L-*myo*-INOSITOL-1-PHOSPHATE SYNTHASE FROM *MARCHANTIA NEPALENSIS*: PARTIAL PURIFICATION AND PROPERTIES

D. R. Chhetri[1]*, S. Yonzone[1], A. K. Mukherjee[2], J. Adhikar[3]

[1]Plant Physiology and Biochemistry Laboratory, Post Graduate Department of Botany, Darjeeling Government College, Darjeeling-734 101, WB, India

[2]Department of Biotechnology, Bengal College of Engineering and Technology Bidhan Nagar, Durgapur-713 212, WB, India

[3]Biochemistry Laboratory, Department of Botany, Presidency College, College Street, Kolkata, WB, India

Received 12 December 2006

Summary. For the first time the enzyme, L-*myo*-inositol-1-phosphate synthase has been partially purified to about 33-fold with approximately 21% recovery from the reproductive part bearing thallus of *Marchantia nepalensis*. The bryophytic synthase specifically utilized D-glucose-6-phosphate and NAD^+ as a substrate and co-factor, respectively. It showed a pH optimum between 7.0 and 7.5 while the temperature optimum was 30ºC. The enzyme activity was slightly stimulated by Mg^{2+} and Ca^{2+}, remarkably stimulated by NH_4^+, slightly inhibited by Mn^{2+} and highly inhibited by Cu^{2+}, Zn^{2+} and Hg^{2+}. The K_m values for D-glucose-6-phosphate and NAD^+ were found to be 0.42 and 0.05 mM, respectively. The V_{max} values were 2.1 and 1.11 mM for D-glucose-6-phosphate and NAD^+, respectively.

Keywords: Bryophytes, inositol synthase, L-*myo*-inositol-1-phosphate synthase, *Marchantia nepalensis*, *myo*-inositol

Abbreviations: G-6-P - D-glucose-6-phosphate, I-1-P - Inositol-1-phosphate, ME - 2-mercaptoethanol, MIPS - L-*myo*-inositol-1-phosphate synthase

* Corresponding author, e-mail**:** munal@sancharnet.in

INTRODUCTION

Inositols are 6-carbon cyclohexane cyclitols found ubiquitously in biological kingdom**.** The essential role of inositol in many cellular processes including membrane formation, cell wall biogenesis, stress response and signal transduction has been well documented (Lackey et al., 2003)**.** *Myo*-inositol is the precursor of all inositol containing compounds including phosphoinositides, inositol phosphates and cell wall polysaccharides. It is formed by the conversion of D-glucose-6-phosphate (G-6-P) to L-*myo*-inositol-1-phosphate (I-1-P) catalyzed by the enzyme L-*myo*-inositol-1-phosphate synthase (MIPS; EC 5.5.1.4). I-1-P is subsequently dephosphorylated to *myo*-inositol. The MIPS reaction has been reported in archea (Chen et al., 2000), bacteria (Bachhawat and Mande, 1999, 2000), protozoa (Lohia et al., 1999), animals (Maeda and Eisenberg, 1980; Mauck et al., 1980; Biswas et al., 1981), humans (Adhikari and Majumder, 1988). Among plants the occurrence of MIPS has been described and characterized in algae (Dasgupta et al., 1984; RayChaudhuri et al., 1997), fungi (Donahue and Henry, 1981; Escamilla et al., 1982; Dasgupta et al., 1984), pteridophytes (Chhetri et al., 2005, 2006), gymnosperm (Gumber et al., 1984; Chhetri and Chiu, 2004) and angiosperm (Loewus and Loewus, 1971; Johnson and Sussex, 1995; Johnson and Wang, 1996; RayChaudhuri et al., 1997). The present study is the first report on the partial purification and characterization of MIPS from *Marchantia nepalensis* which is one of the commonly occurring bryophytes in Eastern Himalayas.

MATERIALS AND METHODS

Plant material

Fresh specimens of *Marchantia nepalensis* Lehm. and Lindb. were collected from their natural habitat in and around Darjeeling hills (ca 2134 m asl) situated between 87º 59′ - 88º 53′ E and 26º 31′ - 27º 13′ N in Eastern Himalayas.

