Functional site of bukatoxin, an α-type sodium channel neurotoxin from the Chinese scorpion (*Buthus martensi* Karsch) venom: probable role of the ^{52}PDKVP^{56} loop

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Abstract **α-Toxins from scorpion venoms prolong the action potential of excitable cells by blocking sodium channel inactivation. We have purified bukatoxin, an α-toxin from scorpion (*Buthus martensi* Karsch) venom, to homogeneity. Bukatoxin produced marked relaxant responses in the carbachol-precontracted rat anococcygeus muscle (ACM), which were mediated through the L-arginine–nitric oxide synthase–nitric oxide pathway, consequent to a neuronal release of nitric oxide. Based on the presence of proline residues in the flanking segments of protein–protein interaction sites, we predicted the site between ^{52}PP^{56} to be the potential interaction site of bukatoxin. A homology model of bukatoxin indicated the presence of this site on the surface. Buka11, a synthetic peptide designed based on this predicted site, produced a concentration-dependent nitric oxide-mediated relaxant response in ACM. Using alanine-substituted peptides, we have shown the importance ^{53}DKV^{55} flanked by proline residues in the functional site of bukatoxin.**

Key words: Scorpion toxin; Sodium channel; Nitric oxide; Homology model; *Buthus martensi* Karsch

1. Introduction

Scorpion venoms are well-known sources of neurotoxins. Most of these toxins affect voltage-gated sodium channels that are responsible for the depolarization phase of the action potential in excitable cells [1]. These sodium channel toxins are single-chain polypeptides composed of 60–70 amino acid residues linked by four disulfide bridges [2]. They are classified into two groups based on their pharmacological effect on the kinetics of sodium channels. α-Toxins bind to site 3 and prolong the action potential by blocking sodium channel inactivation [3–5], while β-toxins bind to site 4 and shift the voltage of activation towards more negative potentials thereby affecting the sodium channel activation leading to spontaneous and repetitive firing [5,6]. The interaction of β-toxins is voltage-independent, unlike the voltage-dependent interaction of α-toxins. However, they have similar three-dimensional structures; they are α/β proteins with one or two short segments of α-helices and a triple-stranded β-sheet, connected by variable regions forming loops [7]. Thus, this structural similarity and functional divergence in sodium channel toxins is intriguing. Based on structural comparison, chemical modifications and site-directed mutagenesis, a multisite interaction for the α-toxin receptor has been suggested [8–11]. However, the site(s) involved in molecular recognition and binding of sodium channel toxins are yet to be identified.

Protein–protein interactions are highly specific and are due to molecular recognition sites. Proline residues have a hypothetical structural role in the flanking segments of interaction sites [12,13]. They act as barriers between the interaction site and their neighboring secondary structural elements in protecting its integrity and conformation. They also help in the presentation of the interaction site to its complementary protein and thus facilitate protein–protein interaction [12,13]. Based on this principle, Kini and Evans [14] developed a method to identify protein–protein interaction sites directly from the amino acid sequences. Using this method, protein–protein interaction sites in several proteins that are unrelated in their structure, function and phylogeny have been identified [15,16].

In this paper we report the purification, characterization and determination of the complete amino acid sequence of bukatoxin, a 65-residue peptide from *Buthus martensi* Karsch venom and the identification of the tripeptide, ^{53}DKV^{55} that could contribute to the function of the toxin.

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Abbreviations: ACM, anococcygeus muscle; CCh, carbachol; CE, capillary electrophoresis; EFS, electrical field stimulation; ESI/MS, electrospray ionization mass spectrometry; HPLC, high-performance liquid chromatography; L-NAME, *N*ω-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; TTX, tetrodotoxin; ODQ, 1*H*-(1,2,4)oxadiazolo(4,3-*a*)quinoxalin-1-one; SNP, sodium nitroprusside; TFA, trifluoroacetic acid

