# Purification and Characterization of Proteases from Milkfish Muscle (*Chanos chanos*)†

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Proteases in acetone powder prepared from milkfish (*Chanos chanos*) muscle were extracted. Three fractions with caseinolytic activity, named A, B, and C, were obtained from CM-Sepharose CL-6B and DEAE-Sephadex A-50 chromatography. Three proteases were purified to electrophoretic homogeneity. Substrate specificity and inhibitor studies indicated that proteases A and C were cathepsin A like; protease B was trypsinlike enzymes. The optimal temperatures of proteases A, B, and C for hydrolysis of casein were found to be 50, 60, and 40 °C, respectively. The optimal pH of proteases A and C for hydrolysis of CBZ-Gly-Phe was 7.0; that of protease for hydrolysis of *p*-toyl-L-arginine methyl ester (TAME) was 8.0. The temperatures which inactivated 50% of enzymes in 5 min were 46 °C for protease A, 51 °C for protease B, and 48 °C for protease C. The molecular weights of proteases A, B, and C were 15 600, 33 000, and 35 600.

## INTRODUCTION

Degradation of Antarctic krill and mackerel after harvest was considered to be due to the high activity of proteases in muscle and viscera (Chen et al., 1978; Seki et al., 1975; Kuwano et al., 1976; Kawabata and Suzuki, 1959). Deterioration of minced fish products was also ascribed to the action of alkaline proteases during setting process (Makinotan, 1984). This evidence suggests that the proteases involved in muscle affect the quality of fish and shellfish during storage and processing.

Many studies have evaluated the application of partial freezing (Uchiyama and Kato, 1974), addition of chemicals (Niwa, 1978; Bailey and Fieger, 1954; Farber, 1954; Fieger et al., 1956), mixtures of crushed ice and NaCl (Ho et al., 1986; Jiang and Lee, 1988), and modified atmosphere storage (Lannelongue et al., 1982; Layrisse and Matches, 1984; Reppond and Collins, 1983) to fish and shellfish to extend the storage life. However, little is known about the mechanism by which these methods influence quality during storage. To investigate the effects of storage methods on proteolysis of fish muscle and viscera, and subsequently the mechanism of maintaining quality by these storage methods, proteases from milkfish muscle were purified and characterized in this study.

## MATERIALS AND METHODS

**Materials.** Milkfish (*Chanos chanos*, 400–600 g/fish) were purchased from a commercial aquatic farm and transported to the laboratory immediately by icing. Acetone powder was prepared from muscle as soon as possible and stored at −20 °C until use. Milkfish muscle was homogenized in prechilled acetone (1:9 w/v). The homogenate was filtered. The acetone-insoluble material was washed several times with acetone and finally once with ether and air-dried at room temperature overnight.

**Purification of Proteases.** The acetone powder (50 g) was homogenized with 18 volumes of deionized water at 5 °C and centrifuged at 10000*g* for 30 min. The supernatant was treated with solid ammonium sulfate. Fractions precipitated between 0.2 and 0.8 saturation were collected and dissolved in a minimal volume of 10 mM phosphate buffer (pH 7.0). The residual ammonium sulfate was removed by dialyzing against the same buffer overnight.

**Sephadex G-75 Column Chromatography.** The 20–80% ammonium sulfate fraction was chromatographed on a Sephadex G-75 column (2.6 × 85 cm) with 10 mM phosphate buffer (pH 7.0) at a flow rate of 0.72 mL/min. Fractions of 5.0 mL were collected.

**DEAE-Sephadex A-50 Column Chromatography.** Fractions with caseinolytic activity, obtained from Sephadex G-75 column chromatography, were concentrated with an ultrafiltration cell (Amicon) and loaded onto a DEAE-Sephadex A-50 column (2.6 × 30 cm). The column was washed with 500 mL of 0.1 M NaCl in 10 mM Tris-HCl buffer (pH 8.0) and eluted with a linear gradient of 0.1–2.0 M NaCl in 10 mM Tris-HCl buffer (pH 8.0) at a flow rate of 0.5 mL/min (2 L total volume). Fractions of 5.0 mL were collected.

