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Isolation and characterization of Hainantoxin-II, a new neurotoxic peptide from the Chinese bird spider (*Haplopelma hainanum*)

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Abstract: Hainantoxin-II (HnTx-II), a novel neurotoxin, was isolated from the venom of the Chinese bird spider (*Haplopelma hainanum*) by cation exchange chromatography and reverse-phase HPLC. The toxin was a single chain polypeptide with calculated molecular weight of 4 253.135 obtained by mass spectrometry. The complete amino acid sequence of HnTx-II was determined by Edman degradation and found to contain 37 residues with three disulfide bonds. Results showed HnTx-II can reversibly paralyze cockroaches for several hours after intra-abdominal injection with ED_{50} of 16 μg/g and kill the insects immediately at a dose of 60 μg/g. It was also shown to kill mice at a LD_{50} value of 1.41μg/g after intracerebroventricular injection. Hainantoxin-II shares 91% sequence homology with Huwentoxin-II (HwTx-II), an insecticidal peptide from another bird spider (*Haplopelma schmidti*) with a unique scaffold. While HnTx-II and HwTx-II both exhibit toxic activities in insects and mammals, HnTx-II shows higher insecticidal activity and lower lethiferous activity of mammals than HwTx-II. These results help clarify structural-functional relationships of the polypeptide toxin.

Key words: HnTx-II; Neurotoxin; Insecticidal peptide; Spider venom; *Haplopelma hainanum*

Hainantoxin-Ⅱ的分离纯化及其结构与功能分析

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摘要：从分布于我国海南省的海南捕鸟蛛 (*Haplopelma hainanum*) 毒素中, 利用阳离子交换色谱和反相高效液相色谱分离得到一种多肽神经毒素, 命名为 Hainantoxin-Ⅱ(HnTx-Ⅱ)。MALDI-TOF 质谱分析表明该多肽毒素相对分子质量为 4 253.135, 其氨基酸序列经 Edman 降解测序为 LFECS VSCEI EKEGN KDCKK KKCKG GWKCK FNMCV KV, 其中的 6 个 Cys 形成 3 对二硫键。同源性搜索表明, HnTx-Ⅱ与 Huwentoxin-Ⅱ(HwTx-Ⅱ)的同源性高达 91%, 仅有 3 个氨基酸残基不同。HwTx-Ⅱ为从虎纹捕鸟蛛中提取的杀虫肽, 该多肽具有独特的结构模体。活性分析表明 HnTx-Ⅱ与 HwTx-Ⅱ具有相似的生物学活性。经腹腔注射 HnTx-Ⅱ, 美洲蜚蠊可被麻痹, 其 ED_{50} 为 16 μg/g; 而当剂量增加到 60 μg/g 时, 可立即杀死美洲蜚蠊, 其杀死昆虫活性明显强于 HwTx-Ⅱ。此外, HnTx-Ⅱ经脑室注射可杀死小鼠, 其 LD_{50} 为 1.41 μg/g, 该活性却明显低于 HwTx-Ⅱ。这两种多肽毒素的结构与功能的差异为进一步阐明多肽毒素的结构与功能之间的关系提供良好的研究模型。

关键词：HnTx-Ⅱ; 神经毒素; 杀虫肽; 蜘蛛毒素; 海南捕鸟蛛
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Spider venom is a source of natural products with diverse biological activities on both insects and mammals (Rash & Hodgson, 2002), and are of interest as potential pharmacological tools (Ashok et al, 2000; Fletcher et al, 1997; Liang et al, 1993), drug discovery structures (Yan & Michael, 1998), and determinants of the role and diversity of neuronal ion channels (Escoubas, 2006). Theraphosidaes, commonly called tarantulas or

