Purification and Characterization of a Proteinase Identified as Cathepsin D from Tilapia Muscle (*Tilapia nilotica* × *Tilapia aurea*)[†]

Shann-Tzong Jiang,*[,‡] Yuh-Tai Wang,[‡] and Ching-San Chen[§]

Graduate School of Marine Food Science, National Taiwan Ocean University, Keelung, Taiwan 20224, ROC, and Institute of Botany, Academia Sinica, Nankung, Taipei 11529, ROC

For understanding the characteristics of lysosomal enzymes, cathepsin D was first purified by heat treatment, concanavalin A-Sepharose and Sephadex G-150 chromatographies, and finally preparative electrophoresis. A purification of 244-fold against the crude extract was achieved. The recovery was 2%. The purified enzyme appeared to be electrophoretically homogeneous and had a molecular weight of 55 000. Inhibitor study and molecular weight determination indicated this enzyme to be an aspartic proteinase, cathepsin D. The optimal pH and temperature were 3.5 and 37 °C, respectively. This proteinase was inhibited by Hg^{2+} and Fe^{3+} but activated by Ca^{2+}, Ni^{2+}, and Mg^{2+} and slightly activated by Zn^{2+} and Cd^{2+}. The purified cathepsin D was completely inhibited by pepstatin, partially inhibited by *p*-(chloromercuri)benzoate (PCMB), and ethylenediaminetetraacetic acid (EDTA), and almost not affected by 2-mercaptoethanol.

INTRODUCTION

Cathepsin D (EC 3.4.23.5) is a lysosomal aspartic proteinase, widely distributed in mammalian cells and fish muscle (Doke et al., 1980; Makinodan et al., 1982; Turk et al., 1981). Being different from the other aspartic proteinases, cathepsin D is an intracellular proteinase. The main physiological role of cathepsin D is the breakdown of tissue proteins (Chewale et al., 1985; Turk et al., 1981).

Recently, many studies indicated that lysosomal enzymes (cathepsins) did not participate in muscle tenderization (Koohmaraie et al., 1986, 1988a,b). However, some papers suggested that cathepsins A, B, D, H, and L could degrade muscle proteins (Asghar and Bhatti, 1987; Bond and Butler, 1987; Goll et al., 1983; Matsumoto et al., 1983; Zeece and Katoh, 1989). According to a previous study (Jiang et al., 1990), the pepstatin-sensitive proteases (mainly cathepsin D) degraded the post-mortem myofibrils at pH 5.5 and 6.0. For investigating which pepstatin-sensitive proteinases are involved in post-mortem myofibril degradation, the cathepsin D from tilapia muscle was first purified and characterized.

MATERIALS AND METHODS

Materials. Tilapia (*Tilapia nilotica* × *T. aurea*) were purchased from a commercial aquatic farm in southern Taiwan and transported to the laboratory in oxygenated water (bubbling oxygen gas). Samples were headed, eviscerated, and washed. The dorsal muscle was used for enzyme preparation.

Purification of Tilapia Muscle Cathepsin D. *Preparation of Crude Enzyme.* The acetone powder of tilapia muscle was prepared by homogenizing with 9 volumes of prechilled acetone (-20 °C) and then filtering with a Büchner funnel to remove acetone. After being washed with 3 volumes of acetone twice, the resulting samples were air-dried in a hood for 8-10 h at room temperature. This step was to remove fat, moisture, and some pigments, etc. The acetone powder was then stored at -20 °C until use. The crude enzyme was extracted with 5 volumes of buffer I (20 mM sodium phosphate, pH 7.0) from 40 g of acetone powder. After the samples were homogenized with a Waring blender subjoined with a baffler for 1 min, centrifugation was performed under 25000*g* for 30 min at 0 °C. The supernatant was used as crude enzyme in this experiment. All the preparation procedures were carried out at 0-4 °C.

