A serine proteinase from sarcoplasmic fraction of red sea bream *Pagrus major* is possibly derived from blood

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**ABSTRACT**

Collagen degradation is known to be involved in post mortem tenderization of fish muscle. A serine proteinase which is assumed to be related to collagen degradation after fish death was purified from sarcoplasmic fraction of red sea bream *Pagrus major* by ammonium sulfate fractionation and column chromatographies on Sephacryl S-300, Q Sepharose and Phenyl Sepharose CL-4B. The enzyme hydrolyzed gelatin and was obtained as a protein band of approximately 38 kDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing condition. N-terminal amino acid sequence of the enzyme was determined for 32 residues. The protein having the same N-terminal amino acid sequence of the enzyme for 10 residues was purified from the serum of red sea bream and showed the same characteristics as the enzyme. Therefore, the serine proteinase was suggested to migrate from blood to muscle and to degrade muscle proteins after fish death.

**INTRODUCTION**

The degradation of collagen, one of the muscle structural proteins, is known as a cause of post mortem

muscle softening. Earlier reports have focused on collagen degradation in fish muscle after death. The degradation of

type I collagen by cathepsin L caused the muscle softening in chum salmon [1, 2]. The solubilization of type V collagen

was reported to be involved in softening of sardine muscle during chilled storage [3]. Also, gelatinolytic enzymes which

degrades gelatin, denatured collagen, have been investigated in various fish [4-8]. Metalloproteinases and serine

proteinases have been detected in various organs of yellowtail [4] and Atlantic cod [5]. Gelatinase-like proteinases were

purified from the dark muscle of common carp, and their activities were inhibited by metalloproteinase inhibitors [6].

Gelatinolytic serine proteinases were detected in the red stingray wing muscle by gelatin zymography [7]. In our

previous study, we also found gelatinolytic enzymes including a trypsin-type serine proteinase (G1) and two

metalloproteinases (G2 and G3) in red sea bream muscle [8]. These enzymes showed gelatinolytic activity under a

slightly acidic condition and 0-10°C, suggesting that they can degrade muscle proteins during transportation and storage

of fish, and are related to post mortem tenderization of fish muscle. Among them, G1 has the strongest gelatinolytic

activity, but its structure had not yet been clarified.

Hence, in this study, we purified G1 and determined its N-terminal amino acid sequence. From the homology

search of the sequence data, G1 was estimated to have a kringle domain and trypsin specific domain, indicating that it

may be involved in blood clotting or fibrinolysis. We hypothesized that G1 would be present in the blood and migrate to

the muscle after fish death. Thus, we searched G1-like enzyme from the serum of red sea bream and investigated its

N-terminal amino acid sequence in order to confirm where G1 derived from.

## MATERIALS AND METHODS

**Fish.**     Cultured red sea bream *Pagrus major* (body weight 2 kg) was purchased from Nagasaki Prefectural Federation of Fisheries Cooperative Association, Japan. 1,200 g of skeletal muscle was excised as previously described [8] and used for purification of a serine proteinase. G1-like enzyme was purified from the serum, which was separated from the blood by centrifugation.

**Chemicals.**      Sephacryl S-300, Q Sepharose and Phenyl Sepharose CL-4B were obtained from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Leupeptin, Pepstatin A and E-64 were purchased from Peptide Institute (Osaka, Japan). Pefabloc SC was purchased from Merck KGaA (Darmstadt, Germany). *o*-phenanthroline was from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Protein standard for gelatin zymography and SDS-PAGE were from Bio-rad (Hercules, CA, USA). All other chemicals used were of analytical grades.

**Assay of gelatinolytic activity.** Gelatinolytic activities of enzymes were analyzed by gelatin zymography. Gelatin zymography was performed as previously described [8].

