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DETERMINATION OF MOLECULAR MASS AND PARTIAL PEPTIDE CONFIRMATION OF SHORT NEUROTOXINS USING CHROMATOGRAPHIC TECHNIQUES AND REVERSE PHASE HPLC

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ABSTRACT: The aim of present work was to investigate the purification of a novel protein (low molecular weight) from Indian cobra *Naja naja* by Cation exchange chromatography on CM-Sephadex C-25 and followed by Gelfiltration chromatography on Sephadex G-100. Fraction numbers 26, 27, and 28 were obtained from CM Sephadex C-25. From all the fractions, the protein concentration was calculated and it was applied onto the Sephadex G-100 Gelfiltration chromatography. Fraction No-11 obtained from Sephadex G-100 was used for the determination of molecular weight of the short neurotoxins by SDS-PAGE and Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF). This SDS-PAGE is corresponding to the gel filtration chromatography which resolved a thick band of ~6-7 kDa proteins, and the MALDI-TOF resolved 6668.530, 7447.438 and 19928.929 Da. Further, this fraction was selected for preparative HPLC by using C_{18} column, in which two major peaks (retention time 30.793 and 32.846) were found. The peak with retention time of 30.793 was preferred for molecular mass determination by MALDI-TOF showed a single sharp peak of 6815.471 Da. It was digested with trypsin enzymatic cleavage, which explored approximately 26 peptides and their masses were renowned. The scores of all these 26 peptides were compared with online mascot analysis and BLAST sequence and it did not match with any other peptides and proteins. Among these 26 peptides, since only two peptides score as 886.648 and 943.690 Da were identical with short neurotoxin -1 from *Naja oxiana*, and short neurotoxin -3 from *Naja mossambica*. Moreover the 6815.471 Da protein was used for hemolytic activity and it did not induce RBC lysis. All this observations suggested that the newly purified protein is a short neurotoxin. This essential information will support us to find out the structural information of short neurotoxin for its application in anti-venom development, antitumor and also for analgesic effects.

Keywords: Snake venom; Indian cobra *Naja naja;* Peptide mapping; Short neurotoxin

INTRODUCTION

Snake bites are remained as an important medical problem in both developing and developed countries (Chugh, 1989 and Warrell, 1996). It has been annually estimated that more than 10,000-15,000 deaths occur in India (Mukerjee, and Maity, 2002). A survey of the literature shows that cobra and viper are the two most categories of snakes in India responsible for maximum snake bite mortality (Baht et al., 1991). The Indian cobra *Naja naja,* (family Elapidae) venom generally has large quantity of neurotoxins and cyto/cardiotoxins (Ponnappa et al., 2008). Neurotoxins are small molecular weight protein divided into three major groups, long chain 65-72 amino acid residues with five disulfide bridges (Nakai et al., 1971) , weak chain 62-65 amino acid residues with five disulfide bridges (Utkin et al., 2001) and short chain 60-62 amino acid residues with four disulfide bridges neurotoxins (Dufton et al., 1983).

However all those neurotoxin consist of three finger loop structure (loop-I, II and III) (Tsetlin, 1999). In general neurotoxin binds to muscular and neuronal nicotinic acetylcholine receptor, which is composed of different acidic sub units, produces peripheral paralysis by blocking neuromuscular transmission at the postsynaptic site (Tu, 1973). Residues from loop-I and II are known to be having a great contribution in the binding and loop-III has a less contribution. Recently three homologous short neurotoxins named NT1, NT2 and NT3 have been identified from the venom of monocloed cobra (*Naja kouthia*) (Yuan Cheng et al., 2002). The amino acid sequence of NT1 and NT3 are indistinguishable with cobrotoxin (CBT) and cobrotoxin b from *Naja naja atra*, respectively (Yu et al., 1993 and Chang et al., 1997). The NT2 has a 61 residue toxin which is identical with the neurotoxin II from *Naja oxiana* (Golovanov et al., 1993). Most of the neurotoxins from snake venom have significant role of antitumor and antinociceptive effects, tested in animal pain model and also it could afford a new tool for combating pain (Adriana et al., 1998). The aim of present study was to carry out for purification and partial sequencing of the small molecular weight protein from Indian cobra *Naja naja*. The newly purified protein masses and its sequences are partially identical with short neurotoxin from, *Naja oxiana, Naja mosambica,* and *Naja pallid.* In addition to the above information, the inclusive sequence and structural information are needed to understand the toxic effect of short neurotoxin for the applications antitumor, analgesic and antivenin production in the field of medical biotechnology.

