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Kinetic mechanism and some properties of glucose-6-phosphate dehydrogenase from sheep brain cortex

[Koyun beyin korteks glukoz-6-fosfat dehidrogenazının kinetik mekanizması ve bazı özellikleri]

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ABSTRACT

Objective: Glucose-6-phosphate dehydrogenase is an important key enzyme which catalyzes the first oxidative step of the pentose phosphate pathway. The objectives of this study are to determine some properties and kinetic mechanism of glucose-6-phosphate dehydrogenase.

Method: In this study we used the enzyme that was purified from sheep brain cortex. The kinetic analysis and other studies were performed within three days after the purification to prevent any loss of enzyme activity.

Results: The subunit molecular weight was determined as 61.2 kDa by SDS-PAGE and 64.5 kDa by Sephadex G-200 gel filtration chromatography. The activation energy (Ea) of the reaction was calculated from the Arrhenius plot as 6.689 kcal/mol. We have calculated the temperature coefficient as 1.913 from the integrated form of the Arrhenius equation (for the temperatures between 30 - 40 ºC). Optimum pH of the enzyme was determined as 8. Kinetic constants estimated by a nonlinear curve-fitting program are as follows: Vm= 2.182 ± 0.064 mmol/min/mg enzyme; Km_{NADP+} = 0.064 ± 0.001 mM; Km_{G6P} = 0.0729 ± 0.006 mM; Ki_{G6P} = 0.074 ± 0.003 mM; Ki_{NADP+} = 0.056 ± 0.001mM.

Conclusion: Sheep brain cortex glucose-6-phosphate dehydrogenase exhibits classical Michaelis-Menten kinetics. Product inhibition studies were undertaken to distinguish the type of the mechanism. We have found that G6PD enzyme obeys 'Theorell-Chance' mechanism. The kinetic behavior, molecular weight, optimum temperature and pH of the enzyme were in accordance with our previous studies.

Key Words: Glucose-6-phosphate dehydrogenase, temperature coefficient, product inhibition**,** Michaelis-Menten kinetics**,** Theorell-Chance mechanism, molecular weight

Conflict of Interest: The authors have declared that no conflict of interest exists.

ÖZET

Amaç: Glukoz-6-fosfat dehidrogenaz pentoz fosfat metabolik yolunun birinci oksidatif basamağını katalizleyen önemli bir enzimdir. Bu makalenin amacı glukoz-6-fosfat dehidrogenaz enziminin kinetik mekanizmasını ve bazı özelliklerinin saptanmasıdır.

Yöntem: Bu çalışmamızda koyun beyin korteksinden saflaştırılmış olan glukoz-6-fosfat dehidrogeneaz enzimini kullandık. Saflaştırılan enzimin yapısal ve kinetik özelikleri enzimde aktivite kaybı olmadan üç gün içinde saptandı.

Bulgular: Enzimin moleküler ağırlığı Sephadex G-200 kromatografisi kullanılarak 64.5 kDa, SDS poliakrilamid jel elektroforezinde ise 61.2 kDa olarak saptandı. Enzim tarafından katalizlenen tepkimenin aktivasyon enerjisi 'Arrhenius' grafiğinden E_a, 6.689 kcal/mol olarak hesaplandı. Arrhenius denkleminden sıcaklık katsayısını 1.913 olarak hesapladık (30 - 40 ºC arasındaki sıcaklıklar için). Optimum pH 8 olarak belirlendi. Enzimin kinetik parametreleri: Vm = 2.182 ± 0.064 mmol/dak/mg enzim; Km_{NADP+} = 0.064 ± 0.001 mM; Km_{G6P} = 0.073 ± 0.006 mM; Ki_{G6P} = 0.074 ± 0.003 mM; Ki_{NADP+} = 0.056 ± 0.001mM olarak hesaplandı.

Sonuç: Koyun beyin korteks glukoz-6-fosfat dehidrogenazı klasik Michaelis-Menten kinetiğine uyduğu saptandı. Ürün inhibisyonu deneyleri ile mekanizmanın 'Theorell-Chance' mekanizmasına uyduğu saptandı. Kinetik mekanizma, molekül ağırlığı, optimum sıcaklık ve pH bulgularının önceki çalışmalarımızla uyumlu olduğu görüldü.

