Isolation and biochemical characterization of L-*myo* inositol-1-phosphate synthase from *Asterella khasiana* (Griff.) Grolle and *Sphagnum junghuhnianum* Doz. & Molk. of Darjeeling Hills

Thesis submitted to
The University of North Bengal for the Award of the Degree of Doctor of Philosophy in Botany

By
Sachina Yonzone

Under the Supervision of
Dr. Subhas Chandra. Roy (Supervisor)
Dr. Dhani Raj Chhetri (Co-supervisor)

Department of Botany,
University of North Bengal
INDIA

June, 2018

DECLARATION

I declare that the thesis entitled **"Isolation and biochemical characterization of bryophytic L-*myo*-inositol-1-phosphate synthase from - *Asterella khasiana* (Griff.) Grolle and *Sphagnum junghuhnianum* Doz. & Molk. of Darjeeling Hills''** has been prepared by me under the joint supervision of Dr. Subhas Chandra Roy, Associate Professor, Department of Botany, University of North Bengal and Dr. Dhani Raj Chhetri, Associate Professor, Department of Botany, School of Life Sciences, Sikkim University, Sikkim. No part of this thesis has formed the basis of any previously awarded degree or fellowship.

Date June 18

Sachina Yonzone

Sachina Yonzone
Department of Botany
University of North Bengal
Raja Rammohunpur, Siliguri

UNIVERSITY OF NORTH BENGAL
DRS DEPARTMENT OF BOTANY (UGC-SAP & DST-FIST assisted)
SILIGURI 734013, WEST BENGAL, INDIA Phone: +91-353-2776337, FAX: +91-353-2699001

CERTIFICATE

I certify that Ms. Sachina Yonzone has prepared the thesis entitled **"Isolation and biochemical characterization of bryophytic L-*myo*-inositol-1-phosphate synthase from - *Asterella khasiana* (Griff.) Grolle and *Sphagnum junghuhnianum* Doz. & Molk. of Darjeeling Hills"** for the award of Ph.D degree in Botany of the University of North Bengal, under my guidance. She has carried out the work at the post graduate Department of Botany, Darjeeling Government College, Darjeeling under the University of North Bengal. No part of this work has ever been used for the award of any other degree.

29.5.18

Dr. Subhas Chandra Roy (Supervisor)
Associate Professor,
Department of Botany,
University of North Bengal,
Dist.Darjeeling

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I do hereby certify that Ms. Sachina Yonzone has prepared the thesis entitled "Isolation and biochemical characterization of bryophytic L-*myo*-inositol-1-phosphate synthase from *Asterella khasiana* (Griff.) Grolle and *Sphagnum junghuhnianum* Doz. & Molk. of Darjeeling Hills'' for the award of Ph.D degree in Botany of the University of North Bengal, under my supervision. She has carried out the work at the post graduate Department of Botany, Darjeeling Government College, Darjeeling under the University of North Bengal. This work presented in this thesis has neither been published nor used for the award of any other degree.

Dhani Raj Chhetri

Dr. Dhani Raj Chhetri (Co-supervisor)
Department of Botany, School of Life Sciences
Sikkim University

6 माईल, सामदुर, तादोंग, गंगटोक - 737102 सिक्किम, भारत
दूराभाष : 00-91-3592 - 251067, 251403, फैक्स :- 251067/251757
6th Mile, Samdur, PO Tadong 737102, Gangtok, Sikkim, India
Phones : 00-91-3592-251067, 251403, Fax - 251067/251757
website : www.sikkimuniversity.in/www.sikkimuniversity.ac.in
Email : sikkimuniversity@gmail.com

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ABSTRACT

The fundamental screening of L-*myo*-inositol-1-phosphate synthase (EC 5.5.1.4) activity from the representative bryophyte species of Darjeeling hills was undertaken, which showed that the enzyme is distributed in all species of this cryptogrammic group studied. Similarly, the end product of the enzymatic reaction i.e., free *myo*-inositol was also found across the bryophytes of the different families under the study conditions. The reproductive part bearing plant bodies were richer sources of this enzyme as well as free *myo*-inositol in the selected samples, *Asterella khasiana* and *Sphagnum junghuhnianum*. L-*myo*-inositol-1-phosphate synthase (MIPS) was partially purified from the reproductive part bearing thallus of *Asterella khasiana* and *Sphagnum junghuhnianum.* The purification procedure involved homogenization, low speed centrifugation, streptomycin sulphate precipitation, ammonium sulphate fractionation, chromatography on DEAE cellulose, Hexylagarose and Bio Gel-A 0.5 m. The enzyme was purified to about 46.34 fold over the homogenate fraction with 36.35% recovery from the liverwort, *A. khasiana*. Similarly, from the moss, *Sphagnum junghuhnianum*, a purification of about 58.67 fold over its homogenate fraction with about 32.86% recovery based on total activity was achieved. Apparent molecular weight of bryophytic MIPS from *Asterella khasiana was* approximately 183kDa and that from *Sphagnum junghuhnianum* was determined as approximately 174 KDa.

D-glucose-6-phosphate was found to be the specific substrate for MIPS from the bryophytes. Among the other hexose phosphates, D-glucose-6-phosphate, D-glucose-1-phosphate, D-fructose-6-phosphate and D-fructose-1,6-bisphosphate could not act as substrates for this enzyme. However, the enzyme showed a little bit of activity in

presence of D-galactose-6-phosphate and mannose-6-phosphate. With the increase in substrate (D-glucose-6-phosphate) concentration from 0 to 6 mM and coenzyme (NAD^+) concentration from 0 to 0.8 mM the activity of *A. khasiana* MIPS increased linearly. The *A. khasiana* MIPS showed K_m values of 3.56 mMand 0.56 mM for D-glucose-6-phosphate and NAD^+ respectively while the V_{max} values were found to be 0.71 mM and 0.68 mM for D-glucose-6-phosphate and NAD^+ respectively. In comparison, the activity of *S. junghuhnianum* MIPS increased linearly with the increase of D-glucose-6-phosphate concentration from 0 to 6 mM and coenzyme (NAD^+) concentration from 0 to 0.6 mM. The K_m for D-glucose-6-phosphate and NAD^+ were 1.81mM and 0.25mM respectively while the V_{max} values were worked out to be 1.42 mM and 1.12 mM for D-glucose-6-phosphate and NAD^+ respectively.

Stability of the bryophytic MIPS varied with the enzyme preparation at different stages of purification. In *Asterella khasiana*, the low speed supernatant remained active for 7-8 days and the BioGel purified fractions for 3-4 days when stored at −20 ºC. Comparatively, the low speed supernatant and BioGel purified fractions from *Sphagnum junghuhnianum* maintained its activity for 10-12 days and 5-7 days respectively, when stored at identical temperature. The deduction of NH_4Cl and ME reduced MIPS activity to 31.80% and 34.51% respectively in of *A. khasiana* and the same caused 40.01% and 33.35% loss of activity respectively in *S. junghuhnianum.* No enzyme activity was observed in absence of glucose-6-phosphate (substrate) and in case of heat-killed enzyme in either of the samples *Asterella khasiana* and *Sphagnum junghuhnianum*. However, when NAD^+ was deducted from the reaction mixture, the enzyme from *A. khasiana* exhibited approximately 59.17% loss of activity and that from *S. junghuhnianum* exhibited about 68.41% loss of activity.

The activity of bryophytic MIPS was directly proportional to the time of incubation upto 90 minutes in both cases and with respect to protein concentration upto 250µg in *A. khasiana* and 300 µg in *S. junghuhnianum*. The bryophytic MIPS operated between pH ranges of 7.0 to 7.5. However, the maximum activity was found at pH 7.0. Though MIPS from both the sources showed temperature maxima at 30^{o}C, it showed significant activity at temperatures as low as 20^{o}C in *A. khasiana* and 10^{o}C in case of *S. junghuhnianum.*

NH_4Cl was a strong stimulator of the enzyme and increased the rate of reaction in a concentration guided manner by 7.5 and 9.3 fold in *A. khasiana* and *S. junghuhnianum* respectively. Similarly, $MgCl_2$ acted as a mild stimulator, increasing the enzyme activity up to 1.6 fold and 1.2 fold in *Asterella khasiana* and *S. junghuhnianum* respectively. EDTA acted as a mild inhibitor in both the cases. Among the monovalent cations, K^+ was mildly stimulatory, Na^+ mildly inhibitory, while Li^+ was strongly inhibitory to the enzyme. Among the divalent cations studied, Ca^{2+} was mildly stimulatory, while Mn^{2+}, Cu^{2+} and Cd^{2+} had varying degree of inhibitory effect from mild to medium. Zn^{2+} was a strong inhibitor of the enzyme while Hg^{2+} showed extreme inhibitory property towards the bryophytic MIPS.

PREFACE

At the outset, I would like an attempt to express my deepest gratitude and endless thanks to my research supervisor, Dr Subhas Chandra Roy, Department of Botany, University of North Bengal for his supervision, encouragement and continuous support for my research. I would also like to express my deep sense of gratitude to my second supervisor, Dr Dhani Raj Chhetri, Department of Botany, School of Life Sciences, Sikkim University for his conceptualization, overall guidance and support regarding the entire work. His drive for scientific excellence has constantly pushed me during my research and it was a great pleasure for me to have gotten a chance of working with him.

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Department of Botany
University of North Bengal SachinaYonzone
Date :

C O N T E N T S

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1. INTRODUCTION

Darjeeling Himalaya is a large repository of plants belonging to different phylogenetic groups with medicinal and other importance. Bryophytes comprise the predominating flora from the cryptogamic group in Darjeeling. From the biochemical standpoint, most of these plants have not been studied seriously anywhere in the world. The physiological and biochemical activities of all plants are predominantly maintained by enzyme-driven reactions. The study of enzymes, therefore, becomes an important aspect to understand the fundamental metabolic processes. *Myo*-inositol is considered as an essential metabolite cum vitamin. The ubiquitous nature of inositols, in general, has been well known for several decades. Existence of this compound, either in free or conjugate form has been reported from a large number of plants, prokaryotes and eukaryotes. The biosynthesis of *myo*-inositol is critical for the production of a functionally diverse group of compounds. *Myo*-inositol is biosynthesized through the conversion of D-glucose-6-phosphate by a NAD-dependent oxido-reductase, L-*myo*-inositol-1-phosphate synthase (MIPS).

Myo-inositol and its derivatives are essential for the survival of eukaryotic cells, including plants, as various metabolic roles emanates from these compounds. A number of possible roles of *myo*-inositol and its derivatives have been reported in plants, including growth regulation, membrane biogenesis, osmotolerance etc (Loewus and Murthy, 2000; Stevenson *et al*., 2000). It also serves as an essential nutrient for growth and development, an integral part of milk and causes an inhibition of cell division in other plants under tissue culture conditions. *Myo*-inositol is also used in sustained cell

division in the cabbage protoplast culture system for regeneration of somatic hybrids or cybrids through asymmetric protoplast fusion (Jie *et al.*, 2011). As phosphorylated derivatives, its role as a phosphorus store and as a "*second messenger*" in signal transduction pathway has long been recognized (Gross and Boss 1993; Nunes *et al.*, 2006). At least three different messenger molecules are known to be produced from phosphoinositides: arachidonic acid, inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). These messenger molecules are marked as turning point in the studies of hormone function (Boss *et al*., 2006).

Inositol is associated with the resistance to both abiotic and biotic responses (Taji *et al*., 2006) under salt stress (Taji *et al*., 2006; Sengupta *et al*., 2008). Different derivatives of *myo*-inositol also serve as compatible solutes and signaling molecules to response salt stress in plants (Kido *et al*., 2013). *Myo*-inositol is also reported to be utilized in the abiotic stress induced galactinol and raffinose synthesis (Taji *et al*., 2002). In addition to inositol, its methylated derivatives, pinitol, ononitol, sorbitol etc. were shown to be involved in stress responses in some plants (Paul and Cockburn, 1989; Ishitani *et al*., 1996; Sheveleva *et al*., 1997; Shen *et al*., 1999). It is also suggested that inositol and the *O*-methyl inositol esters function as osmoprotectants possibly by protecting cellular structure from reactive oxygen species and by controlling turgor pressure (Loewus and Murthy, 2000) and a high concentration of cyclitols like pinitol and ononitol are found in osmotolerant plants. An increase in the production of inositol as a consequence of coordinate transcriptional induction of the MIPS coding gene in response to stress, has been reported from some plants such as *Mesembryanthemum crystallinum*, *Cicer arietinum* etc (Ishitani *et al*.,1996; Boominathan *et al*., 2004). It has also been reported

that the introgression of MIPS-coding gene from *Porteresia coarctata (PcINO1*), in tobacco, rice and *Brasica juncea* generated transgenic lines carrying salt tolerance with concomitantly increased inositol production (Majee *et al.*, 2004; Das Chatterjee *et al.*, 2006).

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; Roy Chaudhuri *et al*., 1997), fungi (Donahue and Henry, 1981a, 1981b); 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; RayChaudhuri *et al*., 1997). During a screening for the activity of the enzyme across a wide array of phylogenetically diverse groups, the activity of the enzyme has been detected in a few liverworts (Dasgupta *et al*., 1984). However, detailed study of the enzyme in the light of metabolic regulation and the isolation and characterization of the same in different groups of bryophytes is still lacking.

The bryophytic flora of Darjeeling Himalayan region in India is very rich, growing on the barren land, denuded forests, rock surfaces etc. Therefore, this work has been undertaken to investigate *myo*-inositol biosynthesis and purification followed by attempts for biochemical characterization of the enzyme from *Asterella khasiana* (Griff.) Grolle and *Sphagnum junghuhnianum* Doz. & Molk. of Darjeeling Hills. Both

of these bryophytes are extremely drought resistant and they grow as lithophytes all over the Darjeeling Himalaya. Moreover *Asterella* has been found to be desiccation resistant resurrection plant while quite a few drought resistant characteristics have already been found in some species of mosses. These factors has emboldened me to select the above two species of bryophytes, one from the liverworts and the other from mosses as my experimental material.

The biosynthesis of *myo*-inositol from glucose-6-phosphate has been documented from a number of biological systems as mentioned above. The major study on the inositol synthase from plant sources were confined to the fungal and angiospermic enzymes. Furthermore, despite *myo*-inositol and several other isomeric inositols or their conjugates having been found in several plant systems, studies of *myo*-inositol biosynthesis in an important phylogenetic group, the bryophytes have not been seriously taken up by investigators. Therefore, the presented research work on bryophytic L-myo-inositol-1-phosphate synthase was carried out with the following objectives:

1. To screen for the activity of the enzyme, L-myo-inositol-1-phosphate synthase from the common bryophytes belonging to different families viz., *Asterella khasiana* (Griff.) Grolle, *Marchantia polymorpha* Linn., *Riccia cruciata* Kash, *Anthoceros angustus* Steph., *Bryum argenteum* Linn., *Brachymenium bryoides* Hook. ex Schwaegr., *Lyellia crispa* R. Br., *Funaria wallichii* (Mitt.) Broth. and *Sphagnum junghuhnianum* Doz. & Molk. available in Darjeelling hills. Also to screen for the occurrence of free *myo*-inositol, the end product of myo-inositol synthase reaction from these bryophytes.

2. To partially purify and compare the L-myo-inositol-1-phosphate synthase from one liverwort, *Asterella khasiana* and one moss, *Sphagnum junghuhnianum*, both showing tolerance to osmotic and desiccation stress and to estimate the enzyme activity at each stage of purification.

3. To characterize the partially purified enzyme from *Asterella khasiana* and *Sphagnum junghuhnianum* from biochemical point of view in order to understand its fundamental metabolic regulation.

4. To study the possible role of the bryophytic enzyme in stress tolerance by analyzing whether the enzyme from these two sources exhibits any cold tolerance characteristics.

5. To contribute towards the existing knowledge on L-myo-inositol-1-phosphate synthase in bryophytes and to establish a continuous line of enzymological study with respect to the above enzyme across the evolutionary scale in plant kingdom.

2. LITERATURE REVIEW

2.1. Nomenclature and chemistry of *myo*-inositol and its derivatives

2.1.1. What is *myo*-inositol?

The inositols constitute an important group of naturally occurring polyhydric alcohols found in most plants and animals. There are nine isomers of inositols of which seven are *meso* forms and one DL pair. *Myo*-inositol is the most widely distributed inositol in plants and probably occurs in all living organisms. It occupies a central position in carbohydrate metabolism being the precursor of a number of metabolic products viz., inositol phosphates, phosphoinositides, cell wall polysaccharides, methylated derivatives and IAA conjugates. As a free cyclitol, *myo*-inositol has been found essential for normal growth and development of plant tissues. Lack of cellular level of inositol has been identified as the cause of "inositol-less-death" in *Saccharomyces cerevisiae.*

Myo-inositols serve as an essential nutrient for growth and development of all living organisms, like formation of sex units, an integral part of milk and precursors of principal storage phosphate in seeds. It also forms cell wall polysaccharides and an essential component of myelin in central nervous system. To account for the significance of diverse metabolic pathways centered around *myo*-inositol, studies on L-*myo*-inositol-1-phosphate synthase, the prime enzyme of *myo*-inositol biosynthesis is a prerequisite.

2.1.2. Chemical configuration of *myo*-inositol and its different forms

Inositols are hexahydroxy-cyclohexanes (cyclitols) containing three or more hydroxyl groups. The planar structures of the different isomers of this compound are presented in Fig-2.1. Out of nine forms of inositols, seven occur in nature, the exeptions being *epi*- and *allo*-inositol. *Myo*-, *chiro*- and *scyllo*-inositols constitute the major stereoisomers in plants (Valluru and Ende, 2011). Numbering of '*myo*-inositol' molecule is done clockwise and the carbons that bear hydroxyls projecting above the plane of the ring are assigned the lowest possible numbers (Loewus and Loewus, 1980). The strain free chair form (Fig-2.2) having an axial substitution at C-2 is the most stable form of *myo*-inositol (Anderson, 1972). The three dimensional molecular structure of *myo*-inositol-2-phosphate indicates that the phosphate group is attached to the axial oxygen atom of the molecule.

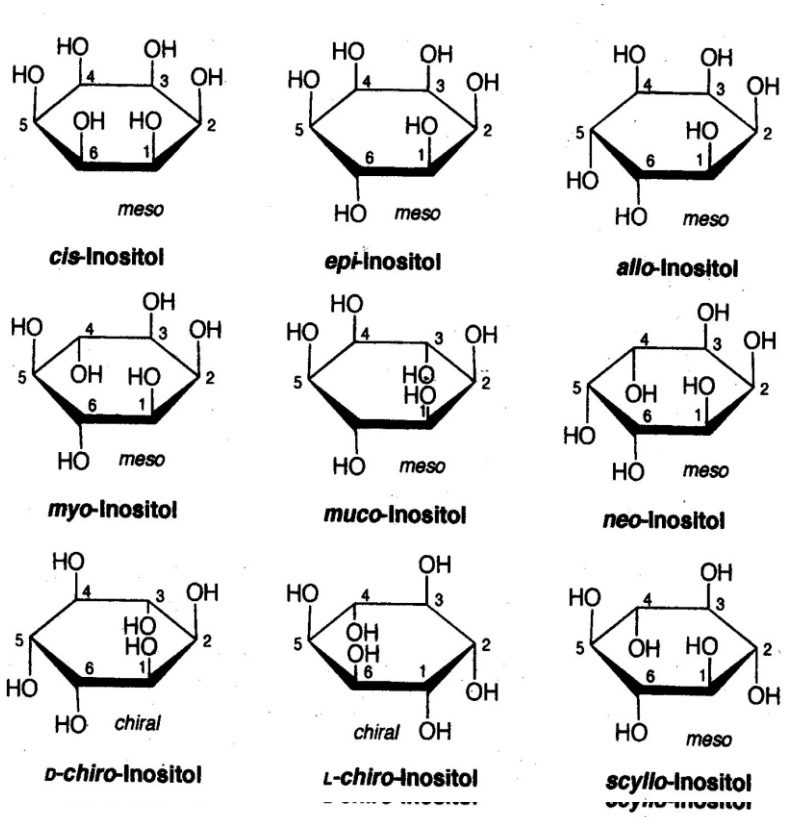


Fig-2.1 Structures of nine possible stereoisomers of inositol

Myo-inositol possesses hydroxyl groups which are secondary alcoholic groups and as such one molecule of *myo*-inositol can be esterified by a maximum of six phosphoric acid molecules by six phosphomonoester bonds generating inositol hexaphosphoric acid (phytic acid). Each *myo*-inositol molecule contains one axial hydroxyl group at C2 (Figure 2.1) and five equatorial hydroxyl groups. When reacted with enzymes or acid, the phytic acid leaves the last phosphate ester at C2, suggesting that the axial phosphate ester is most stable to hydrolysis (Tomlinson and Ballou, 1962).

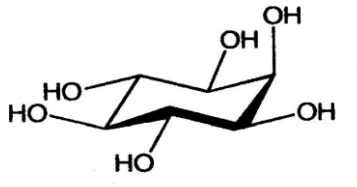
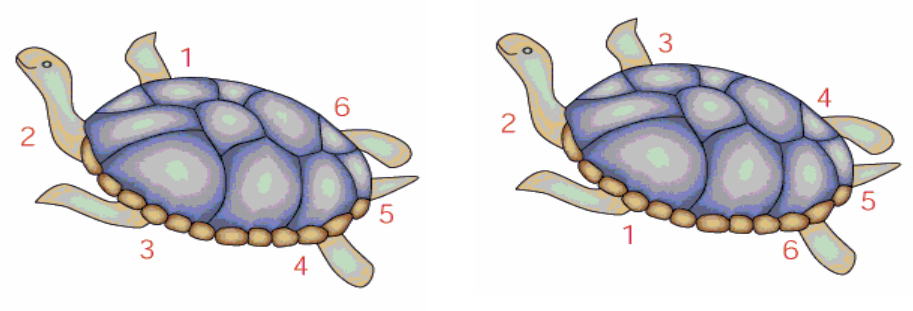


Fig-2.2 Chair form of *myo*-inositol

Numbering of *myo*-inositol has been simplified by visualizing an image of turtle (Fig.2.3) where the head of the turtle is defined as 2- position and is occupied by the axial hydroxyl group, while the five equatorial hydroxyl groups are symbolized by the four limbs and the tail of the turtle. Through this image, the D-ring numbering is assigned by using the right front limb of the turtle as the D-1 position, the head as D-2 position, the left front limb as D-3 position and so on (Agranoff, 1978), when the image is considered through anticlockwise direction. Similarly L-ring numbering is assigned by denoting the left front limb as the L1, head of the rurtle as L2 and so on, while proceeding clockwise.



D-numbering *L-numbering*

Fig-2. 3 Agranoff's turtle

As per Ogawa (1999) the sequential action of several phosphorylases synthesized phytic acid from *myo*-inositol -2-phosphate *in-vivo* from phytic acid or IP_6. Both plant and animal systems contain considerable quantity of phospholipid pool of the cell in the form of phosphoinositides or inositol phospholipid in which phosphatidylinositol or monophosphoinositide is the major *myo*-inositol lipid. Animal cells is recorded to contain about 2-12% of the total phospholipid (White, 1973) along with considerably lower amount of diphosphoinositide (phosphatidyl inositol-4-phosphate and triphosphoinositide (phosphatidyl inositol-4, 5- bisphosphate (Fig.2.4) [Dittmer and Douglas, 1969].

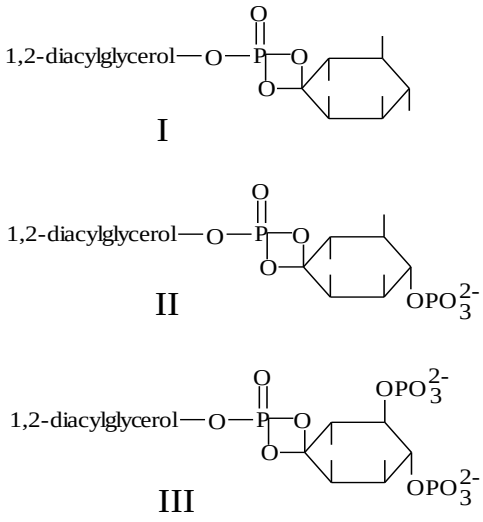


Fig-2.4 Structures of phosphatidylinositols: I. Monophosphoinositide; II. Diphosphoinositide and III. Triphosphoinositide

As compared to animal cells, most of the unsaturated fatty acid groups present in plant are phosphoinositides where phosphatidylinositol acts as a predominant phospholipid (Galliard, 1973). Similar compounds were reported from fungi and higher plants (Loewus and Loewus, 1980), while from tobacco leaves another six phosphosphingolipids were reported (Kaul and Lester, 1978).

2.1.3. Methyl esters of *myo*-inositol

Combined forms like the methyl esters of *myo*-, *scyllo*-, D-and L-*chiro*-and *muco*-inositols have been reported in plants which are considered to be the secondary metabolites and are important in the classification of plants (Plouvier, 1963, 1966; Dittrich *et al.*, 1972). Quite a few monomethyl esters viz., D/L bornesitol, D-ononitol and sequoyitol and three dimethyl esters among which are dimethyl esters are known to occur in plant tissues (Drobak, 1992).

Among the methyl esters of inositol, D-pinitol has been obtained from sugar pine and L-quebrachitol from rubber tree. According to Ogawa (1999) D-pinitol and L-quebrachitol are readily available in large quantities and useful as versatile raw materials for organic synthesis. Naturally occurring methyl derivatives of inositol have been presented in Fig-2.5.

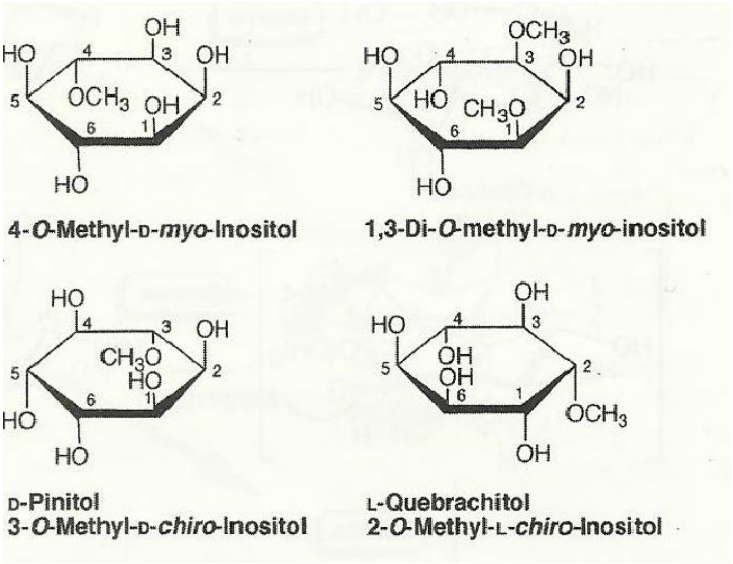


Fig-2.5 Naturally occurring inositol methyl esters

Pinitol (3-O-methyl D-*chiro*-inositol) is one example of an inositol methyl ester which is known as antihyperglycemic agent. This compound has been suggested to form part of the structure of a bovine liver-derived inositol phosphogylcans (IPGs) (Fonteles *et al*., 1996). Furthermore, pinitol has been found to be present in the leaves of *Bougainvillea*, a plant that has been used to treat diabetes mellitus in India (Narayanan *et al*., 1987).

2.1.4. Indole-3 acetyl esters of *myo*-inositol

Indole-3-acetyl esters of *myo*-inositol, 2-O-(IAA)-*myo*-inositol, 1D1-1-O-(IAA)-*myo* inositol viz., di-O-(IAA)-*myo*-inositol and tri-O-(IAA) *myo*-inositol are the indole-3-acetic acid (IAA) esters which are important IAA conjugates of *myo*-inositol (IAAMI) having physiological effects on the plants (Bandurski, 1978). 30% of the lower molecular weight derivatives of IAA the indole-3-acetyl-*myo*-inositol (IAAMI) are present in seeds of *Zea mays* and its transport from endosperm to shoot was 400 times in comparison to the rate of free IAA. This transport is thus adequate to serve as the

seed auxin precursor for the free IAA diffusing downward from the shoot-tip. The first identified seed auxin precursor was indole-3-acetyl-*myo*-inositol. Interconversion between esterified IAA and free IAA occurs in the growing shoot. Free IAA may be limiting for plant growth, but the fact that this free hormone remains in equilibrium with its conjugates suggests a new paradigm that influences plant growth (Basak, 2013). It is proposed that the non-hormone moiety is involved in targeting of IAA within the plant or itself act as messenger (Michalczuk and Bandurski, 1982).

2.1.5. Inositol derived antibiotics

Streptomycin and Kanamycin are some antibiotics which contains diamino and diaminodeoxy derivatives of scyllo-inositol. Production of such aminoglycoside antibiotics is the reason for the development of an era of synthetic carbohydrates (Umezawa, 1974). Other antibiotic like Hygromycin is an aminodeoxy derivative of neo-inositol, while Kasugamycin has (+) chiro-inositol as its constituent. The derivatives of *myo*-inositol added for the chemical modification of these antibiotics in order to enhance the potency of these drugs (Ogawa, 1999).

Aminoglycosides containing a 2-deoxystreptamine unit are a large class of clinically important antibiotics. It has been found that deoxytriptamine is derived from D-glucose and that 2-deoxy-*scyllo*-inosose alongwith 2-deoxy-*scyllo*-inosamine is the two key intermediates in the biosynthetic pathway for 2-deoxystreptamine containing aminoglycosides synthesis (Goda and Akhtar, 1987). An efficient route for the synthesis of these two key intermediates of deosystreptamine has been established (Yu and Spencer, 2001).

Hygromycin-A produced by *Streptomyces hygroscopicus* provides distinct carbon skeletons for the development of antibacterial agents. The Hygromycin- A biosynthetic gene cluster has been identified, cloned and sequenced. The gene cluster has 29 ORFs of which one is assigned to the biosynthesis of one of the three key moieties of hygromycin A, 2L-2amino-2-deoxy-4,5-*O*-methylene-neo-inositol (Palaniappan *et al*., 2006). The importance of Hygromycin-A lies in the fact that it offeres a disticnt carbon skeleton and binding mode for other antibiotics that targets the ribosome of pathogen bacteria. As such, it has the potential to generate new antibiotics against drug resistant pathogens. Thus, semisynthetic programs based on hygromycin A have become attractive (Hayashi *et al*., 1997; Jaynes *et al*., 1992). It may be safely concluded that *myo*-inositol and its derivatives have great potential as starting materials for design of new bioactive compounds (Ogawa, 1999).

2.2. *Myo*-inositol biosynthesis

Biosynthesis of *myo*-inositol takes place by irreversible isomerization of D-glucose-6-phosphate to L-*myo*-inositol-1-phosphate by a NAD-dependent oxido-reductase, L-*myo*-inositol-1-phosphate synthase (D-Glucose-6-phosphate-1 L-*myo*-inositol-phosphate syntase; EC 5.5.1.4), hereinafter called 'MIPS.' Free *myo*-inositol is generated as the end product of this enzymic reaction by cleaving the intermediate product, L-*myo*-inositol-1-phosphate by a phsopahatse, L-*myo*-inositol-1-phosphate phosphatase (L-*myo*-ionositol-1-phosphate phosphohydrolase; EC 3.1.3.25). Lateron, *myo*-inositol may also be converted to chiro-inositol by epimerization of its C3 hydroxyl group (Larner *et al.,* 2010) [Fig-2.6].

It is proposed that the *myo*-inositol-1-phosphate synthase reaction is consisted of three partial reactions, with the formation of 5-keto-glucose-6-phosphate and *myo*-inosoe-2, 1-phosphate, the two postulated enzyme-bound intermediates. The existence of *myo*-inososе-2, 1-phosphate, is evidenced from the study of partially purified testis enzyme which exhibited the presence of myo-inososе 2, 1-phosphate (Chen and Eisenberg, 1975) that proves the formation of 5-keto-glucose-6-phosphate (Kiely and Fletcher, 1969). Subsequently, an intramolecular aldol condendation of 5-keto-D-glucose-6-phosphate formed by the oxidation of D-glucose-6-phosphate by NAD^+ leads to the cyclization of the molecule and also explains NAD^+ requirement (Kiely and Sharman, 1975).

It has been found that *myo*-inososе-2, 1-phsophate as one of the intermediates, which arose from the cyclization of 5-ketoglucose 6-phosphate and that the rate of cyclization of 5-ketoglucose 6-phosphate was far greater than that of reduction of *myo*-inososе-2, 1-phosphate by NADH (Kiely *et al*., 1974).

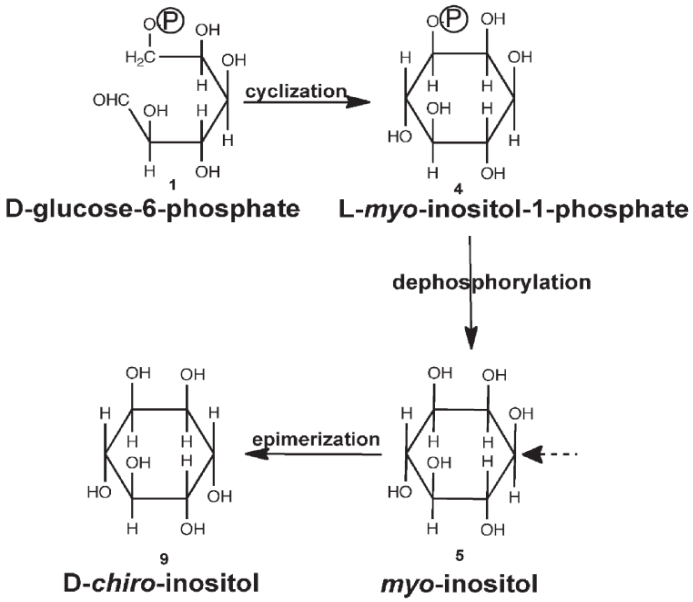


Fig-2.6 Conversion of D-glucose-6-phosphate to *myo*-inositol-1-phosphate by cyclization catalyzed by *myo*-inositol-1-phosphate synthase. *Myo*-inositol-1-phosphate is then dephosphorylated to *myo*-inositol by *myo*-nositol-1-phosphate phosphatase. *Myo*-inositol may be converted by epimerization to D-chiro-inositol.

In lily pollen and wheat germ, the mechanism of producing 1L-*myo*-inositol-1-phosphate (I-1-P) have been studied and it was found that I-1-P was produced *de-novo* by the involvement of MIPS as well as Mg^{2+} dependent ATP kinase respectively. Since MIPS is the sole pathway for biosynthesis of *myo*-inositol from hexose phosphate, the MI kinase pathway functions as salvage mechanism which supplies the I-1-P requirement of the plant cells from stored myo-inositol (Loewus *et al*., 1982).

Myo-inositol is synthesized through cyclization of glucose-6-phosphate molecule (Eisenberg, 1967), it could also be produced by cyclic synthesis (Agranoff *et al*., 1958; Paulus and Kennedy, 1960) and by the hydrolysis of phosphatidylinositol which makes its biosynthesis a unique process. In the synthetic and cyclic pathways, two different forms of compounds are produced, the L-enantiomer and D-enantiomer of *myo*-inositol

respectively though the intermediate, *myo*-inositol 1-phosphate is same in both pathways (Parthasarathy and Eisesnberg, 1986). Both these compounds are hydrolyzed by *myo*-inositol-1-phosphatase (Eisenberg, 1967) generating *myo*-inositol in the process. Thus *myo*-inositol-1-phosphatase (EC: 3.1.3.25) is crucial in the biosynthesis of free *myo*-inositol from inositol-1-phosphate (Nigou and Besra, 2002).

The *de-novo* generation of *myo*-inositol occurs by a universal mechanism that is conserved throughout the phylogenetic domain (Majumder *et al.*, 2003). L-*myo*-inositol-1-phosphate synthase (EC: 5.5.1.4) is a rate-limiting enzyme that catalyzes the first step in the biosynthesis of all *myo*-inositol containing compounds (Seelan *et al.*, 2009). It converts D-glucose-6-phosphate (G-6-P) to L-*myo*-inositol-1-phosphate (I-1-P). The phosphate moiety in I-1-P is subsequently removed by inositol monophosphatase 1 (IMPase) to produce free *myo*-inositol (Majumder and Biswas, 2006).

2.3. L-*myo*-Inositol-1-phosphate synthase from plant system

2.3.1. Occurrence of L-*myo*-Inositol-1-phosphate synthase in different plants

L-*myo*-inositol-1-phosphate has been detected from a number of genera across the divisions in plant kingdom (Table 2.1). Among algae, the enzyme has been detected in *Euglena gracilis*, *Chlorella vulgaris*, *Spirogyra maxima* and *Microspora willeana* while 7 day old culture *Euglena gracilis* produced noteworthy synthase activity (Dasgupta *et al.*, 1984). The enzyme has been purified to electrophoretic homogeneity from *Spirulina platensis* and *Euglena gracilis* (RayChoudhuri *et al.*, 1997). From fungi, inositol synthase activity was recorded in *Saccharomyces cerevisiae* (Donahue and Henry, 1981a), *Neurospora crassa* (Pina *et al.*, 1978), *Polyporus anthelminticus, Ganoderma lucidum, Irpex flavus*, *Agaricus compestris, Schizophyllum commune, Lentinus*

subnudus and *Scleroderma sp*. (Dasgupta *et al*., 1984). In the fungus *Cryptococcus neoformans* both the synthesis and catabolism of *myo*-inositol has been studied (Molina *et al.*, 1999).

Among bryophytes, e.g. *Lunularia sp., Targionia sp., Marchantia polymorpha, Dumortiera sp*, of the order Marchantiales, appreciable degrees of *myo*-inositol synthase (MIPS) were recorded. In *Marchantia polymorpha*, the synthase activity was found to be associated with the development of the reproductive structures. Similarly, MIPS activity was detected in *Marchantia nepalensis* in the reproductive part bearing thallus (Chhetri *et al*., 2006). The enzyme was also detected from from another liverwort, *Lunularia cruciata* (Chhetri *et al*., 2009). The presence of *myo*-inositol synthesizing activity in algae, fungi and bryophytes suggests a more ubiquitous occurrence of this cyclitol and a more general physiological significance of the same in the lower plant groups (Dasgupta *et al*., 1984). From the pteridophytes, inositol synthase activity was detected from the reproductive pinnules of *Diplopterygium glaucum* (Chhetri *et al*., 2006). The enzyme was also isolated from other pteridophytes lik *Azolla filiculoides* (Benaroya *et al*., 2004), *Lycopodium clavatum* and *Selaginella monospora* (Basak *et al*., 2012). Among gymnosperms, the activity of L-*myo*-inositol-1-phosphate synthase was reported from the pollen grains of *Pinus ponderosa* (Gumber *et al*., 1984). The enzyme was partially isolated from the leaves of *Taxus baccata* L. (Chhetri and Chiu, 2004). L-*myo*-inositol-1-phosphate synthase activity has been detected from a wide number of angiosperm genera viz., *Vigna radiata* (Majumder and Biswas, 1973b; Adhikari and Majumder, 1983), *Lemna gibba* (Ogunyemi *et al*., 1978), *Lilium longiflorum* (Sherman *et al*., 1981), *Citrus paradisi* (Abu-abied and Holland, 1994), *Arabidopsis thaliana*

(Johnson and Sussex, 1995), *Phaseolus vulgaris* (Johnson and Wang, 1996), *Thymus vulgaris* (Ray Choudhuri *et al.,* (1997), *Swertia bimaculata* (Chhetri *et al*., 2008) etc.

Most of the L-*myo*-inositol-1-phosphate synthase activity in plants is present in the soluble or cytosolic fractions of the cell. However, about 10% of phosphoinositides (membrane phospholipids) are present in the chloroplast of green plant (Imhoff and Bourdu, 1973), which requires an endogenous pool of *myo*-inositol for their synthesis. Chloroplast membrane is impermeable to the cyclitol (Wang and Nobel, 1971) and points towards the fact that the chloroplast may be the site for the synthesis of *myo*-inositol. Labelled glucose was incorporated into *myo*-inositol in chloroplast preparation from pea (Imhoff and Bourdu, 1973). Similarly chloroplastic inositol synthase has also been reported from *Euglena gracilis* (Loewus *et al*., 1986; Adhikari *et al*., 1987), *Vigna radiata* seedlings (5 to 7 days old) grown under alternate light/dark conditions (Adhikari *et al*., (1987) and *Diplopterygium glaucum* (Chhetri *et al*., 2006c). Moreover, plant species belonging to thallophytes, monocots and dicots contains both chloroplastic and cytosolic forms of *myo*-inositol synthase (Ray Choudhury *et al*., 1997). Tissue specific MIPS activity localized in the outer integumentary areas of developing embryo in soybean seeds have been detected using immunolocalization techniques.This enzyme activity was associated with oxalate crystal idioblasts (Cheira and Grabau, 2007). Expression of MIPS activity was also found in different organs e.g pollen grains, ovules, leaves and in different stages of seed development. The activity showed differential expression under heat and cold stress conditions suggesting its role in abiotic stress response (Abreu and Aragao, 2007). During embryogenesis of mungbean seeds, the expression of MIPS activity was found to be recorded 7-9 days after flowering (Wongkaew *et al*., 2010).

Table -2.1. Distribution of L-*myo*-inisitol-1-phosphate synthase in plant kingdom

Origin	Source	Tissue	Reported by
ALGAE	*Euglena gracilis*	Whole plant	Dasgupta *et al.,* (1984)
	Chlorella vulgaris	Whole plant	Dasgupta *et al*., (1984)
	Spirogyra maxima	Whole plant	Dasgupta *et al*., (1984)
	Microspora willeana	Whole plant	Dasgupta *et al.,* (1984)
	Spirulina platensis	Cultured cells	Ray Choudhuri *et al.,* (1997)
	Enteromorpha linza	Vegetative	Loewus & Loewus (1971)
FUNGI	*Saccharomyces cerevisiae*	Whole plant	Donahue & Henry (1981)
	Neurospora crassa	Whole plant	Pina *et al.* (1978)
	Polyporus anthelminticus	Whole plant	Dasgupta *et al.,*(1984)
	Ganoderma lucidum	Whole plant	Dasgupta *et al*., (1984)
	Irpex flavus	Whole plant	Dasgupta *et al*., (1984)
	Agaricus compestris	Whole plant	Dasgupta *et al*., (1984)
	Schizophyllum commune	Whole plant	Dasgupta *et al*., (1984)
	Lentinus subnudus	Whole plant	Dasgupta *et al*., (1984)
	Scleroderma sp.	Whole plant	Dasgupta *et al.,* (1984)
	Cryptococcus neoformans	Whole plant	Molina *et al*., (1999)
BRYOPHYTES	*Lunulariacruciata*	Whole plant,	Dasgupta *et al*., (1984)
	Lunularia cruciata	Reproductive thallus	Chhetri *et al.,* (2009)
	Targigonia sp.	Whole plant	Dasgupta *et al,* (1984)
	Marchantia polymorpha	Sex organs	Dasgupta *et al.,* (1984)
	Dumortiera sp.	Whole plant	Dasgupta *et al*., (1984)
	Marchantia nepalensis	Reproductive thallus	Chhetri *et al*., (2006)
	Brachymenium bryoides	Whole plant	Yonzone *et al.,* (2018)
PTERIDOPHYTES	*Diplopterygium glaucum*	Reporoductive pinnules	Chhetri *et al.,* (2006, 2007)
GYMNOSPERMS	*Pinus ponderosa*	Pollen grains	Gumber *et al*., (1984)
	Taxus baccata	Leaves	Chhetri and Chiu (2004)
ANGIOSPERMS	*Vigna radiata*	Seed	Majumder & Biswas (1973b)
	Acer pseudoplatanus	Cultured cells	Loewus & Loewus (1971)
	Lemna gibba	Whole plant	Ogunyemi *et al*., (1978)
	Lilium longiflorum	Pollen	Sherman *et al*., (1981)
	Phaseolus vulgaris	Embryo,root,leaf	Loewus & Loewus (1971)
	Oryza sativa	Callus	Johnson & Wang (1996)
	Oryza sativa	Leaves	Funkhouser & Loewus (1975)
	Hevea latex	Latex serum	Ray Choudhuri *et al* (1997)
	Vigna radiata	Leaves	Loewus *et al.,* (1986)
	Thymus vulgaris	Leaves	Ray Choudhuri *et al.,* (1997)
	Rosemarinus officinalis	Leaves	Loewus & Loewus (1971)
	Petroselinum crispum	Leaves	Loewus & Loewus (1971)
	Lemna perpusilla	Whole plant	Loewus & Loewus (1971)
	Salvia officinalis	Leaves	Loewus & Loewus (1971)
	Swertia bimaculata	Leaves	Chhetri *et al.,* 2008

2.3.2 Isolation and characterization of L-*myo*-inositol-1-phosphate synthase of plant origin

L-*myo*-inositol-1-phosphate synthase was purified and characterized from *Saccharomyces cerevisiae* by conventional enzyme purification techniques (Donahue and Henry, 1981b). The M_r of the enzyme determined by gel-filtration chromatography was 240 kDa with a subunit molecular weight of approximately 62 kDa. Some mutants of *Saccharomyces cerevisiae* auxotrophic for inositol which cannot synthesize inositol containing phospholipids exhibited 'inositol-less-death' when deprived of inositol (Culbertson and Henry, 1975). This may be due to the important role played by inositol lipids during the growth of the fungi (Strauss, 1958). Inositol synthase was also purified to homogeneity from another fungus, *Neurospora crassa* by Escamilla *et al*., (1982). The M_r estimated was 345 kDa and the subunit M_r was 59 kDa and the enzyme showed a hexameric structure which was stimulated by 10 mM $(NH_4)_2SO_4$ and also by 50 mM KCl. The same enzyme purified from *Acer pseudoplatanus* had M_r of about 150 kDa (Loewus and Loewus, 1971) while the enzyme from *Lemna gibba* had a M_r of about 135 kDa (Ogunyemi *et al*., 1978).

The enzyme was partially purified and characterized from a gymnosperm, *Pinus ponderosa* pollen grains (Gumber *et al*., 1984). The gymnospermic MIPS showed maximum activity at pH 7.25 to 7.75. The K_m for its substrate, D-glucose-6-phosphate was 0.33 mM. Inhibition by parachloromercurobenzoate and N-ethyl-maleimide and partial protection against this inhibition by G-6-P in the presence of NAD^+ suggested that there was a SH-group involvement at the substrate-binding site. Gymnospermic MIPS was also purified from the needles of *Taxus baccata* L. and the enzyme was

found to be highly stimulated by NH_4^+ , the V_{max} and K_m of the enzyme for its substrate, glucose-6-phosphate and NAD^+were 2.95mM and 1.05 mM respectively (Chhetri and Chiu, 2004). L-*myo*-inositol-1-phosphate synthase was isolated and partially purified from the alga, *Euglena gracilis* by Dasgupta *et al*., (1984). The enzyme exhibited total inhibition by SO_4^{2+}, a pH optimum of 7.5 and the K_m for G-6-P was 2.1 mM. Deduction of endogenous NAD^+, reduced the enzyme activity to about 30% suggesting the occurrence and important role played by NAD^+ in the enzyme from the experimental plant.

L-*myo*-inositol-1-phosphate synthase had also been partially purified from chloroplasts of 6-day-old *Vigna radiata* seedlings. The enzyme exhibited its optimum activity at a pH of 7.5 to 7.75 and the presence of NH_4Cl (9 mM) caused a 2-fold stimulation of the enzyme activity. The chloroplastic MIPS showed K_m of 1.8 mM and 0.13 mM for G-6-P and NAD^+ respectively (Adhikari *et al.*, 1987). Cytosolic and chloroplastic forms of inositol synthase from *Euglena gracilis*, *Oryza sativa* and *Vigna radiata* have been isolated, purified and characterized. The enzyme from these sources differ only in the molecular mass of the chloroplastic and cytosolic native holoenzymes, which appeared to be due to the association of either three or four equal subunits constituting the holoenzyme (Ray Choudhuri *et al*., 1997). From pteridophytic source, MIPS have been isolated and characterizd for the first time from *Diplopterygeum glaucum*. The pteridphytic MIPS showed pH optima between 7.0 and 7.5 while the temperature maxima was 35^oC. The *Km* for its substrate G-6-P and co-factor NAD^+ were found to be 0.83mM and 0.44 mM respectively. Similarly, the V_{max} values were 1.42 and 1.8mM for G-6-P and NAD^+ respectively (Chhetri *et al*., 2006a, 2006b, 2006c).

2.4. Metabolism of inositol phosphates

2.4.1. Various *myo*-inositol phosphates in plants: From mono- to hexakisphosphate

Biosynthesis of phosphate derivatives of *myo*-inositol has been studied in quite details by several wokers (Loewus, 1968; Molinary and Hoffman-Ostenhof, 1968; Asada *et al*., 1969; Tanaka *et al*., 1976; Stephens and Irvine, 1990; Mandal and Biswas, 1970) and it has been reported that higher inositol phosphates are directly produced by the phosphorylation of lower inositol phosphates in germinating mung bean seeds. The enzyme, phosphoinositol kinase was found activated during the formation of seeds, but at a later stage of maturation it declines (Majumder *et al*., 1972). *Myo*-inositol monophosphate can be phosphorylated to higher derivatives upto IP_6 by the same enzyme in presence of ATP serving as the phosphate donor. The reaction kinetics of phosphoinositol kinase suggests that accumulation of *myo*-inositol phosphate is not significant in quantity during the biosynthesis of hexaphosphate. This observation is in consonance with the *in vivo* experiment where appreciable accumulation of other *myo*-inositol phosphates but IP_6 has been found. This inhibitor of phosphoinositol kinase accumulates during last stages of ripening and is destroyed or becomes ineffective during early hours of germination (Majumder and Biswas, 1973b).

