Purification and characterization of a short insect toxin from the venom of the scorpion *Buthus tamulus*

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Abstract **A short chain peptide has been isolated from the venom of a red scorpion of Indian origin, *Buthus tamulus*. This peptide was purified using ion exchange and reverse phase chromatography and was characterized by molecular weight determination and amino acid sequence. The primary structure analysis shows that BtITx3 is a short peptide of 35 amino acid residues having a molecular weight of 3796 Da. The toxin shows toxicity towards the Lepidopteran species of insect *Helicoverpa armigera* causing flaccid paralysis and even death within 24 h. It shows more than 50% homology with the short insectotoxins having four disulfide bridges, which suggests that the toxin belongs to the class of short chain toxins blocking the chloride ion channels. This sequence homology study has also helped to bring out the structure–function relationship between the various short toxins. Homology modeling done by using template structure of a known toxin indicated that this toxin consists of a similar α/β scaffold, as present in other scorpion toxins.** © **2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Scorpion; Toxin; Homology; Insectotoxin; *Buthus tamulus*; *Helicoverpa armigera*

1. Introduction

Scorpion venom consists of numerous polypeptides [1], many of which can interfere with the activity of ion channels and modulate their functional properties. These polypeptides have been used as tools to isolate ion channels and to study the pharmacology [2] and molecular mechanism of action of the ion channels. They possess similar structural scaffolds but have different binding properties, which can be used as powerful tools for probing the structural aspect of toxin–receptor interactions. The sodium channel inactivating toxins acting on rats as well as insects have similar active sites in spite of their structural and pharmacological differences [3]. These polypeptides have different physiological and pharmacological activities [4]. They are specific towards mammals, insects [5] and crustaceans [6]. The anti-insect toxins have been largely used as a means for biological insect control [7,8]. Scorpions belonging to the Buthidae family are medicinally important and more toxic than the other scorpion species.

Scorpion toxins can be divided into four groups depending on their physiological and pharmacological activities. The first class belongs to the toxins acting on the Na^+ channels, which consists of 60–70 amino acid residues and four intermolecular disulfide bonds. These long chain toxins modulate the activation or the inactivation mechanism of the Na^+ channels and are classified into 10 subfamilies [9]. The second class of toxins acting on the K^+ channel has been further divided into three more classes – α-KTx with 23–40 amino acid residues having three or four disulfide bonds [10], β-KTx consisting of peptides with 60–64 amino acid residues and only three disulfide bonds [11] and γ-KTx having a peptide named Erg-Tx1 [12]. The third class of scorpion toxins acting on the chloride channels has 35–37 amino acids residues with four disulfide bonds [13]. The fourth class includes toxins that act on Ca^{2+} channels. There are two peptides in this class consisting of 33 and 27 amino acid residues respectively, which modify the rayodine-sensitive Ca^{2+} channels [14,15]. Potassium channel toxins have been studied more extensively as compared to chloride channel toxins [16]. Chlorotoxin, a chloride channel blocking toxin isolated from the scorpion *Leiureus quinquestriatus*, shows highest homology with short insect toxins [17]. This toxin has provided some lead in the therapy of brain cancer [18]. All these scorpion toxins have long been studied to reveal the structure of the ion channels and to understand the mechanism of toxin–receptor interaction.

The Indian scorpion (*Mesobuthus tamulus*) is a dangerously venomous species, especially for children [19]. It is medically the most important species of scorpion on the Indian subcontinent. There are reports on the characterization of active fractions from the scorpion venom *Buthus tamulus* that not only act on various ion channels but are also found to be protease inhibitors and histamine releasers [20]. Iberiotoxin isolated from *B. tamulus* venom is a unique and highly specific blocker of high conductance calcium-activated potassium channel and has been completely characterized [21]. Tamulustoxin is another toxin isolated from *B. tamulus* venom, which is active on the K^+ channel [22]. Bt-II, a mammalian-specific toxin [23], and ButaIT, an insect-specific toxin, are also isolated from *B. tamulus* [24].

