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α- AND β-FIBRINOGENASES FROM *TRIMERESURUS GRAMINEUS* SNAKE VENOM

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Summary

By means of DEAE-Sephadex A-50 column chromatography, *Trimeresurus gramineus* venom was separated into twelve fractions. The fibrinogenolytic activities were distributed in Fractions 1 and 10. These enzymes were further purified by gel filtration and were homogeneous as judged by cellulose acetate membrane, sodium dodecyl sulfate polyacrylamide gel electrophoresis and ultracentrifugal analysis. Both of them were single peptide chains. The sedimentation constants of α- (Fraction 1) and β-fibrinogenases (Fraction 10) were 2.20 and 3.60, respectively. The molecular weights of α- and β-fibrinogenases were 23 500 and 25 000 respectively. The contents of proline and glycine were higher in β-fibrinogenase than in α-fibrinogenase. The isoelectric points of α-fibrinogenase and β-fibrinogenase were pH > 10 and 4.5, respectively. The optimal pH of α-fibrinogenase was approx. 7.4 and that of β-fibrinogenase was approx. 9.0. The activity of α-fibrinogenase was completely destroyed after 30 min at 60°C, pH 5.4, 7.4 and 9.0, while that of β-fibrinogenase was much less affected by the same treatment. The specific fibrinogenolytic activity of α-fibrinogenase was 31 mg fibrinogen/min per mg protein, while that of β-fibrinogenase was 9 mg fibrinogen/min per mg protein. α-Fibrinogenase cleaved specifically the α(A) chain of monomeric fibrinogen without cleaving the β(B) chain and γ-chain. β-fibrinogenase preferentially cleaved the β(B) chain, and the α(A) chain was also partially cleaved by β-fibrinogenase, if the incubation time was prolonged. Both enzymes showed proteolytic activities toward fibrinogen, fibrin and casein, but were devoid of phospholipase A, alkaline phosphomonoesterase and phosphodiesterase activities found in the crude venom. The tosyl-L-arginine methylester esterase activity of β-fibrinogenase was about 14 times that of crude venom, while α-fibrinogenase was completely devoid of this activity. The fibrinogenolytic activity of α-fibrinogenase was markedly inhibited by EDTA and cysteine, while that of β-fibrinogenase

was inhibited markedly by phenylmethanesulfonylfluoride. α- and β-fibrinogenases exert their fibrinogenolytic activity by a direct action on fibrinogen or fibrin without activation of plasminogen.

Introduction

Snake venoms, especially from Crotalid and Viperid, are well known to be abundant in proteolytic enzymes [1,2]. Recently. Ouyang and his associates [3—6] first purified the fibrino(geno)lytic enzymes from the snake venoms of *Trimeresurus mucrosquamatus* and *Agkistrodon acutus* and studied their physicochemical properties, enzymatic activities and modes of actions. Owing to the specificity of cleaving the α(A) chain or β(B) chain of fibrinogen, the fibrinogenolytic enzymes were named as α- and β-fibrinogenases (EC 3.4.21.5) [5]. In 1957 Ouyang [7] found that, in addition to the thrombin-like action and the inhibitory action of prothrombin activation, the venom of *Trimeresurus gramineus* had a marked fibrinolytic action. The thrombin-like enzyme and the prothrombin activation inhibitor have been purified by Ouyang and Yang [24,36]. In the present study, we tried to purify the fibrino(geno)lytic principles from this snake venom, and studied the physicochemical properties, comparing with those of the venoms of *T. mucrosquamatus* and *A. acutus*.

Materials and Methods

Materials. The venom of *T. gramineus* was collected, centrifuged, lyophilized and stored in a desiccator containing anhydrous $CaCl_2$ at −20°C. Bovine fibrinogen (90%, clottable) was purchased from Pentex Co. Bovine thrombin was purchased from Park, Davis and Co. and dissolved in 50% glycerol to give a stock solution of 1000 NIH units/ml. DEAE-Sephadex A-50, Sephadex 25, 50 and 75 were purchased from Pharmacia, Sweden. α-Casein, tosyl-L-arginine methylester, α-L-phosphatidyl choline, calcium bis-*p*-nitrophenyl phosphate, disodium *p*-nitrophenyl phosphate, cysteine, *N*-ethylmaleimide, tosyl-L-lysine chloromethylketone ($TosLysCH_2Cl$), phenylmethylsulfonylfluoride, ε-aminocaproic acid, soybean trypsin inhibitor, disodium EDTA, trypsin, serum albumin, ovalbumin, pepsin, plasminogen and cytochrome *c* were purchased from Sigma Chem. Co. Trasylol was purchased from FBA Pharmaceuticals. Other chemical reagents were of analytical grade from Wako Pure Chemical Industries.

DEAE-Sephadex A-50 column chromatography. 1 g of the venom was applied to a column (3.2 × 100 cm) packed with DEAE-Sephadex A-50. The first stage gradient elution was carried out with 1000 ml of 0.005 M ammonium acetate (pH was adjusted to 8.0 with 3.5 M NaOH) in the mixing vessel and 1000 ml of 0.25 M ammonium acetate (pH was adjusted to 6.0 with conc. HCl) in the reservoir. The second stage gradient elution was carried out with 800 ml of 0.25 M ammonium acetate (pH 6.0) in the mixing vessel and 1000 ml of 1 M ammonium acetate (pH 5.2) in the reservoir. The flow rate was adjusted to 16—18 ml/h, and eluates of 6 ml per tube were collected. The effluent was monitored continuously at 278 nm and 5°C with an LKB Uvicord.

