Isolation and characterization of a novel type of neurotoxic peptide from the venom of the South African scorpion *Parabuthus transvaalicus* (Buthidae)

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The venom of the South African scorpion *Parabuthus transvaalicus* was characterized using a combination of mass spectrometry and RP-HPLC separation and bioassays. The crude venom was initially separated into 10 fractions. A novel, moderately toxic but very high abundance peptide (birtoxin) of 58 amino-acid residues was isolated, identified and characterized. Each purification step was followed by bioassays and mass spectroscopy. First a C4 RP-HPLC column was used, then a C18 RP Microbore column purification resulted in >95% purity in the case of birtoxin from a starting material of 230 μg of crude venom. About 12–14% of the D214 absorbance of the total venom as observed after the first chromatography step was composed of birtoxin. This peptide was lethal to mice at low microgram quantities and it induced serious symptoms including tremors, which lasted up to 24 h post injection, at submicrogram amounts. At least seven other fractions that showed different activities including one fraction with specificity against blowfly larvae were identified. Identification of potent components is an important step in designing and obtaining effective antivenom. Antibodies raised against the critical toxic components have the potential to block the toxic effects and reduce the pain associated with the scorpion envenomation. The discovery of birtoxin, a bioactive long chain neurotoxin peptide with only three disulfide bridges, offers new insight into understanding the role of conserved disulfide bridges with respect to scorpion toxin structure and function.

*Keywords*: *Parabuthus transvaalicus*; scorpion; toxin; disulfide; birtoxin.

Scorpion venoms are a rich source of neurotoxic peptides with diverse modes of action. Within the complex mixture of those venoms studied, peptides have been found to possess the majority of the biological effect towards the target organisms. Numerous peptides toxic against mammals and insects have been identified. The potent peptides are usually low in abundance [1]. *P. transvaalicus* is a large (up to 150 mm), South African scorpion species from the family Buthidae and considered to be medically important [2]. The victims of envenomation by *P. transvaalicus* suffer from neurotoxic effects and prolonged pain lasting from 1 day in minor cases up to 1 week in severe cases. Symptoms include abnormal reflexes, bladder symptoms, dysphagia, sweating and hypersalivation [2].

Venom of *P. transvaalicus* can be considered a more ‘simple’ venom compared to other scorpion venoms as it contains less than 100 major peptides [3]. The venom can be characterized by defining the individual components of the system (identification of peptide toxins), analysis of the structure of the components (primary, secondary and tertiary structure determination), analysis of the function of each component (determination of the mode of action), analysis of the relationships between these components (synergism) and the target sites or the environment (binding sites and kinetics). The knowledge of a phenomenon depends on the way in which it is observed. Here we have limited our variables to neurotoxic peptides possessing activity against mice or insects.

Recently inhibition of T-type voltage-gated calcium channels by a novel toxin, kurtoxin, from the venom of *P. transvaalicus* has been reported [4]. Furthermore the venoms of three *Parabuthus* species have been compared and partially characterized previously [5], primarily by gel filtration chromatography. In this study we are reporting a more detailed characterization of the venom of *P. transvaalicus*, including the determination of seven different toxic fractions with molecular masses determined by a combination of MALDI and ESI-TOF techniques. The low potency of venom against insects and high potency against mammals was an unexpected result that led to the identification, isolation and characterization of a novel, moderately toxic but very high abundance peptide, birtoxin.

Birtoxin is 58 residues long, close to the 60- to 70-residue range of the ‘long chain neurotoxin’ peptide family [3,6–8]. However, this family of peptides are known to contain eight cysteine residues. The second family of scorpion toxins is the ‘short chain neurotoxins’ which are 30- to 40-residue peptides with six or eight cysteine residues. Birtoxin, having six cysteines bridges the gap between these two distinct groups, perhaps as an evolutionary link. We have also examined the 3D NMR structure of closely related toxin peptides from *C. exilicauda*, *C. sculpturatus* and *C. noxius* [9–11] and overlaid our primary sequence onto these structures. The results indicate a novel structural alternative...
to the fourth disulfide bridge that is currently unique for birtoxin.

