Original Article

Molecular Characterization of a Three-disulfide Bridges Beta-like Neurotoxin from *Androctonus crassicauda* Scorpion Venom

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ABSTRACT

Scorpion venom is the richest source of peptide toxins with high levels of specific interactions with different ion-channel membrane proteins. The present study involved the amplification and sequencing of a 310-bp cDNA fragment encoding a beta-like neurotoxin active on sodium ion-channel from the venom glands of scorpion *Androctonus crassicauda* belonging to the Buthidae family using reverse transcription polymerase chain reaction (RT-PCR) technique. The amplified complementary DNA (cDNA) fragment had a coding sequence of 240 bp. The deduced precursor open-reading frame was composed of 80 amino acid residues contain a signal peptide of 22 amino acid residues, followed by a mature toxin of 58 amino acids. It had a molecular mass of 6.84 kDa and isoelectric point of 4.58. The sequence similarity search revealed several matches with the scorpion toxin-like domain of toxin-3 superfamily with a homology range of 35-75%. Multiple alignments and secondary structure prediction demonstrated that the toxin peptide deduced from the amplified cDNA was related to the long-chain neurotoxins in size but stabilized by three disulfide bridges instead of four. The level of difference implies that the corresponding genes have originated from a common ancestor. This level of difference may also confirm an evolutionary link between the 'short-chain' and 'long-chain' toxins. The analysis showed one major segment within this neurotoxin with maximal hydrophilicity which was predicted to be antigenic by inducing an antibody response.

Keywords: *Androctonus crassicauda*, Beta-neurotoxin, Disulfide bridges

Caractérisation Moléculaire d'un Tri-disulfure Pontant la Neurotoxine de Type Bêta du Venin de Scorpion *Androctonus crassicauda*

Résumé: Le venin de scorpion est la source la plus riche en toxines peptidiques avec des niveaux élevés d'interactions spécifiques avec différentes protéines membranaires des canaux ioniques. La présente étude porte sur l'amplification et le séquençage par la technique de transcription en chaîne à la polymérase (RT-PCR) d'un fragment d'ADNc de 310 pb codant une neurotoxine de type bêta active exprimée au niveau du canal ionique sodique de la glande à venin du scorpion *Androctonus crassicauda* appartenant à la famille des *Buthidae* eL'ADN complémentaire amplifié (ADNc) avait une séquence codante de 240 pb. Le cadre de lecture ouverte du précurseur déduit contenait 80 résidus d'acides aminés composés d'un peptide signal de 22 résidus d'acides aminés et d'une toxine mature de 58 acides aminés. Cette toxine avait une masse moléculaire de 6,84 kDa et un point isoélectrique de 4,58. La recherche de similarité de séquence a révélé plusieurs correspondances avec un domaine d'une toxine de scorpion appartenant à la superfamille de la toxine-3 avec une plage d'homologie de 35 à 75%. Les alignements multiples et la prédiction de la structure secondaire ont démontré que le peptide de la

toxine déduit de l'ADNc amplifié était lié aux neurotoxines à longue chaîne, mais stabilisé par trois ponts disulfures au lieu de quatre. Le niveau de différence implique que les gènes correspondants proviennent d'un ancêtre commun. Ce niveau de différence peut également confirmer un lien évolutif entre les toxines «à chaîne courte» et «à chaîne longue». Nos analyses ont montré qu'un segment majeur de cette neurotoxine présentant une hydrophilie maximale était antigénique et induisait une réponse anticorps.
Mots-clés: *Androctonus crassicauda*, Bêta-neurotoxine, Ponts disulfure

