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Purification and Characterization of Trypsin from the Spleen of Tongol Tuna (*Thunnus tonggol*)

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Trypsin from tongol tuna (*Thunnus tonggol*) spleen was purified to 402-fold by ammonium sulfate precipitation, followed by a series of chromatographic separations. The molecular mass of trypsin was estimated to be 24 kDa by size-exclusion chromatography and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Trypsin appearing as a single band on native PAGE showed the maximal activity at pH 8.5 and 65 °C. It was stable in a wide pH range of 6−11 but unstable at the temperatures greater than 50 °C. The enzyme required calcium ion for thermal stability. The activity was strongly inhibited by 1.0 g/L soybean trypsin inhibitor and 5 mM TLCK and partially inhibited by 2 mM ethylenediaminetetraacetic acid. Activity was lowered with an increasing NaCl concentration (0−30%). The enzyme had a K_m for N^{α}-*p*-tosyl-L-arginine methyl ester hydrochloride of 0.25 mM and a K_{cat} of 200 s^{-1}. The N-terminal amino acid sequence of trypsin was determined as IVGGYECQAHSQPHQVSLNA and was very homologous to other trypsins.

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

INTRODUCTION

Because of the limited biological resources and increased environmental concerns, the interest in full utilization of seafood-processing wastes has been increasing. Fish viscera, a byproduct of the fishery industries, have been recognized as the potential source of different enzymes, especially proteases (*1*). Proteinases are of potential use for industrial applications, e.g., in the detergent, food, pharmaceutical, leather, and silk industries (*2*). Trypsin (EC 3.4.21.4) is one of serine proteinases in fish viscera. The trypsins have been used increasingly because they are both stable and active under harsh conditions, such as at temperatures of 50−60 °C, high pH values, and in the presence of surfactants or oxidizing agents (*2*). Fish trypsins have been isolated and characterized thoroughly based on their physiochemical and enzymatic properties from different sources such as the hepatopancreas of crayfish (*Procambarus clarkii*) (*3*), pyloric caeca of tambaqui (*Colossoma macropomum*) (*4*) and arabesque greenling (*Pleuroprammus azonus*) (*5*), viscera of true sardine (*Sardinops melanostictus*) (*5*) and Japanese anchovy (*Engraulis japonica*) (*6*), and spleen of skipjack tuna (*Katsuwonus pelamis*) (*7*) and yellowfin tuna (*Thunnus albacores*) (*8*).

Tongol tuna (*Thunnus tonggol*) is one of the important species commonly used for canning production in Thailand (*1*). Viscera are generally produced during processing and become byproducts, which are commonly used as low-value animal feeds. Recently, the spleen from tongol tuna has been found to contain trypsin-like serine proteinases with the maximal activity at pH 9.0 and 55 °C (*1*). Nevertheless, no information on the molecular and biochemical characteristics of trypsin from tongol tuna spleen has been reported. Therefore, this investigation aimed to purify and characterize the trypsin from tongol tuna spleen.

MATERIALS AND METHODS

Sample Preparation. Internal organs from tongol tuna (*Thunnus tonggol*) were obtained from Chotiwat Industrial Co., Ltd., Songkhla, Thailand. Those samples (5 kg) were packed in a polyethylene bag, 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, Thailand, within 30 min. Pooled internal organs were then excised, and only the spleen was collected. Defatted spleen powder was then prepared according to the method of Klomklao et al. (*8*). The defatted powder was stored at −20 °C until used.

Preparation of the Crude Extract. Defatted spleen powder was suspended in 10 mM Tris-HCl at pH 8.0 containing 1 mM $CaCl_2$,

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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 used, the lyophilized sample was dissolved with cold distilled water (4 °C) at a ratio of 1:5 (w/v) and referred to as the "crude extract".

Trypsin Purification. All purification processes were carried out in a walk-in cold room (4 °C). During purification, the protein concentration was measured at 280 nm and the trypsin activity was determined using N^{α}-*p*-tosyl-L-arginine methyl ester hydrochloride (TAME) as a substrate.

