Purification and Characterization of Cathepsin B from Ordinary Muscle of Mackerel (*Scomber australasicus*)†

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Cathepsin B from mackerel muscle was purified to electrophoretic homogeneity by HiLoad DEAE-Sepharose, S Sepharose, and FPLC Superdex 75 chromatographies. The molecular weight (MW) of the purified enzyme was 28 000 estimated by SDS–polyacrylamide gel electrophoresis and Superdex 75 chromatography. The purified proteinase hydrolyzed Z-Phe-Arg-MCA and Z-Arg-Arg-MCA but did not hydrolyze Z-Arg-MCA and L-Arg-MCA. From the MW and substrate specificity analysis, it was, accordingly, identified as cathepsin B. The optimal pH and temperature of this proteinase for the activity assay were 6.5 and 55 °C, respectively. It was activated by dithiothreitol, cysteine, glutathione, and β-mercaptoethanol. The thiol-dependent proteolytic activity was substantially inhibited by E-64 [1-(L-*trans*-epoxysuccinylleucylamino-4-guanidinobutane], antipain, chymostatin, iodoacetic acid, leupeptin, tosyllysine chloromethyl ketone, Cu^{2+}, and Hg^{2+}. The inactivation rate constant (K_D) at 50 °C was 8.5×10^{-5} s^{-1}.

INTRODUCTION

The endogenous proteinases play an important role in the protein turnover and protein degradation of postmortem fish muscle. The properties of the proteinase existing in muscular tissues are good references for developing preservation and processing technologies for fish or shellfish. Calpain II (Jiang et al., 1991a), cathepsin D (Jiang et al., 1991b; Jiang et al., 1992; Jiang et al., 1993) and cathepsin L (Lee et al., 1993) have already been purified and well-characterized from tilapia, milkfish, mackerel, grass shrimp, and banded shrimp. Cathepsin B (EC 3.4.22.1), a lysosomal cysteine proteinase, so far has been purified and characterized from the muscle of rat (Hardy and Pennington 1979), monkey (Hirao et al., 1984), gray mullet (Bonete et al., 1984), rabbit (Okitani et al., 1988), tilapia (Sherekar et al., 1988), carp (Hara et al., 1988a), and common mackerel (Matsumiya et al., 1989). In the purification of lysosomal cysteine proteinases from the extract of whole tissue, it is hard to separate successfully the enzyme from endogenous inhibitors, since the enzymes are frequently bonded with endogenous inhibitors during extraction. Ammonium sulfate precipitation at acid pH (Hirao et al., 1984) and acetone fractionation (Schwartz and Barrett, 1980) have been used to separate the enzyme from enzyme–inhibitor complex. Further purification are then carried out by various chromatographies such as Sephdex G-75 (Bonete et al., 1984), CM-cellulose (Sherekar et al., 1988), DEAE-Sephacel (Hara et al., 1988a), Sephadex G-100 (Okitani et al., 1988), and Sephacryl S-100 (Matsumiya et al., 1989).

Studies on the mechanism of postmortem tenderization of muscle by proteinases and investigations on cathepsins B and L have been increasing (Schwartz and Bird, 1977; Yates et al., 1983; Locker and Wild, 1984; Orcutt and Dutson, 1985; Asghar and Bhatti, 1987), since both enzymes have optimal pH around the postmortem condition of animal tissues (Matsukura et al., 1981; Noda et al., 1981; Ouali et al., 1987; Etherington et al., 1987; Mikami et al., 1987; Lee et al., 1993). In the previous study (Lee et al., 1993), cathepsin L was purified from mackerel muscle. Susceptibilities of muscle proteins against mackerel cathepsins B and L are being investigated, with the attempt to clarify the roles of both proteinases in postmortem tenderization of fish muscle. This study aims to purify and characterize the cathepsin B from the mackerel acetone powder.

MATERIALS AND METHODS

Materials. DEAE-Sepharose Fast Flow, S Sepharose Fast Flow, Superdex 75 HR 10/30 column, and electrophoresis and gel filtration calibration kits were purchased from Pharmacia (Uppsala, Sweden). Acrylamide, bis(acrylamide), Coomassie brilliant blue G-250, and dithiothreitol (DTT) were obtained from Merck (Darmstadt, Germany). Glutathione (GSH), β-mercaptoethanol (β-Me), cysteine, antipain, chymostatin, 1-(L-*trans*-epoxysuccinylleucylamino-4-guanidinobutane (E-64), iodoacetic acid (IAA), leupeptin, *N*-ethylmaleimide (NEM), *p*-(chloromercuri)benzoate (*p*CMB), pepstatin, phenylmethanesulfonyl fluoride (PMSF), soybean trypsin inhibitor, tosyllysine chloromethyl ketone (TLCK), and tosylphenylalanine chloromethyl ketone (TPCK) were products of Sigma (St. Louis, MO). Dye reagent concentrate for protein assay was obtained from Bio-Rad (Richmond, CA). (Benzyloxycarbonyl)arginylarginine-7-(4-methyl)-coumarylamide (Z-Arg-Arg-MCA), Z-Phe-Arg-MCA, Z-Arg-MCA, and L-Arg-MCA were purchased from Peptide Institute Inc. (Osaka, Japan).

