Production of Recombinant Alpha Neurotoxin of Scorpion Venom Mesobuthus eupeus and Analysis of its Immunogenicity

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1. Background
Scorpion venom is a major public health problem in many tropical and subtropical countries, as several species of these animals are highly toxic. This results in significant adult morbidity due to severe systemic envenomation, as well as essential pediatric mortality (1). Furthermore, the scorpion venom represents a naturally occurring vast arsenal (~100,000) of bioactive peptides. Out of these, only 1% is currently known which have been widely used as pharmacological tools to probe the molecular mechanisms for the functions, biosynthesis, assembly and localization of various ion channels in normal or disease states due to their ability to potently and specifically affect channel functions (2). Naturally occurring neurotoxins can be considered as valuable and useful tools not only for unraveling structure-function relationship of ion channels but also serving as lead compounds in the development of novel drugs. In this context, the scorpion-derived peptide neurotoxins specific to sodium channels have shown promise as potential therapeutic targets for the treatment of various human diseases.

Different scorpion venoms have been widely used as pharmacological tools to probe the molecular mechanisms for the functions, biosynthesis, assembly and localization of various ion channels in normal or disease states due to their ability to potently and specifically affect channel functions (2). Naturally occurring neurotoxins can be considered as valuable and useful tools not only for unraveling structure-function relationship of ion channels but also serving as lead compounds in the development of novel drugs. In this context, the scorpion-derived peptide neurotoxins specific to voltage-gated sodium channels have shown promise as potential therapeutic targets for the treatment of various human diseases ranging from pain syndrome, asthma, diabetes, cardiac ischemia and hypertension to chronic inflammation, autoimmune disease and cancer (3, 4). In recent decades, sodium channel scorpion toxins have thus stimulated considerable interest among scientists in pharmaceutical science and neurobiology.

Voltage-gated sodium channels (VGSCs) are large integral membrane proteins, which are critical for the ini-
vation and propagation of action potential in excitable cells. They are composed of a pore-forming α-subunit associated with up to four auxiliary β-subunits (5). To date, seven topologically distinct receptor sites for neurotoxins on the α-subunit of VGSCs have been identified, all of which are linked to specific effects on channel function (6). Venom of Buthidae family scorpions contains a large quantity of long-chain neurotoxins assumed to modulate the sodium channel function; however, detailed data on its venom compounds exist only for a small fraction of it (7). As mentioned above, in tropical countries including Southern parts of Iran and especially Khuzestan province, envenomation by scorpion stings presents a major public health problem due to Mesobuthus eupeus of the family Buthidae (8, 9). Antivenom immunotherapy, which still remains the unique specific treatment against scorpion envenomation, is another major area of interest to study buthid toxic components to better understand the envenomation syndrome and improve serotherapy.

In recent years, modern molecular biology techniques have provided powerful tools for understanding the complexity of venomous animals. Particularly, the strategy of cDNA library construction followed by molecular cloning and gene expression in heterologous systems has been and still is the most widely employed approach for the characterization of venom toxin precursor-encoding genes (10). Bioengineered scorpion toxins have been monumental to the evolution of channel science, and are now serving as templates for the development of important experimental molecular therapeutics.

2. Objectives

The main aim of the work is molecular characterization and recombinant production of a novel α-neurotoxin BMK from the Iranian scorpion Mesobuthus eupeus venom, with further assessment of its immunogenicity. The structure-and-function analyses of α-toxin BMK are crucial for the development of new therapeutics, especially with improved immunogenic properties.

3. Materials and Methods

3.1. Isolation of MeNaTxα-4 Family of α-neurotoxin in Mesobuthus eupeus

The study was performed at the Razi Reference Laboratory of Scorpion Research in Iran. The specimens of scorpions were collected in Khuzestan and transported alive to reference laboratory. RNA was isolated from the venom glands (telson) of twenty scorpions of the species Scorpion Buthidae Mesobuthus eupeus, Khuzestan (Iran). Semi-nested RT-PCR technique was used to enable the amplification of cDNAs. The first round of PCR was performed using modF-R (5’- cccagatctcgagtctcg-3’), BMK-R 5’- cgcGAATTCCTCGTAAACCGGTTCAAAATG -3’) primers.

The second round of PCR was performed using BMK-F and BMK-R 5’- cgcAAGCTTACCGCCATTCTCACTTTTCT-3’) 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 for 5 min, followed by 35 cycles of denaturation at 94°C for 40 sec, annealing at 56°C for 90 sec and extension at 72°C for 1 min, with a final extension at 72°C for 10 min.

3.2. Bacterial Strains

The E. coli DH5-α was used routinely for molecular cloning and plasmid transformations and E. coli BL-21 was used for BMK gene expression. Bacteria were grown at 37°C in LB. It was supplemented with 100 μg/ml ampicillin where appropriate. The expression vector pMAL-c2x (New England Biolabs) was used to clone the BMK gene sequence for expression in bacteria.