**Purification of serine proteinase G1.** The crude enzyme was prepared from 1,200 g of red sea bream muscle and subsequently G1 was purified by column cromatographies on Sephacryl S-300, Q Sepharose and Phenyl Sepharose CL-4B in the same manner as previously described [8]. All purification steps were performed at 4°C. G1 was examined for purity using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

**Purification of G1-like enzyme from the serum of red sea bream.** G1-like enzyme was partially purified from 9 ml of the serum by column chromatographies in the same manner as purification of G1 from the muscle. Subsequently, the active fraction of Phenyl Sepharose was purified by Arginine Sepharose 4B affinity chromatography. The adsorbed fractions were eluted with a linear gradient of KCl from 0 to 0.6 M. All purification steps were performed at 4°C. G1-like enzyme was examined for purity by SDS-PAGE.

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis.** SDS-PAGE was performed according to the method of Laemmli [9] using 10 % polyacrylamide gel. After the run, the gel was stained with Coomassie Brilliant Blue R-250 and destained.

**Protein concentration.** Protein in column elutes were determined by measuring the absorbance at 280 nm. The protein concentration was determined by method of Lowry *et al.* [10] using bovine serum albumin as the standard.

**Determination of N-terminal amino acid sequence.** The purified enzyme was loaded to a polyacrylamide gel, and transferred to a polyvinylidene difluoride (PVDF) membrane. After brief staining with coomassie brilliant blue R-250, the protease band estimated from the molecular weight of gelatinolytic activity band was cut out and analyzed by Procise 492 protein sequencer (Applied Biosystemes, CA, USA).

**RESULTS AND DISCUSSION**

In this study, we purified G1 by ammonium sulfate fractionation, gel filtration, anion exchange chromatography and hydrophobic chromatography. The chromatographic profile on Phenyl Sepharose CL-4B column is shown in Fig. 1. Within adsorbed fractions, a single protein peak corresponded to an enzymatic active peak (Fraction No. 59-61). Hence, active fractions in this peak were pooled and then checked for purity by SDS-PAGE (Fig. 2). The main protein band was detected at approximately 85 kDa on SDS-PAGE under non-reducing condition (Fig. 2, lane 2, a black arrow-head) and the gelatinolytic band of G1 was detected at approximately 90 kDa on gelatin zymography. When the polyacrylamide gel containing 0.1% bovine gelatin was used in the SDS-PAGE, the protein band of G1 was estimated at approximately 90 kDa, the same molecular weight as the activity band of G1 on gelatin zymography (data not shown). It may be caused by the affinity of G1 to the gelatin in the polyacrylamide gel. The result suggests that the gelatinolytic enzyme was electrophoresed on gelatin zymography more slowly than on SDS-PAGE. Therefore, the 85

5

kDa protein band was confirmed as the protein band of G1. Under reducing condition, the protein band of G1 was detected at approximately 38 kDa (Fig. 2, lane 3, a white arrow-head). The two protein bands were transferred to a PVDF membrane and analyzed by a protein sequencer. Both N-terminal amino acid sequences completely corresponded with each other. These results suggest that G1 consist of two homologous subunits connected by disulfide bond. Previously, Yanagihara et al. reported that WSP, the serine proteinase from white croaker (Argyrosomus argentatus) skeletal muscle, was homodimer because its molecular weight was estimated by SDS-PAGE to be 32,000 and 71,000 with and without the reducing regent, respectively [11]. Therefore, G1 was thought to have the same subunits structure as WSP. On the other hand, there were some reports of serine proteinases having subunit structures different from G1. Kinoshita et al. reported that the serine proteinase from the sarcoplasma of threadfin-bream had monomeric subunit structure with a molecular weight of 77,000 [12]. The serine proteinase from the skeletal muscle of white croaker (Micropogon opercularis) was reported by Folco et al. to consist of two different subunits with molecular weights of 20,000 (α-subunit) and 15,500 (β-subunit) [13]. In this study, the N-terminal amino acid sequence of G1 was determined for 32 residues (Fig. 3). G1 showed homologies of 28.1% to carp trypsin [14], 15.6% to bovine trypsin (NCBI Protein, accession number CAA38513) and 25.0% to bovine thrombin (NCBI Protein, accession number AAA30781). Their homologies were relatively low. On the contrary, the sequence of G1 has a 70.4% identity with the sequence of WSP [15]. Both proteinases of G1 and WSP were purified from the sarcoplasmic protein. Therefore, G1 was assumed to be the same kind of proteinase as WSP. Also, G1 has a 67% identity with fugu e_gw2.53.94.1 (JGI Fugu rubripes v4.0, protein ID 558408), which is similar to hyaluronic acid binding protein 2 (HABP2) or urokinase (uPA: urinary type plasminogen activator). Those proteins are serine proteases and involved in fibrinolysis. Choi-Miura et al. reported that HABP2 converted the inactive single-chain uPA to the active two-chain uPA [16]. uPA is known as a fibrinolytic enzyme to generate plasmin from plasminogen. Both enzymes have one kringle domain and one trypsin