Preparation of Acetone Powder. Mackerel (*Scomber australasicus*) was obtained from a fisheries market in northern Taiwan. Fish samples were caught from the Pacific Ocean and iced for 3 days before experiment. The dorsal muscle was removed and minced. Ten volumes of chilled acetone (around −40 °C) was added to the minces. After 2 min of homogenizing, samples were filtered using a funnel filter. The residues were resuspended in 3 volumes of chilled acetone, homogenized, and filtered twice. Finally, the filtrates were collected and kept in a ventilation hood for 2–4 h to eliminate acetone. The acetone powder was then packed in a polyethylene bag and stored at −40 °C until use.

HiLoad DEAE-Sepharose Chromatography. Crude enzymes were extracted from acetone powder using 12.5 volumes of 20 mM sodium acetate buffer, pH 5.5, containing 5 mM β-Me and 1 mM ethylenediaminetetraacetic acid (EDTA). After 30 min of centrifuging at 25000*g*, the supernatant was filtered using a filter paper (Toyo No. 5B). The resulting sample was then

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loaded on a DEAE-Sepharose column (5.0 × 22 cm) which was equilibrated with 20 mM sodium acetate buffer, pH 5.5, containing 1 mM EDTA (buffer I). The column was then washed with about 10 bed volumes of buffer I and eluted at a linear gradient of 0–0.5 M NaCl in buffer I. Fractions of 20 mL were collected using a fraction collector (RediFrac) at a flow rate of 10 mL/min. The main fractions with Z-Phe-Arg-MCA hydrolytic activity were collected and further purified with an S Sepharose column.

HiLoad S Sepharose Chromatography. The enzyme solution with Z-Phe-Arg-MCA hydrolytic activity on the DEAE-Sepharose column was concentrated and equilibrated against 20 mM sodium acetate buffer, pH 4.5 (buffer II), using an Amicon ultrafiltration over a YM10 membrane (cutoff 10 000). The resulting sample was applied to an S Sepharose column (1.6 × 22 cm) which was equilibrated with buffer II. The column was then washed with about 5 bed volumes of buffer II and eluted with a linear gradient of 0–0.7 M NaCl in buffer II. Fractions of 3 mL were collected with a fraction collector (RediFrac) at a flow rate of 2 mL/min. Fractions with Z-Phe-Arg-MCA hydrolytic activity were collected and further purified by a Superdex 75 HR column.

FPLC Superdex 75 Chromatography. Fractions with hydrolytic activity against Z-Phe-Arg-MCA obtained from the S Sepharose column were concentrated and equilibrated against 20 mM sodium phosphate buffer, pH 6.5, containing 0.1 M NaCl and 0.5 mM NaN_3 (buffer III) using Amicon ultrafiltration. The resulting sample was chromatographed on a Superdex 75 HR 10/30 column. Elution was performed using buffer III at a flow rate of 30 mL/h. Fractions of 0.25 mL were collected using a fraction collector (FRAC-100). Fractions with Z-Phe-Arg-MCA hydrolytic activity were pooled and subjected to the determination of molecular weight.

Determination of Molecular Weight. The molecular weight (MW) of the purified proteinase was estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Superdex 75 HR gel filtration. SDS–PAGE was conducted according to the method of Laemmli (1970). The acrylamide concentrations of the resolving and stacking gels were 15% and 3.75%, respectively. After electrophoretic running, the gels were fixed, stained, and destained according to the procedure of Neuhoff et al. (1988). Phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and α-lactalbumin (14 400) were used as standard markers. The Superdex 75 HR 10/30 column was equilibrated and eluted with buffer III at a flow rate of 30 mL/h. Ribonuclease A (13 700), chymotrypsinogen A (25 000), ovalbumin (43 000), and albumin (67 000) were used as standard markers.

Assay of Enzyme Activity. The enzyme activity was measured using Z-Phe-Arg-MCA as a substrate (Barrett and Kirschke, 1981). The Z-Phe-Arg-MCA was dissolved in 0.4 M sodium phosphate buffer, pH 6.0, containing 4 mM EDTA and 8 mM cysteine. After 10 min of incubation at 40 °C, the reaction was terminated by the addition of 0.1 M sodium acetate buffer, pH 4.3, containing 0.1 M sodium monochloroacetate. The supernatant were collected by centrifugation at 4 000*g* for 15 min and measured by the intensity of fluorescence at an excitation of 350 nm and an emission of 460 nm. One unit of enzyme activity was expressed as the amount of enzyme that can hydrolyze Z-Phe-Arg-MCA and release 1 μmol aminomethylcoumarin within 1 min of reaction at 40 °C. The hydrolytic activities of the purified enzyme (26 ng, 3845 units/mg) for Z-Arg-Arg-MCA, Z-Arg-MCA, and L-Arg-MCA were also determined in the same assay condition.

Determination of Protein Concentration. Protein concentration was determined using the protein–dye binding method of Bradford (1976). Bovine serum albumin was used as a standard protein.