Heat Treatment. The crude enzyme was heated at 45 °C for 3 min by using a water bath with stirring. After cooling to 0-4 °C, samples were centrifuged at 25000*g* for 30 min. The supernatant was removed for the following purifications.

Concanavalin A-Sepharose Chromatography. The supernatant was concentrated by a Minitan ultrafiltration system (Millipore Co.) with a PTGC membrane (cutoff 10 000). The concentrated sample was chromatographed on a concanavalin A-Sepharose (Con A-Sepharose) column (1.6 × 33 cm), equilibrated with about 6 bed volumes of buffer II (20 mM sodium phosphate containing 0.6 M NaCl and 1 mM 2-mercaptoethanol, 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 20 mL/h. Fractions of 5 mL were collected.

Determination of the Activity of Proteinase. The hemoglobin (Hb) hydrolytic activity of the proteinase was determined according to the method of Makinodan and Ikeda (1976). The acid-denatured hemoglobin, which was prepared by dissolving 5% native hemoglobin (Difco Laboratories, Detroit, MI) in 0.06 N HCl and dialyzing against deionized water for 8-10 h, was employed as substrate. The enzyme solution (0.5 mL) was mixed with 0.5 mL of 5% acid-denatured hemoglobin and 1.5 mL of McIlvaine buffer (pH 3.0). After incubation at 37 °C for 1 h, the reaction was stopped by adding 2.5 mL of 5% trichloroacetic acid (TCA). The reaction mixtures were settled at room temperature for 45 min and filtered through Whatman No. 42 filter paper. Nonprotein nitrogenous compounds in the filtrate were determined according to the method of Lowry et al. (1951). One nanomole of tyrosine equivalent released within 1 h at pH 3.0 was defined as 1 unit.

Gel Filtration on Sephadex G-150. Fractions with Hb hydrolytic activity on Con A-Sepharose chromatography were collected and concentrated by polyethylene glycol 20 000 (PEG 20 000). After being dialyzed against buffer I for 8-10 h, the sample was chromatographed on a Sephadex G-150 column (1.6 × 81 cm) which was equilibrated with about 5 bed volumes of buffer I and eluted with the same buffer at a flow rate of 12 mL/h. Fractions of 5 mL were collected.

Preparative Electrophoresis. Fractions with Hb hydrolytic activity were collected and concentrated with PEG 20 000. The resulting samples were separated by an electroendosmotic

* To whom correspondence should be addressed.

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[‡] National Taiwan Ocean University.

[§] Academia Sinica.

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preparative electrophoresis unit (ELFE) (Genofit Co. Ltd.) with 12.5% acrylamide separating gel and collected by using a fractional collector with 0.1 mL/tube. The fractions with Hb hydrolytic activity were characterized.

Characterization of Cathepsin D. *Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).* Disc SDS-PAGE was performed according to the method of Laemmli and Favre (1973). The thickness and concentration of acrylamide of the slab gel were 0.75 mm and 7.5% or 12.5%, respectively. The concentration of the stacking gel was 3.75%. Silver staining was performed according to the method of Rabilloud et al. (1988).

Determination of Molecular Weight. The molecular weight of the purified enzyme was determined by using SDS-PAGE. 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) were used as standards.

Optimal pH and Temperature. The purified proteinase in McIlvaine buffer (0.2 M sodium phosphate-0.1 M citric acid) at various pHs (2.6-6.0) was 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 is expressed as percentage ratio of the specific acitivity (units/milligram) of cathepsin D measured at various pHs to that measured at pH 3.5.

The purified proteinase in McIlvaine buffer (pH 3.5) was incubated with acid-denatured Hb at various temperatures (15-50 °C) for 1 h. The Hb hydrolytic activity was then measured to determine the optimal temperature for Hb hydrolysis. The relative activity is expressed as percentage ratio of the specific activity (units/milligram) of cathepsin D measured at various temperatures to that measured at 37 °C.

