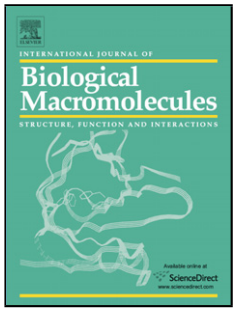

Title: Two trypsin isoforms from albacore tuna (*Thunnus alalunga*) liver: Purification and physicochemical and biochemical characterization

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Two trypsin isoforms from albacore tuna (*Thunnus alalunga*) liver:

Purification and physicochemical and biochemical characterization

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Highlight

- Two trypsins from albacore tuna liver were purified and characterized.
- The molecular weights of the purified trypsins were estimated to be 21 and 24 kDa by SDS-PAGE.
- Both trypsins were extremely stable in the pH range of 7.0-12.0 and highly stable at low temperatures.

ABSTRACT

Two trypsins (A and B) from the liver of albacore tuna (*Thunnus alalunga*) were purified to homogeneity using a series of column chromatographies including Sephacryl S-200, Sephadex G-50 and Diethylaminoethyl-cellulose. Purity was increased to 80.35- and 101.23-fold with approximately 3.1 and 19.2% yield for trypsins A and B, respectively. The molecular weights of trypsins A and B were

estimated to be 21 and 24 kDa, respectively, by SDS-PAGE and size exclusion chromatography. Both trypsins showed only one band on native-PAGE. Trypsins A and B exhibited the maximal activity at 60 °C and 55 °C, respectively, and had the same optimal pH at 8.5 using N^{α}-*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME) as a substrate. Stabilities of both trypsins were well maintained at a temperature up to 50°C and in the pH range of 7.0 to 11.0 and were highly dependent on the presence of calcium ion. The inhibition test demonstrated strong inhibition by soybean trypsin inhibitor and TLCK. Activity of both trypsins continuously decreased with increasing NaCl concentration (0-30%). The N-terminal amino acid sequence of 20 residues of the two trypsin isoforms had homology when compared to those of other fish trypsins.

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

1. Introduction

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

Trypsin (EC 3.4.21.4) is one of the main digestive proteinases found in fish viscera, especially pyloric ceca and intestine [2]. It is a serine proteinase, which is produced as an inactive precursor. It has a function in the hydrolysis of target proteins at the amino acids arginine and lysine [3]. Trypsin has various industrial applications, especially in food industries, due to its high stability and activity under harsh conditions, such as in the presence of surfactants and oxidative agents [4]. Trypsins have been extracted and characterized thoroughly based on their biochemical properties from several species of fish, e.g. the viscera of striped sebream (*Lithognathus mormyrus*) [5], Goby (*Zosterisessor*

ophiocephalus) [6], the intestine of Grey triggerfish (*Balistes capriscus*) [7], the spleen of skipjack tuna (*Katsuwonus pelamis*) [8], yellowfin tuna (*Thunnus albacores*) [9] and the pyloric ceca of Chinook salmon (*Oncorhynchus tshawytscha*) [10]. Recently, Sripokar et al. [11] reported that albacore tuna liver contained high proteolytic activity and the major proteinases were heat-activated alkaline proteinases, most likely trypsin-like serine proteinases. However, the molecular and the biochemical characteristics of trypsin or trypsin-like enzymes in albacore tuna liver still remain unknown. Therefore, in the present study, we attempted to isolate and characterize trypsins from the liver of albacore tuna and obtain basic information about their biochemical and kinetic properties.

2. Materials and methods

2.1 Chemicals

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

2.2 Liver extract preparation

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

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

2.3 Trypsin purification from albacore tuna liver

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

2.3.1 Sephacryl S-200 column chromatography

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

2.3.2 Sephadex G-50 column chromatography

Lyopholized fractions with TAME activity after Sephacryl S-200 column chromatography were dissolved in distilled water and loaded onto a Sephadex G-50 column (3.9×64 cm) previously equilibrated with approximately two bed volumes of SB. The elution was performed with the same

buffer at a flow rate of 0.5 ml/min. Fractions of 3 ml were collected and those with TAME activity were pooled and further purified by anion exchanger DEAE-cellulose chromatography.

