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PURIFICATION OF NON TOXIC ACIDIC PHOSPHOLIPASE A_2 FROM INDIAN COBRA (*NAJA NAJA*) VENOM

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ABSTRACT

Acidic phospholipase A_2 (NND-IV-PLA_2) was isolated from *NajaNaja*(Southern India) venom, was purified by anion and cation exchange chromatography. The acidic PLA_2 profile of eastern and southern region venom is distinctly different from that of the western regional venom, Southern regional acidic phospholipase A_2 did not inducemyotoxicity or anticoagulant activity and didn't induced edema. However, the acidic PLA_2 from all the regions follow the pattern of increasing catalytic activity with increase in the acidic nature of the PLA_2 isoforms.

Key words: Acidic phospholipase A_2,Hemolytic activity, Edema, Myotoxicity, LD_{50}, Anticoagulant activity.

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INTRODUCTION

Asia is the habitat of various species of venomous snakes including Elapidae, Hydrophilidae and Viperidae. According to statistics, the rate of snake bite and envenomation in Asia has been the highest among the world [1] .The characterization of snake venom components is important because a suitable medical treatment depends on a better understanding of the site and mode of action of the venom components. Amino acid sequences of many PLA_{2s} have been determined, with most being about120 amino acids long and having 14 Cys residues forming seven di sulfide bonds. Overall these proteins are closely related (>45% identity), with key residues that are required for catalysis and structure to be conserved [2-8]. Phospholipase A_2 (PLA_2) enzymes (EC 3.1.1.4) are among the best characterized components of snake venoms. PLA_2s catalyze the hydrolysis of glycerol phospholipids at the Sn-2 position, releasing lyso phospholipids and fatty acids kini[9]. They can be classified, based on their cellular localization, as: (A) cytosolic ($cPLA_2$); (B) secretory Ca+2-dependent ($sPLA_2$); and (C) intracellular Ca^{+2}-independent ($iPLA_2$) Chakraborti[10],Balsinde[11],Higuchi [12]. The $sPLA_2$s from various sources belong to one of the several characteristic groups such as IA, IB, IIA, IIB, IIC, IID, IIE, IIF, III, V, IX, X, XIA, XIB, XII, XIII and XIV(Burke and Dennis[13]. Snake venom PLA_2s are proteins belonging to the groups I and II. Those of the group I are found in the Elapidae family (Elapinae and Hydrophiinae), whereas those in group II are found in the Viperidae family (Viperinae and Crotalinae). The latter can be further subdivided into two types: Asp49 PLA_2s, which are catalytically active, and PLA_2homologues, which possess most commonly a Lys49 residue anddo not have catalytic activity Arni and Ward[14], Kini[15], Lomonte[16]. Venom PLA2s display a wide range of isoelectric points, from acidic to highly basic Kini[15], Andrião-Escarso[17]. All acidic PLA_2s purified from Viperidae venoms present an Asp residue at position 49. These isoforms usually have a higher catalytic activity than basic PLA_2s upon conventional substrates in vitro Fernandez[18]. In spite of this, many acidic PLA_2s are not lethal or show a weak lethal potency in mice Araújo; Andrião- Escarso[17].Nevertheless, some of these enzymes can induce significant pharmacological effects such as inhibition of platelet aggregation, hypotension, bactericidal, and antitumor Serrano[19].In other cases, acidicPLA2s appear to lack a significant toxic effect, which suggests that their role in snake venoms might be related to a digestive function. So we purified the acidic PLA_2 from south Indian cobra venom and tested for its non-toxic nature.

MATERIALS AND METHODS

*NajaNaja*venom was milked, lyophilized from Western Ghats of SouthIndia (Hassan). CM-Sephadex C-25, Sephadex G-50, DEAE-Sephadex A-50parabromophenacyl bromide (pBPB), Linoleic acid and egg Phosphatidyl Choline (PC) were from Sigma chemical company (St. Louis, USA) All other reagents were of analytical grade. Fresh human blood samples were collected from healthy volunteers from the Department of Biochemistry, University of Mysore, India, who were non-smokers and were not on drugs ten days prior to blood withdrawal. Male Swiss Wistar mice weighing 20-25 g were used for pharmacological studies.

