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Investigation of Sialidase of *Plasmodium berghei* Infected Albino Mice in the Pathology of Malaria.

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ABSTRACT

Malaria is a major public health problem in the world in general and developing countries in particular, and it is becoming more difficult to manage particularly in areas of multi-drug resistance. Hence, the study was designed to carry out the sialidase activity in the *Plasmodium berghei* infected albino mice. Albino mice free from infection were experimentally infected with *Plasmodium berghei*. The animals showed detectable parasitemia on day 4 post-infection with about 30 % of parasitemia recorded before death of animal. Packed cell volume (PCV) level decreased with increase in the post infection days. Erythrocyte surface sialic acid (ESA) decreased while sialic acid from the serum and brain increased with increase in the level of parasitemia. The activity of the sialidase also increased both during infection and treatment compared to the pre-infection values. In addition, it was found that *Plasmodium berghei* produced sialidase. The partially purified enzyme showed a single band on SDS PAGE with molecular weight of 54.8 kDa. Inhibitory study of the neem leaf and stem bark on the sialidase revealed mixed competitive inhibition pattern.
Keywords: Malaria, *Plasmodium berghei,* parasitemia, sialic acid, sialidase.

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INTRODUCTION

Sialic acids are a family of carboxylated saccharides containing nine carbon atoms and are negatively charged. Sialic acid as a terminal saccharide residue on cell surface glycoconjugates plays important role in a variety of biological processes (Schauer, 2000). Sialic acid occurs on the RBC, masking galactosyl residue since galactose is the immediate sugar after sialic acid (Schauer, 1991). Removal of sialic acids by whatever means which is usually by actions of sialidase from microorganisms results in the destruction of such cells (Jancik *et al*., 1978; Kolb and Kolb-Bachofen, 1978; Kuster and Schauer, 1981; Muler *et al*., 1981, 1983).

Sialidases (EC 3.2.1.18) are the key enzymes of sialic acid catabolism, which hydrolyse the glycosidic linkage between sialic acid and the underlying sugars (Pereira, 1983; Roggentin *et al*., 1994). These enzymes are widely spread in microorganisms like fungi, bacteria and protozoa (Corfield, 1992; Engstler *et al*., 1993) which mostly lack sialic acids. The sialidase cleaves erythrocyte-surface sialic acids thereby, exposing the masked galactosyl residues.

The enzyme sialidase acts on RBC desialylating them, leading to asialo-RBC. These galactosyl residues are then recognised by galactose specific lectins on macrophages, leading to opsonization and erythrophagocytosis or sequestraion of these RBCs from circulation (Jancik *et al*., 1978; Muller *et al*., 1981). Hence, reduction in number of circulating RBC, low PCV, leading to a condition called anemia.

Plasmodium berghei is a unicellular protozoan parasite and is one of the many species of malaria parasites that infect mammals other than human which have been described in African murine rodents (Carter and Diggs, 1977). *Plasmodium berghei* belongs to a group of four plasmodium species that infect murine rodents from Central Africa. These species are *P. vinckei*, *P. chabaudi, P. yoelli and P. berghei* (Killick-Kendrick, 1978). They are not of direct practical concern to man or his domestic animals. The interest of these parasites is that they are practical model organisms in the laboratory for the experimental study of human malaria (Killick -Kendrick, 1978, Carter and Diggs, 1977).

Plasmodium berghei originates from the forests of Central Africa where its natural cyclic hosts are thicket rat *Grammonys surdaster* and the mosquito *Anopheles dureni* (Killick -Kendrick, 1978; Wikipedia, 2007). *Plasmodium berghei* infections are lethal to laboratory rodents.

Like all malaria parasites of mammals, including the four human malaria parasites, *P. berghei* is transmitted by Anopheles mosquitoes and it infects the liver after being injected into bloodstream by a bite of an infected female mosquito. After few days of development and multiplication, these parasites leave the liver and invade erythrocytes (red blood cells). The multiplication of the parasite in the blood causes the pathology such as anaemia and damage to essential organs of the host such as lungs, liver and spleen. *P. berghei* infections may also affect the brain and can be the cause of cerebral complications in laboratory mice (Daily, 2006; Killick -Kendrick, 1978).

The current study aimed to investigate the effect of sialidase enzyme in the plasmodium berghei infected albino mice.

MATERIALS AND METHODS

Experimental Animals

Mature Swiss albino mice (20-25g) of either sex free from infection, were obtained from the animal house facility from the department of Pharmacology and Clinical Pharmacy, Ahmadu Bello University, Zaria, Nigeria. They were stabilized for seven days in the laboratory before being used for experiments. The animals were maintained in a well ventilated room, with temperature of 25 ± 1°C, fed on Excel feeds (Feed Masters Ilorin) and water ad libitum.

Parasite Strain

Malaria parasite (*Plasmodium berghei*, Anka strain) was obtained from Prof. Jacob Golenser of Kuvin Medical Centre, Hebrew University of Jerusalem. The strain was maintained in the laboratory by serial blood passage from mouse to mouse.

Inoculation of Mice and parasite counting

A donor mouse with a rising parasitemia of 20 % was sacrificed and its blood was collected in heparinized syringe and diluted in phosphate buffered saline to10^8 parasitized erythrocytes/ml. The infection of mice was initiated by needle passage of the *Plasmodium berghei* parasite preparation from the donor mouse to healthy test mice via an intraperitoneal route (Peter and Anatoli, 1998; David, *et al* 2004)). Each mouse received 0.2 ml (total of 2 x 10^7 parasitized erythrocytes) of dilute infected whole blood. Parasitemia was monitored by microscopic Giemsa-stained thin blood smears. The number of parasitized erythrocytes in each of the ten such fields was counted thrice and the average was computed to give the level of parasitemia of each mouse (Peter and Anatoli, 1998; Dawit *et al*., 2006).

