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# Enzymatic Properties of Protease from the Hepatopancreas of Shrimp, *Penaeus japonicus*

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A protease purified from hepatopancreas of shrimp, *Penaeus japonicus*, had maximum activity at 70℃ and in neutral and alkaline pH ranges. Specific activity at optimum reaction condition of the protease was estimated to be approximately 12 U/mg/min. The protease was stable in neutral and alkaline pH ranges and activity was retained after heat treatment at 50℃ for 30 min. Apparent $K_m$ and $V_{max}$ value against casein substrate were estimated to be 0.29% and 7.8 $sec^{-1}$, respectively, and those against N-CBZ-L-tyrosine p-nitrophenyl ester (CBZ-Tyr-NE) were 0.38 mM and 2,400 $sec^{-1}$, respectively. The N-terminal sequence of the protease showed high homology to the trypsin from same species and the proteases from shrimp. Myosin heavy chain (MHC) from shrimp tail meat was the most susceptible to the protease and actin/tropomyosin were degraded progressively during 4 hr incubation, but to a lesser degree than MHC.

Key words: Hepatopancreas, Kinetics, *Penaeus japonicus*, pH dependence, Shrimp protease, Temperature dependence, Amino acid sequence

## Introduction

Texture softness of shrimp tail meat mainly occurs after a relatively short period of iced storage of fresh meat, although deterioration during extended frozen storage may also occur. The deterioration is presumed to be due to degradation of shrimp muscle proteins associated with hepatopancreatic proteases, since it is most prominent in the anterior section of the tail. In previous papers, we purified a protease responsible to degradation of shrimp tail meat from the hepatopancreas of shrimp, *Penaeus japonicus* (Choi et al., 1998). The protease, without tryptic and chymotryptic activities, was not inhibited by a specific inhibitor of chymotrypsin, and was only partially inhibited by trypsin or pepsin specific inhibitors. However, the enzyme had specificity against the carboxy terminals of tryosine, tryptophan, and proline, which would classified it as a new protease.

Many proteolytic enzymes have been purified from the digestive organs of decapods, and characterized as to their enzymatic properties. In crustacea, one of the major enzymes has been identified as trypsin-like and its physicochemical and enzymatic properties have been characterized in shrimp (Kim et al., 1996b; Oh et al., 2000), crab (Dendinger and O'Connor, 1990), lobster (Galgani and Nagayama, 1987), and crayfish (Kim et al., 1992; 1994; 1996a). Other enzyme such low molecular weight protease (Pfleiderer et al., 1967) also was demonstrated to play an important role in digestion and might be involved in postmortem muscle degradation. Recently, a proteases without tryptic or chymotryptic activity was isolated from the hepatopancreas of *Penaeus orientalis*, and the protease was found to be responsible for degradation of shrimp tail meat (Oh et al., 1999; 2000). Such enzyme may act in a compensatory manner since there is an apparent absence of peptidic and chymotryptic activities in shrimp. Similar enzymes have been observed in the digestive organs of decapods such as lobster (Galgani and Nagayama, 1987) and shrimp (Galgani et al.,

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1984).

The objective of this research was to characterize enzymatic properties of protease from the hepatopancreas of shrimp, *P. japonicus*. and to clarify the effects of hepatopancreatic protease in the degradation of myofibrillar proteins that may lead to development of textural softness.

## Materials and Methods

### Materials

Protease was purified from the hepatopancreas of shrimp, *P. japonicus*, as described by Choi et al. (1998). Molecular Weight Standard Kit and N CBZ-tyrosine p-nitrophenyl ester (CBZ-Tyr-NE) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Hammersten casein was purchased from USB (Cleveland, OH, USA).

### Protein Measurement

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

### Assay for Enzyme Activity

Caseinolytic activity of protease 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. Activity of protease for CBZ-Tyr-NE was measured at 410 nm at 25℃. One unit of activity for the synthetic substrate was expressed as 1 μmole of nitrophenyl released per minute.

### pH and Temperature Dependence

Caseinolytic activity of the protease was determined using casein at pH range from 4.0 to 10.0 at 50℃. Activities were determined in different buffers with overlapping pH points to exclude the possibility of an influence exerted by the buffers. Buffers used were 0.1 M sodium citrate (pH 4.0~6.0), 0.1 M sodium phosphate (pH 6.0~7.6), Tris-HCl (pH 7.5~8.5), and Sodium carbonate buffer (pH 8.5~10.0). The optimum temperature of the protease against casein hydrolysis was measured at pH 8.1 over a temperature range 25~80℃ with the same concentration of enzyme solution used in pH dependence.