Anahtar Kelimeler: Glukoz-6-fosfat dehidrogenaz, sıcaklık katsayısı, inhibisyon**,** Michaelis-Menten kinetiği**,** Theorell-Chance mekanizması, molekül ağırlığı

Çıkar Çatışması: Hiçbir çıkar çatışmamız olmadığını bildiririz.

Introduction

Glucose-6-phosphate dehydrogenase (G6PD; E.C. 1.1.1.49) is the first and the rate-limiting enzyme of the pentose phosphate pathway. This anabolic pathway is responsible for the reduction of $NADP^+$ to $NADPH+H^+$, synthesis of ribose 5-phosphate and sugar phosphates. $NADPH + H^+$ is one of the essential components, which is used for the numerous biosynthetic reactions and protection of the cells from free radical damage [1]. Ribose 5-phosphate is precursor of nucleic acids, flavin coenzymes FMN, FAD and CoA. In some cellular conditions; the pentose phosphate pathway ends with synthesis of this sugar phosphate [1, 2]. G6PD deficiency is the most common enzymopathy, which is an X-linked hereditary disease resulting in nonimmune hemolytic anemia, jaundice, neonatal jaundice, drug or infection-mediated hemolytic crisis, favism and, less commonly, chronic non-spherocytic hemolytic anemia. G6PD have significant effects on erythrocyte function and survival in human [3, 4]. Because of the enzymes' critical and vital role in maintaining the cellular redox status in red cell metabolism, the studies on G6PD deficiency was documented in about five thousand of manuscripts by now. On the other hand, few molecular and kinetic studies have been reported.

There are many factors affecting the enzyme rate. The factors affecting the kinetic mechanisms should be clarified for fully understanding the properties of G6PD. Kinetic mechanisms of an enzyme may change from one organism to another. The purpose of this study is to analyze the kinetic mechanism and some properties of G6PD from sheep brain cortex.

Materials and Methods

Chemicals:

Glucose-6-phosphate (G6P), nicotinamide adenine dinucleotide phosphate ($NADP^+$), 6-phosphogluconate (6- PGA), and DEAE Sepharose Fast Flow, Sephadex G-200, Tris [Tris (hydroxymethyl) amino methane] were obtained from Sigma Chemical Co., MO, USA. 2-, 5-ADP Sepharose 4B is from Pharmacia Fine Chemicals, Uppsala, Sweden. Bovine serum albumin (BSA) is from British Drug Houses Ltd. Potassium phosphate and orthophosphoric acid are from BDH Chemical (Poole, UK). All other chemicals were analytical grade and obtained from Sigma, USA.

Enzyme:

G6PD was purified as previously described (5-8). The method included 2', 5'-ADP- Sepharose 4B affinity chromatography and DEAE Sepharose Fast Flow ion exchange chromatographies after ultracentrifugation.

The enzyme was obtained with a yield of 68.33% and had a specific activity of 51.25 U/mg protein. The overall purification was approximately 9440-fold. The activity of the purified enzyme was stable at 4 °C more than one week. Therefore, the kinetic analysis and other structural studies were performed within three days after purification before any loss of enzyme activity.

Assays of G6PD

Enzyme activities were determined spectrophotometrically using an LKB Ultraspec Plus (4054 UV/visible) spectrophotometer, by monitoring the NADPH production at 340 nm at 37 °C [9]. The assay mixture contained 10 mM $MgCl_2$, 0.2 mM $NADP^+$ and 0.6 mM G6P in 100 mM Tris/HCl buffer, pH 8.0 in 500 μl. The assay was initiated by addition of the enzyme. Assays were carried out in duplicate and the activities were followed for 60 s. The reaction was linear during this time. In the kinetic studies, the assays were performed in 10 mM potassium phosphate buffer at pH 8 and 37 °C temperature. One unit (U) of activity is the amount of enzyme required to reduce 1 μmol of $NADP^+$ per minute under the assay conditions. Specific activity is defined as units per milligram of protein. The rate of the reduction of $NADP^+$ was calculated using $\varepsilon_{340} = 6.22\ mM^{-1}.cm^{-1}$.

