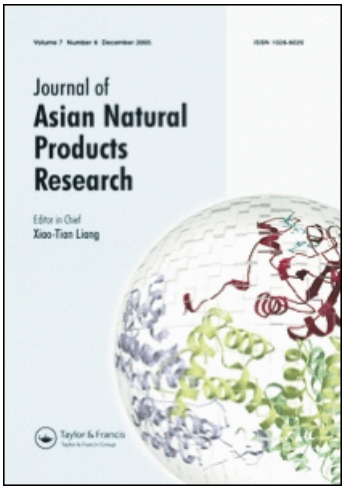

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^{1}H-NMR signal assignments and secondary structure analysis of martentoxin

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^{1}H-NMR signal assignments and secondary structure analysis of martentoxin

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Martentoxin is a peptide of 37 amino acid residues purified from the venom of the Chinese scorpion Buthus martensi Karch, which has been demonstrated to be an inhibitor of voltage-dependent sodium channel and voltage-dependent delayed rectifier potassium channel. To elucidate the molecular mechanism of this interaction, the structure of martentoxin was studied by 2D-NMR. The secondary structure of martentoxin consists of a triple-stranded β-sheet connected to a α-helical structure. This helix encompasses 10 residues from Ser11 to Lys20. The three strands of β-sheet probably comprise residues Gly2-Asp5, Q27-N30 and Glu33-Cys36, Cys30-Asn33 with a type I′β turn centered on Asn31-Asn32. The results indicate that martentoxin possesses the conserved β α β β structure of all the potassium channel toxins.

Keywords: Scorpion venom; Martentoxin; 2D-NMR; Sequential assignment; Secondary structure analysis

1. Introduction

The peptides derived from the venom of scorpions have been classified into two distinct families according to their primary structures and biological function: the first one with four disulfide bridges contains 60–70 amino acid residues and is active against sodium channel [1,2]; the second is composed of 29–40 amino acids containing three or four disulfide bridges which interact specifically with potassium, calcium or chloride channels [3–6]. These scorpion toxins are essential tools to study the interactions between peptide blockers and ion channels [7]. Despite their difference in number of amino acid residues, mode of action and functional site, the three-dimensional structures present a general motif: a short α-helix and a two- or three-stranded β-sheet, stabilized by three or four disulfide bridges.

One novel peptide named martentoxin was purified and identified from *Buthus martensi* Karch. Martentxion consists of 37 amino acid residues, and it shares high sequence similarities with Lgh15-1 that are closely related to the CTX family [8]. In a previous study,

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martentoxin was proved to be the inhibitor of the voltage-dependant sodium channel and voltage-dependent delayed rectifier potassium channel [8]. In order to improve our knowledge of structure-function relationships, the structure of martentoxin was studied by NMR methods. In this paper, the result of sequential assignment and secondary structure analysis is reported.

2. Results and discussion

2.1 Sequential assignment

The sequential assignments of resonances of martentoxin were accomplished in two steps (spin system identification and sequential assignment) according to the general procedure described by Wüthrich. The cross-peaks between HN and H_α were assigned by examinations of DQF-COSY spectrum. The side-chain spin systems were identified on the basis of the TOCSY spectra.

2.1.1 Spin system identification. The four Gly residues were first recognized unambiguously with DQF-COSY in H_2O. Ala, Thr and Val residues also were easily distinguished from other residues on the basis of the strong intensity and narrow width peaks in the methyl group region. In the fingerprint region of the TOCSY spectrum (298K, H_2O, 120 ms), the assignments of these residues were mostly straightforward. The H_α of Val residue has cross-peaks with H_β and two CH_3-γ. The Thr residue has cross-peaks with the H_β and only one CH_3-γ. The remaining methyls belong to the unique Ile, Leu. Therefore, the Leu3, Ile4 were identified according to the patterns of their cross-peaks in TOCSY and DQF-COSY. Ser11, Ser12 were distinguished from the other AMX spin systems on the basis of their two H_β in the lower field region of the TOCSY, while Asn was identified using intra-residual NOE cross-peaks between its two H_β and NH_δ protons. The aromatic protons of Trp5 and Tyr37 were identified with the DQF-COSY and TOCSY in D_2O. The H_δ of Lys residues has cross-peaks with NH, while H_ϵ of Arg residues has cross-peaks with NH in TOCSY spectra in H_2O. Figure 1 shows the fingerprint of DQF-COSY spectrum of

