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Purification and Characterization of Gelatinase-like Proteinases from the Dark Muscle of Common Carp (*Cyprinus carpio*)

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Gelatinolytic proteinases from common carp dark muscle were purified by 30–60% ammonium sulfate fractionation and a combination of chromatographic steps including ion exchange on DEAE-Sephacel, gel filtration on Sephacryl S-200, ion exchange on High-Q, and affinity on gelatin-Sepharose. The molecular masses of these proteinases as estimated by SDS-PAGE were 75, 67, and 64 kDa under nonreducing conditions. The enzymes revealed high activity at a slightly alkaline pH range, and their activities were investigated using gelatin as substrate. Metalloproteinase inhibitors, EDTA, EGTA, and 1,10-phenanthroline, almost completely suppressed the gelatinolytic activity, whereas other proteinase inhibitors did not show any inhibitory effect. Divalent metal ion Ca^{2+} is essential for the gelatinolytic activity. Furthermore, these gelatinolytic proteinases hydrolyze native type I collagen effectively even at 4 °C, strongly suggesting their involvement in the texture softening of fish muscle during the post-mortem stage.

KEYWORDS: Common carp; gelatin zymography; gelatinolytic proteinase; matrix metalloproteinase; purification

INTRODUCTION

The matrix metalloproteinases (MMPs) belong to a large family of endopeptidases that can degrade the resident proteins of basement membranes and the extracellular matrices (ECM) under both physiological and pathological conditions (*1–3*). Tissue metalloproteinases are normally found only in tissues undergoing remodeling or breakdown. Moreover, the enzymes are difficult to extract, because they are not dislodged by detergents, and it was thus proposed that they might bind to matrix components such as collagens (*3*). Until now, around 30 MMPs have been discovered in vertebrates, and they can be grouped into at least four subclasses based on substrate specificity and domain structure: interstitial collagenase, stromelysin, membrane-type MMPs, and gelatinase (*4–7*). MMPs such as gelatinase, collagenase, and membrane-type MMPs are widely distributed in vertebrates and play essential roles in hydrolyzing both gelatins and collagens (*5–8*). The gelatinase subclass consists of two members: MMP-2 (EC 3.4.24.24; gelatinase A) and MMP-9 (EC 3.4.24.35; gelatinase B); enzymes of this family are around 72 and 95 kDa in molecular mass, and both hydrolyze denatured collagen (gelatin) and pepsin-solubilized collagen types IV and V with high efficiency (*6*).

Gelatinolytic proteinases with properties like those of MMPs have been proposed to participate in the metabolism of collagens physiologically and in the post-mortem degradation of fish muscle during cold storage (*9–11*). Such proteinases have been identified in Pacific rockfish (*Sebastes* sp.) (*10*), ayu (*Plecoglossus altivelis*) (*12*), Atlantic cod (*Gadus morhua*), spotted wolfish (*Anarhichas minor*), and Atlantic salmon (*Salmo salar*) (*13*). cDNA of MMPs has been cloned from rainbow trout (*Oncorhynchus mykiss*) (*11*), medaka (*Oryzias latipes*) (*14*), common carp (*Cyprinius carpio*) (*15*), Japanese flounder (*Parali chthys olivaceus*) (*8*), and zebrafish (*Danio rerio*) (*16*). Gelatinolytic proteinases from humans (*17*), chickens (*18*), and cows (*19*) have been purified to homogeneity.

Generally, fish muscle consists of two types, white and dark. White muscle works mainly in aerobic metabolism and generates a short but strong contraction force, whereas dark muscle generates a slow twitch and continuous contraction for a long period (*20*). In most species of fish, white muscle occupies the largest proportion of the body, whereas dark muscle that localizes just under the lateral line makes up only a small part. Previous studies on mackerel (*21*), yellowtail (*22*), and carp (*20*) have indicated that ATPase activities in white muscle were higher those that in dark muscle, suggesting their different physiological roles. However, identification of MMPs from different species of fish were done using dorsal muscle (white muscle) or a mixture of white muscle and dark muscle, and

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isolation of such proteinases to purity has not been performed (*10, 12, 13*).

To elucidate the precise mechanism of fish muscle tenderization during the post-mortem stage, a detailed study of gelatinolytic proteinases with properties like those of MMPs is necessary. Our primary examination revealed that the activity of gelatinolytic proteinases with properties like those of MMPs in dark muscle is much higher than that in white muscle, which makes it advantageous for the detection of enzymatic activity by zymography. Thus, in the present study, we tried to purify gelatinolytic proteinases from common carp dark muscle and investigate their characteristics.

