INTERNATIONAL RESEARCH JOURNAL OF PHARMACY **ISSN 2230 – 8407**
Available online http://www.irjponline.com
Research Article

COBROTOXIN ASSOCIATED WITH HIGH PHOSPHOLIPASE A_2 ACTIVITY DISCOVERED FROM THE VENOM OF Naja naja (INDIAN COBRA)

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Article Received on: 10/12/10 Revised on: 02/01/11 Approved for publication: 12/01/11

ABSTRACT
A toxin with high in vitro neurotoxicity and Phospholipase A_2 activity has been isolated from the venom of the Naja naja (Indian cobra) by column chromatography.

KEY WORDS: Cobrotoxins, column chromatography.

INTRODUCTION
Braganca and Quastel[1] had observed a correlation between the toxicity and Phospholipase A_2 activity in the heated venom of the Naja naja (Indian cobra). Subsequently, two well characterized Cobrotoxins were isolated, their amino acid sequences, and the positions of the disulfide bridges determined[2-4]. In the present investigation, a toxin with high Phospholipase A_2 activity and in -vitro neurotoxicity but relatively less in- vivo lethality in mice is reported.

MATERIALS AND METHODS
Cobra venom was a lyophilized product of the Haffkine Institute, Bombay, India. CM-cellulose chromatography was carried out as described before[5]. CM-Cellulose chromatography, of crude Naja naja venom yielded three distinctly separated peaks, which were subsequently lyophilized and analyzed by disc electrophoresis as described by Panyim and Chalkley[6] in the presence of 8M urea, their protein contents were also determined. Certainly lethal dose (CLD_{100}) was determined by intravenous injection of the crude venom and their components, dissolved in physiological saline in white (approximately 20 gram, Kasauli strain) mice. A dilution which killed all the six mice in a set was the CLD_{100} dosage expressed as µg protein. Phospholipase A_2 activity determined by the method of Ramsey et.al[7], using egg yolk suspension, as the substrate. One unit of the enzyme produces a clearance (Δ) at 925nm of the egg yolk suspension of one/min at 37^0C. The specific activity of the Phospholipase A_2 was expressed as units/mg of protein. Protein was estimated by the method of Lowry et.al[8]. In-vitro neurotoxicity was measured by rat phrenic nerve hemidiaphragm method of Bulbring[9] on a physiograph with a 25.0ml bath, on the E & M instruments company, Texas, U.S.A. The minimum µg of protein /ml required to produce the pharmacological effect was taken as a unit of neurotoxicity.

Fractionation System
4g of CM Cellulose swelled initially in distilled water was treated with 1N HCl for 30 minutes. After HCl treatment, cellulose was washed with distilled water till it was free from acid, and was subsequently equilibrated with 0.05M Sodium acetate. After initial equilibration this slurry was packed in a column 25cm x 6mm, and again equilibrated using the same buffer (0.05M sodium acetate). Sample consisting of 60mg of the crude Naja naja venom dissolved in 2ml of the 0.05M sodium acetate was applied on the column, and eluted using 80ml of 0.05M Sodium acetate in a smooth gradient system.2ml fractions were collected at18^o -20^0C with a flow rate of 30ml per hour .These fractions were diluted (1:1) with ice cold distilled water and their absorbance was measured at 280 nm on a Bechman DU2 spectrophotometer.

Elution profile is given in figure **1 .**These fractions were tested for Phospholipase A_2 activity. The result is summarized in table1. Electrophoretic pattern in the presence of 8M urea is depicted in **fig 2.** Fractions obtained were tested for their enzyme activities and the results are summarized in **table 2**.

Toxicity Studies

Kasauli strain white mice (20g) from Haffkine Institute Bombay were used as experimental animals'. Different fractions in 0.5 ml quantities were injected intravenously in the tail vein of these mice. Lethal effect or otherwise was observed in these mice for 24 hours .Sterile normal saline was used to dilute or dissolve these fractions when required. Lyophilized peak II was subjected to acrylamide gel electrophoresis in the presence of 8M urea as described earlier.

