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Comparative Biochemical Properties of Proteinases from the Hepatopancreas of Shrimp.

I. Purification of Protease from the Hepatopancreas of *Penaeus japonicus*

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A protease, which had no tryptic and chymotryptic activity, was purified from the hepatopancreas of shrimp, *P. japonicus*, through ammonium sulfate fractionation, Q-Sepharose ionic exchange, benzamidine Sepharose 6B affinity, and Sephacryl S-100 gel chromatography. Molecular weight (M.W.) of the protease was estimated to be 24 kDa by gel filtration and showed a single peptide band by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protease had a low ratio of acidic to basic amino acids, which is different with proteases from marine animals. The enzyme was partially inhibited by benzamidine, tosyl-L-lysine chloromethyl ketone (TLCK), phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), and pepstatin. The enzyme did not have any activity against benzoyl-D,L-arginine p-nitroanilide (BAPNA) or benzoyl-L-tyrosine ethyl ester (BTEE) which is a specific substrate of trypsin and chymotrypsin, respectively. However, the enzyme showed activity forward N-CBZ-L-tyrosine p-nitrophenyl ester (CBZ-Tyr-pNE), N-CBZ-L-tryptophan p-nitrophenyl ester (CBZ-Trp-pNE), and N-CBZ-L-proline p-nitrophenyl ester (CBZ-Pro-pNE). The protease did not showed tryptic and chymotryptic activity, which was not reported in shrimp hepatopancreas.

Key words: shrimp protease, *Peneaus japonicus*, protease purification, amino acid composition. substrate specificity.

Introduction

Mushy texture in shrimp tail meat is not caused by an endogenous proteolytic system in the muscle since a little degradation of myofibrillar proteins is observed in tail segment which turned mushy on cooking (Lindner et al., 1988). Hepatopancreatic proteases of shrimp may be responsible for the onset of tissue deterioration leading to mushiness of shrimp tail meat because this phenomenon is most prevalent in the anterior section of the tail.

Many digestive proteolytic enzymes have been isolated from the internal organs of decapods, and have been characterized thoroughly as to their physicochemical and enzymatic properties. In decapods, one of the major proteolytic enzymes is trypsin-like enzyme, and its physicochemical and enzymatic properties have been characterized in crayfish (Kim et al., 1989; 1992; 1994), crab (Dendinger and O'Connor, 1990), lobster (Galgani and Nagayama, 1987b), and shrimp (Gates and Travis, 1969; Kim et al., 1996). The molecular weights (M.W.) of trypsin-like enzymes from crustacea vary widely (22~35 kDa) and are similar to those from fish intestine. However, an extremely low M.W. (11~13 kDa) of protease, which has similar enzymatic properties to trypsin, has been found in the digestive organ of decapods and might be a member of an unknown family of proteases (Pfleiderer et al., 1967). Such enzymes may act in a compensatory manner since there is an apparent absence of peptidic and chymotryptic endopeptidases in decapods (Devillez, 1975). These enzymes have been observed in the digestive organs of crustacea such as lobster (Brockerhoff et al., 1970; Galgani and Nagayama, 1987b), shrimp *P.*

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spp (Galgani et al., 1984), and crayfish, *Orconectes virilis* (Armstrong and Devillez, 1978). Purified protease from shrimp hepatopancreas, however, has been rarely reported so far, and needs to be purified for characterization on its enzymatic properties.

The objective of this paper was to confirm the existence of protease, which has no tryptic and chymotryptic activity and to establish a purification procedure for the protease from shrimp hepatopancreas.

Materials and Methods

Materials

Fresh shrimp (*P. japonicus*) was purchased in a local fish market, and the collected hepatopancreas was stored at −85℃ until used for protease purification.

Protein Concentration

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

Assay for Enzyme Activity

Proteolytic activity was determined according to the method of Kim et al. (1992). 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 ℃. Hydrolysis of benzoyl L-tyrosine ethyl ester (BTEE) was monitored at 253 nm at 25℃. 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 $CaCl_2$.

