ISOLATION AND PARTIAL CHARACTERIZATION OF TWO MUSCLE NECROTIC TOXINS FROM THE VENOM OF THE CENTRAL AMERICAN RATTLESNAKE, *CROTALUS DURISSUS DURISSUS*

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NOMENCLATURE

A_{280} - Absorbance at 280 nm

BAEE - N-benzoyl-L-arginine ethyl ester

BAPNA - N-benzoyl-DL-arginine-p-nitroanilide

CMS - carboxymethyl Sephadex

disc - discontinuous

EDTA - ethylene diaminotetraacetic acid

hr - hour(s)

i.m. - intramuscular injection

i.p. - intraperitoneal injection

i.v. - intravenous injection

kdal - kilodalton

l - liter

LD_{50} - lethal dose for 50 percent of treated animals

mA - milliampere

min - minute(s)

ml - milliliter

nm - nanometer

PAGE - polyacrylamide gel electrophoresis

pI - isoelectric point

PSS - physiological saline

SDS - sodium dodecylsulfate

sec - second(s)

TAME - p-toluene-sulfonyl-L-arginine methyl ester

TEMED - N,N,N',N',tetramethylethylenediamine

TRIS - tris(hydroxymethyl)-amino methane

μg - microgram

μl - microliter

CHAPTER I

INTRODUCTION

Myonecrosis as the result of rattlesnake bite is a well documented clinical occurrence (1-3). Although most victims of snakebite survive, such massive local tissue damage can be induced that in a high percentage of cases the result is either dysfunction or loss of a finger, hand, or other limb. Serious tissue destruction can result from the bite of even the youngest snakes (4). Even the treatment of the victim with antivenin does not prevent necrosis in most cases (5). Numerous assays for myonecrosis have been performed on the venom of various species of rattlesnakes. In every study reported, myonecrosis was induced in laboratory animals injected with the venom of every species investigated (6-10). Despite the clinical consequences and the obvious widespread occurrence of myonecrotic components in rattlesnake venoms, until recently little work has been done to isolate and characterize these muscle damaging proteins of rattlesnake venoms.

The first isolation of a myonecrotic toxin from a rattlesnake venom was reported in 1977 by Cameron and Tu (11). The protein, named myotoxin _a_, was purified from the venom of the North American prairie rattlesnake, _Crotalus viridis viridis_. With a molecular weight of 4621 daltons, myotoxin _a_ is composed of 42 amino acid residues, six of these half cystines, forming the molecule's three disulfide bridges (12). The combination of arginine, lysine, and

histidine accounts for 13 of the residues, with a correspondingly basic isoelectric point of 9.6 demonstrated by isoelectric focusing. The secondary structure of myotoxin a as determined by circular dichroism and raman spectroscopy, shows significant amounts of β-sheet and β-turn structures with small areas of random coil and/or α-helix (13).

The pathenogenesis of myotoxin a has been investigated by Stringer et al. (14) and more recently by Ownby et al. (15). Tissues from animals injected with 1.5 μg/g body weight of the pure toxin intramuscularly shows partial vacuolization of muscle cells at 6, 12, and 24 hours and complete vacuolization and loss of striations at 48 and 72 hours by light microscopy. No evidence of hemorrhage, hemolysis, or damage to connective tissue cells was found. Electron microscopy showed dialation of the sacroplasmic reticulium and the perinuclear space as the cause of the vacuolization. Other ultrastructural features of the affected cells were disintegrated by myofibrils and extreme distortion of mitochondria. It was concluded that the primary site of action was on the sarcoplasmic reticulum either directly or indirectly through the sacrolemma. It was proposed that myotoxin a might affect transport of ions by inhibiting the Na^+/K^+ ATPase.

Ownby et al. (16) has reported the production of an antibody to myotoxin a from rabbits. The antiserum was reacted with myotoxin a in agar-gel double-diffusion plates, and one precipitin line was formed. It was also shown that commercial crotalidae antivenin contained no myotoxin antibodies. This verifies clinical observations that the polyvalent crotalidae antivenin in use is ineffective in

detering myonecrosis (4). In a later study (17), it was shown that the antimyotoxin serum neutralized myotoxin a when mixed with it prior to intramuscular injection up to a dose of 0.75 μg/g.

Another myonecrotic toxin, crotamine, has been isolated from the venom of Crotalus durissus terrificus, the South American rattlesnake. The protein was first reported in 1947 (18), and was described as a neurotoxin (19). In 1971, however, it was shown that crotamine caused depolarization of muscle cell membranes (20). In 1978, Cameron and Tu (21) demonstrated that the toxin caused vacuolization of muscle cells qualitatively similar to that of myotoxin a on the light microscope level. Chemically, crotamine is strikingly similar to myotoxin a. It is also composed of 42 amino acids, possesses three disulfide bonds, and shows an isoelectric point of > 9.0. In fact, amino acid sequence studies shows only three amino acid residue differences between the primary structure of crotamine and myotoxin a . (12, 22).

In 1981, Pool et al. (23) reported the isolation of two myotoxins from the venom of Crotalus viridis concolor, the midget faded rattlesnake of North America. Amino acid analysis indicates 39 amino acid residues for myotoxin I and 35 residues for myotoxin II. Both proteins exhibit high contents of basic amino acids and isoelectric points near 10. Mice injected with either protein exhibited immediate symptomology including extreme tonic hypertension of the hind limbs and labored breathing. The toxins also demonstrate myonecrosis similar to myotoxin a and crotamine.

Russell et al. (8) has found a myonecrotic toxin in the venom of Crotalus viridis helleri. The peptide is composed of 43 amino acid

residues. Highly significant characteristics it displays are six half-cystine residues, a high lysine content, and an NH_2-terminal tyrosine residue. Only five amino acid substitutions plus an additional COOH-terminal residue distinguish it from crotamine from Crotalus durissus terrificus.

Evidence of myotoxins have been shown in two Venezualan rattlesnake species by Rodriguez and Scannone. Crotalus durissus cumanensis (9) contains a highly basic peptide of approximately 5200 daltons. Mice injected with the toxin show the characteristic spastic paralysis of a myonecrotic toxin. The venom of Crotalus vegrandis also shows evidence of a low molecular weight basic toxin, which shows symptomology identical with that of known myonecrotic toxins (10).

Recently, a second myonecrotic component was isolated from the venom of Crotalus viridis viridis (24). Named viriditoxin, its structure varies radically from the rattlesnake myotoxins discussed earlier. It is comprised of approximately 1018 amino acid residues, showing a molecular weight of 115,000. The protein has an isoelectric point of 4.6, confirming the abundance of acidic amino acid residues. The toxin induces myonecrosis similar to myotoxin a under the light microscope, showing loss of muscle fiber striations and severe vacuolization of muscle cells. In addition, viriditoxin also produces hemorrhage following subcutaneous injections, with a minimum hemorrhagic dose of 1.5 μg in 20 gram mice.

It is the purpose of the present study to isolate and characterize the myonecrotic component from the venom of the Central American rattlesnake, Crotalus durissus durissus.

CHAPTER II

LITERATURE REVIEW

The Central American rattlesnake, *Crotalus durissus durissus*, or "La Cascabella" as it is known locally, was first described by Linnaeus in 1758. It is one of nine rattlesnakes of the *durissus* group, a subdivision of the genus *Crotalus*. The *durissus* species, one of six in the durissus group, is the only species of rattlesnake, either of the genus *Crotalus* or *Sistrusus*, that extends south of the southern Mexican states. *Durissus durissus* is thus the only rattlesnake found in Central America, with the other member of the species, *durissus terrificus*, the South American rattlesnake, occurring no further north than Panama. The distribution of *durissus durissus* has been defined as occurring from southern Mexico, in the area of Oaxaca and Vericruz, to the more arid regions of Central America through Guatemala, Salvadore, Honduras, and the west coasts of Nicaragua and Costa Rica (25).

The venom of *C. d. durissus* has been subjected to numerous biochemical and physiological assays by various investigators.

In a study of 17 snake venoms, Friederich and Tu (26) studied the role of metals in hemorrhagic, esterase, and proteolytic activities. It was found that *C. d. durissus* venom contained calcium, zinc, magnesium, sodium, and potassium, as did the other rattlesnake venoms tested. The venom showed a lack of copper, manganese, and iron,

metals present in Viperdae, Elapidae and some Crotalidae venoms, but lacking in the Crotalus genus. When crude venom was dialyzed in EDTA treated dialysis tubing against distilled water, hemorrhagic activity ceased. Addition of manganese restored 15% activity of the untreated venom, zinc restored 4% activity, and added calcium had no effect. Enzymatic activity of protease was measured with casein as substrate; TAME and BAEE were used in determination of esterase activity. EDTA treatment of the crude venom had no effect on esterase activity, while proteolytic activity fell to a fraction of non EDTA-treated activity. Addition of calcium, zinc, or magnesium had little effect on the restoration of activity. No LD_{50} was found for the treated or untreated venom.

In a related study, Kocholaty et al. (27) investigated enzymatic activities and toxicities of venoms from various species in the Elapidae, Viperidae, and Crotalidae families. C. d. durissus venom showed a 75% protein content by the Biuret method. Toxicity levels were found to be 667 and 1244 μg/kg for injections by the i.p. and i.v. routes, respectively. Levels of amino acid oxidase and phosphodiesterase activities were found to be close with that reported for other venoms of the Crotalidae family. However, phospholipase A and proteinase (casein) levels were lower than that of the majority of the crotalids. Marked trypsin activity was found, using BAPNA and TAME as substrates.

In a study by Bolaños (28), it was again shown that injection of the crude venom by the i.p. route to be two to three times as toxic as was via i.v. injection, an inconsistency when compared to similar experiments with other venoms, showing a lower LD_{50} for intravenous

injections. The study also showed the average venom yield per speciman for Crotalus durissus durissus to be 76.8 mg.