Myo-inositol monophosphate is the precursor, from which *myo*-inositol hexaphosphate is biosynthesized, utilizing two enzymes, *myo*-inositol-1-phosphate synthase and *myo*-inositol kinase. The existence of inositol synthase in plant system has been well documented. The existence of inositol kinase has been reported from germinating mung bean seeds (Dietz and Albersheim, 1965) and the formation of *myo*-inositol-1-phosphate

from *myo*-inositol kinase was concluded to be the initial step during the biosynthesis of inositol hexakisphosphate in seeds (English *et al*., 1966).

In plants, different isomers of inositol monophosphate are found viz. I(1)P, I(2)P, I(3P) and I(4)P. The biosynthesis of I(3)P takes place by inositol synthase as well as by an inositol kinase. The hydrolysis of $I(1,4)P_2$ produced the two forms of inositol monophosphate, I(1)P and I(4)P (Joseph *et al*., 1989; Memon *et al*., 1989; Drobak *et al*., 1991; Martinoia *et al*., 1993). Furthermore, I(2)P, is formed as a consequecce of acid–phytase catalyzed hydrolysis of phytic acid (Cosgrove, 1980).

In plants, inositol bisphosphate (IP_2) are of different types of which $I(1,2)P_2$, $I(1,4)P_2$ and $I(4,5)P_2$ are formed by the hydrolysis of phytic acid while $I(1,4)P_2$ and $I(4,5)P_2$ are formed as a breakdown product of $I(1,4,5)P_3$. In addition, the action of PI-PLC on PI(4)P also produces $I(1,4)P_2$ (Murthy, 1996).

The interest in the study of inositol-triphosphate generated with the discovery of second messenger role played by inositol triphosphate, $I(1,4,5)P_3$ (Drobak *et al*., 1991; Martinoia *et al*., 1993). This triphosphate $I(1,4,5)P_3$, is formed by the action of PI-PLC on $PI(4,5)P_2$ (Huang *et al*., 1994). Beside $I(1,4,5)P_3$, other two triphosphates, $I(1,2,3)P_3$ and $I(1,2,6)P_3$ are also formed as the acid phytase catalyzed intermediate products of phytic acid (Cosgrove, 1980). The 1,4,5-triphosphate [$Ins1,4,5)P_3$] is metabolized to inositol 1,3,4,5-tetraphosphate [$Ins(1,3,4,5)P_4$] by a 3-kinase which subsequently by the inositolphosphate-5-phosphomonoesterase converted to three different inositol triphosphate isomers, $Ins(1,3,5)P_3$, $Ins(1,3,4)P_3$. $Ins(1,3,4)P_3$, which in turn gets

converted to inositol 3,4-bisphosphate by a different enzyme, inositol polyphosphate-1-phosphatase. This bisphosphate is further converted to inositol-3-phosphate. Similarly, the existence of Ins(1,2,3) P_3, Ins(3,4,6) P_3, Ins(2,4,5) P_3, Ins(3,5,6) P_3, Ins(1,2,6) P_3, Ins(1,5,6) P_3 etc in aleurone tissue in *S. polyrhiza* and *Arabidopsis* suggest that all these isomers are the products of Ins P_6 metabolism *in-vivo.* Action of kinase over I(1,4,5)P_3 produces inositol-tetrakisphosphates [I(1,4,5,6)P_4] in plants. Different other isomers of inositol-tetrakisphosphates viz., I(1,2,3,6)P_4, I(1,2,5,6)P_4 and D or L-I(1,2,3,4)P_4 are reported to be produced in pants by enzyme catalyzed hydrolysis of phytic acid (Barrientos *et al*., 1994). Ins(1,3,4,5)P_4, another inositol-tetraphosphate is produced by phosphorylation of Ins(1,4,5) P_3 by an enzyme β-kinase (Irvine,1999). Ins(1,3,4)P_3 may be converted to (1,3,4,5)P_4 or (1,3,4,6)P_4 by the action of Ins 1,3,4-triphosphate kinase-2 (Marathe *et al.*, 2018).

Myo-inositol (1,3,4,5,6) pentakisphosphate may be produced from I(1,4,5)P_3 (Stephens *et al*., 1988) by phosphorylating I(3,4,5,6)P_4, It was found that I(1,3,4,5)P_4, I(1,3,4,6)P_4 and I(1,3,4)P_3 are the intermediates in inositol pentakisphosphate synthesis pathway. Most probably, either I(3,4,5,6)P_4 was synthesized from I(1,4,5)P_3 by an unknown route or a second IP_4 might also act as an intermediate in the formation of IP_5 (Stephens *et al*., 1988).

Inositol hexakisphosphate or phytic acid is the major form of phosphorous accumulating in large amounts in the storage tissues and it may limit the availibity of minerals (Aggarwal *et al.*, 2018). 75% of the phosphate is stored as phytin in matured seeds and during germination this stored phytin is decationized and hydrolyzed by phytases for the

germination of embryo (Raboy, 2003). The biosynthesis of phytic acid (inositol hexakisphosphate) may take place either by the sequential phosphorylation of inositol by kinases (Majumder and Biswas, 1973a, 1973c; Biswas *et al.*, 1978; Igaue *et al*., 1980) or by the ultimate hydrolyis of sequential phosphorylation of an inositol derivative (Raboy, 1990; Drobak, 1992). In general, there are two $InsP_6$ generating pathways, a) a lipid- dependent pathway via PLC and b) a lipid-independent pathway via direct phosphorylation of MIPS- generated Ins3P (Raboy 2003; Stevenson-Paulik *et al.*, 2005) [Fig-2.7].

As per lipid dependent pathway, PtdIns(4,5)P_2 formed from *myo*-inositol is hydrolyzed by PLC generating Ins(1,4,5)P_3, which is phosphorylated into IP_6 in stepwise manner by IPP multikinase2(IPK2), inositolpolyphosphate 3-/5-/6-kinase, IPK1, inositol polyphosphate 2-kinase in sequence (Stevenson-Paulik *et al*., 2005; Sweetman *et al*., 2006; Murphy *et al*., 2008). Through the lipid-independent pathway the Ins3P formed by MIPS by the cyclization of G-6-P is directly phosphorylated to Ins(3,4)P_2 and further via Ins(3,4,6)P_3 to Ins(3,4,5,6)P_4 and Ins(1,3,4,5,6)P_5 and finally to $InsP_6$. This system describes the endogenous inositol phosphates in any plant tissues. This sequence differs from that reported in the slime mould *Dictyostelium discoideum* (Brearley and Hanke, 1996) where IP_6 synthesis is catalyzed by a series of soluble ATP dependent kinases.

Besides these pathways, other possible pathways are there in different plant organs and across species to generate IP_6. The mammalian cells seem to use the lipid-dependent pathway, to synthesize $InsP_6$ while in plants it may be synthesized by either pathways based on the tissue type (Irvine, 2003, 2005; York, 2006; Alcazar-Roman and Wente, 2008; Letchar *et al*., 2008; Shears, 2001, 2009).

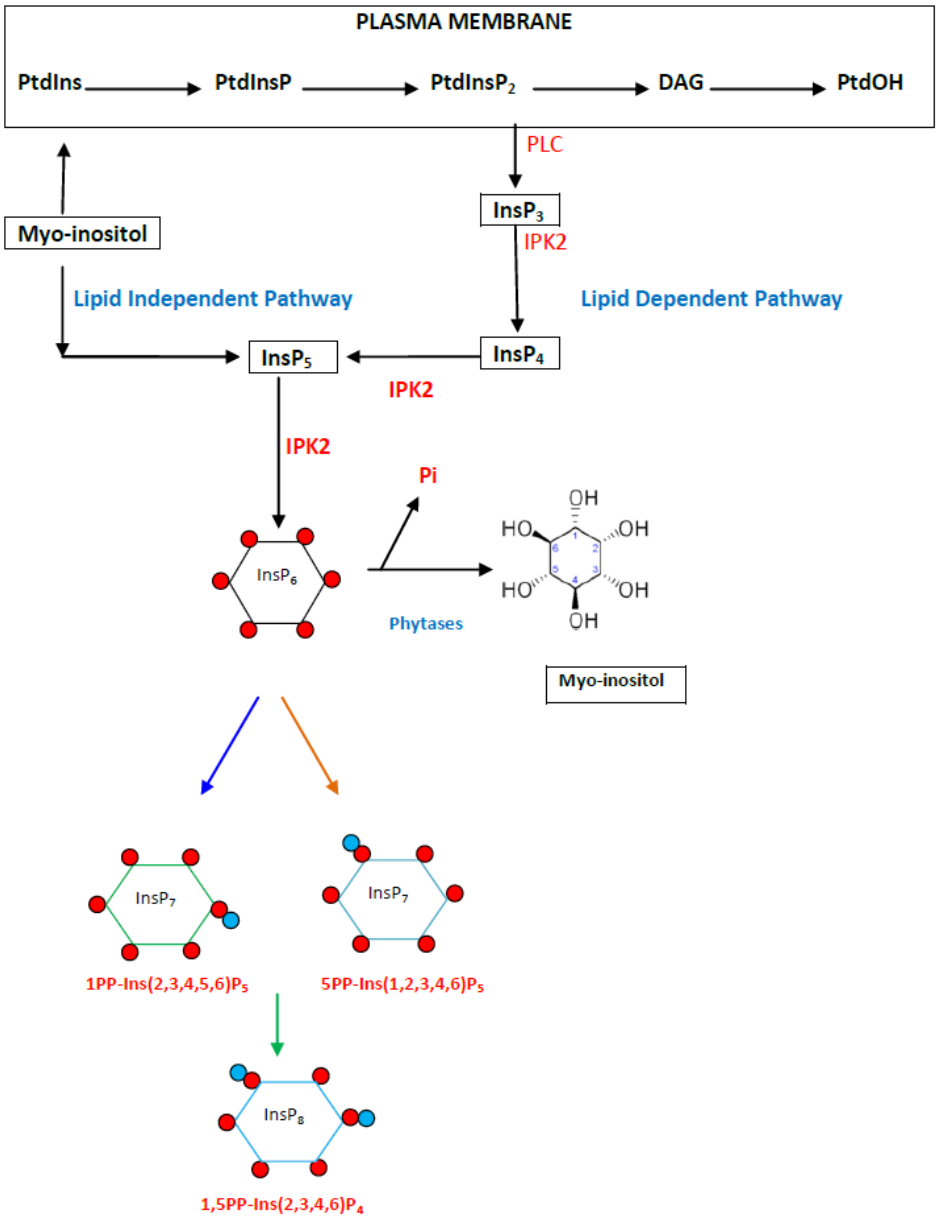


Fig-2.7 Pathway for the generation of $IP_{6,}$ other inositol phosphates, inositol pyrophsphate

2.4.2. Occurrence of other *myo*-inositol phosphates

Formation of inositol pyrophosphates, diphosphoinositol pentakisphosphate (PP-IP_5) and bis-diphosphoinositol tetrakisphosphate [$(PP)_2$-IP_4], containing seven and eight phosphates respectively in inositol ring indicates that even after the formation of $IP_{6,}$ it

may be metabolized to other moleculesis having higher number of phosphate groups. This phenomenon is supported by the discovery of an IP_6-kinase with a high affinity and specificity for IP_6 and the capacity to transfer a phosphate from PP-IP_5 to ADP to form ATP. This ATP synthase activity demonstrates a high phosphoryl group transfer potential for PP-IP_5 suggesting its physiological role (Voglmaier *et al.*, 1997). Pyrophosphate containing inositol molecules have been detected by Stephens *et al.*, (1993) from *Dictyostelium discoideum*, a slime mold. Inositol pyrophosphates are largely synthesized from $IP_{6,}$ generating IP_7 and IP_8. These IP_7 pyrophosphate also link to metabolism at the molecular (phosphate homoeostasis), cellular (energetic) and organismal (insulin signaling) levels (Wilson *et al*., 2013).

The identification of inositol pyrophosphates (inositol with diphosphate groups), with different arrangement of phosphate, diphosphoinositol pentaphosphate [IP_7 or PP-IP_5 or $(PP)_2$- $InsP_3$] (Draskovic *et al.*, 2008; Wilson *et al.*, 2013) and two diphosphate and four monophosphates [IP_8 or $(PP)_2$-IP_4] (Shears, 2009) or with one triphosphate and five monophosphates [IP_8 or (PPP)-IP_5] with seven and eight phosphate groups attached to the six-carbon inositol ring, thus possessing one and two pyrophosphate moieties respectively indicates that IP_6 is the precursor of these pyrophosphates (Draskovic *et al.*, 2008). Inositol pyrophosphates are largely synthesized from IP_6 generating IP_7 and IP_8. Two isomeric forms of IP_8 has been presented in mammalian cells, $[(1,5)PP]_2$-IP_4 and [$(PP)_2$-IP_4] but the plant *D. discoideum* contains $[(5,6)PP]_2$-IP_4 isomers of IP_8. Therefore, taking into account the diverse substrates and the formation of pyrophosphate moiety at different positions of the inositol ring, inositol pyrophosphate

have the potential to become a very large family of molecule (Losito, 2009; Wilson et al., 2013).

2.5. Signalling in plants through inositol phosphates

2.5.1. Signal transduction in plant cells

Signalling pathway in general commences with the binding of the effecter molecules (i.e. growth factor, hormone, neurotransmitters etc) to its cognate receptor on the cell surface. This binding activates a protein kinase and enzymes that leads to the activation of signalling molecules, eventually resulting in the transcription of specific genes (Barik, 1996). Many signal transduction processes occurs when plants are challenged with environmental stresses. Thus low/high temperature, drought and high salinity are very complex stimuli that possesses many different yet related attributes, each of which may provide the plant cell with quite different information for example, low temperature may immediately result in mechanical constraints, changes in activities of macromolecules and reduced osmotic potential in the cellular milieu. High salinity includes both an ionic (chemical) and an osmotic (physical) component. The multiplicity of information embedded in abiotic stress signals underlines the complexity of stress signaling.

On the basis of this multiplicity, it is unlikely that there is only one sensor that perceives the stress condition and controls all subsequent signalling. Rather a single sensor might only regulate branches of the signalling cascade that are initiated by one aspect of the stress condition. For example, low temperature is known to change membrane fluidity (Murate and Los, 1997). A sensor defeacting this change would initiate a signalling

cascade control initiated by an intracellular protein whose conformation/activity is directly altered by low temperature. Thus, there may be multiple primary sensors that perceive the initial stress signal.

Secondary signals (i.e. hormones and second messengers) can initiate another cascade of signalling events, which can differ from the primary signalling in time (i.e. lag behind) and in space (e.g. the signals may diffuse within or among cells, and their receptors may be in different sub-cellular locations from the primary sensors). These secondary signals may also differ in specificity from primary stimuli, may be shared by different stress pathways, and may underline the interaction among signalling pathways for different stresses and stress cross-protection. Therefore, one primary stress may activate multiple signalling pathways differing in time, space and outputs. These pathways may connect or interact with one another using shared components generating interwtined networks. Arachidonic acid, inositol 1.4.5-triphosphate (IP_3) and 1.2-diacylglycerol (DAG) are three different messenger molecules known to be produced from phosphoinositides. All three different messenger molecules signal differently. Arachidonate is oxygenated to form other signaling mediators like IP_3 which mobilize Ca^{2+} from its intracellular storage and DAG which performs the function of an essential cofactor of protein kinase-C (Majerus *et al.*, 1986).

2.5.2. Inositol triphosphate and calcium signalling

One early response to low temperature, drought and salinity stress in plant cells is a transient increase in cytosolic Ca^{2+}, derived from internal stores (Sanders *et al*., 1999; Knight, 2000). In the cascade of events Ca^{2+} plays a pivotal role for the conversion of an external stimulus into biological responses in plant cells (Ranjeva *et al.*, 1998). Internal

Ca^{2+}, release is controlled by ligand sensitive Ca^{2+} channels. It is thus crucial to modulate the entry of external calcium through the plasma membrane and the release of Ca^{2+} from intracellular stores to maintain its cytosolic concentration. Ca^{2+} is released from its intracellular storage which is accompanied by the breakdown of phosphatidylinositol 4.5-bisphosphate to release IP_3. The role of IP_3 coupled to the intracellular Ca^{2+} mobilization was determined from a preparation of saponin permeabilized hepatocytes. Ca^{2+} was released from non-mitochondrial vesicular store and the induction of Ca^{2+} release by IP_3 along with its kinetics and properties strongly suggests that this compound is a second messenger (Joseph *et al*., 1984).

Inositol polyphosphates, cyclic ADP ribose and nicotinic acid, adenine dinucleotide phosphate has been found to be able to induce Ca^{2+} release in plant cells and in general and guard cells in particular (Schroeder *et al*., 2001). An important feature of the role of Ca^{2+} as a signalling molecule is the presence of repetitive Ca^{2+} transients. These transients may be generated both by first round of second messengers and by other signalling molecules such as ABA that may themselves be produced as Ca^{2+} signals may have quite different signalling consequences and therefore, physiological meaning.

In plant cells, there is more than one membrane that serves as a major compartment for the sequestration and storage of Ca^{2+} with steep electrochemical gradients. Plant vascular membranes (tonoplast) possess an electrogenic H^+ pumping-ATPase (inwardly directed) that energize for the Ca^{2+} accumulation via a H^+/ Ca^{2+} antiport mechanism (Schumaker, 1986). ER may also serve as an intracellular calcium store and act as an alternative source of mobile calcium within the same cell. At least 3μM Ca^{2+}

concentration has been measured within isolated ER vesicles from aleurone cells (Bush *et al.*, 1989). Franklin-Tong *et al*., (1996) showed that Ca^{2+} waves in pollen tubes are triggered by photolysis of caged $InsP_3$ which is initiated in the nuclear-RER. Therefore there is some evidence that calcium release may occur from the ER in response to $InsP_3$ (Martinec *et al*., 2000, Choi *et al.,* 2018).

Calcium is responsible for signal transduction cascades which manifests as different physiological responses in plantss (Gilroy *et al*., 1993). In plant cells, though all the elements of calcium based transduction system are there still its detection is difficult. The IP_3 based signaling system is detectable only during the development of guard cells, pulvini and stem epidermal cells that are constantly responding to stimuli (Trewavas and Gilroy, 1991). Abscisic acid activates the channels allowing Ca^{2+} influx to the cytosol through the plasma membrane (Schroeder and Hagiwara, 1990). Free Ca^{2+} is a major structural component of the cell wall in plants and the concentration of free Ca^{2+} in the cell wall regulates growth, development and physiological responses of plants to external stimuli (Trewavas and Gilroy, 1991).

IP_3 acts to mobilize calcium from intracellular stores and constitutes an IP_3/Ca^{2+} pathway. Thus IP_3 plays a central role in the second messenger system by its ability to release Ca^{2+} (Berridge, 1987). Besides mobilization of internal calcium through IP_3, the concentration of Ca^{2+} may also increase due to the influx of external calcium. Both IP_3 and IP_4 (inositol 1,3,4,5-tretrakisphosphate) may be responsible in the entry of external calcium (Berridge and Irvine, 1989). It is known that the calcium directly flows into the cytosol through channels in the plasma membrane. However, it may also ener into the ER before entering the cytosol. The entry of external Ca^{2+} only happens when the

internal pool of Ca^{2+} is emptied. Therefore, the entry of external Ca^{2+} is coupled to the IP_3 mediated emptying of Ca^{2+} from its storage.

Fig-2.8 describes a general scheme to explain the events from the binding of ligand to its receptor leading to the formation of IP_3, DAG etc. and release of of Ca^{2+} from intracellular storage to the cytosol (Karp, 2013).

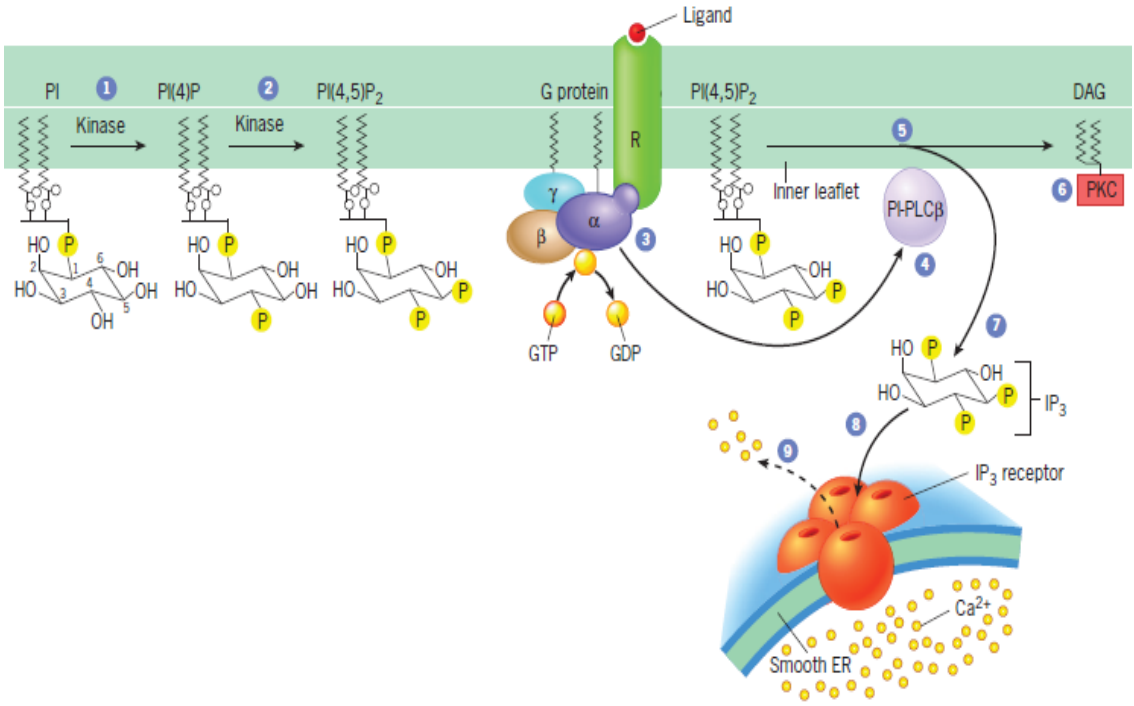


Fig-2.8 Signalling events caused by ligand induced activation of PI in the cell membrabe.Formation of PIP by the addition of phosphate group to PI by kinase (1); formation of PIP_2 by the addition of phosphate group to PIP by another kinase (2); activation of G-protein (3); activation ofe PI-specific phospholipase-C (4); breakdown of PIP_2 to IP_3 and DAG by PI-specific phospholipase-C (5); activation of protein kinase-C (PKC) in the membrane by DAG (6); release of IP_3 in the cytosol (7); binding of IP_3 to its receptor and Ca^{2+} channel in the SER membrane (8) and release of Ca^{2+} in the cytosol (9).

Drobak and Ferguson (1985) first indicated that Ins (1,4,5)P_3 was able to release Ca^{2+} from intercellular stores in plant cells. This release of Ca^{2+} was highly dependent on the

extra vesicular Ca^{2+} activity, which is accompanied by an inward flux of K^+, and the release has been shown to be sensitive to K^+ channel blockers (Canut *et al*., 1985). Gilroy *et al*., 1990 showed a direct evidence for Ins(1,4,5)P_3 -induced Ca^{2+} release in stomatal guard cells.

Besides Ins(1,4,5)P_3, there are other triphosphates (Ins P_3 species) namely Ins(1,3,4)P_3, Ins(1,5,6)P_3 and Ins(2,4,5)P_3, which may execute Ca^{2+} mobilization from microsomes/ vacuoles as well as liposomes to some extent *in-vitro*. For Ca^{2+} release and its mobilization, the prime requirement is the ability of any InsP_3 to bind to its receptor (InsP_3R). In plants the receptor consists of four subunits like its animal counterparts and it binds to both Ins(1,4,5)P_3 and Ins (2,4,5)P_3 with little difference in their affinities. However, the nature of the conformational changes in the InsP_3R induced by Ins(1,4,5)P_3 and Ins (2,4,5)P_3 are different. This conformational change is involved in a membrane-spanning helical domain in InsP_3R, which is required for its property as a Ca^{2+} channel. Ins(1,4,5)P_3 is about four times more effective than Ins(2,4,5)P_3 in Ca^{2+} mobilization from microsomes/ vacuoles in mung bean while Ins(1,3,4)P_3/Ins(1,5,6)P_3 cause an insignificant release of Ca^{2+} because of their poor interactions with the receptor (Gupta *et al*., 1997).

In plants, the level of intracellular I(1,4,5) P_3 increases in resposnse to a variety of extracellular stimuli such as light, pathogens, cell fusion, H_2O_2 and ethanol (Drobak, 1992; Chapman, 1998; Stevenson *et al*., 2000). Transient formation of IP_3 was observed in winter oil-seed rape leaf discs, 30 minutes after exposure to freezing temperature (Smolenska-Sym and Karpareska, 1996). Transient and long term increase in IP_3 was

also seen in *Arabidopsis* cell suspension culture exposed to cold temperature at $0^{o}C$ (Ruelland *et al*., 2002).

Chages in IP_3 level in response to hyperosmotic shock and salinity were also observed in *A. thaliana* cultured cells (T87 cells) [Taji *et al*., 2006]. A rapid and momentary increase in Ca^{+2} within a few seconds of exposure to osmotic stress has suggested that cytoplasmic increase in Ca^{+2} level in response to hyperosmotic stress is due to influx of extracellular stores (Knight *et al*., 1997).

IP_3 formation in plants has also been found to be influenced by phytohormones. Abscissic acid is involved in the plant adaptation to different environmental stress conditions. Endogenous ABA level increases significantly in many plants under water deficit conditions resulting in the tolerance to water stress (Finkelstein *et al.*, 2002; Himmelbach *et al*., 2003). ABA is responsible for inducing the stomatal closure (Schroeder *et al*., 2003). Microinjection of IP_3 in guard cells has been shown to induce stomatal closure (Gilroy *et al*., 1990). A sudden increase in IP_3 level in guard cells has been found in *Vicia faba* in response to ABA treatment (Lee *et al*., 1996). ABA induced closure of stomata under dehydaration conditions is triggered for optimization of loss of water vapour (Hethrington, 2001).

In *Phaseolus coccineus* L. leaf pulvinus, inositol triphosphate [$Ins(1,4,5)P_3$] level increases during darkness (blue light). IP_3 is the second messenger in the PI signalling cascade which increases intracellular Ca^{2+} that in turn increases membrane conductance to close K^+ channel. This intracellular mobilization of Ca^{2+} causes cells to shrink. While

in red light, the second messenger, inositol triphosphate [Ins(1,4,5)P_3] level was decreased so the mobilization of Ca^{2+} was also decreased thereby opening K^+ channel. Therefore, it indicates that in *Phaseolus coccineus*, increased inositol triphosphate production in dark leads to Ca^{2+} mobilization with the shrinking of the cells while light induces swelling the cell by the extracellular Ca^{2+} in the extensor protoplast. Therefore, this movement of the ions in and out of the cell changes turgor of the motor cells within the pulvinus which consequently leads to leaf movement (Morse *et al*., 1987, Cote, 1995; Mayer *et al*., 1997).

2.5.3. Involvement of IP_4, IP_5 and IP_6 in cell signalling.

It is believed that IP_4 controls the transfer of calcium between intracellular pools (Irvine *et al.*, 1988). A specific high affinity receptor for IP_4 has been isolated suggesting that this polyphosphate may have a messenger function (Hawthorne, 1996). IP_4 increases intracellular calcium concentration, provided calcium-mobilizing IP_3 is present. It is believed that this molecule controls the transfer of calcium between intracellular pools (Irvine *et al*., 1988) and stimulates nuclear calcium influx (Theibert *et al*., 1997). Signalling functions attributed to both IP_5 and IP_6 including Ca^{2+} uptake, neurotransmitter release and modulation of desensitization in agonist-stimulated cells have also been postulated (Menniti *et al*., 1993; Sasakawa *et al*., 1995). $InsP_6$ also acts as a signaling molecule (Lemitiri-Chlich *et al*., 2000, 2003). $InsP_6$ which is formed rapidly in response to ABA was found to be about 100 times more potent in releasing Ca^{2+} than $InsP_3$.

The enzyme inositol 5' phosphatase removes a 5' phosphate from several potential second messengers and thus terminates the signal transduction events. A gene encoding

inositol 5' phosphatase activity (At5PTase1) has been described from *Arabidopsis.* When expressed transiently in *Arabidopsis* leaf tissue or ectopically in transgenic plants, At5Ptase1 causes the hydrolysis of $I(1,4,5)P_3$ and $I(1,3,4,5)P_4$ but the enzyme does not hydrolyze I-1-P, $I(1,4)P_2$ or $PI(4,5)P_2$. The multiplicity of At5PTases indicates that these enzymes may have different substrate specificities and play different roles in signal termination (Berdy *et al*., 2001).

Inositol 1,2,3,4,5,6 hexakisphosphate (IP_6) is the most abundant inositol phosphate in cells. This molecule is involved in ABA signal transduction in guard cells and thus plays role in osmostic stress response. IP_6 is also involved in environmental stress response and in *Schizosaccharomyces pombe*, the level of IP_6 increases more than threefold in response to hyperosmotic stress (Ongusaha *et al*., 1998). Increase in IP_6 level in plants in response to dehydration leads to disruption in the activity of ion channels in guard cells. In the guard cells of *Solanum tuberosum* the IP_6 level increased more than five fold with the treatment of ABA. Here IP_6 mimics the effects of ABA that inhibits plasmalemma inward K^+ channel in the guard cells in a Ca^{+2} dependent manner (Lemitiri-Chlich *et al*., 2000). Release of IP_6 in guard cell protoplasts of *Vicia faba* has been shown to induce transient increase in Ca^{+2}. IP_6 did not affect the Ca^{2+} permeable channels in plasma membrane and that it could inhibit the plasmalemma inward K^+ channel without external Ca^{2+} suggests that IP_6 induced transient Ca^{2+} increase within guard cells was not due to external influx of Ca^{2+} but due to the release of Ca^{2+} from internal stores. Therefore, it may be concluded that IP_6 functions as a signaling mediator in ABA signal transuction in guard cells triggering Ca^{2+} release that inhibits inward K^+ channels (Lemitiri-Chlich *et al*., 2003).

2.6. Signalling in plants through phosphoinositides

2.6.1. Occurrence of phosphoinositide system in plants

Phosphoinositides in plants was first reported by Boss and Massel (1985). Since then, a number of reports have appeared indicating the presence of the compound in whole plants and the tissue cultured cells (Lehle, 1990). The occurrence of polyphosphoinositides in plant cells is now an established fact (Strasser *et al*., 1986; Heim and Wagner, 1986; Morse *et al*., 1987; Drobak *et al*., 1988). In plants, plasma membrane is the enriched source of PIP and PIP_2. The membrane-localized phosphoinositides were separated from the pulvinar extract of *Samanea saman*, and the ratio of PI, PIP and PIP_2 was found to be 32: 8:1 (Morse *et al*., 1987). The main phospholipid precursor $PI(4,5)P_2$ has been identified in plant tissues and green algal cells. Kinases that catalyze the sysnthesis of this lipid from phosphatidylinositol have been demonstrated and phosphoinositide specific phospholipase-C which hydrolyze $PI(4,5)P_2$ to yield IP_3 and DAG have been reported (Cote and Crain, 1993).

It has been reported that the plants have the same set of polyphosphoinositols as those of animals (Irvine *et al*., 1989). However, there is a difference between phosphoinositides of higher plants and animal origin in their fatty acid composition. The main fatty acids of plant inositol lipids are palmitic acid, linoleic acid and linolenic acid (Mudd, 1980; Helsper *et al*., 1987). However, there is no conclusive evidence that arachidonic acid is also a component in the higher plants (Lehle, 1990).

The enzyme, phospholipase C (PLC) is responsible for the agonist induced hydrolysis of PIP_2 (phosphatidylinositol 4,5-bisphosphate) to give two important molecules IP_3

(inositol 1,4,5-triphosphate) which acts as a second messenger and DAG. PLC activities have been detected from different plant species namely celery (Irvine *et al*., 1980; McMurray and Irvine, 1988), pollen tubes of *Lilium* (Helsper *et al*., 1987) and soybean and bush beans (Pfaffmann *et al*., 1987), green alga, *Dunaliella salina* (Einspahr *et al*., 1989), *Arabidopsis thaliana* (Hunt *et al*., 2004; Tasma *et al*., 2008, Gaude *et al*., 2008, Munnik and Testerink, 2009), tomato (Vossen *et al*., 2010), potato and rice (Singh *et al*., 2013). PLC activities from all the plant species was stimulated by Ca^{+2} in contrast to the enzymes from animal sources.

The activity of protein kinase C (PKC) is to phosphorylate different proteins. It is important in the sense that one of its substrates is IP_3-phosphatase that terminates the second messenger function of IP_3 (Connolly *et al*., 1986). PKC function in plants has been reported from zucchini (Schafer *et al*., 1985), *Amaranthus tricolor* (Elliot and Skinner, 1986) and *Neurospora crassa* (Favre and Turian, 1987).

Diacylglycerol kinase is another enzyme, which converts DAG into phosphatidic acid (PA) for which the *Arabidopsis thaliana* genome encodes seven genes (Mueller *et al*., 2002; Arisz *et al*., 2009). During the conversion, phosphoinositide synthesis may be initiated while also controlling the intracellular concentration of DAG. A membrane associated DAG-kinase of plant origin has been isolated. This enzyme having a 51 kDa protein has been identified which requires divalent cations but are completely devoid of activity in absence of phospholipid (Wissing *et al*., 1989). PA can also be generated via a separate, PLD signaling pathway (Munnik and Vermeer, 2010). PA which is produced can be further phosphorylated to diacylglycerol pyrophosphate (DGPP), which is

catalyzed by a so- called PA kinase (PAK). This enzyme and DGPP are both implicated in osmotic stress signalling (Schooten *et al*., 2006). The presence of *scyllo*-inositol containing phosphatidylinositol in the plant cells points towards the involvement of this compound in the phosphoinositide and inositol phosphate metabolism in the plant system (Kinnard *et al*., 1995).

$PI(4,5)P_2$ is an important signal mediator in stomatal opening and it is synthesized by the enzyme PIP(5)K4. When irradiated with white light, the level of $PI(4,5)P_2$ increases more at the plasma membrane than in the cytosol of guard cells possibly due to light induced increase in biosyntheisis or decresse in hydrolysis of $PI(4,5)P_2$. The synthesis of $PI(4,5)P_2$ is the rate limiting step in the plant phosphoinositide pathway (Im *et al*., 2007). Another hydrolysis product of $PI(4,5)P_2$ is IP_3 which is already famous for its role on stomatal closing (Gilroy *et al*., 1990, Blatt *et al*., 1990). It is clear that PIP5K4 is responsible for the synthesis of $PI(4,5)P_2$ and sufficient $PI(4,5)P_2$ must be there in the guard cell plasma membrane for stomatal opening to happen (Lee *et al*., 2007). PI-PLC has already been implicatd in the ABA signal transuction in guard cells (Lee *et al*., 2003)

After the discovery of PI system in plants, there have been many studies on its role in stress tolerance and stress signalling (Cho *et al*., 1993; Pical *et al*., 1999; Drobak and Watkins, 2000; Dewald *et al*., 2001; Perera *et al*., 2004; Zonia and Munnik, 2006; Konig *et al*., 2007; Leshem *et al*., 2007; Nishizawa *et al*., 2008; Wang *et al*., 2008; Yang *et al*., 2008; Zhu *et al*., 2009; Munnik and Vermeer, 2010).

2.6.2. Phospholipase-C (PLC) in plants

Phosphoinositide specific phospholipase C (PI-PLC) hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) with the production of two important molecules, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). These two molecules are second messengers that can activate protein kinase C and trigger Ca^{2+} release respectively. Second messenger IP_3 which is produced, is a soluble compound in the cytosol that triggers transient increases in the cytosolic Ca^{2+} level, and a lipid diacylglycerol (DAG) stays within the plasma membrane and activates PKC (Berridge, 1993). It is evident that IP_3 mediated Ca^{2+} release occurs in plant cell (Drobak 1992, 1993; Cote and Crain, 1993, 1994).

The presence of a PtdIns-specific phosphodiesterase (phospholipase C, PLC) in plant tissues was reported by Irvine *et al*., (1980) who found a soluble form of the enzyme in celery stems capable of hydrolysing PtdIns, the resulting products being Ins1P and cyclic Ins(1:2)P. Pfaffmann *et al*., (1987) found a PtdIns-specific PLC activity in plant stems with 90% of the activity being in a soluble form and the remainder associated with membranes. Melin *et al*., (1987) has reported plasma membrane associated PLC activity capable of hydrolysing all three phosphoinositides. This enzyme had a 5-20 times greater activity towards PtdIns4P and PtdIns(4,5)P_2 than the PtdIns. There are two main groups of PLC in plants PLC type I and II. PLC type I is predominantly soluble (cytosolic/ vacuolar) which is reported by Irvine *et al.*,(1980), has a clear preference for PtdIns over PtdIns4P and PtdIn(4,5)P_2 and requires millmolar concentration of Ca^{2+} for full activity, while PLC type II is predominantly associated with the plasma membrane,

which shows marked preference for polyphosphoinositides versus PtsIns and is fully activated by low µM concentration of Ca^{2+} (Drobak,1992).

Molecular cloning of cDNA encoding multiple PI-PLC isoforms have been reported from *Arabidopsis thaliana* (Yamamoto *et al.*, 1995; Hirayama *et al*., 1995, 1997) and *Glycine max* (Shi *et al.*, 1995). *Arabidopsis thaliana* contains nine PLCs (Roeber and Pical 2002; Munnik and Testerink, 2009), but out of nine, only seven are likely to be catalytically active (Hunt *et al.*, 2004; Tasma *et al.*, 2008). *Arabidopsis thaliana* contains six of them, which are called NPCs for non specific PLC (Gaude *et al.*, 2008). Similarly, tomato contains six (Vossen *et al*., 2010), rice contains nine (Singh *et al*., 2013), and potato contains three phosphoinositide-specific phospholipase C isoforms.

PLC in eukaryotes are classified into six different subfamilies, PLCβ,γ,ε,π,δ and ζ (Munnik and Testerink, 2009). Out of these six different subfamilies, PLCβ isoform is activated by the heterotrimeric regulatory GTP-binding protein belonging to the Gq subfamily (Wu *et al.*, 1993), while in *Dunaliella salina* , the plasma membrane polyphosphoinositide-specific PLC is activated by 100 µM –GTPγS over a range of free Ca^{2+} concentration . Evidence in favour of involvement of G-protein in PLC activation has also been presented by Dillenschneider *et al.*, (1986) and it was who found that guanine nucleotide stimulated the release of inositol phosphates from ^{3}H-inositol labelled membranes isolated from cultured sycamore cells.

Environmental signals controls the aperture of stomatal pore by activiating guard cell signalling pathways that result in alterations to guard cell turgor (Schroeder *et al*.,

2001). The control of guard stomatal opening is mediated by ABA which acts by increasing cytosolic Ca^{2+} concentration (Schroeder and Hagiwara, 1990; Blatt, 2000). ABA may generate the increased level of guard cell Ca^{2+} concentration by the involvement of phosphoinositide specific phospholipase-C (Gilroy, 1990; Lee *et al.*, 1996, Staxen *et al*., 1999). It is believed that in addition of IP_3 and possibly IP_6 (Lemitiri-Chlieh *et al*., 2000) may act as Ca^{2+} mobillising compound in guard cell ABA signaling (MacRobbie, 2000). On the other hand, PLD has also been found to be involved in ABA induced stomatal closure (Sang *et al*., 2001), which is interestingly independend to elevation in Ca^{2+} level (Jacob *et al.*, 1999). In ABA induced guard cell signalling, one of the six different mechanisms may be utilized for the increase in cytosolic Ca^{2+} concentration which in turn is dependent on ABA concentration (MacRobbie, 2000). In this view, it may be safely concluded that guard cell ABA signaling pathway is characterized by flexible connections rather than being hardwired (Lee *et al.*, 2003).

2.6.3. Phosphoinositide signalling during osmotic stress

Osmotic stress is a sudden change in the solute concentration around a cell causing a rapid change in the movement of water across its cell membrane. There are two types of osmotic stress: hypo and hyperosmotic stresses. All organisms have mechanisms to respond to osmotic shock depending on the nature and level of stress with sensors and signal transduction networks providing information to the cell about the osmolarity of its surroundings, these signals activate responses to deal with extreme conditions and

thus re-establish the osmotic balance. Different phosphoinositide isomers have been identified in plant cells which help to maintain osmotic equilibrium, (Munnik, 2010).

Plants have evolved several mechanisms to respond to changes in the extracellular osmotic potential. Sudden shifts of extracellular osmotic gradients induce dynamic changes in ion fluxes across the plasma membrane as an early osmoregulatory response (Schroeder and Hagiwarra 1989; Ivashikina *et al.*, 2001). Osmoregulatory ion fluxes are also regulated by specific inositol polyphosphate signal and by PI(4,5)P_2 dependent phospholipase C (PLC) signalling. Osmotically triggered PI(3,5)P_2 responses have been reported from *Chlamydomonas*, tobacco pollen tube, epidermal strips of pea leaves and suspension cultured cells of tomato and alfalfa (Meijer *et al.*, 1999; Zanig and Munnik, 2004). In *Arabidopsis thaliana* PtdIns(4,5)P_2 levels was increased by 8-25 times when cells are subjected to hyperosmotic stress. PtdIns(4,5)P_2 produced is then hydrolyzed to Ins(1,4,5)P_3 and diacylglycerol. These two second messengers rapidly increases with the increased salinity and hyperosmotic stress in *Arabidopsis thaliana* cells and induces Ca^{2+} release from the internal stores. Besides this, they actively participate to regulate the dynamics of the actin cytoskeleton through the interaction with actin binding proteins and activate protein kinase C and PI-PLC. A new-3-phosphorylated phosphoinositides phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P_2] was also identified in *Arabidopsis* and yeast. This [PtdIns(3,5)P_2] also proposed to play a role in membrane trafficking in the endosomal/lysosomal system by regulating the fission of endolysomal subcompartments, which is accelerated by hyperosmotic stress (Dove *et al*., 2009).

Hyperosmotic stress induces increase in the levels of $PI(4,5)P_2$ in plant cells (Pical *et al.*, 1999; Takahashi *et al.*, 2001; Dewald, 2001) and in some cases it is correlated with an increase in IP_3 (Drobak and Watkins, 2000; Takashashi *et al.*, 2001) which may have a role in mobilizing cytosolic Ca^{2+} (Knight *et al*., 1997; Kiegle *et al.*, 2000). However, the studies of Lemitri-Chlich *et al.*, (2000, 2003) suggest that higher order inositol polyphosphates may induce the mobilization of cytosolic Ca^{2+}. Under hyperosmotic stress yeast cells induces the production of $PI(3,5)P_2$ which is supposed to play a role in membrane trafficking in the endosomal/lysosomal system. $PI(3,5)P_2$ regulated the fission of endosomal subcompartments which is accelerated in yeast in response to hyperosmotic stress in different cells (Leshem *et al.*, 2007; Zonia and Munnik, 2008). Plant cells rapidly responds to hyperosmotic stress with a battery of signals including PI(5)P, $PI(3,5)P_2$, $PI(4,5)P_2$, PA (phsphatidic acid), DGPP and IP_3 (Xiong *et al.*, 2002, Meijer and Munnik, 2003). These signals have been identified in a number of different plant species and tissues either singly or in combination and appear to be correlated with the severeity of the stress (Zonia and Munnik, 2006). The varieties of signals elicited by hyperosmotic stress indicate the osmotic status of the plant cells and are critical for the survival strategies of the plants.

In *Chlamydomona*s, different phospholipid signaling pathways were stimulated by the degree of severity of stress. The PtdOH generated in response to a strong hyperosmotic signal appears to be an outcome from activation of PLC-DGK and PLD pathways. Hyperosmotic stress also increased the production of numerous phosphinoside phosphates (Munnik and Meijer, 2000). Increase in $PI(4,5)P_2$ occurred at the expense of

PI(4)P and not PI(5)P suggesting that PI(3,5)P_2 may be synthesized from PI(5)P (Meijer *et al.*, 2001).

But during hypo-osmotic stress, PLD activity was rapidly stimulated and induces increase in PA with change in Cl^- anion flux across the plasma membrane (Teodoro *et al.*, 1998; Barber-Brygoo *et al.*, 2000; Shabata *et al.*, 2000). An evidence of phosphoinositide mediated responses to hypotonic stress comes from experiments with the green alga, *Dunaliella salina*, a species that survives in salinities between 0.05 and 0.5M. On long term basis, the plant maintains osmotic equilibrium by adjusting glycerol levls. In the short term, rapid changes in ion flux and cell volume protects the cells. Under hypotonic conditions, in *D. salina* PI(4,5)P_2 is rapidly hydrolyzed as seen in the decrease in radioactive phosphates in PI(4,5)P_2 and PI(4) (Einspahr *et al.*, 1988). In plants, incresed Ca^{2+} level have been implicated in cell responses to low osmolarity (Okazaki and Tazawa, 1990). In green algae, phosphoinositide turnover have been found to trigger Ca^{2+} mobilization, which mediates responses to hypotonic stress (Cote *et al*., 1996). *Laniprothamnium succinum* responds to hypotonic stress with an influx of Cl^- (Okazaki and Iwasaki, 1992) which depolarizes the plasma membrane and triggers K^+ efflux through depolarization activated K^+ channels. The efflux of K^+ and Cl^- decreases cytosolic osmotic concentration and balances the environmental osmotic strength. These changes in ion permeability may be triggered by Ca^{2+} mobilization because they are preceeded by an increase in cytoplasmic Ca^{2+} levels (Okazaki and Iwasaki, 1992).

2.6.4. Signalling in plants by wounding

In natural habitats plants are exposed to frequent stress by herbivory, insect attack, pathogen attack, physical injury or mechanical wounding etc. Therefore plants develop systemic defence responses as well as express several sets of defence-related genes that are involved in healing damaged tissues (Lawton and Lamb, 1987; Brederode *et al.*, 1991; Memelink *et al.*, 1993). These genes are activated through signalling pathways that include various protein kinases (Seo *et al.*, 1995; Mizoguchi *et al.*, 1996; Bogre *et al.*, 1997; Zhang and Klessig, 1998a). Plant responses to wounding are diverse and many plant species demonstrate a systemic increase in phosphatidic acid (PA) and lysophospholipid levels. In the wounded tomato leaf, phosphatidic acid increased approximately four fold within five minutes whereas lysophosphatidylcholine and lysophosphatidylethanol-amine increased two fold within 15 min of wounding. Similarly phosphatidic acid levels were increased upon wounding in broad bean, soybean, sunflower and pepper seedlings (Lee 1997). In soybean (*Glycine max* L), phosphatidic acid (PA) acts as a second messenger in wound signalling, as levels of PA increases rapidly and transiently at the wound site (Lee *et al.*, 1997).

Mitogen-activated protein kinase (MAPKs) may also be important in wound signal transduction in plants. Two different MAPKs, WIPK (wound induced protein kinase) and SIPK (Salicylic acid-induced protein kinase) are activated in tobacco plants after wounding (Zhang and Klessing 1998a). The kinase activity and mRNA levels of WIPK (tobacco) and its orthologs from alfalfa (SAMK) and *Arabidopsis* (*A*tMPK3) increase upon mechanical stress (Seo *et al.*, 1995; Mizoguchi *et al.*, 1996; Bogre *et al.*, 1997). Similar to tobacco SIPK, the alfalfa and *Arabidopsis* orthologs SIMK and AtMPK6 are also activated in response to wounding (Zhang and Klessing, 1998b; Romeir *et al.*,

1999). In soybean (*Glycine max*.L), wounding activates the SIMK- like MAPK. The wound activation of the MAPK is inhibited when PA production is suppressed and exogenously applied PA specifically activates the MAPK in soybean cells. This suggests that PA participates as a second messenger in wound signal transduction by activating a specific MAPK cascade in soybean (*Glycine max*.L) (Lee *et al.*, 2001).

In *Arabidopsis*, wound signalling is implicated by various biochemical signals, including jasmonic acid (JA), salicylic acid (SA), auxin and Ca^{2+}. The levels of inositol 1,4,5 triphosphate ($InsP_3$) increased four to five fold within 30 min of mechanical wounding accompanied by transient decrease in the precursor lipids PtdIns, PtdIns4P and PtdIns(4,5)P_2, in *Arabidopsis.* Jasmonic acid (JA), formation is necessary for the production of wounding-induced $InsP_3$ signals. Exogenous methyl-JA may cause increased production of $InsP_3$ in wounded *Arabidopsis.* Similar increased levels of $InsP_3$ in *Arabidopsis* rosettes treated with sorbitol have been observed, suggesting that $InsP_3$ may have been formed upon release of endogenous JA (Lobler and Lee, 1998; Stenzel *et al.*, 2003).

Induction of various defence-related genes induced by wounding suggests the role of phosphoinositides in the regulation of defence gene expression. Growth of herbivorous caterpillars was increased on plants with attenuated phosphoinositide signalling. These results establish the involvement of the phosphoinositide system in signal-transduction events leading to the induction of defence responses after mechanical wounding in *Arabidopsis* leaves (Mosblech *et al.*, 2008).

2.6.5. Phosphoinositide signalling in response to pathogen attack

The phosphoinositides are minor phospholipids present in all eukaryotic cells. They are storage forms of messenger molecules that transmit signals across the cell membrane and evoke responses to extracellular agonists. The phosphoinositides break down to liberate messenger molecules in response to occupancy of receptors by specific agonists. All three phosphoinositides are degraded by Phospholipase C (PLC) to form IP_3 and DAG that are messenger molecules serving different functions.

Plants are constantly challenged by pathogens such as bacteria or fungi and become a major threat to plants. Therefore, to cope with the challenges, sophisticated defence mechanisms are erected that resulted in a highly structured plant immune system (Wirthmueller *et al.*, 2013). There are two components of plant immunity: a pathogen-associated molecular pattern triggered immunity (PTI) and an effector triggered immunity (ETI) (Ruelland *et al.*, 2014). A PTI is activated upon recognition of receptor (PRR), whereas a more specialised ETI is responsible for recognition of race-specific pathogen effectors by nucleotide-binding to leucine-rich repeat (NB-LRR) proteins (Kumar and Mysore, 2013). Both PTI and ETI acts simultaneously, which leads to an expression of defence genes and facultatively, to a hypersensitive cell death response (HR) that restricts pathogen propagation at the infection site.

