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Original Article

# Isolation from *Gloydius blomhoffii siniticus* Venom of a Fibrin(ogen)olytic Enzyme Consisting of Two Heterogenous Polypeptides

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**Key Words**

Fibrin(ogen)olytic enzyme, *Gloydius blomhoffii siniticus,* serine proteinase, snake venom

**Abstract**

**Objective:** This study was undertaken to isolate a fibrin(ogen)olytic enzyme from the snake venom of *Gloydius blomhoffii siniticus* and to investigate the enzymatic characteristics and hemorrhagic activity of the isolated enzyme as a potential pharmacopuncture agent.

**Methods:** The fibrinolytic enzyme was isolated by using chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and fibrin plate assay. The characteristics of the enzyme were determined by using fibrin plate assay, protein hydrolysis analysis, and hemorrhage assay. Its amino acid composition was determined.

**Results:** The fibrin(ogen)olytic enzyme with the molecular weight of 27 kDa (FE-27kDa) isolated from *G. b. siniticus* venom consisted of two heterogenous disulfide bond-linked polypeptides with the molecular weights of 15 kDa and 18 kDa. When more than 20 μg of FE-27kDa was applied on the fibrin plate, fibrinolysis zone was formed as indicating its fibrinolytic activity. The fibrinolytic activity was inhibited completely by phenylmethanesulfonylfluoride (PMSF) and ethylenediaminetetraacetic acid (EDTA) and partially by thiothreitol and cysteine. Metal ions such as $Hg^{2+}$ and $Fe^{2+}$ inhibited the fibrinolytic activity completely, but $Mn^{2+}$ did not. FE-27kDa preferentially hydrolyzed $\alpha$-chain of fibrinogen and slowly hydrolyzed $\beta$-chain, but did not hydrolyze $\gamma$-chain. High-molecular-weight polypeptides of gelatin were hydrolyzed partially into polypeptides with molecular weights of more than 45 kDa. A dosage of more than 10 μg of FE-27kDa per mouse was required to induce hemorrhage beneath the skin.

**Conclusion:** FE-27kDa was a serine proteinase consisting of two heterogeneous polypeptides, hydrolyzed fibrin, fibrinogen, and gelatin, and caused hemorrhage beneath the skin of mouse. This study suggests that the potential of FE-27kDa as pharmacopuncture agent should be limited due to low fibrinolytic activity and a possible side effect of hemorrhage.

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## 1. Introduction

The venoms from rattlesnakes in America and

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This paper meets the requirements of KS X ISO 9706, ISO 9706-1994 and ANSI/NISO Z39.48-1992 (Permanence of Paper).

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from salmusa in Asia have toxic effects on the vascular tract and affect blood circulation [1]. The poisonous components in the snake venom, either enzymes or non-enzymatic substances, cause drastic changes in the circulation of blood and thus lead to outbreaks of hemorrhage. The activities of the toxins include coagulation of blood, inhibition of blood coagulation, platelet activation, anti-platelet function, fibrinolysis activation, and hemorrhage. Blood coagulation has been reported to be inhibited by fibrin(ogen)olytic enzymes from the snake venom of the families of *Viperidae, Elapidae*, and *Crotalida* [1, 2].

The fibrin(ogen)olytic enzyme in the snake venom is defined as an enzyme that can hydrolyze fibrin and fibrinogen to liquify insoluble fibrin-rich clot and to remove fibrinogen from blood [2]. The enzyme is also an endoproteinase, which can be classified as either serine proteinase or metalloproteinase based on the structure of active site. The metalloproteinase mostly requires zinc at the active site and is mostly on $\alpha$-chain fibrinogenase [1], but there are some exceptions [3]. However, most serine fibrin(ogen)olytic enzymes preferentially hydrolyze $\beta$-chain over $\alpha$-chain of fibrinogen [4-6].

The fibrin(ogen)olytic enzyme from snake venom has received attention as a therapeutic agent of oriental pharmacopuncture medicine as well as Western medicine to treat obstructive thrombosis and acute stroke by removing fibrinogen and fibrin clots from the blood [7]. Thus, numerous fibrin(ogen)olytic enzymes have been isolated from snake venoms, and their abilities to treat cardiovascular disease without side effects have been evaluated [1]. One of the potential side effects of fibrin(ogen)olytic enzymes for clinical application is hemorrhage [2].

*Gloydius blomhoffii,* called salmusa and mamushi in Korea and in Japan, respectively, is a venomous viper found in China, Japan, and Korea. Its synonyms include *Agkistrodon blomhoffii blomhoffii, Agkistrodon halys blomhoffii,* etc. There are four subspecies: *G. b. blomhoffii, G. b. brevicaudus, G. b. dubitatus,* and *G. b. siniticus* [8]. The geographical location of *G. b. siniticus,* called Yangtze mamushi, is from Shandong, Jiang Su and Anhui provinces, south to the Ch'ang Chiang Basin, eastern Sichuan, Jiangxi, and Hunan [9].

