Purification, structure determination and synthesis of covalitoxin-II, a short insect-specific neurotoxic peptide from the venom of the *Coremiocnemis validus* (Singapore tarantula)

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Abstract Spider venoms contain toxins that specifically immobilize and kill insects. We report the purification and characterization of a new insect-specific toxin named covalitoxin-II (Cvtx-II; mass, 3406.24 ± 0.64), from *Coremiocnemis validus* (Singapore tarantula) venom. The complete 31 amino acid sequence of Cvtx-II has been determined and it shows less than 40% identity with spider toxins. However, Cvtx-II has conserved cystine motif analogous to other spider and ω-conotoxins. Cvtx-II was chemically synthesized and identified with the native Cvtx-II. Synthetic Cvtx-II induced insect-specific non-lethal excitatory activity when injected into crickets, but not in cockroaches and mice.

Key words: Spider; Spider venom; Reversible neurotoxin; *Conus* toxin; Cystine motif

1. Introduction

Spiders are active predators and most of them have developed venoms to capture their prey. The diversity of spiders and their venoms (not all spiders are venomous) have attracted the attention of the scientific community in their search for insecticidal toxins [1–4] and neurotoxins [5,6]. Such toxins have potential as selective biochemical and/or pharmacological tools [6–8] and as lead molecules in drug discovery [9].

Only a small percentage of the 40 000 known spiders have been well characterized. Among the best known of all spiders are Theraphosidae, commonly called 'tarantulas'. We have purified three new peptide toxins (named covalitoxin (Cvtx)-I, -II and -III) from the venom gland extract of *Coremiocnemis validus*, a species of spider found in South East Asian tropical forests. In this paper, we report the purification and biochemical properties of Cvtx-II. Cvtx-II was purified by reverse phase high-pressure liquid chromatography (HPLC), its molecular mass and complete amino acid sequence were determined by electrospray ionization mass spectrometry (ESI/MS) and Edman degradation, respectively. The toxin was chemically synthesized by 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. Cvtx-II is a non-lethal insect-specific neurotoxic peptide. The toxin's structural similarities with other short spider toxins suggest that modulation of ion channel(s) could be responsible for its action.

2. Materials and methods

2.1. Materials

Live tarantulas (*C. validus*) were from our collection maintained at the Laboratory of the Venom and Toxin Research Programme, Faculty of Medicine, National University of Singapore. A reverse phase HPLC column (Sephasil C18; 2.1 mm × 100 mm) was obtained from Pharmacia Biotech (Uppsala, Sweden). Reagents for N-terminal sequencing were from Applied Biosystems (Foster City, CA, USA). The chromatographic solvent acetonitrile (ACN) was purchased from Fisher Scientific (Fair Lawn, NJ, USA), 4-vinylpyridine and trifluoroacetic acid (TFA) were from Fluka Chemika-Biochemika (Buchs, Switzerland). All other chemicals used were of analytical grade and purchased from Sigma Chemical Company (St. Louis, MO, USA). Sterile, non-pyrogen 0.9% NaCl injection B.P. was from B/Braun, Melsungen AG (Malaysia). Fmoc-Tyr(tBu)-OPfp was purchased from Novabiochem (Tokyo, Japan) and H-Lys(Boc)-OH from Kokusan Chemicals Ltd. Fmoc-amino acids, Fmoc-Pro-resin and other reagents used on the peptide synthesizer were obtained from Perkin-Elmer Applied Biosystems Japan (Chiba, Japan).

2.2. Preparation and extraction of venom

The venom glands were dissected from cold anaesthetized spiders and carefully separated from the muscle tissue and exoskeleton. The venom gland extract was prepared by crushing 20 glands using a glass rod into 2 ml of 0.9% normal saline. This was stored at −15°C.

