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Huwentoxin-V, a novel insecticidal peptide toxin from the spider *Selenocosmia huwena,* and a natural mutant of the toxin: indicates the key amino acid residues related to the biological activity

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Abstract

A neurotoxin peptide (named Huwentoxin-V) was purified from the venom of the Chinese bird spider *Selenocosmia huwena* by a combination of ion exchange chromatography and reverse phase HPLC. HWTX-V has 35 amino acid residues, and is in perfect agreement with the molecular mass 4111.4 Da identified by mass spectrometry. A natural mutant of the toxin (called mHuwentoxin-V) was also isolated from the venom. mHWTX-V was only truncated two amino acid residues from the C-terminus of HWTX-V, and its molecular weight is 3877.1 Da determined by mass spectrometry. The six cysteine residues in each sequence of the two peptides suggest three disulfide bridges, the present of which was demonstrated by mass spectrometry after dithiothreiotol reduce and S-carboxymethylation. The primary structure of the two toxins exhibits sequence identity with other spider toxins such as ProTx-I (64%), SGTx (57%), SNX-482 (55%), and Hanatoxin (54%). HWTX-V can reversibly paralyze locusts and cockroaches for several hours with a ED_{50} value as 16 ± 5 μg/g to locusts, and a larger dose of the toxin can cause death. However, mHWTX-V shows no significant effect on locusts and cockroaches. The structure–activity relationship indicates that the residues Phe^{34} and Ser^{35} in the C-terminus of HWTX-V are the key residues of the biological activity.

Keywords: Huwentoxin-V; The mutant of Huwentoxin-V; Key amino acid residues; Insecticidal toxin

1. Introduction

Spider venoms are known to contain multitude components with different biological activities. Among them, neurotoxins acting on different receptors and ion channels are of interests as tools for studying neurophysiology and as potential lead structures for insecticides and pharmaceuticals (Atkinson and Wright, 1992; Escoubas et al., 2000; Crinshin, 1999; Crinshin, 1998). The spider *Selenocosmia huwena* was identified as a species of genus *Selenocosmia* (Wang et al., 1993). In our previous work, we have isolated and characterized several kinds of peptide components from the venom of the spider *Selenocosmia huwena*. HWTX-I has 33 amino acid residues and three disulfide bridges, which selectively inhibited N-type Ca^{2+}channel and only a weak effect on L-type Ca^{2+}channel in prostaglandin E_1 differentiated NG108-15 cell (Liang et al., 1993; Zhou et al., 1997; Penk et al., 2001). HWTX-II contains 37 amino acid

Abbreviations: HWTX-I, huwentoxin-I; HWTX-II, huwentoxin-II; HWTX-IV, huwentoxin-IV; SHL-I, *Selenocosmia huwen* lectin-like peptide-I; HWTX-V, huwentoxin-V; mHWTX-V, the mutant of huwentoxin-V; HWTX-VI, huwentoxin-VI; HPLC, high performance liquid chromatography; MALDI-TOF, matrix assisted laser desorption/ionization time-of-flight; TFA, trifluoroacetic acid.

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residues and can reversibly paralyze insects, block neuromuscular transmission in an isolated mouse phrenic nerve diaphragm preparation and act cooperatively with the potential activity of HWTX-I (Shu and Liang, 1999; Shu et al., 2002). HWTX-IV consists of 35 amino acid residues, which can specifically inhibit the neuronal tetredotoxin-sensitive (TTX-S) voltage-gated Na^+ channel with the IC_{50} value as 30 nM in adult rat dorsal root ganglion neurons (Peng et al., 2002). SHL-I is a peptide with haemagglutination activity, and includes 32 amino acid residues containing three disulfide bonds (Liang and Pan, 1995). The three-dimensional structures of HWTX-I (Qu et al., 1997), SHL-I (Lü et al., 1999), HWTX-IV (Peng et al., 2002) and HWTX-II (Shu et al., 2002), solved by 2D-NMR, reveal that they belong to the Inhibitory Cysteine Knot motif except for HWTX-II, which adopts a unique structure with the disulfide-directed β-hair motif. In this paper, we will describe a rapid procedure of combination of ion exchange and reverse phase high performance liquid chromatography that used to isolate a novel insecticidal neurotoxic peptide (named Huwentoxin-V) and its natural mutant of the toxin from the venom of *Selenocosmia huwena*. In addition, their complete amino acid sequence, the partial biological properties and the possible key amino acid residues in HWTX-V are also presented.

