Effect of Increased Creatine Kinase Levels in Rabbits Due to Phospholipase A_2 of *Naja nigricollis* Venom

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Abstract

Snakebite envenomation is an important public health problem among rural communities in northern Nigeria. Phospholipase A_2 was isolated from the venom of *Naja nigricollis* by a combination of gel filtration on Sephadex G-75 and ion-exchange chromatography on DEAE cellulose. Sodium dodecyl sulfate-polyacrylamide gel filtration showed the enzyme to be a dimer with molecular weight of 65 kDa. The effect of the enzyme Phospholipase A_2 when administered intramuscularly (1.00-5.00µg/g at a given volume of 50µl) exhibited increase in plasma Creatinine Kinases activity that resulted in Myonecrosis due to intense destruction of musclular fiber which involves local infiltration of inflammatory cells that was dose and time-dependent. Rabbits (n=16) were grouped into four according to the dosage and time. Serum CK activity increased from a lower level at different time of 1,3 and 6 hours after envenomation 120.22 ± 10.83 IU/L to 2281.80 ± 609.43 IU/L. Necrosis was more extensive when higher doses of Phospholipase A_2 were injected and also, there was a corresponding increase in serum CK levels from 382.31 ± 122.70 to 818.31 ± 133.32 IU/L progressively. These findings suggest that Phospholipase A_2 induce acute muscle cell damage and oedema upon intramuscular injection in rabbit, as evidenced by increase in serum CK activity. Also, CK was measured as an index of myotoxicity. This findings provide a useful molecular basis in improved treatment and management of envenomation by *Naja nigricollis*.

Keywords: *Naja nigricollis*, Phospholipase A_2, Creatine Kinase.

Introduction

The venom of Black-Neck Spitting Cobra snake, *Naja nigricollis* is known to induce many toxic effects that may lead to death of the victim. In Nigeria, more severe cases of snakebite envenomation are inflicted by cobra species of the family elapidae. Surveys conducted in the more densely populated northern savanna show that *Naja nigricollis* is the predominant species of medical importance. It is a major medical problem among rural communities of the savanna region of West Africa, notably in Benin, Burkina-Faso, Cameroon, Ghana, Nigeria and Togo. West Africa bears the heaviest burden of snakebite, with an estimated incidence of 500 bites and 50 deaths/100,000 population reported in rural Nigeria alone.[1]

Snake venoms are particularly rich in s and contain multiple forms of Phospholipase A_2 (PLA_2) enzymes, [2] that are similar in molecular size, isoelecteric points and even amino terminal sequences.[3] However, (PLA_2) isoenzymes may exhibit a specific pharmacological effect, such as pre-synaptic or post-synaptic neurotoxicity, cardiotoxicity, myotoxicity, platelet aggregation, oedema formation, haemolysis, anticoagulation, convulsion, and hypotension .[4,5]

PLA_2 from snake venoms exhibit a wide variety of pharmacological effects by interfering with normal physiological processes.[6] PLA_2 triggers a cascade of inflammatory events characterized by increased microvascular permeability and oedema formation, leukocyte recruitment into tissues, nociception and release of inflammatory mediators which mimic a number of systemic and local inflammatory disorders in humans .[7]

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Information on the effect of Creatine Kinase and Phospholipase A_2 from *Naja nigricollis* venom found in this locality and its potential role in the pathophysiology of envenomation is scanty. It is well established that the analyses of serum enzymes can help to detect some abnormalities in the liver and heart.

This study is aimed to assess the effect of isolated enzyme at different time interval and, doses of Phospholipase A_2 on Creatine Kinase from African black-necked spitting cobra (*Naja nigricollis*) venom.

Materials and Methods

Chemicals

General laboratory and inorganic chemicals were obtained from Aldrich Chemical Company, and were of analytical grade.

Animals

Sixteen young healthy rabbits (300-400kg), seven months old were purchased from the Department of Biological Sciences, Faculty of Science, Bayero University, Kano. The animals were de-wormed and allowed to acclimatize for a week before the commencement of the experiment.

Naja nigricollis was caught from the wild at Gombe in the northern part of Nigeria. It was identified in the Zoology unit of the Biological Sciences Department of Ahmadu Bello University, Zaria, Nigeria.

