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Amino acid sequence of versutoxin, a lethal neurotoxin from the venom of the funnel-web spider *Atrax versutus*

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The complete amino acid sequence of versutoxin, a lethal neurotoxic polypeptide isolated from the venom of male and female funnel-web spiders of the species *Atrax versutus*, was determined. Sequencing was performed in a gas-phase protein sequencer by automated Edman degradation of the *S*-carboxymethylated toxin and fragments of it produced by reaction with CNBr. Versutoxin consisted of a single chain of 42 amino acid residues. It was found to have a high proportion of basic residues and of cystine. The primary structure showed marked homology with that of robustoxin, a novel neurotoxin recently isolated from the venom of another funnel-web-spider species, *Atrax robustus*.

INTRODUCTION

Funnel-web spiders (*Atrax* sp.) are a group of venomous arachnids indigenous to Australia. In all, 33 species have so far been described [1]. Of the 14 human fatalities so far reported after envenomation by funnel-web spiders, all have been attributed to male *A. robustus* [2]. The characterization of the constituents of the venom of male *A. robustus* was recently undertaken in our laboratories, and a lethal neurotoxin, termed robustoxin, a polypeptide of 42 residues, was isolated and its amino acid sequence determined [3]. The venom of female *A. robustus*, however, was found to lack this toxin.

A major factor in the lower incidence of cases of serious envenomation of humans by other funnel-web species is clearly related to distribution. *A. robustus* inhabits the Sydney metropolitan area (Australia's largest city), whereas other species that contain venom of equal or greater potency (as determined by standard lethality assays with newborn mice [4]) occur in regions of lower human population densities. One such species is *A. versutus*, which is located in the hilly areas west of Sydney. Investigations have shown that the venom of both male and female *A. versutus* contained a polypeptide toxin having chromatographic and electrophoretic properties similar to, but not identical with, robustoxin [4]. We now report on the purification and characterization of this neurotoxin, which we name 'versutoxin' and which proved to be the sole lethal toxin in male and female *A. versutus* venom. The primary structure of versutoxin was determined by sequencing the entire reduced *S*-carboxymethylated toxin. Confirmation of the structure was obtained by sequencing CNBr fragments of the toxin derivative. We compare the sequence obtained with that of robustoxin.

EXPERIMENTAL

Materials

The venom was collected from mature male and female *Atrax sp.* by aspiration of the venom from the tip of live spiders' fangs as described previously [4]. Robustoxin was purified from the venom of male *A. robustus* by cation-exchange chromatography [4,5]. All glassware used in the collection and treatment of venoms and toxins was silylated by treatment with Coatasil (Ajax Chemicals, Auburn, N.S.W., Australia).

Fractionation of the venom of female *A. versutus*

Venom samples from female *A. versutus* were first fractionated by ion-exchange chromatography by using a column (1.6 cm × 30 cm) of CM-Sephadex C-25 equilibrated with 0.1 M-ammonium acetate (Fig. 1). The column effluent was monitored at 280 nm, and fractions (5 ml) were collected. Fractions selected on the basis of the elution profile were pooled and freeze-dried after dilution with 2 vol. of distilled water. The dried fractions generally required dissolution in a minimum volume of water and re-freeze-drying a further two times to remove all the eluant salt. Fractions were assayed for lethality in

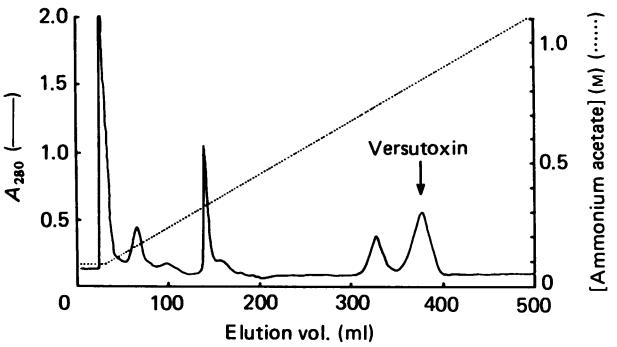

Fig. 1. Ion-exchange chromatography of crude female *A. versutus* venom

Venom (120 mg) was dissolved in 100 ml of distilled water, the pH adjusted to 7.0 with concentrated aq. NH_3 and applied to a column (1.6 cm × 30 cm) of CM-Sephadex C-25. Elution was carried out with a linear ionic-strength gradient of 0.1–1.1 M-ammonium acetate solution, pH 7.0, using a flow rate of 20 ml/h. Fractions of volume 5 ml were collected.