2.3. HPLC purification

The venom gland extract was cleared of tissue debris by centrifugation at 14 000 rpm for 10 min. The supernatant was then applied onto a reverse phase HPLC column Sephasil C18 (2.1 mm × 100 mm) equilibrated with 0.1% TFA using a Smart System (Pharmacia). Bound peptides were eluted using a linear gradient of ACN. The flow rate used was 200 μl/min. Elution of peptides was monitored at 215 nm.

2.4. Electrospray mass spectrometry

Molecular masses of peptides were determined by ESI/MS on a

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Abbreviations: Cvtx, covalitoxin; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high-pressure liquid chromatography; ESI/MS, electrospray ionization mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PTH, phenylthiohydantoin; ODS, octadecylsilane; TFA, trifluoroacetic acid; ACN, acetonitrile

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Perkin-Elmer Sciex API III triple quadrupole instrument equipped with a ionspray interface (Sciex, Thornton, Canada). The ionspray voltage was 4600 V, the ring voltage 350 V and the orifice voltage 45 V. A Shimadzu series LC10-AD quaternary pump was used for solvent delivery. The sample was infused into the mass spectrometer by flow injection at 50 μl/min.

2.5. Reduction and pyridylethylation of the peptide

The peptide was dissolved in 100 μl of the denaturant buffer (6.0 M guanidine hydrochloride, 0.13 M Tris, 1 mM EDTA, pH 8.0) containing 0.07 M β-mercaptoethanol. The mixture was incubated at 37°C for 2 h under nitrogen. Subsequently, 1.5-fold molar excess (over sulfhydryl groups) of 4-vinylpyridine was added and incubated at room temperature under nitrogen. The samples were then immediately desalted on the Sephasil C18 column (2.1 mm × 100 mm).

2.6. Amino acid sequencing by automated Edman degradation

Amino acid sequencing was carried out by automated Edman degradation using an Applied Biosystems 494 pulsed-liquid-phase sequencer. Phenylthiohydantoin (PTH) amino acids were identified using on-line reverse phase HPLC on a PTH-C18 column on an Applied Biosystems 140C analyzer.

2.7. Chemical synthesis of the peptide toxin

A linear precursor of Cvtx-II was synthesized by solid phase methodology with Fmoc chemistry on a Perkin-Elmer Applied Biosystems model 433A peptide synthesizer. To prevent formation of diketopiperazine, protected dipeptide Fmoc-Tyr(tBu)-Lys(Boc)-OH was coupled with Pro-resin in the first step. Fmoc-Tyr(tBu)-Lys(Boc)-OH was synthesized from Fmoc-Tyr(tBu)-OPfp and H-Lys(Boc)-OH in a liquid phase. Six cysteines were protected by trityl groups. All protective groups were removed during cleavage from the resin using 82.5% TFA containing 5% water, 2.5% ethanedithiol, 7.5% phenol and 5% thioanisole. The peptide was recovered as a precipitate by the addition of ether, collected on a glass filter and extracted with 2 M acetic acid. The extract was diluted to a final peptide concentration of 0.05 mM in 1 M ammonium acetate (pH 7.9) containing 0.5 mM oxidized glutathione, 5 mM reduced glutathione and 1 mM EDTA, and the resultant solution was stirred at 4°C for 48 h [10–14]. After acidification of the mixture, the oxidized peptide was desalted and concentrated on an octadecylsilane (ODS) column with a medium pressure pump. The peptide mixture was subjected to gel filtration on a Sephadex G-50F (column: 5 cm × 100 cm, solvent: 30% acetic acid) to obtain folded monomer followed by ion exchange chromatography on a CM-cellulose CM52 column (1.8 cm × 20 cm). The CM52 column was equilibrated with 0.01 M ammonium acetate (pH 4.6) and the peptide was eluted using a linear gradient of the eluting buffer, 0.4 M ammonium acetate, pH 6.5. Finally, Cvtx-II was purified by preparative reverse phase HPLC (Shimadzu LC-8A system) using an ODS column (20 mm × 250 mm). The molecular mass and homogeneity of the peptide were determined by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) on a PerSeptive Biosystems Voyager Linear DE mass spectrometer using α-cyano-4-hydroxycinnamic acid as a matrix. To determine whether the peptide was folded similar to the native toxin, it was co-injected with native Cvtx-II onto an analytical ODS column (4.6 mm × 250 mm) using Shimadzu LC-6A HPLC system. To ensure proper folding, the native and synthetic Cvtx-II were co-eluted using either a shallow linear gradient (5–30% ACN with aqueous 0.1% TFA) or isocratic (16% ACN with aqueous 0.1% TFA) elution conditions.

