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Effects of Storage Conditions, Detergents, Organic Solvents and Metal Ions on the Activity of Soluble Cysteine Protease from *Plasmodium berghei*

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Abstract: As a survival mechanism, plasmodium parasites rely throughout their life cycle on, the hydrolytic products of their host's haemoglobin by the action of cysteine proteases making them good target for drug design. Owing to increasing resistance to current antimalarial drugs, the search for more promising compounds will require screening for inhibitory activity of plasmodium proteases. The goal of this work was to assess the effect of various parameters on the activity of crude cysteine protease from *Plasmodium berghei*. At high parasitaemia (~ 24-29%), parasite isolates were prepared from the blood of infected mice using saponin (0.1% w/v) and Triton-X 100. The major parameters evaluated included the effect of temperature and glycerol during enzyme storage as well as protease's relative residual activities in the presence of various detergents, organic solvents and metal ions. The activity of the enzyme was retained during storage at -20°C storage condition with glycerol (20% v/v) used as preservative. The enzyme was inhibited by Cu^{2+}, Hg^{2+}, Cu^{2+}, Fe^{2+} Co^{2+}; but activated by Zn^{2+} and insensitive to chloroacetamide and ethylene diaminetetra acetic acid. Acetone and dimethyl sulphoxide had minimal effect on enzyme activity compared to the other organic solvents. Incubation of the enzyme with 20 % (v/v) sodium dodecyl sulfate, Triton-X 100 and Tween 80 gave 81%, 51% and 34% reduction in enzyme activity respectively. The relevance of these findings in malarial research is discussed.

KEYWORDS: Cysteine protease, *Plasmodium berghei*, plasmodium proteases

1.0 Introduction

Malaria, a disease caused by several species of obligate intracellular parasitic protozoan of the phylum Apicomplexa and genus *Plasmodium*, has remained an important public health problem globally. Successes have, however, been recorded in recent years in terms of reduced morbidity and mortality due to the use of artemisinin-based combination therapy. Despite these developments, the disease is still transmitted to people throughout tropical and subtropical areas, where 40% of the world's population is at risk of infection (WHO, 2010). In 2015, there were 212 million new malaria cases and 429,000 deaths globally (WHO, 2017).

Efforts at controlling the disease are targeted at the various developmental stages of the parasite while the preventive measures involve the use of nets, closing of doors/windows against mosquitoes, mosquito repellents and drugs (Emmanuel and Uche, 2008). The increasing resistance of malaria parasites to the mainstay antimalarial drugs is a major contributor to the reemergence of the disease as a major public health problem and its spread in new locations and populations (Rosenthal, 2002). The re-emergence of malaria is attributed, largely to the spread of drug-resistant parasite strains, insecticide-resistant mosquitoes, decay of health-care infrastructure and difficulties in implementing and maintaining vector control programmes in many developing countries (Stephanie and Louis, 1999). Because of malaria's growing global burden, its control is essential and it highlights the continued need for new antimalarial agents. Thus, there is a great need for the identification of novel chemotherapeutic targets and drugs, ideally directed against these new targets.

Among the potential targets for the new chemotherapeutic agents are *Plasmodium*

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proteases. Proteases which catalyze the hydrolysis of amide bond of proteins (Rawlings and Barret, 2004), are grouped into exopeptidases and endopeptidases depending upon their sites of action and also based upon functional group at the active site: serine, threonine, cysteine, aspartic acid, glutamic acid and metallo-proteases (Rawlings and Barret, 2004). Plasmodium proteases have been shown to play essential roles during the life cycle of the parasite. They appear to be required for a number of important functions in erythrocytic stage of the parasite including haemoglobin hydrolysis, erythrocyte rupture, erythrocyte invasion, and processing of precursors for synthesis of proteins by the parasite (Rosenthal, 1998, Rosenthal 2001: Klemba and Golberg, 2001). Proteases are thus expected to be promising targets for antimalarial chemotherapy. Owing to increasing resistance to current drugs, the search for more new antimalarial agents is a never ending enterprise.

