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GLUCOSE-6-PHOSPHATASE ACTIVITY IN A SOLUBLE FRACTION FROM COTYLEDON TISSUE OF *BRASSICA NIGRA*

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SUMMARY

A soluble fraction from cotyledon tissue of black mustard *(Brassica nigra)* was found to catalyze the hydrolysis of glucose 6-phosphate. In an attempt to determine whether this reaction was catalyzed by a distinct glucose-6-phosphatase (D-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9) or by an acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2), various characteristics of glucose 6-phosphate and *p*-nitrophenyl phosphate hydrolysis were compared. Both phosphatase activities exhibited a similar distribution pattern in subcellular fractions and in fractions obtained by precipitation with $(NH_4)_2SO_4$. The activities also were compared with respect to the effects of reaction mixture pH in the absence and presence of 1 mM KF–4 mM EDTA and the effect of partial inactivation by various enzyme pretreatments. Glucose 6-phosphate and *p*-nitrophenyl phosphate were mutually competitive inhibitors for the hydrolysis of the other compound. The K_m and K_i values were, respectively, 4.6 mM and 4.6 mM for glucose 6-phosphate and 0.72 mM and 0.79 mM for *p*-nitrophenyl phosphate. A substrate-specificity study indicated that *p*-nitrophenyl phosphate and phenyl phosphate were hydrolyzed more rapidly than glucose 6-phosphate. The soluble enzyme preparation did not exhibit PP_i–glucose phosphotransferase activity. Glucose did not inhibit the hydrolysis of glucose 6-phosphate. It is concluded that the observed hydrolysis of glucose 6-phosphate was catalyzed by an acid phosphatase. The possible existence in plants of a distinct glucose-6-phosphatase is discussed.

INTRODUCTION

Glucose-6-phosphatase (D-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9; Reaction 1) has been reported in such plants as peas[1,2], cactus[3,4], grasses[5], bean[6], black locust[7], and corn[8]. Concomitant PP_i–glucose phosphotransferase activity (Reaction 2), which is associated with mammalian glucose-6-phosphatases (see Nord-

lie[9]) was not reported for any plant glucose-6-phosphatase. In some instances,

$$\text{Glucose-6-}P + H_2O \rightarrow \text{glucose} + P_i \quad (1)$$
$$PP_i + \text{glucose} \rightarrow \text{glucose-6-}P + P_i \quad (2)$$

the observed hydrolysis of glucose-6-*P* at an appropriate pH (6 to 6.5) and substrate concentration was considered sufficient evidence to demonstrate the presence of a glucose-6-phosphatase. Nordlie[9] has suggested that those reports of a distinct glucose-6-phosphatase in plants (and some other sources) may be premature because the possible contribution of acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) and alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) activities to the observed hydrolysis of glucose-6-*P* was not fully assessed. One approach used to minimize this problem is to assay glucose-6-*P* hydrolysis in the presence of KF and EDTA, which were reported[10,11] to inhibit almost completely intestinal acid and alkaline phosphatases but not glucose-6-phosphatase. Thompson[6] also used this criterion in his recent report of a soluble glucose-6-phosphatase in cotyledon tissue of *Phaseolus vulgaris*.

We have obtained a soluble preparation from the cotyledon tissue of *Brassica nigra* which catalyzes the hydrolysis of glucose-6-*P* in the presence of KF and EDTA. In order to determine whether this activity was attributable to a distinct glucose-6-phosphatase, we have examined the enzyme in greater detail than in any previous study of a glucose-6-phosphatase activity from plants.

MATERIALS AND METHODS

Substrates and buffer salts were purchased from Sigma Chemical Company, St. Louis, Mo. All additional chemicals were stock chemicals of reagent grade. Glucose 6-phosphate dehydrogenase and glucose oxidase were obtained, respectively, from Boehringer Mannheim Corporation, New York, N.Y., and Worthington Biochemical Corporation, Freehold, N.J. Seeds of *Brassica nigra* were purchased from Montana Mustard Seed Company, Conrad, Mont.

