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The purification and amino acid sequences of four Tx2 neurotoxins from the venom of the Brazilian 'armed' spider *Phoneutria nigriventer* (Keys)

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Four neurotoxic polypeptides (Tx2-1, Txt2-5, Tx2-6 and Tx2-9) were purified from the venom of the South American 'armed' spider *Phoneutria nigriventer* (Keys) by gel filtration and reverse phase FPLC and HPLC. These cysteine-rich polypeptides exhibited different levels of neurotoxicity in mice after intracerebroventricular injection. Tx2-1, Tx2-5 and Tx2-6 caused spastic paralysis and death, but the less toxic Tx2-9 produced only tail erection and scratching. The molecular weights of the polypeptides as determined by desorption mass spectroscoopy were 5838.8 for Tx2-1, 5116.6 (Tx2-5), 5291.3 (Tx2-6) and 3742.1 (Tx2-9). The complete amino acid sequences of the neurotoxins were determined by automated Edman degradation and by manual DABITC-PITC microseqeunce analysis of peptides obtained after digestions with various proteases. The amino acid sequences of Tx2-1 (53 residues), Tx2-5 (49 residues) and Tx2-6 (48 residues) were homologous, but had only limited similarities to the less toxic Tx2-9 (32 residues). All four polypeptides had varying sequence identities with other neurotoxins from different spider species and biologically active peptides from scorpions, a sea snail and seeds of *Mirabilis jalapa.*

Spider venom; Amino acid sequence; Neurotoxin; *Phoneutria nigriventer*

1. INTRODUCTION

The venom of the aggressive South American solitary 'armed' or wandering spider *Phoneutria nigriventer* contains potent neurotoxins [1–4]. We recently described the purification by gel filtration and reverse phase chromatography of four separate types (Tx1, Tx2, Tx3 and M) of neurotoxic polypeptides from the venom of this spider [5], and have reported the complete amino acid sequence of the lethal neurotoxin Tx1 with a M_r of 8,216 [6].

The smaller neurotoxins of the Tx2 type caused excitatory symptoms such as salivation, lachrymation, priapism, convulsions, spastic paralysis of the anterior and posterior extremities and death following intracerebroventricular injection in mice. They also produced a slow contraction of the guinea pig ileum smooth muscle which was not reversed by washing, and was antagonised completely by atropine and tetrodotoxin [5]. These effects appeared to be due to the activation by these Tx2 toxins of the voltage dependent sodium channel in nerve and muscle cell membranes (Diniz et al., unpublished results).

We now report the complete amino acid sequences of the four main polypeptides purified from the Tx2 fraction by reverse phase HPLC.

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2. MATERIALS AND METHODS

2.1. *Separation of Tx2 neurotoxins from the venom*

The methods used for collection and storage of venom, and the preliminary separation of the Tx2 type neurotoxins from the other venom components by gel filtration on Sephadex G-50 and Superose 12 columns, and reverse phase FPLC on PEP-RPC and PRO-RPC columns were as described previously in [5].

2.2. *Purification of Tx2 neurotoxins*

The individual neurotoxins in the fraction PhTx2 obtained from the reverse phase FPLC on a PRO-RPC column [5] were separated and purified by reverse phase HPLC on an analytical column (4.6 mm × 25 cm) of Vydac C18 (218 TP 54, Technicol Ltd., Stockport, UK) using extended linear gradients of acetonitrile in 0.1% (v/v) aqueous trifluoroacetic acid.

2.3. *Assessment of biological activities*

Fractions were tested for toxicity in mice by intracerebroventricular injection as described in [5,6]. Guinea pig ileum was used for the assay of smooth muscle contraction as in [5].

2.4. *Electrophoresis*

SDS-PAGE was carried out using the Schagger and von Jagow tricine system with 22% gels [7]. Propionic acid/urea PAGE was performed as described in [8].

