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ARTICLE

Purification and Characterization of a Gelatinolytic Metalloproteinase from the Skeletal Muscle of Red Sea Bream (*Pagrus major*)

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A gelatinolytic metalloproteinase (gMP) from red sea bream (*Pagrus major*) skeletal muscle was highly purified by ammonium sulfate fractionation and column chromatographies including (diethylamino)ethyl (DEAE)-Sephacel, phenyl-Sepharose, and gelatin-Sepharose. Purified gMP revealed two bands with molecular masses of 52 and 55 kDa as estimated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) under reducing conditions. The 55 kDa band is quite possibly a glycosylated form of the 52 kDa band. The proteinase revealed optimal activity at 40 °C and pH 8.0. Metalloproteinase inhibitors including ethylenediaminetetraacetic acid (EDTA), ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 1,10-phenanthroline specifically suppressed its activity. gMP was also significantly inhibited by cysteine and dithiothreitol. Divalent metal ion Ca^{2+} is essential for its gelatinolytic activity. Thus, the proteinase is regarded as a matrix metalloproteinase-like proteinase. Furthermore, gMP hydrolyzed gelatin and type-I collagen effectively even at 4 °C, suggesting the possibility of its involvement in the texture tenderization of fish muscle during the post-mortem stage.

KEYWORDS: Red sea bream; gelatin zymography; gelatinolytic proteinase; matrix metalloproteinase; purification

INTRODUCTION

The matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent endopeptidases that play central roles in degrading the resident proteins of basement membranes and the extracellular matrix (ECM) under both physiological and pathological conditions (*1*, *2*). Until now, more than 30 MMPs have been discovered in vertebrates, and they can be classified into six subgroups: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs. MMPs are synthesized as pre-pro-MMPs, and most of them are secreted from cells as zymogens (pro-MMPs), which need to be activated outside the cell. The major activation pathways are proteolytic processes *in vivo*, and they can also be activated *in vitro* by a SH-reactive agent, such as 4-aminophenylmercuric acetate (APMA), sodium dodecyl sulfate (SDS), reactive oxygen, and heat treatment. MMPs are widely distributed in vertebrates, are involved in the metabolism of ECM and non-ECM proteins, and can be inhibited by chelating agents, such as ethylenediaminetetraacetic acid (EDTA) and 1,10-phenanthroline (*1*−*3*).

Texture is considered to be one of the most important quality indices attributed to the commercial value of fish and meat. Generally, post-mortem tenderization of fish meat during cold storage occurs much faster than domestic animals and is one of the most unfavorable quality changes (*4*−*6*). The rigor mortis is completed between 3 and 18 h after killing in the case of pelagic fish while 24 h for bovine (*4*). It is a general concept that post-mortem muscle softening is caused by the proteolysis of muscle structural proteins, such as myofibrillar and ECM proteins (*7*−*9*). Seki and Watanabe (*8*) revealed that changes in myofibrils caused softening of carp muscle during post-mortem storage. In fact, the integrity of fish muscle is also maintained by ECM components, such as collagen fibrils and other proteins mainly localized in the myocommata region (*10*, *11*). Among collagens, type I and V are typical and major fibrillar collagen molecules in the intramuscular connective tissue, playing important roles in meat texture, and contributing a fixed amount of background toughness (*6*, *12*). Consequently, proteolytic breakdown of extracellular macromolecular proteins, such as collagens, by metalloproteinases is supposed to be a major cause for fish meat tenderization (*6*, *13*). Gelatinolytic proteinases with properties similar to MMPs have been proposed to participate in the metabolism of collagens physiologically and in the post-mortem degradation of fish muscle during cold storage. Such fish included squid (*Loligo opalescens*) (*14*), Pacific rockfish (*Sebastes* sp.) (*15*), yellowtail and ayu (*13*, *16*), Atlantic cod (*Gadus morhua*), spotted wolfish (*Anarhichas minor*), and Atlantic salmon (*Salmo salar*) (*17*). More recently, we reported that gelatinase-like metalloproteinases from common carp (*Cyprinus carpio*) dark muscle could hydrolyze native type-I collagen effectively even at 4 °C (*18*). Although

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much effort has been made on the identification of gelatinolytic metalloproteinases (gMPs) from fish muscle (*13*, *15*, *17*, *18*), to our knowledge, however, isolation of such proteinases to purity has not been performed from marine fish.

Red sea bream (*Pagrus major*) is one of the important economic marine fish species widely cultured in China and Japan. Such a kind of fish is sold either alive or on ice. Thus, understanding the characteristics of proteinases and especially such gMPs in fish whole muscle will surely be beneficial for elucidating the mechanism of muscle tenderization during cold storage. In this paper, we tried to purify gMP from red sea bream skeletal muscle and investigate its characteristics.

