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Biochemical Properties of Anionic Trypsin Acting at High Concentration of NaCl Purified from the Intestine of a Carnivorous Fish: Smooth Hound (*Mustelus mustelus*)

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Trypsin from the intestine of smooth hound (*Mustelus mustelus*) was purified by fractionation with ammonium sulfate, Sephadex G-75 gel filtration, and DEAE-cellulose ion exchange chromatography, with a 65-fold increase in specific activity and 15% recovery. The molecular weight of the purified trypsin was estimated to be 24 kDa using size exclusion chromatography and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). The purified enzyme showed esterase-specific activity on N^{α}-*p*-tosyl-L-arginine methyl ester hydrochloride (TAME) that was four times greater than its amidase-specific activity on Nα-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA). The optimum pH and temperature for the trypsin activity were pH 8.5 and 50 °C, respectively, using TAME as a substrate. The enzyme was extremely stable in the pH range of 7.0−9.0 and highly stable up to 40 °C after 1 h of incubation. The purified enzyme was strongly inhibited by soybean trypsin inhibitor (SBTI) and *N*-*p*-tosyl-1-lysine chloromethyl ketone (TLCK), specific inhibitors for trypsin. In addition, smooth hound trypsin showed higher proteolytic activity at high NaCl concentration, demonstrating its potential for protein hydrolysis at high salt content. The N-terminal amino acid sequence of the first 12 amino acids of the purified trypsin was IVGGYECKPHSQ. This sequence showed high homology with trypsins from marine vertebrates and invertebrates. Purified trypsin had a Michaelis−Menten constant (K_m) and catalytic constant (K_{cat}) of 0.387 ± 0.02 mM and 2.62 ± 0.11 s^{-1}, respectively, when BAPNA was used as a substrate. For the hydrolysis of TAME, K_m and K_{cat} were 0.156 ± 0.01 mM and 59.15 ± 2.2 s^{-1}, respectively.

KEYWORDS: Trypsin; smooth hound; *Mustelus mustelus*; intestine; purification; biochemical characterization; N-terminal amino acid sequence

INTRODUCTION

Trypsin (EC 3.4.21.4) is an important pancreatic serine protease synthesized as a proenzyme in the pancreatic acinar cells and secreted into the intestine of mammals. Trypsin acts as a digestive enzyme in the intestine, and it is also responsible for activating all of the pancreatic enzymes, including itself (*1*). Enzymatic and structural properties of mammalian pancreatic trypsins have been extensively characterized (*2*, *3*).

Trypsin and trypsin-like proteolytic enzymes have been isolated and characterized from the viscera of some marine invertebrates and a wide range of cold water and warm water fish, including the digestive gland (hepatopancreas) of the white shrimp (*Penaeus setiferus*) (*4*), crayfish (*Astacus fluviatilis*) (*5*), and cuttlefish (*Sepia officinalis*) (*6*); the spleen of skipjack tuna (*Katsuwonus pelamis*) (*7*) and yellowfin tuna (*Thunnus albacores*) (*8*); the pyloric ceca of chinook salmon (*Oncorhynchus tshawytscha*) (*9*), tambaqui (*Colossoma macropomum*) (*10*), and Monterey sardine (*Sardinops sagax caerulea*) (*11*); and the entire viscera of true sardine (*Sardinops melanostictus*) (*12*), Japanese anchovy (*Engraulis japonica*) (*13*), sardine (*Sardina pilchardus*) (*14*), Gray triggerfish (*Balistes capriscus*) (*15*), striped seabream (*Lithognathus mormyrus*) (*16*), bogue (*Boops boops*) (*17*), and sardinelle (*Sardinella aurita*) (*18*).

Tunisia's marine fisheries produce several thousand metric tons of fish and shellfish annually, mainly for exportation. Smooth hound (*Mustelus mustelus*) is Tunisia's new leading economically important fish species. The smooth hound is a hound shark of the family Trikidae. The smooth hound (*M. mustelus*) is a moderately sized, rather slender shark with two high dorsal fins; gray−brown with no prominent white spots on the dorsum. Smooth hound is utilized for human consumption.

We have earlier reported the purification of pepsin (*19*), a low molecular weight alkaline protease (*20*) and trypsin-like protease (*21*), from the stomach and intestines of smooth hound. These proteases were successfully used for the production of biologically active fish protein hydrolysates (*22*, *23*). In the present study, we describe the purification of trypsin from the intestines of smooth hound and provide basic information about its main biochemical and kinetic characteristics.

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MATERIALS AND METHODS

Reagents. Casein sodium salt from bovine milk, ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), TAME, TPCK, and *N*-*p*-tosyl-l-lysine chloromethyl ketone (TLCK), benzamidine, glycine, ammonium sulfate, bovine serum albumin, and protein markers for molecular weights 14,000−66,000 Da were purchased from Sigma Chemical Co. (St. Louis MO, USA). SBTI and BAPNA were obtained from Fluka Biochemica (USA). Sodium dodecyl sulfate (SDS), acrylamide, ammonium persulphate, *N*,*N*,*N*′,*N*′-tetramethyl ethylenediamine (TEMED), and Coomassie Brilliant Blue R-250 were from Bio-Rad Laboratories (Mexico). Sephadex G-75 and diethylaminoethyl (DEAE)-cellulose were from Pharmacia Biotech (Uppsala, Sweden). Polyvinylidene difluoride (PVDF) membrane was purchased from Applied Biosystems (Roissy France). All other reagents were of analytical grade.