Denson et al. (29) has characterized the coagulant activity of a number of snake venoms. The purpose of the study was to classify the activity into one of four general groups: (1) Activation of factor X of blood coagulation reactions; (2) Conversion of prothrombin to thrombin in the presence of factor V; (3) Conversion of prothrombin to thrombin in the absence of factor V; and (4) The direct conversion of fibrinogen to fibrin, characteristic of Crotalus durissus terrificus venom. Crotalus durissus durissus venom showed the ability to clot citrated fresh human plasma at 3, 6, and 9 minutes. It also showed fibrinolytic activity, but no esterolytic activity. The clotting time of bovine fibrinogen increased as a function of crude venom dilution, thus indicating the ability of the venom to convert fibrinogen to fibrin (thrombin-like activity). The venom did fail to show the ability to activate factor X or activate prothrombin, regardless of the absence or presence of factor V.

Kocholaty et al. (30) did a study on the immunogenic response of Crotalus durissus durissus venom following photooxidative detoxification. Non-photooxidized venom showed proteolytic, esterolytic, and phospholipase activities, as found in other studies. The LD_{50} values were 1.20 g/kg for i.v. injection, and 0.67 g/kg for interperitoneal injection, as shown in other studies. Crude venom was then photooxidized under visible light in a respirometer in the presence of methylene blue. It was found that the three enzymatic activities decreased and the LD_{50} dosage increased as a function of

exposure to the procedure. Rabbits were immunized with photooxidized venom, and the resulting gamma-globulins isolated. It was found that the gamma-globulins gave a high degree of protection to mice injected with crude venom.

In a study by Sosa et al. (31), it was found that Crotalus durissus durissus venom does not contain a direct hemolytic factor, an agent which by itself can hemolyze a red blood cell. Results did show the presence of an indirect lytic factor, an activity which can be stimulated by the presence of lysophosphatidic derivatives. This phenomena has been identified as phospholipase activity. However, the investigators concluded that there was an indication that other agents might be involved in the hemolytic process.

The presence of myonecrotic component(s) in Crotalus durissus durissus venom has been documented. Homma and Tu (6) have studied the morphology of local tissue damage created by the venoms of 37 snakes, nine of them rattlesnakes, in laboratory mice. It was demonstrated that C. d. durissus venom caused a 17% swelling of the injected leg. There was also evidence of hemorrhage near the sight of injection. Muscle necrosis was divided into three groups: (1) coagulative necrosis; (2) myolysis; and (3) mixed type, showing evidence of both coagulative necrosis and myolysis. Crotalus durissus durissus induced the mixed type of necrosis, as did the venom of Crotalus durissus terrificus. Arterial lesions were also induced, which showed several prominent histological changes: (1) disintegration of the endothelial cells; (2) leucocytic infiltration beneath the endothelium; (3) necrosis and disappearance of the smooth muscle fibers in the media, (4) hemorrhage and insudation of a fibrin-like

substance into the subendothelial and medial layer, and (5) formation of mural thrombi.

Myonecrotic action in the venom of *Crotalus durissus durissus* has also been reported by Gutierrez et al. (32). The study also investigated proteolytic and hemorrhagic activities, both of which were evident in *C. d. durissus* venom. It was also found that the crude venom exhibited an i.v. LD_{50} of 31.2 ± 3.4 µg.

Bolaños et al. (33) has studied 149 snakebite cases from the Pacific region of Costa Rica. It was found that 14% of these could be attributed to *Crotalus durissus durissus*. No neurological signs, alterations in the blood pressure or renal complications were found in even the most severe cases; however, the victims did show symptoms of pain, edema, local temperature, mild bleeding, and local necrosis and alteration of the blood coagulation test.

CHAPTER III

EXPERIMENTAL PROCEDURES

A. Materials

Crude, lyophilized _Crotalus durissus durissus_ venom was obtained from the Miami Serpentarium Laboratories, Miami, Fl., as was crude, lyophilized _Crotalus viridis viridis_ venom from which myotoxin _a_ was purified. Sephadex G-50 and G-75 gel filtration gels, carboxymethyl Sephadex C-25 cation exchange gel, protein standards, polyacrylamide monomer, and benzoylated dialysis tubing were obtained from Sigma Chemical Co., St. Louis, Mo. TRIS and KCl were obtained from Fisher Scientific Co., Chemical Manufacturing Division, Fair Lawn, N.J. Gel filtration columns and associated equipment were obtained from Glenco Scientific, Inc., Houston, Tx., and constructed in the OSU glass shop.

B. Sephadex G-75 Gel Filtration

Initial fractionation of crude _Crotalus durissus durissus_ venom was performed using a Sephadex G-75 gel filtration column. Aliquots of 200 mg of the venom were dissolved in 1 ml of an elution buffer consisting of 0.05 M TRIS at pH 10 and 0.1 M KCl. This was loaded onto a Sephadex G-75 gel filtration column (1.5 x 110 cm), equilibrated with the elution buffer. All fractionation steps were performed at 2-4°C in a walk-in cold room. The column was developed at a flow rate of 4.0 ml/hr and 2.5 ml fractions were collected on an Instrumentation

Specialties Co. Model 1200 fraction collector. Absorbance of each tube was measured at 280 nm on a Perkin-Elmer Coleman 101 spectrophotometer. Appropriate tubes for each fraction were pooled and lyophilized. The fractions were then placed in benzoylated dialysis bags (molecular weight limit of 2000 daltons) and desalted by dialysis against distilled water under stirring for 24 hrs. These fractions were lyophilized, weighed, and stored at -20°C.

C. Cation Exchange Chromatography

G-75 fractions 3 and 4 were further fractionated independently by cation exchange chromatography according to the following procedure: The respective desalted, lyophilized G-75 fraction was dissolved in 1 ml of an elution buffer of 0.1 M KCl/0.05 M TRIS (pH 10) and applied to a carboxymethyl Sephadex C-25 cation exchange column equilibrated with the same buffer. The column was developed with a flow rate of 0.3 ml/min with a three-step continuous KCl salt gradient from 0.1 M-2.0 M in the 0.05 M TRIS (pH 10) buffer system. Fractions of 2.5 ml were collected on an Instrumentation Specialties Co. model 1200 fraction collector. Determination of the absorbance of each tube at 280 nm was performed on a Perkin-Elmer Coleman 101 spectrophotometer, and the conductivity of each tube measured on a London type COM2d conductivity meter. The tubes corresponding to each fraction were pooled and lyophilized. These fractions were then dialyzed against distilled water under stirring for 24 hrs using benzoylated dialysis tubing. The desalted fractions were then lyophilized, weighed, and stored at -20°C until used in further tests.

D. Assay for Myotoxicity

Assays for myonecrotic activity on the crude venom and fractions thereof were performed by the method of Ownby et al. (15). 20-24 gram Swiss Webster white mice were injected in the medial aspect of the thigh with 2.5 μg per gram body weight of the crude venom or fractions in physiological saline (0.085% NaCl w/v). Controls were injected only with physiological saline. The animals were sacrificed at 24 hrs by cervical dislocation, and cubical tissue samples of approximately 1 mm per side were taken from the opposite side of the injected area to avoid any damage arising from injection. The samples were immediately fixed in 2% glutaraldehyde for 2 hrs, followed by fixing in 1% osmium tetraoxide for one hour. Dehydration was accomplished by immersion of the tissue in increasing concentrations of acetone, followed by propylene oxide overnight. The tissue was then embedded in Poly/Bed 812, and allowed to polymerize at 60°C in a vacuum oven. The embedded tissue was subsequently sectioned into 1 μm sections on an LKB Ultra Microtome, and stained with Mallory's Ozure II methylene blue. Photographs were taken on a Zeiss photomicroscope using a tungsten light source.

E. Disc Polyacrylamide Gel Electrophoresis

Disc polyacrylamide gel electrophoresis at pH 4.3 was performed by the method of Reisfeld et al. (34), with the compositions of the gel components as follows:

STACKING GEL: 5% acrylamide, 1.25% BIS, 0.5% riboflavin, 0.24 ml 1 N KOH, 0.014 ml glacial acetic acid, 0.0023 ml TEMED/4 ml stacking gel solution.

RESOLVING GEL: 15% acrylamide, 0.1% BIS, 0.1% ammonium persulfate, 0.72 ml 1 N KOH, 0.258 ml glacial acetic acid, 0.06 ml TEMED/12 ml resolving gel solution.

SAMPLE SOLUTION: 5 µl 2.7% KOH/2.9% glacial acetic acid solution; 5 µl 0.005% bromophenol blue solution; 10 µl 40% sucrose solution; 20 µl sample liquid.

The gels were electrophoresed with a Buchler 3-1500 power source at 4 mA/tube for 3 hours, and were stained immediately following electrophoresis with 0.5% aniline blue black in 7% acetic acid for 30 min, and destained in 7% acetic acid. The gels were scanned on a Helena Laboratories Auto Scanner.

F. Slab Polyacrylamide Gel Electrophoresis

Slab polyacrylamide gel electrophoresis was performed using the pH 4.3 system of Reisfeld et al. (34), with the slab technique described by Ames et al. (35). The composition of the gel components was as follows:

STACKING GEL: 5% acrylamide, 1.25% BIS, 0.5% riboflavin, 0.24 ml 1 N KOH, 0.014 ml glacial acetic acid, 0.0023 ml TEMED/4 ml stacking gel solution.

RESOLVING GEL: 15% acrylamide, 0.1% BIS, 0.1% ammonium persulfate, 0.72 ml 1 N KOH, 0.258 ml glacial acetic acid, 0.06 ml TEMED/12 ml resolving gel solution.

SAMPLE SOLUTION: 5 µl 2.7% KOH/2.9% glacial acetic acid solution; 5 µl 0.005% bromophenol blue solution; 10 µl 40% sucrose solution; 20 µl sample liquid.

The slab gel was electrophoresed in a Protean Slab Electrophoresis Cell by BioRad at 20 ma for 1½ hrs followed by 40 ma for 5 hrs, stained immediately with 0.5% analine blue black in 7% acetic acid for 30 min, followed by destaining in 7% acetic acid.