3.3. Enzymes and Plasmids

All the restriction enzymes and buffers were purchased from CinnaGen (IR Iran). The enzymes involved were BamHI, HindIII and T4 DNA Ligase. To isolate plasmid from the bacteria E. coli DH5-α, QIAGEN Plasmid Mini Kit [QIAquick Kit = K0513] was employed. The isolation was performed according to the manual.

3.4. DNA Sequencing and Bioinformatics Analysis

Sequence similarity analysis against GenBank database entries was performed using BLAST at the NCBI website (http://www.ncbi.nlm.nih.gov). The theoretical molecular mass for the putative mature peptides and theoretical pI value were estimated by using ProtParam plMw tool program (http://www.expasy.org/tools/protparam). The 3D structure prediction was performed by the Phyre 2 program.

3.5. Cloning and Construction of MeNaTxα-4 for Expression in Bacteria

The instruction of competent cells was followed by Sambrook.et al (11) finally; the frozen competent cells were stored at -80°C for long-term usage. The Insert 6 µl of the ligation reaction were used to transfact competent E. coli DH5-α cell. The plasmids of positive colonies were purified by means of the High Pure Plasmid Isolation Kit (QIAGEN). Plasmids were verified by sequencing both sites to confirm the reading frame and to conserve restriction sites. The clone finally obtained was called MeNaTxα-4. E. Coli of the strain Bl-21 were transfected with the corresponding plasmid by transformation heat shock and recovered 30 min a 37°C in LB medium containing 100 μg/ml of ampicillin. In order to confirm expression and to verify the best producing strain of E. coli, a small scale culture of 5 ml each was grown in LB medium and the re-
sults analyzed. After the absorbance at OD 600 reached 0.5 units, the cultures were induced with 1 mM IPTG (iso-
propyl-b-D-thiogalactopyranoside) at 37°C for 3 hours. 
Cells were harvested and analyzed in SDS-PAGE denatur-
ing gel.

3.6. Immunological Assays
The bacteria expressing MBP-BMK recombinant protein 
was boiled in SDS-PAGE sample buffer and loaded in mul-
tiple wells of a 10% polyacrylamide gel. After electropho-
resis, the proteins were revealed through immersion of 
the gel in cold 0.1M KCl and a horizontal strip of the gel 
containing the recombinant protein was cut to use it for 
immunization of mice. The gel strip was homogenized 
in a mortar and resuspended in complete Freund’s ad-
juvant and mixed by vigorous vortexing at room tem-
perature for 2-3 minutes. Two hundred µl of this mixture 
containing the recombinant protein was injected intra-
peritoneally to four female mice. Injections were repeat-
ed three time intervals (14, 28 and 42 days) by incomplete 
Freund’s adjuvant. To determine the production of anti-
body against BMK protein, the blood was collected 1 week 
after the last injection by cutting the end of animals’ tails.
Sera was separated from the coagulated blood and tested 
by Western blotting and dot blotting, against the recom-
binant protein and the crude venom separated from 
Iranian scorpion M. eupeus (obtained from the reference 
laboratory of the Razi Institute), respectively.

4. Results

4.1. Cloning Full-length Fragments Into Expression 
PMAL-c2x

Amplification of the BMK gene from M. eupeus was car-
ried out by RT-PCR using BMK-F and BMK-R primers as 
described in materials and methods. After the analysis of 
PCR products, a fragment corresponded to the expected 
PCR product size 273 bp. The PCR product was purified 
and used as template for cloning into an expression vec-
tor. BMK fragment of α-toxin was digested with BamHI 
and HindIII. The DNA was subsequently cloned in-frame 
into BamHI and HindIII sites of expression vector pMAL-
c2x. The presence of 273 bp insert DNA fragment was 
shown after restriction enzyme digestion (Figure 1).

Analyzing toxin 3D structures are important because 
toxin function is related to its structural folding. Inclu-
sion of 3D structural information to toxin sequence 
analysis facilitates identification of residues that are im-
portant for structure and function. According to the mo-
lecular model of BMK (MeNaTxa-4), the fold of the poly-
peptide chain is similar to that of the other long-chain 
toxins. The protein spatial organization was visualized by 
its 3D-structure model (Figure 2).

According to the computational model, the peptide 
is folded into one α-helical conformation and a three-
stranded anti-parallel β-sheet. The α-helix is linked to 
β-strand3 by two disulfide bridges, Cys41-Cys65 and 
Cys45-Cys67. The loop between β-strand1 and the α-helix 
is linked to the core of the molecule by a third disulfide 
bridge, Cys35-Cys55. The fourth disulfide bridge, Cys31-
Cys82, links the loop to the C-terminus (Figure 2). It is sug-
gested that the pharmacological versatility displayed by 
different groups of long chain neurotoxins might have 
been achieved along evolution via structural reconfigu-
ration of the C-tail. The expression system pMAL-c2x facilitates the production of target protein by fusing it to MBP in the plasmid as detailed in Materials and methods. In our experiment the soluble fraction of MBP-BMK fusion protein was expressed in the *E. coli* BL21 heterologous system and tested by SDS-PAGE (Figure 3).