2.3.3 DEAE-cellulose chromatography

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

2.4 Trypsin activity assay

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

2.5 pH and temperature profile

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

2.6 pH and thermal stability

The effect of pH on enzyme stability was evaluated by measuring the residual activity after incubation at various pHs for 30 min at 30°C. Different buffers were used from the above mentioned experiment. For thermal stability, enzyme solution was diluted with 100 mM Tris-HCl, pH 8.0 at a ratio of 1:1 (v/v) and incubated at different temperatures (20, 30, 40, 50, 60, 70 and 80°C) for 15 min in a temperature controlled water bath (Memmert, Germany). Thereafter, the treated samples were suddenly cooled in iced water. The residual activity was assayed using TAME as a substrate at pH 8.0 and 30°C for 20 min. The effect of $CaCl_2$ on thermal stability was also determined by heating the enzyme dissolved in 50 mM Tris-HCl, pH 8.0 in the presence of 2 mM EDTA or 2 mM $CaCl_2$, at 40°C for different times (0, 0.5, 1, 2, 4, 6 and 8 h). At the time designated, the samples were cooled in iced water and assayed for remaining activity.

2.7 Determination of molecular weight

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

2.8 Effect of NaCl

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

2.9 Effect of inhibitors

The effect of inhibitors on trypsin activity was determined according to the method of Klomklao et al. [13] by incubating enzyme solution with an equal volume of proteinase inhibitor solution to obtain the final concentration designated (0.1 mM E-64, 1 mM N-ethylmaleimide, 1 mM iodoacetic acid, 1.0 g/l soybean trypsin inhibitor, 5 mM TLCK, 5 mM TPCK, 1 mM pepstatin A and 2 mM EDTA). The mixture was allowed to stand at room temperature (26-28°C) for 15 min. Thereafter, the remaining activity was measured and percent inhibition was calculated.

2.10 Polyacrylamide gel electrophoresis

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

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

2.11 Determination of N-terminal amino acid sequence

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

2.12 Kinetic studies

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

2.13 Protein determination

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

3. Results and discussion

3.1 Trypsin purification from albacore tuna liver

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

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

For SDS-PAGE, each purified trypsin gave a single band and the apparent molecular weights of trypsins A and B were estimated to be 21 and 24 kDa, respectively, corresponding to that measured by gel filtration using Sephacryl S-200 (data not shown). The results confirm that trypsins A and B are monomeric proteins. The molecular weight of both trypsins was similar to those of mammalian and fish trypsins. Generally, fish trypsins were found to have molecular masses in the range of 20 and 28 kDa [16]. Trypsin from zebra blenny viscera had an apparent molecular weight of 27 kDa as estimated by SDS-PAGE and gel filtration [17]. Nasri et al. [6] reported that the molecular weight of an alkaline calcium dependent trypsin from the viscera of Goby was approximately 23.2 kDa using SDS-PAGE and gel filtration. The apparent molecular weight of trypsin A and B from yellowfin tuna spleen was estimated to be 24 kDa by size exclusion chromatography and SDS-PAGE [9].

3.2 Optimal pH and temperature

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

Temperature activity profiles of trypsins A and B are shown in Fig. 3b. Trypsins A and B had optimal temperatures of 60 and 55°C, respectively. At temperatures above 70°C, an appreciable decrease in activity of both trypsins was observed, presumably due to thermal inactivation. Inactivation of the enzymes at high temperature was possibly due to unfolding of the molecules.

Trypsin from zebra blenny viscera had a temperature optimum of 60°C [17]. Klomklao et al. [9] reported that trypsin A and B from the spleen of yellowfin tuna had the maximal activity at 55 and 60°C, respectively. The optimum temperature for trypsin activity from golden grey mullet viscera was 50°C [18]. Nevertheless, these optimal temperatures were higher than those reported for trypsin from Grey triggerfish [7], vermiculated sailfin catfish [19] and carp [20], which had the optimum temperatures in the range of 30-40°C. The difference might be related to the different temperatures of water where the fish inhabited.

3.3 pH and thermal stability

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

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

The thermal stability of the purified trypsin A and B was also investigated by incubating the enzymes in the presence of 2 mM EDTA or 2 mM $CaCl_2$, at 40°C for different times (0, 0.5, 1, 2, 4, 6

and 8 h). As depicted in Fig. 5, in the presence of 2 mM calcium ion, approximately 85% of the initial activities remained after 8 h of incubation at 40°C. However, the trypsin activity of both enzymes decreased with increasing time in the presence of 2 mM EDTA. Purified trypsin B was rather stable in the presence of 2 mM EDTA than purified trypsin A, especially when the incubation time increased. These results indicated that trypsin A and B from the liver of albacore tuna were most likely stabilized by calcium ion. The presence of calcium ions activates trypsinogen to trypsin and increases the thermal stability of the enzyme. This stabilizing effect is accomplished by a conformational change in the molecule of trypsin, resulting in a more compact structure [13]. Klomklao et al. [22] reported that trypsin purified from the viscera of hybrid catfish was stabilized by calcium ion. El Hadj Ali et al. [5] also found that the stability of trypsin from striped seabream viscera was enhanced by the addition of $CaCl_2$. On the other hand, trypsin from Nile tilapia intestine was not stabilized by calcium ion [24]. These finding suggest a difference in the structure of the primary calcium binding site among different marine fish trypsins.

