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A simple protocol for venom peptide barcoding in scorpions

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ABSTRACT

Scorpion venoms contain many species-specific peptides which target ion channels in cell membranes. Without harming the scorpions, these peptides can easily be extracted and detected by MALDI-TOF mass spectrometry. So far, only few studies compared the venom of different species solely for taxonomic purposes. Here, we describe a very simple protocol for venom extraction and mass fingerprinting that was developed for peptide barcoding (venom code for species identification) and facilitates reproducibility if sample preparation is performed under field conditions. This approach may serve as suitable basis for a taxonomy-oriented scorpion toxin database that interacts with MALDI-TOF mass spectra.

1. Introduction

Scorpions belong to a group of arthropods which phenotype remained largely unchanged during the last 400 million years. The evolutionary success of these predators is largely associated with their very potent venom, which is used for deterring predators and for immobilizing their prey [1]. Major compounds of the venom are peptides [2] and many of these peptides interact with K^+ ion channels ("short chain" peptides [3]) and Na^+ ion channels ("long-chain" peptides [4]). It is hypothesized that the numerous scorpion venom peptides with their divergent effects may have evolved from a common ancestor gene [5].

As it is typical of venom toxins from other animals [e.g. 6–9], composition and efficacy of peptide toxins tend to be taxon-specific in scorpions, although basic types of peptides can be found in different clades [10]. The rapid diversification at the molecular level results from evolutionary adaptations of ion-channels of local prey and predators [11]. Scorpions are becoming helpless if predators resist the scorpions sting [12] and have to adapt their toxin efficacy or die out. For certain species it was even shown that different populations can be differentiated based on AA-substitutions within peptide sequences [13–16].

Information about specific peptide sequences can be used to explore the pharmaceutical potential [17] but might also be useful to confirm species identity. Most of the toxic peptides contain less than 100 AAs and are therefore well detectable by mass spectrometry, which is the method of choice for a fast venom screening [18,19]. For a number of scorpion species, particularly medically important species of Buthidae, comprehensive data exist already, that reflect the peptide complement of the venom [20]. The peptidome of the venom glands from these species consists of mature peptides but also many breakdown products of larger polypeptides [21]. Only few studies compared the venom of different species solely for taxonomic purposes [e.g. 22,23]. The general idea

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behind that strategy was described already by Dyason et al. [22] who pointed out that it is, for species-identification, not important to obtain a complete map of existing peptides but a typical venom profile of ion signals for a given taxon. In the chemotaxonomic studies, different types of mass spectrometers, different mass ranges, different settings (e.g. linear or reflectron mode) and also different protocols for venom extraction have been used.

In our study, we tested several scenarios to obtain mass fingerprints of venom peptides as easy and repetitive as possible. The protocol that turned out to be the most convincing one is described herein and may serve as suitable basis for a taxonomy-oriented scorpion toxin database that interacts with MALDI-TOF mass spectra.

2. Materials and methods

2.1. *Venom extraction*

Heterometrus cyaneus (Vietnam) were obtained from the Pet Factory (Hülsede, Germany); all other scorpions tested in this study were collected during field trips and released following second venom extraction. For electrical stimulation of the membrane anterior to the telson, we used the transcutaneous electrical nerve stimulation unit Promed tens 1000 s (Promed GmbH, Farchant, Germany) connected with modified tweezers to apply power. To improve stimulation, the tweezers were lubricated with conduct gel. The intensity of stimulation current was adjusted for each scorpion; usually a pulse width from 200 to 250 μs and a pulse rate of 60–130 Hz was applied. The extracted venom was collected with a glass capillary (inner diameter of 1 mm; Hilgenberg GmbH, Malsfeld, Germany) that was attached to a flexible silicone tube and a disposable syringe, and transferred into a 0.5 ml Eppendorf microtube containing 200 μl of 35% ethanol/0.1 TFA.

