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CLEAVAGE OF THE Aα-CHAIN OF FIBRINOGEN AND THE α-POLYMER OF FIBRIN BY THE VENOM OF SPITTING COBRA *(NAJA NIGRICOLLIS)*

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The effect of *Naja nigricollis* venom on fibrinogen and highly crosslinked fibrin was examined by SDS-polyacrylamide gel electrophoresis of the reduced products of venom treatment The venom contains a proteolytic activity which degraded the Aα-chain of fibrinogen, but had no apparent effect on the Bβ- or γ-chains of the molecule The venom also readily degraded the α-polymer of highly crosslinked fibrin, without apparent cleavage of the β-chain or the γ-dimer of fibrin The venom had no observed effect on plasminogen, indicating that the effects on the Aα-chain and the α-polymer are by direct action of the venom, and not due to activation of plasminogen The fibrinogenolysis was inhibited by EDTA or 1,10-phenanthroline Inhibition with EDTA could be reversed by the addition of Zn^{2+} The fibrinogenolysis was optimal between pH 7 and 8, consistent with the expected pH optimum for a Zn^{2+} metalloproteinase.

Introduction

Many snake venoms contain enzymes which affect blood coagulation [1] Thrombin-like proteinases, which cleave fibrinopeptide A from the Aα-chain of fibrinogen and initiate clot formation, have been found in several venoms [2] Ancrod, from the venom of *Agkistrodon rhodostoma*, and batroxobin, from the venom of *Bothrops atrox*, are the most studied of these thrombin-like enzymes, and are used clinically as defibrinogenating agents

Some venoms also show fibrinogenolytic activity, which leads to degradation of the fibrinogen molecule without concomitant clot formation The fibrinogenolytic activities of venoms have not been widely studied Although fibrinogenolytic enzymes, like thrombin-like enzymes, might also have the potential to deplete fibrinogen levels in clinical situations, they are generally felt to be proteinases with broad specificity Fibrinogenolytic proteinases have been demonstrated in the venoms of snakes of the *Crotalidae* and *Viperidae* families [3–7] These enzymes inhibit coagulation, possibly due to extensive degradation of the fibrinogen molecule, although two proteinases isolated from *Trimeresurus mucrosquamatus* were shown to cause limited cleavage of fibrinogen [8]

Venoms from the *Elapidae* family of snakes, which includes cobras, characteristically have little or no proteinase activity as detected by the digestion of casein [9–14] Studies of the fibrinogenolytic activity of cobra venoms have yielded conflicting results [15–17] In studies of the in vitro anticoagulant effect of the venom of spitting cobra, *Naja nigricollis*, we found that the major anticoagulant proteins of the venom are phospholipases A_2 [18] In this paper, I present evidence for cleavage of the Aα-chain of fibrinogen and the α-polymer of highly crosslinked fibrin by *N nigricollis* venom The cleavage of the Aα-chain of fibrinogen was associated with a prolongation of the thrombin time which could contribute to the in vivo anticoagulant effect of this venom.

Materials and Methods

Materials *N nigricollis* venom was obtained from Miami Serpentarium Human fibrinogen was prepared from normal plasma by the glycine precipitation procedure [19], or obtained from Kabi Group, Inc (Greenwich, CT) Topical thrombin was obtained from Parke-Davis Prothrombin-deficient human plasma was prepared by the procedure of Biggs and MacFarlane [20] Human plasminogen was purified by affinity chromatography on lysine-Sepharose [21] All chemicals used were reagent grade or better

SDS-polyacrylamide gel electrophoresis Cylindrical gels containing 5 or 7 5% polyacrylamide and 5 3 M urea were prepared by a modification [22] of the Weber and Osborn procedure [23] Samples containing 10% 2-mercaptoethanol/1% SDS/about 10 μg protein were heated at 100°C for 3 min prior to electrophoresis After electrophoresis at 3 mA/gel for 16 h, gels were stained and destained by the procedure of Fairbanks et al [24]

Thrombin clotting time Thrombin was diluted with 0 03 M ammonium acetate, pH 7 4, to give 1 2 NIH units/ml Clotting times were determined by transferring 200 μl fibrinogen sample into the reaction cup of a Baltimore Biological Laboratories fibrometer, and starting the fibrometer by the addition of 100 μl diluted thrombin

