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ARTICLE *in* COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY PART B BIOCHEMISTRY AND MOLECULAR BIOLOGY · JUNE 2006

Impact Factor: 1.55 · DOI: 10.1016/j.cbpb.2006.01.006 · Source: PubMed

Trypsins from yellowfin tuna (*Thunnus albacores*) spleen: Purification and characterization

Sappasith Klomklao [a], Soottawat Benjakul [a,*], Wonnop Visessanguan [b], Hideki Kishimura [c], Benjamin K. Simpson [d], Hiroki Saeki [c]

[a] *Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand*
[b] *National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, 113 Paholayothin Road, Klong 1, Klong Luang, Pathumthani, 12120, Thailand*
[c] *Laboratory of Marine Products and Food Science, Research Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido, 041-8611, Japan*
[d] *Department of Food Science and Agricultural Chemistry, McGill University, Macdonald campus, 21111 Lakeshore Road, Ste. Anne de Bellevue, Quebec, Canada H9X 3V9*

Abstract

Two anionic trypsins (A and B) were purified to homogeneity from yellowfin tuna (*Thunnus albacores*) spleen by a series of column chromatographies including Sephacryl S-200, Sephadex G-50 and DEAE-cellulose. Purity was increased to 70.6- and 91.5-fold with approximately 2.8% and 15.6% yield for trypsin A and B, respectively. The apparent molecular weight of both trypsins was estimated to be 24 kDa by size exclusion chromatography and SDS-PAGE. Both trypsin A and B appeared as a single band on native-PAGE. Trypsin A and B exhibited the maximal activity at 55 and 65 °C, respectively, and had the same optimal pH at 8.5 using TAME as a substrate. Both trypsins were stable to heat treatment up to 50 °C and in the pH range of 6.0 to 11.0. Both trypsin A and B were stabilized by calcium ion. The activities were inhibited effectively by soybean trypsin inhibitor, TLCK and partially inhibited by EDTA, but were not inhibited by E-64, N-ethylmaleimide, iodoacetic acid, TPCK and pepstatin A. Activity of both trypsins continuously decreased with increasing NaCl concentration (0–30%). Apparent K_m and K_{cat} of trypsin A and B for TAME were 0.2–0.33 mM and 66.7–80 S^{-1}, respectively. The N-terminal amino acid sequences of trypsin A, IVGGYECQAHSQPHQVSLNA, and trypsin B, IVGGYECQAHSQPPQVSLNA, indicated the high homology between both enzymes.

Keywords: Trypsin; Serine proteinase; Tuna; Spleen; Purification; Isolation; Viscera; N-terminal amino acid sequence

1. Introduction

Trypsins (EC 3.4.21.4), members of a large family of serine proteinases, specifically hydrolyze proteins and peptides at the carboxyl side of arginine and lysine residues and play the major roles in biological process including digestion, activation of zymogens of chymotrypsin and other enzymes (Klomklao et al., in press-a; Cao et al., 2000). Trypsins are digestive enzymes that have many biochemical and industrial applications due to their high specificity allowing a controlled proteolysis. Among all trypsins, fish trypsins are of immense interest because they exhibit higher catalytic activity than their mammalian counterparts, making them more suitable for a number of biotechnological and food processing applications (Macouzet et al., 2005; Simpson and Haard, 1987; Simpson, 2000). Trypsins have been isolated and characterized thoroughly based on their physiochemical and enzymatic properties from several species of fish and shellfish, e.g. crayfish (*Procambarus clarkii*) (Kim et al., 1992, 1994), mackerel (*Scomber japonicus*) (Kim and Pyeun, 1986), carp (*Cyprinus carpio*) (Cao et al., 2000), crawfish (*Procambarus clarkii*) (Jeong et al., 2000), capelin (*Mallotus villosus*) (Hjelmeland and Raa, 1982), tambaqui (*Colossoma macropomum*) (Bezerra et al., 2001), true sardine (*Sardinops melanostictus*) and arabesque greenling (*Pleuroprammus azonus*) (Kishimura et al., 2006). Recently, Klomklao et al. (in press-a) purified and characterized three trypsin isoforms from skipjack tuna (*Katsuwonus pelamis*) spleen.

Fish viscera generated during the processing is a potential source of enzyme such as proteinases that may have some unique properties for industrial application (Ooshiro, 1971; Haard, 1992; Klomklao et al., 2005). Among fish industries, tuna including yellowfin, skipjack and tongol have been the important species for canning in Thailand with a large volume of raw materials used. Approximately two-thirds of the whole fish are utilized and the remaining involving the viscera becomes the waste. Accordingly, those viscera, which are the essential sources of potential proteinases, can be recovered for further uses. Based on our previous study, yellowfin tuna spleen contained high proteolytic activity, which was identified as trypsin-like serine proteinase (Klomklao et al., 2004). However, no information regarding the molecular and biochemical characteristic of yellowfin tuna spleen proteinases has been reported. Our objective was to purify and study the physicochemical and biochemical properties of trypsin from yellowfin tuna spleen.

2. Materials and methods

2.1. Chemicals

Ethylenediaminetetraacetic acid (EDTA), pepstatin A, soybean trypsin inhibitor, iodoacetic acid, *N*-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenyl-alanine chloromethyl ketone (TPCK), 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), *N*-ethylmaleimide, β-mercaptoethanol (βME) and bovine serum albumin were procured from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA.). Sephacryl S-200 and Sephadex G-50 were purchased from Pharmacia Biotech (Uppsala, Sweden). Diethylaminoethyl (DEAE)-cellulose was obtained from Whatman (Maidstone, England). N^{α}-*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME) was purchased from Wako Pure Chemicals (Osaka, Japan). NaCl, Tris (hydroxymethyl) aminomethane and Folin–Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), Coomassie brilliant blue R-250 and *N*,*N*,*N*′,*N*′-tetramethyl ethylene diamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Fish sample preparation

Internal organs from yellowfin tuna (*Thunnus albacores*) were obtained from Chotiwat Industrial Co. (Thailand) Ltd., Songkhla. Samples (5 kg) were packed in polyethylene bag, kept in ice with the sample/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 30 min. Pooled internal organs were then excised and separated into individual organs. Only spleen was collected, immediately frozen and stored at −20 °C.