This paper describes the isolation, purification, and sequence determination of a short toxin, BtITx3, isolated from the Indian scorpion *B. tamulus*. This toxin shows significant homology with other short insectotoxins like I5A, chlorotoxin etc. It is toxic towards the insects belonging to the Lepidopteran species *Helicoverpa armigera*. An attempt has been made to compare the amino acid sequences of short chain toxins which are K^+ and Cl^- ion channel blockers with that of BtITx3 to understand the structure–activity relationship of the isolated peptide reported here. The three-di-

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mensional structure was generated with the help of Swiss PDB modeler to study the structural scaffold of the toxins, which along with the knowledge of the net charge from the primary sequence helps us to identify the residues responsible for selectivity for the insect chloride channel. This further helps to delineate the difference in the binding capacity of the toxin to various ion channels.

2. Materials and methods

2.1. Materials

All solvents used for separation were of high performance liquid chromatography (HPLC) grade obtained from Spectrochem (India). The chemicals used for sequencing were purchased from Wako (Japan). Bovine serum albumin used as a standard for protein assay was obtained from SRL (India). All chemicals used for preparation of buffers were of analytical grade. All buffers were filtered through 0.45 μm filters (Sartorius, USA). The crude venom was filtered using 0.2 μm filters (Pall Gelman Labs, USA). Peptide sequencing was done on a gas phase sequencer (PPSQ-10) from Shimadzu, consisting of a HPLC system with a reverse phase C18 column (4.6×250 mm), a SPD-10 UV detector and a multi-window chromatogram integrator CR-7A. Absorbance was measured on a UV-265 spectrophotometer from Shimadzu. The polyvinylidene difluoride (PVDF) membrane used for immobilizing the protein was Immobilon-PSQ from Millipore. Centrifugation was done in a Beckman J2-21M/E using a J-18 rotor, and in a Beckman micro-centrifuge using a fixed angle rotor. The column used for ion exchange chromatography was a strong cationic column, Resource S (1 ml) (6.4×30 mm) from Pharmacia, and reverse phase HPLC was done on C4 and C18 semi-preparative columns (22×250 mm) from Vydac. A speed vac concentrator (Savant) was used for lyophilization of the protein samples.

2.2. Sample preparation

Venom from the scorpion *B. tamulus* was obtained in a lyophilized state, from Haffkine Institute, Parel, Mumbai, India. The crude venom (60 mg) was weighed and dissolved in 20 ml of water and stirred at 4°C for 4 h. The solution was centrifuged at 13 300×*g* at 4°C for 20 min to separate the mucous material of the venom. The clear supernatant was separated and the pellet was resuspended in 20 ml water and stirred for 4 h and again centrifuged. The two supernatants were pooled and filtered through a 0.2 μm filter and then lyophilized in a speed vac concentrator. Protein estimation was done by Lowry's method using bovine serum albumin as standard [25]. This aqueous extract was used for further purification.

2.3. Purification

The filtered aqueous crude venom extract was used for isolation of various peptides. The aqueous extract was applied on a Resource S (1 ml) (Pharmacia, 6.4×30 mm) cation exchange HPLC column, equilibrated with 20 mM sodium acetate buffer, pH 5. The column was eluted with 20 mM sodium acetate buffer, pH 5 using a linear gradient of 0–0.5 M NaCl at a flow rate of 0.5 ml/min. The elution profile showed several peaks (S1–S9). These were collected separately and the absorbance was measured at 215 nm. The fraction eluting at 25 min was one of the major peaks (S4). This fraction was collected and lyophilized in a speed vac concentrator and was subjected to further purification on a C4 Vydac semipreparative reverse phase HPLC column (22×250 mm), equilibrated with 0.1% of trifluoroacetic acid (TFA) in water [26]. Separation was done using a linear gradient of 0.1% TFA in acetonitrile from 15 to 100%. The absorbance was measured at 215 nm as well as 280 nm. Various fractions eluting from the column were collected. These fractions were lyophilized and applied on a C18 (Vydac) semi-preparative reverse phase HPLC column for final purification. Separation was done using a linear gradient of 0.1% TFA in acetonitrile starting from 15 to 100%. The eluted fractions were collected and once again lyophilized.

