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Research Article

Purification of Glucose 6-Phosphate Dehydrogenase From Goose Erythrocytes and Kinetic Properties

Şükrü BEYDEMİR
Atatürk University, Arts and Science Faculty, Department of Chemistry, Erzurum - TURKEY
Hayrullah YILMAZ
Dicle University, Faculty of Education, Department of Chemistry, Diyarbakır - TURKEY
Mehmet ÇİFTÇİ
Atatürk University, Biotechnology Application and Research Center, Erzurum - TURKEY
Ebubekir BAKAN
Atatürk University, Medical Faculty, Department of Biochemistry, Erzurum - TURKEY
Ö. İrfan KÜFREVİOĞLU
Atatürk University, Arts and Science Faculty, Department of Chemistry, Erzurum - TURKEY

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Abstract: Glucose 6-phosphate dehydrogenase (G6PD) was purified from goose erythrocytes and some characteristics of the enzyme were investigated. The purification procedure was composed of 3 steps: hemolysate preparation, ammonium sulfate precipitation, and 2', 5'-ADP Sepharose 4B affinity gel chromatography. Thanks to the 3 consecutive procedures, the enzyme, having a specific activity of 36.2 EU/mg protein, was purified for a yield of 68.79% and 3892 folds; to ascertain enzyme purity, SDS-PAGE was performed. Optimal pH, stable pH, optimal temperature, molecular weight, and K_m and V_{max} values for $NADP^+$ and glucose 6-phosphate (G6-P) substrates were also determined for the enzyme. In addition, K_i values and inhibition type were determined by means of Lineweaver-Burk graphs obtained for such inhibitors as ATP, ADP and NADPH. These materials inhibited the enzyme in a noncompetitive manner.

Key Words: Goose, purification, glucose 6-phosphate dehydrogenase, erythrocyte

Kaz Eritrositlerinden Glukoz 6-Fosfat Dehidrogenaz Enziminin Saflaştırılması ve Kinetik Özellikleri

Özet: Glukoz 6-fosfat dehidrogenaz (G6PD) enzimi kaz eritrositlerinden saflaştırıldı ve bazı karakteristik özellikleri araştırıldı. Saflaştırma prosedürü; hemolizatın hazırlanması, amonyum sülfat çöktürmesi ve 2', 5'-ADP Sepharose 4B afinite kromatografisi şeklinde üç basamaktan ibarettir. Bu üç basamak neticesinde 36,2 EÜ/mg protein spesifik aktivitesine sahip olan enzim, % 68,79 verimle 3.892 kat saflaştırıldı ve enzim saflığının kontrol edilmesi için SDS-PAGE yapıldı. Enzim için optimum pH, stabil pH, optimum sıcaklık, molekül ağırlığı, $NADP^+$ için K_m ve V_{max} değerleri, G6-P için K_m ve V_{max} değerleri hesaplandı. Ayrıca ATP, ADP ve NADPH inhibitörleri için Lineweaver-Burk grafikleri çizilerek K_i değerleri ve inhibisyon tipleri tespit edildi. Bu maddeler enzimi yarışmasız olarak inhibe etti.

Anahtar Sözcükler: Kaz, saflaştırma, glukoz 6-fosfat dehidrogenaz, eritrosit

Introduction

Glucose 6-phosphate dehydrogenase (E.C.1.1.49; G6PD) is the key and first enzyme of the pentose phosphate metabolic pathway, an alternative metabolic pathway for glucose metabolism. The enzyme catalyzes the conversion of glucose 6-phosphate to 6-phosphogluconate in the presence of $NADP^+$ (1-4). This reaction yields NADPH, which protects the cell from oxidant agents by producing reduced glutathione. NADPH is also needed for the maintenance of a reducing atmosphere in cells exposed to high concentrations of oxygene radicals, including erythrocytes in the lens and cornea of the eye, and phagocytic cells, which generate peroxide and superoxide anions during the process of killing bacteria. In the case of a lack of NADPH, the concentration of reduced glutathione in the living system

reduces, resulting in cell death. NADPH is also a synthetic coenzyme used in several biomolecules such as fatty acids, steroids and some amino acids. The pentose phosphate pathway is active in a wide variety of cell types, particularly those that have a high rate of nucleotide synthesis or utilize NADPH in large amounts (5-7).

