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Characterization of Partial Purified Trypsin and Chymotrypsin from Viscera of Nile Tilapia (*Oreochromis niloticus* Linneaus)

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ABSTRACT

The tryptic and chymotryptic activities of extraction of spleen, liver, stomach, intestine and mixed viscera of Nile tilapia (*Oreochromis niloticus* Linneaus) were compared. Intestine was the best sources for trypsin and chymotrypsin. Trypsin and chymotrypsin fractions were extracted from intestine of Nile tilapia by 30-70% saturated ammonium sulfate precipitation, dialyzed, acetone precipitation and separated by SBTI affinity chromatography column. Specific activities of trypsin and chymotrypsin were 0.529 and 0.380 unit/mg protein, respectively. The purities of trypsin and chymotrypsin fraction were increased by 5.56 and 3.62 folds, respectively. The optimum temperature and pH of trypsin fraction were 80°C and pH 9.0. The optimum temperature and pH of chymotrypsin fraction were 60°C and pH 9.0. Trypsin fraction was stable at 0-60°C and chymotrypsin was stable at 0-50°C for 30 min at pH 8.0.
Key words: tilapia, *Oreochromis niloticus*, protease, trypsin, chymotrypsin

INTRODUCTION

Fish processing generates large amount of wastes. Normally, more than half of the raw material weight is unused. However, fishery by-products are typically used as feeds and fertilizers that have a low value. There is growing interest in obtaining higher valued biochemicals and pharmaceuticals from fishery wastes, notably enzymes (El-Beltagy *et al*., 2004).

Fisheries processing industry is the big industry in Thailand. The quantities of fishery products exported in 2003 and 2004 were 1,642,033 tons and 1,676,378 tons (Fisheries Foreign Affairs Division, 2005). Nile tilapia "Chitalada" strain (*Oreochromis niloticus* Linneaus) is the accepted international strain from Thailand. This fish is an important freshwater aquacultured fish in Thailand, exported to Japan, USA, Italy etc. The export items are frozen whole fish, fresh and frozen fillet. The total volume of Nile tilapia exportation in 2003 and 2004 were 4,708 tons and 7,622 tons (Fisheries Foreign Affairs Division, 2005). This is an interesting industry that will give large volume of waste to be source of enzyme. The purpose of the present study was to purify and characterized trypsin and chymotrypsin from viscera of Nile tilapia.

MATERIALS AND METHODS

Sample preparation

Nile tilapia "Chitalada" strain (*O. niloticus* Linn.) were obtained from a local fish

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farm in Panatnikom, Chonburi province, Thailand, stored in iced during transportation to laboratory. The viscera were separated into individual organ consisted of spleen, liver, stomach, intestine and mixed viscera and stored at –40°C prior to extraction.

Preparation of crude enzyme extract

Enzyme was extracted from the viscera, using the method described by Simpson and Haard (1984). All procedures were performed at 4°C unless otherwise stated. The individual organs were separated and mixed with extraction buffer (0.05 M Tris-HCl, pH 7.0) at a ratio of 1: 5 (w/v). The slurry was stirred slowly for 3 hr and centrifuged at 3,000 g for 30 min. The supernatant (crude extract) was collected.

Purification of trypsin

Crude enzyme extract was fractionated with solid ammonium sulfate and the precipitate forming between 30 and 70% saturation was dissolved in a minimum amount of extraction buffer to obtain ammonium sulfate fraction. The fraction was dialyzed against about 100 volumes of extraction buffer for 16 hr changing of buffer twice to obtain dialyzed fraction. The dialysis fraction was added three volumes of cold acetone (-20°C) and then kept at -20°C for 3 hr. The precipitate form was collected by centrifugation at 6,000 g for 30 min. The acetone precipitate was suspended in a minimum amount of extraction buffer to obtain acetone fraction.

Five milliliter of the acetone fraction was loaded into affinity chromatography column (1x10 cm) packed with 8 ml of SBTI-Sepharose at a flow rate of 0.25 ml/min and equilibrated with the extraction buffer. Unabsorbed proteins were washed with extraction buffer and 4.8 ml fractions were collected until no additional fractions absorbing at 280 nm appeared. The trypsin fraction was eluted from the column using 5 mM HCl. The fractions with the highest absorption peak were collected.

Purification of chymotrypsin

The unabsorbed fraction of the SBTI-Sepharose column in trypsin purification was used for chymotrypsin purification. The highest absorbed fractions were collected.

Assay of enzyme activity

Nile tilapia trypsin activity was assayed using N-α-benzoyl-arginine –*p*- nitroanilide (BAPNA; Sigma Chemicals, USA) as substrate (Erlanger *et al*., 1961) and Nile tilapia chymotrypsin activity was assayed using N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (SAPPNA; Sigma Chemicals, USA) as substrate (Rungruangsak-Torrisen and Sundbly, 2000).