**MATERIALS AND METHODS**

**Sample preparation**

Scorpion venom was obtained from captive scorpions by passing a small electric shock through the telson to contract the muscles. The venom was collected in a cryo tube as a pooled sample from three milkings at the South African Vaccine Producers Ltd facilities, dried and sent to the University of California Davis (UC Davis). Dried venom was resuspended in sterile water at 10 mg·mL⁻¹ by vigorous vortexing and filtered through a 0.45-µm filter.

**HPLC purification**

Filtered venom sample was injected into a Vydac (Hesperia, CA, USA) RP C₄ Analytical HPLC column (4.6 mm internal diameter × 25 mm length) connected to a Hewlett-Packard HP1100 system coupled to a diode array detector and a computer running the Chemstation® software. A gradient was formed with the following conditions: 5–65% solvent A in 60 min, 65% solvent A for another 20 min for a total of 80 min at a flow rate of 600 µL per minute (solvent A, 95% acetonitrile, 5% water, 0.1% trifluoroacetic acid; solvent B, 95% water, 5% acetonitrile, 0.1% trifluoroacetic acid). Elution was monitored by following the UV trace at 214 and 280 nm. Fractions were collected manually into tubes pretreated with BSA (1 mg·mL⁻¹) and washed with 1 : 1 (v/v) acetone/water, 1 : 1 (v/v) methanol/water and water, respectively. Biologically active fractions were further separated using a Michrome Magic 2002 Microbore HPLC system equipped with a RP C₁₈ column and an online 5 µ peptide trap (Michrome BioResources, Inc. Auburn, CA, USA) with a linear gradient from 5 to 70% solvent A in 23 min at a flow rate of 50 µL·min⁻¹. Fractions were collected manually into BSA pretreated tubes by following the UV trace.

**Bioassays**

Male Swiss-Webster mice were purchased from Charles River Inc., and housed at the Animal Housing Facility, UC Davis. Blowfly (Sarcophaga spp.), crickets (Acheta domestica) and anole lizards (Anoles caroliensis) were purchased from Carolina Biologicals (Burlington, NC, USA), cotton bollworms (Heliothis virescens) were obtained from USDA/ARS (Stoneville, MI, USA) and reared on artificial diet. Collected fractions were tested on mice by intracerebroventricular injections and by injecting last instar blowfly larvae. Third instar cotton bollworms and adult common house crickets were also used in screening for insecticidal components. Briefly fractions were concentrated to dryness using a Heto Speed Vac (ATR, Inc. Emeryville, CA, USA). Dried samples were resuspended in 10 µL of 20 mm ammonium acetate buffer with 1 µg·µL⁻¹ BSA and incubated overnight at 4 °C to reduce variability in toxicity before injection to the test animals. Mice were anesthetized using ethyl ether and intracerebroventricular injections of peptide solutions were executed immediately. Control animals injected with BSA in buffer did not show any symptoms when recovering from anesthesia. All symptoms were observed and recorded up to 24 h post injection. All experiments were carried out in accordance with the guidelines laid down by the National Institutes of Health in the USA regarding the care and use of experimental animals.

**Mass spectroscopy**

Mass spectra of crude venom, separated fractions and isolated peptide were analyzed off-line in a Biflex III (Bruker Daltonics, Bremen, Germany) MALDI-TOF instrument in positive ion mode. The instrument was equipped with a nitrogen laser operating at an output of 337.1 nm with a pulse width of 3 ns, and a repetition rate of 6 Hz. Experiments were conducted in reflector mode with an acceleration potential of 19.3 kV, a reflector potential of 20.0 kV, a time base of 2–4 ns, and a delay of 10–30 ms operated at R < 3000 (full-width half maximum definition). The output signal of the detector was digitized at a sampling rate of 500 MHz per channel using a 1 GHz Lecroy digitizer. A camera mounted on a microscope facilitated inspecting the sample crystallization and selecting the largest crystals for analysis. External calibration was performed using angiotensin II (1046.53 Da, monoisotopic), somatostatin 28 (3147.47 Da, monoisotopic), and human recombinant insulin (5808.6 Da, average) from Sigma. For analysis, matrix solutions consisting of sinapinic acid, 3,5-dimethoxy-4-hydroxycinnamic acid, or α-cyano-4-hydroxycinnamic acid, were mixed in a 1 : 1 (v/v) ratio with samples, spotted on the target and allowed to dry. MASSLYNX (Micromass UK Limited, Manchester, UK) software was used for data processing and analysis.