The objective of this study were to isolate a fibrin(ogen)olytic enzyme from the snake venom of *Gloydius blomhoffii siniticus* and to investigate its enzymatic characteristics and hemorrhagic activity.

## 2. Materials and methods

### 2.1. Isolation of fibrinolytic enzyme from snake venom

The snake venom of *Gloydius blomhoffii siniticus* was obtained from a snake farm in China. An aliquot (10 g) of lyophilized snake venom powder was dissolved in 100 ml of 50 mM Tris-HCl, pH 7.6, and centrifuged at 10,000 g for 30 min. The supernatant was dialyzed in a buffer and was injected into a column (5 cm x 15 cm) of Q-Sepharose (GE, USA) that had been equilibrated with the buffer. The column was washed with 600 ml of the buffer and was then eluted with a linear concentration gradient from 0 M NaCl to 0.35 M NaCl. The total volume of gradient elution was 600 ml. The column was then finally eluted with 200 ml of the buffer containing 0.35 M NaCl. The flow rate of the eluant was 21 ml/h, and the volume of a fraction was 7 ml. The fractions showing the fibrinolytic activity were collected, combined, and concentrated in dialysis tubing in polyethylene glycol. The concentrated solution was dialyzed in 50 mM Tris-HCl, pH 7.6, 0.15 M-NaCl and was then injected into a column (2.5 cm x 109 cm) of Sephadex G-75 (GE, USA). The column was eluted with 50 mM Tris-HCl, 0.15 M NaCl. The flow rate of the eluant was 14 ml/h, and the volume of a fraction was 7 ml. The fractions showing fibrinolytic activity were collected, combined, and dialyzed in 50 mM Tris-HCl, pH 7.6. The solution was then injected into a column (2.5 cm x 10 cm) of DEAE-Sepharose (GE, USA). The column was washed with 20 ml of 50 mM Tris-HCl, pH 7.6, and was eluted with a linear concentration gradient from 0 M NaCl to 0.3 M NaCl. The total volume of the gradient elution was 500 ml. All the chromatography procedures were performed at 4℃. Absorbance at 280 nm of diluted fractions from the chromatographys was determined using the spectrophotometer (Pharmacia, Sweden) and then was multiplied by the dilution factor to estimate the protein concentration. The fibrinolytic activity of the fraction was observed by measuring area of fibrinolysis zone formed in the fibrin plate assay which was described by Astrup and Mullertz [10] and then modified by Choi [11].

### 2.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the procedure described by Laemmli [12]. The molecular weight markers (Bio-Rad, USA) for SDS-PAGE were phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic

anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). β-Mercaptoethanol (5%) was added to the sample buffer used for all SDS-PAGE except for that in Fig 5, lane C. Iodoacetate (0.15 M), instead of β-mercaptoethanol (5%), was added to the sample buffer in Fig 5, lane C. Iodoacetate (0.15 M) was added after heating at 100℃ for 5 min in Fig. 5, lane B.

### 2.3. Protein concentration determination

The protein concentration was determined using BCA Protein Assay Reagent (Pierce, USA). Bovine serum albumin was used for the calibration line.

### 2.4. Protein hydrolysis analysis

Fibrinogen and gelatin was hydrolyzed using the isolated fibrinolytic enzyme and then subjected to SDS-PAGE. An aliquot (0.2 ml) of 10 mg/ml proteins in 50 mM Tris-HCl pH 7.6, 0.15 M NaCl was mixed with 0.05 ml of 0.2 mg/ml fibrinolytic enzyme. The mixture was incubated at 37℃ for 6 h. An aliquot (10 μl) was taken at 0.5, 1, 2, 4, and 6 h, and was added to the sample buffer containing β-mercaptoethanol. The mixture was heated at 100℃ for 5 min before SDS-PAGE.

### 2.5. Hemorrhage assay

The experiment to determine the effect of the fibrinolytic enzyme on hemorrhage reaction beneath back skins of mice was approved by the Animal Experiment Ethics Committee of Sangji University (Approval document No. 2102-9). An aliquot (0.1 ml) of diluted fibrinolytic enzyme in 50 mM Tris-HCl pH 7.6, 0.15 NaCl was injected subcutaneously to Institute of Cancer Research (ICR) mice of 6-week-old (Daehan Biolink, Korea). The mice were sacrificed using cervical dislocation after 6 h and the skin around the injection site was stripped off to determine the diameter of the hemorrhage zone formed beneath the skin. Two diameters ($r_1$ and $r_2$) at a right angle were measured. The hemorrhagic area was calculated following formula of $0.785 r_1 r_2$. The hemorrhagic areas were measured in duplicate by injecting two mice, and the average was calculated.

