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# Identification of a novel gelatinolytic metalloproteinase (GMP) in the body wall of sea cucumber (*Stichopus japonicus*) and its involvement in collagen degradation

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ABSTRACT

Body wall that mainly consists of collagen and polysaccharides is the edible part of sea cucumber and is easy to go autolysis, while the proteinase(s) responsible for autolysis remains unclear. In the present study, a gelatinolytic metalloproteinase (GMP) from the body wall of sea cucumber *Stichopus japonicus* was purified to homogeneity by a combination of ammonium sulfate fractionation and chromatographic steps including DEAE-Sephacel, Sephacryl S-200, Q-Sepharose and Phenyl-Sepharose. The molecular mass of GMP as estimated by SDS-PAGE and gelatin zymography was 45 kDa. The enzyme revealed high activity at a slightly alkaline pH range (8.0–9.0) and the optimal temperature was at 40–45 °C. Metalloproteinase inhibitors, EDTA, EGTA, and 1,10-phenanthroline, almost completely suppressed the activity, whereas other proteinase inhibitors did not show any effect. Peptide mass fingerprinting of the enzyme obtained 3 peptide fragments with a total of 58 amino acid residues, which was 91.4% identical to an alkaline metalloprotease from *Pseudomonas fluorescens*, strongly suggesting it is a metalloproteinase. Divalent metal ion $Ca^{2+}$ is essential for its activity, indicating it is a calcium-dependent metalloproteinase. Furthermore, GMP hydrolyzed collagen effectively at 37 °C and gradually even at 4 °C, suggesting its involvement in the autolysis of sea cucumber.



## 1. Introduction

Sea cucumber (*Stichopus japonicus*) is one of the important aquatic species with abundant nutritional and tonic value. The sea cucumber farming has been a prosperous sector of marine economy, and its production in China reached about 138,000 tons in 2011 [1]. However, sea cucumbers are easy to go autolysis in response to a variety of environmental and mechanical factors. The autolysis of sea cucumber, resulting in huge nutritional and economic losses, has been becoming a major problem during processing and preservation.

As a special kind of seafood with high protein and low fat content, collagen accounts for about 70% of the total body wall protein of sea cucumber [2]. Autolysis of sea cucumber always accompanies with the degradation of collagen. Different endogenous proteinases have been reported to be responsible for the autolysis, including a highly alkaline protease from digestive tract [3]. During sea cucumber processing, it is of interest to notice that even if the viscera are removed, the body wall is still easy to go autolysis. Thus, we propose that endogenous proteinase(s) existing in the body wall play an important role in the autolysis by hydrolyzing the major protein component collagen. Though a cysteine-like protease, a cathepsin L-like enzyme and an acid phosphatase have been identified in the body wall [4–6], no much information is available for their effects on collagen degradation.

On the other hand, matrix metalloproteinases (MMPs), including collagenases, gelatinases, and stromelysins, distribute widely in vertebrates and are responsible for the degradation of collagen [7–10]. Gelatinolytic proteinases with properties like MMPs have also been proposed to participate in the metabolism of collagen physiologically in different species of fish [11–14]. Recently, we reported gelatinase-like metalloproteinases from common carp (*Cyprinus carpio*) dark muscle [15] and red sea bream (*Pagrus major*) skeletal muscle [16], which could hydrolyze native type I collagen effectively. A prolyl endopeptidase from the skeletal muscle of common carp (*Cyprinus carpio*) was also reported to involve in the degradation of collagen peptide fragments acquired from MMP hydrolyzing [17].

Gelatinolytic proteases have also been detected in sea urchins and are responsible for the degradation of extracellular matrix (ECM) involved in tissue and matrix remolding that occurs during

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sea urchin development [18,19]. As a unique organism, metalloproteinase was detected in the mesentery during intestine regeneration in the sea cucumber *Holothuria glaberrima* [20]. However, till now, no information concerning gelatinolytic metalloproteinase from the body wall of sea cucumber has been reported.

To elucidate the precise cause of autolysis, in the present study, we tried to purify a gelatinolytic metalloproteinase (GMP) from the body wall of sea cucumber and characterize its biochemical properties, especially its involvement in collagen degradation.

