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Purification and Characterization of Trypsin from the Intestine of Hybrid Tilapia (*Oreochromis niloticus* × *O.aureus*)

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Trypsin from the intestine of hybrid tilapia (*Oreochromis niloticus* × *O.aureus*) was purified by the following techniques: acetone precipitation, ammonium sulfate fractionation, Sephacryl S-200 gel filtration, and DEAE-sephacel ion exchange chromatography. The purified enzyme was determined to be homogeneous by polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)−PAGE. The molecular weight was estimated as 22,000 Da. The optimum pH and temperature of the enzyme for the hydrolysis of casein were determined to be 9.0 and 60 °C, respectively. The enzyme was stable over a broad pH range from 7.0 to 12.0 at 30 °C, and the enzyme was inactive at temperatures above 50 °C. The behavior of the enzyme for the hydrolysis of casein followed Michaelis−Menten kinetics with K_m of 0.46 mg/mL. The purified enzyme was inhibited by the general serine protease inhibitor phenyl methyl sulphonyl fluoride (PMSF) and also by the specific trypsin inhibitor *N-p*-tosyl-L-lysine chloromethyl ketone (TLCK) using N_α-CBZ-L-lysine *p*-nitrophenyl ester hydrochloride (CBZ-Lys·pNP) as a substrate. The protease was inhibited by the following ions in decreasing order: $Zn^{2+} > Fe^{3+} > Cu^{2+} > Al^{3+} > Co^{2+} = Pb^{2+} > Cd^{2+} > Mn^{2+}$. The ions Li^+, Na^+, K^+, Mg^{2+}, and Ba^{2+} had little effect on the enzyme, and Ca^{2+} can partially promote its activity at low concentration.

KEYWORDS: Trypsin; purification; characterization; intestine; hybrid tilapia

INTRODUCTION

Fish processing generates large amounts of solid and liquid wastes, leading to potential pollution problems. Many different byproducts have been produced from fish processing wastes, among which, fish viscera account for approximately 5−10% of total mass, are one of the most important byproducts of the fisheries industry, and are recognized as potential sources of various enzymes that may have some unique properties of interest for both basic research and industrial applications (*1*).

A variety of digestive proteolytic enzymes have been isolated from the internal organs of fish. The most important proteolytic enzymes from fish viscera are the aspartic protease pepsin and serine proteases trypsin, chymotrypsin, and elastase. Acidic proteases from the fish stomach display high activity between pH 2.0 and 4.0, while alkaline digestive proteases, such as trypsin, are most active between pH 8.0 and 10.0 (*2*). Trypsin has many industrial applications, and it is a very important enzyme in the food industry because it is 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 (*3*). Fish trypsins have been isolated and characterized from the viscera from different species of fish including tongol tuna (*Thunnus tonggol*) (*4*), sardine (*Sardina pilchardus*) (*2*), spotted goatfish (*Pseudupeneus maculatus*) (*5*), jacopever (*Sebastes schlegelii*), elkhorn sculpin (*Alcichthys alcicornis*) (*6*), chinook salmon (*Oncorhynchus tshawytscha*) (*7*), bluefish (*Pomatomus saltatrix*) (*8*), skipjack tuna (*Katsuwonus pelamis*) (*9*), mandarin fish (*Siniperca chuatsi*) (*10*), monterey sardine (*Sardinops sagaxcaerulea*) (*11*), spotted mackerel (*Scomber australasicus*) (*12*), yellow tail (*Seriola quinqueradiata*), brown hakeling (*Physiculus japonicus*) (*13*), Japanese anchovy (*Engraulis japonica*) (*14*), Pacific cod (*G. macrocephalus*), saffron cod (*E. gracilis*) (*15*), and masu salmon (*Oncorhynchus masou*) (*16*).

