

Mannitol Synthesis in Higher Plants¹

Evidence for the Role and Characterization of a NADPH-Dependent Mannose 6-Phosphate Reductase

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ABSTRACT

Mannitol is a major photosynthetic product in many algae and higher plants. Photosynthetic pulse and pulse-chase ¹⁴C-radiolabeling studies with the mannitol-synthesizing species, celery (*Apium graveolens* L.) and privet (*Ligustrum vulgare* L.), showed that mannose 6-phosphate (M6P) and mannitol 1-phosphate were among the early photosynthetic products. A NADPH-dependent M6P reductase was detected in these species (representing two different higher plant families), and the enzyme was purified to apparent homogeneity (68-fold with a 22% yield) and characterized from celery leaf extracts. The celery enzyme had a monomeric molecular mass, estimated from mobilities on sodium dodecyl sulfate-polyacrylamide gels, of 35 kilodaltons. The isoelectric point was pH 4.9; the apparent K_m (M6P) was 15.8 millimolar, but the apparent K_m (mannitol 1-phosphate) averaged threefold higher; pH optima were 7.5 with M6P/NADPH and 8.5 with mannitol 1-phosphate/NADP as substrates. Substrate and cofactor requirements were quite specific. NADH did not substitute for NADPH, and there was no detectable activity with fructose 6-phosphate, glucose 6-phosphate, fructose 1-phosphate, mannose 1-phosphate, mannose, or mannitol. NAD only partially substituted for NADP. Mg^{2+} , Ca^{2+} , Zn^{2+} , and fructose-2,6-bisphosphate had no apparent effects on the purified enzyme's activity. *In vivo* radiolabeling results and the enzyme's kinetics, specificity, and distribution (in two-plant families) all suggest that NADPH-dependent M6P reductase plays an important role in mannitol biosynthesis in higher plants.

Sugar alcohols (acyclic polyols or alditols) are obtained when the aldo or keto group of a sugar is reduced to a hydroxyl. Mannitol, the most frequently occurring sugar alcohol in plants, is particularly abundant in algae and has been detected in at least 70 higher plant families. It is a major carbohydrate in many members of some dicot families, *e.g.* the Scrophulariaceae, Oleaceae, Rubiaceae, and Apiaceae (2). Until recently, however, little information has been available on mannitol's role in higher plants (16, 17). It is now known

that it is an early photosynthetic product (27, 29) and present in phloem tissue or phloem exudates of celery (family Apiaceae) (9) and species in many other families, *e.g.* the Oleaceae (30). Other physiological roles have been proposed, including osmoregulation, storage and recycling of reducing power, and service as a compatible solute (16, 17), but very little is known of mannitol metabolism in higher plants. A M6PR² has been reported as being located in the cytosol of mesophyll protoplasts from celery (27). Preliminary labeling data derived from celery and privet were responsible for the initial assays for reductase activity with M6P and mannitol 1-P as substrates. Here we demonstrate the formation of M6P and mannitol 1-P as early photosynthetic products in celery and privet (family Oleaceae). We also report evidence for the role and importance of M6PR and its characteristics in mannitol biosynthesis in celery.

MATERIALS AND METHODS

Plant Material

For pulse and pulse-chase labeling experiments, privet (*Ligustrum vulgare* L.) shoots (collected midmorning locally and quickly recut under water) were left in water under a mercury vapor lamp (minimum of 700 $\mu E m^{-2} s^{-1}$) for 45 min prior to labeling. Celery (*Apium graveolens* L., Giant Pascal) leaves (still attached to pot-grown plants) were similarly treated. Celery-growing conditions have been previously described (8). For enzyme extractions, leaves were collected midmorning and held briefly on ice prior to use.

Radioisotope Labeling

Three terminal celery leaflets or two pairs of privet leaves were enclosed in plastic bags (approximate volume 1.0 L) with room air and gelatin capsules containing droplets of 50 $\mu L NaH^{14}CO_3$ (50 μCi) and 100 μL 30% (v/v) lactic acid. At time zero, the capsule was broken and photosynthesis continued (mercury vapor lamp, minimum of 700 $\mu E m^{-2} s^{-1}$) at

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² Abbreviations: M6PR, NADPH-dependent mannose 6-phosphate reductase; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; M6P, mannose 6-phosphate; mannitol 1-P, mannitol 1-phosphate; 1 mU = 1 nmol NADPH oxidized/min at 30°C.