Protease Extraction and 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 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 (3×15 cm) previously equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl, and eluted with 500 mℓ linear gradient ranging from 0.1~2 M NaCl. The protease fractions without trypsin activity were pooled and diluted with 3 volumes of 20 mM Tris-HCl buffer (pH 7.5). Appropriated diluted protease solution was loaded on a second Q-Sepharose column (2.5×10 cm) and eluted with 400 mℓ linear gradient ranging from 0.1~1.0 M NaCl. The protease fraction was pooled and diluted with 3 volumes of 20 mM Tris-HCl buffer. The diluted sample was applied onto a third Q-Sepharose column (1.5×10 cm) and eluted with 200 mℓ linear gradient ranging from 0.1~1 M NaCl. The main fraction showing protease activity was pooled and adjusted to a final concentration of 0.5 M NaCl, 5 mM $CaCl_2$, 1 mM benzamidine, and 1 mM EDTA. A mixed solution was loaded onto a benzamidine Sepharose-6B column (1.5×5 cm) equilibrated with an equilibration buffer (20 mM Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl, 5 mM $CaCl_2$, 1 mM benzamidine, and 1 mM EDTA) and rinsed with 10 mℓ of the equilibration buffer. The flow-through was dialyzed against 20 mM Tris-HCl (pH 6.8) containing 0.1 M NaCl and 1 m EDTA. The dialysate was precipitated with cold acetone and the precipitate was dissolved with the same buffer. The protease solution was applied on a Sephacryl S-100 column (1.6×60 cm) and eluted with 20 mM Tris-HCl (pH 6.8) containing 0.1 M NaCl and 1 mM EDTA. Fractions having high proteolytic activity were pooled and stored at −85℃.

Molecular Weight Determination

Molecular weight of the purified protease was determined using a Superose 6 column (HR 10/30) chromatography according to the method of Whitaker (1963). M.W. of denatured protease was determined using SDS-PAGE by the method of Laemmli (1970).

Amino Acid Composition

The amino acid composition of the enzyme was 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 were computed from the best fit to the molecular weight as determined by gel filtration.

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 solution were incubated at 25℃ for 30 min, and residual activity was measured with 5% casein solution at pH 8.1 and 45℃.

Substrate Specificity

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

Results and Discussion

Protease Purification

The purification method was designed to purify a protease without tryptic and chymotryptic activity. The A.S. fraction with 30~70% saturation was loaded onto a first Q-Sepharose column. Two main fractions showing proteolytic activity were separated with the first Q-Sepharose column chromatography and one of them did not have tryptic activity as shown in Fig. 1. These fractions (tube No. 20~36) were pooled and diluted with 3 volumes of 20 mM Tris-HCl buffer (pH 7.5). The enzyme fraction was further separated by a second Q-Sepharose column chromatography. As shown in Fig. 2, a large quantity of chromogenic compounds, which may have resulted from polyphenol oxidase, were separated with protease. The purity was increased by 11-fold with a yield of 20%. However, the second Q-Sepharose fraction still had chromogenic compounds and a lot of contaminated proteins showed in SDS-PAGE (data not shown). Therefore, enzyme fractions (tube No. 57~66) were pooled and applied onto a third Q-Sepharose column and eluted (Fig. 3). The third Q-Sepharose fraction had a low level of tryptic activity for BAPNA and contaminated trypsin activity needed to be removed by affinity chromatography. Thus, the enzyme solution was passed through a benzamidine Sepharose-6B column and successfully removed the trypsin-like enzymes. This step was very efficient in removing the trypsin-like enzymes and the purity increased by 54.7-fold. The pattern of SDS-PAGE

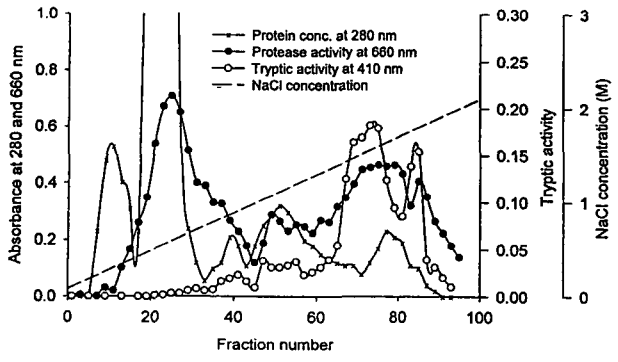

Fig. 1. Chromatogram of a first Q-Sepharose ionic exchange chromatography of 30~70% A.S. fraction. Flow rate was 40 mℓ/hr and fraction volume 5 mℓ per tube.