Smooth Hound Intestines. Smooth hound (*M. mustelus*) was obtained from the fish market of Sfax City, Tunisia. The samples were packed in polyethylene bags, placed in ice with the sample/ice ratio of approximately 1:3 (w/w), and transported to the research laboratory within 30 min. After the fish were washed with water, the internal organs were separated, and only the intestines were collected and then stored in sealed plastic bags at −20 °C until they were used for enzyme extraction.

Preparation of Alkaline Crude Protease Extract. Intestines from *M. mustelus* were washed with tap water and then with buffer A (10 mM Tris-HCl, pH 8.0). Intestines (100 g) were defatted by homogenization with cold acetone (−20 °C) for 30 s in a tissue homogenizer. The homogenate was filtered using Whatman No. 4 filter paper. The acetone insoluble material was washed several times with cold acetone and then dried at room temperature overnight. The acetone dried powder was suspended in buffer A at a ratio of 1:10 (w/v) and stirred continuously for 2 h at 4 °C. The homogenate was centrifuged at 8,500*g* for 30 min at 4 °C. The pellet was discarded, and the supernatant was collected and used as the crude protease extract.

Trypsin Purification. *Ammonium Sulfate Precipitation*. The crude protease extract was first subjected to ammonium sulfate fractionation: 0−20%, 20−40%, 40−60%, and 60−80%. The precipitates obtained after centrifugation at 13,000*g* for 30 min were suspended in buffer A and dialyzed for 24 h at 4 °C against repeated changes in the same buffer (after 8 and 16 h).

Sephadex G-75 Gel Filtration. The 20−40% (w/v) ammonium sulfate fraction was subjected to gel filtration on a Sephadex G-75 column (2.5 cm ×100 cm) equilibrated with buffer B (25 mM Tris-HCl, pH 8.0, containing 0.5‰ Triton X-100). Fractions of 5 mL were eluted at a flow rate of 30 mL/h with the same buffer. Protein content (Abs 280 nm) and trypsin activity were determined. Fractions showing trypsin activity were pooled.

DEAE-Cellulose Anion Exchange Chromatography. The active fractions pooled from Sephadex G-75 were applied to a DEAE-cellulose column (2 cm ×35 cm) previously equilibrated with buffer C (25 mM Tris-HCl, pH 8.0). After being washed with the same buffer, bound proteins were eluted with a linear gradient of sodium chloride in the range of 0−0.5 M in the equilibrating buffer. Fractions of 5 mL were collected at a flow rate of 70 mL/h and analyzed for trypsin activity and protein concentration. The fractions with high trypsin activity were pooled, concentrated by lyophilization, and used for characterization. All of the purification steps were conducted at temperature not exceeding 4 °C.

Assay for Trypsin Activity. *Amidase Activity*. Amidase activity was measured according to the method of Erlanger et al. (*24*) modified by Benjakul et al. (*25*) using BAPNA as a substrate. To initiate the reaction, 200 μL of trypsin with an appropriate dilution was added to the preincubated reaction mixture containing 1000 μL of 0.5 mM of BAPNA in reaction buffer (0.1 M Tris-HCl, pH 8.5) and 200 μL of distilled water, and mixed thoroughly. The mixture was incubated for 10 min at 25 °C. The enzymatic reaction was terminated by adding 200 μL of 30% (v/v) acetic acid. The reaction mixture was centrifuged at 8000*g* for 3 min at room temperature. Trypsin activity was measured by the absorbance at 410 nm due to the release of *p*-nitroaniline. Trypsin amidase activity was then calculated using the following formula:

$$\text{amidase activity (U/mL)} = \frac{(A - A_0) \times \text{final volume of the mixture (mL)} \times 1,000}{8,800 \times \text{time of the reaction (min)} \times 0.2\ \text{(mL)}}$$

where 8800 M^{-1} cm^{-1} is the extinction coefficient of *p*-nitroaniline; *A* and A_0 are A_{410} of the sample and the blank, respectively. One unit of activity was defined as that releasing 1 nmol of *p*-nitroaniline per min. Values are the means of three independent experiments.

Esterase Activity. The esterase activity of trypsin was determined using TAME as the substrate on the basis of the method of Hummel (*26*) with slight modifications. The reaction mixture comprised 2.8 mL of the substrate (1 mM TAME in 0.05 M Tris-HCl buffer, pH 8.5) and 200 μL of the enzyme. The reaction mixture was thoroughly mixed, and the release of tosyl arginine was measured at 247 nm.

$$\text{Esterase activity (U/mL)} = \frac{(A - A_0) \times 3 \times 1,000}{540 \times \text{time of the reaction (min)} \times 0.2\ \text{(mL)}}$$

where 540 M^{-1} cm^{-1} is the extinction coefficient of *p*-nitroaniline; *A* and A_0 are A_{247} of the sample and the blank, respectively. One unit of activity was defined as the amount causing an increase of 1.0 in absorbance at 247 nm per minute. Values are the means of three independent experiments.

