Plant Physiol. (1997) 115: 727–736

L-*myo*-Inositol 1-Phosphate Synthase from Plant Sources[1]

Characteristics of the Chloroplastic and Cytosolic Enzymes

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L-*myo*-inositol 1-phosphate synthase (EC 5.5.1.4) from cyanobacterial (*Spirulina platensis*), algal (*Euglena gracilis*), and higher plant (*Oryza sativa, Vigna radiata*) sources was purified to electrophoretic homogeneity, biochemically characterized, and compared. Both chloroplastic and cytosolic forms of the enzyme were detected in *E. gracilis, O. sativa,* and *V. radiata,* whereas only the cytosolic form was detected in streptomycin-bleached or chloroplastic mutants of *E. gracilis* and in *S. platensis.* Both the chloroplastic and cytosolic forms from different sources could be purified following the same three-step chromatographic protocol. L-*myo*-inositol 1-phosphate synthases purified from these different sources do not differ significantly with respect to biochemical and kinetic parameters except for the molecular mass of the chloroplastic and cytosolic native holoenzymes, which appear to be homotetrameric and homotrimeric associations of their constituent subunits, respectively. Monovalent and divalent cations, sugar alcohols, and sugar phosphates are inhibitory to the enzyme activity. *N*-ethylmaleimide inhibition of synthase activity could be protected by the combined presence of the substrate glucose-6-phosphate and cofactor NAD^+. Antibody raised against the cytosolic enzyme from *E. gracilis* immunoprecipitates and cross-reacts with both chloroplastic and cytosolic forms from the other sources studied.

Inositols are 6-C cyclohexane hexitols found ubiquitously in biological systems. Of the eight possible geometrical isomers, *myo*-inositol is the most abundant and occupies a central position in carbohydrate metabolism, being the precursor of a number of metabolic products such as inositol phosphates, phosphoinositides, cell wall polysaccharides, a number of methylated derivatives, and IAA conjugates. As a free cyclitol, *myo*-inositol has been identified as a compound required for normal growth and development of fungal and plant tissue culture cells (Loewus and Dickinson, 1982; Loewus and Loewus, 1983; Biswas et al., 1984; Loewus, 1990).

Depletion of the normal cellular level of inositol has been shown to lead to a loss of cell viability in the yeast *Saccharomyces cerevisiae,* a phenomenon termed "inositol-less death" (Henry et al., 1977), whereas in cultured plant cells a reduction in the level of intracellular *myo*-inositol inhibits cell division (Biffen and Hanke, 1990). Inositol and its methylated derivative pinitol have been correlated with salt responses in plants (Vernon and Bohnert, 1992; Bohnert et al., 1995; Ishitani et al., 1996; RayChaudhuri and Majumder, 1996). As the phosphate esters inositol(1,4,5) trisphosphate and inositol(1,3,4,5)tetrakisphosphate, inositol is considered an important component for signal transduction in both plant and animal systems (Nishizuka, 1988; Drobak, 1992).

Although a number of metabolic routes emanate from *myo*-inositol, synthesis of this cyclitol is carried out by only one set of enzymatic reactions throughout biological systems. I-1-P synthase (EC 5.5.1.4) cyclizes Glc-6-P to I-1-P by three distinct partial reactions involving reduction and subsequent oxidation of the cofactor NAD^+ (Barnett et al., 1973; Chen and Eisenberg, 1975). A Mg^{2+}-dependent I-1-Pase (EC 3.1.3.25) hydrolyzes the I-1-P synthase product to produce *myo*-inositol (Eisenberg, 1967). This basic mechanism is apparently followed by all *myo*-inositol-producing organisms (Loewus and Dickinson, 1982; Loewus and Loewus, 1983; Biswas et al., 1984; Loewus, 1990).

I-1-P synthase has so far been reported to be widely distributed among the various plant groups and animals. Cytosolic I-1-P synthase from plant sources has been purified to various degrees, and purification of the enzyme to homogeneity followed by detailed characterization was achieved for fungal (Zsindley et al., 1977; Donahue and Henry, 1981; Escamilla et al., 1982) and animal sources (Pittner et al., 1974; Maeda and Eisenberg, 1980; Mauck et al., 1980). The genetics of inositol biosynthesis and its regulation have been elaborately studied in *S. cerevisiae* (Henry et al., 1984; Nikoloff and Henry, 1991; White et al., 1991). The structural gene for the cytosolic I-1-P synthase was identified as *Ino1* for the first time in *S. cerevisiae* (Donahue and Henry, 1981; Majumder et al., 1981). Subsequently, the *Ino1* gene was cloned and sequenced from a number of fungal and plant sources (Klig and Henry, 1984; Johnson and Henry, 1989; Smart and Fleming, 1993; Abu-Abied and

[1] This work was supported by research grants from the Council of Scientific and Industrial Research and Department of Science and Technology, Government of India. A.R.C. is a Senior Research Fellow of the Bose Institute.

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Abbreviations: I-1-P, L-*myo*-inositol 1-phosphate; I-1-Pase, I-1-P phosphatase; ME, 2-mercaptoethanol; NEM, *N*-ethylmaleimide; PCMB, *p*-chloropmercuribenzoate.

Holland, 1994; Klig et al., 1994; Johnson and Sussex, 1995; Ishitani et al., 1996).

Although cytosolic I-1-P synthase has been reported from a wide range of plant and animal systems, the organellar form of the enzyme is the one reported from chloroplasts of *Euglena gracilis*, *Vigna radiata* (Adhikari et al., 1987), and *O. sativa* (RayChaudhuri and Majumder, 1996). In both systems the enhanced activity of the chloroplastic I-1-P synthase was coincident with the photodifferentiation of the plastid. Whether the synthases of the two compartments are biochemically distinct or share identical properties remains a pertinent question.

