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Proteomics of the neurotoxic fraction from the sea anemone *Bunodosoma cangicum* venom: Novel peptides belonging to new classes of toxins

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ABSTRACT

In contrast to the many studies on the venoms of scorpions, spiders, snakes and cone snails, up to now there has been no report of the proteomic analysis of sea anemones venoms. In this work we report for the first time the peptide mass fingerprint and some novel peptides in the neurotoxic fraction (Fr III) of the sea anemone *Bunodosoma cangicum* venom. Fr III is neurotoxic to crabs and was purified by rp-HPLC in a C-18 column, yielding 41 fractions. By checking their molecular masses by ESI-Q-Tof and MALDI-Tof MS we found 81 components ranging from near 250 amu to approximately 6000 amu. Some of the peptidic molecules were partially sequenced through the automated Edman technique. Three of them are peptides with near 4500 amu belonging to the class of the BcIV, BDS-I, BDS-II, APETx1, APETx2 and Am-II toxins. Another three peptides represent a novel group of toxins (~3200 amu). A further three molecules (~~4900 amu) belong to the group of type 1 sodium channel neurotoxins. When assayed over the crab leg nerve compound action potentials, one of the BcIV- and APETx-like peptides exhibits an action similar to the type 1 sodium channel toxins in this preparation, suggesting the same target in this assay. On the other hand one of the novel peptides, with 3176 amu, displayed an action similar to potassium channel blockage in this experiment. In summary, the proteomic analysis and mass fingerprint of fractions from sea anemone venoms through MS are valuable tools, allowing us to rapidly predict the occurrence of different groups of toxins and facilitating the search and characterization of novel molecules without the need of full characterization of individual components by broader assays and bioassay-guided purifications. It also shows that sea anemones employ dozens of components for prey capture and defense.

1. Introduction

Living exclusively in aquatic environments, cnidarians represent the first group of venomous animals. Among the 5 classes of the phylum Cnidaria, the class Anthozoa is represented by the sea anemones, sessile animals that employ a variety of proteic (peptides, proteins, enzymes and proteinase inhibitors) substances as natural weapons (Malpezzi et al., 1993; Schweitz et al., 1995; Grotendorst and Hessinger, 2000; Anderluh and Macek, 2002). These animals are characterized by the presence of stinging organelles, the nematocysts, employed for defense against predators, in competition for substrate and also in prey capture. Nematocysts possess a high concentration of polypeptides and proteins that act as neurotoxins, hemolysins and phospholipase A2 (PLA2) enzymes, and are responsible for a variety of pathological responses (cardiotoxicity, dermatitis, local itching, swelling, erythema, paralysis, pain and necrosis) (Norton, 1991; Anderluh and Macek, 2002; Nevalainen et al., 2004; Watters and Stommel, 2004).

Despite the fact that sea anemones produce their toxins in the restricted and specialized nematocysts, it is interesting to note that most of the papers that describe the isolation and characterization of these molecules follow bioassay-guided purifications from total body extracts (Beress and Beress, 1975; Barhanin et al., 1981; Castañeda et al., 1995; Bruhn et al., 2001; Salceda et al., 2002). Recently, the novel sea anemone toxins APETx1 and APETx2, a "Human Ether-a-gogo Related Gene" (HERG) modulator and an "Acid-Sensing Ion Channel 3" (ASIC3) blocker, respectively, were reported by this approach (Diochot et al., 2003; Diochot et al., 2004).

Abbreviations: amu, atomic mass unit; ASIC, acid-sensing ion channel; CAPs, compound action potentials; CH_3CN, acetonitrile; ESI-Q-TOF MS/MS, Electrospray ionisation quadrupole tandem mass spectrometry; HERG, human ether-a-gogo related gene potassium channel; Kv, voltage-gated potassium channel; LC, liquid chromatography; MALDI-Tof MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MS, mass spectrometry; Nav, voltage-gated sodium channel; rp-HPLC, reversed-phase high performance liquid chromatography; TFA, trifluoroacetic acid.

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On the other hand, our group developed a technique to milk the sea anemone *Bunodosoma caissarum* by electric stimuli and to obtain a rich polypeptidic mixture from its nematocyst venom (Malpezzi et al., 1993). Further purifications of this venom by gel-filtration yielded some fractions with hemolytic and neurotoxic activities (Oliveira et al., 2004; de Oliveira et al., 2006; Oliveira et al., 2006). Applying the same technique to the sea anemone *Bunodosoma cangicum*, other work (Lagos et al., 2001) reported that the so-called fraction III (Fr III) eluted from Sephadex G50 gel-filtration chromatography contained neurotoxins that target voltage-gated sodium and potassium channels. This methodology ensures that the active components are coming directly from the electrically-discharged nematocysts and not from other animal body parts.

In order to verify and partially characterize the composition of the toxins presented in the neurotoxic fraction of *B. cangicum* species, we performed two steps of fractionation by chromatography. The venom was initially submitted to molecular sieving by Sephadex G-50 and the neurotoxic fraction (Fr III) was further fractionated by reversed-phase high performance liquid chromatography (rp-HPLC). The peptide mass fingerprint of Fr III was carried out by ESI-Q-Tof and MALDI-Tof MS analyses offline. This proteomic approach, either through nano-LC-MS or LC-MS and MALDI-Tof offline has previously been done with many venomous organisms, such as cone snails (Vianna Braga et al., 2005; Jakubowski et al., 2006; Quinton et al., 2006), spiders (Machado et al., 2005; Guette et al., 2006; Wilson and Alewood, 2006), snakes (Nawarak et al., 2003; Li et al., 2004; Fox et al., 2006) and scorpions (Pimenta et al., 2003; Batista et al., 2004; Barona et al., 2006; Batista et al., 2006; Favreau et al., 2006; Nascimento et al., 2006), leading to the characterization of many novel components and also to the mass profile of their venoms.

