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Purification and characterization of huwentoxin-II, a neurotoxic peptide from the venom of the Chinese bird spider *Selenocosmia huwena*

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Key words: amino acid sequence; huwentoxin-II; neurotoxin; *Selenocosmia huwena*; spider venom

Abstract: A neurotoxic peptide, huwentoxin-II (HWTX-II), was purified from the venom of the Chinese bird spider *Selenocosmia huwena* by ion exchange chromatography and reversed phase HPLC. The toxin can reversibly paralyse cockroaches for several hours, with an ED_{50} of 127 $\pm$ 54 μg/g. HWTX-II blocks neuromuscular transmission in an isolated mouse phrenic nerve diaphragm preparation and acts cooperatively to potentiate the activity of huwentoxin-I. The complete amino sequence of HWTX-II was determined and found to consist of 37 amino acid residues, including six Cys residues. There is microheterogeneity (Ile/Gln) in position 10, and mass spectrometry indicated that the two isoproteins have a tendency to dimerize. It was determined by mass spectrometry that the six Cys residues are involved in three disulphide bonds. The sequence of HWTX-II is highly homologous with ESTX, a toxin from the tarantula *Eurypefina californicum.*

Abbreviations: HWTX, huwentoxin; SHL-I, *Selenocasmia huwena* lectin-like peptide; HPLC, high perfomance liquid chromatography; MALDI-TOF, matrix assisted laser desorption ionization time-of-flight; MSA, methanesulphonic acid; PTH, phenylthiohydantoin.

The venom from the Chinese bird spider *Selenocosmia huwena,* which is distributed in the hilly areas of Yunnan and Guangxi in the south of China, contains a mixture of compounds with different types of biological activity (1). In our previous work we isolated and characterized two peptide components from the venom. One is a neurotoxin named huwentoxin-I (HWTX-I) which has 33 amino acid residues

and three disulphide bonds (2, 3). The second is a peptide with haemagglutination activity named *S. huwena* lectin-I(SHL-I) which contains 32 residues and three disulphide bonds (4). Spider venoms from different species were proved to contain divergent toxin families (5–8). Bird spiders, which are the biggest among the spider kingdom, are general predators of arthropods and small vertebrates. To be effective, their venom must contain components with adequate paralytic activity against a variety of potential prey species. A single toxin is unlikely to be sufficient, since different types of prey may vary widely in their sensitivities to a given toxin. Huwentoxin-I, the major component in the crude venom of *S. huwena*, is of high toxicity to vertebrates such as mice, birds and frogs but is of very low toxicity to insects. Since insects are also prey of this spider, its venom should also contain some toxin with activities paralytic to insects. In this paper we report the purification and characterization of a new insecticidal neurotoxic peptide named huwentoxin-II from the venom of *S. huwena*, including the complete amino acid sequence and determination of some of its biological properties.

Experimental Procedures

Venom and animals

The venom from adult female *S. huwena* spiders was collected as described in our previous work. Kunming albino mice were obtained from Hunan Medical University and cockroaches (*Periplaneta americana*) from Peking University.

Chemicals

Sequencing grade phenylisothiocyanate, trifluoroacetic acid and dithiothreitol were from Sigma; aminophenyl glass beads were from CPG Inc.; 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochlotide was from Aldrich; methanesulphonic acid and tryptamine were from Pierce; and HPLC grade acetonitrile was from Linhai Chemicals. All other reagents were of analytical grade.

Venom fractionation

Ion exchange chromatography was performed using a Waters Protein-Pak CM 8 H column (5 mm × 50 mm) on a Waters 650 Advanced Protein Purification system equipped with a model 486 detector. Fractions from ion exchange chromatography were further fractionated by reversed phase HPLC on a YWG-pak C l8 column (3.9 mm × 300 mm) using a Waters 510 pump with a model 996 photodiode array detector.

Bioactivity assay

The toxin was assayed qualitatively by intra-abdominal injection of 50 μL solutions of toxin in 0.9% (w/v) NaCl into male cockroaches (*P. americana*) of the same age and with body weights of about 0.35 g. Pharmacological experiments using mouse phrenic neuromuscular transmission preparations were carried out as described previously (2).

Mass spectrometry

Mass spectra were obtained on a Hewlett-Packard G2025 A MALDI-TOF system. Insulin was used as an internal standard, and sinapinic acid as the matrix.

Reduction and carboxymethylation of cysteines

The native protein was reduced and carboxymethylated as described previously (3).

