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CONCANAVALIN A-BINDING ENZYMES OF *CROTALUS SCUTULATUS SCUTULATUS* VENOM

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ABSTRACT

Crotalus scutulatus scutulatus crude venom was separated into two fractions by Concanavalin A Sepharose 4B affinity chromatography. The proteins binding to Con A exhibited phosphomonoesterase (orthophosphoric monoester phosphohydrolase EC 3.1.3.2), phosphodiesterase, 5′-nucleotidase (5′-ribonucleotide phosphohydrolase EC 3.1.3.5), phospholipase A (phosphatidate 2-acylhydrolase EC 3.1.1.4), hyaluronidase (hyaluronate glycanohydrolase EC 3.2.1.d), N-benzoyl-L-arginine ethyl esterase, p-toluenesulfonyl-L-arginine methyl esterase, L-amino acid oxidase (L-amino acid: O_2 oxidoreductase [deaminating] EC 1.4.3.2), and caseinolytic activities. Thrombin-like and NAD nucleosidase (5′-ribonucleotide phosphohydrolase EC 3.1.3.5) activities were not observed.

The crude venom and the fraction containing the glycoproteins which bound to Con A were fractionated by DEAE Sephadex A-50 ion exchange chromatography. Each of these samples yielded fractions having caseinolytic activities.

INTRODUCTION

The Mojave rattlesnake *(Crotalus scutulatus scutulatus)* is a medium sized snake found in a diagonal band from the Mojave Desert in California to northern Guanajuato and northwestern Querétaro in Mexico (Klauber, 1972). *C. s. scutulatus* venom is among the most toxic of the rattlesnake venoms of the numerous species comprising the genus *Crotalus* occurring in the United States (Pattabhriaman *et al.*, 1978; Glenn and Straight, 1978). Venom toxicity (Glenn and Straight, 1978), venom properties (Glenn *et al.*, 1983) and clinical symptoms of envenomation (Hardy, 1983), however, may vary with the geographical distribution of this species.

Rattlesnake venoms are complex mixtures consisting mostly of proteins having enzymatic activity. Although enzymes contribute to the deleterious properties of the crude venoms, the lethal components are reported as peptides and nonenzymatic proteins (Dubnoff and Russell, 1971; Bonilla and Fiero, 1971; Russell *et al.*, 1976). To substantiate this further, Pattabhriaman *et al.* (1978) used Sephadex G-100 (Fine) and DEAE Sephadex A-50 to obtain lethal fractions from *C. s. scutulatus* venom. Of these fractions, toxic fraction C (obtained by gel filtration) was a basic polypeptide (MW 9,000) while toxic fraction K (obtained by ion exchange chromatography of gel filtration peak B) had a molecular weight of 20,000. Bieber *et al.* (1975) described the lethal toxin of Mojave rattlesnake venom as one of the nine fractions obtained by chromatography of the crude venom using DEAE Sephadex A-50. The lethal toxin was further characterized as being a protein cardiotoxin having a molecular weight of about 22,000 and consisting of two subunits. Cate and Bieber (1978) reconstituted Mojave toxin from an acidic subunit and a basic subunit. The basic subunit had phospholipase A_2 activity. Castilonia *et al.* (1981) and Ho and Lee (1981) reported that Mojave toxin, like other snake toxins having phospholipase A_2 activity, has a presynaptic site of activity.

This study involves *C. s. scutulatus* venom enzymes. Mojave rattlesnake crude venom and the venom proteins having an affinity for Concanavalin A are analyzed for a variety of enzymes with special emphasis being placed on proteinase (caseinolytic) activity.

MATERIALS AND METHODS

Lyophilized *C. s. scutulatus* venom was provided by Dr. H. L. Stahnke of Arizona State University. N-benzoyl-L-arginine ethyl ester (BAEE), p-toluenesulfonyl-L-arginine methyl ester (TAME), 5′-adenylic acid, bis-p-nitrophenyl phosphate sodium salt, beef plasma thrombin, β-NAD^+, bovine fibrinogen (F-4000), and bovine albumin Fraction V were purchased from Sigma Chemical Company; disodium p-nitrophenyl phosphate from Nutritional Biochemicals Corporation; Tris-(hydroxymethyl)aminomethane, glycine, ammonium molybdate, hydroquinone, sodium sulfite, magnesium chloride, L-leucine, trichloroacetic acid (TCA), potassium cyanide, potassium hydrogen phosphate, and calcium chloride were purchased from Fisher Scientific Company; sodium hydrogen sulfite from J. T. Baker Chemical Company; hyaluronic acid from Worthington Biochemical Corporation; magnesium sulfate and sodium hydrogen phosphate from Mallinckrodt Chemical Works; casein from ICN Pharmaceuticals, Inc.; Sephadex G-25, Concanavalin A-Sepharose 4B (Con A), DEAE A-50, and columns from Pharmacia, Uppsala I, Sweden.

