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Purification and characterization of raventoxin-I and raventoxin-III, two neurotoxic peptides from the venom of the spider *Macrothele raveni*

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Abstract

The spider *Macrothele raveni* was recently identified as a new species of Genus *Macrothele*. The crude venom from *M. raveni* was found to be neurotoxic to mice and the LD_{50} of the crude venom in mice was 2.852 mg/kg. Two neurotoxic peptides, raventoxin-I and raventoxin-III, were isolated from the crude venom by ion-exchange and reverse phase high performance liquid chromatography. Raventoxin-I was the most abundant toxic component in the venom, while raventoxin-III was a lower abundant component. Both toxins can kill mice and block neuromuscular transmission in an isolated mouse phrenic nerve diaphragm preparation, but have no effect on cockroaches. The LD_{50} of raventoxin-I in mice is 0.772 mg/kg. The complete amino acid sequences of raventoxin-I and raventoxin-III were determined and found to consist of 43 and 29 amino acid residues, respectively. It was determined by mass spectrometry that all Cys residues from raventoxin-I and raventoxin-III are involved in disulphide bonds. raventoxin-III showed no significant sequence homology with any presently known neurotoxins in the protein/DNA databases, while raventoxin-I has limited sequence identity with δ-AcTx-Hv1 and δ-AcTx-Ar1, which target both mammalian and insect sodium channels. Both raventoxin-I and raventoxin-III only work on vertebrates, but not on insects. Moreover, raventoxin-I could exert an effect of first exciting and then inhibiting the contraction of mouse diaphragm muscle caused by electrically stimulating the phrenic nerve, but raventoxin-III could not.
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Keywords: *Macrothele raveni*; Raventoxin-I; Raventoxin-III; Neurotoxins; Spider venom; Amino acid sequences

1. Introduction

Spider venoms are well known to contain several classes of neurotoxins, which are of interest as tools for studying neurophysiology and as potential lead structures in designing and obtaining insecticides and pharmaceuticals. Furthermore, antibodies raised against the critical toxic components have the potential to block the toxic effects and reduce the pain associated with the spider envenomation. The spider *Macrothele raveni* was recently identified as a new species of Genus *Macrothele* (Zhu et al., 2000). It is distributed in the hilly areas of Ningming County, Guangxi Province, China. This hairy spider has a body-length of 2–3 cm, which is smaller than that of *Selenocosmia huwena*. It has been found that the venom from the spider *M. raveni* contains a mixture of compounds with different types of biological activity. In this paper, by combination of mass spectrometry and reverse phase high performance liquid chromatography (HPLC) separation and bioassays, we report the purification and characterization of two novel neurotoxic peptides, named raventoxin-I and raventoxin-III, from the venom of the *M. raveni*, including

Abbreviations: HWTX-I, huwentoxin-I; SHL-I, *Selenocosmia huwena* lectin-like peptide; HPLC, high performance liquid chromatography; MALDI-TOF, matrix assisted laser desorption ionization time-of-hight.

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the complete amino acid sequences and their partial biological properties.

2. Materials and methods

2.1. Venom and animals

Three hundred and eighty adult female *M. raveni* spiders were collected in the hilly area of Ningming county, Guangxi, China. Spiders were identified by Professor M.S. Zhu (College of Life Science, Hebei University). The animals were kept in plastic buckets with mud covered with nylon net and given water daily. Cockroaches and *Tenebrio molitors* L were used to feed the spiders. The venom was obtained by electrical stimulation of spiders every 3–4 weeks, and the lyophilized crude venom was stored at −20 °C prior to analysis. Kunming albino mice were obtained from Central South University Xiang Ya School of Medicine. Cockroaches (*Periplaneta americana*) were from Peking University.

2.2. Chemicals

Reagents for N-terminal sequencing were from Applied Biosystems; guanidine hydrochloride, dithiothreitol, *N*-methylmorpholine and iodoacetamide were from Sigma; and HPLC grade acetonitrile was from Lin hai Chemicals. All other reagents were of analytical grade.

