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Purification, cDNA cloning and function assessment of BmK abT, a unique component from the Old World scorpion species[1]

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Abstract A new neurotoxic component named BmK abT was purified from the venom of Chinese scorpion *Buthus martensi* Karsch. The molecular weight of BmK abT was determined to be 7212 Da on a mass spectrum. The minimum lethal dose of BmK abT was tested to be about 1.5 μg per mouse by intracerebroventricular injection, and the dose induced significant paralysis effect on cockroach was about 5 μg by i.p. injection. The partial amino acid sequence indicated that it was a distinctive polypeptide in the scorpion neurotoxin family. Thereafter, the whole amino acid sequence of mature BmK abT was deduced from cDNA sequence by 5′- and 3′-rapid amplification of cDNA ends. Finally, it was defined to be composed of 63 residues with amidation at the C-terminal residue. By sequence comparison, BmK abT was found to be most similar to Ts VII, a β-toxin from the New World scorpion. The patch-clamp recording on DRG neurons, unexpectedly, showed this toxin could prolong the action potential and increase the amplitude of the peak Na^+ currents, which are the typical characters of α-toxin. These results suggested that BmK abT was a new toxic component found in the Old World scorpion species structurally similar to β-toxins, but functionally similar to α-toxins.

Key words: BmK abT; Sodium channel-ligand; cDNA sequence; Patch-clamp recording

1. Introduction

Most long-chain scorpion neurotoxic polypeptides composed of 60–70 amino acid residues have been extensively demonstrated to be voltage-gated Na^+ channel-specific ligands in mammals, insects and other vertebrates [1–3]. Therefore, they are considered to be powerful tools for understanding and correlating structure and mechanisms of voltage-gated Na^+ channel activation or inactivation. According to biological specificity in vivo and pharmacological and electrophysiological activity, these scorpion neurotoxins can be popularly divided into mammalian α- or β-neurotoxins and excitatory or depressant insect-selective neurotoxins [4–6].

The Asian scorpion *Buthus martensi* Karsch (BmK) is a species widely distributed from northwestern China to Mongolia and Korea. The venom of this species is considered to be a rather active toxin from the Buthidae family [7]. Up to now, at least 10 long-chain Na^+ channel-specific ligands and seven short-chain K^+ channel-blocking peptides have been isolated and characterized from the venom of BmK [8–12], implying BmK venom is a rich source of various active peptides. Here we describe the purification, cDNA cloning and function assessment of BmK abT, a novel component from the venom of BmK.

2. Materials and methods

2.1. Isolation and purification

The venom of BmK, collected by electrical stimulation, was purchased from a local scorpion farm in Suzhou, Jiangsu Province, China. About 0.2 g crude venom was dissolved in 1 ml of 2 M acetic acid solution, and then centrifuged at 12 500 rpm for 25 min at 4°C. The supernatant was filtrated on Sephadex G-50 column (2.5×120 cm), followed by chromatography on DEAE-Sephadex A-50 column (2×100 cm). The purification was performed by repeated reverse-phase HPLC (Waters Model 510, USA) with a Cosmosil C_8 column (4.6×150 mm, Nakarai Chemicals, Japan) until a single peak was achieved.

2.2. Determination of purity and molecular weight (MW)

The purity and MW of the component were assessed by a Finnigan LCQ electrospray iontrap mass spectrometer (Finnigan Corporation, USA) coupled with HPLC.

2.3. Toxicity assays

According to methods described by Ji et al. [10], toxicity was assayed on 18±2 g Kunming white mice by a intracerebroventricular (i.c.v.) injection with a volume of 5 μl modified Harreveld solution, the minimum lethal dose was determined during 12 h after injection. In addition, cockroaches, *Periplaneta americana* (0.8–1.0 g), were tested by injection of 2.5 μl solution into the abdominal segment. Three to five insects were taken for each dose. The paralysis response in insects was scored during 24 h after injection.

