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Intraspecific variation of *Centruroides sculpturatus* scorpion venom from two regions of Arizona

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ABSTRACT

This study investigated geographic variability in the venom of *Centruroides sculpturatus* scorpions from different biotopes. Venom from scorpions collected from two different regions in Arizona; Santa Rita Foothills (SR) and Yarnell (Yar) were analyzed. We found differences between venoms, mainly in the two most abundant peptides; SR (CsEv2e and CsEv1f) and Yar (CsEv2 and CsEv1c) identified as natural variants of CsEv1 and CsEv2. Sequence analyses of these peptides revealed conservative amino acid changes between variants, which may underlie biological activity against arthropods. A third peptide (CsEv6) was highly abundant in the Yar venom compared to the SR venom. CsEv6 is a 67 amino acid peptide with 8 cysteines. CsEv6 did not exhibit toxicity to the three animal models tested. However, both venoms shared similarities in peptides that are predicted to deter predators. For example, both venoms expressed CsEI (lethal to chick) in similar abundance, while CsEd and CsEM1a (toxic to mammals) displayed only moderate variation in their abundance. Electrophysiological evaluation of CsEd and CsEM1a showed that both toxins act on the human sodium-channel subtype 1.6 (hNav 1.6). Complete sequencing revealed that both toxins are structurally similar to beta-toxins isolated from different *Centruroides* species that also target hNav 1.6.

1. Introduction

Scorpions are one of the oldest arthropods in the world belonging to the class Arachnida. They have existed for more than 430 millions of years without substantive morphological changes [1]. While they are distributed worldwide, scorpions prefer tropical and desert areas. Over time, scorpions evolved as predators by developing venom components that are biologically active against a number of other animals. The venom is a complex mixture of biomolecules including peptides and proteins that play a fundamental role in the toxic activity. These toxic peptides specifically interact with Na^+-, K^+- and Ca^{++}-channels of excitable membranes [2]. Scorpion toxins are species-specific, either functioning in prey capture or predator deterence.

In general, scorpions dangerous to humans belong to the family Buthidae, with more than 1053 species known under 92 genera [3]. From this, the *Centruroides* genus, by far the most diverse in the Buthidae family, is distributed from the southern part of North America to Central America. *Centruroides sculpturatus* (different from *C. exilicauda*) [4], commonly called Bark scorpion, is widely distributed in the southwest of USA (mainly in Arizona) and the northwest of Mexico (Sonora state). *C. sculpturatus* is responsible for most scorpion envenomations in the US requiring medical treatment, as it is the only scorpion with venom potentially lethal to humans. Clinical manifestations of envenomation range from local pain and paresthesia to autonomic effects (tachycardia, hypersalivation, diaphoresis and bronchoconstriction) and in serious cases neurologic, respiratory and cardiovascular collapse [5–7]. Several toxins have been identified in *C. sculpturatus* venom, from which only the CsEM1 has been reported toxic to mammalians [8].

The topographic diversity across Arizona supports a heterogeneous distribution of *C. sculpturatus*. Populations inhabit a range of biotopes, from low-elevation xeric desert in the West to high-elevation forested uplands in the north and East. Different biotopes are critical determinants of the behavior and physiology of ectoderms [9–11]. For example, previous work documented differences in the body temperature and body size of female *C. sculpturatus* at two different biotopes. Studies

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demonstrated that smaller size is related to increased elevation and cooler temperatures [12]. Moreover, there are reports of biotope-specific intraspecific variation in venom composition from other scorpion species [13–15]. The study of intraspecific venom variation is critical for improving therapy to treat scorpion envenomation, and for drug discovery.

Currently, there are no reports documenting the biotope-specific differences in the venom composition of *C. sculpturatus*. Here, we compared the biochemical composition of the venom from *C. sculpturatus* inhabiting two different biotopes in Arizona: the low-elevation desert region of the surrounding areas of Yarnell and the oak woodland and pine forest of Santa Rita. Using chromatographic analysis, peptide isolation and amino acid sequencing, we examine the biochemical differences between venoms of distinct populations of *C. sculpturatus*. Our results highlight the structure and function of two peptides expressed by both venoms that are toxic to mammals; CsEd is a novel toxin, and CsEM1a is a toxin previously described as CsEM1, for which we provide a corrected sequence.

2. Material and methods

2.1. Scorpion venom

For this study, we evaluated the venom from *C. sculpturatus* scorpions collected at two different areas. Venom from Yarnell (Yar) and surrounding areas was purchased from Spider Pharm and venom from Santa Rita Foothills (SR) and surrounding areas was provided by Dr. Ashlee Rowe from Michigan State University. Venom was obtained by electrical stimulation, lyophilized and stored at −20 °C until use.