**CM-Sepharose CL-6B Column Chromatography.** The sample collected from the washed portion during the DEAE-Sephadex A-50 column chromatography was loaded onto a CM-Sepharose CL-6B column (1.6 × 30 cm) previously equilibrated with 10 mM potassium phosphate buffer (pH 6.0). The column was washed with 3 volumes of the same buffer and further eluted with a linear gradient of 0.0–1.0 M NaCl in 10 mM phosphate buffer (pH 6.0) at a flow rate of 0.72 mL/min (1 L total volume). Fractions of 5.0 mL were collected.

**Disc Polyacrylamide Gel Electrophoresis (Disc-PAGE).** The polyacrylamide gel systems of pH 8.3 and 6.8 with 7.5% polyacrylamide gel were used (Gabriel, 1971). The current per tube was 2–3 mA. Protein was stained with 0.12% Coomassie brilliant blue in 50% methanol/9.2% acetic acid and destained with 50% methanol/7.5% acetic acid.

**Determination of Molecular Weights.** The molecular weights of purified enzymes were determined by using Sephadex G-150 gel filtration. Albumin (67 000), ovalbumin (43 000), chymotrypsinogen A (25 000), and ribonuclease A (13 700) were used as standards.

**Determination of Protein Concentration.** Protein concentration was determined by the Lowry method (Lowry et al., 1951) using crystalline bovine serum albumin as standard.

**Assay of Proteolytic Activity.** The proteolytic activity was

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**Table I. Summary of the Purification of Milkfish Muscle Proteases**

| | total volume, mL | total protein, mg | total act., units | sp act., units/mg | recovery, % | purifn, x-fold |
|---|---|---|---|---|---|---|
| acetone powder | 700[a] | 14470 | 43.4 | 0.03 | 100 | 1 |
| ammonium sulfate (20–80%) | 440 | 11396 | 42.2 | 0.04 | 97.2 | 1.3 |
| Sephadex G-75 | 305 | 129 | 32.0 | 2.48 | 74.3 | 82.7 |
| CM-Sepharose CL-6B, gradient with 0.0–1.0 NaCl | | | | | | |
| A | 67 | 0.709 | 20.8 | 22.29 | 48.2 | 743 |
| DEAE-Sephadex A-50, gradient with 0.1–2.0 M NaCl | | | | | | |
| B | 64 | 0.836 | 12.8 | 15.31 | 29.7 | 510 |
| C | 38 | 0.362 | 15.2 | 41.99 | 35.2 | 1400 |

[a] Casein at a final concentration of 0.5% was used as substrate.

determined by using denatured casein (0.5% final concentration) as substrate according to the method of Kunitz (1946). The enzymatic reaction was terminated by the addition of trichloroacetic acid (TCA) to a final concentration of 3%. The soluble materials content in the supernatant was measured spectrophotometrically at 280 nm. One unit of enzyme activity was defined as the amount of protease that caused an increase in absorbance at 280 nm of 1.0 in 10 min. Specific activity was expressed as units of enzymatic activity per milligram of protein.

**Assay of Trypsin Esterase Activity.** *1. Benzoyl-L-arginine Ethyl Ester (BAEE) as Substrate (Rick, 1965).* To a 3.0-mL cuvette was added 2.8 mL of 1 mM BAEE in 50 mM Tris-HCl buffer, pH 8.0, containing 20 mM $CaCl_2$. A 0.2-mL aliquot of properly diluted enzyme solution was then added at zero time and mixed immediately. Absorbance at 253 nm was measured continuously for 10 min. One unit of BAEE activity was defined as the amount of trypsin or trypsinlike enzymes that resulted in an increase of 1 absorbance unit at 253 nm in 10 min. Specific activity was expressed as units of enzymatic activity per milligram of g protein.

*2. p-Tosyl-L-arginine Methyl Ester (TAME) as Substrate (Walsh, 1970).* Tris-HCl (2.6 mL, 46 mM), pH 8.1, containing 11.5 mM $CaCl_2$ was measured into a cuvette followed by 0.3 mL of 10 mM TAME. One-tenth milliliter of properly diluted enzyme solution was added at zero time and mixed immediately. Absorbance at 247 nm was measured continuously for 10 min. One unit of TAME activity was defined as the amount of trypsin or trypsinlike enzymes that resulted in an increase of 1 absorbance unit at 247 nm in 10 min. Specific activity was expressed as units of enzymatic activity per milligram of protein.

