Preliminary study on isolation, purification and partial characterization of phospholipase A$_2$ from a Nigerian *Naja nigricolis* venom

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Abstract

Phospholipase A$_2$ in *Naja* species of snake is an enzyme involved in venom toxicity. It hydrolyzes phospholipids, affecting cell membranes. Understanding its properties can contribute to venom research, offering insights into potential medical applications or antivenom development. In this study, isolation and purification of phospholipase A$_2$ from Nigerian *Naja nigricolis* venom was achieved with the use of SDS-PAGE, Resource-S-Cation-Exchange Chromatography and Reverse-phase HPLC. The crude venom was also assessed for PLA$_2$ optimum pH and temperature. A successful isolation and purification was achieved with a notable yield of 768 µg. The enzyme has a molecular weight of 13 kDa and exhibits optimal activity at a pH of 8.0 and a temperature of 50^{0}C. This purification method proves effective in isolating the enzyme, offering potential applications in research and furthering our understanding of the Nigerian *Naja nigricolis* venom phospholipase A$_2$. These findings provide valuable insights into the characteristics of *Naja nigricolis* phospholipase A$_2$ for potential applications in venomics and antivenomics.

Key words: *Naja nigricolis,* Nigerian, isolation, Purification, Phospholipase A_2

Introduction

Envenomation by snakes leads to various pathological conditions due to a complex mix of proteins present in snake venom, which is primarily composed of enzymatic and non-enzymatic proteins, making up approximately 90% of the venom's content.(Tasoulis and Isbister, 2017) These proteins include, but not limited to, serine proteases, metalloproteinases, L-amino acid oxidases, and notably, phospholipase A_2 (PLA_2). The process of isolating, purifying, and characterizing these venom proteins is intricate, often tailored to the specific complexity of the venom and the proteins involved. Structurally and functionally, these proteins can be categorized into super-families, each defined by unique pharmacological effects despite their structural similarities. Within these classifications, PLA_2 enzymes are distinguished into acidic and basic forms based on their amino acid sequences and the nature of their catalytic activities, with the latter playing a more pronounced role in contributing to venom toxicity (Resende et al., 2017).

PLA_2 enzymes, prevalent in various venomous snake families, are pivotal due to their primary function in the hydrolysis of biological membranes, which is crucial for their role in venom's pathogenic effects (Bashir et al., 2020). Notably, some PLA_2 enzymes exhibit neurotoxic effects by targeting the presynaptic junctions, leading to severe neurological impairments (Resende et al., 2017). The enzymatic action of PLA_2 on membrane glycerophospholipids, specifically at the *sn*-2 position, facilitates increased cell membrane permeability. This activity can trigger an uncontrolled influx of Ca^{2+} ions into cells, setting off a cascade of biological events that can culminate in cellular dysfunction (Oliveirar et al., 2022). The release of free fatty acids by PLA_2, including arachidonic acid, further compounds the enzyme's effects by serving as precursors for signalling molecules such as prostaglandins and leukotrienes, which are key mediators in inflammation and pain (Castro-Amorim et al., 2023; Burke and Dennis 2009).

Moreover, the transformation of lysophospholipids by PLA_2 into clotting factors, notably platelet-activating factors, introduces a range of physiological implications, particularly within the immune system (Burke and Dennis 2009). Given the capacity of snake venom components to

disrupt normal physiological processes and chemical balances within the body, they can inflict lasting or temporary pathological conditions (Resiere, et al., 2022). This research seeks to delve into the purification and partial characterization of PLA$_2$ derived from the venom of *Naja nigricolis* in Nigeria. Emphasizing the significance of advancing our understanding through comprehensive studies, including *in silico* and *in vitro/ex vivo* approaches, this study aims to identify potential inhibitors of PLA$_2$. By uncovering inhibitors, we could significantly advance the development of therapeutic interventions to mitigate venom toxicity, marking a pivotal step towards neutralizing the adverse effects of snake venom on human health.

Methodology

Venom Collection and Preparation

The study utilized already extracted and lyophilized venom of *Naja nigricollis*, sourced from the Biochemistry Department at the University of Borno, Nigeria. This initial step ensured a consistent and replicable basis for assessing the venom's phospholipase A$_2$ activity.

