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SEQUENCE CHARACTERIZATION OF A NOVEL α-NEUROTOXIN FROM THE KING COBRA (*Ophiophagus hannah*) VENOM[1]

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Several postsynaptic neurotoxins (α-neurotoxins) with distinct pharmacological and biochemical properties were isolated and purified from the King cobra venom (*Ophiophagus hannah*) by employing sequentially preparative-scale cation-exchange chromatography on SP-Sephadex C-25 coupled with gel filtration and reversed-phase HPLC. The complete sequence of one neurotoxin was determined by N-terminal Edman degradation with the automatic pulsed-liquid phase sequencer on some peptide fragments generated from the endopeptidases, *i. e.* trypsin, *S. aureus* V_8 protease and lysyl endopeptidase. This novel neurotoxin is a basic polypeptide of pI 9.05, consisting of 72 amino-acid residues with 10 cysteine residues. It is found to share about 60 % sequence homology with Toxins a and b isolated from the same venom and the well established α-bungarotoxin, a major postsynaptic toxic ligand for acetylcholine receptor isolated from *Bungarus multicinctus*. The characterized α-neurotoxin molecules were also shown to bind specifically with nicotinic acetylcholine receptors of the electric eel, *Torpedo californica*.

The King cobra of elapid family is supposedly the world's largest poisonous snake. Relatively fewer studies were done on the neurotoxins in this venom as compared with those obtained from some other cobras of the Naja genus. It is well known that the venoms of most elapid snakes generally contain components of three major categories based on their structures and activities, *i.e.* (A) phospholipases A_2 (B) neurotoxins and (C) cardiotoxins (or called cytotoxins) [1,2]. Extensive structural isoforms also exist amongst each class of toxins.

Joubert [3] first fractionated and sequenced two long-chain neurotoxins, designated as Toxins a and b from the King cobra venom. In this communication we have obtained an improved separation of venom toxins for a complete and unambiguous fractionation of crude venom. Moreover we have achieved the determination of one postsynaptic α-neurotoxin by the facile N-terminal sequence analysis of the intact toxin and its protease-digested fragments using a pulsed-liquid phase protein sequencer. The determined sequence is compared with some reported neurotoxins characterized from venoms of closely related cobras of Elapidae family.

[1]The protein sequence of the reported α-neurotoxin has been deposited in the SWISS-PROT sequence database and on EMBL Data Library under the accession number P80156.

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MATERIALS AND METHODS

Isolation and purification of venom toxins

Lyophilized venom of the King cobra snake was obtained from the southern region of mainland China. One gram was dissolved in 10 ml of 0.05 M ammonium acetate buffer, pH 5.8 and applied to a column of SP-Sephadex C-25 (2.2 x 95 cm) equilibrated with the same buffer. After washing the column with 800 ml of the same buffer, elution was carried out using two linear gradients of ammonium acetate buffer; initially with 0.05 M, pH 5.8 and gradually increased to 0.5 M, pH 7.0, followed by 0.5-1.0 M, pH 7.0 gradient. The major fractions having higher lethal toxicity for mice were pooled separately and purified by gel filtration on a Sephadex G-50 column. The main fractions were further purified by chromatography on a CM-52 column with a gradient of initial concentration of 0.05 M ammonium acetate, pH 5.8 to 0.3 M or 0.6 M ammonium acetate, pH 7.0. The final step of purification before sequencing work was carried out on a reversed-phase HPLC (RP-HPLC). Six α-neurotoxins designated as Oh-4, 5, 6A, 6B, 7 and 8 (**Fig. 1A**) were isolated in an apparent homogeneous form as shown by being single bands in SDS-gel electrophoresis. The percent yields for these components were 1.5, 0.9, 0.9, 1.8, 4.8 and 4.1 % as calculated from the final recovered dry-weights for each purified component.

Acetylcholine receptor binding assay

Preparation of the electric eel organs and nicotinic acetylcholine receptor (nAChR)-enriched membrane fragments from *Torpedo californica* was essentially according to the previous report [4]. The purified toxins were labeled with carrier-free $Na^{125}I$ according to chloramine-T method [5,6]. The toxin-binding activities of various purified toxins were expressed as the specific activities, *i.e.* nmols of ^{125}I-toxin bound to the membrane per mg of membrane protein. Lethal toxicity was based on LD_{50} (in μg toxin/g mouse) according to the method of Reed and Muench [7].

