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Trypsin from the Pyloric Ceca of Pectoral Rattail (*Coryphaenoides pectoralis*): Purification and Characterization

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Trypsin from the pyloric ceca of pectoral rattail (*Coryphaenoides pectoralis*) was purified and characterized. Purification was carried out by ammonium sulfate precipitation, followed by column chromatographies on Sephacryl S-200, DEAE-cellulose and Sephadex G-50. The enzyme was purified 89-fold with a yield of 2.2%. Purified trypsin had an apparent molecular weight of 24 kDa when analyzed using SDS−PAGE and size exclusion chromatography. Optimal profiles of pH and temperature of the enzyme were 8.5 and 45 °C, respectively, using N^{α}-*p*-tosyl-L-arginine methyl ester hydrochloride as a substrate. It was stable in a wide pH range of 6−11 but unstable at a temperature greater than 40 °C. Trypsin was stabilized by calcium ion. The activity of purified trypsin was effectively inhibited by soybean trypsin inhibitor and TLCK and was partially inhibited by EDTA. Activity continuously decreased with increasing NaCl concentration (0−30%). The kinetic trypsin constants K_m and K_{cat} were 0.15 mM and 210 s^{-1}, respectively, while the catalytic efficiency (K_{cat}/K_m) was 1400 s^{-1} mM^{-1}. The N-terminal amino acid sequence of trypsin was determined to be 12 residues (IVGGYECQEHSQ), and the sequence showed high homology to other fish trypsins.

KEYWORDS: Trypsin; purification; isolation; pyloric ceca; N-terminal amino acid sequence

INTRODUCTION

Cold-adapted organisms may possess enzymatic adaptabilities in different ways (*1*). The reduced enzymatic rates as a consequence of low temperatures may be compensated by increased enzyme production, change of the type of enzyme present in the system, or adaptability of preexisting enzymes (*1*). In the last case it would be expected that natural selection through evolution would favor enzymes with increased catalytic potential at low temperatures. The observed higher catalytic efficiency of cold-adapted enzymes was due to a more flexible tertiary structure, which enables the enzymes to lower the activation energy barrier during catalysis (*1*). Among this group of enzymes, the cold-adapted fish trypsins are of immense interest because they exhibit higher catalytic activity than their mammalian counterparts and preserve a high degree of activity at lower temperatures, making them more suitable for a number of biotechnological and food processing applications (*2*−*4*).

Trypsins (EC 3.4.21.4), a member of a large family of serine proteinases, specifically hydrolyze proteins and peptides at the carboxyl side of arginine and lysine residues and play major roles in biological process including digestion and activation of zymogens of chymotrypsin and other enzymes (*5*, *6*). Trypsins have been isolated and characterized thoroughly on the basis of their physiochemical and enzymatic properties in a wide range of cold water as well as warm water fish such as crayfish (*Procambarus clarkii*) (*7*), mackerel (*Scomber japonicus*) (*8*), crawfish (*Procambarus clarkii*) (*9*), tambaqui (*Colossoma macropomum*) (*10*), and true sardine (*Sardinops melanostictus*) and arabesque greenling (*Pleuroprammus azonus*) (*11*). Recently, Klomklao et al. (*5*) purified and characterized three trypsin isoforms from skipjack tuna (*Katsuwonus pelamis*) spleen.

Pectoral rattail (*Coryphaenoides pectoralis*) is one of fish species adapted to temperatures below 4 °C and found in 600−1500 m depth around the northern parts of Pacific Ocean. Thus, this fish species may provide a source of specific cold adapted enzymes. On the basis of our preliminary study, the pyloric ceca from pectoral rattail showed high trypsin activity. However, the information regarding the molecular and biochemical characteristic of trypsin from the pyloric ceca of pectoral rattail has not been reported. The aims of this study were to purify and to characterize the biochemical properties of trypsin from the pyloric ceca of pectoral rattail.

MATERIALS AND METHODS

Chemicals. Ethylenediaminetetraacetic acid (EDTA), pepstatin A, soybean trypsin inhibitor, iodoacetic acid, *N*-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 1-(L-*trans*-epoxysuccinylleucylamino)-4-guanidinobutane (E-64), *N*-ethylmaleimide, β-mercaptoethanol (βME), and bovine serum albumin were procured from Sigma Chemical Co. (St. Louis, MO). Sephacryl S-200 and

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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*′-tetramethylethylenediamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA).

