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CDNA Cloning and Sequence Analysis of an Alpha Neurotoxin-Like Gene BMK from the Scorpion *Mesobuthus eupeus* Venom Glands Using Semi-Nested RT-PCR

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Abstract: This work aimed to study the characterization and isolation of a alpha neurotoxin- BmK, from the venom glands of Scorpion Buthida Mesobuthus eupeus Kuzestan (Iran). Scorpions *Mesobuthus eupeus* were collected from the Khuzestan province. Using RNXTM solution, the total RNA was extracted from the twenty separated venom glands. cDNA was synthesized with extracted total RNA as template and modified oligo(dT) as primer. In order to amplify cDNA encoding a BMK neurotoxin, Semi-Nested RT-PCR was done with the specific primers. Follow amplification, the fragment was sequenced. The length of the coding region was 255 bp, encoding a polypeptide of 85 amino acid residues with a calculated molecular weight of 9.565 kDa and theoretical isoelectric point of 4.69.The open reading frame encoded 85 amino acid residues corresponding to the BMK precursor Alpha neurotoxin from M. eupeus belongs to the Toxin_3 super family. The size of peptide is close to the "long chain neurotoxin" peptide family.

Key words: Alpha Neurotoxin · Semi-Nested RT-PCR · Scorpion Venom

INTRODUCTION

Scorpion venom is contains a mixture of various toxic proteins with different functions. The toxins can be divided according to their host specificity: toxins that are active on vertebrates (mammals) and those that are active on invertebrates (arthropods). They modify the opening or closing of the channels in nerve, muscle and heart cells, thus increasing the depolarization of the membrane and leading to the release of neurotransmitters [1]. Scorpion toxins that are active on vertebrate Na+ channels can be divided into two types according to their electrophysiological behaviour, differences in their binding sites and ion flux: (i) α-toxins, which slow down the inactivation of the channel and prolong the action potential and (ii) β-toxins, which cause a shift of the voltage-activated activation to more negative membrane potentials, leading to repetitive firing in both muscles and nerves [2,3]. Many of them can interfere with the activity of ion channels and modulate their functional properties. Four different families of toxins have been described, which are associated with the ion channels: Na^+, K^+, Cl^- and $Ca2^+$. The best-studied peptides are long chain toxins containing 60-70 amino acid residues cross-linked by four disulfide bridges. These peptides are mainly active on sodium channels [2, 4]. Classical α-toxins are also mainly found in the venoms of *Androctonus mauretanicus mauretanicus* (Ammo), *Leiurus quinquestriatus quinquestriatus* (Lqq) and other subspecies of *Buthus* from Africa and Asia [5, 6]. The most potent toxin of M. eupeus is the neurotoxin, of which two general classes, alpha-and beta-neurotoxins exist. The mode of action of these two classes are different. Both of these classes are heat-stable, have low molecular weight and are responsible for causing cell impairment in nerves, muscles and the heart by altering ion channel permeability. Many study deal with the scorpions of Iran to some extent, but a comprehensive study of the scorpion *Buthida Mesobuthus eupeus* Khuzestan (BMK) fauna has been lacking. Our interest in this area has been focused at cloning the genes that code for these toxins for further studies of structure-function relationships by carrying out specifc amino acid substitutions in toxin molecules through recombinant DNA techniques. This work aimed to study the characterization and isolation of a alpha neurotoxin- BmK, from the venom glands of Scorpion *Buthida Mesobuthus eupeus* Khuzestan (Iran).

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MATERIALS AND METHODS

Scorpion Samples: The species *Mesobuthus eupeus* from the Scorpion *Buthida* family is endemic of the Khuzestan province Iran. The Scorpions transported to the reference laboratory of the Razi Institute. They were killed two days after manual extraction of the venom to allow the toxin producing cells of the venom glands to enter into a secretory phase. Twenty separated venom glands were used for total RNA extraction.

Total RNA Extraction: Total RNA was extracted from the venom glands (48 h after stimulation) by use RNA™ cDNA synthesis kit. (Cinagene, Iran), is according to the manufacture procedure using the SMARTTM cDNA synthesis kit. The RNA pellets were dissolved in DEPC-ddH2O and used for cDNA synthesis immediately.

cDNA library Synthesis: cDNA was synthesized from the extracted total RNA as template and modT (modified oligo-dT) (5'-cgcggatccatgcaaaggaatctggtcgt-3') as primer. ModT was added to the extracted RNA and incubated at 70°C for 5 minutes and immediately transferred into ice for 2 minutes. The mixture of 5× buffer, dNTPs, Ribolock, Reverse transcriptase enzyme and ddH2O was added to the samples following by incubation at 42°C for 60 minutes. The samples were incubated at 70°C for 10 minutes and immediately transferred into ice.