In China, tilapia is one of the most important farmed fish. Catches of tilapia were about 1210,000 tonnes in 2007, accounting for 49% of the world's production. The species can be living in both fresh water and brackish water, whose optimum habitat temperature is 24−32 °C. It is predominantly herbivorous and able to produce high quality protein for human consumption (*17*). China has been the largest tilapia producing and exporting country in the world, and tilapia is commonly exploited as a raw material for canning production and chilled fillets. During processing, the tilapia fishery industry in China produces large quantities of waste (including viscera) each year, which are not currently put to good use and discarded. These wastes, which represent an environmental problem to the fishing industry, are composed of important sources of proteolytic enzymes, such as

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trypsin, pepsin, chymotrypsin, and elastase. In order to make good use of the potential resources, the aims of our study were to isolate and purify trypsin from the intestine of the hybrid tilpia (*Oreochromis niloticus* × *O.aureus*). In this article, we describe the purification and biochemical characterization of trypsin from the intestine of hybrid tilapia.

MATERIALS AND METHODS

Biological Materials. Hybrid tilapia (*Oreochromis niloticus* × *O.aureus*) with an average body mass of 0.5 kg and length of 0.25 m was purchased from the local market at Xiamen City, China. The fish were kept in ice and transported to the research laboratory within 1 h. The fish were eviscerated, and the intestines were dissected and cautiously cleaned with cold deionized water. Then the defatted intestine powder was prepared with cold acetone according to the method of Klomklao et al. (*18*). The powder was stored at −20 °C until used.

Reagents. DEAE-sephacel and Sephacryl S-200 HR were purchased from Pharmacia Biotech (Uppsala, Sweden). Ethylenediaminetetraacetic acid (EDTA), 1-(L-*trans*-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), *N*-ethylmaleimide, iodoacetic acid, *N*-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), phenylmethanesulfonyl fluoride (PMSF), pepstatin A, and N_α-CBZ-L-lysine *p*-nitrophenyl ester hydrochloride (CBZ-Lys·pNP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Casein was from Sino-American Biotechnology Co. (Shanghai, China). All other reagents were of analytical grade. The water used was redistilled and ion-free.

Preparation of Crude Extract. Defatted intestine powder was suspended in 50 mM Tris-HCl at pH 8.0, referred to as buffer A at a ratio of 1:50 (w/v), and stirred continuously at 4 °C for 3 h. The mixture was centrifuged at 10,000*g* for 20 min at 4 °C using a Sigma Laborzentrifugen refrigerated centrifuge (Osterode Harz, Germany) to remove the tissue debris. The supernatant was collected and referred to as the crude extract.

Trypsin Purification. The crude enzyme extract was subjected to ammonium sulfate fractionation, and the precipitate in the 30−70% saturation range was collected by centrifugation 20 min at 10,000*g*. The precipitate was dissolved in a minimal volume of buffer A and dialyzed at 4 °C against this buffer. The enzyme dialysate was then applied onto a Sephacryl S-200 column (1.65 × 60 cm) pre-equilibrated with buffer B (10 mM Tris-HCl buffer at pH 8.0). The column was then eluted with the same buffer at a flow rate of 0.5 mL/min, and fractions of 4 mL were collected. Protein content (Abs 280 nm) and protease activity were measured. Fractions showing protease activities were pooled. Pooled fractions were dialyzed against buffer B and were condensed by the polyethylene glycol 20,000. The condensed sample was then chromatographed using a DEAE-sephacel column (1.65 × 26 cm) pre-equilibrated with buffer B. The column was washed with the same buffer at a flow rate of 0.8 mL/min until A_{280} was less than 0.05 and then eluted with buffer B containing NaCl; the elution was carried out by a linear gradient of 0.0−1.0 M NaCl in buffer B at a flow rate of 0.8 mL/min, and fractions of 4 mL were collected. Fractions showing protease activities were pooled. Pooled fractions were dialyzed against buffer B and then stored at −20 °C for further research. All purification processes were performed in a chromatographic ice cuber at a temperature of 4 °C.