Fish Sample Preparation. Pectoral rattail (*C. pectoralis*) was harvested along the Hakodate coast, Japan, stored in ice, and off-loaded approximately 24−26 h after catching. Fish were then transported in ice with a fish/ice ratio of 1:2 (w/w) to the Laboratory. Viscera were then excised and separated into individual organs. Only pyloric ceca was collected, immediately frozen, and stored at −20 °C until used.

Preparation of Crude Enzyme Extract. Frozen pyloric ceca 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 in three volumes of acetone at −20 °C for 30 min according to the method of Klomklao et al. (*12*). The homogenate was filtrated in vacuo on Whatman no. 4 filter paper. The residue obtained was then homogenized in two volumes of acetone at −20 °C for 30 min, and then the residue was air-dried at room temperature until dry and free of acetone odor.

To prepare the crude extract, the pyloric ceca 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 20 min at 4 °C at 20000*g* (H-200, Kokusan, Tokyo, Japan) to remove the tissue debris, and then the supernatant was lyophilized. Before use, the lyophilized sample (10 g) was dissolved with 50 mL of cold distilled water (4 °C) and referred to as "crude extract".

Trypsin Purification. All purification steps were carried out at 4 °C. Crude extract was subjected to ammonium sulfate precipitation at 30−70% saturation and allowed to stand for 2 h. The precipitate was collected by centrifugation at 25000*g* for 20 min. The pellet obtained was dissolved in a minimal volume of SB and dialyzed with 10 volumes of SB with three changes overnight. The dialysate was then applied to a Sephacryl S-200 column (3.9 cm × 64 cm), which was equilibrated with SB. The separation was conducted using the SB at a flow rate of 0.5 mL/min, and 5 mL fractions were collected. The fractions containing trypsin were pooled. Pooled Sephacryl S-200 column fractions were dialyzed against SB for 10−12 h. The sample was then chromatographed on DEAE-cellulose (Whatman, England) column (2.2 cm × 18 cm) equilibrated with SB. The sample was loaded onto the column at a flow rate of 0.5 mL/min. The column was washed with SB until A_{280} was below 0.05 and then eluted with a linear gradient of 0−0.25 M NaCl in SB at a flow rate of 0.5 mL/min. Fractions of 5 mL were collected, and those with TAME activity were pooled. Pooled fractions were dialyzed with SB with 10−12 h and then concentrated by lyophilization. After the first DEAE-cellulose chromatography, trypsin fractions were rechromatographed on the same column equilibrated with SB. The elution was performed with stepwise process using SB containing different NaCl concentrations: 0, 0.1, and 0.2 M, respectively, at a flow rate of 0.5 mL/min. Fractions of 5 mL were collected, and those with high trypsin activity were combined. Pooled fractions with TAME activity from second DEAE-cellulose column were dialyzed against SB for 10−12 h. The dialysate was lyophilized and then dissolved in distilled water prior to Sephadex G-50 column (3.9 cm × 64 cm) chromatography. Sample was loaded to the column and then eluted with the same buffer at a flow rate of 0.5 mL/min. Fractions of 5 mL were collected, and fractions with TAME activity were pooled and used for further study.

During purification, protein concentration was measured at 280 nm and trypsin activity was determined using TAME as a substrate.

Trypsin Activity Assay. Trypsin activity was measured by the method of Hummel (*13*) as modified by Klomklao et al. (*14*) 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*-tosylarginine 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.

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 at pH 8.0.

pH and Thermal Stability. The effect of pH on enzyme stability was evaluated by measuring the residual activity after incubation at various pH values for 30 min at 30 °C. Different buffers used were mentioned above. 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.

Determination of Molecular Weight. The molecular weight of purified trypsin was determined using a size exclusion chromatography on Sephacryl S-200 column. The trypsin separated by size exclusion chromatography was estimated for its molecular weight by plotting the 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).

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 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.

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.

Effect of Inhibitors. The effect of inhibitors on trypsin activity was determined according to the method of Klomklao et al. (*15*) 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.

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 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 manner similar to that for SDS−PAGE except that the sample was not heated and SDS and reducing agent were left out.

