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Nuclear Magnetic Resonance Studies on Huwentoxin-XI from the Chinese Bird Spider *Ornithoctonus huwena*: ^{15}N Labeling and Sequence-specific ^{1}H, ^{15}N Nuclear Magnetic Resonance Assignments

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Abstract Huwentoxin-XI purified from the Chinese bird spider *Ornithoctonus huwena* is a toxin with both antiprotease activity and potassium channel blocking activity. To determine its solution structure, huwentoxin-XI was expressed in a yeast eukaryotic expression system and studied by NMR. The ^{15}N labeling strategy was used to facilitate the process of resonance assignments. The nearly complete sequence-specific assignments of proton and nitrogen resonances were obtained by analyzing a series of two-dimensional (2D) and three-dimensional (3D) spectra, including DQF-COSY, TOCSY, NOESY, ^{15}N-^{1}H HSQC, ^{15}N-^{1}H HNHA, ^{15}N-^{1}H HNHB, ^{15}N-^{1}H TOCSY-HSQC and ^{15}N-^{1}H NOESY-HSQC spectra. Secondary structure analysis of huwentoxin-XI showed that it mainly contains an N-terminal 3_{10}-helix from Thr3 to Arg5 and a C-terminal α-helix from Gln45 to Cys52, plus a triple-stranded antiparallel β-sheet of Glu18-Asn23, Thr26-Ile31 and Asn40-Lys41. These studies provide a solid basis for the final structure determination of huwentoxin-XI.

Key words huwentoxin-XI; toxin; nuclear magnetic resonance; ^{15}N labeling; sequence-specific assignment

Proteinase inhibitors exist in numerous life-forms including plants, animals and microorganisms, playing significant roles for regulating the proteolytic activity of their targeted proteinases, for blocking them in emergency cases, or for signaling receptor interactions [1]. Among them, Kunitz/BPTI proteinase inhibitors are the most extensively investigated. These are usually peptides of approximately 60 amino acids in length, stabilized by three disulfide bridges, with the conserved connection pattern I-VI, II-IV, III-V [2]. The side chain of K15, namely the P1 site [3], of BPTI is the primary determinant of anti-protease specificity, which interacts with Asp189 located in the S1 pocket of trypsin [4].

Dendrotoxins are homologous in sequence and tertiary structure to the Kunitz/BPTI proteinase inhibitors. However, they target K^+ channels instead of proteinases [5]. Conversely, proteinase inhibitors do not show K^+ channel blocking activity [5]. Point mutation in dendrotoxin K from *Dendroaspis polylepis* [6] and acetylation of the lysines in dendrotoxin I from *D. polylepis* [7] revealed that the N-terminal helix and the surrounding β-turn of dendrotoxins might be involved in K^+ channel binding.

Three proteins isolated from the sea anemone *Anemonia sulcata*, named kalicludines [8], were found to be homologous to the Kunitz/BPTI proteinase inhibitors, and interestingly they showed inhibitory effects on both voltage sensitive K^+ channels and trypsin, which is unusual for a toxic member of the family.

Huwentoxin-XI (HWTX-XI) was recently purified from the Chinese bird spider *Ornithoctonus huwena*, and similar to kalicludines, it shows both K^+ channel blocking activity and antiprotease activity (unpublished data). In order to clarify the individual structural basis for the dual bio-activities of HWTX-XI, nuclear magnetic resonance (NMR) spectroscopy was used to elucidate its solution structure.

Here we report the ^{15}N labeling of HWTX-XI and sequence-specific assignments of ^{1}H, ^{15}N resonances

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facilitated by heteronuclear NMR methods.

Materials and Methods

Protein expression and sample preparation

The gene encoding HWTX-XI was cloned through the *Xba*I and *Hin*dIII restriction sites into the yeast expression vector pVT102U, and the recombinant plasmid was transformed into the *Saccharomyces cerevisiae* strain S-78.

To get unlabeled HWTX-XI, several transformants (>2 mm in diameter) were transferred directly into 500 ml Yeast-Peptone-Dextrose (YPD) media (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) and grown at 30 ºC for 3 d. The culture was centrifuged and HWTX-XI was purified from the supernatant by a combined use of carboxymethyl (CM) ion-exchange chromatography (in NaAc buffer, pH 4.2; the target component was eluted at 0.3 M NaCl) and C18 reverse phase HPLC.

To get ^{15}N-labeled HWTX-XI, 10 transformants (>2 mm in diameter) were first transferred directly into 400 ml YPD media and grown for 2 d, then the cells were centrifuged and transferred into 400 ml minimal Yeast synthetic dropout (YSD) media {1.7 g/L yeast nitrogen base (YNB), 25 g/L glucose, 400 mg/L leucine, 200 mg/L adenine, 400 mg/L inositol, 0.5 g/L yeast extract, 2 g/L [^{15}N]NH_4Cl}. The cells were cultured and reloaded to fresh minimal YSD media (800 ml, 1.6 liters in turn) every day by centrifugation and the supernatants collected were stored at –20 ºC for later combined purification (in the same way as the unlabeled HWTX-XI).

