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Structure–activity relationship of an α-toxin Bs-Tx28 from scorpion (*Buthus sindicus*) venom suggests a new α-toxin subfamily ☆

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Abstract

Scorpion venoms are among the most widely known source of peptidyl neurotoxins used for callipering different ion channels, e.g., for Na^+, K^+, Ca^+ or Cl^-. An α-toxin (Bs-Tx28) has been purified from the venom of scorpion *Buthus sindicus*, a common yellow scorpion of Sindh, Pakistan. The primary structure of Bs-Tx28 was established using a combination of MALDI-TOF-MS, LC-ESI-MS, and automated Edman degradation analysis. Bs-Tx28 consists of 65 amino acid residues (7274.3 ± 2Da), including eight cysteine residues, and shows very high sequence identity (82–94%) with other long-chain α-neurotoxins, active against receptor site-3 of mammalian (e.g., Lqq-IV and Lqh-IV from scorpions Leiurus sp.) and insect (e.g., BJα-IT and Od-1 from *Buthotus judaicus* and *Odonthobuthus doriae*, respectively) voltage-gated Na^+ channels. Multiple sequence alignment and phylogenetic analysis of Bs-Tx28 with other known α- and α-like toxins suggests the presence of a new and separate subfamily of scorpion α-toxins. Bs-Tx28 which is weakly active in both, mammals and insects (LD_{50} 0.088 and 14.3 μg/g, respectively), shows strong induction of the rat afferent nerve discharge in a dose-dependent fashion ($EC_{50} = 0.01$ μg/mL) which was completely abolished in the presence of tetrodotoxin suggesting the binding of Bs-Tx28 to the TTX-sensitive Na^+-channel. Three-dimensional structural features of Bs-Tx28, established by homology modeling, were compared with other known classical α-mammal (AaH-II), α-insect (Lqh-αIT), and α-like (BmK-M4) toxins and revealed subtle variations in the Nt-, Core-, and RT-CT-domains (functional domains) which constitute a ''necklace-like'' structure differing significantly in all α-toxin subfamilies. On the other hand, a high level of conservation has been observed in the conserved hydrophobic surface with the only substitution of W43 (Y43/42) and an additional hydrophobic character at position F40 (L40/A/V/G39), as compared to the other mentioned α-toxins. Despite major differences within the primary structure and activities of Bs-Tx28, it shares a common structural and functional motif (e.g., *trans*RT-*far*CT) within the RT-CT domain which is characteristic of scorpion α-mammal toxins.

Keywords: Afferent nerve discharge; α-Neurotoxin; Killer-necklace; Peptidyl inhibitors; Scorpions; Sodium channel; Venoms

It is generally known that scorpion venom is a complex mixture composed of a wide array of proteins (e.g., hyaluronidases and phospholipases), peptides (neurotoxins), and non-proteinaceous substances (e.g., mucopolysaccharides, serotonin, histamine, and protease inhibitors). Scorpion venoms are in particular a rich source of small, mainly neurotoxic proteins and/or peptides modulating various ionic channels (such as K^+, Na^+, Ca^{2+}, and Cl^-) in excitable membranes. Since the ion channels are vital machineries for the activity of living cells, targeting these membrane-bound proteins is thus an efficient means for venomous animals to capture their preys or to defend themselves against enemies [1,2].

☆ The protein sequence of α-toxin (Bs-Tx28) reported in this paper has been deposited in the European Bioinformatics Institute database and will appear in UniProt knowledgebase under Accession No. P84614.

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Scorpion neuropeptides, modulating the voltage-gated Na^+ channels (VGSCs)[2] of vertebrates (mammals) and invertebrates (arthropods), belong to the homologous family of long-chain neurotoxins (LCNs) composed of 61–76 amino acid residues and stabilized by four intrachain disulfide bridges to construct a compact and highly conserved structural scaffold termed as cysteine-stabilized α-helix-β-sheet-motif containing a βαββ-type sequence. Based on their mode of action and binding properties, LCNs affecting VGSCs are divided into two major classes, namely α- and β-neurotoxins [2–4]. β-Toxins (e.g., CsEv1–3 from Mexican scorpion *Centruroides sculpturatus* Ewing venom) shift the voltage dependence of activation from a more negative membrane potential upon binding to receptor site-4 which is mapped to the S3–S4 external loop within the D2-domain of VGSCs [2,5]. Conversely, α-toxins (e.g., AaH-II from scorpion *Androctonus australis* Hector venom) decrease or inhibit the sodium current inactivation, when binding to receptor site-3. The involvement of the extracellular loops S3–S4 of domain D4 and S5–S6 of D1 and D4 in formation of receptor site-3 on VGSCs was recognized [6–8]. Furthermore, some structurally unrelated peptide toxins from sea anemone (e.g., ATX-II from *Anemonia sulcata*) and spider (e.g., δ-atracotoxins from *Atrax robustus*) compete also with scorpion α-toxins for site-3 on vertebrate and invertebrate VGSCs, suggesting the similarity of this receptor site in phylogenetically distant organisms [9,10]. However, explicit differences in the potency and maximum efficacy of various α-toxins toward insects and mammals were observed in vivo by toxin administration and in vitro by competition binding assays applied to insect and rat-brain neuronal preparations [6,9,11–14].