All enzyme assays were performed with concentrations which had been adjusted to produce linear rates of substrate hydrolysis during the incubation intervals. In the crude venom enzyme assays, concentrations of one to two mg per ml were used. Enzyme assays were performed at pH intervals of 0.5 to determine the effects of pH on enzyme activities. For protein estimations and in assays for esterase, phosphomonoesterase, phosphodiesterase, 5′-nucleotidase, and proteinase a Beckman Acta C III spectrophotometer was used to measure absorbance. A Spectronic 20 was used to measure absorbance in the phospholipase A assays. The factors used by Sulkowski *et al.* (1963), Björk (1963), and Richards *et al.* (1965) for converting phosphomonoesterase, phosphodiesterase, and 5′-nucleotidase activities measured at 37 °C to values at 25 °C were used.

Phosphomonoesterase (Richards *et al.*, 1965), phosphodiesterase (Richards *et al.*, 1965), 5′-nucleotidase (Lo *et al.*, 1966; Ging, 1956), phospholipase A (Marinetti, 1965), N-benzoyl-L-arginine ethyl esterase (BAEEase) and p-toluenesulfonyl-L-arginine methyl esterase (TAMEase) (Tu *et al.*, 1965; Schwert and Takenaka, 1955), thrombin-like (Sato *et al.*, 1965), proteinase (Kunitz, 1947; Rick, 1965), and L-amino acid oxidase (Paik and Kim, 1965) assay procedures included the minor modifications used in a previous work (Sifford and Johnson, 1978). Assays for NAD nucleosidase (NADase) were performed by the procedure described by Colowick *et al.* (1951) and Kaplan *et al.* (1951). Hyaluronidase activity was measured by the turbidimetric methods of Kass and Seastone (1944).

Separations using Con A gel were performed by the method of Iscove *et al.* (1974) and Aspberg and Porath (1970). A 425 mg sample of crude venom was applied to a Con A column (2.5 x 15 cm), at 4 °C, which had been equilibrated with 500 ml of 0.05 M ammonium acetate

Table 1. Mean specific enzyme activities of *Crotalus scutulatus scutulatus* crude venom at various pH values.

Enzyme	pH / Specific Activity								
Phosphomonoesterase	7.0	7.5	8.0	8.5	9.0	9.5	10.0	10.5	
	1.14	2.44	3.60	5.36	6.83	7.67	6.17	2.37	
Phosphodiesterase	7.0	7.5	7.8	8.5	9.0				
	0.44	1.07	1.6	1.73	1.2				
Phospholipase A	physiological saline								
	200								
Thrombin-like			8.0						
			0.0						
5'-Nucleotidase		7.5	8.0	8.5	9.0	9.5	10.0	10.5	11.0
		0.0	0.19	1.00	1.80	1.30	0.94	0.67	0.03
Proteinase			8.0	8.5	9.0	9.5	10.0	10.5	11.0
			0.035	0.068	0.063	0.034	0.026	0.020	0.013
Hyaluronidase*	hyaluronic acid solution								
	120								
NADase	0.0 at 2 mg/ml								
TAMEase		7.5	8.0	8.5	9.0	9.5			
		688	800	850	576	304			
BAEEase	7.0	7.5	8.0	8.5	9.0	9.5			
	320	1720	2560	2600	2520	2448			
L-amino acid oxidase**			8.0						
			62.0						

* Hyaluronidase activity is expressed as Turbidity Reducing Units/mg.
** L-amino acid oxidase activity is expressed as µl O_2/mg/hr.

buffer (pH 7.0) containing 0.5 M NaCl and 0.5 mM each of $CaCl_2$, $MnCl_2$, and $MgCl_2$. The bound glycoproteins were eluted with 0.05 M ammonium acetate buffer (pH 7.0) containing 0.5 M NaCl and 0.1 M α-methyl-D-mannoside. Fractions were collected using a Buchler Fraction Collector. Eluates of 4.5 ml each were collected at a flow rate of 17 ml/hr. The eluates were stored at −20°C within 2 hrs after collection.

