Solid-phase synthesis of huwentoxin-I and its structure and bioactivity analysis*

LIANG Songping (梁宋平), XIA Zanxian (夏赞贤) and XIE Jinyun (谢锦云)

(Department of Biology, Hunan Normal University, Changsha 410081, China)

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Abstract Huwentoxin-I, a neurotoxic peptide from the spider *Selenocosmia huwena*, was synthesized by solid-phase method with Fluorenylmethoxycarbonyl amino acid pentafluorophenyl esters (Fmoc-AA-OPfp). The carboxyl and the hydroxy groups were protected by tBu; the side chains of Lys and His were protected by Boc; the guanidine group of Arg was protected by Mtr and the mercaptan group of Cys was protected by Trt. The solid-phase carrier was ethylene diamine-polyethylene-polystyrene (DEA-PEG-PS) resin. The synthetic peptide was cleaved from the resin and deprotected by a 90% TFA solution containing 5% thioanisole, 3% ethanedithiol and 2% anisole. The product was reduced with DTT and then incubated with GSSG and GSH to form the correct disulfide bond linkages. The synthetic peptide was purified by HPLC and then characterized by amino acid composition and sequence analysis, peptide mapping and NMR. The biological activity of the synthetic product was tested by electrophysiological method using the isolated mouse phrenic nerve diaphragm preparation. The results indicated that the synthetic huwentoxin-I has the same chemical and conformational structure and biological activity as those of the native huwentoxin-I from the spider.

Keywords: huwentoxin-I, solid-phase method.

Huwentoxin-I (HWTX-I) is a neurotoxin isolated from the venom of the Chinese bird spider *Selenocosmia henna* which has recently been identified as a new species of genus *Selenocosmia*[1]. This toxin can reversibly block the neuromuscular transmission in an isolated mouse phrenic nerve-diaphragm preparation. The intraperitoneal and intracisternal LD_{50} values of the toxin in mice are 0.70 mg/kg and 9.40 μg/kg, respectively. The experiment on Xenopus embryonic myocytes indicated that HWTX-I reduced the open probability of acetylcholine induced channel activity and finally blocked the channel, which demonstrated that nicotinic acetylcholine receptor was the action site of HWTX-I[2]. The sequence of 33 amino acid residues and the positions of the three disulfide bonds of this toxin have been determined as in fig. 1[3] and were confirmed by 2D-NMR spectropic methodology[4]. No homologies were found when the sequence was compared with those of the proteins stored in the Data Bank, which indicated that the HWTX-I is a novel neurotoxic peptide different from other spider toxins known so far. In this paper we report the solid-phase chemical synthesis of HWTX-I with the Fmoc method. The objectives of this work are to provide sufficient materials to enable extensive studies of this toxin and to establish a procedure to synthesize the analogies of this toxin for the study of the relationship of the structure and function. We also report here the investigation of the optimal conditions for the correct formation of

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Abbreviations: Boc, butyloxycarbonyl; DTT, dithiothreitol; DCC, dicyclohexylcarbodiimide; Fmoc-AA-OPfp, fluorenylmethoxycabonyl-amino acid-pentafluorophenyl ester; EDC, 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide hydrochloride; HOBt, hydroxybenzotriazole; HPLC, high performance liquid chromatography; EDA-PEG-PS resin, ethylenediamine polyethylene glycol-polystyrene resin; Mtr, methoxytrimethylbenzene sulfonyl; Trt, trytyl; tBu, tertiary-butyl ether; NMP, N-methylpyrolidone.

the three disulfide bonds of the synthesized HWTX-I. The results of the chemical and conformational structure analysis and the biological activity testing of the final product are also reported.

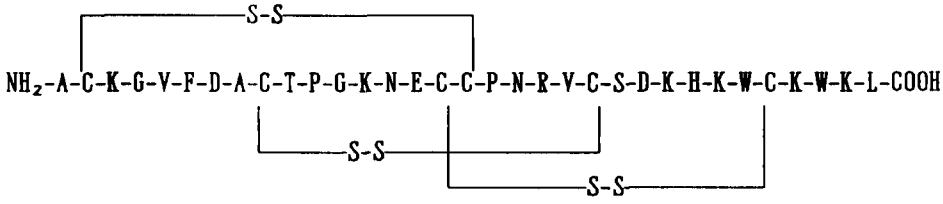

Fig. 1. Amino acid sequence and disulfide bond arrangements in huwentoxin-I.

