PHOTOSYNTHETICA 39 (2): 299-304, 2001

Purification and some catalytic properties of glucose-6-phosphate dehydrogenase isoforms from barley leaves

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Abstract

The cytosolic and chloroplastic isoforms of glucose-6-phosphate dehydrogenase (G_6PDH) were separated and purified from barley leaves (*Hordeum vulgare* L.). In etiolated leaves, only the cytosolic isoform was expressed. The molecular mass of the cytosolic enzyme, G_6PDH_1, was 112±8 kDa and that of the chloroplast enzyme, G_6PDH_2, was 136±7 kDa. The K_m values for glucose-6-phosphate and NADP were 0.133 and 0.041 mM for G_6PDH_1, and 0.275 and 0.062 mM for G_6PDH_2, respectively. The pH optimum was 8.2 for G_6PDH_1 and 7.8 for G_6PDH_2. The enzyme is absolutely specific for NADP. NADPH is a competitive inhibitor of the G_6PDH_1 in respect to glucose-6-phosphate (G_6P) and NADP (K_i = 0.050 and 0.025 mM, respectively). NADPH is a competitive inhibitor of the G_6PDH_2 in respect to NADP (K_i = 0.010 mM), but a non-competitive inhibitor in respect to the G_6P. ADP, AMP, UTP, NAD, and NADH had no effect on the activity of G_6PDH. ATP inhibited the G_6PDH_2 activity.

Additional key words: competitive and non-competitive inhibition; cytosolic and chloroplastic forms; etiolated leaves; *Hordeum vulgare*; K_i; K_m; NADP.

Introduction

G_6PDH (EC 1.1.1.49) catalyses the conversion of G_6P to 6-phosphoglucono-δ-lactone. G_6PDH is a key limiting enzyme in the pentose phosphate pathway (Williams 1980, Copeland and Turner 1987). In several C_3-plants, many enzyme activities of sugar phosphate metabolism have been demonstrated in both the chloroplasts and the cytosol. All such enzymes of green leaf cells could be separated into two isoforms, one located in the chloroplasts and the other in the cytosol (Schnarrenberger *et al.* 1992, 1995). The plastid G_6PDH isoenzyme seems to resemble the cytoplasmic form in several general properties, including molecular mass, kinetic properties, and control of certain effectors in green leaves such as spinach (Lendzian 1980, Schnarrenberger *et al.* 1995) and in non-green plant tissue such as castor bean endosperm (Miernyk 1992). Although the plant enzyme is less studied, the available data indicate different regulatory mechanisms as compared to animal tissues and microorganisms. In particular, the chloroplast G_6PDH is regulated by irradiation through redox reactions of thiol groups—inversely to the other irradiation-regulated enzymes of the Calvin cycle, but the cytosol G_6PDH is not (Scheibe and Anderson 1981, Graeve *et al.* 1994).

However, comparative characterisation of cytosolic and plastidic isoforms of G_6PDH from plant tissues is very rare. Nevertheless, elucidation of peculiarities of different isoform function may explain some aspects of saccharide metabolism in cytosol and chloroplast.

Hence, in the present paper we describe the separation of two isoforms of G_6PDH from barley leaves and their purification to homogenous state. That provides comparison of catalytic properties and possibilities of activity regulation of cytosolic and plastidic G_6PDH isolated from leaf tissue of the same monocotyledonous plant.

Received 15 January 2001, *accepted* 26 April 2001.
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Abbreviations: G_6P – glucose-6-phosphate; G_6PDH – glucose-6-phosphate dehydrogenase.

Materials and methods

The leaves of 10-d-old barley leaves (*Hordeum vulgare* L.) grown in a greenhouse under hydroponic conditions and daylight irradiation at photon flux density of 0.15 J s^{-1} m^{-2} and at 25 °C were used.

