Electrophoretic studies on Glutathione-S-Transferase and its partial purification from *P.berghei* infected rodent tissue.

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ABSTRACT

Malaria is an infectious disease affecting millions of people around the globe. It is caused by intracellular protozoan parasite *Plasmodium* sp. Among the various biochemical strategies to combat the parasite, glutathione metabolism is of prime importance. The antioxidant enzyme such as Glutathione-S-Transferase (GST) protects cells from oxidative stress. During the present study GST activity was estimated electrophoretically in normal and parasitized fractions of blood of White Swiss Mice *Mus musculus*. The *P.berghei* infected blood showed broader achromatic zones than normal blood indicating higher GST activity than the normal blood. At the same time partial purification of GST was done using Ammonium Sulphate precipitation and Sephadex G-50.

Key Words: Malaria, Glutathione, Glutathione-S-Transferase, Sephadex G-50

INTRODUCTION

Malaria is a complex mosquito borne disease caused by protozoan parasite *Plasmodium* sp. It affects millions of people around the globe especially in sub-tropical and tropical regions of the world. Every year thousands of people lose their lives worldwide and millions get infected. There were an estimated 219 million cases and 4, 35,000 deaths in the year 2017 **(WHO: World Malaria Report, 2018).** The biochemistry of this protozoan parasite, *Plasmodium* and host tissues is of great importance and has been worked out extensively. Metabolic pathways of the parasites have been identified as potential drug targets. Glutathione metabolism plays important role in antioxidant defense in erythrocytes parasitized by *P.falciparum* (Atamana and Ginsberg, 1997).Reduced glutathione is considered to be the chief component of glutathione cycle (Beutler and Dale, 1989; Meister, 1974). Its principal role in defense of cell against free radicals has been described (Sies, 1986). Rapport *et al*. (1968) has reported oxidized glutathione (GSSG) to be toxic to the cells. Reduced Glutathione (GSH) is oxidized to GSSG during elimination of H_2O_2 and lipid peroxides by activity of glutathione peroxidase.

The glutathione transferases (GSTs; E.C. 2.5.1.18) are group of multifunctional enzymes, which plays important role in detoxification of xenobiotics (Jakoby,1978).GSTS are involved in various catalytic functions, viz. conjugation, peroxide reduction and isomerization whereas non-catalytic functions include binding transport and storage. The conjugation of –SH group of GSH to electrophilic center of second substrate like CDNB forms thiother (Chasseaud,1979; Jakoby and Habig,1980).

Ricci *et al.* (1984) have described GST activity on polyacrylamide gels. His method was based on reduction of nitro blue tetrazolium salt by glutathione. He has shown that blue formazan appear on the gel accept GST area and activity is detectable with 0.005 unit of enzyme.

In the present study the activity of GST was determined using polyacrylamide gel electrophoresis and enzyme was partially purified from the *P.berghei* infected spleen tissue by ammonium sulphate precipitation and Sephadex G-50. The *P.berghei* was research model because of its similarity in structure, physiology and life cycle with human malarial parasites along with similar molecular basis of drug sensitivity and resistance and easy *in vitro* and *in vivo* study.

MATERIAL AND METHODS

MAINTENANCE OF THE PARASITE

Plasmodium berghei (NK-65 strain), a rodent malaria parasite, was maintained in white Swiss mice, *Mus musculus* (Balb/C) strain as per guidelines of Institutional Animal Ethics Committee (IAEC) of Himachal Pradesh University, Shimla. The parasite was maintained by passing the infection from infected to normal mice intraperitoneally (i.p.) with $1x10^5$ *P. berghei*-infected erythrocytes in citrate saline (Banyal *et al*., 1991).

COLLECTION OF BLOOD

Normal and infected mice were anesthetized with diethyl ether. The blood was collected in citrate saline (2:1) by jugular vein incision and stored at -20^0 C (Ultra low freezer U 41085) till further use.

COLLECTION OF NORMAL AND INFECTED ERYTHROCYTES THEIR FRACTIONS AND CELL FREE PARASITE

The citrated blood was centrifuged at 2000 rpm for 10 minutes at 4^0 C (Plastocrafts, supervin R-V/Fm). Plasma was separated and stored at -20^0 C till further use. The pellet was suspended in equal volume of phosphate buffer saline (0.01 M, pH 7.2). Further it was loaded on CF-11 cellulose (whatmann) column (20 cms x1.5cms). The eluted leucocyte free blood was centrifuged at 2000 rpm for 10 minutes at 4^0 C. supernatant was discarded and the pellet was washed thrice in phosphate buffer saline (0.01M, pH-7.2) and suspended in equal volume of saponin (0.2% w/v) in phosphate buffer saline (pH-7.2). The suspension was incubated at 4^0C for 30 minutes with intermittent shaking after every 5 minutes. It was then centrifuged at 10000 rpm for 20 minutes at 4^0 C. Hemolysate was aspirated and stored at -20^0C till further study. Erythrocytes membranes overlying the cell free parasite were removed and washed separately in phosphate buffer saline (0.01M, pH-7.2) by centrifugation at 10000 rpm for 20 minutes. The recovered pellet was stored at -20^0 C till further use.

In infected erythrocyte samples the lowermost layer was of cell-free parasite. This pellet was resuspended in phosphate buffer saline (0.01M, pH-7.2) and centrifuged twice at 10000 rpm for 20 minutes at4^0 C and the pellet was stored at -20^0 C till further study.

