotoxicity of the selected strain was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay [21] according to the published standard methods of BS-EN30993-5 and ISO10993-5 [22] using human colon adenocarcinoma (Caco2), human liver hepatocarcinoma (HepG2), and human larynx epithelial (HEp-2) cells as the target cell lines. The MTT assay monitors a reduction of yellow tetrazolium salt by mitochondrial dehydrogenase enzymes of metabolically active/viable cells to purple formazan crystals. The formation of formazan can be analysed spectrophotometrically at a wavelength of 570 nm with a plate reader after solubilization with DMSO. To prepare whole cell extract, 500 mg of wet cell paste of HDS3-1 was resuspended in 50 mM Tris–HCl, pH 7.0 containing 4.5 M NaCl at a ratio of 1:1 (w/v). The cell suspension was sonicated for 20 s, followed by a 40 s rest interval with a total of 2 min sonication by a Vibra Cell VCX60 (Sonics and Materials Inc., Newtown, CT, USA). The obtained suspension was freeze-dried, dissolved in 0.1N NaOH at 37 °C for 1 h and then centrifuged at $15,000 \times g$ for 30 min. The supernatant was collected and adjusted to give a stock concentration of 25 mg dry extract/ml. The sample extract was diluted with the growth medium to give the final concentrations ranging from 2 to 250 $\mu\text{g ml}^{-1}$ and added to the target cells previously grown for 48 h in a 96-well plate. After incubation for 24 h, the tested sample was removed. Cells were incubated for 24 h in fresh medium and then tested with MTT assay. Briefly, 50 μl of MTT in 0.1 M phosphate buffer containing 0.9% NaCl (PBS) at 5 mg ml^{-1} was added and the cells were incubated for 4 h. Medium and MTT were then aspirated from the wells. Formazan formed was solubilised with 200 μl of DMSO and 25 μl of Sorensen's Glycine, pH 10.5. The optical density was read with a microplate reader (Molecular Devices) at a wavelength of 570 nm. A dose–response curve was derived from the plot of percentage of cell viability against the concentration of sample. IC_{50} or the concentration required to kill 50% of the cells compared to control was analysed with the SoftMax Program (Molecular Devices).

2.7. Localization of the histamine-degrading activity

The localization of the histamine-degrading activity was determined by using the method of Vyazmensky et al. [23] with some modifications. Ten millilitres of cultured broth were prepared as previously described. The cell suspension was centrifuged at $15,000 \times g$ for 30 min and the supernatant was collected and referred to as 'extracellular fraction'. To obtain membrane and intracellular fractions, the cells were washed twice with 4.5 M NaCl. The cells were resuspended in 50 mM Tris–HCl, pH 7.0 containing 3.5 M KCl at a ratio of 1:1 (w/v). The cell suspension was sonicated for 20 s, followed by a 40 s rest interval with a total of 2 min sonication by a Vibra Cell VCX60 (Sonics and Materials Inc., Newtown, CT, USA). The supernatant was collected by centrifugation at $15,000 \times g$ for 30 min. The obtained fraction was referred to as 'intracellular fraction'. The membrane pellet was washed twice with 50 mM Tris–HCl buffer (pH 7.0) containing 3.5 M KCl and then extracted with 2% (v/v) Triton X-100 in the same buffer by stirring for 45 min at 4 °C. The membrane associated fraction was collected by centrifugation at $15,000 \times g$ for 30 min at 4 °C. All fractions were stored at 4 °C until used.

2.8. Histamine-degrading activity assay

Histamine-degrading activity was determined by measuring the decreased amount of histamine after reaction. The sample with an appropriate dilution (100 μl) was mixed with 0.9 ml of reaction mixture consisting of 55 mM Tris–HCl, pH 7.0, 5.0 M NaCl, 5.0 mM histamine dihydrochloride (free-base), and 555 μM 1-methoxy PMS. The reaction was incubated at 37 °C for 5, 10, 15, 20, 30, 60, and 90 min. At the time designated, the reaction was terminated by adding 1 ml of 0.1N HCl. The concentrations of histamine were estimated by HPLC and the fluorometric method of AOAC [14]. A blank was run in the same manner, except the sample was added after addition of 0.1N HCl. One Unit of enzyme activity was defined as the amount of enzyme that catalyzed a reduction of 1 μmole of histamine per min under the specified conditions.

2.9. Protein determination

Protein concentration was determined by the method of Bradford [24]. Bovine serum albumin was used as a standard.

3. Characterization of the histamine-degrading activity of selected strain

3.1. Substrate preference

The substrate preference of the crude enzyme for degradation of various amines including tryptamine, β -phenylethylamine, putrescine, cadaverine, tyramine, spermidine, and spermine was determined by replacing histamine in the reaction mixture with those amines. The reactions were determined at 45 °C in an incubator shaker with a speed of 200 rpm for 1 h. The reactions were terminated by adding 1 ml of 0.4 M perchloric acid. The mixture was centrifuged at 10,000 $\times$ g for 15 min. Blank was done in the same manner, except the crude enzyme was added after addition of 1 ml 0.4 M perchloric acid. The content of biogenic amines in reaction mixture and blank were extracted, derivatized according to the procedure of Eerola et al. [20] with a slight modification, and determined by a HPLC as described above.

3.2. Effect of salt concentration on activity and stability

To remove salt, crude enzyme was dialyzed against 100 volumes of 50 mM Tris–HCl, pH 7.0 for 12 h with two changes of dialysis buffer at 4 °C. The activity of desalted sample was assayed at pH 7.0, 37 °C in the presence of NaCl at various concentrations (0–5 M). To study the effect of NaCl on stability, the desalted sample was dialyzed against 100 volumes of buffers containing different NaCl concentrations (0–5 M) at room temperature for 24 h. The remaining activity was assayed at pH 7.0, 37 °C in the presence of NaCl of 4.5 M.

3.3. Effect of pH on activity and stability

The activity was assayed at 37 °C over the pH range of 4–9 (50 mM acetate buffer (pH 4.0–5.5); 50 mM phosphate buffer (pH 6.0–6.5); and 50 mM Tris–HCl buffer (pH 7.0–9.0)) in the presence of 4.5 M NaCl. To study the effect of pH on stability, the enzyme solution was mixed with the same buffers but their concentrations were changed to 100 mM in an equal volume and incubated for 24 h at 30 °C. The remaining activity was assayed at pH 7.0, 37 °C in the presence of NaCl of 4.5 M.

3.4. Effect of temperature on activity and stability

Enzyme activity was assayed at different temperatures in the range of 30–80 °C as previously described. To study the effect of temperature on enzyme stability, the dialyzed enzyme in 50 mM Tris–HCl buffer, pH 7.0 was incubated at various temperatures ranging from 4 to 80 °C for 1 h in the absence and presence of 4.5 M NaCl. Thereafter, the enzyme mixture was immediately cooled in an ice bath, and the remaining activity was assayed at pH 7.0, 37 °C in the presence of NaCl of 4.5 M. Relative activity of the enzyme was calculated, in comparison with that without heating.

3.5. Statistic analysis

All experiments were run in triplicate. 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 [25]. Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for Windows: SPSS Inc., Chicago, IL, USA).

4. Results and discussion

4.1. Screening and identification of histamine-degrading halophilic archaea

A total of 156 halophilic archaea strains were isolated from various salt-fermented fishery products made in Thailand. Of 60 strains that exhibited histamine-degrading activity, HDS3-1 from 3-month fermented fish sauce exhibited the highest histamine-degrading activity followed by HDS1-1, HPC1-2, and HIS40-3, isolated from anchovy fish sauce fermented for 1 month, *pla-ra* (salt-fermented fish product of Thailand), and an anchovy fish sauce sample fermented for 40 days, respectively. All of the selected strains showed characteristics of extremely halophilic archaea that required salt at concentrations higher than 1.7 M NaCl to prevent cell from lysis and showed the maximum growth rates at 2.6–4.3 M NaCl (data not shown). Analysis of 16S rRNA sequences of these selected strains indicated that all isolates were in the phylogenetic branch of the *Halobacteriales* (Fig. 1). Strain HPC1-2 was placed with the genus *Halobacterium* in which HPC1-2 was designated as *Halobacterium piscisalsi* [26]. On the other hand, strains HDS3-1, HDS1-1, and HIS40-3 were placed in a single cluster affiliated to the genus *Natrinema* in which strains HIS40-3 and HDS3-1 constituted a new species, for which the name *Natrinema gari* was proposed [27]. Due to some differences from other reference species of *Natrinema*, strain HDS1-1 was likely to be a novel member in this genus. Other genotypic and chemo-taxonomic characteristics were under investigation.

The ability of microorganisms to degrade histamine has been mainly observed among members of *Lactobacillus*, *Micrococcus*, *Klebsiella*, and *Staphylococcus* [28]. However, histamine-degrading activity from these microbial sources might decline rapidly due to high salt content (25% NaCl) and acidic pH (5.5) of fish sauce. Therefore, the addition of these microorganisms as a source of histamine-degrading enzyme might not be an effective means to degrade histamine. To our knowledge, there was only one study that demonstrated the histamine-degrading activity of *Virgibacillus halodenitrificans* SK33, moderately halophilic bacteria isolated from Thai fish sauce under the high salt condition [29]. Although 50% of histamine level was reduced, the addition of this strain in fish sauce fermentation resulted in the increase in tyramine content, almost 2 times higher than the control without inoculation, during the course of 4-month fermentation. Tyramine is a vasoactive monoamine thought to be the cause of the majority of pharmacologic adverse food reactions. Excess levels of tyramine exert an indirect sympathomimetic effect and may result in migraine headaches, palpitations and even hypertensive crisis in sensitive individuals [30].

4.2. Amino acid decarboxylation

The amine producing capacity of these strains was tested in halophilic liquid medium supplemented with 1% (w/v) of the corresponding amino acid substrates. No formation of histamine, tryptamine, β -phenylethylamine, putrescine, cadaverine, tyramine, spermidine or spermine which showed the toxic effects was detected at 7 and 14 days of incubation (data not shown). The ability of microorganisms to decarboxylate amino acids is highly variable, depending on species, strain, and environmental conditions [31]. It was noted that no undesirable compounds especially biogenic amines were formed by the use of these selected microorganisms. Since, HDS3-1 exhibited the highest histamine-degrading activity among strains tested and did not produce any biogenic amines, it was selected for further study.

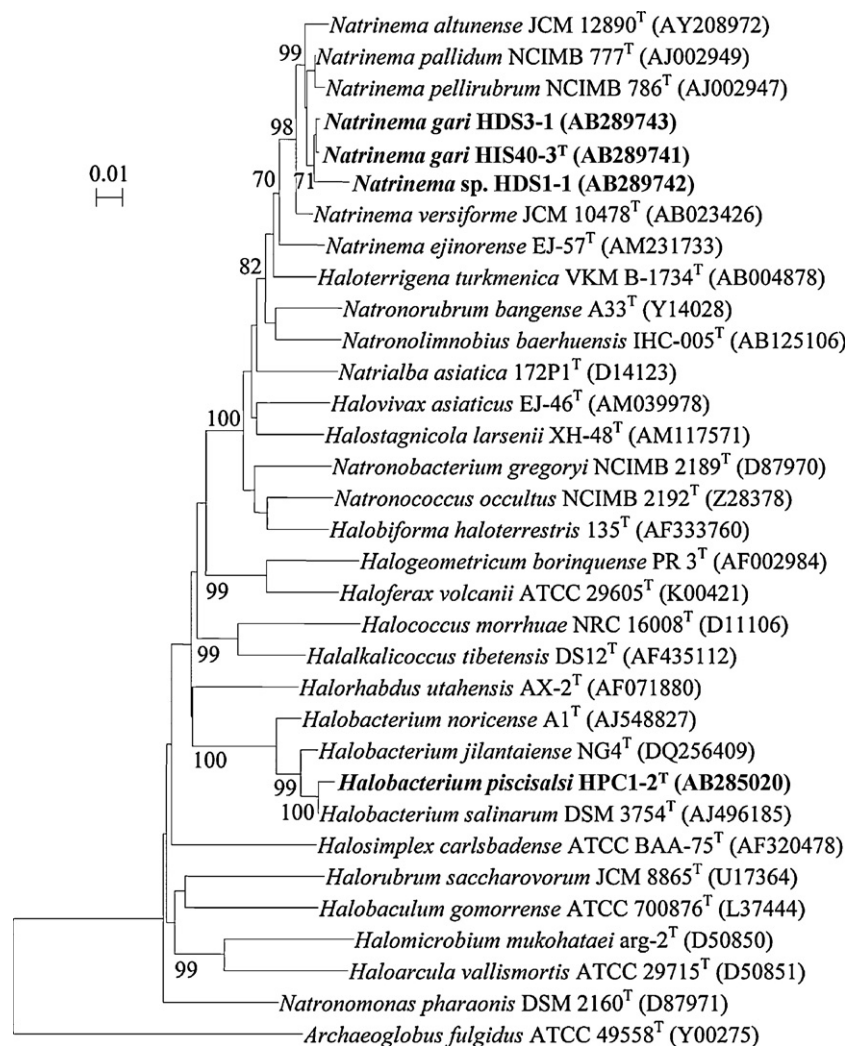


Fig. 1. Phylogenetic tree showing the relationships between strain HIS40-3, HDS1-1, HDS 3-1, HPC1-2, and related bacterial species, based on 16S rRNA gene sequences. The branching pattern was generated by the neighbour-joining method. Bootstrap values higher than 70 out of 100 subreplicates are indicated at the respective bifurcations. The bar represents 0.01 substitutions per nucleotide.

4.3. Cytotoxicity test

The *in vitro* cytotoxic potential of strain HDS3-1 towards Caco2, HepG2, and HEp-2 cells was assessed by measuring total cellular metabolic activity using the MTT assay (Table 1). From the cytotoxicity profiles, the treatment with whole cell extract (2–250 μg for 24 h) to all cells tested resulted in dose-dependent inhibitions of the cell growth. The IC_{50} values in all cell lines tested were higher than 250 $\mu\text{g ml}^{-1}$. However, HEp-2 seemed to be the most sensitive to HDS3-1 cell extracts compared to Caco-2 and HepG2 which widely used with *in vitro* assays to predict the absorption rate of drug compounds across the intestinal epithelial cell barrier and to examine the regulation of a wide variety of liver-specific metabolic

functions, respectively [32,33]. Differential sensitivities of different cell lines have been reported, however little is known so far for the mechanism. While the cytotoxicity tests are useful for screening chemicals for their intrinsic and relative toxicities, to correctly predict oral systematic toxicity *in vivo*, additional data should be collected using *in vivo* studies.

4.4. Localization of histamine-degrading enzymes from *Natrinema* sp. HDS3-1

To examine the cellular location of histamine-degrading activity of HDS3-1, extracellular, intracellular, and membrane-bound fractions were assayed in the presence and absence of 1-methoxy PMS.

Table 1
Cytotoxicity of whole cell extract of strain HDS3-1.

Target cell line	Whole cell extract of strain HDS3-1 ($\mu\text{g ml}^{-1}$)								IC_{50} ($\mu\text{g ml}^{-1}$)
	250	125	62.5	31.25	15.625	7.8125	3.906	1.953	
Caco2	71 $\pm$ 0.3	88 $\pm$ 0.2	79 $\pm$ 0.1	87 $\pm$ 0.5	85 $\pm$ 0.2	90 $\pm$ 0.2	92 $\pm$ 0.2	98 $\pm$ 0.1	>250
HepG2	81 $\pm$ 0.4	83 $\pm$ 0.1	87 $\pm$ 0.5	92 $\pm$ 0.2	96 $\pm$ 0.1	97 $\pm$ 0.2	95 $\pm$ 0.2	92 $\pm$ 0.1	>250
HEp-2	56 $\pm$ 0.2	57 $\pm$ 0.2	63 $\pm$ 0.3	61 $\pm$ 0.1	69 $\pm$ 0.1	74 $\pm$ 0.3	84 $\pm$ 0.2	99 $\pm$ 0.9	>250

Abbreviations: human colon adenocarcinoma (Caco2); human liver hepatocarcinoma (HepG2); and human larynx epithelial carcinoma (HEp-2) cells. MTT results are expressed in percent of cells' viability compared to the control.

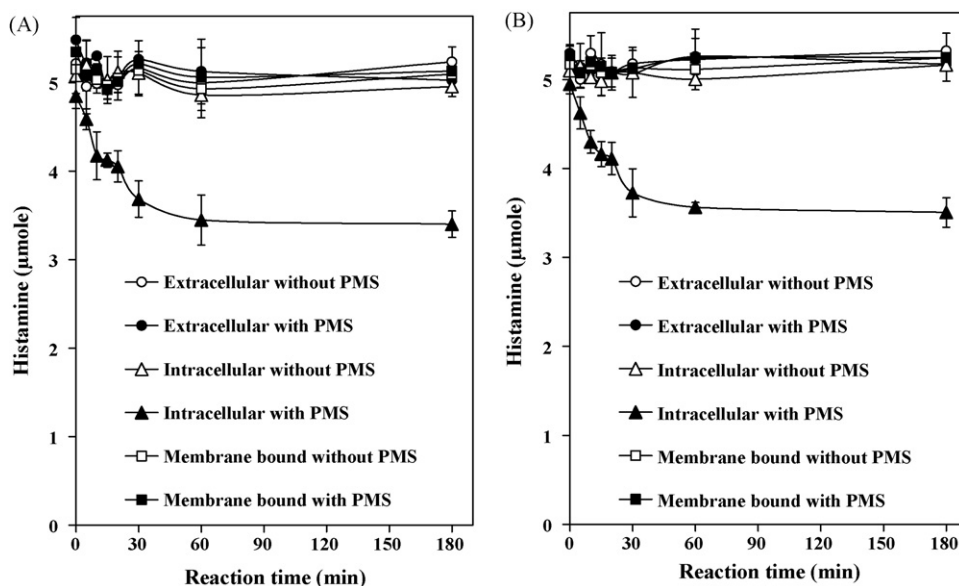


Fig. 2. Time course of histamine-degrading activity from various cellular fractions of HDS3-1 measured by the HPLC (A) and AOAC fluorometric detection (B). Bars represent the standard deviation ($n = 3$). The degradation of histamine was analyzed for 180 min at pH 7.0 and 45 °C in the presence of 4.5 M NaCl. The histamine-degrading activity for the intracellular fraction was 65.0 ± 12.8 Units ml^{-1} (38.4 ± 7.5 Units mg^{-1}).

By using both HPLC and AOAC methods, the histamine content in the reaction mixture of intracellular fraction in the presence of 1-methoxy PMS decreased about one-third of its initial concentration within 30 min and remained constant until the end of 90 min incubation (Fig. 2). In the absence of 1-methoxy PMS, no decrease in histamine content was observed. Since the intracellular fraction of HDS3-1 required the presence of 1-methoxy PMS, a versatile electron carrier that mediates electron transfer between NADH and various electron acceptors in electron transfer systems [34], the results suggested a link to the activity of dehydrogenase.

In other microorganisms, amine dehydrogenases were found to be localized in the various locations of the cells; the periplasmic space, membrane, as well as cytoplasmic space. Amine dehydrogenase is known to be enzyme that catalyzes the oxidative deamination of amines to its corresponding aldehydes and ammonia. Various amine dehydrogenases have been reported so far mostly in prokaryotes such as *Methylomonas* sp. [35], *Paracoccus denitrificans* [36], *Nocardioides simplex* [9], and *Alcaligenes xylosoxidans* [37]. Moreover, these enzymes were found to be both constitutive and inducible. Although many bacteria possess the enzymes for primary amine deamination, the toxicity of the aldehydes generated during the oxidation processes is of the major drawback [38].

