J. Fish. Sci. Tech. 1(2), 209~215, 1998

Comparative Biochemical Properties of Proteinases from the Hepatopancreas of Shrimp.

II. Purification of Trypsin from the Hepatopancreas of *Penaeus orientalis*

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(Received April 1998, Accepted June 1998)

Trypsin-like enzyme was purified from shrimp hepatopancreas through Q-Sepharose ionic exchange, benzamidine Sepharose-6B affinity, and Superdex 75 gel chromatography. Purity of trypsin-like enzyme was increased 69-fold with 44% yield. The enzyme consisted of a single polypeptide chain with a molecular weight (M.W.) of 32 kDa judged by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme was completely inactivated by serine enzyme inhibitors such as soybean trypsin inhibitor (SBTI), tosyl-L-lysine chloromethyl ketone (TLCK), and leupeptin. However, the enzyme was not affected by tosyl-L-phenylalanine chloromethyl ketone (TPCK) which is a chymotrypsin specific inhibitor. The enzyme had no activity against benzoyl-tyrosine ethyl ester (BTEE) which is a chymotrypsin specific substrate. The enzyme showed high activity on the carboxyl terminal of Phe, Tyr. Glu, Arg, and Asp. However. no activity was detected against the carboxyl terminal of Pro, Trp, Cys, Gly, Val, and Ala.

Key words: shrimp protease, *Penaeus orientalis*, 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 prominent in the anterior section of the tail. High activity of protease in intestine of finfish accelerates autolytic degradation of abdominal tissues. 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 on other enzymes (Martinez et al., 1988). Hence, understanding the properties of trypsin is important in studies of the digestive system and degradation of muscle proteins.

A variety of digestive proteolytic enzymes have been isolated from the internal organs of fish and crustacea, and these have been characterized thoroughly as to their physicochemical and enzymatic properties. In crustacea, one of the most active proteolytic enzymes is trypsin-like, and its physicochemical and enzymatic properties have been characterized in crayfish (Kim et al., 1992; 1994), crab (Dendinger and O'Connor, 1990), lobster (Galgani and Nagayama, 1987b), and shrimp (Kim et al., 1996). 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, an 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 rapid purification procedure for trypsin-like enzyme from the hepatopancreas of shrimp and to characterize its substrate specificity and inhibition.

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Materials and Methods

Materials

Hepatopancreases from fresh shrimp (*Penaeus orientalis*) were collected and stored at −80℃ until used for crude enzyme extraction.

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 $\mu\ell$ 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 $\mu\ell$ 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

Shrimp hepatopancreas (50 g) was homogenized with 50 mℓ of 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA and 1 mM $CaCl_2$ by an Ultra-Turrax type tissue grinder (T25B type, Ika, Germany). The homogenate was centrifuged at 12,000xg for 15 min. The supernatant was treated with 0.2 vol. of tetrachloromethane to remove lipids, and the crude enzyme solution was obtained by taking the supernatant after centrifugation.

The crude enzyme solution was applied onto a Q-Sepharose column (3×20 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl and eluted with a 500 mℓ linear gradient ranging from 0.1∼1.0 M NaCl. Trypsin fraction was pooled and adjusted to a final concenration of 0.5 M NaCl, 5 mM $CaCl_2$, 1 mM benzamidine, and 1 mM EDTA. The enzyme solution 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 other protein. The trypsin-like enzyme was then eluted from the column using 20 mM Tris-HCl (pH 6.8) containing 0.5 M NaCl, 5 mM $CaCl_2$, 125 mM benzamidine, and 1 mM EDTA. The trypsin fraction was dialyzed against 20 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl for 3 hr and dialysate was precipitated with 2 volumes of cold acetone. The precipitated enzyme was dissolved with 20 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl and applied to a Superdex 75 gel filtration column (16×75 cm) equilibrated with the previous buffer solution. Fractions with high tryptic activity were concentrated by Centricon™ (Amicon, MWCO, 10K) and stored at −80℃.

Molecular Weight Determination

Molecular weight of the purified trypsin was determined by SDS-PAGE according to the method of Laemmli (1970) using phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), and lactalbumin (14.2 kDa).