In the present communication we have described two forms of I-1-P synthase from both algal (*E. gracilis*) and higher plant (*O. sativa* and *V. radiata*) sources. The two forms of the enzyme from these sources have been purified to electrophoretic homogeneity, characterized, and compared with respect to their biochemical and immunological properties. In addition, we report here experimental results with a single form of I-1-P synthase from an up-until-now unreported cyanobacterial source, *Spirulina platensis*.

MATERIALS AND METHODS

Chemicals

Glc-6-P, Fru-6-P, Gal-6-P, glucitol-6-P, dGlc-6-P, NAD^+, PMSF, Suc, Percoll, NEM, complete and incomplete Freund's adjuvants, gel-filtration media, and protein markers were purchased from Sigma. Ultrogel AcA 34, DEAE-Sephacel, and Superose 12 were from Pharmacia, and Bio-Gel A-0.5m was from Bio-Rad. All other reagents used were of analytical reagent grade.

Plant Materials and Growth Conditions

Seeds of *Oryza sativa* L., obtained from the Chinsurah Rice Research Station (West Bengal, India) and the Central Rice Research Institute (Cuttack, India), were surface-sterilized with 0.1% (w/v) $HgCl_2$, allowed to germinate in complete darkness for 3 d at 30°C, and then grown in 12-h alternating light (2000 μE m^{-2} s^{-1}) and dark conditions. Seeds of *Vigna radiata* var B1, obtained from the Oil and Pulse Research Station (Berhampore, West Bengal, India), were germinated as described by Adhikari et al. (1987).

Pure cultures of *Euglena gracilis* Z., obtained from the National Chemical Laboratory (Pune, India), were maintained under laboratory conditions by routine culture in media containing 0.1% (w/v) beef extract, 0.1% (w/v) tryptone, and 0.1% (w/v) dextrose with 15 mM cyanocobalamine, pH 3.6, and grown under 2000 μE m^{-2} s^{-1} with a light period of 12 h at 26 to 30°C, as described by DasGupta et al. (1984). Bleached *E. gracilis* cells were obtained by treatment of the green cells with 1 mg mL^{-1} streptomycin sulfate, as described by Hunter and Provasoli (1951), and maintained at 28°C under a 12-h light/dark period or in complete darkness. Other strains and mutants of *E. gracilis* were obtained as gifts from the late Professor J.A. Schiff (Brandeis University, Waltham, MA).

A pure culture of *Spirulina platensis* was obtained and maintained as described by Chattopadhyay et al. (1996) in Zarrouk's medium in laboratory conditions at 20 to 25°C in 16-h light (2000 μE m^{-2} s^{-1})/8-h dark cycles.

Extraction of Cytosolic and Chloroplastic I-1-P Synthase

Chloroplasts were isolated from *E. gracilis* cells or from leaves of *O. sativa* and *V. radiata* immediately after the dark period of d 6 or within 1 h of the start of the d 7 photoperiod, following the method of Rathnam and Edwards (1976) with modifications. Cells or leaves were homogenized in 5 volumes of chloroplast isolation buffer (0.33 M Suc, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 1 mM each of $MgCl_2$ and $MnCl_2$) in a prechilled blender for 5 min. The homogenate was passed through four layers of cheesecloth and centrifuged at 180*g* for 10 min at 4°C. The supernatant fraction was centrifuged at 12,300*g* for 20 min to obtain the crude chloroplast pellet (fraction I). The supernatant obtained after pelleting the crude chloroplast (fraction II) was recentrifuged at 28,000*g* for 30 min at 4°C. This latter supernatant fraction was dialyzed against three 2-L changes of buffer A (20 mM Tris-HCl, pH 7.5, 10 mM each of NH_4Cl and ME, and 2 mM PMSF) for 6 h at 4°C and was used as the source of cytosolic I-1-P synthase (fraction III).

For isolation of cytosolic I-1-P synthase from *S. platensis*, about 5 g of a 14-d-old cultured algal mass was homogenized in buffer A in a chilled mortar and pestle. The homogenate was passed through four layers of cheesecloth and centrifuged at 28,000*g* for 45 min at 4°C. The supernatant fraction was used as the source of cytosolic I-1-P synthase in *S. platensis*. The crude chloroplast pellets from *O. sativa*, *E. gracilis*, and *V. radiata* (fraction I) were suspended gently in chloroplast isolation buffer with a paintbrush, washed two times with the same buffer, and purified through a Percoll gradient, according to the method of Salomon et al. (1987). The crude chloroplast suspension was layered over a gradient of 80, 40, and 10% (v/v) Percoll in chloroplast isolation buffer. Intact chloroplasts were separated from the 80/40% Percoll interface after centrifugation at 18,000*g* for 20 min in a swing-out rotor.

The intact chloroplast band was suspended in 10 times its volume in chloroplast isolation buffer and centrifuged at 28,000*g* for 15 min to obtain the chloroplast pellet. Identification of the isolated organelles as intact chloroplasts was made by phase-contrast and electron microscopy and by Hill reaction assays using dichlorophenol-indophenol, as described by Adhikari et al.(1987). Marker enzyme assays for both chloroplastic and cytosolic fractions were as described by RayChaudhuri and Majumder (1996). Intact chloroplasts were broken with glass powder in three times their volume in buffer A and centrifuged at 28,000*g* for 30 min at 4°C. The supernatant fraction was dialyzed for 6 h against three 2-L changes of buffer A at 4°C, a step required for complete removal of interfering Pi from the enzyme assay. The dialyzed fraction was utilized as the source of chloroplastic I-1-P synthase (fraction IV).