2.2. Toxins purification

The purification strategy was performed as previously described with modification for scaling the methods to minimal venom requirement [16]. First, soluble venom (approximately 25 mg, Abs. 280 nm, $\varepsilon = 1\ (mg/mL)^{-1}$) from each group of scorpions was solubilized in 1 mL using 20 mM ammonium acetate buffer (pH 4.7) and then centrifuged at 14,000 g/10 min in order to remove protein aggregates and mucoproteins. Samples were independently applied to a HiPrep 26/60 Sephacryl S-100 HR column previously equilibrated with the acetate buffer. Chromatographic run was performed at constant flow rate of 1 mL/min with collection of 8 mL fractions. Fractions eluted were separated and pooled according to the principal peaks showed in the chromatogram. Then, these pooled fractions were separated using a 1.8 × 2.5 cm column packed with carboxymethylcellulose (CMC) previously equilibrated with 20 mM ammonium acetate buffer pH 4.7. Chromatography was conducted at 2 mL/min flow rate with a linear gradient (0–100%; in 300 min) of 500 mM ammonium acetate buffer pH 7.4 and collection of 4 mL fractions. Peak fractions were pooled and concentrated by freeze-drying. All resulted sub-fractions were separated by RP-HPLC using a C18 semi-preparative reverse-phase HPLC column (Vydac, Hysperia, CA), and eluted with a linear gradient from 100% of solution A (0.12% trifluoroacetic acid (TFA) in water) to 60% of solution B (0.10% TFA in acetonitrile) in 60 min at 1 mL/min. Principal peaks were analyzed by mass spectrometry (MS).

2.3. Toxic activity

Principal isolated peptides were tested using CD1 mice (*Mus musculus*), house crickets (*Acheta domesticus*) and chicks (*Gallus gallus*). Animal experimentation was performed following bioethical standards, using a reduced number of animals and with the approval of the Animal Welfare Committee of our Institute. Dose administration was different in each animal; for mice was intraperitoneal injection using a sample volume of 100 µL, for crickets was an injection intraabdominal between the 4th and 5th spiracles using a sample volume of 10 µL and for chick was a subcutaneous injection under the left wing using a sample volume of 100 µL. Animals were observed the first hour seeking for envenomation symptoms and lethality was discarded after 24 h of survival. All principal peptides were tested in H_2O at different doses up to 5 mg/kg. We reported the lowest doses lethal to the animals, inferring that the lethal dose is equal or less than the amount tested.

2.4. Electrophysiology analysis

Peptides toxic to mammals were tested against human voltage gated-sodium channels of the sub-types hNav1.1 - hNav1.7, all expressed in HEK cells except hNav1.7 that was expressed in CHO cells. Peptides were tested at concentration of 200 nM according to Abs. 280 nm, $\varepsilon = 1\ (mg/mL)^{-1}$. Electrophysiology protocol was performed as previously described [17]. Sodium currents were acquired during step depolarization increased in order of 10 mV steps during 100 ms, from −120 to 40 mV, followed by 50 ms step at full-activation potential. Resting potential was set at −120 mV with a short pre-pulse (5 ms at 50 mV) applied 50 ms before depolarization steps. Current reads were performed using a MultiClamp 700 B amplifier coupled to an analog-digital converter Digidata 1440A and software pCalmp10 (Molecular Devices, Sunnyvale CA, USA). Data represents the mean of three depolarization protocols that were recorded by triplicate in different cells for each channel sub-type. In order to study the activation process, the channel conductance (G) was calculated for each depolarization step using the relation: $G = I/(Vm\text{-}E_{Na})$ where "I" is the current exhibited at "Vm" potential; "E_{Na}" represent the experimental Nernst equilibrium potential for Na^+ calculated for each cell. Conductance was normalized (G/Gmax) and data was fitted to the Boltzmann charge-voltage [18] function using the software Clampfit10.

2.5. Amino acid sequencing

Automatic sequencing was performed using a Shimadzu Protein Sequencer PPSQ-31A/33A. N-Terminus sequence was obtained for the principal isolated peptides by Edman degradation as previously described [19]. Peptide identification was performed by comparing the N-Terminus with sequences already reported. Peptides toxic to mammals were digested with endoproteinase GluC and complete sequenced, following methodology earlier described [20].

3. Results

3.1. Purification and comparison of principal peptides

Whole freeze-dried venoms from both locations were initially solubilized in 1 mL of 20 mM ammonium acetate at pH 4.7 and then centrifuged at 14,000 × g/10 min in order to remove insoluble material. The supernatant of the soluble venom was separated independently by Sephacryl S-100 into three pooled fractions (Fig. 1A). There were significant differences in the abundance and shape of the peaks between venoms, especially in the third fraction (FIII) that resulted larger in Yar venom. Usually, enzymes, large proteins and aggregates are present in FI in the first 25 tubes collected. Most of the venom proteins were present in fractions FII and FIII, which are equivalent to the FII of Sephadex G-50 in past protocols used [17,19,21]. Further purification of pooled fractions FII and FIII, by cation-exchange chromatography and RP-HPLC, allowed us to isolate the principal peptides from Yar and SR venoms. There were 5 and 6 principal peptides corresponding to approximately 38% and 48% of total SR and Yar venoms respectively. All principal peptides were identified by N-terminus sequence and MS analysis. Results are summarized in Table 1, where peptides are displayed in ascending order of abundance.