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Purification and Characterization of Glucose 6-Phosphate Dehydrogenase from Sheep Liver

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Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: $NADP^+$ oxidoreductase, EC 1.1.1.49; G6PD) was purified from sheep liver by a simple and rapid method. The purification process consisted of two steps: preparation of the homogenate, and 2′, 5′-adenosine diphosphate (ADP) Sepharose 4B affinity chromatography.

Through the use of these two consecutive steps, the enzyme was purified with a yield of 35.6% and 1,920 fold, having the specific activity of 11.2 enzyme units (EU/mg protein). A K_M of 0.176 mM and a $V_{\max}$ of 0.0179 EU/ml were obtained for G6-P, and 0.0194 mM and 0.0223 EU/ml for $NADP^+$. Enzymatic activity was measured spectrophotometrically according to Beutler's method at 340 nm and optimal pH and assay temperature were determined. By means of a Lineweaver-Burk plot, the inhibitor constant for NADPH was determined to be K_i, 4.707 ± 0.49 μM and it was shown to inhibit the enzyme in a non-competitive manner.

The purification of enzyme was monitored by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE showed a single band at ∼55 kDa for the enzyme and gel filtration chromatography indicated it to be a dimer.

Key Words: Glucose 6-phosphate dehydrogenase, Purification, Sheep, Liver.

Introduction

Glucose 6-phosphate dehydrogenase (G6PD) is present in almost all mammalian tissues, plants, and microorganisms. The enzyme is located in the cytosol and mitochondria of animal cells and in the cytosol and chloroplasts of green plants[1−4]. It is widely expressed in all tissues and blood cells and it is a housekeeping enzyme[5].

G6PD is the key enzyme that catalyzes first the step of the hexose monophosphate (HMP) pathway[6]. The principal source of cytoplasmic NADPH in many cells is the hexose monophosphate pathway and is

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specifically catalyzed by the two deydrogenases; glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. These reactions appear to be necessary for the maintenance of cellular integrity. NADPH production is decreased in G6PD deficiency. In mammalian red blood cells, the hydrogen peroxide produced as a result of endogenous mechanisms and environmental substances is offset by a mechanism requiring glutathione and, in turn, NADPH. Glutathione, a tripeptide, has a free thiol group in its reduced state. This form acts as an antioxidant, keeping cystein residues of hemoglobin and other proteins of cells in reduced form. Normally, the ratio of reduced to oxidized glutathione is approximately 500. Reduced glutathione also plays a role in some detoxification reactions by reducing inorganic and organic peroxide[6,7].

G6PD was first isolated from human erythrocytes by Yoshida[8]. In the following years, the enzyme was highly purified by ion-exchange materials utilizing its natural substrates G6-P and $NADP^{+}$[1,9]. Initially used by De Flora and co-workers, affinity chromatography (2′, 5′-ADP Sepharose 4B) is the common technique for G6PD purification[10]. This method consisted of three steps: DEAE Sephadex, Phosphocellulose (P11), chromatography and subsequently, affinity chromatography on 2′, 5′-ADP Sepharose 4B. Ninfali et al. modified the method by omitting the first step[11]. With this procedure, the enzyme can be purified from 180 to 200 ml of whole blood within 1.5 days. In our study, the first two steps of De Flora's method were omitted. After preparing the homogenate, the sample was directly applied to a 2′, 5′-ADP Sepharose 4B column. By our procedure, the enzyme can be purified from 160 ml of homogenate within 7 or 8 h. Moreover, we used 0.5 mM $NADP^{+}$ instead of 0.2 mM as used by Ninfali et al for elution[11].

In this study, we have established this technique in order to purify the enzyme within 7-8 h. The kinetic behavior of the enzyme was also investigated.

Materials and Methods

Materials

2′, 5′-ADP Sepharose 4B was purchased from Pharmacia Fine Chem. (Sweden). $NADP^{+}$, glucose 6-phosphate and protein assay reagent were of purchased Sigma Chem. Co. (USA). All other chemicals used were analytical grade and purchased from either Sigma Chem. Co. (USA) or Merck AG (Germany).

