

Purification and partial characterization of a ‘short’ insectotoxin-like peptide from the venom of the scorpion *Parabuthus schlechteri*

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Abstract A disulfide-rich, low-molecular-mass toxin-like peptide has been isolated from *Parabuthus schlechteri* venom using gel filtration, ion exchange, and reversed phase chromatography. Partial characterization of this peptide reveals a relationship with four-disulfide bridge proteins belonging to the family of ‘short’ insectotoxins (44% residue identity). In recognition hereof, the peptide was named PBITx1 (sITx10). Our work also reports on the deduced sequences of two other ‘short’ insectotoxins from *Buthus eupeus*, I₃ and I₄, and it provides a consensus sequence and nomenclature for all known ‘short’ insectotoxins. Finally, sequence similarities with K⁺ channel blockers (charybdotoxin, κ-conotoxin), and a Cl[−] channel blocker (chlorotoxin) are highlighted.

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Key words: Scorpion; Venom; Toxin; Peptide; Insectotoxin; *Parabuthus*

1. Introduction

Venoms of insects, scorpions, snakes and other species provide a rich source of molecules that can interact with membrane receptors and ion channels with high affinity and selectivity. It is well known that scorpion venoms contain a variety of peptides toxic to man [1], insects [2], and crustaceans [3,4]. These toxins isolated from the venom are useful tools for probing the structures of different ion channels and evaluating their physiological contribution to cell and organ behavior. In addition, elucidation of their mechanisms of action, knowledge of their three-dimensional structure and the discovery of common scaffolds between sometimes unrelated toxins open wide perspectives in designing various drugs [5].

In general, scorpion toxins can be divided into two main groups according to their molecular size. The first group represents the long-chain toxins with 60–70 residues cross-linked by four disulfide bridges. Most of these toxins affect voltage-dependent Na⁺ channels in excitable cells [6,7]. The second group contains the short-chain toxins with 30–40 residues cross-linked by three or four disulfide bridges. Their toxicity is based on potent block of several types of K⁺ channels, voltage-dependent and calcium-activated, in a wide variety of cell types [8,9]. Some of the scorpion toxins belonging to the first group display high selectivity towards animals be-

longing to different phyla [10]. For instance, AaHIT from *Androctonus australis hector* [11] and LqhIT2 from *Leiurus quinquestriatus hebraeus* [12] are insect-selective Na⁺ channel toxins, respectively with an excitatory and depressant effect. These toxins are also called ‘long’ insectotoxins (ITs) and were recently reviewed together with the large family of mammalian-selective Na⁺ channel toxins [7]. ‘Short’ insectotoxins have been reported in the literature as well, based on the selective paralytic activity on insects of these ~4 kDa peptides [13]. Analogous structures have been labeled accordingly later on, but sometimes they are also called ‘toxin-like’ peptides. In any case, ‘short’ insectotoxins have received much less attention in the past than the ‘long’ chain toxins, since (i) the number of well-characterized ‘short’ insectotoxins known today is limited (fewer than 10 ‘short’ versus more than 50 ‘long’ chain insectotoxins), (ii) the solution structure by NMR has only been determined for I_{5A} [14], chlorotoxin [15], and Lqh-8/6 [16], and (iii) the biological function mostly remains unknown. In fact, only for chlorotoxin, a basic peptide of ~4 kDa with considerable sequence identity to ‘short’ insectotoxins and purified from the venom of *Leiurus quinquestriatus quinquestriatus*, has block of small-conductance Cl[−] channels been reported [17].

Here we report the purification and partial characterization of a ‘short’ insectotoxin-like peptide in the venom of the scorpion *Parabuthus schlechteri*, a species occurring in southern Africa. During investigation of other short-chain toxins active on voltage-dependent K⁺ channels, such as PBTx1 from *Parabuthus transvaalicus* and PBTx2 from *Parabuthus villosus* [18,19], we discovered by chance in the venom of *P. schlechteri* a ‘short’ insectotoxin-like peptide, which we named PBITx1 (sITx10, see further). Although the biological activity and the C-terminal part of PBITx1 could not be determined yet (because of lack of material), the high level of sequence identity between PBITx1 and other ‘short’ insectotoxins is evident. This work also reports on the deduced sequences of two other ‘short’ insectotoxins from the venom of the scorpion *Buthus eupeus*, I₃ and I₄ [20], and it provides a consensus sequence and uniform nomenclature for all known ‘short’ insectotoxins. The similarities between PBITx1 and chlorotoxin suggest that Cl[−] channels could be the peptides’ natural target.