The crude extract was subjected to ammonium sulfate precipitation at 30−70% saturation. The obtained pellet was dissolved in a minimal volume of SB and dialyzed against SB overnight. The dialysate was then applied onto a Sephacryl S-200 column (3.9 × 64 cm) equilibrated with approximately two bed volumes of SB. The column was then eluted with the same buffer at a flow rate of 0.5 mL/min. Fractions of 5 mL were collected, and those with TAME activity were pooled. Pooled Sephacryl S-200 fractions were dialyzed against SB for 10−12 h. The sample was then chromatographed using a DEAE-cellulose (Whatman, U.K.) column (2.2 × 18 cm) equilibrated with SB. The sample was loaded onto the column at a flow rate of 0.5 mL/min. The column was washed with SB until A_{280} was less than 0.05 and then eluted with a stepwise process using SB containing different NaCl concentrations: 0, 0.25, 0.255, and 0.26 M, respectively. Fractions of 5 mL were collected, and those with TAME activity were pooled. After the first DEAE-cellulose chromatography, trypsin fractions were rechromatographed using the same column equilibrated with SB. The elution was performed by a linear gradient of 0.25−0.255 M NaCl in SB at a flow rate of 0.5 mL/min. Fractions of 5 mL were collected, and the fractions with TAME activity were pooled. Pooled second DEAE-cellulose fractions were dialyzed against SB for 10−12 h. The dialysate was lyophilized and then dissolved in distilled water. The sample was loaded onto a Sephadex G-50 (3.9 × 64 cm) column previously equilibrated with approximately two bed volumes of SB at a flow rate of 0.5 mL/min. Fractions of 3 mL were collected, and those with TAME activity were pooled and used for further study.

Trypsin Activity Assay. Trypsin activity was measured by the method of Hummel (*9*) as modified by Klomklao et al. (*8*) 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 at pH 8.0 and incubated at 30 °C for 20 min. The production of *p*-tosyl-arginine was measured by monitoring the increment in absorbance at 247 nm (A_{247}). One unit of activity was defined as the amount causing an increase of 1.0 in A_{247} per minute.

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

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, the enzyme dissolved in 100 mM Tris-HCl at pH 8.0 was incubated at different temperatures (20, 30, 40, 50, 60, 70, and 80 °C) for 15 min in a temperature-controlled water bath (Memmert, Schwabach, Germany). Thereafter, the treated samples were suddenly cooled in ice water. The residual activity was assayed using TAME as a substrate at pH 8.0 and 30 °C for 20 min.

Determination of the Molecular Weight. The molecular weight of purified trypsin 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 the available partition coefficient (K_{av}) against the logarithm of the molecular weight of the protein standards. The elution volume (V_e) was measured for each protein standard and trypsin. The 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 purified enzyme was incubated in the presence of 2 mM ethylenediaminetetraacetic acid (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 the remaining activity.

Effect of NaCl. The activity was assayed in the presence of NaCl at varying concentrations [0−30% (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. Inhibition of trypsin by different inhibitors was measured according to the method of Klomklao et al. (*1*). The enzyme solution was incubated 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 determined, and percent inhibition was calculated.

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

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

Determination of the N-Terminal Amino Acid Sequence. The purified trypsin was subjected to SDS−PAGE under a reducing condition and electrophoretically transferred to polyvinylidene difluoride (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).

Kinetic Studies. The activity was assayed with different final concentrations of TAME ranging from 0.01 to 0.10 mM. The final enzyme concentration for the assay was 0.1 mg/mL. The determinations were repeated twice, and the respective kinetic parameters including V_{max} and K_m were evaluated by plotting the data on a Lineweaver−Burk double-reciprocal graph (*11*). The turnover number (K_{cat}) was calculated from the following equation: $V_{max}/[E] = K_{cat}$, where [E] is the active enzyme concentration.

