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Partial purification and characterization of L-*myo*-Inositol-1-phosphate synthase of pteridophytic origin

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Key words: D-glucose-6-phosphate, *Diplopterygium glaucum*, enzyme purification, *myo*-Inositol, Pteridophytes

Abstract

Myo-Inositol is an important metabolite for normal growth and development of all living organisms. The cellular level of *myo*-inositol is controlled by the enzyme L-*myo*- inositol--1-phosphate synthase (MIPS) [EC 5.5.1.4]. Appreciable level of MIPS activity was detected from the common pteridophytes like *Dicranopteris, Diplazium, Diplopterygium, Equisetum, Lycopodium, Polypodium, Pteridium, Selaginella etc.* available in Darjeeling Himalayas. The enzyme was partially purified from the reproductive pinnules of *Diplopterygium glaucum* (Thunb.) Nakai. The purification obtained was about 81 fold and the recovery was about 13.5 %. The final enzyme preparation specifically utilized D-Glucose-6-phosphte and NAD+ as its substrate and co-factor respectively. It shows pH optima between 7.0 and 7.5 while the temperature maximum was at 35 °C. The enzyme activity was slightly inhibited by Na^+ and Cd^{2+} and highly inhibited by Li^+ and Hg^{2+}. The K_m values for D-glucose-6-phosphate and NAD+ was found to be as 0.83 mM and 0.44 mM respectively while the V_{max} values were 1.42 mM and 1.8 mM for D-glucose-6-phosphate and NAD+ respectively. The present study indicates the universal occurrence of this enzyme in all plant groups.

List of abbreviations: MIPS=L-myo-inositol-1-phosphate synthase, G-6-P=Glucose-6-phosphate, ME= 2-mercaptoethanol, I-1-P=Inositol-1-phosphate

Introduction

Myo-Inositol is an essential nutrient for growth and development of all living organism, it helps in the formation of sex units and acts as an integral part of storage phosphates in seeds (Murashige and Skoog 1962, Maiti and Loewus 1978, Chiu *et al.* 2003). It also forms cell wall polysaccharides and essential component of myelin in the central nervous system (Adhikari and Majumder, 1988). The biosynthesis of *myo*-Inositol from Glucose-6-phosphate was documented from a number of biological systems (Loewus and Loewus 1983). D-glucose-6-phosphate (G-6-P) irreversibly isomerizes to L-*myo* -inositol-1-phosphate by L-*myo*-inositol-1-phosphate synthase (MIPS). The product of this enzyme generates free *myo*-inositol on dephosphorylation (Loewus and Loewus 1973). The enzyme, MIPS was isolated and characterized from a number of

systems including bacteria (Pittner *et al.* 1979), protozoa (Lohia *et al.* 1999), lower plants (Loewus and Loewus 1971, Donahue and Henry 1981a,b, Dasgupta *et al.* 1984, RayChoudhuri *et al.* 1997), higher plants and animals (Loewus and Loewus 1983, Gumber *et al.* 1984, Majumder *et al.* 1997). However, one important group the pteridophytes has so far been completely left out in the study of *myo*-inositol.

The present study is concerned with the study on the occurrences of free *myo*-inositol and MIPS (EC: 5.5.1.4) from pteridophytes. Purification of MIPS and characterization of its properties are reported here from a suitable pteridophyte studied, *Diplopterygium glaucum* (Thunb.) Nakai (Family - Gleicheniaceae) for the first time.

Materials and Methods

Fresh specimens of pteridophytes, *Dicranopteris linearis* Bedd., *Diplazium dilatatum* Bl., *Diplopterygium glaucum* (Thunb.) Nakai, *Equisetum debile* Roxb., *Lycopodium cernuum* L., *Polypodium quercifolium* L., *Pteridium aquilinum* (L.) Kuhn. *Selaginella megaphylla* Bak. *etc.* were collected from their natural habitats in and around Darjeeling hills (*ca* 2134 m asl) in the Eastern Himalayas. All collections were done in the morning before 9.00 A.M.