### Determination of pH Stability

Purified protease (20 μg/mL) was incubated at 25 ℃ for 30 min in the same volume of various buffers. After preincubation, aliquots were withdrawn from the incubated samples and assayed for proteolytic activity at pH 8.1 and 50℃ using 5% casein solution as a substrate.

### Determination of Thermal Stability

Thermal stability was measured through incubation of the protease in 50 mM Tris-HCl buffer, pH 7.0, at various temperatures for 30 min. The remaining activities after heat treatment were determined using 5% casein solution as a substrate at pH 8.1 and 50℃.

### Determination of Kinetic Parameters

Apparent Michaelis-Menten constant ($K_m$) and substrate turnover number ($V_{max}$) were determined using the Enzfitter Program by Leatherbarrow (1987). Kinetic data with CBZ-Tyr-NE as a substrate were obtained with substrate concentration of 0.05, 0.1, 0.25, 0.5, and 1 mM at pH 8.1 and at 25℃. Kinetic data with casein as a substrate for protease reaction were obtained with substrate concentrations of 0.1, 0.25, 0.5, 1, and 2% casein. The catalytic activity for protease was defined as the ratio $V_{max}/K_m$. Molar concentrations of the enzyme were estimated using molecular weight determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and protein concentrations.

### N-terminal Sequencing

The purified protease was separated on a 10% SDS-PAGE gel and electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad, Richmond, CA, USA) as described by Choli et al. (1989). N-terminal sequencing was determined by Edman degradation in an Applied Biosystems pulsed liquid-gas phase sequencer (model 477A, Foster City, CA, USA) equipped with a phenylthiohydantoin amino acid analyzer (model 120).

### Extraction and Hydrolysis of Myofibrillar Proteins

Myofibrillar proteins were prepared from shrimp

tail meat according to the procedure of Lindner et al. (1988). The isolated myofibrillar proteins were denatured by dialyzing against 8 M urea overnight and then dialyzed against 40 mM Tris-HCl, pH 7.0, containing 500 mM KCl for 3 days before use as a substrate for proteolysis.

Extracted myofibrillar proteins were subjected to proteolysis by the shrimp protease. The purified protease (5 μg) was added to a reaction mixture containing 1 mg of protein substrate in 500 μL of 20 mM Tris-HCl buffer, pH 7.0. The mixture was incubated at 45℃ for 0, 1, 2, 5, 10, 30, 60, 120, and 240 min. At each reaction interval, 30 μL of the reaction mixture was taken and added to 10 mL of SDS-PAGE sample buffer and immediately boiled for 1 min to stop proteolysis. Samples containing 20 μg of proteins were applied to a 7.5% SDS-PAGE gel. SDS-PAGE was performed according to the method of Laemmli (1970).

### Statistical Analysis

All experiments were replicated three times. Data in figures are means of three replications with standard deviation as error bars, calculated by the graphics program Sigmaplot 5.0 (Jandel Corp., San Rafael, CA, USA). Where error bars are not obvious the standard deviation was less than the area covered by data points.

## Results

### pH and Temperature Optima

Effect of pH on the activity of protease was examined between pH 4.0 and 10.0. Maximum caseinolytic activity of the protease occurred at pH 7.5; however, high proteolytic activity was retained in the broad pH range of 6.0 to 9.5 (Fig. 1). At physiological pH (6.8), relative activity of the protease was similar to the maximum activity. This suggested that protease activity was not sensitive to pH changes in both neutral and alkaline ranges. Higher activities occurred in the Tris-HCl buffer solution than phosphate and glycine-NaOH buffer solutions at the same pH.

The protease showed optimum temperature for hydrolysis of casein at 70℃ (Fig. 2). The maximum specific activity of the protease was 11.5 U/mg/min at optimum temperature. Specific activity of the enzyme at ambient temperature (25℃) was exhibited about 20% of maximal activity. The enzyme was remarkably thermostable, with 83% of maximum activity exerted at 75℃.

