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# CHARACTERIZATION OF A FIBRINOGENASE FROM NORTHERN COPPERHEAD *(AGKISTRODON CONTORTRIX MOKASEN)* VENOM

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## Summary

One of the fractions obtained by the carboxymethylcellulose ion-exchange chromatography of northern copperhead (*Agkistrodon contortrix mokasen*) venom prevented the thrombin-induced clotting of fibrinogen by proteolytically degrading the fibrinogen. The active component has been further purified to apparent electrophoretic homogeneity by molecular sieve chromatography. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis indicated a molecular weight of 22 900 ± 600 for the purified enzyme. In addition to its fibrinogenase activity, it catalyzed the hydrolysis of hide powder azure and had an intraperitoneal $LD_{50}$ value in mice of less than 5.1 μg/g body weight. The enzyme rapidly destroyed fibrinogen's ability to form clots. Electrophoresis of fibrinogen which had been incubated only a few minutes with the fibrinogenase revealed the rapid disappearance of the α-chain and the appearance of lower molecular weight fragments. The neutral pH optimum and ethylenediaminetetraacetic acid (EDTA) and dithiothreitol sensitivity indicated that this enzyme belonged to the class metalloproteinases. Atomic absorption studies have revealed one zinc atom per molecule of protein. The apoenzyme's activity was restored by incubation with $ZnCl_2$.

## Introduction

Ouyang and his associates [1—10] have isolated several factors from Asian crotalid venoms which prevent the normal clotting of fibrinogen Some of these

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have no proteolytic activity, but rather interact with prothrombin or with phospholipids to inhibit clotting. Others are fibrino(geno)lytic proteinases which appear to have either a specific action on the $\alpha$-chain ($\alpha$-fibrinogenases) or preferentially attack the $\beta$-chain ($\beta$-fibrinogenases) of fibrinogen. Anticoagulant proteins from *Naja nigricollis* have been associated with phospholipase $A_2$ activity [11] Viperid venoms contain both fibrinogenase and phospholipase anticoagulants [12,13]

Although whole northern copperhead (*Agkistrodon contortrix mokasen*) venom has been shown to cause both whole plasma and purified fibrinogen to clot [14], evidence presented in this report will show that this venom also has a component which has an adverse effect upon clotting. Moran and Geren [15] have previously reported the separation of northern copperhead venom into distinct fractions by ion-exchange chromatography Using their nomenclature, the fraction of interest in this report is the FII fraction. This fraction has been previously shown to contain no L-amino acid oxidase, phospholipase or arginine-ester hydrolytic activity under the conditions assayed, but it does have proteinase activity [15]. This is true regardless of the subspecies of copperhead venom which is used as the initial source of FII [16,17]. The current report describes the presence of a fibrinogenase activity in this fraction, the further purification of the compound responsible for this activity from FII, and finally, the properties of this enzyme.

## Materials and Methods

Lyophilized northern copperhead (*A c mokasen*) venom was purchased from both Sigma Chemical Co., St. Louis, MO and the Miami Serpentarium, Miami, FL. The FII fraction of this venom was prepared by CM-cellulose ion-exchange chromatography as previously described [16]

Molecular sieve gels and reagents for SDS and discontinuous polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories, Richmond, CA. Bovine fibrinogen type I (78% clottable), bovine serum albumin, bovine hemoglobin, cross-linked hemoglobin electrophoresis standard, soybean trypsin inhibitor, egg white trypsin inhibitor, dithiothreitol, *N*-ethylmaleimide and phenylmethylsulfonyl fluoride were obtained from Sigma Chemical Co , St Louis, MO. Hide powder azure, thrombin-free bovine fibrinogen (98% clottable), human thrombin (approx. 2500 NIH units per mg protein) and pronase were purchased from Calbiochem, La Jolla, CA. All other reagents were of analytical quality

SDS gel electrophoresis was performed by the method of Weber and Osborn [18], while discontinuous polyacrylamide gel electrophoresis was by the method of Ornstein [19] and Davis [20].

Spectrophotometric determinations were performed with a Gilford Model 252 up-dated Beckman DU. Generally, protein concentrations were estimated by absorbance at 280 nm ($A_{280}$), assuming an extinction coefficient of 1.0 for a 1.0 mg/ml solution. However, an extinction coefficient of 1.95 was used to estimate thrombin concentration [21].

