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Abstract

Thrombin-like activities were not observed in *Crotalus basiliscus, C. molossus* and *C. scutulatus scutulatus* crude venoms. 5′-Nucleotidase specific activities of 0.863, 0.273 and 5.520 units/mg of crude venom protein were observed in *C. basiliscus, C. molossus* and *C. s. scutulatus* venoms, respectively. Concanavalin A Sepharose 4 B (Con A) affinity chromatography yielded two fractions from each of the crude venoms. In each instance, both fractions exhibited 5′-nucleotidase activities and the Con A-binding proteins had higher activities than the Con A-nonbinding proteins. 5′-Nucleotidase activities in the DEAE Sephadex A-50 chromatographic fractions were localized in the first elution fraction and the last fraction(s) to elute. EDTA had no effect on the 5′-nucleotidase activities of the crude venoms.

Introduction

Crotalus basiliscus (Cope), the Mexican west-coast rattlesnake is one of the world's largest rattlesnakes. Specimens exceeding 150 cm are common, and the maximum size reported is a little over 200 cm. The range of this species extends from the Rio del Fuerte drainage in extreme southern Sonora, southward along the plains, foothills, and valleys of Sinalo, Nayarit, Jalisco, Colima, and northwestern Michoacan, including the middle Rio Tapalcatepec Valley. The ranges of *C. basiliscus* and *C. molossus* overlap and they freely hybridize where they come in contact (Campbell and Lamar, 1989).

C. molossus, the blacktail rattlesnake, is one of the most beautiful rattlesnakes. Its maximum body length is 126 cm. The darkest individuals are usually from habitats featuring dark substrates. *C. molossus* is found from central and west-central Texas northwest through the southern half of New Mexico to northern and extreme western Arizona southward to the southern edge of Mexican Plateau and Mesa del sur, Ocaca. It also occurs on the Tiburon and San Esteban islands in The Gulf of California (Ernst, 1992).

C. scutulatus, the Mojave rattlesnake, has a maximum length of 129 cm (Ernst, 1992). This crotalid species is probably the most dangerous rattlesnake, as its venom is neurotoxic (Minton, 1956; Johnson et al., 1966). *C. scutulatus* is found from southwestern Washington county, Utah, Lincoln and Clark counties, Nevada, and Kern, Los Angeles, and San Bernardino counties in California, southeast through Arizona and southward from Transpeces, Texas, and southwestern Hildalgo and Otero counties in New Mexico, to the southern edge of the Mexican Plateau in Puebla and adjacent Vera Cruz (Campbell and Lamar, 1989).

Crotalid venoms are very complex with the majority of the venom consisting of numerous protein components having enzymatic activities. The concentrations of proteins, and presumably of different toxins, vary between individual snakes (Johnson et al., 1968). At least 18 different enzymatic activities have been identified in crotalid venoms (Iwanaga and Suzuki, 1979). Among these, there are at least four enzymes involved in the hydrolysis of phosphate bonds. These include endonuclease, alkaline nonspecific phosphatase, 5′-nucleotidase, and phosphodiesterase (Tu, 1977). A specific phosphomonoesterase, 5′-nucleotidase, specifically hydrolyzes phosphate monoester which links at the 5′position of DNA and RNA. Upon dialysis, crude venom 5′-nucleotidase activities increased two or three times that of the nondialyzed samples, suggesting that some substance, probably zinc ions which are abundant in crude venoms, inhibits the activity (Suzuki and Iwanaga, 1958).

Many venoms exert profound effects on the blood coagulation system. Some accelerate the coagulation process and others retard it. Thus snake venoms are often divided into two types, coagulant and anticoagulant. Some venoms contain both coagulant and anticoagulant factors simultaneously, and sometimes a venom becomes coagulant or anticoagulant depending on the concentration used (Tu, 1977). Also, various mechanisms can induce procoagulation or anticoagulation. A venom may act as a coagulant for the following reasons: it has thromboplastin-like activity, it contains a factor X activator, it activates factor V, it activates prothrombin, or it has a thrombin-like activity. A venom may also act as an antico-

agulant for various reasons: it has fibrinolytic activity, it can activate plasminogen to release plasmin, it has antithrombic action, and it has inhibitory or destructive action toward any of the blood coagulation factors preceding thrombin (Tu, 1977).

The purpose of this work was to determine the distribution of 5′-nucleotidase and thrombin-like activities in the Concanavalin A Sepharose 4B (Con A) affinity chromatographic fractions and the DEAE Sephadex A-50 ion exchange chromatographic fractions of *C. basiliscus, C. molossus* and *C. s. scutulatus* crude venoms. This work is a continuation of previous works centered on phosphodiesterase (Beasley et al., 1993) and proteinase, N-benzoyl-L-arginine ethyl esterase and p-tosyl-L-arginine methyl esterase (Stegall et al., 1994).