Gel filtration. Sephadex was prepared in 0.005 M ammonium bicarbonate

(pH 7.8) and packed in columns of various size according to the amount of the venom. The elution was carried out with 0.005 M ammonium bicarbonate (pH 7.8). The flow rate was adjusted to 18 ml/h and eluates of 3 ml per tube were collected. Desalting was performed with Sephadex G-25, eluted with distilled water.

SDS-polyacrylamide gel electrophoresis. The freshly prepared aqueous sample solution at 1 mg/ml was added to equal amount of 0.02 M phosphate buffer (pH 7.4), containing 10 M urea/4% sodium dodecyl sulfate in the presence or absence of 4% β-mercaptoethanol and incubated at 37°C for 2 h. 5 μl of tracking dye (bromophenol blue) was mixed with the protein solution before the sample was applied to the gel. A 10% acrylamide gel was prepared according to the method of Weber and Osborn [8]. The anode was in the lower chamber. The electrophoresis was run at a constant current 6 mA per gel for about 5 h until the dye moved to about three-quarters of the gel length. The removed gel was stained with Coomassie brilliant blue (dissolved in a mixture of 454 ml of 50% methanol and 46 ml of glacial acetic acid) for at least 12 h. Destaining was performed by several rinsings in a mixture of 5% methanol and 7.5% glacial acetic acid. The mobility was calculated as

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{distance of dye migration}} .$$

The mobility was plotted against the molecular weights of some standard proteins (serum albumin, 68 000; ovalbumin, 43 000; pepsin, 35 000; soybean trypsin inhibitor, 21 000; and cytochrome *c*, 12 000) on a semi-log scale and the molecular weight of unknown sample could be estimated by interpolation.

Fibrinogenolytic activity. A modified method of Ware et al. [9] was used. An equal part of fibrinogen solution (2%) and venom solution (both dissolved in imidazole/saline buffer, pH 7.4) were mixed and incubated at 37°C for various time intervals. 0.2 ml of the above incubation mixture was taken for assay of clottable fibrinogen. In the meantime, 0.1 ml of the incubation mixture was pipetted into a small test tube, frozen in an acetone/solid CO_2 bath and lyophilized. Then, the lyophilized sample was dissolved in 1 ml of the 0.01 M sodium phosphate buffer (pH 7.4) containing 5 M urea/2% SDS/2% β-mercaptoethanol and left to stand overnight. 0.05 ml of this solution was applied to the top of 7.5% acrylamide gel and electrophoresis was performed in the same way.

Fibrinolytic activity. The method of Astrup and Müllertz [10] was employed. The final concentration of fibrinogen was 0.2% and sample of 0.025 ml was applied to the clotted surface with a micropipette. Plates was incubated for 20 h at 37°C. The lyzed zone was measured as the product of the two perpendicular diameters in mm^2.

Caseinolytic activity. The method of Maeno and Mitsuhashi [11] was modified as following: 0.1 ml of the test solution was mixed with 0.4 ml of 0.1 M phosphate buffer (pH 7.4) and warmed up to 37°C for 5 min. 1 ml of casein (1%) was added and incubated at 37°C for various times. 2 ml of 0.4 M trichloroacetic acid was used to stop the reaction. After centrifugation at 2000 rev./min for 15 min and filtering through No- 42 filter paper, 2 ml of 20% Na_2CO_3 and 0.5 ml of phenol reagent were added to the filtrate and mixed

well. After standing for 20 min at room temperature, the absorbance was measured at 540 nm by colorimeter. According to a standard tyrosine curve, the caseinolytic activity could be calculated.

Sedimentation study. This was carried out in a Spinco Medol E Analytical Ultracentrifuge, at protein concentrations of 11.68 mg/ml (α-fibrinogenase) and 11.3 mg/ml (β-fibrinogenase) in 0.1 M KCl. The rotor speed was 59 780 rev./min. Photographs were taken at various times after the rotor had attained correct speed.

Amino acid analysis. This was carried out on a Beckman Model M121 analyzer, using a two column system. The hydrolyses were carried out in a 110°C oven under a nitrogen vacuum atmosphere for 22 h. Tryptophan was determined by the colorimetric method of Fischl [12].

Estimation of carbohydrate. This was estimated by the method of Dubois et al. [13] using dextrose as standard.

Esterase activity. The method of Hestrin [14] modified by Habermann [15] was followed. 0.05 M tosyl-L-arginine methylester (Tos-Arg-OMe) was used as substrate.

Phospholipase A activity. This was determined by the method of Brown and Bowles [16].

Alkaline phosphomonoesterase and phosphodiesterase activities. These were measured by the method of Suzuki and Iwanaga [17] as modified by Lo et al. [18].

Effects of enzyme inhibitors on fibrinogenolytic and fibrinolytic activities. Equal amounts of both fibrinogenase and enzyme inhibitor solution (pH 7.4) were preincubated at 37°C for 30 min. The above mixture were taken as the starting material for the enzymatic assays on fibrinolytic and fibrinogenolytic activities. Effects of the inhibitors were presented as percentage inhibition of the fibrinogen-destructing activities or the fibrin-lysed zone area of these fibrinogenolytic enzymes.

