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Purification and Characterization of Protease from the Hepatopancreas of Shrimp, *Penaeus orientalis*

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A protease without tryptic and chymotryptic activities was purified from the hepatopancreas of shrimp, *Penaeus orientalis*, using Q-Sepharose ionic exchange, benzamidine Sepharose-6B affinity, Mono-Q, and gel chromatography. Molecular weight (M.W.) of the protease was estimated to be 27 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The amino acid composition of the protease was different from that of protease from *P. japonicus* or trypsin from *P. orientalis*. The protease was completely inhibited by benzamidine, Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK), and phenylmethylsulfonyl fluoride (PMSF) and was not affected by leupeptin, pepstatin, N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), iodoacetate, and ethylenediamine tetraacetate (EDTA). The enzyme did not have any activity against Nα-benzoyl-DL-arginine p-nitroanilide (BAPNA) or N-benzoyl-L-tyrosine ethyl ester (BTEE) which are specific substrates of trypsin and chymotrypsin, respectively. However, the protease showed hydrolytic activity for a carboxyl terminal of Tyr, Trp, Phe, Glu, and Cys.

Key words: shrimp protease, hepatopancreas, purification, inhibition, substrate specificity

Introduction

With the rapid growth of fast-food industry in Korea, a large quantity of shrimp production has been needed to meet the domestic demand. Among shrimp species, *Penaeus orientalis* is the most favorite species for an aquacultural production in Korea. The majority of harvested shrimp is distributed as fresh meat on ice. During distribution and handling, fresh tail meat becomes extremely soft only after relatively short periods of iced storage.

Texture softness 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 turns mushy on cooking (Lindner et al., 1988). 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. Hepatopancreas of crustacea may be responsible for the onset of tissue deterioration leading to softness of shrimp tail meat because the tissue contains high activity of trypsin-like protease (Honjo et al., 1990; Kim et al., 1994; 1996a; 1996b).

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., 1992; 1994), crab (Dendinger and O'Connor, 1990), lobster (Galgani and Nagayama,

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1987b), and shrimp (Gates and Travis, 1969; Kim et al., 1996b). 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. (11,000~13,000) 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 endopeptidase activities in decapods. These enzymes have been observed in the digestive organs of crustacea such as lobster (Galgani and Nagayama, 1987b), shrimp *P.* spp (Galgani et al., 1984), and crayfish *Orconectes virilis* (Armstrong and Devillez, 1978). Trypsin-like proteases in the hepatopancreas of crustacea are primarily responsible for the degradation of tail meat and those were purified and characterized on their enzymatic properties. However, a protease without tryptic and chymotryptic activity has not been reported, and it is necessary that the enzyme should be purified for the characterization of its enzymatic properties.

The objective of this study was to establish a purification protocol for the protease without tryptic and chymotryptic activity from shrimp hepatopancreas and to characterize its biochemical properties.

Materials and Methods

Materials

Fresh shrimp (*Penaeus orientalis*) was purchased at an aquaculture farm, and the hepatopancreas was collected and stored at 85℃ until used for protease purification. N-CBZ-L-tyrosine p-nitrophenyl ester (CBZ-Tyr-NE), N-CBZ-L-phenylalanine pnitrophenyl ester (CBZ-Phe-NE), N-CBZ-L-cysteine p-nitrophenyl ester (CBZ-Cys-NE), N-CBZ-L-tryptophan p-nitrophenyl ester (CBZ-Trp-NE), N-CBZ-L-glutamic acid pnitrophenyl ester (CBZ-Gln-NE), N-CBZ-L-arginine p-nitrophenyl ester (CBZ-Arg-NE), N-CBZ-L-aspartic acid p-nitrophenyl ester (CBZ-Asp-NE), N-CBZ-L-proline p-nitrophenyl ester (CBZ-Pro-NE), N-CBZ-L-glycine p-nitrophenyl ester (CBZ-Gly-NE), N-CBZ-L-valine p-nitrophenyl ester (CBZ-Val-NE), N-CBZ-L-alanine p-nitrophenyl ester (CBZ-Ala-NE), Nα-benzoyl-DL-arginine p-nitroanilide (BAPNA), N-benzoyl-L-tyrosine ethyl ester (BTEE), Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), benzamidine, phenylmethylsulfonyl fluoride (PMSF), leupeptin, soybean trypsin inhibitor (SBTI), pepstatin, benzamidine Sepharose-6B were purchased from Sigma Chemical Co. (St Louis, USA). Q-Sepharose and Superdex 75 were purchased from Pharmacia (Uppsala, Sweden). Hammersten casein was purchased from USB (Cleveland, USA)