2.3. Venom fractionation

Ion exchange chromatography was performed using a Waters protein-Pak CM 8H column (10 mm × 100 mm) on a Waters™ 650 Advanced Protein Purification system equipped with a model 486 detector. Fractions from ion exchange chromatography were further fractionated by reverse phase HPLC on a Vydac C18 column (4.6 mm × 250 mm) on a Waters™ Alliance 2690 HPLC system with a model 996 photodiode array detector.

2.4. Mass spectrometry

Molecular masses of peptides were determined by a Bruker ProFlex III matrix assisted laser desorption ionization time-of-hight (MALDI-TOF) system. Huwentoxin-I (HWTX-I) was used as an internal standard, and α-cyano-4-hydroxy-cinnamic acid as the matrix. Saturated matrix solutions were prepared in a 50% (v/v) solution of acetonitrile and aqueous 0.1% trifluoroacetic acid, mixed in a 8:2 ratio with toxin samples, applied to the stainless steel sample plate and dried before loading into the mass spectromer. All molecular mass and PI calculations were carried out by using ExPASy Proteomics tools (http://www.expasy.ch/).

2.5. Reduction and carbamidomethylation of cysteines

The native peptide (about 0.2 μmol) was dissolved in 100 μl of the denaturant buffer (0.5 M *N*-methylmorpoline acetate, pH 8.3), and 100 mg guanidine hydrochloride and 1.4 mg dithiothreitol added into it. The mixture was incubated at 25 °C for 4 h under nitrogen. Subsequently, 2.2 mg iodoacetamide was added and incubated at room temperature for 12 h under nitrogen. The samples were then immediately desalted on the Vydac C18 column (4.6 mm × 250 mm).

2.6. Amino acid sequence analysis

Amino acid sequencing was carried out by automated Edman degradation using an Applied Biosystems 491 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 140 analyzer.

Sequence homologies were determined using sequences obtained from a search of protein databases, via the BLAST server (BLAST program http://www.ncbi.nlm.nih.gov/)

2.7. Biology assays

The vertebrate toxicity of raventoxins was assayed by intra-peritoneal injection of 50 μl solutions of toxin in 0.9% (w/v) NaCl into 18–20 g mice of both sexes. The control animals were injected with 50 μl of 0.9% normal saline and each toxin was tested on three mice. The LD_{50} was determined in six mice at each of five dosage levels and observed 24 h following intraperitoneal injection according to the method of Schweitz (1984).

The insect toxicity was determined by intra-abdominal injection of 20 μl solutions of toxin in 0.9% NaCl into male cockroaches (*P. americana*) of the same age and with body weights of about 0.35 g (Shu and Liang, 1999). Each assessment was done on three cockroaches.

Pharmacological experiments were carried out using mouse phrenic neuromuscular transmission preparations. After dissection, the preparation was placed in a small Plexiglas chamber and immersed in Tyrode's solution or toxin dissolved in Tyrode's solutions bubbled with 95% O_2, 5% CO_2 and kept at 30–32 °C. Electrical stimulation was applied indirectly to the phrenic nerve with a suction electrode or directly to the muscle at a frequency of 0.2 Hz (supramaximal, 0.2 ms, square wave). The twitch responses were transformed into electric signals by a mechanical–electric transducer made from a semiconductor stain gauge. The signals were amplified and recorded by a Rm6240B four-channel physiological recorder. HWTX-I, a presynaptic N-type Ca^{2+} channel inhibitor, was used as a positive control (Peng et al., 2001; Liang et al., 2000) SHL-I, a non-toxic *S. huwena* lectin-like peptide, was used as a negative control (Liang et al., 1995).