Polyacrylamide Gel Electrophoresis and Zymography. SDS−PAGE was carried out for the determination of purity and molecular weight of the enzyme as described by Laemmli (*27*), using 5% (w/v) stacking and 15% (w/v) separating gels. Samples were prepared by mixing the purified enzyme at a 1:5 (v/v) ratio with the SDS−PAGE sample buffer (10 mM Tris-HCl (pH 8.0), 2.5% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.002% bromophenol blue). Samples were heated at 100 °C for 5 min before loading in the gel. After electrophoresis, the gel was stained with 0.25% Coomassie Brilliant Blue R-250 in 45% ethanol−10% acetic acid and destained with 5% ethanol−7.5% acetic acid. The molecular weight of the purified enzyme was estimated using a low molecular weight calibration kit as markers. The molecular mass markers used are bovine serum albumin (66,000 Da), egg white ovalbumin (45,000 Da), glyceraldehyde-3-P dehydrogenase (36,000 Da), bovine trypsinogen (24,000 Da), soybean trypsin inhibitor (20,100 Da), and bovine α-lactalbumin (14,100 Da).

Native-PAGE was performed according to the procedure of Laemmli (*27*), except that the sample was not heated, and SDS and reducing agent were left out.

Casein-zymography was performed on native-PAGE according to the method reported by Garcia-Carreno et al. (*28*). Briefly, after electrophoresis, the gel was submerged in 100 mL of 1% (w/v) casein in 100 mM Tris-HCl buffer, (pH 8.0) and incubated for 60 min at 40 °C. After washing, the gel was stained with Coomassie Brilliant Blue R-250 for zymography analysis. Development of a clear zone on the blue background of the gel indicated the presence of protease activity.

Determination of the N-Terminal Amino Acid Sequence of *M. mustelus* Trypsin. The purified enzyme, from DEAE-cellulose anion exchange chromatography, was applied to SDS−PAGE and then electrophoretically transferred to a PVDF membrane. After brief staining with Coomassie Brilliant Blue R-250, the PVDF band corresponding to the protease was excised, and the N-terminal amino acid sequence was determined by the Edman degradation method on an ABI Procise 494 protein sequencer (Applied Biosystems).

Protein Determination. Protein concentration was determined by the method of Bradford (*29*) using bovine serum albumin as a standard and during the course of enzyme purification by measuring the absorbance at 280 nm.

Biochemical Properties. *Effect of pH on the Activity and Stability of Trypsin*. Trypsin activity was assayed over the pH range of 4.0 to 11.0 at 30 °C for 20 min using TAME as a substrate. The effect of pH on enzyme stability was evaluated by measuring the residual enzyme activity after incubation at various pH values for 60 min at 25 °C. The following buffer systems were used: 100 mM sodium acetate buffer, pH 4.0−6.0; 100 mM phosphate buffer, pH 7.0; 100 mM Tris-HCl buffer, pH 8.0; 100 mM glycine-NaOH, pH 9.0−11.0; and 100 mM KCl-NaOH, pH 12.0.

Effect of Temperature on the Activity and Stability of Trypsin. Enzyme activity was assayed at different temperatures ranging from 20 to 70 °C using TAME as a substrate. The assay was conducted at pH 8.5 for 20 min. For thermal stability, the enzyme was incubated at different temperatures for 60 min. The residual activity was assayed using TAME as a substrate

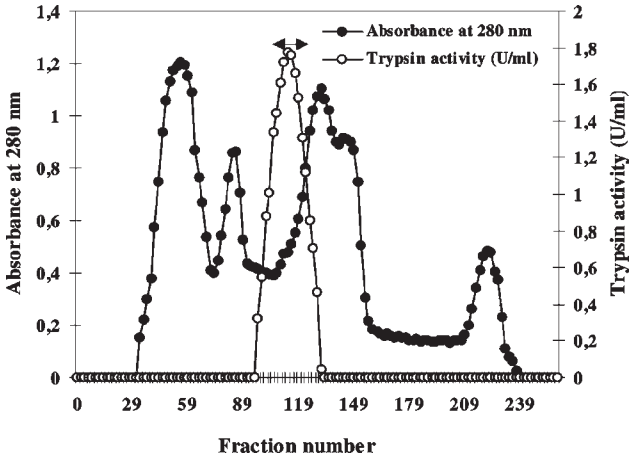

Figure 1. Purification profile of trypsin from smooth hound (*M. mustelus*) by gel filtration on a Sephadex G-75 column. The enzyme preparation (20−40% (w/v) saturation with ammonium sulfate) was applied to a 2.5 × 100 cm column, equilibrated, and eluted with buffer B at a flow rate of 30 mL h^{-1}. Fractions (5 mL) collected from the column were assayed for protein content at 280 nm and trypsin activity.