Results

The effect of *N nigricollis* venom on human fibrinogen was studied by incubation of the venom with fibrinogen at 37°C for 24 h Samples from the incubation mixture were removed at timed intervals for electrophoresis The results (Fig 1) show progressive degradation of the Aα-chain of fibrinogen by incubation with the venom A low proportion of intact Aα-chains remained after 2 h incubation There was no apparent effect of venom on the Bβ- or γ-chains of fibrinogen, even after 24 h incubation (data not shown) With the degradation of the Aα-chain, a cleavage product appeared as a diffuse band on the gels, at a position corresponding to a molecular weight of about 30 000 A second peptide, with a molecular weight of about 18 000 was also seen, partially obscured by one of the venom components

Plasma fibronectin is a contaminant of the commercial fibrinogen used in these studies Fibronectin is visible as a sharp band near the top of the gels, and is partially degraded by *N nigricollis* venom (Fig 1) Subsequent studies with purified plasma fibronectin and fibronectin-free fibrinogen have confirmed that the venom-generated peptides, which migrate faster than the reduced fibrinogen chains in the SDS-polyacrylamide gel patterns, come from the Aα-chain of fibrinogen rather than fibronectin Fibronectin appears as a minor contaminant with normal sample loads, as seen in later figures

The venom-treated fibrinogen remained clottable by thrombin throughout the 2 h period while the Aα-chain was being degraded, although the clotting time gradually increased to over 3-times the control level (Fig 1) After 30 min incubation of the fibrinogen with venom, a flocculent precipitate was visible in the incubation mixture, but a firm clot was not formed unless thrombin was added

The effect of the venom on highly crosslinked clots was also tested Thrombin-formed clots of prothrombin-deficient plasma were incubated for 4 h to allow the fibrin-stabilizing factor of the plasma to crosslink the γ-chains to form dimers and the α-chains to form polymers, as seen in the control gel (Fig 2) Treatment of the highly crosslinked clot with *N nigricollis* venom resulted in degradation of the α-polymer, but there was no apparent effect on the β-chain or γ-dimer of crosslinked fibrin The major cleavage product(s) from the α-polymer appeared in a diffuse band slightly below the position of the intact β-chain on the reduced SDS-polyacrylamide gels, indicating a molecular weight of about 45 000

Clearly, the effect of *N nigricollis* venom on fibrinogen and fibrin might be explained as the indirect result of venom activation of plasminogen contaminating the fibrinogen preparation To test this possibility, human plasminogen was incubated with *N nigricollis* venom or with streptokinase, and the products were examined on SDS-polyacrylamide gels (Fig 3) Treatment with streptokinase converted the plasminogen to plasmin, as indicated by the characteristic split products observed on the gel In contrast, *N nigricollis* venom had no apparent effect on plasminogen

Several proteinase inhibitors were tested to see if any could prevent the degradation of the Aα-chain of

