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Further Studies on *myo*-Inositol-1-phosphatase from the Pollen of *Lilium longiflorum* Thunb[1]

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ABSTRACT

***myo*-Inositol-1-phosphatase has been purified to homogeneity from *Lilium longiflorum* pollen using an alternative procedure which includes pH change and phenyl Sepharose column chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis shows that the enzyme is a dimer (subunit molecular weight, 29,000 daltons). The enzyme is stable at low pH values and is inactivated only below pH 3.0. In addition to 1L-and 1D-*myo*-inositol-1-phosphate, it shows high specificity for 1L-*chiro*-inositol-3-phosphate. As observed earlier with other primary phosphate esters, D-glucitol-6-phosphate and D-mannitol-6-phosphate are hydrolyzed very slowly. No activity is observed with inorganic pyrophosphate or *myo*-inositol pentaphosphate as substrate. The enzyme is inhibited by fluoride, sulfate, molybdate, and thiol-directed reagents. Partial protection against *N*-ethylmaleimide inhibition by substrate and Mg^{2+} together suggests sulfhydryl involvement at the active site.**

MI-1-phosphatase[2] from lily pollen readily hydrolyzes the enantiomeric forms of MI-1-P (16) and in this respect resembles similar MI-1-phosphatases from yeast (4), rat testis (7), and bovine brain (11). It also hydrolyzes MI-2-P as does the MI-1-P phosphatase from chick erythrocytes (20). In so far as all these Mg^{2+}-dependent, alkaline phosphatases have been examined, their molecular and catalytic properties are quite similar; the notable exception being specificity toward MI-2-P as substrate (16).

Here, we describe an alternative method for purification to homogeneity of MI-1-phosphatase from lily pollen and examine subunit structure, substrate specificity, response to inhibitors, and stability of enzyme to heat and pH. Studies on sulfhydryl involvement at the active site are also presented.

MATERIALS AND METHODS

Chemicals. β-Glycerol-P, D-glucose-6-P, D-glucitol-6-P, D-mannitol-6-P, phenyl Sepharose and polyethylene glycol (P-2139) were purchased from Sigma Chemical Co. PHMB, NEM, and 2-mercaptoethanol were purchased from Aldrich Chemical Co and MI-pentaphosphate from Calbiochem-Behring. 1L-*chiro*-Inositol-3-P was kindly supplied by Professor William R. Sherman, Washington University, St. Louis, MO. All other chemicals used in this study were analytical grade. Tris-acetate buffer consisted of 0.02 M Tris-acetate, pH 8.0.

Purification of MI-1-Phosphatase. The procedure previously described (16) was used through the heat treatment step that followed DEAE-cellulose chromatography to obtain a partially purified MI-1-phosphatase. The DEAE-cellulose step was slightly modified in that the enzyme-loaded DEAE-cellulose column was washed with 0.12 M NaCl in Tris-acetate buffer (300 ml) prior to gradient elution. This pregradient wash removed considerable inactive protein previously encountered in gradient fractions. The gradient was initiated with buffer containing 0.12 M NaCl instead of 0.10 M NaCl as previously described.

Heat-treated enzyme (80 ml, 60°C, 20 min) was dialyzed against 10 mM citrate buffer (2 L, pH 3.5) overnight at 4°C. The dialyzed suspension was centrifuged (25,000*g*, 15 min, 4°C) to remove precipitated protein. NaCl was added to a final concentration of 0.5 M. The enzyme was loaded on a phenyl Sepharose column (8 × 0.6 cm) which had been preequilibrated with 0.5 M NaCl in Tris-acetate. The column was washed with 0.1 M NaCl in Tris-acetate buffer (25 ml) and then the enzyme was eluted with straight buffer. Active fractions were pooled, concentrated by osmodialysis with polyethylene glycol (average mol wt 8,000) to 5 ml, loaded on a column of Ultrogel AcA 34 (90 × 1.2 cm), and washed with 50 mM Tris-acetate, pH 8.0 (150 ml). The MI-1-phosphatase obtained from this column was homogenous by electrophoretic standards.