A 5 mM unlabeled HWTX-XI sample and a 3 mM ^{15}N-HWTX-XI sample were prepared in 50 mM K_2HPO_4-KH_2PO_4 buffer (adjusted to pH 4.0 using H_3PO_4), with 5% D_2O for field lock and a small amount of azide for stabilization. The chemical shifts were referenced to internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

NMR experiments

The homonuclear ^1H 2D NMR spectra double quantum filtered correlation spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY) were recorded on a Bruker Avance 800 MHz spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) with the unlabeled HWTX-XI sample. In addition, natural abundance ^{13}C-^1H heteronuclear single quantum correlation (HSQC) spectrum was recorded on a Bruker Avance 500 MHz spectrometer equipped with a cryoprobe. The heteronuclear multidimensional spectra, including 2D ^{15}N-^1H HSQC, 3D ^{15}N-^1H HNHA, 3D ^{15}N-^1H HNHB, 3D ^{15}N-^1H TOCSY-HSQC and 3D ^{15}N-^1H NOESY-HSQC were recorded on a Bruker Avance 600 MHz spectrometer. All NMR experiments were carried out at 303 K. The spectra were processed with NMRpipe (http://spin.niddk.nih.gov/NMRPipe/) [9] and analyzed by NMRview (http://onemoonscientific.com/nmrview/download.html) [10].

Results

^{15}N labeling of HWTX-XI

For the structure determination of HWTX-XI with 55 amino acid residues, traditional homonuclear ^1H 2D NMR methods are rather challenging, although still applicable. Heteronuclear NMR approaches using isotope labeling might be an ideal solution to shorten the time-consuming process of distinguishing overlapped cross-peaks in ^1H 2D NMR spectra.

As **Table 1** shown, despite that recombinant HWTX-

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Table 1 Optimization of recombinant huwentoxin-XI expression for ^{15}N labeling

Media	Time (d)	Yield (mg/L)	Supplement
YPD †	3	10–12	None
Minimal YSD ‡	3	~0	1 g/L NH_4Cl
Minimal YSD	5	~0	1 g/L NH_4Cl
Minimal YSD	2	~1	1.0–2.5 g/L NH_4Cl and 20–50 g/L glucose
Minimal YSD §	1	3–4	2 g/L NH_4Cl, 25 g/L glucose, and 0.5 g/L yeast extract

† YPD media contained yeast extract (10 g/L), peptone (20 g/L) and glucose (20 g/L). ‡ Minimal YSD media contain YNB (1.7 g/L), glucose (25 g/L), leucine (400 mg/L), adenine (200 mg/L) and inositol (400 mg/L). § Ten transformants (>2 mm in diameter) were grown in 400 ml YPD for 2 d. After centrifugation, cells were transferred to 400 ml minimal YSD media and cultured for 1 d, centrifuged, amplified to 800 ml fresh media for 1 d, then centrifuged again and amplified to 1600 ml fresh media for 1d. The whole culture lasted for 5 d and the supernatants collected during the culture were stored at –20 ºC for later combined purification.

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XI reaches an expression level of 10–12 mg/L in YPD media, changing the culture media to minimal YSD media supplemented with NH_4Cl unfortunately resulted in a very poor protein yield (almost 0 mg/L). Prolonging the culture time or raising the amount of nitrogen/carbon resources did not bring much improvement. Using YPD media for cell amplification then transferring the cells into YSD media for short-period labeling gave a much better outcome. Daily reloading of the cells into fresh labeling media resulted in a target protein yield of about 3–4 mg/L, which is sufficient for preparing a several millimole ^{15}N-labeled HWTX-XI sample from 3 liters of culture.

Spin system identification

In traditional homonuclear ^1H 2D NMR methods, the spin system identification is carried out by analyzing scalar coupling patterns of specific residues from TOCSY and DQF-COSY spectra. However, as shown in **Fig. 1**, the amide-aliphatic regions of TOCSY and DQF-COSY spectra recorded on the 800 MHz NMR spectrometer are still too crowded to make reliable coupling pattern analysis. For example, there are cross-peaks from at least four residues overlapped at the same H_N chemical shift 8.2 judged from the DQF-COSY spectrum [**Fig. 1(A)**], and at the corresponding position in the TOCSY spectrum [**Fig. 1 (B)**] there are more than 10 cross-peaks awaiting careful assignment.

However, with the ^{15}N labeling heteronuclear strategy, the spin system identification was done by analyzing a series of heteronuclear multidimensional spectra including 2D ^{15}N-^1H HSQC, 3D ^{15}N-^1H HNHA, ^{15}N-^1H HNHB and ^{15}N-^1H TOCSY-HSQC. The overlapped H_N chemical shifts were distinguished by their individual ^{15}N chemical shifts in 2D ^{15}N-^1H HSQC [**Fig. 2(A)**], and the undistinguished overlapping cross-peaks in TOCSY were also distributed to different 2D planes of 3D ^{15}N-^1H HNHA, HNHB TOCSY-HSQC, according to their individual correlating ^{15}N chemical shifts [**Fig. 2(B)**]. However, this strategy is far from complete, and the identification of residue types or the corresponding position in the protein sequence is impossible without the aid of sequential assignment.

