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Insecticidal peptides from the theraposid spider *Brachypelma albiceps*: An NMR-based model of Ba2☆

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ABSTRACT

Soluble venom and purified fractions of the theraposid spider *Brachypelma albiceps* were screened for insecticidal peptides based on toxicity to crickets. Two insecticidal peptides, named Ba1 and Ba2, were obtained after the soluble venom was separated by high performance liquid chromatography and cation exchange chromatography. The two insecticidal peptides contain 39 amino acid residues and three disulfide bonds, and based on their amino acid sequence, they are highly identical to the insecticidal peptides from the theraposid spiders *Aphonopelma* sp. from the USA and *Haplopelma huwenum* from China indicating a relationship among these genera. Although Ba1 and Ba2 were not able to modify currents in insect and vertebrate cloned voltage-gated sodium ion channels, they have noteworthy insecticidal activities compared to classical arachnid insecticidal toxins indicating that they might target unknown receptors in insect species. The most abundant insecticidal peptide Ba2 was submitted to NMR spectroscopy to determine its 3-D structure; a remarkable characteristic of Ba2 is a cluster of basic residues, which might be important for receptor recognition.

1. Introduction

Spider venoms are complex mixtures of components, among which are highly selective toxins for insects [1]. In this respect, spiders are a very diversified group of insect predators (ca. 40,700 described species [2]) indicating the possibility to discover in their venoms novel insect-selective toxins [1,3]. Comparative screening shows that spider as well as scorpion venoms have been shown to contain the most lethal components against pest insects [4,5]. Mass fingerprinting analyses of spider venoms demonstrated that spider venoms may contain more than 200 different components; among which are toxins to both vertebrates and arthropods, mainly insects [6,7]. Therefore, spider venom represents an attractive and diverse pool of peptides with a great potential for drug discovery [8].

However, pharmacological data in insect cells are still scarce for two main reasons: receptor sites present in arthropods are poorly studied compared with the huge amount of vertebrate specific receptors already described (i.e. sodium ion channel receptors paraNav1 in insects versus Nav1.1–1.8 in vertebrates) [5], and second the lack of strong and specific ligands for the search of potential receptors [9,10]. The precise targeting of insect receptors by robust ligands, such as spider toxins, could be useful for understanding the molecular basis of toxin selectivity at the receptor level. This could also lead to the design of more effective and safer pesticides.

In Mexico, spiders of the theraposid group are large and diverse [11]. However, few Mexican theraposids have been studied, and they may contain several insecticidal peptides that represent a potential for application as biopesticides for agro-industrial or horticultural protection. In this work, we report the isolation and amino acid sequence of two insecticidal peptides from the theraposid *Brachypelma albiceps*, and the solution structure by NMR of the one named Ba2.

Abbreviations: TFA, trifluoroacetic acid; ESI, electro spray ionization; LD_{50}, dose that kills 50% of the animals; Ba1 and Ba2, toxins1 and 2 from the spider *Brachypelma albiceps*

☆ The protein sequence reported in this paper has been submitted to the Swiss Protein Database under the accession numbers P85497 and P85504 for Ba1 and Ba2, respectively. The structural coordinates of Ba2 have been deposited in the Protein Data Bank with accession number 2KGH.

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2. Materials and methods

2.1. Biological materials

B. albiceps venom was obtained by electrical stimulation of field-collected spiders from the state of Morelos in Mexico. The crude venom was frozen and stored at −20 °C until use. The spiders were collected and maintained alive following the Mexican official requirements and licenses (SEMARNAT FAUT-0184 and MOR-IN-166-0704). The theraposids are maintained in excellent conditions (well fed, healthy and have been living in the laboratory for many years).

2.2. Peptide purification

B. albiceps freeze-dried venom (5 mg) was dissolved in 0.1% aqueous trifluoroacetic acid (TFA) containing 5% acetonitrile (100 µl), and the insoluble material was removed by centrifugation at 14,000 *g* for 5 min. Diluted venom was first fractionated using a reverse-phase semi-preparative C_{18} column (5C_{18}MS, 10×250 mm, Nacalai Tesque Japan) equilibrated in 0.1% TFA, and eluted with a linear gradient of acetonitrile/0.1% TFA (0 to 60% in 60 min), at a flow rate of 2 ml/min. The absorbance of the effluent was monitored at 230 nm. Fractions with insecticidal activity were further fractionated by cation exchange HPLC on a TSK-gel sulfopropyl column (SP-5PW; 7.5×75 mm, Tosoh Japan), equilibrated in 0.5 M acetic acid (pH 2.9). A linear gradient of 0.5 M acetic acid in 2 M ammonium acetate (pH 5.9) was applied (0 to 20% in 20 min) at a flow rate of 1.5 ml/min. The active fractions were finally purified on a reverse-phase C_{18} column (4.6×250 mm, Nacalai Tesque, Japan) using the same gradient system as above, with a flow rate of 1 ml/min.

