Nigerian Journal of Biochemistry and Molecular Biology 2019; 34(1&2): 18-27

0189-4757/96 $3.0 + 0.00
Printed in Nigeria

Available online at http://www.nsbmb.com.ng

NJBMB/012/19

Partial Purification and Evaluation of Substrates-preference of Cysteine Protease from *Plasmodium berghei*

***Bulus, T. and Paul, A.**

Department of Biochemistry, Kaduna State University, Kaduna, Nigeria.

Abstract: Malaria, a disease caused by a parasitic protozoan of the phylum apicomplexan, remains an important disease of the tropics particularly Africa, where pregnant women and children constitute most affected population. Plasmodial cysteine proteases play important roles in the progression of malaria and have constituted key chemotherapeutic targets for screening potential antimalarial compounds. This work was designed to evaluate the success of two strategies for possible release of cysteine protease from *Plasmodium berghei* isolates and to partially purify the enzyme molecule. Parasite isolates were prepared from the blood of 25 infected mice at high parasitaemia (~ 24-29%) using saponin treatment. Release of the crude soluble enzyme from the parasite isolates using freezing and thawing and Triton-X100 treatments were compared. The enzyme assay was carried out by incubating the enzyme with 3% gelatin (as substrate), at pH 5.5 and 37^{o}C for 60 minutes terminated using 40% trichloroacetic acid. The enzyme activity was taken as the amount of tyrosine (measured at 260 nm) released in 60 minutes. Enzyme assay revealed significantly higher enzyme activity in Triton-X100 fractions (TX1) than in frozen and thawed fractions ($p < 0.05$). Dithiothreitol (DTT) and cysteine displayed varying stimulatory actions on the soluble enzyme. Variation in the preference of TX1 for various peptide substrates was observed, with maximum activities recorded with Gelatin after incubation at 37^{o}C for 60 minutes. The pooled TX1 fractions (~16ml) were partially purified by ammonium sulfate precipitation (80% saturation), dialysis and gel filtration (Sephadex-G200) resulting in purification of approximately four fold and 82.0% yield. The significance of these findings was discussed against the background of the chemotherapy of malaria.

KEYWORDS: Cysteine protease, *Plasmodium berghei*, Malaria

1.0 Introduction

Malaria has continued to place burden on the economy of the nation. In 2015, malaria financing totaled US$2.9 billion (WHO, 2017). But in the same year, the global tally of malaria was 212 million new cases and 429,000 deaths (WHO, 2017). There is a long way to go in the fight against the disease.

Malaria is a blood-borne parasitic disease caused by intracellular protozoa, the *Plasmodium*, whose life cycle involves penetration of hepatocytes and red cells, a process known to be mediated largely by the activities of various parasitic proteases (Caldeira *et al*., 2009; Dixit and Pande, 2012). In humans the parasite, *Plasmodium falciparum*, is transmitted through the bite of the female anopheles mosquito as it ingests blood meal from its host (Caldeira *et al*., 2009; Dixit and Pande, 2012). Infection due to *Plasmodium berghei* in mice is known to exhibit identical symptoms to that seen in human, with its progression similarly accompanied by release of parasite proteases, and so *Plasmodium berghei* has continued to serve as alternative to *P. falciparum* especially in *in vivo* studies (Albay *et al*., 1999).

Malaria is usually characterized by chills, anaemia, fever, splenomegaly and damage to organs like brain and liver (Ukwe, 2003). Annually, about one million deaths are known to be associated with malaria globally and currently, 40% of the world's population live in malaria endemic areas most of which are Asian or African countries. World Health Organization reported the success achieved in the fight against

***Corresponding Author**
Tel.: +234 8051282789
E-mail: tbulus@yahoo.com

malaria in 91 endemic countries (WHO, 2017). The success was in large part due to the use of insecticide-treated mosquito nets, early diagnostic for children, preventive treatment for pregnant women and the adoption of arteminisin-based combined therapy.

Despite successes achieved in malaria control strategies, some of which involve attempts to develop effective malaria vaccine, eradication of adult mosquito vectors and the breeding habitat for their larvae, and chemotherapy (Oaks *et al.,* 1991, Olliaro *et al.,* 1996; Emmanuel and Uche 2008), the increasing resistance of malaria parasites to the mainstay antimalarial drugs is thought to be, among many other factors, a major contributor to the reemergence of the disease as a major public health problem and its spread in new locations and populations (Rosenthal, 1998, 2002). Thus the use of drugs to prevent malaria or treat the disease is a very effective management approach (Emmanuel and Uche, 2008). Research efforts made at developing promising malarial vaccines are yet to reach the market (Emmanuel and Uche, 2008).

