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Molecular cloning and functional identification of analgesic peptides from *Buthus martensii* Karsch

[*Buthus martensii* (Karsch)'deki analjezik peptidlerinin moleküler klonlanması ve fonksiyonel tanımlanmaları]*

Jianhua Shao[1],
Chunchao Zhao[1],
Changye Hui[2],
Rui Zhao[1],
Xiaoyang Ruan[3]

[1]Yangzhou University, College Of Bioscience And Biotechnology, Yangzhou Jiangsu Province, Pr China
[2]Shenzhen Prevention And Treatment Center For Occupational Disease, Shenzhen, PR China
[3]Health Science Research, Mayo Clinic, Rochester, Mn, USA

Yazışma Adresi
[Correspondence Address]

Jianhua Shao

Yangzhou University, College Of Bioscience And Biotechnology, Yangzhou Jiangsu Province, PR China
Tel. 8618252783535
E-mail. sjhsjh9@yahoo.com.cn

*** Translated by [Çeviri]** Ebru Karabal

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ABSTRACT

Aim: To clone genes encoding analgesic peptides from the cDNA pool of scorpion telson, to obtain recombinant peptides by prokaryotic expression system and examine their analgesic activity in mouse twisting test.

Material and methods: The genes encoding analgesic peptides were cloned from the cDNA pool of scorpion telson by nested PCR. Positive clones were sequenced after screened by PCR-SSCP. The recombinant peptides were obtained by functionally expression in *E. coli* and purified by metal chelating chromatography. The bioactivity was assayed in mouse twisting test.

Results: Two nucleotide sequences encoding potential analgesic peptides were obtained. They were named as BmK 22 and BmK 9. BmK 22 was a new peptide with only one amino acid at site 54 different with BmK 9. In mouse-twisting test, both of the two recombinant peptides exhibited analgesic activity, and BmK 9 showed a stronger activity in pain relieving. Further, when considering structure factors by homology modeling, we speculated that the Arg residue at site 54 of BmK 9 may play an important role in target recognition and influence the analgesic activity. **Conclusion:** Venoms from scorpions contain extremely rich bioactive peptides. The strategy in this paper involving molecular cloning, functional expression and bioactivity identification of BmK 9 and BmK 22 provided a rapid route to discover scorpion toxins with special bioactivity such as analgesics.

Key Words: *Buthus martensii* Karsch, cDNA cloning, analgesic activity, SSCP, homology modelling

Conflict of Interest: There is no conflict of interest in respect of this manuscript.

ÖZET

Amaç: Analjezik peptidleri kodlayan genlerin akrep iğnesinin cDNA havuzundan klonlanması, prokaryotik ekspresyon sistemi aracılığıyla rekombinant peptidlerin elde edilmesi ve peptidlerin analjezik aktivitelerinin fare kıvranma deneyi ile incelenmesi.

Metodlar: Analjezik peptidleri kodlayan genler, akrep iğnesinin cDNA havuzundan nested PCR ile klonlandı. PCR-SSCP (PCR-Tek Zincir Konformasyon Polimorfizmi) ile tarandıktan sonra, pozitif klonlar sekanslandı. Rekombinanat peptidler, *E. coli*'de fonksiyonel ekspresyon ile elde edilip metal şelatlaştırıcı kromatografi ile saflaştırıldı. Biyo-aktivite fare kıvranma deneyi ile belirlendi.

Bulgular: Potansiyel analjezik peptidleri kodlayan 2 nükleotid sekansı elde edildi. BmK 22 ve BmK 9 olarak adlandırıldılar. Yeni bir peptid olan BmK 22'nin, BmK 9'dan sadece 54. pozisyondaki bir amino asidi farklıydı. Fare kıvranma deneyi sonucunda, her iki rekombinant peptid analjezik aktivite gösterdi ve BmK 9'un ağrı azaltmada daha kuvvetli bir etkisi vardı. Ayrıca, homoloji modelleme ile yapısal faktörlerin değerlendirilmesi sonucunda, BmK 9'un pozisyon 54'ündeki arjininin hedef tanımada önemli bir rol oynadığını ve analjezik aktiviteyi etkilediğini düşünüyoruz.

