

Isolation and Characterization of a 32-kDa Fibrinolytic Enzyme (FE-32kDa) from *Gloydius blomhoffii siniticus* Venom

-Fibrinolytic Enzyme from *Gloydius blomhoffii siniticus* Venom-

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Key Words

fibrinolytic enzyme, metalloprotease, *Gloydius blomhoffii siniticus*, snake venom

Abstract

Objectives: This study was undertaken to isolate a fibrinolytic enzyme from the snake venom of *Gloydius blomhoffii siniticus* and to investigate its enzymatic characteristics and hemorrhagic activity 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 investigated using fibrin plate assay, protein hydrolysis analysis, and hemorrhage assay. Its amino acid composition was determined.

Results: The fibrinolytic enzyme with the molecular weight of 32kDa (FE-32kDa) from *Gloydius blomhoffii siniticus* showed a fibrin hydrolysis zone at the concentration of 0.2 mg/mL in the fibrin plate assay. The fibrin hydrolysis activity of the enzyme was inhibited com-

pletely by ethylenediaminetetraacetic acid (EDTA), ethyleneglycoltetraacetic acid (EGTA), and 1, 10-phenanthroline, thiothreitol and cysteine, and partially by phenylmethanesulfonylfluoride (PMSF). Metal ions such as Fe²⁺ and Hg²⁺ inhibited the fibrin hydrolysis completely, but Zn²⁺ enhanced it. FE-32kDa hydrolyzed α -chain but did not hydrolyze β -chain and γ -chain of fibrinogen. High-molecular-weight polypeptides of gelatin were hydrolyzed partially into low-molecular-weight polypeptides, but the extent of hydrolysis was limited. FE-32kDa induced hemorrhage beneath back skin of mice at the dose of 2 μ g.

Conclusions: FE-32kDa is a α -fibrin(ogen)olytic metalloprotease that requires Zn²⁺ for fibrinolytic activity and causes hemorrhage, suggesting that the enzyme is not appropriate for use as a clinical pharmacopuncture.

1. Introduction

The fibrinolytic enzyme in snake venom is defined as an enzyme that can hydrolyze fibrin to liquidize insoluble fibrin-rich clots but not form fibrin clots from fibrinogen [1]. The fibrinolytic enzymes are classified as serine protease and metalloprotease based on the structure of the active site and in α - and β -fibrinoge-

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nase based on the substrate specificity to α - and β -chain of fibrinogen. The metalloproteases from snake venom which mostly require zinc at the active site are classified in classes PI to PIV based on their multidomain organizations, which include a signal peptide, a prodomain, and a metalloprotease domain which is the structure of class PI and additional domains of disintegrin, cysteine-rich, and type C depending on the classes from PII to PIV [2]. The enzymes act mainly as hemorrhagic factors, which exert their effects by degradation of basement membrane proteins, such as laminin, fibronectin, and collagen [3, 4]. Hydrolysis of fibrinogen and inhibition of platelet aggregation by the metalloproteases also enhance hemorrhaging.

The fibrin(ogen)olytic enzyme from snake venom has received attention as a medical agent to treat obstructive thrombosis and acute stroke by removing fibrinogen and fibrin from the blood. Thus, many kinds of the enzymes have been isolated and reported [5]. However, the enzyme for clinical application should not cause hemorrhage [1]. Fibrolase from *Agkistrodon contortrix contortrix* (Southern copperhead snake) is a fibrinolytic enzyme which belongs to zinc metalloprotease and consists of 203 amino acid residues. Alfineprase, a recombinant fibrolase, was used successfully in phase I and II clinical trials to treat distal arterial occlusive disease, but target level was not attained, and so drug development was terminated [6].

Gloydius blomhoffii, called salmusa in Korea and known as mamushi in Japan, 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* [7].