5. Characterization of histamine-degrading activity of selected strain

5.1. Substrate preference

The intracellular fraction of HDS3-1 preferably catalyzed histamine rather than other amines tested (Fig. 3). Relative activities of 20% or less were observed for other biogenic amines. Many amine dehydrogenases found in various bacteria showed different specificity [35–37]. The purified amine dehydrogenase from *Pseudomonas* AM1 had a wide specificity, oxidizing many compounds containing a primary amino group, notably ethanolamine and histamine [38]. Whereas, the purified histamine dehydrogenase from *N. simplex* IFO 12069 showed high activity toward histamine, but it still oxidized agmatine and putrescine at a rate of 30%, compared to that of histamine [9]. Among 20 biogenic amines including

histamine studied by Bakke et al. [8], the purified recombinant histamine dehydrogenase from *Rhizobium* sp. 4-9 was very specific toward histamine and oxidized agmatine and 1,3-diaminopropane with the rate of 10 and 13% of that of histamine, respectively. Fujieda et al. [39] indicated that the clear difference between histamine dehydrogenase and other amine dehydrogenases is the substrate specificity. Histamine dehydrogenase is active almost exclusively toward histamine, while low activity is observed for other amines. The histamine-degrading activity of HDS3-1 was thought to be mediated by histamine dehydrogenase, though the concomitant production of the degradation products, i.e. imidazole acetaldehyde and ammonia [39], was not demonstrated in the present study.

5.2. Effect of salt concentration on activity and stability

The highest histamine-degrading activity of the intracellular fraction from HDS3-1 was observed in the presence of NaCl at the concentration range of 3.5–5 M (Fig. 4A). In the absence of salt, the activity was less than 20% of that obtained under optimum

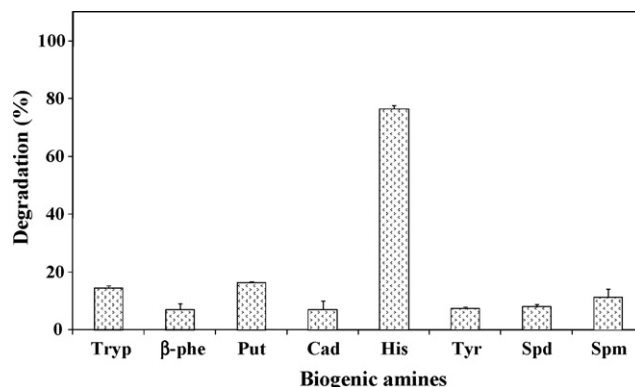


Fig. 3. Degradation of biogenic amines by an intracellular fraction of HDS3-1. Reaction was carried out in the presence of 4.5 M NaCl at 45 °C, pH 7 under shaking condition (200 rpm) for 1 h. Bars represent the standard deviation ($n = 3$). Tryp, tryptamine; β -phe, β -phenylethylamine; Put, putrescine; Cad, cadaverine; His, histamine; Tyr, tyramine; Spd, spermidine; Spm, spermine. Bars represent the standard deviation ($n = 3$).

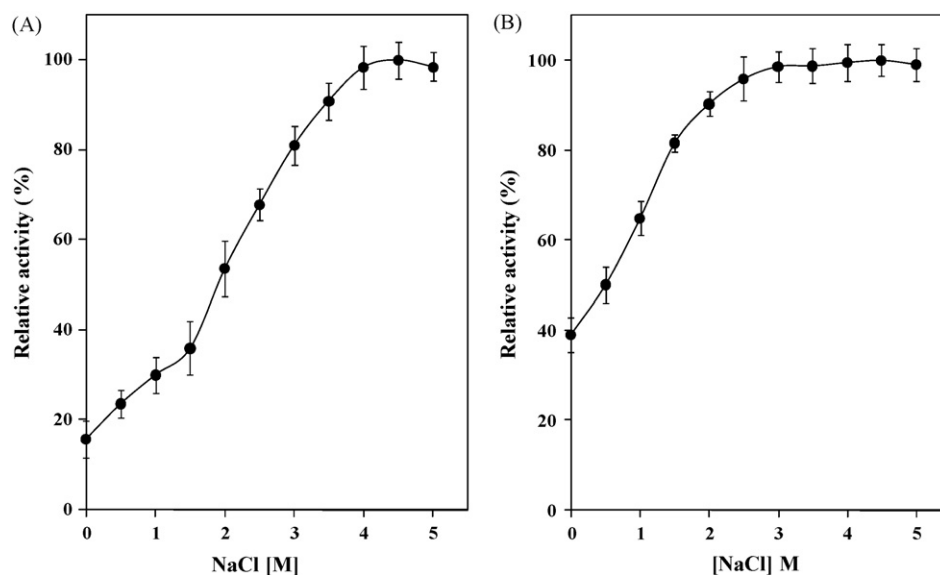


Fig. 4. Effect of NaCl concentration on histamine-degrading activity (A) and stability (B) of HDS3-1. Bars represent the standard deviation ($n = 3$). (A) Residual activity was analyzed using histamine as a substrate for 30 min at pH 7.0 and 37 °C at the various NaCl concentrations. (B) For the stability test, the enzyme was incubated at 30 °C for 24 h in 50 mM Tris–HCl buffer (pH 7.0) containing NaCl at various concentrations. Residual activity was analyzed using histamine as a substrate for 30 min, pH 7.0 in the presence of 4.5 M NaCl at 37 °C.

conditions. This result clearly indicated that the histamine-degrading activity was salt-dependent. The histamine-degrading activity was stable for at least 24 h when incubated in the presence of NaCl at the concentration higher than 2.5 M (Fig. 4B). Less than 50% of the residual activity was detected at the NaCl concentrations below 1 M. In general, many halophilic enzymes require NaCl concentrations in the range of 1–4 M for optimal activity and stability [38]. On the basis of salt requirement, it was also noticed that the histamine-degrading activity of HDS3-1 could be regained upon reestablishment of the high salt concentration. This was similar to several halophilic enzymes of various extreme halophiles [11–13]. Halophilic proteins employ different adaptation mechanisms. Proteins from halophilic organisms have a biased amino acid composition in order to remain stable and active at high ionic strength. Halophilic proteins typically have an excess of acidic

amino acids (i.e. glutamate and aspartate) on their surface [11–13]. Negative charges on the halophilic proteins bind to hydrated ions, thereby reducing their surface hydrophobicity and decreasing the tendency to aggregate at high salt concentration. At the molecular level, the high flexibility of protein structure could allow the rapid change from the fully active form in the presence of salt to an inactive form in the absence of salt. Although there was no explanation for the reestablishment of histamine-degrading activity when the enzyme was restored to high salt concentrations, but it appeared that the enzyme is a trimeric protein (unpublished data).

5.3. Effect of pH on activity and stability

The highest histamine-degrading activity was observed in the pH range of 6.5–8 (Fig. 5A). At pH 4–4.5, no activity was detected.

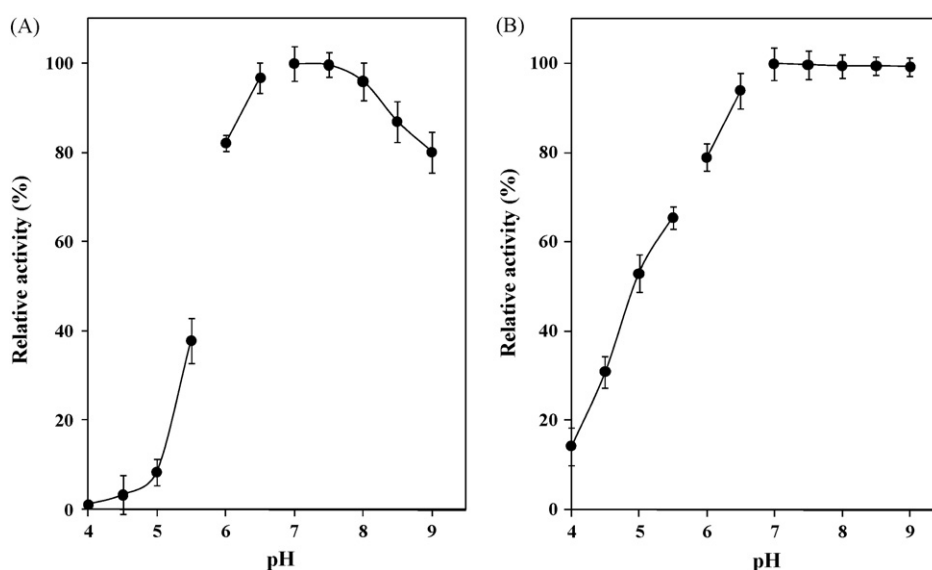


Fig. 5. pH profile (A) and stability (B) of histamine-degrading activity of HDS3-1. Bars represent the standard deviation ($n = 3$). (A) Residual activity was analyzed using histamine as a substrate for 30 min at 37 °C in the presence of 4.5 M NaCl at various pHs. (B) For the stability test, the enzyme was incubated at 30 °C for 24 h in the desired pH buffer at various pH values in the presence of 4.5 M NaCl. Residual activity was analyzed using histamine as a substrate for 30 min at 37 °C, pH 7.0 in the presence of 4.5 M NaCl.

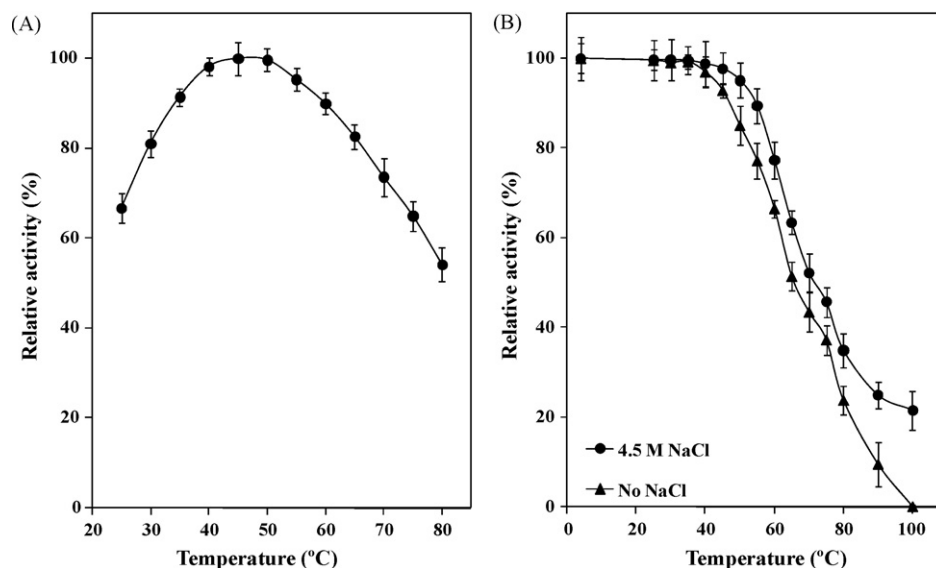


Fig. 6. Temperature profile (A) and thermal stability (B) of histamine-degrading enzyme from HDS3-1. Bars represent the standard deviation ($n = 3$). (A) Residual activity was analyzed using histamine as a substrate for 30 min at pH 7.0 in the presence of 4.5 M NaCl at various temperatures. (B) For the stability test, the enzyme was incubated for 1 h at the temperatures indicated in 50 mM Tris–HCl buffer (pH 7.0) in the presence or absence of 4.5 M NaCl, and then cooled on ice. Residual activity was analyzed using histamine as a substrate for 30 min at pH 7.0 in the presence of 4.5 M NaCl at 45 °C.

As the pH increased, the activity increased rapidly until reaching the maximum. Above pH 8, the activity decreased. However, 80% activity was retained at pH 9. The pH optimum of the histamine dehydrogenase from the HDS3-1 was lower than those from *A. xylosoxidans* (pH 8.0) [37], *N. simplex* IFO 12069 (pH 8.5) [9], and *Rhizobium* sp. 4-9 (pH 9.0) [8]. The histamine-degrading activity was stable for at least 24 h when incubated in the buffers at pH 6.5–9 (Fig. 5B). Nevertheless, the activity gradually decreased under acidic condition in which the residual activity was lower than 50% at pH below 5. This reflected a pH dependent stability of the enzyme. The stability over the wide pH range was reported for aromatic amine dehydrogenase from *A. xylosoxidans* (pH 5–12) [37], methylamine dehydrogenases from *Methylobacterium extorquens* AM1 (pH 2.6–10.6) [35], *Methylomonas* sp. (pH 4.4–10.6) [35], and *Paracoccus denitrificans* (pH 3.0–10.5) [36].

5.4. Effect of temperature on activity and stability

The highest histamine-degrading activity was observed at 40–55 °C (Fig. 6A). The enzyme retained more than 50% at 80 °C. The enzyme activity was quite stable at 50 °C with residual activities of 95% and 80% for incubation with and without 4.5 M NaCl, respectively. However, at higher temperatures, the effect of NaCl was more pronounced as demonstrated by the divergence of the two profiles (Fig. 6B). No activity was detected in the salt free preparation incubated at 100 °C for 1 h while the preparation incubated with NaCl still had a residual activity of 22%. Although it was interesting that there was reasonable thermal stability up to 80 °C under the condition without salt, the histamine-degrading activity was very low. So, only the optimum temperature in the presence of salt was studied. These results clearly showed that the thermal stability of the histamine-degrading activity of HDS3-1 increased in the presence of NaCl. This characteristic was similar to that of most enzymes from halophilic archaea. The effect of salt on the thermal stability of enzymes was variable in non-halophilic archaea [11]. However, it was frequently found that thermal stability of both intracellular and extracellular enzymes of the halophilic archaeons could be improved by NaCl [11,13]. Reversible refolding after high temperature treatment has been demonstrated for the β -lactamase of the moderately halophilic eubacterium *Chro-*

mohalobacter by Tokunaga et al. [40], who claimed that irreversible aggregation was prevented by the abundance of acidic amino acids, which in turn is a characteristic feature of halophilic enzymes [41].

6. Conclusion

Halophilic archaeon, strain HDS3-1, isolated from fish sauce exhibited the ability to degrade histamine in hypersaline condition. The histamine-degrading activity of HDS3-1 was located in the intracellular fraction and required an electron acceptor. The histamine-degrading activity was highest at pH 6.5–8, in the presence of NaCl at 3.5–5 M, and at 40–55 °C. The activity was found to be stable at pH 6.5–9, in the presence of NaCl above 2.5 M, and at temperature lower than 50 °C. The results suggest that histamine-degrading activity of HDS3-1 was mediated by salt-tolerant and thermo-neutrophilic histamine dehydrogenase.

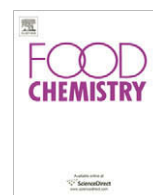
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Whole cell immobilisation of *Natrinema gari* BCC 24369 for histamine degradation

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ABSTRACT

Whole cells of *Natrinema gari* BCC 24369, a novel halophilic archaeon, were immobilised on various matrices by different techniques. Among all matrices tested, Celite showed the highest immobilisation yield. The immobilised whole cells retained histamine-degrading activity as high as 94% of the original activity detected in free whole cells. The catalytic properties of the immobilised whole cells on the Celite support were similar to the corresponding free whole cells, including optimal NaCl concentration (4.0–5.0 M), optimal pH (6.5–7.5), and optimal temperature (40–55 °C). Histamine-degrading activity, either in the presence of NaCl at elevated concentrations or at elevated temperatures, became more stable due to immobilisation. Both free and immobilised whole cells were able to degrade histamine specifically. The immobilised whole cells could be reused for the degradation of histamine up to seven cycles without any significant loss in activity. The immobilised whole cells have the potential to be applied for the degradation of histamine in products high in salt.

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1. Introduction

The presence of high levels of histamine in seafoods and seafood products may cause the rejection for import and is also associated with the food poisoning. Histamine is heat stable and not detectable through organoleptic analysis, even by trained panelists (Arnold, Price, & Brown, 1980). Except for gamma irradiation, no other food processing methods are available for histamine degradation (Etkind, Wilson, Gallagher, & Cournoyer, 1987; Kim et al., 2004).

Natrinema gari BCC 24369 is a novel halophilic archaeon (Tapingkae et al., 2008) that can reduce histamine in culture broth during cultivation in the presence of 4.3 M NaCl. The histamine-degrading activity was mediated through the intracellular enzyme and needed an electron acceptor for its activity (Tapingkae, 2009). Due to the limitations of applying *Nmn. gari* BCC 24369 cells and its enzyme caused by the slow growth rate, low yield of enzyme, and the cost of cell or enzyme preparation, the immobilisation of whole cells might provide potential advantages over applications of free cells and enzyme systems.

Cell immobilisation is the physical confinement or localisation of intact cells to a certain defined region of space with preservation

of some desired catalytic activity (Karel, Libicki, & Robertson, 1985). The use of immobilised whole microbial cells and/or organelles eliminates the often tedious, time-consuming, and expensive steps involved in isolation and purification of intracellular enzymes (Ohmiya, Ohashi, Kobayashi, & Shimizu, 1977). Additionally, the stability of enzyme can be enhanced by retaining its natural catalytic surroundings during immobilisation and subsequent continuous operation (Karel et al., 1985). Furthermore, metabolically active cell immobilisation is particularly preferred where co-factor regeneration machinery is necessary for the catalytic reaction (Bernal, Sevilla, Cánovas, & Iborra, 2007).

Immobilisation is commonly accomplished by using four techniques, physical entrapment within a porous matrix, attachment or adsorption to a pre-formed carrier, self-aggregation by flocculation and cell contained behind barrier (Pilkington, Margaritis, Mensour, & Russell, 1998). Nevertheless, a few critical parameters such as the cost of immobilisation, mass transport limitations and applicability to a specific end-product should be taken into consideration. To maximise the use of whole cells of the *Nmn. gari* BCC 24369 for degradation of histamine in the presence of high salt concentrations without cell disintegration and complex purification of the enzyme, the appropriate immobilisation technique should be applied. The objectives of this study were to select the most effective immobilisation method for whole cells of *Nmn. gari* BCC 24369 and to study the properties of both free and immobilised whole cells.

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2. Materials and methods

2.1. Culture and growth condition for *Nmn. gari* BCC 24369

Nmn. gari BCC 24369 was grown on an agar plate of halophilic medium (pH 7.2). The medium (1 l) contained 250 g NaCl, 5 g caseamino acid, 5 g yeast extract, 1 g sodium glutamate, 2 g KCl, 3 g trisodium citrate, 20 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.036 g $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 0.00036 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ and 20 g agar. The inoculum was prepared by inoculating a loopful of culture into 5 ml of halophilic broth and incubating at 37 °C in a shaker incubator (Sartorius, Certomat® BS-1, Goettingen, Germany) at 200 rpm for 7 days. Cells were cultivated by inoculating 5% (v/v) of seed culture into 200 ml of halophilic medium containing 500 ppm of histamine (free-base) in a 500-ml Erlenmeyer flask, and incubated at 37 °C in a shaker incubator at 200 rpm for 7 days. Cells were harvested in the exponential phase ($A_{600} \sim 5.0$) by centrifugation at 15,000g for 10 min and washed twice with 50 mM Tris-HCl buffer, pH 7.0 containing 4.3 M NaCl. The cell pellet was recovered by centrifugation at 15,000g for 10 min and referred to as wet cell paste.

2.2. Immobilisation of whole cells

2.2.1. Adsorption

Pig bone: The procedure of Negishi, Sato, Mukataka, and Takahashi (1989) was used with some modifications. Pig bone powder was washed with ethanol five times, followed by distilled water. Five grams of washed pig bone powder were added to 10 ml of 50 mM Tris-HCl buffer, pH 7.0, containing 4.5 M NaCl (referred to as standard buffer) and 1 g of wet cell paste. The mixture was agitated (100 rpm) for 1 h at 0–4 °C. The immobilised whole cells were filtered using Whatman paper No. 1, washed with a standard buffer until no protein was released, dried under vacuum for 3 h at room temperature and stored at 4 °C until used.

Chitosan flakes: The procedure of Pereira, Zanin, and Castro (2003) was applied with some modifications. Five grams of chitosan flakes (Bioshell Inc., OR) were soaked in hexane under agitation conditions (100 rpm) for 1 h, and air dried. The soaked chitosan flakes were added to 10 ml of standard buffer containing 1 g of wet cell paste. The mixture was agitated (100 rpm) for 3 h at room temperature, and allowed to stand for 18 h at 4 °C. The immobilised whole cells were filtered, washed, dried, and stored at 4 °C until used.

