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Cloning and functional expression of a synthetic gene encoding huwentoxin-I, a neurotoxin from the Chinese bird spider (*Selenocosmia huwena*)

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Abstract

Cloning and functional expression of a synthetic gene encoding huwentoxin-I, a neurotoxin from the Chinese bird spider *Selenocosmia huwena*. A gene encoding huwentoxin-I, a peptide neurotoxin consisted of 33 amino acid residues from the venom of the Chinese bird spider *Selenocosmia huwena*, was designed, synthesized and expressed in *Escherichia coli* as a hybrid protein fused with glutathione S-transferase at the N-terminal. The fusion protein was purified by GSH-Sepharose 4B affinity column chromatography and cleaved by thrombin to release the toxin peptide. The amino acid sequence of the recombinant toxin was consistent with the designed one by sequence determination and MALDI-TOF mass analysis, suggesting that the recombinant huwentoxin-I produced the same expression product as the native one. After reduction and renaturation, the biological activity of the recombinant toxin was identical with that of the native huwentoxin-I by electrophysiological method.

1. Introduction

Spider neurotoxins are of interest as tools for studying neurophysiology and as potential lead structures for insecticides and pharmaceuticals. Huwentoxin-I

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(HWTX-I) is a neurotoxin isolated from the venom of the Chinese bird spider, *Selenocosmia huwena*, which has recently been identified as a new species of the *Selenocosmia* genus (Liang et al., 1993). This toxin can reversibly block the neuromuscular transmission in an isolated mouse phrenic nerve–diaphragm preparation. The intraperitoneal and intracisternal LD_{50} of the toxin in mice are 0.70 mg/kg and 9.40 μg/kg, respectively. Experiments on Xenopus embryonic myocytes indicated that HWTX-I reduced the open probability of acetylcholine induced channel activity and finally blocked the channel, indicating that nicotinic acetylcholine receptor may be the site of action of this toxin (Zhou et al., 1997). HWTX-I contains 33 amino acid residues including six cysteines which are involved in three disulfide bridges (Zhang and Liang, 1993). The three dimensional structure of HWTX-I was recently determined by 2D-NMR (Qu et al., 1995). The molecule adopts a compact structure consisting of a small triple-stranded antiparallel *β*-sheet and a disulfide-binding knot. In this paper we report the designing, synthesis and functional expression of the HWTX-I gene. The goal of this work is to provide the necessary materials in order to engage in further studies of possible applications for the toxin and establish a procedure to get the analogues of this toxin for the study of the relationship of the structure and function.

2. Materials and methods

2.1. Reagents

Restriction enzymes, T4 DNA ligase, protein molecular weight standard, sodium dodecyl sulfate (SDS) were provided by Promega (Madison, WI, USA); 5-bromo-4-chloro-3-indoly 1-*β*-*D*-galactoside (X-gal), isopropylthio-*β*-*D*-galactoside (IPTG), phenylmethylsulfonyl fluoride (PMSF), acrylamide, *N, N′*-methylen-*bis*-acrylamide, CSH, human thrombin were obtained from Sigma (St. Louis, MO, USA); glutathione-Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden); RNase A, DNase I, lysozyme were from SABC (Luo Yang, People's Republic of China).

2.2. Bacterial strain and plasmids

Escherichia coli DH 5α was used for plasmid propagation and expression. Phagemid pBlusecript was used as a cloning vector and pGEX-KT was a prokaryotic gene fusion vector which produced the fusion gene with glutathione S-transferase (GST).

2.3. Construction of pGH plasmid

The HWTX-I gene was constructed using two synthetic oligonucleotides that were synthesized using a Beckman Oligo 1000 DNA synthesizer (Applied

Biosystem). The sequence of the two 108 mers are shown in Fig. 1. The codon usage was optimized to obtain a high level of expression in *E. coli* (Sharp et al., 1988). The oligonucleotides were annealed and ligated into pBluecscript using standard techniques (Ausubel et al., 1989). After confirmation of the sequence of the synthetic gene with ABI 376 sequencer by dideoxy sequencing, the HWTX-I gene was ligated into pGEX-KT, GST gene fusion vector. The recombinant plasmid was named pGH. The resulting construct, encoding a fusion protein of GST and HWTX-I (GST-HWTX-I) was separated by a thrombin cleavage site.

