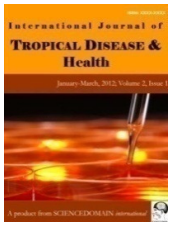

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Purification, Characterization and Vaccine Potential of *Trypanosoma brucei brucei* Glycosyl Phosphatidyl Inositol Specific Phospholipase C

A. S. Abdullahi[1], H. C. Nzelibe[1*] and S. E. Atawodi[1]

[1]Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author HCN designed the study and wrote the protocol. Author ASA conducted the research, statistical analysis, and wrote the draft of the manuscript. Author SEA managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To investigate possible use of Glycosylphosphatidylinositol-specific phospholipase C (GPI-PLC) as a target protein for the development of vaccine against *Trypanosoma brucei brucei* infection was investigated.
Study Design: GPI-PLC from *T. brucei brucei* was purified, characterized and the protein was used as antigen in raising antibody against the parasite
Place and Duration: Department of Biochemistry, Ahmadu Bello University Zaria-Nigeria, between September 2011 and October 2012
Methodology: GPI-PLC was isolated from *T. brucei brucei* and purified by ammonium sulphate precipitation, gel filtration and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The GPI-PLC was further used to raise antisera in rabbits, which was subsequently used to immunize rats for 14 and 21 days pre-infection to investigate the possible use of *T. b. brucei*

**Corresponding author: Email: zeechuks@yahoo.com;*

GPI-PLC as target protein in vaccine production against *T. b. brucei* infection.
Results: An overall yield of 48.76% and purification fold of 10.86 were recorded after gel filtration. The result from SDS-PAGE showed the enzyme to be a 39.585 kDa protein with optimum temperature, optimum pH and activation energy to be 35℃, 8.1 and 19.494 kJ/ mol respectively. The Vmax and Km values were 6.67×10^{-3} µmol/hr and 2.67×10^{-3} µM respectively when 212.5 µg of enzyme was used in the reaction mixture. Immunization with anti GPI-PLC for 14 and 21 days pre-infection significantly lowered the Packed Cell Volume (PCV). Result for the time course of parasitemia following infection with 7.9 x 105 Cells/ml showed a decrease in parasitemia level, thus leading to lowering of mortality rates in Groups immunized with GPI-PLC for 14 and 21 days pre-infection by 20% and 40% respectively relative to Group infected but not treated.
Conclusion: These results suggest that GPI-PLC as a target protein significantly reduced the progression of the *T. b. brucei* infection.

Keywords: Purification; characterization; antigen; vaccine; T. b. brucei; GPI-PLC.

1. INTRODUCTION

African trypanosomes are digenetic parasites whose lifecycle alternates between the midgut of the tsetse fly vector and the bloodstream of mammalian hosts. Native and recently isolated stocks of trypanosomes are pleomorphic in the mammalian bloodstream, transforming from a replicating long slender form into a non-dividing short stumpy form pre-adapted for transmission into the tsetse fly. Once in the fly midgut, the short stumpy form differentiates into a replicating procyclic form. In the bloodstream, *T. brucei* is covered with a variant surface glycoprotein (VSG) whereas in a tsetse fly the parasite expresses procyclin (PARP) as the major surface protein. Differentiation of bloodstream to insect stage (procyclic) *T. brucei* [1] is characterized by increased procyclin expression and loss of VSG within 6 hours of initiating transformation [2]. The most prominent marker for differentiation of bloodstream parasites into procyclic forms is the exchange of the main surface antigens: variant surface glycoprotein (VSG) in the bloodstream stage [3] and procyclin in the procyclic stage [4]. Both proteins are attached to the cell surface of their respective lifecycle stages by a glycosylphosphatidylinositol (GPI)-anchor [5]. It is through the regulated expression of distinct VSG genes, or antigenic variation, that the parasite is able to evade the host immune response [6].

T. brucei contains an endogenous phospholipase C (PLC) known as the GPI-phospholipase C (GPI-PLC) [7-9] that hydrolyses the GPI-anchor on the VSG, releasing dimyristyl glycerol [10]. Glycosylphosphatidylinositol specific-phospholipase C (GPI-PLC) is expressed in bloodstream form *T. brucei*. GPI-PLC is a virulence factor in pleomorphic *T. brucei* [11-12]. The enzyme stimulates endocytosis of transferrin in bloodstream *T. brucei* [13], and is activated by mild acid or hypotonic conditions to cleave GPI at the endoplasmic reticulum [14]. The biological functions of GPI-PLC in non-differentiating bloodstream forms *T. brucei* is not resolved.

In this work, *T. b. b*. GPI-PLC protein was partially purified and characterized, then antibody was raised against the protein. The antibody raised was then passively inoculated in to some experimental animals in order to test the efficacy of *T. b. b* GPI-PLC as a target protein against trypanosomiasis.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Sample

Bloodstream *T. brucei brucei* (Federie) rat adapted strain was obtained from the Department of Biological Sciences Ahmadu Bello University, Zaria, Nigeria. The strain was originally obtained from the Nigerian Institute for Trypanosomiasis Research, Kaduna, Nigeria where it was identified as *T. brucei brucei* (Federie).

2.2 Growing/Harvesting of the Bloodstream Forms of *T. brucei*

The strain was grown by infecting ten rats intraperitoneally with infected host blood contained in a normal saline solution. The parasitemia was monitored according to Herbert and Lumsden [15]. At the peak of infection, the rats were sacrificed under chloroform anaesthesia with 3.8% sodium citrate as anticoagulant, and the blood was collected. The blood from the rats was diluted 1:3 with the PSG buffer so as to reduce the number of red blood

cells that pass through the gel. The diluted blood was then centrifuged at 2000 × g for 5 minutes and the supernatant containing the trypanosomes decanted. The supernatant was then poured onto the column with DE 52 cellulose [16]. The eluates were collected into a flask placed in an ice block while more PSG buffer was added until all the blood had been added and the parasites completely washed out of the column. Films of the eluate were made at intervals and viewed under the microscope for the presence of live trypanonosomes. The combined eluates were spun at 5000 ×g for 10 minutes to concentrate the parasite. The trypanosome-free supernatant decanted. Cells were then frozen at -4℃ until GPI-PLC enzyme was assayed.