Assays for glucose-6-phosphatase and PP_i–glucose phosphotransferase activities were carried out as described by Nordlie and Arion[12]. All phosphatase activities routinely were measured by assay for orthophosphate according to the method of Fiske and SubbaRow[13]. In combined substrate studies glucose-6-phosphatase activity was determined with glucose oxidase[14] and *p*-nitrophenyl phosphatase activity was monitored by assay for *p*-nitrophenol[15]. Phosphatase reactions were initiated by the addition of enzyme and were terminated with 1 ml of 10% (w/v) trichloroacetic acid except in combined substrate studies. In the latter case, reactions were terminated by 4 ml of 0.1 M NaOH (*p*-nitrophenyl phosphatase activity) or 0.5 ml of 12% (v/v) $HClO_4$ (glucose-6-phosphatase activity). The $HClO_4$ was neutralized with an appropriate volume of 6 M KOH prior to assay with glucose oxidase. Reaction mixture compositions and further experimental details are given in the legends to tables and figures and in the text. Protein concentrations were determined according to Lowry *et al.*[16]. It was demonstrated in supplementary experiments that all activities measured were linear with respect to incubation time and protein concentration. All assays were carried out in duplicate. Since several separate enzyme preparations employed in the studies described differed slightly in activity, velocities presented in

this paper have been normalized to 0.028 unit* of glucose 6-phosphate phosphohydrolase activity.

Brassica nigra seeds were germinated on moist filter paper in the dark at 25 °C for growth periods up to 8 days. A growth period of 5 days was used for most enzyme preparations. The cotyledon tissue was washed with tap water and rinsed with distilled water. All subsequent operations were carried out at 4 °C. A precooled solution of 0.25 M sucrose was then added to a ratio of approximately 1 ml sucrose solution per g cotyledon tissue. This suspension was homogenized in a Waring blendor for 1 min. The homogenate was twice filtered through six layers of cheesecloth prior to subcellular fractionation by a method similar to standard procedures used for plant tissues[17–19]. The filtrate was centrifuged at 36 900 × *g* for 30 min to sediment nuclei, cellular debris, and mitochondria. The sedimented material was resuspended in a small volume of 0.25 M sucrose with a Potter–Elvehjem homogenizer. The supernatant fraction was further centrifuged at 123 000 × *g* for 45 min. The microsomal pellet was resuspended as described above. The remaining supernatant fraction was brought to 50% saturation of $(NH_4)_2SO_4$ solution at 4 °C by addition of a saturated solution of $(NH_4)_2SO_4$ (pH 7.0). After an additional 10 min of stirring, the suspension was centrifuged at 28 700 × *g* for 15 min. The resulting supernatant fraction was brought to 70% $(NH_4)_2SO_4$ saturation and centrifuged as described above. The centrifuged pellets from each fraction were homogenized in a small volume of 0.25 M sucrose. All enzyme preparations were assayed immediately or stored at −20 °C for subsequent use. The frozen enzyme preparations were quite stable for several months.

RESULTS

Cotyledon tissue from seedlings of *Brassica nigra* at various stages of germination was homogenized and subcellular fractions were obtained by differential centrifugation. Glucose-6-phosphatase activity was assayed in the presence of KF and EDTA, which were found to inhibit acid and alkaline phosphatase activities in *Phaseolus vulgaris* (Thompson[6]) and mammalian intestine[10,11]. Results of this study are compiled in Table I. The supernatant fraction contained the highest specific activity and number of units of glucose-6-phosphatase activity. The optimal growth period, approximately 5 days, was used for all subsequent enzyme preparations.

The subcellular fractions of 5-day-old cotyledon tissue and fractions of the supernatant preparation, obtained by precipitation with $(NH_4)_2SO_4$, were assayed with glucose-6-*P* and *p*-nitrophenyl-*P* as substrates. The results are presented in Table II. Substantial hydrolysis of both substrates occurred at pH 6.0 in the presence of 1 mM KF–4 mM EDTA. The enzyme fraction precipitated between 50 and 70% $(NH_4)_2SO_4$ saturation exhibited the highest specific activity with each substrate. The ratio of glucose-6-phosphatase to *p*-nitrophenyl phosphatase activity was similar for all fractions except the microsomes. This suggests that a single enzyme in the soluble fractions may catalyze the hydrolysis of both substrates tested.