Results

Fractionation of T. gramineus venom. By means of DEAE-Sephadex A-50 column chromatography, the venom was separated into twelve fractions (Fig. 1). Eight fractions were obtained in the first stage elution, while the other four were obtained in the second stage elution. The fibrinogenolytic activities were distributed in Fractions 1 and 10.

Refractionation of Fractions 1 and 10. Fractions 1 and 10 were refractionated on Sephadex G-75. The main peaks possessing the fibrinogenolytic activity were refractionated on Sephadex G-75 twice and Sephadex G-50 once and a single peak was obtained.

The homogeneity and molecular weight of the purified Fractions 1 and 10. Fractions 1 and 10, obtained from the gel filtration, were desalted on Sephadex G-25 and then lyophilized. The desalted proteins were homogeneous as judged by microzone electrophoresis on a cellulose acetate membrane, their SDS-polyacrylamide gel patterns (Fig. 2) and ultracentrifugation (Figs. 3 and 4). These two purified fractions were single peptide chain since the mobilities were the same in the presence or absence of 2% β-mercaptoethanol. In the following, we

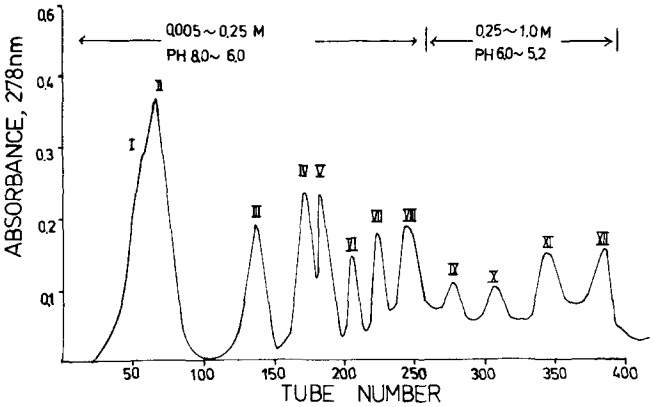

Fig. 1. DEAE-Sephadex A-50 column chromatography of *T. gramineus* venom.

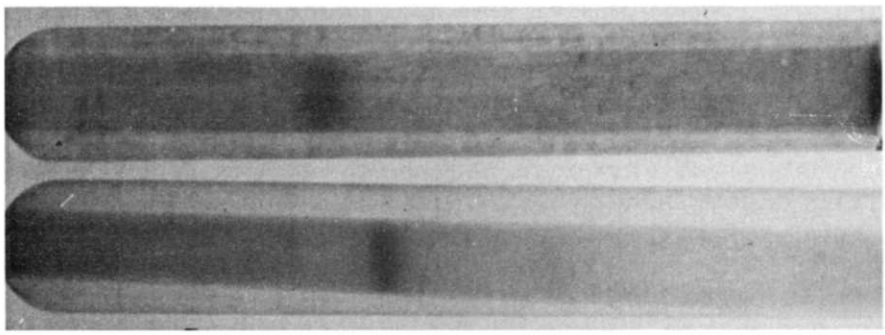

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the purified Fraction 1 (top) and Fraction 10 (bottom). 25 μg of protein was applied to each gel.

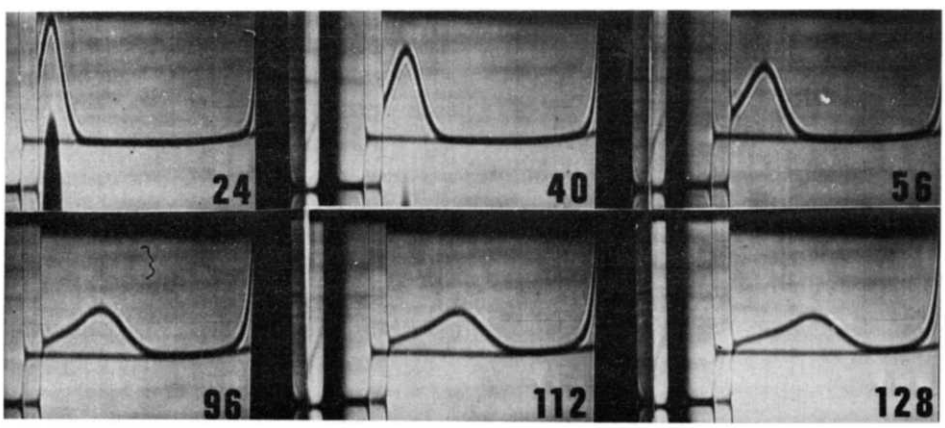

Fig. 3. Schlieren patterns of α-fibrinogenase of *T. gramineus* venom obtained with Spinco analytical ultracentrifuge with rotor speed at 59 780 rev./min. Sedimentation from left to right. The figures on chart refer to time when photograph was taken.