CM-Sephadex C-25 column chromatography

Southern regional Indian Cobra (*NajaNaja*) venom was fractionated on a CM-Sephadex C-25 column. A solution containing 200 mg of lyophilized whole venom in 1 ml of 0.02 M Sodium phosphate buffer, pH 7.0 was loaded on a CM-Sephadex C-25 column (4 cm X 20 cm) equilibrated with the same buffer. The column was Eluted by a stepwise

gradient of phosphate buffers of various molars and pH as indicated in Fig.1. The flow rate was adjusted to 30 ml per hour and 3 ml fractions were collected. Protein elution was monitored at 280 nm spectrophotometrically. Peak fractions were pooled, desalted, lyophilized and stored at -20^{0}C.

DEAE Sephadex A-50 column chromatography

The NN-I peak was further fractionated on DEAE Sephadex A-50 column (2 cm X 20 cm). The column was equilibrated with 0.01 M tris-HCI buffer (pH .0). A solution of 15 mg of the NN-I fraction in equilibration buffer was loaded onto the column. The column was eluted with stepwise NaCI gradient as indicated in Fig. 2. Two ml fractions were collected at a flow rate of 20 ml/h. Elution was monitored at 280 nm. The PLA_2 fractions were pooled individually, desalted, lyophilized and stored at -20^{0}C.

High performance liquid chromatography

Purified NND-IV PLA_2 was subjected to RP-HPLC on a Vydac C_{18} (250 X 4.6 mm, 10 um particle size, 300 A pore size) column in a Shimadzu LC-10AVP system with dual wavelength detector. The column was equilibrated with 0.1% Trifluoroacetic acid (TFA) and eluted using a linear gradient of 70% Acetonitrile in 0.1% TFA at a flow rate of 1 ml/min.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was carried out for whole venom, NN-I fraction and the purified PLA_2 (NND-I PLA_2) on 12.5% polyacrylamide get usedTris-Glycine buffer (pH 8.3) according to the method of Davis [26]. SDS-PAGE was carried out for the same samples according to the method of Laemmle[27] on 12.5% polyacrylamide gel containing o. 1% SDS. The bands were visualized by staining with Coomassie brilliant blue R-250.

Phospholipase A_2 assay

PLA_2 activity was assayed by estimating the free fatty acid released by the action of the enzyme on egg Phosphatidyl choline (PC) as described earlier[25]. The reaction mixture contained 1 µmol of egg PC, 0.5 ml of ether, 50 mMTris-HCI buffer (pH 7.5), 40 µmol of Ca^{2+} and 0.1 µg of enzyme in a final volume of 1 ml. The enzyme activity was expressed as micromole equivalents of linoleic acid released per minute per milligram protein.

Hemolytic activity

Direct and indirect hemolytic activities were assayed as described by Bowman and Kalletta[31.] The substrate for the direct hemolytic activity was prepared by suspending 1 ml of packed fresh human RBC in 9 ml of phosphate buffered saline (PBS). For indirect hemolytic activity 1ml of the fresh hen's egg yolk was included in the above suspension. One ml of the suspension was incubated with different concentrations of PLA_2 for 45 min at 37^{0}C. The reaction was stopped by adding 9 ml of ice cold PBS. The suspension was centrifuged at 2000 rpm for 20 min. The released hemoglobin was read at 530 nm.

Edema inducing activity, anticoagulant activity, myotoxicity and lethal toxicity,

Lethal potency (LD_{50}) was determined according to the method of Meier and Theakston [20]. PLA_2 dissolved in saline was injected intraperitoneally (i.p.) In mice in doses ranging from 4 to 20 mg per Kg body weight. The animals were observed up to 24 h for signs of toxicity. Edema inducting activity was determined according to the method of Yamakawa et al. [21]as modified by Vishwanath[22]. Anticoagulant activity was assayed by estimating the prothrombin time according to the method of Quick[23]. Myotoxicity was assessed by following the change in the serum lactate dehydrogenase and Creatine phosphokinase activities in mice injected (i.p.) with the PLA_2 as

described earlier[24]. The experimental protocol for the animal toxicity study has been cleared by the animal ethics committee, Government of India.

