Comparative Study on the Cathepsin D from Banded Shrimp (*Penaeus japonicus*) and Grass Shrimp (*Penaeus monodon*)

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Muscle proteases from banded and grass shrimps were purified to electrophoretical homogeneity by concanavalin A-Sepharose, ultrafiltration, and preparative isoelectric focusing electrophoresis. These proteases appear to be an aspartic protease, cathepsin D (EC 3.4.23.5). The cathepsin D obtained from banded and grass shrimps, respectively, had p*I* values of 4.9 and 5.2, optimal temperatures at 50 and 45 °C, and a molecular weight for both at 61 000. Both proteases appear to have two identical subunits of 30 kDa each. Ethylenediaminetetraacetic acid and mercaptoethanol did not affect the cathepsin D from grass shrimp, but they inhibited that from banded shrimp. Both cathepsins D were highly inhibited by Ba^{2+}, Mn^{2+}, Fe^{2+}, and Hg^{2+} but activated by K^{+}, Co^{2+}, Zn^{2+}, Cd^{2+}, and Fe^{3+}. The thermostability of the cathepsin D from grass shrimp was higher than that from banded shrimp. Although both cathepsins D had similar optimal pH near 3, banded shrimp had broader pH stability than grass shrimp.

INTRODUCTION

Many endogenous proteases are recognized to accelerate the biochemical and physical changes of muscle during post-mortem storage and processing, which consequently affect the quality of stored and/or processed meat products. These proteases play important roles in post-mortem degradation of myofibrils and muscle proteins (Jiang et al., 1990). Among these proteases, cathepsin D and calpain were considered to be the most important proteases on post-mortem degradation of muscle. Cathepsin D (EC 3.4.23.5) is a lysosomal protease and is widely distributed in animal tissues (Doke et al., 1980; Makinodan et al., 1982; Turk et al., 1981). Although it has been purified from many terrestrial animal tissues, there have been no reports of cathepsin D from marine species such as banded and grass shrimps. In addition, according to some papers (Chung, 1977; Flick and Lovell, 1972), shrimps have a very short or no rigor mortis period and degrade faster than fishes. According to our previous studies (Jiang et al., 1990, 1991), the pepstatin-sensitive proteases (mainly cathepsin D) degraded the post-mortem myofibrils of tilapia at pH 5.5 and 6.0. For understanding the properties of cathepsin D obtained from different shrimp species, cathepsins D from banded and grass shrimp muscle were purified and characterized.

MATERIALS AND METHODS

Materials. Banded shrimp (*Penaeus japonicus*) and grass shrimp (*Penaeus monodon*) were purchased from a commercial aquatic farm in southern Taiwan and transported to the laboratory in oxygenated water (bubbling oxygen gas). All living shrimps were ice-shocked with immersion in ice–water. Samples were then peeled and used for enzyme preparation.

Purification of Shrimp Muscle Cathepsin D. Preparation of Crude Enzyme. Acetone powder was prepared by homogenizing shrimp muscles with 9 volumes of prechilled acetone (−40 °C) and filtered (Büchner funnel). After washing (2×) with 3 volumes of acetone, samples were air-dried in a hood. The acetone powder was then stored at −20 °C until use. Crude enzyme was extracted from 40 g of acetone powder with 10 volumes of buffer I (20 mM sodium phosphate, pH 7.0) containing 0.5 mM dithiothreitol (DTT). Samples were homogenized for 2 min using a Waring blender coupled with a baffler and centrifuged at 25000*g* for 30 min at 4 °C. The supernatant was used as crude enzyme in this experiment. All of the preparation procedures were carried out at 0–4 °C.

Determination of the Activity of Protease. The hemoglobin (Hb) hydrolytic activity of the protease was determined according to the method of Makinodan and Ikeda (1976). The acid-denatured Hb, which was prepared by dissolving 5% native Hb (Difco Laboratories, Detroit, MI) in 0.06 N HCl and dialyzing against deionized water for 8–10 h, was used as substrate. The enzyme solution (0.2 mL) was mixed with 0.2 mL of 5% acid-denatured Hb and 0.8 mL of McIlvaine buffer (pH 3.0). After incubation at 37 °C for 1 h, the reaction was stopped by adding 0.5 mL of 10% trichloroacetic acid (TCA). After holding at 25 °C for 10 min, the reaction mixtures were then centrifuged at 7000 rpm (centrifuge Model 5415C, Eppendorf Co. Ltd.) for 15 min. Nonproteinous nitrogen in the supernatants was determined according to the method of Lowry et al. (1951). One unit of the specific activity was defined as the amount of protease releasing 1 nmol of tyrosine equivalent within 1 h of reaction of pH 3.0.