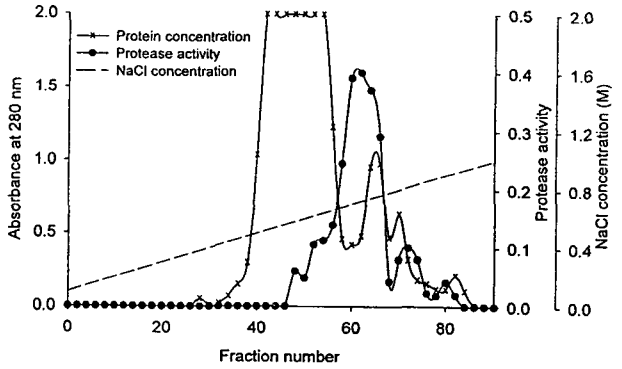

Fig. 2. Chromatogram of a second Q-Sepharose ionic exchange chromatography from the protease fraction of the first Q-Sepharose chromatography. Flow rate was 40 mℓ/hr and fraction volume 5 mℓ per tube.

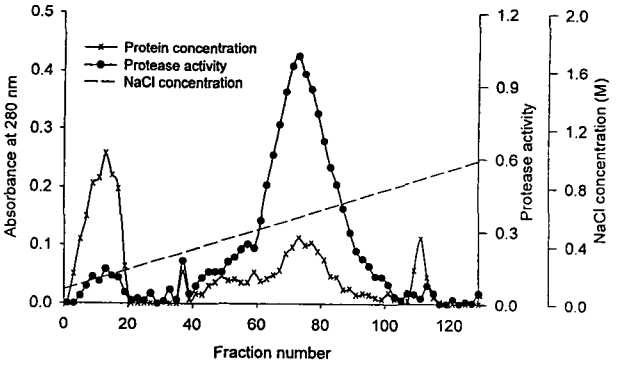

Fig. 3. Chromatogram of a third Q-Sepharose ionic exchange chromatography from the protease fraction of the second Q-Sepharose chromatography. Flow rate was 40 mℓ/hr and fraction volume 1.6 mℓ per tube.

Table 1. Purification of protease from hepatopancreas of shrimp, *Penaeus japonicus*

	Vol. (mℓ)	Protein (mg/mℓ)	Activity (U/mℓ)	Purity (-fold)	Yield (%)
Crude extract	120	9.80	3.76	1	100
1'st Q-Sepharose fraction	85	2.72	1.33	1.27	25.2
2'nd Q-Sepharose fraction	42	0.51	2.14	10.9	20.0
3'rd Q-Sepharose fraction	32	0.16	2.22	36.1	15.7
Flow through of Benzamidine Sepharose 6B	32	0.08	1.68	54.7	11.9
Sephacryl S-100 fraction	6	0.21	5.14	63.7	6.8

Activity was determined with 5% casein at 45°C and pH 8.1

had a little amount of high molecular weight protein band and was successfully removed by Sephacryl S-100 gel filtration (Fig. 4).

Results of the purification of the protease, based on the proteolytic activity for casein, are summarized in Table 1. Only 1.2 mg of protease were recovered from 50 g of shrimp hepatopancreas by these purification procedure. The purity of protease were increased by approximately 64-fold with a yield of 6.8%.

Molecular Weight

The molecular weight of native protease was determined to be 24 kDa by Superose 6 gel filtration (Fig. 6). The major proteolytic enzyme in the hepatopancreas of decapods has been identified to be a trypsin-like enzyme. A trypsin-like enzyme estimated to have a M.W. of 24 kDa has been isolated from white shrimp, *P. setiferus* (Gates and Travis, 1969) and Japanese spiny lobster, *Panulirus japonicus*, (Galgani and Nagayama, 1987b). The M.W. of the trypsin-like enzymes estimated by gel filtration were reported to be 25 kDa from lobster (Brockerhoff et al., 1970) and shrimp (Galgani et al., 1984), and 29 kDa from crab, *Eriocheir japonicus* (Muramatsu and Morita, 1981). Also, a slightly higher M.W. for trypsin-like enzymes from marine invertebrate has been reported, i.e., 32 kDa from *P. japonicus* (Kim et al., 1996) and 33.5 kDa from *Callinectes sapidus* (Dendinger and O'Connor, 1990).