2.4. Mass spectroscopy

MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) analysis was performed on a Kratos Analytical system (Manchester, UK). The method used for sample preparation was the dried drop method. A volume of 0.5 μl (1.0 pmol/μl) was spotted on the plate, followed by 0.5 μl of the matrix CHCA (α-cyano-4-hydroxycinnamic acid) solution and dried at room temperature. The nitrogen laser used for the desorption of the sample was kept at 337 nm.

2.5. Sequence analysis

The primary sequence of the toxin was determined by Edman degradation reaction using the PPSQ-10 sequencer from Shimadzu equipped with an LC-10A HPLC system and C18 reverse phase HPLC column. The scorpion toxins are known to have three to four disulfide bonds, hence to determine the position of cysteine residues the peptides have to be first reduced and alkylated. The toxin was immobilized on Immobilon-PSQ PVDF (Millipore) and 5 μl of the mixture of 0.6% tri-*n*-butylphosphine and 1.2 μl of 4-vinylpyridine in 80% isopropanol was added for the simultaneous reduction and alkylation of the toxin successively followed by Edman degradation reaction. The optimal reaction temperature and time for the reaction were 60°C and 50 min respectively.

2.6. Toxicity test

The toxicity test was done on the cotton bollworm *H. armigera* that is a serious polyphagous noctuid that damages a number of crops in India. The larva used for test was in the early fourth instar stage, 25 mm long and weight ranging from 90 to 120 mg. The crude venom was dissolved in water and injected in various concentrations to determine the LD_{50}. The purified fraction was also dissolved in water and injected into the thoracic region of the larva. The control was injected with 5 μl of water. Symptoms of contraction for 5–10 s were taken as a positive response and paralysis was observed continuously for a period of 30 min. The insect was kept under observation for 24 h, post injection.

2.7. Homology modeling

The multiple sequence alignment was done using CLUSTALW. One-dimensional homology modeling of the sequence was done using the BLASTP algorithm at the URL http://sdmc.krdl.org.sg:8080/scorpion/ over the Scorpion toxin database. The sequences were also aligned using the BLASTP algorithm [27]. Scoring was done using the BLOSUM62 matrices [28]. We used Swiss Modeler to generate the model. This model was then subjected for check to the commonly used WHAT IF [29] and PROCHECK programs [30]. The final model structure thus obtained was then subjected to refinement by energy minimization in Swiss Modeler using a GROMOS96 force field.

3. Results

3.1. Purification of the toxin

The crude venom (60 mg) was fractionated using a strong cationic Resource S column, which gave nine fractions. The nine eluted fractions were collected and numbered S1–S9. These fractions were tested for biological activity in *H. armigera*. Fraction S4, one of the major peaks eluting at 25 min and also active against *H. armigera*, was taken up for further purification. This fraction was loaded on a C4 reverse phase HPLC column and eluted using a linear gradient of acetonitrile (Fig. 1A). Three major peaks were isolated from this column. Of these three peaks, the peak that eluted at 17.8 min was further purified to homogeneity by reverse phase HPLC on a C18 column. The final purified protein eluted at 21.4 min (Fig. 1B) and was named BtITx3. About 40–50 μg (0.15%) of the purified fractions was obtained from 60 mg of the crude venom.

3.2. Bioassay

The crude venom was injected into the thoracic region of fourth instar larvae of the insect *H. armigera*, in different concentrations, and its LD_{50} was found to be 5.31 μg/100 mg of larva. The larvae showed symptoms of contraction for 5–10 s after injection leading to severe paralysis, which was monitored for 30 min. The purified fraction was also