MATERIALS AND METHODS

Lyophilized snake venom (Indian cobra *Naja naja*) was purchased from Irula snake catchers' Industrial co-operative society limited, Chennai, India. G-100 was purchased from Sigma Aldrich (US). CM-Sephadex C-25 was purchased from Pharmacia Sweden. Filtration membranes were purchased from Millipore USA. All other reagents were of analytical grades.

CM-Sephadex C-25 column chromatography

The lyophilized crude venom (200 mg) was dissolved in 1ml of 0.025 M, pH 7.5 phosphate buffer and centrifuged at 3000 g for 5 minutes. Then the supernatant was loaded on to CM-Sepahdex C-25 column (0.5 cm x 45 cm). Unbound proteins were washed out with 0.025 M phosphate buffer pH 7.5. Bounded molecules were eluted with same buffer and then a gradient of 0-05-0.5 M of NaCl at a flow rate of 45 ml /h. The protein was monitored at 280 nm using an Amersham Bioscience spectrophotometer. Targeted fractions were collected, pooled, and desalted by amicon filtration (10 kDa and 6 kDa) membrane for further experiments.

Sephadex G-100 column chromatography

Proteins (6 mg) collected from CM Sephadex C-25 chromatography was loaded on Sephadex G-100 column (0.9 cm x 45 cm).Fractions were collected with 0.02 M of Tris-HCl pH 8.0 and flow rate was 45 ml /h. Fraction number 11 (17.36 mAu) was pooled and concentrated using filtration (10 kDa and 6 kDa) membrane for further experiments.

Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the method (Laemmli, 1970) and 15% gels were used. Molecular mass standards (Bovine serum albumin 66 kDa, Amylase 54 kDa, Horse radish peroxidase 40 kDa and lysozyme 14.3 kDa) were employed. Gel was stained with 0.1% Coomassie brilliant blue R-250.

Hemolytic activity

Hemolytic activity was measured according to the method described (Boman and Kaletta, 1957). Short neurotoxin (50 µg) was added to the reaction mixture contains RBC with PBS and incubated for 45 minutes at room temperature followed by centrifugation at 5000 g for 10 minutes. Supernatant was taken for absorbance at 540 nm.

The amount of hemoglobin released in the supernatant was measured. Phosphate buffer saline with RBC was referred as control.

Protein estimation.

Protein concentration was determined according to the method (Lowry et al., 1951) using bovine serum albumin (BSA) as standard

Molecular mass analysis by MALDI-TOF/ Mass Spectrometry

MALDI-TOF-MS analysis was carried out with Ultraflex TOF/TOF (Bruker Daltonics, Bremen Germany) mass spectrometer, equipped with nitrogen laser (337 nm). The protein sample was mixed with equal volume of saturated matrix solution (sinapinic acid in 50% acetronitrile/ H_2O with 0.1% trifluroacetic acid). This mixture (1 µl) was deposited on the probe plate and dried by steam air of air and the spectra were recorded in the reflectron positive ion mode using Bruker Daltonics FLEX analysis software.

Purification of short neurotoxin by Reverse phase HPLC

Sample of 40µl (80µg) was loaded onto C_{18} revrse phase column (4.6 x 150 mM, ZORBAX-C_8) using a linear gradient of solvent.B 20-95% achieved in 45 minutes. The solvent system consisting A; 0.1% formic acid in water and solvent B; acetonitrile in 0.1% formic acid was used. The protein was monitored at 280 nm.

Trypsin digestion and peptides mapping of short neurotoxin

The short neurotoxin was subjected to tryptic/chymotryptic digestion as described by (Rosenfeld et al., 1992) with some modification. The digested protein was subjected to MALDI-TOF. All the peptides were mapping with other proteins by mascot analysis and BLAST sequence.