Sonuç: Akrep zehri çok zengin biyo-aktif peptidler içermektedir. Bu çalışmadaki BmK 9 ve BmK 22'nin moleküler klonlama, fonksiyonel ekspresyon ve biyo-aktivite belirlenmesi aşamaları, analjezikler gibi özel biyo-aktiviteye sahip akrep toksinlerinin belirlenmesi için hızlı bir yöntem sağlamaktadır.

Anahtar Kelimeler: *Buthus martensii* (Karsch), cDNA klonlama, analjezik aktivite, tek zincir konformasyon polimorfizmi, homoloji modelleme

Çıkar Çatışması: Hiçbir çıkar çatışması yoktur.

Introduction

Scorpions have survived successfully over millions of years without detectable changes in their morphology. Instead, they have developed an efficient gland generating a large variety of toxic polypeptides to support their needs for prey and defense [1]. Their venoms contain a diversity of neurotoxins, which represent a tremendous hitherto partially unexplored resource not only for understanding ion channels but also for use in drug design and development [2]. In China, the scorpion, *Buthus martensii* Karsch, has been a key constituent of Chinese traditional medicines for thousands of years since the Song Dynasty. It was clinically used to treat all sorts of pains and no addiction was found [3].

So far, more than 10 kinds of analgesic peptides had been separated and identified from the venoms of *Buthus martensii* Karsch by chromatographic technique (Fig. 1) [4]. The conventional project to get analgesic peptides from scorpion venoms was fractionation and purification by chromatography guided by bioactivity assay, while the rare venom existing in nature and the large quantity required for bioassay restricted the discovery of new active peptides [5-7]. It is also remarkable to note that the venom of a single scorpion species contains a large number of closely related toxins, so the isolation and purification of sufficient toxins from scorpion venoms remains to be difficult [8]. Screening cDNA library of the scorpion telson combined with peptide synthesis or recombinant peptide production techniques is actually a rapid and efficient strategy for molecular dissection of scorpion venoms.

In this work, based on the sequence homology analysis of amino acids and nucleotide sequences of known analgesic scorpion peptides, the cDNAs encoding two new analgesic peptides were isolated from the venom gland cDNA pool of the Chinese scorpion *Buthus martensii Karsch*. Both of them were functionally expressed in *E. coli*, and showed analgesic activity in mouse twisting test. By homology modeling and 3D structure comparison, we speculated that the arginine at position 54 may be essential to analgesic activity. The strategy in this paper provided a rapid route to discover and investigate scorpion toxins with value as therapeutics. Further as the result of natural selection and accelerated evolution, active peptides in scorpion venoms with same pharmacological function but different strength provided sequences and structures information for the research of structure and function relationships.

Materials and methods

Strains, plasmids, materials and animals

Plasmids pGEM-T vector and pSYPU, E.coli strains JM109 and BL21 (λDE3) were kept in our laboratory. Restriction endonuclease, T4 DNA ligase and DNA polymerase were obtained from TaKaRa (Japan). Scorpions were presented by Wuhan hope star breeding technology Co. The mice used for analgesic activity assay were Kunming mice (Specified pathogen-free level) from the Institute of Military Medical Science Center for Experimental Animals. All animal protocols were approved by the Institutional Animal Care and Use Committee.