**Assay of Chymotrypsin Esterase Activity.** *1. N-Acetyl-L-tyrosine Ethyl Ester (ATEE) as Substrate.* To a 3.0-mL cuvette was added 2.8 mL of 1.0 mM ATEE in 50 mM phosphate buffer, pH 7.0. A 0.2-mL aliquot of properly diluted enzyme solution was then added at zero time and mixed immediately. Absorbance at 237 nm was measured continuously for 10 min. One unit of ATEE activity was defined as the amount of chymotrypsinlike enzyme that resulted in a decrease of 0.001 unit/min.

*2. N-Benzoyl-L-tyrosine Ethyl Ester (BTEE) as Substrate.* To a 3.0-mL cuvette were added 1.5 mL of 80 mM Tris-HCl buffer, pH 7.8, containing 0.1 M $CaCl_2$, and 1.4 mL of 1.07 mM BTEE in 50% methanol solution. A 0.1-mL aliquot of properly diluted enzyme solution was then added at zero time and mixed immediately. Absorbance at 256 nm was measured at 30-s intervals. One unit of BTEE activity was defined as the amount of chymotrypsinlike enzyme that resulted in an increase of 1 absorbance unit in 10 min.

**Assay of Carboxypeptidase A Esterase Activity.** The esterase activity of carboxypeptidase A was determined by either hippuryl-L-phenylalanine (HPA) or carbobenzoxyglycyl-L-phenylalanine (CGP) as substrate according to the method of Bergmeyer et al. (1974) and Appel (1974), respectively. One unit of enzyme activity was defined as the amount of carboxypeptidase A causing an increase in absorbance at 280 nm by 1.0 in 10 min. Specific activity was expressed as units of enzymatic activity per milligram of protein.

**Assay of Cathepsin A and D Activities.** Cathepsin A activity was determined with CBZ-Glu-Phe as substrate by the colorimetric ninhydrin procedure of Moore and Stein (1954). The reaction mixtures containing 0.6 mL of McIlvaine buffer (pH 5.0), 0.2 mL of 35 mM substrate solution, and 0.2 mL of enzyme solution were incubated at 37 °C for 1 h. The reaction was stopped by the addition of 1 mL of 5% TCA and heated for 10 min at 90 °C. The mixture were brought to 5 mL by adding deionized water and filtered through a Toyo 5C filter paper. Controls were prepared in the same manner, except the reaction mixture without substrate was incubated and then TCA and substrate were added to the mixture in this order. One milliliter of filtrate was used for the colorimetric determination. The specific activity was expressed as nanomoles of phenylalanine released per milligram of protein within 1 min of reaction at 37 °C.

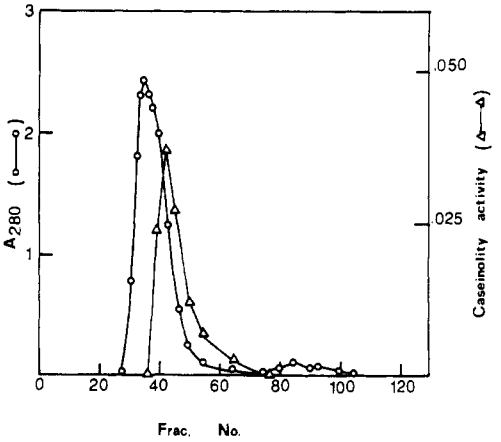


**Figure 1.** Sephadex G-75 gel filtration of milkfish muscle proteases (experimental details are given under Materials and Methods). Caseinolytic activity is expressed as units per milliliter of the solution in fractions.

Cathepsin D activity was determined by a modified Anson's technique (1937). The reaction mixtures containing 1.5 mL of McIlvaine buffer (pH 3.0), 0.5 mL of 5% acid denatured hemoglobin (pH 3.0), and 0.5 mL of enzyme solution were incubated at 37 °C for 1 h. The reaction was stopped with 2.5 mL of 5% TCA. After standing at room temperature for 45 min, the solution was filtered through a Toyo 5C filter paper. Controls were prepared in the same manner, except that hemoglobin was incubated separately from the other components of the reaction mixture and then combined after TCA was added. Five milliliters of 0.55 M $Na_2CO_3$ and 1 mL of Folin phenol reagent were added to 2 mL of TCA filtrate. The absorbance at 660 nm was measured. The specific activity was expressed as nanomoles of tyrosine released per milligram of protein within 1 min of reaction at 37 °C.

