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Inhibition of toxic actions of phospholipase A_2 isolated & characterized from the Indian Banded Krait (*Bungarus fasciatus*) venom by synthetic herbal compounds

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***Background & objectives*: Phospholipase A_2 (PLA_2) is one of the major constituents of krait venom associated with several pathophysiological actions like myotoxicity, cardiotoxicity, neurotoxicity, *etc.* As there was no specific antiserum available against *Bungarus fasciatus* venom, this study was done with synthetic herbal compounds, anti PLA_2 rabbit antiserum and commercial polyvalent snake venom antiserum to neutralize the PLA_2 induced toxicities in experimental models.**

***Methods*: *B. fasciatus* venom phospholipase A_2 fraction 38 (BF-38) was isolated by ion exchange chromatography, molecular weight was determined by mass spectrometry and its N terminal amino acid sequence was identified. Monospecific rabbit antiserum was raised against the PLA_2 in presence of Freund complete adjuvant. The neutralization of PLA_2 induced toxicities was done in *in vitro* and in *in vivo* models using synthetic herbal compounds, anti PLA_2 rabbit antiserum and commercial polyvalent snake venom antiserum.**

***Results*: A toxic PLA_2 (BF-38) was purified from the *B. fasciatus* venom by CM-cellulose and HPLC, of 13.17 kDa and a minor band of 7.3 kDa using ESI-MS. The 13.17 kDa PLA_2 sequence was NLYQFKNMIQC. The 7.3 kDa toxin sequence was RKCLTKYSQDNES and was found to be <10 per cent w/w. Anti PLA_2 rabbit antiserum produced faint precipitant band in immunogel diffusion and showed low titre value. The commercial polyvalent snake venom antiserum, anti PLA_2 rabbit antiserum and the synthetic herbal compounds neutralized the PLA_2 induced toxicities at different intensities.**

***Interpretation & conclusions*: Our results suggested that synthetic herbal compound (BA) along with antiserum might provide effective protection against PLA_2 induced toxicities of *B. fasciatus* venom.**

Key words Banded krait - *Bungarus fasciatus* - herbal antagonists - phospholipase A_2

Kraits are one of the most common poisonous snakes of the eastern India. Their geographical distribution ranges from Pakistan through Southern Asia to Indonesia and Central China[1,2]. There are about 12 species of krait in Asia, among those the golden banded krait (*Bungarus fasciatus*; or common name

Shankhamuti) is very common in West Bengal, India. One of the major components of *B. fasciatus* venom is phospholipase A_2 (PLA_2s), which constitutes about 65 per cent of the total venom proteins[3,4]. The PLA_2 exerts its biological effects by hydrolyzing an acyl group at sn-2 position of the glycerophospholipids leading to the release of fatty acids and lysophospholipids which in turn either act as a second messenger or as pro-inflammatory agent. Amino acid sequences of >280 different PLA_2 enzymes from snake venom have been identified so far (*http://sdmc.lit.org.sg/Templar/DB/snaketoxin _PLA2/index.html)*[5]. Venom PLA_2s exert their pathophysiological actions such as myotoxicity, cardiotoxicity, alteration in blood pressure, oedema, haemolytic activity, platelet aggregation, and neurotoxicity, which are often fatal[6].

In India, there is no specific antiserum available against *B. fasciatus* venom. The commercial polyvalent snake venom antiserum raised against *Naja naja, B. caeruleus,* Russell's viper*, Echis carinatus* is used for *B. fasciatus* snakebite treatment. In the traditional and folk medicine, several herbs (*Andrographis paniculata*, *Leucas linifolia, etc*.) have also been used to treat *B. fasciatus* envenomation[7]. In view of the limited information available, this study was undertaken to purify a PLA_2 from the eastern Indian *B. fasciatus* venom and to neutralize its toxicity and biological activity with synthetic herbal compounds, anti PLA_2 rabbit antiserum and commercial polyvalent snake venom antiserum in *in vitro* and *in vivo* animal models.

Material & Methods

Chemicals and reagents: Lyophilized *Bungarus fasciatus* venom (BFV) was purchased from Calcutta Snake Park, Kolkata, India. Carboxy methyl cellulose, Freund complete adjuvant (FCA), sodium azide (Sigma, USA), Protein pack 60 column, Waters, Division of Millipore (Tokyo, Japan), acrylamide, bis-acrylamide, sodium dodecyl sulphate, coomasie brilliant blue, tri sodium citrate, ammonium per sulphate, glycine, prestained molecular weight marker (Fermentas, USA), were purchased. Commercial polyvalent snake venom antiserum (Batch no. 926) was a gift from Haffkine Institute, Mumbai, India. Synthetic herbal compounds 2-hydroxy-4-methoxy benzoic acid (BA) (Aldrich Sigma, USA), p-anisic acid (AA) (SRL, India) and salicylic acid (SA) (Loba Chemie, India) were purchased.