2.4. Electrophysiological recording

Experiments were performed on neurons acutely isolated from dorsal root ganglia (DRG) of adult rats. Adult male Sprague-Dawley rats were killed, and DRG were rapidly dissected, minced and dissociated by incubation in trypsin III (0.75 mg/ml, Sigma), collagenase 1A (1.25 mg/ml, Sigma) and DNase IV (0.125 mg/ml, Sigma) in Dulbecco's modified Eagle's medium at 35°C for 1 h. The enzymatic treatment cells were then isolated by gentle pipetting in normal extracellular solution. Whole-cell patch-clamp and current-clamp recording were carried out at room temperature with an EPC-9 amplifier, and operated by Pulse+PulseFit software (both from Heka Elektronik, Lambrecht, Germany). Membrane currents usually were filtered at 5 kHz and sampled at 20 kHz. The normal external solution contained (in mM): 150 NaCl, 5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 D-glucose. The facilitated sodium current external solution con-

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[1] The cDNA sequence of BmK abT has been deposited in GenBank under accession number AF241269.

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tained (in mM): 50 choline-Cl, 65 NaCl, 20 TEACl, 5 KCl, 5 $MgCl_2$, 0.01 $CaCl_2$, 5 glucose, 5 HEPES. In voltage-clamp experiments, the patch-pipette (internal) solution contained (in mM): 140 CsCl, 0.5 $CaCl_2$, 3 Na-ATP, 0.1 leupeptin, 10 HEPES, 5 EGTA, 1 $MgCl_2$. All solution pH were adjusted to 7.4.

2.5. Determination of amino acid sequence

Determination of the amino acid sequence was performed by Edman degradation on a protein sequencer (Model 477A, Applied Biosystems, USA).

2.6. Preparation of total RNA from venom glands

1 g fresh telsons containing venom glands of BmK scorpion were homogenized in Trizol reagent (Promega, USA). Total RNA was extracted according to the instructions indicated by the supplier.

2.7. Reverse transcription and 3′-rapid amplification of cDNA ends (RACE)

5 μg total RNA was taken to convert mRNA into cDNA using Superscript II reverse transcriptase (Gibco BRL, USA) and a universal Oligo (dT)-adapter primer (5′-ATTGAAGCTTACGCG-TCGACTATA$(dT)_{18}$-3′). The synthesized cDNAs were used as templates in 3′-RACE. A degenerate primer 1 (5′-ATTAGGATCCAA-(A/G)AA(A/G)AG(T/C)GG(T/G/C)TA-3′) was designed corresponding to the N-terminal 1–5 residues (KKSGY) of mature toxin. The cDNA of mature toxin was amplified using primer 1 and an adapter primer (5′-ATTGAAGCTTACGCGTCGACTATATT-3′). PCR was performed on a PE-2400 (Perkin-Elmer, USA).

2.8. 5′-RACE

Based on the partial sequence determined by 3′-RACE, the antisense primers were designed and synthesized. Gene specific primer 2 of BmK abT (5′-AGGATCCGAACCCAATTATG-3′) corresponded to the 48–52 residue (HNWVP).

The first strand cDNAs synthesized in the previous step were purified on a Glassmax column (Gibco BRL USA), homopolymeric dC tails were then added to their 3′-ends by terminal deoxynucleotidyl transferase. The dC tailed cDNAs were first amplified with primer 2 and an abridged anchor primer (5′-GGCCACGCGTCGACTAG-TACGGGIIGGGIIGGGIIG-3′) complementary to the dC tails. In order to obtain a higher yield of the specific cDNAs, the first PCR product was diluted and used as a template for the second PCR amplification, with primer 2 and an abridged universal amplification primer (5′-GGGCACGCGTCGACTAGTAC-3′).

2.9. Cloning and sequencing of cDNA

The amplified PCR fragments were purified and directly ligated into pGEM-T easy Vector (Promega, USA), then transformed into DH-5α competent cells. The recombinant double-strand cDNA were purified and auto-sequenced on an ABI PRISM 377 DNA sequencer (Perkin-Elmer, USA) with T7 primer.

2.10. Comparison of sequence identity

Amino acid sequences of sodium channel-specific scorpion toxins were obtained from NCBI GenBank database, USA. VNTIsuite 5.5 software (Informax, USA) was used for detecting the sequence identity.