According to Canonne *et al*., (2011), plant phospholipases are essential components for PTI-response. In tomato cells, a significant increase of PA production, implicating both PLD and PLC/DGK pathway was observed after 30 min of treatment with Chitosan, a polysaccharide elicitor (Raho *et al.*, 2011). Similarly, a rapid accumulation of

lysophosphatidylcholine by the activation of PLA2 in cultured cells of California poppy elicited with a yeast glycoprotein was reported (Viehweger *et al*., 2002). These findings suggest that each phospholipase has its specific role in plant immunity. Similar implication of lipid signalling to ETI response is also evident. In *Arabidopsis thaliana* high accumulation of PA has been observed in response to wounding and thus may play a role in biotic stress signalling necessary for resistance to herbivore attack (Bargmann *et al.*, 2009).

In rice cells, the application of exogenous PA or (DAG) could mimic the effects of the N-acetylchitooligosaccharide elicitor as suggested by the triggering of ROS generation or expression of defence-related genes (Yamaguchi *et al.*, 2005), while in tomato PA application is sufficient to induce the accumulation of phytoalexin (Wang *et al*., 2013). Therefore, PA acts as an active molecule in the immune response.

Several types of phospholipases in plant immunity have been participated at the molecular level also. A role in *Arabidopsis thaliana* resistance to powdery mildew fungus (*Blumer graminis* f sp *Hordei*) has been specifically ascribed to PLDδ that accumulated in the plasma membrane near the site of fungal attack (Pinosa *et al.*, 2013). Analysis of PLDδ deficient plants demonstrated a declining resistance to fungal spore's penetration. The chitin induced expression of defence genes was also retarded in pldδ mutant. In pepper, silencing of the CaPLP1 gene, encoding a pPLA2, activity resulted in loss of resistance to *Xanthomonas campestris* pv. *vesicatoria* (Kim *et al.*, 2014). The expression of CaPLP1 *in A. thaliana* (under the CaMV 35S promoter) has rendered plants more tolerant to the pathogen infection. This effect was accompanied by an

increased ROS generation and an enhanced expression of several defence-related genes upon infection (Kim *et al.*, 2014). In contrast, the activity of some of the plant phospholipases seems to favour the resting state of plant defences. Suppression of PLDˇ1 expression in rice resulted in the activation of defence like reactions that include accumulation of ROS and phytoalexins and expression of defence-related genes, contributing to elevated resistance to pathogenic fungi. These defence reactions are thus constitutively repressed by a basal PLDˇ1 activity (Yamaguchi *et al.*, 2009).

2.7. Molecular biology of MIPS gene

2.7.1. Evolution of MIPS gene

Duplication of gene is one of the key driving forces in the evolution of gene and important features of genomic architecture of living organisms including plants. Gene duplication which is followed by its divergence and adoptive specialization of the pre-existing genes may have given diversity to plants (Zhang 2003; Flagel and Wendel, 2009). Therefore, gene duplication followed by functional divergence possibly results in two alternative evolutionary fates i) Neo-functionalization, where one copy acquires an entirely new function whereas the other copy maintains the original function. ii) Sub-functionalization, where each duplicate gene copy adopts part of the task of their parental gene (Ohno, 1970; Jenesen, 1976; Orgel, 1977; Hughes, 1994; Nowak *et al*., 1997). Among these two evolutionary fates, subfunctionalization is reported as a more prevalent outcome in nature because gene duplicate diverge mostly through the partitioning of gene expression and it can also take place at the protein function level leading to functional specialization when one of the duplicated gene becomes better at

performing one of the original functions of the progenitor gene (Hughes, 1994; Gu *et al.*, 2002; Zhang *et al.*, 2002; Conant and Wolfe, 2008).

Structural and functional genomics along with bioinformatics throws ample light in explaining the evolution of genes across divergent phylogenetic groups among prokaryotes and eukaryotes. Biosynthesis of iositol has been considered as an evolutionarily conserved pathway. Free inositol occupies the central position in inositol metabolism because this free inositol can be chanellized to various metabolic routes and produce different inositol derivatives (Fig 2.9)

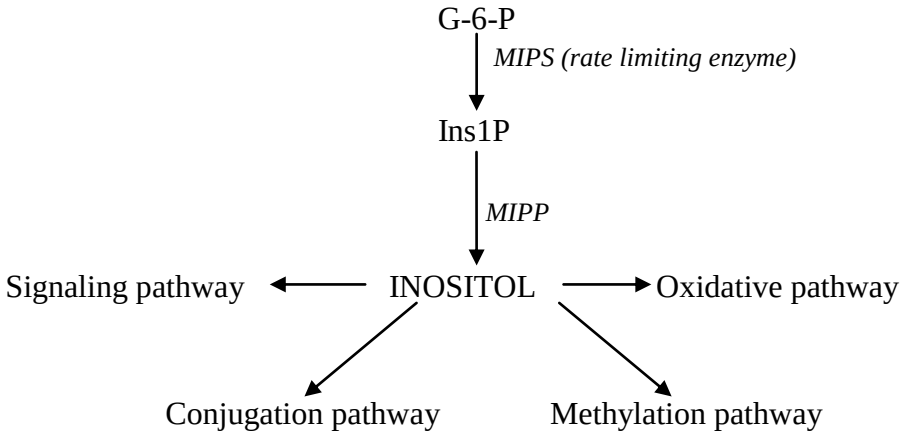


Fig-2.9 Outline of inositol biosynthesis and its utilization in other pathways

This free inositol and its derivatives have acquired different functions over the course of evolution e.g. inositol containing phospholipids are the important constituents of many archaea. With the emergence and diversification of eukaryotes, inositol is shown to be involved in growth regulation, membrane biogenesis, hormone regulation, signal transduction, pathogen resistance, stress adaptation etc. (Loewus and Murthy, 2000;

Stevenson *et al.*, 2000; Michael, 2008). MIPS enzyme is considered to be an ancient protein/gene. Few higher plants and algae are reported to have cytosolic and chloroplastic form of MIPS. However, the enzymatic and biochemical properties of these two forms do not differ significantly between each other. Since the identification of first structural gene for MIPS (*INO1*) from a fungus (Donahue and Henry, 1981a; many MIPS genes has been reported from various sources including prokaryotes and eukaryotes. Majumder *et al*., (2003) have highlighted the evolution and diversification of MIPS in which prokaryotic MIPS protein sequences were quite divergent among themselves and significantly distinct than any other known eukaryotic sequences. In contrast, the eukaryotic MIPS sequences show remarkable similarities among each other. In the biological kingdom, diverse organisms present an overall evolutionary divergence of the MIPS sequences to produce a phylogenetic tree. Therefore, one close subgroup constitutes higher plants and the other subgroup in the eukaryotic cluster constitutes fungi and other organisms.

The phylogenetic tree constructed on the basis of multiple alignments of MIPS gene shows clear segregation of these genes into monocotyledons and dicotyledons. Therefore, a divergence of MIPS gene happened after the divergence of monocots and dicots. Alignments of the DNA sequence of the gene encoding plant MIPS revealed remarkable evolutionary conservation. The presence of conserved sequences and conservative changes observed in a wide range of organisms indicate the central role that enzyme plays in biological systems (Majumder *et al*., 2003). The evolution of the MIPS gene is more diverse and complex among the prokaryotes than amongst the eukaryotes. More than 80 genes homologous to *INO1* have been reported till date and it

has been found that a core catalytic structure is conserved across evolutionarily divergent taxa (Majee and Kaur, 2011).

2.7.2 Identification of *INO1*gene from *Saccharomyces*

Culbertson and Henry (1975) have reported several mutants of *Saccharomyces cerevisiae*, which lack the enzyme MIPS. These mutants are auxotrophic for inositol and are therefore, conditionally defective in the synthesis of inositol containing phospholipids. These mutants have been categorized into 10 different genetic complementation groups termed ino-1 through ino-10 of which the *INO1* locus has been identified as the structural gene for inositol synthase (Donahue and Henry, 1981a). Other loci have been considered to have regulatory function (Culbertson and Henry, 1975). Of these, special mentions may be made of the mutants of ino-5 class, which has been designated as the model organism for the proposed 'rescue-synthesis' of *myo*-inositol (Majumder, 1981). Almost all of these mutants exhibited 'inositol-less-death' when deprived of inositol. Inositol requiring mutants of some other fungi also exhibit this property (Strauss, 1958). The fact that 'inositol-less-death' is observed in a variety of fungal mutants and strains suggests that the phenomenon is basically related to the role of inositol containing lipids during the growth of fungi in media.

The enzyme MIPS (*myo*-inositol-1-phosphate synthase) is a large multimer (240 kDa) consisting of identical subunits of 62 kDa. The gene *INO1*, encoding the enzyme *myo*-inositol-1-phosphate synthase from *Saccharomyces cerevisiae* was isolated by genetic complementation. The *INO1* gene was fully regulated when its gene was located extrachromosomally on the autonomously replicating plasmid. The cloned sequence was shown to complement two independent INO alleles (*ino1-5* and *ino1-13*). Out of

these two, *ino1-5* fail to make any material that is cross reactive with antibody to the wild type inositol-1-phosphate synthase (Klig and Henry, 1984). Of all the inositol auxotrophs isolated, approximately 70% are shown to be alleles of the *ino1* locus, the structural gene for inositol-1-phosphate synthase. Alleles of two other loci, *ino2* and *ino4* comprise 9% of total mutants, with the remainder representing unique loci or complementation group. The expression of the *INO1* gene from *Saccharomyces cerevisiae* is regulated by factors that affect phospholipid synthesis (Hirsch and Henry, 1986) but are not regulated by the products of the *INO2*, *INO4* and *OPI1* (Graves and Henry, 1999), however, *INO2* expression in *Saccharomyces cerevisiae* is controlled by positive and negative promoter elements and an upstream ORF (Eijnhamer, 2001). Sequence analysis shows that *INO1-100* had a 239bp deletion in the INO protein. It was constitutive and independent of these regulators (Swifts and McGraw, 1995).

2.7.3. Occurrence of *INO1* in other plants and microbes

Molecular characterization of inositol synthase structural genes was undertaken from several organisms (Table-2.2) which revealed the biochemistry governing inositol synthase regulation (Majumder *et al.*, 1997). In the aquatic angiosperm, *Spirodela polyrrhiza*, inositol synthase expression was upregulated due to ABA induced morphogenetic response (Smart and Fleming, 1993). The synthase gene transcript increases in response to ABA treatment, accompanied by 3-fold increase in free *myo*-inositol. This is followed by sequential increase in inositol phosphates and in the accumulation of IP_6 (Flores and Smart, 2000). Hitz *et al.*, (2002) studied a mutation in soybean (*Glycine max* L. Merr.) involving a single base change in the third base of the codon that encodes the amino acid residue 396 of the peptide of a seed expressed MIPS

gene. The base substitution causes the residue 396 to change from lysine to asparagine and the change decreases the seed expressed MIPS activity by about 90%. With the decrease in this enzyme activity, the synthesis of *myo*-inositol hexakisphosphate also decreases while the level of inorganic phosphate increases.

In response to salinity stress, the inositol synthase expression is upregulated in *Mesenbryanthemum crystallinum*, (Ishitani *et al*., 1996). A cDNA encoding In *Arabidopsis thaliana* MIPS was produced by Johnson (1994). The expression of the corresponding gene was found to be regulated by inositol concentration (Johnson and Sussex, 1995). The homology of cDNA between *S. polyrrhiza* and *A. thaliana* sequences indicate a high degree of conservation in synthase gene (Johnson, 1994). Yoshida *et al.*, (1999) reported a cDNA clone, *pRINO1* from rice (*O. sativa*), that is highly homologous to the inositol synthase from yeast. The full-length *pRINO1* cDNA sequence revealed an ORF of 510 amino acids. Homology studies revealed an approximate 50% identity of this *INO1* gene to that from *S. cerevisiae* and 86-88% identity with those from *S. polyrrhiza, B. napus, A. thaliana* and *P. vulgaris* (Yoshida *et al*., 1999). It was also found that under the influence of either sucrose or ABA, a higher level of *pRINO1* expression was found to occur in the cultured cells, from scutellum of mature rice seeds. When the said culture was treated with both sucrose and ABA together, a much higher level of gene expression occurred suggesting a synergistic induction of the inositol synthase gene (Yoshida *et al*., 2002).

In case of *Candida albicans*, the sequence of inositol biosynthetic gene, *CaINO1* has been determined. Subsequently, the largest ORF was found to have a coding sequence

of 1560 base pairs, corresponding to a protein of 521 amino acids. The deduced amino acid sequence of *C. albicans INO1* gene product with its homolog in *S. cerevisiae* shows 64% identity and 77% similarity (Klig *et al*., 1994). Similarly, a monocotyledonous resurrection plant, *Xerophyta viscosa* expresses an *INO1* gene termed *XINO1* having striking homology (70-99%) with a numbers of *INO1* genes from other plant sources. This *XINO1* gene is catalytically active at lower range of temperature between 10 ^{o}C and 40^{o}C as opposed to the *RINO1* gene-product making this protein a unique among all MIPS proteins reported so far (Majee *et al*., 2005).

Table-2.2: List of some important organisms from which *INO1* gene homologs have been identified, cloned and sequenced

Organism	**Reference**
Aeropyrum pernix	Majumder *et al.,* 2003
Actinidia arguta	Vizzotto and Falchi, 2016
Actinidia eriantha	Vizzotto and Falchi, 2016
Actinidia rufa	Vizzotto and Falchi, 2016
Actinidia deliciosa	Vizzotto and Falchi, 2016
Anopheles gambii	Majumder *et al.,* 2003
Aster tripolium	Majumder *et al.,* 2003
Avena sativa	Majumder *et al*., 2003
Avicennia marina	Majumder *et al.,* 2003
Arabidopsis thaliana	Johnson(1994);Johnson and Sussex(1995); Johnson and Burk(1995)
Archaeoglobus fulgidus	Chen *et al*., (2000)
Brassica juncea	Majee *et al*., (2004); Das Chatterjee *et al.,* (2006)
Brachypodium distachyon	Nussbaumer *et al.,* (2013)
Candida albicans	Klig *et al*., (1994)
Cicer arietinum	Ishitani *et al.,* (1996); Boominathan *et al.,* (1996)
Citrus paradisi	Abu Abied and Holland (1994)
Drosophila melanogaster	Park *et al.,* (2000)
Eleusine coracana	Reddy (2013)
Entamoeba gracilis	Lohia *et al*., (1999)
Entamoeba histolytica	Lohia *et al*., (1999)
Euglena gracilis	RayChaudhuri *et al.,* (1997)
Glycine max	Hegeman *et al*., (2001); Chiera and Grabou (2007)
Homo sapiens	Ju *et al.,* (2004)
Hordeum vulgare	Larson and Kaboy (1999)

Ipomoea batatas	Zhai *et al.*, (2015)
Jatropha curcas	Wang *et al.*, (2011)
Medicago falcata	Tan *et al.*, (2013)
Medicago sativa	Tan *et al.*, (2013)
Medicago truncatula	Nussbaumer *et al.*, (2013)
Mesenbryanthemum crystallinum	Ishitani *et al.*, (1996)
Mycobacterium tuberculosis	Bachhawat and Mande (1999)
Nicotiana tobacum	Hara *et al.*, (2000)
Neurospora crassa	Mewes *et al.*, (2002)
Oryza sativa	Yoshida *et al*., (1999)
Passiflora caerulea	Abreu and Aragao (2007)
Passiflora coccinea	Abreu and Aragao (2007)
Passiflora edulis	Abreu and Aragao (2007)
Passiflora eichleriana	Abreu and Aragao (2007)
Passiflora nitida	Abreu and Aragao (2007)
Phaseolus vulgaris	Johnson and Wang (1996); Lackey *et al.*, (2003)
Porteresia caorctata	Majee *et al.*, 2004
Ricinus communis L.	Wei *et al.*, (2010)
Saccharomyces cerevisiae	Donahue and Henry(1981); Klig and Henry (1984); Johnson and Henry (1989)
Solanum tuberosum	Keller *et al.*, (1998)
Solanum lycopersicum	Keller *et al.*, (1998), Nussbaumer *et al.*, (2013)
Sorghum bicolor	Nussbaumer *et al.*, (2013)
Sesamum indicum	Chun *et al.*, (2003)
Spartina alterniflora	Joshi *et al.*, (2013)
Spartina alterniflora	Joshi *et al.*, (2013)
Spirodela polyrrhiza	Katsoulou *et al.*, (1996); Smart and Fleming (1993)
Spirulina platensis	RayChaudhuri *et al.*, (1997)
Synechocystis sp	Chatterjee *et al.*, (2004)
Triticum aestivum	Ma *et al.*, (2013)
Xerophyta viscosa	Majee *et al*., 2005
Zea mays	Carson and Raboy (1999)

The *Archaeoglobus fulgidus* inositol synthase gene was cloned and overexpressed in *E. coli*. The native enzyme presented a tetrameric structure of approximately 168 ± 4 kDa with four equal subunits of 44 kDa each. The K_m for G-6-P and NAD^+ were 0.12 ± 0.04 mM and 5.1 ± 0.9 µM respectively (Chen *et al*., 2000).

The *INO1* gene has also been identified, cloned and sequenced from *Mycobacterium tuberculosis*. The *INO1* mutation in yeast can be functionally compensated by *INO1* of this microbe and this gene revealed a distinct class of inositol synthase. The 367 residue-long enzyme from *Mycobacterium tuberculosis* is considerably shorter than the eukaryotic analogues which are at least 500 –550 residue long (Bachhawat and Mande, 1999).

INO1 gene from the *Entamoeba histolytica* has also been cloned and sequenced. The *INO1* gene encodes a 514 amino acid ORF with a molecular mass of 57.44 kDa. *INO1* cDNA clones from *E. histolytica* have been sequenced and its comparison with genomic sequence confirms that INO1 ORF contains no introns. The purified inositol synthase from *E. histolytica* was hybridized with polyclonal antibody raised against cytosolic inositol synthase from *E. gracilis* and and analysed. This pointed towards the structural similarities of inositol synthase across species (Lohia *et al*., 1999).

Eukaryotes use ATP dependent chromatin remodelling complexes to regulate gene expression. Inositol polyphosphates can modulate the activities of several chromatin remodelling complexes in vivo. Mutations in genes encoding inositol polyphosphate kinases that produce IP_4, IP_5 and IP_6 impair transcription in vivo. These results provide a link between inositol polyphosphates, chromatin remodelling and gene expression (Shen *et al*., 2003).

2.7.4. Expression of MIPS at different locations within cells and organs

MIPS, the rate limiting enzyme has been reported from various sources and MIPS-coding sequences have been cloned and characterized from widely different organisms

including plants (Majumder *et al.*, 2003). Expression of the enzyme has been detected in different organs as well as cell organelles. Two forms of MIPS enzyme – chloroplastic forms in some plants and algae and cytosolic form have been reported (Adhikari *et al.*, 1987; RayChaudhury *et al*., 1997; Hait *et al.*, 2002; Chhetri *et al.*, 2006c). Since MIPS enzyme is isolated as a soluble enzyme, it is expected that inositol phosphate biosynthesis is largely restricted to the cytosol. Cytosolic and plastidic MIPS activity has been localized in *Pisum sativum* (Imhoff and Bourdu, 1973), *Vigna radiata* and *Euglena gracilis* (Adhikari *et al.*, 1987), *Citrus paradisi* (Abu-abied, 1994), *Arabidopsis thaliana* (Johnson and Sussex, 1995), *M. crystallinum* (Ishitani *et al.*, 1996), *Phaseolus vulgaris* (Johnson and Wang, 1996), *Oryza stiva* (Ray Chaudhury and Majumder, 1996; Hait *et al.*, 2002), *Zea mays* and *Hordeum vulgare* (Keller *et al.*, 1998), *Brassica napus* (Larson and Raboy, 1999), and *Diplopterygium glaucum* (Chhetri *et al*., 2006a,c). Cytosolic MIPS has been isolated and characterized from many species of plants across the phylogetic divisions (Table-2.1)

Interestingly, in the developing organ of *Phaseolus vulgaris*, MIPS is expressed in several membrane-bound organelles such as plasma membranes, plastids, mitochondria, endoplasmic reticulum, nuclei as well as cell wall of beans (Lackey *et al.*, 2003). Multiple isoforms of MIPS enzyme often reflects the number of subcellular compartments in which the same catalytic reaction is required. Therefore, the detection of the number of MIPS isoforms reflects the distribution of the enzyme to different other cellular compartments in addition to the cytosol and chloroplast (Johnson and Wang, 1996). The *Phaseolus* gene contains upstream ORFs interspersed with consensus RNA splice sites that predict five transit peptides, each with a high probability of

directing the enzyme to different cellular compartments, including the nucleus, thylakoid membranes of chloroplast and microbodies.

MIPS transcript was discovered in the scutellum and aleurone layers of rice embryos by Yoshida *et al.*, (1999). Soybean (*Glycine max*) represents four MIPS genes: in flowers, leaves, roots and germinating cotyledons (Hegeman *et al.*, 2001; Chappelle *et al.*, 2006). Seven homologous MIPS sequence were mapped on different chromosomes in *Zea mays* (Larson and Raboy, 1999). In sesame (*Sesamum indicum*) two or three different sequences were expressed in leaves, stems, roots and developing seeds (Chun *et al*., 2003), but in *Passiflora edulis* MIPS was expressed in seeds (Emanuel, 2007). In developing *Arabidopsis* three isoforms of the MIPS gene have been localized in the cytosol of the endosperms (Mitsuhashi *et al.*, 2008).

The three isoforms of MIPS gene, in the *Arabidopsis* genome are AtMIPS1, AtMIPS2, AtMIPS3 which are predominantly localized to the cytosol within the seed endosperms, with a small amount of MIPS protein in the embryo in the developing seeds (Mitsuhashi *et al.*, 2008). Besides other physiological processes, these AtMIPS gene are also involved in InP_6 synthesis via inositol or phosphatidylinositol synthase.

In *Glycine max*, the MIPS gene, GmMIPS-1 is expressed during the early stages of seed development, in maternal tissue (seed coat) and then transported to the developing embryo i.e. a distinct localization pattern of GmMIPS-1 occurred on one site of the micropyle (Gomes *et al.*, 2005). The localization of the RINO 1 transcript, in developing rice embryo has been detected at the apex of the embryo and then in the scutellum and aleurone layer (Yoshida *et al.*, 1999). Unlike rice, GmMIPS-1 expression

was first detected in the maternal tissues (primary inositol supply for the early embryo) and then in the embryo. Even within the embryo, expression was first observed in the radical rather than the apical regions. Therefore, this localization of GmMIPS-1 in soybean is vitally important for the initial development stages of the embryo.

In chickpea, two divergent MIPS genes- *CaMIPS1* and *CaMIPS2* have been differentially expressed. Both the genes show an overall similar structure consisting of 9 introns and 10 exons. Both the genes (*CaMIPS1* and *CaMIPS2*) showed high similarity (>85%) sequences in their coding regions. Both *CaMIPS1* and *CaMIPS2* encodes functional MIPS enzyme involved in the biosynthesis of inositol in chickpea (Kaur *et al.*, 2008). *CaMIPS1* transcript was found in root, shoot, leaves and flowers in equal amount but was not found in seed, whereas *CaMIPS2* transcript was found in all examined tissues including seeds, suggesting a key role of *CaMIPS2* in phytic acid biosynthesis in seed along with other aspect of inositol metabolism in chickpea. Therefore, these results indicated that there may be a differential regulation of two different genes in different organs to coordinate inositol metabolism in relation to cellular growth.

In rice seeds, phytin is mainly stored in protein bodies as spherical inclusions called globoids (Lott *et al.*, 1995). Localization of globoids or phytin containing particles corresponded well with the accumulation pattern of the *RINO1* transcript. In mature seeds of rice, globoids are observed in most of the embryo tissues and aleurone layer (Wade and Lott, 1997). These two tissues are the ones in which the *RINO1* transcript accumulated most abundantly. These result demonstrated a close relationship between the *RINO* transcript and globoid accumulation, suggesting that I(1)P synthase plays an

important role in phytin biosynthesis in developing seeds of rice. In addition, the apical meristem also accumulated the *RINO1* transcript during early embryogenesis. *RINO1* transcript could be detected in very young leaves of 3-week old plants (Koyama and Yoshida, unpublished data). It has been reported that the apical cells of the rice embryo, at the end of the globular stage are vacuolated and are the probable precursor of the scutellum. The accumulation of the *RINO* transcript in the apical region of the embryo 2DAA is likely to indicate the region expected to form the scutellum.

In view of the aforesaid studies, it is imperative that the research centered on *myo*-inositol and the principal enzyme for its biosynthesis, MIPS is of utmost importance for its various roles in plant systems.

2.7.5. Molecular cloning and overexpression of INO1 gene

The gene encoding *myo*-inositol-1-phosphate synthase was isolated from many plants including Yeast (Klig and Henry, 1984) and *Spirodela polyrrhiza* and as well as in *Citrus paradisi* (Abu-Abied, 1994). A full length cDNA clone from *Citrus paradisii* (Duncan grapefruit) have been isolated which expressed differentially in different developmental stages of citrus seedlings. The deduced amino acid sequence of *INO1* shows 84% identity to the deduced amino sequence of the *Spirodela* enzyme, where tur1 (cDNA) code for a protein which is highly homologous to yeast inositol synthase. The gene *CINO1* from *Citrus paradisi, tur1* from *Spirodela polyrrhiza* and *INO1* from yeast are highly homologous, all encoding for *myo*-inositol-1-phosphate synthase (Abu-Abied, 1994). Similarly, full length MIPS gene (1818bp) from immature embryo of

finger millet (*Eleusine coracana*) designated as *EcMIPS* was cloned which showed a high homology with maize and rice MIPS gene (Reddy, 2013).

The resurrection plant, *Xerophyta viscosa* expresses *INO1* gene termed *XINO1* having striking homology with a number of *INO1* gene from other plant sources. The *INO1* gene from *X. viscosa* was cloned from a cDNA library based on mRNA isolated during cold stress. This *Xerophyta* MIPS, *XINO1* is almost identical to *RINO1* proteins sequences except for only two- three amino acids residues. Overexpression of the *XINO1* was achieved and the bacterially expressed *XINO1* protein was turned out to be active even in a lower temperature of 10^{o}C in striking contrast to the corresponding RINO1 protein having more restricted higher temperature optimum. The *XINO1* gene from *Xerophyta* was catalytically active at lower temperature range between 10^{o}- 40^{o}C than the *RINO1* gene product, which was the first such report from any resurrection plant (Majee, 2005).

There were four isoforms of MIPS gene in *Glycine max* designated as *GmMIPS1*, *GmMIPS2, GmMIPS3* and *GmMIPS4* and one of these MIPS cDNA was shown to express mainly in developing seeds (Hegeman *et al.,* 2001; Chappell *et al.,* 2006), specially in the outer integumentary layer during early seed development (Chiera and Grabau, 2007). A full length *GmMIPS* cDNA with an open reading frame (ORF) of 1533bp has been analyzed which was predicted to encode a polypeptide of 510 amino acid with a 61bp 5′-UTR and 173bp 3′-UTR. Maximum expression of *GmMIPS* was seen in developing seeds and a much lower expression in other vegetative tissues like leaves, flower, roots and stems, where mainly *GmMIPS2, GmMIPS3* and *GmMIPS4*

shows a relatively higher transcript levels (Chappell *et al.*, 2006). Expression of all four isoforms expression confirmed the role of *GmMIPS* isoform in generating L-*myo*-inositol-1-phosphate as a substrate for phytic acid biosynthesis. Therefore, down-regulation of *GmMIPS* using a seed specific promoter may have a great potential for developing of low phytate soybean without affecting the inositol metabolism in other tissues (Kumari, 2013). Similarly, the grass halophyte (*Spartina alterniflora*) encodes *myo*-inositol-1-phosphate synthase MIPS in *Arabidopsis thaliana* and overexpresses as *SaINO1*gene. This *SaINO1* gene is a stress-responsive gene and its constitutive overexpression in *Arabidopsis* provides significantly improved tolerance to salt stress during germination and seedling growth (Joshi *et al.*, 2013) while sweet potato [*Ipomoea batatas* (L) Lam.] expresses *IbMIPS1* gene which is induced by NaCl, polyethylene glycol (PEG), abscisic acid (ABA) and stem nematode. Overexpression of *IbMIPS1* from sweet potato significantly enhances stem nematode resistance besides salt and drought tolerance in transgenic sweet potato by increasing the inositol and proline content and enhancing the rate of photosynthesis. Therefore, overexpression of IbMIPS1 gene has the potential for use to improve the resistance to biotic as well as abiotic stresses in plants (Fei-Bing, 2015; Zhai, 2016).

Jatropha curcas also expresses cDNA encoding *myo*-inositol-1-phosphate synthase (*JcMIPS*) involved in response to abiotic stress (abscisic acid, drought and low temperature). This *JcMIPS* transcript is highly present in seeds and leaf tissues, but at low levels in stem and flower tissues (Wang, 2011). *Ricinus communis* L. also expresses a MIPS cDNA designated as *rcMIPS* with full length of 1669 bp and contains 1533 bp ORF. This *rcMIPS* ORF encodes 510 amino acid residues, which corresponds to a

polypeptide with a molecular mass of approximately 56 KDa. This gene shows homology to the extent of 88.6% with *Zea mays*, 89% with *Arabidopsis*, 91.6% with *Vigna radiata* and 91.4% with *Xerophyta viscosa*. Under abiotic stress conditions, *rcMIPS* gene activity was significantly increased in leaves, stems, and roots of which the expression in levels were higher than those in the stems and roots. This result indicates that the expression of *rcMIPS i*n *R. communis* enhances defensive mechanism against drought stress (Wei *et al.*, 2010). A cDNA encoding *myo*-inositol-1-phosphate synthase from *Sesamum indicum* L. Seeds (*SeMIPS1* cDNA) has been cloned and its functional expression pattern was analyzed in association with salinity stress during seed germination. The *SeMIPS1* cDNA shows deduced amino acid sequences indicating high identity and similarity with other MIPS and the presence of highly conserved functional regions essential for MIPS activity such as co-factor binding and catalysis of the reaction (Majumder *et al.*, 1997; Bachhawat and Mande, 1999). Salt stress during sesame seed germination has an adverse influence on the transcription of *SeMIPS1*. The transcript levels proportionally reduced with the duration of exposure to salinity and the initiation of germination was also collaterally reduced with the increase in salt concentration. This result suggests that expression of *SeMIPS1* is downregulated by salt stress during sesame seed germination (Chun, 2003).

2.7.6. Regulation of INO1 gene

The biosysnthesis of *myo*-inositol is a highly regulated process in *Saccharomyces cerevisiae*. The sysnthesis is mediated by its main biosynthetic enzyme, MIPS which is a repressible cytoplasmic enzyme. In yeast, MIPP is the gene product of the *INO1* gene (Donahue and Henry, 1981), but several other genes influence the action of INO1 gene

(Klig and Henry, 1984). MIPS expression is prevented by *ino2* and *ino4* mutations which are not linked to INO1 gene. In addition, *opi1*, *opi2* and *opi4* mutations results in making the enzyme constitutive (Greenberg *et al.*, 1982). It has also been found that MIPS was regulated by the exogenous inositol and the addition of 50µM *myo*-inositol in the growth medium decreases the specific activity of MIPS by over 50 fold (Culbertson *et al.*, 1976). Cloned *INO1* gene located in the *ino1* cells on a high copy number plasmid extrachromosomally was fully regulated. Thus, positioning of the gene in its normal chromosomal location is not necessary for full regulation of of MIPS (Klig and Henry, 1984). Majority of the mutants lacking the capacity to synthesize MIPS have been found to be the alleles of the *INO1* locus which codes for a 62kDa subunit of MIPS (Donahue and Henry, 1981a). The *opi1, opi2* and *opi4* mutations are recessive and unlinked to *INO1*. It may be suggested that all three gene products are components of a single repressor molecule or maybe, one of them form the repressor and the others helps in either synthesis or activity of the same. One more mutant, *opi3* which does not synthesize MIPS constitutively, but affects phospholipid biosynthesis (Greenberg *et al.*, 1982).

Genetic manipulation of *INO1* gene may disrupt the cellular *myo*-inositol supply thereby affecting various physiological processes. In a study, two methods, based on gene deletion and antisense strategy were used to generate mutants of *Disctystelium discoideum*. These mutants are inositol auxotrophs and they show inability to live by phagocytosis of bacteria which is their exclusive source of nutrition. Tranformation of wild type *D. discoideum* cells (AX2) resulted in three independent transformants by gene disruption (*Ddino1 Δ1* - *Ddino1 Δ3*) and 18 independent transformants by antisense mutagenesis (*Ddino1 as1* - *Ddino1 as18*). These mutants are inositol

auxotrops and they lose their viability when deprived of inositol for longer than 24 hrs. Under inositol deficient conditions, the mutants produced by both the strategies (*Ddino1 Δ1, Ddino1 as1*) accumulate a substance, 2,3-BPG (2,3-bisphosphoglycerate) which is not a primary inositol metabolite (Fischbach *et al.*, 2006). Increase in the level of 2,3BPG may be the result of homoeostasis between IP_3 breakdown and the limited breakdown of PIP_2 or it may be due to delayed degradation of 2,3-BPG in response to *myo*-inositol addition. A link between glycolysis and *myo*-inositol metabolism (Shi *et al*., 2005) may explain the accumulation of BPG as it is essential for the activity of phosphoglycerate mutase in all eukaryotic cells (Fischbach *et al*., 2006).

On analysis of *Arabidopsis thaliana* genome it was found that it carries three isoforms of MIPS, of which MIPS1 seems to be the main player in MI biosysnthesis because mips1 have drastically reduced MI content (Donahue *et al.*, 2010). Analysis of MIPS1 promoter activity in the mips1 mutant background or by addition of MI to the growth medium indicates that MIPS1 protein itself is a positive regulaltor of MIPS1 transcription. MIPS1 recruitment on the MIPS1 promoter is required to interact with histone methyltransferase, ATXR5/6 to expedite DNA methylation and thereby allowing normal transcription. Addition of MI in the growth medium did not restore the activity of the MIPS1 promoter in the mips1 mutant suggesting that the MIPS1 protein itself, but not its catalytic activity, is required for this regulation. During pathogen attack, MIPS1 is downregulated to prevent pathogen proliferation. MIPS1 is released from chromatin to allow ATXR5/6 dependent silencing of MIPS1. Thus, in plants MIPS1 has emerged as a protein that connects cellular metabolism with chromatin functions (Latrasse *et al*., 2013).

Mycobacterium tuberculosis utilizes inositol to produce mycothiol (Fahey, 2001) which is important in protecting the bacteria from ROS and electrophilic toxins (Newton *et al*., 2000). Antisense oligonucleotides (ODNs) which inhibit gene expression are used against human pathogens (Lisziewicz *et al*., 1992). However, ODNs cannot pass through the mycobacterial cell envelope. On the contrary, phosphoorothioate modified oligonucleotides (PS-ODNs) are easily taken up by these bacterial cells (Harth *et al*., 2002). In *M. tuberculosis* MIPS specific PS-ODNs inhibits the expression of ino1mRNA, the production of mycothiol, the proliferation of bacteria and enhances its susceptibity to antibiotics (Li *et al*., 2007).

Reduring the phytic acid content from soybean seeds becomes important in the light that 60-80% of total seed phytate is stored in soybean seeds in the form of phytate which is not readily digestible to the non-ruminant animals, reducing its nutritional value. Induced mutagenesis has been one approach of reducing MIPS encoding gene expression in plants. A single recessive mutation in soybean by changing just one amino acid residue of MIPS gne decresed the specfic activity of seed expressed MIPS enzyme and decreased phytic acid level (Hitz *et al*., 2002). However, complete deletion of gene expression has not been possible due to the presence of multiple genes encoding MIPS in plant genome. Conversely, RNA interference technolaogy has been successful in generating post-transciptional silence to quasi-undetectable levels of mRNA transcripts (Scherer and Rossi, 2003). By this technique multiple copies of the MIPS encoding gene present in soybean genome may be knocked down as these genes have high degree of sequence similarity (Nunes *et al.*, 2006). Therefore, RNAi technology has been used in soybean (*Glycine max*) in order to silence the *myo*-inositol-1-phosphate synthase

(*GmMIPS*) gene, which resulted in a drastic reduction (up to 94.5%) of phytate content in the developed transgenic lines, improved phosphorous availability and inhibited seed germination demonstrating an important correlation between *GmMIPS* gene expression and seed development (Nunes *et al.*, 2006).

RNAi mediated silencing of MIPS gene has also been used in in indica rice cultivar in order to generate low phytate rice. Here, MIPS gene was downregulated by using seed specific promoter, oleosin 18 (Ole18). The rice *INO1* (*RINO1*) cDNA was expressed under the control of Ole18 promoter directing the phytic acid biosynthesis in seeds. The transgenic lines revealed a reduction in phytate levels (reduced by 68%), along with an increased phosphorous content and the plant displayed normal phenotype (Kuwano *et al.*, 2009). Low phytate rice was also achieved by RNAi mediated seed specific silencing of inositol 1,3,4,5,6-pentakisphosphate-2-kinase gene (IPK1), which catalyzes the last step of phytic acid biosynthesis in rice. The transgenic rice revealed 3.85 fold downregulation in IPK1 transcripts in T4 seeds which also showed a significant reduction in phytate levels and a concomitant increase in the amount of inorganic phosphate. IPK1 gene silencing in rice seeds also provide 1.8 fold more iron in the endosperm and do not show any negative effect on seed germination. Thus, silencing of IPK1 gene reduces seed phytate levels without compromising on the growth and development of transgenic rice plants (Ali *et al.*, 2013). RNAi mediated downregulation of IPK1 also led to the development of low phytic acid containing wheat (Aggarwal *et al.*, 2018)

2.8. Role of *myo*-inositol and its metabolites in abiotic stress tolerance:

2.8.1. Stress response

Many plants possess an intrinsic ability to tolerate and survive under adverse environmental conditions (Knight and Knight, 2001). Phosphatidylinositol 4,5 bisphosphate (PIP_2) levels is increased during osmotic stress as found in *Arabidopsis* cells (Pical *et al.*, 1999; Wald *et al.*, 2001) and the time frame for the increase correlates with changes in cytosolic Ca^{2+} levels. The stress hormone ABA also elicits transient increases in IP_3 levels in *Vicia faba* guard cell protoplasts (Lee *et al.*, 1996) and in *Arabidopsis* seedlings (Sanchez and Chu, 2001; Xiong *et al.*, 2001). Transient increases in IP_3 were found in plants upon exposure to light, pathogen, gravity, anoxia or several plant hormones (Munnik *et al.*, 1998; Stevenson *et al*., 2000).

Phospholipase D (PLD) is activated and lead to transient increases in PA levels in plants during drought and hyperosmolarity (Frank *et al.*, 2000, Munnik *et al.*, 2000, Katagiri *et al*., 2001). PLD appears to be activated by osmotic stress through a G-protein (Frank *et al.*, 2000; Katagiri *et al.*, 2001). However, excess PLD activity may have a negative impact on plant stress tolerance. Drought stress induced PLD activities were found to be higher in drought sensitive than in drought tolerant cultivars of cowpea (Maarouf *et al*., 1999) suggesting that al high PLD activity may jeopardize membrane integrity. Consistent with this notion, *Arabidopsis* plants deficient in PLD was found to be more tolerant to freezing stress.

Two component histidine kinases, cyanobacterium histidine kinase HiK33 (Suzuki *et al*., 2000) and the *Bacillus sublitis* histidine kinase Desk (Aguilar *et al.*, 2001) are thermosensors that regulate desaturase gene expression in response to temperature downshifts. In the genome of *Arabidopsis thaliana*, several putative two component histidine kinase have been identified (Urau *et al.*, 2000).

Cold treatment activates some mitogen activated protein kinase (MAPK) by post translational modification in *Arabidopsis* and in *alfalfa* (*Medicago sativa*, Jonak *et al*., 1996). In yeast and in animals MAPK pathways that are activated by receptor sensors such as protein tyrosine kinases, G-protein- coupled receptors and two component histidine kinase, etc. are responsible for the production of compatible osmolytes and antioxidants. Similarly *Arabidopsis histidine* kinase AtHK1 can complement mutations in the yeast two component histidine kinase sensors SLN1 and therefore, may be involved in osmotic stress signal transduction in plants.

Ca^{2+} dependent protein kinase ($CDPK_5$) are serine threonine protein kinase with a C-terminal calmodulin like domain may be involved in abiotic stress signaling (Huang *et al*., 2000). In rice plant $CDPK_5$ was activated by cold treatment (Martin and Busconi, 2001). In addition overexpression of C_5CDPK_7 resulted in increased cold and osmotic stress tolerance in rice (Saijo *et al*., 2000). Thus $CDPK_5$ somehow play roles in the development of stress tolerance. A CDPK was also activated in response to pathogen infection (Romeis *et al*., 2000). Thus $CDPK_5$ somehow play roles in the development of stress tolerance.

2.8.2. Osmolyte accumulation during stress

Accumulation of osmolytes leads to osmotic adjustment which is an important mechanism to tide over the changing environment. Osmolytes help in osmotic adjustment of the cells and also protects the cells and macromolecules by maintaining membrane integrity, preventing protein degradation and protecting against oxidative damage by scavenging free radicals and lowering the T_m value of nucleic acids (Crowe *et al*., 1987; Nomura *et al.,* 1995). Osmolytes not only helps in the osmotic adjustment in the cellular milieu but also acts as scavengers of reactive oxygen species. The presence of D-ononitol and *myo*-inositol are the potential protectants of enzymes and membranes from damage by reactive oxygen species (Sheveleva *et al.*, 1997). Methylated inositols were found to be comparatively more effective quenchers of ROS as compared to glycine-betaine and the like (Orthen *et al*., 1994).

The plant species which are constantly exposed to saline conditions accumulates the cyclic sugar alcohols pinitol and ononitol (Paul and Cockburn, 1989). Thus *Mesembryanthemum crystallinum* accumulates these comounds when subjected to such stress (Bartels and Nelson, 1994). The upregulation of inositol biosynthesis by subjecting plants to salinity stress may be exploited for the enhanced production of *myo*-inositol from glucose-6-phosphate (RayChaudhuri and Majumder, 1996).

2.8.3. Role of *myo*-inositol and its derivatives in temperature Stress

Stress tolerant plants generally possesses a high levels of stress-related metabolites under normal growth conditions and/or accumulate large amounts of protective metabolites under unfavourable conditions, indicating that their metabolism is prepared for adverse growth conditions.

Under freezing conditions, the presence of cyclitol and low molecular weight compounds all together function as cryoprotective solutes, which diminishes the mechanical stress by decreasing the osmotic potential thus reducing the freeze induced shrinkage and accumulation of cryoprotectans prevents this alteration by balancing the concentration of cryotoxic substance during ice formation. Cyclitols like pinitol, quebrachitol, quercitol, O-methyl-*muco*-inositol which have been found to be accumulated at low temperature (Diamantoglou, 1974; Ericsson, 1979; Popp *et al.*, 1997).

In mistletoe (*Viscum album)* more than 25% of its dry matter is occupied by the cyclitols during winter (Richter, 1989). Similarly enhanced storage of cyclitols in the living bark tissue and buds has been found in a number of tree species during the onset of cold season (Poop and Smirnoff, 1995; Popp *et al*., 1997). Even, the transcription of the enzyme *myo*-inositol-o-methyl-transferase (a key enzyme on the biochemical pathway to ononitol and pinitol biosynthesis) has been induced in the Mediterranean species *M.crystallium* when the plant was exposed to 4^{o}C for 78 hr. Similarly, accumulation of pinitol in chickpea in the thylakoid membrane functions as cryoprotective solutes (Orthen, 2000). Proline and raffinose also play a crucial role of compatible solutes in *Arabidopsis* in freezing tolerance (Hannah *et al.*, 2006; Korn *et al.*, 2010).

MIPS-encoding gene cloned from *Passiflora edulis f. flavicarpa* called *PeMIPS1* is differentially transcribed during cold and heat stress, suggesting that it is important for

the environmental stress response. This result correlates with ecological adaptation of yellow passion fruit, a typical species adapted to tropical and subtropical environments that endures winter chills for short periods without injury (Abreu, 2007).

2.8.4 Drought Tolerance

Plants develop complex and dynamic systems like various physiological, morphological, biochemical, and metabolic approaches to respond to water deficit and to adapt to drought conditions (Vincent *et al.*, 2007; Ahuja *et al.*, 2010; Saidi *et al.*, 2011; Walbot *et al*., 2011).

Plants respond to drought stress by the synthesis of different metabolites including polyols in ripe olive fruit, (Martinelli, 2013), grape berry (Conde *et al.*, 2014) etc. Six different polyols (mannitol, sorbitol, galactitol, *myo*-inositol, glycerol and dulcitol) were significantly accumulated in the pulp of grape berries in responses to water deficit. *Myo*-inositol was the most abundant of the quantified polyols in mature leaves and tissues which helps the plant to cope with water deficit, either directly as an osmolyte or indirectly as a precursor of galactinol and raffinose family oligosaccharides (Conde *et al.*, 2014). Though *myo*-inositol is used as an osmolyte like other sugar alcohols, it also functions as a precausor of many metabolites involved in abiotic stress (Kaur *et al*., 2013). *Myo*-inositol is also closely related to the accumulation of RFOs and further stress tolerance (Elsayed *et al*., 2014). There is a close relationship between the metabolism of *myo*-inositol and RFO and the yield performance of maize under drought stress. Galactinol synthase (GolS EC 2.4.1.123) is the key enzyme that catalyzes to produce galactinol from *myo*-inositol. Therefore, galactinol synthase (GolS) has been

assumed to be the key regulatory factor in RFO biosynthesis (Taji *et al.*, 2002; Kerner *et al.*, 2004; Sengupta *et al.*, 2015).

There are seven GolS-related genes in the *Arabidopsis thaliana* and named as *At*GolS1,2,3,4,5,6 and 7 respectively. Among these, *At*GolS1 is the drought responsive gene, which mainly functions in drought stress tolerance (Taji *et al.*, 2002). Similarly, GolS1 from *Boea hygromenic*a enhanced drought tolerance (Wang *et al.*, 2009). The model plant *Ajuga reptans* expresses two distinct GolS, ArGolS1 and ArGolS2 which regulates RFO metabolism (Sprenger and Keller, 2000). RFOs have long been suggested to act as anti-stress agent in both generative and vegetative tissues (Taji *et al.*, 2002; Pennycooke *et al.*, 2003). *Xv*GolS gene encoding galactinol synthase was also identified in the leaves of *Xerophyta viscosa.* This gene shows negative correlation between RFO accumulation and *myo*-inositol depletion which was reversed after rehydration. This suggest that *myo*-ionsitol is channelled into RFO synthesis during water deficit and channelled back to metabolic pathway during rehydration to repair desiccation-induced damages (Peter *et al.*, 2007).

In addition to GolS, MIPS also control the levels of galactinol and raffinose because it controls the production of *myo*-inositol, the galactinol precursor. Therefore, both GolS and MIPS are proved to play important roles in drought–stress tolerance (Taji *et al.*, 2002; Evers *et al.*, 2010).

2.8.5 Salinity stress tolerance

Salinity tolerance is correlated with the increased accumulation of polyols such as mannitol, sorbitol, *myo*-inositol and its methylated derivatives (Loewus and Dickinson, 1982). The pathway from glucose-6-phosphate (G-6-P) to *myo*-inositol-1-phosphate (Ins1-P) and *myo*-inositol is essential for the synthesis of various other cyclic polyols like ononitol, pinitol etc. *Myo*-inositol-O-methyltransferase (*IMT1*) gene has been isolated from *Porteresia coarctata* Roxb. (*PcIMT1*) is implicated in the synthesis of pinitol that in turn enhances salinity stress (Sengupta, 2008). In *Porteresia coarctata* Roxb.(takeoka) (wild halophytic rice) salt-tolerant MIPS gene coded by *PcINO1* and *PcIMT1* generates *myo*-inositol and pinitol respectively even at high salt concentration and so that the inositol pool in the plant is well maintained during salinity stress.

The same gene encoding *myo*-inositol-O-methyltransferase, (*IMT1)* has also been isolated from the facultative halophyte, *Mesembryanthemum crystallinum* (Ishitani *et al.*, 1996). *Mesembyanthemum crystallinum* responds to osmotic stress by shifting from C_3 photosynthesis to crassulacean acid metabolism (CAM). Under salinity stress conditions, the plant also switches on the biosynthesis of a putative osmoprotectant, pinitol. Under salt stress, pinitol did indeed increase reflecting the induction of the gene. Also, low levels of pinitol have been observed previously in unstressed leaf tissue from mature plants (Vernon and Bohnert, 1992). In *Mesembryanthemum crystallinum* sodium uptake is facilitated by the phloem translocation of *myo*-inositol from root to leaves under salt stress. And a 10 fold increase in *myo*-inositol and ononitol was found with large increase in sodium following stress. Therefore *myo*-inositol plays a direct role in

sodium uptake and long distance transport. Thus, besides *myo*-inositol serving as a substrate, it also promotes as a leaf to root signal by sodium uptake (Nelson *et al.*, 1999)

In salt tolerant *Lycopersicon esculentum* plants, *myo*-inositol constituted two-thirds of the soluble carbohydrates in leaves while the most tolerant genotypes was found to have highest, the normal cultivar intermediate while the sensitive genotypes the lowest level of MI after treatment with salt. Thus, *myo*-inositol content is implicated to have an additive effect in salt tolerance in tomato (Sacher and Staples, 1985). *Chiro*-inositol was also isolated and identified in leaves and root tissues of *Limonium* species (Liu, 2009) for salt adaptation. *Chiro*-inositol was found in other halophytes too such as *Aegialitis annulata* L. Gmelinii and *L. latifolium* (Popp, 1984; Murakeozz *et al.*, 2002; Gagneul *et al.*, 2007). *Chiro*-inositol and *myo*-inositol are phloem transportable; therefore, these polyols are readily translocated from leaves to roots. This type of translocation facilitates sodium uptake through xylem to leaves leading to a transition from a non-tolerant to salinity-tolerant state (Nelson *et al.*, 1999).