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) by injection on Spheri-5PTH 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.

Kinetic Studies. The activity was assayed with different final concentrations of TAME ranging from 0.01 to 0.10 mM. The final enzyme

Table 1. Purification of Trypsin from the Pyloric Ceca of Pectoral Rattail

purification steps	total activity (units)[a]	total protein (mg)	specific activity (units mg^{-1} protein)	purity (fold)	yield (%)
crude extract	402.0	3415.0	0.1	1	100
$(NH_4)_2SO_4$ (30−70%)	354.8	548.6	0.6	6	88.3
Sephacryl S-200	133.2	100.9	1.3	13	33.1
first DEAE-cellulose	41.2	8.1	5.1	51	10.2
second DEAE-cellulose	15.6	2.4	6.5	65	3.9
Sephadex G-50	8.9	1.0	8.9	89	2.2

[a] One unit of activity was defined as the amount causing an increase of 1.0 in absorbance at 247 nm per min. Trypsin activity was assayed at pH 8.0, 30 °C for 20 min using TAME as a substrate.

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 (*17*). Values of turnover number (K_{cat}) were calculated from the following equation: $V_{max}/[E] = K_{cat}$, where [E] is the active enzyme concentration and V_{max} is the maximal velocity.

Protein Determination. Protein concentration was measured by the method of Lowry et al. (*18*) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Trypsin Purification. A summary of trypsin purification is presented in **Table 1**. An increase in purity of 6.0-fold was obtained when 30−70% ammonium sulfate was used. The ammonium sulfate precipitation process was able to fractionate the enzyme of interest. Moreover, ammonium sulfate precipitation was normally introduced as an initial step for removal of other proteins in trypsin purification (*19*). Castillo-Yanez et al. (*20*) reported that ammonium sulfate precipitation of trypsin from the pyloric ceca of Montery sardine at 30−70% resulted in the increase in specific activity by 1.3-fold.

Ammonium sulfate fraction was further purified by gel filtration on Sephacryl S-200. Purification fold of 13.0 with a yield of 33.1% was observed after this step. Gel filtration was used to remove other proteins in true sardine viscera trypsin fraction, leading to the higher purity of trypsin (*11*).

Pooled active Sephacryl S-200 fractions were further purified using DEAE-cellulose column. After loading and washing, the column was eluted by using a 0−0.2 M NaCl linear gradient. Only one activity peak was found. A 51-fold increase in purity with a yield of 10.2% was observed. To refine the pooled fraction obtained from previous step, pooled active fractions were subjected to a second DEAE-cellulose column chromatography. After this step, a large amount of contaminated proteins was removed, resulting in a substantial increase in purification fold. Purity was increased by 65 with yields of 3.9%. Yoshinaka et al. (*21*) also chromatographed the trypsin from the eel viscera on an anion exchanger, DEAE-cellulose, twice, and two anionic trypsins were obtained with 22-fold purity. However, it was not completely separated from other contaminating proteins. Therefore, trypsin containing fractions were rechromatographed on Sephadex G-50.

When second DEAE-cellulose sample with trypsin activity was subjected to gel filtration on Sephadex G-50, a single peak was obtained. Purification fold of 89 with a yield of 2.2% was observed. Bezerra et al. (*19*) found that the use of gel filtration on Sephadex G-75 in the final step of the purification process of trypsin from the intestine of Nile tilapia led to an increase in trypsin activity by 21.68-fold.

Purity and Molecular Weight. Purity of the purified trypsin was examined via native gel electrophoresis. The enzyme was observed to migrate as a single band on native PAGE (**Figure 1a**), suggesting the homogeneity of the enzyme. For SDS−PAGE, purified trypsin migrated as a single protein band at 24 kDa (**Figure 1b**). Also, the molecular mass of the enzyme was estimated to be 24 kDa by Sephacryl S-200 gel filtration (data not shown). These results demonstrated that purified trypsin from pectoral rattail pyloric ceca is a monomeric protein with the molecular mass of 24 kDa. Generally, fish trypsin has been reported to have a molecular weight in the range of 23−28 kDa. Purified alkaline protease from the viscera of bolti fish had a molecular weight of 23 kDa (*22*). The molecular mass of trypsin from the pyloric ceca of arabesque greenling was estimated to be approximately 24 kDa by SDS−PAGE (*11*). However, a slightly higher molecular weight (28 kDa) was reported for trypsin of starfish (*23*). Cao et al. (*6*) reported that the molecular weights of two trypsins (A and B) from carp hepatopancreas were estimated to be 28.5 and 28 kDa, respectively.

