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cDNA Cloning, Sequence Analysis and Molecular Modeling of a New Peptide from the Scorpion *Buthotus saulcyi* Venom

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In this study, the cDNA of a new peptide from the venom of the scorpion, *Buthotus saulcyi*, was cloned and sequenced. It codes for a 64 residues peptide (Bsaul1) which shares high sequence similarity with depressant insect toxins of scorpions. The differences between them mainly appear in the loop1 which connects the β-strand1 to the α-helix and seems to be functionally important in long chain scorpion neurotoxins. This loop is three amino acids longer in Bsaul1 compared to other depressant toxins. A comparative amino acid sequence analysis done on Bsaul1 and some of α-, β-, excitatory and depressant toxins of scorpions showed that Bsaul1 contains all the residues which are highly conserved among long chain scorpion neurotoxins. Structural model of Bsaul1 was generated using Ts1 (a β-toxin that competes with the depressant insect toxins for binding to Na^+ channels) as template. According to the molecular model of Bsaul1, the folding of the polypeptide chain is being composed of an anti-parallel three-stranded β-sheet and a stretch of α-helix, tightly bound by a set of four disulfide bridges. A striking similarity in the spatial arrangement of some critical residues was shown by superposition of the backbone conformation of Bsaul1 and Ts1.

Keywords: Amino acid sequence, *Buthotus saulcyi,* Long chain neurotoxins, Scorpion, Three-dimensional structure

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Introduction

Scorpion venom consists of numerous polypeptides (Rochat *et al*., 1970), many of which affect ion channels in excitable membranes. These polypeptides have been used as tools to study the pharmacology (MacKinnon *et al*., 1998) and molecular mechanism of action of the ion channels. The scorpion long chain neurotoxins composed of 60-76 amino acids appear to modify the gating of voltage-sensitive Na^+ channels. These toxins are divided into α and β classes according to their mode of action and binding properties to distinct receptor sites (Jover *et al*., 1980). Among β-toxins, two distinct groups the excitatory and the depressant, show specificity for insects (Zlotkin, 1993). Excitatory toxins cause a fast excitatory paralysis in animals and induce repetitive firing in insect nerves; in contrast, the depressant toxins cause a slow depressor flaccidity due to depolarization of the nerve membrane and blockage of the sodium conductance in axons (Pelhate and Zlotkin, 1982; Zlotkin, 1985). Despite their diverse bioactivity and sequence, all of them, share a highly conserved dense core formed by a cysteine-stabilized α-helix/β-sheet (CSαβ) motif, generally consisting of a three-stranded anti-parallel β-sheet and one α-helix. Three spatially conserved disulfide bridges stabilize the α/β scaffold and a fourth bridge is conserved in all but the excitatory toxins (Fontecilla-Camps *et al*., 1982; Darbon *et al*., 1991; Oren *et al*., 1998). The mutual disposition of the α-helix and the β-sheet in all toxins is similar. However, the carboxy-terminal stretch and the regions connecting the secondary structure elements differ in the various toxins (Gordon *et al*., 1998).

So far, no three-dimensional structure of depressant toxins has been reported. However, the resemblance between depressant and β-toxins in sequence, neurophysiological action and competition for receptor binding site (Gordon *et al*., 1998) permits structural modeling of depressant toxins.

The structural basis of the specificity of long-chain

neurotoxin binding to the Na^+ channel is not yet completely understood. The difficulty of producing active toxins using different expression systems, delayed the determination of the precise location of the binding/toxic sites of the scorpion neurotoxins. It was recently suggested (Karbat *et al*., 2004) that the functional surface of α-toxins is composed of two distinct domains: a conserved "core domain" formed by the residues of the loops connecting the secondary structure elements and a variable "NC domain". In β-toxins, a Glu residue surrounded by a solvent-occluding gasket adjacent to a cluster of non polar residues is suggested to constitute the pharmacophore of these toxins (Cohen *et al*., 2004). The best that can be concluded at present is the general importance of the positively charged residues, of the conserved aromatic residues and of the structural regions such as the N- and C-termini and the loops connecting the secondary structure elements in both α- and β-toxins.

If we assume each of different species of scorpion have around 70 peptides, the overall biodiversity present in about 1500 known species of scorpions in the world should come close to 100,000 distinct polypeptides, among them, at most only 1% is currently known; even then, the function of some remains unknown (Possani *et al*., 1999).