Enzyme Assay. The assay described in our earlier paper was modified slightly. Tris-acetate buffer was 20 mM rather than 100 mM and $MgCl_2$ was 2 mM rather than 4 mM in order to avoid the slight inhibition encountered at the higher concentrations. The reaction was stopped by addition of 6% SDS rather than 30% TCA as used previously. This modification avoided precipitation of protein and allowed an immediate analysis of phosphatase activity. A typical assay (0.5 ml) contained 1.2 μmol β-glycerol-P, 1 μmol $MgCl_2$, 10 μmol Tris-acetate, pH 8.6, and enzyme. The reaction was run 30 min at 37°C, stopped with 0.1 ml of 6% SDS, and analyzed for P_i (5). When PP_i, D-mannitol-6-P, or MI-pentaphosphate was tested as substrate, the reaction was terminated by addition of $HClO_4$ (final concentration, 0.05 M; total volume of reaction mixture, 0.9 ml). P_i was measured (5) under conditions that did not hydrolyze the substrate (9).

Gel Electrophoresis. Enzyme at each step of purification was analyzed on 10% polyacrylamide slab gels by method 1 of Maurer (17). To detect MI-1-phosphatase, the gel was immersed in 50 mM β-glycerol-P containing 10 mM $MgCl_2$, (pH 8.0), for 15 min at 37°C. This was followed by incubation of the gel in phosphomolybdate reagent for 15 to 30 min at 37°C (5) to detect P_i in the region of MI-1-phosphatase activity as a sharp blue

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[2] Abbreviations: MI, *myo*-inositol; MI-1-P, *myo*-inositol-1-phosphate; MI-1-phosphatase, *myo*-inositol-1-phosphatase (EC 3.1.3.25); NEM, *N*-ethylmaleimide; PHMB, *p*-hydroxymercuribenzoate.

band. Subsequently, the gel was stained for protein (3).

SDS-PAGE analysis followed the procedure of King and Laemmli (13) with minor changes. A 6% stacking gel and 12.6% separation gel were used. Separations were run at 150 v for 4 h at 23°C. Gels were fixed overnight in 20% sulfosalicylic acid, a process that leached out the SDS (6). Gels were stained for protein with Coomassie Blue G250 (3) and glycoprotein with periodate-Schiff's reagent (23).

Soluble protein was measured with Coomassie Blue G250 (21).

Reaction with Thiol-Reactive Reagents. To study the protective effect of substrates on inhibition of MI-1-phosphatase by NEM, the enzyme was first incubated with Mg^{2+} and/or the phosphate-ester for 5 min at 30°C and then exposed to NEM. Reaction with NEM was carried out at 30°C but residual enzyme activity was determined at 37°C under standard conditions. Reaction mixtures containing both Mg^{2+} and substrate were assayed for release of P_i during the period of incubation with NEM. Residual enzyme activity was corrected for this value.