MATERIALS AND METHODS

Fish. For each individual experiment, three to four cultured red sea bream (*P. major*) (body weight of 600−700 g) were purchased alive from a fish market in Jimei, Xiamen, China. Fish were obtained from February to July and sacrificed instantly. After decapitation and evisceration, skeletal muscle was immediately obtained and used for the experiment.

Chemicals. (Diethylamino)ethyl (DEAE)-Sephacel, phenyl-Sepharose 6-Fast Flow, and gelatin-Sepharose 4B were purchased from Amersham Biosciences (Uppsala, Sweden). Proteins markers were from Fermentas (Vilnius, Lithuania) and Bio-Rad (Richmond, CA). Bezamidine, 1,10-phenanthroline monohydrate, EDTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), APMA, bovine serum albumin (BSA), and L-amino acids were from Sigma (St. Louis, MO). Bovine gelatin, Triton X-100, and L-3-carboxytrans-2,3-epoxypropionyl-L-leucine-4-guanidinobutylamide (E-64) were products of Amresco (Solon, OH). 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefabloc SC) was a product of Merck (Darmstadt, Germany). Pepstatin A was purchased from Roche (Mannhem, Germany). Type-I collagen from the skeletal muscle of red sea bream was prepared in our laboratory. Other reagents were all of analytical grade.

SDS−Polyacrylamide Gel Electrophoresis (PAGE) and Gelatin Zymography. SDS−PAGE was performed according to the method by Laemmli (*19*), and the gels were stained for proteins with Coomassie Brilliant Blue R-250 (CBB). Enzymatic activity toward gelatin was analyzed by gelatin zymography as described by Wu et al. (*20*), with some modification. Briefly, samples were diluted 3:4 (sample/buffer ratio) in SDS sample buffer (200 mM Tris-HCl at pH 6.8 containing 8% SDS, 0.4% bromophenol blue, and 40% glycerol) and then applied to 12% polyacrylamide gels containing 1 mg/mL bovine gelatin (Amresco, Solon, OH) 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 15 h in 50 mM Tris-HCl containing 5 mM $CaCl_2$ at pH 8.0 (buffer A) and stained with CBB. The area of enzyme activity appeared as a clear band on the CBB-stained dark blue background, and the clearness of the band is in positive correspondence with enzymatic activity.

Purification of gMP. All procedures were performed at 0−4 °C. About 500 g of minced fish muscle was homogenized in 3-fold of 25 mM sodium phosphate buffer (pH 7.5) containing 0.02% NaN_3 (buffer B) and centrifuged at 10000*g* for 15 min. The supernatant was fractionated with 40−70% $(NH_4)_2SO_4$, and the precipitate was dissolved in a minimum volume of buffer B followed by dialysis against the same buffer extensively. The dialyzed solution was subsequently applied to a DEAE-Sephacel column (2.5 × 27 cm) equilibrated with buffer B. Bound proteins were eluted at 1.0 mL/min with a 0−0.5 M linear NaCl gradient in buffer B, and fractions of 5 mL/tube were collected. Gelatinolytic active fractions were pooled, and ammonium sulfate was added to a final concentration of 1 M and applied to a phenyl-Sepharose 6-Fast Flow column (0.8 × 8 cm) pre-equilibrated with buffer B containing 1 M ammonium sulfate. The proteins retained were eluted using a linear decreasing gradient of ammonium sulfate from 1 to 0 M. Active fractions were pooled and dialyzed against buffer B containing 0.1 M NaCl and subsequently applied to an affinity column gelatin-Sepharose 4B (0.6 × 5 cm). Successive stepwise elutions were performed with buffer B 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. Fractions of 1 mL/tube were collected. Active fractions from the final stage were pooled and dialyzed against buffer B and used for enzymatic characterization.

Protein Concentration Determination. The protein concentration was determined by measuring the absorbance at 280 nm of the sample solution on column chromatography or with the method by Lowry et al. (*21*), with bovine serum albumin as the standard.

Effect of Proteinase Inhibitors, Metal Ions, Amino Acids, and Dithiothreitol (DTT). To investigate the effects of different proteinase inhibitors on the proteinase, gels after gelatin zymography electrophoresis were washed and rinsed as described above and subsequently allowed to incubate in buffer A containing 5 mM $CaCl_2$ with corresponding inhibitors (10 mM EDTA, 10 mM EGTA, 2 mM 1,10-phenanthroline, 2 mM Pefabloc SC, 5 mM benzamidine, 15 μM E-64, and 15 μM pepstatin A) at 37 °C for 15 h, respectively, followed by CBB staining. Control tests were performed in the absence of proteinase inhibitors.

To investigate if metal ions are a prerequisite for gelatinolytic activity, it is necessary to examine their effect on enzyme activity. After gelatin zymography electrophoresis, the gels were washed and subsequently allowed to incubate in buffer A containing different divalent metal ions in a final concentration of 5 mM at 37 °C for 15 h, respectively, followed by CBB staining. Control tests were performed in the absence of metal ions.

