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PROPERTIES AND AMINO ACID SEQUENCE OF HUWENTOXIN-I, A NEUROTOXIN PURIFIED FROM THE VENOM OF THE CHINESE BIRD SPIDER *SELENOCOSMIA HUWENA*

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S-P. LIANG, D-Y. ZHANG, X. PAN, Q. CHEN and P-A. ZHOU. Properties and amino acid sequence of huwentoxin-I, a neurotoxin purified from the venom of the Chinese bird spider *Selenocosmia huwena*. *Toxicon* **31**, 969–978, 1993.—By means of reverse phase and ion-exchange high performance liquid chromatography, a neurotoxic peptide named huwentoxin-I was purified from the venom of the Chinese bird spider *Selenocosmia huwena*. The intraperitoneal and intracisternal LD_{50} in mice of the toxin were 0.70 mg/kg and 9.40 μg/kg, respectively. This toxin at the concentration of 1×10^{-5} g/ml can irreversibly block the neuromuscular transmission of the isolated mouse phrenic nerve–diaphragm preparation in 13.4 ± 1.3 min (mean ± S.D., $n = 5$). The isoelectric point is 8.95 determined by isoelectric focusing electrophoresis. It consists of 33 amino acids including 6 Cys and 6 Lys determined by amino acid analysis. The complete amino sequence of huwentoxin-I was determined. The N-terminal and C-terminal residues were Ala and Leu, respectively. The primary structure showed partial homology with that of μ-agatoxins from the funnel-web spider *Agelenopsis aperta*.

INTRODUCTION

SPIDER venoms are known to contain several classes of neurotoxins, which are of interest as tools for studying neurophysiology and as potential lead structures for insecticides and pharmaceuticals. The spider *Selenocosmia huwena* was recently identified as a new species of genus *Selenocosmia* (WANG *et al.*, 1993). It is distributed in the hilly areas of Yunnan and Guangxi in the south of China. This species of spider has been called *Dilaohu* by the local Chinese people, which means earth tiger, because it habitually lives in holes underground and is rather aggressive and venomous. This hairy spider has a bodylength of 6–9 cm (its legs expand it to more than 9–12 cm). The venom fangs extend from 6 to 9 mm and can be deeply introduced into the body of their victims. The venom from the spider *S. huwena* contains a mixture of compounds with different types of biological activity. In our previous work we have found that the crude venom of *S. huwena* was neurotoxic to mice. It can cause paralysis and rapid respiratory failure in mice (LIANG *et al.*, 1993).

In this paper we describe a rapid procedure involving a combination of reverse phase and ion-exchange high performance liquid chromatography that was used to isolate a neurotoxin, named huwentoxin-I, from the venom of the spider *S. huwena*. In addition, the partial biological properties and the complete amino acid sequence of this toxin are presented.

MATERIALS AND METHODS

Venom and animals

Adult female *S. huwena* spiders were collected in the hilly area of Ningming county, Guangxi, China. Spiders were identified by Professor J. F. Wang (Department of Biology, Hunan Normal University). The animals were kept in wooden boxes covered with plastic net and given water daily. Cockroaches, small mice and small frogs were used to feed the spiders. The venom was obtained every 3–4 weeks by the following method. A spider was held with a pair of tweezers and a bundle of flexible polyvinyl plastic tubing (2 mm i.d. × 45 mm), which was held by another pair of tweezers, was used to provoke the spider. The animal would become very aggressive and then grasp the tubing tightly. Finally the venom fangs pierced the tubing and injected venom inside. The venom was taken out of the tubing with a pipetman and immediately freeze-dried. In the biological experiments Kunming albino mice from Hunan Medical University were used.

Chemicals

N,N,N',N'-methylenebis-acrylamide, Coomassie blue R-250, *N,N,N',N'*-tetramethylenediamine (TEMED), carrier ampholyte and isoelectric focusing standards were from Bio-Rad, acrylamide was from Tianjing Chemicals and recrystallized from chloroform; whale myoglobin, phenylisothiocyanate (PITC), trifluoroacetic acid (TFA) were from Sigma, sequencing grade; aminophenyl glass beads were from CPG Inc.; 1-(3-dimethylaminopropyl)-3-ethylcarbdiimide hydrochloride (EDC), dithiothreitol and iodoacetic acid were from Aldrich; methanesulfonic acid (MSA) and tryptamine were from Pierce; acetronitrile was obtained from Huangyan Chemicals, HPLC grade. All other reagents were of analytical grade.

