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Hemorrhagic Component of Northern Copperhead Venom

David Hill
Ouachita Baptist University

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ABSTRACT

The major hemorrhagic component of northern copperhead (Agkistrodon contortrix mokasen) venom was partially purified by ion exchange and molecular sieve column chromatography. TAME and Phospholipase enzymatic activities were shown to be unrelated to hemorrhage. BAEE and procoagulant activities appear to be associated. Hide powder activity seems to correlate with hemorrhagic activity. Both show metal and disulfide dependences.

*Research Director - Dr. Collis R. Geren
**Ouachita Baptist University, Arkadelphia, Arkansas

INTRODUCTION

The northern copperhead (Agkistrodon contortrix mokasen) is commonly found in forests from Massachusetts to Illinois, and in highlands as far south as Georgia and Alabama.[1]

The venom of the northern copperhead has been reported to contain several enzymatic activities as well as a fraction which induces hemorrhage in mice.[2] Whole venom was fractionated by carboxymethyl cellulose ion exchange chromatography into nine distinct fractions. The nonretained material was reported to contain the hemorrhagic activity.[2]

This paper details work toward the separation and characterization of the major hemorrhagic component of northern copperhead venom.

MATERIALS AND METHODS

Lyophilized northern copperhead venom was obtained from Sigma Chemical Company and Miami Serpentarium Laboratories.

Ion exchange celluloses were purchased from Whatman Inc. while molecular sieves were purchased from Bio-Rad laboratories.

Electrophoresis reagents were purchased from Bio-Rad Laboratories. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn.[3] Molecular weights of venom protein components were estimated by their relative migration to the known standard proteins alcohol dehydrogenase, myoglobin, bovine serum albumin, human serum albumin, and α-lactabamin. Disc gel electrophoresis was carried out by the procedure of Ornstein.[4]

Protein solutions were concentrated by means of Centriflo Membrane Cones (Amico Corporation). Solutions were centrifuged in CF-25 cones at 4°C to the volume desired. Molecules with molecular weights above 25,000 should be retained in the cones.

Protein concentrations were estimated by their absorbance at 280nm (A_{280}), assuming an extinction coefficient of 1.0 for a 1.0 mg/ml aqueous protein solution. Conductivity measurements were used to estimate the ionic strengths of gradients.

C3H mice (18-25g) were used for hemorrhage and lethality determinations. Hemorrhage was assayed using the method of Kondo, et al.[5] Lethality was assayed by i.p. injection followed by a 48-hour observation period.

A modified form of the hide powder azure assay of Rinderknecht, et al[6] was used. Ten mg of the hide powder was suspended in 1.4 ml of 20 mM Tris-buffered saline, pH 7.4. For each assay, Venom fractions were then added to each test tube. All were incubated at 37°C for 3 hours. The contents were centrifuged, and the absorbance of the supernatants at 595 nm (A_{595}) was measured.

Phospholipase activity was assayed titrimetrically by the method of Marinetti,[7] which employs soybean lecithin as a substrate.

The titrimetric method of Smith and Parker[8] was utilized to determine Benzoyl-L-arginine ethyl ester (BAEE, Sigma Chem Co) hydrolytic activity. Optimum BAEE activity for whole northern copperhead venom was obtained at pH 8.5, so this was used as the end point in the titration.

The hydrolysis of p-toluene-sulfonyl-L-arginine methyl ester (TAME, Sigma Chem. Co.) was followed spectrophotometrically using a Gilford updated Beckman DU with a multiple positioner.

Blood clotting time assays were similar to those described by Herzig, et al.[9] Human fibrinogen was dissolved in Tris-buffered saline (0.92% NaCl w/v, 20mM Tris, pH 7.4) in a concentration of 3 mg/ml. The fibrinogen solution (0.4ml) was preincubated at 37°C for 10 minutes; then 0.1ml of venom fraction was added to each tube. The tubes were tilted at 15 second intervals until a clot formed. If a clot failed to appear within 10 minutes, the material was considered to have no activity.

Disulfide bond dependence was determined by incubation of 125µl of venom fraction in 10, 1.0, and 0.1mM dithiothreitol (DTT, Sigma) for 15 min at 37°C.