Gel electrophoresis

The purities of the isolated toxins were checked by SDS-polyacrylamide slab gel (5 % stacking/ 14 % resolving gel) as described [8] with some modifications (5 % crosslinking N,N'-methylenebisacrylamide in the gel solution).

Amino acid analysis

The amino-acid compositions were determined with a Beckman 6300 amino acid analyzer using a single-column system based on conventional ion-exchange chromatography system. The special rapid procedure for the preparation of protein hydrolysates using microwave irradiation before amino-acid analysis was essentially according to the previous reports [9,10]. The cysteine and tryptophan contents could be determined with accuracy using the nonvolatile solvent of 4 M methanesulfonic acid containing 3-(2-aminoethyl)indole (Pierce, Rockford, IL, USA) in place of 6 M HCl for the complete amino-acid analysis from a single protein hydrolysate [11].

Protein digestion and sequence analysis

The N-terminal sequences of the isolated peak fractions from HPLC column were carried out by automated Edman degradation with a pulsed-liquid phase protein sequencer (Model 477A, Applied Biosystems, Foster City, CA, USA). The samples each containing about 1-5 nmols of protein were dissolved in 100 μl of 0.1 % trifluoroacetic acid (TFA) and 5 μl each for sequence determinations. For the determination of complete sequences of various neurotoxins, the purified toxins were reduced with β-mercaptoethanol (MSH, 5 folds over toxins) and then reacted with iodoacetic acid (5 folds over MSH). The resulting reduced and carboxylmethylated (RCM-) proteins were then subjected to automated Edman degradation to obtain the N-terminal sequences of intact toxins and confirm the location of cysteine residues along the toxin chains. The RCM-proteins were also digested with trypsin, lysyl endopeptidase and *S. aureus* V_8 protease, and the digests were separated on RP-HPLC column (4.6 x 250 mm, Toyo Soda ODS-120T). The complete sequences for each toxin were obtained by overlapping the partial sequences determined from the isolated fragments of RP-HPLC. The C-terminal sequence was determined by carboxypeptidases A and B (either alone or in combination) digestion at 37 °C for 30 min, 60 min and 90 min in 0.1 M ammonium bicarbonate buffer, pH 7.8 and the released amino acids analyzed by amino-acid analysis.

Sequence comparison and hydropathy profile

A program analysis of the local hydrophilicity of Oh-4 neurotoxin and α-bungarotoxin along their amino-acid sequences based on the Kyte-Doolittle hydropathy scale [12] is carried out on the MacVector sequence analysis software for Macintosh computers (International Biotechnologies, Inc., New Haven, CT). However the signs of the values have been reversed

in order to plot the hydrophilicity instead of hydrophobicity scale. A window of size N=7 was run along the length of peptide segments; for each window, the hydropathy values of the 7 amino acids were summed and divided by 7 to obtain the average hydrophilicity per residue for the window. Values above the axis denote hydrophilic regions which may be exposed on the outside of the protein molecule whereas those values below the axis indicate hydrophobic regions which tend to be buried inside the protein.

RESULTS AND DISCUSSION

There have been various reports on the characterization of varied types of neurotoxins from the Elapidae family of snakes, notably in the Naja genus [1,2]. In contrast to a more thorough understanding of α- and β-neurotoxins from some elapid species, there is still a lack of clear and systematic classification of venom components isolated from some more exotic species of cobras, such as the large King cobra found in Southeastern Asia and China. In this study we have applied multiple-step chromatographies coupled with acetylcholine receptor binding assay for the isolation and characterization of some neurotoxic components from this cobra species. A detailed structural study of one novel α-neurotoxin is reported and its sequence and structure are compared with some well-known neuromuscular blocking toxins.