Protein Concentration and Molecular Weight Determination

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard. SDS-PAGE was performed according to the method of Laemmli (1970). Molecular weight of purified protease was determined by the method of Weber and Osborn (1975) using molecular weight standards (Low Molecular Weight Standard Kit, (SIGMA, St. Louis, MO).

Assay for Enzyme Activity

Proteolytic activity was determined by the method described by Kim et al. (1996a). Activity (U/min/mℓ) was defined as the absorbance equivalent of 1 mmole tyrosine produced /min/mℓ enzyme solution. Specific activity (U/min/mg) was expressed as the absorbance equivalent of 1 mmole tyrosine produced by 1 mg of protease. Amidolytic activity for 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℃. Chymotryptic activity was monitored by hydrolysis of BTEE at 253 nm at 25℃ by the method of Hummel (1959).

Protease Extraction and Purification

Shrimp hepatopancreas (65 g) was homogenized with 65 mℓ of 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 1 mM $CaCl_2$ by an UltraTurrax type tissue grinder (T25B type, Ika, Germany). The homogenate was centrifuged at 12,000×g for 15 min. The supernatant was treated with 0.2 vol. of tetrachloromethane to remove lipids, and the crude enzyme solution was obtained from the supernatant after centrifugation.

The crude enzyme solution was dialyzed overnight against 20 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and 1 mM EDTA. The dialysate was centrifuged at 12,000×g for 30 min and supernatant was applied onto a Q-Sepharose column

(2.5×15 cm) previously equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and 1 mM EDTA. The column was eluted with 300 mℓ linear gradient ranging from 0.1~1.0 M NaCl. The protease fractions without tryptic activity were pooled and diluted with 3 volumes of 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA. Diluted protease solution was loaded on a second Q-Sepharose column (1.5×10 cm) and eluted with 150 mℓ linear gradient ranging from 0.1~0.5 M NaCl. The 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. The adjusted solution was loaded onto a benzamidine Sepharose-6B column (1.0×5 cm) which was equilibrated with an equilibration buffer (20 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl, 5 mM $CaCl_2$, 1 mM benzamidine, and 1 mM EDTA), and rinsed with 5 mℓ of the equilibration buffer. Unbound fraction (flowthrough) was dialyzed against 20 mM Tris-HCl, pH 6.8, containing 1 mM EDTA and further purified by FPLC system (Pharmacia, Sweden) with Mono-Q column. The protease fractions were pooled and precipitated with cold acetone and the precipitate was dissolved in 1.6 mℓ of 20 mM Tris-HCl, pH 6.8, containing 0.1 M NaCl and 1 mM EDTA. The protease solution was applied on a Superdex 75 (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℃.

Amino Acid Composition

The amino acid composition of the purified protease was determined with a Biochrom 20 amino acid analyzer (Pharmacia, Sweden) after hydrolysis in 6 N HCl at 110℃ for 24 and 72 hr. The amino acid residues presented in integral numbers were computed from the best fit to the molecular weight as determined by SDS-PAGE. The residues of serine and threonine were calculated by extrapolating the data obtained from 24 and 72 hr hydrolysates.

