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Review

An overview of peptide toxins from the venom of the Chinese bird spider *Selenocosmia huwena* Wang [= *Ornithoctonus huwena* (Wang)]

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Abstract

The bird spider *Selenocosmia huwena* Wang [= *Ornithoctonus huwena* (Wang)] is one of the most venomous spiders in China. The venom of this spider contains a mixture of compounds with different types of biological activity. About 400 proteins and peptides from the venom can be separated and detected by 2D electrophoresis. Of these, 14 peptide toxins have been purified and characterized from the venom of this spider, with several peptide toxins exhibiting structural similarity but high functional diversity. Most of these huwentoxins (HWTX) contain 30–40 amino acids with three disulfide bonds and adopt an 'inhibitor cystine-knot' (ICK) motif in their three dimensional structure, except for huwentoxin-II (HWTX-II) which adopts a novel scaffold different from the ICK motif. As a group, the toxins possess quite different biological activities including inhibition of voltage-gated calcium and sodium channels, insecticidal activity, lectin-like agglutination, and inhibition of trypsin. Eight cDNAs encoding seven toxins, HWTX-I, -II, -III, -IIIa, -IV -V, and, -VII and one lectin, *S. huwena* lectin-I (SHL-I), have been cloned and sequenced. Comparison of the cDNA sequences of the eight peptides from *S. huwena* indicates that they can be classified into two different superfamilies according to the 'prepro' region of their cDNA sequences.

Keywords: *Selenocosmia huwena*; *Ornithoctonus huwena*; Spider; cDNA; Peptite toxin; Three-dimensional structure

Contents

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1. Introduction

In the summer of 1991, Prof. Jiafu Wang, a zoologist from Hunan Normal University, visited the hilly area of Ninming county of Guanxi Province in the south of China and found a large hairy spider of light brown body color (Fig. 1). This species of spider is commonly called *Dilaohu* by the local Chinese people, which means 'earth tiger', because it habitually lives in holes underground and is rather aggressive and venomous. Prof. Wang identified it as a new species of the family Theraphosidae and named it *Selenocosmia huwena* (Wang et al., 1993).

Recently the taxonomic classification of this spider was reevaluated. Subsequently the spider was proposed to be transferred from the genus *Selenocosmia* to the genus *Ornithoctonus* and was renamed *Ornithoctonus huwena* (Zhu and Song, 2000).

Spider venoms are known to contain several classes of toxins that are used as an efficient means to capture their prey or to defend themselves against predators. These toxins are of interest as tools for studying neurophysiology and as potential lead structures for pharmaceutics and insecticides. (Jackson and Parks, 1989; Escoubas et al., 2000; Rash and Hodgson 2002). Accordingly, since 1991, we have conducted a systematic investigation of the venom of the spider *S. huwena*. This review is focused on the pharmacological and structural characterization of the peptide toxins we have isolated from the venom as well as the cDNAs encoding these toxins.

Fig. 1. *Selenocosmia huwena* Wang [= *Ornithoctonus huwena* (Wang)]. This spider has a normal body length of 6–9 cm (its legs expand it to more than 9–12 cm) and is distributed in the hilly areas of Yunan and Guangxi in the south of China. The larger spider is female and the smaller one is male.

2. Crude venom of S. huwena

2.1. Collection of the venom

Adult female *S huwena* spiders were kept in wooden boxes or plastic pails covered with plastic net and given water daily. Pig liver (cut into pieces of 1–2 cm^3), cockroaches or worms were used to feed the spiders ones every 3 days. The venom was obtained every 2–3 weeks by the following two methods: (1) The 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. (2) The venom was colleted by using an electro-pulse stimulator. The two output electrodes of the stimulator were contacted the both sides of the root part of a chelicerae of the spider. Physiological saline was used to enhance electrical contact. Electrostimulation of 36–80 V, 25–80 Hz with the pulse time of 0.7 ms was applied across the chelicera. Expressed venom was colleted from the fang tips with a glass vial, and was then freeze-dried.

2.2. Properties and biological activities of the crude venom

Despite that we observed several times that a female *S. huwena* can kill a mouse or a sparrow in less than 2 min, there was no record so far that the spider is deadly to human. But there were several cases to show that the bites to human by the spider were very painful and produced severe local swelling. In one case, a man was bitten by the spider at the index finger and felt extremely pain immediately. In about 2 h the swelling was extended to whole palm and

part of the arm. The man felt hyperalgesia in the skin of the palm and the arm and also felt apathetic up to the shoulder. The man was hospitalized and finally recovered in 2 days.

An investigation of the properties of *S. huwena* crude venom found that the venom can block neuromuscular transmission of isolated mouse phrenic nerve-diaphragm and sciatic nerve-sartorius preparations (Liang et al., 1993a). It was also determined that the crude venom of *S. huwena* can inhibit tetrodotoxin (TTX)-sensitive Na^+ currents in undifferentiated NG108-15 cells with an EC_{50} of 3.4 μg/ml, but had no effect on outward delayed-rectifier K^+ currents (Xiao et al., 2001). This indicated that the venom contained at least one neurotoxic component and was the impetus for the isolation and characterization of peptides from the venom. Table 1 summarizes some properties of *S. huwena* venom (Liang et al., 1993).

Using a combination of ion-exchange and reverse-phase high-performance liquid chromatography, about 150–200 component peaks can be detected in chromatograms of the crude venom. Even so, the complexity of the venom is actually underestimated owing to the limits of sensitivity and resolution of the methods used for separation. We found that about 350–400 protein and peptide spots can be detected on a silver-stained 2D electrophoresis gel of *S. huwena* crude venom (Liang et al., 2000a). It follows that the complexity and diversity of the components in the venom is quite impressive.