Construction of cDNA pool, gene cloning and sequencing

A cDNA pool was constructed from total RNA extracted from a single telson of a *Buthus martensii* Karsch scorpion [2]. The consensus regions of the known analgesic peptides were determined after base sequences comparison. Then the foreword primers were designed. The gene encoding the potential analgesic peptides was amplified by nested PCR. The procedures of first PCR were performed by using oligo dT(17) and a gene-specific primer (5′-tgctgcttttccagaaaattcc -3′), the 5` non-coding region of the consensus region of first three analgesic peptides (Fig. 1), as primers. The PCR conditions is a "touch down" procession involving predenaturation at 94 °C for 5 min, two-step cycles of denaturation at 94 °C for 1 min, the first-step cycles annealing for 1 min from 60 °C to 50 °C (decrease 1 °C

```
                    10        20        30        40        50        60        70        80
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
BmK AGAP II     --VKDGYIVDD-KNCAYFCGR-NAYCDDECEKNG-AESGYCQWAGVYGNACWCYKLPDKVPIR-----VPGRCNG------
BmK AGAP        --VRDAYIADD-KNCAYFCGR-NAYCDDECKKNG-AESGYCQWAGVYGNACWCYKLPDKVPIR-----VPGKCNGG-----
BmK αTx11       --VKDGYIADD-RNCPYFCGR-NAYCDGECKKNR-AESGYCQWASKYGNACWCYKLPDDARIM-----KPGRCNGG-----
BmK AngM1       --VRDAYIAKP-ENCVYECGI-TQDCNKLCTENG-AESGYCQWGGKYGNACWCIKLPDSVPIR-----VPGKCQ-------
BmK dITAP3      ----DGYIRGS-NGCKVSCLWGNEGCNKECRAYG-ASYGYCWTWG---LACWCEGLPDDKTWK----SESNTCGGKK----
BmK IT2         ----------K-SGCRVACLIGNQGCLKDCRAYG-ASYGYCWTWG---LACWCEGLPDNKTWK----SESNTCG-------
BmK AS-1        -KADNGYLLNKYTGCKIWCVINNESCNSECKLRR-GNYGYCYFW----LACYCEGAPKESLWA----YETNKCNGKM----
BmK AS          VKTDNGYLLDKYTGCKVWCVINNESCNSECKIRG-GYYGYCYFWK---LACFCQGARKSELWN----YNTNKCNGKL----
BmK I4          -----GYAVDS-SGKVAECLP-NNYCNNECIKVYYADKGYCCLL-----KCYCFGLADDKPVLDIWDSTKNYCDVQII---
BmK IT-AP       --KKNGYAVDS-SGKVAECLF-NNYCNNECTKVYYADKGYCCLL-----KCYCFGLADDKPVLDIWDSTKNYCDVQIIDLS
BmK AngP1       --KKNGYAVDS-SGKVAECLL-NNYCNNICTKVYYATSGYCCLL-----SCYCFGLDDDKAVLKIKDATKSYCDVQII---
```

Figure 1. Primary sequences alignments of BmK analgesic peptides known by Clustal W algorithm

per cycle) and then annealing at 55 °C for the second-step cycles, all elongation at 72 °C for 1.5 min with 25 more cycles, and terminated by a 72 °C for 5 min elongation. After amplification, the PCR product was purified for next PCR. The second nested PCR reaction was carried out by using specific oligonucleotide primers (F: 5`-ctattccatatggttcgggatgcttatattgccaag-3`, and R: oligo dT(17) as second round primers, and the PCR condition were performed as predenaturation at 94 °C for 5 min, cycles of denaturation at 94 °C for 1 min, annealing for 30 sec at 45°C, elongation at 72 °C for 1 min with 25 more cycles, and terminated by a 72 °C for 5 min elongation. Electrophoretic separated PCR products were cloned into the pGEM-T vector (Promega) and transformed into *E. coli* Competent cells JM109 (TaKaRa).