DEAE Sephadex A-50 ion exchange chromatography fractionation was accomplished by the methods of Cheng and Ouyang (1967) and Ouyang *et al.* (1971). The crude venom (425 mg samples) and later the binding protein samples obtained by Con A affinity chromatography were applied to the ion exchange column which had been equilibrated previously with 0.05 M ammonium acetate (pH 8.0). The first stage gradient elution was performed with 800 ml of 0.05 M ammonium acetate (pH 8.0) in the mixing vessel (plastic bottle, 9 cm diameter x 16.5 cm) and 1000 ml of 0.25 M ammonium acetate (pH 6.0) in the reservoir (plastic bottle, 9 cm diameter x 16.5 cm). The second stage gradient elution was performed with 0.25 M ammonium acetate (pH 6.0) in the mixing vessel and 0.9 M ammonium acetate (pH 5.4) in the reservoir. Acetic acid and aqueous ammonia were used to adjust pH. The flow rate from the column was adjusted to 17 ml per hour and an eluate of 3.25 ml per tube was collected by using a Buchler Fraction Collector. These eluates were stored at −20°C within two hrs after collection.

RESULTS AND DISCUSSION

Crude *C. s. scutulatus* venom enzyme activities were influenced by changes in pH. Crude venom phosphomonoesterase, phosphodiesterase, 5'-nucleotidase, BAEEase, TAMEase, proteinase, L-amino acid oxidase, phospholipase A, and hyaluronidase activities were observed. Thrombin-like and NADase activities were not observed in the crude venom (Table 1).

Fractionation of the crude venom with Con A yielded two fractions; Fraction I (F-I) composed of non-binding proteins and Fraction II (F-II) composed of binding glycoproteins (Fig. 1). The bound proteins of F-II were obtained by elution using α-methyl-D-mannoside (Fig. 1). Phosphomonoesterase, phosphodiesterase, 5'-nucleotidase, phospholipase A, L-amino acid oxidase, hyaluronidase, TAMEase, BAEEase, and proteinase enzymes were present in F-II. Neither thrombin-like nor NADase activity was observed in the Con A-binding fraction (Table 2).

Fractionation of the crude venom with DEAE Sephadex A-50 and ammonium acetate buffer at 4°C by two stage elution yielded 12 fractions. Seven fractions eluted during the first stage and five fractions eluted during the second stage (Fig. 2).

After obtaining the above preliminary results, proteinase activity was chosen for more intensive study. A broad distribution of proteinase activity was observed in the crude venom fractions obtained by DEAE Sephadex A-50 chromatography (Fig. 3).

Fraction F-II obtained by Con A affinity chromatography of the crude venom was pooled, lyophilized and desalted by using G-25 Sephadex. After relyophilization, the desalted mixture was applied to a DEAE Sephadex A-50 column. After a two stage fractionation at 4°C using ammonium acetate buffer, the eluates were assayed for proteinase activity. Proteinase activity was found in the first portion of the first elution, while the remaining portion of the fractionation, after tube 75, had lesser activities (Fig. 4).