Metal dependence was investigated by dialysing 150µl of concentrated venom in 450ml of Tris buffered saline against 1500ml of 20mM ethylene diamminetetra-acetic acid (EDTA, Sigma). The sample was dialysed for 3 hours against 500mls of EDTA solution with hourly changes of EDTA.

The effect of proteases upon the venom fraction was determined by the incubation of 500ml of the venom fraction and 55ml of trypsin (1 mg/ml) for 1 hour at 37°C. Pronase was used in a similar manner.

Temperature stability was established by incubating 1.5ml samples of venom fractions for 5 min at 24°C, 50°C, 75°C, and 100°C, followed by the determination of hide powder and hemorrhagic activities.

RESULTS

Northern copperhead venom (375mg) was separated on a 3X9 cm carboxymethyl cellulose (CM) column. The column was equilibrated with 5mM sodium acetate, pH 6.5, and the sample was also dissolved in this buffer. Protein was eluted by a linear gradient of 400ml of application buffer and 400ml of 500mM sodium acetate, pH 6.5. The results were the same as obtained by Moran et al.[2]

The nonretained fraction (CMFI) was further separated on a 2X8 cm diethylaminoethyl (DEAE) cellulose anion exchange column. The column was equilibrated with 5mM sodium acetate, pH 6.5. An elution gradient of 200ml of 5mM sodium acetate, pH 6.5, and 200ml of 200mM sodium acetate, pH 6.5., was used. Figure 1 illustrates such a separation.

All the hemorrhagic activity of the whole fraction could be accounted for by the CMFI fraction of the CM column elution. This activity was further localized in a broad sticking peak of the DEAE cellulose column run. (See Figure 1, indicated area) SDS gel electrophoresis revealed that this hemorrhagic peak was a multicomponent fraction with a major band in the 17,500 molecular weight range.

The DEAE hemorrhagic area was pooled, concentrated, and run on various molecular sieve columns to try to achieve homogeneity of the hemorrhagic component. Fortunately, all of the activity was retained in the Centriflo cones. The best partial purification was achieved on a Bio-Gel P-150 column as shown by Figure 2. The column was 1X100 cm and equilibrated with 100mM Tris, pH 7.4. The arrows on the profile represent the locations of the void and salt volumes respectively. The dotted curve repre-

sents the location of the hemorrhagic activity. The intensities of the lesions induced were scaled on a range of 0 to 4. Figure 3a shows the major part of Figure 2's protein profile with the hide powder hydrolyzing activity of CMFI indicated. Likewise, Figures 3b, 3c, and 3d illustrate the locations of the BAEE and blood clotting, TAME hydrolyzing, and phospholipase activities respectively on the P-150 protein elution profile.

Disc gel electrophoresis revealed that the hemorrhagic activity containing fraction was not homogeneous. Figure 4 compares densitometric scans of disc gel electrophoretic separations of the four peaks obtained from the P-150 elution.

The partially purified hemorrhagin was stable for a 5 minute incubation period at 25°C and 50°C, but not at 75°C or 100°C.

The hemorrhagic activity was retained very well through all of the purification steps (Table 1). Full hemorrhagic activity was still present after incubation with trypsin, and also after incubation with Pronase.

The EDTA experiments revealed that both hide powder azure activity and hemorrhagic activity have a metal dependence. Both also show disulfide bond dependence as illustrated by Table 2.

The DEAE hemorrhagic pool is lethal at 100μg dosage to C3H mice injected i.p.

Figure 5 shows scans of SDS electrophoretic separation of the four P-150 fractions. Figure 6 illustrates the relative migrations of known proteins under the same conditions as the venom fractions shown in Figure 5. The X's marked 3 and 4 represent the migrations of the major components of these fractions.

Long term studies of mice injected with the hemorrhagic fraction revealed loss of hair, calcification of skin, and eventual sloughing. Autopsy one month after injection revealed no permanent damage at the injection site.

