A new small myotoxin from the venom of the prairie rattlesnake (*Crotalus viridis viridis*)

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Fast atom bombardment (FAB) mass spectrometry was used to identify a new small myotoxin from the venom of the prairie rattlesnake (*Crotalus viridis viridis*). FAB mass spectrometry and Edman degradation were used to characterize its structure. This toxin is similar to myotoxin I from *C. v. concolor*, except that it possesses an additional C-terminal asparaginyl-alanine. At 45 residues it is the longest known myotoxin *a* homolog. A myotoxin of 43 residues, identical to myotoxin I from *C. v. concolor*, was also found. To date no other species has been shown to produce more than one length of myotoxin. The present paper documents 42-, 43-, and 45-residue myotoxins from the venom of a single animal.

Myotoxin; Venom; Structure, Fast atom bombardment mass spectrometry; *Crotalus viridis viridis*

1. INTRODUCTION

The small myotoxins are a unique class of basic polypeptides from rattlesnake venoms. They consist of 42–43 residues, have high isoelectric points, and cause severe muscle necrosis by a non-enzymatic mechanism. In contrast to myotoxic phospholipases, the small myotoxins act extremely rapidly and serve two primary biological functions: to limit the flight of prey by causing nearly instantaneous paralysis of the hind limbs, and to promote rapid death by paralysis of the diaphragm. Small myotoxins share essentially no structural attributes with myotoxic phospholipases which are more ubiquitous among crotaline venoms, and which presumably disrupt membranes by hydrolyzing sarcolemma phospholipids.

The first small myotoxin to be discovered was crotamine, a 42-residue polypeptide (p*I* 10.3) from the venom of *Crotalus durissus terrificus* [1,2]. Its primary structure was determined 25 years later by Laure [3]. Several crotamine homologs have since been reported, including myotoxin *a* from the venom of *C. viridis viridis* [4,5], peptide C from the venom of *C. v. helleri* [6], and myotoxins I and II from the venom of *C. v. concolor* [7,8]. Unlike crotamine and myotoxin *a*, peptide C and the two *concolor* myotoxins both consist of 43 residues, rather than 42. The difference results from a C-terminal valyl-asparagine in place of the usual glycine. Another myotoxin called Toxin E has been sequenced from the venom of *C. h. horridus*. It contains 42 residues and is nearly identical in primary structure to crotamine and myotoxin *a* (C.R. Green and J.W. Fox, personal communication). Most recently, a possible dimeric myotoxin has been reported from *C. adamanteus* venom [9].

The present communication reports the discovery of a new small myotoxin from the venom of the prairie rattlesnake (*Crotalus viridis viridis*). This myotoxin was detected by fast atom bombardment (FAB) mass spectrometry [10,11] and sequenced by a combination of FAB mass spectrometry and automated Edman degradation.

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2. MATERIALS AND METHODS

2.1. *Venom extraction and chromatography*

An adult (>1 month) male prairie rattlesnake (*Crotalus viridis viridis*) was captured by SDA, 20 miles east of Meeker, Rio Blanco County, CO, at the eastern and elevational limits for the species. Venom was extracted manually at 2–3 week intervals and kept frozen at −20°C until use. Crude venom was diluted with 100 mM acetic acid/NaOH (pH 4.0), centrifuged at 10 000 rpm for 10 min to remove insoluble material, and fractionated on a 2.5 × 95 cm column of Sephacryl S-200 HR (Pharmacia-LKB) equilibrated in the same buffer. Fraction 5 was subfractionated on a 0.5 × 5 cm Mono S HR (Pharmacia-LKB) column equilibrated in 50 mM formic acid/NH_4OH (pH 4.0). Buffer B was identical except that it contained 2 M NaCl. Further purification was achieved by passing the material from Mono S column over a 0.5 × 5 cm PepRPC HR (Pharmacia-LKB) column equilibrated in 250 mM formic acid/NH_4OH (pH 4.0). Buffer B was identical except that it contained 60% acetonitrile.