## RESULTS AND DISCUSSION

**Purification of Proteases.** *Chromatography on Sephadex G-75.* The protease mixture was fractionated with ammonium sulfate and acetone precipitation (Table I) and then chromatographed on a Sephadex G-75 column. As indicated in Figure 1, the caseinolytic activity was concentrated in the later part of the first protein peak; an 82.7-fold purification was achieved at this step. Fractions containing caseinolytic activity were collected and applied to a DEAE-Sephadex A-50 column.

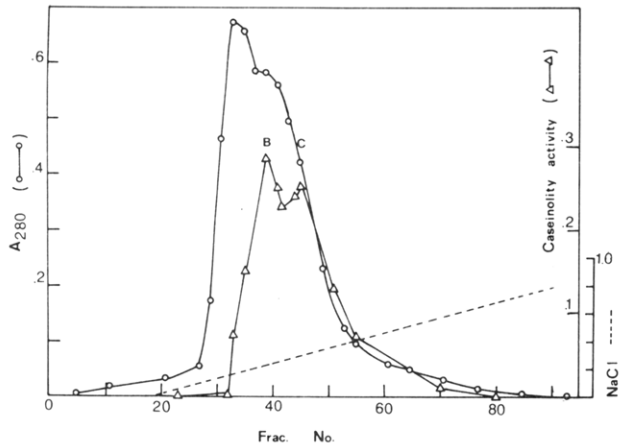


**Figure 2.** DEAE-Sephadex A-50 chromatography of milkfish muscle proteases (eluted with a linear gradient of 0.1–1.0 M NaCl in 10 mM Tris-HCl buffer, pH 8.0). Caseinolytic activity is expressed as units per milliliter.

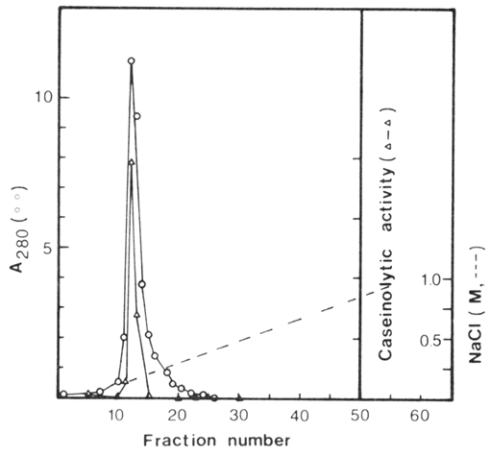


**Figure 3.** CM-Sepharose CL-6B column chromatography of protease A from milkfish muscle. Protease A was collected from the washed portion on DEAE-Sephadex A-50 column chromatography. Caseinolytic activity is expressed as units per milliliter.

*Chromatography on DEAE-Sephadex A-50.* The column was first washed with 500 mL of 0.1 M NaCl/0.01 M Tris-HCl buffer (pH 8.0) and then eluted by using a 0.1–2.0 M NaCl linear gradient. One caseinolytic peak was obtained in the washed portion, designated protease A. Two caseinolytic peaks were obtained during elution and designated proteases B and C (Figure 2). Protease A was purified by CM-Sepharose CL-6B column chromatography with a linear gradient of 0.0–1.0 M NaCl in 10 mM phosphate buffer (pH 6.0) and then by Sephadex G-150 gel filtration (Figure 3). At this step protease A was demonstrated to be homogeneous by disc gel electrophoresis (Figure 4). Proteases B and C were also purified to electrophoretic homogeneity by rechromatography once or twice on a DEAE-Sephadex A-50 column with a linear gradient of 0.1–1.0 M NaCl in 10 mM Tris-HCl buffer (pH 8.7) (Figure 4). The purification of these three milkfish muscle proteases was summarized in Table I. Since 30–50 μg each of proteases A, B, and C was used for the analyses of homogeneity, and there was only a single protein band found in each gel electrophoresis, it is suggested that these proteases were highly purified. The purification of these three proteases was 743-fold for A, 510-fold for B and 1400-fold for C (Table I).