Extraction and partial purification of MIPS from *Marchantia nepalensis*

MIPS was extracted from *Marchantia nepalensis* and partially purified according to the method of Chhetri et al. (2005) with some modifications. The reproductive part bearing *Marchantia nepalensis* thallus (50 g) was collected fresh in the morning, washed twice with cold, sterile distilled water and homogenized in a chilled mortar and pestle in half the volume of 50 mM tris-acetate buffer (pH 7.5) containing 0.2 mM ME. The crude homogenate was passed through four layers of muslin and the liquid was centrifuged at 1,000*g* for 5 min. The supernatant was again centrifuged at 11,400*g* for 20 min and the resulting supernatant collected again, dialyzed overnight against 50 mM tris-acetate buffer (pH 7.5) containing 0.2 mM ME. The clear super-

natant was recovered from the dialysis bag (11,400*g* supernatant) and used as the enzyme source for the initial screening experiments. The 11,400*g* supernatant was subjected to streptomycin sulphate treatment at a final concentration of 2% (w/v) with constant stirring. The mixture was kept in ice-bucket at 0°C for 15 min and then centrifuged at 11,400*g* for 15 min. The supernatant (streptomycin sulphate-treated fraction) was collected and made 0-60% saturated by slowly adding ammonium sulphate. The precipitated protein fraction was dissolved in a minimal volume of tris-acetate buffer (pH 7.5) containing 0.2 mM ME and dialyzed against the same buffer. The dialyzed fraction (ammonium sulphate-treated fraction) was adsorbed for 3 h on DEAE-cellulose (pre-equilibrated with the extraction buffer) and the preparation was loaded in a 8 x 1.2 cm glass column. The column was washed with the extraction buffer and the adsorbed proteins were eluted from the column with a linear gradient of 0 to 0.5 M KCl in 60 ml extraction buffer. Fractions (2.0 ml) were collected at an interval of 8 min. The enzyme was eluted using KCl concentrations between 0.22 to 0.27 M (Fig. 1). The active DEAE-cellulose purified synthase (DEAE-cellulose fraction) was further purified by molecular sieve chromatography on a Sephadex G-200 column (7.5 x 0.8 cm) pre-equilibrated with the extraction buffer. The enzyme was eluted from the column with the same buffer. Fractions of 0.75 ml were collected at

