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ISOLATION, PARTIAL PURIFICATION AND CHARACTERIZATION OF PHOSPHOLIPASE A_2 FROM *Naja katiensis* VENOM

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ABSTRACT
The most effective and acceptable therapy for snakebite victims is the immediate administration of antivenin which is limited by problems of hypersensitivity reactions in some individuals and its inability to resolve the local effects of the venom. The aim of this study was to isolate, partially purify and characterize phospholipase A_2 from Naja Katiensis venom. Phospholipase A_2 was partially purified via a two-step process: gel filtration on Sephadex G-75 and ion exchange chromatography using CM Sephadex, and subjected to SDS-PAGE analysis. From the results, the specific activity of the partially purified PLA_2 decreased from 0.67µmol/min/mg in crude venom to 0.29µmol/min/mg after ion exchange chromatography with a yield of 5% and purification fold of 0.43. The optimum temperature of the purified PLA_2 was found to be 35^oC and optimum p.H of 7. Initial velocity studies for the determination of kinetic constants using L-α-lecithin as substrate revealed a K_m of 1.47mg/ml and V_{max} of 3.32µmoles/min/mg. The sodium dodecyl sulphate polyacrylamide gel electrophoresis of the purified PLA_2 showed a distinct band with molecular weight estimated to be 14KDa. In conclusion, the present study shows that phospholipase A_2 was isolated, purified and characterized. This may serve as a promising candidate for future development of a novel anti-venin drug.
Keywords: Naja Katiensis, Snakebite, Phospholipase A_2, Isolation

INTRODUCTION

Snakebite envenomation is of public health concern as it results in mortality and chronic morbidity especially in the rural communities of the developing world (Harrison *et al.*, 2009). Report has shown that there are as many as 4.5 to 5 million snake bites/year globally, resulting in 2.5 million envenomings, 125,000 deaths and perhaps over 300,000 with permanent disabilities (Chippaux, 1998). The venoms released by snakes after a bite are composed of complex mixture of active substances, mainly peptides and proteins which are able to interfere with the course of several biological processes including thrombosis by affecting platelet aggregation and blood coagulation. Some of these proteins include enzymes like phospholipase A_2 and metalloprotease (Echi Tab, 2008).

Phospholipase A_2 (PLA_2, EC 3.1.1.4) is an enzyme that hydrolyzes the fatty acid ester bond at position 2 of the 1,2-diacyl-sn-phosphoglycerides to release free fatty acid and lysophospholipid. The fatty acid so formed may act as either second messenger or a precursor of eicosanoids, known to be key factors in mammalian tissues, arthropods and in snake venom, based on their source, amino acid sequence, chain length and disulfide bond patterns. Some clinical effects following snakebite are due to phospholipase A_2, which are among the major components of venoms. Although these enzymes share a common catalytic activity and similar structural features with non-toxic mammalian pancreatic enzymes, they also promote a wide variety of pathological symptoms in animals, such as cardiotoxicity, myotoxicity, anticoagulant activity, hemolysis, edema, convulsant activity as well as induction of pre-synaptic toxicity (Warrel, 2008).

Snake venoms are particularly rich in PLA_2s and some of them contain more than one isoform of this enzyme. Many of these isoenzymes have similar molecular weights but can be differentiated by purification methods. Snake venom PLA_2 is able to induce several biological effects and this diversity of physiological functions of PLA_2 isoenzymes from snake venoms is very important for the production of antivenoms. Although, studies have shown that snake bites resulting from *Naja katiensis is* low in Nigeria (Yusuf, 2015), it is pertinent to understand the accelerated evolution of this enzyme in the Elapidae venomas.

Information concerning this enzyme in *Naja katiensis* is scarce and could be important in antivenom design. Therefore, this study aims to partially purify and characterize PLA_2 from the venom of *Naja katiensis*.

MATERIALS AND METHODS

MATERIALS

Equipment

Centrifuge, spectrophotometer, pH metre, water bath, incubator, 5 ml syringes, chromatography column, beakers, conical flasks, measuring cylinders, plane container, glass wool, micro pipettes, glass rods, retort stand, refrigerator, cuvette, weighing balance

Chemicals and Reagents

Lyophilized *Najakatiensis* venom, sephadex G-75, sephadex C-50, Lecithin from egg yolk, deionized water and TCA.

