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Research Article

6-Phosphogluconate Dehydrogenase: Purification, Characterization and Kinetic Properties from Rat Erythrocytes

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Abstract: In this paper, a simple and rapid method for the purification of 6-phosphogluconate dehydrogenase from rat erythrocytes together with an analysis of the kinetic behavior and some properties of the enzyme are considered. The purification steps comprised high-speed centrifugation, 20-50% ammonium sulfate precipitation and 2', 5'-ADP Sepharose 4B affinity gel chromatography. The yield was 78.4% and the specific enzyme activity was 5.15 EU/mg proteins. The molecular mass of the subunit was estimated to be 59,566 Da by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and native enzyme was found to be 111,000 Da by gel filtration column chromatography. The enzyme had an optimal pH at 7.0 and stable pH at 8.0 in 1 M Tris-HCl buffer, and optimal temperature at 45 ºC. K_M and V_{max} for $NADP^+$ and 6-PGA as substrates were also determined. The inhibitor effects of ATP, NADPH and NADH were also examined, and K_i values and the types of inhibition were determined by means of a Lineweaver-Burk graph obtained for them.

Key Words: 6PGD, rat, erythrocyte, purification, kinetic properties

6-Fosfoglukonat Dehidrogenaz: Sıçan Eritrositlerinden Saflaştırılması, Karakterizasyonu ve Kinetik Özellikleri

Özet: Bu çalışmada 6-fosfoglukonat dehidrogenaz'ın sıçan eritrositlerinden saflaştırılması için basit ve hızlı bir metod ile birlikte bu enzimin kinetik davranışı ve özelliklerinin analizi çalışılmıştır. Saflaştırma basamakları yüksek hızda santrifügasyon, % 20-50 amonyum sülfat çöktürmesi ve 2', 5'-ADP Sepharose 4B afinite jel kromatografisini içerdi. Verim % 78,4 oldu ve enzimin spesifik aktivitesi 5,15 EU/mg protein olarak bulundu. Altbirim molekül kütlesi SDS-poliakrilamid jel elektroforezi (SDS-PAGE) vasıtasıyla 59,566 Da olarak hesaplandı ve enzimin tabii halinin molekül kütlesi jel filtrasyon kolon kromatografisi ile 111,000 Da olarak bulundu. Enzim 1 M Tris-HCl tampon çözeltisi içinde pH: 7,0'da optimum, pH: 8,0'da stabil pH'ya ve 45 ºC'de optimum sıcaklığa sahipti. Aynı zamanda $NADP^+$ ve 6-PGA substratları için K_M and V_{max} değerleri belirlendi. ATP, NADPH ve NADH'ın inhibitör etkileri incelendi, K_i değerleri ve inhibisyon tipleri bu inhibitörler için belirlenen Lineweaver-Burk grafikleriyle saptandı.

Anahtar Sözcükler: 6PGD; sıçan; eritrosit; saflaştırma; kinetik özellikler

Introduction

6-Phosphogluconate dehydrogenase (E.C.1.1.1.44; 6PGD) is the third enzyme of the pentose phosphate metabolic pathway, catalyzing the conversion of 6-PGA (6-phosphogluconate) to D-riboluse-5-phosphate in the presence of $NADP^+$ (1). The reaction, catalyzed by 6PGD, yields NADPH, which protects the cell against oxidant agents by producing reduced glutathione (GSH) (2,3). For this reason, 6PGD can be defined as an antioxidant enzyme (4,5). On account of the important role of the enzyme in the pentose phosphate metabolic pathway, it has been extensively studied and purified from numerous sources including sheep liver, *Dicentrarchus labrax* L. liver, rabbit mammary gland, *Drosophila melanogaster*,

the human brain, plants and microorganisms (6-12). The enzyme has also been purified from human erythrocytes, but has not been studied from rat erythrocytes.

