L-*myo*-inositol-1-phosphate synthase: partial purification and characterisation from *Gleichenia glauca*

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Abstract

A screening for the enzyme L-*myo*-inositol-1-phosphate synthase [EC 5.5.1.4] has been made first time in both vegetative and reproductive parts of the representative members of pteridophytes: *Lycopodium*, *Selaginella*, *Equisetum*, *Polypodium*, *Dryopteris*, and *Gleichenia*. The enzyme has been partially purified following low-speed centrifugation, streptomycin sulphate precipitation, ammonium sulphate fractionation, chromatography on DEAE-cellulose and gel-filtration through *Sephadex G-200*, and characterised from the reproductive pinnules of *Gleichenia glauca* Smith. The enzyme has a pH optimum at 7.5. The K_m for glucose-6-P and NAD^+ were 0.922×10^{-3} M and 0.9×10^{-4} M, respectively. A basal activity of the enzyme has been recorded in absence of exogenous NAD^+. The enzyme activity was augmented with NH_4Cl, but heavy metals like Hg^{2+}, Cu^{2+} and Zn^{2+} inactivated it.

Additional key words: inositol synthase, *myo*-inositol, pteridophytes.

Introduction

Existence of *myo*-inositol, either free or in conjugate forms, has been reported from a large number of living organisms with great phylogenetic diversity except in pteridophytes – a significant plant group. L-*myo*-inositol-1-phosphate synthase [D-Glucose-6-phosphate-1-L-*myo*-inositol-1-phosphate synthase; EC 5.5.1.4; hitherto referred to I-1-P synthase], the key enzyme of inositol metabolism, catalyses the synthesis of L-*myo*-inositol-1-phosphate from its only known *de novo* precursor D-glucose-6-phosphate in an NAD^+-dependent oxido-reductase reaction. The L-*myo*-inositol-1-phosphate further generates free *myo*-inositol by *myo*-inositol-1-phosphate phosphatase [EC 3.1.3.25]. Surprisingly, an important plant group, pteridophytes (vascular cryptogams) has been completely left out for the study of *myo*-inositol metabolism. The present investigation includes a systematic study for the presence of I-1-P synthase among the diverse members of pteridophytes along with the characteristics of the partially purified enzyme from *Gleichenia glauca*.

Materials and methods

Chemicals: Tris, β-NAD^+, D-glucose-6-phosphate, D-galactose-6-phosphate, D-fructose-6-phosphate, D-mannose-6-phosphate, bovine serum albumin (BSA), DEAE-cellulose (D-8382), streptomycin sulphate, and sodium metaperiodate were purchased from *Sigma Chemical Company*, St. Louis, USA. *Sephadex G-200* was from *Pharmacia Fine Chemicals*, Piscataway, USA. All other chemicals were of analytical grade and

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Abbreviations: BSA - bovine serum albumin; G-6-P - D-glucose-6-phosphate; I-1-P - L-*myo*-inositol-1-phosphate; I-1-P synthase - L-*myo*-inositol-1-phosphate synthase; NAD - nicotinamide adenine dinucleotide; TCA - trichloroacetic acid.
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purchased from *E. Merck*, Mumbai, India and *SISCO Research Laboratories*, Mumbai, India.

Plants: *Lycopodium clavatum* L., *Selaginella microphylla* (Kunth) Spering, *Equisetum elongatum* Willd., *Gleichenia glauca* (Thumb.) Hook., *Polypodium wallichii* R.Br., and *Dryopteris schemperiana* (Hochst.) C.Chr. were freshly collected from Darjeeling hills and its adjoining areas.

Extraction of I-1-P synthase: All preparations of the enzyme from vegetative and reproductive plant parts were carried out at 0 - 4 °C. The experimental samples were collected, washed twice with chilled distilled water and homogenised individually with a mortar and pestle in double volumes of 50 mM Tris-acetate (pH 7.5) containing 0.2 mM 2-mercaptoethanol. The homogenate was centrifuged at 1 000 *g* and 10 000 *g* for 5 and 20 min, respectively. The 10 000 *g* supernatant fraction(s) was dialysed against the same buffer overnight. The dialysed fraction(s) was used for the screening experiments of I-1-P synthase.

