International Research Journal of BIOLOGICAL SCIENCES

Volume 01 | Issue 02 | 2019

SciRange PUBLICATIONS
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International Research Journal of Biological Sciences

Research Article

pISSN: 2663-5968, eISSN: 2663-5976

Isolation and Characterisation of Glutathione Peroxidase from Malaria Infected Erythrocytes

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ARTICLE INFORMATION

Received: August 28, 2019

Accepted: October 10, 2019

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ABSTRACT

Glutathione Peroxidase (GPx), a selenium-dependent enzyme, plays a major role in the protection of tissues from the toxic effects of reactive oxygen species including H_2O_2 and aids in the maintenance of the glutathione cycle. The aim of this study was to isolate and characterize glutathione peroxidase from erythrocytes of malaria patients using that of non-malaria patients as control. The characterization was done using the optimum pH, temperature, Km and Vmax. Glutathione peroxidase activity was evaluated in 6 malaria patients and compared with 6 controls (non-malaria patients). Decomposition of H_2O_2 was done using O-dianisidine dye as hydrogen donor and the presence of glutathione peroxidase in the haemolysate was detected by the appearance of light brown oxidized O-dianisidine which was measured at 460 nm. The Km and Vmax of GPx from malaria patients and non-malaria patients were 3.125 mM and 3.125 mM/min and 5.0 mM and12.5 mM/min respectively. The maximum activity of GPx from erythrocytes of malaria patients and non-malaria patients was observed at pHs between 6.0-8.0 and 5.0-7.0, respectively. Enzyme activity of erythrocytes of malaria patients and non-malaria patients showed maximal potential to detoxify H_2O_2 between 30-50 °C and 20-40 °C, respectively. At 80 and 90 °C, both enzymes had completely lost their activity.

Key words: Glutathione, glutathione peroxidase, malaria patients, erythrocytes, plasmodium parasite

INTRODUCTION

Glutathione is a thiol (Gly-Cys-Gly) which serves as cofactor for glutathione peroxidases and is the most abundant low molecular mass intracellular thiol in living cells. The reduced glutathione, GSH is the chief component of glutathione cycle[1] and has a principal role in cell defense mechanisms against harmful effects of oxidants, such as free radicals, pro-oxidants or drugs[2]. NADPH is necessary for the function of glutathione peroxidase[3] and for the generation of GSH from its oxidised form, GSSG and subsequently for the maintenance of intracellular pools of glutathione cycle. The

characteristic nature of glutathione system, enables glutathione to play an essential role in many biochemical and pharmacological reactions[4,5].

In particular, reduced glutathione contributes to the viability of erythrocytes by stabilizing thiols in their cell membrane in haemoglobin and in cellular enzymes[6]. Glutathione maintains normal structure, elasticity and integrity of red blood cells and sustains haemoglobin in ferrous state which is essential for transport of oxygen[7] and hence, the protection of red blood cells from oxidative damage and peroxides[8]. Some of the important roles of glutathione include the inactivation of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) through the formation of glutathione disulphide, the conjugation of glutathione with xenobiotics such as acetaminophen, disulfiram (antabuse) and the subsequent elimination of these xenobiotics from the system as mercapturic acid[9,10]. Red blood cells are the host cells for plasmodium parasite where the parasites oxidize NADPH and diminish the level of reduced glutathione, GSH[11].

Glutathione peroxidase (GPx; EC1.11.1.9), is one of the intracellular antioxidant defense enzymes which remove toxic organic hydroperoxides and H_2O_2 through coupling, thus reducing them to their corresponding alcohol (LOH) or H_2O respectively with oxidation of reduced glutathione (GSH) in the glutathione cycle[12,13]. Glutathione peroxidase (located both in the mitochondria and cytosol), plays an important role in preventing cellular oxidative stress, particularly in the peroxisomes that contain various H_2O_2-producing flavoprotein oxidases, for instance, fatty acid oxidases involved in β-oxidation. The glutathione peroxidase activity in the erythrocytes of various healthy organisms/species and in their diseased states has been characterized, however, little information on the characteristic of glutathione peroxidase from erythrocytes of malaria patients has appeared in literature. This research focused on the isolation and characterization of glutathione peroxidase from red blood cells of malaria and non-malaria patients using parameters like optimum pH, temperature, Km and Vmax.