2.3 Cell Lysis

Parasites (2×10^7) were resuspended in 200µl of hypotonic lysis buffer (10mM sodium phosphate, 1 mM EDTA, pH 8) containing leupeptin (2.1 mM), N-tosyl-L-lysine chloromethyl ketone (TLCK) (0.1 mM) and aprotinin (0.4 U) [8,17]. Cells were kept on ice for 20 min. The lysate was then centrifuged (14,000 x g, 4℃ for 10 min.) and the supernatant discarded. The pellet was solubilized in 200µl of 50 mM Tris–HCl, 5 mM EDTA. This served as the crude enzyme for the research.

2.4 Determination of Total Protein Content

Protein concentration was quantified by the Bradford method [18]. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs.

2.5 Determination of GPI-PLC Activity

The enzyme activity was monitored by *p*-Nitrophenylphosphorylcholine (NPPC) hydrolysis as described previously by Katayama et al. [19]. Samples (40µl) were incubated at 35℃ for 2 h in a 1-ml reaction mixture containing 3.6 mM *p*-Nitrophenylphosphorylcholine (NPPC), 0.25 M Tris-HCl (pH 8.0), and 60% glycerol, the absorbance was monitored at 410 nm. To calculate the amount of *p*-nitrophenol produced, a standard plot for *p*-nitrophenol was used in order to determine the enzyme activity as described by Katayama et al. [19].

2.6 Ammonium Sulphate Precipitation

Crude protein (1.5 ml) solution was precipitated at 40%, 50%, 60% and 70% $(NH_4)_2SO_4$ saturations respectively. Each solution was allowed to stand for 1 hour in deep freezer at various saturations. Thereafter, it was centrifuged at 10,000 x g for 15 minutes, the supernatant was collected and the pellet discarded. The supernatant at the end of the 70% $(NH_4)_2SO_4$ saturation precipitation was then tested for activity as described by Katayama et al. [19].

2.7 Gel Chromatography on Sephadex G-75

Gel filtration was carried out using the Sephadex G-75 at a flow rate of 5 ml/7.5 min.

2.8 Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis under denaturing conditions i.e SDS, was performed in 12.5% (w/v) acrylamide disc gel according to the method of Ornstein [20] using a Tris-glycine buffer, pH 8.3. The electrophoresis was carried out on the crude enzyme, pooled supernatant of ammonium sulphate precipitation and on the pooled fractions from Sephadex G-75 column chromatography. Protein bands were located by staining with silver nitrate. The proteins are separated according to their electrophoretic mobility, which is a function of length of polypeptide chain or molecular weight as well as higher order protein folding, posttranslational modifications and other factors.

2.9 Effect of Temperature on Enzyme Activity

The effect of temperature on the activity of the enzyme was monitored at a temperature range of 25-60℃. The reaction mixture (1 ml) was incubated at the various temperatures for 2 hours. The enzyme activity was monitored by *p*-Nitrophenylphosphorylcholine (NPPC) hydrolysis assay as described previously by Katayama et al. [19].

2.10 Determination of Activation Energy

The activation energy was measured for the activity against reciprocal of temperature at 25 to 60℃ in 20 mM phosphate buffer (pH 8.0).

2.11 Effect of pH on Purified Enzyme

The activity profile of the purified enzyme was determined as a function of pH, at a pH range of 7-9 and the optimum pH determined as described by Katayama et al. [19].

2.12 Effect of Substrate Concentration

The substrate concentration was varied over a range of 0.33-1.64 × 10^{-3} µM at a temperature of 35℃ and the various activities determined as described by Katayama et al. [19]. The kinetic constants Michelis constant (K_m) and maximum velocity (V_{max}) were determined from plot of activity against the reciprocal of substrate concentration.

2.13 Raising of GPI-PLC Antisera

Two rabbits with an average weight of 1.35 kg were acclimatized for two weeks at animal house, Faculty of Pharmaceutical Sciences, Ahmadu Bello University before use. The pre-immunized sera from both rabbits were collected. This was designated as day 0. The protein (83 µg) contained in 100 µl PBS was mixed with an equal volume of complete Freund's adjuvant injected intramuscularly on both rabbits. Seven days later same dosage of the protein but this time mixed with an incomplete Freund's adjuvant was inoculated subcutaneously into the rabbits, and designated as the boosters 1, 2, 3 and 4 which were administered at day 7, 14, 21 and 28 respectively. Blood sera from the rabbits on day 14 and 28 were collected and tested for presence of antibodies by ELISA technique.

2.14 Effect of GPI-PLC Antisera on *T. brucei*

To test for the effect of the GPI-PLC antisera on *T. brucei*, infection was established with 7.9 x 10^5 *T. brucei*. In a similar experiment Chiejina et al. [21] used 3 x 10^5 *T. musculi* for infection. The experiment was designed as follows:

Group 1: Animals were infected with the parasite only
Group 2: Animals were inoculated with the antisera and infected with the parasite simultaneously on day 0 of infection
Group 3: Animals were inoculated with GPI-PLC antisera and then infected with *T. brucei* parasite after the antisera has been raised on day 14
Group 4: Animals were inoculated with GPI-PLC antisera and then infected with *T. brucei* parasite after the antisera has been raised on day 21.
Group 5: Animals were inoculated with the antisera only
Group 6: Animals in this group were neither inoculated with GPI-PLC antisera nor infected.

