Fibrinogenase from the Venom of *Trimeresurus mucrosquamatus*

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Abstract. -A new fibrin(ogen)olytic protease (FP) was purified from the venom of *Trimeresurus mucrosquamatus* by DEAE-Sephadex A50, Sephadex-G75, CM-Sepharose CL-6B and mono-s (FPLC) column chromatography. The molecular weight was 22,000 Da and the isoelectric point was 9.2. It was a glycoprotein composed of 194 amino acid residues. FP could hydrolyze casine, finbrin, fibrinogen and also showed hemorrhagic subcutaneously, no phospholipase A activity, arginine esterase activity which existed in the crude venom. The enzyme could be inhibited by ethylenediamine tetra-acetate (EDTA) and cysteine, but not by phenylmethyl sulfonyl fluoride (PMSF). FP cleaved the Bß-chain of fibrinogen first following the Aa-chain. In vivo, thrombolytic activity was tested on artificial thrombus placed in the cerebral artery of rabbits. Thrombolysis was then characterized by angiographic techniques over several intervals. The fibrinolytic activity resulted in thrombolytic recanalization of two dosage groups. Of four rabbits of 0.2 mg/kg, one achieved recanalization in 12 hrs. and three in 24 hrs. Of another four under the dosage of 0.4 mg/kg, three recanalized successfully in 5 hrs. and one in 9 hrs.

Keywords: Venom, Fibrin(ogen)olytic Protease, Thrombolysis.

Introduction

Fibrinolytic and fibrinogenolytic activity had been described in the venoms of a number of snake species, including members of the Crotalinae, Viperinae, and Elapidae families (Ouyang and Teng, 1976; Willis et al., 1988; Daoud et al., 1987; Evans and Barrett, 1988). The fuibrin(ogen)olytic enzymes in snake venoms had also been reviewed previously (Seegers and Ouyang, 1979; Hellmann, 1968; Markland, 1988; Markland, 1991). Several fibrin(ogen)olytic enzymes were isolated from the venom of *Trimeresurus mucrosquamatus*: two fibrinogenases (Ouyang and Teng, 1976), two hemorrhagic principals HT-a and HT-b (Nikai et al., 1985) and three proteinases (Sugihara and Mori, 1985). Willis, et al. (1989) evaluated the thrombolytic potential of anticoagulant proteases in *Crotalus atrox* venom using rats. In the present study, we purified a new fibrinogenase from the venom of Chinese habu snake and studied its characteristics.

Materials And Methods

Lyophilysed Trimeresurus mucrosquamatus venom was obtained from Yuanlin Farm (Hunan, China) and stored at -20°C. Human thrombin was purchased from Shanghai Hospital. Fibrinogen, BAEE (N- benzoyl- L- arginine ethyl ester), PMSF were from the Shanghai Institute of Biochemistry, Academia Sinica. Urokinase (UK) came from Nanjing University. DEAE-Sephadex A50, Sephadex-G75, CM-Sepharose CL-6B and mono-s HR5/5 (FPLC) were purchased from Phrmacia Fine Chemicals (made in Uppsala, Sweden). The other chemicals used were analytical grade from commercial sources.

Isolation Procedure: Isolation of FP was achieved by a combination of gel filtration and ion-exchange chromatography at 4°C (Fig. 1). One gram of crude venom was dissolved in 5 ml of 50 mM Tris, pH 8.8. The insoluble material was removed by centrifugation (2000 g) for 10 min. The supernatant was fractionated thus: first, on DEAE-A50 (3 X 100 cm), 50 mM Tris-HCl pH 8.5; second, Sephadex -G75 (2 X 100 cm), 20 mM Tris-HCl pH 7.5; third, CM-Sepharose CL-6B (2 X 30 cm), 10 mM

