Bioprospecting for L-*myo*-inositol-1-phosphate synthase from cold resistant flora of the Sikkim Himalayas: partial purification and biochemical characterization of the enzyme from *Hippophae salicifolia* D. Don

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By

Raksha Mukhia

Department of Botany
School of Life Sciences
Gangtok – 737102
Sikkim, India

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माइल, सामदुर, तादोंग -737102
गटोक, सिक्किम, भारत
ोन-03592-251212, 251415, 251656
लीफैक्स -251067
वसाइट - www.cus.ac.in

सिक्किम विश्वविद्यालय
SIKKIM UNIVERSITY

6th Mile, Samdur,Tadong -737102
Gangtok, Sikkim, India
Ph. 03592-251212, 251415, 251656
Telefax: 251067
Website: www.cus.ac.in

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Prof. Dhani Raj Chhetri
Ph.D. Supervisor
Department of Botany
Sikkim University, Gangtok

माइल, सामदुर, तादोंग -737102
गटोक, सिक्किम, भारत
ोन-03592-251212, 251415, 251656
लीफैक्स -251067
बसाइट - www.cus.ac.in

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Dr. Dhani Raj Chhetri
Professor and Head,
Department of Botany
Sikkim University,
Gangtok

इल, सामदुर, तादोंग -737102
क, सिक्किम, भारत
-03592-251212, 251415, 251656
फैक्स -251067
ाइट - www.cus.ac.in

6th Mile, Samdur,Tadong -737102
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Ph. 03592-251212, 251415, 251656
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"Bioprospecting for L-myo-inositol-1-phosphate synthase from cold resistant flora of the Sikkim Himalayas: partial purification and biochemical characterization of the enzyme from *Hippophae salicifolia* D. Don"

Submitted by **Raksha Mukhia** under the supervision of **Dr. Dhani Raj Chhetri**, Professor and Head, Department, Botany, School of Life Science, Sikkim University, Gangtok.

Raksha

(Raksha Mukhia)
Ph.D. Scholar

.....

(Prof. Dhani Raj Chhetri)
Ph.D. Supervisor

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Raksha Mukhia
Registration No.: 15/PhD/BOT/03
Department of Botany
School of Life Sciences
Sikkim University, Gangtok

Place: Gangtok
Date: 29/12/ 2023

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DEDICATION

This thesis is dedicated to my beloved parents for their unconditional love, support, encouragement, and inspiration throughout my pursuit.

Thank you for believing in me always.

CONTENTS

ABBREVIATIONS

A_2S	Ammonium Sulphate
CBB	Coomassie Brilliant Blue
cDNA	complementary DNA
DCPIP	2,6-Dichloro-phenolindophenol
DEAE	Diethylaminoethyl
DMSP	Dimethyl sulfoniopropionate
EDTA	Ethylenediamine tetraacetic Acid
Fv/Fm	variable fluorescence/maximum fluorescence
G-6-P	Glucose-6-phosphate
GM	Genetically Modified
Go1S	Galactinol Synthase
HSF	Heat Shock Factor
Hxl	Hexylagarose
I-1-P	L-myo-inositol-1-phosphate
Ins	Inositols
IUB	International Union of Biochemistry
IUPAC	International Union for Pure and Applied Chemistry
K_m	Michaelis constant
MDA	Malondialdehyde
ME	2-Mercaptoethanol
MI	Myo-inositol

M_r	Relative Molecular Mass
pCMB	p-chloromercuribenzoic acid
PEG	Polyethylene Glycol
PI	Phosphatidylinositol
PI_4P	Phosphatidylinositol-4-phosphate
RFO	Raffinose family of Oligosaccharide
ROS	Reactive Oxygen /species
SOD	Superoxide Dismutase
SS	Streptomycin Sulphate
TEMED	Tetra methylethylene diamine
Tm	Melting Temperature
V_{max}	Maximum Velocity
WT	Wild Type

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1. INTRODUCTION

Myo-Inositol (MI) is a sugar alcohol that is defined as a 6-carbon cyclohexane or hexitol that plays diverse roles in plant metabolism. It exists in free or conjugated form and performs versatile roles as signalling molecules, as components of reproductive units, in growth regulation, membrane biogenesis, hormone regulation, pathogen resistance, biosynthesis of pollen cell wall polysaccharides, phosphate storage in seeds, and stress adaptation in higher plants (Murashige & Skoog, 1962; Maiti & Loewus, 1978; Sekhar & Hokin, 1986; Loewus & Murthy, 2000; Stevenson *et al*., 2000; Chiu *et al.*, 2003; Majumder & Biswas, 2006; Michell,2008; Saxena *et al.*, 2013).

Throughout history, man has been attempting to comprehend and elucidate the complexity behind protein metabolism and its evolution. Over a century has passed since the discovery of enzymes. The Swedish chemist Jon Jakob Berzelius conducted some of the first studies, referring to their chemical activity as catalytic in 1835. However, it was not until 1926 that James B. Sumner of Cornell University succeeded in obtaining the first enzyme in purified form. From the jack bean, Sumner was able to separate and crystallize the urease enzyme (Bennett and Frieden, 1969; Holum, 1968). Since then, numerous investigations and enzymology-related discoveries have been made, *myo*-inositol phosphate synthase being one of them.