3. Neurotoxic peptides

As the primary purpose of the venom of *S. huwena* is to paralyse prey, it is not surprising that the spider produces a variety of neurotoxic peptides that affect the nervous system of vertebrates or insects. More than 30 peptide components, most of them are neurotoxins, have been isolated from the venom of *S. huwena* so far. At present 14 of these peptides have undergone peptide sequencing and have had their biological activities thoroughly investigated (see Fig. 2). The remainder have not been extensively characterized due either to the lack of specific methods for bioactivity determination or a limited amount of available toxin.

Table 1
Properties of *S. huwena* venom

Dry weight	0.23 mg/μl venom
Protein content	0.774 mg/mg crude venom
LD_{50} (mice) i.p.	1.16 mg/kg
LD_{50} (cockroach) i.p.	300 μg/g
Hyaluronidase activity	109 U/mg
Alkaline phosphatase activity	2.26 mU/mg
Protease	6.6 mU/mg
DNAse activity	5.58 U/mg
Phospholipase A_2 activity	0 U/mg
Cholinesterase activity	0 U/mg

3.1. Toxins targeting voltage-gated calcium channels

3.1.1. Huwentoxin-I (HWTX-I)

Huwentoxin-I is the most abundant toxic component in the crude venom of *S. huwena.* The molecular weight of HWTX-IV is 3750 Da and it consists of 33 residues, including six cysteines that form three disulfide bridges (Liang et al., 1993c) (see Fig. 2). Using a combination of tryptic digestion of the native toxin and sequence analysis of both intact and S-carboxymethylated toxin, the disulfide bridge pattern was determined to be Cys^2-Cys^{17}, Cys^9-Cys^{22} and Cys^{16}-Cys^{29} (Zhang and Liang, 1993). The 3D solution structure of native HWTX-I was subsequently determined from 2D ^1H NMR data, which showed that it adopts a compact structure consisting of a small triple-stranded antiparallel β-sheet (Asp^7-Csy^9, Val^{21}-Ser^{23} and Lys^{27}-Lys^{30}) and five β-turns (Gly^4-Asp^7, Thr^{10}-Lys^{13}, Pro^{11}-Asn^{14}, Cys^{17}-Arg^{20} and Asp^{24}-Lys^{27}) (Qu et al., 1997, PDB file 1QK6, Fig. 3). A small hydrophobic patch consisting of Phe^6, Trp^{28} and Trp^{31} is located on the one side of the molecule while all six lysine residues are distributed on the molecular surface. The three disulfide bridges are buried within the molecule. Under different pH conditions and after heating at 80 °C for 20 min, secondary structure elements of HWTX-I were not significantly altered, indicating that the structure of HWTX-I is very stable (Liang et al., 1993b). Further analysis revealed that the structure of HWTX-I contains an 'inhibitor cystine-knot' (ICK) motif (Pallaghy et al., 1994; Norton and Pallaghy, 1998) with a 1–4, 2–5, 3–6 disulfide bonding pattern. This disulphide connectivity is adopted by many toxins targeting ion channels and receptors from diverse sources, including spiders and marine snails, as well as structurally related polypeptides from plants and fungi (Qu et al., 1997; Norton et al., 1998).

The intraperitoneal (i.p.) and intracisternal LD_{50} in mice of HWTX-I were 0.7 mg/kg and 9.40 μg/kg, respectively. The neurotoxic symptoms induced by i.p. injection of HWTX-I (100-200 μg/kg) were asynergia (uncoordinated movements), gasping, wearily creeping and spastic paralysis of the hindlimbs. HWTX-I also reversibly blocked the nerve-evoked twitch responses of the isolated mouse phrenic nerve-diaphragm preparation. Following complete blockade of nerve-evoked twitch contractions, responses induced by direct stimulation were unaffected. Neurotransmission could only be partially restored by prolonged and repeated washing with toxin-free solution (Zhou et al., 1996). Using a variety of isolated smooth muscle preparations, HWTX-I was determined to possess presynaptic activity that affects the release of neurotransmitters from autonomic nerve endings of both cholinergic and adrenergic neuroeffector junctions (Liang et al., 2000b). By employing whole-cell patch-clamp methods, it was found that HWTX-I has no significant effect on TTX-sensitive Na^+ currents or delayed-rectifier K^+ currents in cultured NG108-15 cells. However HWTX-I potently inhibited high-voltage-activated (HVA) Ca^{2+} channels expressed in prostaglandin E_1