Measurement of protein concentration

Protein concentrations were determined by the method of Bradford [10] using BSA as a standard. Enzyme activities and protein content were determined spectrophotometrically using an Ultraspec 2100 Pro UV/visible spectrophotometer, (Amersham, Biosciences).

Statistical Analysis of Kinetic Data

The data was analyzed and the kinetic constants were calculated by means of a nonlinear curve-fitting program Statistica, StatSoft Version 9.1, USA.

Results

We have previously described a purification protocol for G6PD from various tissues; bovine lens, sheep and lamb kidney cortex [6-8]. In this study, we have determined the kinetic mechanism and few properties of the G6PD which was purified from sheep brain cortex [5].

Molecular weight, optimal temperature, pH

We have determined the molecular weight of the sheep brain cortex G6PD by two methods. In the first method the molecular weight of the enzyme was estimated by Sephadex G-200 gel filtration according to the method of Andrews [11]. The void volume of the column was measured by using Blue Dextran 2000. The molecular weight of the G6PD was calculated as 64.5 kDa by gel filtration method. In the second method SDS-polyacrylamide gel electrophoresis on 10 % acrylamide was used [12]. We have calculated the molecular weight of G6PD as 61.2 kDa.

To obtain Arrhenius plot, the activities of the sheep brain cortex G6PD initial velocities was measured between 30ºC and 55ºC. From the slope of the log v versus 1/T

plot Ea (activation energy) was determined to be, 6.689 kcal/mol (Figure 1). It is known that the velocity of any homogenous chemical reaction depends on the frequency of collisions between molecules. Arrhenius equation given by [13]:

($k=A.e^{-Ea/R.T}$)

or it can be written in linear form:

$$\log k = \frac{-Ea}{2.303R} x \frac{1}{T} + \log A$$

where *Ea* is activation energy, *T* is temperature (Kelvin), *R* is the gas constant (1.98 cal/ mole. degree), *A* is the Arrhenius constant, *k* is the reaction rate constant.

Temperature coefficient shows the increase in reaction rate with a 10°C rise in temperature. We have calculated Q_{10} as 1.913 from the integrated form of the Arrhenius equation (for the temperatures between 30 - 40 ºC). We have chosen this interval because of the moderate temperature for the full biological function of the enzyme.

The integrated form of the equation one is:

$$\log Q = \frac{10 x E_a}{2.303 x R x T x T_2}$$

We have determined the $\Delta H^{\ddagger}$ (activation enthalpy) of sheep brain cortex G6PD's from the formula, $\Delta H^{\ddagger} = E_a - R \times T$ as 6.026 kcal/mol [13].

For the optimum pH determination, the enzyme activity was measured in the presence of 10 mM potassium phosphate buffer within the pH range 5-12. The enzyme was found to be stable at pH 5 - 12 and the optimal activity was at pH 8 (Figure 2).

Kinetic studies

In this study we have measured the initial velocities to determine kinetic characterization of enzyme. The data were analyzed and the kinetic constants were calculated by means of a nonlinear curve-fitting program of the Statistica. We have used a wider substrate (G6P and $NADP^+$) concentration range to determine if the enzyme is allosteric. Detailed kinetic analyses indicated that sheep brain cortex G6PD exhibit classical Michaelis-Menten kinetics (Figure 3). It has been reported in previous studies that many factors are affecting the kinetic properties of G6PD such as; pH, ionic strength, temperature, substrate concentration and properties of the enzyme (purified enzyme or crude extract) [14, 15]. We have observed classical kinetics under our assay conditions.

The main aim of this study was to determine the kinetic mechanism of the sheep brain cortex G6PD so we had to observe and analyze the effects of substrate depletion and product accumulation in our assay system. Lineweaver-Burk plots obtained by: first holding [G6P] constant and determining the initial velocities when the second substrate [$NADP^+$] is varied. Then, holding [$NADP^+$] constant and changing [G6P] and determined the initial velocities (Figure 4). Afterward, we have established the Km and Vm values of the sheep brain cortex G6PD, which are shown in Table 1.