G6PD was first isolated from human erythrocytes by Yoshida and Huang (8). Later, the enzyme was purified from ion-exchange materials by using the natural substrates G6-P and $NADP^+$. Affinity chromatography (2', 5'-ADP Sepharose 4B), used first by De Flora (9), is a common technique. Some modifications in this technique were made for goose erythrocyte G6PD purification. No studies on the subject were encountered in previous reports.

The aim of this study is to purify glucose 6-phosphate dehydrogenase (G6PD) from goose erythrocytes and to investigate some characteristics of the enzyme.

Materials and Methods

Preparation of the hemolysate

Blood samples collected in EDTA were centrifuged (15 min, 2,500 x g), and the plasma was removed. The pack of red cells was washed 3 times with KCl (0.16 M) and hemolyzed with 5 volumes of ice-cold water and then centrifuged (4 °C, 10,000 x g, for 30 min) to remove the ghosts and intact cells (9).

Ammonium sulfate precipitation and dialysis

The hemolysate was subjected to orderly precipitation with ammonium sulfate (10-20%, 20-30%, 30-40%, 40-50%, 50-60%, and 60-70%). Ammonium sulfate was slowly added to the hemolysate for complete dissolution. This mixture was centrifuged at 10,000 x g for 15 min and the precipitate was dissolved in 50 mM of phosphate buffer (pH 7.0). For each respective precipitation, enzyme activity was determined both in supernatant and in precipitate. The enzyme was observed to precipitate at 40-60% precipitation. The enzyme solution was dialyzed in 50 mM of K-acetate plus 50 mM of K-phosphate buffer (pH 7.0) for 2 h with 2 changes of buffer (9).

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. After several washings, the impurities were removed and the gel conditioned. After removal of the air in the gel, it was resuspended in 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 x 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 dialyzed 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). Washing with 0.1 M KCl + 0.1 M K-phosphate, (pH 7.85) was continued until the final absorbance difference became 0.05. Finally, the enzyme was eluted with a solution of 80 mM K-phosphate + 80 mM KCl + 0.5 mM $NADP^+$ + 10 mM EDTA (pH 7.85). Enzyme activity was measured in final fractions, and the tubes exhibiting activity were collected together. In the resultant solution, the protein was determined. During all procedures, the temperature was kept at 4 °C (7,9,10).

Activity determination

Enzymatic activity was measured by Beutler's method (11). One enzyme unit was defined as the enzyme amount reducing 1 µ mol $NADP^+$ per 1 min.

Protein determination

Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method, with bovine serum albumin being used as a standard (12).

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The control of enzyme purity, using Laemmli's procedure, was carried out in 3% and 8% acryl amide concentrations for running and stacking gel, respectively. To the gel solution was added 10% SDS. The gel was stabilized in the solution containing 50% propanol + 10% TCA + 40% distilled water for 30 min. The staining was performed for about 2 h in a solution of 0.1% Coomassie Brilliant Blue R-250 + 50% methanol + 10% acetic acid. Finally, the washing was carried out in a solution of 50% methanol + 10% acetic acid + 40% distilled water until the protein bands were cleared (13).

Optimal pH determination

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

Stable pH determination

For this purpose, enzyme activity was determined in 1 M Tris-HCl buffers at pH 7.0, 8.0 and 9.0, and in 1 M phosphate buffers at pH 5.0, 6.0, 7.0 and 8.0. In each experiment, equal volumes of buffer and enzyme solution were mixed and kept refrigerated (4 °C). Activity determinations were made at intervals of 8 h for 24 h.

The effect of temperature on G6PD activity

Enzyme activity was measured between 20 and 55 °C at optimal pH.

Molecular weight determination

Sephadex G-200 gel filtration chromatography

The molecular weight of the enzyme was determined on the basis of Andrews' method (14). The void volume of the column was determined with Blue Dextrane 2000. Horse heart cytochrome C (12,400), bovine erythrocyte carbonic anhydrase (29,000), bovine serum albumin (66,000), yeast alcohol dehydrogenase (150,000) and sweet potato β-amylase (200,000) were used as standards (Sigma: MW-GF-200).

SDS-PAGE

The subunit determination was made by SDS-PAGE (13). Rabbit myosin (205,000), *E. coli* β-galactosidase (116,000), rabbit phosphorylase B (97,400), bovine albumin (66,000), chicken ovalbumin (45,000) and bovine carbonic anhydrase (29,000) were used as standards (Sigma: MW-SDS-200).