2.3. Preparation of spleen extract

Frozen spleens were thawed under 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 3 vol. of acetone at −20 °C for 30 min according to the method of Kishimura and Hayashi (2002) and Klomklao et al. (in press-a). The homogenate was filtered in vacuo on Whatman No. 4 filter paper. The residue obtained was then homogenized in two vol. of acetone at −20 °C for 30 min, and then the residue was air-dried at room temperature until dried and free of acetone odor.

To prepare the splenic extract, spleen 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 used, the lyophilized sample (10 g) was dissolved with 50 mL of cold distilled water (4 °C) and referred to as "splenic extract."

2.4. Purification of yellowfin tuna spleen trypsin

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

Splenic extract was chromatographed on a Sephacryl S-200 column (3.9 × 64 cm), equilibrated with approximately two bed volumes of SB. Sample was loaded onto 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 those with TAME activity were pooled and lyophilized. Sephacryl S-200 power was dissolved in distilled water and loaded onto a Sephadex G-50 column (3.9 × 64 cm) previously equilibrated with approximately two bed volumes of SB. The elution and TAME activity determination were carried out as previously described.

Pooled Sephadex G-50 column fractions were dialyzed against SB for 10–12 h. The sample was then chromatographed on DEAE-cellulose (Whatman, England) column (2.2 × 18 cm) equilibrated with SB. The sample was loaded onto the column at a flow rate of 0.5 mL/min. The column was washed with SB until A_{280} was below 0.05 and then eluted with a linear gradient of 0–0.5 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. Two activity peaks (trypsin A and B) were obtained and pooled fractions from each peak were dialyzed with SB for 10–12 h and then concentrated by lyophilization. After the first DEAE-cellulose chromatography, trypsin B fractions were rechromatographed on the same column equilibrated with SB. The elution was performed using linear gradient of 0.425–0.43 M NaCl in SB at a flow rate of 0.5 mL/min. Fractions (5 mL) were collected and those with TAME activity were pooled and used for further study.

2.5. Trypsin activity assay

Trypsin activity was measured by the method of Hummel (1959) as modified by Klomklao et al. (in press-a) using TAME as substrate. Enzyme solution with an appropriate

dilution (20 μL) was mixed with 3.0 mL of 1 mM TAME in 10 mM Tris–HCl buffer, pH 8.0 and incubated at 30 °C for 20 min. Production of *p*-tosyl-arginine was measured by monitoring the increase 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 minute.

2.6. 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 pHs 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.

2.7. pH and thermal stability

The effect of pH on enzyme stability was evaluated by measuring the residual activity after incubation at various pH 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. 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.

2.8. Determination of molecular weight

The molecular weight of purified trypsins was determined using size exclusion chromatography on Sephacryl S-200 column, by plotting available partition coefficient (K_{av}) against the logarithm of molecular weight of the protein standards. The elution volume (V_e) was measured for each protein standard and the trypsins. Void volume (V_o) was estimated by the elution volume of blue dextran (M_r 2,000,000). The standards used included aprotinin (M_r 6,500), trypsinogen (M_r 24,000), bovine serum albumin (M_r 66,000) and catalase (M_r 232,000).

2.9. Effect of $CaCl_2$ on thermal stability

The effect of $CaCl_2$ 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 $CaCl_2$, at 40 °C for different times (0, 0.5, 1, 2, 4, 6 and 8 h). At the time designated, the samples were cooled in iced water and assayed for remaining activity.

2.10. 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.

2.11. 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. The control was conducted in the same manner except that deionized water was used instead of inhibitors.

2.12. Polyacrylamide gel electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970). Protein solutions were mixed at 1 : 1 (v/v) ratio with the SDS-PAGE sample buffer (0.125 M Tris–HCl, pH 6.8; 4% SDS; 20% glycerol; 10% β-mercaptoethanol) and boiled for 3 min. The samples (15 μg) were loaded on the gel made of 4% stacking and 12.5% separating gels and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Protean II Cell apparatus (Atto Co., Tokyo, Japan). After electrophoresis, the gels were stained with 0.1% Coomassie brilliant blue R-250 in 50% methanol and 7% acetic acid and destained with 7% acetic acid.

Native-PAGE was performed using 12.5% separating gels in a similar manner with SDS-PAGE, except that the sample was not heated and SDS and reducing agent were left out.

2.13. Determination of N-terminal amino acid sequence

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

2.14. 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: $V_{max}/[E]=K_{cat}$, where $[E]$ is the active enzyme concentration and V_{max} is the maximal velocity.

2.15. Protein determination

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

3. Results and discussion

3.1. Purification of trypsins from yellowfin tuna spleen

Purification of trypsins from yellowfin tuna spleen was accomplished by a series of chromatographic separations (Table 1). Sephacryl S-200 column chromatography separated trypsin from other proteins with lower molecular weight (Fig. 1a). Approximately 59% of activity were retained and purification fold of 10.1 was obtained. Kishimura et al. (2006) found that the use of Sephacryl S-200 in the first step of purification process of trypsin from true sardine viscera led to an increase in trypsin activity by 9-fold.

Pooled active Sephacryl S-200 fractions were further purified by a size exclusion chromatography using Sephadex G-50 (Fig. 1b). A large amount of proteins was removed with small loss in enzyme activity, leading to 40.6-fold purity. Sephadex G-50 column chromatography was highly effective in separating trypsin from other proteins, but not for resolving the individual trypsins (Kishimura et al., 2005). Therefore, pooled active fractions were subsequently subjected to an ion-exchange chromatography using DEAE-cellulose (Fig. 1c).