Concanavalin A–Sepharose Chromatography. NaCl was added to the crude enzyme solution to a final concentration of 0.6 M and chromatographed on a concanavalin A–Sepharose (Con A–Sepharose) column (2.6 × 40 cm), equilibrated with about 10 bed volumes of buffer II (20 mM sodium phosphate containing 0.6 M NaCl, 5 mM 2-mercaptoethanol, and 0.5 mM sodium azide, pH 7.0). The column was washed with buffer II until the absorbance of eluate at 280 nm was less than 0.05 (about 10 bed volumes) and then eluted with buffer II containing 0.5 M methyl α-D-mannoside at a flow rate of 48 mL/h. Fractions of 5 mL were collected using a fractional collector (Frac-100, Pharmacia Co. Ltd.).

Ultrafiltration. About 280 mL of eluent with Hb hydrolytic activity obtained from Con A–Sepharose chromatography was first ultrafiltered with a Minitan filter (Millipore Co., Bedford, MA) using PTHK membrane (MW cutoff 100 000). During ultrafiltration, chilled buffer I (about 1000 mL) was gradually poured into samples to dilute the concentration of salt and sugar. The resulting filtered sample with a molecular weight lower than 100 000 was further ultrafiltered and concentrated with a Minitan filter using PTTK membrane (MW cutoff 30 000) to remove the components with molecular weight lower than 30 000 and concentrated to a volume of 50 mL. This enzyme solution was further concentrated with an Amicon ultrafiltration cell (Amicon Division, W. R. Grace and Co., Danvers, MA) using a Diaflo membrane (YM30; diameter 43 mm; MW cutoff 30 000) to below 10 mL.

Preparative Isoelectric Focusing Electrophoresis. Ampholyte (2.5 mL of 40% solution of Bio-Lyte 4/6, Bio-Rad Laboratories, Richmond, CA) was added to the enzyme solution (10 mL) obtained from ultrafiltration (using the Minitan filter), and the final volume was adjusted to 50 mL using deionized

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water. After prerunning, enzyme solution with ampholyte was added into the Rotofor cell chamber (Bio-Rad). After 5 h of isoelectric focusing under a condition of 12-W constant power, fractions of sample were harvested according to the manufacturer's directions (Bio-Rad). The resultant samples were concentrated with an Amicon ultrafiltration cell using a Diaflo membrane (YM10; diameter 43 mm; cut off 10 000) to below 5 mL. This step was to eliminate ampholyte and concentrate. The pH of each fraction was measured using a pH meter (HM-30S, TOA Electronics Ltd., Tokyo). The Hb hydrolytic activity of samples was then determined according to the method of Makinodan and Ikeda (1976).

Characterization of Cathepsin D. Determination of Molecular Weight. The molecular weight (MW) of purified proteases obtained from both shrimp species was determined using Sephadex G-150 column chromatography, gradient gel electrophoresis, and disc SDS–polyacrylamide gel electrophoresis (disc SDS–PAGE). The Sephadex G-150 column (2.6 × 80 cm) was equilibrated with 10 bed volumes of buffer I containing 0.5 mM sodium azide and eluted with the same buffer at a flow rate of 24 mL/h. Fractions of 5 mL each were collected. Albumin (67 000), ovalbumin (43 000), chymotrypsinogen (25 000), and ribonuclease A (13 700), purchased from Pharmacia Co., were used as standards for the MW determination.

The gradient gel electrophoresis was performed according to the method of Andrews (1987). The thickness and concentration gradient of the acrylamide slab gel were 0.75 mm and from 5 to 15%, respectively. For determining the subunits of purified proteases, disc SDS–PAGE was performed according to the method of Laemmli and Favre (1973). The thickness and concentration of the acrylamide slab gel were 0.75 mm and 10%, respectively. The concentration of the stacking gel was 3.75%. Gels were stained with Coomassie blue according to the method of Neuhoff et al. (1988). Phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean inhibitor (20 000), and α-lactalbumin (14 400), obtained from Pharmacia, were used as standards.

Optimal pH and Temperature. The purified proteases in McIlvaine (0.2 M sodium phosphate–0.1 M citric acid for pH 2.6–7.5) and 0.2 M KCl–HCl buffers (for pH 1.5–2.0) with various pH values were incubated with acid-denatured Hb at 37 °C for 1 h. The Hb hydrolytic activity was then measured according to the method of Makinodan and Ikeda (1976). The relative activity was expressed as a percentage ratio of the specific activity (units per milligram) of cathepsin D measured at each pH to that measured at pH 3.0 (which had maximum activity).