Kinetic studies

For K_m and V_{max} evaluation, Lineweaver-Burk curves were used (15), and these were obtained in 5 different concentrations of $NADP^+$ (0.004, 0.01, 0.02, 0.03 and 0.04 mM) and in the constant concentration of G6-P, and the same experiments were done for G6-P (in 5 different concentrations of G6-P: 0.03, 0.06, 0.012, 0.018, and 0.42 mM, and in fixed $NADP^+$ concentration (11). All kinetic studies were performed at 25 °C at optimal pH (1 M Tris-HCl, pH 7.0).

The effect of inhibitors

Natural inhibitors such as NADPH, ATP and ADP were selected to determine their effects. For each inhibitor, 3 different concentrations were used (NADPH: 0.1, 0.15 and 0.2 mM; ATP: 1, 5 and 10 mM; ADP: 2, 5 and 7 mM). In these experiments, G6-P was used as substrate at 5 different concentrations. The Lineweaver-Burk curves obtained were used to determine K_i and inhibition type (15).

Results

Table 1 shows the purification characterized with a specific activity of 36.2 EU/mg protein, a yield of 68.75% and a purification coefficient of 3892. Figure 1 exhibits 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 an R_f -Log MW graph (Figure 2) was obtained according to Laemmli's procedure, showing a molecular weight of 73,177 Da for G6PD. The molecular weight of the enzyme was also determined by gel filtration chromatography. A K_{av}-log MW graph was obtained (Figure 3), which showed a molecular weight of

Table 1. Purification scheme of glucose 6-phosphate dehydrogenase from goose 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	0.263	75	28.20	2115	19.725	0.0093	100	1
Ammonium sulfate precipitation(40-60)%	0.781	20	2.11	42.20	15.62	0.3701	79.18	39.79
2′, 5′- ADP sepharose 4B affinity chromatography	0.905	15	0.025	0.375	13.57	36.2	68.79	3,892

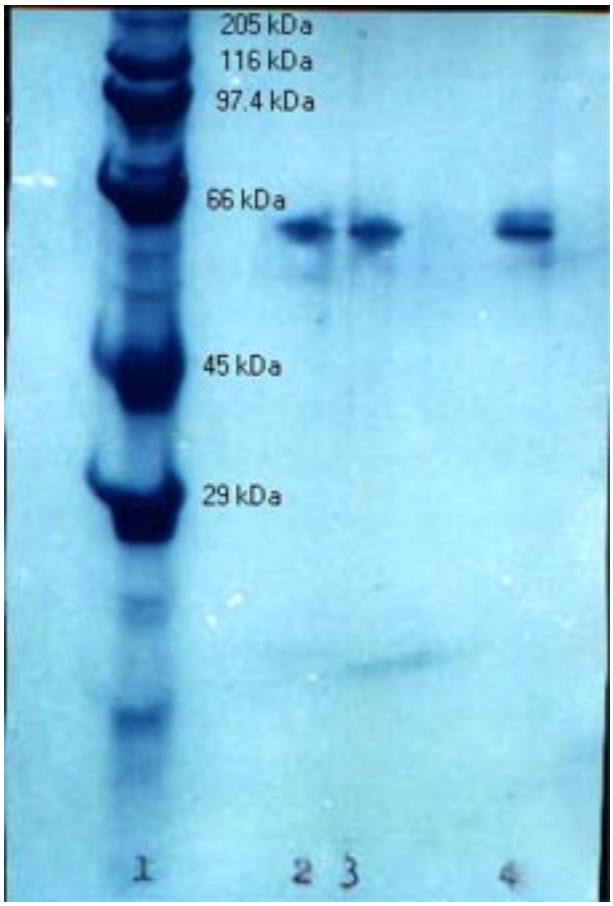

Figure 1. SDS-PAGE bands of G6PD (Lane 1: standard proteins; Lane 2 chicken erythrocytes G6PD, Lane 3: goose erythrocytes G6PD; Lane 4: turkey erythrocytes G6PD).

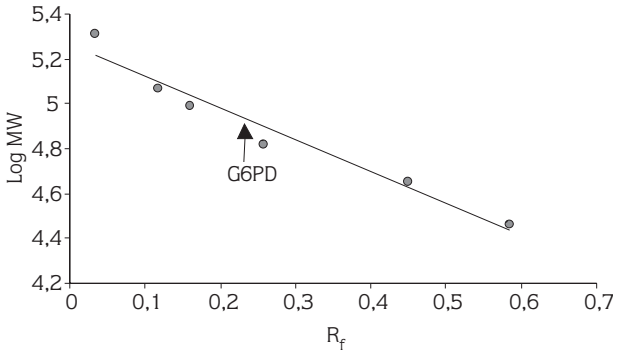

Figure 2. Standard Rf-Log MW graph of G6PD using SDS-PAGE. (Standards: rabbit myosin (205,000), *E. coli* β-galactosidase (116,000), rabbit phosphorylase B (97,400), bovine albumin (66,000), chicken ovalbumin (45,000) and bovine carbonic anhydrase (29,000)).