Effect of Inhibitors

SBTI, TLCK, and benzamidine were dissolved in distilled water. TPCK, leupeptin, pepstatin, and PMSF were dissolved in dimethyl sulfoxide. Equal volumes of diluted inhibitor and purified protease 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

Hydrolysis 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 mmole of a substrate per min per mg of enzyme at 25℃.

Results and Discussion

Protease Purification

The purification method was designed to purify a protease without tryptic activity. Purification steps of protease are summarized in Table 1. The crude enzyme solution was loaded to Q-Sepharose column. Two protease fractions were detected in the Q-Sepharose column chromatography (Fig. 1). The former had protease activity only, however, the latter fraction had protease as well as tryptic activities. Therefore, we targeted to purify the former fraction which had neither tryptic nor chymotryptic activities. With the Q-Sepharose ionic exchange chromatography, the purity of protease increased to 3.81-fold with 40.3% recovery. The low recovery was caused by removal of the tryptic activity in the latter fraction.

The protease fractions (No. 1,428) were pooled and diluted with 3 volumes of 20 mM Tris-HCl buffer, pH 7.5. The protease fraction was further separated by a second Q-Sepharose column chromatography. As shown in Fig. 2, a large quantity of proteins was separated from the protease fractions. With the second Q-Sepharose chromatography, the purity of protease increased to 9.93 fold with 21.2% recovery. The second Q-Sepharose fraction still had low level of tryptic activity against

Table 1. Purification of protease from the hepatopancreas of Shrimp, *p. Orientalis*

	Vol. (mℓ)	Protein (mg/mℓ)	Activity (U/mℓ)	Purity (fold)	Yield (%)
Crude extract	100	9.90	6.17	1	100
1'st Q-Sepharose fraction	75	1.40	3.32	3.81	40.3
2'nd Q-Sepharose fraction	33	0.64	3.96	9.93	21.2
Flow-through of benzamidine Sepharose-6B fraction	38	0.28	3.12	17.9	19.2
MonoQ	1.6	1.46	39.6	43.5	10.3
Superdex 75 fraction	5.0	0.21	7.56	57.8	6.1

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

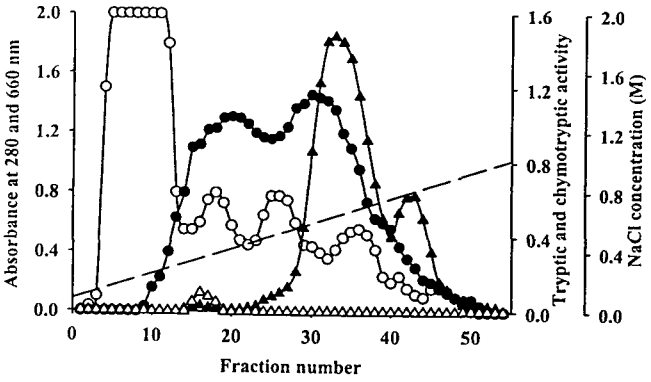

Fig. 1. Chromatogram of a first Q-Sepharose chromatography of crude extract. Flow rate was 40 mℓ/hr and fraction volume was 5 mℓ per tube. The symbols were as follows: —○—, protein concentration at 280 nm; —●—, caseinolytic activity; —▲—, tryptic activity with BAPNA; —△—, chymotryptic activity with BTEE, and ----- , salt concentration.

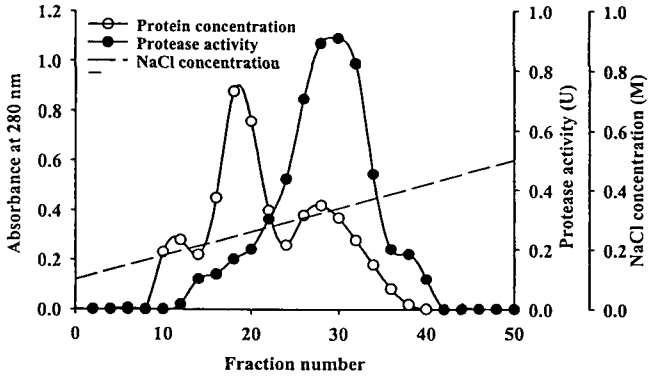

Fig. 2. Chromatogram of a second Q-Sepharose chromatography of protease fraction from the first Q-Sepharose chromatography. Flow rate was 30 mℓ/hr and fraction volume was 3 mℓ per tube.