The purified proteases in McIlvaine buffer (pH 3.0) were incubated with acid-denatured Hb at various temperatures (0–50 °C) for 1 h. The Hb hydrolytic activity was then measured to determine the optimal temperature for Hb hydrolysis. The relative activity was expressed as a percentage ratio of the specific activity (units per milligram) of cathepsin D measured at various temperatures to that measured at 45 °C for grass shrimp and at 50 °C for banded shrimp, respectively (which had maximum activity).

Effect of pH on the Stability of Protease. The purified proteases were incubated at pH values from 3.5 to 7.5 (McIlvaine buffer) at 25 °C for 30 min. After the proteases had cooled to 0 °C, the Hb hydrolytic activity was then measured according to the method of Makinodan and Ikeda (1976). The relative activity was expressed as a percentage ratio of the specific activity (units per milligram) of cathepsin D after 30 min of preincubation at various pH values to that of sample with maximum activity (pH 7.5 for banded shrimp; pH 5.5 for grass shrimp).

Thermostability. For the determination of thermal stability at 0–60 °C, the purified proteases obtained from both shrimps in McIlvaine buffer (pH 3.0) were incubated at various temperatures for 10 min. After the proteases had cooled to 0 °C, the Hb hydrolytic activity was then measured according to the method of Makinodan and Ikeda (1976). The relative activity was expressed as percentage ratio of the specific activity (units per milligram) of cathepsin D after 10 min of preincubation at various temperatures to that without preincubation.

Inhibitor Study. Purified proteases obtained from both shrimps in McIlvaine buffer (pH 3.0) were incubated with *N*-ethylmaleimide (NEM, 1 and 2 mM), leupeptin (1.0 μg/mL), pepstatin (final concentration 1.0 μg/mL), iodoacetic acid (IAA, 1.0 mM), phenylmethanesulfonyl fluoride (PMSF, 1.0 mM), *p*-(chloromercuri)benzoate (PCMB, 1.0 mM), ethylenediaminetetraacetic acid (EDTA, 5.0 mM), and ethylene glycol bis(β-aminoethyl ether *N,N,N',N'*-tetraacetic acid (EGTA, 5.0 mM) at 37 °C for 5 min, respectively. The Hb hydrolytic activity was measured according to the method of Makinodan and Ikeda (1976). The relative activity was expressed as percentage ratio of the specific activity (units per milligram) of cathepsin D with various inhibitors to that without inhibitor.

Effect of Reductants. The purified proteases obtained from both shrimps in McIlvaine buffer (pH 3.0) were incubated with 1.0 mM of dithiothreitol (DTT), 2-mercaptoethanol (2-Me), and glutathione (GSH) at 37 °C for 5 min. The Hb hydrolytic activity was then measured according to the method of Makinodan and Ikeda (1976). The relative activity was expressed as percentage ratio of the specific activity (units per milligram) of cathepsin D with reductants to that without reductants.

Effect of Metal Ions. Purified proteases in McIlvaine buffer (pH 3.0) from shrimp were incubated with 5.0 mM of various metals (Na^+, K^+, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, and Fe^{3+}) at 37 °C for 5 min. The Hb hydrolytic activity was then measured according to the method of Makinodan and Ikeda (1976). The counterion of these metals was Cl^-. The relative activity was expressed as percentage ratio of the specific activity (units per milligram) of cathepsin D with metals to that without metals.

Determination of Protein Concentration. Protein concentration was determined according to the protein dye binding method (Bradford, 1976) using crystalline bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Purification of Protease. The crude enzymes obtained from banded and grass shrimp acetone powder were chromatographed on a Con A–Sepharose column. Fractions with Hb hydrolytic activity were collected (Figure 1). Purifications of 46.3- and 53.1-fold for banded and grass shrimps, respectively, were achieved at this step (Table I). By using the ultrafiltration, the Hb hydrolytic activity appeared on the fractions between cutoff 30 and 100 kDa. The purifications by ultrafiltration as expressed by specific activity were similar to that achieved by gel filtration, but the ultrafiltration was much faster than gel filtration (data not shown). Finally, both enzymes were purified to an electrophoretic homogeneity (Figure 2) using a preparative isoelectric focusing electrophoresis (Rotofor cell, Model 3000 Xi; Ampholyte, Bio-Lyte 4/6, Bio-Rad). The purification is summarized in Table I. The specific activity of the purified protease from grass shrimp was much higher than that of the banded shrimp.

Properties of Protease. Determination of Molecular Weights. The molecular weights (MW) of these purified proteases from banded and grass shrimps were the same, 61 000, estimated by Sephadex G-150 gel filtration and nondissociating gradient PAGE (Figure 3). However, when both purified proteases were subjected to disc SDS–PAGE analysis, both proteases appeared as a single band with MW of 30 000. According to the data obatined in this study, it was considered that both purified proteases were dimers with two identical subunits.

