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# THE INHIBITORY EFFECTS OF SOME PESTICIDES ON HUMAN ERYTHROCYTE GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY (*IN VITRO*)

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## ABSTRACT

Human erythrocyte glucose-6-phosphate dehydrogenase was purified to apparent homogeneity by salting out with ammonium sulfate and applying one chromatographic step with the commercially available resin 2',5'-ADP Sepharose 4B. The enzyme, having a specific activity of 70.7 U/mg protein, was purified 7069 fold with an overall enzyme yield of 33.6%. The purity and molecular mass of the protein was confirmed with SDS-PAGE. The purified glucose-6-phosphate dehydrogenase was effectively active on glucose-6-phosphate and $NADP^+$ with *K*m values of 0.22 and 0.14 mM and *V*max values of 1.94 and 2.76 U/mg, respectively. The *in vitro* effects of commonly used pesticides Glyphosate™ (N-(Phosphonomethyl) glycine) and 2,4-D™ (2,4-Dichlorophenoxyacetic acid) were determined on the purified glucose-6-phosphate dehydrogenase. Both pesticides were effective inhibitors on the activity with $IC_{50}$ values of 32.35 and 38.34 mM, respectively. The interaction kinetics of Glyphosate™ and 2,4-D™ with the purified enzyme indicated uncompetitive and competitive inhibition patterns with *K*i values of 13.45 and 8.45 mM, respectively.

**KEYWORDS:** Glucose-6-phosphate dehydrogenase, purification, pesticide, inhibition.

## 1. INTRODUCTION

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49; G6PD) is the first and key enzyme of the pentose phosphate metabolic pathway, catalysing the conversion of glucose-6phosphate to 6-phosphogluconate in the presence of $NADP^+$. The fundamental physiological function of G6PD is the production of NADPH and ribose-5-phosphate, which are essential for reductive biosynthetic metabolisms and nucleic acid synthesis, respectively [1,2]. The major role of NADPH in erythrocytes is the regeneration of reduced glutathione, which prevents hemoglobin denaturation, preserves the integrity of red blood cells' membrane sulfydryl groups, and detoxifies hydrogen peroxide and oxygen radicals in and on the red blood cells. Moreover, NADPH is also functional in cell membrane protection and cell de-toxification from xenobiotics, through the glutathione re-ductase-peroxidase system and mixed-function oxidases [3-6].

The amount and variety of pesticides used have increased tremendously in recent years. This increase has caused a positive effect on crop production, however, certain pesticides, their residues, metabolites and/or contaminants have created many unforeseen adverse effects on the environment. Under some conditions, pesticides may be present in very low concentrations which have no immediate detectable effect. These small amounts of chemicals can cause sublethal (chronic) damage to organisms and this is more insidious and difficult to define than acute toxicity. Sublethal effects may be further enhanced by persistent pesticides which are accumulated in the organisms and magnified in the food chain [7-10]. Thus, the aim of this present study was mainly to purify G6PD from human erythrocytes and to investigate the *in vitro* inhibitory effects of more commonly used pesticides on this purified enzyme.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals

Glucose-6-phosphate, $NADP^+$, NADPH, protein assay reagents and chemicals for electrophoresis were obtained from Sigma Chem. 2',5'-ADP Sepharose 4B was purchased from Pharmacia. Glyphosate™ (N-(phosphono-

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methyl) glycine) and 2,4-D™ (2,4-Dichlorophenoxyacetic acid) were provided from authorized local pesticide shop. All other chemicals were of the best available grade.

#### 2.2. Preparation of the hemolysate

Appropriate amount of fresh blood from human was collected in the tubes containing anticoagulant EDTA and sample tubes were centrifuged at 3000g for 10 min. The plasma and leukocyte coat were removed by pipette. The package of erythrocytes was washed three times with isotonic KCl solution, then hemolysed with 5 volumes of ice-cold distilled water containing 2.5 mM EDTA and 0.7 mM β-ME. The hemolysate was centrifuged at 15000 g for 20 min at 4 $^0$C to remove the ghost and intact cells [11].

