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Purification and Characterization of Collagenolytic Proteases from the Hepatopancreas of Northern Shrimp (*Pandalus eous*)

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Three gelatinolytic proteases (A1, A2, and B) were purified using a synthetic substrate, DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg, from the hepatopancreas of Northern shrimp (*Pandalus eous*) by several chromatographic steps involving hydroxyapatite column chromatography, gel filtration on Superdex75, and ion-exchange chromatography on a MonoQ column. Collagenolytic proteases A2 and B, but not protease A1, were demonstrated to digest native porcine type I collagen at 25 °C and pH 7.5. Further characterizations of these two collagenolytic proteases showed that the pH optimum of enzyme A2 against DNP-peptide was found to be 11, whereas that of enzyme B was 8.5. The optimum temperature ranged between 40 and 45 °C for both enzymes, although enzyme B appeared to be thermally more stable than enzyme A2 at pH 7.5. Both enzymes were strongly inhibited by PMSF and antipain, which suggests that they belong to collagenolytic serine proteases.

KEYWORDS: Collagenolytic protease; Northern shrimp; *Pandalus eous*; hepatopancreas

INTRODUCTION

Collagenases are defined as proteases capable of degrading the native triple helix of collagen under physiological conditions. Collagenases are classified into two major groups, metallocollagenases and serine collagenases. While the name "collagenase" was first used to describe an enzyme from *Clostridium histolyticum* that degraded collagen (*1*, *2*), other microorganisms are also known to produce collagenases (*3*−*8*). A vertebrate collagenase was first discovered by Gross and Lapiere (*9*) from tadpole tail tissue. This enzyme specifically cleaves triple-helical collagen across α chains at a single point three-quarters of the way from the amino terminus, generating a set of 3/4 and 1/4 fragments (*10*). Many such enzymes have since been isolated from vertebrate tissues and are grouped now under a class of matrix metalloproteinases (MMPs). These vertebrate and bacterial enzymes are metallocollagenases, which contain Zn^{2+} in the active center, require Ca^{2+} for full activity, and are inhibited by chelating agents such as EDTA and 1,10-phenanthroline (*11*).

Serine collagenase, on the other hand, was first isolated from the hepatopancreas of fiddler crabs (*Uca pugilator*) and is capable of cleaving native triple-helical collagen (*12*, *13*). The collagen cleavage sites of this enzyme are located in the protease-sensitive region three-fourths of the length of the collagen helix from the amino terminus (*14*). However, in contrast to the specific Gly-Ile recognition site of metallocollagenases, serine collagenases cleave the triple helix of collagen at multiple loci. The enzymes of the latter type were also purified from a variety of organisms, including Antarctic krill (*Euphausia superba*) (*15*), tropical shrimp (*Penaeus vannamei*) (*16*), grass shrimp (*Penaeus monodon*) (*17*), king crab (*Paralithodes camtschatica*) (*18*), snow crab (*Chionoecetes opilio*) (*19*), greenshore crab (*Carcinus maenas*) (*20*), catfish (*Parasilurus asotus*) (*21*), the fungus *Entomophtora coronata* (*22*), and cattle grub (*Hypoderma lineatum*) (*23*). Unlike metallocollagenases, serine collagenases are thought to be primarily involved in the digestion of foodstuffs rather than in remodeling the extracellular matrix (*12*). Interestingly, these enzymes have been shown to display a catalytic mechanism similar to those of mammalian trypsin, chymotrypsin, and elastase (*24*). These unique properties of serine collagenases could be exploited in the food industry for extensive hydrolysis of various primary food materials.

The hepatopancreas of penaeid shrimp are known to have high proteolytic activities (*16*, *17*, *25*−*29*). Northern shrimp (*Pandalus eous*) is one of the most important crustacean species used in the shrimp-processing industry. With the development and expansion of production, processing, and marketing in response to high consumer demand, wastes of the processing industry such as viscera and wastewater form a cheap and abundant source of various enzymes. However, data concerning the use of Northern shrimp waste as a raw material for the production of enzymes are not available.

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In this paper, we report the purification and preliminary characterization of collagenolytic proteases in the hepatopancreas of Northern shrimp. The results of our study will provide a basis for utilizing these abundant sources for industrial enzymes as byproducts of efficient waste management.

MATERIALS AND METHODS

Chemicals. 2,4-Dinitrophenyl-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg (DNP-peptide), Bz-DL-Arg-*p*NA (BAPA), Bz-Tyr-*p*NA, Suc-$(Ala)_3$-*p*NA (STANA), leupeptin, and antipain were purchased from Peptide Institute, Inc. Porcine skin acid-soluble type I collagen, soybean trypsin inhibitor (SBTI), phenylmethylsulfonyl fluoride (PMSF), *N*-ethylmaleimide (NEM), benzamidine, and 1,10-phenanthroline were purchased from Wako Pure Chemical Industries, Ltd. Other reagents were of analytical grades.