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bird spiders, are among the best known of all spider species (Ashok, 2000). Two Chinese bird spiders, *Haplopelma hainanum* and *Haplopelma schmidti* (*Ornithoctonus huwenum*), have been identified as new species distributed in the hilly areas of southern China and generally inhabiting holes underground. They have a similar morphology, and both kill insects and small vertebrates for food. The crude venom of *H. schmidti* possesses diverse compounds, such as neurotoxin (Liang, 2004), lectin (Li & Liang, 1999; Liang & Lin, 2000), and serine protease inhibitor (Liang, 2004), which cause numerous biological activities in insects and mammals. The structure and function of toxic peptides from *H. schmidti* have been investigated intensively over the last ten years (Liang, 2004). In addition, some components of *H. hainanum* venom have been found to be toxic to mice, and act as tetrodotoxin-sensitive sodium current inhibitors (Xiao & Liang, 2003a) or channel inhibitors (Xiao & Liang, 2003b; Li et al, 2004).

In this study, we report on the isolation, amino acid sequence, and biological activities of a novel toxic peptide, Hainantoxin-II (HnTx-II), from the venom of *H. hainanum*. This peptide shares high sequence homology with Huwentoxin-II (HwTx-II), a peptide from *H. schmidti* with unique disulfide bridge linkage (Shu et al, 2001 & 2002). While both these neurotoxins affect mammals and insects, HnTx-II shows higher insecticidal activity and lower lethiferous activity in mammals than that of HwTx-II. The findings from this study may help provide new insights into structural-functional relationships of the polypeptide toxin.

1 Materials and Methods

1.1 Venom and animals

Adult *H. hainanum* spiders were collected in the hilly area of Tongshi County, Hainan Province in southern China. The venom was obtained by electrical stimulation of the spider's fangs (Qu et al, 1997), and was immediately lyophilized and stored at −20 ℃. Kunming albino mice were obtained from Hunan Medical University and cockroaches (*Periplaneta americana*) from Peking University.

1.2 Chemicals

Reagents for N-terminal sequencing were obtained from Applied Biosystems (USA). The chromatographic solvent acetonitrile (ACN) was purchased from Linhai Chemicals (China), HPLC grade. Trifluoroacetic acid (TFA) was purchased from Sigma Chemical Company (USA). All other reagents were of analytical grade.

1.3 Purification of venom fraction

The lyophilized venom was dissolved in double-distilled water (1 mg/mL) and was centrifuged at 8000 r/min for 15 min. The supernatant was then subjected to cation-exchange chromatography on a 10 mm × 100 mm column initially equilibrated with buffer A containing 0.02 mol/L sodium phosphate buffer, pH 6.5. The column was eluted with a 0−45% linear gradient of buffer B (1 mol/L sodium chloride, 0.02 mol/L sodium phosphate, pH 6.5) over 50 min at a flow rate of 1.0 mL/min. Elution of peptide was monitored at 280 nm. Fraction B from ion-exchange chromatography was further subjected to reverse-phase HPLC on a 3.9 mm × 300 mm C18 column initially equilibrated with 0.1% trifluoroacetic acid in water (buffer A). Elution was performed with a 0−45% linear gradient of buffer B (acetonitrile containing 0.1% trifluoroacetic acid) over 60 min at a flow rate of 0.7 mL/min. Elution of the fraction was monitored at 280 nm. Elution with retention time of 25 min was further fractionated by reverse-phase HPLC at the same conditions as described above.

1.4 Mass spectrometry analysis

Peptide molecular masses were determined by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) with a delayed extraction ion source equipped with a nitrogen laser of 337 nm. The acceleration voltage was set to 20 kV. The matrix used was α-cyano-4-hydroxy-cinnamic acid (CCA, saturated solution in 50% ACN: 0.1% TFA, 1:1).

1.5 Carboxymethylation of peptide and N-terminal sequencing

Peptide reduction and S-carboxymethylation was performed as reported previously (Zhang et al, 1993). The carboxymethylated and native peptide were determined by an Applied Biosystems Model 491 gas-phase Pro-sequencer (Procise^{TM}) according to the Edman degradation procedure.

1.6 Biological activity

The biological activities of HnTx-II were examined by injecting it into cockroaches and mice. Intra-abdominal injections of HnTx-II in 0.9% NaCl solution into mature male cockroaches (BW 0.5 g) (Shu et al, 1999) and intra-abdominal and intracerebroventricular injections into Kunming albino mice (BW 18 − 22 g) were performed (Chen et al, 1995). Mice phrenic neuromuscular transmission preparations were used for pharmacological experiments (Liang et al, 1998).