The following assays were used to determine the effects of the fibrinogenase on the clot-forming ability of fibrinogen Fibrinogen (78% clottable) was dissolved in a 20 mM Tris-HCl-buffered normal saline, pH 7.4, at 5 mg/ml. Frac-

tions collected by molecular sieve chromatography were profiled for fibrinogenase activity by incubating 0.3 ml of the the fibrinogen solution with 25 μl aliquots from sample tubes for 10 min, adding 0.4 μg thrombin and observing the solution for up to 1 h for clot formation. Percent clottable protein was determined by incubating 1 ml of the fibrinogen solution with 10 μg of the fibrinogenase at room temperature and adding 0.75 μg thrombin (1.875 units) at various times. This amount of thrombin consistently induced clotting in 50—60 s in the absence of the fibrinogenase. After an additional 10 min, the solution was centrifuged for 5 min at maximum speed in a clinical centrifuge and the protein concentration of the supernatant was determined spectrophotometrically. Fibrinolytic activity was assayed by incubating clots formed by thrombin in the presence of the fibrinogenase at room temperature for 15 h, crushing the clots to extract the fluid portion, centrifuging for 5 min at maximum speed in a clinical centrifuge and comparing the protein concentration of the supernatant to that of clots formed in the absence of the fibrinogenase which were treated in the same manner.

In other experiments, the fibrinogen solution (98% clottable, 5 mg/ml in 5 mM sodium acetate, pH 6.5) was incubated at room temperature with 10 μg fibrinogenase/ml and aliquots were removed at intervals for SDS electrophoresis. Each aliquot was incubated for 20 h at room temperature with an equal volume of 4% SDS/4% mercaptoethanol/10 M urea prior to electrophoresis [9]. Gels were scanned at 600 nm with a Gilford spectrophotometer equipped with a linear transport accessory. Peaks produced by the α-, β- and γ-chains of fibrinogen were cut out of the chart paper and weighed on an analytical balance in order to quantitate the areas under the peaks.

The hydrolytic activity of FII against hemoglobin and bovine serum albumin was assayed essentially by the acid-soluble protein method of Murata et al. [22], against collagen by the method of Mandl et al. [23] and against elastin by the method of Sachar et al. [24]. Activity against hide powder azure [25] was accomplished as follows the hide powder concentration was 5 mg/ml in 20 mM Tris-HCl-buffered normal saline. Incubation was done routinely at 37°C and for 1 h. Unsolubilized hide powder was removed with a clinical centrifuge and the solubilized material was quantitated by absorbance at 595 nm. This substrate proved convenient for routine assays and was used to optimize the proteolytic activity for pH and temperature. Unless otherwise indicated, hide powder hydrolytic activity is shown simply as the increase in absorbance at 595 nm per h. Enzyme inhibitors and protein modification reagents were tested for effectiveness by incubation with the enzyme for 30 min at room temperature prior to starting the hide powder assay.

The metal dependence of the enzyme was determined by dialysis for 20 h against 100 vol. of 100 mM sodium acetate, pH 6.4, containing 20 mM EDTA. The enzyme was reconstituted by again dialyzing the enzyme for 20 h against 100 vol. of 100 mM sodium acetate, pH 6.5, containing 10 mM $ZnCl_2$.

Atomic absorption spectroscopy for zinc, calcium and magnesium was accomplished with a Perkin-Elmer Model 303 with an air/acetylene flame. Solutions of protein (mg/ml) were used directly, and the metal content was estimated by comparison with standard curves for each cation obtained at the same time. The calcium standard solution was prepared with primary standard

calcium carbonate, magnesium and zinc solutions were prepared by the direct action of hydrochloride on the pure metals. The presence of mg/ml protein did not quench the contribution of known amounts of added standards.

TEX: (ICR) AM albino mice obtained from Timco Breeding Laboratories, Houston, TX, were used for both lethality and hemorrhage studies Lethal dose 50% ($LD_{50}$) was estimated by the method of Reed and Meunch [26]. Hemorrhage was estimated by subcutaneous injection between the shoulder of larger (28—30 g) mice. The buffer was 20 mM Tris-HCl-buffered normal saline, pH 7.4, and the total volume injected was held at 0.5 ml. Animals were killed and autopsied 4 h post-injection. Hemorrhage size was estimated on scale 0—4.