Different samples (100 g. each) were washed twice with cold, sterile, distilled water and homogenized in a chilled mortar and pestle in double volume of 50 mM tris-acetate buffer (pH 7.5) containing 0.2 mM of ME. The crude homogenate was passed through four layers of muslin and the liquid was centrifuged at 1,000 x *g* for 5 minutes in a Plasto Crafts Superspin-R centrifuge. The supernatant was again centrifuged at 11,400 x *g* for 20 minutes and the supernatant collected. The supernatant was dialyzed overnight against 50 mM tris-acetate (pH 7.5) buffer containing 0.2 mM ME and the clear dialyzed supernatant was collected from the dialysis bag (11,400 *g* supernatant).

The MIPS activity was assayed following the procedure of Barnett *et al.* (1970) with slight modifications (Adhikari *et al.* 1987). The assay mixture contained 50 mM tris-acetate (pH 7.5), 14 mM NH_4Cl, 0.8 mM NAD^+, 5 mM ME, 5 mM G-6-P and an appropriate aliquot (100-200 µg) of enzyme protein in a total volume of 500 µl. Suitable blanks (without the enzyme) and zero minute controls were also run. The reaction was terminated by adding 200 µl of 20 % chilled TCA after incubation at 37 °C for 1h. An equal volume of 0.2 M $NaIO_4$ was added to the mixture which was again incubated at 37 °C for 1h that caused the release of inorganic phosphate. The excess periodate was destroyed by 1 M Na_2SO_3. Simultaneously, appropriate non-periodate controls, in which $NaIO_4$ and Na_2SO_3 treatments were omitted were also run. The activity of the enzyme was determined by estimating the product-specific release of inorganic phosphate from *myo*-inositol-1-phosphate by MIPS reaction.

Inorganic phosphate was determined by the method of Chen *et al.* (1956) with slight modifications. A freshly prepared Pi-reagent (2.8 ml.) containing H_2SO_4 (6N), ascorbic acid (10 %, w/v), chilled ammonium molybdate (2. 5 %, w/v) and H_2O mixed in 1:1: 1: 2 ratio was added to the reaction mixture and incubated at 37 °C for 1 h. The blue colour developed was measured at 820 nm in a Beckman DU-64 Spectrophotometer. The inorganic phosphate released was quantified with a standard curve prepared using K_2HPO_4.

Protein was determined according to the method of Bradford (1976) with BSA as a standard. The protein content in fractions obtained from column chromatography was also determined by measuring absorbance at 280 nm.

To purify the enzyme from the reproductive pinnules of *Diplopterygium glaucum* (*D. glaucum*) the clear supernatant (11,400 *g* supernatant) was subjected to Streptomycin sulphate treatment to a final concentration of 2 % (w/v) with constant stirring. The mixture was kept in ice-bucket at 0 °C for 15 minutes and then centrifuged at 11,400 x *g* for 15 minutes. The supernatant (Streptomycin sulphate treated fraction) was collected and fractionated with 0-70 % $(NH_4)_2SO_4$. The precipitated protein fraction was dialyzed against 500 volumes of extraction buffer with 2 changes. The dialyzed fraction (0-70 % Ammonium sulphate fraction) was adsorbed on DEAE-cellulose and the preparation was loaded on a glass column (1.2 x 8.0 cm). The effluent was collected. After this, the column was

washed with one bed volume of the extraction buffer and the adsorbed proteins were eluted from the column with a linear gradient of 0-0.5 M KCl. The active fractions (DEAE-cellulose fraction) were pooled and loaded on top of a column (0.8 x 7.5 cm) of Hexylagarose, which was pre-equilibrated with 50 mM Imidazole-HCl (pH 7.5) containing 0.2 mM ME. The column was eluted with the equilibration buffer. The active fractions (Hexylagarose fraction) were pooled together and loaded in a column (0.8 x 10 cm) of BioGel-A 0.5m, which was pre-equilibrated with 50 mM tris-acetate buffer (pH 7.5) containing 0.2 mM ME. The proteins were eluted with the same buffer and the pooled active fractions (BioGel fraction) was concentrated and used as the ultimate preparation in this experiment.