RESULT

NajaNaja (Southern India) venom upon stepwise fractionation on a CM Sephadex C-25 column resolved into 18 peaks (Fig.1). Eleven of these peaks exhibited PLA_2activity and were numbered from NN-I to NN-XI according to the scheme adopted previously [25]. The unbound fraction (NN-I) accounted for 14.5% of total protein and 88.52% of the total phospholipase activity loaded onto the column. Fraction-I, (NN-I) was subjected to anion exchange chromatography on DEAE Sephadex A-50 column, which resolved it into four well separated peaks designated NND-I to NND-IV (Fig 2). The fourth peak (NND-IV) was the largest fraction and accounted for 42.8% of the total protein and 78.3% of the total phospholipase activity recovered from the DEAE column. It was observed that the specific activity of the four PLA_2s increased with the increase in the acidity (retention in the DEAE column) of the molecule. The summary of purification is tabulated in Table 1.

Fig .3.Shows the RP-HPLC profile of NND-IV which gave a symmetric sharp peak with a retention time of 37.79 min. The Electrophoretic pattern of whole venom, NN-I, fraction and purified NND-IV- PLA_2 when subjected to SDS PAGE and native PAGE under basic conditions. Molecular weight as determined by MALDI mass spectrometry was found to be 14,000 Da as similar to SDS PAGE result. NND-IV released free fatty acid when egg Phosphatidyl choline was used as substrate. The specific activity was 29.19µmoles fatty acid released/min/mg protein. It lysed 50% of RBC in 45 min at as low a concentration as 100ng by indirect lytic activity. However it did not show direct lytic activity. The specific activity of the acidic ISO forms (NND-I to NND-IV) increased with an increase in the retention time of the molecule in DEAE column. NND-I was the least active with specific activity of 0.59 µmoles fatty acid released/min/mg while NND-IV was the most active isoform. NND-IV-PLA_2 was non-toxic to mice up to an i.p. Dose of 20 mg/Kg body weight. The animals did not exhibit signs/symptoms of neurotoxicity. NND-IV- PLA_2 had no effect on prothrombin time and on serum levels of LDH and CPK activities. Edema induced was not significant even at the highest dose of 50 µg tested.

Table 1

Summary of Purification of acidic PLA_2s from Southern Indian Cobra (Naja Naja) venom

Step	Fraction	Total Protein (mg)	Total activity* (µmoles fatty acid released/min)	Specific activity (µmoles fatty acid released/min/mg protein)	Yield* Protein (%)	Activity
CM-Sephadex C-25	Whole venom	200	523	2.615	100	100
	NN-I	29	463	15.96	14.5	88.52
DEAE-Sephadex A-50	NN-I	16	255.4	15.96	100	100
	NND-I	0.32	0.19	0.59	2	0.074
	NND-II	1.85	3.90	2.108	11.56	1.527
	NND-III	3.03	65.2	21.5	18.93	25.528
	NND-IV	6.85	200	29.19	42.8	78.308

Total Enzyme activity was estimated in the pooled peaks.

***** Percent contribution to the total protein and enzyme activity loaded onto the columns.***

Figure.1.
CM-Sephadex C-25 Column chromatography .Fig.2.DEAE Sephadex A-50 Column chromatography