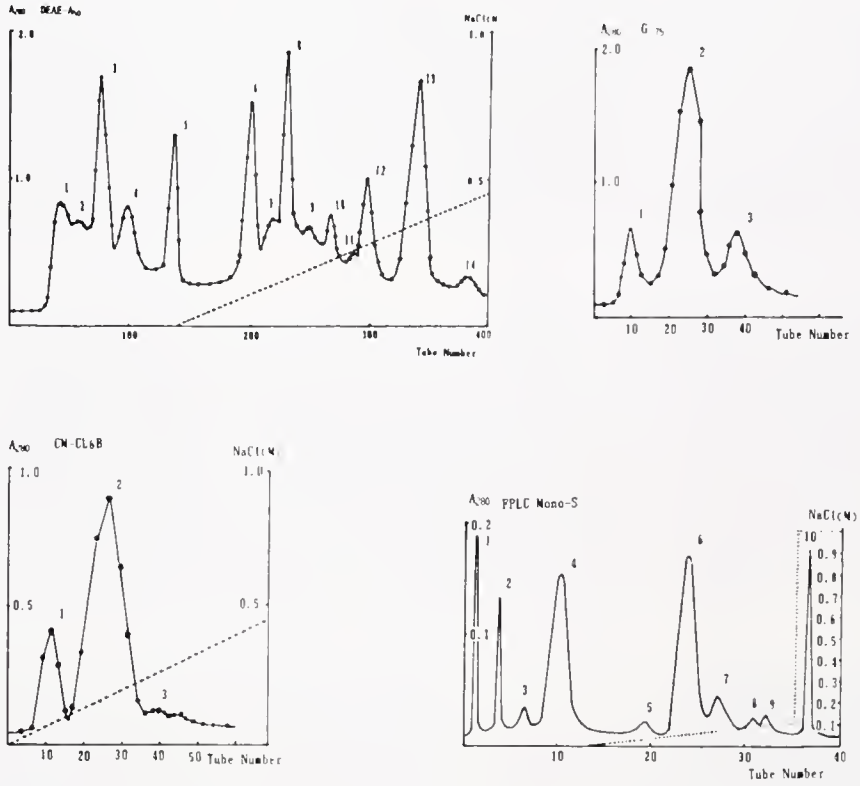

FIG. 1. Fractionation of *Trimeresurus mucrosquamatus* venom. 1st, DEAE-A50 (3X100 cm) anion exchanging, 50mM Tris-HCL pH 8.5; 2nd, Sephadex G-75 gel filtration (2X100 cm), 10mM ammonium acetate pH 7.0; 4th, mono-s cation exchanging, 10mM sodium acetate pH 5.8.

ammonium acetate pH 7.0; and fourth, on mono-s HR 5/5, 10 mM sodium acetate pH 5.8.

Characterization of FP: Assay for hemorrhagic activity assay for gross observation was performed as reported previously (Bjarnason and Fox, 1983). Proteolytic activity was assayed by a method using casine of Kunitz (1947). The inhibition of EDTA, Cysteine, and PMSF was also tested with this method.. Fibrinogenase activity was measured by the method of Ouyang and Huang (1979). Fibrinolytic activity was tested with the fibrin plate method of Astrup and Mullertz (1952), and also with fibrin clot from fibrinogen with thrombin. Arginine ester hydrolytic activity was assayed using BAEE as a substrate. BAEE 50 mM (containing 1 mM CaCl2) was prepared with 50 mM Tris-HCl (pH 8.0) buffer. Trypsin was taken as the control. The phospholipase activity was qualitatively assayed with the substrate of yolk. The pH of the substrate was modulated to 8.0; after the fraction was added, the pH decreased by the phospholipase activity, and was adjusted to that of the original by 10 mM NaOH. The values of sodium hydroxide was taken to present the activity. Amino acid compositions were carried on a Model 835-50 Hitachi high speed automatic analyzer by the method of Simpson et al. (1976). Twenty-four h, 48 h hydrolysates were used. Phanolanaline was used as the minimum residue to calculate the number of amino acid residues.