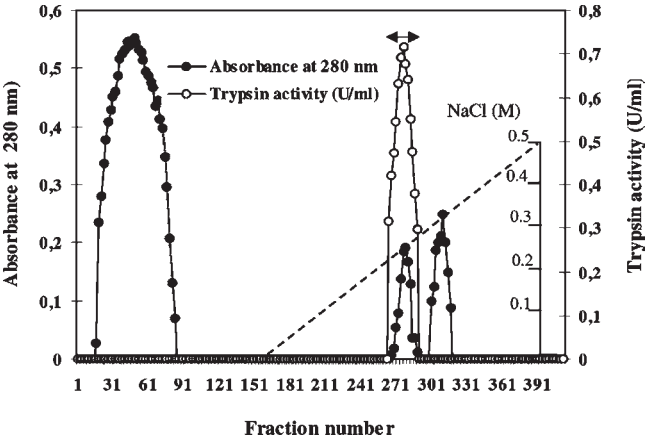

Figure 2. Purification profile of trypsin from smooth hound (*M. mustelus*) by DEAE-cellulose column (2 × 35 cm) pre-equilibrated with buffer C. Elution of adsorbed proteins was carried out with a linear gradient of NaCl (0−0.5 M) in the equilibrating buffer at a flow rate of 70 mL h^{-1}. Fractions (5 mL) collected from the column were assayed for protein content at 280 nm and trypsin activity.

at pH 8.5 and 30 °C for 20 min. The nonheated enzyme was considered as the control (100%).

Effects of Enzyme Inhibitors. The effects of enzyme inhibitors on trypsin activity were studied using PMSF, SBTI, benzamidine, pepstatin A, β-mercaptoethanol, EDTA, TPCK, and TLCK. The purified enzyme was preincubated with each inhibitor for 30 min at 25 °C, and then the remaining enzyme activity was estimated using TAME as a substrate. The activity of the enzyme assayed in the absence of inhibitors was taken as 100%.

Effect of NaCl Concentration. Trypsin activity was assayed in the presence of NaCl at various concentrations (0−30% (w/v)). The relative enzyme activity was determined at 30 °C for 20 min using TAME as a substrate.

Kinetic Studies. The activity of the purified trypsin with different final concentrations of TAME and BAPNA, ranging from 0 to 2000 μM, was evaluated. The final enzyme concentration of the assay was 0.0045 mg/mL. The determinations were repeated twice, and the respective kinetic parameters, including the apparent Michaelis−Menten contant (K_m) and the maximum velocity (V_{max}), were calculated from Lineweaver−Burk plots (*30*). The value of the turnover number (K_{cat}) was calculated from the following equation: $K_{cat} = V_{max}/[E]$, where [E] is the active enzyme concentration.

RESULTS AND DISCUSSION

Trypsin Purification. Trypsin from the intestine of *M. mustelus* was purified by the three-step procedure described in Materials and Methods. In the first step, the crude enzyme extract was fractionated with ammonium sulfate. The fraction F2 (20−40% (w/v) saturation) showed higher specific activity (2.23 U/mg protein), using TAME as a substrate, than fraction F1 (0−20%; 0.018 U/mg), fraction F3 (40−60%; 1.02 U/mg), and fraction F4 (60−80%; 0.026 U/mg). No protease activity was detected in the final supernatant. The 20−40% ammonium sulfate precipitate, which gave the highest specific activity, was then subjected to gel filtration on a Sephadex G-75 column. The elution profiles of the trypsin activity and proteins are shown in **Figure 1**. This procedure yielded a single peak of trypsin activity. Active fractions were pooled and then loaded on a DEAE-cellulose column that had been equilibrated and washed with buffer C. Binding proteins were eluted with a linear gradient of NaCl 0−0.5 M in the same buffer (**Figure 2**). Trypsin activity was detected mainly in the adsorbed fractions.

The results of the purification procedure are summarized in **Table 1**. After the final purification step, trypsin was purified 65.6-fold with a recovery of 15.3%. *M. mustelus* trypsin showed a specific activity of 21.65 U/mg protein on the ester substrate (TAME) and 5 U/mg protein on the amide substrate (BAPNA), indicating a hydrolysis of TAME four times higher and faster than that of BAPNA. These results are in agreement with those of trypsin from sardinelle (*S. aurita*) (*18*) and Montery sardine (*S. sagax caerulea*) (*11*), which showed an esterase-specific activity 4 and 4.5 times higher than their amidase-specific activity, respectively.

Purity and Molecular Weight. The purified trypsin was homogeneous on SDS−PAGE and its molecular weight was estimated to be 24 kDa (**Figure 3a**), corresponding to that determined by gel filtration (data not shown). Fish trypsins have been reported to have molecular weights in the range of 23 to 28 kDa. The molecular weight of *M. mustelus* trypsin was similar to those from other fish species, such as sardine (*S. pilchardus*) (*14*), Gray triggerfish (*B. capriscus*) (*15*), sardinelle (*S. aurita*) (*18*), bogue (*B. boops*) (*17*), striped seabream (*L. mormyrus*) (*14*), walleye pollock (*Theragra chalcogramma*) (*31*), and true sardine (*S. melanostictus*) (*12*).

The purity of the purified trypsin was also evaluated by using native-PAGE. Trypsin migrated as a single protein band on native-PAGE (**Figure 3b**), indicating the homogeneity of the enzyme preparation. The proteolytic activity of this protein band was confirmed by zymogram activity staining. As shown in **Figure 3b**, a unique clear band of casein hydrolysis was observed in the gel, indicating the activity and the homogeneity of the purified trypsin.