2 Results

2.1 Purification and characterization of HnTx-II

The cation-exchange chromatography elution profile of unsexed *H. hainanum* crude venom is shown in Fig.1. Several peaks were observed, which demonstrate that crude venom was relatively complex. Peak B in Fig.1 was collected and further purified by subjection to reverse-phase HPLC, and two peaks with retention time of about 25 and 35 min, respectively, were found (Fig. 2A). The peak with 25 min retention time was found to be toxic to insects and was fractionated again by reverse-phase HPLC. The elution showed one sharp and symmetric peak (Fig. 2B), designated to HnTx-II.

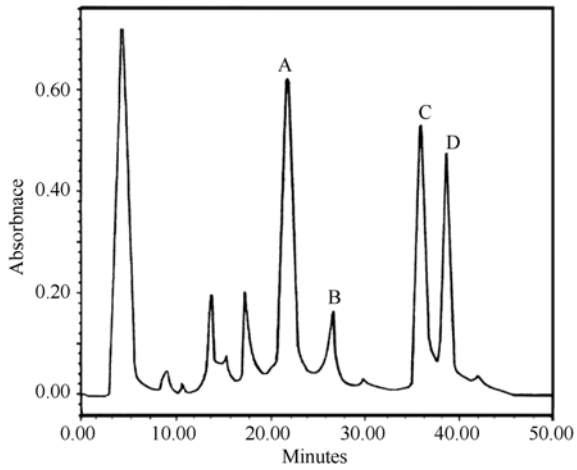

Fig. 1 Cation-exchange HPLC of crude *H. hainanum* venom

Lyophilized venom (1 mg in 1 mL distilled water) was applied to Waters Protein-Pak CM 8 HR Column (10 mm × 100 mm) initially equilibrated with buffer A containing 0.02 mol/L sodium phosphate buffer, pH 6.5. The column was eluted with a 0-45% linear gradient of buffer B (1 mol/L sodium chloride, 0.02 mol/L sodium phosphate, pH 6.5) over 40 min at a flow rate of 1.5 mL/min. Elution of peptide was monitored at 280 nm.

MALDI-TOF mass spectrometry was applied to analyze the molecular weight of native and cysteines carboxymethylation of HnTx-II. The observed molecular masses were 4253.383 (Fig.3A) and 4601.338 (Fig.3B), respectively, which indicated that the toxin contained six cysteines and formed three disulfide bonds. The amino acid sequence was determined by automated Edman degradation of the S-carboxymethylated samples. The intact native toxin was also sequenced for conformation. The complete amino acid sequence obtained was LFECS VSCEI EKEGN KDCKK KKCKG GWKCK FNMCV KV. The theoretical molecular mass calculated from the sequence was 4 259.135 and the mass was in good agreement with mass spectrometry data.

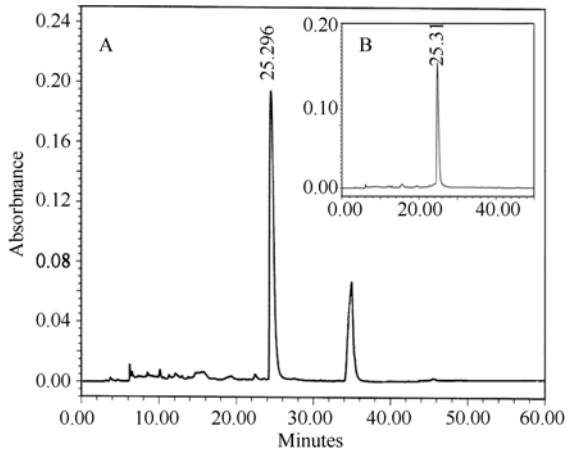

Fig. 2 Purification of HnTx-II by reverse-phase HPLC

(A) The cation exchange HPLC fraction B in Fig.1 was subjected to a Vydac C 18 column (3.9 mm × 300 mm) initially equilibrated with 0.1% trifluoroacetic acid in water (buffer A). Elution was performed with a 0−45% linear gradient of buffer B (acetonitrile containing 0.1% trifluoroacetic acid) over 60 min at a flow rate of 0.7 mL/min. Elution of the fraction was monitored at 280 nm. (B) Elution with retention time of 25 min was further fractionated by reverse-phase HPLC under the same conditions as described above.