The effect of reaction mixture pH on glucose-6-*P* and *p*-nitrophenyl-*P* hydrolysis was examined in the absence and presence of 1 mM KF–4 mM EDTA. The

* 1 unit of glucose 6-phosphate phosphohydrolase activity is 1 μmole of glucose-6-*P* hydrolyzed per min at 30 °C in a reaction mixture containing, in 1.5 ml, 10 mM glucose-6-*P* and 40 mM sodium cacodylate (pH 6.0). Specific activity is units per mg protein.

TABLE I

EFFECT OF GROWTH TIME ON GLUCOSE-6-PHOSPHATASE ACTIVITY

Brassica nigra seeds were germinated and grown for the indicated time periods. Subcellular fractions of the cotyledon tissue were obtained by differential centrifugation and examined for glucose-6-phosphatase activity. Assay mixtures contained, in 1.5 ml, 40 mM sodium cacodylate (pH 6.0), 10 mM glucose-6-*P*, 1 mM KF, 4 mM EDTA, and 0.2 ml of the appropriate subcellular fraction. Reactions were carried out for 5 min at 30 °C. Phosphatase activity was determined by orthophosphate formation. Protein concentrations were determined as described previously. Data are expressed as specific activity.

Fractions	*Specific activity*				
	At day 0	*2*	*4*	*6*	*8*
Homogenate	trace	0.020	0.035	0.037	0.032
Nuclei*	0.000	0.012	0.022	0.030	0.014
Microsomes	0.011	0.000	0.033	0.013	0.044
Supernatant	0.014	0.027	0.045	0.045	0.041

* Also contained nuclear debris and mitochondria.

TABLE II

PARTIAL PURIFICATION OF PHOSPHATASE ACTIVITIES

Enzyme preparations were obtained from cotyledon tissue after 5 days of growth. Assay mixtures and experimental details were identical with those described in Table I, except that in appropriate instances 10 mM *p*-nitrophenyl-*P* replaced the 10 mM glucose-6-*P*. The last column indicates the ratio of specific activity of glucose-6-*P* hydrolysis to that of *p*-nitrophenyl-*P* hydrolysis. Glc-6-*P*, D-glucose 6-phosphate.

Fractions	*Spec. act.*		*Spec. act. Glc-6-P* / *Spec. act. p-nitrophenyl-P*
	Glc-6-P	*p-Nitrophenyl-P*	
Homogenate	0.030	0.088	0.34
Microsomes	0.031	0.057	0.54
Supernatant	0.047	0.143	0.33
0–50% satd $(NH_4)_2SO_4$ fraction	0.029	0.097	0.30
50–70% satd $(NH_4)_2SO_4$ fraction	0.145	0.470	0.32
$(NH_4)_2SO_4$ supernatant fraction	0.000	0.000	—

results are depicted in Figs 1A and 1B. With each substrate substantial inhibition by KF–EDTA was observed only below pH 6.5. In the presence of KF–EDTA, the pH optima were shifted from about pH 5.6 to 6.1 (glucose-6-phosphatase) or 6.5 (*p*-nitrophenyl phosphatase). A significant differential effect of KF–EDTA on the two phosphatase activities was not observed. Consequently, KF–EDTA was not included in subsequent experiments.

In order to further examine whether the observed hydrolysis of glucose-6-*P* was catalyzed by a distinct glucose-6-phosphatase or by an acid phosphatase, the effect of partial inactivation of the enzyme preparation was compared using glucose-6-*P* and *p*-nitrophenyl-*P* as substrates. Glucose-6-phosphatase but not acid phosphatase activities in mammals were reported to be inhibited by preincubation with $ZnCl_2$[20,21] or preincubation at pH 5[22]. The effects of preincubation of the mustard