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℃.

Substrate Specificity

Hydrolysis of the various synthetic substrates was determined as an increase of absorbance at 410 nm resulting from the hydrolysis of ester bond. A reaction mixture was consisted of 3 mℓ of Tris-HCl buffer (pH 7.5) containing 20% ethyl alcohol, 150 $\mu\ell$ of substrate solution, and 10 $\mu\ell$ of enzyme solution. A specific activity represents hydrolysis of nmole of substrate per minute at 25℃ by mg of enzyme.

Results and Discussion

Enzyme Purification

This purification method was designed to purify

trypsin-like enzyme from shrimp hepatopancreas responsible for the degradation of shrimp muscle proteins during prolonged storage. Activity measurement during purification was performed at 25℃ and pH 8.1 on the basis of BAPNA hydrolysis. With a Q-Sepharose column chromatography, a fraction of tryptic activity was separated (Fig. 1). Main tryptic fractions (tube No. 29~39) were pooled and further purified by a benzamidine Sepharose-6B chromatography. After benzamidine Sepharose affinity chromatography, trypsin fraction was dialyzed against 20 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl for 3 hr and dialysate was concentrated by acetone precipitation. The concentrated trypsin was further purified by a Superdex 75 gel filtration. With the gel filtration, a minor contaminant was effectively removed and chromatographic pattern demonstrated a high purity of the protein (Fig. 2). Elution of this enzyme in an anionic exchange chromatography was similar to

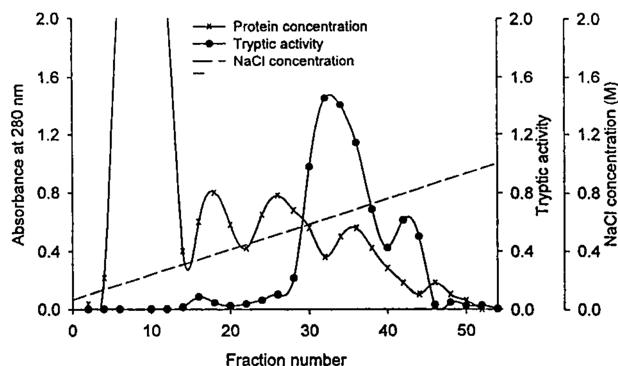

Fig. 1. Chromatogram of a Q-Sepharose chromatography of crude extract. Flow rate was 40 mℓ /hr and fraction volume was 5 mℓ per tube.

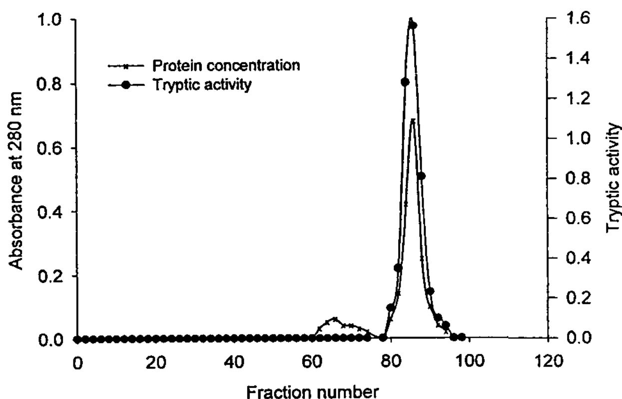

Fig. 2. Chromatogram of Superdex 75 gel filtration from the benzamidine Sepharose-6B bound fraction. Flow rate was 120 mℓ/hr and fraction volume 1 mℓ per tube.

trypsin-like enzymes found in menhaden (Pyeun et al., 1990) and *P. japonicus* (Kim et al., 1996). However, this enzyme was eluted at lower salt concentration in an anionic exchange chromatography of 4 kinds of trypsin from crayfish hepatopancreas (Kim et al., 1992). The fraction obtained with the benzamidine Sepharose 6B chromatography presented the same activity of Q-Sepharose fraction, however, specific activity was increase by 6-fold. This indicates that this step is a highly efficient purification process for the separation of trypsin-like enzymes. The final product after gel filtration were found to be homogenous with SDS-PAGE (Fig. 3).