**Determination of Molecular Weights.** The molecular weight of each protease was determined by Sephadex G-150 column chromatography (1.6 × 60 cm; eluted by 10 mM Tris-HCl buffer, pH 8.0). As indicated in Figure 5, the molecular weights of proteases A, B, and C were 15 600, 33 000, and 35 600, respectively. The molecular weight of protease B was higher than that of bovine trypsin (Sasano and Ota, 1964).

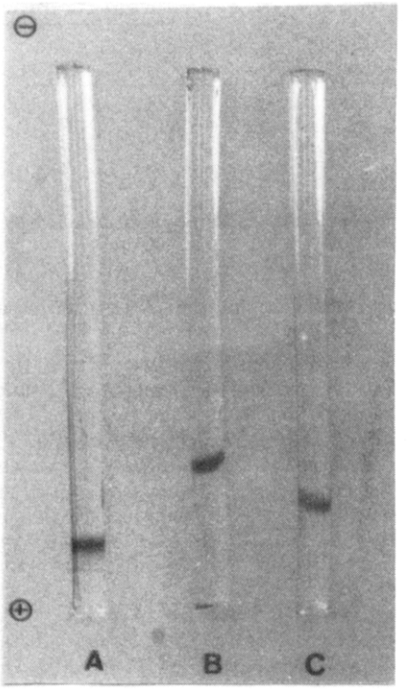


**Figure 4.** Disc gel electrophoretic pattern of purified milkfish muscle proteases (30–50 μg of each proteases was used per tube).

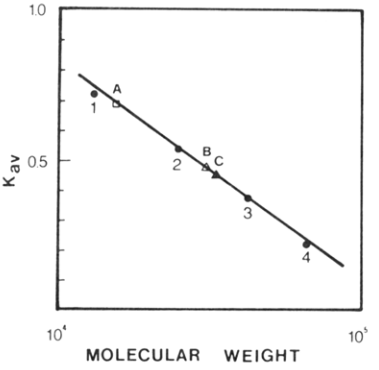


**Figure 5.** Determination of the molecular weights of milkfish muscle proteases by Sephadex G-150 gel filtration. (1) Ribonuclease A, 13 700; (2) chymotrypsinogen A, 25 000; (3) ovalbumin, 43 000; (4) albumin, 67 000. (□) Protease A; (Δ) protease B; (▲) protease C.

**Substrate Specificity.** Table II indicates the activity of each enzyme toward synthetic substrates. Proteases A and C had activity on CBZ-Glu-Tyr, CBZ-Glu-Phe, CBZ-Gly-Phe, and CBZ-Phe-Gly, but not on other synthetic substrates, i.e., TAME, BAEE, ATEE, BTEE, HPA, and CGP. These results suggested that proteases A and C are cathepsin A like (Taylor and Tappel, 1974) and protease B is a trypsinlike protease. This conclusion was further supported by inhibitor studies described under the next section.

**Inhibitor Studies.** As indicated in Table III, protease B was inhibited by 0.1 and 0.5 mM $N^{\alpha}$-tosyl-L-lysine chloromethyl ketone (TLCK), which is a specific inhibitor for trypsin (Schoellman and Shaw, 1963), while protease C was completely inhibited; protease A was partially inhibited by 20 mM pepstatin A. This compound specifically inhibits cathepsins A and D (Makinodan and Ikeda, 1976). Cathepsin D specifically hydrolyzes the pep-

**Table II. Substrate Specificity of Milkfish Muscle Proteases**

| substrate[a] | sp act. A[b] | B[c] | C[b] | trypsin (hog)[c] |
|---|---|---|---|---|
| TAME | 0 | 28.1 | 0 | 32.5 |
| BAEE | 0 | 49.5 | 0 | 43.8 |
| ATEE | 0 | 0 | 0 | 0 |
| BTEE | 0 | 0 | 0 | 10.0 |
| HPA | 0 | 0 | 0 | |
| CGP | 0 | 0 | 0 | |
| CBZ-Glu-Tyr | 2008 (19.0) | | 232.4(2.65) | |
| CBZ-Glu-Phe | 1255 (11.9) | | 320.0(3.64) | |
| CBZ-Gly-Phe | 10570 (100) | | 8783 (100) | |
| CBZ-Phe-Gly | 29290 | 5103 | 41990 | |

