Research Article

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Characterization of Partially Purified Phospholipase A_2 Enzyme of Naja Katiensis Venom

Hassan A U[1]*, Egbe N E[1], Ugochukwu O O[1], Onuh K[1], Kereakede E[1] and Umar S I[2]

[1]*Department of Biotechnology, Nigerian Defence Academy, Kaduna – Nigeria.*

[2]*Department of Microbiology, Bayero University Kano – Nigeria.*

***Corresponding Author**
Hassan AU, Department of Biotechnology, Nigerian Defence Academy, Kaduna – Nigeria.

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Abstract

Snake bite issues has been for long a neglected public health concern in Nigeria and other African countries, causing high number of human fatalities annually. It however remains a serious medical and economic problem. This research is aimed at the characterization of partially purified Phospholipase A_2 (PLA_2) enzyme of Naja katiensis venom. The enzyme is extracted using a two way purification step (Gel filitration using G-75 and ion exchange chromatography on CM-Sephadex). The molecular weight of the enzyme is determine using Sodium Dodecyl-Sulphate Electrophoresis (SDS-Page). The extracted enzyme kinetic parameters were also determine, after which its relative optimum affinity/activity were also determine with related to different ranges of temperature, pH, metal ions and salts. The partially purified PLA_2 gave a total enzyme activity of 3.11 µmol/min and an estimated extrapolated molecular weight of 17.5 KDa. Initial velocity data of the enzyme was used to compute the kinetic parameters of the enzyme, where the Km and Vmax of the enzyme was estimated to be 12.6 mg/ml and 3.32 µmoles/min respectivily. The enzyme optimum temperature and pH was found to be 35oC and 7.0. Ca^{2+} ions was revealed to increase the enzyme activity and affinity. This revealed data could provide an alternative natural way of producing a more friendly pharmaceutical formulation for the management and treatment of snake envenomation.

Keywords: Venom, Envenomation, Antiserum, Kinetic, *Naja Katiensis*, Chromatography

1. Introduction

Snake bite envenomation is of serious detrimental public health concern as it normally results in mortality and chronic morbidity, especially among people from the rural areas. It is also of biomedical importance with social economic impact on developing regions around the world [1]. Snake bite is one of the most human serious health concern up to date as venom-treatment specificity is always a problem when it comes to the management and treatment of envenomation by snakes [2]. Another serious fact is the present treatment storage requirements, which are not really available in most remotes areas in developed and developing countries. Understanding the velocity, nature and contents of snakes' venom is one of the best starting point-way towards seeing an end to this medical and social paradox [3]. *Naja katiensis* is one of the most poisonous predominant kinds of snakes found in the northern Nigeria [4]. The venoms released by snakes after a bite usually composed of complex mixture of active substances, which includes; mainly peptides and proteins (mainly enzymes), which are able to interfere with the natural course of several biological processes including thrombosis by affecting platelet aggregation and blood coagulation [5]. Some of these proteins include enzymes like phospholipase A2, metalloprotease, hyaluronidase, L-amino oxidase and phosphodiesterase [6]. Snakebite detrimental hemostatic effects were shown to been directly involved in proteolytic events caused by the protease and phospholipase A2 enzymes present in snake venom, which subsequently results in blood coagulation, fibrinolysis and platelet aggregation among others. Snake venom proteolytic enzymes interfere with many other biological processes, immune system and organs inflammation among others [7].

Phospholipase A2 also known as lecithinase, is the most widespread of all snakes venom enzymes, basically found in all snake venoms [8]. It usually acts by damaging the body cellular mitochondria, red blood cells, leucocytes, platelets, peripheral nerve endings, skeletal muscle, vascular endothelium and other membranes [9]. Its activity normally produces a kind of presynaptic neurotoxic activity, opiate-like sedative effects which leads to an autopharmacological release of histamine and anti-coagulation [10]. Envenomation by snake is also seen as one of the most neglected public health problems, in Nigeria, Causing around 100,000 human deaths annually [4]. It however remains a serious economic, social and medical problem in Nigeria and the entire African continent.

2. Materials and Method

2.1 Partial-purification of PLA_2 from Naja katiensis venom

Preparation of Crude Venom: - About 30 mg of the *Naja katien-*

sis lyophilized crude venom was dissolved in phosphate buffer (20Mm, pH 7.60), mixed and immediately centrifuged at 3000 xg for 10 minutes and stored at 4ºC until needed [11].