Optimal pH. Purified enzymes in 0.1 M citric acid–0.2 M sodium phosphate buffer, pH 2.5–7.0, and 0.2 M Tris-HCl buffer, pH 7.0–8.5, containing 4 mM EDTA and 8 mM cysteine were incubated with 20 μM Z-Phe-Arg-MCA at 40 °C for 10 min. The intensity of fluorescence of the reaction mixture was measured according to the method of Barrett and Kirschke (1981). The relative activity was expressed as a percentage ratio of the activity measured at various pH values to that with maximum activity.

pH Stability. Purified enzymes in 0.1 M citric acid–0.2 M sodium phosphate buffer, pH 2.5–7.0, and 0.2 M Tris-HCl buffer, pH 7.0–8.5, were incubated at 30 °C for 60 min. After the resultant samples were cooled to 5 °C using ice–water, the residual activity was measured according to the method of Barrett and Kirschke (1981). The remaining activity was expressed as a percentage ratio of the activity of samples incubated at various pH values to that of sample with maximum activity.

Optimal Temperature. Purified enzymes in 0.4 M sodium phosphate buffer, pH 6.0, containing 4 mM EDTA and 8 mM cysteine were incubated with 20 μM Z-Phe-Arg-MCA at various temperatures (5–70 °C) for 10 min. The hydrolytic activity was measured according to the method of Barrett and Kirschke (1981) and expressed as a percentage ratio of the activity measured at various temperatures to that with maximum activity.

Thermal Stability. Purified enzymes in 20 mM sodium phosphate buffer, pH 6.5, were incubated at various temperatures (45–65 °C) for 25 s to 4 h. At definite time intervals, the enzyme solutions were cooled immediately in iced water for 10 min. The remaining activity was measured according to the method of Barrett and Kirschke (1981). The inactivation rate constant (K_D) of the purified proteinase incubated at various temperatures was calculated as follows: $K_D = (\ln A_0 - \ln A_t)/t$, where A_0 and A_t are activities before and after t-s incubation). The activation energy (E_a) was obtained from the slopes (slope = $-E_a/R$) of the inactivation curves in an Arrhenius plot. The thermodynamic parameters were calculated according to Eyring transition-state theory. ΔH^*, ΔG^*, and ΔS^* were calculated using the equations

$$\Delta H^* = E_a - RT \quad (1)$$

$$\Delta G^* = RT(\ln K_b/h + \ln T - \ln K_D) \quad (2)$$

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T \quad (3)$$

where ΔH^*, ΔG^*, and ΔS^* are enthalpy, free energy, and entropy changes, respectively; R, K_b, and h are the gas constant (1.98 cal/deg), Boltzmann constant (1.38×10^{-16} erg/deg), and Plank constant (6.62×10^{-27} erg·s), respectively; and T is the absolute temperature (K).

Effect of Reductants. Purified enzymes in 20 mM sodium phosphate buffer, pH 6.0, were incubated with 0–5 mM cysteine, DTT, GSH, and β-Me at 40 °C for 10 min. The Z-Phe-Arg-MCA hydrolytic activity of the samples was measured according to the method of Barrett and Kirschke (1981).

Effect of Inhibitors. Purified enzymes in 20 mM sodium phosphate buffer, pH 6.0, were incubated with 0.1 mM antipain, iodoacetic acid, NEM, PCMB, pepstatin, PMSF, soybean trypsin inhibitor, TLCK, and TPCK, 0.02 mM leupeptin, and 0.01 mM chymostatin and E-64 at 40 °C for 10 min. The remaining activity of the resulting samples was measured according to the method of Barrett and Kirschke (1981).

Effect of Metal Ions. Purified enzyme in 20 mM phosphate buffer, pH 6.0, was incubated with 1 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 40 °C for 10 min. The remaining activity of the resulting samples was measured according to the method of Barrett and Kirschke (1981). The relative activity was expressed as a percentage ratio of the activity of samples incubated with various metals to that without metal.

RESULTS AND DISCUSSION

Purification. Cathepsin B was purified from acetone powder of mackerel dorsal muscle using HiLoad DEAE-Sepharose (Figure 1), S Sepharose (Figure 2), and FPLC Superdex 75 (Figure 3) chromatographies. The purification of cathepsin B is summarized in Table 1. About 14 000-fold purification was achieved over the crude extract of acetone powder. The yield is 44.6 μg/100 g of mackerel acetone powder (or 10.3 μg/100 g of mackerel muscle). After the major contaminated proteins were washed out of the DEAE-Sepharose column, two peaks with Z-Phe-Arg-MCA hydrolytic activity appeared on the eluting fractions (Figure 1). During the purification of carp cathepsin B by CM-cellulose chromatography (Hara et al., 1988a), there were also two peaks with benzoyl-DL-Arg