This paper reports on purification, gene cloning and molecular modeling of a new peptide from the venom of an Iranian scorpion: *Buthutus saulcyi*. We have predicted by amino acid sequence similarity and molecular modeling studies that the new peptide is possibly a depressant toxin with a unique sequence of loop1 which has been shown to be functionally important in long chain scorpion neurotoxins. We then included the new toxin in a comparative sequence analysis of α-, β-, excitatory and depressant toxins and showed that the new peptide contains some strictly conserved residues, in line with other members of long chain neurotoxins.

Materials and Methods

Toxin purification. The crude venom was initially purified by size-exclusion chromatography on a Sephadex G-50 (Pharmacia) column (150 × 2 cm) followed by further separation using a semi-preparative reversed phase (RP)-HPLC C8 column (250 × 10 mm, 5 μm) Vydac (Hesperia) equilibrated with 0.1% w/v trifluoroacetic acid as reported earlier (Amininasab *et al*., 2004; Talebzadeh-Farooji *et al*., 2004). Elution was carried out with a linear gradient (10-50%) of acetonitrile containing 0.089% w/v trifluoroacetic acid. The flow rate was 2 ml/min and fractions were monitored at 215 nm. Every peak obtained was then collected and rechromatographed on the RP-HPLC column to homogeneity.

The mass spectra of the purified peptides were obtained by electrospray ionization mass spectrometry with a single-stage quadrupole instrument (VG Platform, Micromass). Among the masses obtained one turned out unique and was therefore chosen for further investigation. The N-terminal sequence of the first 9 amino acids of this new peptide was determined on a Procise cLC 492 protein sequencer from Applied Biosystems.

Total RNA extraction. Scorpions were obtained from Razi Vaccine and Serum Research Institute. Total RNA was extracted from 50 mg of scorpion venomous gland (telson), using Total RNA extraction kit.

RT-PCR. The specific primers used for synthesis and amplification of cDNA encoding the new peptide (Bsaul1) gene were 5'-ATT GAA GGA TCC ATT GAA GGA CGC GAC GGC TAT ATA AGA-3' (forward primer) and 5'-ACA GTC CCT GCA GTT AAC CGC ATG TGT TTG TTT CAC-3' (reverse primer). The forward and reverse primers were designed using the N-terminal sequence information of the purified Bsaul1 and the sequence information from LqqIT2 clones (Zaki and Maruniak, 2003) respectively.

The total RNAs (0.5 μg) were converted to cDNA using RevertAid™ M-MuLV Reverse Transcriptase, (Fermentas) and the specific reverse primer at 42°C for 60 min. The resulting cDNA was amplified by PCR using 30 thermal cycles of 94°C (45 s); 55°C (45 s); 72°C (45 s) and a final extension at 72°C for 2 min.

cDNA cloning and sequence analyses. The PCR product was digested by *Bam*HI/*Pst*I (Roche) and cloned into pQE30 vector (Qiagen) and transferred to M15 *E. coli* strain (Qiagen) by electroporation (Eppendorf, EW-36205-05). Positive colonies were selected and their plasmids were isolated for sequencing (Plasmid extraction kit, Qiagen). The cDNA was sequenced using an automatic sequencer (MWG-Biotech AG). The deduced amino acid sequence of Bsaul1 was included in multiple sequence alignments (using ClustalW (Chenna *et al*., 2003)) of different groups of long chain neurotoxins.

Homology modeling studies and molecular dynamics simulation. The homology study was performed using the MODELLER program ver.7v7 (Marti-Renom *et al*., 2000). Initially, the Bsaul1 sequence was aligned against the Ts1 sequence with known structure and we sought a good overall homology. The Ts1 (with protein data bank (PDB) code; 1NPI) was selected as a suitable template to construct a model of Bsaul1. The resulting Bsaul1 structure was relaxed and refined by molecular dynamics simulation with explicit water solvation. MD simulation was performed in the isobaric-isothermal ensemble (NPT) with octahedral periodic boundary condition using the program Amber 8 (Case *et al*., 2004). The initial model was placed in an octahedral box with about 3350 water molecules. The effective water density in the solvation box was 1.02 $g \cdot cm^{-3}$. Two Na^+ charge-balancing-counter ions were added to neutralize charge under protein surface. The simulation began with the 500 steepest descent steps of the energy minimization. Subsequently, MD simulation was performed at 300 K for 1.5 ns. Analysis and comparison of the structures were carried out using Swiss-PdbViewer ver3.7 (Guex and Peitsch, 1997) and MOLMOL (Koradi *et al*., 1996) programs.

