COMPARATIVE STUDIES ON PROTEOLYTIC ACTIVITY OF SPLENIC EXTRACT FROM THREE TUNA SPECIES COMMONLY USED IN THAILAND

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ABSTRACT

Proteolytic activities of splenic extract from three tuna species including skipjack tuna (Katsuwonus pelamis)*, yellowfin tuna* (Thunnus albacores) *and tongol tuna* (Thunnus tonggol) *were studied. Optimal activity of splenic extract from all tuna species was at pH 9.0 and 55C when casein was used as a substrate. Among all species tested, yellowfin tuna showed the highest activity, followed by skipjack tuna and tongol tuna. The proteolytic activity was strongly inhibited by soybean trypsin inhibitor, TLCK and partially inhibited by ethylenediaminetetraacetic acid. E-64, N-ethylmaleimide, iodoacetic acid, TPCK and pepstatin A showed no inhibition. The effect of NaCl and* $CaCl_2$ *on proteolytic activity was also investigated. Activities continuously decreased as NaCl concentration increased, and no activity remained in the presence of 30% NaCl. On the other hand, activities increased as* $CaCl_2$ *concentration increased. The highest activity was obtained in the presence of 1 mM* $CaCl_2$*. SDS-substrate gel electrophoresis revealed that major proteinases in splenic extract from different tuna species were different in apparent molecular weights and sensitivity to TLCK. Although the major activity bands*

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of all species were strongly inhibited by soybean trypsin inhibitor, varying sensitivity to TLCK probably implied the differences in binding characteristic of enzyme to substrate and/or inhibitors. The results suggest that major proteinases in spleen of all tuna species were trypsin-like serine proteinases.

INTRODUCTION

The tuna processing industry, especially canning, is becoming increasingly important since it is one of the income generators for Thailand. In terms of volume, Thailand is the world's largest exporter of canned tuna, with over 20 million cans annually during the past 5 years. Large volumes of raw tuna go through the canning process, in which about two-thirds of the whole fish are utilized. Processing wastes from the tuna canning industry is generated and estimated at 450,000 metric tons annually (Subasinghe 1996). More than 200,000 metric tons of tuna viscera and offal can be collected and used mostly for fishmeal and fish liver powder for animal feed.

Fish viscera is a potential source of enzymes such as proteinases (Haard 1992) that may have some unique properties for industrial applications (Ooshiro 1971; Kawai and Ikeda 1972; Kristjansson 1991). Digestive proteinases have been extensively studied in several species of fish (An and Visessanguan 2000). The most important digestive enzymes are pepsin, secreted from gastric mucosa, and trypsin and chymotrypsin secreted from the pancreas, pyloric caeca and intestine (Simpson 2000). Arunchalam and Haard (1985) isolated and characterized pepsin from polar cod stomach (*Boreagadus saida*). Pepsins from the stomachs of various teleost fish species have been purified and characterized (Noda and Murakami 1981; Sanchez-Chiang *et al.* 1987; Gildberg *et al.* 1990). Most species contained two or three major pepsins with an optimum pH of 2–4 for haemoglobin digestion (Noda and Murakami 1981). Pepsin activity is very dependent on pH values and the type of substrate. Haemoglobin is the substrate most frequently used for determination of pepsin activity (De Vecchi and Coppes 1996). Trypsins have been characterized thoroughly (based on their physicochemical and enzymatic properties) from the intestine of crayfish (Kim *et al.* 1992, 1994), dogfish (Ramakrishna *et al.* 1987), mackerel (Kim and Pyeun 1986) and capelin (Hjelmeland and Raa 1982). Bezerra *et al.* (2001) partially purified trypsin from the pyloric caeca of tambaqui (*Colossoma macropomum*) and found that the enzyme had an optimum pH of 9.5. Trypsin and chymotrypsin from the viscera of anchovy (*Engraulis japonica*) have also been purified and characterized (Heu *et al.* 1995). Recently, Byun *et al.* (2003) purified and characterized serine proteinases from the pyloric caeca of tuna (*Thunnus thynnus*).