Rice (*Oryza sativa*) is sensitive to salinity at the seedling stage and becomes tolerant at the vegetative phase and very susceptible at the reproductive phase (Anbumalarmathi, 2013). Therefore, this stress tolerance is a quantitative trait which is controlled by enhanced activity of chloroplastic form of *myo*-inositol-1-phosphate synthase noticeable only in chloroplast from light grown plants (Raychandhuri, 2002).

Overexpression of *PcMIPS* isolated from halophytic rice, *Porteresia coarctata* enhanced tolerance to salt in transgenic *Brassica juncea* and rice (Chatterjee *et al*.,

2006). It caused tolerance to salt and oxidative stresses in transgenic *Brassica juncea* and increased the seed survival rate under salt and dehydration stresses in transgenic tobacco plants (Goswami *et al.*, 2014). Similarly, overexpression of synthase gene from *Medicago falcata*, *MfMIPS1* improved resistance to chilling, drought and salt stress in transgenic tobacco plants (Tan *et al*., 2013). In the same way, overexpression of the gene IbMIPS1 from *Ipomoea batatas* L. enhances salt tolerance by regulating the expression of salt stress responsive genes, increasing the contents of inositol and proline and enhancing the rate of photosynthesis. Therefore, *IbMIPS1* gene has the potential to be used to improve salt tolerance in sweet potato and other plants (Fei-bing, 2015).

3: MATERIALS AND METHODS

3:1 Materials:

Fresh specimens of bryophytes viz., *Asterella khasiana* (Griff.) Grolle, *Marchantia polymorpha* Linn., *Riccia cruciata* Kash, *Anthoceros angustus* Steph., *Bryum argenteum* Linn., *Brachymenium bryoides* Hook. ex Schwaegr., *Lyellia crispa* R. Br., *Funaria wallichii* (Mitt.) Broth. and *Sphagnum junghuhnianum* Doz. & Molk. 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 Himalayas of India.

D-glucose-6-P (G-6-P, di-sodium salt), β-NAD, D-galactose-6-P (di-sodium salt), D-fructose-6-P (di-sodium salt), D-mannose-6-P (di-sodium salt), L-glucose-6-P (di-sodium salt), D-glucose-1, 6-bisphosphate (di-sodium salt), D-fructose-1, 6-bisphosphate (di-sodium salt), β-NADP, BSA, Coomassie brilliant blue (R), imidazole and *myo*-inositol were obtained from Sigma Chemical Company, St. Louis, Missouri, USA.

2-mercaptoethanol, acrylamide, ammonium molybdate, acetic acid, ammonium sulphate, acetone, ammonium chloride, EDTA (disodium salt), glycerol, glycine, copper chloride, sodium hydroxide, potassium chloride, Tris, TCA, sodium thiosulphate, orthophosphoric acid, dipotassium hydrogen phosphate, magnesium chloride and TEMED were purchased from E. Merck India Ltd., Mumbai, India.

Ascorbic acid, sodium meta-periodate, bromophenol blue, bis-acrylamide, papain, CBB (G-250), alcohol dehydrogenase (from bakers Yeast), DEAE-52 were from Sisco

Research Laboratories, Mumbai, India. Ammonium persulphate, methanol, sodium bicarbonate, sodium sulphite and ammonium chloride were procured from S. D. Fine-Chem Ltd., Mumbai, India.

Amberlite IR-120 (sodium form), Dowex-1-Cl^- (100-200 mesh), manganese chloride, catalase (from bovine liver), dialysis membrane, egg albumin were obtained from Hi Media Laboratories Ltd., Mumbai, India. Boric acid, disodium tetraborate, hydrochloric acid, barium hydroxide, sodium chloride, sulphuric acid and zinc chloride were purchased from Ranbaxy Fine Chemicals Ltd., New Delhi, India. Silver nitrate, mercuric chloride and calcium chloride were from Qualigens Fine Chemicals, Mumbai, India. Ethanol was supplied by Bengal Chemicals & Pharmaceuticals Ltd., Calcutta, India. Sephadex G-200 was from Amersham Pharmacia Biotech marketed by Sisco Research Laboratories, Mumbai, India and chromatography paper (grade 1) was obtained from Whatman International Pvt. Ltd., Kent, England. All other chemicals used were of analytical grade purchased from reputed Indian companies.

3.2 Methods

3.2.1 Isolation of L-*myo*-inositol-1-phosphate synthase from bryophytes:

To assay the *myo*-inositol synthase activity of bryophytes, the enzyme was isolated as per the protocol described below:

(All the operations were carried out at 0ºC to 4ºC)

Different plant samples (50 g each) were collected fresh in the morning, washed twice with cold sterile distilled water and homogenized in a chilled mortar and pestle in equal volume of the extraction buffer (50mM Tris-acetate, pH 7.5 containing 0.2 mM ME). Neutral sand was added in the mortar for facilitating the extraction.

The crude homogenate was passed through 3 layers of cheese cloth and the liquid was centrifuged at 1,000×g for 5 min in a Plastocrafts Superspin-R centrifuge. The pellet was discarded and the supernatant collected. The supernatant was centrifuged at 11,400 ×g for 20 min and the pellet discarded. This preparation was dialyzed overnight against 50 mM tris-acetate (pH 7.5) containing 0.2 mM ME. The dialyzed 11,400 ×g supernatant fraction was recovered from the dialysis bag and used as the enzyme source for the initial screening experiments.

3.2.2 Partial purification of L-*myo*-inositol-1-phosphate synthase from bryophytes: The enzyme was partially purified from the reproductive structure bearing plant bodies of selected bryophytes [*Asterella khasiana* (Griff.) Grolle and *Sphagnum junghuhnianum* Doz. & Molk.] following the method outlined below:

All the operations were carried out between 0 ˚C to 4 ˚C.

3.2.2.1 Preparation of crude extract: About 50 g of freshly collected plant tissues were thoroughly washed with sterile cold distilled water twice. The samples were then homogenized in a mortar and pestle with equal volume of 50 mM tris-acetate buffer (pH 7.5) containing 0.2 mM ME in presence of neutral sand. The resulting slurry was centrifuged at 1,000 ×g for 5 min in a Plastocrafts Superspin-R centrifuge. The pellet

was discarded and the supernatant was designated as crude extract (homogenate). The crude extract was spun again at 11,400 ×g for 20 min and the supernatant was collected (10K supernatant).

3.2.2.2 Streptomycin sulphate precipitation: Streptomycin sulphate powder was added slowly to the 10K supernatant in order to remove nucleic acids in the form of precipitate. The powder was added with constant stirring (using a REMI cyclomixer) to a final concentration of 2 % (w/v). After mixing the required amount of streptomycin sulphate, the mixture was kept in an ice bucket at 0ºC for 15 min and then centrifuged at 11,400 ×g for 15 min. The pellet was discarded and the supernatant collected (SS fraction).

3.2.2.3 Ammonium sulphate fractionation: The streptomycin sulphate treated supernatant (SS-fraction) was made 0-70 % saturated with ammonium sulphate by slowly adding the requisite quantity of the solid salt (43.6 g/100 ml of SS-fraction) with constant stirring (using a REMI magnetic stirrer). The mixture was kept at 0ºC in an ice bucket for 15 min and then centrifuged at 11,400 ×g for 20 min. The supernatant was discarded and the pellet collected. The pellet was dissolved in minimal volume of 50 mM Tris-acetate buffer (pH 7.5) containing 0.2 mM ME, poured into a dialysis sac and dialyzed overnight against 500 vol. of the same buffer. The buffer was changed once and the dialysis procedure was repeated for complete removal of ammonium sulphate. The dialysis sac was prepared earlier by boiling the commercially available membrane for 15 min in a large volume of 2% sodium bicarbonate solution containing 1mM EDTA and rinsing the same thoroughly (5-6 times) in sterile distilled water before

boiling again for 10 min in distilled water. On completion of dialysis, the ammonium sulphate fraction (A_2S-fraction) was recovered from the dialysis sac.

3.2.2.4 Anion exchange chromatography in DEAE cellulose: The dialyzed A_2S fraction was adsorbed in pre-equilibrated DEAE cellulose. DEAE-52 was soaked in about 100 vol of 0.5 N HCl for 30 min. The acid was decanted out and the matrix was washed several times with tap water till the pH was 6.0 to 7.0. The material was washed twice with distilled water and subsequently soaked in 0.5 N NaOH solutions for another 30 min. The NaOH solution was decanted out and the matrix was repeatedly washed with tap water till the pH became 7.0 to 7.5 after which it was again washed twice with sterile distilled water. Finally, the DEAE was soaked for 4 h in 100 vol of 50 mM Tris–acetate buffer containing 0.2 mM ME. Thus, the DEAE became ready.

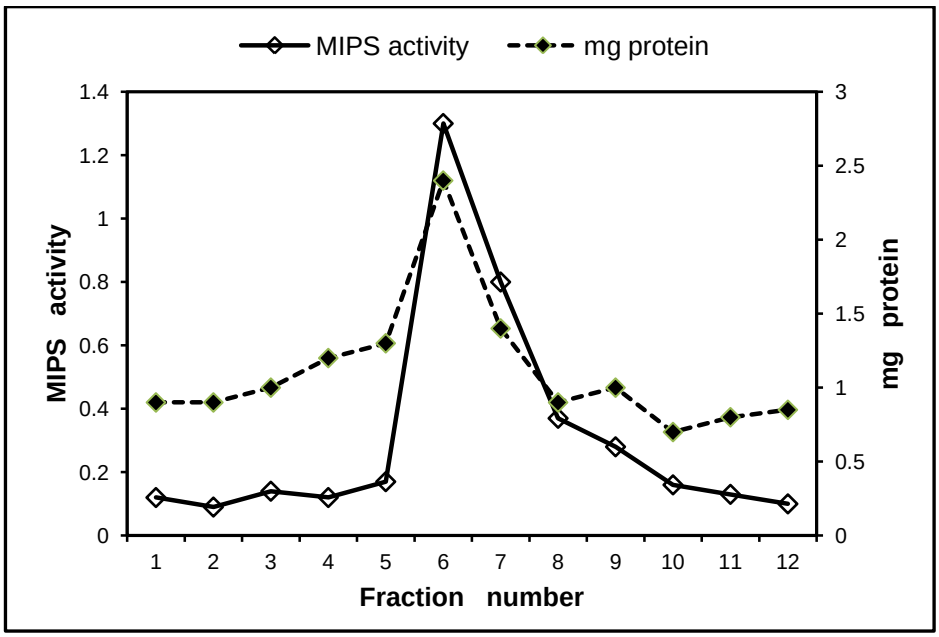


Fig-3.1 Elution profile of cytosolic MIPS from *A. khasiana* in DEAE cellulose column (MIPS activity is expressed as µM I-1-P produced/2ml fraction/hr)

After adsorption for two hours, the preparation was loaded in a glass column (1.2 × 8.0 cm) and the effluent was collected. Subsequently, the column was washed with one bed volume of the same buffer. Lastly, the adsorbed proteins were eluted from the column with a linear gradient of 0 to 0.5 M KCl in 50 mM Tris-acetate buffer (pH 7.5) containing 0.2 mM ME. Fractions at the rate of 2 ml/10 min were collected. Twenty such fractions were collected and assayed for *myo*-inositol synthase activity. The active fractions (DE-fraction) were pooled together and used for the next purification step.

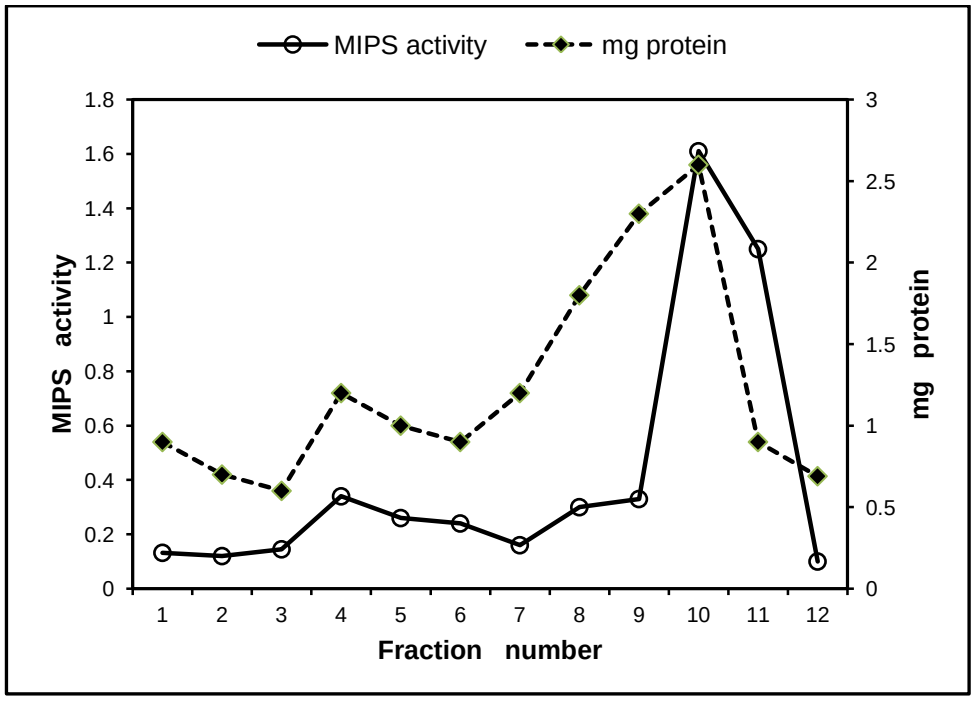


Fig-3.2 Elution profile of cytosolic MIPS from *S. junghuhnianum* in DEAE cellulose column (MIPS activity is expressed as µM I-1-P produced/2ml fraction/hr)

3.2.2.5 Chromatography on Hexylagarose: The pooled active DEAE-cellulose fraction obtained from the previous step was loaded on top of a column (0.8 cm x 7.5 cm) of hexylagarose. After collection of effluents, the column was eluted with the equilibration buffer and fractions of 1.25 ml each were collected at a flow rate of 6-8 min per fraction. Each fraction along with the effluent was assayed for the enzyme activity and the active fractions were pooled and marked as penultimate preparation (Hxl-fraction) of the partially purified enzyme.

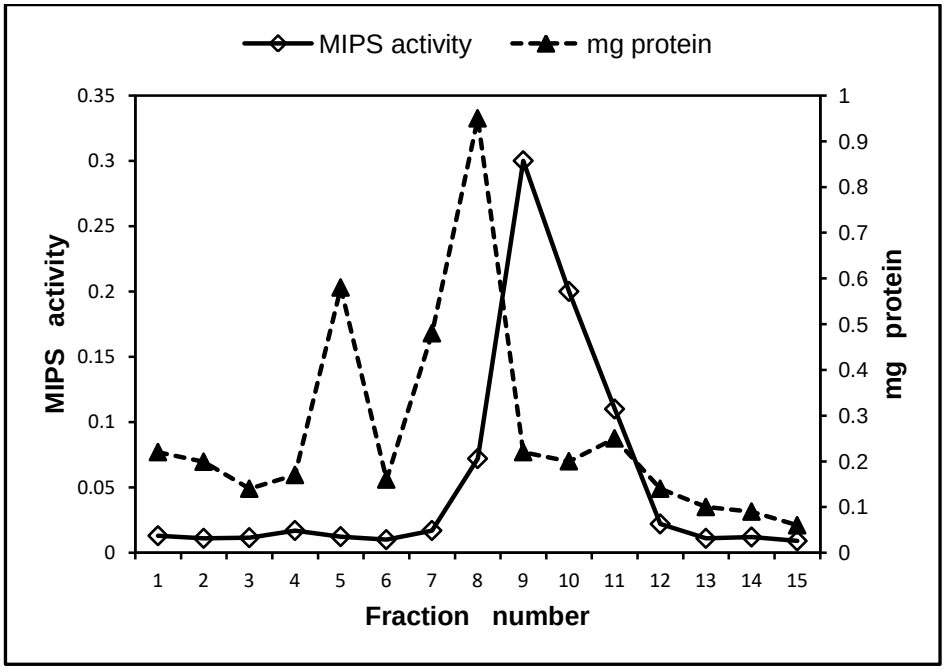


Fig-3.3 Elution profile of cytosolic MIPS from *A. khasiana* in Hexylagarose column (MIPS activity is expressed as µM I-1-P produced/1.25ml fraction/hr)

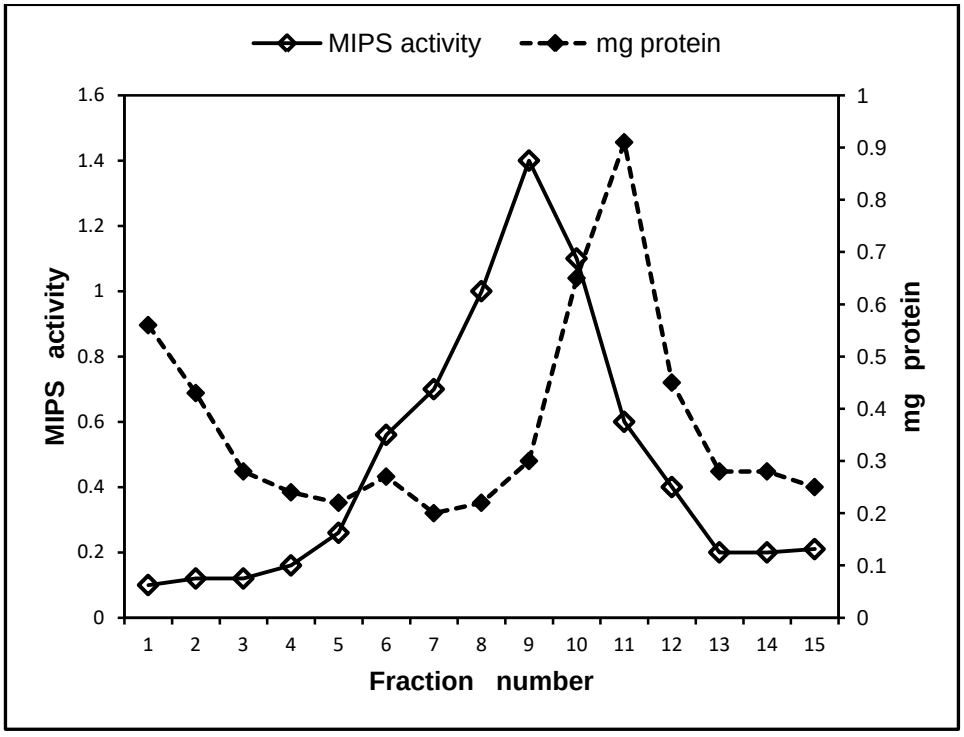


Fig-3.4 Elution profile of cytosolic MIPS from *S. junghuhnianum* in Hexylagarose column (MIPS activity is expressed as µM I-1-P produced/1.25ml fraction/hr)

3.2.2.6 Molecular sieve chromatography through BioGel A-0.5m: The hexylagarose fractions which were found enzymatically active were pooled together and loaded in a column (0.6 x10.0 cm) of BioGel A-0.5m pre-equilibrated with 50 mM Tris-acetate buffer (pH 7.5) containing 0.2 mM ME. Proteins were eluted with the same buffer in fractions of 0.5 ml/5 mins. Fractions containing MIPS activity was pooled and dialyzed against 1.5 L of 50 mM Tris-acetate buffer (pH 7.5) containing 0.2 mM ME. This preparation was concentrated (BioGel-fraction) and used as the enzyme source for further characterization.

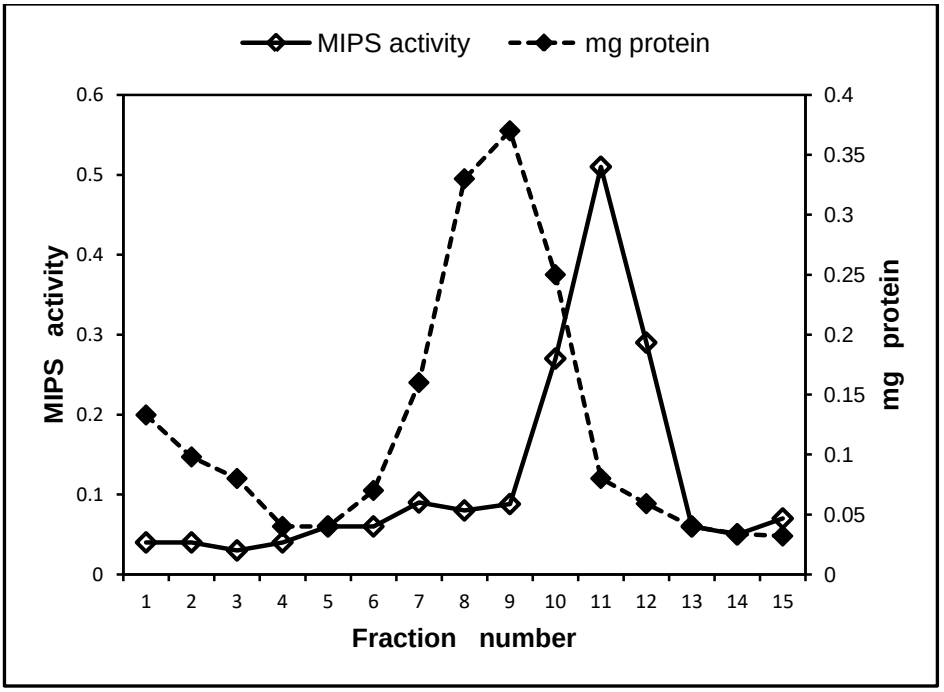


Fig-3.5 Elution profile of cytosolic MIPS from *A. khasiana* in BioGel A-0.5m column (MIPS activity is expressed as µM I-1-P produced/0.5ml fraction/hr)

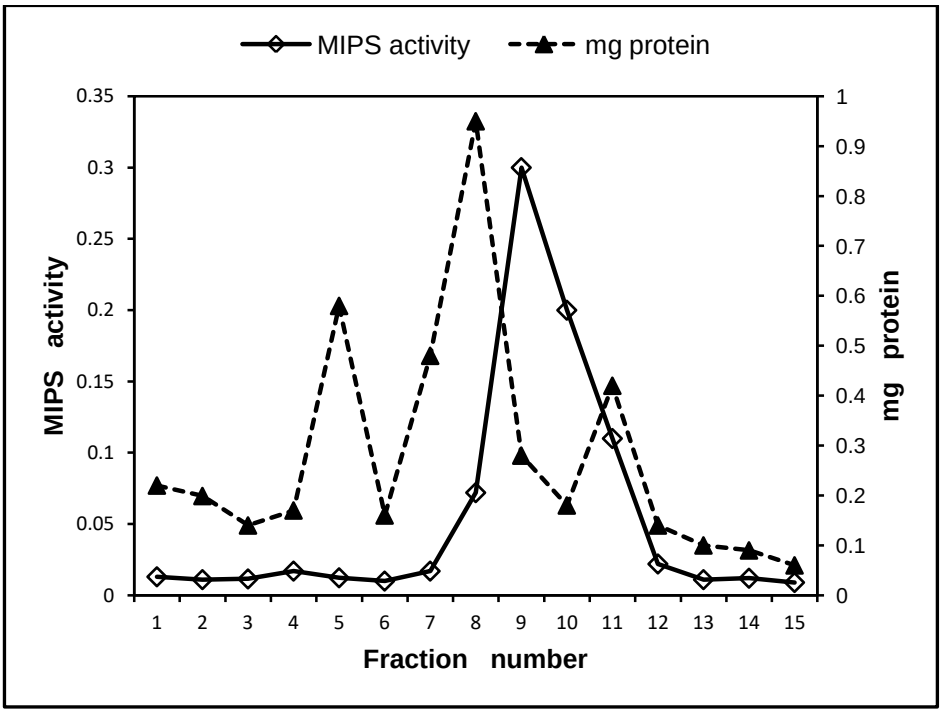


Fig-3.6 Elution profile of cytosolic MIPS from *S. junghuhnianum* in BioGel A-0.5m column (MIPS activity is expressed as µM I-1-P produced/0.5ml fraction/hr)

3.2.2.7 Polyacrylamide gel electrophoresis: Polyacrylamide gel electrophoresis was performed under native conditions following the method of Bollag *et al*., (1996) with slight modifications. The separating gel (8 % acrylamide) was prepared by mixing 8 ml of acrylamide solution (from a mixture of 30 % acrylamide + 0.8 % bis) to 8 ml of 1.5 M Tris buffer (pH adjusted to 8.8 with HCl) and 14 ml of distilled water. To this 50 µL of TEMED and 150 µL of freshly prepared ammonium persulphate (10 %) was added just before pouring. The mixture was then poured in the glass sandwich along the side spacer and overlaid with ethanol. After polymerization for 60 min the overlaying ethanol was decanted off and the stacking gel mixture was poured. The stacking gel (4 %) was prepared by adding 1.1 ml of acrylamide mixture (30 % acrylamide + 0.8 % bisacrylamide) to 2.3 ml of 0.5 M Tris buffer (pH adjusted to 6.8 with HCl) and 4.6 ml of distilled water. To this 60 µL of freshly prepared ammonium persulphate (10 %) and 20 µL of TEMED were added just before pouring. Immediately after the introduction of the stacking gel, a comb was placed on top of the stacking mixture. After polymerization for 60 min, the comb was taken out and the gel wells were washed with running buffer.

The running buffer (containing 3.0 g of Tris-base, 14.4 g of glycine and sufficient quantity of distilled water to adjust the volume to 1L) was poured in the gel apparatus (Biotech regular size vertical slab gel apparatus) and the plates were assembled into it. Thereafter, the samples to be run through electrophoresis were mixed with the sample buffer (5 µL of sample buffer per 20 µL of protein sample). The sample buffer contained 3.1 ml of 1 M Tris-base (pH adjusted to 6.8 with HCl), 5 ml of glycerol, 0.5 ml of bromophenol blue (1 %) and 1.4 ml of distilled water. Then 20 µL of each protein

sample (containing about 30µg of protein) along with the mixed sample buffer was introduced in each well of the stacking gel and the gel was run for 8h at room temperature (Initially at 80 V for 1h and then 100 V).

After completion of the run, the gel was stained overnight by keeping in a staining solution containing 0.1 % CBB (R), 45 % methanol (v/v) and 10 % glacial acetic acid (v/v). Subsequently, destaining was carried out by immersing the gel in a destaining solution containing 10 % methanol (v/v) and 10 % glacial acetic acid (v/v) with 3 changes.

For the inositol synthase assay from the gel, replicate gels were run. One of the gels was stained as described above to visualize the protein bands and the other was sliced into 5 mm fragments. The enzyme from each of the slice was extracted with 250 µL of 50 mM Tris-acetate buffer (pH 7.5) for 30 min at 0ºC to 4ºC. The extracts were then assayed for inositol synthase activity as described in the section 3.3.

3.3 Assay of L-*myo*-inositol-1-phosphate synthase: The inositol synthase activity was assayed by the procedure of Barnett *et al.*, (1970) with slight modifications as per Adhikari *et al.*, (1987).

The following procedure was followed for the assay:

3.3.1 Enzyme incubation: In a total volume of 0.5 ml, the incubation mixture contained 500 mM Tris-acetate (pH 7.5), 140 mM NH_4Cl, 8 mM NAD, 50 mM ME, 50 mM G-6-P and an appropriate protein aliquot (100-200 µg). The reaction was started by the addition of substrate immediately after the addition of the enzyme with proper mixing. Duplicate tubes were run along with an appropriate blank (without enzyme) and

a zero minute control in which 200 µL of 20 % chilled TCA was added prior to the addition of the enzyme, was also run. The enzymatic incubation was carried out for 60 min at 37ºC. After 60 min the reaction was terminated by the addition of 200 µL of chilled TCA (20 %). Two such sets (set I-periodate and set II–non-periodate) were run simultaneously, each having one blank, one zero minute control and two experimental tubes. The quantity of the enzymatic product was estimated by periodate oxidation followed by the estimation of inorganic phosphates.

3.3.2 Oxidation with sodium metaperiodate: After completion of the enzyme incubation, the resultant supernatant was subjected to a treatment with 0.7 ml of 200 mM sodium metaperiodate ($NaIO_4$) and incubated for 60 min at 37ºC. Then, 1.4 ml of 1M Na_2SO_3 (prepared immediately before use) was added in case of set–I to destroy excess of $NaIO_4$. In set-II, water was added instead of $NaIO_4$ and Na_2SO_3 to maintain the volume equal to that of the set-I.

Inorganic phosphate was liberated from *myo*-inositol-1-phosphate during oxidation. Cleavage of G-6-P also took place but it was thought to be extremely low (2 mM of P_i / mol of G-6-P) which could be subtracted considering the blank or zero minute control from the experimental value in set-I. Hydrolysis of phosphate from G-6-P by contaminating phosphatase (if any) was measured by subtracting the value of the blank or zero minute control from the experimental value in set-II. Product specific cleavage of inorganic phosphate was estimated by subtracting the corrected value of set-II from that of set-I.

3.3.3 Estimation of inorganic phosphate: Inorganic phosphate was estimated by the method of Chen *et al.*, (1956) with slight modifications. Pi-reagent (2.8 ml) was added to the reaction mixture (total vol now became 5.6 ml) and incubated at 37ºC for 60 min. The P_i reagent was prepared immediately before use at 10 ^{0}C by adding H_2SO_4 (6N), ascorbic acid (10 % w/v), chilled ammonium molybdate (2.5 % w/v) and H_2O (1:1:1:2) in the same order as mentioned here. After incubation, the blue colour developed was measured at 820 nm in a Beckman DU-64 spectrophotometer. The inorganic phosphate released was estimated with the help of a standard curve prepared from different known quantities (0-20 µg) of phosphorous (using K_2HPO_4).

As 1 mole of myo-inositol-1-phosphate contains 1 mole of inorganic phosphate, the total mole number of inorganic phosphate released was equal to the total mole number of myo-inositol-1-phosphate produced.

3.3.4 Estimation of protein: Protein was estimated by the method of Bradford (1976) with slight modifications, using BSA as standard. Dilutions of known amount of BSA were made with distilled water in the ranges of 0 µg to 250 µg in a total volume of 100 µL. A protein reagent was prepared by dissolving 100 mg of CBB (G-250) in 50 ml of 95% ethanol. To this 100 ml of phosphoric acid (85%) was added and the total volume was made up to 1000 ml with distilled water.

To each aliquot of protein sample (100 µL), 5 ml of Bradford reagent was added, mixed thoroughly and the absorbance was measured at 595 nm with a Beckman DU-64 spectrophotometer after 5 min of incubation. A reagent blank was also prepared with 100 µL of distilled water.

3.3.5 Determination of specific activity of L-*myo*-inositol-1-phosphate synthase: The specific activity of L-*myo*-inositol-1-phosphate synthase was calculated by determining the number of moles of inorganic phosphate released (from *myo*-inositol phosphate) per mg of protein. The activity was defined as µ mol L-*myo*-inositol-1-phosphate (I-1-P) produced per hour per mg of protein i.e., µ mol I-1-P produced (mg protein)$^{-1}$ h^{-1}.

3.3.6 Determination of molecular weight of L-*myo*-inositol-1-phosphate synthase: Approximate molecular weight of the native L-*myo*-inositol-1-phosphate synthase obtained from the plant body of bryophytes *A. khasiana* and *S. junghuhnianum* bearing reproductive structures were determined by gel-filtration through Sephadex G-200 column. 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., ovalbumin (43 kDa), BSA (66 kDa), phosphorylase-b (97.4 kDa), catalase (221.6 kDa) and apoferritin (443 kDa). The void volume was determined with blue dextran 2000 (1 mg / ml). All standards were loaded in the column separately and fractions (0.5 ml) were collected at a flow rate of 0.5 ml/5 min. Each individual protein peak was located by spectrophotometric scanning at 280 nm in a Spectrophotometer. A standard curve was prepared by plotting relative elution volume of proteins against their respective log molecular weights (sections 4.5.3 and 4.8.3). Partially purified enzyme (BioGel A-0.5 m) was loaded into the Sephadex G-200 column under identical conditions. Fractions were assayed for *myo*-inositol synthase activity by the procedure described in Section 3.3. Molecular weight was determined from the relative elution

volume of the active fractions by comparison with the standard curve prepared with the proteins of known molecular weights.

3.4. Extraction and estimation of free *myo*-inositol from bryophytes *Asterella khasiana* (Griff.) Grolle and *Sphagnum junghuhnianum* Doz. & Molk.

3.4.1 Isolation of free *myo*-inositol from bryophytes: Free *myo*-inositol was estimated by the method of Charalampous and Chen (1966) with slight modifications. One gram of bryophytic tissue was homogenized in double volume of 0.154 M KCl in a Remi M-153 homogenizer for 2-3 min. The crude homogenate was centrifuged at 8,500 ×g for 20 min. The supernatant was collected and denatured by boiling in a water bath for 10 minutes, then it was cooled and again centrifuged at 8,500 ×g for 30 min and the supernatant was collected.

The resultant supernatant was demineralized by running through a mixed bed column 0.3 × 1.5 cm each of Dowex-1-Cl^- (100-200 mesh) and Amberlite IR-120 (sodium form). The Dowex was placed on top of the Amberlite and the demineralised solution was collected as effluent. The solution was concentrated to 100 µL by evaporating in a water bath and treated with 100 µL of 0.15 M $Ba(OH)_2$ solution at 100ºC for 15 min. The mixture was again demineralised through another column of the same composition after dilution with 1.3 ml of water. Then, the volume of the above demineralised solution was reduced to 1.0 ml by heating in a water bath and treated with equal volume of 80% ethanol at 4ºC to remove glycogen. The filtrate was collected by low speed centrifugation and the volume was reduced to dryness by boiling in a water bath. The

content was carefully washed and dissolved in 200 µL of water. From this solution inositol and sugars were separated by one-dimensional descending paper chromatography on Whatman No. 1 paper.

From the total of 200 µL of the above solution 50 µL was spotted on a Whatman no. 1 chromatography paper (36 × 12 cm) containing another spot of marker *myo*-inositol (50 µg in 50 µL of H_2O). The chromatography was carried out at room temperature for 3-4h using acetone:water (85:15 v/v) as the solvent. On completion of the solvent run, the chromatography paper was taken out from the chamber and dried. A narrow strip of paper with marker inositol was cut out and stained according to the method of Trevelyan *et al*., (1950). The paper was dipped into solution-I (0.1 ml saturated aqueous solution of $AgNO_3$ in 100 ml of acetone) for about 1 min, dried quickly and dipped into solution-II (0.5 N NaOH solution prepared in 5 % ethanol) for coloration. The background colour was eliminated by dipping the dried strip into solution-III (5 % $Na_2S_2O_3$). Thus, the inositol area became distinct by this stain. From the unstained chromatogram, the paper strip corresponding to the inositol position was eluted slowly with 1ml of H_2O inside a water saturated small chamber in order to isolate the free *myo*-inositol.

3.4.2 Estimation of free *myo*-inositol: Free *myo*-inositol was estimated spectrophotometrically according to the method of Gaitonde and Griffiths (1966) in which inositol was oxidized by sodium metaperiodate and the micro quantity of inositol was determined from the decrease of the quantity of sodium meta-periodate in the reaction mixture after oxidation.

To 1 ml of solution having isolated free *myo*-inositol (method described earlier), 1 ml of 5 mM $NaIO_4$ (sodium metaperiodate) was added and incubated at 37 ^{0}C for 2 h. Then, 200 µL aliquot of the reaction mixture was taken in a stoppered tube and 8 ml of 0.1 M Borate buffer (pH 8.1) was added to it followed by the addition of 2 ml of freshly prepared 2% potassium iodide solution. The solution was mixed thoroughly and the extinction was measured at 352 nm in Beckman DU-64 spectrophotometer after 5 min of incubation. The periodate remaining after the oxidation of *myo*-inositol was estimated by subtracting the extinction of the sample from that of the reagent blank prepared with 1 ml of water. Inositol concentration was determined by extrapolation from a standard curve that was prepared using known concentrations of *myo*-inositol (0-100 µg).

4. RESULTS

4.1 Distribution of L-*myo*-inositol-1-phosphate synthase in different groups of bryophytes from Darjeeling hills:

Experimental bryophytes viz., *Asterella khasiana* (Griff.) Grolle, *Marchantia polymorpha* Linn., *Riccia cruciata* Kash, *Anthoceros angustus* Steph., *Bryum argenteum* Linn., *Brachymenium bryoides* Hook. ex Schwaegr., *Lyellia crispa* R. Br., *Funaria wallichii* (Mitt.) Broth. and *Sphagnum junghuhnianum* Doz. & Molk. etc. were collected fresh as described in Section 3.1. L-*myo*-inositol-1-phosphate synthase was assayed using dialyzed and low speed supernatant as the enzyme source (Section 3.3). The results presented in Table 4.1 show that the enzyme is functional in different genera of bryophytes belonging to different families of all representative species tested during this study with at least a minimum titre of activity. It has also been documented from the same table that the *Asterella khasiana* shows the highest activity among all the species tested along with *Sphagnum junghuhnianum* showing the second highest titre of activity of the enzyme in its vegetative part. This screening result prompted for purification of the enzyme and its characterization from these two species of bryophytes.

4.2 Free *myo*-inositol content in different bryophytic species from Darjeeling hills:

Appreciable quantity of free *myo*-inositol (the final product of *myo*-inositol biosynthesis) was detected in different bryophytic species from Darjeeling hills tested during this study. Samples of different species were collected freshly as described in Section 3.1 for this experiment and the content of total free *myo*-inositol was isolated

and estimated according to the method described in Section 3.4.1 and 3.4.2. The results have been presented in Table 4.2 from which it is clear that the quantity of free *myo*-inositol in almost all bryophytes tested were quantitatively appreciable. Maximum free inositol content was detected in *Bryum argenteum* from among the mosses and *Asterella khasiana* from among the liverworts. However, the content of free *myo*-inositol in vegetative parts of *Anthoceros angustus*, *Brachymenium broides* and *Sphagnum junghuhnianum* were also significant.

Table 4.1 Distribution of L-*myo*-inositol-1-phosphate synthase in some members of bryophytes

[Specific activity defined as μ mol I-1-P produced (mg protein)$^{-1}$ h^{-1} in a dialyzed 11.4 K supernatant. FW = Fresh Weight]

Plant	**Family**	**Plant part used**	**Specific activity [μmol I-1-P produced (mg)$^{-1}$ protein h^{-1}]**
Asterella khasiana (Griff.) Grolle	Aytoniaceae	Vegetative	0.284 ± 0.067
Marchantia polymorpha Linn.	Marchantiaceae	Reproductive	0.105 ± 0.018
Riccia cruciata Kash.	Ricciaceae	Vegetative	0.073 ± 0.001
Anthoceros angustus Steph.	Anthocerotaceae	Whole plant	0.082 ± 0.001
Bryum argenteum Linn	Bryaceae	Whole plant	0.089 ± 0.002
Brachymenium bryoides Hook. ex Schwaegr	Bryaceae	Bryales	0.164 ± 0.005
Lyellia crispa R. Br.	Polytrichaceae	Whole plant	0.078 ± 0.002
Funaria wallichii (Mitt.) Broth.	Funariaceae	Whole plant	0.049 ± 0.001
Sphagnum junghuhnianum Doz. & Molk.	Sphagnaceae	Vegetative	0.193 ± 0.040

Table-4.2. Free *myo*-inositol content in different bryophytes from Darjeeling hills.

Plant	**Family**	**Order**	**Plant part used**	**Free MI [mg (g)$^{-1}$ FW]**
Marchantia polymorpha Linn.	Marchantiaceae	Marchantiales	Reproductive	2.45 ± 0.032
Riccia cruciata Kash.	Ricciaceae	Marchantiales	Vegetative	1.25 ± 0.046
Anthoceros angustus Steph.	Anthocerotaceae	Anthocerotales	Whole plant	2.80 ± 0.026
Asterella khasiana	Aytoniaceae	Marchantiales	Vegetative	2.94 ± 0.136
Bryum argenteum Linn.	Bryaceae	Bryales	Whole plant	3.25 ± 0.135
Brachymenium bryoides Hook. ex Schwaegr	Bryaceae	Bryales	Whole plant	2.60 ± 0.025
Lyellia crispa R. Br.	Polytrichaceae	Polytrichales	Whole plant	1.60 ± 0.025
Funaria wallichii (Mitt.) Broth.	Funariaceae	Funariales	Whole plant	1.95 ± 0.100
Sphagnum junghuhnianum Doz. & Molk	Sphagnaceae	Sphagnales	Vegetative	2.55 ± 0.057

4.3 Activity of L-*myo*-inositol-1-phosphate synthase and content of free *myo*-inositol in different stages in the life cycle of *Asterella khasiana*:

It has been found in many plants including ptridophytes and angiosperms that L-*myo*-inositol-1-phosphate synthase occurs in both vegetative and reproductive structures. In order to find out such eventuality even in case of selected bryophyte from Darjeeling hills, *Asterella khasiana*, screening experiment was designed whereby the activity of the enzyme was assayed in dialyzed homogenate and low-speed supernatant fractions obtained from the vegetative and reproductive parts of this plant species following the methods as described in sections 3.2.1 and 3.3. The results are depicted in Table 4.3. The most striking observation is with the higher activity of this enzyme in low speed supernatant than in homogenate fraction. Moreover, the activity of the enzyme in low

speed supernatant was almost four times higher in reproductive parts as compared to the vegetative structures. In case of homogenate fraction, no such striking result was observed.

Table-4.3 L-myo-inositol-1-phosphate synthase activity in vegetative structures of *A. khasiana* as compared that of the reproductive structures of the same species.

[Specific activity defined as µ mol I-1-P produced (mg protein)$^{-1}$ h^{-1}. FW= fresh weight]

Plant	**Tissue type**	**Enzyme source**	**Specific activity [µmol I-1-P produced (mg)$^{-1}$ protein h^{-1}]**
Asterella khasiana	Vegetative	Homogenate	0.248 ± 0.067
		Low speed supernatant	0.164 ± 0.014
	Reproductive	Homogenate	0.107 ± 0.011
		Low speed supernatant	0.949 ± 0.190

Experiment was also designed to find out whether the L-*myo*-inositol-1-phosphate synthase enzyme product, free *myo*-inositol also occur in the reproductive parts in addition to the vegetative ones. Indeed the occurrence of free myo-inositol was confirmed in the reproductive parts of *Asterella khasiana* and the content of the same in the reproductive part was found to be about 1.36 times higher than that of vegetative part as depiced in Table 4.4.

Table-4. 4 Free *myo*-inositol content in vegetative structures of *A. khasiana* as compared to that of the reproductive structures of the same species. [FW = fresh weight]

Plant	Family	Order	Tissue type	Free myo-inositol [mg $(g)^{-1}$ FW]
Asterella khasiana	Aytoniaceae	Marchatiales	Vegetative	2.94 ± 0.136
			Reproductive	4.00 ± 0.187

4.4 Partial purification of L-*myo*-inositol-1-phosphate synthase from reproductive thallus of *Asterella khasiana*:

The cytosolic enzyme, L-*myo*-inositol-1-phosphate synthase (EC 5.5.1.4.) was isolated and partially purified from the reproductive thallus of freshly collected *Asterella khasiana* by the procedure described in Sections 3.1 and 3.2.2. Table 4.5 represents the outlines of the partial purification. It has become evident from the same table that the enzyme could be purified about 46 fold over the homogenate fraction. The recovery of the enzyme based on total activity was about 36 %.

Table-4.5. Summary of partial purification of L-myo-inositol-1-phosphate synthase from *Asterella khasiana.* [Specific activity defined as µ mol I-1-P produced (mg protein)$^{-1}$ h^{-1}]
(Values are mean ± SE, n=3)

Purification step	Total protein (mg)	Specific activity [µmol I-1-P produced (mg)-1 protein h-1]	Total activity [µmol I-1-P produced (mg)-1 protein h-1]	Recovery (%)	Purification (fold)
Homogenate	51 ± 1.52	0.178 ± 0.002	9.078 ± 0.057	100.0 ± 4.16	1.00 ± 0.06
10K-supernatant	31 ± 1.52	0.245 ± 0.016	7.595 ± 0.253	83.66 ± 3.20	1.37 ± 0.13
SS-fraction	24 ± 1.73	0.288 ± 0.006	6.912 ± 0.141	76.14 ± 2.14	1.61 ± 0.09
A_2S-fraction	08 ± 0.57	0.650 ± 0.060	5.200 ± 0.175	57.28 ± 3.84	3.65 ± 0.57
DE-fraction	04 ± 0.20	1.219 ± 0.009	4.876 ± 0.138	53.71 ± 1.88	6.84 ± 0.29
Hxl-fraction	02 ± 0.14	2.100 ± 0.064	4.200 ± 0.152	46.26 ± 0.90	11.79 ± 1.17
BioGel-fraction	0.4 ± 0.035	8.250 ± 0.408	3.300 ± 0.202	36.35 ± 3.24	46.34 ± 1.53

4.5 The enzymatic characteristics of L-*myo*-inositol-1-phosphate synthase from the reproductive thallus of *Asterella khasiana*:

Partial purification of L-*myo*-inositol-1-phosphate synthase as summarized in Table 4.5 is a time consuming process. Usually, in this laboratory, six to seven days are required

to obtain an active BioGel-A 0.5 m fraction. Furthermore, the BioGel-A 0.5m fraction was moderately stable for a period of a week or so. The active pooled BioGel-A 0.5 m fraction was used for the characterization experiments.

4.5.1 Stability: An important property of the bryophytic L-*myo*-inositol-1-phosphate synthase is the moderate stability of its catalytic activity. Stability varies with the enzyme preparation at different stages of purification. While the low speed supernatant remained active for 7-8 days when stored at −20 ºC, the BioGel 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 dithiothritol (DTT) considerably increased the activity of the enzyme.

4.5.2 PAGE profile and corresponding enzymic activity: The PAGE profile of the protein as well as MIPS activity of the corresponding protein bands (determined from the 5 mm gel slices) from the BioGel A 0.5m fraction of *Asterella khasiana* reproductive thallus after electrophoresis under native condition has been shown in Fig. 4.1. Only the band marked as “active band” had enzymic activity and Fig. 4.1 shows the matching protein band showing the MIPS activity.

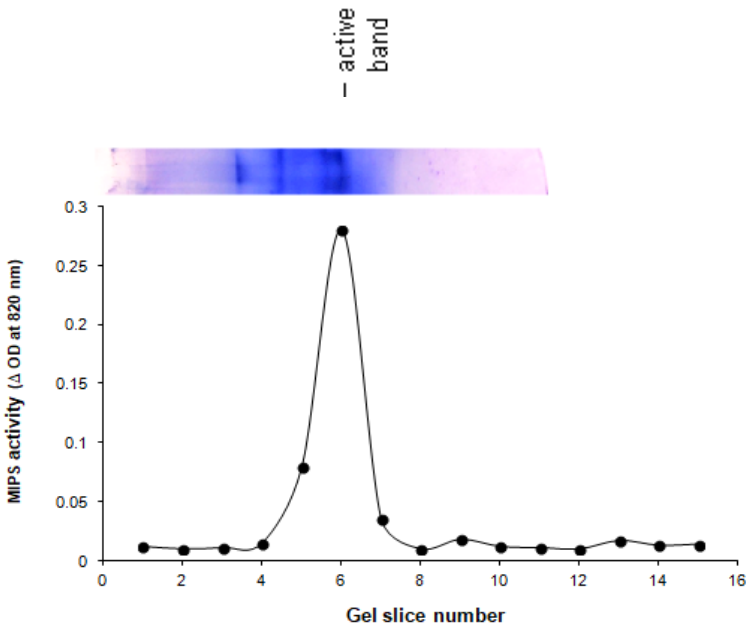


Fig.-4.1 PAGE profile showing MIPS activity in *A. khasiana* when assayed with 5mm gel slice each

4.5.3 Apparent molecular weight of *Asterella khasiana* L-*myo*-inositol-1-phosphate synthase: The apparent molecular weight of *Asterella khasiana* L-*myo*-inositol-1-phosphate synthase was determined following the procedure described in Section 3. It was found that the molecular weight of the enzyme was approximately 183 kDa (Fig. 4.2).

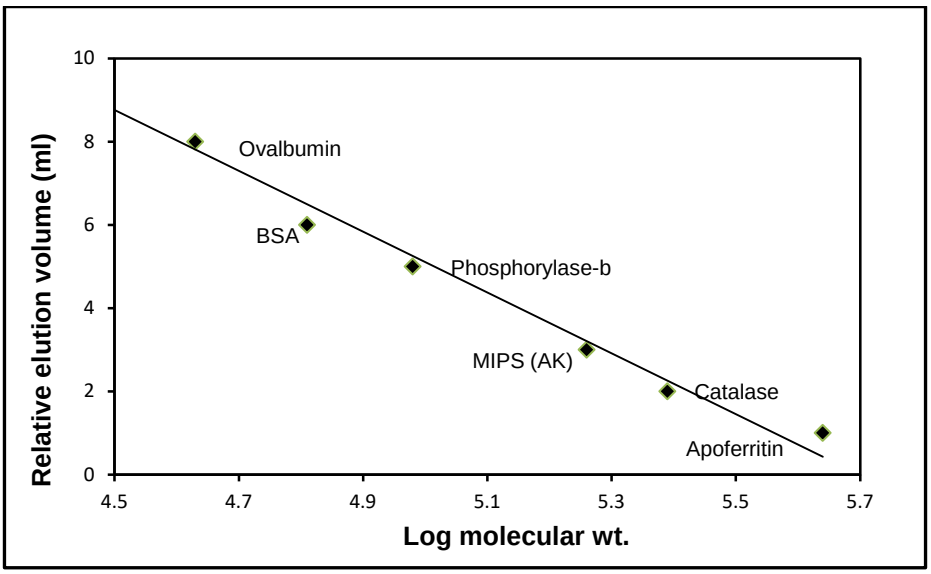


Fig-4.2 Determination of molecular wt. of *Asterella khasiana* (AK) MIPS of by gel filtration chromatography

4.5.4 Requirements for *Asterella khasiana* L-*myo*-inositol-1-phosphate synthase activity:

Asterella khasiana MIPS, when assayed in presence of 50 mM Tris-acetate buffer (pH 7.5), 14 mM NH_4Cl, 0.8 mM NAD, 5 mM ME and 5 mM D-Glucose-6P with an appropriate protein aliquot called the complete set, recorded maximal activity (Table 4.6). When the specific substrate of the enzyme, G-6P was not added in the incubation mixture, enzymatic synthesis of L-*myo*-inositol-1-phosphate could not be detected. About 10 % activity was lost when Tris buffer was omitted from the complete reaction mixture. Deduction of NAD^+, NH_4Cl or ME caused the decrease in MIPS activity by about 59.17%, 31.80% and 34.51% respectively.