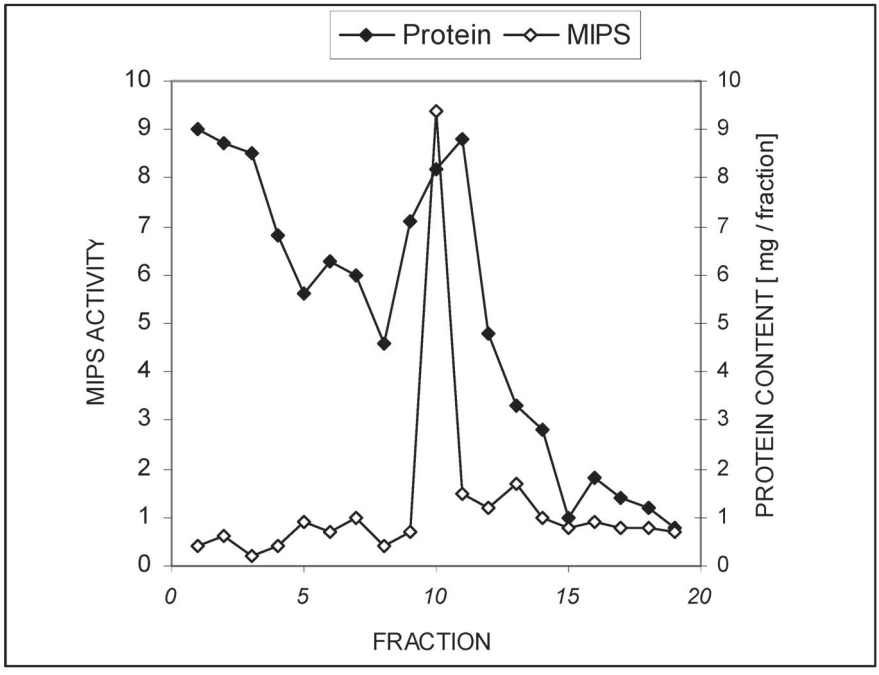

Fig. 1. Elution profile of *Marchantia nepalensis* MIPS on DEAE-cellulose column. MIPS activity is expressed as (μmol (I-1-P) fraction^{-1} h^{-1}).

a flow rate of 10 min fraction^{-1}. The active Sephadex G-200 purified fractions were pooled together (Sephadex G-200 fraction), concentrated and used as the ultimate preparation in this experiment.

Assay of MIPS activity

The MIPS activity was assayed by the procedure of Barnett et al. (1970) with some modifications (Adhikari et al., 1987). The assay mixture contained 50 mM tris-acetate buffer (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 0.5 ml. After incubation at 37 ºC for 1h, the reaction was terminated by the addition of 0.2 ml 20 % chilled TCA. An equal volume of 0.2 M $NaIO_4$ was added to the deproteinized supernatant (0.7 ml) followed by a second incubation at 37 ºC for 1h for the oxidation of the MIPS reaction product, *myo*-insositol-1-phosphate, with a concomitant release of inorganic phosphate. The excess periodate was destroyed by 1M 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 the MIPS reaction. Inorganic phosphate was determined by the method of Chen et al. (1956). 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 the fractions obtained from column chromatography was determined by measuring absorbance at 280 nm.

RESULTS

Purification of the enzyme

MIPS was isolated and purified from freshly collected *M. nepalensis* reproductive thallus employing the techniques of low speed centrifugation, streptomycin sulphate precipitation, ammonium sulphate fractionation, ion-exchange chromatography through DEAE-cellulose and molecular sieve chromatography through Sephadex G-200. The scheme of the purification procedure of MIPS is given in Table 1. The chromatographic profiles of proteins resolved from ammonium sulphate fraction of the reproductive thallus of *M. nepalensis* are shown in Fig. 1 and 2. In the present study, about 33-fold overall purification of the enzyme with about 21% recovery based on enzyme total activity was achieved.

Characterization of the purified enzyme

Table 1. Typical example of partial purification of L-*myo*-inositol-1-phosphate synthase from reproductive part bearing thallus of *Marchantia nepalensis* (50 g). Data are means ± SE.

Fraction	Total protein (mg)	Specific activity [µmol (L-*myo*-inositol-1-phosphate) mg^{-1} (protein)h^{-1}]	Total activity [µmol (L-*myo*-inositol-1-phosphate) h^{-1}]	Recovery [%]	Purification [fold]
Homogenate	129.6 ± 7.53	0.16 ± 0.02	20.73 ± 1.61	100.00 ± 10.02	1.00 ± 0.06
11,400g supernatant	112..0 ± 5.11	0.18 ± 0.02	20.16 ± 2.03	97.25 ± 7.28	1.12 ± 0.13
Streptomycin sulfate treated fraction	47.2 ± 1.51	0.33 ± 0.03	15.56 ± 0.82	75.06 ± 6.10	2.06 ± 0.09
0-60 % ammonium sulfate fraction	22.4 ± 3.45	0.68 ± 0.12	15.23 ± 1.92	73.47 ± 3.38	4.25 ± 0.78
DEAE-cellulose fraction	9.6 ± 1.15	1.5 ± 0.36	14.40 ± 0.80	69.46 ± 5.01	9.37 ± 1.32
Sephadex G- 200 fraction	0.84 ± 0.03	5.22 ± 0.20	4.38 ± 0.86	21.15 ± 2.76	32.62 ± 3.85