Preparation of haemoglobin free erythrocyte membranes (Ghost Cells)

Ghost cells were prepared using the method of Dodge *et al* (1963) on the day of blood collection. The sialic acid was determined using the Periodate-thiobarbituric acid (TBA) Assay for Free Sialic Acids Amminoff (1961). The free sialic acid concentration in the serum samples collected was determined by the TBA assay using 100µl of serum (Engstler *et al.*, 1995). The Bound sialic acid concentration in the serum was determined using 100 µl of serum incubated with 20 µl of 0.1 M HCl for 3 hours to liberate the bound sialic acid. The quantified sialic acid, here, is known as total sialic acid ($[SA]_T$). The difference between the total and free sialic acid represents the bound sialic acid. The distribution of sialic acid is thus;

$$[SA]_T = [SA]_B + [SA]_F.$$

$$\text{Therefore } [SA]_B = [SA]_T - [SA]_F$$

Erythrocytes surface sialic acid (RBC-SA)

Sialic acid on the red cells, was determined (Esievo *et al.* 1982), 100 µl of the washed erythrocyte ghosts was incubated with 100 µl of 0.1 M HCl at 80^{o}C for 1h to liberate the membrane bound sialic acid. The liberated sialic acids concentration was assayed by the TBA method.

Preparation of Parasite Extracts

Red blood cells infected with high parasitemia were incubated with 0.5 % Triton X-100 in Phosphate Buffered Saline (PBS) at 37ºC for 10 min to lyse erythrocyte membrane and were washed three times with ice cold Phosphate Buffered Saline. Released parasites were lysed with 50 mM Phosphate buffer, pH 8.0, and centrifuged at 10,000 g for 30 min, (Raphael *et al*., 2002). The resulting supernatant was referred to as 'parasite extract'.

Isolation of Sialidase

The crude sialidase enzyme was obtained by freeze thawing and treatment of the soluble parasite extract with 0.5% Tween 80 and kept at room temperature for 30 min.

Partial purification of *sialidase* from *P. berghei*

The plasmodium lysate was applied on DE-52 cellulose column (1 cm x 12 cm) which was pre-equilibrated with 50 mM of Phosphate Buffer (pH 7.2). And the column was eluted with a gradient of NaCl (0.0-0.3 M) in 50 mM phosphate Buffer. 3-ml fractions were collected**.** Twenty-two fractions were collected (Lanham and Godfrey, 1970).

Protein determination by a protein Dye-Binding Assay (Bradford, 1976)

The total protein of the purified fractions was determined by employing protein Dye-Binding Assay (Bradford, 1976) using BSA as standard. Each sample (100 µl) was transferred into a test tube and 300 µl of distilled water was added followed by 100 µl of Coomassie dye and then mixed thoroughly. After standing at room temperature for 5min. Absorbance was taken at 595nm.

Size Exclusion Chromatography

Sialidase active fraction from the preceding step with high specific activity was applied onto a Sephadex G-100 column (1.5 cm X 50 cm) pre-equilibrated with 50 mM phosphate buffer, pH 7.2. The column was eluted with the same equilibration buffer and 40 fractions each of 2 ml were collected at a flow rate of 10 ml/ h. Protein peaks were analyzed for sialidase activity (Kleinadam *et al* ., 2001).

Sialidase Assay

Sialidase activity was assayed as described by Kleinadam *et al* (2001) in all the fractions collected at each purification stages. Fifty microlitres of the substrate (4-MU-Neu5Ac) at concentration of 0.7 mM in 50 mM phosphate buffer, pH 7.2, was incubated with 25 µl of serum, intact parasite, or parasite lysate i.e. crude or partially purified sialidase from *P berghei* at 37^{o}C for 1 h. At the end of the incubation period, the reaction was terminated using 500 µl of 0.5M Borate buffer, pH 9.2. The released methylumbelliferone was then measured at 360 nm. For each determination, the spectrophotometer response was calibrated with pure methylumbelliferone as standard and readings were corrected by subtracting serum and substrate blanks. One unit of enzyme activity was defined as the amount of enzyme that hydrolyses 1 µmol of 4-MU-Neu5Ac per minute under the described assay conditions.

SDS-PAGE Analysis of Sialidase fractions

Routine protein electrophoresis, a criterion for enzyme purification was carried out on 12 % polyacrilamide disc gel for fractions with high specific activity (Davis, 1964). Sialidase molecular weight determination was carried out as described by Wiser (2004).

Characterization of the partially purified *sialidase*

Effect of temperature on partially purified *sialidase* activity

The effect of temperature on the rate of 4-MU-Neu5Ac cleavage was measured by incubating the enzyme and substrate at 5^{o}C- 105^{o}C at an interval of 10^{o} C for 30 min, and activity assayed as described above. A plot of enzyme activity against temperature was prepared to determine the optimum temperature.

Effect of pH on partially purified *sialidase* activity

The pH profile was determined by incubating the enzyme and substrate in the following buffers: 60mM acetate pH 4-8 and 50mM phosphate, pH 4 - 9. The enzyme activity was assayed in each case as described earlier. A plot of enzyme activity against pH was prepared to determine the optimum pH.

Kinetic studies of *sialidase*

Fifty microlitres of substrates (4-MU-Neu5Ac) at concentration range of 0.69 mM - 0.05 mM by serial dilution in 100 µl 50 mM phosphate buffer pH 7.2 was incubated with 25 µl of partially purified enzyme at 37^{o} C for 30min. The enzyme activity was determined as described earlier.

Inhibitory studies of *sialidase*

Aliquots of the neem extracts (inhibitors) 50 µl each at concentration range of 1.25 - 10.0 mg/ml were incubated with 25 µl of purified sialidase at 37^{o}C for 30 min. Then four different concentrations of the substrate methylumbelliferyl-N-acetylneuraminic acid at concentration range of 0.05 mM - 0.70 mM in 50 mM

phosphate buffer (pH 7.2) was prepared. Fifty microlitres of the substrates (4-MU-Neu5Ac) were added to a treated enzyme and the mixtures incubated at 37°C for 1 h. The enzyme activity was determined as earlier described.