The activity of G_6PDH was measured spectrophotometrically using the spectrophotometer *SP-46* (*Lomo*, St. Peterburg, Russia) at 340 nm by the rate of NADP reduction during the conversion of G_6P to 6-phosphogluconolactone. The reaction mixture consisted of 0.05 M Tris-HCl, pH 7.5, 2 mM G_6P, 0.25 mM NADP, 0.5 mM Na-EDTA, and 7.5 mM $MgCl_2$. Enzyme reaction was started by adding glucose-6-phosphate into the cuvette. Protein concentration was determined according to Lowry *et al.* (1951).

Electrophoresis was conducted in 7.5 % polyacrylamide gels under non-denaturing conditions according to Davis (1964). Proteins were stained with Coomassie Brilliant Blue *R-250* (Maurer 1968). Gel staining for enzyme detection by activity was performed according to Gabriel (1971). The molecular mass of the enzymes was estimated by chromatography on *Toyopearl HW-55* (*Toyo-Soda*, Tokyo, Japan). The calibration curves were constructed using the following markers: ferritin from horse spleen (450 kDa), aldolase (160 kDa) and phosphoglucomutase (66 kDa) from rabbit muscles, ovalbumin (45 kDa), myoglobin (17.8 kDa), and cytochrome *c* (12.5 kDa).

Original methods were used for purification of cytosolic and chloroplastic isoforms. Purification of G_6PDH_1 was performed as follows. Step *1*: Leaves (15 g) were homogenised using a chilled mortar and pestle in two volumes of extraction buffer containing 0.04 M Tris-HCl, pH 7.5, 1 mM Na-EDTA, 0.05 M 2-mercaptoetanol, 24 mM NADP, 2 kg m^{-3} polyvinylpyrrolidone, and 0.1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 9 000×*g* for 15 min. Step *2*: The enzyme preparation was fractionated by ammonium sulphate precipitation, using the cut between 30 and 60 % saturation at pH 7.5. After centrifugation at 15 000×*g* for 30 min, the precipitate was re-suspended in a minimum volume of the extraction buffer. Step *3*: The protein preparation was purified from low-molecular-mass contaminants by gel-filtration on a *Sephadex G-25* (fine) column (1.4×30 cm). The column was equilibrated and the enzyme was eluted with 10 mM Tris-HCl, pH 7.5, containing 1 mM Na-EDTA and 2 mM dithiothreitol. Step *4*: The enzyme preparation was applied to a DEAE-cellulose column (1.1×10.0 cm) equilibrated with the same column buffer as before. Proteins were eluted with 110 cm^3 of 0 to 0.2 M KCl gradient in column buffer. Fractions of 2-3 cm^3 were collected. Step *5*: The enzyme preparation was then applied to a *Sephadex G-150* column (1.5×75.0 cm) equilibrated with the same column buffer as before. Proteins were eluted using a peristaltic pump (*Pharmacia, LKB* – Pump 1) at a flow rate of 8.33 mm^3 s^{-1}.

Purification of the G_6PDH_2 was the same as for G_6PDH_1 during the first three steps. Subsequently, the enzyme preparation was applied to a DEAE-cellulose column (1.1×10.0 cm) equilibrated with column buffer as described above. Proteins were eluted with 110 cm^3 of 0 to 0.2 M KCl gradient in column buffer and fractions of 2-3 cm^3 were collected. When the first peak of G_6PDH activity was eluted, 10 mM fructose-1,6-bisphosphate was applied into the column buffer, and as a result, the second peak of G_6PDH activity was obtained. The resulting enzyme preparation was applied to *Sephadex G-150* column as described above. All procedures were performed in a cold chamber at 0 to 4 °C.

The statistical processing of values was accomplished by standard procedures (Lloid and Lederman 1984). Programs of linear and parabolic approximations (*Microsoft Exel 97*, *Harvard Graphics*) were used for graphical representations.

Sephadex G-25 and *G-150* were obtained from *Pharmacia* (Uppsala, Sweden). DEAE-32-cellulose was from *Whatman* (Maidstone, United Kingdom). Tris and polyvinylpyrrolidone were purchased from *Serva* (Heidelberg, Germany). NADP, G_6P, and galactose-6-phosphate were obtained from *Sigma* (St. Louis, USA). Phenylmethylsulfonyl fluoride and dithiothreitol were from *Merck* (Darmstadt, Germany); other reagents were obtained from Russian manufacturers.

