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Disulfide Isomers of α-Neurotoxins from King Cobra (*Ophiophagus hannah*) Venom

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Two novel α-neurotoxins, Oh-6A and Oh-6B, isolated from the king cobra (*Ophiophagus hannah*) venom, consist of 70 amino acid residues with 10 cysteine residues and share the same amino acid sequences as determined by Edman degradation on the peptide fragments generated from the proteolytic hydrolysates. Their sequences share 46–53% homology with Oh-4, Oh-5, Toxin a, and Toxin b from the same venom. The finding that Oh-6A and Oh-6B had different retention times in the reversed-phase column suggested that the two toxin molecules should not have the same conformation. Selective reduction on the disulfide bond, Cys26—Cys30, at the tip of their loop II structures resulted in the production of the partially reduced derivatives eluted at the same position. Under redox conditions, the partially reduced Oh-6A and 6B exclusively converted into native Oh-6A as evidenced by HPLC analyses. This suggests that Oh-6A and Oh-6B are disulfide isomers which probably arise from *cis–trans* isomerization of the Cys26—Cys30 disulfide bond. Alternatively, the two toxins exhibited binding activity toward nAChR and lethal toxicity equally. It reflects that the diversity around the extra loop at the loop II structure does not exert a significant effect on the manifestation of the neurotoxicity of Oh-6A and Oh-6B. © 1999 Academic Press

α-Neurotoxins bind specifically to nicotinic acetylcholine receptors (nAChR) on the postsynaptic membrane of skeletal muscles to block the neuromuscular transmission. Approximately 100 α-neurotoxins from *Elapid* and *Hydrophid* snake venoms have been sequenced and they can be structurally classified into two major groups, short and long neurotoxins (1–3). X-ray crystallography of erabutoxin b (4, 5), α-cobratoxin (6), and α-bungarotoxin (7, 8) has provided a model of three-dimensional structures. The sequence homology and common models of toxic action of α-neurotoxins imply that they all possess a similar overall folding, but differ in details such as the extent of secondary structure and positioning of a conserved side chain.

The king cobra, found in southeastern Asia and southern mainland China, belongs to the *Ophiophagus* genus, and is supposed to be the largest poisonous snake in the world. However, fewer studies have been performed on the α-neurotoxins in this venom compared with those obtained from other cobras of the *Naja* genus. In 1973, Joubert first purified and sequenced two long toxins, Toxin a and b, from this venom (9). Recently, six long α-neurotoxins had been isolated from this venom, and two of them, Oh-4 and Oh-5, had been sequenced (10, 11). The roles of the tryptophan residues and cationic groups in their neurotoxicity were also investigated (11–14). Surprisingly, the contribution of these residues to neurotoxicity of Oh-4 and Oh-5 were different from that noted with other long α-neurotoxins. To gain more insight into the structure-function correlation of various α-neurotoxins with similar tertiary structure, one of the ways is to isolate and characterize more toxin isoforms. Comparative studies on α-neurotoxin variants should reveal how structural differences among these toxins affect the functional diversities at the molecular level. In the present study, two novel toxic components, Oh-6A and Oh-6B from the *Ophiophagus hannah* were subjected to sequence and characterization. However, it was surprising to find that the two α-neurotoxins shared the same amino acid sequences, but differed in the retention time on reversed-phase column. Selective reduction on the disulfide bond at the tip of their loop II structure resulted in the production of partially reduced derivatives eluted at the same position. These results suggest that the two neurotoxins are disulfide isomers which could be cis-trans isomerization of one disulfide bond.

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Abbreviations used: DABIA, *N*-(4-dimethylaminoazobenzene-4′)-iodoacetamide; nAChR, nicotinic acetylcholine receptor; RCM, reduced and S-carboxymethylated.