2.5. *Molecular weight determinations*

The molecular weights and purities of the isolated neurotoxins were

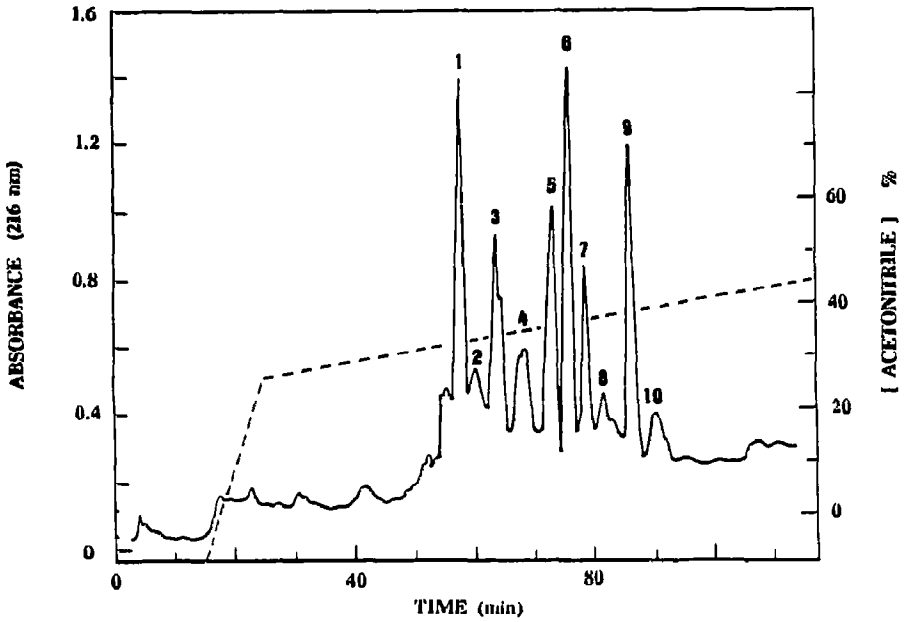

Fig. 1. Reverse phase HPLC separation on Vydac C_{18} of *Phoneutria nigriventer* neurotoxins in the fraction PhTx2 obtained from RP-HPLC on a PRO-FPLC column [5]. The dotted line indicates the gradient of acetonitrile in 0.1% (v/v) aqueous trifluoroacetic acid.

determined using the mass spectroscopy time of flight plasma desorption method [9] in the BioIon system (Applied Biosystems Ltd.).

2.6. *Determination of amino acid sequences*

Samples of the neurotoxins were reduced and *S*-carboxymethylated as described in [10]. The sequences of the intact native and reduced/*S*-carboxymethylated proteins were determined using a model 477A automatic pulsed liquid phase protein sequencer (Applied Biosystems Ltd.) employing standard Edman degradation sequenator programs. Samples (50–100 nmol) of the reduced and *S*-alkylated proteins were also digested separately with trypsin (2% w/w enzyme/substrate in 0.1 M ammonium bicarbonate, pH 8.2, 3 h at 37°C), and/or the Glu-specific endoproteinase from *S. aureus* V8 (2% w/w enzyme/substrate in Na,K-phosphate buffer, pH 8.0, 24 h at 37°C). Peptides from these digests were purified by reverse phase HPLC on analytical columns of Vydac C_{18} as described above. The sequences of these peptides were determined either by the automated method or by the DABITC/PITC manual double coupling microsequencing method [11].

2.7. *Sequence comparisons*

The amino acid sequences of the various Tx2 neurotoxins were compared with one another and with those of other proteins stored in the US National Biomedical Research Foundation Databank (1990) by computer analysis using the FASTA and RDF2 programs [12].

3. RESULTS AND DISCUSSION

Previous examination of the fraction PhTx2 obtained from the venom of the spider *Phoneutria nigriventer* [5] by means of high resolution propionic acid/urea PAGE revealed the presence of at least eight major components. These components were largely resolved during the present investigation by reverse phase HPLC on analytical columns of Vydac C_{18} using extended gradients of acetonitrile (Fig. 1).