Celite: The procedure of Khare and Nakajima (2000) was used with some modifications. Celite® 545 (5 g) (Mallinckrodt Baker Inc., Phillipsburg, NJ) was added to 10 ml of standard buffer containing 1 g of wet cell paste. The mixture was agitated (100 rpm) for 1 h at 0–4 °C. The immobilised whole cells were filtered, washed, dried, and stored at 4 °C until used.

2.2.2. Entrapment

Agar: The procedure of Takeno, Yamaoka, and Sasaki (2005) was used with some modifications. Standard buffer containing 4% (w/v) agar (Difco; BD, Franklin Lakes, NJ) was autoclaved at 121 °C (15 psi) for 15 min. Warm agar solution (50 °C, 2.5 ml) was mixed with 2.5 ml of standard buffer containing 1 g of wet cell paste at 60 °C. This mixture was quickly poured into a petri dish to obtain a thickness of 8 mm and cooled at room temperature. The solidified agar was cut into 8 mm cubes ($\sim 8 \times 8 \times 8$ mm) and washed with standard buffer until no protein was released. The obtained immobilised whole cells were stored at 4 °C until used.

Ca^{2+} -alginate beads: The procedure of Takeno et al. (2005) was used with some modifications. A 2.5-ml aliquot of sodium alginate (Acros Organics, Fair Lawn, NJ) solution (3%, w/v) was mixed with 2.5 ml standard buffer containing 1 g whole cells. The slurry was

added dropwise (diameter ~ 2 mm) into 100 ml of 0.15 M CaCl_2 . After 30 min, the gel beads containing immobilised whole cells were washed and stored at 4 °C until used.

2.2.3. Cross-linking

Alginate-chitosan beads: The procedure of Vidyasagar, Prakash, and Sreeramulu (2006) was used with some modifications. A 2.5-ml aliquot of 3% (w/v) sodium alginate solution was mixed with 2.5 ml of standard buffer containing 1 g of wet cell paste. The slurry was added dropwise (diameter ~ 2 mm) into 100 ml of 0.5% (w/v) chitosan flakes dissolved in 3% (v/v) acetic acid solution containing 0.5% (w/v) CaCl_2 . After 30 min, the gel beads containing immobilised whole cells were washed and stored at 4 °C until used.

Cross-linked alginate beads: The procedure of Vidyasagar et al. (2006) was used with some modifications. A 2-ml aliquot of 3% (w/v) sodium alginate solution was mixed with 2 ml of standard buffer containing 1 g of wet cell paste. One millilitre of glutaraldehyde solution (0.5%, v/v) was added, followed by gentle mixing. The mixture was kept for 90 min at 30 °C. The slurry was added dropwise (diameter ~ 2 mm) into 100 ml of 0.05 M CaCl_2 . After 30 min, the gel beads containing immobilised whole cells were washed and stored at 4 °C until used.

Cross-linked chitosan beads: The procedure of Carrara and Rubiolo (1994) was used with some modifications. Chitosan (3 g) was dissolved in 100 ml of 2% (v/v) acetic acid with continuous stirring for 90 min. The chitosan solution was filtered through cheesecloth to remove insoluble materials and was added dropwise (diameter ~ 4 mm) into 150 ml of 1.5% (w/v) sodium triphosphate solution with gentle stirring. The chitosan beads were washed with standard buffer until neutrality. The beads were activated with standard buffer containing 1% (v/v) glutaraldehyde for 24 h at room temperature. A 5 g sample of the activated chitosan beads was mixed with 10 ml of standard buffer containing 1 g of wet cell paste. The mixture was gently agitated (100 rpm) at room temperature overnight. The beads obtained were washed and stored at 4 °C until used.

Eggshell: The procedure of Venkaiah and Kumar (1994) was used with some modifications. Small pieces of hen eggshell were boiled for 20 min, washed with acetone, and dried in an oven at 60 °C for 12 h. The pieces were crushed in a blender for 5 min and sieved through a 100 mesh. Ground eggshell (5 g) was mixed with 10 ml of standard buffer containing 1 g of wet cell paste. The mixture was gently agitated at 100 rpm and 2 ml of 6% (v/v) glutaraldehyde were slowly added during stirring. The mixture was incubated at 10 °C for 12 h. The residues were obtained by centrifugation at 4 °C, 6000g for 15 min. The immobilised whole cells were washed, dried, and stored at 4 °C until used.

2.3. Histamine-degrading activity assay and protein determination

The histamine-degrading activity was assayed using histamine as substrate. Unless otherwise stated, the free or immobilised whole cells (0.1 g) were added into 1 ml of standard assay mixture consisting of 50 mM Tris-HCl buffer, pH 7.0, 4.3 M NaCl, 500 μM 1-methoxy-5-methylphenazinium methylsulphate (1-methoxy PMS), and 5 mM histamine dihydrochloride. The reaction mixture was incubated at 37 °C for precisely 1 h under static conditions, unless otherwise stated. Enzymatic reaction was terminated by adding 1 ml of 0.1 N HCl. A blank analysis was performed in the same manner, except the free or immobilised whole cells were added after addition of 1 ml 0.1 N HCl. The histamine contents of reaction and blank were determined by the fluorometric method of AOAC. (1995). One unit of activity was defined as the degradation of 1 μmol histamine per hour per gram of matrix under the specified conditions.

Protein was determined according to the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as the standard. The amount of protein bound or retained on the immobilised matrix was determined indirectly from the difference between the amount of protein introduced into the coupling reaction mixture and the total amount of protein in both filtrate and washing solutions.

2.4. Selection of immobilisation method

Whole cells immobilised by different techniques were subjected to determination of histamine-degrading activity, protein loading ratio, and immobilisation yield, which were defined as follows:

$$\text{Histamine-degrading activity (units/g matrix)} = \frac{\text{Activity of immobilised whole cells (units)}}{\text{Actual weight of cell immobilised (g)}}$$

$$\text{Protein loading ratio (\%)} = \frac{\text{Amount of protein bound (mg)}}{\text{Amount of protein loaded (mg)}} \times 100\%$$

$$\text{Immobilisation yield (\%)} = \frac{\text{Total activity of immobilised whole cells (units)}}{\text{Total activity of free whole cells (units)}} \times 100\%$$

The immobilisation method rendering the maximal immobilisation yield was chosen for further study.

2.5. Effect of salt concentration on activity and stability of free and immobilised whole cells

To remove salt, free and immobilised whole cells ($\sim 1 \times 10^6$ CFU or 1 g) were dialysed against 100 volumes of 50 mM Tris–HCl buffer, pH 7.0 at 4 °C. Desalted samples were subjected to activity determination in the presence of NaCl at various concentrations (0–5 M) using the standard assay conditions. To study the effect of NaCl on stability, the desalted samples were dialysed against 100 volumes of standard buffers containing different NaCl concentrations (0–5 M) at room temperature for 24 h. The remaining activity was assayed using the standard assay conditions.

2.6. Effect of pH on activity and stability of free and immobilised whole cells

The activity of free and immobilised whole cells were assayed at 37 °C over the pH range 4–9 (50 mM acetate buffer (pH 4.0–5.5); 50 mM phosphate buffer (pH 6.0–6.5); and 50 mM Tris–HCl buffer (pH 7.0–9.0)). To study the effect of pH on stability, free and immobilised whole cells were incubated in buffers at the designated pH in the presence of 4.5 M NaCl for 24 h at 30 °C. The remaining activity was assayed using the standard assay conditions.

2.7. Effect of temperature on activity and stability of free and immobilised whole cells

Enzyme activity was assayed at different temperatures in the range of 30–80 °C, as previously described. To study the effect of temperature on enzyme stability, the free and immobilised whole cells were subjected to heating at different temperatures ranging from 4 to 80 °C for 1 h, in a temperature-controlled water bath. Thereafter, the enzyme mixture was immediately cooled in an ice bath, and the remaining activity was assayed under the standard assay conditions. Relative activity of free and immobilised

whole cells was calculated, in comparison with that without heating.

2.8. Operational stability of free and immobilised whole cells

The operational stability of the free and immobilised whole cells was assayed by using free and immobilised whole cells ($\sim 1 \times 10^6$ CFU or 1 g) in 10 ml of standard assay mixture containing histamine in successive batches. The samples were kept in an incubator shaker with a shaking speed of 200 rpm at 37 °C for 1 h. Samples were taken and measured for the residual histamine content by using the fluorometric method of AOAC (1995). At the end of each batch, the free and immobilised whole cells were removed from the reaction medium and washed with five volumes of 50 mM Tris–HCl buffer (pH 7.0) containing 4.5 M NaCl to remove any substrate or product retained in the matrix. The free and immobilised whole cells were introduced into a fresh medium. Activities were estimated at the end of each cycle. The above-mentioned steps were repeated until the enzyme lost 50% of its total activity. The residual activity was calculated and expressed relatively to that found for the first cycle.

2.9. Substrate preference of free and immobilised whole cells

The substrate preference of free and immobilised whole cells was determined by measuring the degradation of biogenic amines, including histamine, tryptamine, β -phenylethylamine, putrescine, cadaverine, tyramine, spermidine and spermine. Free and immobilised whole cells ($\sim 1 \times 10^6$ CFU or 1 g) were added to 10 ml of assay mixture containing 50 mM Tris–HCl buffer (pH 7.0), 4.5 M NaCl, 500 μ M 1-methoxy PMS and 5 mM of each biogenic amine. The reaction mixtures were incubated at 45 °C in an incubator shaker with a speed of 200 rpm for 1 h and immediately stopped by adding 1 ml of 0.4 N perchloric acid. The mixture was centrifuged at 15,000g for 10 min. The supernatant was subjected to analysis of biogenic amines. The blank was done in the same manner, except free or immobilised whole cells were added after addition of 1 ml of 0.4 N perchloric acid.

Biogenic amines in supernatant were extracted and derivatised according to the procedure of Eerola, Hinkkanen, Lindfors, and Hirvi (1993) with a slight modification. The supernatant (480 μ l) was added with 20 μ l of 2 mg/ml of 1,7-diaminoheptane as the internal standard and 500 μ l of 0.4 M perchloric acid. The mixtures were mixed thoroughly by a vortex mixer for 5 min and then centrifuged at a speed of 15,000g at 25 °C for 5 min. The obtained supernatant (300 μ l) was mixed with 60 μ l of 2 N NaOH and 90 μ l of saturated sodium bicarbonate. A 600- μ l aliquot of 10 mg/ml dansyl chloride was added to each sample, mixed well, and then incubated for 45 min at 40 °C. Residual dansyl chloride was removed by adding 30 μ l of 25% (v/v) ammonia and centrifuging at 3500g for 5 min. The supernatant was collected and filtered through a 0.45 μ m membrane filter prior to analysis by high performance liquid chromatography (HPLC). A Waters Separation Module 2690 was operated to give a flow rate of 1.5 ml/min, with a mobile phase of 0.1% (v/v) acetic acid (solvent A) and acetonitrile containing 0.1% (v/v) acetic acid (solvent B), as shown in a gradient elution program (Table 1). Separation was achieved using a column of Hyperasil BDS C18 (300 $\times$ 7.8 mm i.d.) set at 40 °C. Samples (20 μ l) were injected and a photodiode array (Model Waters 996), set at a wavelength of 254 nm, was used as the detector. Data were processed and analysed using Millennium 32 software (Waters, Milford, MA).

2.10. Statistic analysis

All experiments were run in triplicate. A completely randomised design was used throughout this study. Data were subjected

Table 1

Gradient elution program for histamine analysis by HPLC.

	Time (min)	Flow (ml/min)	%A	%B
1	0	1.5	55	45
2	15	1.5	40	60
3	25	1.5	10	90
4	30	1.5	10	90
5	32	1.5	55	45
6	37	1.5	55	45

Solvent A; 0.1% (v/v) acetic acid in nanopure water.

Solvent B; 0.1% (v/v) acetic acid in acetonitrile.

to analysis of variance and mean comparison was carried out using Duncan's Multiple Range Test (Steel & Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for Windows: SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Selection of the matrices for immobilisation of *Nmn. gari* BCC 24369

Whole cells of *Nmn. gari* BCC 24369 were immobilised on various matrices by different methods (Table 2). The immobilised whole cells prepared by adsorption on Celite had the highest immobilisation activity (17.5 units/g matrix) and immobilisation yield (94.1%), followed by entrapment in agar. Celite consists of highly porous diatomaceous beads composed of silica (SiO₂) and some other inorganic oxides, which possibly allow bacterial cells to adhere to the surface. Due to its chemical inertness and unique interconnected pore structure, Celite is suitable for physical adsorption (Chang, Chang, Yen, & Shieh, 2007) and was selected as the matrix for entrapment of *Nmn. gari* BCC 24369 cells. The adsorption phenomenon is fundamentally based on weak attraction forces to an inert carrier that has not been chemically derived (Kolot, 1981). However, pig bone powder and chitosan flakes were less effective than Celite on whole cells adsorption. This was probably due to unfavourable morphological and physical characteristics, such as limited porous structures of pig bone powder used in this study. On the other hand, adsorption on chitosan flakes was probably limited by the low affinity to water and excluded volume of chitosan molecules, though the adsorption could be readily caused by interaction between amino groups of chitosan and the external walls of the halophilic archaea which typically contain negatively charged glycoproteins and sulphate (Litchfield, Irby, & Vreeland, 1999).

With the entrapment technique, the cells entrapped in 2% (w/v) agar exhibited maximum immobilisation activity. Higher concen-

trations of agar in the gel decreased the histamine-degrading activity of immobilised cells (data not shown) probably due to smaller pore size in the blocks, which might have interfered with the diffusion of substrate and product in the gel. The apparent activity observed in Ca²⁺-alginate beads was 50% lower than that found in agar support. The decrease in activity might be due to the chemical instability of matrices under high salt concentrations (Horitsu, Maseda, & Kawai, 1990; Martinsen, Skjåk-Bræk, & Smidsrød, 1989). To enhance the chemical resistance and mechanical strength of alginate and chitosan beads, the cross-linked alginate and chitosan beads were prepared using either chitosan or glutaraldehyde as a cross-linking agent. Although better bead characteristics were observed, in which the beads did not dissolve easily in the presence of salt, immobilisation via cross-linking on the alginate or chitosan beads did not significantly improve the immobilisation activity of the whole cells ($p > 0.05$). Moreover, the addition of glutaraldehyde also resulted in a significant decrease in histamine-degrading activity of the immobilised whole cells. A similar effect of glutaraldehyde was observed with eggshell matrix. This was probably attributed to some forms of toxicity or inhibition caused by glutaraldehyde as reported by Nighojkar, Phanse, Sinha, Nighojkar, and Kumar (2006), Ragnitz, Sylatk, and Pietzsch (2001) and Bryjak, Trochimczuk, and Noworyta (1993).

3.2. Characterisation of free and immobilised whole cells from *Nmn. gari* BCC 24369

3.2.1. Effect of salt

The optimum NaCl concentrations of histamine-degrading activities of free and immobilised whole cells were found at 4–5 M and 3–5 M, respectively (Fig. 1A). The decrease in NaCl concentration resulted in lowered enzyme activity. The lowest enzyme activities of free and immobilised whole cells were found in the absence of NaCl, in which 38.1% and 52.0% of activity was obtained, respectively. This result clearly indicates that the histamine-degrading activity of the whole cells was salt dependent. Fig. 1B shows the effect of NaCl concentrations on the stability of free and Celite-immobilised whole cells. The histamine-degrading activity was stable for at least 24 h when incubated in 50 mM Tris–HCl buffer (pH 7.0) in the presence of 3.5–5.0 M NaCl. The enzyme activities of free and immobilised whole cells retained about 50% in the presence of 1.5 and 2.5 M NaCl, respectively. In the absence of salt, the enzyme of immobilised whole cells was more stable than that of free whole cells ($p < 0.05$). The result indicated that the immobilisation improved the stability of the cells to NaCl at sub-optimal level. Many halophilic enzymes require the presence of NaCl or KCl concentrations in the range of 1–4 M for optimum activity and stability (Mevarech, Frolov, & Gloss, 2000). On the basis of salt requirement, it was also noticed that the

Table 2Immobilisation of histamine-degrading enzyme from whole cells of *Nmn. gari* BCC 24369 on various matrices.

Support	Total protein (mg)		Loading efficiency (%)	Unbound activity (units/ml)	Immobilised activity (units/g support)	Immobilisation yield (%)
	Bound	Unbound				
Pig bone	3.7 ± 0.1 ^e	1.48 ± 0.1 ^b	71.6 ± 8.0 ^d	5.0 ± 1.2 ^a	10.9 ± 0.5 ^b	58.5 ± 2.6 ^c
Chitosan flakes	3.6 ± 0.1 ^f	1.63 ± 0.1 ^a	68.7 ± 9.0 ^d	2.1 ± 0.9 ^b	10.8 ± 0.6 ^b	58.2 ± 3.5 ^c
Celite	5.0 ± 0.0 ^b	0.2 ± 0.0 ^e	96.3 ± 1.0 ^b	0.5 ± 0.1 ^c	17.5 ± 0.6 ^a	94.1 ± 3.4 ^a
Agar	5.2 ± 0.0 ^a	0.0 ± 0.0 ^e	100.0 ± 0.0 ^a	0.0 ± 0.0 ^d	15.5 ± 0.9 ^b	83.4 ± 4.7 ^b
Ca ²⁺ -alginate beads	5.2 ± 0.0 ^a	0.0 ± 0.0 ^e	100.0 ± 0.0 ^a	0.0 ± 0.0 ^d	7.7 ± 0.5 ^c	41.1 ± 2.5 ^d
Alginate–chitosan beads	5.2 ± 0.0 ^a	0.0 ± 0.0 ^e	100.0 ± 0.0 ^a	0.0 ± 0.0 ^d	8.3 ± 0.5 ^c	44.5 ± 2.5 ^d
Cross-linked alginate beads	5.2 ± 0.0 ^a	0.0 ± 0.0 ^e	100.0 ± 0.0 ^a	0.0 ± 0.0 ^d	3.0 ± 0.6 ^c	16.2 ± 3.1 ^e
Cross-linked chitosan beads	4.2 ± 0.1 ^d	1.05 ± 0.1 ^c	79.8 ± 12.0 ^{c,d}	1.4 ± 0.4 ^b	3.7 ± 1.2 ^d	19.6 ± 6.5 ^e
Eggshell	4.5 ± 0.0 ^c	0.69 ± 0.0 ^d	86.8 ± 4.0 ^c	0.4 ± 0.1 ^c	3.9 ± 0.7 ^d	20.8 ± 4.0 ^e

* Different superscripts in the same column indicate significant differences ($p < 0.05$).** Mean ± SD ($n = 3$).

Assays were done in the presence of 4.3 M NaCl at 37 °C, pH 7.0 under static condition. Hundred percentage of the immobilised yield is equivalent to the histamine-degrading activity of 18.6 ± 1.2 units/g.

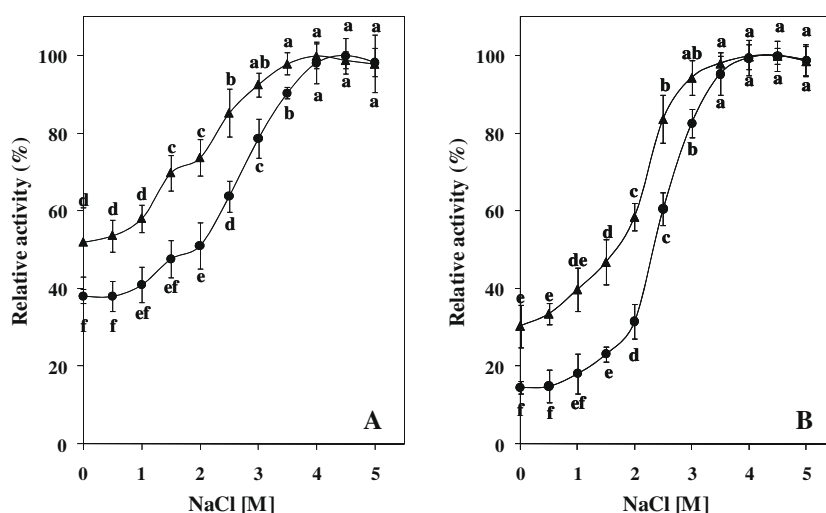


Fig. 1. Effect of NaCl concentration on histamine-degrading activity (A) and stability (B) of free (●) and immobilised whole cells (▲) on Celite support. Histamine-degrading activity was determined by incubating free and immobilised whole cells at 37 °C in 50 mM Tris–HCl buffer, pH 7.0 in the presence of various salt concentrations (0–5 M NaCl) under static condition. Hundred percentage equivalent to 33.5 and 33.2 units/g for free and immobilised whole cells on Celite support, respectively. Bars represent the standard deviation ($n = 3$). Different letters in the same line denote significant differences ($p < 0.05$).