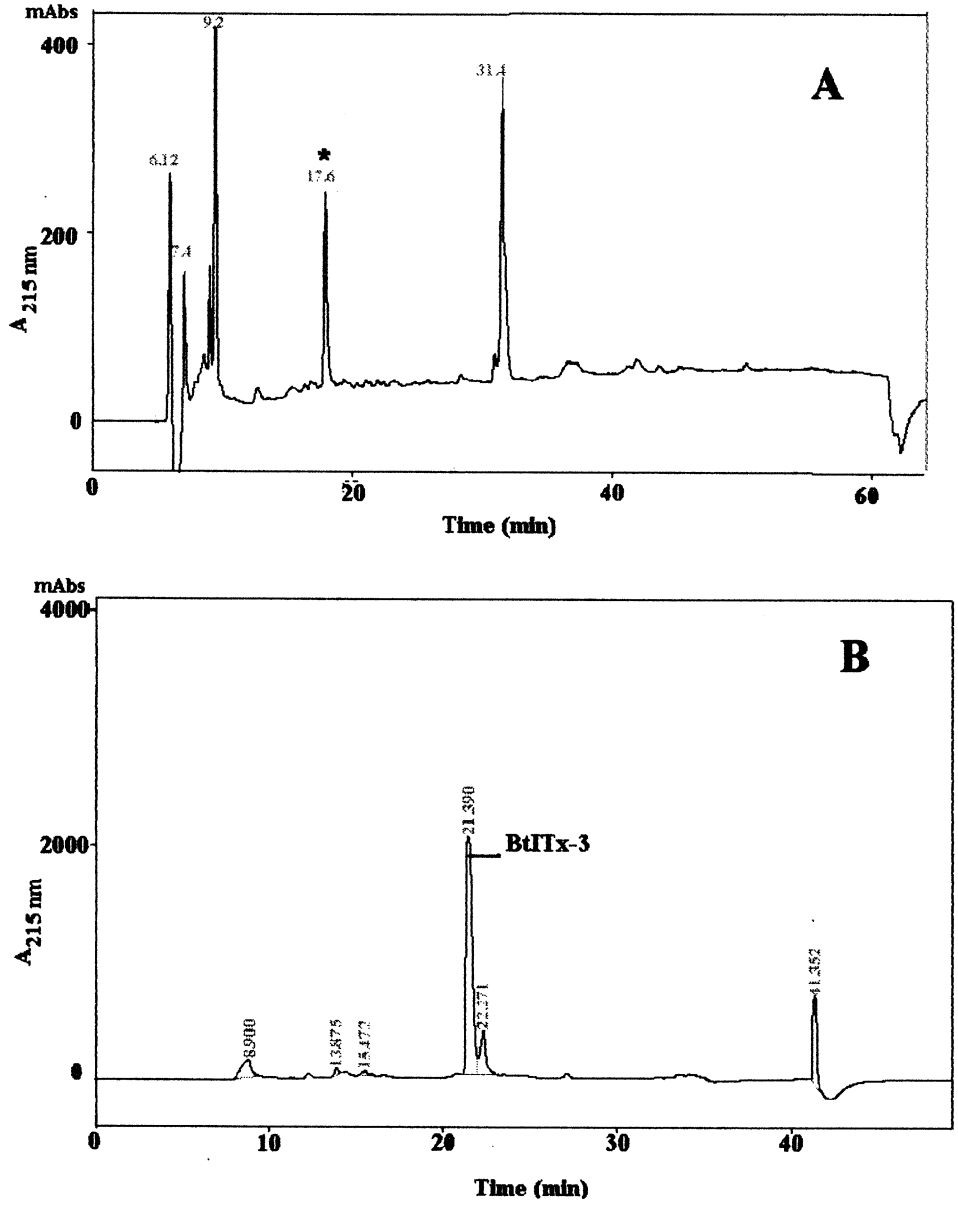

Fig. 1. Purification of the water extract of the crude venom from *B. tamulus*. The crude venom (60 mg) was fractionated using a strong cationic Resource S column, which gives nine fractions, S1–S9. A: The labeled fraction (*) was purified by loading on a Vydac C4 reverse phase HPLC column equilibrated with 0.1% TFA in water. The elution was carried out by a linear gradient of 0.1% TFA in 15–100% acetonitrile. The flow rate was 2.4 ml/min and fractions were monitored at 215 nm. Fractions were collected and lyophilized. B: The final purification was done on a Vydac C18 reverse phase column. The peak labeled BtITx3 was collected and used for further processing.

injected into larvae in a similar manner as the crude venom. The toxin was found to be effective at a dose of 2 μg/100 mg of larva. The effect of the toxin showed initial contraction and then severe paralysis was observed for more than 30 min and the larva was dead within 24 h. Thus BtITx3 was found to be a lethal toxin.