RESULTS

Lyophilized (200 mg) snake venom (Indian cobra *Naja naja*) was centrifuged with phosphate buffer. A supernatant was applied on to CM- Sephadex C-25 chromatography and its protein recovery was calculated. This procedure was repeated and standardized by using different concentration of venom (100 mg, 200 mg and 300 mg). Different venom concentrations of chromatogram profiles were identical with one another (data not shown). In addition to CM-Sephadex C- 25 ion exchange chromatography, approximately eight major peaks were resolved. The fifth peak (Fig.1) constitutes of fraction no 26, 27, and 28 were collected and its molecular weight was screened on SDS-PAGE. All these fractions in the fifth peak contained both high concentration of low molecular weight and low concentration of high molecular weight proteins (Fig.2).

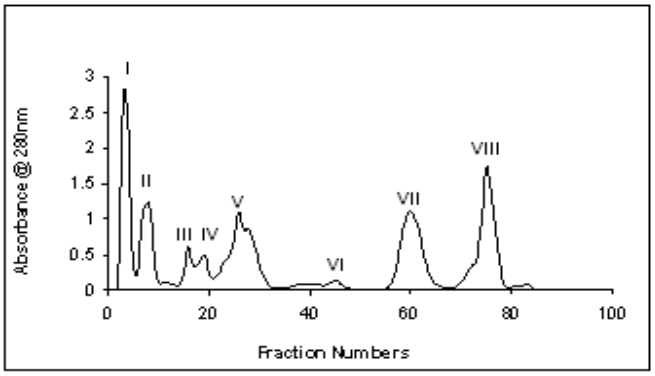

Fig. 1. Purification of short neurotoxin from the venom of *Naja naja*. Venom (200 mg) dissolved in 0.025 M of phosphate buffer pH 7.0 was loaded on CM Sephadex C-25 column (1.8 cm x 43 cm). Bounded proteins were collected with 0.1 M-0.6 M of NaCl gradient and flow rate was 45 ml /h. VI th peak was used for further experiments

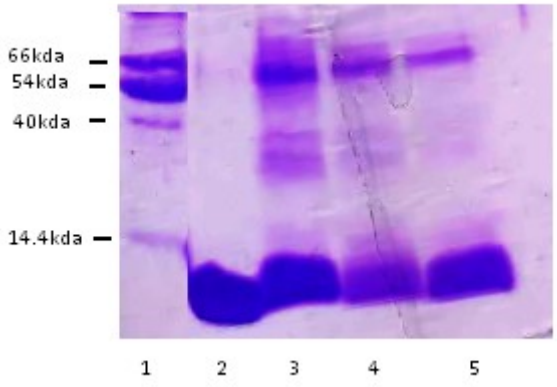

Fig. 2. Identification of molecular weight of target protein on 15% of SDS-PAGE. Molecular weight markers (Lane-1) in kDa (from top to bottom) Bovine serum albumin (66), amylase (54), Horse Radish Peroxidase (40) and lysozyme (14.4). Lane-2 from gelfiltration chromatography. Lane-3 (fraction no-26), lane- 4 (fraction no-27) and lane- 5 (fraction no-28) from CM- Sephadex C-25 chromatography.

The selected fractions were pooled, concentrated and the proteins were separated by gelfiltration chromatography. There were two peaks 6.54 mAu and 17.36 mAu resolved (Fig-3). The major peak 17.36 mAu protein was used for determination of molecular weight and purity by MALDI-TOF (Fig.4) and HPLC (Fig.5).