Inhibitor Study. As shown in Table II, the proteinases purified from both shrimps were inhibited by pepstatin, which is a specific inhibitor for aspartic proteases at acid pH, while leupeptin and NEM did not affect them. Iodoacetic acid (IAA), PMSF, and PCMB partially inhibited these two enzymes. These phenomena suggested that, in addition to the aspartic acid residue, the nonpolar pocket of the active site might also contain cysteine, which consequently affected the corresponding sites of catalytic group and catalytic site on enzyme–substrate complex and

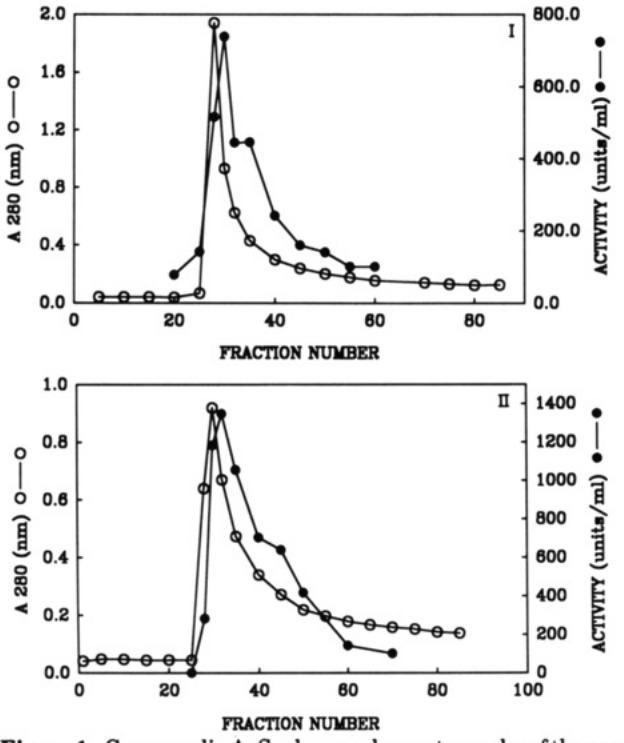

Figure 1. Concanvalin A–Sepharose chromatography of the proteases from banded (I) and grass shrimp muscle (II) [column, 2.6 × 30 cm; equilibrated with buffer II (20 mM sodium phosphate buffer containing 0.6 M NaCl, 5 mM 2-mercaptoethanol, and 0.5 mM sodium azide, pH 7.0); washed with 10 bed volumes of buffer II; eluted with buffer II containing 0.5 M methyl α-D-mannoside; flow rate: 48 mL/h; 5.0 mL/tube]. (O) Absorbance at 280 nM; (●) cathepsin D activity.

Table I. Purification of Cathepsin D from the Banded Shrimp and Grass Shrimp Muscle

purifn step	total protein, mg	total act.,[a] units	sp act., units/mg	recov, %	purifn, x-fold
		Banded Shrimp			
crude extract	3668.5	60 202	16.4	100.0	1.0
Con A eluate	43.3	32 867	760.1	54.6	46.3
ultrafiltration	10.5	16 380	1560.0	27.2	95.1
Rotofor cell[b]	0.42	1 760	4211.5	3.0	256.6
		Grass Shrimp			
crude extract	3461.0	66 267	19.2	100.0	1.0
Con A eluate	35.8	36 500	1019.5	55.1	53.1
ultrafiltration	6.6	17 392	2627.1	26.3	136.8
Rotofor cell[b]	0.47	4 458	9458.7	6.7	492.6

[a] The unit of enzyme activity was expressed as nanomoles of tyrosine liberated per hour. [b] Rotofor cell, isoelectric focusing preparative electrophoresis unit.

retarded the Hb hydrolytic activity at pH 3.0. The protease obtained from grass shrimp was not affected by 5 mM EDTA and EGTA; however, the activity of the banded shrimp cathepsin D was reduced by 70% by 5 mM EDTA.

Although 0.1 mM PCMB did not inhibit the cathepsin D obtained from rat spleen, 1 mM PCMB inhibited about 60% cathepsin D-I and 35% cathepsin D-II activities from the same source. However, 0.1 mM PCMB partially inhibited those from carp and porcine spleen (Cunningham and Tang, 1976; Makinodan et al., 1982; Yamamoto et al., 1979). Some studies indicated that 10 mM EDTA did not affect the Hb hydrolytic activity of cathepsin D from rat spleen, but 100 mM EDTA partially activated that from porcine spleen (Cunningham and Tang, 1976; Yamamoto et al., 1979). A review of these papers shows that the Hb hydrolytic activity of cathepsin D extracted from different animal tissues was completely inhibited by pepstatin. It is recognized that PCMB has high affinity

