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# A NOVEL Kv1.1 POTASSIUM CHANNEL BLOCKING TOXIN FROM THE VENOM OF *Palamneus gravimanus* (INDIAN BLACK SCORPION)

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**ABSTRACT:** A peptide toxin was isolated from the venom of *Palamneus gravimanus*, the Indian black scorpion, to block human Kv1.1 channels expressed in *Xenopus laevis* oocytes. A 4.5 kD peptide (toxin), as confirmed by SDS-PAGE, was purified to homogeneity by ion exchange chromatography using CM-Sephadex C-25 followed by Sephadex G-50 gel filtration. *Palamneus gravimanus* toxin (PGT) selectively blocks the human cloned voltage-gated potassium channel hKv1.1 in a two-electrode voltage-clamp (TEVC) technique. The results obtained indicate that the toxin blocks the hKv1.1 channel at a nanomolar concentration range ($K_i$ value of 10 nM) of the peptide to the external side of the cell. The blockage seems to be voltage-dependent. Comparative structure of PGT (a 4.5 kD peptide) with BTK-2 suggests a close relationship; therefore this toxin can be employed to investigate the hKv1.1 channel structure.

**KEY WORDS:** hKv1.1, $K^+$ channel, PGT, TEVC, *Palamneus gravimanus, Xenopus laevis* oocytes, Indian black scorpion.

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## INTRODUCTION

Many toxins have been isolated from snake and scorpion venoms. Scorpion venoms contain several toxins, which are proved to be high-affinity ligands of ion channels making them useful as pharmacological probes (7). Two main groups of scorpion toxins have been distinguished based on their pharmacological properties. They either modulate the activity of $Na^+$ channels excitable cells or specifically block $K^+$ channels (14). This classification, based on their pharmacological action, also agrees well with the structural properties of this peptide family, since toxins active against $Na^+$ channels are made up of a polypeptide chain of 60-80 amino acid residues reticulated by four disulfide bridges, versus the 30-40 amino acids and three to four disulfide bridges of those active against $K^+$ channels.

Potassium channels are present in all cells and are known to regulate the cell membrane potential. They are particularly important in neuronal cells, where they regulate the repolarization phase of the action potential dependent excitability of the neuron. If the potassium channels are blocked by drugs, the action potential tends to be prolonged, and if this happens at a nerve terminal it results in a prolonged depolarization, allowing calcium channels to remain open for a longer period and cause a greater release of the neurotransmitter substance.

In order to study the role of potassium channels and their subtypes in physiology, it is helpful to have biological tools that interfere with the channel activity. Small molecules like 3.4-diaminopyridine and tetraethyl ammonium, though known to block potassium channels, are not very specific and potent for particular subtypes of channels (9). The most useful tools for studying the potassium channel physiology are naturally occurring small peptides isolated from different scorpion venoms. Hence, studying the physiology and pharmacology of $K^+$ channels by using scorpion toxin probes has gained importance in structural biology and neuropharmacology.

We have purified a novel peptide toxin (PGT) from the venom of the Indian black scorpion *Palamneus gravimanus.* An attempt has been made to characterize this peptide toxin regarding its molecular weight, $LD_{50}$, and electrophysiological action on hKv1.1 potassium channel. PGT isolated from *P. gravimanus* showed its highest homology to BTK-2, a 3.5 kD peptide isolated from *Buthus tamulus* (Indian red scorpion), in general, and its specificity towards inhibition of the hKv1.1 channel, in particular.

## MATERIALS AND METHODS

### *Materials*

*Palamneus gravimanus* lyophilized crude venom was obtained from the Haffkine Institute, Parel, Mumbai, India. CM-Sephadex C-25 column gel (Pharmacia, Sweden) and Sephadex G-50 (Sigma Chemicals, USA) were used. Bovine serum albumin (BSA), used as a standard for protein assay, was obtained from Himedia, Mumbai, India. The chemicals used for buffer preparation were of analytical grade. We also utilized UV-Visible spectrophotometer from Elico (India); acrylamide; bisacrylamide; sodium dodecyl sulphate; Bromophenol Blue; Coomassie Brilliant Blue R-250 (Himedia, Mumbai); TEMED (N,N,N',N'-tetramethylethylenediamine); and broad range molecular weight markers PMW-B (Bangalore, Genei, India).

### *Purification*

Lyophilized crude venom of *Palamneus gravimanus* was weighed (100 mg), dissolved in 20 ml of water and stirred at 4°C for 4h. This was next centrifuged at 10,000 *g* at 4°C for 20 min to separate the mucous from the venom. The clear supernatant was separated and the pellet was resuspended in 20 ml of water, stirred for 4h, and centrifuged; the supernatant was pooled and filtered in a 0.2 µm filter and then lyophilized on a Hindvac speed lyophilizer.

The processed and lyophilized crude venom was fractionated on a CM-Sephadex C-25 column by the method of Ramachandran *et al.* (15). *Palamneus gravimanus* venom (100 mg) dissolved in 5 ml of 0.05M Tris-HCl buffer, pH 8.5, was loaded on a previously equilibrated CM-Sephadex C-25 column (1.5 x 18 cm). After washing the column with 500 ml of 0.05M Tris-HCl buffer (pH 8.5), the venom components were eluted using Tris-HCl buffer, pH 8.5, with a linear gradient of 250 ml 0-0.5 M NaCl at a flow rate of 40 ml/h and 4 ml fractions were collected. Protein elution profile was monitored at 280 nm on a UV-Visible spectrophotometer. Fractions showing the highest toxicity towards white mice were pooled, dialyzed, and lyophilized.

### *Gel filtration on Sephadex G-50*

Fractions showing toxicity towards white mice were pooled, dialyzed, lyophilized, subsequently subjected to gel filtration on a Sephadex G-50 column (1.5 x 60 cm), and eluted with 0.01 M Tris-HCl buffer (pH 8.5). The protein was eluted at a flow rate of 12 ml/h. Fractions of 3 ml were collected and the elution was monitored at 280 nm

on a UV-spectrophotometer. The highest toxic protein peak eluted from the column was pooled, dialyzed, lyophilized and then subjected to SDS-PAGE to confirm its homogeneity, molecular weight, $LD_{50}$, and potassium ($K^+$) channel activity.

Protein concentrations were determined by measuring their absorbance at 280 nm by the method of Lowry *et al.* (12), using bovine serum albumin as standard.