### 2.6. Amino acid composition analysis

The amino acid composition of the fibrinolytic enzyme was analyzed by Korea Basic Science

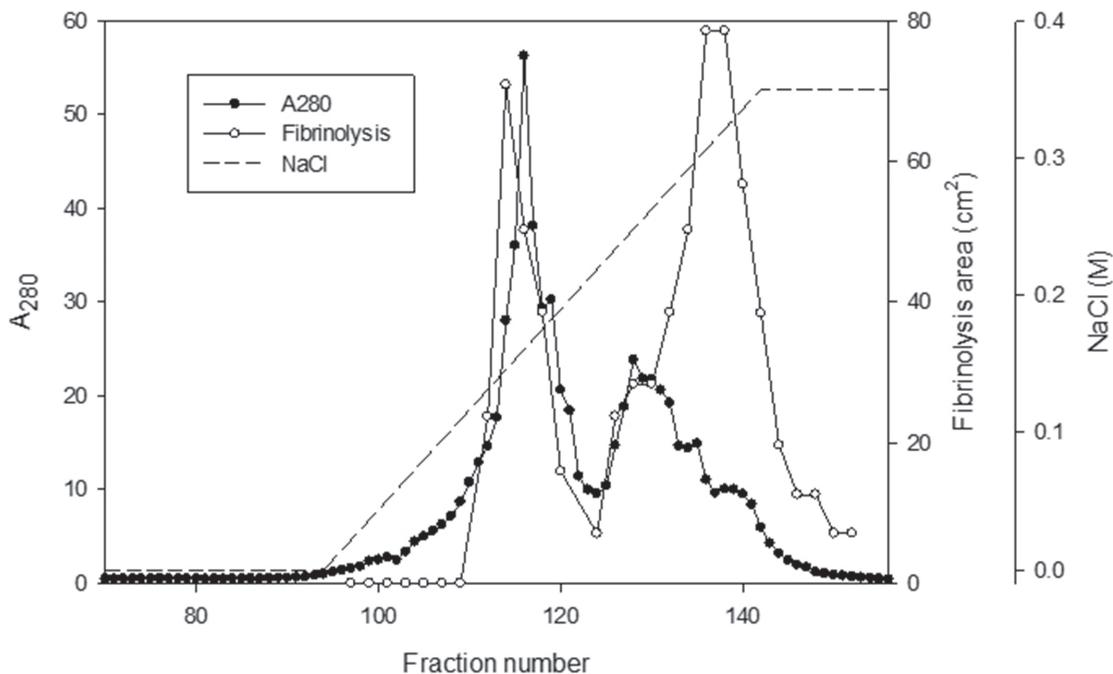


**Figure 1** Ion exchange chromatography of *G. b. siniticus* venom on a Q-Sepharose column (5 cm x 15 cm) equilibrated in 50 mM Tris-HCl, pH 7.6. The flow rate was 21 ml/h and the fraction volume was 7 ml. The combined sample of the fractions 116-120 was subjected to subsequent chromatography in Fig. 2 and to SDS-PAGE in Fig. 4, lane C.

Institute. The PICO-tag procedure of high performance liquid chromatography (HPLC) was used to analyze PITC-labeled amino acids. The HPLC column was Waters Pico-tag Column. The HPLC instruments (Waters Corporation, USA) consisted of 510 HPLC pump, gradient controller, and 2487 UV detector.

## 3. Results and discussion

The sample containing the two polypeptides with the molecular weights of 15 kDa and 18 kDa described by Choi [11] was obtained from chromatography of *Gloydius blomhoffii siniticus* venom. The combined sample of the fractions 115-119 from ion exchange chromatography on the Q-Sepharose column (Fig. 1) still contained many low-molecular-weight polypeptides including the two polypeptides as shown in SDS-PAGE (Fig. 4, lane C). The sample was then subjected to gel filtration chromatography on the Sephadex G-75 column (Fig. 2). The combined sample of the fractions 39-42 was shown to contain the two major polypeptides in addition to a minor polypeptide (Fig. 4, lane D). The sample was then subjected to ion exchange chromatography on the DEAE-Sepharose column (Fig. 3). The pooled sample of the fractions 30-33 was shown to contain only the two polypeptides (Fig. 4, lane E).

When the sample was heated in the sample buffer containing iodooacetate before electrophoresis, a major polypeptide with molecular weight of 27 kDa and a minor polypeptide with molecular weight of 50 kDa were detected in SDS-PAGE (Fig. 5, lane C). The minor polypeptide with the molecular weight of 50 kDa seemed to be a dimer consisting of two FE-27kDa linked by a disulfide bond. Since iodoacetate, one of alkylating agent of sulfhydryl group, prevents formation of linkage between polypeptides and of thus polymeric proteins, the dimeric polypeptide should not be an artifact of SDS-PAGE.

When the sample was heated in the sample buffer containing $\beta$-mercaptoethanol and then iodoacetate was added to the sample before electrophoresis, the two polypeptides with the molecular weights of 15 kDa and 18 kDa were detected in SDS-PAGE (Fig. 5, lane B). These results suggested that the fibrinolytic enzyme with the molecular weight of 27 kDa consisted of the two disulfide bond-linked polypeptides. The fibrinolytic enzyme was then designated FE-27kDa in this study.