Toxins	Amino acid sequences	Bioactivtiy
HWTX-I	ACKGVFDACTP-GKNE-CCPNR--VCSDKHKWCKWKL	A presynaptic inhibitor of N-type calcium currents
HWTX-III	DCAGYMREC---KEKL-CCSGY--VCSSRWKWCVLPAPW	A neurotoxin affecting both mammals and insects
HWTX-IIIa	DCAGYMREC---KEKL-CCSGY--VCSSRWKWCVLPAP	A natural mutant of HWTX-III
HWTX-IV	ECLEIFKACNP-SNDQ-CCKSSKLVCSRKTRWCKYQI*	An inhibitor of TTX-S voltage-gated sodium channels
HWTX-V	ECRWYLGGCSQ-DGD--CCKHL--QCHSNYEWCVWDGTFS	An insecticidal neurotoxin
mHWTX-V	ECRWYLGGCSQ-DGD--CCKHL--QCHSNYEWCVWDGT	A natural mutant of HWTX-V
HWTX-X	KCLPPGKPCYGATQKIPCCG----VCSH--NKCT	An inhibitor of N-type calcium currents
SHL-I	GCLG--DKCDY-NNG--CCSGY--VCSRTWKWCVLAGPW	Hemagglutination activity
HWTX-II	LFECSFSCEIEKEGDKPCKKKKCKGGWKCKFNMCVKV	A neurotoxin affecting both mammals and insects, and acting cooperatively to the activity of HWTX-I
HWTX-VII	-FECSFSCEIEKEGDKPCKKKKCKGGWKCKFNMCVKV	Like that of HWTX-II
HWTX-VIII	-FECSISCEIEKKGE-SCKPKKCKGGWKCKFNMCVKV	Like that of HWTX-II
HWTX-VI	NCIGEQVPCDENDPRCCSGLVVLKKTLHGIWIKSSYCYKCK	A neurotoxin toxic to mammals
HWTX-IX	IICAPEGGPCVAGIGCCAGLRCSGAKLGLAGSCQ	A neurotoxin toxic to mammals
HWTX-XI	IDTCRLPSDRGRCKASFERWYFNGRTCAKFIYGGCGGNGNK FPTQEACMKRCAKA	An inhibitor of trypsin; and a neutotoxin acting on the central neuroal system

Fig. 2. Primary sequences and bioactivities of the peptides isolated from the venom of spider *S. huwena*. All the amino acid sequences were obtained using Edman degradation. The disulfide bridge linkage pattern (1–4, 2–5, 3–6 in HWTX-I, -III, IIIa -IV, -V, -X mHWTX-V and SHL-I; and 1–3, 2–5, 4–6 in HWTX-II, -VII and -VIII) is shown below the sequences. The disulfide bridge linkages of HWTX-I, -II, -IV, -V and SHL-I were assigned by chemical methods, while that of HWTX-III, IIIa-VII, -VIII, m HWTX-V and -X is deduced through sequence similarity analysis. An *asterisk* indicates that the C-terminal carboxyl group is amidated.

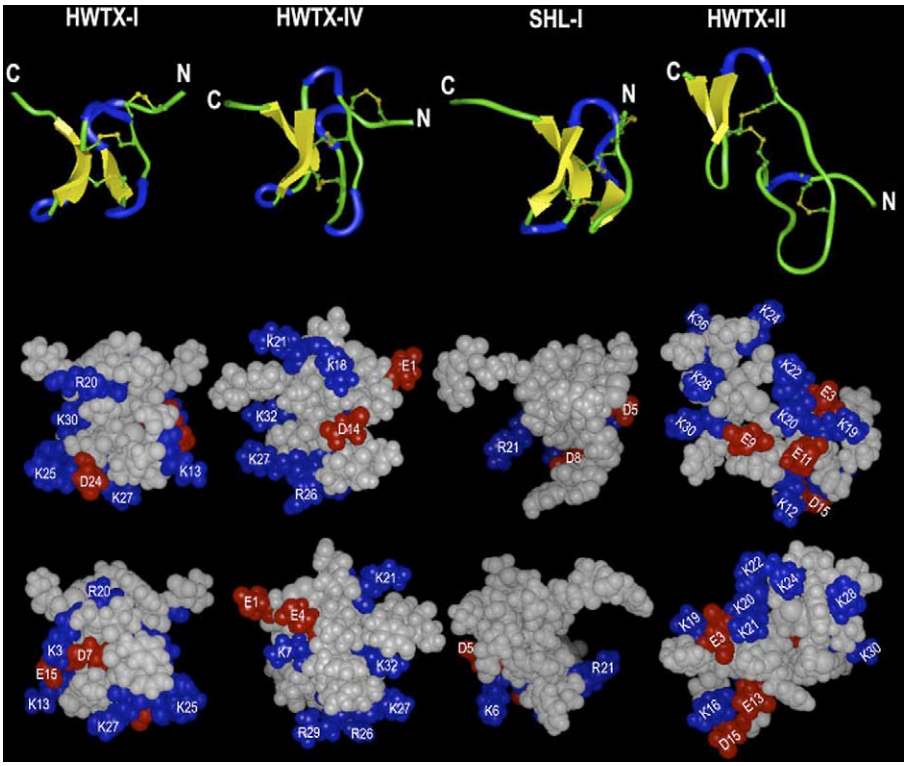

Fig. 3. The NMR structures of HWTX-I, HWTX-IV, SHL-I and HWTX-II. Richardson-style diagrams of the backbone fold of each toxin are shown in the top panel, where the β-sheets, turns and random coils are colored in yellow, blue and green, respectively. The disulfide bridge linkage pattern (1–4, 2–5, 3–6 in HWTX-I, -IV and SHL-I; 1–3, 2–5, 4–6 in HWTX-II) is shown in yellow. The space-filling models in the middle panel show positively and negatively charged residues colored in blue and red, respectively, while the models in the bottom panel show the reverse faces.

differentiated NG108-15 cells ($EC_{50} \approx 100$ nM), with minimal effects on low-voltage-activated (LVA) Ca^{2+} channels. This action on HVA Ca^{2+} channels, was due to a selective inhibition of N-type Ca^{2+} channels with only a very weak inhibition of L-type Ca^{2+} channels. Thus, the molecular target of HWTX-I is very similar to that of the marine snail toxins, ω-conotoxin MVIIA and ω-conotoxin GVIA (Peng et al., 2001).