The effects of different L-amino acids and DTT on the proteinase activity were also detected at a final concentration of 5 mM with a similar method as described in metal ions.

Effects of Temperature and pH. To determine the effect of the temperature on the proteinase activity, gels after gelatin zymography electrophoresis were washed and rinsed as described above and, subsequently, allowed to incubate in buffer A containing 5 mM $CaCl_2$ at different temperatures (4−60 °C) for 15 h, followed by CBB staining.

Optimal pH of the proteinase was also determined by gelatin zymography. Briefly, gels washed and rinsed as described above were allowed to incubate in different pH buffers containing 5 mM $CaCl_2$ at 37 °C for 15 h, followed by CBB staining. The buffers used were 0.2 M sodium acetate buffer (pH 5.0−6.0), Tris-HCl buffer (pH 7.0−9.0), and glycine-NaOH buffer (pH 10.0).

Degradation of Type-I Collagen and Gelatin. To investigate the degradation ability of the proteinase to native proteins, purified proteinase was allowed to incubate with type-I collagen from red sea bream muscle and bovine gelatin, respectively. Type-I collagen was incubated with the proteinase in buffer A containing 5 mM $CaCl_2$ at 37 °C for 0 (control), 1, 3, 6, 12, and 24 h. To test the activity of the proteinase at lower temperatures, type-I collagen degradation reactions were also carried out at 4 °C for 0 (control), 24, 72, 120, 168, and 216 h. On the other hand, gelatin was allowed to react with the proteinase at 37 °C for 0 (control), 1, 3, 6, 9, 12, and 24 h. Samples were applied to 10% gels for electrophoresis followed by CBB staining.

RESULTS

Purification of Gelatinolytic Proteinase. In the present study, a gelatinolytic proteinase from red sea bream skeletal muscle was highly purified through ammonium sulfate fractionation and sequential column chromatographies. Six gelatinolytic active bands with molecular masses in the range of 32 to 200 kDa were detected by gelatin zymography in fractions from DEAE-Sephacel (**Figure 1A**). After phenyl-Sepharose hydrophobic interaction column chromatography, major proteinases with sizes of 52 and 55 kDa were coeluted by 0.3−0.1 M $(NH_4)_2SO_4$ and detected in fractions 125−145, while other proteinases were mostly eluted by 0.9−0.4 M $(NH_4)_2SO_4$ and detected in fractions 30−60 (**Figure 1B**). The major 52 and 55 kDa proteinase fractions were pooled, dialyzed, and loaded onto an affinity gelatin-Sepharose column for further purification. Fractions eluted with buffer B containing 1 M NaCl and 5% DMSO revealed gelatinolytic activity (**Figure 2A**). This fraction migrated as two bands with sizes of 55 and 52 kDa on SDS−PAGE by silver staining under reducing conditions. On gelatin zymography, one major active band (52 kDa) and one minor active band (55 kDa) were detected (lane 2 in **Figure 2B**). We have tried to separate these two