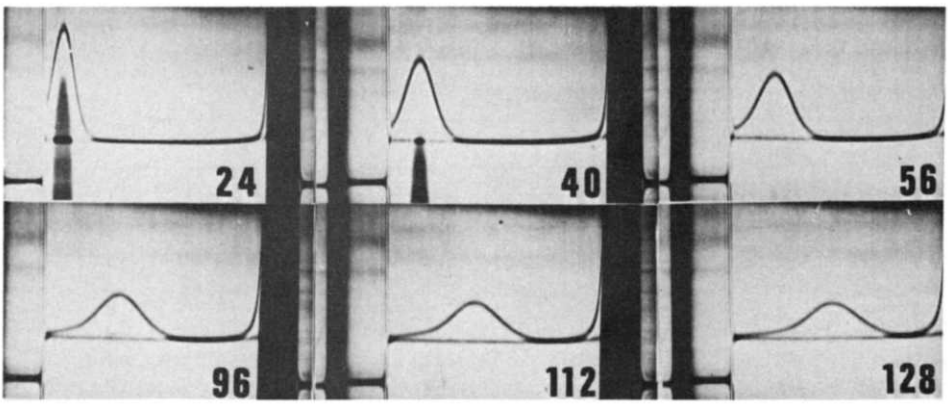

Fig. 4. Schlieren patterns of β-fibrinogenase of *T. gramineus* venom obtained with Spinco analytical ultracentrifuge with rotor speed at 59 780 rev./min. Sedimentation from left to right. The figures on the chart refer to time when photograph was taken.

would like to call the purified Fraction 1 'α-fibrinogenase' and the purified Fraction 10 'β-fibrinogenase' according to their modes of fibrinogenolytic action described hereafter.

Physical properties of α- (Fraction 1) and β-fibrinogenase (Fraction 10). A single symmetrical boundary at 2.20 S (α-fibrinogenase) and 3.60 S (β-fibrinogenase) was obtained by ultracentrifugation (Figs. 3, 4). The molecular weights of α- and β-fibrinogenases estimated by SDS-polyacrylamide electrophoresis were 23 500 and 25 000, and by amino acid analysis 23 986 and 25 303, respectively.

Chemical properties of α- and β-fibrinogenases. The amino acid analysis of the purified α- and β-fibrinogenases is shown in Table I. The number of residues per molecule is expressed as nearest integer. They were found to be composed of 18 amino acids. The carbohydrate contents of α- and β-fibrinogenases were about 4% and 7%, respectively. This indicates that these two enzymes are glycoproteins. The isoelectric points were determined by electrophoresis on cellulose acetate membrane to be pH > 10 (α-fibrinogenase) and pH 4.5 (β-fibrinogenase) in citrate/phosphate buffer.

Optimal pH values of α- and β-fibrinogenases. The optimal pH value of α-fibrinogenase was 7.4, while that of β-fibrinogenase was 9.0.

Effect of heat treatment on α- and β-fibrinogenases. As shown in Table II, the fibrinogenolytic activity of α-fibrinogenase was completely destroyed at 60°C, pH 5.6, 7.4 and 9.0. On the other hand, β-fibrinogenase was only slightly affected at pH 5.6, 7.4, but more profoundly at pH 9.0. Similarly, the fibrinolytic activity of α-fibrinogenase was lost after the heat treatment at different pH values (data not shown).

Fibrinogenolytic activities of α- and β-fibrinogenases. α-Fibrinogenase possessed a potent fibrinogenolytic activity. At a concentration of 5 μg/ml, no clottable fibrinogen could be detected after 30 min incubation at 37°C with a fibrinogen solution of 550 mg%. The activity of β-fibrinogenase was much less potent. The activity of crude venom could not be estimated because the

TABLE I

AMINO ACID COMPOSITIONS OF α- AND β-FIBRINOGENASES OF *T. GRAMINEUS* VENOM (RESIDUES/MOLECULE), IN COMPARISON WITH THOSE OF α, AND β-FIBRINOGENASES FROM *AGKISTRODON ACUTUS* AND *T. MUCROSQUAMATUS*

Amino acid	α-Fibrinogenase			β-Fibrinogenase	
	T. gramineus	*T. mucrosquamatus* [5]	*A. acutus* [4]	*T. gramineus*	*T. mucrosquamatus* [5]
Lysine	19	15	11	11	11
Histidine	8	7	7	5	5
Arginine	8	6	7	9	7
Aspartic acid	26	23	25	27	26
Threonine	13	14	11	9	12
Serine	11	14	23	15	13
Glutamic acid	16	21	21	19	15
Proline	9	7	8	15	18
Glycine	10	9	12	17	24
Alanine	9	9	12	12	12
Half-cystine	8	6	8	12	9
Valine	16	16	10	11	16
Methionine	7	6	6	5	3
Isoleucine	10	9	15	12	11
Leucine	17	17	12	11	19
Tyrosine	7	6	10	12	8
Phenylalanine	7	5	5	7	6
Tryptophan	2	8	5	2	14
Total	203	203	208	211	229

thrombin-like enzyme in crude venom interfered with this testing system [24]. The specific activity could be calculated when the fibrinogenolytic activities of these enzymes were plotted as the rate of fibrinogen destruction against the amount of venoms. As shown in Figs. 5 and 6, the fibrinogenolytic activity of α-fibrinogenase (31 mg/min per mg) was about 3.6 times higher than that of β-fibrinogenase (9 mg/min per mg). On the other hand, the fibrinolytic activity of α-fibrinogenase was six times higher than those of β-fibrinogenase and crude venom. (Fig. 7).

