Purification and Characterization of a Novel Cysteine Proteinase from Mackerel (*Scomber australasicus*)†

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A novel cysteine proteinase from mackerel muscle was purified to electrophoretic homogeneity using HiLoad DEAE-Sepharose, HiLoad S Sepharose, and FPLC Superdex 75 chromatographies. The molecular weight of purified proteinase was 99 000 estimated by Superose 12 gel filtration. This proteinase appeared as two protein bands of 35 000 (designated "a") and 23 000 (designated "b") on SDS−PAGE. This purified proteinase, accordingly, identified to be a trimer of the form a_2b. It hydrolyzed Z-Phe-Arg-MCA and Z-Arg-Arg-MCA but not Z-Arg-MCA and L-Arg-MCA. The optimal pH and temperature for the hydrolysis of Z-Arg-Arg-MCA were 6.0 and 35 °C, respectively. The proteinase was stable at pH 5.5−6.0 but was unstable when the pH was higher than 7.0. The inactivation rate constant (K_D) at 50 °C was 3.8×10^{-1} min^{-1}. This proteinase was activated by dithiothreitol, cysteine, glutathione, and β-mercaptoethanol. The thiol-dependent proteolytic activity was substantially inhibited by 1-(L-trans-epoxysuccinylleucylamino)-4-guanidinobutane, antipain, iodoacetic acid, leupeptin, tosyllysine chloromethyl ketone, Zn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+}, and Fe^{3+}. These results suggest that the cysteine residue is required for the catalytic activity of this proteinase.

Keywords: *Cysteine proteinase; purification; mackerel*

INTRODUCTION

The endogenous proteinases are considered to affect muscle tenderization. Accordingly, the purification and characterization of proteinases from animal muscles have extensively been studied. In fish and shellfish muscles, cathepsin A (Toyohara et al., 1982), cathepsin D (Bonete et al., 1984; Jiang et al., 1991b, 1992, 1993b), cathepsin B (Hara et al., 1988b; Sherekar et al., 1988; Matsumiya et al., 1989; Yamashita and Konagaya, 1990b; Jiang et al., 1994), cathepsin L (Yamashita and Konagaya, 1990a; Lee et al., 1993), calpain (Taneda et al., 1983; Jiang et al., 1991a; Wang et al., 1993), alkaline proteinase (Makinodan et al., 1987; Doke and Ninjoor, 1987), and some peptidases (Osnes and Mohr, 1985; Hara et al., 1988a) have already been identified and characterized. So far, calpains and cathepsins are recognized to be the major contributors to the post-mortem tenderization of muscle (Asghar and Bhatti, 1987; Etherington et al., 1987; Etherington et al., 1990; Jiang et al., 1992a; Wang et al., 1991). However, many unidentified proteinases also exist in the animal muscle. Purification and characterization of these unidentified proteinases should be helpful for the further understanding of post-mortem tenderization of fish muscle.

The post-mortem mackerel deteriorates rapidly at room temperature or even at refrigerated temperature. The autolysis of muscle proteins by endogenous proteinases might be one of the major factors affecting the quality of post-mortem mackerel. For understanding the mechanism of the post-mortem autolysis of mackerel, studies of the distribution, purification, and characterization of muscle proteinases in this species have been done by many researchers (Sakata et al., 1985; Mukundan et al., 1986; Ueno et al., 1986, 1990; Hara et al., 1987; Matsumiya et al., 1989; Matsumiya et al., 1991). In our previous works, cathepsin D (Jiang et al., 1993), cathepsin L and an L-like proteinase (Lee et al., 1993), and cathepsin B (Jiang et al., 1994) from mackerel have already been purified and characterized. The data suggested the implication of these proteinases in the post-mortem tenderization of fish muscle. During the purification of these proteinases, a novel cysteine proteinase with a molecular weight of 99 000 from mackerel was detected by the hydrolysis of Z-Arg-Arg-MCA. This study was to purify, characterize, and discuss the implication of its properties on the post-mortem muscle tenderization.

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MATERIALS AND METHODS

Materials. Mackerel (*Scomber australasicus*), iced for 5−6 h before experimentation, was obtained from a fisheries market in northern Taiwan. DEAE-Sepharose Fast Flow, S Sepharose Fast Flow, Superdex 75, and Superose 12 HR 10/30 prepacked columns, and calibration kits for electrophoresis and gel filtration were purchased from Pharmacia (Uppsala, Sweden). Acrylamide, *N,N'*-methylenebis(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 the 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).