143,557 Da for G6PD. The optimal pH of G6PD was determined to be 7.0 using 1 M Tris-HCl (Figure 4). The pH determined was different from that in the previous

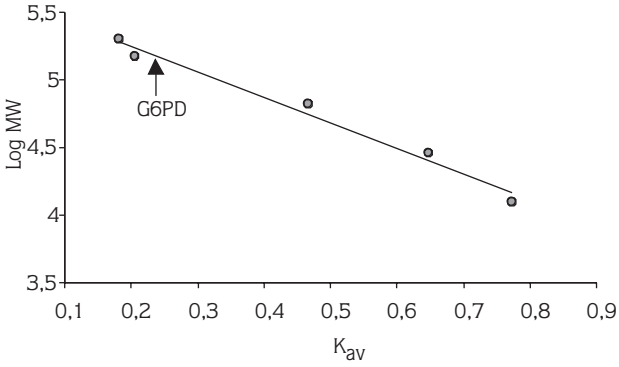

Figure 3. Standard Kav-Log MW graph of G6PD using gel filtration. (Standards: horse heart cytochrome C (12,400), bovine erythrocyte carbonic anhydrase (29,000), bovine serum albumin (66,000), yeast alcohol dehydrogenase (150,000) and sweet potato β-amylase (200,000).

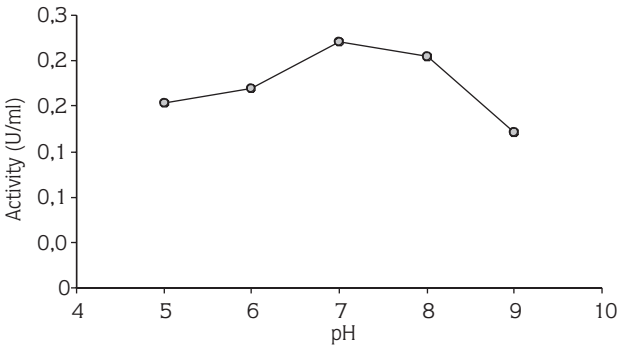

Figure 4. Activity-pH graph of G6PD.

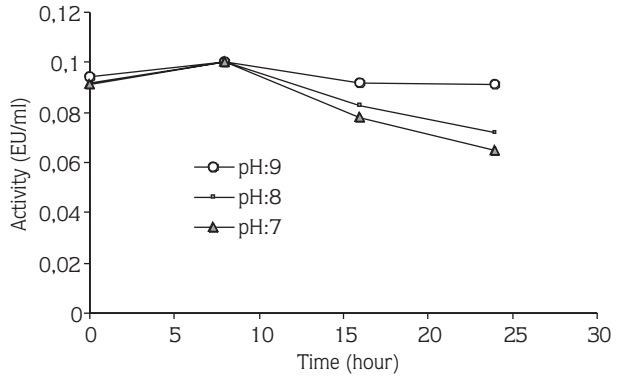

Figure 5. Stable pH graphs of G6PD in 1 M Tris-HCl buffer.

studies. The stable pH of the enzyme was 9.0 in Tris-HCl (Figure 5). The enzyme was seen to show the highest activity at 50 °C (Figure 6) after tests between 20 and 55 °C.

The Lineweaver-Burk graphs, which were constructed for G6-P and $NADP^+$, are shown in Figures 7 and 8. A K_m of 0.0243 mM and a V_{max} of 0.28 EU/ml were obtained for G6-P, and 0.0074 mM and 0.286 EU/ml for $NADP^+$.

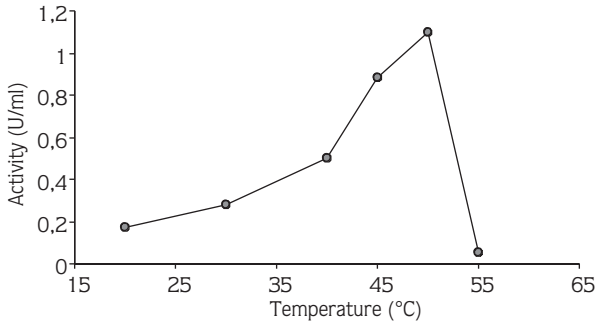

Figure 6. The effect of temperature on G6PD.