Choi [8] detected fibrinolysis activities in the chromatographic fractions containing polypeptides of 59kDa, 54kDa, 46kDa, 32kDa, 18kDa and 15kDa isolated from the snake venom of *Gloydius blomhoffii siniticus*. A fibrin(ogen)olytic enzyme with the molecular weight of 54kDa was isolated and characterized. The enzyme is a metalloprotease which is completely inhibited by ethylenediaminetetraacetic acid (EDTA), ethyleneglycoltetraacetic acid (EGTA) and 1, 10-phenanthroline and which hydrolyzes A α -chain of fibrinogen initially and then slowly B β -chain later. Choi and Lee [9] later reported isolation of a fibrin(ogen)olytic enzyme consisting of two disulfide-linked polypeptides with molecular weights of 18kDa and 15kDa from the same snake venom. The fibrinolytic activity is inhibited completely by phenylmethanesulfonyl fluoride (PMSF) and EDTA and partially by thiothreitol and cysteine, suggesting that the enzyme is a serine protease. The characteristics of the enzyme were similar with those of brevinase isolated from the venom of the Korean snake, *Agkistrodon blomhoffii brevicaudus* [10]. The objectives

of this study were to isolate the fibrinolytic enzyme with a molecular weight of 32kDa from the snake venom of *Gloydius blomhoffii siniticus* and to investigate its enzymatic characteristics and hemorrhagic activities.

2. Materials and methods

2.1. Isolation of fibrinolytic enzyme from snake venom

Ten g of lyophilized venom of *Gloydius blomhoffii siniticus* from a Chinese snake farm was dissolved in 100 mL of 50 mM Tris-HCl, pH 7.6 and centrifuged at 10,000 g for 30 minutes. The supernatant was injected into a column (5 cm \times 15 cm) of Q-Sepharose (GE, USA) equilibrated with 50 mM Tris-HCl, pH 7.6. The column was eluted with 600 mL of the Tris buffer, and then with 600 mL of the Tris buffer containing NaCl with a linear concentration gradient from 0 M to 0.35 M. The column was then finally eluted with 200 mL of the Tris buffer containing 0.35 M NaCl. The flow rate of eluant was 21 mL/hr. The volume of eluant was 7 mL/tube. The fractions showing the fibrin hydrolyzing activity were collected, combined, and concentrated in dialysis tubing which was put into 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 \times 117 cm) of Sephadex G-75 (GE, USA). The column was eluted with 50 mM Tris-HCl, 0.15 M NaCl. The flow rate of eluant was 14 mL/hr and the volume of eluant was 7 mL/tube. The fractions showing fibrin hydrolyzing activity were collected, combined, and dialyzed in 50mM Tris-HCl, pH 7.6. The solution was then injected into a column (2.5 cm \times 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 to 0.3 M NaCl dissolved in the buffer. The total volume of the gradient elution was 500 mL. All the chromatography procedures were performed at 4 $^{\circ}$ C. The absorbance at 280 nm of appropriately diluted fractions from the chromatography was observed. The observed absorbance was multiplied by the dilution factor. The fibrin-hydrolyzing activities of the fractions were observed by using the fibrin plate assays [11], as described by Choi [9].

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 (97.4kDa), serum albumin (66.2kDa), ovalbumin (45.0kDa), carbonic anhydrase (31.0kDa), trypsin inhibitor (21.5kDa), and lysozyme (14.4kDa). The sample buffer

containing 2-mercaptoethanol was added to the sample and heated at 100°C for 5 minutes.

2.3. Protein concentration determination

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

2.4. Protein hydrolysis analysis

Fibrinogen and gelatin were hydrolyzed using the isolated fibrinolytic enzyme and then subjected to SDS-PAGE. An aliquot (200 µl) of 10 mg/mL protein in 50 mM Tris-HCl pH 7.6, 0.15 M NaCl was mixed with 50 µl of 2 µg/mL fibrinolytic enzyme. The mixture was incubated at 37°C for 6 hours. The samples (10 µl) which were taken from the mixture at 0.5, 1, 2, 4, and 6 hours were added to the sample buffer of SDS-PAGE, respectively. The mixture was heated at 100°C for 5 minutes before SDS-PAGE.

2.5. Hemorrhage assay

The experiment to examine the effect of the isolated fibrinolytic enzyme on hemorrhage reaction beneath the 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 fibrinolytic enzyme in 50 mM Tris-HCl, pH 7.6, 0.15 M NaCl at an appropriate concentration was injected subcutaneously into the back skin 6-week-old Institute of Cancer Research (ICR) mice of 6 week old (Daehan Biolink, Korea). The mice were sacrificed using cervical dislocation after 6 hours and the skin around the injection was stripped to determine the diameter of the hemorrhage zone beneath the skin. Two diameters (r_1 and r_2) at a right angle were measured. The hemorrhage area was calculated using the formula of $0.785r_1r_2$. The hemorrhage areas were measured in duplicate by injecting the fibrinolytic enzyme into two mice, and the average was calculated.

