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29 kDa Trypsin from the Pyloric Ceca of Atlantic Bonito (*Sarda sarda*): Recovery and Characterization

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Trypsin from the pyloric ceca of Atlantic bonito (*Sarda sarda*) was purified and characterized with respect to its purity; molecular weight; sensitivity to temperature, pH, and inhibition; and N-terminal sequence. The purified trypsin had a molecular weight of 29 kDa as per sodium dodecyl sulfate polyacrylamide gel electrophoresis, and optimal activity was observed at pH 9 and 65 °C with BAPNA as a substrate. The enzyme was stable to heat treatment up to 50 °C and within the pH range of 7−12. It was stabilized by calcium ions, but its activity was strongly inhibited by soybean trypsin inhibitor, *N*-*p*-tosyl-L-lysine chloromethyl ketone, and phenyl methyl sulfonyl fluoride. The enzyme exhibited a progressive decrease in activity with increasing NaCl concentration (0−30%). The N-terminal 20 amino acid residues of Atlantic bonito trypsin were determined as IVGGYECQAH-SQPWQPVLNS and were homologous with other trypsins.

KEYWORDS: Trypsin; digestive proteinase; viscera; purification; N-terminal amino acid sequence

INTRODUCTION

Atlantic bonito (*Sarda sarda*) is a member of the genus *Sarda* and is distributed along the tropical and temperate coasts of the Atlantic Ocean, Mediterranean Sea, and Black Sea (*1*). The species inhabits pelagic neritic waters at a depth of about 200 m. In Canada, Atlantic bonito is one of the economically important fish species and is commonly used for human consumption. It has also been exploited as a raw material for producing canned tuna. During processing, large quantities of waste are generated. Viscera are one of the most important byproducts of the Atlantic bonito industry and are recognized as a potential source of digestive enzymes, especially proteases (*2*, *3*). The use of alkaline proteases, especially trypsin, has increased remarkably since they are both stable and active under harsh conditions, such as temperatures of 50−60 °C, high pH values (7−12), and in the presence of surfactants or oxidizing against surfactants (*4*).

Trypsins (EC 3.4.21.4) are digestive enzymes that have many biomedical and industrial applications due to their narrow specificity that allows for more controlled proteolysis. Among all trypsins, fish trypsins are of immense interest because they exhibit a high catalytic activity at low reaction temperatures, making them very suitable for a number of biotechnological and food processing applications (*5*, *6*). Trypsins have been isolated and characterized thoroughly based on their physicochemical and enzymatic properties from several species of fish (e.g., skipjack tuna (*3*), yellowfin tuna (*6*), tongol tuna (*7*), chinook salmon (*8*), tambaqui (*9*), Monterey sardine (*10*), true sardine, and arabesque greenling (*11*)). However, trypsin from Atlantic bonito has not been studied. Thus, the objectives of this study were to purify and to characterize this very useful enzyme from Atlantic bonito pyloric ceca.

MATERIALS AND METHODS

Chemicals. Sodium chloride and tris-(hydroxymethyl) aminomethane were obtained from Fisher Scientific (Fairlawn, NJ). N^{α}-*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME), *N*-*p*-tosyl-l-lysine chloromethyl ketone (TLCK), *N*-tosyl-l-phenylalanine chloromethyl ketone (TPCK), β-mercaptoethanol (βME), 1-(l-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), benzoyl-dl-arginine-*p*-nitroanilide (BAPNA), Brij 35, bovine serum albumin (BSA), cyanogen bromide

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(CNBr)-activated Sepharose 4B, ethylenediaminetetraacetic acid (EDTA), Folin−Ciocalteu's phenol reagent, pepstatin A, phenyl methyl sulfonyl fluoride (PMSF), and soybean trypsin inhibitor (SBTI) were procured from Sigma-Aldrich Chemical Co. (St. Louis, MO). *N*,*N*,*N*′,*N*′-Tetramethyl ethylene diamine (TEMED), Coomassie Blue R-250, and sodium dodecyl sulfate (SDS) were purchased from Bio-Rad Laboratories (Mississauga, ON, Canada).

