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Characterization of Glucose-6-Phosphate Dehydrogenase Purified From Lamb Kidney Cortex

[Kuzu Böbrek Korteksinden Saflaştırılan Glukoz-6-fosfat Dehidrogenazın Karakterizasyonu]

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ABSTRACT

This paper describes some properties of glucose-6-phosphate dehydrogenase, which is purified from lamb kidney cortex. Glucose-6-phosphate dehydrogenase catalyses the first step in the pentose phosphate pathway. The enzyme oxidizes glucose-6-phosphate to 6-phosphogluconate, reducing $NADP^+$ to NADPH. Glucose-6-phosphate dehydrogenase has been isolated from a large number of microorganisms, plants and animal tissues. The purification consisted of two steps, 2', 5'-ADP-Sepharose 4B affinity chromatography and DEAE Sepharose Fast Flow ion exchange chromatography. The molecular weight of the enzyme was found to be 67 kDa by Sephadex G-200 gel filtration chromatography. The activation energy of the reaction catalysed by the enzyme was calculated from the Arrhenius plot as 2.67 kcal/mole. Optimum pH of the enzyme was determined as pH 7.7.

Key Words: glucose-6-phosphate dehydrogenase, purification, molecular properties, lamb kidney cortex

ÖZET

Bu makalede kuzu böbrek korteksinden saflaştırılan glukoz-6-fosfat dehidrogenaz'ın bazı özellikleri tanımlanmaktadır. Glukoz-6-fosfat dehidrogenaz pentoz fosfat yolunun ilk basamağını katalizler. Enzim glukoz-6-fosfatı 6-fosfaglukonata oksitlerken, $NADP^+$ nın NADPH' a redüksiyonunu gerçekleştirir. Glukoz-6-fosfat dehidrogenaz birçok mikroorganizma, bitki ve hayvan dokularından izole edilmiştir. Saflaştırma yöntemi ultrasantrifügasyondan sonra 2', 5'-ADP Sepharose 4B affinite ve DEAE Sepharose Fast Flow iyon değiştirici kromatografisi olarak iki basamaktan oluşmaktadır. Enzimin moleküler ağırlığı Sephadex G-200 kromatografisi kullanılarak 67 kDa olarak bulundu. Enzim tarafından katalizlenen tepkimenin aktivasyon enerjisi 'Arrhenius' grafiğinden 2.67 kkal/mol olarak hesaplandı. Enzim için optimum pH 7.7 olarak belirlendi.

Anahtar Kelimeler: glukoz-6-fosfat dehidrogenaz, saflaştırma, moleküler özellikler, kuzu böbrek korteksi

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G-6-PD) (D-glucose 6-phosphate: $NADP^+$ 1-oxidoreductase EC 1.1.1.49) catalyses the oxidation of glucose 6-phosphate in the presence of $NADP^+$ and is the first enzyme in the pentose phosphate pathway. Pentose phosphate pathway provides ribose for nucleoside synthesis and NADPH for reductive biosynthetic reactions and maintenance of glutathione at reduced state (1). Protection against reactive oxygen species (ROS) requires maintenance of endogenous thiol pools, most importantly, reduced glutathione (GSH), by NADPH (2). NADPH is the main source of reducing power in various processes and has enormous physiological significance, including the biosynthesis of L-ascorbic acid, cholesterol, fatty acids, deoxyribonucleotide biosynthesis, and detoxification of xenobiotics, protection against oxidative stress and synthesis of nitric oxide (3). The enzyme was found to show highest activity in the mammary glands, adipose tissue and liver. G-6-PD has a significant activity in the kidney also (4). Because of the vital importance of this enzyme G-6-PD is characterized from various sources including human erythrocyte (1), rabbit liver lumenal endoplasmic reticulum (5), barley roots (6), and *M. Smegmatis* (7). DEAE cellulose, DEAE-Sephadex, CM-Sephadex, Sephadex G-200 and Hydroxyapatite columns have been used frequently for purification of G-6-PD (8, 9). Cibacron blue F3G-A Sephadex, Procion Red HE-3B-Sepharose (10), Procion Yellow HE-3G (11), Matrex gel Orange B (12) dye ligand affinity columns and nickel-chelate column as metal affinity chromatography are also used for purification of the G-6-PD (13). Nowadays, by employing $NADP^+$-Sepharose-4B (14) or 2′-5′-ADP-Sepharose 4B (15) as affinity columns, G-6-PD has been purified very rapidly in high yield. The chromatographic conditions for the purification of G-6-PD were optimised by varying the pH of the buffer; the concentrations of eluting agents, $NADP^+$ (specific elution) and sodium chloride (nonspecific elution); flow rate; retention time of the protein on the column bed; and protein load. Specific elution with $NADP^+$ gave the highest recovery and highest purification factor of the enzyme (16).