Samples. The live Northern shrimp (*P. eous*) used in this study (average body weight of 16.5 g) were procured from a fishery cooperative society in Ishikawa Prefecture, Japan. The hepatopancreas (96 g) was dissected from 150 animals, immediately frozen in liquid nitrogen, and stored at −80 °C until use.

Preparation of Crude Extracts. This and all subsequent purification procedures were performed at 4 °C unless otherwise noted. The frozen hepatopancreas were partially thawed and homogenized with 2 volumes of 50 mM Tris-HCl (pH 7.4) containing 5 mM $CaCl_2$ (buffer A) using a Polytron homogenizer for 5 min. The dispersion was added slowly to 10 volumes of cold acetone (−20 °C) with stirring. The suspension was held at −20 °C for 20 min, and the supernatant was decanted. The precipitate was collected by centrifuging at 19000*g* for 20 min and washed with about 3 volumes of *n*-butanol. After centrifugation, the pellet was dried in a vacuum desiccator for 48 h. The acetone powder (10 g) thus formed was stored at −20 °C until use.

A portion (5 g) of acetone powder was suspended in 100 mL of buffer A by stirring at 4 °C for 1 h. The insoluble material was separated from the extract by centrifuging at 19000*g* for 20 min, and the supernatant was saved. The precipitate was re-extracted with an equal volume of buffer A for 1 h. After centrifugation, the two supernatants were combined and used as the crude extracts.

Purification of Collagenolytic Proteases from the Crude Extracts. Solid ammonium sulfate was slowly added to the crude extracts with stirring to 30% saturation, and stirring was continued gently for 1 h after the final addition of ammonium sulfate. After centrifugation, the supernatant was brought to 70% saturation by further addition of solid ammonium sulfate. The suspension was settled for 1 h and the precipitate collected by centrifugation.

The ammonium sulfate precipitate formed was dissolved in 10 mM potassium phosphate buffer (pH 6.8) and dialyzed against the same buffer. After filtration through a 0.45-μm filter, the dialysate was applied to a hydroxyapatite column (Bio-Rad, 1.6 × 40 cm) equilibrated with the same buffer. The column was washed with the same buffer at a flow rate of 0.5 mL/min, and 15-mL fractions were collected. The proteins remaining on the column were eluted with 400 mM potassium phosphate buffer (pH 6.8). Fractions were monitored for proteolytic activities using DNP-peptide, STANA, BAPA, and Bz-Tyr-*p*NA specific for collagenase, elastase, trypsin, and chymotrypsin-like enzymes, respectively, and gelatin zymography. Flow-through and adsorbed fractions were dialyzed against 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl. Each dialysate was applied to a Superdex75 (Amersham Pharmacia Biotech) gel filtration column (1.6 × 100 cm) equilibrated with 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl at a flow rate of 0.3 mL/min. The active fractions for DNP-peptide were pooled and dialyzed against 20 mM Tris-HCl (pH 7.5) and then loaded onto a MonoQ (Amersham Pharmacia Biotech) column (0.5 × 5 cm) equilibrated with 20 mM Tris-HCl (pH 7.5) at a flow rate of 0.5 mL/min.

Protein Determination. Protein content was monitored at 280 nm during chromatographic separation. At the different steps of the purification procedure, the amount of protein was also determined by the method of Bradford (*30*) using bovine serum albumin as the standard.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was performed according to the method of Laemmli (*31*), using 4−20% gradient polyacrylamide slab gels (TEFCO, Japan). After the run, the gels were stained with 0.05% Coomassie Brilliant Blue in 50% methanol containing 10% acetic acid and destained with 25% methanol containing 7% acetic acid.

Gelatin Zymography. Visualization of gelatinolytic activities was performed according to the method of Heussen and Dowdle (*32*) with slight modifications. Electrophoresis was performed at 4 °C using 15% polyacrylamide slab gels containing 0.1% gelatin (TEFCO, Japan). After electrophoresis, SDS was removed from the gels by washing twice for 30 min in 2.5% Triton X-100. The gels were incubated at room temperature for 1 h in the incubation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 0.02% sodium azide), stained with 0.05% Comassie Brilliant Blue, and destained with 10% acetic acid until pale proteinase bands were clearly visible.

