Partial Characterization of Phospholipase A$_2$ from the Erythrocytic Stage of Plasmodium berghei

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ABSTRACT
Phospholipase A$_2$ (EC. 3.1.1.4) was isolated and partially characterized from the erythrocytic stage of Plasmodium berghei (NK-65) obtained from experimentally infected mice with the objective of studying its kinetic properties and its possible role in the pathogenesis of malaria. The parasite collected by sucrose gradient centrifugation was subjected to lysis to obtain the crude phospholipase A$_2$ which was assayed and subjected to some biochemical characterizations. The enzyme had broad pH and temperature ranges with optima of 7.5 and 37°C, respectively. Initial velocity studies for the determination of kinetic parameters with L-α lecithin as substrate revealed a $K_m$ and $V_{max}$ of 0.68 mg mL$^{-1}$ and 52.60 μmol min$^{-1}$, respectively. The P. berghei PLA$_2$ was slightly activated by Ca$^{2+}$ while Cu$^{2+}$, Zn$^{2+}$ and Mn$^{2+}$ were inhibitory to the enzyme. However, no enzyme activity was detected in the presence of Mg$^{2+}$ and Hg$^{2+}$. Considering the findings of this work, P. berghei can be said to contain PLA$_2$ which has similar properties with some other parasites PLA$_2$ and could be involved in cellular invasion and/or anemia development during cerebral malaria.

Key words: Phospholipase A$_2$, Plasmodium berghei, kinetics, enzyme, parasite

INTRODUCTION
Malaria is a widespread parasitic disease with estimated global incidence of 300-500 million clinical cases each year and mortality estimated to be more than one million. The causative agent of malaria is an intracellular protozoan parasite from the genus Plasmodium (Deregnaucourt and Schrevel, 2000). Of the four common species, Plasmodium berghei (rodent malaria parasite) provides a well established experimental model of malaria infection (Margarida et al., 2006). The life cycle of the malarial parasite Plasmodium forms three invasive stages which have to invade different and specific cells for replication to ensue; this process is vital to parasite survival and consequently proteins responsible for invasion are considered to be vaccine candidates/drug targets (Ecker et al., 2007).

Phospholipase A$_2$ (PLA$_2$) catalyze the hydrolysis of the sn-2 ester bond of glycerophospholipids, leading to the production of non-esterified fatty acids (NEFAs) and lysophospholipids (lysoPLs). The enzyme is involved in diverse processes such as membrane homeostasis, nutrient acquisition and generation of bioactive molecules, intricate modulation of the host’s immune response and tissue invasion (Kohler et al., 2006). The enzyme has been reported in some intracellular parasites; Trichomonas vaginalis, Toxoplasma gondii where it’s required for tissue invasion (Lubick and Burgess, 2004; Cassaing et al., 2000). However, the enzyme play a distinct role in
Trypanosoma congolense where its involved in cleavage of erythrocytes membrane phospholipids leading erythrocytes destruction and hence anemia (Nok et al., 1988). Anemia is also one of the commonest complications of plasmodial infections (Iyawe and Onigbinde, 2009) and therefore provides an important target for research into malarial pathogenesis (Jones et al., 2002). So far, among the pathogenic plasmodium species, PLA₂ has only been described from P. falciparum and the relevance of its inhibition to the therapeutic action of some antimalarial drugs demonstrated (Zidovetzki et al., 1993). Thus, the enzyme may play some significant role in the infection biology of the parasite and could be exploited as a target for deciphering novel chemotherapeutic agents against the disease. Therefore, in this study, we report on biochemical characterization of PLA₂ from P. berghei with the view to obtain preliminary kinetic data for the enzyme.

MATERIALS AND METHODS
Reagents were purchased from Sigma Chemical Company, St. Louis, USA in 2007. Blood-stage samples of P. berghei (NK-65) were obtained from National Institute of Medical Research (NIMR), Lagos, Nigeria in April, 2008 and were maintained by serial passage in mice. Age and sex matched albino mice were obtained from Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Nigeria. The entire study was conducted in the Research Laboratory of the Department of Biochemistry, Ahmadu Bello University, Zaria-Nigeria between August, 2008 and December, 2008.

Experimental infection with Plasmodium berghei: A donor mouse with 20% P. berghei infected erythrocytes was sacrificed and its blood collected in heparinised syringe and diluted in phosphate buffer saline. Five mice were then experimentally infected with of 0.2 mL of the Plasmodium berghei parasite preparation. Blood samples were daily taken from the tail veins of infected mice during the first week of infection to monitor the parasite load by microscopic Giemsa-stained thin blood smears.

Isolation of Plasmodium berghei: The mice were then sacrificed when the number of parasitized erythrocytes was about 20% and the collected blood subjected to sucrose gradient centrifugation as described by Fernandez et al. (1998) to obtain the pure parasites which were then lysed by freeze thawing and treatment with 5% tween 80. The parasites lysate was then used as the crude enzyme.

Phospholipase A₂ (PLA₂) assay: The PLA₂ was assayed by incubating 25 μL of 1 mg mL⁻¹ L α-lecithin substrate with 10 μL of the crude enzyme source for 10 min at 37°C. The reaction was then terminated by immersing the tube in a boiling water bath for 2 min and the amount of released free fatty acid measured titrimetrically at pH 8.0 using NaOH and phenolphthalein as indicator (Bhat and Gowda, 1989). The enzyme activity was expressed as the amount of enzyme that hydrolyzes one μmole of fatty acids from L- α-Lecithin per minute under standard conditions.

pH- and temperature-dependent studies: A pH dependent assay was performed using 50 mM acetate buffer pH 4.0-6.0 and 50 mM phosphate buffer pH 6.5-8.5. A temperature dependent study was also conducted by determining the enzyme activity at varying incubation temperatures ranging from 20-80°C at intervals of 10°C.
**Initial velocity studies:** This was done by incubating the enzyme with varying concentrations of Substrate L-α-Lecithin (0.063-1.0 mg mL⁻¹) to obtain corresponding Vo activity values. The Michaelis constant $K_M$ and Maximum velocity $V_{MAX}$ were computed from Lineweaver-Burk plot of the initial velocity data.