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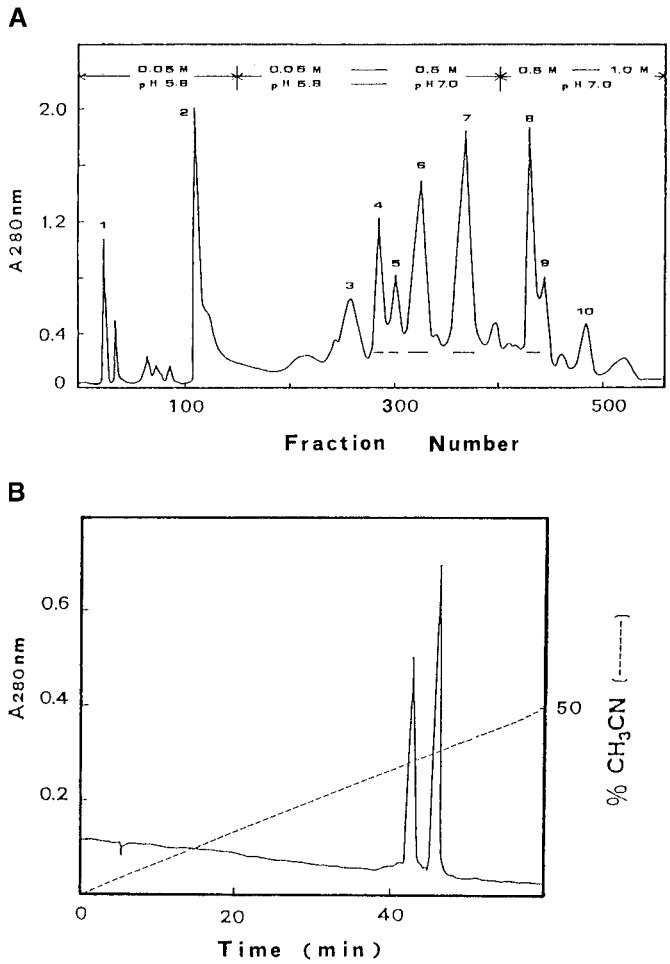

FIG. 1. (A) Chromatography of *Ophiophagus hannah* crude venom on a SP-Sephadex C-25 column. The lyophilized venom (1g) was dissolved in 10 ml 0.05 M ammonium acetate buffer (pH 5.8) and applied to the column (2.2 × 95 cm) previously equilibrated with the same buffer. After the column had been washed with 800 ml of the buffer, the proteins were eluted with a two-stage linear gradient as indicated in the figure. Five ml per fractions was collected and pooled as indicated by bar. (B) Purification of Oh-6A and 6B by reversed-phase HPLC. The major fraction (Oh-6) in A was further separated by a CM-52 column. The fraction, collected from CM-52 column, was chromatographed on a SynChropak RP-P column (0.46 × 25 cm). The column was equilibrated with 0.1% TFA and the proteins were eluted with a linear gradient of 20–60% acetonitrile for 100 min at a flow rate of 1 ml/min.

MATERIALS AND METHODS

Trypsin and *Saccharomyces aureus* V_8 protease were purchased from Sigma Chemical Co. Acetonitrile and trifluoroacetic acid (TFA) were obtained from E. Merck, lysyl endopeptidase was from Wako, Japan, and *N*-(4-dimethylaminoazobenzene-4′)-iodoacetamide (DABIA) was purchased from Fluka, Switzerland. SynChropak RP-P column (0.46 × 25 cm) was purchased from SynChrom Inc. and TSK gel ODS-120T (0.46 × 25 cm) column was obtained from Toyo Soda, Japan. All other reagents were of analytical grade.

Isolation and purification of Oh-6A and 6B. Oh-6A and 6B were isolated from king cobra venom (*Ophiophagus hannah*) according to the procedure described by Chang *et al.* (10). One gram crude venom dissolved in 10 ml of 0.05 M ammonium acetate buffer (pH 5.8) was applied to a column of SP-Sephadex C-25 (2.2 × 95 cm) equilibrated with the same buffer, followed by two linear gradients of 1600 ml ammonium acetate buffer; initially with 0.05 M (pH 5.8) to 0.5 M (pH 7.0), then by 0.5–1.0 M (pH 7.0) gradient. The sixth main fraction (Oh-6) were further purified by chromatography on a CM-52 column with a gradient of 1000 ml, from 0.07 M (pH 5.8) to 0.6 M (pH 7.0) ammonium acetate. Two α-neurotoxins, Oh-6A and 6B, were further separated by reversed-phase HPLC on a SynChropak RP-P column with a linear gradient of 20–60% acetonitrile for 100 min. The flow rate was 1.0 ml/nm and the eluent was monitored at 280 nm.

Protein digestion and sequence analysis. The toxins were reduced and S-carboxymethylated (RCM) according to the procedures described by Crestfield *et al.* (15), followed by proteolytic digestion. RCM-protein (1 mg) was digested with trypsin or V_8 protease in 1 ml of 0.1 M ammonium bicarbonate (pH 8.0), or with lysyl endopeptidase in 0.01 M Tris buffer (pH 9.0) for 6 h at 37°C, at a substrate/enzyme ratio of 50:1 (w/w). The hydrolysates were separated by reversed-phase HPLC on a Toyo Soda ODS-120T column (0.46 × 25 cm) equilibrated with 0.1% TFA as shown in the figure legends of Fig. 2. The peptides were lyophilized for determination amino acid composition and sequence.