Purification and characterization of proteases and their use for screening of potential anti-malarial compound(s) sometimes require storage at low temperature using different cryoprotectants such as glycerol, treatment with organic solvents (since most organic solvents are used in concentrating protein/enzyme preparation by cold precipitation), or even interaction with metal ions which could serve as activators or inhibitors of enzyme's activity. Some of these substances could affect the functionality of proteins and so there is the need to reevaluate the effect of these conditions on the activity of soluble proteases. We report here the effect of glycerol, organic solvents, metal ions, some protease modulators, detergents as well as storage temperatures on the activity of cysteine protease from *Plasmodium berghei*.

2.0 Methods

2.1 Methods

2.1.1 Parasite Inoculation

Briefly, eight donor mice with rising parasitaemia (27-40%) were sacrificed and the blood collected via cardiac puncture in 3.8% (w/v) sodium citrate solution (in PBS, pH 7.2) and diluted (1ml of blood in 0.2ml of citrate buffer solution). The suspension was administered to healthy mice intraperitoneally (0.2ml per mouse) to initiate the infection and 42 mice (mean weight 20 ± 2.7g) were infected and the parasitaemia was monitored thereafter.

2.1.2 Determination of Parasitaemia in Infected Mice

The parasitaemia in the infected mice was monitored by microscopic Giemsa-stained thin blood smear examination as described by Peter and Anatoli (1998). The number of the parasitized red blood cells (RBCs) in every 200 RBCs counted was expressed in percentage and repeated for another field of view and the mean of the two results taken as the measure of the infection.

2.1.3 Preparation of P. berghei from Infected Red cell

When the average rising parasitaemia of the 25 infected mice was between 24-29% ± 5.2, the infected mice were sacrificed and the blood collected in 3.8% (w/v) sodium citrate solution (in 100mM PBS, pH 7.2). The blood from all the mice was pooled together and centrifuged[give model number, manufacturer, city & country] at 2000 rpm for 20 minutes to isolate red blood cells, after which the plasma and the buffy coat were removed by aspiration. The red blood cells were washed four times with ice-cold 0.1M phosphate buffered saline (PBS) pH 7.2 until a clear supernatant was obtained. The supernatant was removed by aspiration and the pellet used for further treatments.

The washed infected erythrocytes (pellet) were lysed by incubation with 0.1% (w/v) of saponin (in PBS, pH 7.2) in the ratio of one part of red blood cells to three parts of saponin solution respectively for 60 minutes at 4°C. After this incubation, the resulting solution consisted of the 100% lysed erythrocytes and was centrifuged maximally at 14,000rpm for 10minutes in an Eppendorf mini-spin plus centrifuge (Germany). The supernatant was discarded and the pellet (dark brown) was washed 3 times in 200mM sodium acetate buffer

pH 5.5. After the washings, the pellet was re-suspended in equal volume of acetate buffer (about 10ml) and this constituted the parasite isolate suspension.

2.1.4 Preparation of Crude Protease from P. berghei Isolates

To release the enzyme from the parasite, the parasite was subjected to three cycles of freezing and thawing followed by treatment with Triton-X100 (0.2% v/v in acetate buffer) and centrifuged at 14,000 rpm for 5 minutes in a refrigerated centrifuge [GL-16C, Longway & Yueping, JiangSU ZhengJi, China] maintained at 4°C. The supernatant was collected as the crude enzyme suspension.

2.1.5 Protein Quantification

The protein was quantified by the binding assay method of Bradford (1976) using bovine serum albumin as standard and Coomassie brilliant blue as dye.

2.1.6 Assay of Protease Activity

The protease activity was determined as described previously by Amlabu *et al.* (2011). In a reaction mixture, 100 µl of gelatin was incubated with 50 µl of the crude enzyme. 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 and incubated at 4°C for 40 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 280nm.

Similarly, a control experiment was carried out in the same manner, except that the enzyme was added after the addition of TCA. All experiments were done in triplicates. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze substrate(s) and release equivalent to 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.

2.1. 7 Effect of Storage on Protease Activity

To assess the effect of storage temperature on the activity of protease in *P. berghei*, two different temperatures were used (4°C and -20°C) over a given time intervals ranging from 6 hrs to one week. After 6hr, 20hrs, 24hrs, 72hrs and 168hrs of enzyme storage at 4°C, the respective residual activities were determined according to Amlabu *et al* (2011) with little modification in substrate (3% gelatin) and enzyme concentrations (100µl each). The experiment was done in triplicates.