METHODS

Preparation of Crude Venom

About 0.03mg of the crude venom was dissolved in phosphate buffer (20Mm, pH 7.60), mixed and immediately centrifuged at 3000x g for 10 minutes and stored at 20ºC until needed.

Gel-filtration on Sephadex G-75

The gel was prepared by dissolving 2g of sephadex G-75 in 50ml ammonium acetate buffer, pH 7.4 for 24 hours at room temperature and mixed with a glass rod. The gel was then poured into a column packed with glass wool at the bottom. The column was first equilibrated with ammonium acetate buffer, pH 7.4, before the sample was applied. Crude venom (0.03 mg) was dissolved in 3ml ammonium acetate buffer, pH 7.4 in a beaker. Thirty (30) fractions at a flow rate of 3ml per minute were collected and analyzed for total protein and enzyme activity. The fractions showing high activity were pooled together and further purified by ion exchange chromatography.

Ion-exchange chromatography on CM Sephadex

The anion-exchanger was prepared by dissolving 2g of C-50 sephadex in 50 ml of Tris buffer, pH 8.2. The gel was then poured into a column. The fractions obtained from the gel-filtration step were pooled together and the sample (3ml) was loaded onto the column and eluted with a linear gradient of sodium chloride solution (0.00, 0.01, 0.15, 0.20, 0.25, and 0.30). Twenty-four fractions were collected 5ml for each concentration, at a flow rate of 5ml/5 minute and analyzed for total protein and enzyme activity. The most active fractions were collected and pooled together.

Determination of PLA_2 Activity

The PLA_2 activity was determined by Habermann and Neumann (1954) method. Briefly, 25uL of 1g /ml L α-lecithin was incubated with 10uL of the partially purified enzyme from *Naja katientsis* venom for 10 mins at 37°C. The reaction was then terminated by immersing the tube in water bath for 2mins and the amount of released free fatty acid measured titrimetrically at pH 8.0 using 20mM NaOH and phenolphthaline indicator. The activity of phospholipase A_2 was defined as the amount of enzymes that hydrolises 1µmol of L α-lecithin per minute under standard conditions

Determination of Protein Content

Content of the protein in the assay was determined according to Bradford (1976) method using Serum Bovine Albumin (BSA) as standard and the concentration was expressed in milligram per milliliter (mg/ml).

Characterization of Partially-purified PLA_2 from *Naja Katiensis* Venom

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To investigate the apparent molecular weight of the partially purified enzyme, sodium dodecyl sulphate polyacrylamide gel electrophoresis carried out using 14% gel in SDS-Tris-glycine buffer, and band was identified by staining with comassie brilliant blue R250 (Lammeli 1970)

Effect of pH and temperature

The effect of temperature on the activity of partially purified PLA_2 was measured at various temperatures from 20°C to 60°C and the effect of pH was as well studied by varying the pH from 2 to 10. Briefly, L-α Lecithin was incubated for 15mins at different temperature, and the reaction was terminated by immersing the tube in a boiling water bath for 2 mins. The amount of released free fatty acid was measured titrimetrically at pH 8.0 using 20mM sodium hydroxide and phenolphthalein indicator, after centrifugation at 1,600g for 10mins; the absorbance of the supernatant was measured at 280nm.

Initial velocity studies

The sample was assayed using various L-α Lecithin concentrations (1% to 5%). Briefly, 100µL of the enzyme were added to the buffer solution. 0.1M Tris-HCl, pH 9.0, and the final volume was adjusted to 250µL, followed by 750µL Of L-α Lecithin (1% to 5%) and incubated for 15mins at different temperatures as stated above, the reaction was terminated by adding 1.5ml of 30% TCA, after centrifugation at 1,600g for 10mins, the absorbance of the supernatant was measured at 280nm. The Lineweaver-Burk plot was used to determine the kinetic parameters K_m and V_{max}.

RESULTS AND DISCUSSION

Partial purification of Phospholipase A_2

Table I shows the results of the partial purification of phospholipase A_2 from *Naja Katiensis* venom. The enzyme was purified via two steps: Gel filtration using G-75 and ion exchange chromatography using CM-Sephadex. From the results, the crude venom had a specific activity of 0.67μmol/min/mg at 100% yield. After gel filtration, it was 0.546 μmol/min/mg at 10% yield. On further purification the specific activity decrease to 0.290 μmol/min/mg at 5% yield with a purification fold of 0.43.

