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CHARACTERIZATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND 6-PHOSPHOGLUCONIC ACID DEHYDROGENASE FROM HAZEL COTYLEDONS

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Key Word Index—*Corylus avellana*; Corylaceae; inherent NADP reduction; pentose phosphate metabolism; glucose-6-phosphate dehydrogenase; 6-phosphogluconic acid dehydrogenase.

Abstract—NADP reduction was shown to occur in a crude cytosolic extract from the cotyledonary material of hazel seed prior to the addition of exogenous dehydrogenase substrate. This activity interfered with the assay of glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase activities. The inherent NADP reduction was removed by ammonium sulphate fractionation. Subsequent de-salting of the resulting partially-purified fraction permitted assay of G6PDH and 6PGDH. Both enzymes were shown to be NADP specific. Typical Michaelis–Menten kinetics were shown for each enzyme towards NADP and their respective substrate.

INTRODUCTION

The existence of glucose-6-phosphate (G6P) breakdown via alternative routes than glycolysis has been demonstrated in a wide range of biological tissues. One such scheme, the pentose phosphate pathway (PPP), leads to the production of a variety of sugar phosphates and concomitant reduction of nicotinamide adenine dinucleotide phosphate (NADP).

Evidence for the occurrence of PPP is mainly derived from studies of the evolution of $^{14}CO_2$ from either glucose-[6-^{14}C] or glucose-[1-^{14}C] yielding a C_6/C_1 ratio which is claimed to reflect the relative contributions of the pentose phosphate and glycolytic pathways. However, another perhaps more valid approach is to isolate the enzymes catalysing individual reactions of one or each pathway.

Qualitative and quantitative information on the initial enzyme of PPP, glucose-6-phosphate dehydrogenase [(D-glucose 6-phosphate: NAD(P) oxidoreductase; EC 1.1.1.49) G6PDH] from higher plants is available [1–7]; however, that the extent of purification effects kinetic studies is apparent from more definitive studies on enzyme preparations from black gram [8] and sweet potato [9].

Controversy also exists in explaining the apparently anomalous ratio between the activities of G6PDH and a subsequent enzyme of PPP, 6-phosphogluconic acid dehydrogenase [(6-phosphogluconic acid: NADP oxidoreductase; EC 1.1.1.44) 6PGDH] in certain tissues [7, 10]. In these reports, G6PDH, the first enzyme of PPP appears to exhibit a much higher activity than 6PGDH, an unusual situation for an enzyme generally believed to be involved in regulating the pathway. The present report describes a source of possible interference in the assay of crude plant extracts, the removal of an inherent NADP reducing component after partial purification and kinetic characterization of G6PDH and 6PGDH from hazel seed cotyledons.

RESULTS

Initial experiments using the supernatant of the 45000 *g* spin (S_{45} fraction) as the source of enzymes demonstrated that NADP reduction occurred prior to the addition of substrate. This activity was found to be only partially diminished by passing the crude extract directly through Sephadex G-25, or by vacuum dialysis; however, all activity was removed by boiling. The reduction was specific to NADP and no reduction occurred with NAD. A full characterization of the inherent NADP-reducing component in the crude cytosolic extract was not attempted, but a number of factors could explain this observation, e.g. the presence of endogenous substrate or perhaps a protein disulphide reductase in conjunction with an electron carrier.

A difference spectrum obtained during the course of the reaction is shown (Fig. 1(a)) to compare with that obtained during NADP reduction by a commercially-purified G6PDH (Fig. 1(b)) and the partially purified hazel cotyledon extract (Fig. 1(c)). There is clearly an increase in absorption over a broad band of wavelengths centred at 340 nm in all the spectra. This is due to the absorption of the reduced nucleotide compared to the oxidized form. In Fig. 1(a), it is noticeable that in addition to the increase at 340 nm there is a significant decrease in absorbance at 275 nm occurring concurrently with an increase in absorbance at 290 nm. Although the NADP concentration may be considered high for this type of experiment and could have interfered with the results at lower wavelengths, it was considered necessary to use a similar concentration to that employed for the enzyme determinations. This fact accompanied by turbidity changes could be the explanation for the lack of the expected spectral changes of NADP → NADPH at 260 nm in Fig. 1(b). It is therefore tempting to speculate from this that a quinone may be present in the crude extract and the reduced NADP is in turn reducing this compound to the quinol form, thus resulting in the