Semi-nested RT-PCR Amplification: For the cDNA amplification Semi-nested RT-PCR technique was used. The first round of PCR was performed using modT-R (5'-cccagatctcgagctcagtg-3'), BMK-F 5-cgcGGATCCTCCGTAAAACGGTTCAAAATG primers and synthesized cDNA as template. The second round of PCR was performed using BMK-F and BMK-R 5-cgcAAGCTTACCGCCATTGCATTTTCCT primers and the PCR products of the initial amplification as templates. The PCR conditions for both rounds were as follows: initial denaturation at 95°C (5 min), followed by 35 cycles of denaturation at 94°C (40 sec), annealing at 56°C (90 sec) and extension at 72°C (1 min), with a final extension at 72°C (10 min). Amplicons were separated by 1% agarose gel electrophoresis and visualized by UV transilluminator.

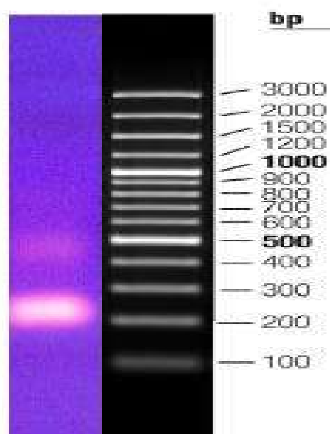
DNA Sequencing and Data Analyses: The PCR products were purified by 1% agarose gel electrophoresis, QIAquick Agarose Gel Extraction kit (www.fermentas.com) and sent to Kawsar Biotech Company for nucleotide sequencing. Sequence similarity analysis against GenBank database entries was performed using BLAST at the NCBI website (http://www.ncbi.nlm.nih.gov). We used online tool software at the Expasy website http://expasy.org/tools for nucleotide sequences to translate into the corresponding amino acids and the predicted signal peptide sequence was identified using online tool software at the Expasy website (http://expasy.org/tools). The program SignalP http://www.cbs.dtu.dk/services/SignalP/ was used to prediction. Multiple sequence alignments were obtained by using the CLUSTAL_W program and edited with the BOXSHADE software http://www.ch.embnet.org/software/BOX_form.html. We applied the SBASE online software http://hydra.icgeb.trieste.it/sbase/ for determines the conserved domains.

RESULTS

In order to characterize mRNA, cDNA was synthesized and the cDNA fragments of about 276 bp encoding an alpha neurotoxin from M. eupeus were amplified using RT-PCR technique (Fig.1).

The length of the coding region was 255 bp, encoding a polypeptide of 85 amino acid residues with a calculated molecular weight of 9.565 kDa and theoretical isoelectric point of 4.69.The open reading frame encoded 85 amino acid residues corresponding to the BMK precursor (Fig. 2).

A putative signal 19-amino-acids signal peptide was identified and the Valine at position 20 was assumed to represent the start of the mature protein (Fig. 2). This sequence is closely related to that of other α-toxins purified from Aau, Lqh, Ab and Amm. We assume that the first ATG codon serves as the translation start codon, as a hydrophobic region that is typical of a signal peptide followed it. This signal peptide was identical with that of the α-toxin Amm [7,8]. Comparison of the cDNA fragment with the GenBank database revealed that the amino acid sequence of alpha neurotoxin- BmK is highly homologous with alpha neurotoxin- BmK of other scorpions and arthropods. It is suggested that alpha neurotoxin- BmK is belongs to the Scorpion *Buthida* family and α-toxins are from the common progenitor. Conserved domains of alpha neurotoxin- BmK was predicted using SBASE online software. As shown in Figure 3, this domain is knottin.



BMK M

Fig. 1: PCR amplification of BMK cDNA and marker (M).

5'ATG AAT TCT TTG GTA ATG ATC AGT TTG GCA CTT CTT GTA ATG ACA

M N S L V M I S L A L L V M T

GGT GTG GAG AGT GTG CGC GAT GGT TAT ATT GCC GAC GAT AAA AAC

G V E S V R D G Y I A D D K N

TGC GCA TAC TTT TGT GGT AGA AAT GCA TAT TAT GAT GAA GAA TGT

C A Y F C G R N A Y Y D E E C

AAG AAG AAT GGT GCC GAG AGT GGC TAC TGC CAA TGG GCA GGT

K K N G A E S G Y C Q W A G

CAA TAC GGA AAC GCC TGC TGG TGC TAT AAG TTG CCT GAT AAA GTA

Q Y G N A C W C Y K L P D K V

CCT ATC AGA GTA CCA GGA AAA TGC CAT GGC GGT 3'

P I R V P G K C H G G

Fig. 2: The full-length cDNA sequence of neurotoxin BmK. The signal peptide is underlined once and highlighted; mature peptide is underlined twice.