**Edman sequencing**

Purity following HPLC was evaluated by MALDI-TOF. For amino-acid sequence determination, the cystine residues of the peptide were reduced and carboxymethylated by incubating in 6 m guanidine hydrochloride, 0.1 m Tris/HCl (pH 8.3), 1 mm EDTA and 20 mm dithiothreitol for 1 h at 37 °C. Iodoacetic acid was then added to a final concentration of 50 mm and incubated for an additional 1 hour at 37 °C in the dark. Finally 300 pm of peptide was subjected to automated Edman sequencing for 60 cycles using a Hewlett-Packard HP GS1000 Sequence Analyzer at the Molecular Structure Facility at UC Davis.

**Peptide quantification and amino-acid analysis**

Amino-acid analysis was conducted by the Molecular Structure Facility at UC Davis with standard methods using a Beckman 6300 Na citrate-based amino acid analyzer. Peptide quantification was accomplished after determination of the molecular mass, primary sequence and amino-acid analysis. The extinction coefficient for birtoxin was calculated according to Gill et al. [12] using the following formula:

\[
\Sigma_{280} = 5690 \times n_{trp} + 1280 \times n_{tyr} + 120 \times n_{ss}
\]

where \(n_{trp} \), \(n_{tyr}\) and \(n_{ss}\) represent the number of tryptophan residues, tyrosine residues and disulfide bonds, respectively. The extinction coefficient for birtoxin was calculated as
13730 m$^{-1} \cdot$cm$^{-1}$, $\Sigma_{280}$ for birtoxin was confirmed experimentally by utilizing the $A_{280}$ and concentration of birtoxin determined by amino-acid analysis according to Beer–Lambert law. Absorbance at 280 nm was determined using a BioRad SmartSpec 3000 Spectrophotometer using a quartz cuvette.

**Structural analysis**

NMR structures for peptide toxins from *C. exilicauda*, *C. sculpturatus* and *C. noxius* were downloaded from the Expasy server (http://www.expasy.ch) in pdb format. The amino-acid sequence of birtoxin was aligned and the backbone atoms were overlaid on the NMR resolved 3D structures using the magic fit and improve fit functions of the Expasy server (http://www.expasy.ch) in pdb format. The amino-acid sequence was determined as described above. Sequence homologies were determined using the CLUSTALW program at the EMBL server (http://www2.ebi.ac.uk/clustalw). Sequence alignments were visualized using the escript tool at the Expasy server.

**RESULTS**

**Characterization of venom**

We initially determined the mass profile of the crude venom using MALDI-TOF. Mass spectroscopy of the crude venom resulted in detection of 72 components within the mass range of 750–7500 Da (Table 1: J. Lango, B. Inceoglu and B. D. Hammock, unpublished results). Optimization of the HPLC gradient conditions resulted in consistent separation of the venom into 10 fractions (Fig. 1). Soluble crude venom and each of the fractions were tested for activity against mice, three insect species, and anole lizards (Table 1). As shown in Table 1 fractions P0, P1, PreP3, P3, P4, P5 and P6 had activity against mice and fractions P2 and P3 had activity against blowfly larvae. Fraction P2 was specific for blowfly only. Interestingly, the crude venom had limited toxicity against insects. The material between the above peaks was collected and assayed, however, it showed little biological activity. The LD$_{99}$ for common house cricket and bollworm were well above 48 $\mu$g per insect for the crude venom. In contrast, the venom was quite potent against mice, with an LD$_{99}$ of 4.8 $\mu$g crude venom per mouse of 20 g with intracerebroventricular injection. Venom was at least five times less toxic by subcutaneous injection and 50 times less toxic when injected intraperitoneally. The crude venom did not show noticeable activity towards adult anole lizards by subcutaneous injection at even high doses (100 $\mu$g venom per lizard of 10 g) except for slowing their motion temporarily for about 3 min.