Assay of I-1-P Synthase

The enzyme was assayed colorimetrically by the periodate oxidation method of Barnett et al. (1970) and the

results were further corroborated by the I-1-Pase assay described previously (Adhikari et al. 1987; RayChaudhuri and Majumder, 1996). The amount of Pi released from the I-1-P synthase product upon periodate oxidation or I-1-Pase hydrolysis was estimated by the method of Chen et al. (1956). Protein was estimated according to the method of Bradford (1976) using BSA as the standard.

Purification of I-1-P Synthases

Purification of the cytosolic and chloroplastic I-1-P synthases was performed as described below. All experiments were at 0 to 4°C.

Gel Filtration through Ultrogel AcA 34

Two milliliters of dialyzed supernatant fluid obtained by homogenization of the chloroplastic (fraction IV) or cytosolic (fraction III) samples was loaded onto an 83-mL bed volume Ultrogel AcA 34 column (1.6 × 41 cm) preequilibrated with buffer B (buffer A containing 20% [v/v] glycerol). Alternatively, Superose 12 was used in place of Ultrogel AcA 34 as the first gel-filtration column. For the cytosolic I-1-P synthase in *O. sativa*, the fraction III protein was made 40 to 70% by saturation with $(NH_4)_2SO_4$, and the precipitated protein was dissolved in 2.5 mL of buffer A, dialyzed in two changes of 1 L of buffer A, and then centrifuged at 28,000*g* for 20 min at 4°C. The supernatant fluid was used as the source of cytosolic I-1-P synthase and this fraction was loaded onto the Ultrogel column. Fractions of 1 mL were eluted with buffer B at the rate of 0.1 mL min^{-1}. Fractions containing I-1-P synthase activity were pooled and dialyzed for 1 h at 4°C in 1 L of buffer C (20 mM Tris-HCl, pH 7.5, 2 mM PMSF, 1 mM ME, and 20% [v/v] glycerol).

Anion-Exchange Chromatography through DEAE-Sephacel

The dialyzed, Ultrogel-pooled fractions were adsorbed on a DEAE-Sephacel column (8-mL bed volume) preequilibrated with 10 bed volumes of buffer C. After 5 h of adsorbtion of the protein onto the column the effluent was collected in 0.5-mL fractions and then washed in buffer C up to nearly 1 bed volume for elution of unbound protein until the A_{280} of the fractions approached 0. Bound proteins were eluted in a 30-mL linear gradient of 0.1 to 0.15 M NH_4Cl in buffer C. Fractions were collected at the rate of 0.4 mL min^{-1}. Fractions with I-1-P synthase activity were pooled, concentrated to reduce the volume to 0.5 mL, and then dialyzed for 6 h at 4°C against one 2-L change of buffer A.

Gel Filtration through BioGel A-0.5m

The dialyzed, concentrated, pooled DEAE fractions were loaded onto a 1- × 8-cm BioGel A-0.5m column preequilibrated with 8 bed volumes of buffer B. Proteins were eluted with buffer B in fractions of 0.4 mL at a flow rate of 0.1 mL min^{-1}. Fractions containing I-1-P synthase activity were pooled, dialyzed against one 2-L change of 20 mM Tris-HCl, pH 7.5, and concentrated by lyophilization.

PAGE Analysis of I-1-P Synthase

Analytical SDS-PAGE was performed according to the method of Laemmli (1970) on 15% (v/v) acrylamide gels at 50 V for 6 h with about 20 μg of purified protein. Protein bands were detected by Coomassie brilliant blue staining, according to the method of Laemmli (1970), or by a silver-staining procedure (Sambrook et al., 1989). Nondenaturing polyacrylamide gels (10%) were prepared similarly but without SDS and run at 50 V for 6 h using 40 μg of purified protein in each lane. Along with the protein markers, two lanes were stained with Coomassie brilliant blue or silver to detect protein bands, and the other lanes were used to detect enzyme activity by slicing each lane of the gel into 5-mm segments. Each slice was crushed in 20 mM Tris-HCl, pH 7.5, and 10 mM ME in a prechilled homogenizer in ice and centrifuged at 12,300*g* for 15 min at 4°C. The supernatant fluid was assayed for I-1-P synthase activity by the methods described above.

Molecular Weight Determination and Subunit Composition

An estimate of the molecular weight of the purified native enzyme was obtained by gel filtration using an Ultrogel AcA 34 column (1.6 × 41 cm) equilibrated in buffer B and calibrated with the reference proteins thyroglobulin (M_r 669,000), ferritin (M_r 440,000), catalase (M_r 232,000), aldolase (M_r 158,000), and albumin (M_r 67,000). The void volume was determined with blue dextran 2000 (1 mg mL^{-1}). All standards were applied to the column in 1.2% of the column volume, and the fractions were eluted at a flow rate of 0.1 mL min^{-1}. Fractions of 1 mL were collected and analyzed for protein content at 280 nm. BioGel A-0.5m-pooled fractions of I-1-P synthase representing 1.2% of the column volume were applied to the calibrated column. Conditions of elution and collection of fractions were identical to the calibration procedure. Determination of the I-1-P synthase elution profile was obtained by enzymatic assay and analysis of the A_{280} profile.

Generation of Polyclonal Antibodies to I-1-P Synthase and Immunoblot Analysis

Antiserum against the purified native cytosolic I-1-P synthase from *E. gracilis* was raised in rabbits. Approximately 100 μg of purified protein emulsified in Freund's complete adjuvant was injected into the leg muscle of a rabbit. After 3 weeks, booster shots with 100 μg of protein in Freund's incomplete adjuvant were given three times at weekly intervals. Three days after the last booster injection, blood was collected and coagulated at 4°C overnight, and the crude serum was obtained by centrifugation at 28,000*g* for 15 min at 4°C.

After SDS-PAGE, protein was transferred to PVDF membranes using a blot apparatus following the methods of Burnette (1981). The blotted membranes were analyzed as

immunoblots by probing with anti-I-1-P synthase antibody (1:500 dilution) raised against the purified cytosolic *E. gracilis* I-1-P synthase using detection kit reagents (ECL, Amersham).

