Purification and characterization of a platelet aggregation inhibitor acidic phospholipase A2 from Indian saw-scaled viper (Echis carinatus) venom

K. Kemparaju\textsuperscript{a}, T.P. Krishnakanth\textsuperscript{b}, T. Veerabasappa Gowda\textsuperscript{a,*}

\textsuperscript{a}Department of Studies in Biochemistry, University of Mysore, Manasagangotri, Mysore, India
\textsuperscript{b}Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore, India

Received 5 August 1998; accepted 9 February 1999

Abstract

An acidic phospholipase A\textsubscript{2} (EC-I-PLA\textsubscript{2}) has been purified from the Indian saw-scaled viper (Echis carinatus) venom through a combination of column chromatography and electrophoresis. EC-I-PLA\textsubscript{2} has a molecular weight of 16000 by SDS-PAGE. It was focussed between pH 4.2 and 4.8 by isoelectro focusing. EC-I-PLA\textsubscript{2} was non-lethal to mice and devoid of neurotoxicity, myotoxicity, anticoagulant activity and cytotoxicity. It induced mild oedema in the foot pads of mice. The purified PLA\textsubscript{2} inhibited ADP, collagen and epinephrine induced human platelet aggregation and the inhibition was both dose and time dependent. © 1999 Elsevier Science Ltd. All rights reserved.

1. Introduction

Phospholipase A\textsubscript{2} (EC.3.1.1.4., PLA\textsubscript{2}) is an esterolytic enzyme which catalyzes the hydrolysis of the 2-acyl groups in sn-3 phosphoglycerides. The hydrolytic

\textsuperscript{*} Corresponding author.
products are fatty acids and lysosphoglycerides (Dennis, 1983). The multimolecular forms of PLA₂ are widely distributed in nature. The richest sources of PLA₂ are the exocrine secretions of mammalian pancreas and the venom of snakes and bees. It is not surprising therefore that the best characterized PLA₂ have been isolated from these sources. PLA₂ enzymes from pancreas and snake venoms undoubtedly serve a digestive function. However, in addition, the latter have evolved into lethal toxins with target specific actions. Hence, these induce a spectrum of pharmacological effects such as pre or postsynaptic neurotoxicity, myotoxicity, cardiotoxicity, initiation or inhibition of platelet aggregation, haemolytic, convulsant, hypotensive, anticoagulant and oedema inducing activities (Kini and Evans, 1989, and references therein).

The saw-scaled vipers (genus *Echis*) have an extensive geographical distribution (for references see Gillissen et al., 1994). Their venom is a complex mixture of predominantly high molecular weight proteins, peptide toxins and glycoproteins (Desmond et al., 1991). There are reports on the variation in the composition of their venom due to geographical distribution. (Iddon et al., 1988; Gillissen et al., 1994). Their bites primarily cause severe bleeding and haemorrhage. Several biologically active components have been isolated and studied from their venoms (for references see Gillissen et al., 1994). However, information regarding the PLA₂ enzymes of *Echis* venoms is limited. In the present study we report the purification and characterization of a platelet aggregation inhibitor PLA₂ from Indian saw-scaled viper (*Echis carinatus*) venom.

2. Materials and methods

Lyophilized *Echis carinatus* venom was purchased from Irula Snake Catcher’s, Madras, India. CM-Sephadex C-25 (4.5 meq/g) and low molecular weight markers were from Pharmacia Fine Chemicals, Uppsala, Sweden. Bio-Gel P-30 was from Bio-Rad Labs., Richmond, USA. Linoleic acid, 2-nitroso-β-naphthol, broad range ampholytes (pH, 3–10) gel filtration molecular weight marker proteins, ADP, collagen and epinephrine were purchased from Sigma, St. Louis, USA. Phosphatidyl choline (PC) from hen’s egg yolk was prepared according to the method of Singleton et al. (1965). Male Swiss Wistar mice (20–22 g) were from Central Animal House, University of Mysore, India. All other chemicals and reagents were of analytical grade and the solvents were redistilled before use.

2.1. CM-Sephadex C-25 column chromatography

*Echis carinatus* venom was fractionated on a CM-Sephadex C-25 column as described previously (Kemparaju et al., 1994). Briefly, whole venom (660 mg) in 4 ml 0.02 M phosphate buffer pH 7.0 was applied to a column (1.2 × 110 cm) previously equilibrated with the same buffer. The column was eluted stepwise using phosphate buffer of various molarities (0.02–0.3 M) and pH 7.0–8.0.
Fractionation was carried out at 20°C at a flow rate of 40 ml/h and 5 ml fractions were collected. The protein elution was monitored at 280 nm. The non-retained acidic fraction is now designated as EC-IC-PLA2.