3.3. Structural analysis

The primary sequence analysis of this peptide shows that this is a short chain peptide with four disulfide bridges. Cysteine identification is typically difficult while sequencing peptides using the Edman degradation reaction, because its phenylthiohydantoin derivative is unstable and thus cysteine is reduced and alkylated with tri-*n*-butylphosphine and 4-vinylpyridine respectively. The primary structure of BtITx3 shows the presence of 35 amino acid residues with eight cysteine residues. The multiple sequence alignment of this peptide shows considerable homology (Fig. 2) with the short insectotoxins, which block the chloride channels. The primary sequence analysis of the peptide gave the following sequence: BtITx3 RCPPCFTTNPNMEADCRKCCGGRGYCASYQ-CICPG.

The molecular mass of the purified peptide BtITx3 as determined by MALDI-TOF was 3796.5 Da (Fig. 3), which is in good agreement with the calculated molecular weight of 3798 Da. The insectotoxins have been divided into long and short

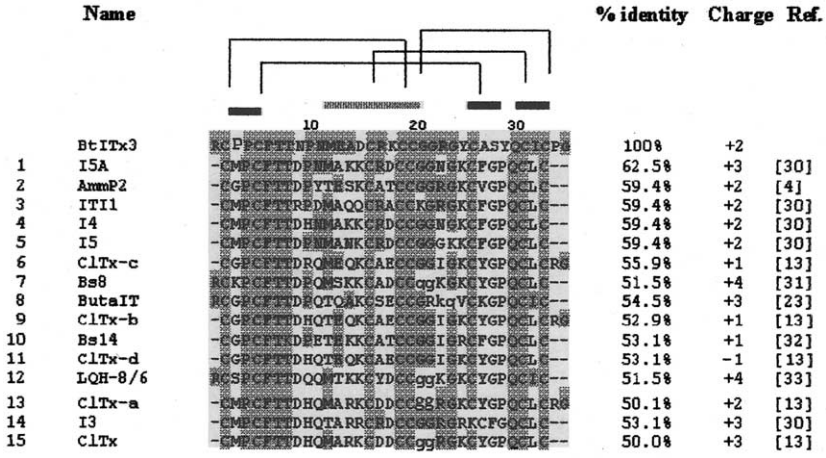

Fig. 2. Amino acid sequence of BtITx3 showing multiple sequence alignment with the short insectotoxin. The target sequence has been aligned based on the positions of cysteine as well as the other conserved residues present in the short insectotoxins. The percentage identity between the toxins and the net charge associated with them are shown on the right. The figure shows homology between the following toxins I5A, ITI1, I4, I5 and I3 from *Buthus eupeus* [31], AmmP2 from *Androctonus mauretanicus* [4,31], ClTx, ClTx-a, b, c and d from *Leiureus quinquestriatus quinquestriatus* [4,13], Bs8 and Bs14 from *Mesobuthus sindicus* [32,33], LQH-8/6 from *L. quinquestriatus hebraeus* [34], ButaIT from *B. tamulus* [24]. The pattern of disulfide bridges as determined by the structure of chlorotoxin is shown in this figure. Regions of secondary structure as α-helix (gray bar) and two antiparallel β-strands (black bar) are also indicated.