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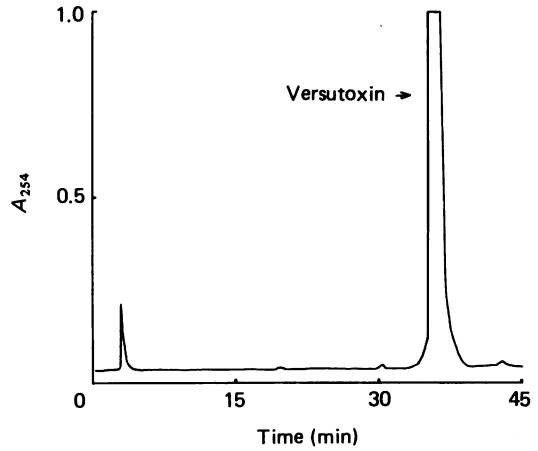

Fig. 2. Reverse-phase h.p.l.c. of partially-purified versutoxin

Sample (3.4 mg/ml) was applied to a Waters C_{18} μBondapak column (7.8 mm × 30 cm) and a linear gradient of 0–60% (v/v) acetonitrile in 0.05% (v/v) trifluoroacetic acid was applied over 45 min at a flow rate of 1.4 ml/min. The versutoxin peak was unsplit when all of it was on scale.

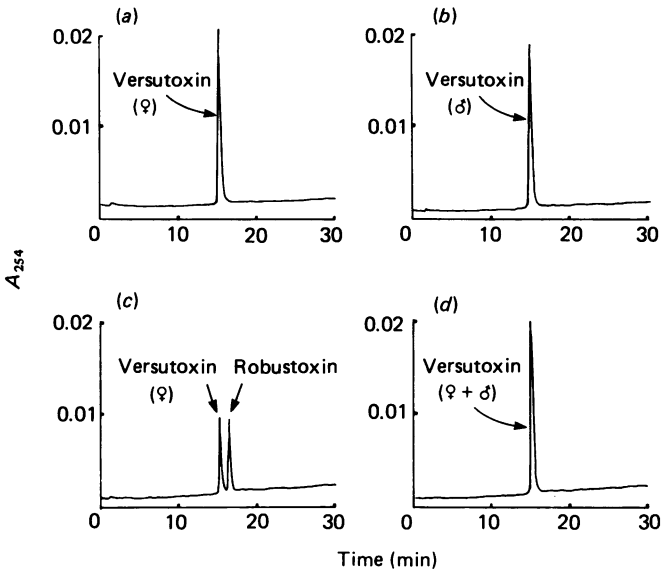

Fig. 3. Analysis of purified samples of versutoxin and robustoxin by h.p.l.c.

Samples (2–10 μg/10 μl) were applied to a TSK TMS-250 C_1 column (4.6 mm × 7.5 cm) and a linear gradient of 0–60% (v/v) acetonitrile in 0.05% (v/v) trifluoroacetic acid was applied over 30 min with a flow rate of 1.0 ml/min. Samples were (*a*) 10 μg of versutoxin from female *A. versutus*, (*b*) 10 μg of versutoxin from male *A. versutus*, (*c*) a mixture containing 5 μg each of versutoxin (from female *A. versutus*) and robustoxin, and (*d*) a mixture containing 5 μg each of versutoxin from male and female *A. versutus*.

newborn mice as described below. Material showing lethality was subjected to further purification by preparative reverse-phase h.p.l.c. using a Waters Associates μBondapak C_{18} (7.8 mm × 30 cm) column (Fig. 2). The venom of male *A. versutus* was fractionated in a manner identical with that described above. However, the stock of venom was limited, so only 20 mg could be processed.