2.2. *N-terminal peptide derivatization using 4-sulfophenyl-isothiocyanate (SPITC)*

For SPITC derivatization, 2 μl of the venom extract in 35% ethanol/0.1 TFA was mixed with 18 μl of 0.1 TFA. SPITC was dissolved in 20 mM $NaHCO_3$ (pH 9.0) at a concentration of 80 mg/ml. 15 μl of the diluted venom extract and 40 μl of the SPITC solution were mixed and the sulfonation reaction performed for 1 h at 55 °C and 300 ppm. After that, the sample was acidified by adding 2.5 μl of 10% acetic acid followed by 50 μl of 0.5% acetic acid. Peptides were loaded in aliquots of 20 μl onto an activated and equilibrated home-made StageTip Empore 3M (IVA Analysentechnik e.K., Meerbusch, Germany) C18 column. The column was then flushed with 2× 20 μl of 0.5% acetic acid. Retained peptides were eluted from the column with 1.3 μl of 10/20/25/30/40/50/60/70/80% acetonitrile in 0.5% acetic acid. Each elution step was performed three times and the eluates spotted onto a MALDI target, respectively. Before drying, matrix solution (CHCA) was added to the samples.

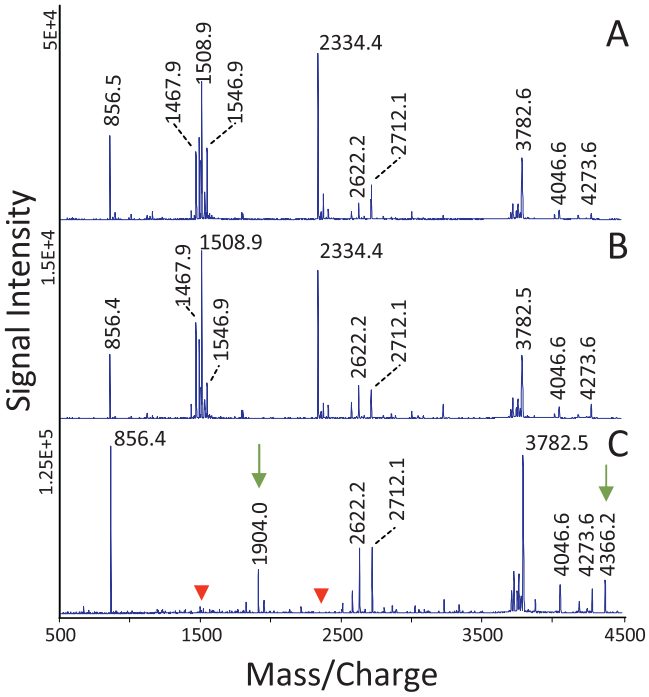

Fig. 1 – MFPs (*m/z* 500–4500) from a venom sample of *Heterometrus cyaneus* stored under different conditions. (A) Venom extract was diluted in 100 μl 0.1 TFA and 0.5 μl of this solution was immediately spotted on a sample plate. Matrix application has been accomplished after 4 weeks; (B) venom sample was diluted in 200 μl 35% ethanol/0.1 TFA and stored for 4 weeks at room temperature. About 0.5 μl of this solution was spotted on the sample plate and matrix application has been accomplished immediately; and (C) remaining venom solution in 0.1 TFA (see A) was stored for 4 weeks at room temperature. About 0.5 μl of this solution was spotted on the sample plate and matrix application has been accomplished immediately. MFPs of samples which were prepared as described in (A) and (B) are almost identical. The sample stored in 0.1 TFA was partially degraded; missing ion signals are marked by arrowheads; additional ion signals are marked by arrows.

2.3. *MALDI-TOF mass spectrometry*

About 0.5 μl of matrix (10 mg/ml 2,5-dihydroxybenzoic acid [DHB; Sigma–Aldrich, Steinheim, Germany] dissolved in 20% acetonitrile/1% formic acid or 10 mg/ml α-cyano-4-hydroxycinnamic acid [CHCA; Sigma–Aldrich] in 60% ethanol/36% acetonitrile) was mixed with the same quantity of venom extract before air drying at room temperature. Samples of venom extract, which were spotted onto the sample plate long before mass spectrometric analysis, were dried without matrix application and matrix was added immediately before analysis.

MALDI-TOF mass spectrometry was performed in positive ion mode on an UltrafleXtreme TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). All spectra, which are discussed here, were acquired in reflectron mode within a range of *m/z* 500–10,000; the settings were optimized to *m/z*