2.6. Amino acid composition analysis

The amino acid composition of the fibrinolytic enzyme was analyzed at the Korea Basic Science Institute. The PITC-labeled amino acids were analyzed using a Pico-Tag Column and the HPLC instrument which consisted of a 510 HPLC pump, a gradient controller, and a 2487 UV detector (Waters Corporation, USA).

3. Results

The fibrinolytic enzyme with a molecular weight of 32kDa (FE-32kDa) described by Choi [8] was obtained from *G. b. siniticus* venom by using several procedures of chromatog-

raphy as follows (Figs. 1, 2, and 3). The combined sample of the fractions 102-107 from the Q-Sepharose column (Fig. 1) contained many polypeptides in addition to FE-32kDa (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 39-44 contained the major polypeptide of FE-32kDa and several minor polypeptides with the molecular weights of 15kDa, 18kDa, and 50kDa (Fig. 4, lane D). The sample was then subjected to ion exchange chromatography on the DEAE-Sepharose column. The combined sample of the fractions 28-30 with high fibrinolytic activity contained only the polypeptide of FE-32kDa (Fig. 4, lane E).

The areas of the fibrinolytic zones in the fibrin plate assay were 0.79 cm², 0.64 cm², 0.50 cm², 0.38 cm² at 0.5 mg/mL, 0.4 mg/mL, 0.3 mg/mL, and 0.2 mg/mL of FE-32kDa, respectively (1-4 in Fig. 5). The fibrin plate assay showed that the minimum concentration of FE-32kDa to form a fibrinolytic zone was ten times lower than that of FE-27kDa and similar with that of FE-54kDa [8, 9].

Protease inhibitors were added to the solution containing FE-32kDa (0.5 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 all of the chelate compounds, including EDTA, EGTA, 1,10-phenanthroline, inhibited fibrinolysis completely. PMSE, a serine protease inhibitor, inhibited fibrinolysis partially and *N*-tosyl-L-lysinechloromethylketone (TLCK), an inhibitor of trypsin and trypsin-like protease, did not show any effect.

Dithiothreitol and cysteine, which cleave disulfide bonds, inhibited fibrinolysis completely. However, iodoacetate, which alkylates sulfhydryl groups, did not show any effect. These results suggest that FE-32kDa is a metalloprotease which has disulfide bonds necessary for the fibrinolytic

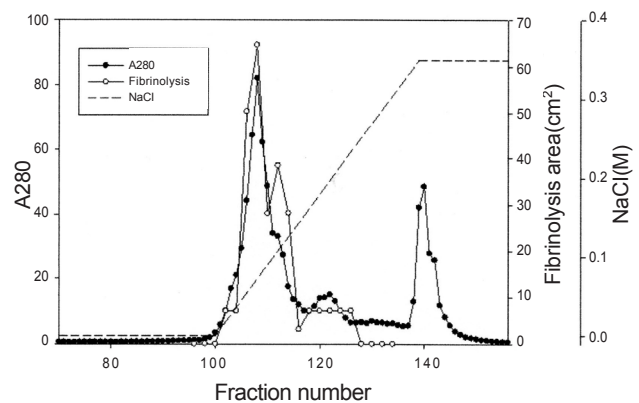


Figure 1 Ion exchange chromatography of *G. b. siniticus* venom on a Q-Sepharose column. The venom sample of fractions 102-107 was subjected to subsequent chromatography (Fig. 2) and to SDS-PAGE (Fig. 4, lane C)

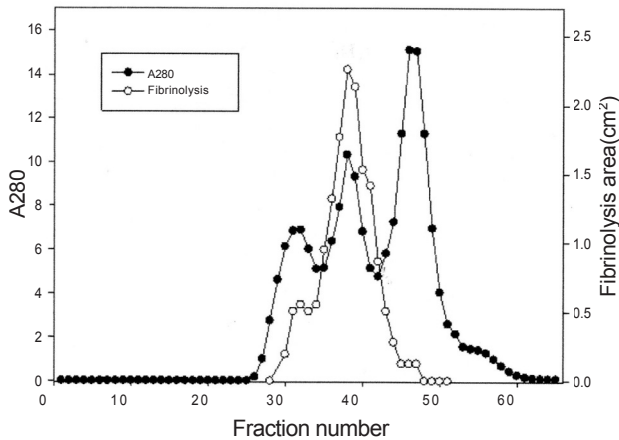


Figure 2 Gel filtration chromatography of the combined sample from the Q-Sepharose column (Fig. 1) on a Sephadex G-75 column. The pooled sample of fractions 39-44 was subjected to subsequent chromatography (Fig. 3) and to SDS-PAGE (Fig. 4, lane D).

