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Purification and partial characterizations of coagulant protein FIa from *Daboia russelli siamensis* (Myanmar) venom[1]

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Key words

snake venoms; *Daboia russelli siamensis*; factor X; blood coagulation

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Abstract

Aim: To purify and characterize the coagulant protein FIa from *Daboia russelli siamensis* (Myanmar) venom. **Methods:** FIa was purified from *Daboia russelli siamensis* (Myanmar) venom by ion-exchange chromatography on CM-Sephadex C-50, and gel filtration on Sephadex G-75 and a Superdex 75 column. The hemostatic activity of FIa was determined by the method of Williams and Esnouf. The specific chromogenic substrates were used respectively to determine the activation of factor X and prothrombin. The fibrinogen-clotting activity of FIa was determined by the method of Gao *et al*. Normal saline was used as a negative control while factor Xa and thrombin were used as positive controls, respectively. **Results:** FIa, a coagulant protein, was achieved by ion-exchange chromatography and gel filtration with a molecular weight of 34 479 and an isoelectric point of 7.2. FIa was shown to have strong hemostatic activity. The hemostatic activity of 0.5 mg FIa was equal to that of 1.5625 u thrombin. FIa primarily activated factor X, however, had no influence on prothrombin, nor did it cleave or clot fibrinogen. **Conclusion:** FIa is a factor X-activating enzyme, which could activate factor X to factor Xa, but has no effect on prothrombin and fibrinogen.

Introduction

Snake venoms are complex mixtures containing many different biologically active proteins and peptides. A number of these proteins affect the mammalian coagulation system by cleaving limited bonds in the blood coagulation factors. Factor V, factor X, prothrombin activators, and thrombin-like enzymes have been separated from different snake venoms[1–3]. Among those, snake venoms of the Viperinae family have been reported to contain strong procoagulants. Because of their biological activities, some of these venom proteins are useful for basic studies of hemostasis and thrombosis and for pharmacological and clinical applications[1,6]. *Daboia russelli siamensis* is widely found over southern China, central and southern Myanmar, and central Thailand. Although we have known that the venom of *Daboia russelli siamensis* also contains some hemostatic fractions, studies about the hemostatic effect and procoagulant mechanism of *Daboia russelli siamensis* (Myanmar) venom are minimal.

In the present investigation, we purified the hemostatic fraction FIa from *Daboia russelli siamensis* (Myanmar) venom, determined the hemostatic activities of FIa and investigated the mechanism of hemostasia and the potential application of FIa.

Materials and methods

Snake venom *Daboia russelli siamensis* (Myanmar) venom was purchased from Guangzhou Medical College (Guangzhou, China) and lyophilized and stored in a desiccator.

Reagents CM-Sephadex C-50, Sephadex G-75, and Superdex 75 were purchased from Pharmacia (Uppasala, Sweden). Human fibrinogen and thrombin were purchased from Sigma (St Louis, MO, USA). Factor X, factor Xa, prothrombin, and the reagent packs for the activity assays of factor Xa, prothrombin, and thrombin were purchased from Merck (Darmstadt, Germany). All other reagents were of

analytical grade and obtained from commercial sources (Guangzhou, China).

Purification of FIa from Daboia russelli siamensis (Myanmar)venom

CM-Sephadex C-50 ion exchange chromatography *Daboia russelli siamensis* (Myanmar) venom (1.0 g) was dissolved in 0.5 mol/L ammonium acetate (pH 5.8), and the supernatant was applied to a column (2.0 cm×80 cm) of CM-Sephadex C-50. The fractions were eluted with a linear gradient consisting of 0.5 mol/L ammonium acetate (pH 5.8) as the starting buffer and 1 mol/L ammonium acetate (pH 8.0) as the limit buffer. The absorbance of the eluates was measured and then the hemostatic fraction was dialyzed and lyophilized.

Sephadex G-75 gel filtration Gel filtration was performed on a Sephadex G-75 column (2.0 cm×100 cm) equilibrated with 0.02 mol/L sodium phosphate buffer (pH 7.4). The first fraction (FI, 100 mg) from CM-Sphadex C-50 chromatography was eluted with the same buffer.