Determination of the Protein Concentration and Assay of Enzymatic Activity. Protein concentration was measured by the method of Lowry et al. (*19*) using bovine serum albumin (BSA) as a standard. Protease activity was measured by the method of Chen et al. (*20*) using casein as a substrate. The enzyme solution (20 μL) was mixed with 1.98 mL of 50 mM Tris-HCl (pH 8.0) containing 1 mL of 1% casein and incubated for 20 min at 45 °C. Then 2 mL of 5% trichloroacetic acid (TCA) was added to stop the reaction. After 10 min, the mixture was filtered to remove the precipitate. The absorbance was measured at 275 nm. A standard curve was generated using solutions of 0−100 μg/mL tyrosine. One unit (U) of enzymatic activity was defined as the amount of enzyme capable of hydrolyzing casein to liberate 1 μg of tyrosine per min under experimental conditions.

Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out according to the method of Laemmli (*21*), using a 5% (w/v) stacking gel and a 12.5% (w/v) separating gel. Samples were prepared by mixing the purified enzyme solution at 1:5 (v/v) ratio with SDS−PAGE sample buffer containing 10 mM Tris-HCl at pH 6.7, 2.5% SDS, 5% β-mercaptoethanol, 20% glycerol, and 0.02% bromophenol blue, and then the samples were boiled for 5 min. After electrophoresis, the gels were stained with 0.25% Coomassie Brilliant Blue R-250 in 45% ethanol and 10% acetic acid. Then the background of the gels was destained with 40% ethanol and 10% acetic acid. The molecular weight of the enzyme was estimated using the protein standards as markers: β-galactosidase (116,000 Da); bovine serum albumin (66,200 Da); ovalbumin (45,000 Da); lactate dehydrogenase (35,000 Da); restriction endonuclease (25,000 Da); bovine milk β-lactoglobulin (18,400 Da); and egg white lysozyme (14,400 Da).

Native−PAGE was performed using 12.5% separating gels in a similar manner, except that the sample was not heated, and the SDS reducing agent was not added to the sample buffer.

Effect of pH on Enzyme Activity. The effect of pH was determined with casein as a substrate. Protease activity was assayed in the pH range of 4.0−13.0 (50 mM acetate buffer for pH values 4.0 to 6.0, 50 mM Tris-HCl buffer for pH values 7.0 to 9.0, and 50 mM glycine-NaOH buffer for pH values 10.0 to 13.0) at a temperature of 45 °C for 20 min. For the evaluation of pH stability, the enzyme was incubated at 30 °C for 30 min in different buffers, and then the residual enzyme activity was measuring under standard assay conditions.

Optimum Temperature and Thermal Stability. The effect of temperature on proteolytic activity was studied from 20 to 80 °C for 20 min at pH 8.0 using 50 mM Tris-HCl buffer. For thermal stability, the enzyme mixed with 50 mM Tris-HCl buffer at pH 8.0 was incubated at different temperatures (20, 30, 40, 50, 60, 70, and 80 °C) for 20 min. After that, the heated samples were rapidly cooled in ice, and the remaining activity was assayed at the standard conditions.

Effects of Some Metal Ions on Enzyme Activity. The effects of various metal ions (2 mM) on enzyme activity were determined using Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}, Fe^{3+}, Al^{3+}, Cd^{2+}, and Pb^{2+}. The samples of the purified enzymes (20 μL) were added to the glass cuvettes mixed with 1.98 mL of 50 mM Tris-HCl (pH 8.0) including 1 mL of 1% casein and 400 μL of 10 mM various metal ions, and incubated for 20 min at 45 °C. The following steps were the same as those in the enzyme assay method as described above.