#### 2.3. Ammonium sulfate fractionation and dialysis

The hemolysate was treated with solid ammonium sulfate to obtain the 30-70% fraction after centrifuging at 15000g for 30 min. The precipitate was dissolved in 50 mM potassium phosphate buffer, pH 7.0, and then dialised at 4 $^0$C in 50 mM potassium acetate/50 mM potassium phosphate buffer, pH 7.0 [12].

#### 2.4. 2',5'-ADP Sepharose 4B affinity chromatography

All purification steps were performed at 4°C unless otherwise stated. Two grams of dry 2',5'-ADP Sepharose 4B for 10 ml of bed volume was several times washed, to remove impurities and conditioning the gel, in 300 ml of distilled water. Following removal of the foreign bodies and air bubbles in the gel, it was resuspended in 0.1 M potassium acetate + 0.1 M potassium phosphate buffer, pH 6.0, at a ratio of 3 gel to1 buffer, and packed in a column (1x10 cm). After setting of the gel, the column was pre-equilibrated with the same buffer prior to loading the enzyme solution. The dialised enzyme solution was loaded on the column and then was sequentially washed with 25 ml of 0.1 M potassium acetate + 0.1 M potassium phosphate buffer, pH 6.0, and 25 ml of 0.1 M potassium acetate + 0.1 M potassium phosphate buffer, pH 7.85. The washing with 0.1 M potassium chloride + 0.1 M potassium phosphate buffer, pH 7.85 was continued until the absorbance becomes almost zero at 280 nm. The enzyme was eluted with a solution of 80 mM potassium phosphate + 80 mM potassium chloride + 0.5 mM $NADP^+$ + 10 mM EDTA (pH 7.85) at a flow rate of 20 ml/h, and 1 ml fractions were collected. The proteins containing the highest glucose-6-phosphate dehydrogenase activity were combined and used as purified enzyme for subsequent studies after confirming homogeneity by gel electrophoresis [2, 12].

#### 2.5. Glucose-6-phosphate dehydrogenase assay and protein determination

The enzyme activity was routinely measured by Beutler's method [11]. One enzyme unit was defined as the enzyme amount that reduces 1μmole $NADP^+$ per min under the assay conditions.

Quantitative protein determination was performed [13] using bovine serum albumin (BSA) as a standard.

#### 2.6. SDS Polyacrylamide gel electrophoresis (SDS-PAGE):

Protein samples were fractionated on 12% SDS-PAGE gels [14] using a Minigel system (Bio-Rad Laboratories, USA). Gels were fixed, stained with Coomassie brilliant blue R-250 (Sigma), and destained using standard methods to detect protein bands.

#### 2.7. Determination of kinetic parameters and i*n vitro* inhibition studies

Various final concentrations of G6P were used to estimate the kinetic parameters *K*m and *V*max. Inhibition experiments were performed using G6P as substrate and different final concentrations of commonly used pesticides Glyphosate™ and 2,4-D™ as inhibitors. The double reciprocal Lineweaver–Burk plot was used to calculate the parameters. Activity % values of glucose-6-phosphate dehydrogenase for five different concentrations of each possible inhibitor were determined by regression analysis. Glucose-6-phosphate dehydrogenase activity without a possible inhibitor was accepted as 100% activity. The inhibitor concentration reduces the enzymatic activity by 50% ($IC_{50}$ values) were determined from the graphs.