MATERIALS AND METHODS

Fish. Cultured common carp (*C. carpio*) with body weights of about 700 g were purchased live from a fish market of Jimei, Xiamen, from February to May. The fish were sacrificed instantly. After decapitation and evisceration, dark muscle and white muscle of the fish were collected. Dark muscle was immediately used for experiment or kept at −70 °C for further use.

Chemicals. DEAE-Sephacel, Sephacryl S-200 HR, and gelatin-Sepharose 4B were purchased from Amersham Biosciences (Uppsala, Sweden); High-Q media, EDTA, and protein standards for SDS-PAGE were from Bio-Rad (Richmond, CA); ethylene glycol bis(2-aminoethylother)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was from Sigma (St. Louis, MO). L-3-Carboxy-*trans*-2,3-epoxypropionyl-L-leucine-4-guanidinobutylamide (E-64), bovine gelatin, and Triton X-100 were products of Amresco (Solon, OH). 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (Pefabloc SC) was a product of Merck (Darmstadt, Germany), and pepstatin A was purchased from Roche (Mannhem, Germany). Soybean trypsin inhibitor (STI) and type I collagen from the skeletal muscle of common carp were prepared in our own laboratory. Other reagents were all of analytical grade.

Purification of Gelatinolytic Proteinase. All procedures were performed at 4 °C. Common carp dark muscle (150 g) was homogenized with 4-fold of 50 mM Tris-HCl (pH 8.0) containing 5 mM $CaCl_2$ (buffer A) using a homogenizer (Kinematica, PT-2100, Switzerland). After centrifugation at 10000*g* for 10 min, the supernatant was collected, and the precipitate was further homogenized with 2-fold of buffer A and centrifuged. The resulting supernatants were collected together and fractionated with ammonium sulfate from 30 to 60% saturation. After centrifugation at 10000*g* for 15 min, the resulting precipitate was dissolved in buffer A and dialyzed against the same buffer extensively. The dialysate was subsequently applied to an ion-exchange column DEAE-Sephacel (2.5 × 17 cm) previously equilibrated with buffer A. After the column had been washed with buffer A until the absorbance at 280 nm reached baseline, binding proteins were eluted with a linear gradient of NaCl from 0 to 0.5 M in a total volume of 500 mL. Two fraction peaks (fractions I and II) with gelatinolytic activity were pooled, respectively. Fraction I was subsequently concentrated by ultrafiltration using a YM-10 membrane (Millipore) and loaded on a gel filtration column of Sephacryl S-200 (1.5 × 98 cm) equilibrated with buffer A and 0.2 M NaCl. Active fractions from Sephacryl S-200 were collected and dialyzed against buffer A and applied to the High-Q column. Binding proteins were eluted with a linear gradient of NaCl from 0 to 0.4 M in a total volume of 100 mL. Gelatinolytic activity fractions were collected and further applied to the affinity column, gelatin-Sepharose 4B (0.6 × 5 cm) equilibrated with buffer A containing 0.1 M NaCl. Successive stepwise elutions were done with buffer A supplemented with 1.0 M NaCl, 1.0 M NaCl containing 5% dimethyl sulfoxide (DMSO), 1.0 M NaCl containing 10% DMSO, and 2.0 M NaCl containing 10% DMSO, respectively. On the other hand, fraction II was further purified by passing through Sephacryl S-200 and gelatin-Sepharose 4B in the same way as fraction I. Active fractions from the final stage were used for enzymatic property analysis.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Gelatin Zymography. SDS-PAGE was performed under nonreducing conditions according to the method of Laemmli (*23*), and the gels were stained for proteins with Coomassie Brilliant Blue (CBB) R-250. Gelatin zymography was performed according to the method of Kleiner and Steler-Stevenson (*24*) with 1 mg/mL gelatin in the gel. Briefly, samples were mixed with one-fourth of SDS sample buffer (200 mM Tris-HCl, pH 6.8, containing 8% SDS, 0.4% bromophenol blue, and 40% glycerol) and then applied to 10% polyacrylamide gels and electrophoresed at 4 °C. After electrophoresis, the gels were washed with 50 mM Tris-HCl (pH 8.0) (buffer B) containing 2.5% (v/v) Triton X-100 for two 30 min periods followed by gentle shaking to remove SDS and rinsing with deionized water. The gels were then incubated at 37 °C for 12–24 h in buffer B containing 10 mM $CaCl_2$ and stained with CBB.

Protein Concentration Determination. Protein concentration was determined by measuring the absorbance at 280 nm of the sample solution or with the method of Lowry et al. (*25*), with bovine serum albumin as standard.

Effect of Temperature. To determine the effect of temperature on the proteinase activity, gels after gelatin zymography electrophoresis was washed and rinsed as described above and subsequently allowed to incubate in buffer B containing 10 mM $CaCl_2$ at different temperatures (10, 20, 30, 40, 50 °C) for 18 h, followed by CBB staining.