Table -4.6 Effect of composition of incubation medium on L-myo-inositol-1-phosphate synthase activity in *Asterella khasiana*

[Specific activity defined as µ mol I-1-P produced (mg protein)$^{-1}$ h^{-1}]

(Values are mean ± SE, n=3)

Conditions	**Specific activity [µmol I-1-P produced (mg)$^{-1}$ protein h^{-1}]**	**Percent activity**
Complete set	1.107 ± 0.118	100 ± 5.03
Without substrate (G-6-P)	0.00	0.00
Without co-enzyme (NAD^+)	0.452 ± 0.009	40.83 ± 3.52
Without NH_4Cl	0.755 ± 0.027	68.20 ± 2.28
Without ME	0.725 ± 0.007	65.49 ± 1.68
Without buffer (tris-acetate)	0.995 ± 0.070	89.88 ± 2.25
Heat killed enzyme	0.00	0.00

4.5.5 Progress of the enzyme reaction with time: Incubation of *Asterella khasiana* L-*myo*-inositol-1-phosphate synthase was carried out for different time periods between 0 and 150 minutes at an interval of 30 minutes each, under standard assay conditions. It was observed that the reaction proceeded linearly with time upto 90 min (Fig. 4.3) beyond which the catalytic activity seemed to have reached a plateau and followed a flat line.

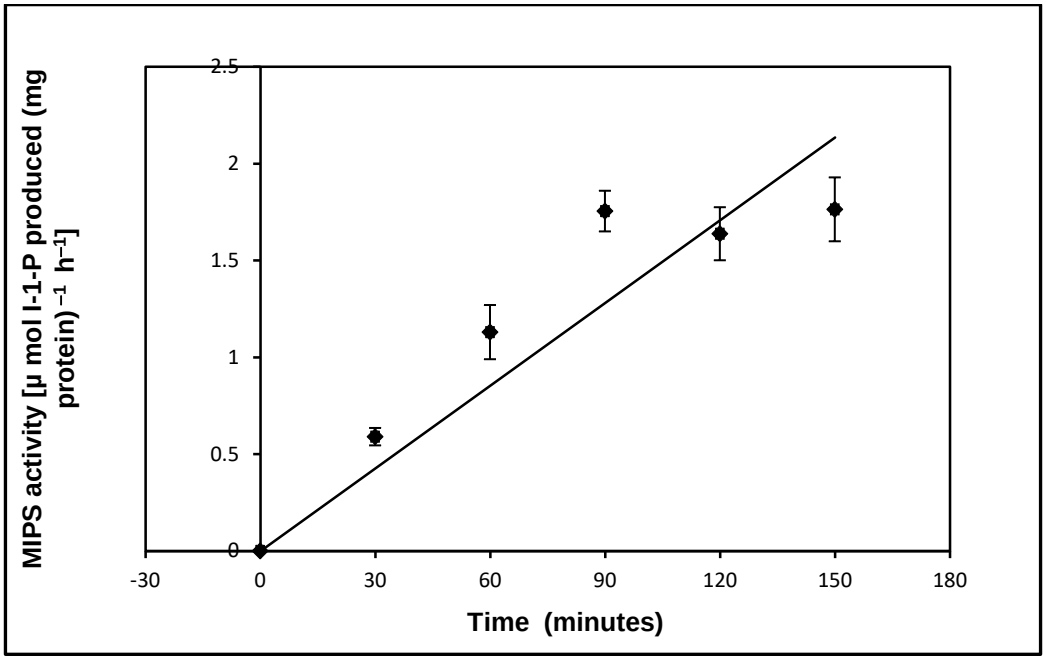


Fig-4.3 Effect of incubation time on the progress of MIPS reaction in *A. khasiana*

4.5.6 Progress of reaction with respect to protein concentration: Using increasing concentrations of *Asterella khasiana* L-*myo*-inositol-1-phosphate synthase enzyme protein (0-400µg), *myo*-inositol synthase assay was carried out under optimal conditions as described in Section 3.3. The results are depicted in Fig. 4.4. It has been revealed that the enzyme activity increased linearly with respect to protein concentration of about 250µg. Increase of protein concentration beyond this level did not increase the enzyme activity.

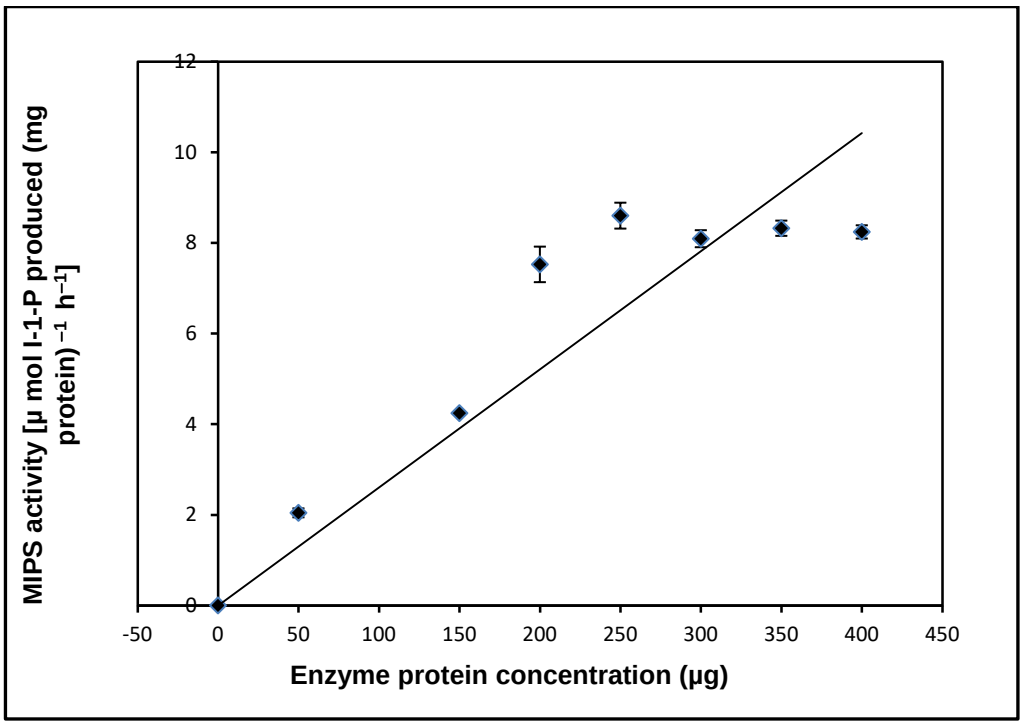


Fig-4.4 Effect of varied enzyme protein concentration on MIPS activity in *A. khasiana*

4.5.7 Thermal stability: In order to find out the relative enzyme activity as influenced by different incubation temperature, *Asterella khasiana* L-*myo*-inositol-1-phosphate synthase enzyme from the BioGel A0.5m fraction was incubated separately for one hour at temperature between the ranges of 0ºC to 60ºC at an interval of ten degrees in presence of standard assay mixture. Results shown in Fig. 4.5, clarifies that the activity of the enzyme was least both at 10ºC and 60ºC. However, the enzyme was remarkably active between the temperature ranges of 20ºC to 50ºC with a maxima at 30ºC. This is an interesting observation on thermal stability of inositol synthase with respect to its stability over a wide range of incubation temperatures.

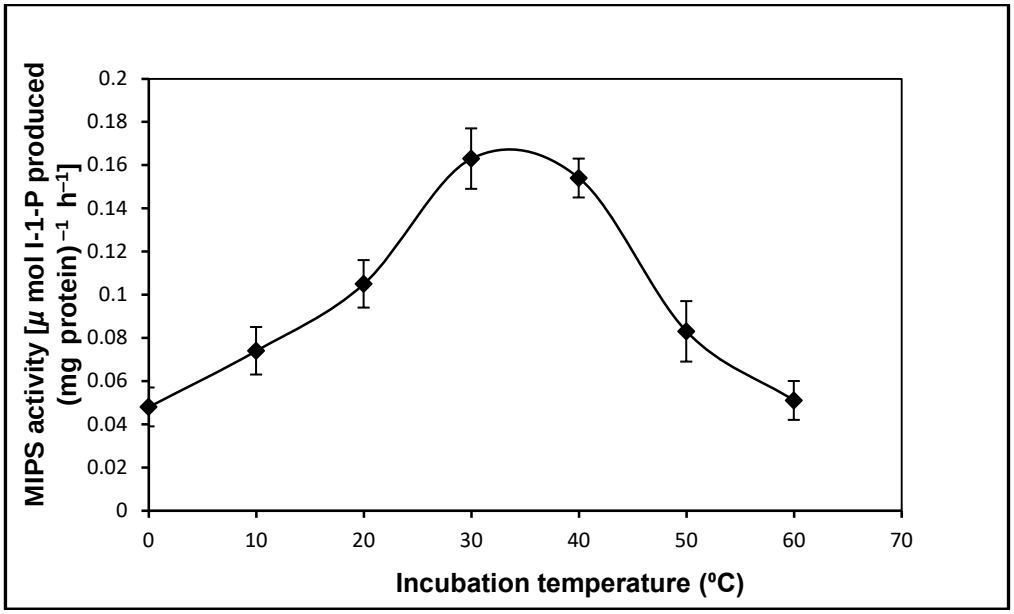


Fig-4.5 Effect of various incubation temperatures on MIPS activity in *Asterella khasiana*

4.5.8 Substrate specificity: The partially purified *Asterella khasiana* L-*myo*-inositol-1-phosphate synthase has been found to utilize specifically D-glucose-6-phosphate as the exclusive substrate for the production of L-*myo*-inositol–1-phosphate. Among other hexose phosphates tested, such as, D-fructose-6-phosphate, D-galactose-6-phosphate, D-mannose-6-phosphate, and hexose bisphosphate like D-fructose-1, 6-bisphosphate used in place of D-glucose-6-phosphate at the identical concentration (5 mM), all were ineffective as substrate for the *Asterella khasiana* L-*myo*-inositol-1-phosphate synthase activity. However, this enzyme could partly utilize D-galactose-6-phosphate and D-mannose-6-phosphate as substrate with about 8.92 % and 3.38 % efficiency, respectively as compared to D-glucose-6-phosphate as substrate (Table 4.7).

Table-4.7. Substrate specificity of L-myo-inositol-1-phosphate synthase from *A. khasiana*

[Specific activity defined as µ lmo I-1-P produced (mg protein)$^{-1}$ h^{-1}]

(Values are mean ± SE, n=3)

Compound	**Concentration (mM)**	**Specific activity [µmol I-1-P produced (mg)$^{-1}$ protein h^{-1}]**	**Percent activity**
D-glucose-6-phosphate	10.0	0.650 ± 0.033	100 ± 8.43
D-fructose 1,6 bisphosphate	10.0	0.00	0.00
Galactose-6-phosphate	10.0	0.058 ± 0.004	8.92 ± 0.16
D-fructose-6-phosphate	10.0	0.00	0.00
D-glucose-1-phosphate	10.0	0.00	0.00
D-mannose-6-phosphate	10.0	0.022 ± 0.0049	3.38 ± 0.25

4.5.9 Effect of D-glucose-6-phosphate (substrate) concentration on *Asterella khasiana* L-*myo*-inositol-1-phosphate synthase activity: Determination of K_m and V_{max} values

4.5.9.1 Effect of substrate concentration: The activity of BioGel A0.5m purified *Asterella khasiana* L-*myo*-inositol-1-phosphate synthase was found to increase with respect to the concentration of D-glucose-6-phosphate upto 6 mM when tried between concentration ranges of 0 to 10 mM substrate. Thereafter, there was no significant change in the enzymatic activity (Fig. 4.7).

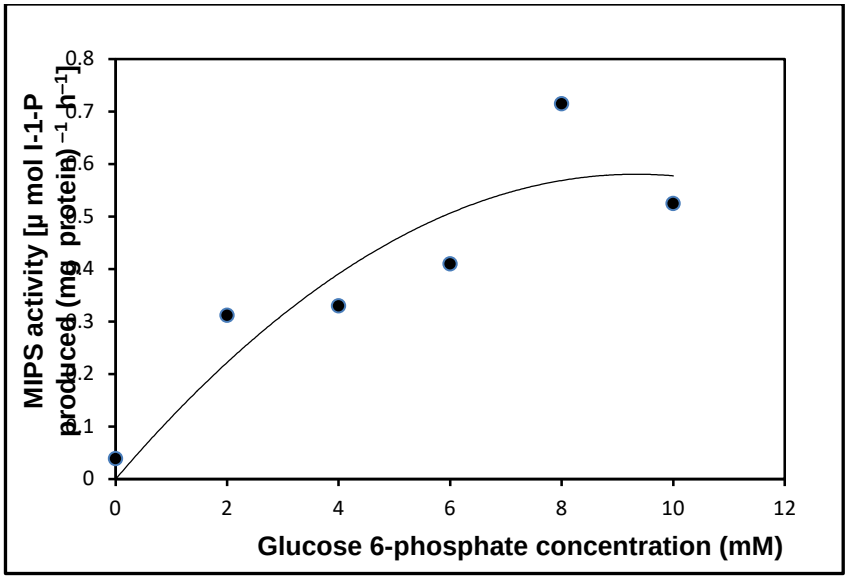


Fig-4.6 Effect of substrate (G-6-P) concentration on *Asterella khasiana* MIPS activity

4.5.9.2 Determination of K_m and V_{max} values : The average K_m for D-Glucose-6-Phospatae for *Asterella khasiana* L-*myo*-inositol-1-phosphate synthase was calculated to be approximately 3.56 mM in accordance with the rate equation of Michaelis-Menten and also by means of Lineweaver-Burk plot for enzyme kinetics. The V_{max} value was calculated as 0.71 mM.

4.5.10 Effect of NAD^+ (co-enzyme) concentration on *Asterella khasiana* L-*myo*-inositol-1-phosphate synthase activity: Determination of K_m and V_{max} values.

4.5.10.1 Effect of coenzyme concentration: Between a concentration range of 0 to 1.0 mM NAD^+, the activity of BioGel-A0.5 m purified enzyme was found to increase with respect to the concentration of NAD+ upto 0.8 mM. Subsequent to that, further enhancement of co-enzyme concentration could not change the enzyme activity (Figure 4.7).

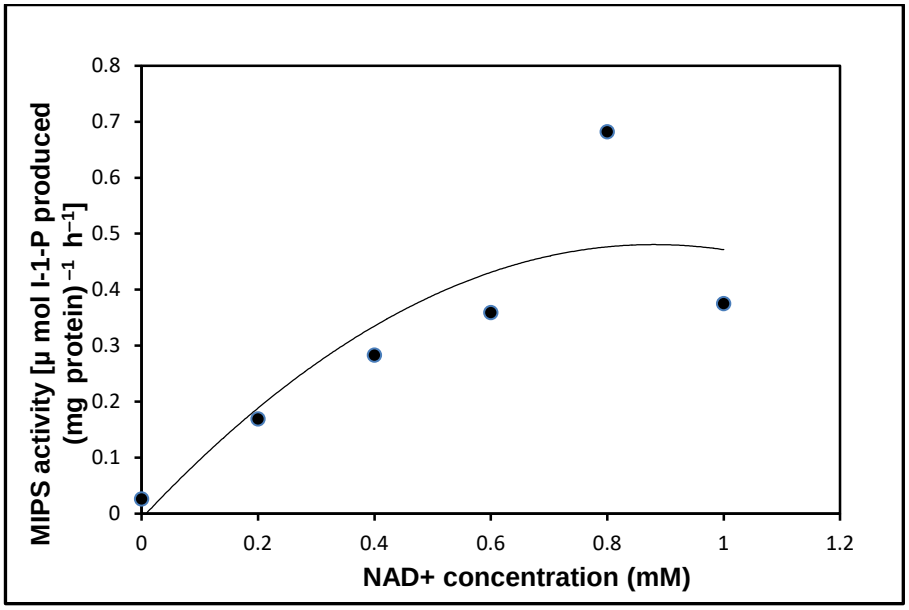


Fig-4.7 Effect of co-enzyme (NAD^+) concentration on *Asterella khasiana* MIPS activity

4.5.10.2 Determination of K_m and V_{max} values: The average K_m for NAD^+ of *Asterella khasiana* L-*myo*-inositol-1-phosphate synthase was calculated to be approximately 0.56 mM in accordance with the rate equation of Michaelis-Menten and also by means of Lineweaver-Burk plot for enzyme kinetics. The V_{max} was calculated as 0.68 mM.

4.5.11 Replacement of NAD^+ with $NADP^+$: L-*myo*-inositol-1-phosphate synthase from most of the reported sources requires NAD^+ as an essential coenzyme for the oxidation-reduction reaction. No other coenzyme has been found to be useful for this enzyme. To ascertain whether the *myo*-inositol synthase from *A. khasiana* is specific for its coenzyme NAD^+, or whether the NAD^+ requirement can be satisfied by almost similar type of coenzyme(s), experiments were performed in which NAD^+ was replaced with $NADP^+$ in the assay mixture. In such experiments the enzyme activity was determined in presence of 0 to 1.0 mM NAD^+ / $NADP^+$ in two parallel experiments. Results of

these are presented in Fig. 4.8. The results revealed that for *myo*-inositol synthase from *Asterella khasiana*, NAD^+ could not be substituted with $NADP^+$ at any concentration. However, there was a minimum basal activity of this enzyme even in the experimental set even with $NADP^+$.

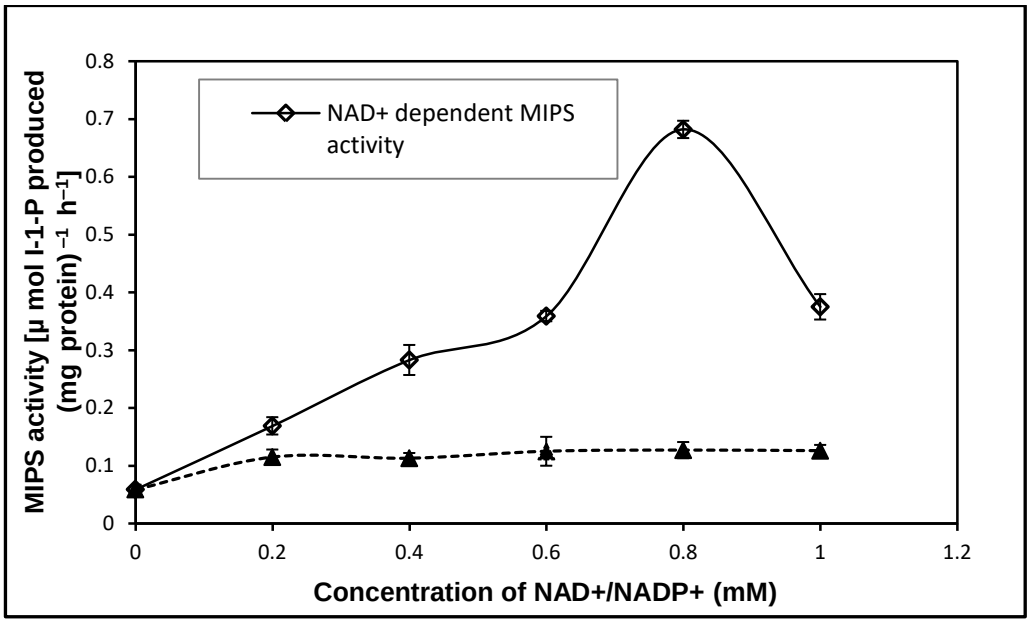


Fig-4.8 Effect of replacement of co-enzyme NAD^+ with $NADP^+$ in *Asterella khasiana* L-*myo*-inositol-1-phosphate synthase activity

4.5.12 Influence of pH on *Asterella khasiana* MIPS activity: The L-*myo*-inositol-1-phosphate synthase, obtained from *Asterella khasiana*, was remarkably influenced by the pH variation. This was worked out under standard assay conditions by using 50 mM Tris-acetate buffer between the pH ranges of 6.0 to 9.0. From the results presented in Fig.4.9, it has become clear that the *Asterella khasiana* enzyme operates appreciably at a narrow pH range of 6.5 to 8.0, having optima at pH 7.0-7.5. Conspicuous decline in the catalytic activity was recorded for the enzyme either below pH 6.5 or above pH 8.0.

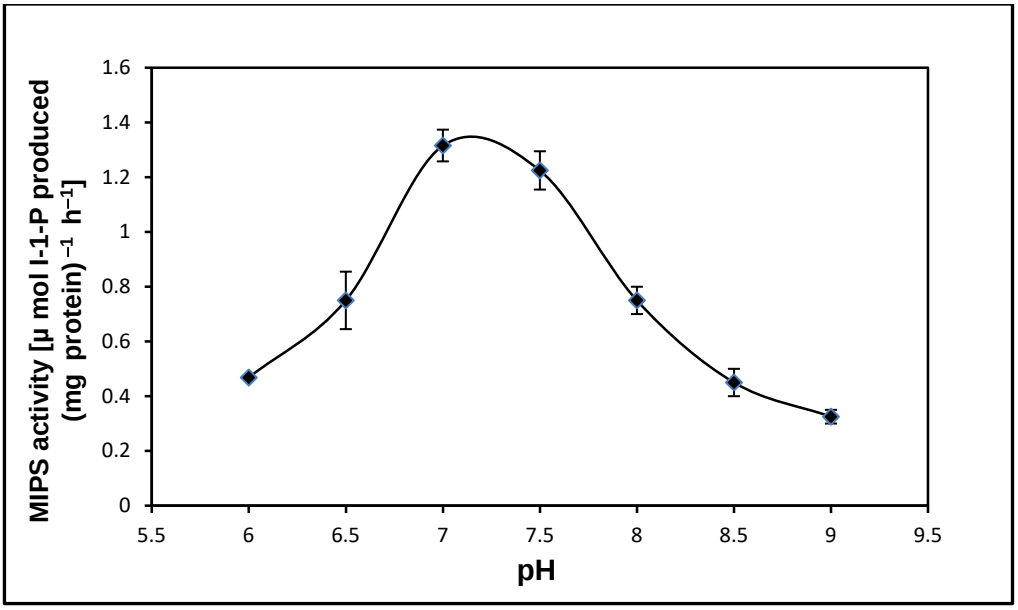


Fig4-9. Influence of pH on *Asterella khasiana* L-*myo*-inositol-1-phosphate synthase activity

4.5.13 Efect of varied concentration of different salts: In order to find out the effects of varied concentration of different salts viz., $MgCl_2$, NH_4Cl and EDTA on *Asterella khasiana* L-*myo*-inositol-1-phosphate synthase, the enzyme was incubated in presence of 0-100mM of the above salts and the enzymatic activities were determined. It was found that the effects of the salts were directly proportional to their respective concentration. Of course the effect was positive in case of $MgCl_2$ and NH_4Cl and negative in case of EDTA. Table-4.8 clearly depicts that the enzyme was stimulated to 1.69 fold and 7.57 fold by the influence of $MgCl_2$ and NH_4Cl respectively at its maximum concentration. On the contrary, the enzyme activity was inhibited to about 57.2% by the influence of EDTA at its maximum concentration of 100mM, during the present studies.

Table-4.8 Effect of varied concentrations of different salts on L-*myo*-inositol-1-phosphate synthase activity in *Asterella khasiana*

[Specific activity defined as μ mol I-1-P produced (mg protein)$^{-1}$ h^{-1}]

(Values are mean ± SE, n=3)

Concentration of salt (mM)	**Type of salts used**		
	$MgCl_2$	**NH_4Cl**	**EDTA**
0	0.105 ± 0.024	0.105 ± 0.024	0.105 ± 0.024
20	0.117 ± 0.015	0.125 ± 0.005	0.191± 0.004
40	0.124 ± 0.012	0.153 ± 0.012	0.150 ± 0.011
60	0.122 ± 0.011	0.648 ± 0.067	0.095 ± 0.020
80	0.145 ± 0.011	0.780 ± 0.086	0.081 ± 0.004
100	0.178 ± 0.28	0.795 ± 0.055	0.060 ± 0.002

4.5.14 Effect of monovalent cations: Effects of some monovalent cations on *Asterella khasiana* L-*myo*-inositol-1-phosphate synthase activity were studied using variable concentrations (0 to 10.0 mM) of chloride salts of K^+, Na^+ and Li^+. To the standard assay mixture, partially purified enzyme preparation was added in presence of the variable concentrations of individual monovalent cations as mentioned above and the enzyme activity were estimated keeping One control set without adding any such cation

was also run. Results of the experiment are presented in Table-4.9. It is evident that the K^+ had a stimulatory effect on this enzyme activity while the Na^+ played a pronounced inhibitory role decreasing the enzyme activity by approximately 47.6% by its 10 mM concentration. Furthermore, Li^+ was a strong inhibitor of this enzyme, which could reduce the specific activity to about 18% at 10 mM concentration.

Table- 4.9. Effect of monovalent cations on L-*myo*-inositol-1-phosphate synthase activity in *Asterella khasiana* (using 5 mM G-6-P as substrate)
[Specific activity defined as μ mol I-1-P produced (mg protein)$^{-1}$ h^{-1}]
(Values are mean ± SE, n=3)

Concentration of cations (mM)	**Specific activity [μmol I-1-P produced (mg)-1 protein h-1]**		
	K^+	**Na^+**	**Li^+**
0	0.105 ± 0.007	0.105 ± 0.007	0.105 ± 0.007
2	0.120 ± 0.011	0.081 ± 0.007	0.184 ± 0.008
4	0.136 ± 0.010	0.030 ± 0.008	0.084 ± 0.003
6	0.108 ± 0.002	0.060 ± 0.003	0.054 ± 0.001
8	0.154 ± 0.004	0.074 ± 0.004	0.038 ± 0.003
10	0.169 ± 0.003	0.055 ± 0.003	0.019 ± 0.002

4.5.15 Effect of Divalent cations: Effects of different divalent cations on *Asterella khasiana* L-*myo*-inositol-1-phosphate synthase activity were studied using variable concentrations (1.0 to 10.0 mM) of chloride salts of Ca^{2+}, Mn^{2+}, Cu^{2+}, Hg^{2+}, Zn^{2+} and Cd^{2+} and Hg^{2+}. Partially purified enzyme was incubated in presence of the variable concentrations of individual divalent cations as mentioned, to the usual assay components and keeping one control set without adding any such cation. Results of such

experiments have been shown in Table-4.10 wherefrom different types of effects of divalent cations were recorded. During the experiment, Ca^{2+} showed a mild stimulatory effect. Mn^{2+}, Cu^{2+} and Cd^{2+} exhibited slightly inhibitory effect while in case of Zn^{2+} the inhibitory effect was more pronounced. On the other hand, Hg^{2+} exhibited a strong inhibition of the enzyme activity restricting it to about 25% of its activity at 10mM concentration. The inhibitory effect of different divalent cations progressed in the order of $Cd^{2+} > Cu^{2+} > Mn^{2+} > Zn^{2+} > Hg^{2+}$.

Table-4.10. Effect of divalent cations on L-*myo*-inositol-1-phosphate synthase activity in *Asterella khasiana* (using 5 mM G-6-P as substrate)
[Specific activity defined as μ mol I-1-P produced (mg protein)$^{-1}$ h^{-1}]
(Values are mean ± SE, n=3)

Conc. of cations (mM)	**Specific activity [μmol I-1-P produced (mg)-1 protein h-1]**					
	Ca^{2+}	**Mn^{2+}**	**Cu^{2+}**	**Hg^{2+}**	**Zn^{2+}**	**Cd^{2+}**
0	0.105± 0.007	0.105± 0.007	0.105± 0.007	0.105± 0.007	0.105± 0.007	0.105± 0.007
2	0.155± 0.003	0.761± 0.022	0.115± 0.010	0.100± 0.009	0.097± 0.007	0.108± 0.009
4	0.181± 0.006	0.217± 0.040	0.122 ± 0.008	0.046± 0.003	0.105 ± 0.007	0.086± 0.006
6	0.362±0.086	0.228 ± 0.012	0.094±0.003	0.071±0.005	0.079±0.005	0.122 ± 0.013
8	0.142±0.009	1.720±0.025	0.100±0.010	0.109±0.008	0.181±0.006	0.130±0.012
10	0.161±0.019	0.095±0.009	0.098±0.009	0.027±0.004	0.086±0.006	0.099±0.010

4.5.16 Effect of Sugar-alcohols: The *Asterella khasiana myo*-inositol-1-phosphate synthase activity from has been found to be erratically influenced by of various concentrations of sugar alcohols like galactitol, mannitol or *myo*-inositol. Results shown in Table-4.11 demonstrated that *myo*-inositol itself upto a concentration of 10 mM was without any significant effect on the enzyme activity.

Table-4.11. Effect of varied concentration of mannitol, galactitol and myo-inositol on L-*myo*-inositol-1-phosphate synthase activity in *Asterella khasiana*
[Specific activity defined as µ mol I-1-P produced (mg protein)$^{-1}$ h^{-1}]
(Values are mean ± SE, n=3)

Type of sugar alcohol	**Concentration (mM)**	**MIPS activity [µmol I-1-P produced (mg)$^{-1}$ protein h^{-1}]**
Mannitol	0	0.246 ± 0.015
	2	0.609 ± 0.025
	4	1.744 ± 0.142
	6	0.822 ± 0.092
	8	1.645 ± 0.182
	10	0.658 ± 0.115
Galactitol	0	0.203 ± 0.012
	2	0.922 ± 0.052
	4	1.315 ± 0.084
	6	0.590 ± 0.024
	8	0.330 ± 0.018
	10	0.133 ± 0.009
Myo-inositol	0	0.261 ± 0.010
	2	0.599 ± 0.078
	4	0.270 ±0.013
	6	0.355 ± 0.010
	8	0.296 ± 0.021
	10	0.302 ± 0.050

With galactitol, the synthase activity is first increased linearly upto a concentration of 4mM and then the activity showed gradual decline culminating in about 35% loss of activity at its 10 mM concentration. However, mannitol, showed stimulation of activity which did not follow a linear relation with increasing concentration but nonetheless enhanced the enzyme activity in each of the sugar alcohol concentration tested.

4.6 Activity of L-*myo*-inositol-1-phosphate synthase and content of free *myo*-inositol in vegetative and reproductive parts of *Sphagnum junghuhnianum*:

Many plants, especially angiosperms exhibit L-*myo*-inositol-1-phosphate synthase activity in both their vegetative and reproductive structures. Screening experiment was designed assuming that the case of the moss, *Sphagnum junghuhnianum* would not be different and the enzyme activity was estimated in vegetative parts as well as in the reproductive parts of the plant. The L-*myo*-inositol-1-phosphate synthase enzyme was estimated in dialyzed homogenate and low-speed supernatant fractions obtained from the vegetative and reproductive parts of this plant species following the methods as described in sections 3.2.1 and 3.3. The results are depicted in Table 4.12.

Table-4.12 L-myo-inositol-1-phosphate synthase activity in vegetative structures of *S. junghuhnianum* a compared that of the reproductive structures of the same species.

[Specific activity defined as µ lmo I-1-P produced (mg protein)$^{-1}$ h^{-1}. FW= fresh weight]

Plant	Tissue type	Enzyme source	Specific activity [µmol I-1-P produced (mg)$^{-1}$ protein h^{-1}]
Sphagnum junghuhnianum	Vegetative	Homogenate	0.193 ± 0.040
		Low speed supernatant	0.072 ± 0.019
	Reproductive	Homogenate	0.164 ± 0.014
		Low speed supernatant	0.374 ± 0.028

The most striking observation is with the higher activity of this enzyme in low speed supernatant than in homogenate fraction. The reproductive parts contained much more titre of enzyme activity as compared to the vegetative part. In the present case, the

activity of the enzyme in the homogenate fraction of the reproductive part was about two times that of vegetative part. Similarly, the activity of the enzyme in low speed supernatant of reproductive part is about two times that of vegetative part.

Experiment was also designed to find out whether the L-*myo*-inositol-1-phosphate synthase enzyme product, free *myo*-inositol also occur in the reproductive parts in addition to the vegetative ones. Indeed the occurrence of free myo-inositol was confirmed in the reproductive parts of *Sphagnum junghuhnianum* just like that in *Asterella khasiana* and the content of the same in the reproductive part of *Sphagnum junghuhnianum* was found to be about 1.32 times higher than that of vegetative part as depiced in Table 4.13.

Table-4.13 Free *myo*-inositol content in vegetative structures of *S. junghuhnianum* as compared that of the reproductive structures of the same species. [FW = fresh weight]

Plant	**Family**	**Order**	**Tissue type**	**Free MI [mg $(g)^{-1}$ FW]**
Sphagnum junghuhnianum	Sphagnaceae	Sphagnales	Vegetative	2.55 ± 0.057
			Reproductive	3.37 ± 0.178

4.7 Partial purification of L-*myo*-inositol-1-phosphate synthase from reproductive parts of *Sphagnum junghuhnianum*: The enzyme, L-*myo*-inositol-1-phosphate synthase (EC 5.5.1.4.) was isolated and partially purified from the reproductive parts of freshly collected *Sphagnum junghuhnianum* by the procedure described in Sections 3.1 and 3.2.2. The enzyme was partially purified by obtaing the low speed supernatant which was subjected to sequential stereptomycin sulphate precipitation, ammonium sulphate cut and run through the steps of column chromatography through DEAE-

cellulose, Hexylagarose and BioGel. Table 4.14 represents the outlines of the partial purification. It has been revealed from the results that the enzyme could be purified about 59 fold over the homogenate fraction and the recovery of the enzyme based on total activity was about 33 %.

Table 4.14 Summary of partial purification of L-myo-inositol-1-phosphate synthase from *Sphagnum junghuhnianum*
[Specific activity defined as µ mol I-1-P produced (mg protein)$^{-1}$ h^{-1}]
(Values are mean ± SE, n=3)

Purification step	Total protein (mg)	Specific activity [µmol I-1-P produced (mg)-1 protein h-1]	Total activity [µmol I-1-P produced (mg)-1 protein h-1]	Recovery (%)	Purification (fold)
Homogenate	50 ± 2.51	0.193 ± 0.010	9.65 ± 1.760	100.0 ± 3.05	1.00 ± 0.11
10K-supernatant	40 ± 2.08	0.224 ± 0.005	8.96 ± 0.057	92.84 ± 1.15	1.16 ± 0.09
SS-fraction	24 ± 1.00	0.335 ± 0.022	8.04 ± 0.206	83.31 ± 4.02	1.73 ± 0.26
A_2S-fraction	15 ± 1.52	0.517± 0.061	7.75 ± 0.132	80.36 ± 2.10	2.67 ± 0.28
DE-fraction	4.5 ± 0.26	1.650 ± 0.086	7.42 ± 0.175	76.94 ± 1.15	8.54 ± 0.70
Hxl-fraction	1.25 ± 0.07	4.616 ± 0.196	5.77 ± 0.334	59.80 ± 2.84	23.91 ± 2.02
BioGel-fraction	0.28 ± 0.01	11.325 ±1.403	3.171 ± 0.264	32.86 ± 1.48	58.67 ± 3.18

4.8 The enzymatic characteristics of L-*myo*-inositol-1-phosphate synthase from the reproductive parts of *Sphagnum junghuhnianum*:

Partial purification of L-*myo*-inositol-1-phosphate synthase is summarized in Table 4.14. To achieve a proper understanding of the enzyme, biochemical characterization of the same was also carried out. Partial purification and biochemical characterization of the enzyme from this plant is new information since there has not so far been any such work anywhere else. The active fractions from BioGel-A 0.5 m fraction was pooled together and used for the characterization experiments.

4.8.1 Stability: An important property of the *Sphagnum junghuhnianum* L-*myo*-inositol-1-phosphate synthase was the moderate stability of its catalytic activity. In this case also, stability varied with the enzyme preparation at different stages of purification. While the low speed supernatant remained active for 10-12 days when stored at −20 ºC, the BioGel purified fractions maintained its activity only up to 5-7 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 dithiothritol (DTT) considerably increased the shelf-life of the enzyme.

4.8.2 PAGE profile and corresponding enzymic activity: The PAGE profile of the protein as well as MIPS activity of the corresponding protein bands (determined from the 5 mm gel slices) from the BioGel A 0.5m fraction of *Sphagnum junghuhnianum* reproductive parts after electrophoresis under native condition has been shown in Fig. 4.10. Only the band marked as “active band” had enzymic activity and Fig. 4.10 shows the matching protein band showing the MIPS activity.

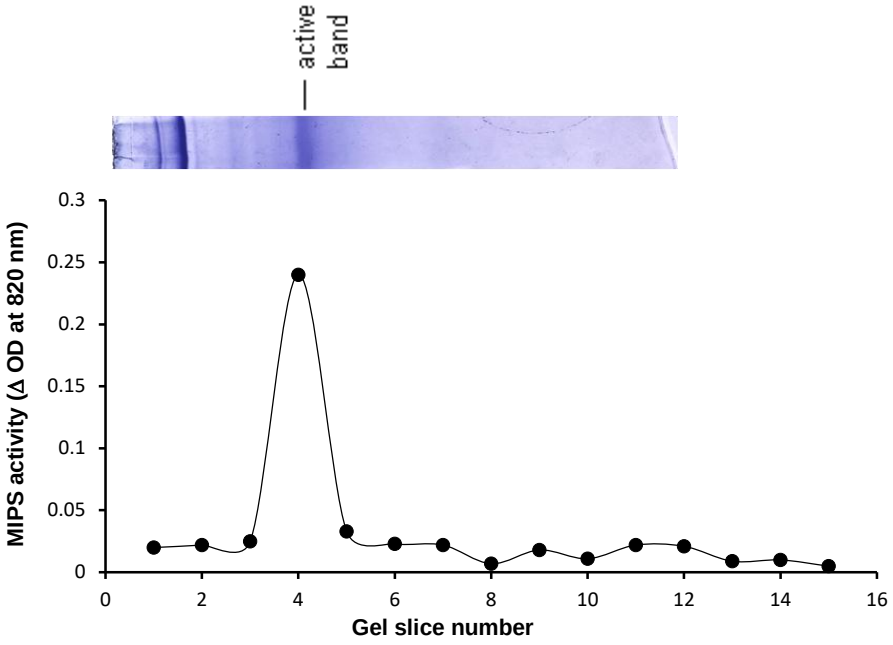


Fig-4.10 PAGE profile showing MIPS activity in *Sphagnum junghuhnianum* when assayed with 5mm gel slice each

4.8.3 Apparent molecular weight of *Sphagnum junghuhnianum* L-*myo*-inositol-1-phosphate synthase: The apparent molecular weight of *Sphagnum junghuhnianum* L-*myo*-inositol-1-phosphate synthase was determined following the procedure described in Section 3. It was found that the molecular weight of the enzyme was approximately 174 kDa (Fig. 4.11).

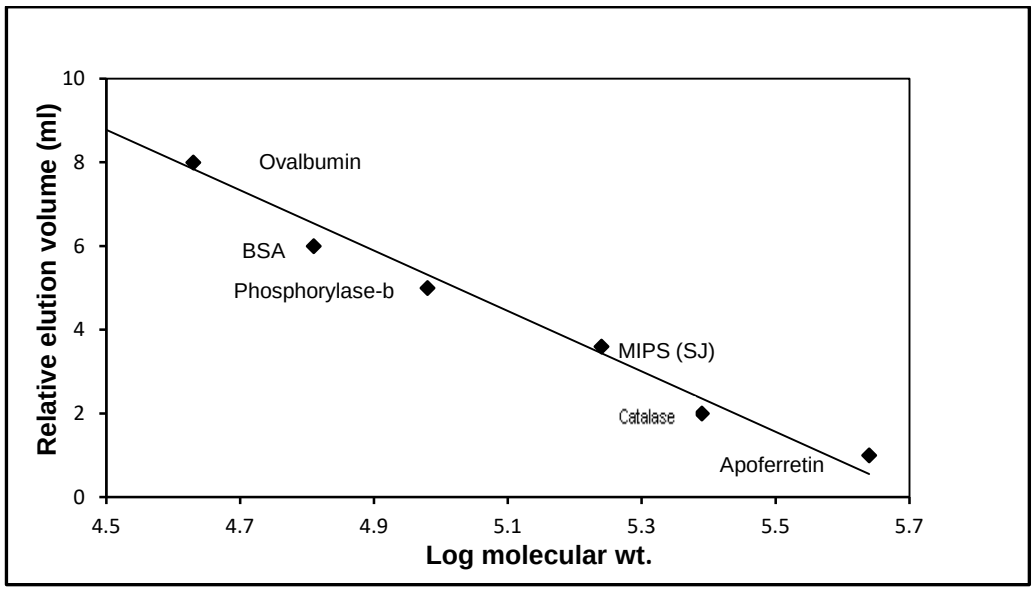


Fig-4.11. Determination of molecular wt. of *S. junghuhnianum* (SJ) MIPS of by gel filtration chromatography

4.8.4 Requirements for *Sphagnum junghuhnianum* L-*myo*-inositol-1-phosphate synthase activity:

Sphagnum junghuhnianum L-*myo*-inositol-1-phosphate synthase, when assayed in presence of standard composition containing 50 mM Tris-acetate buffer (pH 7.5), 14 mM NH_4Cl, 0.8 mM NAD, 5 mM ME and 5 mM D-Glucose-6P with an appropriate protein aliquot called the complete set, recorded maximal activity (Table 4.15). In absence of the specific substrate of the enzyme, G-6-P in the incubation mixture, enzymatic synthesis of L-*myo*-inositol-1-phosphate could not be detected. About 18 % activity was lost when Tris buffer was omitted from the complete reaction mixture. Deduction of NAD^+, NH_4Cl or ME caused the decrease in MIPS activity by about 68.41%, 40.01% and 33.35% respectively.

Table-4.15 Effect of composition of incubation medium on L-myo-inositol-1-phosphate synthase activity in *Sphagnum junghuhnianum*
[Specific activity defined as µ mol I-1-P produced (mg protein)$^{-1}$ h^{-1}]
(Values are mean ± SE, n=3)

Conditions	**Specific activity [µmol I-1-P produced (mg)$^{-1}$ protein h^{-1}]**	**Percent activity**
Complete set	0.997 ± 0.203	100 ± 6.14
Without substrate (G6P)	0.00	0.00
Without NAD^+	0.315 ± 0.010	31.59 ± 1.32
Without NH_4Cl	0.598 ± 0.117	59.99 ± 3.25
Without ME	0. 665 ± 0.143	66.65 ± 4.50
Without buffer (tris-acetate)	0.821 ± 0.020	82.34 ± 6.59
Heat killed enzyme	0.00	0.00

4.8.5 Progress of the enzyme reaction with time: Incubation of *Sphagnum junghuhnianum* L-*myo*-inositol-1-phosphate synthase was carried out for different time periods between 0 and 150 minutes at an interval of 30 minutes each under standard assay conditions. The results showed that the enzymatic reaction proceeded linearly with time upto 90 min (Fig. 4.12) beyond which the catalytic activity seemed to have reached a plateau and did not follow any increase in the rate of reaction.

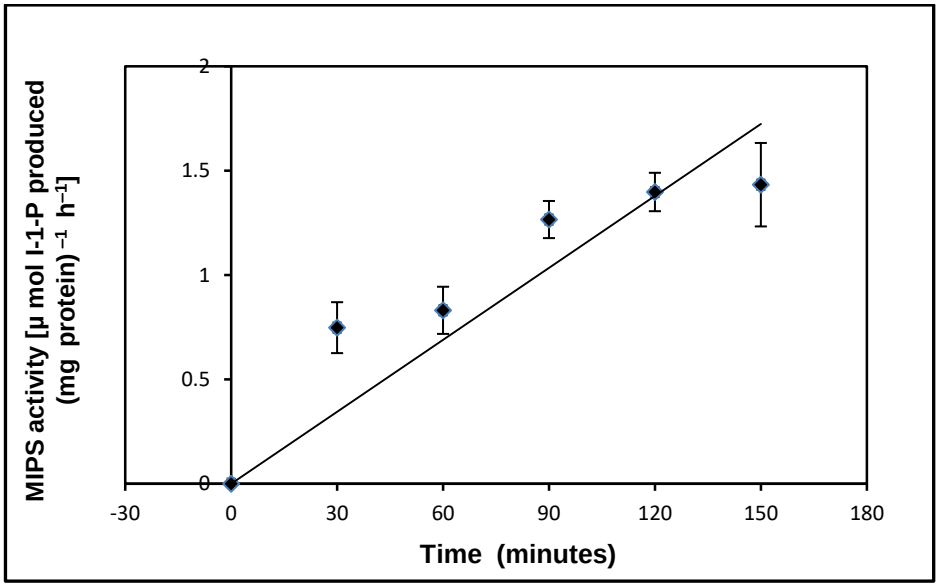


Fig-4.12 Effect of incubation time on the progress of MIPS reaction in *S. junghuhnianum*

4.8.6 Progress of reaction with respect to protein concentration: The isolated L-*myo*-inositol-1-phosphate synthase was assayed in presence of increasing concentrations of *Sphagnum junghuhnianum* L-*myo*-inositol-1-phosphate synthase enzyme protein (0-400µg), with standard assay mixtures under optimal conditions as described in Section 3.3. The results of the experiment as depicted in Fig. 4.13 revealed that the enzyme activity increased linearly with respect to protein concentration to about 300µg. Further increase of protein concentration beyond this level did not increase the enzyme activity.

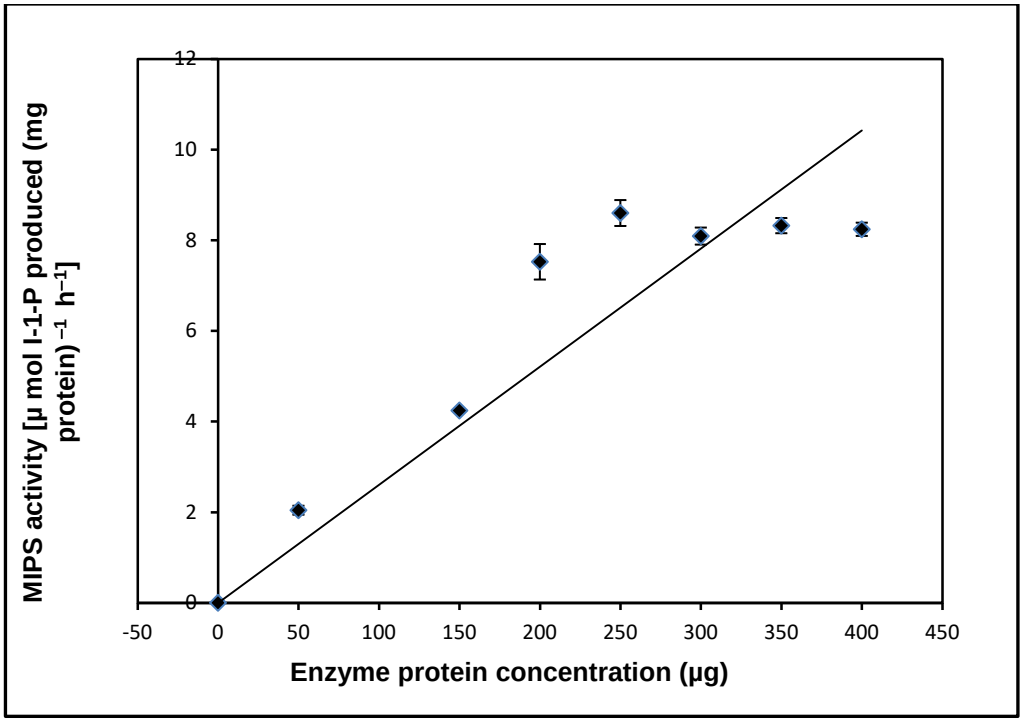


Fig.-4.13 Effect of varied enzyme protein concentration on MIPS activity in *Sphagnum junghuhnianum*

4.8.7 Thermal stability: In order to determine the thermal stability of the enzyme activity across different incubation temperature, *Sphagnum junghuhnianum* L-*myo*-inositol-1-phosphate synthase enzyme from the BioGel A0.5m fraction was incubated separately for one hour at temperature between the ranges of 0ºC and 60ºC at an interval of ten degrees in presence of standard assay mixture. Results shown in Fig. 4.14 confirmed that the activity of the enzyme was least both at 0ºC and 60ºC. However, the enzyme was remarkably active between the temperature ranges of 10ºC to 40ºC with a maximum at about 30ºC. This is an interesting observation on thermal stability of inositol synthase with respect to its stability over a wide range of incubation temperatures.