Purification of PLA_2 Enzyme

The *N. nigricollis*(200 mg) venom dissolved in 1 ml equilibration buffer (0.05 M Tris-HCl, pH 6.8), was loaded on Sephadex G-75 column (2.6 × 50 cm). The sample-dissolving buffer was used for equilibration of the Sephadex column, and elution of the loaded samples. Fractions of 10 ml were collected at a flow rate of 48 ml/hr using fraction collector (Pharmacia LKB, Sweden). Fractions with phospholipase A_2 activity recovered from the previous step were pooled and directly applied to a DEAE Cellulose column (1.6 × 25 cm) pre-equilibrated with the same buffer and eluted with a linear NaClgradient from 0 to 1.3 M in the same buffer.

Phospholipase A_2 Activity

Phospholipase A_2 activity was performed as described by Dole (30). Briefly, crude egg yolk phospholipids (diluted 1:5 in 0.1 M Tris-HCl, 0.01 M CaCl2, pH 8.5) were used as substrate in the presence of 1% Triton X-100. The isolated venom (5, 25 and 50 µg) was added to 10 mL of substrate, incubated at 37°C for 15 minutes, and then the free fatty acids were extracted and titrated with 0.018 M NaOH. Crude venom (10 µg) was utilised as a positive control.

Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The electrophoretic analysis was performed in the Mini-Protean II Dual- Slab Cell (Bio Rad). Preparation of gels, samples, and electrophoresis were performed according to the conditions described by Laemmli[9]. Proteins were stained with 0.2% Coomassie Brilliant Blue R-250 in fixative solution.

In-vivo Myotoxicity

Groups 2-8 rabbits were injected i.m. with different dose (1.00-5.00 µl/kg)of isolated enzyme Phospholipase A_2 dissolved in 0.1 ml of phosphate buffered saline solution, pH 7.2. Three mice were used as control samples, receiving each of them 0.1ml/kg of phosphate buffered saline solution (pH 7.2). After 1, 3, 6 hrsof PLA_2 enzyme injection, rabbits were anaesthetised with chloroform and blood samples were taken from the tail without using anticoagulant. Serum was obtained for the enzymatic determination of Creatine Kinase (CK) levels using akinetic method U. V. (Randox). The CK activity was expressed in international units per liter, with one unit defined as the amount of enzyme that transfers 1.0 mM of phosphate from phosphocreatine to ADP per min at pH 7.4 at 30ºC. Creatine Kinase activity was expressed in units per litre

Blood Sample Collection

Sixteen rabbits were anaesthetized and blood samples were collected. Stored venoms were dissolved in 10 ml of normal saline solution and were injected intramuscularly to the rabbits (300 µg/ kg body weight of rabbit) and their sera at different dose were collected. Blood samples were centrifuged (Sigma centrifuge, Model 6k15) for 10 min at 2500 rpm. Quantitative detection of CK are determined with the help of commercially available kits (Sigma).The results are mean ±SD of three experiments.

Result

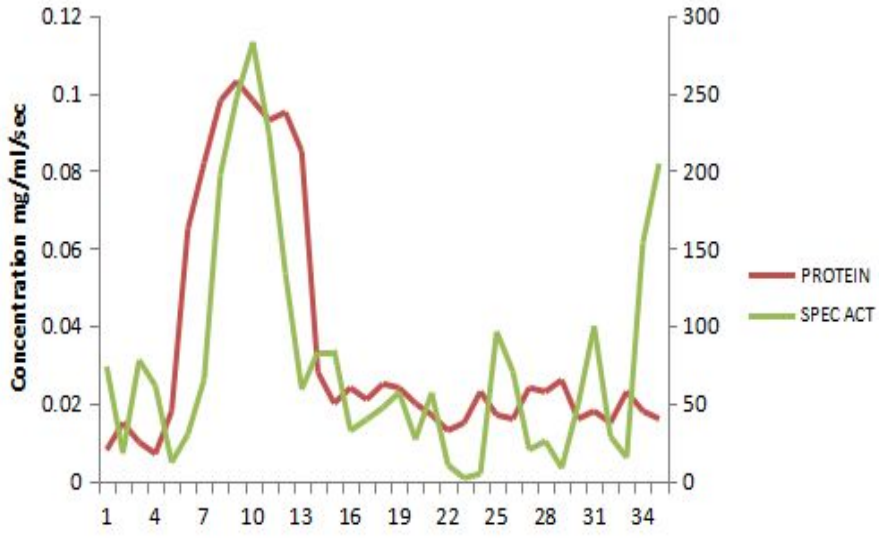


Figure I. Elution profile of *Najanigricollis* Phospholipase A_2 Using Sephadex G75