Infection with *T. b. brucei* in Groups 1, 2, 3 and 4 was carried out on the day which corresponded to the days 14 and 21 of immunization in Group 3 and 4 respectively. The day was termed as Day 0 of infection, which marked the starting day for the daily monitoring of parasitemia level and PCV values.

2.15 Enzyme-Linked Immunosorbent Assay (ELISA)

In the assay, microplate (Linbro/titertek- Flow Labs) consisting of 96 wells was coated overnight at 4℃ with 100 µl of GPI-PLC (3 µg/ml) in Phosphate-Buffered saline (PBS). The plate was washed three times with PBS containing 0.5% tween 20 (PBS/Tween) and unbound sites were blocked for 1 hour at room temperature with 2% bovine albumin in PBS.

The plate was washed three times with PBS/Tween and used immediately for ELISA. To measure the serum titer, 100 µl of 10%, 20% and 40% serial dilutions of serum rabbit anti-sera were added to the plate and incubated for 1 hour at 37℃. The plate was then washed and incubated for 1 hour with 100 µl of an anti-rabbit IgG-peroxidase conjugate (Sigma, 1: 10000 in PBS), followed by further washing. The substrate solution for the peroxidase assay (H_2O_2/OPD) was added and the enzymatic reaction was allowed to proceed for 15 min at room temperature in the dark. The reaction was stopped with 50 µl of 2N H_2SO_4 and the absorbance was read at 492 nm with a Microplate reader (Sigma Diagnostic ELISA Reader).

2.16 Statistical Analysis

The data obtained were expressed as Mean ± Standard Error of mean. Data were analyzed by Duncan One-way ANOVA at $p<0.05$.

3. RESULTS AND DISCUSSION

3.1 Purification of *Trypanosoma brucei* GPI-PLC

T. b. brucei GPI-PLC was isolated using a two-step purification process. The results of the purification of *T. b brucei* GPI-PLC are summarized in Table 1. The crude extract contained approximately 6.044 $\times 10^{-2}$ enzyme units with a specific activity of 0.4133 $\times$ 10^{-2} unit/mg protein. Fractionation of the crude extract with ammonium sulphate, followed by Sephadex G-75 chromatography resulted in a 10.86-fold purification of cysteine protease with a 48.76% recovery.

The purification profile showed that the purification fold after ammonium sulphate precipitation increased by 3.01 compared to Gel filtration step that increased by 10.86. An increase in yield of 107.5% was recorded after ammonium sulphate precipitation, an indication that the unusual increase in enzyme activity might be as a result of removal of a factor by the ammonium sulphate precipitation that must have been inhibiting the activity of the enzyme or dispersal of a complex, thus leading to an increase in total activity for this step. A 10-fold purification and 144% yield was however reported in Spring [22] in the purification of a bacterial cholinesterase at a step involving shaking the extract with *sec*-butanol as precipitant for half an hour at room temperature. However, this may not be possible late in purification when you are trying to remove the last 5% contamination [22]. The purification yielded an overall of 48.76% yield.

The elution profile of *T. b. brucei* GPI-PLC on Sephadex G-75 column is presented in Fig. 1. Fractions 23 – 27 yielded the highest activity, and thus pulled together. The result from the SDS-PAGE Plate 1 revealed a band of 39.59 kDa upon extrapolation from Fig. 2. This is consistent with the value reported by Hanrahan et al. [23] that *T. b. b.* GPI-PLC is approximately a 39 kDa protein. This result strongly validates the report.

3.2 Effect of Temperature on *T. brucei brucei* GPI-PLC

The enzyme activity was measured at different temperatures (25℃, 30℃, 35℃, 40℃, 50℃, 60℃) and the activity was found to be maximum at 35℃ Fig. 3. An optimum temperature is that temperature proteins (enzymes) assume the conformation best suited for catalysis.

3.3 Determination of Activation Energy

The activation energy was determined from a plot of Natural logarithm of activity against the inverse of Kelvin temperature and it was found to be 19.494 kJ/ mol Fig. 4.

3.4 Effect of pH on *T. brucei brucei* GPI-PLC

The effect of pH on the enzyme activity was examined at the pH range 7.5 to 9.0. Maximum activity was at pH 8.1 Fig. 5. The pH value is consistent with the report of Subramanya et al. [13] who reported a pH of 8.0. Perhaps the alkaline pH obtained suggests that the enzyme's active site is made up of negatively charged amino acids when fully ionized at their respective pKa. At this optimal pH, all the carboxyl and ammonium ion species of the amino acids present in the active sites are fully deprotonated leaving a net negative charge.

3.5 Determination of Kinetic Constants

The Michaelis-Menten constant and maximum velocity of the enzyme for the hydrolysis of the substrate at pH 8 at 35℃ is depicted as a Lineweaver-Burk plot in Fig. 6. The Michaelis-Menten constant (K_M) was estimated to be 2.67 $\times$ 10^{-3} µM and the maximum velocity (V_{max}) was estimated to be 6.67 x 10^{-3} µmol/hr when 212.5 µg of the protein reacted with the substrate. The K_m has a slight variation from the report of Hereld et al. [8] and Mensa-Wilmot et al. [17] with a value of 3.6 $\times$ 10^{-3} µM which might be as a result of variation in the assay method**.**

3.6 Qualitative Determination of Amount of Anti-GPI-PLC

A steady increase in the optical density values recorded at a wavelength of 492 nm were observed from week 2 and week 4 with respect to week 0 as 10%, 20% and 40% concentration dilutions of the serum were used Table 2. Steady rise in the Optical Density (OD) from Enzyme Linked Immunosorbent Assay (ELISA) of serum from 0-4 weeks is an indication of the presence and increase in the amount of anti GPI-PLC.

