# Separation of Cobra (Naja naja naja) Venom proteins by Reverse Phase High Performance Liquid Chromatography

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**Summary:** Separation of various proteins from venom of cobra (Naja naja naja) has been achieved by reverse phase high performance liquid chromatography. These proteins include neurotoxins, cytotoxins and phospholipase. Some low and high molecular mass proteins were also separated. The proteins were identified by their biological activities, mobility on SDS-PAGE and N-terminal amino acid sequence analysis.

## Introduction

Snake venom is a complex mixture of various toxic and non toxic proteins. The toxic nature of venom is due to the collective action of all these proteins i.e. neurotoxins, cytotoxins, cardiotoxins, enzymes like phospholipases, protease inhibitor, acetyl cholinestrase etc. Most of the major proteins have been characterized from several species of snakes including cobra.

The traditional methods employed in the separation of venom proteins/peptides include gel filtration, multiple ion exchange chromatography, electrofocusing and reverse phase high performance liquid chromatography (RP-HPLC) [1-7]. In all separation procedures reported till date, multiple separation steps are required for obtaining pure fractions.

In this paper, we present a single step RP-HPLC method for the separation of cobra venom proteins. This method allows the separation of both major and minor protein components from crude venom.

*Materials and Methods:*

The snake was obtained from Thatta, Sind (North West of Karachi) and duly identified. The venom was extracted by squeezing the poison glands in deionized water, lyophilized and kept at -30°C.

The HPLC system used during the study was from JASCO, Japan; consisting of a twincle pump, a gradient programmer model GP A-40, a variable wavelength UVIDEC-100-II for measurement of absorbance and a recorder model RC-250. A Vydac C-18 reverse phase analytical column (Phenomenex, New York, USA) with a pre column (TP 5000, 7.5 x 75 mm from LKB Bromma) was used for separation. The solvents trifluoroacetic acid (TFA) and acetonitrile used were of HPLC grade.

*Separation of venom proteins:*

About 5 mg of lyophilized venom, dissolved in 0.5 ml 0.1% TFA, was subjected to a reverse phase Vydac $C_{18}$ column using a linear gradient

of 0.1% TFA in water (A) and acetonitrile containing 0.1% TFA (B). The absorbance was monitered at 214 nm.

*Biological Activities of separated fractions:*

The peaks separated on RP-HPLC were evaluated for the presence of neurotoxins, cytotoxins and phospholipases by methods reported in the literature [5-11].

*SDS-Polyacrylamide gel electrophoresis (SDS-PAGE):*

SDS-PAGE was carried out in 12.5% and 15% gels according to the method of Laemmli (12).

*N-Terminal amino acid sequencing:*

The N-terminal amino acid sequences of the fractions obtained were carried out by manual DABITC method and DABTH-amino acids were identified by thin layer chromatography [13].

## Results

*Separation of Venom:*

The separation pattern of the crude venom is shown in fig.(1). About 4 major and 7 minor peaks were obtained which were lyophilized and used for further characterization.

*Characterization of separated peaks:*

The neurotoxic activity was found in peak 3, while cytotoxic activity was present in peak 5 and 6. The phospholipase activity was found in peak 4, as determined according to the method of De Haas et al. (11).

*SDS-Polyacrylamide gel electrophoresis:*

The SDS-PAGE of separated peaks from RP-HPLC (Fig.1) is shown in figure (2). These show the presence of proteins in the molecular mass range of 3- 60 kDa. The peak corresponding to neurotoxic activity (peak 3 fig.1) and cytotoxic activity (peak 5 and 6 fig.1) showed a molecular mass range of 3-7 kDa (Fig.2), except that for peak 6 which was slightly contaminated with a 28 kDa protein. Similarly the phospholipase-A containing peak (peak 4 fig.1) showed the presence of a 14 kDa protein and a low molecular mass 3 kDa peptide (Fig.2a,b). The separation also resulted in the isolation of other components (Fig.2) which included; (i) proteins of various molecular masses (peak 1, 7, Fig.1), (ii) a low molecular mass protein (peak 2, fig.1), (iii) a 24 kDa protein (peak 8 fig.2a) and (iv) some proteins in the range of 56 kDa (peaks 9-11, fig.2a).