Analytical h.p.l.c. was utilized to assess the final purity of lethal fractions from male and female *A. versutus* venom and to compare them with robustoxin by a TSK TMS 250 C_1 column (10 μM particle size, 4.6 mm × 7.5 cm; Fig. 3).

Assessment of lethality

Fractions were tested for lethality after subcutaneous injections into newborn mice as previously described [4,6]. Mice (1.5–1.8 g body wt.) received injections of test material from column fractions at doses between 0.1 and 1.0 μg; fractions showing little or no lethality were tested at doses up to 1 mg/mouse.

Reduction and *S*-carboxymethylation of polypeptides

The reduction and *S*-carboxymethylation of polypeptides was performed as described by Kung *et al.* [7], with minor modification. Samples (1 mg), after reduction and *S*-carboxymethylation as described above, were diluted to 10 ml with 0.1% (v/v) trifluoroacetic acid, and then applied to a C_{18} Sep-Pak (Waters Associates) column previously equilibrated with 15 ml of 0.1% trifluoroacetic acid. After loading the sample, a wash was performed with 20 ml of 0.1% trifluoroacetic acid; then the material of interest was eluted with 5 ml of 0.1% trifluoroacetic acid in acetonitrile/water (1:1, v/v). This fraction was collected and freeze-dried.

Amino acid analysis

The method used for the hydrolysis of polypeptides was essentially that described by Simpson *et al.* [8]. To 20 μg of reduced *S*-carboxymethylated polypeptide in a Waters hydrolysis tube (Millipore Corp.) was added 30 μl of 4 M-methanesulphonic acid containing 0.2% (w/v) tryptamine; the solution was then placed in the Waters hydrolysis vessel, together with an additional 500 μl of the acid solution. The vessel was evacuated and placed in an oven at 108 °C for 24 h. The hydrolysate was diluted to 4 ml with water, then 20 μl of triethylamine were added. A 1 ml portion of this solution was dried under vacuum and then redried after the addition of 20 μl of a 'redrying' solution [ethanol/water/triethylamine, 2:2:1 (by vol.)]. The material was then derivatized with phenyl isothiocyanate and analysed by h.p.l.c. using the Waters Pico-Tag method [9].

This analysis gave complete resolution of all the amino acid derivatives, except that it was unable to discriminate between cystine and cysteine. To overcome this limitation, carboxymethylated samples (20 μg; samples were prepared in a fashion identical with that described for reduced *S*-carboxymethylated samples, except that no reducing agents were used) were subjected to hydrolysis as described above. The products were diluted to 2.0 ml, then the solution was carefully adjusted to pH 8.0 with triethylamine. Dithiothreitol (600 μg/200 μl of water) was then added, and the solution was flushed with nitrogen and incubated at 30 °C for 2 h. Then 10 mg of disodium tetrathionate was added and the reaction allowed to proceed overnight at room temperature. Under these conditions cysteine was converted into carboxymethylcysteine, whereas cystine was oxidized to *S*-sulphocysteine. Samples (200 μl and 400 μl) were taken at the end of the incubation, dried, coupled by the Waters Associates Pico-Tag method, and then analysed by h.p.l.c. as described above.

Polypeptides were also analysed after hydrolysis with

Table 1. Amino acid composition of reduced and carboxymethylated versutoxin

Values are given for versutoxin derived from the venoms of both male and female *A. versutus* and after a 24 h hydrolysis by either 6 M-HCl or 4 M-methanesulphonic acid (MSA) containing 0.2% (w/v) tryptamine. All cysteine was found to be in the oxidized (cystine) form. Abbreviation: n.d., not determined.