2. Materials and methods

2.1. Venom and animals

Adult female *Selenocosmia huwena* spiders were purchased from the mountain area of Ningming County, Guangxi, China. The venom from adult female *Selenocosmia huwena* spider was collected as described in our previous work (Liang et al., 1993). Kunming albino mice were obtained from Xiangya Medical College, Central South University, China. Cockroaches (*Periplaneta Americana*) were provided by Peking University. Locusts (*Migratory manieusis*) were collected in Fengqiu County, Henan, China.

2.2. Chemical reagents

Reagents for N-terminal sequencing were from Applied Biosystems; Guanidine hydrochloride, dithiothreitol, *N*-methylmorpholine and iodoacetamide were purchased from Sigma; and HPLC grade acetonitrile was from Li 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

The molecular masses of two peptides determined by using matrix-assisted laser desorption ionization-time of flight (Voyager DE™ MALDI-TOF, US). The laser wavelength was set at 337 nm. Mass spectra were recorded in reflectron mode with an acceleration voltage of 20 kV. The samples were prepared with α-cyano-4-hydroxy-cinnamic acid. External calibration was performed with HWTX-I (molecular weight as 3750.4 Da) (Liang et al., 1993).

2.5. Reduction and S-carboxymethylation of peptides

The native peptide was reduced and S-carboxymethylated using the method described in our previous work (Liang et al., 1993).

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. Phenlthiohydantion (PTH) amino acids were identified using on-line reverse phase HPLC on a PTH-C18 column at an Applied Biosystems 140 analyzer.

2.7. Toxicity assay

The mammalian toxicity of HWTX-V and mHWTX-V was qualitatively assayed by intra-abdominal and intracerebroventricular injection into mice (body weight 18 ± 2 g, both sexes) using solutions in 0.9% (w/v) NaCl for each toxin. The intra-abdominal injection volumes were 50 μl and the intracerebroventricular injection volumes were 20 μl. The controlled mice were injected with corresponding volume of 0.9% normal saline. The injections on mice via intra-abdominal and intracerebroventricular were excised by procedure of Zeng (Zeng and Liang, 2001). The insect toxicity of HWTX-V and mHWTX-V was qualitatively tested by intra-abdominal injection of 20 μl toxin dissolved in insect saline into house male cockroaches (body weight 1.3 ± 0.2 g) and locusts (sex undetermined, body weigh 0.73 ± 0.2 g). Contrasted insects were received injection of insect saline. Observation of paralysis and /or death was made at 5 min, 15 min, 60 min, 24 h and 48 h post-injection. The assay end-point for calculation of ED_{50} was 15 min and ED_{50} were calculated using probit analysis with the SoftTox program (softLabWare Inc.). The injections on insect were

performed according to the method of Anette (Annette, 1989).

2.8. Pharmacological experiments

The experiment was carried out using mouse phrenic nerve-diaphragm preparations. After dissection, the preparation was put into a small Plexiglas chamber immersed in Tyrode's or toxin dissolved in Tyrode's solution (composition as Zhou et al, 1997) bubbled with 95%O_2, 5%CO_2 and kept at 30–32 °C. The electrical stimulation was applied indirectly to the phrenic nerve with a suction electrode. The twitch responses were transformed into electric signals by a mechanical–electric transducer made of semiconductor stain gauge. The signals were amplified and recorded with a pen recorder.

2.9. Database searches and multiple alignments

Sequence homologies were determined using the BLAST server (http://www.ncbi.nlm.nih.gov/). Multiple alignments and percentages of similarity were calculated with identity.

3. Results and discussion

3.1. Purification and chemical structure characterization

A typical ion exchange chromatography elution profile for crude *Selenocosmia huwena*toxin is shown in Fig. 1. The peaks B, C, D and E mainly describe SHL-I, HWTX-II, HWTX-I, and HWTX-IV respectively. The peak A was subjected to reverse phase HPLC (Fig. 2). In this reverse phase chromatography, the peak I is a recently characterized as a neuronal toxin HWTX-VI (unpublished), and the peak