HiLoad DEAE-Sepharose Chromatography. Crude enzyme was extracted from 80 g of mackerel acetone powder

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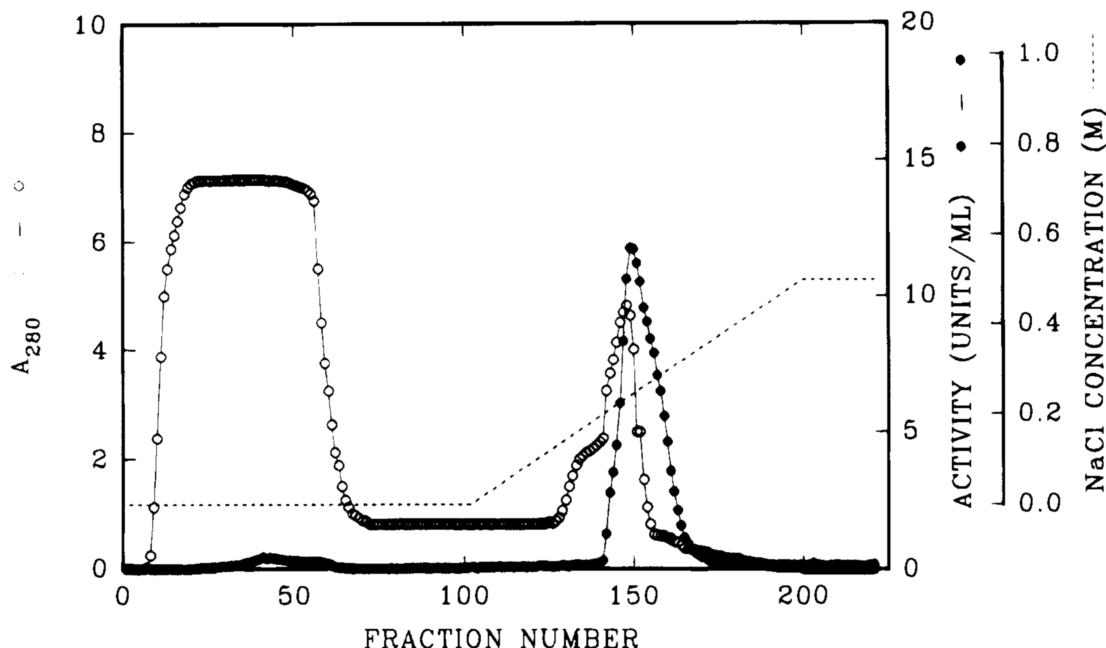

Figure 1. DEAE-Sepharose chromatography of the extract from acetone powder of mackerel ordinary muscle. Crude enzyme was extracted from acetone powder (80 g) using 12.5 volumes of 20 mM sodium acetate buffer (pH 5.5) containing 5 mM β-mercaptoethanol and 1 mM EDTA. After 30 min of centrifuging at 25000g, the supernatant was filtrated by a filter paper. The resulting sample was then 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 5 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 fractional collector (RediFrac) at a flow rate of 5 mL/min. Fractions with Z-Arg-Arg-MCA hydrolytic activity (tubes 145–161) were pooled: (○) absorbance at A_{280}; (●) enzyme activity; (---) NaCl concentration.

according to the method of Lee et al. (1993). The enzyme solution (about 850 mL) was then chromatographed on a DEAE-Sepharose column (5.0 × 22 cm) which was equilibrated previously with 20 mM sodium acetate buffer (pH 5.5) containing 1 mM EDTA (buffer I). After loading, the column was washed with about 10 bed volumes of buffer I and then eluted with 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 5 mL/min. Fractions with Z-Arg-Arg-MCA hydrolytic activity were collected. The extraction of crude enzymes and chromatography of HiLoad DEAE-Sepharose were performed twice to obtain more enzyme sample.

HiLoad S Sepharose Chromatography. Enzyme solution with Z-Arg-Arg-MCA hydrolytic activity on DEAE-Sepharose chromatography was concentrated and equilibrated against 20 mM sodium acetate buffer (pH 4.3) (buffer II) using an Amicon ultrafiltration over a YM10 membrane (cutoff, 10 000). The resulting sample was applied onto the S Sepharose column (1.6 × 22 cm) equilibrated previously with buffer II. The column was then washed with about 5 bed volumes of buffer II and eluted with a linear gradient of 0–0.8 M NaCl in buffer II. Fractions of 3 mL were collected using a fraction collector (RediFrac) at a flow rate of 1 mL/min. Fractions with Z-Arg-Arg-MCA hydrolytic activity were collected and further purified by Superdex 75 chromatography.

Superdex 75 Chromatography. Fractions with Z-Arg-Arg-MCA hydrolytic activity on S Sepharose chromatography were equilibrated with 20 mM phosphate buffer containing 0.1 M NaCl (pH 6.0) (buffer III), concentrated to 1 mL using a stirred cell Model 3, and further concentrated to 200 μL using a Microcone 10. The resulting sample was chromatographed on a Superdex 75 HR 10/30 column equilibrated previously with buffer III. 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-Arg-Arg-MCA hydrolytic activity were pooled and rechromatographed on a Superdex 75 column. Fractions with Z-Arg-Arg-MCA hydrolytic activity were pooled and subjected to the determination of enzymatic properties.

Molecular Weight. The molecular weight (MW) of the purified proteinase was determined by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and Superose 12 gel filtration. SDS–PAGE was conducted in a Laemmli buffer system (Laemmli, 1970). The polyacrylamide concentrations [acrylamide:bis(acrylamide) = 30:0.8 w/w] of stacking and resolving gels were 3.75 and 15%, respectively. After electrophoretic running, the gels were fixed, stained, and destained according to the 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 markers. A Superose 12 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), albumin (67 000), and aldolase (158 000) were used as markers.