Results and discussion

Separation of the two G_6PDHs was achieved by chromatography on DEAE-cellulose. Profiles of the G_6PDH activity and total protein are presented in Fig. 1. G_6PDH_1, the cytosol isoenzyme, was eluted with 0 to 0.2 M KCl gradient, and G_6PDH_2, the chloroplast isoenzyme, with 0.2 M KCl and 10 mM fructose-1,6-bisphosphate. The resulting enzyme preparations were used for further purification (Table 1). The procedure resulted in a 105-fold purification of the G_6PDH_1 with specific activity of 38.00 mkat kg^{-1}(protein). Native PAGE showed that, after gel-chromatography on *Sephadex G-150*, the fractions with maximum activity were homogeneous (Fig. 2). After staining with Coomassie Brilliant Blue, one band with an electrophoretic mobility $R_f \sim 0.10$ was revealed in the gel. On the other hand, the procedure resulted in a 63-fold purification of the G_6PDH_2 with specific activity of 22.52 mkat kg^{-1}(protein). PAGE after gel-chromatography on *Sephadex G-150* revealed a homogeneous protein band in

fractions with maximum enzymatic activity (Fig. 2). Its electrophoretic mobility was $R_f \sim 0.24$. Minor components were not detected. The degree of purification and the yield of both enzymes were low, due to extreme instability of the enzymes, especially of the chloroplast form.

Detection of isoenzymes by enzyme activity in native gels revealed that etiolated barley leaves contained a single band ($R_f \cong 0,1$) of G_6PDH activity, the mobility of which was similar to the R_f obtained for the G_6PDH_1. With green leaves two bands with $R_f \cong 0.10$ and 0.24, respectively, were obtained. The latter value was similar to the R_f of G_6PDH_2. Therefore G_6PDH_1, the cytosolic isoform, may be constitutively expressed and G_6PDH_2, the chloroplast isoform, is expressed only after irradiation (Fig. 2). Similarly, Schaewen *et al.* (1995) found that expression of the plastid enzyme takes place in potato leaves, but not in tuber tissue.

The isolation of purified proteins allowed study of physicochemical properties and regulatory properties of the G_6PDHs from barley leaves. The molecular mass of

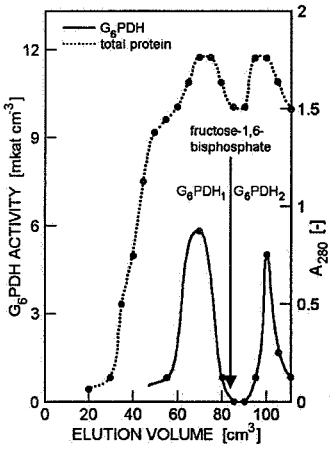

Fig. 1. Chromatography of the enzyme preparation from barley leaves on DEAE-cellulose. Profiles of the total protein and glucose-6-phosphate dehydrogenase (G_6PDH) activity during chromatography are presented.

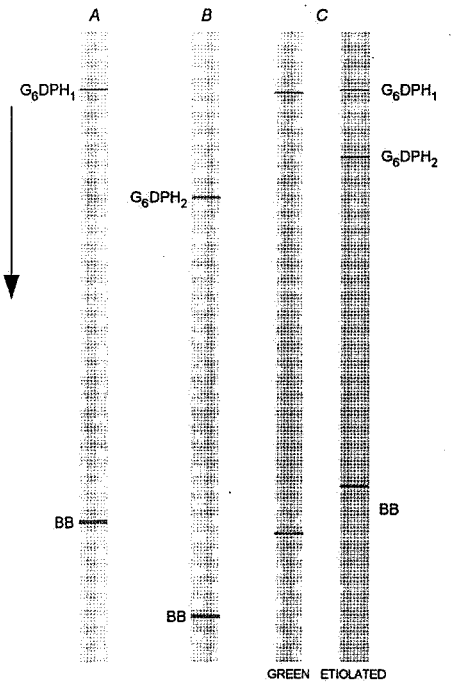

Fig. 2. Electrophoretic separation of (*A*) glucose-6-phosphate dehydrogenase 1 (G_6PDH_1) and (*B*) glucose-6-phosphate dehydrogenase 2 (G_6PDH_2) from barley leaves. *C*: the isoforms in green and etiolated leaves. BB – marker dye bromophenol blue. *Arrow* shows the direction of protein migration.