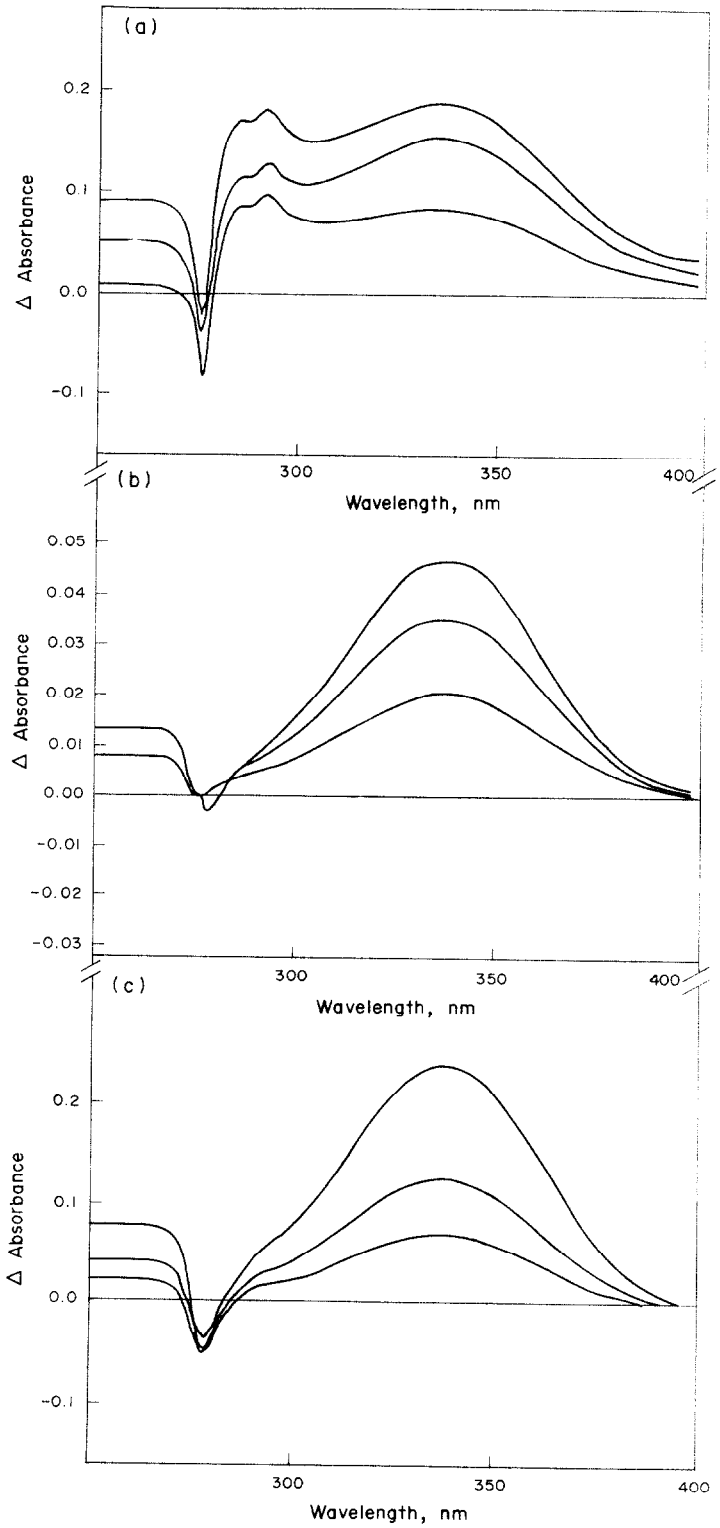

Fig. 1. Difference spectra during (a) inherent NADP reduction by S_{45} cotyledonary extract; (b) NADP reduction by commercially purified G6PDH and (c) G6P dependent NADP reduction by partially purified cytosolic extract.

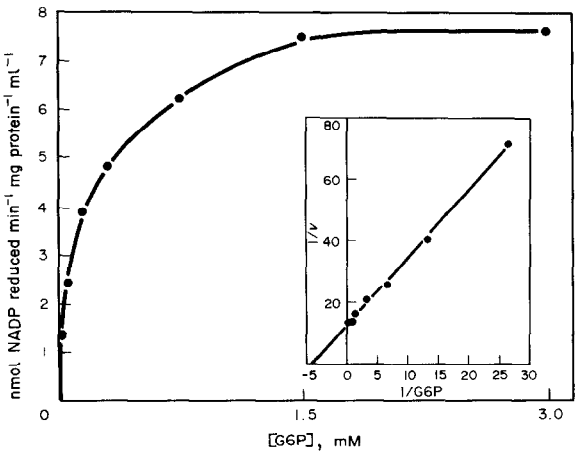

Fig. 2. Effect of G6P concentration on the activity of G6PDH. Inset shows the double-reciprocal plot.