6PGD was first isolated from a mammalian tissue by Villet and Dalziel. They found a seemingly homogeneous preparation from sheep liver. In subsequent years, the enzyme was purified from ion-exchange materials by using the DEAE-Sephadex, CM-Sephadex, DEAE-Cellulose and DEAE-Toyopearl column (6,10,13).

In this report we describe the purification of 6-phosphogluconate dehydrogenase from rat erythrocytes (using 2', 5'-ADP Sepharose 4B affinity column) by a simple and rapid method. With our procedure, the enzyme can be purified within a very short time and with a good yield. In addition, we also state some of its kinetic properties in order to gain a better understanding of the function of the enzyme in this organism.

Materials and Methods

Chemicals and reagents. 2', 5'-ADP Sepharose 4B was purchased from Pharmacia. $NADP^+$, 6-phosphogluconate, and protein assay reagent were purchased from Sigma. All other chemicals used were analytical grade and purchased from either Sigma or Merck.

Preparation of the hemolysate

Fresh blood samples from 6 rats, collected one by one into EDTA-containing tubes, (6 samples) were centrifuged (15 min, 2,500 x g) and plasma and buffy coat (leucocytes) were 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 ^{o}C, 10,000 x g, for 30 min) to remove the ghosts and intact cells (14-16). All steps were carried out at 4 ^{o}C.

Ammonium sulfate precipitation

Ammonium sulfate precipitation was performed according to Ciftci et al. (1).

2', 5'-ADP Sepharose 4B Affinity Chromatography

Two grams of dried 2', 5'-ADP Sepharose 4B gel was used for 10 ml column volume. The gel was washed with 400 ml of distilled water to remove foreign bodies and air was eliminated from the swollen gel. The gel was suspended in 0.1 M K-acetate/0.1 M K-phosphate buffer (pH 6.0) and then packed in a small column (1 x 10 cm) and equilibrated with the same buffer (50 ml). The gel was washed with equilibration buffer. The flow rates for washing and equilibration were adjusted by peristaltic pump at 50 ml/h. The dialyzed sample obtained previously was loaded on 2', 5'-ADP Sepharose 4B affinity column and the flow rate was adjusted to 20 ml/h. Then the column was sequentially washed with 20 ml of 0.1 M K-acetate + 0.1 M K-phosphate (pH: 6.0) until the final absorbance difference became 0.05. Elution was carried out with 80 mM K-phosphate + 80 mM KCl + 10 mM $NADP^+$ + 10 mM EDTA (pH 7.5). The enzyme activity was measured in final fractions, and the activity-containing tubes were collected together. All of the procedures were performed at 4 ^{o}C (17,18).

Activity determination

The enzymatic activity was measured according to Beutler's method (14).

Protein determination

During the purification steps, protein levels were determined spectrophotometrically (595 nm) according to the Bradford method, using bovine serum albumin as the standard (19).

Optimal pH determination

For the optimal pH determination, the enzyme activity was measured in 1 M Tris-HCl and phosphate buffers within a pH 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 a pH of 7.0, 8.0, 8.5 and 9.0, and in 1 M phosphate buffer at a pH of 5.0, 6.0, 7.0 and 8.0. In each experiment, equal volumes of buffer and enzyme solutions were mixed and kept refrigerated (+4 ^{o}C). Activity was determined at 8 h intervals for 32 h.

The effect of temperature on 6PGD activity

The enzyme activity was measured between 20 ^{o}C and 70 ^{o}C at optimal pH for this purpose.

Molecular Mass Determination

Sephadex G-200 gel filtration chromatography

Molecular mass of the native enzyme was determined according to the Andrews method (20). The void volume was observed with Blue Dextrane (2,000 kDa).

SDS-PAGE

The determination of the apparent molecular mass of the subunit, using Laemmli's procedure, was carried out

in 3% and 8% acrylamide concentrations for running and stacking gel, respectively (21).