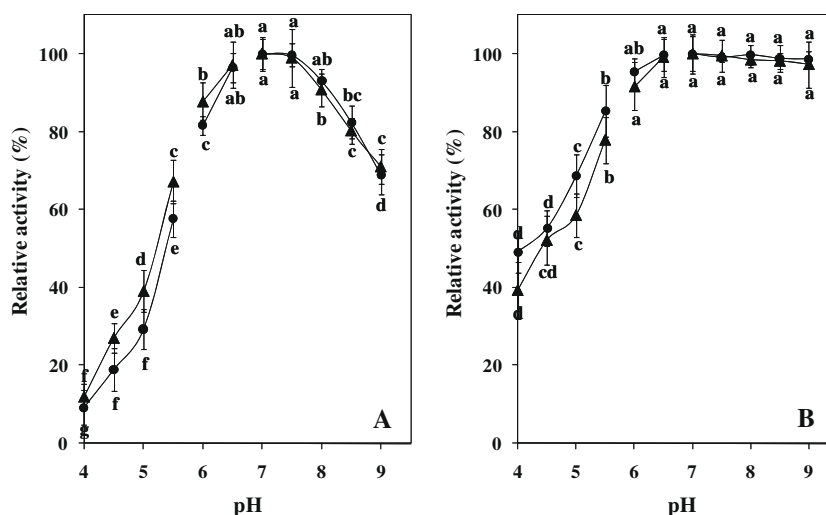


Fig. 2. pH profile (A) and pH stability (B) of free (●) and immobilised whole cells (▲) on Celite support. For the pH profile, histamine-degrading activity was determined by incubating free and immobilised whole cells at 37 °C at various pH values in the presence of 4.5 M NaCl under static condition. For the pH stability, free and immobilised whole cells were incubated in buffers at the designated pH in the presence of 4.5 M NaCl for 24 h at 30 °C. Bars represent the standard deviation ($n = 3$). Hundred percentage equivalent to 33.5 and 33.2 units/g for free and immobilised whole cells on Celite support, respectively. Different letters in the same line denote significant differences ($p < 0.05$).

histamine-degrading activity of *Nmn. gari* BCC 24369 could be regained upon reestablishment of the high salt concentration, similar to several halophilic enzymes of various extreme halophiles (Holmes et al., 1997). In order to remain stable and active at high ionic strength, proteins from halophilic proteins typically have an excess of acidic amino acids (i.e., glutamate and aspartate) on their surface (Holmes et al., 1997). Negative charges on the halophilic proteins bind significant amounts of hydrated ions, thus reducing their surface hydrophobicity and decreasing the tendency to aggregate at high salt concentration. At the molecular level, this probably suggested the high flexibility of protein structure that allowed fast change from the fully active form in the presence of salt to an inactive form in the absence of salt.

3.2.2. Effect of pH

The optimum pH values of the histamine-degrading activity of free and immobilised whole cells were 6.5–7.5 (Fig. 2A). At pH 4, only 10% of activity was found for both free and immobilised whole cells. As the pH increased, the activity increased rapidly, reaching a maximum at pH 7. The activity decreased at pH above 7 and at pH 9 relative activities of 69.0% and 71.0% were obtained for free and immobilised whole cells, respectively. The histamine-degrading activity was stable for at least 24 h when both free and immobilised whole cells were incubated in buffers at pH ranging from 6 to 9 (Fig. 2B). However, the activity decreased markedly at pH below 6 and was lower than 50% at pH 4. There were no significant differences in pH stability between the free and immobilised whole

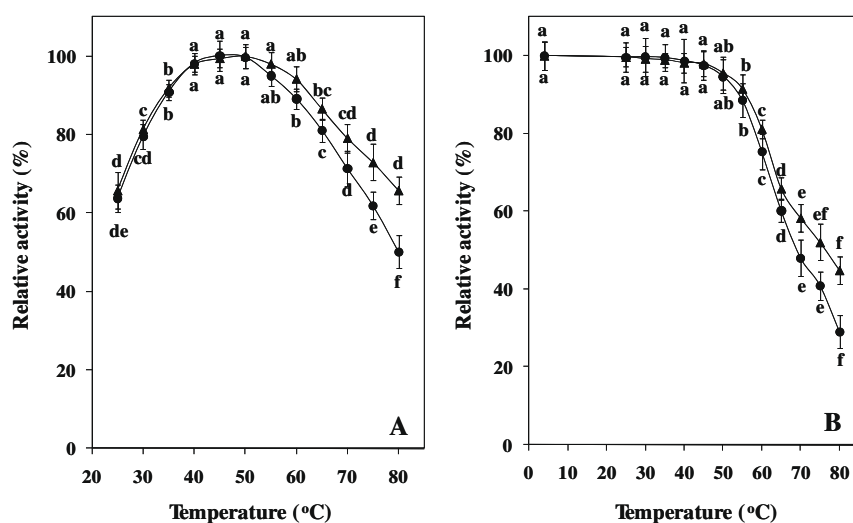


Fig. 3. Temperature profile (A) and thermal stability (B) of free (●) and immobilised whole cells (▲) on Celite support. For the temperature profile, histamine-degrading activity was determined by incubating free and immobilised whole cells at various temperatures, in 50 mM Tris–HCl buffer, pH 7.0 in the presence of 4.5 M NaCl under static condition. For the thermal stability, free and immobilised whole cells were incubated in 50 mM Tris–HCl buffer, pH 7.0 in the presence of 4.5 M NaCl for 24 h at the designated temperature. Hundred percentage equivalent to 33.5 and 33.2 units/g for free and immobilised whole cells on Celite support, respectively. Bars represent standard deviation ($n = 3$). Different letters in the same line denote significant differences ($p < 0.05$).

cells ($p > 0.05$). The histamine-degrading activity of the cell was catalysed by an endogenous enzyme, which would be in a state of fairly constant pH within an intact and viable cell. Thus, the fact that external pH effected the activity significantly was probably due to the loss of cell viability, specially at pH lower than 5.5 (Tapingkae et al., 2008).

3.2.3. Effect of temperature

Fig. 3A shows the effect of temperature on the histamine-degrading activity of free and immobilised whole cells. The maximum activity was observed at temperatures around 40–60 °C for both preparations. At 80 °C, the preparations showed relative activities of 50.1% and 65.8% for free and immobilised whole cells, respectively. The enzyme activities of free and immobilised whole cells were still over 90% after incubating at 4 °C to 50 °C for 1 h (Fig. 3B). The activities of both cell types rapidly decreased after being incubated at temperature higher than 55 °C for 1 h. However, the immobilised whole cells retained more activity than free whole cells when heated at temperature ranging from 65 to 80 °C ($p < 0.05$). Corresponding with the temperature range for growth of this organism at 20–60 °C (Tapingkae et al., 2008), histamine-degrading enzyme was relatively heat stable. The result indicated that immobilisation slightly improved the stability of cell to heat treatment. The higher temperature profile observed in the case of immobilised whole cells may be because of some conformational effects due to adsorption, which protects the cellular proteins against heat denaturation (Dey, Singh, & Banerjee, 2003).

3.2.4. Operational stability of the free and immobilised whole cells

Both free and immobilised whole cells could be used up to five and seven times, respectively, without decreasing their activity (Fig. 4). With increasing repetitions, the activity of immobilised whole cells gradually decreased but still remained over 90% at the 8th cycle ($p > 0.05$). In contrast, the activity of free whole cells rapidly decreased after the 5th cycle and was only 50% at the 8th cycle ($p < 0.05$). At the 10th cycle, the activities of free and immobilised whole cells retained about 30% and 50%, respectively ($p < 0.05$).

Generally, adsorption is a simple physical process in which the forces involved in cell attachment are so weak. Cells that are not

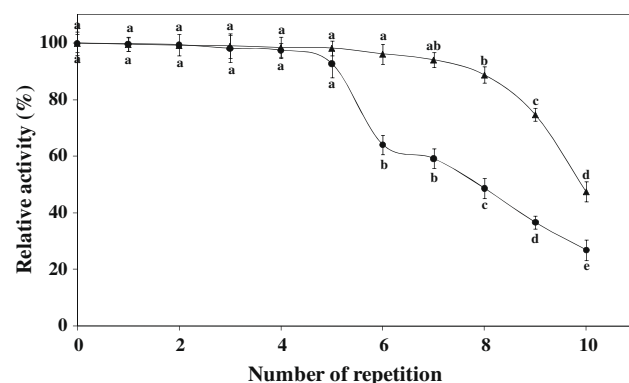


Fig. 4. Effect of reusability on the histamine-degrading activity of free (●) and immobilised (▲) whole cells. Hundred percentage equivalent to 33.5 and 33.2 units/g for free and immobilised whole cells on Celite support, respectively. Bars represent the standard deviation ($n = 3$). Different letters in the same line denote significant differences ($p < 0.05$).

strongly adsorbed are readily desorbed from the support during utilisation of the biocatalyst under the conditions used. The lower effectiveness in the retention of whole cells on Celite compared with other types of immobilisation was reported. Fernandes et al. (2002) indicated that whole cells of *Pseudomonas putida* immobilised on Celite showed lower cells retention than those of microencapsulated in Ca-alginate or κ -carrageenan.

3.2.5. Substrate selectivity

Fig. 5 shows that both free and immobilised whole cells degraded histamine much more efficiently, compared to tryptamine, β -phenylethylamine, putrescine, cadaverine, tyramine, spermidine, and spermine ($p < 0.05$). About 80% of histamine was degraded after treatment with either free or immobilised whole cells. Putrescine and tryptamine were also degraded to some extent. Only small amounts of other biogenic amines determined were degraded. The purified amine dehydrogenase from *Pseudomonas* AM1 had a wide specificity and could oxidise many compounds that contain a primary amino group, notably ethanolamine and histamine (Eady & Large, 1968). Whereas the purified histamine

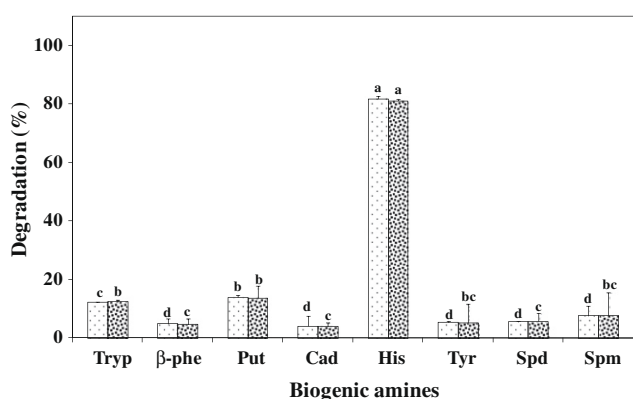


Fig. 5. Substrate preference of free (▨) and immobilised (▨) whole cells on Celite support. The assay was done in 50 mM Tris–HCl buffer, pH 7.0 in the presence of 4.5 M NaCl at 45 °C, under shaking condition (200 rpm) for 1 h. Bars represent the standard deviation ($n = 3$). Different letters in the column denote significant differences ($p < 0.05$). Tryp: tryptamine; β-phe: β-phenylethylamine; Put: putrescine; Cad: cadaverine; His: histamine; Tyr: tyramine; Spd: spermidine and Spm: spermine.

dehydrogenase from *Nocardioides simplex* IFO 12069 showed high activity toward histamine, it still oxidised agmatine and putrescine at a rate of 30%, compared to histamine (Siddiqui, Shoeb, Takayama, Shimizu, & Yorifuji, 2000). Among 20 kinds of biogenic amines studied by Bakke, Sato, Ichikawa, and Nishimura (2005), the purified recombinant histamine dehydrogenase from *Rhizobium* sp. 4–9 was very specific toward histamine and only oxidised agmatine and 1,3-diaminopropane with the rate of 10% and 13% of histamine, respectively. Fujieda, Satoh, Tsuse, Kano, and Ikeda (2004) indicated that the differences between histamine dehydrogenase and other amine dehydrogenases were the specificity toward histamine with low activity to other amines. Since histamine-degrading activity was found only in the presence of 1-methoxy PMS, the result suggested that the enzyme in whole cells was most likely histamine dehydrogenase. Due to the high efficacy in degrading histamine at high salt concentration, immobilised whole cells could be used to remove histamine in fish sauce which contained high salt (~25–30% NaCl).

4. Conclusions

Whole cells adsorption to Celite provided the best compromise between biocatalyst retention and apparent activity in degradation of histamine. The immobilised whole cells of *Nmn. gari* BCC 24369 on the Celite support were more promising than the free whole cells for histamine degradation in that they were stable to high salt, elevated temperatures, and reusable.

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Isolation and screening of lactic acid bacteria from Thai traditional fermented fish (*Plasom*) and production of *Plasom* from selected strains

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ABSTRACT

Lactic acid bacteria (LAB) were isolated from traditional Thai fermented fish, *Plasom* at various fermentation periods. It was found that 138 isolates exhibited a clear zone and growth on MRS agar supplemented with CaCO₃; however, only 133 isolates were identified as LAB. Only 14 strains showed excellent inhibition zone diameters on agar when *Salmonella* sp. was used as an indicator for preliminary detection of antagonistic activity. *Staphylococcus aureus* was used for secondary screening for antagonistic activity of these 14 strains. It was found that only 7 strains exhibited good inhibition zone diameters on agar, and all of them could inhibit *E. coli* as the third indicator. The strains which exhibited widest zones of inhibition against *Escherichia coli*, *S. aureus* and *Salmonella* sp. were LPS04, LPS17, and LD219 and LPS18 respectively. Tested pathogenic strains were inhibited by 4 selected LAB. The strain which showed the best lactic acid production and pH reduction ability was LD219. Using 16s rDNA sequence analysis, LD219 was identified as *Streptococcus salivarius*, LPS04, LPS17 and LPS18 were identified as *Enterococcus faecalis*. *Plasom* was produced by using a mixed culture (LD219, LPS04, LPS17 and LPS18) as a starter culture compared with spontaneous and back-slopping processes. Significant differences ($p < 0.05$) in pH, its titratable acidity as lactic acid and number of total viable counts (TVC), LAB and Enterobacteriaceae were found in these *Plasom* at 0 and 8 days. However, no significant difference ($p > 0.05$) was observed in terms of colour, smell, taste, sour, texture and overall acceptance of *Plasom* produced by non-starter cultured, back-slopping and starter cultured processes.

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1. Introduction

Lactic acid bacteria (LAB) are the most commonly used microorganisms used in food preservation techniques such as fermentation. Lactic acid produced by LAB is a useful compound for food preservation because it maintains the acidic conditions of the fermented products, and is lethal to bacteria that cause food spoilage and food poisoning (Kobayashi et al., 2004). Most fermented foods owe their origin to the fact that the processes used in their production are inhibitory to many microorganisms. As a result, fermented products generally have a longer shelf life than their original substrate and their ultimate spoilage is different in character (Adams & Mitchell, 2002). Past experience has shown that sometimes fermented foods have been attributed to food-borne

illnesses (Nout, 1994), particularly the application of fermentation at the household level (Nout & Motarjemi, 1997). The inhibitory effect of lactic acid depends on the species and loads of pathogenic bacteria, sanitation processes as well as the number of LAB in fermented foods.

According to Caplice and Fitzgerald (1999) and Callewaert, Hugas, and De Vuyst (2000), the use of starter cultures would be an appropriate approach for the control and optimization of the fermentation process. These could be used in order to alleviate the problems of variations in organoleptic quality and microbiological stability observed in indigenous fermented foods. *Plasom* is a traditional fermented fish widely consumed in the south of Thailand. It is made from fish, sugar, salt and roasted rice and is fermented with natural microbial flora. The main microorganisms found in *Plasom* are LAB ranged from 10⁷ to 10⁹ cfu/g depends on salt concentration. Low-salt *Plasom* contained LAB approximately 9.2 log cfu/g and pH around 4.5. The pH of the high-salt *Plasom* was low (5.7) despite the small number of LAB (7.5 log cfu/g)

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(Østergaard, Embarek, Wedel-Neergaard, Huss, & Gram, 1998). No LAB starter cultures are commercially available yet for the small-scale processing of traditional Thai fermented foods (Valyasevi & Rolle, 2002).

Starter cultures for fermented foods are developed today mainly by design rather than by screening. The design principles are based on knowledge of bacterial metabolism and physiology as well as on the interaction with the food product (Ammor & Mayo, 2007; Hansen, 2002; Kaban & Kaya, 2006; Seifu, Buys, & Donkin, 2003) that was derived from screening studies of relevant desired bacterial strains. The development of starter cultures is one of the prerequisites for the establishment of small-scale industrial production of fermented foods in Thailand. The primary consideration before introducing starter cultures for traditional small-scale fermentations should be whether these would significantly contribute to an improvement of processing conditions and product quality. This is particularly so with respect to: rapid or accelerated acidification; an improved and more predictable fermentation process; desirable sensory attributes; and improved safety and reduction of hygienic risks (Holzapfel, 1997). Therefore, a thorough understanding of the fermentation process is required. The knowledge gained from using controlled starter cultures may also benefit those operating at a very small-scale and practicing the use of the back-slopping process (Mugula, Narvhus, & Sorhaug, 2003).

The objectives of this study were to screen LAB isolated from *Plasom*, naturally fermented fish, at different stages of the fermentation process and to select the most suitable strains for this. These were selected according to their technological characteristics. These included their effectiveness in lactic acid production and pH reduction ability, as well as antimicrobial activity against food-borne pathogens, when used as starter cultures.

2. Materials and methods

2.1. *Plasom* preparation

The original formula of *Plasom* consists of 1 kg fish (*Oreochromis niloticus*), 100 g table salt and 200 g sugar. The mixtures were transferred to a bucket and covered with polyethylene film. The containers were incubated at room temperature (30 ± 2 °C) for 8 days (Fig.1). During fermentation, each bucket was taken for LAB isolation every two days. Prior to isolation the selected sample was blended and used as a composite sample.

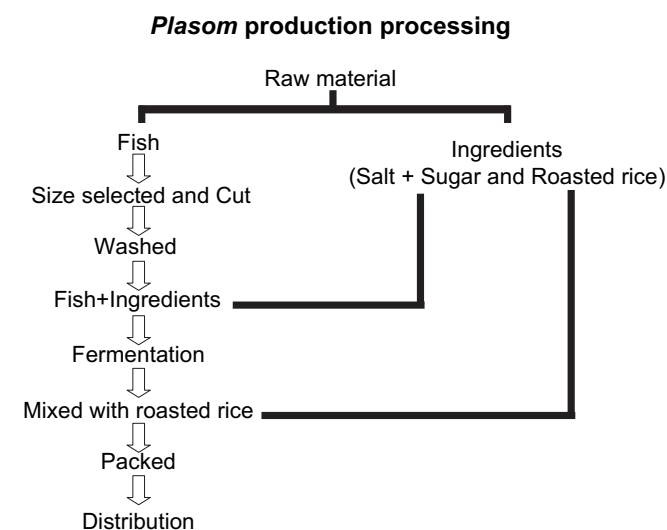


Fig. 1. *Plasom* production processing. Hwanhlem et al.