insect toxins [26], which block the sodium and the chloride channel respectively. The multiple sequence alignment of the isolated toxin was done on the basis of similar cysteine positions, and other conserved N- and C-terminal residues. The toxin was found to be highly homologous to the short insect toxins, which belong to the chloride channel blocking category. The toxin BtITx3 shows the highest homology (Fig. 2) with the insectotoxins I5A, ITI1, I4, I5 and I3 (62.5%, 59.4%, 59.4%, 59.4% and 53.1% respectively) from *Buthus eupeus* [31], AmmP2 (59.4%) from *Androctonus mauretanicus* [4], ClTx, ClTx-a, b, c and d (50.0%, 50.1%, 52.9%, 55.9% and 53.1% respectively) from *Leiureus quinquestriatus quinquestriatus* [13], Bs8 (51.5%) and Bs14(53.1%) from *Mesobuthus sindicus* [32,33] and LQH-8/6. (51.5%) from *L. quinquestriatus hebraeus* [34]. Short insectotoxins have a net charge ranging from +2 to +5, making them basic peptides. The net charge observed on this peptide is +2, which also falls in the same range.

The alignment of the primary sequence of BtITx3 shows 50–60% homology with the short insect toxins based on which the three-dimensional structure of I5A (1SIS) was taken as the template to build the model structure of the desired toxin. BtITx3 was modeled with the template structure of the *Mesobuthus eupeus* toxin (I5A) [35] previously determined by nuclear magnetic resonance (NMR) (1SIS), showing one α-helix from Asn11 to Asp18 and a β-sheet consisting of three β-strands, one from Met3 to Cys5, the second from Lys25 to Phe27 and the third from Pro29 to Leu32 (Fig. 4). The final modeled structure also has one α-helix from Asn11 to Lys18 and a β-sheet consisting of three β-strands, one of them from Pro3 to Cys5, the second from Tyr25 to Ala27 and the last one from Tyr29 to Ile32. The quality of the model for BtITx3 was assessed using the program WHAT IF, which showed that the model was as good as that of the homologous toxin structure 1SIS, implying good stereochemical quality. The PROCHECK report shows that the model has a Ramachandran score of 75.5, which is close to that of the template 76.9. Other stereochemical properties are also in agreement with that of the template, which indicates that the model structure of BtITx3 shows a similar scaffold as possessed by the other short insectotoxins blocking the chloride channel.

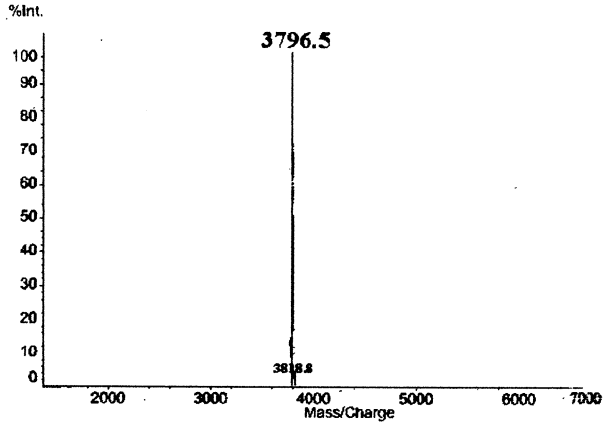

Fig. 3. MALDI-TOF mass spectrum of BtITx3.

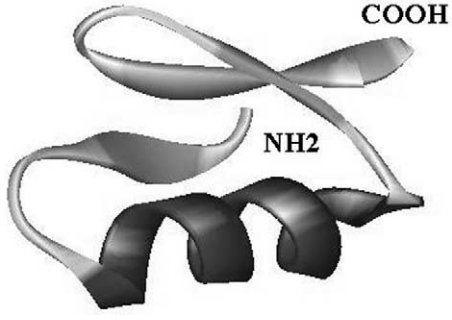

Fig. 4. The three-dimensional structure of BtITx3 derived by Swiss Modeler taking the NMR coordinates of insectotoxin I5A. The structure was visualized on Weblab viewer. The structure shows a α/β scaffold with one α-helix and three β-strands.

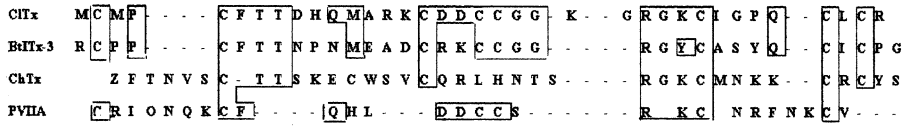

Fig. 5. Primary structure comparison between BtITx3 and other short chain scorpion toxins like chlorotoxin (ClTx), charybdotoxin (ChTx) and conotoxin (PVIIA). The alignment was done manually based on the conserved cysteine residues. The identical sequences between these toxins are shown (boxed).