2.8. Biological assays

The toxicity of Cvtx-II was determined using the following bioassays. Synthetic Cvtx-II was injected into unsexed crickets (*Gryllotalpa hexadactyla*), German cockroaches (*Blattella germanica*, $n = 8$, 0.3–0.8 g, intrathoracical injection) and 2 week old Swiss mice ($n = 4$, 6–8 g body weight, intracerebral injection). The sample was dissolved in 0.9% normal saline. The insects were restrained by their wings and Cvtx-II was injected (20–50 μl volume, 0.1–1.0 μmol/g insect) into their thoracic body cavity. The injections were performed with a fine capillary Hamilton microsyringe. Mice were injected intracerebrally with 20 μl of the samples ($n = 4$, maximal concentration 0.1 μmol/mouse). The animals were observed at regular intervals up to 6 h post-injection for any behavioral changes or death. Control animals were injected with 20 μl of 0.9% normal saline.

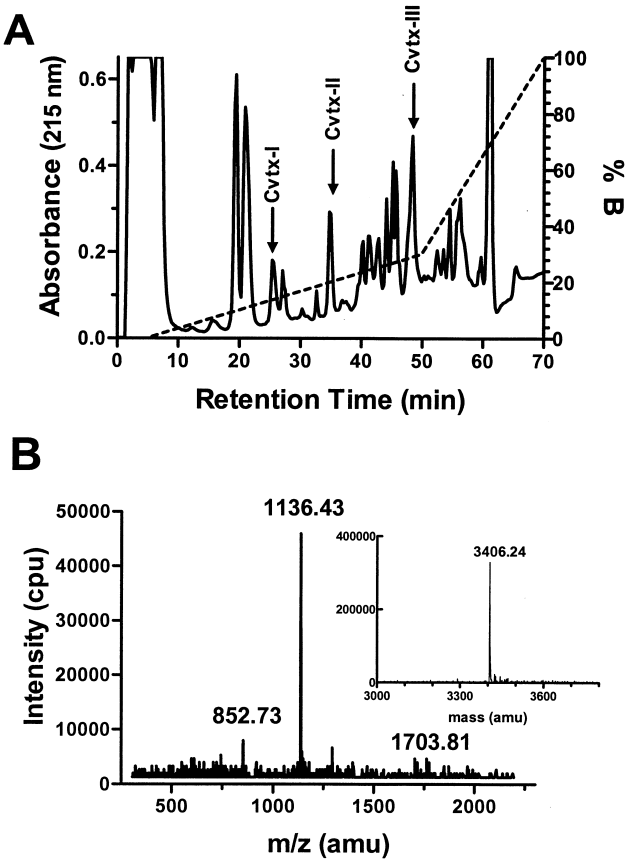

Fig. 1. Purification, homogeneity and mass spectrum of Cvtx-II. A: HPLC chromatography of *C. validus* venom on Sephasil C18 column (2.1 mm × 100 mm). The column was equilibrated and washed with 0.1% TFA and bound peptides were eluted using a linear gradient of 0–60% for 45 min and 60–100% for 20 min ACN in aqueous 0.1% TFA at a flow rate of 200 μl/min. Elution of peptides was monitored at 215 nm. B: Determination of homogeneity and molecular mass of Cvtx-II by ESI/MS. Ionspray voltage = 4600 V; orifice voltage = 45 V; ring voltage = 350 V; inset: reconstructed mass spectrum of the peptide.