Thus, a considerable amount of information has been accumulated about structure, catalytic and regulatory functions of the enzyme and its variants (17 -20).

MATERIALS AND METHODS

Lamb kidney was obtained from a local slaughterhouse, kept in ice and processed within 2–3 h after death.

Tris [Tris (hydroxymethyl) aminomethane], glucose-6-phosphate dehydrogenase (G-6-P), $NADP^+$, 6-phosphogluconate (6-PGA), DEAE Sepharose Fast Flow, Sephadex G-200, were obtained from Sigma Chemical Co., MO, USA. 2', 5'-ADP-Sepharose 4B, Blue Dextran 2000 were from Pharmacia Fine Chemicals, Uppsala, Sweden. Bovine serum albumin (BSA) was from British Drug Houses Ltd.

All other chemicals were analytical grade and obtained from Sigma, USA.

Assay of Glucose-6-phosphate dehydrogenase

Enzyme activities were determined spectrophotometrically using a LKB Ultraspec Plus (4054 UV/visible) spectrophotometer, by monitoring the NADPH production at 340 nm and at 37°C (ε_{340} = 6.22 $mM^{-1}cm^{-1}$) (21). The assay mixture contained 10 mM $MgCl_2$, 0.2 mM $NADP^+$ and 0.6 mM G-6-P in 100 mM Tris/HCl buffer, pH 8.0. Assays were carried out in duplicate and the activities were followed for 60 seconds. The reaction was linear during this time period.

One unit (U) of activity is the amount of enzyme required to reduce one μmol of $NADP^+$ per min under the assay conditions. Specific activity is defined as units per mg of protein.

Assay of 6-phosphogluconate dehydrogenase

6-phosphogluconate dehydrogenase (6-PGD) activities were measured by substituting 0.6 mM 6-PGA as substrate in the assay mixture given above for G-6-PD measurement (22).

Since 6-PGD also catalyses the production of NADPH, in the earlier steps of the purification, both G-6-PD and 6-PGD activities were measured as a sum and the initial velocities of G-6-PD were calculated by subtracting the 6-PGD activities.

Protein assay

Protein concentrations in column fractions were determined measuring the absorbance at 280 nm and, protein concentrations of the purification steps were determined by Bradford's method (23) using bovine serum albumin as standard.

Purification of G-6-PD from lamb kidney cortex

We have previously described a purification method for G-6-PD from bovine lens (24). In this study we have used approximately the same method for the purification of G-6-PD from lamb kidney cortex. The purification procedure consisted of two steps after ultracentrifugation: 2', 5'-ADP Sepharose 4B affinity and DEAE Sepharose Fast Flow anion exchange chromatography steps. All the procedures were carried out at +4°C. The medulla of the kidney was removed and the cortex was minced with scissors after washing with serum physiologic and homogenized using an IKA ultra-turrax homogeniser with S18N-10G probe at 22 000 1/min approximately 3 minutes with 3 volumes of 10 mM Tris/HCI buffer, pH 7.6, containing 1 mM 2-mercaptoethanol and 1mM EDTA (buffer A). The homogenate was centrifuged at 105 000 × g for 60 min. The supernatant obtained was loaded onto 2', 5'-ADP-Sepharose 4B column (1.5 × 6.7