Preparation of the homogenate

A 35 g sample of fresh sheep liver was cut with a knife. Excess blood, foreign tissues and membranes were removed from the samples. Tissue was suspended in 100 ml of 5 mM phosphate buffer (pH 7.4) containing 458 mM saccharose, and was homogenized using a mixer at top speed for 3 min. Then, the material was homogenized by an ultrasonic homogenizer for 40 min. Afterwards, the homogenate was centrifuged at 12,100 rpm (21,200 x g) for 60 min, and the supernatant was removed. This process was repeated three times; temperature was maintained at 4 °C during the homogenization process[12].

Preparation of Affinity Gel

Two grams of dried 2′, 5′-ADP Sepharose 4B gel was used for a column with a 10 ml volume. The gel was washed with 400 ml distilled water to remove foreign bodies and any air in the swollen gel was eliminated. The gel was suspended in a buffer of 0.1 M K-acetate and 0.1 M K-phosphate buffer (200 ml, pH 6.0), and then packed in a small column (1 x 10 cm) and equilibrated with the same buffer. The gel was washed with

equilibration buffer. A peristaltic pump at a 50 ml/h flow rate was utilized for washing and equilibration by a peristaltic pump[11,13].

Purification of G6PD by Affinity Chromatography

The homogenate obtained previously was loaded on the 2′, 5′-ADP Sepharose 4B affinity column and the flow rate was adjusted to 20 ml/h[11]. Then, the column was sequentially washed with 25 ml a buffer of 0.1 M K-acetate and 0.1 M K-phosphate (pH 6.0) and 25 ml of a buffer of 0.1 M K-acetate and 0.1 M K-phosphate (pH 7.85). The wash with the second buffer was continued until the final absorbance difference became 0.05[9]. Elution of the enzyme was carried out with a mixture of 80 mM K-phosphate and 80 mM KCl and 0.5 mM $NADP^+$ and 10 mM EDTA (pH 7.85)[11]. The enzyme activity was measured in all fractions, and the activity-containing tubes were pooled and then dialyzed in 50 mM K-acetate +50 mM K-phosphate buffer (500 ml, pH 7.0) for 2 h with two changes of buffer[11]. All procedures were performed at 4 °C.

Activity Determination

The enzymatic activity was measured by Beutler's method[14]. One EU was defined as the enzyme reducing 1 μmol $NADP^+$ per min at 25 °C and optimal pH (pH 8.0).

Protein Determination

Protein was determined by Bradford's method[15] by using bovine serum albumin as a standard.

Optimal pH Determination

For the optimal pH determination, the enzyme activity was measured in 1 M Tris-HCl and phosphate buffers within the pH of 7.2 to 8.9 and of 4.9 to 8.0, respectively. In brief, a 50 μl enzyme sample (specific activity 11.2 EU/mg protein, concentration 0.034 mg protein/ml) was added to incubation mixture containing 100 μl 1 M Tris-HCl (or phosphate buffer) buffer at different pH values, 100 μl of 100 mM $MgCl_2$, 100 μl of 2 mM $NADP^+$ and 100 μl of 6 mM glucose 6-phosphate. Then final volume of the mixture was made up to 1000 μl with distilled water and incubated for 5 min at 4 °C.

The Effect of Temperature on G6PD Activity

Enzyme activity was measured between 25 and 60 °C at optimal pH for this purpose. The enzyme activity was measured as follows: a 50 μl enzyme sample (specific activity 11.2 EU/mg protein, concentration 0.034 mg protein/ml) was added to the incubation mixture containing 100 μl of 1 M Tris-HCl buffer (pH 8.0), 100 μl of 100 mM $MgCl_2$, 100 μl of 2 mM $NADP^+$ and 100 μl of 6 mM glucose 6-phosphate. The total volume of mixture was again made up to 1000 μl with distilled water and incubated for 3 min.