***Molecular weight determination by gel filtration chromatography***

The molecular weight of the isolated toxin was estimated by gel filtration chromatography, according to the method of Andrews (2), on Sephadex G-75 calibrated columns, using 0.05 M Tris-HCl buffer (pH 8.5).

Sephadex G-75 was suspended in 0.05 M Tris-HCl buffer (pH 8.5) containing 100 mM NaCl and allowed to swell for 24 hours. Fine particles were then removed by decanting the supernatant, and the swollen gel was deaerated overnight in a vacuum desiccator. The gel was packed in a column (1.5 x 60 cm) and equilibrated with the same buffer. The flow rate was adjusted at 12 ml/h using a peristaltic pump. Void volume ($V_o$) of the column was determined by using Blue Dextran (2 mg/ml in an equilibration buffer containing 3% sucrose). The column was calibrated with standard molecular weight markers. Each standard protein (2 mg/ml) of the buffer (containing 3% sucrose) was layered on the gel. The elution was carried out with the same buffer at a constant flow rate of 12 ml/h. Fractions of 3 ml were collected and the protein elution was monitored by determining the absorbance at 280 nm using a Hitachi 150-20 spectrophotometer. The total volume of the eluent up to the fraction having maximum absorbance was considered as the elution volume of the protein ($V_e$). The elution volumes of different standard proteins of known molecular weights and the purified toxin were determined under similar conditions.

A calibration curve was obtained by plotting $V_e/V_0$ against their respective logarithmic molecular weights. Insulin, aprotinin, lysozyme, chymotrypsinogen A, carbonic anhydrase, ovalbumin, and bovine serum albumin were used as standard proteins to obtain the calibration curve. From this calibration curve, the molecular weight of the purified toxin was determined.

### *Molecular weight determination by the sodium dodecyl sulphate polyacrylamide gel electrophoresis*

The molecular weight was determined on SDS-PAGE, according to the method of Laemmli (10).

Molecular weight markers, the crude venom, and the toxin samples after each purification step were subjected to 6-16% gradient SDS-Polyacrylamide gel electrophoresis at pH 6.8 using Tris-HCl buffer, stained with Coomassie Brilliant Blue R-250 for 2 hours and subsequently distained overnight with methanol:acetic acid:water (30:10:60 v/v).

The migration distances of the individual bands of the standard proteins, toxin samples, and those of the tracking dye from the origin of the separating gel were measured. Relative mobility ($R_f$) of the individual proteins was determined by the help of equation.

A calibration curve was obtained by plotting the relative mobility values ($R_f$) of standard proteins against logarithms of their molecular weights. The molecular weight of the toxin was calculated from this calibration curve.

### *Toxicity studies*

Albino mice (*Mus musculus*, 20-40 g body weight, 10 months old), crude venom, *Palamneus gravimanus* purified toxin, and saline (0.9 % NaCl) were used in the toxicity studies performed according to the method of Reed and Meunch (16).

Mice that had fasted the previous night were used in the present study. Three groups, each comprised of six animals were treated as follows: Group 1: saline - control group; Group 2: purified toxin; Group 3: crude venom.

The control group received 4 ml of saline. Whereas varying doses of purified fraction and crude venom were intraperitoneally injected into the other groups. All the animals were observed for 48 h.

### *Activity of purified toxic peptide on potassium channels*

In the present investigation, $K^+$ channel activity of the scorpion purified toxin was determined according to the method of Ritu Dhawan *et al.* (4).

### *Isolation and maintenance of Xenopus* oocytes

Adult female frogs (*Xenopus laevis)* were acquired from Xenopus Express (Plant City, FL, USA), and their colony was maintained in a temperature-controlled room (20°C) with 12 hours of light and dark cycle.

Oocytes were isolated by mini-laparotomy from adult female *Xenopus laevis*. The frogs were anesthetized by immersion in 0.04% benzocaine (Sigma Chemicals, USA) for 15-20 min and then placed on a wet platform during dissection and removal of the ovarian lobes. The incision was sutured and closed, and the frog was allowed to recover for about two months before removal of another batch of oocytes. Ovarian lobes were manually divided into smaller clusters of oocytes and were subsequently treated with 1 mg/ml type 1A collagenase (Sigma Chemicals, USA) in OR-Mg solution (82 mM NaCl, 2 mM KCl, and 5 mM HEPES [pH 7.7]). The isolated stage V and VI oocytes were then incubated for microinjection at 18°C in a ND (96 mM)-HS solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 2.5 mM Na-Pyruvate [pH 7.7]), supplemented with 100 U/ml of Penicillin–Streptomycin solution (Sigma chemicals USA) and 5% heat inactivated horse serum.

### *Potassium channel expression in Xenopus* oocytes

In the present investigation the human cloned voltage-gated potassium channel hKv1.1 was expressed in *Xenopus laevis* oocytes by the method of Baumann *et al.* (3)

Kv1.1 channel was present in a pGEM-3Z vector (Promega) modified to contain untranslated sequences of *Xenopus* β-globulin gene to enhance expression in oocytes (11). Kv1.1 channel had a single amino acid difference (Ser 357 ALA) when compared to the published sequence of the native channel. RNA transcription using T7 polymerase and *Xenopus* oocyte injections were carried out using the protocols previously described by MacKinnon *et al.* (13).

### *Microinjection*

The stock solution (> 1 mg/ml) of $_C$RNA was diluted to 150-300 mg/ml concentrations in diethyl pyrocarbonate (DEPC) treated water. Then, 46 ml of this $_C$RNA solution was microinjected into each oocyte, using a Nanojet automated oocyte injector (Drummond Scientific, Broomall, PA, USA) containing a glass microcapillary with a terminal diameter of 10-15 Å. The injected oocytes were maintained in ND96-HS

solution at 18°C, and electrophysiological recordings were determined 2-7 days after the injection.