Figure 1 shows the result of the gel filtration, of which fractions with highest enzyme activity were pooled together for purification. The pooled fractions from gel filtration were subjected to ion exchange chromatography using CM-Sephadex, andeluted at a linear gradient of sodium chloride solution (0.00, 0.01, 0.15, 0.20, 0.25, and 0.30). Fraction 23 had the highest activity (Figure 2).

Table I: Purification table of phospholipase A_2 from *Naja katiensis* venom

PURIFICATION STEPS	TOTAL PROTEIN (mg)	TOTAL ACTIVITY (µmol/min)	SPECIFIC ACTIVITY (µmol/min/mg)	YEILD (%)	PURIFICATION FOLD
Crude venom	3.00	2.00	0.67	100	1
Sephadex G-75	0.3657	0.2	0.546	10	0.816
CM- sephadex	0.3436	0.100	0.29	5	0.43

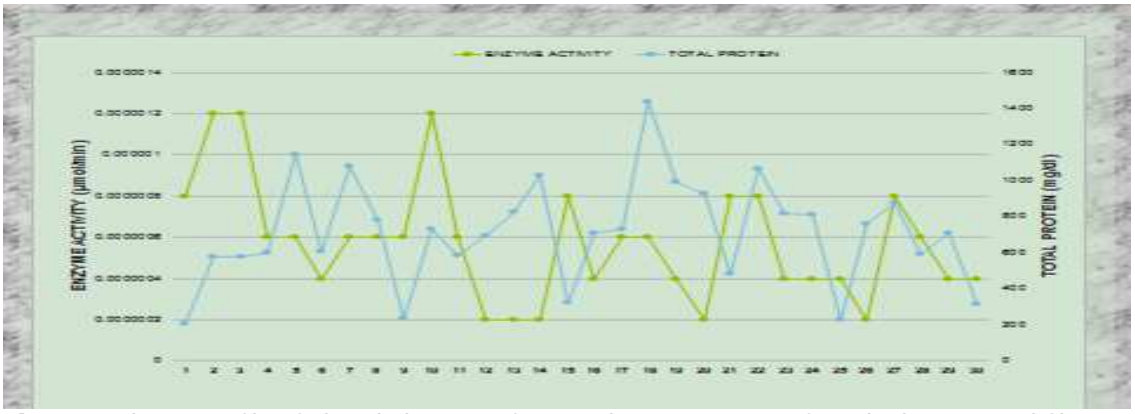

Figure1: Elution profile of Phospholipase A_2 from *N. katiensis* venom aftersphadex G-75 gel filtration chromatography

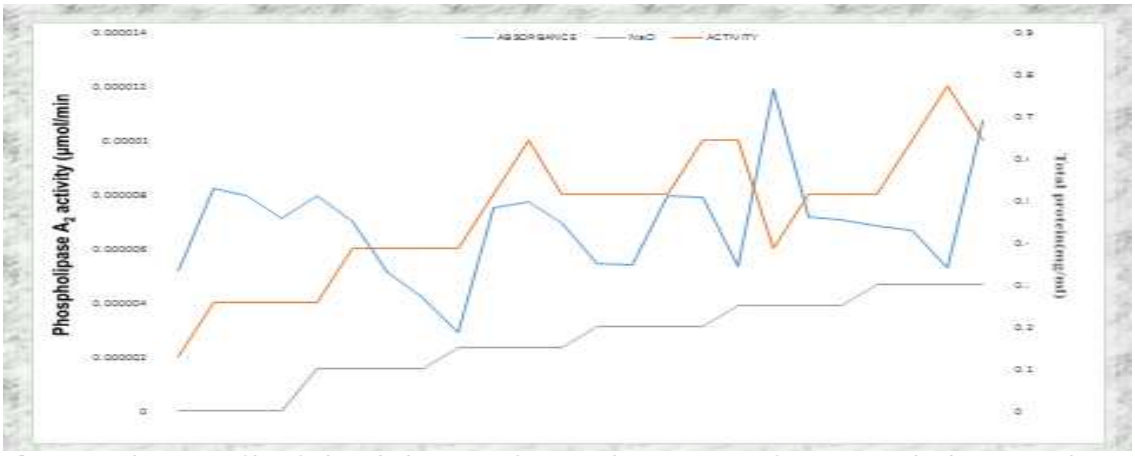

Figure2: Elution profile of Phospholipase A_2 from *N. katiensis*venm from CM-sephadex ion exchange chromatography