The distribution of proteinase varies, depending on species and organs. Torrissen (1984) reported that proteinase activity from the intestine of rainbow trout (*Salmo gairdneri*) was higher than that from Atlantic salmon (*Salmo salar*). *Pyloric caeca* of Chinook salmon (*Oncorhynchus tschawytscha*) yielded proteinase with a higher activity than that from rainbow trout (Dimes *et al.* 1994). For discus fish (*Symphysodon aequifasciata*), proteinase activity in the intestine was higher than that in stomach (Chong *et al.* 2002). The distribution of protease activity in individual viscera organ of three tuna species including skipjack tuna (*Katsuwonus pelamis*), yellowfin tuna (*Thunnus albacares*) and tongol tuna (*Thunnus tonggol*) commercially used in the Thai tuna industries was investigated (Klomklao and Benjakul 2003). The activity per wet tissue weight was highest in spleen and decreased in the order: spleen, pancreas, stomach and liver. However, no information regarding the characteristics and properties of proteolytic enzymes from spleen of different species of tuna has been reported. This study aimed to characterize some molecular and enzymatic properties of major proteinases in splenic extract of three tuna species commonly used in the Thai tuna industries.

MATERIALS AND METHODS

Chemicals

Sodium caseinate, bovine haemoglobin, ethylenediaminetetraacetic acid (EDTA), pepstatin A, soybean trypsin inhibitor, iodoacetic acid, *N*-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenyl-alanine chloromethyl ketone (TPCK), 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), *N*-ethylmaleimide, 2-mercaptoethanol (βME), L-tyrosine and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Trichloroacetic acid, 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 purchased from Bio-Rad Laboratories (Hercules, CA).

Fish Sample Preparation

Internal organs from three species of tuna including skipjack tuna (*Katsuwonus pelamis*), yellowfin tuna (*Thunnus albacares*) and tongol tuna (*Thunnus tonggol*) were obtained from Chotiwat Industrial Co. (Thailand) Ltd., Songkhla. Those samples (5 kg) were packed in polyethylene bags, kept in ice 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 the spleen was collected, immediately frozen and stored at −20C until used.

Preparation of Splenic Extract

Frozen spleens were thawed using running water (26–28C) until the core temperature reached −2 to 0C. The samples were cut into pieces with a thickness of 1–1.5 cm. Samples were ground into powder in liquid nitrogen using a National Model MX-T2GN blender (Taipei, Taiwan) according to the method of Simpson and Haard (1984) as modified by Garcia-Carreno *et al.* (1993).

Spleen powder was suspended in distilled water at a ratio of 1 : 9 (w/v) and stirred continuously at 4C for 15 min. The suspension was centrifuged for 15 min at 4C at 5,000 *g* using a Sorvall Model RC-B Plus centrifuge (Newtown, CT) to remove the tissue debris. The supernatant was collected and referred to as "tuna splenic extract".

Enzyme Assay

Proteinase activity of spleen extract from each tuna was determined using haemoglobin and casein as substrates according to the method of Kunitz (1947) and An *et al.* (1994) with a slight modification. Different buffers were used for different pH conditions: 0.2 M McIlvaine's buffer (0.2 M sodium phosphate–0.1 M sodium citrate) for pH 2.0–7.5 and 0.1 M glycine–NaOH for pH 8.0–11.0 (Glass *et al.* 1989; Sabapathy and Teo 1993; Munilla-Moran and Rey 1996; Hidalgo *et al.* 1999). To initiate the reaction, 200 µL of 200× diluted splenic extract was added into assay mixtures containing 2 mg of casein, 200 µL of distilled water and 625 µL of reaction buffer. The enzymatic reaction was terminated by adding 200 µL of 50% (w/v) trichloroacetic acid (TCA). Unhydrolyzed protein substrate was allowed to precipitate for 15 min at 4C, followed by centrifuging at 7,000 *g* for 10 min. The oligopeptide content in the supernatant was determined by the Lowry assay (Lowry *et al.* 1951) using tyrosine as a standard. Activity was expressed as tyrosine equivalents in TCA-supernatant. One unit of activity was defined as that releasing 1 mmole of tyrosine per min (mmol/Tyr/min). A blank was run in the same manner, except the enzyme was added after addition of 50% TCA (w/v).

pH and Temperature Profile

Proteolytic activity was measured using casein-TCA-Lowry assay (An *et al.* 1994) at pH 3.0, 5.0, 7.0 (using 0.2 M McIlvaine's buffer) and pH 9.0 (using 0.1 M glycine–NaOH) at various temperatures (30, 40, 50, 55, 60, 65 and 70C). The optimal pH was determined at 55C over the pH range of 2.0–

11.0 (0.2 M McIlvaine's buffer for pH 2.0–7.5 and 0.1 M glycine–NaOH for pH 8.0–11.0).

