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Purification, Characterization and Kinetic Properties of Glucose 6-Phosphate Dehydrogenase from *Polygonum cognatum* Meissn Leaves

HÜLYA DEMIR*, SÜKRÜ BEYDEMIR† and MEHMET ÇIFTÇI†
Department of Chemistry, Atatürk Meslek Yüksekokulu, Gazi University, Ankara, Turkey
Tel: (90)(533)2809520; E-mail: h.demir@gazi.edu.tr

In the present studies, the isolation, purification and kinetic properties of glucose-6-phosphate dehydrogenase (G6PD) in the *Polygonum cognatum* Meissn leaves were investigated. The purification procedure was composed of three steps *viz.*, homogenate preparation, ammonium sulfate precipitation and DEAE-Sephadex A50 ion exchange chromatography. The enzyme, having the specific activity of 1.896 EU/mg proteins, was purified with a yield of 57.6 % and 124.08 fold at 4 ºC. Stable pH, optimum pH, optimum temperature, subunit molecular weight, native form molecular weight, K_m and ν_{max} values for $NADP^+$ and glucose 6- phosphate (G6-P) substrates were also determined for the enzyme. Enzymatic activity was spectrophotometrically measured according to Beutler's method at 340 nm.

Key Words: *Polygonum cognatum* Meissn, Glucose 6-phosphate dehydrogenase, Characterization.

INTRODUCTION

Polygonum cognatum Meissn called 'madimak' in Turkish is an edible wild plant. It is a widely grown plant and is used as a vegetable at cuisines[1]. This plant grows on roadsides, slopes and cliffs at altitudes of 720-3000 m[2,3]. In Turkish folk medicine the plant has been used for various purposes, for example its diuretic effects and for the treatment of diabetes mellitus[1,3,4].

The cells have four major NADPH production systems corresponding to the activities of four cytoplasmic enzymes: Glucose 6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD) belonging to the pentose phosphate pathway; malic enzyme (ME) and NADPH depended isocytrate dehydrogenase (NADP-IDH)[5].Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: $NADP^+$ oxidoreductase, EC 1.1.1.49; G6PD) is the key enzyme, which take place in oxidative phase of hexose monophosphate

†Department of Chemistry, Arts and Science Faculty, Atatürk University, 25240, Erzurum, Turkey.

shunt (HMPS) and catalyzes the oxidation of D-glucose 6-phosphate to D-glucose-O-lactone 6-phosphate in the presence[6,7] of $NADP^+$.

It is reported that G6PD is found in animal tissues, plants and microorganisms[8-10]. In animal tissues, the enzyme is localized in cytosol and mitochondria and in green plants in cytosol and chloroplast[7,11,12]. The principal source of cytoplasmic NADPH in many cells is the hexose monophosphate shunt and specifically the two deydrogenase glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. NADPH is widely used in biosynthesis and also plays an important role in maintaining glutathione in the reduced state, in drug detoxification and in the removal of peroxides. Ribose 5-phosphate that plays a role in the nucleotide biosynthesis is one of the important product of this metabolic pathway[13-16].

G6PD has been purified and characterized a number of plant tissues up to now such as parsley (*Petroselinum hortense*) leaves[17]. Chloroplastic isozyme from spinach and from pea has already been partially characterized and purification from pea chloroplasts has been reported[18]. In addition, purification, characterization and cDNA sequence of this enzyme was investigated from potato by Graeve *et al.*[19]. It is not known about the properties and purification procedure of *Polygonum cognatum* Meissn leaves G6PD.

In this work, the detailed purification, characterization of the $NADP^+$-dependent glucose 6-phosphate dehydrogenase enzyme from *Polygonum cognatum* Meissn leaves by means of ammonium sulphate fractionation and on DEAE-Sephadex A50 ion exchange chromatography, some molecular properties and kinetic behaviour of enzyme, were described.

EXPERIMENTAL

$NADP^+$, glucose 6-phosphate, DEAE-Sephadex A50, protein assay reagent and chemicals for electrophoresis were purchased from Sigma Chem. Co. All other chemicals used for analytical grade were purchased from either Sigma or Merck.