3. Results and discussion

Spiders use their venoms to incapacitate their prey. The well studied components in spider venom have been broadly classified into two types: polyamines [15] and polypeptide components [16]. Most research on spider toxins has focused on the analysis of neurotoxic components, especially neurotoxic polypeptides [17–20]. Toxins from the venoms of scorpions, spiders and wasps can provide novel molecules for the design and development of new insecticides.

Crude venom from *C. validus* was fractionated by reverse phase chromatography on a Sephasil C18 column (Fig. 1A). Protein elution was monitored at 215 nm and 25 peaks were observed on the chromatogram. All peaks were tested for their purity by ESI/MS. Three peaks were homogeneous and were named Cvtx-I, -II and -III (with the Roman numericals indicating their order of elution). Cvtx-II was eluted as a sharp peak between 30 and 40 min interval at a gradient less than 30% of solvent B (80% ACN in 0.1% TFA) (Fig. 1A). The mass spectrum of Cvtx-II showed three multiple-charged ions with $m/z = 852.73$ (M^{4+}), 1136.43 (M^{3+}) and

1703.81 (M^{2+}) (Fig. 1B). The deconvoluted mass spectrum (Fig. 1B, inset) indicated that it has a molecular mass of 3406.24 ± 0.64.

The amino acid sequence of native Cvtx-II was determined end to end by automated Edman degradation. All positions were unequivocally assigned except for positions 2, 9, 15, 16, 21 and 28, which showed no new peak. The peptide was reduced, pyridylethylated and resequenced to determine the positions of cysteine residues. Pyridylethyl-cysteines were identified at each of the previously unidentified positions. The complete primary amino acid sequence of Cvtx-II was determined as ACSRAGENCYKSGRCCDGLYCKAYVVT-CYKP. Cvtx-II has 31 residues including six cysteine residues at position 2, 9, 15, 16, 21 and 28 forming three disulphide bonds. The calculated molecular mass (3406.91) of Cvtx-II matched the observed molecular mass (3406.24 ± 0.64), indicating the presence of a free carboxyl-terminal. The peptide has an almost equal distribution of hydrophilic (35.5%), hydrophobic (32.25%) and neutral (32.25%) amino acids. A BLAST search [21] of the protein sequence database showed low homology with two peptides, viz. spider peptide PLTX-II (44 amino acids, presynaptic calcium channel blocker from *Plectreurys tristis*; 37% identity; 47% homology) and conotoxin MrVIB (31 amino acids, sodium channel blocker from *Conus marmoreus*; 40% identity; 47% homology). The cystine motif of Cvtx-II is analogous to other spider toxins (Fig. 2) and 'ω-toxins' isolated from *Conus* venom, which block voltage-activated calcium channels [22,34–48]. Among these families of toxins, the disulphide bonding pattern is well conserved (C1–C4, C2–C5 and C3–C6) [6,22,23]. Moreover, these families share a common structural motif, viz. antiparallel β-sheet structure comprising three β-strands and flexible loops, and the structure is stabilized by the cystine knot, which forms the molecular nucleus [24]. However, despite possessing this common structure, the members of this family exhibit distinctly diverse and specific biological effects by targeting a variety of ionic channels (Fig. 2). The difference in intercysteine loop structures appears to distinguish the affinity for different target proteins. In spider toxins including Cvtx-II, conserved basic residues located close to the hydrophobic C-terminus form a positively charged surface. This charged surface is responsible for ion channel interaction in some spider toxins [16].