2.1.8 Effect of Glycerol on Stability of Protease during Storage

The stability of the protease in different concentrations of glycerol was evaluated by storing the enzyme (suspended in different concentrations of glycerol) at -4°C for 30 days. During the course of this storage, the state of the enzyme in glycerol was monitored. At the 30th day of storage, the enzyme's activity was assayed. The control was treated the same way except that no glycerol was added and this was taken as 100%.

2.1.9 Effects of Metal Ions and Inhibitors on the Activity of Partially Purified Protease

Using the procedure of Abidi *et al.* (2011), the effects of metals and inhibitors on the protease activity were examined. The partially purified protease was pre-incubated (at 4°C for 3 hours) with each metal ion at final concentrations of 5mM and 10mM and the residual protease activity was assayed as usual with the introduction of the substrate. The activity in the absence of any additive was taken as 100%. The experiments were carried out in duplicates.

2.1.10 Effects of Surfactants and Detergents on the Activity of Partially Purified Protease

The effects of some surfactants (Triton-X100, saponin, Tween-20, Tween-80, SDS, detergents) on protease stability were studied by pre-incubating the enzyme with the various detergents (final concentrations of 10% or 20%)

for 2 hours at 4°C after which the residual activity was determined as usual at pH 5.5.

2.1.11 Effect of Organic Solvents on Protease Activity

This was done according to Abidi *et al.*, (2011). The proteolytic activity of the protease was measured in the presence of 15% and 30% (v/v) organic solvents. Ethanol, methanol, acetone, isopropanol and dimethylsulfoxide (DMSO) were used as the organic solvents in the reaction mixture. Each experiment was done in duplicates. The stability was expressed as the remaining proteolytic activity relative to non-solvent containing control (100%).

2.1.12 Statistical Analysis

Statistical Analysis was carried out at $p < 0.05$ using Statistical Package for Social Sciences (SPSS) Version 16.

3.0 Results

As shown in Figure 1, the activity at the start of the experiment was taken as hundred percent and subsequent experiments were presented relative to the activity at the start. It can be seen that the activity of the enzyme stored at -20°C was fairly constant throughout the period of the experiment. On the contrary, there was an observed continuous gradual decline in the enzyme's activity which resulted in less than 20% residual activity at day seven of the experiment. The difference in these activities (between 0^{th} and 8^{th} hour and between 72^{nd} and 168^{th} hour) is significant ($p < 0.05$).

The effect of glycerol as a preservative in the storage medium of the protease enzyme as presented in Table 1 showed that 20% (v/v) of glycerol and above is able to keep solution from freezing at -20°C and total activity of the enzyme retained. The result also revealed that the activity of the enzyme is retained at all concentrations of glycerol in enzyme solution tested.

The result on Table 2 shows the various effects of different metal ions on the enzyme activity. The partially purified enzyme was stable in the presence of EDTA, Ca^{2+} but shows decrease in activity in the presence of Cu^{2+}, and Fe^{2+} sulfates.

The difference between residual activities between different concentrations of each organic solvent is significant ($p < 0.05$) as presented in Figure 2. The enzyme shows less stability to methanol amongst other organic solvents investigated.

Effects of surfactant and detergents on the protease activity are shown in Table 3. The results show that the enzyme activity was substantially reduced by the ionic detergent, SDS and least affected by saponin.

4.0 Discussion

In this study we report the effects of various treatments on the crude and partially purified suspension of protease partially purified *Plasmodium berghei*. The enzyme was extracted following a combination of rounds of freezing-thawing and treatment with Triton-X100 on the parasite isolates. Previous preliminary studies revealed the preference of the enzyme for Gelatin among other substrates and that 3% (w/v) of Gelatin when used for 60 minutes were optimum for the enzyme activity (Amlabu *et al.*, 2011). The report by Amlabu *et al.* (2011), also gave the pH and temperature optima of 5.0 and 40°C respectively. On the basis of this report, the above conditions were adopted for all the activity assays conducted in this work.