### *Electrophysiology*

*Xenopus* oocytes expressing $K^+$ channels were voltage-clamped by using a two-electrode voltage-clamp electrophysiology rig. OC-725 oocyte clamp amplifier (Warner Instruments, Hamden, CT, USA) was utilized to maintain the holding potentials and record membrane currents. The microelectrodes that were pulled by using a P-97 micropipette puller (Sutter Instruments, Novato, CA, USA) were filled with 3 M KCl and had an initial tip resistance of 0.4 to 1.5 MW. The external recording solution was modified ND96 (96 mM sodium gluconate, 2 mM potassium gluconate, 1.8 mM calcium gluconate, 1 mM magnesium gluconate, and 5 mM HEPES [pH 7.7]). Solution exchange was achieved by gravity flow. Analogue data from the amplifier was sampled at 5-25 kHz and filtered at 2-10 kHz in a low pass filter (LPF-100, Warner Instruments), digitized by a TL-1 series of digitizers (Axon Instruments). A software package was used to generate voltage-clamp commands, obtain membrane current, and analyze digitized data. All electrophysiological experiments were performed at room temperature

## RESULTS

### *CM-Sephadex C-25 column chromatography*

*Palamneus gravimanus* crude venom was resolved into four bound and one unbound protein peak on a CM-Sephadex C-25 column (Figure 1). Fractions number 70-95, which showed maximum toxicity to white mice, were pooled, lyophilized, subjected to gel filtration on Sephadex G-50 column, and fractionated into three peaks. Peak II (Figure 2) had the highest protein concentration and toxicity. The summary of the purification procedure is given in Table 1.

### *Criteria for purity and molecular weight determination*

Sephadex G-50 purified toxin was homogeneous on SDS-PAGE, as shown in Figure 3.

The molecular weight of the toxin was determined by SDS-PAGE and gel filtration on Sephadex G-75 using standard protein molecular weight markers (Figures 4 and 5). It was about 4.5 ± 0.1 kD.

### *Toxicity Studies*

Intravenous administration of *Palamneus gravimanus* purified toxin produced typical hypertensive symptoms and showed a $LD_{50}$ value of 2 mg/kg mouse. Group 1 (control group) was administered saline. Dosage of the purified toxin was calculated on the basis of total protein content. *Palamneus gravimanus* crude venom showed a $LD_{50}$ value of 800 μg/kg mice, which died with typical neurotoxic symptoms.

### *Purified toxin activity on human cloned potassium channel (hKv1.1)*

Potassium channels are one of the most important molecular targets of scorpion toxins. The reaction of an isolated peptide toxin on human cloned potassium channels was determined using *Xenopus laevis* oocyte system for homologous channel expression and a standard two-electrode voltage-clamp set up for $K^+$ current recording. hKv1.1 was voltage-clamped at +20 to -70 mV and stopped to a range of test potentials.

Application of *Palamneus gravimanus* venom toxin to these oocytes resulted in the reduction of hKv1.1 currents. Figure 6a shows control oocytes before addition of purified venom toxin at +10 mV; the maximum current observed was 1098 nA. While for the same oocyte, after addition of 10 nM purified venom toxin and after 30 minutes, the same maximum current at +10 mV was now reduced to 523 nA, which was almost a 50% reduction in the current (Figure 6b).

In figure 6c, the graph shows the voltage-dependence of channel blocking by *Palamneus gravimanus* venom toxin. The same concentration of toxin was used for this assay and was tested at different membrane potentials after a gap of 4 minutes for the toxin to act. A “relative channel blocking” of 0.95 % was observed at -20 mV.

The graph in Figure 6d shows channel blocking as a function of the *Palamneus gravimanus* toxin concentration. Relative channel blockage is calculated by dividing the current produced in the presence of toxin by the control current (i.e. the current produced in the absence of the toxin). The results obtained indicate that the toxin blocks hKv1.1 channel in a nanomolar concentration range (10 nM). The block probably seems to be voltage-dependent as indicated by the graph (Figure 6e).

In Figure 6f, the graph shows the effect of *Palamneus gravimanus* toxin at a given concentration (20 nM) at a single potential of +20 mV at different time intervals (time-dependent toxin action).

Table 1: Summary of the purification of toxins from scorpion (*Palamneus gravimanus*) crude venom

| No. | Purification steps | Amount of protein * (mg) | Protein recovery (%) |
|---|---|---|---|
| 1 | Crude venom (100 mg) | 85 | 100 |
| 2 | CM-Sephadex C-25 | 36 | 42.3 |
| 3 | Sephadex G-50 | 11.5 | 31.9 |

*Protein concentration was assayed by the method of Lowry *et al*. (1951)(12).

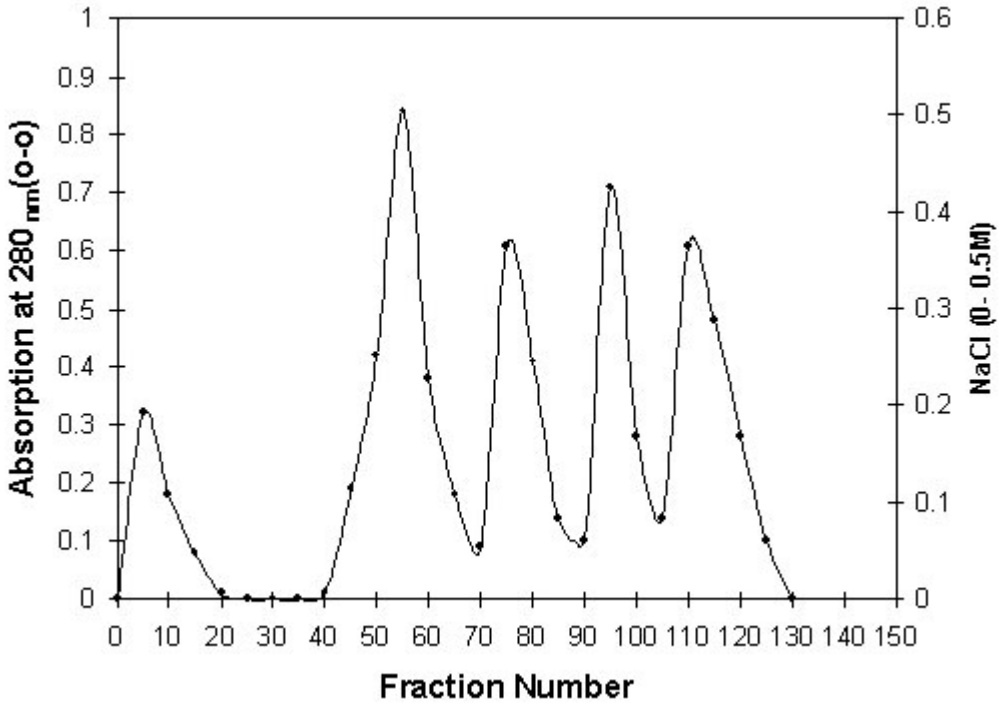


Figure 1: CM-Sephadex C-25 column chromatography of *Palamneus gravimanus* venom. The crude venom was dissolved in 5 ml of 0.05M Tris-HCl buffer, pH 8.5, and loaded on a previously equilibrated CM-Sephadex C-25 column (1.5 x 18 cm). After washing the column with 500 ml of the Tris-HCl buffer, pH 8.5, the venom components were eluted using Tris-HCl with a linear gradient of 250 ml 0-0.5 M NaCl at a flow rate of 40 ml/h, and 4.0 ml fractions were collected. Proteins of the fractions were monitored at 280 nm.