Effects of Inhibitors on Enzyme Activity. The effects of inhibitors on trypsin activity were studied using CBZ-Lys·pNP as a substrate. The enzyme solution (20 μL) was mixed with 2.98 mL of 20 mM CBZ-Lys·pNP and different concentration inhibitors in 0.1 M NaAc−HAc (pH 5.8), and we mearsured the absorbance of 348 nm changing with time using a UV−visible spectrophotometer (Varian Australia Pty LTD) under room temperature.

Assay of Kinetic Parameters of Enzyme. The activity was assayed with different final concentrations of casein ranging from 0.1 to 4.0 mg/mL. The final enzyme concentration for the assay was 0.2 mg/mL. The respective kinetic parameters including the Michaelis−Menten constant (K_m) and maximum velocity (V_m) were evaluated by plotting the data on a Lineweaver−Burk double-reciprocal graph (*22*).

RESULTS AND DISCUSSION

Purification of Trypsin. Purification of trypsin from hybrid-tilapia intestine is summarized in **Table 1**. An increase in purity of 2.7-fold was obtained with the yield of 71.4% by ammonium

Table 1. Summary of the Purification of Trypsin from Hybrid Tilapia

purification steps	total activity (U)	total protein (mg)	specific activity (U/mg)	purification (fold)	yield (%)
scetone precipitation	686166	247	2778	1	100
$(NH_4)_2SO_4$ (30−70%)	489786	66	7421	2.7	71.4
Sephacryl S-200	197649	19	10403	3.8	28.8
DEAE-sephacel	155200	5	31040	11.2	22.6

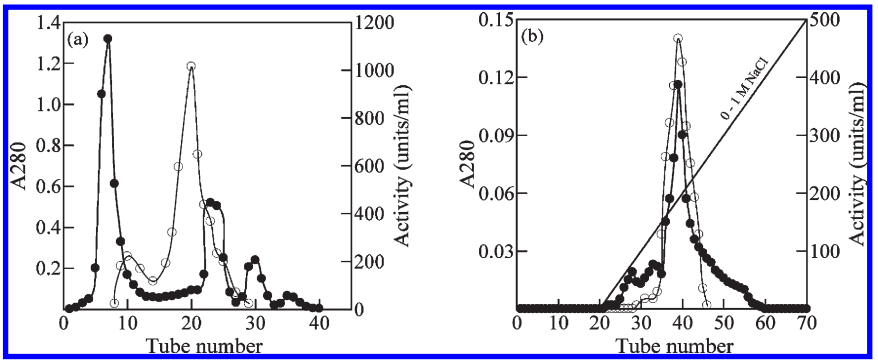

Figure 1. Column chromatography of typsin from hybrid tilapia intestine on Sephacryl S-200 (**a**) and DEAE-sephacel (**b**). -○-, enzyme activity; -●-, protein concentration.

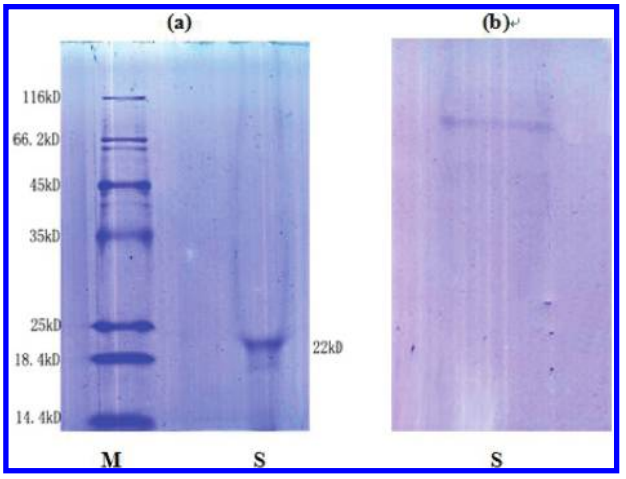

Figure 2. Electrophoresis of purified trypsin from tilapia intestine determined by SDS−PAGE (**a**) and native-PAGE (**b**). M, standard protein markers (kDa); S, sample of purified trypsin.