The effect of epidural administration of HWTX-I in rats with chronic neuropathic pain induced by a sciatic constriction injury was investigated. The results showed that 20 μg/kg HWTX-I blocked the heat hyperalgesia and mechanical allodynia in the injured hindpaw of rats, indicating that epidurally administered HWTX-I could alleviate neuropathic pain (Luo et al., 2002). Given the fact that other Ca^{2+} channel blocking toxins, such as ω-conotoxin MVIIA from *Conus magus*, are currently undergoing investigation for the treatment of opiate-resistant intractable pain, spider venom provides a novel source for potential analgesic pharmaceutics.

The pathway of reduction and oxidative refolding of native HWTX-I was also studied. It was found that the completely reduced peptide could refold and about 90% of full bioactivity could be restored under optimized conditions (0.1 mM reduced glutathione and 1 mM oxidative glutathione at pH 8.5 and 4 °C for 24 h) (Liang et al., 1999). HWTX-I has more recently been synthesized using solid-phase peptide synthesis methods, with the synthetic peptide demonstrating the same bioactivity and 1D NMR spectra, implying that it has the same tertiary structure as that of native toxin (Liang et al., 1997). Further mutation studies arising from this work indicated that Lys^3 and Arg^{20} might be important for the bioactivity of HWTX-I, while Ala^1 is not important (Wang et al., 1998; 2000a,b).

3.1.2. Huwentoxin-X (HWTX-X)

The amino acid sequence of Huwentoxin-X (Fig. 2) contains 28 residues with six cysteines forming three disulfide bonds. The toxin shows less than 30% homology with other huwentoxins but more than 50% with Ptu1 from a bug *Peirates turpis* (Bernard et al., 2001) and ω-conotoxin SVIA from *Conus atriatus*, two N-type Ca^{2+} channel blockers. In the isolated mouse phrenic nerve-diaphragm preparation, HWTX-X partially blocks neuromuscular transmission and paralyzes mice when injected by i.c.v. injection. Under whole-cell voltage-clamp conditions in rat dorsal root ganglion neurons, HWTX-X (10 μM) blocks N-type, but not L-type, calcium channels. When injected i.p., it has no toxic effect on locusts (unpublished data).

3.2. Toxins targeting voltage-gated sodium channels

Huwentoxin-IV (HWTX-IV), a 4108 Da toxin, contains 35 residues with three disulfide bridges. The C-terminal carboxyl group of this toxin is amidated. Using a chemical strategy including partial reduction of the toxin and sequence analysis of the modified intermediates the disulfide bonding pattern was determined to be Cys^2-Cys^{17}, Cys^9-Cys^{24}, and Cys^{16}-Cys^{31}, forming a 1–4, 2–5, 3–6 disulfide connectivity. It specifically inhibits neuronal TTX-sensitive voltage-gated sodium channels with an IC_{50} value of 30 nM in adult rat dorsal root ganglion neurons, while having no significant effect on TTX-resistant voltage-gated sodium channels. Importantly it suppresses peak sodium current without altering activation or inactivation kinetics (Peng et al., 2002). The 3D structure of HWTX-IV was determined using 2D ^{1}H-NMR. The resulting structure is composed of a double-stranded antiparallel β-sheet (Leu^{22}-Ser^{25} and Trp^{30}-Tyr^{33}) and four turns (Glu^4-Lys^7, Pro^{11}-Asp^{14}, Lys^{18}-Lys^{21} and Arg^{26}-Arg^{29}) and belongs to the ICK structural family (PDB file 1MB6). After comparison with other toxins purified from the same species, it is likely that the positively charged residues of loop IV (residues 25–29), especially Arg^{26}, are crucial for binding to the neuronal TTX-sensitive voltage-gated sodium channel (Peng et al., 2002). This toxin would therefore seems to interact with neurotoxin receptor site 1 on the voltage-gated sodium channel to block sodium conductance via a mechanism similar to that of TTX and saxitoxin. As such it represents a novel class of peptide toxins from spider venom.

3.3. Neurotoxins affecting both mammals and insects

3.3.1. Huwentoxin-II (HWTX-II)

Huwentoxin-II consists of 37 amino acid residues including six cysteines involved in three disulfide bridges. The sequence of HWTX-II is highly homologous with ESTX, a toxin from the tarantula *Eurypefina californicum* (Anette, 1989). There is microheterogeneity (Ile/Gln) at position 10 in the sequence of HWTX-II and mass spectrometry indicated that the two isoproteins have a tendency to dimerize (Shu et al., 2001a). HWTX-II was able to reversibly paralyze cockroaches for several hours, with a median knockdown dose ED_{50} of 29 ± 12 nmol/g (body weight). HWTX-II also blocks neuromuscular transmission in the isolated mouse phrenic nerve-diaphragm preparation and acted cooperatively to potentiate the activity of HWTX-I (Shu and Liang, 1999). Using a novel strategy combining Edman degradation sequencing and stepwise thiol modification, the disulfide bridge linkage of HWTX-II was assigned as Cys^4-Cys^{18}, Cys^8-Cys^{29} and Cys^{23}-Cys^{34}, forming a 1–3, 2–5, 4–6 disulfide connectivity which is different from that of HWTX-I and other peptides with an ICK motif (Shu et al., 2001b). The three-dimensional structure of HWTX-II, determined using 2D ^{1}H NMR, revealed two β-turns (Cys^4-Ser^7 and Lys^{24}-Trp^{27}) and a double-stranded antiparallel β-sheet (Trp^{27}-Cys^{29} and Cys^{34}-Lys^{36}) (Shu et al., 2001b, 2002). Although the C-terminal double-stranded antiparallel β-sheet is cross-linked by two disulfide bridges and is conserved in HWTX-II and other HWTXs containing an ICK motif, the structure of HWTX-II unexpectedly does not contain a cystine-knot because of its unique disulfide linkage (2–5, 4–6 in HWTX-II vs. 2–5,