Lethality and toxicity assay

The toxicity of the various chromatographic fractions was qualitatively assayed by i.p. and i.c.m. injection into 18–20 g mice of both sexes using 50–100 μl of solutions in 0.9% (w/v) NaCl for each fraction. The amount of protein injected per fraction corresponded to 0.2 ml of a solution of $A_{1.0cm}$ = 1.000 at 280 nm. The LD_{50} was determined in six mice at each of five dosage levels and observed during a 24 hr period following i.p. and i.c.m. injection. The injections via i.c.m. were performed according to the method of SCHWEITZ (1984).

Venom fractionation

One milligram of lyophilized venom dissolved in 200 μl double-distilled water was first subjected to reverse phase liquid chromatography on a Delta pak C_4 300 Å column (30 × 0.39 cm) using a Waters 600E HPLC system with a 490 model u.v. detector. The major toxic fraction was further subjected to ion-exchange HPLC on a Shim-pak WCX-1 column (5 × 0.4 cm). The column was previously equilibrated with 0.02 M sodium phosphate buffer, pH 6.6 (buffer A). Elution was performed with a linear gradient from 0–45% buffer B (1 M NaAc, 0.02 M phosphate buffer, pH 7.0) over 30 min. at a flow rate of 0.8 ml/min. The toxic fractions from WCX-1 ion-exchange column were further purified by rechromatography with a Nova-pak C_{18} column (15 × 0.46 cm) under lineal gradient of 0-50% acetonitrile in 0.1% TFA.

SDS-polyacrylamide gel electrophoresis

The apparent molecular weight of purified toxin was estimated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) according to the method of SWANK and MUNKRES (1971) using 12.5% acrylamide gels containing 1% SDS and 8M urea in H_3PO_4–Tris system, pH 5.0.

Thin layer gel isoelectric focusing

The gel casting and isoelectric focusing were performed with a horizontal electrophoresis cell of Bio-Rad Inc., 7.5% polyacrylamide gel (Acr : Bis = 19.4 : 0.6) were used. The pH range of carrier ampholyte was 3.0–10.0. The gel was pre-run for 0.5 hr under voltage of 300 V. One-hundred millilitres of 50 μg/μl venom or toxin

solution was added on a 5 × 5 mm piece of filter paper and was laid on the gel surface. Focusing was performed for 3 hr under voltage of 1000 V and the starting current was 3 mA.

After focusing was completed, the gel plate was placed in 20% trichloroacetic acid solution for 1 min and then stained with 0.2% Coomassie blue in 20% (v/v) methanol and 15% (v/v) glacial acetic acid.

Reduction and S-carboxymethylation of peptides

The procedure of CHI *et al.* (1985) was used for the reduction and *S*-carboxymethylation of the toxin. The *S*-carboxymethylated sample was applied to a Nova-pak C_{18} column (15 × 0.46 cm) previously equilibrated with 0.1% trifluoroacetic acid until the u.v. absorbance was near zero; then the *S*-carboxymethylated peptide was eluted with 50% acetonitrile in water (v/v) containing 0.1% trifluoroacetic acid and then freeze-dried.

Amino acid analysis

Two-hundred milligrams of native or *S*-carboxymethylated polypeptide was dissolved in 100 μl constant-boiling HCl, 0.02% phenol. The glass tubes were flushed with N_2 and heat sealed *in vacuo*. The hydrolysates were dried and derivatized with phenylisothiocyanate. The PTC-amino acids were analysed by HPLC using the Pico-Tag method of WATERS (1984). Polypeptides were also analysed after hydrolysis with 4 M methanesulfonic acid (MSA) containing 0.2% (w/v) tryptamine.