MI biosynthesis is dependent on two enzymes. First, NAD^+-dependent *myo*-inositol-1-phosphate synthase (MIPS; EC 5.5.1.4) that catalyses the cyclization of D-glucose-6-phosphate to L-*myo*-inositol-1-phosphate (I-1-P) and then inositol monophosphatase which dephosphorylates I-1-P to free *myo*-inositol (Loewus & Loewus, 1983; Loewus, 1990; Majumder,1997). Of these the former is the rate-limiting step in the production of *myo*-inositol. Beyond biosynthesis, the metabolic processing of MI makes more stereo-forms of inositol and its derivatives that mediates a variety of useful functions. Thus, MI

acts as a precursor for all inositol-containing compounds including phosphoinositides, inositol phosphates and cell wall polysaccharides (Biswas *et al.*, 1984; Loewus and Murthy, 2000). So far, the enzyme MIPS has been documented from archaea (Chen *et al.*, 2000); bacteria (Bachhawat and Mande, 1999; 2000; Yi *et al.*, 2020); protozoa (Lohia *et al.*, 1999); animals (Maeda and Eisenberg, 1980; Mauck *et al.*, 1980; Biswas *et al.*, 1981); humans (Adhikari and Majumder, 1988, Chhetri *et al.*, 2012) 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, 1981; Escamilla *et al.*, 1982; Dasgupta *et al.*, 1984; Suliman *et al.*, 2021); bryophytes (Chhetri *et al.*, 2006a, 2009, 2023); pteridophytes (Chhetri *et al.*, 2005, 2006b; Basak *et al.*, 2012); gymnosperm (Gumber *et al.*, 1984; Chhetri and Chiu, 2004) and angiosperm (Loewus and Loewus, 1971; Johnson and Sussex, 1995; Johnson and Wang, 1996; Ray Chaudhuri *et al.*, 1997; Kuserda, *et al.*, 2015).

Plants demonstrate a diverse range of response to abiotic stress that enable them to tolerate and survive adverse conditions (Cavatte *et al.*, 2012). Typically, plants deal with stress through a variety of mechanisms, including control of cellular osmotic pressure, detoxification of reactive oxygen species, preservation of membrane integrity, and protein stability (Bohnert and Jensen, 1996; Mechri *et al*., 2015; Per *et al.*, 2017). Accumulation of osmoprotectants shield cellular constituents from osmotic damage and studies have brought into limelight the role of numerous metabolites including cyclitols in various stress conditions. (Merchant *et al*., 2006; Tarczynski *et al.*, 1993; Kishor *et al.*, 1995; Holmström *et al.*, 1996; Hayashi *et al.*, 1997; Sheveleva *et al.*, 1997).

Inositol provides resistance to both abiotic and biotic stress conditions in plants (Taji *et al.*, 2006). MI is known to serve as an osmolyte or substitute for the production of compatible solutes (Nelson *et al.*, 1999). MI or their derivatives have been reported to

enhance tolerance against salinity, drought, osmotic, cold and high-temperature stress (Majee *et al.*, 2004; Joshi *et. al.*, 2013; Tan *et al.*, 2013; Conde *et al*., 2015; Khurana *et al*., 2017; Basu, 2019;). Thus, the enzyme MIPS which is the principal enzyme for the biosynthesis of MI is reputed to be a protagonist in the stress response in both prokaryotic and eukaryotic organisms (Chun *et al.*, 2003). It has been observed that MI is utilized in the synthesis of raffinose and galactinol under abiotic stress (Taji *et al.*, 2002). Often the involvement of inositol and its methylated derivatives such as pinitol, ononitol, sorbitol, etc are known to play a role in plant stress (Paul and Cockburn, 1989; Ishitani *et al.*, 1996; Sheveleva *et al.*, 1997; Shen *et al.*, 1999).

The floral diversity of the Sikkim Himalayan region in India is very well known. *Hippophae salicifolia* D. Don (*H. salicifolia*) is an important plant found in the high altitude of this region and is commonly known as Seabuckthorn (vernacular-Chuk, Achook, Lhala, Tarobo). This deciduous tree is a member of the family Elaeagnaceae in India and is exclusively distributed in patches in Sikkim, Jammu and Kashmir, Uttar Pradesh, and Himachal Pradesh (Singh, 1998). *H. salicifolia* thrives in Sikkim along riverbanks, torrential sides, vertical hills, and slopes, primarily on the south-east side of the Lachen and Lachung valley at an elevation between 2391 and 3111m (Basistha *et al.*, 2010). The classification of this species involves various taxonomic aspects that contribute to our understanding of its evolutionary relationships and ecological significance. The classification of this plant can be elucidated through its taxonomic hierarchy. Within the Plant Kingdom, this species is categorized under the division (or phylum) Magnoliophyta, class Magnoliopsida, order Rosales, and family Elaeagnaceae. It belongs to genus, *Hippophae*, and species *Hippophae salicifolia*. It is highly known for its medicinal value.

The plant is packed with high amounts of vitamins A, C, B1, B12, E, K, and polyphenols which account for its vast nutraceutical properties (Lu, 1992; Gupta *et. al*., 2011). Certain studies have established the superiority of *H. salicifolia* over other closely related plants in terms of bioactive components. It is highly effective in treating, gastric ulcers, high blood pressure, respiratory infections, lung problems, menstrual disorders, and heart and digestive issues (Ranjith *et al.,* 2006; Mingyu *et al.*, 1991). Additionally, its aqueous extracts demonstrated adaptogenic activity and may help to counteract the negative effects of hypoxia and cold (Sharma *et al.*, 2015). It is often referred to as a miracle plant due to its variable uses. (Tomar *et al.,* 2011). Apart from its adaptability to soil acidity and alkalinity (Lu, 1992), this plant is known to withstand freezing temperatures of as low as -43°C (Chettri *et al*., 2018). Despite all of its valuable properties, the plant has an ignored status both commercially and ecologically. Additionally, ignorance of the significance of *H. sailcifolia* on the part of the growers is contributing to the decline of its populations (Pant *et al*., 2014).