specific domain in common. The trypsin specific domain includes active residues of peptidase S1 family. In this study, the N-terminal amino acid sequence of G1 begins with the trypsin specific domain. Also, fugu e_gw2.53.94.1 contains a kringle domain upstream of the trypsin specific domain. Therefore, we think G1 has one trypsin specific domain and may have one kringle domain upstream of the trypsin specific domain. The kringle domain of G1 was assumed to have been already cleaved, because the sequence of a kringle domain wasn't detected in the N-terminal amino acid sequence of G1. Those domains that G1 may have are found throughout blood clotting and fibrinolytic proteins, consequently, we hypothesized that G1 derived from blood. To confirm the origin of G1 we searched G1-like enzyme from the serum of red sea bream and investigated the inhibitor spectrum and the N-terminal amino acid sequence.

A gelatinolytic activity was detected in the serum of red sea bream at approximately 97 kDa, a molecular weight similar to that of G1 (Fig. 4). The gelatinolytic activity was inhibited by Pefabloc SC, a proteinase inhibitor for serine protease (Fig. 4, lane 5). The gelatinolytic enzyme was therefore assumed to be a serine proteinase and a G1-like enzyme, and consequently termed G1-S. G1-S was partially purified from the serum of red sea bream. The chromatographic profile on Arginine Sepharose 4B column is shown in Fig. 5. A single protein peak in the adsorbed fraction was found to correspond to the enzymatic active peak. This peak was checked for purity by using SDS-PAGE (data not shown). The protein band of G1-S was detected in the peak, but was contaminated by several other proteins. Thus, the active fractions (Fraction No. 63-79) except for the protein peak were pooled and analyzed by SDS-PAGE. When proteins contained in the polyacrylamide gel were transferred to a PVDF membrane, the protein band of G1-S was detected at approximately 94 kDa, a lower molecular weight than its activity band (Fig. 6, lane 2). Since gelatinolytic enzymes are electrophoresed on gelatin-gel more slowly than on SDS-PAGE, the 94 kDa protein band was assumed to be the protein band of G1-S. The N-terminal amino acid sequence for 10 amino acid residues of G1-S was determined by a protein sequencer. As shown in Fig. 7, the sequence of G1-S was completely identical to the

N-terminus of G1. Consequently, G1, the gelatinolytic enzyme from the muscle, was thought to have been present in the blood of red sea bream and may have been accidentally isolated from muscle homogenate as a sarcoplasmic proteinase. Although there is a difference of the molecular weight between G1 (85 kDa: Fig.2, lane 2) and G1-S (94 kDa: Fig.6, lane 2), their N-terminal amino acid sequence corresponded. Hence, the C-terminal region of G1 may have been degraded during purification or migration from blood to muscle.

The mRNA of G1 must be studied to clarify how G1 is synthesized and migrates to muscle. HABP2 was isolated from human plasma and the mRNA of it was detected in human liver, pancreas and kidney [17]. Kristensen *et al*. reported that the ploteolytic activity and mRNA of uPA were detected in mouse kidney [18]. Therefore, G1 are assumed to be synthesized in the hepatopancreas or kidney. We are currently conducting complementary DNA cloning of G1 from the hepatopancreas or kidney of red sea bream to confirm where G1 are synthesized and to determine its primary structure.

