

# The Effect of Safranin on Glutathione Reductase and Glucose 6-Phosphate Dehydrogenase Enzymes

## Glutasyon Redüktaz ve Glukoz 6-Fosfat Dehidrogenaz Enzimleri Üzerine Safraninin Etkisi

Research Article / Araştırma Makalesi

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### ABSTRACT

The aim of this study was to assess inhibitory effect of safranin on human erythrocyte glutathione reductase (GR) and glucose 6-phosphate dehydrogenase (G6PD). For this purpose, human erythrocyte glutathione reductase was purified 2175-fold (31.35% yield) using 2',5'-ADP Sepharose 4B affinity gel and Sephadex G-200 gel filtration chromatography and erythrocyte glucose 6-phosphate dehydrogenase was purified 8681.3-fold (24.77% yield) using 2',5'-ADP Sepharose 4B affinity gel.

Safranin exhibited inhibitory effects on GR and G6PD enzymes *in vitro*. The IC<sub>50</sub> values of safranin were 1.15 mM for GR and 3.22 mM for G6PD, respectively, and the K<sub>i</sub> constants were 2.63 ± 0.13 and 4.53 ± 0.17 mM, respectively. Safranin displayed noncompetitive inhibition for GR and G6PD.

### Key Words

Safranin; glutathione reductase; glucose 6-phosphate dehydrogenase.

### ÖZET

Bu çalışmanın amacı safraninin insan eritrosit glutasyon redüktaz (GR) ve glukoz 6-fosfat dehidrogenaz (G6PD) üzerindeki inhibitör etkilerini değerlendirmektir. Bu amaçla, 2',5'-ADP Sepharose 4B afinite jeli ve Sephadex G-200 jel filtrasyon kromatografisi kullanılarak insan eritrosit glutasyon redüktaz 2175-kat (%31.35 verim) ve 2',5'-ADP Sepharose 4B afinite jeli kullanılarak eritrosit glukoz 6-fosfat dehidrogenaz 8681.3-kat (%24.77 verim) ile saflaştırıldı.

Safranin *in vitro* GR ve G6PD inhibitör etkisi gösterdi. Safraninde IC<sub>50</sub> değerleri, sırası ile GR için 1.15 mM ve G6PD için 3.22 mM, ve K<sub>i</sub> sabitleri sırası ile 2.63 ± 0.13 ve 4.53 ± 0.17 mM oldu. Safranin GR ve G6PD için yarışmasız inhibisyon gösterdi.

### Anahtar Kelimeler

Safranin, glutasyon redüktaz, glukoz 6-fosfat dehidrogenaz.

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## INTRODUCTION

Glutathione reductase (glutathione: NADP<sup>+</sup> oxidoreductase, EC 1.8.1.7; GR) plays a central role in glutathione metabolism. This flavin enzyme is essential for reducing glutathione disulfide (GSSG) to the tripeptide form. GSH not only counteracts oxidative agents but also protects the sulfhydryl groups of intracellular proteins in the erythrocytes, necessary for protection of cells against oxidative stress [1]. In addition, recent results suggest that GSH is essential for cell proliferation [2]. Alternatively, high GSSG concentrations inhibit a number of important enzyme systems, including protein synthesis [3]. GR enables several vital functions of the cell, such as the detoxification of free radicals and reactive oxygen species as well as protein and DNA biosynthesis, by maintaining a high ratio of GSH/GSSG [4].

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP<sup>+</sup> oxidoreductase EC 1.1.1.49; G6PD) oxidizes glucose-6-phosphate to 6-phosphogluconolactone, reducing NADP<sup>+</sup> to NADPH. The principal physiological function of the pentose phosphate pathway is the production of NADPH and ribose 5-phosphate, which are essential for reductive biosynthesis and nucleic acid synthesis [5]. The main function of the pathway seems to protect the erythrocytes against oxidative damage that is caused by free radical in the number of molecules in cells, including membrane lipids, proteins, and nucleic acids [6,7]. Formation of these harmful radicals is an occurring intracellular metabolic process [8,9]. If the generation of free radicals in cells impairs antioxidant defenses or exceeds the ability of the antioxidant defense system to eliminate them, oxidative stress occurs [10]. The harmful effects of free radicals are checked by the cellular antioxidant defense systems [11].

