Complete amino acid sequence of a new type of lethal neurotoxin from the venom of the funnel-web spider *Atrax robustus*

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Robustoxin, the lethal neurotoxin isolated from the venom of the male Sydney funnel-web spider, *Atrax robustus*, is of unique structural type and physiological mode of action. The primary structure of this 42-residue peptide was determined to be H_2N-Cys-Ala-Lys-Lys-Arg-Asn-Trp-Cys-Gly-Lys[18]-Asn-Glu-Asp-Cys-Cys-Cys-Pro-Met-Lys-Cys[28]-Ile-Tyr-Ala-Trp-Tyr-Asn-Gln-Gln-Gly-Ser[39]-Cys-Gln-Thr-Thr-Ile-Thr-Gly-Leu-Phe-Lys[40]-Lys-Cys-H. The dispostition of disulphide-bridged cysteine residues at both the amino- and carboxy-termini and as a triplet at residues 14–16 appears to have no precedent amongst neurotoxins.

Spider venom *Amino acid sequence* *Polypeptide neurotoxin* Atrax robustus *Robustoxin*

1. INTRODUCTION

The Sydney funnel-web spider, *Atrax robustus*, may be considered to be the world's most dangerous spider because of its strength and aggressiveness, the lethal potency of its venom, its nocturnal habit, and its penchant for invading houses. At least 13 deaths attributed to the bite of the male spider have been recorded since 1927 [1]. Its distribution coincides with the metropolitan area of Australia's largest city. Unlike other poisonous spiders, male rather than female *A. robustus* is dangerous to man [2]. Male *A. robustus* envenomation is also unusual in that it has little effect on non-primates, but is potentially lethal to newborn mice and primates, including man. The symptoms of human envenomation are bizarre and include [3] lacrimation, salivation, muscle fasciculation, massive pulmonary oedema, profound vasoconstriction and hypertension followed by hypotension, leading ultimately to death.

Recently, we isolated the lethal neurotoxin, robustoxin, from the venom of male *A. robustus* [4] and obtained its N-terminal sequence. The venom of female spiders did not appear to contain significant quantities of robustoxin [2]. In monkeys, robustoxin displayed all of the activities observed due to envenomation by crude venom. Its LD_{50}, determined by subcutaneous injection in newborn mice, was 0.16 $mg \cdot kg^{-1}$. We have now determined its complete amino acid sequence and found that this sequence does not appear to have homology with other neurotoxins and that robustoxin is of unique structural type amongst neurotoxins. We had previously found [4] by gel permeation chromatography that robustoxin had an apparent M_r of 5800, but had discounted this result as robustoxin was a very basic peptide (p*I* 10.2), and such peptides tend to give a low result in this type of determination. Based on yields obtained in amino acid analyses, we had tentatively assigned an M_r of 9650 to robustoxin. No free cys-

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Abbreviation: RCM, reduced carboxymethylated

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teine residues were detected. The extensive disulphide bridging indicated that robustoxin was a highly folded peptide.

2. EXPERIMENTAL AND RESULTS

Robustoxin was isolated and purified from crude male *A. robustus* venom as previously described [4]. It was converted to its RCM-derivative according to the procedure of Kung et al. [5]. The amino acid sequence of RCM-robustoxin was determined directly with a model 470A automatic gas-phase protein sequencer (Applied Biosystems Inc., Foster City, CA) essentially by the methods of Hewick et al. [6]. Their standard Edman degradation sequenator program with only minor changes was used in our first run. 20 nmol of RCM-robustoxin in 0.1% (v/v) trifluoroacetic acid was used in each of two uninterrupted runs. In the second run the temperature in the sequencer cartridge was held at 50°C (44°C in first run) and the time of cleavage for the proline at residue 17 was increased from 15 min to 2 h. Essentially the same results were obtained in the two runs, however. Sequencing ceased after the 42nd residue and it was concluded that robustoxin contains only 42 amino acid residues, half the number previously considered [4], and four disulphide bridges. The complete amino acid sequence of robustoxin (M_r 4887) was found to be H_2N-Cys-Ala-Lys-Lys-Arg-Asn-Trp-Cys-Gly-Lys^{10}-Asn-Glu-Asp-Cys-Cys-Cys-Pro-Met-Lys-Cys^{20}-Ile-Tyr-Ala-Trp-Tyr-Asn-Gln-Gln-Gly-Ser^{30}-Cys-Gln-Thr-Thr-Ile-Thr-Gly-Leu-Phe-Lys^{40}-Lys-Cys-H. The earlier N-terminal sequence [4] is corrected by this complete one in two respects. Residue 15 (the N-terminus is residue 1) was shown to be Cys instead of Ile, and residue 17 was Pro rather than Thr. The amino acid composition (table 1) of robustoxin was in complete agreement with the sequence.