INTRODUCTION

Scorpion venom is the richest source of polypeptide neurotoxins with various types of actions. Most of the scorpion envenomations in most area of Iran, especially Khouzestan province, are caused by Buthidae family members, and 41% of the cases belong to *Androctonus* species. Scorpion venoms have a large range of small peptide toxins which can be classified into two types, namely disulfide-bridged peptides (DBPs; Zeng et al., 2006) and nondisulfide-bridged peptides (NDBPs; Zeng et al., 2004; Zeng et al., 2005; Farajzadeh-Sheikh et al., 2013). These peptide toxins recognize and specifically interact with the ion-channel membrane proteins for sodium, potassium (Carbone et al., 1982), chloride (DeBin et al., 1993), and calcium (Valdivia and Possani, 1998). They have been confirmed as important tools for the investigation of the physiological functions of ion channels in cells and highly powerful instruments in ion-channel structure/function studies (Srinivasan et al., 2002). Scorpion peptide toxins are divided into two large classes based on their length. All known scorpion sodium channel-specific toxins are long-chain polypeptides consisting of 60-70 amino acid residues with four disulfide bridges (Bougis et al., 1989; Loret et al., 1990; Nakagawa et al., 1997), compared with the shorter chain-length potassium channel toxins composed of 29-39 amino acid residues with three or four disulfide bridges (Lebrun et al., 1997). The long-chain polypeptides can also be further subdivided into two general classes of alpha and beta toxins depending upon their effects on sodium channel (Jover et al., 1980). The venom of *Androctonus australis* living in North Africa has been widely studied. There are a number of studies purifying and sequencing peptide toxins active on mammals (AaH neurotoxins I, II, and III; Rochat et al., 1972; Kopeyan et al., 1979), one toxic active on insects (Zlotkin et al., 1971), and another toxic active crustaceans (Zlotkin et al., 1975). For the first time, a new anti-insect peptide toxin named AaBTX-L1 was purified from the venom of the *A. australis* (Martin-Eauclaire et al., 2005). This was similar in size to long-chain neurotoxins which interacted with the sodium channel. However, AaBTX-L1was closely packed by only three disulfide bridges, instead of four. With this background in mind, the present study was targeted toward performing the sequence characterization of a cDNA encoding a beta neurotoxin cross-linked by three disulfide bridges isolated from the venom glands of scorpion *A. crassicauda*.

MATERIAL AND METHODS

Scorpion venom gland total RNA extraction. For the purpose of the study, the scorpions of *A. crassicauda* species were collected from Khuzestan area, Iran. In scorpions, the last postabdominal segment, named telson, contains a pair of venomous glands connected to the stinger. The collected scorpions were transported alive to the laboratory where they were stimulated by electricity for the extraction of their

venom. After 3 days (a period that allowed the toxin-producing cells of the venom glands to enter the secretory phase), the animals were sacrificed, and the last segment of the tail containing the two venom glands were cut off and frozen immediately in liquid nitrogen. The total RNA (50 mg) obtained from the scorpion telson was prepared using RNX solution (Cinagen, Iran) according to the standard protocol. Concentrations of the RNA samples were evaluated by a spectrophotometer at 260 nm.

Reverse transcription polymerase chain reaction amplification. The total RNAs (0.5 μg) were converted to complementary DNA (cDNA) using Reverse Transcriptase (Fermentas, Iran) and the oligo(dT) primer at 42 °C for 60 min. The PCR amplification was carried out on the aliquots of *A. crassicauda* telson cDNA as a template using a sequence-specific primer. The PCR process included initial denaturation for 5 min at 95 °C, followed by 35 cycles of 45 s at 94 °C, 1 min at 50 °C, and 1 min at 72 °C, and finally, 7 min of incubation at 72 °C in a final reaction volume of 25 μl containing 50 mMKCl, 10mMTris-HCl (pH=8.3), 20 pmol of each primers, deoxynucleotides (each at 220 μM), 1.5 mM MgCl$_2$, 10 ng cDNA, and 1 U *Taq* polymerase. The primers used for the amplification of cDNA were designed according to the sequence information from *Mesobuthus martensii* insect beta-neurotoxin (GenBank accession number: AF151798). The primers were AcF 5'-ATGATGAAATTTTTGTTA TTTGGTA (forward primer) and AcR 5'-TAATGGT GACGTCTTTATCTTC (reverse primer). The amplified PCR products were separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide, and visualized by ultraviolet transillumination.

DNA sequencing and sequence analysis. The amplified cDNA fragments were extracted from agarose gel prior to submission for DNA sequencing according to the dideoxy termination method using an Applied Biosystems 373 DNA sequencer. The sequence was determined for both strands by using overlapping fragments. The comparison of *A.*