Single-strand conformation polymorphism (SSCP) and DNA sequencing

56 positive clones were screened by the PCR-SSCP (polymerase chain reaction-single-strand conformation polymorphism) as described by Hayashi [9] before being sequenced. Briefly, after PCR amplification, 4 µl of every PCR product (the recombinant plasmids as templates) was mixed with 8 µl of loading dye (98% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). The samples were denatured at 95 °C for 10 min and immediately chilled in ice for 5 min. Then, 2 µl of the above samples were electrophoresed through a non-denaturing 5% polyacrylamide and 0.25% agarose composite gel containing 3% glycerol. Electrophoresis was performed at 250 V for 18-20 h at 8 °C. Positive clones with different electrophoretic bands were sequenced on an ABI PRISM 373 instrument (Applied Biosystems). The nucleotide sequences obtained in this work were deposited in GenBank under accession numbers DQ981785 and DQ981786. The nucleotide sequences and deduced amino acid sequences alignment was performed by BioEdit [10].

Construction, expression and purification of BmK 9 and BmK 22

The gene fragments encoding the mature peptide of BmK 9 and BmK 22 were subcloned into expression plasmid pSYPU, respectively. The recombinant BL21 (λDE3) cells, and positive clones were identified via clony PCR screening and confirmed by DNA sequencing with T7 primers. The construction procedure was performed as described elsewhere [11]. Take BmK 9 for example, the purification procedure of the recombinant peptides was indicated. *E. coli* BL21 (DE3) cells harboring expression vector pSYPU-BmK 9 were grown in LB medium. By using ultrasonic waves to break the cell, the supernatant of the lysate was loaded on to an Ni-NTA agarose column previously equilibrated with buffer A (0.1 M, Na2HPO4/NaH2PO4, pH 8.0). The column was thoroughly washed by each wash buffer, at a flow rate of 1.0 ml/min. (buffer 1: 8 M urea in 0.1 M, pH 8.0 phosphate buffer, buffer 2: 8 M urea in 0.1 M, pH 6.0 phosphate buffer, buffer 3: 8 M urea, in 0.1 M, pH 4.0 phosphate buffer). After removal of urea by gradient elution with buffer A, the recombinant BmK 9 was eluted by buffer B (0.5 M imidazole, pH 9.0) and was desalted and concentrated by ultra-filtration. Each of the elution fractions were analyzed on 15% SDS-PAGE and stained with Coomassie Brilliant Blue R-250. The protein concentration was determined as described by Bradford with bovine serum albumin as a standard.

Analgesic activity

The mouse-twisting assay was carried out as described by Fennessy and Lee [12]. Mice (Kunming mice), 18-20 g, were injected intraperitoneally with 0.2 ml 0.6% acetic acid per 20 g body weight to induce extensive and long-lasting pain in their internal organs. In response, the mice keep twisting their bodies, so that the twisting actions could be counted to reflect the intensity of the pain. To perform the bioassay, 0.2 ml toxin solutions were injected into the tail vein of the mice, using 0.9% NaCl as a control. Twenty minutes later, 0.2 ml 0.6% acetic acid solution was then injected intraperitoneally. Five minutes later, the number of twisting actions was counted for 10 min. A pain-killer, morphine, was used as a control.

Molecular modeling

The tertiary structures of BmK 9 and BmK 22 were constructed by the homology modeling method using the crystal structure of homologous protein. The first step is to search for a number of related sequences to find a related protein as a template using SWISS-MODEL templates (http://swissmodel.expasy.org/). BmK M4 (PDB entry: 1sn4A) having a close sequence identity to the target sequence with a known three-dimensional structure, was selected for homology modeling. The 3D structure model of BmK 9 and BmK 22 was examined using a Swiss-Pdb viewer 4.01. [13].

Results

Cloning of analgesic peptides

By two circles of PCR-SSCP screen, the fifty six positive clones were divided into two groups according to their different electrophoresis behaviors (Fig. 2). Three clones from each group were selected and sequenced randomly. Two kinds of gene fragments were identified to encode two potential analgesic peptides: BmK 9 and BmK 22. BmK 22 was a new peptide with only one amino acid at site 54 different to BmK 9 (Fig. 3). The cDNA sequences of BmK 22 were submitted to GenBank with the accession number ABL62807.