Carboxy-terminal analysis of RCM-robustoxin was performed by a modification of the method of Liberatore et al. [9] using carboxypeptidase Y immobilized on Sepharose 4B (Pierce Chemical Co.) at pH 6.0, since the RCM-toxin was not sufficiently soluble at the usual pH for this enzyme of 8.0. The RCM-toxin (0.5 mg) was dissolved in 2.5 cm^3 of 0.2 M acetic acid and then buffered to pH 6.0 with *N*-ethylmorpholine. Carboxypeptidase Y (25 μg) was added and the mixture was held at

Table 1

Amino acid composition of robustoxin

Amino acid	From the sequence	Molar ratio (hydrolysis time, h)			
		24[a]	24	48	60
Lys	6	5.6	5.6	5.8	5.3
Arg	1	1.2	1.1	1.1	1.0
Asx	4	3.9	3.8	3.7	3.9
Thr	3	3.2	3.1	3.0	2.6
Ser	1	0.7	0.6	0.6	0.5
Glx	4	4.2	3.9	4.1	4.1
Pro	1	1.8[c]	1.6[c]	1.6[c]	1.5[c]
Gly	3	3.2	3.3	3.1	3.2
Ala	2	2.0	2.1	2.0	2.2
Cys	8	7.6	7.4	6.6	5.8
Met	1	[b]	1.1	1.0	1.1
Ile	2	1.9	1.8	1.9	1.8
Leu	1	1.0	1.0	1.0	1.0
Tyr	2	2.1	2.0	2.1	2.0
Phe	1	1.1	1.1	1.0	1.1
Trp	2		1.8		
Total	42				

[a] Oxidised with performic acid before hydrolysis
[b] Not quantified
[c] Not accurate in this analysis. A value of 1.0 for proline was found [4] by ninhydrin detection, when allowance is made for halving the apparent M_r of robustoxin assigned previously

RCM-robustoxin (0.5 mg) was mixed with 2 cm^3 of constant boiling HCl (Pierce Chemical Co.) and 5 mg·cm^{-3} of phenol. Hydrolysis was carried out in evacuated sealed tubes at 108°C. The hydrolysate was dried in a stream of ultrapure nitrogen, dissolved in 0.2 M sodium citrate at pH 3.1, and centrifuged. The supernatant was subjected to analysis using the Waters Associates amino acid analysis HPLC systems through post-column derivatization with *o*-phthalaldehyde [7]. Blank hydrolyses were performed in the absence of robustoxin. The tryptophan content was analysed spectrophotometrically by the procedure of Goodwin and Morton [8]. The content of cysteine was determined after performic acid oxidation of the toxin. Performic acid was prepared by mixing 1 vol 30% (w/w) hydrogen peroxide with 9 vols 98% (w/w) formic acid. Robustoxin (0.5 mg) was dissolved in 2 cm^3 of the performic acid and the mixture was held at ambient temperature for 18 h. It was diluted with water, lyophilised, hydrolysed and subjected to amino acid analysis as above

35°C. Aliquots of the mixture were removed, lyophilized and redissolved in 0.2 M sodium citrate buffer at pH 3.1. These were analysed for free amino acids by HPLC [7]. After 3 days incubation only carboxymethylcysteine and lysine were detected in the digest in agreement with the carboxy terminal of -Lys-Cys-H found in the sequence of robustoxin.

There does not appear to be any precedent, at least among neurotoxins, for the location of disulphide-bridged cysteine residues at both the amino- and carboxy-termini of a polypeptide and for the grouping of three of these consecutively (at residues 14–16). These features and the low M_r of robustoxin distinguish it as a neurotoxin of a new structural type. Drs W. Barker and L. Hunt searched the database of the National Biomedical Research Foundation at Georgetown University, USA, and found no significant homology to any sequence there, although there were some weak similarities to other cysteine-rich neurotoxins and phospholipases from various venoms. Our investigations are continuing into the secondary structure of this potent neurotoxin and to the relationship between its structure and physiological activity.

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