2.2. Electrophoresis

EC-IC-PLA2 was subjected to electrophoresis on 7.5% polyacrylamide gels (0.3 × 13 × 15 cm) as described by Davis (1964). A total of 330 mg (each time 30 mg in 1.5 ml saline) was subjected to PAGE using Tris–glycine buffer pH 8.3 with a current of 50 mA at 20°C for 6 h. After electrophoresis, a portion of the gel (about 2 cm) was sliced longitudinally and stained for proteins with 0.1% Coomassie brilliant blue R-250. The non-stained gel portion was sliced corresponding to the protein bands of the stained gel piece. Proteins from the gel slices were separately electroeluted providing similar conditions for 12 h. Proteins were collected in 3000 cut off dialysis bags. The most acidic protein fraction which moved along with the dye band was called EC-IE-PLA2.

2.3. Bio-Gel P-30 column chromatography

EC-IE-PLA2 (12 mg) in 4 ml 0.1 M NaCl containing 2% sodium dodecyl sulfate (SDS) was thoroughly mixed and kept at 37°C for 1 h. It was then loaded onto Bio-Gel P-30 column (1.5 × 70 cm) which was pre-equilibrated with 0.1 M NaCl. The column was eluted using 0.1 M NaCl with a flow rate of 20 ml/h and 3 ml fractions were collected at 20°C. Protein elution was monitored at 280 nm using a spectrophotometer. Individual protein peaks were pooled and dialyzed in saline before use.

2.4. Phospholipase A2 activity

The assay mixture (1 ml) contained 1 μmol egg PC, 0.3 ml diethyl ether, 50 mM Tris–HCl buffer pH 7.5, 40 μmol Ca2+ and 25 μg enzyme. The free fatty acid released by the action of enzyme on PC was estimated as described by Bhat and Gowda (1989). The specific activity was expressed as nmol free fatty acid released/min/mg protein. For K_m determination, 0–2.2 μmol PC was used in the assay mixture.

The positional specificity was determined using autoclaved E. coli cells with the phospholipids labeled at sn-2 position with (14C) oleate as described by Franson et al. (1974).
2.5. Molecular weight determination

The molecular weight of purified PL\(\text{A}_2\) was determined by gel filtration on a Sephadex G-50 column (90 × 1 cm) according to the method of Andrews (1964). Insulin (6000), lysozyme (14,000), soybean trypsin inhibitor (20,100) and carbonic anhydrase (29,000) were used as standard markers. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 15%) was carried out according to the method of Laemmli (1970). Low molecular weight marker proteins (14–90 kDa) were used. The gel was stained with 0.1% Coomassie brilliant blue R-250.

2.6. Isoelectric focussing

Isoelectric focussing was carried out using Pharmacia 2117 multiphor II electrophoresis system on 7.5% polyacrylamide gel using ampholine with a pH range of 3–10 (Vesterberg, 1972). A portion of the gel was stained for the protein and the remaining portion was cut into 0.5 cm transverse slabs and soaked in 4 ml distilled water for pH determination.

2.7. Fluorescence and protein determination

Fluorescence emission of 28 nmol protein in 2 ml saline was recorded after exciting at 280 nm in a Shimadzu RF 5000 spectrofluorimeter. Protein concentration was determined as described by Lowry et al. (1951).

2.8. Preparation of platelet rich and platelet poor plasma

The method of Ardlie and Han (1974) was employed. Human blood was collected from the vein of healthy donors who were non-smokers and had not taken any medication for the previous 15 days. Nine volumes of the blood were collected into 1 volume of acid citrate dextrose (93 mM sodium citrate, 7 mM citric acid and 140 mM glucose pH 6.5). The citrated blood was immediately centrifuged for 10 min at 90 × g at room temperature. The supernatant was called platelet rich plasma (PRP). The remaining blood was centrifuged for 15 min at 500 × g and the supernatant obtained was the platelet poor plasma (PPP). The platelet concentration of PRP was adjusted with PPP to 225,000 ± 25,000 platelets/μl. The PRP was kept at 37°C and was used within 2 h. All the above preparations were carried out using plasticwares or siliconized glasswares.