Effect of Inhibitors on Proteinase Activity

The effect of inhibitors on proteinase activity was determined by incubating splenic extract 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, 0.1 and 1.0 g/l soybean trypsin inhibitor, 1 and 5 mM TLCK, 1 and 5 mM TPCK, 1 mM pepstatin A and 2 mM EDTA). After incubating the mixture at room temperature (26–28C) for 15 min, the remaining activity was measured by the TCA-Lowry method (An *et al.* 1994). Percent inhibition was then calculated.

Effect of NaCl on Proteinase Activity

Effect of NaCl on proteinase activity was studied. NaCl was added to the standard reaction assay to obtain the final concentration of 0, 5, 10, 15, 20, 25 and 30% (w/v). The residual activity was determined at 55C and pH 9.0 for 15 min using casein as a substrate.

Effect of $CaCl_2$ on Proteinase Activity

To study the effect of $CaCl_2$ on proteinase activity, $CaCl_2$ was added to the standard reaction assay to obtain the different final concentrations (0, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} and 10^{-2} M). The activity was determined at 55C and pH 9.0 for 15 min using casein as a substrate.

Activity Staining

Splenic extracts were separated on SDS-PAGE, followed by activity staining according to the method of Garcia-Carreno *et al.* (1993). Splenic extracts were mixed with sample buffer (0.125 M Tris–HCl, pH 6.8 containing 20% (v/v) glycerol) with and without βME at a ratio of 1 : 1 (v/v). Two μg of proteins were loaded into the gel made of 4% stacking and 12% separating gels. The proteins were subjected to electrophoresis at a constant current of 15 mA per gel by a Mini-Protean II Cell apparatus. After electrophoresis, gels were immersed in 100 ml of 2% casein (w/v) in 50 mM Tris–HCl buffer, pH 7.5 for 1 h with constant agitation at 0C to allow the substrate to penetrate into the gels. The gels were then transferred to 2% casein (w/v) in 0.1 M glycine–NaOH, pH 9.0 and incubated at 55C for 15 min with constant agitation to develop the activity zone. The gels were fixed and stained with 0.125% Coomassie blue R-250 in 45% ethanol and 10% acetic acid and destained in 30% methanol and 10% acetic acid. Development of clear zones on blue

background indicated proteolytic activity. For the inhibitor study, splenic extracts were incubated with an equal volume of proteinase inhibitor solutions to obtain the final concentration designated (1.0 g/l soybean trypsin inhibitor and 5 mM TLCK) for 15 min at room temperature. After incubation, the mixtures were mixed with sample buffer at a ratio of 1 : 1 (v/v). The mixtures were loaded into the gel and activity staining was performed as previously described.

Protein Determination

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

Statistical Analysis

A completely randomized design was used throughout this study. Data were subjected to analysis of variance (ANOVA) and mean comparison between tuna species was carried out using Duncan's Multiple Range Test (Steel and Torrie 1980). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for Windows; SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Temperature and pH Profiles of Tuna Splenic Extracts

The temperature profiles of all tuna splenic extracts are shown in Fig. 1. Proteolytic activity assayed at pH 3.0, 5.0, 7.0 and 9.0 increased markedly from 25C to the highest peak at 55C before rapid inactivation at higher temperatures. At any particular temperature tested, the activity assayed at pH 9.0 was the highest while the activity was negligible in acidic pH ranges. Further characterization confirmed that the optimum pH of all splenic proteinases assayed at 55C was 9.0 (Fig. 2). The activity of crude proteinases markedly decreased in acidic and alkaline pH ranges. Based on the optimum temperature and pH, the major splenic proteinases of all tuna species were characterized as heat-activated alkaline proteinases.

The presence of alkaline proteinases in internal organs of various fish species has been reported. Alkaline serine proteinase from the pyloric caeca of tambaqui (*Colossoma macropomum*) exhibited optimal activity at 60C and pH 9.5 when azocasein was used as a substrate (Bezerra *et al.* 2001). Two anionic trypsins (A and B) obtained from carp hepatopancreases had an optimal activity at pH 9.0 and 45C on Boc-Phe-Ser-Arg-MCA (Cao *et al.* 2000). Alkaline proteinase from the intestinal section of discus fish