Animals: The study was conducted in Laboratory of Toxinology and Experimental Pharmacodynamics, Department of Physiology, University of Calcutta, Kolkata, Male albino New Zealand strain rabbits (1.5 ± 0.2 kg), BALBc mice (20 ± 2 g) and male toad (40 ± 5 g) were used. All the animals were kept in standard laboratory conditions except toads; 12/12 h light-dark cycle, food and water were given *ad libidum*. Animal ethical clearance of Department of Physiology, University of Calcutta, Kolkata was obtained for the study protocol. Each experimental group was matched with a parallel control group treated only with saline solution (0.9%). Experiments were carried out at a laboratory temperature (25 ± 3°C).

Ion exchange chromatography: *B. fasciatus* venom (25 mg) was dissolved in 0.02 M working phosphate buffer, *p*H 7.2 and applied to a column (5.6 x 1.6 cm) of carboxy methyl (CM) -cellulose equilibrated with the buffer. Different protein fractions were collected (5 ml each) with step-wise NaCl gradient of increasing molarities of (0-1 M) in 0.02 M phosphate buffer. All the fractions collected were subjected to protein analysis[8] and PLA_2 activity assay[9].

High performance liquid chromatography (HPLC): The PLA_2 fraction obtained from ion exchange chromatography was desalted by 1KD centripep (3000 rpm, 4°C, 3 h) and further purified by a Protein pack 60 column (7.8 x 300 mm). The column was eluted with 50 mM Na-K phosphate buffer, *p*H 7.2 containing 0.15 M NaCl, the flow rate was 0.8 ml/min and the peak were monitored at 280 nm.

Molecular weight and sequence determination of PLA_2: The molecular weight of the PLA_2 was determined through SDS-PAGE and confirmed through electron-spray-ionization mass spectrometry (ESI-MS) (Sciex API100, Perkin Elmer, USA) in Proteomics and Structural Biology Research (Academia Sinica, Taiwan). N-terminal amino acid sequences of the PLA_2 were determined by Edman-degradation with the sample on a gas-phase amino acid sequencer coupled with a henylthiohydantoin amino acid analyzer (Perkin Elmer, USA).

Lethality determination: The minimum lethal dose (MLD) was assessed by injecting different concentration of the PLA_2 into the tail vein of mice (20 ± 2 g, n=6), observed for 24 h and mortality was recorded[10].

Hyperimmunization of rabbit: Antiserum was prepared against the PLA_2 by hyperimmunization of male albino New Zealand rabbit (1.5 ± 0.2 kg, n = 4). The PLA_2 (48 µg/kg) was mixed with FCA (1:1), and administered intramuscularly in the first, second and

third week of immunization followed by booster dose of PLA_2 (without FCA) for another three weeks[11].

Immunogel diffusion and indirect haemagglutination titre: Immunogel diffusion was done[12]. The commercial polyvalent snake venom antiserum (10 µl)/anti PLA_2 rabbit antiserum (20 µl) was applied in central well and the peripheral wells contained crude *B. fasciatus* venom (10 µl)/PLA_2 (10 µl). It was kept at 4°C for 48 h and the precipitant bands were visualized. The antiserum titre was determined with indirect haemagglutination assay[13].

Neutralization technique: PLA_2 activity was determined by egg yolk coagulation time[9]. One PLA_2 unit was defined as the minimum amount of PLA_2 required to increase the clotting time of the egg yolk emulsion by 1 min against saline control. Neutralization was done by mixing different amount of PLA_2 with a fixed amount of anti-PLA_2 rabbit antiserum/commercial polyvalent snake venom antiserum (10 mg/ml)/synthetic herbal compounds (5 mg/ml) incubating at 37°C x 1 h and the clotting time was recorded[14].

The minimum oedema dose (MED) of the PLA_2 was assessed by injecting 0.01 ml of different concentrations of PLA_2, sub-plantar (s.p) in the paw of male albino mice and the oedema was recorded within 2 h of observation[15]. One MED unit was defined as the minimum amount of PLA_2 required to induce inflammation in mouse paw by 100 per cent within 30 ± 10 min, when administered subplantarly. Neutralization was done by mixing different amount of PLA_2 with a fixed amount anti PLA_2 rabbit antiserum / commercial polyvalent snake venom antiserum (10 mg/ ml) / synthetic herbal compounds (5 mg/ml) incubating at 37°C x 1 h and the decrease in inflammation was recorded by digital calipers (Mitutoyo, Japan)[14].