BAPNA and the contaminating tryptic activity needed to be removed by the affinity chromatography. Therefore, enzyme fractions (tube No. 2,535) were pooled and adjusted to a final concentration of 0.5 M NaCl, 5 mM $CaCl_2$, 1 mM benzamidine, and 1 mM EDTA for benzamidine Sepharose-6B affinity chromatography and was passed through benzamidine Sepharose-6B column. This affinity chromatography was very efficient to remove trypsin-like enzymes. After the benzamidine affinity chromatography, trypsin activity was not detected in non-adsorbed fraction of the column. The purity of the protease increased to 17.9 times with 19.2% recovery.

The non-adsorbed fraction was dialyzed against 20 mM Tris-HCl, pH 6.8, containing 1 mM EDTA to further purify the protease with Mono-Q column. After Mono-Q column chromatography, the purity increased to 43.5-fold with 10.3% recovery. Mono-Q fractions were precipitated with cold acetone and the precipitate was dissolved in 1.6 mℓ of 20 mM Tris-HCl, pH 6.8, containing 0.1 M NaCl and 1 mM EDTA for size exclusion chromatography. After gel filtration on Superdex 75 column (Fig. 3), the purity of protease increased to 57.8-fold with 6.1% recovery.

Molecular Weight

The molecular weight of protease was determined to be 27 kDa by SDS-PAGE (Fig. 4). The major proteolytic enzyme in the hepatopancreas of decapods has been identified to be a trypsin-like enzyme. Trypsin-like enzymes from marine organisms, either vertebrates or invertebrates, in most instances have M.W. in the range of 24~32 kDa. The M.W. of trypsin-like enzymes from crustacea varies widely from those of fish. Trypsinlike enzymes with a M. W. of 24~25 kDa have been isolated from white shrimp, *P. setiferus* (Gates and Travis, 1969), *P. kerathurus* (Galgani et al., 1984), and Japanese spiny lobster, *Panulirus japonicus*, (Galgani and Nagayama, 1987b). Also, trypsin-like enzymes with slightly higher M.W. has been reported from marine invertebrates: 32 kDa from *P. japonicus* (Kim et al., 1996b) and 33.5 kDa from *Callinectes sapidus* (Dendinger and O'Connor, 1990). Uncharacteristically low M.W. (11~13 kDa) proteases which had partial properties

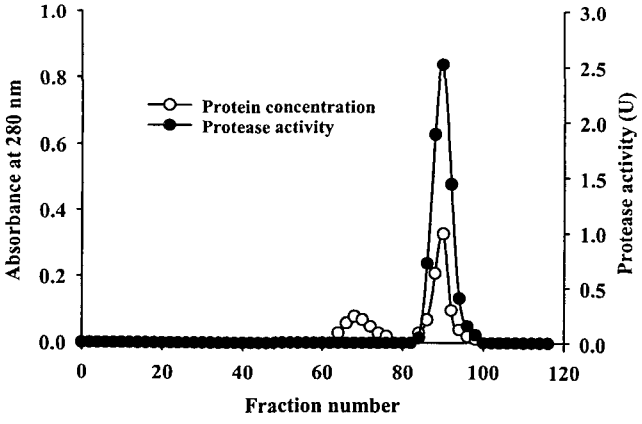

Fig. 3. Chromatogram of Superdex 75 gel filtration from the acetone precipitated fraction. Flow rate was 120 mℓ/hr and fraction volume was 1 mℓ per tube.

