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Biochemical properties of two isoforms of trypsin purified from the Intestine of skipjack tuna (*Katsuwonus pelamis*)

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ABSTRACT

Two trypsins (A and B) from the intestine of skipjack tuna (*Katsuwonus pelamis*) were purified by Sephacryl S-200, Sephadex G-50 and DEAE-cellulose with a 177- and 257-fold increase in specific activity and 23% and 21% recovery for trypsin A and B, respectively. Purified trypsins revealed a single band on native-PAGE. The molecular weights of both trypsins were 24 kDa as estimated by size exclusion chromatography and SDS–PAGE. Trypsin A and B exhibited the maximal activity at 55 °C and 60 °C, respectively, and had the same optimal pH at 9.0. Both trypsins were stable up to 50 °C and in the pH range from 6.0 to 11.0. Both trypsin A and B were stabilised by calcium ion. Activity of both trypsins continuously decreased with increasing NaCl concentration (0–30%) and were inhibited by the specific trypsin inhibitors – soybean trypsin inhibitor and *N*-*p*-tosyl-L-lysine chloromethyl ketone. Apparent K_m and K_{cat} of trypsin A and B were 0.22–0.31 mM and 69.5–82.5 S^{-1}, respectively. The *N*-terminal amino acid sequences of the first 20 amino acids of trypsin A and B were IVGGYECQAHSQPPQVSLNA and IVGGYECQAHSQPPQVSLNS, respectively.

1. Introduction

Due to the limited biological resources and increased environmental concerns, the interest in full utilisation of seafood processing wastes has been increasing. Fish viscera, one of the most important by-products of fishing industry, are known to be a rich source of digestive enzymes, especially proteases with high activity over a wide range of pH and temperature conditions (Gildberg, 1992; Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2006a). High protease activity of fish viscera enzymes may be interesting for many biotechnological and food processing applications (Haard, 1992). A variety of digestive proteolytic enzymes have been isolated from the internal organs of fish. One of the main digestive proteinases detected in fish viscera, especially in the pyloric ceca and intestine, is trypsins (EC 3.4.21.4). Trypsin, a member of a large family of serine proteinases, specifically hydrolyse proteins and peptides at the carboxyl side of arginine and lysine residues and play major roles in biological process including digestion, activation of zymogens of chymotrypsin and other enzymes (Cao et al., 2000; Klomklao et al., 2006a). Trypsins have been isolated and characterised thoroughly based on their physiochemical and enzymatic properties from several species of fish, e.g. crayfish (*Procambarus clarkii*) (Kim, Meyers, Pyeun, & Godber, 1994), carp (*Cyprinus carpio*) (Cao et al., 2000), capelin (*Mallotus villosus*) (Hjelmeland & Raa, 1982), tambaqui (*Colossoma macropomum*) (Bezerra et al., 2001), true sardine (*Sardinops melanostictus*) and arabesque greenling (*Pleuroprammus azonus*) (Kishimura, Hayashi, Miyashita, & Nonami, 2006). Recently, Kishimura, Klomklao, Benjakul and Chun (2008) isolated and characterised trypsin from the pyloric ceca of walleye pollock (*Theragra chalcogramma*).

Skipjack tuna (*Katusuwonus pelamis*) is one of the most important species commonly used for canning production in Thailand (Klomklao, Benjakul, & Visessanguan, 2004). Approximately two-thirds of the whole fish are utilised and the remaining involving the viscera becomes the waste. Accordingly, those viscera which are the essential source of potential proteinases can be recovered for further uses. Based on our previous study, skipjack tuna intestine contained high proteolytic activity, which was identified as trypsin-like serine proteinase. However, no information regarding the molecular and biochemical properties of skipjack tuna intestine proteinases has been reported. Our objective was to purify and study the physicochemical and biochemical characteristic of trypsin from skipjack tuna intestine.

2. Materials and methods

2.1. Chemicals

Ethylenediaminetetraacetic acid (EDTA), pepstatin A, soybean trypsin inhibitor, iodoacetic acid, *N*-*p*-tosyl-L-lysine chloromethyl

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ketone (TLCK), *N*-tosyl-L-phenyl-alanine chloromethyl ketone (TPCK), 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), *N*-ethylmaleimide, β-mercaptoethanol (βME), porcine pancreatic trypsin and bovine serum albumin were procured from Sigma Chemical Co. (St. Louis, MO, USA). Sephacryl S-200 and Sephadex G-50 were purchased from Pharmacia Biotech (Uppsala, Sweden). Diethylaminoethyl (DEAE)-cellulose was obtained from Whatman (Maidstone, England). N^{α}-*p*-tosyl-L-arginine methyl ester hydrochloride (TAME) was purchased from Wako Pure Chemicals (Osaka, Japan). Sodium chloride, tris (hydroxymethyl) aminomethane and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (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 skipjack tuna (*Katsuwonus pelamis*) were obtained from Chotiwat Industrial Co. (Thailand) Ltd., Songkhla. Those samples (5 kg) were packed in polyethylene bag, kept in ice and transported to the Department of Food Science and Technology, Thaksin University, Phattalung within 30 min. Pooled internal organs were then excised and separated into individual organs. Only intestine was collected, immediately frozen and stored at −20 °C until used.

