

L-*myo*-Inositol-1-phosphate synthase from bryophytes: purification and characterization of the enzyme from *Lunularia cruciata* (L.) Dum.

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ABSTRACT

L-*myo*-inositol-1-phosphate synthase (MIPS; EC: 5.5.1.4) catalyzes the conversion of D-glucose-6-phosphate to 1L-*myo*-inositol-1-phosphate, the rate limiting step in the biosynthesis of all inositol containing compounds. *Myo*-inositol and its derivatives are implicated in membrane biogenesis, cell signaling, salinity stress tolerance and a number of other metabolic reactions in different organisms. This enzyme has been reported from a number of bacteria, fungi, plants and animals. In the present study some bryophytes available in the Eastern Himalaya have been screened for free *myo*-inositol content. It is seen that *Bryum coronatum*, a bryopsid shows the highest content of free *myo*-inositol among the species screened. Subsequently, the enzyme MIPS has been partially purified to the tune of about 70 fold with approximately 18% recovery from the reproductive part bearing gametophytes of *Lunularia cruciata*. The *L. cruciata* synthase specifically utilized D-glucose-6-phosphate and NAD⁺ as its substrate and co-factor respectively. The optimum pH shown was 7.0 while the temperature maximum was at 30 °C. The enzyme activity was slightly stimulated by Mg²⁺ and Ca²⁺; remarkably stimulated by NH₄⁺; slightly inhibited by Mn²⁺; highly inhibited by Cu²⁺, Zn²⁺ and Hg²⁺. The *K_m* values for D-glucose-6-phosphate and NAD⁺ was found to be 0.80 and 0.034 mM respectively while the *V_{max}* values were 2.8 and 1.21 mM for D-glucose-6-phosphate and NAD⁺ respectively.

Key words: D-glucose-6-phosphate, inositol monophosphatase, inositol synthase, *myo*-inositol, L-*myo*-inositol-1-phosphate,

Abbreviations: G-6-P = D-glucose-6-phosphate, I-1-P = Inositol-1-phosphate

ME = 2-mercaptoethanol, MIPS = L-*myo*-inositol-1-phosphate synthase

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 have 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 (Biswas et al., 1984; Loewus and Murthy, 2000). The *de-novo* synthesis of *myo*-inositol takes place by the conversion of D-glucose-6-phosphate (G-6-P) to L-*myo*-inositol-1-phosphate (I-1-P) by the enzyme L-*myo*-inositol-1-phosphate synthase (MIPS; EC 5.5.1.4) which is subsequently dephosphorylated by a specific

Mg⁺² dependent inositol monophosphatase to *myo*-inositol. The MIPS reaction has been reported from 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) and plants. Among plants the occurrence of MIPS has been described and characterized from algae (Dasgupta et al., 1984; RayChaudhuri et al., 1997); fungi (Donahue and Henry, 1981a,b; Escamilla et al. 1982; Dasgupta et al. 1984); pteridophytes (Chhetri et al. 2005, 2006a); 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 detailing the partial purification and characterization of MIPS from a bryophyte, *Lunularia cruciata* occurring in the Darjeeling hills of Eastern Himalayas.

MATERIALS AND METHODS

Plant material: Fresh specimens of bryophytes like *Marchantia nepalensis* Lehm. & Lindenb., *Lunularia cruciata* (L.) Dum., *Asterella tenella* (L.) Beauv., *Notothylas indica* Kash. and *Bryum coronatum* Schwaegr were collected from the localities in and around Darjeeling hills (circa 2134 m amsl.) situated between 87°59' - 88°53' E and 26°31' - 27°13' N in the Eastern Himalaya of India.

Free *myo*-inositol determination: Free *myo*-inositol was isolated by the method of Charalampous and Chen (1966). The extracted sample was passed through a mixed bed column of Dowex-1-Cl (100-200 mesh) and Amberlite IR-120 (Na-form) and the free *myo*-inositol was ultimately isolated by one dimensional descending chromatography through Whatman No.1 paper. The content of free *myo*-inositol was estimated spectrophotometrically (Gaitonde and Griffiths, 1966) using a standard curve prepared using known concentrations of pure *myo*-inositol.