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. Thirty 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). Thirty 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 [9].

2.2 Determination of PLA_2 Activity

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 370C. 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 A2 was defined as the amount of enzymes that hydrolises 1umol of L α-lecithin per minute under standard conditions.

2.3 Determination of Total Protein Content

Content of the protein in the clear supernatants was determined according to Bradford method using Serum Bovine Albumin (BSA) as standard and the concentration was expressed in milligram per milliliter (mg/ml) [12].

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

Effect of Temperature

The effect of temperature on the activity of partially purified PLA_2 was measured at various temperatures (20◦C to 60◦C). 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 [13].

2.5 Effect of pH

The effect of pH on the activity of partially purified PLA2 was measured at various pH (2, 4, 6, 8 and 10). Briefly, L-α Lecithin was incubated for 15mins at different pH mention above. The reaction was terminated by immersing the tube in a boiling water bath for 2 mins and the amount of released free fatty acid 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 [14].

2.6 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 temperature mention 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 Km and V_{max} [4].

2.7 Effect of Some Metal Ions on Partially-purified PLA_2 Activity

Various metals were dissolved in deionized water and about 1ml of sample added to 1ml of each metal. This was incubated for 20 minutes at room temperature before determining the activity of serine protease [15].

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

SDS page electrophoresis was done using a 12.5% polyacrylamide slab gel with a Tris-HCl buffer at pH 8.9. A 0.1% SDS-14% polyacrylamide slab gel is used, following the method of Laemmili. The gel was then stained with 0.1% coomassie Brilliant blue R-250 in 50% methanol 7% acetic acid and the background of the gel was de-stained with 7% acetic acid [16].

3. Result

Phospholipase A_2 enzyme was partial purification from the venom of *Naja Katiensis* using a two way purification step; Gel filitration using G-75 and ion exchange chromatography using CM-Sephadex (Figure 1 & 2). The crude venom was found to have a specific activity 12.20 mol/min/mg at 100% yield. After gel filitration, it was 21.94 µmol/min/mg at 93% yield. On further purification the specific activity decrease to 34.56 µmol/min/mg at 86% yield with a purification fold of 2.05 (Table 1). Thirty fractions were collected at a flow rate of 1ml/108sec at 25oC from the gel-filtration chromatography. Fraction 5 & 6 were found to have the highest enzyme activity, which were then pooled and subjected to an ion-exchange chromatography on CM-Sephadex eluted with 90ml of NaCl gradient 0.1-1.2M, at a flow rate of 1ml/120sec at 25oC (Figure 2).

Characterization of the extracted enzyme reveals the activity of the partially purified phospholipase A2 enzyme, increased as temperature increased, up to an optimum temperature of 35oC. Above this temperature, activity decreased as temperature increased (Figure 3). Moreover the activity of purified Phospholi-

pase A2 increased with increasing pH up until an optimum pH of 7, where the activity was highest, after which increases in pH resulted in further decrease in in the enzyme activity (Figure 4). Initial velocity data of the enzyme was used to compute the kinetic parameters of the enzyme, where the Km of the partially purified PLA2 enzyme was estimated to be 12.6 mg/ml, while it Vmax is 3.32 µmoles/min. SDS-PAGE Electrophoresis done on the partially purified Phospholipase A_2 from Naja katiensis venom estimate its molecular weight is shown on lane 3, with an extrapolated weight of 17.5 kDa (Figure 5). Meanwhile effects of different kinds of metal ions and salt was ascertain as shown in table 2.

Enzyme	Step	Total Protein (mg/ml)	Total Enzyme Activity (µmol/min)	Specific Activity (µmol/min/mg)	Purification Fold	Yield (%)
PLA_2	Crude	0.35	4.27	12.20	1.00	100
	Gel filtration on sephadex G-75	0.18	3.95	21.94	1.70	93
	Ion exchange on CM-Sephadex	0.09	3.11	34.56	2.05	86