1 Materials and methods

1.1 Materials

DTT, DCC, trifluoroacetic acid (TFA), glutathione (GSSH) and reduced glutathione (GSH) were products of Sigma. Fmoc-AA-OPfp was from Millipore Ltd; EDC, hydroxybenzotriazole (HOBt), NMP, dimethylaminopyridine (DMAP) and piperidine were from Aldrich; EDA-PEG-PS resin was kindly given by Laursen of Boston University; acetonitrile and methanol (HPLC grade) were purchased from Linhai Chemicals. All other reagents were of analytical grade.

1.2 Solid-phase synthesis of HWTX-I

Solid-phase synthesis, using Fmoc amino acid pentafluorophenyl esters, was performed manually on a laboratory-made solid phase synthesis station which consists of a shake and several valves controlling the deliveries of nitrogen and solvents. The activation of the EDA-PEG-PS resin and the general synthetic protocols (fig. 2) were basically according to the procedure described by Atherton *et al*.[5]. The resin was activated by the reaction with 4-hydroxymethylphenoxyacetic acid-OPfp and HOBt. The first amino acid (leucine) was coupled to the hydroxy groups of the activated resin by the catalysis of dimethylaminopyridine (DMAP). The coupling yield was determined with the method of Sarin *et al*.[6]. 200 mg Fmoc-Leucine-resin (0.23 mmol/g) was used as the starting material in the following procedure of the synthesis according to the sequence of HWTX-I. The Fmoc amino acid pentafluorophenyl esters (3—4 times in excess) dissolved in DMF were used in each coupling step with the HOBt as the catalyst and 20% of piperidine as the deprotecting reagent. A sample of peptide-resin was removed for ninhydrin analysis before every coupling reaction to determine the extent of the coupling and the percentage of the uncoupled amine and to decide whether or not a second coupling was needed. During the synthesis the Trp3, Lys4, Glu19, Gly30 and Ala33 were coupled twice and the Val13 was coupled for three times. After 32 steps of coupling the 33 residues peptide-resin was obtained and a sample of the peptide resin was removed for amino acid analysis.

The procedure of cleavage of peptide from the resin is as in ref. [5], using the mixture trifluoroacetic acid-thioanisole-ethanedithiol-anisole (95:5:3:2; *v*/*v*/*v*/*v*). The reaction proceeded under nitrogen in the dark for 4 h. The resin was then filtered through a sintered glass funnel and washed 3 or 4 times with trifluoroacetic acid. The filtrate was collected and evaporated under vacuum. The cleaved and deprotected peptide product was washed with anhydrous ether and then

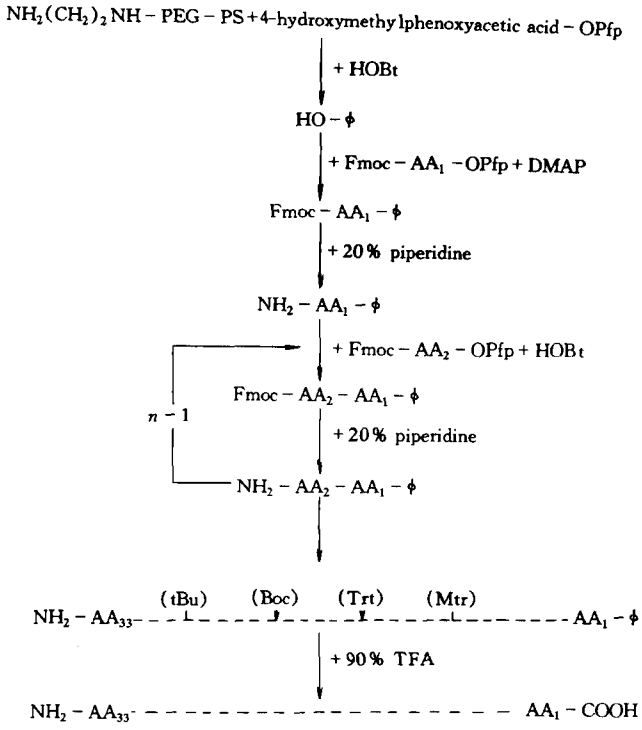

Fig. 2. Synthetic procedure of huwentoxin-I, ϕ stands for solid-phase resin.

evaporated under high vacuum.

