A lethal neurotoxic protein from Indian king cobra
(Opophagus hannah) venom

Pallabi De, S C Dasgupta¹ & A Gomes*

Laboratory of Toxicology and Experimental Pharmacodynamics, Department of Physiology, University of Calcutta

Received 27 June 2001; revised 9 September 2002

A lethal neurotoxic protein (Toxin CM36) was isolated and purified from the Indian King Cobra (Opophagus hannah) venom by CM-Sephadex ion exchange chromatography and HPLC. The purified toxin had a SDS- molecular weight of 15 ± 0.5 kD. The UV absorption spectra of Toxin CM36 showed a peak at 280 nm and an E280 of 343.8 nm, when excited at 280 nm fluorescence. Toxin CM36 had an LD50 of 3.5 µg/20 g (iv) in male albino mice. It exhibited neurotoxicity and produced irreversible blockade of isolated chick biventer cervicis and rat phrenic nerve diaphragm. The neurotoxicity was found to be Ca²⁺ dependent. Toxin CM36 had no significant effect on isolated guinea pig heart and atriule. It also had no effect on blood pressure of cat and rat but produced respiratory apnoea in rat and guinea pig. Toxin CM36 lacked phospholipase activity.

The king cobra of the elapidae family is the world’s largest venomous snake. It is known that elapidae snake are extremely poisonous and have strong neurotoxic action. Recently, Lin et al., isolated two novel neurotoxins OH-6A and OH-6B from the king cobra (Opophagus hannah) venom. Gomes et al.² isolated and purified a lethal protein toxin from the Indian king cobra venom having cardiototoxic and haemorrhagic activity. The present investigation describes the isolation and purification of a lethal, neurotoxic protein toxin from the Indian king cobra venom. Some of the pharmacological actions of the toxin were examined to understand its mode of action.

Material and Method

Lyophilized whole venom of captive adult king cobra (Opophagus hannah) was purchased from Calcutta Snake Park (Calcutta, India). The following chemicals were used. CM-sephadex C-50 and sephadex G-10 (Pharmacia fine Chemicals, Sweden), low molecular weight markers, Coomassie brilliant blue R-250, amido black 10B, chloralose, carbacirol (Sigma, USA) acrylamide, bisacrylamide, tetramethylethlenediamine, sodium dodecyl sulfate, TRIS-hydroxy methylaminomethane (SRL, India), ethylendiamine tetraaceteticacid (Qualigen, India), acetylcholine chloride, 4-aminopyridine, urethane (E. Merck, India). Chemicals and solvents otherwise not mentioned were of analytical grade.

Animals were purchased commercially from M/s. B.N. Ghosh & Company, Calcutta, India. Albino mice(Swiss) and rats (Wistar) were given synthetic diet (pellets, Ashirwd Industries, Chandigarh, India), guinea pigs were given green leafy vegetables and soaked grains and cats were given rice milk and fish. Chicks were given poultry feed and water ad libitum.

CM-Sephadex C-50 column chromatography

Lyophilized cobra venom (90mg) was dissolved in 3ml of the equilibrating buffer (phosphate buffer 0.02M, pH 7.2) and applied to a CM sephadex C-50 column (150x25 mm) which was eluted stepwise using phosphate buffer containing NaCl from 0.05 to 0.8M. The flow rate was adjusted to 25ml hr⁻¹ and 10ml fractions were collected at room temperature. Protein was estimated according to Lowry et al.³. The fraction was desalted by passing through a sephadex G-10 column (100x10mm) and was subsequently lyophilized.

High performance liquid chromatography (HPLC)

HPLC was performed with CM sephadex purified toxin using a Waters protein pak 60(fractionating size 2000-800,000) column (7.8x360mm), equilibrated with 100 mM phosphate and 0.1 mM phosphate buffer in a stepwise gradient. Elution of protein was monitored at 280 nm.
Electrophoresis and determination of molecular weight

Polyacrylamide gel electrophoresis (PAGE) was carried out with the purified toxin on 7.5% polyacrylamide rod gels using tris glycine buffer 1M (pH 8.3) according to the method of Davis. The gels were stained with 0.2% amido black 10B and destained with 7% acetic acid. Sodium dodecyl sulphate (SDS-PAGE) was carried out according to the method of Laemmli on 10% acrylamide slab gel containing 0.1% SDS. Low molecular weight (6500-66,000 Dalton) marker kit from Sigma (USA) was used. The gel was stained with 0.25% Coomassie Brilliant Blue R250 and destained with 7.5% acetic acid containing 5% methanol. The zones of protein bands were recorded on the basis of the relative mobility to the marker protein of unknown molecular weight. The molecular weight of the unknown protein was determined graphically.