Kinetic studies

For K_M and V_{max} evaluation, Lineweaver-Burk curves were used (22), obtained in 5 different concentrations of $NADP^+$ (0.02, 0.04, 0.10, 0.13 and 0.20 mM) and in the constant concentration of 6-PGA, and the same experiments were performed for 6-PGA (in 5 different concentrations of 6-PGA; 0.06, 0.18, 0.30, 0.40 and 0.60 mM), and in a fixed $NADP^+$ concentration (14). All kinetic studies were performed at 25 °C and at optimal pH (1 M Tris-HCl, pH: 7.0).

The effect of inhibitors

To determine K_i values, ATP, NADPH and NADH were used as inhibitors. For each inhibitor, 3 different concentrations were tested (ATP: 1.0, 3.0 and 4.0 mM; NADPH: 0.060, 0.100 and 0.140 mM; NADH: 0.025, 0.050 and 0.100 mM). In these experiments, 6-PGA was used as substrate in 5 different concentrations (0.06, 0.18, 0.30, 0.42 and 0.60 mM for NADPH; 0.006, 0.018, 0.030, 0.042 and 0.060 mM for ATP, and NADH). The Lineweaver-Burk curves obtained were used for the determination of K_i and inhibitor type (22). Analysis of the data obtained was made by t test, and was given as ± SD.

Results

The enzyme was purified 2575-fold with a specific activity of 5.15 EU/mg protein, 78.4% yield, by the combination of ammonium sulfate fractionation (20-50%) and 2', 5'-ADP Sepharose 4B affinity gel chromatography (Table 1). Figure 1 shows the SDS-PAGE performed for the purity and subunit molecular weight of the enzyme. For the standard proteins and 6PGD, R_f values were calculated, and a R_f -Log MW graph (Figure 2) was obtained according to Laemmli procedure, showing a molecular mass of 59,566 Da for 6PGD. The molecular mass of the native enzyme was also determined by gel filtration chromatography. A K_{av}-log MW graph was obtained (Figure 3), which showed a molecular mass of 111,000 Da for 6PGD. Optimal pH of 6PGD was determined as 7.0 using 1 M Tris-HCl (Figure 4). The stable pH of the enzyme was 8.0 in Tris-HCl (Figure 5). The enzyme was seen to show the highest activity at 45 °C (Figure 6).

The Lineweaver-Burk graphs are shown in Figures 7 and 8, which were constructed for $NADP^+$ and 6-PGA. A K_M of 0.059 mM and a V_{max} of 0.063 EU/ml were obtained for $NADP^+$, and 0.194 mM and 0.054 EU/ml for 6-PGA (Table 2).

In addition, K_i constants were 2.500 ± 0.866 mM, 0.052 ± 0.007 mM, and 0.070 ± 0.007 mM for ATP, NADPH and NADH, respectively (Table 3) (Figures 9,10 and 11).

Discussion

The importance of G6PD and 6PGD in metabolism has been well known for many years. This is because NADPH generation is provided by the activities of the 2 enzymes in the oxidative phase of the pentose phosphate metabolic pathway. Therefore, we think that the easier purification methods of 6PGD enzyme make it possible that investigations into the subject can be easily performed.

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

Purification Steps	Activity (EU/ml)	Total volume	Protein (mg/ml) (ml)	Total protein	Total activity (mg)	Specific activity (EU/mg)	Yield (%)	Purification fold
Hemolysate	0.025	20	11.98	236.8	0.500	0.002	100	1
Ammonium sulfate precipitation (20-50)%	0.040	10	0.2550	2.55	0.40	0.157	80	78.500
2',5'- ADP Sepharose 4B chromatography	0.098	4	0.019	0.076	0.392	5.150	78.400	2575