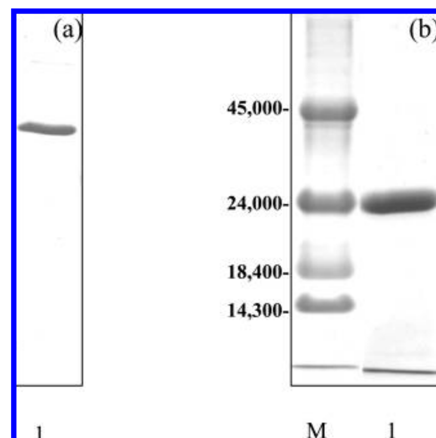

Figure 1. Protein pattern of purified trypsin from the pyloric ceca of pectoral rattail by native PAGE (**a**) and SDS−PAGE (**b**): M, molecular weight standards of egg albumin (45 kDa), bovine pancreatic trypsinogen (24 kDa), bovine-milk-betalactoglobulin (18.4 kDa), and egg white lysozyme (14.3 kDa); lane 1, purified trypsin.

Optimal pH and Temperature. Trypsin from the pyloric ceca from pectoral rattail exhibited a maximal activity at pH 8.5 (**Figure 2a**). The activity of purified trypsin sharply decreased above optimum pH because of the denaturation of enzyme. The decrease in activity was also found in acidic pH ranges. Under acidic and alkaline pHs, the changes in enzyme conformation were possibly caused by charge repulsion. At extreme acidic or alkaline pH, protein or enzyme unfolds because of a decrease in electrostatic bonds (*24*). The optimum pH value of purified trypsin was similar to that of trypsin isolated from Monterey sardine pyloric ceca, which had an optimum of pH 8.0 when BAPNA was used as a substrate (*20*). Bezerra et al. (*19*) found that trypsin-like enzyme from Nile tilapia intestine had optimal pH of 8.0 when azocasein was used.

The effect of various temperatures on trypsin activity is depicted in **Figure 2b**. Purified trypsin showed the highest activity

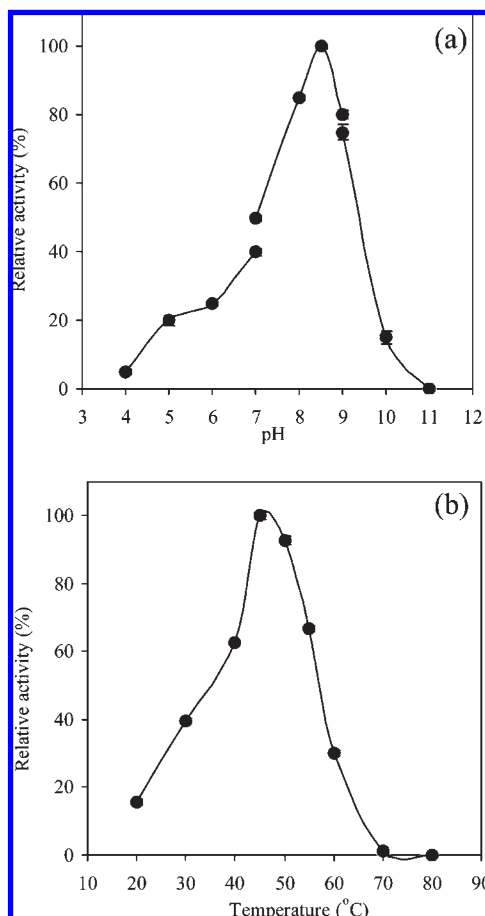

Figure 2. pH (**a**) and temperature (**b**) profiles of purified trypsin from the pyloric ceca of pectoral rattail. Bar indicates standard deviation from triplicate determination.