Enzyme activity was determined as described by Simpson and Haard (1985). Two hundred microliters of enzyme fraction was added to 2.8 ml of 40°C preheated specific subtrate (1 mM BAPNA or 0.2 mM SAPPNA) in 0.05 M Tris-HCl (pH 8.2) containing 0.02 M $CaCl_2$ and the release of *p*-nitroaniline was measured at 410 nm at 30°C at 15 sec intervals with the use of Thermospectronic Helios Gamma UV-Vis Spectrophotometer (USA)

Enzyme activity (unit/ ml) =

$$\frac{A410 / \ \text{min} \times \text{ml of total volume} \times 1000}{8,800 \times \text{ml of enzyme solution}}$$

Enzyme specific activity = Enzyme activity (unit/ ml)/ protein concentration (mg/ml)

where 8,800 is the molar extinction coeffecient of *p*-nitroaniline

Protein concentration

Protein concentration was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

Optimum pH and stability

The optimum pH and stability of Nile tilapia trypsin and chymotrypsin fractions were

determined as described by Simpson and Haard (1985). The compositions of the buffer solutions (Stoll and Blanchard, 1990) used were: 0.1M citrate-phosphate buffer (pH 4.0-7.0); 0.1M Tris-HCl, pH 8.0 and 0.1M glycine-NaOH (pH 9.0-10.0).

The optimum pH of trypsin and chymotrypsin fraction was determined by preincubating the specific substrate in above buffer solutions at 30°C for for 10 min and applying to the enzyme fraction to assay for the residual enzyme activity.

The influence of pH on the stability of trypsin and chymotrypsin fractions was determined by preincubating the enzyme fractions with the above buffer solutions at 0°C for 30 min and then applied to the specific substrate to assay for the residual enzyme activity.

Temperature optimum and stability

The optimum temperature and stability of Nile tilapia trypsin and chymotrypsin fractions were determined as described by Simpson and Haard (1985). The optimum temperature of the trypsin and chymotrypsin fraction was determined by preincubating the specific substrate in pH 8.0 buffer solutions at various temperatures from 0 to 80°C at 10°C intervals for 10 min. The residual enzyme activity was determined after applying substrate buffer to the enzyme fraction.

The temperature stability of the trypsin and chymotrypsin fraction was determined by preincubating the enzyme fraction in pH 8.0 buffer solutions at the above temperature for 30 min and immediately cooling on ice. Residual enzyme activity was determined after applying to the specific substrate.

Effect of inhibitors on tryptic and chymotryptic activities

The lists of inhibitors from Sigma Chemicals (USA) were as follows: 1 mM phenylmethanesul-fonyl fluoride (PMSF) in ethanol and 1.5 mM aprotinin bovine lung in 0.9% NaCl for serine protease inhibition; 100 mM soybean trypsin inhibitor (SBTI) and 50 mM N∝-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) in 1 mM HCl for trypsin inhibition; 5 mM N-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) in DMSO for chymotrypsin inhibition.

Enzyme fraction was incubated with the respective inhibitor at a ratio (v/v) of 1: 1 for 30 min at 40°C. The residual enzyme activity was determined as described by Simpson and Haard (1985). A control assay was included by adding distilled water instead of inhibitor solution.

RESULTS AND DISCUSSION

The fish weight was about 282-338 g and viscera weight was about 6 % of body weight. The viscera was separated into individual organ that consisted of spleen, liver, stomach and intestine, giving the percentage values based on the body weight of 0.08, 1.39, 0.81 and 2.49 %, respectively.

Activity of the enzymes extracted from each viscera organ of Nile tilapia is given in Table 1. Spleen gave the highest trypsin activity (1.68 unit/ml) followed by intestine, liver, mixed viscera and stomach whereas intestine gave the highest chymotrypsin activity (0.17 unit/ml) followed by spleen, mix viscera, liver and stomach. Comparing enzyme activity with body weight, it was found that intestine gave the highest activity for trypsin and chymotrypsin fractions at 7.76 and 0.107 % (activity/body weight). Therefore the intestine was selected to be a source for enzyme extraction.

Purification of Nile tilapia trypsin and chymotrypsin

The activities of enzyme during purification of trypsin and chymotrypsin fractions from Nile tilapia intestine were summarized in Table 2. Trypsin fraction had 0.21 mg protein/g intestine tissue. Enzyme activities and protein decreased at each stage hence specific activity was

Table 1 Comparison on activity of trypsin and chymotrypsin from Nile tilapia.