Results

The crude venom of scorpion Buthotus saulcyi was first separated on Sephadex G-50 and further fractionated on C8 RP-HPLC column (Fig. 1). A peptide with a unique molecular mass

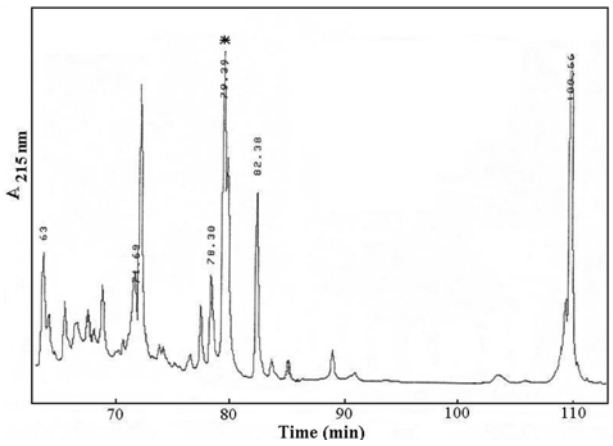

Fig. 1. Reverse-phase HPLC as the second step of the chromatographic purification of Bsaul1. The purification was done on a Vydac semi-preparative reversed phase C8 HPLC column equilibrated with 0.1% w/v TFA in water. The elution was carried out by a linear gradient of 0.089% TFA in 10-50% of acetonitrile. The flow rate was 2 ml/min and fractions were monitored at 215 nm. The labeled (*) fraction corresponds to Bsaul1. Every peak obtained was then collected and rechromatographed on the RP-HPLC column to homogeneity.

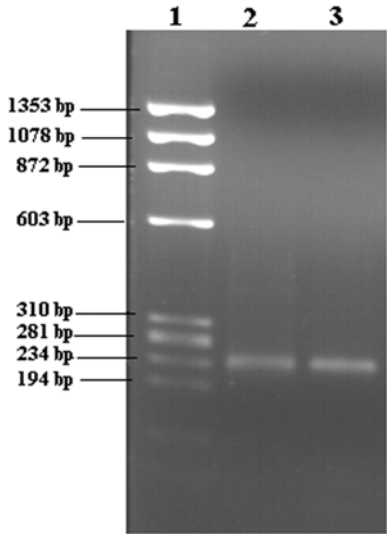

Fig. 2. Amplified cDNA of Bsaul1 gene on 1.5% agarose gel. From left to right, lane1, DNA size marker; lane 2, 3 PCR amplified Bsaul1.

(7419.75 ± 0.27) was then subjected to sequencing. N-terminal partial sequencing revealed the amino acid sequence of the first 9 amino acids of the purified peptide as: DGYIRKRDG, which is identical to N-terminal region of a depressant neurotoxin, LqqIT2. We named this new peptide as Bsaul1.

Amplification, cloning and sequencing of cDNA of Bsaul1. DNA fragment of 240 bp was amplified from the total RNAs of the venomous gland (Fig. 2). This fragment was then

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1                                                  10
GAC GGC TAT ATA AGA AAG AGA GAC GGA TGC AAG CTT TCA TGC AGT
D   G   Y   I   R   K   R   D   G   C   K   L   S   C   S

                   20                                  30
TTA GCA TGG TTG GAT AAT GAA AAT TGC CGT CAA GAG TGC ATT TCC
L   A   W   L   D   N   E   N   C   R   Q   E   C   I   S

                                   40
TAC GGT GGC ACT TAT GGA TAT TGC TGG TTC TGG GGA CTT GCG TGC
Y   G   G   T   Y   G   Y   C   W   F   W   G   L   A   C

                   50                                  60
TGG TGT GAA AAC CTA CCA GAC GAA AAG ACA TGG AAG AGT GAA ACA
W   C   E   N   L   P   D   E   K   T   W   K   S   E   T

AAC ACA TGC GGT TAA
N   T   C   G   *
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Fig. 3. The nucleotide and deduced amino acid sequences of Bsaul1 cDNA.

cloned and sequenced. According to the sequencing results, the peptide coding sequence was 192bp in length and codes a 64-residue peptide with 8 cysteines (Fig. 3). The sequence was deposited in GenBank: accession number **AY770502**.