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2. Materials and methods

2.1. Materials

Lyophilized *B. martensi* Karsch venom was obtained from the Huazhen Pharmaceutical Animal Research Institute (Guangzhou, China). Prepacked Superdex 75 and Sephasil C18 columns were from Pharmacia Biotech (Uppsala, Sweden). Sprague–Dawley rats (280–350 g) were from the Animal Holding Unit, National University of Singapore. Acetonitrile (ACN) and trifluoroacetic acid (TFA) were purchased from Fisher Scientific (Leicestershire, UK) and Fluka Chemika-Biochemika (Buchs, Switzerland), respectively. α-Cyano-4-hydroxycinnamic acid and all reagents for peptide synthesis and sequencing were purchased from Perkin-Elmer Applied Biosystems (Chiba, Japan). Drugs and chemicals used for pharmacological studies were from Sigma Chemicals (St. Louis, MO, USA).

2.2. Purification of bukatoxin

Bukatoxin was purified from the crude venom by gel filtration and reverse-phase high-performance liquid chromatography (HPLC). The lyophilized crude venom was applied on to a Superdex 75 Hiload (16/60) column equilibrated with 50 mM Tris buffer pH 7.5. Proteins eluted were monitored at 280 nm. The biologically active fraction was then applied onto a Sephasil C18 reverse-phase column, equilibrated with 0.1% TFA. The bound peptides were eluted using a linear gradient of ACN. Elution was monitored at 215 nm.

2.3. Electrospray ionization mass spectrometry (ESI/MS) and capillary electrophoresis (CE)

Samples were analyzed using Perkin-Elmer Sciex API 300 triple quadrupole mass spectrometry equipped with an ion spray interface (Sciex, Thronton, Canada). The ion spray voltage was set at 4600 V and the orifice voltage was set at 30 V. The mass was determined by flow injection analysis at a flow rate of 50 μl/min using Shimadzu 10 AD pumps as the solvent delivery system. CE was performed on a BioFocus 3000 system (Bio-Rad, Singapore). The sample was injected to a 25 μm×24 cm coated capillary using pressure mode (5 psi/s) and run in 0.1 M phosphate buffer (pH 2.5) under 12.00 kV from + to − at 15°C for 10 min. Migration was monitored at 200 nm.

2.4. N-terminal amino acid sequence

The N-terminal amino acid sequence of the peptide was determined by automated Edman degradation using an Applied Biosystem 494 pulsed liquid-phase sequencer equipped with an online 785A PTH amino acid analyzer.

2.5. Pharmacological studies

The pair of anococcygeus muscles (ACM) from male Sprague–Dawley rats was isolated and mounted under 1 g resting tension in a 4-ml organ bath containing Krebs physiological salt solution [17]. The solution was maintained at 37°C and aerated with 5% carbon dioxide in oxygen. Carbachol (CCh) 3 μM was used to increase the tone of the ACM, which was pretreated with 5 μM of phentolamine to block the adrenergic response. Relaxant responses of the CCh-precontracted ACM were evoked by electrical field stimulation (EFS) (20–30 V, 1 ms, 10 Hz for 10 s) using a Grass stimulator (Model S88) and the data were recorded in a MacLab system8™ via a force-displacement transducer (model FT03). The effects of tetrodotoxin (TTX) 2 μM, nitro-L-arginine methyl ester hydrochloride (L-NAME) 50 μM, D-NAME 50 μM, 1*H*-(1,2,4)oxadiazolo(4,3-*a*)quinoxalin-1-one (ODQ) 1 μM or hemoglobin (Hb) 1 μM on the relaxant responses of the ACM produced by bukatoxin (0.001–3 μM), buka11 (1–200 μM), EFS or sodium nitroprusside (SNP) 1 μM were studied.