Thrombosis assay by angiography: For FP dosage determination, 0.2 ml rabbit plasma made clot with 3U thrombin, 50μg FP was used to test in vitro activity. In 4.5-5.5 hours, the milky white

TABLE 1. Summary of purified FP from *T. mucrosquamatus* venom (n=6).

	Fraction in step				
	Crude venom	DAEA-A50 3	G-75 2	CMCL-6B 2	mono-s 6
Recovered protein (mg)	1000	107	93.1	72.6	19.1
Hemorrhage (mm X mm) (25±3 gm mice 100 µg sample)	16.3	6.25	7.20	7.80	8.64
Caseinolytic activity Units/mg.min	0.64	0.42	0.46	0.58	0.48
Fibrinolytic activity (Fibrin heated plate mm^2)	270	225	240	288	216
Arginie esterase activity	+	-	-	-	-
Phospholipase A activity	+	-	-	-	-

TABLE 2. Effects of chemical factors on FP proteolytic activity (n=6).

%	Na+	Ca++	Mg++	Cu++	Zn++	Al+++	Fe+++	Co6+	EDTA	Cyst.	PMSF
Acti.	100	180	115	230	335	305	310	390	0.1	0.1	98

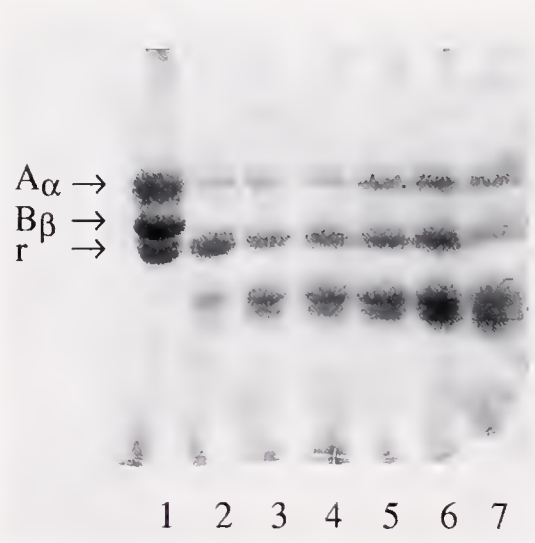

FIG. 2. SDS-PAGE of reduced fibrinogen after incubation with FP (10ug) lane 1; fibrinogen; lane 2-7; fibrinogen with FP for 5, 10, 20, 30; 45; and 60 min. respectively.

clot could become clear. Thrombolytic activity by FP was tested on artificial thrombus catheterized in the rabbit. Sixteen rabbits, ranging from 2.2 to 2.7 kg, were used. 0.5 ml of blood was drawn from the ear vein of the rabbits and made thrombus as 20 mm (long) X 1.00 mm (diameter). The rabbits were then anesthetized with an intravenous injection of a ketamine at a dosage of 2 mg/kg weight. The left common carotid artery was isolated, and a polythylene catheter (1.00 ID X 1.40 OD) was inserted into the artery. By digital subtraction angiology (DSA, Angiotran cmp, Siemens, Germany), normal angiographic was recorded with angiografin solution (meglucamine diatrizoate 65% 30 ml X "Schering AG", made in Germany) of 3 ml. The thrombus was catheterized into the artery, then the thrombosis graph was recorded. Thirty minute after the thrombus induction, the 16 rabbits, separated into four groups, were injected with saline, U.K. 1,000 IU/kg weight, 0.2 mg FP/kg weight, 0.4 mg FP/kg weight transcatheterically respectively, then the four angiogrames were taken after 5, 9, 12 and 24 hours.