## 3. RESULTS AND DISCUSSION

Human erythrocyte glucose-6-phosphate dehydrogenase was purified to apparent homogeneity by salting out with ammonium sulfate and subsequentially using 2',5'-ADP Sepharose 4B affinity chromatography. Upon fractionation of the G6PD active fractions with ammonium sulfate, 68% of the activity was obtained in the fraction saturated with 30-70% ammonium sulfate. This step removed the greater part of the contaminants and considerably decreased total protein amount. The precipitate with G6PD activity was dissolved and applied on 2',5'-ADP Sepharose 4B affinity chromatography column. Figure 1 shows the typical elution pattern of the enzyme activity on this affinity column. The enzyme activity and total protein concentrations were determined from all fractions collected. The fractions with the highest G6PD activity and the relatively lower protein contents were pooled. In order to reduce the number of the purification steps, this affinity chromatography purified the enzyme from remaining contaminants to apparent homogeneity, retaining 50% of the activity from the previous step. The G6PD was purified 7069 fold with an overall enzyme yield of 33.6% and a specific activity of 70.7 U/mg (Table 1).

SDS-PAGE analysis of the purified enzyme showed the presence of a single band with an apparent molecular mass of ca. 59 kDa, when stained with Coomassie brilliant blue (Figure 2).

The reaction kinetics of the purified G6PD were determined from Lineweaver–Burk plots using glucose-6-phosphate and $NADP^+$ as substrates with *K*m values of

0.22 and 0.14 mM and *V*max values of 1.94 and 2.76 U/mg, respectively. (Table 2). Affinity of the enzyme for $NADP^+$ was considerably higher than for glucose-6-phosphate. The higher G6PD affinity for $NADP^+$ have also been reported [15]. The inhibition kinetic experiments of the enzyme were performed using glucose-6-phosphate as substrate, while commonly used pesticides Glyphosate™ and 2,4-D™ as inhibitors. Table 3 shows that the enzyme was uncompetitively and competitively inhibited by Glyphosate™ and

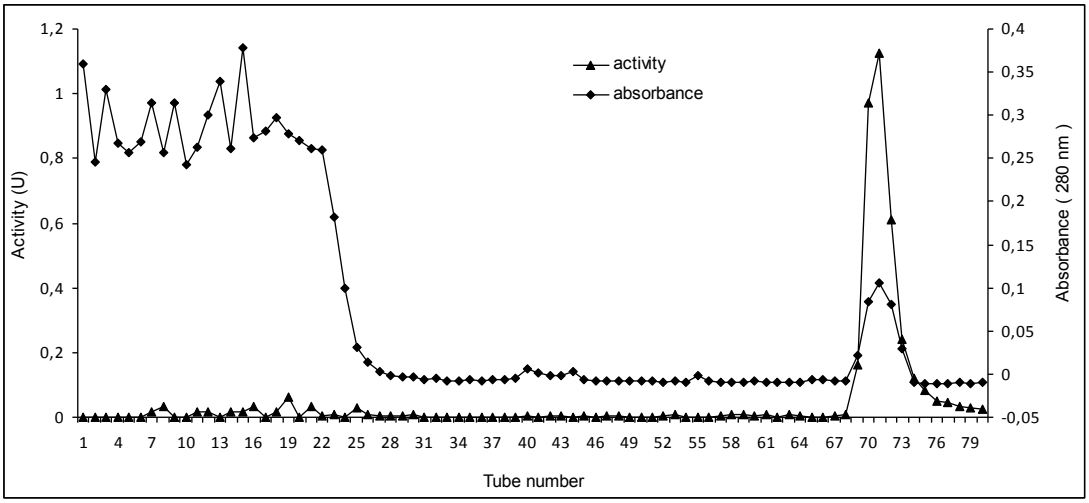


**FIGURE 1 - Purification of human erythrocyte glucose-6-phosphate dehydrogenase by affinity chromatography. The enzyme activity and total protein concentrations were determined, from all fractions collected, as described in Methods section. The enzyme activity was expressed as the enzyme amount that reduces 1µmole $NADP^+$ per min under the assay conditions.**