Extraction and partial purification of MIPS from *Lunularia cruciata*: Reproductive part bearing *Lunularia cruciata* 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 (pH 7.5) buffer 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 centrifuged at 11,400×g for 20 min and the resulting supernatant was collected again, dialyzed overnight against 50 mM tris-acetate (pH 7.5) buffer containing 0.2 mM ME and the clear supernatant recovered from the dialysis bag (11,400×g supernatant) was used as the enzyme source for the initial screening experiments. The 11,400×g supernatant from *L. cruciata* 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 min and then centrifuged at 11,400×g for 15 min. The supernatant (streptomycin sulphate treated fraction) was collected which and was made 0-60% saturated by slowly adding ammonium sulphate. The precipitated protein fraction was dissolved in minimal volume of tris-acetate buffer (pH 7.5) containing 0.2 mM ME and dialyzed against the same buffer with one change. 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 8×1.2 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 cm³ extraction buffer. Fractions (2.0 cm³) were collected at an interval of 8 minutes. The enzyme was eluted between KCl concentrations of 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 (7.5×0.8 cm) pre-equilibrated with the extraction buffer and the enzyme was eluted from the column with the same buffer. Fractions of 0.75 cm³ were collected at 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: The MIPS activity was assayed by 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 NH₄Cl, 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 cm³. After incubation at 37 °C for 1h, the reaction was terminated by 0.2 cm³ of 20 % chilled TCA. An equal volume of 0.2 M NaIO₄ was added to the deproteinized supernatant (0.7 cm³) followed by a second incubation at 37 °C for 1h for the oxidation

of MIPS reaction product, *myo*-inositol-1-phosphate, with concomitant release of inorganic phosphate. The excess of periodate was destroyed by 1M Na₂SO₃. Simultaneously, appropriate non-periodate controls, in which NaIO₄ and Na₂SO₃ 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). The inorganic phosphate released was quantified with a standard curve prepared using K₂HPO₄. 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 determined by measuring absorbance at 280 nm.

RESULTS

Determination of free myo-inositol from bryophytes:

Appreciable quantity of free *myo*-inositol (the final product of *myo*-inositol biosynthesis) was detected from vegetative and reproductive parts of different bryophytic species (Table 1). It was revealed that the quantities of free *myo*-inositol in almost all plant parts were moderately high. Free *myo*-inositol content was detected in relatively large quantities in the reproductive part bearing thallus of *Marchantia nepalensis*, *Bryum coronatum* and *Notothylas indica* while the same in the vegetative thallus of *Bryum coronatum* was also noteworthy (Table 1). Different inositol derivatives are known to be essential for all life forms (Majumder et al., 2003) especially in the formation of sex units. Hence, the detection of free *myo*-inositol in these bryophytes with higher content of the same in the reproductive parts is justified.

Table 1. Distribution of free *myo*-inositol in vegetative and reproductive structures of different bryophytic species (values are mean \pm SE), FW = fresh weight.

Class	Family	Plant species	Plant part	Free <i>myo</i> -inositol content (mg/g FW)
Hepaticopsida	Marchantiaceae	<i>Marchantia nepalensis</i>	Vegetative	1.23 \pm 0.10
			Reproductive	2.88 \pm 0.23
Hepaticopsida	Marchantiaceae	<i>Lunularia cruciata</i>	Vegetative	0.50 \pm 0.10
			Reproductive	0.80 \pm 0.09
Hepaticopsida	Aytoniaceae	<i>Asterella tenella</i>	Vegetative	1.52 \pm 0.14
			Reproductive	1.00 \pm 0.17
Anthocerotopsida	Notothylaceae	<i>Notothylas indica</i>	Vegetative	0.55 \pm 0.04
			Reproductive	2.00 \pm 0.20
Bryopsida	Bryaceae	<i>Bryum coronatum</i>	Vegetative	2.10 \pm 0.11
			Reproductive	2.25 \pm 0.22

Purification of the enzyme: The enzyme MIPS was isolated and purified from the reproductive thallus of freshly collected *L. cruciata* 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 summary of the purification of MIPS

is given in Table 2. Chromatographic profiles of proteins resolved from ammonium sulphate fraction of the reproductive thallus of *L. cruciata* are shown in Figures 1 and 2. An overall purification of the enzyme to about 70 fold with about 18 % recovery based on total activity could be achieved in the present study.