This plant is known to grow in challenging environments of high elevation under conditions of cold temperature and very less water availability (Gupta *et al.,* 2012). It is found in the rocky mountain areas of Sikkim Himalayas which is able to survive extremely low temperatures, high winds, and desiccation. Osmotic regulation, desiccation tolerance, and photo-inhibition tolerance are discovered to be some essential elements of cold adaptation of plants (Wei *et al.,* 2005). The physiological and biochemical activities of all plants are predominantly maintained by enzyme-driven reactions. As a result, studying enzymes becomes crucial in comprehending the basic metabolic processes. Production of inositol has previously been linked to instances of abiotic stress (Majee *et al.*, 2004, 2005). Thus, the prime enzyme for *myo*-inositol biosynthesis, L-*myo*-inositol-1-phosphate synthase (MIPS), is important to be investigated in order to understand the

relevance of *myo*-inositol. This makes the plant an apt model for studying MIPS enzyme in relation to the plants available in colder regions. Though the MIPS enzyme has been studied from more than 60 different evolutionarily diverse organisms (Majumder *et al.*, 2003), it has not been particularly studied in cold-resistant flora so far. Therefore, this research aims to bridge the lacuna.

The isolation, partial purification, and biochemical characterization of the enzyme, L-*myo*-Inositol-1-phosphate synthase from *Hippophae salicifolia* D. Don and its role in the cold stress tolerance along with the screening for the presence of MIPS found in other plants from three different altitudinal zone viz. alpine plants, *Primula denticulata* (Primulaceae), *Bistorta emodi* (Polygonaceae), *Pedicularis siphonantha* (Scrophulariaceae), temperate plants viz. *Argentina lineata* (Trevir.) Soják. (Rosaceae), *Koenigia mollis* (D.Don) T.M.Schust. & Reveal (Polygonaceae), *Hellenia speciosa* (J. Koenig) S.R. Dutta (Costaceae), and those from tropical regions viz. *Houttuynia cordata* (Saururaceae), *Euphorbia hirta* (Euphorbiacaea), and *Solanum viarum* (Solanaceae) of Sikkim Himalayas have been carried out in this research. These plants from different altitudinal zones were chosen to make a basic comparative screening of MIPs enzyme. Therefore, this work has been undertaken to investigate *myo*-inositol biosynthesis and partial purification of the enzyme followed by biochemical characterization of the same from *H. salicifolia* from Sikkim Himalayas. This will help us enhance our knowledge of cold stress tolerance in plants while enlightening us about the enzymology of hitherto unstudied MIPS from a cold-tolerant plant.

The research work presented here on the study of possible occurrence of MIPS among the mountain flora of Sikkim Himalaya followed by its purification and characterization from *Hippophae salicifolia* was carried out with the following specific objectives:

OBJECTIVES

1. To screen the activity of the enzymes involved in *myo*-inositol biosynthesis from some cold resistant plants of Sikkim.

2. To identify L-*myo*-Inositol -1-phosphate synthase from cold stressed *H. salicifolia* of Sikkim Himalayan region at the biochemical level.

3. Purification of the enzyme, L-*myo*-inositol-1-phosphate synthase from *H. salicifolia* and biochemical characterization of the same for its characterization.

4. To determine whether the L-*myo*-inositol-1-phosphate synthase protein from *H. salicifolia* plays any role in cold stress tolerance.

2. LITERATURE REVIEW

2.1. The Inositols

The most notable aspect of investigations over time is arguably the fact that stress responses in plants are universal. Changes in ROS scavenging and metabolic modifications that affect the redox state are all reactions that are common to all or nearly all plant species and are driven by the network of interactions between various signaling channels that are formed in a plant-specific manner (Farooq *et al.,* 2019). Diverse orders, families, and species have undergone evolutionary processes via a variety of pathways, thereby attaining protective mechanisms through the modification of their biochemical adaptations (Hochachka *et al.,* 2002). They are variations of glycine betaine, DMSP, ectoine, methyl-inositol, or amino acid accumulation. Inositol serves a crucial function in the control of a wide range of physiological characteristics, acting as indispensable constituents of the messenger molecules which are accountable for cell signal transduction. (Mato *et. al.,* 1987; Alberts *et al.,* 2002).

Inositols (Ins) are naturally occurring substances that are found in a wide range of plants and animals (Siracusa, 2022). Chemically, it is characterized as a cyclic carbohydrate having six hydroxyl groups one on each of the carbon atom of the hexose ring forming a cyclohexanehexol. These compounds tend to have a structure very identical to the cyclic form of monosaccharides like glucose and are termed sugar alcohols accordingly (Ehlers *et al*., 2019). Considering the detailed structural arrangement constituting six chiral centers, the occurrence of 64 possible stereoisomers is possible only theoretically but the actual isomers are only nine as represented in Figure 2.1.

