Comparative characteristics of cytosolic and chloroplastidial D/L- *myo*-Inositol-1-phosphate phosphatase partially purified from *Enteromorpha intestinalis* (L.) Nees (a marine macro alga) grown under high salinity niche

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Abstract

The present investigation describes a protocol for partial purification and basic characterization of one of the key enzymes of *myo*-inositol biosynthesis, D/L- *myo*-Inositol-1-phosphate phosphatase (MIPP), from a marine macro alga (*Enteromorpha intestinalis*) growing under varying salinity conditions of Chilika lagoon (Odisha, India). Cytosolic and chloroplastidial forms of MIPP were partially purified to about 55- and 16- folds respectively following low-speed centrifugation, high-speed centrifugation, 0-80% ammonium sulfate precipitation, successive chromatography through CM-cellulose, Sephadex G-200 and UltrogelAcA 34 columns. The apparent molecular weights of the native cytosolic and plastidial forms of MIPP were determined to be 146 and 148 kDa respectively. Cytosolic and plastidial MIPP were remarkably active within the temperature range of 20-40°C and function within a narrow pH range (7.0-7.5). Using nonlinear regression kinetics, the Km value for D-MIP of the cytosolic MIPP and its plastidial iso-form were 0.07277 mM and 0.07332 mM respectively. Different monovalent as well as divalent cations exhibited variable effects on enzyme activity of either preparation. The activity of MIPP was remarkably found to increase proportionately with the salinity of Chilika lagoon.

Introduction

Myo-Inositol is a 6 carbon cyclohexane hexitol which has a diverse role in plant biology. It is required, either in its free form or in different conjugated forms, as a major component of reproductive units; precursors of storage phosphates in seeds, pollen wall polysaccharides.[1,2] and signal molecules.[3] In addition to these it is also a precursor and substrate of many crucial metabolites in plants.[4] The biosynthesis of this indispensable sugar alcohol is essentially reliant on two enzymes, out of which the first one is a rate limiting NAD^+-dependent oxidoreductase (L-*myo*-Inositol-1-phosphate synthase) and the second one is a Mg^{+2} dependent phosphatase (D/L- *myo*-Inositol-1-phosphate phosphatase).

D/L- *myo*-Inositol-1-phosphate phosphatase (MIPP; EC 3.1.3.25) dephosphorylates *myo*-Inositol-1-phosphate (MIP) in order to maintain the cellular inositol pool which is essential for many metabolic and signalling pathways in plants.[4] It has been reported earlier that many plants accumulate organic osmoprotectants/osmolytes in response to salinity stress and evidence of high level of osmolyte accumulation in response to salinity stress are available for many plant species.[5,6] Thus *myo*- Inositol in its free form has been recognized to play a crucial role in salt tolerance of plants.[5] Taking these facts collectively in consideration, attempts have been made in the present work for a comparative study of purification and characterization between cytosolic and plastidial forms of MIPP after its basic identification from a Chlorophycean marine macro alga *Enteromorpha intestinalis* which is found to grow profusely in Chilika lagoon throughout all seasons under different salinity regimes.

The MIPP reaction has been reported from several prokaryotic as well as eukaryotic organisms such as *Escherichia coli*,[7] *Synechocystis* sp,[8] yeast,[9] vascular cryptogams,[10,11] higher plants[12,13] and animals.[14-16] It is noteworthy to mention here that although the inositol biosynthetic pathway is necessarily functional in several plant groups, supporting evidence are primarily based only on purification and characterization of MIPS but extensive information regarding MIPP is still very meagre. Perusal of available literature clearly reveals that although MIPP activity has been previously reported from some flowering plant species[12,13] and also from some vascular cryptogams[10,11] so far no work has been carried out regarding its activity in non-vascular cryptogams, particularly its role in salt-stress physiology. Thus the present investigation attempts to bridge the existing gap towards understanding the basic regulatory principles underlying inositol biosynthesis in the marine macro algae *E. intestinalis* as a model.

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Contributions: SBa carried out the responsibility of plant material collection, identification of the plant sample, took part in some characterization experiments, determination of PAGE profile and preparation of the manuscript. AB carried out the experiments of MIPP purification, partial characterization of the enzyme and estimated free myo-inositol contents. DSM carried out some experiments of partial characterization. SBh carried out some experiments of partial characterization. JA designed the entire project, isolated chloroplasts, performed Kinetic data analysis and assisted in manuscript preparation.