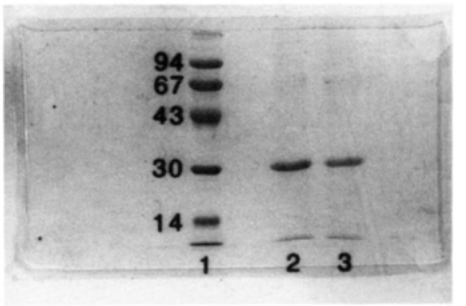

Figure 2. Disc SDS–polyacrylamide electrophoresis of purified cathepsin D from banded and grass shrimps (10.0% acrylamide gel, using dissociating system. (Lane 1) Standards [phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), and α-lactalbumin (14 400)]; (lanes 2 and 3) purified cathepsin D from banded and grass shrimps, respectively.

for SH groups and can rapidly react with enzymes containing SH groups and subsequently inhibit the activity. Although the properties of cathepsin E are similar to those of cathepsin D, the MW of cathepsin E is around 90 000–100 000, much higher than that of cathepsin D (Yamamoto et al., 1978, 1979, 1985; Yonezawa et al., 1987; Zeece and Katoh, 1989). The p*I* values of the purified proteases from banded and grass shrimps were 4.9 and 5.0, respectively, determined by preparative isoelectric focusing electrophoresis. These p*I* values were higher than that of cathepsin E (p*I* < 3.0) (Matsumoto et al., 1983; Asghar and Bhatti, 1987; Goll et al., 1983). Accordingly, from the inhibitor studies, p*I* values, and MW, the proteases obtained in this study were identified to be cathepsin D. The active sites of these cathepsins D might contain aspartic acid and cysteine.

Effect of Reductants. The Hb hydrolytic activity of cathepsin D from grass shrimp was not affected by 1 mM DTT, 2-Me, and GSH, but that from banded shrimp was partially inhibited by 1 mM 2-Me (Table III). Yamamoto et al. (1979) reported that 10 mM 2-Me did not activate the cathepsin D of rat spleen; it did activate 40% activity of that from carp muscle (Makinodan et al., 1982). On the other hand, Kirschke and Barrett (1987) reported that cathepsin D was irreversibly inactivated by DTT, and the degree of inhibition was dependent on species.

Effect of Metal Ions. The Hb hydrolytic activity of the purified cathepsin D from both shrimps was activated by K^+, Co^{2+}, Zn^{2+}, Cd^{2+}, and Fe^{3+} ions, while it was inhibited or partially inhibited by Ba^{2+}, Mn^{2+}, Fe^{2+}, and Hg^{2+} ions. Na^+, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ni^{2+}, and Cu^{2+} ions activated the cathepsin D from banded shrimp but did not affect that from grass shrimp (Table IV). According to Takeda et al. (1986) and Yamamoto et al. (1979, 1985), cathepsin D from rat spleen and human erythrocyte membrane acid proteinase (EMAP) were inhibited by Hg^{2+} and Fe^{3+}. Mg^{2+}, Ca^{2+}, and Ni^{2+} (10 mM) did not affect the activity of cathepsin D from rat spleen but significantly activated that from tilapia muscle. Hg^{2+} has been reported to bind to SH groups of the target enzyme and to subsequently inhibit the enzymatic activity (Klee, 1988). Inhibition of shrimp cathepsin D by IAA, PMSF, PCMB, and Hg^{2+} suggests that the active site of these proteases might also contain cysteine.

Effect of pH and Temperature on the Cathepsin D Activity. The pH curve for grass shrimp cathepsin D was broader than that for banded shrimp cathepsin D, with maximum activity occurring at pH 3.0 for both (Figure 4). Almost no activity was detected when the pH of the reaction mixture was near neutral. It is recognized that