3–6 in ICK molecules). This suggests that HWTX-II should adopt a novel scaffold different from the ICK motif. The structure of HWTX-II may be considered as the first representative of a novel structural scaffold found in spider toxins (Shu et al., 2002).

3.3.2. Huwentoxin-VII (HWTX-VII) and Huwentoxin-VIII (HWTX-VIII)

Another two insecticidal peptides, which share high sequence identity with HWTX-II, have been purified from the venom of *S. huwena* (Dai et al., 2003).The amino acid sequence of HWTX-VII, composed of 35 amino acid residues including six cysteines, is similar to that of HWTX-II except that only six residues are different (see Fig. 2). HWTX-VIII has 36 residues including six cysteines. Its amino acid sequence is similar to that of HWTX-II except for the truncated N-terminal leucine residue (see Fig. 2). The bioactivities of the two peptides are similar to that of HWTX-II. Both of them paralyze locusts and kill mice following i.c.v. injection. They block neuromuscular transmission in the isolated mouse phrenic nerve-diaphragm preparation and act cooperatively with HWTX-I. Based on the high sequence identity with that of HWTX-II, these two peptides might adopt the same disulfide bridge pattern and structural scaffold as HWTX-II (Dai and Liang, 2003).

3.3.3. Huwentoxin-III (HWTX-III)

Huwentoxin-III (3853 Da) has 33 residues including six cysteine residues, which form three disulfide bridges (Huang et al., 2004). Although it shares 60% identity with *S. huwena* lectin-I (SHL-I) (see Section 4.1), it cannot agglutinate human erythrocytes. Huwentoxin-III can reversibly paralyze cockroaches for several hours with an ED_{50} value of 50 ± 30 nmol/g. In addition, HWTX-III can enhance smooth muscle contractions elicited by nerve stimulation as well as cause spontaneous contractions of isolated rat vas deferens smooth muscle (Huang et al., 2004). A natural mutant of HWTX-III (named HWTX-IIIa), which is only truncated an amino acid residues from the C-terminus of HWTX-III, was also isolated from the venom. Interestingly, IIWTX-IIIa did not show the same activity as that of HWTX-III, which suggested that the C-terminal residue (Trp) of HWTX-III should be a key residue to the bioactivity of HWTX-III. (Huang et al. 2002).

3.4. Neurotoxins active on insects

3.4.1. Huwentoxin-V (HWTX-V)

Huwentoxin-V contains 35 residues, while its natural mutant (named mHWTX-V) is truncated two amino acid residues from the C-terminus of HWTX-V. The molecular masses of the two peptides are 4111 and 3877 Da, respectively. The six cysteine residues in each sequence form three disulfide bridges with a 1–4, 2–5, 3–6 connectivity, which is same as that in ICK peptides. Huwentoxin-V can reversibly paralyze locusts and cockroaches for several hours with an ED_{50} of 3.9 ± 1.2 nmol/g in locusts, and a large dose of this toxin can cause death. However, mHWTX-V shows no significant effect on locusts and cockroaches, which indicates that the two residues Phe^{34} and Ser^{35} at the C-terminus of HWTX-V are key residues required for biological activity (Zhang and Liang, 2003).

3.5. Neurotoxins active on mammals

3.5.1. Huwentoxin-VI (HWTX-VI)

Huwentoxin-VI contains 40 residues with a molecular weight of 4440 Da. The molecule has six cysteine residues forming three disulfide bonds. HWTX-VI 5 μg/mouse injected via i.c.v. into adult mouse brain can paralyze mice in 1.5 min with complete recovery after 20 h. Using whole-cell patch clamp experiments in NG108-15 cells, it was found that HWTX-VI (10 μM) has no effect on voltage-gated Na^+ currents (Yi et al., 2002). So far the specific target of this toxin has not been determined.

3.5.2. Huwentoxin-IX (HWTX-IX)

Huwentoxin-IX contains 34 residues with a molecular weight of 3137 Da. The molecule has six cysteine residues forming three disulfide bonds. The sequence of HWTX-IX, containing eight glycine residues, has low homologue with that of other toxins in the database. It can paralyze mice for 12 h by intracisternal injection of 4 μg. Owing to the very limited amount of the available toxin, HWTX-IX has not been further investigated yet for its biological activities.