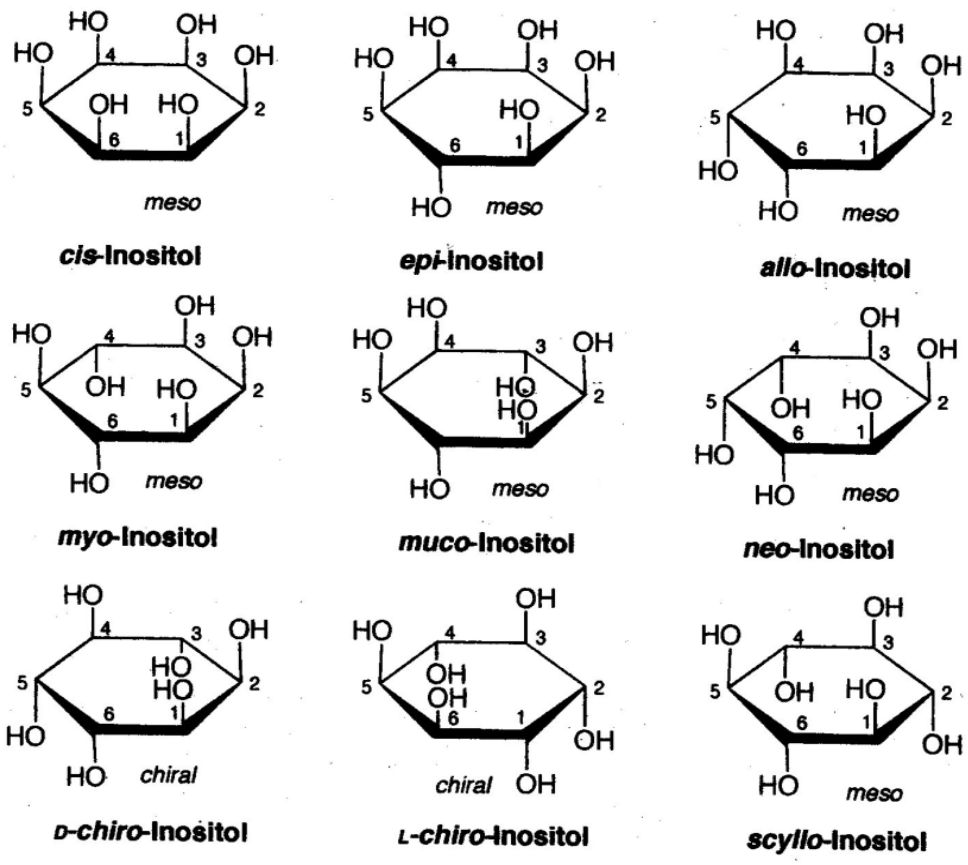


Figure 2.1. Structures of nine stereoisomers of inositol

Natural isomers of inositol are *myo*-, D-*chiro*-, L-*chiro*-, *muco*-, *scyllo*-, and *neo*-, while synthetic molecules constitutes *allo*-, *cis*-, and *epi*- (Pani *et al.,* 2020). Amongst these, *myo*-inositol (MI) is the earliest and the most common form, followed by *chiro*-inositol (Ehler *et al.,* 2019; Ruiz-Aceituno *et al.,* 2014; Li *et al.,* 2021). MI is widely distributed inositol in plants but is present in all living organisms. In 1850, Scherer isolated it from muscle extracts and termed it 'inositol' that implies muscle in Greek (Posternak, 1965). Furthermore, inositol derivatives are represented by inositol methyl ester viz., bornesitol, ononitol, and sequoytol are monomethyl esters of MI, while viscumitol and dombonitol

are dimethyl esters of MI (Ford, 1985; Ritcher, 1992). D-pinitol, quebrachitol, and pinpollitol are methyl- and dimethyl-esters of D-*chiro*-inositol whereas brahol is a monomethyl ether of *allo*-inositol (Owczarczyk-Saczonek *et al.,* 2018; Ahmad *et al.,* 1998). The structure of the aforementioned methyl ester compounds are shown in Figure 2.2. Further, inositol is a representative of other derivatives as well.

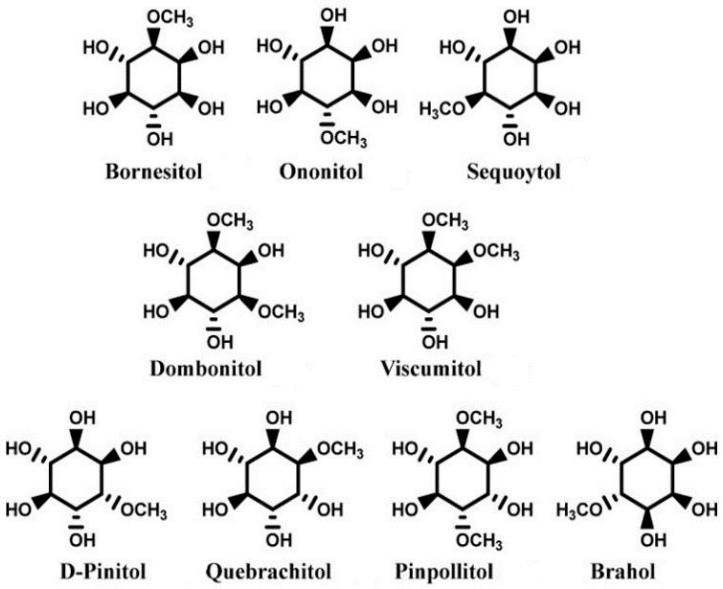


Figure 2.2 Methyl ether derivative of inositols

2.2. Stereochemistry

Stereochemical isomerism of inositol is very sophisticated that includes, chiral, prochiral, and conformational forms (Parthasarathy and Eisenberg, 1986, 1991; Posternak, 1965). The nine stereoisomeric forms of the inositol is an outcome of the arrangement of the six secondary hydroxyl groups on the cyclohexane ring in one of two directions: axial or equatorial (Figure 2.1). The *myo*-isomer of inositol has one axial hydroxyl group, the *epi-*, *chiro-*, and *neo*-isomers have two, and the *allo-, cis-,* and *muco*-isomers have three axial

hydroxyl groups. Of the nine stereoisomers of inositol (Figure 2.1), six are non-chiral isomers (*scyllo-, myo-, epi-, neo-, cis-,* and *muco*-isomers) as the molecules containing them have one or more planes of symmetry, thus making them meso compounds. In addition D- and L-*chiro* isomers are mirror images of one another. The *allo*-isomer is the conformational isomer as shown in fig.2.3 (i) and exists as its enantiomers 2.3 (ii) and 2.3 (iii). *Allo*-inositol is chiral as it is 50/50 mixture of the two enantiomers at room temperature, nevertheless the chemical is optically inactive.