Effect of pH. To determine the proteinase activity at different pH values, gels after gelatin zymography electrophoresis were first washed with corresponding pH value buffers ranging from pH 4.0 to 11.0 and rinsed with deionized water. The gels were subsequently allowed to incubate in their appropriate pH value buffers for 18 h at 37 °C, followed by CBB staining. The buffers used were 0.2 M sodium acetate−HCl buffer (pH 4.0), sodium acetate buffer (pH 5.0–6.0), Tris−HCl buffer (pH 7.0–9.0), and glycine−NaOH buffer (pH 10.0–11.0).

Effect of Protein Inhibitors. To determine which kind of proteinases are found, it was necessary to investigate the inhibitory effect of different kinds of proteinase inhibitors on these enzymes. After gelatin zymography electrophoresis, gels were washed as described above and incubated in buffer B containing inhibitors with different final concentrations (EDTA, 10 mM; EGTA, 6 mM, 1,10-phenanthroline, 10 mM; STI, 20 μg/mL; Pefabloc SC, 1 mM; benzamidine 5 mM; E-64, 20 μM; pepstatin, 20 μM) at 37 °C for 18 h, followed by CBB staining.

Effect of Metal Ions. To investigate if metal ions are prerequisite for gelatinolytic activity, it is necessary to examine their effect on enzyme activity. Following gelatin zymography electrophoresis, the gels were washed as described above and then exposed to one of the following five treatment buffers at 37 °C for 18 h: 1, buffer B; 2, buffer B with 10 mM $CaCl_2$, 5 mM $MgCl_2$, and 100 μM $ZnCl_2$; 3, buffer B with 10 mM $CaCl_2$, 5 mM $MgCl_2$, 100 μM $ZnCl_2$, and 10 mM EDTA; 4, buffer B with 10 mM $CaCl_2$; 5, buffer B with 5 mM $MgCl_2$; 6, buffer B with 100 μM $ZnCl_2$.

Digestion of Type I Collagen and Gelatin. To investigate the digestive ability of these proteinases to native proteins, purified gelatinolytic proteinases were allowed to incubate with type I collagen purified from carp muscle and bovine gelatin, respectively. Type I collagen was incubated with an appropriate amount of activated proteinase in buffer B at 30 °C for 0, 0.5, 1, 3, and 6 h. Similarly, type I collagen degradation reactions were also carried out at 4 °C for 0, 3, 12, 24, 48, 96, and 168 h. On the other hand, gelatin was allowed to react with the proteinases at 37 °C for 0, 0.5, 1, 3, and 6 h. Samples were applied to 8% gels for electrophoresis followed by CBB staining.

RESULTS

Purification of Gelatinolytic Proteinases. In the present study, gelatinolytic proteinases were purified from the dark muscle of common carp by ammonium sulfate fractionation and sequential column chromatographies. Two gelatinolytic activity peaks (I, II) were detected by gelatin zymography in the fractions from DEAE-Sephacel (**Figure 1A**). Fraction I was further subjected to Sephacryl S-200 gel filtration, High-Q ion-exchange, and gelatin-Sepharose 4B affinity column. Fractions eluted with buffer A containing 1 M NaCl and 5% DMSO revealed gelatinolytic activity (**Figure 1B**). This fraction

