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Purification and characterisation of trypsins from the spleen of skipjack tuna (*Katsuwonus pelamis*)

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Abstract

Three trypsin isoforms, trypsins A, B and C, from the spleen of skipjack tuna (*Katsuwonus pelamis*) were purified by a series of chromatographies including Sephacryl S-200, Sephadex G-50 and diethylaminoethyl-cellulose to obtain a single band on native-PAGE and SDS–PAGE. The molecular mass of all the trypsin isoforms was estimated to be 24 kDa by size exclusion chromatography and SDS–PAGE. The optimum pH and temperature of the three isoforms for the hydrolysis of N^{α}-*p*-tosyl-L-arginine methyl ester hydrochloride were 8.5 and 60 °C, respectively. Trypsins were stable to heat treatment up to 50 °C, and over a pH range of 6.0–11.0. All isoforms were stabilised by calcium ions. The trypsin activities were effectively inhibited by soybean trypsin inhibitor, TLCK and partially inhibited by ethylenediaminetetraacetic acid, while E-64, *N*-ethylmaleimide, iodoacetic acid, TPCK and pepstatin A showed no inhibitory effect. Activities decreased continuously as NaCl concentration (0–30%) increased. Trypsins A, B and C showed K_m of 0.11–0.29 mM and K_{cat} of 57.1–114 s^{-1}. The N-terminal amino acid sequence of 20 residues of three trypsin isoforms was IVGGYECQAHSQPHQVSLNS and had high homology to those of other fish trypsins.

Keywords: Trypsin; Proteinase; Spleen; Skipjack tuna; Purification; N-terminal amino acid sequence

1. Introduction

The tuna processing industry, especially canning, has become increasingly important as an income generator for Thailand. In terms of volume, Thailand is the world's largest exporter of canned tuna with over 20 million cans produced annually (Klomklao, Benjakul, & Visessanguan, 2004; Subasinghe, 1996). Large volumes of raw tuna go through the canning process, for which about two-thirds of the whole fish are utilized. Processing wastes containing viscera and offal from the tuna canning industry are inevitably generated and estimated at 450,000 metric tons annually (Subasinghe, 1996).

Fish viscera, accounting for 5% of the total mass, have wide biotechnological potential as a source of digestive enzymes, especially digestive proteases that have high activity over a wide temperature range (Gildberg, 1992; Simpson & Haard, 1987). Those proteases may have some unique properties for industrial applications, such as in the detergent, food, pharmaceutical, leather and silk industries (Haard, 1992; Kawai & Ikeda, 1972; Ooshiro, 1971). The use of alkaline proteinases has increased remarkably since they are both stable and active under harsh conditions,

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such as at temperatures of 50–60 °C, high pHs and in the presence of surfactants or oxidising agents (Joo, Park, Kim, Paik, & Chang, 2001; Klomklao, Benjakul, Visessanguan, Simpson, & Kishimura, 2005).

Trypsins (EC 3.4.21.4) specifically hydrolyse proteins and peptides at the carboxyl side of arginine and lysine residues and play major roles in biological processes including digestion, activation of zymogens of chymotrypsin and other enzymes. Trypsins have been characterised thoroughly based on their physicochemical and enzymatic properties from the intestine of crayfish (Kim, Meyers, & Godber, 1992; Kim, Meyers, Pyeun, & Godber, 1994), dogfish (Ramakrishna, Hultin, & Atallah, 1987), mackerel (Kim & Pyeun, 1986) and capelin (Hjelmeland & Raa, 1982). Bezerra et al. (2001) partially purified trypsin from pyloric caeca of tambaqui (*Colossoma macropomum*) and found that the enzyme had an optimal pH of 9.5. Byun, Park, Sung, and Kim (2003) also purified and characterised serine proteinases from pyloric caeca of tuna (*Thunnus thynnus*). Recently, skipjack tuna spleen has been reported to possess high proteolytic activity and those proteinases were classified to be trypsin-like serine proteinases with optimal activity at pH 9.0 and 55 °C (Klomklao et al., 2004). However, molecular and biochemical properties of trypsin in skipjack tuna spleen, have not been studied. The objectives of this study were to purify and to characterise the trypsin from skipjack 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 purchased 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. 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 bags, 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 until used.

2.3. Preparation of spleen extract

Frozen spleens 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 homogenised into powder in three volumes of acetone at −20 °C for 30 min according to the method of Kishimura and Hayashi (2002) with a slight modification. The homogenate was filtered in vacuo on ADVANTEC 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.

To prepare the 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 lyophilised. Before use, the lyophilised sample was dissolved in distilled water and referred to as "splenic extract". All preparation procedures were carried out at 4 °C.

2.4. Purification of skipjack tuna spleen trypsin

The splenic extract was chromatographed on a Sephacryl S-200 column (3.9 × 64 cm), which was equilibrated with approximately two bed volumes of SB. The sample was loaded onto the column and then eluted with the same buffer at a flow rate of 0.5 ml/min at 4 °C. Fractions of 5 ml were collected and those with TAME activity were pooled and further purified by Sephadex G-50 column. Absorbance at 280 nm (A_{280}) of each fraction was also measured.