cm) equilibrated with buffer A. The column was washed with the same buffer (the flow rate was 10.8 ml/h) until the absorbance at 280 nm decreased to 0.021 to remove all the non-specifically bound compounds. 6-PGD did not bound to the affinity column and eluted while washing the column with buffer A. G-6-PD was eluted with buffer A containing 0.1 mM $NADP^+$ with a flow rate of 10.8 ml/h. Active G-6-PD fractions were combined and loaded onto DEAE Sepharose Fast Flow column (1.5 × 7.5 cm) equilibrated with 5 mM potassium phosphate buffer, pH 6.9 (buffer B). The flow rate was maintained at 16.8 ml/h and the column was washed with buffer B until the absorbance at 280 nm decreased to 0.003 O.D. and G-6-PD was eluted with buffer B containing 225 mM KCl.

Molecular weight determination

The molecular weight of the enzyme was estimated by Sephadex G-200 gel filtration chromatography according to the method of Andrews (25). The elution volume of the enzyme was determined by measuring the enzyme activity. The void volume of the column was measured by using Blue Dextran 2000.

RESULTS AND DISCUSSION

Purification of G-6-PD

This study present the characterisation of the G-6-PD purified from lamb kidney cortex. The purification procedure is slightly differs from the other purification methods. Although kidney G-6-PD has been previously partially purified from rat (26), rabbit (27), mouse (28) kidney cortex, and there is no report on lamb kidney cortex G-6-PD. For partial purification of G-6-PD from rat kidney-cortex, cytosol was obtained by high-speed centrifugation, 40-55 % ammonium sulphate fractionation and Sephadex G-25 chromatography were used (26). Rabbit microsomal enzyme was purified by hydroxyapatite, CM-Sepharose and finally 2', 5'-ADP-Sepharose 4B affinity chromatography steps (5). Human G-6-PD expressed in *Escherichia coli* was purified to homogeneity, using a simple one-step fractionation on 2', 5'-ADP-Sepharose (15). Two different isoforms of G-6-PD was partially purified from barley roots. The procedure included an ammonium sulphate step, Q-Sepharose and Reactive Blue agarose chromatography, and led to 60-fold and 150-fold purification for the two isoforms, respectively (6).

In this study a rapid procedure to purify G-6-PD from lamb kidney cortex is presented. 6-PGD did not bind to the affinty column and eluted with the run through fraction (Fig.1.), G-6-PD was eluted with buffer A containing 0.1 mM $NADP^+$. Elution profile of G-6-PD from DEAE Sepharose Fast Flow ion exchange chromatography is given in Fig.2. In purification procedure we prefered to use affinity column before ion exhange chromatography to get a high purification fold in a very short time. In

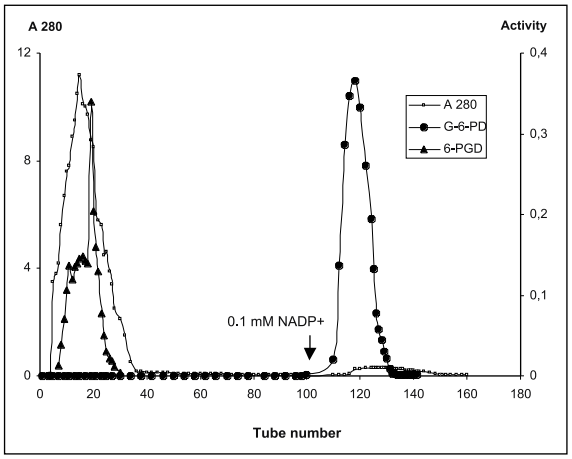

Figure 1. Affinity chromatography of G-6-PD and 6-PGD on 2', 5'-ADP-Sepharose 4B. Column size, 1.5 × 6.7 ml; column equilibration and washing buffer, 10 mM Tris/HCl pH 7.6 containing 1 mM 2-ME and EDTA. Enzyme elution buffer: same as the washing buffer containing 0.1 mM $NADP^+$; flow rate, 10.8 ml/h. Fractions of 1.12 ml were collected.