However, the apparent molecular weight of 27 kDa

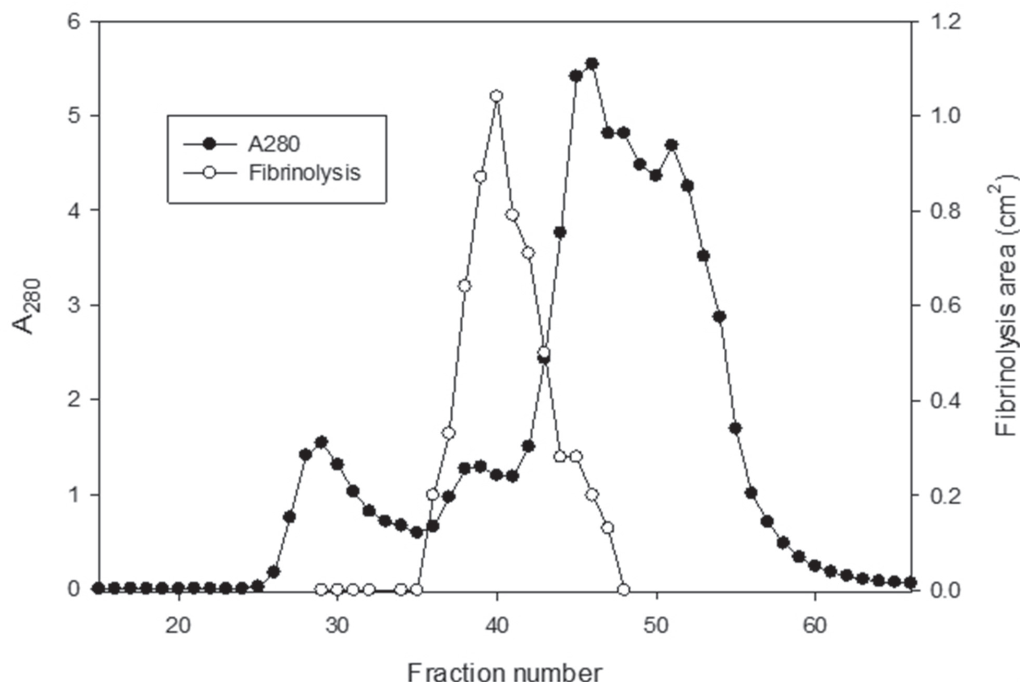


**Figure 2** Gel filtration chromatography of the combined sample from the Q-Sepharose column (Fig. 1) on a Sephadex G-75 column (2.5 cm x 109 cm) equilibrated in 50 mM Tris-HCl, pH 7.6, 0.15 M NaCl. The flow rate was 14 ml/hr and the fraction volume was 7 ml. The pooled sample of the fractions 39-42 was subjected to subsequent chromatography in Fig. 3 and to SDS-PAGE in Fig. 4, lane D.

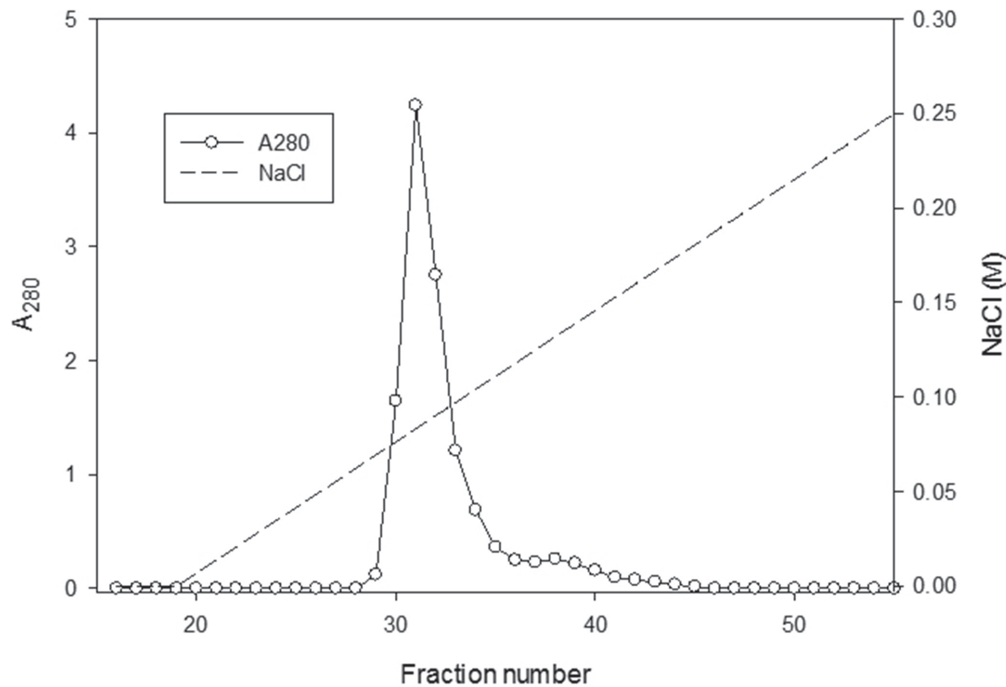


**Figure 3** Ion exchange chromatography of the pooled sample obtained from the Sephadex G-75 column (Fig. 2) on a DEAE Sepharose column (2.5 cm x 10 cm) equilibrated in 50 mM Tris-HCl, pH 7.6. The flow rate was 30 ml/h and the fraction volume was 7 ml. The pooled sample of the fractions 30-33 was subjected to SDS-PAGE in Fig. 4, lane E and Fig. 5.