crassicauda cDNA sequences with the GenBank database sequences was accomplished using the blastn and blastx algorithm programs (Altschul et al., 1990) to identify the putative functions of the cDNA fragments. The theoretical molecular mass and isoelectric point were calculated by means of the Compute pI/MW tool software available at the Expasy website (ca.expasy.org/tools/pi_tool.html). In addition, the Pfam database search software was used to determine the conserved domains (Thompson et al., 1994). The alignments of multiple sequences were obtained using the CLUSTAL_W program (Finn et al., 2008) and edited by the BOXSHADE software (www.ch.embnet.org/software/BOX_form.html). The signal peptide was predicted with the SignalP program, version3.0 (www.cbs.dty.dk/sevices/SignalP). The secondary structure of the protein was predicted using the PSIPRED Protein Sequence Analysis server (bioinf.cs.ucl.ac.uk/psipred). The three-dimensional structure prediction was performed by the Phyre 2 program (Kelley and Sternberg, 2009). Antigenic epitopes were determined using the Bepipred Linear Epitope Prediction program (Kolaskar and Tongaonkar, 1990).

RESULTS

Amplification and sequence analysis. The use of 10 fresh telsons giving 0.05 g of tissue resulted in the achievement of 5 μg total RNA. The cDNA was synthesized by oligo(dT)-primed reverse transcriptase. For the purpose of amplification, a cDNA, encoding beta-neurotoxin specific primers, was designed based on the sequence information from *Mesobuthus martensii*. One cDNA fragment obtained from the total RNAs of the venomous gland was subjected to amplification using RT-PCR (Figure1). According to the sequencing results, the cDNA of 243 bp contained a single open reading frame of 240 bp. The deduced precursor open reading frame is composed of 80 amino acid residues that consist of a signal peptide of approximately 22 amino acid residues.
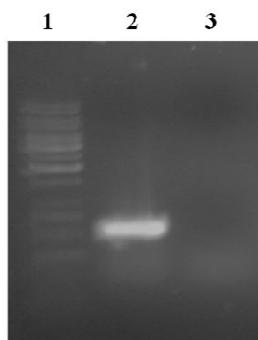
Figure 1. 1% agarose gel electrophoresis of reverse transcription polymerase chain reaction (RT-PCR)of peptide toxin isolated from scorpion *A. crasicauda*; lane 1) 100 bp DNA size marker, lane 2) second round of RT-PCR amplification product, and lane 3) negative control (water. Each lane was loaded with 8 μl of the total reaction.

The cleavage site of the signal peptide is located at a small residue Gly. These features are similar to those of the most scorpion toxin precursors described previously (Martin-Eauclaire et al., 2005; Zeng et al., 2006; Zhu and Gao, 2006). The mature peptide coding sequence was 174 bp in length and coded a 58-residue peptide stabilized by three disulfide bridges with a calculated molecular mass of 6.84 kDa and theoretical pI of 4.58.

Analysis of amino acid sequence alignments. Comparison with the sequences retrieved from the GenBank revealed similarity (35-76%) with several beta-toxins like precursors described in several scorpions.The highest level of identity was scored with a toxin Acra III-2 (76%), toxin Acra II-2 (71%), toxin Acra1 and AcraI-1 precursor (68%), and toxin AcraI-2 precursor (64%) obtained from *A. Crassicauda*. A cDNA sequence analysis also showed identity to toxin KBT precursor *Mesobuthus martensii* putative beta-like toxin Tx814 obtained from *Buthus occitanus* Israelis (73%), followed by *M. eupeusvenom* lipolysis activating peptide alpha subunit (68%). Some representatives of these matched beta-neurotoxins were chosen for a multiple sequence alignment using the Clustal_W program. As shown in Figure 2, the isolated cDNA sequence was aligned with the protein sequences recorded in Table 1. Since the amplified cDNA sequence shows a significant sequence identity with the described toxins, it can also be a beta-toxin acting on sodium channels. The Pfam database search showed the e-values of 3.2e-07 and 2.43e-03 with the conserved scorpion toxin-like domain of Toxin-3 family (amino acid: 37-76) and Knot1 family (amino

acid resides: 46-70), respectively (Figure 2). These families contain both neurotoxins and plant defensins. In addition, the "Scorpion toxin signature" motif 57[GVSYGYCYNSQ]67, which is highly conserved in all known scorpion neurotoxins is present within this region (Figure 2).