Table 1: Purification table of phospholipase A_2 from *Naja katiensis* venom

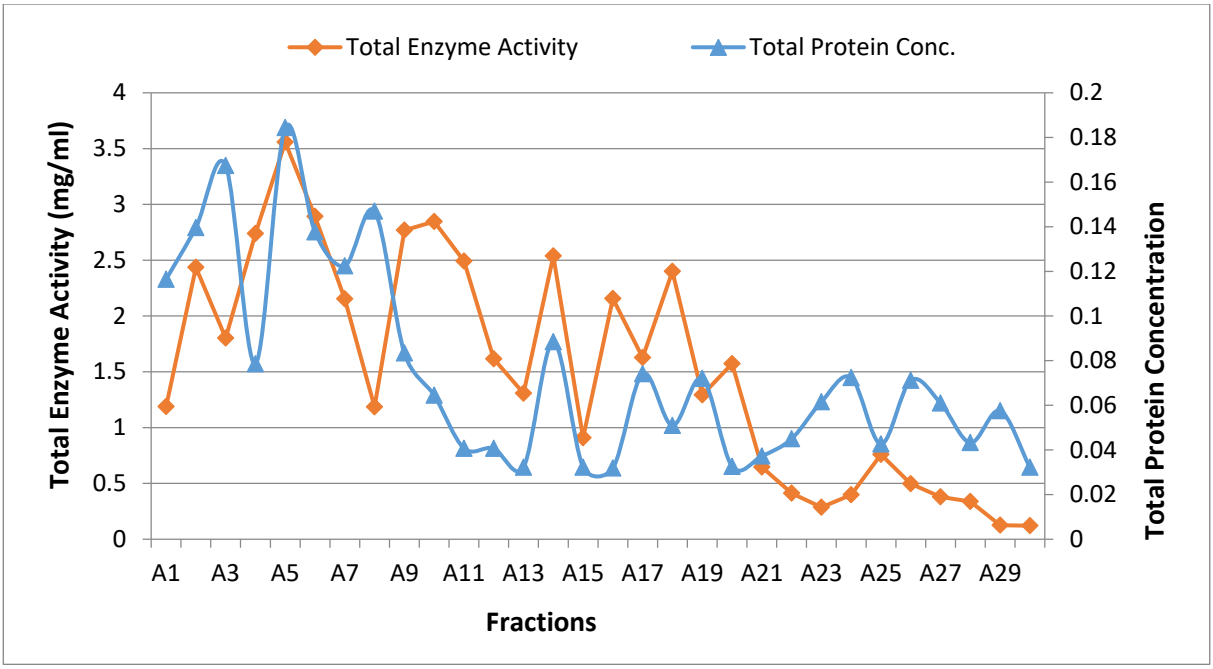


Figure 1: Elution Profile of Phospholipase A_2 on Sephadex G-75

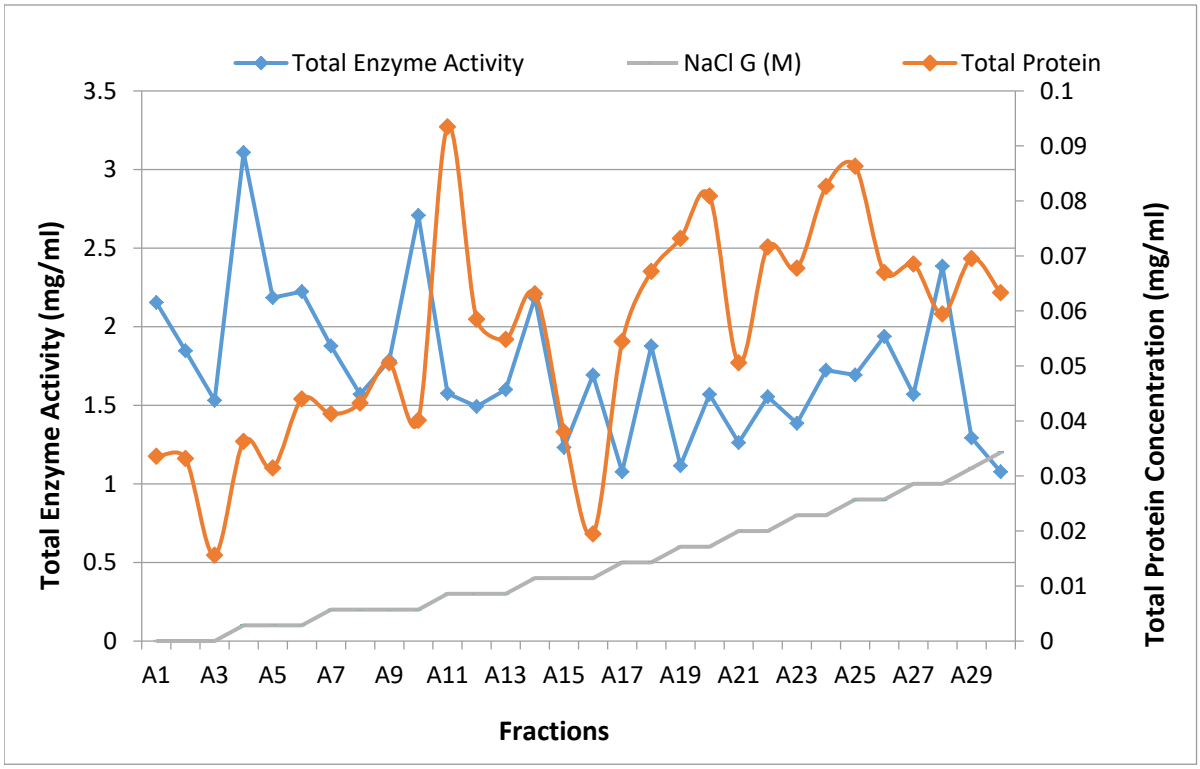