Pooled fractions with TAME activity from the Sephacryl S-200 column were concentrated by lyophilisation and then dissolved in distilled water prior to size exclusion chromatography. The sample was applied to a Sephadex G-50 column (3.9 × 64 cm) previously equilibrated with approximately two bed volumes of SB. The sample was loaded onto the column at 4 °C and then eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions of 5 ml were collected and subjected to A_{280} measurement. The fractions with TAME activity were pooled and dialysed against SB for 10–12 h at 4 °C.

After Sephadex G-50 chromatography, the sample was then chromatographed on a 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 at 4 °C. The column was washed with SB until A_{280} was less than 0.05. The elution was performed with step-wise process using SB containing different NaCl con-

centrations: 0, 0.2, 0.225 and 0.25 M, respectively. Fractions of 5 ml were collected and A_{280} was read. The fractions with TAME activity were pooled and used for further studies.

2.5. Trypsin activity assay

Trypsin activity was measured by the method of Hummel (1959) and Kishimura and Hayashi (2002) with a slight modification using TAME as a substrate. The 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 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 pH 4.0–7.0, 50 mM Tris–HCl buffer for pH 7.0–9.0 and 50 mM glycine–NaOH for pH 9.0–11.0) at 30 °C for 20 min. For temperature profile study, the activity was assayed at different temperatures (20, 30, 40, 50, 55, 60, 65, 70 and 80 °C) for 20 min under the optimal pH.

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. Different buffers used included 50 mM acetate buffer for pH 4.0–6.0, 50 mM Tris–HCl buffer for pH 7.0–9.0 and 50 mM glycine-NaOH for pH 10.0–11.0. For thermal stability, enzyme solution was diluted with 100 mM Tris–HCl, pH 8.0 at a ratio of 1:1 and incubated at different temperatures (20, 30, 40, 50, 60, 70 and 80 °C) for 15 min in a temperature controlled water bath (Memmert, Germany). Thereafter, treated samples were suddenly cooled in iced water. The residual activity was assayed using TAME as a substrate at pH 8.0 and 30 °C for 20 min.

2.8. 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 the percent inhibition was calculated.

2.9. Effect of NaCl

The effect of NaCl on trypsin activity was studied. NaCl was added into the standard reaction assay to obtain 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.

2.10. 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. After a specific heating time (0, 0.5, 1, 2, 4, 6 and 8 h), the samples were cooled in iced water and assayed for remaining activity.

2.11. 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. 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 the addition of SDS and reducing agent was omitted.

2.12. Size exclusion chromatography

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

2.13. Determination of N-terminal amino acid sequence

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

2.14. Kinetic studies

Trypsin solutions were diluted with distilled water to obtain a concentration of 0.1 mg/ml. Then, the activity was assayed with 3 ml of different final concentrations of TAME ranging from 0.01 to 0.10 mM. The determinations were repeated twice and the respective kinetic parameters 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 spleen

Purification of trypsins from skipjack tuna spleen is summarised in Table 1. After Sephacryl S-200, total activity of approximately 59.6% was retained, while 91.5% of protein was removed. Gel filtration was used to remove other proteins in true sardine viscera trypsin fraction, leading to the higher purity of trypsin (Kishimura, Hayashi, Miyashita, & Nonami, 2006). From the result, purity of 6.8-fold was obtained after subjecting to Sephacryl S-200 column.

To refine the pooled fraction obtained from previous step, pooled active fractions were subjected to a Sephadex G-50 column. Purification of 49.3-fold with a yield of 33.1 was obtained. Kishimura and Hayashi (2002) found that the use of Sephadex G-50 for the purification of trypsin from starfish pyloric caeca led to an increase in activity by 34-fold.

Pooled active Sephadex G-50 fractions were further purified using a DEAE-cellulose column. Pooled Sephadex G-50 fractions were dialysed against 10 mM Tris–HCl, pH 8.0 containing 1 mM $CaCl_2$ for 24 h at 4 °C, prior to loading onto an ion exchanger, DEAE-cellulose. After loading and washing, the column was eluted by a step-wise process using different NaCl concentrations and three trypsin activity peaks were found (Fig. 1). These trypsin isoforms were tentatively designated as trypsin A, B and C based on the elution order obtained in this purification step. Fractions of each isoform were pooled. After DEAE–cellulose chromatography, a large amount of contaminated proteins was removed, resulting in a substantial increase in purification fold. Purities of trypsins A, B and C were increased by 112.5-, 57- and 51.3-fold with the yields of 7.5%, 3.4% and 2.5% respectively. Ion exchange chromatography was used to remove contaminating protein and to separate different trypsin isoforms. Two trypsins from the digestive tract of anchovy (*Engraulis encrasicholus*) were purified by using an ion exchanger, DEAE-sepharose at the final step of purification (Martinez, Olsen, & Serra, 1988). Purification folds of 12.5 and 23.5 were obtained for trypsin type I and II, respectively. Kishimura, Hayashi, Miyashita, and Nonami (2005) also isolated two trypsin isozymes from visera of Japanese anchovy (*Engraulis japonica*) using DEAE-cellulose in the final step, leading to the increases in trypsin activity by 37- and 73-fold.