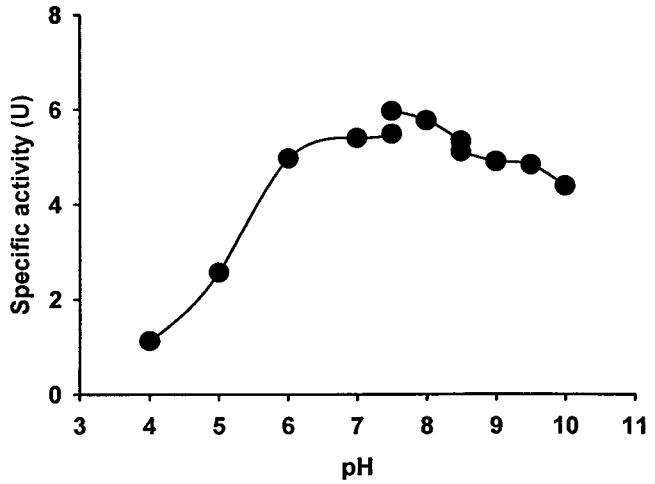


Fig. 1. Effect of pH on hydrolysis of casein by purified protease from shrimp hepatopancreas. Buffers used were 0.1 M sodium acetate (pH 4.0~6.0), 0.1 M sodium phosphate (pH 6.0~7.5), 0.1 M Tris-HCl (pH 7.5~8.5), and 0.1 M glycine-NaOH (pH 8.5~10.0).

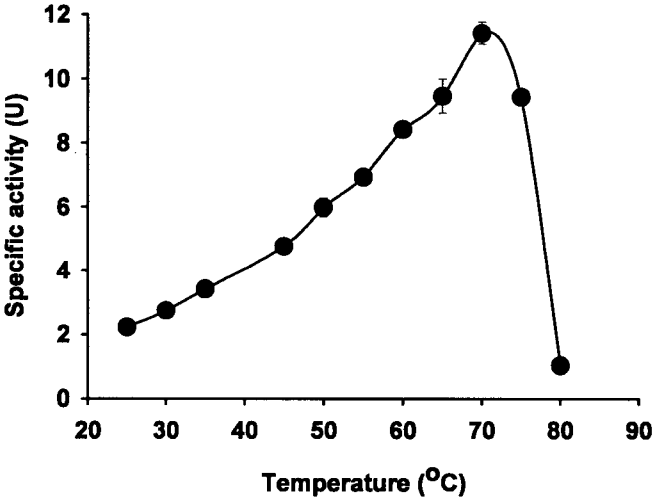


Fig. 2. Effect of temperature on hydrolysis of casein by purified protease from shrimp hepatopancreas. Reaction was performed at pH 8.1 and at indicated temperature.

### pH and Temperature Stability

The protease was stable over the range of pH 6.0~9.0, but unstable in the acidic region, with total loss of activity following incubation at pH 4.0 and 3.0 for 30 min (Fig. 3). At pH 4.5 and 10.0, activities of protease decreased to 25 and 60% of original activity, respectively.

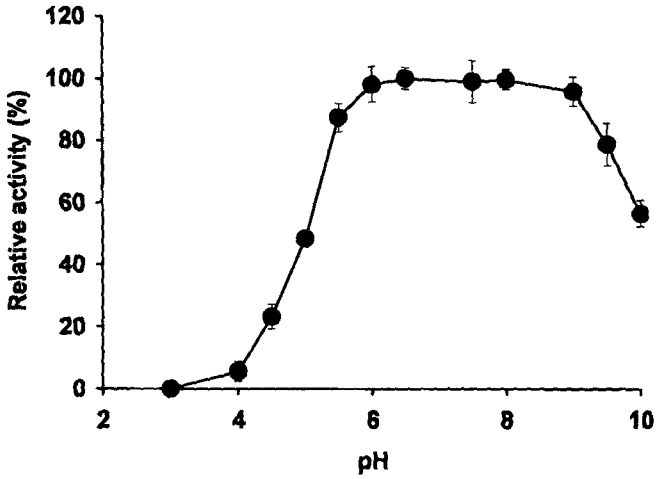


Fig. 3. pH stability of purified shrimp hepatopancreatic protease. The purified protease was incubated at indicated pH for 30 min at 25℃ and residual activities were determined with 5% casein at pH 8.1 and at 50℃.

