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ISOENZYMES OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM TOBACCO CELLS

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; glucose-6-phosphate dehydrogenase; isoenzymes.

Abstract—Two anodic isoenzymes of glucose-6-phosphate dehydrogenase (G6PDH) were isolated from tobacco suspension culture WR-132, utilizing fractional ammonium sulfate precipitation and DEAE-cellulose chromatography. The pH optimum was 9.0 for isoenzyme G6PDH I and 8.0–8.3 for G6PDH IV. Isoenzyme G6PDH I exhibited Michaelis–Menten kinetics for both substrates, G6P and $NADP^+$, with K_m's of 0.22 mM and 0.06 mM, respectively. G6PDH IV exhibited Michaelis–Menten kinetics for G6P with a K_m of 0.31 mM. The $NADP^+$ double reciprocal plot showed an abrupt transition between two linear sections. This transition corresponds to an abrupt increase in the apparent K_m and V_{max} values with increasing $NADP^+$, denoting negative cooperativity. The two K_m's for high and low $NADP^+$ concentrations were 0.06 mM and 0.015 mM, respectively. MWs of the isoenzymes as determined by SDS disc gel electrophoresis were 85000–91000 for G6PDH I and 54000–59000 for G6PDH IV. Gel filtration chromatography on Sephadex G-150 showed MW's of 91000 for G6PDH I and 115000 for G6PDH IV. A probable dimeric structure for IV is suggested, with two $NADP^+$ binding sites.

INTRODUCTION

Isoenzymes of glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ 1-oxidoreductase, EC 1.1.1.49), the first enzyme in the hexose monophosphate pathway, have also been reported recently in higher plants, for example, in pea [1] and spinach leaves [2]. The isoenzymes from these two sources appear to have similar kinetic and physical properties. In this paper, the separation and characterization of two glucose-6-phosphate dehydrogenase (G6PDH) isoenzymes from WR-132 tobacco suspension cultures are reported.

RESULTS

When the G6PDH preparation from tobacco suspension culture was subjected to both anodic and cathodic polyacrylamide gel electrophoresis, 4 anodic isoenzymes were visible. These 4 isoenzymes have been designated G6PDH I, G6PDH II, G6PDH III and G6PDH IV, with the corresponding mobilities of 0.12, 0.20, 0.28 and 0.38 in the anodic polyacrylamide system. Starch gel electrophoresis also confirmed the existence of only the 4 isoenzymes.

The enzyme preparation was applied to a DEAE-cellulose column which had been pre-equilibrated with 10^{-5} M $NADP^+$, 30 mM β-mercaptoethanol, and 100 mM imidazole–HCl buffer (pH 6.5). G6PDH I eluted from the column after an initial major protein band. Following G6PDH I elution, G6PDH IV could be eluted from the column with the same buffer containing 50 mM NaCl. G6PDH II and III could not be retrieved in active form by this procedure. G6PDH I and II were most prominent in younger cells, while G6PDH III and IV became most prominent as the cells aged. Thus, in later studies, G6PDH I was isolated by the above procedure from young cells (less than 5 day growth period) and 10-day-old cells were harvested for G6PDH IV isolation.

The gel filtration method of Andrews [3] indicated MWs of 91000 and 115000 for G6PDH I and IV, respectively. However, MW determination for G6PDH IV had to be accomplished in the presence of $NADP^+$, since no activity for G6PDH IV could be detected following gel filtration in the absence of $NADP^+$. On the other hand, the SDS electrophoresis procedure of Weber *et al.* [4] yielded apparent MW's of 85000–91000 and 54000–59000 for G6PDH I and IV, respectively.

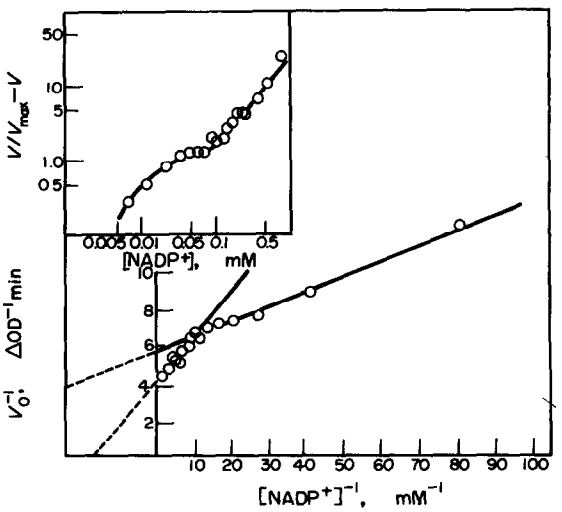

Fig. 1. $NADP^+$ double reciprocal plot of G6PDH IV. $NADP^+$ Hill plot of G6PDH IV. The larger V_{max} is used to plot the graph.

G6PDH I obtained from DEAE-cellulose chromatography had a sharp pH profile with maximum activity at pH 9. The saturation curve for both substrates and the Lineweaver–Burk [5] plot of these data indicated that G6PDH I is a typical Michaelis–Menten type enzyme. The apparent K_m was 220 μM and 60 μM for G6P and $NADP^+$, respectively. However, G6PDH IV had a much broader pH profile with a lower pH optimum between 8 and 8.3. G6P saturation data indicated that with respect to this substrate, G6PDH IV was very similar to G6PDH I, with an apparent K_m of 310 μM. But, as indicated in Fig. 1, the Lineweaver–Burk plot for $NADP^+$ saturation data contained an abrupt transition between two linear sections. This transition corresponds to an abrupt increase in the apparent K_m from 15 μM to 60 μM and V_{max} values with increasing $NADP^+$. Using the V_{max} value corresponding to the higher K_m, a Hill plot (Fig. 1) indicated that a change had occurred. For $NADP^+$ concentrations greater than 0.1 mM, the Hill coefficient was 1.05, while for concentrations less than 0.1 mM, the line was non-linear.