It is believed that the complexity of malarial life cycle reflects a series of evolutionary adaptations that optimised its ability to exploit its hosts and this, together with the parasite's lager genome has contributed to the difficulty in developing potent vaccine against the disease (Aravind *et al*., 2003). These limitations not only highlight the continual relevance of chemotherapy in the fight against malaria, but also that new effective, nontoxic and easily affordable antimalarial agents are needed.

Among potential new targets for chemotherapy are *Plasmodium* proteases (Rosenthal, 1998, 2001, 2004) which mediate invasion of cellular barriers (e.g. hepatocyte and RBCs) by the parasite, host cellular protein degradation (e.g. haemoglobin), erythrocyte rupture, evasion of immune response and processing of precursors for synthesis of proteins by the parasite (Rosenthal, 1998, Rosenthal, 2001, Klemba *et al*., 2001).

Generally, parasite proteases are involved in numerous physiological processes that include food digestion, cell maintenance, cell signalling, wound healing, cell differentiation inflammation, fertilization, allergic reactions, cell growth and death, blood clotting, tumor growth, and bone remodeling (Goll *et al*., 2003). Therefore, this class of proteases has been prime targets of inhibitor design and has been identified as therapeutic targets for many diseases and conditions including malaria. The active site of cysteine proteases consists of catalytic triad (Cys, His and Asn) that is and a subsite binding pockets where orientation of the substrate for catalysis occurs via interaction between subsites of the enzyme and amino acid residues of the substrate (Janna, 2007).

Purification and characterization of these proteases is a prerequisite for *in vitro* screening of compounds suspected to possess antimalarial activity as well as for the study of mechanisms of action of such agents. Previous effort to purify cysteine protease from *Plasmodium berghei* had employed a more cumbersome temperature-induced phase separation strategy (Amlabu *et al*., 2011). In this study, we evaluated the release of plasmodium protease using a more direct extraction procedure involving two different strategies (repeated freeze-thaw cycles and treatment with detergents). In addition, the soluble enzyme was assessed for its preference for various peptide substrates and thereafter partially purified.

2.0 Materials and Methods

2.1 Materials

2.2.1 Chemicals and Reagents

Coomassie Brilliant Blue, Dialysis tubing, Triton-X100, Dithiothreitol, Sephadex-G200, gelatin and casein were obtained from Sigma-Aldrich, USA. Other reagents were of analytical grade.

2.2.2 Experimental Animals

Twenty five mice (of both sexes) with average weight of 20 ± 2.7 g were used. The mice were inbred and kept in the Animal housing facility of the Department of Biochemistry, Kaduna State University, Kaduna-Nigeria.

2.2.3 Collection of Plasmodium berghei

Plasmodium berghei, was obtained from the Nigerian Institute for Medical Research (NIMR), Lagos. Mice were inoculated with infected blood from a donor mouse, transported to the Department of Biochemistry Kaduna State University, Kaduna, Nigeria where the parasite was maintained continually in young (4-6 weeks old, of 18-23 g body weight) mice through continuous serial passage.

2.2 Methods

2.2.1 Animal Inoculation and Microscopic Monitoring of Parasitaemia

Five donor mice with rising parasitaemia (27-35%) were sacrificed and the infected blood collected in 2 ml syringe containing 0. 2ml of 3.8% (w/v) sodium citrate solution (in PBS, pH 7.2) via cardiac puncture. The suspension was administered intraperitoneally to twenty five (20 ± 2.7g mean body weights per mouse) apparently healthy experimental mice (0.2 ml of suspension per mouse) to initiate the infection and their parasitaemia were monitored thereafter every day post infection. The parasitaemia in the infected mice were determined by microscopic examination of Giemsa-stained thin blood smears as described by Peter and Anatoli, (1998). Briefly, after sterilization with methylated spirit, blood was collected from mouse's tail on a glass slide and made into a thin blood smear. Dried slides were fixed with methanol (30 seconds immersion), stained with Giemsa (for 45 minutes), washed, dried and subsequently viewed under compound microscope - Olympus CH30, India (X-1000 magnification). Parasitaemia was expressed as the percentage of parasitized red blood cells (RBCs) in every 200 RBCs counted (Moody, 2002). Mean of two counts was taken as the measure of the infection.