Sample Preparation. Atlantic bonito (*S. sarda*) samples were purchased from a local market (Waldman Plus, Montreal). The fish were stored in ice with a fish/ice ratio of 1:2 (w/w) and transported to the laboratory within 30 min of purchase. The fish were eviscerated, and the pyloric ceca were excised and powdered with liquid nitrogen in a waring blender. The powder was defatted with acetone according to the method of Klomklao et al. (*6*) and stored at −20 °C until use.

Preparation of Crude Extract. Defatted pyloric ceca powder was stirred continuously in the extraction buffer (EB, i.e., 0.05 M tris-HCl, pH 7.8 containing 0.02 M $CaCl_2$) at a ratio of 1:9 (w/v) at 4 °C for 3 h. The homogenate was centrifuged for 30 min at 4 °C at 10 000*g* using a Beckman J2−21 refrigerated centrifuge (Beckman Coulter, Inc., Fullerton, CA) to remove insoluble material, and the supernatant thus obtained was designated as the crude enzyme extract.

Trypsin Purification. All purification steps were carried out at 4 ± 1 °C in a cold room. During purification, the protein concentration was measured at 280 nm, and the trypsin activity was assayed with BAPNA as a substrate (*12*, *13*).

The crude enzyme extract was made up to 0.2% with Brij 35 and held overnight prior to centrifugation at 10 000*g* for 30 min. The supernatant obtained was fractionated with solid ammonium sulfate, and the fraction precipitating between 40−60% saturation was collected by centrifugation at 10 000*g* for 30 min to recover the pellet that was subsequently dissolved in 15 mL of EB and dialyzed against 20 volumes of EB overnight with three changes of the dialysis buffer. The dialysate was treated with 3 times its volume of cold acetone (−20 °C) and left to stand for 3 h at −20 °C before centrifugation at 10 000*g* for 30 min. The acetone pellet was then dissolved in 10 mL of EB to produce the acetone fraction for the affinity chromatography step.

The SBTI-Sepharose 4B affinity matrix was prepared and packed in a borosilicate column as per the method of Simpson and Haard (*2*). The acetone fraction was applied onto the SBTI-Sepharose 4B column (1 cm × 10 cm) that had been pre-equilibrated with two bed volumes of EB. The column was then washed thoroughly with the same buffer at a flow rate of 0.5 mL/min until A_{280} was less than 0.005. The elution of the bound trypsin was accomplished with 5 mM HCl at a flow rate of 1 mL/min, and fractions of 1 mL were collected dropwise and directly into 2 mL of EB to neutralize the acid and adjust the pH to ∼7.8. The fractions with BAPNA activity were pooled and simultaneously concentrated and desalted by centrifugation using 10 kDa MWCO Amicon Ultra centrifugal filter devices (Millipore Corporation, Billerica, MA). The concentrated sample was then loaded onto a Sephadex G-75 (1.5 cm × 75 cm) column previously equilibrated with approximately two bed volumes of EB at a flow rate of 0.1 mL/min. Fractions of 3 mL/tube were collected, and those with BAPNA activity were pooled and used for the study as described in this paper.

Protein Determination. Protein concentrations of the samples were determined by the method of Lowry et al. (*14*) using bovine serum albumin as a standard.

Trypsin Activity Assay. Amidase Activity Assay. Trypsin amidase activity was assayed with BAPNA as a substrate using a modified version of the method of Erlanger et al. (*12*), as described by Klomklao et al. (*13*). For the enzyme activity assay, 2.8 mL of the substrate (1 mM BAPNA in 0.05 M tris-HCl buffer, pH 8.2 containing 0.02 M $CaCl_2$) were mixed with 200 μL of the enzyme solution, and the release of *p*-nitroaniline from BAPNA was measured at 410 nm using a Hitachi U 2000 spectrophotometer (Tokyo, Japan). One BAPNA unit of activity was defined as $\Delta A_{410nm/min} \times 3 \times 1000/8800$, where 8800 is the extinction coefficient of *p*-nitroaniline at 410 nm and 3 is the total volume of the reaction mixture (*12*).