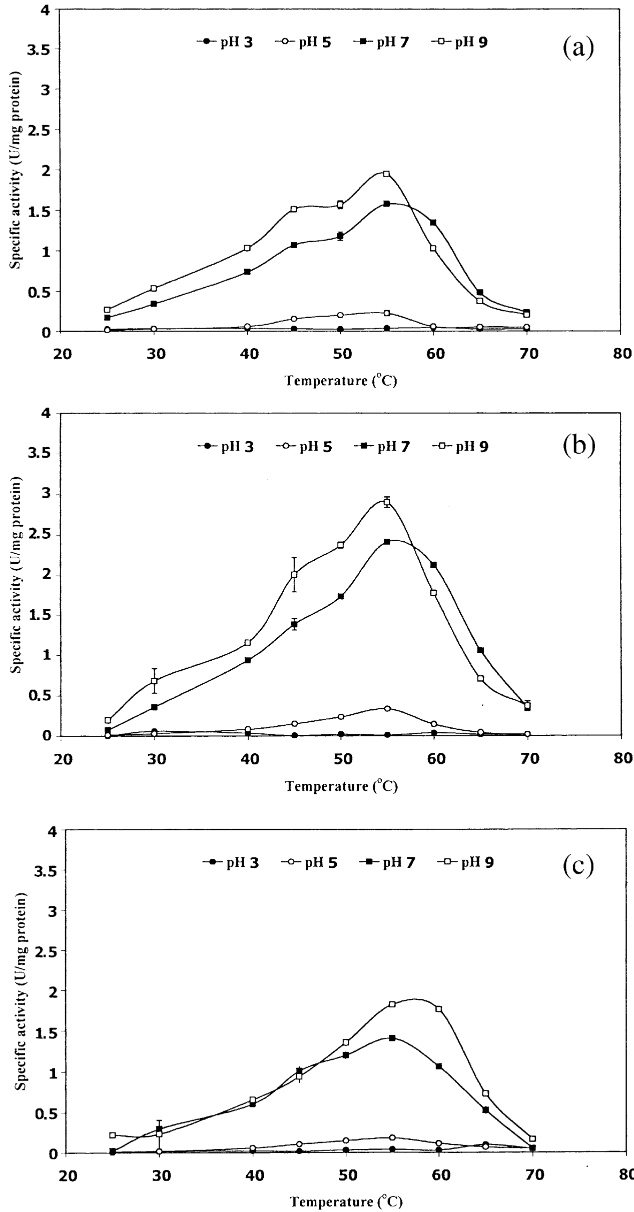

FIG. 1. TEMPERATURE PROFILES OF SPLEEN PROTEINASES FROM DIFFERENT TUNA SPECIES
(a) skipjack tuna; (b) yellowfin tuna; (c) tongol tuna. Bars indicate standard deviation from triplicate determinations.

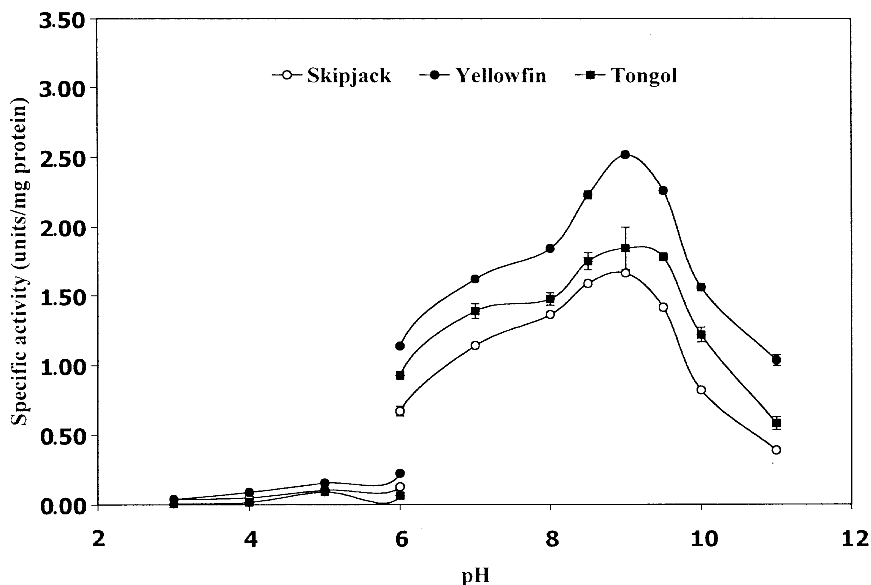

FIG. 2. pH PROFILES OF SPLEEN PROTEINASES FROM DIFFERENT TUNA SPECIES
Bars indicate standard deviation from triplicate determinations.

(*Symphysodon aequifasciata*) had an optimum pH ranging from 7.5 to 9.0 and from 11.5 to 12.5 when casein was used as a substrate (Chong *et al.* 2002). Kristjansson (1991) reported that trypsin from the pyloric caeca of rainbow trout (*Oncorhynchus mykiss*) had optimum pH of 9–10 on BAPNA. Heu *et al.* (1995) also reported that trypsin and chymotrypsin from anchovy viscera had optimum activity at pH 9.0 and 8.0 when casein and synthetic substrates (BAPNA and BTEE) were used as substrates, respectively. Based on the optimum activity at pH 9.0 and 55C, it was concluded that tuna splenic proteinase resemble trypsin or trypsin-like proteinases.

