Purification and Characterization of Trypsins Affecting on the Autolysis of Shrimp, *Penaeus japonicus*

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Two trypsins were purified from shrimp hepatopancreas through ammonium sulfate fractionation, Q-Sepharose ionic exchange, benzamidine Sepharose-6B affinity, and Sephacryl S-300 gel chromatography. Both enzymes had a single polypeptide chain with a molecular weight (M.W.) of 32 kDa by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), although trypsin A and B were estimated to be a molecular weight of 27.2 and 22.8 kDa, respectively, using Sephacryl S-300 gel filtration. Both trypsins had similar amino acid compositions and rich in glycine, valine, alanine, aspartic acid, and glutamic acid, but low in methionine and basic amino acids. Both enzymes were completely inactivated by soybean trypsin inhibitor (SBTI), phenylmethylsulfonyl fluoride (PMSF), tosyl-L-lysine chloromethyl ketone (TLCK), benzamidine, leupeptin, however, not affected by tosyl-L-phenylalanine chloromethyl ketone (TPCK) and pepstatin.

Key words : shrimp protease, shrimp trypsin, trypsin purification, trypsin inhibition

Introduction

Texture softness in shrimp tail meat is attributed to degradation of shrimp muscle proteins associated with hepatopancreatic proteases, since this phenomenon is most prevalent in the anterior section of the tail meat. High activity of protease in intestine of finfish accelerates autolytic degradation of abdominal tissues (Kim and Pyeun, 1986). Trypsin is quantitatively important in the digestive system. Moreover, participation of other proteases in muscle degradation is affected by the presence of trypsin due to its unique activation function for other enzymes (Martinez et al., 1988). Hence, understanding properties of trypsin may support an information for the texture softness in shrimp tail meat.

A variety of digestive proteolytic enzymes have been isolated from the internal organs of fish and crustacea, and have been characterized thoroughly as to their physicochemical and enzymatic properties. In crustacea, one of the major proteolytic enzymes is trypsin-like, and its physicochemical and enzymatic properties have been characterized in crayfish (Kim et al. 1989; 1994; 1996; Zwilling and Neurath, 1981), crab (Dendinger and O'Connor, 1990), lobster (Galgani and Nagayama, 1987), and shrimp (Gates and Travis, 1969). Although most characteristics of trypsin-like enzymes from crustacea are largely similar to those of vertebrate origin, some properties are markedly dissimilar. These differences include calcium ion requirements for enzymatic catalysis and pH stability, extremely low isoelectric point, low level of basic amino acids, and a wide range of molecular weights (Zwilling and Neurath, 1981).

The objective of this paper was to establish a purification procedure for trypsins responsible for degradation of muscle proteins, and to characterize and compare their physicochemical properties from shrimp hepatopancreas.

Materials and Methods

Materials

The hepatopancreas from shrimp (*Penaeus japonicus*) was collected, and stored at −85℃ until used for crude enzyme extraction. Preparation of the crude extract followed the procedure of Kim et al. (1989).

Determination of Protein Concentration

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Assay for Enzyme Activity

Amidolytic activity for benzoyl-D,L-arginine p-nitroanilide (BAPNA) was measured using the assay method of Erlanger et al. (1961). Fifty μl of enzyme solution was mixed with 1.0 ml of 1 mM BAPNA dissolved in 0.05 M Tris-HCl buffer, pH 8.1, containing 1 mM $CaCl_2$. The hydrolysis of BAPNA was monitored at 410 nm at 25℃. One enzyme unit was defined as the amount of enzyme that hydrolyzed 1 mmole of BAPNA per min per 1 ml of enzyme solution. Specific activity was expressed as enzyme units per 1 mg of enzyme.

Hydrolysis of benzoyl L-tyrosine ethyl ester (BTEE) was monitored at 253 nm. Fifty μl of an appropriately diluted enzyme solution was mixed with 1.0 ml of 1 mM BTEE dissolved in 0.05 M Tris-HCl buffer, pH 8.1, containing 1 mM $CaCl_2$.