Results

Fourteen fractions were achieved after DEAE-Sephadex A50 chromatography, and three fractions, which contained fibrinolytic activity, were further fractionated. Fraction 3 was equilibrated with 20 mM Tris-HCl buffer (pH 7.5) by dialysis and loaded onto the Sephadex-G75 column. Fraction 2

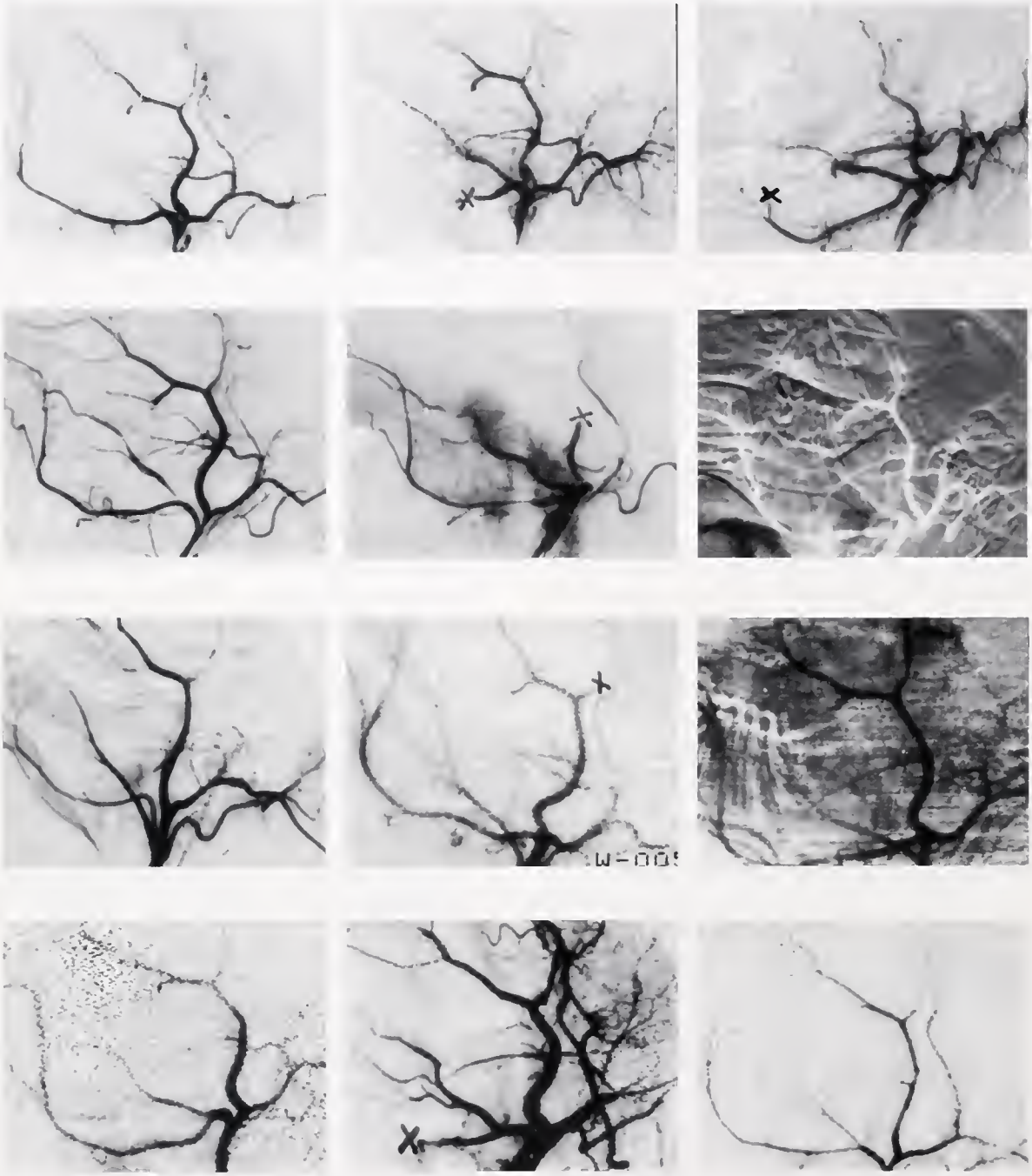

FIG. 3. Aniographs of in vivo thrombolysis (from left). Control group: A., Normal graph. B, Thrombosis performed (X). C, Treated with saline, 24 hours. FP group: D and G, Normal graphs. E and H, Thrombosis performed (X). F, Treated with FP at 0.2 mg/kg dosage, recanalization occured in 24 hrs. I, Treated with FP at 0.4 mg/kg dosage, recanalization occured in 5 hrs. U. K group (positive control): J, Normal graph. K, Thrombosis performed (X). L, Recanalized within 9 hours of U. K administration (1,000 IU/kg).

obtained in this step yielded fibrinolytic activity. This fraction was dialyzed against 10 mM aminium acetate (pH 7.0) and loaded on the CM-Sepharose CL-6B column and was separated into three fractions, the fibrinolytic activity was located in the 2nd, this fraction was rechromatographied on FPLC mono-s column and eluted with three phases of gradient: 0-30 ml (0 M NaCl); 30-80 ml (0-0.1 M NaCl) and 80-100 ml

TABLE 3. Proteases from the venom of *T. mucrosquamatus*.

Amino Acid Resid.	Proteases								
	MuA	ßFP	*a*FP	HTa	HTb	P-1	P-2	P-3	FP*
Asx									28
Asp	86	26	28	12	25	28	29	29	
Thr	36	12	14	4	11	16	14	12	12
Ser	94	13	14	14	28	16	17	15	11
Glx	110	15	21	19	33	15	17	17	21
Pro	27	18	7	2	7	5	1	2	6
Gly	56	24	9	10	25	11	14	13	8
Ala	48	12	9	12	12	8	10	11	6
Val	69	16	16	8	1	16	14	15	14