2.4. Expression and purification of the recombinant HWTX-I

E. coli DH 5α harbouring pGH were cultured and induced with IPTG. In brief, bacteria were grown to $O.D_{550\ nm}$ 1.0–1.5 and then induced with 0.1 mM IPTG for 4 h at 37°C. Cells were harvested at 4000 g for 10 min, washed once with phosphate-buffered saline (PBS, 140 mmol/l NaCl, 2.7 mmol/l KCL, 10 mmol/l Na_2HPO_4, 1.8 mmol/l NaH_2PO_4), then suspended in lysis buffer consisting 1% Triton X-100, PBS, 2 mM EDTA and the protease inhibitor PMSF (1 mM) and incubated with 1 mg/ml lysozyme for 30 min. After removal of nucleic acids with

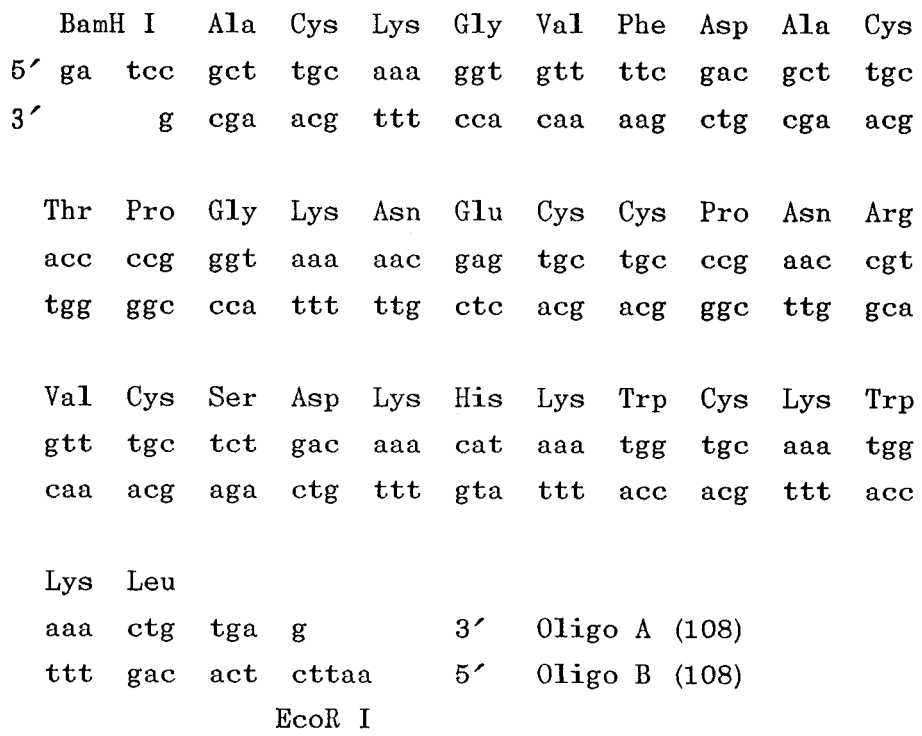

Fig. 1. DNA sequence of synthetic HWTX-I peptide.

DNas I, the lysate was centrifuged at 27,000 ***g*** for 30 min. Purification of the fusion protein (GST-HWTX-I) was achieved in a one-step procedure using affinity chromatography with glutathione–Sepharose 4B under the conditions as described (Smith and Corcoran, 1990). The GST-HWTX-I was eluted with eluate consisting of 5 mM GSH, 50 mM Tris-HCl, pH 8.0. The cleavage of GST-HWTX-I by thrombin was performed essentially as described (Smith and Johnson, 1988). The rHWTX-I was purified from the cleavage mixture using size-exclusion HPLC on a Shimpac Diol-150 7.9×250 mm column. Elution was performed with 0.2 M NH_4AC, pH 6.0, at a flow rate of 0.6 ml/min. The fraction collected was further purified with reverse-phase HPLC in a Vydac 4.6×250 mm C4 column. Elution was performed with a linear gradient of 30–65% acetonitrile containing 0.1% TFA at a flow rate of 1.0 ml/min.