Protein Determination. The protein concentration was measured by the method of Lowry et al. (*12*) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Trypsin Purification. Purification of trypsin from tongol tuna spleen is summarized in **Table 1**. An increase in purity of 28.1-fold was obtained by ammonium sulfate precipitation (30−70%). Ammonium sulfate precipitation was introduced as an initial step to remove other proteins in the crude extract. Kristjansson (*13*) found that ammonium sulfate precipitation (30−70%) of trypsin from rainbow trout pyloric caeca resulted in a 4.9-fold increase in specific activity. Klomklao et al. (*8*) also reported that size-exclusion chromatography on a Sephacryl S-200 column was highly effective in purifying trypsins from yellowfin tuna spleen. After the protein was chromatographed on a Sephacryl S-200 column, a large amount of the protein was removed with a small loss in activity, leading to an increase in purity by 49.6-fold (**Table 1**). Pooled active Sephacryl S-200 fractions were further purified using a DEAE-cellulose column. After loading and washing, the column was subjected to

Table 1. Purification of Trypsin from Tongol Tuna Spleen

purification steps	total activity (units)[a]	total protein (mg)	specific activity (units/mg of protein)	purity (fold)	yield (%)
crude extract	283.0	3100	0.09	1	100
$(NH_4)_2SO_4$ (30–70%)	251.2	99.11	2.53	28.1	88.8
Sephacryl S-200	168.0	37.67	4.46	49.6	59.4
first DEAE-cellulose	68.0	8.82	7.71	85.7	24.0
second DEAE-cellulose	24.2	1.29	18.76	208.4	8.6
Sephadex G-50	18.8	0.52	36.15	401.7	6.6

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

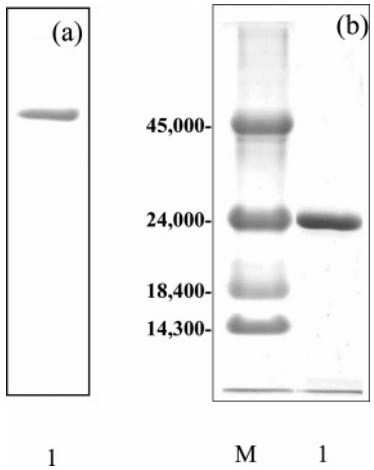

Figure 1. Protein pattern of trypsin from tongol tuna spleen determined by native PAGE (**a**) and SDS–PAGE (**b**). M, molecular weight standard; lane 1, purified trypsin.

stepwise elution using different NaCl concentrations. A 85.7-fold increase in purity was obtained. To refine the pooled fraction obtained from previous steps, pooled active fractions were loaded onto the second DEAE-cellulose column and eluted using a linear gradient of 0.25−0.255 M NaCl. A purification fold of 208.4 with a yield of 8.6% was obtained after this step. Yoshinaka et al. (*14*) chromatographed the trypsin from the eel viscera on an anion exchanger, DEAE-cellulose, twice, and two anionic trypsins (1 and 2) were obtained with 22-fold purity. When second DEAE-cellulose fractions with TAME activity were subjected to gel filtration on Sephadex G-50, a single protein peak was found. A purification fold of 401.7 with a yield of 6.6% was observed. The use of the gel filtration on Sephadex G-50 in the final step of the purification process of trypsin from true sardine viscera and arabesque greenling pyloric caeca led to an increase in trypsin activity by 117- and 20-fold, respectively (*5*).

Electrophoretic Pattern. Purified trypsin appeared as a single band on the native PAGE (**Figure 1a**), indicating that the trypsin obtained was a monomer. For SDS−PAGE, a single band with the molecular weight of 24 kDa was observed (**Figure 1b**). Also, the molecular weight of the enzyme was estimated to be approximately 24 kDa by gel filtration using a Sephacryl S-200 column (data not shown). The results confirm that trypsin from tongol tuna spleen had a monomeric structure with a molecular weight of 24 kDa. The molecular weight of tongol tuna trypsin was similar to those reported for other fish trypsins, such as Japanese anchovy (*6*), skipjack tuna (*7*), yellowfin tuna (*8*), true sardine, and arabesque greenling (*5*). Trypsins isolated from various fish species have been reported to have the molecular weight ranging from 20 to 30 kDa (*4*, *13*).

pH Optimum and Stability. Trypsin from tongol tuna spleen showed the maximum activity at pH 8.5 (**Figure 2a**). The negligible activity was observed in both acidic and alkaline pH