Partial purification of I-1-P synthase from *Gleichenia glauca*: The 10 000 *g* supernatant was precipitated with streptomycin sulphate to a final concentration of 2 %. After 15 min, the suspension was centrifuged at 10 000 *g* for 20 min. The pellet was discarded. The supernatant of the previous step was made 70 % saturated with solid $(NH_4)_2SO_4$. After 20 min, the precipitated proteins were collected by centrifugation at 10 000 *g* for 20 min. The 0 - 70 % $(NH_4)_2SO_4$ fraction thus obtained was dissolved in minimal volume of 50 mM Tris-acetate (pH 7.5) containing 0.2 mM 2-mercaptoethanol and dialysed against the same buffer overnight. The dialysed $(NH_4)_2SO_4$ fraction was adsorbed on DEAE-cellulose, previously equilibrated with 50 mM Tris-acetate (pH 7.5) buffer containing 5 mM 2-mercaptoethanol (hereafter called as standard buffer) for 2 h and packed in a 7.5 × 1.2 cm glass column. The column was washed with two bed volumes of standard buffer. I-1-P synthase was eluted from the column with a linear gradient of 0 to 0.5 M KCl in 60 cm^3 standard buffer. Fractions (2.0 cm^3) were collected at an interval of 10 min. The enzyme was eluted between KCl concentrations of 0.25 to 0.3 M (Fig. 1). The DEAE-cellulose purified I-1-P synthase was further purified by molecular sieve chromatography on *Sephadex G-200* column (8 × 1 cm) previously equilibrated with the standard buffer. Fractions of 0.75 cm^3 were collected at a flow rate of 15 min $fraction^{-1}$ (Fig. 2).

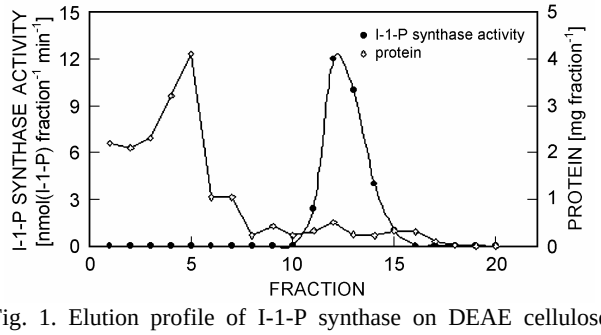

Fig. 1. Elution profile of I-1-P synthase on DEAE cellulose column.

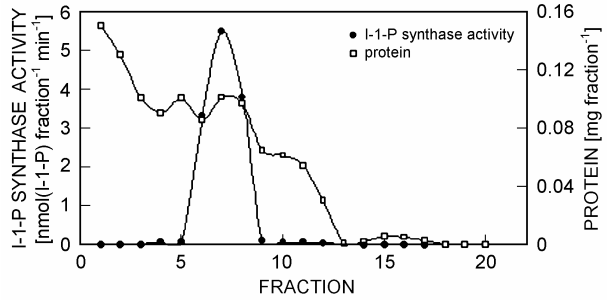

Fig. 2. Elution profile of I-1-P synthase on Sephadex G-200 column.

Assay of I-1-P synthase was done according to the method of Barnett *et al*. (1970) with slight modifications (Dasgupta *et al*. 1984, Adhikari and Majumdar 1988). The assay mixture contained 50 mM Tris-acetate (pH 7.5), 14 mM NH_4Cl, 0.8 mM NAD^+, 5 mM 2-mercaptoethanol, 5 mM D-glucose-6-phosphate and enzyme in a total volume of 0.5 cm^3. Suitable blanks (in which addition of enzyme was omitted), and zero minute controls were also run. After incubation at 37 °C for 1 h, the reaction was terminated by the addition of 0.2 cm^3 of 20 % chilled trichloroacetic acid (TCA). An equal volume of 0.2 M $NaIO_4$ was added to the deproteinised supernatant (0.7 cm^3) followed by an incubation at 37 °C for 1 h for oxidation of the I-1-P synthase reaction product, *myo*-inositol-1-phosphate, with concomitant release of its free form and inorganic phosphate. The excess periodate was destroyed by 1 M Na_2SO_3. Subsequently with the periodate set, appropriate non-periodate controls, made by identical volume of H_2O (in which $NaIO_4$ and Na_2SO_3 treatment was omitted) were also run. Inorganic phosphate was estimated from both sets (periodate and non-periodate) by the method of Chen *et al*. (1956). The set without $NaIO_4$ indicated the amount of inorganic phosphate released by non-specific phosphatases, and the difference between the sets, with and without $NaIO_4$, showed the actual I-1-P synthase activity measured in terms of nmol(*myo*-inositol-1-phosphate produced) mg^{-1}(protein) min^{-1}. Protein was estimated according to the method of Bradford (1976) using BSA as standard.

Results

Various representatives of pteridophytes were screened for the presence of I-1-P synthase (both vegetative and reproductive parts). The enzyme was functional in vegetative as well as in reproductive parts of all the species studied (Table 1). However, the reproductive parts showed about three-fold higher activity in comparison with the vegetative parts of the same species except in case of *Lycopodium clavatum*. The highest enzyme activity was found in *Polypodium wallichii* and *Gleichenia glauca*. The I-1-P synthase from *Gleichenia glauca* was purified up to 24-fold (Table 2).