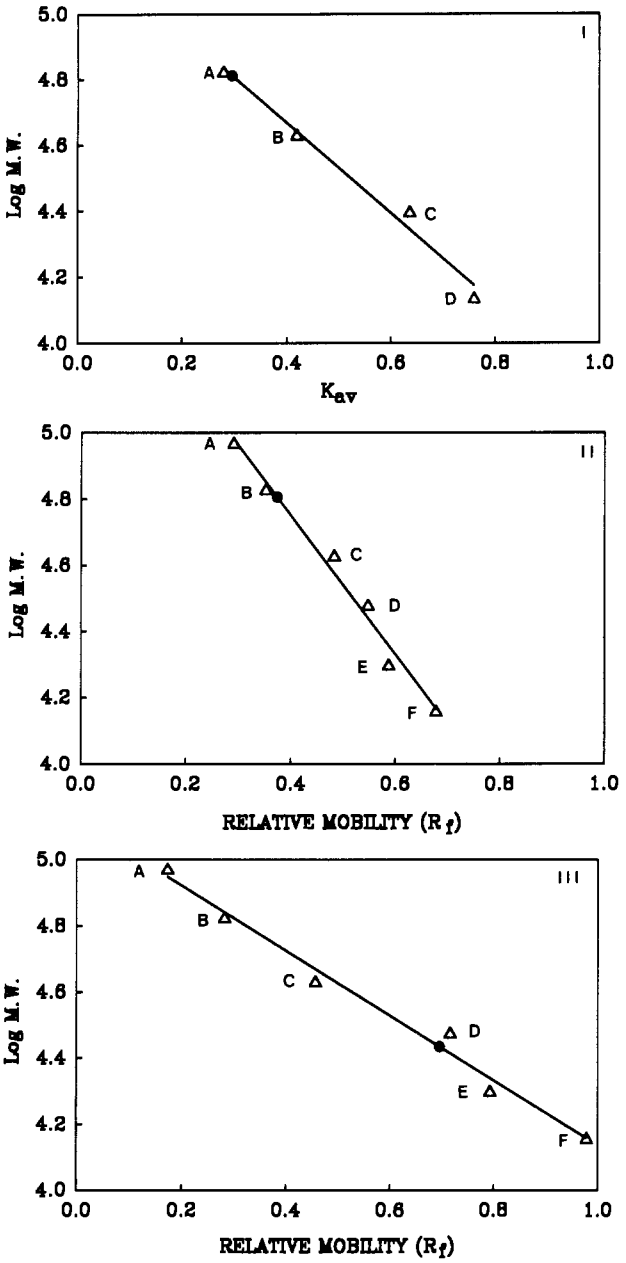

Figure 3. Calibration curves for the determination of molecular weight. (I) Sephadex G-150 column chromatography (2.6 × 80 cm; equilibrated and eluted with 20 mM sodium phosphate buffer, pH 7.0, containing 0.5 mM sodium azide; flow rate, 24 mL/h; tube, 5 mL); (A) albumin, 67 kDa; (B) ovalbumin, 43 kDa; (C) chymotrypsinogen, 25 kDa; (D) ribonuclease A, 13.7 kDa; (○) from banded shrimp; (●) from grass shrimp). (II and III) Using 5–15% gradient PAGE and SDS–PAGE on 10% polyacrylamide gel; (A) phosphorylase *b* subunit, 94 kDa; (B) bovine serum albumin, 67 kDa; (C) ovalbumin, 43 kDa; (D) carbonic anhydrase, 30 kDa; (E) soybean inhibitor, 20 kDa; (F) α-lactalbumin, 14.4 kDa; (○) from banded shrimp; (●) from grass shrimp.

most of the cathepsins D from different sources have an optimum pH of 3.0–5.0 (Asghar and Henrickson, 1982; Asghar and Bhatti, 1987; Bond and Butler, 1987; Iodice et al., 1966; Makinodan et al., 1982; Takeda et al., 1986; Zeece and Katoh, 1989). However, some cathepsin D had two optimal pH values (Iodice et al., 1966; Makinodan et al., 1982; Takeda et al., 1986). The pH curves for cathepsin D obtained from different sources were different. This phenomenon suggests that the pK_a values for the carboxyl groups were different. According to the present data and studies on the other species, the catalytic groups on the active site of cathepsin D from different sources were aspartate, but the residues around the active site might not be similar.

Table II. Effect of Various Inhibitors on the Hb Hydrolytic Activity of Cathepsin D

inhibitor[a]	concn	rel act.,[b] % banded shrimp	rel act.,[b] % grass shrimp
none		100	100
leupeptin	1 μg/mL	97.5	97.1
pepstatin	1 μg/mL	5.6	8.6
NEM	1 mM	100	100
NEM	2 mM	100	94.3
iodoacetic acid	1 mM	79.5	94.3
PMSF	1 mM	63.1	77.1
PCMB	1 mM	28.9	65.7
EDTA	5 mM	30.9	100
EGTA	5 mM	98.8	100

[a] NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride; PCMB, *p*-(chloromercuri)benzoate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid. [b] The relative activity was expressed as percentage ratio of the specific activity (units/mg) of cathepsin D with various inhibitors to that without inhibitor.

Table III. Effect of Various Reductants on the Hb Hydrolytic Activity of Cathepsin D

reductant[a]	concn, mM	rel act.,[b] % banded shrimp	rel act.,[b] % grass shrimp
none		100	100
DTT	1	97.3	100
2-Me	1	67.9	100
GSH	1	93.8	100

[a] DTT, dithiothreitol; 2-Me, 2-mercaptoethanol; GSH, glutathione. [b] The relative activity was expressed as percentage ratio of the specific activity (units/mg) of cathepsin D with various reductants to that without reductants.