RESULTS

Purification of lily pollen MI-1-phosphatase is summarized in Table I. Modifications to the original procedure (16) are: (a) deletion of chromatofocusing following recovery of heat-treated MI-1-phosphatase from the DEAE-cellulose step; (b) addition of two steps involving a treatment at pH 3.5 followed by hydrophobic affinity chromatography of heat-treated MI-1-phosphatase from DEAE-cellulose; and (c) postponement of gel filtration until after affinity chromatography. Since MI-1-phosphatase is heat stable (4, 7, 16, 19, 20), the effects of this treatment were examined briefly. At the DEAE-cellulose step, lily pollen enzyme was stable at 60°C for 30 min and then slowly lost activity ($t_{½}$ = 108 min) (Fig. 1). Further purification increased this rate. Magnesium chloride provided some protection against inactivation. Results obtained with 2 mM $MgCl_2$ were similar to data shown in Figure 1 at 10 mM. At 50°C, enzyme from the Ultrogel AcA step lost 25% of its activity in the first 30 min with no further loss (data not shown). When the pH was lowered to 3.5, considerable protein was precipitated while 90% of the MI-1-phosphatase remained soluble (Fig. 2). Additional purification was achieved by loading the pH 3.5-treated enzyme on a phenyl Sepharose column that had been equilibrated with buffered 0.5 M NaCl. A small amount of MI-1-phosphatase activity was released by buffered 0.1 M NaCl but the bulk of the enzyme was eluted by straight buffer (Fig. 3). Other hydrophobic affinity columns (ethyl, butyl, hexyl, and octyl) were tested but none retained the enzymic activity. The enzymically active peak that eluted from phenyl Sepharose contained two PAGE-detectable protein bands (Fig. 4, gel no. 3) of which the faster band accounted for all MI-1-phosphatase activity. Gel filtration on Ultrogel AcA 34 separated the contaminating higher mol wt component from the enzyme (Fig. 4, gel no. 4). A test for glycoprotein by periodic acid-Schiff's reagent on a gel containing approximately 20 μg of MI-1-phosphatase was negative. Under the same conditions, a positive test for glycoprotein was observed in the band corresponding to contaminating higher mol wt protein from the phenyl Sepharose step.

Table I. *Purification of MI-1-phosphatase*

Treatment	Protein	Activity	Specific Activity
	mg	*nkats*	*nkats/mg*
Homogenate[a]	2829	ND[b]	
Crude extract	2560	ND	
Ammonium sulfate (35–55%)	876	ND	
DEAE-cellulose	49	18.87	0.385
pH (3.5)	7	15.09	2.16
Phenyl sepharose	0.5	7.55	15.10
Ultrogel AcA 34	0.2	6.04	30.2

[a] Freezer-stored mature pollen (30g) from *Lilium longiflorum* Thunb. cv Nellie White, harvested in 1981.

[b] Not determined due to the presence of nonspecific phosphatases.

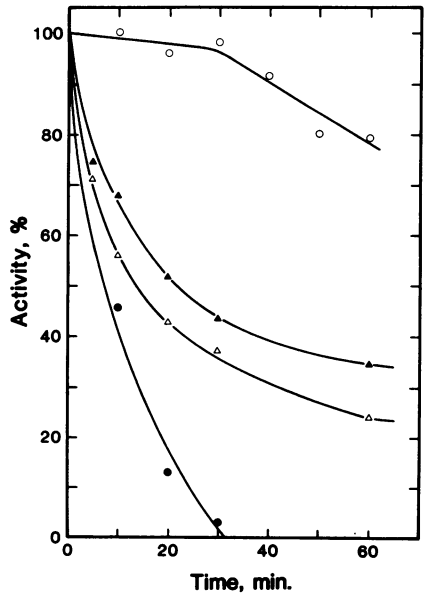

FIG. 1. Heat stability of MI-1-phosphatase. In each determination, the enzyme was held at the selected temperature and aliquots were assayed at intervals for activity at 37°C standard assay. DEAE-cellulose-treated enzyme was held at 60°C (○) or 70°C (●). Pure enzyme from Ultrogel AcA 34 was held at 60°C with (▲) or without (△) 10 mM $MgCl_2$.