2.3. Preparation of intestine extract

Frozen intestines were thawed using a 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 homogenised into powder in three volumes of acetone at −20 °C for 30 min using an IKA homogeniser (Model T25, Selangor, Malaysia) according to the method of Klomklao et al. (2006a). The homogenate was filtrated in vacuo on Whatman No. 4 filter paper. The residue obtained was then homogenised 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 odour.

To prepare the intestine extract, intestine 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 lyophilised. Before use, the lyophilised sample (10 g) was dissolved with 50 ml of cold distilled water (4 °C) and referred to as "crude extract".

2.4. Purification of skipjack tuna intestine trypsin

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

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

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

Pooled fractions with TAME activity from Sephadex G-50 column chromatography were collected and lyophilised. The lyophilised fractions were dissolved in distilled water and dialysed 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 less than 0.05 and then eluted with a linear gradient of 0.05–0.4 M NaCl in SB at a flow rate of 0.5 ml/min. Fractions of 3 ml were collected and the fractions with TAME activity were pooled. Two activity peaks (trypsin A and B) were obtained and pooled fractions from each peak were dialysed with SB for 10–12 h and then concentrated by lyophilisation and used for further study.

2.5. Trypsin activity assay

Trypsin activity was measured by the method of Hummel (1959) using TAME as a substrate. Enzyme solution with an appropriate dilution (20 µl) was mixed with 3.0 ml of 1 mM TAME in 10 mM Tris–HCl buffer, pH 8.0 and incubated at 30 °C for 20 min. Production of *p*-tosyl-arginine was measured by monitoring the increment in absorbance at 247 nm. One unit of activity was defined as the amount causing an increase of 1.0 in absorbance per min.

2.6. pH and temperature profile

Trypsin activity was assayed over the pH range of 4.0–11.0 (50 mM acetate buffer for pHs 4.0–7.0; 50 mM Tris–HCl buffer for pHs 7.0–9.0 and 50 mM glycine-NaOH for pHs 9.0–11.0) at 30 °C for 20 min. For temperature profile study, the activity was assayed at different temperatures (20 °C, 30 °C, 40 °C, 50 °C, 55 °C, 60 °C, 65 °C, 70 °C 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 pHs for 30 min at 30 °C. A different buffers used was above mentioned. 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 (W350, Memmert, Schwabach, 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.

2.8. Determination of molecular weight

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

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 10 mM EDTA or 10 mM $CaCl_2$, at 30 °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 as a substrate.

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.

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, except that the sample was not heated and SDS and reducing agent was 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 & 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.

2.15. Protein determination

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

3. Results and discussion

3.1. Purification of trypsins from skipjack tuna intestine

The results of each purification step are presented in Table 1. After Sephacryl S-200 column chromatography (Fig. 1a), total activity of approximately 58% was remained, while 67.2% of protein was removed. Kishimura et al. (2008) reported that the use of Sephacryl S-200 in the first step of purification process of trypsin from the pyloric ceca of walleye pollock led to an increased in trypsin activity by 18-fold. From the result, purity of 2.0-fold was obtained with Sephacryl S-200 chromatography.

To refine the pooled fraction obtained from previous step, pooled active fractions were subjected to Sephadex G-50 column chromatography. Sephadex G-50 chromatography separated trypsin from other proteins with lower molecular weight (Fig. 1b). Purification fold of 6.5 with a yield of 37% was observed. Sepha dex G-50 column chromatography was highly effective in separating trypsin from other proteins, but not for resolving the individual trypsins (Kishimura, Hayashi, Miyashita, & Nonami, 2005). Hence, pooled active fractions were subsequently subjected to an ion-exchange chromatography using DEAE-cellulose.

Pooled Sephadex G-50 fractions were dialysed against 10 mM Tris–HCl, pH 8.0 containing 1 mM $CaCl_2$ (SB), prior to anion exchanger, DEAE-cellulose column. After loading the sample and washing with SB, column was eluted by using a 0.05–0.4 M NaCl linear gradient (Fig. 1c). Two trypsin activity peaks were found (Fig. 1c). These enzymes were assigned as trypsin A and B based on the elution order. Purification fold of 177 and 257 were obtained for trypsin A and B, respectively. Ion exchange chromatography was used to remove the contaminating proteins and to separate different trypsin isoforms. Kishimura et al. (2005) used DEAE-cellulose in the final step for isolation of two trypsin isozymes from Japanese anchovy viscera, leading to the increases in purity by 37-fold and 73-fold. Cao et al. (2000) also purified two anionic trypsins from carp hepatopancreas by using anion exchange, Q-Sepharose column.