3. Results

3.1. Purification and identification of BmK abT

The supernatant of BmK venom was fractionated into at least five fractions upon Sephadex G-50 column as shown in Fig. 1A. The third fraction was separated into five further subfractions on a DEAE-Sephadex A-50 column (Fig. 1B). The subfraction 3-2 was purified by reverse-phase HPLC as shown in Fig. 1C. Finally, a polypeptide subfraction 3-2-4, named as BmK abT was obtained as a single peak by repeated reverse-phase HPLC (Fig. 1D). The purity of BmK abT was determined to be a single peak on mass spectrum with a precise MW of 7212 Da (Fig. 2). The toxicity assays showed that the minimum lethal dose of BmK abT was about 1.5 μg per mouse by i.c.v. injection, and a significant paralysis effect occurred at a dose of 5 μg in cockroach *P. americana* by i.p. injection. The first 40 residues from the N-terminal end of the toxin were identified by subjecting native BmK abT (about 0.2 nmol) to amino acid sequencing (shown in Fig. 3).

3.2. Effects of BmK abT on DRG sodium channels

Whole-cell patch-clamp recording revealed that peak sodium currents of DRG neurons ($n=7$) were significantly increased from −10 to 40 mV and the inactivation of the peak current was slowed (Fig. 4A.b) in the presence of BmK abT (10 μM). The effect could be partially reversed by perfusing cells with the control external solution (Fig. 4A.c). The current–voltage (I–V) relationship is shown in Fig. 4B.

The current-clamp recording showed that the action potential of DRG neurons ($n=4$) was prolonged after application of 10 μM BmK abT (Fig. 5), which indicates that BmK abT could slow the inactivation process of DRG sodium channels.

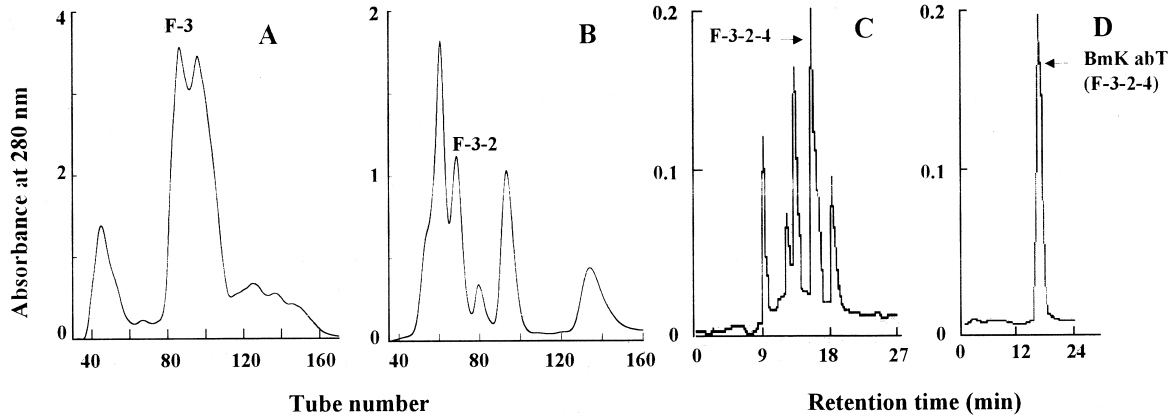

Fig. 1. Isolation and purification patterns of BmK abT. A: Chromatography of BmK venom (500 mg) on a Sephadex G-50 column (2.5×120 cm). The column was equilibrated followed with elution by 0.05 M ammonium acetate (pH 7.5). Flow rate: 12 ml/h, 5 ml/tube. B: Chromatography of the fraction F-3 (120 $OD_{280\ nm}$) on DEAE Sephadex A-50 (2×100 cm). The column was equilibrated followed with elution by 0.1 M ammonium acetate (pH 8.5). Flow rate: 12 ml/h, 4.8 ml/tube. C: HPLC profile of subfraction F-3-2: elution was performed by a linear pattern from 15% for 7 min, then by linear gradient pattern from 15 to 30% in 25 min, to 33% in 27 min. D: HPLC profile of subfraction F-3-2-4 (BmK abT). Elution was performed by a linear form from 15% for 7 min, then by linear gradient form from 15 to 31% in 21 min, to 32% in 23 min. Solvent A: 0.1% TFA. Solvent B: acetonitrile. Flow rate: 1 ml/min at 25°C.