Amino acid analysis

Native or S-carboxymethylated polypeptide (100 μg) was hydrolyzed in 100 μL of constant boiling HCl or in 50 μL of 4 N methanesulphonic acid containing 0.29% (w/v) tryptarnine at 110°C for 24 h. The hydrolysates were dried and derivatized with phenyl isothiocyanate, and the resulting phenylthiocarbamyl amino acids were analysed by reversed phase HPLC using a YWG-pak C 18 column.

Amino acid sequence analy.sis

Peptides were immobilized on capillary columns prepacked with aminophenyl glass beads (9) and sequenced by solid-phase Edman degradation on a MilliGen/Biosearch Model 6600 Prosequencer.

Results and Discussion

Venom fractionation

A typical ion exchange chromatography elution profile for crude *S. huwena* toxin is shown in Fig. 1. The peak

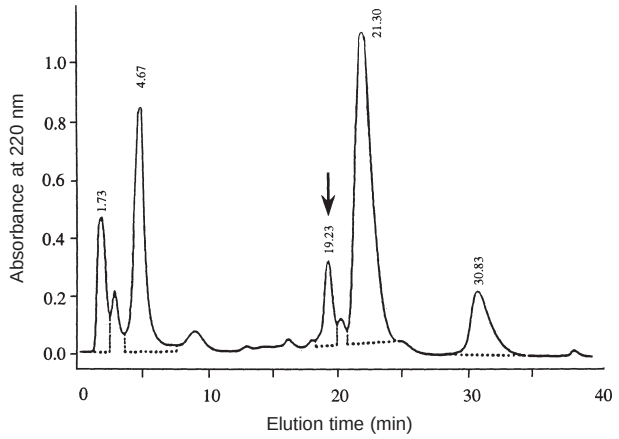

Figure 1. Ion exchange HPLC of crude *S.* huwena venom. Lyophilized venom (1 mg in 1 mL distilled water) was applied to a Waters Protein-Pak CM 8 H ion exchange column (5 mm × 50 mm) initially equilibrated with 0.02 M sodium phosphate buffer, pH 6.25 (buffer A). The column was eluted with a linear gradient of 0–50% of buffer B (I M sodium chloride, 0.02 M sodium phosphate, pH 6.25) over 40 min at a flow rate of 0.7 mL/min.

with retention time of 4.76 min is SHL-I, which has the sequence of Gly-Cys-Leu-Gly-Asp-Lys-Cys-Asp-Tyr-Asn-Asn-Gly-Cys-Cys-Ser-Gly-Tyr-Val-Cys-Ser-Arg-Thr-Trp-Lys-Trp-Cys-Val-Leu-Ala-Gly-Pro-Trp. The main peak with the retention time of 21.3 min contains HWTX-I, which has the sequence of Ala-Cys-Lys-Gly-Val-Phe-Asp-Ala-Cys-Thr-Pro-Gly-Leu-Asn-Glu-Cys-Cys-Pro-Asn-Arg-Val-Cys-Ser-Asp-Lys-His-Lys-Trp-Cys-Lys-Trp-Lys-Leu. The peak having a retention time of 19.23 min was subjected to reversed phase HPLC (Fig. 2), and the peptide eluting at 31.58 min was collected and lyophilized. This peptide was found to be toxic to insects and named huwentoxin-II (HWTX-II).

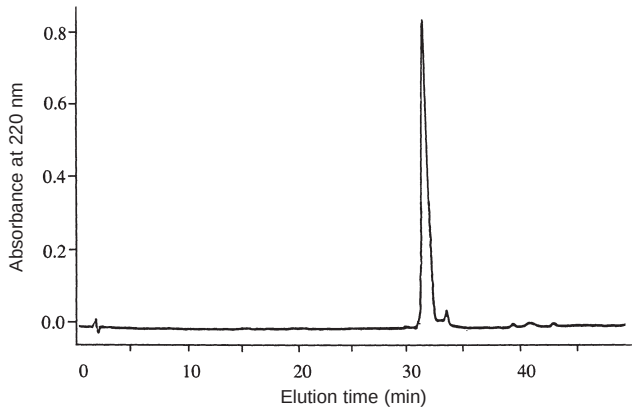

Figure 2. Purification of huwentoxin-II by reversed phase HPLC. The ion exchange HPLC fraction eluting at 19.23 min (Fig. 1), and representing 2 mg of crude venom, was applied to a YWG C18 column (3.9 mm × 300 mm) initially equilibrated with 0.1% trifluoroacetic acid in water (buffer A). Elution was performed with a linear gradient of 0–40% of buffer B (acetonitrile containing 0.1% trifluoroacetic acid) over 40 min at a flow rate of 0.7 m1/min.