Activity Stained by Z-Arg-Arg-MCA. The purified enzyme in 20 mM phosphate buffer (pH 6.0) (buffer IV) was applied on the nondissociating continuous PAGE with an ascending gradient from 5 to 20% polyacrylamide [acrylamide: bis(acrylamide) = 30:0.8 w/w]. The electrophoresis was performed in buffer IV at 100 V for 6 h. After electrophoretic running, 0.5 mL of reaction mixture containing 10 mM Z-Arg-Arg-MCA, 4 mM cysteine, and 1 mM EDTA was spread on the surface of gels. After a 10-min incubation at 25 °C, the active band with fluorescence was detected by ultraviolet light at 366 nm. The gel was finally fixed, stained, and destained according to the procedure of Neuhoff et al. (1988).

Enzyme Activity. The enzyme activity was measured using Z-Arg-Arg-MCA as substrate according to the method of Barrett and Kirschke (1981). The reaction was performed at 35 °C for 10 min. The hydrolytic specificity of the purified enzyme against Z-Phe-Arg-MCA, Z-Arg-MCA, and L-Arg-MCA was also determined using identical conditions. One unit of enzyme activity was expressed as the amount of the enzyme that can hydrolyze the substrate and release 1 μmol of aminomethylcoumarin within 1 min of reaction at 35 °C.

Protein Concentration. Protein concentration was determined using the protein–dye binding method (Bradford, 1976). The bovine serum albumin was used as a standard protein.

Optimal pH and Temperature. Purified proteinase in 0.2 M sodium phosphate–0.1 M citric acid buffer with various pH values (3.0–7.5) containing 4 mM EDTA and 8 mM cysteine was incubated with 20 μM Z-Arg-Arg-MCA at 35 °C for 10 min and then the intensity of fluorescence was measured (Barrett and Kirschke, 1981). The relative activity was

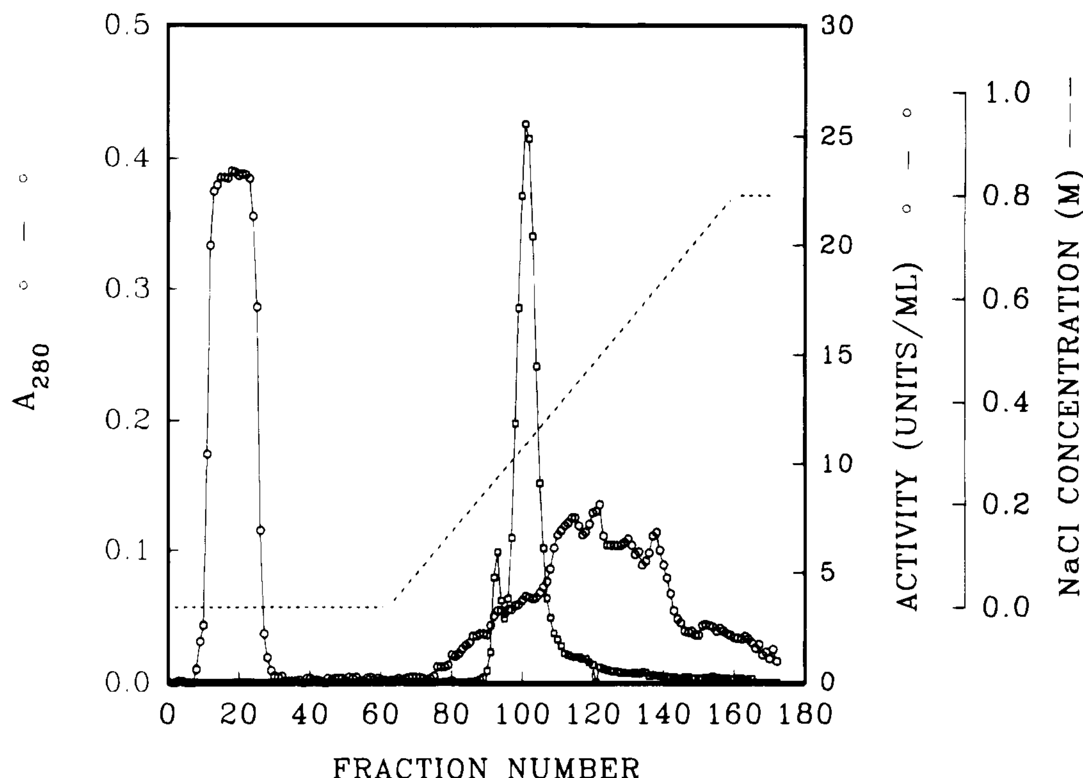

Figure 2. HiLoad S Sepharose chromatography of the proteinase solution obtained from DEAE-Sepharose chromatography. The enzyme solution obtained from DEAE-Sepharose chromatography was concentrated and equilibrated against 20 mM sodium acetate buffer (pH 4.3) (buffer II) using an Amicon ultrafiltration over a YM10 membrane (cutoff, 10 000). The resulting sample was applied to the 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 0–0.8 M NaCl in buffer II. Fractions of 5 mL were collected with a fraction collector (RediFrac) at a flow rate of 1 mL/min. The major peak with Z-Arg-Arg-MCA hydrolytic activity (tubes 97–106) was pooled: (○) absorbance at A_{280}; (●) enzyme activity; (---) NaCl concentration.

expressed as a percentage ratio of the specific activity measured at each pH to the maximum activity.