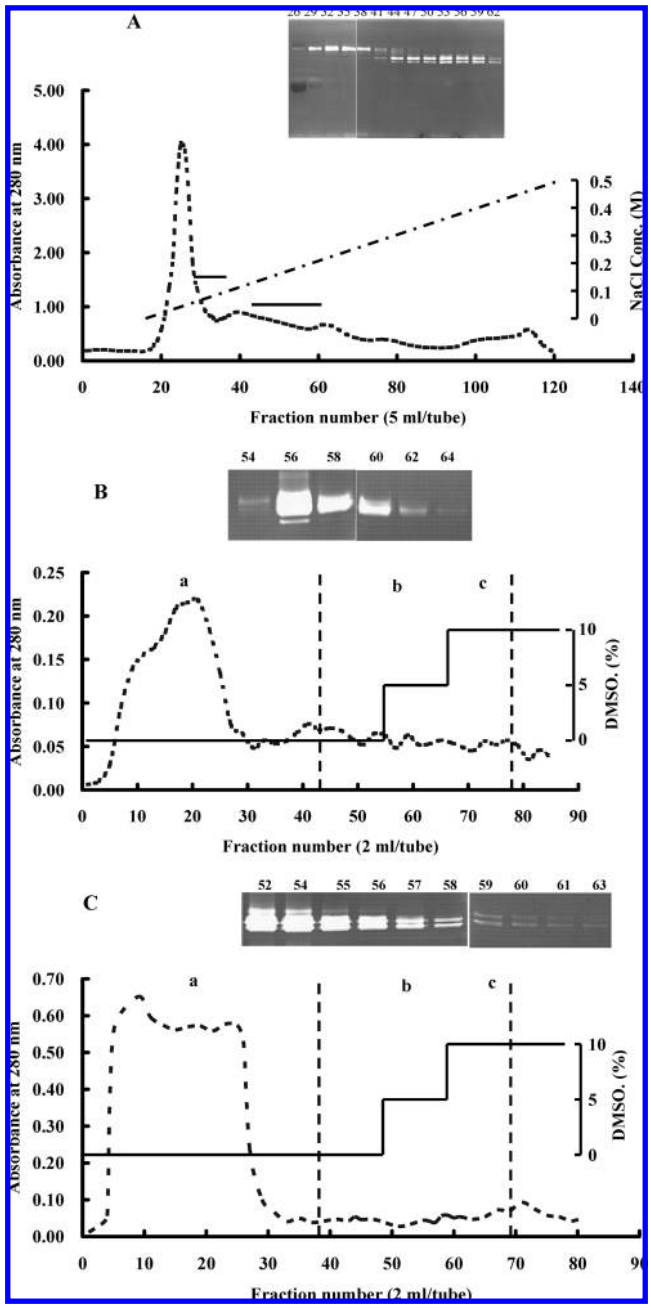

Figure 1. Column chromatography purification of common carp GP-I: (**A**) DEAE-Sephacel chromatography; (**B**) gelatin-Sepharose 4B chromatography of GP-I; (**C**) gelatin-Sepharose 4B chromatography of GP-II, III; (a) buffer A containing 0.1 M NaCl; (b) buffer A containing 1 M NaCl; (c) buffer A containing 2 M NaCl.

migrated as a single band with a molecular mass of 75 kDa on SDS-PAGE under nonreducing conditions. Gelatin zymography analysis showed the corresponding gelatinolytic active band, suggesting the purified enzyme (named GP-I) is a gelatinolytic proteinase (**Figure 2A**). On the other hand, fraction II from DEAE-Sephacel was also further subjected to Sephacryl S-200 gel filtration and gelatin-Sepharose 4B affinity column. Similar to GP-I, gelatinolytic active fraction II were also eluted out with buffer A supplemented with 1 M NaCl and 5% DMSO from the gelatin-Sepharose column (**Figure 1C**). On SDS-PAGE, however, two protein bands with molecular masses of 67 and 64 kDa were detected, and on gelatin zymography, two corresponding distinct zones of lysis with sizes of 69 and 65 kDa in gelatin substrate gels were detected (**Figure 2B**). These

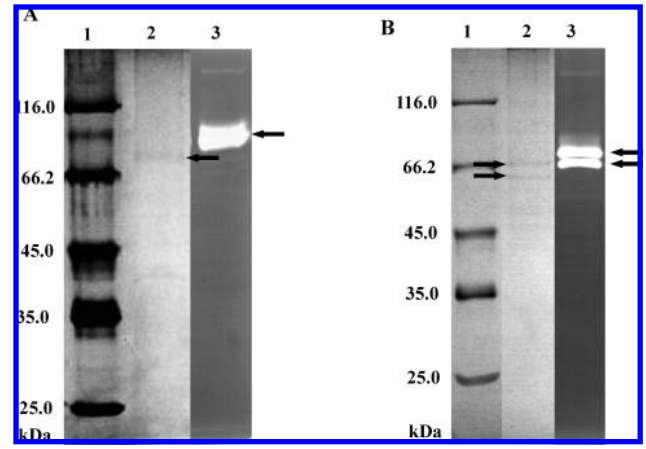

Figure 2. SDS-PAGE (lane 2) and gelatin zymography (lane 3) of purified gelatinolytic proteinases: (**A**) GP-I; (**B**) GP-II, III; lane 1, protein marker; lane 2, purified gelatinolytic proteinase was subjected to electrophoresis on a 10% gel followed by silver staining; lane 3, purified proteinase was subjected to gelatin zymography. The protein standards were β-galactosidase (116.0 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate degydrogenase (35.0 kDa), and restriction endonuclease (25.0 kDa). Arrowheads indicate the positions of protein bands.