Analysis of amino acid sequence alignments. A collection of depressant toxin sequences was used for comparative analysis. Multiple alignments of these sequences and Bsaul1 were done by ClustalW program (Fig. 4). The amino acid sequences of Bsaul1 and aligned depressant toxins share 66-82% similarity. Like other depressant toxins, Bsaul1 starts with an acidic residue and lack the preceding basic residue, which is an apparent characteristic of classical β-toxins (Polikarpov *et al*., 1999). All of these peptides share eight highly conserved cysteines. Also, the residues involved in the formation of β-strands are almost conserved. Differences are mainly observed in loop1, between β-strand1 and the α-helix. Moreover, the carboxy terminal region, starting after the third β-strand and protruding out of the α/β scaffold, is highly conserved among these toxins. Also, the amino acid content of α-helix in Bsaul1 is different compared to other similar depressant toxins, especially in replacement of the starting Gly with Asn, which is not a conservative replacement especially from side chain point of view.

Comparative analysis of Bsaul1 and other toxins sequences. Bsaul1 was included in a comparative analysis of α-, β-, excitatory and depressant amino acid toxin sequences (Fig. 5). All of these toxins share the disulfide bridges: Cys14-Cys38, Cys24-Cys45 and Cys28-Cys47, the strictly conserved sequences Gly36-Tyr37-Cys38 and Cys45-Tyr/Trp46-Cys47, the strictly conserved residues Gly2 (except for LqhαIT) Tyr3, Leu50 and Glu/Asp52 (all residues named according to Bsaul1 numbering). Cys10, responsible for anchoring the C-terminus to the CSαβ motif in classical β-toxins, is not conserved in the E-toxins. Gly9 and Glu27 are conserved among all β-, D-, and E-toxins. Glu27 is in the middle of a positively charged area and fully exposed to the solvent in the three-dimensional structures of β-toxins (Polikarpov *et al*.,