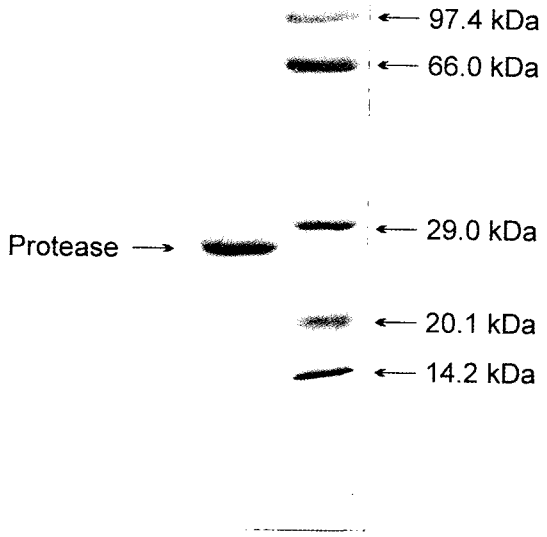

Fig. 4. SDS-PAGE of purified protease from the hepatopancreas of shrimp, *P. orientalis*.

of trypsin, have been identified in the digestive organ of decapods such as lobster (Galgani and Nagayama, 1987b), shrimp, *P. spp.* (Galgani et al., 1984), and crayfish, Orconectes virilis, (Armstrong and Devillez, 1978). These may be a member of unknown family of proteases which have not been reported in vertebrates (Zwilling and Neurath, 1981). A protease was purified from the hepatopancreas of *P. japonicus* (Choi et al., 1998) and the M. W. was estimated to be 24 kDa which is lower than trypsin-like enzyme isolated from same species (Oh et al., 1998).

Amino Acid Composition

Amino acid composition of purified protease was compared with protease from the hepatopancreas of *P. japonicus*, trypsin from the hepatopancreas of *P. orientalis*, and bovine trypsin (Table 2). The amino acid composition of shrimp protease was different from that of protease from *P. japonicus* (Choi et al., 1998) or trypsin from *P. orientalis* (Oh et al., 1998). Asp, Glx, and Ser residues in the protease from *P. orientalis* were higher than that from *P. japonicus*. However, nonpolar amino acids such as Ala, Pro, Leu, Ile, and Phe and Tyr were rich in the protease of *P. japonicus* compared to that of *P. orientalis*. Simialr amino acid composition except Arg, Thr, Ala, and Tyr were observed between the protease and trypsin from *P. orientalis*. The ratio of acidic

Table 2. Comparison of amino acid compositions of shrimp hepatopancreatic proteases and trypsin and bovine trypsin

AA	Protease		Trypsin	
	POP	PJP[1]	POT[2]	Bovine[3]
Asx	36	22	40	22
Glx	23	9	32	14
Ser	21	9	28	33
Gly	29	25	40	25
His	8	8	8	3
Arg	7	6	4	2
Thr	19	14	10	10
Ala	13	27	24	14
Pro	19	24	22	9
Tyr	8	11	14	10
Val	19	22	19	17
Met	4	3	3	2
Ile	11	14	13	15
Leu	13	16	16	14
Phe	7	11	11	3
Lys	6	6	6	14
Cys/2	13	ND	14	12
Ratio of acidic to basic amino acids	2.8	1.6	3.7	1.9
Total residues	256	227	303	223
M.W	27 kDa	24 kDa	32 kDa	24 kDa

The values are given in residues per molecule.
ND: not determined.
[1]Choi et al., 1998. J. Fish. Sci. Tech. 1, 201.
[2]Oh et al., 1998. J. Fish. Sci. Tech. 1, 209.
[3]Walsh and Neurath, 1964. Proc. Nat. Acad. Sci. U.S.A. 51, 884.

to basic amino acids of protease was lower than those of trypsins from crustaceae, suggesting a low anionic properties of the enzyme (Kim et al., 1992; 1996b).