MATERIALS AND METHODS

Study subjects: The study was carried out on 6 malaria and 6 non-malaria patients attending the laboratory at the University of Cape Coast Hospital in the Central Region of Ghana. The subjects were selected without any known bias. All patients were in a stable clinical condition at the time of testing. Pre-study evaluations included a complete medical history, physical examination and a urine and blood drug screen for alcohol and drug use. Although control subjects were not on hospital admission, they were asked to adhere to a low-monoamine, alcohol-free and caffeine-restricted diet for 1 week preceding the blood draw. All persons gave their informed consent for the use of their blood for the study.

Preparation of erythrocyte lysates and enzyme extraction: A volume of five milliliters of whole blood drawn from the cubital median vein of the subjects into EDTA tubes were used. The blood samples were centrifuged at 4000 x g for 7 min at 4°C and the overlapping plasma and the white layer (buff coat) were carefully taken with a pipette into an Eppendorf tube. Sediments of erythrocytes were rinsed 3 times with 10 mL of isotonic (0.9% g mL^{-1}) NaCl-solution and the tubes were inverted several times and centrifuged at 4000 x g for 7 min. The supernatant was removed and the procedure repeated two more times. The erythrocytes were haemolysed with distilled water and centrifuged at 4,000 x g for 10 min at 4°C. The supernatants were collected into ice chambers and stored at 0°C prior to their use.

Enzyme assays: The reaction mixture consisted of 50 µL crude enzyme extract, 0.5 mL of 1.5% H_2O_2, 2.4 mL of 0.1 M sodium phosphate buffer pH 7.0 and 50 µL of 1% o-dianisidine in 20 mL of 70% methanol in a total volume of 3 mL. The reaction was stopped by the addition of 50 µL Conc. H_3PO_4 after 5 min. The molar extinction coefficient of O-dianisidine of 11.3 mM^{-1} cm^{-1} at 460 nm was used to determine glutathione peroxidase activity. The activity was calculated from the Eq. 1 as the decrease in absorbance at 460 nm (ΔA_{460} nm min^{-1}) was directly proportional to the GPx activity.

$$\text{Activity}(\text{M/min}) = \left(\frac{\Delta A \times d \times V(mL)}{\varepsilon \times v \times t}\right) \quad (1)$$

Where:

- ΔA = Change in Absorbance between $A_{460\,nm}$ (Start) – $A_{460\,nm}$ (Stop)/min
- d = Dilution factor of original sample
- V (mL) = Reaction volume
- v = Volume of the tested sample
- t = Reaction time
- ε = 11. 3 mM^{-1} cm^{-1}; Molar extinction coefficient of o-dianisidine at 460 nm

Protein concentration estimation: Protein content in each extract was estimated using the Biuret method. An amount of 3 mL of Biuret reagent was added to each test tube with 2 mL of the extract. The absorbance of the developed purple colour was taken at 595 nm. A blank was also run parallel by replacing enzyme extract with 2 mL of distilled water. Protein concentration in the sample was estimated from the standard curve of Bovine Serum Albumin (BSA).

Effect of pH: Several tubes containing 0.1 M sodium phosphate buffer with different pH (2.0 to 10.0) and crude enzyme were incubated at 25°C for 5 min. After which GPx activity was determined for the contents of each test tube as described above.

Effect of temperature: Several tubes containing crude enzyme extract and 0.1 M sodium phosphate buffer at pH of 7 were incubated at different temperatures (20-90°C) for 5 min. Each reaction was stopped and enzyme activity measured as described above.

Enzyme kinetics determination: GPx activity in the crude enzyme extract was measured using H_2O_2 as substrate at varying concentrations (130-650 mM) at 460 nm. Kinetic constants, V_{max} and K_M values were determined according to the method of Lineweaver and Burk[14].

RESULTS

Protein content of crude enzyme extracts of malaria and non-malaria patients: The protein content of crude GPx extracts from erythrocytes of malaria infected patients was significantly higher than that of non-malaria patients as shown in Table 1.

Optimum pH: The optimal pH for GPx from the erythrocytes of malaria and non-malaria patients was found to be 7.0 and 6.0, respectively as depicted in Fig. 1.

Optimum temperature: The highest GPx activity occurred at 40°C for malaria patients and 30°C for non-malaria patients (Fig. 2).