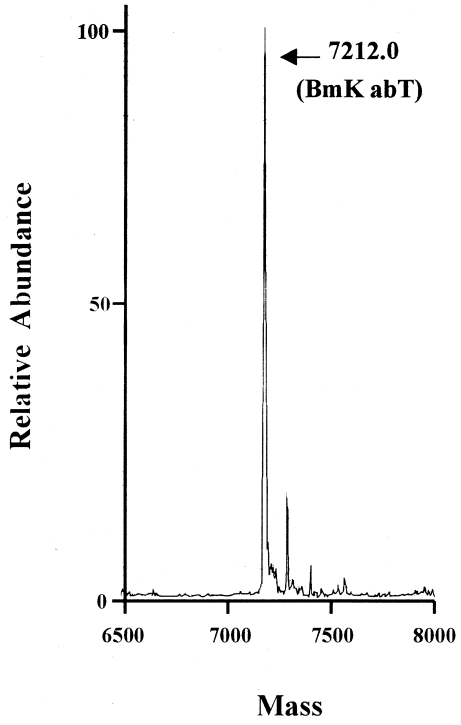

Fig. 2. The determination of purity and MW of BmK abT by mass spectrometry. The mass spectrometry showed BmK abT to be a single peak with a MW of 7212 Da.

3.3. Cloning and sequencing of BmK abT cDNA

The whole cDNA sequence of BmK abT was completed by overlapping 3′- and 5′-RACE results. Besides 249 bp of ORF, it extended 40 bp of the 5′-untranslated region (UTR) and the 3′-UTR up to the poly A tail. The 3′-UTR included a polyadenylation signal (AATAAA), located 35 bp downstream the stop codon (Fig. 3). A precursor composed of a signal peptide of 19 residues and a mature peptide of 64 residues was deduced from the cDNA sequence. However, the MW of the deduced mature peptide (7340.72 Da) disagreed with that of native BmK abT (7212 Da) on the mass spectrum, the latter seems to lack one amino acid residue.

3.4. Sequence comparison of BmK abT with other scorpion toxins

As shown in Fig. 6, BmK abT showed poor structural identity (30–35%) with some known toxins (BmK I, BmK IT and BmK IT2) from the same venom [8,10,13], and also very low similarity (20–30%) with many other scorpion toxins such as Css II, a typical β-type toxin [16]; Lqq V and AaH I, two α-type toxins [17,18]; CsE V and Ts IV-5, two α-type toxins from the New World [21,22]; Lqh α-IT and Lqq III, two α-like toxins [23,24]; Lqq IT1 and AaH IT1, two excitatory anti-insect toxins [14,19], but 40% structural identity with BmK AS, BmK AS-1 and Lqq IT2 [11,14], and unexpectedly, 46.2% identity with Ts VII, a distinctive β-type toxin from the New World scorpion species [15].

4. Discussion

In this study, a new active polypeptide named as BmK abT was purified from the venom of Chinese scorpion BmK. The mature BmK abT was finally defined to be composed of 63 amino acid residues by the analysis of cDNA sequence and mass spectrum. The MW experimentally determined was 7212, but the evaluation of the mass contributed by the mature toxin deduced from cDNA gave a value of 7340.72, thus the missing amino acid should have a mass of 146.72, and most possibly be Glu (147), Gln (146) or Lys (146). In the