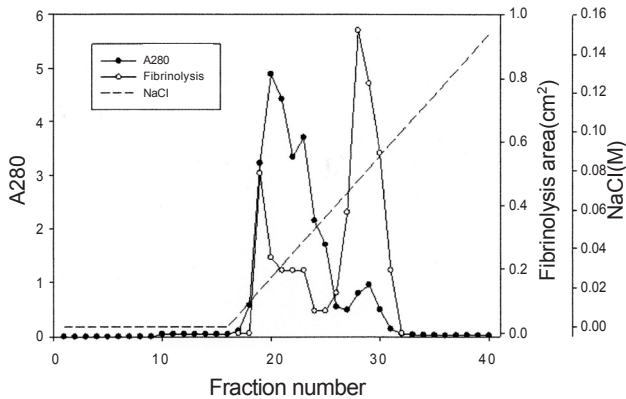


Figure 3 Ion exchange chromatography of the venom sample obtained from the Sephadex G-75 column (Fig. 2) on a DEAE Sepharose column. The combined sample of fractions 28-30 was used for SDS-PAGE (Fig. 4, lane E).

Table 1 Effects of protease inhibitors on the fibrinolytic activity of FE-32kDa from *G. b. siniticus* venom

Protease inhibitors	Concentration (mM)	Relative fibrinolysis (%)
Control	0	100
EDTA	10	0
EGTA	3	0
1,10-Phenanthroline	10	0
PMSF	10	43
TLCK	1	110
Cysteine	10	0
Dithiothreitol	10	0
Iodoacetate	1	110

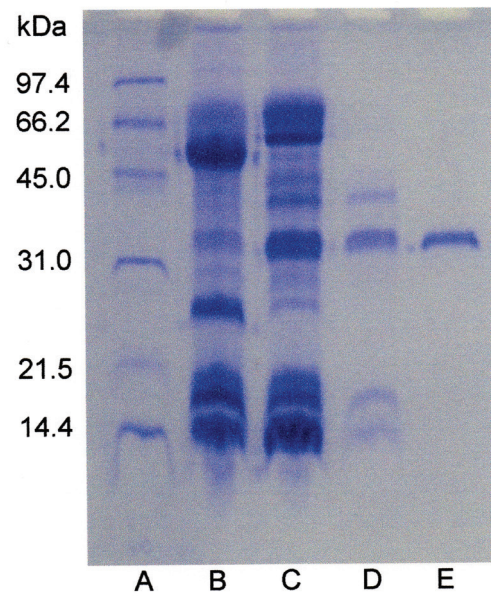


Figure 4 SDS-PAGE of *G. b. siniticus* venom and its chromatography fractions. (lane A: molecular weight marker, lane B: crude venom, lane C: sample from the Q-Sepharose column (Fig. 1), lane D: sample from the Sephadex G-75 column (Fig. 2), and lane E: sample from the DEAE-Sepharose column (Fig. 3)). The samples were heated in a sample buffer containing β -mercaptoethanol before SDS-PAGE.

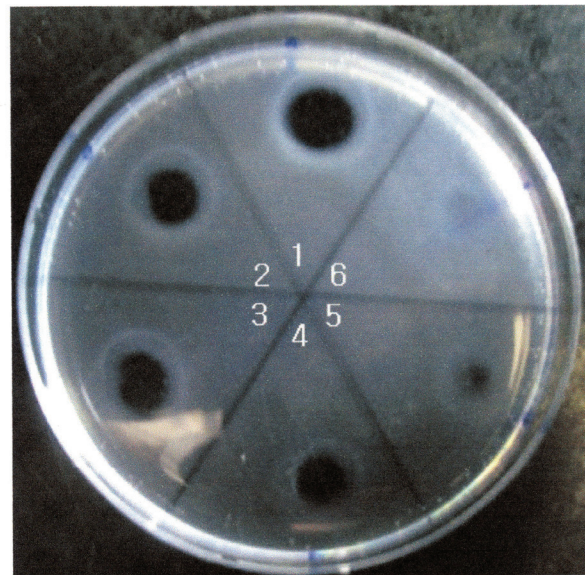


Figure 5 Fibrin plate assay to determine the fibrinolytic activity of FE-32kDa from *G. b. siniticus* venom. (1: 0.5 mg/mL, 2: 0.4 mg/mL, 3: 0.3 mg/mL, 4: 0.2 mg/mL, 5: 0.1 mg/mL, and 6: 0.05 mg/mL).

activity.

