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Enantiomeric Form of *myo*-Inositol-1-Phosphate Produced by *myo*-Inositol-1-Phosphate Synthase and *myo*-Inositol Kinase in Higher Plants[1]

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ABSTRACT

The product of *myo*-inositol-1-phosphate synthase, EC 5.5.1.4, from mature pollen of *Lilium longiflorum* Thunb., cv Ace (Easter lily) and that of *myo*-inositol kinase, EC 2.7.1.64, from wheat germ has been identified as 1L-*myo*-inositol-1-phosphate by gas chromatography of its trimethylsilyl-methyl phosphate derivative on a glass capillary column bearing a chiral phase.

MI-1-P[4] synthase, EC 5.5.1.4, catalyzes the conversion of glucose-6-P to MI-1-P. Product of synthase from rat testis (3) and from yeast (2) was assigned the 1L configuration on the basis of its optical properties (1, 5). (A numerical prefix precedes the enantiomeric assignment in order to avoid ambiguity regarding the numbering of ring carbon atoms of inositol.) The recent discovery that DL-MI-1-P is resolved upon GC of its trimethylsilyl-methyl phosphate derivative on a chiral phase-coated capillary column has shown that product of bovine testis synthase is also the 1L enantiomer (8). In plants, MI-1-P synthase is the sole source of *de novo* MI-1-P biosynthesis and, in conjunction with a Mg^{2+}-dependent alkaline MI monophosphatase, EC 3.1.3.25 (10), provides free MI to the many requirements for this latter compound (9). Plants also contain a MI kinase, EC 2.7.1.64, that converts MI to MI-1-P (4). The chiral configuration of this product has yet to be established.[5] If the kinase-derived product is 1L-MI-1-P, MI kinase may have an important metabolic role as a mechanism for recycling MI into MI-1-P after the release of free MI from stored or translocated forms of bound MI. Because of this need for information regarding the chiral form of MI-1-P produced by MI-1-P synthase and MI kinase in higher plants, studies were undertaken that apply the gas chromatographic procedures of Leavitt and Sherman (7, 8) to this problem.

MATERIALS AND METHODS

Chemicals. MI, MI-2-P, glucose-6-P, DTT, and ATP (No. A-6144 from equine muscle) were obtained from Sigma Chemical Company. [1-^{14}C]Glucose-6-P was obtained from New England Nuclear Corp. [2-^{3}H]MI was prepared and purified in this laboratory (14). *N,O*-bis(Trimethylsilyl)-trifluoroacetamide with 10% trimethylsilyl chloride was obtained from the Regis Chemical Co., Morton Grove, IL. Diazald was from Aldrich Chemical Co., Milwaukee, WI.

MI-1-P from MI-1-P Synthase. Stored mature pollen (100 g) from *Lilium longiflorum* Thunb. cv Ace (Easter lily) was suspended in 500 ml Tris-acetate buffer (pH 8) containing 1 mM DTT and ground with a glass-glass homogenizer. The extract was treated as described earlier (11) to obtain a partially purified MI-1-P synthase. Only the leading half of the peak of synthase activity as eluted from Ultragel AcA34 was used in this experiment in order to obtain a preparation low in phosphatase activity. This sample was incubated with 2.8 mM glucose-6-P (87 μCi of [1-^{14}C]glucose-6-P), 1 mM NAD^+, 40 mM Tris, 0.5 mM ammonium acetate, 3 mM NaN_3 in a total volume of 200 ml (pH 8) for 24 h at 30°C. The reaction was terminated by pouring the solution into 750 ml of hot 95% ethyl alcohol. Suspended material was removed by centrifugation and the supernatant evaporated to a volume of 30 ml. This was passed through a 1.2-cm diameter column containing 7 g (wet weight) Dowex 50x8 H^+ (200–400 mesh) exchange resin. The effluent and washes were evaporated to a volume of 5.5 ml, slurried with Whatman CC-31 microcrystalline cellulose, dried under reduced pressure to a powder, and loaded on a prefilled (2 × 51 cm) column of the same cellulose. The column was eluted with 1 M ammonium acetate:95% ethyl alcohol (3:7, v/v). Glucose-6-P appeared in fractions from 700 to 1,000 ml and MI-1-P, 1,200 to 1,600 ml. Paper chromatography of an aliquot of separated MI-1-P using the same solvent system showed that this product was devoid of glucose-6-P. As a further check on the purity of the MI-1-P, an aliquot was dephosphorylated and analyzed by descending paper chromatography in ethyl acetate:pyridine:water (10:6:5, v/v) for 18 h. A single radioactive peak comigrating with MI appeared. Based on recovery of ^{14}C, about 2% of the substrate was converted to product to give 3.3 mg MI-1-P.