G. Amino Acid Analysis

Amino acid compositions were determined by the method of Moore and Stein (36). Hydrolysis was performed with 6 N HCl at 110°C for 24, 48, and 72 hrs. Analysis was accomplished using a Beckman Model 120 C automatic amino acid analyzer modified by the method of Liao et al. (37). Myotoxin a was also hydrolyzed in the same manner and used as a reference. The values for half-cystine were calculated by extrapolation to zero time.

H. SDS Gel Electrophoresis

SDS slab gel electrophoresis was performed by the method of Laemmli et al. (38). The composition of the gel components were as follows:

RESOLVING GEL (pH 8.8): 3.6 ml 1 N HCl, 2.7 g TRIS, 0.017 ml TEMED, 4.2 g acrylamide, 0.11 g BIS, 0.3 ml 10% SDS, 0.01 g ammonium persulfate/29.8 ml resolving gel solution.

STACKING GEL (pH 6.8): 1.2 ml 1 N HCl, 0.15 g TRIS, 0.012 ml TEMED, 0.7 g acrylamide, 0.018 g BIS, 0.2 ml 10% SDS, 0.0035 g ammonium persulfate/7.7 ml stacking gel solution.

The proteins were electrophoresed in a 15% gel at pH 8.9 at 10 mA for two hrs, followed by 20 mA for four hrs on a Bio-Rad Protean electrophoresis cell. Protein standards used were bovine serum albumin (66 kdal), ovalbumin (45 kdal), carbonic anhydrase (31 kdal), chymotrypsinogen (25 kdal), soybean trypsin inhibitor (21.5 kdal), ribonuclease A (13.7 kdal), lysozyme (14.4 kdal), insulin (5.7 kdal) and myotoxin a (4.6 kdal). The gel was fixed in a solution containing 10% acetic acid and 25% isopropanol; stained with a 0.02% coomassie blue 7% acetic acid solution; and destained with a solution containing 25%

methanol and 10% acetic acid.

Molecular weights were estimated by the construction of a graph plotting log of molecular weight vs. relative mobility.

I. Sephadex G-50 Gel Filtration

Molecular weight of the toxins were estimated by a Sephadex G-50 gel filtration column (100 x 1.5 cm) by the method of Andrews et al. (39), using a buffer with 0.05 M TRIS (pH 10) and 0.1 M KCl. Polypeptide standards used were chymotrypsinogen a (23.2 kdal), cytochrome c (12.4 kdal), salamine (7 kdal), myotoxin _a_ (4.6 kdal) and clupeine (4 kdal). The void volume was marked with Dextran Blue. From the elution profile obtained, a graph plotting log of molecular weight vs. the ratio of the elution volume over the void volume was constructed, and the appropriate molecular weights of the toxins were calculated from their respective elution volumes.

CHAPTER IV

RESULTS AND DISCUSSION

A. Isolation of Venom Myotoxins

The isolation of myotoxins I and II from crude *Crotalus durissus durissus* venom appears in Figure 1. From the initial fractionation of the venom on a Sephadex G-75-40 gel filtration column, six peaks were obtained, as shown in the elution profile in Figure 2. These peaks represent fractions 1 through 6. Fraction 3 from Sephadex G-75 was further fractionated on a CMS cation exchange column, with resolution into three peaks, shown in Figure 3. Fraction 3C from CMS proved to be essentially homogeneous by electrophoresis and displayed myonecrotic activity, and was termed myotoxin I.

Fraction 4 from Sephadex G-75 was also subjected to cation exchange chromatography, resulting in its separation into four peaks, as seen in Figure 4. CMS fraction 4D, after demonstrating myonecrotic activity and homogeneity by electrophoresis was named myotoxin II. The relative composition of the venom with respect to each fraction and the two myotoxins is summarized in Table I. The active myotoxic fractions 3 and 4 from the Sephadex fractionation represents 4 and 26%, respectively, of the crude venom. Myotoxins I and II are minor constituents of this venom, and are not neutralized by the antisera presently commercially available (40). However, Ownby (41) reports that the myonecrotic components in *Crotalus durissus durissus* venom react with

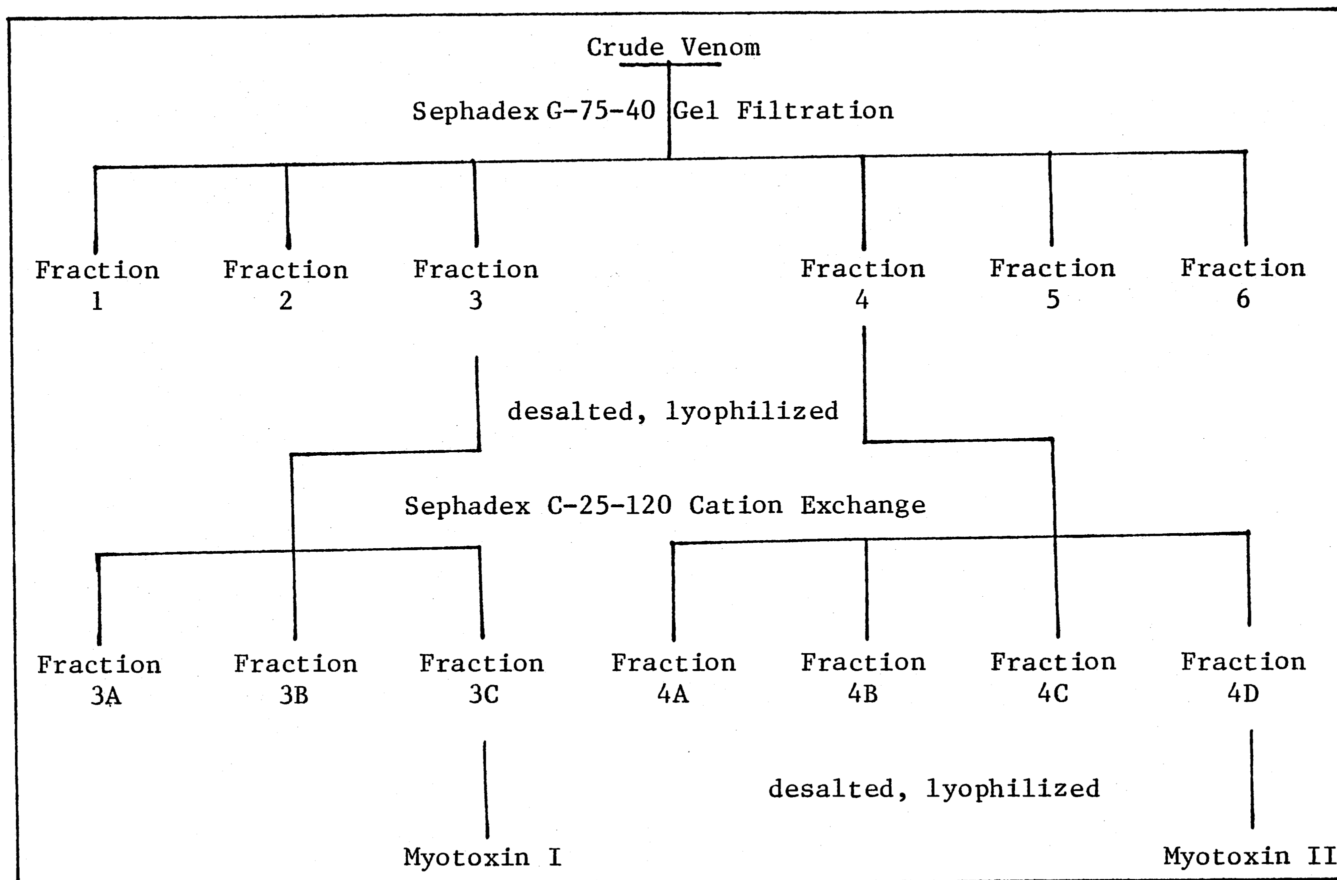

Figure 1. Isolation Procedure of Myotoxins I and II from Crotalus durissus durissus Venom.