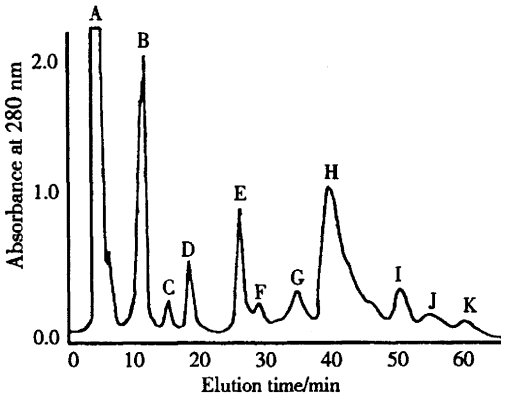

Fig. 1. Ion exchange HPLC of crude *M. raveni* venom. Lyophilized venom (5 mg in 1 ml distilled water) was applied to a Waters Protein-Pak CM 8H ion exchange column (10 mm × 100 mm) initially equilibrated with 0.02 M sodium phosphate buffer, pH 6.3 (buffer A).The column was eluted with a linear gradient of 0–80% of buffer B (1 M sodium chloride, 0.02 M sodium phosphate, pH 6.3) over 60 min at a rate of 2.0 ml/min.

3. Results and discussion

3.1. Venom fractionation and mass spectrometry

Spiders use their venoms to paralyse or kill their prey. The well studied components in spider venom have been broadly classified into two types: polypeptide components and polyamines (Rash and Hodgson, 2002). Most research on spider toxins has focused on the analysis of neurotoxic polypeptides in order to determine the role and diversity of neuronal ion channels, the process of exocytosis and the potentiality for the use of insect-specific toxins from animal sources in agriculture.

Following preliminary testing, the crude venom from the spider *M. raveni* was found to be toxic to mice and was selected for further fractionation. A typical ion exchange chromatography elution profile for the crude venom of the spider *M. raveni* is shown in Fig. 1. The fraction A contained raventoxin-II, which was a insect-specific neurotoxin; another four mammalian neurotoxins, named raventoxin-I, raventoxin-III, raventoxin-IV, and raventoxin-V, respectively, were isolated from Fraction E, J, H and I. Both Fraction E and J were subjected to reverse phase HPLC, and the peptides eluting at 23.64 min (raventoxin-I) and 17.95 min (raventoxin-III) were collected and lyophilized, respectively, (Fig. 2). Toxins were obtained in a high degree of purity as confirmed by analytical chromatography and MALDI-TOF MS. Raventoxin-I was the most abundant toxic component in the venom, while raventoxin-III was a lower abundant component. The MALDI-TOF mass spectra of raventoxin-I and raventoxin-III in the presence of an internal HWTX-I standard ($M + H^+ = 3751.45$; calc. $M = 3750.45$) are shown in Fig. 3. The observed mass ($M + H^+$) of raventoxin-I is 4841.11 Da, while that of raventoxin-III is 3287.58 Da. In other words, the molecular mass of raventoxin-I is 4840.11 Da, while that of raventoxin-III is 3286.58 Da.

3.2. Amino acid sequence analysis

The N-terminal 35 amino acid residues sequence of raventoxin-I was determined by automated Edman degradation and its C-terminal 12 amino acid residues were determined by Staphylococcal protease V_8 digestion combined with N-terminal sequencing. In addition,