***Table 1.* Amino acid composition of huwentoxin-II**

	Residues/molecule			
	From hydrolysis with		From sequence	
Amino acid	6M HCl	4M MSA	Isoprotein a	Isoprotein b
Asx	2.1 (2)[a]	1.9 (2)[a]	2	2
Glx	5.2 (5)	4.7 (5)	4	5
Ser	2.2 (2)	2.1 (2)	2	2
Gly	3.1 (3)	2.9 (3)	3	3
His	0.0 (0)	0.0 (0)	0	0
Arg	0.0 (0)	0.0 (0)	0	0
Thr	0.2 (0)	0.0 (0)	0	0
Ala	0.0 (0)	0.0 (0)	0	0
Pro	1.2 (1)	0.8 (1)	1	1
Tyr	0.1 (0)	0.0 (0)	0	0
Val	1.9 (2)	1.7 (2)	2	2
Met	1.0 (1)	0.8 (1)	1	1
Cys	6.4 (6)	5.8 (6)	6	6
Ile	1.1 (1)	0.9 (1)	1	0
Leu	1.1 (1)	1.0 (1)	1	1
Phe	2.8 (3)	3.1 (3)	3	3
Lys	9.7 (10)	10.7 (10)	10	10
Tr	n.d.	0.9 (1)	1	1
Total	37	38	37	37

[a]Parentheses indicate nearest integral value.

Amino acid sequence of huwentoxin-II

The results of amino acid analysis of S-carboxymethylated HWTX-II after hydrolysis with 6 N HCl or 4 N methanesulphonic acid are given in Table 1. Of the 37 amino acid residues in the molecule, the most notable are the 10 Lys residues, which indicate that the peptide is basic, and the six Cys residues which, like related toxins, are probably involved in disulphide bonds.

Sequence analysis of HWTX-II was performed by immobilizing 20 μg of native or S-carboxymethylated protein on aminophenyl glass beads packed in a capillary column (9) and sequencing through 40 cycles on a MilliGerd Biosearch Prosequencer. The N-terminal amino acid was determined to be Leu. After cycle 37 (Val) three blank cycles were produced, indicating that Val-37 is the C-terminal residue. Single phenylthiohydantoin (PTH) signals were detected at each cycle, except for position lo, at which two signals corresponding to PTH-Ile and PTH-Gln were observed. The complete amino acid sequence of HWTX-II is: NH_2-Leu-Phe-Glu-Cys-Ser-Phe-Ser-Cys-Glu-IIe/Gin-Glu-Lys-Glu-Gly-Asp-Lys-Pro-Cys-Lys-Lys-Lys-Lys-Cys-Lys-Gly-Gly-Trp-Lys-Cys-Lys-Phe-Asn-Met-Cys-Val-