**TABLE 1 - Purification of human erythrocyte glucose-6-phosphate dehydrogenase**

| Step | Total protein (mg) | Specific activity (U/mg) | Yield (%) | Purification factor (fold) |
|---|---|---|---|---|
| Hemolysate | 732,27 | 0,01 | 100 | - |
| Ammonium sulfate | 391,12 | 0,012 | 68,18 | 1,2 |
| 2'5'-ADP Sepharose 4B affinity chromatography | 0,035 | 70.69 | 33.65 | 7068,9 |

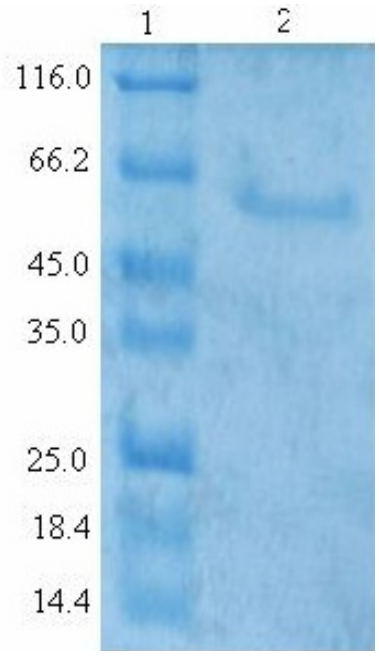


**FIGURE 2 - SDS-PAGE of purified human erythrocyte glucose-6-phosphate dehydrogenase. The enzyme was electrophoresed at pH 8.3 on a 12% polyacrylamide gel and stained with Coomassie brilliant blue R-250. Lanes: 1, molecular weight standards (*β*-galactosidase,116 kDa; bovine serum albumin, 66.2 kDa; egg albumin, 45 kDa; lactate dehyrogenase, 35 kDa; Rease Bsp981 (*E.coli*), 25 kDa; *β*-lactoglobulin, 18.4 kDa; Lysozyme, 14.4 kDa; 2, purified human erythrocyte glucose-6-phosphate dehydrogenase).**

**TABLE 2 - Kinetic parameters of human erythrocyte glucose-6-phosphate dehydrogenase**

| Substrate | *K*m (mM) | *V*max (U/mg) |
|---|---|---|
| G6P | 0,22 | 1,94 |
| $NADP^+$ | 0,14 | 2,76 |

**TABLE 3 - Inhibitory effects of Glyphosate ™ and 2,4-D ™ on human erythrocyte glucose-6-phosphate dehydrogenase**

| Pesticide | Inhibition type | *K*i (mM) | $IC_{50}$ (mM) |
|---|---|---|---|
| Glyphosate™ | Uncompetitive | 13,45 | 32,35 |
| 2,4-D™ | Competitive | 8,45 | 38,34 |

2,4-D™, respectively. Glyphosate™ which is a widely used herbicide in agricultural fields, was the most effective inhibitor of the enzymatic activity with *K*i value of 13.45 mM (Figure 3) and $IC_{50}$ of 32.35 mM (Figure 4). As shown in

Figures 5 and 6 the obtained *K*i and $IC_{50}$ values of 2,4-D™ were 8.45 and 38.34 mM, respectively.

The essential role of G6PD activity in overall metabolism of organisms has been well known for several years. Glutathione is used by antioxidant defense mechanisms and its production depends on NADPH to be synthesized in the pentose phosphate metabolic pathway mainly with G6PD catalytic activity. Thus, G6PD has been considered as an antioxidant enzyme [16]. Many chemicals at relatively low dosages affect the metabolism of biota by altering normal enzyme activity. In some of these interactions there is high reactivity involving a high degree effect