Purified proteinase in 0.1 M phosphate buffer (pH 6.0) containing 4 mM EDTA and 8 mM cysteine was incubated with 20 μM Z-Arg-Arg-MCA at various temperatures (5–70 °C) for 10 min, and the hydrolytic activity was measured (Barrett and Kirschke, 1981). The relative activity was expressed as a percentage ratio of the specific activity measured at various temperatures to the maximum activity.

Effect of pH on Stability. Purified proteinase in 0.2 M sodium phosphate–0.1 M citric acid buffer with various pH (3.0–7.5) was preincubated at 30 °C for 60 min. After cooling to 5 °C in iced water, the remaining activities were then measured (Barrett and Kirschke, 1981) and expressed as a percentage ratio of the specific activity of samples incubated at various pH values to the sample with maximum activity.

Thermal Stability. Purified proteinase in 20 mM phosphate buffer (pH 6.0) was preincubated at various temperatures (30–50 °C) for 30 min. At definite time intervals, the enzyme solutions were cooled immediately in iced water for 10 min. The remaining activities were then measured (Barrett and Kirschke, 1981). The inactivation rate constant (K_D) of the purified proteinase incubated at various temperatures was calculated as follows: $K_D = (\ln A_0 - \ln A_t)/t$ (A_0 and A_t are the activities before and after t min of 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 as follows:

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

$$\Delta G^* = RT(\ln K_0/h + \ln T - \ln K_D) \quad (2)$$

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

ΔH^*, ΔG^*, and ΔS^* are enthalpy (kcal·mol^{-1}), free energy (kcal·mol^{-1}), and entropy changes (cal·K^{-1}·mol^{-1}), respectively; the R, K_b, and h are the gas constant (1.98 cal·deg^{-1}), the Boltzmann constant (1.38 × 10^{-16} erg·deg^{-1}), and the Plank constant (6.62 × 10^{-27} erg·s), respectively. T is the absolute temperature (K).

Effect of Reductants. Purified proteinase in 0.1 M sodium potassium phosphate buffer (pH 6.0) was incubated with 0–5 mM cysteine, DTT, GSH, and β-Me at 35 °C for 10 min. The Z-Phe-Arg-MCA hydrolytic activity of the resulting samples was measured according to the method of Barrett and Kirschke (1981).

Effect of Inhibitors. Purified proteinase in 20 mM phosphate buffer (pH 6.0) was incubated with 0.1 mM antipain, chymostatin, E-64, IAA, leupeptin, NEM, pCMB, pepstatin, PMSF, soybean trypsin inhibitor, TLCK, and TPCK at 30 °C for 20 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 specific activities of samples with various inhibitors to that without inhibitor. Finally, the inhibition percentage was expressed as the relative percentage with inhibitors subtracted from that without inhibitors (100%).

Effect of Metal Ions. Purified proteinase in 20 mM phosphate buffer (pH 6.0) was incubated with 5 mM concentrations 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 30 °C for 20 min. The remaining activities of the resulting samples were measured according to the method of Barrett and Kirschke (1981). The relative activity was expressed as a percentage ratio of the specific activity of samples with various metals to that without metal.

RESULTS AND DISCUSSION

Purification. A cysteine proteinase with Z-Arg-Arg-MCA hydrolytic activity from mackerel muscle was purified to electrophoretic homogeneity using DEAE-Sepharose (Figure 1), S Sepharose (Figure 2), and