Carboxypeptides Y hydrolysis

One-hundred micrograms of toxin was dissolved in 300 μl of 0.05 M pyridine–acetic acid buffer (pH 5.5), and the 20 μg of carboxypeptidase Y was added (TSCHESHE, 1977). Samples of 20 μl were withdrawn at zero time and 30 sec, 1 min, 2 min, 4 min, 8 min, 15 min, 0.5 h, 1 hr and 2 hr. Twenty microlitres of 1 N HCl was added immediately to the sample to stop further digestion. Samples were lyophilized and derivatized with PITC and loaded on a Nova-pak C_{18} HPLC column for amino acid analysis. In the controls, active enzyme was replaced with boiled carboxypeptidase Y.

Amino acid sequence analysis

Peptides were sequenced by Solid-phase Edman degradation on prototypes of MilliGen/Biosearch Model 6600 ProSequencer (LAURSEN *et al.*, 1989) using prepacked aminophenyl glass beads capillary column for immobilization and sequencing (LIANG and LAURSEN, 1990).

Pharmacological experiments

The experiments were 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 solutions 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 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 of semiconductor stain gauge. The signals were amplified and recorded with a pen recorder.

RESULTS

A typical reverse phase C_4 300 Å column chromatographic separation of 1 mg *S. huwena* crude venom is shown in Fig. 1. There are about 23 peaks of u.v. absorption at 220 nm. Each peak was individually collected and lyophilized. Four fractions (A, B, C and D in Fig. 1) were found toxic to mice and killed the mice within 1 hr after injection. The other peaks did not show a toxic effect to mice with the same amount injected. The toxic fraction A was further purified through ion-exchange HPLC on a WCX-1 column. Two peaks were observed as shown in Fig. 2 and were collected. The major peak (R.T. = 21.5 min) was directly applied to a Nova-pak C_{18} column preequilibrated with 0.1% TFA in water, and then eluted with a linear gradient of 0–50% acetonitrile in constant 0.1% TFA. A sharp and symmetric peak at about 25% acetronitrile was obtained (Fig. 3). The sample was collected and lyophilized. This fraction was found to be toxic to mice and was named

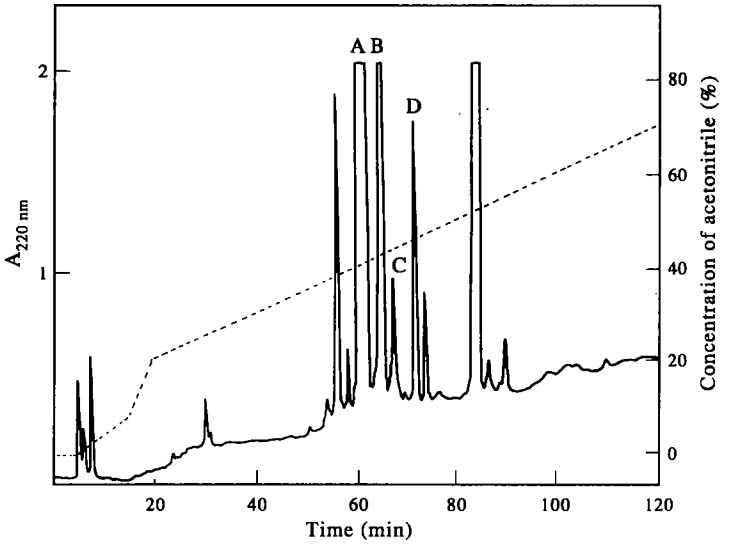

FIG. 1. REVERSE-PHASE HPLC OF *S. huwena* VENOM ON C_4 COLUMN.
One milligram of crude venom from *S. huwena* was applied to a Delta-pak C_4 300 Å column (0.39 × 30 cm) and a linear gradient to 70% acetonitrile in a constant 0.1% trifluoroacetic acid over 120 min was used. The flow rate was 0.7 ml/min; the column temperature was 40°C.