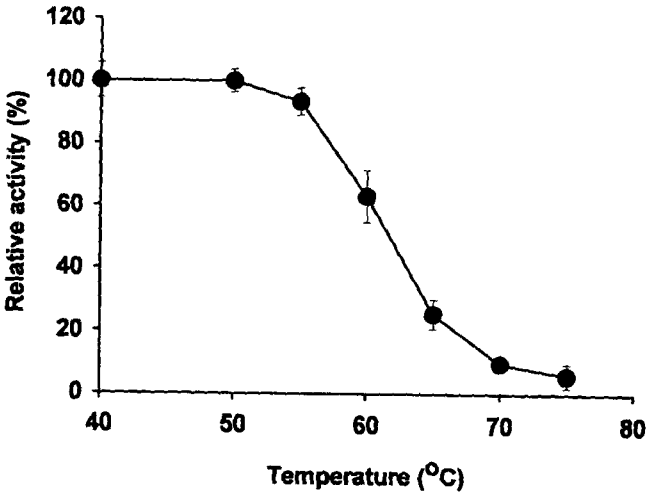


Fig. 4. Thermal stability of purified shrimp hepatopancreatic protease. The purified protease was incubated for 30 min at indicated temperatures and residual activities were determined with 5% casein at pH 8.1 and at 50℃.

The residual activity for casein was examined after heat treatment for 30 min (Fig. 4). Treatment up to 50℃ for 30 min did not affect protease activity. With heat treatment at 60℃ there was approximately a 40% reduction of protease activity. The protease was almost completely inactivated by heat treatment at 70℃.

### N-Terminal Sequence

The N-terminal amino acid sequence of the protease from *P. japonicus* is given in the top line of Table 1 and compared to those of related proteases from marine animals and bovine. The sequence of the protease from *P. japonicus* showed considerable similarity with trypsin from same species and *P. orientalis*. Seven out of 10 amino acid residues of protease were identical to those of trypsin from *P. monodon*. However, N-terminal sequence of shrimp showed low similarity with trypsin from bovine and dogfish. Shrimp proteases had two region of sequence conservation, $I^1$-V-G-$G^4$- and $T^8$-$P^9$; the former sequence was known as the proteolytic cleavage site in inactive trypsinogen. Therefore, the protease should be initially synthesized as an inactive proenzyme, then processed with proteolytic cleavage into the active enzyme.

Table 1. The N-terminal amino acid sequence of protease of shrimp, *P. japonicus*, compared to proteases from other species

| Species | Sequence | Reference |
|---|---|---|
| Protease of *P. japonicus* | I-V-G-G-S-D-A-T-P-G | |
| Protease of *P. orientalis* | I-V-G-G-V-A-E-T-P-H | 1 |
| Trypsin of *P. japonicus* | I-V-G-G-S-E-A-T-P-F | 2 |
| Trypsin of *P. orientalis* | I-V-G-G-T-D-V-T-P-G | 2 |
| Trypsin of *P. monodon* | I-V-G-G-T-A-V-T-P-G | 3 |
| Trypsin of bovine | I-V-G-G-Y-T-C-G-A-N | 4 |
| Trypsin of dogfish | I-V-G-G-Y-E-C-P-K-H | 5 |

1) Oh et al., 2000
2) Unpublished observation
3) Lu et al., 1990
4) Walsh, 1970
5) Titani et al., 1975

### Kinetic Properties

Hydrolysis of casein at pH 7.0 and at 45℃ and of CBZ-Try-NE at pH 8.1 and at 25℃, were examined at different substrate concentrations and the result are presented in Table 2. Apparent Michaelis-Menten ($K_m$) and turnover number ($V_{max}$) for casein hydrolysis were determined to be 0.29% and 7.8 $sec^{-1}$, respectively. Also the $K_m$ and $V_{max}$ of protease for CBZ-Tyr-NE were determined to be 0.38 mM and 2,400 $sec^{-1}$, respectively.