After loading the dialyzed active Sephadex G-50 fractions onto anion exchanger, DEAE-cellulose column, the column was washed and then eluted using a 0–0.5 M NaCl linear gradient. DEAE-cellulose separated the Sephadex G-50 fractions into two activity peaks (Fig. 1c). These enzymes were assigned as trypsin A and B based on the elution order. Purity of trypsin A and B increased approximately 70.6- and 85.8-fold, respectively. Ion exchange chromatography was used to remove the contaminating proteins and to separate different trypsin isoforms. Purification of two anionic trypsins from carp hepatopancreas was achieved by using anion exchanger, Q-Sepharose column (Cao et al., 2000). Kishimura et al. (2005) also isolated two trypsin isozymes from viscera of Japanese anchovy (*Engraulis japonica*) using DEAE-cellulose in the final step, leading to the increases in purity by 37-fold and 73-fold. However, trypsin B was not completely separated from trypsin A and other contaminating proteins. Therefore, trypsin B containing fractions were rechromatographed on a second DEAE-cellulose chromatography.

Dialyzed trypsin B containing fractions loaded onto the second DEAE-cellulose was eluted using a linear gradient of 0.425–0.43 M NaCl (Fig. 1d). From the result, this step effectively separated trypsin B from the contaminants. The 91.5-fold increase in purity with a yield of 15.6% was observed at this step. Yoshinaka et al. (1985) also chromatographed the trypsin from the eel viscera on an anion exchanger, DEAE-cellulose, twice and two anionic trypsins (1 and 2) were obtained with 22-fold purity.

Table 1
Purification of trypsins from yellowfin tuna spleen

Purification steps	Total activity (U)*	Total protein (mg)	Specific activity (U/mg protein)	Purity (fold)	Yield (%)
Crude extract	361.1	3150	0.11	1	100
Sephacryl S-200	212.5	191	1.11	10.1	58.8
Sephadex G-50	192	43	4.47	40.6	53.2
1st DEAE-cellulose					
Trypsin A	10.1	1.3	7.77	70.6	2.8
Trypsin B	79.3	8.4	9.44	85.8	22.0
2nd DEAE-cellulose					
Trypsin B	56.4	5.6	10.07	91.5	15.6

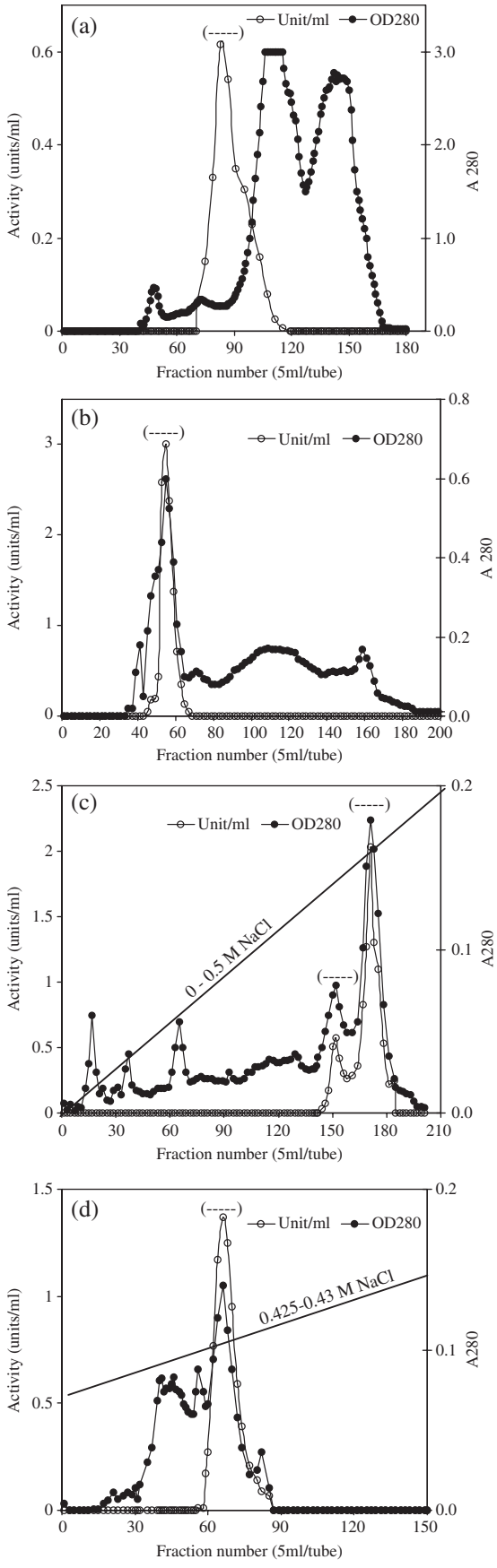
*One unit of activity was defined as the amount causing an increase of 1.0 in absorbance at 247 nm per minute. Trypsin activity was assayed at pH 8.0, 30 °C for 20 min using TAME as a substrate.

3.2. Purity and molecular weight

Purity of the purified trypsins was evaluated by using native gel electrophoresis. As shown in Fig. 2a, both trypsin A and B migrated as a single protein band and showed the different mobilities in native-PAGE, indicating the homogeneity of both enzymes. Both enzymes also appeared as a single band on SDS-PAGE with the molecular mass of 24 kDa (Fig. 2b), corresponding to that determined by Sephacryl S-200 gel filtration (data not shown). The results indicated that trypsin A and B are the monomeric protein with a molecular mass of 24 kDa. The molecular weight of trypsin A and B was similar to those of mammalian and fish trypsin. Trypsins were reported to have a molecular masses between 20 and 25 kDa (Keil, 1971; Kim et al., 1992). However, those from marine organisms have molecular masses in the range of 24–30 kDa. The molecular mass of trypsin from pyloric caeca of arabesque greenling and viscera of true sardine was estimated to be 24 kDa by SDS-PAGE (Kishimura et al., 2006). Two trypsins (I and II) from pyloric caeca of Japanese anchovy had a molecular mass of 24 kDa as estimated by SDS-PAGE (Kishimura et al., 2005). Cao et al. (2000) reported that the molecular masses of two trypsin enzymes from carp hepatopancreas were approximately 28.5 and 28 kDa by SDS-PAGE and gel filtration, respectively. Molecular masses of four trypsins from hepatopancreas of crayfish were estimated by gel filtration to be approximately 23.8, 27.9, 24.8 and 31.4 kDa, respectively (Kim et al., 1992).