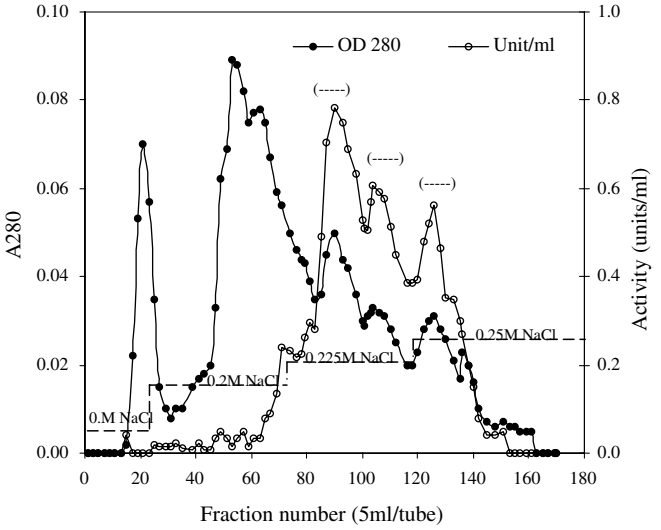

Fig. 1. Elution profile of trypsins on DEAE-cellulose column. Pooled fractions from Sephadex G-50 column were dialysed against SB and applied onto a DEAE-cellulose column. Step-wise elution was carried out using SB containing different NaCl concentrations.

Table 1
Purification of trypsins from the spleen of skipjack tuna

Purification steps	Total activity (units)[a]	Total protein (mg)	Specific activity (units/mg protein)	Purity (fold)	Yield (%)
Crude extract	720.8	4940.5	0.15	1.0	100.0
Sephacryl S-200	429.5	421.1	1.02	6.8	59.6
Sephadex G-50	238.7	32.3	7.39	49.3	33.1
DEAE-cellulose					
Trypsin A	54.0	3.2	16.88	112.5	7.5
Trypsin B	24.8	2.9	8.55	57.0	3.4
Trypsin C	17.7	2.3	7.70	51.3	2.5

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

3.2. SDS and native-PAGE

When three different DEAE-cellulose fractions, designated as trypsin A, B and C were analysed on SDS–PAGE, a single protein band with an estimated molecular mass of 24 kDa was found for all isoforms. The result revealed that all trypsin isoforms had the same molecular mass (Fig. 2a). Different molecular weights have been reported for purified trypsin from fish digestive organs, depending on the fish species. The molecular mass of a trypsin from pyloric caeca of arabesque greenling was estimated to be approximately 24 kDa by SDS–PAGE (Kishimura et al., 2006). The molecular weights of two trypsins enzymes (A and B) from carp hepatopancreas were estimated to be approximately 28.5 and 28 kDa, respectively (Cao et al., 2000). A trypsin from pyloric caeca of Monterey sardine (*Sardinops sagax caerulea*) had a molecular weight of 25 kDa as estimated by SDS–PAGE (Castillo-Yanez, Pacheco-Aguilar, Garcia-Carreno, & Toro, 2005). Kim et al. (1992) reported that the molecular weights of four trypsin enzymes from hepatopancreas of crayfish were estimated to be approximately 23.8, 27.9, 24.8 and 31.4 kDa, respectively, by gel filtration.

For native-PAGE, trypsins A, B and C showed a single band but with different mobilities (Fig. 2b). The result suggested that all these isoforms were different in molecular property as evidenced by the different positions on the native PAGE. However, trypsin B and C had similar mobility on the native PAGE, indicating the homogeneity of both isoforms. Since the single band was observed on both SDS–PAGE and native-PAGE for each isoform, it was most likely that those trypsin isoforms were present as monomers. According to the size exclusion result, the molecular mass of all isoforms was estimated to be 24 kDa (data not shown). All results confirmed that all trypsin isoforms were present as monomers in nature.