Isolation and purification of α-neurotoxins from the King cobra, (Ophiophagus hannah)

Fig. 1A shows the general elution pattern of the crude venom on an SP-Sephadex C-25 cation-exchange column. The fractions indicated by bars (Fractions Oh-4 to Oh-8) exhibited higher lethal toxicity as assessed by LD_{50} on the mice were further purified. To further purify **Oh-4**, a second purification step of the lyophilized fraction was performed on the Sephadex G-50 gel filtration, followed by another CM-52 cation-exchange chromatography (**Fig. 1B**). Final purification was done on a reversed-phase HPLC (**Fig. 1C**), resulting in an essentially pure toxin in a yield of about 1.5 %. The toxin showed one single band under denaturing SDS-gel conditions with a molecular mass of about 8 kDa (data not shown), corroborating that it is a single polypeptide chain without extra subunits. Since this protein shows comparable lethal activity (LD_{50} of 0.25 μg/ g mouse) (**Table 1**) and binding activity to nAChR (**Table 1, Fig. 2**) when compared with some neurotoxins characterized from the snakes of same Elapidae family, we have therefore carried out a sequence analysis on this toxin in the endeavor to make a systematic comparison with the well-known α-neurotoxins based on their structures.

Acetylcholine receptor binding activity of Oh-4

Preparation of the electric organs and nicotinic acetylcholine receptor (nAChR)-enriched membrane fragments from *Torpedo californica* was essentially according to the previous report [4]. The purified toxins were labeled with carrier-free $Na^{125}I$ according to chloramine-T method [5,6]. As shown in Table 1 the binding activities of various purified neurotoxins were found to correlate with the indices of lethal toxicity, LD_{50} (in μg toxin/g mouse). Oh-4 was actually found to be the weakest toxin among six neurotoxins purified in this report. Nevertheless it is still more toxic than the crude venom in terms of their respective LD_{50} on

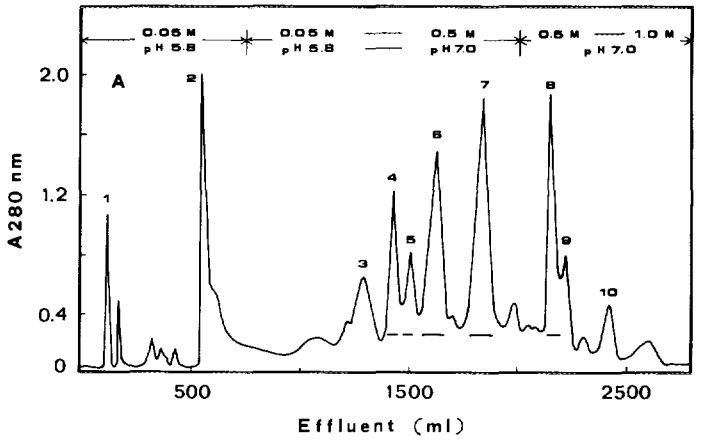

Fig. 1A. Chromatography of crude venom of *Ophiophagus hannah* on a SP-Sephadex C-25 column. The lyophilized venom (1 g) was dissolved in 10 ml of 0.05 M ammonium acetate buffer (pH 5.8) and applied to the column (2.2 x 95 cm) previously equilibrated with the same buffer. After the column had been washed with 800 ml of the buffer, the proteins adsorbed were eluted with a two-stage linear gradient as indicated in the figure. Each chamber contained 800 ml of elution buffers and the flow rate was 50 ml per hr.