Uncharacteristically low M.W. (11-13 kDa) proteases have been identified in the digestive organ of decapods such as lobster (Galgani and

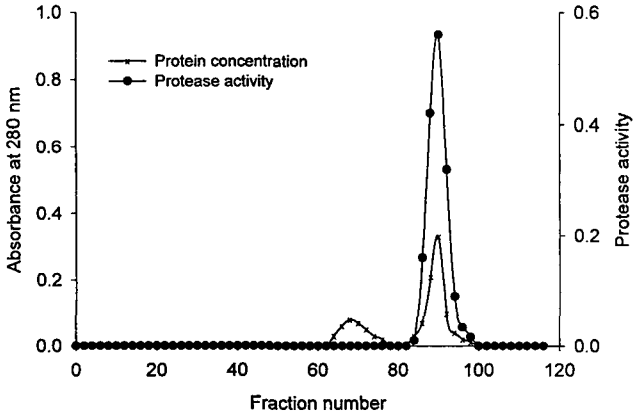

Fig. 4. Chromatogram of Sephacryl S-100 gel filtration from the protease fraction of benzamidine Sepharose-6B affinity chromatography. Flow rate was 120 mℓ/hr and fraction volume 1 mℓ per tube.

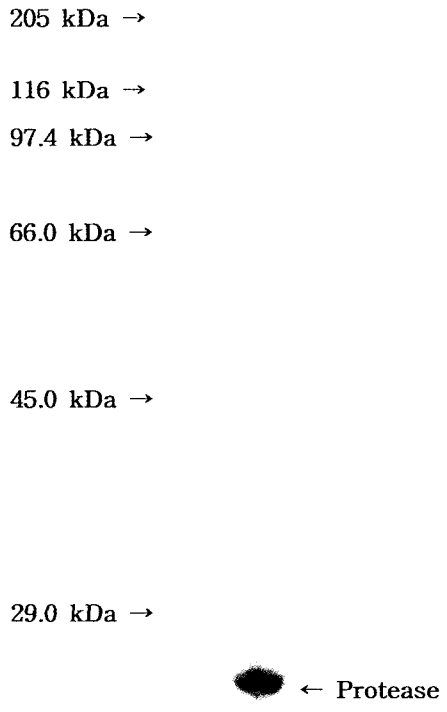

Fig. 5. SDS-PAGE (10%) of purified protease from the hepatopancreas of shrimp (*P. japonicus*).

Nagayama, 1987b), shrimp, *P. spp.*, (Galgani et al., 1984), and crayfish, *Orconectes virilis*, (Armstrong and Devillez, 1978). These may be a member of an unknown family of proteases which have not been reported in vertebrates (Zwilling and Neurath, 1981). Although the M.W. of protease purified in this study was similar to the trypsin-like enzymes, the properties of the enzyme were similar to low M.W. of preteases found in the digestive organ of decapods.

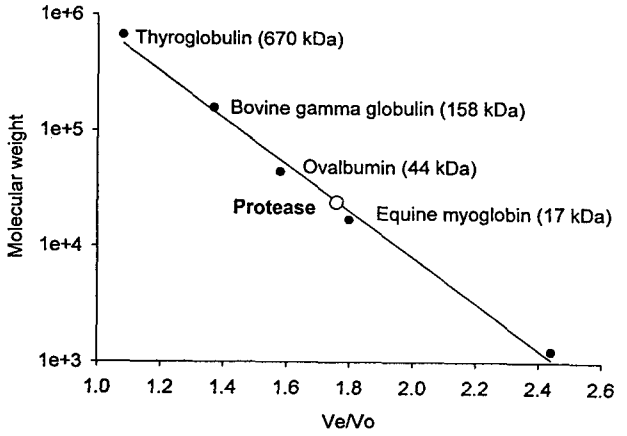

Fig. 6. Determination of molecular weight of purified protease from the hepatopancreas of shrimp, *Penaeus japonicus*, by Superose 6 gel filtration.

Amino Acid Composition

Amino acid composition of purified protease was compared with trypsin-like enzymes from crayfish, *Procambarus clarkii*, shrimp, *P. japonicus*, and bovine and given in Table 2. Amino acid composition of shrimp protease was different with trypsins from shrimp, *P. japonicus*, and crayfish. Shrimp protease was rich in aspartate, glycine, alanine, proline, and valine residues. The ratio of acidic to basic amino acids of protease was lower than those of trypsins from decapods, suggesting a low anionic properties of the enzyme.