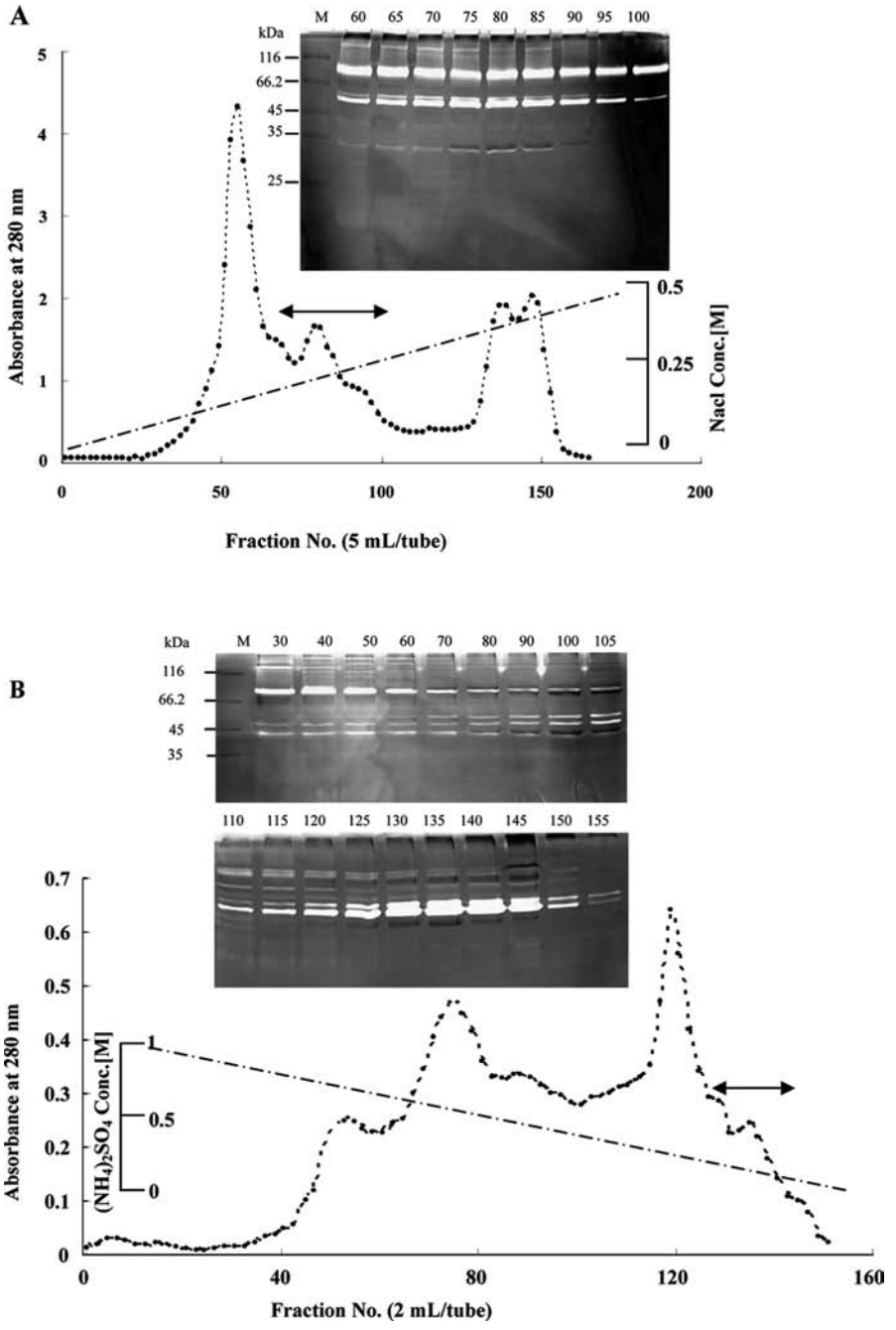

Figure 1. Column chromatography purification of gMP and gelatin zymography. (A) DEAE-Sephacel chromatography and (B) phenyl-Sepharose chromatography. The numbers on top of the lanes correspond to the fraction number.

proteinases individually but were not successful. Because of its effectiveness in degrading gelatin and its characteristics as a metalloproteinase as described below, the proteinase was thus named gMP. As a result, approximately 30 μg of highly purified gMP was obtained from 500 g of red sea bream skeletal muscle.

Effect of Protease Inhibitors and Metal Ions and Chemicals. The gelatinolytic activity of gMP was almost completely suppressed by EDTA, EGTA, and 1,10-phenanthroline, while inhibitors to the other three types of proteinases (serine, cysteine, and asparatic) did not reveal any inhibitory effect (**Figure 3**). These results strongly suggested that gMP is a metalloproteinase.

To further characterize gMP as a metalloproteinase, it is essential to demonstrate which metal ion(s) is required for the gelatinolytic activity. As shown in **Figure 4A**, the highest gelatinolytic activity of gMP was observed when the gel was incubated in buffer containing $CaCl_2$. The enzymatic activity was slightly activated by $BaCl_2$. However, gelatinolytic activity of gMP was faintly evident in the absence of metal ion (control) and also less evident in the presence of $MgCl_2$, $MnCl_2$, $FeSO_4$, $CdCl_2$, $ZnSO_4$, and $CuSO_4$, respectively. It is noteworthy that at least 0.2 mM $CaCl_2$ was needed for full activation of the proteinase (**Figure 4B**). These results gave the evidence that Ca^{2+} is essential for the activity of gMP.

Amino acids and DTT were also used in the present study to confirm the properties of gMP. As shown in **Figure 4C**, gelatinolytic activity of the enzyme was completely suppressed by DTT and strongly inhibited by L-cystein (Cys), respectively. In contrast, the other 19 L-amino acids did not show much inhibitory effects (data not shown).