As the search for lead compounds and novel pharmacological tools are increasingly based on the naturally evolved venom molecules (Lewis and Garcia, 2003; Terlau and Olivera, 2004), we believe it is extremely important to survey a sea anemone venom by using the proteomic approach, combining the use of venom fractionation by rp-HPLC with the employment of ESI and MALDI-Tof mass spectrometry. Our findings lead to the discovery of 9 novel peptides, including 6 novel ones from 2 new classes of toxins. Another 3 toxins are from the group of type 1 sodium channel toxins. Furthermore, our data shows that at least 81 molecules are eluted in the neurotoxic fraction of the sea anemone *B. cangicum* venom and may be employed as active peptides during stings.

2. Materials and methods

2.1. Venom collection and fractionation

Twenty *B. cangicum* specimens were collected on the northern coast of São Paulo State, Brazil, and venom was obtained by electrical stimulation of animals as previously described (Malpezzi et al., 1993). The *B. cangicum* venom (approximately 70 mg) was fractionated by gel-filtration chromatography using a Sephadex G-50 column (1.9×131 cm, Amersham Biosciences, Uppsala, Sweden), according to Lagos et al. (2001) and Oliveira et al. (2004). The neurotoxic fraction (1 mg), eluted in the third peak (Fr III), was submitted to rp-HPLC chromatography in an ÄKTA Purifier machine (Amersham Biosciences, Uppsala, Sweden) using a semipreparative CAPCELL PAK C-18, 10×250 mm (Shiseido Corp., Kyoto, Japan) column. The HPLC conditions used were: 0.1% trifluoroacetic acid (TFA) in water (solvent A) and acetonitrile containing 0.1% TFA (solvent B). The separations were performed at a flow rate of 2.5 mL/min and a 10–60% gradient of solvent B over 40 min and the peptides were monitored at UV 214 nm. Each of the individual sub-fractions from Fr III were manually collected and lyophilized or concentrated for further molecular mass assessments by either ESI-Q-Tof or MALDI-Tof mass spectrometry. In the case of individual peak repurification, different gradients of the solvents A and B described above and flow rate conditions were adjusted to best fit the purified peak to a proper symmetry. The protein content of either the neurotoxic fraction or the pure peptide samples was estimated by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA) following the manufacturer's instructions.

2.2. Mass spectrometry analyses

Analyses of rp-HPLC fractions from the venom Fr III were performed on an Ettan MALDI-TOF/Pro (Amersham Biosciences, Uppsala, Sweden) equipped with 337 nm pulsed nitrogen laser under reflectron mode. The accelerating voltage was 20 kV. Matrix, α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich Co., USA), was prepared at a concentration of 10 mg/mL in 1:1 CH_3CN/0.1% TFA. External calibration was performed with [Ile^7]-angiotensin III (*m*/*z* 897.51, monoisotopic, Sigma) and human ACTH fragment 18–39 (*m*/*z* 2465.19, monoisotopic, Sigma). The sample solution (0.5 μL) dropped onto the MALDI sample plate was added to the matrix solution (0.5 μL) and allowed to dry at room temperature. The same samples were also analysed on a Q-TOF Ultima API instrument fitted with an electrospray ion source (Micromass, Manchester, UK) under positive ionization mode. The aqueous sample solutions (2 μL) were directly injected using a Rheodyne 7010 sample loop coupled to a LC-10A VP Shimadzu pump at 20 μL/min constant flow rate. External calibration was performed with NaI (Fluka) over *m*/*z* 100–2000.

2.3. Partial primary structure determination of *B. cangicum* Fr III peptides

The partial amino acid sequences of some pure native peptides were determined by Automated Edman degradation through a gas-phase sequencer PPSQ-10 (Shimadzu Corp., Kyoto, Japan). Cysteines' positions were considered based on blank cycles.

2.4. Crab nerve assay (sucrose-gap)

The nerve preparation was obtained from the crab leg sensory nerve, as described previously (Lagos et al., 2001). A walking leg was isolated from an adult blue crab *Callinectes danae* and its nerve exposed by cutting the membranes and articulations of the leg as described by Malpezzi et al. (1993). The nerve was placed in a groove of an acrylic chamber across five interconnected compartments, each one isolated with vaseline plugs. The electrodes for stimulation (platinum–iridium) were connected to compartments 1 (positive) and 2 (negative), and the recording electrode (silver chloride) to compartments 3 and 5. Compartments 1, 2, 3 and 5 contained physiological solution for crabs, while compartment 4 contained 1 M of sucrose. The test substance was added to compartment 3, which contained 100 μL of physiological solution. Extracellular compound action potentials were evoked by single supra-maximal stimuli (18–20 V) at 0.1 Hz and lasting 0.05 ms (Grass S8800 Stimulator, Grass Instruments, Warwick, USA). The action potentials of the nerve were amplified with a pre-amplifier (CP-511 AC, Grass Instruments, Warwick, USA), and cut-off frequency 3 to30 Hz. The data were recorded and saved on a PC hard disk using the WinWCP V 3.1.6 software (Whole Cell Analysis Program, version 3.1.6, Strathclyde Electrophysiology Software, John Dempster, University of Strathclyde, Scotland), controlling an Analogical/Digital board (National Instruments—NIDAQ, model Lab-PC+ 28). The sampling interval was 0.2 ms and the record size was 512 samples within each record. Action potentials measured before each treatment were used as controls. The effect of 1 μM of synthetic ShK peptide (Peptides International, Louisville, USA) was studied as a control on action potentials in order to verify the effect over voltage-gated potassium channels blockage in this preparation.