Table 1. Purification profile for *T. brucei* GPI-PLC

Stage	Total protein(mg)	Total Activity ($\times10^{-2}$ Units)[a]	Specific Activity ($\times10^{-2}$ Units/mg)	% Yield	Purification fold
Crude	14.625	6.044	0.4133	100	1
Ammonium sulphate precipitation	5.225	6.5027	1.2445	107.59	3.01
Gel filteration	0.657	2.9480	4.4871	48.76	10.86

[a]*One unit of T. b brucei GPI-PLC activity was defined as the amount of enzyme that hydrolyses 1.0 µmol of p-Nitrophenylphosphorylcholine per hour at pH 8 and temperature of 35°C.*

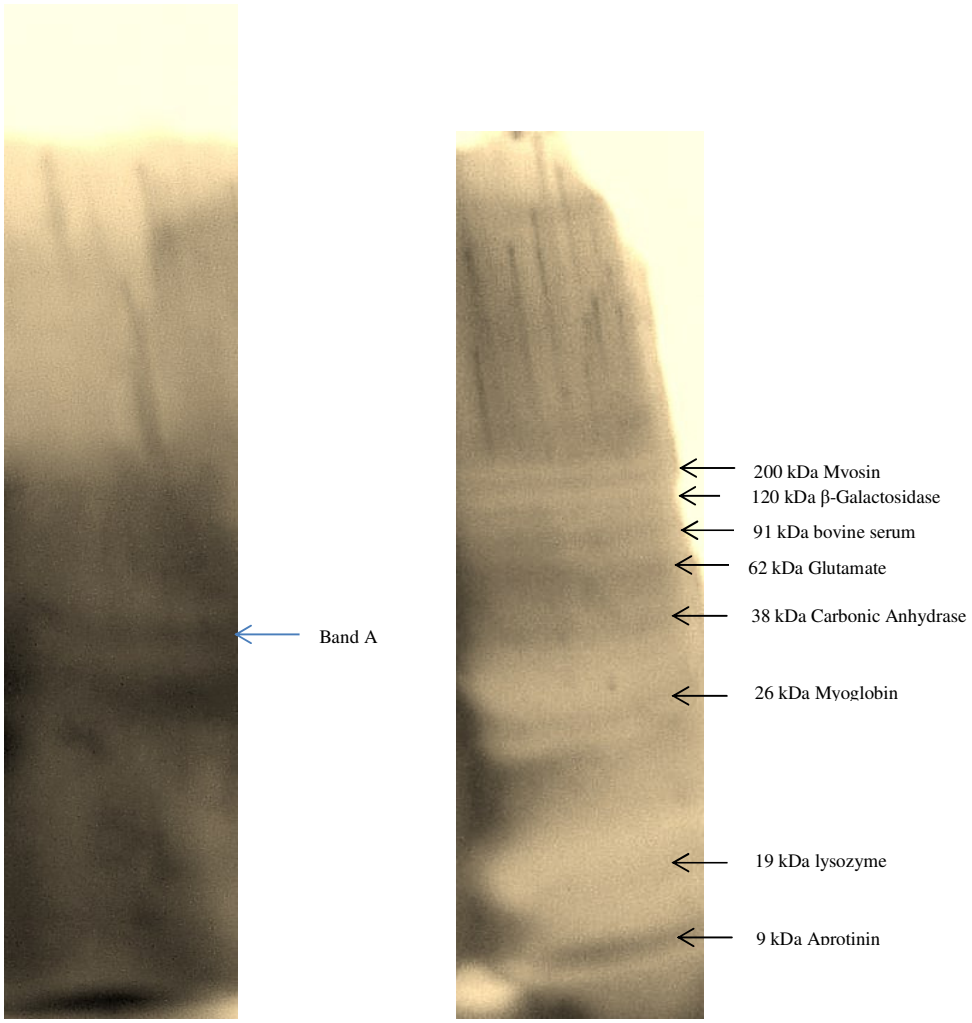

Plate I. Electrophoretic patterns for *T. brucei* GPI-PLC on polyacrylamide gel using Silver nitrate staining (Lane A; Band from sephadex G-75 purification step lane B; marker proteins)