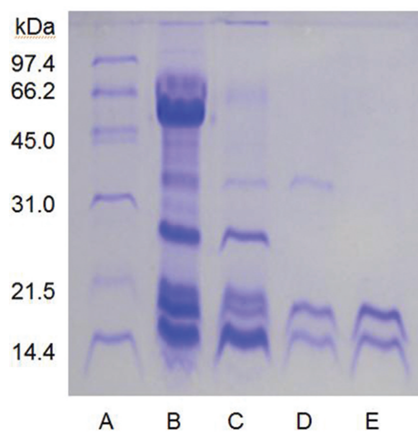


**Figure 4** SDS-PAGE of *G. b. siniticus* venom and its chromatography fractions. Lane A: molecular weight marker, Lane B: crude venom, Lane C: sample from the Q-Sepharose column, Lane D: sample from the Sephadex G-75 column, Lane E: sample from the DEAE-Sepharose column. The samples were heated in the sample buffer containing $\beta$-mercaptoethanol before SDS-PAGE.

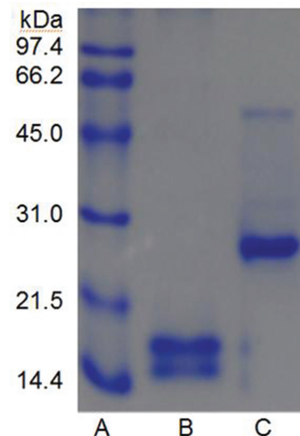


**Figure 5** SDS-PAGE of FE-27kDa from *G. b. siniticus* venom. Lane A: molecular weight marker, Lane B: sample which was heated in the sample buffer containing $\beta$-mercaptoethanol and then added with iodoacetate after heating, and Lane C: sample which was heated in the sample buffer containing iodoacetate.

obtained from SDS-PAGE was less than the calculated molecular weight of 33 kDa obtained by adding the molecular weights of the two polypeptides. The discrepancy between the two molecular weights may be due to the fact that the two polypeptides linked by a disulfide bond could not be converted to a fully-extended linear structure of a polypeptide with the same molecular weight, but were converted to a linear structure lined side by side with the two polypeptides in the gel of SDS-PAGE without $\beta$-mercaptoethanol. Thus, the hydrodynamic volume of the linear structure lined with the two polypeptides should be less than that of the fully-extended linear structure, which would result in a lower apparent molecular weight of 27 kDa.

The structure of FE-27kDa was similar to that of brevinase isolated from the snake venom of a Korean snake, *Agkistrodon blomhoffii brevicaudus* by Lee et al. [6]. Brevinase consists of two heterogeneous polypeptides with the molecular weights of 16.5 kDa and 17 kDa that are linked through a disulfide bond. The amino acid sequence obtained from the cDNA sequence of the brevinase gene [13] showed a high degree of homology with those of mucofibrase [14], ancrod [15], and calobin [16].

The areas of the fibrinolysis zones in the fibrin plate assay were 0.71 $cm^2$ and 0.33 $cm^2$ at 3 mg/ml and 2 mg/ml of FE-27kDa, respectively (Fig. 6, 1 and 2). The fibrin plate assay showed that the minimum concentration of FE-27kDa to form a fibrinolysis zone was about seven times higher than that (0.3 mg/ml) of the fibrinolytic enzyme with a molecular weight of 54 kDa (FE-54kDa) [11], suggesting that the fibrinolytic activity of FE-27kDa was weaker than that of FE-54kDa.

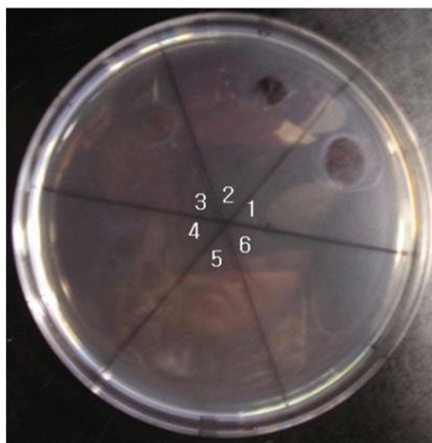


**Figure 6** Fibrin plate assay to determine the fibrinolytic activity of FE-27kDa from *G. b. siniticus* venom: 1: 3 mg/ml, 2: 2 mg/ml, 3: 1.5 mg/ml, 4: 1 mg/ml, 5: 0.5 mg/ml, and 6: 0.25 mg/ml.

