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Enzymological characterization of FII_a, a fibrinolytic enzyme from *Agkistrodon acutus* venom[1]

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Key words

snake venoms; *Agkistrodon acutus*; fibrinolysis; metalloproteinase

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Abstract

Aim: To study the enzymological characterization of a fibrinolytic enzyme (FII_a) from *Agkistrodon acutus* venom. **Methods:** The fibrinogenolytic effect and the influences of several protease inhibitors, chelating agents, and metal ions on fibrinogenolytic activity were visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The metal content of FII_a was determined by atomic absorption spectroscopy. **Results:** After incubation with FII_a (0.25 g/L), Aα-, Bβ- and γ-chains of fibrinogen disappeared within 5 min, 30 min, and 8 h , respectively. The molecular weights of major degradation products were 45 000 and 41 000, which were different from those bands produced by plasmin. The fibrinogenolytic activity of FII_a was strongly inhibited by ethylenediamine tetraacetic acid (EDTA), ethyleneglycol tetraacetic acid (EGTA), dithiothreitol and cysteine, but not by phenylmethyl-sulfonyl fluoride and soybean trypsin inhibitor. Zinc (3171±25 mg/kg), potassium (489±17 mg/kg) and calcium (319±13 mg/kg) were found in FII_a. Zn^{2+}, Ca^{2+} and Mg^{2+} could recover the fibrinogenolytic activity of FII_a, which was inhibited by EDTA. Only Ca^{2+} could recover the fibrinogenolytic activity inhibited by EGTA. **Conclusion:** FII_a can degrade the Aα-, Bβ- and γ-chains of fibrinogen. FII_a is a metalloproteinase, and Zn^{2+}, Ca^{2+}, and disulfide bonds are necessary for its fibrinogenolytic activity.

Introduction

Studies on snake venoms have been proceeding for a long time. It is known that fractions of snake venom exhibit a number of biological activities, such as fibrinogenolysis and/or fibrinolysis, and anti-platelet aggregation[1]. Approximately 3 kinds of enzymes from snake venoms can degrade fibrinogen, these are thrombin-like enzyme (TLE)[2], plasminogen activator[3], and fibrinolytic enzyme. Among them, fibrinolytic enzymes can directly degrade not only fibrinogen but also fibrin *in vitro* and *in vivo*. Furthermore, they are not inhibited by proteinase inhibitors in human blood. With their potential use for treating thrombotic disease the fibrinolytic enzymes have been widely investigated. The fibrinolytic enzymes have been purified from the venoms of *Agkistrodon acutus*[4], *A piscivorus piscivorus*[5], *A contortrix*[6], *A rhodostoma*[7], *Bothrops jararaca*[8], *Crotalus atrox*[9], *Trimeresurus mucrosquamatus*[10] and *Vipera lebetina*[11]. More than 70 kinds of fibrinolytic enzymes have been isolated, and novel fibrinolytic enzymes continue to be reported.

The fibrinolytic enzyme from Taiwanese *Agkistrodon acutus* venom was first isolated by Ou-yang and Huang[12]. In our previous work, another fibrinolytic enzyme called FII_a was purified from Anhui *Agkistrodon acutus* venom. FII_a can degrade fibrin and fibrinogen *in vitro*, and solubilize thrombus *in vivo*[4,13]. However, the enzymological characteristics of FII_a have not been shown clearly. In the present investigation, we mainly investigate the influences of several protease inhibitors, chelating agents, and metal ions on the fibrinogenolytic activity of FII_a. The metal content was also determined.

Materials and methods

Snake venom Lyophilized *Agkistrodon acutus* venom

was collected from Qimen Snake Farm (Anhui, China).

Reagents DEAE-Sephadex A-50, Sephadex G-75, ethylenediamine tetracetic acid (EDTA), ethyleneglycol tetraacetic acid (EGTA), phenylmethylsulfonylfluoride (PMSF) and soybean trypsin inhibitor (SBTi) were purchased from GE Health Care (Little Chalfont, UK). Bovine fibrinogen and plasmin were from Sigma (St Louis, MO, USA). Molecular weight protein standards were from NEB (Beverly, MA, USA). All other chemicals and solvents were of analytical grade from commercial sources.

Purification of the enzyme FII_a, a fibrinolytic enzyme from *Agkistrodon acutus* venom, was prepared according to the method described by Liang *et al*[4].