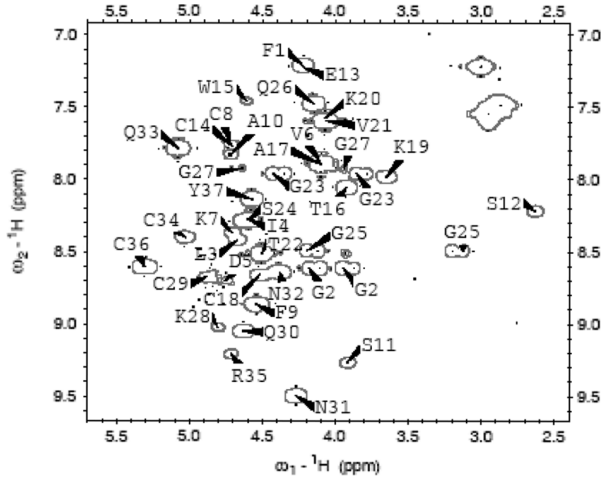

Figure 1. Fingerprint region of DQF-COSY spectrum of martentoxin (H_2O, pH = 3.6, 298K).

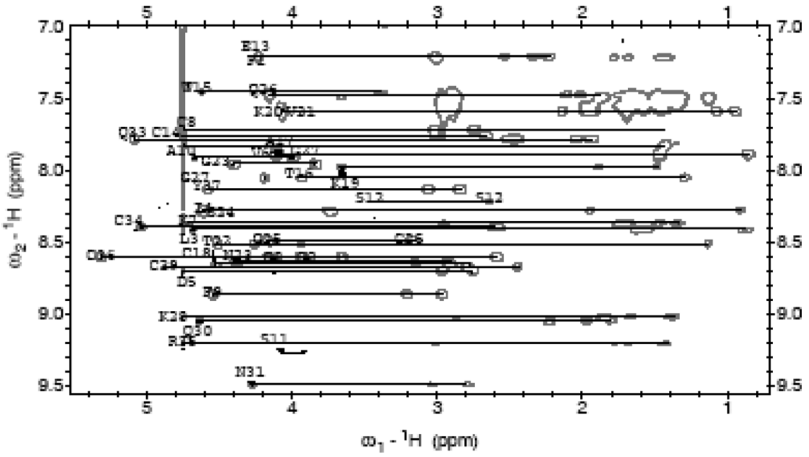

Figure 2. Fingerprint region of TOCSY spectrum of martentoxin with spin-lock time of 120 ms (H_2O, pH = 3.6, 298K).

martentoxin and figure 2 shows the fingerprint of TOCSY spectrum of martintoxin with a mixing time of 120 ms.

2.1.2 The sequential assignment. The spin systems were connected in sequence by virtue of HN-HN$_{i,i+1}$ and H$_\alpha$-HN$_{i,i+1}$, connectivities in NOESY spectra (figures 3 and 4). Starting from Thr, Gly was linked to it on the basis of one medium NOE cross-peak, and the unique two spin systems Thr22-Gly23 adjacent was identified. The connectivities were extended up to Gln26, which was assigned using H$_\gamma$-HN, and down to Lys19 (each assignment was confirmed by either medium H$_\alpha$-HN$_{i,i+1}$, strong HN-HN$_{i,i+1}$ and weak H$_\beta$-HN$_{i,I+1}$ correlation peaks). From Gln26, medium H$_\alpha$-HN$_{i,i+1}$ connectivity offers the connectivities up to Lys 28 which