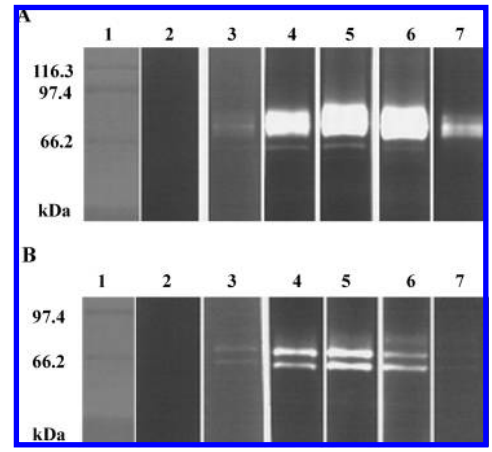

Figure 3. Gelatin zymography to detect the effect of temperature on gelatinolytic proteinases: (**A**) GP-I; (**B**) GP-II, III; lane 1, protein marker; lane 2, 10 °C; lane 3, 20 °C; lane 4, 30 °C; lane 5, 40 °C; lane 6, 50 °C; lane 7, 60 °C. Gelatin-incorporated gels were allowed to incubate in buffer B containing 10 mM $CaCl_2$ for 18 h at 10, 20, 30, 40, 50, and 60 °C, followed by CBB staining.

two proteinases were named GP-II and GP-III, respectively. We have tried to separate these two proteinases individually, but were not successful. Quite possibly, GP-III is a product from GP-II after autolytic cleavage as prolonged incubation resulted in only one active band corresponding to the size of GP-III on zymography (data not shown). As a result, starting from 150 g of carp dark muscle, approximately 16 μg of GP-I and 19 μg of GP-II and GP-III were obtained.

Temperature Optimum. The gelatinolytic activity at different temperatures was detected by gelatin zymography. Clear proteolytic bands of PG-I were revealed from 30 to 60 °C, and the band was clearer at 30 °C than that at 60 °C (**Figure 3A**). At 40 and 50 °C, the gelatinolytic activities were higher as the bands were much brighter under these two temperatures, suggesting the optimum temperature of GP-I was between 40 and 50 °C. The gelatinolytic active bands of GP-II and GP-III were clear from 30 to 50 °C, and the clearest bands were observed at 40 °C. Thus, the optimum temperatures of GP-II and III were both around 40 °C (**Figure 3B**).

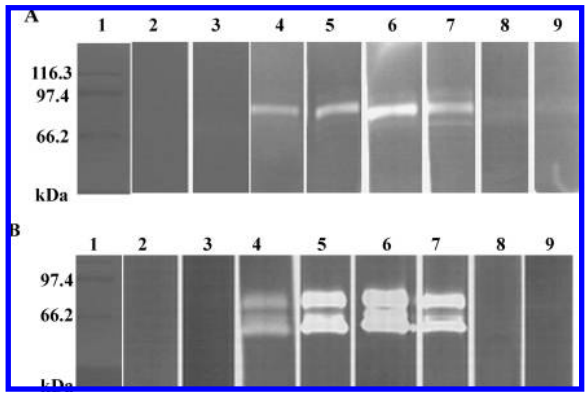

Figure 4. Gelatin zymography to detect the effect of pH on gelatinolytic activity: (**A**) GP-I; (**B**) GP-II, III; lane 1, protein marker; lanes 2–9 correspond to pH 4.0–11.0. Gelatin-incorporated gels were allowed to incubate at 37 °C for 18 h in different buffers with pH values of 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0, respectively, followed by CBB staining.

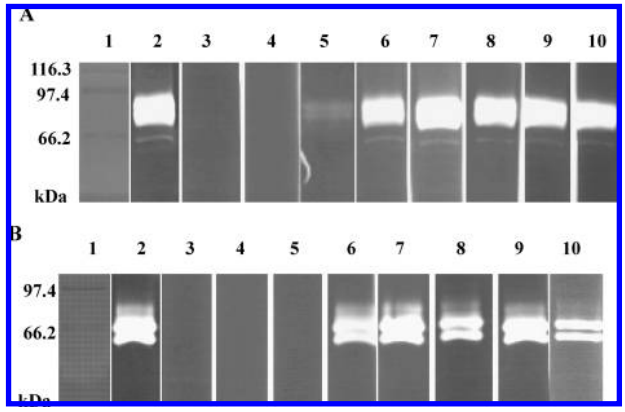

Figure 5. Gelatin zymography to investigate the inhibitory effect of proteinase inhibitors on gelatinolytic proteinases: (**A**) GP-I; (**B**) GP-II, III; lane 1, protein marker; lane 2, control (without inhibitor); lane 3, EDTA (10 mM); lane 4, EGTA (5 mM); lane 5, 1,10-phenanthroline (10 mM); lane 6, benzamidine (5 mM); lane 7, Pefabloc SC (1 mM); lane 8, STI (20 μg/mL); lane 9, E-64 (20 μM); lane 10, pepstatin A (20 μM). Gelatin-incorporated gels were allowed to incubate with proteinase inhibitors at 37 °C for 18 h in buffer B containing 10 mM $CaCl_2$, followed by CBB staining.

pH Optimum. Gelatinolytic activity at various pH values was also determined by gelatin zymography. As shown in **Figure 4A**, GP-I revealed high proteolytic activity from pH 6.0 to 9.0, and the highest activity was around pH 8.0. Similar to GP-I, GP-II and GP-III showed the same results (**Figure 4B**). These results indicated that all of these gelatinolytic enzymes are alkaline proteinases and their optimum pH values were around 8.0.