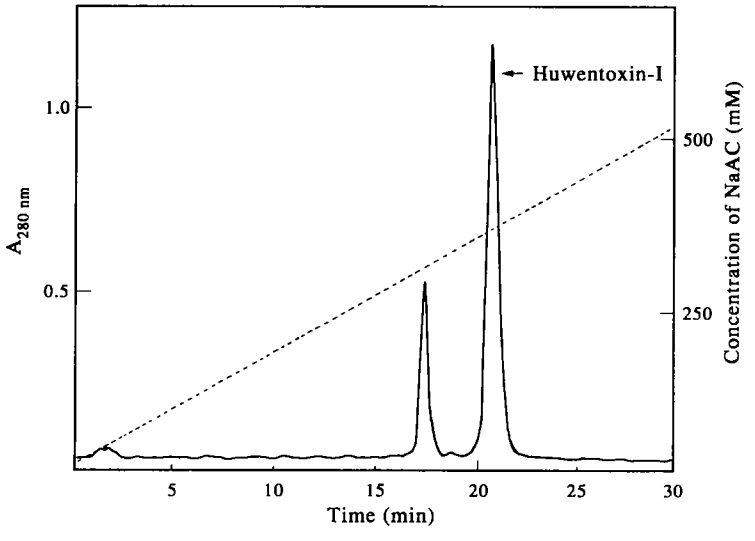

FIG. 2. ION-EXCHANGE HPLC OF PARTIALLY PURIFIED TOXINS.
About 0.5 mg of fraction A (Fig. 1) obtained by C_4 RP-HPLC was applied to a Shim-pak WCX-1 ion-exchange column (0.4 × 5 cm). The column was previously equilibrated with 0.02 M sodium phosphate buffer, pH 6.6. The elution was performed with a linear gradient from 0 to 50% 1 M NaAc in a constant 0.02 M phosphate buffer, pH 7.0, over 30 min at room temperature.

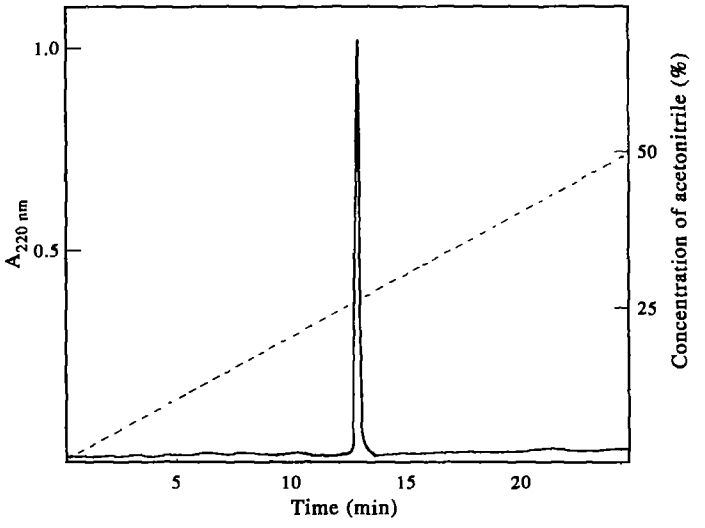

FIG. 3. REVERSE-PHASE HPLC OF HUWENTOXIN-I.
Two-hundred microlitres of the fraction containing huwentoxin-I after ion-exchange HPLC was applied to a Nova-pak C_{18} column (0.44 × 15 cm) previously equilibrated with 0.1% TFA. The elution was performed with a linear gradient from 0 to 50% acetonitrile in a constant 0.1% TFA over 25 min at 40°C.

huwentoxin-I. The smaller peak (R.T. = 17.5 min) in Fig. 4 was also found to be toxic to mice and was further purified by HPLC on the Nova-pak C_{18} column. We named this toxin huwentoxin-II. To determine the homogeneity and the apparent mol. wt of huwentoxin-I, about 20 μg of the toxin was subjected to SDS polyacrylamide gel electrophoresis (Fig. 4). Whale myoglobin and its CNBr cleavage fragments were used as references for mol. wt. The toxin yielded a single band indicating the purity. The apparent mol. wt of the toxin was estimated to be about 4500. The homogeneity of huwentoxin-I was also confirmed by isoelectric focusing gel electrophoresis (Fig. 5).

Since huwentoxin-I is the most abundant toxic component in the venom of *S. huwena*, further experiments have been performed on it for the characterization.