Effect of temperature and pH on the activity of the partially purified phospholipase A_2

The activity of the partially purified phospholipase A_2 increased as temperature increased from 20°C up to a temperature of 35°C. However, decreased in activity was observed as the temperature increased (Figure 3). The activity of the partially purified Phospholipase A_2 was found to increase with increasing pH until it reached pH 7, where the activity was highest. After which increases in pH resulted in further decrease in activity (Figure 4).

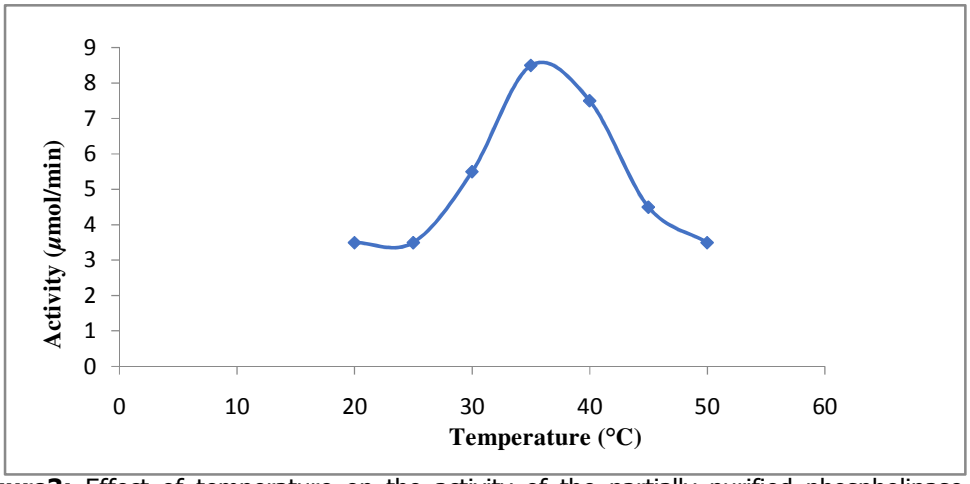

Figure3: Effect of temperature on the activity of the partially purified phospholipase A_2 from *N. katiensis* venom.

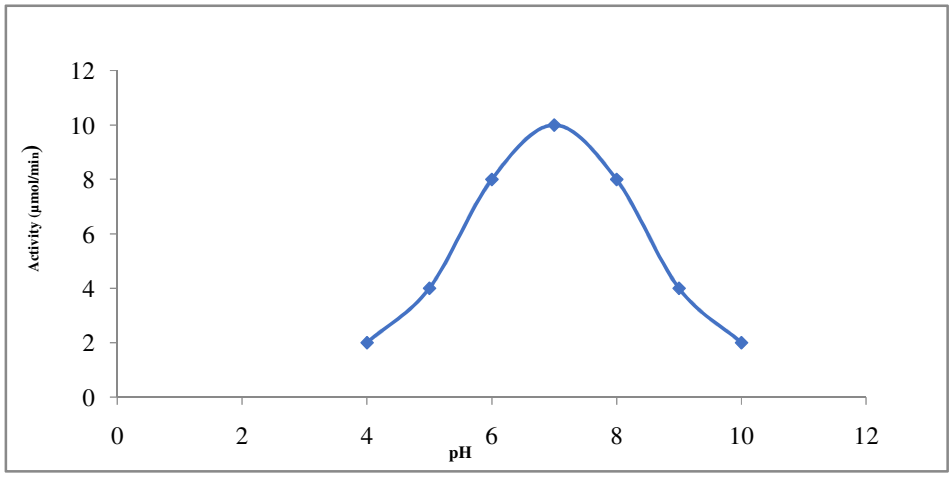

Figure 4: Effect of pH on the activity of the partially purified phospholipase A_2 from *N. Katiensis* venom

KINETIC STUDY

Initial velocity data were used to compute the kinetic parameters of the enzyme. The Km of the partially purified PLA_2 enzyme of 12.6mg/ml and Vmax of 3.32 µmoles/min were obtained.