2.2.2 Preparation of Plasmodium berghei Isolate from Infected Red Cells

At rising parasitaemia between 24 and 29% (usually around the 16th day post infection), infected mice were sacrificed after chloroform anaesthesia and the blood collected in 2 ml syringe containing 0.2ml of 3.8% (w/v) Sodium Citrate solution (in 100mM PBS, pH 7.2). The pooled blood was centrifuged (2000 rpm for 20 min.), the plasma and the buffy coat were removed by aspiration using Pasteur pipette and the pellet (red blood cells) washed four times with ice-cold 0.1M phosphate buffered saline (PBS, pH 7.2) until a transparent supernatant was observed. The supernatant was removed by aspiration and the residual RBC re-suspended in 3 part (1:3) cold erythrocyte lysis solution (0.1% w/v) of saponin in PBS, pH 7.2) for 60 min. at 4^{o}C (Ferone, 1970). After incubation, the suspension was again centrifuged (14,000 rpm for 10min. using Eppendorf Mini spin-Plus centrifuge (Germany). The supernatant was discarded and the pellet (dark brown parasite isolate) washed three times in 200mM sodium acetate buffer, pH 5.5. Thereafter, the pellet was re-suspended in 10 ml of acetate buffer to constitute the parasite isolate suspension.

2.2.3 Preparation of Haemoglobin

Hemoglobin was prepared from Carmel according to the procedure of Dodge *et al.* (1963). The blood from the above mentioned sources were collected into 2% EDTA solution and the erythrocytes were isolated by centrifugation at 2000 rpm for 20 minutes after which the plasma and the buffy coat were removed by aspiration. The erythrocytes were washed three times and re-suspended in an equal volume of isotonic phosphate buffer (310 ideal milliosmolar). The washed red cells were hemolyzed by hypotonic buffer (20 ideal milliosmolar) in the ratio of 1:14 washed red blood cells to hypotonic buffer respectively and mixed by gentle swirling. The hemolysed red blood cells solution was then maximally centrifuged at 16,000 rpm for 40 minutes after which the supanatant (which is essentially hemoglobin) was collected and dried

2.2.4 Preparation of Crude Enzyme from P. berghei Isolate

Soluble protein/enzyme from the parasite was released using three approaches: first, the parasite suspension was subjected to three cycles

of freezing and thawing with subsequent centrifugation (14,000rpm for 5 minutes 4°C). The supernatant was collected and referred to as the crude enzyme of the freezing-thawing phase (FT). For the second approach, the residual pellet from the first approach was re-suspended in 0.2% (v/v) Triton-X100 in acetate buffer, incubated at 4°C for 90 min. and centrifuged cold at 14,000 rpm for 5 min. The supernatant was collected as crude enzyme for the triton x-100 treatment. The third approach employed a combination of freezing and thawing cycles and triton-X100 treatments. The centrifugation was carried out as described before and the supernatant was referred to as triton phase-2. The crude enzymes from these phases of extraction were labeled and stored in aliquots of 1000µL at -20°C.

2.2.5 Determination of Protein Content

The protein concentrations of the various enzyme suspensions were determined using the method of Bradford (1976) with bovine serum albumin (BSA) as standard.

2.2.6 Assay for Protease Activity

The protease activity of the various enzyme suspensions was determined individually following the procedure described by Amlabu *et al* (2011). In a reaction mixture of 1ml, 100 µl of 0.2M sodium acetate buffer, pH 5.5 and 100 µl of gelatin, haemoglobin, bovine serum albumin (BSA) were independently incubated with 50µl of crude enzyme and the volume adjusted to 1000 µl with acetate buffer. The incubation was carried out at 37°C for 60 minutes after which the reaction was rapidly stopped by the addition of 500 µl of 40% trichloroacetic acid (TCA) and allowed to stand at 4°C for 30 minutes. The precipitated protein was removed by centrifugation at 14,000 rpm for 10 minutes. The supernatant was collected and the absorbance was read at 280 nm. A control experiment was carried out in the same manner, except that the enzyme was heat-inactivated at 100°C for 10 minutes before the introduction of the substrate. All experiments were done in triplicate. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze substrate(s) and release equivalent of 1.0 µg of tyrosine within 1 min reaction at 37°C. The number of units of activity per mg protein was taken as the specific activity of the enzyme.

2.2.6.1 Study on the Effect of the Soluble Protease on various Peptide Substrates

To determine the suitable substrate for the entire assay, proteolytic activity of the crude extract was tested against different concentrations (1, 2 and 3% [w/v]) of gelatin, BSA and bovine haemoglobin as substrates. The procedure was essentially same with that described under assay of protease.

2.2.6.2 Study on the Effect of varying Substrate Concentration and Incubation Time on Protease Activity

To establish the optimum substrate concentration and time of incubation, gelatin at concentrations of 2, 3 and 5% in acetate buffer was used as the substrate and the assay was carried out with 50 µl of the crude enzyme over an incubation time range of 15-120 minutes. The assay for the enzyme was performed as previously described.