RESULTS

Changes in serum and Erythrocyte sialic Acid at different days of post infection

There was an increase in the level of free sialic acid in the serum of the infected mice with increase in the days of post infection (figure 1) which corresponds to a fall in PCV (figure 2). The erythrocyte surface sialic acid (RBC-SA) at different level of parasitemia showed a gradual decrease in the level of mean RBC-SA (figure 3).

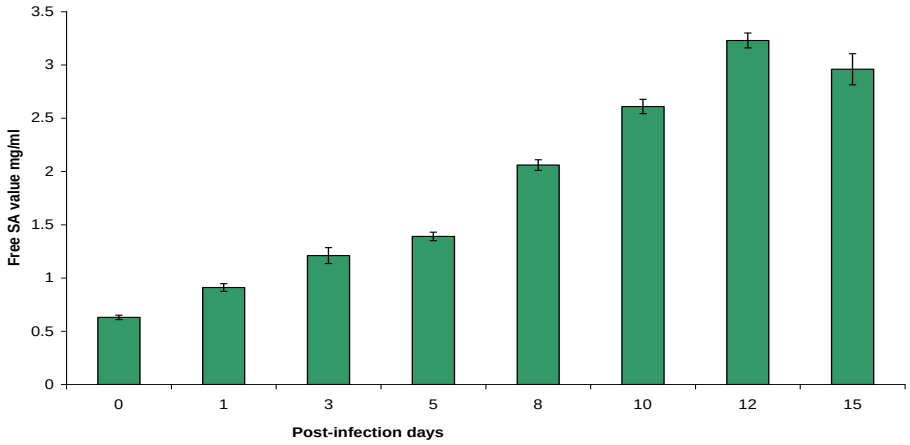

Figure 1: Free sialic acid profile of the serum of *P. berghei* infecte mice at different days of post infection

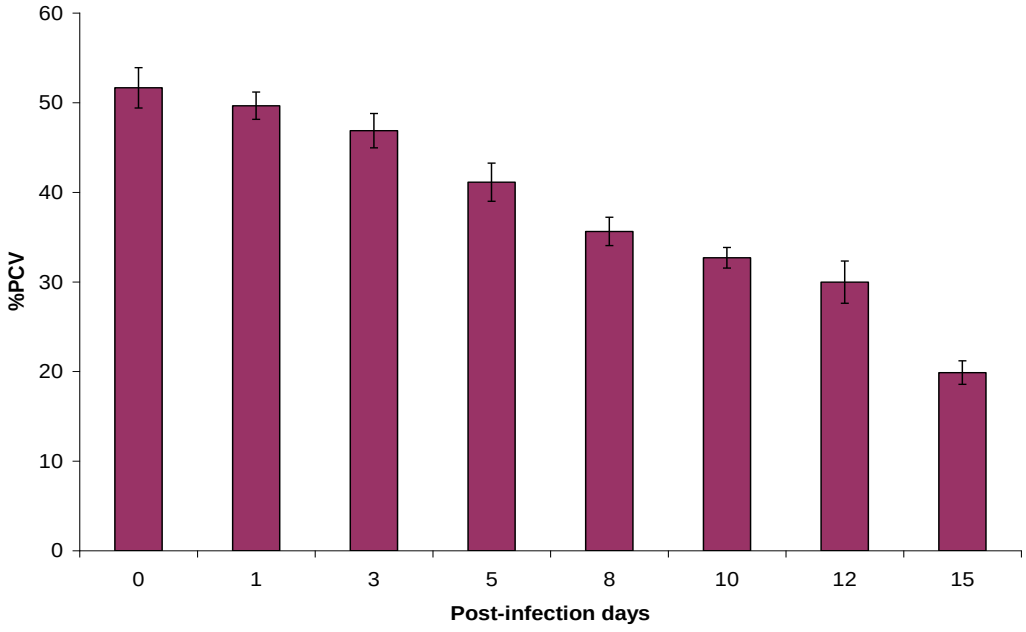

Figure 2: Packed cell volume of *P. berghei* infecte mice blood at different days of post infection

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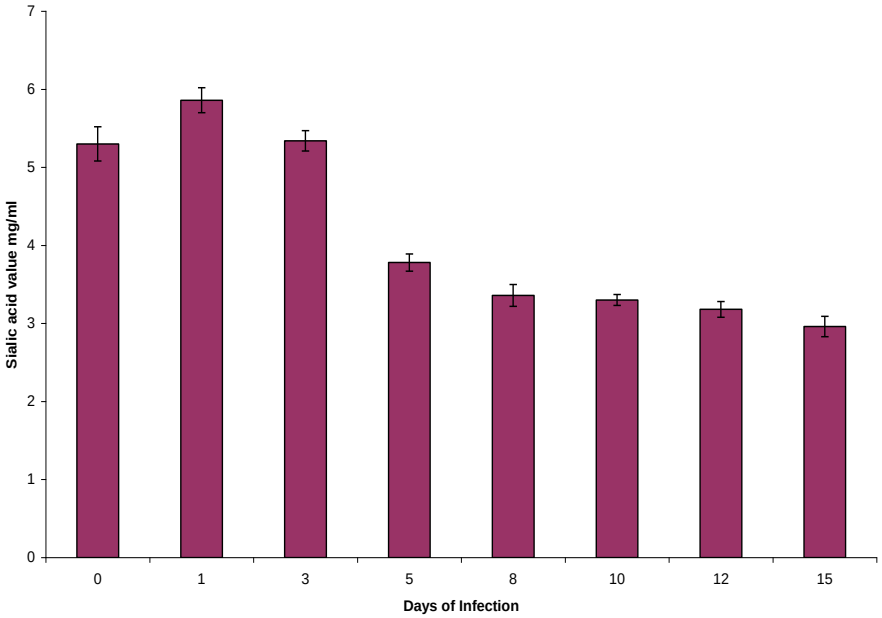

Figure 3: Erytrocyte Sialic aicd (RBC-SA) of *P. berghei* infecte mice at different days of post Infection

***Sialidase* Activity in the serum**

The level of serum sialidase was observed to increase progressively with increase in the level of parasitemia (figure 4). In the serum of control mice (uninfected), there was no detectable sialidase activity. The summation of these observations strongly points to the parasite as the source of sialidase in infected mice.