Esterase Activity Assay. The esterase activity of trypsin was determined using TAME as the substrate based on the method of Hummel (*15*) with slight modifications. The reaction mixture comprised 2.8 mL of the substrate (1 mM TAME in 0.05 M tris-HCl buffer, pH 8.2 containing 0.02 M $CaCl_2$) and 200 μL of the enzyme. The reaction mixture was thoroughly mixed, and the release of tosyl arginine was measured at 247 nm with a Hitachi U 2000 spectrophotometer (Tokyo, Japan). One TAME unit of activity was defined as $\Delta A_{247nm/min} \times 3 \times 1000/540$, where 540 is the extinction coefficient of tosyl arginine at 247 nm (*15*).

Table 1. Purification of Trypsin from Pyloric Ceca of Atlantic Bonito

purification steps	total activity (units)[a]	total protein (mg)	specific activity (units/mg of protein)	purification (-fold)	yield (%)
crude extract	85.90	1289.5	0.067	1	100
Brij 35	66.87	913.3	0.073	1.1	77.8
$(NH_4)_2SO_4$ (40–60%)	23.93	127.2	0.188	2.8	27.9
acetone precipitation	23.89	81.2	0.294	4.4	27.8
SBTI-Sepharose 4B	17.75	11.8	1.504	22.4	20.7
Sephadex G-75	10.2	3.2	3.188	47.6	11.9

[a] Trypsin activity was assayed at pH 8.2 and 25 °C using BAPNA as a substrate.

pH Optimum and Stability. Trypsin activity was assayed within the pH range of 4.0−12.0 by preparing the BAPNA substrate in various buffer solutions (i.e., 0.1 M citrate-NaOH buffer for pH 4.0−6.0; 0.1 M tris-HCl buffer for pH 7.0−9.5; and 0.1 M glycine-NaOH for pH 10.0−12.0) prior to addition of the enzyme at 25 °C. The effect of pH on enzyme stability was evaluated by measuring the residual enzyme activity after incubation of the enzyme at various pH values for 30 min at 25 °C prior to addition to the substrate (1 mM BAPNA in 0.05 M tris-HCl buffer, pH 8.2 containing 0.02 M $CaCl_2$). The compositions of the buffers used for the pH stability study were the same as described previously.

Temperature Optimum and Thermal Stability. Enzyme activity was assayed at different temperatures ranging from 20 to 80 °C using BAPNA as a substrate. The assay was conducted at pH 8.2 using 0.05 M tris-HCl buffer containing 0.02 M $CaCl_2$. For thermal stability, the enzyme was incubated at different temperatures (20, 30, 40, 50, 60, 70, and 80 °C) for 15 min in a temperature controlled water bath (Precision Scientific Shaking Water bath 25, Chicago, IL). Thereafter, the heat treated samples were rapidly cooled in an ice bath, and residual activity was assayed using BAPNA as a substrate at pH 8.2 and 25 °C, as previously described.

Effect of $CaCl_2$ on Thermal Stability. The purified trypsin was incubated in the presence of 2 mM EDTA or with 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 ice−water and assayed for remaining activity, using BAPNA as a substrate.

Effect of NaCl. Trypsin activity was assayed in the presence of NaCl at various concentrations (0−30% (w/v)). The residual enzyme activity was determined at 25 °C and pH 8.2, using TAME as a substrate.

Effect of Inhibitors. Inhibition of bonito trypsin by different inhibitors was measured according to the method of Klomklao et al. (*6*). The enzyme solution was incubated with an equal volume of the inhibitor solution. The final concentrations of the inhibition solution are specified in **Table 2**. The mixture was left to stand at room temperature (26−28 °C) for 30 min. Thereafter, the remaining activity was determined at 25 °C and pH 8.2, using BAPNA as a substrate, and percent inhibition was calculated.