2. Materials and methods

2.1. Venom collection

P. schlechteri scorpions were captured in South Africa. Venoms were collected by electrical stimulation and lyophilized after dilution in a saline buffer or distilled water.

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2.2. Venom purification

Lyophilized venom was dissolved in 100 mM ammonium acetate, pH 7 (Merck, Germany). After vortexing, the sample was clarified by centrifugation at 14000 rpm for 15 min and its supernatant was then applied onto a Superdex 30 prep grade HiLoad 16/60 FPLC column (Pharmacia LKB Biotech, Sweden) equilibrated with 100 mM ammonium acetate, pH 7. The material was eluted with the same buffer at a flow rate of 0.2 ml/min. Absorbance of the eluate was monitored at 280 nm and 4 ml fractions were collected automatically. The fraction containing PBITx1 was recovered, lyophilized and applied on a PepRPC HR 5/5 C₂/C₁₈ reversed phase FPLC column (Pharmacia, Sweden) equilibrated with 0.1% trifluoroacetic acid (TFA) in distilled water. Separation was performed by using a linear gradient of 0–100% acetonitrile (supplemented with 0.1% TFA) for 60 min. The flow rate was 0.5 ml/min and the absorbance was measured at 214 nm. Fractions eluting at 20–27 min with potential short-chain toxins were recovered, dried (Speed Vac Plus, Savant, USA), and applied on a monomeric 238TP54 C₁₈ reversed phase HPLC column (Vydac, USA) equilibrated with 0.1% TFA in distilled water. Separation was performed with the following gradient: after 4 min an immediate step to 5% acetonitrile (supplemented with 0.1% TFA), followed by a linear gradient to 30% acetonitrile for the next 5 min, and a linear gradient to 60% for the last 11 min (total run: 20 min). The flow rate was 0.75 ml/min and the absorbance was simultaneously measured at 214, 254 and 280 nm. The fraction containing PBITx1 was recovered, dried (Speed Vac Plus, Savant, USA), and applied to a Mono S PC 1.6/5 cation exchange HPLC column (Pharmacia, Sweden) equilibrated with 50 mM phosphate buffer, pH 6.8. Separation was performed using a linear gradient of 0–100% 1 M NaCl (supplemented with 50 mM phosphate buffer, pH 6.8). The flow rate was 0.1 ml/min and the absorbance was simultaneously measured at 214, 254 and 280 nm. Desalting of the material was performed with the monomeric 238TP54 C₁₈ reversed phase HPLC column (Vydac, USA) using the same experimental reversed phase conditions as above. Additional confirmation of sample purity was accomplished by application of the peptide to a 218TP104 C₁₈ analytical reversed phase column (Vydac, USA). A gradient of 0–60% acetonitrile in the presence of 0.1% TFA was run for 60 min. The component eluting at 25.32 min corresponded to the pure peptide and was used for sequence determination.

2.3. Sequence determination

The automated Edman degradation procedure was used in a Beckman LF3000 protein sequencer. Approximately 50 pmol of pure PBITx1 peptide was loaded into the sequencer. Unequivocal sequence was obtained for the 22 amino acid residues located at the N-terminal segment of the native peptide.

3. Results and discussion

Crude venom was fractionated by gel filtration as detailed in Section 2 to produce three typical groups of components (Fig. 1A). The occurrence of three groups is reminiscent of the venom fractionation of another closely related *Parabuthus* scorpion, *P. transvaalicus*. This observation points to a clear interspecies relationship within the genus, corroborating our previous work [18]. In our quest of finding novel short-chain toxins in the venom active on voltage-dependent K⁺ channels, such as PBTx1 from *P. transvaalicus* and PBTx2 from *P. villosus* [18,19], we recovered fractions eluting at 20–27 min during the reversed phase FPLC run (Fig. 1B). Final purification of PBITx1 was achieved by means of consecutive reversed phase, cation exchange and again reversed phase HPLC runs (Fig. 1C–E). Although the same and typical gel filtration fingerprint was obtained for the *P. schlechteri* scorpions used in this study ($n=9$), regardless of milking time interval and span in captivity, it was found that the presence of PBITx1 in the venom of this species was very scarce and that PBITx1 represents only a minor fraction of the venom constituents. In fact, the only successful isolation we have