First equation:

$$\frac{v}{Vm} = \frac{[A][B]}{Km_B[A] + Km_A[B] + [A][B]}$$

Second equation:

$$\frac{v}{Vm} = \frac{[A][B]}{Kia \cdot Km_B + Km_B[A] + Km_A[B] + [A][B}$$

According to the criteria of Mannervik, loss function of

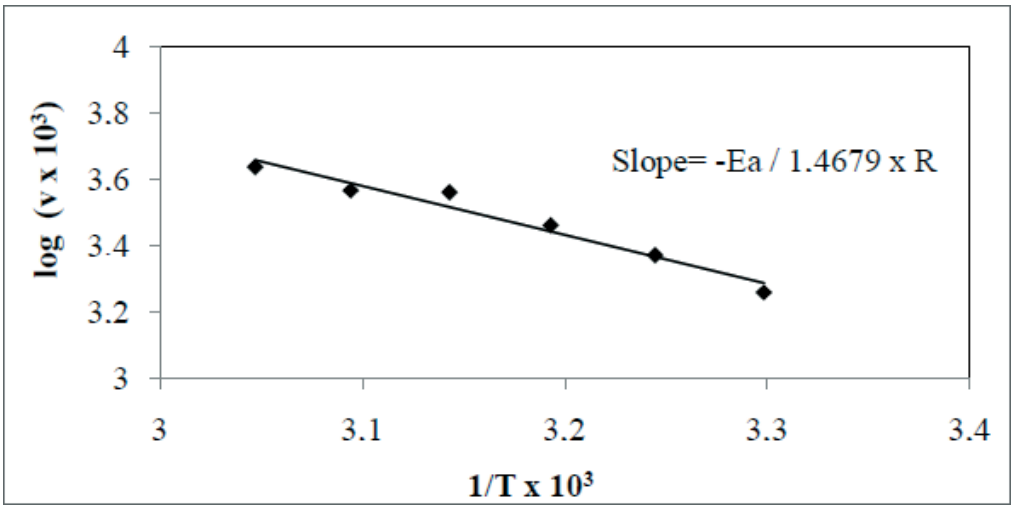

Figure 1. Arrhenius plot. G6PD activities were carried out by using 0.2 $NADP^+$ and 0.6mM G6P final concentration between between 30ºC and 55ºC.

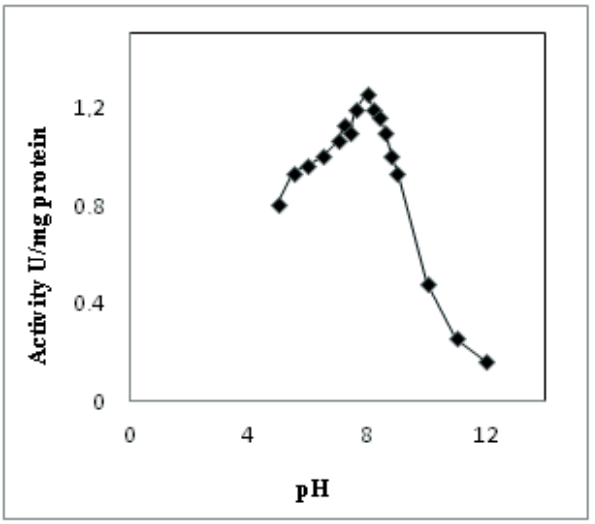

Figure 2. Optimum pH plot. The assay was performed in 10mM potassium phosphate buffer between pH 5 and pH 12.

the best fitted mechanism must be the lowest among the models tested [16]. However in this study the loss functions obtained are so close to each other. Therefore we have done inhibition studies to conclude definitely on the type of the kinetic mechanism.

Product inhibition patterns to distinguish different kinetic mechanisms are given in Table 2. Furthermore, we have determined the kinetic mechanism of the G6PD from the product inhibition plots (Figure 5 and 6). Product inhibition studies revealed that sheep brain cortex G6PD follows a Theorell-Chance mechanism. In this mechanism enzyme follows a sequential reaction mechanism in which two substrates react to form two products, where the substrates bind and products are released in an ordered fashion as seen in figure 7.