Preparation of the homogenate: *Polygonum cognatum* Meissn plants with leaves were harvested in May in the Erzurum. The leaves of plants were harvested and stored frozen in plastic bags until use. The homogenate was prepared from frozen 10 g plant leaves by homogenizing in liquid nitrogen with a minimal volume of buffer 0.1 M *tris*-maleat (pH 8.0) (about 0.6 g/mL). The homogenate was filtered through four layers of cheesecloth and pulverized polyethylene glycol (4/100 mL) was added. After being stirred for 20 min the insoluble material was removed by centrifugation[18] at 13,000 × g for 0.5 h.

Ammonium sulfate precipitation: The homogenate was subjected to precipitation with ammonium sulfate (between 0 and 60 %). Solid ammonium sulfate was slowly added to homogenate and stirred up to 0.5 h ot 4 ºC for

completely dissolution. This mixture was centrifuged at 15,000 × g for 0.5 h and pellet was resuspended in a minimum volume of 0.1 M of *tris*-maleat buffer (pH 8.0). The enzyme activity was determined both in supernatant and in precipitate for each respective precipitation. The enzyme was observed to precipitate at 20-40 % precipitation step. The resultant solution was clear and contained partially purified enzyme.

Dialysis: The obtained partially purified enzyme was dialyzed at 4 ºC in 0.1 M *tris*-maleat buffer (pH 8.0), for 2 h with two changes of buffer.

Ion exchange chromatography: Ion exchange chromatography material was prepared from DEAE-Sephadex A50. 10 g dry DEAE-Sephadex A50 gel was used for 50 mL column volume. The gel was to blow up with distilled water at the 80-90 ºC and to remove foreign bodies and air of swollen gel was eliminated. The gel was suspended in 0.1 M *tris*-maleat buffer (pH 8.0), then packed in a column (3 cm × 30 cm) and equilibrated and washed with same buffer. The flow rates for washing and equilibration were adjusted[18] by peristaltic pump 25 mL/h.

Purification of glucose 6-phosphate dehydrogenase: The G6PD enzyme, dialyzed and filtered, was loaded on DEAE-Sephadex A50 column and the gel was washed with 0.1 M *tris*-maleat buffer (pH 8.0) until the absorbance of column eluate at 280 nm is < 0.05. Bound protein was eluted with a gradient of 0 to 500 mM sodium chloride in 0.1 M *tris*-maleat buffer (pH 8.0) at 20 mL/h flow rate. Eluates were collected in 2 mL tubes and their activity and absorbance were separately measured at 340 nm and 280 nm, respectively. Active fractions were collected. All of the procedures[18] were performed at 4 ºC.

Activity determination: The enzymatic activity was measured by monitoring the change in absorbance at 340 nm due to the reduction[20] of $NADP^+$. In this spectrophotometric measurement, the reaction medium maintained at 25 ºC contained 0.1 mM *tris*-maleat (pH = 8.0) with 0.5 mM EDTA, 10 mM $MgCl_2$, 0.2 mM $NADP^+$ and 0.6 mM (G6-P) in a total volume of 1 mL. One unit of enzyme (EU) activity was defined as the enzyme amount reducing 1 m mol $NADP^+$ per 1 min at 25 ºC, pH 8.0.

Protein determination: Protein concentration was determined spectrophotometrically (595 nm) using method of Bradford[21] with bovine serum albumin as the protein standard.

Stable pH determination: For this purpose, the enzyme activity was determined in 0.1 M *tris*-maleat buffer at pH of 6.0, 7.0, 7.5, 8.0 and 8.5. In each experiment, the equal volumes of buffer and enzyme solutions were mixed and kept refrigerated (at 4 ºC). Activity determinations were made with an interval of 4 h for 12 h.

Optimum pH determination: For the optimum pH determination, the enzyme activity was measured in 0.1 M *tris*-maleat buffers within the pH of 6.0 to 8.5.

Effect of temperature on G6PD activity: The enzyme activity was measured between 10 and 60 ºC at optimum pH for this purpose.

Molecular weight determination

SDS polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGE was performed essentially according to Laemmli's procedure[22]. It was carried out in 4 and 10 % acrylamid concentrations for running and stacking gel, respectively[20]. The molecular-mass markers, *E. coli* β-galactosidase (116 kDa), rabbit phosphorylase B (97,4 kDa), bovine albumin (66 kDa), chicken ovalbumin (45 kDa) and bovine carbonic anhydrase (29 kDa) were used as standards (Sigma: MW-SDS-200).