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Materials and Methods

Plant material

Experimental macro alga *Enteromorpha intestinalis* (L.) Nees, was collected from Rambha, Chilika lagoon, Odisha (geographic location: 19° 50' N and 85 °30' E), India. The samples were kept frozen under -20°C until use.

Isolation of chloroplasts

Chloroplasts of *Enteromorpha intestinalis* were isolated following the method of Hachtel (1976)[17] with minor modifications as suggested by Adhikari *et al.* (1987).[18] The authenticity of the isolated chloroplasts was established as described by Adhikari *et al.*[18]

Purification and characterization of D/L- *myo*-Inositol-1-phosphate phosphatase

In order to purify the MIPP from the experimental sample separately, all operations were carried out at 0-4°C following identical methods for both the soluble (cytosolic) and particulate (chloroplastidial) enzyme forms. About 75-100 g of plant tissues (for cytosolic enzyme) / 10-15 ml chloroplast pellet (obtained from 80-100 g tissues for plastidial enzyme) were thoroughly washed with sterile cold distilled water twice. The tissues were then homogenized in a mortar and pestle with 2 volumes of extraction buffer [50 mMTris-HCl (pH 7.2), containing 0.2 mM of 2-Mercaptoethanol (standard buffer)] in presence of neutral sand. The slurry was centrifuged at 800 g for 5 minutes in a Remi C-24 BL cold centrifuge. The homogenate was spun again at 11,400 g for 30 minutes in a refrigerated centrifuge (Remi C-24 BL) and the supernatant was collected (Low-speed supernatant). The resultant low-speed supernatant was then centrifuged at 1, 10,000 g in an ultracentrifuge (Hitachi GX Series, Model: CS 120G X 2 Micro Ultracentrifuge, Rotor No. 26) for 2 hours. On completion of centrifugation, the clear supernatant (High-speed supernatant) was collected from the centrifuge tubes and were kept ready for the next step.The high-speed supernatant was fractionated with ammonium sulfate [$(NH_4)_2SO_4$]: 20-80% (for cytosolic) / 0-80% (for chloroplastidial) saturation]. The resultant $(NH_4)_2SO_4$ pellet was dissolved in minimal volume of standard buffer and dialyzed overnight against the same (at least against 700 volumes). Dialyzed fraction was chromatographed using a cation-exchange matrix CM-cellulose (obtained from GENEI, Bangalore, India) in a glass column (1.2×15.0 cm). The effluent was collected and the column was washed with one bed volume of the standard buffer. The elution of adsorbed proteins was made by a linear gradient of 0 to 0.5 M KCl prepared in standard buffer. The MIPP active CM-cellulose fractions were pooled together and the subsequent column chromatography was carried out through Sephadex G-200 (obtained from Amersham Pharmacia Biotech, UK) (0.8×14.0 cm). The MIPP active Sephadex G-200 fractions were pooled together and finally molecular sieve chromatography was completed through UltrogelAcA 34 column (obtained from Bio Rad, USA) (0.8×8.0 cm). The MIPP active fractions obtained from Ultrogel AcA 34 were pooled together and used as the enzyme preparation for biochemical characterization of MIPP(s). All experiments were repeated twice with multiple replicates.

Assay of *myo*-inositol-1-phosphate phosphatase

MIPP was assayed by the method of Eisenberg Jr. (1967)[14] with slight modifications.[10] Inorganic phosphate was estimated following the method of Chen *et al.* (1956).[19] The specific activity was defined either as n mol *myo*-inositol- 1-phosphate released $(mg)^{-1}$ protein h^{-1} or n mol P_i released mg^{-1} protein h^{-1}.