3.3. Optimal pH and temperature

The effects of pH on the trypsin activities are shown in Fig. 3a. The optimal pH for TAME hydrolysis of all trypsin isoforms was 8.5. The activity of purified trypins sharply decreased at very alkaline pH, probably due to the denaturation of enzymes. The decrease in activities was also found in the acidic pH ranges. Under acidic and alkaline pHs, the changes in enzyme conformation was possibly caused by charge repulsion, which is associated with a decrease in electrostatic bonds (Benjakul, Visessanguan, & Tanaka, 2003b; Vojdani, 1996). The optimum pH value of three trypsin isoforms was similar to that of trypsin obtained from Monterey sardine pyloric ceaca which had an optimal pH of 8.0 on BAPNA (Castillo-Yanez et al., 2005). The anionic trypsin (I and II) from the Arctic fish capelin had the optimal pH of 8–9 when BAPNA was used as a substrate (Hjelmeland & Raa, 1982). Trypsin from the pyloric caeca of the starfish (*Asterina pecinifera*) had an

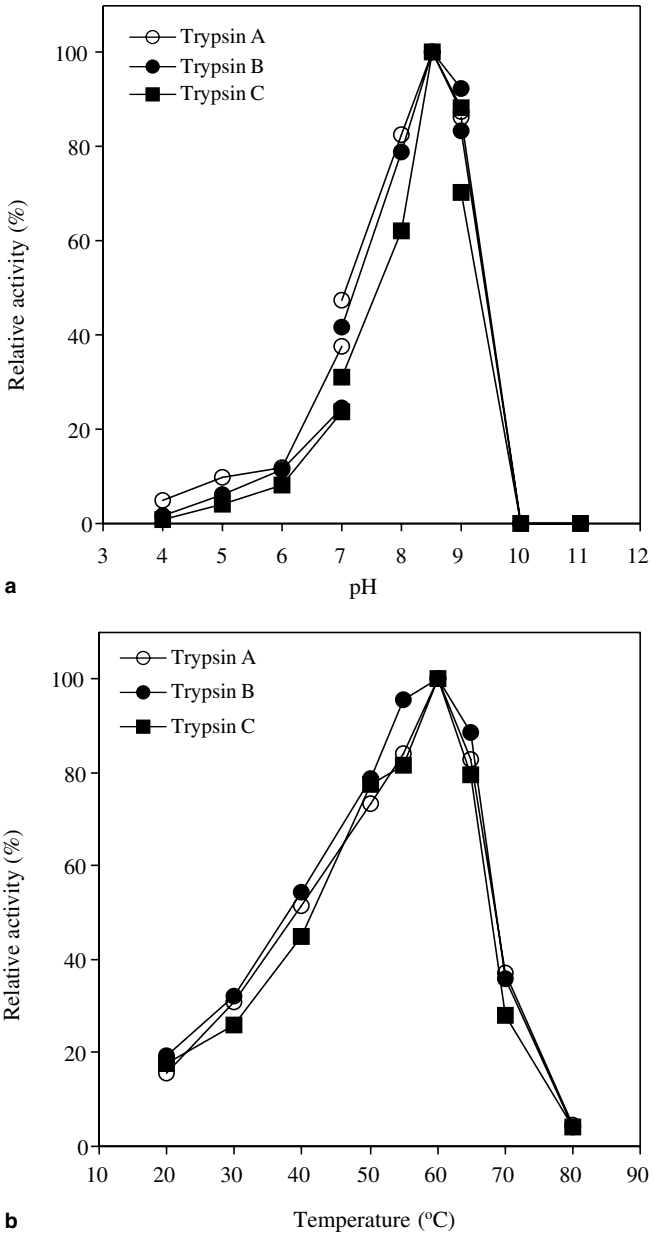

Fig. 3. pH (a) and temperature (b) profiles of purified trypsins A, B and C from skipjack tuna spleen.

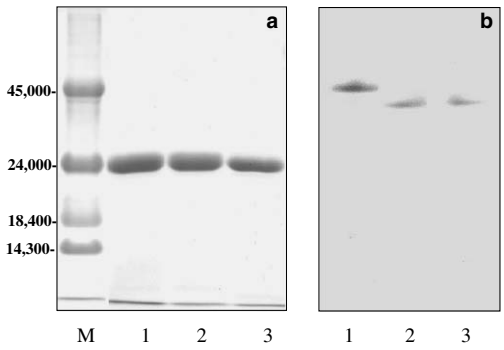

Fig. 2. Protein pattern from SDS–PAGE (a) and Native-PAGE (b) of trypsins A, B and C from skipjack tuna spleen. M, molecular weight standard; lane 1, trypsins A; lane 2, trypsins B; lane 3, trypsins C.

optimal pH of 8.0 when TAME was used as a substrate (Kishimura & Hayashi, 2002).

The effects of various temperatures on the activity of three trypsin isoforms from skipjack tuna spleen are depicted in Fig. 3b. Trypsins A, B and C showed the highest activity at 60 °C. A sharp decrease in activity was observed at temperatures above 60 °C. The optimal temperature of purified trypsins was similar to that of trypsin from pyloric caeca of rainbow trout (*Oncorhynchus mykiss*) with a temperature optimum of 60 °C (Krisjansson, 1991). Bezerra et al. (2001) also reported that alkaline serine proteinase from the pyloric caeca of tambaqui (*C. macropomum*) exhibited optimal activity at 60 °C. However, the purified trypsins from skipjack tuna spleen had slightly higher optimal temperatures than trypsin from other species, which had the optimal temperatures in the ranges of 40–45 °C (Cao et al., 2000; Heu, Kim, & Pyeun, 1995; Martinez et al., 1988; Simpson, 2000; Simpson & Haard, 1984). The difference might be related to the difference in the temperature of the water that the fish inhabited.