Materials and Methods

Lyophilized *C. molossus* and *C. basiliscus* venoms were provided by Dr. H.L. Stahnke of Arizona State University. *C. s. scutulatus* venoms, adenosine 5′-monophosphate, fibrinogen, and thrombin were purchased from Sigma Chemical Company. Ammonium molybdate, hydroquinone, and sodium sulfite were purchased from Fisher Scientific Company. Magnesium sulfate, sulfuric acid, and tris (hydroxymethyl) aminomethane (TRIS) were purchased from Mallinckrodt Chemical Works. Glycine was purchased from Matheson, Coleman and Bell. Sodium sulfate was purchased from J.T. Baker Chemical Company. Sephadex G-25, Concanavalin A-Sepharose 4B (Con A), DEAE Sephadex A-50, and columns were purchased from Pharmacia, Uppsala 1, Sweden.

Fractionations using Con A gel were performed using 425 mg samples of whole venom on columns which had been equilibrated with 0.05 M ammonium acetate buffer (pH 7.0) (Iscove et al., 1974; Aspberg and Porath, 1970; Hinson et al., 1985=5). The Con A-nonbinding proteins (FI) were eluted at 4 °C with 500 mL of 0.05 M ammonium acetate (pH 7.0) containing 0.5 M NaCl. The Con A-binding proteins (FII) were eluted at 4 °C with 500 mL of 0.05 M ammonium acetate buffer containing 0.5 M NaCl and 0.25 M α- methyl-D-mannoside. Eluates of 110 drops (~4 mL) per tube were collected and stored at -12 °C within 2 hrs after collection (Beasley et al., 1993 and Stegall et al., 1994).

DEAE Sephadex A-50 ion exchange chromatography was performed on 400 mg samples of whole venom dissolved in 0.05 M ammonium acetate buffer (pH 8.0) (Cheng and Ouyang, 1967; Ouyang et al., 1971; Sifford and Johnson, 1978; Hintson et al., 1985). Two stage gradient elutions were performed on each venom using ammonium acetate buffers. The first stage gradient elution was performed with 250 mL of 0.05 M ammonium acetate (pH 8.0) in the mixing vessel and 310 mL of 0.9 M ammonium acetate (pH 6.0) in the reservoir. The second stage gradient elution was performed with 240 mL of 0.3 M ammonium acetate (pH 6.0) in the mixing vessel and 310 mL of 0.9 M ammonium acetate (pH 5.4) in the reservoir. The column (2.5 cm X 56 cm) was maintained at 4 °C and eluates were collected in 5 mL fractions. All fractions were stored at -12 °C within 2 hrs of collection (Beasley et al., 1993).

For protein concentration estimations and 5′-nucleotidase assays, a Beckman DB spectrophotometer and a Spectronic 1201 were used. Protein concentrations of the fractions were estimated from their absorbances at 280 nm. An absorbance at 280 nm of 1.0 for 1.0 mg of venom per mL was used (Sifford and Johnson, 1978).

5′-Nucleotidase activities were determined by the method of Ging (1956), Lo et al. (1966), Sifford and Johnson (1978) and Hinson et al. (1985). The factor (0.250) used by Ging (1956) and Lo et al. (1966) for converting 5′-nucleotidase activities which were measured at 37 °C to values at 25 °C was used. Thrombin-like activities were determined by the method of Sato et al. (1965) with the minor modification used in the works by Sifford and Johnson (1978) and Hinson et al. (1985).

Metal requirements for each enzyme were investigated by incubating 0.3 mL of venom or venom fraction with an equal volume of ethylenediaminetetraacetic acid (EDTA) solution for 10 minutes at 37 °C (Friederich and Tu, 1971; Goucher and Flowers, 1964; Stegall et al., 1994). This mixture was diluted with water to yield a final concentration of 0.6 m EDTA in a total volume of 1.0 mL. Aliquots of this mixture were assayed for 5′-nucleotidase with control samples having water in place of EDTA.

Results and Discussion

Thrombin-like activities were not observed in *C. basiliscus, C. molossus,* and *C. s. scutulatus* crude venoms. Thus, subsequent assays for thrombin-like activities were not performed on the venom fractions obtained by affinity and ion exchange chromatography. Thrombin-like activities have been reported in other crotalid venoms, e.g., *Agkistrodon acutus, Trimeresurus gramineus, T. okinavensis, T. flavoriridis, C. horridus horridus,* and *C. adamanteus* (Tu, 1977). Markland and Damus (1971) have extensively studied the chemical properties of purified *C. adamanteus* venom thrombin-like enzyme.

Con A fractionation yielded two fractions for each of the whole venoms: Fraction I (FI) was composed of Con A-nonbinding proteins and Fraction II (FII) was composed of Con A-binding proteins. In each instance, the crude venom 5′-nucleotidase activities were higher than the activities in the corresponding FI and FII proteins

(Table 1).

Table 1. 5′-Nucleotidase specific activities of selected crotalid venoms.

