

Full Length Research Paper

Characterization of phospholipase A₂ (PLA₂) from *Echis ocellatus* venom

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Phospholipase A₂ (EC. 3.1.1.4) was isolated and partially characterized from the venom of *Echis ocellatus*. The enzyme was purified 13.5-fold with a yield of 86.69% on DEAE-Sephadex G-75 column. The PLA₂ from *E. ocellatus* venom had broad pH and temperature ranges with optima of 7.5 and 40°C respectively. Initial velocity studies for the determination of kinetic constants with L- α -lecithin as substrate revealed a K_m and V_{max} of 1mg/ml and 0.35 μ moles/min respectively. The enzyme activity was enhanced by Ca²⁺ and strongly inhibited by Mg²⁺ and Co²⁺. Cu²⁺ was fairly inhibitory to the enzyme. The relevance of these findings towards understanding the biochemistry of *E. ocellatus* envenomation and development of antivenom for *E. ocellatus* venom is discussed.

Key words: *Echis ocellatus*, PLA₂, venom.

INTRODUCTION

The saw-scaled viper *Echis ocellatus* is among the commonest cause of envenomation in West Africa, being responsible for approximately 95% of the reported cases in northern Nigeria causing several hundreds of deaths annually (Bharati et al., 2003; Hasson et al., 2003). Snake venoms are composed of complex mixture of active substances, mainly peptide and proteins which are able to interfere with the course of several biological processes including thrombosis by affecting platelet aggregation and blood coagulation (Rossing and Tans, 1992). Some of these proteins include enzymes like phospholipase A₂ and metalloproteases (Sallau et al., 2005).

Phospholipase A₂ (PLA₂) are enzymes found to catalyze the hydrolysis of fatty ester in the 2-position of 3-sn-phospholipid to release fatty acid and lysophospholipid; the fatty acid so formed may act as either a second messenger or a precursor of eicosanoids (Fuly et al., 2002; Huang and Mackessy, 2004). The enzyme from snake venoms is primarily used for trophic and defense functions in most species but show wide range of pharmacological activities such as neurotoxicity, myotoxicity, cardiotoxicity, but with a greater impact on platelet aggregation and blood coagulation (Bharati et al., 2003; Hasson et al., 2003; Shashidharamurthy and Kempa-

raju, 2006; Higuchi et al., 2007). The enzyme is therefore a highly interesting molecule to venom researchers because in addition to digesting the prey, it mimics the pathological action of the whole venom poisoning (Shashidharamurthy and Kemparaju, 2006).

PLA₂ have been purified and characterized from *Lachesis muta*, *Naja naja* and *Bothrops leucurus* snake venoms (Fuly et al., 2002; Shashidharamurthy and Kemparaju, 2006; Higuchi, et al., 2007) and its inhibition in *Echis carinatum* venom by *Guiera senegalensis* extract has been demonstrated (Sallau et al., 2005). However, information concerning the counterpart enzyme in *E. ocellatus* that could be important in antivenom design is scanty. Therefore, in this study, we report on the purification and characterization of PLA₂ from *E. ocellatus* venom with the view to obtain kinetic data for the enzyme and establish other characteristics that would be important in achieving the said objective.

MATERIALS AND METHODS

Reagents were purchased from Sigma Chemical Company, St. Louis, USA. Freeze dried *E. ocellatus* venom was a gift from Dr. Y.P. Ofemile of the Department of Physiology and Pharmacology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria.