Sephadex G-200 Gel filtration chromatography: Molecular weight of the native enzyme was determined by the gel filtration in a column of Sephadex G-200[23] with the following molecular-mass markers, yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), bovine erythrocyte carbonic anhydrase (29 kDa) and horse heart cytochrome C (12.4 kDa) were used as standards (Sigma: MW-GF-200). The void volume was observed with Blue Dextrane (2,000 kDa).

Kinetic studies: For K_m and V_{max} evaluation, Lineweaver-Burk curves were used[24], which were obtained in five different concentrations of $NADP^+$ (0.01, 0.02, 0.03, 0.04 and 0.05 mM) and in the constant concentration of G6-P (0.6 mM). The same experiments were done for G6-P in five different concentrations of G6-P (0.03, 0.06, 0.09, 0.12 and 0.15 mM) and in fixed $NADP^+$ concentration (0.2 mM)[20]. All kinetic studies were performed at 25 ºC and in optimum pH (0.1 M *tris*-maleat pH 8.0).

RESULTS AND DISCUSSION

There are enzymes which improve the total antioxidative defence capacity of the organism, *e.g.* superoxide dismutase, glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase and nitric oxide synthase[25]. The housekeeping enzyme glucose-6-phosphate dehydrogenase (G6PD) is a particularly important enzyme in carbon catabolism in the chloroplasts of higher plants[8]. This enzyme catalyzes the first and rate-limiting reaction step in the oxidative hexose monophosphate shunt which supplies reduced $NADP^+$ for a variety of biosynthetic processes. The generated NADPH has been shown to be essential for the protection of cells against oxidative damage[26,27]. It is known that NADPH has an important role in detoxification reactions. This mechanism can be described as GSH peroxidase metabolized H_2O_2 and lipid peroxides to non-toxic products. It leads to the oxidation of GSH to GSSG. GSSG is reduced to GSH by GSSG reductase. This reaction enzymatically, requires NADPH produced by glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase[17]. The G6PD is known to

be regulated by light. However, the dehydrogenase from plants has been difficult to purify and there is less information on kinetics and mechanism of deactivation.

G6PD is first discovered in red blood cells and yeast by Yoshida[28]. In following years, the enzyme was purified through ion-exchange materials by using the natural substrates: G6-P and $NADP^+$, resulting in a high degree of purification[29,30]. For example, the enzyme has been purified and characterized from different animal tissues[6,15,31]. However, G6PD enzyme has also been purified and characterized a number of plant tissues up to now. Previously, G6PD was partially purified from spinach leaves[32]. Then Fickenscher and Scheibe purified the G6PD enzyme from pea leaves using a method consisted of two steps (a) Remazol Brilliant Gelb GL-Sepharose CL 4B affinity chromatography and (b) DEAE-Sephadex A 50 ion exchange chromatography in 1986[18]. Esposito and co-workers[33] did partially purification of the enzyme from barley roots using ammonium sulfate step, Q-Sepharose and Reactive Blue agarose chromatography. However, Çoban *et al.*[17] have reported that parsley (*petroselinum hortense*) leaves G6PD enzyme had been purified with a spesific activity 2.146, 58-fold using ammonium sulphate (20-60 %) precipitation and DEAE-Sephadex A 50 ion exchange column chromatography.

The presented purification procedure was included mainly two steps and allowed easily the purification of the enzyme. DEAE-Sephadex A 50 ion exchange column after the ammonium sulphate (20-40 %) precipitation were directly applied. The purification steps of G6PD from *Polygonum cognatum* Meissn leaves are summarized in Table-1.

TABLE-L
PURIFICATION SCHEME OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE FROM *Polygonum cognatum* Meissn LEAVES

Steps	Activity (U/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification (fold)
AA	0.0167	25	1.0936	27.3400	0.4180	0.01528	100.0	1.00
BB	0.1065	3.0	1.6796	5.0388	0.3195	0.06341	76.4	4.15
CC	0.0963	2.5	0.0508	0.1269	0.2407	1.89600	57.6	124.08

AA = Homogenate; BB = Ammonium sulfate precipitation (20-40 %);
CC = DEAE-Sephadex A50 ion exchangechromatography.