2.2. *Cyanogen bromide cleavage*

Cleavage with cyanogen bromide was achieved by adding 50 μg CNBr (Sigma) to 100 μl of 0.1% trifluoroacetic acid containing 10 μg of native myotoxin. After 12 h at room temperature in the dark, the reaction was stopped by the addition of two volumes of distilled water.

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2.3. *Fractionation of CNBr peptides*

Peptide mixtures were fractionated by HPLC on an RP-300 aquapore reverse phase column (C_8, 300 Å, 2.1 × 100 mm, Applied Biosystems) using a Brownleee Microgradient HPLC syringe pump equipped with a Rheodyne 7125 injector. Column effluent was monitored at 214 nm using a Kratos 783 UV detector and fractions were collected by hand.

2.4. *Mass spectrometry*

Samples were analyzed on a JEOL JMS-HX110HF/HX110HF tandem double-focusing mass spectrometer, using MS-1 only. The instrument was operated at 10 kV accelerating voltage with resolution of 1:500 and the mass axis was calibrated with cesium iodide with an accurary of ± 0.3 Da. Sample (1 μl) was mixed with 1 μl of m-nitrobenzyl alcohol, on a gold-plated stainless steel FAB probe tip, and ions were formed by FAB using 10–20 keV Cs^+. Data were acquired as raw profiles using the ACM program of the JEOL DA-5000 data system. Masses are given as the chemical average mass of the peak centroid.

2.5. *Protein sequencing*

Automated Edman degradation was performed with an Applied Biosystems 470A gas phase sequencer equipped with a 120A PTH online amino acid analyzer. Phenylthiodydantoin-amino acid peaks were integrated with a Nelson Analytical model 2600 data system and sequence data were interpreted on a VAX 8650 according to the method of Henzel et al. [11].

3. RESULTS AND DISCUSSION

3.1. *Chromatography*

Fractionation of crude venom over Sephacryl S-200 HR resulted in 7 fractions (Fig. 1). Fraction 5 contained myotoxin *a* and the new myotoxin with relatively few contaminants. When subfactionated over Mono S at pH 4.0, 3 fractions resulted. Contaminating material from S-200 fractions 4 and 6 eluted in a heterogeneous peak early in the gradient (Fig. 2). The major, somewhat assymetric second peak contained myotoxin *a*, and the new myotoxin eluted last.

Reverse phase chromatography of Mono S fraction 3 yielded 4 fractions (Fig. 3). Fractions 1 and 2 both consisted of myotoxin *a* of identical mass, while fractions 3 and 4 both contained the new myotoxin, also of identical mass. When fraction 3 was passed back over PepPRC under identical conditions, contaminating myotoxin *a* was eliminated, but large amounts of fraction 4 were generated from fraction 3 (not shown). This suggests that fractions 3 and 4 may represent different charge or conformational states of the same primary structure. Glutamate residues may be implicated since the peak-doubling phenomenon is not seen when myotoxins are fractionated at pH 5.5.

3.2. *Amino acid sequence analysis*

Analysis of PepRPC fractions 1 and 2 (Fig. 3) by FAB mass spectrometry afforded mass spectra (not shown) containing abundant protonated molecular ions (MH^+) at *m/z* 4824.0 (Table I). The predicted MH^+ for authentic myotoxin *a* is 4822.8, thus, PepRPC fractions 1 and 2 were identified as myotoxin *a*. Analysis of PepRPC fractions 3 and 4 afforded the mass spectrum

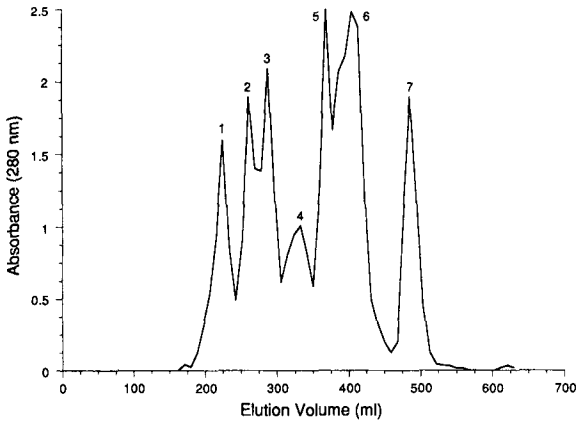

Fig. 1. Fractionation of crude prairie rattlesnake venom over a 2.5 × 95 cm column of Sephacryl S-200 HR. Myotoxin *a* and the new myotoxin eluted in fraction 5.

