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myo-Inositol-1-Phosphatase from the Pollen of *Lilium longiflorum* Thunb.[1]

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ABSTRACT

A Mg^{2+}-dependent, alkaline phosphatase has been isolated from mature pollen of *Lilium longiflorum* Thunb., cv. Ace and partially purified. It hydrolyzes 1L- and 1D-*myo*-inositol 1-phosphate, *myo*-inositol 2-phosphate, and β-glycerophosphate at rates decreasing in the order named. The affinity of the enzyme for 1L- and 1D-*myo*-inositol 1-phosphate is approximately 10-fold greater than its affinity for *myo*-inositol 2-phosphate. Little or no activity is found with phytate, D-glucose 6-phosphate, D-glucose 1-phosphate, D-fructose 1-phosphate, D-fructose 6-phosphate, D-mannose 6-phosphate, or *p*-nitrophenyl phosphate. 3-Phosphosphoglycerate is a weak competitive inhibitor. *myo*-Inositol does not inhibit the reaction. Optimal activity is obtained at pH 8.5 and requires the presence of Mg^{2+}. At 4 millimolar, Co^{2+}, Fe^{2+} or Mn^{2+} are less effective. Substantial inhibition is obtained with 0.25 molar Li^+. With β-glycerophosphate as substrate the K_m is 0.06 millimolar and the reaction remains linear at least 2 hours. In 0.1 molar Tris, β-glycerophosphate yields equivalent amounts of glycerol and inorganic phosphate, evidence that transphosphorylation does not occur.

In higher plants this *myo*-inositol-1-phosphatase links *myo*-inositol biosynthesis to the *myo*-inositol oxidation pathway to produce an alternative path from D-glucose 6-phosphate to UDP-D-glucuronate that bypasses UDP-D-glucose dehydrogenase. *myo*-Inositol-1-phosphatase also furnishes free *myo*-inositol for reactions that lead to other cyclitols and cyclitol-containing compounds of biosynthetic and/or regulatory significance in plant growth and development.

1L-MI-1-P,[3] the product of 1L-MI-1-P synthase (EC 5.5.1.4) is hydrolyzed to MI and Pi by MI-1-phosphatase (EC 3.1.3.5),[4] a Mg^{2+}-dependent enzyme that occurs in yeast (3), rat testis (8), rat mammary gland (21), and bovine brain (12). The epimeric form 1D-MI-1-P,[5] also is hydrolyzed by MI-1-phosphatase at 80% to 90% of the rate of the 1L isomer. In a recent study of MI-1-phosphatase from chick erythrocytes (24) only the 1D isomer was used as substrate but the properties of this enzyme indicate that it resembles those which hydrolyze both epimers of MI-1-P.

Conversion of D-glucose to MI in plants involves enzymic steps similar to those encountered in animal tissues and fungi, and the epimeric form of the intermediate, MI-1-P, has been established as 1L (MW Loewus, K Sasaki, AL Leavitt, L Munsell, WR Sherman, and FA Loewus, manuscript in preparation). Free MI is a common constituent of plants, probably ubiquitous, and it is utilized in numerous metabolic processes of which the MI oxidation pathway has emerged as highly significant due to its biosynthetic role as a source of UDP-D-glucuronate (9, 13, 14). Oxidation of MI to D-glucuronate is probably the first committed step in UDP-D-glucuronate biosynthesis via MI but we choose to include MI biosynthesis in the overall process since MI-1-P synthase and MI-1-phosphatase directly link conversion of glucose-6-P to UDP-D-glucuronate formation by processes operating independently of UDP-D-glucose dehydrogenase (EC 1.1.1.22) (17, 26). In this regard, a Mg^{2+}-dependent MI-1-phosphatase was detected in cell-free extract of *Acer pseudoplatanus* suspension cell culture (15). Subsequently, thi enzyme was found to be present in crude cell-free MI-1-P synthase preparations from other plant sources, notably that of lily pollen. This paper describes the isolation, purification, and properties of MI-1-phosphatase from lily pollen. A preliminary report appeared earlier (18).