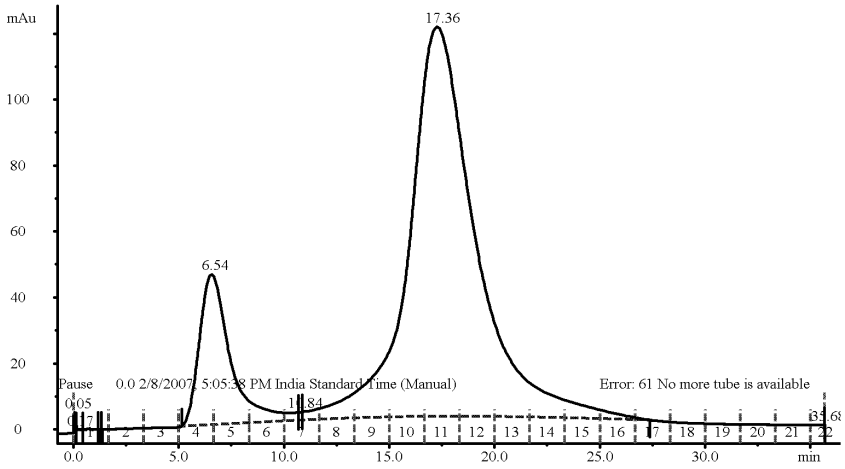

Fig. 3. Purification of short neurotoxin by gelfiltration chromatography. Proteins (6 mg) collected from CM-Sephadex C-25 chromatography was loaded onto Sephadex G-100 gelfiltration column (0.9 cm x 45 cm). Fractions were collected with 0.02 M Tris-HCl pH 8.0 and flow rate was 45 ml /h. Fraction number 11 (17.36 mAu) was used for molecular weight determination

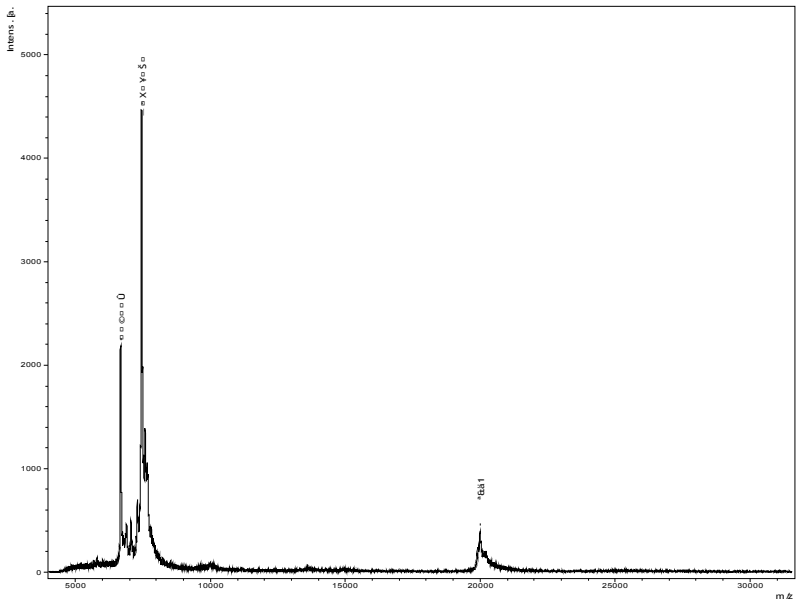

Fig. 4. Molecular mass analysis by MALDI-TOF. The first peak of molecular mass of 6668.530 Da was selected for identification of short neurotoxin.