observed spectral changes. The only satisfactory method found for eliminating this inherent NADP reduction, which also allowed later enzyme determinations, was ammonium sulphate precipitation followed by de-salting of the re-suspended protein, since both dehydrogenases are subject to sulphate ion inhibition. This partially purified fraction (PDS) was used as the source of G6PDH and 6PGDH in the study of the effects of G6P, 6PG and NADP concentrations on their respective activities.

The effect of G6P concentration on the activity of G6PDH is shown in Fig. 2. A Lineweaver–Burk plot of $1/V$ vs $1/[G6P]$ (Fig. 2) extrapolates to a K_m value of 1.8×10^{-5} M. The effect of NADP concentration on the enzyme is shown in Fig. 3. Typical Michaelis–Menten type saturation was exhibited and the K_m value for NADP calculated from the Lineweaver–Burk plot (Fig. 3) was 3.1×10^{-5} M.

Similar results are shown for the effects of 6PG concentration (Fig. 4) and NADP concentration (Fig. 5) on the activity of 6PGDH. The K_m value for 6PG was 11×10^{-5} M and for NADP 1.6×10^{-5} M. Both enzymes were specific for NADP and were unable to utilize NAD.

DISCUSSION

Most other studies of dehydrogenase activity in crude enzyme preparations have used double-beam spectrophotometers (e.g. [2, 3, 11]) incorporating extract plus NADP only in the blank cuvette and omitting substrate. Thus any inherent activity would be concealed. That this inherent activity exists in tissues other than hazel cotyledons is apparent from the work of Kovacs and Simpson [1]. They estimated G6PDH activity in wild oat seeds by calculating the difference in the rates of reduction of NADP between separate assays, one containing and one omitting G6P; this is interpreted to mean that inherent activity was present. We, therefore, considered it important to check that there was no interference between G6PDH and 6PGDH activities monitored in the presence of inherent activity compared to the same activities assayed in the partially purified extract. Experience with a large number of samples over a long period showed that results were more reproducible with the partially purified fraction. The explanation for this is apparent after taking into account the difference spectra results. Inherent NADP reduction by the crude cytosolic extract reflects a rate proportional to the formation of NADPH by an unknown component and also the subsequent oxidation of NADPH, possibly by a quinone. Since both these reactions will occur after the addition of G6P or 6PG, their kinetics will be superimposed on the kinetics of the G6PDH and 6PGDH enzymes, thus confounding any accurate quantification of the enzymic rates of reaction alone.

In this study, the PDS extract contained both G6PDH and 6PGDH, so that for every mol of G6P entering PPP 2 mol of reduced NADP were eventually formed. The rapidity of NADP reduction was determined by the combined rates of reaction of the two enzymes (see Fig. 6). If the rate of reaction 1 is much faster than that of 2, the number of mol of NADP reduced in a given time interval will be only slightly greater than the number of mol of G6P converted to 6PG. At the other extreme, if reaction 1 is the rate-limiting step, the number of mol of NADP reduced will be double the number of mol of G6P broken down. The addition of a commercially purified G6PDH to the assay cuvette showed, by the increased rate of reduction of NADP, that with G6P as substrate,

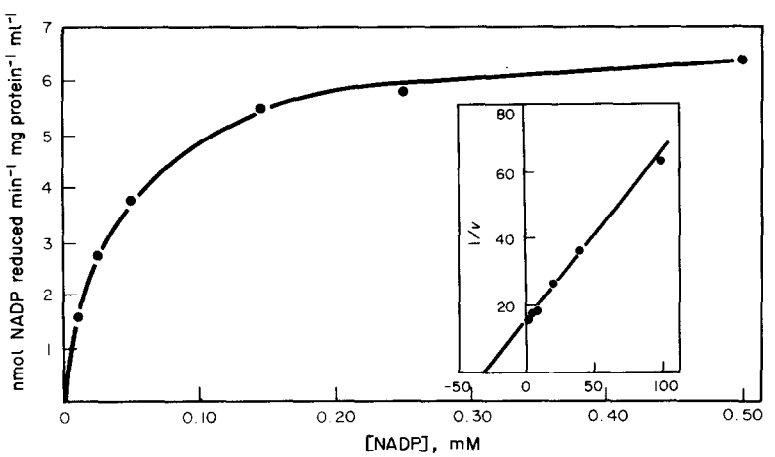

Fig. 3. Effect of NADP concentration on the activity of G6PDH. Inset shows the double-reciprocal plot.