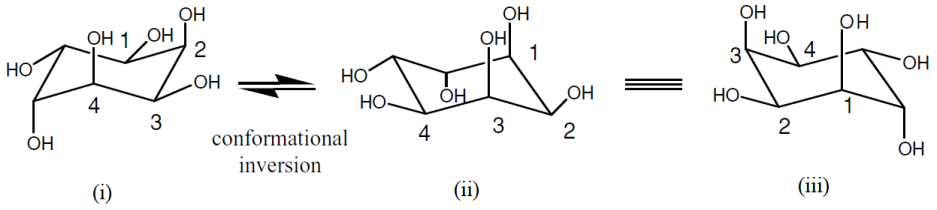


Fig. 2.3. *Allo*- inositol: Conformational isomer and enantiomers

MI can be divided into two mirror-image halves (Figure 2.4.i) – a perpendicular plane passes through C-2 and C-5 and divides the molecule into non-superimposable mirror images. C-1 is enantiopic to C-3 and C-6 is enantiotopic to C-4, so the product of a substitution at C-1 (Fig. 2.4.ii) will be the enantiomer of the same reaction at C-3 (Fig. 2.4.iii). Though the two halves of MI are stereochemically non-equivalent, they are chemically equivalent to an achiral molecule. But, a chiral molecule, such as an enzyme, can readily differentiate between C-1 and C-3 as well as C-4 and C-6 and preferentially react with one enantiotopic carbon or another. For instance, MI kinase phosphorylates MI specifically at the D-3 position and yields the chiral product, 1D-*myo*-inositol-3-monophosphate (Fig. 2.4. iii) (Deitz and Albersheim, 1965; Loewus *et al.,* 1982). This is the same isomer produced from glucose-6-phosphate by *myo*-inositol phosphate synthase (Loewus, 1990). The only route by which the enantiomer 1D-*myo*-inositol-1-

monophosphate (Fig. 2.4. ii) is biosynthesized is through dephosphorylation of 1D-*myo*-inositol-1,4-bisphosphate by phosphatase. Enzymes react selectively with one enantiomer or another, thus the spatial differences amongst enantiotopic carbons resulting in significant biological consequences.

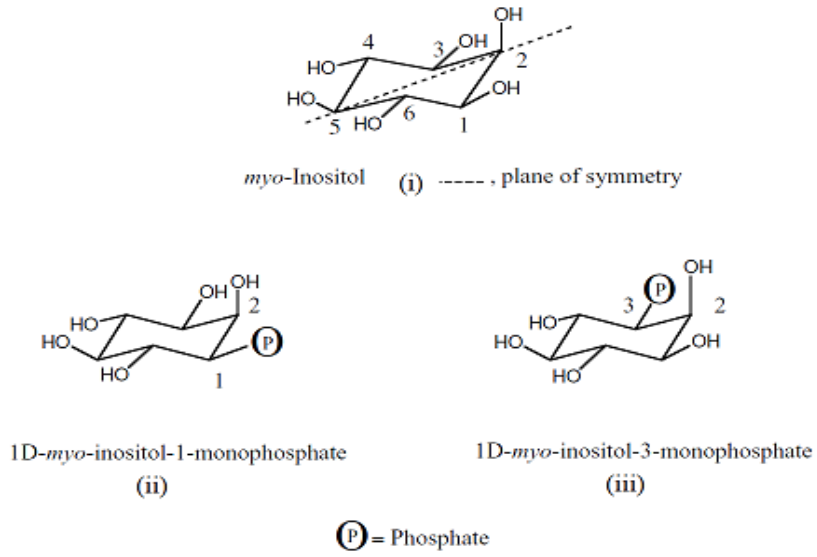


Figure 2.4. *Myo*-inositol and phosphorylated derivatives

2.3. Nomenclature

As already discussed, the *myo*-isomer of inositol is the most profuse form in nature and occupies an exclusive place in inositol metabolism because this is the only isomer synthesized *de novo* from D-glucose-6-phosphate. Apart from this, all other isomers are derived from MI (Loewus, 1990; Loewus and Murthy, 2000). In the beginning of 1900s, MI was referred to as meso-inositol, likely due to its lack of optical activity and inability to be separated into optical isomers (Posternak, 1965). But, six other isomers also did not exhibit optical activity so the name appeared to be unsuitable. In the year 1954, the appellation *myo*-inositol was established – a name that was not appropriately coined due to its pleonastic nature with both *myo* and inositol meaning muscle. Yet, the name continued.

For sometime, confusion loomed over the names of inositol and its derivatives. This was partially a result of adjustments made to the numbering regulations by the IUPAC (International Union of Pure and Applied Chemistry, 1976) and IUB (International Union of Biochemistry), and because of the stereochemical complexity of all cyclitols, along with inositols (1989). MI that was already known as a meso compound having mirror image halves. IUPAC came up with the recommendations I-4 (IUPAC, 1976), numbers and the direction of numbering in inositols were then arranged with reference to the spatial relations and nature of substituents on the ring. In MI, substituents are assigned to two sets, substituents above the ring were assigned to one set and those below to another. The lowest number is assigned to the set with more substituents. For, MI (Figure 2.5. iii), there are four hydroxyls (C-1, C-2, C-3, and C-5) in one set and two (C-4 and C-6) in the other (Murthy, 1996; Parthasarathy and Eisenberg, 1986; IUB, 1989).

