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*, I_3 and I_4, 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.

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 belonging 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*, I_3 and I_4 [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 14 000 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 C_2/C_{18} 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 C_{18} 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 C_{18} 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 C_{18} 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

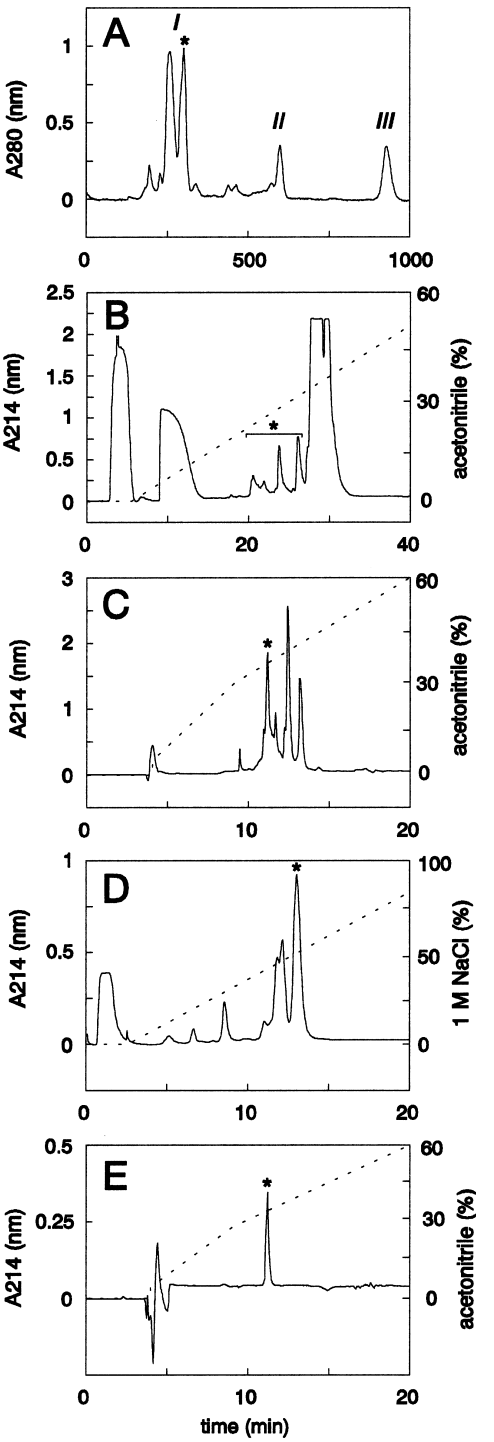
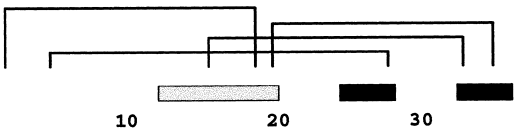


Fig. 1. Purification of PBITx1 from *Parabuthus schlechteri* venom. A: Crude venom was first fractionated by FPLC gel filtration as indicated in Section 2, yielding three typical groups of peaks (I–III). The labeled fraction (★) in group I was recovered and lyophilized. Based on a constructed gel filtration 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 C_2/C_{18} 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 C_{18} 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 C_{18} reversed phase column. See Section 2 for experimental details during the different purification steps.