Functional expression and purification

The gene of BmK 9 and BmK 22 were cloned into pSYPU and expressed in *E.coli* in soluble form, respectively. The solubility of product was greatly increased in the vector

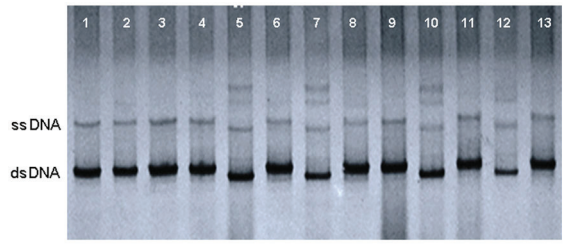

Figure 2. The electrophoresis patterns of PCR-SSCP from different clones. Among which the clones of 1, 2, 3, 4, 6, 8, 9, 11, 13 were identical and coded for BmK 9, and the sequences of clones 5, 7, 10, 12 coded for a new peptide BmK 22.

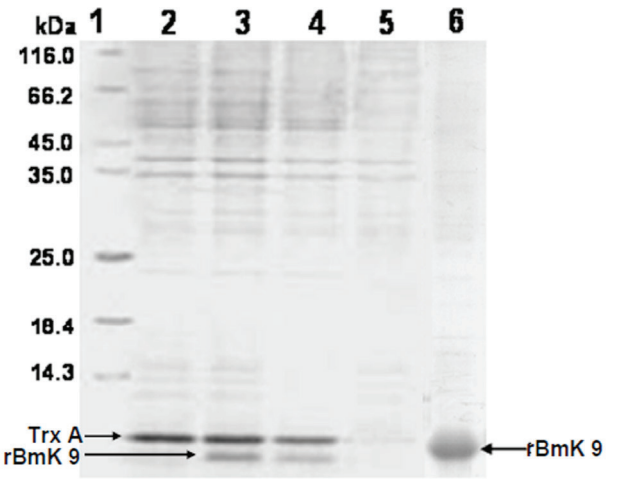

Figure 4. Coomassie Bright Blue R250-stained SDS-PAGE gel (15%) of the expression and purification process of BmK 9. Lane 1: a low molecular weight marker; Lane 2: IPTG induced pSYPU expressed in *E.coli* BL21(DE3); Lane 3: IPTG induced pSYPU-BmK 9 expressed in *E.coli* BL21(DE3); Lane 4: the supernatant part of cell lysate of *E.coli* BL21(DE3) containing pSYPU-BmK 9; Lane 5: the precipitation part of cell lysate; Lane 6: the purified BmK 9

of pSYPU due to co-expression of soluble thioredoxin (Trx A) in an unfusion form, which is required for cytoplasm disulfide bond formation in *E. coli* strain. For simplicity, the purification and detection of BmK 9 was used as an example. After expressed in *E. coli* BL21 (λDE3), 90% of the recombine proteins were in soluble form. The recombinant proteins were isolated from soluble fractions and purified to homogeneity on a Nickel metal chelating affinity column. The elution fractions were analyzed by 15% SDS-PAGE. The theory molecular weights of recombinant BmK 9 and Trx A are 10.8 kDa and 11.7 kDa, respectively, as shown in Fig. 4.