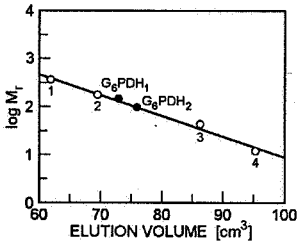

Fig. 3. Determination of molecular mass of glucose-6-phosphate dehydrogenase isoforms G_6PDH_1 and G_6PDH_2 from barley leaves by chromatography on *Toyopearl HW-65*. Marker proteins: ferritin from horse spleen (450 kDa) (*1*), aldolase from rabbit muscles (160 kDa) (*2*), ovalbumin (45 kDa) (*3*), and cytochrome *c* (12.5 kDa) (*4*).

the enzymes determined by gel-chromatography on *Toyopearl HW-65* was 112±8 for G_6PDH_1 and 136±7 kDa for G_6PDH_2 (Fig. 3). The molecular masses of mi-crobial G_6PDH range between 100 and 120 kDa and of mammalian enzymes between 120 and 130 kDa (Levy 1979).

The pH optimum for the reaction catalysed by the G_6PDH_1 was 8.2. The pK of ionogenic groups estimated

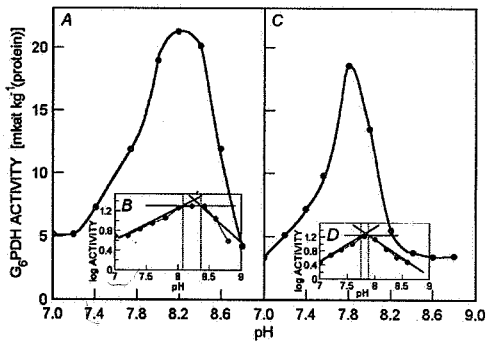

Fig. 4. pH dependence of glucose-6-phosphate dehydrogenase isoforms activity (*A, C*) and determination of pK values of functional groups from {logV: pH} plot (*B, D*) for glucose-6-phosphate dehydrogenase 1, G_6PDH_1 (*A, B*) and glucose-6-phosphate dehydrogenase 2, G_6PDH_2 (*C, D*) (0.05 M Tris-HCl, pH 7.5, containing 0.5 mM Na-EDTA, 7.5 mM $MgCl_2$, 0.25 mM NADP, and 2 mM glucose-6-phosphate).

from dependence of log activity *versus* pH were 8.05 and 8.45. This is close to the pK of cysteine sulfhydryl groups. The G_6PDH_2 has pH optimum of 7.8. Values of pK for ionogenic groups calculated from the (log activity; pH) dependence were 7.75 and 7.95. The first is close to the pK of imidazole group of histidine whereas the second is close to the cysteine sulfhydryl group (Fig. 4).