Amino acid	Composition (residues/molecule)				
	From hydrolysis with:				
	6 M-HCl		4 M-MSA		From sequence
	Male	Female	Male	Female	
Asx	1.9	2.4	3.1	2.9	3
Glx	3.6	3.8	4.0	4.0	4
Ser	2.9	2.9	3.2	2.9	3
Gly	2.1	2.3	2.2	2.0	2
His	0.0	0.0	0.0	0.0	0
Arg	1.1	1.2	1.1	1.0	1
Thr	1.9	1.9	1.8	1.8	2
Ala	3.0	3.1	2.9	3.0	3
Pro	1.0	1.3	1.2	1.1	1
Tyr	1.7	1.9	1.8	1.8	2
Val	0.9	0.9	0.8	0.9	1
Met	0.8	1.0	1.0	1.1	1
Cys	6.4	6.5	6.7	6.8	8
Ile	1.1	1.1	0.9	1.0	1
Leu	0.9	1.1	1.2	1.0	1
Phe	0.0	0.0	0.0	0.0	0
Lys	5.5	6.1	5.5	5.9	6
Trp	n.d.	n.d.	2.4	2.5	3

constant-boiling HCl. Fractions (20 μg) were hydrolysed by incubation at 108 °C in the presence of 6 M-HCl and 0.02% (v/v) phenol for 24 h. The hydrolysates were dried *in vacuo*, then derivatized with phenyl isothiocyanate and analysed as described above. The results are given in Table 1.

CNBr cleavage of reduced *S*-carboxymethylated polypeptides

The cleavage of reduced *S*-carboxymethylated peptides with CNBr was performed by the method of Hempel *et al.* [10]. The sample (1 mg) was dissolved in 150 μl of 70% (v/v) formic acid; then 4 mg of CNBr was added. The reaction vial was sealed, and the contents allowed to react at room temperature for 24 h. The solution was then diluted with 5 ml of ice-cold water and freeze-dried. The freeze-dried material was then fractionated by preparative reverse-phase h.p.l.c. (Fig. 4). Fractions were collected, freeze-dried and subjected to amino acid sequencing.

Amino acid sequence determination

The amino acid sequence of reduced *S*-carboxymethylated peptides was determined directly with a model 470A automatic gas-phase polypeptide sequencer (Applied Biosystems, Foster City, CA, U.S.A.) essentially by the methods of Hewick *et al.* [11] and using a standard Edman-degradation sequenator program. Samples in 0.1% (v/v) trifluoroacetic acid were applied to the filter,

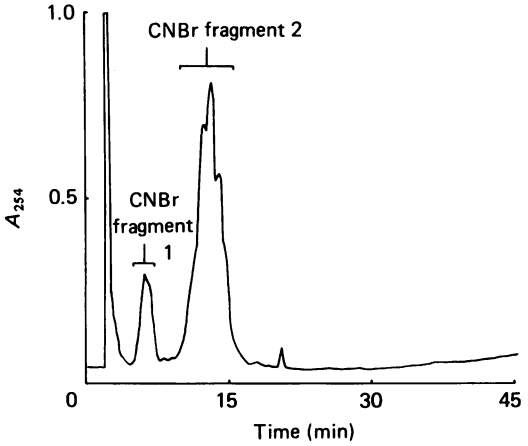

Fig. 4. H.p.l.c. separation of fragments produced by CNBr treatment of reduced and carboxymethylated versutoxin

The sample (1 mg/ml) was applied to a Waters C_{18} μBondapak column (7.8 mm × 30 cm), and a linear gradient of 0–60% (v/v) acetonitrile in 0.05% (v/v) trifluoroacetic acid was applied over 45 min at a flow rate of 1.4 ml/min.

Table 2. Repetitive yields for amino acid sequence of reduced and *S*-carboxymethylated versutoxin

Residue	Cycle nos.	Repetitive yield (%)
C	1, 8	94.0
C	1, 14	90.3
C	1, 31	89.2
A	2, 23	91.9
A	2, 37	87.7
K	3, 10	92.0
K	3, 19	90.5
K	3, 40	88.1
N	6, 26	92.0
W	7, 24	91.1
W	7, 39	85.4
G	9, 29	92.0
E	12, 27	90.3
	Average . . .	90.3

which had been conditioned with trifluoroacetic acid and Biobrene (Applied Biosystems). Sequencing was then commenced, and continued until four consecutive blank cycles were encountered. The average repetitive yield was 90.3% (Table 2).