Secondary structure and a three dimensional-structure model. The PSIPRED protein sequence analysis method predicted the secondary structure of the toxin peptide. Each residue was assigned values for alpha helix, beta sheet, and coils using a window of 7 residues (Figure 3A). The use of these information parameters, the likelihood of a given residue calculated, and the conformation with the largest conformation confidence were assigned to the residues. Based on the secondary structure data and computational three-dimensionalstructure model (Figure 3B), the mature peptide was folded into one alpha-helical 25[QFCRQICKI]33 conformation and a three-stranded antiparallel-beta-sheet, namely 15[LYNCTI]20, 38[YGYCY]42, and 45[QCWCEY]50.The alpha-helix is connected to the long outer strand of the beta-sheet (C-terminus) by a pair of disulfide bonds involving Cys27-Cys46 and Cys31-Cys48. The beta-sheet loop prior to the alpha-helix at N-terminus part is linked to the middle strand of the beta-sheet located after the alpha-helix by a disulfide bond between Cys18 and Cys41 (Figure 3A). The loops extend out of this dense core of the secondary structure, and the largest loop was joined to the N-terminus of the molecule. Another typical feature of this peptide was the presence of two proline residues separated by three amino acids at the N-terminus part, which may contribute to the formation of a double-turn structure.

Antigenic B-cell epitope prediction. For the prediction of antigenic epitopes, the antigenic elements were predicted by finding the area with the greatest hydrophilicity. These epitopes were expected to be recognized by the antibodies of the immune system. The Bepipred linear epitope prediction program was used to predict the locations of the antigenic part in a protein, assuming that the antigenic regions would be

exposed on the surface of the protein, and therefore would be located in hydrophilic regions (Figure 4). The scale was basically a hydrophilic index with polar residues assigned negative values.

DISCUSSION

Scorpion venom contains a large variety of venom neurotoxin peptides. Each species of scorpion may have more than 100 different venom peptides with high levels of specific interactions with various ion-channel membrane proteins. The cDNA sequences described in this study encoded 80 amino acid residues with a putative hydrophobic signal peptide of 22 residues, thereby meeting the conditions of von Heijne rule (Leu [-3]/Gly[-1]) (von Heijne, 1986). An interesting finding in the present study was the high degree of primary structural conservation between the putative signal peptides among four different species illustrated in Figure 2. The Phe-4 position in the isolated cDNA and AAQ22733 was occupied by Leu in all other toxins.

Table 1. A list of protein sequences with homology to the amplified complementary DNA

protein	Organism	Accession #
Toxin KBT precursor	*Mesobuthus martensii*	AAQ22733
Birtoxin	*Parabuthus transvaalicus*	P58752
Birtoxin analog peptide AaF1CA25	*Androctonus australis*	AJ781834
Beta-toxin AaBTxL1 precursor	*A. australis*	QLCS8
Toxin Acra1 precursor	*A. crassicauda*	P0C292
Toxin AcraI-1 precursor	*A. crassicauda*	POC293
Toxin AcraI-2 precursor	*A. crassicauda*	POC294
Beta-toxin KAaH1 precursor	*A. australis*	Q4LCT0
Beta-toxin KAaH2 precursor	*A. australis*	Q4LCS9
Toxin-like peptide AaF1CA7 precursor	*A. australis*	Q4LCT1

Table 2. Antigenic epitopes from the isolated complementary DNA sequence obtained from *Androctonus crassicauda*