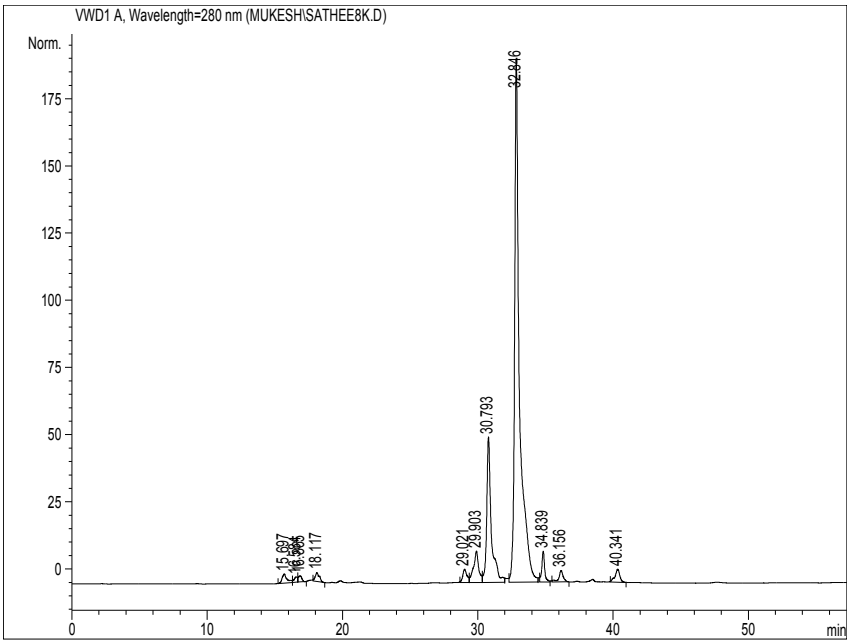

Fig. 5. Purification of short neurotoxin from the Mono Q column by C_{18} reverse phase HPLC. The retention time of 30.793 peak was selected for identification of short neurotoxin.

Based on the recovery of different retention time of fractions were collected from HPLC. A specific fraction of 30.793 minutes retention time was selected individually for molecular mass analysis by MALDI-TOF. The selected fraction has shown a single peak of molecular mass of 6815.475 Da on the MALDI-TOF (Fig-6). The 6815.475 Da protein was exploited for direct hemolytic and acetylcholinesterase activity. It did not accelerate or induce both hemolytic and acetylecholinesterase activity (data not shown) confirmed, it is neither cardiotoxin nor enzymatic protein. After this concrete information, the 6815.475 Da protein was digested with trypsin enzyme, showed 26 peptides and theirs scores (Fig-7). All the peptides scores were compared with BLAST sequence and mascot analysis did not competent with none of the proteins. Only the peptides score of 886.648 and 943.690 Da were identical with short neurotoxin -1 from *Naja oxiana*, and short neurotoxin -3 from *Naja mossambica* (Fig-8).

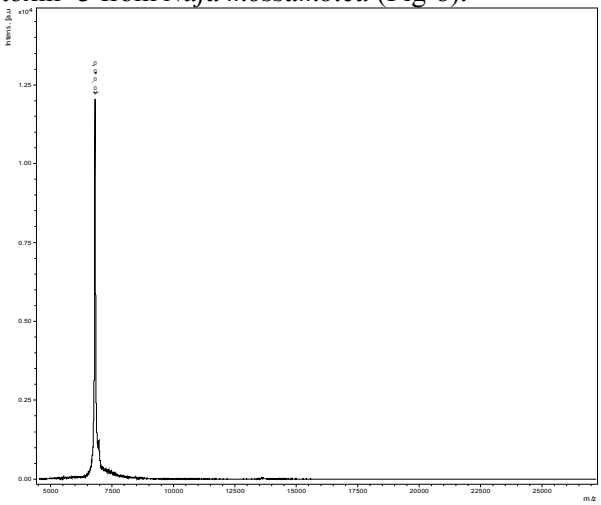

Fig. 6. Molecular mass analysis of short neurotoxin by MALDI-TOF. A single symmetrical peak of molecular mass of 6815.471 Da was selected for peptide digestions.

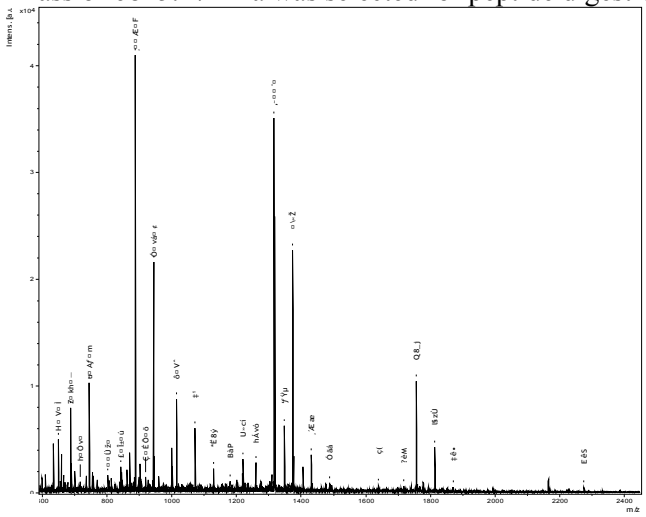

Fig. 7. Tryptic digestion of short neurotoxin. Twenty six peptides and theirs scores were obtained by trypsin digestion. The peptide scores of 886.648 and 943.690 Da were utilized for identification of short neurotoxin.