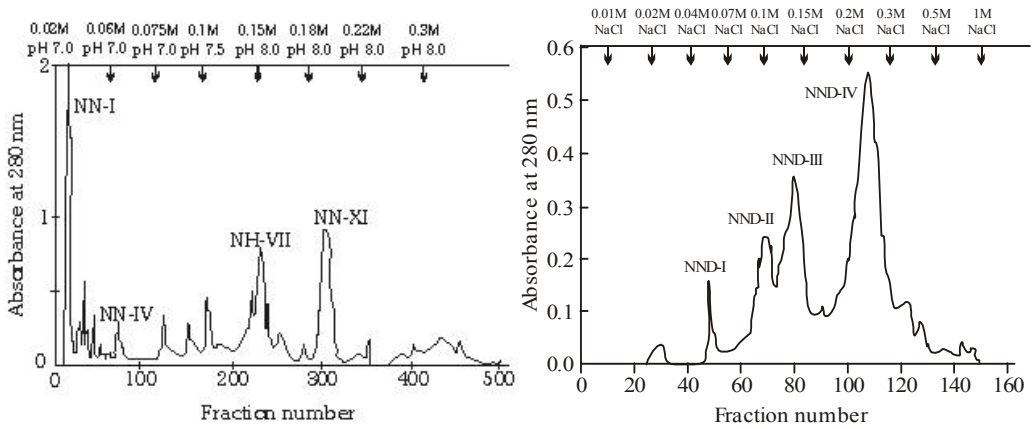
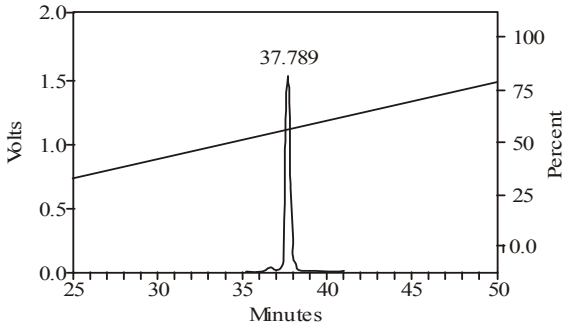


Figure.3.
RP-HPLC of NND-IV PLA$_2$



DISCUSSION

Variation in the composition of acidic PLA_2 in Indian cobra venom has not been studied. Though the protein content of the acidic fraction of the two regions is almost the same, the eastern regional *NajaNaja* venom acidic fraction is twice as active as the corresponding western regional acidic fraction. The western regional venom has been reported to have five acidic PLA_2isoform[9]. A similar fractionation protocol for southern regional venom gave four distinctly separable acidic PLA_2isoenzymes. Representation of the different isoforms in the two regional venoms was very different. In the eastern region venom the most acidic NND-IV- PLA_2, as deduced from the retention in the DEAE column as well as mobility in native PAGE, accounted for 76% of the PLA_2 activity recovered from the anion exchange column. The only comparable isoform in terms of protein content to this PLA_2 in the western region was NN-I_{2d}, which constituted 33% of the protein. However it shows only one fifth of the catalytic activity of NND-IV PLA_2. Though NN-I_{2e} showed highest specific activity (16 µmoles) in the western regional venom, it constituted only 18.7% of the acidic fractionof protein and contributed about 20% of the enzyme activity. This is not comparable to either the protein content or activity of NND-IV- PLA_2. However, the specific activity of NND-IV- PLA_2 is far less compared to the acidic PLA_2 (2180 µmoles)

isolated by Hazlett and Dennis [26] from *Najanaja* venom. Further investigation of this aspect of enzyme is required. PLA_{2s} widely vary in catalytic potencies. A proper explanation for such a variation has not been given. Acidic PLA_2isoforms are considered catalytically more active than the basic ones. In the present investigation we have noted that among acidic PLA_{2s}, the enzyme activity increases with an increase in the acidic nature of the molecule. This may be due to the faster release of the product fatty acids by the presence of repulsive acidic groups on the enzyme surface. Similar relationship between enzyme activity and the acidic nature of the molecule is also seen in acidic NND-IV-PLA_2s of Najanaja (Western region, India) venom [28] and in the various Pa-PLA_2 isoforms of Pseudechisaustrali venom [30].

Acidic PLA_2 are generally considered less toxic with few or no pharmacological activities. Some of the pharmacological properties reported in acidic NND-IV- PLA_2s are cardiotoxic[32], myotoxic[33], and edemainducing activities [22]. NND-IV-PLA_2(southern region, India) was non-toxic to mice similar to the acidic PLA_2s isolated from western regional venom [28] and eastern region34. It did not induce either myotoxicity or anticoagulant activity. However, while NND-IV- PLA_2 was non edematic, all the acidic NND-IV- PLA_2s isolated from the western regional venom induced edema [28].

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