Carbon-1 could be assigned to either of two enantiotopic carbons (i) and (ii) or (iii) and (iv) (Figure 2.5). IUPAC recommended in 1976 that if the molecule is viewed in the vertical (Fischer – Tollens) projection with C-1 at the top with C-2 and C-3 on the front edge of the ring (Fig. 2.5), the configuration is assigned D if the hydroxyl group or other substituent at the lowest- numbered chiral center projects to the right (i) and L if it projects to the left (ii). Usually, a horizontal projection is used and the structure is drawn (iii) and (iv), so that if the substituent on the lowest-numbered asymmetric carbon is above the plane of the ring and the numbering is counterclockwise, the configuration is assigned D (iii), and if clockwise, the configuration is L (iv). Thus, in substituted inositol phosphates (v) and (vi), the initial point could be either of the enantiotopic carbons. The number 1 precedes the D or L to direct C-1 as the chiral center considered to define the configuration.

Of the two potential outcomes, the recommendation put forth by IUPAC - IUB in 1976 stated that the designation of L should be used for meso compounds, particularly MI. The IUPAC (1976) rules required the compound (Fig. 2.6. i) to be tagged as 1D-*myo*-inositol-1,3,4,5-tetrakisphosphate, to facilitate the attachment of the substituent to carbons with lower numbers, whereas (Fig. 2.6.ii) should be labeled 1L-*myo*-inositol-1,5,6-trisphosphate. Therefore, the dephosphorylation reaction (Figure 2.6) shows that 1D-*myo*-inositol-1,3,4,5-tetrakisphoshphate is dephosphorylated to 1L-*myo*-inositol-1,5,6-trisphosphate. On the other hand, 1D-*myo*-inositol-1,3,4,5-tetrakisphosphate is dephosphorylated to 1D-*myo*inositol-3,4,5-trisphosphate, that indicates dephosphorylation occurring at C-1. IUB (1989) recommended that either the 1D or the 1L numbering be allowed so long as the prefix 1D or 1L is specified. Henceforth, the author could use either numbering depending on the relationships that were being stressed. It was further recommended that the symbol "Ins" be taken to mean *myo*-inositol with numbering proceeding counterclockwise. These recommendations were in relation to the big number of MI phosphates of 1D configuration that were being discovered as hydrolytic products of phosphoinositides (IUB, 1989). To retain the nomenclature of MI phosphates, it is imperative to recall the numerical order in which., Agranoff (1978) suggested the similarity of the chair conformation to a turtle (Figure 4) and recommended a mnemonic. The head of the turtle represented the C-2 axial hydroxyl and the four limbs and tail represented the five equatorial groups. The right-hand limb was labelled the D-1 position and the proceeding counterclockwise, the head is D-2, etc. The left front limb was D-3. On the other hand, if the L stereospecific numbering was implied then the left front limb was L-1 and the numbering proceeded clockwise as given in (iv) (Figure 2.5). In phosphoinositides, the right limb carried the diacylglycerol group. MI derivatives that are currently designated D configuration were assigned L configuration and the other way

around in the literature published before 1968. Circa in 1960, employed different nomenclature in the seminal work conducted to determine the chirality of *myo*-inositol monophosphate, the isomer (Fig. 2.4. ii) which resulted from hydrolysis of D-galactinol and was labelled as D-*myo*-inositol-1-monophosphate (Ballou and Pizer, 1960). The *myo*-inositol monophosphate isomer (Fig. 2.4.ii), derived from the hydrolysis of phosphatidylinositides was allocated L as it showed optical rotation opposite to that of *myo*-inositol-1-monophosphate (Fig. 2.4.iii) allocated as D (Ballou and Pizer, 1960). In 1968, the rule was changed so that the lowest numbered stereo-genic carbon now specifies configuration.

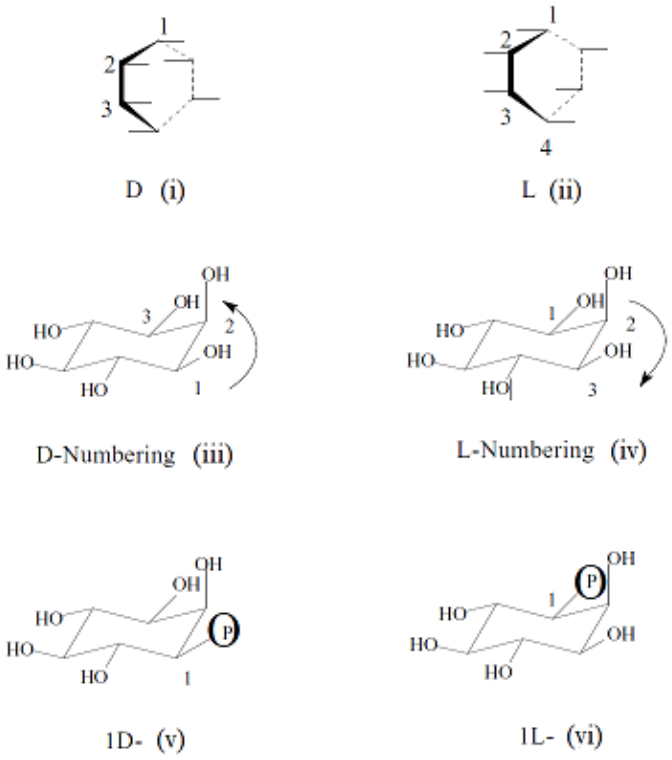
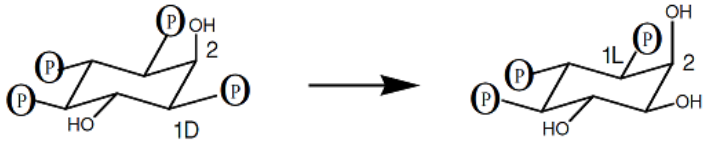


Figure 2.5. Nomenclature of *myo*-inositol and its derivatives



1D-*myo*-inositol-1,3,4,5-tetrakisphosphate

(i)

1L-*myo*-inositol-1,3,5,6-tetrakisphosphate

1L-*myo*-inositol-1,5,6-trisphosphate

(ii)

1D-*myo*-inositol-3,4,5-trisphosphate

Figure 2.6. Hydrolysis of *myo*-inositol phosphate alternative numbering

2.4. *Myo*-inositol biosynthesis

The most notable biochemical phenomenon taking place during the metabolism of MI is its biosynthesis by conversion of glucose-6-phosphate to the *myo*-inositol-1-phosphate through the multistep *myo*-inositol-1-phosphate synthase reaction (Geiger and Jin, 2006).