Thermostability. For determining the thermal stability at 25-60 °C, the purified proteinase in McIlvaine buffer (pH 3.5) was incubated at various temperatures for 30 min. After the resulting enzyme solutions were cooled to 0 °C, the Hb hydrolytic activity was then measured according to the method of Makinodan and Ikeda (1976). The relative activity is expressed as percentage ratio of the specific activity (units/milligram) of cathepsin D after 30 min of preincubation at various temperatures to that without preincubation.

Inhibitor Study. The purified proteinase in McIlvaine buffer (pH 3.5) was incubated with pepstatin (final concentration 1.0 μg/mL), 2-mercaptoethanol (10 mM), EDTA (10 mM), and PCMB (1.0 mM) at 37 °C for 5 min, respectively. The Hb hydrolytic activity was then measured according to the method of Makinodan and Ikeda (1976). The relative activity is expressed as percentage ratio of the specific activity (units/milligram) of cathepsin D with various inhibitors to that without inhibitor.

Effect of Metal Ions. The purified proteinase in McIlvaine buffer (pH 3.5) was incubated with various metals (10 mM Na^+, K^+, Mg^{2+}, Ca^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, and Fe^{3+}) at 37 °C for 5 min. Then the Hb hydrolytic activity was measured according to the method of Makinodan and Ikeda (1976). The counterion of these metals was Cl^-. The relative activity is expressed as percentage ratio of the specific activity (units/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 Proteinase. In the preliminary study, the Hb hydrolytic activity of crude proteinase extracted from the acetone powder of tilapia muscle increased linearly during a 90-min incubation at 37 °C. After heating at 45 °C for 3 min, the crude enzyme was chromatographed on a Con A-Sepharose column. Fractions with Hb hydrolytic activity were collected (Figure 1). A 60-fold purification was achieved at this step (Table I). After chromatography on a Sephadex G-150 column, the Hb hydrolytic activity appeared on the second protein peak (Figure 2). Finally, samples with Hb hydrolytic activity were purified to an electrophoretic homogeneity (Figure 3) by using a 12.5% polyacrylamide gel preparative electrophoresis (ELFE). A 244-fold purification was obtained. The recovery was 2% in this study. The purification of cathepsin D from tilapia muscle is summarized in Table I.

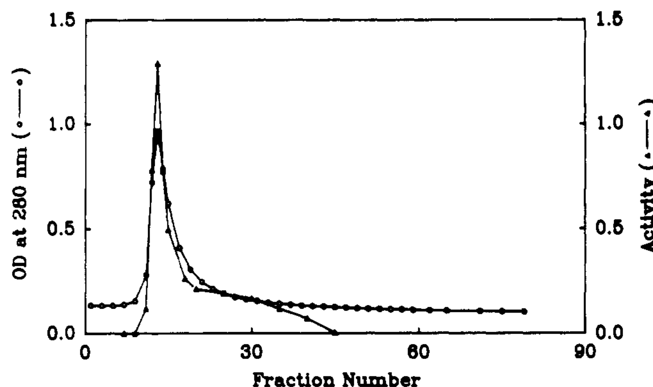

Figure 1. Concanavalin A-Sepharose chromatography of the proteinase after heat treatment [1.6 × 33 cm; equilibrated with buffer II (20 mM sodium phosphate buffer containing 0.6 M NaCl and 1 mM 2-mercaptoethanol, pH 7.0); washed with 10 bed volumes of buffer II; eluted with buffer II containing 0.5 M α-D-mannoside; flow rate 20 mL/h; 5.0 mL/tube].