The effects of metal ions on the fibrinolytic activity of FE-32kDa were determined (Table 2). The concentration of metal ions added to FE-32kDa (0.5mg/mL) was 10 mM. Table 2 shows that Fe^{2+} and Hg^{2+} inhibited the fibrinolytic activity completely and that Co^{2+} inhibited it strongly. Ca^{2+} ,

Mn²⁺, and Cu²⁺ inhibited the fibrinolytic activity weakly and Mg²⁺ and Cs²⁺ had little effects on the fibrinolytic activity. However, Zn²⁺ increased the fibrinolytic activity by up to 40%. These results suggest that FE-32kDa is a metalloproteinase which requires Zn²⁺ at the active site of the enzyme. The zinc ion at the active site of the enzyme seems to have been partially lost during the purification procedures of the enzyme, and subsequent addition of Zn²⁺ in the fibrin plate assay seems to have increased the enzymatic activity.

Fibrinogen (16 mg/mL) was mixed with FE-32kDa (1.6 µg/mL) and incubated at 37°C for a certain time. The sam-

Table 2 Effects of metal salts on the fibrinolytic activity of FE-32kDa from *G. b. siniticus* venom.

Salts	Concentration (mM)	Relative fibrinolysis (%)
Control	0	100
MgCl ₂	10	110
CaCl ₂	10	82
MnCl ₂	10	77
FeCl ₂	10	0
CoCl ₂	10	41
CuCl ₂	10	76
ZnCl ₂	10	140
CsCl	10	95
HgCl ₂	10	0

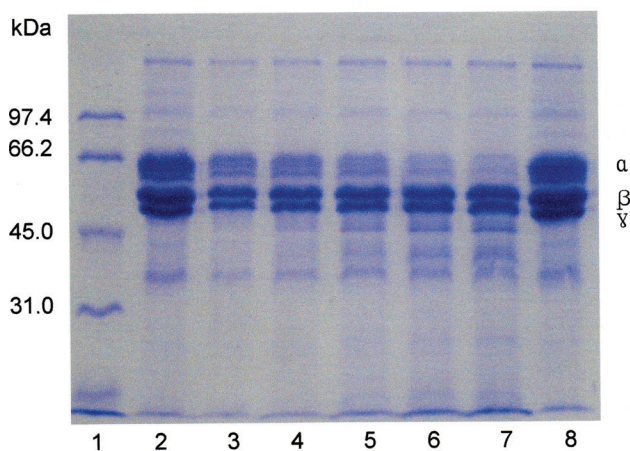


Figure 6 SDS-PAGE of fibrinogen treated with FE-32kDa from *G. l. siniticus* venom. (lane 1: molecular weight marker, lanes 2-7: fibrinogen treated with FE-32kDa for 0, 30, 60, 120, 240, and 360 minutes at 37°C, respectively, and lane 8: fibrinogen treated without the enzyme for 360 minutes). The greek letters at the right side are the names of the fibrinogen chains.

ple was subjected to SDS-PAGE to determine the fibrinogen hydrolysis pattern of FE-32kDa (Fig. 6). The SDS-PAGE pattern showed that the intensity of α -chain of fibrinogen decreased continuously throughout the incubation and was very low after 4 hours (Fig. 6, lane 6, 7). However, no change was noted for β -chain and γ -chain of fibrinogen after 6 hours. Gelatin (16 mg/mL) was mixed with FE-32kDa (1.6 µg/mL) and incubated at 37°C. The sample was subjected to SDS-PAGE to determine the fibrinogen hydrolysis pattern of FE-32kDa (Fig. 7). The gelatin polypeptides with high molecular weights located beneath the border between the stacking gel and the separating gel decreased after 4 hours (Fig. 7, lane 6, 7). However, no further degradation to the polypeptides with molecular weight less than 50 kDa was observed.