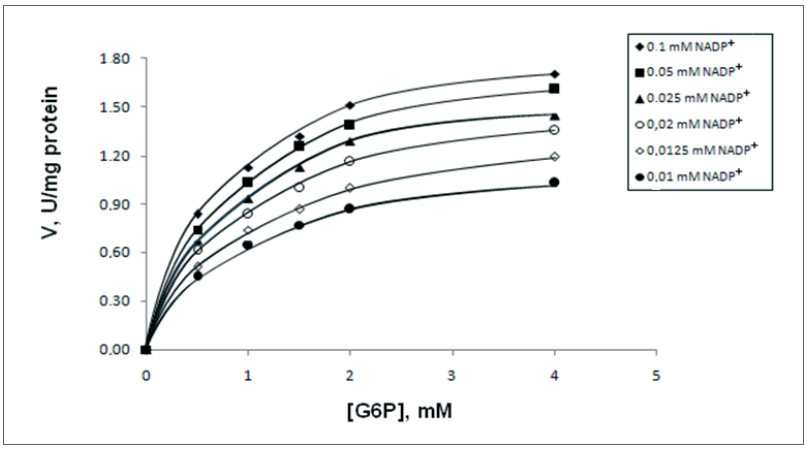

Figure 3. Michaelis-Menten plot of initial velocity against G6P as varied substrate at different fixed $NADP^+$ concentrations for the reaction catalyzed by G6PD from sheep brain cortex. The velocities were determined in 100 mM Tris/HCl buffer, pH 8.0. Michaelis-Menten plot of initial velocity against G6P as varied substrate (0.05 - 0.4 mM). Full diamond 0.1 mM $NADP^+$, full square 0.05 mM $NADP^+$, full triangle 0.025mM $NADP^+$, empty circle 0.02 mM $NADP^+$, empty diamond 0.0125 mM $NADP^+$, full circle 0.01 mM$NADP^+$.

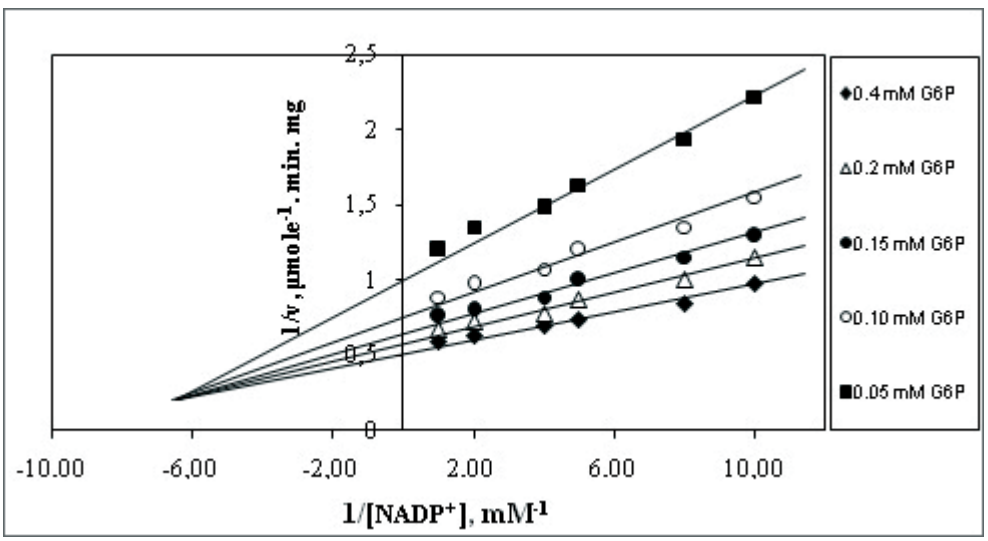

Figure 4. Double-reciprocal plot of initial velocity against $NADP^+$ as varied substrate at different fixed G6P concentrations for the reaction catalyzed by G6PD from sheep brain cortex. The velocities were determined in 100 mM Tris/HCl buffer, pH 8.0. Full diamond 0.4 mM G6P, empty triangle 0.20 mM G6P, full circle 0.15 mM G6P, empty circle 0.10 mM G6P, full square 0.05 mM G6P.

Table 1. Regression analysis of the kinetic data of G6PD from sheep brain cortex.

Mechanism	Kinetic values	Loss value
Ping-Pong Bi Bi	*Vm = 2.55 ± 0.105 **Km $_{NADP+}$ = 0.0123 ± 0.001 **Km $_{G6P}$ = 0.120 ± 0.010	0.082
Sequential mechanisms	*Vm = 2.182 ± 0,064 **Km $_{NADP+}$ = 0.064 ± 0.001 **Km $_{G6P}$ = 0.073 ± 0.006 **Ki $_{G6P}$ = 0.074 ± 0.003 **Ki $_{NADP+}$ = 0.056 ± 0.001	0.023

*(mmol/min/mg enzyme)
**mM

Table 2. Product inhibition patterns for the reactions of catalyzed by G6PD from sheep brain cortex.