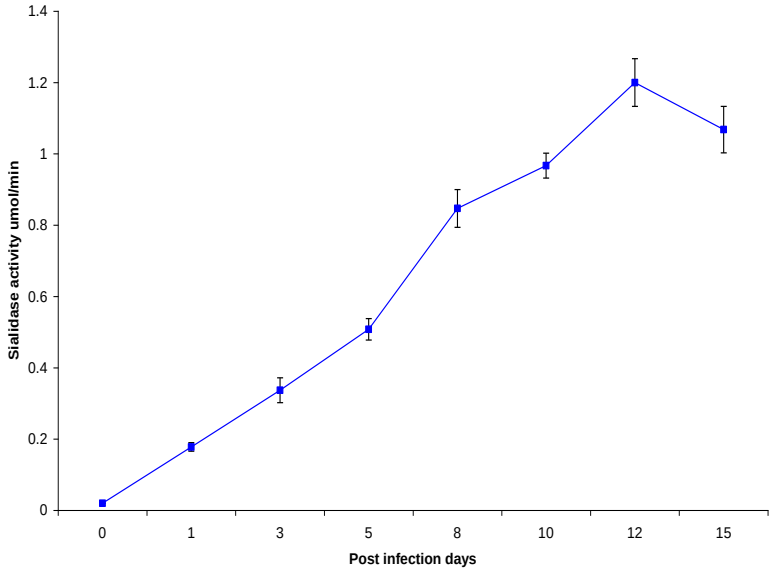

Figure 4: Sialidase activity at differtent different days of post infection in serum of *P. berghei* infected mice

Purification of *sialidase* from Plasmodium berghei

Three protein peaks were observed after eluting the parasite extract from the DE-52 Cellulose column with each peak having activities (figure 5). Fraction number 5, 12 and 20 showed highest sialidase activity. They were then subjected to UV light of which only fraction 20 flouresced (plate 1). This fraction was then used

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for gel filtration chromatography. Two active protein peaks were eluted which had high sialidase activity (figure 6) and only fraction 21 flouresced. The protein electrophoresis showed one protein band with molecular weight of 54.8 kDa (plate 2) on SDS-treated polyacrylamide gel. The activities and yields of partially purified sialidase during purification steps are shown in Table 1. The crude extract contained 10.06 mg/ml of proteins with a specific activity o.147 µmol/min. The final purified sialidase obtained by gel filtration yielded high specific activity (0.737µmol/min).