2.2. Isolation of LAB

Twenty-five grams of the sample were added to 225 ml of sterile 0.05 M potassium phosphate buffer pH 7.2 containing 1% NaCl (PBS) and shaken for 5 min. Appropriate decimal dilutions were prepared in PBS buffer and poured into a sterile petri dish on de Man Rogosa and Sharpe (MRS, Labscan Asia Co., Ltd., Thailand) agar containing 0.3% (w/v) CaCO_3 (Maragkoudakis et al., 2006). This was subjected to incubation at 37 °C for 24 h. Bacterial colonies that exhibited clear zone on the plates were individually picked and streaked on MRS agar containing 0.3% (w/v) CaCO_3 . This procedure was repeated in order to purify the isolates. Each of the isolates was first tested for catalase by placing a drop of 3% hydrogen peroxide solution on the cells. Immediate formation of bubbles indicated the presence of catalase in the cells. Only those isolates which were catalase negative were Gram-stained, and only those which were Gram-positive were maintained in MRS broth containing 25% glycerol at -20 °C. For routine analysis, strains were subcultured twice in MRS broth for 24 h at 37 °C. Selected LAB were identified by 16S rDNA analysis.

2.3. Detection of antagonistic activity

For detection of antagonistic activity, an agar spot test was used. The agar spot test was a modification of that described by Schillinger and Lucke (1989). Approximately 10^4 CFU/ml of the strains to be tested for production of an antimicrobial compound were spotted onto the surface of MRS agar plates (1.5% agar) and incubated for 48 h at 37 °C. This allowed colonies to develop. The plates were overlaid with 10 ml of Nutrient soft agar (0.75% agar). The overlay agar was seeded with 10^4 cfu/ml of the pathogenic bacteria to be tested for sensitivity (*Escherichia coli*, *Staphylococcus aureus* and *Salmonella* sp.). After incubation for 24 h at 37 °C the plates were checked for inhibition zone diameters. Inhibition was scored as positive if the width of the clear zone around the colonies of the producer strain was 10 mm or larger.

Strains exhibiting antagonistic activities against pathogenic bacteria were investigated for their antimicrobial compounds as described by Aslim, Yuksekdog, Sarikaya, and Beyatli (2005). Strains of LAB were grown for 24 h at 37 °C in 10 ml of MRS broth and then centrifuged at 9500g for 10 min. Three samples of the supernatant were taken from each strain of LAB. Samples 1 and 2 were adjusted to pH 6.5 with NaOH (1 N) to rule out acid inhibition. For sample 2, a catalase solution (Fluka, Germany) (300 U/ml in PBS buffer, pH 7) was added to rule out inhibition from hydrogen peroxide. For sample 3, the supernatant was used as a control. The antagonistic activity of the three samples was detected using the well diffusion agar method (Papamalonis, Tzanetakis, Tzanetaki, & Kotzekidou, 2003). Twenty ml of soft BHI agar (containing 1.0% agar) were poured on sterile plates and inoculated with an overnight culture of pathogenic bacteria (about 10^7 cfu/ml). After solidification, wells were perforated with a sterile 7 mm cork borer. The culture filtrates (50 μ l) were placed in each well. The plates were incubated at 37 °C and examined after 24 h for clear zones showing the pathogenic bacteria inhibition. The diameters of the inhibition zones were measured and the diameter of the well, 7 mm, was subtracted from the total zone diameter.

2.4. Lactic acid production and pH reduction ability

The effectiveness of selected LAB on the growth of pathogenic bacteria was further studied for lactic acid production. Each selected strain was cultured for 48 h at 37 °C in MRS broth. Approximately 10^7 cfu/ml of the selected LAB were inoculated into 40 ml MRS broth. The cultures were incubated for 48 h at 37 °C, and

aliquots were taken for the measurements of the pH of the media. The pH was directly measured using a pH meter (420A, Orion, Hungary). A cell-free solution was obtained by centrifuging (9500g, 10 min, and 4 °C) the culture broth and used for total acidity to determine the lactic acid (AOAC, 2000).

2.5. Comparison of spontaneous, back-slopping and inoculated Plasom

Plasom was prepared according to the formula described above for the Plasom preparation. Selected LAB (LD219, LPS04, LPS17 and LPS18) were cultivated in 10 ml MRS broth at 37 °C for 18 h. The bacterial cells were harvested by centrifugation (9500g, 10 min, and 4 °C). Cell pellets were then washed and suspended in 10 ml of saline solution. The starter culture for Plasom production was then prepared by mixing each LAB strain in the ratio 1:1:1:1. Three different samples of Plasom were prepared using back-slopping, a starter culture (10^7 cfu/g) and without a starter culture. The pH, total acidity as lactic acid and LAB, Enterobacteriaceae and total bacteria analysis of Plasom was determined at 0 and after 8 days of incubation.

2.6. Sensory evaluation of Plasom

Samples were mixed with roasted rice and fried with palm oil at a cooking temperature (150 °C). The sensory evaluation was carried out by 30 panelists using a 9-point hedonic scale (1 = dislike extremely, 9 = like extremely) (Lawless & Heymann, 1999; Meilgaard, Civille, & Carr, 1999). The panelists were asked to evaluate samples fermented for 8 days for colour, odour, flavour, texture and overall likeness.

2.7. Statistical analysis

Statistical analysis was performed using one-way ANOVA with post hoc Duncan's test; $p < 0.05$ was considered significant. All data are expressed as mean $\pm$ S.D.

3. Results and discussion

3.1. Isolation of LAB

The isolation of LAB from Plasom at various fermentation periods was performed. MRS agar containing 0.3% (w/v) CaCO_3 was used as a preliminary screening medium for LAB. It was found that 138 isolates exhibited a clear zone and growth on MRS agar supplemented with CaCO_3 . Calcium carbonate was used as an indicator for acid-producing strains since it dissolved when interact with acid then a clear zone is observed (Onda et al., 2002). However, 133 isolates were identified as LAB using the criteria of being Gram-positive and catalase negative. Of these 133 isolates, 25 isolates were cocci; 75 isolates were short rods and 33 isolates were rods (data not shown).

The selection of suitable starter cultures among strains naturally present in fermented products should be based on certain characteristics. These include: the ability to ferment by lowering pH, increasing the titratable acidity; increasing the number of LAB; and decreasing the Enterobacteriaceae with fermentation time (Mugula et al., 2003).

3.2. Detection of antagonistic activity

The potential of starter cultures to produce antibacterial compound has attracted much attention as they can be used to prevent food spoilage and to inhibit the growth of food pathogens (Hammes

& Hertel, 1998). The antibacterial activity of 133 LAB isolates toward *Salmonella* sp. was firstly determined by an agar spot test. It was found that 112 isolates exhibited antibacterial activity (data not shown). However, only 14 strains showed excellent inhibition zones diameters larger than 40 mm (Table 1). Strain LPS18 produced the widest inhibition zone diameters on agar. The 14 LAB strains were then secondary screened for *S. aureus* inhibition by the agar spot test. The antagonistic effects of the LAB are presented in Table 1.

It was found that antagonistic activity toward *S. aureus* of all 14 LAB strains were lower than this capability against *Salmonella* sp. *S. aureus* is Gram-positive bacteria which have a thick mesh-like cell wall made of peptidoglycan (50–90% of cell wall), while *Salmonella* sp. is Gram-negative bacteria with a thinner layer (10% of cell wall). Gram-negative bacteria also have an additional outer membrane which contains lipids, and is separated from the cell wall by the periplasmic space. The antibacterial action of lactic acid is largely, but not totally, assigned to its ability in the undissociated form to penetrate the cytoplasmic membrane. This results in reduced intracellular pH and disruption of the transmembrane proton motive force (Ray & Sandine, 1992). The relative efficacy of lactic acid against Gram-negative bacteria is not unexpected. This is because the small water-soluble molecule lactic acid gains access to the periplasm through the water-filled porin proteins of the outer membrane (OM), as reviewed by Nikaido (1996).

The OM, however, functions as an efficient permeability barrier that is able to exclude macromolecules (such as bacteriocins or enzymes) and hydrophobic substances (such as hydrophobic antibiotics). The permeability barrier property of the OM is largely due to the presence of a specific lipopolysaccharide (LPS) layer on the membrane surface. LPS molecules consist of a lipid part, termed lipid A, and a hydrophilic heteropolysaccharide chain protruding outward and providing the cell with a hydrophilic surface (Helander, Mäkelä, Westphal, & Rietschel, 1996).

In the present study it was found that LPS17 and LD219 produced the widest inhibition zones when *S. aureus* was used as an indicator. In addition, for a third screening against *E. coli* inhibition, only 7 strains exhibited inhibition zone diameters on agar (Table 1). The strains which showed widest zones of inhibition against *E. coli* were LPS04. Roth and Keenan (1971) reported that lactic acid is able to cause sub-lethal injury to *E. coli*, and similar properties have also been assigned to acetic acid (Przybylski & Witter, 1979). Indirect evidence suggests that such injury involves disruption of the LPS layer. A permeabilizer function of lactic acid

Table 1

Inhibition zone diameters of LAB isolated from Plasom on agar when *Salmonella* sp., *S. aureus* and *E. coli* were used as indicator.

Isolated no.	Inhibition zone (mm)		
	<i>Salmonella</i> sp.	<i>S. aureus</i>	<i>E. coli</i>
LD002	52.7 $\pm$ 4.6*	30.0 $\pm$ 5.0	53.7 $\pm$ 2.9
LD012	49.7 $\pm$ 4.0	29.0 $\pm$ 1.7	ND
LD016	51.3 $\pm$ 6.1	32.3 $\pm$ 0.6	53.7 $\pm$ 1.2
LD219	49.3 $\pm$ 2.1	38.3 $\pm$ 2.9	18.00 $\pm$ 2.0
LD804	55.3 $\pm$ 9.5	27.3 $\pm$ 2.0	ND
LD817	51.3 $\pm$ 1.1	37.0 $\pm$ 5.6	54.7 $\pm$ 0.6
LNM01	55.3 $\pm$ 9.0	ND	ND
LNM05	52.0 $\pm$ 7.2	20.3 $\pm$ 2.0	ND
LNM06	54.0 $\pm$ 8.2	23.3 $\pm$ 2.0	ND
LPS04	52.3 $\pm$ 8.3	31.0 $\pm$ 1.0	62.0 $\pm$ 12.2
LPS12	51.3 $\pm$ 1.5	30.0 $\pm$ 0.0	56.0 $\pm$ 4.6
LPS17	49.3 $\pm$ 3.1	38.3 $\pm$ 8.5	53.7 $\pm$ 2.1
LPS18	59.7 $\pm$ 9.5	28.0 $\pm$ 1.0	ND
LPS20	52.7 $\pm$ 2.5	26.0 $\pm$ 4.0	ND

* Mean $\pm$ SD from triplicate determinations.

ND: not determined.

Table 2
pH values and titratable acidity of cell-free supernatant of LAB isolated from *Plasom*.

Isolated no.	pH	Titratable acidity (%)
LD002	4.3	1.2 ± 0.1
LD016	4.6	1.0 ± 0.1
LD219	4.4	1.3 ± 0.1
LD817	4.5	1.1 ± 0.1
LPS04	4.5	1.2 ± 0.1
LPS12	4.6	1.1 ± 0.1
LPS17	4.6	1.1 ± 0.1

* Mean ± SD from triplicate determinations.

would not only be utilizable in decontamination procedures and in protective cultures. It would also provide a mechanistic explanation for the antimicrobial and health-promoting effects of probiotic lactic acid bacteria (Salminen, Deighton, Benno, & Gorbach, 1998). Lactic acid, in addition to its antimicrobial property due to the lowering of the pH, also functions as a permeabilizer of the gram-negative bacterial outer membrane. It may also act as a potentiator of the effects of other antimicrobial substances (Alakomi et al., 2000).

Four LAB strains (LD219, LPS04, LPS17 and LPS18) which exhibited inhibitory efficiency toward tested pathogenic bacteria were analyzed for their production of antibacterial compounds. Supernatants obtained from all strains did not exhibit the inhibition zones after pH adjustment to 6.5. It indicated that the inhibitory effect toward pathogenic bacteria mainly because of organic acid (data not shown). LAB produce various compounds such as organic acids, diacetyl, hydrogen peroxide, and bacteriocin or bactericidal proteins during lactic fermentations. The bacteriocins from them are generally recognized as safe (GRAS) and LAB have attracted a great deal of attention as a novel approach to control pathogens in food-stuffs (Savado, Ouattara, Bassole, & Traore, 2004). Levels and types of organic acids produced during the fermentation process depend on LAB species or strains, culture composition and growth conditions (Lindgren & Dobrogosz, 1990). Hydrogen peroxide is produced by LAB in the presence of oxygen as a result of the action of flavoprotein oxidases or nicotinamide adenine dinucleotide (NADH) peroxidase. The antimicrobial effect of hydrogen peroxide may result from the oxidation of sulfhydryl groups causing denaturing of a number of enzymes, and from the peroxidation of membrane lipids thus increasing membrane permeability (Condon, 1987; Kong & Davison, 1980).

The antimicrobial effect of organic acids lies in the reduction of pH, as well as the undissociated form of the molecules. It has been proposed that the low external pH causes acidification of the cell cytoplasm, while the undissociated acid, being lipophilic, can diffuse passively across the membrane. The undissociated acid acts by collapsing the electrochemical proton gradient, or by altering the cell membrane permeability which results in disruption of substrate transport systems (Ammor, Tauveron, Dofour, & Chevallier, 2006). In this research, it has been demonstrated that isolated LAB had the

ability to ferment by lowering pH and increasing the titratable acidity against the pathogenic bacteria.

3.3. Lactic acid production and pH reduction ability

Lactic acid produced by LAB is a useful compound for preserving food because it maintains the acidic conditions of the fermented products, and is antagonistic to food spoilage and food poisoning bacteria. Most species of LAB promotes the decarboxylation of L-malate to lactate and forms CO₂ if an energy source such as glucose is present. A proton is taken up in the reaction, which prevents the decrease of pH in the growth medium caused by lactic acid production from glucose fermentation (Daeschel, 1988). Seven strains of LAB, which showed excellent inhibition zone diameters larger than 30 mm on the growth of *S. aureus* (Table 1), were chosen for study in lactic acid production and reduction of pH. All of them were cultured in MRS broth including D-glucose (dextrose) 20 mg/ml which was used as the carbon source. The strain LD219 was the best for lactic acid production and pH reducing ability at 37 °C for 48 h (Table 2). The homofermentative LAB converted more than 90% of glucose to lactate via the Emden–Myerhoff–Parnas (EMP) pathway under anaerobic conditions. In this pathway, two molecules of pyruvate and NADH are generated from one molecule of glucose. The pyruvate generated acts as the endogenous final electron acceptor for the oxidation of NADH catalyzed by lactate dehydrogenase (LDH).

LAB can also use exogenous electron acceptors as well (Hayashi, Okada, & Kozaki, 1982; Warriner & Morris, 1995). Among such electron acceptors, dioxygen (O₂) is mostly discussed in terms of O₂ use by fermentative anaerobes (Lucey & Condon, 1986). Homo-fermentative LAB are facultative anaerobes and can be cultivated aerobically, although growth is slower than in anaerobic cultivation. Under anaerobic conditions, the microbes produce acetate, acetoin or both, as well as lactate, because they use O₂ as the final electron acceptor (Murphy & Condon, 1984a, 1984b; Coccagn-Bousquet, Garrigues, Loubiere, & Lindley, 1996). O₂ reduction is catalyzed by flavoprotein oxidase such as NADH oxidase and pyruvate oxidase (Murphy & Condon, 1984a, 1984b; Coccagn-Bousquet et al., 1996; Kaneko, Takahashi, & Suzuki, 1990). Hydrogen peroxide, generated in the two-electron reduction of O₂ by NADH oxidase, also can participate in NADH oxidation catalyzed by NADH peroxidase (Murphy & Condon, 1984b).

3.4. Identification of selected LAB

The strains which exhibited widest zones of inhibition against *E. coli* (LPS04), *S. aureus* (LPS17, LD219) and *Salmonella* sp. (LPS17) were identified to a genus level. LD219 was identified as *Streptococcus salivarius*, LPS04, LPS17 and LPS18 were identified as *Enterococcus faecalis* based on their morphological properties, biochemical tests and 16S rDNA analysis. The rDNA sequences were deposited in DDBJ/EMBL/Genbank as accession numbers AB530698,

Table 3
The changes in pH, titratable acidity, TVC, LAB and Enterobacteriaceae of spontaneous, back-slopping and inoculated *Plasom* at 0 and 8 days of fermentation.

<i>Plasom</i>	Fermentation times									
	0 Day					8 Day				
	pH	Titratable acidity (%)	TVC (log cfu/g)	LAB (log cfu/g)	Enterobacteriaceae (log cfu/g)	pH	Titratable acidity (%)	TVC (log cfu/g)	LAB (log cfu/g)	Enterobacteriaceae (log cfu/g)
spontaneous	5.6 ± 0.1 ^{b*}	0.3 ± 0.1 ^a	7.6 ± 0.1 ^b	7.7 ± 0.1 ^b	5.6 ± 0.2 ^a	5.7 ± 0.1 ^c	0.5 ± 0.1 ^a	7.7 ± 0.3 ^b	5.7 ± 0.1 ^a	4.7 ± 0.1 ^c
back-slopping	5.1 ± 0.1 ^a	0.5 ± 0.1 ^b	6.3 ± 0.1 ^a	6.3 ± 0.1 ^a	5.4 ± 0.1 ^a	4.6 ± 0.1 ^a	0.9 ± 0.1 ^c	5.6 ± 0.1 ^a	5.7 ± 0.1 ^a	3.9 ± 0.1 ^a
inoculated	5.8 ± 0.1 ^b	0.3 ± 0.1 ^a	8.4 ± 0.1 ^c	8.6 ± 0.1 ^c	5.3 ± 0.1 ^a	5.0 ± 0.1 ^b	0.7 ± 0.1 ^b	7.8 ± 0.1 ^b	7.8 ± 0.1 ^b	4.2 ± 0.1 ^b

* Mean ± SD from triplicate determinations.

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

Table 4Sensory evaluation liking scores of spontaneous, back-slopping and inoculated *Plasom*.

<i>Plasom</i>	Liking scores of sensory characteristics					
	colour	smell	taste	sour	texture	overall acceptance
spontaneous	6.3 ± 1.4 ^{a*}	5.9 ± 1.9 ^a	6.3 ± 1.9 ^a	5.7 ± 2.2 ^a	6.6 ± 1.7 ^a	6.7 ± 1.7 ^a
back-slopping	6.2 ± 1.6 ^a	5.8 ± 1.9 ^a	5.3 ± 2.0 ^a	5.4 ± 1.7 ^a	6.0 ± 1.5 ^a	6.3 ± 1.5 ^a
inoculated	6.6 ± 1.3 ^a	5.4 ± 1.9 ^a	5.7 ± 2.0 ^a	5.6 ± 1.6 ^a	6.6 ± 1.6 ^a	6.5 ± 1.7 ^a

*Mean ± SD from 30 panelists.

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

AB530696, AB530699 and AB530697, respectively. These strains were Gram-positive, non spore-forming cocci, catalase negative and formed off-white colonies. *S. salivarius* was isolated from various fermented foods such as yoghurt and cheese (Thian & Hartman, 1981; Quiberoni et al., 2003). *S. salivarius* is a Gram-positive coccus that forms chains and produces L-lactate from glucose. It is essential to milk fermentation, especially in the yoghurt and cheese making processes, to provide optimal conditions for the development of texture and flavour (Zoon & Allersma, 1996). In addition, *S. salivarius* or some lactic acid bacteria has the great potential use as a starter in the production of Gamma-aminobutyric acid (GABA)-containing yoghurt, fermented milk, cheese and other functional fermented foods (Irigoyen, Ortigosa, Juansaras, Oneca, & Torre, 2007; Lee et al., 2010; Minervini, Bilancia, Siragusa, Gobbetti, & Caponio, 2009; Nomura, Kimoto, Someya, Furukawa, & Suzuki, 1998; Park & Oh, 2007; Yang et al., 2008). *E. faecalis* was isolated from various fermented foods such as Tunisian fermented dough, dairy products, fermented capers, vegetable foods and fermented milk (Burgos, López, Abriouel, Omar, & Gálvez, 2009; Cariolato, Andrichetto, & Lombardi, 2008; Gardini et al., 2008; M'hir et al., 2008; McGowan-Spicer et al., 2008; Pérez-Pulido et al., 2006).