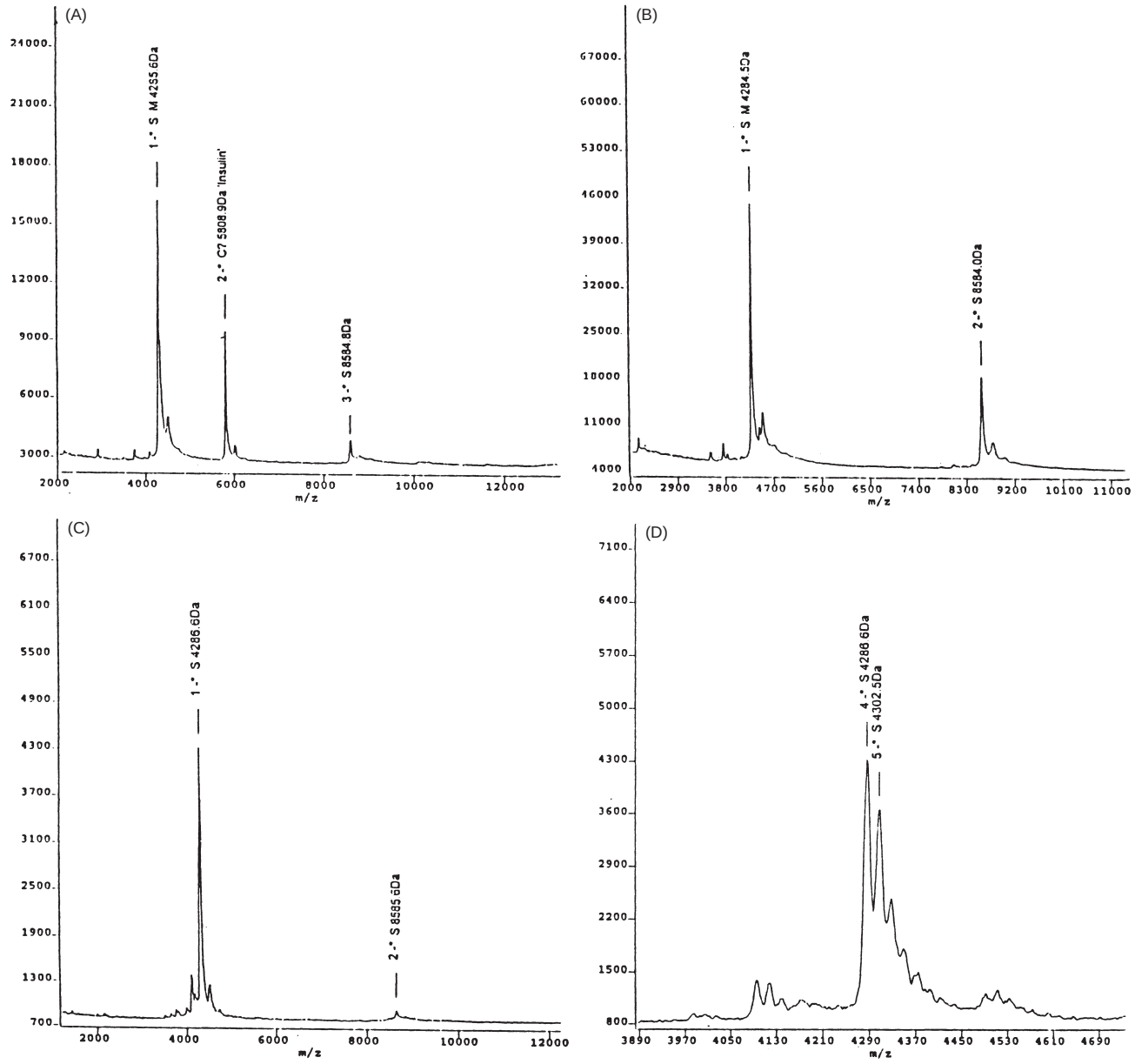

Figure 3. MALDI-TOF mass spectra of huwentoxin-II. Panels A, B and C show spectra of three different batches of HWTX-II. Panel D is an expanded view of the major peak in panel C.

Lys-Val-COOH. Comparison of the sequence of HWTX-II with that of HWTX-I indicated that there is very low homology between the two toxins in spite of both peptides containing six Cys residues. This result is not surprising because the biological activities of the two toxins are quite different.

Folded structure of huwentoxin-II

The venoms and toxins of many organisms are typically tightly cross-linked by disulphide bonds. MALDI-TOF mass spectrometry was used to analyse the oxidation state of the six Cys residues in HWTX-II (Fig. 3). The calculated masses

Figure 4. Sequence comparison between huwentoxin-II and *Eurypelma californicum* ESTX neurotoxin. Gaps have been inserted to achieve the best alignment. Residues identical to HWTX-II are boxed.

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ESTX:   IFECVFSCDIEKEG-KPCKPKGEKKCTSGGWKCKIKLCLKI
HWTX-II: LFECSFSCEIQEKEGDKPCK-K--KKCKGGWKCKFNMCVKV
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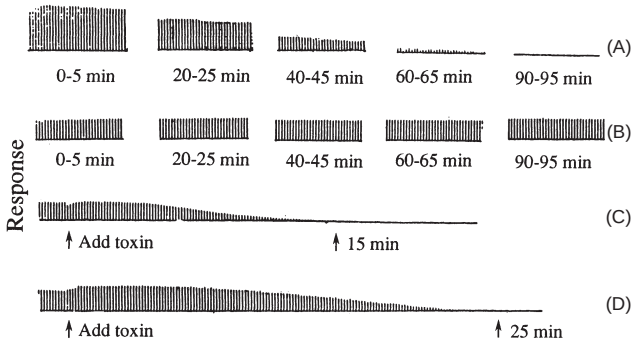