Determination of Crude Phospholipase A$_2$ Activity

To assess the activity of Phospholipase A$_2$ (PLA$_2$) in the crude venom, a modified version of the classic egg yolk coagulation technique, originally developed by Habermann and Neumann (Habermann and Neumann 1954) was employed, with some adaptations. This method is known for its sensitivity and specificity to PLA$_2$ activity. In the experimental setup, a precisely measured volume of 10µl of the venom was mixed with an equal volume of 50 mM Tris/HCl buffer at a pH of 8.0. This mixture was then combined with 1000µl of egg yolk-derived L-α-lecithin substrate solution. The resulting mixture was incubated at 37±1^{0}C, a condition optimized to closely mimic physiological temperatures, thereby providing a relevant context for assessing PLA$_2$ activity. Subsequently, the enzymatic reaction was stopped by immersing the mixture in boiling water for 2 minutes. This allows for the accurate quantification of the liberated fatty acids, which are the reaction products indicative of PLA$_2$ activity.

The quantification of these fatty acids was accomplished through titration against 20mM NaOH, using phenolphthalein as an indicator. This colorimetric endpoint titration was conducted to determine the precise amount of

enzyme (PLA_2) necessary to catalyze the hydrolysis of 1µmole of free fatty acids from the L-α-lecithin substrate under the standardized experimental conditions.

Optimum Temperature Estimation

Identifying the optimum temperature for PLA_2 activity involved conducting a series of assays across a temperature gradient ranging from 30 to 80 ºC. This broad spectrum was strategically chosen to capture the possible environmental and physiological conditions that could affect the enzyme's activity. By incrementally adjusting the experimental temperatures within this range and observing the corresponding enzyme activity, the study aimed to pinpoint the specific temperature at which PLA_2 displays its highest enzymatic efficiency. This systematic approach is fundamental for assessing the enzyme's thermal stability and adaptability, which are essential attributes for its potential use in medical and biotechnological applications.

Optimum pH Determination

Similar to temperature optimization, the experiment extended to determining the ideal pH for PLA_2 activity. Recognizing that enzymatic activity is profoundly influenced by the hydrogen ion concentration, the assay explored a wide pH range from 2 to 12. The methodology employed an array of 0.1M buffer solutions—Acetate Buffer for pH 5, Phosphate Buffer for pH 6, and Tris-HCl Buffer for the pH range of 7-12, supplemented with HCl to cover the entire spectrum. These buffers facilitated precise adjustments of the reaction milieu, ensuring that each assay reflected the enzyme's performance across the pH spectrum accurately.

Isolation and Purification of PLA_2

Fractionation of Venom with Resource-S-Cation-Exchange Chromatography

The process of isolating specific components from cobra venom, particularly Phospholipase A_2 (PLA_2), utilized a sophisticated approach involving Resource-S-cation-exchange chromatography, integrated within the high-performance liquid chromatography (HPLC) system Shimadzu model LC-20AD, California, US. Initially, the venom underwent reconstitution in eluent A, composed of 20mM 2-(N-morpholino) ethanesulfonic acid (MES) at a pH of 6, preparing it for the fractionation process. The cation-exchanger was pre-calibrated with eluent A. After this, eluent B, containing 0.8M sodium chloride in 20mM MES at pH 6, was introduced which initiated the elution process, employing an optimized linear-gradient flow maintained at a flow rate of 1ml/min. Protein elution was monitored via

measurement of absorbance at 280nm using UV-VIS Spectrophotometer SP-UVG757. This enabled the identification and collection of fractions rich in PLA_2, based on their enzymatic activity using a fraction collector LP30FC and comparative analysis with existing cation-exchange chromatograms of cobra venoms. After the successful fractionation, the PLA_2-enriched fractions were then processed using diafiltration with Vivaspin. This step concentrated the fractions and removed impurities, preparing the sample for freeze-drying. The freeze-dried samples represented a concentrated form of PLA_2, which was then ready for further stages of purification and subsequent protein identification.