Concanavalin A-Binding Enzymes of *Crotalus scutulatus scutulatus* Venom

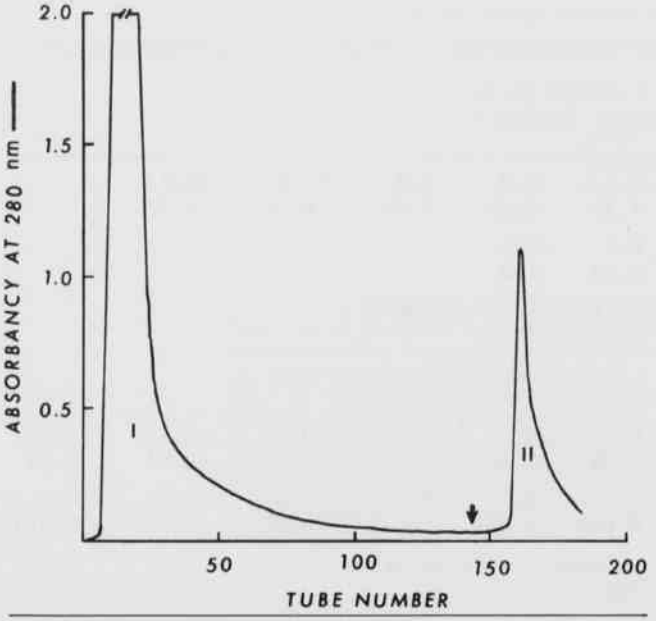

Figure 1. Chromatography of *Crotalus scutulatus scutulatus* crude venom (425 mg) on Concanavalin A Sepharose 4-B column (2.5 x 15 cm) by two stage elution. The arrow indicates the start of the second stage elution (using α-methyl-D-mannoside).

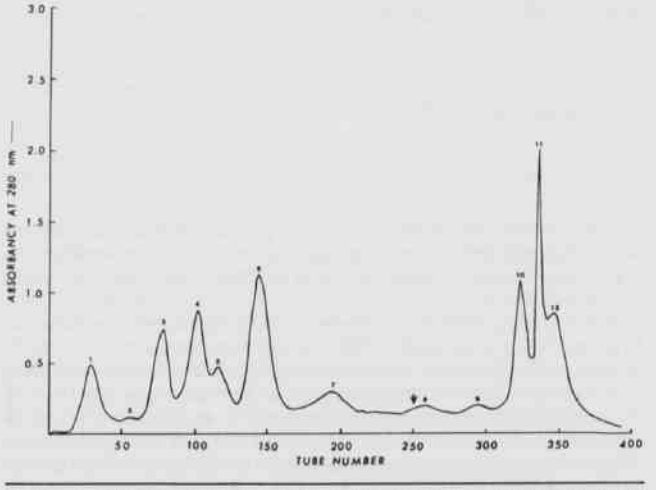

Figure 2. Chromatography of *Crotalus scutulatus scutulatus* crude venom (425 mg) on DEAE Sephadex A-50 column (2.5 x 60 cm) at 4 °C by two stage gradient elution with ammonium acetate buffer. The arrow indicates the start of the second stage elution.

Table 2. Mean specific activities of Concanavalin A-binding enzymes of *Crotalus scutulatus scutulatus* venom*.

Fraction II Enzymes	Specific Activities
Phosphomonesterase	49.7
Phosphodiesterase	26.2
Phospholipase A	500
Thrombin-like	0.0
5"-Nucleotidase	8.7
Proteinase	0.23
Hyaluronidase**	25
NADase	0.0
TAMEase	710
BAEEase	2000
L-amino acid oxidase***	327

* All enzyme assays were performed at the optimum pH obtained by using the crude venom.
** Hyaluronidase activity is expressed as Turbidity Reducing Units/mg
*** L-amino acid oxidase is expressed as µl/hr/mg.

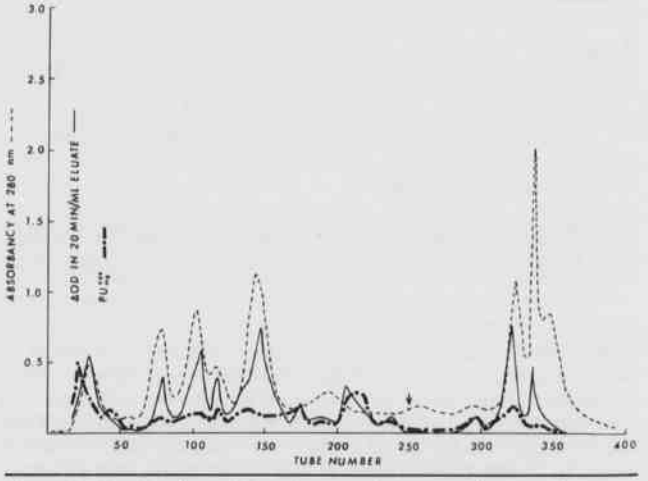

Figure 3. Distribution of *Crotalus scutulatus scutulatus* crude venom proteinase activity in the eluates obtained by DEAE Sephadex A-50 ion exchange chromatography. The arrow indicates the start of the second stage elution. --- indicates protein content estimated by absorbancy at 280 nm. Rates of substrate hydrolysis in 20 min/ml eluate as indicated by change in optical density (ΔOD) in 20 min is shown by —. Specific activity expressed as proteinase units hydrolyzed per mg of venom ($PU^{c\ as}_{mg}$) is indicated by —•—.