Safranin (also Safranin O or 2,8-dimethyl-3,7-diamino-phenazine) is a biological stain used in histology and cytology. Safranin is used as a counterstain in some staining protocols, colouring all cell nuclei red. This is the classic counterstain in a Gram stain. It can also be used for the detection of cartilage, mucin and mast cell granules [12].

Many chemicals or drugs are being used in therapies which are potent inhibitor or activator of specific enzymes [13-15]. Thus, we aimed in this study to purify GR and G6PD from human erythrocytes and analyse the inhibitory potential of safranin on the enzyme activity.

## MATERIALS AND METHODS

### Materials

Sephadex G-200, NADPH, GSSG, protein assay reagents and chemicals for electrophoresis were obtained from Sigma Chem. Co. 2',5'-ADP-Sepharose 4B was obtained from Pharmacia. All other chemicals used were of analytical grade and obtained from either Sigma-Aldrich or Merck.

### Preparation of hemolysate

Blood samples were obtained from Atatürk University, Medicinal Faculty Blood Centre according to the protocol approved by the ethics committee of Atatürk University, Medicinal Faculty. Fresh human blood samples were collected in tubes containing EDTA, then centrifuged (15 min, 2500xg) and plasma and buffy coat (leucocytes) were removed. The packed red cells were washed three times with KCl (0.16 M) and hemolyzed with 5 volume of ice-cold water then centrifuged for 30 min (4°C, 10000xg) to remove the ghosts and intact cells [15].

### Ammonium sulphate precipitation and dialysis

The hemolysate was brought to 35-65% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> saturation with solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The precipitate was separated by centrifugation at 5000 g for 15 min and dissolved in a small amount of 50 mM phosphate buffer pH 7.0, and then enzyme activities were determined both in the supernatant and in the precipitate for each respective precipitation. The precipitate was dissolved in phosphate buffer (50 mM; pH: 7.0). The resultant solution was clear, and contained partially purified enzyme. Then, it was dialyzed at 4°C in 1 mM EDTA+10 mM potassium phosphate buffer (pH: 7.5) for 2 h with twice refreshment of the buffer [15]. Partially purified enzyme solution was kept at 4°C.

### **Purification of GR and G6PD Enzymes 2',5'-ADP Sepharose-4B affinity chromatography**

Two grams of dried 2',5'-ADP Sepharose-4B was used for a column (1 x 10 cm) of 10 mL bed volume. The gel was washed with 300 mL of distilled water to remove foreign bodies and air, suspended in 0.1 M potassium acetate + 0.1 M potassium phosphate buffer (pH: 6.0), and packed in the column. After settling of the gel, the column was equilibrated by means of a peristaltic pump with 50 mM potassium phosphate buffer including EDTA (1 mM pH: 6.0). The flow rates for washing and equilibration were adjusted to 20 mL/h [14].

**Purification of GR:** The previously obtained dialyzed sample was loaded onto the 2',5'-ADP Sepharose-4B affinity column and the column was washed with 25 mL of 0.1 M potassium acetate + 0.1 M potassium phosphate (pH: 6), and 25 mL of 0.1 M potassium acetate+0.1 M potassium phosphate (pH: 7.85). The washing procedure was continued with 50 mM potassium phosphate buffer including 1 mM EDTA, pH: 7.0, until the final absorbance difference became 0.05 at 280 nm. The enzyme was eluted with a gradient mixture of 0 to 0.5 mM GSH+1 mM NADPH in 50 mM potassium phosphate, containing 1 mM EDTA (pH: 7.0). Active fractions were collected and dialyzed with equilibration buffer. All of the procedures were performed at 4°C [15].

### **Sephadex G-200 gel filtration chromatography**

Dried Sephadex G-200 (2 g) was used for a 165 mL column (2x50 cm) bed volume. The gel was incubated in distilled water at 90°C for 5 h. After removal of the air from the gel, it was loaded onto the column. Flow rate was adjusted to 15 mL/h by means of a peristaltic pump. Then, the column was equilibrated with 50 mM Tris-HCl + 50 mM KCl buffer, pH: 7.0, until the final absorbance difference became 0 at 280 nm. The dialyzed GR enzyme sample was mixed with 5% glycerol and loaded onto the column. Elutions were collected in 2-mL amounts. In each fraction, enzyme activity was determined at 340 nm. Active fractions were collected and stored at -20°C for testing the enzyme purity by electrophoresis [15,16].