Sequence-specific assignments

Complete spin system identification for Gly, Ala and

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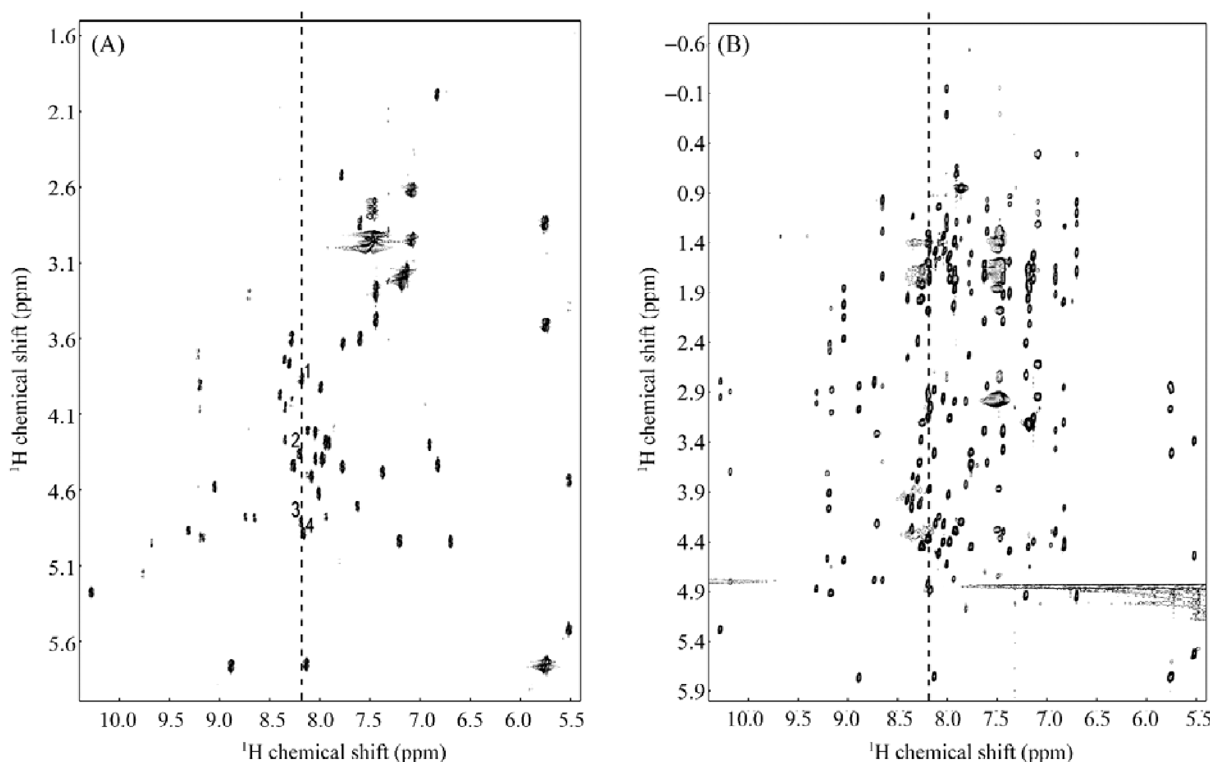

Fig. 1 Amide-aliphatic regions in DQF-COSY and TOCSY spectra of huwentoxin-XI
(A) DQF-COSY spectrum. (B) TOCSY spectrum. The dashed lines illustrate overlapping cross-peaks from at least four spin systems at H_N chemical shift 8.2.

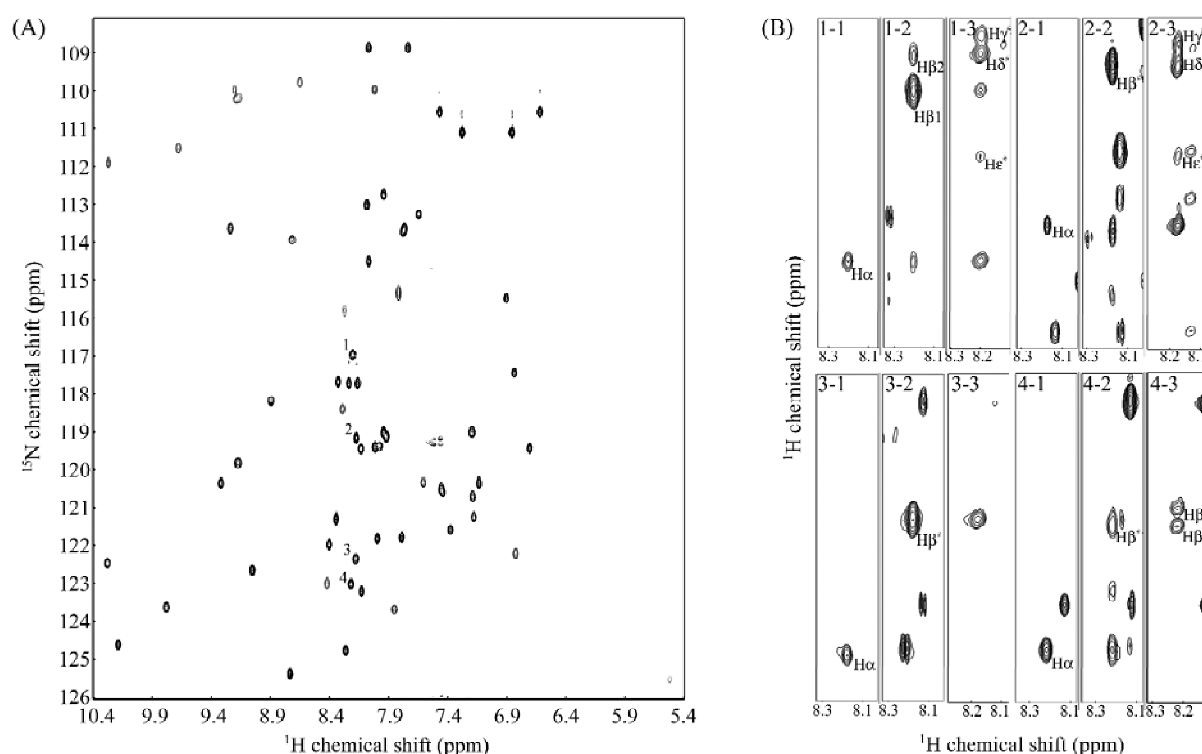

Fig. 2 Spin system identification by heteronuclear approaches

(A) 2D ^{15}N-^{1}H HSQC spectrum. (B) NMRview strips for corresponding numbered spin systems from 3D ^{15}N-^{1}H HNHA (*-1), ^{15}N-^{1}H HNHB (*-2) and ^{15}N-^{1}H TOCSY-HSQC (*-3). In the 2D ^{15}N-^{1}H HSQC spectrum, overlapping cross-peaks (the denoted numbers do not refer to those in **Fig. 1**) at H_N chemical shift 8.2 give their individual ^{15}N chemical shift value. The α, β and other side-chain proton resonance assignments of each spin system were roughly identified from 3D ^{15}N-^{1}H HNHA (*-1), ^{15}N-^{1}H HNHB (*-2) and ^{15}N-^{1}H TOCSY-HSQC (*-3), respectively. With the aid of 3D ^{15}N-^{1}H HNHA, HNHB, the cross-peak 1,2 in 2D ^{15}N-^{1}H HSQC spectrum were identified as resonances from long side-chain residues and cross-peak 3,4 from residues of AMX type. # overlapping proton resonances.