Figure 2: Elution Profile of Phospholipase A2 on CM-Sephadex

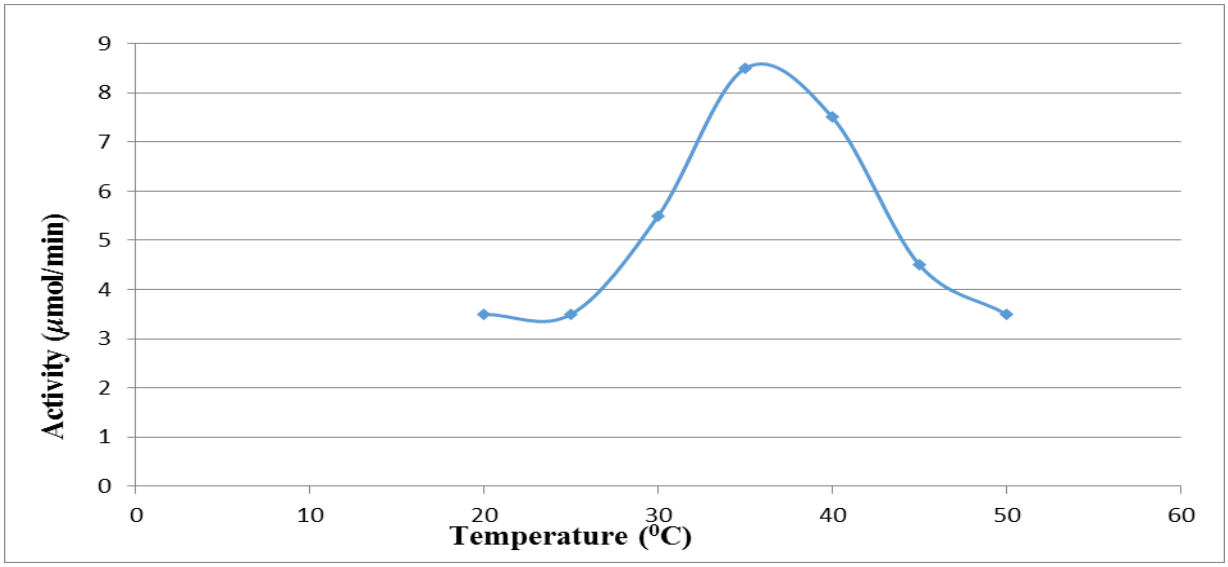


Figure 3: Effect of temperature on the activity of the partially purified phospholipase A_2 from *N. katiensis* venom.