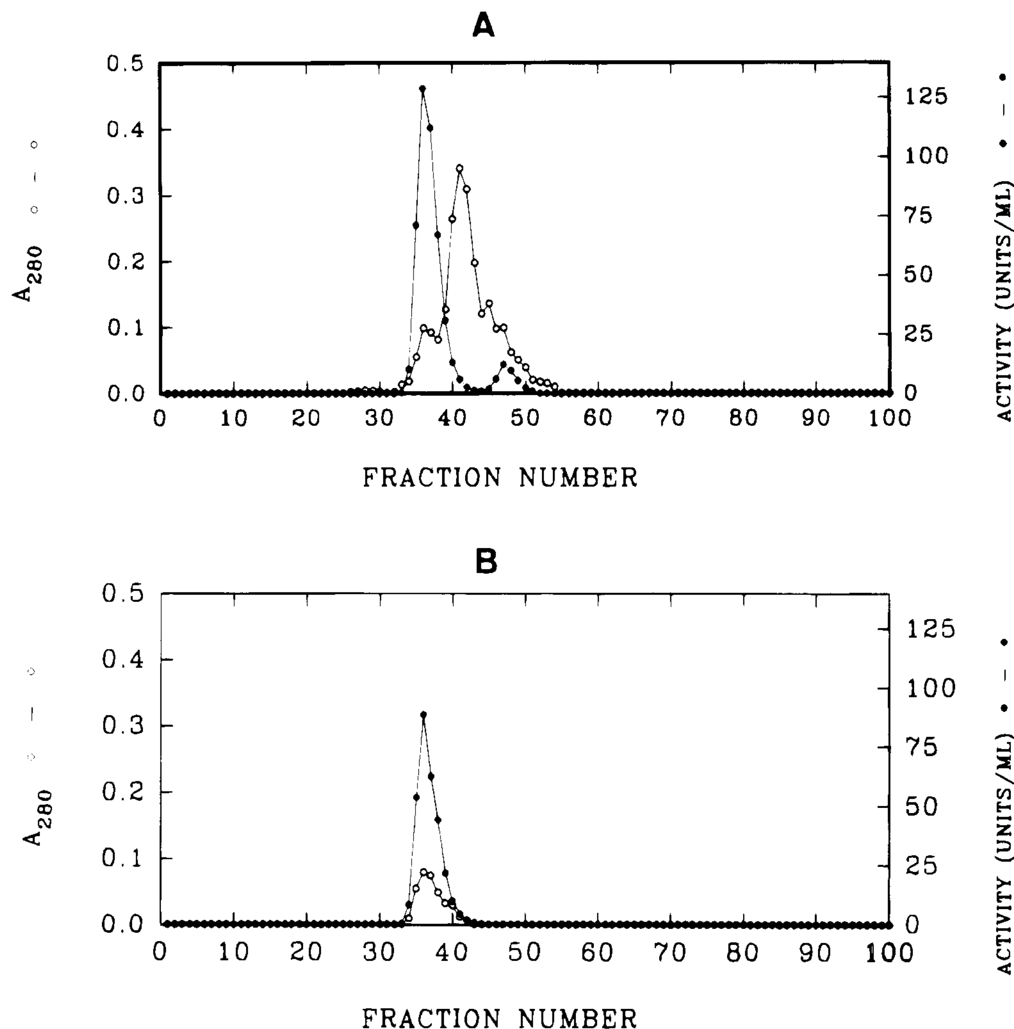

Figure 3. FPLC Superdex 75 chromatography of the proteinase solution. The enzyme solution obtained from S Sepharose chromatography was concentrated and equilibrated against 20 mM phosphate buffer (pH 6.0) containing 0.1 M NaCl (buffer III) using Amicon ultrafiltration. The resulting sample was chromatographed on Superdex 75 HR 10/30 column (A, first chromatography). Fractions with Z-Arg-Arg-MCA hydrolytic activity (tubes 34–39) were collected, concentrated and rechromatographed on a second Superdex 75 HR 10/30 column (B, second chromatography). 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): (○) absorbance at A_{280}; (●) enzyme activity.

Table 1. Summary of the Purification of a 99-kDa Cysteine Proteinase from Mackerel

procedure	total protein (mg)	total act. (units)	sp act. (units/mg)	yield (%)	purification (fold)
crude extract	27798.336	6734.85	0.24	100.0	1
DEAE-Sepharose	107.111	3776.00	35.25	56.1	147
S Sepharose	0.620	668.00	1077.42	9.9	4489
first Superdex 75	0.023	97.95	4258.70	1.5	17745
second Superdex 75	0.011	64.77	5888.18	1.0	24534

Superdex 75 (Figure 3) chromatographies. According to the analysis of PAGE, a single fluorescent band active-stained by Z-Arg-Arg-MCA (Figure 4A), which corresponded to a blue band stained by Coomassie brilliant blue G-250 (Figure 4B) on nondenaturing gradient PAGE, was observed. However, two protein bands on SDS–PAGE were obtained (Figure 5). According to the PAGE analysis the purified proteinase involved at least two subunits. The purification of this proteinase is summarized in Table 1. The yield sharply decreased from 56.1 to 9.9% after S Sepharose chromatography (Table 1). This might be due to the loss of a minor peak with Z-Arg-Arg-MCA hydrolytic activity on S Sepharose chromatography (Figure 2). The minor peak was purified previously and identified to be a cathepsin B (Jiang et al., 1994). According to the study by Ueno et al. (1990), there were two fractions with Z-Arg-Arg-MCA hydrolytic activity from mackerel muscle found on Q Sepharose chromatography. Of them, one was identified as cathepsin B, while the other one was considered to be a new cathepsin B. In our preliminary experiments, the crude extract was separated into two fractions with Z-Arg-Arg-MCA hydrolytic activity on DEAE-Sephacel chromatography (2.6 × 31 cm; data not shown), but this is not in the case for DEAE-Sepharose chromatography (5 × 22.3 cm; Figure 1). This might result from the lower flow rate and the higher packing gel of DEAE-Sephacel than of DEAE-Sepharose. In an attempt to increase the quantity of enzyme sample, DEAE-Sepharose chromatography on a HiLoad system was employed instead of DEAE-Sephacel.