N-Terminal Amino Acid Sequence of *M. mustelus* Trypsin. The N-terminal 12 amino acids of the *M. mustelus* trypsin, was determined to be IVGGYECKPHSQ. The N-terminal amino acid sequence of smooth hound trypsin showed uniformity indicating that it was isolated in a pure form and that if other isoforms are present, their amounts must be small.

The N-terminal amino acid sequence of *M. mustelus* trypsin was aligned with the sequences of other animal trypsins (**Figure 4**). The sequence of *M. mustelus* trypsin showed high homology with trypsins from fish and marine invertebrates. The trypsin from *M. mustelus* differs from that of jacopever (*Sebastes schlegelii*) by only one amino acid (*32*). The His_{10} in *M. mustelus* trypsin

Table 1. Summary of the Purification of Trypsin from Smooth Hound (*M. mustelus*) Intestine[a]

purification steps	total activity (U)	total protein (mg)	specific activity (U/mg)	recovery (%)	purity (fold)
crude extract	417.2	1264.24	0.33	100	1
ammonium sulfate precipitation (20−40%)	326.5	146.41	2.23	78.29	6.75
Sephadex G-75	202.1	17.93	11.27	48.44	34.15
DEAE-cellulose	63.68	2.95	21.65	15.30	65.60

[a] All operations were carried out at 4°C. The only precipitate formed between 20−40% saturation with ammonium sulphate was subjected to gel filtration on Sephadex G-75.

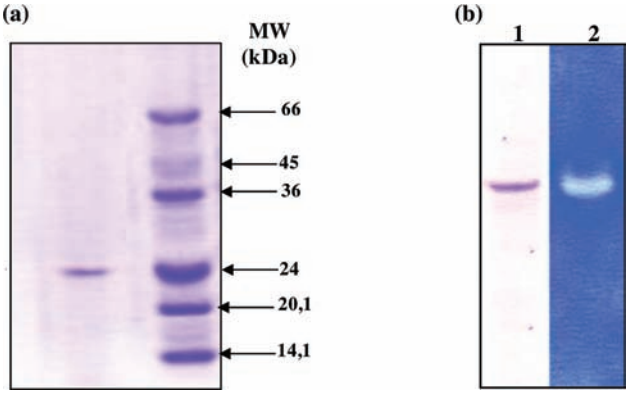

Figure 3. (**a**) SDS−PAGE of the purified trypsin from *M. mustelus* intestines. Lane 1, standard proteins marker; lane 2, purified trypsin. (**b**) Native-PAGE (lane 1) and zymogram detection of proteolytic activity (lane 2) of the purified trypsin from *M. mustelus* intestines.

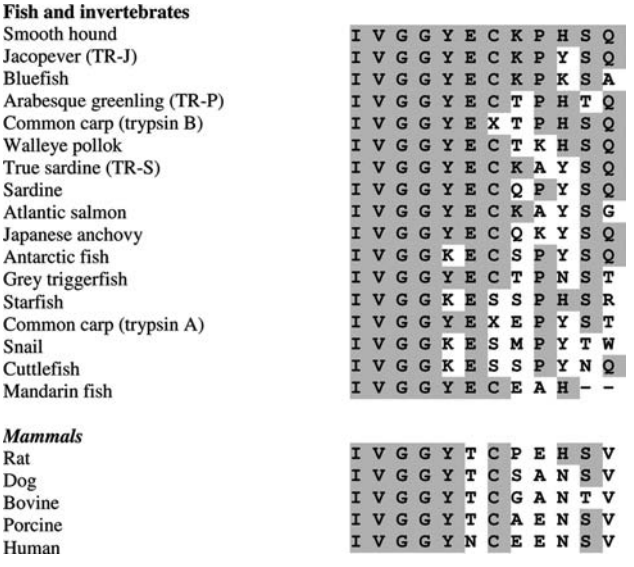

Figure 4. Alignment of the N-terminal amino acid sequence of the purified trypsin from smooth hound with the sequences of other trypsins. Residues identical with *M. mustelus* trypsin are shaded. Smooth hound, *M. mustelus* trypsin (this study); starfish (*Asterias amurensis*) (*40*); starfish (*A. pectinifera*) (*41*); snail (*Biomphalaria glabrata*) (*42*); Antarctic fish (*P. magellanica*) (*43*); sardine (*S. pilchardus*) (*14*); Japanese anchovy (TR-II) (*E. japonica*) (*13*); true sardine (TR-S) (*S. melanostictus*) and arabesque greenling (TR-P) (*P. azonus*) (*12*); jacopever (TR-J) (*Sebastes schlegellii*) (*32*); common carp (*Cyprinus carpio*) (*33*); mandarin fish (*Siniperca chuasti*) (*44*); walleye pollock (*T. chalcogramma*) (*31*); bluefish (*Pomatomus saltatrix*) (*34*); rat (*45*); dog (*46*); bovine(*47*); porcine (*48*); and human (*49*).

sequence was replaced by Tyr_{10} in Jacopever trypsin. However, there are two amino acid residues in the 12-terminal sequence that differ from trypsins from common carp (*Cyprinus carpio*) (*33*), bluefish (*Pomatomus saltatrix*) (*34*), arabesque greenling (TR-P) (*P. azonus*) (*12*), walleye pollock (*T. chalcogramma*) (*31*), true sardine (TR-S) (*S. melanostictus*) (*12*), and sardine (*S. pilchardus*) (*14*).