at 45 °C. A very sharp decrease in activity was observed above 50 °C. When assayed at 70 °C, no activity was found, presumably as a result of thermal inactivation. Enzymes were inactivated at high temperature, possibly because of the partial unfolding of the enzyme molecule. This result was similar to that of alkaline protease from the viscera of bolti fish, which had optimum temperatures of 45 °C (*22*). Heu et al. (*25*) also reported that trypsin from the viscera of anchovy had optimum temperature at 45 °C. However, the optimal temperature of the purified trypsin from the pyloric ceca of pectoral rattail is lower than that reported for trypsin from the pyloric ceca of bluefish, which had maximal activity at 55 °C (*14*). Bezerra et al. (*10*) reported that alkaline serine proteinase from tambaqui pyloric ceca exhibited optimal activity at 60 °C. The difference might be related to the difference in the temperatures of water where fish inhabited.

pH and Thermal Stability. The purified enzyme was stable over a wide pH range (6−11) (**Figure 3a**). However, at pH 5.0, the activity was slightly decreased and no activity was detected after incubation at pH 4.0. The stability of trypsin at a particular pH might be related to the net charge of the enzyme at that pH (*20*). Diminished stability at acidic pHs was found for trypsin from several fish species (*4*, *5*, *11*). From the result, the trypsin might undergo the denaturation under the acidic condition, where the conformational change took place and enzyme could not bind to the substrate properly.

The thermal stability of the purified trypsin was examined for 15 min in the temperature range of 20−80 °C (**Figure 3b**). The result showed that purified trypsin was stable up to 40 °C and slightly lost its stability at 50 °C. The purified trypsin was not

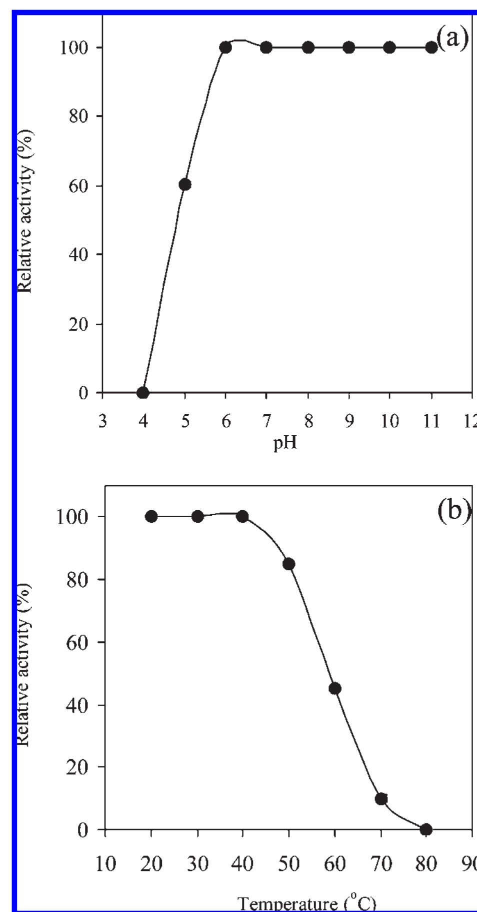

Figure 3. pH (**a**) and thermal (**b**) stability of purified trypsin from the pyloric ceca of pectoral rattail. Bar indicates standard deviation from triplicate determination.

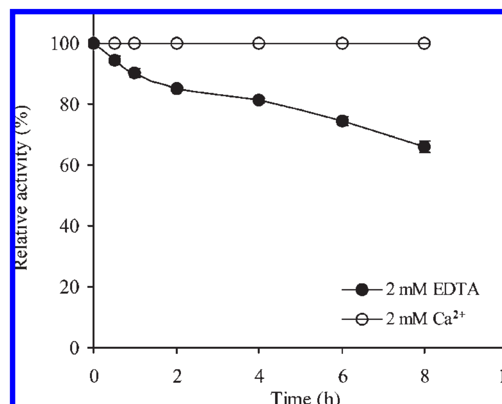

Figure 4. Effect of calcium ion and EDTA on the stability of purified trypsin from the pyloric ceca of pectoral rattail. The stability was tested at 40 °C for different times. Bar indicates standard deviation from triplicate determination.

stable at higher temperature. Approximately 45% of activity was retained after heating at 60 °C, but no trypsin activity was remained when heated to 80 °C. At high temperature, the enzyme possibly underwent denaturation and lost its activity. The result was in agreement with Castillo-Yanez et al. (*20*) who reported that trypsin from the pyloric ceca of Monterey sardine was stable up to 40 °C but unstable at temperatures higher than 40 °C. Trypsin from the viscera of anchovy was not stable at temperatures higher than 45 °C (*25*).