2.5. SDS–polyacrylamide gel electrophoresis

The molecular weight of expressed toxin was estimated by polyacrylamide gel electrophoresis in SDS according to the method of Laemmli (1970) using 16.5% acrylamide gels.

2.6. Amino acid sequence determination

The amino acid sequence of the purified rHWTX-I was analysed using the method described by Liang and Laursen (1990). Briefly, Edman degradation was performed on prototype of Milli Gen/Biosearch model 6600 ProSequencer using a prepacked aminophenyl glass beads capillary column for immobilization and sequencing. The native HWTX-I was used as control.

2.7. MALDI-TOF mass spectrometry

Mass spectrometry analysis of the rHWTX-I was performed using a MALDI-TOF mass spectrometer by Micromass Corp.

2.8. Reduction and renaturation of the rHWTX-I

The rHWTX-I was dissolved in a solution consisting of 0.1 M Tris-HCl pH 8.0, 6 M guanidine HCl, 2 mM EDTA and 100 mM DTT and the concentration of the rHWTX-I was 1 mg/ml. The reduction was performed for 2 h at room temperature. For the renaturation, the solution system consisted of 0.1 M Tris-HCl pH 8.0, 1 mM EDTA and 4 mM GSSG was formed gradually in reduced peptide solution diluted 100 times. The final concentration of guanidine HCl reached 1 M. The sample was stirred slowly for 24 h at 4°C. Renatured rHWTX-I was purified further by reverse-phase HPLC on a Vydac 2.1×150 mm C 18 column. Elution was performed with a linear gradient of 0–37% acetonitrile containing 0.1% TFA at a flow rate of 0.6 ml/min. All the HPLC reactions were performed using a Waters 2010 HPLC separation system with a 990 model UV detector.

2.9. Biological activity determination

Experiments to determine activity of renatured rHWTX-I were carried out using mouse phrenic nerve-diaphragm preparations as previously described (Liang et al., 1993). The concentration of sample was 1×10^{-5} g/ml. The native HWTX-I was used as control.

3. Results

3.1. Construction of the HWTX-I gene expression system

The nucleotide sequence encoding HWTX-I was designed to obtain codons maximizing the expression level in *E. coli* (Fig. 1). *Bam*H I and *Eco*R I sites were added to the 5′ end of each, in order to facilitate cutting with restriction enzymes. In addition, a terminator codon TGA was inserted at the 3′ end of the synthetic HWTX-I gene.

After annealing, ligation and transformation of the HWTX-I gene, the structure of the cloned HWTX-I gene was verified using dideoxy sequencing. Subsequently, the confirmed HWTX-I gene was inserted into *Bam*H I and *Eco*R I sites of the