The hemorrhagic activity of FE-32kDa was determined by injecting 100 µl of FE-32kDa at various concentrations subcutaneously into the shaved back skin of mice. The mice were sacrificed after six hour and then the area of the hemorrhage zone (Fig. 8) formed beneath the skin was determined. When FE-32kDa was administered to a mouse at the dosages of 20 µg, 10 µg, 5 µg, and 2 µg, the areas of the hemorrhage zone were 1.8 cm², 2.2 cm², 1.4 cm², and 1.0 cm², respectively (A-D in Fig. 8). The hemorrhagic activity of FE-32kDa at the low dosage suggests that the enzyme is not appropriate for clinical treatment of obstructive thrombosis and acute stroke.

The amino acid composition of FE-32kDa is shown in Table 3. The enzyme contains cysteine residues which may be relevant to inactivation of the enzyme by cysteine and dithiothreitol (Table 2). The contents of aspartic acid and leucine which are the major amino acid residues in FE-

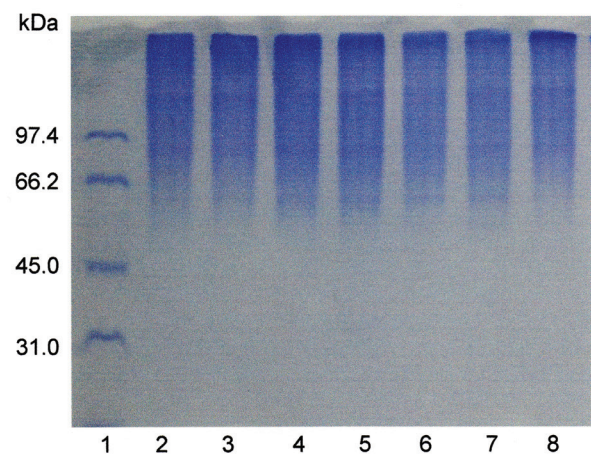


Figure 7 SDS-PAGE of gelatin treated with FE-32kDa from *G. l. siniticus* venom. (lane 1: molecular weight marker, lanes 2-7: gelatin treated with the enzyme for 0, 30, 60, 120, 240, and 360 minutes at 37°C, respectively, and lane 8: gelatin treated without the enzyme for 360 minutes).

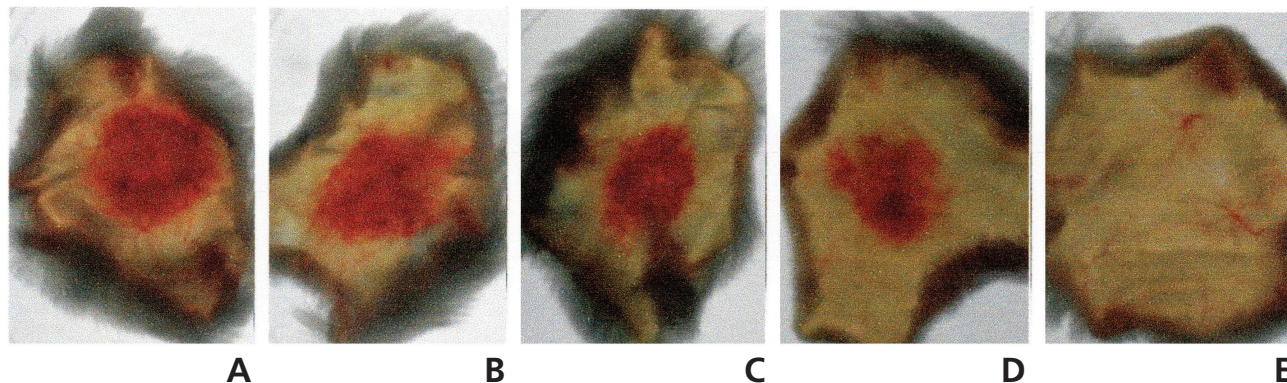


Figure 8 Hemorrhagic activity of FE-32kDa from *G. l. siniticus* venom. (A: 20 µg, B: 10 µg, C: 5 µg, D: 2 µg, and E: 0 µg per dose of 100 µl).

32kDa are 11.2% and 8.8%, respectively (Table 3).