**Purification of G6PD:** Dialyzed sample was loaded on 2',5' ADP-Sepharose 4B affinity column and the gel was washed with 25 mL of 0.1 M K-acetate/0.1 M K-phosphate (pH 6.0), with 25 mL of 0.1 M K-acetate/0.1 M K-phosphate (pH 7.85), and finally, with 0.1 M KCl/0.1 M K-phosphate (pH 7.85). Elution was carried out with 80 mM K-phosphate + 80 mM KCl + 0.5 mM NADP<sup>+</sup> + 10 mM EDTA (pH 7.85) solution at 20 mL/h flow rate. Eluates were collected in 2 mL tubes and each of their activity was separately calculated. All of the procedures were performed at 4°C [14].

### **Protein determination**

In all samples, the protein content was quantified spectrophotometrically at 595 nm according to Bradford's method using bovine serum albumin as standard [7,17].

### **SDS polyacrylamide gel electrophoresis**

The control of enzyme purity was carried out using Laemmli's procedure [18] with 8% and 3% acrylamide concentrations for running and stacking gel, respectively. This method was well-described previously [13-15].

### **Effects of safranin**

In order to determine the effects of safranin on human GR and G6PD, different safranin concentrations (from 0.5 to 1.5 mM for GR, 1.0 to 5.0 mM for G6PD) were added into the reaction medium. The enzyme activity was measured, and an experiment in the absence of safranin was used as control (100% activity) [19]. The IC<sub>50</sub> values were obtained from activity (%) vs. safranin concentration plots. To determine GR K<sub>i</sub> constants in the media with inhibitor, the substrate (GSSG) concentrations were 0.015, 0.04, 0.07, 0.10, and 0.15 mM, and G6PD K<sub>i</sub> constants in the media with inhibitor, the substrate (G6PD) concentrations were 0.024, 0.048, 0.072, 0.120, and 0.192 mM. Inhibitor solution was added into the reaction medium, resulting in 3 different fixed concentrations of inhibitors in 1 mL of total reaction volume. Lineweaver-Burk graphs [20] were drawn using 1/V vs. 1/[S] values, and K<sub>i</sub> constants were calculated from these graphs. Regression analysis graphs were drawn for IC<sub>50</sub> using inhibition % values by a statistical package (SPSS-for windows; version 10.0) on a computer (student *t*-test; n: 3).

**Table 1.** Purification scheme of GR from human erythrocyte.

Purification step	Activity (EU/ml)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Hemolysate	0.37	75	36.1	2707.5	27.75	0.01	100	1
Ammonium sulfate precipitation (35-65)%	0.69	20	38.4	768	13.8	0.018	49.73	1.80
2',5'-ADP Sepharose 4B affinity chromatography	1.35	11	0.34	3.74	14.85	3.97	53.51	397
Sephadex G-200 gel filtration chromatography	0.87	10	0.04	0.40	8.70	21.75	31.35	2175

**Table 2.** Purification scheme of G6PD from human erythrocytes.

Purification step	Activity (EU/ml)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Hemolysate	0.41	70	19.5	1365	28.7	0.021	100	1
Ammonium sulfate precipitation (35-65)%	1.27	11	1.14	12.54	13.97	1.114	48.67	53.05
2',5'-ADP Sepharose 4B affinity chromatography	2.37	3	0.013	0.039	7.11	169.3	24.77	8681.3

## RESULTS

In this study, human erythrocyte GR and G6PD were purified by the following steps: hemolysate preparation, ammonium sulphate precipitation, 2',5'-ADP Sepharose-4B affinity chromatography, and Sephadex G-200 gel filtration chromatography. As seen in Table 1 and 2, the purified GR enzyme was characterized with a specific activity of 21.75 EU/mg proteins, a yield of 31.35% and a purification coefficient of 2175; and G6PD with a specific activity of 196.3 EU/mg proteins, a yield of 24.77% and a purification coefficient of 8681.3, respectively.