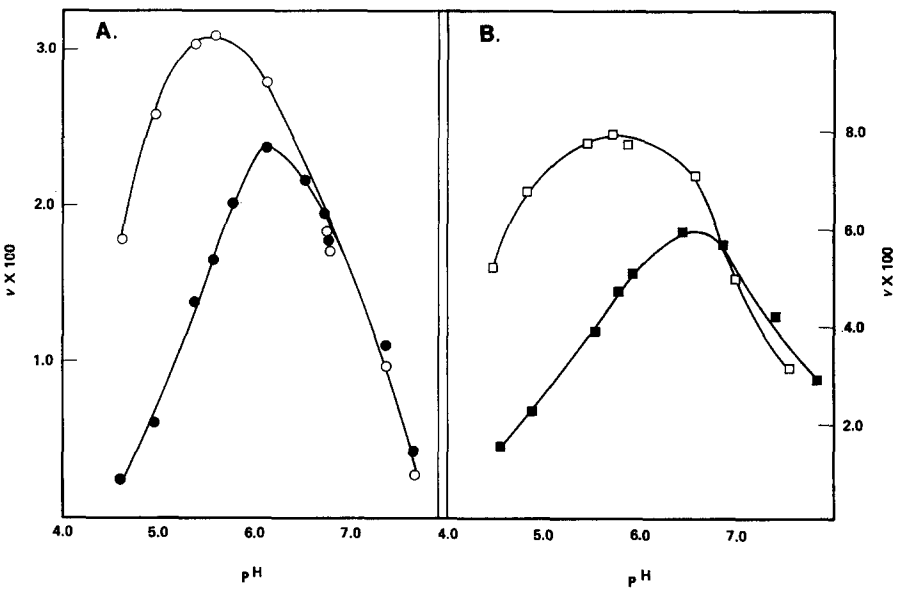

Fig. 1. Effect of reaction mixture pH on the hydrolysis of (A) glucose-6-*P*, and (B) *p*-nitrophenyl-*P*. Reaction mixtures contained, in 1.5 ml, 0.2 ml of partially purified enzyme preparation, 10 mM phosphate substrate, 40 mM buffer (sodium acetate, pH 4–5; sodium cacodylate, pH 5–7; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7–8), and 1 mM KF–4 mM EDTA (●, ■) or an equal volume of water (○, □). Reaction mixtures for each data point were prepared in triplicate and one sample of each was used to determine the actual pH with a Beckman, model Century SS-1, pH meter equipped with microelectrodes. Reactions were carried out at 30 °C for 5 min. Aliquots from the terminated reaction mixtures were assayed for orthophosphate formation. Velocity (v) is μmoles of substrate hydrolyzed per min.

preparation at acidic pH in the presence of $ZnCl_2$ are compiled in Table III. A similar decrease in phosphatase activity was observed with both substrates. In another experiment, the enzyme preparation was preincubated at various temperatures. The results are depicted in Fig. 2. The glucose-6-phosphatase activity was considerably more heat labile than *p*-nitrophenyl phosphatase activity. This pattern resembles that found in certain mammalian tissues where glucose-6-phosphatases were more susceptible to thermal inactivation than acid phosphatases[23–25].

TABLE III

EFFECT ON PHOSPHATASE ACTIVITIES OF ENZYME PREINCUBATION WITH $ZnCl_2$

The partially purified enzyme preparation was preincubated at 0 °C for 45 min with either $ZnCl_2$ solutions or equal volumes of water. Zn Cl_2 concentrations and pH values listed refer to preincubation conditions. Phosphatase activities then were assayed with reaction mixtures containing, in 1.5 ml, 10 mM phosphate substrate, 40 mM sodium cacodylate (pH 6.0), and 0.2 ml of preincubated enzyme preparation. Reactions were carried out for 5 min at 30 °C and then assayed for orthophosphate formation. Data are expressed as percentage of inactivation relative to activity exhibited by the preparation preincubated with water, which was considered to represent 0% inactivation.