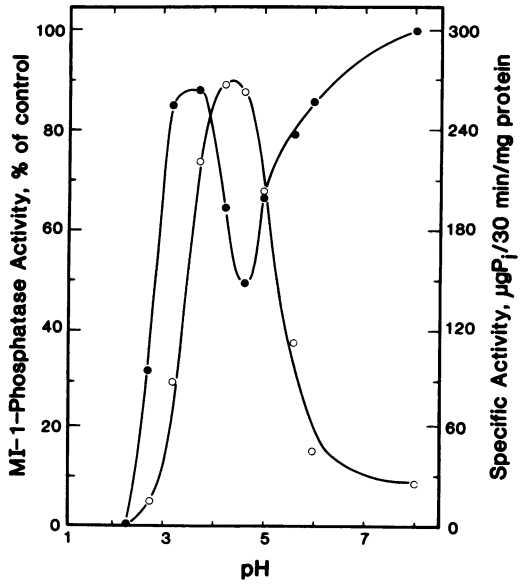

FIG. 2. pH stability of MI-1-phosphatase. For each point, DEAE-cellulose enzyme (5 ml) was dialyzed overnight at 4°C against 10 mM citrate (pH 3.0–6.0) or glycine-HCl (pH 2.2 and 2.65) buffer of desired pH (250 ml). The enzyme was centrifuged (25,000*g*, 20 min, 4°C) and precipitate was discarded. Supernatant was readjusted to pH 8.0 and assayed for activity and protein. Plot shows MI-1-phosphatase activity (●) as per cent of control (activity at pH 8 = 100%) and as specific activity (○).

Analysis of MI-1-phosphatase by SDS-PAGE after Ultrogel AcA 34 column chromatography revealed a single band with a mol wt of 29,000 ± 2,000 D when log mol wt was plotted against electrophoretic mobility for a set of mol wt markers.

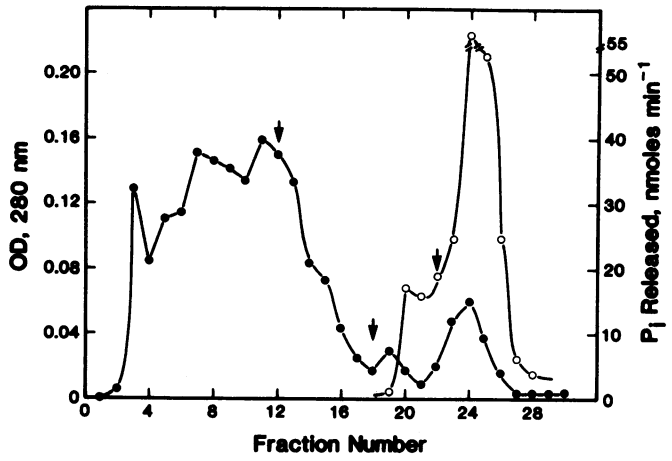

FIG. 3. Purification of MI-1-phosphatase on a phenyl Sepharose column. Arrows mark starting points for washing the column with 0.5 M and 0.1 M NaCl in Tris-acetate buffer and with straight buffer. The bulk of inactive protein (●) eluted ahead of MI-1-phosphatase (○).

Pure enzyme stored in 50 mM Tris-acetate, pH 8.0, for 1 month at 4°C lost no activity. When the enzyme solutions that were used to obtain data for Figure 2 were stored at their respective pH values for 1 month at 4°C, samples held at pH 4 to 5 lost 50% of their total activity. The remaining samples lost no activity. Attempts to store purified enzyme in the presence of 1 mM 2-mercaptoethanol or DTT at 4°C resulted in complete loss of activity within 4 d. Fifty mM 2-mercaptoethanol or DTT failed to reactivate the inactive enzyme. Enzyme from the phenyl Sepharose step was stable overnight at 23°C. The activity was not significantly affected by ionic strength in 20 to 300 mM Tris-acetate, pH 8.6.

In addition to substrates whose K_m and relative V_{max} values were determined previously (16), other substrates were tested. 1L-*chiro*-Inositol-3-P had a K_m of 0.044 mM and a V_{max} of 54 relative to 1L-MI-1-P as 100. The corresponding values for D-glucitol-6-P were 2 mM and 25, and for D-mannitol-6-P, 0.82 mM and 20, respectively. PP_i and MI-pentaphosphate were inactive as substrates.