Assay of Hydrolytic Activity. Synthetic substrate DNP-peptide was used for the study of collagenolytic protease activities. Hydrolysis of DNP-labeled peptide was measured spectrophotometrically as described by Masui et al. (*33*). One hundred microliters of 1 mM peptide in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 10 mM $CaCl_2$ was incubated with an equal volume of enzyme solutions at 25 °C for 10 min. The reaction was terminated by the addition of 0.5 mL of 1 M HCl. The DNP-peptide fragments were extracted with 1 mL of ethyl acetate containing *n*-butanol (1:0.15, v/v) by vigorous shaking, followed by centrifugation, after which hydrolysis was measured at 365 nm. One unit of enzyme activity was defined as the amount of protein required for the hydrolysis of 1 μmol of substrate per minute, taking the molecular extinction coefficient of the reaction product DNP-Pro-Gln-Gly-OH to be 17.3×10^3 M^{-1} cm^{-1}.

Reactions against STANA and BAPA were carried out at 25 °C for 5 min in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 10 mM $CaCl_2$, and 1% dimethyl sulfoxide with enzyme solutions. Because Bz-Tyr-*p*NA was insoluble, the reaction was carried out in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 10 mM $CaCl_2$, and 10% methanol (*24*). Final concentrations of all substrates were 0.5 mM. Hydrolyses of these substrates liberate the chromogenic *p*-nitroaniline moieties, which can be monitored spectrophotometrically at 405 nm using an extinction coefficient of 9920 M^{-1} cm^{-1} (*34*). The initial rate of release of *p*-nitroaniline was measured for each of these substrates. One unit of enzyme activity (U) was defined as 1 μmol of *p*-nitroaniline released per minute under the assay conditions.

Hydrolysis of native collagen was observed by SDS−PAGE. Porcine skin acid-soluble type I collagen (1.5 mg/mL) in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 10 mM $CaCl_2$, and 1 M glucose was incubated with an equal volume of enzyme solutions at 25 °C for 0.5, 1, and 2 h. The reaction mixture was analyzed for degradation by SDS−PAGE.

Effects of pH, Temperature, and Thermal Stability. Activities were measured with DNP-peptide. For pH studies, the experimental conditions were 10 min of incubation at 25 °C in the Britton−Robinson buffer system (*35*) over the pH range of 4 to 13.5.

To determine the effect of temperature on the activity, the reaction buffer of 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl and 10 mM $CaCl_2$ was preincubated for 5 min in the range from 0 to 80 °C. After the enzyme solution was added, the reaction solution was incubated for 10 min at respective temperatures.

To determine thermal inactivation of the enzymes, they were incubated for 30 and 60 min at 20−70 °C in 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl and 10 mM $CaCl_2$ and immediately cooled on ice before the remaining activities were measured at 25 °C for 10 min.

Effects of Various Inhibitors. Each enzyme solution was preincubated with different compounds (PMSF, leupeptin, antipain, SBTI, benzamidine, EDTA, 1,10-phenanthroline, NEM, 2-mercaptoethanol) in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl at 25 °C for 30 min, followed by the addition of an equal volume of DNP-peptide solution. The residual activity was determined at 25 °C for 10 min.

