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Synthetic peptide antigens derived from long-chain alpha-neurotoxins: Immunogenicity effect against elapid venoms

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ABSTRACT

Three-finger toxins (3FTXs), especially α-neurotoxins, are the most poorly neutralized elapid snake toxins by current antivenoms. In this work, the conserved structural similarity and motif arrangements of long-chain α-neurotoxins led us to design peptides with consensus sequences. Eight long-chain α-neurotoxins (also known as Type II) were used to generate a consensus sequence from which two peptides were chemically synthesized, LCP1 and LCP2. Rabbit sera raised against them were able to generate partially-neutralizing antibodies, which delayed mice mortality in neutralization assays against *Naja haje*, *Dendrospis polylepis* and *Ophiophagus hannah* venoms.

1. Introduction

Snake venoms are a rich cocktail of bioactive molecules; among them, proteins and peptides play an essential role in prey hunting and defense against predators. Nonetheless, snakebites are a serious health problem, mainly in rural areas of tropical countries, where antivenom availability represents a big issue [1]. Particularly, proteins found in venoms, which can be nested in super-protein families, such as phopholipases A_2 (PLA_2), snake venom metalloproteinases (SVMP), serine proteinases and three-finger toxins (3FTXs), for instance, are the main toxins responsible for snake envenomation. Concerning elapid venoms, the low immunogenicity of 3FTXs makes generating homogeneous antivenoms difficult [2]. The elapid 3FTXs are peptides with associated non-enzymatic activity, ranging from 60 to 85 amino acids. They contain eight highly conserved cysteine residues that form 4 disulfide bridges that stabilize their hydrophobic core, from which emerge three loops that bear 3–5 antiparallel beta-strands. Besides, some 3FTXs also contain an extra pair of cysteine residues that forms one more disulfide bridge located at one of the loops. 3FTXs encompass many proteins with diverse functions like cytotoxicity (e.g. cardiotoxins) [3,4] and neurotoxicity (e.g. α-neurotoxins, fasciculins, muscarinic toxins, L-type calcium channel blockers) [5–7]. Snake venom composition from elapids and from their related colubrids show that PLA_2 and 3FTXs are not only the most abundant protein families [8,9], but also, the most toxic ones [10]. There are many reports in the literature that have led us to identify elapid 3FTXs with high toxicity and low immunogenicity when compared to other elapid toxins [11,12]. Among the 3FTXs, the three finger α-neurotoxins play an important role in envenomation as curare mimetic toxins. They bind to the nicotinic acetylcholine receptor (nACR), which prevents the union with the neurotransmitter, favors a resting state of the nACR, and disrupts neurotransmission [13]. Four- and five-disulfide bridge α-neurotoxins have been reported to antagonize muscle and neuronal nACRs, respectively. Interestingly, some long-chain α-neurotoxins (five disulfide bridged) can antagonize a wide variety of subtypes of acetylcholine receptors [14]. It has been demonstrated that the α-neurotoxin loops are crucial for activity, specificity and affinity; for instance, the second loop and the region close to the disulfide bridge at the tip of it, are essential for selectivity [6,15]. Furthermore, α-neurotoxins from elapid venoms are the main target in the antivenom industries. They represent a challenge because many of the current elapid antivenoms have shown low antibody recognition toward these neurotoxins when compared with other antivenoms [10].

In this work, we analyzed eight elapid long-chain α-neurotoxin sequences that were reported to be among the most toxic

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elapid venom components. A consensus long-chain α-neurotoxin sequence was determined based on multiple sequence alignments, and then two peptides containing the second loop sequence were chemically synthesized. Both peptides were used to immunize New Zealand rabbits, and their sera effectiveness was evaluated against elapid venoms. Model structures were generated for both peptides, to rationalize their effectiveness at generating protective antibodies.

2. Material and methods

2.1. Long-chain α-neurotoxin alignment

The amino acid sequences of the most toxic long-chain α-neurotoxins reported were retrieved from the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/pubmed). Based on a multiple sequence alignment, a consensus amino acid sequence was determined [16]. We focused on the second loop region, which is located from the second to the sixth Cys residue. Such region has been reported to be crucial for the nACR interaction and specificity [17]. As a result, two peptide sequences were selected and named LCP1 and LCP2.

2.2. Peptide models

LCP1 and LCP2 sequences were submitted to the PEP-FOLD3 server (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/), and all the generated models were retrieved for analysis [18–20]. The structures were filtered requiring the formation of the characteristic disulfide bond at the tip of the second finger and another one linking the N- and C-termini of the peptides. The resulting structures were refined by enforcing the disulfide bonds in CHARMM-GUI [21,22], followed by a brief steepest descent energy minimization (200 steps for hydrogen atoms and 400 steps for all atoms) in CHARMM40 [23] with the CHARMM36 [24] force field. The structure with the best overall energy was chosen for LCP1 and LCP2. The refined structures were compared to the α-cobratoxin in complex with the acetylcholine-binding protein (DOI 10.2210/pdb1yi5/pdb) and the α-bungarotoxin in complex with the α7 nicotinic receptor chimera (DOI 10.2210/pdb4hqp/pdb) in the PDB [25], and visualized in VMD (http://www.ks.uiuc.edu) [26].