2.6. Molecular modeling

The three-dimensional structure of *Leiurus quinquestriatus hebraeus* toxin (PDB ID: 1LQI) and *Androctonus australis hector* toxin (1AHO) were used as homology templates for molecular modeling of bukatoxin. The sequence was submitted to the PredictProtein server for secondary structure prediction [18,19] and the information from the PredictProtein was used to check the homology match. The first NMR structure of 1LQI (out of 29) was structurally aligned with the crystal structure of 1AHO. The pair-wise sequence identities between bukatoxin and the templates were 66% and 64% respectively. The homology model was constructed using the automated MODELER routine (Insight II, Molecular Simulations Inc., USA). Since this version of MODELER fits only the backbone, the side chains were re-packed using the rotamer library supplied by Insight. Except for the loop regions where the accuracy of the backbone atoms is expected to be lower, the side chains are positioned well by this packing procedure. Finally the model was refined by energy minimization using molecular mechanics.

2.7. Peptide synthesis

Buka11 (YKLPDKVPIRV), scrambled peptide (YPKPVRDIKVL)

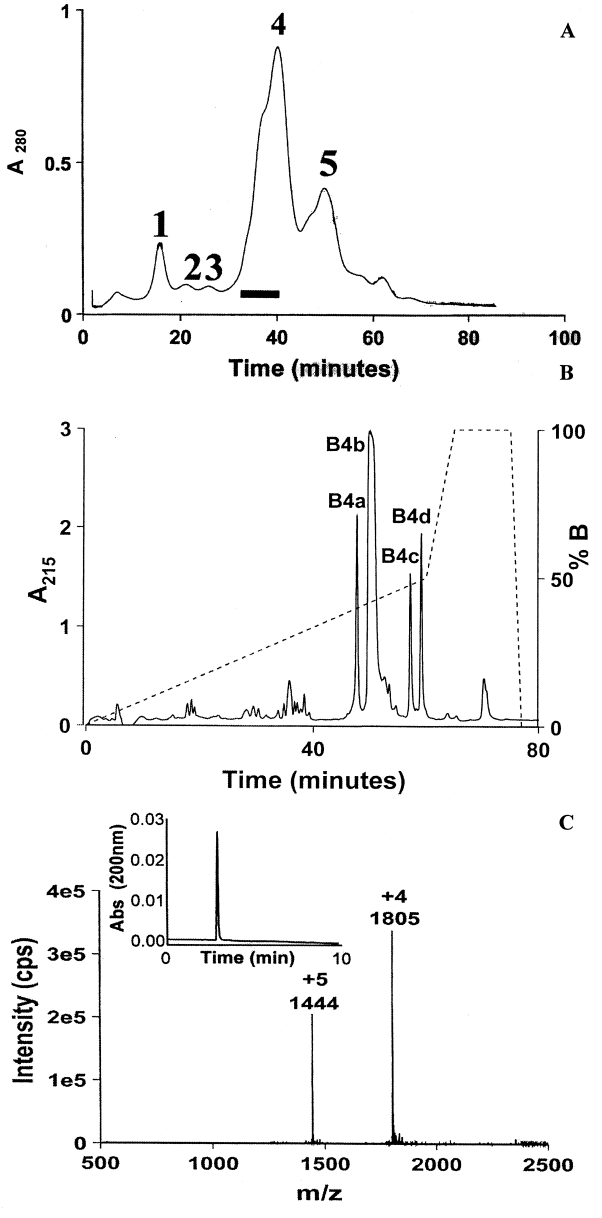

Fig. 1. Purification and homogeneity of bukatoxin. A: Gel filtration of *B. martensi* Karsch venom on a Superdex 75 column equilibrated with 50 mM Tris buffer. The crude venom (200 mg) was dissolved in 1 ml of 50 mM Tris buffer and centrifuged at 3000 rpm for 10 min. Supernatant was then loaded onto the column. Elution was monitored at 280 nm. B: Reverse-phase HPLC of fraction 4 on a Sephasil C18 column. The column was equilibrated with 0.1% TFA and eluted with a linear gradient of 80% acetonitrile in 0.1% TFA. Elution was monitored at 215 nm. The horizontal bars indicate pooled peaks. C: The mass spectrum determined by ESI/MS shows multiple charged ions related to molecules bearing four and five protons. Inset shows the electropherogram of bukatoxin as determined by CE.