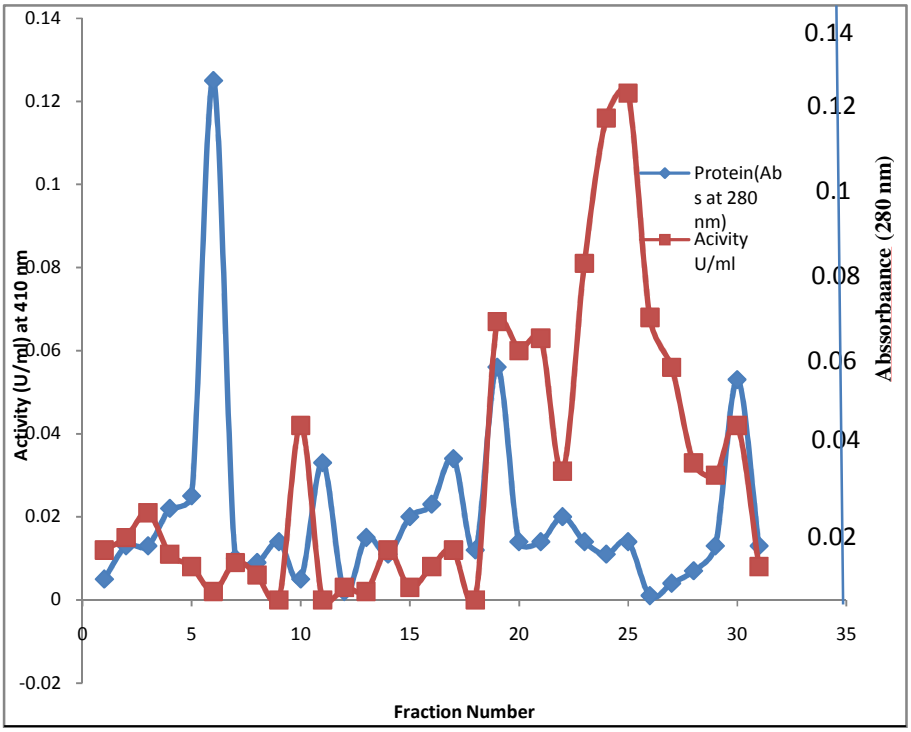

Fig. 1. Elution profile of *T. brucei* GPI-PLC on sephadex G-75 column

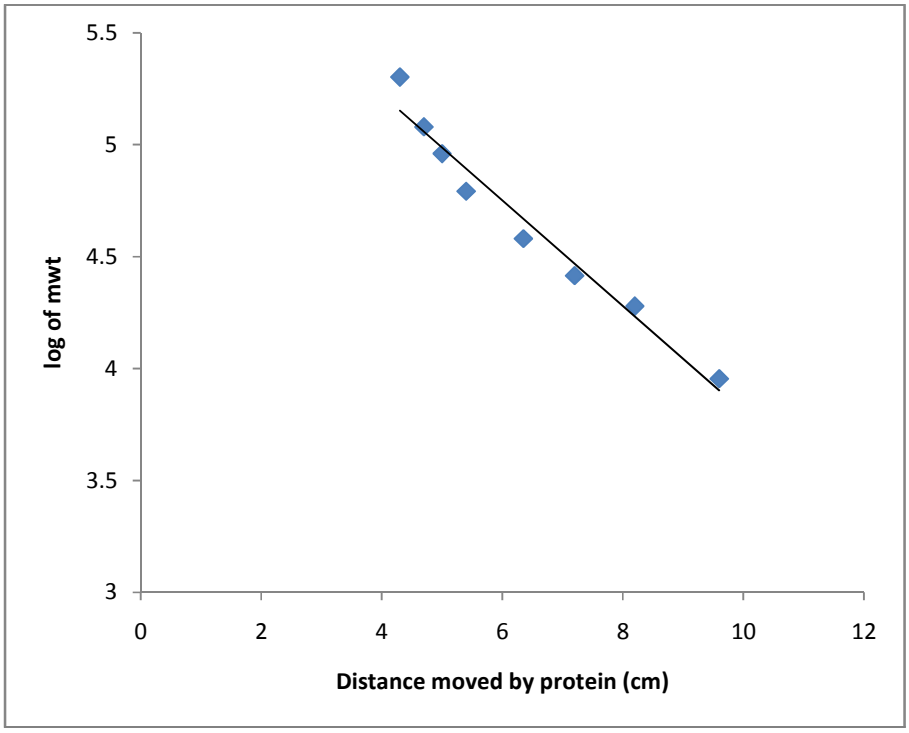

Fig. 2. Determination of molecular weight of *T. b. brucei* GPI-PLC using protein standards on SDS-PAGE and staining with $AgNO_3$ (Band A is 39, 585 Da. Distance moved by Band A = 6.65cm)