Thr residues in HWTX-XI was obtained by analyzing 3D ^{15}N-^{1}H HNHA, HNHB and TOCSY-HSQC spectra, and they were taken as the starting points for screening $d_{\alpha N}$ and d_{NN} connectivities in 3D ^{15}N-^{1}H NOESY-HSQC spectra recorded with a mixing time of 150 ms. Nearly complete sequential connectivities were observed throughout the sequence of HWTX-XI except for I1, P7, G34 and P43 (**Fig. 3**). The spin systems of residues Pro7 and Pro43 were identified by the observation of strong sequential NOE cross-peaks between the δ protons of the proline and the α proton of the previous residue in the ^{1}H 2D NOESY spectra (200 ms of mixing time), which also indicated the *trans* conformation for the two prolines. **Fig. 4** shows the 2D ^{15}N-^{1}H HSQC spectrum with all amide HN and side-chain HN cross-peaks based on the resonance assignments.

Confirmation of resonance assignments

By analyzing 3D ^{15}N-^{1}H HNHA, HNHB and TOCSY-HSQC spectra, unambiguous proton resonance assignments were obtained for most short side-chain residues in HWTX-XI. Assignments of side-chain protons in long side-chain residues such as Lys and Arg were achieved by analyzing their scalar coupling patterns in TOCSY and DQF-COSY spectra in combination with heteronuclear spectra.

Based on the characteristic ^{13}C chemical shift distribution of -CH, -CH_2 and -CH_3 groups of different amino acids, assignments of α protons (especially α protons of Gly), β protons of Ser and Thr, and methyl protons of Ala, Thr, Ile, Leu and Met were further confirmed by the ^{13}C-^{1}H HSQC spectrum (**Fig. 5**).

Resonance assignments of side-chain -NH_2 were obtained by checking in 3D ^{15}N-^{1}H NOESY-HSQC spectra the shared cross-peaks (H_N-H_γ for Gln, H_N-H_β for Asn) through the amide HN and side-chain HN planes (**Fig. 6**). For resonance assignments of Arg side-chain ε-NH, shared H_N-H_δ or H_N-H_γ cross-peaks were also observed in 3D

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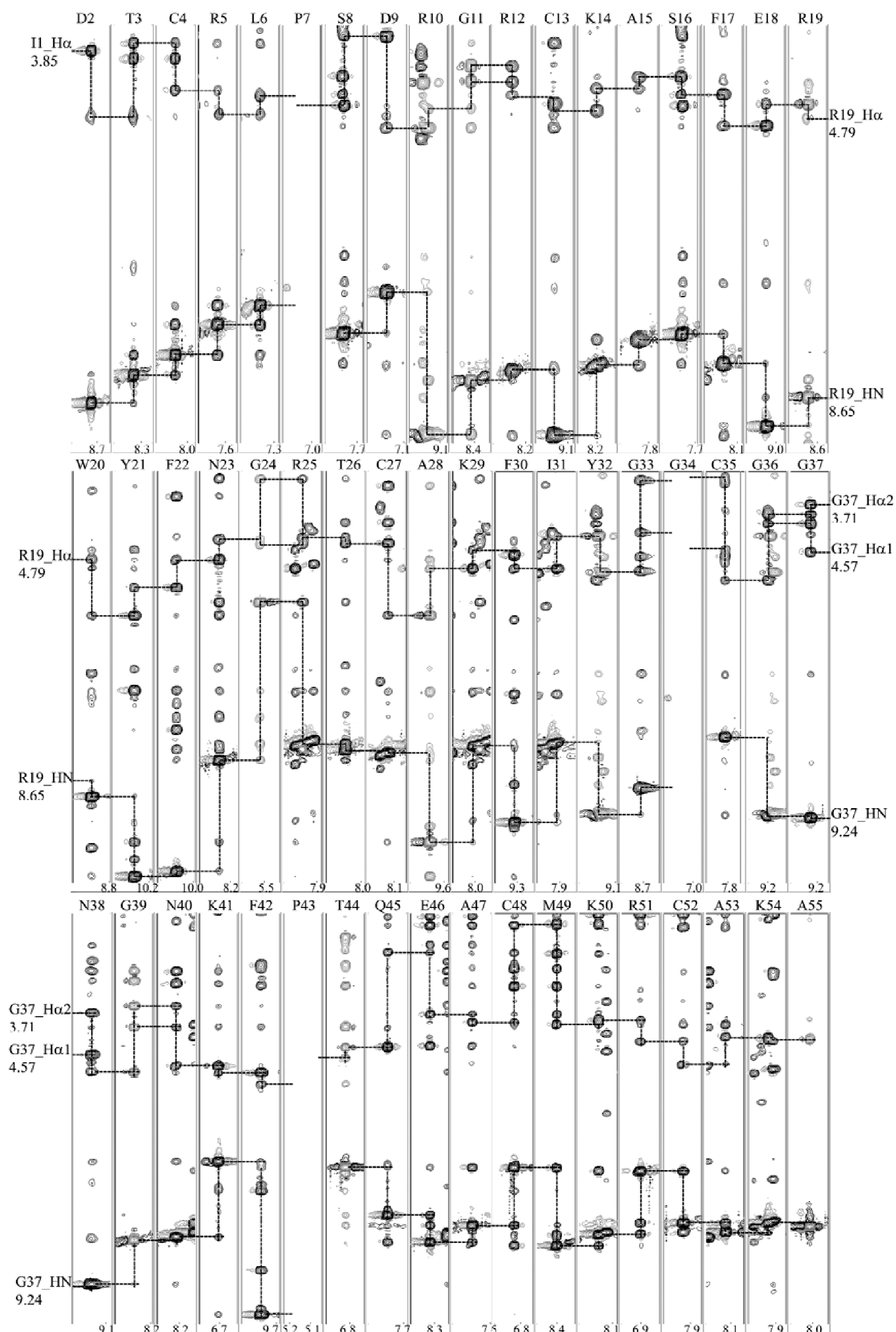