Plasma recalcification time using goat plasma was determined[10]. One minimum plasma recalcification dose (MPRD) unit was defined as the minimum amount of PLA_2 required to increase the clotting time of the plasma by 100 per cent against saline control. Neutralization was done by mixing different amounts of the PLA_2 with a fixed amount of anti PLA_2 rabbit antiserum/commercial polyvalent snake venom antiserum (10 mg/ ml)/synthetic herbal compounds (5 mg/ml) incubating at 37°C x 1 h and the clotting time was recorded[14].

One minimum cardiotoxic dose (MCTD) was defined as the minimum amount of PLA_2 required to cause the onset of cardiac arrhythmia within 10 ± 2 min upon its administration on isolated toad heart suspended in Ringer amphibian fluid at room temperature (28 ± 1°C). Neutralization was done by mixing different amount of the PLA_2 with a fixed amount of anti PLA_2 rabbit antiserum/commercial polyvalent snake venom antiserum (10 mg/ ml)/synthetic herbal compounds (5 mg/ml) incubating at 37°C x 1 h to study the inhibition of the cardiac arrhythmia[16].

Statistical analysis: The results were expressed as mean of observations (n=6); MLD was expressed as mean ± SE.

Results

On ion exchange chromatography, *B. fasciatus* venom showed six protein peaks. Fraction 38 showed phospholipase activity (Fig. 1, Table I). Further purification of fraction 38 by HPLC showed a major sharp peak followed by a small peak at 280 nm, with retention time of 11.8 and 13.7 min, respectively (Fig. 1, inset A).

SDS-PAGE of fraction 38 showed two bands corresponding to around 14 and 7 kDa mass in the ratio of 9:1 (densitometric analysis by ImageJ software, data not shown). The exact molecular weight was confirmed using the mass spectroscopy on ESI-MS found to be 13.17 and 7.3 kDa (Fig. 2). The N terminal sequence of

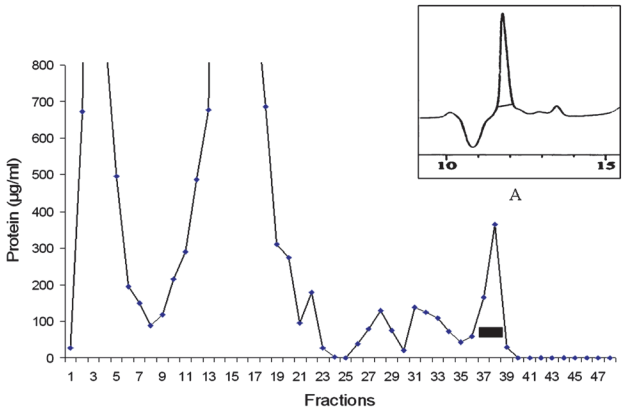

Fig. 1. Purification of PLA_2 from *Bungarus fasciatus* snake venom by carboxy methyl-ion exchange chromatography. *B. fasciatus* venom (25 mg) was applied in a column (5.6 x 1.6 cm) packed with CM- cellulose. Elution was done with 0.02 M phosphate buffer and in a stepwise gradient of NaCl (0 – 1 M). ▬ = Fraction 38 was collected and used as 'BF-38'. Inset A: HPLC Chromatogram of Fraction 38 using Protein pack 60 column (7.8 x 300 mm). The column was run with 50 mM Na-K phosphate buffer containing 0.15 M NaCl (*p*H 7.2, flow rate 0.8 ml/min). Retention time was 11.8 min.

Table I. Summary of two step purification of *Bungarus fasciatus* snake venom PLA_2 (BF-38)

Steps	Initial protein (mg)	Total PLA_2 unit	One PLA_2 unit (µg)	Fold of purification	% Recovery	% Loss
1. Crude venom	25	5000	5	--	--	-
2. BF-38	1.5	200	8	25	94.9	5.1

the first 11 amino acids of the 13.17 kDa band was found to be NLYQFKNMIQC and first 13 amino acids of the 7.3 kDa band was found to be RKCLTKYSQDNES. The PLA_2 was named as BF-38 (*Bungarus fasciatus* fraction 38).

