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Functional and immuno-reactive characterization of a previously undescribed peptide from the venom of the scorpion *Centruroides limpidus*

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ABSTRACT

A previously undescribed toxic peptide named Cl13 was purified from the venom of the Mexican scorpion *Centruroides limpidus*. It contains 66 amino acid residues, including four disulfide bonds. The physiological effects assayed in 7 different subtypes of voltage gated Na^+-channels, showed that it belongs to the β-scorpion toxin type. The most notorious effects were observed in subtypes Nav1.4, Nav1.5 and Nav1.6. Although having important sequence similarities with two other lethal toxins from this scorpion species (Cll1m and Cll2), the recently developed single chain antibody fragments (scFv) of human origin were not capable of protecting against Cl13. At the amino acid sequence level, in 3 stretches of peptide Cl13 (positions 7–9, 30–38 and 62–66) some differences with respect to other similar toxins are observed. Some of these differences coincide with contact points with the human antibody fragments.

1. Introduction

In several countries of the world scorpion stings are considered to be a public health problem [5]. Mexico is one of them, where just in the year 2015 over 265,000 human accidents were registered. For the State of Morelos, where our Institute is located, in the same year, about 31,500 cases were reported [30]. From the 281 different species of scorpions identified in Mexico, one of the most dangerous is *Centruroides limpidus*, earlier known as *Centruroides limpidus limpidus* and now re-classified simply as *Centruroides limpidus* [27]. The venom of this species has been largely studied [1,7,13,18,26]. Many different peptides were previously purified from de venom of *C. limpidus*, and their amino acid sequences and specificities were determined; for instance insect and crustacean specific peptides were described, such as the first three dimensional structure of an arthropod scorpion toxin resolved by NMR studies [12]. Also one of the first antimicrobial peptides was identified and described from this venom [24]. Among the well-known toxic components are Cll1m and Cll2, two potent toxins acting on mammals [1,18,29]. Various aspects of intoxication were evaluated in experimental mice [16,17], as well as some pharmacokinetic data in rabbits were previously reported [2]. More recently, a complete transcriptomic analysis of the venom glands of male and female *C. limpidus* was performed showing at least 20 different peptides, with similar amino acid sequences, and surmised to be specific for binding to Na^+-channels [3]. Apart from the knowledge on the diversity of peptides and functions associated to venom components of *C. limpidus*, the development of new anti-venoms against this species is the object of intense work, by our research group. For this purpose, we have used in the past 3 basic experimental approaches: 1) the construction of recombinant single-chain Fv (scFv) libraries of human origin displayed on filamentous phages, 2) rational screening of the libraries using specific toxins as antigens, and 3) *in vitro* maturation of the best binders. This technology allows producing antibody libraries with high diversity from which antibodies with desired specificities can be obtained. This technology has permitted the selection of several single chain antibodies (scFv), capable of recognizing and protecting against the deleterious effect of toxins Cll1m and Cll2 [21,23] (and unpublished results). However, when tested

Abbreviations: aa, amino acid; Cll, *Centruroides limpidus*; Cll1m, toxin 1 from *C. limpidus*; Cll2, Toxin 2 from *C. limpidus* and Cl13 Toxin 13 from *C.limpidus*; hNav, human voltage gated sodium channels; scFv, single chain antibody fragment.

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against the whole soluble venom still some adverse intoxication symptoms were observed. On the contrary, another scFv that was fully capable of protecting mice against Cn2, the main toxin from *C. noxius*, was also able to protect against the whole soluble venom of this scorpion species [19]. Thus, we knew that having identified the proper type of toxins present in a given species of scorpion and having developed the adequate neutralizing single-chain antibody, it should be sufficient and appropriate for eliminating the effects of the whole venom. For this reason, we decided to conduct a more rigorous search on the soluble venom of *C. limpidus* species. The various fractions obtained by separation of the venom by chromatographic procedures permitted the identification of an unknown component very toxic to mice. After assaying the various fractions of the venom, one of them was toxic and its structure was unknown. This component was further purified and characterized. Here we describe this previously undescribed peptide that was called Cl13 and we report its effects on Na^+-channels. It affects the function of these channels in a similar manner to other beta-scorpion toxins (β-ScTx), but with unusual alpha effect on hNav 1.6. Some immune-reactive aspects and additional comparative analysis with other toxins are also included in this communication.

2. Material and methods

2.1. Venom source, chemical and reagents

Scorpions collected in the States of Morelos and Guerrero (Mexico), under official permission of SEMARNAT (# MOR-IN-166-07-04) were milked for venom by electrical stimulation [1]. The venom was dissolved in water, and centrifuged at 10,000*g* for 15 min. The supernatant was freeze-dried and kept at −20 °C until use. All chemicals and reagents were analytical grade substances. Double distilled water was used through all the procedures. The protein content of the samples was estimated based on the absorbance at λ = 280 nm. A solution displaying one unit of absorbance at this wavelength was assumed to correspond to 1 mg/mL protein content.