Purification with C_{18} Reverse-Phase HPLC

Following the initial stage, the fractions rich in Phospholipase A_2 (PLA_2) underwent a secondary purification stage employing Reverse-Phase High-Performance Liquid Chromatography (HPLC) Shimadzu LC2050C 3D, California, US. This process commenced with the reconstitution of the PLA_2-containing fractions in Solvent A, a mixture of 0.1% trifluoroacetic acid (TFA) in ultrapure water. The reconstituted fractions were introduced into a LiChrospher WP 300 C18 reverse-phase column of the Shimadzu LC-20AD HPLC system. Prior to the elution process, the column was carefully pre-equilibrated with Solvent A. The elution process was carried out using Solvent B, consisting of 0.1% TFA in acetonitrile. Monitoring of the protein elution was conducted at an absorbance of 215nm using UV-VIS Spectrophotometer SP-UVG757. Following their successful separation, these fractions were then subjected to lyophilization, yielding a dry, concentrated powder of the purified PLA_2 which was then stored at a temperature of −20°C, until it was ready for further analysis.

Validation of PLA_2 with Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For this purpose, SDS-PAGE by Laemmli 1971 was adopted. A 15% polyacrylamide gel was prepared. The sample was mixed with SDS sample buffer constituted with 125mM Tris HCL, 4% SDS, 20% glycerol, 0.02% bromophenol blue, 10% 2 β-mercaptoethanol and 50mM DTT), boiled at 95°C for 5 minutes, and loaded into the wells of the gel. Electrophoresis was performed in Tris-Glycine running buffer (pH 8.3) at constant voltage of 90V applied for 2.5 hours until the dye front reached the bottom. The gel was

stained with Coomassie Brilliant Blue to visualize proteins.

Results

Characterization of Optimal Activity Conditions

Our investigation revealed that the PLA_2 isolated from *Naja nigricollis* venom exhibits its highest enzymatic activity under ~~specific conditions:~~ an optimal pH of 8 and a temperature of 50°C. The enzyme activity declined after reaching the peak at pH 8.0 with lowest activity observed at pH 11.0 (Figure 1). In Figure 2, the highest activity of the enzyme was recorded at 50°C while lowest was at 80°C. There seemed to be a linear increase in activity with increase in temperature until it reached the optimal temperature and thereafter, a linear decrease in activity was observed at higher temperatures. These findings highlight the enzyme's adaptability and potential resilience in various applications, from pharmacological research to therapeutic development.