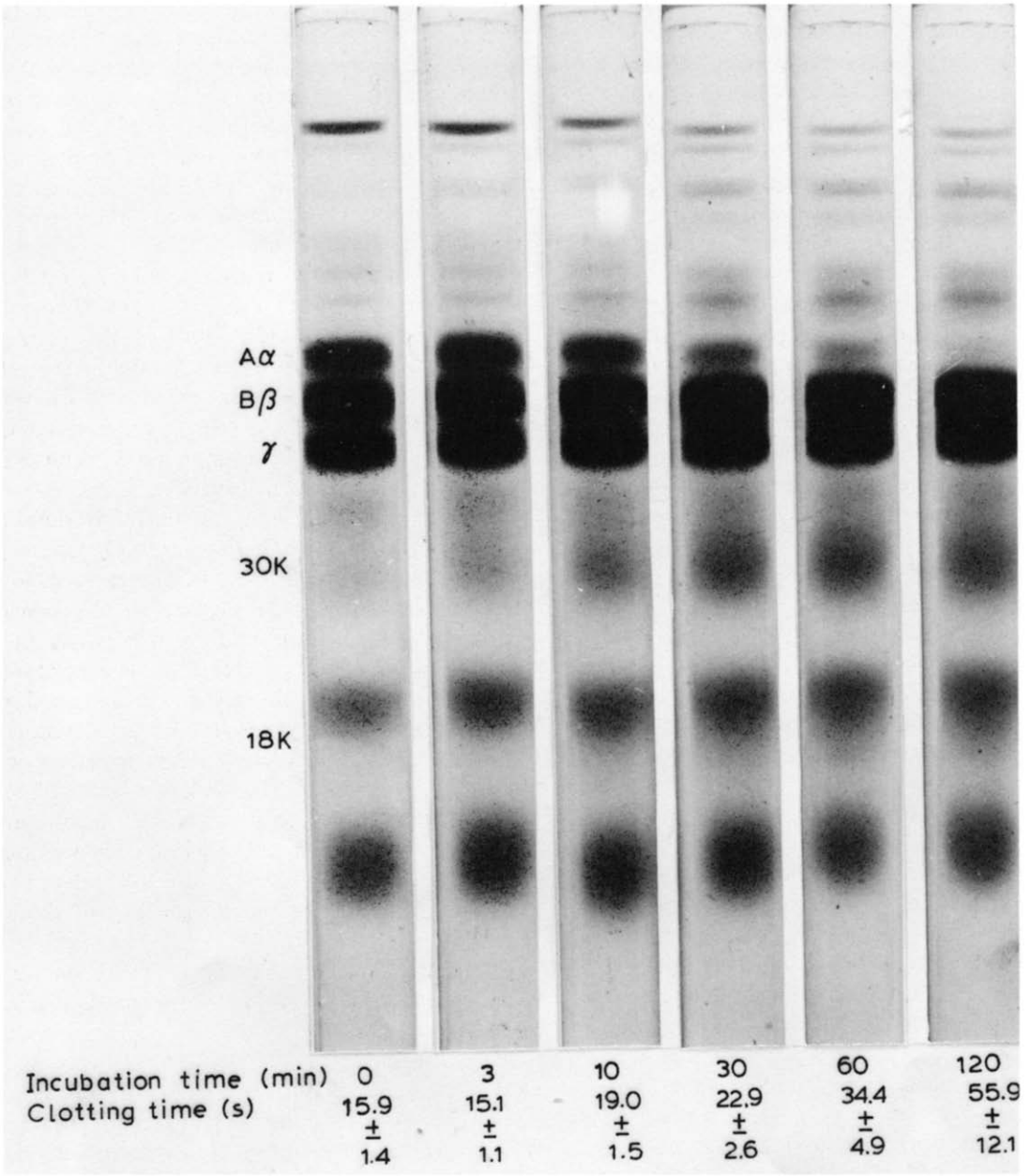