No.	Start posotion	End position	Peptide	Peptide length
1	1	10	AYVPGNYPLY	10
2	56	57	VT	2
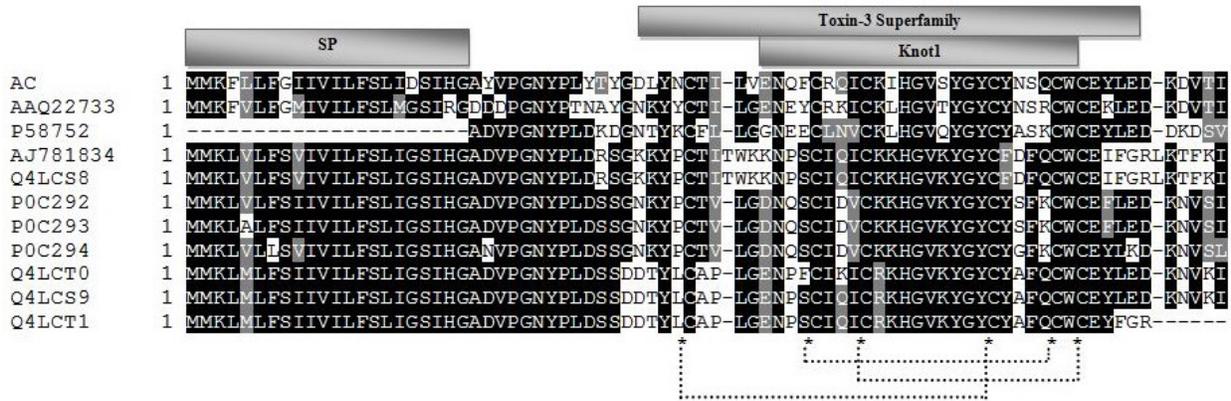
Figure 2. Multiple sequence alignments of the isolated complementary DNA sequence obtained from Androctonus crassicauda (Ac) and other scorpion beta-toxinsThe amino acid sequence of the isolated cDNA was aligned with toxin KBT precursor M. martensii (AAQ22733), Birtoxin from South African fat tail scorpion *Parabuthus transvaalicus* (P58752), toxin-like peptide AaF1CA25 from Sahara scorpion *A. australis* (AJ781834), beta-toxin AaBTxL1 precursor *A. australis* (QLCS8), toxin Acra1 precursor *A. crassicauda* (P0C292), toxin AcraI-1 precursor *A. crassicauda* (POC293),), toxin AcraI-2 precursor *A. crassicauda* (POC294), beta-toxin KAaH1 precursor *A. australis* (Q4LCT0), beta-toxin KAaH2 precursor *A. australis* (Q4LCS9), and toxin-like peptide AaF1CA7 Precursor *A. australis* (Q4LCT1). The amino acids are denoted by one-letter symbols. Shading indicates identity (black) or conservative substitutions (grey) relative to the isolated cDNA (Ac). Gaps represented by dashes were introduced to maximize the alignment. Disulfide bridges are indicated at the bottom by dashed lines and marked by stars below the sequence. The signal peptide and domains are indicated.

The Gly-8 position was replaced by Ser in nearly all toxins. In addition, the Asp-18 was occupied by Gly in all other toxins. Histidine was also located in position 21 in all of toxins, except for AAQ22733 which is replaced by Arg. Multiple sequence alignments revealed that our toxin peptide shared 57% similarity with Birtoxin, which was the first member of anti-insect toxin purified from the venom of the South African scorpion *Parabuthus transvaalicus*.
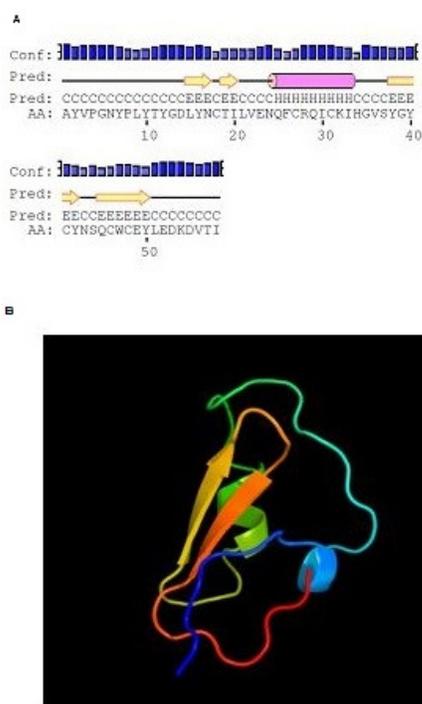
Figure 3. Secondary structure of peptide toxin from the isolated complementary DNA obtained from *Androctonus crassicauda; aschematic* representation of the secondary structure (A) and three-dimensional model (B) of peptide toxin. The amino acid sequences predicted to form amphipathic alpha-helix (H), beta-sheets (E), and random coils (C); Conf: Confidence of prediction, pred: Predicted secondary structure, and AA: target sequence.