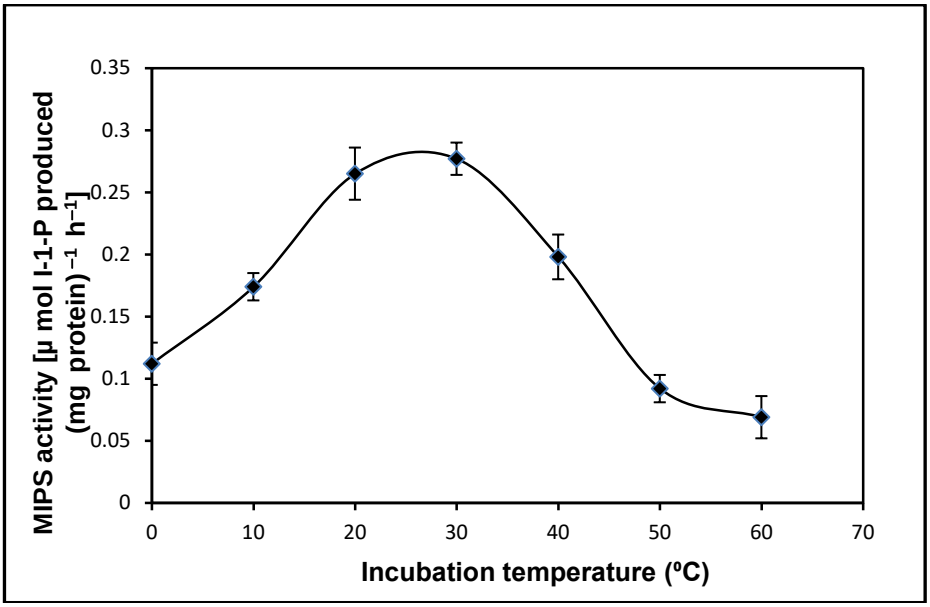


Fig-4.14 Effect of various incubation temperatures on MIPS activity in *Sphagnum junghuhnianum*

4.8.8 Substrate specificity: The partially purified *Sphagnum junghuhnianum* L-*myo*-inositol-1-phosphate synthase has been found to exclusively utilize D-glucose-6-phosphate as the exclusive substrate for the production of L-*myo*-inositol–1-phosphate. Among other hexose phosphates tested, such as: D-fructose-6-phosphate, D-galactose-6-phosphate, D-mannose-6-phosphate, and hexose bisphosphates like D-fructose-1, 6-bisphosphate used in place of D-glucose-6-phosphate at the identical concentration (5 mM), all were ineffective as substrate for the *Sphagnum junghuhnianum* L-*myo*-inositol-1-phosphate synthase activity. However, this enzyme could partially utilize D-galactose-6-phosphate and D-mannose-6-phosphate as substrate with about 11.23 % and 3.81 % efficiency respectively, when compared to D-glucose-6-phosphate as substrate (Table 4.16).

Table-4.16. Substrate specificity of L-myo-inositol-1-phosphate synthase from *Sphagnum junghuhnianum* [Specific activity defined as µ mol I-1-P produced (mg protein)$^{-1}$ h^{-1}] *(Values are mean ± SE, n=3)*

Compound	Concentration (mM)	Specific activity [µmol I-1-P produced (mg)$^{-1}$ protein h^{-1}]	Percent activity
D-glucose-6-phosphate	10.0	0.997 ± 0.074	100 ± 10.02
D-fructose 1,6 bisphosphate	10.0	0.00	0.00
Galactose-6-phosphate	10.0	0.112 ± 0.016	11.23 ± 0.90
D-fructose-6-phosphate	10.0	0.00	0.00
D-glucose-1-phosphate	10.0	0.00	0.00
D-mannose-6-phosphate	10.0	0.038 ± 0.002	3.81 ± 0.32

4.8.9 Effect of D-glucose-6-phosphate (substrate) concentration on *Sphagnum junghuhnianum* L-*myo*-inositol-1-phosphate synthase activity: Determination of K_m and V_{max} values

4.8.9.1 Effect of substrate concentration: The activity of BioGel A-0.5m purified *Sphagnum junghuhnianum* L-*myo*-inositol-1-phosphate synthase was found to increase with respect to the concentration of D-glucose-6-phosphate upto 6 mM, when tried between concentration ranges of 0 to 10 mM substrate. Thereafter, there was no significant change in the enzymatic activity (Fig. 4.15).

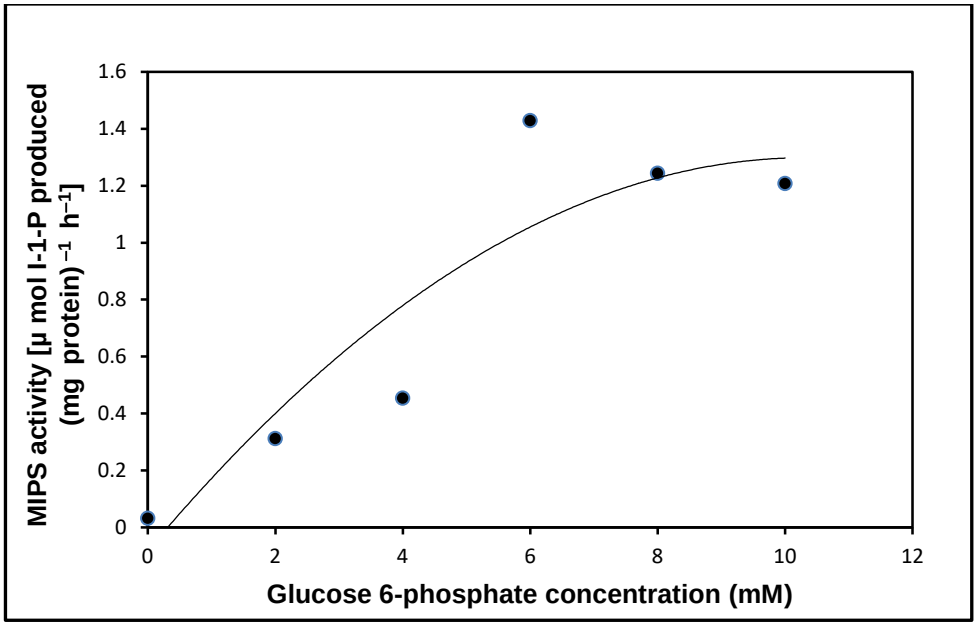


Fig-4.15 Effect of substrate (G-6-P) concentration on *S. junghuhnianum* MIPS activity

4.8.9.2 Determination of K_m and V_{max} values: The average K_m for D-Glucose-6-Phospatae for *Sphagnum junghuhnianum* L-*myo*-inositol-1-phosphate synthase was calculated to be approximately 1.81 mM in accordance with the rate equation of Michaelis-Menten and also by means of Lineweaver-Burk plot for enzyme kinetics. The V_{max} value was calculated as 1.42 mM.

4.8.10 Effect of NAD^+ (co-enzyme) concentration on L-*myo*-inositol-1-phosphate synthase activity in *Sphagnum junghuhnianum*: Determination of K_m and V_{max} values.

4.8.10.1 Effect of coenzyme concentration: Between a concentration range of 0 to 1.0 mM NAD^+, the activity of BioGel-A0.5 m purified enzyme was found to increase with respect to the concentration of NAD^+ upto 0.6 mM. Subsequent to that, further enhancement of co-enzyme concentration could not increase the enzyme activity (Figure 4.16).

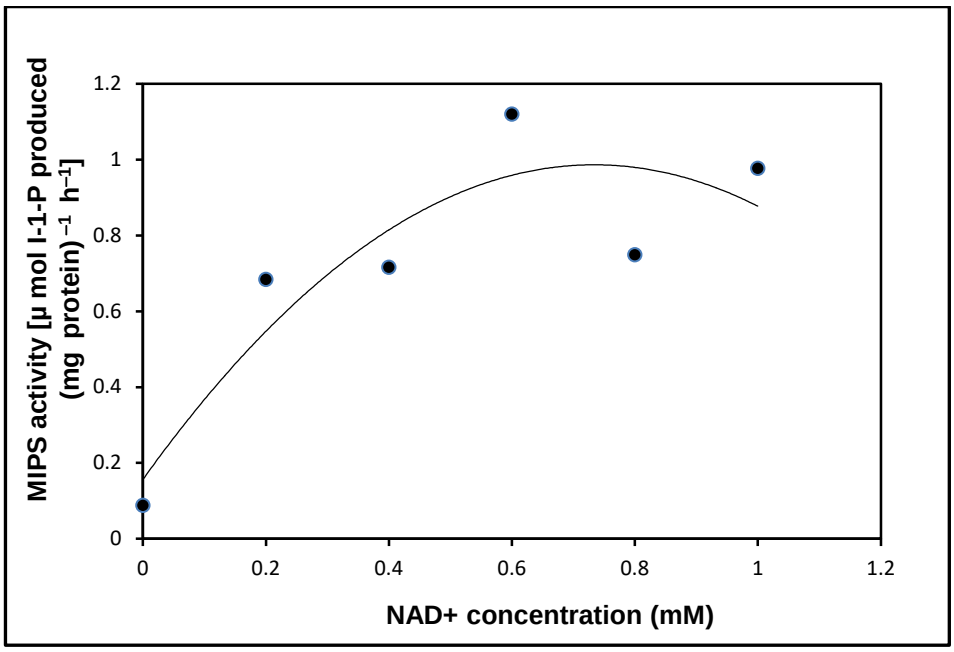


Fig-4.16 Effect of co-enzyme (NAD) concentration on *S. junghuhnianum* MIPS activity

4.8.10.2 Determination of K_m and V_{max} values: The average K_m for NAD^+ of L-*myo*-inositol-1-phosphate synthase from *Sphagnum junghuhnianum* was calculated to be approximately 0.25 mM in accordance with the rate equation of Michaelis-Menten and also by means of Lineweaver-Burk plot for enzyme kinetics. The V_{max} was calculated as 1.12 mM.

4.8.11 Effect of replacement of NAD^+ with $NADP^+$: Efforts were made to find out whether NAD^+ is the exclusive coenzyme for *myo*-inositol synthase from *Sphagnum junghuhnianum* or whether the NAD^+ requirement can be bypassed by adding other similar type of coenzyme(s), and experiments were designed in which NAD^+ was replaced with $NADP^+$ in the assay mixture. In such experiments the enzyme activity was determined in presence of 0 to 1.0 mM NAD^+ / $NADP^+$ in two parallel experiments. Results of these are presented in Fig. 4.17. The results revealed that for *myo*-inositol

synthase from *Asterella khasiana*, NAD^+ could not be substituted with $NADP^+$ at any concentration. However, in this case also, there was a minimum basal activity of this enzyme even in the experimental set with $NADP^+$.

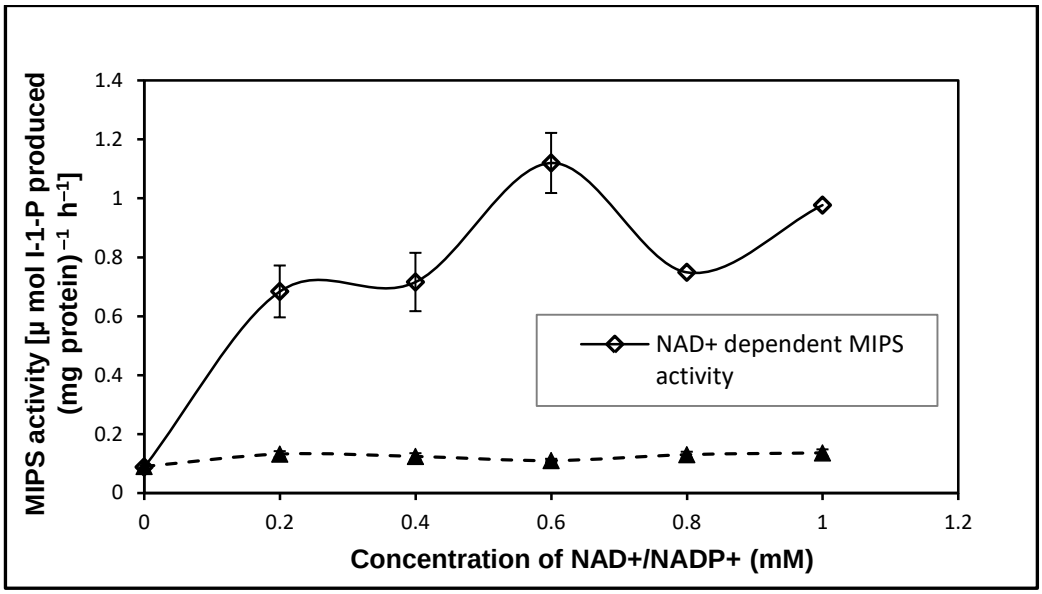


Fig-4.17 Effect of replacement of co-enzyme NAD^+ with $NADP^+$ on L-*myo*-inositol-1-phosphate synthase activity from *Sphagnum junghuhnianum*

4.8.12 Influence of pH on *Sphagnum junghuhnianum* MIPS activity: The L-*myo*-inositol-1-phosphate synthase, obtained from *Sphagnum junghuhnianum*, was significantly influenced by the pH variation. This was worked out under standard assay conditions by using 50 mM Tris-acetate buffer between the pH ranges of 6.0 and 9.0. From the results presented in Fig.4.18, it has become clear that the *Sphagnum junghuhnianum* enzyme operates appreciably at a narrow pH range of 7.0 to 7.5 having optima at pH 7.0. Conspicuous decline in the catalytic activity was recorded for the enzyme either below pH 7.0 or above pH 8.0.

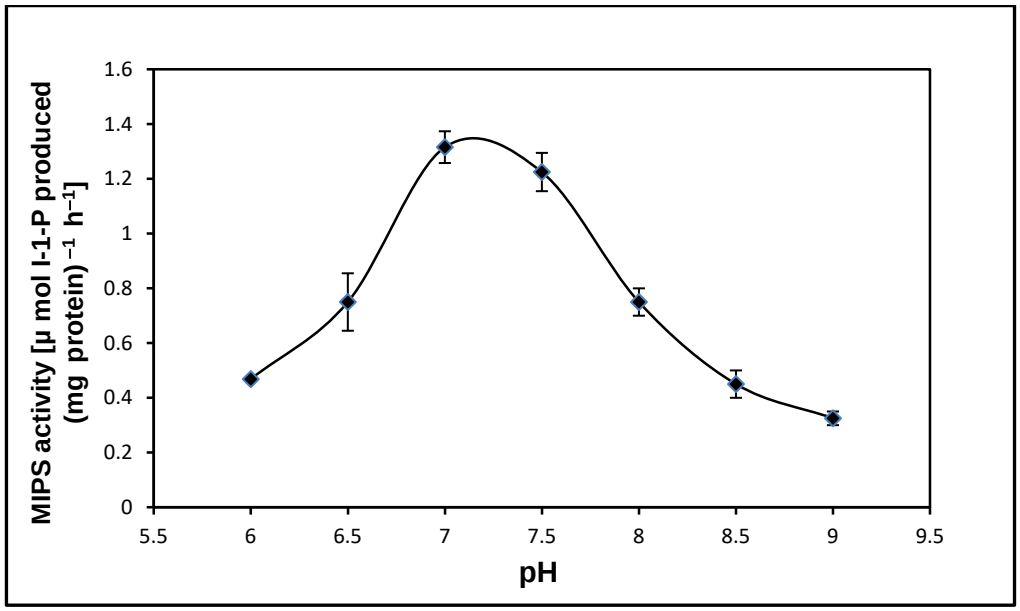


Fig-4.18 Influence of pH on *Sphagnum junghuhnianum* L-*myo*-inositol-1-phosphate synthase activity

4.8.13 Efect of varied concentration of different salts: In order to find out the effects of varied concentration of different salts viz., $MgCl_2$, NH_4Cl and EDTA on *Sphagnum junghuhnianum* L-*myo*-inositol-1-phosphate synthase, the enzyme was incubated in presence of 0-100mM of the above salts and the enzymatic activities were determined. It was found that the effects of the salts were directly proportional to their respective concentration. Different types of effects were exhibited by different types of salts on the enzyme activity. The effect of $MgCl_2$ was slightly stimulatory causing about 1.3 fold increase in enzyme activity, while NH_4Cl was extremely stimulatory increasing the enzyme activity by up to 9.3 fold. On the other hand, EDTA exhibited inhibitory effect causing a decrease in the enzyme activity to about 53.66% at its highest concentration (Table-4.17).

Table-4.17 Effect of varied concentrations of different salts on L-myo-inositol-1-phosphate synthase activity in *Sphagnum junghuhnianum*
[Specific activity defined as µ mol I-1-P produced (mg protein)$^{-1}$ h^{-1}]
(Values are mean ± SE, n=3)

Concentration of salt (mM)	**Type of salts used**		
	$MgCl_2$	**NH_4Cl**	**EDTA**
0	0.150 ± 0.029	0.150 ± 0.029	0.150 ± 0.029
20	0.168 ± 0.017	0.158 ± 0.018	0.107 ± 0.007
40	0.180 ± 0.010	0.578 ± 0.032	0.140 ± 0.016
60	0.238 ± 0.028	0.608 ± 0.037	0.108 ± 0.007
80	0.175 ± 0.014	1.065 ± 0.498	0.091 ± 0.001
100	0.197 ± 0.013	1.399 ± 0.233	0.079 ± 0.003

4.8.14 Effect of monovalent cations: Effects of some monovalent cations on *Sphagnum junghuhnianum* L-*myo*-inositol-1-phosphate synthase activity were studied using variable concentrations (0 to 10.0 mM) of chloride salts of K^+, Na^+ and Li^+. To the standard assay mixture, partially purified enzyme preparation was added in presence of variable concentrations of individual monovalent cations as mentioned above and the enzyme activity was estimated keeping one control set without adding any such cation. Results of the experiment are presented in Table-4.18. It is evident that the K^+ had a stimulatory effect on this enzyme activity which increases the enzyme activity by almost 1.8 fold while the Na^+ played a mild inhibitory role. On the other hand, Li^+ was a strong inhibitor of this enzyme, which could reduce the specific activity to about 24% at 10 mM concentration.

Table-4.18. Effect of monovalent cations on L-*myo*-inositol-1-phosphate synthase activity in *Sphagnum junghuhnianum* (using 5 mM G-6-P as substrate)
[Specific activity defined as µ mol I-1-P produced (mg protein)$^{-1}$ h^{-1}]
(Values are mean ± SE, n=3)

Concentration of cations (mM)	**Specific activity [µmol I-1-P produced (mg)-1 protein h-1]**		
	K^+	**Na^+**	**Li^+**
0	0.105 ± 0.007	0.105 ± 0.007	0.105 ± 0.007
2	0.130 ± 0.010	0.045 ± 0.003	0.180 ± 0.018
4	0.116 ± 0.005	0.023 ± 0.005	0.066 ± 0.002
6	0.141± 0.005	0.099 ± 0.006	0.065 ± 0.002
8	0.166 ± 0.006	0.130 ± 0.003	0.039 ± 0.001
10	0.199 ± 0.006	0.086 ± 0.005	0.025 ± 0.006

4.8.15 Effect of Divalent cations : Effects of different divalent cations on *Sphagnum junghuhnianum* L-*myo*-inositol-1-phosphate synthase activity were studied using variable concentrations (1.0 to 10.0 mM) of chloride salts of Ca^{2+}, Mn^{2+}, Cu^{2+}, Hg^{2+}, Zn^{2+} and Cd^{2+} and Hg^{2+}. Partially purified enzyme was incubated in presence of the variable concentrations of individual divalent cations as mentioned, to the usual assay components and keeping one control set without adding any such cation. Results of such experiments have been shown in Table-4.19.

Table-4.19. Effect of divalent cations on L-*myo*-inositol-1-phosphate synthase activity in *Sphagnum junghuhnianum* (using 5 mM G-6-P as substrate)
[Specific activity defined as µ mol I-1-P produced (mg protein)$^{-1}$ h^{-1}]
(Values are mean ± SE, n=3)

Conc. of cations (mM)	**Specific activity [µmol I-1-P produced (mg)$^{-1}$ protein h^{-1}]**					
	Ca^{2+}	**Mn^{2+}**	**Cu^{2+}**	**Hg^{2+}**	**Zn^{2+}**	**Cd^{2+}**
0	0.105± 0.007	0.105± 0.007	0.105± 0.007	0.105± 0.007	0.105± 0.007	0.105± 0.007
2	0.080± 0.004	0.048± 0.002	0.148± 0.015	0.096± 0.005	0.182± 0.010	0.098± 0.006
4	0.124± 0.013	0.251± 0.032	0.226± 0.023	0.069± 0.008	0.175±0.012	0.075±0.008
6	0.128±0.011	0.314±0.029	0.110±0.009	0.071±0.005	0.085±0.005	0.161±0.025
8	0.139±0.020	0.079±0.006	0.105±0.002	0.087±0.006	0.075±0.009	0.124±0.023
10	0.153±0.016	0.073±0.001	0.099±0.003	0.019±0.006	0.059±0.005	0.085±0.005

The results shows four different types of effects of divalent cations were recorded. In this case, Cu^{2+} at the experimental concentration showed almost no effect or minute inhibitory effect, Ca^{2+} showed stimulatory effect enhancing the enzyme activity by about 46%, in case of Cd^{2+}, Mn^{2+} and Zn^{2+} there was distinct inhibitory effects causing

the loss of activity by about 19%, 30% and 44% respectively, while Hg^{2+} exhibited extremely toxic effect towards the enzyme activity and caused about 82% loss in activity at its 10mM concentration. The inhibitory effect of different divalent cations progressed in the order of $Cu^{2+} > Cd^{2+} > Mn^{2+} > Zn^{2+} > Hg^{2+}$.

4.8.16 Effect of Sugar-alcohols: The effect of sugar alcohol including myo-inostol, the end product of MIPS reaction was determined experimentally. The *Sphagnum junghuhnianum myo*-inositol-1-phosphate synthase activity has been found to have no influence or inderminate influence by various concentrations of sugar alcohols like galactitol, mannitol or *myo*-inositol. Results presented in Table-4.19 demonstrated that *myo*-inositol upto a concentration of 10 mM was slightly stimulatory on the enzyme activity. With galactitol, the synthase activity is first increased linearly upto a concentration of 4mM and then the activity showed gradual decline culminating in about 34% loss of activity at its 10 mM concentration. However, mannitol, showed stimulatory activity which enhanced the enzyme activity by upto 2.19 folds in a concentration dependent manner.

Table-4.20 Effect of varied concentration of mannitol, galactitol and myo-inositol on L-*myo*-inositol-1-phosphate synthase activity in *Sphagnum junghuhnianum*

[Specific activity defined as µ mol I-1-P produced (mg protein)$^{-1}$ h^{-1}]

(Values are mean ± SE, n=3)

Type of sugar alcohol	**Concentration (mM)**	**MIPS activity [µmol I-1-P produced (mg)$^{-1}$ protein h^{-1}]**
Mannitol	0	0.230 ± 0.018
	2	0.179 ± 0.009
	4	0.095 ± 0.003
	6	0.171 ± 0.010
	8	0.535 ± 0.042
	10	0.505 ± 0.096
Galactitol	0	0.172 ± 0.011
	2	0.295 ±0.009
	4	0.358 ± 0.012
	6	0.120 ± 0.010
	8	0.234 ± 0.036
	10	0.113 ± 0.009
Myo-inositol	0	0.195 ±0.011
	2	0.474 ± 0.052
	4	0.143 ± 0.013
	6	0.155 ± 0.042
	8	0.501 ± 0.064
	10	0.298 ± 0.039

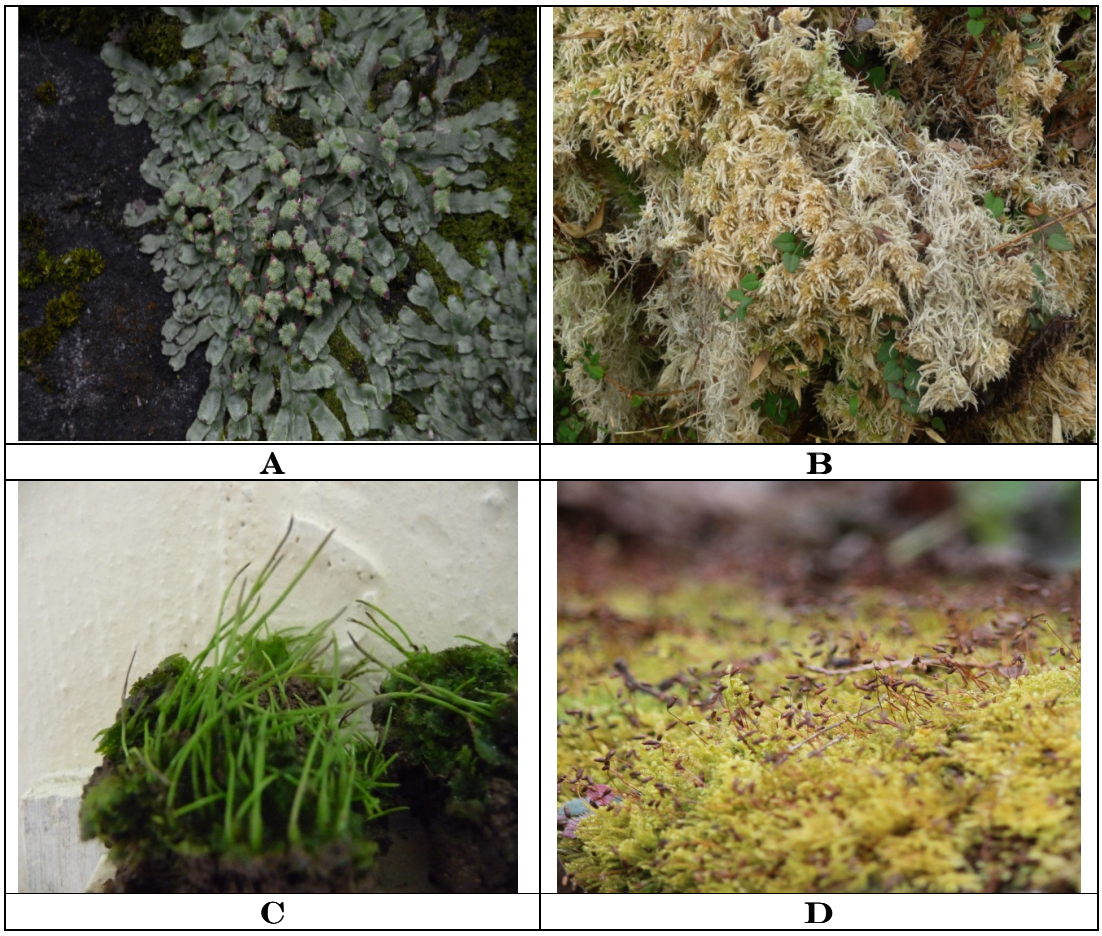


Plate-1. (A). *Asterella khasiana* (Griff.) Grolle; (B) *Sphagnum junghuhnianum* Doz. & Molk. (C) *Anthoceros angustus* Steph.; (D). *Brachymenium bryoides* Hook. ex Schwaegr.

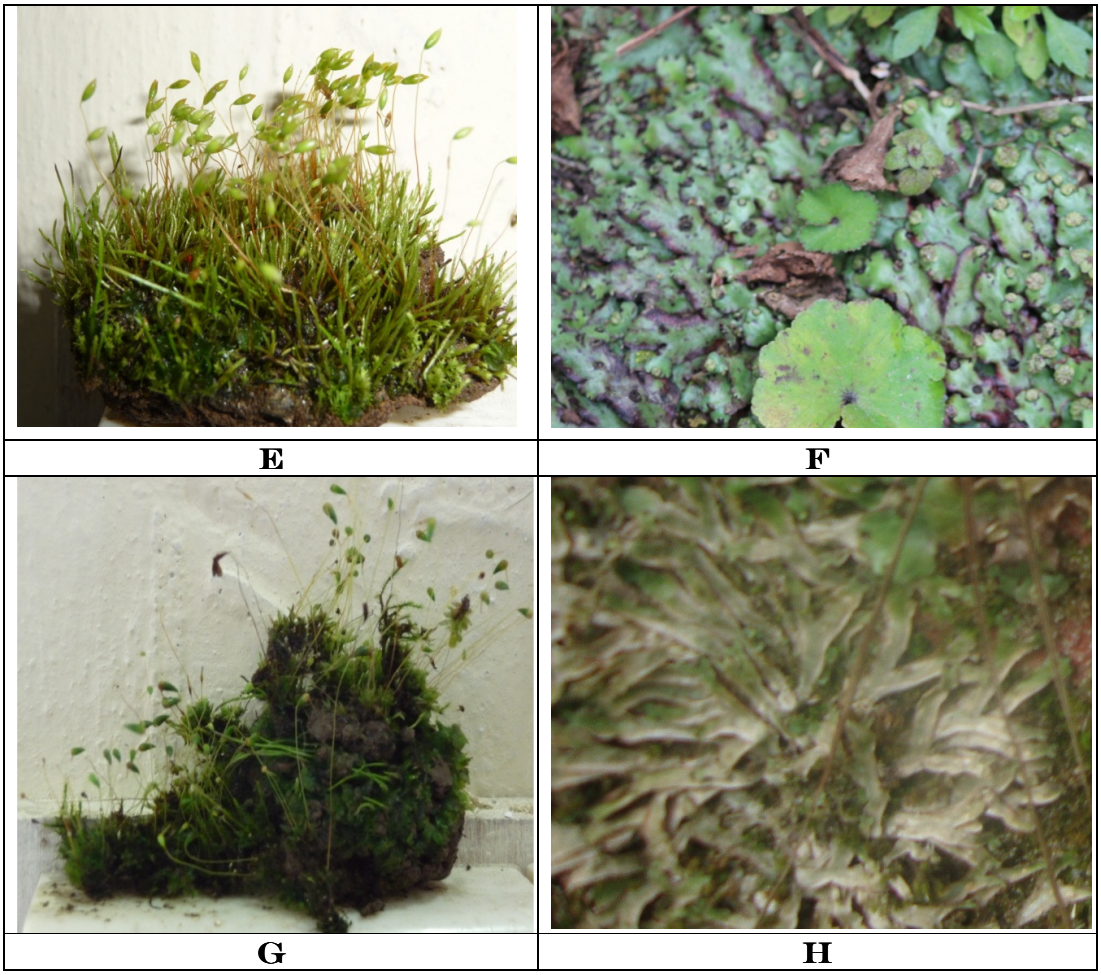


Plate-2. (E). *Lyellia crispa* R. Br.; (F). *Marchantia polymorpha* Linn.
(G). *Funaria wallichii* (Mitt.) Broth. ; (H). *Riccia cruciata* Kash.

5. DISCUSSION

When we see evolution in the plant kingdom from a simpler life forms to a more complex life forms, the position of the plant group bryophytes comes somewhere in between in the scheme. *Myo*-inositol and by extension the principal enzyme for its biosynthesis, L-*myo*-inositol-1-phosphate synthase is essential for various vital cellular functions in the life of plants including membrane formation, cell wall biogenesis, stress response and signal transduction (Lackey *et al*., 2003). Therefore, it's no surprise that the occurrence of the enzyme have been documented in different groups of plants viz., algae (Dasgupta *et al*., 1984); fungi (Donahue and Henry, 1981; Dasgupta *et al.*, 1984); ptridophytes (Benaroya *et al*., 2004; Chhetri *et al.*, 2006a, 2006b, 2006c), gymnosperms (Gumber *et al.*, 1980; Chhetri and Chiu, 2004) and angiosperms (Majumder and Biswas, 1973; Loewus and Loewus, 1980; Johnson and Wang, 1996; Ray Choudhuri *et al*., 1997; Majee *et al*., 2004; Majumder and Biswas, 2006 etc.). In the evolutionary scheme of things, the plants must have progressed through the groups like bryophytes to ptridophytes, gymnosperms and angiosperms (Fig-5.1). Naturally, the occurrence of *myo*-inositol in this group too is a foregone conclusion. This is the *raison d'etre* of the present work. Indeed, there have been sporadic reports regarding the occurrence of the enzyme L-myo inositol-1-phosphate synthase in bryophytes (Dasgupta *et al.*, 1984; Chhetri *et al.*, 2006, 2009). These studies were preliminary in nature and represented only a couple of thalloid bryophytes. However, complete study of the enzyme involving its functional biochemical characterization and the end product of the pathway, free *myo*-inositol is still lacking. This present work endeavours to fill that gap.

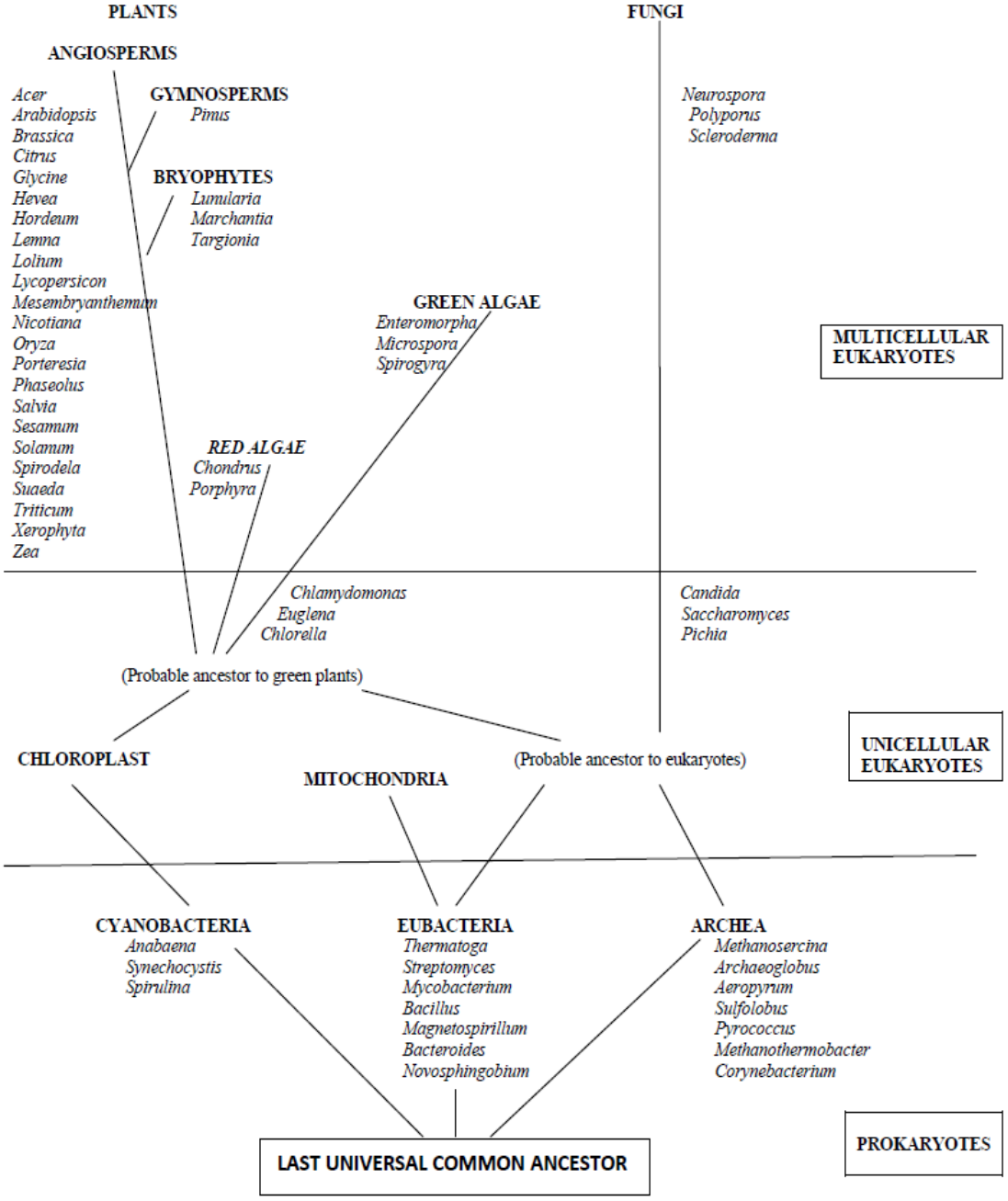


Fig-5.1: Distribution of MIPS across different life forms in plants

(Modified from Majumder *et al.*, 2003)

This study enumerates the basic physico-chemical properties of the enzyme isolated from a two species of bryophytes available in Darjeeling hills, *A. khasiana* and *S. junghuhnianum* belonging to the group liverworts and mosses respectively. The fundamental question of occurrence of *myo*-inositol-1-phosphate synthase in different phylogenetically related families of bryophytes have been included in Table 4.1 which satisfied the preliminary answer about the occurrence of the enzyme MIPS in different groups of bryophytes. The results showed the highest titre of activity of the enzyme with consistent output in *A. khasiana* and *S. junghuhnianum* hence their utilization for further purification and characterization experiments. Activity of the enzyme presented in Table 4.1, clarifies that the enzyme may remain functional in the whole plant body, the vegetative part or the reproductive part among the tested organisms. Furthermore, it has been revealed that the titre of the enzyme-protein does not follow any cardinal rule regarding its activity in one or the other group of bryophytes. Hence, the activity is highest in *A. khasiana*, a liverwort and *S. junghuhnianum*, a moss. Additionally, it has been revealed that the enzyme exhibited significantly higher activity in the reproductive parts than in the vegetative portion of the same plant which is the general feature of other plants also (Majumder and Biswas, 1973; Donahue and Henry, 1981; Das Gupta *et al.*, 1984; Gumber *el al.*, 1984; Chhetri *et al.*, 2005, 2006a). Although these experiments have been made with relatively crude enzyme preparations, the unequivocal presence of the enzyme across different groups throws light on its universal prevalence, especially the distribution of MIPS among various bryophytic groups.

A. khasiana showed a four-fold higher enzyme activity and *S. junghuhnianum* showed a two-fold higher activity in its reproductive parts as compared to the vegetative parts

(Table-4.3 and Table-4.12). The product of this enzyme, L-*myo*-inositol-1-phosphate (the immediate precursor of free *myo*-inositol) fulfils the essential requirement for the formation of sex-linked structures and also the requirements during the development of life in many stages. This indicates a greater degree of inositol demand in reproductive plant parts than in the vegetative structures of bryophytes like other living organisms. Though greater specific activity of this enzyme has been recorded in the reproductive parts of *A. khasiana* and *S. junghuhnianum*, the enzyme MIPS may have some other metabolic significance in the vegetative parts. In ptridophytes, similar results of greater activity of the enzyme in reproductive parts as compared to the vegetative structures have already been reported (Chhetri *et al.*, 2005; Basak, 2013).

The vegetative and reproductive structures are characteristically associated with the production and utilization of *myo*-inositol. Therefore, this study has undertaken to estimate the free myo inositol also in addition to the activity of the enzyme MIPS. During the present studies, appreciable level of free *myo*-inositol has been detected in all bryophytes studied. However, the content was higher in *Bryum argenteum, Asterella khasiana* and *Anthoceros angustus* etc. studied (Table-4.2). The content of free myo-inositol was in general higher in the mosses as compared to the thalloid bryophytes. Whether this higher concentration of myo-inositol in mosses is due to more biosynthesis of the same or whether in the thalloid bryophytes the free myo-inositol is readily converted to other metabolites is still a lingering question.

As in the case of MIPS activity, the content of free *myo*-inositol, the end product of MIPs reaction was much higher in the reproductive parts when compared to the

vegetative parts. Thus the reproductive parts of *A. khasiana* showed 1.36 fold more *myo*-inositol than the vegetative parts (Table-4.4) and the reproductive parts of *S. junghuhnianum* showed 1.32 fold more *myo*-inositol than the vegetative parts (Table-4.13). Naturally, reproductive parts were chosen for the partial purification of the enzyme from the species concerned. The content of *myo*-inositol may point towards the status of *myo*-inositol biosynthesis of each species under the period of plant collection.

Biochemical characterization of the enzyme, MIPS is a prerequisite for deciphering the fundamental metabolic regulation with respect to the biosynthesis of inositol in the target organisms, *A. khasiana* and *S. junghuhnianum*. In turn isolation and purification of the L-*myo*-inositol-1-phosphate synthase from the above two bryophytic samples, *A. khasiana* and *S. junghuhnianum* was carried out to the extent possible which is presented in sections 4.4 and 4.7. Consequent upon the partial purification of the enzyme concerned from the target samples, study of the different parameters of the enzyme and it behaviour under the influence of altering internal and external environment was undertaken. The enzyme, MIPS was purified up to about 46.34 fold over the homogenate fraction in case of the liverwort, *A. khasiana* by employing low-speed centrifugation, streptomycin sulphate precipitation, ammonium sulphate fractionation, and successive chromatography on DEAE cellulose, hexylagarose and one final molecular sieve chromatography through BioGel A 0.5m (Table-4.5). The recovery of the enzyme based on total activity was about 36.35 % at this stage of purification. A comparable yield of 49 fold purification with 34% recovery has been reported from *Ulva lactuta* (Basak, 2013). Similarly, in the moss, *S. junghuhnianum*, the same techniques as described above including the different chromatography steps were

employed for partial purification of the enzyme. As a result the enzyme could be purified to about 58.67 fold over its homogenate fraction and the recovery in this case was about 32.86% based on total activity at this stage of purification (Table-4.14). Similarly, 61 fold purification and 54% recovery had been reported from pteridophyte, *Lycopodium clavatum* while 80.9 fold purification and 13.4% recovery had been reported from *Diplopterygium glaucum* (Chhetri *et al.*, 2006a; Basak *et al.*, 2012).

A question may naturally arise, why the enzyme was only partially purified? Though extremely desirable, purification of an enzyme at the level of homogeneity demands extensive standardization of several successive techniques. Such experiment in plant, a hitherto unknown system demands extremely sophisticated laboratories. Under the present laboratory conditions, several attempts were taken to purify this enzyme further by employing other chromatographic matrix like Superdex-200 and also by affinity chromatography using epoxy-activated glucose-6-phosphate, sepharose CL 6B etc. as the matrix. However, these techniques in the present bryophytic enzymes did not exhibit a very good result as was with the animal enzyme (Maeda and Eisenberg Jr., 1980). It may be mentioned here that the number of homogeneous preparation of this enzyme is still very little covering plant, animal and microbial systems and only a few homogeneous enzyme from the plants have been recovered either from natural populations or when it was overexpressed in microbial systems (Ogunyemi *et al.*, 1978; Pittner *et al*., 1979; Mauck *et al.*, 1980; Maeda and Eisenberg Jr., 1980; Donahue and Henry, 1981; Escamilla *et al*., 1982; Johnson and Sussex, 1995; RayChaudhuri *et al*., 1997; Smart and Flores, 1997; Majee *et al.*, 2005)

L-*myo*-inositol-1-phosphate synthase has been purified and characterized by conventional enzyme purification techniques such as ammonium sulphate precipitation, ion-exchange chromatography, gel filtration chromatography etc. Loewus and Loewus (1971) have purified this enzyme from *Acer pseudoplatanus* and the M_r of this preparation came to about 150 kDa. However, plant MIPS purified by employing the techniques of gel-filtration chromatography through Ultrogel AcA-34, anion exchange chromatography through DEAE-Sephacel, another gel filtration through BioGel A-0.5m found that the molecular weight of the native cytosolic MIPS from *Euglena gracilis* and *Oryza sativa* were 179KDa while that from *Spirulina platensis* was 200.32 kDa (RayChaudhuri *et al.*, 1997). In the pteridophyte, *Diplopterygium glaucum,* the apparent M_r of the enzyme was determined as 170.8 (Chhetri *et al*., 2006a). The apparent molecular weights of the cytosolic MIPS from the sample bryophytes during the present studies were found to be 183 kDa for *Asterella khasiana* and 174 KDa for *Sphagnum junghuhnianum.* This result was in consonance with the previous studies which describes the apparent molecular weight of the cytosolic enzyme from plant sources ranging from 135 kDa in *Lemna gibba* (Ogunyemi *et al*., 1978), 155 kDa in *Pinus ponderosa* (Gumber *et al*., 1984), 157 kDa in *Lilium longiflorum* (Loewus *et al*., 1984), 240 kDa in *Saccharomyces sp.* (Donahue and Henry, 1981) to 260 kDa in *Neurospora crassa* (Escamilla *et al.*, 1982). The native PAGE profiles of both the enzyme preparations exhibited 3-5 major bands (Fig-4.1 and Fig-4.10) which indicated that the preparations were not homogeneous. Therefore, no further attempts were made to determine its subunit molecular weight through SDS-PAGE.

MIPS obtained from the bryophytes did not exhibit any activity in absence of its substrate glucose-6-phosphate, both in case of *A. khasiana* and *S. junghuhnianum*. This is in consonance with the behaviour of the same enzyme obtained from pteridophytes (Chhetri *et al*., 2006a, 2006b), gymnosperms (Chhetri and Chiu, 2004), angiosperms (Chhetri *et al*., 2008) and human foetus (Chhetri *et al*., 2012) since G-6-P is the specific substrate of these enzymes. Presence of the co-enzyme NAD^+ was found to be essential in order for the enzyme to express its 100% activity. In the present instance, when the specific co-enzyme NAD^+ was deducted from the reaction mixture, the enzyme from *A. khasiana* exhibited approximately 59.17% loss of activity (Table-4.6) and that from *S. junghuhnianum* exhibited about 68.41% reduction of activity (Table-4.15). However, there was discernible MIPS activity to the tune of approximately 41% and 32% in case of *A. khasiana* and *S. junghuhnianum* respectively. This points towards the existence of some bound NAD^+ in the molecular architecture of this enzyme (Chhetri *et al*., 2012). Earlier studies on MIPS have shown that the enzyme from a number of other sources also exhibited variable degree of NAD^+ independent activity which is responsible for the residual enzyme activity even in absence of added NAD^+ in the reaction mixture, obviously due to the presence of endogenous NAD^+ in the enzyme system (Pittner and Hoffmann-Ostenhof, 1976; Barnet *et al*., 1970; DasGupta *et al*., 1984, Adhikari and Majumder, 1988). Deduction of NH_4Cl and ME was responsible for the loss of MIPS activity to the tune of 31.80% and 34.51% respectively in case of *A. khasiana* (Table-4.6) and the same caused the loss of 40.01% and 33.35% activity respectively in case of *S. junghuhnianum* (Table-4.15).

L-*myo*-inositol-1-phosphate synthase activity in both *A. khasiana* and *S. junghuhnianum* increased in a linear fashion with respect to time at least upto 90 minutes of incubation at 37°C (Fig-4.3 and Fig-4.12). Similarly, pteridophytic MIPS from *Diplopterygium glaucum* showed time linearity of 90 minutes (Chhetri *et al.*, 2006a) while the same from *Lycopodium clavatum* and *Selaginella monospora* exhibited time linearity between 75 and 90 minutes (Basak, 2013)

MIPS activity also increased linearly with respect to the concentration of enzyme protein in the assay mixture at least up to a concentration of 250 µg in case of *A. khasiana* enzyme and 300 µg of protein under optimal assay condition in case of *S. junghuhnianum* (Fig. 4.4 and Fig-4.13). The enzyme protein linearity *vis-a-vis* MIPS activity in *Diplopterygium glaucum* was found to be 280µg (Chhetri *et al*., 2006a) and the same effect in relation to the enzyme concentration in *Lunularia cruciata* was 300µg (Chhetri *et al*., 2009). These results were in consonance with the present results. However, the value for the same in the MIPS from algae was divergent in having its optimal activity at approximately 120pg (Basak, 2013).

The bryophytes MIPS from both *A. khasiana* and *S. junghuhnianum* were highly specific for its substrate, glucose-6-phosphate which is universal for the enzyme from different sources. However, *A. khasiana* MIPS exhibited 8.92 % activity when glucose-6-phosphate was replaced with galactose-6-phosphate and 3.38% activity when the same specific substrate was replaced with mannose-6-phosphate in comparison to the standard assay mixture containing glucose-6-phosphate as substrate (Table-4.7). Similarly, in case of *S. junghuhnianum*, replacement of glucose-6-phosphate with

galactose-6-phosphate and mannose-6-phosphate resulted in 11.23% and 3.81% enzyme activity respectively as compared to that when glucose-6-phosphate was used as substrate (Table-4.16). In the same scale, 9.38% activity of the enzyme MIPS was recorded with galactose-6-phosphate and 1.42% with mannose-6-phosphate as substrates in a leafy pteridophyte while the activity in *Selaginella monospora* was found to be 21.34% and 13.23% respectively, when galactose-6-phosphate and mannose-6-phosphate were used as substrates. However, the mammalian enzyme from human fetal liver was completely dependent on glucose-6-phosphate as its substrate and the above two hexose-6-phosphate could not act as substitutes of the enzyme (Chhetri *et al*., 2006a, Chhetri *et al*., 2012, Basak, 2013).

It has been found that a NAD^+-dependent oxido-reductase which is functionally identical with the L-*myo*-inositol -1-phosphate synthase is able to isomerize galactose-6-phosphate to *muco*-inositol-1-phosphate (Adhikari and Majumdar, 1988). In plants, the occurrence of different esters of *muco*-inositol is not uncommon. In addition, the utilization of mannose-6-phosphate as substrate, even to a limited extent also indicates the synthesis of the same or another isomer of inositol. Thus in the bryophytic systems studied, the probability of *muco*-inositol biosynthesis through a metabolic by-pass cannot be ruled out. It is pertinent to mention here that different isomers of *myo*-inositol are readily interconvertible by rotation of the C-C bonds (Ogawa, 1999) which may explain the existence of some activity of MIPS in presence of galactose-6-phosphate and mannose-6-phosphate even when glucose-6-phosphate was not there in the assay mixture. The identification of the isomeric *myo*-inositol product of galactose-6-

phosphate and mannose-6-phosphate through chemical studies may throw some light on the biosynthesis of inositol in bryophytes.