Organ	Trypsin			Chymotrypsin		
	Activity (U/ml)	Specific activity (U/mg)	Activity/ body weight (%)	Activity (U/ml)	Specific activity (U/mg)	Activity/ body weight (%)
Spleen	1.68	0.19	0.27	0.15	0.01	0.001
Liver	0.58	0.08	0.98	0.07	0.01	0.002
Stomach	0.03	0.01	0.06	0.07	0.01	0.0001
Intestine	0.77	0.17	7.76	0.17	0.03	0.107
Mixed viscera	0.14	0.03	2.28	0.15	0.03	0.011

Table 2 Purification of trypsin and chymotrypsin from intestine of Nile tilapia.

Purification step	Total protein (mg/ml)	Trypsin				Chymotrypsin			
		Activity (U/ml)	Specific act. (U/mg)	Yield (%)	Purity (fold)	Activity (unit/ml)	Specific act. (U/mg)	Yield (%)	Purity (fold)
Crude extract	6.66	0.63	0.095	100.00	1	0.7	0.105	100.00	1
NH_4SO_4 fraction	6.22	0.6	0.096	95.24	1.02	0.67	0.108	95.71	1.03
Dialyzed fraction	5.51	0.58	0.105	92.06	1.11	0.6	0.109	85.71	1.04
Acetone fraction	4.77	0.54	0.113	85.71	1.19	0.53	0.111	75.71	1.06
Tris fraction	1.06	-	-	-	-	0.41	0.38	58.57	3.62
HCl fraction	0.21	0.111	0.529	17.62	5.56	-	-		-

higher. The yield was 17.62% with a 5.56-fold increase in specific activity. The total activity and specific activity of trypsin fraction were 0.111 units/ml and 0.529 units/mg protein, respectively. El-Shemy and Levin (1997) reported enzyme activity of hybrid tilapia at 0.0049 units/ml and 0.2045 units/mg protein, respectively.

Chymotrypsin fraction had 1.06 mg protein/ g intestine tissue. Enzyme activities and protein content decreased at each stage. The yield was 58.57% with a 3.62-fold increase in specific activity. The total activity and specific activity of chymotrypsin fraction were 0.41 units/ml and 0.38 units/mg protein.

Optimum pH and stability

The optimum pH condition for hydrolysis of BAPNA by Nile tilapia intestinal trypsin fraction and SAAPPNA by chymotrypsin fraction was 9.0 (Figure 1). Acidic pH values were more inhibitory to the enzyme than alkaline pH values. Similar results have been reported for trypsin from intestine of hybrid tilapia which has an optimum of pH 9.0 (El-Shemy and Levin, 1997), bovine trypsin has an optimum pH of 8.2 (Erlanger *et al*., 1961), Yellowfin tuna trypsin and chymotrypsin have an optimum pH of 8.0 (Jantaro, 2000) and anchovy trypsin and chymotrypsin was in the pH range of 8.0-9.0 and 7.5-8.5, respectively (Heu *et al*., 1995)

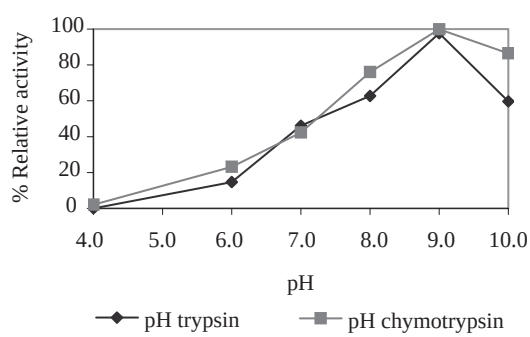

Figure 1 optimum pH at 30°C.

The optimum pH condition for stability of Nile tilapia intestinal trypsin and chymotrypsin fraction at 30°C were 10.0 and 9.0, respectively (Figure 2). Both enzymes were notably more stable towards alkaline than acidic pH values. The trypsin and chymotrypsin activities were maintained at pH 8.0-10.0 (more than 80 % of maximum activity). These results are similar to hybrid tilapia trypsin that stable at pH 8.0 (El-Shemy and Levin, 1997) and yellowfin tuna trypsin and chymotrypsin were stable at the pH range of 7.0-8.0 (Jantaro, 2000)

Optimum temperature and stability

The optimum temperature for Nile tilapia intestinal trypsin and chymotrypsin activities at pH 8.0 were 80 and 60°C, respectively (Figure 3). The pattern of Nile tilapia trypsin activity was increased when the temperature was increased, it was different from hybrid tilapia trypsin which decreased when the temperature was up to 50°C (El-Shemy and Levin, 1997). Anchovy trypsin and chymotrypsin had maximum activity for both specific substrates at 45°C and decreased when the temperature was up to 50°C and inactive at 65°C (Heu *et al*., 1995). It indicated that Nile tilapia trypsin and chymotrypsin activities were maintained even at ambient temperature to 80°C.