Under standard assay conditions, lily pollen MI-1-phosphatase was inhibited 45% by 1 mM NaF, 95% by 1 mM $HgCl_2$, and 30% by 10 mM iodoacetate, 10 mM $(NH_4)_2SO_4$, or 20 mM Na_2MoO_4, respectively. Sulfate inhibited competitively (data not shown). Other compounds that failed to inhibit or stimulate phosphatase activity included MI, *myo*-inosose, D-*chiro*-inositol, L-*chiro*-inositol, DL-bornesitol, sequoyitol, *scyllo*-inositol, deoxy-*scyllo*-inositol, L-phenylalanine, L-glutamic acid, glycine, Na_2CO_3, H_3BO_3, and D-glucuronic acid. Cyclitols were tested at 20 mM and other compounds at 10 mM.

Inhibition by $HgCl_2$, iodoacetate, 2-mercaptoethanol, and DTT revealed a sulfhydryl requirement for enzymic activity. This effect was investigated further by reaction of the enzyme with NEM and PHMB. Figure 5 shows the pseudo-first order inactivation of MI-1-phosphatase at several concentrations of NEM. At the higher concentrations, 0.6 and 1.17 mM, a biphasic inactivation is observed. The enzyme always exhibits a residual activity (10–15%) even after treatment with 9 mM NEM. The apparent first order rate constant (k') can be calculated from $t_{1/2}$ of inactivation, for the appropriate NEM concentration. The inset of Figure 5 shows a plot of k' *versus* log [NEM] which gives a straight line of slope 1. Studies involving the protection from NEM inactivation of enzyme by preincubation with substrate(s) were also conducted (Table II). Mg^{2+} alone gave no protection. β-Glycerol-P alone accelerated inactivation while 1L-MI-1-P alone gave some protection. β-Glycerol-P or 1L-MI-1-P provided substantial protection in the presence of Mg^{2+}. MI-2-P was less effective than either β-glycerol-P or 1L-MI-1-P.

PHMB inactivated the enzyme so rapidly that kinetic studies were impossible. Preincubation of enzyme with β-glycerol-P and/or Mg^{2+} gave no protection against inhibition by PHMB. To show that NEM and PHMB reacted with the same groups, the enzyme was treated first with 100 μM PHMB and then with 5 mM NEM (Fig. 6). Addition of 16 mM L-cysteine restored activity completely whether the enzyme was treated with PHMB alone or with PHMB followed by NEM.