The i.p. LD_{50} in mice of huwentoxin-I is 0.70 mg/kg (body weight). The neurotoxic symptoms induced by i.p. injection of huwentoxin-I were excitation, gasping and spastic paralysis of the posterior extremities. No salivation or lachrymation was observed. The i.c.m. LD_{50} in mice for huwentoxin-I was determined to be 9.40 μg/kg. Because the i.c.m. injection was under pentobarbital anaesthesia, usually no apparent behavioural or motor defects were noted except for the gasping before the death. Figure 6 shows the effect of the huwentoxin-I on the neuromuscular transmission of the isolated mouse phrenic nerve–diaphragm preparation. With 1×10^{-5} g/ml huwentoxin-I, the amplitude of the twitch response to indirect stimulation decreased gradually and eventually disappeared. The transmission was irreversibly blocked in 13.4±1.3 min (mean±S.D., $n = 5$). After blockage, however, the twitch responses to direct stimulation were unaffected, and there was no sign of recovery after washing with Tyrode's solution for 30 min.

The results of amino acid analysis of *S*-carboxymethylated huwentoxin-I after hydrolysis with 6 N HCl and 4 N mecaptoethane sulfonic acid (MSA) are given in Table 1. Among the 33 amino acid residues per molecule of the toxin there are 6 Lys, 1 Arg and 6

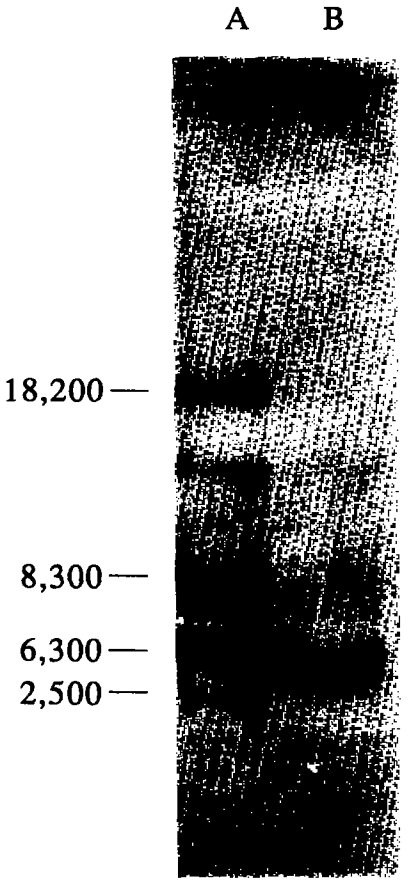

FIG. 4. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF HUWENTOXIN-I FROM *S. huwena*.
(A) Intact whale myoglobin (mol. wt 18,200) and its CNBr cleavage fragments (mol. wt 8300, 6300 and 2500) as low mol. wt standards; (B) the purified huwentoxin-I.

Cys. The high content of basic amino acids is coincident with the high isoelectric point (p*I* = 8.95) of the toxin determined by isoelectric focusing electrophoresis (Fig. 5).

The N-terminal residue of huwentoxin-I, as determined by DABITC/PITC double coupling method and also by solid-phase Edman degradation with MilliGen 6600 ProSequencer, is alanine. Leucine was determined by carboxypeptidase Y hydrolysis to be the C-terminal residue of the toxin and lysine is the second residue from the C-terminal. In order to determine the complete amino acid sequence of huwentoxin-I, 20 μg (5 nmole) of native and *S*-carboxymethylated toxin were immobilized to aminophenyl glass capillary column (1.3 mm i.d. × 32 mm) individually according to the method described above, and then placed in the column holder in the MilliGen/Biosearch model 6600 ProSequencer. A programme of 38 Edman degradation cycles plus three precycles for phenylthiohydantoin (PTH) standards was set and then performed. The complete amino acid sequence of huwentoxin-I (Fig. 7) was determined from the *S*-carboxymethylated sample. The intact native toxin was also sequenced for confirmation. After cycle 33 (leucine), five blank cycles were produced. The result that the 33rd residue (Leu) is the C-terminal residue is