DISCUSSION

The hemorrhagic activity of whole northern copperhead venom can be accounted for largely by one fraction. This fraction is not homogeneously as of now, but a P-150 column elution profile allows dissociation of several enzymatic activities from

the hemorrhagic activity. A significantly purified phospholipase eluted in a distinct peak separate from the hemorrhagic peak. The two intermediate peaks showed both procoagulent and BAEE hydrolytic activity. The smaller of these appears to be homogeneous upon SDS gel electrophoresis.

Hemorrhagic activity and hide powder azure proteolytic activity were both present in the void peak. The two activities seem to be associated. Both show metal dependence and correlate well in disulfide bond dependence at all 3 DTT concentrations used.

The comparison of SDS to disc patterns reveals that the proteins in the hemorrhagic fraction are subunit proteins, and these subunits are dissociated by the SDS alone.

Further work will involve purification of the hemorrhagic component to homogeneity and continuance of its characterization.

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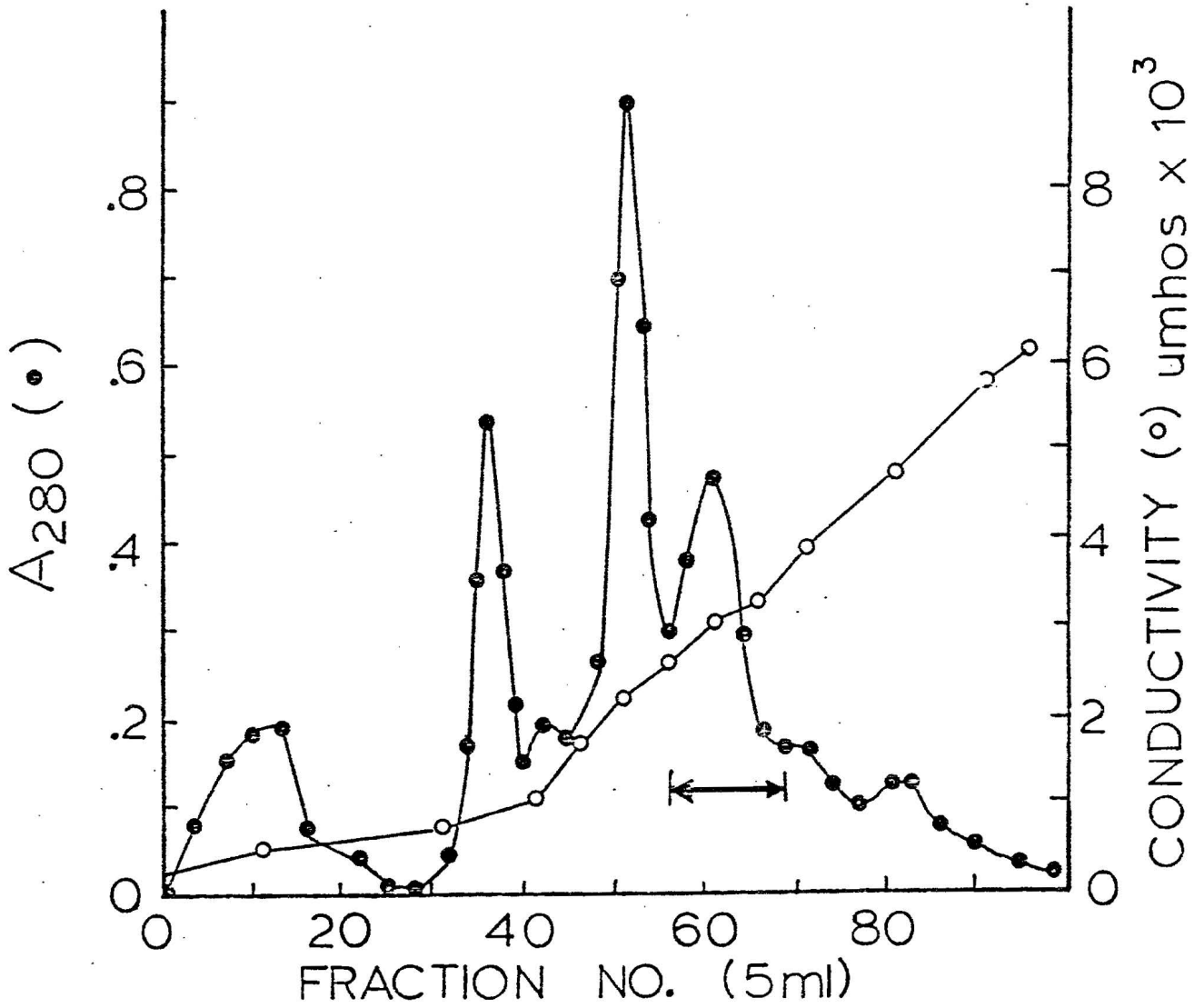
A280 (•)
.8
.6
.4
.2
0
0
20
40
60
80
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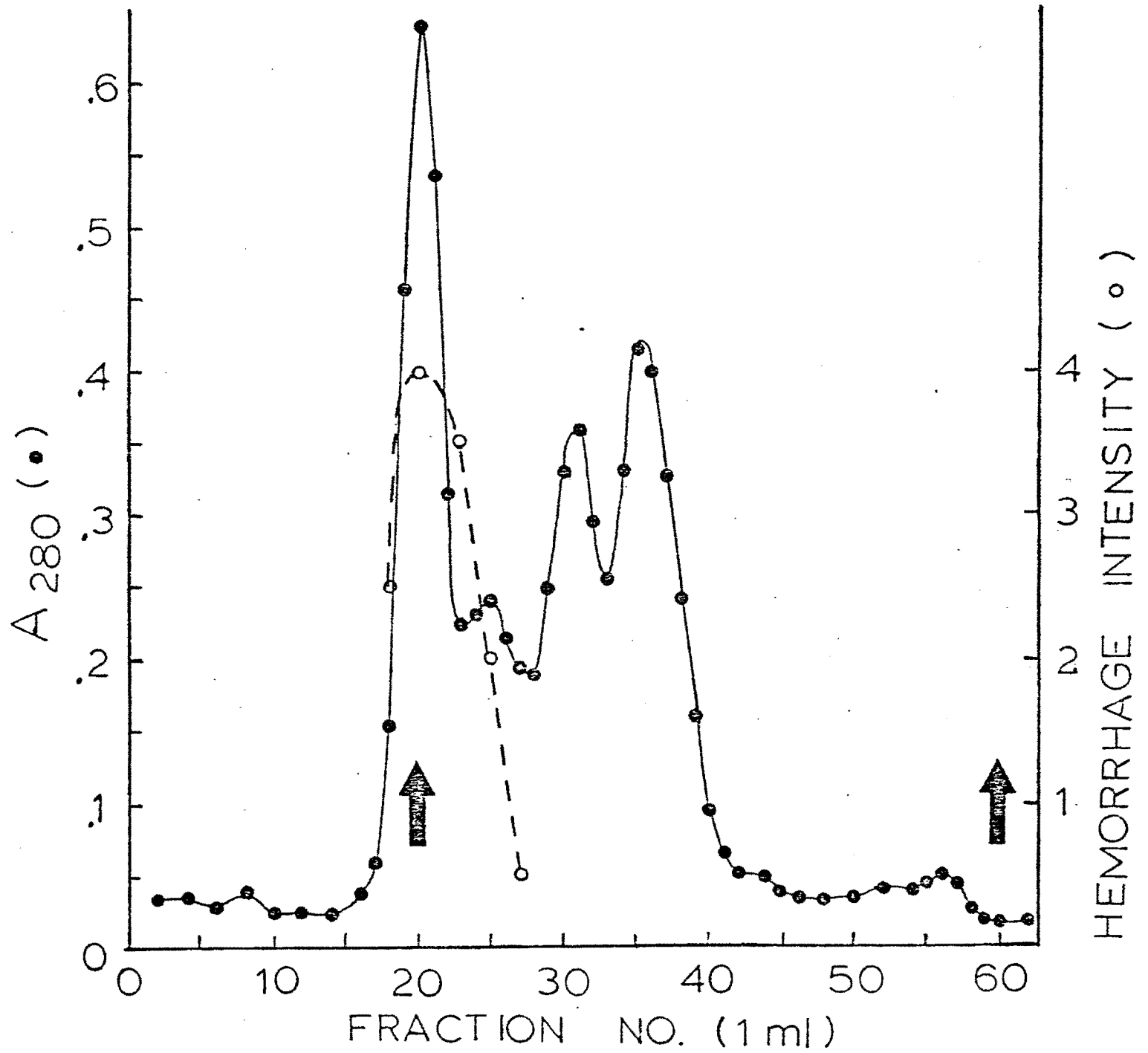
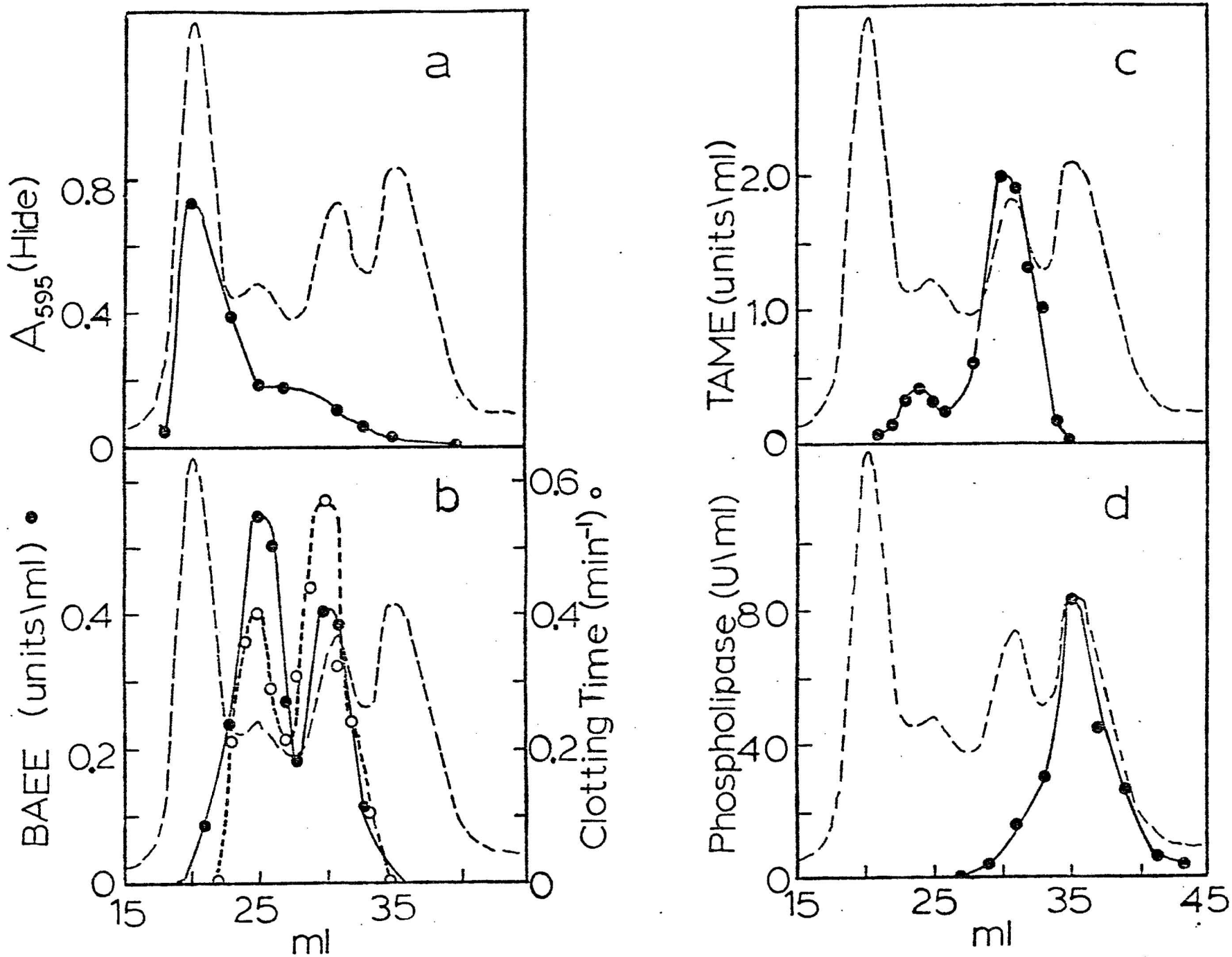
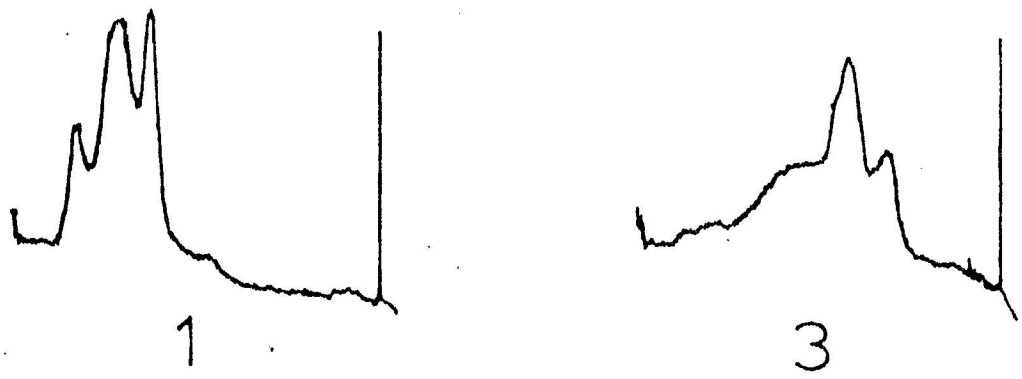
CONDUCTIVITY (○) umhos x 10³
8
6
4
2
0