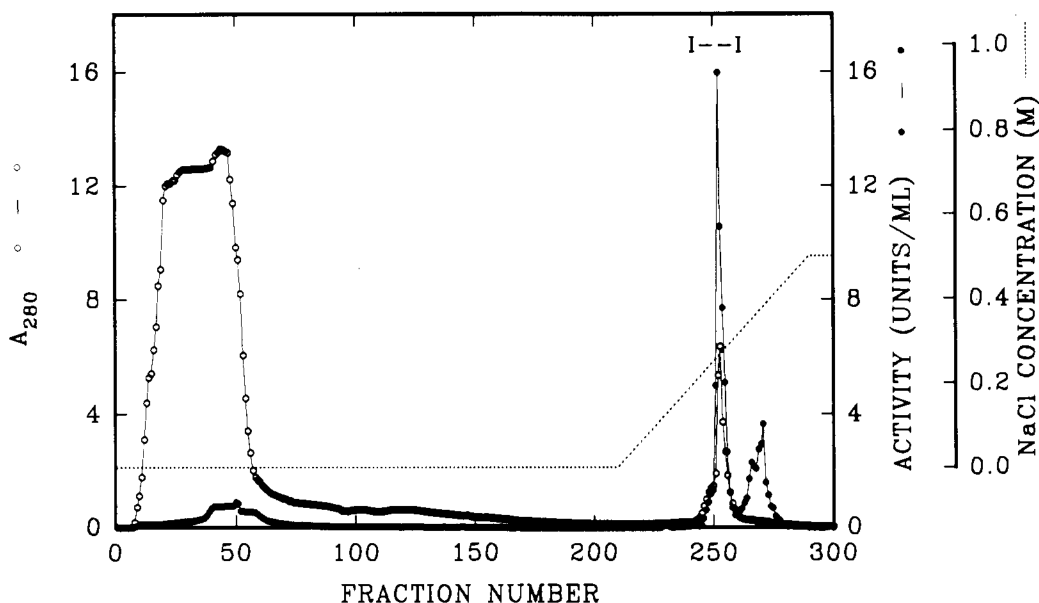

Figure 1. HiLoad DEAE-Sepharose chromatography of the crude enzyme extracted from acetone powder of mackerel muscle. The crude enzyme (910 mL) was applied to a DEAE-Sepharose column (5.0 × 22 cm) equilibrated with 20 mM sodium acetate buffer, pH 5.5, containing 1 mM EDTA and 5 mM β-Me (buffer I). The column was then washed with buffer I and eluted at a linear gradient of 0–0.5 M NaCl in buffer I. Fractions of 20 mL were collected at a flow rate of 10 mL/min. (○) Absorbance at 280 nm; (●) activity (units/mL); (- - -) NaCl concentration.

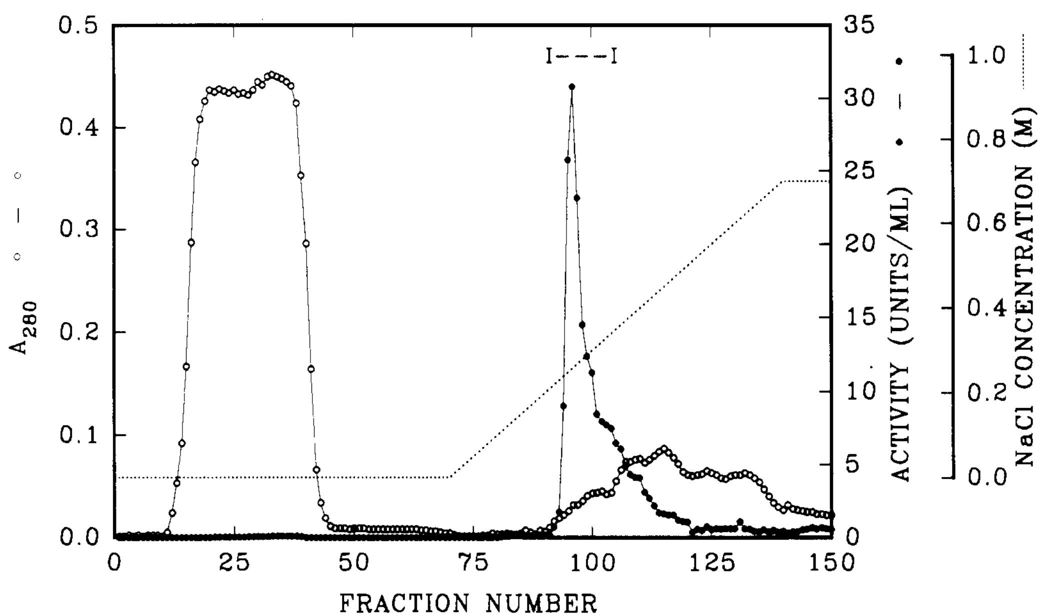

Figure 2. HiLoad S Sepharose chromatography of the fractions with Z-Phe-Arg-MCA hydrolyzing activity after DEAE-Sepharose column. The enzyme solution was concentrated and equilibrated against 20 mM sodium acetate buffer, pH 4.5 (buffer II), using an Amicon ultrafiltration. The resulting sample was applied to an S Sepharose column (1.6 × 22 cm) equilibrated with buffer II. The column was then washed with buffer II and eluted using a linear gradient of 0–0.7 M NaCl in buffer II. Fractions of 3 mL were collected at a flow rate of 2 mL/min. (○) Absorbance at 280 nm; (●) activity (units/mL); (- - -) NaCl concentration.

β-naphthylamide hydrolytic activity. The main fractions (tubes 217-258) with Z-Phe-Arg-MCA hydrolytic activity were collected. Since the activity peak came out before the protein peak, the p*I* of the enzyme might be higher than that of the contaminated proteins. After the pH of the resulting sample was adjusted to 4.5 and the sample was chromatographed on a S Sepharose column, the predominant contaminated proteins were washed out by using buffer II, and only one peak with Z-Phe-Arg-MCA hydrolytic activity was eluted at about 0.3 M NaCl on S Sepharose chromatography (Figure 2). The fractions from tubes 92–104 were collected, concentrated, and applied to a Superdex 75 column. The fractions from tubes 44–49 on Superdex 75 chromatography (Figure 3) were pooled. It appeared as a single protein band on SDS–PAGE (Figure 4). Although some affinity chromatographies such as Sepharose 4B-α-*N*-benzoyl-L-argininamide (Ogino and Nakashima, 1974), agarose–hemoglobin (Bonete et al., 1984), Sepharose–Ahx-Gly-Phe-Glysc (Rich et al., 1986), peptide-conjugated Sepharose (Okitani et al., 1988), and organomercurial agarose (Sherekar et al., 1988) have been employed to replace ion-exchange chromatography for the isolation of cathepsin B, ion-exchange chromatography was still the most effective method for the purification of cathepsin B. There was also a high-performance liquid chromatography procedure with ionic exchanger used in the simultaneous isolation of cathepsins B, H, and L from human liver (Dalet-Fumeron et al., 1991).