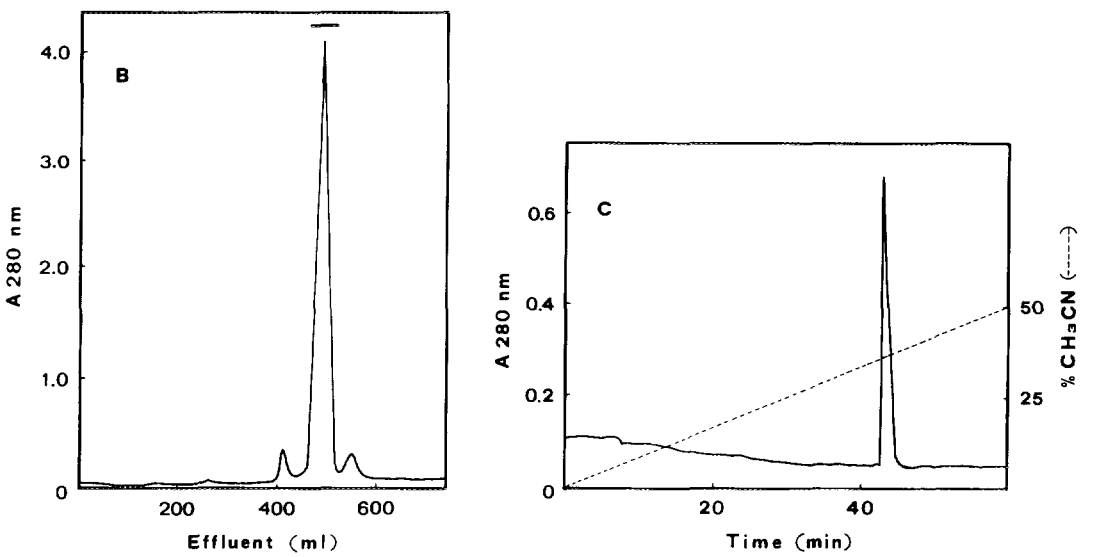

Fig. 1B. Chromatography of **Oh-4** on a CM-52 column. The fraction 4 in **Fig. 1A** was lyophilized, gel-filtrated on a Sephadex G-50 column and further purified on a CM-52 column (2.5 x 45 cm) with a linear gradient from 0.05 M, pH 5.8 to 0.3 M, pH 7.0 ammonium acetate buffer.

Fig. 1C. The final purification of **Oh-4** by RP-HPLC. The major fraction in **Fig. 1B** was lyophilized, desalted by Sephadex G-50 and chromatographed on a RP-HPLC column (TSK gel ODS-120 T, 0.46 x 25 cm). The column was equilibrated with 0.1 % TFA and the proteins adsorbed were eluted with a linear gradient of 0-50 % acetonitrile for 60 min at a rate of 1 ml/min.

Table 1. Lethality and binding activity to nAChR of α-neurotoxins isolated from *Ophiophagus hannah* venom

Preparation	Lethal toxicity* LD_{50} (μg/g)	Binding activity** 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)

* Measured i.p. with mice weighing 18 ± 1 g. ** 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. ND: not determined.

lethal toxicity tests. It is to be noted that acetylcholine-receptor binding assay should be a suitable and more sensitive alternative for toxicity tests on experimental animals regarding neurotoxicity studies of some animal and plant toxins.

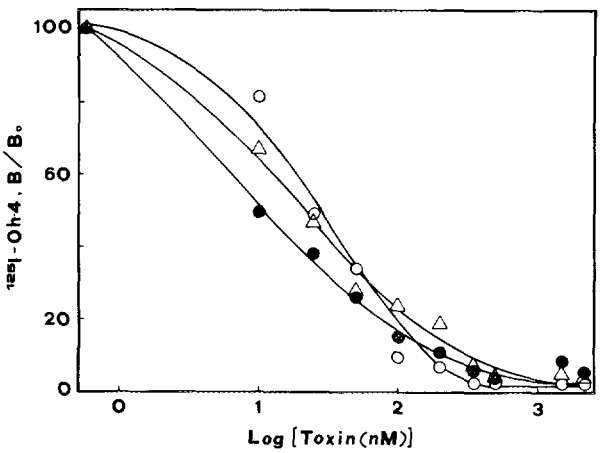

Fig. 2. Competitive binding experiments of Oh-toxins on nAChR with ^{125}I-Oh-4. B and B_0 are bound radioactivities in the presence and absence of unlabeled toxins, respectively. O, Oh-4; ●, Oh-7; Δ, Oh-8.