Estimation of protein

Protein was determined according to the method of Bradford (1976)[20] with BSA as a standard. Polyacrylamide gel electrophoresis of the active Ultrogel AcA 34 fractions were performed under non denaturing conditions following the method of Bollag *et al.* (1996)[21] with necessary modifications as elucidated by Banerjee *et al.* (2007).[10] For the MIPP assay from the native PAGE, replicate gels were run. One of the gels was stained after completion of the run by Coomassie Brilliant Blue (R-250) to visualize the protein bands and the other was sliced successively to 5 mm fragments using a gel slicer. The enzyme from each of the slice was then extracted in relevantly marked eppendorf tube with 250 µl of 50 mM Tris-acetate buffer (pH 7.5) and kept overnight at 0°C to 4°C. The eppendorf tubes were centrifuged at 11,400 g for 30 minutes using Remi RM- 02 Plus mini centrifuge. The pellets were discarded and the supernatants were eventually transferred to respective test tubes for enzyme assay. The MIPP activity was assayed following the assay method described earlier.

Molecular weight determination of native *myo*-inositol-1-phosphate phosphatase

Approximate molecular weight of the native MIPP obtained from the respective samples was determined by gel-filtration through Sephadex G-200. The Sephadex G-200 was suspended in 50 mM Tris- acetate (pH 7.5) and packed in a column of suitable size and calibrated with 1 ml each of marker proteins *e.g.*, catalase (221.6 kDa); bovine serum albumin dimer (66.5kDa); phosphorylase-b (97.4 kDa); ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and lysozyme (14.3 kDa). The void volume was determined with blue dextran 2000 (1 mg / ml). All standards were loaded in the column separately and fractions (0.75 ml) were collected at a flow rate of 0.75 ml / 6 min. Each individual protein peak was located by spectrophotometric scanning at 280 nm in a Jasco V-5 UV-Vis Spectrophotometer. A standard curve was prepared by plotting relative elution volume of proteins against their respective log molecular weights.

Kinetic analysis

As far as the evaluation of the kinetic parameters of MIPP is concerned, the Michaelis–Menten constant (Km) was analysed. In order to establish relation between the substrate (D/L-MIP) and the rate of the enzyme catalysed reaction, all the parameters were calculated by nonlinear regression method using the software Prism 5 (Graph Pad).

Isolation and quantification of free Inositol

Free *myo*-inositol was isolated by the method of Charalampous and Chen (1966)[22] with minor modifications. It was quantified spectrophotometrically following the method of Gaitonde and Griffiths (1966).[23]

Table 1. Screening of cytosolic and plastidial MIPP activity and free *myo*-inositol content in the experimental macro alga *Enteromorpha intestinalis* grown under different salinity conditions.

Experimental sample	Salinity of water (PSU)	Free *myo*-inositol content (mg)/(g) FW ± S.E		Specific Activity of MIPP (n mole P_i released $(mg)^{-1}$ Protein) h^{-1}	
		Cytosol	Chloroplast	Cytosolic	Chloroplastidial
Enteromorpha intestinalis (sample collected in rainy season)	6.41 to 7.21	0.83±0.36	1.34±0.27	12.78±2.66	145.23±15.48
Enteromorpha intestinalis (sample collected in winter season)	12.4 to 13.7	1.43±0.52	4.16±0.77	17.14±4.16	270.65±21.43

Determination of salinity

The measurement of practical salinity units values of water, collected from Chilika lagoon (in separate sterile containers) in rainy and winter seasons were measured using Systronics Water Analyzer (Model no: 571).

Results

The results obtained in the present study clearly reveal the existence of two forms of MIPP (cytosolic and plastidial) in the experimental alga *E. intestinalis*. Although appreciable activity has been detected in both cytosolic as well as plastidial MIPP, the plastidial isoform exhibited greater activity in comparison to its cytosolic counterpart (Table 1). A marked increase in both cytosolic and plastidial MIPP activity (from now on termed as MIPPs) could be recorded in thalli collected in the winter season (under higher salinity of lagoon water) and this high plastidial MIPP activity detected in the winter thalli of *E. intestinalis* was also correlated with a substantial increase in the content of free *myo*- inositol in the chloroplasts of *E. intestinalis* collected in the post monsoon season (Table 1).

Summary of the purification of MIPPs has been provided in Table 2. The overall purification of cytosolic MIPP was recorded to be around 55- fold with about 32% yield while that of the plastidial MIPP was found to be 16- fold with 26% recovery. Column chromatography using Ultrogel AcA 34 revealed that MIPPs from *E. intestinalis* were retained and eluted effectively with the extraction buffer.