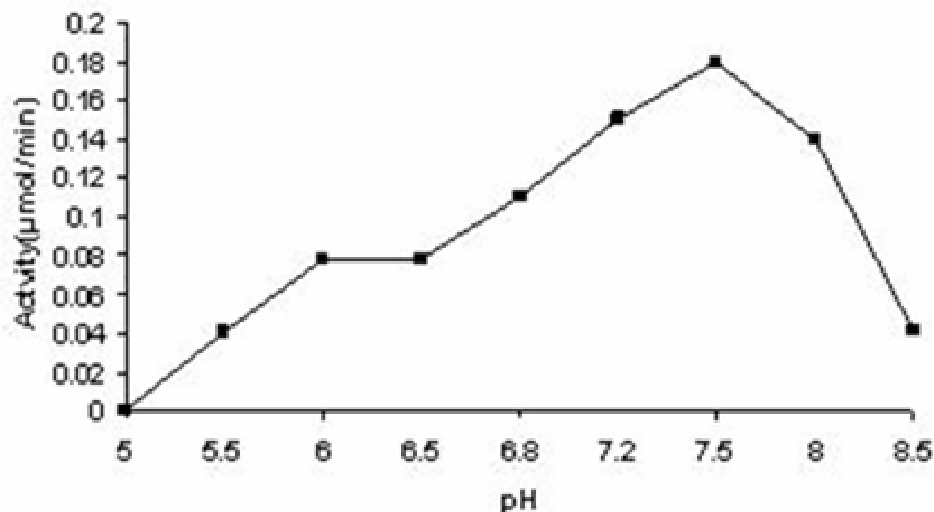
Partial purification of phospholipase A₂

Two ml of 10 mg/ml of crude *E. ocellatus* venom was loaded onto DEAE cellulose column (1.5 x 33 cm) pre-equilibrated with 50 mM

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Table 1. Purification profile of phospholipase A₂ isolated from *Echis ocellatus* venom.

Purification step	Protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min/mg)	Purification fold	Yield (%)
Crude	6.70	16.00	2.39	1.00	1.00
Ion Exchange on DEAE- cellulose	1.20	8.00	6.67	2.80	50.00
Gel filtration on Sephadex G-75	0.43	13.87	32.26	13.50	86.69

**Figure 1.** Effect of pH on PLA₂ activity.

phosphate buffer pH 6.8. The column was eluted stepwise with NaCl gradient (0.01 – 0.1 M) at a flow rate of 1ml/min. Twenty, 2 ml fractions were collected and assayed for Phospholipase A₂ activity and total protein. The PLA₂ active fractions were pooled together and loaded on DEAE Sephadex G-75 column equilibrated with phosphate buffer (pH 6.5). The column was eluted with the same buffer, maintaining a flow rate of 2ml/min. Fractions were collected and assayed for PLA₂ and total protein concentration.

PLA₂ Assay

The PLA₂ activity was assayed as described by (Bhat and Gowda, 1989). Briefly, 25 μl of 1 mg/ml L α-lecithin substrate was incubated with 10 μl of the enzyme for 10 min at 37°C. The reaction was then terminated by immersing the tube in a boiling water bath for 2min and the amount of released free fatty acid measured titrimetrically at pH 8.0. The activity of Phospholipase A₂ was defined as the amount of enzyme that hydrolyzes 1 μmole of fatty acids from L- α-Lecithin per minute under standard conditions.

Effect of pH and temperature on PLA₂ activity

A pH-dependent assay of the partially purified enzyme was performed using 50mM acetate buffer pH 5.0-6.5, 50mM phosphate buffer pH 6.8 - 7.2 and Tris HCl buffer pH 7.5 - 8.5. Also, the effect of temperature was determined by assaying the enzyme activity at varying temperatures ranging from (25 - 70°C).

Initial velocity studies: The enzyme was assayed with varying concentrations of the substrate (0.25 – 1 mg/ml) L-α-lecithin. Initial velocity values obtained were then used to plot Lineweaver-Burk's plot to determine the Km and Vmax.

Effects of some divalent cations on PLA₂ activity: The enzyme assay described above was performed in the presence of chloride salts of the following divalent ions; Ca²⁺, Co²⁺, Mg²⁺, and Cu²⁺ at 10mM final concentrations.

RESULTS

The purification profile of PLA₂ from *E. ocellatus* venom is summarized in Table 1. The *E. ocellatus* venom was found to possess PLA₂ activity. The crude enzyme had a specific activity of 2.39 μmol/min/mg, which was increased to 6.67 and 32.26 μmol/min/mg after passing through DEAE cellulose and DEAE sephadex 75 columns respectively.