When Nile tilapia intestinal trypsin and chymotrypsin was held at 0 to 60°C for 30 min, activities were still remained (Figure 4). However, no activity remained after holding the enzyme at 70°C for 30 min which was similar to hybrid tilapia trypsin (El-Shemy and Levin, 1997). Anchovy trypsin and chymotrypsin were stable at 0-50°C and loss activities at 60°C (Heu *et al*., 1995).

Effect of inhibitors on tryptic and chymotryptic activities

Nile tilapia intestinal trypsin and chymotrypsin were serine proteases because both enzymes were inhibited by PMSF and aprotinin, serine protease inhibitors, even though trypsin fraction was inhibited by PMSF only at 17.45% while 31.58% chymotrypsin was inhibited by aprotinin (Table 3). Nile tilapia intestinal trypsin was completely inhibited by SBTI and TLCK (trypsin inhibitors), but about 90.55% was

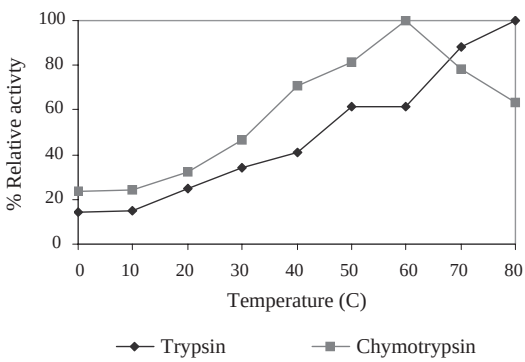

Figure 3 Optimum temperature at pH 8.0.

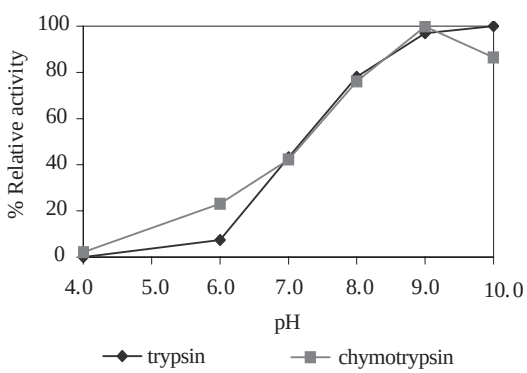

Figure 2 pH Stability at 30°C.

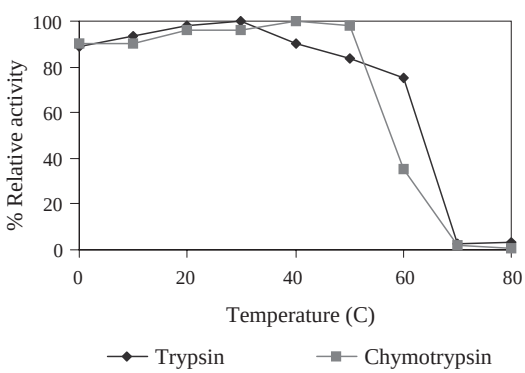

Figure 4 Thermal stability at pH 8.0.

Table 3 Effect of inhibitors on tryptic and chymotryptic activities.

Inhibitor	Conc. (mM)	Trypsin inhibition (%)	Chymotrypsin inhibition (%)
PMSF	1.0	17.45	98.02
Aprotinin	1.5	99.29	31.58
SBTI	100.0	98.08	94.73
TLCK	50.0	98.52	59.17
TPCK	5.0	90.55	67.40

inhibited by TPCK (chymotrypsin inhibitor). Notably this fraction should be trypsin-like enzyme.

Nile tilapia intestinal chymotrypsin was inhibited by TPCK (67.4%), notably this fraction should be chymotrypsin-like because it was also inhibited by SBTI (94.73%) and TLCK (59.17%). The result was similar to trypsin and chymotrypsin from viscera of anchovy (Heu *et al*., 1995) and trypsin from digestive tract of discus fish (Chong *et al.*, 2002). Anchovy trypsin and chymotrypsin were inhibited by TPCK at 0 and 36%, respectively (Heu *et al*., 1995).

CONCLUSION

The viscera of Nile tilapia was used for enzyme extraction. Intestine was found to be the best sources for trypsin and chymotrypsin when compared by activity per body weight. Trypsin and chymotrypsin fraction gave 0.529 and 0.380 unit/mg protein, respectively. Trypsin fraction had the optimum temperature and pH at 80°C and 9.0, respectively. Chymotrypsin fraction had the optimum temperature and pH at 60°C and 9.0, respectively. Trypsin fraction was stable at 0-60° C and chymotrypsin was stable at 0-50°C at pH 8.0 for 30 min. Tryptic activity was inhibited by Aprotinin, SBTI, TLCK and TPCK whereas chymotryptic activity was inhibited by PMSF, SBTI, TPCK and TLCK.

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