POLYACRYLAMIDE GEL ELECTROPHORESIS

Non reducing, non denaturating PAGE was performed by modified method of Laemmli (1970) in mini vertical slab gel electrophoresis apparatus (Genei, Bangalore) using 5% stacking and 10% separating gels. Gels were pre run at 100V for 1 hour. The samples preincubated with DTT (2.0) were loaded on the gel.

SPECIFIC ENZYME STAINING OF GLUTATHIONE TRANSFERASE

After electrophoresis, gels were placed in potassium phosphate buffer (0.1 M, pH 6.5) containing GSH (4.5mM), CDNB (1.0mM) and nitro blue tetrazolium chloride (NBT,1.0 mM). Gels were incubated at 37 0C for 10 minutes. When the non-enzymatic areas on the gels turned blue the reaction was stopped by washing gels in water. The stained gels were photographed and stored in NaCl (1.0M).

PARTIAL PURIFICATION OF ENZYME BY AMMONIUM SULPHATE PRECIPITATION AND SEPHADEX G-50

10% homogenate of spleen was prepared and subjected for ammonium sulphate 355 (w/v) precipitation for two hours.the clear precipitate was obtained by centrifugation at 10000 rpm for 30 minutes.the precipitates were resuspended in phosphate buffer (0.02M, pH 7.2).The enzyme was eluted through Sephadex G-50 column (8 cmsX1.7 cms). The eluted fractions were assayed for enzyme activity by method of Habig *et al*, 1974.

PROTEIN ESTIMATION

Protein concentration was determined by slightly modified method of Lowry *et al.* (1951) using bovine serum albumen as standard.

RESULTS

SPECIFIC ENZYME STAINING OF GLUTATHIONE TRANSFERASE

GST activity in total erythrocytes infected with *P.berghei* and in normal erythrocytes was observed as an achromatic zone with higher intensity in the *P.berghei* infected erythrocytes than achromatic zone of normal erythrocytes. A similar achromatic zone was observed in cell-free *P.berghei.* (Fig.1)

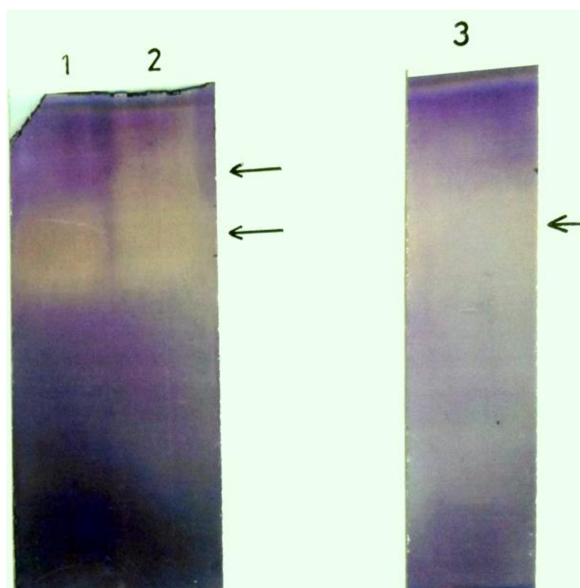

Fig-1: Native gel stained for glutathione transferase activity
(**Lane 1**-Normal erythrocytes, **Lane2**- Infected Erythrocytes, **Lane 3**-Cell-free Parasite)

PARTIAL PURIFICATION OF GLUTATHIONE TRANSFERASE

GST was partially purified from spleen of *P.berghei* –infected mice using two step purification. In first step GST was precipitated at 35% $(NH_4)_2\ SO_2$ (final concentration) and in second step the gel filtration chromatography using (Sephadex G-50) was employed. The combination of these two steps resulted in 3.3 fold purification of enzyme. (Table-1)

Table 1: Partial purification of glutathione transferase from *P.berghei* infected spleen by $(NH_4)_2SO_4$ and Sephadex G-50.

GLUTATHIONE TRANSFERASE

Steps	Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Sp.Activity (U/mg)	Purification (× Fold)
Crude	10	0.238	8.27	0.028	1.0
$(NH_4)_2SO_4$ Fractionation 35%	3	0.483	7.518	0.064	2.3
Sephadex (G-50) Fraction 1-5	5	0.351	4.887	0.071	2.5
Fraction-2	1	0.937	10.057	0.093	3.3

DISCUSSION

In the present study, GST activity was estimated electrophoretically in *P.berghei* infected erythrocytes and normal erythrocytes in which achromatic zones were observed with higher intensity in case of *P.berghei*-infected erythrocytes indicating higher activity of GST in *P.berghei* infected erythrocytes. Similar achromatic zones were observed in cell-free *P.berghei* showing the role of GST.

It was observed that GSH caused reduction of nitroblue tetrazolium chloride (NBT) when incubated in the presence of phenazine methosulphate which resulted blue formazan formation.However, when GSH was incubated with glutathione transferase and 1-chloro2, 4-dinitrobenzene (CDNB), the extent of NBT reduction was depressed owing to enzymatic conjugation of sulfhydryl function, which resulted in formation of achromatic zone in glutathione transferase area on the gel. Our results were similar to the results of Ricci *et al.*(1984).They have observed such GST specific achromatic zones on the gel using tissue extracts of heart and placenta of humans and bovine kidney.

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