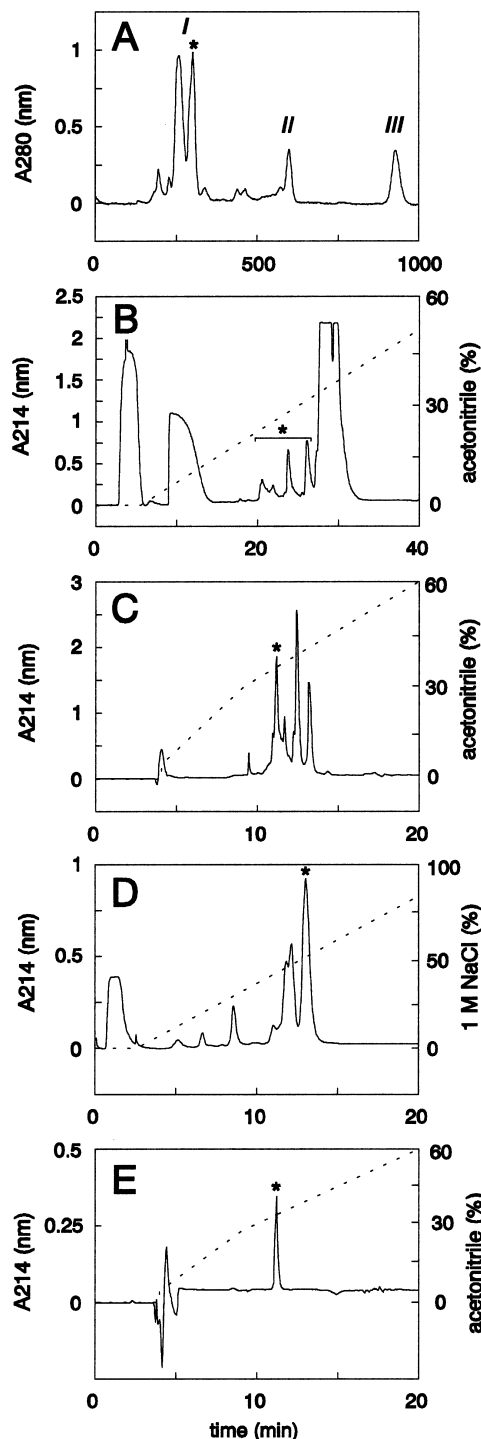


Fig. 1. Purification of PBITx1 from *Parabuthus schlechteri* venom. A: Crude venom was first fractionated by FPLC gel filtration as indicated in Section 2, yielding three typical groups of peaks (I–III). The labeled fraction (*) in group I was recovered and lyophilized. Based on a constructed gel filtration calibration curve, the molecular mass of the material in this fraction ranged from 3 to 6 kDa [18]. B: The second purification step was carried out using a FPLC C₂/C₁₈ reversed phase column. Fractions eluting at 20–27 min (*) with 'potential' short-chain toxins were recovered and dried. C: The third step involved a HPLC C₁₈ reversed phase purification. The labeled fraction (*) was recovered and dried. D: Cation exchange purification step on a Mono S HPLC column. The labeled fraction (*) was recovered. E: Final purification and desalting on a HPLC C₁₈ reversed phase column. See Section 2 for experimental details during the different purification steps.

Member		sITx Name	Ident. (%)	Charge	Ref.	
1102030						
I ₁		1	100	44	+3	[13]
AmmP2		2	60	56	+2	[1,2]
I ₃	*	3	72	52	+3	[20]
I ₄	**	4	69	60	+3	[20]
I ₅		5	71	64	+3	[20]
I _{5A}		6	74	64	+3	[20]
Peptide I Bs		7	60	88	+5	[21]
chlorotoxin		8	64	60	+3	[17]
Lqh-8/6		9	50	68	+4	[16]
PBITx1		10	44	100	+3?	
consensus	-C-P CFTT-----C--CC-G----KC-G-QC-C---					

Fig. 2. Partial amino acid sequence of PBITx1. The primary structure (in single letter code) of PBITx1 is compared with similar 'short' insectotoxins, after manual alignment of the cysteine residues. The consensus sequence is given below. For the 10 similar sequences known thus far, a uniform nomenclature is proposed: the family of sITx peptides (from short insectotoxin). Numbering within this family has been assigned according to date of publication. Therefore, peptide I₁ and PBITx1 are designated sITx1 and sITx10, respectively. The percentage identity between these peptides is also shown, either as compared to peptide I₁ (left column), or as compared to PBITx1 (right column). The net charge of the insectotoxins ranges from +2 to +5, indicating that they are basic molecules at physiological pH. The pattern of disulfide bridges determined for I_{5A} [14], chlorotoxin [15], and Lqh-8/6 [16] is indicated. Regions of secondary structures: α -helix (gray bar) and two antiparallel β -strands (black bar). Sequences for I₃ (★) and I₄ (★★), which are deduced sequences, are shown in italics and are based on amino acid analysis results combined with clear sequence identity (> 50%) with I₁, I₅ and I_{5A} (see also text) [20]. For peptide I₄, residues at positions 14 and 23 are shown in small caps because we cannot exclude a reversed occurrence in the native peptide.