Fibrinogenolytic activity assay FII_a (1 g/L, 150 μL) was incubated with 450 μL of bovine fibrinogen (1 g/L) at 37 ºC. Aliquots were taken at 5 min, 15 min, 30 min, 45 min, 1 h, 4 h and 8 h intervals, and 600 μL of a denaturing solution (10 mol/L urea, 4% sodium dodecylsulfate and 4% β-mercaptoethanol) was added and the mixture was incubated at 100 ºC for 4 min. Each sample (20 μL) was analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) using a 4% spacer gel and a 12% separation gel[14]. Human plasmin (50 U/L) was used as positive control.

Effect of inhibitiors on fibrinogenolytic activity The effects of EDTA (5 mmol/L), EGTA (5 mmol/L), PMSF (5 mmol/L), SBTi (0.15 g/L), dithiothreitiol (DTT; 5 mmol/L) and cysteine (5 mmol/L) on fibrinogenolytic activity were examined by incubation with FII_a (1 g/L) at 37 ºC for 1 h. After adding bovine fibrinogen (1 g/L), the mixture was incubated for a further 1 h. Each sample (20 μL) was analyzed by SDS-PAGE.

Reactivation by metal ions on fibrinogenolytic activity FII_a (1 g/L, 150 μL) was incubated with EDTA (final concentration: 5 mmol/L) at 37 ºC for 1 h. $MgCl_2$, $CaCl_2$ and $ZnCl_2$ (final concentrations: 5 mmol/L) were added to the incubation solution, and the mixture was incubated for a further 1 h. The fibrinogenolytic activity was examined by SDS-PAGE after a 1-h incubation with 450 μL of bovine fibrinogen (1 g/L). The same experiment was performed with EGTA (final concentration: 5 mmol/L) instead of EDTA.

Metal content assay Metal content was determined using an atomic absorption spectrophotometer. The absorbances of standard solutions were used to draw standard graphs. The metal content of FII_a was estimated by comparison with the standard curve[14].

Results

FII_a degraded the Aα-chain preferentially, followed by the Bβ-chain of fibrinogen, but the γ-chain was the most insusceptible to the enzyme. At a molar ration of 3:1 (fibrinogen: FII_a), the Aα-chain was totally degraded within 5 min, with relatively lower activity for the Bβ-chain, which disappeared within 30 min. The γ-chain was only degraded following a prolonged 8-h incubation with FII_a (Figure 1A). Concomitant with the digestion of fibrinogen, major fragments of M_r approximately 45 000 and 41 000 were observed.

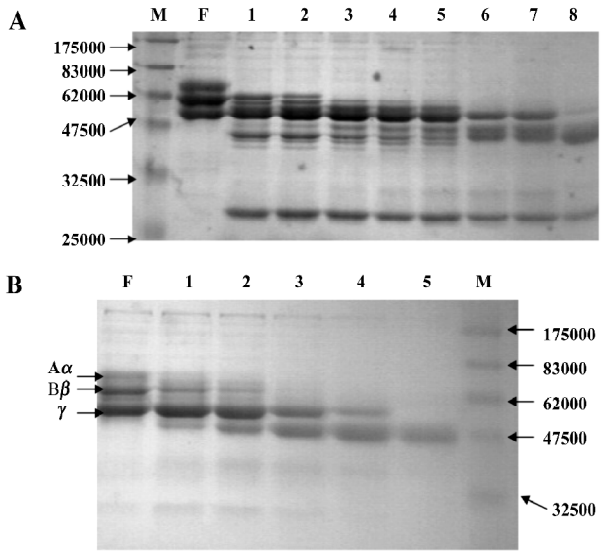

Figure 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of degraded fibrinogen (1 g/L, 450 μL) by (A) FII_a (1 g/L, 150 μL) and (B) plasmin (50 U/L) at 37 ºC. (A) Lanes 1–8: FII_a+fibrinogen incubated for 5 min, 15 min, 30 min, 45 min, 1 h, 2 h, 4 h and 8 h. (B) Lanes 1–5: plasmin+fibrinogen incubated for 5 min, 15 min, 30 min, 45 min and 1 h. M, marker; F, fibrinogen.

When fibrinogen was incubated with human plasmin, the Aα- and Bβ-chains disappeared within 15 min, while the γ-chain disappeared within 1 h. The major digestion fragment observed was at M_r 45 000, of which the cleavage pattern was different from that of FII_a (Figure 1B).

The fibrinogenolytic activity of FII_a was inhibited by EDTA, EGTA, DTT and cysteine, but not by PMSF or SBTi (Table 1). The fibrinogen was still intact after incubation with FII_a pretreated with EDTA, EGTA, DTT, and cysteine (Figure 2). However, the fibrinogen was degraded after incubation with FII_a pretreated with PMSF and SBTi (Figure 2). Zn^{2+}, Ca^{2+}, and Mg^{2+}, at concentrations of 5 mmol/L, could restore the fibrinogenolytic activity of EDTA-treated FII_a. Only Ca^{2+} could restore the fibrinogenolytic activity of EGTA-treated FII_a. Both 1 mmol/L and 5 mmol/L Ca^{2+} were effective (Figure 3).