Trypsins from decapods have similar ratios of acidic to basic amino acids in the range of 3.1 to 5.3 (Table 2). This is quite different from mammalian trypsins and shrimp protease, which have basic isoelectric points. Of particular interest, glutamate content in shrimp protease and bovine trypsin is significantly lower than that in decapod trypsins. The low ratio of acidic to basic amino acid residues supports the observation that shrimp protease was bound weakly to the anionic exchange resin and eluted in a low salt concentration.

Table 2. Comparison of amino acid compositions of protease from the hepatopancreas of shrimp with trypsins from shrimp, crayfish, and bovine. The values are given in residues per molecule.

AA	Shrimp protease	Trypsin		
		Shrimp[1]	Crayfish[2]	Bovine[3]
Asp	21	40	46	22
Glu	9	44	28	14
Ser	9	33	21	33
Gly	24	44	59	25
His	7	9	7	3
Arg	6	7	2	2
Thr	13	16	20	10
Ala	25	28	29	14
Pro	22	19	17	9
Tyr	10	9	11	10
Val	21	30	22	17
Met	3	1	3	2
Ile	13	20	18	15
Leu	15	20	19	14
Phe	10	12	6	3
Lys	6	10	5	14
Ratio of acidic to basic amino acids				
	1.5	3.2	5.3	1.9
Residues	214	342	313	207

[1] Kim et al., 1996. J. Korean Fish. Soc. 29 (6); 797.
[2] Kim et al., 1992. Comp. Biochem. Physiol. 103B; 391.
[3] Walsh and Neurath, 1964. Proc. Nat. Acad. Sci., 52; 884.

The anionic nature of the trypsin-like enzymes would result from the amino acid compositions, which show a very high proportion of acidic amino acid residues. Anionic trypsins seem to be quite common in marine organisms, as described in *P. setferus* trypsin (Gates and Travis, 1969), *P. japonicus* trypsin (Kim et al., 1992), and crayfish trypsins (Kim et al., 1996). The amino acid compositions of trypsins from different species are well known; including humans and bovine (Walsh and Neurath, 1964). The amino acid composition of decapods has some marked differences from that of bovine trypsin (Bradshaw et al., 1970; Walsh and Neurath, 1964). Specifically, decapod trypsin, like anionic trypsin from fish intestine, contains low levels of basic amino acid residues compared to mammalian trypsin.

Effect of Inhibitors

Activity of protease was strongly inhibited by 10 mM benzamidine and SBTI (Table 3). SBTI and benzamidine are well known as trypsin specific inhibitors. The enzyme activity was reduced 47%

by 1 mM benzamidine, with complete inhibition by 10 mM benzamidine. The enzyme was inhibited to a lesser degree by PMSF and TLCK. However, TPCK and EDTA were not effected on the activity of the protease.

TLCK deactivates only trypsin-like enzymes whose specificity corresponds to the reagent structure, i.e. 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 portion of the active center in the molecule (Severin and Tomasek, 1965). However, inhibition by TLCK is difficult to interpret safely because of a possible side reaction which is the reagent has the possibility of side reaction, particularly at -SH groups. Therefore, the possibility of finding histidine in the active site of shrimp protease was rare since the inhibition by TLCK was not complete. Four trypsins from crayfish (Kim et al., 1992) and two trypsins from *P. japonicus* (Kim et al., 1996) were completely inactivated by addition of 1 mM TLCK.

Although benzamidine is well known as a trypsin specific inhibitor, protease activity was reduced 47% of original activity by 1 mM benzamidine, with complete inhibition by 10 mM benzamidine. Trypsins from krill (Osnes and Mohr, 1985b) and *P. japonicus* (Kim et al., 1996) were almost entirely inhibited by 1 mM benzamidine, but trypsin from shrimp *P indicus* (Honjo et al., 1990) was not inactivated even though higher concentrations of benzamidine were applied.