## Results

FII, as obtained by CM-cellulose ion-exchange chromatography [16], caused mild hemorrhage with 90 μg protein, causing a 1.5 and 135 μg a 2.5 hemorrhagic lesion, respectively. FII had an intraperitoneal $LD_{50}$ in mice of 5.1 μg/g body weight. Eight mice were used for this determination and three survived. It did have proteolytic activity as reported earlier [15—17]

Fig. 1 illustrates a typical further purification of FII by molecular sieve chromatography on Bio-Gel P-100. The lower molecular weight peak (centering on fraction numbers 35 and 36) obtained by this procedure contained all of the proteinase activity of this fraction as determined by hide powder azure hydrolysis and prevented the thrombin-induced clotting of fibrinogen. This material was lethal, but it caused no hemorrhages in mice. That activity resided in the higher molecular weight peak. As shown in Fig. 1, the specific activity for the hide powder hydrolytic activity was fairly constant across the peak. SDS and

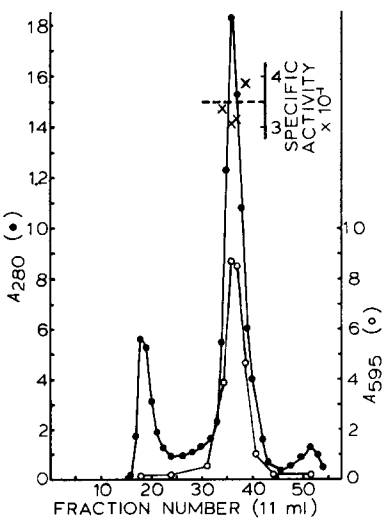


Fig 1 Separation of FII by molecular sieve chromatography 15 mg protein was applied to a 1 X 100 cm P-100 column and eluted with 100 mM sodium acetate, pH 6 5, in 1 2 ml fractions The location of hide powder hydrolytic activity is indicated by ○, while X shows the hide powder activity (in $A_{595}$ solubilized/h) per mg of enzyme The column was run at room temperature

discontinuous polyacrylamide electrophoresis gels of the proteolytic peak showed a single band. The SDS system indicated a molecular weight for this protein of 22 900 ± 600 as compared to the migration of cross-linked hemoglobin standards. This number is the average with 1 S.D. of four measurements. The reduced enzyme had the same apparent molecular weight, so the protein consists of a single polypeptide chain. Molecular sieve chromatography (P-100) gave a molecular weight of 23 500 based on the separation of blue dextran, bovine serum albumin, β-lactoglobulin, myoglobin and potassium ferricyanide.

26 mg of FII was obtained from 300 mg of whole venom. From the 26 mg of FII, 17 mg of purified proteinase was obtained, and the specific activity of the hide powder hydrolytic activity increased from 39.7 to 63.7 $A_{595}$ solubilized/h per mg. (Some variation in this value occurred among the several times this procedure was completed. For example, the specific activity across the peak in Fig. 1 is approx. 35). The recovery of the hydrolytic activity was essentially 100%. An $LD_{50}$ value was not determined for the highly purified enzyme, but two mice injected intraperitoneally with 5.6 μg of protein/g body weight and one with 8.0 μg/g died, while two injected with 2.8 μg/g survived. The $LD_{50}$ for FII as reported here was 5.1 μg/g and the value for whole venom was 9.1 μg/g as reported earlier [15].

Fibrinogenase activity coincided with the proteinase and was not found in the higher molecular weight protein peak. Table I shows the effect of this enzyme on the ability of fibrinogen to form thrombin-induced clots Although the fibrinogenase apparently has a rapid effect, when thrombin and the fibrinogenase were added simultaneously to fibrinogen, clotting occurred in the same length of time as when thrombin was added in the absence of the fibrinogenase. In addition, incubation of thrombin with the fibrinogenase (0.75 μg thrombin/10 μg fibrinogenase) for up to 0.5 h at room temperature did not affect thrombin's capacity to induce clots. Clots formed in the presence of the