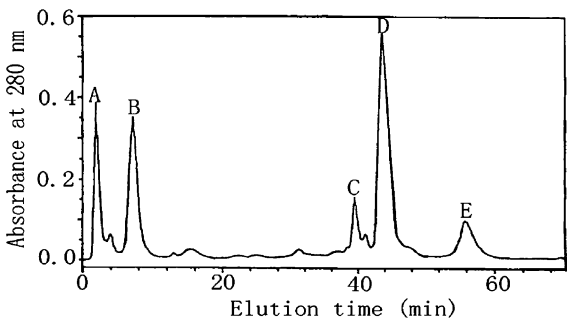

Fig. 1. Ion exchange HPLC of crude *Selenocosmi huwena*. Lyophilized venom (5 mg in 1 ml distilled water) was applied to a Waters Protein Pak CM 8 H exchange column (10 mm × 100 mm) initially equilibrated with buffer A (0.02 M sodium phosphate buffer, pH 6.25). The column was eluted with a linear gradient of 0–80% 0f buffer B (1 M sodium chloride, 0.02 M sodium phosphate, pH 6.25) over 40 min at a rate of 1.5 ml/min.

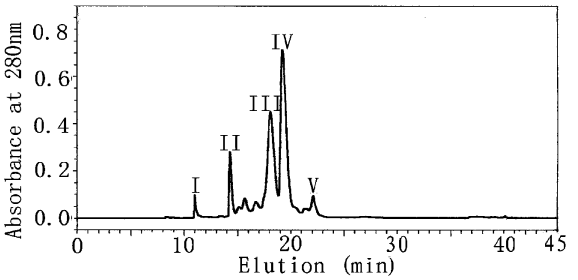

Fig. 2. The reverse phase HPLC of the peak A in ion exchange procedure. The ion exchange peak A (Fig. 1) eluting at 5 min was applied to a Vydac C18 column (4.6 mm × 250 mm) initially equilibrated with 0.1% trifluoroacetic acid in distilled water (buffer A). Elution was performed with a linear gradient of 25-40% buffer B (acetonitrile containing 0.1% trifluoroacetic acid) over 45 min at a flow rate of 1 ml/min at 40 °C. Elution of peptides was monitored at 280 nm.

II is the mutant of SHL-I (unpublished). The peak III (eluting at 18.0 min) was collected and lyophilized and further purified by reverse phase HPLC (Fig. 3C) to show a single fraction. We named this toxin the mutant of Huwentoxin-V (mHWTX-V). The peak IV (retention time at 19.2 min) was also collected, lyophilized, and further purified by reverse phase HPLC (Fig. 3A). This peptide toxin was found to be toxic to insects and called HWTX-V. The peak V has not been characterized yet. The molecular masses of HWTX-V and mHWTX-V were determined by MALDI-TOF mass spectrometry to be 4111.4 ± 0.4 Da (Fig. 3B) and 3877.1 ± 0.4 Da (Fig. 3D). After dithiothreitol reduction and S-carboxymethylation, the masses of the two peptides increase to 4460.2 ± 0.4 Da and 4225.1 ± 0.4 Da respectively (data not shown). From their mass units shift, we can conclude that HWTX-V and mHWTX-V contained six cysteines. The complete amino acid sequence of HWTX-V is NH_2-ECRWYLGGC-SQDGDCCKHLQCHSNYEWCVWDGTFS-COOH determined by automated Edman degradation. There were about 6 mass units different from the exact mass (4111.4 Da) and the theoretical mass (4117.5 Da), indicating that, like many other toxic peptides from the venom of spider, all the six cysteine residues were involved in three disulfide bonds. The amino acid sequence of mHWTX-V was determined to be H_2-ECRWYLGGCSQDGDCCKHLQCHSNYEWCVW DGT-COOH. The sequences of two peptides are almost identical except that mHWTX-V was truncated two amino acid residues (Phe^{34} and Ser^{35}) in the C-terminus of HW TX-V. It is unclear whether HWTX-V and mHWTX-V are expressed from the same gene. Comparison of mHWTX-V calculated molecular mass of 3883.2 Da (or 3877.2 Da if all cysteines are involved in disulfide bridges) with the experimentally determined molecular mass obtained by MALDI-TOF MS, confirmed the presence of three disulfide bridges.