Serine proteinase inhibitors (serpins) are proteins regulating proteolytic events associated with blood coagulation, fibrinolysis and connective tissue turnover. Serine proteinase inhibitors have been found in some teleost fish such as carp and rainbow trout [19-22]. Serpins involved in G1 need to study for understanding the regulation of its proteolytic activity. Serpins involved in G1 need to study for understanding the regulation of its proteolytic activity.

In this study, it is suggested that G1 is present in blood, migrates to the muscle through the capillary and participates in the collagen degradation with the matrix metalloproteinases in the post-mortem muscle of red sea bream. Further studies are needed to determine the primary structure and origin of G1. The study on collagenase is also important to clarify the initiation of collagen degradation.

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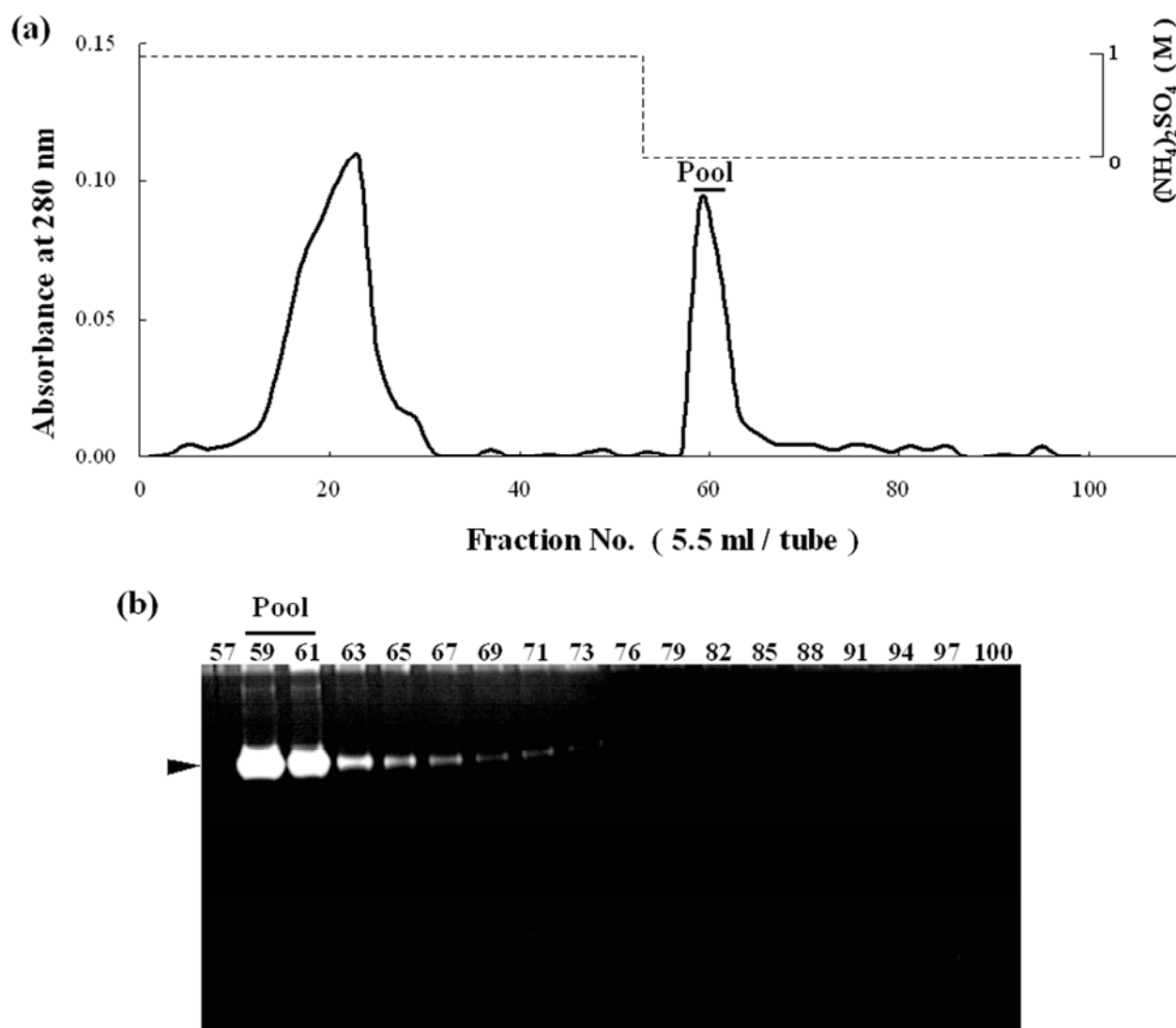