UV and fluorescence spectra

UV absorption spectrum of the purified toxin was recorded in the range of 240-400nm in a Hitachi U 3200 spectrophotometer. Fluorescence emission spectrum of the purified toxin was recorded in the range of 300-400 nm after exciting the protein solution at 280nm in a Hitachi F4020 spectrophotometer.

Phospholipase activity

The PLA2 activity was assayed by the egg coagulation methods of Habermann and Neumann.

Lethality in mice

LD50 was computed according to the method of WHO. The protein toxin was administered into male albino mice (20gm) through caudal vein. Mortality was recorded up to 24 hr of observation.

Isolated chick biventer cervicis and rat phrenic nerve diaphragm

Isolated chick (Leghorn strain, 14 days old, 80±5 g, n=6) biventer cervicis were prepared after Ginsborg and Warriner and male albino (180±10 g, n=6) rat phrenic nerve diaphragm were prepared after Bulbring. The toxin was infused through jugular vein.

Blood pressure

Arterial blood pressure was recorded via indwelling arterial cannula from the common carotid artery of diethyl ether chloralose (80mg/kg, iv) anaesthetized male cat (2±0.2 kg, n=4) and urethane (1.75 gm/kg, ip) anaesthetized male albino rat (180±10 g, n=4) by a mercury manometer on a rotating smoked drum. The toxin was administered through femoral/jugular vein.

Respiration

Respiration was recorded on a smoked drum from urethane (1.75 mg/kg, ip) anaesthetized male albino rats (180±10 g, n=6) and guineapig (22±10 g, n=6) according to the method of Gaddum. The toxin was infused through jugular vein.

Results

CM sephadex ion exchange chromatography

Ophiophagus hannah venom was resolved into four major peaks on a CM-sephadex C-50 column
DE et al.: NEUROTOXIC PROTEIN FROM INDIAN KING COBRA

(Fig. 1). Peak II eluted with 0.2M NaCl in phosphate buffer possessed lethal and neurotoxic activity. 1022-fold of purification was achieved by this process. The yield of the protein was 75.70% (Table 1). The fraction was provisionally named as Toxin CM36. Passing it through a sephadex G-10 column and lyophilized subsequently desalted the fraction.

**HPLC**

Toxin CM36 was further purified by HPLC using Waters protein pak 60 column. It was eluted as a single symmetrical peak with a retention time of 14.2 min (Fig. 2).

**Homogeneity, SDS-molecular weight and characterization**

HPLC fraction of Toxin CM36 was homogenous as confirmed by native PAGE and SDS-PAGE. The SDS-molecular weight of the toxin was calculated to be 15±0.5 kD (Fig. 3). The UV spectrum showed a peak at 280nm and fluorescence spectrum showed an emission maxima at 343.8nm (Fig. 4).

**Lethality in mice**

Intravenous LD50 of Toxin CM36 was 3.56 μg/20 g in male albino mice (Fiducial limit 5.45-2.32).

**Action on isolated heart and auricle**

Toxin CM 36 (10 and 25 μg) did not alter significantly the rate and amplitude of isolated guineapig heart observed up to 180 min (n=6).

Toxin CM36 (3 and 8 μg/ml) did not alter the rate and amplitude of contraction of isolated guineapig auricle observed up to 180 min (n=6).

**Action on blood pressure**

Toxin CM36 (50 and 500 μg/kg, iv. bolus) did not produce any prominent effect on cat and rat blood pressure observed upto 240 min (n=4).