25 to 27°C. For pulse labeling (20 s–8 min), the bag and leaves were freeze-clamped (liquid N₂) and the frozen leaf tissue was dropped into 10 mL of methanol:chloroform:H₂O:formic acid (12:5:2:1, v/v), which was immediately frozen (liquid N₂) and then stored overnight at –20°C. In pulse-chase studies, the leaves were labeled for 4 min, followed by a chase in room air (1–120 min), and then frozen as in the pulse studies.

Extraction, Fractionation, and Autoradiography of Radiolabeled Products

All tissues were extracted (3) and metabolites separated on columns of SP- and QAE-Sephadex (23) into sugar, amino acid, organic acid, and phosphate ester fractions. The latter fraction was also passed through a second SP-Sephadex (H⁺) column to remove cations before it and the sugar fraction were further analyzed on thin-layer cellulose plates. Sugars were chromatographed in the first dimension in methyl ethyl ketone:pyridine:H₂O:acetic acid (70:15:15:2, v/v) followed by *n*-propanol:H₂O:*n*-propyl acetate:acetic acid:pyridine (120:60:20:4:1, v/v) and in the second dimension in methyl ethyl ketone:acetic acid:saturated boric acid in H₂O (9:1:1.5, v/v). Phosphate esters were separated by two-dimensional TLC (1). Developed plates were autoradiographed and radioactive components extracted according to Redgwell *et al.* (25).

Trimethylsilyl derivatives of sugars, polyols, and phosphatase-treated phosphate esters were analyzed by GC/flame ionization detection after conversion of the sugars to their respective oximes (6). For isolation and identification of mannitol 1-P, celery and privet leaves (17.5 and 21.5 g fresh weight, respectively) were labeled as above with 500 μCi ¹⁴C₂ for 4 min, then immersed in liquid N₂ and killed in 120 mL methanol:chloroform:H₂O:formic acid (12:5:2:1, v/v). Leaves were homogenized immediately in a blender and stored overnight at –20°C. Isolation of mannitol 1-P and other phosphate esters was similar to the procedure of Redgwell and Bielecki (24) for sorbitol 6-phosphate. Sugar phosphates were isolated by anion-exchange column chromatography on QAE-Sephadex. A 0.05 to 0.5 M gradient of NH₄HCO₃ was used to elute fractions that were monitored by thin-layer electrophoresis for sugar phosphates. A pooled fraction was dried and applied to Whatman No. 1 paper and chromatographed in *tert*-butanol:H₂O:saturated aqueous picric acid:boric acid (40:10:2:1, v/v/v/w) with standard mannitol 1-P (Sigma) applied to the margin as a marker. Autoradiography revealed three bands (G6P, M6P, F6P). The standard mannitol 1-P co-migrated with F6P, and this band was cut out, eluted with H₂O, and rechromatographed on paper in butanol:acetic acid:H₂O:pyridine (55:15:45:45, v/v). Two radioactive bands were detected, F6P and a faint band of mannitol 1-P, each of which was eluted separately with H₂O and subjected to partition column chromatography on LH20 Sephadex. Radioactive fractions were combined.

Identification of the hexitol phosphate was done by mixing the radioactive fraction with a solution of standard mannitol 1-P and then subjecting the mixture to two-dimensional TLC for phosphate esters (1). Autoradiography revealed a single radioactive spot that co-migrated with the standard mannitol 1-P detected by molybdate spray reagent. The hexitol phosphate was dephosphorylated by treatment with phosphatase

(24) and the products separated by two-dimensional TLC as used for sugars. Autoradiography revealed a single radioactive spot that coincided with standard mannitol.

Privet neutral sugars and oligosaccharides were identified by applying neutral sugar extracts (from a 16-min pulse) as bands across TLC plates that were then chromatographed in one dimension as above, yielding five bands. These were each eluted and separately rechromatographed, again eluted, hydrolyzed in 0.5 N TFA, and the products derivatized and quantified as the alditol acetates via GC/flame ionization detection.

To determine specific radioactivities, radioactivities were obtained from the two-dimensional TLC sugar and the thin-layer electrophoresis phosphate ester separations as described by Redgwell *et al.* (25). Sugars were quantified by GC/flame ionization detection. Phosphate esters, after recovery from the paper chromatogram, were further purified by LH20 Sephadex fractionation, subjected to phosphatase, and the Pi analyzed by the procedure of Penny (21).