2.9. Platelet aggregation

The turbidimetric method of Born (1962) was followed using a chronolog dual channel aggregometer connected to a omniscribe dual pen recorder to record the
light transmission as a function of time. Aliquots of PRP (0.45 ml) were preincubated with PLA₂ toxin (0–20 μg/0.45 ml PRP) for 2 min in a siliconized cylindrical glass cuvette under constant stirring. The aggregation was initiated by the addition of agonists such as ADP (76 μM), collagen (approximately 2 μg/ml) and epinephrine (22 μM) separately. The aggregation was followed for a period of 4 min. The aggregation trace is the plot of light transmission between PRP and PPP baseline, which represent 0% and 100% aggregation, respectively. Percent inhibition of aggregation was calculated using the value of control (without PLA₂ toxin) to be 100%.

2.10. Oedema inducing activity

The procedure of Yamakawa et al. (1976), modified by Vishwanath et al. (1987) was followed. Groups of six mice were injected in the right foot pads with different doses of PLA₂ in 20 μl saline. The left foot pads received 20 μl saline which served as controls. The increase in weight due to oedema was calculated as the oedema ratio by the following equation:

\[
\text{Oedema ratio} = \frac{\text{weight of the oedematous leg} \times 100}{\text{weight of normal leg}}.
\]

Minimum oedema dose is the amount of protein required to cause an oedema ratio of 120%.

2.11. Determination of LD₅₀, myotoxicity, cytotoxicity and anticoagulant activity

The method of Meier and Theakston (1986) was followed for LD₅₀ determination. Myotoxicity was determined by the method of Gutierrez et al. (1989). EC-I-PLA₂ as i.p. dose of 14 mg/kg body weight was injected into a group of six mice. After 2 h blood samples were drawn by retroorbital puncture and lactate dehydrogenase activity was determined in the serum samples using Span diagnostic kit. Cytotoxicity was determined based on trypan blue exclusion using Ehrlich ascites tumor cells grown in the peritoneal cavity of Swiss albino mice according to the method of Chwetzoff et al. (1989). Anticoagulant activity was measured by prothrombin times according to the method of Quick (1966).

2.12. Haemolytic activity

Direct and indirect haemolytic activity was assayed by the method of Boman and Kauttla (1957). Freshly collected human red blood cells from healthy donors were used for the assays. Activity was expressed as percentage of haemolysis.
3. Results and discussion

An acidic PLA₂, EC-I-PLA₂, has been purified from Indian saw-scaled viper venom. The purification was achieved using CM-Sephadex C-25 column chromatography, PAGE followed by gel filtration on a Bio-Gel P-30 column (Fig. 1). PAGE pattern of EC-IC-PLA₂ recovered from CM-Sephadex C-25 column is shown in Fig. 2A. After electroelution, about 90% of the protein and 125% of the phospholipase activity were recovered from the gel slices (data not shown). The fast moving protein band, EC-IE-PLA₂, contributed for about 4% of the protein and 31% of the phospholipase activity loaded onto PAGE. EC-IE-PLA₂, although it gives a single band in PAGE (Fig. 2B), showed 2 bands in SDS-PAGE (Fig. 3B). EC-IE-PLA₂, when subjected to gel filtration on a Bio-Gel P-30 column, and in the presence of 8 M urea or 7 M guanidinium hydrochloride eluted as a single peak (not shown). However, in the presence of 2% SDS upon gel filtration, EC-IE-PLA₂ was resolved into three peaks (Fig.1). This suggested that EC-IE-PLA₂ is an association of three proteins with different molecular weight. The first and last peak did not show phospholipase activity, but contributed for about 4% and <1% of the protein loaded on to the column, respectively. The middle major peak designated as EC-I-PLA₂ showed phospholipase activity. About 89% of the protein and 132% of the activity loaded on to the column were recovered in this peak. A summary of the purification of EC-I-PLA₂ is given in Table 1. The proteins corresponding to the first and second peak resolved in gel filtration are also seen in the SDS-PAGE pattern of EC-IE-

![Fig. 1. Bio-Gel P-30 column chromatography of EC-IE-PLA₂. (—) corresponds to EC-I-PLA₂.](image-url)
PLA₂, but the protein of the third peak was not noticed in the SDS-PAGE pattern of EC-IE-PLA₂. This could possibly be due to its occurrence in trace amount (<1%).