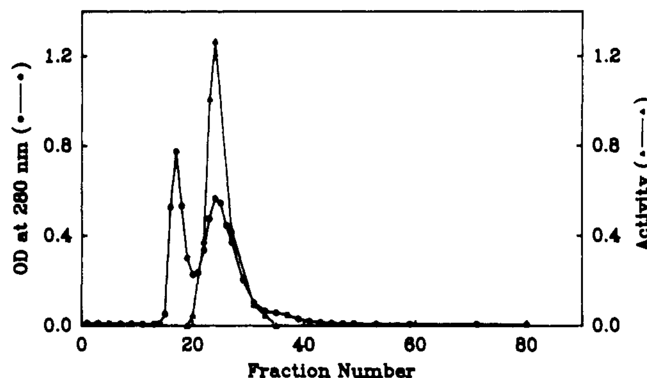

Figure 2. Profiles of the proteinase on Sephadex G-150 chromatography (1.6 × 81 cm; equilibrated with 20 mM sodium phosphate buffer, pH 7.0; eluted with the same buffer; flow rate 12 mL/h; 5.0 mL/tube).

Table I. Purification of Cathepsin D from the Skeletal Muscle of Tilapia

purifn steps	total protein, mg	total act.,[a] units	sp act., units/mg	recov, %	purifn, x-fold
crude extract	5204.59	99 311	19.08	100.00	1.00
heat treatment	4740.12	93 827	19.79	94.48	1.04
Con A eluate	39.65	45 514	1147.90	45.83	60.16
Sephadex G-150	18.26	30 947	1694.85	31.16	88.83
ELFE[b]	0.43	1 999	4649.72	2.01	243.70

[a] The unit of enzyme activity is expressed as nanomoles of tyrosine liberated per hour. [b] ELFE; electroendosmotic preparative electrophoresis unit.

Properties of Proteinase. *Determination of Molecular Weights.* According to the dissociating and nondissociating PAGE (Figure 3), the purified proteinase was a monomer with a molecular weight of 55 000. The molecular weight of glycoproteins was frequently overestimated by using the gel filtration because of the conformation obstacle to filtration (Andrew, 1965; Beeley, 1985). The proteinase obtained in this study was a glycoprotein, since it was separated with Con A-Sepharose chromatography. The prosthetic groups would interfere with the determination of molecular weight. However, Leach et al. (1980a,b) investigated the mobility of glycoprotein on

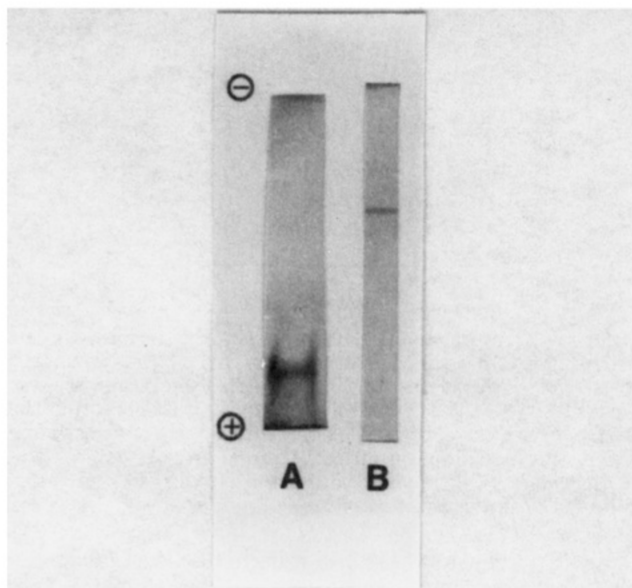

Figure 3. Disc SDS-PAGE of purified cathepsin D from tilapia muscle. (A) 7.5% acrylamide gel, using nondissociating system; (B) 12.5% acrylamide gel, using dissociating system.

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

chemicals	concn	act.,[a] %
none		100
2-mercaptoethanol	10 mM	100
EDTA[b]	10 mM	88
PCMB[c]	1 mM	55
pepstatin	1 μg/mL	0

[a] The relative activity is expressed as percentage ratio of the specific activity (units per milligram) of cathepsin D with various inhibitors to that without inhibitor. [b] EDTA, ethylenediaminetetraacetic acid. [c] PCMB, p-(chloromercuri)benzoate.