Trypsins from decapods have similar ratios of acidic to basic amino acids in the range of 3.1 to 5.3 (Kim et al., 1992). This is quite different from mammalian trypsins and the shrimp protease, which have basic isoelectric points. The low ratio of acidic to basic amino acid residues supports the observation that the protease was bound weakly to the anionic exchange resin and eluted at lower salt concentration compared to trypsin. The anionic nature of the trypsin-like enzymes might result from the amino acid compositions, which show a very high proportion of acidic amino acid residues. Anionic property seems to be quite common in marine organisms, as described for *P. setiferus* (Gates and Travis, 1969), *P. japonicus* (Kim et al., 1996b), and crayfish trypsins (Kim et al., 1992). The amino acid compositions of the decapod trypsins

have some marked differences from that of bovine trypsin (Walsh and Neurath, 1964). Specifically, decapod trypsins, like the anionic trypsin from fish intestine, contain low levels of basic amino acid residues compared to mammalian trypsin.

Effect of Inhibitors

Activity of protease was completely inhibited by 1 mM TLCK and 10 mM benzamidine (Table 3). It was also strongly inactivated by 1 mM PMSF and 0.1 mM SBTI. However, TPCK, pepstatin, and EDTA did not affect the activity of the protease.

TLCK is a well known trypsin-specific inhibitor and inactivates only trypsin-like enzymes whose specificity corresponds to the reagent by alkylation of the active center histidine. The reagent is known to form a covalent bond with histidine residue in the catalytic site of enzyme and 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, particularly at SH groups. Degree of protease inhibition by 0.1 mM TLCK was less than those of four trypsins from crayfish (Kim et al., 1992) and trypsins from *P. japonicus* (Kim et al., 1996b), but similar to the protease from *P. japonicus* (Choi et al., 1998).

Benzamidine is well known as a trypsin-specific inhibitor; however, 12% of the original activity of the protease was inhibited by 1 mM benzamidine, and completely inhibited by 10 mM benzamidine. This inhibition level is lower than those of the protease from the hepatopancreas of *P. japonicus* (Choi et al., 1998). Trypsins from menhaden (Pyeun et al., 1990) and *P. japonicus* (Kim et al., 1996b) were almost entirely inactivated by 1 mM benzamidine, but trypsin from shrimp, *P. indicus* (Honjo et al., 1990) was not affected even at higher concentrations of benzamidine applied.

PMSF and SBTI have been described as strong inhibitors for serine proteases, with variable inhibitory effects on trypsins from decapods and other animals such as Atlantic blue crab (Dendinger and O'Connor, 1990) and shrimp (Honjo et al., 1990). The protease from *P. orientalis* was strongly inactivated by PMSF like trypsins from *P. japonicus* (Kim et al., 1996b). The latter was more easily inactivated by PMSF than the protease from *P. japonicus* (Choi et al., 1998).

Pepstatin specifically inhibits acid proteases and the inhibitory activity can be explained by the strong binding of pepstatin to the active site of pepsin. This result was similar to trypsin from *P. japonicus* (Kim et al., 1996b), *P. orientalis* (Oh et al., 1998), and four trypsins from *P. clarkii* (Kim et al., 1992).

The protease was not affected by leupeptin, pepstatin, TPCK, and EDTA. Leupeptin has the arginine residue at their terminal carbon that inhibits trypsin and papain and are able to inactivate the proteolytic activity of trypsin. Trypsin inhibitors such as TLCK, SBTI, PMSF and benzamidine, had similar inhibitory activity on the trypsin-like protease (Kim et al., 1992; 1996b; Oh et al., 1998). However, the protease was not affected by leupeptin even though it was strongly inhibited by other trypsin inhibitors. The protease was not

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

Inhibitor	Conc.	Inhibition (%)
Control		0
TLCK	1.0 mM	100
	0.1 mM	40
Benzamidine	10 mM	100
	1.0 mM	12
PMSF	1.0 mM	95
Leupeptin	0.1 mM	9
SBTI	0.1 nM	89
Pepstatin	0.1 mM	0
TPCK	0.1 mM	0
EDTA	0.1 mM	0
Iodoacetate	1.0 mM	0

Enzyme solution was incubated with same volume of inhibitor at 25℃ for 30 min and residual activity was measured with 5% casein 45℃ and pH 8.1.