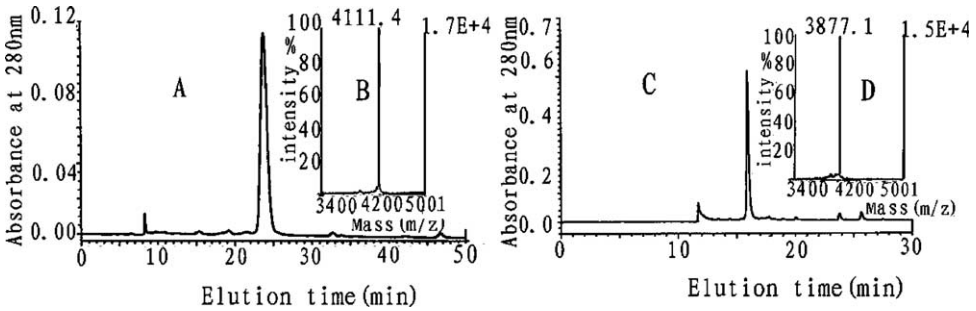

Fig. 3. (A) Further purification of HWTX-V by reverse phase HPLC. The fraction IV (Fig. 2) eluting at 19.2 min was further purified on a Vydac C4 column (4.6 mm × 250 mm) previous equilibrates with buffer A (water containing 0.1% TFA). The elution was performed with a linear gradient from 20 to 35% buffer B (acetonitrile containing 0.1% TFA) over 50 min at a flow rate 0.7 ml/min at 40 °C. Elution of peptide was monitored at 280 nm. (B) The mass spectrum of HWTX-V determined by Voyager DE™ STR MALDI-TOF system. (C) Further purification of mHWTX-V by reverse phase HPLC. The fraction III (Fig. 2) eluting at 18 min was further purified on a Vydac C4 column (4.6 mm × 250 mm) previous equilibrates with buffer A (water containing 0.1% TFA). The elution was performed with a linear gradient from 25 to 35% buffer B (acetonitrile containing 0.1% TFA) over 30 min at a flow rate 0.7 ml/min at 40 °C. Elution of peptide was monitored at 280 nm. (D) The mass spectrum of mHWTX-V determined by Voyager DE™ STR MALDI-TOF system.

3.2. Primary bioactivity assay

Spiders use their venoms to paralyze or kill their preys. The well-studied components in the venom of spiders have been broadly classified into two types: polypeptide components and polyamines. In order to determine the roles and diversities of neuronal ion channel, the process of exocytosis and potentiality for the use of insect specific from animal in agriculture, most researches on spider toxin have focused on the analysis of neurotoxin polypeptide. Preliminary studies of the bioactivitical activity showed that HWTX-V immobilized locusts and cockroaches within 15 min, and caused a dose-dependent reversible paralysis that lasted about 6–8 h. The intra-abdominal injection ED_{50} value in locust (*Migratory manieusis Meyen*) of HWTX-V is 16 ± 5 μg/g ($P = 0.95$), and a larger dose (≥100 μg/g) can cause insects to die. The neurotoxic symptoms induced by intra-abdominal injection of Huwentoxin-V were firstly paralysis, then immobilization and last gradually recovery. This paralysis activity might have significant meanings for the spiders preying. HWTX-V was not shown to block neuromuscular transmission in isolated mouse phrenic nerve diaphragm preparation. The signals of experiments had no change within 100 min at a concentration of 5×10^{-5} g/ml (data not shown). HWTX-V also had no evident effect on mice by intra-abdominal injection with high dose of 200 μg/g and by intracerbroventricular injection at the level of 30 μg/g. We can conclude that Huwentoxin-V is a potent insecticidal toxin, which has insect-specific toxic activity. Comparison of the sequence of HWTX-V with that of HWTX-II (another insecticidal toxin from the same spider) indicated that there is very low homology between two toxins (Fig. 4). This result was not surprising because the biological activities of two toxins are quite different. As the toxicity of HWTX-V is eight times as strong as that of HWTX-II (ED_{50} value as 127 ± 54 μg/g)(Shu and Liang, 1999), HWTX-V appears to have an interesting potential for agriculture application to kill pests. The activity in physiology supports the possibility to develop HWTX-V as a new insecticide for agriculture through the modification by molecular biology and/or chemical approach. Surprisingly, mHWTX-V showed no evident effect on locusts and cockroaches. mHWTX-V also had not effect on mice by intra-abdominal injection at a dose as high as 200 μg/g and by intracerebroventricular injection at the level of 30 μg/g, and no effect on neuromuscular transmission in isolated mouse phrenic nerve diaphragm preparation at the 5×10^{-5} g/ml concentration within 95 min(data not shown). Interestingly, although HWTX-V and mHWTX-V have high homology, there are marked differences between two toxins in biological activity. The precise mechanism of Huwentoxin-V remains to be elucidated. Further studies on the functional mechanism of HWTX-V and mHWTX-V at the molecular level are in progress.