Substrate Specificity. The purified enzyme could hydrolyze Z-Phe-Arg-MCA and Z-Arg-Arg-MCA but did not hydrolyze Z-Arg-MCA and L-Arg-MCA (Table 2). The substrate specificity of the purified enzyme was similar to that of the cathepsin B from common mackerel (Matsumiya et al., 1989). Although Barrett and Kirschke (1981)

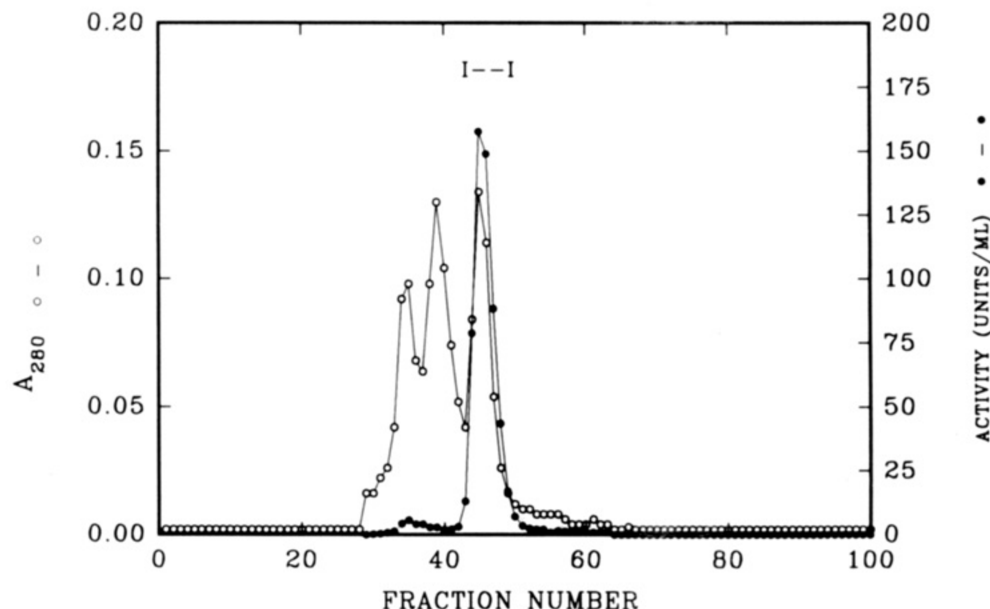

Figure 3. FPLC Superdex 75 chromatography of the fractions with Z-Phe-Arg-MCA hydrolytic activity obtained from the S Sepharose column. The enzyme solution was concentrated and equilibrated against 20 mM sodium phosphate buffer, pH 6.5, containing 0.1 M NaCl (buffer III) by Amicon ultrafiltration. The resulting sample was applied to a Superdex 75 HR 10/30 column. Elution was performed using buffer III at a flow rate of 30 mL/h. Fractions of 2.5 mL were collected.

Table 1. Purification of Cathepsin B from Mackerel

procedure	total protein (mg)	total act. (units)	sp act. (units/mg)	yield (%)	purification (fold)
crude extract	11230.31	2798.25	0.249	100.0	1
DEAE-Sepharose	44.33	946.20	21.344	33.8	86
S Sepharose	0.24	329.16	1371.500	11.8	5508
Superdex 75	0.04	139.40	3485.000	5.0	13996

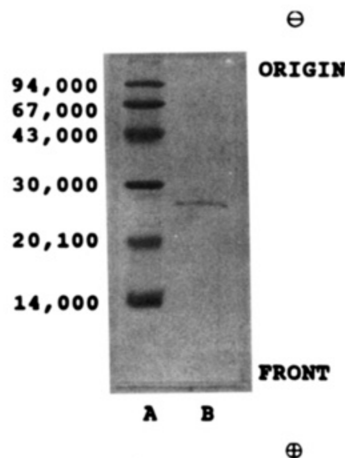

Figure 4. Electrophoretic pattern of cathepsin B using 15% sodium dodecyl sulfate–polyacrylamide electrophoresis. (A) Standard; (B) cathepsin B.

