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Purification, characterization and structural study of the neuro-peptides from scorpion *Buthus martensi* Karsch*

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Abstract: Total 40 peptides were purified from the venom of *B. martensi* Karsch, and characterized on the basis of their molecular weight (ESI-MS), N-terminal sequence (5 residues) and the toxicity to mammals and insects. Among 29 long chain peptides (60–70 residues) a novel anti-insect toxin BmKαIT1 and a new anti-mammal toxin BmK IV were identified and their primary structures were determined by Edman degradation. Besides, from 11 short chain peptides (28–40 residues), three K^+ channel inhibitors were identified and their action modes were investigated by the assay in acutely dissociated rat hippocampal neurons using whole-cell patch-clamp configuration. The solution structure of BmK I, the main toxin, was determined by 2D ^{1}H-NMR spectroscopy and molecular modeling techniques.

Scorpion venoms are rich sources of fascinating neuro-peptides, which bind with high affinity and specificity to various ion channels and thus widely serve as useful tools in probing the protein mapping of ion channels and clarifying the molecular mechanisms involved in the signal transduction and channel gating. Chinese scorpion *Buthus martensi* Karsch, which is a species belonging to the *Buthidae* family, has been used as a Chinese traditional medicine since Song Dynasty and is still used as a drug even at the present time to treat neurological symptoms such as incomplete paralysis and mimetic paralysis. To date, several neurotoxins such as anti-mammal toxin BmK I, II and BmK M8 [1,2], anti-insect toxin BmKIT1∼IT5 [3], as well as the K^+ channel inhibitor BmP01∼03 have been reported from the venom [4]. Thus, it is worth studying the entire spectrum of scorpion neuro-peptides available in this species, in order to understand the full span of their variability in structures and diversity in pharmacological activities. This paper reports the systematic isolation and characterization of total 40 neuro-peptides, inhibitory effects of BmP01 on K^+ channel and the primary structures of BmKαIT1, a novel anti-insect toxin, and BmK IV, a new anti-mammal toxin, from the venom. The overall fold of BmK I, a main toxin, is also presented.

ISOLATION OF THE NEURO-PEPTIDES

Crude venom of scorpion *B. martensi* was collected from local culture farms in Henan Province, China. The separation and purification of neuro-peptides from the crude venom were carried out by a hybrid chromatographic procedure consisted of gel-filtration, ion-exchange and reverse-phase HPLC. Freeze-dried venom was chromatographied on a Sephadex G-50 column to give four fractions. The toxin containing fractions (F3 and F4) were separated by FPLC into 5 (F301-F305) and 12 (F401-F412) subfractions on a Mono S cation exchange column, respectively. Further separation of each subfraction was performed by gel-filtration on Sephadex G-50 column, and/or FPLC on Mono S column and finally

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by HPLC on C18 column. At last, total 40 components were isolated from the venom. Their chemical purity and characteristics were assessed by HPLC, capillary electrophoresis, molecular weight and amino acid analysis, as well as the N-terminal sequence (five residues, determined by DABITC/PITC double-coupling manual method). The results summarized in Table 1 revealed the presence of 29 long-chain peptides (5 known neuro-peptides and 24 new components) and 11 short-chain peptides (8 new components along with 3 known K^+ channel inhibitors: BmP01~BmP03) in the venom.