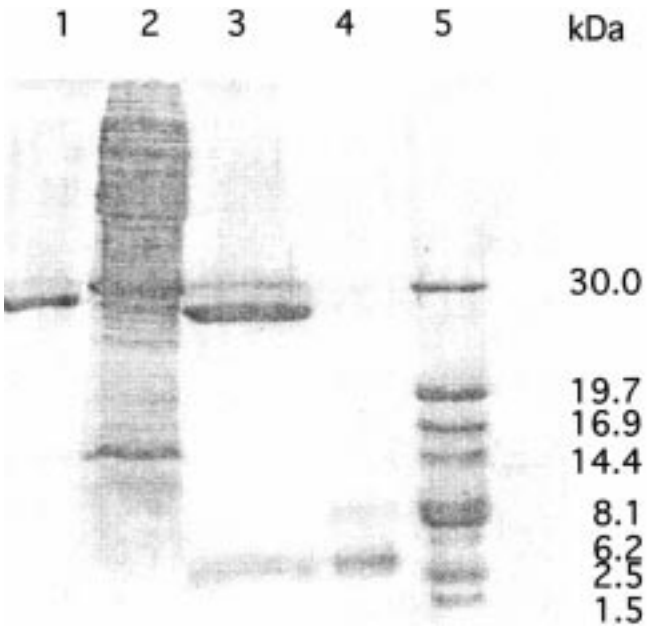

Fig. 2. Fusion protein cleaved with thrombin was analyzed by SDS-PAGE electrophoresis. Lanes: 1, GST; 2, lysate; 3, GST-HWTX-I after thrombin cleavage; 4, native HWTX-I; 5, standard molecular weights (low range).

fusion vector pGEX-KT. The sequence encoding HWTX-I was fused onto the C-terminal portion of the GST.

3.2. Expression and purification of the rHWTX-I

In order to obtained significant quantities of the peptide, the HWTX-I was expressed in *E. coli* DH 5α as part of fusion protein with the GST gene using IPTG as an inducer. After partial purification of the GST-HWTX-I, affinity chromatography with GSH–Sepharose 4B was performed so that GST-HWTX-I was purified to homogeneity. The yield of GST-HWTX-I was approximately 12.5–15.0 mg/l of culture. Purified GST-HWTX-I was cleaved with thrombin, a sequence-specific protease. SDS gel electrophoresis and Coomassie Blue staining of the digested mixture revealed specific bands at the expected molecular weight (Fig. 2). The fusion protein band at about 30 kDa was cleaved into two major products, a 26 kDa band representing GST and a small band of 4 kDa, representing the rHWTX-I.

The rHWTX-I was separated from the digested mixture by size-exclusion HPLC. Determination of the amino acid sequence for the rHWTX-I showed that the sequence of the first 25 amino acids were as follows: G-S-A-C-K-G-V-F-D-A-C-T-P-G-K-N-E-C-C-P-N-R-V-C-S, which is identical to the native peptide except

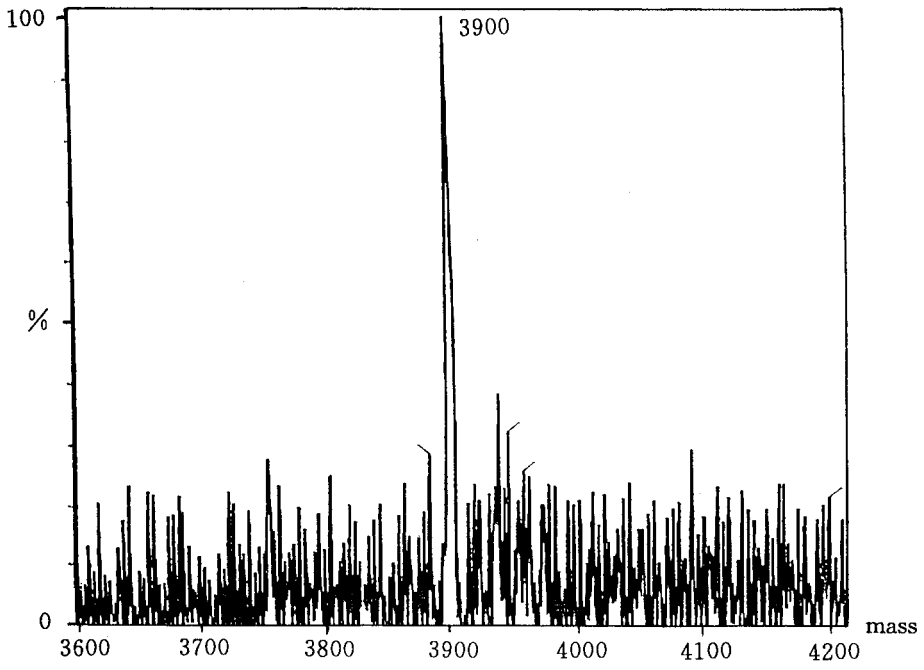

Fig. 3. MALDI-TOF mass spectrometry of the rHWTX-I.

for the two excess amino acids, G-S (data not shown). In addition, the exact mass was 3900.0 Da which corresponds well with the calculated mass of the reduced rHWTX-I, being 3900.2 Da (Fig. 3): 3750.00 Da + Gly 75.07 + Ser 105.09 + 6−36 ($2H_2O$) = 3900.16 Da.