Table 1. L-myo-inositol-1-phosphate synthase specific activity and protein content in some species of the pteridophytes (nd - not determined). Means ± SE.

Family	Plant species	Plant parts	Protein content [mg g^{-1}(f.m.)]	I-1-P synthase [nmol(I-1-P) mg^{-1}(protein) min^{-1}]
Lycopodiaceae	*Lycopodium clavatum*	vegetative	0.482 ± 0.08	1.466 ± 0.22
		strobili	1.070 ± 0.05	1.616 ± 0.27
Selaginellaceae	*Selaginella microphylla*	vegetative	0.495 ± 0.08	0.250 ± 0.18
		strobili	0.823 ± 0.03	0.980 ± 0.06
Equisetaceae	*Equisetum elongatum*	vegetative	0.356 ± 0.12	0
		strobili	nd	nd
Polypodiaceae	*Polypodium wallichii*	vegetative	0.945 ± 0.07	0.600 ± 0.07
		sori	1.262 ± 0.11	2.033 ± 0.41
Dryopteridaceae	*Dryopteris schemperiana*	vegetative	0.865 ± 0.45	0.483 ± 0.11
		sori	1.127 ± 0.43	1.566 ± 0.25
Gleicheniaceae	*Gleichenia glauca*	vegetative	0.883 ± 0.05	0.566 ± 0.08
		sori	1.145 ± 0.07	1.900 ± 0.36

Table 2. Typical example of partial purification of L-myo-inositol-1-phosphate synthase from *Gleichenia glauca* reproductive pinnules (33 g). Means ± SE.

Fraction	Total protein [mg]	Specific activity [nmol(I-1-P) mg^{-1}(protein) min^{-1}]	Total activity [nmol (I-1-P) min^{-1}]	Recovery [%]	Purification [fold]
Homogenate	37.05 ± 6.01	1.973 ± 0.21	73.099 ± 7.18	100.00 ± 9.83	1.00 ± 2.03
10 000 *g* supernatant	30.66 ± 3.88	2.238 ± 0.30	68.617 ± 5.69	93.86 ± 7.78	1.13 ± 1.98
Streptomycin sulfate treated fraction	26.86 ± 2.61	2.368 ± 0.34	63.604 ± 4.02	87.01 ± 5.50	1.20 ± 1.96
0 - 70 % ammonium sulfate fraction	19.45 ± 0.14	3.110 ± 0.59	60.489 ± 2.98	82.74 ± 4.08	1.57 ± 1.84
DEAE-cellulose fraction	1.10 ± 5.97	27.326 ± 8.66	30.167 ± 7.12	41.26 ± 9.75	13.84 ± 4.61
Sephadex G- 200 fraction	0.28 ± 6.24	46.927 ± 15.19	13.280 ± 12.75	18.16 ± 17.45	23.78 ± 5.56

Table 3. Substrate specificity of L-*myo*-inositol-1-phosphate synthase from *Gleichenia glauca.* Means ± SE.

Substrate	Concentration [mM]	Specific activity [nmol(I-1-P) mg^{-1}(protein) min^{-1}]
Glucose-6-P	5	3.202 ± 0.62
Fructose-6-P	5	0.00
Galactose-6-P	5	0.467 ± 0.29
Mannose-6-P	5	0.00

As the *Sephadex G-200* fraction was stable for less than 24 h, the partially purified 0 - 70 % $(NH_4)_2SO_4$ fraction was used for the characterisation of the enzyme *Gleichenia* enzyme is absolutely specific for the substrate glucose-6-phosphate (Table 3) and the K_m for G-6-P, as determined by Lineweaver-Burk plot, is 0.922×10^{-3} M. Like the inositol synthase reported from all other sources (Pina *et al.* 1978, Loewus and Loewus 1980, Maeda and Eisenberg 1980, Donahue and Henry 1981, Dasgupta *et al.* 1984, Gumber *et al.* 1984, Ray Choudhury *et al.* 1997), the partially purified *Gleichenia* enzyme has been found to be maximum active in presence of added NAD^+; depletion of this coenzyme in the assay mixture reduced the enzyme activity to about 35 %. The K_m for NAD^+ was calculated as 0.9×10^{-4} M. Absence of either ammonium ion or 2-mercaptoethanol decreased the activity to about 62 and 71 % respectively, as compared to the complete

Table 4. Effect on composition of incubation medium on L-*myo*-inositol-1-phosphate synthase activity. Means ± SE.