Caseinolytic activity was determined according to the method of Kim and Pyeun (1986).

Trypsin Purification

Purification of trypsins from crude enzyme solution was undertaken by first employing ammonium sulfate (A.S) fractionation with 30~70% saturation. The A.S fraction was dissolved in 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA. The sample was applied onto a Q-Sepharose column (3×20 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 0.2 M NaCl and elute with a 500 ml linear gradient ranging from 0.2~2.0 M NaCl. Trypsin fraction was pooled and dialyzed against 20 mM Tris-HCl buffer, pH 6.8, containing 0.5 M NaCl, 5 mM $CaCl_2$, and 1 mM EDTA. The dialyzate was loaded onto a benzamidine Sepharose 6B column (1.5×5 cm) equilibrated with the above buffer. The column was washed with the equilibration buffer until the effluent did not contain protein. Trypsin-like enzymes were then eluted from the column using 20 mM Tris-HCl, pH 6.8, containing 0.5 M NaCl, 5 mM $CaCl_2$ and 125 mM benzamidine. The trypsin fraction was dialyzed against 20 mM Tris-HCl buffer, pH 7.5, containing 0.5 M NaCl and applied to a Q-Sephacel column (2×15 cm) equilibrated with the previous buffer solution. The column was eluted with a 300 ml linear gradient ranging from 0.5 to 1.0 M NaCl. Each trypsin fraction was concentrated by Centricon™ (Amicon, MWCO, 10 K) with several replacement of 0.02 M Tris-HCl, pH 7.5, containing 0.1 M NaCl. The concentrated solution was applied to a Sephacryl S-300 column (2.5×60 cm) equilibrated with the above buffer. Fractions with high tryptic activity were concentrated by ultrafiltration and stored at −20℃ until used in subsequent characterization.

Molecular Weight Determination

Molecular weight of the purified trypsin was determined by Sephacryl S-300 gel filtration according to the method of Andrews (1964) and SDS-PAGE by the method of Laemmli (1970).

Amino Acid Composition

The amino acid compositions of the enzymes were determined with a LKB 4150 type amino acid analyzer after hydrolysis in 6 N HCl at 110℃ for 24 hr. The amino acid residues presented in integral numbers which were computed from the best fit to their molecular weights as determined by SDS-PAGE.

Effect of Inhibitors

Soybean trypsin inhibitor (SBTI), tosyl-L-lysine chloromethyl ketone (TLCK), benzamidine, and dithiothreitol (DTT) were dissolved in distilled water.

Tosyl-L-phenylalanine chloromethyl ketone (TPCK), leupeptin, pepstatin, and phenylmethylsulfonyl fluoride (PMSF) were dissolved in dimethyl sulfoxide. Equal volumes of diluted inhibitor and purified enzyme solutions were incubated at 25℃ for 30 min, and residual activities were measured with 1 mM BAPNA at pH 8.1 and 25℃.

Results and Discussion

Trypsin Purification

With the first Q-Sepharose column chromatography, two fractions of tryptic activities and one fraction of proteolytic activity were separated (Fig. 1). Tryptic fractions (tube No. 65~90) were pooled and dialyzed for benzamidine Sepharose-6B chromatography. After

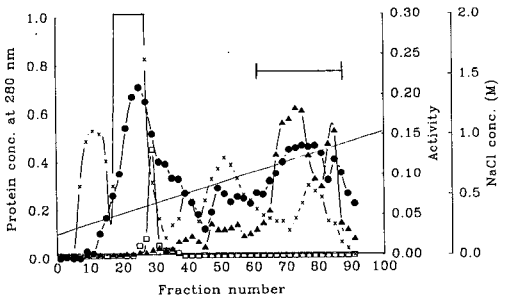

Fig. 1. Chromatogram of a first Q-Sepharose chromatography of 30~70% ammonium sulfate fraction. Flow rate was 40 ml/hr and fraction volume was 5 ml per tube. The symbols were as follows; −×−, protein concentration at 280 nm, −●−, caseinolytic activity; −▲−, tryptic activity with BAPNA; −□−, chymotrytic activity with BTEE, and −−−, salt concentration.