The G6PD activity in leaves was almost completely separated (99 %) from 6-phosphogluconate dehydrogenase activity by subjecting the supernatant to a 20-40 % saturated ammonium sulphate fractionation. After ammonium sulfate fractionation, pellet obtained was dissolved with 0.1 M of *tris*-maleat

(pH 8.0) buffer. DEAE-Sephadex A50 ion exchange column chromatography was done after the ammonium sulfate fractionation. The enzyme was obtained with a yield of 57.6 % and had a specific activity of 1.896 U/mg proteins. The overall purification was about 124.08-fold. Temperature (4 ºC) was maintained during the purification process (Table-1). It is stated that this method used for the purification of G6PD from *Polygonum cognatum* Meissn leaves is a rapid purification method, which can be performed in a day.

Fig. 1 exhibits the SDS-PAGE made for the purity and subunit molecular weight of the enzyme.

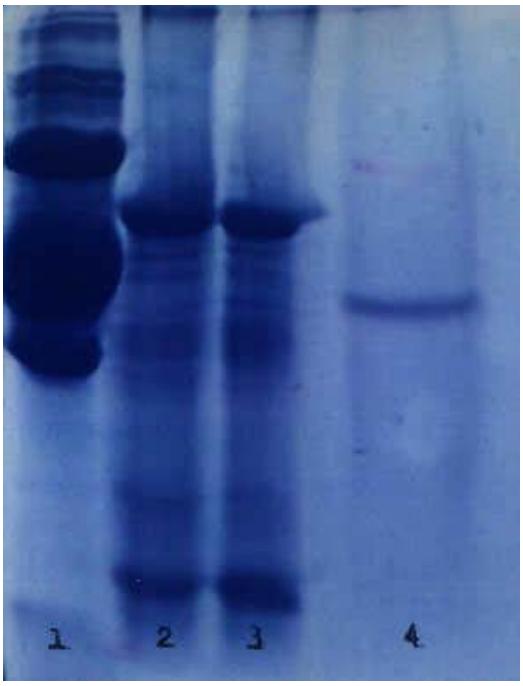

Fig. 1. SDS-polyacrylamide gel electrophoresis of G6PD purified using ion exchange column. (Lane 1: Standard proteins; Lane 2: Homogenate; Lane 3: Ammonium sulfate fractionation; lane 4: G6PD)

For the standard proteins and G6PD, R_f values were calculated and R_f -log MW (mol. wt.) graph (Fig. 2) was obtained according to Laemmli's procedure[2], showing a subunit molecular weight of 74.4 kDa for G6PD.

The molecular weight of the native enzyme was also determined by gel filtration. K_{av} – log MW graph was obtained (Fig. 3), which showed a molecular weight of 73.2 kDa for G6PD. The two graphs showed the molecular weights similar to each other. G6PD enzyme can be form dimers, trimers, tetramers and hexamers of the identical subunits.

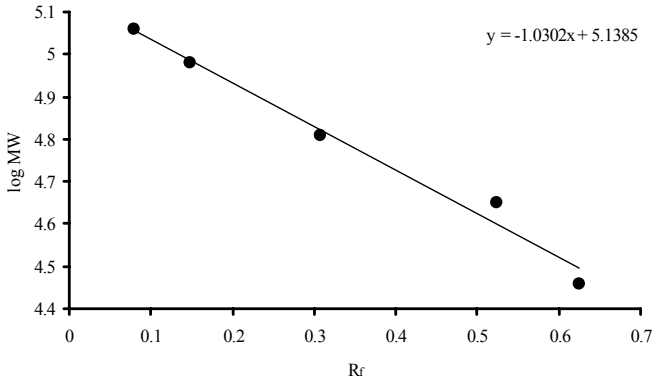

Fig. 2. Standard R_f - log MW (mol. wt.) graph of G6PD using SDS-PAGE. [Standards: *E. coli* β-galactosidase (116,000), rabbit phosphorylase B (97,400), bovine albumin (66,000), chicken ovalbumin (45,000) and bovine carbonic anhydrase (29,000)].