Table 1 Characterization of the peptides from *B. martensi* K

Fraction no.	N-terminal Sequence	MW (Da)	Amount (mg)	Possible assignment
31131	VGCEE	2948	0.6	BmP02
31132	DGYGY	8147	2.4	
31133	DGYIR	6874	3.9	
31311	VGCEE	2939	8.1	BmP03
31331	ATCED	3177	14.4	BmP01
3211	IPSGY	7635	10.1	
3212	DGYIR	6728	2.9	
3221	VRDAY	7044	8.5	
34121	VRDAY	7027	1.0	
34122	VRDGY	7265	2.0	
3421	DGYIR	6731	16.6	
3431	DGYIR	6737	11.7	
3512	DNGYL	7721	13.0	BKAS1
3531	DGYIR	6731	8.0	
3533	DNGYL	7692	1.0	BmKAS
4112	TPYPV	3327	7.0	
4114	ATCED	3178	3.1	
4115	VRDAY	7036	2.2	
4122	AVPCG	3460	6.8	
4311	VRDAY	7042	13.2	
4312	VRDAY	7048	5.6	
4322	TPFAI	3245	8.0	
4411	VRDGY	7221	27.1	
4511	VRDGY	7214	45.0	
4521	GRDAY	7040	28.1	BmK M8
4531	TPF	3658	10.2	
4611	VGDGY	3752	12.3	
4622	FGIID	4062	17.0	
4623	VRDGY	7222	12.2	
4631	FGIID	4062	2.7	
46322	VRDGY	7226	1.4	
46323	VRDGY	7216	1.4	
47-21	VRDAY	7026	23.0	
47-31	VKDGY	7393	12.6	
49-11	VGDGY	7394	5.1	
49-21	ARDAY	7023	9.5	
49-31	VRDAY	7267	5.1	
49-32	GRDAY	7113	1.6	
49-4	DGYIR	6732	2.1	
410-11	VRDAY	7217	15.2	BmK II
410-3	DGYIG	6731	1.1	
411-12	GRDAY	7115	5.9	
412-1	VD-SV	4171	12.0	
412-2	VRDAY	7232	23.0	BmK I
413-11	VRDAY	7181	14.0	BmKαIT

ELECTROPHYSIOLOGIC ASSAY OF BmP01 AND BmP03

The inhibitory effects of BmP01 and BmP03 on K^+ channels were investigated by the assay in acutely dissociated hippocampal pyramidal neurons and the preliminary results on BmP03 were reported [5]. As shown in Fig. 1A, when depolarization voltage pulses were applied from a holding potential of −60 mV, outward K^+ currents were evoked and were recorded under whole-cell patch-clamp configuration. The outward K^+ currents activated rapidly, inactivated transiently and then reached steady level. The application of BmP01 at 10 μmol/L markedly reduced the peak currents. Complete recovery was observed when the peptide was washed out. The current-voltage relationship of K^+ currents at peak and steady state level (Fig. 1B) indicates that BmP01 reduced the peak outward currents by 42 ± 3.9%. In comparison, BmP03 exhibited more potential effects on the K^+ channel (63%). These results electro-physiologically demonstrated that they are K^+ channel blockers.

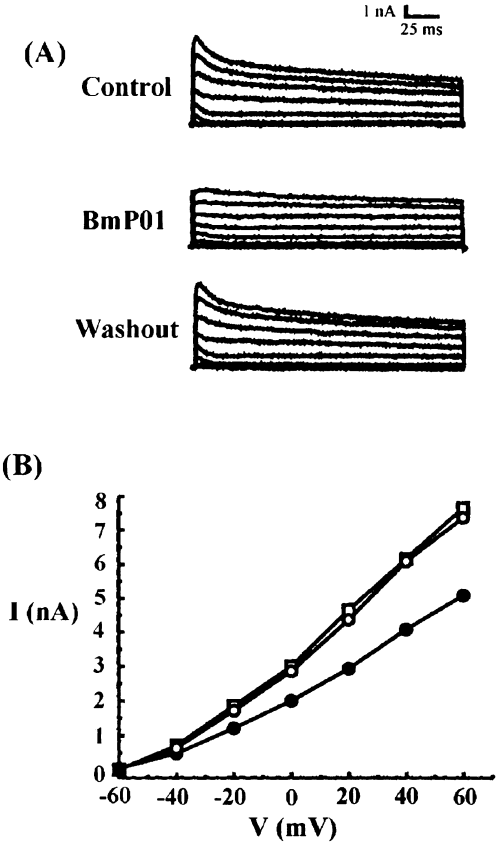

Fig. 1 Effects of BmP 01 on K^+ channel currents in hippocampal pyramidal neurons. Control: □, BmP01: ●, washout: ○.