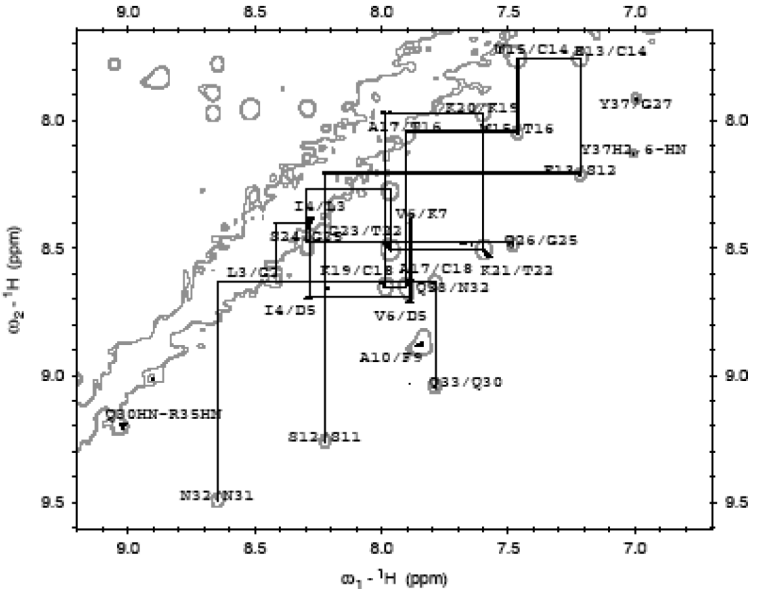

Figure 3. HN-HN$_{i,i+1}$ region of NOESY spectrum of martentoxin with mixing time of 140 ms (H_2O, pH = 3.6, 298K).

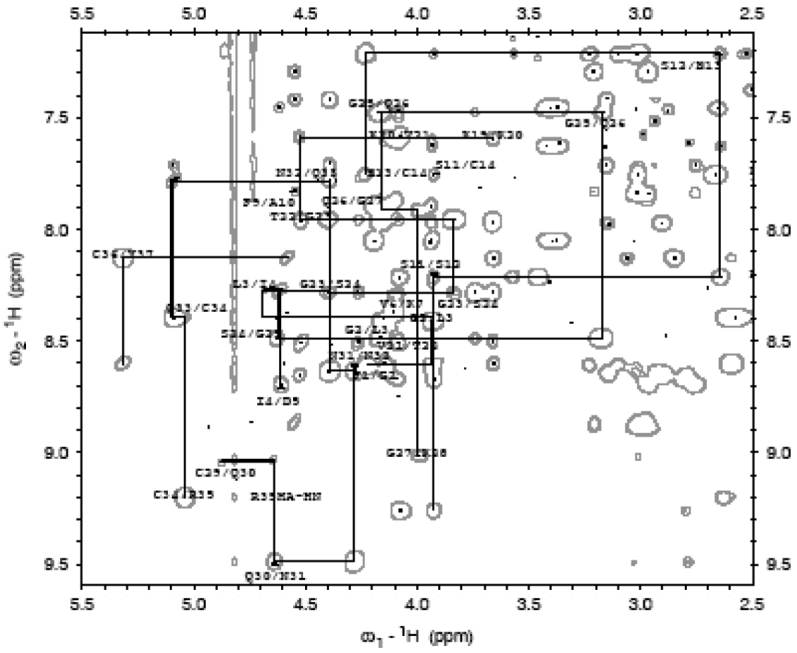

Figure 4. H_α-$HN_{i,i+1}$ region of NOESY spectrum of martentoxin with mixing time of 140 ms (H_2O, pH = 3.6, 298K).