2.2.7 Enzyme Purification

Ammonium sulfate crystals were added gently to the ice-cold supernatant kept in ice-cold water bath and was continuously stirred to dissolve the added crystals until 80% saturation level was achieved (Nooralabettu, 2014). This step took about 40 minutes and further stirred for 20 minutes and left over night at 4°C. The precipitate was collected by centrifugation (using Eppendorf refrigerated centrifuge set at 4°C) at 15,000 x g for 10 minutes. The supernatant was decanted and the precipitate (enzyme) was recovered by re-suspending the protein pellet in 10 ml of 200 mM acetate buffer pH 5.5. The protease activity and the protein content at this stage were determined as described earlier. The ammonium sulfates from the precipitate were removed by dialysis.

Briefly, dialysis-tube was prepared by boiling the tubes in 10 mM sodium bicarbonate containing 1mM ethylene diamine tetra acetic

acid (EDTA) for 30 minutes as described by Krishna (2014). The tubes were extensively washed inside and out with distilled water. One end of the dialysis tube was closed by knotting and the tube was checked for leaks after which the reconstituted pellet was introduced into the tube and the other end of the tube was knotted keeping sufficient space above the sample and placed in a beaker containing about ten times volume of 0.1M acetate buffer, pH 5.5 kept at 4^oC for 48 hours with two changes of external buffer.

The dialysate was purified on Sephadex G-200 (Sigma-Aldrich, USA) column (1.6cm x 20cm). The sephadex column was equilibrated with 100mM sodium acetate buffer (pH 5.5). The enzyme was loaded onto sephadex G-200 column and eluted with the same buffer. Fractions each of 5ml were collected (at flow rate of 38 ml/hr) and their enzyme activities and protein content (absorbance at 280nm) determined. The active fractions were pooled, concentrated and stored at -4^oC for further studies.

3.0 Results

Figure 1 shows the micrograph of the Giemsa-stained thin smear of blood of infected mice. Sixteen day post infection, most red cells were found to habour the trophozoite stage of the parasite.

Attempt were made to monitor the release of the protease protein by different means; including repeated freezing-thawing cycles, Triton-X100 treatments and the combination of both methods. More of the protein was released by freeze-thaw cycles while relatively higher ($P< 0.05$) protease activities were associated with the detergent treatment (Figure 2).

Addition of both cysteine and Dithiotreitol (DTT) at the final concentrations of 10mM (Figure 3) raised the enzyme activity by close to 100% while inclusion of EDTA at same concentration reduced the enzyme's activity.

The result in Figure 4 reveals a high preference of the enzyme for gelatin amongst other peptide substrates.

The pH (5.5) and temperature optima of 5.5 and 37^oC respectively were retained as conditions for the assay of plasmodia cysteine protease. To determine the suitable concentration and the corresponding incubation time, various concentrations of Gelatin in acetate buffer were prepared and assayed for proteolysis as described earlier. Figure 5 shows that 3% (w/v) Gelatin at 60 minutes incubation is optimum for the protease activity assay.

Cysteine protease was partially purified by a combination of ammonium sulfate precipitation, and gel filtration chromatography that resulted to 3.50 fold purification and 82% recovery as shown in Table 1 and the chromatogram for gel filtration chromatography presented in Figure 6.

4.0 Discussion

In this study, attempt was made to evaluate the extraction of soluble protease from the isolate of *P. berghei* obtained at high parasitaemia, when the infection was at advanced stage and the protease activities were at optimal level. Previous report on the isolation and purification of cysteine protease by Amlabu *et al* (2011) employed a more cumbersome procedure of temperature-induce phase separation. This study employed a simpler and more direct approach of freezing and thawing and detergent solubilization since both approaches, when used previously, was effective methods of protein extraction (Cross, 1984; Nok and Balogun, 2003). The study compared the ability of these strategies to release enzyme molecule from the parasite isolate. The repeated cycles of freezing and thawing released more of the protein, while more of the protease activity was observed in the enzyme solution prepared by Triton-X 100 treatment. This is consistent with the findings of Amlabu *et al* (2011) and suggests the existence of protease as membrane bound molecule probably anchored to glycoprotein moieties.

Cysteine protease has been reported to be localized to the acidic vacuole of plasmodium (Francis *et al*., 1997). Usually, membrane-bound enzymes are released from the membrane by treatment with detergent which is known to disrupt the lipid bilayer (Nok and Balogun, 2003). Thus, a combination of Tixton-X 100 treatment and few cycles of freezing and

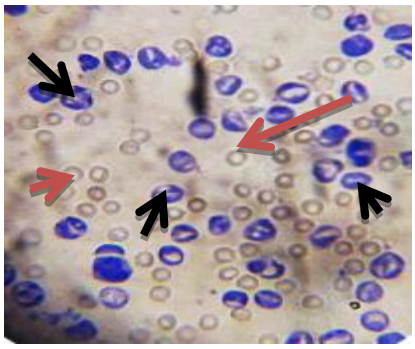

Figure 1: Erythrocytic Stage of *Plasmodium berghei* infection in Mice
[Black arrows point to Giemsa-stained infected erythrocytes at tropozoite stage while red arrows show normal uninfected mice red blood cells. Thin blood smears prepared from blood 16 days post infection and viewed under x1000 magnification]