Pharmacological Studies

Neuromuscular Transmission

In the present investigation, the action of the venoms of Naja naja and its components was studied on an isolated rat phrenic nerve hemidiaphragm preparation (Bulbring 1946). Rat phrenic nerve hemidiaphragm preparation was isolated as described by Seth et.al. (1972). The preparation mounted on a Perspex electrode was set in an organ bath of 25ml capacity containing Kreb's solution, maintained at 37^0C and aerated with carbogen. The response in the form of a quick sharp downward twitch was recorded on Physiograph four, E and M instrument Co. Texas, U.S.A. under the following conditions, voltage: 11 Volts, duration 5milliseconds, frequency 1Hz.To begin with ,the twitches were allowed to stabilize ,after which the sample, (dissolved in Kreb's solution), was added to the organ bath. Reduction in the height of the twitch was indicative of blockade of neuromuscular transmission.

Neostigmine was used to see, if the neuromuscular blocks could be reversed? Physiograph pattern of the crude venom toxin isolated from peak **II** is shown in **fig -03** the results of which are summarized in **table 03** for the venom of Naja naja.

RESULT

CM-Cellulose chromatography[5] of 60mg of the crude Naja naja venom yielded two distinctly well separated peaks. Disc electrophoretic profile[6] showed that the second peak had two toxins. The second peak contained the presently reported neurotoxin (table1) The Phospholipase A_2 activity in vitro neurotoxicity and CLD_{100} are tabulated in table 1.

DISCUSSION

The new cobrotoxin reported in the present investigation is less lethal in vivo, but highly neurotoxic in vitro and has a high Phospholipase A_2 activity (Table.1).

PLA_2 plays a very important role not only in the neurotoxin traversing the lipid bilayer particularly of the skin (site of the bite) but is also known to orient the neurotoxin to bring about its proper alignment with the acetylcholine receptor thus bringing about the receptor mediated interaction. In the present investigation, association of high levels of PLA_2 with the neurotoxin would bring about an efficient interaction of the cobra neurotoxin with the Acetylcholine receptor in turn bringing about the much intended nerve conduction block, ultimately resulting in selective muscle paralysis. **Thus making this cobrotoxin a very important therapeutic agent to treat movement disorders in general and alleviating pain in human beings in particular.**

ACKNOWLEDGEMENT

The gift of a Bechman DU spectrophotometer by the Humboldt-Striftung is gratefully acknowledged. The authors thank Prof. U. C. Seth, of Seth. G. S. medical College for providing facilities for the Pharmacological experiments.

REFERENCES

1. Branganca BM and Quastel JH. Nature 1952;169: 695-697.
2. Karlson, E., Arnberg, H. and Eaker, D.(1971) Eur.J.Biochem.21, 1-16.
3. Ohta., M and Hayashi, K. (1974) Biochem.Biophys.Res.Comm.57, 973-979.
4. Nakai, K., Sasaki, T .and Hayashi, K, (1974) Biochem.Biophys. Res. Comm 44,893- 897

5. Bolar, H.V. and Master and R.W.P (1976) Biochem .Biophys. Res.comm70, 573- 577
6. Panyim, S. and Chalkley, R. (1969) Arch. Biochem and Biophys.130, 337-346
7. Ramsey, H. W., nyder ,G. K., Kitchen,H.and Taylor, W.J.(1972) Toxicon,193, 265 - 275.
8. Lowry, O. H., Rosebrough, N.J. Farr, A. L. and Randall, A.(1951) J. Biol .Chem 193,265 - 275.
9. Bulbring, E. (1946) Br.J.Pharmach.Chem.other.1, 38-61.

Table 1: Specific activity of Phospholipase A_2, *in vitro* Neurotoxicity and CLD_{100} of the cobrotoxin

Components	Phospholipase A_2*specific activity	In vitro Neurotoxicity * µg protein	CLD_{100} µg protein
Whole venom	0. 13	80	13.3
Cobrotoxin (Peak II)	22.1	2.0	1580

*for units please see the text.