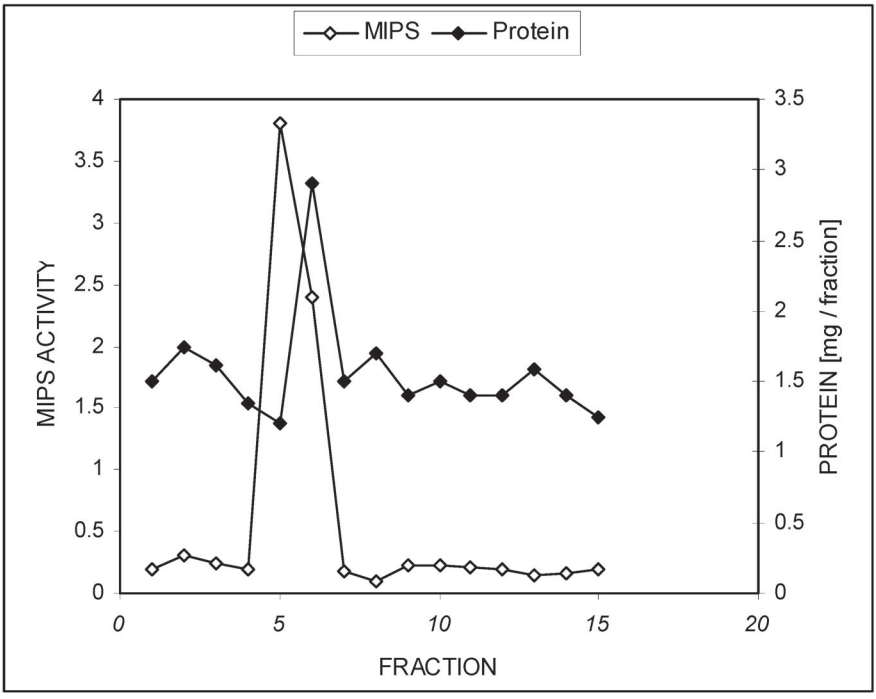

Fig. 2. Elution profile of *Marchantia nepalensis* MIPS on Sephadex G-200 column. MIPS activity is expressed as (mol (I-1-P) $fraction^{-1}$ h^{-1}).

Table 2. Effect of the composition of the incubation medium on *Marchantia nepalensis* L-*myo*-inositol-1-phosphate synthase activity. Data are means ± SE.

Conditions	Specific activity [µmol (L-*myo*-inositol-1-phosphate) mg^{-1}(protein)h^{-1}]	Percent activity
Complete set	11.86 ± 0.89	100.00 ± 11.07
Without substrate (G-6-P)	0.0	0.0
Without buffer (tris-acetate)	8.1 ± 0.15	68.72 ± 3.55
Without co-factor (NAD^+)	3.7 ± 0.10	31.19 ± 2.26
Without NH_4Cl	4.0 ± 0.45	33.75 ± 1.81
Without 2-mercaptoethanol	6.2 ± 0.43	52.27 ± 4.54
Heat-killed enzyme	0.0	0.0

The maximal activity of MIPS was recorded when assayed in the presence of 50 mM Tris-acetate buffer (pH 7.5), 14 mM NH_4Cl, 0.8 mM NAD^+, 5 mM ME and 5 mM G-6-P (Table 2). If the specific substrate (G-6-P) was not added in the incubation mixture, the enzymatic synthesis of L-*myo*-inositol -1-phosphate could not be detected. The K_m and V_{max} values for G-6-P, as determined by Lineweaver-Burk plot were 0.42 and 2.1 mM, respectively. About 31% activity was lost when Tris-buffer was omitted from the reaction mixture. Deduction of NAD^+ (co-enzyme) resulted in the loss of enzyme activity by about 69% while the increase in the co-enzyme concentration up to 0.4 mM resulted in an enhancement of enzyme activity. K_m of NAD^+ was 0.05 while V_{max} was 1.11 mM as determined by the Lineweaver-Burk double reciprocal plot. The absence of either ammonium ions or ME decreased the enzyme activity to about 34% and 52%, respectively as compared to the complete set.