Table 2. Effects of Various Enzyme Inhibitors on the Activity of Purified Trypsin from the Intestine of Smooth Hound (*M. mustelus*)[a]

inhibitors	concentration (mM)	remaining activity (%)
none		100
SBTI	1 mg/mL	8.5 ± 0.1
PMSF	5	11.2 ± 0.5
TLCK	5	12.0 ± 0.2
TPCK	5	95.5 ± 2.7
benzamidine	5	46.4 ± 3.1
EDTA	5	96 ± 2.8
β-mercaptoethanol	5	100
pepstatin A	1	100

[a] Purified enzyme was pre-incubated with various enzyme inhibitors for 30 min at 25 °C, and the remaining activity was determined at pH 8.5 and 30 °C using TAME as a substrate. Enzyme activity measured in the absence of any inhibitor was taken as 100%.

Effects of Enzyme Inhibitors on Trypsin Activity. Proteases can be classified by their sensitivity to various inhibitors (*35*). In order to determine the nature of the purified protease, the effects of a variety of enzyme inhibitors, including a chelating agent and a specific group reagent, on protease activity were investigated (**Table 2**). SBTI and TLCK, trypsin specific inhibitors, strongly inhibited the enzyme. β-mercaptoethanol, pepstatin A, an aspartic protease ihibitor, TPCK, a specific inhibitor for chymotrypsin, and EDTA, a metalloprotease inhibitor, did not affect trypsin activity. TLCK inactivates only trypsin-like enzymes by forming a covalent bond with histidine at the catalytic portion of the molecule and then blocking the substrate-binding portion at the active center (*8*). Therefore, these results confirmed that the purified enzyme is a serine proteinase, mostly likely trypsin.

Effect of pH on the Activity and Stability of Smooth Hound Trypsin. The pH activity profile of the purified smooth hound trypsin is shown in **Figure 5a**. The purified enzyme was active between pH 7.0 and 10.0, with an optimum around pH 8.5. The relative activities at pH 7.0, 9.0, and 10.0 were about 40, 83, and 32%, respectively, of that at pH 8.5. However, as shown in **Figure 5a**, trypsin activity decreased significantly above pH 10.0 and was only 4% at pH 11.0. The sharp decrease in hydrolysis of TAME by purified trypsin at low and high pH values might be attributed to irreversible protein denaturation.

The pH stability showed that the smooth hound trypsin is highly stable over a broad pH range, maintaining over 100% of its original activity between pH 7.5 and 9.0, and more than 80% and 55% of its activity at pH 10.0 and 11.0, respectively (**Figure 5b**). The pH stability of smooth hound trypsin was similar to that reported by Khantaphant and Benjakul (*36*) for brownstripe red snapper trypsin and was higher than Monterey sardine trypsin, which was stable in the pH range from 7.0 to 8.0 (*11*). However, smooth hound trypsin was unstable below pH 6.0. No activity was found after incubation at pH 4.0 for 60 min, suggesting irreversible denaturation of trypsin at acidic pH values.

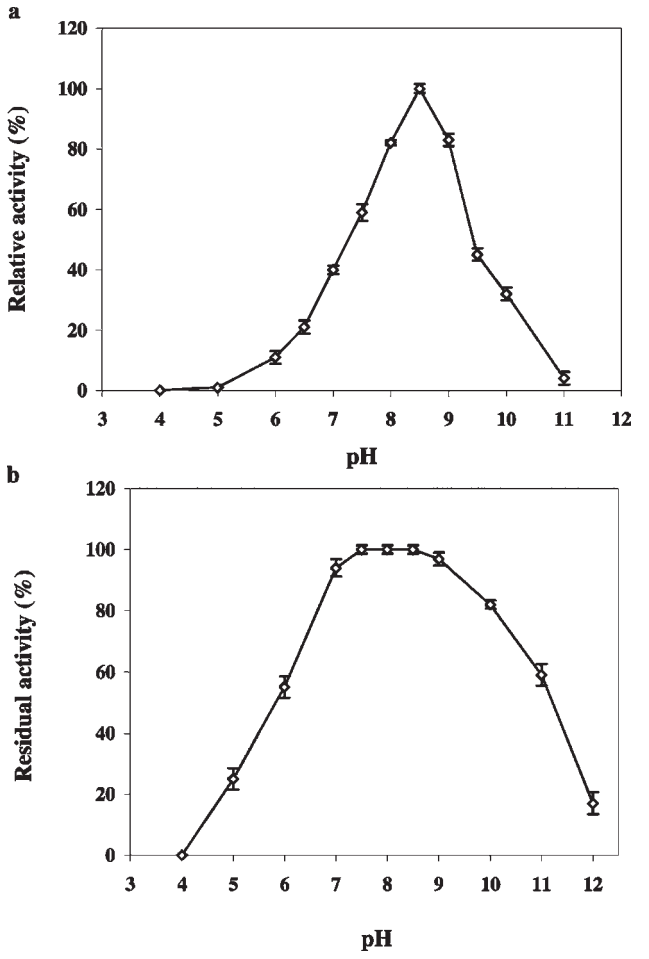

Figure 5. pH profile (**a**) and pH stability (**b**) of the purified trypsin from the intestine of smooth hound. Trypsin activity was assayed in the pH range of 4.0 to 12.0 at 30 °C. The maximum activity obtained at pH 8.5 was considered as 100% activity. The pH stability of the enzyme was determined by incubating the enzyme in different buffers for 60 min at 25 °C, and the residual enzyme activity was determined at pH 8.5 and 30 °C using TAME as a substrate. The activity of the enzyme before incubation was taken as 100%. Buffer solutions used for pH activity and stability are presented in Materials and Methods.