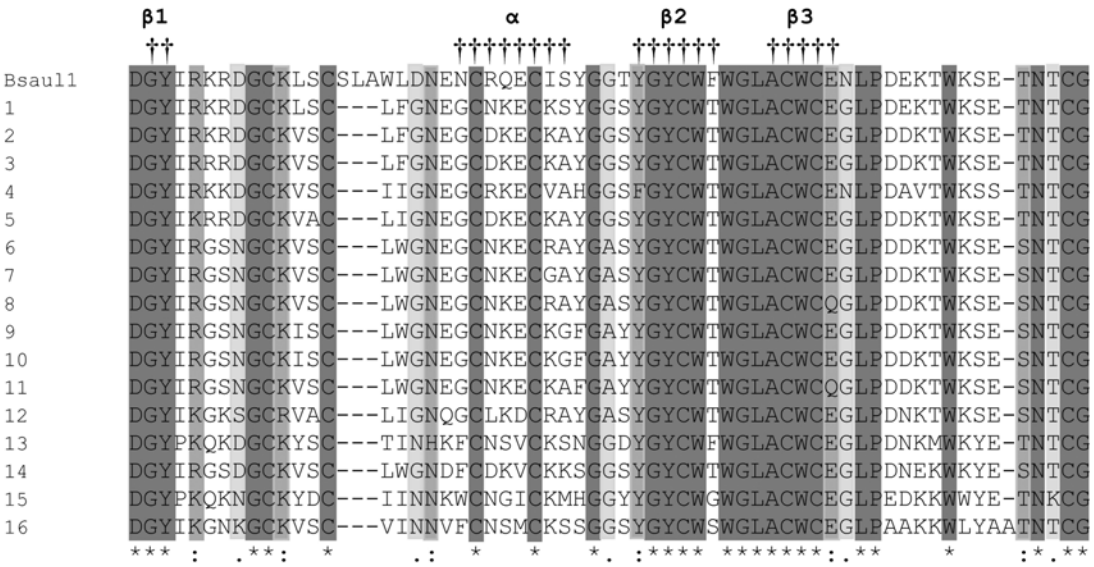

Fig. 4. Multiple sequence alignment of Bsaul1 and depressant toxins. Columns marked with " * " and colored by dark gray are the residues which are identical in all sequences in the alignment, " : " and medium gray mean that conserved substitutions have been observed in that column and semi-conserved substitutions are marked with " . " and colored by light gray. The alignment was performed using the program CLUSTALW. The names and accession numbers of sequences are as following:
1-Depressant insect neurotoxin, LqqIT2 (**AAB25388**); 2-Insect toxin 5, BotIT5 (**P55904**)
3-Insect toxin 4, BotIT4 (**P55903**); 4-Depressant insect neurotoxin, BjIT2 (**AAB25389**)
5-Depressant insect neurotoxin, LqhIT2 (**AAB25387**); 6-Insect toxin 3 (**CAA63059**)
7-Depressant insect toxin BmK ITa1 (**AAM09097**); 8-Depressant insect toxin BmK ITa (**AAD31592**)
9-Depressant scorpion toxin KIM2 (**AAN59783**); 10-Insect-toxin 4, BmkIT4 (**CAA63431**)
11-Depressant insect neurotoxin, BmK ITb (**AAF77063**); 12-Depressant insect toxin BmK IT2 (**P68727**)
13-Insecticidal toxin (**CAA07277**); 14-Depressant insect neurotoxin BmK ITc (**AAD4164**)
15-Insect toxin 6, BotIT6 (**P59864**); 16-Depressant insect-specific toxin BsIT4 (**D59352**)

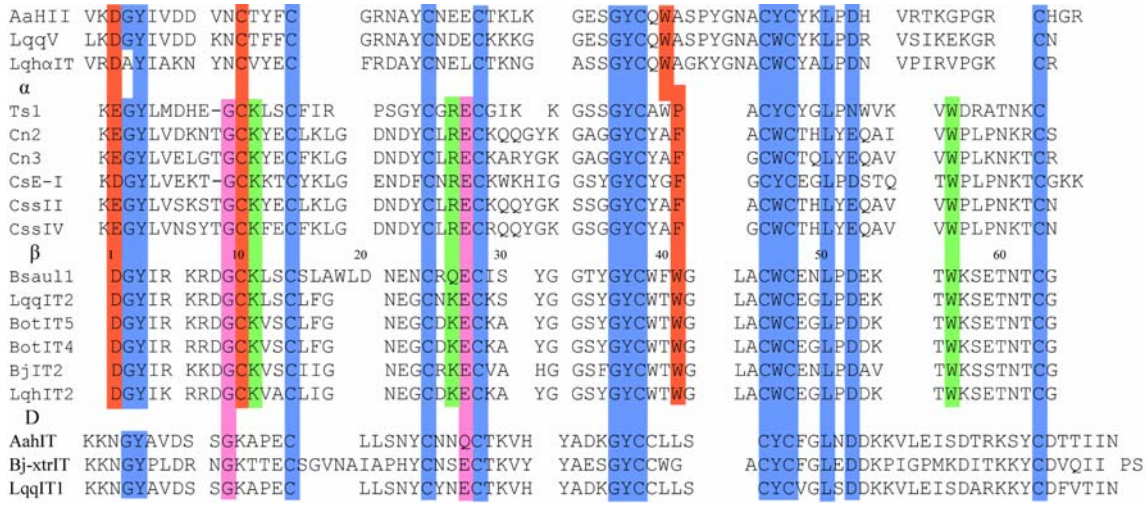

Fig. 5. Multiple sequence alignment of α- and β-, depressant (D) and excitatory (E) toxins. Columns colored blue, red, green, and pink indicated residues that are globally invariant, conserved in α/β and D, conserved in β and D and conserved in β/D/E, respectively. The alignment was performed using the program CLUSTALW and adjusted manually.

1999). All β- and D-toxins possess the highly conserved triad Gly9-Cys10-Lys11, the conserved Tyr/Trp56 and Lys/Arg26; however, this last position is occupied by Gln in Bsaul1.