The HPLC profile shown in Fig. 4 indicates that the reduced and renatured rHWTX-I accounted for more than 30% of the purified rHWTX-I under the given conditions. The amino acid peptide from the main peak (Fig. 4B, retention time 30 min) was further collected and lyophilized.

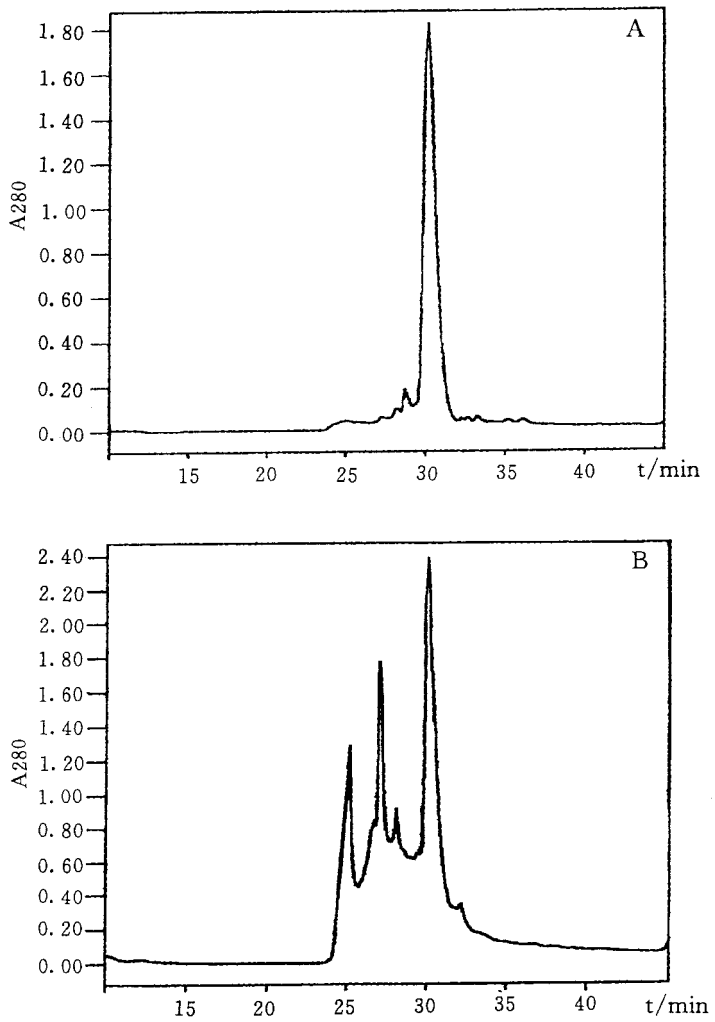

Fig. 4. Purification of renatured rHWTX-I using reverse-phase HPLC. A, native rHWTX-I; B, renatured rHWTX-I.

3.3. Biological activity of the rHWTX-I

Importantly, rHWTX-I displayed the same potency as native HWTX-I for blockade of the neuromuscular transmission. Fig. 5 shows the effect of the recombinant and native HWTX-I with the concentration of 1×10^{-5} g/ml on the neuromuscular transmission of the isolated mouse phreneic nerve-diaphragm preparation. The amplitude of the twitch responding to indirect stimulation was reduced and finally lost. The time of transmission block was 14.3 ± 3.2 min (mean $\pm$ SD, $n = 4$) for the native HWTX-I, while it was 14.9 ± 4.3 min (mean $\pm$ SD, $n = 4$) for rHTWX-I.