[a] TAME, tosyl-L-arginine methyl ester. BAEE, benzoyl-L-arginine ethyl ester. ATEE, acetyl-L-tyrosine ethyl ester. BTEE, benzoyl-L-tyrosine ethyl ester. HPA, hippuryl-L-phenylalanine. CGP, carbobenzoxyglycyl-L-phenylalanine. [b] Specific activity is expressed as nanomoles of released FAA $min^{-1}$ (mg of protein)$^{-1}$; values in parentheses are the percentage ratios relative to the substrate of CBZ-Gly-Phe. [c] Specific activity is expressed as unit 10 $min^{-1}$ (mg of protein)$^{-1}$.

**Table III. Effect of Some Inhibitors on the Activity of Milkfish Muscle Proteases**

| inhibitor | concn, mM | rel act., % A[a] | B | C | pepsin | cathepsin D |
|---|---|---|---|---|---|---|
| control | | 100 | 100 | 100 | 100 | 100 |
| PCMB[b] | 0.001 | 81.2 | 100 | 90.1 | 108 | 98 |
| TPCK[b] | 1.0 | 47 | 86 | 57 | 90 | 67 |
| TLCK[b] | 0.1 | 100 | 37 | 102 | 94 | 100 |
| TLCK[b] | 0.5 | 95 | 20 | 89 | 102 | 98 |
| iodoacetic acid | 0.1 | 97 | 21 | 100 | 98 | 104 |
| pepstatin A | 20.0 | 57 | | 0.0 | 10.9 | 50 |
| leupeptin | 20.0 | 100 | | 100 | | |

[a] The amounts of the proteases, in the assay mixture (2 mL) are (A) 1.1 μg ($7.43 \times 10^{-5}$ μmol), (B) 1.07 μg ($6.37 \times 10^{-5}$ μmol), and (C) 3.58 μg ($1.44 \times 10^{-4}$ μmol). [b] PCMB, *p*-(chloromercuri)benzenesulfonic acid. TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone. TLCK, $N^{\alpha}$-p-tosyllysine chloromethyl ketone.

tides containing at least five amino acid residues (Turk et al., 1984). Accordingly, from the substrate specificity and inhibitor studies, proteases A and C were considered to be cathepsin A like and protease B was considered to be trypsinlike.

**Effect of Metal Ions.** Some studies indicated that the carboxypeptidase A contained in the pyloric coeca of mackerel *Scomber japonicus* was activated 5-fold by cobalt at 1 mM (Ooshiro, 1962), and the carboxypeptidase A from Antarctic krill *Euphausia superba* and milkfish viscera was also activated by cobalt (Chen et al., 1978, 1989). In addition, we are interested in nontoxic protease inhibitors, which might be good candidates for use in prevention of the deterioration of fish and shellfish muscle. Therefore, the effects of metal ions on the purified proteases were examined.

Each protease was incubated with a variety of metal ions at 50 °C (which was near the optimal temperature for the activities of these purified proteases) for 2 min. Substrate (proteases A and C, CBZ-Gly-Phe; B, TAME) was then added to assay the activity. The results are shown in Table IV. Mercuric ion at 0.1 and 0.5 mM partially inhibited proteases A and C. Zinc, copper, and manganese ions partially inhibited the activity of protease B, but had no effect on proteases A and C. The sodium, potassium, cobalt, calcium, and magnesium ions showed no effects on the milkfish muscle proteases. All the chelating agents used in this study had no effects on the proteases from milkfish muscle. Since copper, zinc, and manganese ions