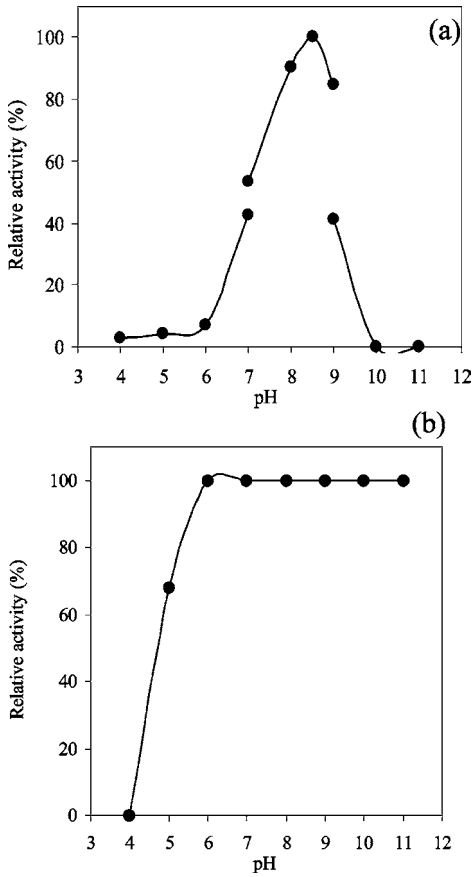

Figure 2. pH profile (**a**) and pH stability (**b**) of purified trypsin from tongol tuna spleen.

values. A change in pH affects both the substrate and enzyme by changing the charge distribution and conformation of the molecules (*15*). Most enzymes undergo irreversible denaturation in a very acid and alkaline solution, causing the loss of stability (*15*). The optimal pH of trypsin from tongol tuna spleen was similar to those of trypsins from yellowfin tuna spleen and skipjack tuna spleen (*7*, *8*). For pH stability, trypsin showed the high stability in the pH range of 6−11, but the inactivation was more pronounced at pH values below 6 (**Figure 2b**). A complete loss in activity was found after incubating trypsin at pH 4. The stability of trypsin at a particular pH might be related to the net charge of the enzyme at that pH (*8*). A similar pH effect on activity has been reported for trypsin from several fish species (*5*−*8*, *13*).

Temperature Optimum and Thermal Stability. The maximal activity of the purified trypsin was observed at 65 °C (**Figure 3a**). A sharp decrease in activity was found at temperatures above 70 °C, possibly because of the thermal denaturation. Klomklao et al. (*8*) reported that trypsins A and B from yellowfin tuna spleen had the optimal temperatures at 55 and 65 °C, respectively. Alkaline serine proteinase from the

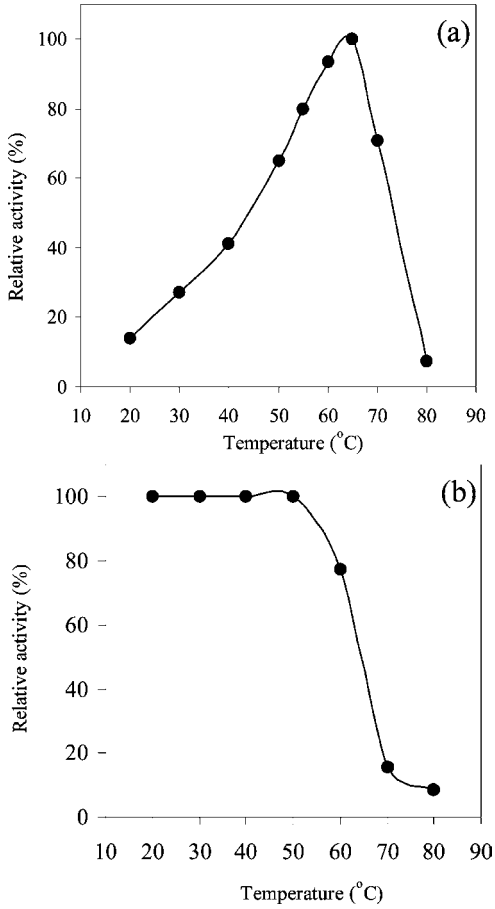

Figure 3. Temperature profile (**a**) and thermal stability (**b**) of purified trypsin from tongol tuna spleen.