Table 4. Substrate specificity of protease from the hepatopancreas of shrimp, *penaeus orientalis*

Substrate	Specific activity (μmole/mg protein)
CBZ-Tyr-pNE	10.6
CBZ-Phe-pNE	10.1
CBZ-Cys-pNE	4.74
CBZ-Trp-pNE	2.13
CBZ-Gln-pNE	0.91
CBZ-Arg-pNE	0.00
CBZ-Asp-pNE	0.00
CBZ-Pro-pNE	0.00
CBZ-Gly-pNE	0.00
CBZ-Val-pNE	0.00
CBZ-Ala-pNE	0.00
BAPNA	0.00
BTEE	0.00

Activity was determined with 1 mM of substratesat 25℃ and pH 8.1.

affected by pepstatin, which suggests the absence of acidic amino acids in the active site of the enzyme. TPCK is a specific inhibitor of chymotrypsin, which suggests the protease did not have chymotryptic properties. In the inhibition studies, the protease exhibited properties of trypsin-like enzymes but did not show any chymotryptic and peptidic properties.

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 substrates such as CBZ-Tyr-NE, CBZ-Phe-NE, CBZ-Cys-NE, CBZ-Trp-NE, and CBZ-Gln-NE.

The protease showed partial properties of trypsin in the inhibition studies. However, the enzyme has an hydrolytic activity against substrates containing aromatic amino acids such as Tyr, Phe. and Trp. This result brings some discrepancy to the results of inhibitor studies and thus more studies are required on the substrate specificity of the enzyme. 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 secrets pepsin to denature food proteins before trypsin and other proteases exert their catalytic activity. However, chymotrypsin activity was not detected in the hepatopancreas and digestive tract of shrimp studied among the five species of crustacea (Galgani and Nagayama, 1987 a) nor in the shrimp used in this study. The absence of peptidic and chymotryptic endopeptidases in crustacea might be compensated by the low M.W. protease, which has similar enzymatic properties to trypsin and has been found in the digestive organs 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. japonicus* (Fig. 1). Therefore, hydrolytic activity for Tyr, Phe, and Trp by the shrimp protease might be a compensatory activity for to the absence of chymotrypsin.

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References

Armstrong, J.R. and E.J. Devillez. 1978. Protease of low molecular weight in the digestive fluids of decapods. Can. J. Zool. 56, 2225~2229.

Bradfors, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of proteindye binding. Anal. Biochem. 72, 248~254.

Choi, S.M., E.S. Oh, D.S. Kim, J.H. Pyeun, D.M. Choi, C. B. Ahn and H.R. Kim. 1998 . Comparative biochemical properties of proteinase from the hepatopancreas of shrimp. I. Purification of protease from the hepatopancreas of *Penaeus japonicus*. J. Fish. Sci. Tech. 1, 201~208.

Dendinger, J.E. and K.L. O'Connor. 1990. Purification and characterization of a trypsin-like enzyme from the midgut gland of the atlantic blue crab, *Callinectes. sapidus*. Comp. Biochem. Physiol. 95B, 525~530.

Erlanger, B.F., N. Kokowsky and W. Cohen. 1961. The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95, 271~278.

Galgani, F., Y. Benyamin and H.J. Ceccaldi. 1984. Identification of digestive proteinases of *Penaeus kerathurus* (Forskal): A comparison with *Penaeus japonicus* bate. Comp. Biochem. Physiol. 78B, 355~361.