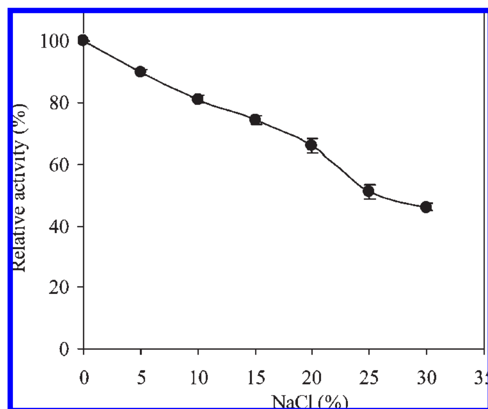

Figure 5. Effect of NaCl concentrations on the activity of purified trypsin from the pyloric ceca of pectoral rattail. Bar indicates standard deviation from triplicate determination.

Effect of Calcium Ions on Thermal Stability. The thermal stability of the purified trypsin from pectoral rattail pyloric ceca was highly dependent on the presence of calcium (**Figure 4**). Total activity was retained throughout 8 h of incubation at 40 °C in the presence of 2 mM calcium ion, while in the presence of 2 mM EDTA, the activity of purified trypsin decreased with increasing the time. These results indicated that pectoral rattail required calcium ion for its stability. Fish trypsin was stabilized by the calcium ion similarly to porcine pancreatic trypsin (*11*). Two calcium binding sites are presented in the 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 the zymogen (*4*, *5*, *11*). The occupancy of the primary calcium-binding site stabilizes the protein toward thermal denaturation or autolysis (*11*). From the result, it can be concluded that purified trypsin from pyloric ceca of pectoral rattail possesses a primary calcium-binding site as reported in trypsin from tongol tuna (*26*), skipjack tuna (*5*), yellowfin tuna (*4*), and true sardine (*11*). Therefore, the stability of trypsin from pectoral rattail can be maintained by the addition of calcium ion.

Effect of NaCl. The activity of purified trypsin decreased with increasing NaCl concentration (0−30%) (**Figure 5**). Remaining activity of the enzyme at 30% NaCl was approximately 48%. The activity loss might be due to the denaturation of trypsin caused by the "salting out" effect (*27*). NaCl at higher concentration possibly competed with enzyme in water binding, resulting in a stronger protein−protein interaction, which was possibly associated with precipitation (*24*). This led to a loss in activity in the presence of high NaCl concentration. Ishida et al. (*28*) reported that thermostable proteinase in salted anchovy muscle was still active and able to degrade myofibrillar protein in commercial salted fillets containing 16−17% NaCl. From the result, the activity of 50% remained at 20% NaCl. Thus, trypsin from the pyloric ceca of pectoral rattail may have potential in accelerating the fish sauce fermentation.

Effect of Inhibitors. To confirm the identity of the purified trypsin from pectoral rattail pyloric ceca, the effect of various protease inhibitors on its activity was determined and is shown in **Table 2**. Among all proteinase inhibitors, soybean trypsin inhibitor and TLCK exhibited the highest inhibitor (91−100%). Inhibitors for chymotrypsin (TPCK), aspartic (pepstatin A), and cysteine (E-64, *N*-ethylmaleimide, iodoacetic acid) proteinases had no inhibitory effect on trypsin activity. The inhibition pattern of purified trypsin was similar to trypsins from other fish species, such as anchovy and Monterey sardine (*20*, *25*). Bezerra et al. (*10*) also reported that trypsin-like enzyme from tambaqui pyloric ceca was inhibited by some trypsin inhibitors, such as PMSF, benzamidine, and TLCK. From the result, it was noted that approximately 10% inhibition was found with 2 mM EDTA, indicating that the enzyme required metal ions for its activity. EDTA possibly affected the structure of enzyme or active site conformation, leading to the reduced activity. Moreover, EDTA could chelate the ion required for activity of enzymes. This might lead to the less availability of these ions. The inhibitory results confirmed that purified enzyme was trypsin, which possibly required metal ions for its activity.

