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MULTIPLE MYOTOXIN SEQUENCES FROM THE VENOM OF A SINGLE PRAIRIE RATTLESNAKE (*CROTALUS VIRIDIS VIRIDIS*)

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S. D. AIRD, W. G. KRUGGEL and I. I. KAISER. Multiple myotoxin sequences from the venom of a single prairie rattlesnake (*Crotalus viridis viridis*). *Toxicon* **29**, 265–268, 1991.—Multiple myotoxin *a* sequences have been determined from the venom of a single adult male prairie rattlesnake (*Crotalus viridis viridis*). This is the first time such individual variation has been reported for this toxin class and the number of isoforms suggest that myotoxin *a* is the product of a duplicated locus.

CROTAMINE, a basic polypeptide (p*I* 10.3) of 42 residues from the venom of *Crotalus durissus terrificus* was first reported by GONÇALVES and VIEIRA (1950). Its primary structure was determined 25 years later by LAURE (1975). In recent years, several crotamine homologs have been reported, including myotoxin *a* (CAMERON and TU, 1977; FOX *et al.*, 1979) from the venom of *C. viridis viridis*, peptide C (MAEDA *et al.*, 1978) from the venom of *C. v. helleri*, myotoxins I and II from the venom of *C. v. concolor* (ENGLE *et al.*, 1983; BIEBER *et al.*, 1986), and myotoxin from the venom of *C. adamanteus* (SAMEJIMA *et al.*, 1987). The studies on *C. v. concolor* were the first to report the presence of more than one myotoxin primary structure from the venom of a single species. In contrast to crotamine and myotoxin *a*, which contain 42 residues, peptide C and the two *concolor* myotoxins consist of 43 residues, and the *adamanteus* myotoxin is comprised of at least 44 residues. The differences result from a C-terminal valyl-asparagine in place of glycine-42 in the 43-residue myotoxins, and a C-terminal valyl-asparagyl-asparagine in the *adamanteus* myotoxin. A myotoxin called toxin E also has been sequenced from the venom of *Crotalus h. horridus*. It contains 42 residues and is nearly identical in primary structure to crotamine and myotoxin *a* (C. R. GEREN and J. W. FOX, personal communication).

AIRD and DESSAUER (Geographic variation in venom and blood proteins of *Crotalus viridis*. Abstracts, 57th Annual Meeting of the American Society of Ichthyologists and Herpetologists, 1977) observed the presence of 3–4 poorly resolved, but intensely stained myotoxin bands in individual venoms of several *Crotalus viridis* subspecies using starch gel

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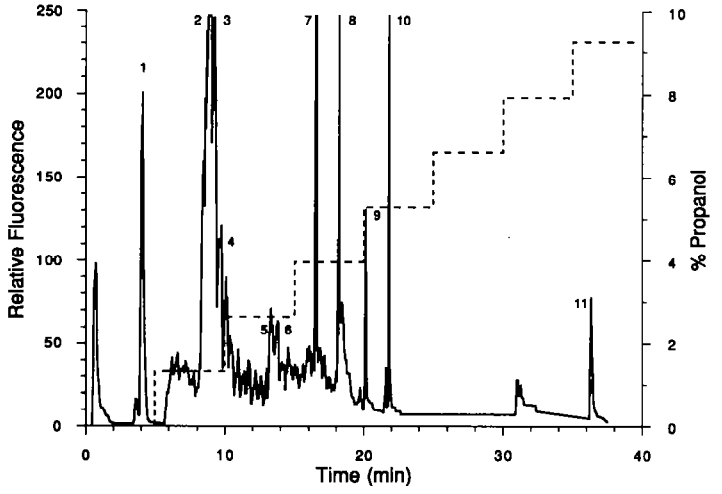

FIG. 1. REVERSE PHASE (C_{18} HPLC PROFILE OF S-200 FRACTION 4).
Buffer A contained 0.5 M acetic acid/pyridine (pH 4.0). Buffer B was identical except that it contained 40% 1-propanol. Peaks 1–6 were sequenced. Undampened pump pulses in this home-made HPLC system resulted in considerable baseline fluctuation.

electrophoresis, although at that time the identity of the bands was unknown. These early findings have been confirmed subsequently with non-denaturing polyacrylamide gel electrophoresis in 18% gels and with reverse phase HPLC (AIRD, unpublished results). Using PAGE it is nearly impossible to obtain discrete myotoxin bands even with SDS and in the presence of dithiothreitol or β-mercaptoethanol.