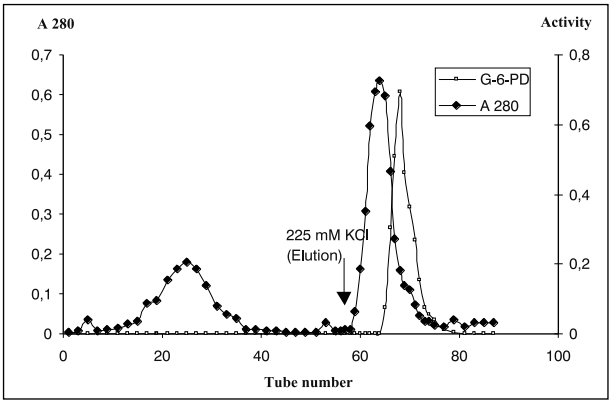

Figure 2. Elution profile of G-6-PD from DEAE Sepharose Fast Flow. Column size, 1.5 × 7.5 cm. G-6-PD was eluted with 225 mM KCl prepared in 5 mM potassium phosphate buffer, pH 6.9 at a flow rate of 16.8 ml/h.

addition, G-6-PD was separated well from 6-PGD at the end of the purification procedure. Also 6-PGD was not bind to the affinity column presumably because this enzyme has a lower affinity to the 2',5'-ADP Sepharose 4B column as compared G-6-PD. The enzyme was eluted with 225 mM KCl in buffer B. The whole purification procedure took three working days. After this two-step purification, the specific activity of the enzyme was determined to be 16.81 U/mg protein, and 4202-fold increase in the purity was obtained. The overall yield was about 26.4. A summary of a typical purification is presented in Table I.

Some properties of lamb kidney cortex G-6-PD

Optimum pH

G-6-PD was found to be active in a wide range of pH. The enzyme conveniently assayed between pH 6-10, using 10 mM potassium phosphate buffer. The optimum pH of the enzyme was determined to be between pH 7.6-7.8 (Fig. 3). This type of curve may be seen for polyprotic

Table I. Summary of a typical purification procedure.

Purification step	Volume (ml)	G-6-PD (U/ml)	Total G-6-PD (U/ml)	Protein (mg/ml)	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Homogenate	27	0.36	9.72	78.780	2127	0.004	100	1
105 000 x g supernatant	22	0.35	7.70	24.000	528	0.014	78.6	3.5
2',5'-ADP Sepharose 4B	15	0.26	3.90	0.140	2.10	1.85	39.8	462.5
DEAE Sepharose Fast Flow	7	0.37	2.59	0.022	0.15	16.810	26.4	4202

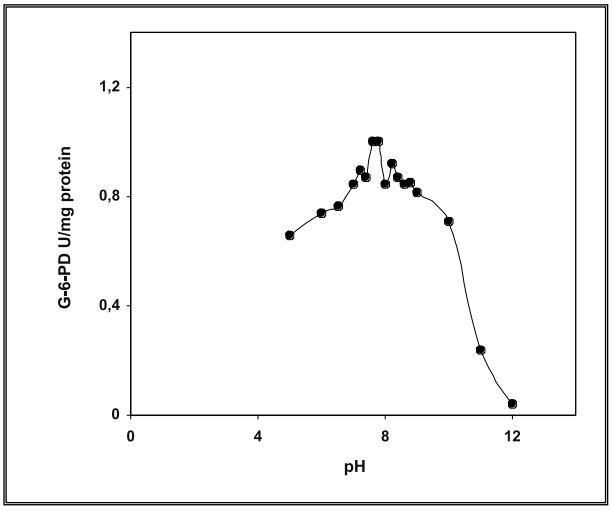

Figure 3. The effect of pH on G-6-PD activity. The assay was performed in 10 mM potassium phosphate buffer between pH 5 and pH 12.

systems and indicate that the active site of the enzyme contains several ionisable groups (29). In thermophilic bacterium *Aquifex aeolicus* optimum pH of the G-6-PD was determined to be between pH 3-10.5 at 25 °C (13). The enzyme purified from parsley (*Petroselinum hortense*) leaves, pH optima are found as 6.0, and 8.0 (30). The optimum pH of rat liver and kidney cortex G-6-PDH was 9.4 (26). In our previous study with the bovine lens enzyme we obtained more than one pH optimum (mainly at pH 7.7 and pH 9.6) (24).