(FIGURE 1)

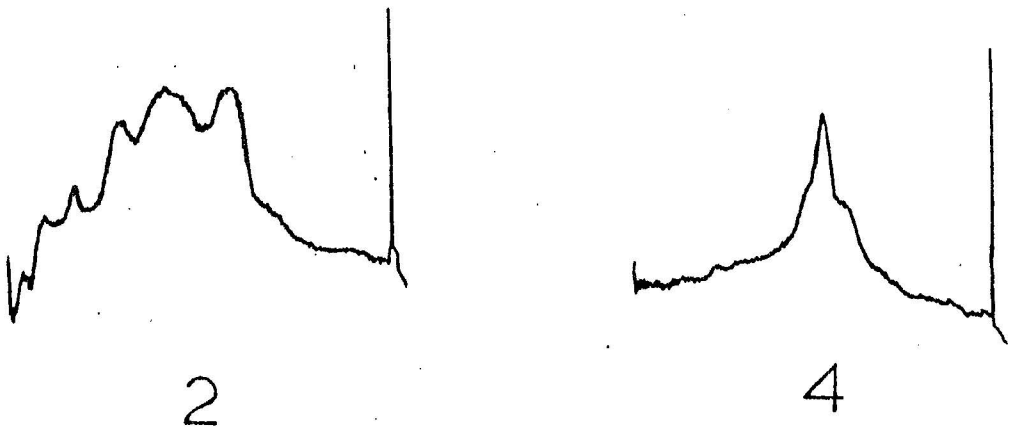

A 280 (•)
.6
.5
.4
.3
.2
.1
0
0
10
20
30
40
50
60
FRACTION NO. (1 ml)
HEMORRHAGE INTENSITY (○)
4
3
2
1


a
b
c
d
A_{595} (Hide)
BAEE (units\ml) •
Clotting Time (min^{-1}) ○
TAME (units\ml)
Phospholipase (U\ml)
0.8
0.4
0
0.6
0.2
2.0
1.0
80
40
15
25
35
45
ml