1.3 Reduction and oxidation of the synthetic product

The formation of the three disulfide bonds of the synthetic peptide was achieved by using the procedure described by Saxena *et al*.[7]. 28 mg synthesized product was first dissolved in 2.5 mL Tris-HCl buffer (50 mmol/L, pH 6.2) containing 6 mol/L guanidine hydrochloride and 200 mmol/L DTT and then was incubated for 40 min at 37℃. 1 mL of the above reaction solution was then added to 1 mL Tris-HCl buffer (1.5 mol/L, pH 8.0) containing 0.3 mmol/L oxidized glutathione and 3.0 mmol/L reduced glutathione. The mixture solution was then incubated at 4℃ and stirred with a magnetic stirrer for 5 d. The peptide was finally purified by HPLC.

1.4 Purification of the synthetic peptide by HPLC

The synthesized sample, after reduction and oxidation, was first desalted by a reverse phase Vydac C_4 column (15×0.46 cm) using a Waters 2010 HPLC station with a 996 photodiode array detector. 50 mL sample solution was applied to the column by pump A and then the column was washed with 0.1% TFA (buffer A) until the u.v. absorbtion 230 nm was near zero. The peptide was eluted out with 50% buffer A and 50% buffer B (acetonitrile, 0.1% TFA). The major peak was collected and further purified by ion-exchange HPLC using a TASK-Gel-CM cation-exchange column (25×0.4 cm). The column was previously equilibrated with 0.02 mol/L sodium phosphate buffer, pH 6.6 (buffer A). Elution was performed with a linear gradient from 0%—60% buffer B (1 mol/L NaAc, 0.02 mol/L phosphate buffer, pH 7.0) over 30 min. The major peak was collected and was finally purified by a reverse-phase HPLC using a YWG-C_{18} column.

1.5 Reduction and S-carboxymethylation of peptide

The procedure of Zhang *et al*.[3] was used for the reduction and S-carboxymethylation of the synthesized peptide. The S-carboxylated peptide was purified by revese phase HPLC as in section 1.4.

1.6 Amino acid composition and sequence analysis

The amino acid composition analysis was performed according to ref. [2]. The sequence of the S-carboxymethylated peptide was analysed in a MilliGen Moddel 6600 ProSequencer using the method of Liang *et al*.[8].

1.7 Tryptic digestion and peptide mapping

The S-carboxymethylated sample was digested by trypsin and then analysed by HPLC using the procedure as in our previous paper[3].

1.8 Proton NMR analysis

The synthetic and the native HWTX-I were dissolved in 0.5 mL of 20 mmol/L phosphate buffer (90% H_2O/10% D_2O) with a final concentration of 4 mmol/L in the peptides. The 1D-NMR analysis was performed at 500 MHz on a Bruker AM-500 NMR spectrometer equipped with an Aspect 3000 computer. ^1H-NMR spectra were recorded at 27℃ and 36 000 data points were acquired (128 scans per increment). The spectral width was 6 494 Hz (12.987 ppm). Solvent suppression was carried out by using the presaturation method.

1.9 Assay of biological activity

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 sample dissolved in Tyrode's solutions bubbled with 95% O_2, 5% CO_2 and kept at 30—32℃. 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 signal by a mechanical-electric transducer made of semiconductor stain gauge. The signals were amplified and recorded with a pen recorder.