4. Discussion

Three fibrinolytic enzymes, FE-54kDa [8], FE-27kDa [9], and FE-32kDa, were isolated from the venom of *G. b. siniticus*. FE-27kDa was a serine proteinase whose fibrinolytic enzyme activity was inhibited by PMSF [9]. However, both FE-54kDa and FE-32kDa were metalloproteases whose enzyme activities were inhibited by EDTA, EGTA, and 1, 10-phenanthroline [8]. The fibrinolytic activities of FE-54kDa and FE-32kDa were promoted by calcium and zinc, respectively. FE-54kDa hydrolyzed preferentially α -chain of fibrinogen and than β -chain slowly. However, FE-32kDa hydrolyzed α -chain only. FE-54kDa hydrolyzed most polypeptides of gelatin extensively into polypeptides with molecular weights less than 45kDa [8]. However, the hydrolysis of gelatin by FE-32kDa produced polypeptides with molecular weights more than 50kDa (Fig. 7). The areas of the fibrinolysis zones formed by FE-32kDa were larger than those formed by FE-54kDa [8]. These results show that FE-32kDa is more active in fibrin hydrolysis than FE-54kDa.

Brevilysin H1 from *Gloydius blomhoffii brevicaudus* is a metalloprotease which hydrolyzes α -chain of fibrinogen faster than β -chain [13]. The enzyme has a molecular weight of 55kDa in the reducing condition and 110 kDa in the non-reducing condition of SDS-PAGE, consists of 420 amino acid residues, and is a PIII-class metalloprotease. Because FE-54kDa and brevilysin H1, which were isolated from the snakes of the same species, had similar molecular weights, they should be very similar molecules. The molecular weights of FE-54kDa suggests that the enzyme also belongs to the PIII class.

Terada and his colleagues studied the metalloproteases, such as brevilysin H6 [14, 15], brevilysin L4 [16, 17] and brevilysin L6 [18], isolated from the venom of Chinese mamushi, *Agkistrodon halys brevicaudus*, which is clas-

Table 3 Amino acid composition of FE-32kDa.

Amino acid	Composition (%)
Cysteine	3.8
Aspartic acid	11.2
Glutamic acid	6.9
Serine	4.7
Glycine	3.8
Histidine	3.9
Arginine	5.6
Threonine	5.7
Alanine	2.4
Proline	3.5
Tyrosine	6.7
Valine	6.1
Methionine	4.5
Isoleucine	7.6
Leucine	8.8
Phenylalanine	3.6
Tryptophane	4.5
Lysine	6.7
Total	100

sified currently in *Gloydius ussuriensis* [19]. The molecular weight of brevilysin H6 is 60kDa and Zn⁺ is located at the active site of the enzyme. The enzyme belongs to class PIII which is composed of metalloprotease, disintegrin-like, and cysteine-rich domains and is degraded auto-catalytically into 29 kDa and 45 kDa fragments, both of which show no proteolytic activity [15]. Addition of Ca²⁺ increases its proteolytic activity and heat stability. The en-

zyme hydrolyzes A α -chain of fibrinogen preferentially and B β -chain slowly [14]. These results show that FE-54kDa is similar with brevilysin H6 in the aspects of the positive effects of Ca²⁺ on the fibrinolytic activity, the specificities to fibrinogen chains, and the molecular weights.

The molecular weights of brevilysin L4 and L6 were 22kDa and 21.5kDa, respectively [16, 17]. Hydrolysis of fibrinogen by brevilysin L6 was slower than brevilysin L4. The complete amino acid sequence of brevilysin L4 indicates that the enzyme is a PI class metalloprotease [18]. The nucleotide sequence of a cDNA clone encoding brevilysin L4 has revealed that the gene of brevilysin L4 encodes a PII-class metalloprotease, which undergo post-translational autolysis to split into brevilysin L4 and disintegrin.

5. Conclusion

The molecular weight of FE-32kDa suggests that the enzyme should belong to the PII class, which may also be split into a PI-class metalloprotease similar with non-hemorrhagic brevilysin L4. Further studies are required to isolate a non-hemorrhagic brevilysin L4-like fibrinolytic enzyme from *Gloydius blomhoffii siniticus* venom. However, the hemorrhagic activity of FE-32kDa in mice skin suggests that the enzyme is not appropriate for use as a clinical pharmacopuncture.

Disclosure Statement

The authors declare that there are no conflicts of interest.

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