The " knottin" fold is stable cysteine-rich scaffold, in which one disulfide bridge crosses the macrocycle made by two other disulfide bridges and the connecting backbone segments. Members include plant lectins/ antimicrobial peptides, plant proteinase/amylase inhibitors, plant gamma-thionins and arthropod defensins.

The amino acid sequence of neurotoxin BmK was aligned with alpha-neurotoxin from species of scorpions including *Androctonus australis* (accession Q9BLM4,)

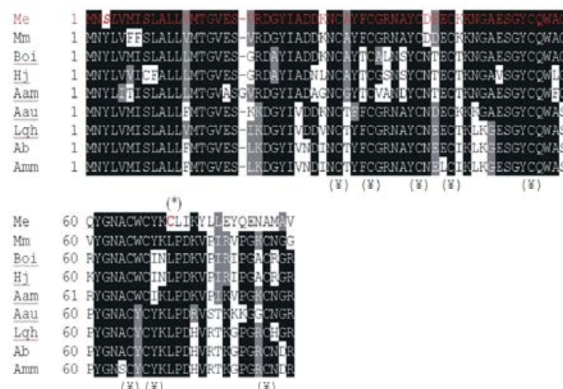


Fig. 3: Sequence alignment of neurotoxin BmK (Me) sequence from species of scorpions. Gaps are indicated by (-), conserved cysteines are indicated by (¥), the position of two catalytic residues is indicated by (#), the position of other active site residues is indicated by (*). homologous amino acids are shaded in grey and Fully conserved amino acids are shaded in black. Me(bmk): *Mesobuthus* eupeus from Khuzestan, Mm: *Mesobuthus martensii*, Boi: *Buthus occitanus israelis*, Aau: *Androctonus amoreuxi*, Lqh:*Leiurus quinquestriatus*, Ab : *Androctonus bicolor*, Amm : *Androctonus mauretanicus mauretanicus*"

Leiurus quinquestriatus hebraeus (accession GQ335456.1,), *Buthus occitanus israeli*s (accession FJ360776.1) *androctonus bicolor* (accession GQ335448.1) *androctonus mauretanicus mauretanicus* (accession Q7YXD3), Hottentotta judaicus (accession Q56TT9), *Mesobuthus martensii* (accession AF370023) and *Androctonus amoreuxi* (accession Q86SE0) as shown in Figure 3.

Alpha toxins bind voltage-independently at site-3 of sodium channels and inhibits the inactivation of the activated channels, thereby blocking neuronal transmission (By similarity).These toxins all bind to site 3 of the voltage activated Na+ channels with high affinity and are all highly toxic in mouse by s.c. injection [1,9,10]. BmK (Me) sequence and Mm were more similar (92% sequence homology) than BmK (Me) and Amm (78%). The main difference was the extra C-terminal acidic residue (ser,bold) only found in Me(Bmk). All the analgesic peptides contain eight cysteine residues cross-linked by four disulfide bridges and the cysteine positions are highly conserved [(¥),

inset]. Me (Bmk) had an acid residue, Ser, which is unusual compared with the other α-toxins, instead those of has an acid residue (tyr).

DISSCUSION

In this study, alpha neurotoxin BmK from venom glands of *M. eupeus* scorpion from Khuzestan province was identified. The amino acid sequence of alpha-neurotoxin BmK are 85 residues long and cross linked by four disulphide bridges that belongs to the long scorpion toxin super family Sodium channel inhibitor family and Alpha subfamily [11,12] all known toxins in the long chain neurotoxin family have four disulfide bridges that is similarity with this report. On the other hand, its C-terminal region is not similar with other toxins. Three of these constitute the core of the structure, two disulfides bonding the a helix to the antiparallel b sheet and a third disulfide links the b sheet to an extended segment preceding the helix [8,9,13,14,15] was compared to alpha neurotoxins from several scorpions, which revealed higher homology within these species. *M. martensii* alpha neurotoxin BmK has demonstrated the highest homology with alpha neurotoxin BmK determined in this study, whereas the least similarity was found with *Androctonus mauretanicus mauretanicus* alpha neurotoxin.

According to the differences between *M. martensii* and *Androctonus mauretanicus mauretanicus,* it is concluded that *M. eupeus* of Khuzestan and east Mediterranean *M. eupeus* belong to the different subspecies. The alpha neurotoxin from *M. eupeus* belongs to the Toxin_3 superfamily. The length of the peptide is close to the "long chain neurotoxin" peptide family. BmK, which was described as the α-toxin of reference, shares 92% similarity with Mm, the most potent toxin from Mm venom. The toxin belong to the same structural α-toxin group. Its complete final sequence was compared with those of the other scorpion α-toxins that belong to the same structural Aau, Lqh, Ab and Amm.

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