Effect of Temperature on the Activity and Stability of Smooth Hound Trypsin. The effect of temperature on activity was determined by assaying enzyme activity at different temperatures (**Figure 6a**). The trypsin from smooth hound was active at temperatures from 20 to 70 °C with an optimum around 50 °C. The relative activities at 40, 55, and 60 °C were about 50, 89, and 62%, respectively, of that at 50 °C. The optimum temperature of *M. mustelus* trypsin was similar to that from the viscera of bogue (*B. boops*) (*17*), lower than that described by Balti et al. (*6*) for cuttlefish trypsin (70 °C), and higher than that of trypsin from Gray triggerfish, which had the optimal temperature of 40 °C (*15*).

The thermal stability profile of the purified trypsin showed that the enzyme is highly stable at temperatures below 40 °C but was inactivated at higher temperatures (**Figure 6b**). The enzyme retained more than 60 and 40% of its initial activity after 60 min of incubation at 50 and 55 °C, respectively. The thermal stability of *M. mustelus* trypsin is similar to that of other marine vertebrate and invertebrate trypsins. *M. mustelus* trypsin was more stable than that from the pyloric ceca of chinook salmon (*O. tshawytscha*) (*9*), which lost over 90% of its activity after 30 min at 60 °C.

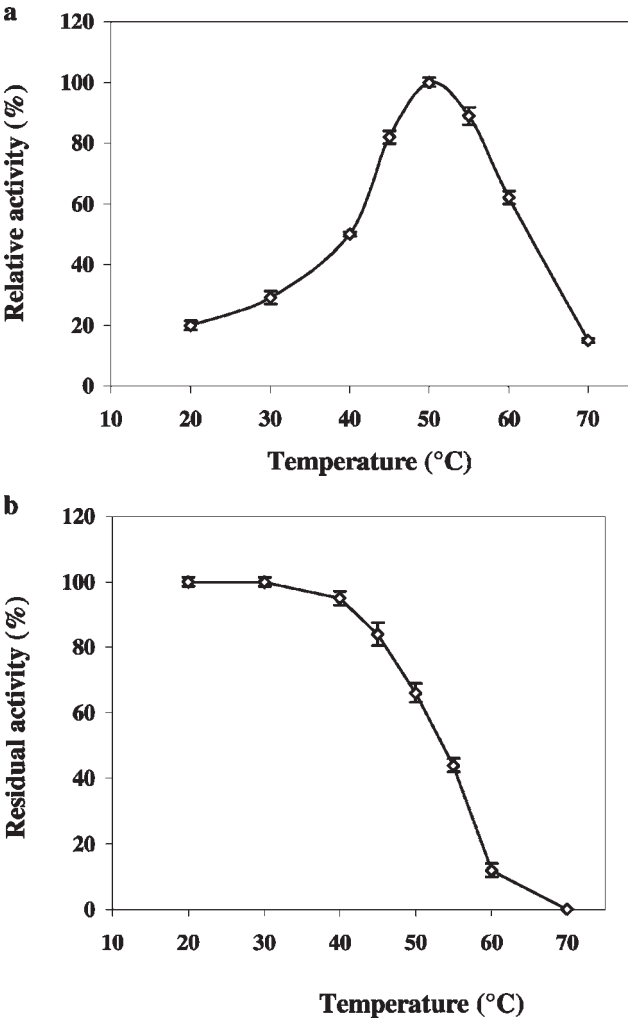

Figure 6. Temperature profile (**a**) and thermal stability (**b**) of the purified trypsin from the intestine of smooth hound. Enzyme activity was assayed at different temperatures ranging from 20 to 70 °C at pH 8.5 using TAME as a substrate. The activity of the enzyme at 50 °C was taken as 100%. For thermal stability, the enzyme was incubated at different temperatures for 60 min. The residual activity was assayed at pH 8.5 and 30 °C. The nonheated enzyme was considered as the control (100%).

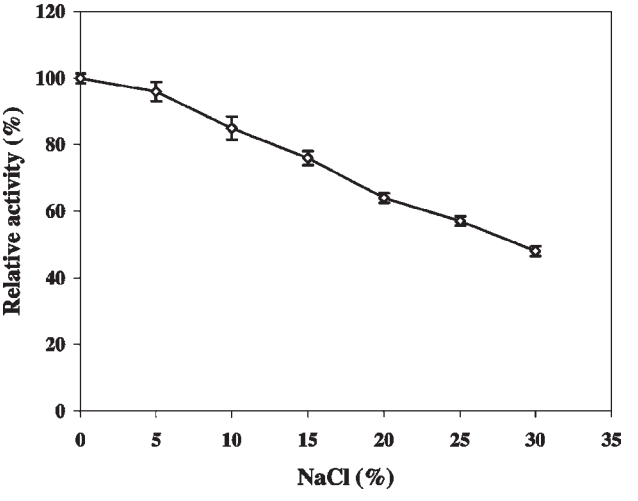

Figure 7. Effect of NaCl concentration on the activity of purified trypsin.