The apparent molecular weight (Mr) of the native MIPPs, determined by gel filtration on a Sephadex G-200 column was 146 k Da (cytosolic) and 148 k Da (plastidial).

By means of native PAGE gel slice assay, the major band of MIPPs was found to coincide with the enzymatic activity (Figure 1) as described earlier in the materials and in the method section.

In order to determine the requirements of MIPP for its catalysis, it was revealed that in presence of all the assay components the experimental *E. intestinalis* MIPP recorded a maximum activity of 100%. However, notable reduction of the activity of MIPPs could be recorded when Tris buffer and KCl were omitted from the reaction mixture. The MIPP activity dropped to 79% and 96% in absence of the buffer, 91% and 94% in absence of KCl for cytosolic and chloroplastidial enzymes respectively. Regarding substrate specificity, either L-MIP or D-MIP was found to be the exclusive substrate and dephosphorylated almost with equal efficiency by MIPPs. Other hexose monophosphates viz., D-glucose- 6-phosphate, D-fructose- 6- phosphate, D-galactose- 6- phosphate under identical concentration (0.2 mM) was completely ineffective as substrates. Under standard assay conditions it was observed that the MIPPs reaction proceeded linearly with time up to a maximum of 60-75 min in both the cases (Figure 2A, B).

The linearity of MIPP reaction under increasing protein concentration (0-80µg) indicated 30 and 60 µg respectively for cytosolic and plastidial enzymes (Figure 2C, D). The effect of substrate concentration and kinetic analyses of MIPPs were carried out using D-MIP in the concentration range of 0.0-1.0 mM. The reaction rate was noted to increase with respect to D-MIP concentration of 0.1 to 0.2 mM. The Km value for D-MIP of cytosolic MIPP was 0.07277 mM and that of the chloroplastidial MIPP was 0.07332 mM (Figure 2E, F). MIPPs function remarkably active between the temperature ranges of 20-40°C. The temperature maxima for cytosolic MIPP were found to be 40°C, while that of the plastidial one was 35°C (Figure 3A, B). The algal MIPPs exhibited optimum activity at a pH range of 7.0 (cytosolic) and 7.5 (plastidial) when 50mM Tris-HCl buffer was used at a pH range of 6.0-9.0 (Figure 3C, D). Among the monovalent cations tested, K^+ had slight stimulatory role and Li^+ was strongly inhibitory (41-56%). Using the analogous concentrations of other divalent cations, it was revealed that Mg^{2+} played