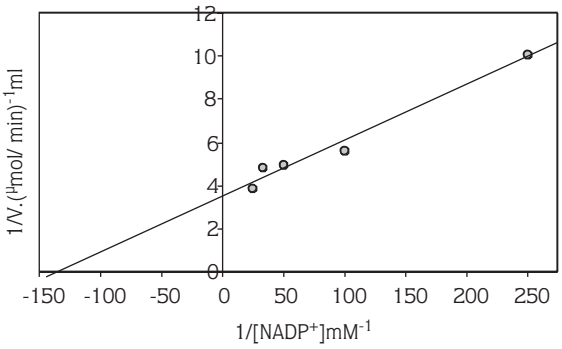

Figure 7. Lineweaver-Burk graph in 5 different $NADP^+$ concentrations and in constant G6-P concentration.

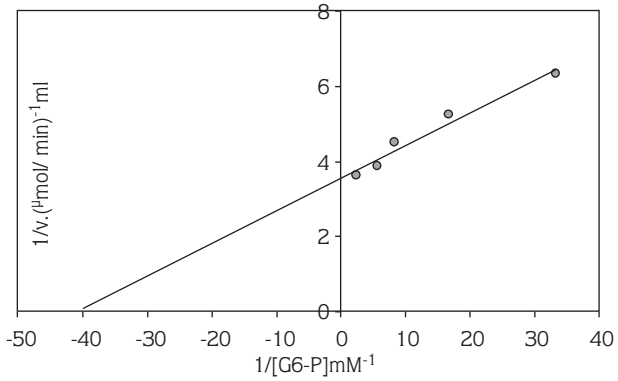

Figure 8. Lineweaver-Burk graph in 5 different G6-P concentrations and at constant $NADP^+$ concentration.

Discussion

Goose G6PD was purified and characterized for the first time in this study. The purification steps included hemolysate preparation, ammonium sulfate precipitation and 2', 5'-ADP Sepharose 4B affinity gel chromatography. This procedure is a modified method of Ninfali et al., which they used for mammalian erythrocyte G6PD purification (9). The purification characteristics show that the procedure used in the study is reliable enough to be used by other investigators. This purification procedure also has the advantage of being relatively fast, as it takes only 6 to 7 h. The enzyme, having a specific activity of 36.2 EU/mg proteins, has been purified at a 68.75% yield, 3897-fold. The specific activity mentioned above for G6PD is higher than that reported in some studies (16-18) and lower than that in others (9,19,20). The same is true for the purification fold. The yield reported is higher than that in previous studies (9,16-20). The results suggest that the purification method of G6PD from goose red cells is convenient.

The optimal pH of G6PD was 7.0 using 1 M Tris-HCl (Figure 4). The pH determined was different from that in human erythrocytes (9,11), bovine lens (16), chicken erythrocytes (17), sheep erythrocytes (18), dog liver (19,20) and rat liver and kidney cortex (21). The stable pH of the enzyme was 9.0 in Tris-HCl (Figure 5). This pH was similar to that of sheep erythrocytes (18), and it was different from those of chicken erythrocytes (17) and dog liver (19,20). The enzyme's highest activity point was at 50 °C (Figure 6) after trials between 20 and 55 °C. The optimum temperature determined was similar to those of human erythrocytes (11), bovine lens (16) and rat liver and kidney cortex (21), and it was different from those of chicken erythrocytes (17) and parsley leaves (22).

Figure 1 exhibits the SDS-PAGE performed for the purity and molecular weight of the enzyme. A high purity of the enzyme was obtained. For standard proteins and G6PD, the R_f values were calculated, and the R_f -Log MW graph (Figure 2) was obtained according to Laemmli (13), showing a molecular weight of 73,177 Da for G6PD. The molecular weight of the enzyme was also determined by gel filtration. A Kav-Log MW graph was obtained (Figure 3), which showed a molecular weight of 143,557 Da for G6PD. The 2 graphs did not show similar molecular weights, suggesting the enzyme may be a

dimer in an active state. G6PDs of different origin have similar molecular weights as follows: 59 kDa from human erythrocytes (dimer), 65 kDa from human liver (dimer), 64 kDa from rat erythrocytes (23), and in human placenta (24) and in bovine lens (monomer) (16), 119,662 kDa from sheep erythrocytes (dimer) (18) and in dog liver (monomer) (20), as well as in rat cortex and liver (monomer) (21).