2.2. Purification of peptides

The soluble venom was initially fractionated by gel filtration using a Sephadex G-50 column, following the methodology earlier described [12,18]. The column was equilibrated and run in 20 mM ammonium acetate buffer, pH 4.7. The principal fraction containing toxic peptides was further separated using ion-exchange column prepared with carboxy-methyl-cellulose (CM-cellulose) resins. The same buffer was used and the sub-fractions were eluted using a linear gradient of sodium chloride from 0 to 0.5 M. Finally, at the end of the run the column was washed with 1 M NaCl. The most basic fraction was lyophilized and further separated by high performance liquid chromatography (HPLC), using an analytical C18 reverse-phase column (4.6 × 250 mm from Vydac, Hysperia, CA, USA). The solution A contains 0.12% trifluoroacetic acid (TFA) in water. In this condition the NaCl salt is eluted from the column during loading of the solubilized sub-fractions. The elution of the various peptides from the reverse-phase column is obtained by application of a linear gradient from solution A to 60% solution B (0.10% TFA in acetonitrile); over 60 min. Fractions absorbing at λ = 230 nm were monitored and manually collected, freeze-dried using a Savant SpeedVac dryer and used for chemical and functional characterization as described below. During purification protein content was evaluated based on absorbance at 280 nm, assuming that one unit of absorbance corresponds to 1 mg/mL protein content. Once the peptide was obtained in pure form and the amino acid sequence determined, the extinction coefficient was evaluated theoretically and found to be 19,410 for the case of Cl13. It means that a solution containing 1 mg/mL protein, gives 3 absorbance units at 280 nm.

2.3. Biological effects on mice

Mice (CD1 strain) were used for lethality tests, by intraperitoneal injection. The use of a reduced number of mice was approved by the Animal Welfare Committee of our Institute.

The mice were injected with different toxin concentrations diluted in PBS up to a volume of 100 μL. Mice were observed for the symptoms during the subsequent 24 h.

2.4. Sequence by Edman degradation and mass spectrometry analysis

The peptides obtained from the HPLC separation were analyzed by mass spectrometry using an apparatus model LCQ*Fleet* from Thermo Fisher Scientific Inc. (San Jose, CA, USA). Edman degradation was performed using a PPSQ-31A Protein Sequencer from Shimadzu Scientific Instruments, Inc. (Columbia, MD, USA). A sample of native peptide (*circa* 0.5 nmol) was directly loaded for sequence and a reduced and alkylated sample of the same peptide was additionally sequenced for identification of the cysteine residues. For determination of the full amino acid sequence it was necessary to digest the pure reduced and alkylated peptide with enzymes, separate the corresponding fragments and sequenced them, as mentioned below.

2.5. Alkylation and enzymatic digestion of the peptide

Reduction and alkylation with iodoacetamide of the pure peptide was performed in the same conditions as earlier described for other toxic peptides, according to Olamendi-Portugal et al. [15]. The reduced and alkylated peptide was applied to a C18 reverse-phase column for elimination of reagents (similar conditions than described). The clean peptide was collected and used for direct sequence determination. In order to obtain the full amino acid sequence this peptide was treated with two enzymes: trypsin and AspN protease (both from Roche Mammheim. Germany). Briefly, the following procedure was used: two independent samples containing 75 μg of reduced and alkylated peptide were dissolved separately in 100 mM Tris-HCl buffer, pH 8.0 and added with either 1 μg of AspN protease or 2 μg of trypsin and allowed to digest over-night at 37 °C. The solutions were separated by HPLC, using a C18 reverse-phase column in the same conditions described in Section 2.2 above. The various peptides were directly loaded into the sequencer for Edman degradation (see Section of Results and Discussion).

The protein sequence data reported in this paper will appear in the UniProt Knowledgebase under the accession number: C0HK69 (for Toxin Cl13 from *Centruroides limpidus*).

2.6. Electro-physiological characterization of the peptide

HEK cells expressing human voltage gated Na^+ channels (hNav 1.1 to Nav1.6) and CHO cells expressing hNav 1.7 were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, Toluca, Mexico) supplemented with Fetal Bovine Serum (FBS) (Byproductos, Guadalajara Mexico), at 37 °C with 5% CO_2. Cells were a kind gift from Professor Enzo Wanke from University of Milano Bicocca, Milan, Italy.

Extracellular solution contained in mM: 130 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 5 glucose, pH 7.3 adjusted with NaOH. Intracellular solution contained in mM: 105 CsF, 27 CsCl, 5 NaCl, 2 $MgCl_2$, 10 EGTA, 10 HEPES, pH 7.3 adjusted with CsOH.