4. Peptides with other biological activities

4.1. Selenocosmia huwena lectin-I (SHL-I)

Selenocosmia huwena lectin-I, composed of 32 residues, is a relatively abundant component in the venom of this spider. The disulfide bridge linkage of SHL-I was identified as Cys^2-Cys^{14}, Cys^7-Cys^{19} and Cys^{13}-Cys^{26} by employing a strategy combining multi-enzymatic digestion and partial reduction (Li and Liang, 1999). *S. huwena* lectinwas found to have haemagglutination activity, while it showed very low toxicity in both mammals and insects. It can agglutinate human and mice erythrocytes at a minimum concentration of 125 and 31 μg/ml, respectively (Liang and Pan, 1995), which means that the haemagglutination activity of SHL-I is relatively low. The agglutination activity of SHL-I can be inhibited by D-mannosamine (Liang and Lin, 2000). The 3D structure of SHL-I was determined using 2D ^{1}H-NMR. The characteristic structural feature of SHL-I is a triple-stranded antiparallel β-sheet composed of residues Asp^5-Cys^7, Tyr^{17}-Ser^{20} and Trp^{25}-Leu^{28} and three β turns composed of residues Cys^2-Asp^5, Cys^{14}-Tyr^{17} and Arg^{21}-Lys^{24}. The C-terminal fragment from Leu^{28} to Trp^{32} adopts two sets of conformations corresponding to the *cis* and *trans*

conformations of Pro^{31} (PDB file 1QK7). The structure of SHL-I has high similarity with that of the 3D-structure of the N-terminus of hevein, a lectin from rubber-tree latex. In the corresponding type I turn region of hevein, Trp^{21} and Trp^{23} had been reported contribute to the van de Waals paking of hydrophobic sugar surface against aromatic amino acid side chains. In SHL-I, there are also two aromatic residues (Trp 23 and Trp 25). Compared with hevein, it is reasonable that residues in this loop contribute to the activity or binding of SHL-I (Lu et al., 1999).

4.2. Huwentoxin-XI (HWTX-XI)

Huwentoxin XI, a serine protease inhibitor, consists of 55 residues with three disulfide bridges. The toxin was isolated from the venom by ion-exchange chromatography and reverse phase high performance liquid chromatography. The molecular weight of HWTX-XI is 6166 Da, determined by MALDI-TOF mass spectrometry, which is identical with the calculated mass based on the complete amino acid sequence determined by automated Edman degradation (Fig. 2). The sequence of HWTX-XI is highly homologous with that of dendrotoxin-I, a potassium channel inhibitor from snake (Nishio et al., 1999) and that of kalicludine 3, a potassium channel inhibitor and a trypsin inhibitor from sea anemone (Schweitz et al., 1995). The inhibition of trypsin by HWTX-XI was competitive, with a K_i value of 67.8 nM determined using spectrophotometry with BAPNA as the substrate. The binding properties of HWTX-XI on trypsin and α-chymotrypsin were measured using a BIAcore binding assay system. The results showed that HWTX-XI could bind to trypsin with a dissociation constant of 5.54×10^{-3} M. The inhibition profiles of trypsin by HWTX-XI also revealed a 1:1 binding stoichiometry. Huwentoxin-XI also has a strong biological activity with a LD_{50} of 256 ± 23 μg/kg when injected into the fourth ventricle of the adult mouse brain (unpublished data).

5. Comparision of the 3D structures of huwentoxins

Four NMR structures of huwentoxins, HWTX-I (Qu et al., 1997), SHL-I (Lu et al., 1999), HWTX-II (Shu et al., 2001a), and HWTX-IV (Peng et al., 2002), have been reported (Fig. 3). The structures of HWTX-I, HWTX-IV and SHL-I adopt the typical ICK motif, which incorporates a small triple-stranded antiparallel β-sheet with a topology of +2x, −1 and a cystine-knot formed by three disulfides with a linkage pattern of 1–4, 2–5, 3–6. There are three more toxins in Fig. 2 (HWTX-III, -V, -X), which have the same disulfide pattern of 1–4, 2–5, 3–6. We could reasonably propose that they also adopt the ICK motif in their 3D structures.

Structural comparison shows that the R.M.S.D between HWTX-I, -IV and SHL-I are about 1.3–1.4 Å for trace Cα atoms. This indicates that the 3D structures of HWTX-I, -IV and SHL-I are highly similar although their biological functions are completely different. It seems that there is a divergent evolution mechanism in the spider *S. huwena*, by which an ancient conserved scaffold was used to develop multiple diverse functions. It is generally accepted that the positively or negatively changed residues on the surface of a neurotoxins usually play an important role in their binding to targets. We can see from Fig. 3 that there are more charged residues, especially positively charged residues, on the surfaces of the neurotoxins HWTX-I and HWTX-IV than that of SHL-I, which is a lectin-like peptide.

Due to its unique 1–3, 2–5, 4–6 disulfide bond-pairing pattern (Fig. 2), HWTX-II does not form a cystine-knot, but it shows similarities in structure with the ICK motif molecules. The C-terminus in all the four structures (Fig. 3), especially the double-stranded antiparallel β-sheet and the two associated disulfide bridges, are conserved. The disulfide bonds 2–5 and 4–6 in HWTX-II (shown in yellow) are similar to the 2–5 and 3–6 in the ICK motif molecules, respectively. The significant difference between HWTX-II and the ICK motif molecules is the N-terminal portion of the molecule, contributed from the 1–3 disulfide bond of HWTX-II.

Because HWTX-II does not form a cystine-knot, the structure of HWTX-II may be considered as the first representative of a novel structural scaffold found in spider toxins. Moreover, this fold may also be adopted by other molecules in addition to HWTX-II. The primary structure of HWTX-II (Shu and Liang, 1999) is highly homologous with HWTX-VII and -VIII from the same spider. The high homology of the primary sequence of these three toxins may imply that the molecular folding of HWTX-VII and -VIII could be similar to HWTX-II.