3.3. Optimal pH and temperature

The pH activity curves of trypsin A and B from yellowfin tuna spleen are shown in Fig. 3a. Both trypsins exhibited the maximal activity towards TAME at pH 8.5. The activities of both purified trypsins were high in pH range of 7.0–9.0 but considerable loss of activity was observed at very acidic and alkaline pHs. No activity was found at pH 10.0 and 11.0. The sharp decrease in hydrolysis of TAME by purified trypsin at low and high pH might be attributed to irreversible denaturation. Trypsin from Monterey sardine pyloric caeca had an optimal pH at 8.0 when BAPNA was used as a substrate (Castillo-Yanez et al., 2005). Maximal activity towards TAME of trypsin from pyloric caeca of starfish (*Asterina pectinifera*) was obtained at pH 8.0 (Kishimura and Hayashi, 2002). Bezerra et al. (2005) reported that trypsin-like enzyme from intestine of Nile tilapia had optimal pH of 8.0 when azocasein was used as a substrate. Trypsin purified from the pyloric caeca of tambaqui also



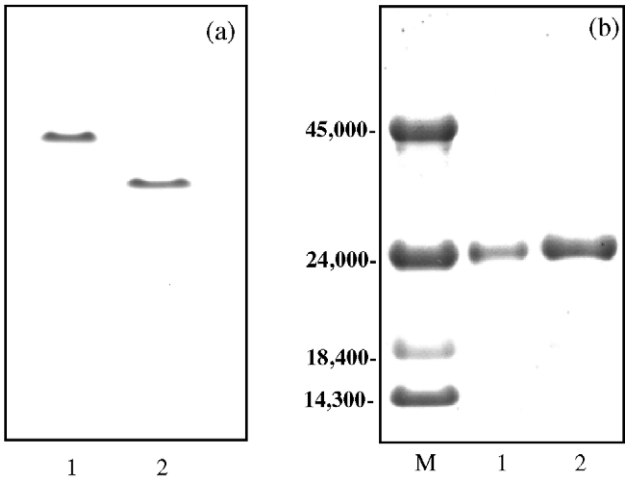


Fig. 2. Protein pattern of purified trypsins A and B from yellowfin tuna spleen determined by native-PAGE (a) and SDS-PAGE (b). M, molecular weight standard; lane 1, trypsin A; lane 2, trypsin B.

exhibited the high activity at alkaline pH (Bezerra et al., 2001). Optimal pH can be varied, depending upon protein substrates (Benjakul et al., 2003). Two trypsin-like enzymes (A and B) from digestive tract of anchovy had the optimal pH at 8.0 and 9.0 for hydrolysis of BAPNA and at 9.5 for the hydrolysis of casein as well as myofibrillar protein (Martinez et al., 1988). Maximal amidolytic and esterolytic activities of crayfish hapatopancreatic trypsins occurred in a pH range of 7.5–8.5 (Kim et al., 1994). The differences in optimal pH were attributed to the accessibility of the substrate to the active site as affected by charge on the substrate and on the active site at the particular pH of the medium (Benjakul et al., 2003). Therefore, optimal pH of two trypsins can be changed when other substrates are used instead of TAME.

The temperature profiles of two trypsins are presented in Fig. 3b. Trypsin A and trypsin B had optimal temperatures of 55 and 65 °C, respectively. An appreciable decrease in activity of both enzymes was observed at temperatures above 70 °C, presumably as a result of thermal inactivation. Enzymes were inactivated at high temperature, possibly due to the partial unfolding of the enzyme molecule. Trypsin from the pyloric caeca of rainbow trout and tambaqui had a temperature optimum of 60 °C (Kristjansson, 1991; Bezerra et al., 2001). Trypsins from crayfish hepatopancreas also had an optimal temperature of 60 °C (Kim et al., 1994). Trypsin activity from intestine of Nile tilapia had the maximal activity at 50 °C (Bezerra et al., 2005). Jeong et al. (2000) reported that four trypsins from hepatopancreas of crawfish had optimal temperatures in the range of 60–70 °C. However, these optimal temperatures were higher than those reported for trypsin from Greenland cod (Simpson and Haard, 1984), capelin (Hjelmeland and Raa, 1982), sardine (Murakami and

Fig. 1. Purification of trypsins from yellowfin tuna spleen. (a) Elution profile of trypsins on Sephacryl S-200 column; (b) elution profile of trypsins on Sephadex G-50 column; (c) elution profile of trypsins on the 1st DEAE-cellulose column. Elution was carried out with a linear gradient of 0–0.5 M NaCl in SB; (d) elution profile of trypsin B on the 2nd DEAE-cellulose column. Elution was carried out with a linear gradient of 0.425–0.43 NaCl.

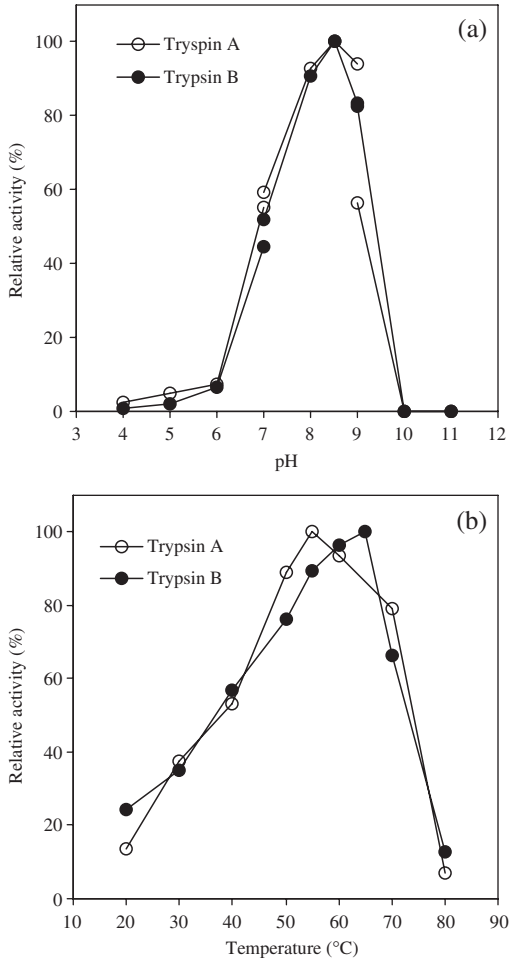


Fig. 3. pH (a) and temperature (b) profiles of purified trypsin A and B from yellowfin tuna spleen.