Table 1. L-*myo*-inositol-1-phosphate synthase activity in pteridophytes [specific activity defined as µmol I-1-P produced mg^{-1}·protein·h^{-1}] in a dialyzed 11,400 *g* supernatant. Means ± SE. FW = Fresh Weight

Family	Plant species	Plant part	Protein content [mg g^{-1} FW]	Sp. Activity [µ mol I-1-P produced $(mg)^{-1}$ protein h^{-1}]
Gleicheniaceae	*Dicranopteris linearis* Bedd.	Vegetative	0.543 ± 0.027	0.055 ± 0.006
		Sori	1.005 ± 0.052	0.103 ± 0.008
Gleicheniaceae	*Diplopterygium glaucum* (Thunb.) Nakai	Vegetative	0.886 ± 0.054	0.042 ± 0.004
		Sori	1.138 ± 0.040	0.115 ± 0.013
Dryopteridaceae	*Diplazium dilatatum* Bl.	Vegetative	0.873 ± 0.007	0.032 ± 0.003
		Sori	1.084 ± 0.010	0.089 ± 0.008
Equisetaceae	*Equisetum debile* Roxb.	Vegetative	0.363 ± 0.003	0.0
		Strobili	0.619 ± 0.015	0.012 ± 0.002
Lycopodiaceae	*Lycopodium cernuum* L.	Vegetative	0.474 ± 0.03	0.086 ± 0.004
		Strobili	1.068 ± 0.09	0.095 ± 0.007
Polypodiaceae	*Polypodium quercifolium* L.	Vegetative	0.985 ± 0.072	0.053 ± 0.006
		Sori	1.112 ± 0.115	0.101 ± 0.012
Hypolepidaceae	*Pteridium aquilinum* (L.) Kuhn.	Vegetative	0.663 ± 0.010	0.040 ± 0.007
		Sori	0.981 ± 0.092	0.068 ± 0.019
Selaginellaceae	*Selaginella megaphylla* Bak.	Vegetative	0.492 ± 0.009	0.021 ± 0.004
		Strobili	0.887 ± 0.113	0.045 ± 0.013

Results and Discussion

MIPS was assayed using the low-speed centrifuged and dialyzed supernatant of different experimental pteridophytes *viz. Dicranopteris linearis* Bedd., *Diplazium dilatatum* Bl., *Diplopterygium glaucum* (Thunb.) Nakai, *Equisetum debile* Roxb., *Lycopodium cernuum* L., *Polypodium quercifolium* L., *Pteridium aquilinum* (L.) Kuhn. *Selaginella megaphylla* Bak: etc. The enzyme was functional in vegetative as well as reproductive (strobili/sori) parts of the experimental species with at least a minimum titre of activity (Table 1). Moreover. the strobili or sori displayed about 2-fold higher activity in comparison to the vegetative parts except in case of *L. cernuum*.

Purification of enzyme

The cytosolic MIPS was isolated and purified from the reproductive pinnules of *Diplopterygium glaucum* (*D. glaucum*) by low speed centrifugation, streptomycin sulphate precipitation, ammonium sulphate fractionation, and chromatography on DEAE-cellulose, Hexylagarose and BioGel-A 0.5 m. The summary on the purification of MIPS is given in Table 2. The ultimate chromatography on BioGel-A 0.5m column resulted in about two-fold purification over the penultimate purification step employing Hexylagarose column. An overall purification of the enzyme to about 81 fold with about 13.5 % recovery based on total activity could be achieved in the present study.

Characterization of the purified enzyme

The *D. glaucum* MIPS recorded maximum activity when assayed in presence of 50 mM tris-acetate buffer (pH 7.5), 14 mM NH_4Cl, 0.8 mM NAD^+, 5 mM 2-mercaptoethanol (ME) and 5 mM glucose-6-phosphate (G-6-P). The enzymatic synthesis of L-*myo*-inositol-1-phosphate could not be detected in absence of G-6-P. About 17 % activity was lost when tris buffer was omitted from the reaction mixture. Deduction of NAD+, NH_4Cl or ME resulted in the loss of enzyme activity by about 67 %, 49 % and 32 % respectively (Table 3).

In *D. glaucum* MIPS, G-6-P has been found as the exclusive substrate for the production of L-*myo*

Table 2. Partial purification of L-*myo*-inositol-1-phosphate synthase from *D. glaucum*. Means ± SE (in the parenthesis).