Scorpion α-toxins

Toxin	Sequence alignment	% I	Ref
BKTx	VRDGYIADDK NCAYFCGRNA YCDEECIING AESGYCQQAG VYGNACWCYK LPDKVPIRVS --GECQQ	100	
Neurotoxin X	VRDGYIADDK DCAYFCGRNA YCDEEC-KKG AESGKCWYAG QYGNACWCYK LPDWVPIKQK VSGKCN-	78	(20)
NeurotoxinIV	VRDAYIADDK NCVYTCGSNS YCNTECTKNG AESGYCQWLG KYGNACWCIK LPDKVPIRIP --GKCR-	72	(21)
Neurotoxin V	-RDAYIADSE NCTYTCALNP YCNDLCTKNG AKSGYCQWAG RYGNACWCID LPDKVPIRIS --GSCR-	67	(22)
Insect toxin	VRDAYIAKNY NCVYECFRDA YCNELCTKNG ASSGYCQWAG KYGNACWCYA LPDNVPIRVP --GKCR-	68	(23–25)
Neurotoxin	VRDAYIAKNY NCVYECFRDA YCNELCTKNG ASSGYCQWAG KYGNACWCYA LPDNVPIRVP --GKCR-	67	(23–25)
Makatoxin	-RDAYIADSE NCTYTCALNP YCNDLCTKNG AKSGYCQWAG RYGNACWCYD LPDKVPIRIS --GSCR-	64	(26)
NeurotoxinXI	-KDGYIVDDR NCTYFCGTNA YCNEECVKLK GESGYCQWVG RYGNACWCIK LPDHVRTVQA --GRCRS	63	(27)

Scorpion β-toxins

BKTx	VRDGYIADDK N-CAYFC--- GRNAYCDEEC IIN--GAESG YCQQAGVYGN ACWCYKLPDK VPIRVSG --ECQQ	100	
CsEv2	-KEGYLVNKS TGCKYGCLKL GENEGCDKEC KAKNQGGSYG YC-----YAF ACWCEGLPES TPTYPLP NK-CS	33	(28)
CsEv3	-KEGYLVKKS DGCKYGCLKL GENEGCDTEC KAKNQGGSYG YC-----YAF ACWCEGLPES TPTYPLP NKSC-	32	(29)
CsEv1	-KEGYLVKKS DGCKYDCFWL GKNEHCNTEC KAKNQGGSYG YC-----YAF ACWCEGLPES TPTYPLP NKSC-	31	(28)
Cll1	-KEGYLVNKS TGCKYGCFWL GKNENCDKEC KAKNQGGSYG YC-----YAF GCWCEGLPES TPTYPLP NKSCS	30	(30)
Cn2	-KEGYLVDKS TGCKYECLKL GDNDYCLREC KQQYGKGAGG YC-----YAF ACWCTHLYEQ AIVWPLP NKRCS	30	(31)
CssII	-KEGYLVNKS TGCKYGCLKL GDNDYCLREC KQQYGKSSGG YC-----YAF ACWCTHLYEQ AVVWPLP NKTCN	29	(32)
Cii1	-KEGYLVNKS TGCKYGCLKL GDNDYCLREC KQQYGKGAGG YC-----YAF GCWCTHLYEQ AVVWPLP KKTCN	28	(33)

Fig. 2. Sequence identities of bukatoxin with other scorpion α- [20–27] and β-toxins [28–33]. Sequences were aligned with cysteine residues and gaps (–) were introduced for maximum accuracy. % I is the percentage identity of BKTx (bukatoxin) with other scorpion α- and β-toxins.

and the three alanine analogues (D5A Buka11; K6A Buka11 and V7A Buka11) were synthesized by solid-phase methodology using 9-fluorenylmethoxycarbonyl chemistry on an Applied Biosystems model 433A peptide synthesizer and were purified by HPLC. The purified synthetic peptides were characterized by matrix-assisted laser desorption/ionization time of flight mass spectrometry measurements.