Due to the scarcity of *C. validus* venom and very low yield of Cvtx-II during purification, larger amounts of Cvtx-II needed for activity studies were chemically synthesized. A linear precursor of Cvtx-II was synthesized by solid phase methodology with Fmoc chemistry. A peptide lacking C-terminal Lys-Pro was a dominant product when Pro-preloaded 4-hydroxymethyl-phenoxy-methyl-copolystyrene–1% divinylbenzene resin was used, indicating formation of diketopiperazine. The application of 2-chlorotrityl resin was also ineffective, causing formation of many truncated peptides. Finally, the linear precursor was successfully obtained by use of a protected dipeptide (Fmoc-Tyr(tBu)-Lys(Boc)-OH) in the first coupling step. After oxidative folding reaction (Fig. 3A), synthetic Cvtx-II was purified by gel filtration, anion exchange and reverse phase HPLC. Its identity with native Cvtx-II was verified by MALDI-TOF-MS analysis. Its mass was determined as 3407.86 (MH^+). Co-elution of the synthetic and native Cvtx-II from an ODS column using both gradient (Fig. 3B) and isocratic conditions (data not shown) revealed that the synthetic Cvtx-II has the same three-dimensional structure as the native Cvtx-II.

Source	Protein	Amino acid sequence	Length	% I	Activity	Ref.
Coremiocnemis validus	Cvtx-II	ACSRAGENCYKSGR--CCDGLYCKAYVVT-------CYKP	31	--	Insect Neurotoxic	
Grammostola spatulata	ω-GsTx SIA	DCVRFWGKCSQTSD--CCPHLACKSKWPRNI-----CVWDGSV	36	40	Ca2+ Channel blocker	[34]
Grammostola spatulata	HaTx1	ECRYLFGGCKTTSD--CCKHLGCKFRDKY-------CAWDFTFS	35	37	K+ Channel blocker	[35]
Heteropoda venatoria	Seq-5	ECGTLFSGCSTHAD--CCEGFICKLW----------CRYERTW	31	36	Ca2+ Channel blocker	[36]
Agelena opulenta	Agelenin	GGCLPHNRFCNALSGPRCCSGLKCKELSIWDSR----CL	35	36	Ca2+ Channel blocker	[37]
Scodra griseipes	SGTx1	TCRYLFGGCKTTAD--CCKHLACRSDGKY-------CAWDGTF	34	35	K+ Channel blocker	[38]
Grammostola spatulata	HaTx2	ECRYLFGGCKTTAD--CCKHLGCKFRDKY-------CAWDFTFS	35	35	K+ Channel blocker	[35]
Grammostola spatulata	Seq-1	YCQKWLWTCDSERK--CCEDMVCRLW----------CKKRX	29	35	Analgesic	[39]
Phrixotrichus auratus	PaTx1	YCQKWMWTCDSARK--CCEGLVCRLW----------CKKII	29	35	K+ Channel blocker	[40]