The Bsaul1 three dimensional structure model studies. Bsaul1 structural model was constructed using Ts1 as template. Ts1 (Pinheiro *et al*., 2003) is a classical β-toxin that

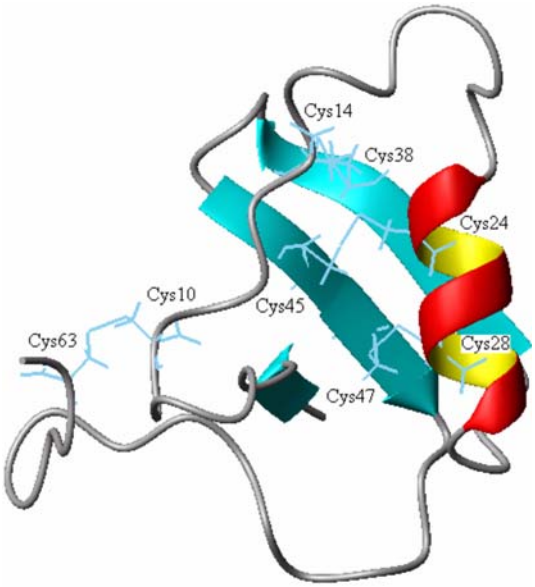

Fig. 6. Ribbon representation of the three-dimensional model of Bsaul1. The secondary structure elements of the core and the four disulfide bridges are shown.

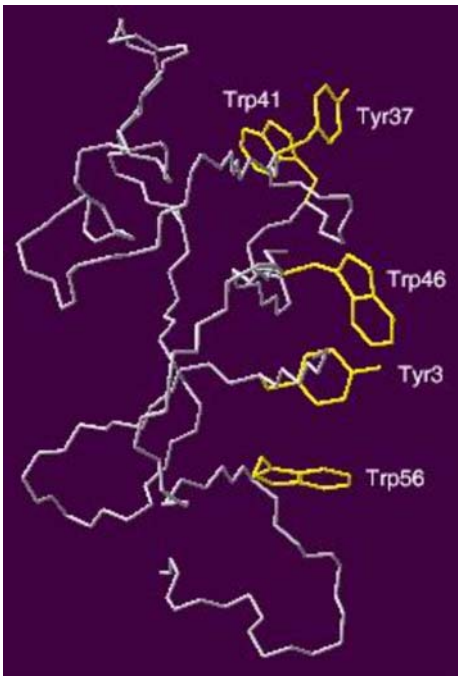

Fig. 7. Side view of three-dimensional model of Bsaul1 presenting the conserved aromatic cluster.

competes with the depressant insect-specific LqhIT2 and with the excitatory insect-specific AaHIT toxins, binding to closely correlated but distinct receptor sites of the sodium channel (Barhanin *et al*., 1984; de Lima *et al*., 1986; Gordon *et al*., 1992; Zilberberg *et al*., 1997; Oren *et al*., 1998; Froy *et al*., 1998).

According to the molecular model of Bsaul1, the fold of the polypeptide chain is similar to that of the other long-chain toxins. A schematic view of the Bsaul1 model is given in (Fig. 6). According to the computational model, Bsaul1 is folded into a three-stranded antiparallel β-sheet (residues 2-3, 35-40 and 44-48) and one α-helix (residues 23-30). The α-helix is linked to β-strand3 by two disulfide bridges, Cys24-Cys45 and Cys28-Cys47, which are conserved in all long chain toxins. Loop 1 between β-strand1 and the α-helix is linked to the core of the molecule by a third invariant disulfide bridge, Cys14-Cys38. The fourth disulfide bridge, Cys10-Cys63, links this loop to the C-terminus.

The Bsaul1 peptide can be visualized as having two distinct triangular flat faces opposite to each other. One face contains the conserved aromatic cluster (Fontecilla-Camps *et al*., 1981) formed by the residues Tyr3, Tyr37, Trp41, Trp46 and Trp56 (Fig. 7). In fact only Tyr3 and Tyr37 (in Bsaul1 numbering) are strictly conserved in all toxins. The aromatic ring of Trp43 in Ts1 makes van der Waals contacts with the side chains of Tyr4 and Tyr36 being roughly perpendicular to both of them (Polikarpov *et al*., 1999). The relative orientation adopted by the side chains of residues Tyr3, Tyr37, and Trp46 leads to the formation of a 'herringbone' pattern, which is the spatial arrangement corresponding to the lowest energy for relatively solvent-exposed aromatic rings (Burley and Petsko, 1985).