Table 2. Substrate Specificity of Cathepsin B

substrate[a] (5 μM)	act. (units mL^{-1} min^{-1})	substrate[a] (5 μM)	act. (units mL^{-1} min^{-1})
Z-Phe-Arg-MCA	4.065	Z-Arg-MCA	–[b]
Z-Arg-Arg-MCA	1.355	L-Arg-MCA	–[b]

[a] Z, benzyloxycarbonyl; MCA, 4-methyl-7-coumarylamide. [b] Not detected.

recommended that the substrates Z-Arg-Arg-MCA, Arg-MCA, and Z-Phe-Arg-MCA be used for the determination of the activities of cathepsins B, H, and L, respectively, Z-Arg-Arg β-naphthylamide, Z-Phe-Arg-MCA, and Z-Arg-Arg-MCA have long been used to determine the activity of cathepsin B (Barrett, 1980; Knight, 1980). According to our previous experiment, since the Z-Phe-Arg-MCA was more sensitive than Z-Arg-Arg-MCA for the purified enzyme (Table 2), it was used as substrate in this study. This substrate was also used to determine the activity of monkey cathepsin B (Hirao et al., 1984). Although the specificity of the endopeptidase activity of cathepsin B is still unclear, that from rat liver (Towatari and Katunuma, 1983), monkey muscle (Hirao et al., 1984), and carp muscle (Hara et al., 1988a) revealed dipeptidyl carboxypeptidase activity. Investigation of the specificity of the endopeptidase activity of the purified cathepsin B is ongoing using various specific peptides and proteins.

Molecular Weight. The MW of purified proteinase was estimated to be 28 000 by SDS–PAGE and Superdex 75 chromatography (data not shown). The MW of muscle cathepsin B was reported to be 25 000 from rat (Hardy and Pennington, 1979) and gray mullet (Bonete et al., 1984), 24 000 from monkey (Hirao et al., 1984), 23 500 from tilapia (Sherekar et al., 1988), 29 000 from carp (Hara et al., 1988a), 27 000 from rabbit (Okitani et al., 1988), and 23 000 from common mackerel (Matsumiya et al., 1989). The MW of the purified enzyme was similar to that of the enzyme purified from carp and rabbit muscles. However, the enzyme purified from porcine spleen was found to be a dimer with 22 000 and 5000 subunits (Takahashi et al., 1984). According to the above data, the cathepsin B enzymes purified from various sources were monomer or/and dimer with MW from 23 000 to 29 000.

According to the study on porcine liver (Takahashi et al., 1979), there were three forms of cathepsin B with identical MW but different p*I*. On the SDS–PAGE analysis, form III appeared as a single band at 29 000, while forms I and II appeared as three bands at 29 000, 25 000, and 4000. The authors conjectured that forms I and II might result from the intracellular processing of form III. Hara et al. (1988b) proved that cysteine proteinase inhibitors, such as E-64 and leupeptin, could inhibit the proteolysis of the single-chain form of cathepsin B into the two-chain form in the intracellular process using

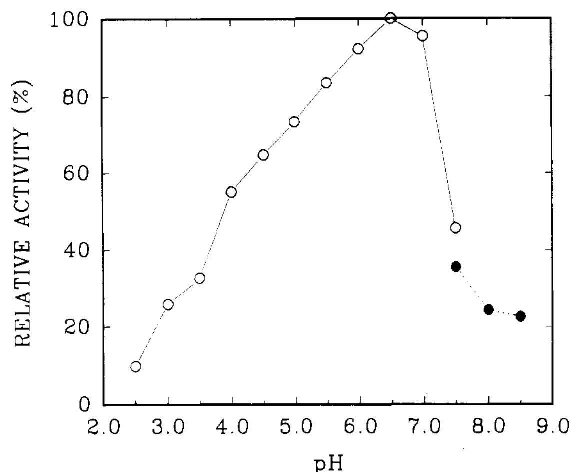

Figure 5. Effect of pH on Z-Phe-Arg-MCA hydrolyzing activity of cathepsin B.

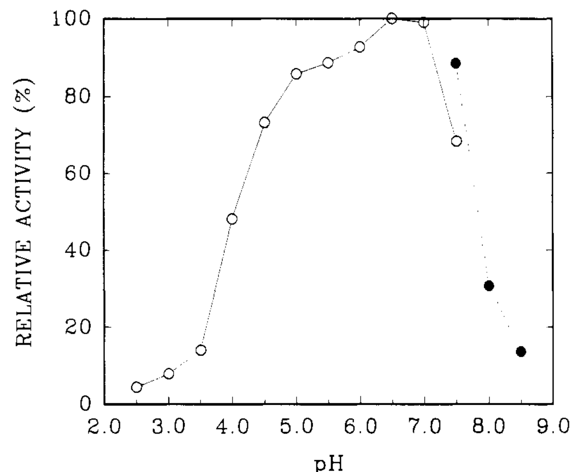

Figure 6. pH stability of cathepsin B.

pulse-chase experiments. In the investigation of the minor peak with Z-Phe-Arg-MCA hydrolytic activity (tubes 270-280) observed on DEAE-Sepharose chromatography in this study (Figure 1), a fraction isolated after S Sepharose and Superdex 75 chromatographies was obtained and showed four bands with MW of 33 000, 28 000, 25 000, and 20 000 on SDS-PAGE (data not shown). The further purification of this minor peak is ongoing. Accordingly, the type of isolated cathepsin B might be dependent on the purification conditions in vitro.