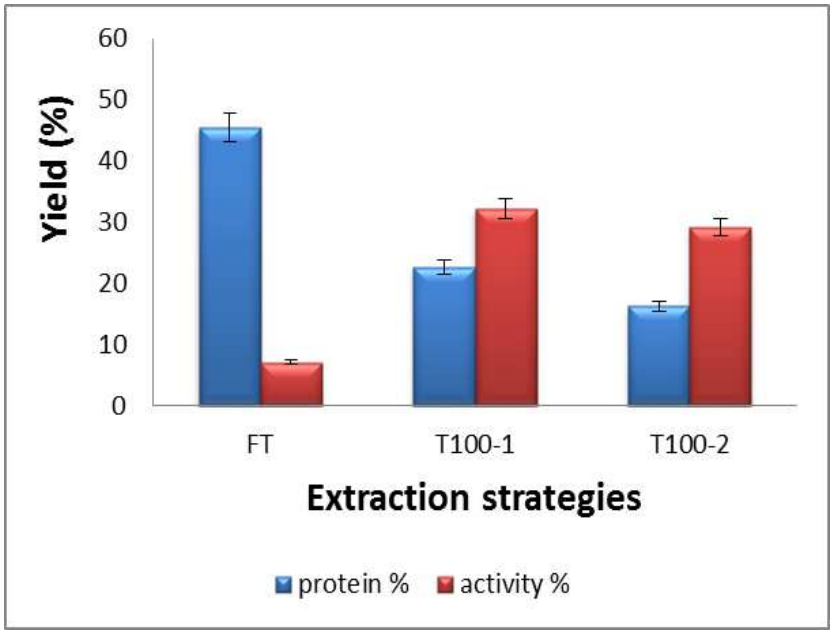

Figure 2: Release of Protein and soluble Enzyme from *P. berghei* isolate after different extraction procedures
[Each bar represents mean of triplicate experiments. The result of the enzyme activity test has been corrected with the value of an appropriate control experiment conducted. FT; supernatant obtained after three cycles of freezing and thawing; T100-1; supernatant obtained following the treatment of pellet from FT (14,000xg, 90 minutes, at 4^{o}C) with Triton X-100; and T100-2; the supernatant obtained after subjecting pellet of T100-1 to a combination of Triton-x100 treatment and three cycles of freezing and thawing. Enzyme activities and protein contents of each extraction phase are expressed in percentage of the total activity and protein contents].

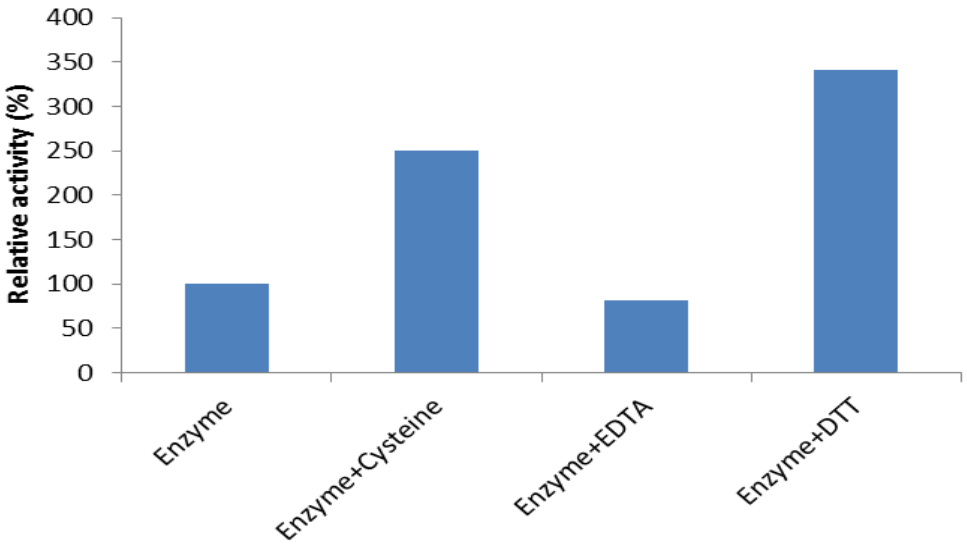

Figure 3: Effect of EDTA, Cysteine and DTT on the activity of soluble protease from *P. berghei*
[The assay was carried out in the absence of the modulators of the enzyme (EDTA (ethylenediaminetetraaceticacid), Cysteine and DTT (dithiothreitol)) was taken as 100%. Each Bar represents the mean of triplicate experiments]