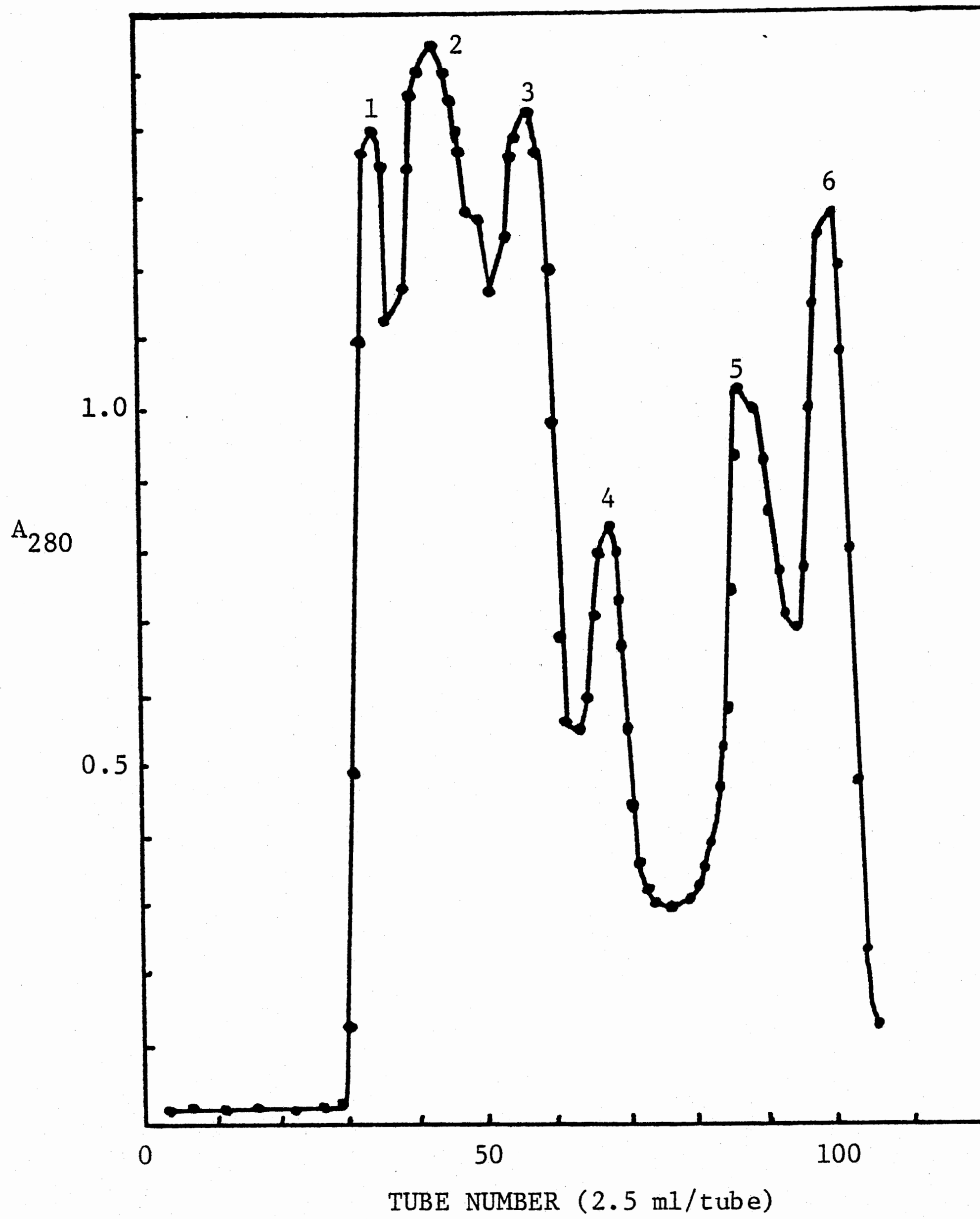

Figure 2. Sephadex G-75 Elution Profile of Crotalus durissus durissus Venom.

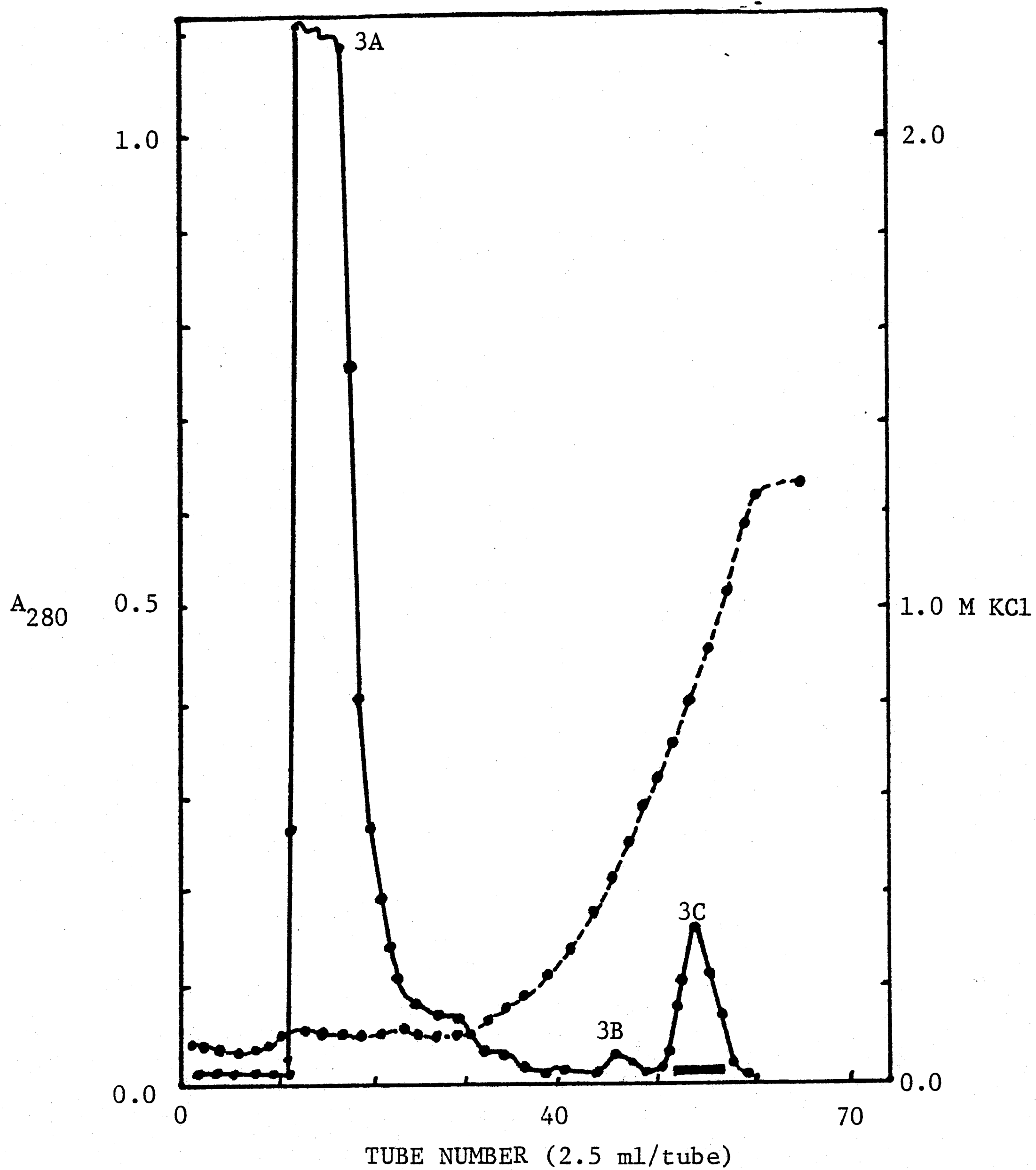

Figure 3. Sephadex C-25 Cation Exchange Chromatography of Sephadex G-75 Fraction 3.

Marked peak indicates the fraction composed of myotoxin I.

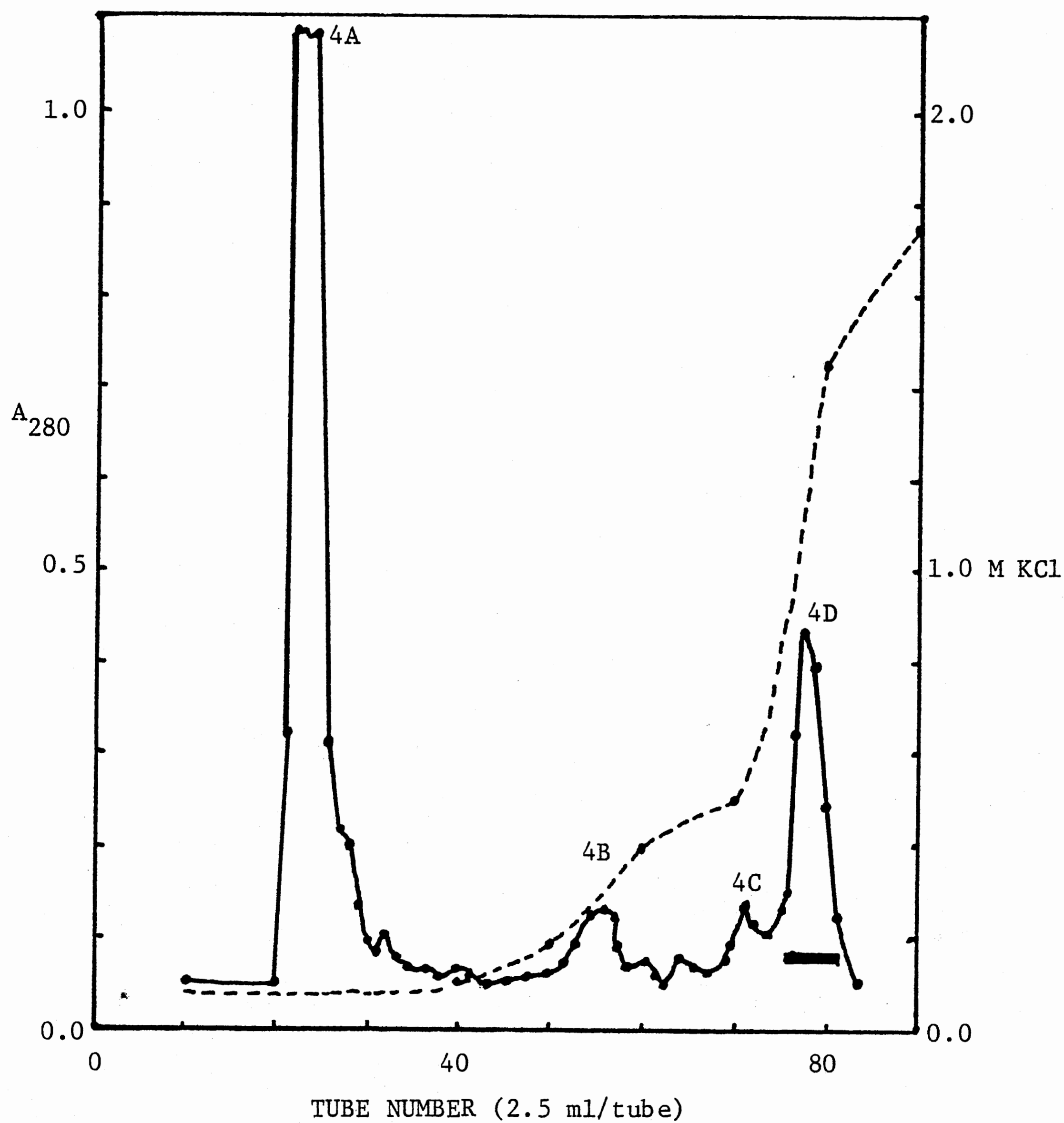

Figure 4. Sephadex C-25 Cation Exchange Chromatography of Sephadex G-75 Fraction 4.

Marked peak indicates the fraction composed of myotoxin II.