Table 1
Purification of trypsins from skipjack tuna intestine.

Purification steps	Total activity (units)[a]	Total protein (mg)	Specific activity (units/mg protein)	Purity (fold)	Yield (%)
Crude extract	420	5826	0.07	1	100
Sephacryl S-200	242	1910	0.13	2	58
Sephadex G-50	152	33	4.6	65	37
DEAE-cellulose					
Trypsin A	96.4	7.7	12.5	177	23
Trypsin B	86.4	4.7	18.4	257	21

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

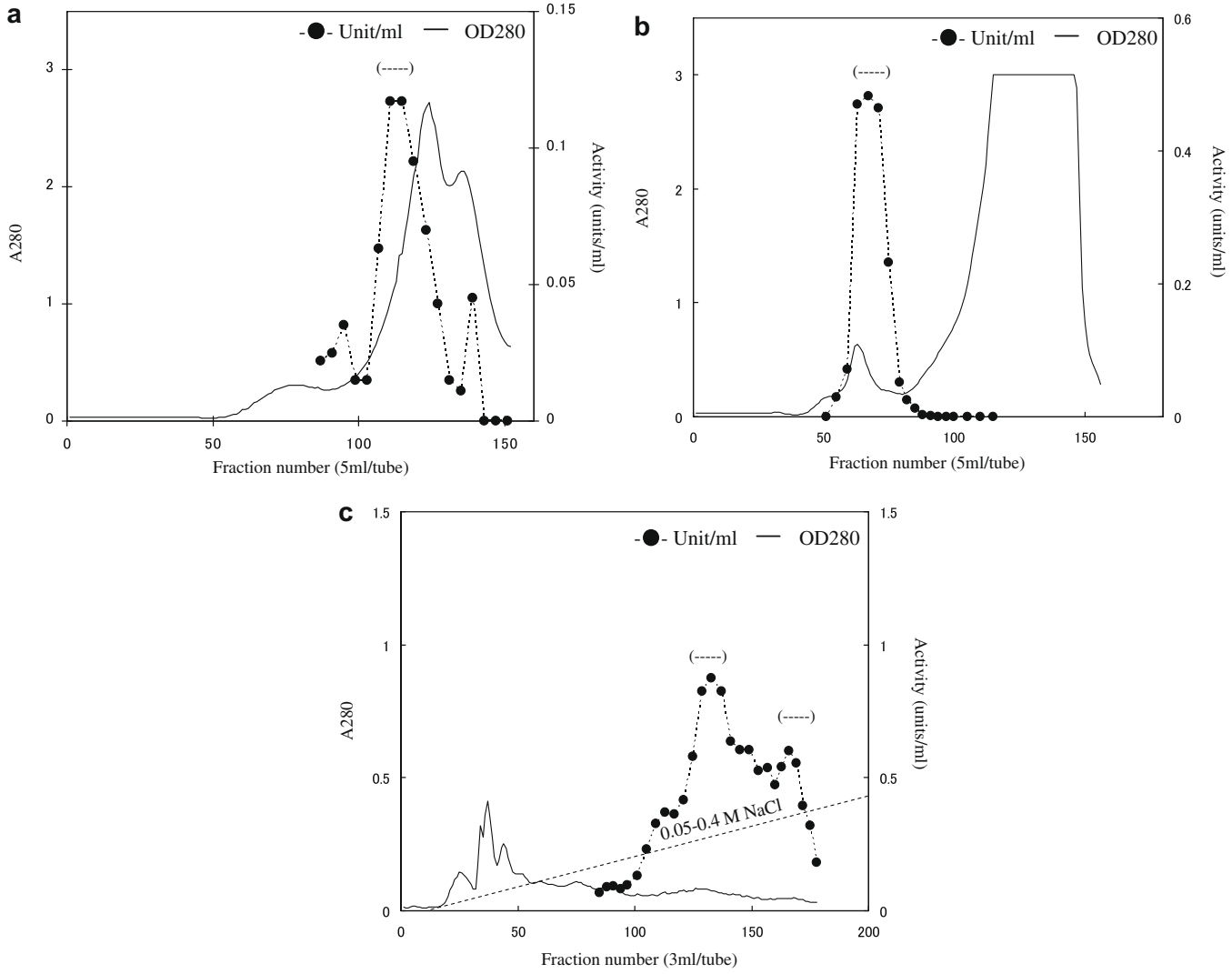

Fig. 1. Purification of trypsins from skipjack tuna intestine. (a) Elution profile of trypsins on Sephacryl S-200 column; (b) elution profile of trypsins on Sephadex G-50 column; (c) elution profile of trypsins on the DEAE-cellulose column. Elution was carried out with a linear gradient of 0.05–0.4 M NaCl in SB.