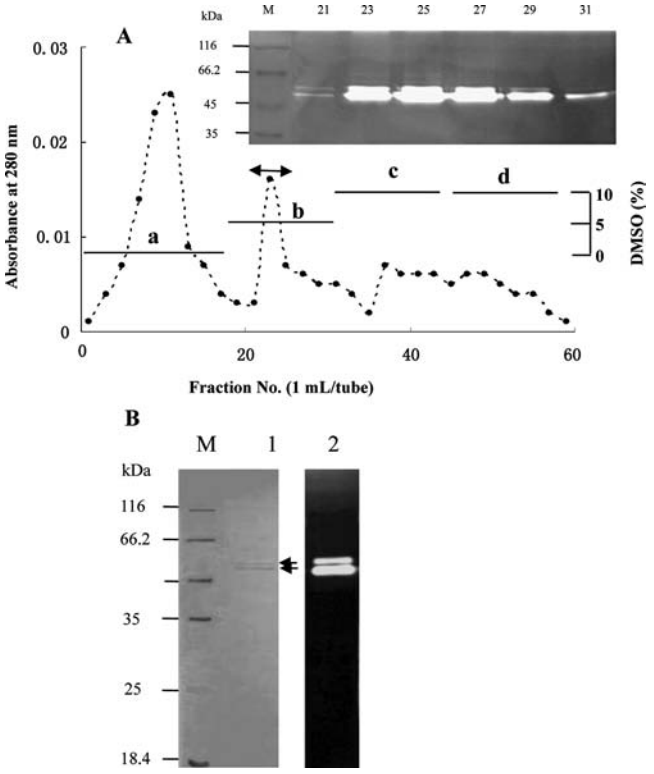

Figure 2. Gelatin-Sepharose affinity chromatography purification of gMP and gelatin zymography. (A) Column chromatography. The numbers on top of the lanes correspond to the fraction number. (a) Buffer B containing 1 M NaCl, (b) buffer B containing 1 M NaCl and 5% DMSO, (c) buffer B containing 2 M NaCl and 5% DMSO, and (d) buffer B containing 2 M NaCl and 10% DMSO. (B) SDS−PAGE followed by silver staining (lane 1), gelatin zymography (lane 2). Lane M, protein marker.

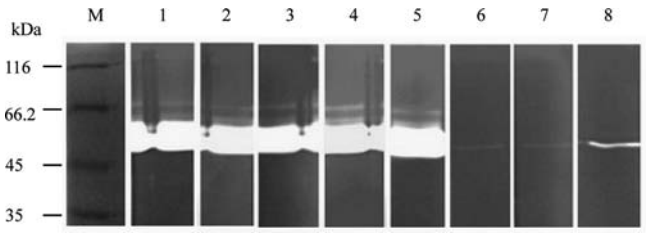

Figure 3. Gelatin zymography to investigate the inhibitory effect of proteinase inhibitors on gMP. Lane M, protein marker; lane 1, control (without inhibitor); lane 2, Pefabloc SC (2 mM); lane 3, benzamidine (5 mM); lane 4, E-64 (15 μM); lane 5, pepstatin A (15 μM); Lane 6, EDTA (10 mM); lane 7, EGTA (10 mM); and lane 8, 1,10-phenathroline (2 mM). Gelatin-incorporated gels were allowed to incubate in buffer A with different proteinase inhibitors at 37 °C for 15 h, followed by CBB staining.

Effects of Temperature and pH. Gelatinolytic activity of gMP at different temperatures was detected. A clear proteolytic band of gMP was revealed from 30 to 45 °C, and the optimal temperature of the present proteinase was around 40 °C (**Figure 5A**). However, when the incubation temperature was over 45 °C, the intensity of the active band decreased obviously. It is noteworthy that the proteinase retained gelatinolytic activity to somewhat degree even at 4 °C. Gelatinolytic activity at various pH values was also determined by gelatin zymography. As shown in **Figure 5B**, gMP revealed high proteolytic activity from pH 6.5 to 9 and the highest activity was around pH 8.0, suggesting it is an alkaline proteinase.

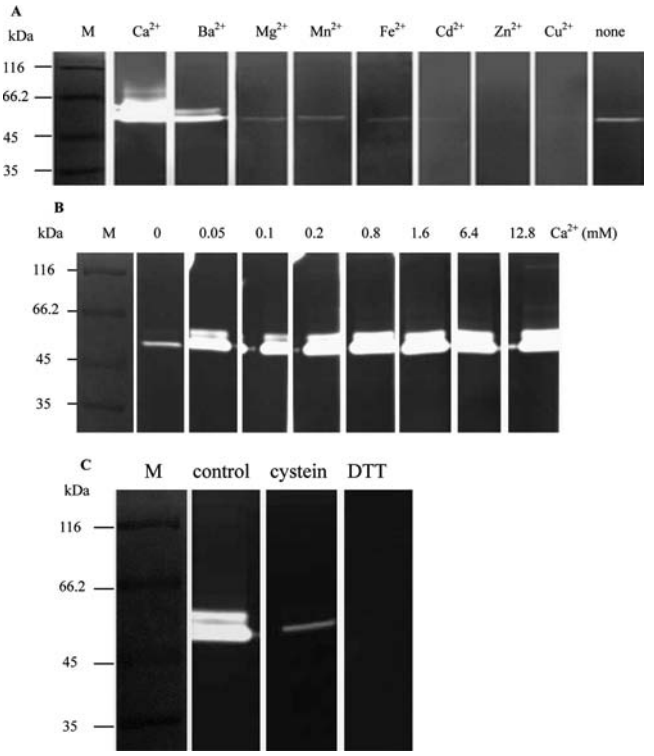

Figure 4. Gelatin zymography to investigate the effect of (A) metal ions, (B) Ca^{2+}, and (C) DTT and cystein on gMP. Gelatin-incorporated gels were allowed to incubate at 37 °C for 15 h, followed by CBB staining.