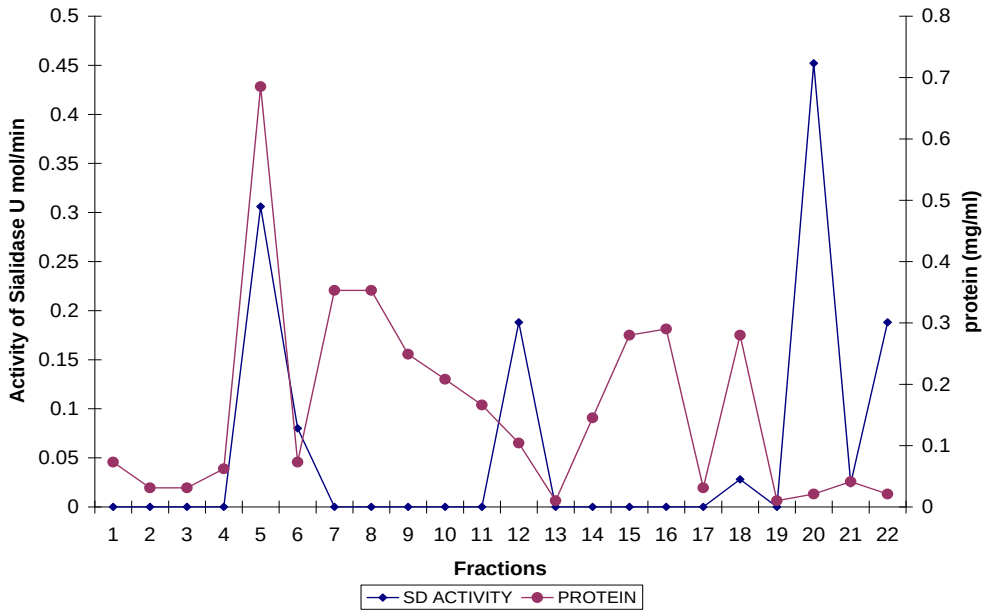

Figure 5: Elution Profile of *P. berghei* Sialidase from DE-52 Column

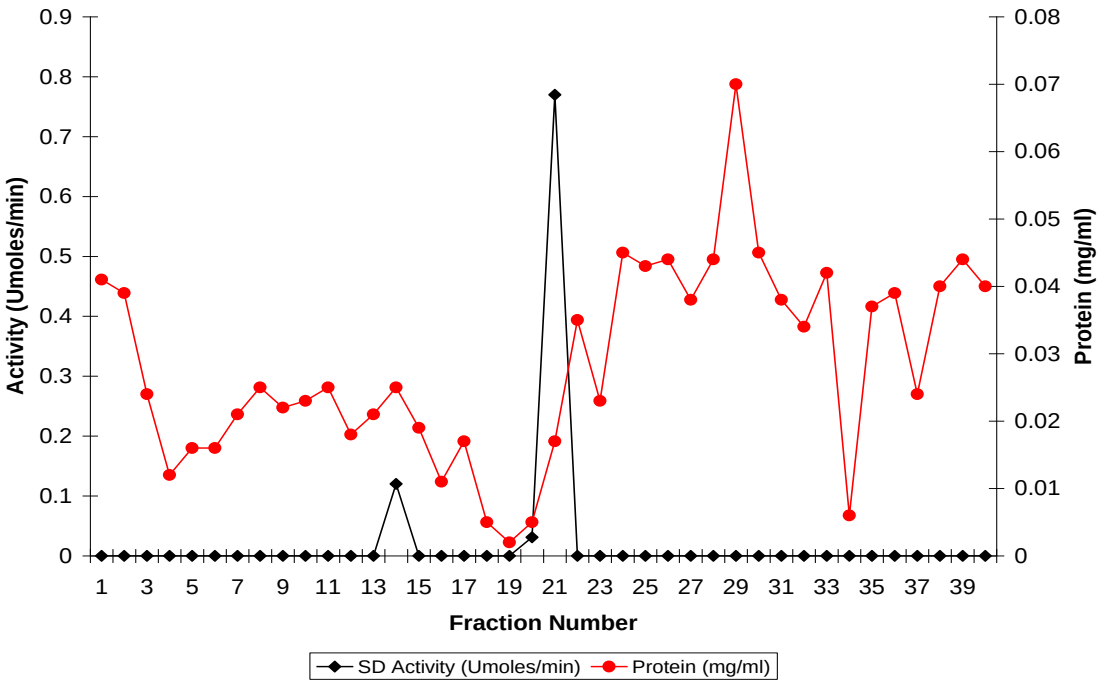

Figure 6: Gel filtration elution profile of *Plasmodium berghei* Sialidase on Sephadex G-100 column

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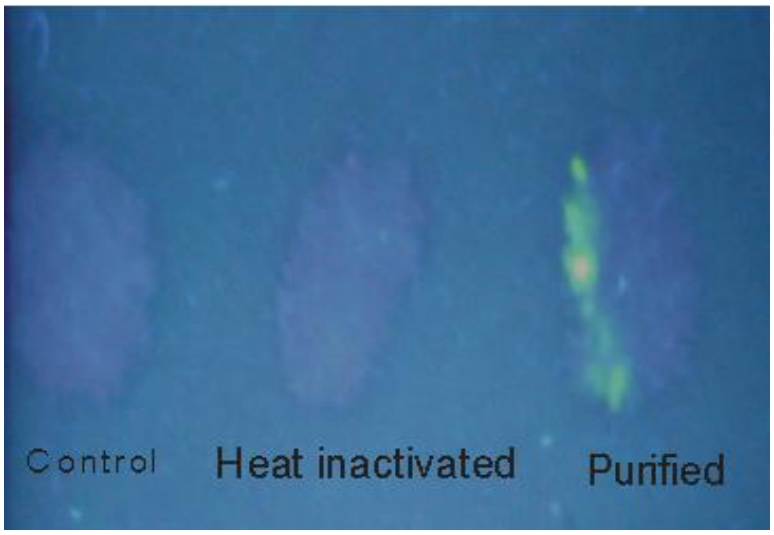

Plate 1: Flourescence picture of Sialidase in the isolated parasite

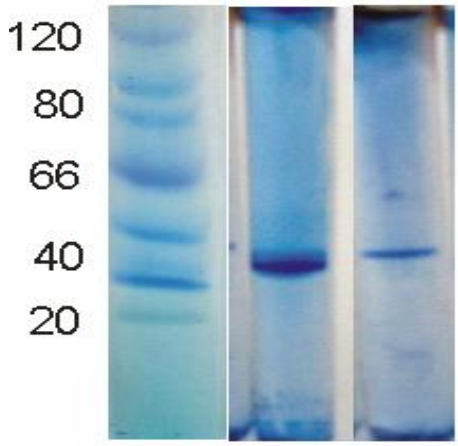

Plate 2: SDS-PAGE analysis of partially purified *Plasmodium berghei* Sialidase on 12 % acrylamide gels. Lane A: Molecular weight standards (6.5-120 kDa), Lane B: Crude enzyme, Lane C: Purified Sialidase (54.8 kDa)

Table 1: Purification table

Purification Steps	Protein (mg/ml)	Total activity (µmol/min)	Specific activity (µmol/min/mg protein)	Yield (%)	Purification fold
Crude	10.06	1.48	0.147	100	1.0
DE-52	3.52	1.266	0.360	70.21	2.45
Sephadex G-100	1.25	0.921	0.737	62.16	5.01

Optimum Temperature Determination

Temperature dependent studies showed that the *Plasmodium berghei* sialidase was optimally active at 35^{o}C (Figure 7). At temperature between 10^{o}C and 30^{o}C, the enzyme activity was found to double for every 10^{o}C rise in temperature. An over 60 % loss of sialidase activity accompanied a rise in temperature from optimum temperature to 50^{o}C.

Optimum pH Determination

The optimum pH activity of the enzyme showed maximum proteolytic activity at pH 6.0 (Figure 8). The activity of the sialidase is higher in the acidic region than was seen in the alkaline range.

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Lineweaver-Burk Plot for Sialidase Activity

Lineweaver Burk plots of initial velocity data of *Plasmodium berghei* sialidase reaction velocity to methylumbelleferyl - N – acetylneuraminic acid concentration showed that the enzyme has a K_m and V_{max} of 0.067 mM and 0.286 µmol/min respectively (Figure 9).

