BIOCHEMICAL AND KINETIC PROPERTIES OF CRUDE PHOSPHOLIPASE A₂ FROM NAJA NIGRICOLLIS VENOM

1Laila Miftah Garba, 1Abdullahi Balarabe Sallau, 2Mujaheed Abubakar, 2Abdulrazak Muhammad Hussain 2Zurki Ibrahim and 3Zaharaddeen Muhammad

1Department of Biochemistry, Ahmadu Bello University, P.M.B. 1045, Zaria – Nigeria.
2Department of Biological Sciences, Sule Lamido University, P.M.B. 048, Kafin Hausa – Nigeria.
3Department of Chemistry, Sule Lamido University, P.M.B. 048, Kafin Hausa – Nigeria.

*Corresponding Author Email Address mujaheedabubakar88@gmail.com or mujahid.abubakar@jsu.edu.ng Phone: +2348039654644

ABSTRACT
Snake envenomation has been a source of concern worldwide due to potential mortality risks associated with it. This study was carried out to investigate the biochemical and kinetic properties of crude phospholipase A₂ (PLA₂) from Naja nigricollis venom. Biochemical and kinetic properties of the venom was determined using standard procedure. Though the results showed that the enzyme PLA₂ is stable at pH 4 and 6, the optimum pH of activity of the PLA₂ is 3, while the optimum temperature of activity of the enzyme is 70 °C. Of all the activators used (CoCl₂, CaCl₂, MnCl₂ and MgCl₂), MgCl₂ was the highest activator of the enzyme activity at 5 mM. The suggestive amino acids in the active site of the PLA₂ were aspartate and glutamate. The kinetic parameters, Kᵣ and Vₘₐₓ of the PLA₂ were found to be 0.6 mg/ml and 3.3 μmol/min respectively. These findings could lend support to more understanding of biochemical and kinetic properties of crude Naja nigricollis PLA₂.

Keywords: Snake envenomation, Naja nigricollis, Phospholipase A₂, Biochemical properties.

INTRODUCTION
Naja nigricollis snake belongs to the Viperidae family and is one of the most poisonous snakes found in Nigeria (Nasidi, 2007). Their envenomations are characterized by conspicuous local tissues damage and in severe cases by systemic bleeding, haemorrhagic and cardiovascular shock (Nok, et al., 2001). The venoms of vipersid snakes are rich sources of proteins with activity against various factors involved in coagulation and fibrinolysis (Faure et al., 2000). Snake venoms are complex mixture of enzymatic and toxic proteins, which include phospholipase A₂ (PLA₂), myotoxins, hemorrhagic metallo-proteasea and other proteolytic enzymes, coagulant components, cardiotoxins, cytokotins and neurotoxins (Aird, 2002; Soares et al., 2005). The composition and effects of venom varies considerably between species to species. The venom leads to the disruption of normal cellular functions by these enzymes and toxins. The main enzymes of snake venom include the PLA₂, hydrolases, hyaluronidases, phosphatases, esterases, phosphodiesterase, neuclosidases and metalloproteases (Sallau et al., 2008).

PLA₂ is the commonest enzyme found in snake venom. It is a lipolytic enzyme that hydrolyses the fatty acyl ester bonds at the 2-sn position of membrane phospholipids producing equimolar amounts of free fatty acid (FFA) and lysophospholipid, mainly arachidonic acid (AA); these products then become available for conversion to potent pro inflammatory mediators, such as platelet activating factor and eicosanoids (Hasson et al., 2003). The enzyme from snake venom is primarily use for trophic and defense functions in most species but show wide range of pharmacological activities such as neurotoxicity, myotocytosis, cardiotoxicity, with a greater impact on platelets aggregation and blood coagulation (Higuchi et al., 2007). Some components of snake venom have great potential for medical use because of the specific actions of such components (Koh et al., 2006). Snake venom components are also used in basic research in physiology, biochemistry and immunology. Venom components allow researchers to examine the operation of the process and to develop drugs to correct malfunctions due to diseases. For instance, venoms are currently being investigated for their potential use as antiviral and antibacterial agents (Petkovic et al., 1991). Actions of different snake venom are broad and the understanding of their multiple poisoning processes is desirable in the formulation of a satisfactory antidote. In this study, we conducted a biochemical and kinetic studies of PLA₂ from Naja nigricollis venom.