*N-Terminal amino acid sequence:*

The N-terminal amino acid sequence of peak 3, 4, 5 and 6 (fig.1) and their identification by comparison with known sequences further confirmed the presence of neurotoxin, cytotoxins and phospholipase-A containing peaks, as shown in the table.

## Discussion

The separation on reverse phase Vydac C-18 column described in the present paper is a single step rapid separation procedure for the protein components present in cobra venom.

The protein components isolated during the present investigation were identified by their biological activities, molecular mass and N-terminal amino acid sequences. The main aim of the study has been the separation of major and minor venom proteins. The separated major components were identified and found to be cytotoxins, neurotoxins and phospholipase. It was also observed that some high molecular

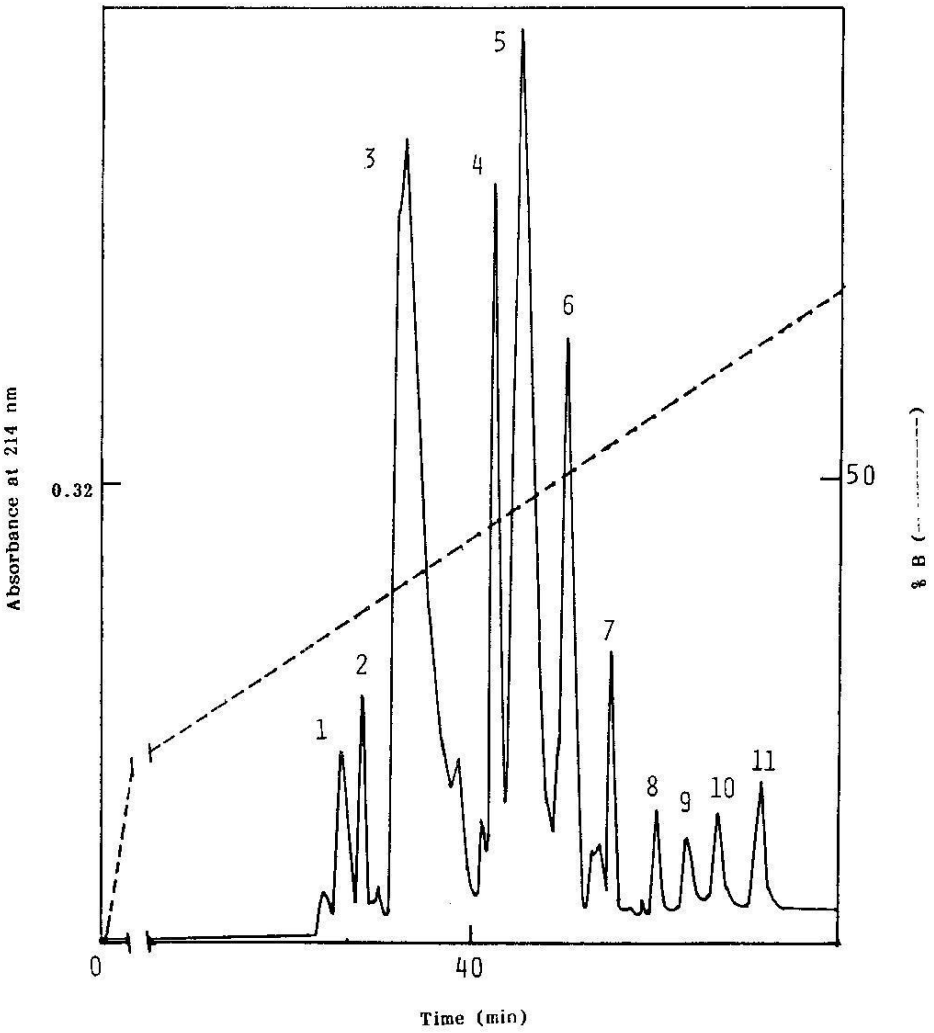


Fig.1: Separation of cobra venom by RP-HPLC on a Vydac C-18 column. A linear gradient of 0.1% TFA in acetonitrile containing 0.1% TFA was employed. Absorbance was recorded at 214 nm. Broken line represents gradient. Peptide designations are mentioned in the text.

mass proteins in purified form were also present in cobra venom, as judged by SDS-PAGE (Fig.2a).