Optimum temperature

We have found that the optimal temperature of the lamb kidney cortex G-6-PD is 45°C. But, in our experiments, we preferred to study at physiological temperature, 37°C. The optimal temperature of thermophilic bacterium *Aquifex aeolicus* G-6-PD was determined to be between 40°-105°C (13). Optimum temperature of G-6-PD from parsley (*Petroselinum hortense*) leaves was 60°C (30) and the optimum temperature of the lens G-6-PD was found to be 25°C. To obtain Arrhenius plot, the activities of the enzyme were measured between 20-50°C. Linearity shows that the enzyme conformation is stable between these temperatures (Fig. 4.). Activation energy (Ea) was determined from the slope of the plot as 2.67 kcal / mole. In our previous study Ea was determined to be 5.88 kcal/mol (24).

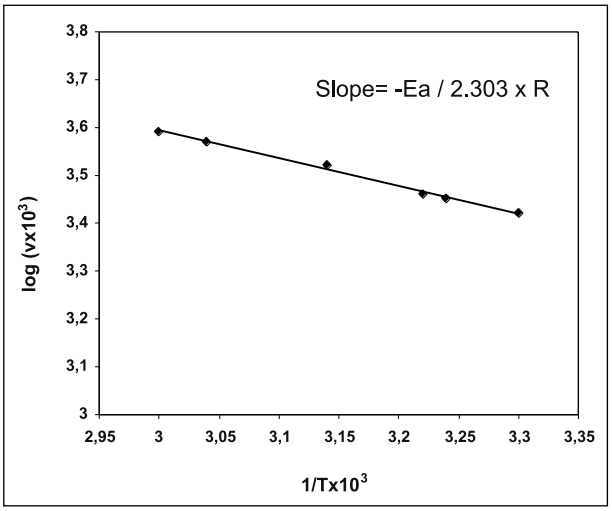

Figure 4. Arrhenius plot. Ea was determined from the slope of the plot as Ea= 2.67 Kcal / mole. The enzyme assays were carried out between 20 and 50°C.

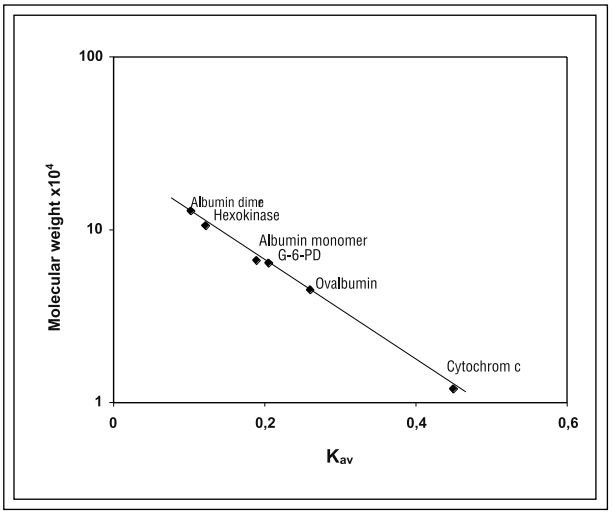

Figure 5. Estimation of the molecular weight of G-6-PD by gel filtration on Sephadex G-200. Column size, 1.7 × 55.5 cm; eluant buffer, 50 mM potassium phosphate, pH 7.5 containing 0.1 M KCl. The protein standards: yeast type VI hexokinase 105,000 Da; bovine serum albumin, monomer 66,000 Da, ovalbumin 45, 000, cytochrom c 12,384 The molecular weight of G-6-PD was found to be 67,000 Da.

Molecular weight

Estimation of the molecular weight of G-6-PD from bovine lens by gel filtration on Sephadex G-200 was found to be 62 ± 3 kDa (25). G-6-PD subunit molecular weight from *Candida boidini* has been calculated as 61 kDa (31). In this study the molecular weight of the enzyme was estimated as 67 kDa by Sephadex G-200 gel filtration chromatography (Fig. 5.). In our gel filtration chromatography study, only a single protein peak is found as G-6-PD. This also shows the purity of the enzyme.

The results presented here are in concurrence with the previously published results obtained from different sources.

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