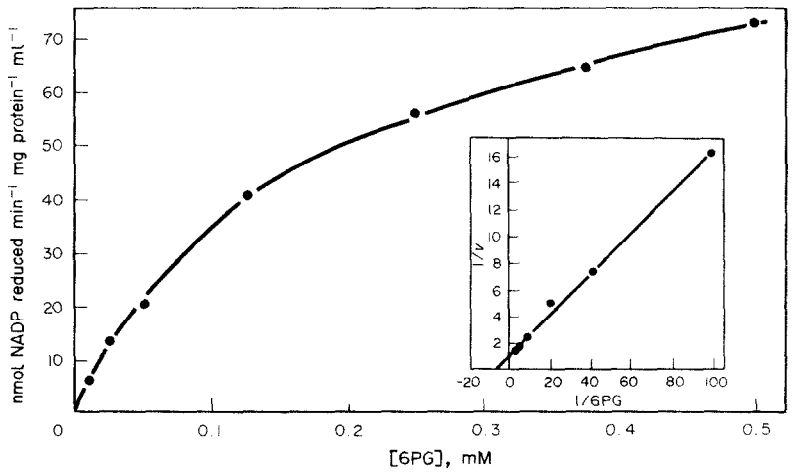

Fig. 4. Effect of 6PG concentration on the activity of 6PGDH. Inset shows the double-reciprocal plot.

half the rate of reduction of NADP reflected the G6PDH activity of the PDS extract. This point has been neglected by some workers, though Brown and Wray [11] and Lendzian and Bassham [12] have used other methods to demonstrate its validity. G6PDH activities reported in this paper are presented with this correction.

The nucleotide specificity of G6PDH from various sources has been tested [13] and compared [8], and it would appear that with the exception of work performed by Mayer *et al.* [4] on lettuce seedlings, all plant G6PDH enzymes, including that isolated from hazel cotyledons, are NADP specific.

Kinetic studies on G6PDH from sweet potato [9], rat mammary gland [14], yeast [15] and black gram [8] reveal from the double-reciprocal plot that the Hill coefficient is less than unity and that in the presence of NADP, negative co-operativity exists between the enzyme and G6P. No such feature is apparent for the hazel enzyme though this was not purified to the same extent as the above, and contaminants may have obscured the effect.

Michaelis constants for 6PGDH have been reported from several sources, e.g. *Neurospora* [16], bacteria [17], pig liver [18] and black gram [7]. The activity has been previously detected as isozymes in higher plant extracts [6, 19, 20]. Detailed physical and chemical characterization of the enzyme in plants have not been so thoroughly examined.

The work of Sapag-Hager *et al.* [10] showed an inbalance of activities between G6PDH and 6PGDH in rat liver. Ashihara and Komamine [7], using the same assay system, also demonstrated a faster rate of reduction of NADP by G6PDH than by 6PGDH. In this latter case, the results may either reflect the technique itself or the fact that different volumes of extract may have been used, since activity is expressed in terms of ΔA/min. Kovacs and Simpson [1], on the other hand, show that the activity of G6PDH is lower than 6PGDH, even though they appear to make no correction for the contribution of 6PGDH activity after the addition of G6P.

In this work, the 6PGDH enzyme reduced NADP at an apparent rate 12 times greater than G6PDH at

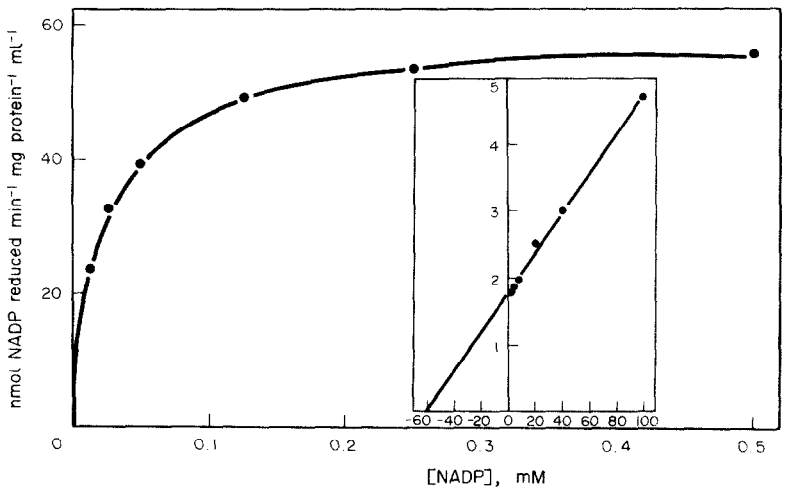

Fig. 5. Effect of NADP concentration on the activity of 6PGDH. Inset shows the double-reciprocal plot.