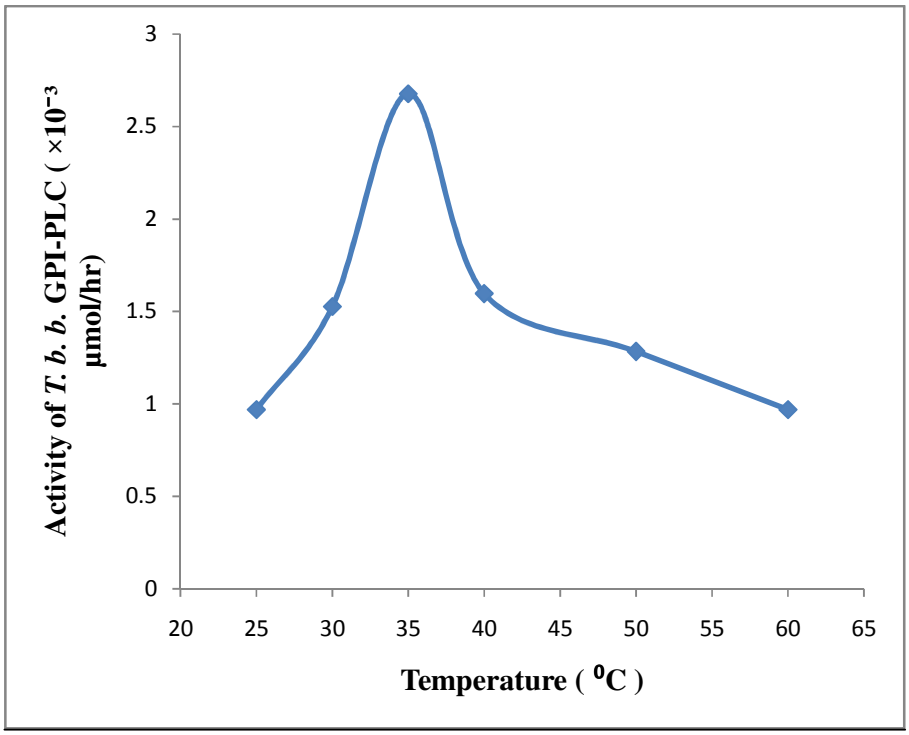

Fig. 3. Effect of temperature on *T. brucei brucei* GPI-PLC activity

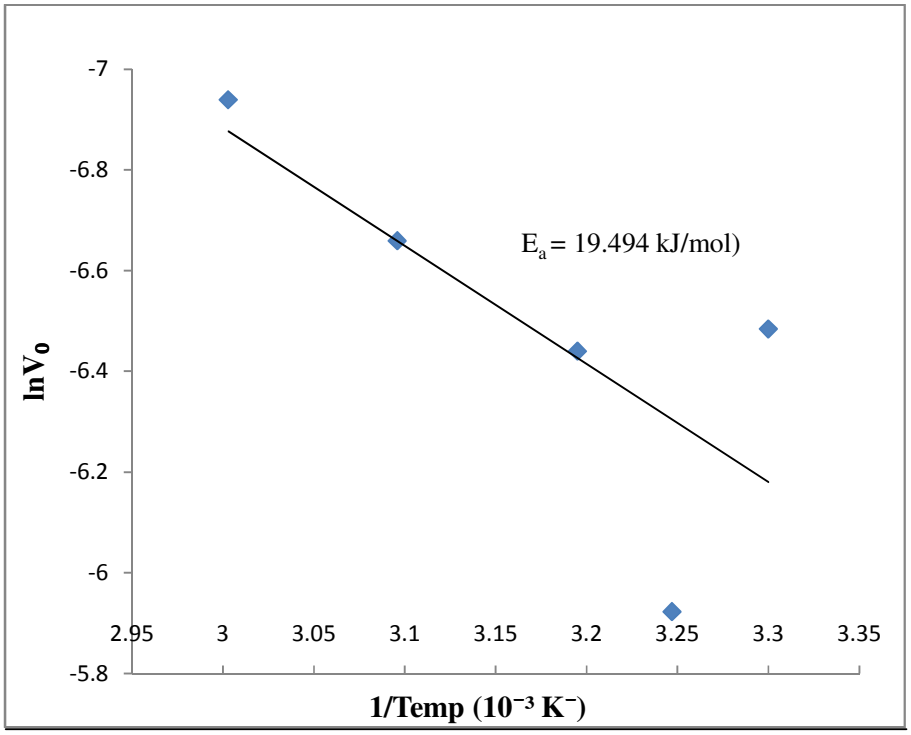

Fig. 4. Arrhenius plot for *T. brucei brucei* GPI-PLC

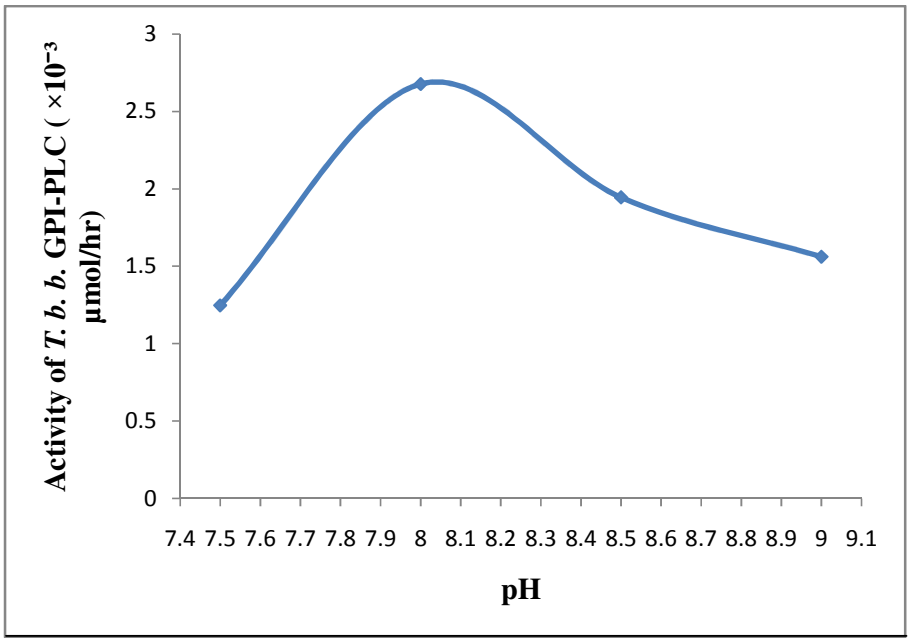

Fig. 5. Effect of pH on *T. brucei brucei* GPI-PLC activity

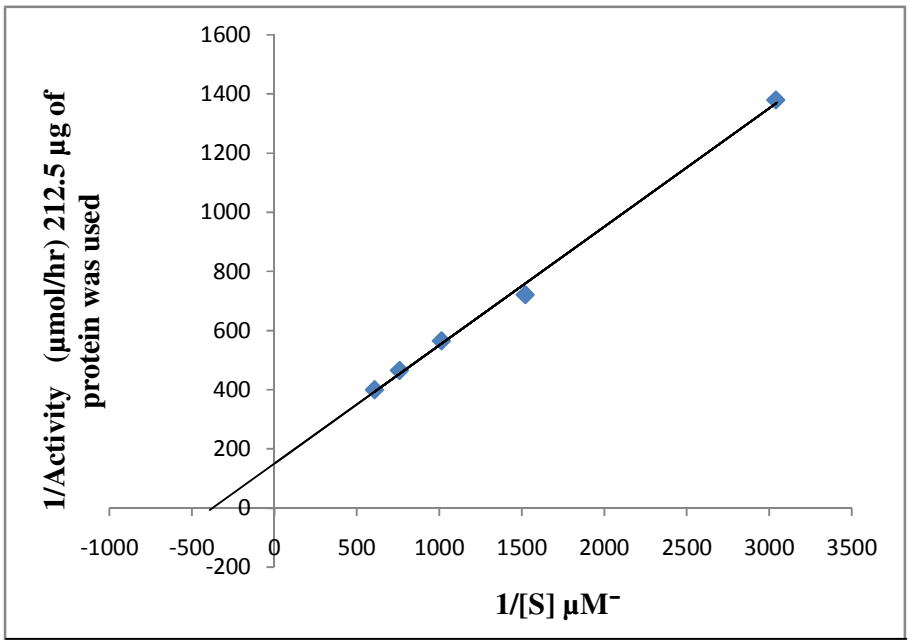

Fig. 6. Lineweaver Burke plot for *T. b. brucei* GPI-PLC

3.7 Packed Cell Volume (PCV) Data Obtained From Six Experimental Groups

PCV data obtained from the six experimental groups (Group 1-6) on days 0, 1, 3 and 5 post-infection are shown on Table 3. There was no significant difference observed in the PCV values of the six groups till day 5 post infection. Immunization with anti GPI-PLC had a significantly positive effect on the Packed Cell Volume (PCV) values as no significant difference was recorded across the groups from Day 0 through Day 1 post-infection, but from Day 3

post-infection through Day 5 post-infection, group with infection only had a significantly lower PCV value relative to groups having 14 and 21 days immunization with anti GPI-PLC pre-infection, indicating that immunization with *T. b. b.* anti GPI-PLC significantly reduced the degree of anaemia.

3.8 Time-course of Parasitemia

Following infection with 7.9 x10^5 *T. b. brucei*, the parasitemia in groups 1-4 was monitored, it was observed that bloodstream forms of the parasite (*T. b. brucei*) were first viewed microscopically on day 3 post-infection and the parasitemia peaked on day 6 post-infection Fig. 7. Result of the time course of parasitemia following infection with 7.9 x 10^5 Cells/ml revealed that on the first day (Day 3 post-infection) bloodstream *T. b. b.* were viewed on the microscope at a 400X magnifications, group 1 and group 4 had the highest parasitemia, while group 3 and group 2 had the least parasitemia respectively. At the peak of parasitemia on Day 6, groups having 14 and 21 days immunization pre-infection had the least parasitemia levels, while the group with simultaneous immunization and infection and the group with infection only, had the highest parasitemia levels. This is an indication that the immune system responded to the presence of GPI-PLC by raising antibodies against it. The phenomenon of immune suppression associated with African trypanosomiasis is a well-documented, although not well-understood, aspect of infection. It is clear that infection with trypanosomes is accompanied by the development of increasing immunosuppression that results in a progressive inability of the host to control parasite growth, leading ultimately to death [24-25].