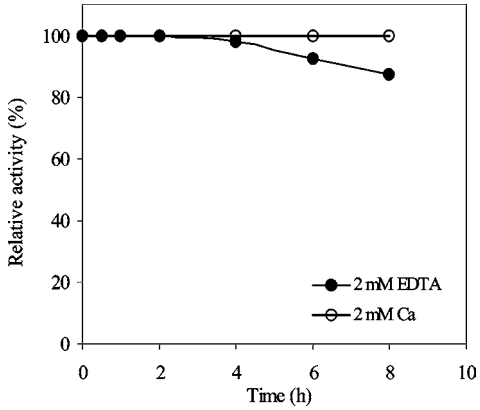

Figure 4. Effect of calcium ion and EDTA on the stability of purified trypsin from tongol tuna spleen. The stability was tested by incubating the enzyme at 40 °C for different times.

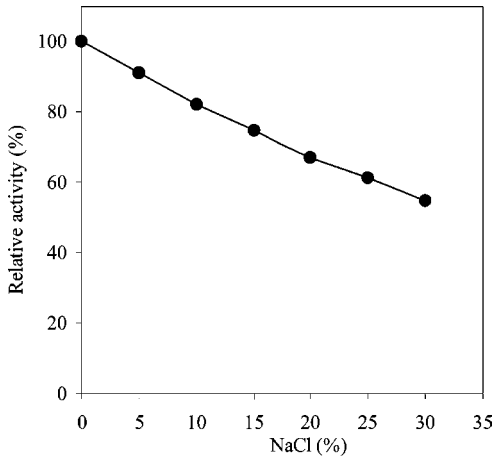

Figure 5. Effect of NaCl concentrations on activities of purified trypsin from tongol tuna spleen.

pyloric caeca of tambaqui exhibited optimal activity at 60 °C (*4*). The temperature optimum of the purified trypsin was higher than that of trypsin from cold-water fish, which had the optimal temperatures in the range of 40−45 °C (*16*). The difference might be related to the different temperature of water, where fish inhabited. For thermal stability, trypsin from tongol tuna spleen was stable below 50 °C, but the activity sharply decreased above 60 °C (**Figure 3b**). The enzyme was almost completely inactivated at 80 °C. At high temperatures, enzymes most likely underwent denaturation and lost their activity (*7*). Trypsin from tongol tuna spleen exhibited the similar thermal stability to those of other fish species (*5*−*8*).

Effect of Ca^{2+} on Thermal Stability. The total activity was retained throughout 8 h at 40 °C in the presence of 2 mM calcium ion, while the activity decreased with increasing time in the presence of 2 mM EDTA (**Figure 4**). The results indicate that trypsin from tongol tuna spleen was stabilized by calcium ions. Binding of calcium to a single binding site in bovine trypsin significantly stabilized the enzyme against denaturation (*13*). In the presence of calcium ions, trypsin is believed to undergo a conformational change, resulting in a more compact structure, which is more resistant to autolysis (*1*, *6*). Stabilization against thermal inactivation by calcium ion has also been found for the trypsins from yellowfin tuna (*8*), skipjack tuna (*7*), true sardine, and arabesque greenling (*5*).

Effect of NaCl. Trypsin activity continuously decreased with increasing NaCl (**Figure 5**). The activity in the presence of 30% NaCl was approximately 55% of that without NaCl. 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 the competition for water of ionic salts, leading to the induced enzyme precipitation (*7*, *8*). At 25% NaCl, 63% of activity remained. Therefore, trypsin from tongol tuna spleen may have a potential in accelerating the hydrolysis of high-salt products, such as fish sauce.

Effect of Inhibitors. Trypsin was markedly inhibited by trypsin inhibitors such as soybean trypsin inhibitor (93.75% inhibition) and TLCK (90.50% inhibition). Inhibitors for cysteine and aspartic proteinases involving E-64, *N*-ethylmaleimide, iodoacetic acid, and pepstatin A did not show inhibitory effects toward trypsin activity. No inhibition was also observed when TPCK, a specific inhibitor for chymotrypsin, was used (*1*). The result confirms that this purified enzyme was serine proteinase, mostly likely trypsin. The trypsin-like enzyme from tambaqui pyloric caeca was inhibited by some trypsin inhibitors including PMSF, benzamidine, and TLCK (*4*). Two trypsin isoforms from yellowfin tuna spleen were inhibited by soybean trypsin inhibitor and TLCK (*8*). From the result, EDTA, which chelates the metal ions required for the enzyme, partially lowered trypsin activity (7.18% inhibition). Removal of calcium ion might affect the enzyme structure, resulting in some losses in activity. The result suggests that trypsin most likely required metal ions as cofactors for activity.