Amino-acid and sequence analysis of Oh-4

Intact Oh-4 protein was first directly sequenced without modification by sensitive pulsed-liquid phase protein sequencer. The chemically-modified RCM-Oh-4 was then subjected to automated Edman degradation to confirm the location of cysteine residues along the toxin chain. The RCM-toxin was also digested with trypsin, lysyl endopeptidase and *S. aureus* V_8 protease, and the resulting peptides were separated by RP-HPLC as shown in **Fig. 3**. Four tryptic peptides (T_1 to T_4), six peptides derived from lysyl endopeptidase (L_1 to L_6) and four overlapping peptides (V_1 to V_4) derived from V_8 protease digestion were obtained. In order to definitely determine the actual size and C-terminal residue of this toxin, C-terminal sequencing by carboxypeptidases A and B (either alone or in combination) was carried out to complement automatic N-terminal sequencing analysis. It was found that glutamine had been released first, followed by lysine and methionine in agreement with the residues 70-72 determined for the $4L_3$ fragment. From the amino-acid compositions (data not shown) and sequence analyses (**Table 2**) of isolated peptides, the complete amino-acid sequence of Oh-4 is presented in **Fig.**

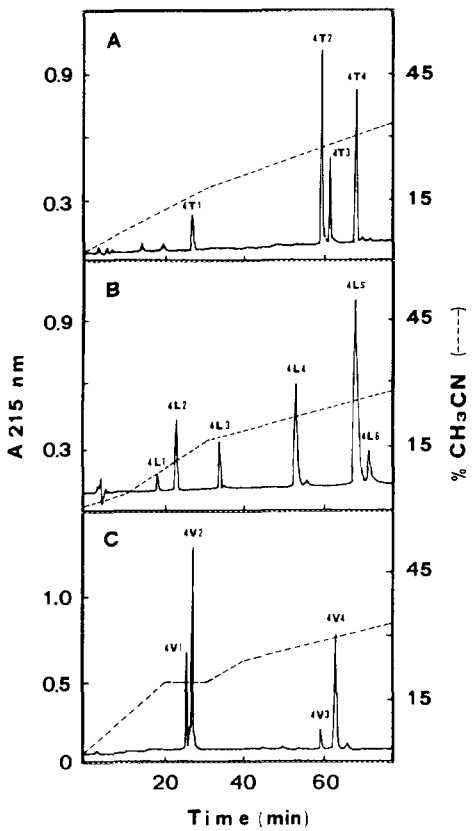

Fig. 3. Separation of protease-digested peptides of **RCM-Oh-4** by RP-HPLC. A. Trypsin; B. Lysyl endopeptidase; C. *S. aureus* V8 protease. The peptides were chromatographed on a TSK-gel ODS-120T column (0.46 x 25 cm, Toyo Soda) at a rate of 1 ml/min with a linear gradient of 0-50 % CH_3CN in 0.1% TFA as shown by a dotted line.

Table 2. Amino-acid sequences of the proteolytic peptides derived from RCM-Oh-4

Peptide	Sequence	Position
4T2	VIELGCTATCPTVKPHEQITCCSTDNCNPHPK	38-69
4T3	IISEACPPGQDLCYMK	10-25
4T4	TWCDVFCGTR	26-35
4V1	TKCYKTGDRIISE	1-13
4V2	LGCTATCPTVKPHEQICCSTDNCNPHPKMKQ	41-72
4V4	ACPPGQDLCYMKTWCDVFCGTRGRVIE	14-40
4L3	MKQ	70-72
4L4	TGDRIISEACPPGQDLCYMK	6-25

The listed peptide fragments correspond to those isolated from RP-HPLC (Fig. 3) and were subjected to automated Edman degradation to obtain the N-terminal sequences. For the fragment 4L3, C-terminal sequence analysis was carried out by carboxypeptidases A and B (either alone or in combination) to complement the N-terminal sequence analysis.

4. Oh-4 belongs to the long-chain α-neurotoxin consisting of 72 amino-acid residues with 10 half-cystine residues. The preliminary determination of N-terminal sequences of Oh-7 and Oh-8 up to the 30th residues suggests that they are probably identical to the Toxins a and b reported by Joubert [3], respectively. Oh-4 did not cross-react immunologically with Oh-7 and Oh-8 (data not shown). In **Table 3**, the amino-acid sequence of Oh-4 is compared with those of Toxins a and b as well as other well-established long α-neurotoxins from the snakes of same or different genera. It is found that Oh-4 sequence differs by 30 and 26 amino-acid residues from those of Toxins a and b, respectively. A degree of homology ranging from 56 to 62% between Oh-4 and α-bungarotoxin from *Bungarus multicinctus* [13,14], α-cobratoxin from *Naja naja siamensis* [15] and Toxin A from *Naja naja* [16] is also observed.