The partially purified PLA₂ enzyme was most active at pH 7.5 (Figure 1) with complete cessation of activity below pH 5.0 and above pH 9.0 while maximum activity was observed at 40°C (Figure 2). Initial velocity data were used to compute the kinetic parameters of the enzyme. The Km of 1mg/ml and Vmax of 0.35 μmoles/min were obtained (Figure 3). Ca²⁺ was found to increase PLA₂ activity two-fold while Cu²⁺, Mg²⁺ and Co²⁺ were inhibitory to the enzyme (Figure 4).

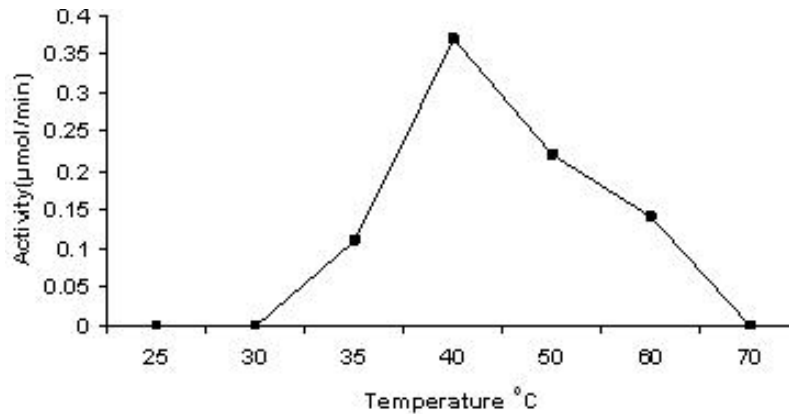


Figure 2. Effect of temperature on PLA₂ activity

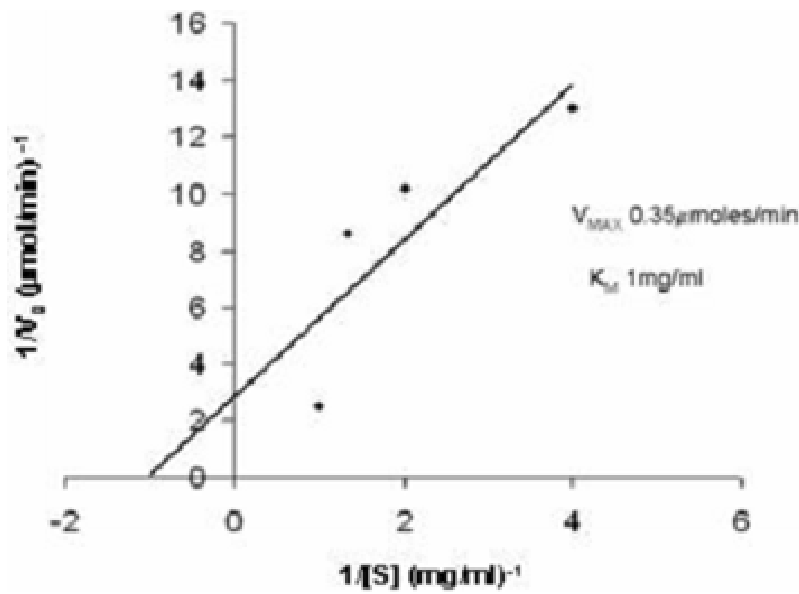


Figure 3. Lineweaver Burk's plot of PLA₂ from *Echis ocellatus* venom.