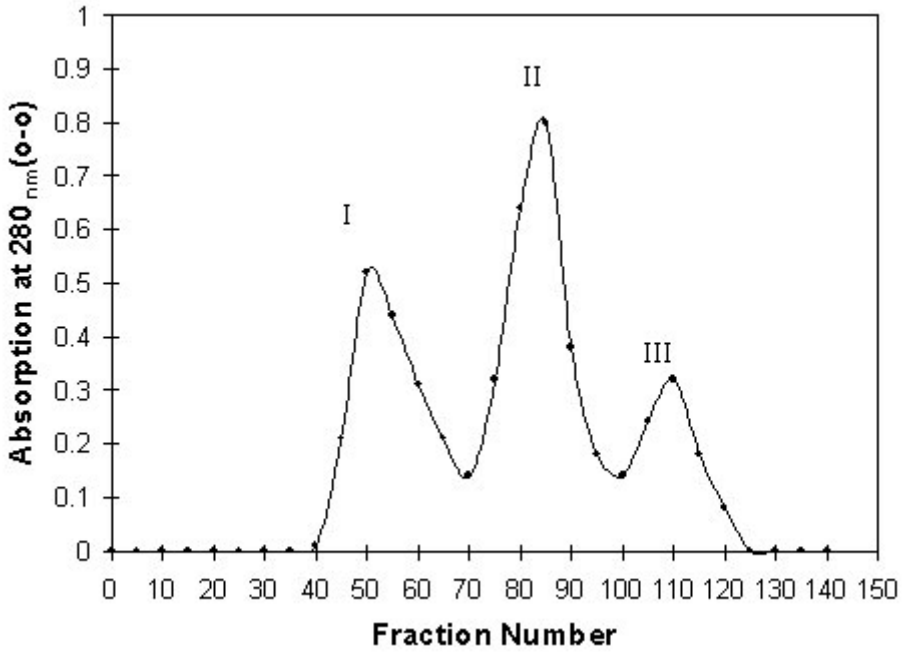


Figure 2: Gel filtration chromatography of Peak-III from Figure 3 (Lane D) on a Sephadex G-50 column (1.5 x 60 cm). Fractions were eluted with 0.01 M Tris-HCl, pH 8.5, at a flow rate of 12 ml/h, and those fractions of 3.0 ml were collected.

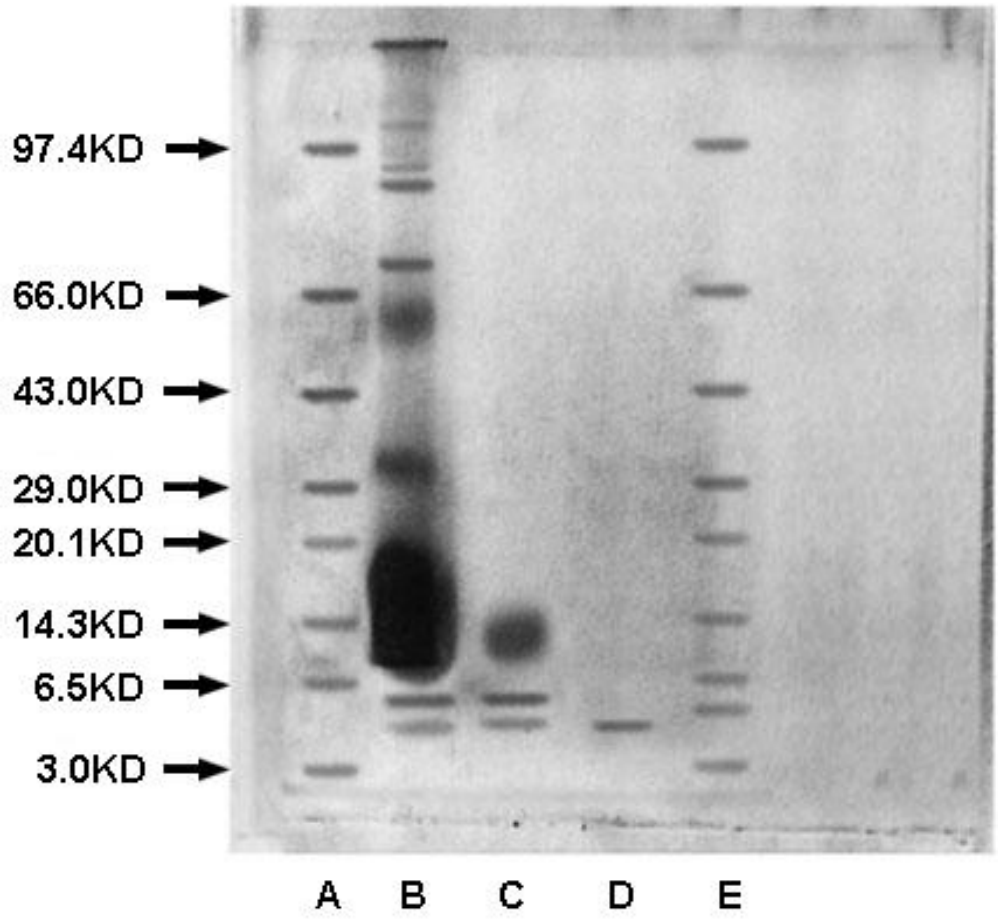


Figure 3: SDS-PAGE of the purified *Palamneus gravimanus* toxin. Lane A: molecular weight markers; Lane B: *Palamneus gravimanus* crude venom; Lane C: purified toxin after CM-Sephadex C-25 column; Lane D: purified toxin after Sephadex G-50; Lane E: molecular weight markers.