TABLE II

THE EFFECTS OF HEAT TREATMENT ON THE FIBRINOGENOLYTIC ACTIVITIES OF α- AND β-FIBRINOGENASES FROM *T. GRAMINEUS* VENOM

Both enzymes were in a concentration of 1 mg/ml and heated at 60°C, pH 5.6, 7.4 and 9.0 for 30 min. After proper dilution with imidazole-saline (pH 7.4), the remaining fibrinogenolytic activities were assayed and the residual activities were calculated as percentage of initial values (means of three determinations are presented).

	Residual fibrinogenolytic activity (%) at pH		
	5.6	7.4	9.0
α-Fibrinogenase	0	0	0
β-Fibronogenase	93	83	54

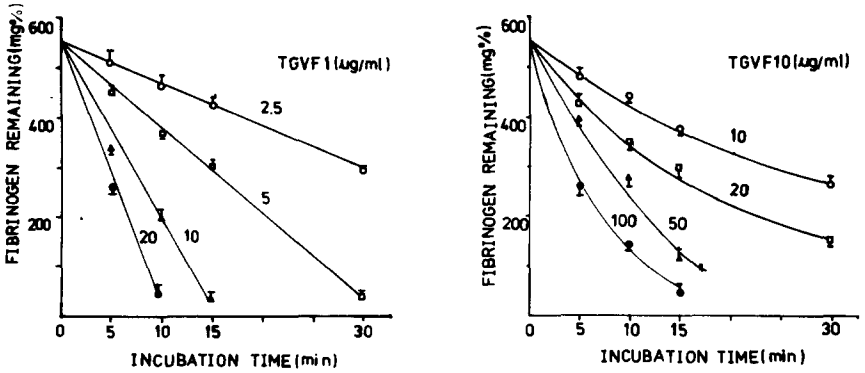

Fig. 5. The fibrinogenolytic activities of Fractions 1 (α-fibrinogenase) and 10 (β-fibrinogenase). Values of means ± S.E. are presented (n = 3). TGV: *T. gramineus* venom.

Mode of action of fibrinogenolysis induced by α- and β-fibrinogenases. Reduced SDS-polyacrylamide gel electrophoresis was performed in the presence of β-mercaptoethanol to examine which subunit of fibrinogen was affected by α- and β-fibrinogenases. As shown in Fig. 8, α-fibrinogenase (5 μg/ml) completely digested the α(A) chain without affecting the β(B) and γ-chains even after 2 h incubation. As shown in Fig. 9, β-fibrinogenase degraded preferentially β(B) chain first and the α(A) chain was also degraded if the incubation time was prolonged.

Enzymatic properties of α- and β-fibrinogenases. The enzymatic activities of purified α- and β-fibrinogenases were compared with those of crude venom. As

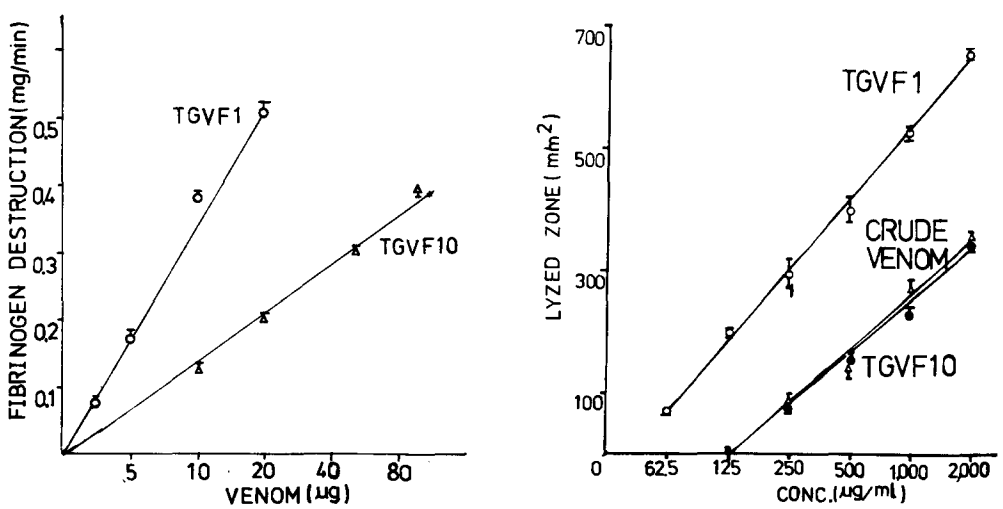

Fig. 6. Kinetic study of the fibrinogenolytic activities of Fractions 1 (α-fibrinogenase) and 10 (β-fibrinogenase): The specific fibrinogenolytic activities calculated were: TGVF1: 31 mg/min per mg, TGVF10: 9 mg/min per mg.

Fig. 7. The fibrinolytic activities of α-(TGVF1), β-(TGVF10) fibrinogenases and crude venom. Each plate contains 10 ml of 0.2% fibrinogen solution. 25 μl of testing material was applied to the fibrin surface.