Venom	Activity*
Crotalus basiliscus:	
Crude Venom	0.863
Concanavalin A-nonbinding proteins (F1)	0.265
Concanavalin A-binding proteins (F11)	0.740
Crotalus molossus	
Crude venom	0.273
Concanavalin A-nonbinding proteins (F1)	0.024
Concanavalin A-binding proteins (F11)	0.036
Crotalus scutulatus scutulatus	
Crude venom	5.520
Concanavalin A-nonbinding proteins (F1)	0.131
Concanavalin A-binding proteins (F11)	1.010

*5′-Nucleotidase activities are reported as mean µmoles substrate hydrolyzed/min/mg venom

DEAE Sephadex A-50 chromatography of *C. basiliscus, C. molossus,* and *C. s. scutulatus* venoms yielded several fractions from each of the whole venoms. 5′-Nucleotidase activities were localized, for the most part, in the first fraction obtained during the first stage elution and in the last few fractions obtained during the second stage elution. The highest specific activities occurred in the first fraction (around tube 20) to elute (Figs. 1-3). These results were comparable to distributions of phosphodiesterase and 5′-nucleotidase activities in the DEAE Sephadex A-50 fractions of *A. bilineatus* venom in which these enzymes eluted in the first fraction (Sifford and Johnson, 1978). *C. basiliscus* and *C. s. scutulatus* venoms yielded only two peaks of activity for 5′-nucleotidase. *C. molossus* venom's 5′-nucleotidase activity was much lower than the activities observed in *C. basiliscus* and *C. s. scutulatus* venoms.

The peak activities for 5′-nucleotidase observed in the DEAE Sephadex A-50 fractions were less than the activities in the crude venoms, suggesting that this enzyme is heat labile or that a necessary activator might have been removed. The mean 5′-nucleotidase activity for *C. s. scutulatus* crude venom in this work was somewhat higher than the activities reported by Hinson et al. (1985) and Childs et al. (1986). The 5′-nucleotidase activities in our Con A fractions, however, were lower than those reported by either Hinson et al. (1985) or Childs et al. (1986).

EDTA had no effect on the 5′-nucleotidase activities of the crude venoms. Thus, no EDTA assays were performed on the venom fractions.

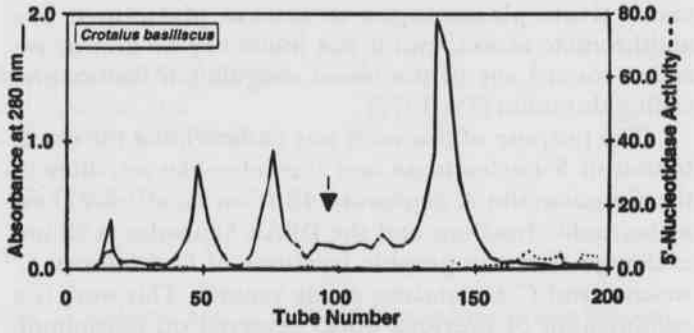

Fig. 1. 5′-Nucleotidase activities in the eluates from DEAE Sephadex A-50 column chromatography of whole *Crotalus basiliscus* venom. The arrow indicates the start of the second stage elution.

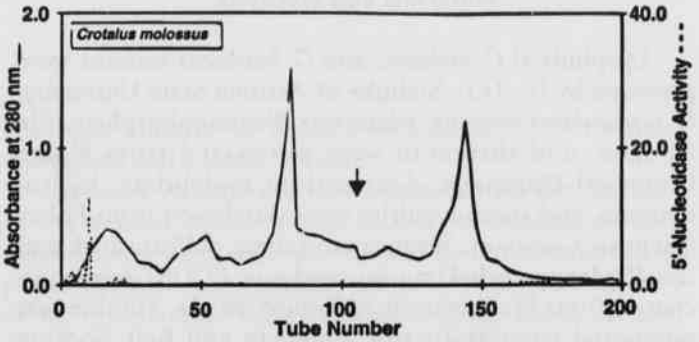

Fig. 2. 5′-Nucleotidase activities in the eluates from DEAE Sephadex A-50 column chromatography of whole *Crotalus molossus* venom. The arrow indicates the start of the second stage elution.

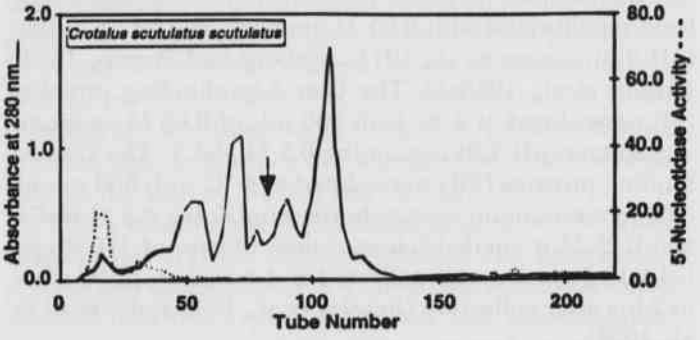

Fig. 3. 5′-Nucleotidase activities in the eluates for DEAE Sephadex A-50 column chromatography of whole *Crotalus scutulatus scutulatus* venom. The arrow indicates the start of the second stage elution.

Additional purification will be necessary to determine whether the 5′-nucleotidase activities observed in the DEAE Sephadex A-50 fractions were the result of one

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enzyme or several. This question was also raised by Stegall et al. (1994) in their work with proteinases and esterases in *C. basiliscus, C. molossus,* and *C. s. scutulatus* venoms.

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