Noda, 1981) and Atlantic croaker (Pavlisko et al., 1997), which had the optimal temperatures in the range of 40–45 °C. The difference might be related to the different temperatures of water, where the fish inhabited.

3.4. pH and thermal stability

The effect of pH on the stability of two trypsins from yellowfin tuna spleen is depicted in Fig. 4a. Both enzymes were stable over a broad pH range (pH 6–11). However, some losses in activities were observed at slightly acidic pH for both enzymes and slightly decreased activity was found for trypsin A at alkaline pH. No activity was found for trypsin B after incubation at pH 4.0, while about 50% of trypsin A were remained at this pH. The results indicated that trypsin A was more stable in the acidic pH than trypsin B. The stability of trypsins at particular pH may be relevant to the net charge of the enzyme at that pH (Castillo-Yanez et al., 2005; Martinez et al., 1988; Klomklao et al., in press-a). At extreme pH, strong intramolecular electrostatic repulsion caused by high net charge results in swelling and unfolding of the protein molecules (Damodaran, 1996; Benjakul et al., 2000). Inactivation of enzyme activity at acidic pH was also reported for the anionic trypsins from capelin (Hjelmeland and Raa, 1982), Atlantic blue crab (Dendinger and O'Connor, 1990), true sardine (Kishimura et al., 2006) and Atlantic white croaker (Pavlisko et al., 1997).

The residual esterolytic activities of two trypsins were examined with TAME at 30 °C and pH 8.0 after heat treatment at various temperatures for 15 min (Fig. 4b). With heat treatment up to 50 °C for 15 min, no changes in activity were observed for both enzymes. However, at 60 °C, activities of trypsin A and B were reduced by 20 and 10%, respectively. All enzymes were almost completely inactivated at 80 °C. Although thermal stabilities of both trypsins were not distinctively different, trypsin B exhibited slightly higher thermal stability. Similar results were obtained for the thermal stability of trypsin obtained from starfish (Kishimura and Hayashi, 2002), Atlantic blue crab (Dendinger and O'Connor, 1990), rainbow trout (Kristjansson, 1991) and true sardine (Kishimura et al., 2006). Although the trypsins had a high maximal activity (55 and 65 °C for trypsin A and B, respectively), 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. Differences in thermal stability of enzymes might be determined by bondings stabilizing enzyme structure. More disulfide linkages as well as stronger

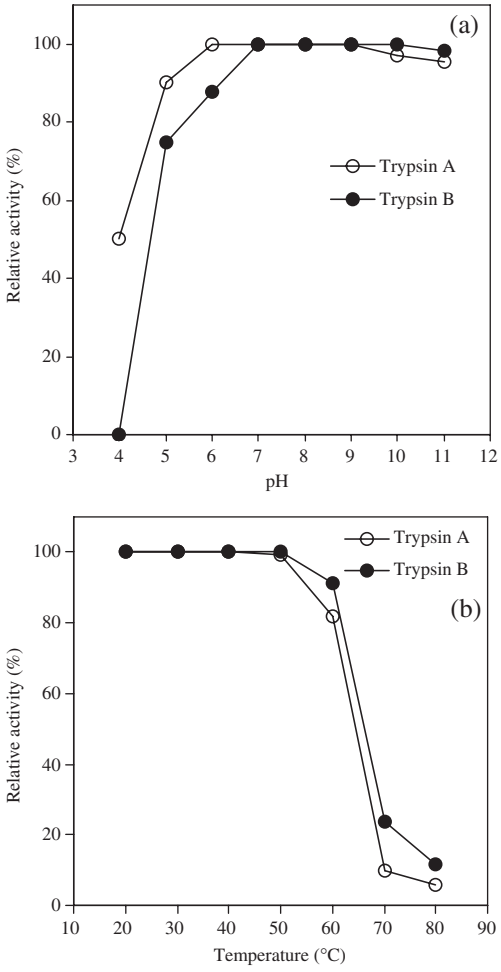


Fig. 4. pH (a) and thermal (b) stability of purified trypsin A and B from yellowfin tuna spleen.

hydrophobic interactions in the interior of protein contribute to the greater thermostability of proteins. Higher thermostability was associated with a higher number of intramolecular disulfide bonds in protease (Simpson and Haard, 1984; Heu et al., 1995; Klomklao et al., in press-a).

3.5. Effect of calcium ions on the thermal stability

The stability of trypsin A and B was highly dependent on the presence of calcium (Fig. 5). In the presence of 2 mM EDTA, the activity of both trypsins decreased with increasing time. However, in the presence of 2 mM calcium ion, approximately 90% of the original activities were remained after 8 h of incubation at 40 °C. Trypsin A was rather stable in the presence of 2 mM EDTA than trypsin B, particularly when the incubation time increased. These results indicated that both trypsins from yellowfin tuna spleen were most likely stabilized by calcium ion. The presence of calcium ions activates trypsinogen to trypsin and increases the thermal stability of the enzyme. This stabilizing effect is accomplished by a conformational change in the trypsin molecule, resulting in a more compact structure (Kim et al., 1994; Klomklao et al., 2004). Stabilization against thermal inactivation by calcium was also reported for the trypsins from true sardine (Kishimura et al., 2006), eel (Yoshinaka et al., 1985) and rainbow trout (Kristjansson, 1991). Nevertheless, calcium ion did not show the enhancing effect on stability of trypsins from sardine (Murakami and Noda, 1981), capelin (Hjelmeland and Raa, 1982) and Nile tilapia (Bezerra et al., 2005). These findings suggest a difference in the structure of the primary calcium binding site among different marine fish trypsins.