Molecular Weight Determination

Sephadex G-200 Gel Filtration

The molecular weight of the enzyme was determined according to Andrew's method[16]. The void volume was observed with Blue Dextran 2000. Horse heart cytochrome c (MW 12,400 Da), bovine erythrocyte carbonic anhydrase (MW 29,000 Da), bovine serum albumin (MW 66,000 Da), yeast alcohol dehydrogenase (MW 150,000 Da), and sweet potato β-amylase (MW 200,000 Da) were used as standards (Sigma: MW-GF-200).

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

In order to control the enzyme purity using Laemmli's procedure[17], SDS-PAGE was carried out in 4% and 10% acrylamide concentrations for stacking and running gel, respectively. The gel solution contained 10% SDS. The gel was stabilized in the solution containing a mixture of 50% propanol, 10% TCA and 40% distilled water for 30 min. The staining was made for about 2 h in a mixture of of 0.1% Coomassie Brilliant Blue R-250, 50% methanol and 10% acetic acid. Finally, the washing was carried out in the a mixture of 50% methanol, 10% acetic acid and 40% distilled water until the protein bands were clearly observable.

Kinetic Studies

K_M and V_{max} were estimated separately for $NADP^+$ and G6-P. Enzyme activity was measured as follows: 50 μl enzyme sample (specific activity 11.2 EU/mg protein, concentration 0.034 mg protein/ml) was added to the incubation mixture containing 100 μl of 1 M Tris-HCl buffer (pH 8.0), 100 μl of 100 mM $MgCl_2$, at six different substrate cuvette concentrations for $NADP^+$ (0.01, 0.02, 0.04, 0.066, 0.1 and 0.2 mM) at a fixed concentration of G6-P (0.60 mM). Then the total volume of mixture was completed to 1000 μl with distilled water and incubated for 5 min at 25 °C. The same procedure was repeated for G6-P (0.03, 0.06, 0.12, 0.18, 0.30 and 0.60 mM) in fixed $NADP^+$ (0.20 mM) concentration, and K_M and V_{max} values were determined by means of a Lineweaver-Burk plot[18]. All kinetic studies were performed at 25 °C at optimal pH (1 M Tris-HCl, pH 8.0).

Effect of NADPH

The effect of three different concentrations of NADPH (0.005, 0.0125, and 0.025 mM) on the kinetic parameters of G6PD when G6-P (0.03, 0.06, 0.12, 0.18, and 0.30 mM) was used as a substrate were also investigated. The Lineweaver-Burk curves obtained were used for the determination of K_i and inhibition type[18]. The enzyme activity was measured as follows: a 50 μl enzyme sample (Specific activity 11.2 EU/mg protein, Concentration 0.034 mg protein/ml) was added to the incubation mixture containing 100μl of 1 M Tris-HCl buffer (pH 8.0), 100 μl of 100 mM $MgCl_2$, 100 μl of 2 mM $NADP^+$ and 100 μl of 6 mM glucose 6-phosphate. Then the total volume of this mixture was completed to 1000 μl with distilled water and incubated for 5 min at 25 °C.

Results and Discussion

Enzyme Purification

Initially in this study, sheep liver G6PD was purified and characterized. The purification steps included preparation of homogenate, and 2′, 5′-ADP Sepharose 4B affinity chromatography. This procedure is a modification of the method Ninfali and co-workers used the purification of for mammalian erythrocyte G6PD[11]. Table shows a purification characterized with a specific activity of 11.2 EU/mg protein, a yield of 35.6% and a purification coefficient of 1920. This yield is higher than those obtained in bovine lens[19] (13.7%), dog liver[20] (18%), and parsley leaves[21] (8.79%) and is lower than those obtained rabbit[11] (56%), chicken[22] (54.68%) and human erythrocytes[11] (41%), as well as pig liver[12] (40%) and human placenta[23] (62%). These values suggests that the procedure used in the study is good enough for G6PD purification. This purification procedure also has the advantage of a short experiment duration of about 6 to 7 h. The elution profile of 2′, 5′-ADP Sepharose 4B affinity chromatography shown in Figure 1.

Table. Purification scheme of glucose 6-phosphate dehydrogenase from sheep liver.