**Purification and characterization of birtoxin**

Fraction P4 (Fig. 1) from the C$_4$ column gave severe symptoms when injected to mice. The injected mice were first stunned then started to tremble, the tremors severity increased with time and lasted up to 24 h. Also the paws were contracted and the body took the shape of hunchback with frequent whole body jerks. The symptoms did not initiate immediately after the injection was completed but increased in intensity for 30 min after a 10-min lag. Control animals injected with BSA in buffer rapidly recovered within three minutes. From the UV absorbance of the C$_4$ column profile, birtoxin was estimated to constitute 12–14% of the crude venom. We further purified the fraction P4 from the first column by injecting into a C$_18$ Microbore column. Toxicity was only seen in the UV dense fraction collected from the microbore run. This second step resulted in more than 97% purity as determined by MALDI fraction (Fig. 2). This fraction was also injected to mice and biological activity was confirmed. Injection of 1 $\mu$g of pure birtoxin induced severe neurotoxic symptoms in mice up to 24 h but was not lethal. However 2 $\mu$g of pure peptide was lethal. The peptide was then reduced, carboxymethylated and the amino-acid sequence was determined as described above.

**Table 1. Biological activity of the C4 fractions of the venom of Parabuthus transvaalicus and the major molecular masses detected from each fraction.** HPLC fractions from the C4 column were tested for bioactivity in mice and insects. Relative toxicity is denoted as: –, nontoxic; +, toxic; ++, moderately toxic; and ++++, highly toxic. Molecular masses from each fraction which are above 2000 Da are also given. The birtoxin mass is in bold. IC, intracerebroventricular.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Mouse (IC injection)</th>
<th>Larvae Blow fly</th>
<th>H. virescens</th>
<th>Molecular ion masses detected (M + H)$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>None detected above 2000 Da</td>
</tr>
<tr>
<td>P1</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>None detected above 2000 Da</td>
</tr>
<tr>
<td>P2</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>3492, 3640, 3769, 3912, 4092$^a$, 4297, 4512, 5757, 6544$^b$, 6615$^b$, 6635, 7221, 7215, 7261</td>
</tr>
<tr>
<td>PreP3</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>Not determined</td>
</tr>
<tr>
<td>P3</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
<td>2437, 5258, 5441, 5648, 6057, 6356, 6526, 6543$^b$, 6574</td>
</tr>
<tr>
<td>P4</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>5543, 5752, 6160, 6647$^a$, 6854, 7225, 7303, 6603$^a$</td>
</tr>
<tr>
<td>P5</td>
<td>+++</td>
<td>–</td>
<td>–</td>
<td>None detected above 2000 Da</td>
</tr>
<tr>
<td>P6</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>Not determined</td>
</tr>
<tr>
<td>P7</td>
<td>+++</td>
<td>–</td>
<td>–</td>
<td>Not determined</td>
</tr>
<tr>
<td>P8</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

Venom LD$_{99}$ is 4.8 $\mu$g crude venom/20 g mouse. LD$_{99}$ for blowfly is 1.2 $\mu$g venom/100 mg larvae.

* Most abundant species.
The average molecular mass \((\text{M} + \text{H})^+\) for native birtoxin is determined to be 6543.6 Da using MALDI-TOF. Birtoxin has 58 amino-acid residues. Amino-acid sequencing resulted in 57 amino acids giving a calculated average mass of 6455.2 Da. Serine 57 was not detected by Edman degradation due to the low conversion efficiency and the low amount of peptide left on the column, but valine 58 was detected clearly. Amino-acid analysis revealed the presence of an extra serine, which was assigned to position 57. The calculated and expected mass were in agreement after assigning the 57th amino acid as a serine residue. The presence of six cysteine residues was confirmed by measuring the molecular mass of reduced and carboxymethylated birtoxin (Fig. 2B). Homology searching revealed over 40 matches of scorpion toxins with identity ranging from 22% to 57%. The best of these matches were selected for multiple sequence alignment (Fig. 3). Birtoxin shows significant identity to the previously discovered sodium channel blocker toxins from the scorpions *C. exilicauda*, *C. sculpturatus* and *C. noxius*.