Kinetic Study. Kinetic constants for TAME hydrolysis by tongol tuna spleen trypsin were determined using Lineweaver−Burk plots. K_m and K_{cat} of tongol tuna spleen trypsin were calculated to be 0.25 mM and 200 s^{-1}, respectively. The K_m

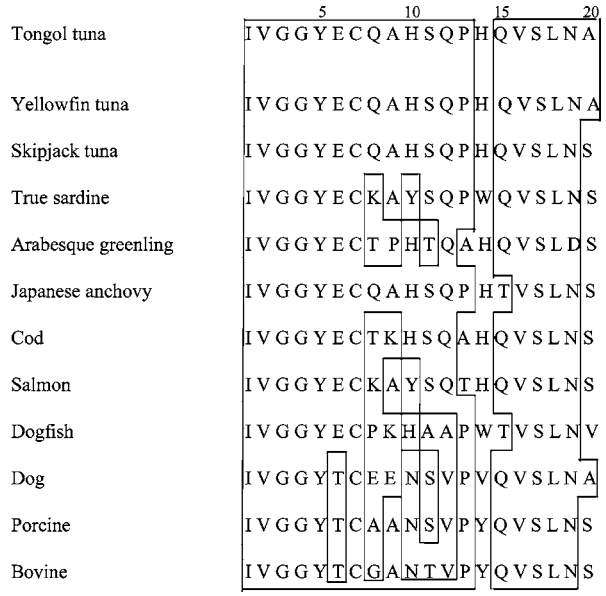

Figure 6. Comparison of the N-terminal amino acid sequence of the purified trypsin from tongol tuna spleen with other enzymes: yellowfin tuna (*8*), skipjack tuna (*7*), true sardine, arabesque greenling (*5*), Japanese anchovy (*6*), cod (*17*), salmon (*18*), dogfish (*19*), dog (*20*), porcine (*21*), and bovine (*22*). Amino acid residues different from those of trypsin are boxed.

obtained for tongol tuna trypsin was similar to those reported for trypsin from yellowfin tuna (*8*), skipjack tuna (*7*), and crayfish (*3*). Moreover, the enzyme had a slightly lower K_m value than trypsin from bovine, which had K_m in the range of 0.3−0.33 mM (*3*). The result indicates that tongol tuna trypsin had a higher affinity for TAME than did bovine trypsin. Additionally, the catalytic efficiency (800 s^{-1} mM^{-1}) of tongol tuna spleen trypsin was greater than that of mammalian trypsin (*16*). From the result, it was suggested that trypsin from tuna tongol spleen had more flexible structures than trypsin from warm-blooded animals

N-Terminal Sequence. The N-terminal amino acid sequence of trypsin from tongol tuna spleen was determined to be IVGGYECQAHSQPHQVSLNA (**Figure 6**). The result indicates that the N terminus of the enzyme was unblocked. The N-terminal amino acid sequence of the trypsin from tongol tuna spleen was aligned with those of other animal trypsins (**Figure 6**). Being similar to other fish trypsins, the enzyme had a charged Glu residue at position 6, where Thr is most common in mammalian pancreatic trypsins (**Figure 6**). Coincidentally, N-terminal sequence of tongol tuna spleen trypsin exhibited similarity to those of skipjack tuna spleen and yellowfin tuna spleen, suggesting the possibility that they were genetically evolved from a common ancestor. From the results, the N-terminal sequence clearly showed that trypsin from tongol tuna spleen was most likely a member of the trypsin family. In addition, the present data of the N-terminal amino acid sequence may be useful for designing primers for the cDNA cloning of trypsin.

In summary, the enzyme purified from tongol tuna spleen with the maximal activity at 65 °C and pH 8.5 can be classified as trypsin on the basis of substrate specificity, molecular weight, inhibitor study, and N-terminal sequencing. Trypsin was salt-tolerant and could be used in a high-salt environment.

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