Zn^{2+}, K^+ and Ca^{2+} were found in significant quantities, at 3171±25 mg/kg, 489±17 mg/kg and 319±13 mg/kg, respectively. The concentrations of Mg^{2+}, Fe^{2+} and Cu^{2+} were only

Table 1. Effect of inhibitors on the fibrinogenolytic activity of FII_a.

Inhibitor	Concentration/mmol·L^{-1}	Fibrinogenolytic activity
EDTA	1.0	–
	0.1	–
	0.01	+/–
EGTA	5	–
DTT	5	–
Cysteine	5	–
SBTi	0.15 g/L	+
PMSF	5	+

DTT, dithiothreitol; EDTA, ethylenediamine tetracetic acid; EGTA, ethyleneglycol tetraacetic acid; PMSF, phenylmethylsulfonylfluoride; SBTi, soybean trypsin inhibitor.

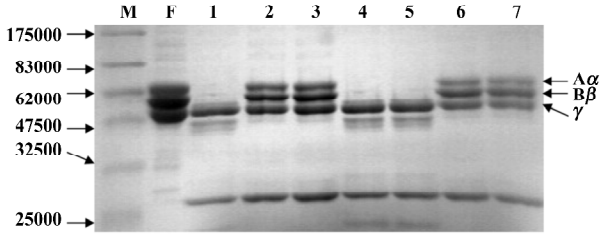

Figure 2. The effect of inhibitors on the fibrinogenolytic activity of FII_a by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. Lane 1, FII_a+fibrinogen; lane 2, FII_a+5 mmol/L EDTA+fibrinogen; lane 3, FII_a+5 mmol/L EGTA+fibrinogen; lane 4, FII_a+5 mmol/L PMSF+fibrinogen; lane 5, FII_a+0.15 g/L SBTi+fibrinogen; lane 6, FII_a+5 mmol/L DTT+fibrinogen; lane7, FII_a+5 mmol/L cysteine+fibrinogen. M, marker; F, fibrinogen.

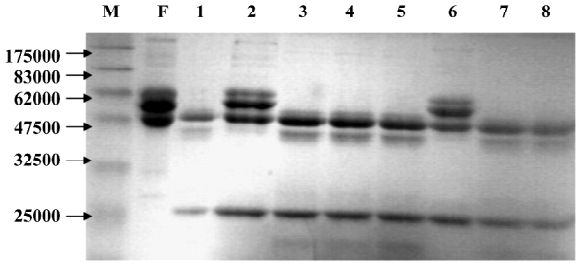

Figure 3. Reactivation by metal ions on fibrinogenolytic activity of FII_a by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. Lane 1, FII_a+fibrinogen; lane 2, FII_a+1 mmol/L EDTA+fibrinogen; lane 3, FII_a+1 mmol/L EDTA+$ZnCl_2$+fibrinogen; lane 4, FII_a+1 mmol/L EDTA+$MgCl_2$+fibrinogen; lane 5, FII_a+1 mmol/L EDTA+$CaCl_2$+fibrinogen; lane 6, FII_a+1 mmol/L EGTA+fibrinogen; lane 7, FII_a+1 mmol/L EGTA+1 mmol/L $CaCl_2$+fibrinogen ; lane 8, FII_a+1 mmol/L EGTA+5 mmol/L $CaCl_2$+fibrinogen. M, marker; F, fibrinogen.

at trace amounts (Table 2). For each mole of FII_a, there was approximately 1 mole of Zn^{2+}, 0.3 mole of K^+ and 0.2 mole of Ca^{2+}.

Table 2. The metal contents of FII_α. n=3. Mean±SD.