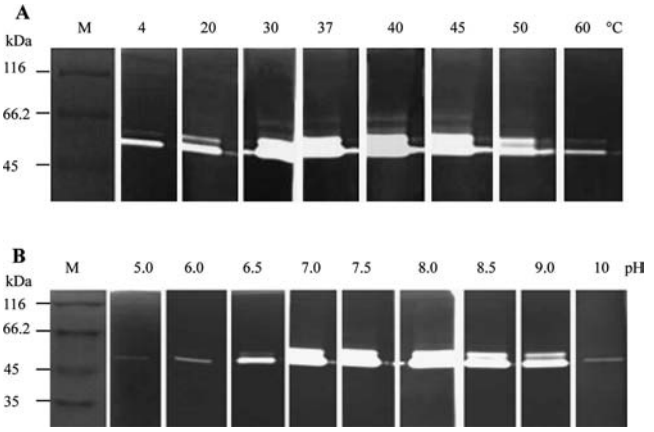

Figure 5. Optimal temperature and optimal pH of gMP. (A) Gelatin zymography to detect the effect of the temperature on gMP. Gelatin-incorporated gels were allowed to incubate in buffer A for 15 h at 4−60 °C, respectively, followed by CBB staining. (B) Gelatin zymography to detect the effect of pH on gMP. Gelatin-incorporated gels were allowed to incubate at 37 °C for 15 h in different buffers with pH values of 5.0−10, respectively, followed by CBB staining.

Digestion of Collagen and Gelatin. To identify the degradation effect of gMP, it is necessary to investigate its hydrolyzing ability on different native substrates. As shown in **Figure 6A**, red sea bream native type-I collagen was degraded by the proteinase with efficiency. After incubation at 37 °C for 12 h, the β chain was digested greatly and the α_1 chain was also hydrolyzed. Approximately 80% of the original collagen was degraded after 24 h. Such degradation was also observed even at 4 °C, and after 216 h, the β chain disappeared completely, although about 20% of the original α_1 chain remained (**Figure 6B**). The digestion of gMP to bovine gelatin was also investigated. As shown in **Figure 6C**,