Fig. 1 Phenyl Sepharose CL-4B hydrophobic chromatography of the active fraction from Q Sepharose. **a** The elution pattern of proteins; **b** gelatinolytic activity of each fraction. The enzyme solution (47.5 mg protein) was applied to a column (1.46 × 12 cm) equilibrated 50 mM Tris-HCl (pH 7.5) containing 5 mM CaCl$_2$ and 1 M (NH$_4$)$_2$SO$_4$, and eluted by the same buffer without (NH$_4$)$_2$SO$_4$. Three-milliliter of Fractions were collected. A bar indicates pooled fractions. A black arrow-head indicates the activity band of G1. The gelatin gel was incubated at 37°C for 18 h
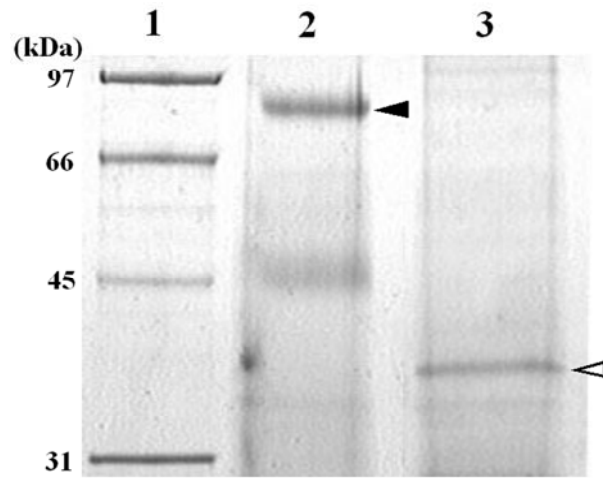
Fig. 2    SDS-PAGE of purified G1. The purified G1 was applied to 10 % polyacrylamide gel electrophoresis and stained with CBB. Lane 1, protein standard; lane 2, 67 μg protein of purified G1 (non-reducing); lane 3, 44 μg protein of purified G1 (reducing). Black and white arrow-heads indicate protein bands of G1 under non-reducing and reducing conditions, respectively