Electrophoresis. SDS-PAGE was performed according to the method of Laemmli (*16*). 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 0.3% bromophenol blue) and boiled for 3 min. The samples (∼15 μg) were loaded onto the gels made of 4% stacking and 12% separating gels and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Protean II Cell apparatus (Bio-Rad, Mississauga, ON, Canada). After electrophoresis, the gels were stained with 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 7.5% acetic acid for 3 h and destained with a 50% methanol and 7.5% acetic acid solution.

Table 2. Effect of Various Inhibitors on Activity of Purified Trypsin from Atlantic Bonito Pyloric Ceca[a]

inhibitors	concentration	% inhibition[b]
Control		0
E-64	0.1 mM	6.09 ± 0.3
SBTI	1.0 mg/mL	84.2 ± 70.8
TLCK	5 mM	100
TPCK	5 mM	5.34 ± 0.1
PMSF	1 mM	89.5 ± 10.4
pepstatin A	0.01 mM	0
EDTA	2 mM	6.85 ± 1.8

[a] Each enzyme solution was incubated with the same volume of inhibitor solution at 25 °C for 20 min, prior to measuring residual activity using BAPNA as a substrate. [b] Mean ± SD from triplicate determinations.

Native-PAGE was performed using 12% separating gels in a similar manner, except that the sample was not heated and SDS and the reducing agent were excluded from the sample buffer.

Determination of N-Terminal Amino Acid Sequence. The purified trypsin was subjected to SDS-PAGE under reducing conditions and electrophoretically transferred to a polyvinylidenedifluoride (PVDF) membrane. After the membrane was briefly stained by Coomassie Brilliant Blue, it was sent to the Sheldon Biotechnology Laboratory (McGill University) for analysis. For the N-terminal analysis, the band of protein was applied to a protein sequencer, Procise 492 (Perkin-Elmer, Foster, CA) by injection on a Spheri-5 PTH column (220 mm × 2.1 mm) linked to an HPLC system. The system was calibrated using a PTH residue standard kit (Sigma-Aldrich, St. Louis, MO) containing all the 20 common amino acids.

RESULTS AND DISCUSSION

Trypsin Purification. Trypsin from Atlantic bonito pyloric ceca was extracted and purified successively using 40−60% ammonium sulfate precipitation, acetone precipitation, affinity chromatography, and gel filtration chromatography (**Table 1**). An increase in purity of 2.8-fold was obtained by ammonium sulfate precipitation (40−60%). Kurtovic et al. (*8*) obtained an increase in purity of trypsin from chinook salmon pyloric ceca by 4.0-fold with 40−60% ammonium sulfate precipitation. The ammonium sulfate fraction was further fractionated with cold acetone. A large amount of proteins was removed with some loss in enzyme activity, leading to 4.4-fold purity. Similar to Kurtovic et al. (*8*), affinity chromatography on SBTI-Sepharose 4B was highly effective in concentrating trypsin activity (**Figure 1a**). From the results, the specific activity increased approximately 5 times, as compared to that obtained for the acetone fraction (**Table 1**). To refine the affinity fractions, the active fractions were subjected to gel filtration on a Sephadex G-75 column (**Figure 1b**). Purification of 47.6-fold with a yield of 11.9% was obtained. In a previous study, the use of gel filtration on Sephadex G-50 in the final step of the purification process of trypsin from tongol tuna spleen led to an increase in trypsin activity by 401.1-fold (*7*). The differences in fold purification between the last study (*7*) and the present study may relate to different source materials as well as the different purification techniques used.