3.2. Electrophoretic pattern

Trypsin A and B appeared as a single band on the native-PAGE (Fig. 2a) and showed the different mobilities in native-PAGE, indicating the homogeneity of both enzymes. For SDS–PAGE, a single band with the molecular weight of 24 kDa was observed for both enzymes (Fig. 2b). Also, the molecular weight of the enzyme was estimated to be approximately 24 kDa by gel filtration using Sephacryl S-200 (Fig. 3). The results confirm that trypsin A and B are the monomeric protein with a molecular weight of 24 kDa. Generally, fish trypsins have been reported to have molecular weights in the range of 23–28 kDa. Two trypsins (I and II) from the pyloric ceca of Japanese anchovy had a molecular weight of 24 kDa as estimated by SDS–PAGE (Kishimura et al., 2005). Kurtovic, Marshall and Simpson (2006) reported that purified trypsin from the pyloric ceca of chinook salmon had a molecular weight of 28 kDa by SDS–PAGE. Two trypsins (A and B) from carp hepatopancreas had the molecular weights of 28.5 and 28 kDa by SDS–PAGE and gel filtration, respectively (Cao et al., 2000). Molecular weights of trypsin A and B from spleen of yellowfin tuna were estimated by SDS–PAGE and gel filtration to be approximately 24 kDa (Klomklao et al., 2006b).

3.3. Optimal pH and temperature

The effect of pH on the rate of TAME hydrolysis was measured and the results are presented in Fig. 4a. Trypsin A and B exhibited the maximal activity at pH 9.0, which was higher than that of porcine pancreatic trypsin (pH 8.0). Trypsin A, B and porcine trypsin showed the same behaviour between pH 4 and 11. The activities of both trypsins and porcine trypsin were high in pH range of 7.0–9.0 but considerable loss of activity was observed at very acidic and alkaline pHs. A change in pH affects both the substrate and enzyme by changing the charge distribution and conformation of the molecules (Klomklao et al., 2006b). Most enzymes undergo irreversible denaturation in a very acid and alkaline solution, causing the loss of stability. The optimum pH of both trypsins from skipjack tuna intestine was similar to those of trypsins from yellowfin tuna spleen (Klomklao et al., 2006b) and tongol tuna spleen (Klomklao et al., 2006a).

The activity of trypsin A, B and porcine pancreatic trypsin increased with temperature up to an optimum of 55, 60 and 60 °C, respectively, when assayed against TAME (Fig. 4b). A sharp decrease in activity was found at temperature above 60 °C, possibly due to the thermal denaturation. Generally, Trypsin A and B

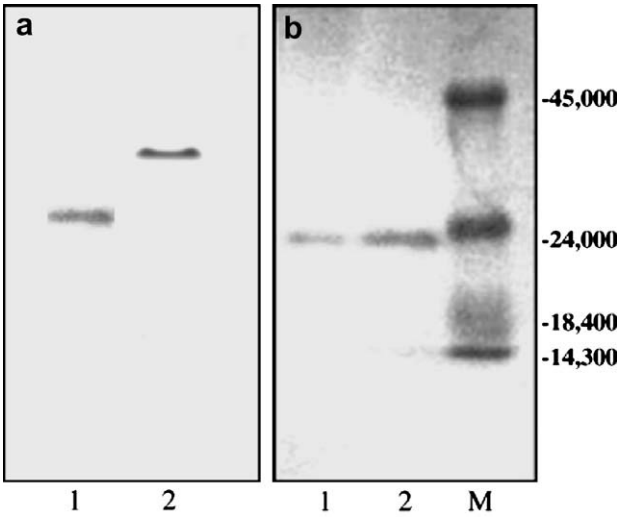

Fig. 2. Protein pattern from native-PAGE (a) and SDS–PAGE (b) of purified trypsin A and B from skipjack tuna intestine. *M*, molecular weight standard; lane 1, trypsin A; lane 2, trypsin B.

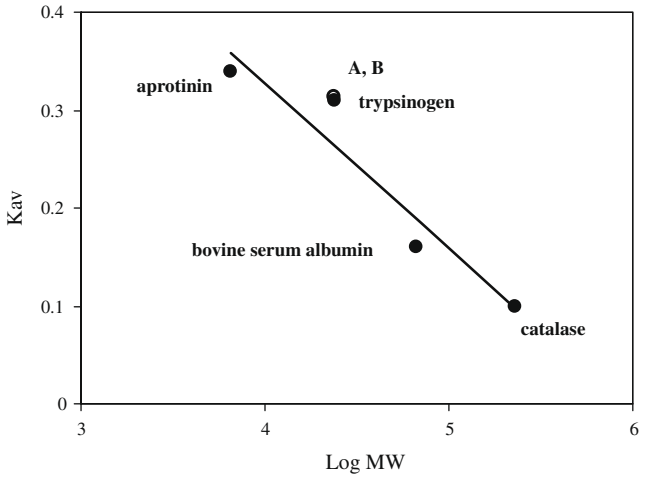

Fig. 3. Calibration curve for the molecular weight determination of the purified trypsin A and B on Sephacryl S-200 chromatography. (A) Trypsin A; (B) trypsin B.