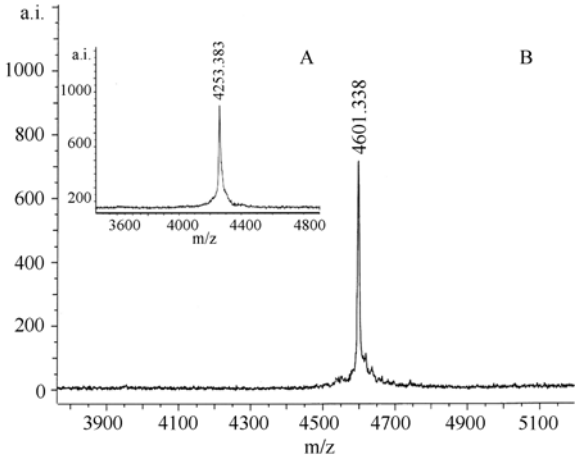

Fig. 3 MALDI-TOF mass spectra of the HnTx-II

The mass spectra showed molecular masses with 4253.383 for native toxin (A) and 4601.338 for S-carboxymethylation toxin (B).

Homologous peptides were searched and HnTx-II was found to share high homology with HwTx-II, an insecticidal peptide and mammalian neurotoxin from the Chinese bird spider *Haplopelma schmidti* (*Selenocosmia huwena*) (Shu et al, 1999), ESTx1 from the tarantula *Aphonopelma Californicum* (*Eurypelma Californicum*) (Savel-Niemann, 1989), and TXP1 from the Mexican red knee tarantula *Brachypelma smithii* (Kaiser et al, 1994). Their sequence alignment and identities are shown in Tab. 1.

Tab. 1 Sequence alignment of HnTx-II, HwTx-II, ESTx1 and TXP1

Species	Toxin	Amino acid sequence	Identity (%)
H. hainanum	HnTx-II	LFECSVSCEIEKEGNKDCK-K--KKCKGGWKCKFNMCVKV	-
H. schmidti	HwTx-II	LFECSFSCEIEKEGDKPCK-K--KKCKGGWKCKFNMCVKV	91
A. californicum	ESTx1	IFECVFSCDIEKEG-KPCKPKGEKKCTGGWKCKIKLCLKI	60
B. smithii	TXP1	IFECVFSCDIEKEG-KPCKPKGEKKCSGGWKCKIKLCLKI	60

HwTx-II was from the venom of the Chinese bird spider *H. schmidti* (Shu et al, 1999), ESTx1 was from the American tarantula *A. californicum* (Savel-Niemann, 1989), and TXP1 was from the Mexican red tarantula *B. smithii* (Kaiser et al, 1994).

Gaps were inserted to achieve best alignment. Residues conserved for HnTx-II to HwTx-II, ESTx1 and TXP1 were given a gray background, and residues strictly conserved were given a black background.

2.2 Biological activity assays

HnTx-II is a multi-activity peptide both in insects and mammals, likely due to its similar amino acid sequence to HwTx-II. Cockroaches and Kunming albino mice were used to verify HnTx-II's biological activity. Flexible cockroaches were quickly paralyzed after intra-abdominal injection of 20 μg/g HnTx-II compared to the control insects injected with non-toxic peptide, *H. schmidti* lectin-I (SHL-I). The paralytic effect lasted over 10 hours. Larger doses (40 μg/g body weight) killed cockroaches half a day after the injection, while increasing the dosage to 60 μg/g body weight, killed the cockroaches within a few minutes. The dose required to paralyzed half of the insects (ED_{50}) was 16 μg/g (Tab. 2).