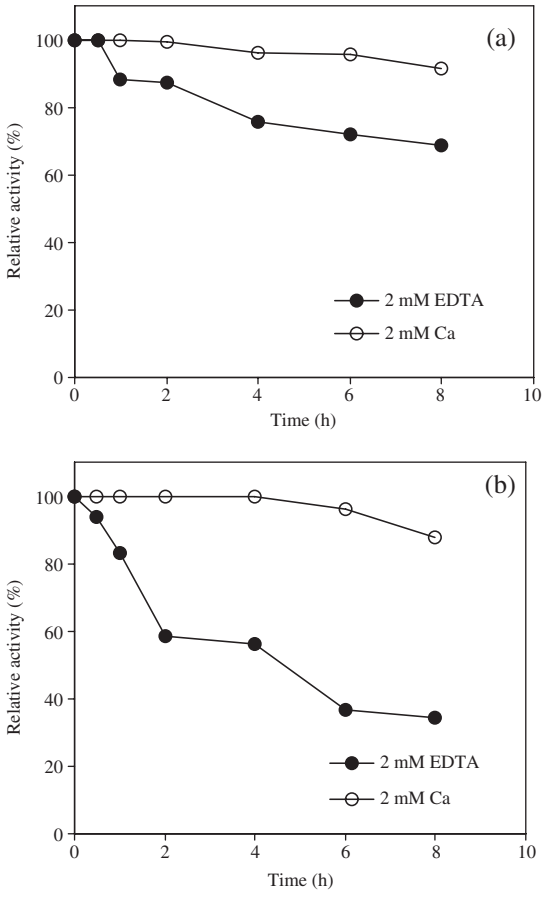


Fig. 5. Effect of calcium ion and EDTA on the stability of purified trypsin A (a) and B (b) from yellowfin tuna spleen. The stability was tested at 40 °C for different times.

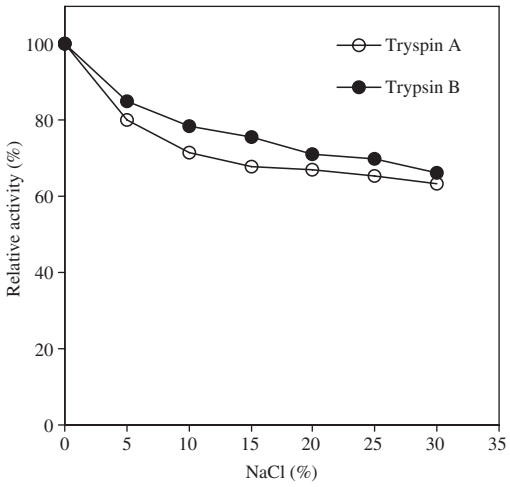


Fig. 6. Effect of NaCl concentrations on activities of purified trypsin A and B from yellowfin tuna spleen.

3.6. Effect of NaCl

The activities of both trypsins decreased gradually with increasing NaCl (Fig. 6). Trypsin B showed slightly higher activity than trypsin A in the presence of NaCl ranging from 5% to 30%. This result indicated that trypsin B was more tolerant to NaCl than trypsin A. Remaining activities of trypsin A and B at 30% NaCl were approximately 64% and 67%, respectively. 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 trypsin molecule by salt, leading to the aggregation of those enzymes (Klomklao et al., 2004; Klomklao et al., in press-c). Thermostable proteinase in salted anchovy muscle was still active and able to degrade myofibrillar protein in commercial salted fillets containing 16–17% NaCl (Ishida et al., 1994). Klomklao et al. (in press-b) also reported that natural actomyosin in sardine muscle containing 25% NaCl was degraded by partially purified trypsin from skipjack tuna spleen. From the results, more than 60% of activity was remained for both trypsins in the presence of high salt concentration (30%). Hence, these trypsins can involve in hydrolysis of proteins in high salt fermented fish products such as fish sauce.

3.7. Effect of inhibitors

The activities of both trypsins were completely inhibited by TLCK and were almost completely inhibited by soybean trypsin inhibitor (Table 2). EDTA showed the partial inhibition towards both trypsins. None of trypsins were inhibited by TPCK, a

Table 2
Effect of various inhibitors on the activity of purified trypsins from yellowfin tuna spleen*

Inhibitors	Concentration	% Inhibition**	
		Trypsin A	Trypsin B
Control		0	0
E-64	0.1 mM	0	0
N-ethylmaleimide	1 mM	0	0
Iodoacetic acid	1 mM	0	0
Soybean trypsin inhibitor	1.0 g/L	96.2±0.8	95.8±1.1
TLCK	5 mM	100	100
TPCK	5 mM	0	0
Pepstatin A	0.01 mM	0	0
EDTA	2 mM	11.8±1.8	29.9±2.1

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

specific inhibitor for chymotrypsin (Klomklao et al., 2004). Also, proteinase inhibitors for cysteine and aspartic proteinases showed no inhibitory effects on the activity of both trypsins. The result confirmed that trypsin A and B were serine proteinases, most likely trypsin. TLCK is well known as a trypsin specific inhibitor. TLCK inactivates only trypsin-like enzymes by forming a covalent bond with histidine at the catalytic portion of molecule and then blocking the substrate-binding portion at the active center (Jeong et al., 2000). Two kinds of trypsins from the hepatopancreas of carp were inhibited by some trypsin inhibitors, such as soybean trypsin inhibitor, aprotinin, benzamidine and TLCK (Cao et al., 2000). Trypsin-like enzyme from tambaqui pyloric caeca was inhibited by some trypsin inhibitors, such as PMSF, benzamidine and TLCK (Bezerra et al., 2001). From the results, it was noted that EDTA partially inhibited the activities of both trypsin A and B with the different degrees. Trypsin B was inhibited by EDTA to a greater extent, compared with trypsin A. Results indicate that these enzymes require metal ions as cofactor for activity. Therefore, two enzymes from yellowfin tuna spleen were trypsin-like proteinase, which possibly required metal ions for their activities.