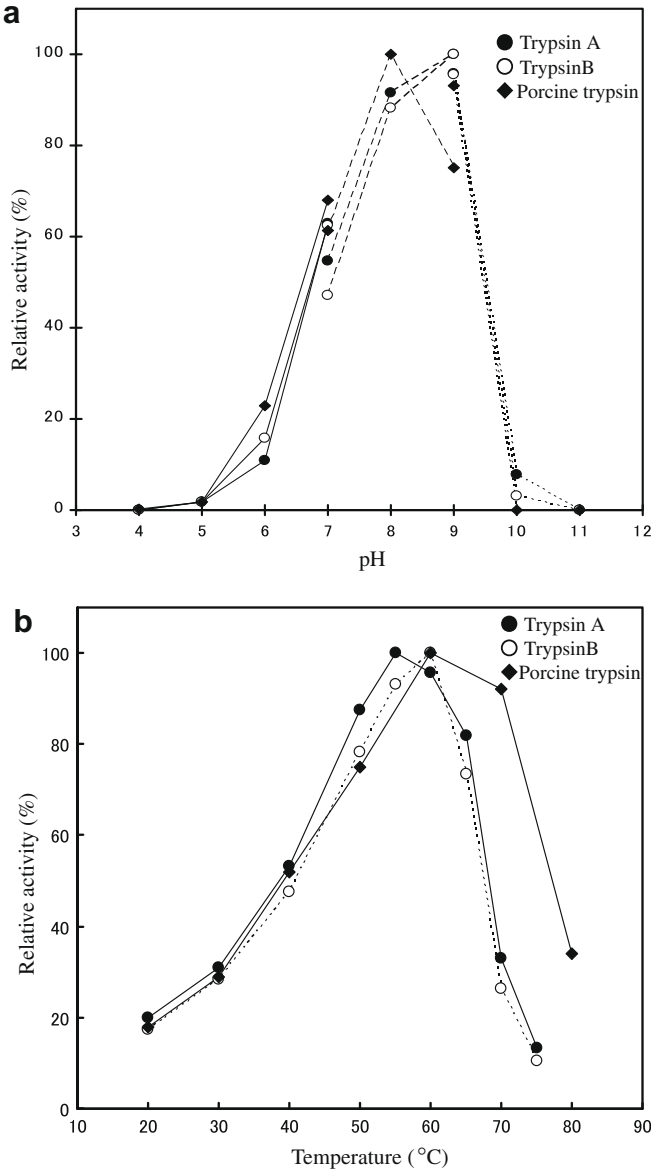

Fig. 4. pH (a) and temperature (b) profiles of purified trypsin A, and B from skipjack tuna intestine and porcine trypsin.

showed high activity in the range from 20 to 50 °C while porcine trypsin was more active in the range from 60 to 80 °C. This behaviour is in accordance to the fact that mammalian trypsins are more active at higher temperatures than fish trypsins (Simpson, 2000). Studies on trypsins from other fish species like yellowfin tuna, tongol tuna, crayfish and rainbow trout showed similar profiles on activity toward temperature with slight differences on the optimum temperature (Kim et al., 1994; Klomklao et al., 2006a, 2006b; Krisjansson, 1991).

3.4. pH and thermal stability

The effect of pH on the stability of two trypsins from skipjack tuna intestine and porcine pancreatic trypsin is showed in Fig. 5a. Trypsin A, B and porcine trypsin exhibited different pH stability. Trypsin A and B showed high stability in the pH range of 6.0–11.0, but the inactivation was more pronounce at pH below 6 (Fig. 5a). A completely loss in activity was observed after incubating both trypsins at pH 4, while porcine trypsin has stability at acidic and alkaline pHs. Simpson (2000) reported that trypsin from warm blooded organism are extremely stable at acidic and alkaline pHs while fish trypsins are stable only to alkaline pH. The stability of trypsin at particular pH might be related to the net charge of the enzyme at that pH (Castillo-Yanez, Pacheco-Aguilar, Garcia-Carreno, & Toro, 2005). A similar pH effect on activity has been reported for trypsin from several fish species (Castillo-Yanez et al., 2005; Kishimura et al., 2006; Klomklao et al., 2006a, 2006b).