MATERIALS AND METHODS

Chemicals and Reagent
The substrate (egg yolk), sodium hydroxide, calcium chloride, manganese chloride, magnesium chloride, conc. Hydrochloric acid, Tris base, cobalt chloride, phenolphthalein. All chemicals and reagent used were of analytically pure grade.

Snake Venom
Naja nigricollis venom crystals were a gift from Dr. Y. P. Osemefie of the department of Toxicology and pharmacology faculty of veterinary medicine Ahmadu Bello University Zaria. Stock solution of 10 mg/ml of the venom in a Tris/HCL buffer was prepared when needed for use.

PLA₂ Assay
The PLA₂ activity of the crude venom was determined by modified egg yolk coagulation method by Habermann and Neumann (1954). Briefly, fresh egg yolk (L-a-lecithin) was homogenized in distilled water to yield a concentration of 100mg/ml. The venom (10 µl) and 10 µl of 50 mM Tris/HCl buffer (pH 8.0) were incubated with 1000 µl substrate (L-a-lecithin) at 37±1 °C. At the end of 10 minutes incubation, the mixture was immersed in boiling water for 2 mins stop the reaction. The liberated fatty acids were titrated against 20 mM NaOH using phenolphthalein as indicator. The PLA₂ activity was determined as the amount of enzyme required to hydrolyze 1µmole of free fatty acids from lecithin under standard condition.
Biochemical and Kinetic Properties of Crude Phospholipase A₂ from Naja nigricollis Venom

Determination of the Effect of pH on the Activity of PLA₂
Optimum pH for activity of PLA₂ was determined by assaying for PLA₂ activity varying pH values: 3, 4, 5, 6, 7, 8 and 9. To test tubes arranged in duplicates for each 10 µl pH bufffer, 10 µl of the crude venom and 1000 µl of the substrate (L-α-icethin) of fibrinogen solution was added. The mixture was incubated for 10 minutes at 37 °C and the reaction is stopped by immersing the mixture in boiling water for 2 minutes. Free fatty acids (FFA) released was then titrated against NaOH solution.

Determination of the Effect of Temperature on the Activity of PLA₂
The effect of temperature on the activity of the enzyme was determined by assaying for PLA₂ activity at different temperatures: 20 °C, 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, 90 °C and 100 °C. PLA₂ activity was determined by titrating FFA released.

Determination of the Kinetic Properties of Crude PLA₂
This was done via initial velocity studies according to the method described by Sallau et al. (2008) with some modifications. Briefly, the activity of PLA₂ on crude venom was determined by varying the concentration of α–Lecithin of 0.1 g/ml, 0.05 g/ml and 0.025 g/ml was incubated for 10 mins with the crude enzyme in the presence of buffer. After incubation, PLA₂ activity was then obtained by titration, FFA released using NaOH solution to obtain initial velocity data. The data was then used to plot the Lineweaver-Burk plot that was used to determine the kinetic parameters Kₘ and Vₖₐₓ.

Determination of the Amino Acids on the Active Site of the Enzyme (PLA₂)
This was done using Tris/HCL buffer of pH 3, 4 and 5 and varying the substrate concentration in each buffer (0.1 g/ml, 0.05 g/ml and 0.025 g/ml of the substrate) (Sallau et al., 2008). The PLA₂ activity was determined in each case using the procedure stated earlier.

Determination of the Effect of Divalent Cat-ions on the Activity of PLA₂
10 mM, 5 mM and 1 mM concentrations of the divalent cat-ions; Mg²⁺, Ca²⁺, Co²⁺, and Mn²⁺ were prepared using Tris-HCL buffer (pH 8.0). The PLA₂ activity was then determined using these buffers respectively

RESULTS

Effect of pH on the Activity of PLA₂
The effect of pH on crude Naja nigricollis PLA₂ activity was presented in Figure 1 which shows that the venom had a broad range of pH (from 3 to 9) with optimum pH at 3. The enzyme was highly active over the narrow pH range of 3 to 6. Low enzyme activities were observed in alkaline pH above 6.

Figure 1: Effect of pH on PLA₂ activity

Effect of Temperature on the Activity of PLA₂
The effect of temperature on crude Naja nigricollis PLA₂ activity showed a broad temperature range of activity with an optimum value of 70 °C (Figure 2). The enzyme continued to show some activity even at temperatures above 70 °C but in a declined manner representing a drop of more than 40%.