Molecular Weight. The MW of the purified proteinase was 99 000, estimated by Superose 12 gel filtration (Figure 6). According to the SDS–PAGE (Figure 5), two heterogeneous subunits with MW of 35 000 (designated the "a" chain) and 23 000 (designated the "b" chain) were obtained. The ratio of the area of a to b bands on SDS–PAGE was about 2:1, which was

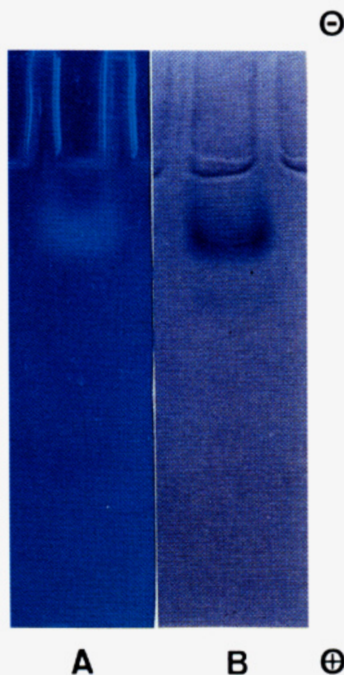

Figure 4. Activity staining of the purified proteinase on nondissociating continuous PAGE with an ascending gradient from 5 to 20% polyacrylamide: (A) active-stained by Z-Arg-Arg-MCA, the active band with fluorescence was detected by ultraviolet light at 366 nm; (B) stained by Coomassie brilliant blue G-250.

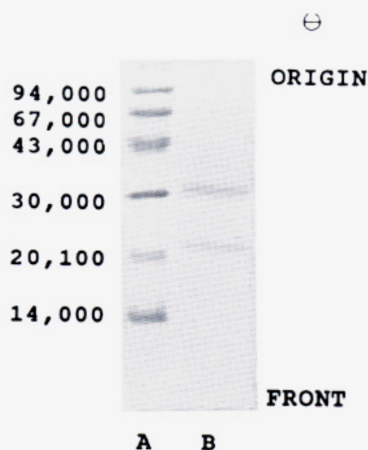

Figure 5. Profiles of sodium dodecyl sulfate–polyacrylamide gel electrophoresis: (A) low MW standard; (B) the purified proteinase. The concentration of acrylamide was 15%.

determined by a thin-layer chromatoscanner (Shimadzu, Model CS-930). Accordingly, the purified proteinase might be a trimer composed of two a and one b polypeptide chains. Although some forms of cathepsin B with relatively higher MW (33 000–40 000) have already been isolated from nonmuscle tissues (Docherty and Phillips, 1988), they were identified as monomers. The purified proteinase had an a_2b structure and was not similar to cathepsin B.

Substrate Specificity. The purified proteinase could strongly hydrolyze Z-Arg-Arg-MCA with an activity of 11.49 units/mL and moderately hydrolyze Z-Phe-Arg-MCA with an activity of 0.23 units/mL but did not hydrolyze Z-Arg-MCA and L-Arg-MCA. This phenomenon suggests that the purified proteinase seems likely to be an endopeptidase. The K_m values for the hydrolysis of Z-Arg-Arg-MCA and Z-Phe-Arg-MCA were estimated to be 0.9 and 4.2 μM, respectively. The substrate specificity against Z-Arg-Arg-MCA and Z-Phe-Arg-MCA was very similar to that of the new type of cathepsin B reported by Ueno et al. (1990). Z-Arg-Arg-MCA was reported to be also hydrolyzed by cathepsin B and cathepsin L (Barrett, 1980; Barrett and Kirschke, 1981). Considering the MW of the subunits, the a and b polypeptides seem likely to correspond to cathepsin L (Lee et al., 1993) and cathepsin B (Matsumiya et al., 1989) from mackerel, respectively. The properties of the purified proteinase seem to be proteasome-like. Multicatalytic activity studies of the proteinase are ongoing.

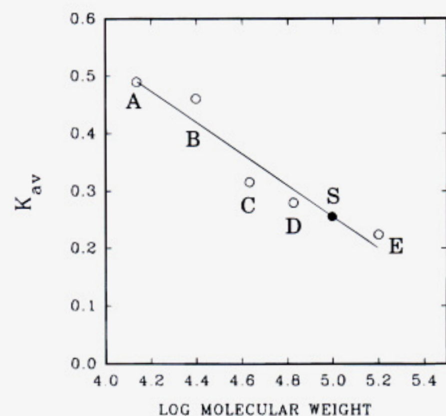

Figure 6. Calibration curve for the molecular weight determination of the purified proteinase on Superose 12 HR 10/30 chromatography. S, the purified proteinase, 99 000; A, ribonuclease A, 13 700; B, chymotrypsinogen A, 25 000; C, ovalbumin, 43 000; D, albumin, 67 000; E, aldolase, 158 000.

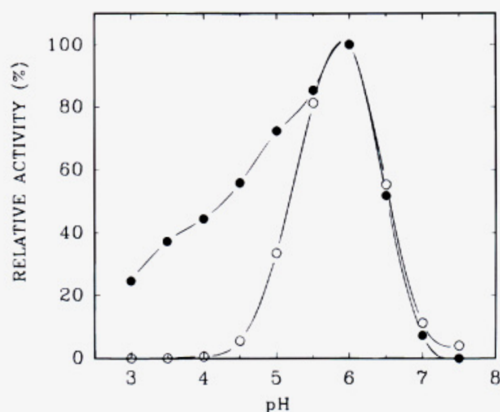

Figure 7. Effect of pH on Z-Arg-Arg-MCA hydrolyzing activity of the purified proteinase: (○) profile of pH-dependent activity; (●) profile of pH stability.