The minimum lethal dose of the PLA_2 was found to be 17.3 mg/kg i.v., in male albino mice. One PLA_2 unit was found to be 8 µg, the minimum oedema dose (MED) was 6 µg, the minimum plasma recalcification dose (MPRD) was 12 µg and the minimum cardiotoxic dose (MCTD) was 38 µg (Table II). BA (5 mg/ml) neutralized PLA_2 activity up to 2 fold, MED up to 1.5 fold, plasma recalcification up to 1 fold and MCTD 1 fold. AA (5 mg/ ml) neutralized PLA_2 activity up to 2 fold, MED up to 1.5 fold, MPRD 1 fold, but gave no protection against PLA_2 induced cardiotoxicity. SA (5 mg/ml) neutralized the PLA_2 activity up to 1.5 fold, MED up to 1 fold, MPRD up to 1 fold and MCTD up to 1 fold (Table II).

Anti PLA_2 rabbit antiserum developed low titre value (1:32) assessed through haemagglutination assay. The anti PLA_2 rabbit antiserum showed faint precipitant band in immune gel diffusion.

Commercial polyvalent snake venom antiserum neutralized PLA_2 activity up to 1 fold, it gave no protection against MED, MPRD and MCTD (Table III). Anti PLA_2 rabbit antiserum neutralized PLA_2 activity up to 3.5 fold, MED up to 1 fold, MPRD up to 2 fold and MCTD up to 1 fold (Table III).

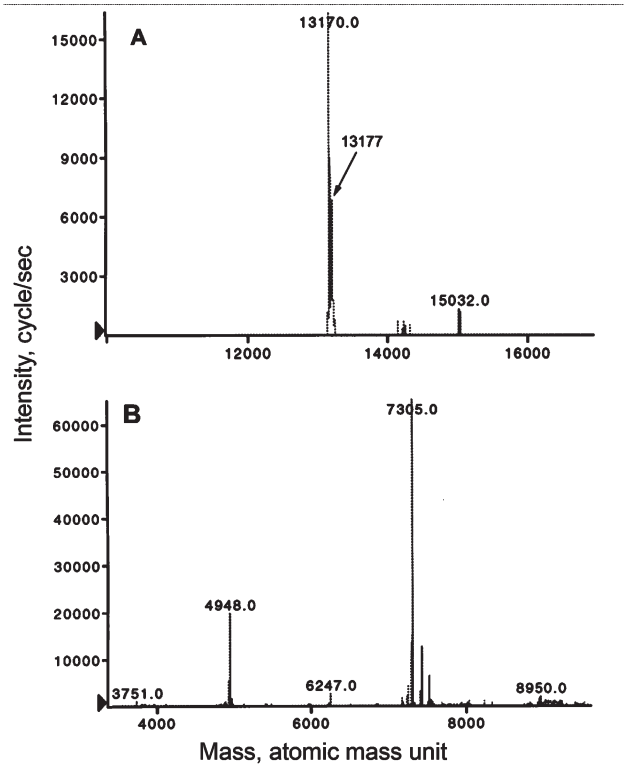

Fig. 2. Determination of molecular weight of BF-38 by electron-spray-ionization mass spectrometry (ESI-MS). A. Mass of the PLA_2 was 13177 Daltons. B. Mass of the 3FTx was 7305 Daltons.

Table II. Neutralization of PLA_2 induced activities by synthetic herbal compounds

Activities	Synthetic herbal compounds + PLA_2 incubate in µg		
	BA + PLA_2	AA + PLA_2	SA + PLA_2
PLA_2 activity (1 unit = 8µg)	*16 (2) 20 (NP)	*16 (2) 20 (NP)	*12 (1.5) 16 (NP)
Minimum oedema dose (1 MED=6µg)	*9 (1.5) 12 (NP)	*9 (1.5) 12 (NP)	*6 (1) 9 (NP)
Minimum plasma recalcification dose (1 MPRD= 12µg)	*12 (1) 24 (NP)	*12 (1) 24 (NP)	*12 (1) 24 (NP)
Minimum cardiotoxic dose (1MCTD= 38 µg)	*38 (1) 76 (NP)	38 (NP) --	38 (1) 76 (NP)

Values shown are mean of six experiments. *Protection offered, values within parentheses indicate fold of protection; NP, no protection; BA, 2- hydroxy -4- methoxy benzoic acid; AA, *p*- anisic acid; SA, salicylic acid

Table III. Neutralization of PLA_2 induced toxicities by commercial polyvalent anti-snake venom antiserum and anti PLA_2 rabbit antiserum