TABLE I

DECREASE IN CLOTTABLE PROTEIN WHEN FIBRINOGEN IS INCUBATED WITH THE FIBRINOGENASE

Type I bovine fibrinogen, 5 mg/ml in 20 mM Tris-HCl-buffered saline, pH 7 4, was incubated at room temperature with 10 μg/ml of anticoagulant protein The absorbance at 280 nm of 5 mg fibrinogen/ml in the absence of either anticoagulant or thrombin is 4 80, and 78% of this protein is clottable Thrombin (0 75 μg, 1 875 NIH units) was added after the indicated times of incubation with the anticoagulant protein Data are the average of duplicate or quadruplicate samples Percentage of clottable protein was calculated by the formula $(1\ 00 - A_{280}/4\ 8) \times 100$

| Time (min) | Effect of thrombin | $A_{280}$ of supernatant | % Clottable protein |
|---|---|---|---|
| 0 | Hard clot in 50—60 s | 1 07 | 77 7 |
| 1 | Hard clot in 60—70 s | 1 00 | 79 2 |
| 5 | Soft clot in 3—4 min | 1 35 | 71 9 |
| 10 | Increase in turbidity, but no clot | 1 94 | 59 6 |
| 15 | | 2 49 | 48 0 |
| 20 | | 2 85 | 40 6 |
| 30 | | 3 32 | 30 8 |
| 60 | No increase in turbidity | 4 41 | 8 0 |

fibrinogenase were allowed to incubate an additional 15 h at room temperature and compared to clots incubated for the same length of time but formed in the absence of the fibrinogenase. The $A_{280}$ of the supernatant of the former was 2.40 ± 0.20 as compared to 1.05 ± 0.08 for the controls, indicating that some fibrinolysis did occur

Fig 2 illustrates that a direct effect of the enzyme on fibrinogen can be observed by SDS-polyacrylamide gel electrophoresis after only a few minutes of incubation at room temperature. The molecular weights of the different subunit chains of fibrinogen obtained in these experiments agreed favorably with data reported by Pizzo et al [27]. A comparison of the results illustrated by Fig. 2 with the data in Table I shows that the rapid disappearance of the $\alpha$-subunit of fibrinogen in the presence of the fibrinogenase is correlated with the decreased ability of fibrinogen to form thrombin-induced clots. Coincident with the disappearance of the $\alpha$-chains was the appearance of a doublet of molecular weights 44 000 and 42 000 and a smaller 23 000 molecular weight fragment.

Quantitation of the areas under the peaks of the electrophoresis gel scans revealed no decrease in either the $\beta$- or $\gamma$-chains over the 60 min time of the experiment, showing that the doublet and the 23 000 dalton fragment result

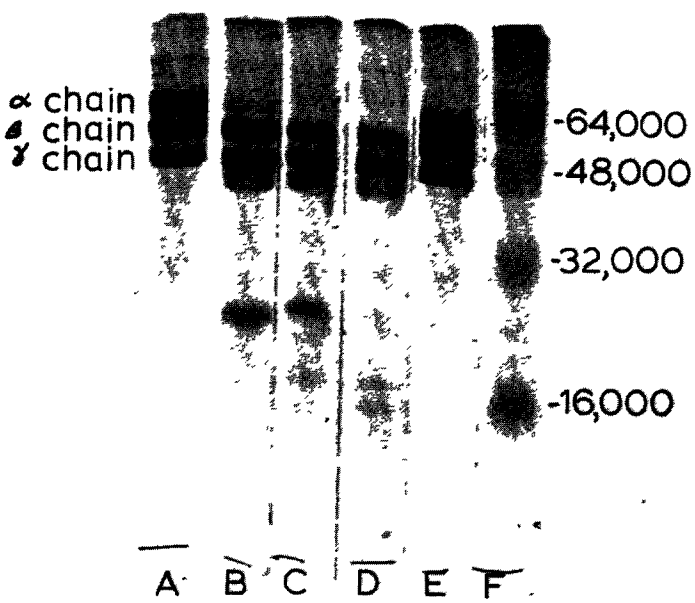


Fig 2 Time-dependent effect of the fibrinogenase on fibrinogen subunits as revealed by SDS-polyacrylamide gel electrophoresis Bovine fibrinogen (98% clottable, 5 mg/ml 5 mM sodium acetate, pH 6 5) was incubated with 10 $\mu$g anticoagulant/ml for 1 h at room temperature Protein samples, 125 $\mu$g each, were removed from the incubation mixture at various times and compared to fibrinogen incubated in the absence of the anticoagulant The pins near the bottoms of the gels indicate the migration of the bromphenol blue tracking dye Gel A, 0 time incubation, B, 1 min, C, 5 min, D, 60 min, gel E shows fibrinogen incubated 60 min in the absence of the anticoagulant, and gel F shows the migration of cross-linked hemoglobin standards

primarily from the degradation of the α-chains.