LETHALITY AND PARALYSIS TEST

The biological specificity and toxicity were determined by a series of paralysis and lethality assays using albino mice (Kuming strain, male, 18–20 g) and *Blattella germanica* cockroach (60 ± 5 mg), respectively. The lethality of various peptides to mice was determined by subcutaneous injection of different amount of peptides (from 5 to 200 μg) in 50 μL 0.15 M NaCl and 1% BSA buffer. The paralysis to cockroach was monitored by the injection of the peptide solution (2 μL at five different concentrations) into the intersegmental membranes of ventral. For mice, the symptoms induced by the toxin injection

were sialorrhea, polyuria, exophthalmia, diarrhea, lacrimation, dyspnea, reduction of movements, loss of equilibrium and convulsions. Here 'lethal' means the animal died after showing some or all of the above symptoms. Mortality was scored after 72 h. For cockroach, after a few seconds of uncoordinated motion, it fell on its dorsal side, violent trembling of the body and the legs. As shown in Table 2, five fractions are toxic to both mice and cockroach, four fractions are toxic only to cockroach and one fraction is toxic only to mice, other fractions show weak or no toxicity. The fraction 413-11 and 4411 were named as anti-insect toxin BmKαIT01 and anti-mammal toxin BmK IV, respectively, and submitted to the protease digestion and the sequence determination.

Table 2 Toxicity data of the peptides from *B. martensi* K

Fraction no.	LD_{50} on mice (μg/20g)	PU_{50} on cockroach (ng/60 mg)
3211	>107	11.7
3212	>257	35
3431	>342	11.2
4411	7.5	>6250
4721	64	22.5
410-11	12.5	3.25
410-3	>157	50
411-12	94	195
412-2	4.87	1.75
413-11	90	0.31

SEQUENCES OF BmKαIT01 AND BmK IV

BmKαIT01 and BmK IV were reduced by ditioethreitol and then alkylated with iodoacetic acid. The reduced and alkylated peptides were digested with TPCK-trypsin and endoproteinase Glu-C or α-chymotripsin, respectively. The peptide fragments liberated from the enzyme digestion were separated by HPLC on C18 column and sequenced by Beckman LF3200 Sequencer directly. The full sequence of BmKαIT01 except for Ala17 and His42 was determined by combination of Edman degradation results of TPCK-trypsin and endoproteinase Glu-C digestion products. The initial ambiguity at position 17 and 42 was clarified by repeated measurement and the full sequence was confirmed by ESI tandem mass spectroscopy. The sequence of BmK IV was determined by Edman degradation using peptide fragments derived from TPCK-trypsin and α-chymotripsin, except for the C-terminal (residue 61-65), which remains unknown due to the peptide segment missing during the HPLC separation. The sequences of BmKαIT01 and BmK IV were schematically shown in Scheme 1. It is interesting that the sequence of BmKαIT01 is more close to that of LqhαIT from *L. quinquestriatus hebraeus* other than that of BmK IT2 [7]. While the difference in the sequence between BmK IV and BmK I, especially at position 8, 32 and 50 may account for their distinct specificity to cockroach.

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                    10        20        30        40        50        60
BmKαIT1  VRDAYIAQNYNCVYHCARDAY-CNELCTKDGAKSGSCPYLGEHKFACYCKDLPDNVPIRVPGKCH
LqhαIT   VRDAYIAKNYNCVYECFRDAYCNELCTKNGASSGYCQWAGKYGNACWCYALPDNVPERVPGKCR
BmKIT2   DGYIKGKSGCRVACLIGNQGCLKDCRAYGASYGYCWTWGLA---CWCEGLPDNKTWKSESNTCG
BmK I    VRDAYIAKPHNCVYECARNEY-CNDLCTKDGAKSGYCQWVGKYGNGCWCIELPDNVPIRVPGKCH
BmK IV   VRDGYIADDKNCAYFCGRNAYCDDECKKNGAESGYCQWAGVYGNACWCYKLPDKVPIRVP- - - - -
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Scheme 1 The sequences of BmKαIT01 and BmK IV in comparison to some similar toxins.