Electrophoresis. Purity of the isolated trypsin was evaluated by using native-PAGE. The trypsin migrated as a single protein band in native-PAGE (**Figure 2a**), confirming the homogeneity of the enzyme preparation. The enzyme also appeared as a single band on SDS-PAGE with a molecular weight of 29 kDa (**Figure 2b**). Different molecular weights have been reported for purifed trypsins from fish digestive organs, depending on the fish species. The molecular weights of two trypsins enzymes (A and B) from carp hepatopancreas were estimated to be approximately

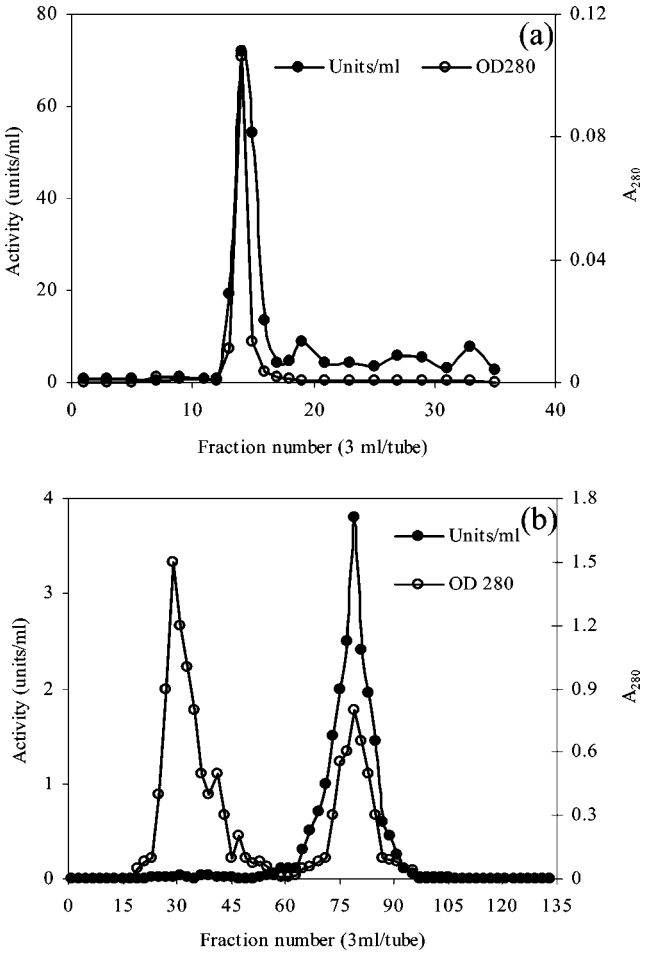

Figure 1. Purification of trypsin from Atlantic bonito pyloric ceca. (**a**) Elution profile of trypsin on SBTI-Sepharose 4B column and (**b**) elution profile of trypsin on Sephadex G-75 column.

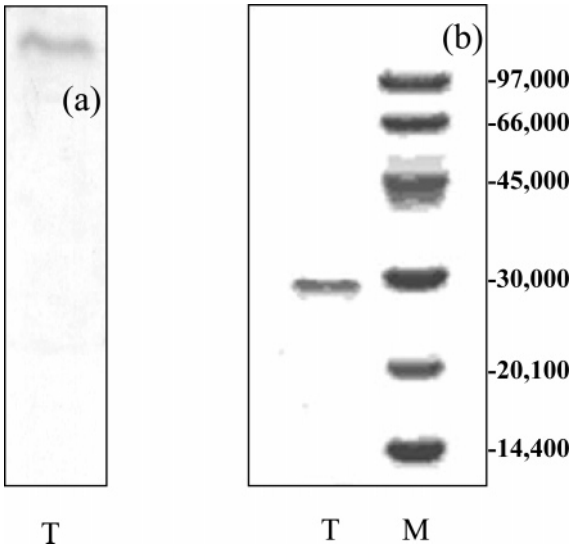

Figure 2. Electrophoretic pattern of trypsin from Atlantic bonito pyloric ceca determined by (**a**) native-PAGE and (**b**) SDS-PAGE. T = purified trypsin and M = low molecular weight standards.

28.5 and 28 kDa, respectively (*17*). A trypsin from pyloric ceca of Monterey sardine (*Sardinops sagax caerulea*) had a molecular weight of 25 kDa as estimated by SDS-PAGE (*10*). Kim et al. (*18*) reported that the molecular weights of four trypsin enzymes from hepatopancreas of crayfish were approximately 23.8, 27.9, 24.8, and 31.4 kDa, respectively, estimated by gel filtration.