Metal ion	Content/mg·kg^{-1}
Zn^{2+}	3171±25
K^+	489±17
Ca^{2+}	319±13
Mg^{2+}	26±7
Fe^{2+}	26±2
Cu^{2+}	24±3

Discussion

FII_a is a α,β-fibrinogenase because it degraded both the Aα-chain and the Bβ-chain of fibrinogen[15]. The Aα-chain of fibrinogen was very susceptible to FII_a, and it was completely degraded within 5 min. Cleavage of the γ-chain of fibrinogen was observed only with a prolonged incubation time. Thus far there have been few reports of fibrin(ogen)olytic snake venom enzymes that cleave of the γ-chain. No enzyme reported has shown cleavage specificity directed solely at the γ-chain[16]. Because the γ-chain of fibrinogen was stable when was incubated with snake venom fibrin(ogen)olytic enzyme, we postulated that the degradation might occur at either an increased incubation time or at an increased concentration. In our previous study, the γ-chain was unaffected after a 2-h incubation. However, in the present study FII_a appeared to degrade the γ-chain after prolonged (8 h) incubation. The same phenomenon was noticed for cerastase F-4 (from *Cerastes cerastes* venom) and a fibrin(ogen)olytic enzyme from *V lebetina* venom, and they appeared to degrade the γ-chain following 48-h and 24-h incubations, respectively[17,18]. Plasmin also cleavages the Aα-, Bβ-, and γ-chains of fibrinogen, but the patterns are different from those observed when cleaved by FII_a. It is interesting that various fibrin(ogen)olytic enzymes seem to produce different degradation patterns for fibrinogen. For example, FII_a mainly yields fragments of 45 kDa and 41 kDa, while basilase produces fragments of 45 kDa, 36 kDa and 10 kDa, and atroxase gives fragments of 45 kDa and 38 kDa[19]. The studies on some fibrin(ogen)olytic enzymes reveal that their cleavage preference is commonly directed to the amino-terminal side of hydrophobic amino acid residues. They display distinct and unique cleavage characteristics with fibrinogen.

The fibrin(ogen)olytic enzymes from snake venoms can be classified as metalloproteinases or serine proteinases. Chelating agents (EDTA, EGTA) completely inhibited FII_a, while serine protease inhibitors (PMSF, SBTi) were

ineffective, indicating that it belongs to the metalloproteinase group. This was supported by data from atomic absorption spectroscopy. For each mole of FII_a there was approximately 1 mole of Zn^{2+}, 0.3 mole of K^+ and 0.2 mole of Ca^{2+}. Like many of the venom fibrinolytic enzymes, FII_a is a zinc metalloproteinase. Besides Zn^{2+}, Ca^{2+} is another metal ion often found in venom with fibrinolytic enzymes. Metal analysis has indicated that the calcium content of atroxase (from western diamondback rattlesnake venom)[9] and lebetase (from *V. lebetina* snake venom)[20] is 0.3 mol/mol and 1 mol/mol, respectively. In adamalysin from *C. adamanteus*[21] and atrolysin c(d) from *C. atrox*[22] it was found that except Zinc-binding site, a calcium ion is bound near the carboxy-terminus of the enzyme. Thus far, only atroxase was reported to contain 1 mol/mol of K^+, while FII_a contains 0.3 mol/mol of K^+. The functions of calcium and potassium have not been elucidated, but they may play a role in retaining the stability of the protein.

Zn^{2+}, Ca^{2+} and Mg^{2+} were effective in restoring the activity of EDTA-treated FII_a, while only Ca^{2+} could restore the activity of EGTA-treated FII_a. The mechanism for this is not clear. It is reported that snake venom metalloproteinases have Zn^{2+}-dependent activities, but some are more active in the presence of Ca^{2+}[23,24]. This seems probably responsible in part for this phenomenon. The effect of Mg^{2+} on the activity of FII_a needs to be elucidated. FII_a is inhibited by DTT and cysteine, suggesting that disulfide bonds are necessary for holding the structure.

In conclusion, like many venom fibrin(ogen)olytic enzymes, FII_a is a metalloproteinase. Both Zn^{2+} and Ca^{2+} play important roles in the fibrinogenolytic activity of FII_a.

References

1 Tseng YL, Lee CJ, Huang TF. Effects of a snake venom metalloproteinase, triflamp, on platelet aggregation, platelet-neutrophil and neutrophil-neutrophil interactions: involvement of platelet GPIbα and neutrophil PSGL-1. Thromb Haemost 2004; 91: 315–24.

2 Tatematsu R, Komori Y, Nikai T. A new thrombin-like enzyme, flavoviridiobin from the venom of *Trimeresurus flavoviridis* (habu). J Nat Toxins 2000; 9: 327–39.

3 Zhang Y, Wisner A, Xiong Y, Bon C. A novel plasminogen activator from snake venom. Purification, characterization, and molecular cloning. J Biol Chem 1995; 270: 10 246–55.

4 Liang XX, Chen JS, Zhou YN, Qiu PX, Yan GM. Purification and biochemical characterization of FIIa, a fibrinolytic enzyme from *Agkistrodon acutus* venom. Toxicon 2001; 39: 1133–9.