Galgani, F. and F. Nagayama. 1987a. Digestive proteinases in the five species of Lithodidae (Crustacea, Decapoda). Comp. Biochem. Physiol. 87B, 103~107.

Galgani, F. and F. Nagayama. 1987b. Digestive proteinases in the Japanese lobster *Panulirus japonicus*. Comp. Biochem. Physiol. 87B, 889~893.

Gates, B.J. and J. Travis. 1969. Isolation and comparative properties of shrimp trypsin. Biochemistry 8, 4483~4489.

Honjo, I., S. Kimura and M. Nonaka. 1990. Purification and characterization of trypsin-like enzyme from shrimp *Penaeus indicus*. Nippon Suisan Gakkaishi. 56, 1627~1634.

Hummel, B.C. 1959. A modified spectrophotometric determination of chymotrypsin, trypsin, and thrombin. Can. J. Physiol. 37, 1393~1399.

Kim, H.R., S.P. Meyers and J.S. Godber. 1992. Purification and characterization of anionic trypsins from hepatopancreas of crayfish, *Procambarus clarkii*. Comp. Biochem. Physiol. 103B, 391~398.

Kim, H.R., S.P. Meyers and J.S. Godber. 1994. Enzymatic properties of anionic trypsins from the hepatopancreas of crayfish, *Procambarus clarkii*. Comp. Biochem. Physiol. 107B, 197~203.

Kim, H.R., S.P. Meyers. and J.S. Godber. 1996a. Anionic trypsins from crayfish hepatopancreas: Effects

on protein degradation of tail meat. J. Food Sci. 61, 78~80, 96.

Kim, H.R., D.S. Kim, C.B. Ahn and J.H. Pyeun. 1996b. Purification and characterization of trypsins affecting on the autolysis of shrimp, *Penaeus japonicus*. J. Korean Fish. Soc. 29, 797~804.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature 227, 680~686.

Lindner, O., S. Angel, Z.R. Weinberg and R. Grant. 1988. Study of factors inducing mushiness in stored prawns. Food Chem. 29, 119~132.

Oh, E.S., D.S. Kim, K.J. Jung, J.H. Pyeun, M.S. Heu and H.R. Kim. 1998. Comparative biochemical properties of proteinase from the hepatopancreas of shrimp. II. Purification of trypsin from the hepatopancreas of *Penaeus orientalis*. J. Fish. Sci. Tech. 1, 209~215.

Pfleiderer, G., R. Zwilling and H.H. Sonneborn. 1967. Eine protease vom molekulargewicht 11,000 und eine trypsinahnliche Fraktion aus Astacus fluviatilis Fabr. Z. Physiol. Chem. 348, 1319~.

Pyeun, J.H., H.R. Kim and J.S. Godber. 1990. Comparative studies on the enzymatic properties of two trypsin-like enzymes from menhaden, *Brevoortia tyranus*. Bull. Korean. Fish. Soc. 23, 12~24.

Severin, E.S. and V. Tomasak. 1965. Studies on active site of trypsin. I. The reaction of trypsin with specific inhibitor NatosylLlysilchloromethane and certain pseudosubstrates. Biochem. Biophys. Res. Comm. 20, 496~502.

Walsh, K. and H. Neurath. 1964. Trypsinogen and chymotrypsinogen as homologous proteins. Proc. Natl. Acad. Sci. U.S. 52, 884~889.

Weber, K. and M. Osborn. 1975. Proeins and sodium dodecyl sulfate: Molecular weight determination of polyacrylamide gels and related procedures. Ch. 3. In *The proteins*, 3'rd ed., (H. Neurath and R.L. Hill, ed.), p. 179~223. Acad. Press, New York.

Zwilling, R. and H. Neurrath. 1981. Inverterate proteases. In *Methods in enzymology*, Vol. 80, (L. Lorand, ed.) pp. 633~664, Acad. Press, New York.