Substrate	*Inactivation (%)*		
	$ZnCl_2$ absent, pH 5.5	*2.5 mM $ZnCl_2$, pH 4.6*	*5.0 mM $ZnCl_2$, pH 4.4*
Glucose-6-*P*	0	68	82
p-Nitrophenyl-*P*	0	69	79

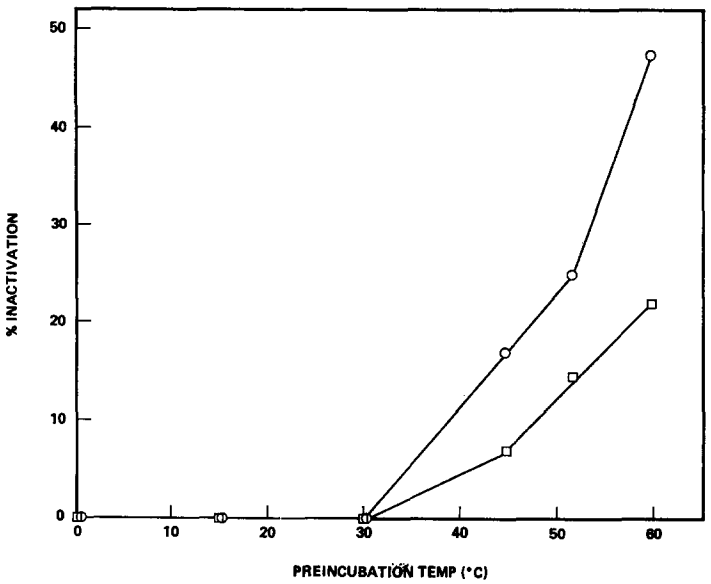

Fig. 2. Partial thermal inactivation of glucose-6-phosphatase (○) and *p*-nitrophenyl phosphatase (□) activities. Aliquots of partially purified enzyme preparation were preincubated at the indicated temperatures for 30 min, cooled, and then assayed for phosphatase activities. Reaction mixture composition and other experimental details were as described in Table III. Data are expressed as percentage of inactivation relative to the enzyme preparation preincubated at 0 °C, which was considered to represent 0% inactivation.

In order to determine more clearly whether the observed hydrolysis of glucose-6-*P* is attributable to an acid phosphatase, kinetic studies were carried out at pH 6.0. Experimental details and results of the combined substrate studies are given in Figs 3A and 3B. Data are presented as conventional double reciprocal plots. Michaelis and inhibitor constants were calculated as described by Dixon and Webb[26]. When two compounds serve as alternate substrates for the same enzyme, each will competitively inhibit the reaction involving the other[27]. As can be seen in Figs 3A and 3B, glucose-6-*P* acted as a competitive inhibitor of *p*-nitrophenyl-*P* hydrolysis and *p*-nitrophenyl-*P* behaved similarly towards the hydrolysis of the sugar phosphate. The K_m and K_i values for glucose-6-*P* were both 4.6 mM. The K_m and K_i values for *p*-nitrophenyl-*P* were 0.72 mM and 0.79 mM, respectively. The close agreement of K_m and K_i values for each compound is consistent with the catalysis of glucose-6-*P* and *p*-nitrophenyl-*P* hydrolysis by the same active site of a single enzyme.

The substrate specificity of the enzyme preparation was examined at pH 6.0 with a variety of phosphoryl compounds. As shown in Table IV, *p*-nitrophenyl-*P* and phenyl-*P* were the preferred substrates under the experimental conditions employed. The relative activity with respect to glucose-6-*P* was 43% of that observed with *p*-nitrophenyl-*P*. All the sugar phosphates tested were relatively poor substrates.

Since mammalian glucose-6-phosphatases possess associated PP_i–glucose phosphotransferase activity (see Nordlie[9]), the partially purified preparation from mustard was assayed for this activity. No phosphotransferase activity was observed