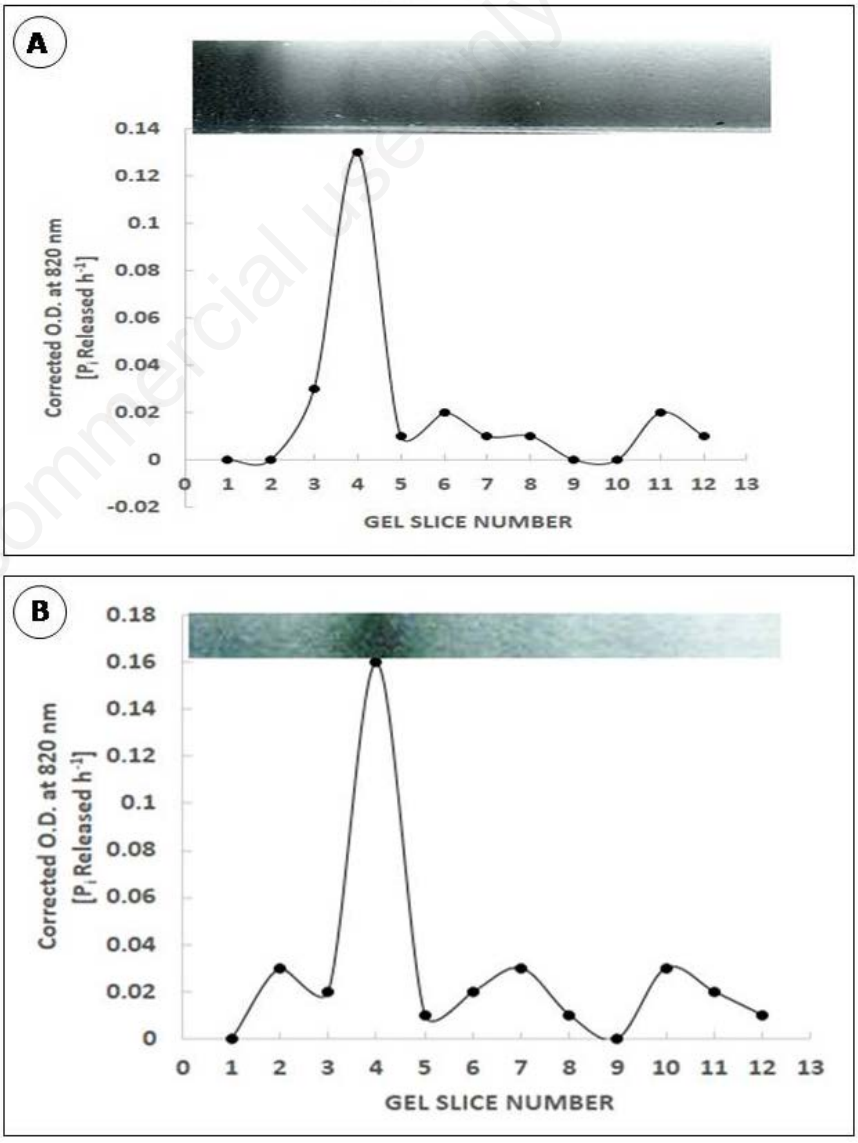

Figure 1. Native PAGE profile showing the *myo*-inositol 1-phosphate phosphatase (MIPP) activity of the corresponding band: (A) cytosolic and (B) plastidial.

appreciable stimulatory role and Ca^{2+} exhibited inhibitory effect at higher concentrations (Figure 3E, F). When used between the concentration ranges of 0-50mM EDTA, it was also revealed that EDTA had significant inhibitory role on MIPPs.

Discussion and Conclusions

Perusal of available literature clearly reveals that although purification of MIPP has been carried out dominantly in animal systems,[14-16] reports describing preparation of cytosolic/plastitidial forms of MIPP from plant systems is still very inadequate. Only a few reports describing purification and characterization of MIPP are available primarily from angiosperms[12,13] and few from vascular cryptogams.[10,11] Therefore, the presence of MIPPs in *E. intestinalis* adds further strength to the ubiquitous metabolism of *myo*-inositol biosynthesis in non-vascular cryptogams too. The partial purification of MIPPs from *E. intestinalis* and its preliminary characterization paves the way towards understanding its role in non- vascular cryptogams.

The results obtained in this work clearly reveals that the activity of MIPPs gets enhanced in response to high salinity levels of Chilika lagoon and this enhanced MIPPs activity is also correlated with a proportional increase in the content of free *myo*- inositol. It has been reported earlier that salt stress results in alterations in plant metabolism, reduced water potential, ion imbalance and toxicity.[24] Moreover, it has also been reported earlier that in addition to bringing about disturbances in ionic homeostasis salt stress also causes considerable damage to the membrane architecture due to enhanced accumulation of ROS. Disorganization of the thylakoidal structure of chloroplasts is also brought about by salt stress.[25] Thus taking these facts into contemplation the enhanced activity of the organelle- bound MIPP specifically in response to salt stress seems logical. Work done by previous workers are indicative of the fact that the overproduction of cyclitols in transgenic tobacco, *Arabidopsis* and wheat plants have resulted in maintenance of optimal ionic homeostasis and cellular integrity.[26] It has been reported earlier that Chilika lagoon undergoes a cyclical variation in salinity throughout the year and the distribution of flora and fauna in Chilika is also influenced greatly by salinity fluctuations.[27] Since inositol and its derivatives act as osmolytes/osmoprotectants and are able to protect cells from the effects of osmotic imbalance imposed by high salinity levels, higher activity of MIPPs along with higher level of free inositol detected in the thalli of *E. intestinalis* housed at higher salinity seems logical. However, it must be taken into account that although salt stress has been associated with a number of interconnected physiological responses, the underlying mechanisms of salt tolerance in plants are still poorly understood. Since algae are inhabitants of biotopes characterized by changing salinities they can serve as model organisms for a better understanding of the salt acclimation phenomenon in higher plants.[28,29]