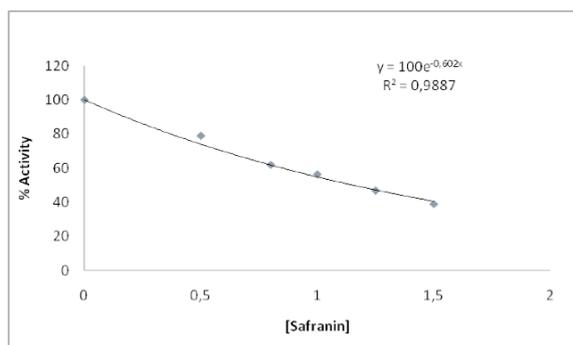
Also, it was observed that safranin effectively inhibited human erythrocyte GR and G6PD (Figure 1 and 2). To show inhibitory effects, whereas, the most suitable parameter is the  $K_i$  constant, some researchers use the  $IC_{50}$  value.  $K_i$  value was calculated from Lineweaver-Burk graphs (Figure 3 and 4). Therefore both  $K_i$  and  $IC_{50}$  parameters of the drug were determined in this study. As shown in Table 3 and Figures 3, 4; the  $K_i$  values for safranin were found as  $2.63 \pm 0.13$  and  $4.53 \pm 0.17$  mM and the corresponding  $IC_{50}$  value was calculated as 1.15 and 3.22 mM for GR and G6PD, respectively.

## DISCUSSION

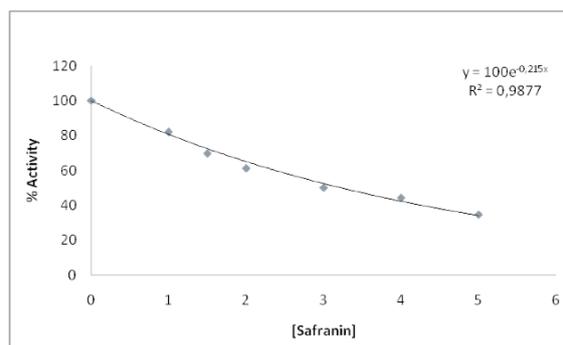
Many chemicals affect metabolism at relatively low doses by altering normal enzyme activity, particularly a specific enzyme [13-15,18]. Some chemicals and drugs, such as Sodium ceftizoxime, sodium ampicillin, sodium cefuroxime, sodium ceftazolin, sodium cefoperazone, streptomycin sulphate, gentamicin sulphate, and netilmicin sulphate inhibit glucose-6-phosphate dehydrogenase enzyme activity [21]. It was reported that 6-hydroxy-3-oxo-3H-xanthene-9-propionic acid (XAN) causes noncompetitive inhibition by glutathione reductase in FAD bonding region. Poly-cyclic aromatic compounds can either be strong or weak inhibitors of erythrocyte glutathione reductase [22]. Similar

**Table 3.**  $K_i$  and  $IC_{50}$  values obtained from regression analysis graphs for GR and G6PD in the presence of safranin in different concentrations.

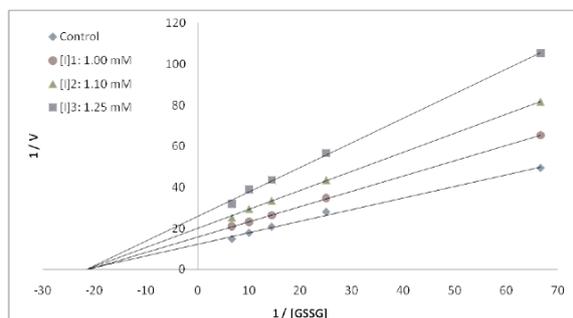
Enzyme	$IC_{50}$	$K_i$ , mM	Inhibition type
GR	1.15 mM	$2.63 \pm 0.13$	Non-competitive
G6PD	3.22 mM	$4.53 \pm 0.17$	Non-competitive