3.4. pH and thermal stability

Skipjack tuna spleen trysins were stable in the pH ranging from 6.0 to 11.0 (Fig. 4a). However, three trypsins were unstable at pH below 5.0. No activity of all trypsin isoforms was found after incubation at pH 4.0. The stability of trypsins at a particular pH might be related to the net charge of the enzyme at that pH (Castillo-Yanez et al., 2005). Diminished stability at acidic pHs was observed for trypsin from several fish species (Hjelmeland & Raa, 1982; Kishimura et al., 2005, 2006; Krisjansson, 1991; Simpson, Smith, Yaylayan, & Haard, 1989). From the result, the trypsin might undergo denaturation under acidic conditions, where the conformational change took place and enzyme could not bind to the substrate properly.

Trypsins A, B and C from skipjack tuna spleen were stable for 15 min when heated up to 50 °C (Fig. 4b). No trypsin activities were remained after the heat treatment at 80 °C. After heat treatment at 60 °C, activities of approximately 68% were retained for all isoforms. At high temperature, the enzymes possibly underwent denaturation and lost their activity. Trypsin from Atlantic blue carp was stable at temperature ranging from 30 to 50 °C for 30 min but activity was rapidly lost at temperature above 50 °C (Dendinger & O'Connor, 1990). Kishimura and Hayashi (2002) found that the trypsin activity of starfish pyloric caeca was stable up to 50 °C and activity almost disappeared when heated at temperature higher than 70 °C. Enzymes are inactivated at high temperature due to the partial unfolding of the enzyme molecule. The mechanism for increasing thermal stability of proteins appears to be due to strengthening of hydrophobic interactions and disulfide bonding in the interior of the protein molecule (Kim et al., 1992). Numerous disulfide linkages, as well as stronger hydrophobic interactions in the interior of the protein contribute to protein thermal stability. Disulfide bonds may stabilise a

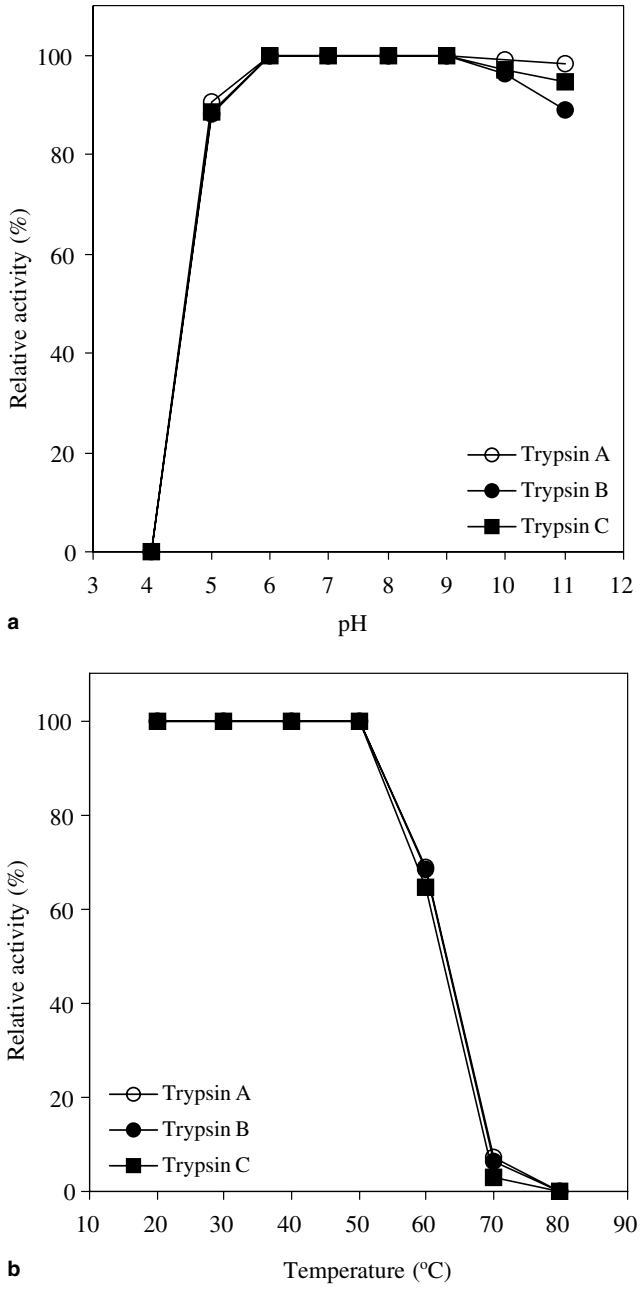

Fig. 4. pH (a) and thermal (b) stability of purified trypsin A, B and C from skipjack tuna spleen.

folded conformation (Creighto, 1983). Fish enzyme was less stable than bovine enzyme at temperatures above 40 °C, possibly caused by a lower number of maximal intramolecular disulfide bonds in fish trypsin compared with bovine trypsin (Simpson & Haard, 1984).