MATERIALS AND METHODS

Chemicals. L- and DL-α-glycerophosphate, β-glycerophosphate, 2′-AMP, 3′-AMP, 5′-AMP, the various sugar phosphates and glycerol dehydrogenase (EC 1.1.1.6) were purchased from Sigma Chemical Co. 1D- and 1L-MI-1-P were generously provided by Professor William R. Sherman, Washigton Univ., St. Louis, MO.

Isolation and Purification of MI-1-Phosphatase. Freezer-stored mature pollen (30 g) from *Lilium longiflorum* Thunb. cv. Ace was suspended in 0.02 M Tris-acetate (200 ml) containing 0.5 mM GSH, pH 8.0. Pollenkitt, a gummy orange residue associated with stored lily pollen, was removed by stirring the suspension with a glass rod until all pollenkitt adhered to the rod. The suspension was ground in a Kontes Duall glass-glass homogenizer (50 strokes) and centrifuged at 27,000*g* for 30 min. To the supernatant was added solid ammonium sulfate to 35% saturation. After equilibration for 20 min at 4°C, the precipitate was removed and additional ammonium sulfate added to bring the solution to 55% saturation. Material precipitating between 35% to 55% saturation was recovered (12,000*g*, 15 min), redissolved in the 0.02 M Tris-acetate buffer described above (20–30 g/100 ml), and dialyzed against two changes of the same buffer (4 h, 4°C).

This dialyzed material was applied to a column of DEAE cellulose (2 × 30 cm, Cellex D; Bio-Rad) that had been previously equilibrated with the same buffer. After loading, the column was washed with additional buffer (300 ml) and then eluted by a

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[3] Abbreviations: MI, *myo*-inositol; MI-1-P, *myo*-inositol-1-phosphate; MI-1-phosphatase, *myo*-inositol-1-phosphatase; glucose-6-P, D-glucose-6-phosphate.

[4] In accord with a recent suggestion (12), the term MI-1-phosphatase is used rather than the more specific term 1L-MI-1-phosphatase.

[5] Prior to 1968, systems of cyclitol nomenclature other than the one currently in use (1) were used to describe substituted inositols. Thus, the product of 1L-MI-1-P synthase sometimes was designated D-MI-1-P or simply MI-3-P in contrast to the currently acceptable term, 1L-MI-1-P.

concave gradient of three interconnected reservoirs (200 ml each) containing 0.02 M Tris-acetate—0.5 mM GSH—0.1 M NaCl in the first two reservoirs and 0.02 Tris-acetate—0.5 mM GSH—0.6 M NaCl in the third reservoir. All solutions were pH 8.0 and 4°C. MI-1-phosphatase was eluted at 250 to 370 ml (0.19–0.29 M NaCl) which was well beyond the point at which MI-1-P synthase appeared (158–216 ml, 0.125–0.165 M NaCl). Nonspecific glucose-6-P activity was removed from fractions containing MI-1-phosphatase by heating (60°C, 15 min), repeating the step if necessary to lower glucose-6-P phosphatase activity to <10% of the total MI-1-phosphatase activity. Unless otherwise noted, all studies on the properties of MI-1-phosphatase were conducted with the heat-treated DEAE cellulose-purified enzyme. Mol wt was determined by comparison of enzyme and macromolecular standards using gel filtration and by plotting $K_{av} = (V_e - V_o)/(V_t - V_o)$ against $\log_{10}$ of the mol wt. The measured volumes were the elution volume of the enzyme or standard, V_e; the volume of the column, V_t; and the void volumn, V_o. The standards were Cyt *c*, chymotrypsinogen, ovalbumin, and BSA.