6. cDNAs of huwentoxins

Eight cDNAs encoding seven toxins, HWTX-I, -II, -III, -IIIa, -IV, -V, VII and one lectin, SHL-I, from the spider *S. huwena*, were cloned and sequenced. Fig. 4 shows the two different cDNAs of HWTX-I and HWTX-II. Based on the amino acid sequences of HWTX-I and -II, 3′ and 5′ RACE primers were designed and synthesized. By overlapping the two partial cDNA sequences obtained by 3′ and 5′ RACE, the full-length cDNA sequences of these two toxins were obtained (Diao and Liang, 2003).

The signal sequences of the eight peptides are all 21 or 24 amino acids in length. They have all the characteristics of a typical signal peptide, including a hydrophobic core rich in valine and leucine, and a consensus cleavage point for a signal peptidase. The intervening propeptide regions of these toxins are between 27 and 29 amino acids in length. This segment is rich in glutamate residues as in other spider toxins such as *Phoneutria nigriventer* and *Agelenopsis*

cDNA sequence of HWTX-I

```
atcagtaact gaagttcacc gtaacactct cgtctcagaa gattattgct tttccgtggt
           -48                     -40
tgtgccgaac ATG AGA GCG TCA ATG TTT TTG GCC TTG GCA GGA TTA GTT CTG CTT
            M   R   A   S   M   F   L   A   L   A   G   L   V   L   L
           -30                                         -20
TTT GTT GTT TGC TAT GCC TCG GAA TCT GAG GAA AAA GAA TTC CCC AGA GAA CTG
 F   V   V   C   Y   A   S   E   S   E   E   K   E   F   P   R   E   L
                   -10                                     -1   1
CTT TTC AAG TTT TTT GCA GTT GAT GAC TTC AAA GGC GAA GAA AGG GCG TGC AAA
 L   F   K   F   F   A   V   D   D   F   K   G   E   E   R   A   C   K
                       10                                      20
GGG GTT TTT GAT GCA TGC ACA CCT GGA AAG AAT GAG TGC TGT CCA AAC CGT GTT
 G   V   F   D   A   C   T   P   G   K   N   E   C   C   P   N   R   V
                           30          33
TGT AGT GAT AAA CAC AAG TGG TGT AAA TGG AAA TTA TAG gcaaatgaga tcaat
 C   S   D   K   H   K   W   C   K   W   K   L  End
gtatg cagtccagtt ttctaactgg atgtatttgg ccaatgatgg tgttacgtga
agtacttcat ctgcctggca aaaaatgaga attaataaac attattcccc tttaa
caaaa aaaaaaaaaa aa
```

cDNA sequence of HWTX-II

```
tgtaggtgat tgctttcgta gtagatctga ttccatagtt agacaacttc agctagttct
                           -48                             -40
 ggtcaattgc aggagaattt gaaagaca ATG AAG GTG ACA TTG ATT GCC ATT CTG
                                M   K   V   T   L   I   A   I   L
                               -30
ACA TGC GCT GCA GTG TTA GTT CTT CAC ACA ACA GCA GCA GAA GAA CTC GAA GCA
 T   C   A   A   V   L   V   L   H   T   T   A   A   E   E   L   E   A
    -20                                         -10
GAA AGT CAG CTG ATG GAA GTT GGT ATG CCC GAT ACA GAA TTA GCA GCT GTG GAT
 E   S   Q   L   M   E   V   G   M   P   D   T   E   L   A   A   V   D
        -1   1                                   10
GAA GAA AGA CTC TTC GAA TGC TCT TTT TCA TGC GAA ATT GAG AAA GAA GGC GAC
 E   E   R   L   F   E   C   S   F   S   C   E   I   E   K   E   G   D
            20                                      30
AAA CCA TGC AAA AAG AAG AAA TGT AAA GGT GGA TGG AAA TGC AAA TTC AAT ATG
 K   P   C   K   K   K   K   C   K   G   G   W   K   C   K   F   N   M
            37
TGT GTG AAG GTT TAA agtgccgtaa tcatcacgac aaaattcgga atgaaaagtg
 C   V   K   V  End
gagagatttg tccttggtga cccattggaa agtacgcatg cgtccttcaa tcattaaccg
caatattgtt tgaatctctt ctgtttctga aacgaattct caataaaatt gttaaaacat
ttcaaaaaaa aaaaaaaaaa aaaaa
```

Fig. 4. cDNA and deduced amino acid sequences of HWTX-I and HWTX-II. The amino acids are denoted by one-letter symbols. The cDNAs encoding the mature peptide are underlined. The 'aataaa' polyadenylation signals are double underlined.

aperta (Penaforte et al., 2000; Santos et al., 1992). From analysis of the charged residues of the pro- and mature toxin regions, we find that the mature peptides are rich in basic residues except for SHL-I, which is a neutral molecule. Importantly, it is known that basic amino acids, such as lysine and arginine, are key residues involved in the binding of the certain toxins including the scorpion toxin LqhαIT (Tugarinov et al., 1997) and the sea anemone toxin anthopleurin-B (Monks et al., 1995) to their target. The pro- region, rich in acidic residues, may therefore have two main potential functions: (1) to neutralize the basic residues of mature peptide so as to stabilize the toxin precursor in the