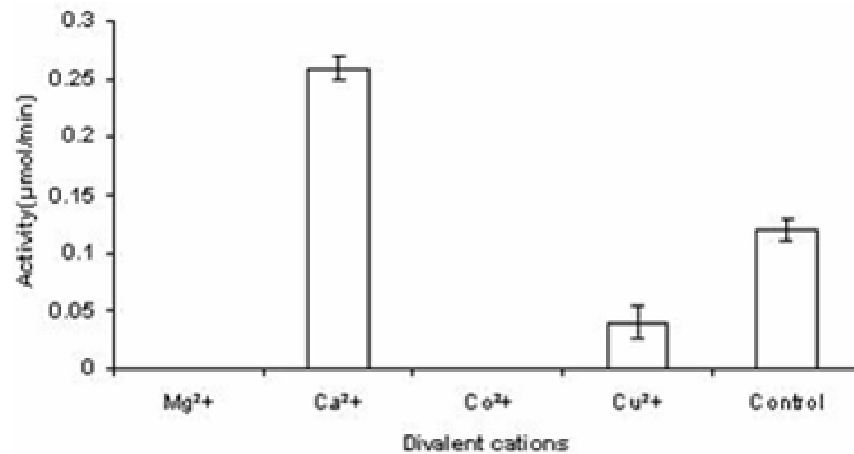


Figure 4. Effects of some divalent cations on PLA₂ activity.

DISCUSSION

Understanding the characteristics of PLA₂ from snake venoms is a serious concern for venom researchers, as it would help the production of effective therapeutic anti-venoms, considering the role of the enzyme in envenomations. This report reveals the isolation and partial characterization of PLA₂ from *E. ocellatus* venom which belongs to the Class II PLA₂ predominantly found in viperid snakes (Yang, 1994; Scott and Sigler, 1994).

The increase in specific activity of the crude PLA₂ after the two purification steps could be due to the removal of other synergistically interacting components of the venom. The very high percentage yield obtained was higher than those obtained from ostrich pancreatic PLA₂ and dromedary PLA₂ (Bacha et al., 2007) which could mean that the purification steps used here were more appropriate for this enzyme.

The *E. ocellatus* venom PLA₂ optimal activity at pH 7.5 was found to be similar to that reported for *L. muta* and *N. naja* venoms PLA₂ (Fuly et al., 2002; Shashidaramurthy and Kemparaju, 2006) and could be attributed to the pathological role of the enzyme in envenomations as basic pH contributes to the enhancement of the enzymatic reaction by general base catalytic mechanism. General base-mediated attack on productively bound substrates has been described by Scott and Sigler (1994) to be the first out of the three essential steps in catalysis of PLA₂ enzymes as deduced from crystallographic studies on the enzyme. The PLA₂ optimum temperature agrees with other findings by Shashidaramurthy and Kemparaju, (2006); Bacha et al., (2007). The increase in body temperature of snake bitten victims above the physiological temperature could also make the condition favorable for PLA₂ to exert its hydrolytic function effectively.

The fairly low Km value is an indication of moderately high affinity of the enzyme for phospholipids which further substantiates the observed toxicities in viperid snakes as a result of PLA₂. Furthermore, the Vmax obtained implies that at the end of 1min. post *E. ocellatus* envenomation, at least 0.35µmoles of free fatty acids would have been excised from the victim's red blood cells leading to haemolysis of red blood cells observed after envenomations (Rosenberg, 1979). It could also lead to liberation of free fatty acids from other membrane phospholipids making the milieu highly acidic with the lysophospholipids generated exhibiting a detergent-like action that leads to degeneration of muscle fibres (myotoxicity) and/or inhibiting the release of neurotransmitters (neurotoxicity), both of which are major forms of viperid PLA₂ toxicity (Caratsch et al., 1985). The increase in activity of *E. ocellatus* PLA₂ in the presence of Ca²⁺ shows that the enzyme is Ca²⁺ dependent. Studies have shown that snake venoms PLA₂ need submicromolar concentrations of Ca²⁺ to be catalytically active and also increase in Ca²⁺ is needed for both enzyme binding and catalysis (Shashidaramurthy and Kemparaju, 2006; Higuchi et al.,

2007). Yang (1994) has described the calcium binding loop in myotoxicity of PLA₂ and that calcium binds to His48 of PLA₂ located at the catalytic site of the enzyme activating it towards its toxic function.. The observed effect of copper, magnesium and cobalt, although divalent as calcium, may suggest a probable modification of enzyme protein conformation after binding differently from the pattern calcium does, and hence causing a decrease in the enzyme action. The observation could also give a guide towards inclusion of effective quantity of these inhibiting divalent cations in antivenom design for *E. ocellatus* venom.

It was therefore concluded that *E. ocellatus* venom contains PLA₂ with biochemical characters similar to other venoms PLA₂.

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