For thermal stability, both trypsins from skipjack tuna intestine was stable below 55 °C, but the activity sharply decreased above 60 °C (Fig. 5b). The enzyme was almost completely inactivated at 75 °C. At high temperatures, enzymes most likely underwent denaturation and lost their activity (Klomklao et al., 2006a). Trypsins from skipjack tuna intestine exhibited the similar thermal stability to those of other fish species (Castillo-Yanez et al., 2005; Kishimura et al., 2006; Klomklao et al., 2006a, 2006b) However, the temperature stability of trypsin A and B from skipjack tuna intestine was more unstable than trypsin from porcine trypsin (Fig. 5b). Differences in thermal stability of enzymes might be determined by bonding stabilised enzyme structure. More disulphide linkages as

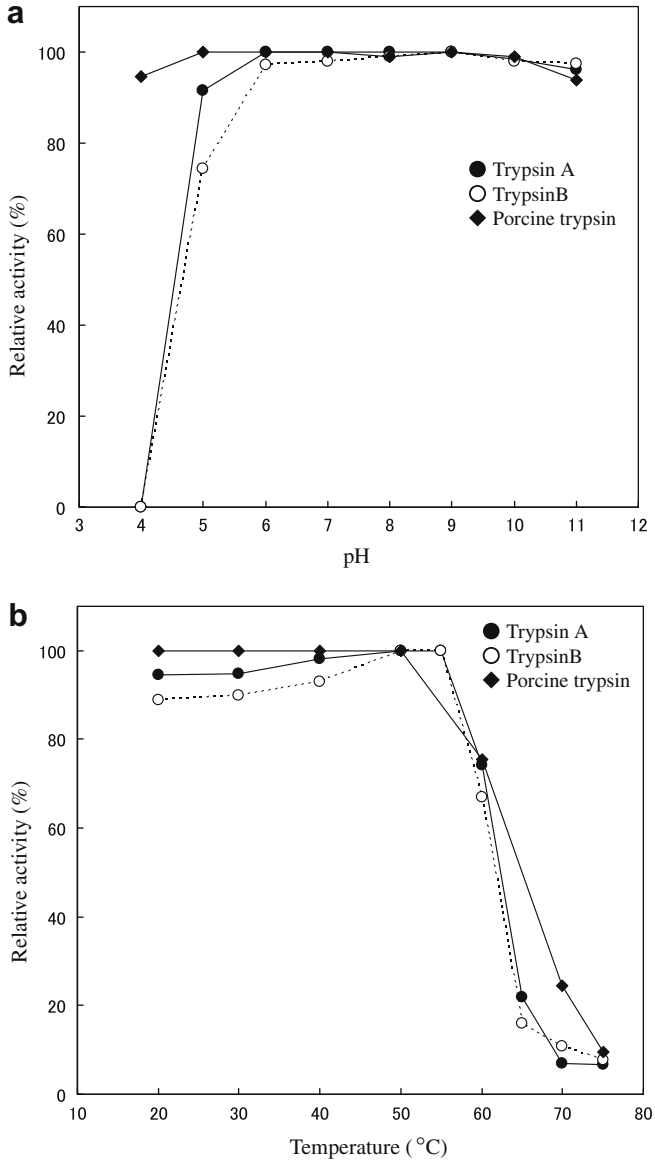

Fig. 5. pH (a) and thermal (b) stability of purified trypsin A and B from skipjack tuna intestine and porcine trypsin.

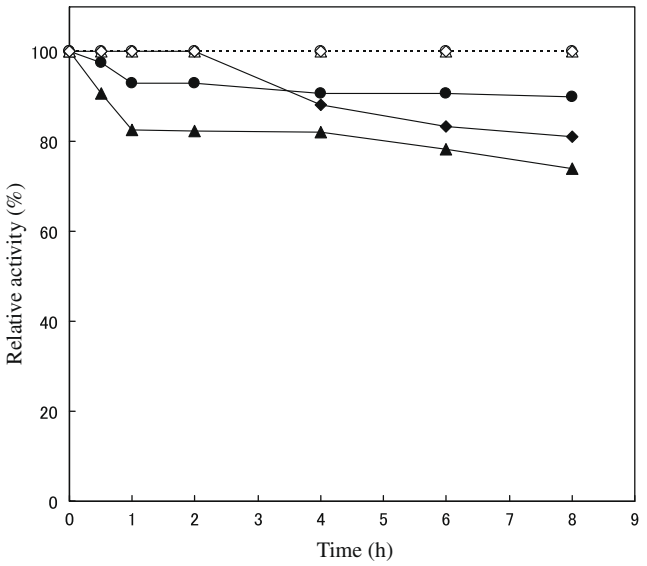

Fig. 6. Effect of calcium ion and EDTA on the stability of purified trypsin A and B from skipjack tuna intestine and porcine trypsin. The enzymes were kept at 30 °C for 0–8 h in the presence of 10 mM $CaCl_2$ (open symbols) or 10 mM EDTA (closed symbols), and then the remaining activities at 30 °C were determined. Trypsin A (circle); trypsin B (triangle); porcine trypsin (diamond).

well as stronger hydrophobic interactions in the interior of protein contribute to the greater stability of proteins. Higher thermostability was associated with a higher number of intramolecular disulphide bonds in protease (Klomklao et al., 2006a; Simpson, 2000).