Enterococci are ubiquitous bacteria that have a predominant habitat in the digestive tract of humans and animals. They may also be present in soil, surface waters and on plants and vegetables. They can also occur in foods, especially in those of animal origin such as meat, fermented sausages and cheeses (Giraffa, 2002). It is well known that enterococci are a relevant component of the bacterial population of a variety of cheeses, mostly artisanal cheeses (Cariolato et al., 2008). In addition, it was reported that enterocin produced from *E. faecalis* were antagonistic to *Salmonella enterica*, *L. monocytogenes*, *S. aureus*, and *B. cereus* (Izquierdo, Wagner, Marchioni, Aoude-Werner, & Ennahar, 2009; Molinos et al., 2009). Gálvez et al., 1998 reported that enterocin produced from *E. faecalis* could not inhibit *E. coli* similarly to the inhibitory spectrum of cell-free supernatants from *E. faecalis* C901 against bacterial strains from human origin. Those belonging to our collection assayed on the spot found that *E. coli* was resistant. However, in the present study *E. coli* was inhibited by LPS04. Inhibition zone diameters on agar come from organic acids during lactic fermentations.

3.5. Comparison of spontaneous, back-slopping and inoculated *Plasom*

Plasom was produced by using a mixed culture (LD219, LPS04, LPS17 and LPS18) as a starter culture in the ratio 1:1:1:1 and comparing this with the control and back-slopping processes. Differences in pH, titratable acidity as lactic acid and number of total viable count (TVC) were found in these *Plasom* at both 0 and after 8 days of fermentation ($p < 0.05$). However, the amount of Enterobacteriaceae at 0 day were not different ($p > 0.05$) (Table 3). With the finished *Plasom*, the highest amount of Enterobacteriaceae was found in spontaneous *Plasom* ($p < 0.05$). Although the highest amount of LAB was found in inoculated *Plasom* ($p < 0.05$), the lowest

pH and highest lactic acid were found in back-slopping *Plasom* ($p < 0.05$). This was in agreement with Hu, Xia, and Ge (2008) who studied the characteristic of fermented silver carp sausages inoculated with LAB as a mixed starter culture. They found that Enterobacteriaceae in fermented silver carp sausages produced by a starter culture was lower than the control (without a starter culture). In a similar study, Kimaryo, Massawe, Olasupo, and Holzapfel (2000) reported the number of Enterobacteriaceae in kivunde, a Tanzanian traditional fermented cassava, produced with *Lactobacillus plantarum* as a starter culture, was lower than spontaneous fermentation. In addition, in the present study the number of Enterobacteriaceae counts in inoculated *Plasom* did not lower than in back-slopping *Plasom* as a direct consequence of the number of inoculated LAB in inoculated *Plasom* was only about 1–2 log cfu higher than in the back-slopping *Plasom*.

Normally, starter cultures were used for contributing to the development of characteristic properties of fermented food such as taste, aroma, visual appearance, texture, shelf life, nutritional value and safety (Holzapfel, 1997). In the present study, consumers preferred spontaneously fermented foods rather than inoculated fermented foods. This is because they typically showed results from a variety of microorganisms which contribute to the development of unique characteristics (Holzapfel, 2002). However, statistical analysis was undertaken using one-way ANOVA with post hoc Duncan's test. This indicated that the panelists could not detect any significant differences ($p > 0.05$) among spontaneous, back-slopping and inoculated *Plasom* when colour, smell, taste, sour, texture and overall acceptance of *Plasom* were studied (Table 4). Sobowale, Olurin, and Oyewole (2007) found that the sensory characteristic of traditional and starter cultures used for fermented cassava and fufu flour were not significantly different in terms of colour, odour, and texture. However, the cassava fufu flour produced by using the starter culture SL19 had the highest overall acceptability ($p < 0.05$). Hu, Xia, and Ge (2007) also reported no significant differences for taste, texture, and appearance in silver carp sausages produced by different combinations of LAB and *S. xylosus* used as starter cultures.

4. Conclusion

S. salivarius LD219, *E. faecalis* LPS04, *Ent. faecalis* LPS17 and *Ent. faecalis* LPS18 had good antimicrobial activity against food-borne pathogens (*Salmonella* sp., *S. aureus* and *E. coli*) as well as had good acidifying activity. The inhibitory effect toward pathogenic bacteria mainly comes from organic acid. These four strains originated from the dominant non-starter LAB of fermented fish, *Plasom* and were able to produce *Plasom* with similar quality as *Plasom* fermented with the back-slopping technique. It indicated that these four LAB strains can be used as starters to produce fermented fish, *Plasom*.

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Original article

Probiotic lactic acid bacteria from *Kung-Som*: isolation, screening, inhibition of pathogenic bacteria

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Summary Lactic acid bacteria (LAB) were isolated from *Kung-Som* at various fermentation periods. Only ten strains, named D2SM22, D6SM3, D6SM24, D6SM26, D8SM21, D10SM5, D10SM11, D10SM16, D10SM20 and D16SM26 showed a survival rate of more than 50% under the simulated gastric juice. After being subjected to simulated gastric juice, four strains (D6SM3, D8SM21, D10SM16 and D10SM20) showed a survival rate of more than 50% in simulated small intestinal juices. Growth of strain D6SM3, D8SM21 and D10SM16 under micro-aerobic and anaerobic conditions was not different. Tested pathogenic strains (*Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Vibrio parahaemolyticus* and *Salmonella* sp.) were inhibited by probiotic LAB. However, none of strains could produce bacteriocins. All strains were identified as *Lactobacillus plantarum*. No differences in pH, acidity, LAB count and liking scores between *Kung-Som* produced with starter culture and conventional method were observed ($P > 0.01$).

Keywords Fermented shrimp, lactic acid bacteria, *Lactobacillus plantarum*, pathogenic bacteria, probiotic.

Introduction

Fermentations have been used traditionally to preserve foods due to the inhibitory effect toward many microorganisms. Lactic acid bacteria (LAB) have been reported to be the predominant microorganisms in fermented foods (Nout, 1994). Inhibitory effect of lactic acid depends on species and load of pathogenic bacteria, sanitation process as well as number of LAB in fermented foods. According to Caplice & Fitzgerald (1999) and Callewaert *et al.* (2000), the use of starter cultures would be an appropriate approach for the control and optimisation of the fermentation process in order to alleviate the problems of variations in organoleptic quality and microbiological stability observed in indigenous fermented foods.

Although the concept of using bacteria as biotherapeutic agents is not new, probiotic LAB have attracted enormous attention only in recent years. Generally, probiotics are presumed to be live to exert a positive effect on the health and the well-being of the host (Ouwehand & Salminen, 1998). High tolerance to low

pH and bile salts considered as important selection criteria (Schillinger *et al.*, 2005; Maragkoudakis *et al.*, 2006). Another criterion of the probiotic culture includes the ability to inhibit pathogenic bacteria (Ouwehand *et al.*, 1999; Maragkoudakis *et al.*, 2006).

Dairy products such as fermented milk and yoghurt are often used as carrier for probiotic cultures. Recently, attention has been directed to use other fermented foods such as sausages, juice, fermented gruel or beverages as food carrier since these products are not heated and harbour high numbers of LAB (Mugula *et al.*, 2003; Pennacchia *et al.*, 2004; Fleet, 2007; Sheehan *et al.*, 2007). *Kung-Som* is a traditional fermented shrimp widely distributed in the south of Thailand. It is made from shrimp, sugar, salt and water and is fermented with the natural microbial flora. The main microorganisms found in *Kung-Som* are LAB (Tanasupawat *et al.*, 1998; Kobayashi *et al.*, 2003). In order to use probiotics as starter cultures for fermented shrimp such as *Kung-Som*, probiotic culture should be well-adapted to the fermentation conditions and becomes dominant in the final product.

The objectives of this work were to characterise probiotic LAB isolated from *Kung-Som*, naturally fermented shrimp at different stages of the fermentation and to select the most suitable strains, according to their

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technological characteristics including probiotic properties and antimicrobial activity against food-borne pathogens, to be used as starter cultures.

Materials and methods

Kung-Som preparation

The original formula of *Kung-Som* consisted of 1 kg shrimp, 75 g table salt, 300 g sugar and 250 mL water. The mixtures were transferred to glass jar (nine bottles) and covered with polyethylene film. Rubber band was used to hold the film in place. The containers were incubated at room temperature for 16 days. During fermentation, each jar was taken for LAB isolation every 2 days. Prior to isolation the selected sample was blended and used as a composite sample.

Isolation of LAB

Samples (25 g) were added to 225 mL of sterile 0.05 M potassium phosphate buffer pH 7.2, containing 10 mg mL⁻¹ NaCl (PBS) and shaken for 5 min. Appropriate decimal dilutions (10⁻⁵ to 10⁻⁶) were prepared in PBS buffer and poured into sterile petri dish on de Man Rogosa and Sharpe (MRS; Labscan Asia Co., Ltd, Bangkok, Thailand) agar containing 3 mg mL⁻¹ CaCO₃ and 1.5 mg mL⁻¹ ox bile salt (Difco, Detroit, MI, USA) (Maragkoudakis *et al.*, 2006) and subjected to incubation at 37 °C for 24 h. Bacterial colonies that exhibited clear zone on the plates were individually picked and streaked on MRS agar containing 3 mg mL⁻¹ CaCO₃. This procedure was repeated in order to purify the isolates. Each of the isolates was first tested for catalase by placing a drop of 3% hydrogen peroxide solution on the cells. Immediate formation of bubbles indicated the presence of catalase in the cells. Only those isolates which were catalase-negative were Gram-stained, and only those which were Gram-positive were maintained in MRS broth containing 25% glycerol at -20 °C. For routine analysis, strains were subcultured twice in MRS broth for 24 h at 37 °C. Selected LAB were identified by the API 50 CHL identification kit (Biomérieux, Marcy l'Etoile, France) and 16S rRNA analysis.

Survival of LAB under simulated stomach condition

The acid tolerance of LAB was studied in different pH solutions. Bacterial cells from 24 h incubation were harvested by centrifuging at 9500 g for 10 min at 4 °C. Cells were washed twice with PBS buffer before being resuspended in PBS solution, adjusted to pH 2.5 or 3 and added with 3 mg mL⁻¹ pepsin (Fluka, St Louis, USA) (Maragkoudakis *et al.*, 2006). Tolerant LAB was assessed in terms of viable colony counts and enumerated after incubation at 37 °C in water bath for 4 h.

Survival of LAB under simulated intestinal juice after sequential incubation in simulated gastric juice

After 4 h in being subjected to simulated gastric juice, the strains were centrifuged and washed twice with PBS solution before being resuspended in PBS solution, pH 8 containing 1 mg mL⁻¹ pancreatin (Sigma, St Louis, USA) and 3 mg mL⁻¹ or 5 mg mL⁻¹ ox bile salts. Tolerant LAB were assessed in terms of viable colony counts and enumerated after incubation at 37 °C in water bath for 4 h (De Angelis *et al.*, 2006; Maragkoudakis *et al.*, 2006).

Effect of micro-aerobic and anaerobic condition on growth of LAB

One percent of 24 h LAB was inoculated to 10 mL MRS broth (micro-aerobic condition) and 10 mL MRS broth containing 0.5 mg mL⁻¹ L-cysteine covered with liquid paraffin (anaerobic condition) (Talwalkar *et al.*, 2001). The samples were incubated at 37 °C for 24 h. For micro-aerobic condition, cell were enumerated on MRS agar and incubated at 37 °C for 24 h. On the other hand, cells were dropped on MRS agar containing 0.5 mg mL⁻¹ L-cysteine and overlaid with 15 g L⁻¹ agar, followed by incubation at 37 °C for 24 h in anaerobic jar.

Inhibitory effect in co-cultivation

The effectiveness of selected LAB on the growth of pathogenic bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Vibrio parahaemolyticus* and *Salmonella* sp.) was further studied in a co-cultivation under anaerobic condition. Each 4 mL of MRS broth and BHI broth containing 0.5 mg mL⁻¹ L-cysteine was placed into test tube. One mL of each pathogen active inoculums in BHI broth (10⁶ CFU mL⁻¹) was transferred into the test tube. Immediately, 1 mL of the active culture of LAB in MRS broth (10⁶ CFU mL⁻¹) was aseptically transferred to the same test tube and overlaid with liquid paraffin. The controls of pathogenic strains and LAB were separately transferred into test tube containing Brain Heart Infusion broth (BHI, HiMedia Laboratores Pvt. Ltd, Mumbai, India) and MRS broth, respectively and then were incubated for 48 h at 37 °C in a water bath. Aliquots from each test tube were analysed every 12 h for pH and survival of each LAB and pathogenic bacteria. The individual total bacteria count for each LAB and tested pathogen was estimated by preparing 10-fold serial dilutions. The dilution was spreaded onto the MacConkey agar (HiMedia Laboratores Pvt. Ltd) for *E. coli*, on Baird Parker agar (HiMedia Laboratores Pvt. Ltd) for *S. aureus*, on Mannitol-egg yolk polymyxin agar (HiMedia Laboratores Pvt. Ltd) for *B. cereus*, on

thiosulfate–citrate–bile salts–sucrose agar (HiMedia Laboratores Pvt. Ltd) for *V. parahaemolyticus*, on Salmonella–Shigella agar (HiMedia Laboratores Pvt. Ltd) for *Salmonella* sp., and dropped on MRS agar in order to check the survival of LAB. Plates were incubated at 37 °C for 15 h, and then the colonies were counted and expressed as CFU mL⁻¹.

Strains exhibiting antagonistic activities against pathogenic bacteria were investigated for their antimicrobial compounds as described by Aslim *et al.* (2005). Strains of LAB were grown for 24 h at 37 °C in 10 mL of MRS broth and then centrifuged at 9500 g for 10 min. Three samples of the supernatant were taken from each strain of LAB. Samples 1 and 2 were adjusted to pH 6.5 with NaOH (1 N) to rule out acid inhibition. For sample 2, a catalase solution (Fluka, Germany) (300 U mL⁻¹ in PBS buffer, pH 7) was added to rule out inhibition from hydrogen peroxide. For sample 3, the supernatant was used as a control. The antagonistic activity of the three samples was detected using the well diffusion agar method (Papamanoli *et al.*, 2003). The sterile plates were poured with 20 mL of soft BHI agar (containing 1.0% agar) inoculated with overnight culture of pathogenic bacteria (about 10⁷ CFU mL⁻¹). After solidification, wells were perforated with a sterile 7 mm cork porer. The culture filtrates (50 µL) were placed into each well. The plates were incubated at 37 °C and examined after 24 h for clear zones of the pathogenic bacteria inhibition. The diameters of the inhibition zones were measured and the diameter of the well, 7 mm, was subtracted from the total zone diameter.

Comparison of spontaneous and probiotics LAB fermented *Kung-Som*

Kung-Som was prepared according to the formula described in *Kung-Som* preparation. Selected probiotic LAB was cultivated in 10 mL MRS broth at 37 °C for 18 h. The bacterial cells were harvested by centrifugation (9500 g, 10 min, and 4 °C). Then, cell pellets were washed and suspended in 10 mL of saline solution. Two different *Kung-Som* were prepared with starter culture (10⁷ CFU g⁻¹) and without starter culture, respectively. The pH, total acidity as lactic acid (AOAC, 2000), LAB, *Enterobacteriaceae* and total bacteria analysis of *Kung-Som* were determined every 2 days.

Sensory evaluation of *Kung-Som*

The sensory evaluation was carried out with 9-point hedonic scale (1 = dislike extremely, 9 = like extremely) (Lawless & Heymann, 1999; Meilgaard *et al.*, 1999) by thirty panelists. Panelists were asked to evaluate for colour, odour, flavour, texture and overall likeness of samples fermented for 10 days.

Statistical analysis

Differences in means were analysed by two-tailed paired *t*-test using Statistic Package for the Social Science (SPSS 10.0 for Windows, SPSS Inc., Chicago, IL, USA).

Results and discussion

Isolation of LAB

When MRS agar containing 3 mg mL⁻¹ CaCO₃ and 1.5 mg mL⁻¹ ox bile salt was used as a preliminary screening medium for LAB and their probiotic properties, 311 isolates exhibited a clear zone and growth on MRS agar supplemented with CaCO₃ and bile salt. However, only 274 isolates were identified as LAB using the criteria of being Gram positive and catalase negative. Of these 274 isolates, 120 isolates were cocci; 111 isolates were short rods and 43 isolates were rods.

The selection of suitable probiotic starter culture among strains naturally present in fermented products should be emphasised. Hammes & Hertel (1998) highlighted the importance of using dominant strains of meat products as probiotics because they are more competitive than LAB from other sources due to their adaptation to the meat environment. Bile salt was added into the MRS agar in order to preliminary screening of probiotic property. From isolation and screening of LAB from *Kung-Som* at various fermentation periods, it was found that 311 isolates exhibited clear zone on MRS agar supplemented with bile salt and CaCO₃. However, only 274 isolates were identified as LAB. LAB grew slowly on bile salt-containing MRS agar. Bile salts affected the cell membrane of the microorganisms which were composed of lipids and fatty acids. Bile acids can inhibit growth or kill many bacteria. The protonated (non-dissociated) form of the bile salts exhibits toxicity through the same mechanism as organic acids, by causing intracellular acidification and collapse of the proton motive force, which in turn results in inhibition of nutrient transport. However, some LAB are able to hydrolyse bile salts with bile salt hydrolase. This weakens their detergent effect (Erkkila & Petaja, 2000). In addition, calcium carbonate was used as an indicator for acid-producing strain since it dissolved when interacts with acid and the clear zone was observed (Onda *et al.*, 2002).

Survival of LAB under simulated the stomach condition

The gastrointestinal transit tolerance of 274 LAB isolates was determined by exposing washed cell suspensions to simulated gastric juice (pH 3.0) containing pepsin (3 mg mL⁻¹) at 37 °C for 4 h, mimicking the stomach condition. Under this condition, only ten strains, named D10SM20, D10SM5, D16SM26,

D10SM16, D6SM26, D6SM3, D8SM21, D6SM24, D2SM22 and D10SM11 had a survival rate of more than 50% (Table S1). However, the survival rate at pH 2.5 in the presence of pepsin (3 mg mL⁻¹) of these ten isolates was very low (Table S2).

In order to survive and establish within the human gastrointestinal tract, some properties of probiotics including their ability to resist the very acidic pHs (pH 2.5–pH 3.5) of the stomach (Holzapfel *et al.*, 1998) and the exposure to bile in the upper part of the intestine should be studied. Ten isolates out of 274 isolates retained their viability higher than 50% after 4 h of exposure to pH 3 in the presence of pepsin (3 mg mL⁻¹). The survival rate was lowered at pH 2.5. Survival at pH 3 is significant as ingestion with food or dairy products raises the pH in stomach to 3.0 or higher (Martini *et al.*, 1987; Mainville *et al.*, 2005). In similar studies, Goldin *et al.* (1992) reported the survival of *Lactobacillus* GG at pH 3 and Charteris *et al.* (1998) reported almost complete loss of viability for *L. casei* 212.3 and F19 strains *Lactobacillus* GG at 3 h interval at pH 2.5. Jacobsen *et al.* (1999) reported that out of forty-four *Lactobacillus*, none of strain could replicate at pH 2.5. The results were in accordance with previous study that the tolerance of *L. casei* to acid was highly strain specific. Out of seven strains screened, only three, C1, Y and NCDC17, exhibited fairly acid tolerance and survived at pH 2 and/or 3 (Mishra & Prasad, 2005). However it was not clear whether the decrease of viability was caused by pepsin alone or by synergy with high acidity. Generally, those probiotic bacteria are mainly consumed in the presence of foods such as milk or meat. Milk proteins have a protective effect on the starters and thus support bacterial survival in the acidic environment of the stomach (Conway *et al.*, 1987; Charteris *et al.*, 1998; Fernandez *et al.*, 2003).

Survival of LAB under simulated intestinal juice after sequential incubation in simulated gastric juice

The cells of LAB which survived from simulated gastric juice (pH 3.0, 3 mg mL⁻¹ of pepsin) at 37 °C for 4 h were further tested for bile salt tolerance. Only four strains (D10SM20, D8SM21, D10SM16 and D6SM3) showed a survival rate of more than 50% at pH 8.0 containing 3 mg mL⁻¹ bile salt and pancreatin (1 mg mL⁻¹) (Table S3). The survival rate of f strains decreased to some extent when 5 mg mL⁻¹ of bile salt was used (data not shown).