Kinetic studies on bryophytic L-*myo*-inositol-1-phosphate synthase was performed and the rate of reaction was found to increase with respect to its specific substrate (glucose-6-phosphate) concentration upto 6 mM in case of both the liverwort (*A. khasiana*) and the moss (*S. junghuhnianum*) (Fig-4.6 and Fig-4.15). The K_m value for glucose-6-phosphate of this enzyme from *A. khasiana* was calculated and was about 3.56mM while the same from *S. junghuhnianum* was found to be about 1.81 mM. Thus the K_m for glucose-6-phosphate in case of *A. khasiana* MIPS was comparable to that of rat testis enzyme which was about 3.89 (Maeda and Eisenberg Jr., 1980) and the K_m in case of *S. junghuhnianum* MIPS was somewhat similar to that of *Euglena gracilis* enzyme which was about 2.1 mM (DasGupta *et al*., 1984). In contrast, the pteridophytic MIPS from *Diplopterygium glaucum* showed a K_m for glucose-6-phosphate of about 0.83 mM (Chhetri *et al*., 2006a) and the K_m for the same for MIPS from the gymnosperm, *Taxus baccata* was about 1.05mM (Chhetri and Chiu, 2004). The V_{max} values for the substrate glucose-6-phosphate have also been worked out and it was found that the same for MIPS from *A. khasiana* was 0.71mM while the MIPS from *S. junghuhnianum* showed a value of 1.42 mM. Thus the V_{max} value for glucose-6-phosphate in case of MIPS obtained from *S. junghuhnianum* was exactly identical to that of the V_{max} value of pteridophytic enzyme from *Diplopterygium glaucum* calculated as 1.42 mM but less than those of yeast enzyme calculated at 1.6 mM (Donahue and Henry, 1981), *Euglena gracilis* at 4.0, *Oryza sativa* at 4.42 and *Spirulina platensis* at 5.05 (RayChaudhury *et al*., 1997). The V_{max} for glucose-6-phosphate in case of MIPS from *A. khasiana* at 0.71

was less than that from yeast (Donahue and Henry, 1981), human fetal brain and rat brain (Adhikari and Majumder, 1988) but more than those from the enzymes from other plant sources mentioned above.

The influence of the co-enzyme NAD^+ with respect to its concentration on this enzyme activity was determined and found that the rate of reaction increased with the increase in the concentration NAD^+ in the reaction mixture reaction upto its concentration of 0.8 mM in case of *A. khasiana* (Fig-4.7) and up to a concentration of 0.6mM in case of *S. junghuhnianum* (Fig-4.16). The K_m for the coenzyme, NAD^+ was found to be about 0.56 mM for MIPS from *A. khasiana* and the K_m for the MIPS from *S. junghuhnianum* was about 0.25 mM. These values were somewhat comparable to that of pteridophytic MIPS from *Diplopterygium glaucum* which showed K_m of about 0.44mM (Chhetri, 2004). In contrast, the K_m for NAD^+ for MIPS from many other plants were quite different e.g., 8mM for the yeast enzyme (Donahue and Henry, 1981), 0.11mM for the *Spirulina platensis*, 0.16mM for *Euglena gracilis*, 0.13mM for *Oryza sativa* (RayChaudhuri *et al*., 1997). Strangely enough, the K_m for NAD^+ for the same enzyme from human fetal brain showed a comparable value at 0.45mM (Adhikari and Majumder, 1988). The V_{max} for NAD^+ for the enzyme isolated from the bryophytes was also determined and found to be 0.68mM for the *A. khasiana* enzyme which was less than those from other plant sources like *Euglena gracilis* at 3.98 mM, *Spirulina platensis* at 4.24mM and *Oryza sativa* at 5.08 mM (RayChaudhuri *et al*., 1997) but more than those from some other sources (Majumder *et al*., 1997). The V_{max} for NAD^+ for the enzyme isolated from *S. junghuhnianum* was found to be about 1.12mM/hr which was comparable to that of 1.14 mM for the same obtained for the yeast enzyme (Donahue and Henry, 1981). Similarly,

the pteridophytic enzyme from *Diplopterygium glaucum* showed a V_{max} *of* 1.8 mM (Chhetri, 2004). Earlier studies have shown the V_{max} for NAD^+ for the same enzyme from *Marchantia nepalensis* to be about 1.11mM (Chhetri *et al*., 2006d) and *Lunularia cruciata* to about 1.21mM (Chhetri *et al*., 2009) which is in consonance with the present studies at least in case of *S. junghuhnianum.*

The bryophytic L-*myo*-inositol-1-phosphate synthase isolated from *A. khasiana* and *S. junghuhnianum* was specific for NAD^+. However, trials were made to obtain conclusive evidence for the same and from the results it was found that NAD^+ was not replaceable by $NADP^+$ in any of the two cases (Fig-4.8 and Fig-4.17). Interestingly, even in absence of the added NAD^+ some basal enzyme activity was observable, making the case for enzyme-bound endogenous NAD^+ more reasonable as already discussed. The residual enzyme activity, in presence of $NADP^+$ certifies the hypothesis.

The bryophytic MIPS from *A. khasiana* and *S. junghuhnianum* showed that it operates significantly through a narrow pH range i.e., from pH 7.0 to 7.5 with maximum at pH 7.0 in both the cases (Fig-4.9 and Fig-4.18). In earlier studies the pH optima for the same was found to be at pH 7.0 for the enzyme from yeast (Donahue and Henry, 1981), pH 7.2 from rat mammary gland (Naccarato *et al*., 1974), pH 7.5 from *Euglena gracilis* (RayChaudhuri *et al*., 1997), rat brain and human fetal brain (Adhikari and Majumder, 1988), pH 7.6 from *Entamoeba histolytica* (Lohia *et al*., 1999), pH 7.7 from *Lemna gibba* (Ogunyemi *et al*., 1978), rat testis (Maeda and Eisenberg, Jr., 1980) and *Neurospora* (Escamilla *et al*., 1982), pH 7.8 from *Spirulina platensis* (RayChaudhuri *et al*., 1997), pH 8.0 from *Acer pseudoplatanus* (Loewus and Loewus, 1971), pH 8.4 from

Oryza sativa (Funkhouser and Loewus, 1975). Similarly, the enzyme from *Diplopterygium glaucum* showed pH optima at pH 7.0 to 7.5 (Chhetri *et al*., 2006a) while those from *Pinus ponderosa* pollen and *Lilium longiflorum* pollen showed pH optima between pH 7.2-7.7 and pH 7.8-8.5 respectively (Gumber *et al*., 1984; Loewus *et al*., 1984).

The effects of varying concentrations (0-100mM) of different salts viz., $MgCl_2$, NH_4Cl and EDTA on bryophytic L-*myo*-inositol-1-phosphate synthase activity have been worked out (Table-4.8 and Table-4.17) and found that $MgCl_2$ acted as a mild stimulator, increasing the enzyme activity up to 1.6 fold and 1.2 fold in *Asterella khasiana* and *S. junghuhnianum* respectively. NH_4Cl was a strong stimulator of the enzyme and increased the rate of reaction by 7.5 and 9.3 fold in *A. khasiana* and *S. junghuhnianum* respectively. Earlier 1.3 fold stimulation of enzyme activity by the influence of 12mM NH_4Cl was recorded in case of pteridophytic MIPS (Chhetri *et al*., 2006a). In *Euglena gracilis*, the enzyme activity was stimulated 2.0 fold by the effect of NH_4Cl (Dasgupta *et al*., 1984) while in *Acer pseudoplatanus*, the enzyme activity was stimulated 2.3 fold by NH_4Cl (Loewus and Loewus, 1971). In both cytosolic and chloroplastic forms of MIPS from *Oryza sativa*, *Vigna radiata* and *Euglena gracilis*, NH_4Cl produced about 5.0 fold stimulation of activity (RayChaudhuri *et al*., 1997). On the other hand, EDTA acted as a mild inhibitor and at its highest concentration (100mM), it decreased the enzyme activity by 42.8% and 47.3% in *A. khasiana* and *S. junghuhnianum* respectively, though up to a concentration of 40mM the salt did effect any change in the enzyme activity in the bryophytes studied. In ptridophytes too, EDTA affected the enzyme activity in similar concentration dependent manner (Chhetri, 2004) which is an

established character of this enzyme in different plant species (Loewus and Loewus, 1980; Dasgupta *et al*., 1984). Ogunyemi *et al*., (1978) have found that MIPS from *Lemna gibba* could be inhibited by 30% by the influence of 1mM EDTA while 10mM EDTA completely inhibited the enzyme activity. EDTA at a concentration of 10mM did not cause any stimulation or inhibition of the enzyme activity; however, at 50mM it caused 1.5 fold stimulation of bovine MIPS (Maeda and Eisenberg Jr., 1980). The enzyme MIPS isolated from *Streptomyces griseus* was found to be completely inhibited by 60mM EDTA, however, this inhibition could be reversed by Mg^{2+} (Pittner *et al*., 1979). It appears that the bryophytic MIPS from both the sources were not affected by low concentration of EDTA, while at higher concentration it played an inhibitory role. Mg^{2+} is required for MIPS activity and addition of EDTA hinders the MIPS activity probably by inactivation of Mg^{2+} concentration (Sipos and Szabo, 1989). Mg^{2+} is an absolute necessity for conversion of *myo*-inositol-1-phosphate to *myo*-inositol in *Acer pseudoplatanus* (Loewus and Loewus, 1971). In *Neurospora crassa* 2.0 fold stimulation of the enzyme activity occur in presence of 2mM of Mg^{2+} (Sherman *et al*., 1981). Bryophytic MIPS was stimulated by 1.6 fold in *A. khasiana* and 1.3 fold in *S. junghuhnianum* (Table-4.8 and Table-4.17) by $MgCl_2$ in a concentration dependent manner. At very high concentrations, the role of Mg^{2+} has been found to be inhibitory in case of *Neurospora crassa* (Escamilla *et al*., 1982). Similar effects have also been observed in case of MIPS from rat testis (Maeda and Eisenberg, 1980). However, the bryophytic enzyme did not behave in the same way even at quite high a concentration.

Effects of other monovalent cations were also studied whereby the assay mixture contained varying concentration (0-10mM) of different chloride salts. It was found that the monovalent cation, K^+ showed stimulatory effect on bryophytic L-*myo*-inositol-1-

phosphate synthase enhancing the rate of reaction by 1.6 fold in *A. khasiana* and 1.8 fold in *S. junghuhnianum* in a concentration dependent manner. This is in consonance with 2.0 fold stimulation of the enzyme activity in presence of K^+ in MIPS from bovine testis (Mauck *et al*., 1980). In the present instance, Na^+ acted as a mild inhibitor of this enzyme from bryophytes, inhibiting the activity up to 47.6% in *A. khasiana* and 22.0% in *S. junghuhnianum* in a concentration dependent manner. High concentration of Na^+ inhibited the enzyme activity in case of *Neurospora crassa* (Escamilla *et al*., 1982). On the other hand, like in other cases, Li^+ acted as a strong inhibitor of the enzyme inhibiting the activity of the same by up to 81.9% and 76.1% in *A. khasiana* and *S. junghuhnianum* respectively. Li^+ exhibited this exclusive inhibition in a concentration dependent manner (Table-4.9 and Table-4.18). Rat testes enzyme had shown identical character (Maeda and Eisenberg, Jr., 1980; Loewus and Loewus, 1980). Li^+ was also inhibitory to *Oryza sativa, Vigna radiata* and *Euglena gracilis* enzyme in a concentration dependent manner and even its 5mM concentration proved to be detrimental to the enzyme activity (RayChaudhuri *et al*., 1997). In *Diplopterygium glaucum,* Li^+ inhibited the MIPS activity by 60% at 10 mM concentration (Chhetri, 2004).

Influence of some other divalent cations on the bryophytic MIPS was also studied. The studies revealed that Ca^{2+} was a mild stimulator of this enzyme causing 1.53 fold and 1.45 fold stimulation of the enzyme activity in *A. khasiana* and *S. junghuhnianum* respectively. At the same time, Mn^{2+}, Cu^{2+} and Cd^{2+} had some inhibitory effect which inhibited the enzyme activity by 9.5%, 6.6% and 5.7% respectively in the liverwort, *A. khasiana*. In case of MIPS isolated from the moss, *S. junghuhnianum,* Cu^{2+} showed a

comparable inhibition of the enzyme activity, slowing down the reaction by 5.7% while $Cd^{2+}$and Mn^{2+}were slightly more toxic which inhibited the enzyme activity by 19.0% and 30.4% respectively. The action of divalent cations Ca^{2+}, Mg^{2+} and Mn^{2+} were found to be inhibitory to different degrees at 5mM in some other plants (RayChaudhuri *et al*., 1997). On the bryophytic MIPS, Zn^{2+} also exhibited a pronounced inhibitory activity slowing down the enzyme reaction by 18.0% and 43.8% in *A. khasiana* and *S. junghuhnianum* respectively. As expected, the heavy metal Hg^{2+} acted as a strong inhibitor of the bryophytic L-*myo*-inositol-1-phosphate synthase which caused the inhibition of *A. khasiana* enzyme by approximately 74.2% and that of *S. junghuhnianum* enzyme by about 81.9% (Table-4.10 and Table-4.19). MIPS isolated from *Archaeglobus* have been found to be active in presence of divalent cations such as Zn^{2+} and Mn^{2+} (Majumder *et al*., 2003). Similarly Zn^{2+}has been found as an integral part of MIPS in *Mycobacterium* conferring proper structural conformation to its presumed active site (Norman *et al*., 2002). In general, the eukaryotic MIPS showed a preference for NH_4^+ over divalent cations for its optimal activity. However, whether bryophytic MIPS is a Class-I aldolase where an intermediate Schiff base is formed between an amine group on the enzyme and a carbonyl group on the substrate; a Class-II aldolase that require divalent metals for its reaction or a Class-III aldolase requiring NH_4^+ for its optimal activity is an open question. Interestingly, the bryophytic MIPS isolated from *A. khasiana* as well as from *S. junghuhnianum* exhibited appreciable stimulation of activity both in the presence of NH_4^+ as well as that of Mg^{2+}. Maybe, it belongs to a completely different category showing dual characters of Class-II and Class-III aldolases.

The sugar alcohol, *myo*-inositol itself, up to the experimental concentration of 10 mM showed very little influence in either *A. khasiana* as well as in *S. junghuhnianum* enzyme which is in consonance with the studies on the *Diplopterygium glaucum* L-*myo*-inositol-1-phosphate synthase activity (Chhetri, 2004). Another sugar alcohol, galactitol showed enhancement of the enzyme activity up to a concentration of 4 mM in both *A. khasiana* and *S. junghuhnianum* beyond which the compound became toxic causing about 35% and 34% loss of activity respectively in the above two bryophytes at a concentration of 10mM. However, mannitol did not follow any pattern in its general stimulatory activity on *A. khasiana* MIPS, though in *S. junghuhnianum* it followed somewhat concentration dependent influence in its stimulatory role (Table-4.11 and Table-4.20). In contrast, sugar alcohols such as inositol, mannitol and sorbitol inhibited both chlorophastic and cytosolic forms of MIPS from other plant sources at 4mM. Moreover, *Euglena gracilis* cells under culture showed reduced MIPS activity in even lesser concentration of sugar alcohols (RayChaudhuri *et al*., 1997).

Preliminary studies on L-*myo*-inositol-1-phosphate synthase of bryophytic origin have been started by Chhhetri *et al*., (2006c, 2009) and the present investigator was a member of the group. The presented study conclusively proves the occurrence of L-*myo*-inositol-1-phosphate synthase in bryophytes and thus completes the phylogenetic tree regarding the occurrence of the same in different plant groups starting from algae to angiosperms. The screening of the enzyme and its end product, *myo*-inositol in different families of bryophytes indicates the universal occurrence and activity of the enzyme across different bryophytic groups. Since the detailed studies relating to L-*myo*-inositol-1-phosphate synthase from a hitherto neglected plant group, the bryophyte has been

undertaken, it is a prerequisite that its metabolic regulation is also studied with different parameters of biochemical characterization. Consequently, isolation of L-*myo*-inositol-1-phosphate synthase from the vegetative as well as reproductive parts of the bryophytes was undertaken. During these screening experiments, it was found out that the vegetative tissues showed a higher titre of MIPS activity in homogenate fraction over the low speed supernatant fraction, specifically in case of vegetative tissues in both the bryophytes studied (Table-4.3 and Table-4.12). This provides circumstantial evidences explaining the probability of existence of different forms of L-*myo*-inositol-1-phosphate synthase in bryophytic system, with some form being cytosolic and some other being particulate in nature. Such differential forms of MIPS have already been reported in pteridophytes (Chhetri *et al*., 2006d). Earlier, chloroplastic forms of MIPS in addition to the cytosolic forms have been reported by different groups (Imhoff and Bourdu, 1973; Adhikari *et al*., 1987; RayChaudhuri *et al*., 1997). Different types of particulate MIPS have been isolated from membrane bound organelles like chloroplasts, mitochondria, plasma membrane, plastids, endoplasmic reticulum, nuclei etc. in *Phaseolus vulgaris* (Lackey *et al*., 2003). Naturally, multiple forms of MIPS cannot be ruled out in case of bryophytes too. However, this was beyond the purview of present studies and as such the present study remained restricted to the cytosolic MIPS in bryophytes, the most predominant source of the enzyme from different other sources too.

Experiments as described in Section-4.5.7 and Section-4.8.7 determine the thermal stability of the enzyme MIPS from *A. khasiana* and *S. junghuhnianum* respectively. Of the sample bryophytes, *A. khasiana* showed significant activity of L-*myo*-inositol-1-phosphate synthase between 20^{o}C and 50^{o}C with the temperature maxima at 30^{o}C

(Fig.4.5), similarly, the activity of L-*myo*-inositol-1-phosphate synthase isolated from *S. junghuhnianum* exhibited remarkable activity between temperatures of 10^{o}C and 40^{o}C with the temperature maxima at 30^{o}C (Fig-4.14). The temperature maxima of MIPS in both the bryophytes were 30^{o}C which is comparatively lower than the enzyme, isolated from a number of other sources as described in section-2 e.g., 35^{o}C for *Entamoeba histolytica* (Lohia *et al*., 1999) and some plants (RayChaudhuri *et al*., 1997), 40^{o}C for human fetal liver enzyme (Chhetri *et al*., 2012). Unlike the MIPS from other sources, the *A. khasiana* MIPS shows appreciable activity at the minimum temperature of 20ºC and the *S. junghuhnianum* MIPS exhibits its activation at even lower temperature of 10ºC. This may be called a unique feature of the bryophytic MIPS from these two sources though both exhibited temperature maxima at 30ºC. The activity of the enzyme in temperature as low as 10ºC is a significant information which has so far not been reported save from one source, *Xerophyta viscosa*, which is a resurrection plant and is extremely tolerant to osmotic and low temperature stress (Majee *et al*., 2005). This activity of bryophytic MIPS in comparatively lower temperature satisfies our hypothesis that bryophytic MIPS may have some cold temperature tolerance characteristics and it may be a potential future source of mining cold resistance specific MIPS genes. Studies have suggested that tolerance of MIPS to higher temperature is associated with the NAD^{+} bound to the enzyme (Adhikari and Majumder, 1983, Chhetri *et al*., 2006c). Whether bound NAD^{+} is also responsible for the activity of MIPS at lower temperature needs to be found out through further research.

Myo-inositol-1-phosphate synthase isolated from *Mycobacterium tuberculosis* H37Rv could functionally compensate for the yeast *INO1* mutation though the *INO1* of

Mycobacterium and other homologous prokaryotic genes revealed a distinct class of this enzyme (Bachhawat and Mande, 1999). *Myo*-inositol lipids are major components of mycobacterial plasma membrane and cell wall. Thus, *myo*-inositol is an essential metabolite that forms the building blocks for the synthesis of glycolipid and other metabolites in mycobacteria (Morita *et al*., 2011). Therefore, controlling these synthetic pathways is critical for controlling mycobacterial pathogenesis; as such this enzyme can be a potential drug target.

Disruption of inositol homeostasis has been associated with a number of illnesses, including metabolic syndrome, Alzheimer's disease, diabetes, epilepsy etc. (Gelber *et al*., 2001; Wang and Raleigh, 2014; Scioscia *et al*., 2007; Chang *et al*., 2014). Dysregulation of inositol levels has been reported in a wide variety of biomedical studies. Understanding the metabolism of myo-inositol will provide insight into the mechanism underlying these diseases (Frej *et al*., 2016). Altered inositol levels have also been demonstrated in patients with bipolar disorder (Shimon *et al*., 1997), major depressive disorder (Coupland *et al*., 2005) and schizophrenia (Shimon *et al*., 1998). For these reasons, modulating *myo*-inositol level was proposed as a therapy for these disorders (Chengappa *et al*., 2000; Palatnik *et al*., 2001).

MIPS expression during early stages of seed development is closely related to the essentiality of inositol biosynthesis for several biochemical pathways in plants (Downes *et al*., 2005). In *Passiflora edulis* the MIPS synthesizing gene was upregulated after a short exposure to cold stress (5ºC) (Abreu and Aragao, 2007), in *M. crystallinum*, salinity stress induced upregulation of MIPS mRNA expression by 5-fold and free inositol accumulation by approximately 10-fold (Ishitani *et al*., 1996). Indeed high and

low no-freezing temperatures and osmotic stresses appear to have several features in common (Abreu and Aragao, 2007). Thus MIPS plays role in basic metabolism as well as during the response of plants to environmental stress.

In plants, multiple physiological and biochemical characters are controlled by MIPS and in turn plants also possess multiple MIPS genes suggesting that different activities may be controlled by different MIPS genes (Valluru and Ende, 2011). Indeed two isoforms of MIPS gene were found in ice-plant (Ishitani *et al*., 1996), two in rice genome (Yoshida *et al*., 1999; Suzuki *et al*., 2007), seven in maize (Larson and Raboy, 1999), four in soybean (Hegeman *et al*., 2001), three in *Sesamum indicum* (Chun *et al*., 2003), two in chickpea (Kaur *et al*., 2008), three in *Arabidopsis* (Luo *et al*., 2011) and three in *Phaseolus vulgaris* (2012). The fate of the cell may be linked to differential gene regulation. MIPS may be involved in transcriptional regulation of chromatin metabolism in addition to being involved in myo-inositol biosynthesis. Thus in plants MIPS appear to have evolved as a protein that connects cellular metabolism, pathogen response and chromatin remodelling (Latrasse *et al*., 2013).

The thesis presented describes the fundamental evidence for the occurrence of L-*myo*-inositol-1-phosphate synthase in a different group of plants, the bryophytes thus completing the report on the occurrence of the enzyme in diverse groups from algae to angiosperms. Since a long time, the distribution, physiological role, biochemical characteristics and metabolic regulation of this enzyme have been documented from bacteria, algae, fungi, ptridophytes, gymnosperms, angiosperms and mammals. However, the information was meagre regarding the occurrence and function of *myo*-

inositol synthesizing potentiality from the amphibians of the plant kingdom, the bryophytes. Therefore, the presented study completes that gap of information regarding the biosynthesis and regulation of *myo*-inositol covering all plant groups through the evolutionary scale.

6. CONCLUSION

Myo-inositol is the most widely distributed inositol which occurs in all living organisms. It is the central component of several biochemical pathways and its products are important in several cellular processes. Lack of cellular level of inositol has been identified as the cause of "inositol-less-death" in *Saccharomyces cerevisiae*. Inositol phosphates are essential for signalling in almost all organisms; in plants, inositol hexakisphosphate contributes to phosphate storage. *Myo*-Inositol acts as a precursor for making phosphatidyl inositol, which is essential in all eukaryotes, including pathogenic fungi and protozoa, as well as in a small but very significant group of eubacterial pathogens that includes the mycobacteria. Galactinol synthesized from UDP-galactose and inositol is the basic substrate for the raffinose series of sugars in plants. These sugars have been implicated in stress tolerance and in carbohydrate transport. It is known that *myo*-inositol is a compatible osmolyte, and the molecule aids in maintaining an ideal osmotic state when a cell is placed in a hypertonic environment. Inositol may also be conjugated to auxins, preventing biological activity and allowing long-distance transport within the plant.

The present study elucidates the study of MIPS concerning its occurrence and fundamental biochemical characterization in bryophytes. During the study, appreciable L-*myo*-inositol-1-phosphate synthase activity has been detected in two bryophytes, *Asterella khasiana* and *Sphagnum junghuhnianum* exhibiting maximum titre of activity at reproductive stages. *A. khasiana* showed a four-fold higher enzyme activity and *S. junghuhnianum* showed a two-fold higher activity in its reproductive parts as compared to the vegetative parts. The enzymes isolated from both organisms showed almost equal

molecular wt of about 180kDa suggesting a trimeric structure of the protein as in many other cases of multicellular organisms. D-glucose-6-phosphate was found to be the specific substrate for MIPS from the bryophytes. However, the enzyme showed a little bit of activity in presence of D-galactose-6-phosphate and mannose-6-phosphate. The *A. khasiana* MIPS showed a K_m of 3.56 mM and 0.56 mM for D-glucose-6-phosphate and NAD^+ respectively while the V_{max} were found to be 0.71 mM and 0.68 mM for D-glucose-6-phosphate and NAD^+ respectively. In comparison, in *S. junghuhnianum* MIPS the K_m for D-glucose-6-phosphate and NAD^+ were 1.81mM and 0.25mM respectively while the V_{max} were 1.42 mM and 1.12 mM for D-glucose-6-phosphate and NAD^+ respectively.

The deduction of NH_4Cl and ME reduced MIPS activity to 31.80% and 34.51% respectively in of *A. khasiana* and 40.01% and 33.35% loss of activity respectively in *S. junghuhnianum.* No enzyme activity was found in absence of glucose-6-phosphate (substrate) in either *Asterella khasiana* or *Sphagnum junghuhnianum*. When NAD^+ was deducted from the reaction mixture, the enzyme from *A. khasiana* exhibited approximately 59.17% loss of activity and that from *S. junghuhnianum* exhibited about 68.41% loss of activity. The bryophytic MIPS operated between a pH ranges of 7.0 to 7.5. However, the maximum activity was found at pH 7.0. The MIPS from experimental bryophytes showed catalytic activity at temperatures 20ºC in *A. khasiana* and 10ºC in case of *S. junghuhnianum.* The activity of the enzyme in temperature as low as 10ºC, is a significant information which may be a good subject for future studies. The isolation of MIPS gene and the analysis of its homology *vis-à-vis* other stress tolerant MIPS genes may provide some interesting insight and pave the way for the use of the same in

the improvement of crop plants through the application of biotechnology. However, such studies were beyond the scope of this present research and will be taken up in near future. Whether the tolerance to cold stress in these plants is also associated with the NAD^+ bound to the enzyme as in the case higher temperature tolerance needs also to be found out through further research.

NH_4Cl was a strong stimulator of the enzyme and increased the rate of reaction in a concentration guided manner by 7.5 and 9.3 fold in *A. khasiana* and *S. junghuhnianum* respectively. K^+ showed stimulatory effect on bryophytic L-*myo*-inositol-1-phosphate synthase enhancing the rate of reaction by 1.6 fold in *A. khasiana* and 1.8 fold in *S. junghuhnianum* in a concentration dependent manner. Na^+ acted as a mild inhibitor of this enzyme from bryophytes, Li^+ acted as a strong inhibitor inhibiting the activity of the same by almost 81.9% and 76.1% in *A. khasiana* and *S. junghuhnianum* respectively. However, $MgCl_2$ acted as a stimulator, increasing the enzyme activity up to 1.6 fold and 1.2 fold in *Asterella khasiana* and *S. junghuhnianum* respectively. Among other divalent cations studied, Ca^{2+} was mildly stimulatory, while Mn^{2+}, Cu^{2+} and Cd^{2+} had varying degree of inhibitory effect from mild to medium. Zn^{2+} and Hg^{2+} showed extreme inhibitory property towards the bryophytic MIPS. Thus the bryophytic MIPS showed both the characteristics of Class-II aldolase that require divalent metals and a Class-III aldolase requiring NH_4^+ for its optimal activity. The finding that both plants exhibited stimulation of activity in presence of NH_4^+ as well as Mg^{2+} pointing to the dual character of MIPS from these amphibian plants is quite interesting. It cannot be ruled out that these plants may belong to a completely different category showing both the characters of Class-II and Class-III aldolases.

The enzyme, MIPS was purified up to about 46.34 fold over the homogenate fraction in case of the liverwort, *A. khasiana* and 58.67 fold from *S. junghuhnianum* over its homogenate fraction. In plants, multiple physiological and biochemical characters are controlled by MIPS and in turn plants also possess multiple MIPS genes suggesting that different activities may be controlled by different MIPS genes. Naturally, the presence of multiple forms of MIPS may not be ruled out in bryophytes also.

Further studies should explore both cytosolic and the particulate form of the enzyme and the enzyme expressed in different parts of the plants as well as different cell organelles at different developmental stages. Effort should also be made to purify the enzyme to homogeneity, to isolate and sequence the gene, to determine its homology with MIPS from other plants, to confirm the cold and drought tolerance properties of bryophytic MIPS, if any, and to overexpress the gene in order to find out whether transgenics expresses desirable traits in terms of stress tolerance and productivity.

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APPENDIX- A: LIST OF PUBLICATIONS

Yonzone S, Chhetri DR, Roy SC: Isolation and characterization of an enzyme L-*myo*-inositol-1-phosphate synthase from bryopsida *Brachymenium bryoides* Hook. ex Schwagr. found in Darjeeling hills. *Int. J. Creat. Res.Thoug.* 6: 1625-1631 (2018).

Yonzone S, Jha S, Chhetri DR: Gluconeogenic Fructose 1, 6-bisphosphatase from *Ginkgo biloba* L: Isolation and characterization. *Ann. Plant. Sci.* 4: 1116-1122 (2015).

Chhetri DR, Yonzone S, Tamang S, Mukherjee AK : L-*myo*-inositol-1-phosphate synthase from bryophytes: purification and characterization of the enzyme from *Lunularia cruciata*(L) Dum. *Braz. J. Plant.* 21: 243-250 (2009).

Chhetri DR, Yonzone S, Mukherjee AK, Adhikari J : L-*myo*-inositol-1-phosphate synthase from *Marchantia nepalensis*: partial purification and properties. *Gen. Appl. Plant. Physiol.* 32: 153-164 (2006).

APPENDIX- B: LIST OF CONFERENCES / SEMINARS ATTENDED

1. Oral presentation on "International conference on Contemporary Issues in Integrating Climate- The Emerging Areas of Agriculture, Horticulture, Biodiversity, Forestry, Engineering, Fundamental/ Applied Science and Business Management for Sustainable Development (AGROTECH-2017)" on 11th and 12th May, 2017, organised by Himalayan Scientific Society for Fundamental and Applied Research in collaboration with Kalimpong Science Centre, Kalimpong and UBKV, Kalimpong, Darjeeling.

2. Oral presentation on UGC sponsored national seminar "Advances in Biology: Eastern Himalayan Perspective" on 3rd and 4th Oct 2015, organised by Department of Botany and Department of Zoology, Kalimpong College, Kalimpong, Darjeeling.

3. Oral presentation as Young Scientist in "National Conference on New Frontiers in Medicinal Plant Research and Special Meeting on Medicinal Plants for Livelihood Security & Community Empowerment in Eastern Himalayas" held on 3,4,5 Oct 2013 0rganized by Sikkim university, Sikkim.

4. Poster presentation on "National Symposium on Recent Trends in Plant and Microbial Research "held on 22nd-23rd March 2013, organized by DST (FIST) & UGC-SAP assisted DRS, Department of Botany, University of North Bengal, Siliguri, Darjeeling.

5. Participated on "National Conference on Biological and Bioinformatics of Economically Important Plants and Microbes" held from 17th-19th Feb 2012 jointly organized by Department of Botany & Bioinformatics facility, University of North Bengal, Siliguri, Darjeeling

6. Participated in the National Workshop on "Bio-Physical Chemistry for Cancer Research" organized jointly by St Josephs college, Darjeeling, Darjeeling Government college, Darjeeling held from 29th Oct to 2nd Nov 2011 at, Darjeeling Government college, Darjeeling.

7. Participated in "Seminar Cum Workshop on Bioinformatics" held from 15th-17th Jan 2010 at Bioinformatics Facility, University of North Bengal, Siliguri, Darjeeling.

APPENDIX- C: LIST OF ABBREVIATIONS

A. khasiana	*Asterella khasiana*
ABA	Abscisic acid
DAG	Diacylglycerol
DCI	D-chiro-inositol
DEAE	Diethylaminoethyl
DGK	Diacylglycerol kinase
DGPP	Diacylglycerol pyrophosphate
DTT	Dithiothritol
EC	Enzyme commission
EDTA	Ethylene diamine tetraacetate
F-6-P	Fructose-6-phosphate
FBP/F1,6P /Fru-P_2	Fructose 1,6-bisphosphate
Fru-P_2ase	Fructose 1,6-bisphosphatase
G-1-P	Glucose-1-phosphate
G-6-P	Glucose-6-phosphate
G-6-PD	Glucose-6-phosphate dehydrogenase
Gal-6-P	Galactose-6-phosphate
GDP	Guanosine diphosphate
GMP	Guanosine monophosphate
GoIS	Galactinol synthase
IMPase	Inositol monophosphatase
IMT	Inositol-O-methyltransferase
IPC synthase	Inositol phosphorylceramide synthase
IP_1	*Myo*-inositol monophosphate
IP_2	*Myo*-inositol bisphosphate
IP_3	*Myo*-inositol trisphosphate
IP_4	*Myo*-inositol tetrakisphosphate
IP_5	*Myo*-inositol pentakisphosphate
IP_6 / $InsP_6$	*Myo*-inositol hexakisphosphate / phytic acid

IPG	Inositol phosphoglycan
IPK	Inositol-1,3,4,5,6-pentakisphosphate kinase
IUPAC	International union for pure and applied chemistry
kDa	Kilodaltons
LCI	L-chiro-inositol
LRR	Leucine rich repeat
Man-6-P	Mannose-6-phosphate
M	Molar
MAPK	Mitogen activated protein kinase
ME	2-mercaptoethanol
MI	*Myo*-inositol
MIPS/inositol synthase	L-*myo*-inositol-1-phosphate synthase
MIP/InsP	*Myo*-inositol-1-phosphate
MIPD	*Myo*-inositol-1-phosphate dehydrogenase
MMO	1-O-C-methylene *myo*-inositol
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
OS	Oxidative stress
ORF	Open reading frame
pCMB	para-chloromercurobenzoate
PA	Phosphatidic acid
PAGE	Polyacryladime gel electrophoresis
PAK	Phosphatidic acid kinase
PI/PtdIns	Phosphatidylinositol
PI kinase	Phosphoinositol kinase
PKC	Protein kinase C
PIP	Phosphatidyl inositol 4-phosphate
PI-PLC	Phosphoinositide-specific phospholipase C
PLC	Phospholipase C
PMSF	Phenylmethanesulfonylfluoride
PP-IP_5	Diphosphoinositol pentakisphosphate

PP_2-IP_4	Bisdiphosphoinositol tetrakisphosphate
PIP_2	Phosphatidyl inositol 4,5-bisphosphate
PIP_3	Phosphatidyl inositol 3,4,5-trisphosphate
RFO	Raffinose family oligosaccharide
RIL	Recombinant inbred line
ROS	Reactive oxygen species
Ru5P	Ribulose-5-phosphate
RuBP	Ribulose 1,5-bisphosphate
SDS	Sodium dodecyl sulphate
SIPK	Salicylic acid induced protein kinase
SMIT	Na^+/*myo*-inositol co-transporter
S. junghuhnianum	*Sphagnum junghuhnianum*
StaS	Stachyose synthase
TBA-RS	Thiobarbituric acid reacting substances
TCA	Trichloro acetic acid
TEMED	N, N, N´, N´-tetramethylethylene diamine
TLC	Thin layer chromatography
Tris	Tris (hydroxymethyl) aminomethane
UDP-Gal	UDP-D-galactose
VS	Verbascose synthase
WIPK	Wound induced protein kinase

ISOLATION AND CHARACTERIZATION OF AN ENZYME L-*MYO*-INOSITOL-1-PHOSPHATE SYNTHASE FROM BRYOPSIDA: *Brachymenium bryoides* HOOK. EX SCHWAGR. FOUND IN DARJEELING HILLS

[1]Sachina Yonzone, [2]Dhani Raj Chhetri, [3]Subhas Chandra Roy

[1]Research scholar, [2,3]Associate professor
[1]Plant Physiology and Biochemistry,
Post Graduate Department of Botany,
Darjeeling Government College, 734101, West Bengal, India.

***Abstract*: Brachymenium bryoides is an important genus of moss generally regarded as an indicator plant to understand the effects of climate change. In Darjeeling Himalayan region, like any other biodiversity hotspots, the moss flora is very rich, although neglected. Any biochemical work on these plants, especially the present species is absolutely lacking. The presence of enzyme L-myo-inositol-1-phosphate synthase (MIPS) is essential for the survival of this moss like any other groups of plants since it forms the cell walls as well as the sexual units in plants. The enzyme MIPS from Brachymenium bryoides utilizes D-glucose-6-phosphate as a substrate NAD^+ as a co-factor respectively. It showed a pH optimum at 7.5 while the temperature maximum was at $40S^0C$. The enzyme activity was remarkably stimulated by Ca^{2+}, NH_4^+ and Mg^{2+} and extremely inhibited by Zn^{2+}, Cd^{2+}, Mn^{2+} and Hg^{2+}. The K_{rn} values for D-glucose-6-phosphate and NAD^+ were found to be as 6.72 mM and 0.78 mM respectively while the V_{rnax} values were 1.62 mM and 1.11 mM for D-glucose-6-phosphate and NAD^+ respectively.**

***Keywords*: Brachymenium bryoides. myo-inositol, L-myo-inositol-1-phosphate synthase (MIPS), inositol synthase, UDP-galactose, D-glucose-6-phosphate**

1. Introduction

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 [15]. The enzyme, MIPS has been isolated and characterized from a number of systems including bacteria [25], protozoa [19], lower plants [18,10,9,26], higher plants and animals [16,12,21]

In plants, *myo*-inositol becomes incorporated in a number of metabolic products viz., inositol phosphates, phosphoinositides, cell wall polysaccharides etc. As a free cyclitol, *myo*-Inositol has been proved essential for normal growth and development of plant tissue. Lack of cellular level of inositol has been identified as the cause of loss of viability in *myo*-inositol requiring mutants of *Saccharomyces cerevisiae* [8] and the same causes an inhibition of cell division in other plants under tissue culture conditions and the phenomenon has been termed as "inositol-less-death".

Inositol is associated with the resistance to both abiotic and biotic responses [29] specifically in halophytes under salt stress [30,28] active as an osmolyte. Different derivatives of *myo*-inositol also serve as compatible solutes and signalling molecules in response to salt stress in plants [13], oxidative stress, temperature stress, drought and desiccation stress etc. The phosphorylated derivates of inositol are considered as the important component of signal transduction in plants and animals [2]. The breakdown product of *myo*-inositol, D-glucuronic acid, is utilized for synthesis of cell wall pectic non-cellulosic compounds [14]. Intact plant tissue readily utilizes the glucuronic acid and rapidly converts it to cell wall polysaccharides and other products of glucuronic acid metabolism [17].

The present study is concerned with the study on the occurrences of MIPS (EC: 5.5.1.4) from a moss from Darjeeling hills. Partial purification of MIPS and characterization of its properties from *Brachymenium bryoides* have been reported here.

2. Materials and Methods

Fresh specimens of bryophyte *Brachymenium bryoides* Hook. ex Schwagr (*B.bryoides*) of Darjeeling Hills were collected from the Birch Hill area in Darjeeling hills (circa 2134 m amsl.) in $87^0$59' - $88^0$53' E and $26^0$31' - $27^0$13' N in the Eastern Himalayas of India.

Purification of L-*myo*-inositol-1-phosphate synthase from *B.bryoides*

The enzyme L-*myo*-inositol-1-phosphate synthase was purified from *B.bryoides* following the method outlined below:
All the operations were carried out between 0˚ C to 4˚ C

Preparation of crude extract: About 100g of freshly collected plant material was thoroughly washed with sterile distilled water twice. The sample was then homogenized in a mortar and pestle with 2 vol of 50 mM Tris-acetate buffer (p^H7.5) containing 0.2 mM ME in presence of neutral sand. The slurry was centrifuged at 1,000×g for 5 min in a Plasto Crafts Superspin-R centrifuge. The pellet discarded and the supernatant designated as crude extract (1K).The crude extract was spun again at 11,400×g for 20 min and the supernatant was collected (10K).

Streptomycin sulphate precipitation: To the 10K sample**, s**treptomycin sulphate powder was added slowly with constant stirring to a final concentration of 2% (w/v) to the 10K supernatant in order to remove nucleic acids. After mixing the required amount of streptomycin sulphate, the mixture was kept in an ice-bucket at 0^{o}C for 15 min and then centrifuged at 11,4000×g for 15 min. The pellet was discarded and the supernatant collected (SS).

Ammonium sulphate fractionation: The streptomycin sulphate treated supernatant (SS) was then made 0-70 % saturated with ammonium sulphate by adding the requisite quantity of the solid salt (43.6g/100 ml) slowly with constant stirring. The mixture was kept in an ice-bucket for 15 min and then centrifuged at 11,400×g for 20 min. The supernatant was discarded and the pellet collected. The pellet was dissolved in minimal volume of 50mM tris-acetate buffer (pH7.5) containing 0.2 mM ME, poured into a dialysis sac and dialyzed against 500 volumes of the same buffer. On completion of dialysis, the ammonium sulphate fraction (A_2S) was recovered from the dialysis sac.

Anion exchange chromatography with DEAE cellulose: The dialyzed A_2S fraction was adsorbed in pre-equilibrated DEAE cellulose. After adsorption for two hours, the preparation was loaded in a glass column (1.2×8.0 cm) and the effluent was collected. After this, the column was washed with one bed volume of the same buffer. Lastly, the adsorbed protein was eluted from the column with the linear gradient of 0-0.5 M KCl in 50mM Tris acetate buffer (p^H7.5) containing 0.2 mM ME. Fractions at the rate of 1.1 ml/10 min were collected. Each fraction was assayed for *myo*-inositol synthase activity. The active fractions (DE) was pooled and used for the next purification step.

Molecular sieve chromatography through BioGel A-0.5 m: The enzymatically active fractions from the previous step was pooled together and loaded in a column (0.8 X 10.0 cm) of BioGel A-0.5m pre-equilibrated with 50 mM Tris-acetate buffer (pH 7.5) containing 0.2 mM ME. Proteins were eluted with the same buffer in fractions of 0.75 ml/8 mins. Fractions containing MIPS activity were pooled and dialyzed against 1.5 L of 50 mM Tris-acetate buffer (pH 7.5) containing 0.2 mM ME (BioGel fraction). This preparation was concentrated and used as the enzyme source for further characterization.

Enzyme activity assay: The MIPS activity was assayed by the procedure of Barnett *et al.*, (1970) with slight modifications. 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. 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 determination: 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 ºC for 1h. The absorbance was measured at 820 nm and the inorganic phosphate released was quantified with a standard curve prepared using K_2HPO_4.

Protein determination: 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
.

Determination of specific activity of inositol synthase: The specific activity of L-*myo*-inositol-1-phosphate synthase will be calculated by determining the number of moles of inorganic phosphate released (from *myo*-inositol phosphate) per mg of protein. The activity may be defined as nmol L-*myo*-inositol-1-phosphate (I-1-P) produced per hour per mg of protein i.e. nmol I-1-P produced (mg protein)$^{-1}h^{-1}$**.**

3. Result and Discussion

Purification of L-myo-inositol-1-phosphate synthase

The enzyme, L-*myo*- inositol-1-phosphate synthase was isolated and purified from the thallus of *Brachymenium bryoides.* The activity of the enzyme was much more pronounced in the reproductive part bearing thallus than in vegetative thallus (Table-1). This follows the common trend as in other bryophytes [7]. The summary on the purification of MIPS is given in Table 2. The ultimate chromatography on BioGel A 0.5m column resulted in about 22 fold purification of the enzyme with about 22 fold recovery over the crude homogenate fraction in the present study.

Characterization of the purified enzyme

Requirements for B. bryoides MIPS activity: The *B. bryoides* MIPS, 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) 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. About 33% activity was lost when tris buffer was omitted from the reaction

mixture. Similarly, 31% of enzyme activity was lost in absence of tris-buffer from the reaction mixture in case of *Marchantia nepalensis* [6]. Deduction of NH_4Cl or ME resulted in the loss of enzyme activity by about 23% and 25% respectively. In comparison, the absence of NAD^+, NH_4Cl and ME decreased the activity of *Euglena gracilis* MIPS by 70%, 23% and 30% respectively [9]. In hepaticopsid bryophyte, *Lunularia cruciata*, the omission of Glucose-6-phosphate (G-6-P), tris buffer, NH_4Cl and ME causes the loss of activity by 100%, 25%, 40% and 27% respectively [7]. Curiously enough, in the present study, both the NH_4Cl and ME exert almost equal influence to the enzyme activity.

Substrate specificity: The *B. bryoides* MIPS have been found to utilize G-6-P as the exclusive substrate for the production of L-*myo*-inositol-1-phosphate (MI-1-P). Among other hexose phosphates tested, such as D-fructose-6-phosphate, D-fructose-1, 6-bisphosphate and D-glucose-1,6-bisphosphate used in place of G-6-P at identical concentrations (5 mM), all were ineffective. This result is in conformity with MIPS from other sources [26]. Nevertheless, a basal level of approx 3-4% enzyme activity in presence of all these substrate isomers was seen which may be the result due to non *myo*-inositol specific phosphate release in the assay reaction (Table-4)

Stability of the enzyme: An important feature of *B. bryoides* MIPS is the moderate stability of its catalytic activity. Stability varies with the enzyme at different stages of purification. While the low speed supernatant remained active for 7-8 days when stored at −20 ºC, the BioGel 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 dithiothritol (DTT) considerably increased the activity of the enzyme.

Enzyme and time linearity: The *B. bryoides* MIPS exhibited enzyme linearity upto 350 µg of protein concentration under optimal assay conditions (Fig-1). In contrast, the *Diplopterygium glaucum* MIPS showed enzyme linearity upto 280 µg and in *Marchantia nepalensis* it was upto 250 µg [6,4]. The rate of enzyme reaction proceeded linearly upto 60 minutes with G-6-P as the substrate (Fig-2). This is quite different from MIPS from that of the *Acer pseudoplatanus* cell culture that shows time linearity upto 150 minutes [18].

pH – activity relationship: The *B. bryoides* 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 – 8.5 were employed (Fig-3). This value is a little less in comparison to the pH optima for MIPS from other species like *Spirulina platensis* -7.8, *Euglena gracilis*-8.2 [26] and rice cell culture-8.4 [11]. However, it is in conformity with earlier studies with *Swertia bimaculata* enzyme [5].

Effect of temperature: The effect of temperature was studied in the temperature range of 0 - 60ºC at 10ºC intervals. The activity of the enzyme was negligible at 10ºC and gradually increased after 20ºC reaching the maximum 40ºC (Fig-4). This optimum temperature is relatively high as compared to that of *Spirulina platensis, Euglena gracilis, Oryza sativa and Vigna radiata* [26].

Effect of metal ions: Effect of different metal ions was tested in 5 mM concentrations using chloride salts of metals. Of the monovalent cations tested K^+ and Li^+ had little effect, while NH_4^+ was an appreciable stimulator of the enzyme. NH_4^+stimulation of *B. bryoides* MIPS was to the tune of 1.86 times in contrast to the *Acer pseudoplatanus* MIPS which is stimulated 2.3 times with NH_4^+ [18]. The remarkable feature of *B. bryoides* MIPS was its stimulation by divalent cations. Using the similar concentrations of divalent cations it was revealed that Ca^{2+} caused the stimulation of the enzyme by 2.39 times while Mg^{2+} was slightly stimulatory to the enzyme activity. Of the other divalent cations Cu^{2+}, Zn^{2+}, Mn^{2+}, Cd^{2+} and Hg^{2+} strongly inhibitory (34%, 64%, 67%, 68%, and 86% respectively) (Table-5). In general, the effects of monovalent and divalent cations are similar to those obtained from other sources except for the effect of Ca^{2+} which is highly stimulatory to the MIPS activity in this case.

Reaction rate-substrate concentration relationship: Kinetic studies were carried out using G-6-P (substrate) in different concentrations. The reaction rate was found to increase with respect to G-6-P upto a concentration of 10 mM. The K_m value for G-6-P calculated from Michaelis-Menten plot was 6.72 mM and the V_{max} value was calculated as 1.61 mM. The K_m value for G-6-P is quite far away from that of pine pollen (K_m=0.33) [12], but somewhat closed for that from animal sources e.g., 2.7 for bovine testis enzyme [22]; 3.89 for rat testis enzyme [20] and 4.4 for rat brain enzyme [1].

Reaction rate-co-enzyme concentration relationship: Between concentrations of 0-1.0 mM of NAD (co-enzyme) the activity of purified enzyme was found to increase only upto 0.2 mM concentration. With more increase in co-enzyme concentration the activity could not be increased. The K_m value for NAD was 0.78 and the V_{max} value was calculated as 1.11 mM from the Michaelis-Menten equation. The K_m value for NAD+ was entirely different from that obtained from other sources e.g., *Euglena gracilis* (K_m= 0.16-0.20) [26] and *Entamoeba histolytica* (K_m= 0.66) [9].

The present study reports the occurrence and partial purification of MIPS for the first time from *B. bryoides*. Table-2 summarizes the partial purification of this enzyme from the moss. The enzyme from *B. bryoides* was highly specific for G-6-P. Though the enzyme exhibits its optimal activity in presence of co-enzyme NAD^+, still it could maintain about one third of the total activity when NAD^+ was not added externally (data not shown). This indicates the presence of bound NAD+ in the molecular architecture of this enzyme which has also been reported earlier [9]. 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 the various biochemical activities of the phosphate esters of *myo*-inositol [23,31,24]. This indicates an important function played by this enzyme in metabolism and makes this study worthwhile in understanding the basic metabolism in plants [27]. In *B. Bryoides*, the ultimate chromatography on BioGel A-0.5m yielded a 22 fold increase in the enzyme activity with about 11% recovery. This study may lead to further research to look for homogeneous MIPS preparation from *B. bryoides*, sequencing the responsible gene and analysis of sequence homology.