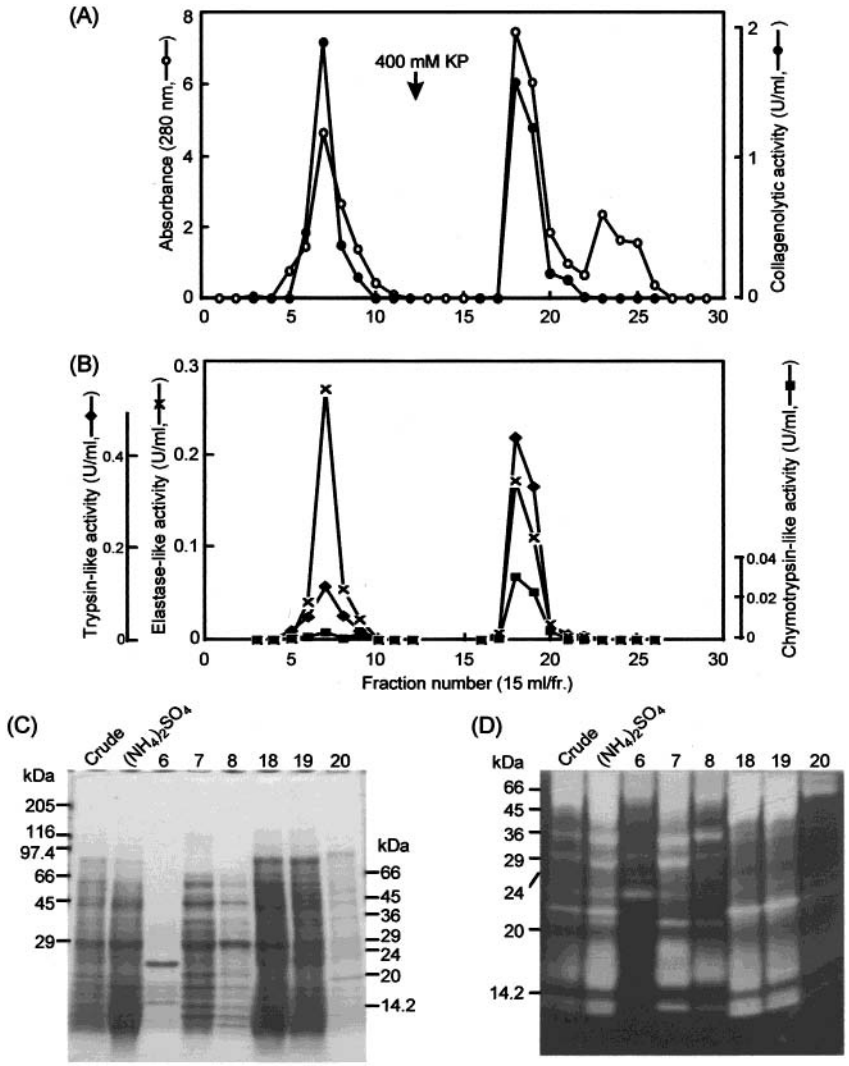

Figure 1. Elution profile on hydroxyapatite column chromatography of an ammonium sulfate fraction of Northern shrimp (*P. eous*). Experimental conditions are described in Materials and Methods. (A) ○, absorption at 280 nm. ●, collagenolytic activity with DNP-peptide as a substrate. (B) Substrates employed were Bz-Arg-*p*NA for trypsin-like activity (◆), Suc-$(Ala)_3$-*p*NA for elastase-like activity (×), and Bz-Tyr-*p*NA for chymotrypsin-like activity (■). The active fractions subjected to SDS–PAGE analysis (C) and their corresponding gelatin zymography pattern (D) are shown.

RESULTS

Purification. Approximately 10 g of acetone powder was obtained from the hepatopancreas (96 g) of 150 shrimp (average body weight of 16.5 g). A portion (5 g) of acetone powder was used to prepare crude extracts. Eisen et al. (*12*) reported that no increase in the activity was observed when crude extracts treated with trypsin were examined for collagenase-, trypsin-, or chymotrypsin-like activities. Therefore, the crude extracts were used as a starting material for the enzyme purification without any activation, which showed a considerable activity against porcine skin acid-soluble type I collagen.

Collagenolytic activities were observed in both flow-through and adsorbed fractions loaded on a hydroxyapatite column (**Figure 1A**). The two fractions also contained trypsin- and elastase-like activities, with a slight chymotrypsin-like activity only in the adsorbed fractions (**Figure 1B**). However, it should be mentioned that the organic solvent (10% methanol) used to dissolve the chymotrypsin-specific substrate Bz-Tyr-*p*Na might have some inhibitory effects, resulting in lower chymotrypsin-like activity. The SDS−PAGE pattern of the fractions corresponding to these two peaks revealed some major protein bands (**Figure 1C**), the proteolytic activities of which were further observed by activity staining on gelatin zymography gel (**Figure 1D**). Therefore, each of these two peak fractions was pooled and passed through a Superdex75 gel filtration column. As shown in **Figure 2**, single major peaks of collagenolytic activity are obtained from both pools of fractions and named peaks I and II. The active fractions were pooled and further applied to a MonoQ ion-exchange column. Fractions corresponding to peak I were separated into two major peaks, whereas a single peak of collagenolytic activity was obtained from those belonging to peak II. These three active fractions were designated as A1, A2 (**Figure 3A**), and B (**Figure 3B**), which were further rechromatographed on the same column before subsequent characterization.