Scorpion α-toxins, modulating the receptor site-3 of VGSCs, were further divided into at least three distinct pharmacological subfamilies according to their preferential activities against mammals and insects: (1) classical α-mammal toxins (e.g., AaH-II or Lqh-II [15–17]) which bind with high affinity to rat brain VGSCs (0.2–0.4 nM) and are practically non-toxic to insects; (2) α-toxins, highly active on insects (e.g., Lqh α-IT or Lqq-III [18–20]) which bind with high affinity to insect VGSCs (0.03–0.07 nM), but are over three orders of magnitude less potent in mammalian brain as compared to AaH-II; and (3) α-like toxins (e.g., BmK-M1, Bom-III-IV, or Lqh-III) which are toxic in both, mammals (~2–60 μM) and insects (~0.43–30 nM), although at much higher concentrations [10,21–23]. In spite of differences described above, these pharmacologically distinct subfamilies of scorpion α-toxins (with the exception of Amm-VIII [24]) are equally toxic to mice by subcutaneous injection (SC) and affect with similar affinity to rat skeletal muscle VGSCs [18]. Thus, variations in toxicity and binding profiles of scorpion α-toxins demonstrate the heterogeneity, not only in toxin structure and function, but also in their ultimate targets [7,25]. Therefore, elucidation of the inherent ability of α-toxins of the various scorpions to differentiate between insect and mammalian VGSCs or among subtypes in mammalian excitable tissues is of potential value for the future design of selective drugs and insecticides [6,9,11].

Despite accumulated details on the structure and function of few representatives of scorpion α-toxins, a huge wealth of new natural venom resources (99.98% [2]) is still untouched and available for exploring the mode of action of this structurally homologous, but functionally different class of neuropeptides. With our ongoing efforts for this goal, we have established the structure and activity relationship of a new toxin from scorpion *Buthus sindicus* venom which significantly extends the information for an almost neglected, but unique and newly proposed subfamily of scorpion α-toxins. These toxins are active against both, insects and mammals, but exert their main toxic effects either on mammals (e.g., Lqq-IV, Bs-Tx28, and Lqh-IV) or insects (e.g., Bjα-IT and Od-1).

Materials and methods

Isolation and purification

The scorpions (*Bu. sindicus*; family *Buthidae*) were collected from the province of Sindh, Pakistan. The pre-venom was collected by electrical stimulation of the telson in deionized water and centrifuged at 15,000 rpm for 10 min. The supernatant was freeze-dried and stored at −20 °C until used [26].

The single step purification of Bs-Tx28 was achieved by subjecting the whole pre-venom to a Nucleosil $7C_{18}$ reverse-phase high-performance liquid chromatographic column (250 × 10 mm; Macherey-Nagel, Düren, Germany). The following conditions for RP HPLC separation were used: eluent A, 0.1% trifluoroacetic acid in water (v/v); eluent B, 100% acetonitrile containing 0.05% TFA (v/v); gradient program: 15% B for 5 min, followed by 70% B in 90 min at a flow rate of 1 ml/min [27]. The UV absorbance of the eluate was monitored at 230 nm. The protein concentration was determined according to the method of Bradford [28] using BSA as standard protein reference.

Capillary electrophoresis

Capillary electrophoresis was performed on a BioFocus 3000 system (Bio-Rad, Hercules, USA). Samples were

[2] *Abbreviations used:* AaH, *Androctonus australis* Hector; Amm, *Androctonus mauretanicus mauretanicus*; AUC, area under curve; Be, *Buthus eupeus*; Bj, *Buthotus judaicus*; BmK, *Buthus martensii* Karsch; Bom, *Buthus occitanus mardochei*; Bot, *Buthus occitanus tunetanus*; Bs, *Buthus sindicus*; CsEv, *Centruroides sculpturatus* Ewing; ESI MS, electrospray ionization mass spectrometry; ICV, intracerebro-ventricular injection; LCNs, long-chain neurotoxins; LD_{50}, half lethal dose; Lqh, *Leiurus quinquestriatus hebraeus*; Lqq, *Leiurus quinquestriatus quinquestriatus*; Od, *Odonthobuthus doriae*; Os, *Orthochirus scrobiculosus*; MALDI-TOF-MS, matrix-assisted laser desorption time of flight mass spectrometry; MkTxI, Makatoxin I from *Buthus martensii* Karsch; RMSD, root mean squared deviation; RT-CT, reverse turn-C-terminal domain; SC, subcutaneous injection; TTX, tetrodotoxin; Tx, toxin; VGSC(s), voltage-gated Na^+ channel(s).