3.8. Kinetic study

K_m and K_{cat} values of two trypsins for hydrolysis of TAME were calculated using Lineweaver–Burk plots and are presented in Table 3. K_m of trypsin A and B were 0.33 and 0.20 mM, respectively. K_{cat} of trypsin A and B were 80 and 66.7 S^{-1}, respectively. The K_m value of trypsin B was lower than that of trypsin A. This result suggests that trypsin B has higher affinity to TAME, compared with trypsin A. K_m values for TAME with trypsins from crayfish hepatopancreas were determined to be 0.22–0.42 mM (Kim et al., 1994), which were in agreement with the present results. The low K_m values were also found for carp trypsin (Cohen et al., 1981) and menhaden trypsin (Pyeun et al., 1990). For turnover number (K_{cat}), trypsin A had a higher value than trypsin B. However, the catalytic efficiency value for trypsin B was higher than those of trypsin A. This result suggests that trypsin B would be more efficient in transforming the substrate to product. Moreover, the catalytic efficiency of yellowfin tuna spleen trypsins was higher than those of mammalian trypsins reported (Simpson, 2000).

Table 3
Kinetic properties of yellowfin tuna spleen trypsins for the hydrolysis of TAME

Enzyme	K_m (mM)*	K_{cat} (S^{-1})*	K_{cat}/K_m (S^{-1} mM^{-1})*
Trypsin A	0.33±0.1	80.0±0.1	242.42±0.1
Trypsin B	0.20±0.3	66.7±0.2	333.5±0.2

K_m, 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.
*Mean±SD from triplicate determinations.

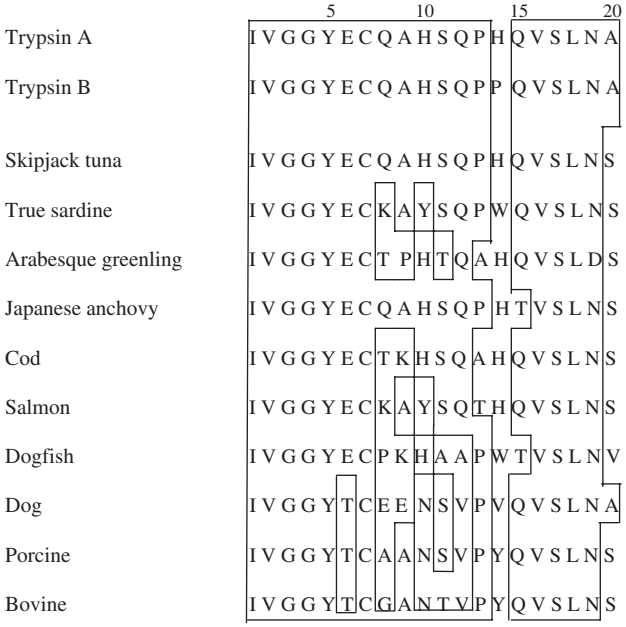


Fig. 7. Comparison of N-terminal amino acid sequences of the purified trypsin A and B from yellowfin tuna spleen with other enzymes: skipjack tuna (Klomklao et al., in press-a), true sardine, arabesque greenling (Kishimura et al., 2006), Japanese anchovy (Kishimura et al., 2005), cod (Gudmundsdottir 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 (Walsch, 1970). Amino acid residues different from those of trypsin A and B are boxed.

3.9. N-terminal sequencing

The N-terminal 20 amino acids of trypsin A and B were IVGGYECQAHSQPHQVSLNA and IVGGYECQAHSQPPQ VSLNA, respectively (Fig. 7). N-terminal amino acid sequences of two trypsins were compared with those of other teleosts, an elasmobranch and three mammals. The N-terminal of trypsins A and B exhibited high homology and showed similarity to that of skipjack tuna spleen. The highly conserved amino acid sequences between yellowfin tuna and skipjack tuna suggest that they are genetically evolved from a common ancestor, not entirely surprising for two scombroid fish. Moreover, the sequences of two trypsins from yellowfin tuna spleen and other trypsins started with IVGG after the proteolytic cleavage of inactive trypsinogen.

4. Conclusion

Two enzymes from yellowfin tuna spleen with a maximal activity at pH 8.5 and 55–65 °C were purified and identified to be trypsin based on molecular weight, substrate specificity, inhibitor study and N-terminal sequencing. Enzymatic properties were essentially consistent with those of trypsin from other species.

Acknowledgments

This research was supported by Thailand Research Fund under the Royal Golden Jubilee Ph.D. Programme to Sappasith Klomklao (Grant No. PHD/0216/2546) and a Ph.D. scholarship from the Ministry of Education of Thailand.

References

Benjakul, S., Visessanguan, W., Thummaratwasik, P., 2000. Isolation and characterization of trypsin inhibitors from some Thai legume seeds. J. Food Biochem. 24, 107–127.

Benjakul, S., Visessanguan, W., Leelapongwattana, K., 2003. Purification and characterization of heat-stable alkaline proteinase from bigeye snapper (*Priacanthus macracanthus*) muscle. Comp. Biochem. Physiol. B 124, 107–127.

Bezerra, R.S., Santo, J.F., Paiva, P.M.G., Correia, M.T.S., Coelho, L.C.B.B., Vieira, V.L.A., Carvalho, L.B., 2001. Partial purification and characterization of a thermostable trypsin from pyloric caeca of tambaqui (*Colossoma macropomum*). J. Food Biochem. 25, 199–210.

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

Cao, M.J., Osatomi, K., Suzuki, M., Hara, K., Tachibana, K., Ishihara, T., 2000. Purification and characterization of two anionic trypsins from the hepatopancreas of carp. Fish. Sci. 66, 1172–1179.

Castillo-Yanez, F.J., Pacheco-Aguilar, R., Garcia-Carreno, F.L., Toro, M.A.N., 2005. Isolation and characterization of trypsin from pyloric caeca of Monterey sardine *Sardinops sagax caerulea*. Comp. Biochem. Physiol. B 140, 91–98.

Cohen, T., Gertler, A., Birk, Y., 1981. Pancreatic proteolytic enzyme from carp (*Cyprinus carpio*) II. Kinetic properties and inhibition studies of trypsin, chymotrypsin and elastase. Comp. Biochem. Physiol. B 69, 647–653.

Damodaran, S., 1996. Amino acids, peptides, and proteins. In: Fennema, O.R. (Ed.), Food Chemistry Quality. Marcel Dekker, New York, pp. 322–429.

Dendinger, J.E., O'Connor, K.L., 1990. Purification and characterization of a trypsin-like enzyme from the midgut gland of the Atlantic blue crab *Callinectes sapidus*. Comp. Biochem. Physiol. B 95, 525–530.