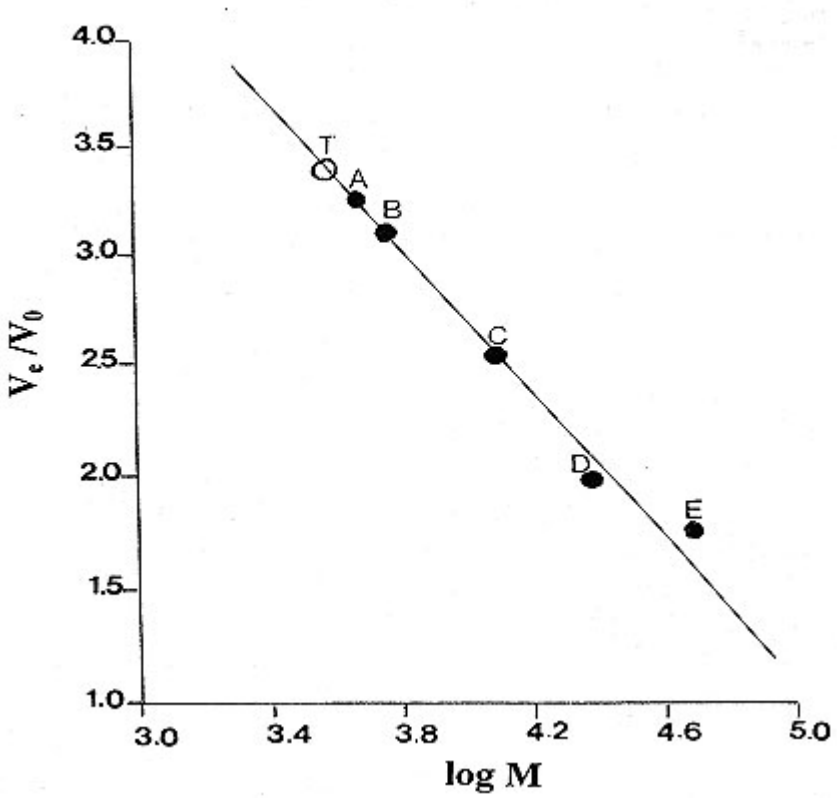


Figure 4: Calibration curve for the determination of the toxin molecular weight at pH 8.5 by gel filtration chromatography on Sephadex G-75. Marker proteins used for calibration: A: insulin (5,600); B: aprotinin (6,500); C: lysozyme (14,300); D: carbonic anhydrase (29,000); E: ovalbumin (45,000); T: toxin.

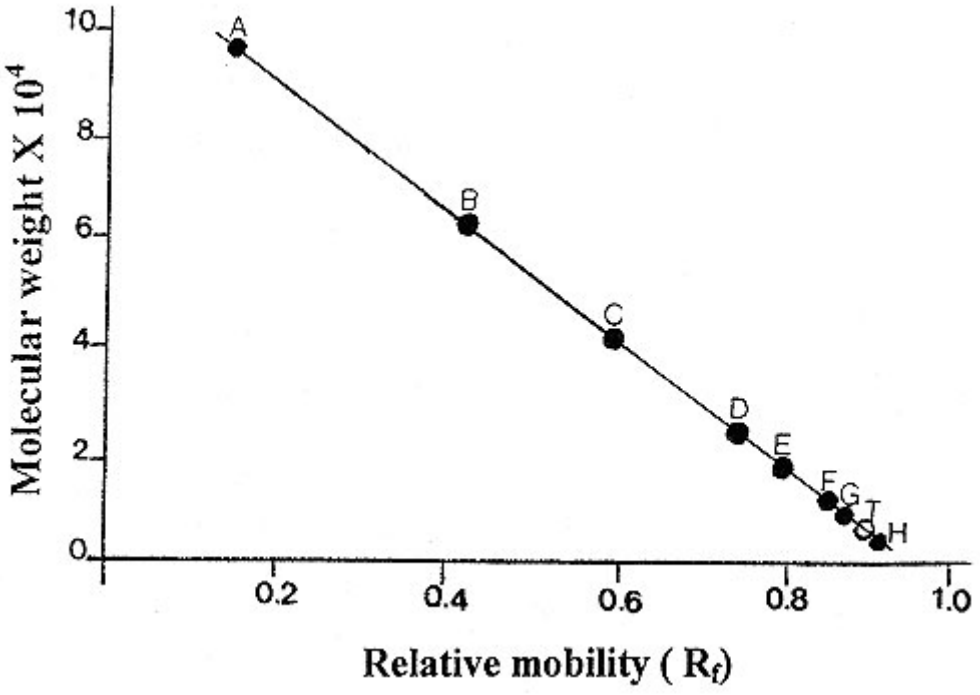


Figure 5: Calibration curve for the determination of the toxin molecular weight by SDS-PAGE (6-16%). Marker proteins used for calibration: A: Phosphorylase b (97,400); B: bovine serum albumin (66,000); C: ovalbumin (43,000); D: carbonic anhydrase (29,000); E: soyabean trypsin inhibitor (20,100); F: lysozyme (14,300); G: aprotinin (6,500); H: insulin; T: toxin.