was assigned by NH-H_δ cross-peak in TOCSY. The sequential assignments were extended from Lys19 to Cys14 by strong HN-$HN_{i,i+1}$ and weak H_β-$HN_{i,i+1}$. Using medium H_α-$HN_{i,i+1}$ and strong HN-$HN_{i,i+1}$ cross-peaks, the connection is extended down to Ser11. The unique Ile4 was linked to Gly2 through Leu3 and to Asp5 through medium H_α-$HN_{i,i+1}$ and weak HN-$HN_{i,i+1}$. Val6 was connected using NOE cross-peak between its HN and HN of the Asp5 residue. A long chain spin system had weak H_α-$HN_{i,i+1}$ and HN-$HN_{i,i+1}$ connectivity with Val6. This allows us to put Lys7 into the sequence. Next, starting from the other Ala10, we linked it down to Phe9 by a weak H_α-$HN_{i,i+1}$ connectivity. Note that Phe9 has medium H_β-HN $_{i,i+1}$ connectivity with one AMX spin system. The spin system was identified as Cys8. Lys7 was connected using H_β-HN $_{i,i+1}$ cross-peak with Cys8. A group of AMX-AMX-AM(PT)X spin systems Gln31-Asn32-Gln33 were connected in sequence by virtue of strong H_α-$HN_{i,i+1}$, HN-$HN_{i,i+1}$ and HN-CH of their side-chain cross-peaks. Gln30 was connected with Asn31 by a strong H_α-$HN_{i,i+1}$ connectivity. Starting from Gln32, we link it up to Arg35, and an AMX spin system Cys36 was assigned using medium H_β-$HN_{i,i+1}$, while Tyr37 was identified using weak H_α-$HN_{i,i+1}$ and H_β-$HN_{i,i+1}$. The last AMX spin system was assigned as Phe1. Phe1 has medium H_β-$HN_{i,i+1}$ with Gly2.

Finally, complete assignments of all the residues were obtained (figure 5, table 1).

2.2 Structure determination

2.2.1 Hydrogen bonds. The exchange rates of amide protons of martentoxin with the solvent were measured. Amide protons still present after 7 h of exchange were considered as an indication of engaging in hydrogen bonds. Most of the slowly exchanged amide protons occurred in regular secondary structures such as HN 14, 16 and 17 in helix and HN 29,30,32,33,34,35 in the β-sheet.

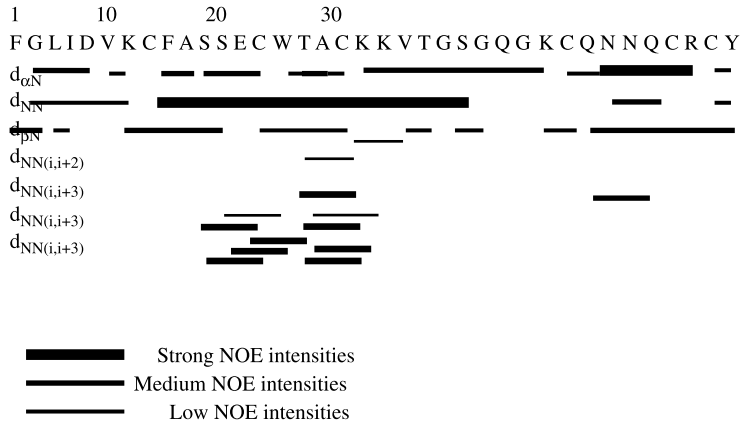

Figure 5. Summary of the sequential NOE connectivity's observed for martentoxin.

Table 1. ^{1}H Chemical shifts for martentoxin at pH 3.6 and 298K.