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                                                                  -19
TATTGCAGTTCATCATAAAGTAACGAAAACTGACTTCACT ATG AAA GCA GCA CTT TTA TTG GTT ATT TTC
                                        + M   K   A   A   L   L   L   V   I   F
                                                -1                          10
TCA TTA ATG TTG ATA GGA GTC CTG ACT AAG AAA AGT GGC TAT CCA ACT GAT CAT GAA GGA TGC
 S   L   M   L   I   G   V   L   T   K   K   S   G   Y   P   T   D   H   E   G   C
                                    # K   K   S   G   Y   P   T   D   H   E   G   C
                            20                                          30
AAG AAT TGG TGC GTT TTA AAT CAT TCT TGT GGG ATA TTG TGT GAA GGT TAT GGA GGA TCT GGT
 K   N   W   C   V   L   N   H   S   C   G   I   L   C   E   G   Y   G   G   S   G
 K   N   W   C   V   L   N   H   S   C   G   I   L   C   E   G   Y   G   G   S   G
                        40                                          50
TAT TGC TAT TTT TGG AAA TTG GCA TGT TGG TGT GAT GAT ATT CAT AAT TGG GTT CCA ACT TGG
 Y   C   Y   F   W   K   L   A   C   W   C   D   D   I   H   N   W   V   P   T   W
 Y   C   Y   F   W   K   L   -   -   -   -   -   -   -   -   -   -   -   -   -   -
                    60
TCT AGA GCG ACC AAC AAA TGT CGT GCA AAA TAA TACTTCGATATATGTAACTTTTTAAATGAGTGCTAATAA
 S   R   A   T   N   K   C   R   A   K   *
 -   -   -   -   -   -   -   -   -   -

AATATATCACCACAAAAAAAAAAAAAAAAA
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Fig. 3. The cDNA sequence of BmK abT. +: The precursor amino acid sequence deduced from cDNA; #: the partial amino acid sequence (40 residues) determined by Edman degradation. The C-terminal residue Lys (italic) in the precursor would be cleaved off during post-translation modification. The signal peptide (−19 to −1) and a potential polyadenylation signal of AATAAA are underlined.

residues from residue 41 to the C-terminus, there was no Glu nor Gln, so the missing one could only be Lysine. As illustrated above, with respect to the similar case of many other scorpion toxins such as AaH II and Ts VII [20,15], the C-terminal Lys-64 was cleaved by a basic residue-specific carboxypeptidase during post-translation modification. Additionally, in respect of the odds of 0.72, native BmK abT might be thus amidated at the Ala-63 residue.

Electrophysiological recording showed that BmK abT could increase the peak sodium currents, slowed down the inactivation of sodium channels, and prolong the action potential of DRG neurons. The results strongly indicated that BmK abT worked on sodium channels in a similar manner as classical α-toxins [25].

The geographical distribution of scorpion toxins shows that the α-type toxins are mainly from the Old World, whereas β-type toxins are generally from the New World. Amino acid sequence alignment showed that BmK abT differed extensively from those classical α- and β-toxins, as well as the excitatory anti-insect toxins (20–30%), and have a considerable sequence identity with the β-subtype toxins such as depressant anti-insect toxins, BmK AS and BmK AS-1 (about 40%), and mostly with Ts VII (46.2%). The structural similarity to β-subtype toxins, and the functional similarity to α-toxins as mentioned above, indicated that BmK abT might be a distinctive component and make a link between α- and β-toxins.

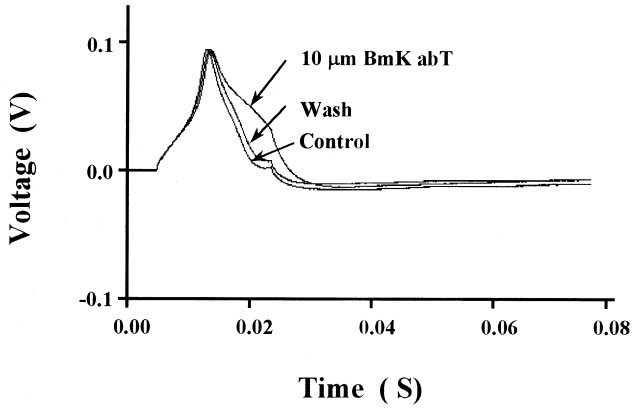

Fig. 5. Effect of BmK abT on DRG action potentials. Application of BmK abT (10 μM) on DRG neurons prolonged the action potentials.