Proteinase Activity in Spleen from Different Tuna Species

Splenic proteinase activities of all tuna species tested are shown in Table 1. Under the same optimal condition, splenic extract of yellowfin tuna showed the highest specific activity when casein was used as a substrate, followed by those of skipjack tuna and tongol tuna, respectively. Due to limited information particularly on the role and biological importance of proteolysis in spleen, proteinase may be involved in the maintenance of normal spleen functions e.g. recycling of red blood cells and in the immune defense system (Koshikawa *et al.* 1998). It is also speculated that differences

TABLE 1.
ACTIVITY OF SPLENIC PROTEINASES FROM DIFFERENT TUNA SPECIES*

Types	Proteinase activity (U/g tissue)***
Skipjack tuna	515.27 ± 4.75b**
Yellowfin tuna	629.03 ± 8.58c
Tongol tuna	444.14 ± 8.46a

* Activity was analyzed using casein as a substrate for 15 min at pH 9.0 and 55C.
** The different letters in the same column denote significant differences (<0.05).
*** Mean ± SD from triplicate determinations.

TABLE 2.
EFFECT OF VARIOUS INHIBITORS ON THE ACTIVITY OF SPLENIC PROTEINASES FROM DIFFERENT TUNA SPECIES*

Inhibitors	Concentration	% Inhibition***		
		Skipjack tuna	Yellowfin tuna	Tongol tuna
Control		0a	0a	0a
E-64	0.1 mM	0a	0.1 ± 2.3a	0a
N-ethylmaleimide	1 mM	0.7 ± 2.1a**	0.8 ± 4.7a	0.5 ± 1.2a
Iodoacetic acid	1 mM	0.9 ± 1.7a	0.2 ± 3.4a	0.7 ± 0.6a
Soybean trypsin inhibitor	0.1 g/l	68.0 ± 1.4d	72.1 ± 1.6e	72.1 ± 0.6e
	1.0 g/l	76.3 ± 0.8e	79.8 ± 0.8f	79.9 ± 0.9f
TLCK	1 mM	37.4 ± 1.7c	39.1 ± 3.1c	34.6 ± 1.8c
	5 mM	68.2 ± 2.1d	65.3 ± 9.5d	65.3 ± 2.7d
TPCK	1 mM	12.3 ± 0.9b	2.2 ± 0.5a	3.5 ± 0.7a
	5 mM	13.5 ± 4.7b	3.5 ± 2.4a	3.5 ± 1.1a
Pepstatin A	0.01 mM	0a	3.1 ± 3.2a	5.1 ± 1.4a
EDTA	2 mM	38.6 ± 1.9c	19.7 ± 2.2b	22.2 ± 2.9b

* Activity was analyzed using casein as a substrate for 15 min at pH 9.0 and 55C.
** The different letters in the same column denote the significant differences (<0.05).
*** Mean ± SD from triplicate determinations.

in the level of splenic proteinase activity among tuna species might be related to growth and physiological change in fish such as metabolism, season, age, size, habitat temperature and depth, spawning phase and diet (Haard 2000; Simpson 2000).

Effect of Inhibitors on Splenic Proteinases

The effect of various inhibitors on splenic proteinase activity from all tuna species was determined (Table 2). Spleen proteinase activity from all tuna species was strongly inhibited by 1.0 g/L soybean trypsin inhibitor (76–80%)

and 5 mM TLCK (65–68%) and partially inhibited by EDTA. Specific inhibitors of cysteine proteinases (E-64, *N*-ethymaleimide, iodoacetic acid), chymotrypsin (TPCK) and aspartic proteinase (pepstatin A) had no inhibitory effect on splenic proteinase activity. The result indicated that major proteinases from the spleens of the three tuna species were serine proteinases, particularly trypsin or trypsin-like enzymes (Bezerra *et al.* 2001). The serine proteinase from the intestine of discus fish was inhibited by soybean trypsin inhibitor, PMSF and TLCK while TPCK and EDTA showed partial inhibition (Chong *et al.* 2002). Trypsin-like enzyme from tambaqui pyloric caeca was inhibited by some trypsin inhibitors, such as PMSF, benzamidine and TLCK (Bezerra *et al.* 2001). EDTA, specific for metalloproteinase (Tsuchiya *et al.* 1994), had a slight inhibitory effect on proteolytic activity with approximately 20–40% inhibition. The result suggested that the spleen proteinases from all tuna species were classified to be the trypsin-like serine proteinases, which possibly required metal ions for activity.