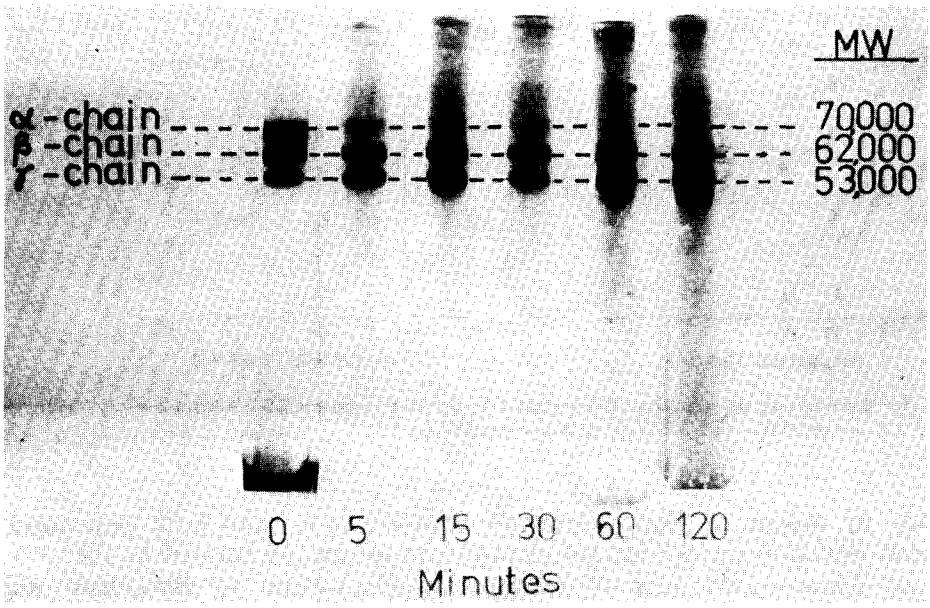

Fig. 8. Reduced dodecyl sulfate polyacrylamide gel electrophoretic patterns of fibrinogen incubated with α-fibrinogenase (5 μg/ml) at the time periods shown beneath each gel.

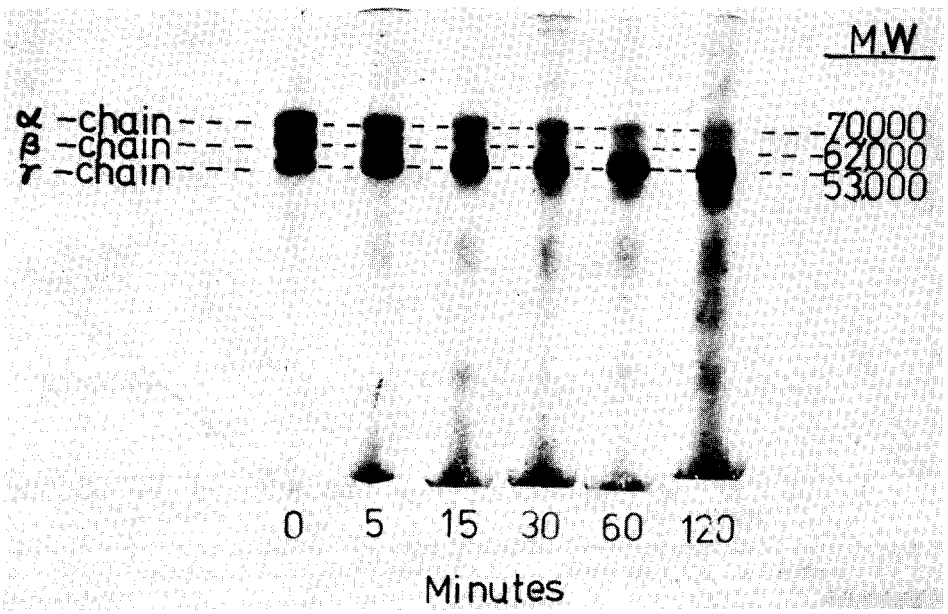

Fig. 9. Reduced dodecyl sulfate polyacrylamide gel electrophoresis patterns of fibrinogen incubated with β-fibrinogenase (25 μg/ml) at the time periods shown beneath each gel.

TABLE III

COMPARISON OF THE ENZYMATIC ACTIVITIES OF THE PURIFIED α- AND β-FIBRINOGENASES AND THE CRUDE VENOM OF *T. GRAMINEUS*

	Crude venom	α-Fibrinogenase	β-Fibrinogenase
Fibrinogenolysis (mg/min per mg protein)	— *	31 ± 3	9 ± 2
Caseinolysis (μmol/min per mg protein)	0.04 ± 0.01	0.09 ± 0.02	<0.01
Tos-Arg-OMe esterase (μmol/min per mg protein)	37 ± 5	0	507 ± 32

* The fibrinogenolytic activity of crude venom could not be measured, because crude venom contained the thrombin-like enzyme.

shown in Table III, α-fibrinogenase possessed the strongest fibrinolytic and caseinolytic activities. Concerning the esterase activity, β-fibrinogenase was about 14 times higher than that of crude venom, while α-fibrinogenase was completely devoid of this activity. Both α and β-fibrinogenases were devoid of phospholipase A, phosphodiesterase and alkaline phosphomonoesterase activities found in crude venom (data not shown).