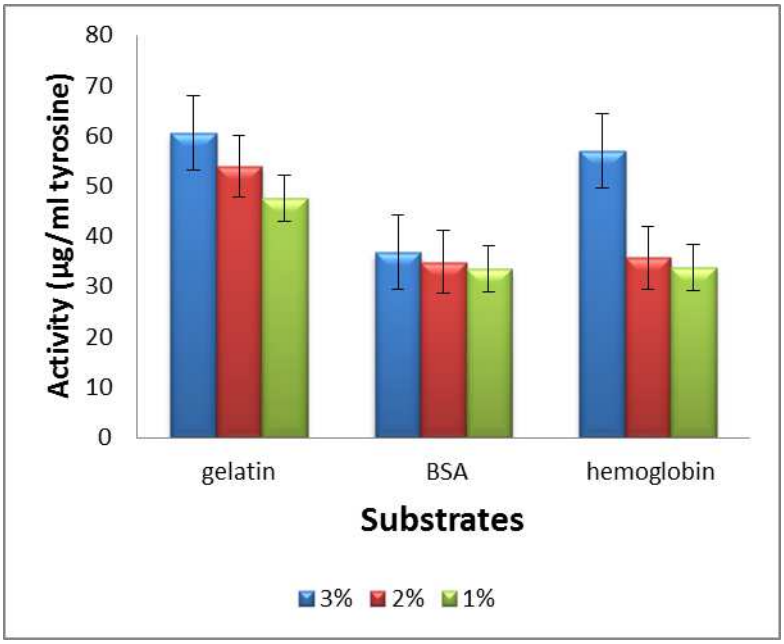

Figure 4: Hydrolysis of peptide substrate by soluble protease from *P. berghei* during 60 minutes of incubation

[The assay was carried out in triplicate according to the procedure mentioned earlier and the mean value of the triplicate independent experiment is presented above and the error bar was generated using standard error. The result of the enzyme assay has been corrected with the value of an appropriate control experiment conducted].

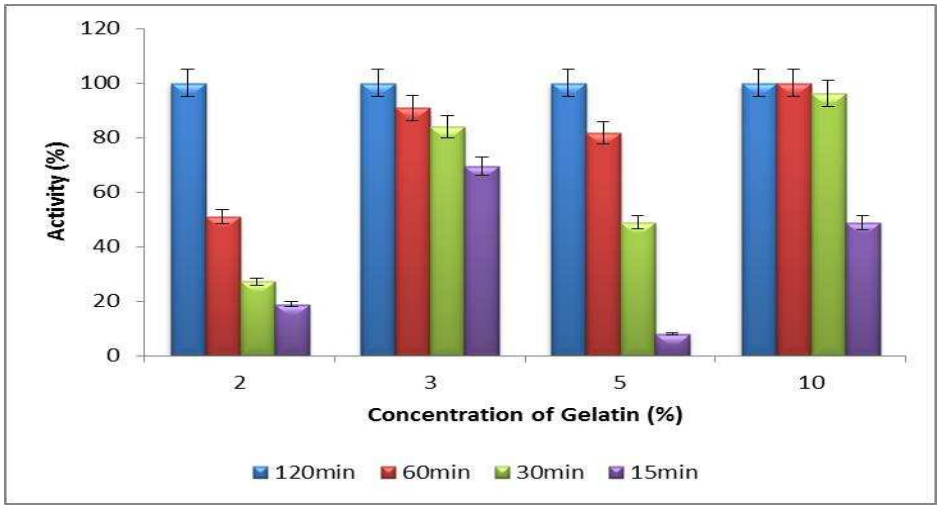

Figure 5: The effect of the concentration of gelatin (substrate) on the enzyme activity
[The values presented are control-corrected values and are the means of triplicate experiments]

Table 1: Purification Profile of Soluble Protease from *Plasmodium berghei*

Purification steps	Total protein (mg)	Total activity (µg/min)	Specific activity (µg/min/mg)	Purification (fold)	Recovery (%)
Supernatant	2.00	0.28	0.139	1.00	100.0
$(NH_4)_2SO_4$ (80%)	0.86	0.24	0.281	2.02	85.7
Sephadex G200	0.48	0.23	0.487	3.50	82.1

Recovery and fold purification were calculated on the basis of total activity and specific activity respectively