EC-I-PLA₂ was homogeneous as it showed a single band in PAGE (Fig.2C), SDS-PAGE (Fig. 3C) and isoelectro focussing (not shown). The estimated molecular weight was 16,000 determined by SDS-PAGE and 15,800 by gel filtration on a Sephadex G-50 column. The molecular weight agrees with the range generally reported for monomeric snake venom PLA₂ (Desmond et al., 1991; Kemparaju et al., 1994). The isoelectric point (pI) was estimated to be between 4.2 and 4.8. This agrees with the pI values reported for two acidic PLA₂ (pI 4.6) of Kenyan E. carinatus leakeyi venom (Desmond et al., 1991). EC-I-PLA₂ exhibited a fluorescence emission maximum at 348 nm when excited at 280 nm, suggesting the exposed tryptophan residues in the molecule.

EC-I-PLA₂ released radiolabeled fatty acid from E. coli cells containing phospholipids specifically labeled at the sn-2 position with (14C) oleate. EC-I-PLA₂ showing a specific activity of 544 nmol/min/mg. Kₘ determination by double reciprocal plot (Lineweaver and Burk, 1934) was found to be 1.66 mM. The specific activity and Kₘ values of EC-I-PLA₂ are greater than the values reported for EC-IV-PLA₂ (400 nmol/min/mg and 0.25 mM respectively) which is a basic PLA₂ isolated from the same venom (Kemparaju et al., 1994). This
suggested that EC-I-PLA2 appears to have less affinity towards the substrate PC when compared with EC-IV-PLA2. The pH and temperature optima (pH 7.5 and 37°C, respectively) are comparable with the values reported for most of the venom PLA2.

EC-I-PLA2 exhibited indirect haemolytic activity as it converts lecithin into lysolecithin which in turn lyses the erythrocyte membrane. About 38% haemolysis was observed when 25 μg enzyme was incubated for 10 min in the reaction mixture. EC-I-PLA2 did not show direct haemolytic activity, possibly due to the inability of the enzyme to hydrolyze inner membrane phospholipids of erythrocytes (Rosenberg, 1986). EC-I-PLA2 was not lethal to experimental mice up to an i.p. dose of 14 mg/kg body weight. In contrast, the basic PLA2 isolated from the same venom (Kemparaju et al., 1994) was toxic with an i.p. LD50 value of 5 mg/kg body weight. Although this result supports the view that acidic PLA2 is less toxic and enzymatically more active than basic PLA2 (Rosenberg, 1986), lethal potencies of venom PLA2 can neither be correlated to its catalytic property nor to its isoelectric point (Dhillon et al., 1987). For instance, an acidic PLA2 from N. naja sputatrix venom (Tan and Arunmozhiarasi, 1989) possess an i.v. LD50 value of 0.86 mg/kg/body weight in mice, and enzyme activity of 2130 μmol/min/mg. EC-I-PLA2 induced non-haemorrhagic oedema in the foot pads of mice.
<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Protein recovery (%)</th>
<th>Total activity(^a) (nmol fatty acid released/min)</th>
<th>Specific activity (nmol fatty acid released/min/mg protein)</th>
<th>Fold purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrophoresis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC-IC-PLA(_2)</td>
<td>330.0</td>
<td>100.0</td>
<td>18810</td>
<td>57</td>
<td>1.0</td>
</tr>
<tr>
<td>EC-IE-PLA(_2)</td>
<td>15.2</td>
<td>4.6</td>
<td>6019</td>
<td>396</td>
<td>6.9</td>
</tr>
<tr>
<td>Bio-gel P-30 column</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC-IE-PLA(_2)</td>
<td>12.0</td>
<td>100.0</td>
<td>4752</td>
<td>396</td>
<td>1.0</td>
</tr>
<tr>
<td>EC-I-PLA(_2)</td>
<td>10.7</td>
<td>89.0</td>
<td>5821</td>
<td>544</td>
<td>1.4</td>
</tr>
</tbody>
</table>

\(^a\) Total enzyme activity was estimated in the electroeluted/pooled fractions.

EC-IC-PLA\(_2\) = fraction from CM-Sephadex C-25 column; EC-IE-PLA\(_2\) = fraction from electrophoresis. Values represent the mean of two experiments only.
The minimum oedema dose (2.25 μg) agrees with the values reported for the acidic PLA$_2$ TFV PL-Ia (2.2 μg) and TFV PL-Ib (3 μg) of _T. flavoviridis_ venom (Vishwanath et al., 1987) and NN-I2c-PLA$_2$ (2.5 μg) and NN-I2e-PLA$_2$ (2.7 μg) of _N. naja naja_ venom (Rudrammaji and Gowda, 1998).