TABLE I

DISTRIBUTION OF PROTEIN AND MYONECROSIS OF FRACTIONS FROM SEPHADEX G-75 AND C-25 FRACTIONATION OF *CROTALUS DURISSUS DURISSUS* VENOM

Fractional Step	Fraction	Myonecrosis[1]	% of Protein in Crude Venom
G-75 Gel Filtration	1	-	6.2
	2	-	37.2
	3	++	3.9
	4	+++	26.2
	5	-	25.6
	6	-	0.9
C-25 Ion Exchange	3A	-	3.2
	3B	+	0.2
	3C	++	0.5
	4A	-	7.6
	4B	-	2.2
	4C	+	0.4
	4D	+++	15.9

[1]Legend: (-) = No evidence of myonecrosis; (+) = mild myonecrosis; (++) = moderate myonecrosis; (+++) = severe myonecrosis.

antimyotoxin *a* (produced in rabbits) and identify completely in gel diffusion with myotoxin *a* and crotamine.

B. Identification of Myonecrotic Activity

The results of the assays for myonecrosis performed on the crude venom, as well as on the various fractions and subfractions, is shown in Table I. Muscle tissue removed from an animal injected with crude venom is shown in Figure 5, which can be compared to tissue injected only with PSS in Figure 6.

It can be seen from these photographs that crude *Crotalus durissus durissus* venom induces massive hemorrhage, seen as small dark points, which are erythrocytes which have escaped the surrounding capillaries. Figure 5 also shows large areas of necrosis, as demonstrated by the vacuolization of the individual muscle cells.

From Table I it can be seen that both fractions 3 and 4 show strong myonecrotic activity. Therefore, these fractions were further fractionated and the subfractions assayed for myonecrotic activity.

In the testing of subfractions 3A-3C and 4A-4D, it was discovered that fractions 3C and 4D contained the higher activities. The small degree of necrosis arising from fractions 3B and 4C is considered to result from some overlap with fractions 3C and 4D, respectively.

Figures 7 and 8 show tissue from animals injected with myotoxins I and II. These can be compared to the activity of myotoxin *a* from *Crotalus viridis viridis* appearing in Figure 9. The lack of erythrocytes in these photographs indicate that myotoxins I and II are not responsible for the hemorrhagic activity shown by the crude venom. The large areas of vacuolization of cell nuclei are readily apparent

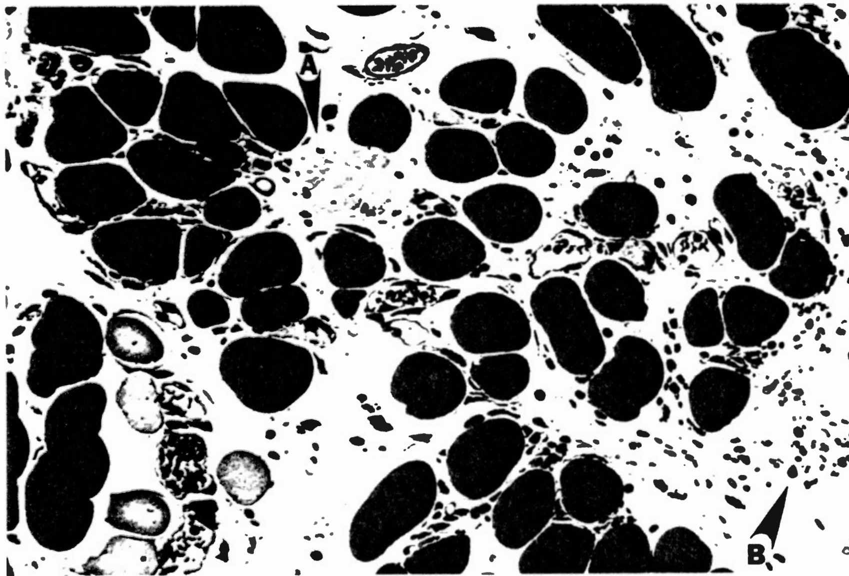

Figure 5. Photomicrograph of Muscle Tissue Injected with Crude Crotalus durissus durissus Venom.

Arrow A points to muscle cell which has been destroyed as a result of myonecrotic components present in the crude venom. Arrow B points to erythrocytes that have escaped through nearby capillaries. (Tissue magnified 192 times.)

Figure 6. Photomicrograph of Muscle Tissue from Control Animal Injected with Physiological Saline.

Arrow points to normal muscle cell. (Tissue magnified 192 times.)

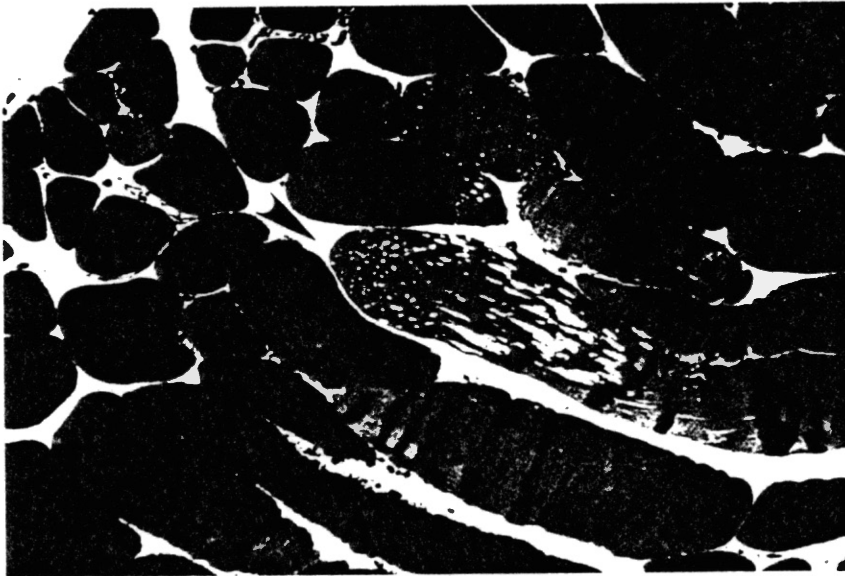

Figure 7. Photomicrograph of Muscle Tissue Injected with Purified Myotoxin I.

Arrow points to damaged muscle cell. Lighter areas in cell are vacuoles formed as the result of the action of myotoxin I. (Tissue magnified 192 times.)

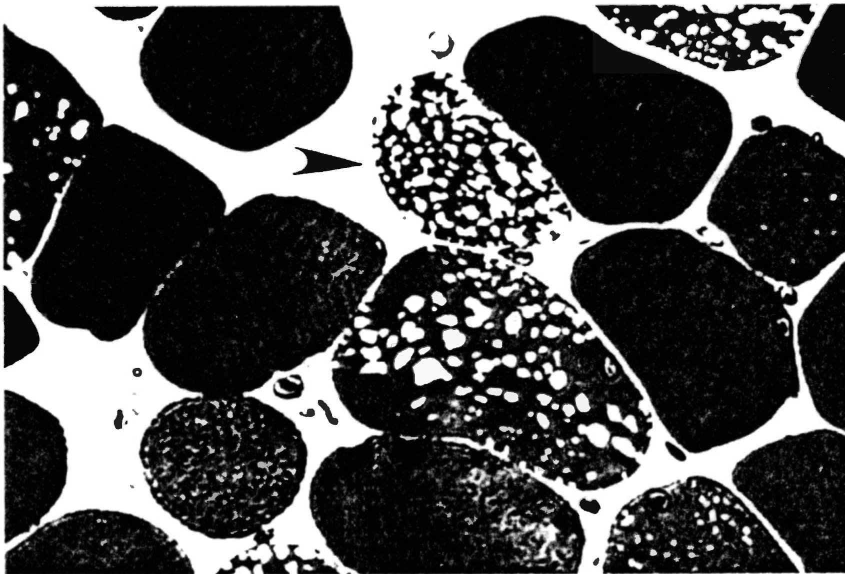

Figure 8. Photomicrograph of Muscle Tissue Injected with Purified Myotoxin II.

Arrow points to cell which shows the extreme vacuolization characteristic of rattlesnake myonecrotic toxins. (Tissue magnified 480 times.)

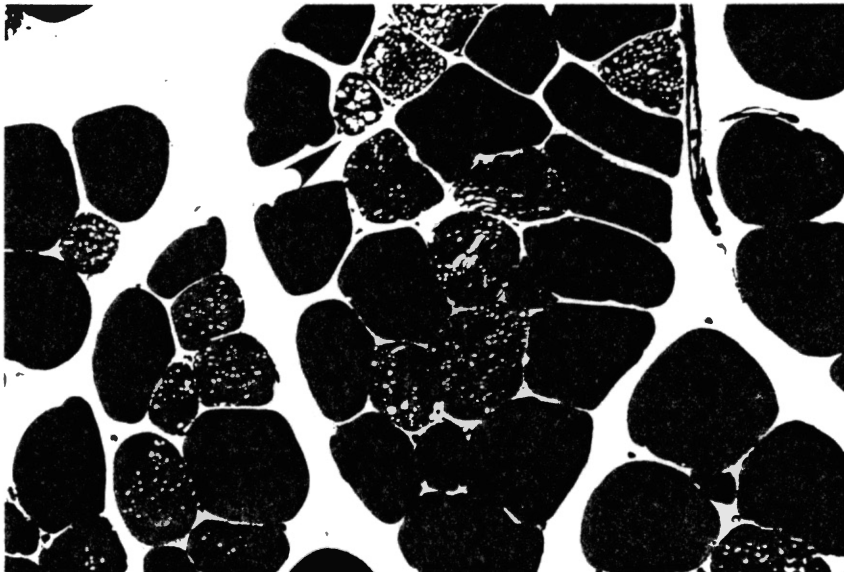

Figure 9. Photomicrograph of Muscle Tissue Injected with Myotoxin _a_ from _Crotalus viridis viridis_.