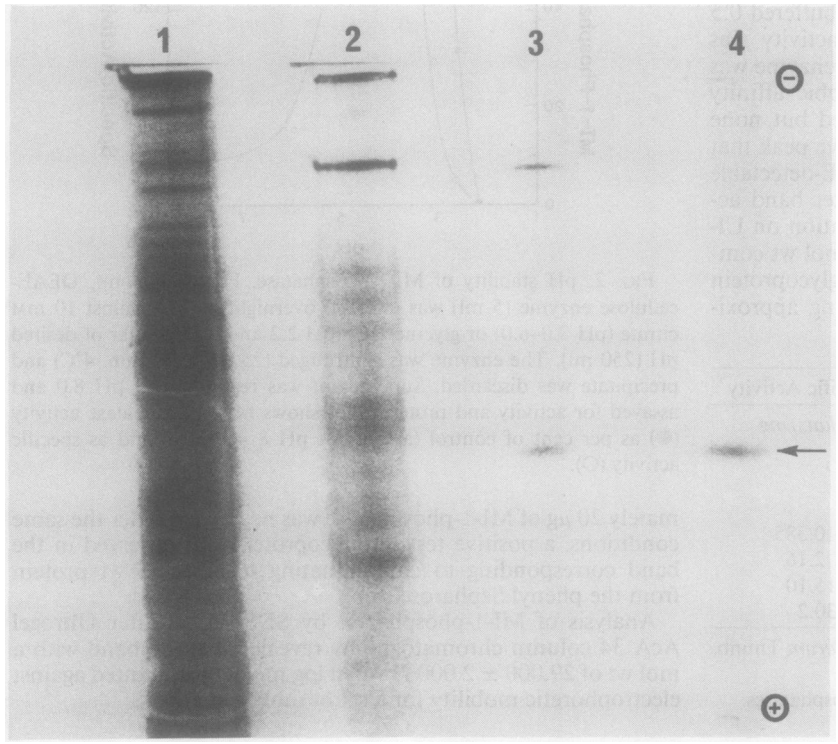

FIG. 4. PAGE analysis of MI-1-phosphatase during purification. The stages of purification include (1) DEAE-cellulose, (2) pH, (3) phenyl Sepharose, and (4) Ultrogel AcA 34. MI-1-phosphatase activity is located in the single band of gel No. 4 (arrow).

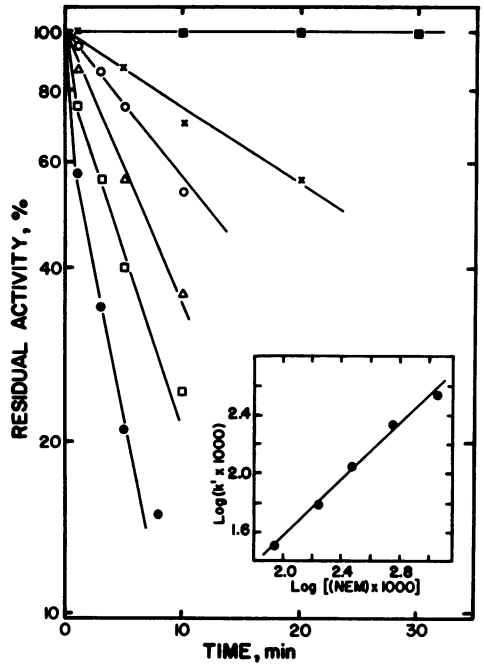

FIG. 5. Concentration dependence of MI-1-phosphatase inactivation by NEM. Purified enzyme (150 μl, 1.5 μg) was incubated in 50 mM Tris-acetate, pH 7.0 at 30°C. NEM was added to required concentration and aliquots (25 μl) were withdrawn to test for residual activity. The concentrations (in mM) of NEM used were 0.00 (■), 0.09 (×), 0.18 (○), 0.29 (△), 0.60 (□), and 1.17 (●). Inset: Order of MI-1-phosphatase inactivation with respect to NEM concentration. The apparent first-order rate constant (k') for the inactivation was calculated from slopes of the lines in Figure 5 for each NEM concentration employed.

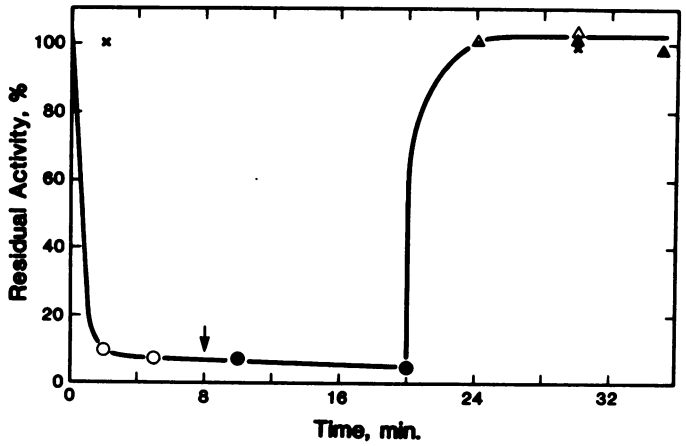

FIG. 6. Sequential inactivation of MI-1-phosphatase by PHMB and NEM. Six hundred μl of enzyme (approximately 6.1 μg), pH 8.0, was treated with PHMB (final concentration, 89 μM) at 30°C and aliquots (75 μl) were withdrawn to test for residual enzyme activity (○). After 6 min, a 150-μl aliquot was removed and held at 30°C. To remaining enzyme was added NEM (final concentration, 3.8 mM) and again aliquots (75 μl) were withdrawn for assay (●). At 20 min, L-cysteine was added (final concentration, 16 mM) to NEM-treated (▲) and untreated (△) enzyme, and aliquots were removed for assay of MI-1-phosphatase. A control to which nothing was added is included (×).