Chromatofocusing was accomplished on a column of polybuffer exchange gel (0.9 × 30 cm, Pharmacia PBE-94). The loading buffer was 25 mM histidine-HCl (pH 6.55). The eluting buffer was Pharmacia Polybuffer-74 (pH 4.0). All pH measurements were made at 4°C on an Orion model 601 pH-meter. Gel filtration was accomplished on columns (1.2 × 90 cm) of Sephadex G-100 or Sephadex G-200. Protein fractions from the Sephadex G-200 step and the chromatofocusing step were analyzed by gel slab electrophoresis in 6% and 10% polyacrylamide gel, respectively, by method 1 of Maurer (19).

Separation of MI and MI-1-P. This mixture was resolved on a column (1.2 × 95 cm) of Sephadex G-15 with 0.1% acetic acid as solvent. Elution was run at 8 ml/h (25). MI-1-P eluted ahead of MI (peaks at 64 and 78 ml, respectively), a condition useful in experiments in which highly radioactive [2-^{3}H]MI was used to measure conversion of MI to MI-1-P.

Phosphatase Assays. In order to conserve relatively rare supplies of 1L- and 1D-MI-1-P, most assays were run with β-glycerophosphate. The reaction mixture (0.5 ml) contained 0.6 μmol β-glycerophosphate, 2 μmol $MgCl_2$, 50 μmol Tris-HCl (pH 8.5) and enzyme. Samples were incubated for 1 h at 37°C. The reaction was stopped with 30% TCA (0.1 ml), centrifuged to remove precipitate, and analyzed for Pi (5). The reaction was linear beyond 20% consumption of substrate. Shorter intervals were used for kinetic studies and consumption of substrate was <10%, well within the linear range. Assays for enzyme activity with other substrates including MI-1-P followed the same procedure.

The pH profile of the enzyme was obtained by standard assay in the following 0.1 M buffers: sodium acetate (pH 4.8); Mes (pH 6); and Tris-acetate (pH 7–9.5).

To assay a reaction mixture for glycerol, the reaction was stopped with 9.2 M $HClO_4$ (50 μl). After removal of precipitate by centrifugation, the solution was neutralized with KOH and the resulting $KClO_4$ removed by another centrifugation. The supernatant was applied to a small ion exchange column (pasteur pipette with 0.3 ml, bed volume, of Bio-Rad AG1 × 8, formate form, 200–400 mesh) and washed with 0.3 ml H_2O. The effluent and wash were combined, neutralized and adjusted to 1.0 ml with water. Aliquots (0.2–0.3 ml) were analyzed for glycerol (11).

RESULTS

The presence of nonspecific phosphatases in early steps of purification precluded an assay for MI-1-phosphatase until fractionation on DEAE cellulose and heat treatment (Table I). In this table, the raw extract refers to homogenized pollen preceding centrifugation and the crude extract to the supernatant following centrifugation. About 10- to 15-fold increase in specific activity was achieved in the two steps subsequent to DEAE cellulose chromatography. The elution profile of protein from DEAE cellulose (Fig. 1) shows a large 280 nm-absorbing peak in the first 100 ml of eluent. It contained a heat-labile phosphatase, active on β-glycerophosphate and glucose-6-P. A heat-stable Mg^{2+}-dependent phosphatase, active on β-glycerophosphate appeared in fractions eluted with 0.19 to 0.29 M NaCl. Heat treatment of this material lowered its glucose-6-P activity to <10% of its MI-1-P activity.

Table I. *Purification of myo-Inositol-1-Phosphatase*

Treatment	Protein	Activity[a]	Specific Activity
	mg	*nkat*	*nkat/mg*
Homogenate	7,943	ND[b]	
Crude extract	7,190	ND	
Ammonium sulfate (35–55%)	2.956	ND	
DEAE cellulose	102	30.4	0.298
Sephadex G-200	46	22.4	0.488
Gel electrophoresis[c]		24.0	

[a] Calculated for 30 g pollen and assayed after one heat treatment (60°C, 15 min). A typical assay for activity was 2 to 9 pkat.