shown in Fig. 4. The major peak was at *m/z* 5240.0, 185 Da higher than the predicted mass of any known myotoxin.

Amino terminal sequence analysis using automated Edman degradation was performed on an aliquot of PepRPC fraction 3, identifying the first 29 residues (Table II). The derived amino terminal sequence was identical to those of *C. v. concolor* myotoxins I and II [8]. Heterogeneity at position 8, with similar amounts of both glycine and glutamic acid, was also reported in the *C. v. concolor* myotoxins [8].

The carboxyl terminus of the new myotoxin was sequenced after CNBr cleavage and disulfide bond reduction. The resulting peptides were separated by reverse phase HPLC. Analysis of CNBr fractions 1 and 2 by FAB mass spectrometry afforded two mass spectra with

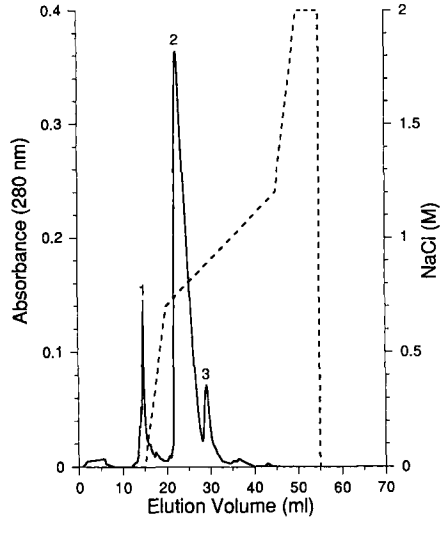

Fig. 2. Subfractionation of Sephacryl S-200 HR fraction 5 over a 0.5 × 5 cm Mono S HR column. Fraction 1 contained contaminating material from S-200 fractions 4 and 6. Fraction 2 contained myotoxin *a* while the new myotoxin eluted in fraction 3.

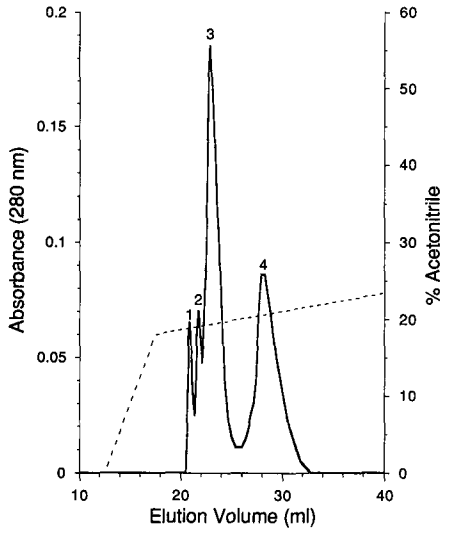

Fig. 3. Subfractionation of Mono S fraction 3 over a 0.5 × 5 cm PepRPC HR column. Fractions 1 and 2 both contained myotoxin *a* of identical mass (4926 Da). Fractions 3 and 4 both contained the novel myotoxin (5250 Da).

abundant MH^+ ions at *m/z* 2026.8 and 3189.2, respectively (Table I). The combined molecular masses observed for these two peptides sum to the observed mass of the novel myotoxin, 5040.2 [2026.8 (CNBr-1) + 3189.2 (CNBr-2) − 101 (homoserine lactone) + 131.1 (methionine) − 6 (minus 6 protons to form 3 disulfides) = 5040.1]. The mass of CNBr fraction 2 (3189.2), corresponds to the amino acid sequence of residues 1–28. CNBr fraction 1 was sequenced directly by automated Edman degradation and the resulting amino acid sequence is shown in Table II. This sequence corresponds to residues 29–45 and the observed mass of this peptide (2026.8) is in close agreement with the predicted mass (2027.4). Thus the novel myotoxin ends in asparaginyl-alanine and has a total of 45 amino acid residues, the longest small myotoxin presently known.