3.5. Effect of calcium ions on the thermal stability

The effect of $CaCl_2$ on the stability of the skipjack tuna trypsins was determined in the presence of 10 mM EDTA or 10 mM $CaCl_2$. The total activity was retained throughout 8 h at 30 °C in the presence of 10 mM calcium ion, while the activities decreased with increasing time in the presence of 10 mM EDTA (Fig. 6). Trypsin A was rather stable in the presence of 10 mM EDTA than trypsin B, particularly when the incubation time increased. The results indicated that trypsin A and B were stabilised by calcium ion similar to porcine pancreatic trypsin (Fig. 6). Binding of calcium to a single binding site in bovine trypsin significantly stabilised the enzyme against denaturation (Klomklao et al., 2004). In the presence of calcium ions, trypsin is believed to undergo a conformational change, resulting in a more compact structure, which is more resistant to autolysis (Kim et al., 1994). Stabilisation against thermal inactivation by calcium ion has also been found for the trypsins from yellowfin tuna (Klomklao et al., 2006b), true sardine and arabesque greenling (Kishimura et al., 2006). However, calcium ion did not show the enhancing effect on stability of trypsins from sardine (Noda & Murakami, 1981) 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.

3.6. Effect of NaCl

The activities of trypsin A and B from skipjack tuna intestine continuously decreased with increasing NaCl (Fig. 7). Trypsin B showed slightly higher activity than trypsin A in the presence of NaCl ranging from 5% to 30%, indicating that trypsin B was more tolerant to NaCl than trypsin A. The activities of trypsin A and B in the presence of 30% NaCl was approximately 35% and 45% of that without NaCl, respectively. The decrease in activity could be described by the salting out phenomenon. An increase in ionic strength causes a reduction in enzyme activity by an enhanced hydrophobic–hydrophobic interaction between protein chains, and the competition for water of ionic salts, leading to the induced enzyme precipitation (Klomklao et al., 2004). At 25% NaCl, 40–50% of activities was remained. Therefore, trypsins from skipjack tuna intestine may have a potential in accelerating the hydrolysis of high-salt products, such as fish sauce.

3.7. Effect of inhibitors

The influence of several well known protease inhibitors on the activity of the skipjack tuna enzymes is summarised in Table 2. Soybean trypsin inhibitor and TLCK strongly inhibited the enzymes. Inhibitors for cysteine and aspartic protease involving E-64, *N*-ethylmaleimide, iodoacetic acid and pepstatin A did not show inhibitory effects towards trypsin activity. However, TPCK, a specific inhibitor for chymotrypsin, and EDTA, which chelates the metal ions required for enzyme, partially lowered trypsin activity. Soybean trypsin inhibitor binds strongly to the active site

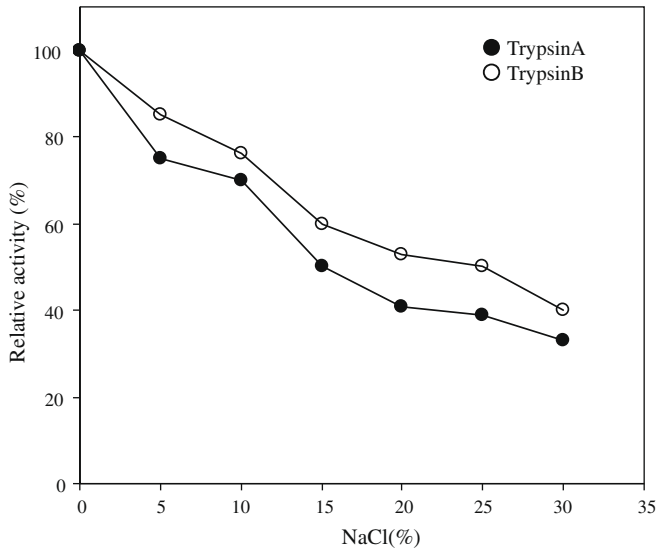

Fig. 7. Effect of NaCl concentrations on activities of purified trypsin A and B from skipjack tuna intestine.

Table 2
Effect of various inhibitors on the activity of purified trypins from skipjack tuna intestine[a].

Inhibitors	Concentration	% Inhibition	
		Trypsin A	Trypsin B
Control		0	0
E-64	0.1 mM	0	0
N-ethylmaleimide	1 mM	0	0
Iodoacetic acid	1 mM	6.6 ± 1.08	4.9 ± 0.05
Soybean trypsin inhibitor	1.0 g/l	88.01 ± 2.1	91.06 ± 1.35
TLCK	5 mM	81.09 ± 0.06	85.98 ± 1.36
TPCK	5 mM	5.78 ± 1.12	9.98 ± 0.87
Pepstatin A	0.01 mM	0	0
EDTA	2 mM	0.88 ± 2.53	1.34 ± 1.04

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

Table 3
Kinetic properties of skipjack tuna intestine trypsins for the hydrolysis of TAME.