**Table IV. Effect of Metal Ions and Chelating Agents on the Activity of Milkfish Muscle Proteases**

| metal ion | concn, mM | rel act., % A[a] | B | C |
|---|---|---|---|---|
| control | | 100 | 100 | 100 |
| $Na^{+}$ [b] | 1.0 | 100 | 100 | 100 |
| $K^{+}$ | 1.0 | 102 | 100 | 98 |
| $Hg^{2+}$ | 0.1 | 53 | 79 | 64 |
| $Hg^{2+}$ | 0.5 | 25 | | 42 |
| $Cu^{2+}$ | 0.1 | 104 | 74 | 100 |
| $Cu^{2+}$ | 0.5 | 108 | 26 | 100 |
| $Zn^{2+}$ | 0.1 | 100 | 63 | 94 |
| $Mn^{2+}$ | 1.0 | 82 | 82 | 102 |
| $Mn^{2+}$ | 5.0 | 82 | 26 | 96 |
| $Co^{2+}$ | 0.1 | 92 | 90 | 103 |
| $Ca^{2+}$ | 1.0 | 100 | 100 | 102 |
| $Mg^{2+}$ | 1.0 | 98 | 110 | 95 |
| chelating agents | | | | |
| control | | 100 | 100 | 100 |
| EDTA | 1.0 | 100 | 93 | 96 |
| salicylaldoxime | 0.1 | 98 | 107 | 100 |
| α-dimethyldithiocarbamate | 0.1 | 92 | 100 | 90 |

[a] The amounts of the proteases used are the same as those in Table III. [b] Chloride ion is the counterion of all these metal ions.

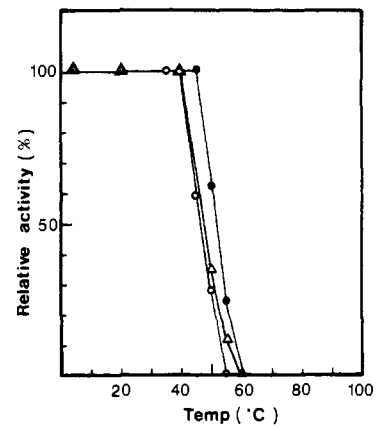


**Figure 6.** Thermostability of milkfish muscle proteases. (○) Protease A; (●) protease B; (△) protease C. The substrates were CBZ-Gly-Phe for proteases A and C, TAME for protease B.

significantly inhibited protease B and mercuric ion also inhibited proteases A and C, zinc and manganese might be good candidates for use in the preservation of fish and shellfish.

**Thermostability.** The purified milkfish muscle proteases A, B, and C in 0.01 M Tris-HCl buffer containing 0.1 M NaCl, pH 8.0, were heated for 5 min at different temperatures. After cooling, the remaining activity was determined by using synthetic substrates indicated in the legend of Figure 6. The temperatures at which half of the activity of proteases A, B, and C were inactivated under the experimental conditions were 46, 51, and 48 °C, respectively. The thermostability of milkfish muscle trypsinlike protease B was similar to that of trypsins from brine shrimp *Artemia salina* (Olalla et al., 1978), Antarctic krill *E. superba*, and milkfish viscera (Chen et al., 1978, 1989) examined under similar conditions. Protease B, trypsinlike protease, was more stable at high temperature than cathepsin A like proteases A and C, which are very heat labile enzymes in milkfish muscle. The instability of these proteases at elevated temperatures might be due to autolysis and/or denaturation. Since proteases A and C are very heat labile, their molecular structures might not be as compact as that of protease B.

**Optimal Temperature and pH.** The optimal temperatures for the activities of the purified milkfish muscle proteases A, B, and C for hydrolysis of casein were

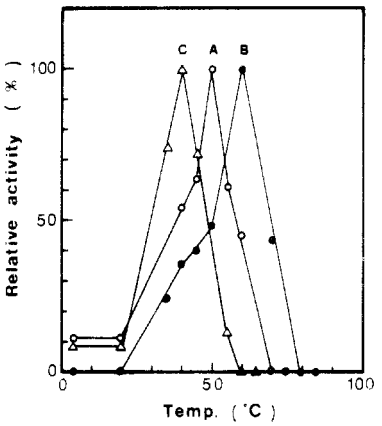


**Figure 7.** Temperature–activity profiles of milkfish muscle proteases (substrate, casein). (○) Protease A; (●) protease B; (△) protease C).