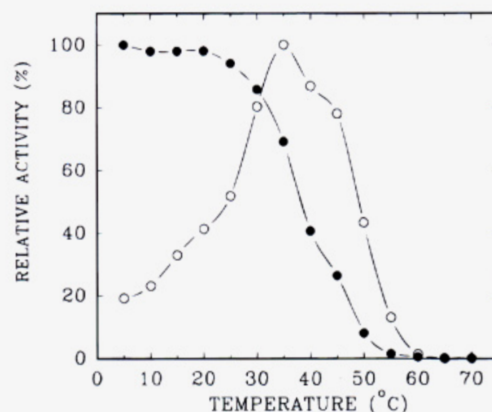

Figure 8. Effect of temperature on Z-Arg-Arg-MCA hydrolyzing activity of the purified proteinase: (○) profile of temperature-dependent activity; (●) profile of thermal stability.

Table 2. Thermodynamic Parameters[a] for Thermal Inactivation of the Purified Proteinase

temp (°C)	$K_D \times 10^3$ (min^{-1})	ΔG^* (kcal/mol)	E_a (kcal/mol)	ΔS^* (cal/K·mol)	ΔH^* (kcal/mol)
30	3.1	22.22	46.85	79.31	46.25
35	10.3	21.86	46.85	79.16	46.24
40	27.9	21.60	46.85	78.69	46.23
45	119.0	21.05	46.85	79.15	46.22
50	380.0	20.65	46.85	79.13	46.21

[a] K_D, thermal inactivation rate constant; ΔG^*, change in free energy; E_a, activation energy; ΔS^*, change in entropy; ΔH^*, change in enthalpy.

Table 3. Effect of Reductants[a] on the Purified Proteinase

	proteolytic act. (μmol mL^{-1} min^{-1})			
concn (mM)	cysteine	DTT	GSH	β-Me
0.0	ND[b]	ND	ND	ND
1.0	6.335	4.800	0.520	1.695
2.0	7.975	6.460	0.890	2.615
3.0	8.695	7.195	1.160	3.175
4.0	9.355	8.160	1.405	3.570
5.0	9.560	8.755	1.675	4.145

[a] DTT, dithiothreitol; GSH, glutathione; β-Me, β-mercaptoethanol. [b] ND, not detected.

Effect of pH. The optimal pH of the purified proteinase for the hydrolysis of Z-Arg-Arg-MCA was 6.0 (Figure 7). When the enzyme was preincubated at various pH values at 30 °C for 60 min, it was also the most stable at pH 6.0. However, its stability significantly decreased when the pH was higher than 7.0 (Figure 7). These properties were similar to those of cathepsin B (Hara et al., 1988b; Matsumiya et al., 1989; Yamashita and Konagaya, 1990b; Jiang et al., 1994) or cathepsin L (Yamashita and Konagaya, 1990a; Lee et al., 1993; Turk et al., 1993), which were known to be inactive under alkaline conditions.

Effect of Temperature. The optimal temperature and T_m value (temperature for 50% inactivation after 30 min of incubation) were about 35 and 38 °C, respectively (Figure 8). After 30 min of incubation at various temperatures (5–70 °C), no significant change in the activity of purified proteinase was observed between 5 and 20 °C. However, when the preincubation temperatures were higher than 25 °C, the hydrolytic activity decreased significantly (Figure 8). The thermal inactivation rate constant (K_D) and the inactivation energy were calculated to be 0.38 min^{-1} and 46.85 kcal/mol at 50 °C, respectively (Table 2). The thermal stability of purified proteinase was much lower than that of cathepsin B (Jiang et al., 1994) or cathepsin L (Lee et al., 1993) from mackerel. This heat-labile property might result from the dissociation or denaturation of the subunits during incubation.

Effect of Reductants. The hydrolytic activity of the purified proteinase was quite low in the presence of reductants. However, it was effectively activated in the presence of 1–5 mM reductants such as β-Me, GSH, DTT, and cysteine (Table 3). Among them, cysteine was the most effective. This phenomenon was similar to that of lysosomal cysteine proteinases from fish (Hara et al., 1988b; Sherekar et al., 1988; Matsumiya et al., 1989; Yamashita and Konagaya, 1990a,b; Aranishi et al., 1992; Lee et al., 1993).

Effect of Inhibitors. The purified proteinase was completely inhibited by cysteine proteinase inhibitors such as antipain, chymostatin, E-64, IAA, and leupeptin but was not by serine proteinase inhibitors and aspartic proteinase inhibitors such as pepstatin, PMSF, soybean trypsin inhibitor, and TLCK (Table 4). TPCK, which is an inhibitor for chymotrypsin-like serine proteinase, moderately inhibited the purified proteinase (Table 4).