C-Terminal amino acid sequence

Reduced *S*-carboxymethylated polypeptide (100 nmol) was digested with carboxypeptidase Y (Sigma; 13 nmol) in 400 μl of pyridine/acetate buffer, pH 5.6, by the method of Klemm [12]. Aliquots (25 μl) were withdrawn at 0, 1, 2, 5, 10, 30, 60, 120 and 240 min, and after 22 h. Acetic acid (5 μl) was added immediately, and the samples were frozen until they were dried in a centrifugal evaporator and subsequently re-dried after the addition of 20 μl of ethanol/water triethylamine (2:2:1, by vol.). The phenthiocarbamoyl derivatives of amino acids were prepared by addition of 30 μl of ethanol/water/tri-

ethylamine/phenyl isothiocyanate (7:1:1:1, by vol.). After 20 min the samples were dried under vacuum and analysed by h.p.l.c. using the Pico-Tag system. The digestion appeared to proceed more slowly than described in the literature [12]. After 60 min, *S*-carboxymethylcysteine was detected and, after 22 h, lysine was found to be present. Concurrently, as a control, *S*-carboxymethylated lysozyme was also digested with carboxypeptidase Y under the same conditions. The *C*-terminal residue, leucine, followed by arginine, were detected at similar times to the *C*-terminal residues from versutoxin.

RESULTS

Ion-exchange chromatography of *A. versutus* venom

The elution profile of the venom of female *A. versutus* is presented in Fig. 1. This profile was found to be similar in outline to that of the venom of male *A. robustus* [4]. Of the major peaks evident, only one (which had a similar elution volume to that of robustoxin) showed lethality towards newborn mice.

Purification of the fraction possessing lethality

The column fraction showing lethality was further resolved by preparative h.p.l.c. (Fig. 2). The yield of the purified toxin was 3.2 mg from 120 mg (equivalent to 200 'milkings') of crude female *A. versutus* venom. The purity of this final fraction was confirmed by analytical h.p.l.c. It was found to have an elution volume different from that of robustoxin (Fig. 3). The LD_{50} (dose causing death in 50% of the animals treated) for newborn mice of this purified toxin, versutoxin, was found to be 0.22 mg/kg. That previously determined for robustoxin was 0.16 mg/kg [4].

Amino acid analysis of versutoxin

The amino acid composition of vesutoxin determined after hydrolysis of the reduced *S*-carboxymethylated sample both with 4 M-methanesulphonic acid containing 0.2% (w/v) tryptamine and with constant-boiling HCl is given in Table 1. In an additional experiment in which the carboxymethylated toxin was hydrolysed with methanesulphonic acid and then oxidized by treatment with sodium tetrathionate, the proportion of cysteine to cystine was determined. Versutoxin, like robustoxin, was found to have no free cysteine. All cysteine was present in the oxidized form (cystine). This latter analysis did not allow complete quantification of all amino acids, since during h.p.l.c. analysis the tetrathionate was co-eluted with some of the polar amino acids, such as glutamate and aspartate.

Chromatography of male *A. versutus* venom

Fractionation of male *A. versutus* venom by ion-exchange chromatography gave an elution pattern similar to that observed for venom of female *A. versutus*. A component was isolated with an elution volume identical with that of versutoxin from female *A. versutus* venom. This component was the only lethal material in the venom. It had the same lethality in newborn mice, identical elution volume by analytical h.p.l.c. (Fig. 3), and the same amino acid composition as versutoxin isolated from the venom of female spiders. It was concluded that it was identical with versutoxin. The yield of versutoxin from male *A. versutus* was approx. 300 μg from 20 mg of crude dried venom.