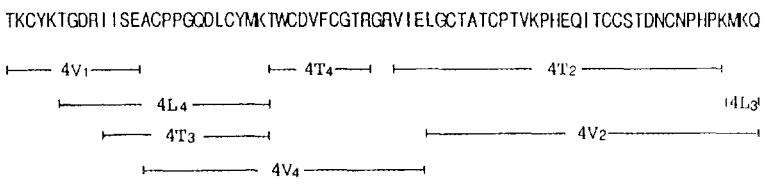

Fig. 4. The complete amino-acid sequence of **Oh-4**. The peptides derived from varied digestions with trypsin, lysyl endopeptidase and *S. aureus* V_8 protease correspond to those listed peptides in Table 2.

Table 3. Comparison of amino-acid sequences of some long-chain α-neurotoxins from snake venoms

α-Neurotoxin	---------10--------20--------30--------40--------50--------60--------70----	% Homology
1. Oh-4	TKCY KTGDRIISEACPPGQDLCYMKTWCDVFCGTRGRVIELGCTATCPTVKPHEQITCCSTDNCNPHPKMKQ	100
2. Toxin a	TKCY VTPD-VKSQTCPAGQDICYTETWCDAWCTSRGKRVNLGCAATCPIVKPGVEIKCCSTDNCNPFPTWRKRP	58
3. Toxin b	TKCY VTPD-ATSQTCPDGQDICYTKTWCDGFCSSRGKRIDLGCAATCPKVKPGVDIKCCSTDNCNPFPTWKRKH	64
4. α-Bungarotoxin	IVCHTTATSP-ISAVTCPPGENLCYRKMWCDAFCSSRGKVVELGCAATCPSKKPYEEVTCCSTDKCNPHPKQRPG	62
5. α-Cobratoxin	IRCF ITPD-ITSKDCPNGH VCYTKTWCDAFCSIRGKRVDLGCAATCPTVKTGVDIQCCSTDNCNPFPTRKRP	57
6. Toxin A	IRCF ITPD-ITSKDCPNGH VCYTKTWCDGFCSIRGKRVDLGCAATCPTVRTGVDIQCCSTDDCDPFPTRKRP	56

Sequences listed were taken from 1.*Ophiophagus hannah* (Oh), this report; 2. and 3. Oh (ref. 3); 4. *Bungarus multicinctus* (ref. 13, 14); 5. *Naja naja siamensis* (ref. 15); 6. *Naja naja* (ref. 16).

*Hydropathy profiles and structural comparison of **Oh-4** and α-bungarotoxin*

In the pair-wise comparison of Oh-4 primary sequence with those published α-neurotoxins in the data banks using software package (DNASTAR Inc., Madison, WI, USA), sequence homology ranging from 55-65 % was found, which is quite low when compared with sequence similarity found between some orthologous toxins (such as several cardiotoxin and phospholipases A_2 isoforms) isolated from some related species of Elapidae family. Therefore it would be of interest to compare the general distribution of surface-charge groups in these functionally related toxins using the popular program analysis of the local hydrophilicity in these sequences based on the Kyte-Doolittle hydropathy scale [12]. **Fig. 5** shows the hydropathy profiles and predicted secondary-structures for Oh-4 and α-bungarotoxin along the whole polypeptide chains. It is quite noteworthy that they are all devoid of α-helical structures and the overall profiles share a great similarity for the distribution of hydrophilic amino acids along the polypeptide chains despite the medial sequence homology between the two (only 62 %). They show similar hydrophilic profiles except the N-terminal amino-acid segments, which may partly account for the low homology found between these two toxins. Especially noteworthy is the highly hydrophobic profile revealed in the N-terminal 10 amino-acid segment of α-bungarotoxin, in contrast to an exposed and relatively hydrophilic amino-terminal region in Oh-4. Further conformational comparison by computer graphics based on the known X-ray structure of α-neurotoxin may be able to shed light on some subtle differences among these structurally and functionally related toxins.

