Purification and characterization of glucose 6-phosphate dehydrogenase from rainbow trout (*Oncorhynchus mykiss*) erythrocytes

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ABSTRACT: Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: $NADP^+$ oxidoreductase, EC 1.1.1.49; G6PD) from rainbow trout (*Oncorhynchus mykiss*) erythrocytes was purified, using a simple and rapid method, and some characteristics of the enzyme were investigated. The purification procedure consisted of three steps: haemolysate preparation, ammonium sulphate precipitation, and 2′,5′-ADP Sepharose 4B affinity gel chromatography, which took one working day. Thanks to the three consecutive procedures, the enzyme, having the specific activity of 14.51 EU/mg proteins, was purified with a yield of 70.40% and 1 271.19-fold. In order to control the purification of the enzyme SDS polyacrylamide gel electrophoresis was carried out. SDS polyacrylamide gel electrophoresis showed a single band for the enzyme. Optimal pH, stable pH, optimal temperature, molecular weight, and K_M and V_{max} values for $NADP^+$ and glucose 6- phosphate (G6-P) were also determined for the enzyme. In addition, the effect of NADPH on the enzyme was investigated and K_i value and the type of inhibition were determined by means of Lineweaver-Burk graph obtained for NADPH.

Keywords: purification; characterization; glucose 6-phosphate dehydrogenase; *Oncorhynchus mykiss*; erythrocyte

The cell has four major NADPH production systems corresponding to the activities of four cytoplasmic enzymes: glucose 6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD) belonging to the pentose phosphate pathway; malic enzyme (ME); and NADPH depended isocitrate dehydrogenase (NADP-IDH) (Barroso et al., 1999). Glucose 6-phosphate dehydrogenase is the key enzyme that catalyses the first step of pentose phosphate metabolic pathway (Shreve and Levy, 1977; Lehninger et al., 2000). It is reported that it is found in animal tissues, plants and microorganisms (Gleason, 1996; Kurutas and Tuncer, 2000; Nemoto and Sasakuma, 2000). In animal tissues, the enzyme is localised in cytosol and mitochondria and in green plants in cytosol and chloroplasts (Schnarrenberger et al., 1973; Levy, 1979; Ocheretina et al., 2000).

G6PD was isolated from human erythrocytes for the first time by Yoshida (Yoshida, 1966). In the next years, the enzyme was purified from ion-exchange materials by using the natural substrates: G6-P and $NADP^+$. The affinity chromatography (2′, 5′-ADP Sepharose 4B) used for the first time by De Flora and co-workers (Ninfali et al., 1990) is a common technique. Some modifications of this technique were made for rainbow trout erythrocyte G6PD purification.

A unique source of NADPH in erythrocytes is the pentose phosphate metabolic pathway and synthesis of NADPH decreases G6PD deficiency (Telefoncu and Zihnioglu, 1989). A major role of NADPH in erythrocytes is the regeneration of reduced glutathione that prevents haemoglobin denaturation, preserves the integrity of red blood cell membrane sulphhydryl groups and detoxifies hydrogen peroxide and oxygen radicals in and on the red blood cells (Deutch, 1990; Weksler et al., 1990). A decrease in G6PD results NADPH and reduced glutathione deficiency in erythrocytes; scarcity of

reduced glutathione in erythrocytes causes early haemolysis in spleen (Andrews and Mooney, 1994). In normal erythrocytes, G6PD activity decreases with aging (Yuregir et al., 1994).

In addition, when one molecule of palmitate is synthesized in the biosynthesis of fatty acids, 14 molecules of NADPH are used. Six molecules of NADPH are synthesized in the pentose phosphate pathway. Essentially, the pentose phosphate pathway is more active in adipose tissue than in muscle tissue. This situation showed that the G6PD enzyme is very important in the biosynthesis of fatty acids (Lehninger et al., 2000).

There is no report available about the purification and characterization of G6PD enzyme in trout erythrocytes. Therefore purification and characterization of trout erythrocyte G6PD were studied in this paper. In addition, we aimed to support future *in vitro* studies.

MATERIAL AND METHODS

Material. 2′, 5′-ADP Sepharose 4B was purchased from Pharmacia. $NADP^+$, glucose 6-phosphate, protein assay reagent, and chemicals for electrophoresis were purchased from Sigma Chem. Co. All other chemicals used for analytical grade were purchased from either Sigma or Merck.