Figure 5. Effect of huwentoxin-II on neuromuscular transmission in isolated mouse phrenic nerve diaphragm preparation. Twitch response of the diaphragm to electrical stimulation in the presence of (A) 5×10^{-5} g/mL HWTX-II; (B) 5×10^{-5} g/mL SHL-I; (C) a mixture of 5×10^{-6} g/mL of HWTX-II and 5×10^{-6} g/mL of HWTX-I; (D) 1×10^{-5} g/mL HWTX-I.

for the Ile-10 and GIn-10 isoforms of HWTX-II are 4290.3 Da and 4305.2 Da, respectively. Assuming that all six Cys residues are involved in disulphide bonds, with a loss of six protons, the masses of the oxidized proteins would be 4284.3 Da (Ile form) and 4299.2 Da (Gln form). Figure 3A shows the MALDI-TOF mass spectrum of HWTX-II in the presence of an internal insulin standard ($M + H^+ = 5808.9$; calc. M = 5807.8). The observed mass ($M + H^+$) for HWTX-II is 4285.6 Da, or exactly that calculated ($M + H^+ = 4285.3$) for the fully oxidized Ile form. These results indicate that, like many related proteins, all the cysteine residues are involved in disulphide cross-links.

Figure 3A,B,C are mass spectra of different batches of HWTX-II which, in addition, show peaks at about 8585 Da. The amount of the 8585-Da peak is variable in relation to the 4285-Da peak. Its mass suggests a dimer of HWTX-II and if one sums the calculated masses of HWTX-II Ile-lo form (4284.3 Da) and Gln-10 form (4299.2 Da) a value of 8583.5 Da is obtained, which is nearly exactly the observed mass (8584 Da) for the HWTX-II (Gin/lle) dimer. These data suggest that the two isoforms of HWTX-II form a dimer with some degree. The isoforms are not resolved by HPLC (Fig. 2), indicating either that they are of similar hydrophobicity, despite the difference in polarity of the Ile and GIn side chains, or that they elute as the dimer. They are, however, poorly resolved by mass spectrometry as seen in Fig. 3D, a higher resolution view of the main peak in Fig. 3C.

The apparent dimerization of HWTX-II finds analogy in the sea anemone toxin BDS-I from *Anemonia vulcata* (10, 11), which exists as a dimer between two 43-residue isoproteins with Leu/Phe microheterogeniety at position 18. Under alkaline conditions, the dimer dissociates. It would be of interest to study the state of aggregation of HWTX-II as a function of pH.

The 38-residue insecticidal peptide ESTX purified from the tarantula *Eurypelma californicum* (12) also displays microheterogeneity (Ser/Thr at position 26) and the amount of Thr form is higher than the Ser form, although there has been no report concerning dimer formation. ESTX shows a high degree of homology with HWTX-II (Fig. 4), including the six conserved Cys and I Trp residues. HWTX-II and ESTX both contains lo Lys, four Glu and one Asp residues, although not always in the same sequence position, so that the two proteins have the same overall charge. The structural similarity of HWTX-II and ESTX is probably related to the fact that the two kinds of spider, *Selenocosmia huwena* and *Eurypelma californicum*, belong to the same family, *Theraphosidae*. It is probable that these two toxins come from the same gene family. Since a great variety of ethnological and ecological factors influence the effectivity and specific mechanisms of spider venom, it would be of interest to compare these two proteins with respect to their active sites and mechanisms of function.

Bioactivity

Preliminary studies of the biological activity showed that HWTX-II can immobilize cockroaches within 10 min and cause a dose-dependent paralysis that lasts about 6 h. The ED_{50} value was 127 ± 54 μg/g ($P = 0.95$). Large doses (200 μg/g) can cause death. The toxin was also shown to be able to block neuromuscular transmission in isolated mouse phrenic nerve diaphragm preparations within 92 ± 5 min (mean $\pm$ SD, $n = 5$) at a concentration of 5×10^{-5} g/mL. The non-toxic control peptide, *S. huwena* lectin-I (SHL-1), at the same concentration had no effect on the neuromuscular transmission. A mixture of HWTX-I and HWTX-II (prepared 30 min before use) showed an obviously enhanced effect. Whereas a 1×10^{-5} g/mL solution of HWTX-I blocked transmission in 25 ± 3 min (mean $\pm$ SD, $n = 5$) (Fig. 5D), a mixture of 5×10^{-6} g/mL each of HWTX-I and HWTX-II blocked transmission in 15 ± 2 min (mean $\pm$ SD, $n = 5$). Thus there is a cooperative relationship between HWTX-I and HWTX-II. Further studies on the functional mechanism of the huwentoxins are in progress.

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