**Effects of some divalent cations on PLA₂ activity:** The enzyme activity was assayed in the presence of chloride salts of the following divalent ions; Ca²⁺, Zn²⁺, Mg²⁺, Cu²⁺, Mn²⁺ and Hg²⁺ at 10 mM final concentrations.

**RESULTS**

The pH dependent profile of the *P. berghei* PLA₂ revealed an optimal peak at pH 7.5. Relatively lower enzyme activities were observed at acidic pHs in comparison to alkaline pH (Fig. 1). The results of the temperature dependent studies showed a typical bell shaped curve with optimum activity at 37°C (Fig. 2). Figure 3 presents the double reciprocal plot of the *P. berghei* PLA₂ with $K_M$ and $V_{MAX}$ of 0.68 mg mL⁻¹ and 52.60 μmol min⁻¹, respectively using L-α lecithin as substrate. In the analysis of metal divalent cations on the activity of the enzyme, Ca²⁺ was found to activate the enzyme whereas Cu²⁺, Zn²⁺ and Mn²⁺ were inhibitory to the enzyme. No enzyme activity was detected in the presence of Mg²⁺ and Hg²⁺ (Fig. 4).

![Fig. 1: Effects of pH on PLA₂ activity from *P. Berghei*](image1)

![Fig. 2: Effects of temperature on PLA₂ activity from *P. Berghei*](image2)
**DISCUSSION**

The role of PLA$_2$ in the pathogenesis of some intracellular parasites have been fully elucidated (Joshi et al., 1987; Zidovetzki et al., 1993; Cassaing et al., 2000; Lubick and Burgess, 2004). So far, among the genus *Plasmodium*, it is only *P. falciparum* reported to produce PLA$_2$ whose inhibition related to the therapeutic action of some antimalarials (Zidovetzki et al., 1993). This report reveals the isolation and some biochemical characterization of PLA$_2$ from the erythrocytic stage of *P. berghei*.

The *P. berghei* PLA$_2$ optimal activity at pH 7.5 was found to be similar to that reported for *P. falciparum* PLA$_2$ (Zidovetzki et al., 1993) and could be attributed to the pathological role of the enzyme in the mammalian host. A basic pH optimum region contributes to the enhancement of the enzymatic reaction by general base catalytic mechanism. Indeed, the optimum pH of 7.5 could implicate more positively charged amino acid at the active site of the enzyme since the ionizable groups in the active site must be in proper ionic forms to maintain conformation of the active site in order to bind the substrate and catalyse the reaction. Moreover, the observed bell shaped curve be due to the unfolding and subsequent denaturation of the enzyme molecule outside the pH region in which the enzyme is stable and active. The temperature dependent studies showed a typical bell shaped curve with optimum activity at 37°C (Fig. 2) which is a favourable temperature for parasitic life in the mammalian host and complete cessation of activity at above 70°C. Optimal activities of
PLA$_2$ from *T. congolense* and *T. vaginalis* PLA$_2$s have been reported to be 37°C (Nok et al., 1993; Lubick and Burgess, 2004). It therefore appears that optimal activity at 37°C is a common property of parasites PLA$_2$s. A close examination of the temperature activity profile could indicate two different effects of temperature operating simultaneously in the *P. berghei* PLA$_2$ catalysed reaction. The two effects are the increase in initial velocity of the enzyme reaction at the lower temperatures and the heat destruction of the enzyme at higher temperatures. The latter effect leads to a continuous decline in enzyme concentration as indicated by the increasing curvature of the progress curve as temperature rises until velocity falls to zero.

The fairly low $K_M$ is an indication of moderately high affinity of the enzyme for cellular phospholipids. Moreover, the $V_{max}$ of 52.60 µmol min$^{-1}$ could indicate that at the end of one hour post *P. berghei* infection about 3156 µmols of free fatty acids would have been excised from the host’s cells which may consequently accelerate the cellular invasion/penetration processes by the parasite and/or anemia development during the disease (Iyawe et al., 2006). It may also lead to generation of free fatty acids in the host system leading to cytotoxicity.

The slight activation of the enzyme by Ca$^{2+}$ contrasts the report of Zidovetzki et al. (1993) and Cassaing et al. (2000) where *P. falciparum* and *T. gondii* PLA$_2$s respectively, were Ca$^{2+}$ independent. Thus, Ca$^{2+}$ requirements by the parasites PLA$_2$s could be organism and/or species related. Studies have shown that some PLA$_2$s need submicromolar concentrations of Ca$^{2+}$ to be catalytically active and also increase in Ca$^{2+}$ is needed for both enzyme binding and catalysis (Sallau et al., 2008). The observed effects of other cations (analysed herein), although divalent as calcium, may suggest a probable modification of enzyme protein conformation after binding differently from the pattern calcium does and hence causing a decrease in the enzyme action. Therefore, the presence of these ions in physiological systems could modulate the activity of this enzyme during the course of *P. berghei* infection.

We therefore conclude that *P. berghei* expresses PLA$_2$ and could be important in the pathogenesis of cerebral malaria. We are presently working on the role of this enzyme in the disease pathogenesis by monitoring the inhibitory effect of some antimalarials on the enzyme.

REFERENCES