Arrow points to a group of necrotic cells which are indistinguishable from cells damaged by myotoxins I and II from _Crotalus durissus durissus_ venom on the light microscope level of investigation. (Tissue magnified 192 times.)

in each of these photographs, and have proved to be indistinguishable from myotoxin a on the light microscopy level of investigation.

C. Electrophoretic Homogeneity

Demonstration of the homogeneity of myotoxins I and II was determined by disc polyacrylamide gel electrophoresis in a 15% gel at pH 4.3. Figure 10 shows a scan of a gel with myotoxin I as the sample compared with a gel containing myotoxin a from Crotalus viridis viridis venom. Myotoxin I shows relative homogeneity with noticeable but minor contaminates. This figure also points out a great deal of chemical similarity between myotoxin I and myotoxin a. Figure 11 shows the same experiment repeated with myotoxin II. Again, the protein appears relatively pure, and indicates similar chemical properties to myotoxin a.

Figure 12 shows the results of slab polyacrylamide gel electrophoresis performed with myotoxins I and II. This was also accomplished in the 15% gel system at pH 4.3. This experiment confirms the fact that myotoxin I and myotoxin II are distinct proteins, with differing migration properties. It can be seen from this gel that myotoxin II migrates further than myotoxin I, which is in good agreement with other data collected, in that myotoxin II is of lower molecular weight than myotoxin I, and also possess a higher charge/mass ratio.

D. Amino Acid Compositions of Toxins I and II

The results of the amino acid analysis of toxins I and II are shown in Tables II and III, respectively. Myotoxin I was found to consist of 42 amino acid residues. It was found that it contained a high number of basic

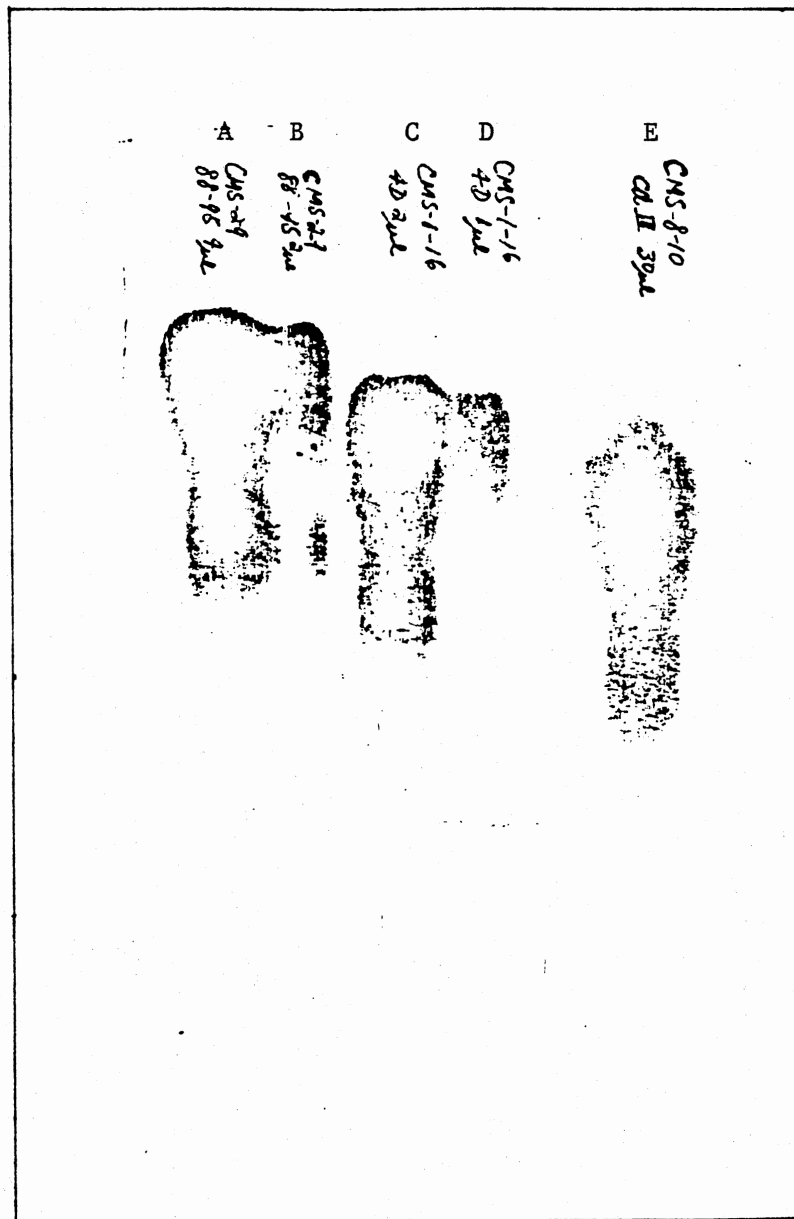

Figure 10. Slab Polyacrylamide Gel Electrophoresis of Purified Myotoxins I and II.

Lanes A and B contain myotoxin a from Crotalus viridis viridis venom; Lanes C and D contain myotoxin II and Lane E myotoxin I, both from Crotalus durissus durissus venom.

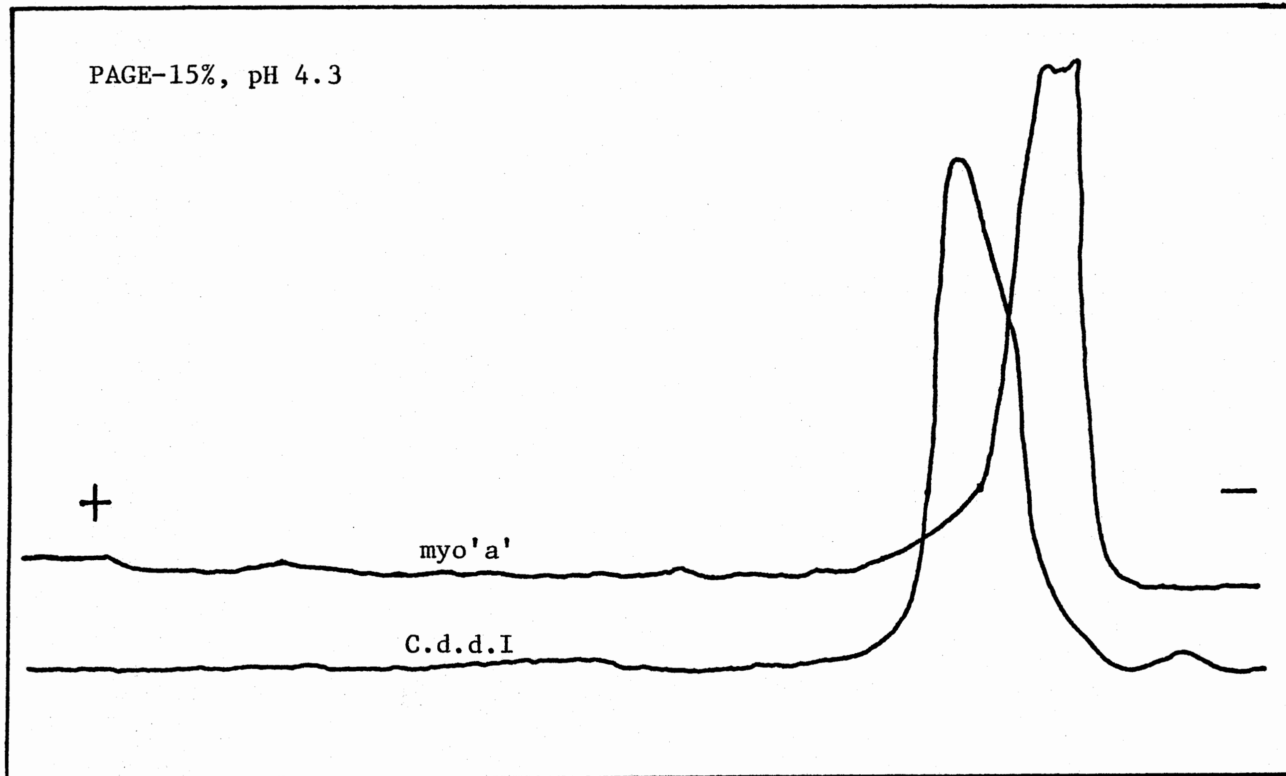

Figure 11. Scan of Disc Polyacrylamide Gel Electrophoresis of Purified Myotoxin I.