### Proteolysis of Myofibrillar Proteins

SDS-PAGE was used to monitor the degradation of shrimp myofibrils by protease from the hepatopancreas of shrimp, *P. japonicus*. The degradation of myofibrillar proteins from shrimp tail meat is

Table 2. Kinetic properties of shrimp hepatopancreatic protease for hydrolysis of casein and CBZ-Tyr-NE

| Substrate | $V_{max}(s^{-1})$ | $K_m(mM)$ | $V_{max}/K_m(mM^{-1}s^{-1})$ |
|---|---|---|---|
| CBZ-Tyr-NE[a] | 2,400 | 0.38 | 6,300 |
| Casein[b,c] | 7.8 | 0.29 | 26.9 |

Data are average values for triplicate determinations.
[a]$V_{max}$ is units per micromole protease, where one unit of activity is defined in the materials and methods section.
[b]Reaction condition was 0.25 mL of various concentra-tions of casein and 50 μL of protease (20 μg/mL) at 50℃ and pH 8.1
[c]$V_{max}$ is tyrosine equivalent units/mg protease.

presented in Fig. 5 at 0, 1, 2, 5, 10, 30, 60, 120, and 240 min incubation. As incubation progressed up to 60 min, myosin heavy chain (MHC) degradation was apparent, accompanied by an increase in protein fragments with M.W. of 75~116 kDa. This might be produced by endopeptidase activity of the protease. After 240 min incubation, MHC and hydrolytic fragments were completely hydrolyzed. Up to 240 min incubation, actin and tropomyosin were degraded progressively, but to a lesser degree than MHC.

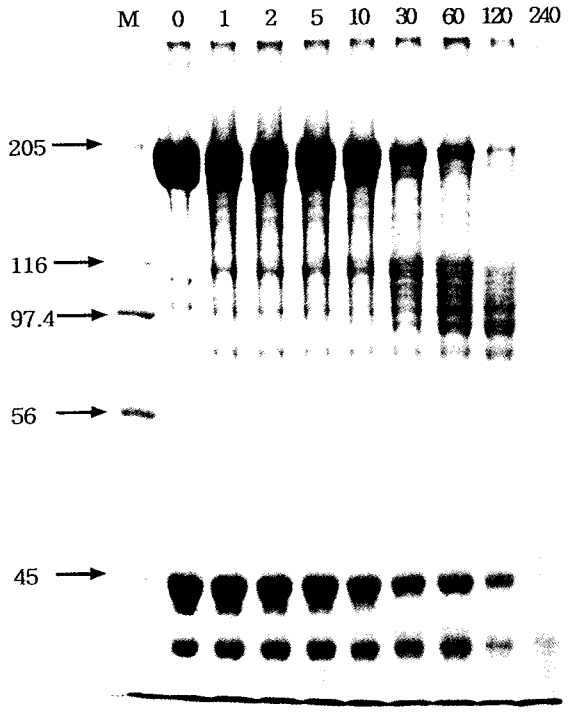


Fig. 5. Hydrolysis of myofibrils from shrimp tail meat by the purified protease at 25℃. Top numbers indicate incubation time (min). After enzymatic reaction, the hydrolysates were analyzed on 7.5% polyacrylamide gel.

## Discussion

A comparison of the proteolytic activity of shrimp protease with other proteases from crustacea or fish is difficult due to different experimental condition or different activity units. The optimum pH for proteolytic activity from the digestive organs of crustacea has been reported to be in the neutral and alkaline ranges (Kim et al, 1994; 1996a; Oh et al., 2000). However, trypsins from fish intestine generally have higher activities at alkaline pH with the highest caseinolytic activities at pH 9.4 to 10.0 as demonstrated by anchovy (Heu et al., 1995). The apparent pH-activity profiles of the protease from *P. japonicus* were similar to pH profiles of trypsin from *P. orientalis* (Oh et al., 2000), *P. indicus* (Honjo et al., 1990), *P. clarkii* (Kim et al., 1996a) and the proteinase from Japanese spiny lobster (Galgani and Nagayama, 1987), which is different from that of trypsins from fish intestine.

The maximum specific activity of the protease was estimated to be 11.5 U/mg/min, which is similar to trypsin A from *P. clarkii* (Kim et al., 1996a). Generally, proteolytic activity from the digestive extract of crustacea have optimum temperature at 45 to 55 ℃, for two species of shrimp (Galgani et al., 1984) and five species of crab (Galgani and Nagayama, 1987). The digestive protease from lobster has been found to have higher optimum temperature (60℃) than that from shrimp (Galgani et al., 1984). Furthermore, the protease purified from the hepatopancreas of *P. japonicus* had higher optimum temperature for hydrolysis of casein, which is similar to trypsin from Atlantic blue crab (Dendinger and O'Connor, 1990) and to protease from *P. orientalis* (Oh et al., 2000). High optimum temperature of the protease creates the potential for autolysis of muscle protein during shrimp processing. If cooking temperature is not high enough to inactivate the protease, it could lead to muscle degradation during subsequent storage or distribution.