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

Haard, N.F., 1992. A review of proteolytic enzymes from marine organisms and their application in the food industry. J. Aquat. Food Prod. Technol. 1 (1), 17–35.

Hermodson, M.A., Ericsson, L.H., Neurath, H., Walsh, K.A., 1973. Determination of the amino sequence of porcine trypsin by sequenator analysis. Biochemistry 12, 3146–3153.

Heu, M.S., Kim, H.R., Pyeun, J.H., 1995. Comparison of trypsin and chymotrypsin from the viscera of anchovy (*Engraulis japonica*). Comp. Biochem. Physiol. B 112, 557–567.

Hjelmeland, K., Raa, J., 1982. Characteristics of two trypsin type isozymes isolated from the Arctic fish capelin (*Mallotus villosus*). Comp. Biochem. Physiol. B 71, 557–562.

Hummel, B.C.W., 1959. A modified spectrophotometric determination of chymotrypsin, trypsin, and thrombin. Can. J. Biochem. Physiol. 37, 1393–1399.

Ishida, M., Niizelei, S., Nagayama, F., 1994. Thermostable proteinase in salted anchovy muscle. J. Food Sci. 59, 781–785.

Jeong, Y., Wei, C.I., Preston, J.F., Marshall, M.R., 2000. Purification and characterization of proteases from hepatopancreas of crawfish (*Procambarus clarkii*). J. Food Biochem. 24, 311–332.

Keil, B., 1971. Trypsin. In: Boyer, P.D. (Ed.), The enzymes. Academic Press, New York, pp. 249–275.

Kim, H.R., Pyeun, J.H., 1986. The proteinase distributed in the intestinal organs of fish II. Characterization of the three alkaline proteinases from the pyloric caeca of mackerel, *Scomber japonicus*. Bull. Jpn. Soc. Sci. Fish. 19, 547–557.

Kim, H.R., Meyers, S.P., Godber, J.S., 1992. Purification and characterization of anionic trypsins from the hepatopancreas of crayfish, *Procambarus clarkii*. Comp. Biochem. Physiol. B 103, 391–398.

Kim, H.R., Meyers, S.P., Pyeun, J.H., Godber, J.S., 1994. Enzymatic properties of anionic trypsins from the hepatopancreas of crayfish, *Procambarus clarkii*. Comp. Biochem. Physiol. B 107, 197–203.

Kishimura, H., Hayashi, K., 2002. Isolation and characteristics of trypsin from pyloric caeca of the starfish *Asterina pectinifera*. Comp. Biochem. Physiol. B 132, 485–490.

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

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

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

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

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., Simpson, B.K., in press-a. Purification and characterization trypsins from skipjack tuna (*Katsuwonus pelamis*) spleen. Food Chem.

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., Simpson, B.K., in press-b. Proteolytic degradation of sardine (*Sardinella gibbosa*) proteins by trypsin from skipjack tuna (*Katsuwonus pelamis*) spleen. Food Chem.

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., Simpson, B.K., in press-c. Effect of the addition of spleen of skipjack tuna (*Katsuwonus pelamis*) on the liquefaction and characteristics of fish sauce made from sardine (*Sardinella gibbosa*). Food Chem.

Kristjansson, M., 1991. Purification and characterization of trypsin from the pyloric caeca of rainbow trout (*Oncorhynchus mykiss*). J. Agric. Food Chem. 39, 1738–1742.

Laemmli, U.K., 1970. Cleavage of structure proteins during the assembly of the head of bacteriophage T_4. Nature 277, 680–685.

Lineweaver, H., Burk, D., 1934. The determination of enzyme dissociation constants. J. Am. Chem. Soc. 56, 665–666.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193, 256–275.

Macouzet, M., Simpson, B.K., Lee, B.H., 2005. Expression of a cold-adapted fish trypsin in *Pichia pastoris*. Fems. Yeast Res. 5, 851–857.

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

Martinez, A., Olsen, R., Serra, J.L., 1988. Purification and characterization of two trypsin-like enzymes from the digestive tract of anchovy, *Engraulis encrasicholus*. Comp. Biochem. Physiol. B 79, 613–622.

Murakami, K., Noda, M., 1981. Studies on proteinases from the digestive organs of sardine-I. Purification and characterization of three alkaline proteinases from the pyloric caeca. Biochim. Biophys. Acta. 658, 17–26.

Ooshiro, Z., 1971. Studies on proteinase in the pyloric caeca of fish. II. Some properties of proteinase purified from the pyloric caeca of mackerel. Bull. Jpn. Soc. Sci. Fish. 26, 685–690.

Pavlisko, A., Rial, A., Coppes, Z., 1997. Characterization of trypsin purified from the pyloric caeca of the southwest Atlantic white croaker *Micropogonias furnieri* (Sciaenidae). J. Food Biochem. 21, 383–400.

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

Pyeun, J.H., Kim, H.R., Godber, J.S., 1990. Comparative studies on the enzymatic properties of two trypsin like enzymes from menhaden, *Brevoortia tyranus*. B. Kor. Fish. Soc. 23, 12–24.

Simpson, B.K., 2000. Digestive proteinases from marine animals. In: Haard, N. M., Simpson, B.K. (Eds.), Seafood enzymes: utilization and influence on postharvest seafood quality. Marcel Dekker, New York, pp. 531–540.

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

Simpson, B.K., Haard, H.F., 1987. Cold-adapted enzymes from fish. In: Knorr, D. (Ed.), Food Biotechnology. Marcel Dekker, New York, pp. 495–528.

Titani, K., Ericsson, L.H., Neurath, H., Walsh, K.A., 1975. Amino acid sequence of dogfish trypsin. Biochemistry 14, 1358–1366.

Walsch, K.A., 1970. Trypsinogens and trypsins of various species. Meths. Enzymol. 19, 41–63.

Yoshinaka, R., Sato, M., Suzuki, T., Ikeda, S., 1985. Purification and some properties of two anionic trypsins from the eel (*Anguilla japonica*). Comp. Biochem. Physiol. B 80, 5–9.