Met	15	3	6	2	5	6	3	3	0
Ile	29	11	9	3	8	8	9	10	10
Leu	42	19	17	6	15	18	16	16	17
Tyr	41	8	6	6	8	5	8	7	9
Phe	42	6	5	8	12	6	5	5	9
His	20	5	7	4	6	8	7	8	8
Lys	62	11	15	7	16	14	11	12	15
Trp	16	14	8	3	2	2	2	2	2
Arg	30	7	6	5	10	5	7	7	9
$CysSO_3$	39	9	6			8	12	10	9
CmCys				6	15				
NH_4									3
Total	862	229	203	131	239	195	196	194	194

TABLE 4. Proteases from the venom of *T. mucrosquamatus*.

Name	pI	M.W.	A.A.	res. on fibrinogen	Author	Date
*a*fibrinogenase	8.1	22,400	203	A*a*	Ouyang et al	1976
ßfibrinogenase	5.7	26,000	229	Bß	Ouyang et al	1976
Mucrotoxin A	4.3	94,000	862	A*a*, Bß	Sugihara et al	1983
HT-a	4.72	15,000	131	Bß	Nikai et al	1985
HT-b	8.9	27,000	239	A*a*	Nikai et al	1985
P1	8.1	23,000	195	Bß, A*a*	Sugihara et al	1985
P2	9.2	23,500	196	A*a*, Bß	Sugihara et al	1985
P3	9.8	23,000	194	A*a*, Bß	Sugihara et al	1985
FP*	9.2	22,000	194	Bß, A*a*	This study	

(1 M NaCl). The fibrinolytic activity was in the 6th fraction and the principal was qualified as FP.

Properties of FP: The molecular weight of the purified FP was determined to be 22,000 Da by SDS-polyacrylamide slab gel electrophoresis. The isoelectric point obtained by isoelectric focusing disc polyacrylamide gel was 9.2. The enzyme is a glycoprotein as shown by periodic acid-Schiff's agent staining after low pH (pH 4.3) polyacrylamide gel electrophoresis. The amino acid residues of FP was 194, the combined number of Glx and Asx were 49. Yet, the isoelectric point of the proteinase was basic. This enzyme possessed proteolytic activity hydrolyzing casine, fibrin and fibrinogen, but did not have BAEE hydrolase,

phospholipase A activity. The enzyme also had hemorrhagic activity (Table 1).