Fraction	Total volume (ml)	Protein (mg/ml)	Sp. Activity [µ mol I-1-P produced $(mg)^{-1}$ protein h^{-1}]	Recovery (%)	Purification (fold)
Homogenate	234.0 (8.98)	0.474 (0.056)	0.120 (0.020)	100.00 (15.29)	1.00 (0.15)
11,400 *g* supernatant	217.0 (14. 71)	0.424 (0.072)	0.130 (0.008)	91.13 (11.34)	1.09 (0.30)
Streptomycin sulphate treated fraction	208.0 (16.11)	0.385 (0.038)	0.140 (0.026)	85.70 (7.68)	1.18 (0.28)
0-70% ammonium sulphate treated fraction	15.5 (1.32)	4.416 (0.601)	0.160 (0.042)	79.96 (9.19)	1.29 (0.17)
DEAE-cellulose fraction	12.0 (2.08)	0.486 (0.055)	1.213 (0.150)	52.66 (6.19)	10.01 (1.61)
Hexylagarose fraction	8.5 (0.76)	0.124 (0.015)	5.02 (0.539)	39.34 (2.44)	41.40 (1.93)
BioGel fraction	4.5 (0.86)	0.041 (0.012	9.981 (2.155)	13.47 (1.088)	80.99 (4.19)

Table 3. Effect of different essential requirements on L-*myo*-inositol-1-phosphate synthase activity in *D. glaucum*. Means ± SE.

Condition	Sp. Activity [μ mol I-1-P produced (mg)$^{-1}$ protein h^{-1}]
Complete set	9.256 ± 1.888
Minus substrate (G-6-P)	0.000
Minus Buffer	7.664 ± 1.350
Minus NAD$^+$	3.071 ± 0.203
Minus NH_4Cl	4.736 ± 1.521
Minus ME	6.281 ± 0.308
Heat-killed enzyme	0.000

Table 4. Substrate specificity of L-*myo*-inositol-1-phosphate synthase from *D. glaucum*. Means ± SE.

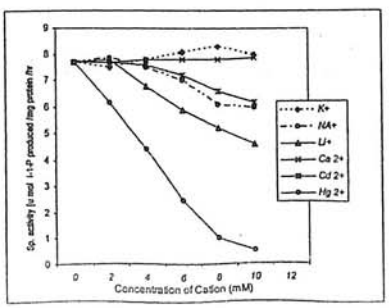
Substrate	Concentration (mM)	Sp. Activity [μ mol I-1-P produced (mg)$^{-1}$ protein h^{-1}]
D-Glucose-6-phosphate	5	9.162 ± 0.987
D-Ribose-5-phosphate	5	0.000
D-Fructose-6-phosphate	5	0.000
D-Galactose-6-phosphate	5	0.860 ± 0.108
D-Mannose-6-phosphate	5	0.130 ± 0.020
D-Glucose-1, 6-bisphosphate	5	0.000
D-Fructose-1, 6-bisphosphate	5	0.000

-inositol-1-phosphate (I-1-P). Other hexose and pentose phosphates, such as D-fructose-6-phosphate, D-ribose-5-phosphate, D-fructose-1, 6-bisphosphate and D-glucose-1, 6-bisphosphate used in place of G-6-P at identical concentrations (5 mM) were ineffective as substrate. However, this enzyme could partially utilize D-galactose-6-phosphate and D-mannose-6-phosphate as substrate with 9.38 % and 1.42 % efficiency respectively as compared to G-6-P (Table 4).

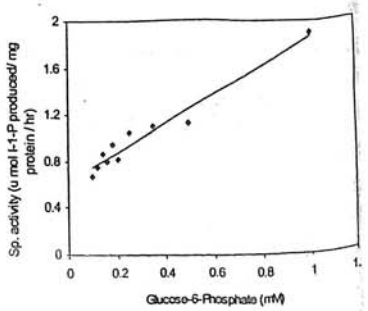
The *D. glaucum* MIPS exhibited optimum activity at a pH range of 7.0-7.5 when 50 mM tris-acetate buffer at a pH range of 6.0-9.0 were employed. It may be noted here that the purified MIPS from other sources recorded almost similar pH optima. The effect of temperature was studied in the temperature range of 0 - 60 °C at 10°C intervals. The activity of the enzyme was least both at 10 °C and 50 °C. However, it was remarkably active between the temperature ranges of 20-40 °C with maxima at 30 °C. This optimum temperature is slightly low as the enzymes isolated from other sources are optimally active between 35° and 37° C (RayChoudhuri *et al.* 1997).