Labeling sulfhydryl groups of partially reduced toxin with DABIA. Toxin was reduced with two-fold molar excess of DTT in 0.1 M Tris buffer (pH 8.6) containing 0.1% EDTA, and the reaction was allowed to proceed for 40 min. Then the sample was desalted through a PD-10 column (Pharmacia Biotech) equilibrated with 0.1 M acetic acid and subjected to lyophilization. The sulfhydryl group of partially reduced toxin was labeled with DABIA according to the procedure described by Chang *et al.* (16). Toxin was dissolved in 600 μl of 0.2 M Tris–HCl buffer (pH 8.4) containing 5 M guanidine chloride and was incubated with 600 μl of DABIA solution (0.5 mg/ml) in dimethyl formamide. Labeling of protein was conducted for 1 h at room temperature in the dark with magnetic stirring. The sample was acidified with 30 μl of TFA, and the excess reagent was removed through a PD-10 column eluted with 50% acetic acid, DABIA labeled protein was subjected to reduction and S-carboxymethylation of the remaining disulfide bonds and followed by enzymatic digestion.

Oxidation of partially reduced toxins. The partially reduced Oh-6A and 6B were dissolved in 50 mM sodium bicarbonate (pH 9.6) containing 2 mM GSH/0.5 mM GSSG, respectively. The reaction mixtures allowed to oxidize at 25°C for 2 h, then immediately mixed with an equal volume of 4% TFA. The samples were further desalted

TABLE 1

Lethality and Binding Activity to nAChR of α-Neurotoxins Isolated from *Ophiophagus hannah* Venom

Preparation	Lethal toxicity[a] LD_{50} (μg/g)	Binding activity[b] IC_{50} (nM)
Crude venom	0.49	ND
Oh-4	0.25	0.23 (0.33)
Oh-5	0.22	0.16 (0.26)
Oh-6A	0.17	0.06 (0.08)
Oh-6B	0.14	0.06 (0.08)
Oh-7	0.14	0.05 (0.08)
Oh-8	0.11	0.06 (0.07)

Note. ND, not determined.

[a] Measured i.p. with mice weighing 18 ± 1 g.

[b] Calculated from the molar concentration to induce the 50% inhibition of [^{125}I]-Oh-7 binding to the nAChR in duplicate determinations. The data in parentheses were obtained from the inhibition of [^{125}I]-Oh-8.

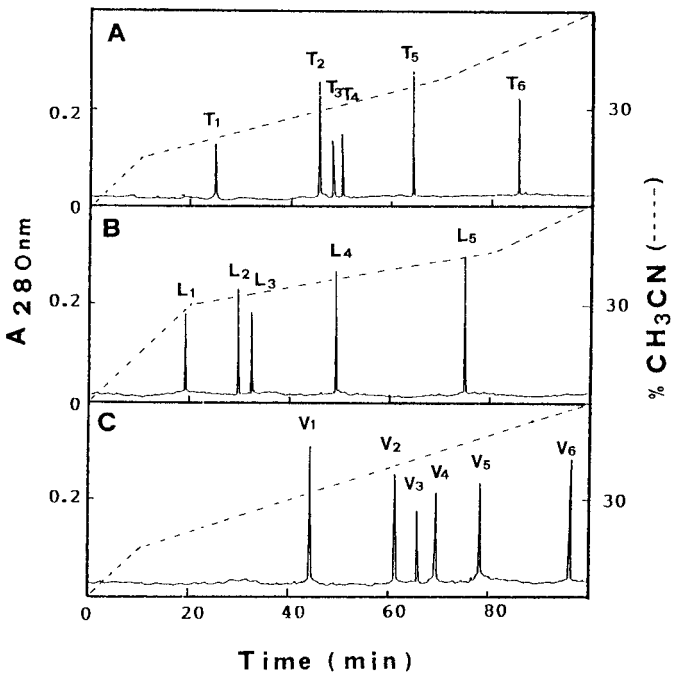

FIG. 2. Separation of protease-digested RCM-Oh-6A and RCM-Oh-6B. The hydrolysates of RCM-Oh-6A and RCM-Oh-6B showed the identical chromatographic profile. (A) Trypsin; (B) Lysyl endopeptidase; (C) *S. aureus* V_8 protease. The peptides were applied on a TSK-gel ODS-120T column (0.46 × 25 cm) at a flow rate of 1 ml/min, and eluted with a linear gradient of acetonitrile as shown by dotted lines.

through a PD-10 column equilibrated with 0.1 M acetic acid. After lyophilization, the samples were analyzed by native gel electrophoresis and HPLC.