Kinetic parameters: From the Line weaver-Burk plot as shown, Km and Vmax values of GPx enzyme for malaria patients and non-malaria patients were found to be 3.125 mM (3.125×10^3 μM) H_2O_2, 3.125 mM min^{-1} and 5.0 mM (5.0×10^3 μM) H_2O_2 and 12.5 mM min^{-1}, respectively as shown in Fig. 3.

Table 1: Protein content of crude enzyme extract from erythrocytes of malaria and non-malaria patients

Sample	Protein content (mg mL^{-1})
Malaria patients	170.5±2.78
Non-malaria patients	51.5±3.04

Values represent the mean of three readings±standard deviation of the mean

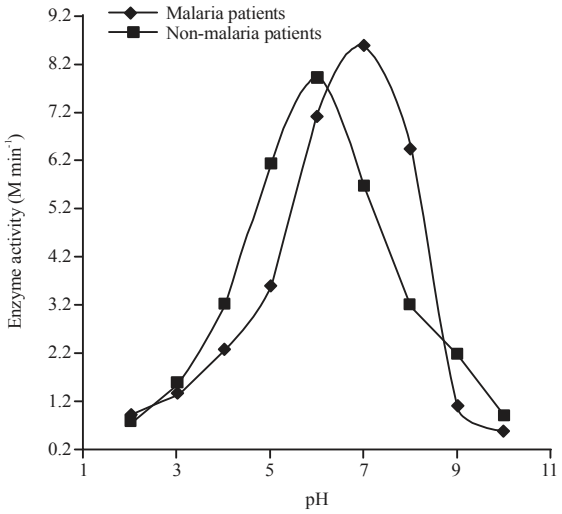

Fig. 1: Effect of pH on the activity of glutathione peroxidase from erythrocytes of malaria and non-malaria patients

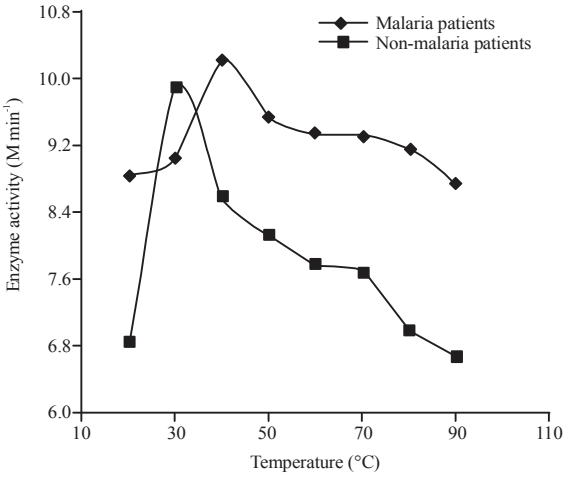

Fig. 2: Effect of temperature on glutathione peroxidase activity of erythrocytes from malaria and non-malaria patients

DISCUSSION

Friedman *et al.*[15] showed by means of spectrophotometric methods that higher levels of methemoglobin occurred during malaria infection. This is an indication that malaria infected RBC is under oxidative stress as stated by Golenser and Chevion[16]. The high protein content of glutathione peroxidase, GPx from erythrocytes of malaria patients may be

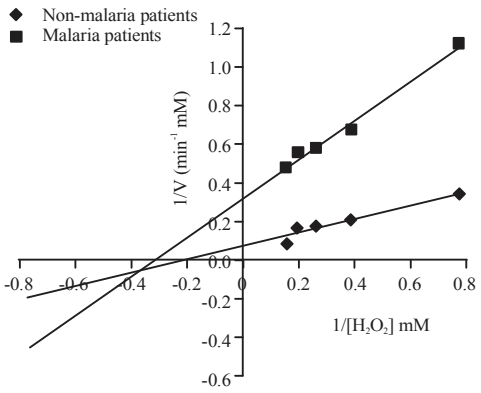

Fig. 3: Lineweaver-Burk plot of glutathione peroxidase from malaria and non-malaria patients indicating their Km and Vmax values