3.9 Mortality Rate

Death of animals in Group 1(Infected only), Group 2 (Simultaneously inoculated and infected), Group 3 (Infected on day 14 post-immunisation) and Group 4 (Infected on day 21 post-immunisation) were first recorded on day 6 post-infection. Groups 1 and 2 had the highest mortality rate of 80%, while groups 3 and 4 had mortality rate of 60% and 40% respectively on day 6 post infection. On day 7 post-infection, 100% mortality was recorded for all the four groups Fig. 8. Mortality across the groups was first recorded on the Day 6 post-infection. The group 1 and group 2 both had mortality rate of 80% on Day 6 post-infection. While groups 3 and 4 had mortality rates of 60% and 40% respectively on Day 6 post-infection. On the Day 7 post-infection 100% mortality rate was however recorded across all groups. The decrease in mortality rates recorded in groups having 14 and 21 days immunization with anti GPI-PLC pre-infection relative to the group with infection only, was an indication that the host's immune system was able to detect GPI-PLC on the surface of the parasite as an antigen there by raising antibodies against it. This was only possible due to the presence of *T. b. b* GPI-PLC on the extracellular face of the membrane. The vaccination provided against the parasite was a passive immunization which ceased to be protective when the inoculum itself was cleared from the host's system. Absolute clearance of the parasite in the host's blood could not be achieved.

This study revealed that GPI-PLC is localized on the plasma membrane which is consistent with the report by Turner [26]. However, our evidence strongly supports the claims that the GPI-PLC is mainly restricted to a small sub-domain of the plasma membrane [23]. Results from this study do not support the localization of the GPI-PLC in the flagellar pocket, in the Golgi, on the cytoplasmic face of intracellular vesicles and in the glycosomes or in the ER, as previously proposed [14,27-28].

Table 2. Qualitative determination of Anti-GPI-PLC (Antigen) raised by ELISA technique at 492 nm wavelength

Concentration (%)	Optical Density		
	Week 0	Week 2	Week 4
	Rabbit	Rabbit	Rabbit
10	0.026	0.041	0.063
20	0.072	0.098	0.132
40	0.161	0.220	0.304

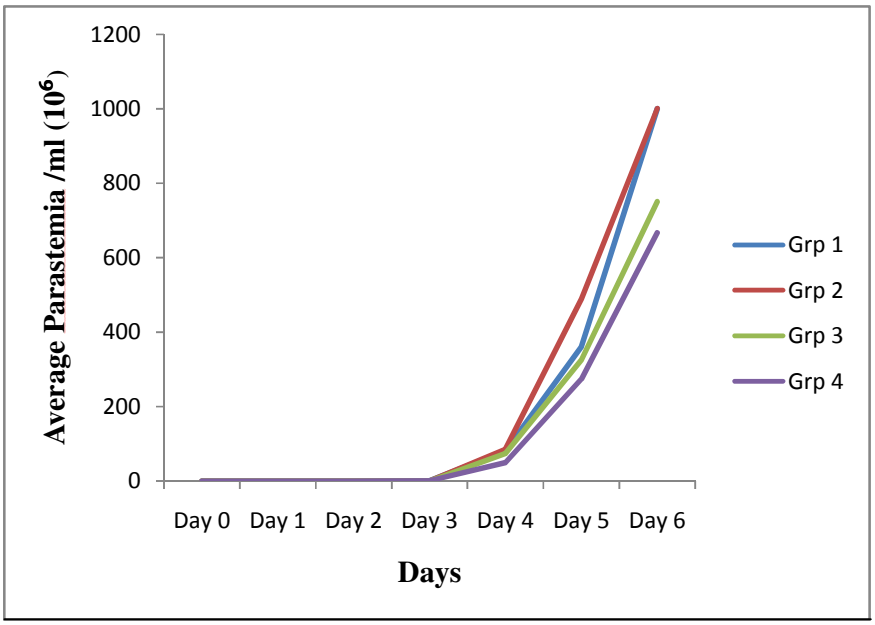

Fig. 7. Time-course of parasitemia following infection with 7.9 x10^5 *T. b. brucei*