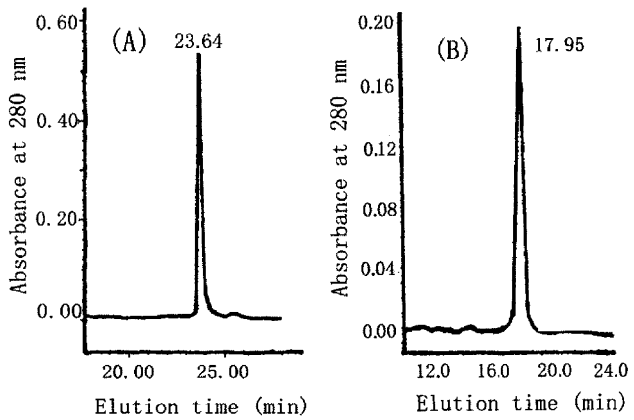

Fig. 2. Purification of raventoxin-I and raventoxin-III by reverse phase HPLC. Both ion-exchange HPLC fraction E and J were applied to a Vydac C-18 column (4.6 mm × 250 mm). The column was equilibrated and washed with 0.1% trifluoroacetic acid in water (buffer A) and bound peptides were eluted using a linear gradient of 0–15% for 5 min, 15–55% for 40 min and 55–100% for 5 min buffer B (acetonitrile containing 0.1% trifluoroacetic acid) at a flow rate of 1.0 ml/min. Elution of peptides was monitored at 280 nm. (A) The peptide eluting at 23.64 min was raventoxin-I. (B) The peptide eluting at 17.95 min was raventoxin-III.

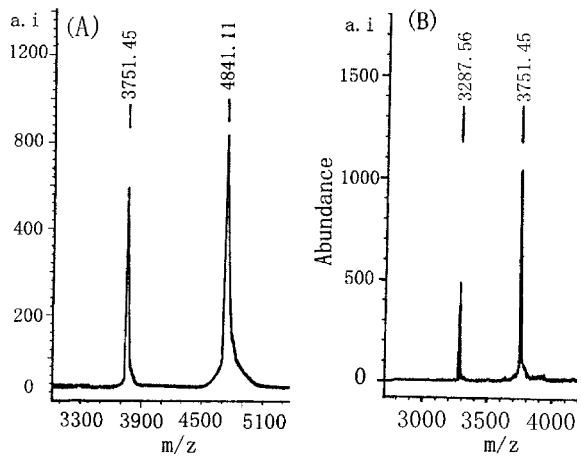

Fig. 3. MALDI-TOF mass spectra of raventoxins. (A) MALDI-TOF mass spectrum of raventoxin-I. (B) MALDI-TOF mass spectrum of raventoxin-III.

the C-terminal residue confirmed by carboxypeptidase Y was cysteine residue. The complete amino acid sequence of raventoxin-I is CGTNRAWCRNAKDHCCCGYSCVKPI-WASKPEDDGYCWKKFGGC. It is composed of a single peptide chain and consists of 43 amino acid residues, including eight cysteine residues and six aromatic amino acid residues. Furthermore, there is a triplet cysteine residues in the middle of the sequence, and at the first and last position, there are also cysteine residues. The sequence was in good agreement with the data obtained from mass spectrometry. The theoretical molecular mass (4848.55) calculated from sequence data was eight more than the molecular mass (4840.11) from MALDI-TOF MS, suggesting that the eight cysteine residues were paired into four disulfide bridges.

A protein database search showed that the isolated raventoxin-I has limited sequence identity with δ-AcTx-Hv1a from *Hadronyche versuta* and δ-AcTx-Ar1 from *Atrax robustus* (Wang et al., 2001), which target both mammalian and insect sodium channels (shown in Fig. 4).

The amino acid sequence of native raventoxin-III was determined by automated Edman degradation. All positions were assigned except for positions 2,9,15,16,21 and 26, which showed no new peak. Then the peptide was reduced, *S*-carbamidomethylated and resequenced to determine the positions of cysteine residues. Carbamidomethylated-cysteines were identified at each of the previously unidentified positions. The complete amino acid sequence of raventoxin-III was GCKLTFWKCKNKKECCGWNA-CALGICMPR. It has only 29 residues including six cysteine residues at position 2,19,15,16,21 and 26, one acidic aspartic acid residue and five basic lysine residues. Hence, raventoxin-III is a basic polypeptide whose isoelectric point based on calculation is 9.13. The theoretical molecular mass of raventoxin-III (3293.04) is six more than the factual molecular mass (3286.58), indicating that, like many related proteins, all the cysteine residues are involved in disulphide cross-links. The peptide has an uneven distribution of hydrophilic (34.48%), hydrophobic (37.93%) and neutral (27.59%) and shows no significantly homology with any presently known toxins in the protein/DNA databases, indicating that raventoxin-III is a novel toxic molecule, and that it would be of interest to study the relation between the structure and functional mechanism of raventoxin-III.