M6PR Isolation

Routine enzyme preparations involved freezing lamina from leaves 5 through 8 from mature, 14- to 17-leaf celery plants in liquid N₂ as described by Davis *et al.* (8) followed by grinding to a powder in a mortar and pestle. The powder, representing 10.0 g fresh weight of lamina, was homogenized at 0°C with a Polytron (2 × 15-s full speed) in 100 mL buffer A (100 mM Tris-HCl, pH 7.5, 10 mM DTT) containing 5.0 g insoluble PVP. This and all other buffers were degassed under vacuum with stirring for at least 1 h prior to use. The homogenate from two such extractions was filtered through Polycloth and centrifuged at 27,500g for 20 min. The supernatant fluid (crude extract) was slowly precipitated with cold (–21°C) acetone (30–60% fraction), with nitrogen gas blowing onto the surface, allowed to stand for 20 min under nitrogen, and the precipitate collected by centrifugation (27,500g for 20 min). The pellet was resuspended in a minimum volume (<5 mL) of buffer B (20 mM Mes, pH 6.5, 2 mM MgCl₂, 1 mM DTT, 0.02% [w/v] NaN₃) while nitrogen gas was blown gently over the surface of the buffer and pellet. The sample was briefly centrifuged (26,800g for 10 min), and the supernatant fraction was applied to a Sephacryl S-200 column (93 × 2.5 cm) preequilibrated and eluted with buffer B. Active fractions could be combined, lyophilized, and stored at 4°C, retaining full activity for at least several months. Otherwise, high activity fractions (>10 mU/100 μL) were pooled and loaded directly onto a column (6.9 × 2.5 cm, 34 mL resin) of Reactive Yellow 86-agarose (Sigma), previously equilibrated with buffer B. The column was washed with buffer B until A₂₅₄ of the column effluent had returned to the base line for two to four fractions (approximately 25 mL). Activity was then eluted with 0.1 mM NADPH in buffer B, and fractions with high M6PR activity (>10 mU/50 μL) were pooled. Purified enzyme, after affinity chromatography, was flash frozen (liquid N₂) and stored at –21°C. In this form, at least 50% of the activity was retained after 1 month. Samples at various stages during the purification were precipitated by adding cold acetone (–21°C) to 70% and leaving at –21°C for 30 min. Precipitated protein was centrifuged, and the

pellet resuspended in SDS-sample buffer (15) prior to loading onto SDS-PAGE gels (5% acrylamide stacking gel, 10 or 12.5% acrylamide resolving gel) (15). Gels were stained with Coomassie brilliant blue R-250. Protein was otherwise assayed following the method of Bradford (5) using BSA as the standard.

Nondenaturing isoelectric focusing was performed on thin gels (approximately 0.7 mm) containing 5% deionized acrylamide, 0.13% *N,N'*-methylenebisacrylamide (British Drug House) essentially as described by Ried and Collmer (26), with pH ranges of 3.0 to 10.0 and 4.0 to 7.0 (at 8°C). Gels were prefocused at 2 W for 20 min prior to sample loading and were focused at 6 W for 2 h after application of the proteins (3–9 µg/application) either directly on the gel surface in a chain of drops (10 µL total, 1 µL/drop) or in Miracloth wicks (5 × 10 mm), which were removed 40 min after the start of the run. When using wicks, the protein remained in the wicks when loaded at the anode end. Gels were either fixed (22) and stained with 0.1% Coomassie brilliant blue G-250 or silver (4) or used for *in situ* activity staining (see below). Gels were calibrated for pH using Pharmacia broad-range standards.

To localize enzyme activity, gel strips were incubated for 1 h to overnight in the dark at 30°C in either 8 mM Tris-HCl, pH 8.5, 90 mM mannitol 1-P, 2.6 mM NADP, 0.7 mM phenazine methosulfate, and 2.6 mM nitro blue tetrazolium, or the same mixture lacking mannitol 1-P.