Protease inhibitors were added to the solution containing FE-27kDa (2.5 mg/ml) in the fibrin plate assay. The relative fibrinolysis was the ratio of the area of the enzyme treated with protease inhibitor to that of a control without treatment (Table 1). Table 1 shows that among the chelate compounds, which remove metal ions from the enzyme, EDTA inhibited fibrinolysis completely, 1, 10-phenanthroline inhibited it strongly, and EGTA did not inhibit it. PMSF, a serine protease inhibitor, inhibited fibrinolysis completely. However, TLCK, an inhibitor of trypsin and trypsin-like protease, did not show any effect. Dithiothreitol and cysteine, which cleave disulfide bonds, inhibited fibrinolysis strongly, but iodoacetate did not have any effect on it. These results suggest that FE-27kDa is not a trypsin-like enzyme, but a serine proteinase. The disulfide bond is required for fibrinolytic activity, and metal ions are needed to promote fibrinolysis. The inhibition pattern of FE-27kDa is similar to that of brevinase reported by Lee et al.[6]. Brevinase is moderately inhibited by aprotinin, bezamidine, EDTA, EGTA, and 1, 10-phenanthroline. The fibrinolytic activity is completely inhibited by PMSF, Pefabloc, and dithiotreitol. These results indicate that brevinase is also a serine proteinase requiring a disulfide bond for its fibrinolytic activity. However, FE-54kDa [11] is a metalloproteinase containing a disulfide linkage. The enzyme is inhibited by chelating agents such as EDTA, EGTA, and 1, 10-phenanthroline and by

**Table 1** Effects of protease inhibitors on the fibrinolytic activity of FE-27kDa from *G. b. siniticus* venom

| Protease inhibitors | Concentration (mM) | Relative fibrinolysis (%) |
|---|---|---|
| Control | 0 | 100 |
| EDTA | 10 | 0 |
| EGTA | 3 | 100 |
| 1,10-phenanthroline | 10 | 21 |
| PMSF | 10 | 0 |
| TLCK | 1 | 100 |
| Cysteine | 10 | 21 |
| Dithiothreitol | 10 | 21 |
| Iodoacetate | 1 | 100 |

**Table 2** Effects of salts on the fibrinolytic activity of FE-27kDa from *G. b. siniticus venom*

| Salts | Concentration (mM) | Relative fibrinolysis (%) |
|---|---|---|
| Control | 0 | 100 |
| $MgCl_2$ | 10 | 70 |
| $CaCl_2$ | 10 | 50 |
| $MnCl_2$ | 10 | 100 |
| $FeCl_2$ | 10 | 0 |
| $CoCl_2$ | 10 | 68 |
| $CuCl_2$ | 10 | 50 |
| $ZnCl_2$ | 10 | 60 |
| CsCl | 10 | 50 |
| $HgCl_2$ | 10 | 0 |

reducing agents such as dithiothreitol and cysteine.

The effects of metal ions on the fibrinolytic activity of FE-27kDa were determined (Table 2). The concentration of salts added to FE-27kDa (2.5 mg/ml) was 10 mM. Table 2 shows that $Hg^{2+}$ and $Fe^{2+}$ inhibited fibrinolysis completely and that $Mg^{2+}$, $Ca^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Zn^{2+}$ and $Cs^{+}$ inhibited it moderately. $Mn^{2+}$ was the only metal ion that did not show any effect on fibrinolysis. The inhibition by metal ions of FE-27kDa activity in this study was different from that of brevinase [6]. The fibrinolytic activity of brevinase is completely inhibited by $Zn^{2+}$, but is unaffected by $Ca^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Fe^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $K^{+}$, and $Na^{+}$.

Fibrinogen was mixed with FE-27kDa and incubated at 37 ℃ for a certain time. The sample was subjected to SDS-PAGE to determine the fibrinogenolytic activity of FE-27kDa (Fig. 7). The SDS-PAGE pattern showed that $\alpha$-chain of fibrinogen decreased initially after 30 min (Fig. 7, lane 3) and disappeared after 360 min (Fig 7, lane 7). The intensity of $\beta$-chain of fibrinogen was weakened after 240 min (Fig. 7, lane 6). However, there was no change for $\gamma$-chain throughout the time of the incubation. These results showed that FE-27kDa hydrolyzed $\alpha$-chain initially and then $\beta$-chain slowly. However, brevinase preferentially cleaves $\beta$-chain of fibrinogen and more slowly $\alpha$-chain [6]. The concentration ratio of FE-27kDa to fibrinogen was 0.5% in the assay, suggesting that the fibrinogenolytic activity of FE-27kDa was weaker than that of brevinase whose concentration ratio to fibrinogen was 0.025%. FE-54kDa [11] preferentially cleaved $\alpha$-chain of fibrinogen and slowly cleaved $\beta$-chain. The concentration ratio of FE-54kDa to fibrinogen was 0.0025%, suggesting that it had very strong fibrinogenolytic activity.