Purification Steps	Total volume (mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Supernatant of Homogenate	160	5480	32	5.84×10^{-3}	100	1
Eluent of 2′, 5′ ADP Sepharose 4B affinity chromatography	30	1.02	11.4	11.2	35.6	1920

Figure 2 shows the SDS-PAGE gel photograph of the purified G6PD. The molecular weight of the enzyme was found to be ∼85 kDa by SDS-PAGE and 176 kDa by gel filtration procedure. These molecular weight values suggest that the enzyme is dimeric. The G6PDs of different origin have similar molecular weights, such as 121 kDa from mouse liver (dimer), 130 kDa from rat liver (dimer), 118 kDa from human liver (dimer), and 133 kDa from pig liver[24].

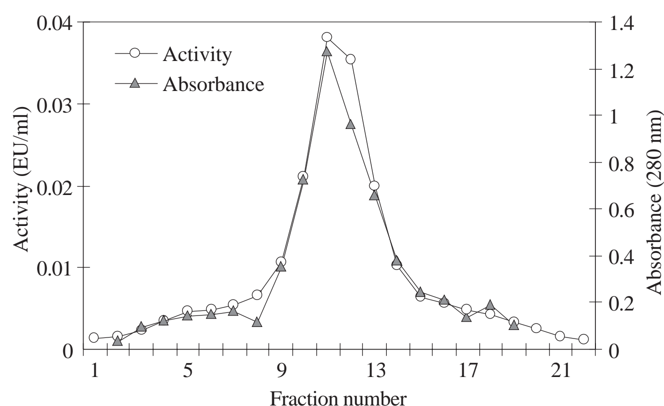

Figure 1. Elution profile of G6PD from 2′, 5′-ADP Sepharose 4B affinity column.

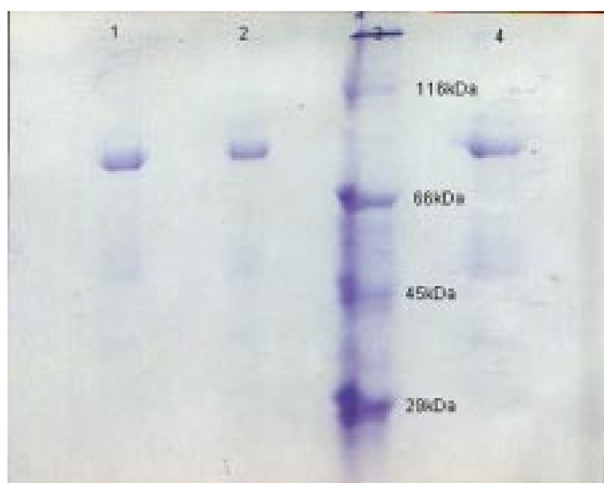

Figure 2. SDS-PAGE of G6PD purified by affinity gel. (Lane 1, 2 and 4: G6PD; Lane 3, Standard proteins; E.Coli β-galactosidase (MW 116,000 Da), bovine albumin (MW 66,000 Da), chicken ovalbumin (MW 45,000 Da), and bovine carbonic anhydrase (MW 29,000 Da).

Effect of Temperature and pH on Enzyme Activity

The effect of pH on G6PD was studied at pH values between 5.5 and 8.9. The optimal pH of G6PD was determined to be 8.0 using 1 M Tris-HCl (Figure 3a). The pH we determined was similar to that given in previous studies[14,19,20]. The effect of temperature was studied at temperature values between 25 and 60 °C. The highest activity was observed between 45 and 50 °C (Figure 3b). The optimum temperature we determined was similar to that given in previous studies[24,25].