Effect of different metal ions was tested in 1-10 mM concentrations using chloride salts of metals. Of the monovalent cations tested K^+ had a little effect, Na^+ played a minor inhibitory role, Li^+ was strongly inhibitory (60 %). Rat testis enzyme had the identical characteristics (Loewus and Loewus 1980). Using the similar concentrations of divalent cations it was revealed that Ca^{2+} exhibited no effect, Cd^{2+} moderately inhibited (25 %) and Hg^{2+} strongly inhibited (90 %) the enzyme activity (Fig. 1). These characters are almost similar to those of earlier reports (Wells *et al.* 1974, Maeda and Eisenberg Jr. 1980, Loewus and Loewus 1980).

Kinetic studies were carried out using G-6-P (substrate) in the range of 0-10 mM. The reaction rate was found to increase with respect to G-6-P upto a concentration of 4 mM (Fig. 2). The K_m value for G-6-P calculated from Lineweaver-Burk double reciprocal plot was 0.83 mM as against 3.89 mM for the rat testis enzyme (Maeda and Eisenberg Jr. 1980), 1.18 mM for the Yeast enzyme (Donahue and Henry 1981b), 2.1 mM for *Euglena gracilis* enzyme (Dasgupta *et al.* 1984), 2.17 mM for *Spirulina platensis* enzyme (RayChoudhuri *et al.* 1997). The V_{max} value of this pteridophytic enzyme was calculated as 1.42 mM in comparison to 1.6 mM for the yeast enzyme (Donahue and Henry 1981b) and 0.07 for the *Oryza sativa* enzyme (RayChoudhuri *et al.* 1997). Between concentrations of 0-1.0 mM of NAD^+ (co-enzyme) the activity of purified enzyme was found to increase upto 0.5 mM concentration. With more increase in co-enzyme concentration the activity could not be increased. The co-enzyme sat-



← Fig. 1. Effect of metal ions on L-*myo*-inositol-1-phosphate synthase activity in *D. glaucum*



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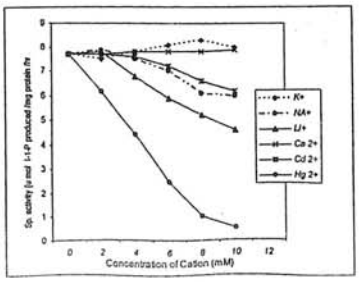

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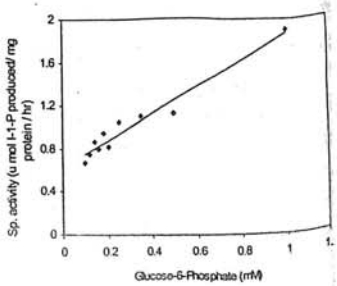


→ Fig. 2. Effect of varied Glucose-6-phosphate concentration (0-10 mM) on L-*myo*-inositol-1-phosphate synthase activity in *D. glaucum* (Lineweaver-Burk plot)

uration curve is hyperbolic in nature. The K_m value for NAD^+ was 0.44 which was quite different from those recorded for the enzyme from other sources *e.g.* 17.9 mM for the testis enzyme (Maeda and Eisenberg Jr. 1980) and 8 mM for the yeast enzyme (Donahue and Henry 1981b). The V_{max} value for NAD^+ for the *D. glaucum* MIPS was calculated from the Lineweaver-Burk double reciprocal plot (Fig. 3) which was found to be 1.8 mM as compared to 1.14 mM for the same obtained from the yeast enzyme (Donahue and Henry 1981b).

MIPS from most of the sources require NAD^+ as an essential coenzyme for the oxidation reduction reaction. To ascertain whether the MIPS from *D. glaucum* is specific for its coenzyme NAD^+, experiments were performed in which the enzyme activity was determined in presence of 0-1.0 mM NAD^+ and $NADP^+$ in two parallel experiments. It was determined that NAD^+ could not be substituted with $NADP^+$ of any concentration. However, there was a basal activity of this enzyme even in the experimental set with $NADP^+$ (Fig. 4).This makes the speculation of enzyme bound NAD^+ more reasonable.

Previous studies on MIPS have shown that the enzyme is operative in thallophytes, bryophytes, gymnosperms as well as angiosperms. The finding of the occurrence of MIPS in pteridophytes indicates a universal distribution of the enzyme and its ancient protein nature.