The solvent system used can be easily removed by lyophilization and as TFA effectively dissolves almost all proteins even at high concentrations of organic solvents [14].

The present separation of cobra venom proteins on Vydac C-18 column resulted in the separation of all major components, including neurotoxins (Peak 3 Fig.1), which comprise of almost identical peptides with relatively close hydrophobicities as judged by their amino acid sequences [15] and are therefore eluted in the same peak.

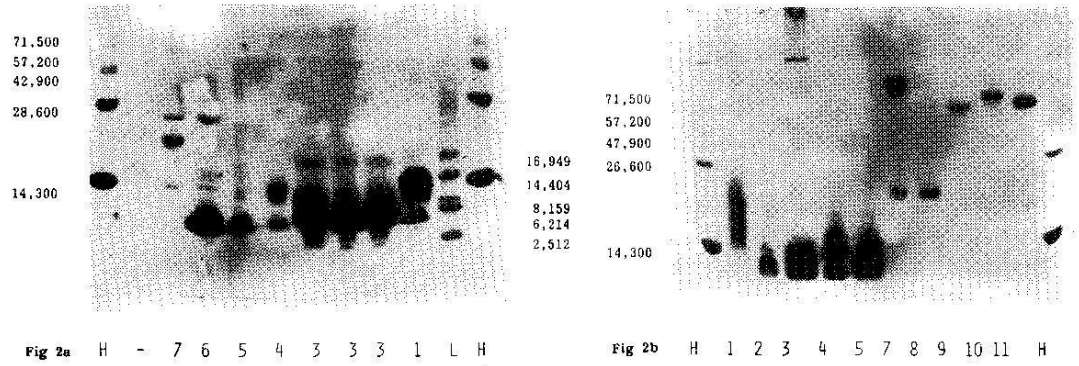


Fig.2: SDS-PAGE of peaks after reverse-phase HPLC in (a) 15% and (b) 12.5% gels. H=High molecualr mass standard (14-70 kDa markers). L=Low molecular mass standard (containing 2.5-17 kDa markers). Numbers represent peaks from Fig.1.

In the case of cytotoxins which have very little differences in their primary structure and have identical size were separated into two major peaks (Peak 5 & 6, fig.1). About three peaks could be expected from those types of cytotoxins present in cobra venom [15], but detection of two peaks indicated that one of the two peaks 5 & 6, (Fig.1), may contain two cytotoxins. SDS-PAGE of peak 9 and peak 11 (fig.1) also gave a single band (Fig.2), thus showing the presence of equal molecular mass proteins.

The phospholipase containing peak (Peak 4 Fig.1) gave two bands on SDS-PAGE (Fig.2a,b). The 14 kDa band corresponds to phospholipase, while the contaminating peptide has a molecular mass of about 3 kDa. Probably these two peptides have identical hydrophobicities, which could explain their co-elution on Vydac C-18 column.

Some minor components were also separated and found to be pure on SDS-PAGE (Fig.2). These proteins contain a low molecular mass protein (Peak 2, Fig.1) and some high molecular mass components (Peak 8-11, Fig.1). Peak 1 and 7 (fig.1) were found to be impure on SDS-PAGE.

The method described in this paper is suitable for the separation of various cobra venom components

Table-1: Identification of presently separated major cobra venom proteins by comparison of their N-terminal sequences with previously characterized proteins: neurotoxin, cytotoxin [15] phospholipase [16].

| Peak No. in Fig.1 | N-terminal Sequence | Identified as |
|---|---|---|
| 3 | Ile-Arg-Cys-Phe-Ile | Neurotoxin |
| 4 | Asn-leu-Tyr-Gln-Phe | Phospholipase |
| 5 | Leu-lys-Cys-Asn-lys | Cytotoxin |
| 6 | Leu-lys-Cys-Asn-lys | Cytotoxin |

which comprise of known proteins and some uncharacterized protein components.

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