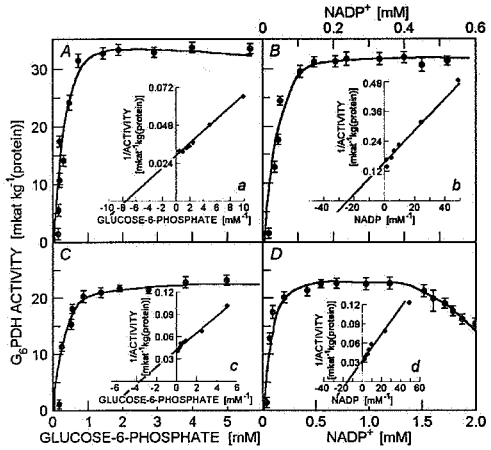

Fig. 5. Dependence of the reaction rate catalysed by glucose-6-phosphate dehydrogenase 1 (G_6PDH_1) (*A, B*) and glucose-6-phosphate dehydrogenase 2 (G_6PDH_2) (*C, D*) from barley leaves on glucose-6-phosphate concentration (*A, C*) (0.05 M Tris-HCl, pH 7.5, containing 0.5 mM Na-EDTA, 7.5 mM $MgCl_2$, and 0.25 mM NADP) and NADP concentration (*B, D*) (0.05 M Tris-HCl, pH 7.5, containing 0.5 mM Na-EDTA, 7.5 mM $MgCl_2$, and 3 mM glucose-6-phosphate). K_m determination by the Lineweaver-Burk method for glucose-6-phosphate (*a, c*) and NADP (*b, d*).

The dependence of the G_6PDH activity on substrate concentration followed the Michaelis-Menten kinetics. The K_m values of G_6PDH_1 and G_6PDH_2 for G_6P were 0.133 and 0.275 mM, respectively. The K_m values of G_6PDH_1 and G_6PDH_2 for NADP were 0.041 and 0.062 mM, respectively (Fig. 5). A substrate inhibition of G_6PDH_2, however, occurred at high concentrations of NADP with a K_i of 0.45 mM (Fig. 6). The reported K_m values for NADP vary from 14 mM (black gram enzyme; Ashihara and Komamine 1976) to 330 mM (wheat germ enzyme; Mirfakhrai and Auleb 1989). The K_m values for G_6P vary from 9 mM (wheat germ enzyme; Mirfakhrai and Auleb 1989) to 18 mM (hazel cotyledons enzyme; Gosling and Ross 1979).

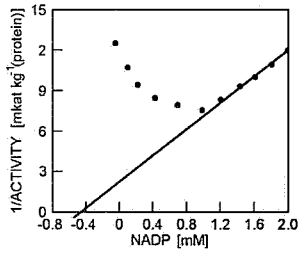

Fig. 6. Determination of the constant of NADP-caused inhibition of glucose-6-phosphate dehydrogenase 2 (G_6PDH_2) from barley leaves (0.05 M Tris-HCl, pH 7.5, containing 0.5 mM Na-EDTA, 7.5 mM $MgCl_2$, and 3 mM glucose-6-phosphate).

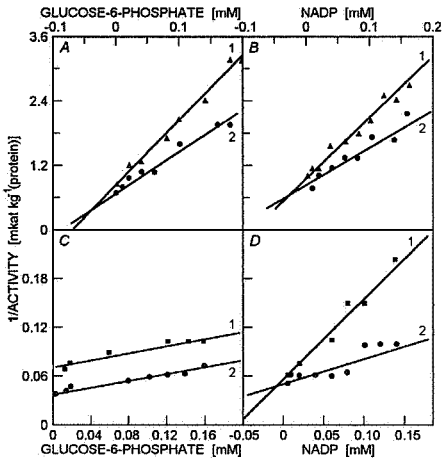

Fig. 7. Determination of the type and constants of NADPH-caused inhibition of glucose-6-phosphate dehydrogenase 1, G_6PDH_1 (*A, B*) and glucose-6-phosphate dehydrogenase 2, G_6PDH_2 (*C, D*) from barley leaves with respect to glucose-6-phosphate [0.05 M Tris-HCl, pH 7.5, containing 0.5 mM Na-EDTA, 7.5 mM $MgCl_2$, 0.25 mM NADP, and the fixed concentrations of glucose-6-phosphate: 1 mM (1) and 2 mM (2)] (*A, C*) or to NADP [0.05 M Tris-HCl, pH 7.5, containing 0.5 mM Na-EDTA, 7.5 mM $MgCl_2$, NADP, 2 mM glucose-6-phosphate, and the fixed concentrations of NADP: 0.10 mM (1) and 0.25 mM (2)] (*B, D*).