Effect of Proteinase Inhibitors. The inhibitory effect of proteinase inhibitors indicated that GP-I, GP-II, and GP-III were typical metalloproteinases as their gelatinolytic activities were almost completely suppressed by the metalloproteinase inhibitors EDTA, EGTA, and 1,10-phenanthroline (**Figure 5**). However, inhibitors for serine proteinases including STI, Pefabloc SC, and benzamidine, an inhibitor for cysteine proteinase, E-64, and an inhibitor for aspartic proteinase, pepstatin A, did not show any significant effects (**Figure 5**).

Effect of Metal Ions. To further characterize GP-I, GP-II, and GP-III as metalloproteinases, it is necessary to demonstrate that metal ions are required for the gelatinolytic activity. Also, it should be shown that chelating agents such as EDTA that

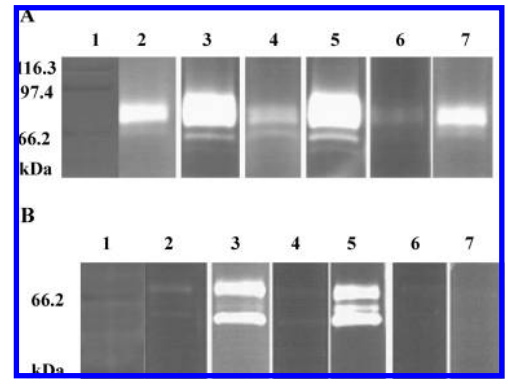

Figure 6. Gelatin zymography to investigate the effect of metal ions on the gelatinolytic proteinases: (**A**) GP-I; (**B**) GP-II, III; lane 1, protein marker; lane 2, control (without metal ions); lane 3, 10 mM $CaCl_2$, 5 mM $MgCl_2$, and 100 μM $ZnCl_2$; lane 4, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 100 μM $ZnCl_2$, and 10 mM EDTA; lane 5, 10 mM $CaCl_2$ only; lane 6, 5 mM $MgCl_2$ only; lane 7, 100 μM $ZnCl_2$ only. All gels were incubated at 37 °C for 18 h in buffer B containing different metal ions as described below, followed by CBB staining.

specifically bind metal ions would inhibit hydrolytic activity. As shown in **Figure 6** gelatinolytic activities of the three enzymes incubated in the presence of $CaCl_2$, $MgCl_2$, and $ZnCl_2$ were observed. However, with the addition of sufficient 10 mM EDTA, gelatinolytic activity was almost completely suppressed. Nevertheless, when $MgCl_2$ or $ZnCl_2$ only was present in the incubation buffer, enzyme activity was faintly evident. In the absence of metal ions, gelatinolyic activities were also less evident. These results strongly suggest that divalent ion Ca^{2+} is essential for the activity of these proteinases.

Digestion of Collagen and Gelatin. To identify the degradation effect of the three purified enzymes, it is necessary to investigate their hydrolyzing ability on different native substrates. As shown in **Figure 7A**, native type I collagen from carp was degraded by GP-I, GP-II, and GP-III with high efficiency. After incubation for 1 h, β-chain was digested greatly and the α_1-chain was also hydrolyzed. Complete degradation was observed after 6 h. Similarly, GP-II and GP-III also digested type I collagen effectively (**Figure 7B**). Such degradation was also observed after 12 h even at 4 °C, and after 168 h, the β-chain disappeared completely, although about 15–20% of the original α_1-chain remained (**Figure 8**). The digestion of these proteinases to gelatin was also investigated. As shown in **Figure 9A**, when GP-I and gelatin were incubated at 37 °C, complete digestion was observed after 6 h. Digestion by GP-II and GP-III was also detected (**Figure 9B**). However, no degradation of carp myofibrilar proteins by GP-I and GP-II, III was observed (data not shown), suggesting that these proteinases are specifically active to native collagens and gelatin.