Studies on the effect of protease modulators on the crude suspension of cysteine protease revealed that the activity of cysteine protease is greatly enhanced by cysteine and Dithiothreitol. This suggests that the majority of the proteolysis observed can be attributable to cysteine protease. Similar observations were reported by Afaf *et al.* (2004), that cysteine protease from *T. aestivum*, is activated by β-mercaptoethanol and dithiothreitol; similar observation was made previously by Amlabu *et al.* (2011).

The inhibitory effect of the protease by Cu^{2+}, Hg^{+} and Fe^{2+}, except Co^{2+} and Ag^{2+} have also been reported by Yin *et al.* (2013) on the activity of cysteine protease. The later authors reported that cysteine protease was never affected by Co^{2+} and Ag^{2+} and their observation was similar to that of Narahara *et al.*, (1984). This discrepancy in the observations above can

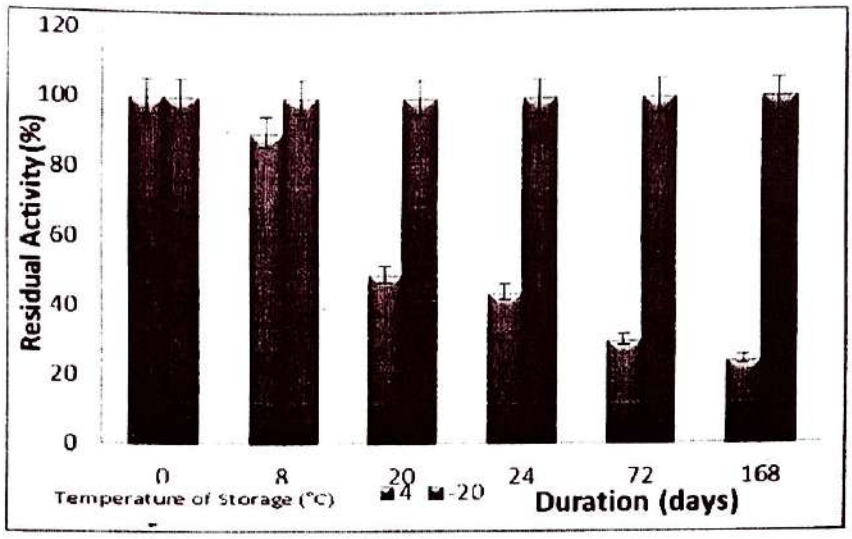

Figure 1: Effect of storage temperature on the protease stability

Table 1: Effects using glycerol as cryopreservative during storage of the soluble plasmodium protease

Concentration of glycerol (%)	Enzyme state	Relative activity (%)
0	Frozen	100
5	Frozen	88
10	Semi-frozen	112
20	Liquid	100
25	Liquid	97
40	Liquid	98
50	Liquid	99

Table 2: Effect of metal ions and inhibitors on the activity of soluble protease from *P. berghei*

Agents	Relative activity (%)	
	Concentration	
	5(mM)	10(mM)
Control	100.0	100.0
$HgCl_2$	72.8	68.8
$MnCl_2$	90.5	89.2
$BaCl_2$	80.9	75.0
$CoCl_2$	89.0	85.0
$ZnCl_2$	102.0	90.0
$CuSO_4$	85.9	61.0
$FeSO_4$	75.5	58..5
$Ca(OH)_2$	93.0	91.0
$AgCl_2$	81.0	85.0
$MgCl_2$	95.0	81.0
EDTA	95.0	95.0
Chloroacetamide	87.0	100.0