3.3. Bioactivity assay

Preliminary biological activity studies showed that the crude venom from *M. raveni* was neurotoxic to mice. The LD_{50} of the crude venom in mice was 2.852 mg/kg (body weight). Both raventoxin-I and raventoxin-III can cause death in mice. The neurotoxic symptoms were excitation, gasping and spastic paralysis, as well as exophthalmos. The breath was short and hasty; the heartbeat was fast. However, salivation was only observed after injection of raventoxin-I, but was not observed after injection of raventoxin-III. The LD_{50} of raventoxin-I in mice by intra-abdominal injection was 0.772 mg/kg (body weight), which was higher than that of δ-AcTx-Hv1a (0.22 mg/kg), δ-AcTx-Ar1 (0.16 mg/kg) (Little et al., 1998a,b) and HWTX-I (0.7 mg/kg) (Liang et al., 1993a,b), indicating that the vertebrate toxicity of raventoxin-I was lower than that of δ-AcTx-Hv1 and δ-AcTx-Ar1, and was also lower than that of HWTX- I reported by our laboratory. Furthermore, no visible symptoms or behavioral changes were observed after injecting both raventoxin-I and raventoxin-III into cockroaches (dose 100 μg/g body weight), which indicated that raventoxin-I and raventoxin-III were mammalian specific. Although raventoxin-I has partial sequence homology with δ-AcTx-Hv1 and δ-AcTx-Ar1, it is of different function.

Fig. 5 shows the effect of raventoxin-I and raventoxin-III on the neuromuscular transmission of the isolated mouse phrenic nerve diaphragm preparation. With 3.0 μM raventoxin-III, the amplitide of the twitch response to indirect stimulation decreased gradually and disappeared eventually.

Toxin	Amino acid sequence	Length	%I
Raventoxin- I :	CGTNRAWCRNAKDH CCCGYS CVKP I WASKPE DDGYCWKKFGGC	43	–
δ-ACTX-Hv1a:	CAKKRNWCGKTED - CCCPMKCVYAWYNEQG SCQS TI SAL WKKC	42	45
δ-ACTX-Ar1 :	CAKKRNWCGKNED - CCCPMKCIYAWYNQQG SCQT TI TG L F KKC	42	40

Fig. 4. Primary sequence comparison among raventoxin- I, δ-AcTx-Hv1a and δ-AcTx-Ar1. Residues identical to raventoxin-I are shaded in black. To maximize identity, the deletions (–) are included in the sequence. %I is percentage identity of raventoxin-I with other spider toxins.