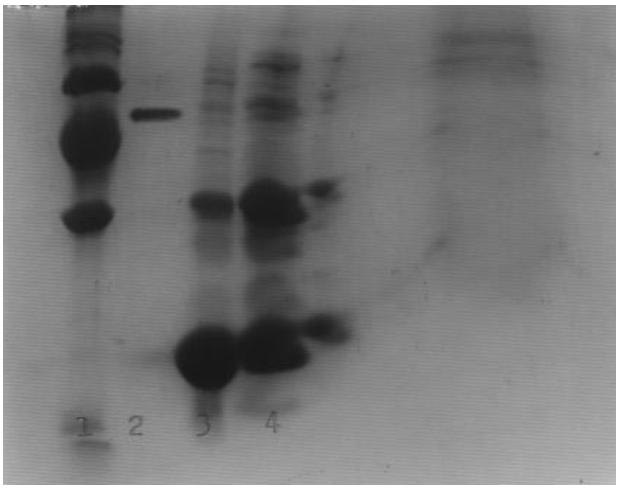

Figure 1. SDS-PAGE bands of 6PGD (Lane 1: Standard proteins; Lane 2: 6PGD; Lane 3: Ammonium sulfate precipitation; Lane 4: Hemolysate).

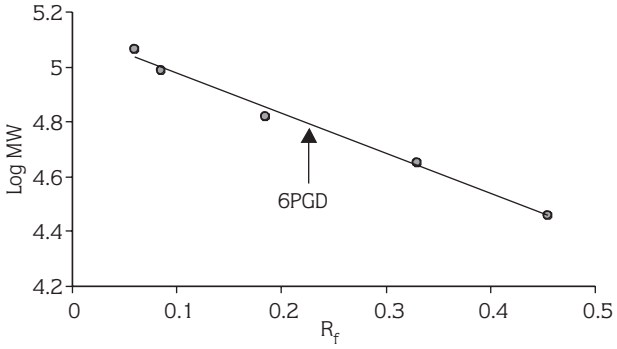

Figure 2. Standard Rf-Log MW graph of 6PGD using SDS-PAGE. (Standards: *E. coli* β-galactosidase (116,000), rabbit phosphorylase B (97,400), bovine albumin (66,000), chicken ovalbumin (45,000) and bovine carbonic anhydrase (29,000).

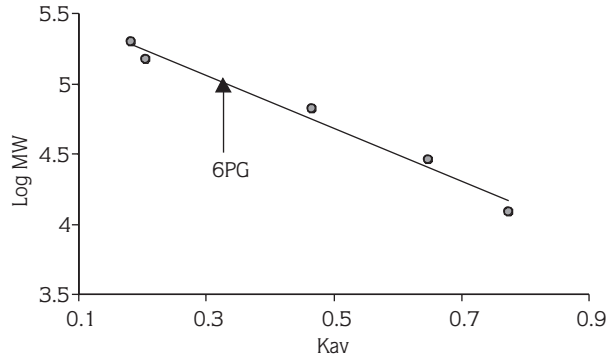

Figure 3. Standard Kav-Log MW graph of 6PGD using gel filtration. (Standards: horse heart cytochrome C (12,400), bovine erythrocyte carbonic anhydrase (29,000), bovine serum albumin (66,000), yeast alcohol dehydrogenase (150,000) and sweet potato b-amylase (200,000).

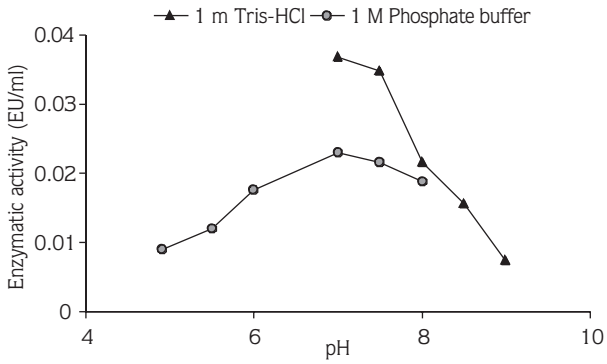

Figure 4. Activity-pH graph of 6PGD.