2.3. Sequence determination

Insecticidal toxins were reduced and alkylated prior to Edman sequencing. Peptides were also subjected to enzymatic hydrolysis. Hydrolysis with type XVII-B endoproteinase Glu-C from *Staphylococcus aureus* V8 was carried out in 0.1 M sodium bicarbonate buffer (pH 7.6), at 37 °C for 3 h, using a 1:20 (w/w) enzyme to substrate ratio. The peptides obtained by endoproteinase Glu-C digestion were fractionated by reversed-phase HPLC using a C_{18} column (4.6×250 mm, Nacalai Tesque, Japan) and a linear gradient of acetonitrile in 0.1% aqueous TFA. All fractions were analyzed by electro spray ionization mass spectrometry using a Finnigan LCQ^{DUO} ion trap mass spectrometer (San Jose, CA, USA). All major endoprotease Glu-C fragments were sequenced by automated Edman degradation using a Beckman LF3000 Protein Sequencer.

2.4. NMR spectroscopy and structure calculation

2.4.1. Sample preparation

The pure toxin Ba2 (2.5 mg) was dissolved in 0.5 ml of H_2O/D_2O (90/10 by vol.) at pH 3.0 and it was used for NMR spectra recordings. Amide proton exchange rate was determined after peptide lyophilization and dissolution in 100% D_2O.

2.4.2. NMR experiments

All ^{1}H spectra were recorded on BRUKER DRX500 spectrometer equipped with a HCN probe. Self-shielded triple axis gradients were used. Two-dimensional NOESY and TOCSY spectra were acquired at 290 K using states-TPPI method [12] to achieve F1 quadrature detection [13]. The spectral width in both dimensions was 6000 Hz. NOESY and TOCSY experiments were recorded with 2048 data points for t2 and 512 points for t1 increments, with 64 transients per experiment. DQF-COSY experiment was recorded with 4096 data points in t2 and 1024 data points in t1. Water suppression was achieved using pre-saturation during the relaxation delay (1.5 s), and during the mixing time in the case of NOESY experiments, or using a water-gate 3-9-19 pulse train [14] using a gradient at the magic angle obtained by applying simultaneous x-, y-, and z-gradients prior to detection. NOESY spectra were acquired using mixing time of 80 ms. TOCSY was performed with a spin locking field strength of 8 kHz and spin lock time of 80 ms. The amide proton exchange experiments were recorded immediately after dissolution of the peptides in D_2O. A series of NOESY spectra with a mixing time of 80 ms were recorded at 290 K, the first one for 1 h, followed by spectra of 12 h each.

2.4.3. Data processing

Spectra were processed with the software NMRPipe. The matrices were transformed to a final size of 2048 points in the acquisition dimension and to 1024 points in the other. Prior to a Fourier transform, the signal was multiplied by a shifted sine bell window in both dimensions and then, a fifth order polynomial baseline correction was applied.

2.4.4. Spectral analysis

Identification of amino acid spin systems and sequential assignment were done using the standard strategy described by Wüthrich [15], which was applied with the graphical software, NMRview [16]. The comparative analysis of COSY and TOCSY spectra recorded in water gave the spin system signatures of the protein. The spin systems were then sequentially connected using the NOESY spectra.

2.4.5. Experimental restraints and structure calculations

The automated assignment of ambiguous restraints and structure calculations of Ba2 were performed with CYANA 2.1 [17]. NOE cross-peaks for sequential, secondary structures and the aromatic side chain restraints were assigned manually and converted in distance restraints with CYANA 2.1. Hydrogen bonds derived from the proton/deuterium exchange analysis were added as structural input during the calculations. The calculation consisted of 7 cycles of iterative automated NOE assignment and structure calculation of 200 conformers in each cycle. At the end of each CYANA run, unambiguously assigned peaks were derived as distance restraints and used as input for the next calculation steps. To keep the same assignment condition, the same NOE calibration parameter, calculated by CYANA during the first run of NOE assignment was used in all the other runs of calculation.

The final structure calculations with CYANA were started from 200 conformers and a simulated annealing with 20,000 time steps per conformer was done using the CYANA torsion angle dynamics algorithm [18,19]. The 40 best structures were refined using a short restrained molecular dynamics simulation in explicit solvent [20] in the program XPLOR-NIH [21]. At the end of the refinement, the 20 lowest energy structures were selected to form the final ensemble. The quality of the structure was analyzed with PROCHECK-NMR [22]. All structure representations were made with the program PYMOL (DeLano Scientific, Palo Alto, California, USA) [23]. The structural coordinates and experimentally derived restraints have been deposited in the Protein Data Bank with accession number 2KGH.

2.5. Electrophysiological recordings in Xenopus laevis oocytes

For the expression in *Xenopus laevis* oocytes, the $Na_v1.5$ and β_1 genes were subcloned into pSP64T [24]. For *in vitro* transcription, $Na_v1.5$/pSP64T was first linearized with XbaI and β_1/pSP64T with EcoRI. Next, capped cRNAs were synthesized using the large-scale SP6 mMESSAGE-mMACHINE transcription kit (Ambion, USA) [25–27]. The Para/pGH19-13-5 vector, tipE/pGH19 vector and $Na_v1.2$/pLCT1 vector were linearized with NotI and transcribed with the T7 mMESSAGE-mMACHINE kit (Ambion, USA).