Table 4. Effect of Various Inhibitors on the Purified Proteinase

inhibitor	final concn (mM)	inhibition (%)
none	0.0	0.0
antipain	0.1	99.1
chymostatin	0.01	13.9
E-64	0.01	99.3
iodoacetic acid	0.1	94.9
leupeptin	0.02	98.3
N-ethylmaleimide	0.1	0.6
p-(chloromercuri)benzoate	0.1	9.9
pepstatin	0.1	0.0
phenylmethanesulfonyl fluoride	0.1	1.3
soybean trypsin inhibitor	0.1	0.0
tosyllysine chloromethyl ketone	0.1	100.0
tosylphenylalanine chloromethyl ketone	0.1	56.4

Table 5. Effect of Metal Ions on the Purified Proteinase

metal ion[a] (1 mM)	relative act. (%)	metal ion[a] (1 mM)	relative act. (%)
none	100.0	Ba^{2+}	119.1
K^+	113.4	Mn^{2+}	38.4
Na^+	113.6	Co^{2+}	0.0
Mg^{2+}	87.5	Ni^{2+}	0.0
Sr^{2+}	75.8	Cu^{2+}	0.0
Zn^{2+}	0.0	Hg^{2+}	0.0
Ca^{2+}	35.1	Fe^{2+}	0.0
Cd^{2+}	0.0	Fe^{3+}	0.0

[a] The counterion of all metals was chloride; cysteine was added to a final concentration of 2.0 mM in all of the reaction mixtures.

According to the inhibitor study and substrate specificity, the purified enzyme was a cysteine proteinase.

Effect of Metal Ions. In the presence of 2.0 mM cysteine and 1.0 mM EDTA, Zn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+}, and Fe^{3+} completely inhibited, while Ca^{2+}, Mn^{2+}, Sr^{2+} and Hg^{2+} moderately inhibited, the activity of purified proteinase (Table 5). However, K^+, Na^+, and Ba^{2+} did not affect the purified proteinase (Table 5). The Zn^{2+}, Cu^{2+}, and Ni^{2+} could interact with histidine residues, while Co^{2+}, Hg^{2+}, Fe^{2+}, and Fe^{3+} could form chelates with cysteine residues (Malmström and Rosenberg, 1960). Accordingly, the active center of the purified proteinase might contain histidine and cysteine residues. The enzymes with more than one subunit, such as transglutaminase (Curtis and Lorand, 1976; Jiang and Lee, 1992) and *m*-calpain (Wang et al., 1993), usually need calcium to activate. The purified proteinase was not activated by any metal ions in the absence of cysteine (data not shown). In contrast to calpains and transglutaminase, the purified proteinase was partially inactivated by Ca^{2+}. The inhibitory effect of Ca^{2+} increased with increase of Ca^{2+} (from 0.1 to 5.0 mM, data not shown). Although the Ca^{2+} level increased gradually from 1 to 100 μM in post-mortem fish muscle (Pearson and Young, 1989), 90% of the activity of the purified proteinase remained even at 100 μM Ca^{2+}. Therefore, this proteinase is still important in the post-mortem tenderization of fish muscle.

According to the study on the degradation of actomyosin by crude proteinases from mackerel (Sakata et al., 1985), no significant difference in the SDS–PAGE profiles between actomyosin–crude proteinase mixture with and without pepstatin was observed. Sakata et al. (1985) thus postulated that the proteinases participating in the autolysis of mackerel muscle were cathepsins B and L and their similar proteinases. However, from the investigation of the autolysis of 16 fish muscle homogenates (including mackerel), Hara et al. (1987) demonstrated that, except for cathepsins B and L, there were other cysteine proteinases involved in the autolysis of muscle homogenates. The purified proteinase might be one of the cysteine proteinases differing from cathepsins B and L which participate in the post-mortem autolysis of mackerel.

According to Asghar and Bhatti (1987), the cysteine proteinases in the skeletal muscle included cathepsins B, H, and L and Ca^{2+}-activated neutral proteinases (CANP). The substrate specificity, thio dependence, optimal pH, and pH stability of the purified proteinase were similar to cathepsin B or cathepsin L, but the molecular conformation with two heterogeneous subunits was relatively similar to CANP. The purified proteinase is a novel cysteine proteinase that is different from cathepsins B, H, and L and CANP. Considering the post-mortem pH of fish muscle, this cysteine proteinase with an optimal pH at 6.0 might play an important role in the autolysis of mackerel muscle. Further study on the proteolysis on myofibrillar proteins by this novel cysteine proteinase is ongoing. The results will be helpful for understanding the participation of this proteinase on post-mortem proteolysis of mackerel muscle.

Although cathepsin B (Matsumiya et al., 1989; Jiang et al., 1994), cathepsin L, and L-like proteinase (Lee et al., 1993) were considered to participate in the post-mortem autolysis of mackerel, the activities of these cysteine proteinases were regulated *in vivo* by indigenous inhibitors (Laskowski and Kato, 1980). Therefore, an appropriate understanding of the regulation of cysteine proteinase by inhibitors is essential for elucidating the mechanism of post-mortem tenderization of fish muscle.

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