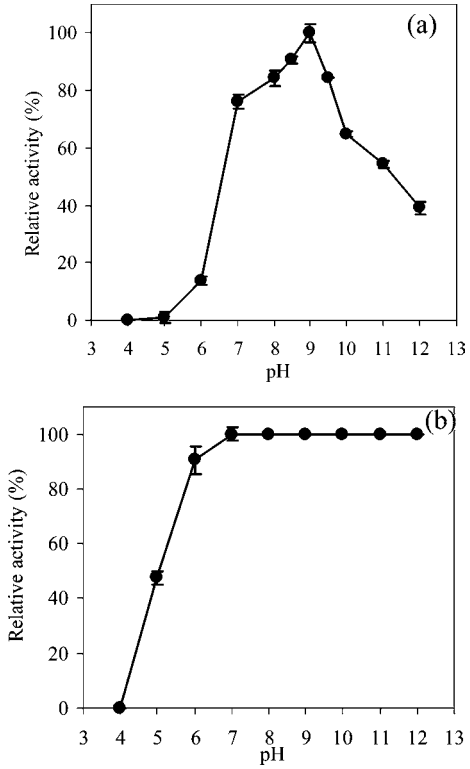

Figure 3. (**a**) pH activity profile and (**b**) pH stability of purified trypsin from Atlantic bonito pyloric ceca. Values are expressed as means ± SD from triplicate determinations.

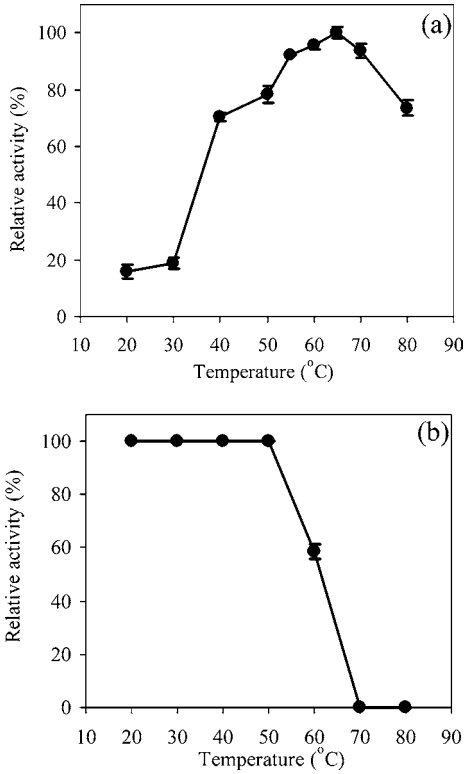

Figure 4. (**a**) Temperature activity profile and (**b**) thermal stability of purified trypsin from Atlantic bonito pyloric ceca. Values are expressed as means ± SD from triplicate determinations.

pH Optimum and Stability. Trypsin from Atlantic bonito pyloric ceca exhibited a maximal activity at pH 9.0 (**Figure 3a**). This pH optimum value was similar to those previously reported for other fish trypsins such as Monterey sardine trypsin (pH 8.0; ref *10*) and yellowfin and skipjack tuna trypsins (pH 9.0; refs *3* and *6*). For pH stability, Atlantic bonito trypsin was relatively more stable in the pH range from 7 to 12 but very unstable below pH 7 (**Figure 3b**). Most marine animal trypsins characterized thus far are known to be highly unstable at acidic pHs but are more stable at a neutral to slightly alkaline pH (*6*−*7*, *11*).