as a result of metabolic activities mediated by malaria parasites upon haemoglobin digestion during the erythrocytic stage[17]. This condition might have produced the high amount of proteins such as methemoglobins and haemoproteins[6,18,19]. Glutathione peroxidase of malaria patients showed activity over a broad optimum pH from 6.0 to 8.0. Maximum enzyme activity of non-malaria patients was observed at pH values between 5.0 and 7.0. Increase in pH results in the disruption of hydrogen bond and charged groups formed between the heme components and amino acid residues in the active site of the enzyme thereby inactivating it. GPx from non-malaria patients exhibited high activity at pH 6.0 close to physiological pH whereas GPx from malaria patients exhibited high activity at neutral pH 7.0. However, the activity of GPx obtained from non-malaria patients was different from the results published by Awasthi *et al.*[20] which had an optimal activity at pH 8.5 for the enzyme from normal human erythrocytes using tert-butyl hydroperoxide as substrate.

Maximum GPx activity for malaria patients was obtained at 40°C whereas maximum GPx activity for non-malaria patients was at 30°C. Optimal temperature of 40°C can be regarded as a steady point in that at extreme conditions such as fever during malaria where the temperature can rise above the normal temperature 37°C, the enzyme can work to reduce erythrocytic oxidative stress from hydrogen peroxide. The enzyme showed an increase in activity as temperature increases from 20 to 40°C after which a decrease in activity was observed for GPx from non-malaria patients. Similarly, the pattern in Fig. 2 for the GPx from malaria patients showed the maximal potential to detoxify hydrogen peroxide occurred at 30°C. Above 50°C, the rate of thermal denaturation and inactivation was faster which made both enzymes lose their conformational structures of the active site and hence activity dropped rapidly. At 80 and 90°C, GPx from both sources have completely lost their activity as most hydrogen bonding and disulphide bridges which maintain the shape of the active site are broken and hence, enzyme-substrate complexes were no longer formed.

The affinity of GPx towards its substrate was determined at optimum pH and temperature with different H_2O_2 concentrations using the Lineweaver-Burk plots (Fig. 3). The kinetic parameters K_M and V_{max} values of GPx from malaria patients were determined to be 3.125×10^3 μM and 3.125 mM min^{-1} whereas that from non-malaria patients were found to be 5.0×10^3 μM and 12.5 mM min^{-1}. Awasthi *et al.*[20] found the K_M value of glutathione peroxidase from normal human erythrocytes to be 4.1×10^3 μM (4.1×10^{-3}M) which was higher than K_M of GPx from malaria patients and lower than K_M value of GPx from non-malaria patients in this study, the disparity might be as a result of crude enzyme used in this present work.

The lower K_M of GPx from erythrocytes of malaria patients indicated its high affinity for H_2O_2 than GPx in normal erythrocytes; this makes it more effective at protecting RBC from oxidative damage. Additionally, Atamna *et al.*[17] observed that erythrocytes infected with *P. falciparum* produced OH^- radicals and H_2O_2 about twice as much compared to normal erythrocytes, hence, the high activity of GPx in malaria-infected erythrocytes can relieve the erythrocytes from the high oxidative burden as compared to GPx of non-malaria-infected erythrocytes. Therefore, it is of no surprise that the parasites live in pro-oxidants environment that contains oxygen and iron which are the key prerequisite for the generation of ROS via Fenton reaction and Haber-Weiss reaction. A research by Gaetani *et al.*[21] suggested that glutathione peroxidase was effective in detoxification of H_2O_2 at high levels in normal human erythrocytes. The unpurified enzyme extracts might have received interferences from other intracellular enzymes such as catalases from the host and thioredoxin peroxidases from the malaria parasites as reported by Nickel *et al.*[22], these enzymes use hydrogen peroxide as their optimal substrate.

CONCLUSION

Crude extract of GPx from malaria-infected erythrocytes gave the highest protein content, recorded optimum activities at higher pH and temperatures values and recorded a lower K_M value. This indicated GPx from malaria-infected erythrocytes has a higher affinity for H_2O_2 than GPx from non-malaria-infected erythrocytes. Further studies are required if possible

using pure form GPx to get a better understanding of the functioning of GPx in malaria-infected erythrocytes.

ACKNOWLEDGMENT

The authors are very grateful to the staff of the Department of Biochemistry Laboratory, University of Cape Coast for their technical support. We are also grateful to the staff of the University of Cape Coast Hospital laboratory for providing us with the blood samples for this study.

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