MI-1-P from MI Kinase. MI kinase was prepared from 50 g

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

[5] Molinari and Hoffmann-Ostenhof (12) suggested that kinase-produced 1L-MI-1-P might be an intermediate in the formation of phytic acid; however, no experimental evidence has been given to establish this point.

wheat germ as described by English *et al.* (4). The 30 to 50% ammonium sulfate precipitate was redissolved in 8 ml 50 mM Tris·HCl buffer (pH 8.2) containing 50 mM DTT and loaded on a column (2 × 90 cm) of Sephadex G-25 to remove ammonium sulfate from the enzyme. The kinase was eluted in the same buffer and stored at −20°C. In separate preparations, protein content ranged from 2 to 16 mg/ml.

To prepare MI-1-P, the reaction mixture consisting of 1 mM ATP, 0.1 mM MI (20 μCi of [2-^{3}H]MI), 1 mM DTT, 4 mM $MgCl_2$, 10 ml enzyme (2–3 mg protein/ml), and 90 ml 50 mM Tris·HCl buffer (final pH 8.6) was incubated 24 h at 23°C, then frozen, and lyophilized. Residues were redissolved in 3 ml H_2O, centrifuged, and loaded on a column (1.2 × 98 cm) of Sephadex G-15 to separate MI-1-P from MI (15). MI-1-P-containing fractions were pooled, neutralized with NH_4OH, and evaporated to dryness. Residues were taken up in 1 ml H_2O and further purified by paper chromatography on Whatman MM3 paper with 1 M ammonium acetate:95% ethyl acetate (7:3, v/v), 50 h descending. A radioscan revealed one peak with mobility slightly less than that of a control spot of MI-2-P and in the region corresponding to MI-1-P. To remove extraneous nonlabeled substances that accompanied the product following elution from the MM3 paper, chromatography was repeated on washed MM3 paper in the same solvent. Based on recovery of ^{3}H, about 13% of the substrate was converted to product but the final recovery was only 0.3 mg of MI-1-P.

Characterization of MI-1-P. Samples were converted to their per(trimethylsilyl) or trimethylsilyl-methyl phosphate derivatives and analyzed by GC on conventional and glass capillary columns as described by Leavitt and Sherman (7, 8).

The per(trimethylsilyl) derivatives were formed by shaking samples overnight with a mixture of *N,O*-bis(trimethylsilyl)-trifluoroacetamide (containing 10% trimethylsilyl chloride) and dry pyridine (1:1, v:v). The final concentration of inositol phosphate was usually about 0.5 μg/μl of reagent. The trimethylsilyl-methyl phosphate was prepared from the per(trimethylsilyl) derivative. In a typical experiment, 60 μg of MI-1-P was trimethylsilylated, taken to dryness in a stream of N_2, and the residue dissolved in 300 μl of 10% methanol in diethyl ether at 0°C. This removes the trimethylsilyl ester groups from the phosphate, generating penta(trimethylsilyl) MI-1-P, which is then methylated. Ethereal diazomethane is added dropwise to the cold methanol-ether solution until the yellow color persists. After 5 min at 0°C, the solvents and diazomethane were removed in a stream of N_2 and the residue briefly subjected to rotary pump vacuum. When thoroughly dry, the residue was taken up again in an appropriate volume of trimethylsilylating reagent, whereupon it was ready for gas chromatographic analysis.

MI monophosphate was characterized as to the position of phosphate substitution as the per(trimethylsilyl) derivative. A 13-m CP Sil 5 glass capillary column (Chrompak, the Netherlands) was used in the synthase study, and a 2 m × 6 mm o.d. glass column packed with 3% OV-17 on 100/120 Gas Chrom Q (Applied Science Division of Milton Roy Co., State College, PA) was used for the kinase analysis. The chiral configuration of MI-1-P, as the trimethylsilyl-methyl phosphate derivative, was determined on a 25-m Chirasil Val glass capillary column (Applied Science). Either a flame ionization detector or a flame photometric detector in phosphorus-selective mode was used (Varian Associates) as indicated in the illustrations.