various concentrations of polyacrylamide gel electrophoresis (5–15%, SDS–PAGE) and concluded that the molecular weight of glycoprotein could be determined by higher acrylamide concentration of SDS–PAGE. Accordingly, the molecular weight of the purified proteinase was considered to be 55 000 in this study. Generally, the difference of molecular weight was employed to identify the cathepsins D and E, since the molecular weight of cathepsin E (90 000–100 000) was much higher than that of cathepsin D (40 000–50 000) (Yamamoto et al., 1978, 1979, 1985; Yonezawa et al., 1987; Zeece and Katoh, 1989). However, various cathepsins D from different sources are easily misidentified as cathepsin E, since the prosthetic groups will affect the determination of molecular weight and the properties of these proteinases are similar.

Inhibitor Study. Pepstatin (1 μg/mL), a specific inhibitor for aspartic proteinases, completely inhibited, while *p*-(chloromercuri)benzoate (PCMB, final concentration of 1 mM) partially inhibited, this enzyme (Table II). This phenomenon 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 the catalytic group and the catalytic site on the enzyme–substrate complex and retarded the Hb hydrolytic activity at pH 3.0. This enzyme was slightly inhibited by 10 mM EDTA; however, it was not affected by 10 mM 2-mercaptoethanol. The PCMB (0.1 mM) partially inhibited the cathepsin D from carp and porcine spleen but did not inhibit that from rat spleen; 1 mM PCMB inhibited 60% of the Hb hydrolytic activity of cathepsin D-I and 35% cathepsin D-II from rat 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 (Cunningham and Tang, 1976; Yamamoto et al., 1979). Mercaptoethanol (10 mM) did not activate the Hb hydrolytic activity of cathepsin D from rat; however, it did activate 40% of the activity of that from carp muscle (Makinodan et al., 1982; Yamamoto et al., 1979). From a review of those papers it can be concluded that the Hb hydrolytic activities of cathepsin D extracted from different animal tissues were completely inhibited by pepstatin. It is recognized that PCMB has high affinity for SH groups and can rapidly react with enzyme containing SH groups and therefore inhibited the activity. From the inhibitor studies and molecular weight determination, the proteinase obtained in this study was identified as cathepsin D. The activity site might contain aspartic acid and cysteine.

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

metal ion[a]	concn, mM	ionic radius, nm	rel act.[b]
none			100
Na^+	10	0.095	100
K^+	10	0.133	100
Mg^{2+}	10	0.065	138
Ca^{2+}	10	0.099	177
Ni^{2+}	10	0.070	176
Zn^{2+}	10	0.074	113
Cd^{2+}	10	0.097	113
Hg^{2+}	10	0.110	0
Fe^{3+}	10	0.064	13

[a] The counterion of all metals was Cl^-. [b] The relative activity is expressed as percentage ratio of the specific activity (units per milligram) of cathepsin D with 10 mM metals to that without metals.

Effect of Metal Ions. The Hb hydrolytic activity of the purified cathepsin D was not affected by Na^+ and K^+ ions (Table III). The divalent metal ions with ionic radius less than 0.1 nm significantly activated this proteinase. However, divalent metal ion with ionic radius more than 0.1 nm, Hg^{2+}, and trivalent metal, Fe^{3+}, almost completely inhibited the activity of this proteinase (Table III). 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 also 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). Combining the facts of inhibition of PCMB and Hg^{2+} on the purified cathepsin D activity further supports the assumption that the active site of this proteinase might also contain cysteine.

Effect of pH and Temperature on the Cathepsin D Activity. The optimum pH value for cathepsin D was very sharp, maximum activity occurring at pH 3.5 (Figure 4). Almost no activity was detected when the pH of the reaction mixture was over 6.0 or lower than 2.5. A very sharp activity decline was observed at pH 3.5–2.5 or 3.5–5.0. It is recognized that most of the cathepsins D from different sources have an optimum pH of 3.5–5.0 (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, there were some cathepsins D with two optimal pHs in this pH range (Iodice et al., 1966; Makinodan et al., 1982; Takeda et al., 1986). The curves for cathepsin D activity as a function of pH from different sources were slightly different. This suggested that the pK_a values for the car-