The lower the K_m, the higher the affinity of the enzyme to the substrate. For this reason, the affinity of $NADP^+$ to G6PD is higher than that of G6-P as reported for chicken erythrocytes (17), sheep erythrocytes (18) and dog liver (19, 20).

ATP, ADP and NADPH inhibit the enzyme in a noncompetitive manner (Figures 9-11 and Table 2). The lower the K_i, the higher the inhibition. Consequently, NADPH has a high rate of inhibition for the enzyme compared to ADP or ATP. Similar results have been noted by others (17). The regulation of G6PD, which is an allosteric enzyme and is at the control step of the pentose phosphate pathway, is carried out by the NADPH/$NADP^+$ ratio, with the increased ratio inhibiting the enzyme, since NADPH is a natural inhibitor of the enzyme.

In conclusion, G6PD, a GSH-producing and an indirect antioxidant enzyme, has been purified from goose erythrocytes by a simple, fast, inexpensive method, and some of its kinetic parameters have been investigated.

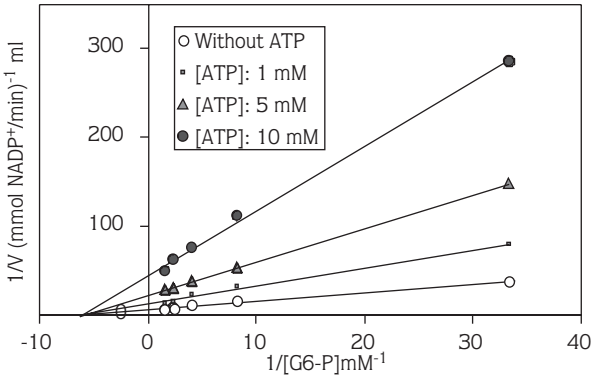

Figure 9. Lineweaver-Burk graph in 5 different substrate (G6-P) concentrations and in 3 different ATP concentrations for the determination of K_i for ATP.

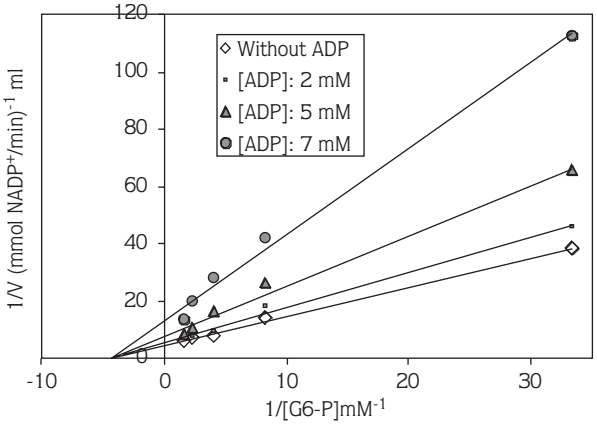

Figure 10. Lineweaver-Burk graph in 5 different substrate (G6-P) concentrations and in 3 different ADP concentrations for the determination of K_i for ADP.

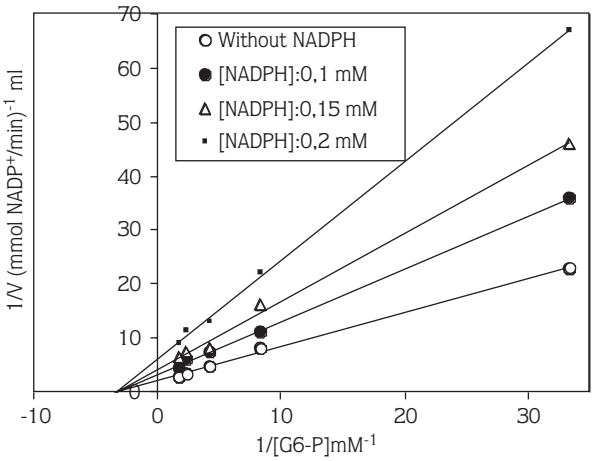

Figure 11. Lineweaver-Burk graph in 5 different substrate (G6-P) concentrations and in 3 different NADPH concentrations for the determination of K_i for NADPH.

Table 2. NADPH, ATP and ADP K_i constants.

Inhibitor	K_i (mM)	Inhibition type
NADPH	0.144	Noncompetitive
ATP	1.598	Noncompetitive
ADP	5.729	Noncompetitive

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