RESULTS

MI-1-P from Lily Pollen MI-1-P Synthase. Gas chromatograms of enzymic product and standards on capillary columns of CP Sil 5 and Chirasil Val are presented in Figure 1. The per(trimethylsilyl) derivative of authentic DL-MI-1-P (6) on CP Sil 5 gave a single peak (R_t = 3.86 min) for the racemic mixture. Acid-treated MI-2-P, an equilibrium mixture of approximately

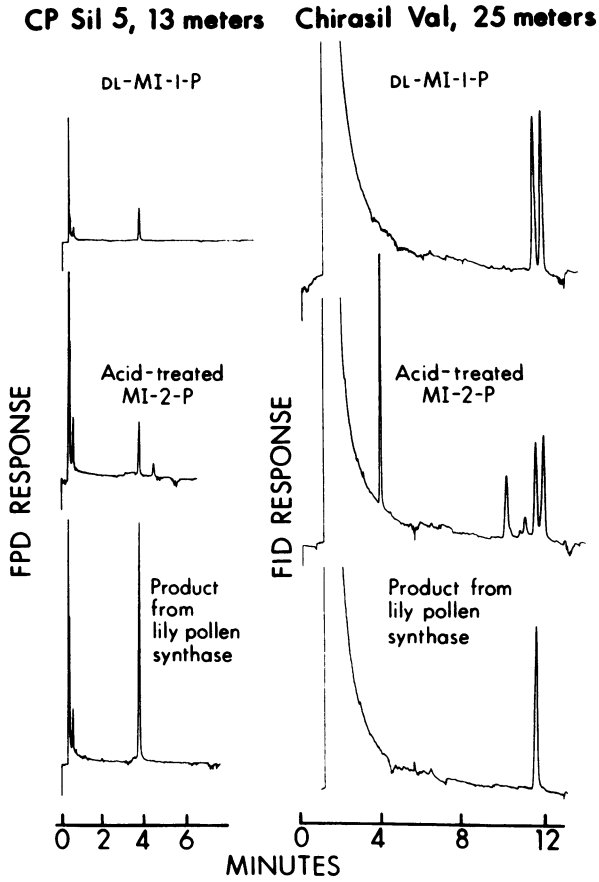

FIG. 1. Gas chromatograms of derivatized DL-MI-1-P, acid-treated MI-2-P, and product from lily pollen MI-1-P synthase. The ordinate gives phosphorus selective flame photometric detector (FPD) or flame ionization detector (FID) response in arbitrary units. See text for details.

30% MI-2-P and 70% DL-MI-1-P (13), produced two peaks (R_t = 3.85 and 4.59 min). The first peak, corresponding to DL-MI-1-P, was 73% of the integrated area under these two peaks. MI monophosphate from lily pollen synthase produced a single peak (R_t = 3.85 min) with the same retention time as DL-MI-1-P.

The trimethylsilyl-methyl phosphate derivative of authentic DL-MI-1-P gave two peaks on Chirasil Val. Acid-treated MI-2-P gave three prominent peaks in the region of MI monophosphate (R_t = 10.09, 11.53, and 11.91 min). Peaks at R_t = 11.53 and 11.91 min correspond to results obtained with DL-MI-1-P. Combined area under these latter peaks was 76% of the total area under all three peaks, a value similar to that of the DL-MI-1-P component of acid-treated MI-2-P as determined on the CP Sil 5 column. The peak (R_t = 3.86 min) was not characterized. MI-1-P from lily pollen synthase gave a single peak (R_t = 11.52 ± 0.02 min) with a retention time identical within experimental error to the first peak produced by DL-MI-1-P and previously identified as L-MI-1-P (8).