Table 1. L-*myo*-inositol-1-phosphate synthase activity in vegetative and reproductive stages of the moss, *B. bryoides.*

Tissue type	Enzyme source	Specific activity [nmol I-1-P produced $(mg)^{-1}$ protein h^{-1}]
Vegetative	Homogenate	154.00
	Low speed supernatant	198.00
Reproductive	Homogenate	162.52
	Low speed supernatant	814.53

Table 2. Summary of partial purification of L-*myo*-inositol-1-phosphate synthase from *B. bryoides.*

Fraction	Total volume(ml)	Specific activity [nmol I-1-P produced (mg)-1 protein h-1]	Total activity [nmol I-1-P produced $(mg)^{-1}$ protein h^{-1}]	Recovery (%)	Purification (fold)
Homogenate	72	26.67	1920.96	100	1.00
10K- supernatant	70	15.76	1103.20	57.42	0.59
SS-fraction	69	45.43	3134.67	163.18	1.70
A_2S-fraction	3	155.86	467.58	24.34	5.84
DE-fraction	2.2	190.86	419.89	7.15	21.85
Bio Gel A 0.5m fraction	1.5	283.87	425.80	10.67	22.16

Table-3. Effect of composition of incubation medium on L-myo-inositol-1-phosphate synthase from *B. Bryoides*

Conditions	Specific activity [nmol I-1-P produced $(mg)^{-1}$ protein h^{-1}]	Percent activity
Complete set	155.86	100
Without substrate (G-6-P)	0.00	0.00
Without NH4Cl	49.62	77.33
Without ME	48.38	74.98
Without buffer (tris-acetate)	43.50	67.42
Heat killed enzyme	0.00	0.00

Table -4. Effect of some substrate isomers on L-myo-inositol-1-phosphate synthase from *B. bryoides.*

Compound	Concentration (mM)	Specific activity [nmol I-1-P produced $(mg)^{-1}$ protein h^{-1}]	Percent activity
D-glucose-6-phosphate	10.0	155.86	100
D-fructose 1,6 bisphosphate	10.0	2.48	3.84
D-fructose-1-phosphate	10.0	1.98	3.06
D-glucose-1-phosphate	10.0	1.86	2.88

Table-5. Effect of monovalent and divalent cations on L-myo-inositol-1-phosphate synthase activity from *B. bryoides.*

Cation	Concentration	Specific activity [nmol I-1-P produced (mg)-1 protein h-1]	Percent activity
Control	0	216.76	100.00
K^+	5	232.97	107.47
Li^+	5	268.81	124.01
NH_4^+	5	403.22	185.88
Ca^{2+}	5	519.71	239.58
Mg^{2+}	5	286.73	132.18
Cu^{2+}	5	143.36	66.08
Zn^{2+}	5	79.21	36.51
Mn^{2+}	5	71.68	33.04
Cd^{2+}	5	68.81	31.72
Hg^{2+}	5	30.46	14.04

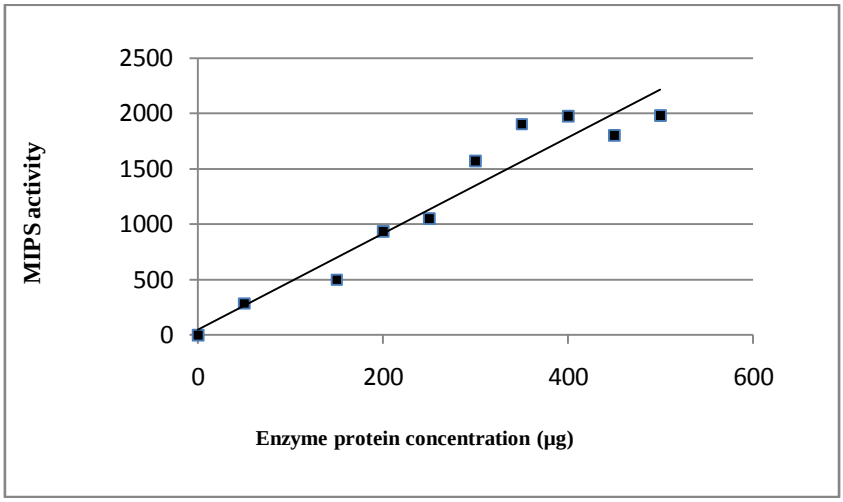

Fig 1. Effect of different enzyme concentration on *B. bryoides* MIPS activity. Enzyme activity is expressed as [nmole of Inositol-1-phosphate produced $(mg)^{-1}$ protein h^{-1}]

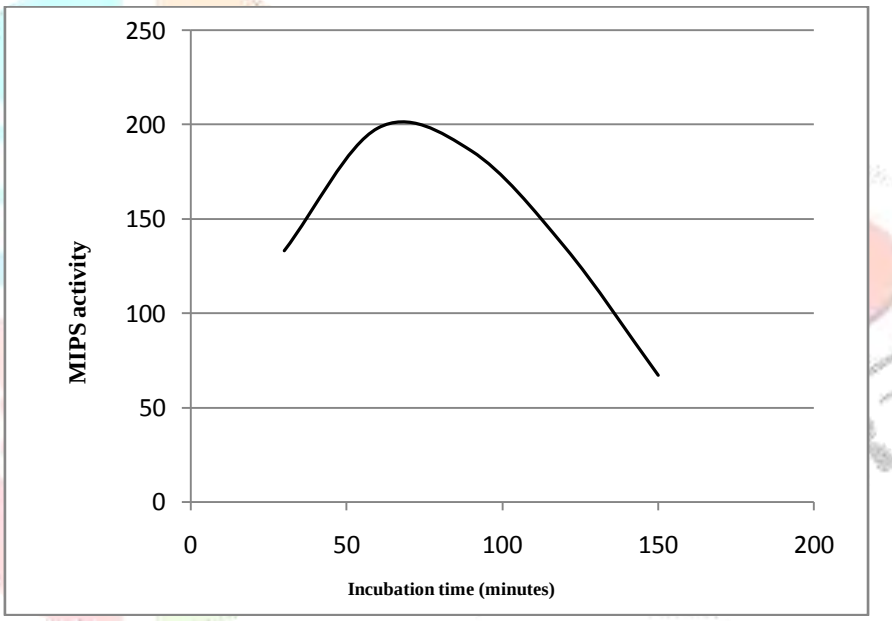

Fig 2. Effect of different incubation time on *B. bryoides* MIPS activity. Enzyme activity is expressed as [nmole of Inositol-1-phosphate produced $(mg)^{-1}$ protein h^{-1}]

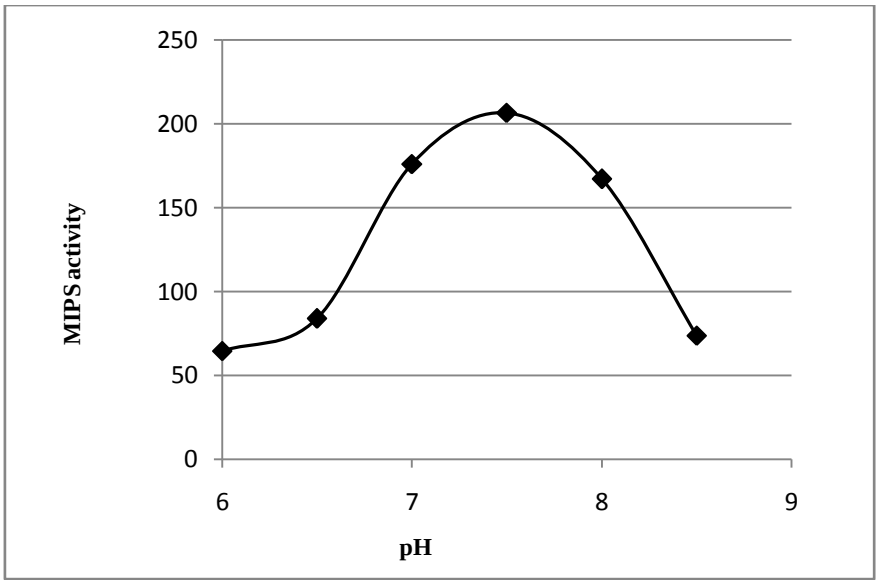

Fig 3. Effect of different pH on MIPS activity in *B. bryoides* MIPS activity. Enzyme activity is expressed as [nmole of Inositol-1-phosphate produced $(mg)^{-1}$ protein h^{-1}]

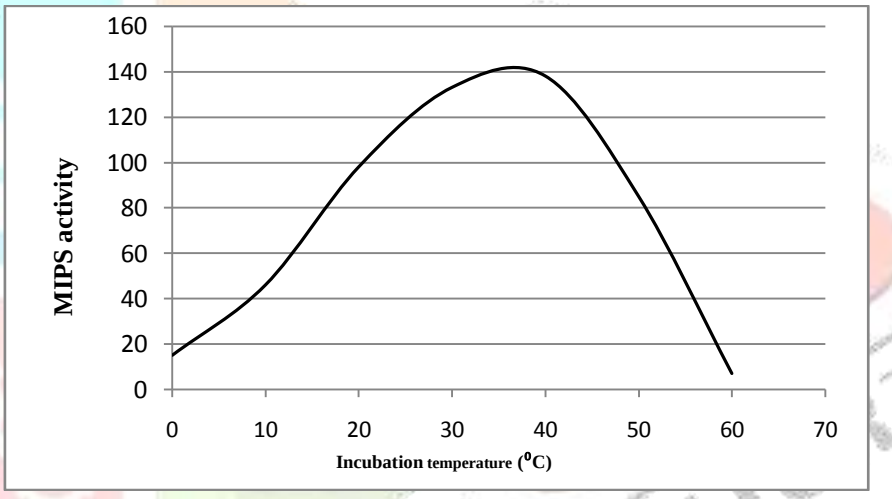

Fig 4. Effect of different reaction temperature on *B. bryoides* MIPS activity. Enzyme activity is expressed as [nmole of Inositol-1-phosphate produced $(mg)^{-1}$ protein h^{-1}]

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Original Research Article

Gluconeogenic Fructose 1, 6-bisphosphatase from *Ginkgo biloba* L.: Isolation and characterization

Sachina Yonzone[1], Suchisree Jha[2] and Dhani Raj Chhetri[3]*

[1]PG Department of BotanyDarjeeling Government College, Darjeeling-734101, India
[2]Department of Botany, North Bengal University, Rajaramohanpur, Darjeeling, India
[3]Department of Botany, Sikkim University, 6th Mile, Gangtok 737102, Sikkim, India.

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Abstract: Fructose 1,6-bisphostase (FBPase) was partially purified from mature leaves of *Ginkgo biloba* which is a rare medicinal plant effective as antioxidant, adaptogenic and memory enhancer. The purification procedure involved homogenization, low speed centrifugation, streptomycin sulphate precipitation, ammonium sulphate fractionation and chromatography on Bio Gel-A 0.5 m. The level of purification was about 22 fold and the recovery was about 56% at the final stage of purification. D-fructose-1,6-bisphosphate (FBP) was found to be the specific substrate for *Ginkgo biloba* FBPase. Among the other hexose phosphates, D-glucose-6-phosphate, D-fructose-6-phosphate and D-galactose-6-phosphate could not act as substrates for this enzyme. However, this enzyme showed traces of activity in presence of D-fructose-1-phosphate. With the increase in substrate (FBP) concentration from 0 to 8 mM the activity of the enzyme increased linearly. The FBPase activity was directly proportional to the time of incubation upto 60 minutes and with respect to protein concentration upto 400 µg. The Bio Gel A 0.5 m purified *G. biloba* FBPase showed a K_m values of 1.86 mM for D-fructose-1,6-bisphosphate. The FBPase required Mg^{2+} as a cofactor. Mn^{2+} and Mg^{2+} appreciably stimulated the FBPase activity in a concentration guided manner. The monovalent cations, K^+ and Na^+ had little effect while Zn^{2+} was extremely inhibitory to the enzyme.

Key wods: *Ginkgo biloba*, enzyme purification, enzyme characterization, fructose 1,6-bisphosphatase, D-fructose-1,6-bisphosphate, gluconeogenesis

Introduction

Fructose -1,6- bisphosphatase enzyme (FBPase, EC: 3.1.3.11) in soluble and particulate forms, is considered to be related with two vital biochemical events namely gluconeogenesis and photosynthesis. This enzyme also plays a key regulatory role in the sucrose biosynthetic pathway (Huber *et al.,* 1985; Daie, 1993). In higher plants, at least two forms of the enzyme have been identified: chloroplastic and cytosolic (Kelly *et al.,* 1976). Of these, the cytosolic form of the enzyme is homologous to the enzyme present in the gluconeogenic pathway (Ladror *et al.,* 1990).

FBPase was originally considered as a specific hexose bisphosphatase. Later on, it was named as fructose-1,6-bisphosphatase (Gomori, 1943) which is markedly specific for fructose -1,6- bisphosphate (FBP) [Mokrasch and McGlivery, 1956] that transforms the substrate to fructos-6 phosphate and inorganic phosphate. The gluconeogenic FBPase is an allosteric enzyme which is strongly inhibited by the negative modulator AMP and stimulated by 3-phosphoglycerate and citrate.

FBPase has so far been isolated and characterized from a considerable number of microbial, plant and animal sources. The enzyme was purified and its properties investigated from a bacteria *Acinobacter iwoffi* (Mukkada and Bell, 1971). Fujita and Freese (1979) have purified and characterized the enzyme from *Bacillus subtilis* and Funayama *et al.,* (1979) purified the same from *Saccharomyces cerevisiae*. In *Mangifera indica* the activity of the enzyme was found to be at the maximum during fruit ripening. It was activated by citrate and inhibited by AMP and Zn^{2+}. Yeast cell grown in sugar medium exhibited FBPase activity (Gancedo and Gancedo, 1971). Activity of the enzyme was also found in germinating seeds of *Sida spinulosa* and appreciable titre of activity was also detected from the cotyledons of *Corylus avellana* (Li and Ross, 1988).

***Corresponding Author:**
Dr. Dhani Raj Chhetri,
Department of Botany,
Sikkim University, Gangtok 737102,
Sikkim, India.

FBPase of chloroplastic origin was isolated from spinach leaves (Baier and Latzko, 1975). The enzyme was strongly activated by light and had a molecular weight of 160 kda with possibly 4 equal subunits. Cytosolic FBPase from spinach leaves have been purified by Zimmermann *et al.,* (1978) which showed a molecular weight of 130 kDa. This cytosolic enzyme regulated sucrose biosynthesis in plant leaves. Khayat *et al.,* (1993) purified cytosolic FBPase to 472 fold from *Beta vulgaris* L. leaves, the activity of which was light dependent in an indirect manner unlike the chloroplastic FBPase which is directly light regulated. The cytosolic enzyme was purified to homogeneity from castor oil seed endosperm (*Ricinus communis* L. cv. Hate) by Moorhead *et al.,* (1994). Cytosolic FBPase was purified from Spinach leaves which resolved to a single band of 39 kDa. This protein showed strong cross reactivity with plant cytosolic FBPase but no cross reactivity with chloroplastic FBPase (Hur and VasConselos, 1998). Cytosolic FBPase was also isolated from apple leaves (Zhou and Cheng, 2004). The enzyme was a homotetramer of 37 kDa units. It was stimulated by Mg^{+2} and Mn^{+2} but competitively inhibited by fructose-2,6-bisphosphate.

Despite many reports of the occurrence of FBPase in different plant systems, the information regarding the enzyme from gymnosperm is lacking. This is an attempt to bridge that gap by reporting the occurrence of the enzyme and its fundamental characterization from a famous gymnosperm *Ginkgo biloba* L. The study may pave ways toward understanding the purification, metabolic regulation cloning and its manipulation leading to new insights that may generate suitable applications.

Materials and Methods

Plant Material

Mature leaves of *Ginkgo biloba* were collected in ice-box and immediately brought to the laboratory for analysis. The plant material was collected from Lloyd's Botanical Garden, Darjeeling between 9.00 and 10.00 AM during the month of August-September. The ambient temperature was 21 ± 2°C with 80 ± 4% RH during the period. However, all experimental procedure was carried out at 0°C.

Partial Purification of gluconeogenic Fructose-1, 6-bisphosphatase from Ginkgo biloba

The isolated leaf tissues of the experimental plant were washed several times with cold distilled water followed by chilled 50 mM Tris-HCl (pH 7.5) buffer containing 0.2 mM ME and the partial purification of fructose-1, 6-bisphosphatase is done following the method outlined below:

Homogenate: 50gms of buffer washed, deribbed, leaf tissues obtained from *Ginkgo biloba was* homogenized with 3-volumes of 50 mM Tris-HCl (pH 7.5) the homogenate was centrifuged at 1000 RPM for 2 minute and the supernatant was collected which was the homogenate fraction.

Low speed supernatant: The homogenate fraction was then centrifuged at 10,000 RPM for 20 minutes in a Remi C-24 centrifuge. The pellet was discarded and the supernatant fraction was recovered from the centrifuge tubes. This fraction was named the 10K supernatant.

Streptomycin sulphate precipitation: In order to remove contaminating nucleic acids (if any) in the form of precipitate, streptomycin sulphate powder was gently added to the low-speed supernatant with constant stirring (by using a Remi cyclomixer) to a final concentration of 1% (w/v). After proper mixing, it was kept in an ice bucket for 20 minutes followed by a spin at 10,000 RPM for 15 minutes. The pellet was discarded and the supernatant was collected (SS fraction).

Ammonium sulphate fractionation: The streptomycin sulphate treated fraction obtained from the previous step was made 0-30% saturated with ammonium sulphate [$(NH_4)_2SO_4$] by adding requisite quantity (17.6 gm/100 ml) solid $(NH_4)_2SO_4$ salt slowly with constant stirring (using a Remi magnetic stirrer). It was kept at 0°C for 15 minutes and then centrifuged at 10000 RPM for 20 minutes. The pellet was discarded and the supernatant fraction was made 30-70% saturated with $(NH_4)_2SO_4$ by adding requisite quantity (27.3 gms per 100 ml) solid salt slowly with constant stirring. It was kept at 0°C for 15 minutes and then centrifuged at 10000 RPM for 20 minutes. The pellet obtained was collected and dissolved in minimal volume of 50 mM Tris-HCl (pH 7.5)

buffer having 0.2 mM ME and dialyzed over-night against the same buffer (500 volumes with one change). On completion of dialysis, the 30-70% $(NH_4)_2SO_4$ fraction was recovered from the dialysis bag (A_2S-fraction).

Molecular sieve chromatography through BioGel A-0.5m: The bulk volume of the dialyzed $(NH_4)_2SO_4$ fraction obtained from the previous step was loaded onto a column (0.6 x 7.0 cm) of BioGel A-0.5m [before this experimentation, the column material was pre-equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 0.2 mM ME.] Proteins were eluted with the same buffer in fractions of 2.0 ml at a flow rate of 10 minutes per tube. Twenty such fractions were collected. Fractions containing Fructose-1, 6-bisphosphatase activity were pooled together and dialyzed against 1.0 litre of 50 mM tris-HCl buffer (pH 7.5) containing 0.2 mm ME. This preparation was concentrated and used as the enzyme source (BioGel-fraction) for the experiments of characterization.

Enzyme activity assay

The FBPase activity was assayed by the procedure of Udvardy *et al.,* (1982) with slight modification. The enzyme assay mixture contained 50 mM tris-acetate (pH 7.5), 1 mM FBP, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM G-6-P and an appropriate aliquot of enzyme protein in a total volume of 1.0 ml. The mixture was incubated at 37 ºC for 1h after which the reaction was terminated by adding 0.2 ml of 20 % chilled TCA. The mixture was spun at 3,000 rpm for 5 minutes and the pellet discarded. A volume of 2.8 ml Pi-reagent was added to the deproteinized supernatant followed by a second incubation at 37 ºC for 1h for the oxidation of FBPase reaction product D-fructose-6-phosphate, with concomitant release of inorganic phosphate. The activity of the enzyme was determined by estimating the product-specific release of inorganic phosphate from D-fructose-6-phosphate by FBPase reaction. The amount of inorganic phosphate released from the FBPase reaction product was estimated by the method of Chen *et al.,* (1956) and the protein was determined according to the method of Bradford (1976) with BSA as a standard. The content of protein in different fractions obtained from column chromatography was also estimated.

Results

Purification of Fructose-1, 6-bisphosphatase from Ginkgo biloba

The enzyme, FBPase was isolated and purified from the mature leaves of *Ginkgo biloba*. The summary on the purification of FBPase is given in Table 1. Chromatographic profile of proteins resolved from $(NH_4)_2SO_4$ fraction of the crude homogenate of *Ginkgo biloba* leaves is shown in Fig. 1. The BioGel A-0.5m chromatography has revealed that the FBPase from *Ginkgo biloba* was retained and eluted in a single peak with the extraction buffer. In the present study, an overall purification of the enzyme to about 22 fold with about 56% recovery based on total activity was achieved (Table 1).

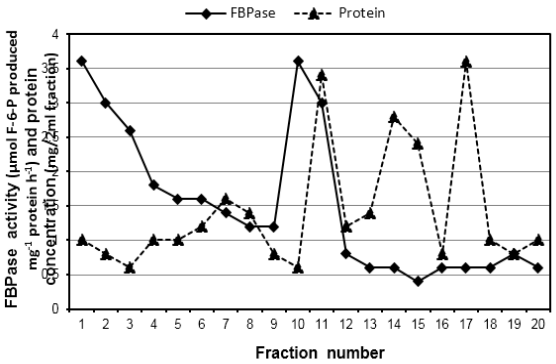

Figure 1: Elution profile of cytosolic FBPase from *Ginkgo biloba* leaves in BioGel A-0.5m column. FBPase activity defined as µ mol of F-6-P produced mg^{-1} protein h^{-1}; protein content expressed as mg/2ml fraction.

Table 1: Summary of partial purification of Fructose-1, 6-bisphosphatase from *Ginkgo biloba* leaves

Purification step	Protein content (mg/ml)	Specific activity [µ mol F-6-P produced $(mg)^{-1}$ protein h^{-1}]	Total activity [µ mol F-6-P produced $(mg)^{-1}$ protein h^{-1}]	Recovery (%)	Purification (fold)
Homogenate	3.4	1.36	740.50	100.00	1.00
10K-supernatant	4.8	1.60	675.84	91.26	1.17
SS-fraction	4.0	2.80	728.00	98.30	2.05
A_2S-fraction	7.2	1.00	13.60	1.83	0.73
BioGel-fraction	3.5	29.60	414.40	55.96	21.76

Characterization of the partially purified enzyme

Requirements for FBP for G. biloba FBPase activity: The *G. biloba* FBPase have been found to utilize FBP as the exclusive substrate and other hexose phosphates could not replace it. When the specific substrate of the enzyme, FBP was not added to the reaction mixture, synthesis of F-6-P could not be detected. However, this enzyme could very slightly utilize D-fructose-1-phosphate (Table 2). Deduction of tris buffer resulted in the loss of enzyme activity by about 58%. Similarly, deduction of $MgCl_2$ and EDTA caused the reduction of enzyme activity by 68% and 50% respectively (Table 3).

Table 2: Effect of some substrate isomers on Fructose-1, 6-bisphosphatase activity from *Ginkgo biloba*

Compound	Concentration (mM)	Specific activity [µ mol F-6-P produced $(mg)^{-1}$ protein h^{-1}]	Percent activity
D-fructose 1,6-bisphosphate	10.0	3.8	100.00
D-fructose-1-phosphate	10.0	0.002	0.05
D-fructose-6-phosphate	10.0	0.00	0.00
D-glucose-1-phosphate	10.0	0.00	0.00
D-glucose-6-phosphate	10.0	0.00	0.00
D-galactose-6-phosphate	10.0	0.00	0.00

Table 3: Effect of composition of incubation medium on *Ginkgo biloba* fructose-1, 6-bisphosphatase activity.

Conditions	Specific activity [µ mol F-6-P produced $(mg)^{-1}$ protein h^{-1}]	Percent activity
Complete set	3.8	100.00
Without substrate (FBP)	0.00	0.00
Without buffer (tris-acetate)	1.60	42.10
Without $MgCl_2$	1.20	31.5
Without EDTA	1.88	49.47
Heat killed enzyme	0.00	0.00

Enzyme stability: Stability of *G. biloba* FBPase varied with the enzyme at different stages of purification. While the low speed supernatant remained active for 10-12 days with insignificant loss of activity when stored at −20 ºC, the BioGel-A 0.5m purified fractions maintained only about 70% of its activity up to 2-3 days when stored at identical temperature. Addition of enzyme stabilizer, 2-mercaptoethanol (ME) or dithiothritol (DTT) increased the shelf-life of the enzyme by 4-5 days.

Enzyme concentration and the time linearity: The *G. biloba* FBPase exhibited enzyme activity linearity up to 400 µg of protein concentration under optimal assay conditions. The rate of enzyme reaction proceeded linearly up to 60 minutes with FBP as the substrate

Effect of temperature and pH: The enzyme was remarkably active between the temperature ranges of 20-40 ºC with 30 ºC as the temperature maximum (Figure 2). Temperature maxima of 35ºC was determined for the cytosolic enzymes from both higher as well as primitive plants. The *G. biloba* FBPase exhibited optimum activity at a pH range of 7.5 – 8.0 (Figure 3). FBPase from other plant sources exhibited similar pH optima.

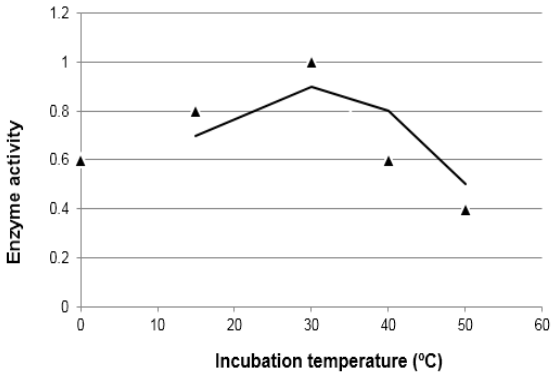

Figure 2: *Ginkgo biloba* FBPase enzyme activity at different incubation temperature (enzyme activity defined as µ mol of F-6-P produced mg^{-1} protein h^{-1}

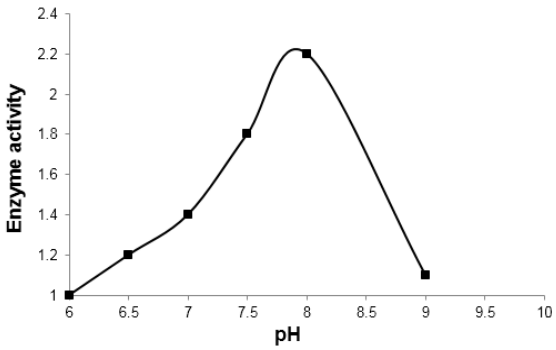

Figure 3: *Ginkgo biloba* FBPase enzyme activity at different pH (enzyme activity defined as µmol of F-6-P produced mg^{-1} protein h^{-1}

Effect of monovalent and divalent cations: Effect of different metal ions on the enzyme activity was tested. Of the monovalent cations tested, K^+ and Na^+ had no effect. Among the divalent cations, Ca^{2+} had no effect, Mg^{2+} and Mn^{2+} were stongly stimulatory while Zn^{2+} was strongly inhibitory to the enzyme activity. Mg^{2+} was stimulatory to the enzyme activity in a concentration dependent manner upto 10 mM while the same was true for Mn^{2+} upto 6 mM concentration. (Table 4).

Table 4: Effect of monovalent and divalent cations on Fructose-1, 6-bisphosphatase activity (using 5 mM FBP as substrate) from *Ginkgo biloba*

Concentration of cations (mM)	Specific activity [µ mol F-6-P produced (mg)$^{-1}$ protein h^{-1}]						
	K^+	Na^+	Ca^{2+}	Mg^{2+}	Mn^{2+}	Cu^{2+}	Zn^{2+}
0	0.60	0.60	0.60	0.60	0.60	0.60	0.60
2	1.20	0.60	1.20	1.00	0.40	0.20	0.20
4	0.60	0.20	1.00	1.00	1.40	0.14	0.12
6	0.20	0.40	1.20	1.80	2.80	0.12	0.10
8	0.40	0.40	1.00	2.60	1.00	0.10	0.10
10	0.60	0.40	0.80	3.20	1.40	0.10	0.08

Determination of K_m value: The activity of the BioGel purified *G. biloba* FBPase was found to increase with respect to the concentration of FBP upto 8 mM when tried between concentration ranges of 0 to 10 mM of substrate. Thereafter, the enzyme activity remained unchanged. The average K_m value for FBP was determined to be approximately 1.86 mM in accordance with the rate equation of Michaelis-Menten (Figure 4).

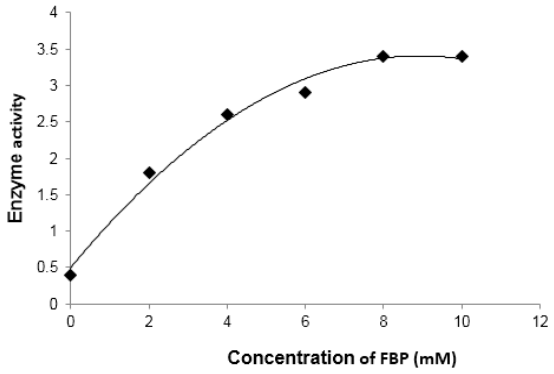

Figure 4: Effect of varied substrate (FBP) concentration on *Ginkgo biloba* FBPase activity (enzyme activity defined as µ mol of F-6-P produced mg^{-1} protein h^{-1}

Effect of varied concentrations different salts: Salts of different chemicals at 0 to 100 mM concentrations were tested. The *G. biloba* FBPase activity was stimulated by NH_4Cl in a concentration guided manner up to a concentration of 50 mM and by $MgCl_2$ upto a concentration of 25 mM. The level of stimulation was 4 times and 6 times respectively. EDTA was stimulatory upto 25 mM, beyond which it seemed to be toxic. Lithium had shown no effect on the enzyme activity (figure-not shown).

Discussion

FBPase activity has so far been reported from almost all groups of plants but gymnosperms. The present work was undertaken with the objective of determining the occurrence of FBPase from *Ginkgo biloba* L., a gymnosperm regarded as a living fossil and to biochemically characterizes the enzyme. The enzyme activity could be detected in the leaves of *Ginkgo biloba* and the cytosolic enzyme from the same was highly specific for its substrate FBP. However, the enzyme could partially utilize fructose-1-phosphate which is in consonance with earlier studies (McGlivery, 1955).

The cytosolic enzyme from *Ginkgo biloba* was highly unstable and the partially purified enzyme could maintain its activity only for 2-3 days. In contrast, the cytosolic FBPase from spinach leaves did not lose its activity upto 2 months of storage in 50% glycerol at -20°C (Herzog *et al.,* 1984). At -20°C, the *Anacystis nidulans* FBPase could be stored for several weeks without any loss of activity (Udvardy *et al.,* 1982).

The monovalent cations used did not show any significant effect on the enzyme activity. However, among divalent cations Mg^{+2} and Mn^{+2} were distinctly stimulatory while Cu^{+2} and Zn^{+2} were decidedly inhibitory of the enzyme function. This effect was not unexpected, since in other cases like *Acinetobacter iowaffi,* the enzyme requires divalent cations, Mg^{2+} and Mn^{2+} for its activity (Mukkada and Bell, 1971). The strong inhibition due to heavy metals suggests that one or more free sulphhydryl groups are present within the active site of the enzyme

(Chhetri *et al.,* 2006). Of the other salts, LiCl and EDTA showed inhibitory action while NH_4Cl was strongly stimulatory to the enzyme. Inhibition by EDTA was also a characteristic feature of FBPase from *Acer pseudoplatanus* (Scala and Semersky, 1971) and inhibition by Zn^{+2} was observed in *Mangifera indica* FBPase activity. Li^{+1} inhibited FBPase activity in *Purococcus furiosus* (Verhees *et al.,* 2002).

In the present case, *Ginkgo biloba* FBPase displayed pH optima of 8.0 which was quite similar to the pH optima towards the alkaline range shown by the same enzyme in bacteria and lower plants viz., pH 8.0-8.5 in *Bacillus licheniformis* (Opheim and Bernlohr, 1975); pH 8.0 in *Bacillus subtilis* (Fujita and Freese, 1979); and it was 8.0 for *Saccharomyces cerevisiae* (Funayama *et al.,* 1979). The pH optimum was found to be towards neutral in FBPase extracted from the storage tissue of different fatty seeds (Youle and Huang, 1976). Cytosolic FBPase of higher plants exhibits neutral pH optima (Botha and Turpin, 1990) in contrast to the alkaline FBPase of *Ginkgo biloba*.

The K_m value for of *Ginkgo biloba* cytosolic FBPase for its substrate FBP was 1.86 which was quite different from the same from other sources as in 3.1µM from apple leaf (Zhou and Cheng, 2004); 20µM from *Bacillus licheniformis* (Opheim and Bernlohr, 1975) and 13µM from *Synechococcus leopoliensis* (Gerbling *et al.,* 1986). However, the value was nearer to the chloroplastic FBPase from spinach leaves having a value of 1.40 mM (Lazaro *et al.,* 1975).

Conclusion

The cytosolic FBPase from *Ginkgo biloba* leaves utilized D-fructose-1,6-bisphosphate (FBP) as its specific substrate. However, this enzyme showed traces of activity in presence of D-fructose-1-phosphate. The *Ginkgo biloba* FBPase was alkaline like that of prokaryotes in contrast to the same from higher plants which were neutral. The stability of enzyme was very short unlike the cytosolic enzymes form higher plants. Thus this enzyme is unique in showing some eukaryotic and some prokaryotic charaters. This may indeed be a considered an evolutionary link between gymnosperms and angiosperms.

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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

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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

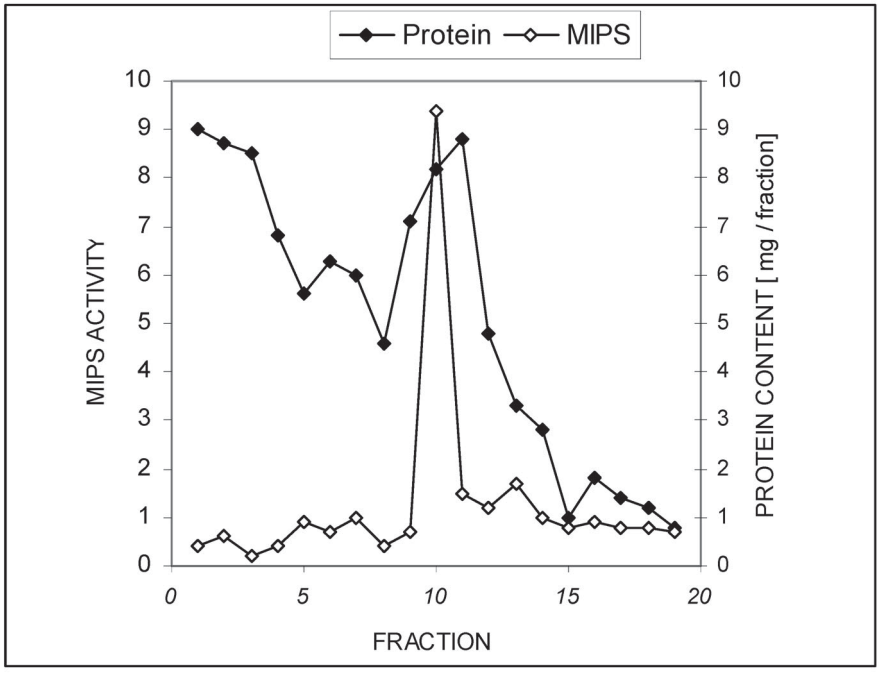

Fig. 1. Elution profile of *Marchantia nepalensis* MIPS on DEAE-cellulose column. MIPS activity is expressed as (μmol (I-1-P) fraction^{-1} h^{-1}).

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

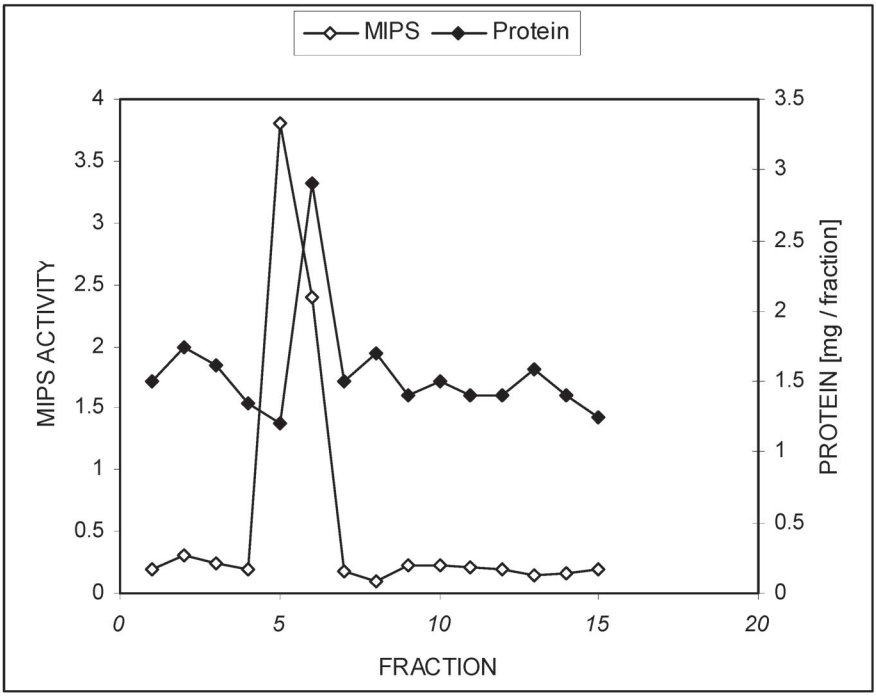

Fig. 2. Elution profile of *Marchantia nepalensis* MIPS on Sephadex G-200 column. MIPS activity is expressed as (mol (I-1-P) $fraction^{-1}$ h^{-1}).

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).

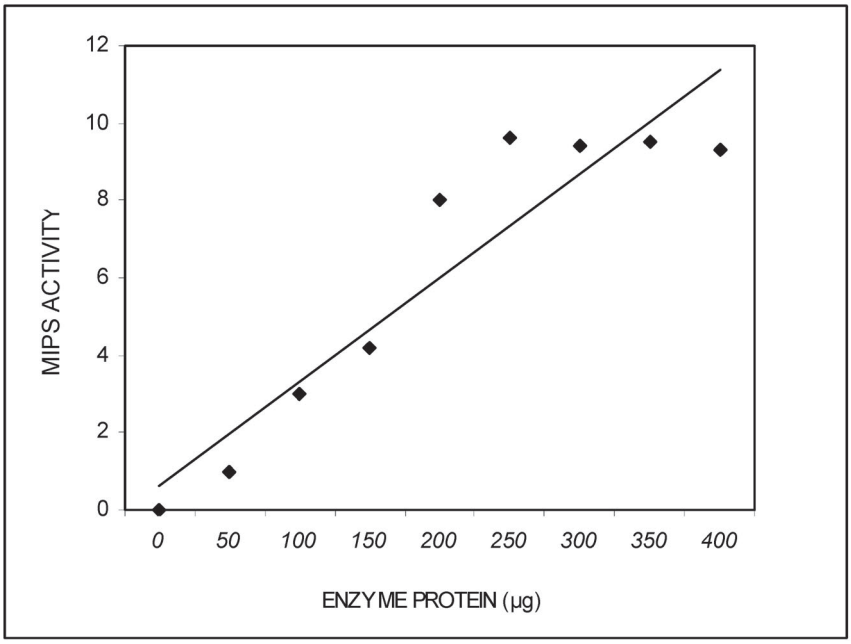

Fig. 3. Effect of different enzyme concentrations on *Marchantia nepalensis* MIPS. MIPS activity is expressed as (mol (I-1-P) mg^{-1}(protein) h^{-1}).

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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RESEARCH ARTICLE

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

Dhani Raj Chhetri[1]*, Sachina Yonzone[1], Sanjeeta Tamang[1] and Asok Kumar Mukherjee[2]

[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.

* Corresponding author. Post box No. 79, Darjeeling-H.P.O., Darjeeling-734 101, WB, India; e-mail: dhanirajc@gmail.com
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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 form 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 Mg^{2+} and Ca^{2+}; remarkably stimulated by NH_4^+; slightly inhibited by Mn^{2+}; highly inhibited by Cu^{2+}, Zn^{2+} and Hg^{2+}. 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 $87^0$59' - $88^0$53' E and $26^0$31' - $27^0$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 cruciate: 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 cm^3 extraction buffer. Fractions (2.0 cm^3) 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 cm^3 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 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 cm^3. After incubation at 37 ºC for 1h, the reaction was terminated by 0.2 cm^3 of 20 % chilled TCA. An equal volume of 0.2 M $NaIO_4$ was added to the deproteinized supernatant (0.7 cm^3) followed by a second incubation at 37 ºC for 1h for the oxidation

of MIPS reaction product, *myo*-insositol-1-phosphate, with concomitant release of inorganic phosphate. The excess of 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 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 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 ± SE), FW = fresh weight.

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

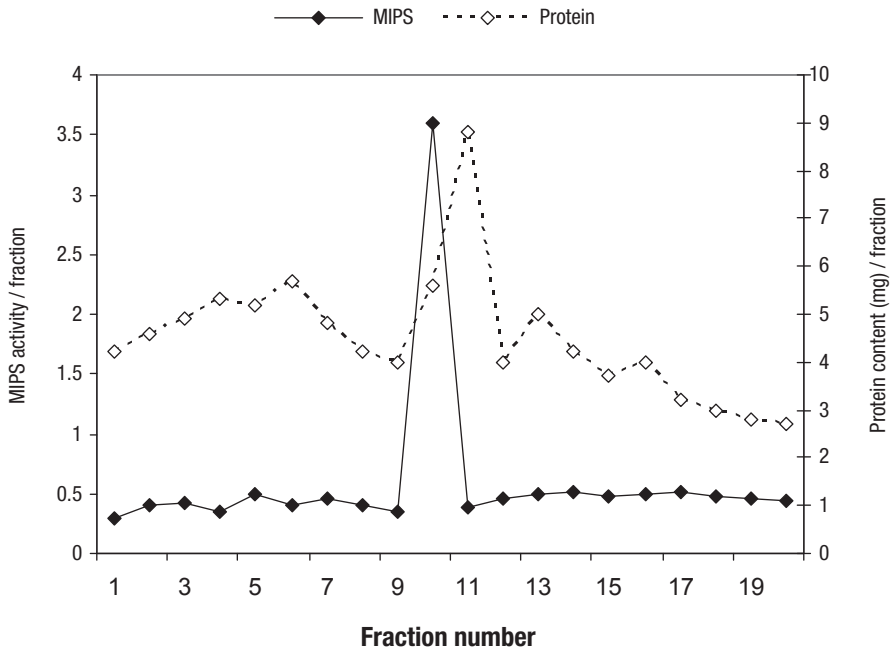

Figure 1. Elution profile of *Lunularia cruciata* MIPS on *DEAE-cellulose* column. MIPS activity is expressed as [μmol (I-1-P) produced fraction^{-1} h^{-1}].

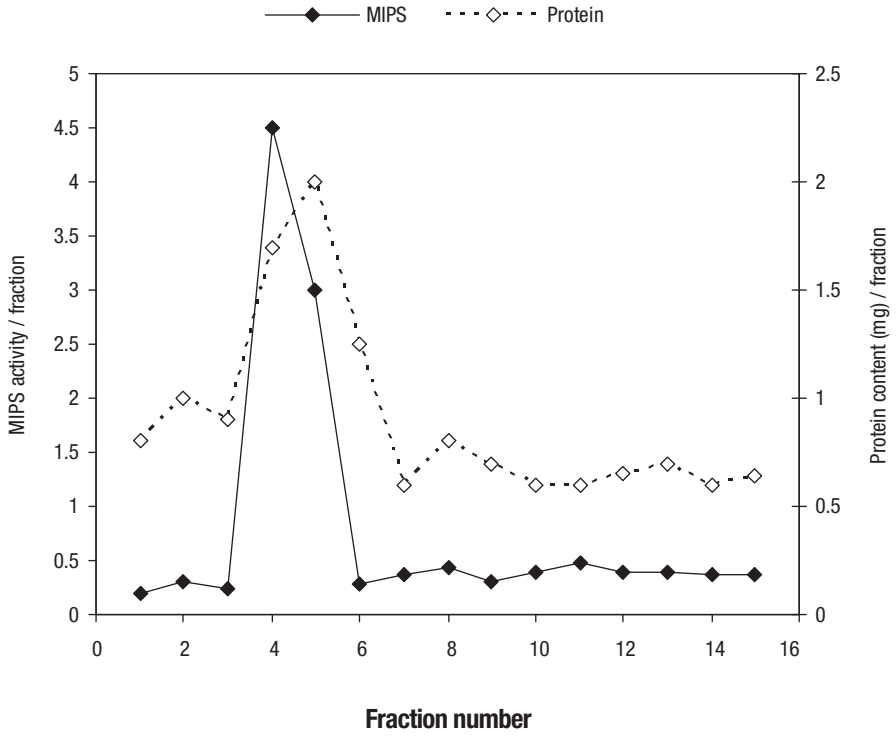

Figure 2. Elution profile of *Lunularia cruciata* MIPS on *Sephadex G-200* column. MIPS activity is expressed as [μmol (I-1-P) produced fraction^{-1} h^{-1}].

Characterization of the purified enzyme: The *L. cruciata* MIPS when assayed in presence of 50 mM tris acetate buffer (pH 7.5), 14 mM NH_4Cl, 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 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 NH_4^+ was an appreciable stimulator of the enzyme. NH_4^+ 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 NH_4^+. Among the divalent cations it was found that Ca^{2+} and Mg^{2+} slightly stimulated; Mn^{2+} slightly inhibited, Cu^{2+}, Zn^{2+} and Hg^{2+} strongly inhibited the enzyme activity with Hg^{2+} acting as the strongest (80%) inhibitor (Table 4).

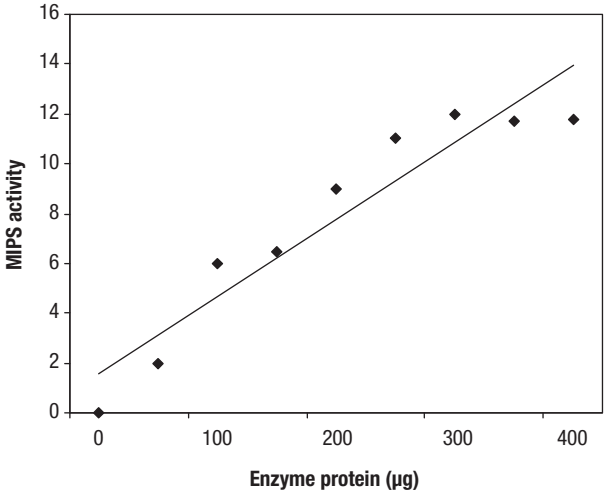

Figure 3. Effect of different enzyme concentration on *Lunularia cruciata* MIPS. MIPS activity is expressed as [μmol (I-1-P) produced mg^{-1}(protein) h^{-1}].

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

Fraction	Total protein [mg]	Specific activity [μ mol (L-*myo*-inositol-1-phosphate) mg^{-1}(protein)h^{1}]	Total activity [μ mol (L-*myo*-inositol-1-phosphate) h^{-1}]	Recovery [%]	Purification [fold]
Homogenate	204.4 ± 9.71	0.12 ± 0.01	24.52± 3.51	100.00 ± 7.63	1.00 ± 0.08
11,400×*g* supernatant	130.0 ± 5.40	0.15 ± 0.02	19.50 ± 1.01	79.52 ± 2.40	1.25 ± 0.14
Streptomycin sulfate treated fraction	79.2 ± 4.37	0.22 ± 0.01	17.42 ± 1.80	71.05 ± 5.02	1.83 ± 0.53
0-60 % ammonium sulfate fraction	12.88 ± 1.42	1.17 ± 0.08	15. 06 ± 1.56	61.41 ± 2.58	9.75 ± 1.20
DEAE-cellulose fraction	1.8 ± 0.20	6.96 ± 0.68	12.52 ± 1.12	51.06 ± 2.69	58.00 ± 2.16
Sephadex G-200 fraction	0.52 ± 0.06	8.41 ± 0.30	4.37 ± 0.67	17.82 ± 2.30	70.08 ± 2.81

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

Condition	Specific activity [μ mol (L-*myo*-inositol-1-phosphate) mg^{-1}(protein)h^{1}]	Percent activity
Complete set	13.1 ± 0.91	100.00 ± 7.36
Without substrate (G-6-P)	0.0	0.0
Without buffer (tris-acetate)	9.9 ± 1.25	75.5 ± 4.08
Without Co-factor (NAD^+)	5.3 ± 0.14	40.45 ± 2.44
Without NH_4Cl	8.1 ± 0.56	61.83 ± 3.77
Without 2-mercaptoethanol	9.6 ± 0.16	73.28 ± 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 ± SE).

Cation	Concentration [mM]	Specific activity [μ mol (L-*myo*-inositol-1-phosphate) mg^{-1}(protein)h^{1}]	Percent activity
Control	0	8.06 ± 0.81	100.00 ± 7.79
K^+	5	8.60 ± 0.71	107.56 ± 8.76
Na^+	5	6.95 ± 0.54	86.22 ± 6.91
NH_4^+	5	11.60 ± 1.41	143.92 ± 10.28
Mg^{2+}	5	9.43 ± .0.69	116.99 ± 8.87
Mn^{2+}	5	7.60 ± 0.21	94.29 ± 8.94
Ca^{2+}	5	9.01 ± 1.44	111.78 ± 4.94
Zn^{2+}	5	4.30 ± 0.65	53.34 ± 2.32
Cu^{2+}	5	5.08 ± 0.11	62.87 ± 1.99
Hg^{2+}	5	1.65 ± 0.12	20.47 ± 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 $Hg^{2+} > Zn^{2+} > Cu^{2+}$with Hg^{2+} limiting the enzyme activity to about 20%. The strong enzyme inhibition due to heavy metals suggests that one or more free sulphydryl 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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