Minor peaks were observed in the mass spectrum of PepRPC fraction 3 (Fig. 3). Peaks observed at *m/z* 5168.9 and 5055.4 correspond to myotoxin sequences 3 and 4, respectively (Table I). Sequence 4 is identical to myotoxin I from *C. v. concolor* [8]; thus, the venom of this single *C. v. viridis* contains at least 3 different small myotoxins, based on length. At present no other species has been shown to possess more than one type. While the 43- and 45-residue myotoxins may simply result from differential post-template processing of the same

Table II

Sequence analysis of PepRPC fraction 3 and CNBr fraction 1.

Cycle	PepRPC fraction 3 Amino acid	Yield (pmol)	CNBr fraction 1 Amino acid	Yield (pmol)
1	Y	20.6	D	124
2	K	9.0	C	(a)
3	R	6.2	R	72.1
4	C	(a)	W	15.6
5	H	4.9	K	58.9
6	K	5.9	W	15.9
7	K	8.2	K	66.3
8	E,G	5.6,4.0	C	(a)
9	G	7.3	C	(a)
10	H	3.9	K	30.0
11	C	(a)	K	39.8
12	F	5.4	G	20.6
13	P	5.4	S	10.8
14	K	2.0	V	11.6
15	T	2.1	N	8.7
16	V	2.6	N	12.4
17	I	1.9	A	2.1
18	C	(a)		
19	L	1.7		
20	P	2.2		
21	P	3.5		
22	S	2.7		
23	S	1.6		
24	D	0.6		
25	F	0.6		
26	G	0.2		
27	K	0.3		
28	M	0.4		
29	D	0.2		

(a) Cysteine was identified by the presence of a dehydroalanine peak and the absence of a serine peak during the cycle.

Table I

Summary of mass spectrometric data obtained on PepRPC fractions 1 through 4 and after cyanogen bromide cleavage of PepRPC fraction 3.

Sequence	Fraction	MH^+ calculated	MH^+ observed
1. YKQCHKKGGHCFPKEKICIPPSSDLGKMDCRWKWKCCKKGSG	PepRPC-1 PepRPC-2	4822.8	4824.0
2. YKRCHKKEGHCFPKTVICLPPSSDFGKMDCRWKWKCCKKGSVNNA	PepRPC-3 PepRPC-4	5241.2	5240.0
3. YKRCHKKGGHCFPKTVICLPPSSDFGKMDCRWKWKCCKKGSVNNA	PepRPC-3 PepRPC-4	5169.2	5168.9
4. YKRCHKKEGHCFPKTVICLPPSSDFGKMDCRWKWKCCKKGSVN	PepRPC-3 PepRPC-4	5056.2	5055.4
5. YKRCHKKEGHCFPKTVICLPPSSDFGK(hS)	CNBr-2	3188.6	3189.2
6. DCRWKWKCCKKGSVNNA	CNBr-1	2027.4	2026.8

Masses are reported as the chemical average of the peak centroid. All observed masses are accurate to within 0.03%.