Other tests. Lethal toxicity and nicotinic acetylcholine receptor (nAChR) binding measurement assay were carried out in the same manner as previously described (17–19).

RESULTS AND DISCUSSION

Isolation and purification of Oh-6A and 6B. Figure 1A showed the elution pattern of the crude venom on a SP-Sephadex C-25 column, and the fractions indicated by bars (fraction Oh-4 to Oh-8) exhibited high lethal toxicity as assessed by LD_{50} on the mice. The Oh-6 was further purified on a Sephadex G-50 column and followed by a CM-52 column (data not shown). Two fractions, Oh-6A and Oh-6B, were separated by reversed-phase HPLC on a SynChropak RP-column (Fig. 1B). The yields of Oh-6A and Oh-6B represented 0.9 and 1.8% of the total venom proteins, respectively. The results of SDS–polyacrylamide gel electrophoresis showed that two toxins were homogenous and had a molecular mass of about 8 kDa (data not shown). Moreover, the two toxins exhibited a nAChR-binding activity (Table 1) as did other neurotoxins from *Elapidae* family. Apparently, the Oh-6A and Oh-6B are neurotoxic proteins.

Amino acid sequence of Oh-6A and 6B. The N-terminal sequence of RCM-Oh-6A and RCM-Oh-6B were determined up to 20 residues, revealing that they had the same N-terminal amino acid sequences. The RCM-toxins were further digested with trypsin, lysyl endopeptidase and *S. aureus* V_8 protease, respectively. The hydrolysates of RCM-Oh-6A and RCM-Oh-6B were separated by a reversed-phase column. Chromatographic profiles of the two RCM-toxins were essentially the same: six tryptic peptides (T_1 to T_6), five lysyl endopeptidase-digested peptides (L_1 to L_5), and six V_8 protease-digested peptides (V_1 to V_6) were separated (Fig. 2). Based on the results of amino acid composition and sequence determination of these peptides, the complete amino acid sequence of Oh-6A and Oh-6B were constructed (Fig. 3). It was found that both Oh-6A and Oh-6B had the same primary structure, and consisted of 70 amino acid residues with 10 cysteine residues. As shown in Fig. 4, Oh-6A and Oh-6B shared approximately 46–54% sequence identity with Oh-4, Oh-5, Toxin a and Toxin b from the same venom (9–11), α-cobratoxin from *Naja naja siamensis* (20), α-bungarotoxin from *Bungarus multicinctus* (21), and Toxin A from *Naja naja* (22).

Isomerization of Cys26—Cys30 of Oh-6A and Oh-6B. Recent study showed that *N. naja atra* cobrotoxin exhibited a tendency to isomerize its disulfide bonds at

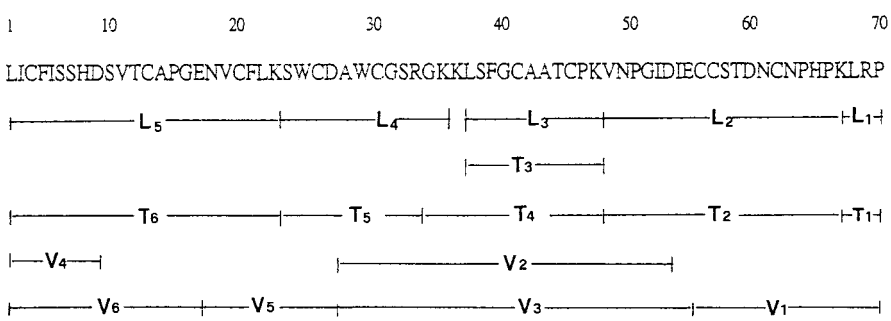

FIG. 3. The complete amino-acid sequence of Oh-6A and Oh-6B. The peptides derived from the hydrolysates of RCM-toxins which were digested with trypsin, lysyl endopeptidase and *S. aureus* V_8 protease, respectively. The designations of peptides are the same as those shown in Fig. 2.