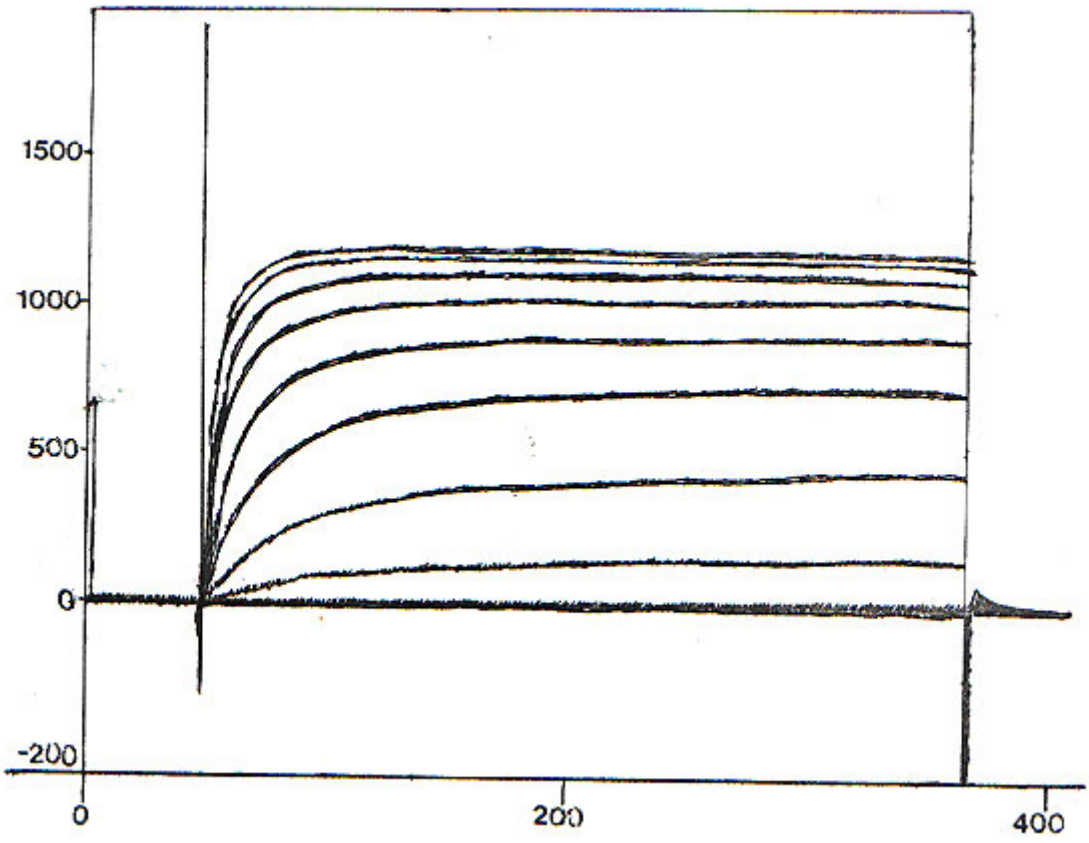


Figure 6a: Control oocytes before the addition of *Palamneus gravimanus* toxin at +10 mV (the maximum current is 1098 nA).

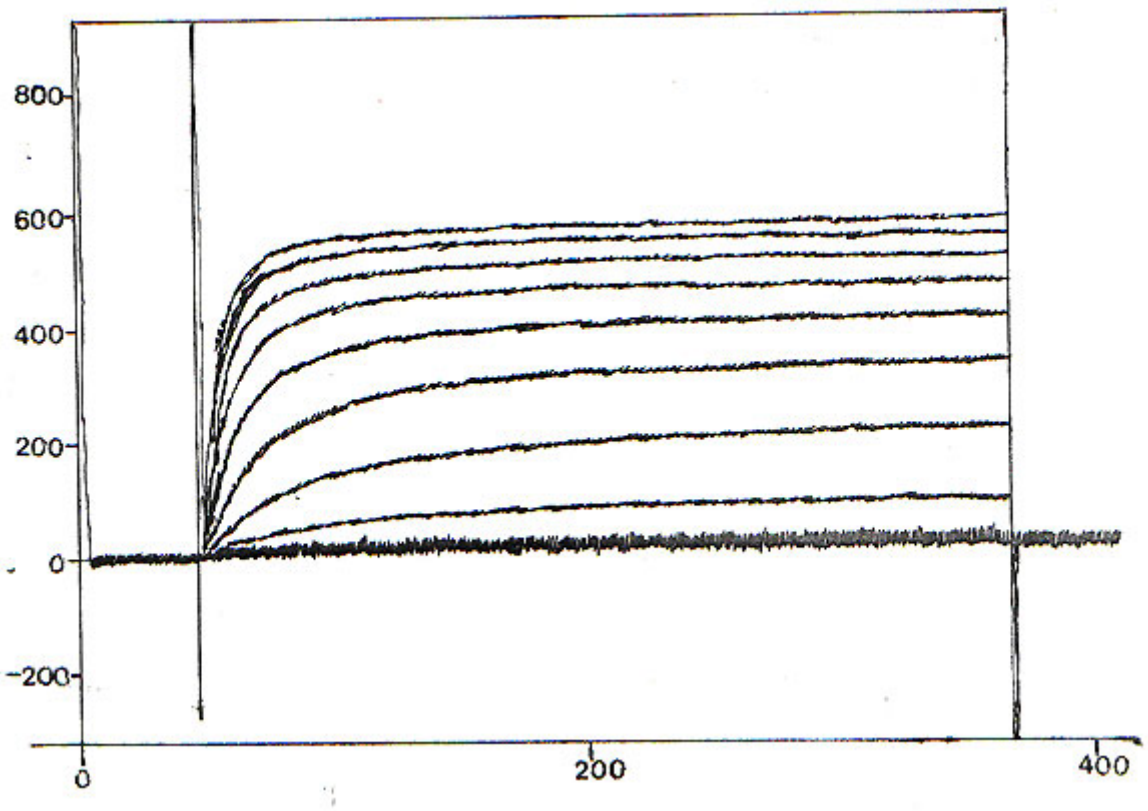


Figure 6b: The same oocyte after the addition of 20 nM *Palamneus gravimanus* toxin. After 30 min, the maximum current at +10 mV reduced to 523 nA.

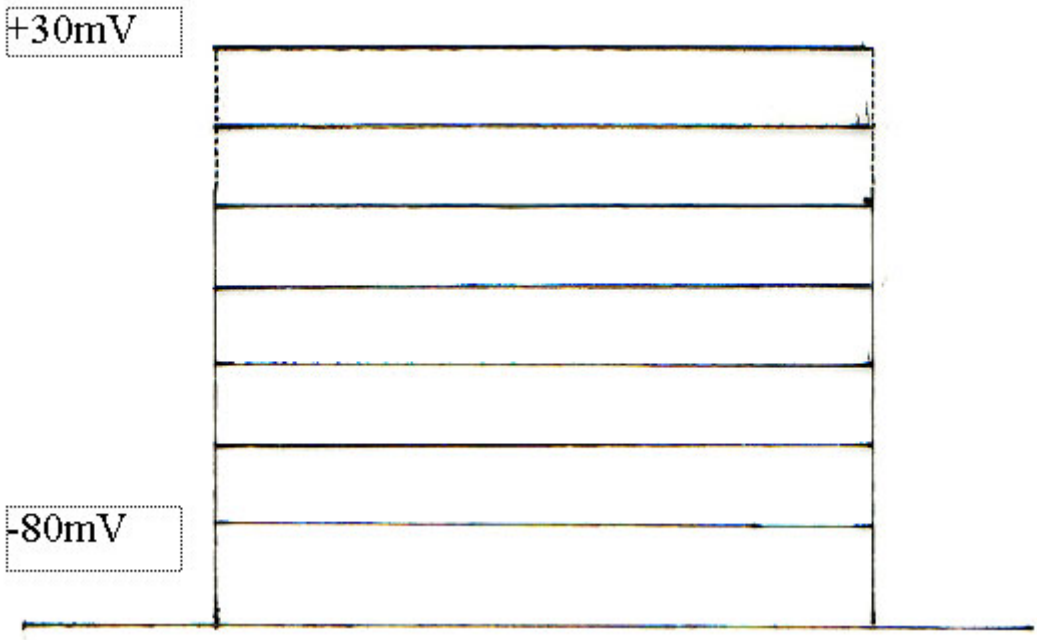


Figure 6c: Pulse protocol used for the measurement of currents.