Proteolytic activity from marine animal is very stable for a long period at neutral pH (Kim et al., 1992). However, it is irreversibly denatured at pH 3.0, which is opposite to that of bovine trypsin. The inactivation of protease from marine animal in an acidic pH may be a conformationally irreversible change, but the inactivation of bovine trypsin might

be caused by autolysis. pH stability of the protease from *P. japonicus* was similar to trypsins from the hepatopancreas of *P. clarkii* (Kim et al., 1992), protease from *P. orientalis* (Oh et al., 2000), and trypsin from the midgut gland of the Atlantic blue crab (Dendinger and O'Connor, 1990). The sharp decrease in hydrolysis of casein by the protease at low pH may be attributed to the irreversible denaturation of the protease as shown in crayfish (Kim et al., 1992). Bovine pancreatic trypsin, on the other hand, is very stable in acidic pH. Apparently, inactivation at acid pH is a phenomenon common to proteases from various species of decapods.

Thermal stability of the protease from *P. japonicus* in this study was not distinctively different from that of *P. clarkii* (Kim et al., 1992) and *P. orientalis* (Oh et al., 2000). Trypsin from Atlantic blue crab was stable up to 50℃ for 30 min of heat treatment but activity was rapidly lost above 50℃ (Dendinger and O'Connor, 1990). This finding is in agreement with the present result. Although, the protease had maximum activity at 70℃ (Fig. 2), incubation at 70℃ without casein substrate induced total loss of activity. This result suggest that the surrounding environment influences thermal stability of the enzyme. Partially purified enzymes were more stable against thermal inactivation than purified enzymes (Kim et al., 1992). Hence, it can be assumed that the presence of other proteins enhanced thermal stability of the protease.

$K_m$ values for casein with a protease from *P. orientalis* were estimated to be 0.31% and 5.21 $s^{-1}$, respectively, which are in agreement with the present result (Oh et al., 2000). However, lower $K_m$ values were observed for casein with bonefish crude protease, which was 0.042% at 25℃ and pH 9.0 (Jany, 1976) and with two proteinase from rotifer, which were 0.044 and 0.052% (Hara et al., 1984). This indicated that shrimp protease had lower affinity for casein than did bonefish proteases.

Autolysis of fish tissue is most likely due to degradation of muscle protein caused by either proteolytic enzymes released from the intestinal tract or proteinases in muscle tissue. Tryptic activity accounted for about 70% of the total proteolytic activity responsible for degradation in capelin muscle tissue (Aksnes and Brekken, 1988). A trypsin-like protease from Peruvian hake degraded major contractile and cytoskeletal constituent proteins of myofibrils at neutral pH (Martone et al., 1991). Also, textural degradation of filefish meat gel accompanied by increased peptides and breakdown of MHC was caused by proteinase associated with myofibrils (Toyohara et al., 1990). More recently, a protease responsible for tissue softening of Pacific whiting was identified to be cathepsin L-like protease and the degradation pattern of myofibrils by the cathepsin L-like protease was the same as the autolytic pattern of surimi (An et al., 1994). Like other protease in muscle tissue, the purified protease in this study may also be responsible for the degradation of shrimp tail meat, with HMC as the most susceptible to degradation by the protease.

## Conclusion

The pH optima and pH stability of the protease purified from the hepatopancreas of shrimp (*P. japonicus*) were similar to those of proteases from crustacea, however the enzyme showed optimum temperature for hydrolysis of casein at 70℃. High optimum temperature of the protease causes the potential for autolysis of muscle proteins during shrimp processing. If cooking temperature is not high enough to inactivate the protease, it could lead to muscle degradation during subsequent storage or distribution. With development of the purification scheme presented previously (Choi et al., 1998), it will be possible to evaluate further the effects of different commercial processing conditions on enzyme activity. This result may aid in establishing optimal processing conditions to maximize storagability of processed shrimp products.

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