DISCUSSION

In the present study, through ammonium sulfate fractionation and a series of column chromatographies, gelatinolytic proteinases (GP-I, GP-II, and GP-III) from carp dark muscle were purified. On DEAE-Sephacel column chromatography, GP-I was eluted at 0.1–0.2 M NaCl, whereas GP-II and GP-III were eluted at 0.2–0.4 M NaCl. Especially, GP-I, GP-II, and GP-III all bound to the gelatin-Sepharose and were eluted under same conditions (**Figure 1**). This result indicated that these gelatinolytic proteinases contain gelatin-binding domains, which are unique to gelatinases. The molecular masses of these proteinases as estimated by SDS-PAGE were 75, 67, and 64 kDa,

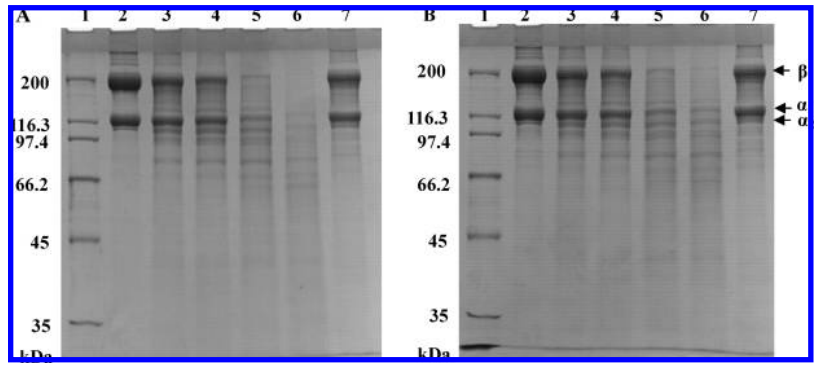

Figure 7. Degradation of type I collagen by GP-I or GP-II, III, respectively, at 30 °C: (**A**) GP-I; (**B**) GP-II, III; lane 1, protein marker; lane 2, 0 h; lane 3, 0.5 h; lane 4, 1 h; lane 5, 3 h; lane 6, 6 h; lane 7, collagen incubated at 30 °C for 6 h in the absence of enzyme. Portions of gelatinolytic proteinases (40 ng) and type I collagen (20 μg) were incubated in buffer B at 30 °C for 0, 0.5, 1, 3, and 6 h, respectively, followed by CBB staining.

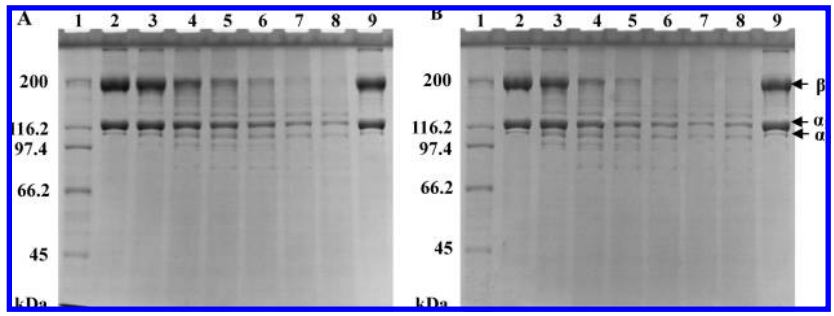

Figure 8. Degradation of type I collagen by GP-I or GP-II, III, respectively, at 4 °C: (**A**) GP-I; (**B**) GP-II, III; lane 1, protein marker; lane 2, 0 h; lane 3, 3 h; lane 4, 12 h; lane 5, 24 h; lane 6, 48 h; lane 7, 96 h; lane 8, 168 h; lane 9, control, collagen incubated at 4 °C for 168 h in the absence of enzyme. Portions of gelatinolytic proteinases (GP-I, 40 ng; GP-II, III, 40 ng) and type I collagen (20 μg) were incubated in buffer B at 4 °C for 0, 3, 12, 24, 48, 96, and 168 h, respectively, followed by CBB staining.

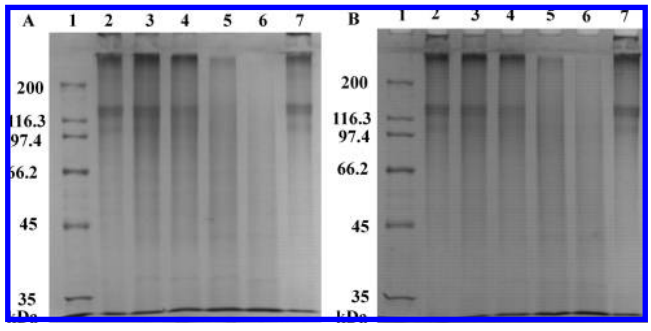

Figure 9. Degradation of gelatin by GP-I or GP-II, III, respectively, at 37 °C: (**A**) GP-I; (**B**) GP-II, III; lane 1, protein marker; lane 2, 0 h; lane 3, 0.5 h; lane 4, 1 h; lane 5, 3 h; lane 6, 6 h; lane 7, gelatin incubated at 37 °C in the absence of enzyme. Portions of proteinases (40 ng) and gelatin (20 μg) were incubated in buffer B at 37 °C for 0, 0.5, 1, 3, and 6 h, respectively, followed by CBB staining.