As the higher molecular weight peak obtained from P-100 chromatography had no hide powder hydrolyzing activity, the following experiments were conducted with the less pure FII in an effort to further characterize the compound which acts on fibrinogen and hide powder azure. Since earlier work [15] had shown that FII had a weak hydrolytic activity with casein, bovine hemoglobin and serum albumin, elastin orecin, elastin Congo red and collagen were tested as substrates. Neither hemoglobin nor albumin appeared to be a particularly good substrate. No activity with elastin or collagen was observed. Thus hide powder azure hydrolysis was used for routine assays. The pH optimum for hide powder azure hydrolysis was in the range 7—8 while the temperature optimum was 43°C. The reaction appeared insensitive to ionic strength in the range 20—200 mM. It should be emphasized that no concerted effort was directed towards total characterization of the optimum conditions for this assay. All of the hide powder azure hydrolytic assays included below were conducted at 37°C in 20 mM Tris-HCl-buffered normal saline, pH 7.4, with an incubation period of 1 h.

Incubation of FII for 30 min at room temperature with egg white and soybean trypsin inhibitors (5 μg inhibitor/μg FII) had no effect on hide powder hydrolysis. Likewise, incubation of FII with 20 μg/ml phenylmethylsulfonyl fluoride for 30 min had no effect. Acetonitrile was used as the carrier for this modification reagent. Incubation for 30 min with *N*-ethylmaleimide at concentrations as high as 10 mM was also ineffective against the enzyme activity. Dithiothreitol, however, completely prevented hide powder hydrolysis at concentrations as low as 0.5 mM. This inactivation occurred rapidly as the enzyme had lost 80% of its activity even when the hide powder assay was initiated immediately after mixing the enzyme with 0.5 mM dithiothreitol. Lower concentrations of dithiothreitol, 0.2 and 0.1 mM, showed some time dependence, indicating that this was inactivation and not inhibition

The following experiments were accomplished with the highly purified fibrinogenase. Extensive dialysis of the enzyme against buffer containing EDTA reduced the hide powder hydrolyzing activity to negligible levels and rendered the enzyme nontoxic to mice at doses of 21 μg of protein/g body weight. Enzyme dialyzed against buffer only retained its hide powder hydrolytic and lethal activities. Atomic absorption analysis of enzyme dialyzed against buffer alone showed that each mole of protein was associated with 2.63 mol calcium, 0.40 mol magnesium and 0.98 mol zinc, using 22 900 as the molecular weight of the protein. Enzyme dialyzed against EDTA contained only 0.09 mol zinc/mol enzyme. No other metal was assayed. Because of the nearly one-to-one relationship between the enzyme and zinc, the enzyme was dialyzed for 20 h against 100 vol. of 100 mM sodium acetate buffer, pH 6.5, containing 10 mM $ZnCl_2$. Adding back the zinc in this fashion restored 87% of the enzyme's original activity. The lethality was also restored.

## Discussion

The proteinase described in this report exerts its effect on clotting by acting directly upon fibrinogen The effect of this enzyme resembles that reported by

Ouyang et al. [4,5,8—10] for the α-fibrinogenases from Asian crotalid venoms in that it proteolytically disrupts the α-chain of fibrinogen. It differs from these enzymes in that a 42 000—44 000 molecular weight doublet appears as the α-chain is degraded. The action of the copperhead proteinase seems highly specific as neither the β- nor γ-chains appear affected, and the doublet produced from the α-chain also appears stable after the original cleavage. Only the 23 000 molecular weight fragment appears to be further hydrolyzed

The substance described in this report should probably be called a proteinase rather than an anticoagulant or a fibrinogenase It does catalyze the limited hydrolysis of some other proteins. Its inactivation by EDTA and dithiothreitol, its neutral pH optimum and lack of sensitivity to active serine and sulfhydryl modification reagents indicates that it is a metalloproteinase (see Ref. 28 for a review). The α-fibrinogenases reported by Ouyang et al. [4,5,8—10] are also probably metalloproteinases. A number of hemorrhagic toxins isolated from western diamondback rattlesnake (*Crotalus atrox*) venom by Bjarnason and Tu [29] are similar in the sense that they are EDTA-sensitive, zinc-dependent enzymes. Additional work in our laboratory indicates that the FII proteinase also occurs in the venom of the four copperhead subspecies not reported in this paper

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