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Tityus serrulatus venom peptidomics: Assessing venom peptide diversity

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ABSTRACT

MALDI-TOF-TOF and *de novo* sequencing were employed to assess the *Tityus serrulatus* venom peptide diversity. Previous works has shown the cornucopia of molecular masses, ranging from 800 to 3000 Da, present in the venom from this and other scorpions species. This work reports the identification/sequencing of several of these peptides. The majority of the peptides found were fragments of larger venom toxins. For instance, 28 peptides could be identified as fragments from Pape proteins, 10 peptides corresponded to N-terminal fragments of the TsKβ (scorpine-like) toxin and fragments of potassium channel toxins (other than the k-beta) were sequenced as well. N-terminal fragments from the *T. serrulatus* hypotensins-I and II and a novel hypotensin-like peptide could also be found. This work also reports the sequencing of novel peptides without sequence similarities to other known molecules.

1. Introduction

The classical approaches for the identification and characterization of bioactive components in scorpion venoms were based on function-to-structure studies which were directed by the ability of the toxins in inducing conspicuous pharmacological effects (Pimenta et al., 2001). Such approaches have successfully built a solid knowledge on the physiological effects and on the structure of several scorpion toxins (Possani et al., 2000; Pimenta et al., 2001; Rodriguez de la Vega and Possani, 2004, 2005; De Lima et al., 2007b). Most scorpion toxins contain three or four disulfide-bridges and can recognize and specifically interact with ion channels including Na^+, K^+, Cl^- and Ca^{2+} channels (DeBin et al., 1993; Tytgat et al., 1999; Possani et al., 2000; Zeng et al., 2001; Goudet et al., 2002; Rodriguez de la Vega and Possani, 2004, 2005; De Lima et al., 2007b). Several hundred ion channel-targeting peptides have been isolated and characterized from nearly 20 species of scorpion (Zeng et al., 2005).

Recently, proteomic approaches have been employed in the assessment of scorpion (Pimenta et al., 2001; Goudet et al., 2002; Pimenta et al., 2003; Chen et al., 2005; Barona et al., 2006; Batista et al., 2006; Batista et al., 2007; Borges and Rojas-Runjaic, 2007; Schwartz et al., 2007; Bringans et al., 2008), other invertebrates (Escoubas et al., 2002; Machado et al., 2005; Pimenta et al., 2005; Favreau et al., 2006; Richardson et al., 2006; Liao et al., 2007; Rates et al., 2007) and vertebrates (Steinborner et al., 1997; Wabnitz et al., 1999; Fry et al., 2002; Fry et al., 2003; Li et al., 2004; Brinkworth et al., 2005; Calvete et al., 2007; Yanes et al., 2007; Olamendi-Portugal et al., 2008) toxin diversity. These modern structure-to-function approaches are conceptually

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distinct from the above mentioned 'classical' approaches, once sequence similarities are used to direct the pharmacological characterization efforts.

Scorpion venoms also contain enzymes, nucleotides, lipids, biogenic amines, and other unidentified components (Almeida et al., 2002; Batista et al., 2006). Small and non-reticulated (without disulfide-bridges) peptides are also present in these venoms although only a limited number of sequences have been determined thus far (Pimenta and De Lima, 2005; Zeng et al., 2005). Such molecules may present a wide range of biological activities, such as bradykynin potentiating, antimicrobial, hemolytic, immune-modulating (Pimenta and De Lima, 2005; Zeng et al., 2005) and hormone-like activities (Pimenta and De Lima, 2005; Pimenta et al., 2005; Verano-Braga et al., 2008).

Due to the development of microscale analytical techniques, such as mass spectrometry, studies focused on peptides in the 800–3000 Da molecular mass range are becoming increasingly abundant. However, when compared to well-established state-of-the-art ion channel-targeting toxins, the structural and biological activity characterizations of small peptides from arthropod venoms are in their early stages (Pimenta and De Lima, 2005).