Activities	Antiserum (µg)	
	Commercial polyvalent snake venom antiserum + PLA_2	Anti PLA_2 rabbit antiserum + PLA_2
PLA_2 (1 unit = 8 µg)	*8 (1) 12 (NP)	*28 (3.5) 32 (NP)
Minimum oedema dose (1 MED=6 µg)	6 (NP) --	*6 (1) 9 (NP)
Minimum plasma recalcification dose (1 MPRD= 12 µg)	12 (NP) --	*24 (2) 36 (NP)
Minimum cardiotoxic dose (1MCTD= 38 µg)	38 (NP) --	*38 (1) 76 (NP)

Values shown are mean of six experiments. *Protection offered, values within parentheses indicate fold of protection; NP, no protection

Discussion

PLA_2 is one of the most common abundant toxic enzymes present in several snake venoms including kraits. Several natural (aristolochic acid, betulinic acid, *etc*.)[17] and synthetic compounds (isoxazolines, diazepinones, acenaphthenes, *etc*.)[18] are responsible for the PLA_2 enzyme inhibition. The antagonists of PLA_2 vary with their origin, and hence generate a poor immune response in the body and are not always effective. The present study was aimed to identify a toxic PLA_2 from the Indian banded krait venom and to neutralize the PLA_2 (BF-38) induced actions through synthetic herbal compounds and anti PLA_2 rabbit antiserum in *in vivo* and *in vitro* models.

Previously, eight variants of PLA_2 have been identified from the *B. fasciatus* venom samples from different regions[3,4] including a homodimeric PLA_2[19]. Apparently, different *B. fasciatus* geographic samples or different batch of the venom may show variations. Earlier, from Kolkata *B. fasciatus* snake venom (KBF), we have purified seven PLA_2, five three finger toxins (3FTx) from cDNAs[4]. In the present study in BF-38, the PLA_2 was identified from BFV which was combined with 3FTx in the ratio 9:1. Both could be identified as previously found PLA-X and 3FTx-RK, respectively[4]. We failed to separate this small amount of 3FTx combination from the PLA_2. The separation of such multimeric toxins (PLA_2 and 3FTx) is largely unexplored and represents an exciting area of toxin molecular evolutionary research as stated earlier by Possani *et al*[20]. They identified similar multimeric 'Taicatoxin' from the *Oxyuranus scutellatus* snake venom, which is composed of an alpha-neurotoxic 3FTx, beta PLA_2 and gamma Kunitz peptide in the ratio 1:1:4. In the present study, we found that BF-38 was lethal (17 mg/kg, i.v, bolus) in male albino mice. The LD_{50} for similar isoforms (PLA-X-1 and XI-2) from pooled *B. fasciatus* venom (purchased from Miami Serpentarium Laboratory, USA) was >50 µg/g mice[21,3]. The present PLA_2 + 3FTx lethality was more than the other PLA_2 purified from *B. fasciatus* venom. This unique association between these two toxic molecules (PLA_2 and 3FTx) providing complex lethal features of *B. fasciatus* envenomation may complicate therapeutic management further.

Krait snakebite management is a problem in South-East Asian countries especially in India, since no specific anti- krait venom antiserum is available. The rural people often use herbal preparations as an antidote[22]. Alam *et al*[23] identified 2- hydroxy -4-methoxy benzoic acid from the root extract of *Hemidesmus indicus*, which neutralized Russells' viper venom induced lethal and haemorrhagic effects in experimental animals. Alam and Gomes[24] and Chatterjee *et al*[16], also confirmed that some synthetic acids (2-hydroxy-4-methoxy benzoic acid, salicylic acid, p-anisic acid) are beneficial in neutralizing snake venom actions in *in vitro* and *in vivo* animal models. Probably due to its low molecular weight, the BF-38 showed poor antigenic property denoted by its low titre (1:32) against anti PLA_2 rabbit antiserum and faint immunogel diffusion bands. In the present study, the fold protection by the three synthetic herbal compounds was in the order BA > AA > SA. However, the folds of protection offered by the synthetic herbal compounds were greater than the commercial polyvalent snake venom antiserum, but it is less effective than anti PLA_2 rabbit antiserum. Combination studies with salicyclic acid, anisic acid and commercial polyvalent snake venom antisera did not produce greater neutralization of PLA_2 induced activities. However, 2-hydroxy-4-methoxy benzoic acid and commercial polyvalent snake venom antiserum produced a marginally by higher fold of protection (data not shown).

Earlier, Alam and Gomes[11] suggested that combination therapy (herbs and commercial polyvalent snake venom antiserum) may provide better protection

against snake envenomation. The findings of the present study also suggest that in case of banded krait envenomation the combination therapy with the right choice of herbal antagonist and commercial polyvalent snake venom antiserum may provide better management.

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