**Structural analysis**

The availability of NMR structures for similar peptides has prompted us to examine these structures and compare birtoxin to these structures [9–11]. The backbone overlay of birtoxin on CeNV1 structure (for definition of toxin abbreviations, see Fig. 3) (Fig. 4A) resulted in a good agreement between the two backbones (calculated rmsd of 0.01 Å between the two aligned backbones). Thus, we were able to visualize the positions of the four disulfides in the CeNV1 toxin with respect to the three disulfides in birtoxin. The aligned structure revealed that both toxins have a conserved core with three disulfide bridges (Fig. 4B). In the CeNV1, cysteines 16–41, 25–46 and 29–48 are disulfide bridged and form the core disulfides whereas in birtoxin all disulfide bridges are at the core. CeNV1 has a fourth disulfide bridge between Cys12 and Cys65 (Fig. 4A). This fourth disulfide is, like the other three, very well conserved among the long chain neurotoxin peptide toxin family (Fig. 3). Birtoxin does not possess an equivalent for Cys12 and does not possess the 9–13 residues at the C-terminus of CeNV1, CsN-3 and CnN2. The NMR structure reveals that the fourth disulfide wraps the N- and C-termini from the outside of the molecule (Fig. 4). Thus we named this the ‘wrapper’ disulfide. The absence of two cysteine residues in birtoxin corresponding to Cys12 and Cys65 in CeNV1 and other long chain neurotoxins indicates the absence of the fourth disulfide. Therefore we concluded that birtoxin has a novel structural organization and an alternative system for keeping the polypeptide chain in a particular three dimensional conformation that retains biological activity without the fourth disulfide bridge.

**DISCUSSION**

Isolation of peptide toxins from scorpions have two major applications. As summarized in the introduction identified potent peptides can be employed in the design and production of superior anti-venom. Also peptide toxins from scorpions are probes for identifying distinct types of ion channels and important tools for understanding their physiology [13,14]. One interesting feature of the venom of *P. transvaalicus* is its specialization towards mammalian activity, only one out of 10 initial fractions showed insect specificity. This might well be because of the larger size of this scorpion, which makes it a more visible prey to mammalian predators such as bats. Moreover the lack of strong insecticidal components can also be explained by the larger size of this scorpion because it does not require a toxin to subdue an insect prey. A further interesting finding from our bioassays on anole lizards indicate that the venom
of *P. transvaalicus* does not possess components that would target reptilian ion channels. Ion channels from reptiles have not been fully characterized, and our finding suggests that they may have different pharmacological properties compared to either mammalian or insect ion channels. The venom of *P. transvaalicus* proved to have less than 100 major peptides. Furthermore we were able to detect at least seven different toxic activities within the crude venom. In-depth characterization of each of the activities present in the venom may reveal a structurally unique family of long chain neurotoxin peptides with three disulfide bridges.

The primary sequence of many peptide toxins from numerous scorpions are known but few of the structures have been resolved and we know even less of how the particular structure affects the activity [9–11]. The experimental determination of the three-dimensional structure of birtoxin may clarify how this toxin retains its 3D conformation and will improve our understanding of the structure and function of peptide toxins. Finally, it is interesting to note that birtoxin shows similarity between birtoxin and toxins from American scorpions contributes to the hypothesis that scorpion toxins originate from a common ancestor [24]. The most similar toxin to birtoxin is Cse-VIII, to which it is 54% identical.
bridge between Cys12–Cys65 in CeNV1 is absent in birtoxin. Birtoxin was overlaid and fitted on to the NMR structure. The disulfide is the 'wrapper' disulfide. In the lower panel the primary sequence of CeNV1 is seen. The disulfide bridge between residues cys12 and cys65 supported by USDA Grant 97-35302-09919 and NIEHS Grant and support during the preparation of this manuscript. This work was acknowledged by Dr Isaac Pessah for the critical review and all Hammock Laboratory members for their support and discussions.

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