	Chemical shifts (ppm)			
Residue	*N-H*	*α-H*	*β-H*	*Others*
F1	7.21	4.21	3.10, 3.24	2,6 H: 7.21*; 3,5 H: 7.14*; 4 H: 7.39*
G2	8.61	4.14, 3.93		
L3	8.42	4.68	1.62	γH: 1.55, δCH3: 0.90,0.82
I4	8.29	4.60	1.94	γCH2: 1.13, 1.23; γCH3: 0.91; δCH3: 0.75
D5	8.71	4.75	2.95, 2.76	
V6	7.89	4.11	1.48	γCH3: 0.86
K7	8.38	4.71	1.47	γCH2: 1.34; δCH2: 1.68, 1.73; ϵCH2: 2.95; ϵNH3: 7.565
C8	7.71	4.78	3.01, 2.74	
F9	8.867	4.55	3.20, 2.97	2,6 H: 7.29*; 3,5 H: 7.39*; 4 H: 7.25*
A10	7.83	4.72	1.43	
S11	9.27	3.92	4.07	
S12	8.217	2.643	3.46, 3.58	
E13	7.21	4.21	2.23	γCH2: 2.54, 2.34
C14	7.76	4.74*	3.00, 2.66	
W15	7.45	4.61	3.39, 3.30	2H: 6.99; 4H: 7.63; 5H: 7.15; 6H: 7.25; 7H: 7.47; NH: 9.93
T16	8.05	3.93	4.18	γCH3: 1.29
A17	7.90	4.12	1.47	
C18	8.66	4.52	3.13, 2.89	
K19	7.98	3.66	1.89, 1.87	γCH2: 1.59; δCH21.48; ϵCH2: 2.90; ϵNH3: 7.49
K20	7.59	4.04	1.93	γCH2: 1.57; δCH2: 1.68; ϵCH2: 2.96; ϵNH3 + : 7.58
V21	7.59	4.07	2.15	γCH3: 1.07, 0.95
T22	8.51	4.51	4.26	γCH3: 1.13
G23	7.96	4.40, 3.88		
S24	8.29	4.62	3.75	
G25	8.49	4.16, 3.18		
Q26	7.47	4.16	1.72, 1.65	γCH2: 2.11, 2.02; δNH: 7.33, 6.75
G27	7.92	3.98, 4.68		
K28	9.02	4.81	1.85	γCH2: 1.41, 1.38; δCH2: 1.67; ϵCH2: 2.87; ϵNH3 + : 7.48
C29	8.67	4.88	2.79, 2.44	
Q30	9.04	4.63	1.96, 1.79	γCH2: 2.22; δNH: 6.89, 7.51
N31	9.50	4.27	3.03, 2.78	γNH2: 7.61, 6.93
N32	8.64	4.38	3.14, 2.91	γNH2: 7.41, 6.97
Q33	7.78	5.08	2.03, 1.93	γCH2: 2.50, 2.45; δNH: 7.38, 6.81
C34	8.40	5.03	2.56, 2.61	
R35	9.21	4.72	1.77, 1.67	γCH2: 1.45, 1.41; δCH2: 3.00
C36	8.61	5.32	3.65, 2.59	
Y37	8.14	4.58	3.06, 2.85	2,6H: 7.00; 3,5H: 6.64

*Values obtained from spectra recorded in 99.96% D_2O at 298K.

Table 2. Coupling constants and Δppm of $^1H_\alpha$ of martentoxin comparing with random coil.

	Chemical shift (ppm) of αH			*$3J_{NH\text{-}\alpha H}$ (Hz)*
	Martentoxin	*Random coil*	*Δppm*	
F1 residue	4.21	4.66	−0.45	7.0
G2	4.14, 3.93	3.94, 3.91	0.20, 0.03	10.0
L3	4.68	4.32	0.36	5.5
I4	4.60	4.20	0.40	8.5
D5	4.75	4.61	−0.14	
V6	4.11	4.13	−0.02	
K7	4.71	4.27	0.44	
C8	4.78*	4.94	−0.16	9
F9	4.55	4.66	−0.11	10.5
A10	4.72	4.26	0.46	
S11	3.92	4.51	−0.59	5.0
S12	2.643	4.51	−1.87	5.0
E13	4.21	4.25	−0.04	6.0
C14	4.74	4.94	−0.20	
W15	4.61	4.77	−0.16	
T16	3.93	4.48	−0.55	5.0
A17	4.12	4.26	−0.14	
C18	4.52	4.94	−0.42	6.5
K19	3.66	4.27	−0.61	4.5
K20	4.04	4.27	−0.23	
V21	4.07	4.13	−0.06	
T22	4.51	4.48	0.03	10
G23	4.40, 3.88	3.94, 3.91	0.46, −0.03	11.5
S24	4.62	4.51	0.11	8
G25	4.16, 3.18	3.94, 3.91	0.22, −0.73	11.5
Q26	4.16	4.29	−0.13	7.5
G27	3.98, 4.68	3.94, 3.91	0.04, 0.77	10
K28	4.81	4.27	0.54	10
C29	4.88	4.94	−0.06	
Q30	4.63	4.29	0.34	10.5
N31	4.27	4.70	−0.43	7.0
N32	4.38	4.70	−0.32	7.0
Q33	5.08	4.29	0.79	10.0
C34	5.03	4.94	0.09	10.0
R35	4.72	4.29	0.51	10.0
C36	5.32	4.94	0.36	9.5
Y37	4.58	4.63	−0.05	8.0

2.2.2 Coupling constants. Twenty-seven $^3J_{HN\alpha}$ values were obtained, as listed in table 2 and converted into ϕ angle constraints.