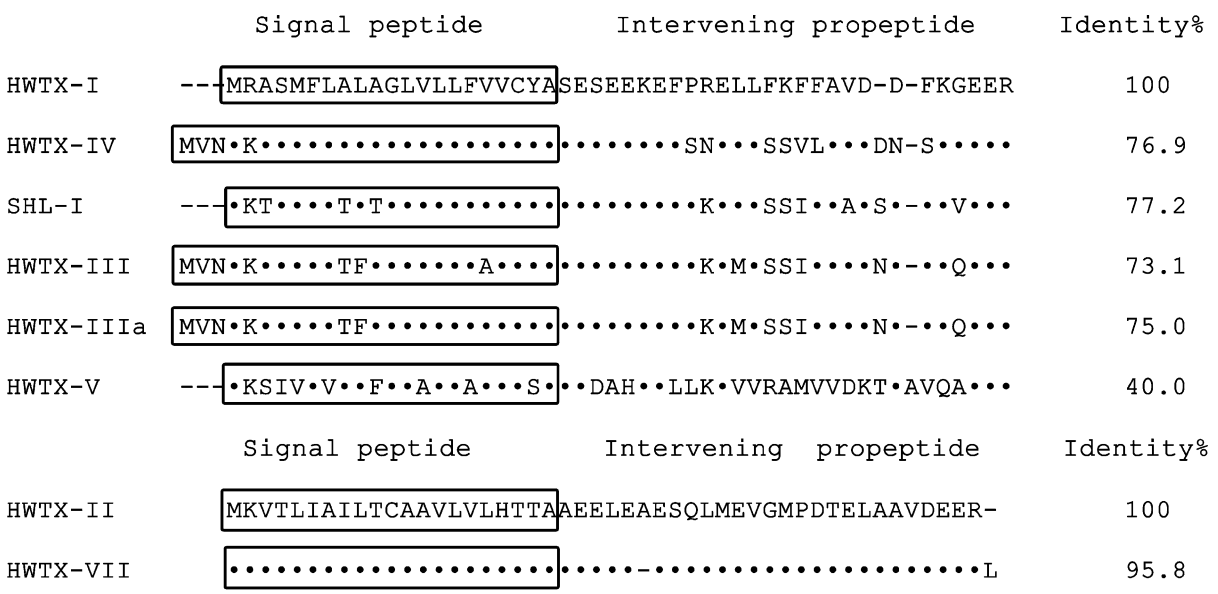

Fig. 5. Alignment of prepro-regions of toxins from *S. huwena* according to the amino acid sequences deduced from their cDNAs. The hydrophobic region (signal peptide) is shown by open boxes. Dots in the first six toxin sequences indicate that the amino acid is identical to that of HWTX-I. Dots in the last two toxin sequences indicate that the amino acid is identical to that of HWTX-II. When there is no similarity the corresponding change is indicated. Gaps (dashes) are introduced to maximize the deduced polypeptide sequence similarities. The identity of the prepro-region is annotated at the end of sequence.

cell cytoplasm, or (2) the acidic residues might form a precursor structure that covers the basic mature functional side, that could prevent the toxins interacting with related molecule before they are transported to venom glands and this maybe the way to prevent the toxicity to themselves. Except for HWTX-IIa, which has an additional leucine, the pro-regions of the seven peptides all end in the amino acid sequence EER, which might be the cleavage signal for the propeptide processing enzyme.

The prepro-regions of HWTX-I, -IIIa, -IV, and SHL-I, have high similarity (over 75%), although their coding sequences have almost no homology and their biological functions are different. HWTX-V also has a certain similarity with the former four toxins (40%), but with low homology to HWTX-II (Fig. 5). It therefore appears that HWTX-I, -IIIa -IV, -V and SHL-I, evolved from the same ancestor. At the same time, the prepro- region of HWTX-II and -VII are highly homologous (over 95%), as well as their coding sequences which also share a high similarity (about 81%). However, the prepro-regions of the latter two toxins have low homology with that of the former five peptides, indicating that HWTX-II and -VII originated from another progenitor. Interestingly, the 3D structures of HWTX-I, -IV and SHL-I all contain a cystine-knot motif, but HWTX-II exhibits a different motif. The similarity in the prepro-sequence and 3D structure indicates that HWTX-I, -IIIa, -IV, -V and SHL-I, belong to the same superfamily, whereas HWTX-II and -VII belong to another superfamily. It appears that during evolution, the peptides in the spider venom underwent hypermutation in the mature toxin region, while conserving the basic structural framework. Moreover, the signal peptide was related to the orientation of the folding of peptides, its residues were important for the sort of structure of molecule, so it became the most conserved region.

7. Conclusions

Spider venoms represent an incredible source of biologically active substances that selectively target a variety of vital physiological functions in both insects and mammals. The author of this review realizes that the array of *S. huwena* toxins mentioned above represent only the tip of the iceberg and that the number of novel *S. huwena* toxins will be expanding in the future. There is a Chinese saying: 90 *li*is only half of 100-*li* journey, which means the going is toughest towards the end of a journey. In the journey of the investigation of the toxins of *S. huwena*, what we finished so far are relatively easy tasks. We face more challenges if we need go further. The more results we got, the more questions we need to answer. For example, we need to determine the binding site of HWTX-IV on sodium channel; we need to investigate the heamagglutination mechanism of SHL-I, the smallest lectin so far found; we need to determine: does HWTX-XI block potassium channels in addition to its action as a trypsin inhibitor? We need to do the alanine-scanning mutagenesis to determine the pharmacophores of HWTX-I and HWTX-II; we also need to do the potential

therapeutic or insecticidal development; finally, there are more than 300 peptides or proteins in the *S. huwena* venom have not been characterized. It is almost impossible to finish the journey of the investigation of *S. huwena* toxins, but we will try to go further.

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