## 2. Materials and methods

### 2.1. Sea cucumber

The sea cucumbers were cultured in plastic cages at depth of 2–3 m in the sea and fed with seaweed (*Laminaria japonica*). Samples (body weight 100–200 g) were purchased alive during January to March, 2012, from a marine culturing base in Xiapu, Fujian province, China and transferred to our laboratory within 6 h. The sea cucumbers were sacrificed instantly and after evisceration, the body wall was immediately used for experiment or kept at −80 °C for further use.

### 2.2. Chemicals

DEAE-Sephacel, Sephacryl S-200 HR, Q-Sepharose and Phenyl-Sepharose 6-Fast Flow were purchased from GE Healthcare (Waukesha, WI, USA). Proteins markers and bovine serum albumin were from Bio-Rad (Richmond, CA, USA). Benzamidine, 1,10-phenanthroline monohydrate, EDTA, EGTA were from Sigma (St. Louis, MO, USA). Bovine gelatin, L-3-carboxytrans-2,3-epoxypropionyl-L-leucine-4-guanidinobutylamide (E-64) and Triton X-100 were products of Amresco (Solon, OH, USA). Pepstatin A was purchased from Roche (Mannhem, Germany), 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefabloc SC) was a product of Merck (Darmstadt, Germany). Porcine pepsin was from Sinopharm (Shanghai, China). Other reagents were all of analytical grade.

### 2.3. Preparation of sea cucumber collagen

Collagen from sea cucumber body wall was extracted as previously described by Liu et al. [21], with some modifications. All steps were performed at 0–4 °C. Sea cucumber body wall (50 g) was minced and washed in distilled water with continuous stirring for 30 min. The process was repeated twice. Washed samples were stirred in 10 volumes of 0.1 M Tris–HCl (pH 8.0) containing 5 mM EDTA and stirred for 24 h. Then, the samples were transferred to 10 volumes of distilled water and further stirred for 24 h. After centrifuged at 10,000 × *g* for 30 min, the precipitate was mixed with 10 volumes of 0.1 M NaOH and stirred for 72 h to dissolve non-collagen components. The collagen was recovered by centrifugation at 10,000 × *g* for 30 min. The insoluble component was washed with distilled water several times until the pH reached 7.0, followed by dissolving in 10 volumes of 0.5 M acetic acid containing 0.5% (w/w) porcine pepsin with continuous stirring for 48 h. The solution was centrifuged at 12,000 × *g* for 60 min, and the supernatant was collected. Collagen in the supernatant was salted out by adding NaCl to a final concentration of 0.8 M and stirred for 24 h. After centrifugation at 10,000 × *g* for 30 min, the collagen was collected and dissolved in 0.5 M acetic acid, and dialyzed against 0.02 M $Na_2HPO_4$. After centrifugation at 8000 × *g* for 10 min, the precipitate was dissolved in 0.5 M acetic acid and dialyzed against 0.5 M acetic acid. The purity of the collagen was checked by SDS-PAGE.

### 2.4. Purification of GMP

All procedures were performed at 0–4 °C. About 400 g of sea cucumber body wall were minced and homogenized with 4-fold of 20 mM Tris–HCl (pH 8.0) containing 5 mM $CaCl_2$ and 0.02% $NaN_3$ (buffer A) and centrifuged at 10,000 × *g* for 15 min. The supernatant was collected as crude enzyme and was fractionated with ammonium sulfate from 30% to 80% saturation. After centrifugation at 10,000 × *g* for 15 min, the precipitate was dissolved in a minimum volume of buffer A and dialyzed against the same buffer extensively. The dialyzed solution was subsequently applied to an ion-exchange column DEAE-Sephacel (2.5 cm × 10 cm) previously equilibrated with buffer A. After washing the column with equilibration buffer, bound proteins were eluted in turn with buffer A contain 0.1, 0.2, 0.3, 0.4, 0.5 M NaCl, and fractions of 5 mL/tube were collected. Gelatinolytic active fractions were pooled and concentrated by ultrafiltration using a YM-10 membrane (Millipore) and loaded on a gel filtration column of Sephacryl S-200 (2.5 cm × 98 cm) equilibrated with buffer A contain 0.15 M NaCl. Active fractions were collected and dialyzed against buffer A and applied to a Q-Sepharose ion exchange column (1.5 cm × 6 cm). Binding proteins were eluted with a 0–0.5 M linear NaCl gradient in buffer A. Gelatinolytic activity fractions were collected, ammonium sulfate was added to a final concentration of 1 M and applied to a Phenyl-Sepharose 6 Fast Flow column (1.5 cm × 5 cm) pre-equilibrated with buffer A 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 A and used for enzymatic characterization. During the whole process of GMP purification, the enzyme activity was detected by gelatin zymography as described below. The purification of GMP was individually repeated for 4 times and similar results were obtained.