Results of purification of the trypsin-like enzyme were summerized in Table 1. The purity of the enzyme was increased 69-fold with a 44% yield.

Molecular Weight

The molecular weight of trypsin-like enzyme was determined to be 32 kDa with a single polypeptide chain by SDS-PAGE (Fig. 4). The molecular weight of the enzyme was slightly higher than is commonly reported for trypsins from mammalia and fish.

Trypsins from mammalia have been reported to have a M.W. between 20 and 25 kDa (Keil, 1971). However, those from marine organisms, either invertebrates or vertebrates, in most cases have M.W.

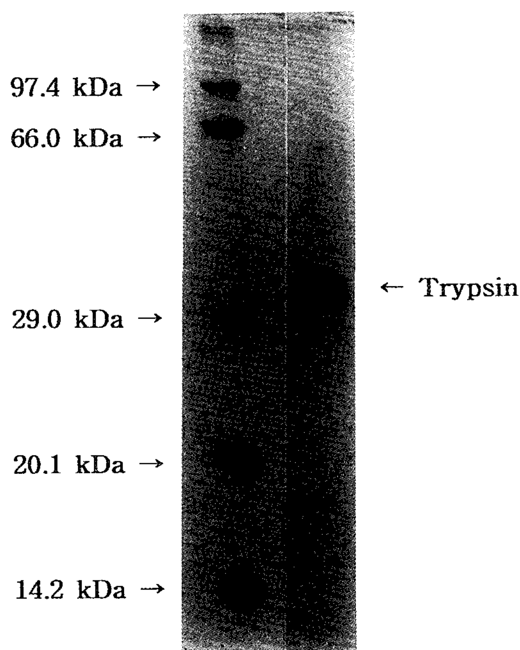

Fig. 3. SDS-PAGE (12.5%) of purified trypsin from the hepatopancreas of shrimp (*P. orientalis*)

Table 1. Purification of trypsin from the hepatopancreas of shrimp, *P. orientalis*

	Vol. (mℓ)	Protein (mg/mℓ)	Activity (U/mℓ)	Purity (-fold)	Yield (%)
Crude extract	80	9.90	2.01	1	100
Q-Sepharose fraction	98	0.95	2.10	10.9	71.7
Benzamidine Sepharose 6 B fraction	44	0.35	4.65	65.5	59.9
Superdex 75 fraction	7	0.462	6.50	69.2	44.0

Activity was determined with 1 mM BAPNA at 25℃ and pH 8.1

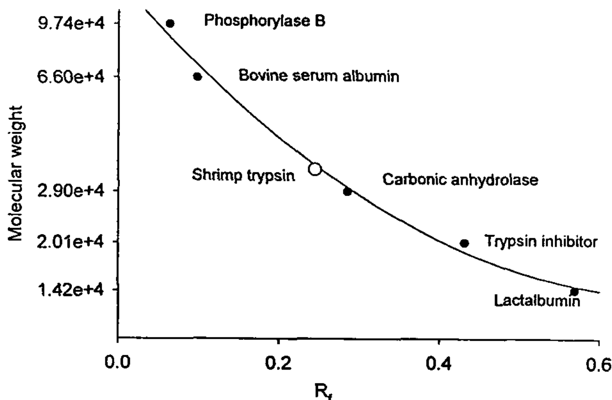

Fig. 4. Determination of molecular weight of purified trypsin from the hepatopancreas of shrimp, *Penaeus orientalis*, by Superose 6 gel filtration.

in the range of 24~30 kDa. The M.W. of trypsin-like enzymes from crustacea varies widely. The M. W. has been found for three trypsins from crab, *Eriocheir japonicus*, of approximately 29 kDa, and the enzymes did not dissociate into subunits in the presence of SDS (Muramatsu and Morita, 1987). Also, trypsins from crustacea 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 32 kDa from shrimp *P. japonicus* (Kim et al., 1996). However, a somewhat lower M.W. was reported in the hepatopancreas of *P. setiferus* (Gates and Travis, 1969), the digestive glands of the Japanese spiny lobster (Galgani and Nagayama, 1987b), and digestive gland of *P. kerathrus* (Galgani et al., 1984).