Condition	Specific activity [nmol(I-1-P) mg^{-1}(protein) min^{-1}]
Complete set	3.916 ± 0.85
Without G-6-P	0
Without buffer	3.112 ± 0.59
Without NAD	1.403 ± 0.02
Without NH_4Cl	2.442 ± 0.36
Without 2-mercaptoethanol	2.783 ± 0.48

medium (Table 4). The reaction was linear with time up to 60 min of incubation at 37 °C. The temperature maximum of the enzyme was around 30 °C and the enzyme was most active at pH 7.0 to 7.5. The *Gleichenia* enzyme showed stimulation of activity in presence of NH_4^+, while K^+ had no significant effect and the Na^+ caused a little inhibition. Among the divalent cations tested, Mg^{2+} and Ca^{2+} showed mild stimulating effect whereas Mn^{2+} was slight inhibitory. Heavy metals like Zn^{2+}, Cu^{2+} and Hg^{2+} significantly inhibited the activity of this enzyme (Table 5).

Table 5. Effect of mono and divalent cations on L-*myo*-Inositol-1-phosphate synthase activity from *Gleichenia glauca.*

Cation	Concentration [mM]	Specific activity [nmol(I-1-P) mg^{-1}(protein) min^{-1}]
Control	0	2.941 ± 0.45
K^+	5	3.065 ± 0.40
Na^+	5	2.672 ± 0.54
NH_4^+	5	4.402 ± 0.04
Mg^{2+}	5	3.583 ± 0.23
Mn^{2+}	5	2.359 ± 0.64
Ca^{2+}	5	3.170 ± 0.37
Zn^{2+}	5	1.464 ± 0.94
Cu^{2+}	5	1.484 ± 0.93
Hg^{2+}	5	0.555 ± 1.24

Discussion

Although reported in almost all groups of plant, no report is so far available regarding the L-*myo*-inositol-1-phosphate synthase activity in pteridophytes. From the result presented here it is revealed that the enzyme is present in different phylogenetically related pteridophytes studied, of which *Gleichenia glauca* is the most distinguished. It also appears that the enzyme remains functional both in vegetative as well as in reproductive plant parts of all the pteridophytes tested. It can further recall the occurrence of this enzyme in different isoforms in plants at least being localized in chloroplast and cytosol (Adhikari *et al*. 1987, Loewus and Loewus 1983, Wang and Johnson 1995). The reproductive parts showed higher enzyme activity compared to the vegetative parts of the same organism that is a universal feature of all life forms (Biswas and Biswas 1996, Dasgupta *et al*. 1984, Donahue and Henry 1981, Gumber *et al.* 1984, Loewus and Loewus 1980, Maeda and Eisenberg 1980, Majumdar and Biswas 1973, Ray Choudhury *et al*. 1997, Stevenson *et al*. 2000).

Previous studies indicate that L-*myo*-inositol-1-phosphate synthase is operative in thallophytes (Dasgupta *et al.* 1984, Pina *et al.* 1978), bryophytes (Dasgupta *et al.* 1984), gymnosperms (Gumber *et al.* 1984) and in angiosperms (Loewus and Loewus 1980, Majumdar and Biswas 1973, Ogunyemi *et al.* 1978). Therefore, the present study definitely stands as an information bridge towards the knowledge of a more universal distribution of the enzyme.

No I-1-P synthase activity was detected in absence of its substrate glucose-6-phosphate. This enzyme exhibits optimal activity in presence of NAD as a co-factor, the absence of which causes about 65 % loss of activity, indicating the probable presence of some bound NAD in the molecular architecture of this enzyme (Barnett *et al.* 1970, Pittner and Hoffman-Ostenhof 1976, Dasgupta *et al.* 1984). The narrow pH optimum (7.5) has been obtained from *Gleichenia glauca* I-1-P synthase activity. It may be noted that purified I-1-P synthase(s) from other sources exhibits almost similar pH optimum around pH 7.0 (Maeda and Eisenberg 1980, Donahue and Henry 1981, Dasgupta *et al.* 1984). Among the monovalent cations, NH_4^+ was a good stimulator of this enzyme while Na^+ showed a little inhibition. Again, Mg^{2+} and Ca^{2+} were mild stimulators; while Mn^{2+} was a mild inhibitor. Heavy metals, like Hg^{2+} Cu^{2+} and Zn^{2+} showed strong inhibition for the enzyme activity.

Recent studies by other workers (Johnson and Sussex 1995, Molina *et al*. 1999, Chen *et al*. 2000, Flores and Smart 2000) have focused on distribution, characterisation and diverse metabolic significance of this protein in addition to the discovery of the crystal structure and mechanism of action of I-1-P synthase in other group(s) of organisms (Stein and Geiger 2002). Therefore, the present fundamental study centered on I-1-P synthase of pteridophytic origin is a prerequisite to extend more uniform information about the phylogenetic diversity of this catalytic protein.

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