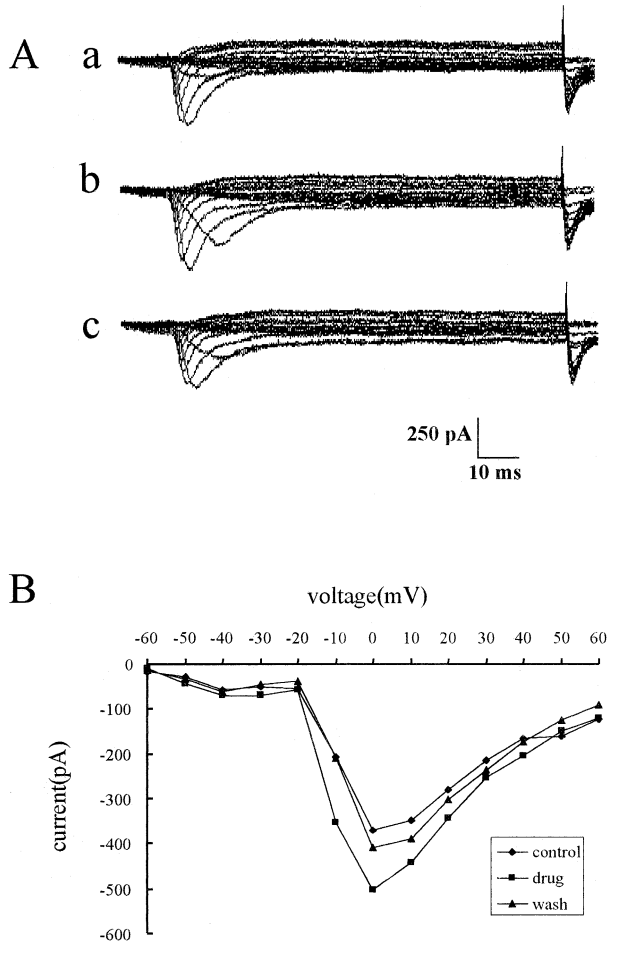

Fig. 4. Effects of BmK abT on DRG peak sodium currents. A: Effect of BmK abT under whole cell patch-clamp recording. a: Control of sodium currents recorded by stepping the membrane from −60 to +60 mV in 10 mV increments from the holding potential of −60 mV on DRG neurons. b: BmK abT (10 μM) increased the peak sodium currents and slowed the inactivation of sodium. channels. c: The sodium currents were partially recovered at 5 min after perfusing the cell with the control external solution. B: The relationship of voltage and sodium currents in the presence and absence of BmK abT.

The arrangement of eight cysteine residues in BmK abT (...-C_{12}-X-X-X-C_{16}-...-C_{22}-X-X-X-C_{26}-...-G_{33}-X-C_{35}-...-C_{42}-X-C_{44}-...-C_{61}-...) is in accordance with all of other sodium channel specific scorpion toxins except excitatory anti-insect toxins. It thus might be possible that the positions of four disulfide bridges in BmK abT were paired as the manners of Cys-12 and Cys-61, Cys-16 and Cys-35, Cys-22 and Cys-42, and Cys-26 and Cys-44. Additionally, the conserved Gly-33 might make a contribution to the stabilization of disulfide bonds.

Although whole sequence of BmK abT was more close to that of a β-toxin Ts VII, the diversity of the local functional motif might have unexpected effects. For example, the length of B-loop between the β2 and the β3 strand was generally regarded as an important element in the activity of α-type toxins [26]. This region in BmK abT was deduced to an intermediate size, shorter than that of classical α-type and α-type anti-insect toxins such AaH I, Lqq V, Lqh αIT and Lqq III; and had the same size as Ts IV-5 and CsE V, which have been defined as two transitional α-type toxins from the New World, and the same size as BmK AS, BmK AS-1 and the depressant anti-insect toxins; but they are longer than β-toxins such as Ts VII and Css II. Moreover, the flexibility of scorpion toxins at either the N- or C-terminal region was deemed to be important for the functional specificity of toxins. It was found that BmK abT, Ts IV-5 and CsE V have a very similar sequence to KK(S/D)GYP$_6$... at the N-terminal region. Especially the residue Pro-6 in these three toxins, which may enable the conformation of the peptide to change, seemed to lack in all of the other types scorpion toxins (Fig. 6).