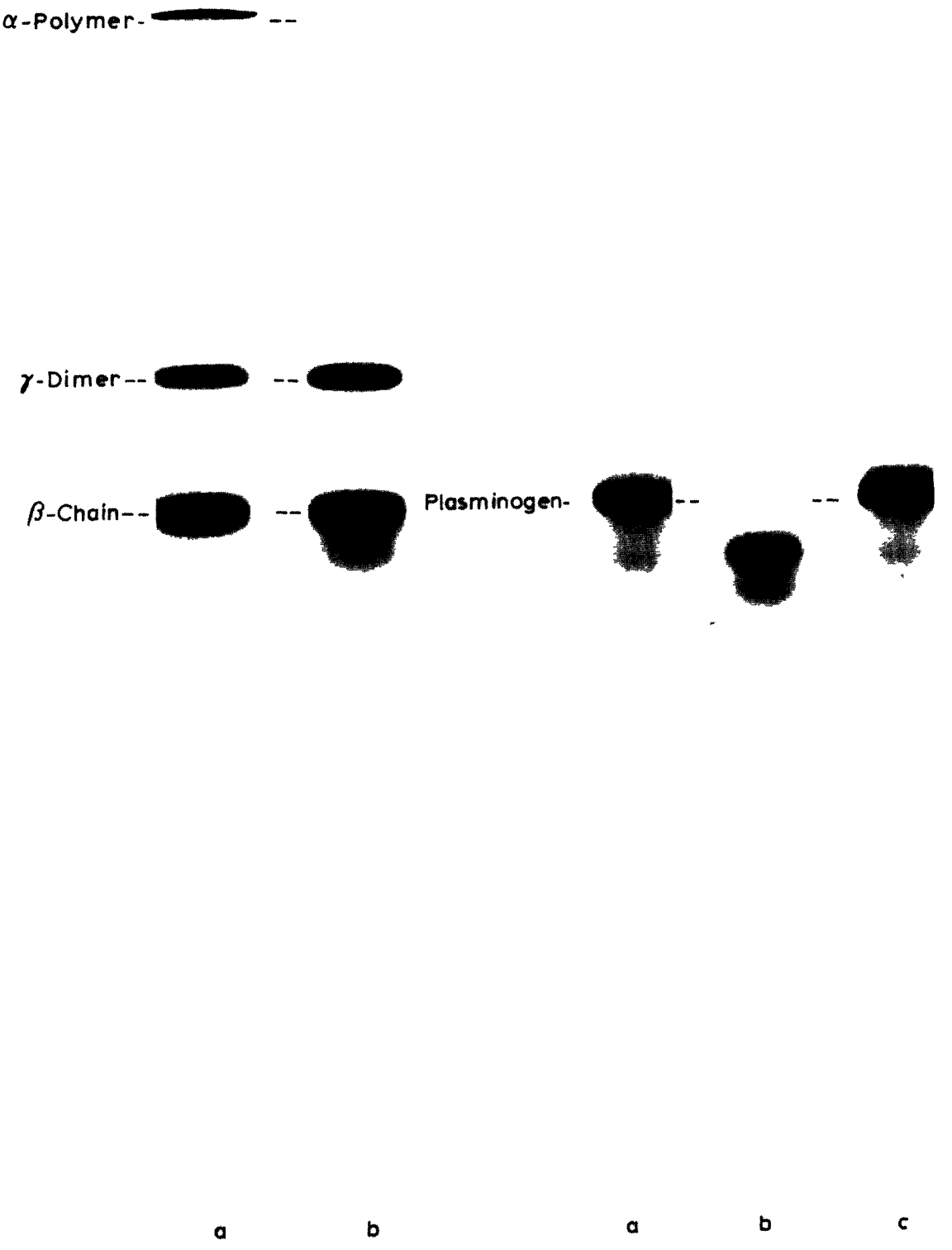