Enzyme Assays

Enzyme activities were routinely monitored by cofactor oxidation or reduction at 340 nm and 30°C in 33 mM Tris-HCl, pH 7.5 (pH 8.5 for reduction), 3 mM DTT, saturating levels (0.4 mM) of NADPH or NADP, 10 mM M6P (or 10 mM mannitol 1-P) (barium salts solubilized immediately prior to use with equimolar K₂SO₄), and enzyme extract (10–100 µL, depending on the stage of purification) in a final volume of 1 mL. Substrate concentrations and enzyme specific activities otherwise varied with the experiment. For pH optimum determinations, the partially purified lyophilized enzyme (from the Sephacryl S-200 eluate) was resuspended in 10 mM Tris-HCl, pH 8.0, and 50 µL (57 µg protein) was added to 800 µL of the buffer being tested, also at 10 mM, with either 1.3 mM NADP or 0.13 mM NADPH (total volume 1 mL). The reaction was initiated by addition of 50 µL M6P or mannitol 1-P (100 mM K salt). Apparent *K_m* for M6P was determined in 10 mM Tris-HCl, pH 7.5, over seven concentrations ranging from 0.1 to 10 mM. Apparent *K_m* for mannitol 1-P was determined in 10 mM Tris-HCl, pH 8.5, over the same concentration range. Substrate specificities were determined with 10 mM of the K salt of the phosphate esters and either 1.3 mM NAD(P) or 0.13 mM NAD(P)H as above.

Procedures for determinations of M6PR activity in crude celery extracts have been described by Davis *et al.* (8). Assays for M6P isomerase (phosphomannose isomerase) and mannitol 1-P phosphatase have been described (27), or were modified here using a different phosphate assay (28) for the phosphatase activity.

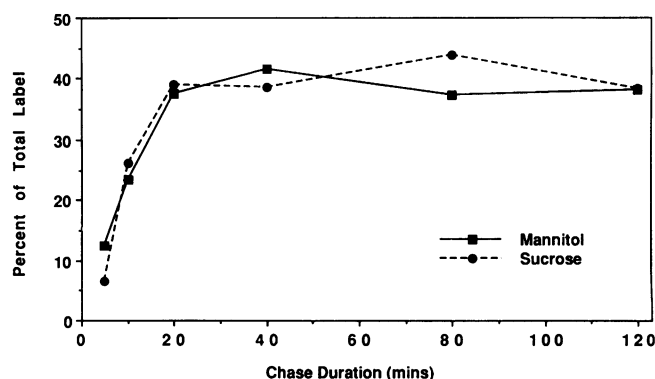


Figure 1. Radiolabeling of mannitol and sucrose in terminal leaflets of nearly fully expanded celery leaves pulsed 4 min with ¹⁴CO₂ and then chased with room air. Each time point represents results from a single plant.

RESULTS

Radiolabeling Studies

Photosynthetic pulse-chase radiolabeling of celery leaves in ¹⁴CO₂ for 4 to 120 min followed by chromatography and autoradiography showed that after 20 min or more, over 80% of the total label was in two products, mannitol and sucrose (Fig. 1). At shorter times with pulse radiolabeling, the proportion of ¹⁴C in these two compounds was much lower in both celery and privet, whereas that in the triose-P and hexose-P esters was much higher (Fig. 2). For example, in celery with a 30-s pulse (no chase), mannitol 1-P and M6P each represented approximately 3% of the total radioactivity, whereas sucrose, mannitol, and triose-P accounted for 0.3, 0.6, and 30%, respectively (Fig. 2). In a separate experiment using a single 4-min pulse (no chase), specific radioactivities for both M6P and mannitol 1-P were high compared with sucrose and mannitol, *e.g.* hexose phosphates were approximately 100× higher and mannitol 1-P 20× higher (Table I). The two main carbohydrates, sucrose and mannitol, had similar specific radioactivities in both celery and privet.

Some labeling patterns, however, were species dependent. Unlike with celery, in the first 60 s of privet pulse-labeling total radioactivity in mannitol 1-P was high compared with that in M6P (Fig. 2), which seems inconsistent with the proposed pathway (see below). This may have been an artifact due to poor air-mixing during these short labeling times. On the other hand, specific radioactivities after a 4-min pulse in both species (Table I) were entirely consistent with the proposed pathway. In privet, radioactivity was also high in galactinol and a raffinose-related compound (Other in Fig. 2). Galactinol was identified from co-migrating standards and hydrolysis, which yielded equimolar amounts of galactose and inositol. The raffinose-related compound was tentatively identified as verbascose because hydrolysis resulted in one equivalent each of fructose and glucose and three of galactose. Galactinol has also been identified as the galactose donor in the biosynthesis of raffinose, stachyose, and verbascose (13). Despite the species differences, the labeling results with both species were otherwise generally consistent with the proposed