pH Optimum and Stability. The optimal pH of the purified cathepsin B for the hydrolysis of Z-Phe-Arg-MCA was 6.5 (Figure 5), which was similar to that of cathepsin B from monkey and rabbit muscles (Hirao et al., 1984; Okitani et al., 1988). According to Matsumiya et al. (1989), the optimal pH of cathepsin B from common mackerel muscle (*Scomber japonicus*) was 5.5 (Matsumiya et al., 1989), which was more acidic than that from mackerel (*S. australasicus*). Although these two mackerel are of the same genus, the MW and optimal pH of cathepsin B from these two species were quite different and the substrate specificity was similar. The optimal pH values of some cathepsin B were 6.0 for that from porcine kidney (Takahashi et al., 1981), human kidney (Baricos et al., 1988), gray mullet muscle (Bonete et al., 1984), tilapia muscle (Sherekar et al., 1988), and carp muscle (Hara et al., 1988a) and 6.2 for that from rabbit liver (Ogino and Nakashima, 1974). The purified proteinase was stable at pH 4.5–7.5 and most stable at pH 6.5 (Figure 6). It remained almost 100% activity at pH 7.0 even after 60 min of incubation at 30 °C. In addition, over 50% of the activity of that from carp (Hara et al., 1988a) was retained after 20 min of incubation at 40 °C, pH 6.5. These data indicate that the cathepsin B is still active at the physiological and postmortem pH of fish muscle. This phenomenon suggested that it might be involved in the tenderization of postmortem muscle.

Optimal Temperature and Thermal Stability. The optimal temperature for the hydrolysis of Z-Phe-Arg-MCA of the purified proteinase was 55 °C (Figure 7). This temperature was higher than that of carp (Hara et al., 1988a) and tilapia (Sherekar et al., 1988), which were reported to be 45 and 42 °C, respectively. The inactivation energy calculated from the inactivation curve in an Arrhenius plot (data not shown) was 67.76 kcal/mol. The thermal denaturation rate constant of this proteinase was 8.5×10^{-5} s^{-1} at 50 °C (Table 3), while those of calpain II of tilapia, cathepsin L-like proteinase, and cathepsin L of mackerel (Lee et al., 1993) were 3.0×10^{-4} s^{-1} (1.8×10^{-2} min^{-1}), 6.9×10^{-4} s^{-1}, and 5.1×10^{-5} s^{-1} at 50 °C, respectively.

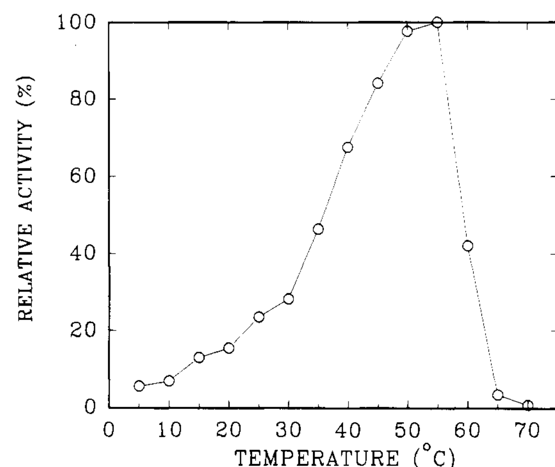

Figure 7. Effect of temperature on Z-Phe-Arg-MCA hydrolyzing activity of cathepsin B.

Table 3. Thermodynamic Parameters for Thermal Inactivation of Cathepsin B

temp (°C)	K_D[a] × 10^6 (s^{-1})	ΔG^*[b] (kcal/mol)	E_a[c] (kcal/mol)	ΔS^*[d] (cal/K·mol)	ΔH^*[e] (kcal/mol)
45	1.5	26.71	67.76	127.11	67.13
50	8.5	26.02	67.76	127.24	67.12
55	52.9	25.25	67.76	127.62	67.11
60	155.0	24.93	67.76	126.64	67.10
65	989.0	24.08	67.76	127.25	67.09

[a] K_D, thermal denaturation rate constant. [b] ΔG^*, change in free energy. [c] E_a, activation energy. [d] ΔS^*, change in entropy. [e] ΔH^*, change in enthalpy.

The thermal stability of the purified cathepsin B was higher than that of calpain II of tilapia and cathepsin L-like proteinase of mackerel but close to that of cathepsin L of mackerel. Accordingly, cathepsins B and L might be more stable than calpain II and cathepsin L-like proteinase and play an important role in postmortem tenderization.

Effect of Reductants. The hydrolytic activity of the purified enzyme was very low in the absence of reductants but effectively activated in the presence of β-Me, GSH, DTT, and cysteine (Table 4). Most cathepsin B purified from other species also needs reductants to activate (Hirao et al., 1984, 1988a; Okitani et al., 1988; Sherekar et al., 1988; Matsumiya et al., 1989). In addition to these thiol reagents, NaCl (Hara et al., 1988a) and phospholipid (Fazili and Qasim, 1986) were also reported to significantly increase the activity of cathepsin B of carp and buffalo liver, respectively. In this study, the activity of the purified cathepsin B was not enhanced by NaCl (0.1–0.9 M) (data not shown).

Table 4. Effect of Reductants on Cathepsin B

concn (mM)	proteolytic act. (μmol mL^{-1} min^{-1})			
	cysteine	DTT[a]	GSH[b]	β-Me[c]
0.0	0.245	0.245	0.245	0.245
1.0	4.350	6.885	1.265	2.110
2.0	5.540	7.605	1.600	3.430
3.0	6.130	7.150	1.925	3.525
4.0	6.545	7.895	2.075	4.410
5.0	7.045	7.835	2.365	4.695

[a] DTT, dithiothreitol. [b] GSH, glutathione. [c] β-Me; β-mercaptoethanol.