Considering all the above elements, it can be concluded that BmK abT, found in the Old World, might be a new transitional member between α- and β-type toxins and serve as a useful ligand to study the sodium channel receptors.

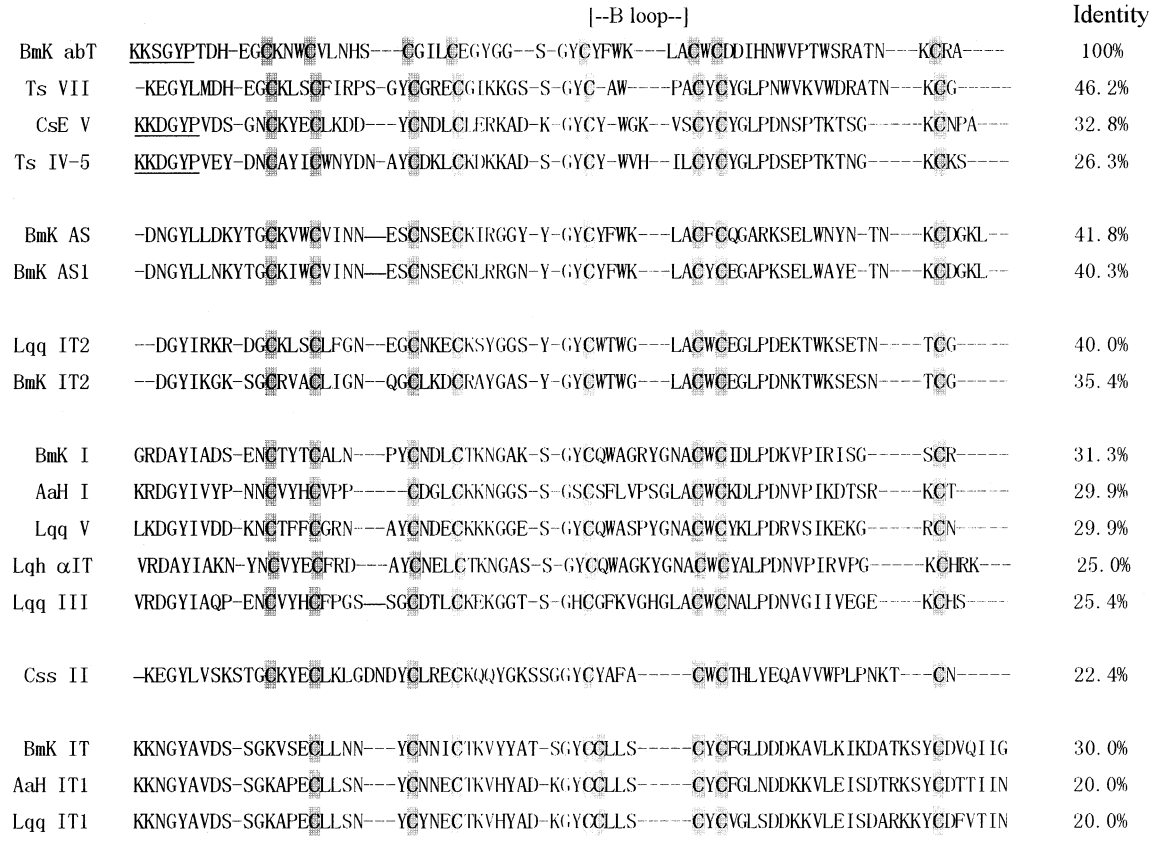

Fig. 6. Comparison of amino acid sequence of BmK abT with that of partial scorpion toxins. The sequences were aligned taking as reference the Cys residues (shadowed). Gaps were introduced in order to maximize similarities. The six N-terminal residues [KK(S/D)GYP$_6$] of BmK abT, CsE V and Ts IV-5 are underlined. The length of the B-loop region was compared (see text). The toxin identity to BmK abT is shown at the right of each sequence (%).

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