Fig. 3 Sequential assignment of huwentoxin-XI by 3D ^{15}N-^{1}H NOESY-HSQC

Nearly complete $d_{\alpha N}$ and d_{NN} sequential connectivity was established throughout the sequence of HWTX-XI except for I1, P7, G34 and P43.

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Fig. 4 2D ^{15}N-^{1}H HSQC spectrum of huwentoxin-XI

Amide NH assignments are annotated with the one-letter amino acid code and the sequence number. Assignments of side-chain NH and NH_2 groups are also shown.

^{15}N-^{1}H NOESY-HSQC spectra for confirmation (**Fig. 7**).

The chemical shifts were obtained for nearly all backbone nitrogens/protons and more than 95% of the side-chain protons of individual residues in HWTX-XI. The results are shown in **Table 2**.

Hydrogen bond and secondary structure analysis

Slow-exchanging amide protons in HWTX-XI were identified by redissolving lyophilized ^{15}N-HWTX-XI sample in pure D_2O and analyzing the remaining cross-peaks in a series of 2D ^{15}N-^{1}H HSQC spectra recorded thereafter (**Fig. 8**), from which 29 hydrogen bonds were confirmed in accordance with the secondary structure analysis. Secondary structure analysis of HWTX-XI was carried out by screening in the 200 ms NOESY spectrum for characteristic NOE connectivity patterns of β-sheet and α-helix with the aid of $^{3}J_{HN\text{-}H\alpha}$ coupling constants calculated from the 3D ^{15}N-^{1}H HNHA spectrum. The results indicate that HWTX-XI mainly consists of an N-terminal 3_{10}-helix from Thr3 to Arg5 and a C-terminal α-helix from Gln45 to Cys52, plus a triple-stranded anti-parallel β-sheet of Glu18-Asn23, Thr26-Ile31 and Asn40-Lys41.

Discussion

To date, those spider toxins with their structures elucidated fall into two main structure motifs, the inhibitor cysteine knot (ICK) motif and the disulfide-directed β-hairpin (DDH) motif. It is generally accepted that the ICK motif originates from the DDH motif [11,12]. The disulfide connection pattern in the DDH motif is I-III, II-V, IV-VI whereas the ICK motif takes the I-IV, II-V, III-VI pattern, a major difference of the two structure motifs. Based on the observation of unambiguous NOE cross-peaks between β protons of Cys4 and Cys52, Cys13 and Cys35, Cys27 and Cys48, the disulfide bridge pattern of HWTX-XI was confirmed as I-VI, II-IV, III-V, which is consistent with the conserved disulfide connection patterns of the Kunitz/BPTI inhibitor family and totally different from either the ICK or the DDH motif.

As HWTX-XI is so different from all known spider toxins, it would be of biological importance to investigate how it has evolved and what the structural basis is for its dual bioactivities. The current work achieved nearly complete chemical shift assignments of HWTX-XI and