2 Results and discussion

2.1 The solid-phase synthesis of HWTX-I

The Leu-resin was obtained by the esterification of the activated DEA-PEG-PS resin with Fmoc-Leu-OPfp (five times in excess), catalyzed by DMAP. The remaing hydroxyl groups were masked by acetic anhydride. The coupling yield of this step was 77%. Although the yield was relatively low, the experimental procedure was very simple. The coupling yield can be improved by using the symmetric anhydride method. Fmoc amino acid pentafluorophenyl esters were used in the following coupling steps. The average coupling yield was 98.3%. After 32 steps of coupling, 235 mg of 33 amino acid residue peptide-resin was obtained and gave a final yield of 44.7%. Because the tryptophan is not stable during the cleavage by acid and the Mrt and Trt

groups are apt to give modification to other amino acids when deprotected by acid, special scavengers are required in the cleavage solution. In our experiment a mixture solution of trifluoroacetic acid-thioanisole-ethanedithiol-anisole (95:5:3:2; $v/v/v/v$) was used for cleavage and deprotection since there were Trp, Arg (Mtr) and Cys (Trt) in the peptide resin. Under this condition the acid liable Boc, tBu and Trt groups were removed. Since it usually needs 8 h to remove the Trt group thoroughly but the tryptophan is easy to be destroyed during long time cleavage, we chose 4 h of the reaction time to meet the coordination and compromise. Through filtration, washing with ether and evaporation under vacuum, 28 mg of the crude peptide product was obtained from 100 mg of peptide-resin.

2.2 Reduction, oxydation and purification of the synthetic peptide

The formation of the three disulfide bonds is necessary for the synthetic HWTX-I to have correct conformation and biological activity. Without suitable treatment, the synthetic peptide after cleavage from the resin was tested to have no biological activity since the three disulfide bonds were not correctly formed. In order to promote the formation of the three disulfide bonds, the synthetic peptide was first reduced by DTT and then incubated in a mixture solution of oxidized and reduced glutathione for 5 d. In this condition the synthetic peptide underwent a transition from one-dimensional to three-dimensional structure that was finally functionally active and this process also involved coupling of sulfhydryl groups. The formation of the three disulfide bonds depends upon the amino acid sequence of the peptide and the reduction and oxidation condition of the environment and is a process of self energy optimization.

The sample, treated through reduction and oxidation, was desalted by reverse-phase HPLC and then was purified by cation exchange HPLC as is shown on fig. 3. The fraction of peak 3 was collected and identified by amino acid analysis and u.v. spectrum. The product was further purified by reverse phase HPLC as in fig. 4. Fig. 5 is the chromatogram of the HPLC of a mixture of

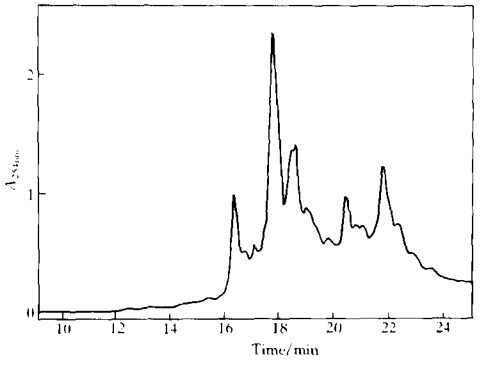

Fig. 3. Ion-exchange HPLC profile of the synthesized huwentoxin-I. Column, TSK Gel-CM (250 × 4.0 mm); eluent: A, 0.02 mol/L sodium phosphate buffer (pH 5.0); B, 1 mol/L sodium acetate buffer (pH 7.0); A/B from 100/0 to 40/60 within 25 min; flow rate, 0.8 mL/min; room temperature.

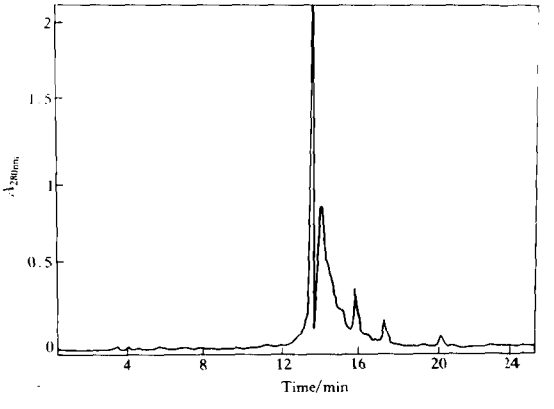

Fig. 4. Reverse-phase HPLC profile of the synthetic huwentoxin-I after ion-exchange HPLC. Column, YWG C18 (300 × 3.9 mm); eluent: A, 0.1% TFA in water; B, 0.1% TFA in acetonitrile; A/B from 100/0 to 40/60 within 30 min; flow rate, 0.8 mL/min; temperature, 45℃.