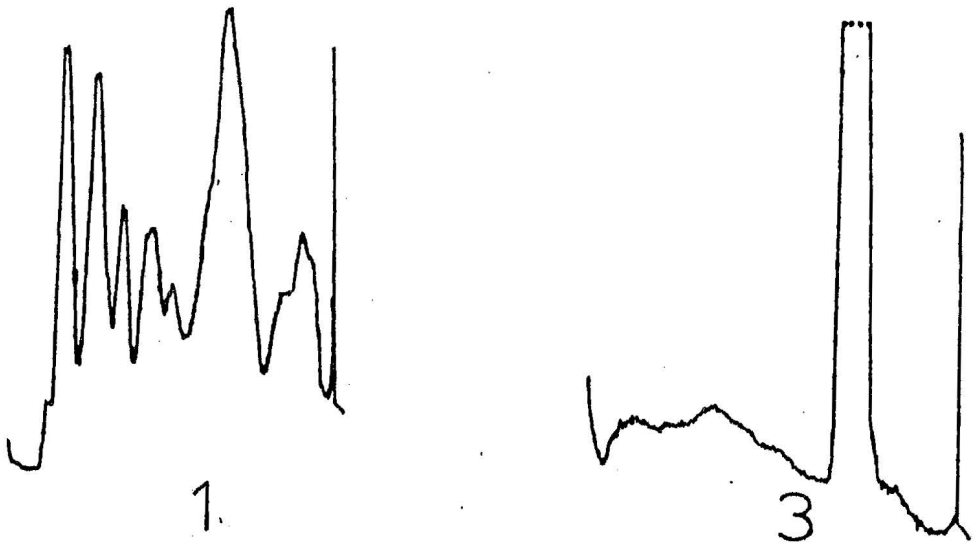
(FIGURE 3)