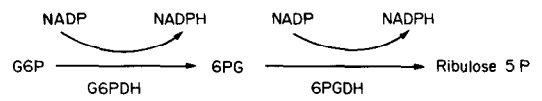

Fig. 6.

equivalent substrate and NADP concentrations. Whether this is due to the presence of a higher proportion of 6PGDH to G6PDH in the extract or a higher catalytic centre activity is not clear.

Many authors suggest that G6PDH is the regulatory enzyme of PPP and reports reviewed by Roberts [21, 22] and Roberts and Smith [23] have implicated PPP in the dormancy breakage mechanism of a number of graminaceous seeds. Whilst concurring with these views, the present authors feel that conclusions based solely on C_6/C_1 decarboxylation ratios, or only on uncharacterized enzymes present in crude extracts, must be treated with reservation. Following the above characterizations, the activities of G6PDH and 6PGDH are being followed throughout the stratification of hazel seeds, a process required to break their dormancy. It is hoped that such a study will help to elucidate the biochemical basis of this common dormancy-breakage mechanism.

EXPERIMENTAL

Hazel nuts (Kent Cobs) *Corylus avellana* L. were obtained at the end of September, 1977, dried and the cupule removed. The seeds were then stored under dry conditions at 5° until required. At the time of use, the pericarps were removed and any damaged or infected nuts rejected. Embryonic axes were removed and the testa and endosperm scraped off; in this way only cotyledonary material was used on the source of enzyme.

Approximately 5–8 g cotyledon tissue were initially ground with a pestle and mortar in 30 ml extraction medium (0.02 M phosphate buffer, pH 7.5 plus 0.48 M mannitol) at 5°. The resulting macerate was transferred to a beaker and further homogenized using a Silverson Laboratory mixer emulsifier. This suspension was filtered through 2 layers of butter muslin into a centrifuge tube and spun at *ca* 2000 *g* at 5°. At frequent intervals, the centrifuge was stopped and as much of the lipid 'cake' removed as possible, until after a total of 1 hr spin little or no lipid layer remained. The supernatant was transferred and spun at 45000 *g* for 25 min. Approximately 15 ml of the resulting aq. phase (S_{45} fraction), between the organellar pellet and the newly formed lipid cake, was removed using a Pasteur pipette. A small aliquot of the S_{45} fraction was set aside for initial assays of the inherent NADP reduction and dehydrogenase assays, while 10 ml was immediately salted out with $(NH_4)_2SO_4$. The 40–80% satn ppt. was collected and re-dissolved in 3 ml of extraction medium. This was de-salted by elution through a 20 × 1.5 cm Sephadex G-25 (coarse) column using 0.02 M phosphate buffer, pH 7.5. The eluant was continuously monitored at 280 nm and the protein fraction collected (PDS fraction). The PDS fraction was stored at 5° and all activities were assayed within 48 hr.

Assay of dehydrogenase enzymes. The activity of the dehydrogenases was measured spectrophotometrically by following the maximal rates of change in absorbance at 340 nm. For the determination of inherent NADP reduction by the S_{45} fraction, the reaction mixture contained 100 µl 4.5×10^2 M NADP, and 200 µl S_4 diluted to 3 ml with assay medium (0.02 M phosphate buffer, pH 7.5 and 0.005 M $MgCl_2$). The activities of G6PDH and 6PGDH were determined using 200 µl PDS fraction varying the G6P, 6PG and NADP concns as shown. G6PDH activity was calculated as explained in the Discussion.

Construction of difference spectra. Difference spectra were obtained between 250 and 400 nm during the reduction of NADP by (i) a commercially purified G6PDH from *Torula* yeast and (ii) the partially purified extract from hazel cotyledons, both in the presence of G6P. A similar spectrum was also drawn for the reaction occurring in the presence of crude cytosolic extract and NADP. A Shimadzu UV 300 double-beam spectrophotometer linked to a Digital memory background corrector was used, reading sample solns against a blank composed of buffer and NADP.

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