**Action on respiration**

Toxin CM36 at a dose (20 μg/100 g, iv. bolus) produce a fall in respiratory rate in rat (50.2±0.51%,

---

**Table 1 — Purification of Toxin CM 36 by CM-Sephadex ion exchange chromatography from Indian king cobra (Ophiophagus hannah) venom**

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Activity MLD (μg/20 gm, iv)</th>
<th>Total activity</th>
<th>Yield (%)</th>
<th>Fold of purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>King cobra venom</td>
<td>3</td>
<td>30</td>
<td>90</td>
<td>4</td>
<td>12</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Toxin CM-36</td>
<td>10</td>
<td>0.5</td>
<td>5</td>
<td>4.5</td>
<td>45</td>
<td>75.70</td>
<td>1022</td>
</tr>
</tbody>
</table>

*MLD=Minimum lethal dose (μg/20 g, iv)*

---

Fig. 1 — Ion-exchange chromatography of king cobra venom on CM-Sephadex C-50 column. The lyophilized venom (90 mg) was dissolved in 3 ml of 0.01M phosphate buffer, pH7.2 and applied to a column (150×25 mm) of CM-Sephadex equilibrated with the buffer. Fractions (10 ml) were collected at 29°C. UA= Unabsorbed peak. PI=Lethal neurotoxic fraction (Toxin CM36)

Fig. 2 — HPLC purification of Toxin CM36 on protein pak 60 column. Toxin CM36 was applied to a protein pak 60 column equilibrated with phosphate buffer. Successive fractions were eluted by stepwise gradient at a flow rate of 0.5 ml/min and was monitored at 280 nm.
n=6) and guinea pig (40.5±0.20%, n=6) which gradually came back to normal within 40.8±0.5 min and 40.5±0.3 min respectively. However, at higher doses (40 μg/100 g, iv, bolus) it produced fall in respiratory rate (83.33±0.75%, n=6) and amplitude (60.5±0.62%, n=6) and produced gasping in rats. The animal died within 34.5±0.2 min (Fig. 5).

**Phospholipase activity**

Toxin CM36 was devoid of phospholipase activity.

**Action on isolated nerve muscle preparation**

On isolated rat phrenic nerve diaphragm preparation, Toxin CM36 (8 μg/ml) produced 100% blockade.
of electrically induced twitch response within 10.2 ±0.30 min (n=6). The blocking effect remained unaltered up to 4±0.25 hr inspite of repeated washing (Fig. 6). On isolated chick biventer cervicis preparation, toxin CM36 (8 µg/ml) blocked the electrically induced twitch response within 20.0±0.48 min (n=6). The blocking effect remained unaltered up to 5±0.15 hr inspite of repeated washing (Fig. 6).

Effect of agonist, antagonist, ions, enzymes & pH on Toxin CM36 activity

On isolated chick biventer cervicis preparation, toxin CM36 (8 µg/ml) abolished the ACh, KCl, and carbachol induced contractile response (n=6). By doubling the K⁺ ion concentration (9.4 mM) of the medium, there was 40.8±0.5% (n=6) increase of neuromuscular blocking time of chick biventer cervicis preparation (Fig. 7). However lowering K⁺ ion concentration (2.3 mM) of medium or the presence of voltage dependent K⁺ channel blocker, 4-aminopyridine (1 µM) failed to alter toxin CM36 activity on this preparation.

Doubling Ca²⁺ ion concentration (5.2 mM) of the medium had no effect on toxin CM36 induced neuromuscular blocking time. However, low Ca²⁺ ion concentration (1.6 mM) and presence of EDTA (1 mM) in the medium significantly reduced the neuromuscular blocking time (10.5±0.2 and 11.6±0.4 min) respectively (Fig. 7). Toxin CM36 lost its neurotoxic activity (100%) when exposed to enzyme action (Trypsin 1 mM). However, acidic (pH 3) and alkaline (pH 10) medium did not alter toxin CM36 action significantly.