DISCUSSION

As the data indicate, G6PDH I and G6PDH IV appear to be two distinct enzymes possessing different physical properties. The two isoenzymes of Tolbert *et al.* from spinach leaves are quite similar and appear to differ only by a few amino acids [2]. The pea leaf isoenzymes of Anderson and co-workers differ in charge, but have almost identical MW's [1]. Our G6PDH I exhibits Michaelis–Menten kinetics for both substrates: G6P and $NADP^+$. The enzyme appears to contain no subunit structure, as both gel filtrations and SDS electrophoresis indicate MW of 85000 to 91000.

G6P as a substrate for G6PDH IV exhibits a typical saturation curve. However, $NADP^+$ produces an abrupt transition in its Lineweaver–Burk plot. Teipel and Koshland [6] attribute this type of phenomena to a multisite enzyme in which the relative magnitude of the binding constants of the sites first decrease and then increase as saturating levels of substrate are reached. This transition corresponds to an abrupt increase in the apparent K_m and V_{max} values with increasing $NADP^+$. The data suggest there are at least 2 $NADP^+$ binding sites, one with a much lower K_m than the other site. The abrupt increase in the apparent K_m and V_{max} values with increasing $NADP^+$ concentrations corresponds to negative cooperativity. This type of phenomena is not uncommon, being reported for glutamate dehydrogenase from pig heart [7], ox liver [8–10] and yeast [11]; for glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle [12, 13], as well as G6PDH from human erythrocytes [14], yeast [1] and sweet potato [15]. Ashihara and Komamine also have observed this behaviour with G6PDH from *Phaseolus mungo* [16], but in contrast to our results, the abrupt transition in their studies occurred with respect to G6P rather than $NADP^+$.

The MW's we obtained suggest that G6PDH IV is a dimer, with a subunit MW of 54000–59000 and a native MW of 115000.

EXPERIMENTAL

Growth of WR-132 tobacco suspension cultures. Tobacco suspension culture line (*Nicotiana tabacum* L., var. Xanthi), was obtained from Dr. A. C. Olson, U.S. Department of Agriculture, Albany, California. The cells were collected by centrifugation and then re-suspended (2 g cells per 10 ml suspension). These suspensions were added to 40 ml of medium and grown at room temp. in continual light (10 lx) on a rotary shaker (190 rpm). Cells were harvested after 10 days growth. A revised medium of ref. [17] was used, in which 2,4-D was substituted for IAA, and kinetin was deleted.

Enzyme preparation Tobacco tissue (60 g) was mixed with 30 g washed glass beads, 30 g pre-treated Polycar AT which had been hydrated 30 min prior to use, 120 ml 2 mM EDTA, 30 mM β-mercaptoethanol (necessary if enzyme activity is to be obtained) and 100 mM Tris–HCl buffer (pH 8.5) and homogenized in a Sorvall Omnimixer at 5000 rpm for 5.5 min. All preparatory operations were carried out at 4°. The resulting homogenate was filtered through cheesecloth and centrifuged at 34000 g for 15 min. Supernatant was saturated to 30% with $(NH_4)_2SO_4$, centrifuged and the pellet discarded. The resulting supernatant was saturated to 70% with $(NH_4)_2SO_4$ and centrifuged at 34000*g* for 10 min. The pellet was then dissolved in a small vol of 30 mM β-mercaptoethanol, 100 mM imidazole–HCl buffer. Dialysis against 100 vol of the same buffer yielded the enzyme preparation used in these studies.

Polyacrylamide gel electrophoresis. This was used to separate the anodic isoenzymes, according to the procedure of ref. [18]. Cathodic electrophoresis was carried out in the same manner except the current was reversed and the running pH was 4.3 with methyl green as the tracking dye. Starch gel electrophoresis was accomplished using the procedure of ref. [19]. After electrophoresis, the isoenzymes were visualized by the method of ref [20].

MW's of the isoenzymes. These were determined by gel filtration chromatography according to the procedure of ref. [3]. The Sephadex G-150 was pre-equilibrated in 30 mM β-mercaptoethanol, 10^{-5} M $NADP^+$ and 100 mM imidazole–HCl buffer (pH 6.5). The column was standardized using γ-globulin, lipoxidase, creatine kinase, serum albumin, ovalbumin and α-chymotrypsinogen A. Enzymatic activity was measured to determine the elution vol of the G6PDH isoenzymes, while either *A* at 280 nm or 230 nm was used to detect the presence of protein. A second method used for determining MW's was SDS polyacrylamide gel-electrophoresis utilizing the method of ref. [4]. After electrophoresis, the gels were stained for protein using 0.25% Coomassie Brilliant Blue in 45.4% methanol/HOAc soln, the protein bands were highly visible. Standards used were β-galactosidase, lipoxidase, serum albumin, catalase, ovalbumin and α-chymotrypsinogen A.

Enzyme assays. The assay procedure of ref. [21] was employed. All assays were initiated with $NADP^+$ and contained 100 mM Tris–HCl buffer (pH 8.0), 5 mM Mg^{2+} enzyme, G6P and $NADP^+$ in a total of 3 ml. Concns of G6P and $NADP^+$ were varied depending on the situation, with saturating levels being 5 mM and 1 mM, respectively.

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