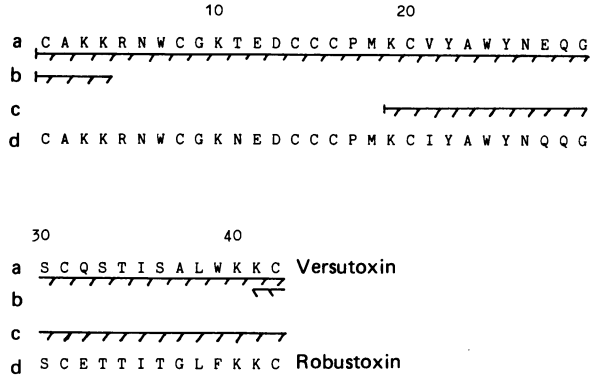

Fig. 5. Amino acid sequence of versutoxin

a, Complete sequence of reduced *S*-carboxymethylated versutoxin (repetitive yields are given in Table 2); b, CNBr fraction 1 (Fig. 4); c, CNBr fraction 2 (Fig. 4); d, robustoxin [3].

Amino acid sequence of versutoxin

The complete amino acid sequence of versutoxin was determined from the entire intact reduced *S*-carboxymethylated toxin. Fragments obtained by CNBr cleavage of the reduced *S*-carboxymethylated toxin were sequenced for confirmation. The results are shown in Fig. 5. Cleavage of the reduced *S*-carboxymethylated polypeptide with CNBr produced two main peptide fragments that were easily separated by h.p.l.c. (Fig. 4). Sequencing of the first-to-be-eluted fragment was hindered by low sequence yields, which were attributed to both incomplete recovery of the fraction after cleavage and to poor retention on the filter within the sequencer. However, the first four residues of this fragment coincided with those of the *N*-terminus obtained by sequencing the intact toxin. The sequence of the second CNBr fragment coincided with the sequence obtained beyond residue 18 in the toxin. No difficulty was encountered in the identification of any amino acids within the sequence of this fragment. After cycle 24 (cysteine), five blank cycles were produced. This fragment, which overlapped the sequence of the toxin, was therefore taken to be the *C*-terminus of versutoxin. The *C*-terminal sequence of versutoxin (-Lys-Cys) was confirmed by digestion of the intact reduced *S*-carboxymethylated toxin with carboxypeptidase Y.

The sequence of versutoxin, derived as above, was totally consistent with its amino acid composition and is shown in Fig. 5, where it is compared with that of robustoxin.

DISCUSSION

The identification of the lethal toxin, versutoxin, in the venoms of both male and female *A. versutus* was achieved after fractionation of the venoms by cation-exchange chromatography and h.p.l.c. The identity of the toxins purified from the venoms of male and female spiders was inferred on the basis of identical chromatographic elution volumes, lethalities and amino acid compositions. The comparable lethal potency of the venoms of male and female *A. versutus* [4] is consistent with this conclusion. These results contrast with those for robustoxin, which

was found only in the venom of male *A. robustus*, not in that of female spiders [4]. Versutoxin was shown to have chromatographic properties and amino acid composition similar to, but distinct from, those of robustoxin.

Versutoxin was found to possess similar lethality to robustoxin, as indicated by the LD_{50} assay in newborn mice. Although the precise basis of action of these toxins has not been fully elucidated, they both appear to be neurotoxic. Injection of robustoxin or versutoxin (10–20 μg intravenously) into anaesthetized monkeys produced symptoms such as severe disturbances in respiration, blood pressure and heart rate, lachrymation, salivation, skeletal-muscle fasciculation, increase in body temperature and increased firing in motor and autonomic nerves [13,14]. However, neither *A. versutus* venom nor versutoxin produced the characteristic sustained hypotension which followed injection of monkeys with male *A. robustus* venom or robustoxin [14].