The typical purification process of the collagenolytic proteases from Northern shrimp hepatopancreas is summarized in **Table 1**. The SDS−PAGE patterns of the purified fractions are shown in **Figure 4A**. In the presence of the reducing agent 2-mercaptoethanol, all three proteases migrated as single prominent bands with additional low-molecular weight proteins. The molecular weights were estimated to be 29 000, 22 000 and 23 000 for proteases A1, A2, and B, respectively. The activities of the crude and pure fractions were visualized by gelatin zymography (**Figure 4B**). All three fractions observed in MonoQ ion-exchange chromatography were able to hydrolyze both DNP-

Table 1. Purification of Collagenolytic Proteases from Northern Shrimp Hepatopancreas

step	total protein[a] (mg)	total activity[b] (U)	specific activity (U/mg)	purification (fold)	yield (%)
acetone powder (5 g)					
crude extract	1687	121.3	0.072	1	100
ammonium sulfate precipitates	914	101.4	0.111	1.5	83.6
hydroxyapatite column					
flow-through	137	45.3	0.331	4.6	37.3
adsorbed	311	36.8	0.118	1.6	30.3
Superdex 75 column					
peak I	15.5	17.5	1.13	15.7	14.4
peak II	20.3	7.2	0.355	4.93	5.9
MonoQ column					
protease A1	1.1	2.5	2.27	31.5	2.1
protease A2	2.7	5.7	2.11	29.3	4.7
protease B	1.3	3.5	2.69	37.4	2.9

[a] Protein concentrations were determined by the Bradford assay using BSA as the standard. [b] One unit of enzyme activity (U) was defined as 1 μmol of DNP-Pro-Gln-Gly-OH released per minute.

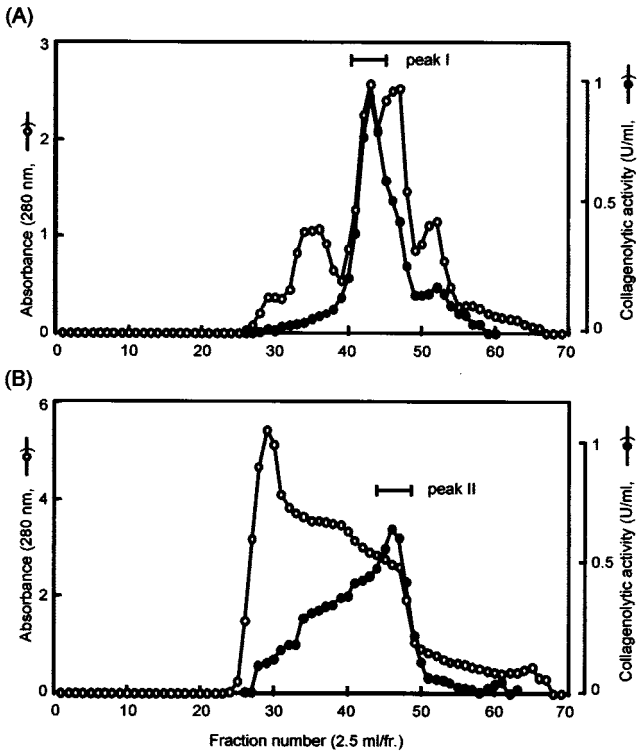

Figure 2. Elution profile on Superdex 75 gel filtration of hydroxyapatite column flow-through (A) and adsorbed fraction (B) Northern shrimp. Experimental conditions are described in Materials and Methods. ○, absorption at 280 nm. ●, collagenolytic activity with DNP-peptide as a substrate.

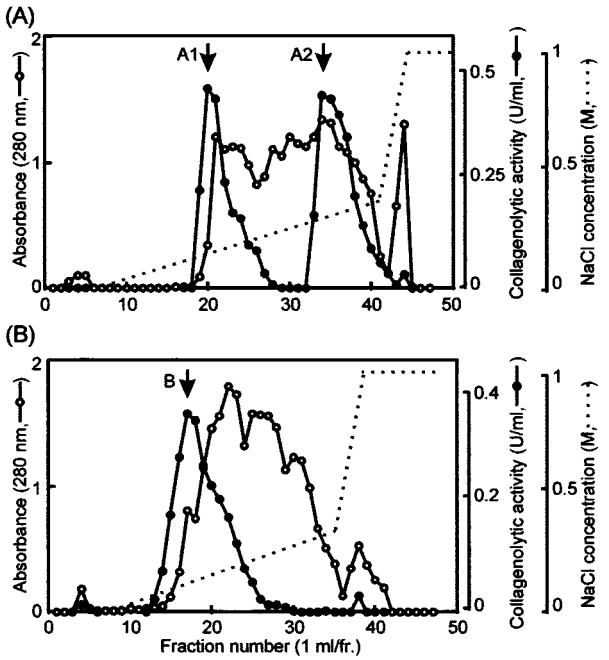

Figure 3. MonoQ ion-exchange column chromatography of the active fractions from Superdex 75 gel filtration of Northern shrimp. Experimental conditions are described in Materials and Methods. Panels A and B show elution profiles for the active fractions of peaks I and II obtained from Superdex 75 gel filtration (see **Figure 2**). ○, absorption at 280 nm. ●, collagenolytic activity with DNP-peptide as a substrate.

peptide and gelatin. However, proteases A2 and B were demonstrated to more clearly cleave porcine skin acid-soluble type I collagen than protease A1 (**Figure 5**). Therefore, collagenolytic properties were further investigated only for proteases A2 and B.