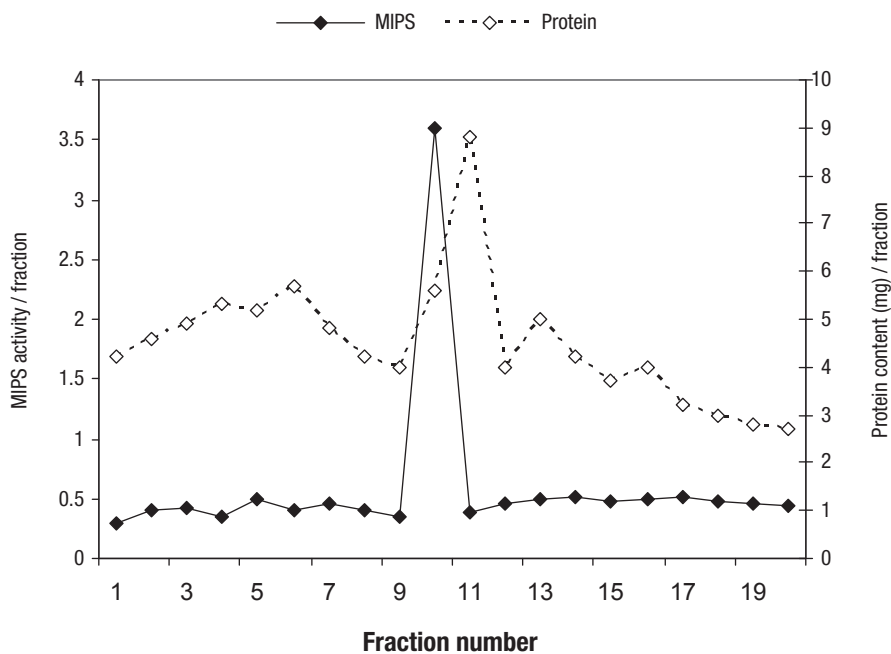


Figure 1. Elution profile of *Lunularia cruciata* MIPS on DEAE-cellulose column. MIPS activity is expressed as $[\mu\text{mol (I-1-P) produced fraction}^{-1} \text{ h}^{-1}]$.

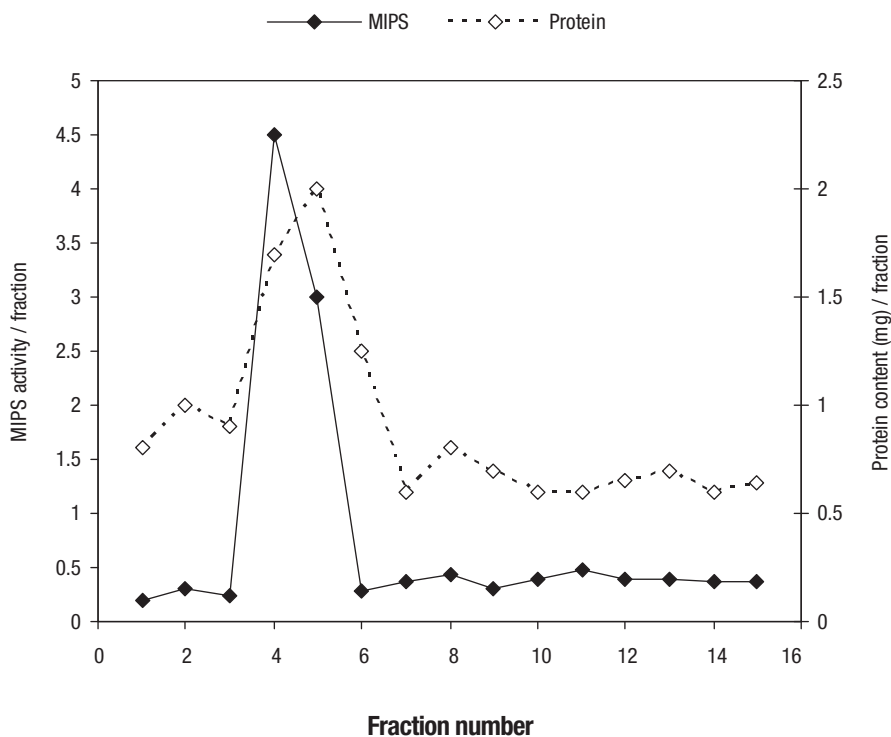


Figure 2. Elution profile of *Lunularia cruciata* MIPS on Sephadex G-200 column. MIPS activity is expressed as $[\mu\text{mol (I-1-P) produced fraction}^{-1} \text{ h}^{-1}]$.