Brachypelma smithii	Protein 5	SCVDFQTKCKKDSD--CCGKLECSSRWKW-------CVYPSPF	34	35	Not known	[41]
Selenocosmia huwena	SHLP-1	GCLGDK--CDYNNG--CCSGYVCSRTWKW-------CVLAGPW	32	34	Haemagglutination	[42]
Hadronyche versuta	ω-ACTX-HV1F	SAVCIPSGQPCPYSKY--CCSGS-CTYKTNENGNSVQRCD	37	34	Ca2+ Channel blocker	[43]
Heteropoda venatoria	Seq-1	DDCGGLFSGCDSNAD--CCEGYVCRLW----------CKYKL	30	34	Ca2+ Channel blocker	[36]
Heteropoda venatoria	Seq-6	DDCGTLFSGCDTSKD--CCEGYVCHLW----------CKYK	29	33	Ca2+ Channel blocker	[36]
Grammostola spatulata	Seq-1	YCQKWMWTCDEERK--CCEGLVCRLW----------CKKKIEW	31	33	Antiarrhythmic	[44]
Grammostola spatulata	Seq-2	YCQKWMWTCDEERK--CCEGLVCRLW----------CKKKIEW	31	33	Analgesic	[39]
Heteropoda venatoria	Seq-8	DDCGKLFSGCDTNAD--CCEGYVCRLW----------CKLDW	30	33	Ca2+ Channel blocker	[36]
Phrixotrichus auratus	PaTx2	YCQKWMWTCDEERK--CCEGLVCRLW----------CKRIINM	31	32	K+ Channel blocker	[40]
Hysterocrates gigas	SNX-482	GVDKAGCRYMPGGCSVNDD--CCPRLGCHSLFSY-------CAWDLTFSD	41	31	Ca2+ Channel blocker	[45]
Selenocosmia huwena	HWTX-I	ACKGVFDACTPGKNE--CCPNRVCSDKHKW-------CKWKL	33	31	nAch Receptor blocker	[46]
Heteropoda venatoria	Seq-2	DCGTIWHYCGTDQSE-CCEGWKCSRQL---------CKYVIDW	33	31	Ca2+ Channel blocker	[36]
Phoneutria nigriventer	Tx2-9	SFCIPPKP-CKSDEN--CCKKFKCKTTGIVKL-----CRW	32	31	Neurotoxic	[47]
Heteropoda venatoria	Seq-4	ADCGWLPHSCESNAD--CCENWACATTGRFRYL----CKYQI	36	30	Ca2+ Channel blocker	[36]
Aptostichus schlingeri	Aptotoxin VII	WLGCARVKEACGPWEWP-CCSGLKCDGSE---------CHPQ	32	30	Insecticide	[48]
Heteropoda venatoria	Seq-3	DDDCGWIMDDCTSDSD--CCPNWVCSKTGFVKNI----CKYEM	37	28	Ca2+ Channel blocker	[36]
Hadronyche versuta	ω-ACTX-HV1A	SPTCIPSGQPCPYNEN--CCSQS-CTFKENENGNTVKRCD	37	28	Ca2+ Channel blocker	[6]
Hadronyche versuta	ω-ACTX-HV1B	SSTCIPSGQPCPYNEN--CCSQS-CTYKENENGNTVKRCD	37	27	Ca2+ Channel blocker	[43]
Hadronyche versuta	ω-ACTX-HV1C	SSTCIPSGQPCPYNEN--CCSQS-CTFKENENGNTVKRCD	37	27	Ca2+ Channel blocker	[43]
Hadronyche versuta	ω-ACTX-HV1D	SPTCIPSGQPCPYNEN--CCSKS-CTYKENENGNTVQRCD	37	27	Ca2+ Channel blocker	[43]
Hadronyche versuta	ω-ACTX-HV1E	SPTCIPSGQPCPYNEN--CCSQS-CTYKENENGNTVKRCD	37	27	Ca2+ Channel blocker	[43]