Effects of pH on the Activity. The pH dependence of the activity of proteases A2 and B is shown in **Figure 6**. The activities against DNP-peptide were measured with a pH range of 4−13.5. The optimum pHs of proteases A2 and B were found to be pH 10−11 and pH 8−8.5, respectively.

Effects of Temperature on the Activity and Thermal Stability. The effects of temperature on the collagenolytic activities of both enzyme preparations are shown in **Figure 7**. The activity of both proteases increased with the increase in temperature, reaching a maximum at 40 °C for protease A2 and 45 °C for protease B. A further increase in temperature resulted in lowering of enzyme activity, apparently due to irreversible thermal inactivation.

The thermal stability of proteases A2 and B was also studied (**Figure 8**). After incubation for 30 min at 40 °C, protease A2 retained only 45% of the initial activity under the experimental conditions, whereas protease B retained 88% of the activity. However, when incubated at 60 °C, protease A2 lost almost all its activity, whereas protease B still retained about 60% of its initial activity. Incubations for longer periods (60 min) also showed similar stability profiles for both proteases.

Effects of Various Inhibitors on the Activity. Both proteases A2 and B were strongly inhibited by serine protease inhibitors, PMSF, leupeptin, and antipain (**Table 2**). They were slightly inhibited by benzamidine and 1,10-phenanthroline. SBTI in-

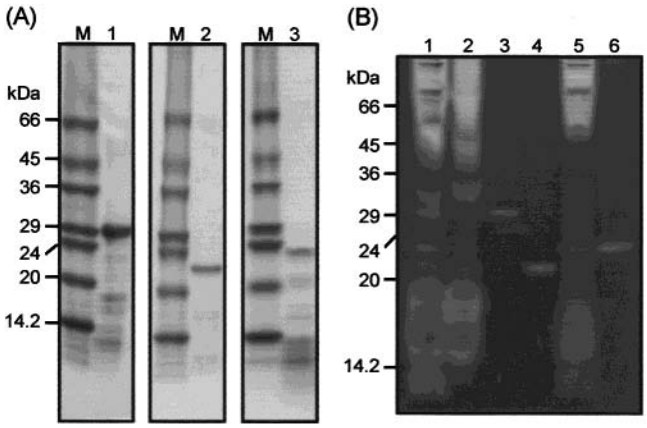

Figure 4. SDS–PAGE (A, 4–20% gradient gel) and gelatin zymography patterns (B, 15% gel) for collagenolytic proteases purified from Northern shrimp. (A) Lane M, low-molecular-weight marker proteins (Sigma); lanes 1–3, proteases A1, A2, and B from the MonoQ ion-exchange column, respectively. (B) Lane 1, ammonium sulfate fraction; lane 2, hydroxyapatite column flow-through; lane 3, protease A1 from the MonoQ ion-exchange column; lane 4, protease A2 from the MonoQ ion-exchange column; lane 5, hydroxyapatite column adsorbed fraction; lane 6, protease B from the MonoQ ion-exchange column.

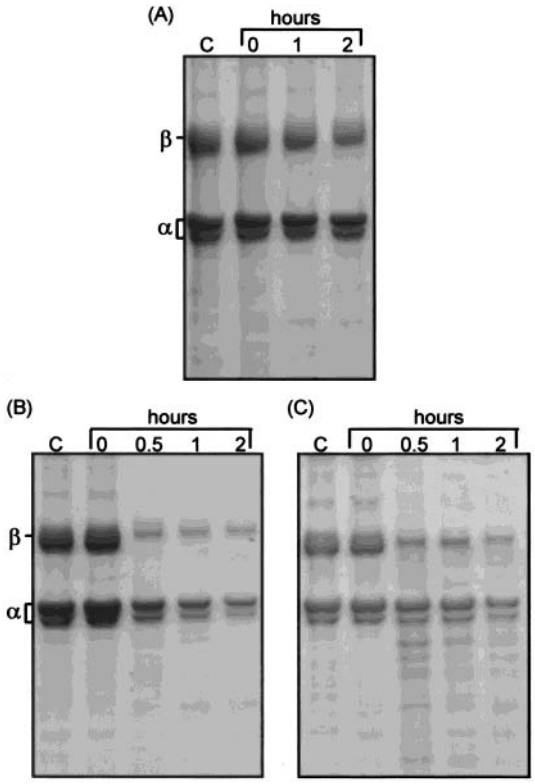

Figure 5. Degradation profiles of porcine skin acid-soluble type I collagen by Northern shrimp proteases A1 (A), A2 (B), and B (C). Lane C, standard porcine skin acid-soluble type I collagen.

hibited the activity of protease B but was totally ineffective against protease A2. Neither of these proteases was inhibited by NEM and EDTA. A 1% solution of 2-mercaptoethanol did not affect the enzyme activities; however, inhibition was observed with 10% 2-mercaptoethanol (**Table 2**).