Effect of NaCl on Splenic Proteinase Activity

The effect of NaCl at different concentrations on proteinase activity in tuna splenic extract is depicted in Fig. 3. The activity decreased with increas-

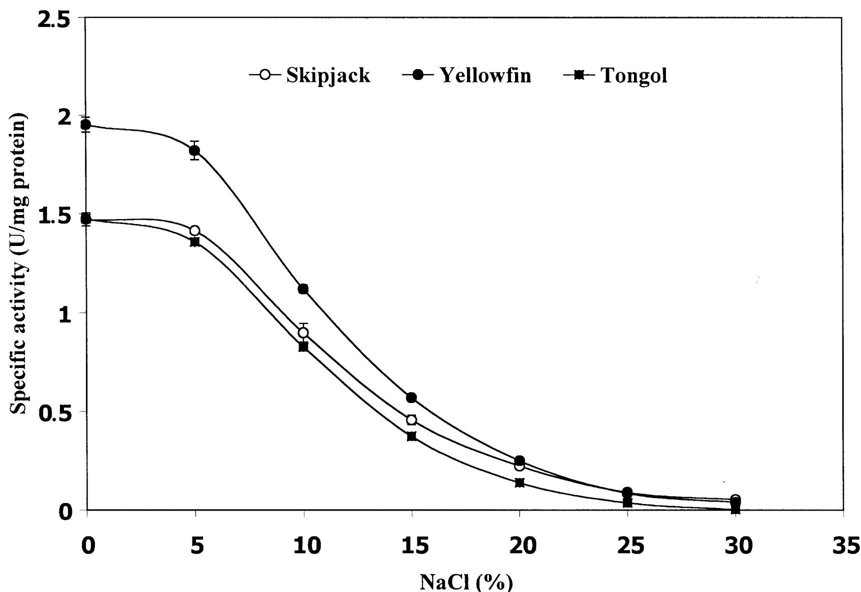

FIG. 3. EFFECT OF NaCl CONCENTRATION ON ACTIVITIES OF SPLEEN PROTEINASES FROM DIFFERENT TUNA SPECIES
Bars indicate standard deviation from triplicate determinations.

ing NaCl concentration. In the presence of NaCl ranging from 1 to 10%, splenic proteinase of yellowfin tuna showed considerably higher activity than those of skipjack tuna and tongol tuna. However, similar activity levels of splenic proteinase of all tuna species were obtained in the presence of 15% NaCl and no activity was observed in the presence of 30% NaCl. Loss of activity might be due to denaturation of proteinases caused by the "salting out" effect. Thermostable proteinase in salted anchovy muscle may be still active and able to degrade myofibrillar protein in commercial salted fillets containing 16–17% NaCl (Ishida *et al.* 1994). The activity of acid proteinases from sardine was reduced with the addition of 3.42 M NaCl (Noda and Murakami 1981). Nevertheless, Fang and Chiou (1989) reported that NaCl up to 3.42 M had no effect on pepsin, trypsin and chymotrypsin activities from tilapia, although the isoenzymic pattern varied depending on acclimatization to different salinities. From the results, more than 50% of splenic proteinase activity remained in the presence of a high concentration of NaCl (10–20%). Thus, these proteinases may contribute to the hydrolysis of proteins in high-salt fermented fish products such as fish sauce.

Effect of $CaCl_2$ on Proteinase Activities

Proteinase activities of splenic extracts from all species increased with the addition of calcium chloride (Fig. 4). At a concentration lower than 1 μM, calcium had no influence on the activity of spleen extracts for all species. When the concentration of calcium was increased from 1 μM to 1 mM, activity apparently increased. However, there was no further increase in the activity with calcium chloride above 1 mM. The result was in accordance with Sipos and Markel (1970) who found that calcium below 1 μM had no influence on bovine trypsin activity, however when the concentration of calcium was increased from 1 μM to 1 mM, the activity increased. It is known that calcium ions promote the formation of active trypsin from trypsinogen and stabilize trypsin against autolysis (Sipos and Markel 1970). Stabilization against thermal inactivation by calcium was also reported for the trypsins from catfish (Yoshinaka *et al.* 1984) and rainbow trout pyloric caeca (Kristjansson 1991). Bode and Schwager (1975) showed that calcium not only protected trypsin against self-digestion, but it also slightly increased its proteolytic activity. Furthermore, Sipos and Markel (1970) concluded that calcium promotes the formation of a calcium–trypsin complex from a reversible inactive form. Delaage *et al.* (1968) reported the existence of a specific calcium binding site on trypsinogen. The binding of calcium to trypsinogen induces a conformational change, which is associated with the formation of an active form. Therefore, calcium ions played an essential role in activation of proteinases from tuna spleen.