Group 1: Animals were infected with the parasite only; Group 2: Animals were inoculated with the antisera and infected with the parasite simultaneously on day 0 of infection; Group 3: Animals were inoculated with GPI-PLC antisera and then infected with T. brucei parasite after the antisera has been raised on day14; Group 4: Animals were inoculated with GPI-PLC antisera and then infected with T. brucei parasite after the antisera has been raised on day 21

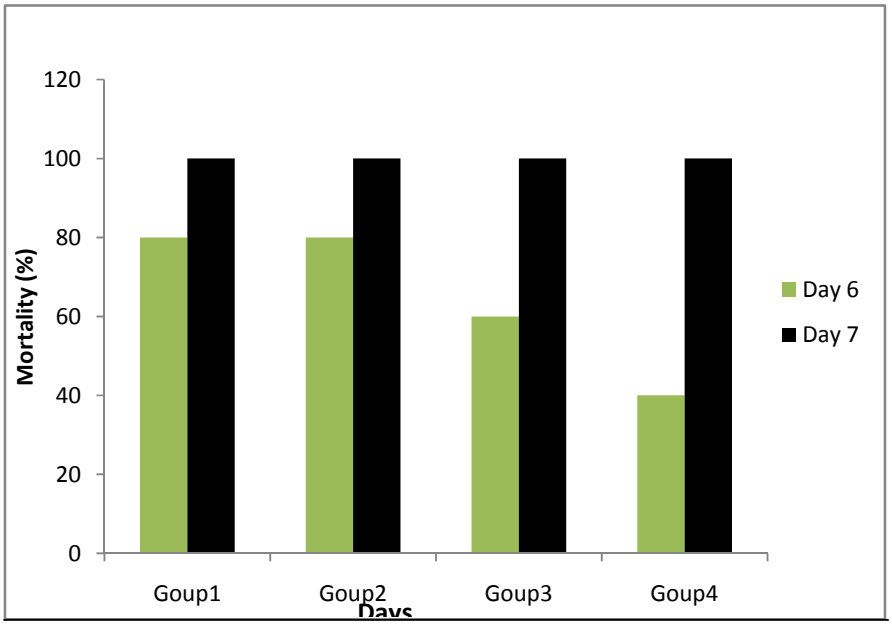

Fig. 8. Effect of anti GPI-PLC on mortality rate of rats after six and seven days post infection

Group 1: Animals were infected with the parasite only; Group 2: Animals were inoculated with the antisera and infected with the parasite simultaneously on day 0 of infection; Group 3: Animals were inoculated with GPI-PLC antisera and then infected with T. brucei parasite after the antisera has been raised on day14; Group 4: Animals were inoculated with GPI-PLC antisera and then infected with T. brucei parasite after the antisera has been raised on day 21

Table 3. Packed Cell Volume of rats infected with *T. b. brucei* and or inoculated with anti GPI-PLC

	Day 0	Day 1	Day 3	Day 5
Group1	46.8±1.43[a]	46.4±1.17[a]	38.4±2.32[b]	39.2±2.89[b]
Group2	52.6±1.03[a]	47.4±1.03[a]	46.8±1.46[a]	42.8±0.97[ab]
Group3	47.6±1.44[a]	46.4±1.17[a]	45.2±1.66[a]	45.4±0.68[a]
Group4	50.2±0.20[a]	47.2±1.02[a]	48.0±0.84[a]	47.8±2.15[a]
Group5	48.2±3.58[a]	45.6±1.75[a]	45.6±2.44[a]	47.6±1.29[a]
Group6	48.6±3.61[a]	45.4±1.44[a]	44.2±1.02[a]	48.0±2.26[a]

Results are Mean ± SEM; Means followed by the same letter within the same column are not significantly different at p<0.05
Legend: Group 1: Animals were infected with the parasite only; Group 2: Animals were inoculated with the antisera and infected with the parasite simultaneously on day 0 of infection; Group 3: Animals were inoculated with GPI-PLC antisera and then infected with T.b. brucei parasite after the antisera has been raised an day 14; Group 4: Animals were inoculated with GPI-PLC antisera and then infected with T.b. brucei parasite after the antisera has been raised on day 21; Group 5: Animals were inoculated with the antisera only; Group 6: Animals were neither inoculated with GPI-PLC antisera nor infected

4. CONCLUSION

Trypanosoma brucei brucei Glycosylphosphatidylinositol Specific Phospholipase C (GPI-PLC) from the experiment was found to be a virulence factor in *T. brucei brucei* infection as immunization with anti GPI-PLC on *T. brucei brucei* infection was found to significantly reduce the degree of anaemia and parasitemia thus, leading to a decrease in mortality rate. Findings from this research tend to agree more with claims that say it is located in a restricted portion of the outer leaflet of the plasma membrane since the immune system was able to recognize the *T. brucei brucei* GPI-PLC contrary to the reports that it is found in either the flagellar pocket, the Golgi, glycosomes, cytoplasmic face of intracellular vesicles or ER as previously proposed.

CONSENT

Human subjects were not used for the study.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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