It has been recommended to use 3 mg mL⁻¹ bile as a suitable concentration for selection of probiotics (Gilliland *et al.*, 1984; Goldin *et al.*, 1992). Four of ten isolates showed survival rate of more than 50% under simulated small intestine. In contrast to pepsin, most strains examined in this study could survive well in a pancreatin solution at pH 8.0 in the presence of bile salts (3 mg mL⁻¹), simulating the small intestine environ-

ment. Most studies have shown that the majority of the strains survived well under such conditions, suggesting a potential recuperation of the initial levels during the passage of the small intestine (Charteris *et al.*, 1998; Du Toit *et al.*, 1998; Jacobsen *et al.*, 1999; Fernandez *et al.*, 2003). However, the susceptibility or resistance of probiotic cultures to bile is species as well as strain specific (Gilliland *et al.*, 1984).

Effect of micro-aerobic and anaerobic condition on growth of LAB

No differences in growth of strain D6SM3, D8SM21 and D10SM16 under micro-aerobic and anaerobic condition were observed ($P > 0.01$) as shown in Table S4. Nevertheless, the growth of strain D10SM20 was different under both conditions ($P < 0.01$).

Probiotic bacteria generally grow and colonise at the small intestine. Under strictly anaerobic condition, probiotic bacteria have been reported to survive under aerobic or micro-aerobic condition (Talwalkar *et al.*, 2001; Talwalkar & Kailasapathy, 2003; Altieri *et al.*, 2008). In the present study, the growth of LAB under micro-aerobic and anaerobic condition was not significantly different except for strain D10SM20. Oxygen toxicity is a major problem in the survival of probiotic bacteria. Screening probiotic bacteria for oxygen tolerance before their incorporation could ensure high cell counts in aerobic condition (Talwalkar *et al.*, 2001).

Inhibitory effect in co-cultivation

The inhibitory effect of these four strains (D10SM20, D8SM21, D10SM16 and D6SM3) on the growth of food borne pathogens (*E. coli*, *S. aureus*, *B. cereus*, *V. parahaemolyticus* and *Salmonella* sp.) was studied in a co-cultivation under anaerobic condition. The growth of *E. coli*, *S. aureus*, *B. cereus* and *Salmonella* sp. cultivated with LAB increased within the first 12 h and dramatically decreased throughout the incubation of 48 h (Figs S1a, S2a, S3a and S4a). The inhibition of pathogens likely coincided with the decreases in pH. However, decreasing pH in the first 12 h had no impact on the growth of those pathogens. Generally, pH lower than 4.4 could inhibit the growth of *E. coli* (Alvarado *et al.*, 2006); pH lower than 3.7 could inhibit *S. aureus* (Alvarado *et al.*, 2006); pH lower than 4.0 inhibited *B. cereus* (Yang *et al.*, 2008) and pH lower than 4.4 was able to inhibit *Salmonella* sp. (Sorrells & Speck, 1970). *E. coli* was inhibited when the pH was lower than 4.4 during 12 h incubation (Fig. S1b). *S. aureus* was inhibited when the pH dropped to 4.2 within 24 h (Fig. S2b). *B. cereus* was inhibited when the pH decreased to 4.2 within 24 h (Fig. S3b) and *Salmonella* sp. was inhibited after 12 h of incubation, in which the pH was lower than 4.4 as shown in Fig. S4b.

The dynamic effect of LAB on the growth of *V. parahaemolyticus* is shown in Fig. S5a. All four strains of LAB completely inhibited the growth of *V. parahaemolyticus* within 24 h of incubation.

The amount of *E. coli*, *S. aureus*, *B. cereus* and *Salmonella* sp. in the control increased continuously and the pH of the control broth was higher than that of the co-cultured broth. The amount of *V. parahaemolyticus* in the control increased within the first 12 h. Thereafter, the continuous decrease was found up to 48 h of incubation (Figs S1–S5).

Four probiotic LAB strains (D10SM20, D8SM21, D10SM16 and D6SM3) were analysed for their production of antibacterial compounds. Supernatants obtained from all strains did not exhibit the inhibition zones after pH adjustment to 6.5 (data not shown). It indicated that the inhibitory effect toward pathogenic bacteria mainly because of organic acids.

The potential of starter cultures to produce antibacterial compound has attracted much attention as they can be used to prevent food spoilage and to inhibit growth of food pathogens (Hammes & Hertel, 1998). The inhibitory effect of four LAB on the growth of food borne pathogenic bacteria including *E. coli*, *S. aureus*, *B. cereus*, *V. parahaemolyticus* and *Salmonella* sp. was studied in a co-cultivation under anaerobic condition. The result showed that all tested pathogenic strains were inhibited by probiotic LAB. Strain D6SM3 exhibited the highest inhibitory effect against all of pathogenic bacteria tested. LAB produce various compounds such as organic acids, diacetyl, hydrogen peroxide, and bacteriocin or bactericidal proteins during lactic fermentations. The bacteriocins from the generally recognised as safe (GRAS) LAB have arisen a great deal of attention as a novel approach to control pathogens in food-stuffs (Savadogo *et al.*, 2004). Levels and types of organic acids produced during the fermentation process depend on LAB species or strains, culture compositions and growth conditions (Lindgren & Dobrogosz, 1990). Hydrogen peroxide is produced by LAB in the presence of oxygen as a result of the action of flavoprotein oxidases or nicotinamide adenine dinucleotide (NADH) peroxidase. The antimicrobial effect of hydrogen peroxide may result from the oxidation of sulphhydryl groups causing denaturing of a number of enzymes, and from the peroxidation of membrane lipids, thus increasing membrane permeability (Kong & Davison, 1980; Condon, 1987). In this experiment, co-cultivation was studied under anaerobic condition. Thus hydrogen peroxide might not affect the growth of pathogenic bacteria. The antimicrobial effect of organic acids lies in the reduction of pH, as well as the undissociated form of the molecules. It has been proposed that the low external pH causes acidification of the cell cytoplasm, while the undissociated acid, being lipophilic, can diffuse passively across the membrane. The undissociated acid

acts by collapsing the electrochemical proton gradient, or by altering the cell membrane permeability, which results in disruption of substrate transport systems (Ammor *et al.*, 2006). Therefore, the isolated LAB had probiotic properties against pathogenic bacteria, mainly due to organic acids produced.

Identification of selected probiotic LAB

Four probiotic LAB strains (D10SM20, D8SM21, D10SM16 and D6SM3) were Gram-positive, non-spore-forming, short rods, catalase negative and off-white colonies. All strains were identified as *Lactobacillus plantarum* based on their morphological properties and biochemical tests by API 50 CHL. The result was confirmed by 16S rRNA gene sequencing.

Four selected LAB isolates were Gram-positive, non spore-forming short rods and catalase negative. These four strains were identified as *Lactobacillus plantarum*. *L. plantarum* was isolated from various fermented foods such as chilli puree, Indonesian fermented food (Adnan & Tan, 2007), kim chi, Korean fermented food (Lee *et al.*, 2006), farmhouse fermented milk (Martinovic *et al.*, 2005), a traditional fermented gruel from Burkina Faso (Omar *et al.*, 2006), and dry fermented sausage (Papamanoli *et al.*, 2003). *Lactobacillus* species were the dominant LAB in the last stage of fermentation because they produce acid more slowly than cocci, but were more tolerant to acid than cocci (Friedemann, 1939; Adnan & Tan, 2007). In addition, *Lactobacilli* have been reported to exhibit probiotic properties (Papamanoli *et al.*, 2003; Klingberg *et al.*, 2005; Schillinger *et al.*, 2005; Maragkoudakis *et al.*, 2006).

Comparison of spontaneous and inoculated fermented shrimp

Kung-Som produced by using *Lactobacillus plantarum* D6SM3 as a starter culture was compared with that with spontaneous fermentation. No differences in pH, titratable acidity and number of LAB were found in both *Kung-Som* (data not shown). Nonetheless, *Enterobacteriaceae* of inoculated *Kung-Som* was lower than spontaneous *Kung-Som* (Fig. S6). In addition, no significant difference ($P > 0.01$) was observed in term of colour, odour, flavour and overall likeness between *Kung-Som* produced without and with starter culture (Table S5).

Kung-Som produced by non-starter cultured and starter cultured had similar pH, acidity and number of LAB ($P > 0.01$). However, a number of *Enterobacteriaceae* of inoculated *Kung-Som* was lower than that found in spontaneous counterpart. This was in agreement with Hu *et al.* (2008) who studied the characteristic of fermented silver carp sausages inoculated with LAB as mixed starter culture and found that *Enterobacteriaceae* in fermented silver carp sausages produced

by starter culture was lower than the control (without starter culture). Kimaryo *et al.* (2000) reported a number of *Enterobacteriaceae* in kivunde, a Tanzanian traditional fermented cassava, produced with *L. plantarum* as starter culture was lower than that found in product obtained from spontaneous fermentation.

Normally, starter cultures contribute to the development of characteristic properties of fermented food such as taste, aroma, visual appearance, texture, shelf life, nutritional value and safety (Holzapfel, 1997). This study indicated that liking scores of *Kung-Som* produced without and with starter culture were not different ($P > 0.01$). However, colour liking scores of inoculated *Kung-Som* was higher than that of spontaneous fermentation. In addition, odour, flavour, texture and overall liking scores of *Kung-Som* produced by starter culture were lower than those of spontaneous fermentation. Consumer may prefer spontaneous fermented foods rather than inoculated fermented foods because a variety of microorganisms contribute to the development of a unique characteristic in the former (Holzapfel, 2002). In accordance with Sobowale *et al.* (2007), the sensory characteristic of traditional and starter culture fermented cassava, fufu flour, was not significantly different in terms of colour, odour, and texture, but the cassava fufu flour produced using starter culture SL19 had the highest overall acceptability.

Conclusions

Four probiotic LAB were isolated from *Kung-Som* showed a survival rate of more than 50% under the simulated gastric juice containing pepsin and simulated small intestinal juices containing bile salt and pancreatin. Growth of 4 strains under micro-aerobic and anaerobic conditions was not different. These 4 probiotic LAB can be inhibited the growth of *E. coli*, *S. aureus*, *B. cereus*, *V. parahaemolyticus* and *Salmonella* sp. None of strains could produce bacteriocins. All strains were identified as *Lactobacillus plantarum*. Characteristics of *Kung-Som* produced using D6SM3 as starter culture were compared with those of traditional *Kung-Som*. No differences in pH, acidity, LAB count and liking scores.

Acknowledgment

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 The changes in *E. coli* growth (a) and pH of medium (b) when co-cultured with probiotic LAB in MRS + BHI broth containing 0.5 mg mL⁻¹ L-cysteine and overlaid with liquid paraffin.

Figure S2 The changes in *S. aureus* growth (a) and pH of medium (b) when co-cultured with probiotic LAB in MRS + BHI broth containing 0.5 mg mL⁻¹ L-cysteine and overlaid with liquid paraffin.

Figure S3 The changes in *B. cereus* growth (a) and pH of medium (b) when co-cultured with probiotic LAB in

MRS + BHI broth containing 0.5 mg mL⁻¹ L-cysteine and overlaid with liquid paraffin.

Figure S4 The changes in *Salmonella* sp. growth (a) and pH of medium (b) when co-cultured with probiotic LAB in MRS + BHI broth containing 0.5 mg mL⁻¹ L-cysteine and overlaid with liquid paraffin.

Figure S5 The changes in *V. parahaemolyticus* growth (a) and pH of medium (b) when co-cultured with probiotic LAB in MRS + BHI broth containing 0.5 mg mL⁻¹ L-cysteine and overlaid with liquid paraffin.

Figure S6 The changes in Enterobacteriaceae growth of spontaneous and inoculated fermented shrimp at 0, 2, 4, 6, 8 and 10 days of fermentation period.

Table S1 Survival rate of LAB in the presence of pepsin (3 mg mL⁻¹) at pH 3.

Table S2 Survival rate of LAB in the presence of pepsin (3 mg mL⁻¹) at pH 2.5.

Table S3 Survival rate of LAB in the presence of 3 mg mL⁻¹ bile salt and pancreatin (1 mg mL⁻¹) at pH 8.0 (4 h) after sequential incubation in simulated gastric juice (4 h).

Table S4 Total counts of LAB in MRS broth (micro-aerobic condition) and MRS broth containing 0.5 mg mL⁻¹ L-cysteine (anaerobic condition).

Table S5 Liking scores of spontaneous and probiotic LAB inoculated *Kung-Som*.

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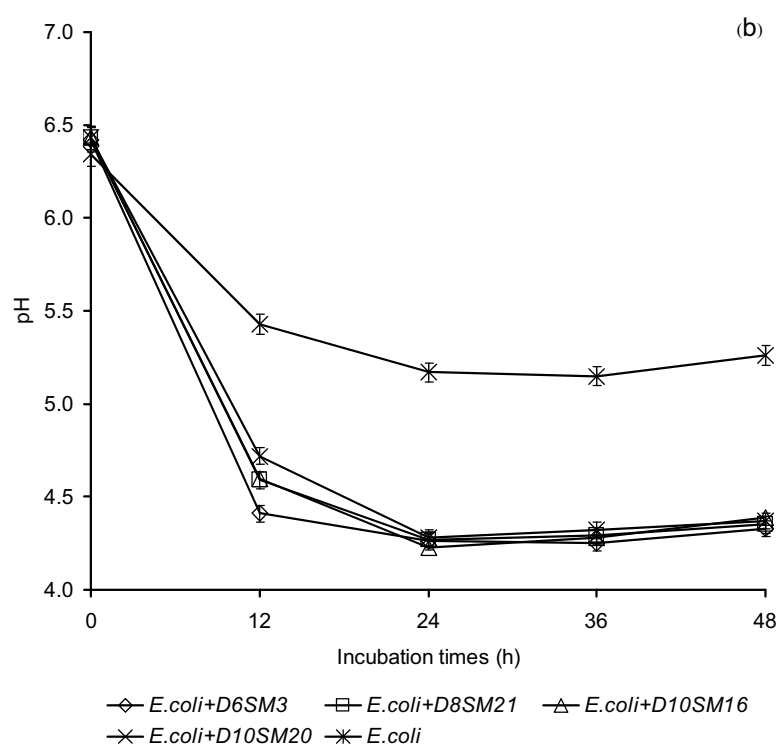
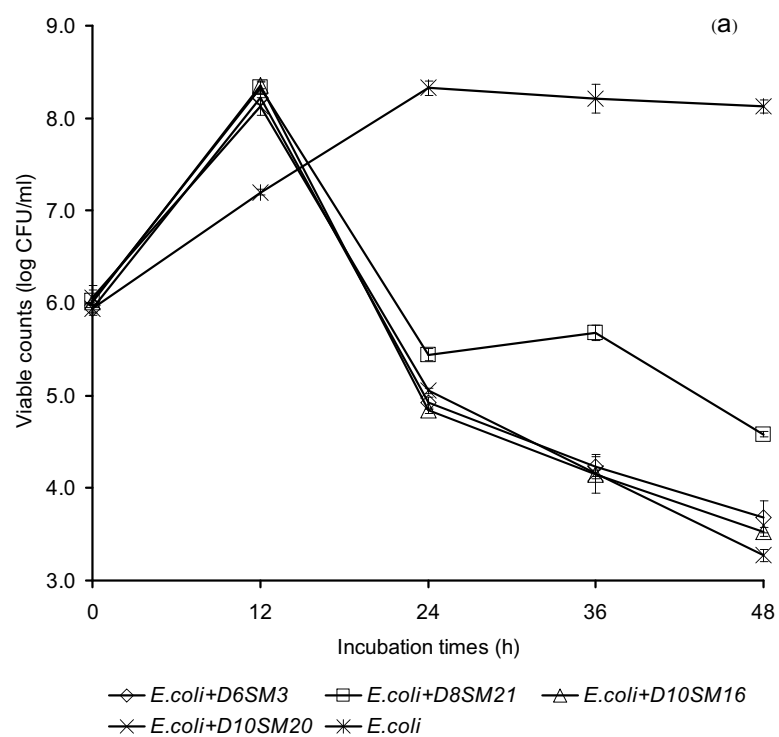


Fig. 1

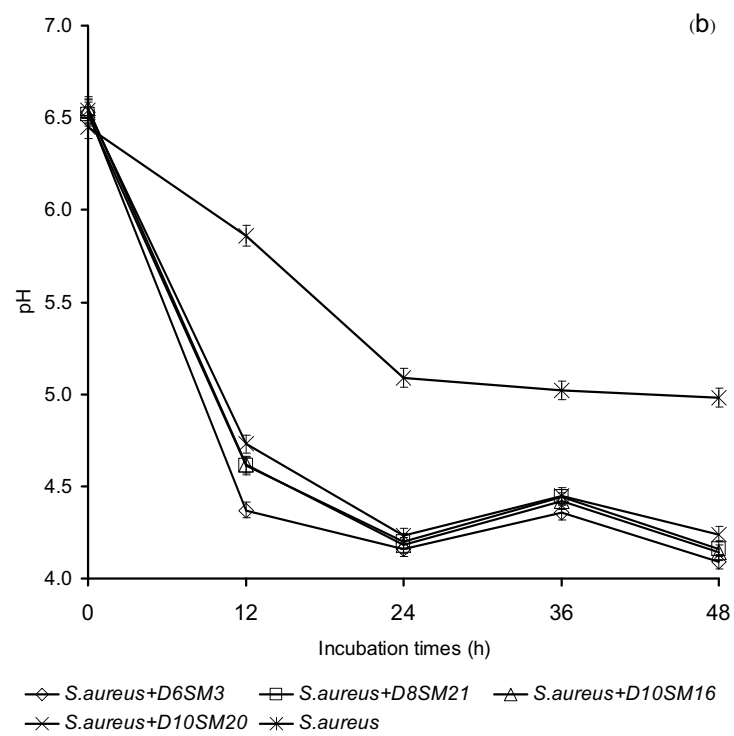
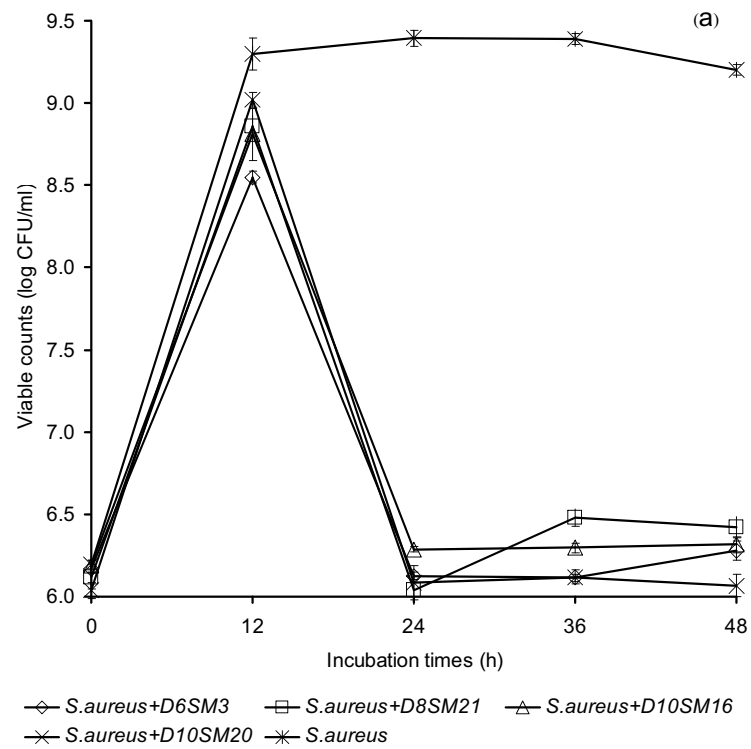