Superdex 75 gel filtration Gel filtration was performed on a Superdex 75 column (1.0 cm×80 cm) equilibrated with 0.02 mol/L sodium phosphate buffer (pH 7.4). The first fraction (FIa, 30 mg) from Sephadex G-75 gel filtration by gel filtration was eluted with the same buffer.

Molecular weight and isoelectric point determination SDS-PAGE was performed according to the method of Laemmli[17]. The molecular weight standards were MBP-G-galactosidase (175 000), MBP-paramyosin (83 000), glutamic dehydrogenase (62 000), aldolase (47 500), triosephosphate isomerase (32 500), G-lactoglobulin A (25 000), lysozyme (16 500), and aprotinin (6 500). Isoelectrofocusing–PAGE was carried out by the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China), with a pH gradient of 3–10 generated by ampholine (pH 3–10; Amersham Biosciences, Uppsala, Sweden).

Measurement of hemostatic activities Three different groups were established at various concentration intervals. The FIa groups were given 0.5, 0.25, 0.125, 0.625, and 0.3125 mg/100 μL and each concentration was tested 6 times. Thrombin was used as a positive control and 25, 12.5, 6.25, 3.125, 1.5625, 0.78125, and 0.390625 U/100 μL thrombin was given. Each concentration was also tested 6 times. Normal saline was used as a negative control.

The hemostatic activities of thrombin and FIa were measured according to the coagulation time determined by the method of Williams and Esnouf[4]. The citrated plasma (100 μL) was incubated with 100 μL of 0.01 mol/L Tris-HCl buffer (pH 7.3) containing 0.15 mol/L NaCl at 37 °C for 3 min. 100 μL of 0.05 mol/L $CaCl_2$ plus thrombin (100 μL) or FIa (100 μL) were added to the pre-incubation mixture respectively. The clotting time of the plasma was then recorded.

Effect of FIa on human blood factor X Using the method of Gowda *et al*[6], the activation of purified human factor X was followed by using the FXa differential chromogenic substance. Purified human blood factor X (100 U) was incubated at 37 °C in 50 mmol/L Tris-HCl (pH 7.4) containing 0.1 mol/L NaCl, 0.01 mol/L $CaCl_2$, and 12 μg of FIa in a total reaction volume of 500 μL. Aliquots (50 μL) were removed at various times. 50 μL of FXa differential chromogenic substance solution [dissolved in 50 mmol/L Tris-HCl (pH 7.4) containing 0.1 mol/L NaCl (final concentration of 0.2 mmol/L) was added into the aliquots. The light absorbance was recorded at 405 nm. FXa was used as a positive control and normal saline was used as a negative control.

Effect of FIa on human prothrombin Using the method of Hofmann and Bon[7], the activation of the purified prothrombin was followed by using the thrombin differential chromogenic substance. Purified human prothrombin (6.67 μg/μL) was incubated at 37 °C in 50 mmol/L Tris-HCl (pH 7.4) containing 0.1 mmol/L NaCl and 10 mmol/L $CaCl_2$ with FIa. Aliquots (50 μL) were removed at various times. 450 μL of thrombin differential chromogenic substance solution [dissolved in 50 mmol/L Tris-HCl (pH 7.4) containing 0.1 mol/L NaCl] was added into the aliquots. The light absorbance was recorded at 405 nm. Thrombin was used as a positive control and normal saline was used as a negative control.

Effect of FIa on fibrinogen Using the method of Gao *et al*[8], fibrinogen-clotting activity was determined by mixing 20 μL of FIa (concentrations were 0.5, 0.25, 0.125, 0.0625, and 0.02125 mg/100 μL, respectively) with 200 μL of human fibrinogen solution (3 mg/mL) in 50 mmol/L Tris-HCl buffer (pH 7.4) and 0.1 mol/L NaCl (containing 10 mmol/L $CaCl_2$) and incubated at 37 °C. The clotting time was recorded. Thrombin was used as a positive control and normal saline was used as a negative control.