The figure clearly shows the presence of an intense protein band with the molecular weight of 51.337 kDa expected for MBP-BMK (9.337 kDa of BMK and 42 kDa of MBP). Thus we concluded that BMK was produced in heterologous *E. coli* system in soluble and functional form. The results also indicated that this type of expression system is particularly well adapted to disulfide rich membrane proteins.

4.2. Immunodot Blot Assay

Western blot analysis with the anti-scorpion neurotoxin antibodies obtained from mice showed that the expressed recombinant BMK occupied the expected position (Figure 4). In the first two lanes, which corresponded to pMalc2x before and after induction with IPTG, no visible protein bands were observed that could attribute to MBP. Whereas a strict protein band was detected in lane 3 corresponding to the recombinant protein MBP-BMK.

It is considered that animal antisera have a number of antibodies reacting with *E. coli* proteins, which may create pseudo-reactions. However, in our study the reaction is likely related to the antibodies raised against BMK and not MBP, since antibodies elicited against MBP had been removed from the antiserum by treating with *E. coli* lysate. Therefore, we concluded that the serum obtained from the immunized mice contains antibodies, which are able to recognize the recombinant BMK protein. Thus, the produced recombinant protein was found to be immunogenic. It is clear that handling, transportation and milking of animals alive always poses some risks, especially when dealing with dangerous species for humans, as it is currently done nowadays (12, 13). In order to determine whether the antiserum obtained from mice can react with native BMK toxin from *M. eupeus* crude venom, an immunodot assay was performed (Figure 5).

The resulted reactions demonstrated that the serum of immunized mice can also react with scorpion crude venom, which corroborated the result that anti-BMK antibodies are able to recognize BMK toxin from the Iranian *M. Eupeus* scorpion. Thus, we produced a recombinant BMK toxin, which was found to be immunogenic, and antibodies produced against recombinant BMK can react with both recombinant and crude venom BMK neurotoxin thereby opening new prospects for the treatment of envenomation. Since this type of peptide contains 4 disulfide bridges, it is expected that the heterologous expression might fold the peptide with possible distinct disulfide arrangements. The variability generated by these various disulfide arrangements should be investigated whether it could present any advantage for the production of neutralizing antibodies.
5. Discussion

Following successful construction and planning, unique BMK gene was isolated, expressed and characterized. The results showed that the alpha toxin gene could be cloned into pMAL-c2x and transformed into E. coli DH5α, and a special E. coli host cells BL21. The bacteria that were used have rare tRNAs as host cell for protein expression. Previous studies have shown that this type of expression system is particularly well adapted to disulfide rich proteins. It has been used to express erabutoxin and BotXIV insect specific toxin of the scorpion *Buthus occitanus tunetanus* (14). Hence, it seems that the entire recombinant product expectedly goes to the inclusion bodies. The result showed that recombinant host cell could express recombinant MBP-BMK protein 51.337 kDa (9,337 kDa BMK and 42 kDa MBP). The fact that this strain produces the recombinant protein as inclusion bodies could be due to the experimental conditions used. The protein that was expressed at 37°C was insoluble, as similarly found in reference (15). Therefore, recombinant BMK gene in pMAL-c2x was transformed into this E. coli strain BL21. After induction, the recombinant E. coli expressed the protein in both soluble and non-soluble fractions. In order to produce higher level of soluble protein, the temperature for bacterial culture was reduced and the result showed that 37°C was appropriate. The soluble recombinant MBP-BMK was expressed. The protein, which was found to be immunogenic, reacted with antibodies against the recombinant MBP-BMK protein. Immunized mice with the recombinant MBP-BMK protein produced an adequate level of anti-sera titer, which was able to recognize not only the pure toxic peptide but also the soluble venom. The western blot and dot blot analyses with anti-scorpion neurotoxin polyclonal antibodies showed that the expressed BMK protein located in the expected position. Scorpion α-toxins constitute a family of peptide modulators that induce a prolongation of the action potential of excitable cells by inhibiting voltage-gated sodium channel (VGSC) inactivation (16). Therefore, according to the previous studies we also found that the BMK gene is a new member of α-toxin from the Iranian scorpion *M. eupeus*. It belongs to the long chain scorpion Toxin-3 superfamily. Regarding all known toxins in this family, MeNaTx-e-4 is sodium channel inhibitor with four conserved disulfide bridges.

These results are quite important, because they open the possibility to scale up the production of the recombinant peptide for immunization purposes using horse (or sheep). This allows production in bigger amounts of anti-sera titer, which was able to recognize not only the pure toxic peptide but also the soluble venom. The western blot and dot blot analyses with anti-scorpion neurotoxin polyclonal antibodies showed that the expressed BMK protein located in the expected position. Scorpion α-toxins constitute a family of peptide modulators that induce a prolongation of the action potential of excitable cells by inhibiting voltage-gated sodium channel (VGSC) inactivation (16). Therefore, according to the previous studies we also found that the BMK gene is a new member of α-toxin from the Iranian scorpion *M. eupeus*. It belongs to the long chain scorpion Toxin-3 superfamily. Regarding all known toxins in this family, MeNaTx-e-4 is sodium channel inhibitor with four conserved disulfide bridges.

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