benzamidine Sepharose affinity chromatography, trypsin fraction was applied on the second Q-Sepharose column. With the chromatography, two kinds of trypsins were eluted separately (Fig. 2). These enzymes were tentatively named trypsin A and B using the elution order. As noted in SDS-PAGE pattern, high molecular weight protein other than the trypsin-like enzyme were still existed after the second Q-Sepharose chromatography. With the Sephacryl S-300 gel fil-

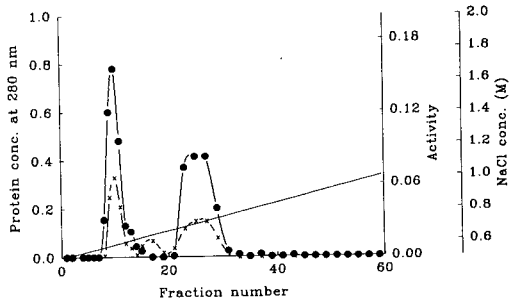

Fig. 2. Chromatogram of a second Q-Sepharose chromatography of benzamidine Sepharose-6B fraction. Flow rate was 40 ml/hr and fraction volume was 5 ml per tube. The symbols were as follows; −×−, protein concentration at 280 nm; −●−, tryptic activity with BAPNA; and −−−, slat concentration.

tration, high molecular weight protein was effectively separated and two trypsins were purified electrophoretically homogenous.

The two trypsin-like enzymes were isolated only in small quantities by these purification procedures. Having started with 50 g of shrimp hepatopancreas, only 2.8 mg of trypsin A and 2.2 mg of trypsin B were obtained. However, the present purification procedure produced trypsins with high purity.

Molecular Weight

The molecular weight of trypsin A and B was determined to be 32 kDa with a single polypeptide chain (Fig. 3), although trypsin A and B showed a molecular weight of 27.2 and 22.8 kDa, respectively, using Sephacryl S-300 gel filtration (Fig. 4).

The discrepancy of M.W. estimated by SDS-PAGE and gel filtration was caused by the existence of a sugar chain, as reported in the proteinases isolated from krill (Kimoto et al., 1983). Trypsin in hepatopancreas from shrimp *Penaeus indicus* had a single peptide with a M.W. of 36 kDa on SDS-PAGE, although the enzyme showed a M.W. of 18 kDa on Sephadex G-100 gel filtration (Honjo et al., 1990). However, they did not find any evidence of glycosylation of enzyme and the interaction between enzyme and Sephadex resin might be the reason for discrepancy of M.W. Tryp-

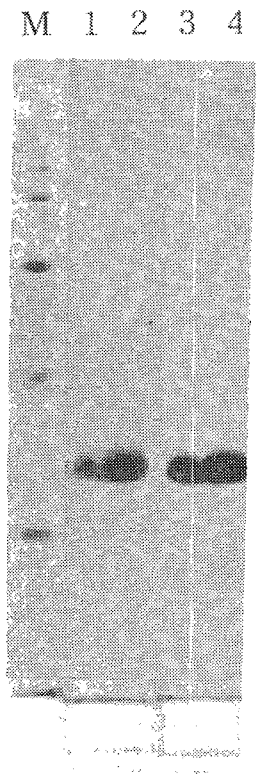

Fig. 3. SDS-polyacrylamide gel electrophoresis (10%) of the purified trypsin A and B. Lane 1 and 2 represent 2 and 10 μg of trypsin A, respectively. Lane 3 and 4 represent 5 and 10 μg of trypsin B, respectively. Lane M contains molecular mass markers as follows; β-galactosidase, 116 kDa; phosphorylase B, 97.4 kDa; bovine serum albumin, 66 kDa, ovalumin, 45 kDa; carbonic anhydrase, 29 kDa.