Heat and pH stability: The proteinase, at a concentration of 40 μg/ml in 10 mM acetate buffer (pH 5.8) containing 10 mM NaCl, were incubated for 30 minutes at various temperatures and then quickly cooled down to room temperature. The caseinolytic activity was then determined. The enzyme was fully active at 37°C, showed little activity at 50°C, and almost lost complete activity at 55°C. Incubation of FP at pH values below 5.0 and above 10.0 for 30 min lead to a sudden decrease in proteolytic activity.

Biological activity: The effects of some reagents on the proteolytic activity of FP were examined. This activity was inhibited by ethylenediamine tetraacetic acid (EDTA), cysteine but not by PMSF. The effects of some divalent and trivalent ions on the proteolytic activity of FP were also assayed. The enzyme (40 μg/ml) and ions at a concentration of 10 mM in 10 mM acetate buffer were first incubated at 37°C for 30 min. before the proteolytic activity was assayed. The proteolytic activity increased in the presence of bivalent ions in the following order: Ca++ < Cu++ < Zn++ < Co++. The increase by Fe+++ and Al+++ were lower than that of Zn++ but higher than that of Cu++, Ca++ (Table 2). When fibrinogen was incubated with FP, this enzyme cleaved the Bb-chain of fibrinogen and followed the Aa-chain as shown in Figure 2. The degrading of fibrinogen by FP was measured as 36.5 mg per mg enzyme in one minute.

Thrombolysis: None of the four rabbits of saline administration reached recanalization. Of the FP 0.2 μg/kg group, one achieved recanalization in 12 hrs. and three in 24 hrs.; of the other four of 0.4 mg/kg, three recanalized successfully in 5 hrs. and one in 9 hrs. Of the four rabbits treated with 1,000 IU/kg of U.K., thrombolytic recanalization occurred in two in 6 hrs. and two in 9 hrs. (Fig. 3).

Discussion

The venom of the Crotalinae species contained much proteinases, which had proteolytic and esterase activities. Several enzymes were isolated from *Trimeresurus mucrosquamatus* venom (Table 3 and 4). Our results showed that FP is a new fibrinogenase existing in *Trimeresurus mucrosquamatus* venom. Compared with the others, this enzyme, is a metallo-proteinase which attacks the Bb-chain of fibrinogen preferentially. As we know, the enzymes which had fibrinogenolytic activity were classified as a-fibrinogenases and b-fibrinogenases; most of the a-fibrinogenases can degrade Aa-chain of fibrinogen and usually are metallo-proteinases. Of most of the b-fibrinogenases degrading the Bb-chain of fibrinogen, little of them could also degrade the Aa-chain inhibited by DFP or PMSF. They are serine proteinases. HT-a, the first example of which hydrolyzed Bb-chain of fibrinogen and was inhibited by EDTA. The followed FP was the second report of these enzymes. Three proteinases from the *Agkistrodon halys blomhoffii* were activated by Ca++ and Co++ (Satake et al., 1963). Ca++ and Zn++ were also needed for the proteolytic activity of protein G from *Bothrops asper* (Ortiz and Gubensak, 1987). FP was activated by Co++ and Zn++. For these metallo-proteinases, these bivalent cations were important for their stability and their activities. FP had marked activity on the plasma clot in vitro, also 0.4 mg FP/kg dosage occurred thrombolysis in 3/4 in vivo. It was a good trial for FP thrombolytic potential. Hemorrhage was caused when injected subcutaneously, but did not occur within the heart, liver, kidney and lung after FP injection in rabbits at the dosage of 1 mg/kg in mice.

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