Table IV. Effect of Metal Ions on the Hb Hydrolytic Activity of Cathepsin D

metal ion[a]	rel act.,[b] % banded shrimp	rel act.,[b] % grass shrimp
none	100	100
Na^+	120.1	106.9
K^+	120.1	120.7
Mg^{2+}	124.4	97.0
Ca^{2+}	135.0	104.1
Sr^{2+}	246.0	106.9
Ba^{2+}	32.5	0.0
Mn^{2+}	30.6	0.0
Fe^{2+}	66.6	66.0
Co^{2+}	250.8	228.7
Ni^{2+}	123.0	93.1
Cu^{2+}	159.5	101.9
Zn^{2+}	226.9	127.6
Cd^{2+}	191.3	244.8
Hg^{2+}	36.5	69.0
Fe^{3+}	139.7	120.0

[a] The counterion of all metals was Cl^-; the concentration of all metals was 5 mM. [b] The relative activity was expressed as percentage ratio of the specific activity (units/mg) of cathepsin D with 5 mM metals to that without metals.

The protease of banded shrimp had greater stability to varied pH than did that of grass shrimp (Figure 5). The banded shrimp cathepsin D was stable from pH 4 to 8, while the grass shrimp cathepsin D was stable over a much narrower range with an optimum at pH 6.

As indicated in Figure 6, the optimal temperatures for cathepsins D from banded and grass shrimps were at 50 and 45 °C, respectively, which is similar to the value for that from porcine spleen, rat spleen, and carp muscle (50 °C) (Cunningham and Tang, 1976; Yamamoto et al., 1979; Doke et al., 1980; Makinodan et al., 1982; Draper and Zeece,

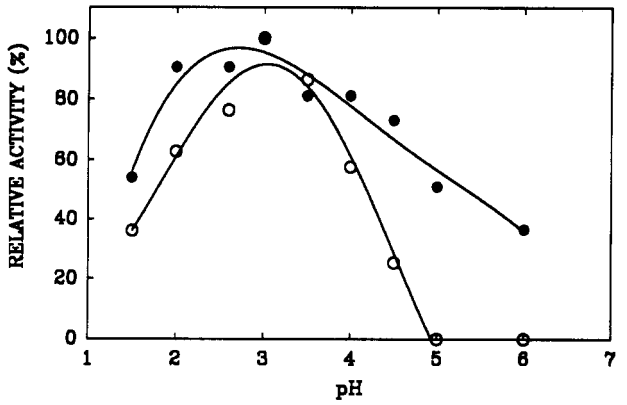

Figure 4. Effect of pH on Hb hydrolytic activity of cathepsin D. (○) Banded shrimp; (●) grass shrimp. Proteinase in McIlvaine buffer with various pH values was incubated with acid-denatured hemoglobin at 37 °C for 1 h. The relative activity was expressed as percentage ratio of the specific activity (units per milligram) of cathepsin D measured at various pH values to that measured at pH 3.0 (banded shrimp, 4033.5 units/mg; grass shrimp, 8071.6 units/mg).

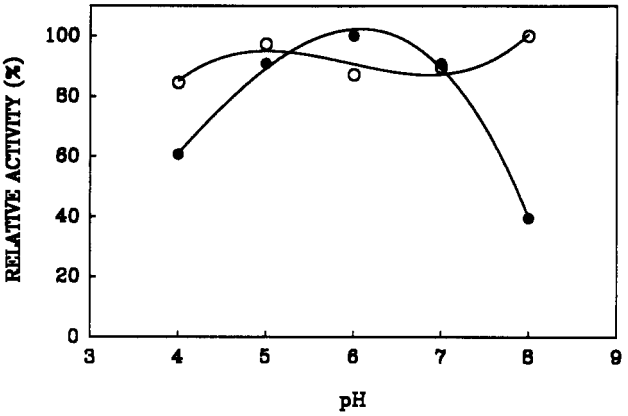

Figure 5. pH stability of cathepsin D. (○) Banded shrimp; (●) grass shrimp. Proteinase in McIlvaine buffer with various pH values was incubated at 25 °C for 30 min and then the activity was assayed. The relative activity was expressed as percentage ratio of the specific activity (units per milligram) of cathepsin D after 30 min of preincubation at various pH values to that of sample with maximum activity (pH 7.5 for banded shrimp; pH 5.5 for grass shrimp).