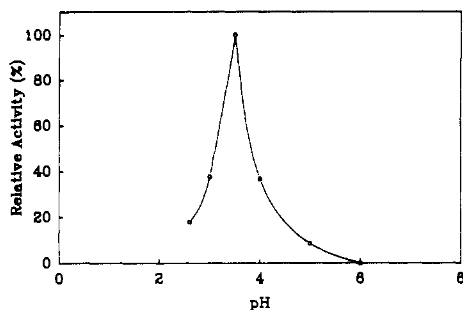

Figure 4. Effect of pH on Hb hydrolytic activity of the cathepsin D. [Proteinase in McIlvaine buffer with various pHs was incubated with acid-denatured hemoglobin at 37 °C for 1 h; the relative activity is expressed as percentage ratio of the specific activity (units/milligram) of cathepsin D measured at various pHs to that measured at pH 3.5 (4643.8 units/mg).]

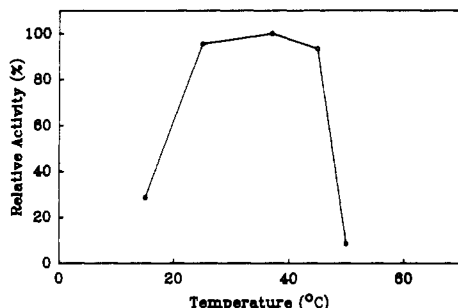

Figure 5. Effect of temperature on Hb hydrolytic activity of the cathepsin D. [Proteinase in McIlvaine buffer, pH 3.5, was incubated with acid-denatured hemoglobin at various temperatures for 1 h; the relative activity is expressed as percentage ratio of the specific activity (units/milligram) of cathepsin D measured at various temperatures to that measured at 37 °C (4647.5 units/mg).]

bonyl groups were slightly different. Accordingly, the catalytic groups in the active site of the corresponding proteinases from different sources might be aspartate residues, but the environment around this residue was not similar. As indicated in Figure 5, the cathepsin D from tilapia was very stable at the reaction temperature around 25–45 °C and had an optimum temperature of 37 °C, which is lower than 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., 1983; Draper and Zeece, 1989). There was a rapid activity decrease at reaction temperatures lower than 20 °C or higher than 45 °C.

When the preincubation temperature was 45 °C, 70% of the Hb hydrolytic acivity of proteinase still remained. However, it decreased rapidly when the temperature was higher than 45 °C (Figure 6). This proteinase was almost completely inactivated during a 30-min preincubation at 65 °C. After the cathepsin D was incubated at 50 °C for 30 min, the Hb hydrolytic activity of that from porcine spleen, rat spleen, and carp muscle remained at 60–70% (Cunningham and Tang, 1976; Yamamoto et al., 1979; Doke et al., 1980; Makinodan et al., 1982; Draper and Zeece, 1989), while that from tilapia remained at only 50% (Figure 6). This suggested that the thermostability of cathepsin D from tilapia was lower than that from carp, rat spleen, and porcine spleen.

Although almost no Hb hydrolytic activity of cathepsin D was detected at pHs higher than 6.0 (Figure 4), the pepstatin-sensitive proteases (mainly cathepsin D) hydrolyzed the α-actinin of tilapia muscle fibrils at pH 6.0 (Jiang et al., 1990). The pH of the environmental conditions and types of substrate would affect the hydrolytic capability of this proteinase. From these studies

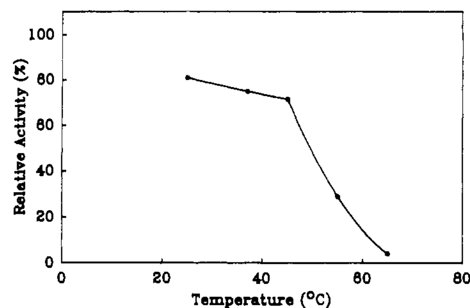

Figure 6. Heat inactivation of cathepsin D at various temperatures. [Proteinase in McIlvaine buffer, pH 3.5, was incubated at various temperatures for 30 min. After the proteinase was cooled to 0 °C, the Hb hydrolytic activity was measured; the relative activity is expressed as percentage ratio of the specific activity (units/milligram) of cathepsin D after 30 min of preincubation at various temperatures to that without preincubation (4649.7 units/mg).]