Inhibitory studies of neem extracts against *Plasmodium berghei Sialidase*

The inhibitory studies of the neem extracts (leaf and stem bark) against the activity of sialidase showed the same type of inhibitory pattern. Both showed mixed competitive inhibition, with K_i and K_I values of 0.097 and 0.066 for neem leaf extract and -0.235 and -1.903 for neem stem bark respectively (Figures 10 and 11).

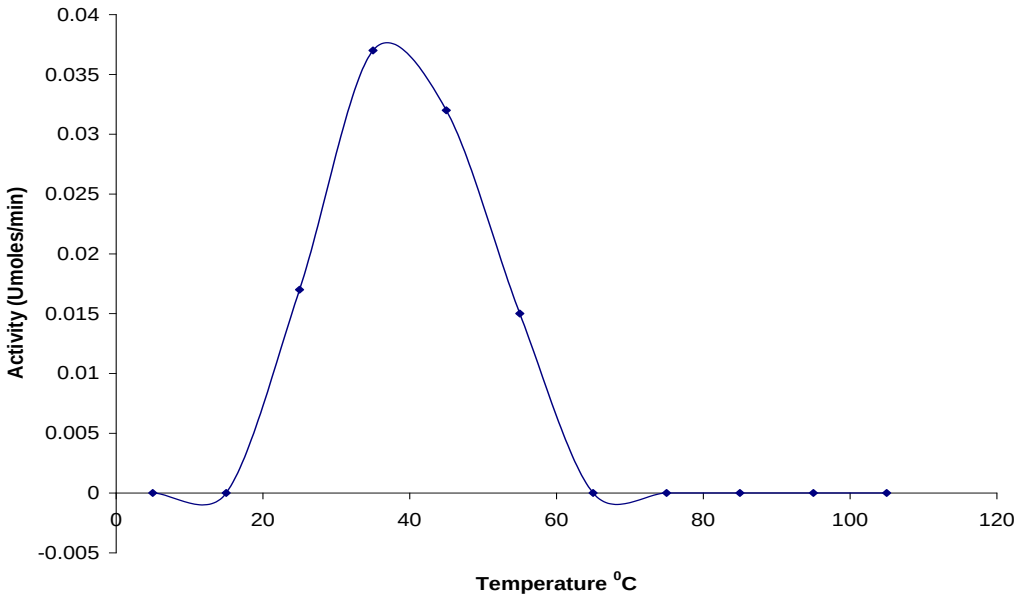

Figure 7: Optimum Temperature determination for the activity of *Plasmodium berghei* Salidase