Characterization of the purified enzyme: The *L. cruciata* MIPS when assayed in presence of 50 mM tris acetate buffer (pH 7.5), 14 mM NH₄Cl, 0.8 mM NAD⁺, 5 mM ME and 5 mM G-6-P recorded maximal activity (Table 3). When 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. Deduction of NAD⁺ (co-enzyme) resulted in the loss of enzyme activity by about 60%. In comparison, the deduction of NAD⁺ resulted in the loss of enzyme activity by 70% in *Euglena gracilis* (Dasgupta *et al.* 1984). About 25 % activity was lost when tris-buffer was omitted from the reaction mixture. Absence of either ammonium ion or ME decreased the enzyme activity to about 62 % and 73 % respectively, as compared to the complete set.

Kinetic studies were carried out using G-6-P (substrate) in the range of 0-8 mM. The reaction rate was found to increase linearly with respect to G-6-P up to a concentration of 4mM. The K_m value for G-6-P, as determined by Lineweaver-Burk plot was 0.80 mM which is comparable to the same for the pteridophytic enzyme having a value of 0.83 (Chhetri *et al.*, 2006a). The V_{max} value of this bryophytic enzyme was calculated as 2.80 mM as against 1.6 mM for the Yeast enzyme (Donahue and Henry, 1981b) and 1.42 mM for the pteridophytic enzyme (Chhetri *et al.* 2006a). Though this value differs widely from other plant species, it corresponds to the V_{max} value of 2.95 reported for *Taxus baccata* (Chhetri and Chiu, 2004). Between concentrations of 0-1.0 mM of NAD⁺ (co-enzyme) the increase in co-enzyme concentration up to 0.5 mM resulted in the enhancement of enzyme activity. The K_m of NAD⁺ was determined as 0.03 which was quite different from those recorded for the enzyme from other sources *e.g.* 8.00 mM for the Yeast enzyme (Donahue and Henry, 1981b) and 0.44 for the pteridophytic enzyme (Chhetri *et al.* 2006a). The V_{max} value of NAD⁺ for the *L. cruciata* MIPS was found to be 1.21 mM which is comparable to that of yeast having a value of 1.14 mM (Donahue and Henry, 1981b) but different from those of pteridophytic enzyme which exhibits a V_{max} value of 1.80 mM (Chhetri *et al.*, 2006a).

Stability of the MIPS enzyme varied at different stages of purification. While the 11,400×g supernatant remained active for 8-10 days when stored at -20°C, the Sephadex G-200 purified fractions maintained its activity only up to 3-4 days when stored at identical temperature. However, repeated

freezing and thawing resulted in remarkable loss of activity. Addition of enzyme stabilizer, 2-mercaptoethanol (ME) or dithiothreitol (DTT) considerably increased the activity of the enzyme.

Enzyme activity linearity of *L. cruciata* MIPS was seen up to 300 µg of protein concentration under standard assay conditions (Fig. 3). The temperature maximum was found to be at 30°C which is slightly low as the enzymes from other sources are optimally active between 35° and 37 °C (RayChoudhuri *et al.*, 1997). The *L. cruciata* enzyme exhibited a pH optima of 7.0 which is too lower as compared to that of other plant species like *Spirulina platensis*-7.8, *Euglena gracilis*-8.2 (RayChoudhuri *et al.* 1997) and *Acer pseudoplatanus*-8.0 (Loewus and Loewus, 1971).