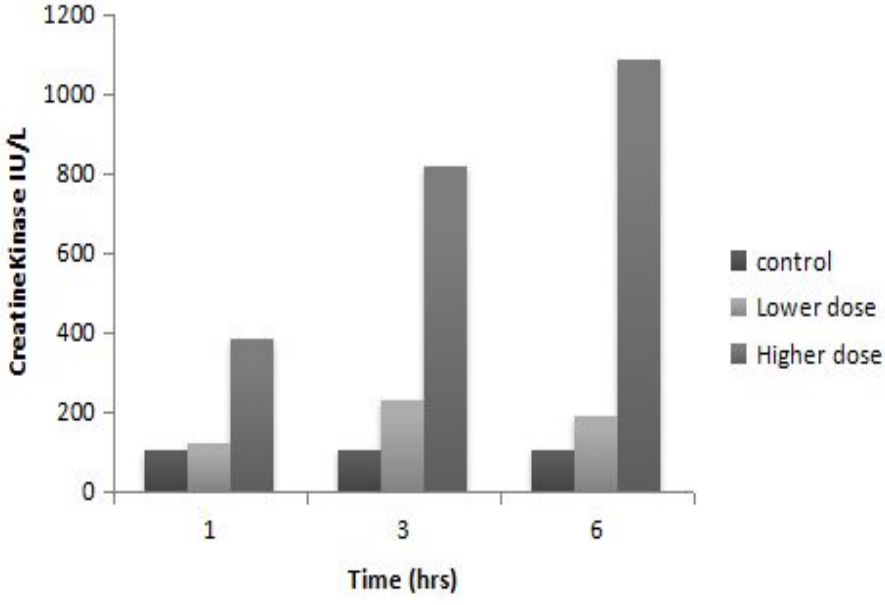


Figure2. Effect of dose and time on in vivo myotoxicity of *Naja nigricollis* venom in Rabbit. The venom phospholipase A_2 dose was increased from 1.0 to 5.0µg/g, From 1.00- 2.5µg/g was referred as the lower dose and 3.00-5.00 µg/g as the higher dose.Plasma Creatinine Kinase was measured after 1, 3.and 6hrs after the venom injection.

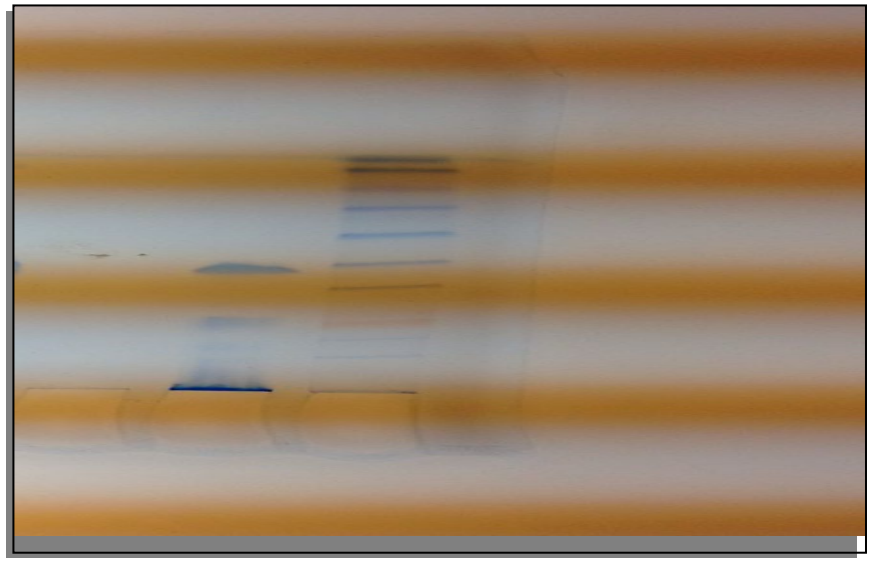
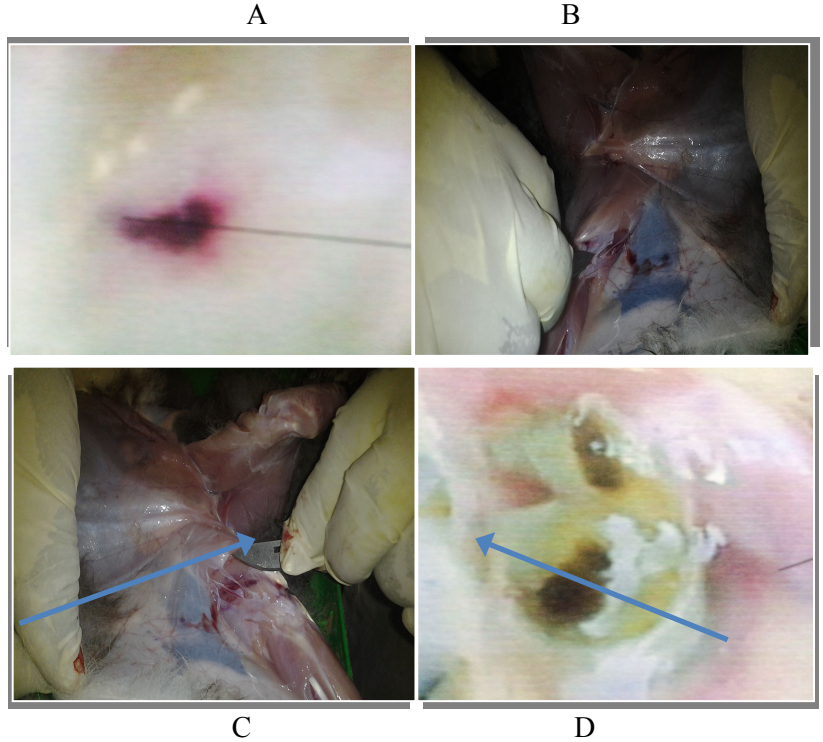