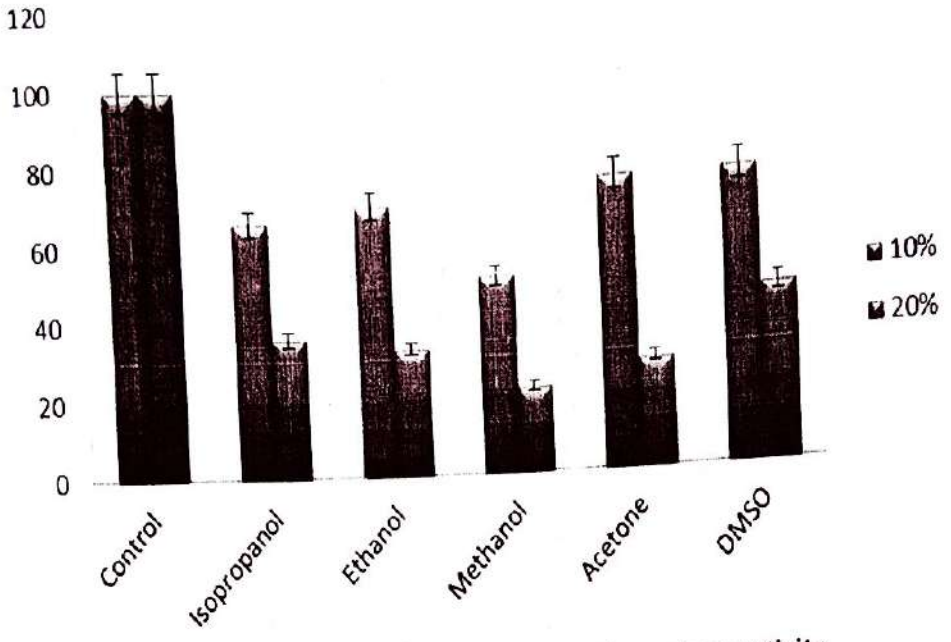

Figure 2: Effect of organic solvents on the protease activity

Table 3: Effect of surfactant and detergents on the activity of *P.berghei* protease

Detergent	Residual activity (%) Concentration (%,v/v)	
	10	20
Tween-80	72	66
Triton-X100	67	49
SDS	37[a]	19[b]
Saponin	93	88.7

be accounted for by taking note of the substrate(s) involved. Metal ions have been reported to affect the enzyme catalysis by direct interaction between the metal ion and the enzyme, either at the active site or on any location at the interaction(s) contained on the tertiary structure of the enzyme thereby altering the enzyme-substrate specificity and/or mediating conformational change of the enzyme (Means and Feeney, 1971; Anders and Rodney, 1991). Indirectly, metal ions also affect protease catalysis by binding to the substrate and altering the kind of interaction and possibly the ionic state of the peptide substrate which is a critical factor in enzyme-substrate interaction (specificity) (Anders and Rodney, 1991). The reports of Jun *et al.* (2007) is not at variance with the result we presented here. The reversibility studies of the effect of these metal ions suggest the possible interaction at the active site. Thus, Regardless of the mechanisms involved, identification of the metal ion binding sites may facilitate development of new types of protease inhibitors (Anders and Rodney, 1991). These compounds would be designed to target the delivery of cation to a site responsible for inhibition of the protease (Anders and Rodney, 1991).

Effect of organic solvents (ethanol, methanol, isopropanol, acetone and DMSO) on the activity and stability of the partially purified protease showed significant variation among solvents used. The enzyme was shown to be fairly stable in the presence of lower concentration of alcohol and/or ketone-containing organic solvents, but at higher concentration (20%) and at room temperature, there was significant decrease in activity. Babu *et al.*, (2001) reported that alcohols have some hydrophilic component, but are only moderate competitors for amide hydrogen bonds and so they tend to disrupt tertiary structure and leave secondary structural interactions largely undisturbed. This might be one of the reasons why methanol has been used as a denaturant in protein folding studies (Babu and Doughlas, 2000). Proteins in hydrophobic solvents are thought to retain their native structure as a result of kinetic trapping, which results from stronger hydrogen bonding between the protein atoms and a more rigid structure in the absence of water (Carla and Dagma, 2001). The structural stability is reportedly due to the fact that in hydrophobic water-immiscible solvents, any water that might be present will tend to stay at the protein surface because of the hydrophobic and hydrophilic nature of the protein surface (Miroliaei and Nemat-Gorgani, 2002). Thus the reduction in enzyme activity in the presence of polar organic solvents might be as a result of their ability to cause substrate inhibition and changes in protein conformation as indicated by kinetic and fluorescence studies (Miroliaei and Nemat-Gorgani, 2002). Dimethylsulfoxide and other polar solvents have been reported to destabilize the tertiary structure of proteins (Fink 1974; Henderson *et al.*, 1975; Fujita *et al.*, 1982; Kharasch 1983; Baker and Hubbard, 1984; Ping *et al.*, 1995; Carla and Dagma, 2001).