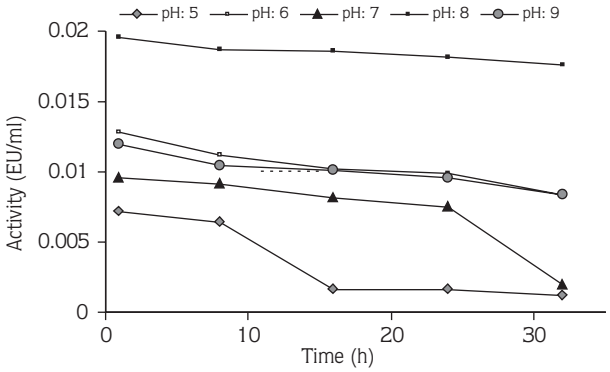

Figure 5. Activity % - [Drug] graph for stable pH graphs of 6PGD in 1 M Tris-HCl buffer. in presence of ceftriaxone.

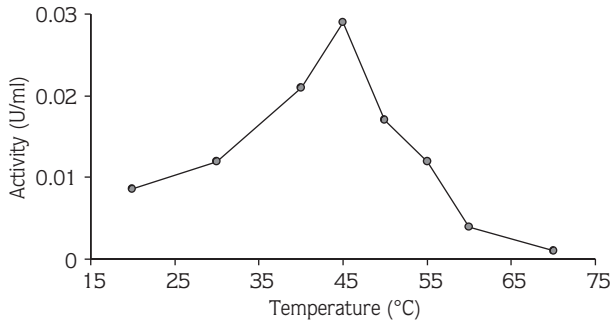

Figure 6. The effect of temperature on 6PGD.

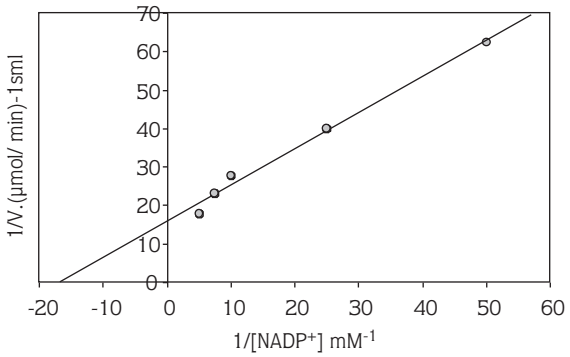

Figure 7. Lineweaver-Burk graph in 5 different $NADP^+$ concentrations and in constant 6-PGA concentration.

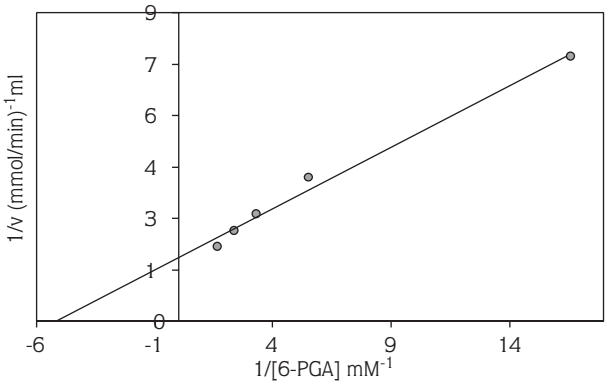

Figure 8. Lineweaver-Burk graph in 5 different 6-PGA concentrations and in constant $NADP^+$ concentration.

Table 2. K_m and V_{max} constants for 6-PGA and $NADP^+$.

Source	$K_M^{6\text{-PGA}}$(mM)	K_M^{NADP+}(mM)	$V_{max}^{6\text{-PGA}}$(EU/ml)	V_{max}^{NADP+}(EU/ml)
Rat 6PGD	0.194	0.059	0.054	0.063

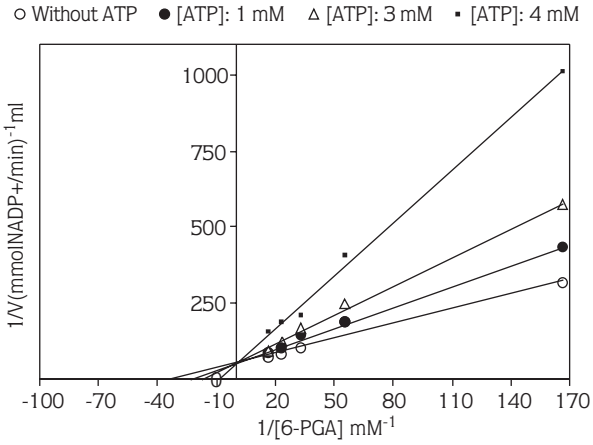

Figure 9. Lineweaver-Burk graph in 5 different substrate (6-PGA) concentrations and in 3 different ATP concentrations for determination of K_i for ATP.