Kinetic mechanism	Product (Inhibitor)	Varied substrate A (G6P) Saturated substrate B ($NADP^+$)	Varied substrate B ($NADP^+$) Unsaturated substrate A (G6P)
Ordered Bi Bi			
	6-PGA (P)	UC	MT
	NADPH (Q)	C	-
Theorell-Chance			
	6-PGA (P)		C
	NADPH (Q)	C	
Random Bi Bi			
	6-PGA (P)	MT	MT
	NADPH (Q)	MT	MT

Note: UC: uncompetitive; C: competitive; MT: mixed type; A: as a first substrate B: as a second substrate

G6PD activities were measured at saturated or unsaturated substrate concentrations. Then we have examined the product inhibition types. Mixed type is key indicator for random Bi Bi; uncompetitive inhibition type is for ordered Bi Bi and finally competitive is for Theorell-Chance mechanisms which were mentioned in Table 2. Product inhibition by 6PGA when $NADP^+$ was used as varied substrate at unsaturated [G6P] showed a competitive inhibition. The product 6PGA concentrations were; 0.4, 0.6, 0.8 mM in the absence of NADPH. Ki was found to be 1.96 ± 0.001mM (Figure 5). Therefore kinetic mechanism of the sheep brain cortex G6PD cannot be Ordered Bi Bi mechanism because we get competitive inhibition. We have to do distinctive and characteristic experiments. We planned to use G6P as varied substrate, at a constant saturated 0.2 mM $NADP^+$ along with the product NADPH at 0.025, 0.05, 0.075, 0.1mM concentrations. It is apparent that NADPH acts as a competitive inhibitor (Figure 6). Therefore kinetic mechanism cannot be Random Bi Bi. The data were analyzed by means of a nonlinear curve fitting program of the statistical software package Statistica and with Ki_{NADPH} calculated as 0.042 ± 0.002 mM. Sheep brain cortex G6PD obeys 'Theorell-chance' mechanism. We have concluded the type of the kinetic mechanism as Theorell-Chance from the product inhibition pattern.

Discussion

This paper describes some properties G6PD, which is purified from sheep brain cortex. In this study, we have also analyzed the kinetic mechanism of the enzyme.

We determined molecular weight of the enzyme by two different methods; SDS-PAGE electrophoresis and Sephadex G-200 gel filtration chromatography. Sheep brain cortex G6PD subunit molecular weight was determined as 61.2 kDa by SDS-PAGE and 64.5 kDa by using Sephadex G-200 gel filtration. According to our findings it appears that sheep brain cortex G6PD is found in monomeric form. But, it is generally accepted that G6PD is catalytically active in dimeric and tetrameric forms of the identical subunits [17, 18]. However, enzyme may be dissociated into its monomers during gel filtration chromatography because of various