Member			sITx Name	Ident. (%)		Charge	Ref.
		1 10 20 30					
I_1		MCMPCFTTRPDMAQQCRACCKGR--GKCFGPQCLCGYD	1	100	44	+3	[13]
AmmP2		CGPCFTTDPYTESKCATCCGGR--GKCVGPQCLCNRI	2	60	56	+2	[1,2]
I_3	*	*MCMPCFTTDHQTARRCRDCCGGRGR-KCFG-QCLCGYD*	3	72	52	+3	[20]
I_4	**	MCMPCFTT*DHNMAkKCRDCCGGn--GKCFGPQCLCNR*	4	69	60	+3	[20]
I_5		MCMPCFTTDPNMANKCRDCCGG-GK-KCFGPQCLCNR	5	71	64	+3	[20]
I_{5A}		MCMPCFTTDPNMAKKCRDCCGGN--GKCFGPQCLCNR	6	74	64	+3	[20]
Peptide I Bs		RCKPCFTTDPQMSKKCADCCGGKGKGKCYGPQCLC	7	60	88	+5	[21]
chlorotoxin		MCMPCFTTDHQMARKCDDCCGGKGRGKCIGPQCLC-R	8	64	60	+3	[17]
Lqh-8/6		RCSPCFTTDQQMTKKCYDCCGGKGKGKCYGPQCICAPY	9	50	68	+4	[16]
PBITx1		RCKPCFTTDPQMSKKCADxCGGx-Kx?	10	44	100	+3?	
consensus		-C-PCFTT-------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 I_1 and PBITx1 are designated sITx1 and sITx10, respectively. The percentage identity between these peptides is also shown, either as compared to peptide I_1 (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 I_3 (★) and I_4 (★★), which are deduced sequences, are shown in italics and are based on amino acid analysis results combined with clear sequence identity (>50%) with I_1, I_5 and I_{5A} (see also text) [20]. For peptide I_4, 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 μg (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 sindicus* [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 I_1, I_5 and I_{5A} from *B. eupeus* (44, 64, 64% respectively) [13,20], AmmP2 from *Androctonus mauretanicus* (56%) [2,6], peptide I from *B. sindicus* (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 I_1 and AmmP2; if it is glycine, it is rather similar to I_5.

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, I_3 and I_4, from the venom of the scorpion *B. eupeus* living in Central Asia to highlight the high degree of identity with I_1, I_5 and I_{5A}. Almost 15 years ago, the amino acid composition of five 'short' insectotoxins (I_1, I_3, I_4, I_5 and I_{5A}) from *B. eupeus* was published, together with the complete sequences of I_1, I_5 and I_{5A} [20]. For I_4, sequence information was available for as far as eight N-terminal residues, whereas no sequence has ever been reported for I_3. 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 I_3 (36 aa) and I_4 (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

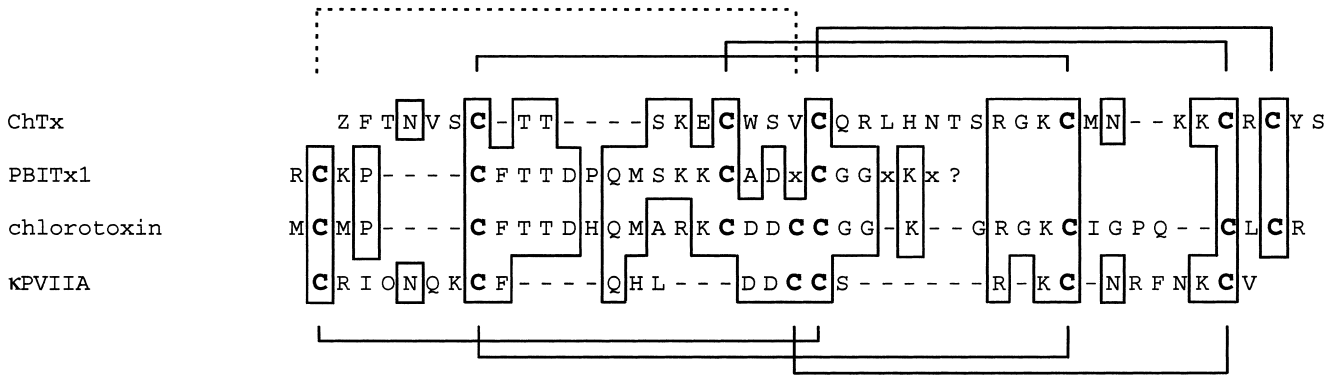


Fig. 3. Primary structure comparison between short-chain toxins from scorpion and snail venom. Manual alignment of cysteine residues reveals identical regions (boxed) between short-chain toxins isolated from scorpion venom, such as ChTx [23], PBITx1 and chlorotoxin [15,17], but also with κ-conotoxin [24,25] which is a toxin isolated from a different phylum. The pattern of disulfide bridges is indicated. It is interesting to note that the fourth disulfide bridge in chlorotoxin (dashed line) represents an 'additional' disulfide pairing as compared to the pairings in ChTx.

(e.g. for I_3: D9 (90%), H10 (30%), Q11 (50%), T12 (20%), R14 (60%), R15 (90%), D18 (80%), G21 (90%), R23 (60%), G24 (50%), R25 (60%), and GYD with I_1 (100%)). The analysis carried out above, together with the published sequences, enabled us to compose a list of all 'short' insectotoxins known thus far and to derive a consensus sequence (see Fig. 2). Together with the eight cysteine residues, eight other residues were found to be 100% conserved for the 10 members of 'short' insectotoxins.