using acid-denatured Hb and muscle fibrils as substrate, the substrate effect may be greater than the pH effect when the hydrolysis occurs in muscle. According to the data obtained in this study, the divalent metals, except Hg^{2+}, significantly activated this proteinase (Table III). This suggested the feasibility of using chelating agents to improve the keeping quality of post-mortem fishes.

LITERATURE CITED

Andrew, P. The gel-filtration behavior of proteins related to their molecular weights over a wide range. *Biochem. J.* **1965**, *96*, 595–606.

Asghar, A.; Bhatti, A. R. Endogenous proteolytic enzymes in skeletal muscle: Their significance in muscle physiology and during postmortem aging events in carcasses. *Adv. Food Res.* **1987**, *31*, 343–451.

Asghar, A.; Henrickson, R. L. Postmortem stimulation of carcasses: Effects on biochemistry, biophysics, microbiology and quality of meat. *Crit. Rev. Food Sci. Nutri.* **1982**, *18*, 1–58.

Beeley, J. G. Physico-chemical characterization. In *Glycoprotein and Proteoglycan Techniques*; Elsevier Science (Biomedical Division): Amsterdam, 1985; pp 63–99.

Bond, J. S.; Butler, P. E. Intracellular proteases. *Annu. Rev. Biochem.* **1987**, *56*, 333–364.

Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

Chewale, J. G.; Takahashi, T.; Tang, J. The primary structure of cathepsin D and the implications for its biological functions. In *Aspartic Proteinases and their Inhibitors*; Kostka, V., Ed.; de Gruyter: Berlin, 1985; pp 101–116.

Cunningham, M.; Tang, J. Purification and properties of cathepsin D from porcine spleen. *J. Biol. Chem.* **1976**, *251*, 4528–4536.

Doke, S. N.; Ninjoor, V.; Nadkarni, G. B. Characterization of cathepsin D. from skeletal muscle of fresh water fish, *Tilapia mossambica*. *Agric. Biol. Chem.* **1980**, *44*, 1521–1528.

Draper, A. M.; Zeece, M. G. Thermal stability of cathepsin D. *J. Food Sci.* **1989**, *54*, 1651–1652.

Goll, D. E.; Otsuka, Y.; Nagainis, P. A.; Shannon, J. D.; Sathe, S. K.; Muguruma, M. Role of muscle proteinases in maintenance of muscle integrity and mass. *J. Food Biochem.* **1983**, *7*, 137–141.

Iodice, A. A.; Leong, V.; Weinstock, L. M. Separation of cathepsin A and D of skeletal muscle. *Arch. Biochim. Biophys.* **1966**, *117*, 477–486.

Jiang, S. T.; Wang, Y. T.; Gau, B. S.; Chen, C. S. Role of pepstatin-sensitive proteases on the postmortem changes of tilapia muscle myofibrils. *J. Agric. Food Chem.* **1990**, *38*, 1664–1668.

Klee, C. B. Interaction of calmodulin with Ca^{++} and target proteins. In *Molecular Aspects of Cellular Regulations; Calmodulin*; Cohen, P., Ed.; Elsevier: Amsterdam, 1988; Vol. 5, Chapter 3, pp 35–56.

Koohmaraie, M.; Schollmeyer, J. E.; Dutson, T. R. Effect of low-calcium-requiring calcium activated factor on myofibrils under varying pH and temperature conditions. *J. Food Sci.* **1986**, *51*, 28–32.