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                                              10              20               30
Red sea bream G1 (muscle)    1   I L G G L K V S P G   S I P W Q V S V Q V   R P Q N S N L P F K   H T   32
White croaker WSPᵃ           1   I F G G L K V P P A   G I P W Q A S V Q V   R P X N S A Q           27
Fugu e_gw2.53.94.1ᵇ        173   I F G G L K V N P G   G I P W Q V S V Q V   K Q K N T N Q I F R   H V  204
Carp trypsin Aᶜ              1   I V G G Y E X E P Y   S T P W Q A S L N S   G Y H F X G G S L V   V E   32
Bovine trypsinᵈ             21   I V G G Y T C G A N   T V P Y Q V S L N S   G Y H F C G G S L I   N S   52
Bovine thrombinᵉ          367   I V E G Q D A E V G   L S P W Q V M L F R   K S P Q E L L C G A   S L  398
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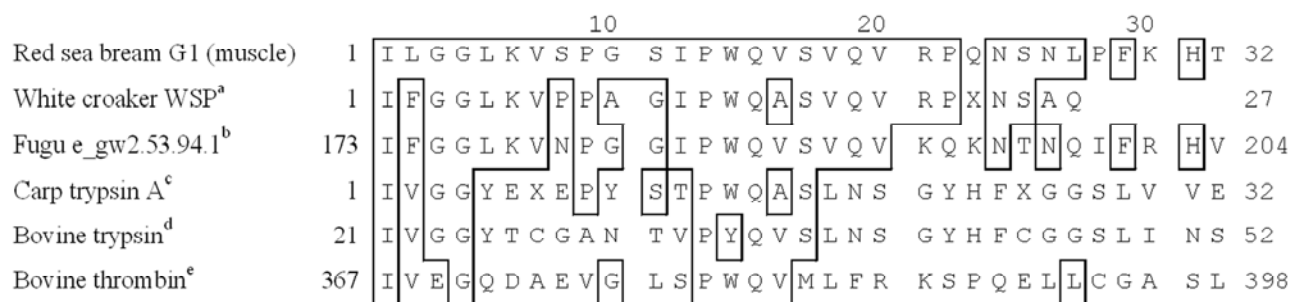
Fig. 3     Alignment of the N-terminal amino acid sequence of G1 with other proteins. Numbers indicate positions of each of the amino acid sequence. Identical residues with G1 were boxed. [a]White croaker WSP (Cao *et al*. [15]); [b]fugu e_gw2.53.94.1 (JGI *Fugu rubripes* v4.0, protein ID 558408); [c]carp trypsin A (Cao *et al*. [14]); [d]bovine trypsin (NCBI Protein, accession number CAA38513); [e]bovine thrombin (NCBI Protein, accession number AAA30781)
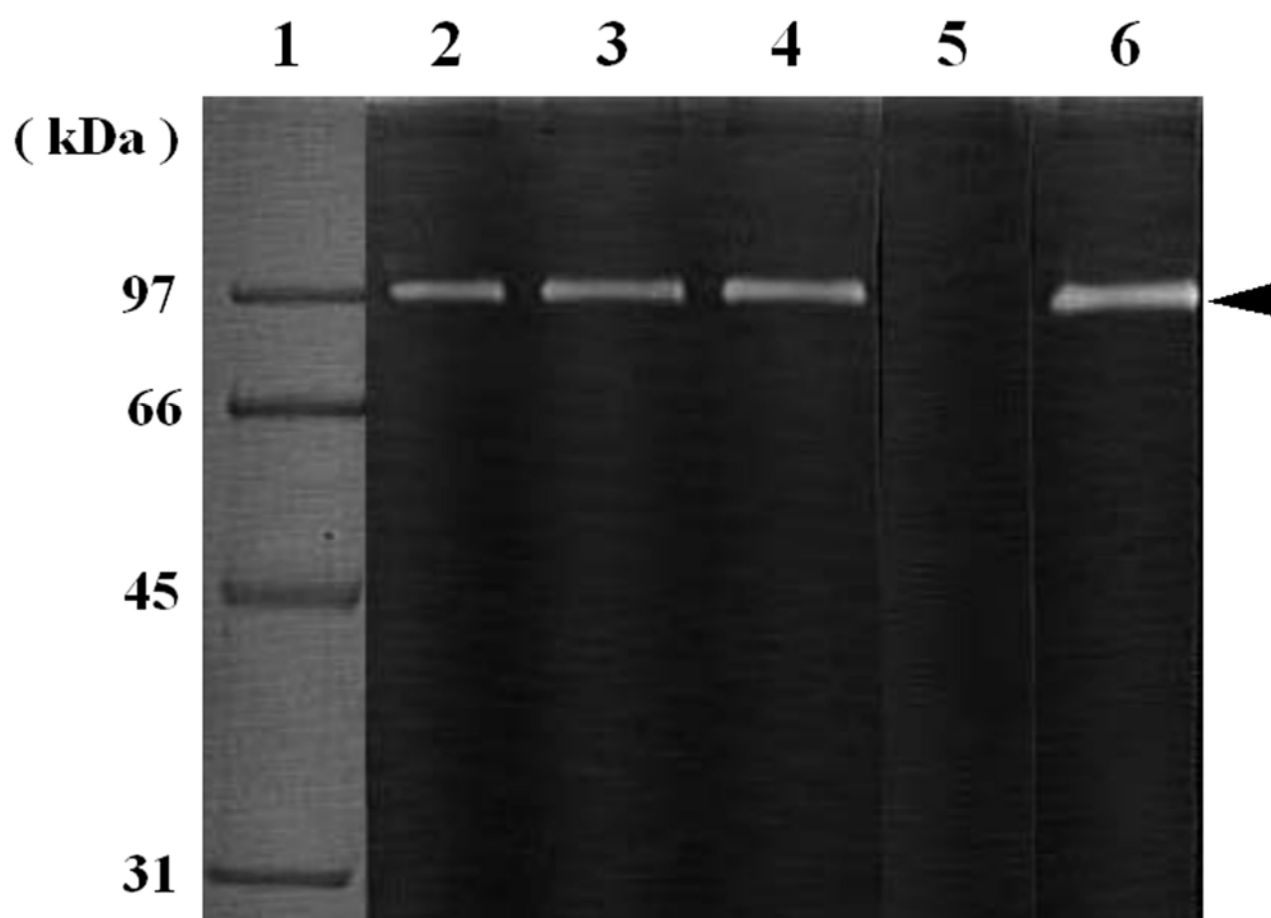
Fig. 4    Effect of proteinase inhibitors on G1-like enzyme using gelatin zymography. After 0.15 µL of the serum was preincubated with the respective inhibitors for 5 min on ice, it was loaded to gelatin gel. The gelatin gel was incubated at 37°C for 2 h. Lane 1, protein standard; lane 2, non-treated serum; lane 3, serum treated with 0.01 mM Pepstatin A; lane 4, with 0.01 mM E-64; lane 5, with 10 mM Pefabloc SC; lane 6, with 10 mM EDTA. A black arrow-head indicates the activity band of G1
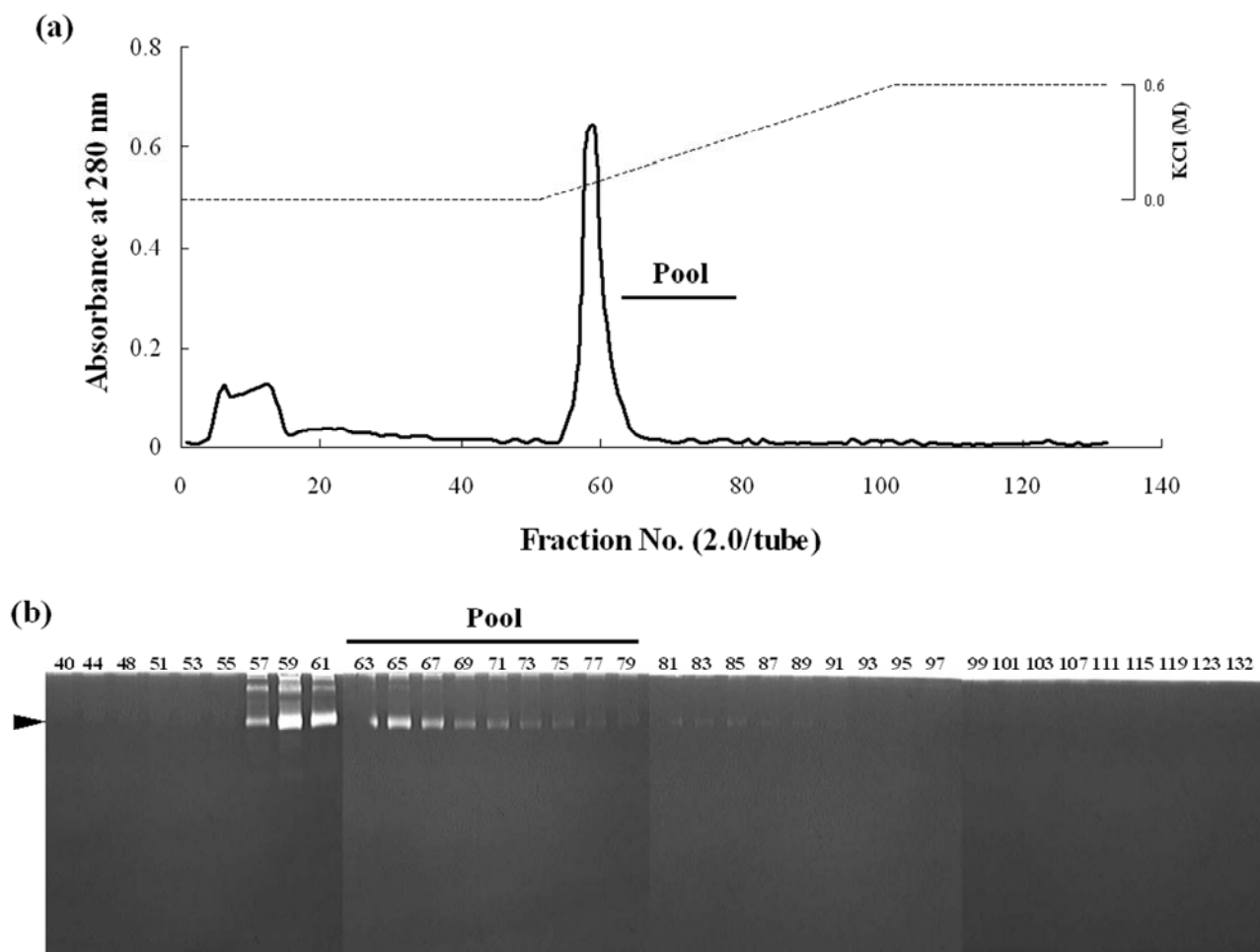
Fig. 5   Arginine Sepharose 4B affinity chromatography of the active fraction from Phenyl Sepharose. **a** The elution patterns of proteins and enzyme activities; **b** gelatinolytic activity of each fraction. The Q-Sepharose fraction (17.2 mg protein) was applied to a column equilibrated with 50 mM Tris-HCl (pH 7.5) containing 