Individual heterogeneity of snake venom toxins has been generally ignored because most toxinologists work with pooled commercial samples. Until ENGLE *et al.* (1983) no one reported any heterogeneity, even in pooled samples, which should contain all of the isomers represented among the constituent individual samples.

This study examined venom of a single adult male prairie rattlesnake (*Crotalus viridis viridis*) collected by SDA at Strawberry Creek, Moffat County, CO, close to the contact zone with the midget faded rattlesnake (*Crotalus viridis concolor*). Venom was extracted manually and frozen at −20°C until use. Prior to use venom was centrifuged to remove insoluble material. Wet crude venom was then diluted slightly with 0.1 M sodium acetate (pH 4.0) and fractionated over a 1.5 × 95 cm column of Sephacryl S-200 equilibrated in the same buffer (AIRD, 1985). The fourth S-200 fraction, which contained primarily myotoxin *a* was then subfractionated by reverse phase HPLC, as previously described (AIRD *et al.*, 1985; LEWIS, 1984) and sequenced on an Applied Biosystems 470A protein sequencer, also as described previously (AIRD *et al.*, 1985).

Reverse phase HPLC of S-200 Fraction 4 yielded multiple peaks, which included myotoxins, hypotensive peptides, and unidentified compounds. Twelve major peaks were separated (Fig. 1); six contained myotoxins (Fig. 2). Additional peaks may also have contained myotoxins, but were not sequenced.

The reverse phase chromatography of these small myotoxins displayed peculiarities that have also been encountered by SMITH and SCHMIDT (1990). In several instances, the same primary sequence appeared in peaks having substantially different relative hydrophobicity. Studies employing mass spectrometry (P. R. GRIFFIN and S. D. AIRD, unpublished results) have suggested that this phenomenon may result from protonated and de-