The scorpion *Tityus serrulatus* is the organism from its group (Scorpionidae) whose venom has been most extensively studied (De Lima and Martin-Eauclaire, 1995; Pimenta et al., 2001). Despite that, no studies have focused on the systematic sequence determination of small (800–3000 Da) un-reticulated peptides. This report, therefore, focuses on the *de novo* sequencing of such molecules through MALDI-TOF-TOF tandem mass spectrometry.

2. Materials and methods

All reagents and chemicals were analytical or spectroscopic grade.

2.1. Animals and venom extraction

Venom was extracted from several individuals by electrical stimulation directly on the hindmost abdominal segment. The venom was transferred to chilled acidified water (0.1% aqueous trifluoroacetic acid) and centrifuged (15,000 rpm for 5 min) to remove cellular debris and a mucous pellet. The supernatant fractions were lyophilized and stored at −20 °C until required.

2.2. Liquid chromatography

Venom fractionation was performed using two different experimental setups. In the first setup, *T. serrulatus* pooled venom (5 mg) was applied to a Superdex Peptide 10/300 GL gel filtration column and eluted with 100 mM ammonium acetate buffer using a 0.5 mL/min flow rate. Elution was monitored by absorbance readings at 280 nm. The obtained fractions were further separated using reverse phase chromatographies (RPC), which were performed by using a Chromolith Performance RP-18 100/4.6 mm column (Merck), with a 4.0 mL/min flow rate. Elution was monitored by absorbance readings at 214 nm.

In the second setup, *T. serrulatus* venom (250 mg) was applied to a Sephadex G-50 (Superfine) column and eluted with 0.15 M ammonium formiate buffer, pH 6.5 using a 0.7 mL/min flow rate. Obtained fractions were pooled according to the chromatographic profile and then lyophilized. These fractions were further separated by high performance liquid chromatography (HPLC) in a preparative C4 reverse phase column (Vydac, USA) (flow rate 1.0 mL/min). These sub-fractions obtained were eluted in a second HPLC separation, using the analytical C4 reverse phase column (Vydac) (flow rate 1.0 mL/min) or were separated in a C18 reverse phase column (Vydac) (flow rate 1.0 mL/min). In this fractionation setup, elution was monitored by absorbance readings at 214 nm.

For all RPC analysis (in both setups) columns were previously equilibrated with 0.1% aqueous trifluoroacetic acid, and the compounds were eluted by a linear gradient 0.1% TFA in acetonitrile. The fractions eluted from the RPC column were subjected to MALDI-TOF-TOF analyses.

2.3. Mass spectrometry and data analyses

MS and tandem MS analysis were performed using a MALDI-TOF-TOF AutoFlex III™ (Bruker Daltonics) instrument in positive/reflector mode controlled by the FlexControl™ software. Instrument calibration was achieved by using Peptide Calibration Standard II (Bruker Daltonics) as reference and α-cyano-4-hydroxycinnamic acid was used as matrix. Samples were spotted to MTP AnchorChip™ 400/384 (Bruker Daltonics) targets using standard protocols for the dried droplet method.

MS data analysis was performed by using the FlexAnalysis™ software (Bruker Daltonics). Peptide *de novo* sequencing was performed using a combination of manual and automatic data interpretation using the softwares FlexAnalysis™ and BioTools™ (Bruker Daltonics). Alternatively, MS/MS data were exported as *.txt files and than converted to the *.dta extension. Then such files were imported into *.psq files which are recognizable by the PepSeq™ (Micromass, UK) software which was also used for manual *de novo* sequencing.

Similarity searches were performed with the obtained sequences using the Fasta3 tool against the Swiss-Prot data Bank, as previously described (Pearson, 1990).

3. Results and discussion

3.1. Venom fractionation

The gel filtration step of the first venom fractionation setup yielded 16 fractions (Fig. 1A), which were further purified by RPC (Fig. 2). The gel filtration step of the second fractionation setup yielded 7 fractions (Fig. 1B) which were, also, further purified by RPC (data not shown). RPC fractions from both setups were submitted to MALDI-TOF-TOF analysis. Ions with sufficient signal intensities were submitted to MS/MS analyses.