Results

The isolation of FIa was achieved by using 3 steps of purification (Figure 1A–1C). Ion exchange chromatography of crude venom using a column of CM-Sephadex C-50 yielded 11 fractions (Figure 1A). The first fraction (FI) had the highest hemostatic activity. Its hemostatic activity, expressed by the thrombin coagulation time, was 430±5 s/31.25 μg (n=6). Further chromatography of FI using a column of Sephadex G-75 resulted in 3 fractions: Fa, Fb, and Fc (Figure 1B). The hemostatic activity of Fa was 350±5 s/31.25 μg (n=6), which

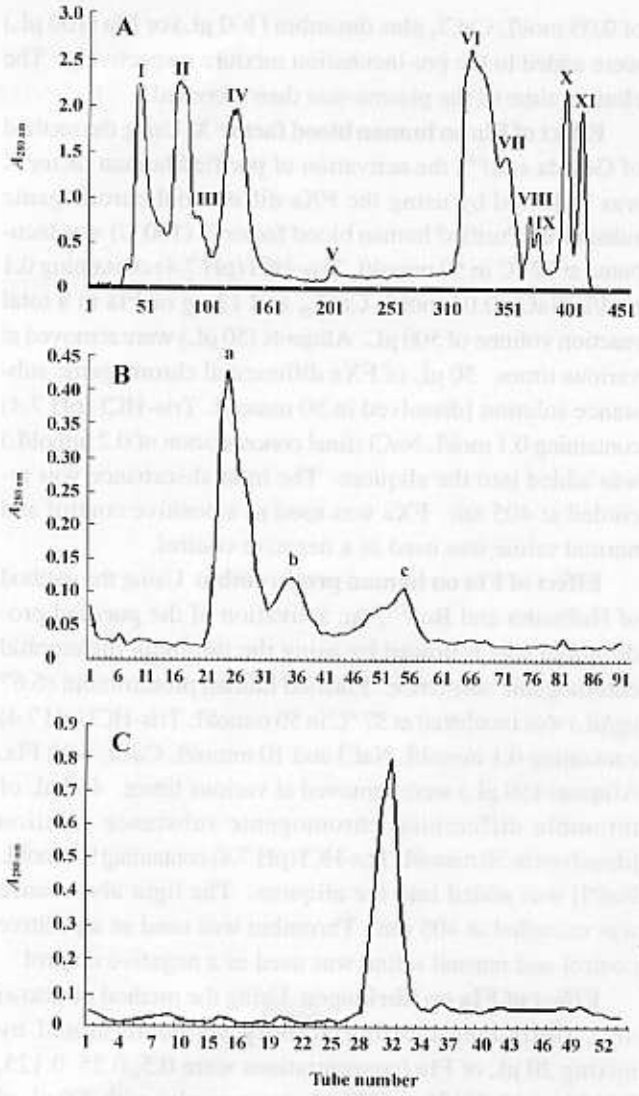

Figure 1. Isolation of F I a by a three-step purification. A, CM-Sephadex C-50 ion exchange chromatography of *Daboia russelli siamensis Myanmar* venom. CM-Sephadex C-50 column (2.0×80 cm) was pre-equilibrated with 0.5 mol/L ammonium acetate buffer, crude venom (1.0 g) was applied in the same buffer (8 mL) and eluted with a gradient of ammonium acetate concentration from 0.05 mol/L (pH 5.8) to 1 mol/L (pH 8.0) at a flow rate of 4 tube/h. The hemostatic activity was highest in fraction I (F I). B, Sephadex G-75 gel filtration of F I. F I,100 mg, was applied on Sephadex G-75 column (2.0×100 cm) equilibrated with 0.02 mol/L sodium phosphate buffer (pH 7.4). The flow rate was 12 mL/h. The hemostatic activity was concentrated in fraction a (Fa). C, Superdex 75 gel filtration of FIa. Superdex 75 column (1.0×80 cm) was pre-equilibrated with 0.02 mol/L sodium phosphate buffer (pH 7.4) and eluted with the same solution at a flow rate of 3 mL/h.

was higher than that of Fb and Fc. The collection of the sharp peak of Fa was then applied to a Superdex 75 column. The single peak obtained was named FIa (Figure 1C). The hemostatic activity of FIa was 290±5 s/31.25 μg (n=6). From 1 g of crude venom, 10.75 mg of FIa was obtained.

FIa was found to be electrophoretically homogeneous by SDS-PAGE (Figure 2) and isoelectric focusing (Figure 3). SDS-PAGE analysis gave a calculated molecular weight of 34 479. Isoelctric focusing studies indicated that FIa was a neutral protein with a pI of 7.2.