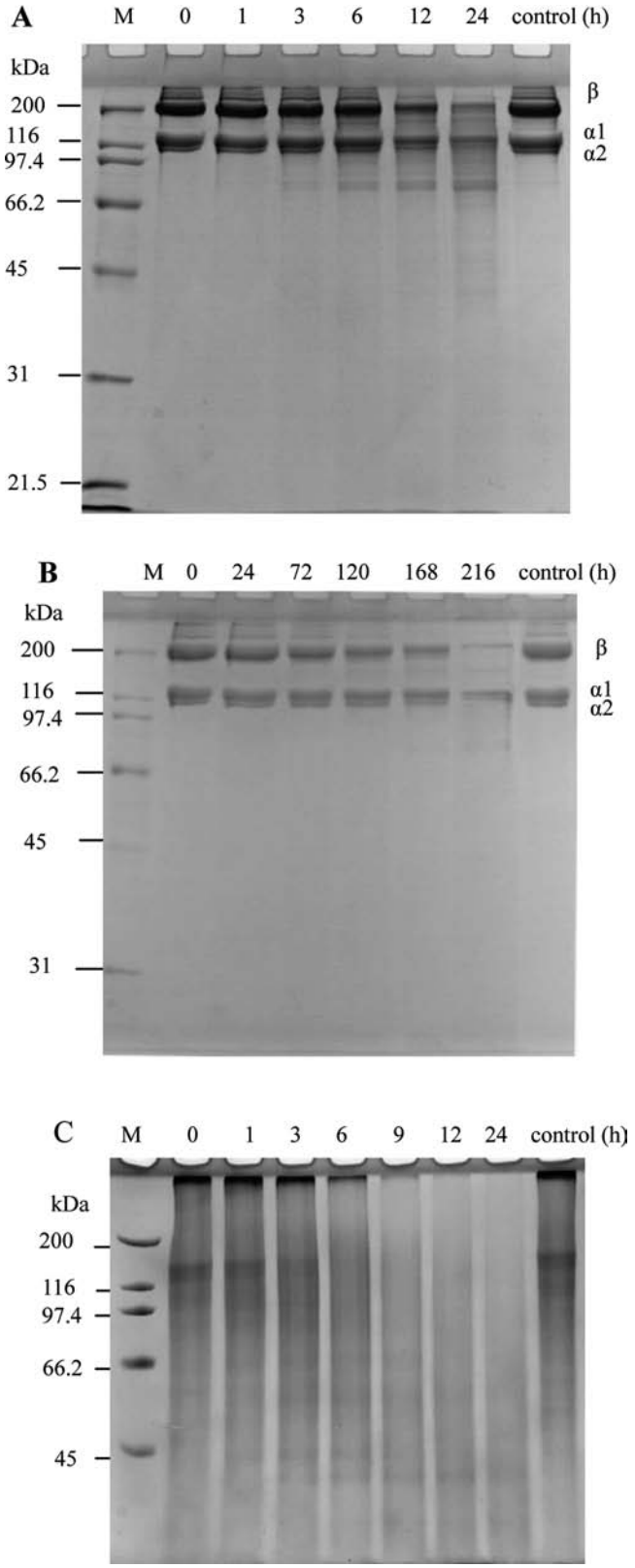

Figure 6. Degradation of type-I collagen at (A) 37 °C and (B) 4 °C and (C) bovine gelatin at 37 °C by gMP. gMP (≈40 ng) and red sea bream type-I collagen (≈20 μg) or gelatin (≈20 μg) were incubated in buffer A containing 5 mM $CaCl_2$ at 37 or 4 °C for different time intervals, respectively, followed by SDS−PAGE and CBB staining.

complete digestion of gelatin was observed after 24 h. In addition, with respect to the hydrolyzation, the proteinase revealed somewhat weaker collagenolytic activity than gelatinolytic activity.

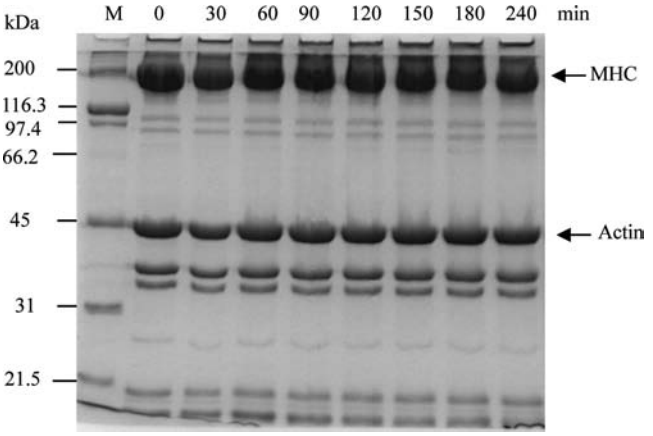

Figure 7. Proteolytic effect of gMP on myofibrillar proteins at 37 °C. gMP (≈40 ng) and myofibrillar proteins (≈40 μg) were incubated in buffer A containing 5 mM $CaCl_2$, 0.5 M NaCl, and 5 mM benzamidine at 37 °C for different time intervals, respectively, followed by SDS−PAGE and CBB staining.

However, degradation of myofibrilar proteins by gMP was not obvious (**Figure 7**), suggesting that the proteinase is specifically active to collagen and gelatin.

DISCUSSION

In the present study, we developed a protocol enabling the purification of a gMP by ammonium sulfate fractionation and column chromatographies including DEAE-Sephacel, phenyl-Sepharose, and gelatin-Sepharose. After phenyl-Sepharose hydrophobic interaction column chromatography, the 55 and 52 kDa active fractions were mostly separated from other gelatinolytic proteinases (**Figure 1B**). Then, using gelatin-Sepharose affinity chromatography, both 55 and 52 kDa active fractions bound to the column were eluted under the same conditions (**Figure 2**). On the other hand, the biochemical property of the 55 kDa fraction was in accordance with that of the 52 kDa fraction, suggesting these two enzymes are actually the same metalloproteinase, while the 55 kDa fraction is quite possibly a glycosylated form or isozyme of the 52 kDa fraction. Several MMPs were identified as glycoproteins. For example, human MMP-9 contains both N- and O-linked carbohydrates and has a differentially glycosylated form in recombinant MMP-9 expressed in insect cells (*22*). In the present study, however, because of the extremely low content and yield of gMP, glycosylation analysis of gMP and which family of MMPs the present enzyme belong to were not performed and remain for further investigation.