3.4 Effect of NaCl

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

3.5 Effect of inhibitors

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

3.6 Kinetic study

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

efficiency of trypsins from albacore tuna liver was higher than those reported for mammalian trypsins [27].

3.7 N-terminal sequencing

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

4. Conclusion

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

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Figure Legends

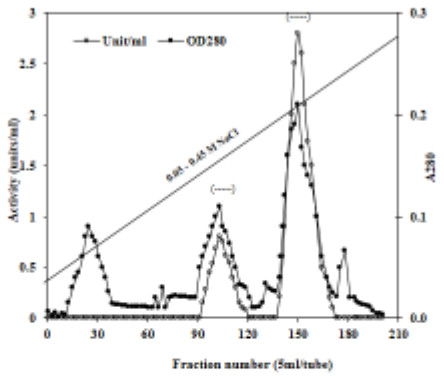

Fig. 1

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

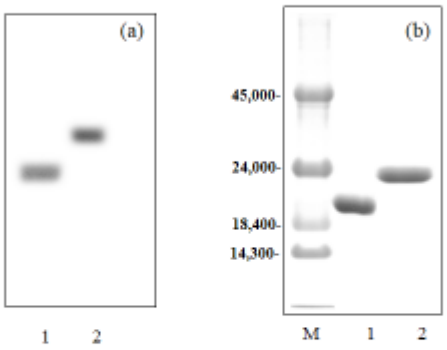

Fig. 2

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

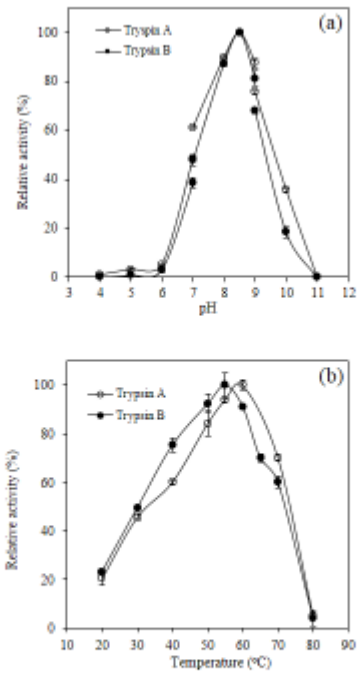

Fig. 3

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

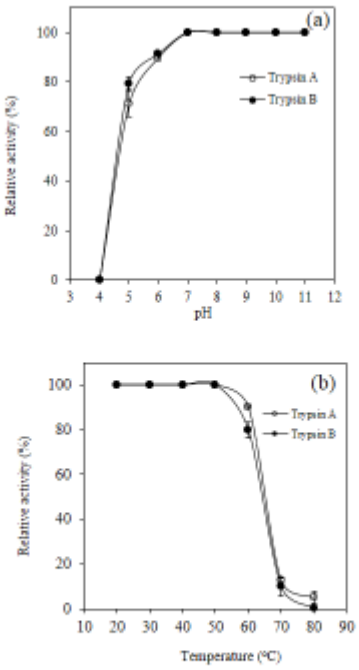

Fig. 4

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

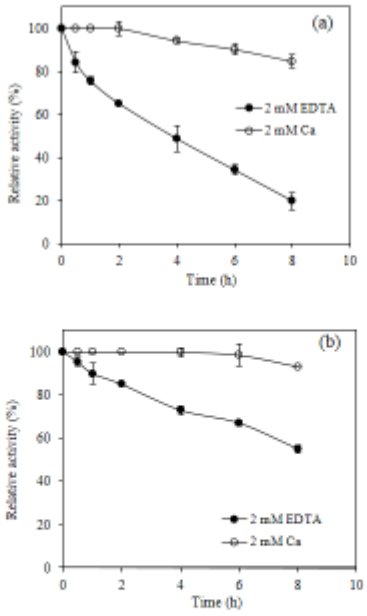

Fig. 5

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

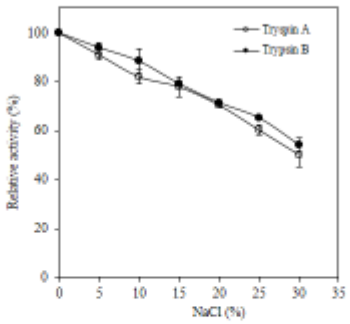

Fig. 6

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

Fig. 7

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

Table 1

Purification of trypsins from the liver of albacore tuna

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

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

Table 2

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

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

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

Table 3

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

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

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