[b] Not determined.

[c] The protein concentration was too low to make a chemical determination. A rough visual estimate of protein concentration in this fraction, of 2 to 10 mg, was obtained by comparing the band width with standards. Specific activity would then be 12 to 2.4 nkat/mg.

Unheated enzyme from the DEAE cellulose step had a mol wt of 43,000 (Sephadex G-200) with no evidence of higher mol wt forms such as found by Naccarato *et al.* (21) in an unheated preparation of mammary gland MI-1-phosphatase. After heat treatment, a mol wt of 54,000 was obtained on Sephadex G-100. When heat-treated MI-1-phosphatase from the Sephadex G-200 step was subjected to polyacrylamide gel electrophoresis, five protein bands were detected of which only the fastest one contained MI-1-phosphatase activity (Fig. 2).

In other experiments, heat-treated MI-1-phosphatase from the DEAE cellulose step was subjected to chromatofocusing with a pH 7 to pH 4 polybuffer at pH 4.2 as the eluent. One pass through this column produced a single peak of activity at pH 4.2 in which 80% of the activity applied to the column was recovered. Inasmuch as the column was inefficient in removing extraneous protein, the MI-1-phosphatase peak was subjected to chromatofocusing again under the same conditions. Again, a single peak (54% of activity applied to the second column) appeared at pH 4.2 with a specific activity of 1.6 nkat/mg protein (protein estimated from $O.D._{280nm}$ absorption). This activity was of the same order as that obtained with purification by gel electrophoresis (Table I). Upon gel electrophoresis, a single protein band appeared in the same position as the active component from the Sephadex G-200 fraction.

A list of compounds which act as substrate to MI-1-phosphatase are given in Table II along with their K_m vaue and the V_{max} (relative to 1L-MI-1-P). These values were measured on the heat-treated, DEAE cellulose-purified enzyme. A few substrates were also tested by standard assay with enzyme from the active band after gel electrophoresis of the Sephadex G-200-purified enzyme. These values are given in the final column of Table II. Pollen enzyme hydrolyzed 1D-MI-1-P at 80% to 90% of the rate obtained with 1L-MI-1-P. MI-2-P was also hydrolyzed but at a much lower rate and the K_m was almost 10-fold greater. Purification beyond the DEAE cellulose step failed to alter enzyme specificity toward the three MI-monophosphates or β-glycerophosphate. The low level of residual glucose-6-P activity that resisted heat treatment was also present in the active band after gel electrophoresis. D-Mannose-6-P, D-glucose-1-P, D-fructose-6-P, D-fructose-1-P, 5′-AMP, and *p*-nitrophenyl phosphate acted neither as substrate nor