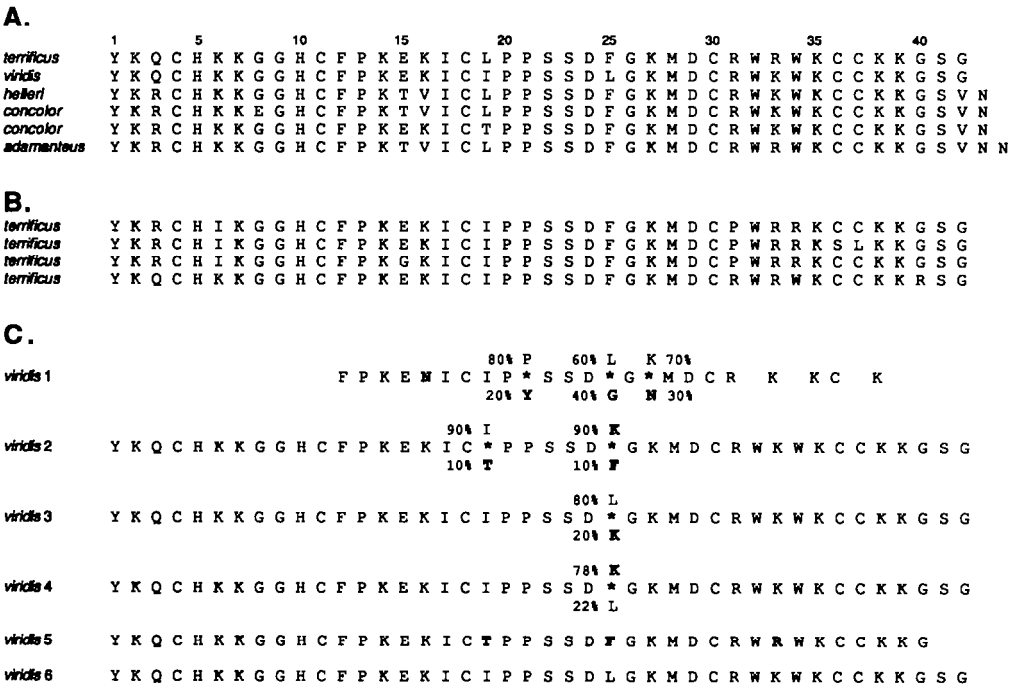

FIG. 2. MYOTOXIN SEQUENCES FROM THE VENOM OF A SINGLE *Crotalus viridis viridis* COMPARED WITH PUBLISHED SEQUENCES.

A. Literature references are as follows: *C. d. terrificus*, LAURE (1975); *C. v. viridis*, FOX *et al.* (1979); *C. v. helleri*, MAEDA *et al.* (1978); *C. v. concolor*, BIEBER *et al.* (1986); *C. adamanteus*, SAMEJIMA *et al.* (1987). B. Four *C. d. terrificus* cDNA sequences from SMITH and SCHMIDT (1990). C. Novel sequences from the present investigation. Previously only positions 3, 6, 8, 15, 16, 19, 25, and 42 were known to be variable, based on protein sequence and considering all taxa together. An asterisk indicates a position where two different amino acid residues were detected at the same position. The approximate percentage of each is indicated. Bold-faced residues are previously unreported for *C. v. viridis*.

protonated glutamate residues. At pH 4 it is possible to isolate well-separated peaks with identical masses, whereas at pH 5.5, duplicate peaks are not seen.

In addition, several myotoxin peaks contained more than one homolog. This would not be surprising in instances where the substitutions involved residues of similar relative hydrophobicity (e.g. lysine for asparagine) (MEEK and ROSSETTI, 1981); however, we found peaks containing mixtures of phenylalanine or leucine with lysine, or leucine with glycine, at position 25 (Fig. 2). In peptides of more than 20 residues, tertiary structure has the potential to minimize or prevent interaction of some buried residues with the stationary phase of the column (MEEK and ROSSETTI, 1981), which may explain the coelution of significantly different isoforms.

One of the six peaks sequenced, which aborted at residue 40 (C-5, Fig. 2), displayed threonine-19, which has been reported previously only in *C. v. concolor*, and phenylalanine-25 which has been found in all myotoxins except myotoxin *a*. It also contained arginine-33 which was previously known only from *C. d. terrificus* and *C. adamanteus*. A second sequence (C-1, Fig. 2) contained an 11-residue contaminating peptide that bears some resemblance to a hypotensive peptide, in that it contained four prolines. However, commencing with residue 12, the sequence was that of a myotoxin. It is possible that the

small peak eluting on the lead edge of Peak 1 (Fig. 1) contained the myotoxin and that the 11-residue peptide comprised Peak 1. This was suggested by a dramatic drop in HPLC peak height commencing with residue 12. Sequence C-1 contained asparagine at position 16, tyrosine-21, glycine-25 and asparagine-27, none of which have been reported previously (Fig. 2). That sequence also aborted prematurely at position 38 and there were some missing residues along the way (Fig. 2).

SMITH and SCHMIDT (1990) reported multiple crotamine sequences from a cDNA library derived from venom glands of three adult *C. d. terrificus*. Among 400,000 plaques, 800 positives were identified. Only four of these were sequenced, but they were all different and they included a number of novel structural variants. These included isoleucine-6, glycine-15, proline-31, arginine-34, serine-36, leucine-37, and arginine-40. Sequencing of pyridylethylated crotamine confirmed the existence of isoleucine-6, and, in fact, a mixture of isoleucine and lysine was found at that position in all crotamine fractions. Arginine-34 was also confirmed by sequencing, but none of the other cDNA sequences were observed suggesting that some potentially dysfunctional genes (serine-36, leucine-37) may not be expressed.

Results obtained in this study suggest that myotoxin *a* is probably the product of a duplicated locus, which may explain the plethora of forms seen in pooled venom samples and the chromatographic and electrophoretic difficulties encountered in working with these molecules.

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