Effect of Inhibitors

Activity of trypsin-like enzyme was inhibited by TLCK, leupeptin, and SBTI, which are specific inhibitors of serine enzymes (Table 2). The enzyme was partially inhibited by benzamidine and PMSF. However, dithiothreitol (DTT) and TPCK were not affected on the activity of the enzyme.

TLCK and benzamidine are well known trypsin specific inhibitors. The enzymatic activity was completely inactivated by TLCK but benzamidine affected half of TLCK effect. 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 in the molecule (Shaw et al., 1965). Tryptic activity was reduced 32% by 1 mM benzamidine, with more inhibition by 10 mM benzamidine. Trypsins from krill (Osnes and Mohr, 1985) and *P. japonicus* (Kim et al., 1996) were almost entirely inhibited by benzamidine, but trypsin from shrimp (Honjo et al., 1990) was not inactivated even though higher concentration of benzamidine was applied. Thus, inhibition of trypsin by benzamidine may vary with the source of the enzyme. PMSF, TLCK, SBTI, 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). However, trypsin from *P. monodon* was not inhibited by PMSF (Lu et al., 1990).

Table 2. Effect of inhibitors on the activity of trypsin from the hepatopancreas of shrimp, *P. orientalis*

Inhibitor	Conc.	Inhibition (%)
Control		0
TLCK	1.0 mM	100
	0.1 mM	50
Benzamidine	10 mM	56
	1.0 mM	32
PMSF	1.0 mM	25
Leupeptin	0.1 mM	100
SBTI	10 mM	100
	1.0 mM	72
Pepstatin	0.1 mM	100
DTT	0.1 mM	0
TPCK	0.1 mM	0
Iodoacetate	0.1 mM	28

Enzyme solution was incubated with same volume of inhibitor at 25℃ for 30 min and residual activity was measured with 1 mM BAPNA at 25℃ and pH 8.1

Leupeptin which has arginine residue at its terminal carbon and inhibits trypsin and papain, is able to inactivate the proteolytic and esterolytic activities of trypsin (Aoyagi et al., 1969). This inhibitor reduced tryptic activity completely for trypsin in this study. Also, leupeptin has been reported to be very efficient inhibitors for trypsins from menhaden (Pyeun et al., 1990), catfish (Yoshinaka et al., 1984), and capelin (Hjelmeland and Raa, 1982).

The trypsin-like enzyme was completely inhibited by pepstatin, which suggests a presence of acidic amino acid in the active site of the enzyme. Pepstatin specifically inhibits acid proteases and the inhibitory activity can be explained by the strong binding of pepstatin to active site of pepsin. This suggestion would confirm and coincide with the data of substrate specificity (Table 3). However, two trypsin from *P. japonicus* (Kim et al., 1996) and four trypsins from *Procambarus clarkii* (Kim et al., 1992) were not affected by pepstatin.

The enzyme isolated was not inhibited by TPCK, a specific inhibitor of chymotrypsin. This suggests that the enzyme is a serine type enzyme, classified as a trypsin-like enzyme that lacks chymotrypsin activity.

Table 3. Substrate specificity of trypsin from the hepatopancreas of shrimp, *P. orientalis*

Substrate	Specific acitivity (nmole/mg)
BAPNA	
CBZ-Phe-pNE	3.05
CBZ-Tyr-pNE	2.69
CBZ-Glu-pNE	2.65
CBZ-Arg-pNE	1.49
CBZ-Asp-pNE	0.96
CBZ-Pro-pNE	0
CBZ-Trp-pNE	0
CBZ-Cys-pNE	0
CBZ-Gly-pNE	0
CBZ-Val-pNE	0
CBZ-Ala-pNE	0
BTEE	0