The characteristics of MIPP recorded in the present investigation are in partial concurrence with all the major MIPP(s) recorded earlier from plant systems.The molecular weight of native cytosolic MIPP from the common fern *Dryopteris filix mas* was reported to be around 94 k Da.[10] However in this work the apparent molecular weight of cytosolic MIPP has been found to be 146 k Da while that of its plastidial isoform has been recorded to be 148 k Da. MIPP obtained from eukaryotic and bacterial sources have been reported to exhibit a broad range of substrate specificity. Nevertheless in case of this experimental alga, D-or L- MIP was found to be the exclusive substrate. The plastidial iso-form on the other hand exhibited feeble activity when D-*myo* inositol -3- phosphate replaced the principal substrate. This is in complete agreement with the results obtained in case of *Dryopteris filix mas.*[10] In the present study the temperature maxima for cytosolic and plastidial MIPP has been found to be 40°C and 35°C respectively (Figure 3A, B). A quick glance on the reports published earlier clearly reveals a

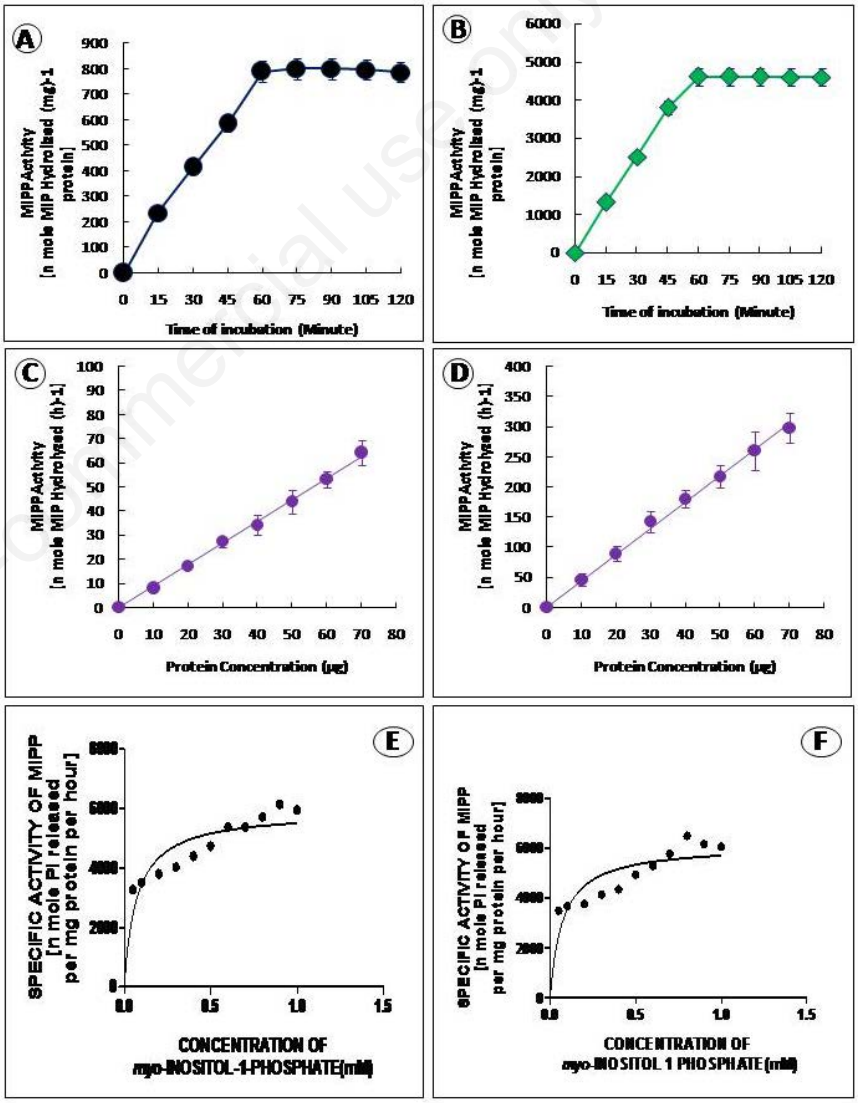

Figure 2. Time course of *E. intestinalis* MIPP: (A) cytosolic and (B) plastidial. Effect of protein concentrations on *E. intestinalis* MIPP: (C) cytosolic and (D) plastidial. Determination of Km value for D-MIP of *E. intestinalis* MIPP by Nonlinear Regression Kinetics: (E) cytosolic and (F) plastidial.