Enzyme	K_m (mM)	K_{cat} (S^{-1})	K_{cat}/K_m (S^{-1} mM^{-1})
Trypsin A	0.22 ± 0.02	82.5 ± 0.01	266.13
Trypsin B	0.31 ± 0.01	69.5 ± 0.05	315.91

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

	5 10 15 20
Trypsin A	I V G G Y E C Q A H S Q P P Q V S L N A
Trypsin B	I V G G Y E C Q A H S Q P P Q V S L N S
Yellowfin tuna	I V G G Y E C Q A H S Q P H Q V S L N A
Tongol tuna	I V G G Y E C Q A H S Q P H Q V S L N A
True sardine	I V G G Y E C K A Y S Q P W Q V S L N S
Arabesque greenling	I V G G Y E C T P H T Q A H Q V S L D S
Japanese anchovy	I V G G Y E C Q A H S Q P H T V S L N S
Cod	I S N L S V Q H A Q S H K T C E Y G G V
Salmon	I V G G Y E C K A Y S Q T H Q V S L N S
Dogfish	I V G G Y E C P K H A A P W T V S L N V
Dog	I V G G Y T C E E N S V P V Q V S L N A
Porcine	I V G G Y T C A A N S V P Y Q V S L N S
Bovine	I V G G Y T C G A N T V P Y Q V S L N S

Fig. 8. Comparison of *N*-terminal amino acid sequences of the purified trypsin A and B from skipjack tuna intestine with other enzymes: yellowfin tuna (Klomklao et al., 2006b), tongol tuna (Klomklao et al., 2006a), true sardine, arabesque greenling (Kishimura et al., 2006), Japanese anchovy (Kishimura et al., 2005), cod (Gudmundsdottir et al., 1993), salmon (Male, Lorens, Smalas, & Torrissen, 1995), dogfish (Titani, Ericsson, Neurath, & Walsh, 1975), dog (Pinsky, Laforge, & Scheele, 1985), porcine (Hermodson, Ericsson, Neurath, & Walsh, 1973), and bovine (Walsh, 1970).

of trypsin to impede catalysis. Soybean trypsin inhibitor has been shown to inhibit trypsins from carp (Cao et al., 2000), tambaqui (Bezerra et al., 2001), chinook salmon (Kurtovic et al., 2006) and yellowfin tuna (Klomklao et al., 2006b). TLCK is well known as a trypsin specific inhibitor that has been shown to inhibit trypsins from carp (Cao et al., 2000), yellowfin tuna (Klomklao et al., 2006b) and tongol tuna (Klomklao et al., 2006a). 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 centre (Klomklao et al., 2006a). The result confirms that these purified enzymes were serine proteinases, mostly likely trypsin.

3.8. Kinetic study

Kinetic constants for TAME hydrolysis by skipjack tuna trypsins (Trypsin A and B) were determined using Lineweaver–Burk plots (Table 3). K_m and K_{cat} were 0.31 mM and 82.5 S^{-1} for skipjack tuna intestine trypsin A; 0.22 mM and 69.5 S^{-1} for skipjack tuna intestine trypsin B, 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. The K_m obtained for skipjack tuna trypsins was similar to those reported for trypsins from yellowfin tuna (Klomklao et al., 2006b), tongol tuna (Klomklao et al., 2006a) and crayfish (Kim et al., 1994). Moreover, the enzyme had slightly lower K_m value than trypsin from bovine, which had K_m in the range of 0.3–0.33 mM (Kim et al., 1994). The result indicates that skipjack tuna trypsins had higher affinity for TAME than bovine trypsin. Additionally, the catalytic efficiency (166.13–315.91 S^{-1} mM^{-1}) of trypsin A and B from skipjack tuna intestine was greater than that of mammalian trypsin (Simpson, 2000). From the result, it was suggested that trypsins from skipjack tuna intestine had more flexible structures than trypsin from warm blooded animals.

3.9. N-Terminal sequencing

The *N*-terminal 20 amino acids of trypsin A and B were IVGGYECQAHSQPPQVSLNA and IVGGYECQAHSQPPQVSLNS, respectively (Fig. 8). The result indicates that the *N*-terminal of the enzymes was unblocked. The *N*-terminal amino acid sequences of two trypsins were compared with those of other animal trypsins (Fig. 8). It was found that *N*-terminal of trypsin A and B exhibited the high homology and showed similarity to that of yellowfin tuna spleen and tongol tuna spleen. The highly conserved amino acid sequence between yellowfin tuna tongol tuna and skipjack tuna suggest that they were genetically evolved from a

common ancestor. Moreover, the sequences of two trypsins from skipjack tuna intestine and other trypsins started with IVGG after the proteolytic cleavage of in inactive trypsinogen. From the results, the *N*-terminal sequence clearly showed that trypsin from skipjack tuna intestine was most likely a member of trypsin family. In addition, the present data of the *N*-terminal amino acid sequence may be useful for designing primers for the cDNA cloning of trypsin.

4. Conclusion

Two trypsins from skipjack tuna intestine were purified and identified based on molecular weight, substrate specificity, inhibitor study and *N*-terminal sequencing. Two isoenzymes were salt toterant and could be used in a high-salt environment.

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