BAPNA; Benzoyl-D,L-arginine p-nitroanilide
CBZ-Phe-pNE; N-CBZ-L-Phenylalanine p-nitrophenyl ester
CBZ-Tyr-pNE; N-CBZ-L-Tyrosine p-nitrophenyl ester
CBZ-Glu-pNE; N-CBZ-β-Benzyl-Glutamate p-nitrophenyl ester
CBZ-Arg-pNE; N-CBZ-L-Arginine p-nitrophenyl ester
CBZ-Asp-pNE; N-CBZ-β-Benzyl-L-Aspartate p-nitrophenyl ester
CBZ-Pro-pNE; N-CBZ-L-Proline p-nitrophenyl ester
CBZ-Trp-pNE; N-CBZ-L-Tryptophan p-nitrophenyl ester
CBZ-Cys-pNE; N-CBZ-L-Cysteine p-nitrophenyl ester
CBZ-Gly-pNE; N-CBZ-L-Glycine p-nitrophenyl ester
CBZ-Val-pNE; N-CBZ-L-Valine p-nitrophenyl ester
CBZ-Ala-pNE; N-CBZ-L-Alanine p-nitrophenyl ester
BTEE; Benzoyl-L-tyrosine ethyl ester.

Substrate Specificity

The trypsin-like enzyme did not show hydrolytic activity for BTEE which is a specific substrate for chymotrypsin. The enzyme had activity on CBZ-Phe-pNE, CBZ-Tyr-pNE, CBZ-Glu-pNE, CBZ-Arg-pNE, and CBZ-Asp-pNE. The specific activity of the trypsin-like enzyme was highest on the forward CBZ-Phe-pNE and was a similar level to CBZ-Tyr-pNE and CBZ-Glu-pNE.

The enzyme was classified as a real trypsin-like enzyme by inhibition studies. However, the enzyme has an activity against peptides containing aromatic amino acids. These results have some discrepancy with the results of inhibitor studies and require more studies on the specificity of synthetic substrates. It also suggests that the structure of active site might be similar to chymotrypsin encompassing the aromatic amino acid residues. Trypsin from *P. monodon* hydrolyzed various synthetic substrates with Arg and Lys at the P1 site, but not the substrates for chymotrypsin and carboxypeptidase. Reactivities of the shrimp trypsin depend on secondary interaction sites beyond the P 1 site as most other trypsins do (Lu et al., 1990).

The alimentary tracts of vertebrates and some fish have an acidic stomach and pepsin to denature food proteins before trypsin and other proteases exert their catalytic activity. Shrimps do not have an acidic stomach. Furthermore, chymotrypsin activity was not detected in the hepatopancreas or digestive tracts of shrimp even though five species of crustacea were examined (Galgani and Nagayama, 1987a). The absence of peptidic and chymotryptic endopeptidases in crustacea might be covered by a compensatory activity of an extremely low M.W. protease, which has similar enzymatic properties to trypsin, has been found in the digestive organ of crawfish (Pfleiderer et al., 1967) and shrimp (Galgani et al., 1984).

Like other crustacea, peptidic and chymotryptic activity was not detected in the hepatopancreas of *P. orientalis* (data not shown). Therefore, hydrolytic acitivity for Phe and Tyr might be a compensatory activity for the absence of chymotrypsin. The enzyme has activity for Glu and Asp which are a sites of hydrolysis by pepsin. This would provide for the presence of acid amino acid residues in the

active site of the enzyme. This result supports the inhibition data in which the enzyme was completely deactivated by pepstatin, a specific inhibitor of pepsin.

Conclusion

The enzyme purified from shrimp hepatopancreas was shown to be a true member of the trypsin family of serine proteases, comparable to trypsin-like enzymes in other crustacea. Confirmation included determination of molecular weight and inhibition studies. Purified enzyme was inhibited by TLCK, leupeptin, SBTI, and pepstatin, indicating that histidine and serine or acid amino acid residue might be play a role in the active site. The enzyme was also partially inhibited by other known trypsin inhibitors, i.e., benzamidine and PMSF, but not by TPCK.

Acknowledgement

This paper was supported by NONDIRECTED RESEARCH FUND, Korea Research Foundation.

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