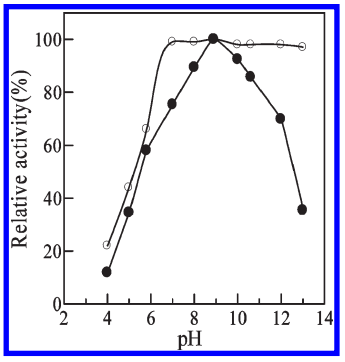

Figure 3. pH optimum and pH stability of the purified trypsin from tilapia intestine (50 mM acetate buffer for pH values 4.0 to 6.0, 50 mM Tris-HCl buffer for pH values 7.0 to 9.0, and 50 mM glycine-NaOH buffer for pH values 10.0 to 13.0). -○-, pH stability; -●-, pH optimum.

sulfate precipitation (30−70%), suggesting the removal of some other proteins in the crude extract. Ammonium sulfate precipitation was introduced as an initial step to remove other proteins in the crude extract. Klomklao et al. (*8*) found that ammonium sulfate precipitation (40−60%) of trypsin from bluefish pyloric ceca resulted in a 8.3-fold increase in specific activity. Then the protein was subjected to gel filtration on a Sephacryl S-200 column (**Figure 1a**), leading to an increase in purity by 3.8-fold with a recovery of 28.8%. Pooled active Sephacryl S-200 fractions were further purified using a DEAE-sephacel column. This step resulted in a considerable increase in the specific activity, and only one active peak was observed (**Figure 1b**). After the final purification step, trypsin was purified by 11.2-fold with a yield of 22.6%, and a specific activity of 31,040 U/mg protein was obtained. Therefore, trypsin could be obtained at low cost from viscera (intestine) produced as waste in hybrid tilapia industrial processing. Purification to homogeneity of trypsin was achieved by acetone precipitation, ammonium sulfate fractionation (30−70%), Sephacryl S-200 gel filtration, and DEAE-sephacel ion exchange chromatography.

Molecular Weight of Enzyme. The molecular weight of the purified enzyme was estimated by SDS−PAGE as 22,000 Da (**Figure 2a**), which was in the range of 20,000−30,000 Da for trypsins purified from various fish species (*23*). The molecular weight of hybrid tilapia trypsin was similar to those reported for other fish trypsins, such as sardine (*2*), spotted goatfish (*5*), tongol tuna (*4*), Nile tilapia (*24*), mandrish fish (*10*), skipjack tuna (*9*), jacopever, elkhorn sculpin (*6*), and Theragra (*25*). Electrophoresis results indicated that trypsin from hybrid tilapia had a monomeric structure with a molecular weight of 22,000 Da.

Optimum pH and Stability. The effect of pH on enzyme activity was determined over a pH range of 4.0−13.0. The purified enzyme was active between pH 7.0 and 11.0, and the maximal activity of the enzyme was observed at pH 9.0 (**Figure 3**). Loss of activity was found at very acidic and high alkaline pH because most enzymes suffer irreversible denaturation in very acidic and alkaline conditions. The optimum pH of trypsin from hybrid tilapia was similar to those of trypsins from the intestine and pyloric ceca of spotted goatfish (*5*), pyloric ceca of bluefish (*8*), and viscera of sardine (*2*).

For pH stability, trypsin was very stable in a broad pH range of 7.0 −13.0 (**Figure 3**), but it was unstable below pH 6.0. After incubating at pH 4.0, the trypsin lost all the enzyme activity. Trypsin in general tended to be more active at alkaline pH (*23*), for example, the trypsin purified from the viscera of sardine (*2*), the pyloric ceca of bluefish (*8*) and carp (*26*), the pyloric ceca of rainbow trout (*27*), and the spleen of skipjack tuna (*9*) also exhibited high activity at alkaline pH.