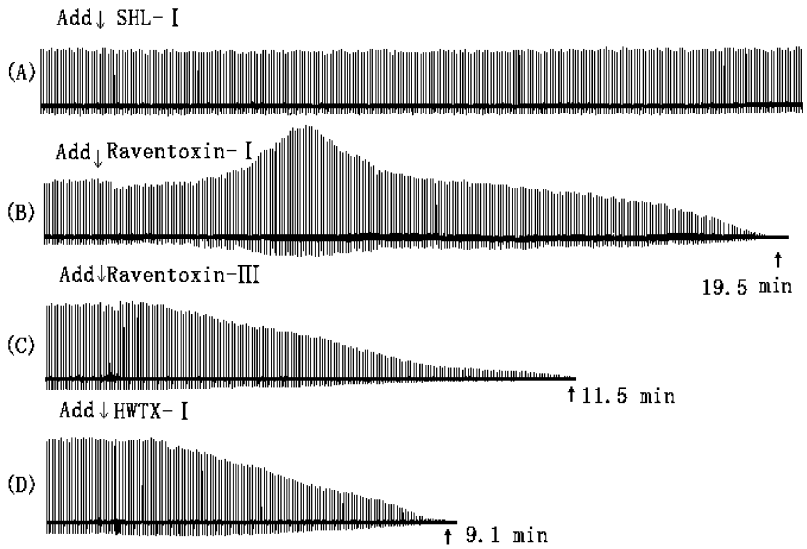

Fig. 5. Effect of raventoxin-I and raventoxin-III on neuromuscular transmission in isolated mouse phrenic nerve diaphragm preparation. Twitch response of the diaphragm to electrical stimulation in the presence of (A) 3.0 μM SHL-I; (B) 3.0 μM raventoxin-I; (C) 3.0 μM raventoxin-III; (D) 3.0 μM HWTX-I. Among them, the non-toxic SHL-I was used as negative control, and HWTX-I was used as positive control.

The transmission was blocked in 11.5 ± 1.3 min (mean ± SD, $n = 6$). After blockage, the direct electrical stimulation of muscle was unaffected and the response of nerve stimulation appeared anew by repeated washing with Tyrode's solution for 4 or 5 times. With less than 1.5 μM raventoxin-III, the amplitide of the twitch response to indirect stimulation began to recover even prior to complete disappearance. It indicated that raventoxin-III could reversibly block the neuromuscular transmission. The non-toxic control peptide, *S. huwena* lectin-I (SHL-I), at the same concentration had no effect on the neuromuscular transmission. With 3.0 μM raventoxin-I, the amplitide of the twitch response to indirect stimulation first increased gradually, then decreased slowly and disappeared eventually. Contraction was blocked in 19.5 ± 1.4 min (mean ± SD, $n = 4$). Interestingly, with 0.5 μM raventoxin-I, the contraction amplitude could increase by 160%, but when the concentration was less than 0.1 μM, the contraction enhancement effect disappeared. The experiments indicated that the excitatory action was related to the concentration of raventoxin-I. After blockage, a little attenuation of the twitch responses to direct muscular stimulation could be observed and the responses could last 40 min prior to disappearing. Also the responses of the directly stimulated diaphragms following exposure to 0.5 μM raventoxin-I could repeat the process: the contraction first increased gradually, then inhibited slowly, but the contraction amplitude could only increase by 100% and the response time could last 80 min. With 0.5 μM raventoxin-I, the transmission blockage time of the indirectly stimulating diaphragms was 36.1 min (mean ± SD, $n = 3$). The experiments suggest that raventoxin-I could not only block the neuromuscular transmission, but also could act on muscle. Presently, no toxins having similar action to raventoxin-I were reported, so the exact mechanism of action raventoxin-I needs to be investigated further. Fig. 6 shows the concentration-response relationship for raventoxin-I on the neuromuscular transmission of the isolated mouse phrenic nerve diaphragm preparation. The experimental results show that raventoxin-I blocks neuromuscular transmission slower than HWTX-I did for the same concentration. These findings suggested that despite coming from the same spider, raventoxin-I and raventoxin-III have different biological functions.

In summary, two novel polypeptide toxins have been purified and characterized from the venom of the spider *M. raveni*. The complete amino acid sequence and the biological activities of both toxins showed that they were two neurotoxic peptides different from other animal toxins

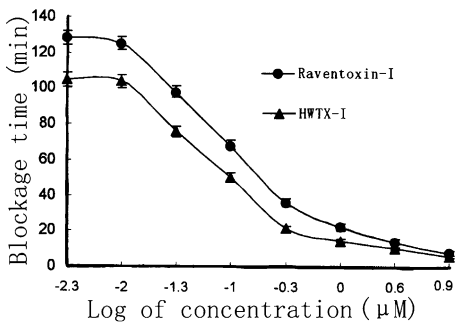

Fig. 6. Concentration–response relationship curve of raventoxin-I on neuromuscular transmission in isolated mouse phrenic nerve diaphragm preparation. Each point represented the average of two independent experiments. Error bars represented the standard devitation. HWTX-I was used as positive control.

known so far. Further studies are in progress to study these toxins as well as other biologically active components in the venom of the spider *M. raveni.*

Acknowledgements

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