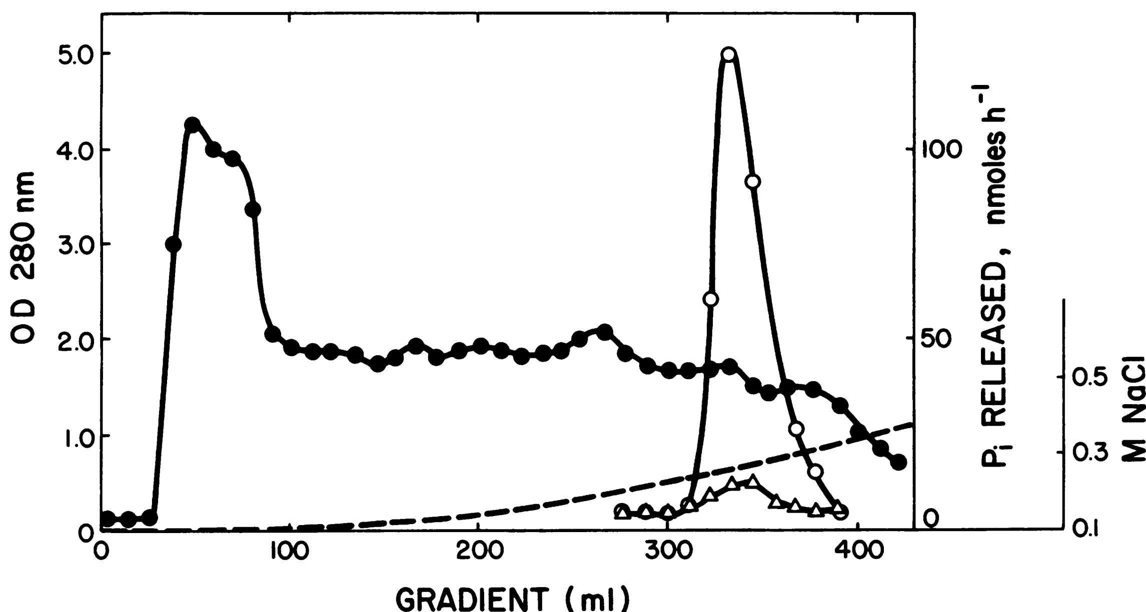

FIG. 1. DEAE cellulose elution profile of MI-1-phosphatase. Fractions were heated to 60°C for 15 min prior to assay. Absorption at 280 nm (●), release of Pi from β-glycerophosphate (○) release of Pi from glucose-6-P (△), NaCl gradient (- - -).

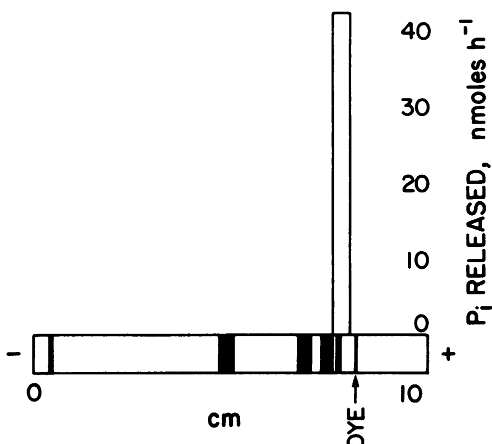

FIG. 2. Polyacrylamide slab gel electrophoresis of MI-1-phosphatase. Activity of enzyme indicated by bar above the diagram of the developed gel.

inhibitor in the reaction. Phytate was not a substrate and MI, a product of the reaction, did not inhibit the reaction. 3-Phosphoglycerate was a weak competitive inhibitor ($K_i \sim 11$ mM).

The activity was tested from pH 4.8 to 9.5 and was optimal at 8.5. The optimum Mg^{2+} concentration increased from 1.1 mM at a substrate concentration of 1.2 mM β-glycerophosphate to 6 mM at a substrate concentration of 0.04 mM (Fig. 3). Co^{2+} and Fe^{2+} partially replaced the Mg^{2+} requirement while Ni^{2+} and Mn^{2+} were less effective (Table III). Other metal ions listed in Table III were poor or ineffective as replacements for Mg^{2+}. Several monovalent cations were tested for their effect on MI-1-phosphatase activity (Table IV). None were stimulatory but Li^+ caused substantial inhibition at 250 mM.

Some alkaline phosphatases function as transphosphorylases (20). Tris, at concentrations as low as 0.1 M, accepts phosphate from substrate donors in the presence of alkaline phosphatase, a process that reduces the amount of Pi released into the medium relative to ester hydrolyzed (7). To test for possible transphosphorylase activity in lily pollen MI-1-phosphatase, production of glycerol and Pi from β-glycerophosphate was measured as a function of time in the presence of 0.1 M Tris·HCl (Fig. 4).