Table II. *Protection of MI-1-phosphatase from NEM Inactivation by Substrate(s)*

Experiment 1: Enzyme (2.7 μg protein) from phenyl Sepharose step, pH 7.0, was incubated for 5 min at 30°C in the presence of substrate(s) and NEM was then added to a final concentration of 2.4 mM. Aliquots (0.5 μg protein) were withdrawn at selected intervals to test for residual activity. Experiment 2: The procedure and conditions of this experiment were as described in experiment 1 except that enzyme (1 μg protein) from Ultrogel AcA 34 step was used and aliquots containing 0.2 μg protein were withdrawn to test for residual activity.

Experiment	Substrate(s) Used for Protection	Per Cent Activity Remaining after[a]	
		3 min	12 min
		%	
1	None	44	19
	Mg^{2+} (50)[b]	43	18
	β-Glycerol-P (60)	16	12
	β-Glycerol-P (60) and Mg^{2+} (50)	75	64
2	None	22	15
	L-MI-1-P (8.7)	34	17
	L-MI-1-P (8.7) and Mg^{2+} (52)	49	36

[a] The control (100% activity) was incubated under similar conditions except that no protecting substrate or NEM was added. The activity of this enzyme was monitored at various intervals.

[b] The numbers in parenthesis indicate the final concentration of substrate (in mM) in the incubation mixture.

DISCUSSION

The purification procedure for MI-1-phosphatase from lily pollen is modified to eliminate the chromatofocusing step and to introduce two new steps, a pH 3.5 treatment that takes advantage of the stability of the enzyme in a relatively acid environment and hydrophobic chromatography on phenyl Sepharose. The new procedure provides a relatively simple route to homogeneous enzyme in quantities sufficient for further studies. On SDS gel, the enzyme has a single protein band corresponding to 29,000 ± 2,000 D. This is approximately one-half of the mol wt of 54,000 D reported for active lily pollen MI-1-phosphatase (16) and leads to the conclusion that this enzyme is a dimer.

The stability of lily pollen enzyme to low pH is much greater than that of bovine brain which is inactivated at pH 5 (11), but its stability to heat treatment (60°C for 15–30 min) is lower than values (70°C for 15–30 min) reported for enzyme from mammalian or avian sources (7, 11, 19, 20).

The ratio V_{max}/K_m provides a means of measuring specificity in terms of discrimination among substrates (22). In addition to substrates tested previously (16; see Table II), L-*chiro*-inositol-3-P (also identified as (−)-inositol-3-P) (4, 7, 11) and D-glucitol-6-P were tested as substrate for lily pollen MI-1-phosphatase. Ratios of V_{max}/K_m for these substrates are: L-*chiro*-inositol-3-P, 1227; L-MI-1-P, 1220; D-MI-1-P, 1038; β-glycerol-P, 693; MI-2-P, 64; α-L-glycerol-P, 32; D-glucitol-6-P, 13; and D-glucose-6-P, 5. Clearly, the first three substrates are best. L-MI-1-P has metabolically significant roles in plants such as intermediate in the MI oxidation pathway and in phytic acid biosynthesis (14, 15). Hallcher and Sherman (11) found D-MI-1-P in brain tissue where it too may have a metabolic role. The recent discovery that MI-1,4,5-trisP acts as second messenger in the mobilization of intracellular calcium in mammalian tissues (12) and undergoes sequential loss of phosphate via MI-1,4-bisP and 1D-MI-1-P to form free MI (2) has yet to be repeated in plant tissues. Should this process be present in lily pollen, the MI-1-phosphatase described here may have an essential role in the cyclic process described by Berridge. L-*chiro*-Inositol-3-P, which was prepared synthetically, does not appear to be a naturally occurring compound.