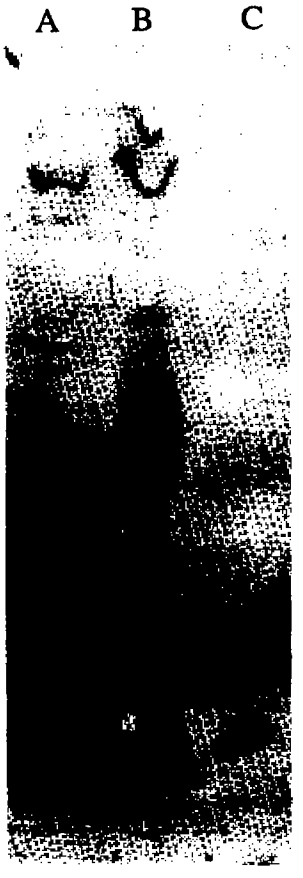

FIG. 5. THIN LAYER IEF-POLYACRYLAMIDE GEL ELECTROPHORESIS OF THE PURIFIED HUWENTOXIN-I AND THE CRUDE VENOM OF *S. huwena*.
(A) The crude venom from *S. huwena*; (B) IEF standards (p*I* 4.65–9.60); (C) the purified huwentoxin-I.

coincident with the result of carboxypeptidase Y hydrolysis. The sequence is also coincident with the result of the amino acid analysis, that there are 6 Lys, 6 Cys, 2 Trp and only 1 Leu, and so on. The repetitive yields for amino acid sequence analysis are listed in Table 2. The average repetitive yield for amino acid PTH derivatives was 91.7%.

DISCUSSION

This is the first report of purification and characterization of a spider neurotoxin from a species of the genus *Selenocosmia*. We have determined the lethality of huwentoxin-I by both peripheral and central nervous system injection routes, confirming that its toxicity is comparable to some other spider toxins such as versutoxin and robustoxin from the funnel-web spiders *Atrax* sp. ((SHEUMACK *et al.*, 1984; BROWN *et al.*, 1988). The amino acid sequence of huwentoxin-I was established by utilizing automated Edman degradation and HPLC identification of the cleaved amino acid phenylthiohydantoin (PTH) derivatives and was in full accord with the amino acid analysis data. The C-terminal sequence of this

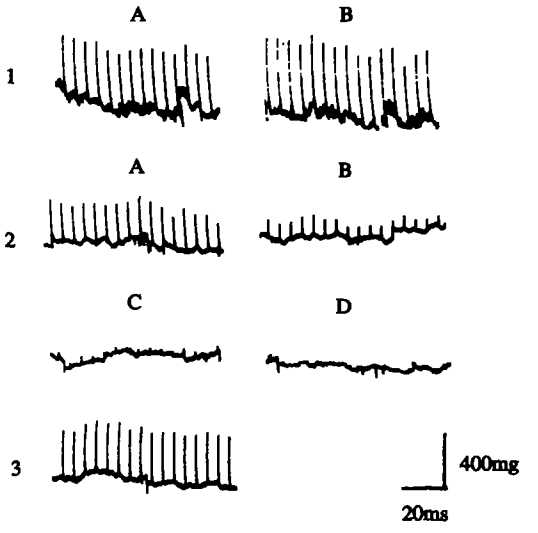

FIG. 6. THE EFFECT OF THE HUWENTOXIN-I ON THE NEUROMUSCULAR TRANSMISSION OF THE ISOLATED MOUSE PHRENIC NERVE–DIAPHRAGM PREPARATION.
(1) The twitch response before 1×10^{-5} g/ml huwentoxin-I was added: A, indirect stimulation; B, direct stimulation; (2) the effect on the twitch response after 1×10^{-5} g/ml huwentoxin-I was added: A–D were the response after the toxin was added at 8, 10, 12 and 14 min, respectively; (3) the twitch response by direct stimulation to the muscle after the neuromuscular transmission was blocked.

TABLE 1. AMINO ACID COMPOSITION OF REDUCED AND CARBOXYMETHYLATED HUWENTOXIN-I FROM *S huwena*

	Composition (residues/molecule)		
	from hydrolysis with		
Amino acid	6 M HCl	4 M MSA	from sequence
Asx	3.46(4)	3.37(4)	4
Glx	1.13(1)	1.20(1)	1
Ser	0.85(1)	0.89(1)	1
Gly	2.05(2)	2.35(2)	2
His	0.91(1)	0.85(1)	1
Arg	0.79(1)	0.80(1)	1
Thr	0.85(1)	0.79(1)	1
Ala	2.00(2)	2.00(2)	2
Pro	1.89(2)	1.68(2)	2
Tyr	0.10(0)	0.05(0)	0
Val	1.87(2)	2.10(2)	2
Met	0.12(0)	0.00(0)	0
Cys	4.66(6)	5.45(6)	6
Ile	0.10(0)	0.00(0)	0
Leu	1.06(1)	0.80(1)	1
Phe	0.90(1)	1.00(1)	1
Lys	5.70(6)	5.65(6)	6
Trp	n.d.	1.60(2)	2
Total	(31)	(33)	33