The hemostatic effect of purified enzyme FIa and throm-

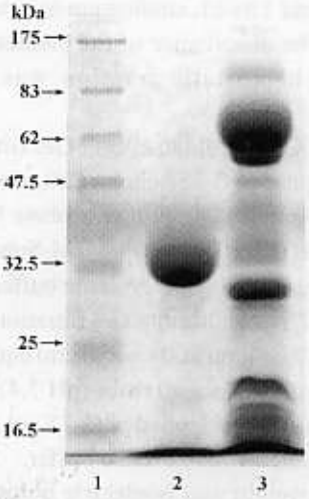

Figure 2. SDS-PAGE of FIa. Lane 1: molecular mass markers; Lane 2: FIa; Lane 3: crude venom. The protein bands were stained with 0.25% coomassie brilliant blue R-250 solution.

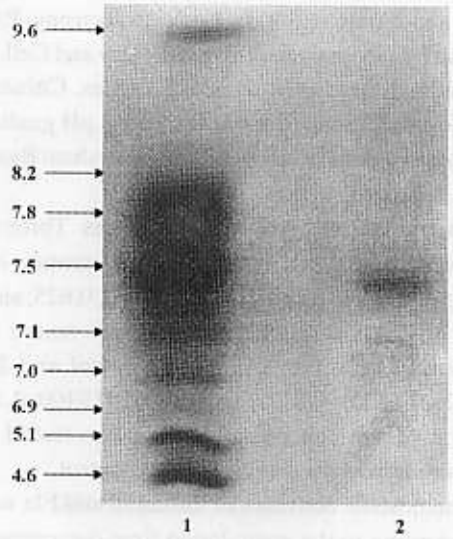

Figure 3. Isoelectric focusing analysis of FIa at different pH ranges. Lane 1: Pharmacia isoelectric focusing calibration kit pH 3–10; Lane 2: FIa.

bin was determined by coagulation time. The clotting time of normal human plasma was 960±5 s (n=6). FIa (100 μL) at several different dosages, 0.5, 0.25, 0.125, 0.0625, and 0.03125 mg, was applied. The clotting times of FIa were 60±5 s, 100±5 s, 142±3 s, 180±4 s, and 290±2 s, respectively (n=6). It was shown the clotting times increased with the decreased dosages (Figure 4A). The hemostatic effect of thrombin (100 μL) at different dosages (5, 12.5, 6.25, 3.125, 1.5625, 0.78125, and 0.390625 u, was also determined. The clotting times of thrombin were 6.88±0.1 s, 12.13±0.15 s, 16.52±0.08 s, 26.56±0.21 s, 64±1.2 s, 89±1.4 s, and 146±3 s, respectively (n=6). It was shown that the clotting times also increased with the decreased dosages of thrombin (Figure 4B).

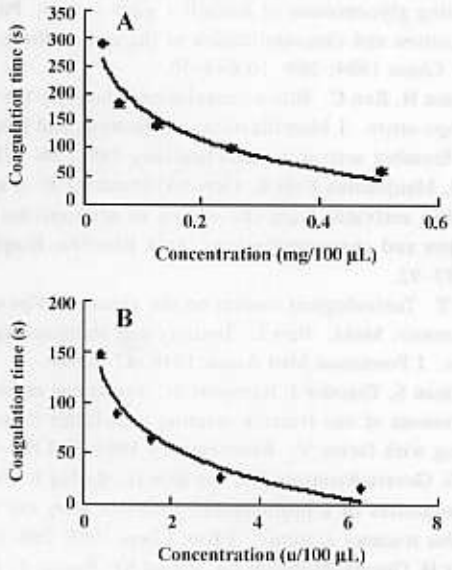

Figure 4. Coagulant activity of FIa and thrombin in different concentrations. A, Coagulaant activity of FIa at different concentrations. B, Coagulant activity of thrombin at different concentrations. The activities were expressed by coagulation time (n=6).

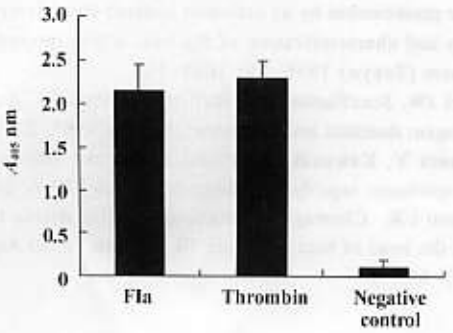

Figure 5. The effect of FIa on human factor X. After adding FIa, the absorbance of the reaction mixture showed statistically significant difference, compared with negative control (n=3). The results were expressed by the light absorbance at 405 nm.