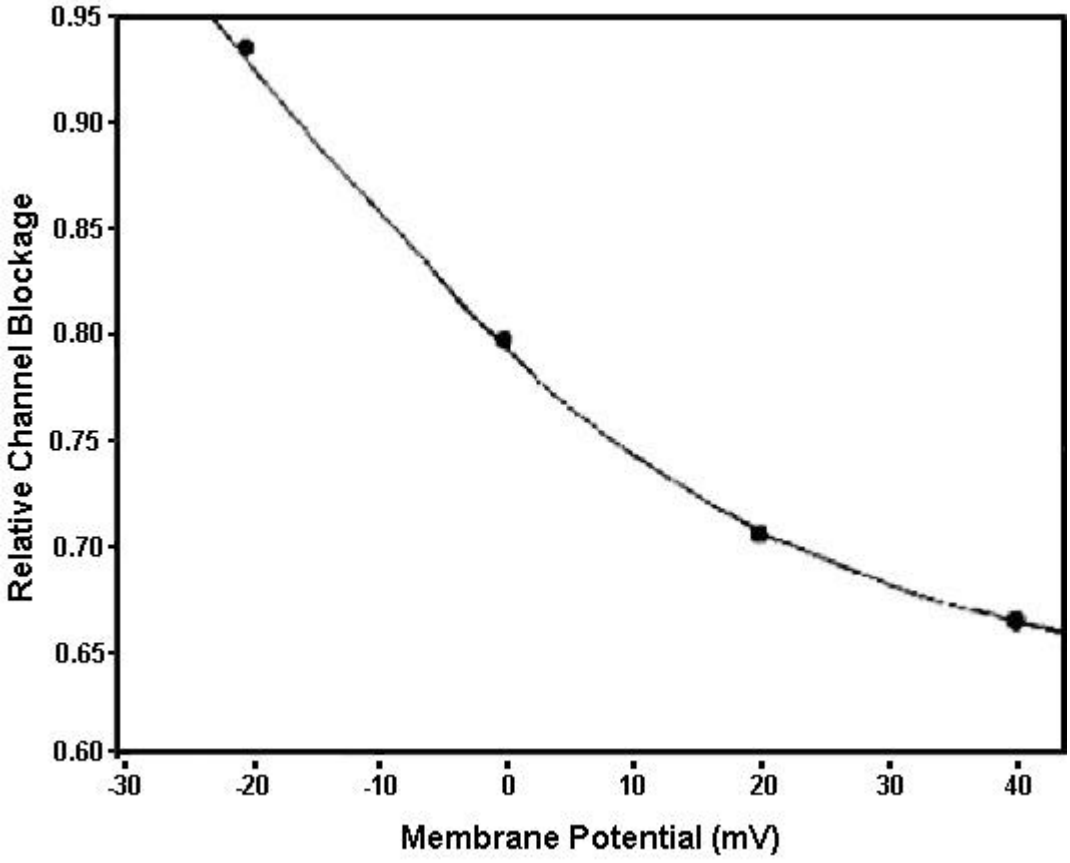


Figure 6d: Voltage-dependence of the channel blocking by *P. gravimanus* toxin.

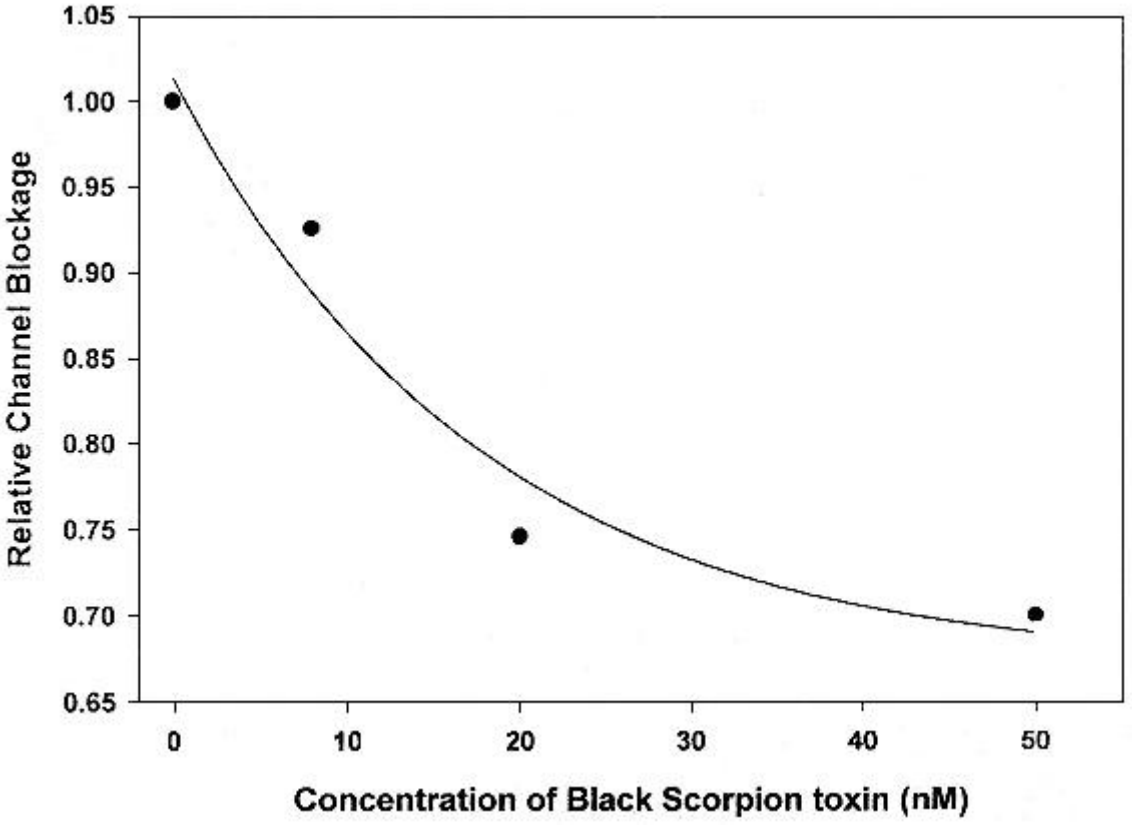


Figure 6e: Channel blockage as a function of the toxin concentration.