Effect of NaCl Concentration. The effect of NaCl on the activity of *M. mustelus* trypsin was studied at pH 8.5 and 30 °C by the

Table 3. Kinetic Parameters of *M. Mustelus* Trypsin and Other Fish Trypsins

substrates	trypsins	K_m[a] (mM)	K_{cat}[a] (s^{-1})	K_{cat}/K_m (s^{-1} mM^{-1})	references
BAPNA	smooth hound (*M. Mustelus*)	0.387	2.62	6.77	this work
	brownstripe red snapper (*Lutjanus vitta*)	0.507	4.71	9.27	*34*
	cuttlefish (*S. officinalis*)	0.064	2.32	36.25	*6*
	striped seabream (*L. mormyrus*)	0.29	1.36	4.68	*16*
	Gray triggerfish (*B. capriscus*)	0.068	2.76	40.58	*15*
	sardinelle (*S. aurita*)	1.67	3.87	2.31	*18*
TAME	smooth hound (*M. Mustelus*)	0.156	59.15	379.16	this work
	skipjack tuna (*Katsuwonus pelamis*)[S]				*7*
	A	0.29	114	393.10	
	B	0.20	61.5	307.50	
	C	0.11	57.1	519.09	
	skipjack tuna (*Katsuwonus pelamis*)[I]				*36*
	A	0.22	82.5	266.13	
	B	0.31	69.5	315.91	
	brownstripe red snapper (*Lutjanus vitta*)	0.328	112	341	*34*
	tongol tuna (*Thunnus tonggol*)	0.25	200	800	*37*

[a] K_m and K_{cat} values of all trypsins were determined at 30 °C under optimal pH, except trypsins from cuttlefish, striped seabream, Gray triggerfish, and sardinelle, which were assayed at 25 °C. S: spleen. I: intestines.

addition of NaCl to the reaction mixture (**Figure 7**). A continuous decrease in trypsin activity was observed with increasing NaCl concentration. The relative activity of trypsin at 30% NaCl was approximately 48%. The trypsin from the intestine of smooth hound may be used to facilitate the hydrolysis of proteins in high salt fermented fish products such as fish sauce.

The decrease in activity might be due to the denaturation of the enzyme caused by the salting out effect with increasing NaCl concentration. NaCl at higher concentration possibly competed with the enzyme in water binding, resulting in a stronger protein−protein interaction, which was possibly associated with precipitation.

Kinetic Properties. Kinetic data for the purified trypsin from smooth hound are summarized in **Table 3**. K_m and K_{cat} for the hydrolysis of BAPNA were 0.387 mM and 2.62 s^{-1}, respectively, and the corresponding values for the hydrolysis of TAME were 0.156 mM and 59.15 s^{-1}, respectively. The catalytic efficiencies (K_{cat}/K_m) for the hydrolysis of BAPNA and TAME were calculated to be 6.77 and 379.16 $s^{-1}mM^{-1}$, respectively.

The results indicated that trypsin from smooth hound had a higher affinity for TAME than did BAPNA. These results were similar to those reported by Khantaphant and Benjakul, (*34*). The kinetic parameters of trypsin from *M. mustelus* were also compared with those of other fish species (**Table 3**). For the BAPNA hydrolysis reaction, trypsin from smooth hound had a higher K_m value than trypsins from cuttlefish (*6*), sardinelle (*18*), Gray triggerfish (*15*), and striped seabream (*16*). K_m is often associated with the affinity of the enzyme for substrate (*37*). Therefore, trypsin from smooth hound had a lower affinity for BAPNA than those from other fish species.

For TAME hydrolysis, a lower K_m was observed for trypsin from smooth hound than those from skipjack tuna (*38*), brownstripe red snapper (*36*), and Tongol tuna (*39*). This indicated that trypsin from smooth hound had a higher affinity for TAME than trypsins from skipjck tuna, brownstripe red snapper, and Tongol tuna.

Catalytic efficiency (K_{cat}/K_m) of trypsin from smooth hound was lower than those of trypsins from cuttlefish (*6*), Gray triggerfish (*15*), and brownstripe red snapper (*36*), when BAPNA was used as a substrate. A higher catalytic efficiency was noticeable, compared with that of trypsin from the intestines of skipjack tuna when TAME was used as a substrate.

Conclusions. In the present study, trypsin from the intestine of smooth hound was purified, and its molecular weight was estimated to be 24 kDa by gel filtration as well as by SDS−PAGE. After the final purification step, the enzyme was purified 65.6-fold with a specific activity of 21.65 U/mg and 15.3% recovery. The enzyme showed an optimum temperature at 50 °C and an optimum pH of 8.5. The enzyme was stable at a pH range of 7.0−10.0 and had a high thermostability at temperatures below 40 °C. The N-terminal sequence of the trypsin showed high homology with vertebrate and invertebrate trypsins. Further research is needed to determine properties of *M. mustelus* trypsin as a possible biotechnological tool in the fish processing and food industries.

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