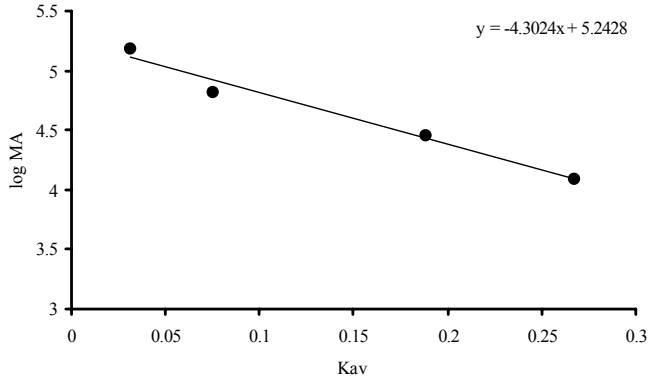

Fig. 3. Standard K_{av} – log MW (mol. wt.) graph of G6PD using gel filtration. [Standard proteins: Horse heart cytochrome C (12,400), bovine erythrocyte carbonic anhydrase (29,000), bovine serum albumin (66,000), yeast alcohol dehydrogenase (150,000)]

The two graphs showed the molecular weights similar to each other. G6PD enzyme can form dimers, trimers, tetramers and hexamers of the identical subunits. The minimum structure, necessary for catalysis, is the dimeric form[34]. On the contrary, the molecular weight of the G6PD from parsley leaves was found to be monomer of the active enzyme by comparison of Sephadex G-150 gel filtration column chromatography with SDS-PAGE. G6PDs of different origin have similar molecular weight as follows: 77.6 and 79.3 from parsley leaves[17], in chicken erythrocyte[6], bovine lens[12], *Trypanosoma brucei*[14] and in human placenta[35] by gel filtration and SDS-PAGE, respectively.

Fig. 1 exhibits the SDS-PAGE made for the purity and molecular weight of the enzyme. A high purity for the enzyme has been obtained. For the standard proteins and G6PD, R_f values were calculated and R_f – log MW graph (Fig. 2) was obtained according to Laemmli procedure[12], showing a subunit molecular weight of 40.7 kDa for G6PD. The molecular weight of the native enzyme was also determined by gel filtration chromatography. K_{av} – log MW graph was obtained (Fig. 3), which showed a molecular weight of 40.5 kDa for G6PD. The two graphs showed the molecular weights similar to each other, suggesting the enzyme to be a monomer in the active state. G6PD enzyme can form dimers, trimers, tetramers and hexamers of the identical subunits. It is known that the minimum structure, necessary for catalysis, is the dimeric form[34]. On the contrary, the molecular weight of the G6PD from parsley leaves was found to be monomer of the active enzyme by comparison of Sephadex G-150 gel filtration column chromatography (77.6 kDa) with SDS-PAGE (79.3 kDa)[17]. G6PDs of different origin have similar molecular weight as follows: In chicken erythrocyte[6], bovine lens[12], *Trypanosoma brucei*[14], in human placenta[35] and in turkey erythrocyte by gel filtration and SDS-PAGE, respectively. The enzyme was stable in the pH 8.0 in 0.1 M *Tris*-maleat (Fig. 4).

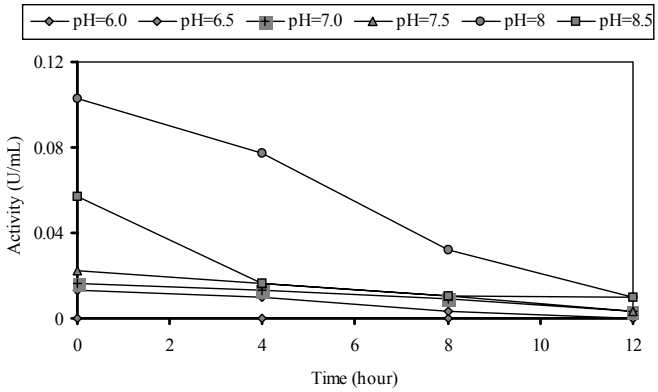

Fig. 4. Stable pH graphs of *Polygonum cognatum* Meissn leaves G6PD in 1 M *tris*-HCl buffer

The stable pH determined was similar to the parsley leaves G6PD[17]. The optimum pH of the purified enzyme has been determined *ca*. 8.0 using 0.1 M *tris*-maleat (Fig. 5).

The pH determined was similar to that in the previous studies[14,15,17,36]. In general, most plants G6PD enzyme shows maximum activity between pH 7.0-9.0[18,32,37]. The purified enzyme showed the highest activity at 40 ºC (Fig. 6).