marked variation in the temperature maxima of MIPP obtained from different sources. In case of the fern pteridophyte *Dryopteris filix mas* temperature maxima for cytosolic MIPP has been recorded to be about 40°C while in case of non- fern pteridophytes (Lycopsida members) the temperature maxima for cytosolic MIPP has been recorded to be about 35°C. MIPP(s) obtained from microbial and mammalian sources have exhibited a temperature maxima of 37°C while MIPP obtained from the cyanobacteria *Synechocystis* has shown a slightly higher temperature maxima (42°C).[8] Most MIPP(s) isolated from various sources have been found to be optimally active in the pH range of 7.8-8.5.[8] However *Enteromorpha* MIPP has been found to be optimally active at pH 7.0 (cytosolic) and 7.5 (Plastidial) (Figure 3C, D). In *Dryopteris filix mas,* cytosolic MIPP has been found to exhibit a pH range of 7.5-8.5[10] while in case of non- fern pteridophytes the pH range has been recorded between 7.0 and 7.5.[11] As far as the kinetic parameters are concerned, the Km value for D-MIP of cytosolic MIPP was recorded to be 0.07277 mM and that of the chloroplastidial MIPP was 0.07332 mM (Figure 2E, F). It is a sharp contrast of Km values detected in prokaryotic as well as in mammalian systems.[8,30] In case of mammalian system Km value for D-MIP has been found to be much higher while prokaryotic systems have exhibited a much lower Km value for D-MIP. Among the monovalent cations tested Li^+ was found to exert a strong inhibitory effect on MIPPs. It has been reported earlier that inhibition by Li^+ is a general characteristic feature of the inositol monophosphatase family enzymes.[1] The results obtained in this study also indi-