MIPS stability varied at the different stages of purification. While the 11,400*g* supernatant remained active for 14-15 days when stored at -20ºC, the Sephadex G-200 purified fractions maintained their activity only up to 5-7 days when stored at identical temperatures. However, repeated freezing and thawing resulted in a remarkable loss of activity. The addition of enzyme stabilizer, 2-mercaptoethanol (ME) or dithiothreitol (DTT) increased considerably the activity of the enzyme. Enzyme activity linearity of *M. nepalensis* MIPS was observed up to 250 g of protein concentration under standard assay conditions (Fig. 3). The temperature optimum was found to be 30ºC and the enzyme was most active within a pH range of 7.0 to 7.5. K^+ had little effect on the *M. nepalensis* MIPS activity and Na^+ played a minor inhibitory role. Among the divalent cations Ca^{2+} and Mg^{2+} slightly stimulated while Cu^{2+}, Zn^{2+} and Hg^{2+} strongly inhibited the enzyme activity with Hg^{2+} acting as the strongest inhibitor (Table 3).

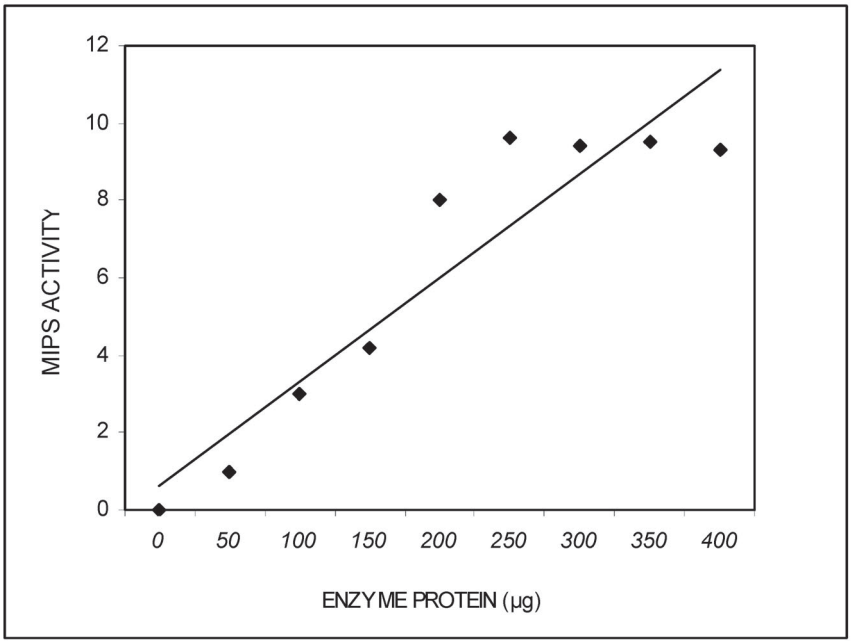

Fig. 3. Effect of different enzyme concentrations on *Marchantia nepalensis* MIPS. MIPS activity is expressed as (mol (I-1-P) mg^{-1}(protein) h^{-1}).

Table 3. Effect of monovalent and divalent cations on *Marchantia nepalensis* L-*myo*-inositol-1-phosphate synthase activity. Data are means ± SE.

Cation	Concentration	Specific activity mg^{-1}(protein)h^{-1}]	Percent activity
Control	0	6.79 ± 0.60	100.00 ± 4.40
K^+	5	7.13 ± 0.70	105.00 ± 5.29
Na^+	5	5.85 ± 0.42	86.17 ± 7.86
NH_4^+	5	10.76 ± 0.62	158.46 ± 9.11
Mg^{2+}	5	7.50 ± 0.37	110.45 ± 8.00
Mn^{2+}	5	5.62 ± 0.52	82.76 ± 6.38
Ca^{2+}	5	7.87 ± 0.21	115.90 ± 5.93
Zn^{2+}	5	3.25 ± 0.10	47.86 ± 4.12
Cu^{2+}	5	3.50 ± 0.78	51.54 ± 1.99
Hg^{2+}	5	1.25 ± 0.11	18.40 ± 2.12