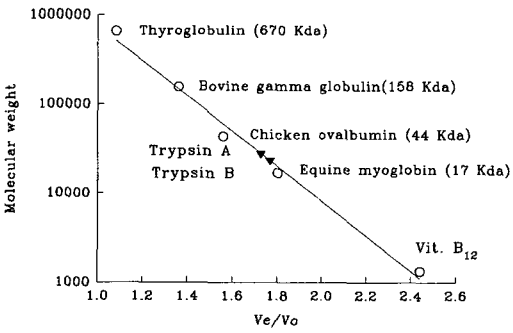

Fig. 4. Determination of molecular weights of shrimp *hepatopancreatic* trypsins by Sephacryl S-300 gel filtration.

sins were found to have higher M.W. i.e., 33.5 kDa from the midgut gland of crab *Callinectes sapidus* (Dendinger and O'Connor, 1990) and 30 to 31 kDa from Antarctic krill (Osnes and Mohr, 1985), which are very similar with this result. Also, three trypsins from crab *Eriocheir japonicus* were reported to a M.W. of approximately 29 kDa. However, trypsins from marine organisms, either invertebrates or vertebrates, in most instances have a M.W. in the range of 24~30 kDa. The M.W. of trypsin-like enzymes from crustacea varies widely. Trypsin from hepatopancreas of white shrimp *Penaeus setiferus* (Gates and Travis, 1969) and that from digestive glands of the Japanese spiny lobster *Panulirus japonicus* (Galgani and Nagayama, 1987) were estimated to be M.W. of 24 kDa by gel filtration.

Amino Acid Composition

Amino acid compositions of purified trypsin A and B compared with analogous enzymes from crayfish *Procambarus clarkii,* shrimp *Penaeus monodon,* and bovine are given in Table 1. Similar amino acid compositions were clearly observed among *Penaeus japonicus* trypsins. The compositions were similar to trypsin from shrimp *Penaeus monodon* except for histidine, arginine, valine, and methionine. Shrimp trypsins were rich in glycine, valine, alanine, and acidic amino acid residues, but low in methionine, and basic amino acids. These results are consistent with anionic properties of other trypsin-like enzymes from fish.

Trypsins from crustacea have similar ratios of acidic to basic amino acids in the range of 3.1 to 5.3 (Table 1). This is quite different from mammalian trypsins, which have basic isoelectric points. Of particular interest, lysine content in bovine trypsin is significantly higher than that in crustacean trypsins. The high ratio of acidic to basic amino acid residues supports the observation that trypsin-like enzymes from shrimp were bound strongly to the anionic exchange resin and eluted in a high salt concentration. The similarities in amino acid compositions among the trypsins

Table 1. Amino acid compositions of shrimp hepatopancreatic trypsins. The values are given in residues per molecule

A.A.	Shrimp		Crayfish[1]	Shrimp[2]	Bovine[3]
	Trypsin A	Trypsin B			
Asp	40	42	46	30	22
Glu	44	48	28	29	14
Ser	33	36	21	28	33
Gly	44	45	59	35	25
His	9	9	7	9	3
Arg	7	7	2	3	2
Thr	16	10	20	8	10
Ala	28	22	29	18	14
Pro	19	15	17	13	9
Tyr	9	13	11	9	0
Val	30	28	22	14	17
Met	1	1	3	2	2
Ile	20	19	18	12	15
Leu	20	19	19	11	14
Phe	12	13	6	8	3
Lys	10	9	5	7	14
Ratio of acidic to basic amino acids					
	3.2	3.6	5.3	3.1	1.9
No of residues	342	336	313	240	207

[1] Kim et al., 1992. Comp. Biochem. Physiol. 103B; 391.
[2] Lu et al., 1990. Biol. Chem. Hoppe-Seyler, 371; 851.
[3] Walsh and Neurath, 1964. Proc. Nat. Acad. Sci., 52; 884.

may reflect considerable homology in primary structure (Zwilling et al., 1975), and are comparable to other anionic trypsins derived from fish. Further analysis of cysteine and tryptophan is needed to obtain the complete amino acid composition of the trypsins.