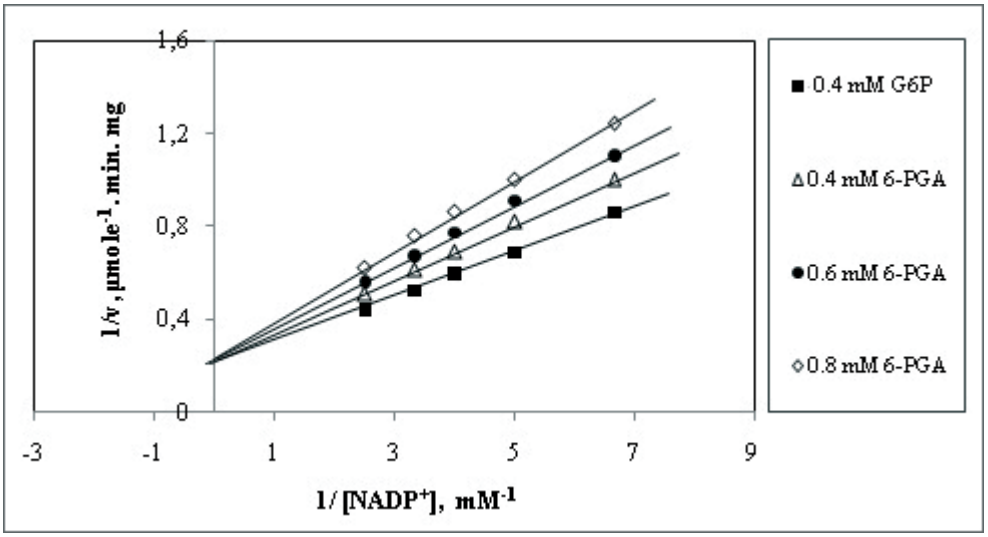

Figure 5. Product inhibition of G6PD from sheep brain cortex by 6PGA: 1/v versus 1 / [$NADP^+$] plot at different fixed 6PGA concentrations at a constant nonsaturating G6P concentration in the absence of NADPH. The velocities were determined in 100 mM Tris/HCl buffer, pH 8.0. Full square 0.4mM G6P (no inhibitor), empty triangle 0.4 mM 6-PGA, full circle 0.6 mM 6-PGA, empty diamond 0.8 mM 6-PGA.

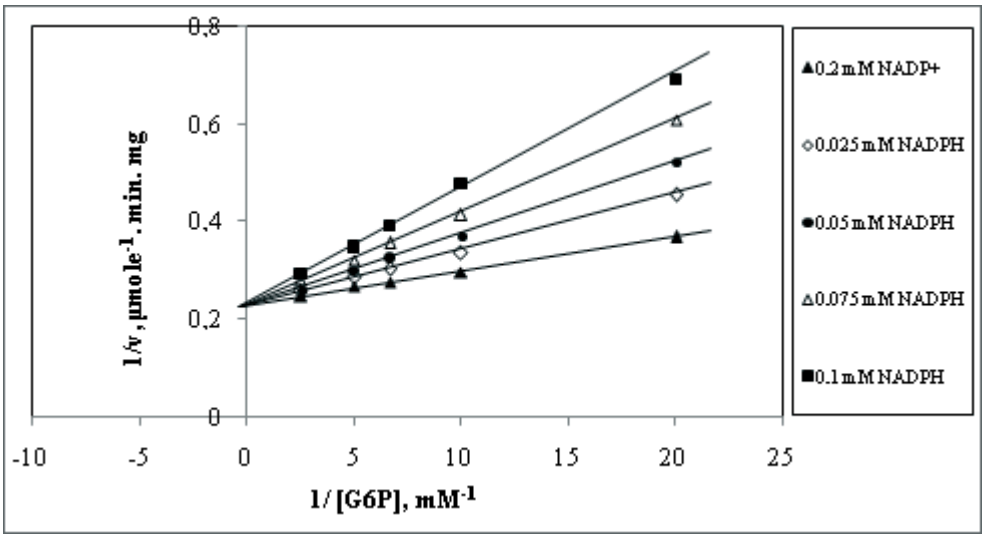

Figure 6. Product inhibition of G6PD from sheep brain cortex by NADPH: 1/v versus 1 / [G6P] plot at different fixed NADPH concentrations at a constant saturating $NADP^+$ concentration in the absence of 6PGA. The velocities were determined in 100 mM Tris/HCl buffer, pH 8.0. Full triangle 0.2 mM $NADP^+$ (no inhibitor), empty diamond 0.025mM NADPH, full circle 0.05mM NADPH, empty triangle 0.075 mM NADPH, full square 0.1 mM NADPH.

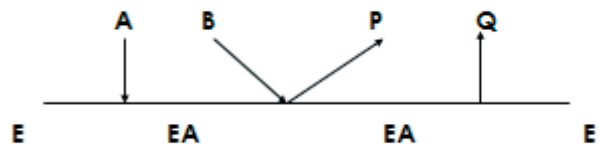

Figure 7. Theorell-Chance mechanism

factors such as; dilution of the enzyme sample, ionic factors (salt) or physical effects. The molecular weight of the G6PD from sheep brain cortex was in accordance with our previous studies [6-8].