3. Results and discussion

The fractionation of the crude venom on a Superdex 75 gel filtration column yielded five fractions (Fig. 1A). Among these fractions, only fraction four was found to mediate relaxation responses in the precontracted rat ACM. This fraction was then further sub-fractionated by reverse-phase HPLC into four major peaks (Fig. 1B). Of the four peaks, peak two was found to possess pharmacological activity. Homogeneity of the toxin was assessed by ESI/MS and CE (Fig. 1C). The molecular weight of the toxin was found to be 7215.6 ± 0.16. This toxin was named bukatoxin. The complete amino acid sequence of bukatoxin (SwissProt accession number P82815) was determined by automated Edman degradation, with a reproducible yield of 95%. During sequencing, blank cycles were encountered at positions 12, 16, 22, 26, 36, 46, 48 and 63. With cysteines at these positions, the calculated molecular weight was 7215.0, which matched the observed molecular weight. Bukatoxin shows a striking structural identity of 78% with neurotoxin X from *Mesobuthus eupeus* [20] and 72% with neurotoxin IV from *L. quinquestriatus quinquestriatus* [21]. The sequence alignment of bukatoxin with other scorpion toxins is given in Fig. 2.

Bukatoxin produced a marked, concentration-dependent relaxation of the CCh-precontracted rat ACM (Fig. 3). The bukatoxin-mediated relaxant responses, like those produced by EFS, were completely inhibited by L-NAME (Fig. 3A), TTX (Fig. 4A), ODQ (Fig. 4B) and Hb but not by D-NAME. In contrast, the relaxation induced by the nitric oxide (NO) donor SNP was inhibited only by ODQ and Hb and not by L-NAME, D-NAME or TTX.

The relaxant responses produced by EFS in the ACM were sensitive to inhibition by TTX, a highly selective blocker of neuronal sodium channels [34], indicating that EFS activated neuronal sodium channels with consequent depolarization of nitrergic nerves and the release of NO. Similarly, the relaxant responses produced by bukatoxin also involve a neuronal release of NO since TTX inhibited these responses. It is likely that bukatoxin mediates this action by modulation of neuronal sodium channels. Furthermore, our results provide strong evidence that the bukatoxin-mediated relaxant responses of

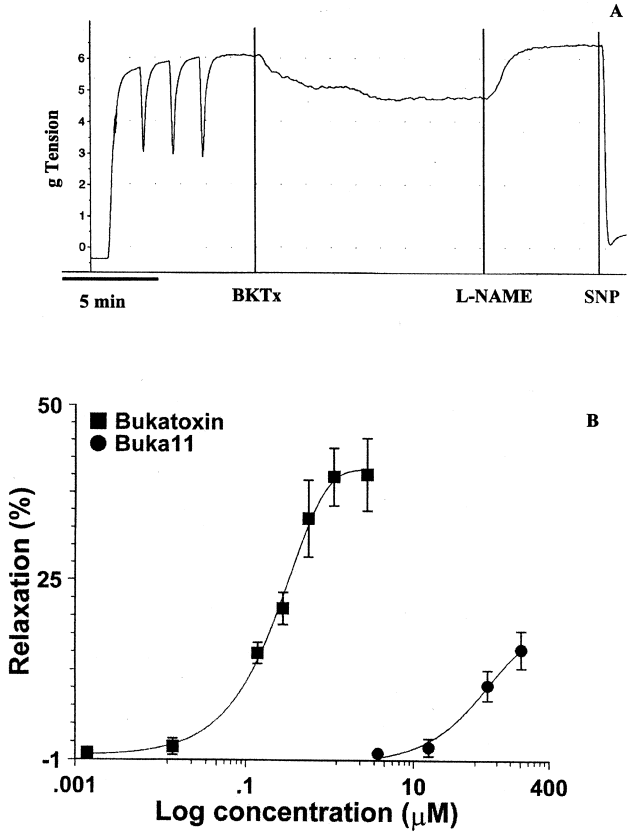

Fig. 3. A: Bukatoxin (0.2 μM) evoked a marked relaxation of the CCh (3 μM)-precontracted rat ACM. B: Concentration–response effect of bukatoxin and Buka11.