5 mM $CaCl_2$, and eluted with 0-0.6 M KCl gradient. Two-milliliter of fractions were collected. A bar indicates pooled fractions. A black arrow-head indicates the activity band of G1. The gelatin gel was incubated at 37°C for 18 h
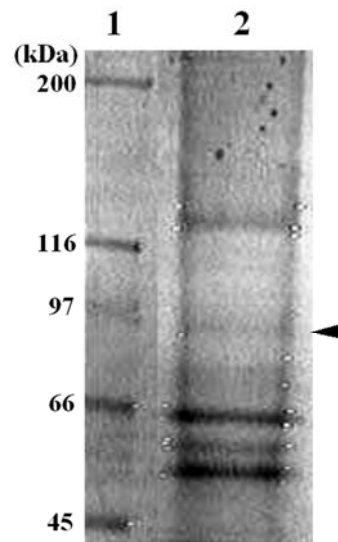
Fig. 6    SDS-PAGE of purified G1-like enzyme. After SDS-PAGE, proteins in the polyacrylamide gel were

transferred to a PVDF membrane, and the membrane was stained with CBB. Lane 1, protein standard; lane 2, 410 μg

protein of purified G1-S (non-reducing). A black arrow-head indicates a protein band of G1

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                                          10                    20                     30
Red sea bream G1-S (serum)  │I L G G L K V S P G│
Red sea bream G1 (muscle)   │I L G G L K V S P G│  S I P W Q V S V Q V   R P Q N S N L P F K   H T
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Fig. 7      Alignment of the N-terminal amino acid sequence of G1 and G1-like enzyme. Numbers indicate positions of

each of the amino acid sequence. Identical residues with G1-S were boxed