In conclusion we have purified and established one of the primary sequences of 6 postsynaptic α-neurotoxins isolated from the King cobra. It is very rare that distinct isoforms of long-chain neurotoxins are present in a single species of cobras such as these multiple neurotoxins reported here. The detailed structural analysis and comparison of these sequences with published neurotoxins may provide some insights into the molecular basis for the divergence and variation of various isotoxins from the same or closely related snake species.

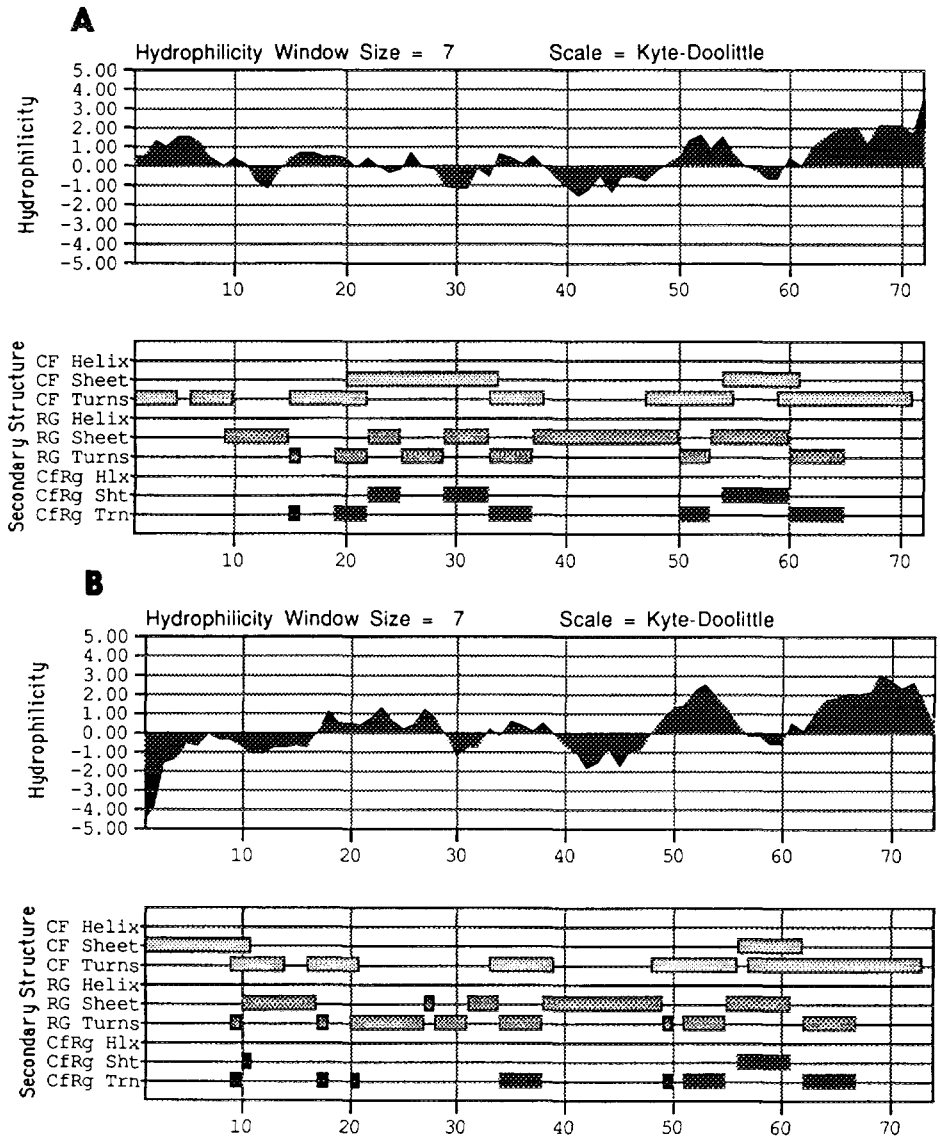

Fig. 5. Hydropathy profiles and secondary-structure predictions for **Oh-4** (A) and α-bungarotoxin (B). The methods for the analysis of the local hydrophilicity and conformational analyses of each structural segments (α-helix, β-sheet and β-turns) along the protein sequences were based on the methods of Kyte and Doolittle [12], Chou and Fasman (CF) [17] and Garnier *et al.* (RG) [18] and a consensus joint result (CfRg).

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