NAD had no appreciable effect on G_6PDH isozymes. G_6PDH was active only with NADP, being consistent with G_6PDH of almost all other plants (Muto and Uritani 1972, Ashihara and Komamine 1976, Gosling and Ross 1979, Mirfakhrai and Auleb 1989). In this respect, plant G_6PDH differs considerably from microbial enzymes, most of which are active both with NAD and NADP (Levy 1979). Sugar phosphates such as fructose-1,6-bisphosphate and glucose-1-phosphate also could not re-

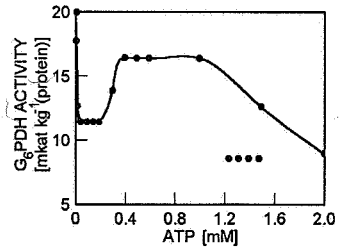

Fig. 8. Effect of ATP on the glucose-6-phosphate dehydrogenase 2, G_6PDH_2 reaction rate (0.05 M Tris-HCl, pH 7.5, containing 0.5 mM Na-EDTA, 7.5 mM $MgCl_2$, 0.25 mM NADP, and 2 mM glucose-6-phosphate).

place G_6P in the standard assay system. NADPH is a competitive inhibitor of the G_6PDH_1 with respect to G_6P and NADP with K_i values of 0.050 and 0.025 mM, respectively. NADPH is a competitive inhibitor of the G_6PDH_2 with respect to the NADP with a K_i of 0.010 mM, but in respect to the G_6P it is a non-competitive inhibitor (Fig. 7). In comparison, K_i for NADPH with respect to NADP is 0.070 mM for the spinach stroma enzyme (Lendzian 1980) and 0.010 mM for the sweet potato enzyme (Muto and Uritani 1972). The concentration of NADPH in spinach chloroplast is about 0.5 mM (Takahama *et al.* 1981). Probably a considerable amount of the nucleotide is a protein bound *in vivo*. If this is so, then the control of activity of the enzyme by reduction charge (Lendzian and Bassham 1975) seems likely. K_i (NADPH) values reported for the enzyme from other sources range from 0.7 μM (*Saccharomyces carlsbergensis*) to 0.15 mM (rat adipose tissue) (Levy 1979).

ATP, ADP, AMP, UTP, NAD, and NADH at concentrations below 2 mM have no effect on the G_6PDH_1 enzyme activity. In contrast, ATP inhibited G_6PDH_2, and its influence was specific. The inhibitory effect increased with increasing concentrations up to 0.4 mM (decrease in enzyme activity by about 36 %). A further increase in ATP concentration up to 1.2 mM decreased its inhibitory effect. At higher concentrations the inhibitory effect took place again, and at a concentration of 2.0 mM the G_6PDH_2 activity decreased twofold (Fig. 8).

Thus, comparison of catalytic properties of cytosolic and plastidic isoforms of G_6PDH indicated some differences, such as pH-optimum and regulation of activity by concentrations of substrate, NADPH, and ATP.

Table 1. Purification of isoforms of glucose-6-phosphate dehydrogenase from barley leaves.

Purification stage	Fraction volume [cm^3]	Total activity [nkat]	Protein content [mg]	Specific activity [mkat kmg^{-1}(protein)]	Degree of purification	Activity yield [%]
Homogenate	30.0	144.50±13.83	405.90±3.44	0.36±0.03	1.00	100.00
Fractionation with $(NH_4)_2SO_4$ (30-60% saturation)	2.5	114.02±9.17	100.65±8.62	1.13±0.17	3.14	78.90
Sephadex G-25	5.6	78.01±14.00	36.96 ± 3.48	2.11±0.17	5.86	53.90
Isoform I						
DEAE-cellulose	7.0	56.01±3.83	6.54±0.35	8.56±0.67	23.78	38.80
Sephadex G-150	6.0	8.17±1.33	0.22±0.02	38.00±1.50	105.56	5.70
Isoform II						
DEAE-*cellulose*	6.0	21.50±2.50	2.10±0.18	10.24±0.50	28.44	14.90
Sephadex G-150	6.0	3.83±1.00	0.17±0.01	22.52±1.33	65.56	2.70

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