On this occasion, we also propose a uniform nomenclature for these peptides, representing a dramatic improvement in the naming of the ever expanding family of short-chain toxins in general over the 'whimsical' names currently in use. By analogy with Miller's nomenclature for K^+ channel blocking toxins, α-KTx [22], we propose to use the name sITx (from short insectotoxin). Thus, instead of using the biological action on ion channels to designate 'short' insectotoxins, such as chlorotoxin [17], or using fraction numbers, like Lqh-8/6 [16], these peptides can be labeled much more easily sITx8 and sITx9, respectively. Using this nomenclature, PBITx1 corresponds to sITx10. Our consensus sequence, as well as our nomenclature, might be a useful tool for future classification and systematic comparison of the ever growing number of novel toxins.

Although a clear relationship between PBITx1 and 'short' insectotoxins is evident, it is interesting to compare the primary structure of PBITx1 with those of other basic short-chain toxins isolated from different arthropods and from animals belonging to distinct phyla (Fig. 3). For instance, the absence of a cysteine pair at positions 19–20 in PBITx1, assuming that residue 19 represents an aspartic acid and not a cysteine residue (see above), contrasts with all other 'short' insectotoxins. It is reminiscent of the CxxxC motif in α-KTx neurotoxins, such as in charybdotoxin (ChTx) from the venom of the scorpion *L. quinquestriatus hebraeus* [23]. In addition, sequence alignment of PBITx1 with ChTx, combined with the introduction of gaps to improve alignment, revealed sequence identity at seven positions (28%). Likewise, comparison between chlorotoxin and ChTx yields identity at 11 positions (30.6%) with a fully conserved stretch of residues R25-G26-K27-C28 residing in one of the β-sheets. Interestingly, K27, known to be crucially involved in the interaction with the pore of voltage-gated K^+ channels, forms part of this stretch of residues. Nevertheless, chlorotoxin block of voltage-gated K^+ channels has never been shown so far. In terms of three-dimensional structure, it is known that all these 'short' scorpion peptides have an α-helix bound by two disulfide bridges to the second strand of the antiparallel β-sheet (see Fig. 2), resulting in very similar scaffolds. In the case of 'short' insectotoxins, a stabilizing role is played by a fourth disulfide bridge connecting the N-terminus of the peptide with the C-terminal part of the α-helix [14–16].

Finally, we have compared the sequences of PBITx1, chlorotoxin, ChTx and κ-conotoxin PVIIA from the venom of *Conus purpurascens* [24]. There are several obvious reasons for such a comparison: (i) a number of sequence identities exist, which deserve closer examination, (ii) κ-conotoxin PVIIA is the first cone snail toxin described to block voltage-gated K^+ channels [24], and (iii) ChTx is one of the best characterized scorpion toxins also acting on voltage-gated K^+ channels (Fig. 3). A search for κ-conotoxin sequence identity reveals identity at six positions with PBITx1 (24%), 12 positions with chlorotoxin (33%), and nine positions with ChTx (24.3%). The presence of residues C8-F9 and D13-D14-C15-C16 in κ-conotoxin is noteworthy, because identical positions can be found in 'short' insectotoxins as well. Furthermore, the sequence R18-K19-C20 in κ-conotoxin is very reminiscent of the R25-K27-C28 stretch in ChTx. Some of these primary structure comparisons may not be functionally relevant, since it is known that the fold adopted by κ-conotoxin PVIIA is unrelated to the fold of short-chain toxins from scorpions [14–16,23,25]. However, in the light of the hypothesis of convergent evolution of animal toxins, i.e. toxins that have unrelated structures but similar functions thanks to conserved key functional residues [26], it will be interesting to see if, for instance, the absence of a conserved diad of functional residues, such as K27-Y36 in ChTx [26] and K7-F9 or F23 in κ-conotoxin [25], can explain the supposed inactivity of 'short' insectotoxins on voltage-gated K^+ channels.

In the absence of strong experimental evidence at present, we can only speculate on the biological activity of 'short' insectotoxin peptides. Based on the literature evidence, a block of small-conductance Cl^- channels derived from epithelial cells has been reported for chlorotoxin [17]. The other 'short' insectotoxins presumably act on the glutamate receptor of the postsynaptic membrane, manifesting a paralyzing effect

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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