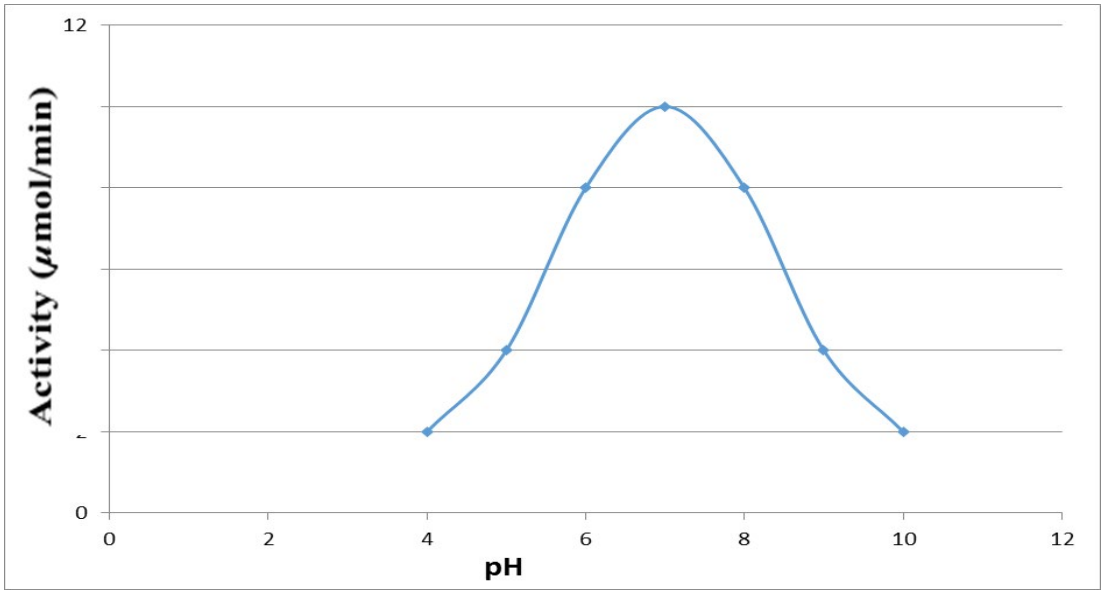
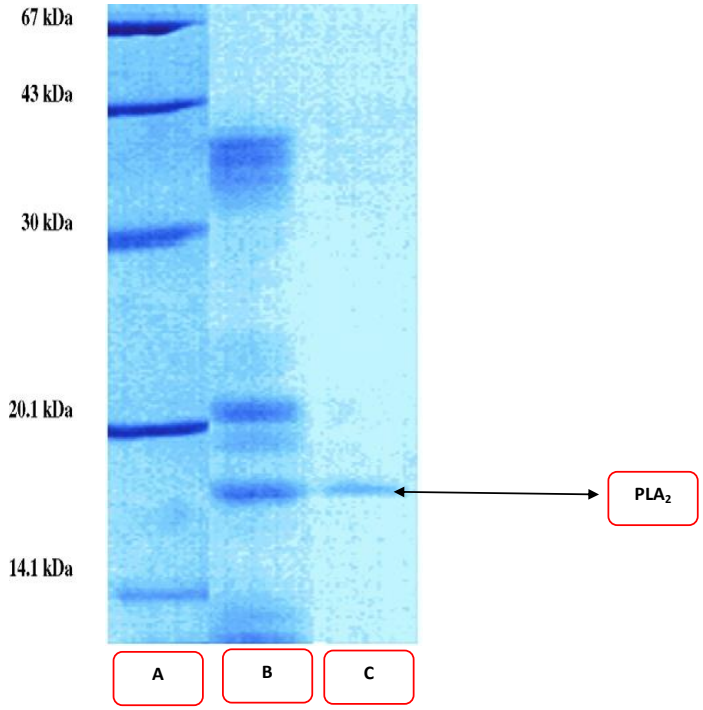


Figure 4: Effect of pH on the activity of the partially purified phospholipase A_2 from *N. katiensis* venom

Ions (10mM)	PLA_2 Activity	
	Control	Activity
Ca^{2+}	3.03 µmol/min	5.28 µmol/min
Mg^{2+}		1.63 µmol/min
Cd^{2+}		0.37 µmol/min
Zn^{2+}		3.12 µmol/min
Mn^{2+}		2.12 µmol/min
Ba^{2+}		1.50 µmol/min

Table 2: Effect of Some Metal Ions and salt on Partially-purified PLA_2 Activity



KEY: Lane A = Molecular weight marker, Lane B = Crude Venom Lane C = Extracted Phospholipase A_2 (extrapolated to be 17.5 kDa)