α-Neurotoxin	1 10 20 30 40 50 60 70	% identity
	+ +. + + + + + +	
Oh-6A(6B)	LICF--ISSHDSVT--CAPGENVCFLKSWCDAWCGSRGKKLSFGCAATCPKVNPGIDIECCSTDNCNPHPKLRP	100
Oh-5	TK.Y--KTGDRIISEA.P..QDL.YM.T...VF..T..RVIEL..T....T.K.HEQ.T.......D..H.MLQ	46
Oh-4	TK.Y--KTGDRIISEA.P..QDK.YM.T...VF..T...VIEI.......T.K.HEQ.T...........NKMQ	50
Toxin a	TK.Y--VTPDV-KSQT.PA.QDI.YTE.......T....RVNL.......I.K..VE.K.........F.TW.KRP	53
Toxin b	TK.Y--VTPDV---ET.PD.QDI.YT.....GF.S....RIDL.......K.K..V..K.........F.TWKRKH	51
α-Cobratoxin	IR..--.TPDI-TSKD.PN.H-..YT......F.SI...RVDL.......T.KT.V..Q.........F.TRKRP	54
α-BuTX	IV.HTTAT.PI-SAVT.P..E.L.YR.M....F.S....VVEL.......SKK.YEEVT.....K......Q..G	54
Toxin A	IR..--.TPDI-TSKD.PN.H-..YT.T...GF.SI...RVDL.......T.RT.V..Q.....D.D.F.TRKRP	49

FIG. 4. Comparison of amino-acid sequence of Oh-6A (6B) with those of other long-chain α-neurotoxins. Oh-4, 5, Toxin a and Toxin b from *Ophiophagus hannah* venom (Refs. 9–11); α-cobratoxin from *Naja naja siamensis* venom (Ref. 20); α-bungarotoxin from *Bungarus multicinctus* venom (Ref. 21); Toxin A from *Naja naja* venom (Ref. 22), respectively. Residues identical to that in the top line were designated with a dot, gaps are marked with hyphens.

the C-terminus (23, 24). Although the disulfide isomers had the same primary structure, they differed in the gross conformation and the retention time in reversed-phase column (23). Thus, it was probable that the isomerization reaction occurred between Oh-6A and Oh-6B as well. Under the conditions of limited DTT reduction, Oh-6A and 6B could be partially reduced with two-fold molar excess of DTT in 0.2 M Tris buffer (pH 8.6) containing 0.1% EDTA at room temperature. HPLC analyses showed that the partially reduced derivatives of Oh-6A and 6B were eluted at the same retention time (data not shown). To determine the positions of selective reduced disulfide bond(s), the sulfhydryl groups of partially reduced Oh-6A and Oh-6B were subjected to labeling with DABIA. After the remaining disulfide bonds were completely reduced and S-carboxymethylated, DABIA-labeled proteins were hydrolyzed with lysyl endopeptidase. The hydrolysates were further separated by reversed-phase HPLC. Comparison of the resulting chromatographic profile with those of the hydrolysates of RCM-toxins (Fig. 2B) showed that, instead of L_4 peptide, a DABIA-labeled peptide appeared. The results of amino acid analysis and sequence determination indicated that DABIA-peptide, corresponding to extra loop region (Ser24–Lys35) of Oh-6A and Oh-6B, had the same amino acid composition and sequence. The sulfhydryl groups of partially reduced derivatives were further allowed to oxidize in 50 mM sodium bicarbonate (pH 9.6) containing 2 mM GSH/0.5 mM GSSG at 25°C for 2 h. The products of the oxidized mixtures were analyzed by reversed-phase HPLC and gel electrophoresis. It showed that the partially reduced Oh-6A and Oh-6B exclusively converted into native Oh-6A. However, the observation that oxidation of partially reduced Oh-6B could not form native Oh-6B under the experimental condition, is likely to reflect that some factors, such as protein disulfide isomerase (25), govern the formation of Oh-6B in venom glands of king cobra. These clearly indicate that the different retention time in reversed-phase column noted with Oh-6A and 6B should be resulted from the Cys26—Cys30 disulfide linkage. One possible explanation for this structural heterogeneity could be cis-trans isomerization of the Cys26—Cys30 disulfide bond.

In summary, in the present study, two novel α-neurotoxin have been isolated from the king cobra. However, our results suggest that the two α-neurotoxins are disulfide isomers, which correlated with Cys26—Cys30 disulfide linkage. To our knowledge, this novel finding is the first report on the disulfide isomers which is probably produced by cis-trans isomerization of one disulfide bond.

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