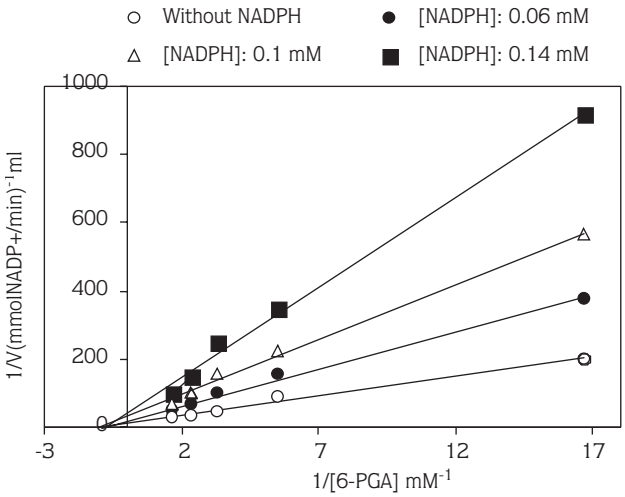

Figure 10. Lineweaver-Burk graph in 5 different substrate (6-PGA) concentrations and in 3 different NADPH concentrations for determination of K_i for NADPH.

In the present study, 6-phosphogluconate dehydrogenase was purified by ammonium sulfate precipitation and 2', 5'-ADP Sepharose 4B affinity gel chromatography. 6-Phosphogluconate dehydrogenase was clearly separated from glucose-6-phosphate dehydrogenase by ammonium sulfate precipitation. The enzyme was purified 2575-fold in 5 or 6 h with a 78.4% yield by the combination of ammonium sulfate fractionation (20-50%) and 2', 5'-ADP Sepharose 4B affinity gel chromatography (Table 1). The procedure is very fast and efficient. Previously, investigators used several consecutive steps to purify the enzyme. Sokolow et al. (23) used 4 successive steps for purification: ammonium sulfate fractionation, Sephacryl-200 gel filtration, and chromatography on DEAE Bio-Gel and phosphocellulose. At the end of these steps the enzyme

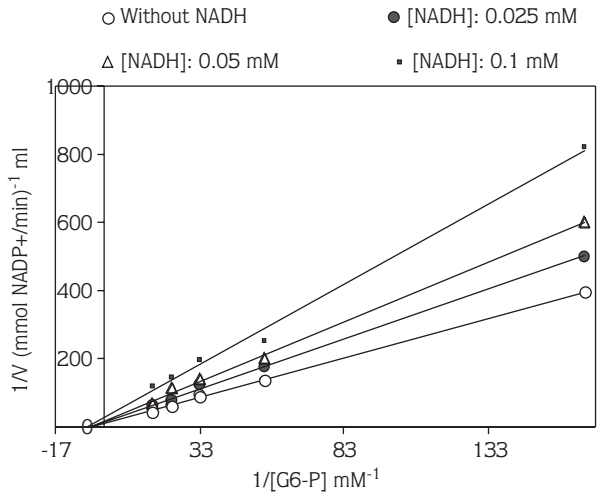

Figure 11. Lineweaver-Burk graph in 5 different substrate (6-PGA) concentrations and in 3 different NADH concentrations for determination of K_i for NADH.

Table 3. K_i constants for ATP, NADPH and NADH.