RESULTS

Cytosolic and Chloroplastic I-1-P Synthase Activities in Various Plant Groups

Earlier studies by this laboratory revealed the presence of a cytosolic form of I-1-P synthase in a number of lower plant groups (DasGupta et al., 1984). In the algae, *E. gracilis* exhibited much higher enzyme activity in comparison with the others tested. Subsequently, detection of a chloroplastic form of I-1-P synthase from *E. gracilis* and *V. radiata* (Adhikari et al., 1987) and a salt- and photo-responsive chloroplastic form of the enzyme in *O. sativa* (RayChaudhuri and Majumder, 1996) prompted us to undertake further study.

The results of our search for the presence of two forms of I-1-P synthase in cyanobacterial, algal, and higher plant systems are given in Table I. Although only a single form of the enzyme was detected in *S. platensis*, both cytosolic and plastidial forms were detected in *E. gracilis* and germinating seedlings of *O. sativa* and *V. radiata*. Although different *E. gracilis* varieties and mutants exhibited appreciable cytosolic I-1-P synthase activity, chloroplastic I-1-P synthase activity could not be detected in either streptomycin-bleached or dark-grown cells or in the mutants O2BX, G1BU, Y3BUD, Y3BUL, and Y1BXD, which lack well-differentiated chloroplasts and exhibit defective plastid constituents (data not presented). In *O. sativa* and *V. radiata* dark-grown and light-/dark-grown seedlings exhibited similar cytosolic I-1-P synthase activity. However, the chloroplastic I-1-P synthase activity was enhanced nearly 3-fold by growth of the seedlings in a light/dark regime, as was reported previously for *E. gracilis* (Adhikari et al., 1987).

Table I. *Cytosolic and plastidial I-1-P synthase activity in S. platensis, E. gracilis and its mutants, and seedlings of O. sativa and V. radiata*

Enzyme assays were performed with a dialyzed $(NH_4)_2SO_4$ fraction obtained from the 28,000*g* supernatant fraction of the cell homogenate (cytosolic inositol synthase) or from isolated plastids (plastidial inositol synthase). Identification of cytosolic and plastidial fractions was made by assay of marker enzymes for both chloroplastic and cytosolic fractions as described by RayChaudhuri and Majumder (1996). Rubisco and NADP-glyceraldehyde 3-P dehydrogenase activities were found to be predominant in the chloroplastic fractions, the former being undetectable and the latter present only to an extent of less than 10% in the cytosolic fractions. The cytosolic fractions, on the other hand, showed appreciable Glc-6-P dehydrogenase activity in contrast to the chloroplastic fraction, in which activity was nearly absent.

Plant Material	Inositol Synthase Activity	
	Cytosolic	Plastidial
	μmol I-1-P h^{-1} mg^{-1} protein	
S. platensis	0.28	–
E. gracilis var Z.	0.25	0.18
E. gracilis var *bacillaris*	0.20	0.15
E. gracilis var Z. (dark-grown)	0.26	0.01
E. gracilis Z (streptomycin-bleached)	0.13	0
Seedlings of *O. sativa*		
Dark-grown	0.13	0.05
Light/dark (12/12 h)-grown	0.14	0.17
V. radiata		
Dark-grown	0.20	0.12
Light/dark (12/12 h)-grown	0.20	0.48

Purification of Cytosolic and Chloroplastic I-1-P Synthases

Following the demonstration of chloroplastic and/or cytosolic forms of I-1-P synthase from cyanobacterial, algal, and higher plant sources, we studied the two forms of the enzyme in greater detail to evaluate their characteristics. Protocols were designed for the purification of the different forms of the enzyme from *S. platensis*, *E. gracilis*, *O. sativa*, and *V. radiata*. The cytosolic and chloroplastic I-1-P synthases had distinctly different elution profiles during gel-filtration chromatography on Ultrogel AcA 34. The *E. gracilis* and *O. sativa* cytosolic I-1-P synthases eluted between the 232-kD (catalase) and the 140-kD (lactate dehydrogenase) markers in fractions 75 to 90, whereas the one from *S. platensis* eluted earlier, in fractions 55 to 80, suggesting that the protein has a higher molecular weight. Furthermore, upon gel filtration through the same Ultrogel AcA 34 column, the chloroplastic 28,000*g* supernatant fraction revealed an I-1-P synthase activity that eluted prior to catalase and between fractions 50 to 70 for *E. gracilis*, *O. sativa*, and *V. radiata*. Such differences in the elution profiles of the cytosolic and chloroplastic I-1-P synthases establishes their nonidentical nature, at least in terms of molecular weight.

Both cytosolic and chloroplastic I-1-P synthases from all sources could be further purified by anion-exchange chromatography on DEAE Sephacel, in which they eluted between 110 and 120 mM NH_4Cl. In each case a contaminating, nonspecific phosphatase that co-eluted in the Ultrogel AcA 34 fractions with I-1-P synthase became negligible in the DEAE-Sephacel fractions. Purification to electrophoretic homogeneity for both forms of I-1-P synthase from each source was achieved by a final BioGel A-0.5m filtration step (Fig. 1). The cytosolic I-1-P synthases from *S. platensis*, *E. gracilis*, and *O. sativa* were purified up to 285-, 277-, and 153-fold, respectively, by the procedure described above. The chloroplastic I-1-P synthases from *E. gracilis*, *O. sativa*, and *V. radiata* were purified up to 179-, 218-, and 95-fold, respectively.