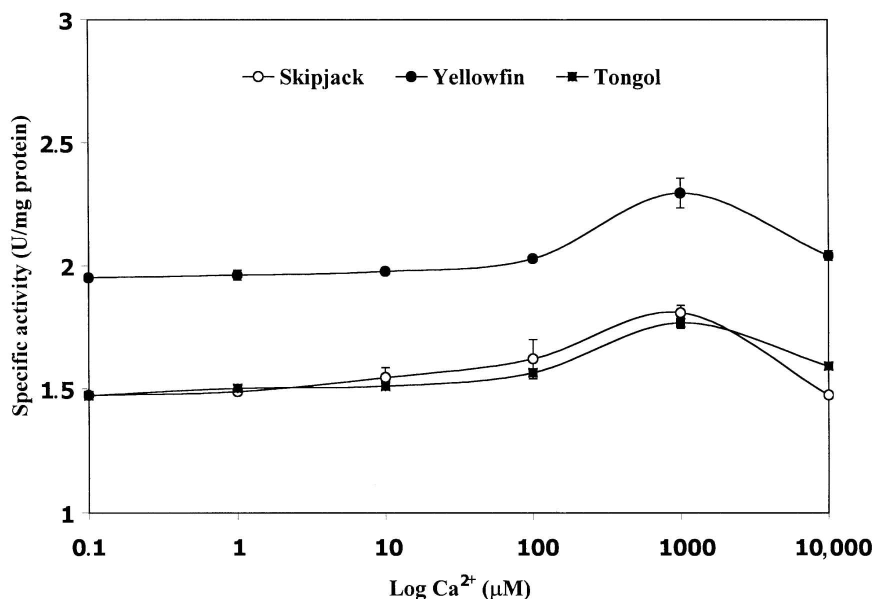

FIG. 4. EFFECT OF $CaCl_2$ CONCENTRATION ON ACTIVITIES OF SPLEEN PROTEINASES FROM DIFFERENT TUNA SPECIES
Bars indicate standard deviation from triplicate determinations.

Activity Staining of Splenic Proteinases

The proteinase activity in tuna splenic extract was identified by separation on SDS-substrate polyacrylamide gels followed by staining for proteolytic activity (Fig. 5). Under both reducing and nonreducing conditions, a similar pattern of activity bands is shown as clear zones on the dark background. The molecular weights of the major activity bands were estimated to be 42 kDa for skipjack tuna and 21 kDa for tongol and yellowfin tuna. However, additional activity bands were observed with apparent MW of 24 kDa for tongol and 30, 29, and 24 kDa for yellowfin, respectively. The results indicated the differences in the major proteinases in splenic extract among all tuna species tested particularly, skipjack and the other two species.

Based on the molecular weight, the activity bands were tentatively identified as trypsin and/or trypsin-like serine proteases. Trypsin consists of a single peptide chain with molecular weight typically of 24 kDa. However, the differences in trypsin may be owing to the genetic variation among species (Torrissen 1984). The molecular weight of trypsin from pyloric caeca of Greenland cod was 23.5 kDa (Simpson and Haard 1984). The molecular weight of the two anionic trypsins serine proteinases (A and B) from carp