respectively, under nonreducing conditions, and on gelatin zymography, gelatin lysis bands at 80, 69, and 66 kDa were also detected (**Figure 2**). These results are in accordance with gelatinolytic enzymes identified in Altantic cod (*9*) and an MMP-2 from bovine pulmonary artery smooth muscle plasma membrane (*19*), but somewhat different from a gelatinolytic enzyme from freshwater fish ayu (*12*). However, gelatinolytic proteinases from Atlantic cod and ayu were identified in dorsal muscle (white muscle), and these enzymes were not purified to homogeneity, so comparison of the characteristics of the present enzymes with other species of fish could not be performed.

The optimal pH values for these gelatinolytic proteinases were around pH 8.0, suggesting they are alkaline enzymes. Similar optimal pH was also reported in metalloproteinases from Pacific rockfish (*Sebastes* sp.) (*10*). The optimal temperature of the present proteinases was around 40 °C (**Figure 3**), which is similar to a matrix metalloproteinase-like form from eastern oyster hemocyte (37 °C) (*26*).

Gelatinolytic activities were completely suppressed by metalloproteinase inhibitors EDTA, EGTA, and 1,10-phenanthroline, whereas serine, cysteine, and asparatic proteinase inhibitors including benzamidine, Pefabloc SC, STI, E-64, and pepstatin A did not show any significant effects (**Figure 5**), indicating that these gelatinolytic proteinases are metalloproteinases. Maximum gelatinolytic activity was detected in the presence of $CaCl_2$, whereas only very weak activity was revealed with Mg^{2+} or Zn^{2+}, and the activities were completely inhibited by EDTA even in the presence of Ca^{2+} (**Figure 6**). These results strongly suggested that Ca^{2+} is necessary for the activity, further demonstrating that these enzymes are metalloproteinases.

Gelatinolytic proteinases can be classified as matrix serine proteinase (MSP) and matrix metalloproteinase (MMP) (*12*). Our present results indicate that gelatinolytic proteinases in common carp dark muscle are quite possibly MMPs on the basis of the following lines of evidence: (1) these proteinases are active in gelatin zymographies and bind specifically to gelatin-Sepharose (**Figure 1**); (2) the enzymatic activities are most potent at pH values close to neutrality or higher (**Figure 4**); (3) the enzymatic activities were completely suppressed by metalloproteinase inhibitors EDTA, EGTA, and 1,10-phenanthroline (**Figure 5**); (4) these enzymes showed maximum activity in the presence of Ca^{2+} (**Figure 6**); and (5) the enzymes digested native type I collagen (**Figures 7** and **8**) and hydrolyzed gelatin (**Figure 9**).

Fish muscle after death and during cold storage degrades more rapidly than mammalian or chicken muscle (*8*). Disintegration of the muscle connective tissues is mainly responsible for the tenderization of fish muscle during chilled storage (*27*). Among

connective tissues, collagen plays a significant role in meat texture and contributes a fixed amount of background toughness (*28*). Four types of collagen (type I, II, V, and XI) have been identified in fish tissues and organs, whereas type I and V collagens are typical fibrillar collagen molecules in the intramuscular connective tissue (*27*). More recent study revealed that post-mortem tenderization processing of fish muscle is directly related to its collagen content (*29*). Thus, proteolytic breakdown of type I or V collagen is most likely relative to the disintegration of connective tissues, and metalloproteinases are proposed to be responsible for this reaction (*30, 31*). Several studies have been carried out in the molecular cloning of different metalloproteinases from different species of fish (*8, 14, 15*). However, to our knowledge, purification of native metalloproteinases from fish muscle has not been performed. Quite possibly, their extremely low content and strong binding with matrix proteins enhanced the difficulty in the isolation and purification of such proteinases. Using recombinant rainbow trout MMP-13, a collaganase, Saito et al. (*11*) reported that this recombinant proteinase cleaved the nonhelical domains of rainbow trout muscle type V collagen as well as rainbow trout skin type I collagen at 10 and 5 °C. More recently, a recombinant human MMP-9 was identified as effective in degrading native collagen types I and III (*32*). However, Kerkvliet et al. (*33*) reported collagen breakdown in soft connective tissue explants is associated with the level of active gelatinase A (MMP-2).

In the present study, we purified gelatinolytic proteinases with properties like those of MMPs from carp dark muscle, which effectively digest type I collagen at 30 °C and even at 4 °C. These results gave direct evidence that these gelatinolytic proteinases are involved in the disintegration of intramuscular connective tissue that induces the post-mortem tenderization of fish muscle during cold storage.

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