The aromatic residue Trp/Tyr56 and Lys11 (in Bsaul1 numbering) are conserved amongst all β-, and D-toxins (Darbon *et al*., 1983). The position and orientation of the side chains of these residues do not differ significantly among the Bsaul1 and Ts1 structures. Also, the spatial arrangement of the Glu27 residue, which is conserved in the α-helix of all depressant, excitatory and β-toxins (Cohen *et al*., 2004), is very similar in Bsaul1 and Ts1 (Fig. 8).

Our further experiments focused on establishment of an efficient expression system for recombinant Bsaul1, and its refolding *in vitro* into a fully functional toxin. Expression of Bsaul1 was carried out in *E. coli* strain M15 and the insoluble BsaulI accumulated within the inclusion bodies. This peptide was purified using Ni-NTA chromatography under denaturing conditions. The refolding of BsaulI was carried out by dilution of the denatured and purified peptide into a refolding buffer. Some initial studies carried out, suggest that the refolded Bsaul1 leads to a characteristic paralysis on blow fly (*Lucilla sericata*) larvae. Secondary structural properties of recombinant Bsaul1 were also confirmed by IR spectroscopy (unpublished work).

Discussion

In this paper we report the cDNA and amino acid sequence of a novel peptide from the scorpion *Buthotus saulcyi* which we named Bsaul1. Sequence alignments analysis showed that Bsaul1 sequence is similar to that of the insect depressant neurotoxins; however there are some differences between Bsaul1 and known scorpion neurotoxins (Fig. 4). The loop1

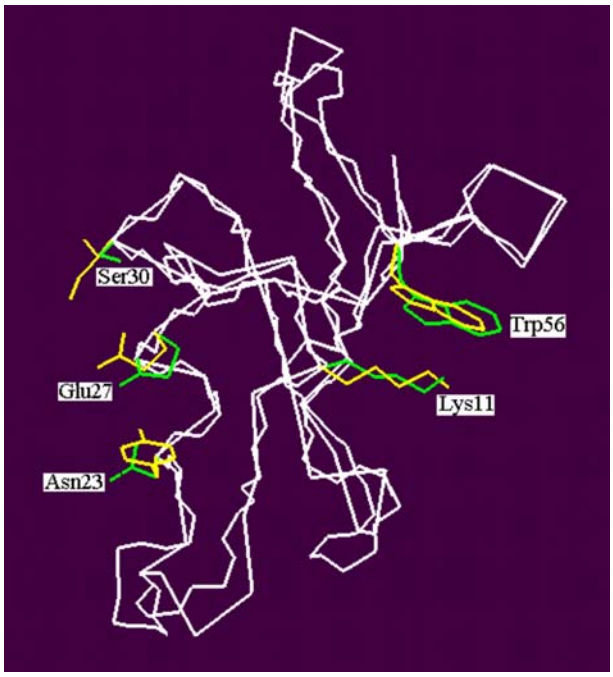

Fig. 8. Best-fit superposition of the backbone conformations of Bsaul1 (green) and Ts1 (yellow). The spatial arrangements of some conserved residues in Bsaul1 and Ts1 are indicated.

(between β-strand1 and the α-helix) is three amino acids longer in Bsaul1 as compared to other similar depressant insect toxins. There is a unique sequence composed of Ser15-Leu16-Ala17-Trp18-Leu19-Asp20 in loop1 region of Bsaul1 (Fig. 3) which involves three inserted amino acids. It has been suggested that the differences in bioactivity of the various toxins and their specificity of interactions with Na^+ channels are dictated by their non similar exterior composed of various amino acid side chains, loops and turns connecting the conserved secondary structure elements and the C-termini (Froy *et al*., 1998; Pinheiro *et al*., 2003). Therefore, it is reasonable to conclude that Bsaul1 may possess novel properties due to its unique loop sequence. The carboxy terminal region, starting after the third β-strand and protruding out of the α/β scaffold, is highly conserved among these toxins. This fact is in agreement with the hypothesis that the pharmacological versatility displayed by different groups of long chain neurotoxins might have been achieved along evolution via structural reconfiguration of the C-tail, in other word; the alterations in this region may lead to the formation of new molecular exteriors and induce a variety of bioactive surfaces (Gurevitz *et al*., 2001).