Discussion

Venoms are complex mixtures of proteins and peptides possessing a variety of biological activities. Elapidae snakes are extremely venomous and have strong neurotoxic actions. The high toxicity of elapidae venom is apparently due to an unusually high content of neurotoxins. Several toxins with neurotoxicity have been isolated from cobra venom. Nakai et al.15 reported the presence of a neurotoxin, toxin A from N. naja (Indian cobra) of India. Karlsson et al.16, reported the presence of a neurotoxins, toxin 3 and toxin 4 from Naja naja naja. (Indian spectacled cobra). Toxin a and b are two neurotoxin reported from King cobra from Thailand17. Till now, no neurotoxin have been reported from the Indian King cobra venom. In the present study, toxin CM 36 a lethal neurotoxin was isolated and purified from the Indian King cobra Ophiophagus hannah venom by a combination of ion-exchange chromatography and HPLC. A single symmetrical peak in HPLC indicated the lack of any other impurities. The SDS-MW of the toxin (15±0.5kD) was found to be higher than that normally exhibited by neurotoxins isolated from cobras or kraits, which show a range of 7000-8000 (ref. 18). UV and fluorescence emission spectra resembled typical of that of a protein. Toxin CM36 had no significant effect on the cardiovascular system since, it produced no effect on blood pressure of cat/ rat, isolated guineapig heart and auricle. However, this toxin like all other neurotoxins produced apnoea and respiratory failure in experimental animals. It has been reported that the main cause of death due to elapidae venom perhaps due to the peripheral respiratory paralysis caused by the neurotoxic properties of the toxin.18 Besides its action on the respiratory system, the
most prominent action of Toxin CM36 was its effect on neuromuscular preparation. It produced irreversible blockade of chick biventer cervices (CBC) and rat phrenic nerve diaphragm (RPND) preparations. Toxin CM36 was devoid of any PLA₂ activity. The degree of sensitivity of the toxin to nerve-muscle preparation was in the following order: CBC > RPND. On CBC preparation, Toxin CM36 abolished Ach, KCl and carbachol induced contractions. This suggested that the toxin probably interacted with the post-synaptic region. Post synaptic neurotoxin (α-neurotoxin) from king cobra (Ophiophagus hannah) have been reported from Taiwan by Chang et al.¹⁹ Ca²⁺ played an important role in Toxin CM36 induced neurotoxicity. Low Ca²⁺ and EDTA reduced the neuromuscular blocking time of Toxin CM36 indicating that Ca²⁺ played role in Toxin CM36 induced neurotoxicity. However, high Ca²⁺ concentration did not have any effect on Toxin CM36 induced neurotoxicity. Though enzyme destroyed Toxin CM36 action, exposure to acidic and alkaline pH did not have any effect on Toxin CM36 activity. Attempt to raise antiserum against Toxin CM36 in rabbit was a failure (data not shown). The toxin was administered by sc, keeping in mind the high toxicity of the toxin. Out of six rabbits immunized, two of them died reasons for which could not be specified. May be lethality of the toxin was a key factor. However, loss of weight, anorexia was observed in surviving rabbits. Antiserum raised against Toxin CM36 in surviving rabbits failed to show precipitation bands in immunoglobulin diffusion and immunoelectrophoresis. It also failed to antagonize lethal action and neurotoxicity induced by Toxin CM36 indicating its poor antigenicity. It has been shown earlier that post-synaptic neurotoxins that are extremely toxic limit the immunizing dose, thus conferring to the low immunogenicity of the toxin as well as the whole venom²⁰.

Lethality contributing factors of Toxin CM36 is probably the neuromuscular blocking activity. Respiratory distress followed by respiratory arrest induced by Toxin CM36 may also probably due to respiratory muscle paralysis induced by neuromuscular blockade by the toxin. It is possible that Toxin CM36 is one of the major lethal components of king cobra venom. However, detailed structural characterization will enlighten the structure-function relation of the neurotoxin.

Acknowledgement
The authors are thankful to CSIR, New Delhi for partial financial assistance (Ref. No. 37/0851/94/EMR II dated 12.12.94).

References
5 Lowry O H, Roscrough N J, Farr A L & Randall R J, Protein measurement with the folin phenol reagents, J. Biol Chem, 193 (1951) 265.
6 Davis B J, Disc electrophoresis II. Method and application to human serum proteins, Ann NY Acad Sci, 121 (1964) 404.
14 Bulbring E, Observation of the isolated phrenic nerve diaphragm preparation of the rat, Br J Pharmacol, 1 (1946) 38.
17 Joubert F J, Snake venom toxins. The amino acid sequences of two toxins from Ophiophagus hannah (King Cobra) venom, Biochem Biophys Acta, 317 (1973) 85.