Fish husbandry and maintenance. The rainbow trout (n = 50) used in this study were mature 4 to 5 years old individuals of an average weight 1.6 to 2.2 kg. The average water temperature was 9 ± 2°C mean ± SD during the tests. At the time of sample collection the fish were fed a commercial pelleted trout feed twice per day at 2% of body weight.

Preparation of the haemolysate. Blood was sampled from the caudal vein using a 10ml plastic syringe. Then each sample (n = 50) was separately transferred into heparinised (5 IU/ml) Vacuntainer® tubes and centrifuged at 2 500 × g for 15 minutes. The plasmas were removed by drip. After the packed column of red cells was washed with KCl solution (0.16 M) three times, the samples were centrifuged at 2 500 × g for each time and supernatants were removed. The erythrocytes were haemolysed with 5 vol. of ice-cold water and centrifuged (+4°C, 10 000 × g) for 30 min to remove ghosts and intact cells (Delgado et al., 1990; Ninfali et al., 1990). Then, all haemolysate samples were pooled in a tube.

Ammonium sulphate fractionation and dialysis. The haemolysate was subjected to precipitation orderly with ammonium sulphate (10–20%, 20–30%, 30–40%, 40–50%, 50–60%, 60–70% and 70–80%). Ammonium sulphate was slowly added to the haemolysate for complete dissolution. This mixture was centrifuged at 5 000 × g for 15 min and the precipitate was dissolved in 50 mM of phosphate buffer (pH 7.0). For each respective precipitation, the enzyme activity was determined both in supernatant and in precipitate. The enzyme was observed to precipitate at 40–65% precipitation. Then, the enzyme solution was dialysed at 4°C in 50 mM K-acetate/5 mM K-phosphate buffer (pH 7.0) for 2 h with two changes of buffer (Ninfali et al., 1990).

2′, 5′-ADP Sepharose 4B affinity chromatography. For 10 ml of bed volume, 2 g of dry 2′, 5′-ADP Sepharose 4B was washed several times in 400 ml of distilled water. With several washings, the impurities were removed and the gel conditioned. After removal of the air in the gel, it was resuspended in the buffer (0.1 M K-acetate + 0.1 M K-phosphate, pH 6.0) at a ratio of 25% buffer and 75% gel and was packed in a column (1 × 10 cm). After precipitation of the gel, it was equilibrated with the same buffer by means of a peristaltic pump (flow rate: 50 ml/h). The dialysed enzyme solution obtained previously was loaded on the column, and the flow rate was adjusted to 20 ml/h. Then, the column was sequentially washed with 25 ml of 0.1 M K-acetate + 0.1 M K-phosphate, (pH 6.0) and 25 ml 0.1 M K-acetate + 0.1 M K-phosphate (pH 7.85). The washing with 0.1 M KCl + 0.1 M K-phosphate (pH 7.85) was continued until the final absorbance difference was 0.05. Finally, the enzyme was eluted with the solution of 80 mM K-phosphate + 80 mM KCl + 0.5 mM $NADP^+$ + 10 mM EDTA (pH 7.85). The enzyme activity was measured in final fractions, and the activity-containing tubes were collected together. The protein was determined in the resultant solution. During all procedures, the temperature was kept at +4°C (Morelli et al., 1978; Muto and Tan, 1985; Ninfali et al., 1990).

Activity determination. The enzymatic activity was measured by Beutler's method (Beutler, 1971). One enzyme unit was defined as the enzyme amount reducing 1 μmol $NADP^+$ per 1 minute.

Protein determination. Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method (Bradford, 1976), with bovine serum albumin being used as a standard.

SDS polyacrylamide gel electrophoresis (SDS-PAGE). The control of enzyme purity, using

Laemmli's procedure (Laemmli, 1970), was carried out in 3% and 8% acrylamide concentrations for stacking and running gel, respectively. 10% SDS was added to the gel solution. The gel was stabilized in the solution containing 50% propanol + 10% TCA + 40% distilled water for 30 minutes. The staining was made for about 2 h in the solution of 0.1% Coommassie Brillant Blue R-250 + 50% methanol + 10% acetic acid. Finally, the washing was carried out in the solution of 50% methanol + 10% acetic acid + 40% distilled water until protein bands were cleared.