It also showed 68% similarity with the already described beta-type Birtoxin analogs AaF1CA25 from Sahara scorpion *A. australis* (Martin-Eauclaire et al., 2005). The Birtoxins are the members of scorpion toxin-like family, which is related to a long chain neurotoxin-like peptide possessing only six cysteine residues. Multiple

sequence alignments revealed that the amino acid residues (i.e., Tyr38, Tyr40, and Trp47) in the amplified cDNA sequence were identical with those amino residues in AaF1CA25. However, only Phe42 was replaced by a well-conserved amino acid residue, namely Tyr42.
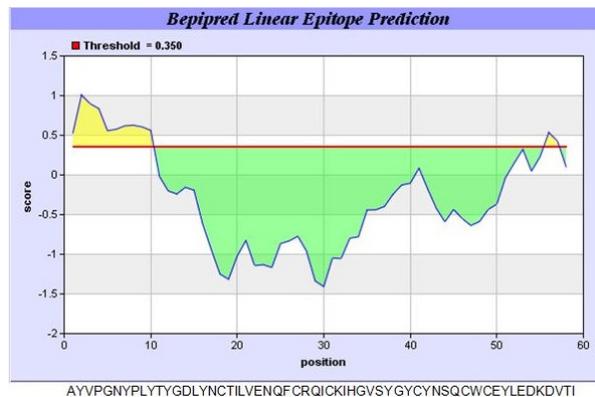
Figure 4. B-cell epitopes sites recognized on the isolated complementary DNA sequence from Androctonus crassicauda by Kolaskar and Tongaonkar antigenicity program

The level of homology implies that our peptide is related to this family of long-chain scorpion toxins with three disulfide bridges. In fact, the same as these two Birtoxins, the peptide toxin in this study was similar to the long-chain toxins in terms of size and was stabilized by three disulfide bridges instead of four. It was suggested that these corresponding genes originate from a common ancestor and may confirm an evolutionary link between the 'short-chain' and 'long-chain' toxins. Primary and secondary structure analyses indicated a similar configuration to the well-known scorpion peptide toxins with the cysteine-stabilized alpha-helical motif, engaging a Cys-X-X-X-Cys stretch of the alpha-helix, connected by two disulfide bridges to a Cys-X-Cys extend in a beta strand belonging to an antiparallel beta-sheet (Kobayashi et al., 1991). It was confirmed that all known toxins in the long-chain neurotoxin family have four disulfide bridges in which the fourth bridge typically crosslinks the first and the last cysteine residues. For this reason, the fourth cysteine bridge is named 'wrapper disulfide bridge'.

Since nearly all known peptide toxins in this category have the wrapper disulfide bridge for keeping the molecule together, it can be considered that the wrapper disulfide must have an essential role for peptide integrity and functionality. As seen in Figure 3, the close proximity of N- and C-termini, rather than the lack of the fourth disulfide bridge in this peptide as wrapper, would allow multiple hydrogen bondings for holding the molecule together. In the present study, it was hypothesized that the close contact between the N- and the C-termini parts might be adequate for putting together the peptide in a way similar to other members in this family with four disulfide bridges. As shown in Birtoxin, which is a very abundant peptide, it is possible to speculate that our peptide was also the major peptide in the venom of *A. crassicauda*. One assumption is that the absence of the fourth disulfide bridge decreases the complexity of folding into the active conformation. Therefore, this peptide can be expressed in higher quantities relative to four disulfide bridged peptide toxins. Peptide toxin shares some common key molecular determinants as a sodium channel blocker rather than a potassium channel blocker. It was proven that potassium channel blockers from the scorpion venom have at least two important residues that create an essential structure for channel-blocking ability. These two residues are Lys27, the side chain of which penetrates into the channel pore, and an aromatic residue, usually Tyr36 (Jouirou et al., 2004). Homology search implied that the locations of these critical residues within the primary structure of our peptide toxin were not conserved. However, the comparison of the primary structures of our peptide with those of homologous sodium channel blocking Birtoxins obtained from the *A. Australis* revealed that it had a high degree of primary and structural similarity with sodium channel blockers. Since the identification of antigenic elements is an essential step in designing subunit peptide for producing synthetic antivenom, the location of antigenic B-cell epitopes was determined using the Bepipred Linear Epitope Prediction program.

The analysis showed one major segment within this toxin peptide with maximal hydrophilicity which can be recognized by the antibodies of the immune system. Table 2 demonstrates the highest pick in the mature peptide. This region located in the N-terminal region of the peptide toxin, which is solvent-accessible with a simple structure, is likely to be appropriate for the recognition of native toxin peptide.

In conclusion, the toxin peptide was similar to the long-chain toxins in terms of size; however, it was stabilized by three disulfide bridges instead of four. This is originated from a common ancestor and may confirm an evolutionary link between the short-chain and long-chain toxins. Our analysis showed one major segment within this toxin peptide which was likely to be recognized by the antibodies of the immune system.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

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