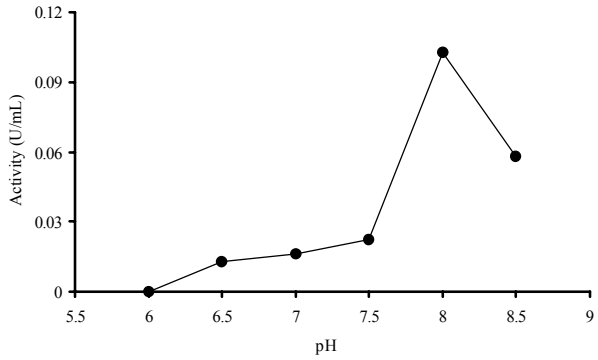

Fig. 5. Activity-pH graph of *Polygonum cognatum* Meissn leaves G6PD

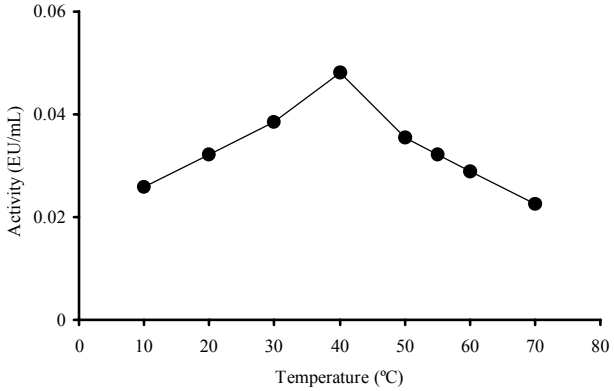

Fig. 6. Effect of temperature on *Polygonum cognatum* Meissn leaves G6PD

The Lineweaver-Burk graphs were shown in Figs. 7 and 8, which were constructed for $NADP^+$ and G6-P. A K_m of 0.011 mM and a ν_{max} of 0.104 EU/mL were obtained for $NADP^+$ and 0.063 mM and 0.050 EU/mL for G6-P (Table-2).

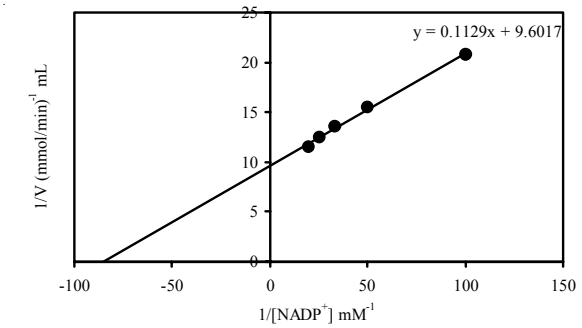

Fig. 7. Lineweaver-Burk graph in 5 different $NADP^+$ concentrations and in constant G6-P concentration

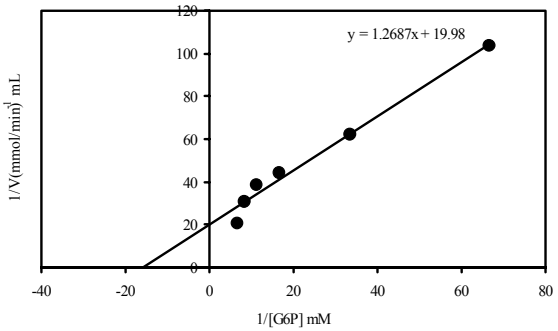

Fig. 8. Lineweaver-Burk graph in 5 different G6-P concentrations and in constant $NADP^+$ concentration

These K_m values are similar to those obtained pea leaves[18], in potato[19], in parsley leaves[17] and in *Acetobacter hansenii*[38]. The K_m for G6P is higher than that for $NADP^+$, suggesting the higher affinity of G6PD to $NADP^+$ when compared with G6P (Table-2).

TABLE-2
K_m AND V_{max} CONSTANTS FOR G6P AND $NADP^+$

Source	K_M^{G6P} (mM)	K_M^{NADP+} (mM)	V_{max}^{G6P} (EU/mL)	V_{max}^{NADP+} (EU/mL)
Polygonum cognatum Meissn leaves.	0.063	0.011	0.050	0.104

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