3.5. Effect of inhibitors

The effect of various proteinase inhibitors on the activity of trypsins A, B and C was determined (Table 2). The activities of all trypsin isoforms were completely inhibited by 1.0 g/l soybean trypsin inhibitor and 5 mM TLCK, a specific inhibitor toward trypsin and partially inhibited by EDTA.

Table 2
Effect of various inhibitors on the activity of purified trypsins from the spleen of skipjack tuna[a]

Inhibitors	Concentration	% Inhibition		
		Trypsin A	Trypsin B	Trypsin C
Control		0	0	0
E-64	0.1 mM	0	0	0
N-ethylmaleimide	1 mM	0	0	0
Iodoacetic acid	1 mM	0	0	0
Soybean trypsin inhibitor	1.0 g/l	100	100	100
TLCK	5 mM	100	100	100
TPCK	5 mM	0	0	0
Pepstatin A	0.01 mM	0	0	0
EDTA	2 mM	25.3	17.5	13.7

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

Specific inhibitors of cysteine proteinases (E-64, *N*-ethylmaleimide, iodoacetic acid), chymotrypsin (TPCK) and aspartic proteinase (pepstatin A) had no inhibitory effect on trypsin activities. The result confirmed that all trypsin isoforms were serine proteinases, most likely trypsin. Trypsin-like enzyme from tambaqui pyloric caeca was inhibited by some trypsin inhibitors, such as PMSF, benzamidine and TLCK (Bezerra et al., 2001). 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). When EDTA, specific for metalloproteinase (Benjakul, Visessanguan, & Leelapongwattana, 2003a; Klomklao et al., 2004), was used, it reduced the esterase activities of all trypsin isoforms with different degrees varying from 14% to 25%. Among all isoforms, trypsin A was inhibited by EDTA to a greater extent, compared with the other trypsin isoforms. This result indicated that these isoforms require metal ions as cofactors for activity. EDTA possibly affected the structure of the enzyme or active site conformation, leading to the reduced activity. Furthermore, EDTA could chelate the ion required for activity of enzymes. This might lead to less availability of these ions. The results reconfirmed that three isoforms were trypsin-like proteinases, which possibly required metal ions for their activities.

3.6. Effect of NaCl

The activity of all trypsin isoforms decreased with increasing NaCl concentration (Fig. 5). In the presence of NaCl ranging from 1% to 20%, the activity of trypsin A was slightly higher than those of trypsins B and C. However, similar activities of all trypsin isoforms were obtained in the presence of 25–30% NaCl. At 30% NaCl concentration, the activities of all isoforms decreased by 30–35%. Loss of activity might be due to the denaturation of trypsins caused by the "salting out" effect (Klomklao et al., 2004; Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, in press). NaCl at higher concentration possibly

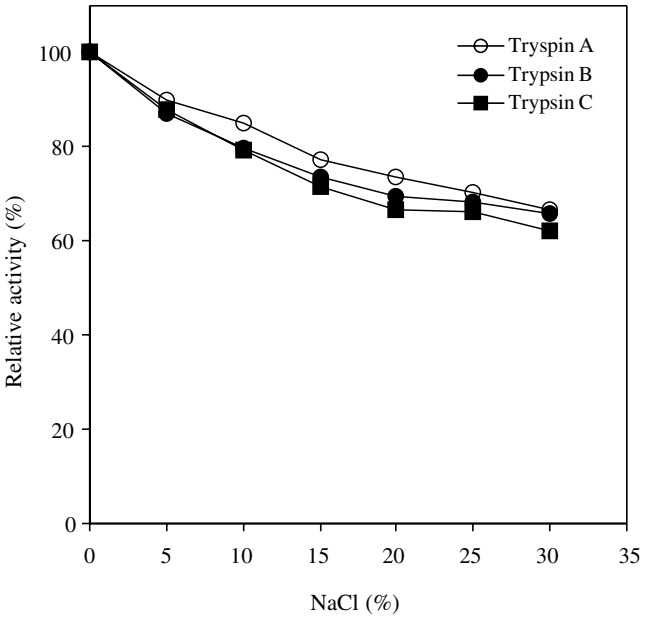

Fig. 5. Effect of NaCl concentrations on activities of purified trypsins A, B and C from skipjack tuna spleen.

competed with the enzyme in water binding, resulting in a stronger protein–protein interaction, which was possibly associated with precipitation (Benjakul et al., 2003a, 2003b; Vojdani, 1996). This led to the loss in activity in the presence of high NaCl concentrations. Klomklao et al. (in press) found that partially purified trypsin from skipjack tuna spleen was still active and able to degrade natural actomyosin in sardine muscle containing 25% NaCl. Therefore, trypsins from tuna spleen might be possible to accelerate the protein hydrolysis in fish sauce production, in which salt at high level (25–30% NaCl) was used.