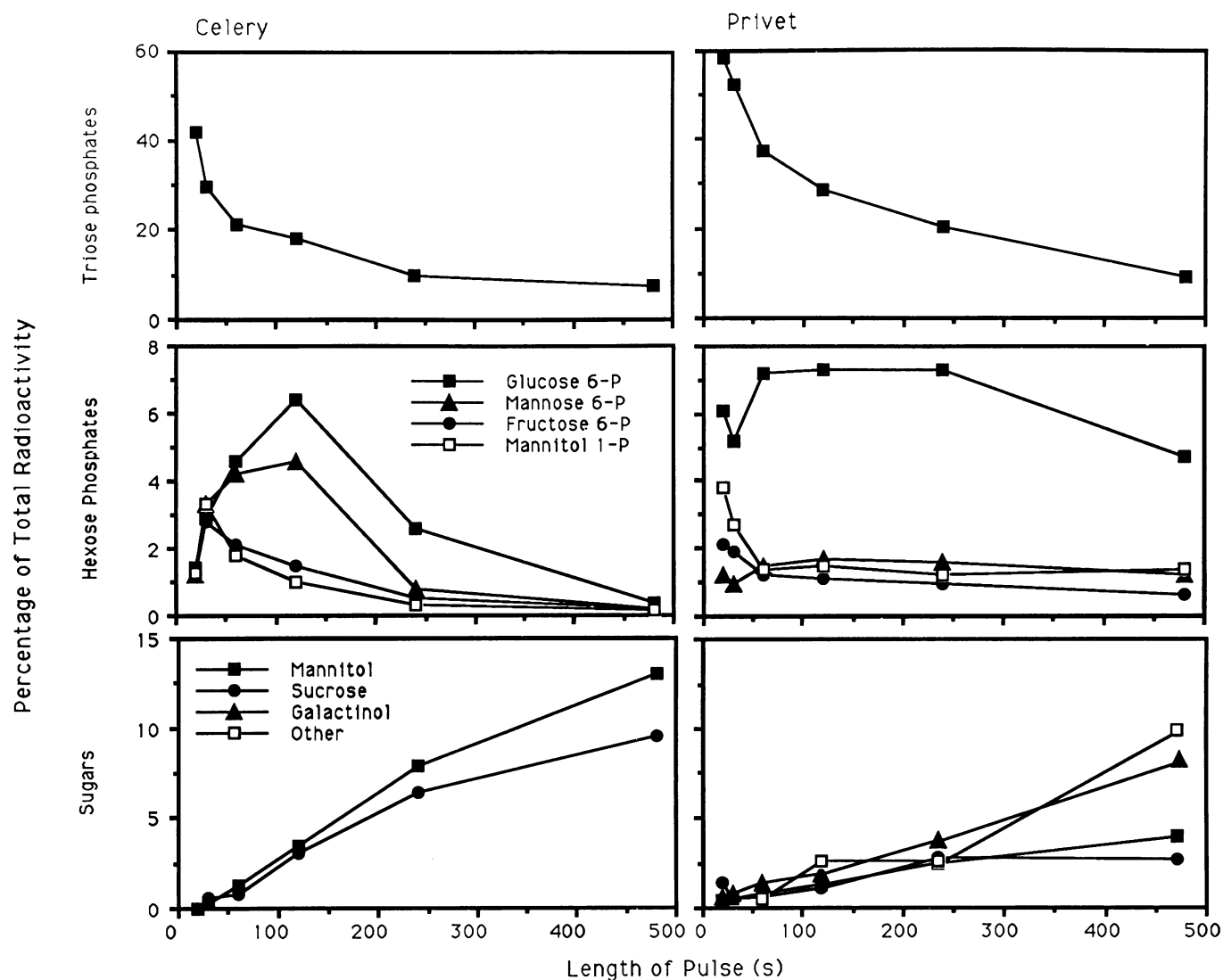


Figure 2. Results of $^{14}\text{CO}_2$ radiolabeling of triose-P, hexose-P, and sugars (pulsed 20 to 480 s, no chase) in terminal leaflets or leaves of celery (left panels) and privet (right panels), respectively. Each time point represents a single observation. "Other" refers to a raffinose-derivative tentatively identified as verbascose.