The enzyme suspension was stored individually in various concentrations of glycerol at -20°C for 30 days after which the residual activity was determined and compared. Statistically, there is no significant difference between the residual activities ($p< 0.05$), but based on physical observation, 20% (v/v) glycerol and above was sufficient to prevent the enzyme from being frozen and the activity retained as well. However, the effect of glycerol in enzyme storage has been reported to affect the catalytic efficiency, V_{max} or K_m but not the overall activity of the enzyme (Yancey, 2005; Olsen *et al*, 2007 and Kulaksiz-Erkmen *et al.*, 2012). According to report of Levy *et al.*, (1966), the response of enzyme to different osmolytes depends largely on the enzyme system.

To assess the stability of the enzyme, the enzyme preparation was stored at 4°C and -20°C and the residual activity tested at different time interval. The result shows that the enzyme was fairly stable at 4°C in the first 8 hour after which there was continuous decrease in activity as the time progressed while enzyme stored at -20°C retained 100% activity (Figure 3). The storage can be improved by inclusion of antimicrobial agents in the soluble enzyme to exclude some interference that can result from microbial contamination and growth. However, the effect of the antimicrobial agent on the enzyme activity should also be evaluated and taken into consideration.

Various detergents can cause reduction in enzyme activity. This reduction in activity was notable in our study with SDS and Triton-X100 and agrees with the report of Wang (1999) that SDS and Triton-X100 which bind to polar and non-polar groups on proteins cause denaturation. Therefore, the lost in activity can be due to the denaturation of the protein. No wonder, lower concentrations of these surfactants have found application in the disruption of membrane to release membrane bound enzyme (Tukmachev *et al* 1979) and conversely higher concentrations during the denaturing condition of gel electrophoresis (Andrew *et al* 2014).

Conclusion

This work highlights the applicability of detergents in extraction and the stability of the protease enzyme during cryopreservation. It also suggests that regardless of the mechanisms involved, identification of the ion binding sites may facilitate development of new types of protease inhibitors. These compounds would be designed to target the delivery of drugs to a site responsible for inhibition of the protease.

References

Abidi, F., Jean-Marc, C., Thomas, H. and Mohamed, N. M. (2011). Purification and biochemical characterization of stable alkaline protease prot-2 from *Botrytis cinerea*. Process Biochemistry 46: 2301-2310.

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.

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.

Anders, R. K. and Rodney, L. L. (1991). Copper inhibits the protease from human immunodeficiency virus 1 by both cysteine-dependent and cysteine-independent mechanisms. Proceedings of the National Academy of Science USA 88: 5552-5556.

Andrew, B. N., William, J. W. and David, H. P. (2014). Native SDS-PAGE: High resolution electrophoretic separation of proteins with retention of native properties including bound metal ions. metallomics 6(5):1068-1078

Babu, K. R. and Doughlas, D. J. (2000). Methanol-induced conformations of myoglobin at pH 4.0. Biochemistry 39:14702-14710.

Babu, K. R., Moradian, A and Douglas, D. J. (2001). The methanol-induced conformational transitions of beta-lactoglobulin, cytochrome c, and ubiquitin at low pH: a study by electrospray ionization mass spectrometry. Journal of American Society of Mass Spectrometry 12:317-328.

Baker, E. N. and Hubbard, R. E. (1984). Hydrogen bonding in globular proteins. Proceedings of Biophysics and Molecular Biology 44: 97-179.

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

Carla, M. and Dagmar, R. (2001). Proteins in organic solvents. Current Opinion in Structural Biology 11:761–764.

Fink, A. L. (1974). The trypsin-catalysed hydrolysis of N^{α}-Benzyloxycarbonyl-L-Lysine p-Nitrophenyl Ester in Dimethylsulfoxide at sub-zero temperatures. Journal of Biological Chemistry 249: 5027-5032.

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

Fujita, Y., Izumiguchic S. and Noda. Y. (1982). Effect of dimethylsulfoxide and its homologues on the thermal denaturation of lysozyme as measured by differential scanning calorimetry. International Journal of Peptide Protein Research 19: 25-31.

Henderson, T. R., Henderson, R. F. and York, J. L. (1975). Effects of dimethyl sulfoxide on subunit proteins. Annals of the New York Academy of Sciences 243(1): 38-53.