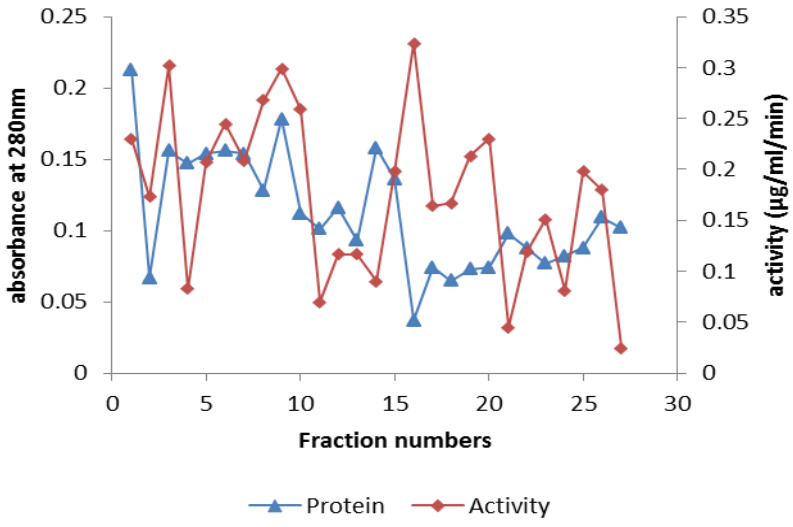

Figure 6: Purification of Cysteine Protease from *Plasmodium berghei* using Sephadex G-200
[The result of the enzyme assay has been corrected with the value of an appropriate control experiment conducted]

thawing could have synergistic effect in the extraction of proteases from plasmodium.

Specificity studies of the enzyme (Figure 3), reveals rapid proteolysis of all the substrates tested. But comparatively higher proteolysis occurred with gelatin as peptide substrate. Thus gelatin was adopted as the most suitable peptide substrate for the enzyme in all the assays. The difference in proteolysis towards different proteins thought to be due to the difference in amino acid sequence and composition at/around the susceptible peptide bond.

The effect of substrate (gelatin) concentration was also evaluated following the test for proteolysis at different concentrations incubated at different times (Figure 4). The result indicates that the saturation effect occurred in the first 30 minutes with 5 and 10% gelatin while the saturation effect with 3% gelatin occurred in the first 60 minutes. Amlabu *et al* (2011) used 3% gelatin and 60 minute incubation time and this was justified by our study.

The effects of DTT, EDTA and Cysteine were evaluated to investigate the proportion of the proteolysis that is attributable to cysteine protease. Since DTT and cysteine are said to modulate cysteine protease activity, higher enzyme activity observed in the presence of DDT is suggestive of the predominance of cysteine pprotease in parasite extract studied. Cysteine protease from *T. aestivum* and cysteine protease from *P. berghei* were reported to be activated by β-mercaptoethanol and dithiothreitol (Afaf *et al.,* 2004; Amlabu *et al.,* 2011). Cysteine and dithiotreitol, as strong reducing agent, are able to keep the active site cysteine residues in their reduced form (-SH) and this is critical for the proteolytic activity (Afaf *et al.,* 2004).

Partial purification of the soluble protease by a combination of ammonium sulfate precipitation, and gel filtration chromatography resulted in 82% recovery and 3.5 fold purification. The soluble protease appeared to be a mixture of several proteases; larger proportion of it being cysteine protease (Afaf *et al.,* 2004; Amlabu *et al.,* 2011). The slight reduction in proteolytic activity due to the presence of EDTA, however, suggests the presence of metalloprotease in smaller proportion relative to cysteine protease. This is in agreement with previous studies (Rosenthal *et al*., 1987; Coombs *et al*., 2001) that suggested different classes of proteases in *Plasmodia*.

Conclusion

In conclusion, this study shows that malarial protease could be extracted through a combination of freezing-thawing cycles with treatment of Triton-X100, the latter playing most important role in enzyme release. The result of this study will aid further purification and determination of kinetic parameters of the pure enzyme. In addition, partially purified soluble protease solution could be used to screen extracts cum synthetic compounds for their inhibitory activities and possibly extend these results to *in vitro* and *in vivo* assessment of their antimalarial effects/efficacies.

References

Afaf, S. F., Ahmed, A. A. and Saleh, A. M. (2004). Characterization of a cysteine protease from wheat *Triticum aestivum* (cv.Giza 164). Bioresource Technology 91: 297-704.

Albay, C., Yucel, A., Ceber, K. and Imir, T. (1999). The *in vitro* effect of *Plasmodium berghei* antigen lysate on Natural Killer (NK) cell cytotoxic Activity. Tropical Journal of Medical Science 29:253-258.

Amlabu, E., Nok, A. J., Inuwa, M., Akin-Osanaiye, B. C. and Haruna, E. (2011). Cysteine protease from the malaria parasite, *Plasmodium berghei*-purification and biochemical characterization. Journal of Cell and Molecular Biology 9(2): 43-49.

Aravind, L., Lakshminarayan, M., I., Thomas. E. W., and Louis H. M. (2003). Plasmodium Biology: Genomic Gleanings. Cell. 115:771-785.