Analysis of Purity

The purified forms of both cytosolic and chloroplastic I-1-P synthases were analyzed by nondenaturing PAGE, and their positions were revealed by enzymatic activity (Fig. 1, insets). For each, a major sharp protein band was resolved, which was coincident with the I-1-P synthase activity and with an R_F value between 0.099 and 0.111 for the chloroplastic I-1-P synthase and between 0.3 and 0.36 for the cytosolic I-1-P synthase. No other protein band was detected in nondenaturing PAGE. In preparations stored

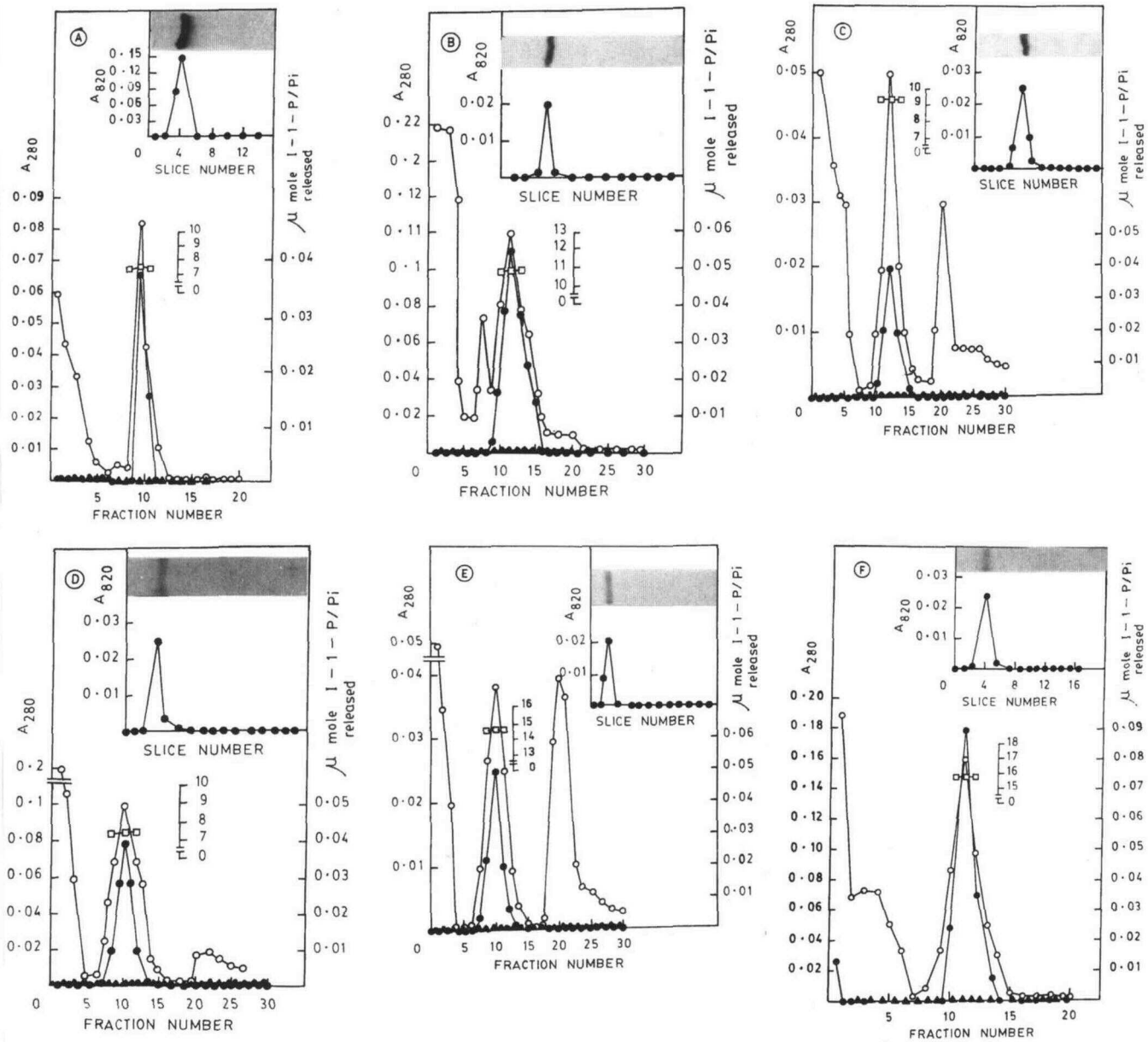

Figure 1. Purification of cytosolic and chloroplastic I-1-P synthases by BioGel A-0.5m gel-filtration chromatography. The dialyzed DEAE pool for cytosolic fractions of *S. platensis* (A), *E. gracilis* (B), and *O. sativa* (C) and chloroplastic fractions from *E. gracilis* (D), *O. sativa* (E), and *V. radiata* (F) were chromatographed through a BioGel A-0.5m column equilibrated in 20 mM Tris-HCl, pH 7.5, 14 mM NH_4Cl, 2 mM PMSF, and 10 mM ME, 20% (v/v) glycerol, as described in "Materials and Methods." Chromatography was monitored by A_{280} (○), I-1-P synthase activity (●), and nonspecific phosphatase activity (▲). □, Specific activity (μmol I-1-P h^{-1} mg^{-1} protein) of the fractions that were ultimately pooled for PAGE analysis. The insets represent nondenaturing PAGE analysis of the pooled peak activity fractions, stained by Coomassie brilliant blue (A–C) or silver (D–F), and the localization of I-1-P synthase activity in gel slices, as described in "Materials and Methods."

for a longer time, a faint band of nearly 60 kD appeared, which was likely a dissociated or degraded form of the native enzyme.