Table I. Amounts, Radioactivities, and Specific Radioactivities of Selected Carbohydrates and Phosphate Esters Extracted from Leaves of Celery and Privet after a 4-min Exposure to $^{14}\text{CO}_2$ in the light

Each number represents a single observation.

Compound	Tissue Content		Radioactivity		Specific Radioactivity	
	Celery	Privet	Celery	Privet	Celery	Privet
	$\mu\text{mol/g fresh wt}$		nCi/g fresh wt		$\text{nCi}/\mu\text{mol C}$	
Sucrose	55.8	21.0	2481	303	3.7	1.2
Mannitol	58.1	58.8	3062	564	8.8	1.6
Glucose	3.2	8.7	16.6	79	0.9	1.5
Galactinol		3.2		1585		41.2
G6P	0.076	0.180	352	382	772	353
F6P	0.022	0.045	126	111	955	411
MGP	0.045	0.035	240	168	889	800
Mannitol 1-P	0.0092	0.0075	11.0	6.2	199	138

Table II. Summary of a Typical Purification of M6P Reductase from 20 g Fresh Wt of Celery Leaves

Step	Volume	Protein	Total Activity	Specific Activity	Yield	Purification
	<i>mL</i>	<i>mg</i>	<i>mU</i>	<i>mU/mg</i>	%	×
Crude extract	201	341.7	18,070	53	100	1
30–60% acetone fractionation	5.2	30.3	11,820	390	65	7
S-200 gel filtration	16.1	8.46	7,230	855	40	16
Reactive Yellow 86-agarose	11.4	1.23	4,620	3,756	26	71

pathway, namely (in brief) triose-P → → F6P → M6P → mannitol 1-P → mannitol (17, 27).

Enzyme Purification and Characterization

Results of the standard purification protocol are summarized in Table II. Typically, final purification, following Reactive Yellow 86 affinity chromatography, averaged 68-fold, whereas yields averaged 22% (range from five purifications, 50–90%, 18–26%, respectively). Best yields were maintained through avoiding oxidation by: (a) using DTT throughout the procedure, (b) using degassed buffers, and (c) solubilizing the final acetone pellet under gaseous nitrogen. SDS-PAGE analysis of the extracts at various stages of purification is illustrated

in Figure 3. M6PR is visible as one of a number of significant bands in the crude extract (note the dominant large and small subunits of Rubisco, at approximately 51 and 15 kD, respectively), but only a single band is evident following affinity chromatography. Isoelectric focusing revealed a single band, or in some cases a doublet (Fig. 4, lanes B, C). These bands coincided with the *in situ* stain for enzyme activity (Fig. 4, lane D). Protein staining indicated an isoelectric point of pH 4.94 (run on a narrow pH range gel) and 4.87 ± 0.03 (four runs on broad range gels). Silver staining showed the affinity-purified protein to be homogeneous on a broad pH range gel

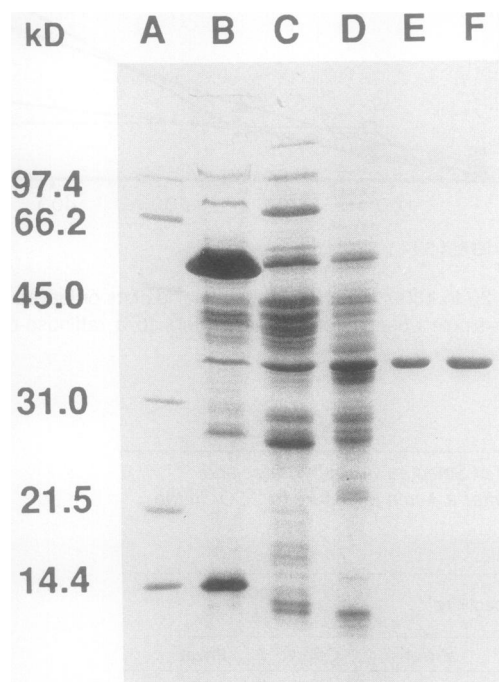


Figure 3. SDS-PAGE gel (12.5% acrylamide) of samples collected after the various purification steps (see Table II). Lanes: A, Bio-Rad low range molecular mass markers; B, 27,500g supernatant fluid of the crude leaf extract (163 μ g protein); C, 30 to 60% acetone fraction (101 μ g); D, post gel-filtration chromatography on Sephacryl S-200 (70 μ g); E and F, post affinity chromatography on Reactive Yellow 86 (4 and 8 μ g, respectively). The gel was stained with Coomassie brilliant blue R-250.