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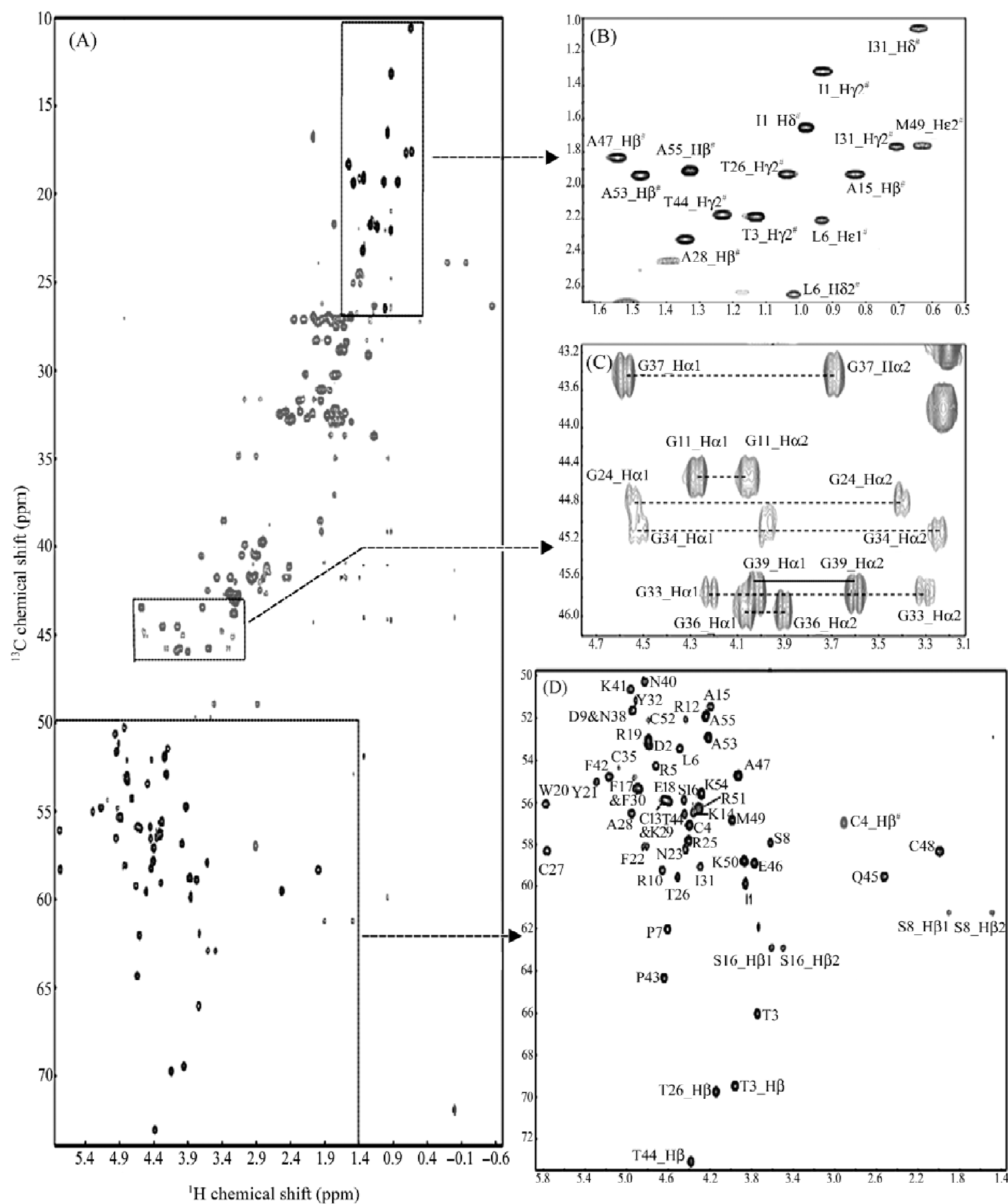

Fig. 5 Resonance assignments for short side-chain residues in huwentoxin-XI using 2D ^{13}C-^{1}H HSQC spectrum

(A) Full spectrum of 2D ^{13}C-^{1}H HSQC. (B) -CH3 region of ^{13}C-^{1}H HSQC. (C) Gly-CH2 region ^{13}C-^{1}H HSQC. (D) -CH region of ^{13}C-^{1}H HSQC. Assignments of all α protons, β protons of Ser and Thr, methyl protons of Ala, Thr, Ile, Leu and Met are shown with the one-letter amino acid code and the sequence number. # overlapping proton resonances.

provided a solid basis for its structure elucidation, which is a pre-requisite for future detailed structure-function relationship study. Furthermore, the ^{15}N-labeling protocols for disulfide-rich peptide toxins used in our work illustrate an effective solution toward structure elucidation of such toxins and might be applicable to similar proteins.

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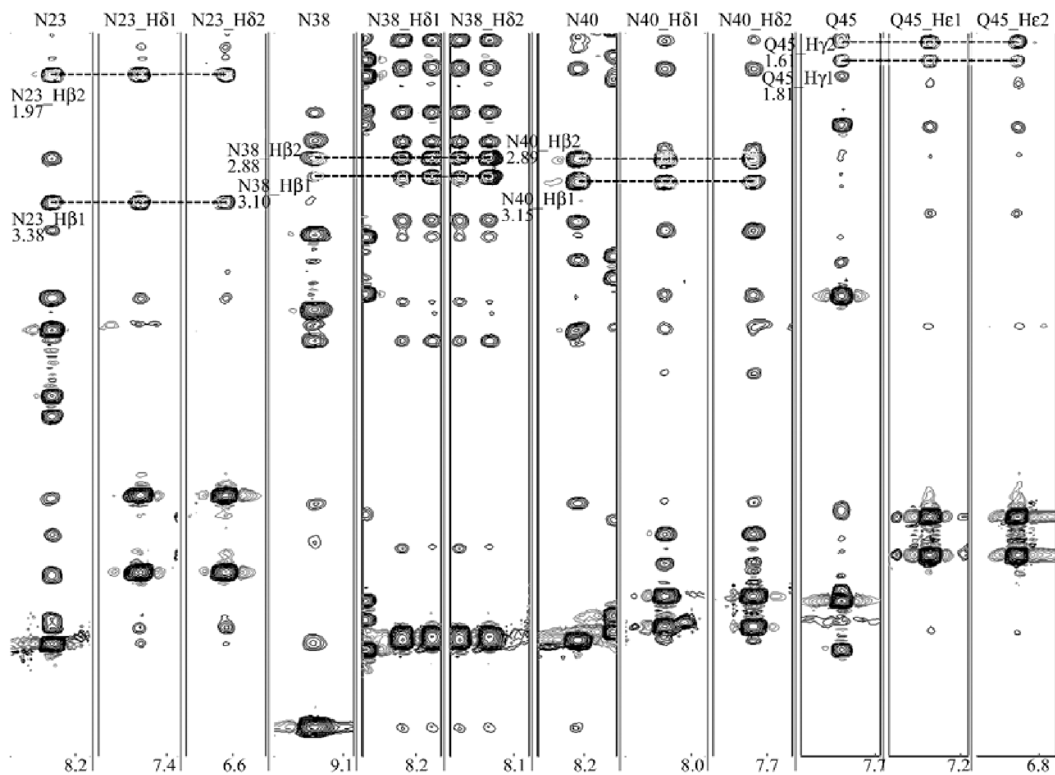

Fig. 6 Side-chain -NH_2 assignments in huwentoxin-XI by 3D ^{15}N-^{1}H NOESY-HSQC

Shared cross-peaks (H_N-H_γ for Gln, H_N-H_β for Asn) at the amide HN and side-chain HN positions are shown by the dashed lines.

