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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 phosphohydrolase EC 3.1.1.4), hyaluronidase (hyaluronate glycanohydrolase EC 3.2.1.1), N-benzoyl-L-arginine ethyl esterase, p-toluene sulfonfyl-L-arginine methyl esterase, L-amino acid oxidase (L-amino acid: O2 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 (Pattabhiraman 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 venom, the lethal components are peptides and nonenzymatic proteins (Dubnoff and Russell, 1971; Bonilla and Fiero, 1971; Russell et al., 1976). To substantiate this further, Pattabhiraman 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. Cizette and Bieber (1978) reconstituted Mojave toxin from an acidic subunit and a basic subunit. The basic subunit had phospholipase A, activity. Castillon et al. (1981) and Ho and Lee (1981) reported that Mojave toxin, like other snake toxins having phospholipase A, 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-toluene sulfonfyl-L-arginine methyl ester (TAME), 5'-adenylic acid, bis-p-nitrophenyl phosphatase sodium salt, beef plasma thrombin, β-NAD+, bovine fibrinogen (F-4000), and bovine albumin Fraction V were purchased from Sigma Chemical Company; diiodium p-nitrophenyl phosphate from Nutritional Biochemicals Corporation; Tris-hydroxymethylaminomethane, glycine, ammonium molybdate, hydroquinone, sodium sulfate, 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 Mallinkrodt Chemical Works; casein from ICN Pharmaceuticals, Inc.; Sephadex G-25, Concanavalin A-Sepharose 4B (Con A), DEAE A-50, and columns from Pharmacia, Uppsala, 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 Suzuki 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 ester (BAEEase) and p-toluene sulfonfyl-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 (Palk and Kim, 1965) assay procedures included the minor modifications used in a previous work (Siford 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 Seasson (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
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. scutulatus* venom enzyme activities were influenced by changes in pH. Crude venom phosphomonoesterase, phosphodiesterase, phospholipase A, thrombin-like, and NADase activities were observed. The phosphomonoesterase and phosphodiesterase activities were not observed in the crude venom (Table 1).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>pH</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphomonoesterase</td>
<td>7.0</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>1.14</td>
<td>2.44</td>
</tr>
<tr>
<td>Phosphodiesterase</td>
<td>7.0</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>0.44</td>
<td>1.07</td>
</tr>
<tr>
<td>Phospholipase A</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>physiological saline</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
</tr>
<tr>
<td>Thrombin-like</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>7.5</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
<td>0.19</td>
</tr>
<tr>
<td>Proteinase</td>
<td>8.0</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>0.035</td>
<td>0.068</td>
</tr>
<tr>
<td>Hyaluronidase*</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.0</td>
<td>0.013</td>
</tr>
<tr>
<td>NaDase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0 at 2 mg/ml</td>
</tr>
<tr>
<td>TAMEase</td>
<td>7.5</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>688</td>
<td>800</td>
</tr>
<tr>
<td>BAEEase</td>
<td>7.0</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>320</td>
<td>1720</td>
</tr>
<tr>
<td>L-amino acid oxidase**</td>
<td>8.0</td>
<td>62.0</td>
</tr>
</tbody>
</table>

* Hyaluronidase activity is expressed as Turbidity Reducing Units/mg.

** L-amino acid oxidase activity is expressed as µl O$_2$/mg/hr.

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

<table>
<thead>
<tr>
<th>Fraction II Enzymes</th>
<th>Specific Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphomonoesterase</td>
<td>49.7</td>
</tr>
<tr>
<td>Phosphodiesterase</td>
<td>26.2</td>
</tr>
<tr>
<td>Phospholipase A</td>
<td>500</td>
</tr>
<tr>
<td>Thrombin-like</td>
<td>0.0</td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>8.7</td>
</tr>
<tr>
<td>Proteinase</td>
<td>0.23</td>
</tr>
<tr>
<td>Hyaluronidase**</td>
<td>25</td>
</tr>
<tr>
<td>NADase</td>
<td>6.0</td>
</tr>
<tr>
<td>TAMEase</td>
<td>710</td>
</tr>
<tr>
<td>BAEEase</td>
<td>2000</td>
</tr>
<tr>
<td>L-amino acid oxidase***</td>
<td>327</td>
</tr>
</tbody>
</table>

*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 µmol/hr/mg.

Phospholipase A₂, 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 (Jimenez-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; Jimenez-Porras, 1970; Shiloah et al., 1973; Maeno and Mitsuhashi, 1961; Saito et al., 1965; Delpierre et al., 1973; Deutsch and Diniz, 1955; Pfeiderer 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,
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, Viper, 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.

ACKNOWLEDGEMENTS

We thank Drs. Anthony T. Tu and M. Laskowski, Sr. for their suggestions after reading the manuscript. Thanks are also due to Mrs. Alice Chandler for typing the manuscript.

LITERATURE CITED


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