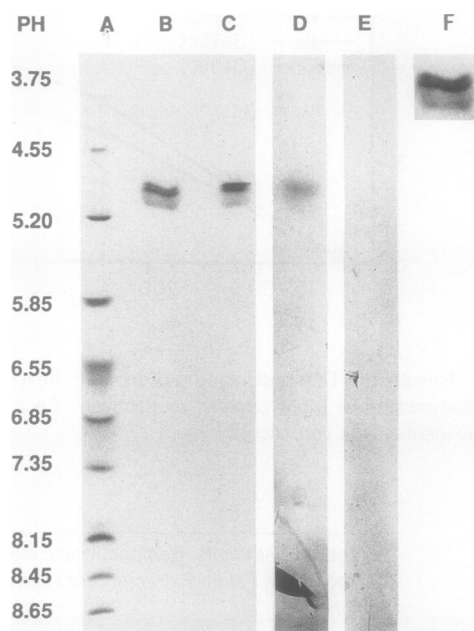


Figure 4. Native isoelectric focusing of affinity-purified M6PR. Lanes: A, Pharmacia broad-range isoelectric focusing markers (the position labeled 3.75 shows the position of methyl red dye that is lost from the gel during the fixation and staining steps). B and C, 3 μ g M6PR loaded in wicks placed 1 cm from the cathode (bottom of the figure) and halfway between the cathode and the anode, respectively (the gel has been cropped so that the edges represent the leading edges of the electrode wicks). Lanes A, B, and C were stained with 0.1% Coomassie brilliant blue G-250 after fixation (see text). Lane D as in C but stained for M6PR activity using mannitol 1-P as substrate. Lane E as in D but with mannose 1-P omitted. Lane F, enlargement of the stained band in B to illustrate that the major protein band appears to be a doublet.

(data not shown). In all cases, M6PR activity stains coincided with the protein-stained bands.

Crude extracts contained two enzymes thought to be involved in mannitol synthesis that could potentially interfere with accurate assay of M6PR activity: M6P isomerase (phosphomannose isomerase) and mannitol 1-P phosphatase (27). The isomerase was unstable in crude extracts and its activity was very low following acetone precipitation, and neither enzyme was present following affinity chromatography (data not shown).

Molecular mass of M6PR was estimated from mobilities on SDS-polyacrylamide gels or on calibrated Sephacryl S-200 or Superose 12 columns. Average monomeric molecular mass on 10% acrylamide was 35.3 ± 0.1 kD, and on 12.5% acrylamide, 34.5 ± 1.0 kD (cf. Fig. 3). With Sephacryl S-200, native molecular mass was 58.3 ± 7.1 kD; with Superose 12 (equilibrated and eluted with buffer B + 150 mM KCl), it was 52.6 kD (single determination). Although these values differed depending on the procedure used, this enzyme may be a dimer of two 35 kD subunits, accounting for the anomalous mobilities. Other characteristics were as follows: the apparent K_m value for M6P was 15.8 mM, but averaged threefold higher for mannitol 1-P; pH optima were 7.5 with M6P and 8.5 with mannitol 1-P (the enzyme was inactivated below pH 6.0, but stable from pH 6.0 to 9.0). Substrate and cofactor requirements of the purified enzyme were quite specific. NADH could not substitute for NADPH in reducing M6P. There was no detectable activity with F6P, G6P, fructose 1-P, mannose 1-P, mannose, or mannitol. NAD only partially substituted in oxidizing mannitol 1-P, at only 8% of the rate with NADP. Magnesium, calcium, and zinc ions at 0.1 to 6.0 mM had no significant effects on activity with either M6P or mannitol 1-P as substrate. Although enzyme activity was reduced by 47% in 100 mM NaCl, desalted preparations regained 100% of control activity. Fructose-2,6-bisphosphate at 1 and 5 μ M had no apparent effect on activity of the purified enzyme.

Incubating pure enzyme with NADPH and 14 C-labeled M6P resulted in formation of labeled mannitol 1-P. The ratio, following phosphatase treatment and chromatography, of mannose/mannitol (cpm) was 1095/1246, but with the boiled enzyme control this ratio was 2407/200. The apparent labeling of mannitol in the control is probably due to fructose tailing into the mannitol spot, because a small amount of labeled F6P was present as a contaminant in the labeled M6P.