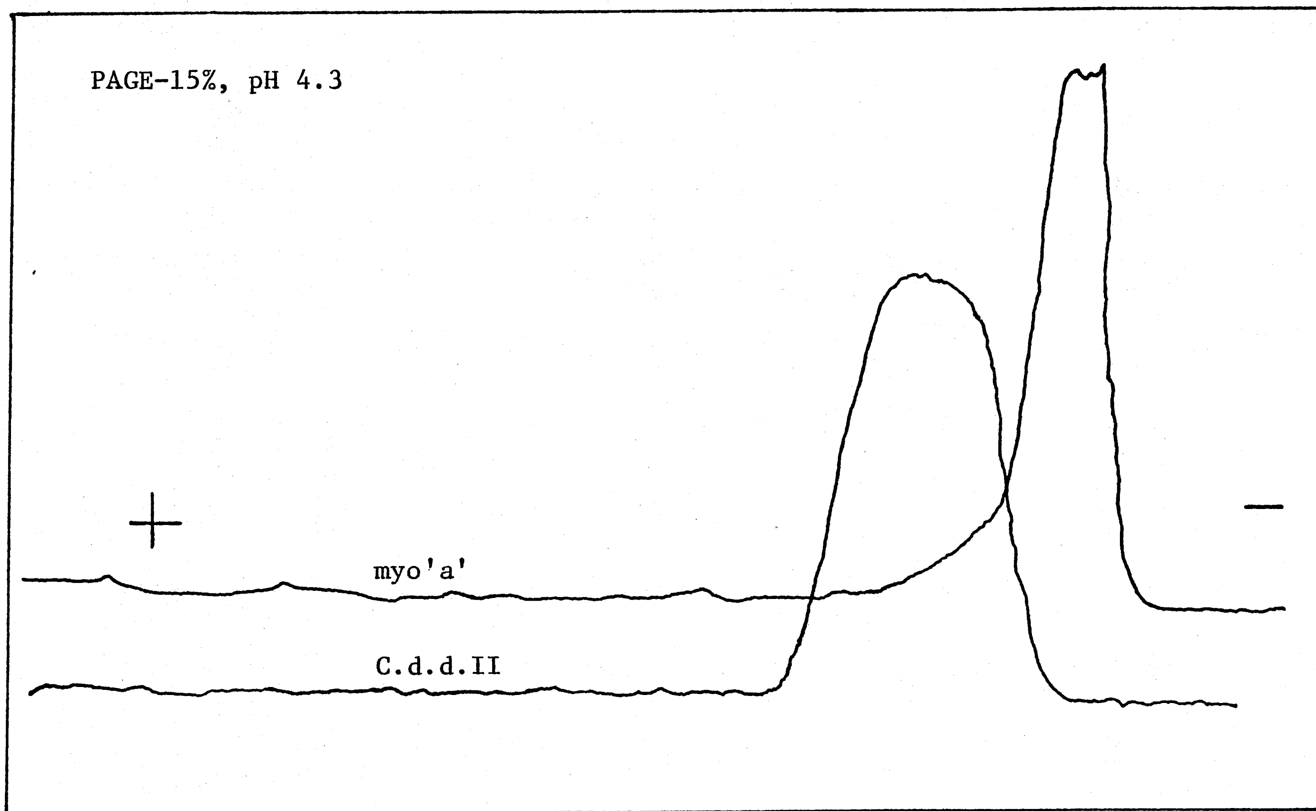

Figure 12. Scan of Disc Polyacrylamide Gel Electrophoresis of Purified Myotoxin II.

TABLE II

AMINO ACID COMPOSITION OF MYOTOXIN I[1,2]

Amino Acid	Corrected[3] Value	Nearest Integer	Weight
asx	2.95	3	345
thr	1.11	1	101
ser	5.68	6	522
glx	2.96	3	387
pro	2.12	2	194
gly	4.91	5	285
ala	1.44	1	71
½-cys	6.30	6	618
val	1.39	1	99
met	0.68	1	131
ile	1.10	1	113
leu	1.07	1	113
tyr	0.76	1	163
phe	1.18	1	147
his	1.76	2	274
lys	5.89	6	768
arg	1.08	1	156
Total		42	4487

[1]Analysis based on comparison to myotoxin a.

[2]Tryptophan not determined.

[3]The value for ½ cystine was corrected by extrapolation to zero time; average values are given for all other amino acids.

TABLE III

AMINO ACID COMPOSITION OF MYOTOXIN II[1,2]

Amino Acid	Corrected[3] Value	Nearest Integer	Weight
asx	2.89	3	345
thr	0.76	1	101
ser	2.51	3	261
glx	1.18	1	129
pro	1.60	2	194
gly	2.74	3	171
ala	0.58	1	71
½-cys	6.35	6	618
val	1.80	2	198
met	0.79	1	131
ile	0.90	1	113
leu	0.89	1	113
tyr	0.74	1	163
phe	1.36	1	147
his	1.38	1	137
lys	5.91	6	768
arg	1.17	1	156
Total		35	3816

[1] Analysis based on comparison to myotoxin a.

[2] Tryptophan not determined.

[3] The value for ½ cystine was corrected by extrapolation to zero time; average values are given for all other amino acids.

residues and six half-cystine residues.

Myotoxin II was found to be composed of 35 amino acids. It also contains a high percentage of basic amino acids, and has six half-cystine residues.

Due to the small amount of myotoxins I and II available, analysis for tryptophan was not performed.

E. Molecular Weight Estimates

The results of an SDS slab gel electrophoresis procedure performed with myotoxin I appears in Figure 13. From this data, it can be seen that myotoxin I shows a molecular weight of approximately 8500. However, myotoxin a from Crotalus viridis viridis venom with a known molecular weight of 4600 also shows a similar migration distance.

This is thought to arise from the inability of the SDS molecules to completely neutralize the excess positive charges in these molecules which arises from their high content of basic amino acid residues. This would result in a retarded migration rate for the molecules, accounting for the discrepancy in molecular weights. Due to these results, the experiment was not repeated with myotoxin II.

The molecular weights of the myotoxins were also determined by Sephadex G-50-40 gel filtration. The column was calibrated, and myotoxins I and II were separately loaded on the column and their elution volumes determined. Figure 14 shows the calibration plot obtained. It can be seen from this figure that myotoxin I shows a molecular weight of 5500, and myotoxin II a molecular weight of 4500.

Minimum molecular weights are also obtainable from the amino acid compositions of myotoxins I and II. From this data, molecular

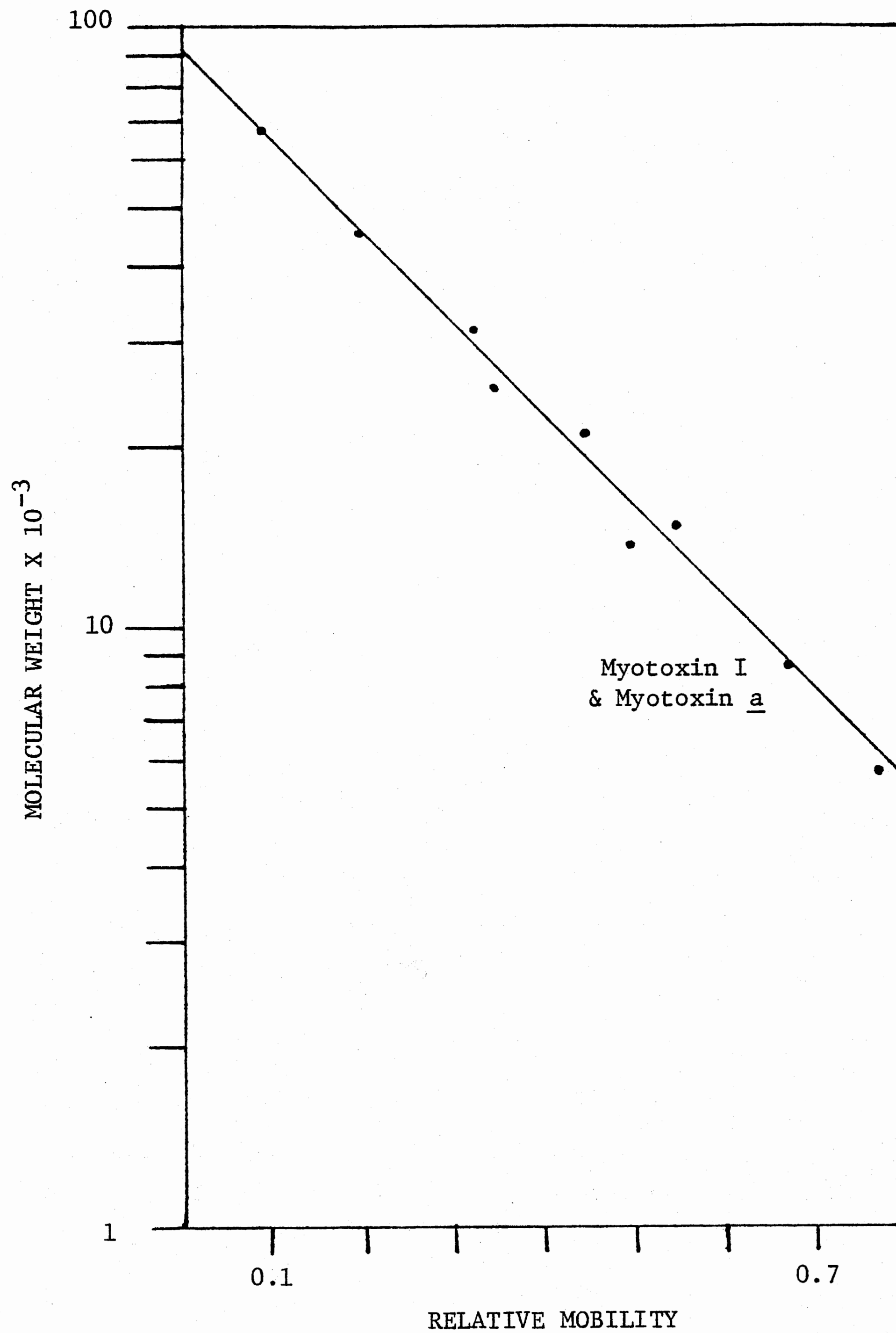

Figure 13. Molecular Weight Determination of Myotoxin I by SDS Gel Electrophoresis.