the ACM, like those to EFS, are mediated through the activation of the L-arginine–nitric oxide synthase (NOS)–NO pathway since the stereoselective and competitive NOS inhibitor L-NAME, but not its D-isomer, produced complete inhibition of these relaxant responses. The binding and sequestration of NO by Hb [35] may account for the inhibition of the bukatoxin-mediated relaxation by Hb. Moreover, the relaxant responses produced by bukatoxin were inhibited by ODQ, a selective inhibitor of NO-sensitive soluble guanylyl cyclase [36], suggesting the NO–cGMP pathway underlies these relaxant responses and that the target enzyme for NO in the ACM is soluble guanylyl cyclase.

To identify the functional site of the sodium channel neurotoxins, we used a method of identifying potential interaction sites based on the presence of proline residues in the flanking segments of protein–protein interaction sites [11,12]. Accordingly, we aligned the amino acid sequences of α- and β-toxins and identified the region between 52 and 56, particularly the tripeptide segment ^{53}DKV^{55}, as the possible interaction site of α-toxins (Fig. 2). Inspection of the molecular model of bukatoxin revealed that this site forms a surface loop and is available for binding interaction (Fig. 5A). This site is independent of face A, face B and site C as proposed by De-Cheng [8]. Therefore we predicted that this segment, in particular ^{53}DKV^{55}, might be functionally important in α-toxins. On the other hand, this tripeptide segment is replaced in all β-toxins (Fig. 2). Overall, α-toxins have a characteristic site that is different from β-toxins.

Based on this predicted site, we synthesized the peptide Buka11, and examined its activity. Buka11, but not the scrambled peptide, produced concentration-dependent relaxant responses in the CCh-precontracted ACM (Figs. 3B and 4C), which were sensitive to inhibition by TTX, L-NAME, ODQ and Hb. These results indicate that the predicted segment could probably play a significant role in the interaction with the neuronal sodium channel. Further, each of the three alanine-substituted analogues of Buka11 failed to produce any relaxant response in the ACM even at a concentration of 1 mM, implying that the three residues (DKV) could play a vital role in peptide–channel interaction. The activity of Buka11 was, however, ~800-fold less than that of the native toxin. This lower activity is probably due to the conformational flexibility of the short peptide. This is the first evidence for the role of the ^{52}PDKVP^{56} loop in the interaction site of