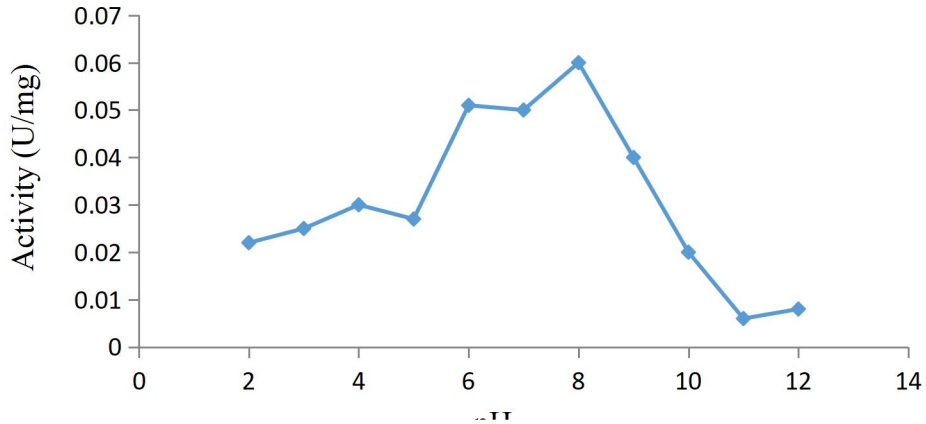


Figure 1. Optimum pH of PLA_2 from *Naja nigricolis* Venom

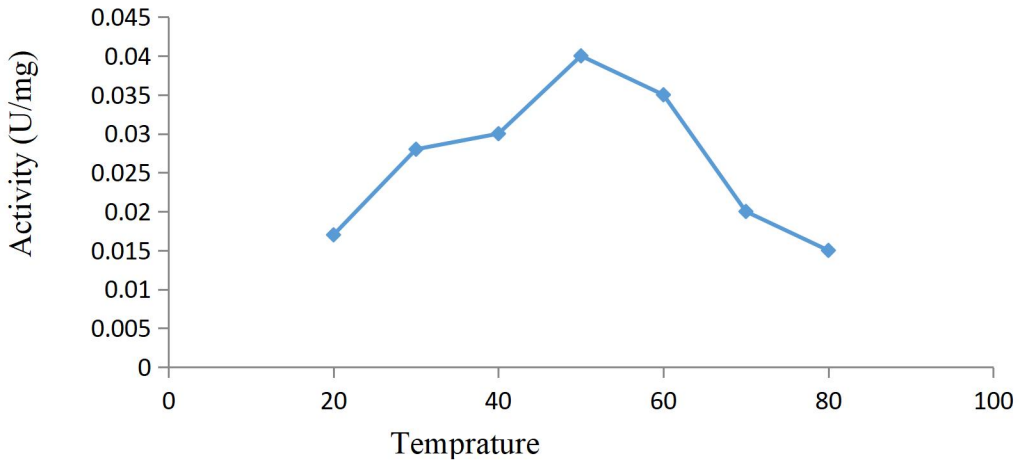


Figure 2: Optimal Temperature of PLA₂ from Nigerian *Naja nigricolis* Venom

Yield from Purification Process

The purification process yielded a significant amount of PLA₂, totalling 768µg from the initial venom sample. This yield underscores the efficiency of the purification techniques employed, marking a successful isolation process that could set a standard for future venom studies.

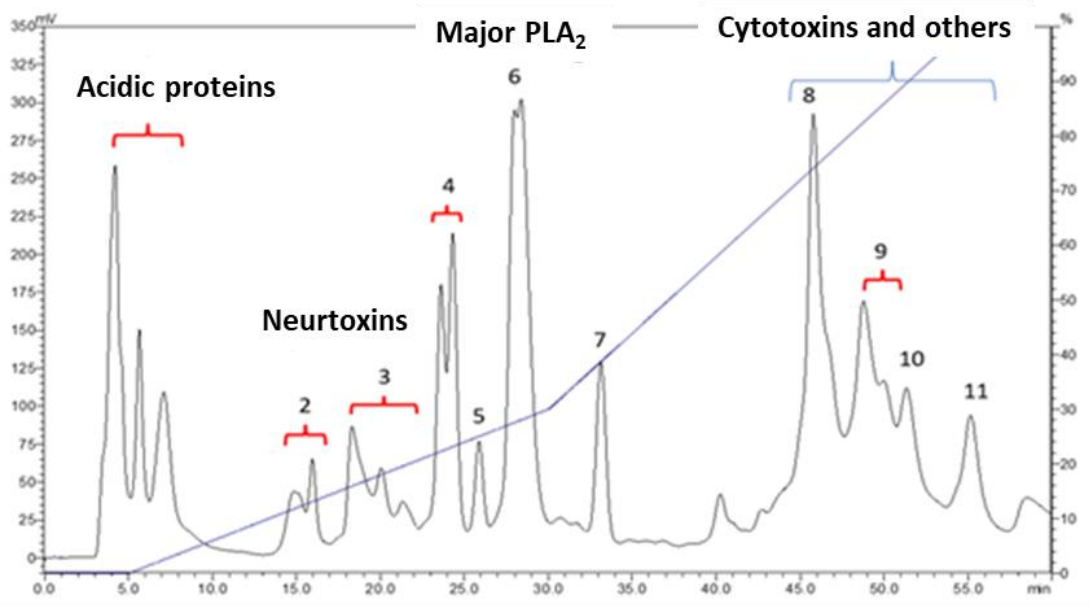


Figure 3. Cation-exchange chromatography of Nigerian *N. nigricollis* venom.

Molecular Weight Determination

Through SDS-PAGE analysis, we determined the molecular weight of the isolated PLA₂ to be approximately 13kDa (Figure 4). This parameter is critical for understanding the enzyme's structural properties and its interaction within the venom's toxicological profile, offering a basis for further structural and functional analysis.

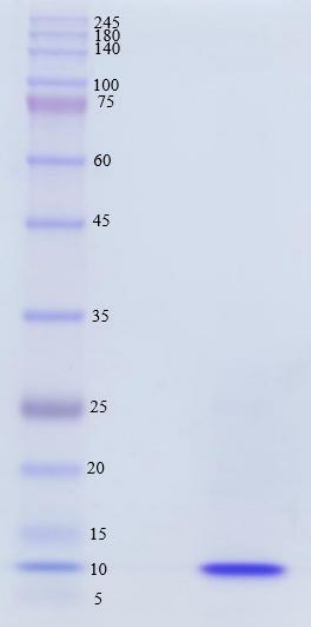


Figure 4. Electrophoretic profile of isolated basic phospholipase A$_2$ (Fraction 6) on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions

Discussion

Venom from *Naja nigricollis*, like that of other venomous species, contains a complex mixture of enzymatic and non-enzymatic proteins, with Phospholipase A$_2$ (PLA$_2$) being a key contributor to its toxic profile. PLA$_2$ hydrolyzes the sn-2 position of glycerophospholipids present in biological membranes, releasing lysophospholipids and fatty acids, which mediate various pharmacological effects such as cell signaling, inflammation and neurotransmission (Adamude et al., 2021; Doley et al., 2010). Previous studies, including those by Kini and Menaldo (2003), emphasized the importance of characterizing and purifying PLA$_2$ to advance our understanding of its functions and mechanisms of action, particularly the basic isoforms, which are largely responsible for the venom's toxic effects (Fernández et al., 2009).

This study established the optimal operational temperature for purified PLA$_2$ from *Naja nigricollis* venom at 50°C. This is consistent with findings by Egbe et al. (2023) for the same species, who identified an optimal temperature of 55°C, and aligns closely with data from Alonazi et al. (2023). However, it contrasts with PLA$_2$ from *Echis ocelatus*, which has an optimal temperature of 40°C

(Sallau et al., 2008). These variations may be attributed to differences in environmental factors and evolutionary adaptations, as highlighted by Zhang (2003) and Kazandjian (2022). The optimal pH for PLA_2 activity was identified as 8.0, which corresponds with earlier report by Resende et al. (2017) for similar enzymes. However, discrepancies exist with studies like that of Bashir et al. (2020), which reported an optimal pH of 7.5, and Alonazi et al. (2023), who observed peak activity at a slightly acidic pH of 5–6. Such differences might be explained by the influence of habitat conditions on enzyme functionality, reinforcing the importance of studying venom components within their ecological contexts (Zheng, 2023).

During the isolation of PLA_2, co-elution with cytotoxins and cardiotoxins presented a challenge, as these proteins eluted alongside PLA_2 in initial chromatography steps. The application of cation-exchange High-Performance Liquid Chromatography (HPLC) successfully separated PLA_2, with its peak identified between 27 and 30 minutes, corresponding to Fraction 6. This fraction, a basic protein, displayed a molecular weight of 12–13 kDa on SDS-PAGE, consistent with PLA_2 from *Naja naja naja* (Deems and Dennis, 1997) and Bothrops atrox (Menaldo, 2015), suggesting a conserved size across species despite potential sequence differences (Fernández et al., 2009). The molecular weight of the isolated PLA_2, approximately 12–13 kDa, is in line with findings from Alonazi et al. (2023), who reported a molecular weight of 14 kDa for PLA_2 from *Cerastes cerastes gasperettii*. This highlights structural conservation across diverse snake species. Additionally, the yield of PLA_2 from *Naja nigricollis* venom was 768 µg, representing 6.4% of the total venom protein. This differs from *Naja melanoleuca*, where PLA_2 made up 12.9% of venom proteins (Lauridsen et al., 2017), underscoring the variability in venom composition across species. Such findings emphasize the need for species-specific approaches to venom research. The results of this study contribute to a deeper understanding of the biochemical requirements such as temperature and pH of PLA_2 from *Naja nigricollis*, revealing key insights into its enzymatic behavior under different conditions. These findings not only provide a basis for future studies on PLA_2's toxicological and therapeutic applications but also highlight the importance of considering environmental and evolutionary factors in venom research. Future investigations should focus on elucidating the molecular mechanisms behind the observed variations in optimal conditions across species.

Furthermore, the study's limitations include the co-elution of toxins during isolation, which could be addressed in future research by refining purification techniques to enhance enzyme purity and yield.

Conclusion

This study successfully optimized the operational conditions and isolated Phospholipase A_2 (PLA_2) from *Naja nigricollis* venom, with an optimal temperature of 50°C and a pH of 8.0. The enzyme, with a molecular weight of 12–13 kDa, showed structural similarities to PLA_2 from other species, though challenges with co-elution of toxins highlighted the need for improved purification techniques. These findings advance our understanding of venom biochemistry and support further research on PLA_2's toxicological and therapeutic potential, particularly in antivenom development. Future studies should focus on refining isolation methods and exploring PLA_2's pharmacological applications.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of Interest

The Authors declare that there is no conflict of interest.

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