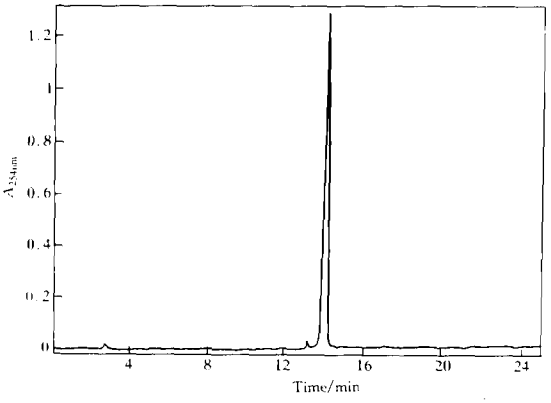

Fig. 5. Reverse-phase HPLC profile of the native and synthetic huwentoxin-I. Column, YWG C18 (300 × 3.9 mm); eluent: A, 0.1% TFA in water; B, 0.1% TFA in acetonitrile; A/B from 100/0 to 40/60 within 30 min; flow rate, 0.8 mL/min; temperature, 45℃.

synthesized and native HWTX-I at an equal amount. Only one sharp and symmetric peak appeared in the HPLC profile, indicating that the synthetsized HWTX-I had identical retaition time as that of the native HWTX-I and was of high purity. 2.9 mg of the purified HWTX-I was obtained from 28 mg crude synthesized product. The reasons for the relatively low yield are mainly the incomplete cleavage and the sample losses during reduction, oxidation and purification. The incomplete cleavage was proved by amino acid analysis of the resin after cleavage. Further optimization of the cleavage condition is needed to improve the yield.

2.3 The structure and bioactivity analysis of the synthesized HWTX-I

2.3.1 The amino acid composition and sequence analysis. The results of the amino acid composition analysis of the synthetic HWTX-I after hydrolysis with 6 N HCl are given as following: Asx 3.20, Glu 1.04, Ser 0.86, Gly 1.97, His 0.90, Thr 0.75, Ala 2.00, Arg 0.88, Pro 2.20, Val 2.06, Leu 1.38, Phe 0.78, Lys 5.79. The mole ratios of the component amino acids were quite satisfactory when compared with the theoretical ratios of the HWTX-I except for Trp and Cys which were destroyed during hydrolysis by HCl. The first 26 cycles of Edman degradation of the synthetic peptide by a 6600 ProSequencer have given correct amino acid PTHs according to the sequence of HWTX-I. No more PTH signals were obtained because the working condition of the sequencer was not quite good after 26 cycles.

2.3.2 The peptide mapping analysis. Figure 6 is the comparison of the tryptic peptide mapping of the native and synthetic HWTX-I after S-carboxymethylation. The two peptide maps are almost identical, indicating that the synthesized HWTX-I has the same primary structure as that of the native toxin.

2.3.3 The 1D-NMR spectra analysis. Since we have already achieved the sequence-specific ^{1}H-NMR assignment of the native HWTX-I[4], it is significant to compare the 1D-NMR spectra of the synthetic HWTX-I and that of the native HWTX-I to investigate their three-dimensional structure. Fig. 7 is the comparison of the 1D-NMR spectra of the synthetic and native HWTX-I at the same concentration under the same experimental condition by a 500 MHz NMR spectrometer. The result is quite satisfactory for the two spectra are very similar to each other. The proton peaks of the nine methyl groups from Ala1, Ala8, Val5, Val21, Thr10 and Leu33 are almost identical in the two spectra. The proton peaks of NH groups from Trp31 and Trp28 at downfield region show the same chemical shifts and strength in the two spectra, although there is a little shift of the baseline. This result indicates that the synthetic HWTX-I possessed the same three-dimensional structure as that of the native HWTX-I.