Molecular Weight Determination and Subunit Composition of Native Enzymes

The relative molecular weight of the I-1-P synthases was estimated by gel-filtration analysis of the BioGel-purified enzyme through Ultrogel AcA 34 in comparison with standard protein markers and determination of the I-1-P synthase elution by enzymatic assay. For cytosolic or chloroplastic I-1-P synthase only one A_{280} peak of the eluted fractions was observed and enzyme activity was coincident with this peak. No enzymatic activity was detected in any other fraction that would have suggested either a lower-molecular-weight form of the enzyme or a higher-

molecular-weight aggregate. The cytosolic and chloroplastic I-1-P synthases eluted at different zones under the same chromatographic conditions. The approximate native molecular mass of the chloroplast enzyme appeared to be 248 kD for *E. gracilis*, 253 kD for *O. sativa*, and 266 kD for *V. radiata*, respectively; whereas the native molecular mass of the cytosolic enzyme appeared to be 179 kD for both *E. gracilis* and *O. sativa* and 200.32 kD for *S. platensis* (data not shown).

Results of the SDS-PAGE analysis of the I-1-P synthases from the different sources studied are shown in Figure 2. The subunit molecular masses were estimated to be approximately 59, 61, and 67 kD for the cytosolic I-1-P synthases in *O. sativa*, *E. gracilis*, and *S. platensis*, respectively, and approximately 60, 62, and 58 kD for the chloroplastic I-1-P synthases in *O. sativa*, *E. gracilis*, and *S. platensis*, respectively (Fig. 2). Based on the estimated subunit molecular weight of the I-1-P synthase proteins isolated from the different sources and the data shown in Figure 1, it appears that, whereas the cytosolic I-1-P synthases from *S. platensis*, *E. gracilis*, and *O. sativa* are possibly trimers of their respective subunits, the chloroplastic I-1-P synthases of *E. gracilis*, *O. sativa*, and *V. radiata* are homotetramers.

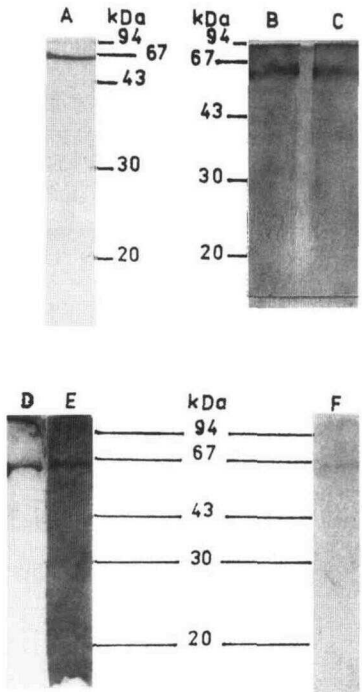

Figure 2. SDS-PAGE analysis of the BioGel A-0.5m-purified proteins. About 20 μg of protein was electrophoresed in a 15% (v/v) acrylamide gel. Protein bands were detected by Coomassie brilliant blue (A and F) or silver (B–E) staining. A to F, Cytosolic I-1-P synthase of *S. platensis* (A), *E. gracilis* (B), and *O. sativa* (E) and chloroplastic I-1-P synthase of *E. gracilis* (C), *O. sativa* (D), and *V. radiata* (F). Molecular mass markers are indicated.

Characteristics of the Purified Cytosolic and Chloroplastic I-1-P Synthases

Substrate Specificity and Cofactor Requirement

The BioGel-purified cytosolic and chloroplastic I-1-P synthases from all sources studied showed absolute substrate specificity for Glc-6-P. Other monophosphates such as Fru-6-P, Fru-1-P, and Glc-1-P could not replace Glc-6-P as a substrate. A basal activity of about 4% was noted using Gal-6-P as a substrate for the chloroplastic or cytosolic forms in *O. sativa*, *E. gracilis*, *V. radiata*, and *S. platensis*.

The I-1-P synthase reaction was NAD^+-dependent and could not be replaced by $NADP^+$. Both cytosolic and chloroplastic I-1-P synthases retained nearly 20% of their activity without the addition of NAD^+ to the assay mixture. The enzymes presumably had tightly bound NAD^+ even after the final purification step, as reported earlier for the adult brain or testis enzyme (Barnett et al., 1973; Adhikari and Majumder, 1983).

pH and Temperature Optima

The activity of the purified I-1-P synthases was assayed as a function of pH by buffering the reaction mixture with Tris-HCl from pH 6.8 to 8.6. The optimum pH was 7.6 for chloroplastic and cytosolic I-1-P synthases in *E. gracilis* and chloroplastic I-1-P synthase in *O. sativa* and pH 7.2 for chloroplastic I-1-P synthase in *V. radiata* (Table II). The pH optima for cytosolic I-1-P synthases in *S. platensis* and *E. gracilis* were 7.8 and 8.2, respectively. All of the I-1-P synthases studied were optimally active between 35 and 37°C.

Kinetic Properties

Estimates of K_m values for the substrate and cofactor were obtained with BioGel A-0.5m-purified enzyme and were determined by Lineweaver-Burk analysis of the I-1-P synthase reactions with increasing concentrations of Glc-6-P (0–6 mM) using 0.8 mM NAD^+ or with increasing concentrations of NAD^+ (0–0.8 mM) using 5 mM Glc-6-P as the substrate, other reaction conditions remaining constant. The K_m values for Glc-6-P and NAD^+ and their corresponding V_{max} values are summarized in Table II.

Reaction with Thiol-Reactive Reagents

Both chloroplastic and cytosolic enzymes from *O. sativa* and *E. gracilis* were strongly inhibited by thiol-reactive reagents such as NEM and PCMB between 0.5 and 2 mM. A sharp decline in enzyme activity was noted between 0.1 and 0.5 mM NEM. At 0.5 mM NEM the decrease was about 30 to 35% in *O. sativa* and about 60% in *E. gracilis*, and at 2 mM it was about 70 to 80% in both cases. Inhibition by NEM, however, could be prevented to an extent of 80 to 100% by preincubation of the enzymes in the combined presence of Glc-6-P (5 mM) and NAD^+ (0.8 mM) but not by either Glc-6-P or NAD^+ alone. Similar results were obtained with the PCMB-treated enzymes. These results suggest that both -SH reagents effect inactivation of the en-

Table II. *Comparison of properties of inositol synthases from S. platensis, E. gracilis, O. sativa, and V. radiata*

The enzymes were purified and assayed as described in "Materials and Methods." Characterization of the enzymes was performed with BioGel A-0.5m-purified preparations. Data are expressed as the averages of at least three independent experiments.