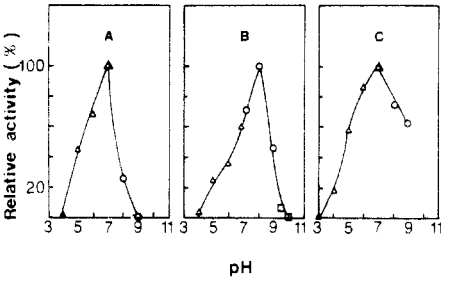


**Figure 8.** pH–activity profiles of milkfish muscle proteases. Lyophilized proteases were dissolved in buffer solutions at various pH values. The activities were determined by using the same buffer. (△) Citrate–phosphate buffer; (□) sodium phosphate buffer; (○) Tris-HCl buffer. (A) Protease A; (B) protease B; (C) protease C. The substrates were CBZ-Gly-Phe for proteases A and C, TAME for protease B.

50, 60, and 40 °C, respectively (Figure 7). Noguchi et al. (1976) observed a lower temperature optimum (40 °C) with the trypsinlike enzyme isolated from krill. The optimal temperature for trypsinlike protease B from milkfish muscle (60 °C) was much higher than that for protease B from krill (Chen et al., 1978), but similar to that for protease B from milkfish viscera (Chen et al., 1989). Proteases A, B, and C showed 50% inactivation at pH 8.0 when temperatures were 46, 51, and 48 °C, respectively (Figure 5), whereas the same proteases had optimal temperatures of 50, 60, and 40 °C at pH 8.0 (Figure 7). Possibly, the better stability of protease B observed in Figure 7 is because of a protective effect of the substrate, e.g., the activity loss of protease B in Figure 5 may be autolytic rather than thermal.

The optimal pH of proteases A and C for hydrolysis of CBZ-Gly-Phe was 7.0, while that for protease B for hydrolysis of TAME was 8.0. (Figure 8). The optimal pH for protease B is similar to that of the bovine and krill counterparts. This suggests that the prototropic groups around the active sites of the corresponding proteases isolated from three different organisms have similar p$K$ values; the environments around the active sites of the corresponding enzymes are similar regardless of the abovementioned organisms. In addition, during storage of milkfish the proteolytic activity caused by trypsinlike could be reduced to a minimum under acidic condition.

**Effect of Reducing Agents.** All of the reducing agents examined except the KCN showed marked inhibitory effect on the activities of proteases A, B, and C. Protease B appeared to be more susceptible to the reducing agents than proteases A and C (Table V). Ascorbic acid and L-cysteine were the most potent inhibitor followed by glutathione. This suggests that disulfide bonds play an important role in maintaining a unique conformation with which the proteases can function properly; ascorbic acid and cysteine might also be used to suppress autolysis during storage of milkfish.

**Table V. Effect of Reducing Agents on the Activity of Milkfish Muscle Proteases**

| reducing agent | concn, mM | rel act., % A[a] | B | C |
|---|---|---|---|---|
| control | | 100 | 100 | 100 |
| KCN | 1.0 | 96 | 90 | 107 |
| L-cysteine | 1.0 | 50 | 36 | 46 |
| ascorbic acid | 0.1 | 43 | 0 | 51 |
| glutathione | 0.1 | 92 | 39 | 84 |
| glutathione | 1.0 | 50 | 61 | 53 |

[a] The amounts of the proteases used are the same as those in Table III.

The trypsinlike proteases are recognized to exist in digestive tracts. It was interesting that the trypsinlike proteases are also present in fish muscle. The high degree of purification of proteases A and C demonstrated low activity in milkfish muscle or incomplete extraction of these proteases. Proteases A and C were markedly inhibited by pepstatin A (Table III) and are considered to be cathepsin A like proteases. These proteases belong to the pepstatin-sensitive proteases. Whether these pepstatin-sensitive proteases are involved in the fish muscle tenderization process or not is worthy of investigation and may lead to an understanding of the mechanism of the tenderization process of postmortem fish and shellfish muscle.

**Summary.** To summarize the present results, milkfish muscle proteases A and C are cathepsin A like with molecular weights of 15 600 and 35 600, respectively. Protease B is a trypsinlike enzyme with a molecular weight of 33 000.

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