Figure 2: Effect of temperature on PLA₂ activity

Kinetic Properties of Crude PLA₂
The Lineweaver Burks plot (Figure 3) shows that the Kₘ and Vₖₐₓ values are 0.6 mg/ml and 3.3 µmol/min respectively.
Biochemical and Kinetic Properties of Crude Phospholipase A$_2$ from Naja nigricollis Venom

**DISCUSSION**

PLA$_2$ is an enzyme that catalyzes 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. 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 (Shao et al., 1993; Adamude et al., 2016). 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 (Sallau et al., 2008). Toxicological properties of snake bite are thought to be associated with enzymes especially PLA$_2$, which is found to be its most toxic component (Adamude et al., 2016). 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 (Shao et al., 1993). The enzyme displayed optimum activity at 70°C and was active over a temperature range of 20 – 100°C. This may be due to the fact that, *Naja nigricollis* is found mostly in sub Saharan Africa where temperature is usually high, hence this specie adapt to and tolerate higher temperatures. Interestingly most snake venom metalloproteases enzymes display optimum activity at 40 – 50°C. The wide range of temperature and pH of activity for the enzyme shows that the enzyme is capable of withstanding extreme conditions which is important for venom toxicity.

As a result, the site of catalysis in the envenomed victim provides the optimum pH for the activity of the enzyme; blood too being alkaline in nature. The observed low $K_m$ value of 0.6 mg/ml is a clear indication of the high kinetic efficiency of the enzyme. Previous studies by Torres et al. (2012) and Chernyshenko et al. (2010) reported $K_m$ values of 14.59 mg/ml and 8.5 μM from Bothrops moojeni and Echis multisquamatis venoms respectively. The observed property of low $k_m$ value, though a disadvantage in terms of envenomation by *Naja nigricollis*, could be considered a great advantage in terms of the clinical usefulness of these enzymes in the treatment of human diseases and as diagnostic reagents. Divalent metal ions are involved in enzyme catalysis in a variety of ways which include activation of electrophiles or nucleophiles, bridging an enzyme with substrate together by means of coordinate bonds as well as holding reacting groups in

**Amino Acids on the Active Site of the Enzyme (PLA$_2$)**

Figure 4 shows the Amino Acids (aspartate and glutamate) could be possibly present on the active site of the PLA$_2$, because the pH optimum observed was acidic.

**Effect of Divalent Cat-ions on the Activity of PLA$_2$**

Figure 5 shows the effect of divalent cat-ions on the activity of crude PLA$_2$ from *Naja nigricollis* Venom, which indicates that MgCl$_2$ has the highest activator for the enzyme activity, followed by CoCl$_2$, CaCl$_2$ and MnCl$_2$.

As a result, the site of catalysis in the envenomed victim provides the optimum pH for the activity of the enzyme; blood too being alkaline in nature. The observed low $K_m$ value of 0.6 mg/ml is a clear indication of the high kinetic efficiency of the enzyme. Previous studies by Torres et al. (2012) and Chernyshenko et al. (2010) reported $K_m$ values of 14.59 mg/ml and 8.5 μM from Bothrops moojeni and Echis multisquamatis venoms respectively. The observed property of low $k_m$ value, though a disadvantage in terms of envenomation by *Naja nigricollis*, could be considered a great advantage in terms of the clinical usefulness of these enzymes in the treatment of human diseases and as diagnostic reagents. Divalent metal ions are involved in enzyme catalysis in a variety of ways which include activation of electrophiles or nucleophiles, bridging an enzyme with substrate together by means of coordinate bonds as well as holding reacting groups in

**Figure 3: Lineweaver Burksplot for Crude PLA$_2$ using lecithin as a substrate**

**Figure 4: Determination of Amino Acid on the active site of the PLA**

**Figure 5: The Effect of Divalent Cations on the Activity of PLA$_2$**
the required three dimensional orientations (Advani et al., 2010). Therefore, it was concluded that crude PLA2 enzyme from Naja nigricollis venom revealed an optimum activity at 70 °C and an optimum pH of 3 with high kinetic efficiency. The Divalent Cations MgCl2 showed the highest activator for the enzyme activity. The suggestive amino acids in the active site of the PLA2 were aspartate and glutamate.

REFERENCES