Gelatin was mixed with FE-27kDa and incubated for a

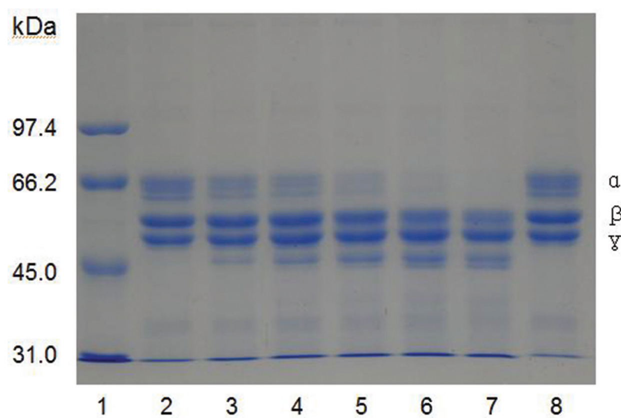


**Figure 7** SDS-PAGE of fibrinogen treated with FE-27kDa from *G. b. siniticus* venom. Lane 1: molecular weight marker, Lanes 2, 3, 4, 5, 6, and 7: fibrinogen treated with the enzyme for 0, 30, 60, 120, 240, and 360 min at 37℃, respectively, and Lane 8: fibrinogen treated without the enzyme for 360 min. The Greek letters at the left side are the names of the fibrinogen chains.

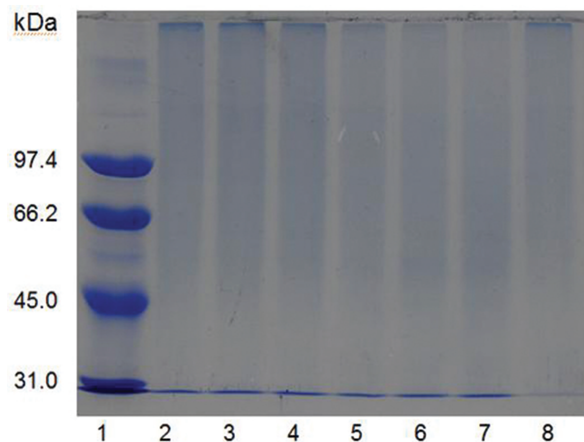


**Figure 8** SDS-PAGE of gelatin treated with FE-27kDa from *G. b. siniticus* venom. Lane 1: molecular weight marker, Lanes 2, 3, 4, 5, 6, and 7: gelatin treated with the enzyme for 0, 30, 60, 120, 240, and 360 min at 37℃, respectively, and Lane 8: gelatin treated without the enzyme for 360 min.

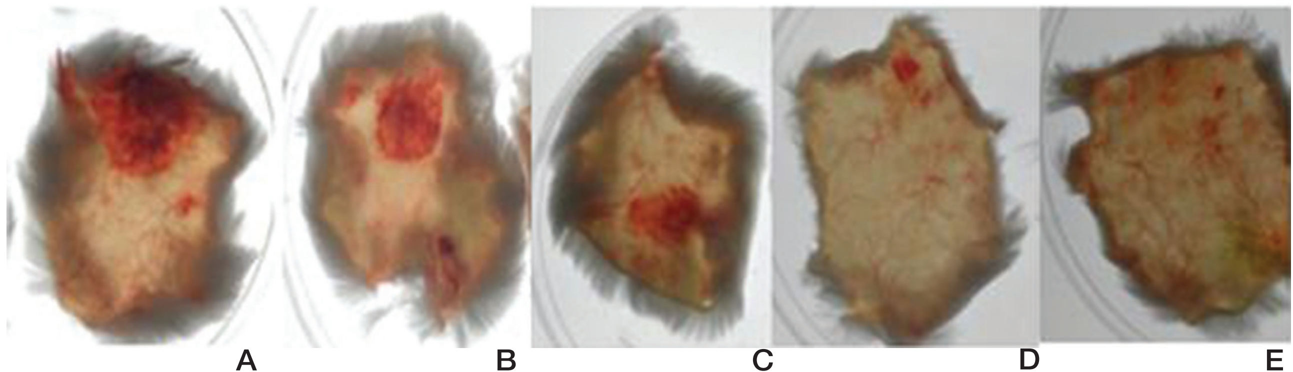


**Figure 9** Hemorrhage assay of FE-27kDa from G. b. siniticus venom. A: 100 µg, B: 50 µg, C: 25 µg, D: 10 µg, and E: 5 µg dosage per mouse.

certain time. The sample was subjected to SDS-PAGE to determine the gelatin hydrolytic activity of FE-27kDa (Fig. 8). According to the SDS-PAGE results, the gelatins incubated for 0 min (Fig. 8, lane 2) and 30 min (Fig. 8, lane 3) and the gelatin that was not incubated (Fig. 7, lane 8) showed smear patterns of polypeptides that were strongly localized at molecular weights of more than 97.4 kDa. However, the polypeptide bands at molecular weights between 45.0 kDa and 66.2 kDa were intensified after more than 4 h, but there were no polypeptides with molecular weights lower than 45.0 kDa. These results suggest that even though the hydrolysis of the gelatin by FE-27kDa was evident, the enzyme did not hydrolyze the gelatin extensively. However, FE-54kDa hydrolyzed high-molecular-weight polypeptides of the gelatin extensively into low-molecular-weight polypeptides with molecular weights less than 45.0 kDa [11].