Neurotoxins Tx2-1, Tx2-5 and Tx2-6 which eluted (peaks 1, 5 and 6) in a homogeneous condition at 33%, 36% and 37% acetonitrile, respectively, were the most toxic to mice after intracerebroventricular injection. All three caused scratching, lachrymation, hypersalivation, sweating and agitation followed by spastic paralysis of the anterior and posterior extremities and death at dose levels of 1.62 μg/mouse for Tx2-1, 0.24 μg/mouse for Tx2-5 and 0.79 μg/mouse for Tx2-6. The other major component (Tx2-9, 38% acetonitrile) which was obtained in a pure state, was much less toxic to mice, causing only tail erection, scratching and a reduction in mobility (after 30 min) at a dose level of 1.40 μg/mouse. The polypeptides contained in the minor peaks 2, 3 and 4 had no toxic effects. Peaks 7 and 8 were found to have toxicity, but in both cases were a mixture of at least two peptides. The full details of the pharmacological effects of these neurotoxins will be published elsewhere (Diniz et al., in preparation); however, a preliminary investigation of the toxins by the patch-clamp technique revealed that only Tx2-6 significantly activated voltage-dependent sodium channels.

The results of SDS-PAGE indicated that the neurotoxins Tx2-1, Tx2-5, Tx2-6 and Tx2-9 had apparent an M_r in the range of 4–7 kDa. More accurate measurements of 5838.8 (Tx2-1), 5116.6 (Tx2-5), 5291.3 (Tx2-6) and 3742.1 (Tx2-9) were obtained from the BioIon time of flight plasma desorption mass spectroscopy method which yielded single peaks for each of the proteins confirming their homogeneity.

Fig. 2 shows the complete amino acid sequences of Tx2-1 (53 amino acids), Tx2-5 (49 residues), Tx2-6 (48

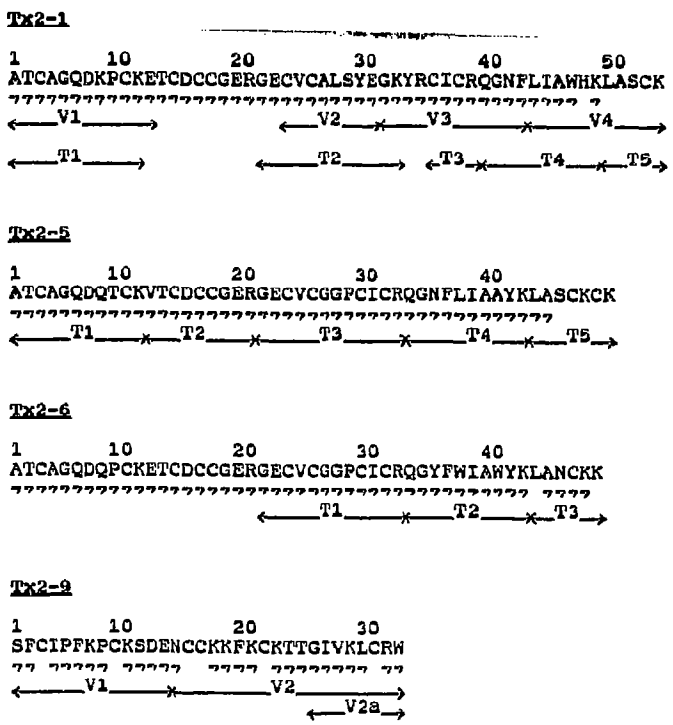

Fig. 2. The amino acid sequences of the neurotoxins Tx2-1, Tx2-5, Tx2-6 and Tx2-9 from the venom of *Phoneutria nigriventer*. (—) Arrows indicate residues determined by automated degradation of the intact native or *S*-reduced and carboxymethylated proteins. Solid lines indicate peptides obtained from digestions with trypsin (T), and/or the Glu-specific protease from *S. aureus* V8 (V) which were sequenced by the manual DABITC/PITC method or by the automated means.