Figure 5: SDS-PAGE of Partially Purified Phospholipase A2 of N. katiensis venom

4. Discussion

Envenomation by snakes has become serious global detrimental issue, especially with the numerous counts of antiserum treatment specificity, storage and availability issues [17]. the only treatment available so far is only the polyvalent antiserum which binds to the components of the venom and neutralizes them but does not have any effects on the damages

already caused by the venom [18]. Another issue with snake antivenom is the fact that it is expensive, high specie-venom specificity, ideal storage condition and facilities, which are really not available especially in rural and even some developed areas of Nigeria and other African countries [4]. Having more information about snake venom enzymes, their features and bio-gram profile is the best and foremost starting focal point of developing other more suitable pharmaceutical formulations that will serve as a better alternative to the current snake antisera.

N. katiensis is one of the most dangerous venomous snakes in Nigeria responsible for maximum deaths. In this study the characterization of Partially Purified Phospholipase A_2 Enzyme of *Naja Katiensis* Venom was done. The presence of PLA_2 enzyme has been described in several human tissues, animal venoms, pathogenic organism and cancers cells [19]. It however has been shown to be present *N. katiensis* venom use in this study. PLA2 being one of the most predominant enzyme in snake venom aids in damaging mitochondria, red blood cells, leucocytes, platelets, peripheral nerve endings, skeletal muscle, vascular endothelium and other membranes, hence produces presynaptic neurotoxic activity, opiate-like sedative effects [20]. In this study enzyme Phospholipase A2 was partial purification using a two-step purification, where the most active fractions from the first step are pooled together and applied to the second step under NaCl gradient. The partially purified PLA2 was active on lecithin, with an activity of 3.11 µmol/min and a purification yield of 86%, which shows that a relatively higher relative extraction was achieved. This correspond with the study of Nwune *et al*. 2016 and Hassan *et al.* 2020 on different isolated snake venom PLA2 enzymes [21].

Kinetic of enzyme inhibition help to show the various kinetics parameters for an enzymes in the presence and absence of an inhibitor. The relative enzyme kinetics study shows that the Km and V_{max} value of the enzyme was 12.6 mg/ml and 3.32 µmoles/min respectively. Characterization studies done shows that the enzyme has an optimum temperature and pH of 35 oC (Figure 3) and 7.0 (Figure 4) respectively. However adjustment of these parameters proof lethal to the venom enzyme. This correspond with a similar snake venom research report by Abdullahi [22]. This enzyme characterization study shows that increasing the biosystem temperature also increased, the enzyme affinity up to an optimum temperature of 35oC as stated above, however higher increase of temperature above this, also decreased the enzyme activity. The same scenario was seen as per pH increase and decrease. Berg *et al* (2007) also reported similar findings as regards to snake venom enzymes kinetics and affecting factors [23]. SDS-PAGE Electrophoresis done on the partially purified Phospholipase A_2 from Naja katiensis venom extrapolated its molecular weight to be of about 17.5 kDa (Figure 5). In a related research done by Marunak et al. (2007) reported that the PLA_2 isolated from snake venom has molecular weight of 15.6KDa, similar to the one isolated from B. Jararacussu from Brazil 16.7KDa and Ketelhut et al. (2003) revealed that PLA_2 isolated has 15 KDa molecular mass [24, 25].

Meanwhile effects of different kinds of metal ions and salt was ascertain as shown in table 2, where the study reveals that most of the metal ions has detrimental effects on the isolated enzyme, with the exception of Ca^{2+} ions which tends to be increasing the enzyme activity and affinity. However
Ba^{2+} and Cd^{2+} metal ions has the highest recorded inhibitory effects on the isolated enzyme. This study was compare to the study by Chinyere et al 2016 on the effects of aqueous root extract of Annona senegalensis on Bitisarietans venom protease and phospholipase A_2 activities. This research also report similar findings with our research even though it involves different kinds of snake venoms.

5. Conclusion

Higher or lower temperature (above 60 oC or below 20 °C) and pH above or below 7.0, plus presence of some heavy metal ions and salt can be used against the activity and affinity of Naja Katiensis PLA2 venom. The data from this research can also aid in formulation of a friendlier therapeutically agent for the treatment and management of snake envenomation.

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