Optimal pH determination. For the optimal pH determination, the enzyme activity was measured in 1 M Tris-HCl and phosphate buffers within the pH values of 7.2 to 8.9 and of 4.9 to 8.0, respectively.

Stable pH determination. For this purpose, the enzyme activity was determined in 1 M Tris-HCl buffer at pH of 7.2, 8.0 and 9.9, and in 1 M phosphate buffer at pH of 4.9, 6.0, 7.0 and 8.0. In each experiment, the equal volumes of buffer and enzyme solutions were mixed and kept refrigerated (+4°C). Activity determinations were made at an interval of 6 h for 24 h.

The effect of temperature on G6PD activity. The enzyme activity was measured between 10 and 80°C at optimal pH for this purpose.

Molecular weight determination. **(a) Sephadex G-200 gel filtration**. The molecular weight of the enzyme was determined on the basis of Andrew's method (Andrews, 1965). The enzyme-containing tube was first determined. The void volume was observed with Blue Dextrane 2000. Yeast hexokinase 100 kDa, rabbit heart creatine phosphokinase 81 kDa, bovine serum albumin 66 kDa, bovine pancrease deoxyribonuclease 31 kDa were used as standards. **(b) SDS-PAGE**. The subunit determination was made by SDS-PAGE (Laemmli, 1970). Standard proteins; yeast hexokinase (100 kDa), rabbit heart creatine phosphokinase (81 kDa), bovine serum albumin (66 kDa), bovine liver glutamic dehydrogenase (55 kDa), bovine spleen deoxyribonuclease (38 kDa) were used as standards.

Kinetic studies. For K_M and V_{max} evaluation, Lineweaver-Burk curves were used (Segel, 1975), which were obtained at five different concentrations of $NADP^+$ (0.004, 0.008, 0.012, 0.016 and 0.02 mM) and at the constant concentration of G6-P (6 mM), and the same experiments were conducted for G6-P (at five different concentrations of G6-P: 0.012, 0.024, 0.036, 0.048 and 0.06 mM, and at fixed $NADP^+$ concentration (2 mM). All kinetic studies were performed at 25°C and optimal pH (1 M Tris-HCl, pH: 8.0). To determine K_i constant and inhibition type for NADPH at optimum pH and 25°C, in the media with NADPH or without NADPH, the substrate (glucose 6-phosphate) at 0.15, 0.3, 0.45, 0.6 and 0.9 mM concentrations was added to the reaction medium, resulting in three different fixed concentrations of NADPH in 1 ml total reaction volume. For the three fixed NADPH (0.15, 0.3, 0.9 mM) concentrations mentioned, the values were obtained by using five substrate concentrations. To draw Lineweaver-Burk graphs by using 1/V vs. 1/[S] value, regression analysis was carried out and equations obtained from regression analysis were used to draw graphs for each fixed NADPH concentration. K_i values were calculated from these Lineweaver-Burk graphs.

Whole experiments were repeated three times.

RESULTS AND DISCUSSION

Several enzymes are important in antioxidative defence because they metabolise either free radicals or reactive oxygen intermediates to non-radical products. Some of the best known of these enzymes include a family of enzymes known as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and glutathione reductase (GSSG-Rd). GSH peroxidase metabolises H_2O_2 and lipid peroxides to non-toxic products and in doing so it leads to the oxidation of GSH to GSSG. GSSG is reduced to GSH by GSSG reductase. Enzymatically, this reaction requires nicotinamide adenine dinucleotide phosphate (NADPH) produced by glucose-6-phosphate dehydrogenase and 6-phospho gluconate dehydrogenase in the pentose phosphate metabolic pathway (Reiter et al., 1997).

The importance of G6PD in metabolism has been well known for many years. We think that the easier purification methods of the enzyme make it possible that investigations on the subject can be done easily. The enzyme has been eluted from ion exchange material by using one of its ligands. Morelli et al. (1978) used 2′, 5′-ADP Sepharose 4B for this purpose for the first time. The investigators also used three consecutive steps for purification: DEAE Sephadex, phosphocellulose (P11), and affinity chromatography on 2′, 5′-ADP Sepharose 4B (Ninfali et al., 1990). The three steps take a long time and thus result in a decline in enzyme activity during the procedure.