GLOBE FOLDING OF BmK I

The sequential resonance assignments and secondary structures of BmK I have been previously reported [6]. The distance geometry using variable target function method (DIANA) was applied to establish 50 preliminary structures. An iterative process combined with the structural calculation was used to assign unidentified NOE cross-peaks and total 770 NOE cross-peaks were unambiguously assigned and converted to distance range constraints. Sixteen structures with least constraint violation and lowest target function were selected and submitted to a hybrid refinement procedure including restrained molecular dynamics, simulated annealing (SA) and repeated energy minimization calculation (using Tripos force field on a SGI Indigo 2 workstation). Figure 2 shows the best-fit superposition of 16 backbone structures. The RMSD value between 16 structures and the averaged structure is 1.0 ± 0.03 Å for the backbone coordinates. However, somewhat high RMSD value in the regions of 8–12, 39–43, especially in the C-terminal indicates those are quite flexible areas. The major secondary structural features of the molecule include a short α-helix (Tyr21-Gly30), a triple-stranded anti-parallel β-sheet (Asp3-Ile6, Cys46-Glu50, Ser33-Gln37), four β-turns (8–11, 30–33, 39–42 and 52–55) and two intervening loops (12–17 and 56–64) connecting above regular secondary structures. As shown in Fig. 3, the tertiary structure of the molecule is quite similar to other scorpion toxins: the α-helix runs roughly parallel with the β-sheet and cross-linked each other by two disulfide linkages, which form a highly condensed core structure. Usually the hydrophobic surface of the molecule involves in the binding with the receptor. The hydrophobic character of the conserved hydrophobic surface of the molecule was determined mostly by the great concentration of aromatic residues. There are two hydrophobic centers in the molecule: a cluster of aromatic residues including Tyr5, Tyr 35, Trp47 and Tyr 42 form a hydrophobic patch at the first side of the molecule. On the opposite side of the molecule, the hydrophobic surface is consisted of less aromatic residues, only Tyr14 and Tyr21. In addition, BmK I shows interesting distribution of aromatic residues at the first hydrophobic patch: the aromatic rings aligned in a herringbone fashion. Since all the long chain scorpion toxins show highly conserved globe folding and hydrophobic character, it is suggested that the difference of electrostatic potential distribution may play a key role to the selectivity of binding site. According to the tertiary structure, the possible salt bridges appear between residues Arg18 and Glu20, Asp24 and Lys28, Lys32 and Glu50. These and some basic residues locating at the flexible regions of the molecule, such as Lys41 and Lys63, may involve in the binding process. The pharmacological diversity of neuro-peptides in this species should be related to differences in their sequences and spatial organization of the hydrophobic patches and charges, as well as the internal dynamics, which remains to further study.

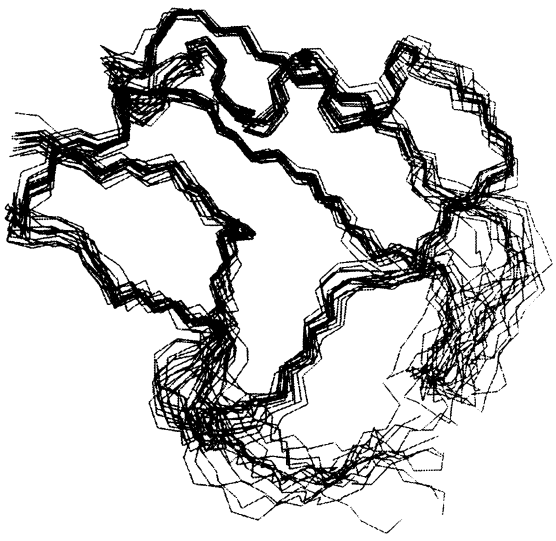

Fig. 2 The best-fit superposition of 16 backbone structures.

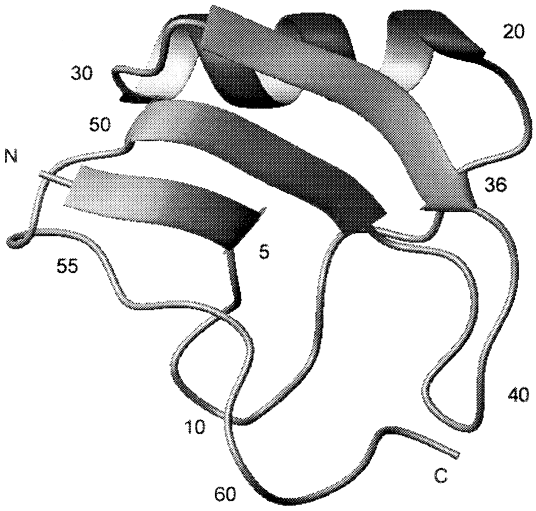

Fig. 3 Schematic view of the backbone of BmK I.

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