Effect of Inhibitors

Activities of both trypsins were inhibited by SBTI and PMSF, which are inhibitors of serine enzymes (Table 2). Trypsin A was inhibited to a lesser degree by PMSF compared with the trypsin B. TLCK and benzamidine are well known as trypsin specific inhibitors. Both enzymes were completely inactivated by TLCK. TLCK deactivates only trypsin-like enzyme by alkylation of the active-center histidine. The reagent is known to form a covalent bond with histidine residue in the catalytic site of the enzyme and to block the substrate-binding of the active center of the molecule (Severin and Tomasek, 1965). Tryptic activities were reduced 31 to 40% by 1 mM benzamidine, with complete inhibition by 10 mM benzamidine.

SBTI, PMSF, DFP, TLCK, and benzamidine have been described as strong inhibitors, with variable inhibitory effects for trypsins from crustacea and other animals such as Atlantic blue crab (Dendinger and O'Connor, 1990), shrimp (Honjo et al., 1990), and Antarctic krill (Osnes and Mohr, 1985). Leupeptin which has the arginine residue at its terminal carbon and inhibits trypsin and papain, is able to inactivate the proteolytic and esterolytic activities of trypsin

Table 2. Effect of inhibitors on the activity of trypsin-like from shrimp hepatopancreas

Inhibitor	Conc.	% Inhibition	
		Trypsin A	Trypsin B
Control		100	100
TLCK	0.1 mM	0	0
Benzamidine	1.0 mM	60	71
	10 mM	0	0
PMSF	1.0 mM	20	0
Leupeptin	0.1 mM	0	0
SBTI	1.0 mM	0	0
DTT	1.0 mM	82	103
Pepstatin	0.1 mM	82	100
TPCK	0.1 mM	100	100

Each enzyme solution was incubated with same volume of inhibitor at 25℃ for 30 min and residual activity was determined with 1 mM BAPNA at 25℃ and pH 8.1.
TLCK : N-p-Tosyl-L-lysine chloromethyl ketone
TPCK : N-p-Tosyl-L-phenyllanine chloromethyl ketone
SBTI : Soybean trypsin inhibitor
PMSF : phenylmethylsulfonylflouride

(Aoyagi et al., 1969). This inhibitor reduced tryptic activity completely for both trypsins in this study.

None of the shrimp enzymes isolated were inhibted by TPCK, a specific inhibitor of chymotrypsin, or pepstatin, a specific inhibitor of acid proteases. This suggests that the two enzymes are serine type enzymes, classified as trypsin-like enzyme that lacks chymotrypsin and pepsin activity.

Disulfide bonds are important to stabilization of protein conformation by increasing protein hydrophobicity (Creighton, 1983). Therefore, addition of reducing agents, which break disulfide bonds, causes a decrease in activity of trypsin-like enzyme. DTT reduced trypsin A activity about 20%, however, that did not change trypsin B activity. The activity of four anionic trypsins from crayfish hepatopancreas were inhibited about 50% by 1 mM DTT (Kim et al., 1992) and Wolz and coworker (1990) reported that sulfhydryl reagents had strong inhibitory effects on *Astacus protease* in order of DTT, 2-mercaptoethanol, and glutathione.

Conclusion

The shrimp enzymes purified in this research were shown to be true members of the trypsin family of serine proteases, comparable to trypsin-like enzymes in other crustacea. Confirmation included determination of molecular weight, amino acid composition, and inhibition studies. Purified enzymes were inhibited by TLCK, DFP, and PMSF, indicating that histidine and serine residue play a role in the catalytic mechanism. The enzymes also were inhibited by other known trypsin inhibitors, i.e., benzamidine, soybean trypsin inhibitor, and leupeptin, but not by TPCK which is a chymotrypsin inhibitor.

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