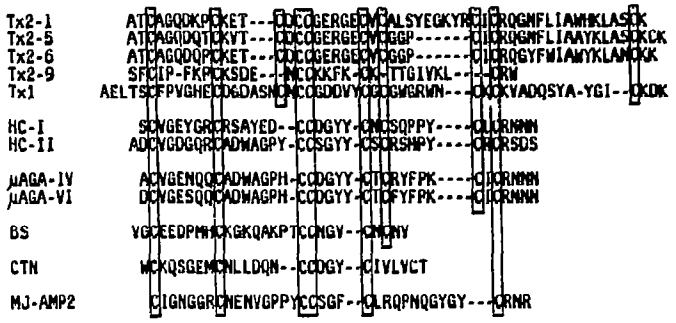

Fig. 3. Comparison of the amino acid sequences of the *Phoneutria nigriventer* neurotoxins Tx2-1, Tx2-5, Tx2-6 and Tx2-9 with the N-terminal sequence of the toxin Tx1 from the venom of the same spider [6]; HCI and HCII, paralytic and insecticidal curtatoxins from the funnel web spider *Hololena curta* [14]; μAGA IV and μAGA V, the μ-agatoxins from *Agelenopsis aperta* [13]; BS, peptide from the venom of the scorpion *Buthus sindicus* [18]; CTN, mollusc-specific toxin from the sea-snail *Conus textile neovicarius* [19]; and MJ-AMP2, an anti-fungal peptide from seeds of *Mirabilis jalapa* [20]. The numbering of residues refers only to Tx2-1. –, indicates gaps inserted in sequences to maximize homology.

residues) and Tx2-9 (32 residues) as determined by a combination of the automated and manual microsequencing methods. The M_r values calculated from these sequences were in close agreement with the estimates obtained from mass spectroscopy: Tx2-1 (5818/5838.8), Tx2-5 (5111/5116.6), Tx2-6 (5298/5291.3) and Tx2-9 (3743/3742.1). No examples of microheterogeneity were detected in any of the four proteins investigated. All four sequences are notable for their high content of CYS residues, a feature common to Tx1 from *Phoneutria nigriventer* [6] and to the neurotoxins from the venoms of other spider species [13–17].

When the sequences of Tx2-1, Tx2-5 and Tx2-6 are aligned (Fig. 3), they exhibit a strong homology to one another. Tx2-5 and Tx2-6 are the most similar with 85.4% sequence identity, but they show 70.9% and 72.2% homology, respectively, when compared with Tx2-1. It is evident that Tx2-1 is larger than Tx2-5 and Tx2-6 due to the insertion/deletion of six amino acids between positions 29 and 34. The smaller, less toxic Tx2-9 has only limited resemblances (approx. 30%) to Tx2-1, Tx2-5 and Tx2-6.

These Tx2 neurotoxins from *Phoneutria nigriventer* also show low levels of sequence identity with the lethal Tx1 from the same spider [6], paralytic and insecticidal curtatoxins from the funnel-web spider *Hololena curta* [14], the μAGA toxins from *Agelenopsis aperta* [13], peptides from the venom of the scorpion *Buthus sindicus* [18], mollusc-specific toxins from the sea-snail *Conus textile neovicarius* [19] and an anti-fungal peptide from the seeds of *Mirabilis jalapa* [20]. These similarities are most evident in the locations of the Cys residues (Fig. 3).

In addition to their toxic effects on mice, the Tx2 neurotoxins from *Phoneutria nigriventer* are known to be insecticidal to the larval and adult forms of the house-fly (*Musca domestica*) (Figueiredo et al., unpublished results). Their weak sequence resemblance to the μAGA toxins from *Agelenopsis aperta* [13] is interesting in that these other spider toxins are also thought to activate sodium channel currents in insect neuronal membranes probably by binding to receptor proteins [21]. However, the Tx2 neurotoxins from *P. nigriventer* do not appear to have sequence homologies with other peptide toxins that act on sodium channels [22,23] such as those purified from the venoms of the scorpions *Leiurus quinquestriatus quinquestriatus* [24], *Androctonus australis* Hector [25], *Centruroides* spp. [26], *Tityus serrulatus* [27] and the nematocysts of the sea-anemones *Anthopleura xantogrammatica* and *Anemone sulcata* [28].

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