No visible symptoms or behavior change were observed, however, after intra-abdominal injection of 50 μg/g HnTx-II into Kunming albino mice. Pharmacological experiments, which were carried out by using isolated mice phrenic nerve-diaphragm preparations treated with HnTx-II at 50 μg/mL, showed that HnTx-II could not block neuromuscular transmission (figure not shown) but could kill mice after intracerebroventricular injection (LD_{50} value of 1.41 μg/g) (Tab. 2). From these results, we inferred that HnTx-II was an insecticidal peptide that affected the central nervous system of mammals.

Tab. 2 The biological activities of HnTx-II and HwTx-II

Activity	HnTx-II	HwTx-II [a]
Affect to insects		
Paralyzed cockroaches (ED_{50})	16 μg/g	127 μg/g
Affect to mammals		
Killed mice (LD_{50})	1.41 μg/g	0.28 μg/g
Blocked neuromuscular transmission in mice at 50 μg/mL	No effect	Within 91 min

[a] The activity values of HwTx-II were from Su et al (1999).

3 Discussion

The Chinese bird spider *H. hainanum* is a newly identified species and its venom has been found to contain a mixture of components. The main component, HnTx-I, (Fig.1A), had no effect on mice and cockroaches after intra-abdominal injection (Li et al, 2003). The other two components, HnTx-III and HnTx-IV (Fig.1C and Fig.1D, respectively), are novel mammalian nerve sodium channel blockers (Xiao & Liang, 2003b). In our study, HnTx-II showed 91% sequence identity with HwTx-II (Tab. 1), and their cysteine residues were highly conserved. HwTx-II has a unique disulfide linkage pattern (C1-C3, C2-C5 and C4-C6) (Fig.4) (Shu et al, 2001), which differs from other spider neurotoxin peptides with an inhibitor cystine knot (ICK) motif (Pallaghy et al, 1994; Norton & Pallaghy, 1998). The three-dimensional structure of HwTx-II is also the first representative of a novel structural scaffold in peptide toxins (Shu et al, 2002). The unique disulfide linkage pattern and structural scaffold of HwTx-II should be valid for HnTx-II owing to its high sequence homology and conserved cysteine residues with HwTx-II.

Fig. 4 The unique disulfide linkage pattern of HWTX-Ⅱ (C1-C3, C2-C5 and C4-C6)

Biological activity assays showed that HnTx-II was an insect neurotoxin with an ED_{50} value of 16 μg/g, which was much lower than that observed for HwTx-II (127 μg/g) (Shu et al, 1999) (Tab. 2). This indicates that HnTx-II has higher insecticidal activity than HwTx-II. Despite its weak insecticidal activity, HwTx-II also blocked neuromuscular transmission in mice of the

phrenic nerve diaphragm preparations within 91 minutes at a concentration of 50 μg/mL (Shu et al, 1999), while HnTx-II had no effect on neuromuscular transmission even at a dose of 500 μg/mL (ten times higher than that of HwTx-II) (Tab. 2). Additionally, though both toxins killed mice after intra-cerebroventricular injection, their LD_{50} values showed a significant difference with the value of HnTx-II being about five times higher than that of HwTx-II (Tab. 2). That is, HnTx-II had lower mammalian neurotoxin activity than HwTx-II.

The roles played by protein or peptide depend on their three-dimensional structures (i.e., based on amino acid composition). The bioactivity variations between HnTx-II and HwTx-II depend, therefore, upon the three amino acid residues mutated at position 6 (Val→Phe), 15 (Asn→Asp), and 16 (Asp→Pro) of the sequences (Tab. 1). Additionally, HnTx-II is a single chain peptide with a calculated molecular mass of 4253, while HwTx-II formed by two subunits of molecular mass 4284 and 4300, respectively (Shu et al, 1999). Further investigation of the variations in chemical structure and biological activity between HnTx-II and HwTx-II should provide insight into the proteins' structure-function relationships, and lead to synthesis or expression of HnTx-II mutants with strong insecticidal activity to serve as useful agricultural drugs.

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