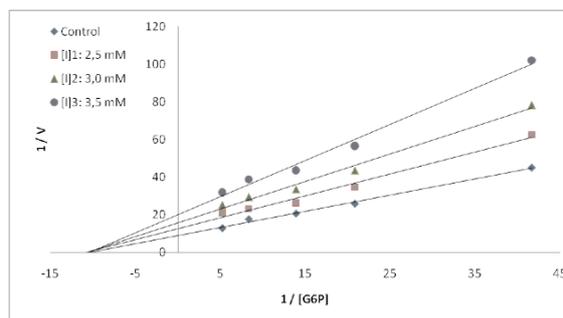
**Figure 1.** The effects of different concentrations of safranin (0.5-1.5 mM) on human erythrocyte glutathione reductase.



**Figure 2.** The effects of different concentrations of safranin (1.0-5.0 mM) on human erythrocyte glucose-6-phosphate dehydrogenase.



**Figure 3.** Lineweaver-Burk graph in 5 different substrate (GSSG) concentrations and in 3 different safranin concentrations for determination of  $K_i$  value.



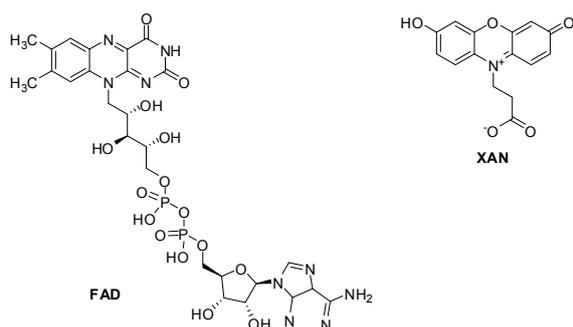
**Figure 4.** Lineweaver-Burk graph in 5 different substrate (G6P) concentrations and in 3 different safranin concentrations for determination of  $K_i$  value.

results were obtained in different studies [23, 24]. Recently, the effect of safranin on the activity of different enzymes has been studied extensively. The effect of safranin on yeast glutathione reductase and human GR were performed before for its X-Ray crystallography, but not determined  $IC_{50}$  value and  $K_i$  constants of them. G6PD is the first key enzyme in the pentose phosphate metabolic pathway and is involved in the generation of NADPH, which is indispensable for biosynthesis of reduced glutathione (GSH) [22]. However, according to our knowledge, there is no information about the effect of safranin on erythrocyte GR and G6PD. In the present study, we investigated the effect of safranin on GR and G6PD from human erythrocytes.

In conclusion, safranin showed high inhibitory effect on human erythrocyte GR and G6PD enzymes activity. According to the results obtained from this study, safranin was demonstrated as a potent inhibitor for GR and G6PD; therefore it could cause some side effects. It was reported that the decrease in GR and G6PD enzymes activity could lead to imbalance in redox status due to

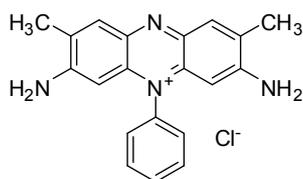
decrements in GSH levels and NADPH in the brain. Some studies have tried to correlate alterations in GSH with neurodegenerative disorders such as Alzheimer's disease, amyotrophic lateral sclerosis, schizophrenia, and Parkinson's disease [25,26]. Furthermore, loss of glutathione and consequent oxidative damage have been suggested early signaling events in apoptotic cell death. A decrease in GSH triggers activation of neuronal lipoxygenase, which leads to production of peroxides, an influx of calcium, and, ultimately, cell death [27]. Safranin and XAN are frequently used in drug industry. The residues lining the XAN binding pocket in hGR are not well conserved among homologs of hGR, so, although compounds binding at this site are not competitive inhibitors, they are reasonable for structure-based drug design. Similarly, safranin both G6PD and GR were shown noncompetitive inhibition.

According to these data, safranin was found to be efficient inhibitor for these enzymes. Owing to widely use of this compound for both human and animals drug development; we thought that this molecule is important agent for G6PD and GR



**Figure 5.** Structure of FAD functional group and 6-hydroxy-3-oxo-3H-xanthen-9-propionic acid (XAN).

activity.



**Figure 6.** Molecular structure of the safranin.

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