Arrhenius graph was a linear, between 30 to 55 °C which proves that there is no conformational change within the assay conditions. An additional finding of this study is the calculation of temperature coefficient, Q_{10} as 1.913. The effect of temperature on an enzyme catalyzed reaction is frequently expressed in terms of a temperature coefficient, Q_{10} [13]. The activation enthalpy of G6PD from sheep brain cortex by the formula in [13] was calculated as 6.026 kcal/mole and these data are consistent with previously calculated data from other sources by other studies [19, 20].

The optimum pH of the sheep brain cortex was also examined and was determined as pH 8. We have determined the initial velocities to determine the kinetic characterization of enzyme. These kinetic analyses indicated that sheep brain cortex G6PD exhibit Michaelis-Menten, classical kinetics. The enzymes which obey Michaelis-Menten kinetics have multiple,

but independent substrate binding sites: the binding of one substrate molecule has no effect on the vacant sites of enzyme which indicates that there is no cooperativity of substrate with the enzyme [21]. The G6PD from the heterocystous cyanobacterium, Anabaena sp. PCC 7120 [22], human erythrocytes [23], exhibit classical kinetic. Another study showed that sigmoid kinetics were observed for both purified and crude human erythrocyte enzyme samples at high pH, temperature, ionic strength, and high concentration of G6P. On the contrary, at low pH, temperature, and ionic strength, the crude enzyme samples exhibit sigmoid kinetics although the purified enzyme exhibit classical kinetics despite the high concentration of G6P. The assay conditions such as high pH, ionic strength, and temperature affected tetramer-dimer transitions of the enzyme [17].

G6PD catalyzes the transformation of G6P to 6-phosphogluconolactone and reduction of co-enzyme nicotinamide adenine dinucleotide phosphate to NADPH by different kinetic mechanisms in different organisms. It has been shown that G6PD catalyzes the reaction by ordered Bi Bi mechanism in *Schizosaccharomyces pombe* [24] mouse liver [25] lamb kidney cortex [26] *Corynebacterium glutamicum* [27] *Acetobacter hansenii* (formerly known as *Acetobacter xylinum*) [28] bovine lens [6]. Random Bi Bi mechanism is investigated in *Plasmodium falciparum* [29], *Taenia crassiceps* (Eucestoda: Cyclophyllidea) [30], *Aspergillus aculeatus*, a filamentous fungus [31], *Aspergillus niger* and *Aspergillus nidulans* [32]. In our previous study we have found that sheep kidney cortex G6PD was to operate according to a Ping Pong Bi Bi mechanism. In the same study we have suggested that the kinetic mechanism was changing by the age [8]. Human erythrocyte G6PD was shown to follow either a random or a Theorell-Chance mechanism [33]. *Leuconostoc mesenteroides* G6PD can catalyze the reaction by NAD^+ or $NADP^+$. Levy and his colleague established in their study that kinetic behavior of *Leuconostoc mesenteroides* changes according to the kind of its coenzyme. They defined that enzyme catalyze the reaction with random mechanism with NAD^+ but an ordered, sequential mechanism in the company of $NADP^+$ linked reaction [34].

Sheep brain cortex G6PD obeys 'Theorell-chance' mechanism. In Theorell-Chance mechanism, no ternary EAB (E.G6P.$NADP^+$) complex is specified, and the reaction behaves as if EA (E.G6P) and B ($NADP^+$) react to form P (6PGA) without B ($NADP^+$) binding to the enzyme. In reality, EAB (E.G6P.$NADP^+$) and EPQ (E.6PGA.NADPH) complexes likely exist transiently at very low concentrations that are kinetically not significant [35]. Furthermore it was formerly observed that, the dimeric G6PD follows either a Random or a Theorell-chance mechanism [34]. The observed kinetic behavior of G6PD from sheep brain cortex is in accordance with the enzyme from other sources [27, 33].

Conclusion

G6PD is providing alternative reaction routes in different organisms. Besides the clinical importance due to its common enzymopathy, the major role of G6PD is reduction of NADPH, which is indispensable for reductive metabolism and maintenance of cellular redox homeostasis. Perhaps another important property is the increase of G6PD activity in cancer tissues. For these reasons we have to know all the properties of this key enzyme perfectly. Numerous kinds of studies have to be done to understand every aspect of this enzyme.

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Conflict of interest: The authors have declared that no conflict of interest exists.

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