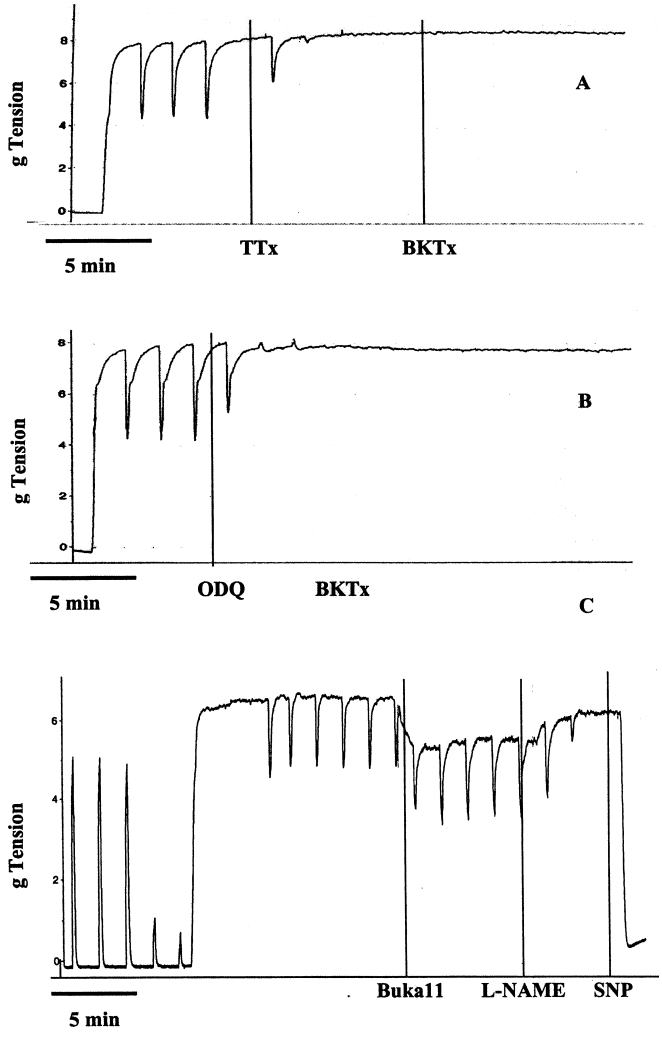

Fig. 4. The relaxant effects of bukatoxin (0.2 μM) and EFS (20–30 V, 1 ms, 10 Hz for 10 s) on the precontracted ACM were completely inhibited by (A) TTX (2 μM) and (B) ODQ (1 μM). SNP (1 μM) produced a marked relaxation of the ACM when added at the end of each experiment, indicating that the muscle remained viable to nitrergic relaxation throughout the experiment. C: The relaxant effect of Buka11.

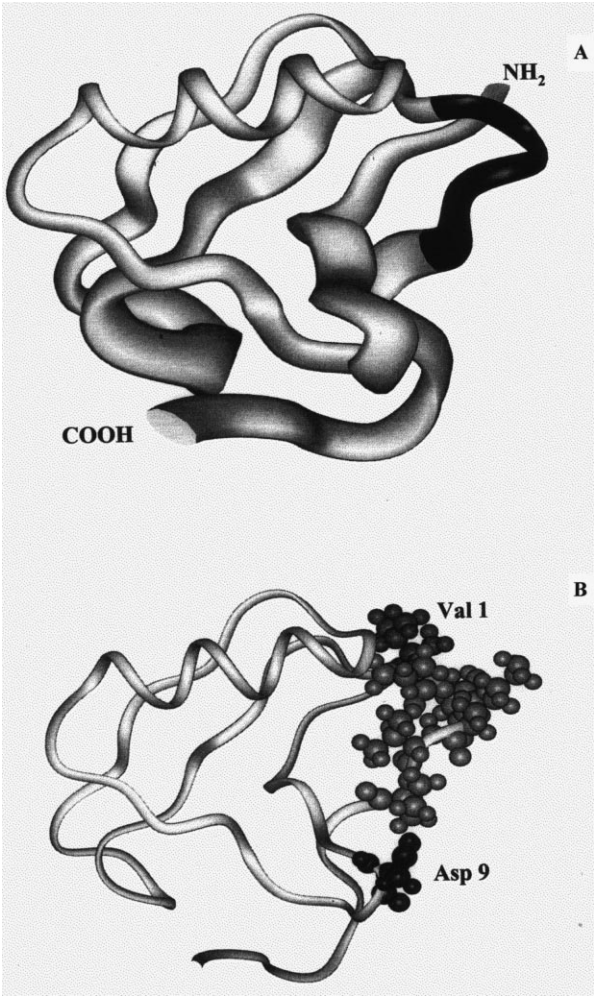

Fig. 5. A: Molecular model of bukatoxin. The regions with lower homology are shown in thick ribbon. The predicted site ^{52}PP^{56} is highlighted black. B: Proposed surface of bukatoxin that may interact with the sodium channel. Based on the surface accessibility, it is proposed that along with the site ^{52}PP^{56}, Val1 and Asp9 may also be responsible for the interaction of the toxin to sodium channel protein.

sodium channel toxins from scorpion venoms. In addition, it is possible that other residues in the vicinity, for example Val1 and Asp9, which are also part of the same surface (Fig. 5B), could contribute to the interaction. Similarly, short segments or a few residues have been reported to be sufficient for specific recognition and interaction between toxins and their target ion channels [15,37,38].

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