Analgesic activity of BmK 9 and BmK 22

Analgesic activity tests were carried out at a dose of 0.14 μmol/Kg and showed that BmK 9 had a stronger analgesic activity than BmK 22 in the mouse twisting test. And both peptides showed stronger analgesic activity than positive control morphine at experimental dose. (Table 1)

The homology modeling of BmK 9 and BmK 22

By homology modeling, BmK 9 and BmK 22 had molecular scaffold common to most long-chain scorpion toxins comprising of an α-helix and a three-stranded antiparallel β-sheet, resulting in a compact core and several loops and γ-turns extending outside. The structural scaffold of the scorpion toxins is highly conserved and is made rigid by a set of intramolecular disulfide bonds. The amino acid at site 54 was on the γ-turn jointing the second and third β-sheets and extended outwards (Fig. 5).

```
BmK 9     1 GTTCGGGATGCTTATATTGCCAAGCCCGAAAACTGTGTATACCATTGTGCTACAAATGAAGGTTGCAAC
BmK 22      ............................................................

BmK 9     1  V  R  D  A  Y  I  A  K  P  E  N  C  V  Y  H  C  A  T  N  E  G  C  N
BmK 22       .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .

BmK 9    70 AAATTATGTACTGACAATGGTGCTGAGAGTGGCTATTGCCAATGGGGAGGTAGATATGGAAATGCCTGC
BmK 22      ............................................................

BmK 9    24  K  L  C  T  D  N  G  A  E  S  G  Y  C  Q  W  G  G  R  Y  G  N  A  C
BmK 22       .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .

BmK 9   139 TGGTGCATAAAGTTGCCCGATAGAGTACCGATTAGAGTACCAGGAAAATGTCATCGCTGA
BmK 22      ......................T.................................

BmK 9    47  W  C  I  K  L  P  D  R  V  P  I  R  V  P  G  K  C  H  R  *
BmK 22       .  .  .  .  .  .  .  S  .  .  .  .  .  .  .  .  .  .  .
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Figure 3. Nucleotide sequences and amino acid sequences of BmK 9 and BmK 22. The differences in nucleotides and amino acids of BmK 22 to BmK 9 were highlighted in red.

Table 1. Analgesic activity of BmK 9 and BmK 22

Groups	Dose (μmol/Kg)	Twisting times ± SD	Inhibition efficiency
Normal saline		43.5 ± 2.2	
BmK 9	0.14	26.7 ± 2.8	61.4%
BmK 22	0.14	22.8 ± 3.5	52.3%
Morphine	3.51	19.1 ± 1.5	44.1%

The analgesic activity of BmK 9 and BmK 22 assayed in mouse-twisting model. The inhibition efficiency is the ratio of $(T_0\text{-}T)/T_0$, in which T_0 is the mean twisting times of control group and T is the mean twisting times of experiment groups with BmK 9, BmK 22 and the pain killer morphine. (Sample size: 10)

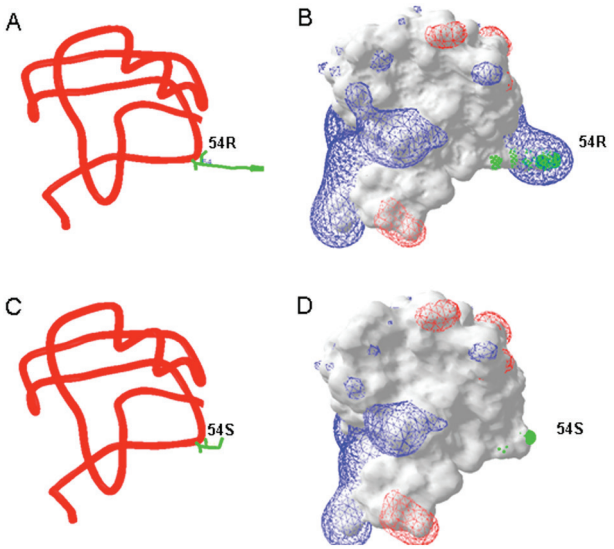

Figure 5. Homology models of BmK 9 and BmK 22. **A.** Homology model of BmK 9, the side chain of Arg at site 54 is colored in green. **B.** Surface model and the electrostatic potential map are also represented. Electronegative and electropositive parts are colored by blue and red, respectively. **C.** Homology model of BmK 22, the side chain of Ser at site 54 is colored in green. **D.** Surface model and the electrostatic potential map are also represented. Electronegative and electropositive parts are colored by blue and red, respectively.

Discussion

The interest of our group had been focused on the separation and isolation of toxins with pharmacological activity from the venom of *Buthus martensii* Karsch. Several analgesic peptides had been identified by traditional chromatography, such as BmK AGAP **[14]**, BmK AS [15], BmK AGP-SYPU1 **[**16] and BmK AGP-SYPU2 [17] and so on. While the fact that limited availability of naturally occurring toxins in crude venom was the main obstacle to pharmacological testing. The strategy in this paper involving the target gene clone, recombinant peptide production techniques accompany with pharmacologic assay is actually rapid and efficient for exploring pharmaceutical peptides from animal venoms. Here, we reported the gene cloning, functional expression and analgesic activity of two scorpion peptides BmK 9 and BmK 22.