Table 5. Effect of Various Inhibitors on Cathepsin B

inhibitor	final concn (mM)	inhibition (%)
antipain	0.1	93.4
chymostatin	0.01	92.7
E-64	0.01	97.9
iodoacetic acid	0.1	85.4
leupeptin	0.02	96.5
N-ethylmaleimide	0.1	0.0
p-(chloromercuri)benzoate	0.1	0.0
pepstatin	0.1	0.0
phenylmethanesulfonyl fluoride	0.1	0.0
soybean trypsin inhibitor	0.1	0.0
tosyllysine chloromethyl ketone	0.1	100.0
tosylphenylalanine chloromethyl ketone	0.1	0.0

Effect of Inhibitors. The purified proteinases were strongly inhibited by antipain, chymostatin, E-64, IAA, leupeptin, and TLCK but not by NEM, *p*CMB, pepstatin, PMSF, soybean trypsin inhibitor, and TPCK (Table 5). This was similar to cathepsin B from monkey (Hirao et al., 1984), carp (Hara et al., 1988a), and common mackerel (Matsumiya et al., 1989). However, that purified from common mackerel could also be completely or partially inhibited by 1 mM TPCK, NEM, and *p*CMB (Matsumiya et al., 1989). The cathepsin B from gray mullet (Bonete et al., 1984) was completely inhibited by 10 mM NEM, while that of tilapia (Sherekar et al., 1988) was moderately inhibited by 1 mM NEM and *p*CMB. Baici and Gyger-Marazzi (1982) reported that leupeptin was a slow but tight-binding inhibitor for cathepsin B from human spleen and rabbit liver. E-64 (Hanada et al., 1978) and IAA (North, 1989) are considered to be cysteine protease inhibitors, while leupeptin, antipain, TLCK, and chymostatin are serine and cysteine proteases (Umezawa and Aoyagi, 1983) and soybean trypsin inhibitor (Birk, 1976), TPCK (Salvesen and Nagase, 1989), and PMSF (North, 1989) are serine protease inhibitors. The pepstatin can inhibit most of the aspartic proteases (Umezawa, 1976). From the above data, the purified cathepsin B could be inhibited by E-64, IAA, leupeptin, antipain, TLCK, and chymostatin but could not be inhibited by soybean trypsin inhibitor, TPCK, and PMSF. The purified cathepsin B, accordingly, is considered to be cysteine proteinase.

Effect of Metal Ions. Cu^{2+} and Hg^{2+} greatly inhibited, while Mn^{2+}, Fe^{2+}, and Fe^{3+} partially inhibited, the purified cathepsin B (Table 6). Other metal ions (K^+, Na^+, Mg^{2+}, Sr^{2+}, Zn^{2+}, Ca^{2+}, Cd^{2+}, Ba^{2+}, Co^{2+}, Ni^{2+}) did not affect or partially increased the activity of purified cathepsin B (Table 6). Hg^{2+} and Cu^{2+} are high affinitive toward the SH groups on proteinase molecules. The inhibition by Hg^{2+} and Cu^{2+} further supported the inhibitor study conclusion that the purified cathepsin B is a cysteine proteinase. According to the studies on cathepsin B of porcine liver (Takahashi et al., 1979), carp muscle (Hara et al., 1988a), and common mackerel muscle (Matsumiya et al., 1989), these proteinases were also inhibited by Hg^{2+}.

From the effects of inhibitors (Table 5) and metal ions (Table 6), the purified proteinase was considered to be cysteine proteinase. According to the analysis of SDS–PAGE (Figure 4) and substrate specificity (Table 2), the purified proteinase was further identified as cathepsin B. Reductants were essential for this proteinase (Table 4). Therefore, degradation of myofibrillar proteins by cathepsin B might depend on the redox potential of the muscle. In general, the redox potentials of the postmortem muscle decreased from initial values of around +100 mV to −100 or even −200 mV (Ahn and Maurer, 1989). The activities of cathepsins B and L significantly increased when the redox potential was below −50 mM (Motilva et al., 1993). On the other hand, the level of cathepsin B of the postmortem muscle was also critical to the degradation of myofibrillar proteins by this proteinase. According to the study on cathepsins B, D, and L and calpains in electrically stimulated chicken muscle (Etherington et al., 1990), the activity of calpains decreased markedly, but the activity of cathepsins B and L did not decrease after 48 h of storage at 15 °C. Considering the optimal pH (Figure 5), pH stability (Figure 6), and thermal stability (Table 3) of the purified cathepsin B, the redox potential (Motilva et al., 1993), and proteinase levels (Etherington et al., 1990), the purified cathepsin B and the previously purified cathepsin L (Lee et al., 1993) might play important roles in the postmortem autolysis of mackerel.

Table 6. Effect of Metal Ions on Cathepsin B

metal ion[a] (1 mM)	rel act. (%)	metal ion[a] (1 mM)	rel act. (%)
none	100.0	Ba^{2+}	104.5
K^+	108.1	Mn^{2+}	68.1
Na^+	116.8	Co^{2+}	101.3
Mg^{2+}	118.3	Ni^{2+}	118.7
Sr^{2+}	120.9	Cu^{2+}	6.8
Zn^{2+}	100.6	Hg^{2+}	7.0
Ca^{2+}	98.7	Fe^{2+}	61.1
Cd^{2+}	99.6	Fe^{3+}	56.0

[a] The counterion of all metals was chloride.

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