Inhibitor	K_i (mM)	Inhibition type
ATP	2.50 ± 0.866	Competitive
NADPH	0.052 ± 0.007	Noncompetitive
NADH	0.07 ± 0.007	Noncompetitive

was purified 400-fold with a 78.4% yield. Purification of human brain 6PGD was performed 530-fold with a 6% yield using ammonium sulfate fractionation, DEAE-cellulose column, Sephacryl-200 gel filtration, Red A matrix gel column and ultrafiltration (10). In another study, Sosa-Saavedra et al. (24) used 6 steps for purification (350-fold with a 27% yield): ammonium sulfate precipitation, thermal treatment, Sephadex G-200, DEAE Sephacel (eluted with a KCl gradient and eluted with a pH gradient) and Cibachrom Blue Sepharose. All these steps take a long time and thus result in a decline in enzyme activity during the procedure. For this reason, we omitted several steps. After the hemolysate was precipitated with ammonium sulfate, the sample was directly applied to 2', 5'-ADP Sepharose 4B column. However, in the present study the enzyme can be purified from 20 ml of whole blood within 5 or 6 h. The purification degree and yield were higher than those of previous studies (8,10-12,23,24). In addition, the advantage of the purification method suggested in the study is that it is less time-consuming and of low cost.

SDS-PAGE of the rat erythrocyte 6PGD displayed a single band and the subunit size of the enzyme was estimated to be 59,566 Da (Figures 1 and 2). The molecular weight of the native enzyme was estimated by Sephadex G-200 gel filtration column chromatography at 111,000 (Figure 3). This indicates that the enzyme is composed of 2 subunits. The molecular weight of rat 6PGD is similar to those reported for the enzyme extracted from *Dicentrarchus labrax* L. liver, rabbit mammary gland, *Drosophila melanogaster*, and rat liver (7-9,25).

The purified enzyme activity was measured in 1 M Tris-HCl and 1 M phosphate buffer at various pH values (Figure 4), and rat 6PGD showed a sharp pH profile around 7.0 in 1 M Tris-HCl, distinct from the enzymes from *Corynebacterium glutamicum,* rat liver and kidney cortex. The pH determined was similar to *Dicentrarchus labrax* L. liver and sheep liver (7,26). To determine the stable pH of the purified enzyme, 6-phosphogluconate dehydrogenase was incubated for 32 h in 1 M Tris-HCl (pH 7.5-8.9) and 1 M phosphate (5.0-8.0) buffers at +4 °C. Enzyme activity was measured every 8 h, and the stable pH of the enzyme was determined as 8.0 in Tris-HCl (Figure 5). In Figure 5 it seems that there is a significant difference between pH 8.0 and the others, and the enzyme activities were higher at pH 8.0 than at the others at all time points. The enzyme was seen to show the highest activity at 45 °C (Figure 6) after tests between 20 and 70 °C.

The apparent K_M and V_{max} values of rat 6PGD for 6-PGA and $NADP^+$ were determined by Lineweaver-Burk graphs (Figures 7 and 8) and (Table 2). The K_M for $NADP^+$ is lower than that for 6-PGA, suggesting the higher affinity of 6PGD to $NADP^+$ when compared with 6-PGA. K_M values are very similar to those obtained in rabbit mammary gland, *Drosophila melanogaster*, and Phormidium sp. (8,9,13). In contrast, Corpas et al. (27) reported that the K_M for $NADP^+$ is higher than that for 6-PGA in rat liver and kidney cortex. In addition, K_i values were calculated for ATP, NADPH and NADH from Lineweaver-Burk graphs and these are given in Table 3. The data given in Figure 9 show that ATP inhibits the enzyme in a competitive manner; NADPH and NADH inhibit it noncompetitively (Figure 10 and 11). NADPH is an important product of reaction, and catalyzed 6PGD.

Consequently, the effect of inhibition of NADPH on this enzyme activity is noteworthy for the important metabolic states related to the pentose phosphate pathway. Because NADPH's K_i value is the smallest, its affinity to 6PGD is the highest (Table 3). K_i values obtained for NADPH are similar to those of *Dicentrarchus labrax* L. liver (7). K_i values for the other inhibitors could not be compared since no study was encountered.

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