Purified enzyme FIa from *Daboia russelli siamensis* (Myanmar) venom readily cleaves a number of commercially available chromogenic substrates that have been designed for human blood factor X. The parameters for chromogenic substrate conversion by the venom activator are higher than by normal saline (Figure 5). It was shown that FIa can active factor X to factor Xa. However, in the test of chromogenic substrates to prothrombin, the parameters of FIa had no change compared to the normal saline group (Figure 6). It showed that FIa had no catalytic efficiency on protrombin.

In the test of fibrinogen-clotting activity, we found that FIa at dosages of up to 0.5 mg/100 μL could not coagulate human fibrinogen, which was the same with the normal saline group. It showed that FIa had no effect on human fibrinogen.

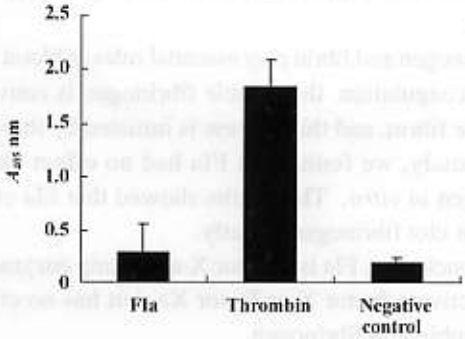

Figure 6. The effect of FIa on human prothrombin. After adding FIa, the absorbance of the reaction mixture did not show any statistical difference, compared with negative group (n=3). The results were expressed by the light absorbance at 405 nm.

Discussion

Studies on Russell's viper venoms have been conducted for a long time. It is known that fractions of Russell's viper venom exhibit a number of hemostatic activities such as the proteases from Russell's viper venom that activate factor X, factor V, and prothrombin[9-12].

In this paper, we have described the purification and characterization of the factor X-activating fraction FIa from *Daboia russelli siamensis* (Myanmar) venom.

With a 3-step procedure, we obtained a purified factor X-activating fraction FIa. Its molecular weight was 34 479 and its pI was 7.2, showing a difference when compared with RVV-X obtained by Esnouf and Williams[4]. It is also different from the factor X-activating fraction obtained by Yang *et al* from Thailand *Daboia russelli siamensis*[5]. Judging from the above facts, FIa is a new factor X-activating protein

found from the venom of *Daboia russelli siamensis* (Myanmar) venom, or these difference is a different derivation of viper *russellii*. We found that FIa might shorten clotting time, and the hemostatic activity of 0.5 mg FIa was equal to that of 1.5625 u of thrombin.

We know that the activation of the extrinsic coagulation pathway initiated by an expression of prothrombinase (prothrombin, FXa) plays an important role in hemostasis. Factor Xa, the physiological activator of prothrombin, converts prothrombin to active thrombin. Thrombin directly activates platelets and cleaves fibrinogen to fibrin monomers[13,14]. In order to understand the relationship between FIa and this process, we determined the activating activities of FIa on factor Xa prothrombin *in vitro*. In light of our data, we found that FIa could increase the activity of factor X, which has a concentration-time relationship. However, we found that FIa could not affect the activity of prothrombin directly.

Fibrinogen and fibrin play essential roles in blood clotting. During coagulation, the soluble fibrinogen is converted to insoluble fibrin, and this process is initiated by thrombin[15]. In this study, we found that FIa had no effect on human fibrinogen *in vitro*. The results showed that FIa could not cleave or clot fibrinogen directly.

In conclusion, FIa is a factor X-activating enzyme, which could activate factor X to factor Xa, but has no effects on prothrombin and fibrinogen.

These activities indicate the potential therapeutic application of FIa for hemostasis. Therefore, the clinical application of FIa remains to be explored and evaluated. Moreover, because habitat, climate, age, and environment influence the venom ingredient and toxicity at different levels, the stability of natural venom is not ideal. It is more interesting to produce medical venom products by molecular cloning and expression. The N-terminus amino acid sequences of FIa have been determined, but the data are not shown in the present paper, which would be very useful in following studies.

Acknowledgement

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