Phospholipase A_2, 5′-nucleotidase, phosphodiesterase, deoxyribonuclease, ribonuclease, adenosine triphosphatase, phosphomonoesterase, NADase, exopeptidase, hyaluronidase, and L-amino acid oxidase are present in snake venoms. Crotalidae venoms are especially noted for their proteinases and L-arginine-ester hydrolases (Jiménez-Porras, 1970; Suzuki, 1966; Tu *et al.*, 1965; Friederich and Tu, 1971). Isozymes of the above enzymes have been purified from several venoms (Doery and Pearson, 1961; Kawauchi *et al.*, 1971; Wells and Hanahan, 1969; Braganca and Sambray, 1967; Saito and Hanahan, 1962; Jiménez-Porras, 1970; Shiloah *et al.*, 1973; Maeno and Mitsuhashi, 1961; Sato *et al.*, 1965; Delpierre *et al.*, 1973; Deutsch and Diniz, 1955; Pfleiderer and Sumyk, 1961; Murata *et al.*, 1963; Maeno *et al.*, 1959; Shaham *et al.*, 1973; Suzuki, 1966; Sifford and Johnson, 1978). Five hemorrhagic toxins with proteolytic activity were isolated from *C. atrox* venom (Bjarnason and Tu, 1978). Thus, multiple proteolytic enzymes are apparently quite common in rattlesnake venoms. The results presented herein substantiate this in that the caseinolytic activities were present in the DEAE A-50 fractions of the *C. s. scutulatus* crude venom and the Con A-binding fraction.

Denson *et al.* (1971) reported the absence of thrombin-like activity in *C. s. scutulatus* venom. In our study using *C. s. scutulatus* venom,

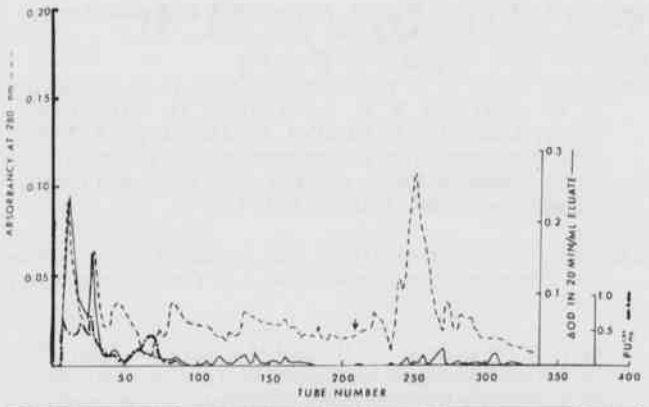

Figure 4. Distribution of proteinase activity in the eluates from DEAE Sephadex A-50 ion exchange chromatography of Fraction II from Concanavalin A-Sepharose 4B affinity chromatography of *Crotalus scutulatus scutulatus* crude venom. The ion exchange chromatography was performed on a column 2.5 x 27 cm at 4 °C by two stage elution. The arrow indicates the start of the second stage elution. Protein content estimated by absorbancy at 280 nm is shown by ---. Rates of substrate hydrolysis in 20 min per ml eluate measured by change in optical density (ΔOD) are indicated by —. Specific activities of Fraction II proteinase ($PU^{c\ at}_{mg}$) are indicated by —•—.

the absence of thrombin-like activity is also reported.

Since a synthetic substrate (bis-p-nitrophenyl phosphate sodium salt) was used for the assay of phosphodiesterase, it was not possible to differentiate whether the enzyme is exonuclease or endonuclease.

NADase activity was not observed in *C. s. scutulatus* venom at a concentration of 2.0 mg/ml. Tatsuki *et al.* (1975) reported, with few exceptions, only very weak NADase activity in the venoms of the genera *Crotalus, Bothrops, Bitis, Vipera,* and *Trimeresurus.* No NADase activity was found for several members of the genus *Crotalus* including *C. adamanteus, C. atrox, C. durissus, C. viridis viridis* and *C. basiliscus.*

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