2.3.4 The assay of biological activity of the synthetic HWTX-I. The biological activity assay is very important in checking the result of the synthesis of the HWTX-I. Four preparations of the mouse phrenic nerve-diaphragm were used. The results (fig. 8) demonstrated that the synthesized HWTX-I can reversibly block the neuromuscular transmission of the isolated mouse phrenic nerve-diaphragm preparations. The average blockage time was about the same as that of the native HWTX-I.

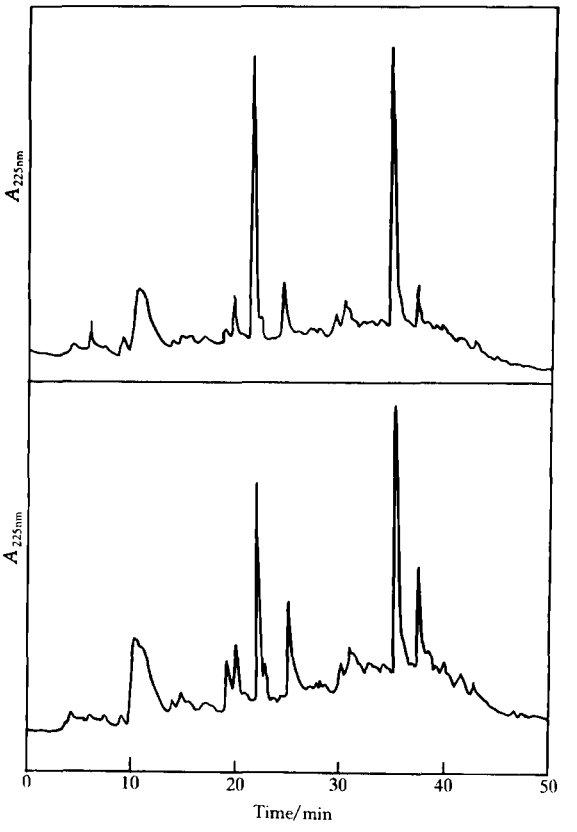

Fig.6. Tryptic peptide mapping of the native and the synthetic huwentoxin-I. Column, YWG C18 (300 × 3.9 mm); eluent: A, 0.1% TFA in water; B, 0.1% TFA in acetonitrile; A/B from 100/0 to 40/60 within 40 min; flow rate, 0.8 mL/min; temperature, 45℃.

In summary, the neurotoxic peptide HWTX-I has been synthetic by solid phase method with Fmoc amino acid pentafluorophenyl esters. The synthesized toxin has been proved to have the same chemical and three-dimensional structure and to possess the same biological activity. By far, about 50 toxic peptides have been isolated from spider venoms of different species, whose amino acid sequences were determined. But only agatoxinI-VA[9] and u-agatoxin-IV[10] from the spider *Agelenopsis aperta* have been chemically synthesized by using the Boc-amino acids and anhydrous HF for cleavage. Here we report the chemical synthesis of HWTX-I by using the Fmoc amino acid pentafluorophenyl esters and TFA for cleavage. The advantage of our procedure is free from the HF which is very toxic and difficult to handle. Instead of using the procedure of stepwise selective formation of disulfide bonds[11], we used the method of reduction and oxidation with DTT and the mixture solution of GSSG and GSH. Although we have not determined the three disulfide bonds directly by chemical method, the correct formation of the three disulfide bridges was proved by the 1D-NMR spectra analysis, as well as by the biological activity analysis of the synthetic HWTX-I. This result is a good example to demonstrate that the formation of disulfide bridges of a protein depends upon the amino acid sequence and the reduction and oxidation condition of the environment of the protein. The result of this experiment has also proved the correctness of determination of the primary structure of HWTX-I . The successful synthesis procedure will also provide us a method to study the relationship between the structure and function of huwentoxin-I.