4. Discussion

The results presented in this paper describe the cloning and expression of a synthetic gene encoding a small peptide of the HWTX-I isolated from the venom of the Chinese bird spider, *Selenocosmia huwena*. According to the amino acid sequence of the native HWTX-I, we synthesized a synthetic gene and attempted to express it using a GST fusion expression system. Fusion of the toxin gene to the C-terminal of GST resulted in a significant level of protein production. The chimerical protein was formed by fusion of the GST with a hexapepide which contains a thrombin cleavage site, followed by the sequence of the HWTX-I peptide.

The ability to produce foreign polypeptides in *E. coli* in the form of fusion proteins with various carrier proteins has made large-scale purification protocols possible (Smith and Johnson, 1988). The pGEX-KT vector (Hakes and Dixon, 1992) has been designed to induce high-level intracellular expression of a gene or gene fragments as fusion with *Schistosoma Japonicum* GST. The pGEX-KT vector

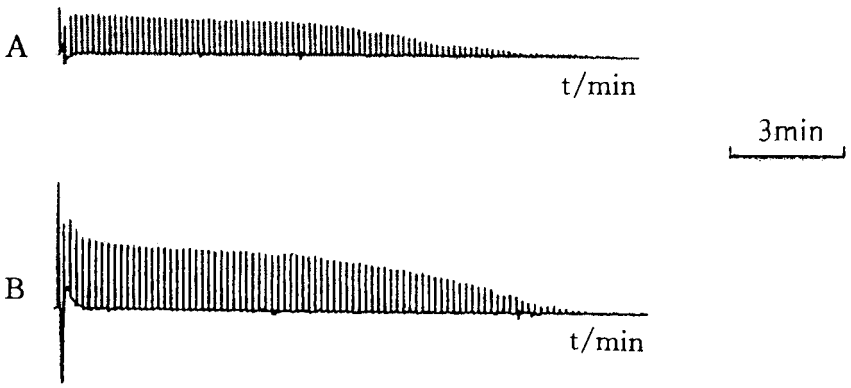

Fig. 5. Blockades of neuromuscular transmission with the rHWTX-I and native HWTX-L. A, renatured rHWTX-I; B, native HWTX-I.

was therefore used for the construction of plasmid pGH expressing HWTX-I fused to the C-terminal end of GST. This vector allowed the introduction of (I) a tac promoter for chemically inducible high-level expression, (II) an internal Lac I^q gene for tight regulation of the GST protein, (III) a thrombin cleavage site in the C-terminal end of the GST protein and (IV) a glycin-rich "kinker" upstream of the thrombin cleavage site which permits rapid and complete cleavage of the fusion protein by the protease. PGH was used to produce the GST-HWTX-I protein in *E. coli* strain DH 5α. Although the yield of fusion protein thus prepared was about 12.5–15.0 mg/l of growth medium, we think that the yield may be improved. There are various possible reasons for the modest level of toxin produced by our system: (I) low rate of protein synthesis; (II) poor protein stability; (III) toxicity of the HWTX-I for host bacteria.

Without reduction and renaturing treatment, a low activity of the rHWTX-I was found. This indicates that most of the rHWTX-I synthesized in *E. coli* remained unactivated, probably due to the fact that the biological activity of the HWTX-I is dependent on the correct formation of three disulfide bridges (Zhang and Liang, 1993).

As a consequence of the DNA sequence of the synthetic HWTX-I gene (Fig. 1), the recombinant toxin contained two extra residues (Gly-Ser) at the amino-terminal of the sequence compared with that of the native HWTX-I. Since the recombinant toxin has proved to have the same biological activity as that of the native HWTX-I, the two extra residues on the N-terminal probably have no influence to the function of the molecule. In addition, previous experiments in our group also confirmed that mutations at the amino-terminal residue of the HWTX-I appeared to have no effect on its biological activity (to be published). The outcome of the essay searching biological activity (Fig. 5) suggested that the recombinant HWTX-I shows the same activity as the native HWTX-I. This allows for the large scale production of this toxin for further studies.

Acknowledgements

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