D-glucose-6-phosphate is irreversibly isomerized to L-*myo*-inositol-1-phosphate by a NAD-dependent oxido-reductase, *myo*-inositol-1-phosphate synthase (MIPS).

The enzymatic reaction produces free *myo*-inositol when L-*myo*-inositol-1-phosphate undergoes hydrolysis through the activity of a specific phosphatase that is dependent on Mg^{2+}. For this hydrolysis reaction, the enzyme L-*myo*-inositol-1-phosphate-phosphatase is accountable. The enzyme, L-*myo*-inositol-1-phosphate synthase (EC: 5.5.1.4) plays a crucial role in the biosynthesis of all MI containing compounds, as it is responsible for catalyzing the first and the rate limiting step in the process (Seelan e*t al.,* 2009). D-glucose-6-phosphate (G-6-P) is converted to L-*myo*-inositol-1-phosphate (I-1-P) by it. This step is followed by dephosphorylation of l-*myo*-inositol-1-phosphate to MI, by *myo*-inositol monophosphatase (IMP; EC 3.1.3.25) (Sherman *et al.,* 1981; Loewus and Loewus, 1983; Torabinejad and Gillaspy, 2006; Torabinejad *et al.,* 2009). These two reactions collectively is known as the Loewus pathway, that was first known to be studied

in plants. Thus, overall, cyclization, dephosphorylation and epimerization are concerned with the conversion of D-glucose to MI. The enzyme originally was termed cyclase or cycloaldolase but now is widely named as *myo*-inositol-1-phosphate synthase (EC 5.5.1.4).

MI is synthesized from the molecule, glucose-6-phosphate (Eisenberg, 1967) by cyclic synthesis (Agranoff *et al.,* 1958; Paulus and Kennedy, 1960) and by the hydrolysis of phosphatidylinositol. In the synthetic and cyclic pathways, two different forms of compounds are formed, the L-enantiomer and D-enantiomer of MI respectively though the intermediate, *myo*-inositol-1-phosphate is the similar in both pathways (Parthasarathy and Eisesnberg, 1986). *Myo*-inositol-1-phosphatase hydrolyses both these compounds (Eisenberg, 1967) producing MI in the process. Thus *myo*-inositol-1-phosphatase (EC: 3.1.3.25) is extremely important in the biosynthesis of free MI from inositol-1-phosphate (Nigou and Besra, 2002).

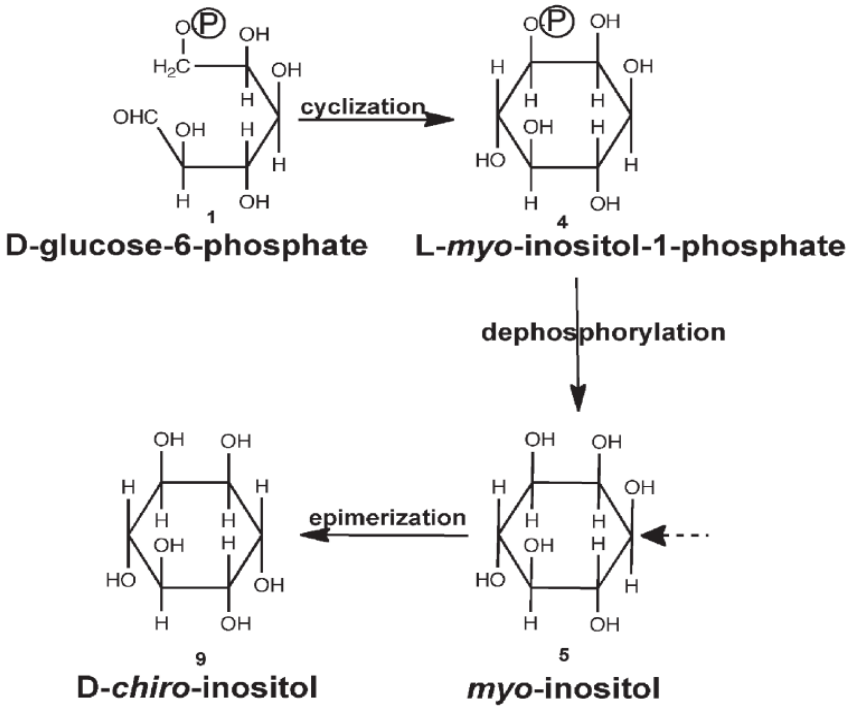


Figure.2.7 Conversion of D-glucose-6-phosphate to *myo*-inositol-1-phosphate by cyclization catalysed by *myo*-inositol-1-phosphate synthase. *Myo*-inositol-1-phosphate is then dephosphorylated to *myo*-inositol by *myo*-nositol-1-phosphate phosphatase. *Myo*-inositol may be converted by epimerization to D-chiro-inositol.

2.5. Purification and characterization of MIPS

The enzyme MIPS is an essentiality when it comes to the *de novo* synthesis of MI, necessary for the growth and development of all living organisms. The enzyme has been reported from remarkable number of life forms, including bacteria, plants and animals. Among plants, this enzyme has been purified and characterized from a numerous species.