Values are given for huwentoxin-I after 24 hr hydrolysis by either 6 M HCl or 4 M methanesulfonic acid (MSA) containing 0.2 (w/v) tryptamine. The hydrolysates were derivatized with PITC and analysed by RP-HPLC.

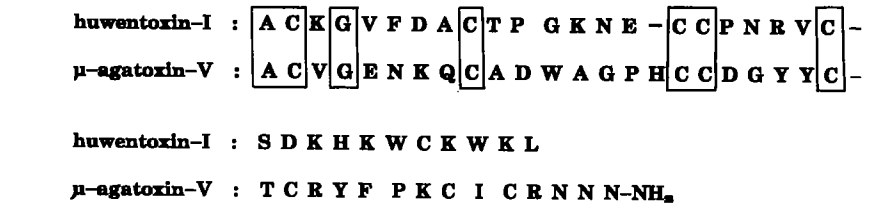

FIG. 7. COMPARISON OF THE SEQUENCE OF HUWENTOXIN-I FROM *S. huwena* AND μ-AGATOXIN-V FROM *A. aperta*.

TABLE 2. REPETITIVE YIELDS FOR AMINO ACID SEQUENCE ANALYSIS OF HUWENTOXIN-I

Residue	Cycle nos	Repetitive yield (%)
Asp	7, 24	92.6
Val	5, 21	90.7
Gly	4, 12	90.1
Pro	11, 18	93.2
Asn	14, 19	88.0
CM-Cys	2, 9	95.1
Lys	3, 13	91.1
	Average	91.7

toxin (-Lys-Leu) was also confirmed by digestion of the intact reduced *S*-carboxymethylated toxin with carboxypeptidase Y. The results indicated that huwentoxin-I is a single-chain peptide. It is similar to many neurotoxins from other spiders and scorpions, in that the huwentoxin-I has a high content of cysteine and lysine residues in the sequence. The six cysteine in the sequence indicated that there are probably three disulfide bond linkages in the molecule of the toxin. It is different from the neurotoxic peptides of funnel-web spiders such as versutoxin, robustoxin and μ-agatoxins which have four disulfide bonds. No homologies were found when the sequence was compared with those of the proteins stored in the U.S. National Biomedical Research Foundation 1990 Data Bank. But there were some homologies when the sequence of huwentoxin-I was compared with that of μ-agatoxin from the funnel-web spider, *Agelenopsis aperta* (SKINNER *et al.*, 1989) (Fig. 7). The μ-agatoxins are known to be insecticidal neurotoxins, which can induce repetitive firing and massive transmitter release from presynaptic stores at the neuromuscular junction of insects (SKINNER *et al.*, 1989). According to our previous work, the LD_{50} of the crude venom from *S. huwena* in cockroaches (*Periplaneta americana*) was 300 μg/g (intraabdominal cavity injection, observed in 24 hr), and 1.16 mg/kg mice (i.p. injection). The crude venom could also block the neuromuscular transmission of the toad sciatic nerve–sartorius muscle, but did not affect the conduction of the nerve (LIANG *et al.*, 1993). Although the precise mechanism of action of huwentoxin-I remains to be elucidated, it irreversibly blocks the neuromuscular transmission of mouse phrenic nerve–diaphragm preparation to indirect muscle stimulation, but not to direct stimulation. Therefore, the biological activity of huwentoxin-I is quite different from that of μ-agatoxins.

In summary, the purification procedure presented above allowed the isolation of huwentoxin-I, a neurotoxin active to mammals, from the venom of *S. huwena*. The

complete amino acid sequence and the biological activities of huwentoxin-I show that it is a neurotoxic peptide different from other spider toxins known so far. Further studies are in progress to characterize this toxin as well as other toxic components in the venom of *S. huwena.*

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