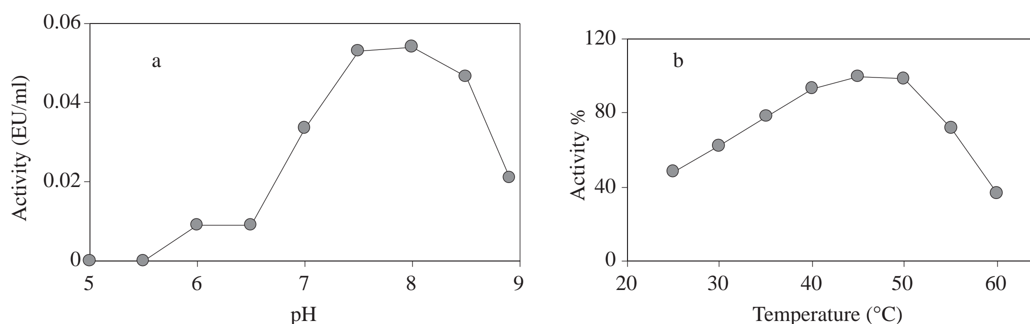

Figure 3. The effects of pH **(3a)** and temperature on the activity of G6PD **(3b)**.

Kinetic Parameters of G6PD

The Lineweaver-Burk plots shown in Figure 4, were constructed for G6-P and $NADP^+$. A K_M of 0.176 mM was obtained for G6-P, and 0.0194 mM for $NADP^+$. These K_M values are very similar to those obtained from dog liver[20], rat cortex and liver[25], and human placenta[26]. 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. A $V_{\max}$ of 0.0179 EU/ml (3.06 EU/mg protein) was obtained for G6-P, and 0.0223 EU/ml (3.818 EU/mg protein) for $NADP^+$. These $V_{\max}$ values are very similar to this obtained in bovine lens[19] but are lower dog liver[27] and in human placenta[23] G6PD.

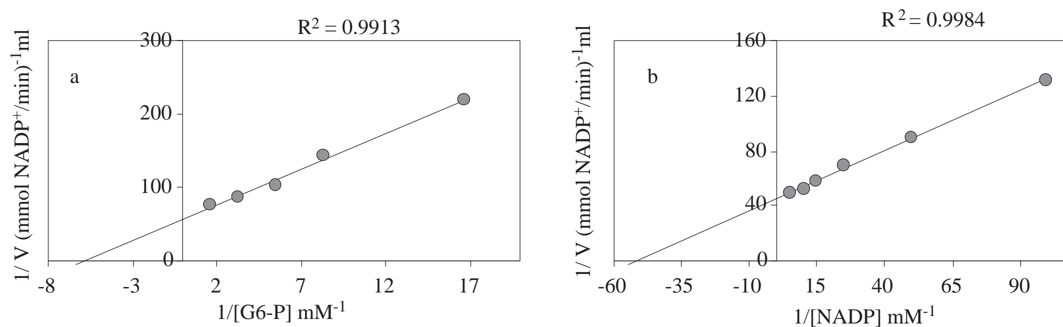

Figure 4. Lineweaver-Burk graphs in 6 different G6-P concentrations and in constant $NADP^+$ concentration **(4a)** and in 6 different $NADP^+$ concentrations and in constant G6-P concentration **(4b)**.

NADPH inhibits the enzyme in a non-competitive manner (Figure 5). The inhibition constant of NADPH was estimated to be 4.707 ± 0.49 μM. This value is similar to those from previous studies[20,25,27].

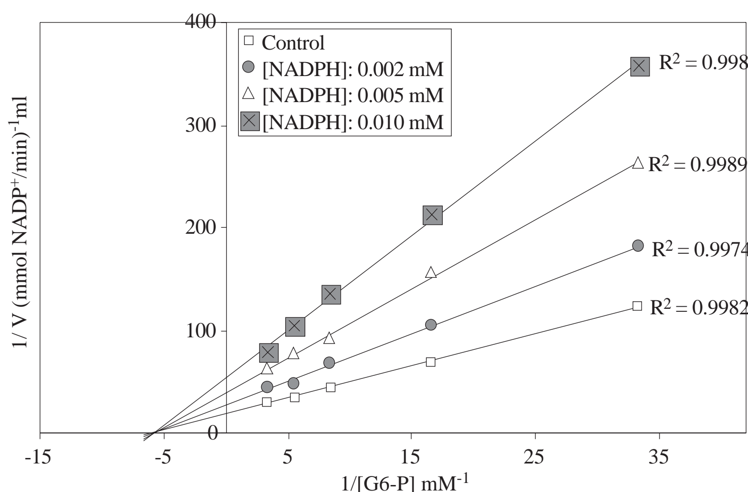

Figure 5. Lineweaver-Burk plot in 5 different substrat (G6-P) concentrations and in 3 different NADPH concentrations for determination of K_i for NADPH.