Table II. *Substrate Specificity of myo-Inositol-1-Phosphatase*

Substrate[a]	Enzyme from Heat-Treated DEAE Cellulose Step		Enzyme from Gel Electrophoresis Step[b]
	K_m	Relative V_{max}[c]	
1L-MI-1-P	0.082	100	100
1D-MI-1-P	0.078	81	90
MI-2-P	0.733	47	59
β-Glycerol-P	0.061	39	57
DL-α-Glycerol-P	0.382	23	
L-α-Glycerol-P	0.562	18	
3′-AMP	0.372	51	
2′-AMP	1.580	23	
Glucose-6-P	1.710	8	9

[a] Other compounds tested which were inactive as substrate or inhibitor: D-glucose-1-P, D-mannose-6-P, D-fructose-6-P, D-fructose-1-P, *p*-nitrophenyl-P, MI.

[b] Relative activity based on standard assay. See "Materials and Methods."

[c] V_{max} value relative to that of 1L-MI-1-P.

Glycerol and Pi were released at the same rate. When the concentration of Tris·HCl was increased, release of Pi remained greater than glycerol, excluding the possibility of its transfer to Tris·HCl (Fig. 4, inset). The fact that MI was not an inhibitor of the reaction is added evidence for absence of transphosphorylase activity. Finally, when the assay for MI-1-phosphatase activity was run in the presence of 1.2 mM [2-^{3}H]MI and products were separated on Sephadex G-15, a method that resolves MI-monophosphate from MI (25), no radioactivity was found in the region of MI-monophosphate.

DISCUSSION

Mature pollen from *L. longiflorum* contains a Mg^{2+}-dependent MI-1-phosphatase that is relatively heat stable as compared to other alkaline phosphatase activities in this plant tissue. Its substrate specificity, although similar to that of MI-1-phosphatase from animal and fungal sources, is not identical (Table V). In so far as MI-1-phosphatase from various sources has been tested,

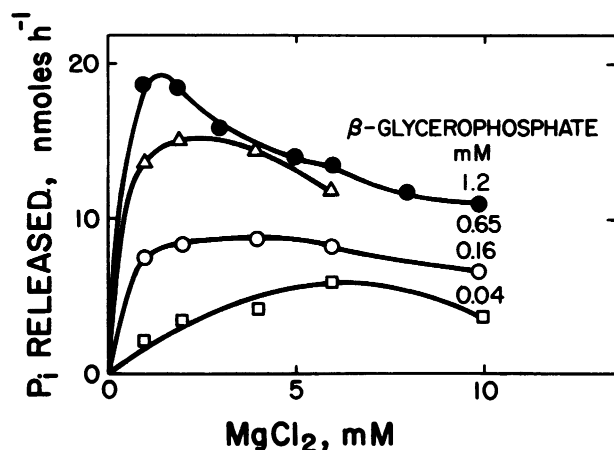

FIG. 3. Mg^{2+} requirement for MI-1-phosphatase from lily pollen.

Table III. *The Metal Ion Requirement of MI-1-Phosphatase*

Metal Salt (4 mM)	Relative Activity[a]
$MgCl_2$	100
$CoSO_4$	28
$FeSO_4$	28
$NiCl_2$	17
$MnCl_2$	8
$CuSO_4$	3
$CdSO_4$	2
$ZnCl_2$	0
$CaCl_2$	0
$FeCl_3$	0

[a] Assay: 1.2 mM β-glycerophosphate in 0.1 M Tris acetate buffer (pH 8.5).

Table IV. *Effect of Monovalent Cations on myo-Inositol-1-Phosphatase Activity*

Cation/mM	Activity[a]				
	0	1	10	50	250
None	100				
Li^+		95	80	56	18
Na^+		92	97	103	90
K^+		94	94	100	94
NH_4^+		87	100	105	80

[a] Relative to control (none).

each will hydrolyze 1D- as well as 1L-MI-1-P, and in each case, the rate of hydrolysis with the 1L isomer exceeds that of the 1D isomer by 5% to 20%. MI-2-P is substrate for plant, avian, and, possibly, yeast MI-1-phosphatase and in this respect unlike the enzyme from mammalian sources. In plants, MI-2-P is the terminal product of phytase, EC 3.1.3.26 (MI-hexakis-P hydrolase) action on phytic acid.