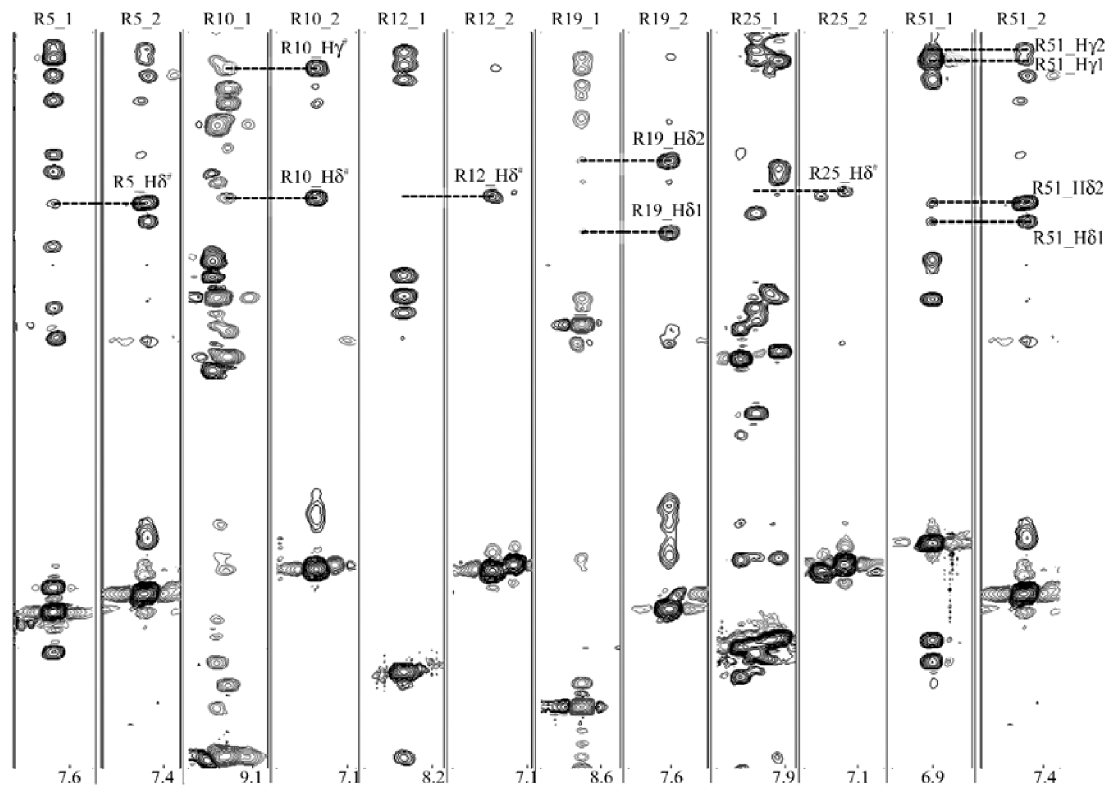

Fig. 7 Side-chain -NH assignments in huwentoxin-XI by 3D ^{15}N-^{1}H NOESY-HSQC

Shared H_N-H_δ or H_N-H_γ cross-peaks of Arg residues are shown by the dashed lines. # overlapping proton resonances.

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Table 2 Chemical shifts of the assigned ^{15}N, ^{1}H resonances of huwentoxin-XI