Table 1. Purification scheme of glucose-6-phosphate dehydrogenase from rainbow trout erythrocytes

Purification step	Activity (U/ml)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor
Haemolysate	0.371	80	32.5	2 600	29.68	0.011	100	1
Ammonium sulphate precipitation (40–65)%	0.864	25	19.3	482.5	21.60	0.044	72.78	3.92
Affinity chromatography	2.612	8	0.18	1.44	20.89	14.51	70.40	1 271.19

For this reason, we omitted the first two steps. After the haemolysate was precipitated with ammonium sulphate, the sample was directly applied to 2′, 5′-ADP Sepharose 4B column. Moreover, we used 0.5 mM $NADP^+$ instead of 0.2 mM used by Ninfali et al. (1990) for elution, resulting in more concentrated enzyme elution.

Thanks to the procedure used in this study, the enzyme, having the specific activity of 14.51 EU/mg proteins, can be purified from 40–50 ml of whole blood within 7 or 8 h with a yield of 70.40% and 1 271.19-fold (Table 1).

Figure 1 shows the SDS-PAGE made for the purity and molecular weight of the enzyme. For the standard proteins and G6PD, R_f values were calculated, and R_f -Log MW graph (Figure 2) was obtained according to Laemmli (1970) procedure,

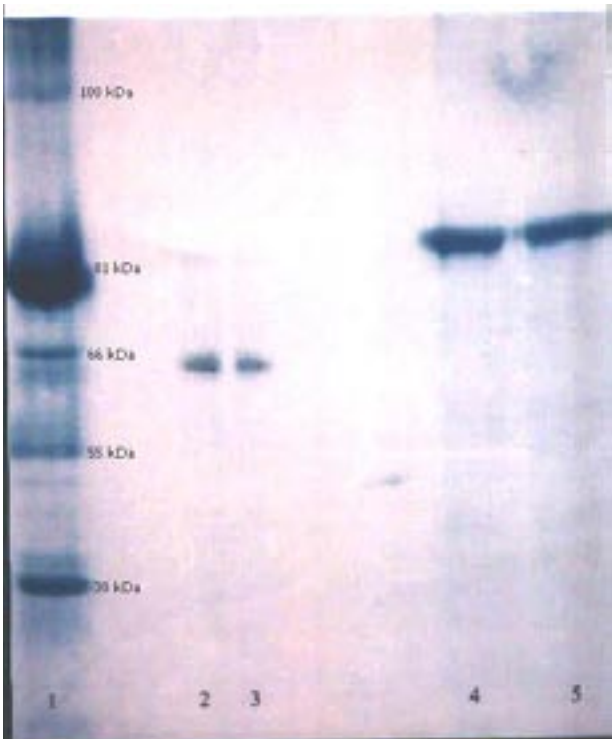

Figure 1. SDS-PAGE bands of G6PD. Lane 1: standard proteins; yeast hexokinase (100 kDa), rabbit heart creatine phosphokinase (81 kDa), bovine serum albumin (66 kDa), bovine liver glutamic dehydrogenase (55 kDa), bovine spleen deoxyribonuclease (38 kDa); lanes 2 and 3 are rainbow trout G6PD

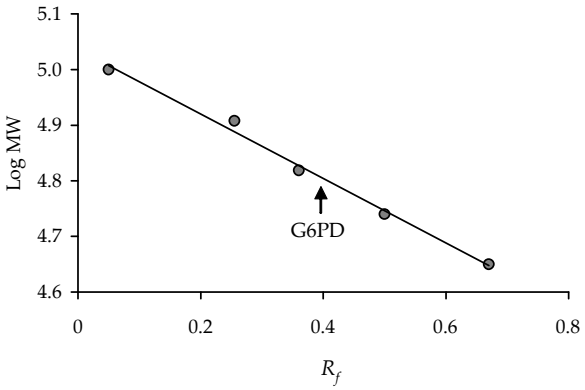

Figure 2. Standard R_f-Log MW graph of G6PD using SDS-PAGE

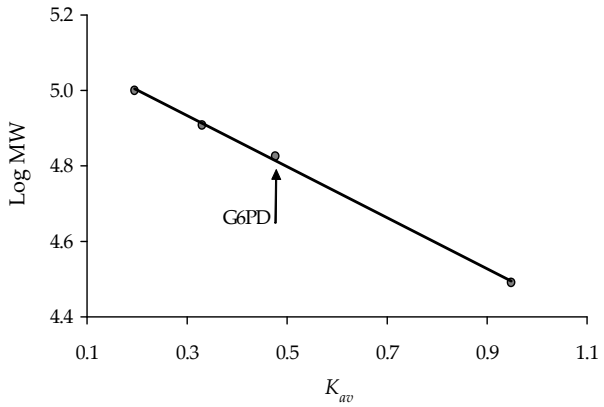

Figure 3. Standard K_{av}-Log MW graph of G6PD using gel filtration. Standards: yeast hexokinase 100 kDa, rabbit heart creatine phosphokinase 81 kDa, bovine serum albumin 66 kDa, bovine pancrease deoxyribonuclease 31 kDa