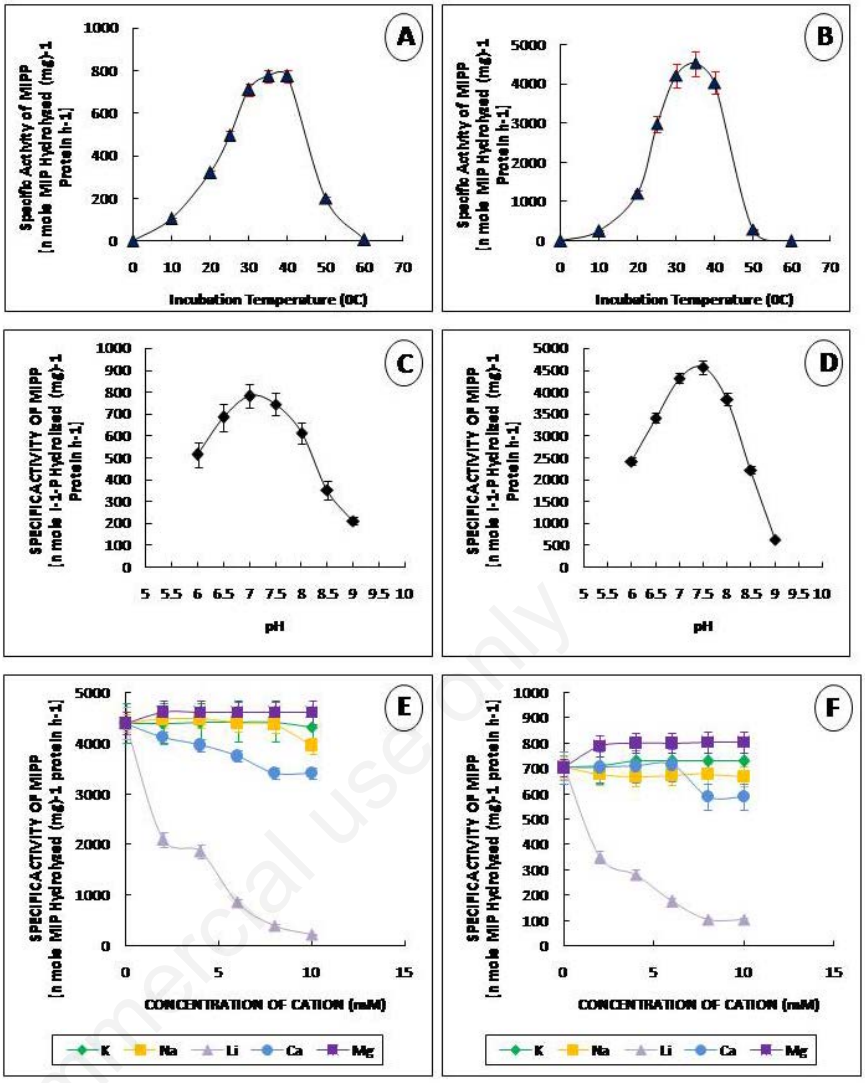

Figure 3. Effect of incubation temperature on *E. intestinalis* MIPP: (A) cytosolic and (B) plastidial. pH dependence of MIPP: (C) cytosolic and (D) plastidial. Effect of different monovalent and divalent cations on *E. intestinalis* MIPP activity: (E) cytosolic and (F) plastidial.

Table 2. Outline of partial purification of (A) cytosolic and (B) chloroplastidial *myo*-inositol-1-phosphate phosphatase from *Enteromorpha intestinalis*.(Values are mean±SE, n=3).

Purification step	(A) CYTOSOLIC MIPP			(B) CHLOROPLASTIDIAL MIPP		
	Specific activity [n mol P_i released (mg)$^{-1}$ protein h^{-1}]	Total activity [n mol P_i released h^{-1}]	Purification (fold)	Specific activity [n mol P_i released (mg)$^{-1}$ protein h^{-1}]	Total activity [n mol P_i released h^{-1}]	Purification (fold)
Homogenate fraction	14.50±4.22	871.88±88.69	1.00±0.07	278.44±17.90	4995.21 ±132.16	1.00±0.14
11,400 g supernatant fraction	16.42±6.90	843.65±89.16	1.13±0.04	281.06±14.22	4154.06±89.16	1.00±0.09
1, 10,000 g supernatant fraction	17.55±7.04	837.13±75.11	1.21±0.11	283.66±19.41	3806.71±75.11	1.01±0.07
20-80 % / 0-80% Ammonium sulfate fraction	23.48±9.92	535.34±82.52	1.61±0.09	286.04±25.63	3195.06±82.52	1.02±0.13
CM-cellulose fraction	136.90±16.82	436.71 ±42.80	9.44±0.36	747.23±36.34	2914.19 ±42.80	2.68±0.46
Sephadex G-200 fraction	188.22±37.11	365.14±41.93	12.98±0.63	1828.77±49.27	2395.68±51.93	6.56±0.61
Ultrogel AcA 34 fraction	803.68±56.13	281.28±53.31	55.42±6.34	4611.27±72.34	1291.15±73.31	16.56±2.06

cate that MIPPs activity is sensitive to metal chelators like EDTA. This is in complete agreement with the results obtained in case of vascular cryptogams by previous workers.[10,11] The possible reason behind this sensitivity towards a metal chelator like EDTA may be attributed to the presence of some 'built-in' Mg^{+2} as suggested earlier.[8]

Thus a comparative analysis of cytosolic and plastidial MIPP obtained from a naturally lodged algal source depicted in this work not only re-establishes the unique metabolic features and widespread distribution of MIPP in the plant kingdom but also reinstates its primordial protein nature. The results obtained in this study thus unveil some unique metabolic features of *myo*-inositol biosynthesis and its regulation in a non-vascular cryptogam for the first time where no laboratory simulation with NaCl was provided. However in order to understand the phylogeny of these proteins in cryptogams, a thorough search for immunologically cross-reactive material in different classes of algae or the analysis of nucleotide sequences with the aid of MIPP gene probes may be carried out in future. Further studies based of proteomics and/or metabolomics are in progress in order to better understand the role of inositol/inositol like osmoprotectants in amelioration of salinity stress among different phylogenetically distant algal groups, such as in members of Phaeophyceae and Rhodophyceae.

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