Fig. 2

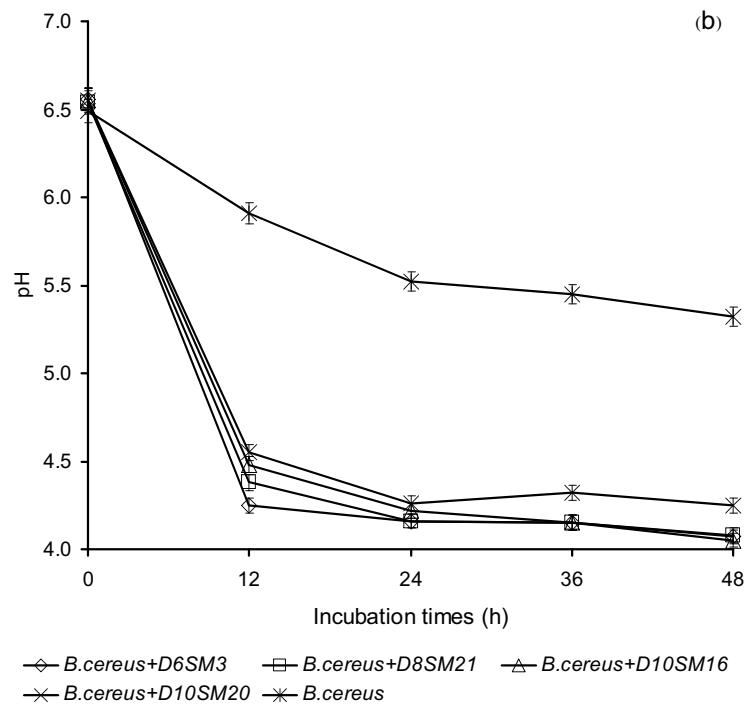
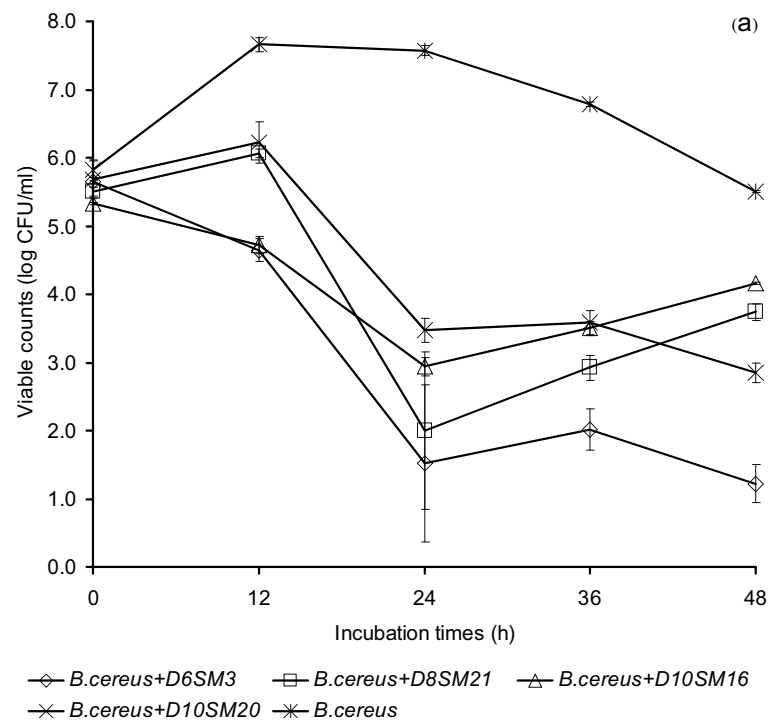


Fig. 3

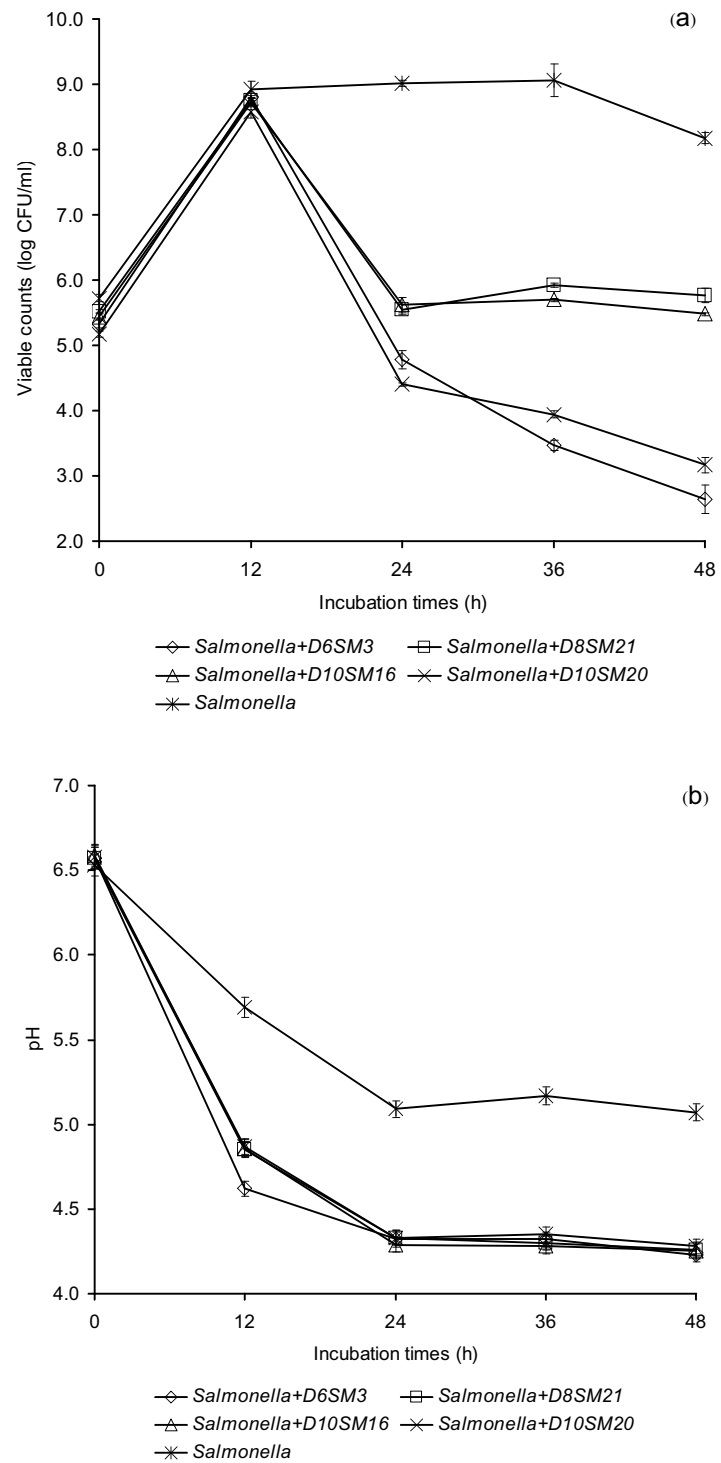


Fig. 4

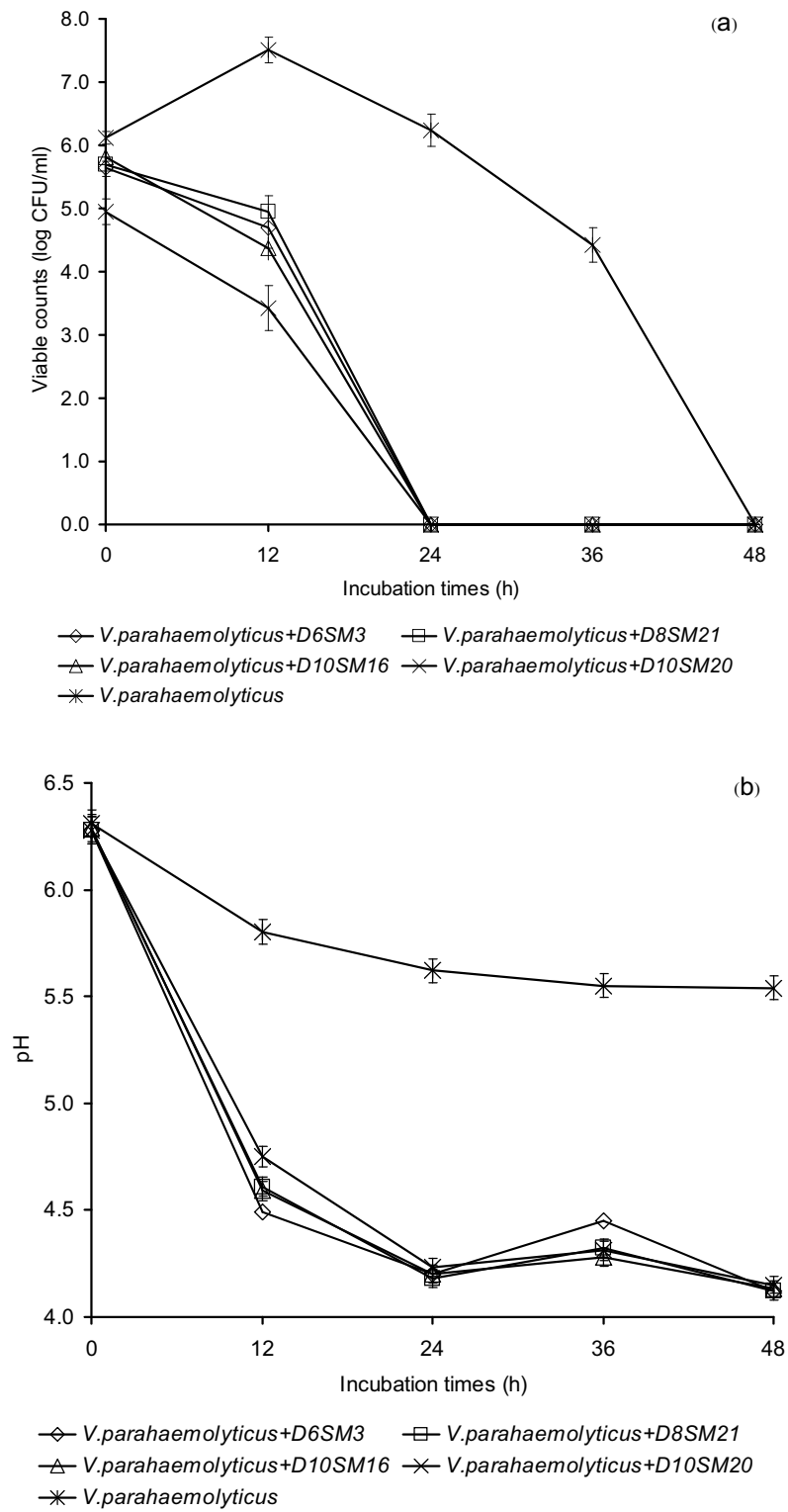


Fig. 5

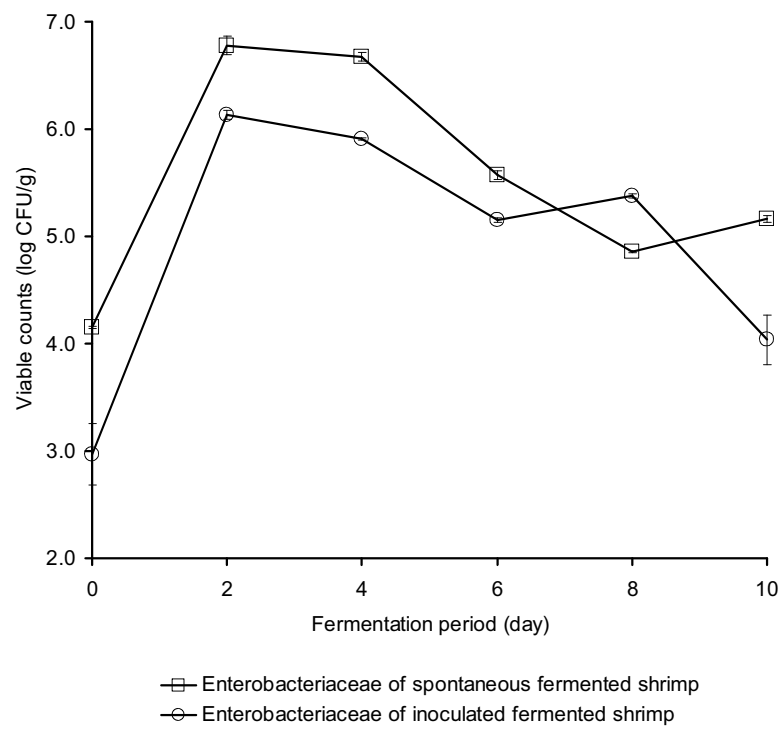


Fig. 6

1 **Table 1** Survival rate of LAB in the presence of pepsin (3 mg ml⁻¹) at pH 3

Isolate no.	log CFU ml ⁻¹ (0 h)	log CFU ml ⁻¹ (4 h)	Survival rate (%)
D10SM20	8.88±0.04*	8.82±0.04	87.45
D10SM5	9.17±0.04	9.09±0.03	82.01
D16SM26	8.86±0.03	8.73±0.02	74.31
D10SM16	8.35±0.04	8.18±0.08	68.45
D6SM26	9.10±0.04	8.91±0.04	63.40
D6SM3	8.93±0.10	8.74±0.04	62.52
D6SM24	8.41±0.01	8.14±0.02	54.17
D2SM22	8.91±0.07	8.65±0.05	53.71
D10SM11	9.00±0.04	8.71±0.08	51.66
D8SM21	9.24±0.02	8.94±0.05	50.00

2 * Mean ± SD from triplicate determinations.

1 **Table 2** Survival rate of LAB in the presence of pepsin (3 mg ml⁻¹) at pH 2.5

Isolate no.	log CFU ml ⁻¹ (0 h)	log CFU ml ⁻¹ (4 h)	Survival rate (%)
D6SM3	8.93±0.10*	6.08±0.03	0.14
D10SM20	8.88±0.04	5.81±0.10	0.09
D10SM5	9.17±0.04	5.57±0.02	0.02
D10SM16	8.35±0.04	4.42±0.05	0.01
D2SM22	8.91±0.07	4.86±0.01	0.01
D8SM21	9.24±0.02	5.15±0.10	0.01
D6SM26	9.10±0.04	4.94±0.14	0.01
D6SM24	8.41±0.01	3.95±0.08	0.00
D16SM26	8.86±0.03	4.37±0.07	0.00
D10SM11	9.00±0.04	4.19±0.09	0.00

2 * Mean ± SD from triplicate determinations.

- 1 **Table 3** Survival rate of LAB in the presence of 3 mg/ml bile salt and pancreatin (1 mg ml⁻¹) at pH
2 8.0 (4 h) after sequential incubation in simulated gastric juice (4 h)

Isolate no.	log CFU ml ⁻¹ (0 h)	log CFU ml ⁻¹ (4 h)	Survival rate (%)
D6SM3	8.74±0.04*	8.69±0.18	94.02
D8SM21	8.94±0.05	8.75±0.02	64.37
D10SM16	8.18±0.08	7.88±0.14	51.42
D10SM20	8.82±0.04	8.53±0.02	51.00
D10SM11	8.71±0.08	8.23±0.04	32.69
D16SM26	8.73±0.02	8.10±0.00	23.46
D10SM5	9.09±0.03	8.43±0.03	22.02
D6SM26	8.91±0.04	6.68±0.07	0.60
D6SM24	8.14±0.02	5.65±0.05	0.33
D2SM22	8.65±0.05	5.74±0.10	0.12

- 3 * Mean ± SD from triplicate determinations.

1 **Table 4** Total counts of LAB in MRS broth (micro-aerobic condition) and MRS broth containing
2 0.5 mg/ml L-cysteine (anaerobic condition)

Isolate no.	log CFU ml ⁻¹ (0 h)	log CFU ml ⁻¹ (24 h)		<i>P</i> **
		Micro-aerobic condition	Anaerobic condition	
D6SM3	7.48	9.18±0.15 ^{a*}	9.41±0.07 ^a	0.127
D8SM21	7.45	9.48±0.04 ^a	9.56±0.08 ^a	0.339
D10SM16	5.86	9.38±0.02 ^a	9.43±0.06 ^a	0.274
D10SM20	7.34	9.24±0.04 ^a	9.36±0.04 ^b	0.003

3 * Mean ± SD from triplicate determinations.

4 ***P* < 0.01 is considered significant difference (paired *t* test, two-tailed).

5 Different superscripts in the same row indicate significant differences (*P* < 0.01).

1 **Table 5** Liking scores of spontaneous and probiotic LAB inoculated *Kung-Som*

Attributes	Liking scores	
	Spontaneous <i>Kung-Som</i>	Inoculated <i>Kung-Som</i>
Colour	7.53±1.48 ^{a*}	7.73±1.20 ^a
Odour	7.30±1.60 ^a	7.00±1.68 ^a
Flavour	7.17±1.70 ^a	6.67±1.65 ^a
Texture	7.57±1.59 ^a	7.17±1.68 ^a
Overall acceptance	7.40±1.67 ^a	6.90±1.77 ^a

2 * Mean ± SD from 30 panelists.

3 Different superscripts in the same row indicate significant differences ($p < 0.01$).

4

Output ของโครงการวิจัย

1. ผลงานตีพิมพ์ในการสารวิชาการนานาชาติ จำนวน 43 เรื่อง

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- 40) Hwanhlem, N., Buradaleng, S., Wattanachant, S., Benjakul, S., Tani, A. and Maneerat, S. 2011. Isolation and screening of lactic acid bacteria from Thai traditional fermented fish (Plasom) and production of Plasom from selected strains. Food Control. 22: 401-407. (Impact factor = 2.812)
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2. การนำผลงานวิจัยไปใช้ประโยชน์

-เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

-ผลิตนักศึกษาบัณฑิตศึกษา

ปริญญาเอก 6 คน

ปริญญาโท 5 คน

-การเข้าสู่ตำแหน่งวิชาการ

ผู้ช่วยศาสตราจารย์ 3 คน

รองศาสตราจารย์ 1 คน

ยื่นขอตำแหน่งศาสตราจารย์ 1 คน

3. อื่น ๆ

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- 2) Tapingkae, W., Tanasupawat, S., Benjakul, S. and Visessanguan, W. 2009. Degradation of histamine in fish sauce by free and immobilized whole cell of *Natrinema gari* BCC 24369. 60th Pacific Fisheries Technologists Annual Meeting. February 22-25, 2009. Downtown Hilton, Portland, Oregon, USA.
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 - 4) Visessanguan, W., Tapingkae, W., Tanasupawat, S., Benjakul, S. and Parkin, K. 2009. Purification and properties of a Haloneutrophilic thermostable histamine dehydrogenase from *Natrinema gari* BCC 24369. 60th Pacific Fisheries Technologists Annual Meeting. February 22-25, 2009. Downtown Hilton, Portland, Oregon, USA.
 - 5) Khantaphant, S. and Benjakul, S. 2009. Antioxidative activity of protein hydrolysate from brownstripe red snapper as affected by prior washing and defatting. International Symposium on Seafood Processing Technology and Safety Control System. October 31-November 2, 2009. Ocean University of China, Qingdao, Shandong, China.
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 - 7). Prodpran, T., Hoque, Md. S. And Benjakul, S. 2009. Properties of cuttlefish skin gelatin film as affected by heat treatment. 3rd Joint Trans-Atlantic Fisheries Technology Conference. 15-18 September 2009, Copenhagen, Denmark.
 - 8). Temdee, W. and Benjakul, S. 2010. Effect of cashew bark and kiam wood extracts on gel properties of gelatin from the skin of cuttlefish. The 13th Food Colloids 2010. March 22-24, 2010. Granada, Spain
 - 9). Aewsiri, T., Benjakul, S., Visessanguan, W., Wierenga, P.A. and Gruppen, H. 2010. Effect of modification of cuttlefish skin gelatin using tannic acid on antioxidative activity and emulsifying properties. The 13th Food Colloids 2010. March 22-24, 2010. Granada, Spain
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 - 11). Riebroy, S., Chaijan, M., Maneerat, S., Noonium, T. and Benjakul, S. 2010. Effect of glucono-delta-lactone on properties and acceptability of Som-fug, a Thai fermented sausage, during refrigerated storage. 1st International Congress on Food Technology. November 3-6, 2010. Antalya, Turkey.
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