Table 3. Effect of inhibitors on the activity of protease from the hepatopancreas of shrimp, *Penaeus japonicus*

Inhibitor	Concentration	Relative activity (%)
Control		100
TLCK	0.1 mM	53
Benzamidine	10 mM	0
	1.0 mM	53
PMSF	1.0 mM	28
Leupeptin	0.1 mM	91
SBTI	1.0 mM	15
Pepstatin	0.1 mM	52
TPCK	0.1 mM	100
EDTA	1.0 mM	92

Protease (70 μg/mℓ) was incubated with same volume of inhibitor at 25℃ for 30 min, and residual activity was determined with 5% casein solution at 50℃ and pH 8.1.

PMSF and SBTI have been described as strong inhibitors for serine enzyme, with variable inhibitory effects on trypsins from dacapods and other animals such as Atlantic blue crab (Dendinger and O'Connor, 1990), shrimp (Honjo et al., 1990), and Antarctic krill (Osnes and Mohr, 1985a). Trypsin from *P. monodon*, however, was not inhibited by PMSF (Lu et al., 1990).

Pepstatin specifically inhibits acid proteases and the inhibitory activity can be explained by the strong binding of pepstatin to the active site of pepsin. The protease was inhibited 48% of original activity by pepstatin, which suggests a presence of acidic amino acid in the active site of the enzyme. However, two trypsins from *P. japonicus* (Kim et al., 1996) and four trypsins from *P. clarkii* (Kim et al., 1992) were not affected by pepstatin.

The protease was not affected by leupeptin, TPCK, and EDTA. Leupeptin has the argininal residue at their terminal carbon. This inhibits the

Table 4. Specificity of the protease against synthetic substrates

(Specific activity, μmol/μg)

Substrate (0.5 mM)	Protease
CBZ-Tyr-p-NE	408
CBZ-Pro-p-NE	335
CBZ-Trp-p-NE	303
CBZ-Gly-p-NE	0
CBZ-Ala-p-NE	0
CBZ-Val-p-NE	0
CBZ-Phe-p-NE	0
CBZ-Cys-p-NE	0
CBZ-Asp-p-NE	0
BAPNA	0
BTEE	0

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
BAPNA; Benzoyl-D,L-arginine p-nitroanilide
BTEE; Benzoyl-L-tyrosine ethyl ester

common enzyme trypsin and papain which cleave the carboxyl side of basic amino acids such as arginine or lysine. TPCK is a specific inhibitor of chymotrypsin. With the inhibition experiment, the protease did not show any chymotryptic activity, but exhibited partial properties of trypsin-like enzymes.

Substrate Specificity

The protease did not show hydrolytic activity on BAPNA and BTEE which are specific substrates for trypsin and chymotrypsin, respectively. The protease, however, showed hydrolytic activity on the synthetic peptides such as CBZ-Tyr-pNE, CBZ-Trp-pNE, and CBZ-Pro-pNE.

The enzyme exhibited partial properties of trypsin in the inhibition studies. However, the enzyme has an hydrolytic activity against peptides containing aromatic amino acids. This result brings some discrepancy to the results of inhibitor studies and thus more studies are required on the specificity of synthetic substrates. It also suggests that the structure of the active site might be similar to chymotrypsin encompassing the aromatic amino acids residue.

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. However, shrimp do not have an acidic stomach. Furthermore, chymotrypsin activity was not detected in the hepatopancreas and digestive tract of shrimp even though there have been studied five species of crustacea (Galgani and Nagayama, 1987a) and shrimp in this study. 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, and has been found in the digestive organ of crawfish (Pfleiderer et al., 1967), crab (Herbold et al., 19 71), and shrimp (Galgani et al., 1984). Like other crustacea, peptidic and chymotryptic activity was not detected in the hepatopancreas of *P. japonicus* (data not shown). Therefore, hydrolytic activity for Phe and Tyr by shrimp protease might be a compensatory activity due to the absence of chymotrypsin. Also, the result of substrate specificity was contradictory to the result of inhibitor studies and require more studies on the specificity of synthetic substrates.

Conclusion

The shrimp protease purified in this research showed no chymotrypsin or trypsin activity against BAEE and BAPNA, respectively. The protease has not been reported untill now and classified as a new member of protease. The enzyme was not deactivated by a specific inhibitor of chymotrypsin, and only partially inhibited by trypsin specific inhibitors and pepsin specific inhibitors. The ratio of acidic to basic amino acid residues was similar to bovine trypsin, which has cationic properties. However, the enzyme had specificity against the carboxyl terminal of tyrosine, tryptophan, and proline, which was similar to that of chymotrypsin.

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