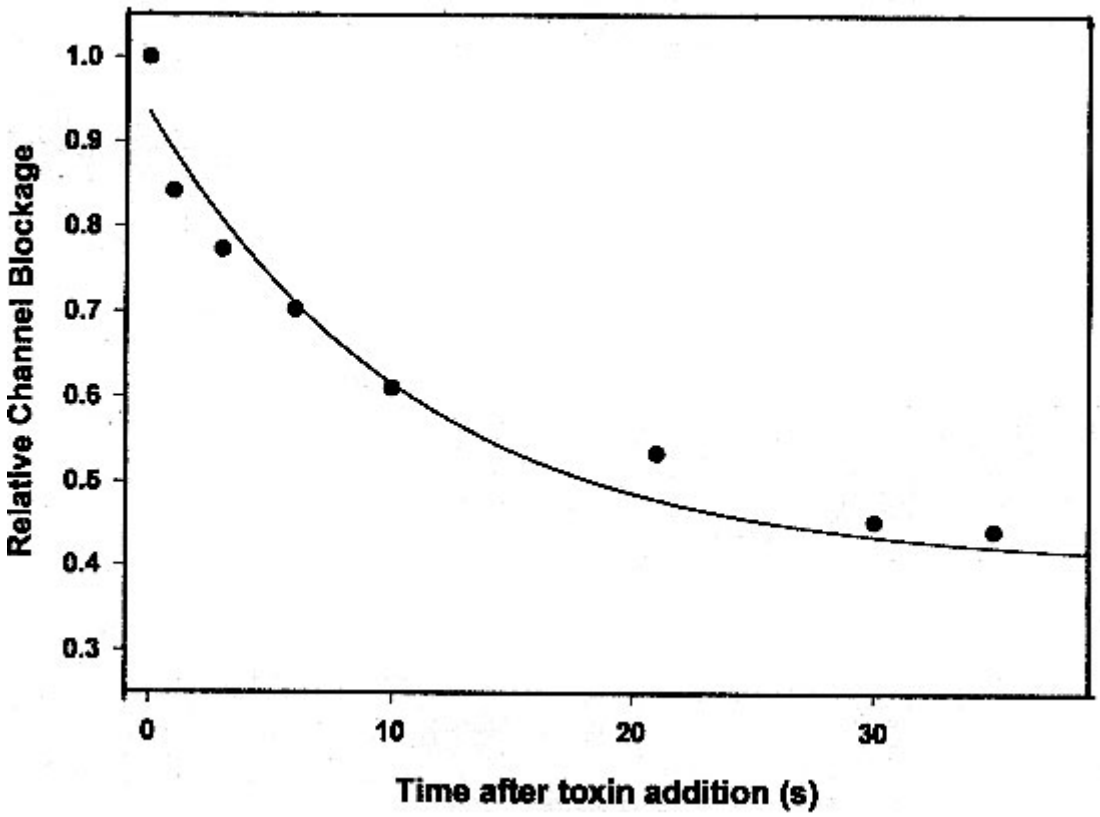


Figure 6f: Effect of the toxin at a given concentration at a single potential at different times. Time-dependence of the toxin action.

## DISCUSSION

In the past, a large number of toxins have been isolated from various scorpion species (19, 8, 18). The toxic action of the scorpion venom is probably due to a small amount of low molecular weight peptide toxins basic in nature (19).

In the present study we have isolated and characterized a novel potassium channel inhibitor from *Palamneus gravimanus* venom in a two-step procedure combining ion exchange and gel filtration chromatography. The molecular weight of the isolated toxin was about 4.5 ± 1 kD, as assayed by SDS-PAGE, and had a $LD_{50}$ value of approximately 2 mg/kg body weight. Earlier researchers had isolated and purified toxins from different scorpion species. Galvez *et al*. (5) purified a 4.3 kD polypeptide, called “Ibtx”, from the venom of *Buthus tamulus*. Romi-Lebrun *et al.* (17) purified three toxins ChTx from the Chinese scorpion *Buthus martensi*, with molecular mass ranges of 3800-4300 Da, and all of them were known to be potent inhibitors of voltage-gated potassium channels. Garcia *et al.* (6) purified a toxin from the venom of

*Leiurus quinquestriatus* showing a molecular mass of about 4.1 kD, a potent inhibitor of shaker $K^+$ channel. Dhawan *et al.* (4) purified a peptide of about 3.5 kD from *Buthus tamulus* (Indian red scorpion), known as BTK-2, which particularly inhibits the Kv1.1 channel. The data on toxins purification and their activities on $K^+$ channel in different scorpion species venoms suggested that the molecular mass of the toxin peptides ranged between 3.5 and 4.5 kD and their activity was usually directed to blocking only Kv1.1 potassium channel without affecting Kv1.2 or Kv1.4, very closely related potassium channels. The most important application of such bioactive peptides is envisaged in neuropharmacological dissection of physiological processes and in drug design to provide templates leading to specific blocks. Further investigations are needed to determine the specific amino acid residues of the peptide toxin, which may be involved in the toxin and hKv1.1 channel interaction, and these toxins could probably be used to determine the architectural difference between the voltage-gated and the calcium activated channel pores.

In conclusion, we have purified and characterized a toxic peptide (toxin) from the venom of the Indian black scorpion *Palamneus gravimanus*. This toxin is a potent inhibitor of the hKv1.1 channel, which closely resembles the toxins Lq2 from *Leiurus quinquestriatus* and BTK-2 from *Buthus tamulus,* with respect to the molecular mass and action on voltage-gated potassium channels. The purified toxin is helpful in designing drug molecules and also as a molecular tool to explore the pore region of the voltage-gated Kv1.3 potassium channels (1).

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