FE-27kDa was injected subcutaneously into shaved back skins of mice. The mice were sacrificed after six hour and then the diameter of the hemorrhage zone (Fig. 9) formed beneath the skin was measured. When FE-27kDa was administered to a mouse at dosages of 100 $\mu$g, 50 $\mu$g, 25 $\mu$g, and 10 $\mu$g, the areas of the hemorrhage zones were 2.5 $cm^2$, 1.5 $cm^2$, 0.9 $cm^2$, and 0.2 $cm^2$, respectively (Fig. 9, A-D). The results showed that the areas and the intensities of the hemorrhage zones were correlated with the dosages of FE-27kDa and that the minimum dosage of FE-27kDa to induce hemorrhage was 10 $\mu$g.

Most of the fibrin(ogen)olytic enzymes from snake venom are able to induce hemorrhage, which results from proteolysis of basement membrane proteins [17, 18]. The hemorrhagic activity of FE27kDa showed a sun-bursting radiating pattern of the hemorrhagic phenomenon beneath the skin [19]. However, the hemorrhagic activity of FE-27kDa was relatively weaker than that of FE-54kDa [11]. When FE-54kDa at dosages of 40 $\mu$g, 20 $\mu$g, 10 $\mu$g, and 5 $\mu$g was administered to the mice, the areas of the hemorrhage zones were 1.1 $cm^2$, 0.9 $cm^2$, 0.36 $cm^2$, and 0.38 $cm^2$, respectively (results not shown). These results showed that FE-54kDa had a higher hemorrhagic activity than FE-27kDa because a hemorrhage zone was still formed at a dosage of 5 $\mu$g FE-54kDa. The strong gelatin hydrolytic activity of FE-54kDa might lead to the formation of a hemorrhage zone at low concentrations. When the activities in fibrinolysis, gelatin hydrolysis and hemorrhage were compared, FE-54kDa was overall more active than FE-27kDa.

The amino acid composition of FE-27kDa is shown in Table 3. FE-27kDa contained cysteine at a concentration of 4.3%, which was in accord with inhibition of fibrinolysis by a reducing agent, such as cysteine and dithiothreitol. Among the amino acids, lysine and glutamic acid (glutamine) were major components with concentrations of more than 10%.

Many studies have been reported the isolation of a non-hemorrhagic fibrin(ogen)olytic enzyme that could be presumably used to treat obstructive thrombosis and acute stroke resulting from cardiovascular diseases. Didisheim and Lewis [20] proposed that the activity to hydrolyze fibrin clots of the snake venom from *Agkistrodon, Crotalus* and *Bothops* could be applied clinically. Fibrolase from *Agkistrodon contortrix contortrix* (southern copperhead snake) is a fibrin(ogen)olytic enzyme that belongs to zinc-metalloproteinase family and consists of 203 amino acid residues. Alfimeprase, an enzyme recombinantly modified from fibrolase, has been successful in phase I and II clinical trials

**Table 3** Amino acid composition of FE-27kDa from *G. b. siniticus* venom

| Amino acid | Composition (%) |
|---|---|
| Cysteine | 4.3 |
| Aspartic acid (Asparagine) | 7.0 |
| Glutamic acid (Glutamine) | 12.6 |
| Serine | 7.0 |
| Glycine | 2.7 |
| Histidine | 2.5 |
| Arginine | 4.5 |
| Threonine | 4.8 |
| Alanine | 2.1 |
| Proline | 2.3 |
| Tyrosine | 7.9 |
| Valine | 5.2 |
| Methionine | 2.0 |
| Isoleucine | 4.0 |
| Leucine | 5.4 |
| Phenylalanine | 7.1 |
| Tryptophane | 8.6 |
| Lysine | 10.0 |
| Total | 100.0 |

to treat distal arterial occlusive disease, but did not attain target levels; thus, the drug's development was terminated [21].

## 4. Conclusion

This study showed that FE-27kDa, a serine proteinase, had fibrin(ogen)olytic activity and gelatin hydrolytic activity and caused hemorrhage beneath the skin of a mouse. The results suggested that the possible use of FE-27kDa as a pharmacopuncture agent in Oriental medicine should be limited due to its relatively low fibrinolytic activity and potential side effects of hemorrhage.

## Acknowledgment

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