DISCUSSION

MIPS has been reported by several authors for almost all groups of plants. Although there is one report regarding the occurrence of the enzyme in some bryophytes (Dasgupta et al., 1984), no report is so far available regarding the purification and functional characterization of MIPS in bryophytes. The present study fills that void towards making a continuous line of information regarding the purification and characterization of MIPS from the most primitive to the most highly evolved organisms. Here we report for the first time the partial purification and characterization of MIPS from *M. nepalensis.* The enzyme isolated from *M. nepalensis* did not show any activity in the absence of its specific substrate G-6-P. Though the enzyme exhibited its optimal activity in the presence of co-enzyme NAD^+, still it could maintain about 31% of the total activity in case NAD^+ was not added externally. This encourages 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 Hoffmann Ostenhof, 1976).

Like in all other eukaryotes, MIPS from *M. nepalensis* required NH_4^+ for its optimal activity in contrast to the divalent cations required for MIPS from prokaryotes (Majumder et al., 2003). This indicates that the bryophytic MIPS is a type–III aldolase. Among the cations, Na^+ and Mn^{2+} were mild inhibitors, Ca^{2+} and Mg^{2+} were mild stimulators and Cu^{2+}, Zn^{2+} and Hg^{2+} were strong inhibitors of *M. nepalensis* MIPS. Thus, the cations can be arranged in the order $Hg^{2+}>Zn^{2+}>Cu^{2+}$with Hg^{2+} reducing the enzyme activity to about 18%. The narrow pH optimum (7.0-7.5) for *M. nepalensis* MIPS was quite similar to that reported for other materials (Donahue and Henry, 1981; Dasgupta et al., 1984; Adhikari and Majumder, 1988; Lohia et al., 1999). The optimum temperature for *M. nepalensis* MIPS (30^{o}C) is slightly less as compared to that for MIPS from *Spirulina platensis*, *Euglena gracilis*, *Oryza sativa* (RayChaudhuri et al., 1997), *Entamoeba histolytica* (Lohia et al., 1999), *Streptomyces griceus* (Sipos and Szabo, 1989) but similar to that from *Gleichenia glauca* (Chhetri et al., 2005).

MIPS isolated from the bryophyte *Marchantia nepalensis* showed approximately 50% less activity as compared to MIPS from the pteridophyte *Diplopterygium glaucum* (Chhetri et al., 2006). The affinity of the bryophytic enzyme to the substrate (G-6-P) and the co-factor (NAD^+) was much higher compared to the pteridophytic enzyme based on the Michaelis constants. On the other hand, MIPS from the above sources showed some similarities. Both MIPS were highly stimulated by NH_4^+, slightly inhibited by Na^+, unaffected by K^+ and strongly inhibited by Hg^{2+}. Both enzymes showed also common pH optima (7.0 - 7.5).

MIPS is involved in the metabolic utilization of G-6-P and generates ribulose-5-phosphtat. It is also related to the activity of fructose-1,6-bisphosphatase as well as

different phosphate esters of *myo*-inositol (Murthy, 1996). The presence of numerous cellular compartments and genetic loci for MIPS indicates the role of this enzyme in the regulation of metabolic flux of inositol (Lackey et al., 2003). Recent studies by other workers have focused on the molecular cloning of the MIPS gene (Ju et al., 2004; Park and Kim, 2004; Majee et al., 2004), its crystal structure determination (Stein and Geiger, 2002; Norman et al., 2002), its role in salinity tolerance (Nelson et al., 1998; Majee et al., 2004), bipolar disorder (Agam et al., 2002), carcinogenesis (Nishino et al., 1999; Wattenberg 1999) and diabetes (Suzuki et al., 1998). Therefore, further investigations on *myo*-inositol and MIPS from different organisms will be of fundamental importance to understand the nature and activity of this enzyme across phylogenetic groups.

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