Fig 1 SDS-polyacrylamide gel electrophoretic patterns of fibrinogen treated with *N nigricollis* venom for the indicated time periods The timed incubation contained 1 mg ml^{-1} human fibrinogen and 0 25 mg ml^{-1} *N nigricollis* venom in 0 03 M ammonium acetate, pH 7 4 At the indicated sampling times, 200-μl aliquots were transferred to a fibrometer cup to determine the thrombin clotting time Clotting time (means ± S D for nine determinations from four separate experiments) are indicated below the sampling times Also at the sampling times, 180-μl aliquots of the incubation were added to test tubes containing 5 μl 0 5 M EDTA Samples containing 25 μg protein were reduced and applied to 5% polyacrylamide gels The gels were intentionally overloaded to show the cleavage products


α-Polymer
γ-Dimer
β-Chain
Plasminogen
a
b
a
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c

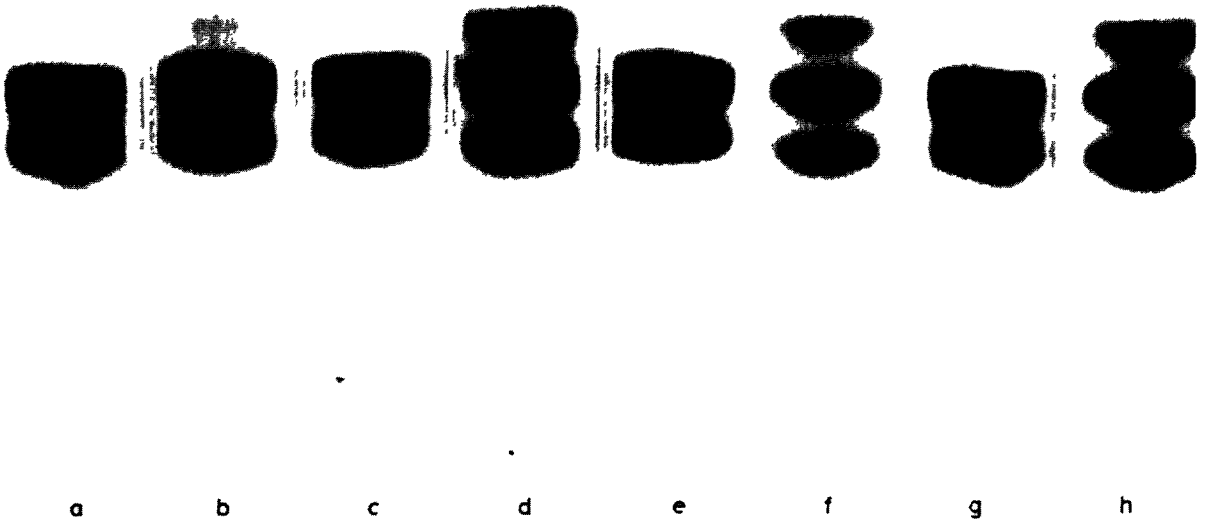

Fig 4 Effects of various proteinase inhibitiors on the fibrinogenolytic activity of *N nigricollis* venom Tubes containing 240 μg venom in 0 5 ml 0 03 M ammonium acetate, pH 7 4, were preincubated for 1 h at 23°C with each inhibitor Fibrinogen (1 mg dissolved in 0 5 ml 0 03 M ammonium acetate, pH 7 4) was added and the tubes were incubated for 2 h at 37°C Samples were then removed for electrophoresis Gel a, no inhibitor, gel b, 2 mM phenylmethanesulfonyl fluoride, gel c, 100 μg/ml soybean trypsin inhibitor, gel d, 1 mM 1,10-phenanthroline, gel e, 1 mM 4-hydroxymercuribenzoate, gel f, 2 mM dithioerythritol and 2 mM EDTA, gel g, 1 μg/ml pepstatin, gel h, no venom

Fig 2 (Left-hand figure) SDS-polyacrylamide gel electrophoretic patterns showing the effect of *N nigricollis* venom on highly crosslinked fibrin Clots were formed by adding 50 μl prothrombin-deficient plasma, 100 μl 0 1 M Tris-HCl, pH 7 5/14 mM $CaCl_2$ and thrombin (1 NIH unit) The clots were kept at 23°C for 4 h, washed with deionized water, blotted and transferred to tubes containing 200 μl 0 5 ml^{-1} *N nigricollis* venom in 0 03 M ammonium acetate buffer, pH 7 4, or 200 μl buffer alone After incubation at 37°C for 4 h, the clots were washed with deionized water, blotted and transferred to 200 μl solution containing 5 0 M urea/0 05 M sodium phosphate, pH 7 0/1% SDS/10% 2-mercaptoethanol 20-μl aliquots of these samples were electrophoresed Gel a, thrombin-formed clot without venom, gel b, thrombin-formed clot with venom Clots which were dissolved in the electrophoresis buffer immediately after the 23°C incubation gave the identical pattern seen in gel a

Fig 3 (Right-hand figure) SDS-polyacrylamide gel electrophoretic patterns showing the lack of effect of *N nigricollis* venom on human plasminogen Solutions containing 100 μg plasminogen and 15 μg venom or 5 μg streptokinase in 100 μl 0 03 M ammonium acetate, pH 7 4, were incubated for 3 h at 37°C Samples containing 10 μg plasminogen were removed from these incubations, reduced and electrophoresed as in Fig 1 Gel a, plasminogen alone, gel b, streptokinase-treated plasminogen, gel c, venom-treated plasminogen The minor bands on gel c were contributed by the venom, as indicated by a control gel with venom alone