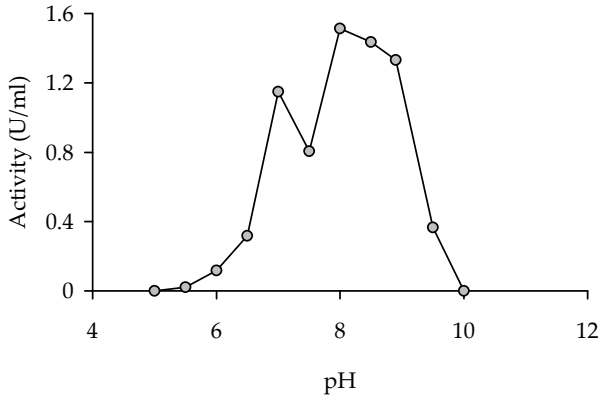

Figure 4. Activity-pH graph of G6PD

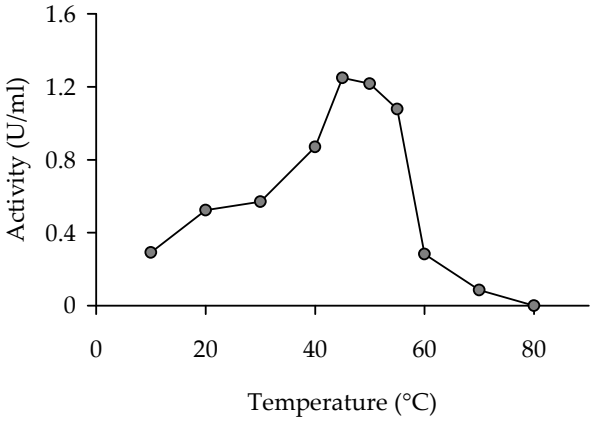

Figure 5. The effect of temperature on G6PD

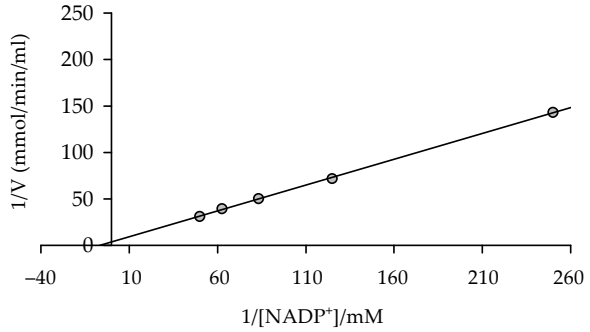

Figure 6. Lineweaver-Burk graph at 5 different G6-P concentrations and at constant $NADP^+$ concentration

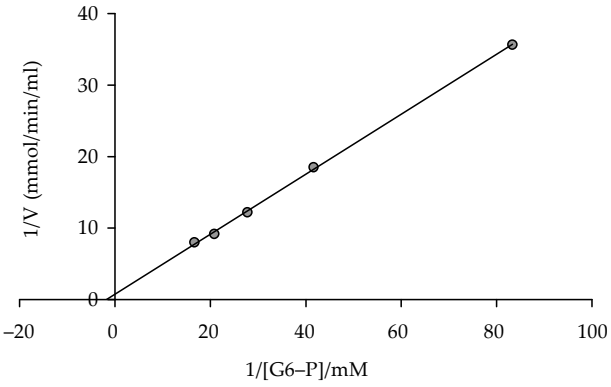

Figure 7. Lineweaver-Burk graph in 5 different $NADP^+$ concentrations and in constant G6-P concentration

showing a molecular weight of 64.26 kDa for G6PD. The molecular weight of the enzyme was also determined by gel filtration chromatography. K_{av}-Log MW graph was obtained (Figure 3), which showed a molecular weight of 66.22 kDa for G6PD. The findings related to the molecular weight studies suggest the enzyme to be a monomer in active state because the molecular weights determined by SDS-PAGE and gel filtration chromatography were approximately the same.