Bradford, M. M. (1976). A rapid and sensitive methods for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

Caldeira, R. L., Goncalves, L. M. D., Martins, T. M., Silveira, H., Novo, C., Rosario, V. D. and Domingos, A. (2009). *Plasmodium chaubudi*: Expression of active recombinant chabaupain-1 and localization studies in Anopheles sp. Experimental Parasitology. 122:97-105.

Coombs, G. H., Goldberg, D. E., Klemba, M., Berry, C., Kay, J. and Mottram, J. C. (2001). Aspartic proteases of *Plasmodium falciparum* and other

parasitic protozoa as drug targets. Trends in Parasitology 17(11):532-537.

Cross, G. A. M. (1984). Release and Purification of *Trypanosoma brucei* Variant Surface Glycoprotein. Journal of Cellular Biochemistry. 24:79-90.

Dixit, R. and Pandey, K. C. (2012). Structure-Function of Faciains: Malarial Cysteine Protease. Journal of Tropical Medicine. doi:10.1155/2012/345195.

Dodge, J. T., Mitchell, C. and Hanahan, D. J. (1963). The Preparation and chemical characteristics of hemoglobin-free Ghosts of human erythrocytes. Archives of Biochemistry and Biophysics 100: 119-130.

Emmanuel, C. I. and Uche, O. (2008). Current trends in malarial chemotherapy. African Journal of Biotechnology 4: 349-356.

Ferone, R. (1970). Dihyrofolate Reductase from pyrimetamine-resistant *Plasmodium berghei*. The Journal of Biological Chemistry 245(4):850-854.

Francis, S. E., Sullivan, D. J. and Goldberg, D. E., (1997). Haemoglobin metabolism in the malaria parasite *Plasmodium falciparum* Annual Review of Microbiology 51:97-123.

Goll, D. E., Thompson, V. F., Li, H., Wei, W. E. I., and Cong, J. (2003). The calpain system. Physiological Reviews 83(3):731-801.

Janna M. M (2007). The design, synthesis and biological assay of cysteine protease specific inhibitors: Unpublished Ph.D. Thesis, University of Canterbury

Klemba, M., Coombs, G. H., Goldberg, D. E., Berry, C., Kay, J. and Mottram, J. (2001) Aspartic proteases of *Plasmodium falciparium* and other parasite protozoa as drug targets. Trends in Parasitology 17: 532-537.

Moody, A. (2002). Rapid diagnostic tests for malaria parasites. Clinical Microbiology Review 15:66-78.

Nok, A. J. and Balogun, E. O. (2003). Bloodstream *Trypanosoma congolense* sialidase could be involved in anemia during experimental trypanosomiasis. Biochemistry 133:725-730.

Nooralabettu, K. P. (2014). Optimisation of ammonium sulfate precipitation method to achieve high throughput concentration of crude alkaline phosphatase from Brown shrimp (*Metapenaeus monoceros*) hepatopancreas. International Journal of Analytical Bio-science *2*(1):7-16.

Oaks, S. C., Mitchell, V. S., Pearson, G. W. and Carpenter, C. C. J. (1991). Malaria: obstacles and opportunities.: Washington, DC: The National Academies Press. pp. 1-328. https://doi.org/10.17226/1812

Olliaro, P., Cattani, J. and Wirth, D. (1996). Malaria, the submerged disease. The Journal of the American Medical Association 275:230-233.

Peter, I. T. and Anatoli, V. K. (1998). Malaria parasite biology and protection. The current global malaria situation. ASM Press. Washington DC. USA. pp. 11-22.

Rosenthal, P. J., Kim, K., McKerrow, J. H. and Leech, J. H. (1987). Identification of three stage-specific proteinases of *Plasmodium falciparum*. Journal of Experimental Medicine. 166:816-821.

Rosenthal, P. J. (1998). Proteases of malaria parasites: new targets for chemotherapy. Emerging Infectious Diseases 4(1):49-57.

Rosenthal, P. J. (2001). Antimalarial chemotherapy: mechanisms of action, resistance, and new directions in drug discovery. Antimalarial chemotherapy: mechanisms of action, resistance, and new directions in drug discovery. 325-345.

Rosenthal, P. J. (2002) Hydrolysis of erythroctye proteins by proteases of malaria parasites. Current Opinion in Haematology 9: 140-145.

Rosenthal, P. J. (2004). Cysteine proteases of malaria parasites. International Journal for Parasitology 34: 1489-1499.

Ukwe, C. V. (2003). Malaria. In: Therapeutic Basis of Clinical Pharmacy in the Tropics. Snaap Press, Enugu, Nigeria. pp. 250.

World Health Organisation (WHO, 2017). Global health experts define malaria work packages. https://www.who.int