SDS-PAGE

The apparent molecular weight of the partially purified enzyme was estimated from results of sodium dodecyl sulphate-polyacrylamide elecotrophoresis (SDS-PAGE) and when compared to the molecular marker, the purified enzyme had a molecular weight approximated to be 14KDa (figure 5).

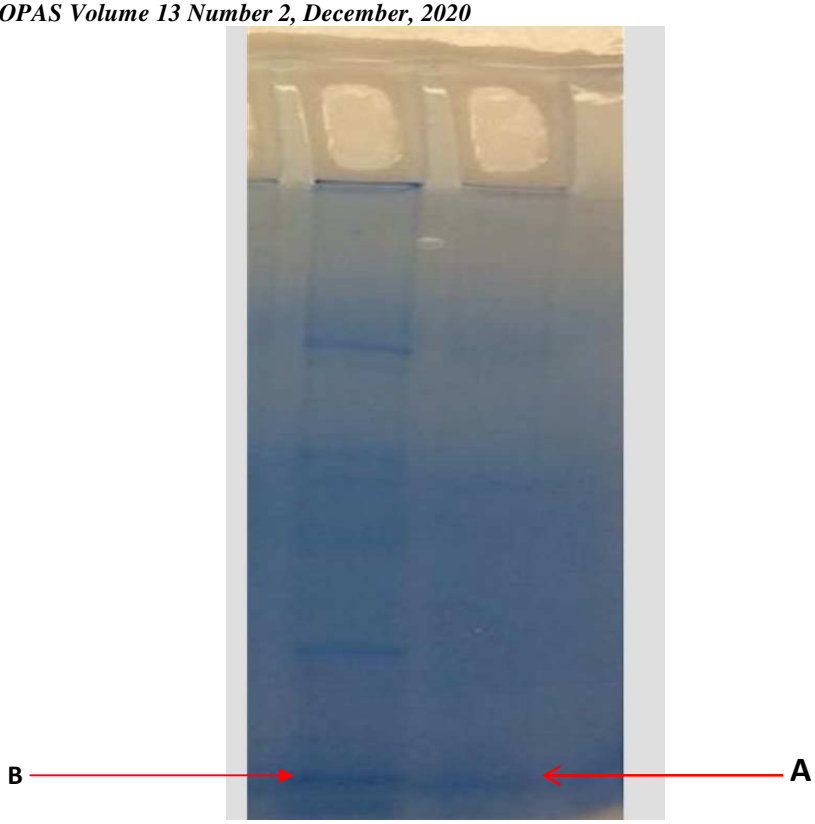

Figure 5: Electrophoregram of partially purified phospholipase A_2 from *N. katiensis* venom on polyacrylamide gel
usingcoomassie brilliant blue staining.
Band A represents partially purified PLA_2 with an estimated molecular weight of 14kDa
Band B molecular marker

DISCUSSION

Toxicological properties of snakebite are thought to be associated with enzymes especially phospholipase A_2 (PLA_2), which is found to be its most toxic component. Considering the role of PLA_2 in envenomation, understanding the characteristics of the enzyme from snake venom has raised concern for venom researchers, as it would help in the production of effective therapeutic antivenins. In this study, *Naja Katiensis* venom which belongs to the class ll PLA_2 predominantly found in *viperidae* snakes was isolated and partially purified. The specific activity of the partially purified PLA_2 from *Naja Katiensis* decreased from 0.67 to 0.29µmin/mg after ion exchange chromatography with a purification fold of 0.43, and 5% yield. Activity of 0.29µmole/min/mg is similar to that found in *Naja siamensis,* and *Naja melanoleuca* but different from *Naja mosambica* (0.47 µmole/min/mg) (William, 1998)*, Naja naja* (0.16 µmole/min/mg) (Pugh, *et al*, 1980)*, and Naja pallida* (0.67 µmole/min/mg) (Aird, 2002).
The maximum activity occurred at 35°C, but activity decreased at 20°C and 50°C which is similar to the activity of *Bothriopsis taeniata* which the maximum velocity was obtained between 35°C and 40°C and decrease at 20°C (Vargas *et al.,* 2014).

CONCLUSION

From this study, Phospholipase $A_2$was partially purified and characterized by docking studies from *Naja katiensis* venom. This may be used to as a promising enzyme to develop a novel anti-venin drug.

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