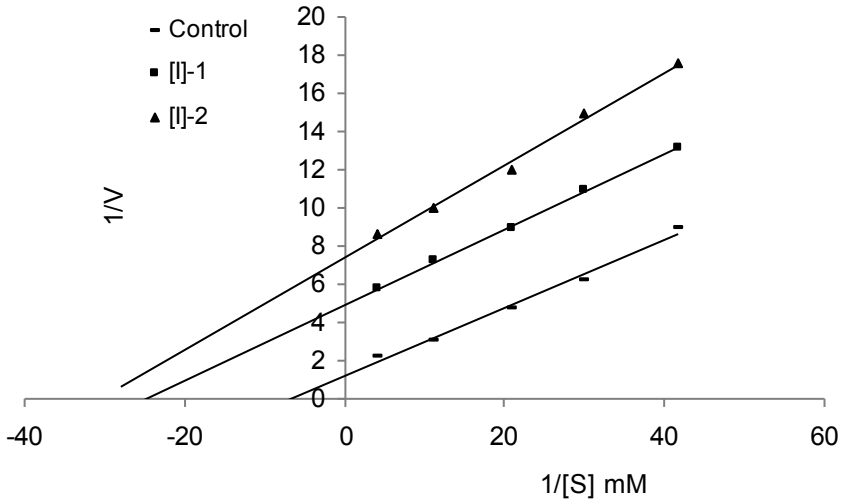


**FIGURE 3 - Lineweaver-Burk graph of G6PD in different glucose-6-phosphate and Glyphosate™ concentrations.**

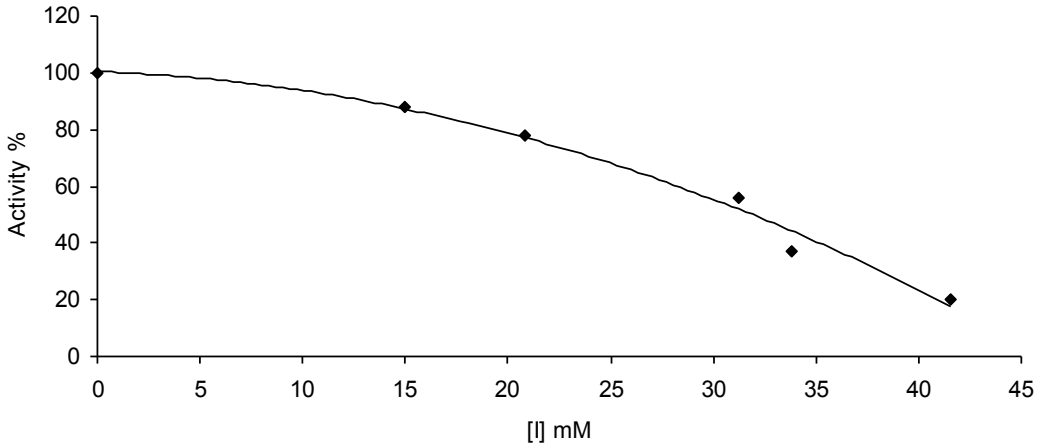


**FIGURE 4 - Activity % curve of G6PD in different Glyphosate™ concentrations.**

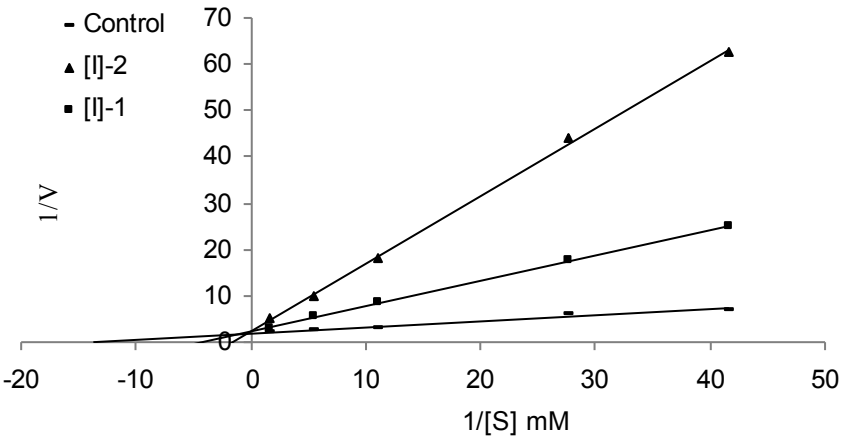


**FIGURE 5 - Lineweaver-Burk graph of G6PD in different glucose-6-phosphate and 2,4-D™ concentrations.**

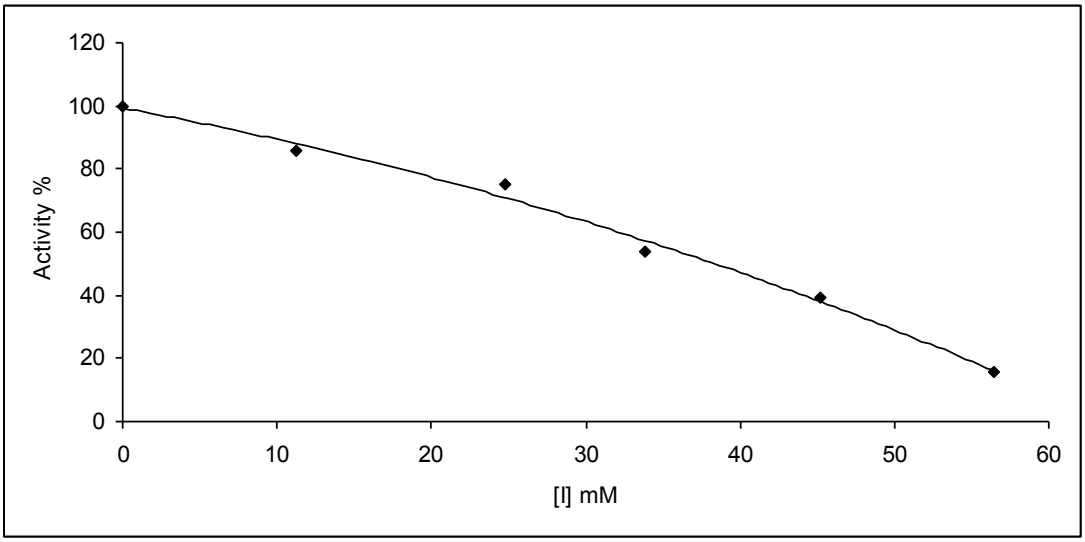


**FIGURE 6 - Activity % curve of G6PD in different 2,4-D™ concentrations.**

on the whole animal or plant. On the other hand, many chemicals affect the activity of many enzymes only to a moderate degree and it is presumed that the ultimate debilitating effect on the whole organism develops from a variety of nonspecific biochemical functions [17]. Therefore, Glyphosate™ and 2,4-D™ were chosen to investigate their effects on the G6PD activity. Glyphosate™ has been reported as the most used herbicide in the USA with 5-8 million pounds [2.5 to 4 kilotonnes] every year on lawns and yards and 85–90 million pounds annually in US agriculture [18]. 2,4-D™ is also the most widely used herbicide in the world, and the third most commonly used in North America [19]. Laboratory toxicology studies suggest that other ingredients combined with Glyphosate ™ may have greater toxicity than Glyphosate ™ alone. For example, a study comparing Glyphosate™ and Roundup™ which is the brand name of a systemic herbicide and contains the active ingredient Glyphosate™, found that Roundup™ had a greater effect on aromatase than Glyphosate™ alone [20]. Another study has shown that Roundup™ formulations and metabolic products cause the death of human embryonic, placental, and umbilical cells *in vitro* even at low concentrations. The effects are not proportional to Glyphosate™ concentrations but dependent on the nature of the adjuvants used in the formulation [21]. Also it has been reported that 2,4-D™ causes cancer in humans and it's exposure substantially increased the risk of Non-Hodgkin's lymphoma and amyotrophic lateral sclerosis [22-24]. Since the strong inhibitory concentrations of these pesticides have been found rather low, both have negative effects on the metabolism through G6PD activity.

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