Effects of enzyme inhibitors on the fibrinogenolytic activities of α- and β-fibrinogenases. Table IV shows the effects of some enzyme inhibitors on the fibrinogenolytic activities of α and β-fibrinogenases, compared with trypsin. It was shown that the fibrinogenolytic activity of α-fibrinogenase was almost completely inhibited by EDTA and to a great extent (59%) by cysteine. The effect of β-fibrinogenase was inhibited markedly (71%) by phenylmethanesulfonylfluoride, while EDTA and cysteine had no inhibitory effect on it. The effect of trypsin was almost completely inhibited by phenylmethylsulfonylfluoride, Trasylol, TosLysCH_2Cl and soybean trypsin inhibitor. Concerning the fibrinolytic activities only EDTA showed a complete inhibition on α-fibrinogenase and crude venom.

TABLE IV

EFFECTS OF SOME ENZYME INHIBITORS ON THE FIBRINOGENOLYTIC ACTIVITIES OF α- AND β-FIBRINOGENASES AND TRYPSIN

Each experiment was performed in triplicate. Percent inhibitions are presented as means ± S.E. Final concentrations: ε-aminocaproic acid (EACA), 10^{-2} M; Trasylol, $5 \cdot 10^{-5}$ M; EDTA, $2.5 \cdot 10^{-4}$ M; Soybean trypsin inhibitor (SBTI), 10^{-4} g/ml; Cysteine *N*-ethylmaleimide (NEM), tosyl-L-lysine chloromethylketone (TLCK) and phenylmethanesulfonylfluoride (PMSF), $2.5 \cdot 10^{-3}$ M; α-fibrinogenase, 5 μg/ml, β-fibrinogenase, 25 μg/ml; trypsin, 1 μg/ml; fibrinogen, 10 mg/ml.

	α-Fibrinogenase	β-Fibrinogenase	Trypsin
EACA	17 ± 3	18 ± 3	7 ± 10
Trasylol	21 ± 2	−15 ± 2	96 ± 6
EDTA	96 ± 3	19 ± 5	8 ± 5
SBTI	−16 ± 5	−22 ± 2	98 ± 6
Cysteine	59 ± 7	3 ± 4	16 ± 8
NEM	16 ± 2	−17 ± 4	10 ± 6
TLCK	17 ± 6	24 ± 3	97 ± 5
PMSF	18 ± 5	71 ± 10	102 ± 8

Discussion

We isolated two fibrinogenolytic enzymes from *T. gramineus* snake venom. The purified Fractions 1 (α-fibrinogenase) and 10 (β-fibrinogenase) were homogeneous as judged by microzone electrophoresis on cellulose acetate membrane, SDS-polyacrylamide gel electrophoresis and ultracentrifugal analysis. These enzymes were single peptide chains since the mobilities on SDS-polyacrylamide gel electrophoresis were the same in the presence or absence of 2% β-mercaptoethanol. However, there were some differences in their physicochemical properties. The molecular weight of β-fibrinogenase (25 000) was a little larger than that of α-fibrinogenase (23 500). This was in accordance with the fibrinogenolytic enzymes from the venom of *T. mucrosquamatus* (Table V). Concerning the amino acid composition, the content of aspartic acid was highest in both enzymes. However, the content of glycine and proline of β-fibrinogenase was much higher than that of α-fibrinogenase. The high content of lysine in α-fibrinogenase might be responsible for its high basicity in nature (p*I* > 10), while the β-fibrinogenase was an acidic protein (p*I* 4.5). There were also marked differences in heat stability and optimal pH values (Table II). The fibrinogenolytic activities of α-fibrinogenase was 31 mg/min per mg protein, while that of β-fibrinogenase was 9 mg/min per mg protein. The relative potencies of the fibrinogenolytic activities of α- and β-fibrinogenases and crude venom were in the ratio of 1 : 0.15 : 0.17. α- and β-fibrinogenase were named by their specific nature in the mode of action of their fibrinogenolytic activities. α-Fibrinogenase digested the α(A) chain selectively, while β-fibrinogenase digested the β(B) chain preferentially and also the α(A) chain if the incubation time was prolonged (Figs. 8, 9). It has been reported that α-chain, containing no detectable carbohydrate, is the most susceptible subunit to be degraded by plasmin. The carbohydrate-containing β-chain is degraded more slowly than the α-chain. The γ-chain of fibrinogen, which also contains carbohydrate is the most resistant subunit to be digested, but on prolonged incubation is cleaved by plasmin too [19]. Arvin, from *Agkistrodon rhodostoma* [20] and the thrombin-like enzyme from *Agkistrodon acutus* also digested the α-chain of fibrin monomer [21]. α-Fibrinogenase was different from trypsin and plasmin in its mode of action on fibrinogen, because it degraded the α-chain only, while β- and γ-chains were intact even when the incubation time was prolonged. β-Fibrinogenase of *T. gramineus* venom was the second enzyme reported to degrade preferentially the β(B) chain of fibrinogen. β-Fibrinogenase possessed a strong Tos-Arg-OMe esterase activity, but it did not have the coagulant action toward fibrinogen possessed by other thrombin-like enzymes of snake venom [22—24].

Concerning the enzymatic properties, α- and β-fibrinogenases possessed proteolytic activities toward fibrinogen, fibrin and casein, but were devoid of the phospholipase A, phosphodiesterase and alkaline phosphomonoesterase activities found in the crude venom. Additionally, β-fibrinogenase also possessed a strong esterase activity toward Tos-L-arginine-methylester (507 μmol/min per mg protein), but α-fibrinogenase was without this activity. The fibrinogenolytic activities of both enzymes were not dependent on plasminogen activation since ε-aminocaproic acid and trasylol did not inhibit their actions.