MI-1-P from Wheat Germ MI Kinase. Scans obtained from chromatography of enzymic product and authentic DL-MI-1-P on a conventional column of OV-17 and a capillary column of Chirasil Val are seen in Figure 2. Per(trimethylsilylated)DL-MI-1-P gave a single peak (R_t, 190°C = 8.00 ± 0.02 min) on OV-17. Under the same conditions, per(trimethylsilyl)MI monophosphate from MI kinase gave two peaks (R_t = 2.25 and 8.01 min), the latter coincident with the retention time of authentic DL-MI-1-P. The identity of the phosphorylated compound at R_t = 2.25 was not determined. If the sensitivity of the phosphorus selective detector was increased, a third peak, R_t = 10.7 min, was seen. It was about 1% of the phosphorylated product and had a retention

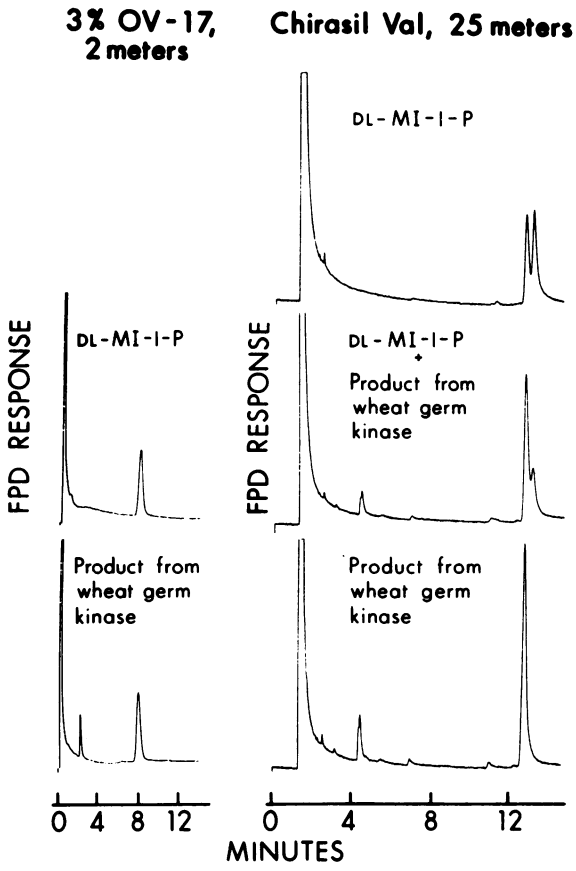

FIG. 2. Gas chromatograms of derivatized DL-MI-1-P and product from wheat germ MI kinase. The ordinate gives phosphorus selective flame photometric detector (FPD) response in arbitrary units. See text for details.

time similar to MI-2-P. The presence of MI-2-P in the chromatogram may be due to isomerization of MI-1-P during GC (7) and thus is not necessarily evidence of its enzymic formation or production during work-up of the enzymic product. The trimethylsilyl-methyl phosphate derivative of DL-MI-1-P gave two peaks (R_t, 195°C = 12.62 and 12.99 min) with the phosphorus selective detector. Under identical conditions, the addition of derivatized product of MI kinase to that of DL-MI-1-P enhanced only one peak, the one corresponding to the 1L isomer. When the derivatized product of MI kinase was injected on the column alone, a single peak (R_t = 12.59 min) was found in the region of the chromatogram corresponding to MI-1-P. If 1D-MI-1-P was present in the product of MI kinase, it was less than 2% by the methods of detection used here. As in the separation performed on OV-17, an unidentified phosphorylated component appeared early (R_t = 4.38 min) in the separation on Chirasil Val. This substance appeared to be partially resolved into two compounds suggesting that this component may consist of a racemic mixture.

DISCUSSION

The results clearly show that lily pollen and wheat germ have two enzymic mechanisms for producing 1L-MI-1-P, a *de novo* process involving 1L-MI-1-P synthase and a Mg^{2+}-ATP dependent MI kinase, respectively. In each, the 1L configuration of the product is unique, *i.e.* within the limits of detection the 1D isomer is not seen. Since 1L-MI-1-P synthase is the sole biosynthetic pathway from hexose phosphate to MI, it follows that MI kinase can function as a salvage mechanism which resupplies the 1L-MI-1-P requirements of the plant cell from stored or translocated forms of MI. Questions concerning intracellular localization of these enzymes, relative levels of enzymic activity at each stage of growth and development, enzymic regulation, and metabolic control by the plant remain to be answered.

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