Biochimie 147 (2018) 114–121

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Cloning and sequencing of three-finger toxins from the venom glands of four *Micrurus* species from Mexico and heterologous expression of an alpha-neurotoxin from *Micrurus diastema*

Jaime Felipe Guerrero-Garzón, Melisa Bénard-Valle, Rita Restano-Cassulini, Fernando Zamudio, Gerardo Corzo, Alejandro Alagón, Alejandro Olvera-Rodríguez*

Instituto de Biotecnología, Universidad Nacional Autónoma de México, Av. Universidad # 2001, Colonia Chamilpa, CP: 62210 Cuernavaca, Morelos, Mexico

ARTICLE INFO

Article history:
Received 4 November 2017
Accepted 24 January 2018
Available online 31 January 2018

Keywords:
Antisera
Elapid
Micrurus
α-neurotoxin
mRNA transcript
Recombinant
Three finger toxins

ABSTRACT

The three-finger toxins (3FTxs) represent an extremely diverse protein family in elapid venoms, where the short chain α-neurotoxins are the most relevant toxin group from the clinical point of view. Essentially, the 3FTxs variability and the low proportions of α-neurotoxins in the venoms of North American coral snakes make it difficult to obtain effective elapid antivenoms against the envenomation symptoms caused mainly by these α-neurotoxins. In this work, thirty 3FTx transcript sequences were obtained from the venom glands of four coral snake species from Mexico (*M. diastema, M. laticollaris, M. browni* and *M. tener*). The transcripts were mined using a forward oligonucleotide based on the highly conserved signal peptide from the 3FTxs, and four of these transcripts, named MlatA1, B.D, B.E and D.H, encoded for short-chain α-neurotoxins. Additionally, one isoform of the D.H α-neurotoxin transcript was identified in the venom of *M. diastema*. The mature α-neurotoxin coded in the D.H transcript was heterologously expressed, and it was found soluble (4.2 mg/l) in the cytoplasm of a bacterial system. The recombinant D.H (rD.H) had an IC_{50} value of 31.5 ± 4.4 nM on nicotinic acetylcholine receptors of the muscular type expressed in rhabdomyosarcoma cells (TE671). The rDH also had an LD_{50} of 0.15 mg/kg mice, and it was evaluated as a potential immunogen in New Zealand rabbits. The protective capacity of rabbit sera was tested against two native coral snake α-neurotoxins, and against rD.H. One of the anti-rD.H rabbit sera was able to neutralize the lethality of all three neurotoxins when tested in groups of CD1 mice. This work contributes to the increasing understanding of the high diversity of 3FTxs, and shows that recombinant coral snake α-neurotoxins are promising supplements for hyperimmunization protocols for coral snake antivenom production.

1. Introduction

In the Americas 200,000 cases of snakebite envenomation per year have been estimated, of which around 3000 are fatal [1]. However, envenomation by coral snakes is relatively rare in humans, generally causing less than 5% of total snakebites [2,3]. Coral snakes are the most diversified group of the family *Elapidae* (related to cobras, kraits and mambas) in the continent, and they are classified in two genera: *Micrurus and Micruroides*. Among them, the *Micrurus* genus has the largest number of species and the widest geographical distribution, including 79 species from southern United States to northeastern Argentina [4,5]. Despite their low incidence, all coral snake envenomations have medical significance given the high toxicity of the venom and the possibility of developing respiratory failure [6]. Symptoms observed after coral snake bites also include local pain, sialorrhea, paresthesia, ptosis, weakness, blurred vision, diplopia, fasciculations and paralysis [7–9].

The most abundant and clinically relevant groups of proteins that have been reported in *Micrurus* venoms are phospholipases A_2 (PLA_2) and the three-finger toxins (3FTx) [10–12]. Molecules from both families are known to act on the neuromuscular endplate, either blocking acetylcholine binding to its postsynaptic receptor, as in the case of some 3FTxs, known as α-neurotoxins, or

* Corresponding author.
E-mail address: aolvera@ibt.unam.mx (A. Olvera-Rodríguez).

https://doi.org/10.1016/j.biochi.2018.01.006
0300-9084/

preventing the presynaptic release of the same neurotransmitter, like some PLA_2s, designated β-neurotoxins. Both α-neurotoxins and β-neurotoxins cause flaccid paralysis of skeletal muscle [13,14].

Coral snake 3FTxs are small molecules with a globular hydrophobic core from which three adjacent loops emerge. They contain from 60 to 75 amino acid residues with four to five disulfide bonds which are cross-linked forming three loops with five antiparallel β-strands that together create a large β-pleated sheet [15–17]. Within α-neurotoxins, those of the short-chain (60–62 amino acids and four disulfide bridges) are the most toxic peptides, with LD_{50} values ranging from 0.04 to 0.3 mg/kg. More than 150 α-neurotoxins (short- and long-chain with four and five disulfide bonds, respectively) have been described from a wide variety of elapid [18] and colubrid venoms [19]. Only 55 short-chain α-neurotoxins have been described from American coral snakes [20].

An interesting aspect of North American *Micrurus* venoms is that they usually contain short-chain α-neurotoxins in low quantities with respect to PLA_2s [21]. This feature, in addition to their small molecular mass, could affect the capacity to generate sufficient neutralizing antibodies against them when the complete venom is used as immunogen. In order to solve this problem, a good strategy could be the enrichment of traditional immunization systems with supplemental quantity of pure α-neurotoxins [22]. However, the low amount of these components is a limitation for this purpose. In the current work, we search for α-neurotoxins in four species of Mexican *Micrurus,* and heterologously express one from *M. diastema* (rD.H) to evaluate its potential as an immunogen.