Most assays of lily pollen MI-1-phosphatase were run with β-glycerophosphate, a substrate often used to identify non-specific alkaline phosphatase activity (20) but in this case acted upon by a relatively specific phosphatase. A Fe^{3+}-stimulated alkaline β-glycerophosphatase occurs in *Phaseolus radiatus* (22). This enzyme was not tested with MI-1-P as substrate. Lily pollen MI-1-phosphatase was not stimulated by Fe^{3+} (Table III), therefore the phosphatases from *P. radiatus* and lily pollen are not the same enzyme. *p*-Nitrophenyl phosphate, an artificial substrate often used in phosphatase assays due to the colorimetric property of *p*-nitrophenol, was not hydrolyzed by the lily pollen enzyme. Residual glucose-6-P activity remained with MI-1-P activity after gel

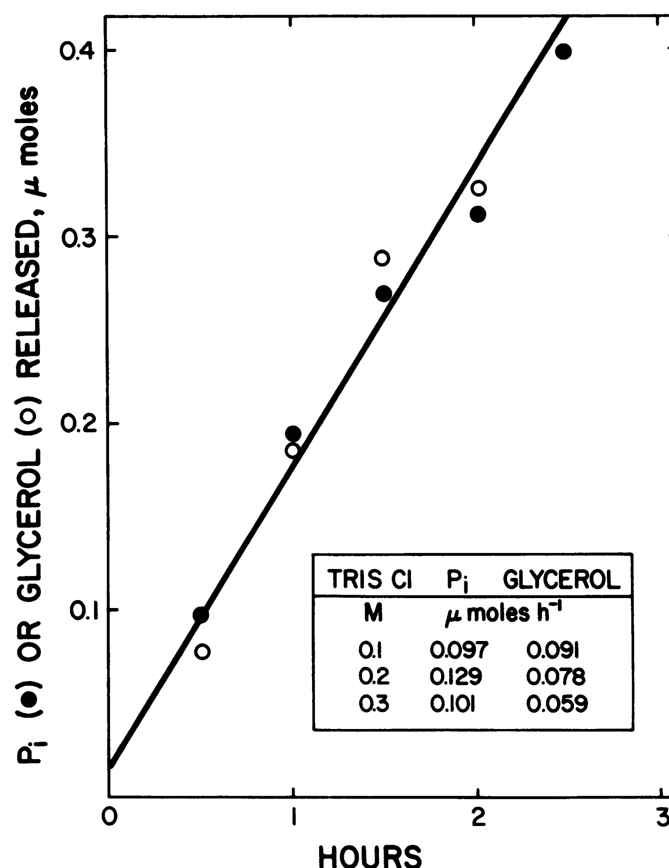

FIG. 4. Release of glycerol and Pi from β-glycerophosphate during hydrolysis with MI-1-phosphatase from lily pollen in the presence of 0.1 M Tris.

electrophoresis and it is possible that this activity is integral to MI-1-phosphatase. Lily pollen enzyme cleaved phosphate esters joined to secondary hydroxyl functions more readily than those joined to primary hydroxyl functions and, as noted by Eisenberg (8) in MI-1-phosphatase from rat testes, the equatorial esters of MI-1-P were cleaved more rapidly than the axial ester of MI-2-P by the pollen enzyme.

Naccarato *et al.* (21) reported that 250 mM Na^+, K^+ and NH_4^+ ions stimulated rat mammary gland MI-1-phosphatase while Li^+ ion completely inhibited this activity. In a more detailed study of the Li^+ ion effect on MI-1-phosphatase from bovine brain, Hallcher and Sherman (12) found 50% inhibition by Li^+ at 0.8 mM. Lily pollen enzyme is also inhibited by Li^+, but the effect occurs at much higher concentrations. There was no evidence for stimulation of activity by Na^+, K^+ or NH_4^+ ions.