Temperature Optimum and Thermal Stability. The optimal temperature of Atlantic bonito trypsin for the hydrolysis of BAPNA was 65 °C (**Figure 4a**). Above 65 °C, the enzyme activity decreased sharply. The loss in activity was presumably caused by thermal denaturation of the enzyme. Trypsin from chinook salmon had an optimum temperature of 60 °C for the hydrolysis of BAPNA (*8*). Klomklao et al. (*7*) reported that the maximal activity of trypsin from tongol tuna spleen was 65 °C. However, the optimal temperature of the purified Atlantic bonito trypsin was higher than those of Greenland cod, sardine, and anchovy, which ranged from 40 to 45 °C (*2*, *19*, *20*). The differences in optimal temperatures may relate to the differences in the habitat temperatures of the animals, as well as differences in substrates or assay conditions used in the different studies. For thermal stability, Atlantic bonito trypsin was quite stable from 20 to 50 °C and retained approximately 60% of its original activity after incubation at 60 °C for 15 min (**Figure 4b**). However, the trypsin was completely inactivated at 70 °C. At high temperatures, the enzyme most likely underwent denaturation and lost its activity (*6*). This finding is consistent with the observation made by De-Vecchi and Coppes (*21*) that the thermal stability of fish trypsin varies with species as well as with experimental conditions.

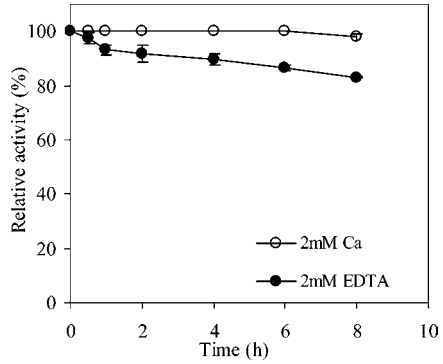

Figure 5. Effect of calcium ions and EDTA on the stability of purified trypsin from Atlantic bonito pyloric ceca. The stability was tested by incubating the enzyme at 40 °C for different times. Values are expressed as means ± SD from triplicate determinations.

Effect of Ca^{2+} on Thermal Stability. A continuous decrease in activity was observed in the presence of 2 mM EDTA with increasing the incubation time (**Figure 5**). On the other hand, no marked changes in activity were found in the presence of 2 mM $CaCl_2$ after incubation for 8 h. Fish trypsin was stabilized by calcium ions similar to mammalian trypsins (*11*). Two Ca^{2+} binding sites are present in bovine trypsinogen. The primary site with a higher affinity for calcium ions is common in trypsinogen and trypsin, and the secondary site is only found in zymogen (*11*). The occupancy of the primary site by Ca^{2+} stabilizes the proteins against thermal denaturation or autolysis. From the result, purified trypsin was stabilized by Ca^{2+}, suggesting that trypsin from the pyloric ceca of Atlantic bonito possibly possesses a primary calcium binding site. Thus, the stability of trypsin from Atlantic bonito pyloric ceca can be maintained by the addition of Ca^{2+} ions.

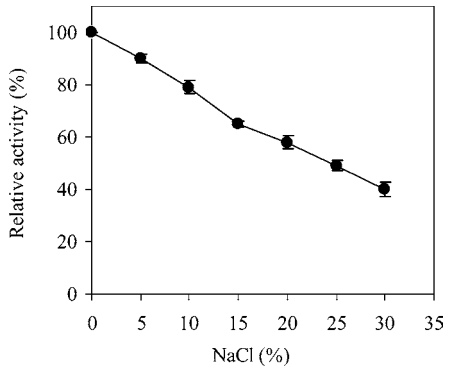

Figure 6. Effect of NaCl concentrations on activities of purified trypsin from Atlantic bonito pyloric ceca. Values are expressed as means ± SD from triplicate determinations.