2. Materials and methods

2.1. Venom and venom glands

The coral snake venoms were collected by manual stimulation from specimens captured in the Mexican States of Veracruz (*M. diastema*) and Morelos (*M. laticollaris*). The venoms were recovered in 20 mM ammonium acetate buffer at pH 4.7, centrifuged at 17,600 *g* to eliminate cell debris as well as insoluble material, lyophilized and stored at −20 °C until use.

Coral snake venom glands were extracted from coral snake specimens (*M. laticollaris*, *M. diastema*, *M. browni* and *M. tener* from the Mexican States of Colima, Veracruz, Chiapas and Tamaulipas, respectively) from our scientific collection that died in captivity of natural causes. The specimens were preserved frozen at −70 °C.

2.2. Experimental animals

CD-1 mice (18–20 g) were purchased from Harlan México, and they were used for toxicity assays. New Zealand rabbits (2.5 kg) were provided by the Animal Facility at the Biotechnology Institute (UNAM), and they were used for immunization. The experimental animals were maintained according to Mexican legislation for the use of laboratory animals (Norma Official Mexicana, 1999, NOM-062-ZOO-1999).

2.3. 3FTx sequences from coral snake venom glands

Total RNA was purified from the venom glands of each specimen. Glands from each coral snake species were separately homogenized in Trizol® Reagent (1 ml/100 mg; Gibco BRL). Then, chloroform was added (1/5 total volume) and the homogenate was centrifuged at 16,800 *g* and 4 °C for 5 min. The organic phase was discarded and isopropanol (1/10) was added to the aqueous phase, incubated at room temperature for 10 min and centrifuged at 16,800 *g* during 5 min. The RNA pellet was washed twice with cold ethanol (75%), dried at room temperature, dissolved in DEPC (diethyl-pyrocarbonate) treated water and stored at −70 °C. The cDNA coding for putative 3FTx's collected from each coral snake species was achieved by the 3′RACE technique (Invitrogen). Briefly, total RNA was reverse-transcribed using a poly(T) primer with an adapter following manufacturer's instructions, and the resulting cDNA was amplified by PCR using an adapter primer and a specific antxPS primer (5′*ATGAAAACTCTGCTGCTGAC 3′;* Carbajal-Saucedo et al., 2013), which encodes for the conserved 3FTx signal peptide. The double-stranded DNA was cloned into pCR TOPO 2.1 vector (Invitrogen) by standard procedures as previously described by our group [23]. DNA sequencing was performed using universal primers and the chain termination method [24] on a ABI 3500 genetic analyzer (Thermo Fisher Scientific, Waltham MA, USA) at the facilities of the Instituto de Biotecnología, UNAM.

2.4. Sequence analysis

The resulting DNA sequences were analyzed using the software 4-picks and Gene Construction Kit, and the protein alignments were done with Clustal W. A database search for similar protein sequences was achieved using the Basic Local Alignment Tool (BLAST) from NCBI (http://ncbi.nlm.nih.gov/Blast).

2.5. Cloning, expression and purification of rD.H

The specific coding sequence for the mature D.H toxin (found in the venom glands of *M. diastema*) was enlarged by PCR using the respective pCR TOPO 2.1 plasmid as the template; the primers antxFPMDH (5′***GGA TCC*** *ATG ATA TGT CAC AAC CAA CAG* 3′) and antxRPMDH30 (5′***AAG CTT*** *TTA AGC GTT GCA TTT GTC TGA TG* 3′), designed previously to obtain the D.H. sequence, were used. These primers included the restriction sites *Bam*HI and *Hind*III (shown in boldface) to allow proper cloning of the product in the expression vector pQE30 (Qiagen, Germany). Competent *Escherichia coli* Origami Gold DE3 (Novagen®) were transformed with the pQE30 vector containing the rD.H clone. The protein expression was induced when the bacterial culture (supplemented with ampicillin and kanamycin) had an A_{600} of 0.6–0.8. The protein expression was induced with 0.1 mM IPTG for 24 h at 16 °C. After cell centrifugation, the total soluble protein from bacteria was removed using the BugBuster® protein extraction reagent (Novagen, Germany). The soluble protein fraction was loaded into an agarose-NiNTA histidine tag purification matrix (Novagen®) and washed with 20 vol of phosphate buffered saline (PBS) that consisted of NaCl (137 mM), KCl (2.7 mM), Na_2HPO_4 (10 mM) and KH_2PO_4 (2 mM) at pH 7.2. The removal of non-specific proteins was carried out using PBS with 25 mM imidazole, while the elution of recombinant protein was achieved with PBS with 250 mM imidazole. Purified recombinant D.H toxin (rD.H) was then dialyzed against PBS (pH 7.2) and stored at −20 °C.

2.6. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Electrophoretic separation was performed on a 17% acrylamide/bisacrylamide gel, using the discontinuous system described by Laemmli [25] under reducing conditions. For molecular weight estimation, 5 μl of Broad Range Protein Markers (10–250 kDa) from New England Bio-Labs were included. The gels were fixed and stained with Coomassie Brilliant Blue R-250 (Bio Rad).

2.7. Western blot

Western blot for recombinant toxin was carried out by electrotransferring samples from 17% SDS–PAGE to nitrocellulose