cDNA libraries were increasingly being used for high-throughput interrogation of animal venoms. In this paper we modified traditional methods by fusion nested PCR, touchdown PCR and PCR-SSCP. Nested PCR was used to amplify the low abundance genes and decrease the mutation rate during amplification. In the touchdown PCR, the initial higher annealing temperature leaded to greater specificity for primer binding, while the lower temperatures permitted more efficient amplification at the end of the reaction. By nested PCR, we amplified target fragments coding for potential analgesic peptides from the cDNA pool of scorpion *Buthus martensii* Karsch. The PCR-SSCP was a rapid and sensitive detection technology for point mutations and DNA polymorphism and had emerged as one of the most popular mutation detection strategies and now was widely using for this purpose **[18]**. By PCR-SSCP six different clones were picked out from the 56 positive clones and sequenced to be two cDNA sequences. PCR-SSCP used in this paper was aimed to improve efficiency of sequencing by screen out different clones.

Almost all scorpion long-chain neurotoxins have eight cysteines, forming a disulfide-knot motif. The redox state of the cytoplasm militates against the formation of disulfide bonds that are required for active scorpion toxins. Our strategy was to produce rBmK toxins as co-expression proteins with thioredoxin A in the form of non-fusion protein. TrxA generates a less-reducing cytoplasmic environment that facilitates disulfide bond formation and plays a key role in the solubility of rBmK toxins by slowing down the misfolding rate and stabilizing the folding intermediate. This expression system provides an easy way to purify the recombinant peptide and also allows for the production of a functional peptide without any refolding steps.

The α-toxins were the major components of old world scorpion venoms. They were long chain toxins containing 60–70 amino acid residues cross-linked by four disulfide bridges [19]. All 3D structures of long chain scorpion toxins reported today showed a common molecular scaffold comprising an α-helix and a three-stranded β-sheet. There are three major functional domains in α-toxins: the first three N-terminal residues; the five-residue turn in combination with the C-tail and

the loop between the β2 and β3 sheets [20-22]. However they exhibited various biological activities which may be determined by the variation of the amino acids on the molecular surface. This kind of phenomena can also be seen in phospholipase A2 enzymes [23]. Several studies on relationships of structure and analgesic activity of scorpion analgesic toxins are reported recently and shed some light on the structural and functional anatomy. They were BmK AGAP [24-26], BmK AS [27] and BmK AGP-SYPU2 [28]. As far as the only one difference residue in site 54 (Ser of BmK 22 and Arg of BmK 9) was concerned, by molecular modeling, this amino acid was just on the surface of C-terminus, and extended outside. The analgesic activity of BmK 9 with a positive charged Arg at site 54 was stronger than BmK 22 with a Ser at the site 54.

In conclusion, two analgesic peptides BmK 9 and BmK 22 were reported here, the positive charge at site 54 would enhance the analgesic activity. While the properties of functional domain and their mode of interaction with the channel remained to be described at molecular level, both in terms of the three-dimensional structure and functionality.

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Conflict of Interest: There is no conflict of interest in respect of this manuscript.

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