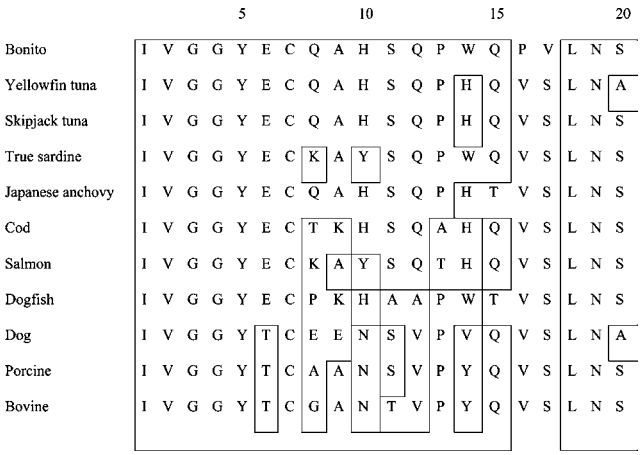

Figure 7. Comparison of N-terminal amino acid sequence of purified trypsin from Atlantic bonito pyloric ceca with other enzymes: yellowfin tuna (*6*), skipjack tuna (*3*), true sardine (*11*), Japanese anchovy (*23*), cod (*24*), salmon (*25*), dogfish (*26*), dog (*27*), porcine (*28*), and bovine (*29*).

Effect of NaCl. A continuous decrease in trypsin activity was observed as the NaCl concentration increased (**Figure 6**). The decrease in activity could be described by the salting out phenomenon. An increase in the ionic strength causes a reduction in enzyme activity by an enhanced hydrophobic−hydrophobic interaction between protein chains and competition for water by the ionic salts, leading to enzyme precipitation (*6*). From this result, the residual activity of trypsin at 30% NaCl was approximately 40%. Generally, fish sauce is produced by adding salt to the fish with ratios of fish/salt of 2:1 or 3:1. The salt content in commercial fish sauce produced in Thailand was 25%, and the pH of fish sauce decreased from neutral pH (∼7) to acidic pH (∼5) (*22*). Thus, the trypsin from Atlantic bonito pyloric ceca may be used to facilitate the hydrolysis of proteins in high salt fermented fish products such as fish sauce.

Effect of Inhibitors. Trypsin from the pyloric ceca of Atlantic bonito was inhibited by the well-known trypsin inhibitors investigated, namely, SBTI and TLCK, and also by PMSF (**Table 2**). On the other hand, E-64, TPCK, pepstatin A, and EDTA (that are specific for cysteine proteinase, chymotrypsin, aspartic acid, and metallo proteinase, respectively) had very little or no effect on bonito trypsin activity. The results confirm that the Atlantic bonito enzyme belongs to the trypsin family of enzymes. Previous studies have shown that two trypsin isoforms from yellowfin tuna spleen were inhibited by SBTI and TLCK (*6*) and that a trypsin-like enzyme from tambaqui pyloric ceca was also inhibited by PMSF, benzamidine, and TLCK (*9*). Kurtovic et al. (*8*) reported that the chinook salmon trypsin was inhibited by PMSF, SBTI, and benzamidine. Also, two kinds of trypsin from carp hepatopancreas inhibited by SBTI, benzamidine, and TLCK were reported (*17*).

N-Terminal Sequence. Figure 7 shows the N-terminal 20 amino acid sequence of Atlantic bonito trypsin aligned with the amino acid sequences of other trypsins from marine animals and mammals. The Atlantic bonito trypsin N-terminal sequence was found to be IVGGYECQAHSQPWQPVLNS. The results indicate that the N-terminal region of the enzyme was unblocked. The N-terminal sequence of Atlantic bonito pyloric ceca trypsin showed high similarities with other trypsins, especially with those of skipjack and yellowfin tuna trypsins, suggesting the possibility that they were genetically evolved from a common ancestor. Generally, the N-terminal region of trypsin, especially from the first to the seventh residues, demonstrates high homology (*17*). However, all fish trypsins had a charged Glu residue at position 6, whereas Thr is most common in mammalian pancreatic trypsin (**Figure 7**). The N-terminal amino acid sequence also lends credence to the notion that the Atlantic bonito enzyme also belongs to the trypsin family of enzymes.

In summary, based on molecular weight, substrate specificity, inhibitor study, and N-terminal sequencing, the purified enzyme in the pyloric ceca from Atlantic bonito was classified as a true trypsin. The enzymatic characteristics were essentially consistent with those of trypsin from other species.

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