3.3. Structural assay

HWTX-V consists of 35 amino acid residues including six cysteine residues involved in three disulfide bridges, and mHWTX-V was only truncated two amino acid residues (Phe^{34} and Ser^{35}) from the C-terminus of HWTX-V. Because the two toxins have five negatively charged residues (three Asp and two Glu for a calculated p*I* of 4.8), they were eluted at first in the CM cation exchange experimental procedure (Fig. 1). The locations of the six

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LFECSFSCEIEKEGDKPCKKKKCKGGWKCKFNMCVKV  HWTX-II
ECRWYLGGCSQDGDCCKHLQCHSNYEWCVWDGTFS    HWTX-V
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Fig. 4. Comparing the sequence of HWTX-II with that of HWTX-V.

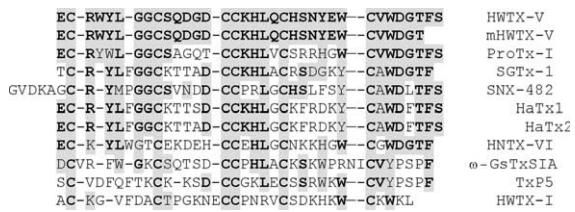

Fig. 5. Sequence alignment of HWTX-V with its homology toxins. The conservative amino acid residues are shaded in black the and similar properties residues are shaded in gray.

cysteine residues in two toxins are well conserved in many neurotoxins from the venom of other spiders (Fig. 5). Computerized database search and multiple alignment calculation shows that the isolated huwentoxin-V had high homology with the ProTx-I (64% identity) toxin isolated from the venom the tarantula spider *Thrixopelma pruriens* (Middleton et al., 2002). HWTX-V also shows similarity with SGTx-1 (57% identity) toxin from the spider *Scodra grisepes* (Marvin et al., 1999), with SNX-482 (55% identity) toxin from the tarantula spider *Hysterocrates gigas* (Newcomb et al., 1998) and with HaTx1 (54% identity)/HaTx2 (54% identity) toxins from the spider *Grammostola spatulata* (Swartz et al., 1995). Fig. 5 also shows that HWTX-V has 48% identity with HNTX-VI (Pan et al., 2002), 43% identity with *ω*-GsTxSIA (Corzo et al., 2000), 34% identity with TxP5 (Pallaghy et al., 1994) and 30% identity with HWTX-I (Liang et al., 1993). Comparing the sequence of HWTX-V with those of its homological toxins, the C-terminal residue Phe^{34} is found to highly conservative. It was previously suggested that the aromatic residues forming a solvent-exposed hydrophobic cluster were crucial for the binding of the protein to its receptor (Darbon et al., 1991). The Phe^{34} in the C-terminal of HWTX-V might play a key role on biological activity via forming a solvent-exposed hydrophobic cluster. The Ser^{35} residue is also conserved well in the alignment sequences. The amino acid residues of Phe^{34} and Ser^{35} should be indispensable for HWTX-V activity formation. The two C-terminal residues (Phe^{34} and Ser^{35}) together with other residues may contribute to the formation activity domain. The C-terminal residues are indispensable for the toxin function like those toxins such as Bm32-VI (Escoubas et al., 2000b), Bm33-I (Escoubas et al., 2000b), Aah IT (Darbon et al., 1991), J-ACTX-Hv1c (Maggio et al., 2002) and ω-Atracotoxin-Hv1a (Tedford et al., 2001). According to toxicity assay experiment, HWTX-V has insect-specific toxic activity, whereas mHWTX-V having no anti-insect toxicity. Structure-activity studies of two toxins indicate that Phe^{34} and Ser^{35} in the C terminus of HWTX-V are the key amino acid residues, which might determine the insect-specific activity of HWTX-V.

In summary, HWTX-V is an insect-specific neurotoxin whose phylogentic specificity derives from its ability to antagonize insects. From the difference between HWTX-V and mHWTX-V, we suggest that Phe^{34} and Ser^{35} in the C terminus are the key amino acid residues of HWTX-V. Further studies are in progress to characterize the two toxins as well as other toxic components in the venom of *Selenocomia huwena*.

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