Gelatinolytic activities were mostly suppressed by metalloproteinase inhibitors EDTA, EGTA, and 1,10-phenanthroline, whereas asparatic, cysteine, and serine proteinase inhibitors, including pepstatin A, E-64, Pefabloc SC, and benzamidine, did not show any significant effects (**Figure 3**). On the other hand, maximum gelatinolytic activity was detected in the presence of $CaCl_2$ and weak activity was observed with $BaCl_2$, whereas only very weak activity was revealed with other bivalent metal ions, including Mg^{2+}, Mn^{2+}, Fe^{2+}, and Zn^{2+} (**Figure 4A**). In addition, 1,10-phenanthroline, a Zn^{2+} chelating agent (*23*), could mostly inhibit the enzymatic activity even in the presence of Ca^{2+} (**Figure 3**), strongly suggesting that the metalloproteinase uses Zn^{2+} as its metal co-factor, which is essential for its activity. However, in the presence of 5 mM Ca^{2+} and 0.1 mM Zn^{2+}, although noticeable enzymatic activity could be identified, the intensity of the gelatinolytic band was relatively fainter than those in the presence of Ca^{2+} only (data not shown). Many zinc

proteinases are inhibited by adding a higher concentration of zinc to the assays. In the case of the endoproteinase thermolysin, the inhibition mechanism is proposed to be due to zinc hydroxide binding the ionized carboxylate of an active-site glutamate residue, which then bridges the catalytic zinc by displacing its bound water (*24*).

The gelatinolytic activity of gMP was strongly inhibited by DTT and L-cystein (Cys) (**Figure 4B**), suggesting that disulfide(s) and the cysteine residue are essential for its activity. MMPs frequently use either disulfides or calcium ion to stabilize their structures, and the cysteine residue coordinates with the catalytic ion Zn^{2+} to maintain the latency of the enzyme (*2*). These results further suggested that gMP is quite possibly an MMP-like metalloproteinase.

Metalloproteinases with gelatinolytic activity have been found in muscles of various fish species, including ayu (*13*), Pacific rockfish (*15*), yellowtail (*16*), Atlantic cod, spotted wolfish (*17*), herring, and sardine (*25*). The molecular masses of MMPs are different because of their groups, zymogens, and active forms among various animals (*2*). In Japanese flounder, pro-MMP-9 and pro-MMP-2 are 78 and 63 kDa and they shift to active forms of 68 and 58 kDa, respectively, in the presence of APMA (*26*). In mammalians, the apparent molecular mass of pro-MMP-9 is 92 kDa (*27*). The molecular mass of chicken pro-MMP-9 is 75 kDa, and its active form is 60 kDa (*28*). Lødemel et al. (*29*) reported gelatinolytic activities at 225, 92, 82, 72, 62, and 52 kDa in Atlantic cod tissues, such as heart and liver. In common carp dark muscle, the 90 kDa metalloproteinase shifted to 75 kDa and the 70 kDa metalloproteinase shifted to active forms of 64 kDa via a new state of 67 kDa after treatment with APMA, respectively (*18*). More recently, Yoshida et al. (*30*) reported that two metalloproteinases G2 and G3 with molecular masses of 65 and 60 kDa were identified in the skeletal muscle of red sea bream. In the present study, the gMP exhibited two active bands with sizes of 55 and 52 kDa. The difference of molecular masses in these proteinases maybe owing to their different subgroups and also may arise from different experimental conditions employed.

The optimal temperature of gMP was around 40 °C (**Figure 5A**), which is similar to those of metalloproteinases from common carp dark muscle (*18*), marine crab hepatopancreas gelatinase (*31*), and a MMP-like proteinase from eastern oyster hemocyte (*32*), while it is different from that of barnacle larvae gelatinase, which was 25 °C (*33*). The optimal pH value for the present proteinase was around pH 8.0 (**Figure 5B**), which is almost the same as those of the above-mentioned metalloproteinases (*18*, *31*−*33*).

Unlike mammalian animal meats in which post-mortem aging is necessary for flavor enhancement, post-mortem tenderization in fish muscle degrades more rapidly than mammalian or chicken muscle and is one of the most unfavorable quality changes (*5*). Disintegration of muscle connective tissues is proposed to be responsible for the tenderization of fish muscle during chilled storage (*6*). A recent study revealed that post-mortem tenderization of fish muscle is adversely related to its collagen content (*5*). Hahn-Dantona et al. (*28*) reported that chicken gelatinase could hydrolyze denatured type-I collagen. Bracho and Haard (*15*) described two metalloproteinases with sizes of 47 and 95 kDa in Pacific rockfish skeletal muscle that could readily hydrolyze collagen and gelatin. A study by Saito et al. (*34*) revealed that type-I collagen was more susceptible to proteolysis by recombinant MMP-13 from rainbow trout. On the other hand, Kubota et al. (*6*) reported that recombinant jfMMP-9 from Japanese flounder did not hydrolyze collagen apparently, but this enzyme enhanced the solubilization of type-I collagen from crude connective tissue at chilled temperatures. More recently, we recognized that GP-I as well as GP-II and GP-III from common carp dark muscle hydrolyze type-I collagen and gelatin effectively (*18*). Thus, the proteolytic breakdown of collagen is most likely relative to the reduction of muscle elasticity. The degradation of collagen and gelatin by gMP (**Figure 6**) strongly suggested that it may play an active role in the degradation of ECMs of fish muscle during the period of post-mortem.

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