### 2.5. SDS-PAGE and gelatin zymography

SDS-PAGE was performed under non-reducing conditions according to the method of Laemmli [22]. Enzyme activity was analyzed by gelatin zymography performed according to the method of Kleiner and Stetlerstevenson [23] with 1 mg/mL gelatin in the gel. Briefly, samples were mixed with a quarter 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 2.5% (v/v) Triton X-100 for 30 min by gently shaking to remove SDS, followed by rinsing with deionized water. The gels were then incubated at 37 °C for 18 h in 50 mM Tris–HCl, pH 8.0 (buffer B) containing 10 mM $CaCl_2$ and stained with Coomassie Brilliant Blue (CBB) R-250. 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 to enzyme activity.

### 2.6. MALDI-TOF/TOF-MS/MS analysis

In order to obtain the protein primary structure information, purified protein was run on a 10% gel and stained with silver nitrate and the target protein band was picked for mass spectrometry analysis [24]. MALDI-TOF mass spectra results were obtained using 4800 Plus MALDI-TOF/TOF-MS/MS Analyzer (Applied Biosystems, Foster, USA) by School of Life Science, Sun Yat-Sen University, China.

### 2.7. 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 of Lowry et al. [25], using bovine serum albumin, fraction V as standard.

### 2.8. Effect of proteinase inhibitors

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 B containing 10 mM $CaCl_2$ with corresponding inhibitors (10 mM EDTA, 10 mM EGTA, 10 mM 1,10-phenanthroline, 5 mM Benzamidine, 10 mM PMSF, 20 μM E-64, and 20 μM Pepstatin A) at 37 °C for 18 h, respectively, followed by CBB staining. Control tests were performed in the absence of proteinase inhibitors.

### 2.9. Effect of metal ions

To investigate if metal ions are prerequisite for gelatinolytic activity, their effects on enzyme activity were examined. Following gelatin zymography electrophoresis, the gels were extensively washed as described above and subsequently allowed to incubate in buffer B containing different divalent metal ions with a final concentration of 10 mM at 37 °C for 18 h. Control test was performed in the absence of metal ions.

### 2.10. Effect of temperature and pH

To determine the effect of temperature on the proteinase activity, gels after gelatin zymography electrophoresis were washed and rinsed as described above and subsequently incubated in buffer B containing 10 mM $CaCl_2$ at different temperatures (20, 30, 40, 45, 50, 60 °C) for 18 h, followed by CBB staining.

The effect of pH on the enzyme activity was also assayed by gelatin zymography. After SDS-PAGE, gels washed and rinsed as described above were allowed to incubate in different pH buffers for 18 h at 37 °C, followed by CBB staining. The buffers used were 0.2 M sodium acetate–HAC buffer (pH 4.0–6.0), Tris–HCl (pH 7.0–9.0) and glycine–NaOH buffer (pH 10.0–11.0).

### 2.11. Degradation of sea cucumber collagen

In order to know the degradation ability of the proteinase to collagen, purified enzyme was used. The experiment was performed using sea cucumber collagen dissolved in buffer B containing 10 mM $CaCl_2$ in the presence of GMP. In the control test (self-degradation), buffer was used to replace GMP. The collagen and GMP mixture was incubated at 37 °C for 0, 0.5, 1, 1.5, and 2 h. After incubation, samples were applied to 8% gel for electrophoresis followed by CBB staining. To test the activity of the proteinase at different temperatures, sea cucumber collagen degradation reactions were also carried out at 25, 27, 28, 29, 32, 35, 37 °C for 2 h. Furthermore, collagen was also allowed to react with the proteinase at 25 °C for 0, 2, 4, 8, 12, 16 h and at 4 °C for 0, 3, 6, 9, 18 d in the presence of 0.02% sodium azide to prevent bacterial growth.