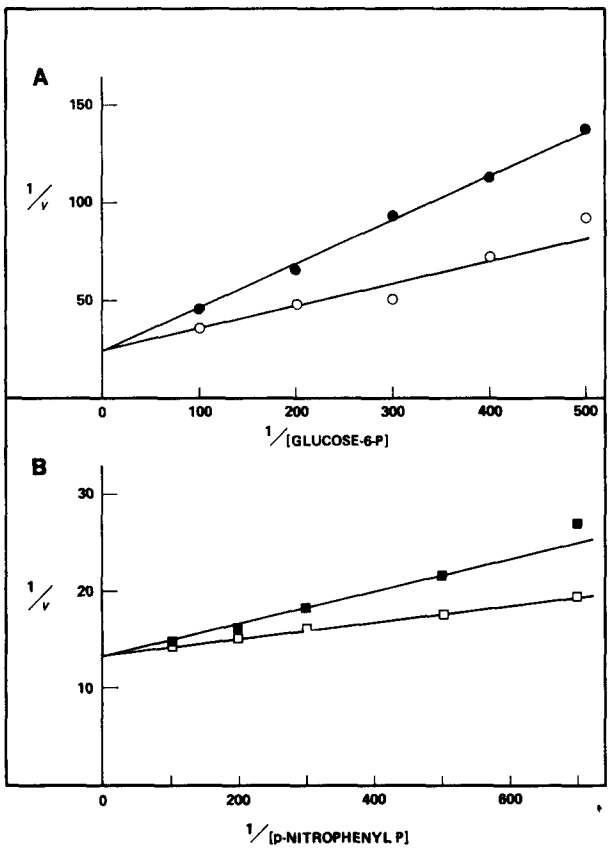

Fig. 3. Kinetics of mutual inhibition by glucose-6-*P* and *p*-nitrophenyl-*P*. Reaction mixtures contained, in 1.5 ml, 0.2 ml of partially purified enzyme preparation, 40 mM sodium cacodylate (pH 6.0), and the indicated, varied concentrations of (A) glucose-6-*P* or (B) *p*-nitrophenyl-*P*. Assays were carried out at 30 °C for 5 min in the absence (○, □) and presence of (A) 0.75 mM *p*-nitrophenyl-*P* (●) or (B) 4.0 mM glucose-6-*P* (■). Aliquots of the terminated reaction mixtures were assayed for (A) glucose or (B) *p*-nitrophenol formation. Velocity (v) is as defined in Fig. 1. The K_m and K_i values were calculated to be, respectively, 4.6 mM and 4.6 mM for glucose-6-*P*, and 0.72 mM and 0.79 mM for *p*-nitrophenyl-*P*.

with reaction mixtures containing 0.2 ml of enzyme preparation, 0.4 M glucose, 20 mM PP_i, and 40 mM buffer (acetate at pH 4.5, cacodylate at pH 6.0, or *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid at pH 7.5) in a total volume of 1.5 ml. In mammalian glucose-6-phosphatases, glucose is a non-competitive inhibitor of glucose-6-*P* hydrolysis[28,29], a finding which is consistent with the observations that phosphotransferase activities are present in those enzymes. However, when the partially purified mustard preparation was examined using concentrations of up to 0.8 M glucose, no inhibition of glucose-6-*P* hydrolysis occurred at pH 4.5, 6.0, or 7.5. This correlated with the lack of phosphotransferase activity exhibited by the mustard preparation.

TABLE IV

SUBSTRATE SPECIFICITY

Reaction mixtures contained, in 1.5 ml, 40 mM sodium cacodylate (pH 6.0), 20 mM substrate, and 0.2 ml of the partially purified enzyme preparation. Reactions were carried out for 5 min at 30 °C and then assayed for orthophosphate formation. Enzyme activities are expressed relative to *p*-nitrophenyl phosphatase activity which was assigned a value of 100%.

Substrate	*Relative activity (%)*
p-Nitrophenyl phosphate	100
Phenyl phosphate	105
Adenosine triphosphate	79
Pyrophosphate	73
β-Glycerophosphate	54
Glucose 6-phosphate	43
Mannose 6-phosphate	30
Fructose 6-phosphate	27
Glucose 1-phosphate	1