Plant Material	pH Optimum	Temperature Optimum	K_m		V_{max}	
			Glc-6-P	NAD^+	Glc-6-P	NAD^+
		°C	mM		μmol min^{-1}	
Cytosolic						
S. platensis	7.8	35	2.17	0.11	0.08	0.07
E. gracilis						
Green cells	7.5	35	2.25	0.16	0.06	0.06
Dark-grown cells	7.6	35	2.51	0.19	0.07	0.08
Streptomycin-bleached cells	7.5	35	1.95	0.20	0.005	0.01
O. sativa	8.2	35	1.97	0.14	0.07	0.09
Chloroplastic						
E. gracilis	7.6	37	1.95	0.12	0.06	0.08
O. sativa	7.6	37	2.14	0.08	0.07	0.10
V. radiata	7.2	35	2.85	0.26	0.02	0.02

zymes by interaction with -SH groups located at or near the active site. Attempts to reactivate the completely inactivated enzymes with either ME or DTT were unsuccessful.

Immunodetection of I-1-P Synthases

Polyclonal antibody raised against purified cytosolic I-1-P synthase from *E. gracilis* was used for immunotitration of cytosolic and chloroplastic I-1-P synthases isolated from *S. platensis*, *E. gracilis*, *O. sativa*, and *V. radiata*. Both forms of the I-1-P synthase isolated from these sources were precipitated by the anti-I-1-P synthase. Although the antiserum was most effective in precipitating the *E. gracilis* cytosolic I-1-P synthase against which it was generated, both cytosolic and chloroplastic I-1-P synthases from *S. platensis*, *E. gracilis*, *O. sativa*, and *V. radiata* were precipitated by the same antiserum, albeit to different degrees (data not presented). The immunoblot analysis showed that the same antibody reacted with only one distinct polypeptide band in crude extracts of cytosolic and/or chloroplastic proteins from *S. platensis*, *E. gracilis*, and *O. sativa* (Fig. 3).

Effects of Various Chemicals and Reagents on Cytosolic and Chloroplastic I-1-P Synthases

A number of chemicals, including monovalent and divalent cations, sugar alcohols, and sugar phosphates, were tested for their influence on the catalytic activity of the cytosolic and chloroplastic I-1-P synthases. Of the monovalent cations, K^+ was marginally stimulatory to both cytosolic and chloroplastic I-1-P synthases in *O. sativa* up to 2.5 mM, whereas at higher concentrations (5 mM) it inhibited enzyme activity by 10 to 15%. In contrast, in *E. gracilis* and *V. radiata*, KCl inhibited enzyme activity in a concentration-dependent manner up to 5 mM. NaCl was inhibitory to both enzyme forms in *E. gracilis* and to the chloroplastic enzyme in *V. radiata* starting at 1 mM, whereas in *O. sativa* enzyme activity was inhibited to a much lesser extent. LiCl was inhibitory in a concentration-dependent manner to both forms of the enzyme and completely inhibited activity at 5 mM in *E. gracilis*, *O. sativa*, and *V. radiata*. Between 10 and 14 mM, NH_4Cl produced an approximately 5-fold stimulation of both forms of the enzyme from *O. sativa*, *E. gracilis*, and *V. radiata*. Other NH_4^+ salts such as NH_4HCO_3 and $(NH_4)_2SO_4$, however, were strong inhibitors of the enzyme activity.

Divalent cations exhibited varying degrees of inhibition of cytosolic and chloroplastic I-1-P synthase. Of the several divalent cations tested, Cl^- salts of Fe^{2+}, Zn^{2+}, Cu^{2+}, and Pb^{2+} were found to be inhibitory, leading to 100% inhibition at approximately 1 mM. The other divalent cations tested (Ca^{2+}, Mg^{2+}, and Mn^{2+}) were also inhibitory to different degrees at 5 mM.

Sugar alcohols such as inositol, mannitol, and sorbitol inhibited both forms of I-1-P synthase activity at 4 mM. *E.*

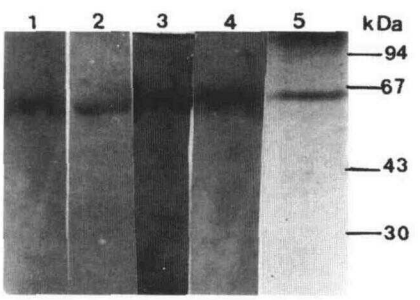

Figure 3. Immunoblot analysis of I-1-P synthase made from gels loaded with 50 μg of protein from crude extracts from various sources. Lane 1, Cytosolic supernatant from *S. platensis*; lane 2, chloroplast protein from *E. gracilis*; lane 3, cytosolic protein from *E. gracilis*; lane 4, chloroplast protein from *O. sativa*; and lane 5, cytosolic protein from *O. sativa*. Extraction of protein and immunoblotting were carried out as described in "Materials and Methods." The protein markers used were phosphorylase *b*, albumin, ovalbumin, and carbonic anhydrase.

gracilis cells grown in the presence of relatively low concentrations of these compounds (0.2–1 mM) exhibited altered profiles of I-1-P synthase activity during growth, accompanied by a reduction in enzyme activity. A number of sugar phosphates were found to act as inhibitors of both forms of I-1-P synthases. Fru-6-P and Gal-6-P at 5 mM inhibited the activities to different degrees. Glucitol-6-P or dGlc-6-P, the two natural substrate analogs, inhibited I-1-P synthase activity to a greater extent, even at 1 mM.