Residue	HN	^{15}N	Hα	Hβ	Hγ	Hδ	Hε	Hζ
I1			3.85	1.94	1.53, 1.24; 0.93†	0.97†		
D2	8.73	125.39	4.77	2.76†				
T3	8.34	121.31	3.73	3.96	1.12†			
C4	8.07	114.51	4.38	2.95†				
R5	7.64	113.28	4.7	2.19, 1.73	1.62†	3.29†	7.44	
L6	7.38	121.59	4.48	1.88, 1.58	1.9	1.01, 0.93		
P7			4.59	2.27, 1.95	2.06, 1.84	3.78, 3.56		
S8	7.76	113.64	3.63	1.90, 1.51				
D9	7.19	119.02	4.93	2.72, 2.40				
R10	9.15	110.15	4.65	2.21, 2.06	1.85†	3.22†	7.18	
G11	8.42	123.01	4.29, 4.06					
R12	8.27	115.82	4.45	1.97, 1.81	1.68, 1.64	3.2†	7.19	
C13	9.17	119.83	4.65	3.36, 2.88				
K14	8.2	116.98	4.36	2.08, 1.60	1.40, 1.30	1.60†	2.96†	7.46
A15	7.85	123.69	4.2	0.84†				
S16	7.77	113.69	4.45	3.62, 3.50				
F17	8.18	122.36	4.89	3.06†		7.07†	7.32†	
E18	9.05	122.65	4.56	2.15, 1.85	2.36, 2.01			
R19	8.65	109.79	4.79	1.73, 0.98	1.05, 1.29	3.61, 2.84	7.61	
W20	8.89	118.2	5.76	3.07, 2.84		Hδ1=6.75	Hε1=10.27	
					Hε3=7.10; Hζ3=7.07; Hη2=7.21; Hζ2=7.52			
Y21	10.28	122.48	5.29	2.95, 2.79		7.06†	6.68†	
F22	10.19	124.62	4.81	3.70, 2.90		7.51†	7.21†	7.29
N23	8.26	124.77	4.43	3.38, 1.97		7.47, 6.62		
G24	5.52	125.52	4.54, 3.39					
R25	7.97	119.38	4.39	1.76, 1.64	1.52†	3.16†	7.14	
T26	8.08	113.02	4.51	4.14	1.04†			
C27	8.13	119.46	5.76	3.51, 2.88				
A28	9.68	111.53	4.95	1.33†				
K29	8.02	119.41	4.63	1.16†	0.11, –0.16	1.26†	2.77, 2.71	7.47
F30	9.32	120.37	4.87	3.01, 2.90		7.06†	7.18†	
I31	7.92	119.13	4.29	1.75	1.43, 1.18; 0.71†	0.64†		
Y32	9.18	110.22	4.91	2.48, 2.41		6.70†	7.71†	
G33	8.72	113.95	4.23, 3.31					
G34	7.71	108.87	4.51, 3.23					
C35	7.82	115.35	5.08	3.84, 2.98				
G36	9.2	109.97	4.07, 3.91					
G37	9.24	113.64	4.57, 3.71					
N38	9.17	119.83	4.92	3.10, 2.88		8.24, 8.16		
G39	8.29	118.41	4.02, 3.59					
N40	8.22	123.02	4.81	3.15, 2.89		8.07, 7.74		
K41	6.71	119.44	4.94	1.68, 1.49	1.09, 0.99	1.22, 0.50	2.95,2.62	7.09
F42	9.78	123.63	5.15	3.19, 2.74		7.29†	6.82†	
P43			4.63	2.46, 2.25	2.33, 2.20	4.05, 4.00		
T44	6.83	122.22	4.4	4.4	1.23†			
Q45	7.79	121.79	2.52	1.17, –0.54	1.81, 1.61		7.28, 6.86	
E46	8.33	117.69	3.76	1.99, 1.79	2.39, 2.36			
A47	8	121.83	3.91	1.55†				
C48	6.84	117.45	1.99	3.22, 2.85				
M49	8.4	121.98	3.97	1.96, 1.91	2.54, 2.06		0.63†	
K50	8.17	119.17	3.87	1.75, 1.71	1.61†	1.47, 1.37	2.93†	7.48
R51	6.9	115.49	4.31	1.91, 1.77	1.72, 1.63	3.47, 3.28	7.4	
C52	7.94	112.75	4.78	2.02, 1.79				
A53	8.13	123.22	4.21	1.48†				
K54	7.94	119.03	4.28	1.85, 1.72	1.38†	1.66†	2.98†	7.49
A55	8.02	109.98	4.2	1.33†				

† stands for overlapping proton resonances from -CH_2, -CH_3 and sidechain protons of Tyr/Phe.

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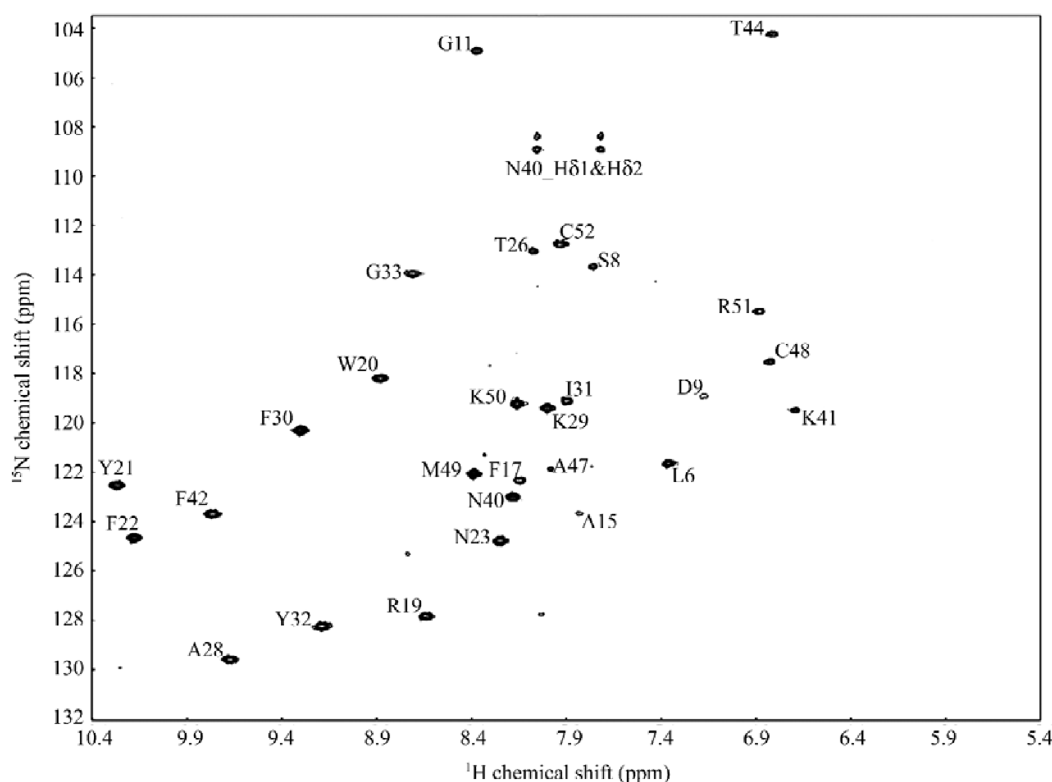

Fig. 8 Hydrogen-deuterium exchanging 2D ^{15}N-^{1}H HSQC spectrum of huwentoxin-XI

Cross-peaks remaining after hydrogen-deuterium exchanging for 20 h are annotated with the one-letter amino acid code and the sequence number.

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