Fig. 2. The primary sequence identities of Cvtx-II with other spider toxins. The amino acid sequences are aligned with their cysteine residues for comparison; to maximize identity, the deletions (-) are included in the sequence. % I is percentage identity of Cvtx-II with other spider toxins.

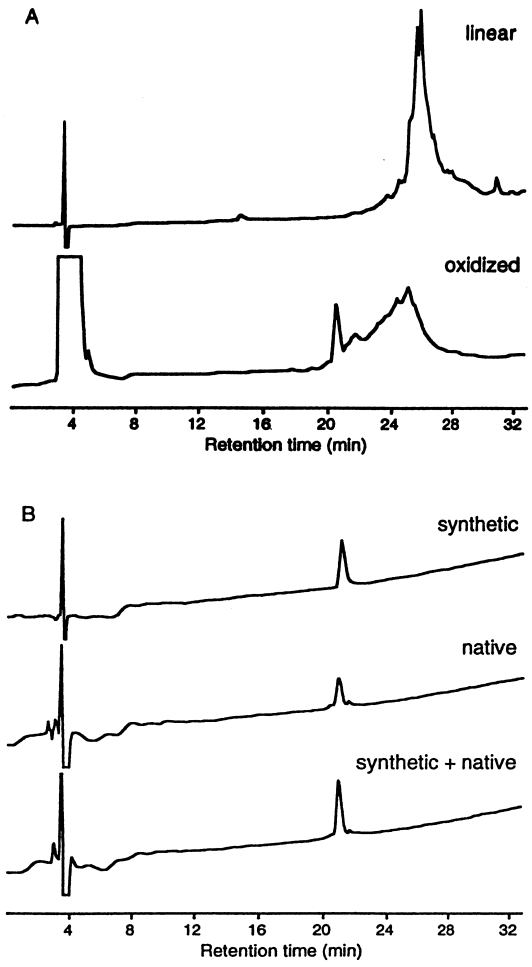

Fig. 3. The chromatographic profiles of synthetic linear and oxidative folded Cvtx-II. Reverse phase HPLC profiles of the linear precursor of Cvtx-II and the oxidative folding mixture (A), and synthetic Cvtx-II, native Cvtx-II and co-injected synthetic and native Cvtx-II (B). The peptides were eluted from an ODS column (4.6 mm×250 mm) in a linear gradient of 5–35% ACN with aqueous 0.1% TFA for 30 min at flow rate of 1 ml/min.

The synthetic Cvtx-II was used for the characterization of biological activity on crickets, cockroaches and young Swiss mice. No visible symptoms or behavioral changes were observed after Cvtx-II was injected in *Dictyoptera* German cockroaches (maximal concentration 0.3 μmol/g) or Swiss mice (maximal concentration 0.1 μmol/mice). However, injection of the peptide into crickets induced excitatory behavioral symptoms such as quivering, jerking and hyperextension of the legs. Rapid movement of antenna, mandibles and maxillae with abdominal contraction and frequent body arching were observed. This was accompanied by loss of righting reflex possibly due to limb weakness. Locomotion was greatly reduced in peptide-injected crickets as compared to the control insects (crickets injected with normal saline). Gradual body tilting to one side was observed within 10–15 min of injection. Escape reflexes on application of cercal stimuli were either weak or could not be elicited, indicating paralysis. The dose required to immobilize or inactivate 50% of insect (ID_{50}) was 0.2 μmol/g (0.16–0.33 μmol/g with 95% fiducial confidence limits calculated using Probit analysis of data from eight groups of at least four animals). The immobilizing effect lasted for 40–60 min with a gradual recovery of normal posture and activity.

The excitatory symptoms induced by Cvtx-II suggest that it is a species-specific anti-insect toxin which is analogous to other anti-insect spider toxins [1] and scorpion toxins [25–28]. Excitatory toxins are only active on insects and induce immediate and reversible spastic–contractive paralysis through sustained repetitive firing as observed in locust neuromuscular preparation [29]. At the molecular level, the excitotoxins retard insect sodium channel inactivation [30]. In light of similarities in biological action with other excitotoxins, we believe that insect ion channel protein could be the target of Cvtx-II. Insect-selective excitatory toxins are valuable tools for the study of the pharmacology of ion channels present in insects and the design of insect-selective biopesticides [31,32].

Understanding the natural predatory behavior of arthropods will provide a better perspective of the biological significance of arthropod venom. In most cases of spider predation, the prey is not eaten immediately after capture, but wrapped in a ball of silk. Most tropical spider species possibly have insect-specific non-lethal/reversible neurotoxic components, which could play a beneficial role in preventing the captured prey from putrefaction in a humid and hot tropical environment. Unlike other species of spiders, tarantulas do not use web-strategy to capture their prey, and they devour the prey within minutes. Hence, the occurrence of such a protein with reversible neurotoxic property in its venom possibly suggests venom co-evolution within the Phyla. These toxin(s) in association with/without other components may act by unknown mechanism(s) to bring down the basal metabolic rates of its victims to a minimum level, causing a natural 'refrigeration effect'. This effect is most commonly utilized by other arthropods, such as wasps and hornets, to store food for the survival of its brood [33].

In summary, a novel peptide toxin has been purified and characterized from the venom of Singapore tarantula *C. validus*. This insect-specific toxin may be used as a tool for understanding molecular mechanisms involved in reversible paralysis with the possibility of providing a better insight into the development of selective bio-insecticides. However, further cellular and molecular studies are needed to understand the mechanism(s) of reversible paralysis of crickets by Cvtx-II.

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