DISCUSSION

The glucose-6-phosphatase activity present in a soluble fraction from mustard cotyledon tissue appears to be catalyzed by an acid phosphatase. Glucose-6-phosphatase and *p*-nitrophenyl phosphatase activities exhibited a generally parallel pattern with respect to subcellular distribution, partial purification with $(NH_4)_2SO_4$, enzyme preincubation with $ZnCl_2$ at acidic pH, and inhibition by 1 mM KF–4 mM EDTA at various pH values. The two activities were not similar in their response to partial thermal inactivation. However, this may be attributable to the presence of a second, heat-stable phosphatase acting on *p*-nitrophenyl-*P* or to a heat-induced conformational change in a single enzyme which differentially altered the two activities. Kinetic studies with the simultaneous presence of glucose-6-*P* and *p*-nitrophenyl-*P* were consistent with the catalysis of both phosphatase activities by one enzyme. The results of all these experiments, taken individually, could be explained on a basis other than the involvement of a single enzyme. Taken collectively, however, these data indicate that a common enzyme catalyzes the hydrolysis of both glucose-6-*P* and *p*-nitrophenyl-*P*.

The substrate specificity study indicated that the enzyme examined is an acid phosphatase rather than a distinct glucose-6-phosphatase. Phenyl-*P*, *p*-nitrophenyl-*P* and β-glycerol-*P* all were hydrolyzed more rapidly than glucose-6-*P* under experimental conditions commonly used to measure glucose-6-phosphatase activity. At pH 6, the K_m value for *p*-nitrophenyl-*P* was approximately 6-fold smaller than that for glucose-6-*P*. Inhibition by 1 mM KF–4 mM EDTA of both phosphatase activities was observed only at or below pH 6.5. KF–EDTA previously was found to inhibit acid phosphatase but not glucose-6-phosphatase activities[6,10,11]. The lack of PP_i–glucose phosphotransferase activity in the mustard preparation and the failure of glucose to inhibit glucose-6-*P* hydrolysis also are in contrast with the properties of well-established mammalian glucose-6-phosphatases.

Glucose-6-phosphatases generally are located in the microsomal fractions of mammalian cells (see Nordlie[9]). In the mustard preparations we observed a constant ratio of glucose-6-phosphatase to *p*-nitrophenyl phosphatase activity in all fractions

except the microsomes, in which a higher ratio was obtained. This indicates the possible presence of a specific glucose-6-phosphatase in the microsomal fraction. In contrast with other reports, a glucose-6-phosphatase recently was reported to be present in the soluble fraction from *Phaseolus vulgaris* (Thompson[6]). However, in that study glucose-6-*P* hydrolysis was measured at pH 6.5 in the presence of 4 mM KF–4 mM EDTA, while the extent of inhibition (85–95%) by KF–EDTA of acid phosphatase (*β*-glycerophosphatase) activity was measured at pH 5.4. With the soluble mustard preparation, we found that the extent of inhibition by KF–EDTA was highly pH dependent. This indicates that the effectiveness of KF–EDTA in masking acid phosphatase activity should be measured at the same pH used to measure glucose-6-*P* hydrolysis. For example, we observed that 1 mM KF–4 mM EDTA caused approximately 55% inhibition of *p*-nitrophenyl phosphatase activity at pH 5.4 and only 2% inhibition at pH 6.5. In a subsequent experiment with 4 mM KF–4 mM EDTA, a 75–80% inhibition was observed at pH 5.5 and the extent of inhibition was the same with *p*-nitrophenyl-*P*, *β*-glycerol-*P*, or glucose-6-*P* as substrate. Hübscher and West[10] found that the full inhibitory effect of KF–EDTA was obtained only when enzyme preparations were preincubated with the inhibitors. Thus the usefulness of KF–EDTA in distinguishing phosphatase activities appears to be dependent on pH, concentration of KF, and enzyme preincubation. Under the experimental conditions employed in the present studies, the observed hydrolysis of glucose-6-*P* at pH 6.5 in the presence of KF–EDTA was an insufficient criterion to establish the presence of a distinct glucose-6-phosphatase in mustard.

To our knowledge, the existence of a true glucose-6-phosphatase has not been definitely established in any plant. The metabolic need in plants for this enzyme is unknown, since glucose is generally synthesized by the hydrolysis of sucrose[30]. We suggest that characteristics such as those presented in this paper should be examined in other reported plant glucose-6-phosphatases in order to determine whether the observed hydrolysis of glucose-6-*P* is catalyzed by an acid phosphatase or by a distinct glucose-6-phosphatase.

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