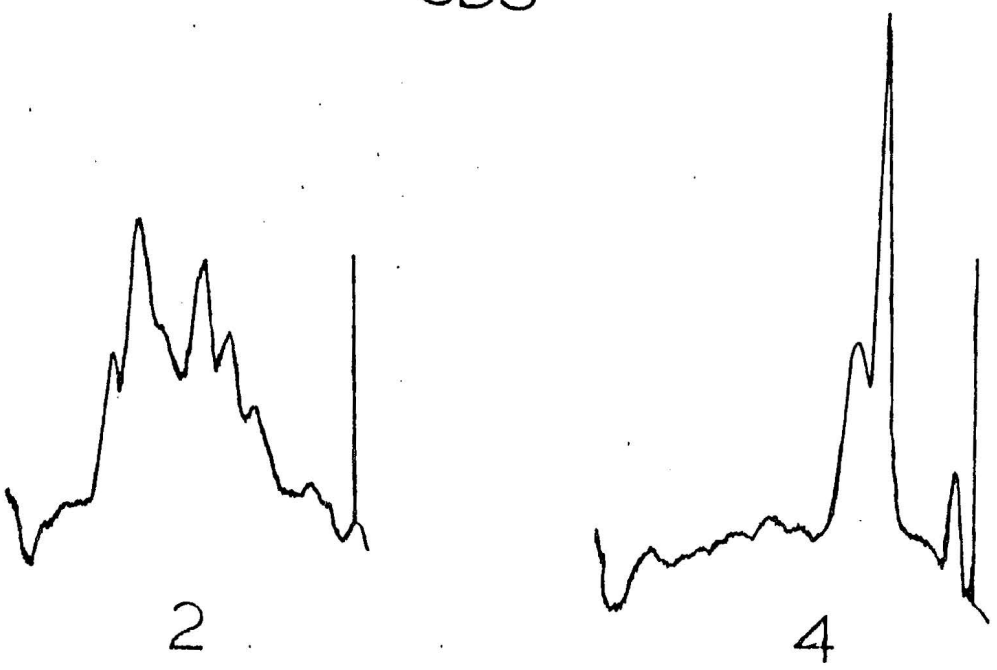
Disc



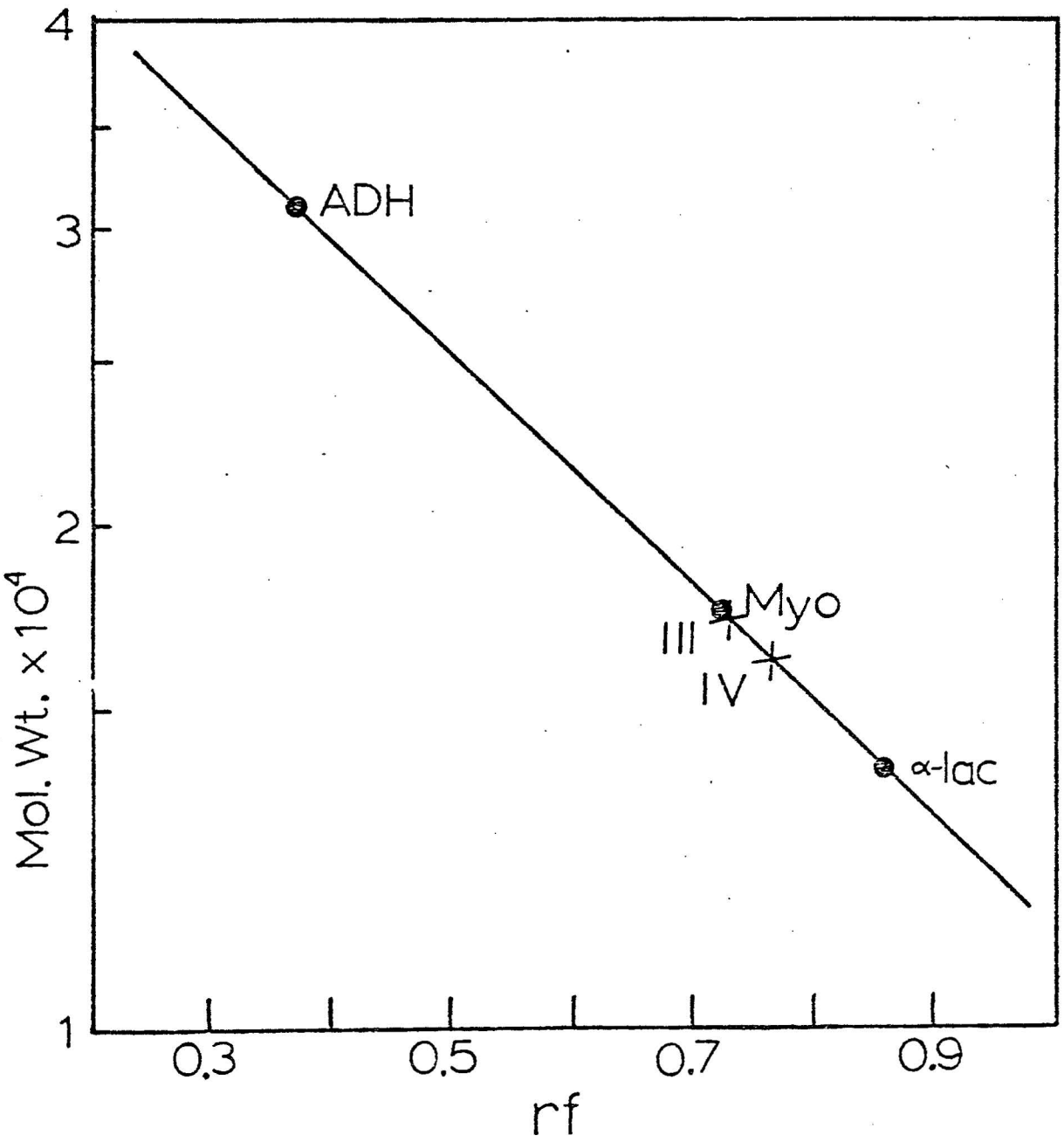
(FIGURE 4)



SDS



(FIGURE 5)



(FIGURE 6)

TABLE I

Purification Table

Step	Dose (μg)[1]	% of Whole Venom[2]	Recovery of Activity	Fold Purification
Whole Venom	100	100	100	1
CMFI[3]	40	40	100	2.5
DEAE D[4]	14.5	14.8	100	6.9
P-150[5]	6.82	4.52	67	14.7

[1] Dose required to give +3 hemorrhage
[2] Based on A_{280} protein
[3] Nonretained fraction from CM cellulose chromatography
[4] Most acidic fraction from DEAE cellulose chromatography
[5] Peak 1 from Bio-Gel P-150 chromatography

TABLE II

Dissulfide Dependence of Copperhead Hemorrhagin

D.T.T. Conc.[1] (mM)	% Retention of Hemorrhagic Activity	% Retention of Hide Powder Activity
0	100	100
0.1	100	97
1.0	40	39
10.0	0	2.8

[1] Purified hemorrhagin was incubated for 15 min at 37°C with the indicated concentrations of dithiothreitol.