Rates of hydrolysis for different substrates by nonspecific alkaline phosphatase from several sources are virtually identical (10, 23), a fact explained by the formation of a phosphoryl enzyme complex whose breakdown is rate-limiting and by a reaction rate not dependent upon release of the various alcohols to be measured by products (6). This is not true of MI-1-phosphatase since the V_{max} values vary considerably among various substrates. 1L-, 1D-MI-1-P, and β-glycerophosphate have similar K_m values but the rate of hydrolysis of the latter is much slower than those of MI-1-P epimers. To the extent that K_m reflects substrate binding, release of Pi is not a rate-limiting step.

There is no evidence of a transphosphorylating activity in MI-1-phosphatase similar to that found in alkaline phosphatases. In the presence of Tris, α-glycerophosphate-derived Pi is released in amounts equal to or greater than glycerol. Whether the decrease in appearance of glycerol at higher concentrations of Tris (Fig. 4,

Table V. *Comparison of Substrate Specificity of MI-1-Phosphatase from Different Sources*

Substrate	Activity[a]						
	Rat testis (8)[c]	Rat mammary gland (21)	Bovine brain (12)	Chick erythrocyte (24)	*Candida utilis* (yeast)		Lily pollen[b]
					(3)	(2)	
1L-MI-1-P	100	100	100		~105[d]		100
1D-MI-1-P	89		70	100	100	100	81
MI-2-P	0	<10		85	0	40	47
(−)-Inositol-3-P	102		80		77	77	
β-Glycerophosphate	41		0	69	70	112	39
L-α-Glycerophosphate		60	0				18
α-Glycerophosphate	32	0[e]	0	27	15		23[e]
2′-AMP	86	<10	20	122	72		23
3′-AMP	0	<10		33	14		51
p-NO_2-phenyl-P	0				13	123	0
Glucose-6-P	0	<10	0	7		76	8

[a] Values relative to 1L-MI-1-P (or in the case of chick erythrocyte and yeast, 1D-MI-1-P).
[b] This paper.
[c] Numbers in parentheses, references.
[d] Calculated from data in Reference 4.
[e] Racemic form is specified.

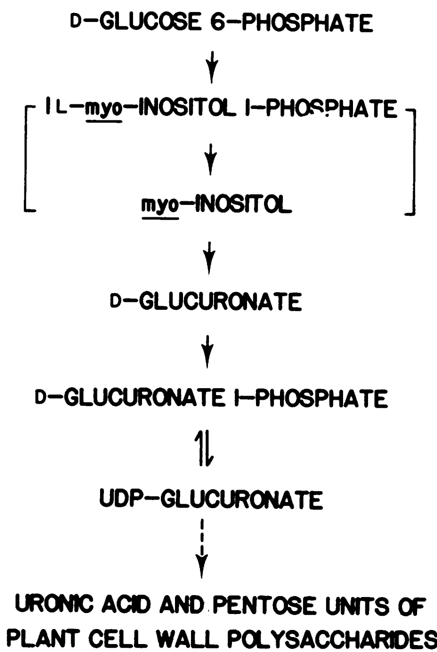

FIG. 5. The MI oxidation pathway. The MI-1-phosphatase step is indicated by brackets.

inset) has significance remains to be established. Additional evidence that MI-1-phosphatase from lily pollen lacks transphosphorylase activity is seen in the lack of inhibition by MI and the failure to find radioactive MI-monophosphate in a reaction mixture that had been supplemented with [2-^{3}H]MI.

The role of MI-1-phosphatase in plants is predicted from inhibitor and *in vivo* labeling experiments on the MI oxidation pathway (16, 17). This pathway (Fig. 5) converts glucose-6-P to UDP-D-glucuronate which ultimately supplies the uronosyl and pentosyl residues for cell wall biosynthesis. Conceivably, MI-1-phosphatase also acts on the product of MI kinase and, as noted earlier, the terminal MI-monophosphate of phytate degradation.

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