Koohmaraie, M.; Babiker, A. S.; Merkel, R. A.; Dutson, T. R. Role of Ca-dependent proteases and lysosomal enzymes in post-mortem changes in bovine skeletal muscle. *J. Food Sci.* **1988a**, *53*, 1253–1257.

Koohmaraie, M.; Babiker, A. S.; Schroeder, A. L.; Merkel, R. A.; Dutson, T. R. Acceleration of postmortem tenderization in ovine carcasses through activation of Ca-dependent proteases. *J. Food Sci.* **1988b**, *53*, 1638–1641.

Laemmli, U. K.; Favre, M. Maturation of the head of bacteriophage T4. I. DNA packaging events. *J. Mol. Biol.* **1973**, *80*, 575–599.

Leach, B. S.; Collawn, J. F.; Fish, W. W. Behavior of glycopolypeptides with empirical molecular weight estimation methods (1). In sodium dodecyl sulfate. *Biochemistry* **1980a**, *19*, 5734–5741.

Leach, B. S.; Collawn, J. F.; Fish, W. W. Behavior of glycopolypeptides with empirical molecular weight estimation methods (2). In random coil producing solvents. *Biochemistry* **1980b**, *19*, 5741–5747.

Lowry, H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.

Makinodan, Y.; Ikeda, S. Studies on fish muscle proteases VI. Separation of carp muscle cathepsins A and D, and some properties of carp muscle cathepsin A. *Bull. Jpn. Soc. Sci. Fish.* **1976**, *42*, 239–247.

Makinodan, Y.; Kyaw, N. N.; Ikeda, S. Intracellular distribution of carp muscle cathepsin D and electron micrograph of muscle lysosomal fraction. *Bull. Jpn. Soc. Sci. Fish.* **1982**, *48*, 1501–1509.

Matsumoto, T.; Okitani, A.; Kitamura, Y.; Katoh, H. Mode of degradation of myofibrillar proteins by rabbit muscle cathepsin D. *Biochim. Biophys. Acta* **1983**, *755*, 76–80.

Rabilloud, T.; Carpentier, G.; Tarroux, P. Improvement and simplification of low-background silver staining of proteins by using sodium dithionite. *Electrophoresis* **1988**, *9*, 288–291.

Takeda, M.; Ueno, E.; Kato, Y.; Yamamoto, K. Isolation and catalytic and immunochemical properties of cathepsin D-like acid proteinase from rat erythrocytes. *J. Biochem.* **1986**, *100*, 1269–1277.

Turk, V.; Puizdar, V.; Lah, T.; Gubensek, F.; Kregar, I. Structural and denaturation studies of cathepsin D and the existence of its possible precursor. In *Proteinases and their Inhibitors*; Turk, V., Vitale, Lj., Eds.; Mladinska Knjiga; Ljubljana, 1981; pp 117–124.

Yamamoto, K.; Katsuda, N.; Kato, K. Affinity purification and properties of cathepsin E-like acid proteinase from rat spleen. *Eur. J. Biochem.* **1978**, 492, 499–508.

Yamamoto, K.; Katsuda, N.; Himeno, M.; Kato, K. Cathepsin D of rat spleen, affinity purification and properties of two types of cathepsin D. *Eur. J. Biochem.* **1979**, *95*, 459–467.

Yamamoto, K.; Takeda, M.; Yamamoto, H.; Tatsumi, M.; Kato, Y. Human erythrocyte membrane acid proteinase (EMAP): sidedness and relation to cathepsin D. *J. Biochem.* **1985**, *97*, 821–830.

Yonezawa, S.; Tanaka, T.; Miyauchi, T. Cathepsin E from rat Neutrophils: Its poperties and possible relations to cathepsin D-like and cathepsin E-like acid proteinases. *Arch. Biochem. Biophys.* **1987**, *256*, 499–508.

Zeece, M. G.; Katoh, K. Cathepsin D and its effects on myofibrillar proteins: A review. *J. Food Biochem.* **1989**, *13*, 157–178.

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