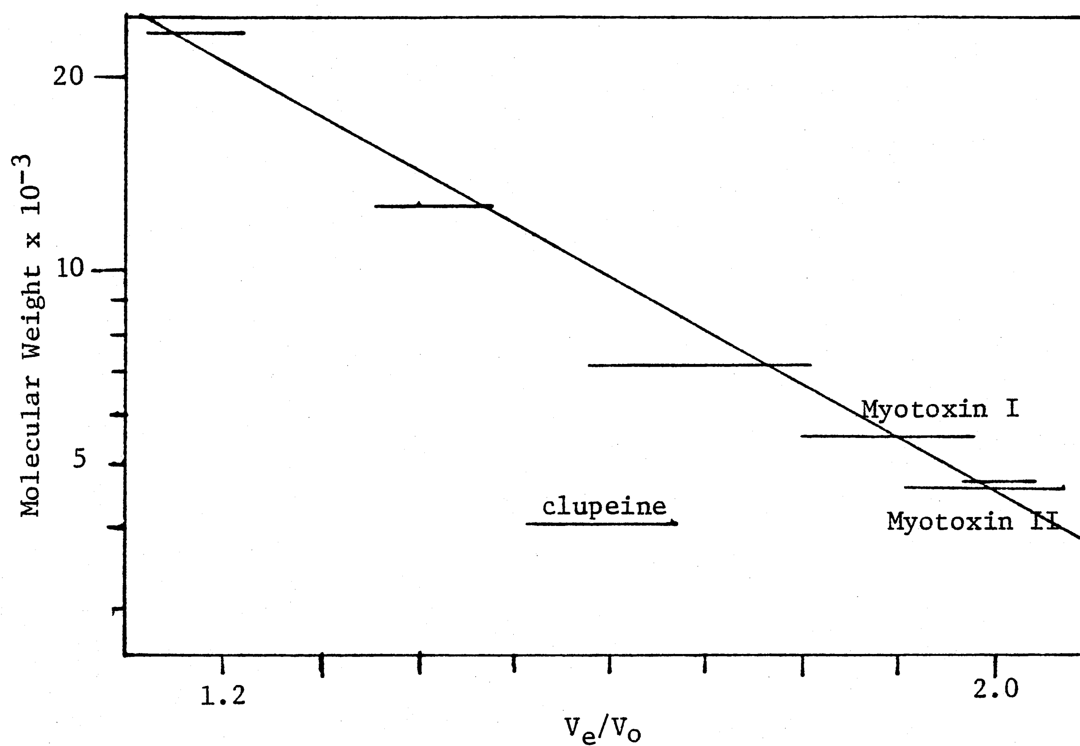

Figure 14. Molecular Weight Determination of Myotoxins I and II by Sephadex G-50 Gel Filtration.

Uncertainty in V_e/V_o indicated by lengths of data bars. As can be seen, only clupeine deviated substantially from the standardization curve.

weights of 4487 and 3816 were calculated for myotoxins I and II, respectively.

F. Comparison of Myotoxin I and II with Other Rattlesnake Myotoxins

A summary of the previously characterized rattlesnake myonecrotic proteins appears in Table IV, as well as the data compiled from this work. It can be seen that all of these toxins, with the exception of viriditoxin, have several strong similarities. First, the molecular weights all span the narrow range from 3700-4700. Secondly, all have unusually high lysine contents, with few arginine residues present. All of these toxins for which a value has been reported possess 6 half cystine residues, with myotoxin _a_, crotamine, and the myotoxin from _Crotalus viridis helleri_ known to possess three disulfide bridges. This indicates a great similarity of their three-dimensional structures, undoubtedly related to the mechanism of their biological actions.

Since the work done in this area to date is limited and incomplete, it is impossible to make distinctions between these various proteins/polypeptides. It is clear though, that these molecules as a whole represent an entirely new class of biological compounds, unique not only in their structure, but their function as well.

TABLE IV

COMPARATIVE DATA OF MYONECROTIC PROTEINS FROM RATTLESNAKE VENOMS

Source	Name	MW	pI	No. of Amino Acids	Disulfide Bridges	Reference
C. viridis viridis	Myotoxin a	4600	9.2	42	3	11
	Viriditoxin	115000	4.8	1018		24
C. durissus terrificus	Crotamine	4600	>9.0	42	3	21
C. viridis concolor	Myotoxin I		>9.0	35		23
	Myotoxin II		>9.0	39		
C. viridis helleri		4700		43	3	8
C. durissus durissus	Myotoxin I	4500		42		Present Inves.
	Myotoxin II	3800		35		

CHAPTER V

SUMMARY

It was the prestated objective of this work to isolate and characterize the myonecrotic components of Crotalus durissus durissus venom.

During the course of this study, two myonecrotic toxins were identified and isolated. These myotoxins have been found to be of low molecular weight, and show a great deal of physical and chemical similarities to previously isolated and characterized polypeptides that demonstrate the same biological activities.

Myotoxin I from Crotalus durissus durissus venom has been shown to possess a minimum molecular weight of 4487, and composed of a minimum of 42 amino acid residues. These include six lysines, which greatly effect the behavior of the molecule. Also present are six half-cystine residues, making possible the existence of three disulfide bridges. Both of these characteristics are present in several of the well characterized myonecrotic polypeptides occurring in rattlesnake venoms (21).

Myotoxin II from Crotalus durissus durissus venom demonstrates a minimum molecular weight of 3816, with a minimum of 35 amino acid residues. It also possesses six lysine residues, had has six half-cystines. Although smaller than myotoxin I, and the two best characterized rattlesnake basic myotoxins (myotoxin a from Crotalus viridis viridis and crotamine from Crotalus durissus terrificus), its smaller

size is not unprecedented (23).

Both of these toxins have demonstrated strong myonecrotic activities. Their injection into test animals has resulted in severe vacuolization of muscle cells indistinguishable from myotoxin *a* and crotamine on the light microscopy level of investigation.

LITERATURE CITED

(1) Tu, A. T. (1977) "Venoms: Chemistry and Molecular Biology." Wiley, New York. pp. 382.

(2) Emery, T. A. and Russell, F. E. (1963) "Venomous and Poisonous Animals and Noxious Plants of the Pacific Region." Pergamon, New York. pp. 409.

(3) Stahnke, H. L. (1966) Arizona State University Bureau of Publications, No. 56247. Tucson, Arizona.

(4) Stahnke, H. L., Allen, F. M., Horan, R. V., and Tenery, J. H. (1957) Am. J. Trop. Med. Hyg. 6, 323.

(5) Minton, S. A. (1954) Am. J. Trop. Med. Hyg. 3, 1077.

(6) Homma, M., and Tu, A. T. (1971) Br. J. Exp. Path. 52, 538.

(7) Pool, W. R. and Bieber, A. L. (1981) Toxicon. 19:4, 517.

(8) Russell, F. E., Pattabhiraman, T. R., Schaeffer, R. C., Gonzales, H., Tamiya, N. and Maeda, N. (1978) "Some Properties of the Venom of Crotalus virdis helleri." Proceedings of the Second Symposium of the European Section of the International Society of Toxicology.

(9) Rodriguez, O. G., and Scannone, H. R. (1976) Toxicon. 14, 400.

(10) Scannone, H. R., Rodriguez, O. G., Lancini, A. R. (1978) "Enzymatic Activities and Other Characteristics of Crotalus Vergrandis Snake Venom." Proceedings of the Fifth International Symposium on Toxicology.

(11) Cameron, D. L. and Tu, A. T. (1977) Biochemistry 6, 2546.

(12) Fox, J. W., Elzinga, M. and Tu, A. T. (1979) Biochemistry 18, 678.

(13) Bailey, G. S., Lee, J. and Tu, A. T. (1979) J. Biol. Chem. 254, 8922.

(14) Stringer, J. M., Kainer, R. A. and Tu, A. T. (1971) Am. J. Pathol. 67, 127.

(15) Ownby, C. L., Cameron, D. and Tu, A. T. (1976) Am. J. Pathol. 85:1, 149.

(16) Ownby, C. L., Wood, W. M., and Odell, G. V. (1979) Toxicon 17, 373.

(17) Ownby, C. L., Odell, G. V., and Wood, W. M. (1980) "Neutralization of Myotoxin a from Rattlesnake (Crotalus viridis viridis) Venom with Anti-Myotoxin Serum." Unpublished.

(18) Goncalves, J. M. and Polson, A. (1947) Arch. Biochem. 13, 253.

(19) Goncalves, J. M. (1956) in "Venoms", pp. 261. Buckley, E. E. and Progres, N., eds. American Association for the Advancement of Science, Washington, D.C.

(20) Cheymol, J., Goncalves, J. M., Bourillet, F. and Roch-Arveiller, M. (1971) Toxicon 9, 279.

(21) Cameron, D. L. and Tu, A. T. (1978) Biochim. Biophys. Acta 532, 147.

(22) Laure, C. J. (1975) Hoppe-Zeylers Z. Physiol. Chem. 356, 215.

(23) Pool, W. R., Engle, C. M., and Bieber, A. L. (1981) "Isolation and Characterization of Two Myotoxins from the Venom of the Midget Faded Rattlesnake (Crotalus viridis concolor)." Federation Proceedings 40:6, 1579.

(24) Fabiano, R. J. and Tu, A. T. (1981) Biochemistry 20, 21.

(25) Gloyd, H. K. (1978) "The Rattlesnakes: Genera Sistrusus and Crotalus." pp. 122. Society for the Study of Amphibians and Reptiles, Tucson, Arizona.

(26) Friederich, C. and Tu, A. T. (1971) Biochem. Pharmacol. 20, 1549.

(27) Kocholaty, W. F., Ledford, E. B., Daly, J. B., and Billings, T. A. (1971) Toxicon 9, 131.

(28) Bolaños, R. (1972) Am. J. Trop. Med. Hyg. 21, 3.

(29) Denson, K. W. E., Russell, F. E., Almagro, D., and Bishop, R. C. (1972) Toxicon 10, 557.

(30) Kocholaty, W. F., Goetz, J. C., Ashley, B. D., Billings, T. A. and Ledford, E. B. (1968) Toxicon 5, 153.

(31) Sosa, B. P., Alagon, A. C., Possani, L. D., and Julia, J. Z. (1979) Biochem. Physiol. 64B, 231.

(32) Gutierrez, J. M., and Chaves, F. (1980) Toxicon 18, 315.

(33) Bolaños, R., Marin, O., Media, E. M., and Alfaro, E. A. (1981) Act. Med. Cost. 24:3, 211.

(34) Reisfeld, A., Lewis, D., and Williams, J. (1962) Nature 195, 281.

(35) Ames, G. F. L. (1974) J. Biol. Chem. 249:2, 634.

(36) Moore, S., and Stein, W. H. (1957) in "Methods in Enzymology." Colowick, S. P. and Kaplan, N. O., eds. Vol. 6, pp. 819. Academic, N.Y.

(37) Liao, T. H., Robinson, G. W., and Salnikow, J. (1973) Anal. Chem. 45, 2286.

(38) Laemmli, U. K. (1970) Nature 227, 680.

(39) Andrews, P. (1964) Biochem. J. 91, 222.

(40) Guiterrez, J. M. (1982) Private Communication.

(41) Ownby, C. L. (1982) Unpublished.

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