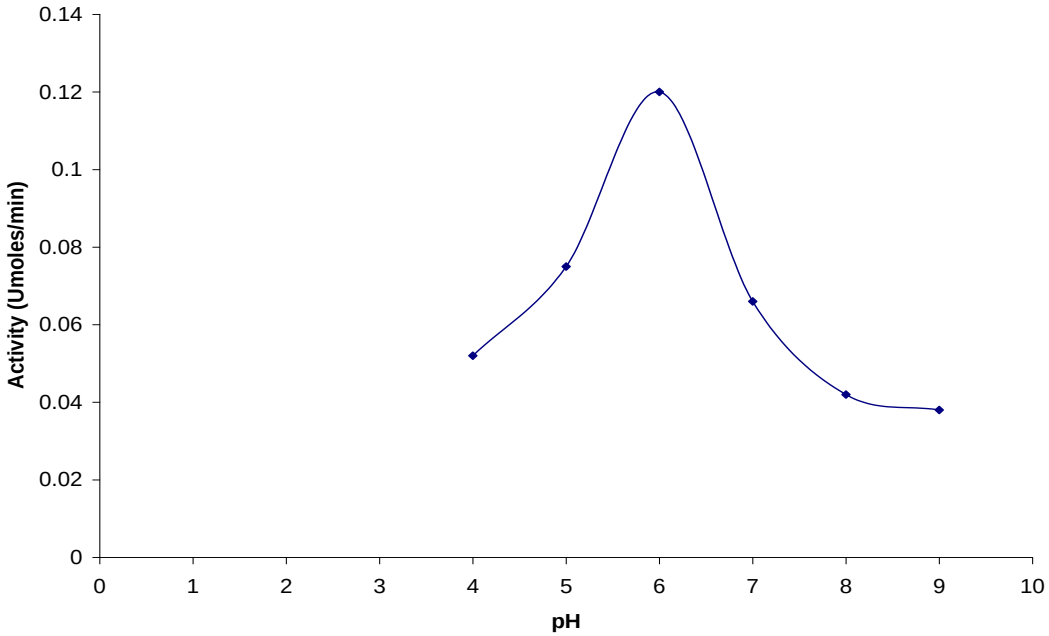

Figure 8: Effect of pH on the activity of *Plasmodium berghei* sialidase

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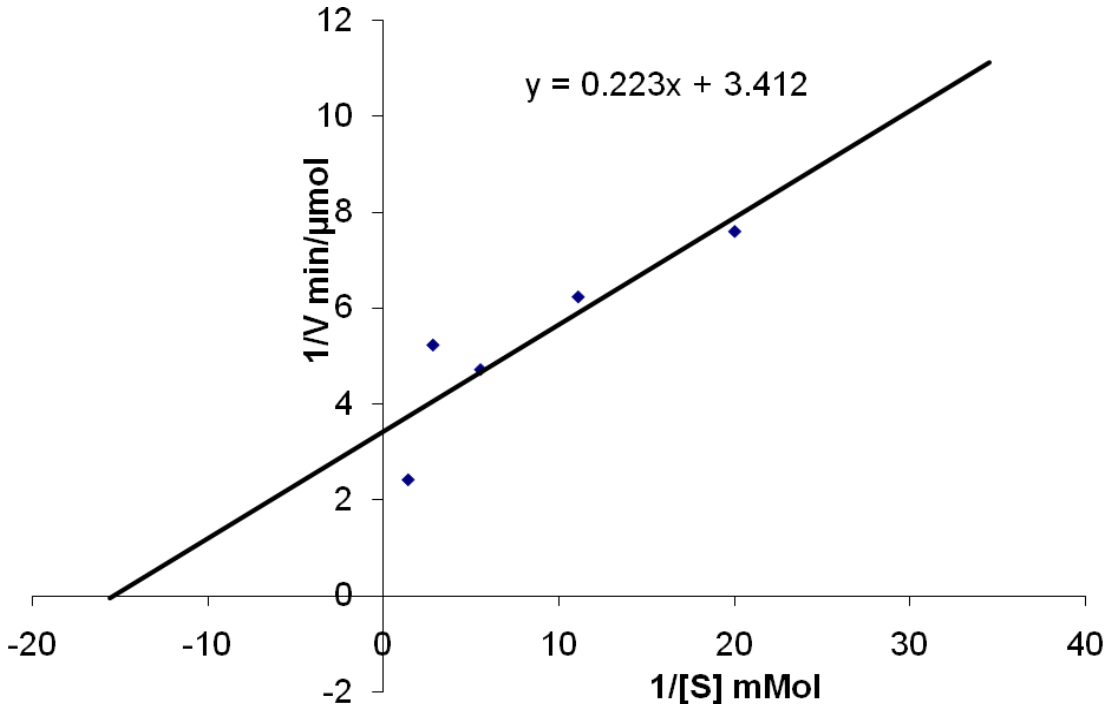

Figure 9: Lineweaver-Burk Plot of *P. berghei* Sialidase with Methylumbelleferyl - N- acetylneuraminic acid

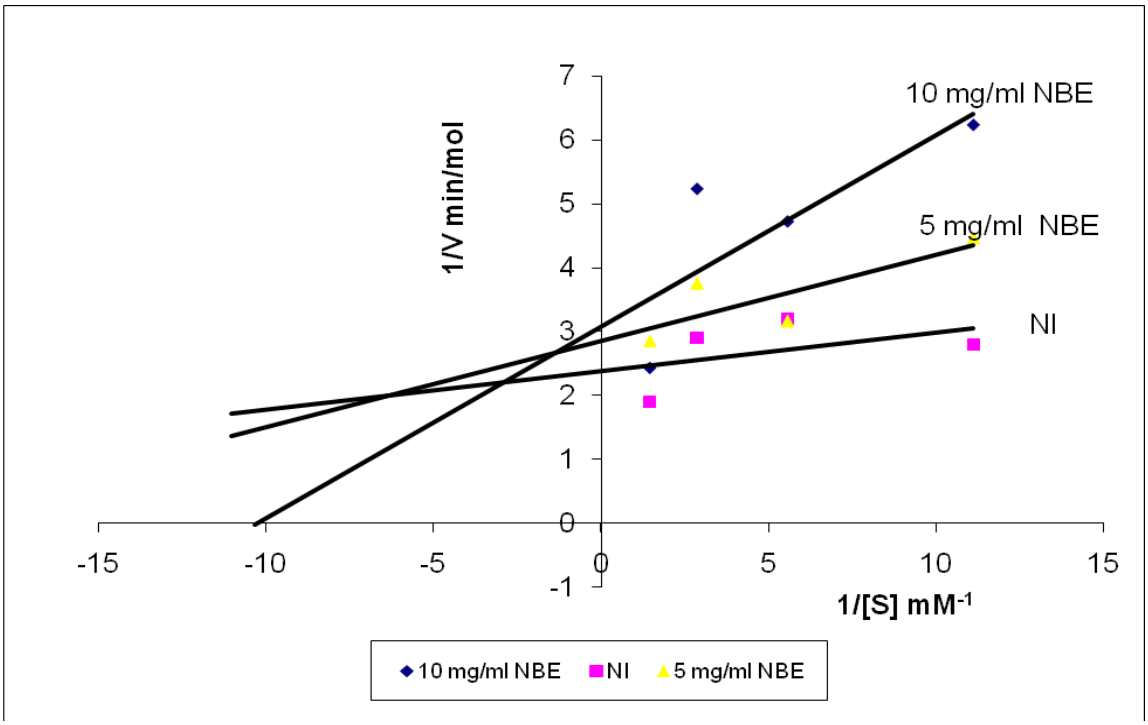

Figure 10: Plot of reciprocal of initial velocity vs the reciprocal of mM concentration of MU- Neu5AC in the absence and presence of 2.5 and 5.0 mg/ml Neem leaf extract

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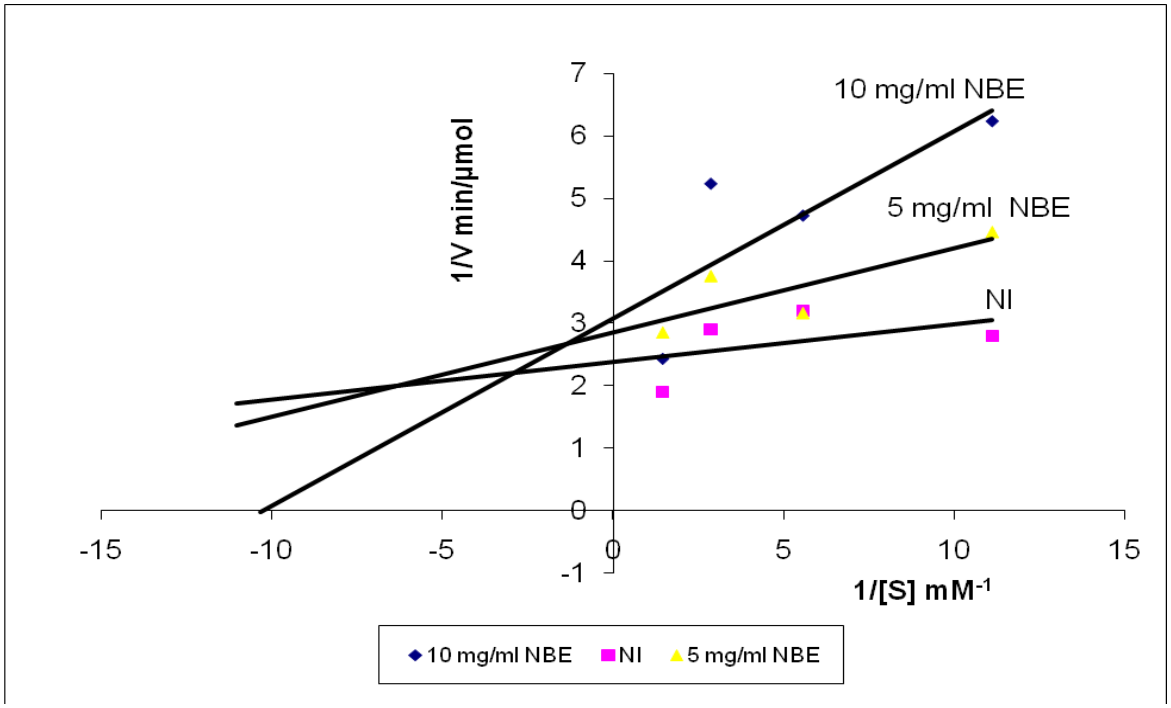