It is reported that the G6PD enzyme can form dimers, trimers, tetramers, and hexamers. The minimum structure necessary for catalysis is the dimeric form (Levy, 1979). But Ulusu et al. (1999) showed that bovine lens G6PD can form a monomer in active state. Similar results were also found for alveolar nodule and adenocarcinoma G6PD in mammary glands of BALB/c mice (Ulusu et al., 1999). The subunits of different origin G6PD enzyme molecular weights are between 50–67 kDa (Levy, 1979). The values obtained for trout erythrocytes G6PD are very similar to these results.

Optimal pH of G6PD has been determined as 8.0 using 1 M Tris-HCl (Figure 4). As shown in Figure 4, the activity vs. pH curve has more than one maximum value (pH 7.0 and 8.0). The results of this type may be seen in diprotic systems and point out the active enzyme may include some ionisable groups. The result of this type was obtained in bovine lens (Segel, 1975; Ulusu et al., 1999).

Stability of the enzyme at different pH was also investigated using 1 M Tris-HCl (pH: 7.0, 7.5, 8.0, 8.5, 8.9) and 1 M phosphate buffers (pH: 5.0, 5.5, 6.0, 6.5, 7.0). G6PD was more stable at pH 8.9 than at other pH values for the duration of 48 hours.

The enzyme was found to show the highest activity at 45°C (Figure 5) after tried between 10–80°C.

The Lineweaver-Burk graphs are shown in Figures 6 and 7, which were constructed for G6-P and $NADP^+$. K_M of 0.50 mM and V_{max} of 1.352 EU/ml

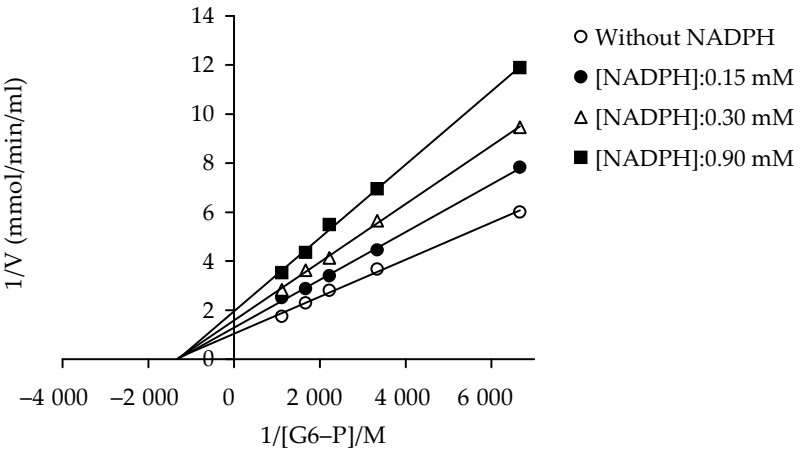

Figure 8. Lineweaver-Burk graph at 5 different substrate (G6-P) concentrations and at 3 different NADPH concentrations for determination of K_i for NADPH

were obtained for G6-P, and 0.166 mM and 0.275 EU per ml for $NADP^+$. The K_M for $NADP^+$ is lower than that for G6-P, suggesting the higher affinity of G6PD to $NADP^+$ when compared with G6-P. Similar results were obtained for G6PD of bovine lens (Ulusu et al., 1999), rat liver and rat kidney cortex (Corpas et al., 1995), dog liver (Bilgi et al., 1995) and human placenta (Ozer et al., 2001).

In addition, K_i constant and inhibition type for NADPH were determined. NADPH markedly inhibited the enzyme and the inhibition constant (K_i) was found to be 0.703 mM by means of Lineweaver-Burk graph (Figure 8). It is reported that NADPH inhibited all G6PDs. For example, G6PDs of different origin have similar K_i values as follows: 0.173, 0.100, 0.025, and 0.0171 mM for bovine lens (Ulusu et al., 1999), rat liver, rat kidney cortex (Corpas et al., 1995) and human placenta (Ozer et al., 2001). It is generally acknowledged that the activity of G6PD enzyme is metabolically controlled by the cytosolic ratio of free $NADP^+$ to NADPH (Corpas et al., 1995).

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