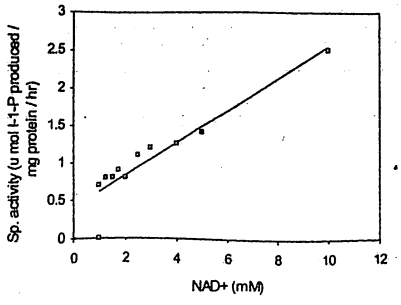
In algae, MIPS was detected from a number of genera and it was purified to electrophoretic homogeneity from *Spirulina platensis* and *Euglena gracilis* (RayChoudhuri *et al.* 1997). The enzyme from different sources did not differ significantly in their biochemical characteristics and followed the trend of the same in other species (Loewus and Loewus 1983, Loewus 1990).

Appreciable MIPS activity was recorded in *Saccharomyces cerevisiae*, where the enzyme was purified and characterized thoroughly (Donahue and Henry 1981b). Escamilla *et al.* (1982) purified MIPS from *Neurospora crassa.* The enzyme was optimally stimulated by 10 mM $(NH_4)_2SO_4$ and 50 mM KCl while NaCl had minor inhibitory effect.

The only report of MIPS from gymnosperm has come from the studies on *Pinus ponderosa* pollen grains. The enzyme exhibited pH optima between 7.25 and 7.75 and the apparent K_m for G-6-P was 0.33 mM (Gumber *et al.* 1984).

The partially purified chloroplastic synthase from *Vigna radiata* was optimally active at a pH of 7.5 to 7.75. A two fold increase in the enzyme activity was found in presence of 9 mM NH_4Cl. The K_m for G-6-P and NAD^+ was determined to be around 1.8 mM and 0.3 mM respectively (Adhikari *et al.* 1987). Both cytosolic and chloroplastic MIPS were purified from *Oryza sativa.* The optimal activity of the enzyme was recorded pH 8.2 and and. 7.6 for the cytosolic and the chloroplastic forms respectively. Both forms of the enzyme were stimulated to about 5 fold by NH_4Cl and inhibited 100 % by 1 mM Cl^- salts of Fe^{2+}, Zn^{2+}, Cu^{2+} and Pb^{2+} (RayChoudhuri *et al.* 1997).

The present communication reports the occurrence and partial purification of MIPS from pteridophytes. Table-1 depicts the presence of this enzyme from different pteridophytes. The reproductive parts show a higher titre of activity which is an universal feature of all life forms (Maeda and Eisenberg Jr. 1980, Dasgupta *et al.* 1984, Gumber *et al.* 1984, Majumder and Biswas 1973a, Donahue and Henry 1981a,b, Loewus and Loewus 1980). The enzyme from *D. glaucum* was highly specific for G-6-P. Though the enzyme exhibits its optimal



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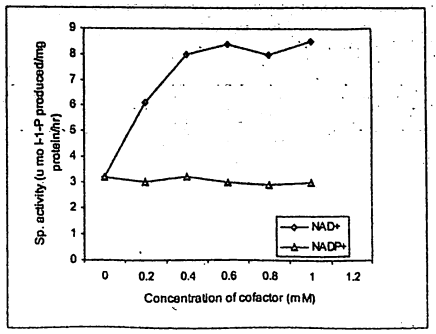
→ Fig. 4. Effect of NAD^+ and $NADP^+$ on L-*myo*-inositol-1--phosphate synthase activity in *D. glaucum*



activity in presence of co-enzyme NAD^+, still it could maintain about one third of the total activity when NAD^+ was not added externally. This emboldens us to conclude the presence of bound NAD^+ in the molecular architecture of this enzyme which has also been reported earlier (Barnett *et al.* 1970, Pittner and Hoffman-Ostenhof 1976, Dasgupta *et al.* 1984). MIPS is involved in the metabolic utilization of G-6-P and it also generates Ribulose-5-phosphate as a product. The activity of this enzyme also seems to be related to that of Fructose-1,6-bisphosphatase and coupled with this are myriad of biochemical activities of the phosphate esters of *myo*-inositol (Joseph *et al.* 1989, Drobak 1992, Martinoia *et al.* 1993, Huang *et al.* 1994, Murthy 1996, Voglmaier *et al.* 1997, Ogawa 1999). All these points towards a central role played by this enzyme in metabolism and make this study worthwhile in understanding the basic metabolism in plants (Loewus *et al.* 1978, Verma and Dougall 1979, De and Biswas 1979, Culbertson and Henry 1975, RayChoudhuri and Majumder 1996, Iqbal *et al.* 2002).

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