4. Discussion

Scorpion toxins show a similar α/β scaffold despite the large variation in their size, origin and functional properties. The three-dimensional structure mainly consists of a small three-stranded antiparallel β-sheet packed against an α-helix (N-terminal), which is roughly parallel to the β-sheet. The two β-strands are linked to the α-helix by three disulfide bonds in the case of the chloride channel toxins and two in the case of the potassium channel toxins [36]. The multiple sequence alignment among the short insect toxins shows a high degree of consensus. The toxin BtITx3 shows a similar pattern of cysteine residues as that found in the chloride channel blockers. There is a remarkable feature of the chloride channel blocking toxins, showing a major difference in the α-helix, with a shift of one turn toward the N-terminus, whereas in the case of potassium channel blockers the central part of the helix has the characteristic C-X-X-X-C motif. Thus, the primary structure shows a C-X-X-X-C pattern in potassium channel blockers and C-X-X-C-C in the case of chloride channel toxins. The fourth disulfide bond in the chloride channel helps to link the N-terminal of the molecule with the rest of the molecule, while in the case of toxins blocking the potassium channel this is done with the help of the hydrophobic residues present in the β-turn, which link with the first residue at the N-terminal.

The difference in the specificity towards various ion channel toxins is dependent on the key residues of the toxin, which bind with the ion channel receptor. The distribution of functionally important residues involved in the toxin–channel interaction is different in chloride channel toxins as compared to potassium channel toxins. The toxins belonging to the potassium channel family like charybdotoxin have their binding residues located in the β-sheet, which is the solvent-exposed part of the toxin. Functionally important residues present in this region of charybdotoxin are Arg25, Lys27 and Arg34, which bind to the potassium ion channel. Chloride channel toxins also show the presence of Arg24 and Lys25, but Arg34 is replaced by Leu or Ile, and the positively charged surface important for binding the receptor is present in the helical region.

The primary sequence comparison of the chloride channel blocking toxin chlorotoxin with that of the potassium ion channel blocker charybdotoxin shows (Fig. 5) 10 conserved residues, and similar residues as Arg25, Gly26, Lys27 and Cys28 residing in one of the β-sheets. Based on the pattern of primary sequence homology BtITx3 shows 10 residues, other than the cysteine residues, which are similar to chlorotoxin and four residues similar to the potassium channel blocker charybdotoxin. The potassium channel toxins are known to interact with the outer vestibule of the channel by the residue Lys27 that occludes the channel [37]. The chloride channel toxins like chlorotoxin have been found to inhibit epithelial chloride channel activity only when applied through the intracellular region. Moreover, the crucial residue Lys27 is also present in chlorotoxin but it does not bind to the voltage-gated ion channel as observed in the case of charybdotoxin. The residues present in the C-terminal β-sheet of chlorotoxin are similar to that of the potassium channel blocking toxin charybdotoxin, i.e. Arg25 and Lys27, except Arg34, which is replaced by neutral residue Leu34. Further in chlorotoxin the N-terminal helix residues Asp17 and Asp18, just before the fourth cysteine residue Cys19, neutralize the positive charge effect of the residues in the C-terminal β-sheet. These functionally important residues in the case of toxin BtITx3 are replaced by Arg23 and Try25. However, residues Arg17 and Lys18 are basic unlike the acidic residues in the case of chlorotoxin. This indicates that toxin BtITx3 might have a different mode of binding the chloride channel. This toxin, however, does not have the most crucial residue Lys27 which is actually responsible for the occlusion of the pore of potassium ion channel.

In conclusion, the insect-selective toxin BtITx3 can be classified as one of the members of the chloride channel toxins and further structural and mutagenesis studies are being carried out to understand its molecular mechanism as well as to exploit its insecticidal activity.

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