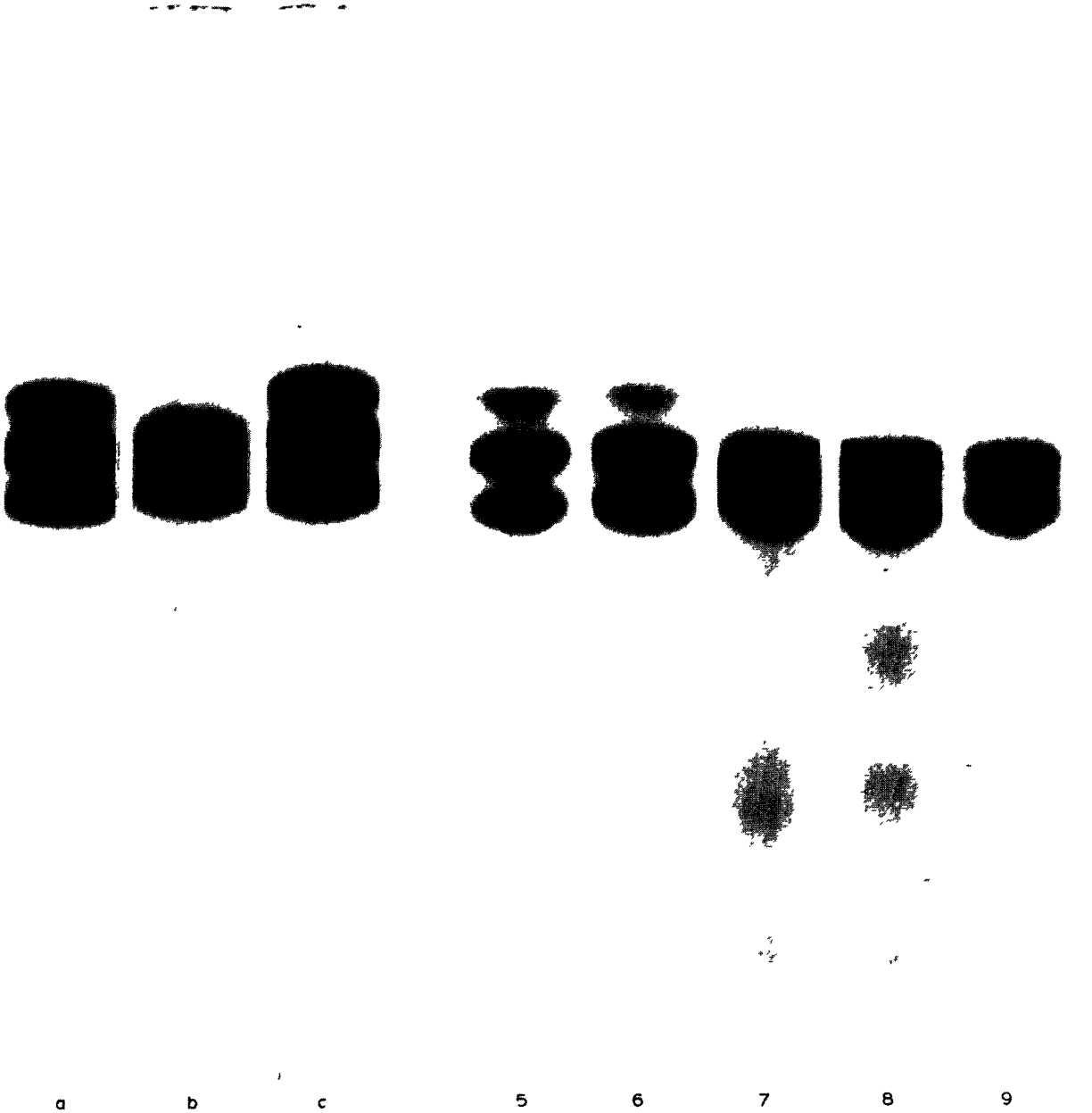

Fig 5 Restoration of fibrinogenolytic activity by addition of Zn^{2+} to EDTA-treated venom Incubations contained 250 μg/ml venom and 1 mg/ml fibrinogen in 0 03 M ammonium acetate, pH 7 4/0 2 mM EDTA/1 0 mM of the chloride salt of various metals After 2 h at 37°C, samples were removed for electrophoresis Gel a, EDTA alone, gel b, EDTA and Zn^{2+}, gel c, EDTA and Ca^{2+}