been able to carry out was achieved with ~100 mg crude venom pooled from different animals. PBITx1 yield from this starting material was ~1.5 μ g (0.0015%), as judged from the absorbance measured at 280 nm and taking into account the molar extinction coefficient of peptide I, a highly homologous peptide isolated from *Buthus indicus* [21].

The primary amino acid sequence of PBITx1 was determined as detailed in Section 2. Most of the positions were unequivocally assigned. It is known that cysteine residues are difficult to identify, unless the sample is alkylated beforehand. However, blank spaces in a clear sequence mean the presence of a cysteinyl residue, which is the case for positions 2, 5, 16 and 20 in PBITx1. Position 19 could be either a cysteine or an aspartic acid. The difficulty in assigning position 19 arises from the fact that the previous residue at position 18 is also an aspartic acid. Additionally, position 23 is either an arginine or a glycine. Again the definitive assignment of this position is hampered by the preceding double glycines at positions 21 and 22. Finally, the last amino acid identified at position 25 is very likely a lysine.

Comparison of these data with other closely related 'short' insectotoxin-like peptides is shown Fig. 2. When cysteine residues are aligned, a search for sequence identity shows a close relationship with insectotoxins I₁, I₅ and I_{5A} from *B. eupeus* (44, 64, 64% respectively) [13,20], AmmP2 from *Androctonus mauretanicus* (56%) [2,6], peptide I from *B. indicus* (88%) [21], chlorotoxin from *L. quinquestriatus quinquestriatus* (60%) [17] and Lqh-8/6 from *L. quinquestriatus hebraeus* (68%) [16]. If

residue 23 of PBITx1 is an arginine, it resembles toxin I₁ and AmmP2; if it is glycine, it is rather similar to I₅.

Another common feature is the net charge of these 'short' insectotoxins, ranging from +2 to +5, making them basic peptides. The strong retention of PBITx1 seen on a cation exchange column (Fig. 1D) confirms the peptide's basic character.

We also provide here the deduced sequences of two insectotoxins, I₃ and I₄, from the venom of the scorpion *B. eupeus* living in Central Asia to highlight the high degree of identity with I₁, I₅ and I_{5A}. Almost 15 years ago, the amino acid composition of five 'short' insectotoxins (I₁, I₃, I₄, I₅ and I_{5A}) from *B. eupeus* was published, together with the complete sequences of I₁, I₅ and I_{5A} [20]. For I₄, sequence information was available for as far as eight N-terminal residues, whereas no sequence has ever been reported for I₃. Thanks to the amino acid analysis data of Ovchinnikov [20] and the strong conservation in primary structure of the 'short' insectotoxins in general, a theoretical sequence analysis as outlined below made it possible to predict the most plausible sequences for I₃ (36 aa) and I₄ (35 aa) (see Fig. 2). Our strategy was the following: (i) alignment of the cysteine residues (eight residues), (ii) fill in the 100% conserved N- and C-terminal regions (in single letter code: MCMPCFTT and KCFG-QCLC, respectively, yielding 11 additional residue positions), (iii) fill in three other 100% conserved positions (A13, R17 and G22), and (iv) conclusion of the sequence by comparing identical and homologous positions between all 'short' insectotoxins

on insects [27]. One of the reasons that the biological activity remains unclear is the scarcity and tiny share of 'short' insectotoxins in scorpion venom, hampering full characterization of these peptides. Moreover, it has been claimed that 'short' insectotoxins are not part of the scorpion's natural secretions, but are found only in venom collected by electrical stimulation [28]. The fact that PBITx1 could only be found in one purification round, although all the venoms from *P. schlechteri* were electrically elicited using the same protocol, does not corroborate the above. Therefore, future experiments, such as analyzing the genomic organization of sITx genes or chemical synthesis of sITx peptides, will be needed to shed light on the biological activity of these interesting molecules.

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