Jun, J., Xiao-Da, Y. and Kui, W. (2007). Inhibition of cysteine protease papain by metal ions and polysulfide complexes, especially mercuric ion Journal of Chinese Pharmaceutical Sciences 16(1): 1-8.

Kharasch, N. and Thyagarajan, B. (1983). .Structural basis for biological activities of dimethyl sulfoxide Annals of New York Academy of Science 411: 391-402

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

Kulaksiz-Erkmen, G., Can, B., Dalmizrak, O., Ogus, I. H. and Ozer, N. (2012). The effect of glycerol in the storage medium on the inhibitory kinetic

behavior of glutathione reductase. Archives of Biological Science, Belgrade, 64 (1): 239-247.

Levy, H. R., Raineri, R. R. and Nevaldine B. H. (1966). On the structure and catalytic function of mammary glucose 6-phosphate dehydrogenase. Journal of Biological Chemistry 25: 2181-2187.

Means, G. E. and Feeney, R. E. (1971). Chemical Modification of Proteins. Holden-Day, San Francisco, USA., pp. 105-204.

Miroliaei, M. and Nemat-Gorgani, M. (2002). Effect of organic solvents on stability and activity of two related alcohol dehydrogenases: a comparative study. Internationai Journal of Biochemistry and Cell Biology 34(2):169-175.

Narahara, H., Koyama, Y., Yoshida, T., Poonsuk, A., and Taguchi, H. (1984). Control of water content in a solid state culture of *Aspergillus oryzae*. Journal of Fermentation Technology 62: 453-459.

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.

Olsen, S. N., Ramlov, H. and Westh, P. (2007). Effects of osmolytes on hexokinase kinetics combined with macromolecular crowding. Test of the osmolyte compatibility hypothesis towards crowded systems. Comparative Biochemistry and Physiology 148: 339-345.

Ping, H., Atchun, D. and Winslow, S. C. (1995). Effects of dimethyl sulfoxide, glycerol and ethylene glycol on secondary structures of cytochrome c and lysozyme as observed by infrared spectroscopy. Journal of Pharmaceutical Science 84 (4): 387-392.

Rawlings, N. D. and Barrett, A. J. (2004). Introduction: Metallopeptidases and their clans. In: Handbook of Proteolytic Enzymes. Academic Press. pp. 231-267.

Rosenthal, P. J. (1998). Proteases of Malaria Parasites: New Targets for Chemotherapy Emerging Infectious Diseases 4(1): 49-57.

Rosenthal, P. J. (2001). Protease inhibitors. In *Antimalarial Chemotherapy*. Humana Press, Totowa, New Jersey. pp. 325-345.

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

Shelby, L. B. and William, B. J. (1972). Glycerol as an enzyme-stabilizing agent: Effects on aldehyde dehydrogenase. Proceedings of the National Academy of Science, USA 69(9): 2373-2376.

Stephanie J. and Louis M. (1999). Malaria Vaccine Development Status Report· Parasitology and International Programs Branch I and Laboratory of Parasitic Diseases National Institute of Allergy and Infectious Diseases .National Institutes of Health Bethesda, MD, USA. Downdoaded from https://www.nature.com/nm/special_focus/malaria/commentaries/malcom.html, 15th September, 2015.

Tukmachev, V. A., Nedospasova, L. V., Zaslavskii, B. and Rogozhin, S. V. (1979). Effect of sodium dodecyl sulfate on biological membranes. Biofizika 24(1):55-60.

Wang, W. (1999). Instability, stabilization and formulation of liquid protein pharmaceuticals International Journal of Pharmacy 185: 129-188.

World Health Organization (2010). World Malaria report. Available: http://www.who.int/malaria/world_malaria_report_2010/en/. Accessed 6 December 2011

World Health Organization (2017). World Malaria Report 2016: Summary, Geneva: (WHO/HTM/GMP/2017.4). License: CC BY-NC-SA 3.0 IGO.

Yancey, P. H. (2005). Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. Journal of Experimental Biology 208: 2819-2830.

Yin, L. J., Chou, Y. H. and Jiang, S. T. (2013). Purification and characterization of acidic protease from *Aspergillus oryzae* BCRC 30118. Journal of Marine Science and Technology 21(1): 105-110.