Effect of different metal ions on *L. cruciata* MIPS activity was tested in 5 mM concentrations using chloride salts of metal ions. Among monovalent cations tested, K⁺ had little effect and Na⁺ played an inhibitory role while NH₄⁺ was an appreciable stimulator of the enzyme. NH₄⁺ stimulation of the enzyme was to the tune of 1.4 times in contrast to the *Acer pseudoplatanus* (Loewus and Loewus, 1971) MIPS which is stimulated by 2.3 times with NH₄⁺. Among the divalent cations it was found that Ca²⁺ and Mg²⁺ slightly stimulated; Mn²⁺ slightly inhibited, Cu²⁺, Zn²⁺ and Hg²⁺ strongly inhibited the enzyme activity with Hg²⁺ acting as the strongest (80%) inhibitor (Table 4).

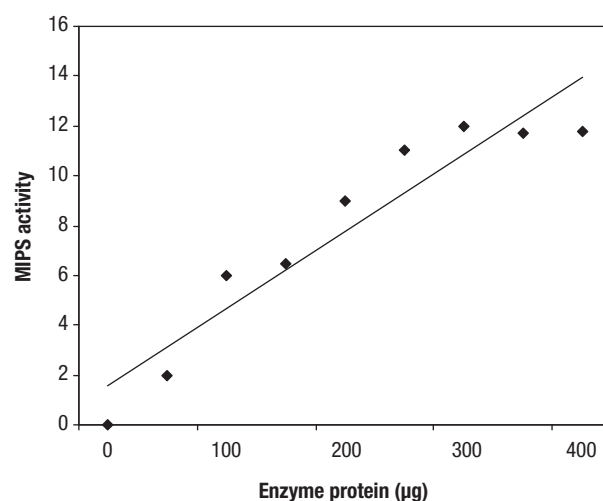


Figure 3. Effect of different enzyme concentration on *Lunularia cruciata* MIPS. MIPS activity is expressed as [µmol (l-1-P) produced mg⁻¹(protein) h⁻¹].

Table 2. Summary of partial purification of L-*myo*-inositol-1-phosphate synthase from reproductive part bearing thallus of *Lunularia cruciata* (values are mean \pm SE).

Fraction	Total protein [mg]	Specific activity		Recovery [%]	Purification [fold]
		$[\mu\text{ mol (L-}myo\text{-inositol-1-phosphate) mg}^{-1}\text{(protein)h}^{-1}]$	Total activity $[\mu\text{ mol (L-}myo\text{-inositol-1-phosphate) h}^{-1}]$		
Homogenate	204.4 \pm 9.71	0.12 \pm 0.01	24.52 \pm 3.51	100.00 \pm 7.63	1.00 \pm 0.08
11,400 \times g supernatant	130.0 \pm 5.40	0.15 \pm 0.02	19.50 \pm 1.01	79.52 \pm 2.40	1.25 \pm 0.14
Streptomycin sulfate treated fraction	79.2 \pm 4.37	0.22 \pm 0.01	17.42 \pm 1.80	71.05 \pm 5.02	1.83 \pm 0.53
0-60 % ammonium sulfate fraction	12.88 \pm 1.42	1.17 \pm 0.08	15.06 \pm 1.56	61.41 \pm 2.58	9.75 \pm 1.20
DEAE-cellulose fraction	1.8 \pm 0.20	6.96 \pm 0.68	12.52 \pm 1.12	51.06 \pm 2.69	58.00 \pm 2.16
Sephadex G-200 fraction	0.52 \pm 0.06	8.41 \pm 0.30	4.37 \pm 0.67	17.82 \pm 2.30	70.08 \pm 2.81

Table 3. Effect of composition of incubation medium on *Lunularia cruciata* L-*myo*-inositol-1-phosphate synthase activity (values are mean \pm SE).

Condition	Specific activity $[\mu\text{ mol (L-}myo\text{-inositol-1-phosphate) mg}^{-1}\text{(protein)h}^{-1}]$	Percent activity
Complete set	13.1 \pm 0.91	100.00 \pm 7.36
Without substrate (G-6-P)	0.0	0.0
Without buffer (tris-acetate)	9.9 \pm 1.25	75.5 \pm 4.08
Without Co-factor (NAD ⁺)	5.3 \pm 0.14	40.45 \pm 2.44
Without NH ₄ Cl	8.1 \pm 0.56	61.83 \pm 3.77
Without 2-mercaptoethanol	9.6 \pm 0.16	73.28 \pm 3.67
Heat-killed enzyme	0.0	0.0

Table 4. Effect of monovalent and divalent cations on *Lunularia cruciata* L-*myo*-inositol-1-phosphate synthase activity (values are mean \pm SE).