3.7. Effect of calcium ions on the stability

The effect of $CaCl_2$ on the stability of trypsin A, B and C from skipjack tuna spleen was examined at pH 8.0 and 40 °C (Fig. 6). In the presence of 2 mM calcium ion, total activities were retained throughout 8 h of incubation at 40 °C, whereas the activity of all trypsin isoforms decreased with increasing time in the presence of 2 mM EDTA. These results indicated that all trypsin isoforms from skipjack tuna spleen require calcium ions for their stability. Three trypsin isoforms were stabilised by calcium ions similarly to porcine pancreatic trypsin (data not shown). Two calcium-binding sites are present in bovine trypsinogen (Kosslakoff, Chambers, Kay, & Stroud, 1977). The primary site, with a higher affinity for calcium ions, is common in trypsinogen and trypsin, and the secondary site is only in the zymogen (Kishimura & Hayashi, 2002). Occupation of the primary calcium-binding site by calcium ions stabilises the protein toward thermal denaturation or autolysis (Kishimura et al., 2005, 2006). The presence of calcium ions not only activates trypsinogen to trypsin but also increases the thermal stability of the enzyme. This stabilis-

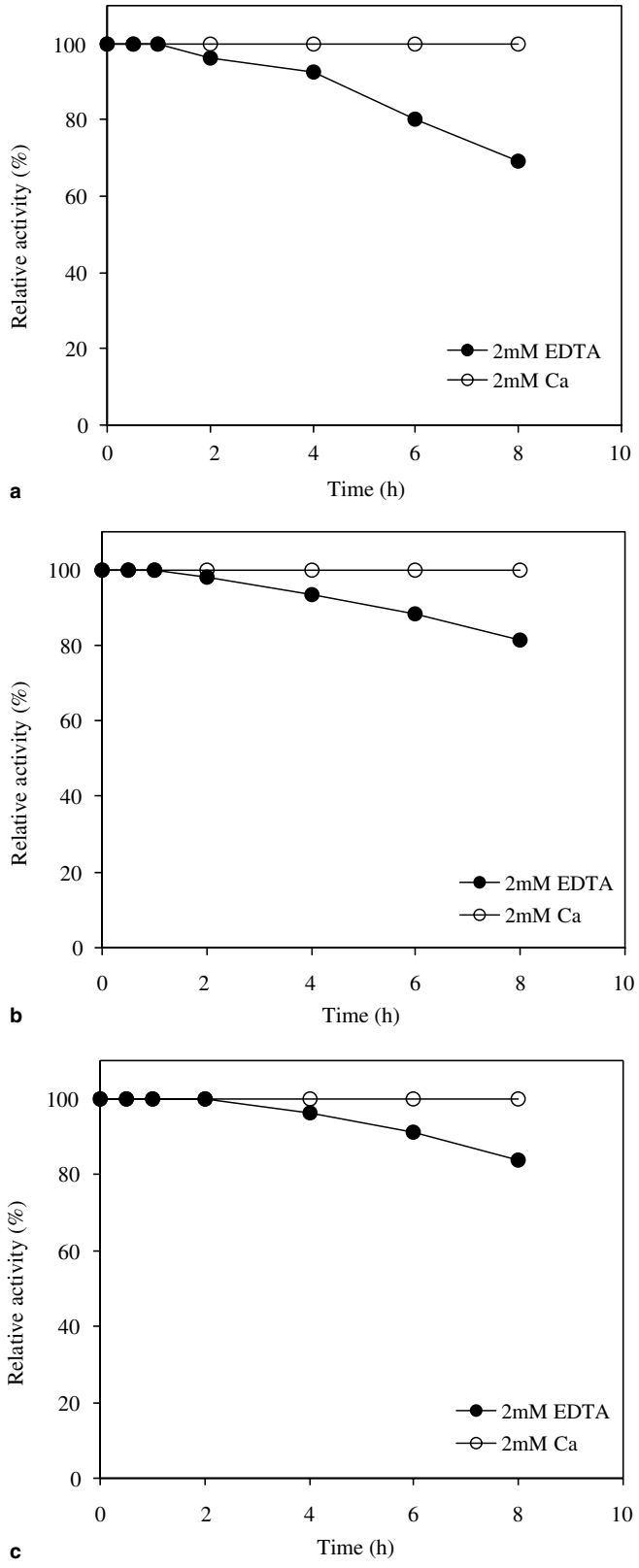

Fig. 6. Effect of calcium ion and EDTA on the stability of purified trypsins A (a), B (b) and C (c) from skipjack tuna spleen. The stability was tested at 40 °C for different times.

ing effect is accomplished by a conformational change in the trypsin molecule, resulting in a more compact structure (Kishimura & Hayashi, 2002). These results suggest that all trypsin isoforms possess a primary calcium-binding site like mammalian pancreatic trypsin and other fish trypsins (Genicot, Rentier-Delrue, Edwards, Van Beeumen, & Gerday, 1996; Kishimura et al., 2006).