2.3. LCP1 and LCP2 peptide synthesis

LCP1 (NVCYTKTWCDAFCSSRGKVVELGCAA) and LCP2 (QTCPPGENVAYTKTWCDAFCSSRGKVVELGCAA) were chemically synthesized using a solid phase peptide synthesis on Wang resin as solid support and 9-fluorenylmethyloxycarbonyl (Fmoc) as amino acid protecting group. In order to eliminate by-products and truncated peptides of the peptide synthesis, the crude peptide was dissolved in 10% aqueous acetonitrile (CH_3CN) solution/0.1% trifluoroacetic acid (TFA) and purified by Reversed-Phase High-performance Liquid Chromatography (RP-HPLC) (Agilent 1100 series; Agilent, CA) on a semi-preparative C_8 column (10 × 250 mm, 5 μm, VYDAC®) using a 10–60% gradient of an aqueous acetonitrile solution/0.1% trifluoroacetic acid (TFA) at a flow rate of 2 mL/min. The peptide fractions corresponding to either LCP1 or LCP2 received a second purification, which was performed loading them onto an analytic C_{18} column (4.6 × 250 mm, VYDAC®) using the same acetonitrile gradient at a flow rate of 1 mL/min. LCP1 and LCP2 isotope-averaged masses were obtained by mass spectrometry.

2.4. Animal immunization and antiserum production

New Zealand rabbits, kept at our facilities (Biotechnology Institute, UNAM, Mexico), were divided in two groups, one corresponding to LCP1 and another to LCP2 (two rabbits per group). They were hyper-immunized in a multi-site subcutaneously way [27] with 100 μg of peptide-adjuvant emulsions starting with Complete Freund Adjuvant and alternating incomplete adjuvant and aluminum hydroxide. Animals were immunized every fifteen days and bled weekly for four months. At the end of the immunization period, sera from each rabbit group were tested by enzyme-linked immunosorbent assay (ELISA) and pooled to perform the sera effectiveness assays.

2.5. Antiserum titration

Specific peptide recognition and antiserum titration was performed by ELISA as follows: flat bottom polystyrene microtiter plates (Maxisorp Nunc) were coated with 100 μL/well of 5 μg/mL of either LCP1 or LCP2 in carbonate/bicarbonate stock solution at pH 9.5, and incubated overnight at 4 °C. Plates were then rinsed 3 times with 200 μL/well of rinsing buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20 and pH 8). After that, it was added 150 μL/well of blocking buffer (50 mM Tris-HCl, 5 mg/mL gelatin, 0.2% Tween 20, and pH 8) and incubated 2 h at 37 °C. After a second rinsing cycle, serum anti-LCP1 or anti-LCP2 was mixed with vehicle buffer (50 mM Tris-HCl, 0.5 M NaCl, 1 mg/mL gelatin, 0.05% Tween 20, and pH 8) diluting 1:30, placed in the first well (200 μL) and further serially diluted 1:3 (with the same buffer for the ELISA plates) from wells 2–11; well 12 contained just vehicle buffer. Plates were incubated 1 h at 37 °C and then rinsed 3 times, followed by incubation with 100 μL/well of peroxidase-conjugated goat anti-rabbit (5×10^{-4} μg/mL, Zymed) for 1 h at 37 °C. Plates were then rinsed 3 times with rising buffer and, finally, 100 μL/well of peroxidase were added with peroxydase chromogenic substrate (soluble BM Blue POD substrate, Roche), and incubated for 10 min at room temperature, in darkness. At the end of the incubation, the reaction was stopped with 100 μL/well SDS 5%. The absorbance of the plates was

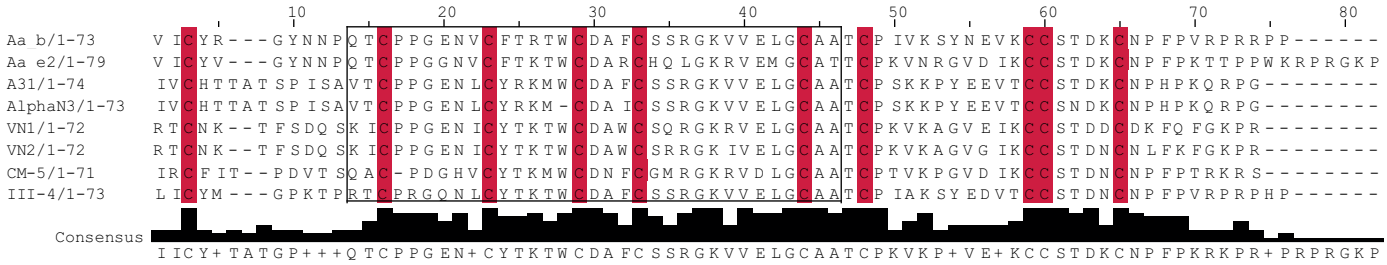

Fig. 1. Multiple sequence alignment of long-chain α-neurotoxins. Multiple sequence alignment of amino acid sequences of eight snake venom long-chain α-neurotoxins. Cysteine residues are highlighted in red. The amino acid sequence from the second to the sixth Cys residues has been reported to be part of the canonical second loop of long-chain α-neurotoxins, which is portrayed in a black box. At the bottom, the consensus amino acid sequence is shown. *Acanthophis antarticus*, Toxin Aa b [30], Toxin Aa e2 [31]; *Bungarus candidus*, A31 [32], AlphaN3 [33]; *Dendroaspis polylepis*, VN1/Dpp2a [34]; VN2/Dpp2b [35]; *Naja haje*, CM-5 [36]; *Notechis scutatus scutatus*, III-4 [37].