Cation	Concentration [mM]	Specific activity $[\mu\text{ mol (L-}myo\text{-inositol-1-phosphate) mg}^{-1}\text{(protein)h}^{-1}]$	Percent activity
Control	0	8.06 \pm 0.81	100.00 \pm 7.79
K ⁺	5	8.60 \pm 0.71	107.56 \pm 8.76
Na ⁺	5	6.95 \pm 0.54	86.22 \pm 6.91
NH ₄ ⁺	5	11.60 \pm 1.41	143.92 \pm 10.28
Mg ²⁺	5	9.43 \pm 0.69	116.99 \pm 8.87
Mn ²⁺	5	7.60 \pm 0.21	94.29 \pm 8.94
Ca ²⁺	5	9.01 \pm 1.44	111.78 \pm 4.94
Zn ²⁺	5	4.30 \pm 0.65	53.34 \pm 2.32
Cu ²⁺	5	5.08 \pm 0.11	62.87 \pm 1.99
Hg ²⁺	5	1.65 \pm 0.12	20.47 \pm 1.69

DISCUSSION

The present study reports the partial purification and characterization of MIPS for the first time from *L. cruciata*. The enzyme from *L. cruciata* does not show any activity in absence of its specific substrate G-6-P. The enzyme exhibits its optimal activity in presence of co-enzyme NAD⁺ and NAD⁺ could not

be substituted by NADP⁺ at any concentration. However, it could maintain about 40% of the total activity when NAD⁺ was not added externally. This proves the presence of endogenous NAD⁺ in the molecular architecture of this enzyme which has also been reported earlier (Adhikari and Majumder, 1983; Chhetri et al., 2006b).

Like all other eukaryotes, the *L. cruciata* MIPS requires NH_4^+ for its optimal activity in contrast to the divalent cation requiring MIPS of 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 *L. cruciata* MIPS in the order of $\text{Hg}^{2+} > \text{Zn}^{2+} > \text{Cu}^{2+}$ with Hg^{2+} limiting the enzyme activity to about 20%. The strong enzyme inhibition due to heavy metals suggests that one or more free sulphhydryl groups are present within the active site of the enzyme (Nelson and Cox, 2000). The narrow pH optima (7.0-7.5) obtained for *L. cruciata* MIPS is quite similar to the same obtained for the MIPS from other sources (Donahue and Henry, 1981; Dasgupta *et al.*, 1984; Adhikari and Majumder, 1988; Lohia *et al.*, 1999). The optimum temperature for *L. cruciata* MIPS was found to be 30 °C which is slightly less as compared to that from *Spirulina platensis*, *Euglena gracilis*, *Oryza sativa* (RayChaudhuri *et al.*, 1997), but similar to that from *Gleichenia glauca* (Chhetri *et al.*, 2005).

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). Free inositol is channeled for the production of different methylated derivatives, which acts as potent osmolytes for amelioration of oxidative damage during osmotic stress (Bohnert *et al.*, 1995). Increased synthesis of inositol by plants has been observed in salt environment by stress tolerant MIPS protein which is able to function under such stress conditions (Ghosh Dastidar *et al.*, 2006). Induction of the increased production of inositols and its methylated derivatives like ononitol and pinitol have been reported in response to salt stress in several plants (Vernon and Boenert, 1992; Ishitani *et al.* 1996; Sheveleva *et al.*, 1997). Studies by other workers have revealed its direct role in salinity tolerance (Nelson *et al.*, 1998; Majee *et al.*, 2004), desiccation tolerance (Majee *et al.*, 2005) and extremely high temperature tolerance (Chen *et al.*, 1998, Lamosa *et al.*, 2006). Bryophytes being a highly desiccation and drought tolerant plants may prove to be an ideal candidate for fishing stress tolerant genes. Considering the essential roles of inositols, the present study detailing the investigation on the biosynthesis and regulation of *myo*-inositol in bryophyte is of fundamental importance.

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