5 Hahn BS, Chang IM, Kim YS. Purification and characterization of piscivorase I and II, the fibrinolytic enzymes from eastern cottonmouth moccasin venom (*Agkistrodon piscivorus*). Toxicon 1995; 33: 929–41.

6 Trikha M, Schmitmeier S, Markland FS. Purification and characterization of fibrolase isoforms from venom of individual southern copperhead (*Agkistrodon contortrix*) snakes. Toxicon 1994; 32: 1521–31.

7 Ouyang C, Hwang LJ, Huang TF. Alpha-fibrinogenase from *Agkistrodon rhodostoma* (Malayan pit viper) snake venom. Toxicon 1983; 21: 25–33.

8 Maruyama M, Sugiki M, Yoshida E, Shimaya K, Mihara H. Broad substrate specificity of snake venom fibrinolytic enzymes: possible role in haemorrhage. Toxicon 1992; 30: 1387–97.

9 Willis TW, Tu AT. Purification and biochemical characterization of atroxase, a nonhemorrhagic fibrinolytic protease from western diamondback rattlesnake venom. Biochemistry 1988; 27: 4769–77.

10 Hung CC, Huang KF, Chiou SH. Characterization of one novel venom protease with beta-fibrinogenase activity from the Taiwan habu (*Trimeresurus mucrosquamatus*): purification and cDNA sequence analysis. Biochem Biophys Res Commun 1994; 205: 1707–15.

11 Siigur E, Siigur J. Purification and characterization of lebetase, a fibrinolytic enzyme from *Vipera lebetina* (snake) venom. Biochim Biophys Acta 1991; 1074: 223–9.

12 Ou-yang C, Huang TF. Purification and characterization of the fibrinolytic principle of *Agkistrodon acutus* venom. Biochim Biophys Acta 1976; 439: 146–53.

13 Chen JS, Liang XX, Qiu PX, Yan GM. Thrombolysis effect with FIIa from *Agkistrodon acutus* venom in different thrombosis model. Acta Pharmacol Sin 2001; 22: 420–2.

14 Gasmi A, Chabchoub A, Guermazi S, Karoui H, Elayeb M, Dellagi K. Further characterization and thrombolytic activity in a rat model of fibrinogenase from *Vipera Lebetina* venom. Thromb Res 1997; 86: 233–42.

15 Pinto AF, Dobrovolski R, Veiga AB, Guimaraes JA. Lonofibrase, a novel α-fibrinogenase from *Lonomia obliqua* caterpillars. Thromb Res 2004; 113: 147–54.

16 Swenson S, Markland FS Jr. Snake venom fibrin(ogen)olytic enzymes. Toxicon 2005; 45: 1021–39.

17 Daoud E, Tu AT, el-Asmar MF. Mechanism of the anticoagulant, Cerastase F-4, isolated from *Cerastes cerastes* (Egyptian sand viper) venom. Thromb Res 1986; 41: 791–9.

18 Gasmi A, Karoui M, Benlasfar Z, Karoui H, el-Ayeb M, Dellagi K. Purification and characterization of a fibrinogenase from *Vipera lebetina* (desert adder) venom. Toxicon 1991; 29: 827–36.

19 Gatta D, Dong A, Witt J, Tu AT. Biochemical characterization of basilase, a fibrinolytic enzyme from *Crotalus basiliscus*. Arch Biochem Biophys 1995; 317: 365–73.

20 Siigur J, Samel M, Tonismagi K, Subbi J, Siigur E, Tu AT. Biochemical characterization of lebetase, a direct-acting fibrinolytic enzyme from *Vipera lebetina* snake venom. Thromb Res 1998; 90: 39–49.

21 Gomis-Ruth FX, Kress LF, Bode W. First structure of a snake venom metalloproteinase: a prototype for matrix metalloproteinase/

collagenase. EMBO J 1993; 12: 4151–7.
22 Zhang D, Botos I, Gomis-Ruth FX, Doll R, Blood C, Njoroge FG *et al*. Structural interaction of natural and synthetic inhibitors with the venom metalloproteinase, atrolysin c (from d). Proc Natl Acad Sci USA 1994; 91: 8447–51.
23 Assakura MT, Reichl AP, Asperti MC, Mandelbaum FR. Isolation of the major proteolytic enzyme from the venom of the snake *Bothrops moojeni* (caissaca). Toxicon 1985; 23: 691–706.
24 De-Camargo-Goncalves LR, Chudzinski-Tavassi AM. High molecular mass kininogen inhibits metalloproteinases of *Bothrops jararaca* snake venom. Biochem Biophys Res Commun 2004; 318: 53–9.

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