Optimum Temperature and Thermal Stability. The effect of temperature on enzyme activity was determined by assaying enzyme activity at different temperatures (**Figure 4**). Trypsin from hybrid tilapia was active at temperatures from 40 to 70 °C and had an optimum at 60 °C. A sharp decrease in activity was found when the temperature was increased above 80 °C, possibly due to thermal denaturation. Similar results have also been reported for trypsins from starfish pyloric ceca, which had the optimal temperature of 55 °C (*28*), skipjack tuna spleen, which had optimum temperature of 60 °C (*9*), and jacopever and elkhorn sculpin pyloric ceca, which had the optimal temperature of 60 and 50 °C (*6*).

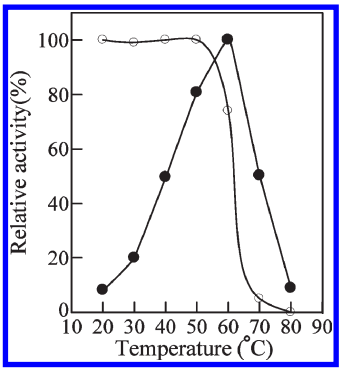

Figure 4. Temperature optimum and thermal stability of the purified trypsin from tilapia intestine. -○-, thermal stability; -●-, temperature optimum.

Table 2. Effects of Various Metal Ions on the Activity of Purified Trypsin from Hybrid Tilapia Intestines

metal ions	concentrations (mM)	relative activity (%)
control		100.0
Li^{+}	2.0	100.0
Na^{+}	2.0	98.5
K^{+}	2.0	99.0
Ca^{2+}	2.0	125.0
Mg^{2+}	2.0	97.8
Ba^{2+}	2.0	96.4
Mn^{2+}	2.0	60.5
Mn^{2+}	4.0	20.4
Cu^{2+}	1.0	55.5
Cu^{2+}	2.0	10.2
Cu^{2+}	4.0	0.0
Co^{2+}	1.0	76.8
Co^{2+}	2.0	43.6
Zn^{2+}	0.05	79.2
Zn^{2+}	1.0	48.9
Zn^{2+}	2.0	0.0
Al^{3+}	1.0	59.3
Al^{3+}	2.0	4.5
Fe^{3+}	1.0	49.5
Fe^{3+}	2.0	0.0
Cd^{2+}	1.0	75.4
Cd^{2+}	2.0	45.5
Pb^{2+}	1.0	79.6
Pb^{2+}	2.0	43.8

For thermal stability, the purified trypsin was highly stable below 50 °C, but the activity sharply decreased when the temperature was above 70 °C (**Figure 4**). The enzyme activity was almost completely lost at 80 °C. Trypsin from hybrid tilapia intestine displayed thermal stability similar to those of other fish species. Kishimura et al. (*29*) found that trypsin activity of true sardine viscera was stable up to 50 °C and that the activity disappeared when it was heated above 70 °C. Trypsin fraction from the pyloric ceca of chinook salmon was stable below 50 °C for 30 min but lost its activity rapidly at temperatures above 60 °C (*7*).

Effects of Some Metal Ions on Enzyme Activity. The effects of some metal ions (2 mM) on the activity of trypsin from hybrid tilapia intestine were shown in **Table 2**. Li^{+}, Na^{+}, K^{+}, Mg^{2+}, and Ba^{2+} showed almost no influence on enzyme activity, but Ca^{2+} slightly improved enzyme activity. It was suggested that the majority of alkali metal ions and alkaline earth metal ions in general had no effect on the enzyme except that Ca^{2+} could activate protease activity. Therefore, the trypsin was fairly salt tolerant, suggesting that the enzyme could be used in a high-salt environment. Similar results have been reported from Nile tilapia

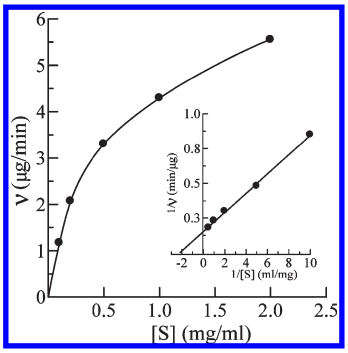

Figure 5. Lineweaver−Burk plot for the determination of K_m and V_m for trypsin from tilapia intestine on the hydrolysis of casein. Conditions were 50 mM Tris-HCl (pH 8.0) with different concentrations of casein at 45 °C. The concentration of enzyme was 0.2 mg/mL.