Table 2: Specific activity of PLA_2

Enzyme	PLA_2 Activity of whole venom	Specific activity of PLA_2 peak II
Phospholipase A_2	8.8	10.2

Table 3: Physiographs of the whole venom; Peak II and toxin isolated from Peak II, of Naja naja (Figure 04)

Sample	Bath concentration µ g/ml	Effect	Time (Minutes)	Effect of Drug (Neostigmine)	Direct Muscle Stimulation
Whole Venom	80.0	Persistent Muscle Contraction	<1.50	Block not reversed	Normal
Peak II	160.0	Total Nerve Conduction Block	2.0	Block not reversed	Normal
Toxin Isolated from Peak II	142.0	Total Nerve Conduction Block	15.0	Block not reversed	Normal

Observations: The whole venom showed persistent contraction, Peak II and isolated toxin from Peak II showed total nerve conduction block, which could not be reversed by Neostigmine. Direct muscle stimulation in all the cases indicated that the muscle function was normal, confirming the action of the crude venom, peak II and toxin isolated from peak II.

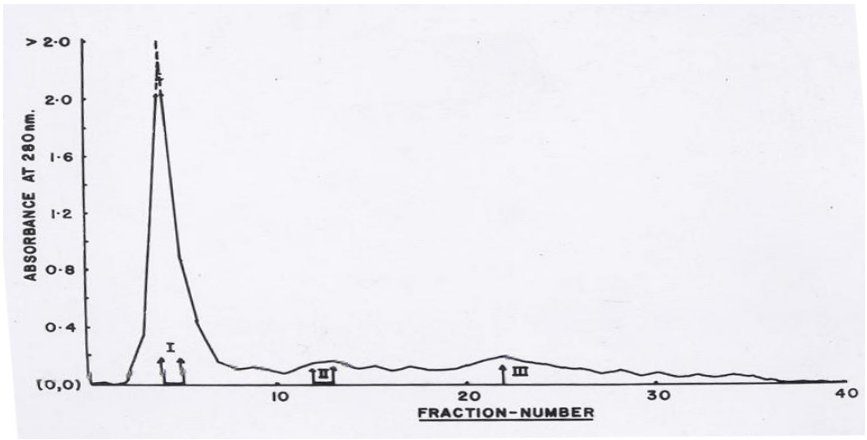

Figure 1: CM Cellulose column chromatography of crude venom of Naja naja (Indian Cobra) Peak –I, Peak –II, Peak III

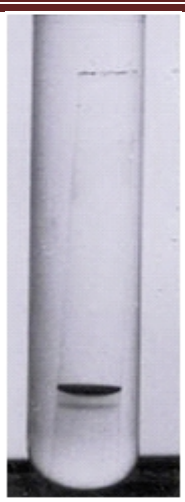

Figure 2: Disc electrophoretic patterns of the isolated toxin in the presence of 8M Urea

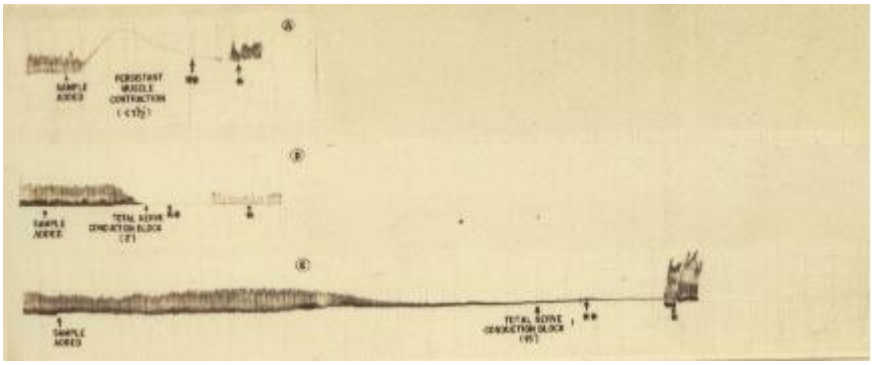

Figure 3: Physiographs of the Naja naja crude venom, peak II and toxin isolated from peak II.
A-Crude venom of Naja naja B- Peak II C- Toxin isolated from peak II
*Direct muscle stimulation ** Drug added

Source of support: Nil, Conflict of interest: None Declared