1989). The cathepsin D from grass shrimp still had 17% activity even at 0 °C, but that from banded shrimp had almost completely lost activity at that temperature (Figure 6). After 5 days of frozen storage at −40 °C, the specific activity of banded shrimp cathepsin D decreased from 410.8 to 245.7 units/mL and was completely lost after 12 days of storage, while grass shrimp cathepsin D still had 13.7% activity remaining even after 16 days of storage at that temperature (Table V).

After preincubation for 10 min, the temperatures for inactivating 50% of the enzyme activity of banded and grass shrimps were 33 and 58.4 °C, respectively (Figure 7). However, the cathepsins D from porcine spleen, rat spleen, carp muscle, and tilapia muscle still had 50–70% activity after the cathepsin D was incubated at 50 °C for 30 min (Cunningham and Tang, 1976; Yamamoto et al., 1979; Doke et al., 1980; Makinodan et al., 1982; Draper and Zeece, 1989). These results suggested that the cathepsins D from both shrimps were more labile than those from land animals and fish.

In summary, cathepsin D obtained from banded and grass shrimp species, respectively, had p*I* values of 4.9 and 5.2, optimal temperatures at 50 and 45 °C, and a molecular weight for both at 61 000. Both proteases appear to have two identical subunits of 30 000 each. The stability of cathepsin D from grass shrimp was greater than that from banded shrimp. Although both cathepsin D had similar optimal pH near 3, the pH curve for grass shrimp cathepsin D was broader than that for banded shrimp enzyme. These data suggest that the degradation of grass shrimp muscle by cathepsin D might be greater than that of banded shrimp muscle during storage and/or processing.

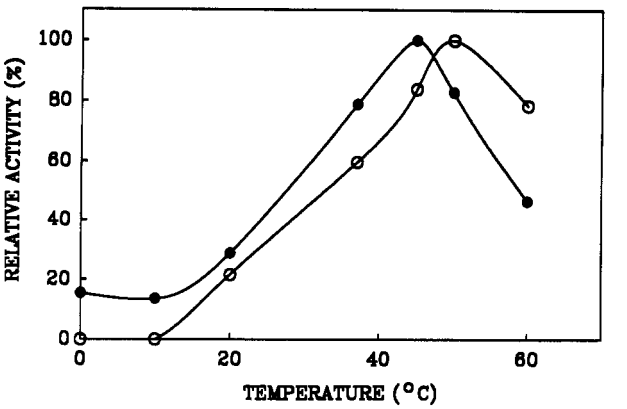

Figure 6. Effect of temperature on Hb hydrolytic activity of cathepsin D. (○) Banded shrimp; (●) grass shrimp. Proteinase in McIlvaine buffer, pH 3.0, was incubated with acid-denatured hemoglobin at various temperatures for 1 h. The relative activity was expressed as percentage ratio of the specific activity (units per milligram) of cathepsin D measured at various temperatures to that measured at 37 °C (banded shrimp, 3929.2 units/mg; grass shrimp, 7691.7 units/mg).

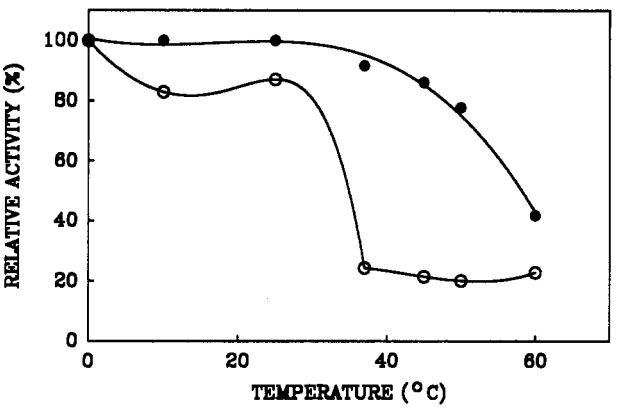

Figure 7. Heat inactivation of cathepsin D at various temperatures. (○) Banded shrimp; (●) grass shrimp. Proteinase in McIlvaine buffer, pH 3.0, was incubated at various temperatures for 10 min. After the proteinase had cooled to 0 °C, the Hb hydrolytic activity was measured. The relative activity was expressed as percentage ratio of the specific activity (units per milligram) of cathepsin D after 10 min of preincubation at various temperatures to that without preincubation (banded shrimp, 4129.0 units/mg; grass shrimp, 6437.5 units/mg).

Table V. Stability of Cathepsin D Obtained from Banded and Grass Shrimp Muscle at −40 °C

	rel act., %	
days at −40 °C	banded shrimp	grass shrimp
0	100	100
5	59.8	35.0
12	7.9	23.5
16	0.0	13.7

[a] The relative activity was expressed as percentage ratio of the specific activity (units/mg) of cathepsin D to that of zero time (grass shrimp, 1126.0 units/mL; banded shrimp, 410.8 units/mL).

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