Table 2. Effect of Various Inhibitors on the Activity of Purified Trypsin from the Pyloric Ceca of Pectoral Rattail[a]

inhibitors	concentration	inhibition (%)[b]
control		0
E-64	0.1 mM	0
N-ethylmaleimide	1 mM	0
iodoacetic acid	1 mM	0
soybean trypsin inhibitor	1.0 g/L	100
TLCK	5 mM	91.1 ± 0.34
TPCK	5 mM	0
pepstatin A	1 mM	0
EDTA	2 mM	10.1 ± 0.03

[a] Each enzyme solution was incubated with the same volume of inhibitor at 25 °C for 15 min, and residual activity was analyzed using TAME as a substrate for 20 min at pH 8.0 and 30 °C. [b] Mean ± SD from triplicate determination.

Table 3. Kinetic Properties of Pectoral Rattail Pyloric Ceca Trypsin for the Hydrolysis of TAME[a]

enzyme	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} mM^{-1})
trypsin	0.15	210	1400

[a] K_m and K_{cat} values were determined using TAME as a substrate at pH 8.0 and 30°C. The final enzyme concentration for the assay was 0.1 mg/mL.

Kinetic Study. The K_m and K_{cat} values were calculated using Lineweaver−Burk plots and are presented in **Table 3**. K_m and K_{cat} were 0.15 mM and 210 s^{-1}, respectively. The low K_m value obtained from pectoral rattail trypsin was close to those reported for trypsins from Atlantic croaker (*29*) and Greenland cod (*3*). In contrast, this K_m value was lower than those of tongol tuna (*26*) and yellowfin tuna (*4*). Therefore, purified trypsin exhibited higher affinity to TAME than those from tongol tuna and yellowfin tuna. The catalytic efficiency (K_{cat}/K_m) of pectoral rattail trypsin, 1400 s^{-1} mM^{-1}, was close to those of trypsins from crayfish (*7*). Furthermore, the catalytic efficiency of purified enzyme was higher than those of mammalian trypsins reported (*30*).

N-Terminal Sequence. As shown in **Figure 6**, 12 residues of the N-terminal amino acid sequence of the purified trypsin were determined. The N-terminal amino acid sequences of purified trypsin were IVGGYECQEHSQ. N-Terminal amino acid sequences of purified enzyme were compared with those of skipjack tuna (*5*), true sardine, arabesque greenling (*11*), cod (*31*), salmon (*32*), dogfish (*33*), dog (*34*), porcine (*35*), and bovine (*36*). It was found that the sequence of the purified trypsin showed high homology to other trypsins, especially the sequence from the first to seventh residues. However, all fish trypsins had a charged Glu residue at position 6, while Thr is most common in mammalian pancreatic trypsin (**Figure 6**). The N-terminal sequence clearly demonstrated that purified trypsin from pectoral rattail pyloric ceca was most likely a member of the trypsin family.

Conclusion. The pectoral rattail enzyme purified in this research was shown to be true member of the trypsin family

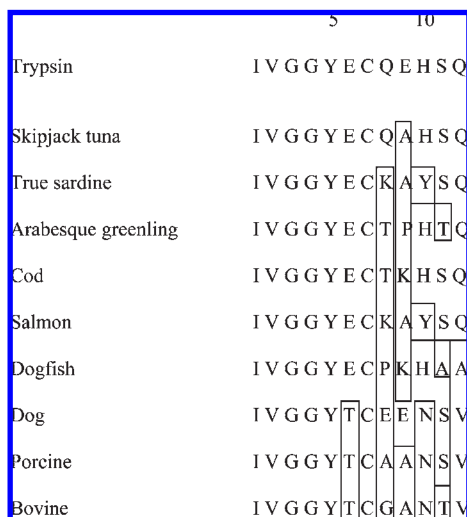

Figure 6. Comparison of N-terminal amino acid sequence of the purified trypsin from the pyloric ceca of pectoral rattail with other enzymes: skipjack tuna (*5*), true sardine, arabesque greenling (*11*), cod (*31*), salmon (*32*), dogfish (*33*), dog (*34*), porcine (*35*), and bovine (*36*).

of serine proteases. Confirmation included determination of molecular weight, substrate specificity, inhibitor study, and N-terminal sequencing. Enzymatic characteristics were essentially consistent with those of trypsin from other species.

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