Table 3. Effects of Various Enzyme Inhibitors on the Activity of Purified Trypsin from Hybrid Tilapia Intestine

inhibitors	concentrations (mM)	inhibition (%)
control		0
E-64	0.05	0
N-ethylmaleimide	1.0	0
iodoacetic acid	1.0	5
TPCK	5.0	3
TLCK	5.0	80
PMSF	5.0	70
pepstatin A	0.1	0
EDTA	2.0	25

intestine (*24*), saridine viscera (*2*), mandarin fish pyloric ceca (*10*), and spotted goatfish pyloric ceca (*5*). However, Mn^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}, Al^{3+}, Fe^{3+}, Cd^{2+}, and Pb^{2+} could inhibit enzyme activity, particularly, among them the Zn^{2+}, Al^{3+}, and Fe^{3+} seriously inhibited enzyme activity. It is known that Co^{2+}, Cd^{2+}, and Hg^{2+} act on sulphydryl residues in proteins (*30*). Inhibition caused by these metal ions suggests that the sulfhydryl residues may be the active side for the catalytic action of this protease. These results also implied that this trypsin may be a metalloprotease that probably maintains some metal-binding sites in its structure. This is confirmed by EDTA inhibition (**Table 3**).

Effects of Inhibitors on Enzyme Avtivity. The effects of various proteinase inhibitors on the activity of hybrid tilapia intestine trypsin were determined (**Table 3**). Trypsin was markedly inhibited by the serine−protease inhibitor PMSF (70% inhibition) and trypsin specific inhibitor TLCK (80% inhibition), while specific inhibitors of cysteine proteinase (E-64, *N*-ethylmaleimide, and iodoacetic acid) and aspartic proteinase (pepstatin A) did not show inhibitory effects toward trypsin activity. Also when using the chymotrypsin specific inhibitor TPCK, there was almost no inhibition observed. These results for molecular weight and effects of inhibitors confirmed that the purified enzyme belonged to serine proteinase, most likely trypsin. From the results, metalloprotease inhibitor EDTA, which chelates the metal ions required for the enzyme, partially inhibited enzyme activity by 25%, indicating the importance of some metal ions in enzyme stabilization. It was suggested that metal ions most likely were also the factors for enzyme activity.

Kinetic Studies. Hybrid tilapia intestine trypsin hydrolyzed casein obeyin the Michealis−Menten equation. Kinetic constants for casein hydrolysis by the enzyme were determined using Lineweaver−Burk double-reciprocal graph (**Figure 5**). K_m and V_m of the trypsin were calculated to be 0.46 mg/mL and 6.6 μg/min, respectively.

In conclusion, the enzyme was similar in many biochemical characteristics to the trypsin of other fish, and these characteristics suggest that the enzyme possibly could be an important biotechnological tool for the fish processing and food industries.

ABBREVIATIONS USED

PMSF, phenyl methyl sulphonyl fluoride; TLCK, *N*-*p*-tosyl-L-lysine chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid; E-64, 1-(L-*trans*-epoxysuccinyl-leucylamino)-4-guanidinobutane; CBZ-Lys·pNP, N_{α}-CBZ-L-lysine *p*-nitrophenyl ester hydrochloride; IC_{50}, inhibitor concentrations leading to 50% activity loss; PBS, sodium phosphate buffer; K_m, Michaelis−Menten constant; V_m, maximum velocity.

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