2.3 Secondary structure

Analysis of the sequential and medium-range NOE intensities together with the chemical shifts index (CSI) [9] and coupling constant values (table 2) helped us to predict the secondary structure of martentoxin. The presence of strong sequential HN-HN NOEs together with medium H_α-$HN_{i,i+3}$ and H_β-$HN_{i,i+3}$ connectivity and small $^3J_{HN\alpha}$ coupling constants suggests the presence of a helical conformation formed by residues 10−20. Three stretches of strong sequential H_α-HN NOES indicated three extended regions running from residues 2 to5, 27 to30 and 33 to36. HN_i-HN_j connectivity was identified between 2 to 5, 27 to 30 and 33 to 36, with decreased intensities. This was confirmed by the relatively large coupling constants. The chemical shifts of H_α exhibit negative shift with respect to the random coil values in postulated helical region and positive shifts in postulated β-strand

regions. The β-sheets 27 to 30 and 33 to 36 of are linked together by a type I′β turn (30–33), centered on residues Asn31-Asn32. The type of turn was proved with the hydrogen bond between the 30 and 33 residues, the strong NOE intensities of $d_{NN(31,32)}$ and $d_{NN(32,33)}$ and $^{3}J_{HN\alpha}$ coupling constants of Asn31 (7 Hz) and Asn32(6.5 Hz).

2.4 Conclusion

Secondary structure of martentoxin was studied by 2D-NMR techniques. The spin systems were identified on the basis of both TOCSY and DQF-COSY spectra in H_2O and D_2O. The sequential assignment of martentoxin was completed by NOESY spectra. The conformation of martentoxin composed of an α-helix (Ser^{11}-Lys^{20}) and three strands of β-sheet (Gly^{2}-Asp^{5}, Q^{27}-N^{30}, Glu^{33}-Cys^{36}). Q^{30} to N^{32} form a type I′β turn linking the last two strands. From this study it was concluded that matentoxin possesses the conserved β α β β structure of all potassium channel toxins.

3. Experimental

3.1 Sample preparation

Martentoxin was purified from the venom of *Buthus martensi* Karch and characterized as described previously [8]. The purified peptide was dissolved to a final concentration of 3.5 mM in 500 μL of H_2O/D_2O (90/10 mixture, V/V. pH 3.6). After a set of NMR experiments, the sample was lyophilized and dissolved in 99.96% D_2O.

3.2 NMR spectroscopy

All NMR measurements were performed on a Varian INOVA-500 spectrometer, and Z-axis gradients were used. The experiments were performed at 298K. Two-dimensional DQF-COSY, TOCSY and NOESY spectra were acquired both in H_2O and D_2O. Typically, 512 free induction decays of 4096 data points were collected per experiment. The spectral width was set to 5228.8 Hz. TOCSY [10] spectra were recorded using the MLEV-17 pulse with spin-lock times of 50, 80, 120 ms, respectively, and NOESY [11] spectrum were acquired with mixing time of 70, 140 and 210 ms, respectively. For the determination of H-D exchange rates, a series of 1D spectra during the first 3 h followed by two TOCSY spectra ($\tau_m = 60$ ms 5 h) were recorded at 298K started immediately after the sample was dissolved in D2O. Presaturation was used to suppress the water peak in all the experiments.

3.3 Data processing

Spectra were processed with VNMR 6.1B software. The matrixes were transformed to a final size of 8192 point in both dimensions. The signal was multiplied by a shifted sine bell window in both dimensions before Fourier transformation.

3.4 Spectral analysis

Spin system identification and sequential assignments were performed using the Wüthrich's strategy [12], aided with the Sparky software.

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