Plate1: Protein Electrophoregrams and Phospholipase A_2 Activity of Purified *N. nigricollis* Key: Lane 1=molecular weight marker, Lane 2=purified enzyme (65KDa),



Dnecrosis

Plate 2 ABCD: A slight haemorrhage when injected (10µl)with the isolated venom (phospholipase A_2) Plate B and C indicated damage of the muscular tissue with a dose of (20µl) after 3hrs. Necrosis and haemorrhagic severe tissue damage at plate D with the dose of (50µl) after 6hrs of intramuscular injection.

Discussion

Phospholipase A_2 was isolated from *N. Nigricollis* snake venom. It was purified to homogeneity by only two simple chromatographic steps. This phospholipase exhibited high edematogenic and myotoxic activities, demonstrating its capacity to contribute to tissue damage after snakebite or envenoming produced by this *Naja nigricolli*s venom. The two highest peaks from (figure 1) indicated the increase in specific activity of the crude PLA^2 after the two purification steps could be due to the removal of other synergistically interacting components of the venom,[10] these two peaks were selected for further characterization of Phospholipase A_2.

The enzyme appeared as a single band at 65 kDa in SDS PAGE, after reduction by 2-mercaptoethanol (plate 1) which agreed with Najanaja.[11]Indeed, myotoxins of snake venoms affect mainly the plasma membrane of muscle cells to which they bind through their cationic sequence .[12] Molecular mechanism by which they caused the muscle tissue damage is not yet fully elucidated. Myonecrosis is due to the myotoxins that induce irreversible damage of skeletal muscle fibers. These molecules bind to the plasma membrane of muscle cells and alter its permeability and integrity (Plate 5). The toxin induced a dose and time dependent myonecrosis upon IM injection in Rabbit, as evidenced by the significant increase in plasma CK activity (plate 2B and C).

Creatinine Kinase level increased since the first hour after injection to the sixth hour with the highest level (Plate A,B,C and D), the morphological characteristic of myonecrosis induced by this toxin are in distinguishable from those previously described for B.aspermyotoxin II, [13 16] suggesting a common mechanism of action. Figure 2 shows the effect of increasing dose of the *Naja nigricollis* venom on plasma CK level, 382.31 ± 122.70 to 818.31 ± 133.32 IU/L, these results indicate that the plasma CK levels depend on the dose and time of venom induction. Several studies show that PLA^2 enzyme of *bothropic* venom are also able to increase the plasma CK level.[14. 15] These data suggest that increasing concentration of venom result in increasing muscles lesion, and further demonstrate that increasing dose of the injection can also increase these plasma CK level.

Conclusion

The phospholipase A_2 exhibited high edematogenic and myotoxic activities, demonstrating its capacity to contribute to tissue damage after snakebite and envenoming produced by the *Naja nigricollis* snake.

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