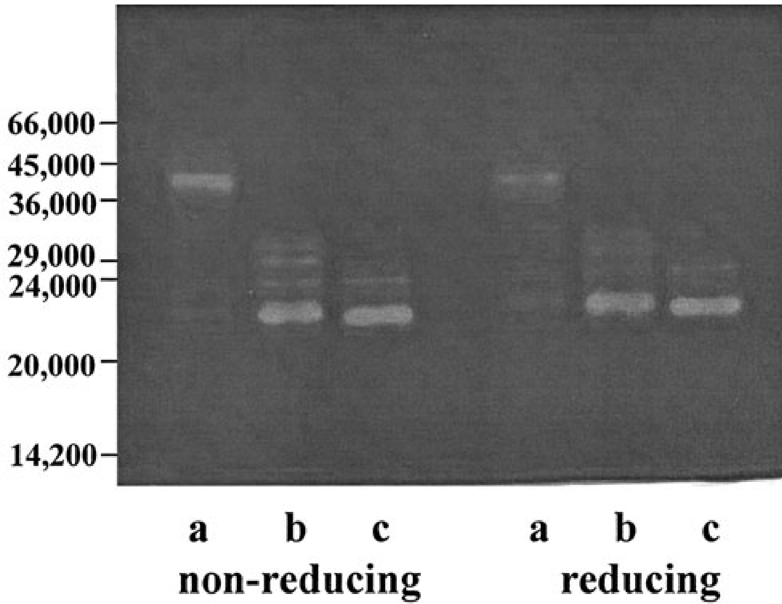

FIG. 5. ACTIVITY STAINING OF SPLEEN PROTEINASES FROM DIFFERENT TUNA SPECIES UNDER REDUCING AND NON-REDUCING CONDITIONS
a: skipjack tuna; b: yellowfin tuna; c: tongol tuna.

hepatopancreases was estimated to be approximately 28.5 kDa and 28 kDa, respectively. Trypsin-like enzyme from pyloric caeca of tambaqui had a molecular weight of 38.5 kDa (Bezerra *et al.* 2001). Two novel trypsin-like serine proteases from mouse spleen consisted of a single polypeptide with a molecular mass of 29 kDa (Fukusen and Aoki 1996). In human and mouse, trypsin is widely expressed in both pancreatic and nonpancreatic tissue. Among nonpancreatic tissues, the spleen was the highest in trypsin expression (Koshikawa *et al.* 1998). It is speculated that splenic trypsin plays some roles in the immune response while in the stomach and intestine, trypsins are secreted and function as digestive enzymes together with the pancreas-derived trypsins (Koshikawa *et al.* 1998).

Figure 6 shows the effect of soybean trypsin inhibitor and TLCK on the activity bands observed on SDS-substrate gel electrophoresis. The major activity bands of all tuna species were strongly inhibited when the extract was treated with soybean trypsin inhibitor. However, TLCK exhibited varying levels of inhibition against these activity bands. From the results, splenic proteinase from skipjack seemed to be the most susceptible to TLCK, followed by those from yellowfin and tongol, respectively. Since TLCK

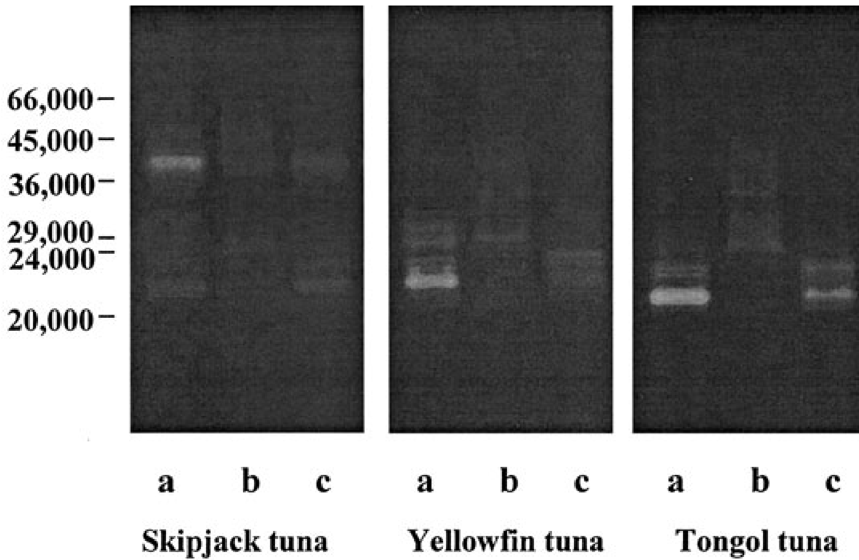

FIG. 6. ACTIVITY STAINING OF SPLEEN PROTEINASES FROM DIFFERENT TUNA SPECIES WITH AND WITHOUT PROTEINASE INHIBITORS
a: control; b: 1 g/l soybean trypsin inhibitor; c: 5 mM TLCK.

specifically attracted trypsin to the active site and it binds in a similar mode to that of a substrate (Wu *et al.* 1999), the results probably implied the differences in substrate/inhibitor binding characteristics of the major proteinases in splenic extracts of the three tuna species.

The major splenic proteinase from each tuna species will be further purified and characterized.

CONCLUSION

Tuna splenic extract contained heat-activated alkaline serine proteinases as the major enzymes with optimum pH and temperature of 9.0 and 55C, respectively. However, spleen from different species contained different proteinases, both in terms of number and molecular weight. Splenic proteinases can be potential novel enzymes for future applications.

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