DISCUSSION

The crude extracts obtained from the hepatopancreas of Northern shrimp showed considerable activity against BAPA, STANA, DNP-peptide, and porcine skin acid-soluble type I collagen. Several kinds of gelatinolytic proteases were found in the crude extracts (**Figure 1D**), and two distinct active fractions were separated by hydroxyapatite column chromatography (**Figure 1**). Three proteases with activity against DNP-peptide were purified from these fractions by Superdex75 and MonoQ column chromatographies. This synthetic peptide, which was originally designed as a substrate for collagenolytic proteases (*33*), is a good substrate for vertebrate metallocolla-

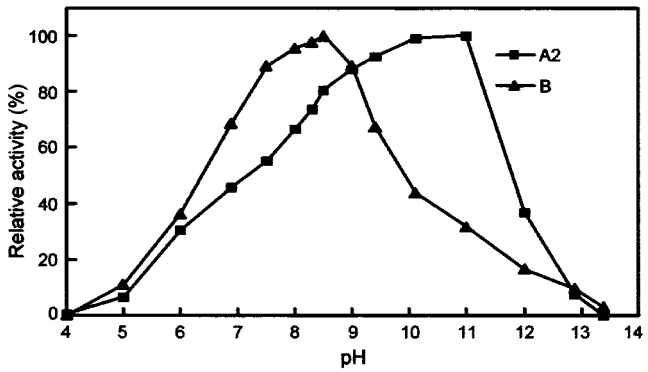

Figure 6. Effects of pH on the activity of Northern shrimp collagenolytic proteases A2 and B. Activities were measured with DNP-peptide dissolved in Britton–Robinson buffers over the pH range of 4–13.5.

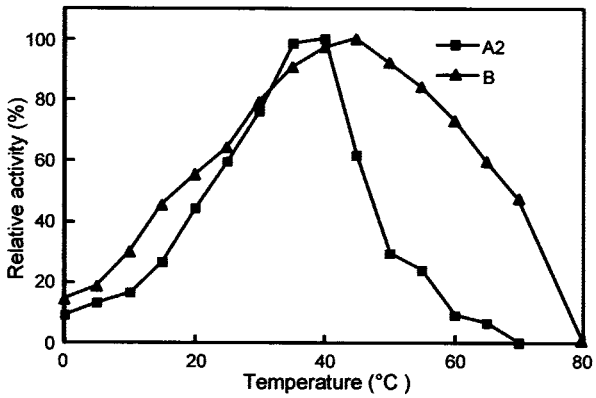

Figure 7. Effects of temperature on the activity of Northern shrimp collagenolytic proteases A2 and B. Activities were measured in 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl and 10 mM $CaCl_2$ with DNP-peptide as a substrate.

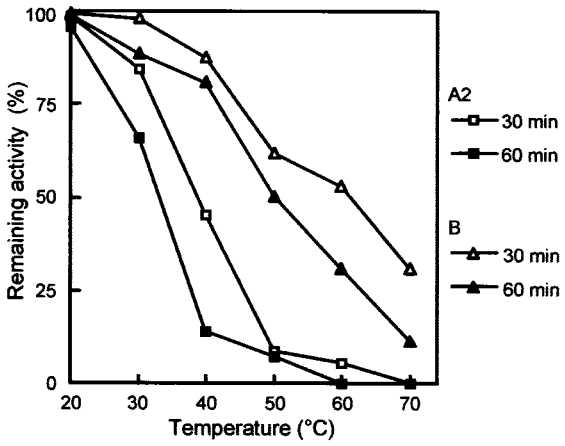

Figure 8. Thermal stability of Northern shrimp collagenolytic proteases A2 and B. Enzyme solutions were incubated for 30 and 60 min at different temperatures, and remaining activities were measured with DNP-peptide as a substrate.