3.8. Kinetic studies

Kinetic constants, K_m and K_{cat}, of three trypsin isoforms from skipjack tuna spleen for hydrolysis of TAME were determined from a Lineweaver–Burk plot (Table 3). K_m and K_{cat} of different skipjack tuna spleen trypsin isoforms for the esterolytic reaction were different. Trypsin C showed the lowest K_m values, followed by trypsin B and A, respectively. These results suggest that trypsin C has higher affinity to TAME, conpared with other isoforms. The low K_m values obtained for skipjack tuna spleen trypsins were similar to values reported for crayfish hepatopancreas trypsins (Kim et al., 1994), carp trypsin (Cohen, Gertler, & Birk, 1981) and menhaden trypsin (Pyeun, Kim, & Godber, 1990). For K_{cat}, trypsin C also exhibited the lowest value, while trypsin A had the highest value. The catalytic efficiency (K_{cat}/K_m) of skipjack tuna spleen trypsins (308–519 S^{-1} mM^{-1}) was similar to that of trypsin from hepatopancreas of crayfish (Kim et al., 1994). Most intracellular enzymes normally function at substrate concentrations similar to or below their K_m. However, K_m of digestive enzymes may not be critical to their functionality, since trypsin may function at extremely high substrate concentrations in the digestive tract and in possible industrial application (Kim et al., 1994). Therefore, trypsin A was probably a better catalyst for hydrolysis of TAME than were the other isoforms.

3.9. N-terminal amino acid sequence

The N-terminal amino acid sequence of three trypsin isoforms from skipjack tuna spleen was determined to be IVGGYECQAHSQPHQVSLNS (Fig. 7). The result indicated that N-terminus of all trypsin isoforms was unblocked. When N-terminal amino acid sequences of three isoforms were compared with those of trypsin from true sardine, arabesque greenling (Kishimura et al., 2006), cod (Gudmundsdottir et al., 1993), salmon (Male, Lorens, Smalas, & Torrissen, 1995), porcine (Hermodson, Ericsson, Neurath, & Walsh, 1973), and bovine (Walsch, 1970), all isoforms exhibited the highest homology and they also shared high identities with other trypsins, espe-

Table 3
Kinetic properties of skipjack 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	0.29	114	393.10
Trypsin B	0.20	61.5	307.50
Trypsin C	0.11	57.1	519.09

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

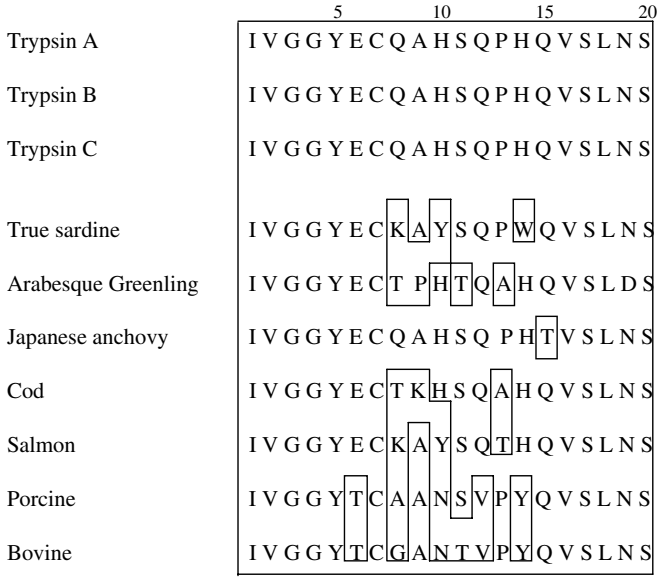

Fig. 7. Comparison of N-terminal amino acid sequence of the purified trypsins A, B and C from skipjack tuna spleen with other enzymes: true sardine, arabesque greenling (Kishimura et al., 2006), cod (Gudmundsdottir et al., 1993), salmon (Male et al., 1995), porcine (Hermodson et al., 1973), and bovine (Walsch, 1970). Amino acid residues different from those of trypsin A, B and C are boxed.

cially with that of Japanese anchovy trypsin. Generally, the N-terminal region of trypsin-like proteinase, especially from first to seventh residues demonstrate high homology (Cao et al., 2000). However, all fish trypsins had a charged Glu residue at position 6, whereas Thr is most common in mammalian pancreatic trypsin (Fig. 7). The N-terminal sequence clearly demonstrated that three trypsin isoforms from skipjack tuna spleen were most likely members of the trypsin family.

4. Conclusion

The skipjack tuna spleen enzymes were purified and identified to be trypsin-like proteinases based on substrate specificity, molecular weight, inhibitor study and N-terminal sequencing. All of trypsins showed maximal activity at pH 8.5 and 60 °C and were salt tolerant. These characteristics suggest that the enzymes could be an important biotechnological tool for the fish processing and food industries, in which high salt concentration is used.

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