DISCUSSION

Purification and Subunit Organization of Chloroplastic and Cytosolic I-1-P Synthases

An important aspect of the present work was the study of the structural and catalytic properties of the I-1-P synthases from the cytosol and chloroplast, with the objective of evaluating the identity of the two forms of the protein. As a prerequisite for such a study, purification of the two enzyme forms was attempted. Here we devised a simple, three-step purification procedure for the I-1-P synthases to obtain homogeneous preparations of one or both forms of the enzyme from *S. platensis*, *E. gracilis*, *O. sativa*, and *V. radiata*. The procedure has been found to be highly reproducible and gives fairly high yields of substantially purified and active enzymes.

Instability of the enzymes was a serious problem during purification, which we addressed by the use of 20% (v/v) glycerol and 2 mM PMSF throughout most of the purification steps. The final preparation in all cases can be stored at −20°C in 20% (v/v) glycerol for several weeks without any appreciable loss of activity. Homogeneity of each of the six native proteins isolated by this procedure was established by the results depicted in Figures 1 and 2. The subunit molecular weight of the polypeptide for both cytosolic and chloroplastic I-1-P synthases (Fig. 2) is within the range of 58,000 to 67,000, similar to that reported from other sources. This is also in agreement with the size of the predicted protein product of the *Ino1* genes so far isolated (Majumder et al., 1997).

Taking the subunit molecular weight into account, the derived native molecular weight of the cytosolic I-1-P synthase (178,500–200,320) is indicative of a trimeric protein structure, as reported earlier for the duck-weed (*Lemna gibba*) (Ogunyemi et al., 1978), lily (*Lilium longifolium*) (Loewus et al., 1984), and rat testis (Maeda and Eisenberg, 1980). In contrast, the molecular weight of the native chloroplast holoenzyme (248,000–266,000) is suggestive of a protein structure with four identical subunits, as in the case of *Neurospora crassa* (Zsindley et al., 1977) and *S. cerevisiae* (Donahue and Henry, 1981).

Biochemical Characteristics of Cytosolic and Chloroplastic I-1-P-Synthases

Biochemical characterization of the cytosolic and chloroplastic enzymes revealed insignificant differences between the two (Table II). Both forms operate optimally within the pH range of 7.2 to 8.2, a range favored by most other I-1-P synthases. The K_m and V_{max} for Glc-6-P and NAD^+ are also similar to other cytosolic forms (Loewus and Loewus, 1983; Loewus, 1990). Both the chloroplastic and cytosolic I-1-P synthases appear to share similar properties with respect to these characters. A striking observation was the much lower V_{max} values for Glc-6-P and NAD^+ of the cytosolic I-1-P synthase from streptomycin-bleached *E. gracilis* (Table II). The effects of monovalent and divalent cations, sugar alcohols, and sugar phosphates were similar to those obtained from other sources.

An important property of the chloroplastic and cytosolic I-1-P synthases is the possible involvement of -SH groups in catalytic activities. Both PCMB and NEM inhibit the chloroplastic and cytosolic I-1-P synthase activities in *E. gracilis* and *O. sativa*. NEM inhibition of I-1-P synthase activity was blocked by prior incubation of the enzyme in the combined presence of Glc-6-P and NAD^+, suggesting that the -SH groups reside close to the active site. Such results are comparable to those obtained for I-1-P synthases from pine pollen (Gumber et al., 1984), yeast (Chen and Charalampous, 1964), and the rat mammary gland (Naccarato et al., 1974). Inositol inhibited the activity of both cytosolic and chloroplastic I-1-P synthases, as reported earlier for yeast (Hirsch and Henry, 1986), Arabidopsis (Johnson and Sussex, 1995; Johnson and Wang, 1996), and *O. sativa* (Funkhouser and Loewus, 1975). This possible regulatory role of inositol in I-1-P synthase activities needs further investigation.

Antiserum raised against the *E. gracilis* cytosolic I-1-P synthase inhibited the activity of both cytosolic and chloroplastic I-1-P synthases from *S. platensis*, *E. gracilis*, *O. sativa*, and *V. radiata* and cross-reacted with a single polypeptide band in each source following SDS-PAGE (Fig. 3). This suggests that both forms may share some common epitopes and that they are conserved among the various divergent organisms. It appears, considering the comparative characterization of the two forms of I-1-P synthase, that the cytosolic and chloroplastic I-1-P synthases, although distinct in their subcellular localization, are similar, if not identical, in their biochemical, immunological, and protein subunit characteristics and differ only in the organization of the subunits in the native holoenzymes.

An obvious next step in this work will be the isolation of the gene(s) for the two forms of I-1-P synthase and localization of the gene for the chloroplastic enzyme in the nuclear or chloroplast genome. Studies conducted so far with protein synthesis inhibitors suggest a nuclear origin of the chloroplastic I-1-P synthase (data not presented). The presence of identical subunits in the two forms, despite the different organization of the respective holoenzymes, also supports such a contention. Verification of this must await further investigations currently in progress.

Isolation of I-1-P synthase from a cyanobacterium such as *S. platensis* establishes the probable antiquity of the inositol biosynthetic pathway in biological systems. This observation, along with the reported presence of I-1-P synthase in *Streptomyces griseus* (Pittner et al., 1979) and *Entamoeba histolytica* (A. Lohia, N.C. Hait, and A.L. Majumder, unpublished data), provides interesting clues toward an understanding of the phylogenetic relationship of the chlo-

roplastic and cytosolic I-1-P synthases from plant, microbial, and animal sources (Majumder et al., 1997).

Received March 25, 1997; accepted June 26, 1997.

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