According to comparative analysis of amino acid sequences of Bsaul1 and α-, β-, excitatory and depressant toxins (Fig. 5) Bsaul1 contains all of the conserved residues, in line with other members of long chain neurotoxins. It may be concluded that the above mentioned conserved structural determinants may play a common role in structural/functional properties of Bsaul1 and other long chain neurotoxins.

According to the three-dimensional structure model (Fig. 5), Bsaul1 is composed of a three-stranded antiparallel β-sheet and one α-helix. The α-helix is linked to β-strand3 by two disulfide bridges, which are conserved in all long chain toxins. This structural motif is known as a cysteine stabilized α-helix/β-sheet (CSαβ) motif (Landon *et al*., 1997) and is found in many proteins exhibiting antibiotic and toxic activities (Landon *et al*., 1996). The presence of secondary structure elements was further confirmed by infrared spectroscopy of Bsaul1 after its expression and refolding.

One of two distinct triangular flat faces of Bsaul1 contains the conserved aromatic cluster (Fontecilla-Camps *et al*., 1981) formed by the residues Tyr3, Tyr37, Trp41, Trp46 and Trp56 (Fig. 6). It has been suggested (Fontecilla-Camps *et al*., 1988; Landon *et al*., 1997) that the hydrophobic patch is a conserved part of all scorpion toxins governing the interactions of these polypeptides with their receptors, where as the adjacent regions are variable and may modulate their selectivity to insects or mammals. However, this hypothesis has not been experimentally verified (Possani *et al*., 1999). Such a flat surface formed by aromatic side chains exists, to a certain extent, in all long-chain neurotoxins and is commonly known as the "conserved hydrophobic surface" (Fontecilla-Camps *et al*., 1981) or "herring bone motif" (Landon *et al*., 1997). It should be stressed that the conserved feature is the surface itself and not the residues forming the surface.

It was shown by the three-dimensional model that the position and orientation of the side chains of Trp/Tyr56 and Lys11 (in Bsaul1 numbering) does not differ significantly among the Bsaul1 and Ts1 structures (Fig. 8). In Ts1, the side chains of residues Lys12, Trp54 and Arg56 coordinate the PhoB ion (Polikarpov *et al*., 1999). The Lys12 and Trp54 are conserved among the β-scorpion long chain toxins and seem to be involved in the binding specificity of the classical β-toxins (Polikarpov *et al*., 1999), but Arg56 is not conserved in the β-toxins. In Bsaul1, Lys11 and Trp56 occur in the same place and orientation as Lys12 and Trp54 in Ts1 and thereby may play a similar role.

It was recently suggested that a Glu residue (Glu30 in Bj-xtrIT, Glu28 in Cn2 and Glu 26 in Ts1), surrounded by a solvent-occluding gasket (its flanking hydrophobic residues), adjacent to a nonpolar cluster constitute the "pharmacophore" in β-toxins (Cohen *et al*., 2004). This Glu residue, conserved in the α-helix of all β-toxins, most likely interacts electrostatically with a positively charged receptor counterpart. This interaction is protected by the hydrophobic seal around Glu residue. Superposition of the three-dimensional model of Bsaul1 and three dimensional structure of Ts1 reveals that the spatial arrangement of Glu27 in Bsaul1 and Glu26 are very similar. It has been suggested that the striking similarity in the spatial arrangement of this Glu residue and its surrounding and adjacent residues in distinct β-toxins may explain the competition among them on binding to Na^+ channels (Cohen *et al*., 2004). The Glu27 flanking residues in Bsaul1 (Asn23 and Ser30) are less hydrophobic than in Ts1 (Tyr22 and Ile29) and Bj-XtrIT

(Tyr26 and Val34). The loop preceding the α-helix of Bsaul1 contains solvent-exposed hydrophobic residues (Leu16, Ala17, Trp18 and Leu19) similar to the corresponding region in Bj-xtrIT (Val19, Ile22, Ala23 and Pro24) and Ts1 (Phe16 and Ile17). Interestingly, the three inserted residues in loop1 of Bsaul1 are located in the above mentioned hydrophobic cluster and are of particular interest for further investigation of binding properties.

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