Protein MW: 6885 **(**Short Neurotoxin-1 from Central Asian cobra *Naja oxiana* **)**
Amino Acid Composition:

LECHNQQSSQ PPTTKTCSGE TNCYKK**WWSD HR**GTIIERGC GCPKVKPGVN LNCCRTDRCNN(*Naja Oxiana*- short neurotoxin-1)

Protein MW: 7218 **(**Short Neurotoxin- 3 from *Naja mossambica*)
Amino acid composition:

LNCHNQMSAQ PPTTTRCSR**W ETNCYKK**RWR DHRGYKTERG CGCPTVKKGI QLHCCTSDNCNN *Naja mossambica* - Short neurotoxin-3)**.**

Number	m/z (mi)	m/z (av)	Modifications	Start	End	Missed Cleavages	Sequence
1	886.3955	886.9507		27	32	0	(K) WWSDHR(G)
2	943.3978	944.0600		20	26	0	(R) WETNCYK(K)

Fig. 8. Peptide mapping analysis for short neurotoxin. The two peptide scores of 886.955 and 943.3978 Da were corresponding with short neurotoxin-1 from central Asian cobra *Naja oxiana* and short neurotoxin -3 from *Naja mossambica* respectively.

DISCUSSION

The prime purpose of venom release is to interfere selectively with critical and essential physiological functions, which can lead to death, and digestion of prey. This is thought to be brought about by neurotoxins and peptides present in the cobra venom. *Naja* neurotoxins actions were well characterized. They are interacting with muscular nicotinic acetylcholine receptor (nAchR) at the post synaptic site and irreversibly inhibit acetylcholine from binding to the receptor, resulting in peripheral flaccid paralysis from neuromuscular transmission blockage. The cobra victim dies primarily produce respiratory failure due to relaxation of diaphragm muscle (Changeux, 1981 and Ruan et al., 1990). Neurotoxins contribute to toxicity, generally posses three finger loop structure. Three finger proteins are rich in elapidae snake and are capable of producing number of highly toxic functions (New and Mene, 1990). Some of the conserved residues in the neurotoxin for instance half cystine, glycine and proline lie towards the globular region of the molecules and most likely they are responsible for determining the tertiary structure. This globular portion of the neurotoxin posses the determinants of neurotoxicity (Low, 1979) On the medical application of neurotoxin has been demonstrated that activation of the central cholinergic system produces antinocicpetion tested in animals. Induction of cholinergic pathway by nicotinic agonists has been explored to exhibit antinociceptive effects were determined in a variety of species and pain tests (Pedigo et al., 1975).

Our preliminary purpose was to isolate short neurotoxin from Indian cobra *Naja naja* constitutes of pre/post synaptic neurotoxin (Kini, 1997). Only long neurotoxin and weak neurotoxins were previously found in this venom (Ohta et al., 1981 and Shafqat et al., 1991). In the present study we have isolated a small molecular weight protein which is identical with short neurotoxin-1 from *Naja oxiana* and short neurotoxin-3 from *Naja mossambica.*

The purified protein was a basic protein and confirmed as a short neurotoxin by molecular weight and partial sequences. Short neurotoxins from elapidae snake venoms have similar molecular weight (~6-7 kDa) and identical amino acid residues. Molecular weight of both long neurotoxins and short neurotoxins could be divergent which facilitate to classification of neurotoxins but the molecular functions have been influenced the similar biological activity.

In summary, the results obtained from present work suggested that the purified protein as biological target of short neurotoxin, and it is ensured by identical molecular mass and partial peptides scores with their relative sequences. The purified short neurotoxin is a member of family of neurotoxins could be interacting with acetylcholine receptor by producing peripheral paralysis. Further the complete sequences and biological activity of purified short neurotoxin are needful to development of antivenin for controlling antinociceptive effects and snake venom envenomation.

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