Figure 11: Plot of reciprocal of initial velocity vs the reciprocal of mM concentration of MU- Neu5AC in the absence and presence of 5 and 10 mg/ml Neem stem bark extract.

DISCUSSIONS

The terminal sialic acid from the sugar residues and glycoproteins is cleaved by the enzyme sailidase, and the detection of sialidase rests on the assay of free sialic acid split from a substrate (Webster and Campbell, 1972. It was observed during this study that *Plasmodium berghei* (ANKA strain) produced sialidase in vitro. The increase in the level of free serum sialic acid accompanied by little decrease in the level of bound serum sialic acid and a high decrease in the erythrocyte surface sialic acid suggests that, the RBC is the source of the released free sialic acid (Akin-Osanaiye *et al*., 2013).

Free serum sialic acid was very high in mice with high parasitemia. It is known that sialidase cleaves sialic acid from the erythrocytes of infected animals (Oladele *et al*., 2002; Esievo 1983); the cleavage could explain why infected animals had very high free serum sialic acids, and relatively low erythrocyte surface sialic acids.

Sialidases have been reported in bacteria and viruses and their pathological significance has been fully elucidated (Corfield, 1992; Hubl, 2000). Clough *et al*. (1996) reported that *Plasmodium falciparum* lacks sialidase acivity. However, in this present study, the enzyme sialidase has been isolated, purified and partially characterized from *P. berghei* parasite. Ononiwu and Useh (2005) had reported the presence of sialidase in *P. berghei* parasite. The lack of this enzyme in *P. falciparum* and its presence in *P. berghei* can be explained by the report of Grobe *et al* (1998), that the genes for sialidases are irregularly distributed among microorganisms, which are closely related to species or even single strains of a given species.

The purification of the sialidase from the *P. berghei* parasite resulted in one active peak which was obtained from Sephadex G-100 column. The protein electrophoregram of the purified sialidase showed one band with molecular weight of 54.8 kDa. Reports on sialidase from bacteria recorded average molecular weights between 50 – 125 kDa (Abrashev and Dulguerova, 2000). The molecular weight obtained in this study was very close to that recorded (56-58 kDa) for *Clostridium perfringens* (Nees *et al*., 1975). The temperature optimum of the sialidase catalysed reaction was 35^{o}C which is within the range reported for most siaidases. Abrashev and Dulguerova (2000) reported the optimum temperature for most sialidases to be 35 - 40 ^{o}C, except in some few cases like in non pathogenic strains of *Arthro-bacte ureafaciens* and *Micromonospora viridifaciens* that showed maximum activity at 50 – 58 ^{o}C (Uchida *et al*., 1979; Aisaka *et al*., 1991). The neuraminidase produced by *Clostridium sordelii* is active between 4 to 55 ^{o}C (Roggentin *et al*., 1987). The pH

optimum of the blood stream *P. berghei* sialidase was 6.0. This was similar to that of *T. vivax*, which is 5-6 (Engstler *et al*., 1995).

The kinetic data and Line - Weaver-Burk plot of the *P. berghei* sialidase showed that the enzyme has high affinity for the substrate methyl umbelliferyl-N- acetyl neuraminic acid (4-MU-Neu5Ac) as evident by low K_m value of 0.067 mMol, V_{max} 0.286 µmol/min.

Inhibition studies carried out on the enzyme using the neem leaf and stem bark extracts indicated that both extracts showed mixed inhibition patterns with K_i and K_I values for neem leaf extract to be 0.097 and 0.066 respectively and -0.235 and -1.903 for neem stem bark respectively. From these results, inhibition binding constant as a result of enzyme-inhibitor complex (Ki) is higher than that for enzyme-substrate-inhibitor complex (KI) showing that there is more enzyme-inhibitor complex intermediate as compared to enzyme-substrate-inhibitor complex for both extracts studied.

CONCLUSION

It was concluded that free sialic acid increased in the serum of the infected mice with increase in parasitemia. The sialidase activity increased with increased rise in parasitemia which showed that *Plasmodium berghei* contains sialidase. Sialidase has been purified through sequential chromatographic methods and characterized. The purified enzyme was also found to have an optimum temperature of 35 ^{0}C and optimum pH of 6.0 with K_M and V_{max} of 0.067 mMol and 0.286 µmol/min respectively. The neem leaf and stem bark extracts inhibited sialidase activity in a mixed competitive pattern with K_i and K_I values for leaf extract to be 0.097 and 0.066 mg/ml respectively while -0.235 and -1.903 mg/ml for neem stem bark respectively.

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