When models of these three substrates are compared by superimposing phosphate groups, only one additional equatorial hydroxy group (equivalent to the one at C-5 in 1L-MI-1-P) has a configuration common to all three structures. The absence of a third polar binding site common to these substrates may explain its lack of enantiomeric specificity. Of course, the possibility exists that MI-1-phosphatase has multiple binding sites which accomodate different hydroxyl groups but this is less likely.

Alternatively, these models superimpose with an axial hydroxyl group above the ring and two equatorial hydroxyl groups below the ring but separated by one ring carbon from the axial hydroxyl group. Here, the phosphate is above the ring, either adjacent (1L- and 1D-MI-1-P) or opposite (1L-*chiro*-inositol-3P) to the carbon bearing the axial hydroxyl. A binding site that catalyzes the cleavage of phosphate ester at any one of three separate positions is indicated in this model.

An interesting property of MI-1-phosphatase is its hydrolysis of MI-2-P. Although the K_m for this substrate is 9-fold greater than those of 1L- or 1D-MI-1-P, hydrolysis of MI-2-P proceeds at 50% of the rate of either enantiomer of MI-1-P. Similar kinetics for hydrolysis of MI-2-P were found in MI-1-phosphatase from chick erythrocytes (20). While the higher K_m may be due to the axial orientation of phosphate in MI-2-P, it can be shown that higher reaction rates are achieved if the enzyme uses its binding energy to increase its V_{max} rather than to lower its K_m (8). To fully understand the structural requirements of the substrate, more detailed studies involving the use of mono- and polyphosphate esters of the inositols are needed.

A Mg^{2+}-dependent specific D-glucitol-6-phosphatase has been isolated from plants (10). This enzyme differs from lily pollen MI-1-phosphatase which hydrolyzes D-glucitol-6-P very slowly as previously noted for other primary phosphate esters (16). Primary phosphates have lower free energy of hydrolysis and thus a lower phosphate transfer potential (18). The nature of the nucleophilic attack on phosphorus of the phosphate group at the active site will, therefore, determine whether an enzyme catalyzes the hydrolysis of primary phosphate as efficiently as it does secondary phosphate.

Fluoride, a common inhibitor of metal-activated enzymes (1), inhibits MI-1-phosphatase from yeast (4), mammalian and avian sources (11, 19, 20), and lily pollen. Although this inhibition has been ascribed to removal of Mg^{2+} (11), the stoichiometry suggests that inhibition may include other effects such as binding with active site groups or replacement of H_2O in the coordination sphere of Mg^{2+} where H_2O may be required for phosphate hydrolysis (1).

Sulfate and molybdate also inhibit MI-1-phosphatase. The competitive nature of SO_4^{2-} inhibition suggests that it binds to the enzyme in the same way as phosphate of the substrate.

MI-1-phosphatase is inactivated by thiol-directed reagents, PHMB and NEM. The inactivation reaction exhibits first order kinetics with respect to NEM concentration (Fig. 5, inset) which suggests that the modification of a single sulfhydryl group causes the observed loss of activity. Whether the residual activity observed after modification is due to activity of the modified enzyme or a form of the enzyme which is resistant to modification is not clear. Partial protection of the enzyme from NEM inactivation by β-glycerol-P or 1L-MI-1-P in the presence of Mg^{2+} indicates the presence of an essential sulfhydryl at or near the active site. Accelerated inactivation in the presence of β-glycerol-P alone is probably due to increased reactivity of essential sulfhydryl upon binding of β-glycerol-P or to increased accessibility of NEM to this group (24) following such treatment.

Restoration of full activity following sequential treatment of MI-1-phosphatase with PHMB and NEM suggests that NEM is reacting with the same groups as PHMB and provides added support for the view that inactivation by PHMB and NEM is due to modification of a sulfhydryl. Complete reactivation by L-cysteine also indicates that no other irreversible side reaction occurred.

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