References

1. H.R. Levy, **Adv. Enzymol., 48,** 97-192 (1979).
2. M. Grunwald and H.Z. Hill, **Biochem J., 159,** 683-687 (1976).
3. J. Ozols, **P. Natl. Acad. Sci. USA, 90,** 5302-5306 (1993).
4. C. Schnarrenberger, A. Oeser and N.E. Tolbert, **Arch. Biochem. Biophys., 154,** 438-448 (1973).
5. G.T. Yüreğir, K. Aksoy, A. Arpacı, İ. Ünlukurt and A. Tuli, **Ann Clin Biochem., 31,** 50-55 (1994).
6. A.L. Lehninger, D.L. Nelson and M.M. Cox (eds.), Principles of Biochemistry, 2nd ed., New York, Worth Publishers Inc., 2000.
7. M.I. Kanji, M.L. Toews and W.R. Carpar, **J. Biol. Chem., 251,** 225-2258 (1976).

8. A. Yoshida, **J. Biol. Chem., 241,** 4966-4975 (1966).
9. A. Morelli, U. Benatti, G.F. Gaetani, A. De Flora and P. Natl, **Acad. Sci. USA, 75,** 1979-1983 (1978).
10. A. De Flora, A. Morelli, U. Benatti and F. Giuliana, **Arch. Biochem. Biophys., 169,** 362-363 (1975).
11. P. Ninfali, T. Orsenigo, L. Baronciani and S. Rapa, **Prep Biochem., 20,** 297-309 (1990).
12. I. Mohammed, M.L. Tuews and W.R. Carpar, **J. Biol. Chem., 251(8),** 2255-2257 (1976).
13. N. Muto and L. Tan, **J. Chromatogr., 326,** 137-146 (1985).
14. E. Beutler (ed.), Red cell metabolism Manual of biochemical methods, 1971, p 68. London, Academic Press., 1971.
15. M.M. Bradford, **Anal. Biochem., 72,** 248-251 (1976).
16. P. Andrews, **Biochem. J., 96,** 595-606 (1965).
17. U.K. Laemmli, **Nature, 227,** 680-683 (1970).
18. H. Lineweaver and D. Burk, **J. Am. Chem. Soc., 57,** 685 (1934).
19. N.N. Ulusu, M.S. Kus, L. Acan and E.F. Tezcan, **Int. J. Biochem. Cell B., 31,** 787-796 (1999).
20. C. Bilgi, İ. Ünsal, N. Özer, M.K. Erbil and L. Karaca, **Tr. J. of Medical Sciences., 24,** 291-295 (1995).
21. A.K. Çoban, M. Çiftçi and Ö.İ. Küfrevioğlu, **Prep. Biochem. Biotech, 32 (2),** 173-187 (2002).
22. H. Yılmaz, M. Çiftçi, Ş. Beydemir and E. Bakan, **Prep. Biochem. Biotech., 32(3),** 287-301 (2002).
23. Y. Aksoy, İ.H. Öğüs and N. Özer, **Protein Exp. Purif., 21,** 286-292 (2001).
24. D.S. Shreve and H.R. Levy, **Biochem. Biophys. Res. Com., 78,** 1369-1375 (1977).
25. F.J. Corpas, G.S. Leticia, J. Peragon and A. Lupianez, **Life Sciences. 56,** 179-189 (1995).
26. N. Özer, Y. Aksoy and İ.H. Ögüs, **Int. J. Biochem. Cell B., 33,** 221-226 (2001).
27. N. Özer, C. Bilgi and İ.H. Öğüs, **Int. J. Biochem. Cell B., 34(3)**, 253-262 (2002).