Fig 6 pH optimum of the fibrinogenolytic activity of *N nigricollis* venom Incubations contained, in 1 ml, 0 034 M Tris-maleate buffer of the appropriate pH/1 mg fibrinogen/0 25 mg venom After 1 h incubation at 37°C, the reactions were terminated by the addition of 20 μl 0 5 M EDTA, and samples were removed for electrophoresis The incubation pH is indicated under each gel

fibrinogen by the venom Fig 4 shows that the serine proteinase inhibitor, phenylmethanesulfonyl fluoride, and the thiol proteinase inhibitor, 4-hydroxymercuribenzoate, slightly inhibited fibrinogenolysis Soybean trypsin inhibitor and pepstatin, on the other hand, had no apparent inhibitory effect Fibrinogenolysis was most inhibited, however, by the metalloproteinase inhibitors, 1,10-phenanthroline and EDTA

The fibrinogenolytic activity was titrated with EDTA, and it was found that a final concentration of 0 1 mM EDTA was sufficient to completely inhibit the cleavage of fibrinogen by the venom An experiment was performed to see if adding back divalent metals to EDTA-treated venom could restore fibrinogenolytic activity and the results are shown in Fig 5 It is obvious that Zn^{2+} is able to restore fibrinogenolytic activity to the EDTA-treated venom when added in slight excess over the amount of EDTA Ca^{2+} was not able to restore the activity (Fig 5), nor could Mg^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+} or Sn^{2+}

The pH optimum of the fibrinogenolytic activity was determined in incubations of fibrinogen and venom buffered over the pH range between pH 5 and 9 The results (Fig 6) show that the fibrinogenolytic effect is optimal in the range of pH 7–8, since no intact Aα-chain remained at the end of the incubation period for incubations buffered at those pH values

Discussion

The Aα-chain of fibrinogen appears to be susceptible to cleavage by many proteinases, including endogenous plasma proteinases [25,26] The novel aspect of the present work is the demonstration that *N nigricollis* venom contains one or more proteinases with the specific abilities to cause a limited cleavage of both the Aα-chain of fibrinogen and the α-polymer of highly crosslinked fibrin These activities were found despite the absence of any generalized proteolytic activity in the venom [9–14]

The venom cleavage of fibrinogen might be compared to the plasmin cleavage of fibrinogen and fibrin, except that the venom activity was limited to only the Aα-chain or the α-polymer, whereas plasmin causes more extensive degradation of these substrates Because of its broad specificity, plasmin can readily be assayed by monitoring its degradation of casein, whereas *N nigricollis* venom has little or no effect on casein However, the venom apparently cleaved the α-polymer just as readily as it cleaved the Aα-chain of fibrinogen Plasmin, on the other hand, does not cleave the α-polymer as readily as the Aα-chain [27], despite its broader specificity and its ultimate ability to extensively cleave highly crosslinked fibrin

The site(s) of cleavage in the Aα-chain and the α-polymer are not known, but some information can be obtained from the electrophoretic patterns of the products The disappearance of the α-polymer band of highly crosslinked fibrin after venom treatment shows that the venom cleaves at least one peptide bond between crosslinking sites of the α-chain Recent evidence indicates that the crosslinks of the α-polymer join acceptor glutamine residues at positions 328 and 366 in the α-chain [28] with donor lysine residues near the carboxy terminus [29] Cleavage of one peptide bond between these acceptor and donor regions would be sufficient to degrade the α-polymer to monomer size fragments, provided the donor residues of one α-chain crosslink only with acceptor residues of one other α-chain It seems likely, however, that the venom acted at more than one peptide bond, since the only observed product had an apparent molecular weight of about 45 000, smaller than the monomer molecular weight of 66 000 This 45 000 molecular weight species might represent a crosslinked peptide containing the two peptides, with molecular weights of 30 000 and 18 000, obtained from venom cleavage of the Aα-chain Smaller soluble peptides might be lost in the clot-washing procedure and thus be undetected on the gels More accurate molecular weight characterization and isolation of the products obtained with the purified proteinase(s) will be required to establish the relationship of the products from cleavage of both fibrinogen and fibrin

The venoms of *Elapids* have not previously been considered sources of fibrinogenolytic and fibrinolytic proteinases because of their weak proteolytic activity The present work establishes the presence of these activities in the venom of *N nigricollis* and indicates that the fibrinogen cleavage is due to a zinc metalloproteinase The purification of the proteinase responsible for these activities is in progress Efforts are also underway to determine the specific peptide bonds cleaved in the Aα-chain and the α-polymer by the venom

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