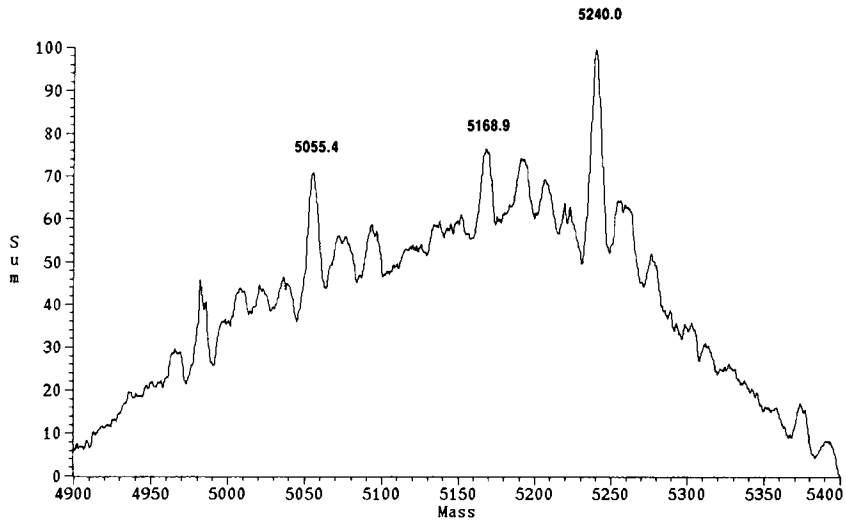

Fig. 4. FAB mass spectrum of PepRPC fraction 3.

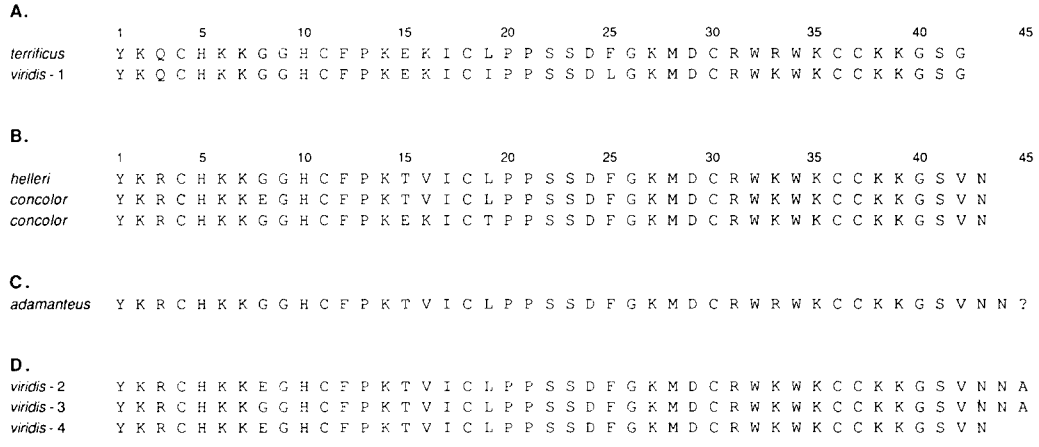

Fig. 5. Amino acid sequences of small myotoxins. The new myotoxin is most similar to myotoxin I from *C. v. concolor* rather than to myotoxin *a* from *C. v. viridis*. Literature references are as follows: (A) 42-residue myotoxins, *C. d. terrificus* [3]; *C. v. viridis* [5]; (B) 43-residue myotoxins, *C. v. helleri* [6]; *C. v. concolor* [8]; (C) Dimeric myotoxin, *C. adamanteus* [9]. (D) New myotoxins from *C. v. viridis* venom, present study.

gene, differential processing cannot account for the presence of myotoxin *a*. The present work lends additional support to the suggestion by Aird et al. [13] that the myotoxin gene has been duplicated in *C. v. viridis*.

3.3. *Nomenclatural suggestions*

Seven different names for small myotoxins are currently established in the toxinological literature, even though no names have been assigned to the increasing number of structural variants of myotoxin *a* [13] and crotamine [14]. It seems unwise to assign different names to simple amino acid substitutions in the same primary structure. However, it may be useful to distinguish between the various lengths of myotoxin, especially in *C. v. viridis*, where several sizes occur. It is also unlikely that *C. v. viridis* will remain an isolated case. It would probably be best to refer to these molecules by length (42-residue myotoxins, 45-residue myotoxins, etc). This would be more descriptive than referring to them as Class 1, 2, 3 or Type A, B, C, etc. We strongly recommend dispensing with trivial names such as crotamine, Peptide C, Toxin E, etc., which indicate neither function nor homology within the group.

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