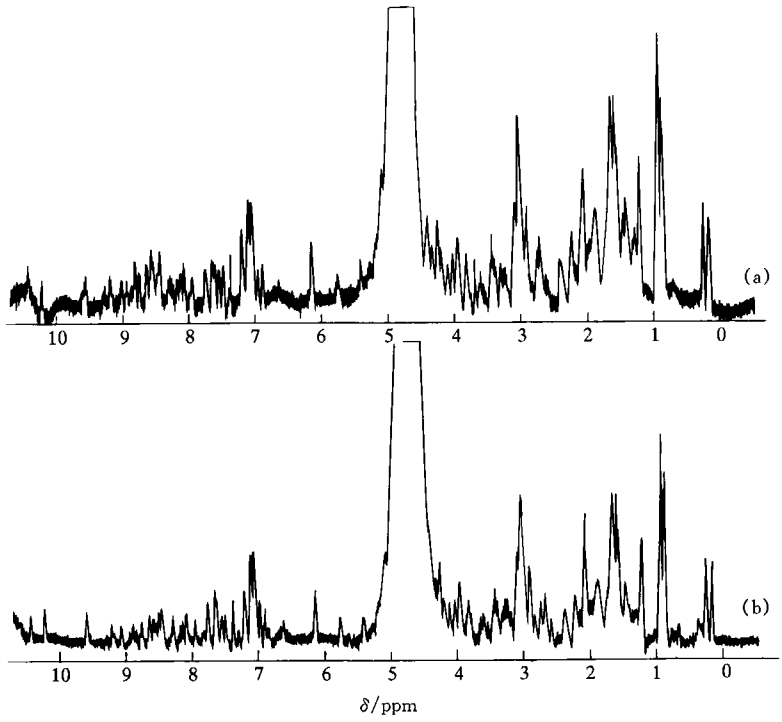

Fig. 7. 1D-NMR spectra of the native and synthetic huwentoxin-I. (a) Synthetic huwentoxin-I (4 mmol/L in phosphate buffer, pH 5.0, 90% H_2O/10% D_2O); (b) native huwentoxin-I (4 mmol/L in phosphate buffer, pH 5.0, 90% H_2O /10% D_2O).

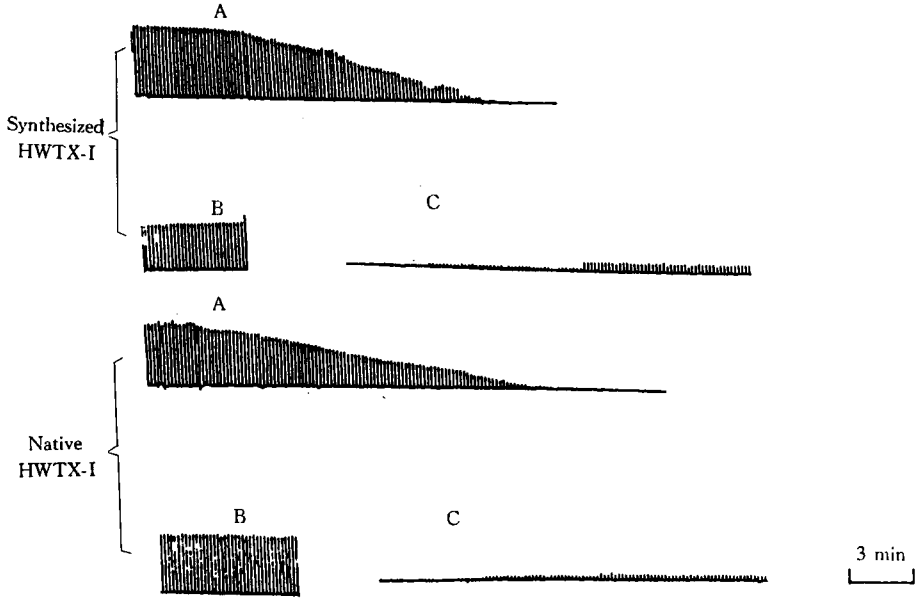

Fig. 8. The effect of the synthetic and native huwentoxin-I on the neuromuscular thansmission of the mouse phrenic nerve diaphragm preparation. A, The effect on the twitch response by indirect stimulation after the sample (1×10^{-5} g/mL) was added; B, the twitch response by direct stimulation after the transmission was blocked; C, the beginning of the recovery of the twitch response by indirect stimulation after washing for 30 min.

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