M6PR Activity in Other Higher Plants

Although no efforts were made to optimize extraction conditions, an attempt was made to extract and assay NADPH-dependent M6PR activity from several mannitol-synthesizing species other than celery, *i.e.* common privet, gardenia (*Gardenia jasminoides*, Rubiaceae), and ngaio (*Myoporum laetum*, Myoporaceae). NADPH-dependent M6PR-like activity was detectable in all these species (data not shown), suggesting that M6PR activity is related to the presence of mannitol. Also, negligible NADPH-dependent M6PR activity was found in two higher plant species that do not produce mannitol (27). On the other hand, NADH-dependent M6PR activity has been reported in brown algal macrophytes where mannitol is a common constituent and the primary

photosynthetic product. Although several attempts were made here to assay NADPH-dependent M6PR in these organisms, none were successful.

DISCUSSION

Although mannitol is the most widely distributed of the sugar alcohols, it is not the only one found in higher plants, nor is M6PR the only reductase forming sugar alcohols in higher plants. A number of different reductases and sugar alcohols occur in unrelated taxa. Sorbitol commonly occurs in the Rosaceae as a primary photosynthetic product, and a NADPH-dependent aldose 6-P reductase is responsible for sorbitol synthesis in leaf (19) and perhaps fruit tissues (12); also, a NAD-dependent sorbitol dehydrogenase may mediate sorbitol degradation in sink tissues (17). Negm has reported a NAD-dependent mannitol 1-P dehydrogenase in *Fraxinus* (Oleaceae) tissue cultures, a ribitol-synthesizing NADPH-dependent ribose 5-P reductase in *Adonis* (Ranunculaceae) (20), and a galactitol-synthesizing NADPH-dependent aldose reductase in *Euonymus* (Celastraceae) leaves (18). Sorbitol has been found and both ketose and aldose reductases have been detected in crude extracts of germinating soybean (*Glycine max*, Fabaceae) seedling axes where these may serve as a possible means of glucose and fructose interconversions (14). Sorbitol (7) and a ketose reductase (10) have also been reported in maize (*Zea mays*, Poaceae). Although the metabolic importance of some of these enzymes and the roles of some sugar alcohols are still being debated, mannitol's importance in photosynthesis is now clear, as is a role for M6PR in mannitol biosynthesis. Approximately 50% of the carbon fixed by photosynthesis was found in mannitol in recently fully expanded celery leaves (8), and mannitol was translocated from the leaf (9). Evidence here (Fig. 1) also shows that mannitol is an important photosynthetic product and strongly suggests that conversion of M6P to mannitol 1-P via M6PR is an important step in mannitol biosynthesis. Evidence for both M6P and mannitol 1-P in any higher plant has not previously been reported, but their presence here in two mannitol-synthesizing species is entirely consistent with the proposed pathway, triose-P \rightarrow F6P \rightarrow M6P \rightarrow mannitol 1-P \rightarrow mannitol.

The data are also consistent with previous evidence showing that the NADPH-dependent M6PR is located in the cytoplasm of celery leaves (27). This is supported by the enzyme's substrate specificities, with M6P apparently derived from the same cytoplasmic hexose-P pool as for sucrose biosynthesis, and the pH optimum, *e.g.* pH 7.5 for M6P reduction. The data are also compatible with the enzyme's activity changes during celery leaf development, increasing in parallel with photosynthetic capacity and export of mannitol from source leaves (8, 9). In addition, the extracted activity of the celery M6PR, reported here (Table II) and elsewhere (8, 27), is adequate to account for observed rates of mannitol biosynthesis, even at the unusually high photosynthetic rates observed in celery (11).

These enzyme activities, characteristics, developmental changes, and tissue distribution and substrate requirements may indicate the general importance for a NADPH-dependent M6PR in mannitol-synthesizing higher plants. Although

other data are perhaps only suggestive, *i.e.* sorbitol biosynthesis is accomplished via an equivalent NADPH-dependent aldose 6-P reductase using G6P as substrate (19), the labeling patterns reported here for species from two different families are entirely consistent with the hypothesis that mannitol is derived from M6P and mannitol 1-P. When considering mannitol's occurrence in 70 higher plant families, and sometimes in high concentrations, a careful survey of higher plant taxa where mannitol is an important photosynthetic product may show that M6PR and these substrates are widespread and generally important steps in mannitol biosynthesis in higher plants.

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