The amino acid sequence of versutoxin was established by utilizing automated Edman degradation and h.p.l.c. identification of the cleaved amino acid phenylthiohydantoin derivatives [11] and was in full accord with the amino-acid-analysis data. The sequence of versutoxin was found to have close homology with robustoxin. Both toxins consist of 42 amino acid residues (M_r of versutoxin: 4852; that of robustoxin: 4854), have a high proportion of basic residues and contain four cystine residues with no free cysteine. This last property indicates that versutoxin, like robustoxin, is a tightly folded polypeptide. Also, disulphide-bridged cysteine residues at both the *N*- and *C*-termini and as a triplet at residues 14–16 are common features of the toxins, and which appear to have no precedent among other neurotoxins [3]. In total, eight of the 42 residues were found to differ between the two toxins; these occurred at residues 11, 21, 27, 32, 33, 36 and 39. All observed differences were conservative, in that the differing residues had side chains of a similar nature. Moreover, all differences, with one exception, could be explained by point mutations from an ancestral gene [15]. The sole exception was residue 39 (tryptophan in versutoxin, and phenylalanine in robustoxin). Continued investigations into the sequences of toxins of other funnel-web species that are, as yet, uncharacterized may provide further insight into the phylogeny of the genus *Atrax*.

Hydrophilicity [16,17] and antigenicity [18] plots of the amino acid sequences of versutoxin and robustoxin indicated that the *N*-terminal region, which is identical for the first ten residues in each toxin, is a likely antigenic site. We therefore predict antigenic cross-reactivity between the two toxins.

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REFERENCES

1. Gray, M. R. (1981) Aust. Nat. Hist. **20**, 256–270
2. Gray, M. R. & Sutherland, S. K. (1978) in Arthropod Venoms (Bettini, S., ed.), pp. 121–148, Springer-Verlag, Berlin
3. Sheumack, D. D., Claassens, R., Howden, M. E. H. & Whiteley, N. M. (1985) FEBS Lett. **181**, 154–156
4. Sheumack, D. D., Baldo, B. A., Carroll, P. R., Hampson, F., Howden, M. E. H. & Skorulis, A. (1984) Comp. Biochem. Physiol. **78C**, 55–68
5. Sheumack, D. D., Carroll, P. R., Hampson, F., Howden, M. E. H., Inglis, A. S., Roxburgh, C. M., Skorulis, A. & Strike, P. M. (1983) Toxicon Suppl. **3**, 397–400
6. Sutherland, S. K. (1980) Med. J. Aust. **2**, 437–441
7. Kung, S. D., Katsuhiro, S. & Wildman, S. G. (1974) Biochim. Biophys. Acta **365**, 138–147
8. Simpson, R. J., Neuberger, M. R. & Liu, T. Y. (1976) J. Biol. Chem. **251**, 1936–1940
9. Waters Associates (1984) Operator's Manual no. 88140 (Pico-Tag Amino Acid Analysis System), Waters Associates, Milford, MA
10. Hempel, J., Buhler, R., Kaiser, R., Holmquist, B., De Zalenski, C., Von Wartburg, J.-P., Vallee, B. & Jornvall, H. (1984) Eur. J. Biochem. **145**, 437–445
11. Hewick, R. M., Hunkapiller, M. W., Hood, L. E. & Dreyer, W. J. (1981) J. Biol. Chem. **256**, 7990–7997
12. Klemm, P. (1984) in Methods in Molecular Biology (Walker, J. M., ed.), vol. 1, pp. 255–259, Humana Press, Clifton, NJ
13. Mylecharane, E. J., Spence, I., Sheumack, D. D. & Howden, M. E. H. (1984) Clin. Exp. Pharmacol. Physiol., Suppl. **8**, 126–127
14. Phillips, C. A., Spence, I., Mylecharane, E. J., Brown, M. R., Sheumack, D. D., Claassens, R. & Howden, M. E. H. (1987) Clin. Exp. Pharmacol. Physiol. Suppl. **10**, 14–15
15. Min Jou, W., Haegeman, G., Ysebaert, M. & Fiers, W. (1972) Nature (London) **237**, 83
16. Hop, T. P. (1986) J. Immunol. Methods **88**, 1–18
17. Parker, J. M. R., Guo, D. & Hodges, R. S. (1986) Biochemistry **25**, 5425–5432
18. Welling, G. W., Weijer, W. J., van der Zee, R. & Welling-Wester, S. (1985) FEBS Lett. **188**, 215–218