Table 2. Residual Activity (%) of Purified Northern Shrimp Collagenolytic Proteases in the Presence of Various Chemical Compounds

compound	concn	residual activity (%)[a] protease A2	protease B
control		100	100
PMSF	1 mM	7	5
	10 mM	3	2
leupeptin	0.1 mM	79	64
	1 mM	10	4
antipain	0.1 mM	15	2
	1 mM	5	0
SBTI	0.1 mg/mL	100	32
	1 mg/mL	100	12
benzamidine	1 mM	74	100
	10 mM	63	81
EDTA	1 mM	100	100
	10 mM	100	100
1,10-phenanthroline	1 mM	91	90
	10 mM	85	62
NEM	1 mM	100	100
	10 mM	100	94
2-mercaptoethanol	1%	100	100
	10%	17	22

[a] Each enzyme solution was preincubated with the inhibitors or chemical compounds at 25 °C for 30 min, and the residual activity was determined at 25 °C with DNP-peptide as a substrate in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl.

genases (*33*) and fiddler crab serine collagenase (*24*). Vertebrate metallocollagenases specifically cleave this peptide at the Gly-Ile bond (*33*, *36*). On the other hand, fiddler crab serine collagenase cleaves this peptide at the Gln-Gly and Ile-Ala bonds rather than at the Gly-Ile bond (*24*). As shown in **Figure 4A**, three proteases migrated on SDS−PAGE gels, in the apparent molecular weight range between 22 000 and 30 000, in good agreement with the molecular size ranges of most collagenolytic serine proteases. Since rechromatography of all three fractions could not eliminate low-molecular-weight proteins, it is plausible that these fragments might be produced by inter- and/or intramolecular autolytic cleavage. However, artifacts resulting from the column resolution cannot be ruled out. Two of the three Northern shrimp proteases (A2 and B) shown to be active toward DNP-peptide were collagenolytic, as evidenced by their ability to cleave both α and β chains of native collagen. The other one (A1), being able to cleave gelatin, as observed by activity staining on gelatin zymography gel, showed activity toward the β chain of native collagen (see **Figure 5**). However, since we were particularly interested in isolating collagenolytic proteases from Northern shrimp-processing wastes, protease A1 was not included for subsequent enzyme characterization.

The pH optima of collagenolytic proteases were evaluated with DNP-peptide as a substrate at 25 °C. The maximum activity of protease B was observed at pH 8.5, whereas the activity of protease A2 increased with increasing pH up to 11 (**Figure 6**). The optimum pH for protease B was slightly alkaline relative to those for other crustacean collagenolytic proteases (*12*, *18*, *20*), whereas the highly optimum pH for protease A2 was comparable to those obtained with trypsin-like serine proteases from marine fish species (*37*).

The optimum temperatures for collagenolytic proteases A2 and B were 40 and 45 °C, respectively; irreversible inactivation occurred at higher temperatures. These considerably lower optimum temperatures were in accordance with that obtained for protease activity in Antarctic krill (*Euphausia superba*) (*15*) but differed from that for grass shrimp (*Penaeus monodon*), which was 55−60 °C (*27*). The structural basis for this temperature adaptation of proteolytic enzymes isolated from cold-adapted fish species has been a subject of recent study (*38*, *39*). However, it seems that these enzymes have less rigid tertiary structures, which would allow them to maintain the conformational flexibility required for efficient catalysis at low temperatures. The shrimp species used in the present study is a variety (*eous*) of *Pandalus borealis* (*40*), which has been considered as a synonym of *P. eous* (*41*). The latter species has been found at as low as −1.6 °C, and the most common temperature range is between 0 and 5 °C (*42*). The shrimp that live at such extreme temperatures might have adapted their enzyme systems to work efficiently at low temperatures. This observation should be of great importance in view of the potential use of these enzymes in food industries, where low reaction temperatures are beneficial in terms of both food quality and energy minimization (*43*).

The activity of various classes of inhibitors against Northern shrimp proteases is shown in **Table 2**. Proteases A2 and B appeared to be serine proteases since they were inhibited by PMSF, a well-known serine protease inhibitor. A slight inhibition was observed with 1,10-phenanthroline, as was also observed for green shore crab collagenase (*20*), which could be due to the hydrophobicity of 1,10-phenanthroline (*44*). SBTI inhibited protease B to a certain extent, as in the case of collagenolytic serine proteases from fiddler crab, greenshore crab, and Antarctic krill (*12*, *15*, *20*).

In conclusion, the Northern shrimp proteases described in this study exhibited enzymatic properties similar to those of serine collagenases reported previously. However, the exact nature of the interaction of these enzymes with the substrate collagen must be known to better understand their precise functions. Investigation of the substrate specificity using both synthetic and natural substrates will be the subject of further studies, which might aid in their rational use for food product development.

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