The plasminogen molecule was apparently not cleaved by these enzymes as shown on SDS-polyacrylamide gel electrophoresis even if the plasminogen were incubated with these enzymes at a concentration as high as 100 μg/ml at 37°C for 1 h. Thus, their fibrinogenolytic action is directly on fibrinogen just as is the case with the fibrinogenolytic enzymes from *A. acutus* and *T. mucrosquamatus* venoms [5,6]. Trypsin and plasmin can digest both α(A) and β(B) chains and, on prolonged incubation, also the γ-chain of fibrinogen [20,25]. From these results, it is suggested that the enzymatic action of both fibrinogenolytic enzymes on fibrinogen are more specific than those of trypsin and plasmin.

As shown in Table IV, EDTA almost completely inhibited the fibrinogenolytic activity of α-fibrinogenase. However, the fibrinogenolytic activity of β-fibrinogenase was not affected significantly by EDTA. The most notable difference between both enzymes and trypsin was that the fibrinogenolytic activities of both enzymes were unaffected by soybean trypsin inhibitor and trasylol in a concentration that produced complete inhibition of the fibrinogenolytic activity of trypsin.

Cysteine inactivated partially the fibrinogenolytic activity of α-fibrinogenase, but not that of β-fibrinogenase. The importance of disulfide bridges for fibrinogenolytic activity was also reported with thrombin [26], thrombin-like enzyme [27] and fibrinolytic enzymes [5,6,28].

Neither α- nor β-fibrinogenas was inhibited by sulfhydryl group reagent *N*-ethylmaleimide. This indicates that neither of the enzymes is a thiol enzyme such as ficin, bromelain and papain [29]. Similarly, neither enzyme was profoundly inhibited by Tos-LysCH_2Cl, which has been used as a histidine-reactive reagent to elucidate the role of this amino acid in the active center of plasmin [30], trypsin [31], thrombin [26] and thrombin-like enzymes [28, 32,33].

Sulfonation with phenylmethylsulfonyl fluoride (PMSF) inhibited the fibrinogenolytic activity of β-fibrinogenase, but not that of α-fibrinogenase. Sulfonation also inhibited trypsin, chymotrypsin [34], thrombin [35] and thrombin-like enzymes [32].

The physicochemical properties and some enzymatic activities of the fibrinogenases from the venom of *T. gramineus* are summarized in comparison with those from the venoms of *A. acutus* and *T. mucrosquamatus* in Table V. We could classify these enzymes into two groups: (1) α-fibrinogenases, including one acidic and two basic polypeptides, are single peptide chains, heat labile, EDTA- and cysteine-sensitive and their optimal pH were 7.4 in fibrinogenolytic activities. They possessed strong proteolytic activities toward fibrinogen, fibrin and casein, but were devoid of Tos-Arg-OMe esterase activity. They digested selectively the α(A) chain of fibrinogen, leaving β(B) and γ-chain unaffected. (2) β-Fibrinogenases including two acidic polypeptides, are single peptide chains, heat stable, EDTA- and cysteine-insensitive and their optimal pH were in the range 8.5—9.0. They possessed a rather weak fibrinogenolytic, fibrinolytic and caseinolytic activities. However, they have a very potent Tos-Arg-OMe esterase activity without coagulating fibrinogen. Sulfonation with phenylmethylsulfonyl fluoride inactivated their activities. They cleaved preferentially the β(B) chain of fibrinogen, but also α(A) chain if the incubation time was prolonged.

TABLE V

SOME PHYSICOCHEMICAL AND ENZYMATIC PROPERTIES OF α- AND β-FIBRINOGENASES FROM VENOMS OF *T. GRAMINEUS*, *T. MUCROSQUAMATUS* AND *A. ACUTUS*

PMSF, phenylmethylsulfonylfluoride.

	α-Fibrinogenase			β-Fibrinogenase	
	T. gramineus	*T. mucrosquamatus* [3,5]	*A. acutus* [4]	*T. gramineus*	*T. mucrosquamatus* [3,5]
Molecular weight	23 500	22 400	24 100	25 000	26 000
$s_{20,w}$	2.20	2.52	2.44	3.60	3.04
Amino acid residues/molecule	203	203	208	211	229
Isoelectric point	>10	8.1	3.8	4.5	5.7
Carbohydrate content	4%	2%	<1%	7%	2%
Heat stability	labile	labile	labile	stable	stable
Enzyme inhibitor	EDTA, cysteine	EDTA, cysteine	EDTA, cysteine	PMSF	PMSF
Optimal pH	7.4	7.4	7.4	9.0	8.5
Fibrinogenolysis (mg/min per mg protein)	31 ± 3	120 ± 4	— *	9 ± 2	20 ± 5
Caseinolysis (μmol/min per mg protein)	0.09 ± 0.02	0.10 ± 0.02	0.10 ± 0.02	<0.01	0.03 ± 0.01
Tos-Arg-OMe esterase (μmol/min per mg protein)	0	0	0	507 ± 32	1500 ± 210

* The fibrinogen (10 mg/ml) became unclottable after 15 min preincubation with α-fibrinogenase of *A. acutus* venom (10 μg/ml).

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