EC-I-PLA$_2$ inhibited human platelet aggregation when induced separately by ADP, collagen and epinephrine. The inhibition was both concentration (Fig. 4) and time (Fig. 5) dependent in all the cases studied. The inhibition was pronounced with epinephrine and collagen when compared with ADP. At a concentration of 20 μg EC-I-PLA$_2$, the percent inhibition was 82 ± 5, 72 ± 7 and 33 ± 2 (values are mean ± S.D.; _n_ = 3), respectively, for epinephrine, collagen and ADP induced aggregation. The calculated IC$_{50}$ values ware 12.8 and 13.8 μg for collagen and epinephrine induced aggregation, respectively. For ADP induced aggregation the IC$_{50}$ value could not be calculated as there was only 33% inhibition observed when 20 μg EC-I-PLA$_2$ was used in the reaction mixture. The platelet aggregation inhibition was not tested beyond 20 μg EC-I-PLA$_2$ in all the cases. Fig. 6 shows the typical aggregation pattern when stimulated by collagen.

Our results agree well with the aggregation properties of two acidic PLA$_2$ isolated from _T. gramineus_ (Ouyang and Huang, 1983) and _A. halys_ (Ouyang et al., 1983) venoms, which inhibited thrombin, ADP, collagen, and sodium arachidonate induced rabbit platelet aggregation. Three acidic PLA$_2$ of _N. naja naja_ venom also inhibited ADP, collagen and epinephrine induced human platelet aggregation (Rudrammaji, personal communication). However, EC-I-PLA$_2$ differs from the aggregation properties of an acidic PLA$_2$ of _A. acutus_ venom (Chen and Chen, 1983).

Fig. 4. Effect of EC-I-PLA$_2$ on platelet aggregation induced by ADP (□), collagen (○) and epinephrine (●). EC-I-PLA$_2$ (0–20 μg/0.45 ml PRP) was preincubated for 2 min at 37°C. Platelet aggregation was initiated separately by the addition of ADP (76 μM), collagen (approximately 2 μg/ml) and epinephrine (22 μM) and was recorded using platelet aggregometer. Values represent mean ± S.E.M. of three experiments.
Fig. 5. Effect of preincubation time on platelet aggregation induced by ADP (■), collagen (○) and epinephrine (●). EC-I-PLA₂ (5 μg/0.45 ml PRP) was preincubated for different time intervals (2–20 min) at 37°C. Platelet aggregation was initiated separately by adding ADP (76 μM), collagen (approximately 2 μg/ml) and epinephrine (22 μM). Aggregation was recorded using platelet aggregometer. Values represent mean ± S.E.M. of three experiments.

Fig. 6. Platelet aggregation trace for inhibition of collagen induced aggregation by EC-I-PLA₂. EC-I-PLA₂ (0–20 μg/0.45 ml PRP) was initiated by adding collagen (approximately 2 μg/ml) and recorded using platelet aggregometer. Each trace is a representative of three experiments.
1989) which showed biphasic effects on human platelet aggregation. Several PLA₂ interfering in platelet function have been isolated and studied from snake venoms (Kini and Evans, 1990, and references therein). PLA₂ which affect platelet aggregation includes two classes (Kini and Evans, 1989). Class A enzymes exhibit biphasic effects, where, under certain conditions, they both inhibit and initiate aggregation, whereas class B PLA₂ only inhibit platelet aggregation. Thus EC-I-PLA₂ belongs to the latter class. EC-I-PLA₂ is devoid of myotoxicity, anticoagulant activity and cytotoxicity (on EAT cells).

Thus, in conclusion, EC-I-PLA₂, being a non-lethal PLA₂, appears to play a role in the digestion of the prey. However, synergism with other components of the venom cannot be ruled out in inducing toxic effects.

References


Chen, R.H., Chen, Y.C., 1989. Isolation of an acidic phospholipase A₂ from the venom of Agkistrodon acutus (Five pace snake) and its effect on platelet aggregation. Toxicon 27, 675–682.


of neutralizing the haemorrhagic activity of West African *Echis carinatus* (carpet viper) venom. Toxicon 26, 167–179.