In 1971, Loewus and Loewus, purified and characterized the enzyme, L-*myo*-inositol-1-phosphate synthase from *Acer pseudoplatanus* and its M_r was calculated to be about 150 kDa. The enzyme isolated from *Lemna gibba* had almost same M_r of about 135 kDa (Ogunyemi *et al.,* 1978). However, the enzyme purified and characterized from *Saccharomyces cerevisiae* through conventional purification method exhibited a molecular weight of 240 kDa along with a subunit molecular weight of approximately 62 kDa (Donahue and Henry, 1981), directing towards a tetrameric structure of the enzyme.

MIPS was isolated and partially purified from the alga, *Euglena gracilis* (Gupta *et al.,* 1984). The enzyme exhibited total inhibition by $(NH_4)_2\ SO_4\ SO_4^{2+}$, a pH optimum of 7.5 and the K_m for the substrate, G-6-P was recorded to be 2.1 mM. It was also partially purified from the macro-alga, *Enteromorpha intestinalis* (L.) Nees, to about 41-fold over

the homogenate. The *Mr* of the native enzyme was calculated to be about 164 kDa and, the temperature and pH optima recorded to be 35°C and 7.5 respectively. Km value was 0.1761 mM for the substrate, G-6-P and 0.1695 mM for the coenzyme β-NAD. The monovalent cation, K^+ was found to have a enhancing influence while Li^+ was inhibited strongly. Amongst all the divalent cations, Ca^{2+} showed a slight stimulatory effect whereas salts of Cu^{2+}, Cd^{2+} and Hg^{2+} were found to be strong inhibitors, Hg^{2+} being the potent among the inhibitors (Basu, *et al*., 2018).

From amongst gymnosperm, the enzyme was purified from the pollen grains of *Pinus ponderosa* (Gumber *et al*., 1984). The gymnospermic MIPS recorded a maximum activity at pH 7.25 to 7.75. The K_m for G-6-P was 0.33 mM. Purification of this enzyme was also done from another gymnosperm, *Taxus baccata* L., where the enzyme was found to be highly stimulated by NH_4^+, the V_{max} and K_m of the enzyme for its substrate i.e., G-6-P was found to be 2.95mM and 1.05 mM respectively (Chhetri and Chiu, 2004). From the angiosperms, *Vigna radiata* seedlings were used for the purification of the enzyme and it exhibited an 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 the enzyme from *Euglena gracilis, Oryza sativa* and *Vigna radiata* have also been isolated, purified and characterized. The vital difference between the native holoenzymes from these sources is their molecular mass, that might have been caused by the association of either three or four equal subunits that constitution the holoenzyme (RayChaudhuri *et al.,* 1997).

This enzyme has been partially purified from *Diplopterygium glaucum* (Thunb.) Nakai, a pteridophyte from the hills of the Darjeeling Himalayas. The enzyme showed a recovery rate of 13.5% when it was purified to roughly 81-fold. The ideal pH range was recorded

to be 7.0 - 7.5, and the optimum temperature recorded was 30 °C. The enzyme activity was considerably inhibited by Li^{+}, Zn^{2+}, and Hg^{2+} and stimulated by NH_4^{+}, EDTA, pCMB, and a few substrate isomers like glucose-1-phosphate and fructose-6-phosphate was also seen to inhibit the activity. The molecular weight of MIPS was found to be approximately 171 kDa in this pteridophyte (Chhetri *et al.,* 2006b).

Among bryophytes, it was partially purified from *Marchantia nepalensis* to about 33-fold with approximately 21% recovery. The enzyme activity was observed to be slightly stimulated by Mg^{2+} and Ca^{2+}, significantly stimulated by NH_4^{+}, slightly inhibited by Mn^{2+} and remarkably inhibited by Cu^{2+}, Zn^{2+} and Hg^{2+}. The K_m values for the substrate and co-enzyme were found to be 0.42 and 0.05 mM, respectively. The *Vmax* values were 2.1 and 1.11 mM for G-6-P and NAD^{+}, respectively (Chettri *et al.,* 2006a).

Since the enzyme is considered to be essential for the survival of mosses, like any other group of plants, hence, the enzyme MIPS was isolated and characterized from *Sphagnum junghuhnianum* that was found to record an optimum pH at 7.0 and the temperature maxima at 30ºC. The MIPS activity was seen to be remarkably stimulated by NH_4^{+} and Ca^{2+} and K^{+} also stimulated the enzyme to some extent. The enzyme was also inhibited by Zn^{2+}, Cd^{2+}, Mn^{2+} and Hg^{2+} ultimately showed more than 80% inhibition. The K_m values for G-6-P and NAD^{+} were found to be as 1.81 mM and 0.25 mM respectively while the V_{max} values were 1.42 mM and 1.12 mM for the substrate and co-enzyme respectively (Chhetri *et al.,* 2023).

2.6. Distribution of MIPS, MI and its isomers in plants

Wide distribution of inositol is known in the the plant kingdom. MI is the most widely distributed inositol in plants and probably occurs in all living organisms (Majumder *et. al.,* 1997). Phylogenetic, L-*myo*-inositol-1-phosphate has been distinguished from a

number of genera across the divisions. The MIPS reaction has been known from archaea (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, Chhetri *et al*., 2012) and plants. Among plants MIPS occurence has been illustrated and characterized from algae (Dasgupta *et al.,* 1984; RayChaudhuri *et al.,* 1997); fungi (Donahue and Henry, 1981; Escamilla et al. 1982; Gupta *et al*., 1984); bryophytes (Chhetri *et al.,* 2006a, 2009); pteridophytes (Chhetri *et al.,* 2005, 2006b); gymnosperm (Gumber *et al.,* 1984; Chhetri and Chiu, 2004) and angiosperm (Loewus and Loewus, 1971; Johnson and Sussex, 1995; Johnson and Wang, 1996; Ray Chaudhuri *et al.,* 1997) [Table 2.1].

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

Origin	**Source**	**Tissue**	**Reported by**
ALGAE	*Euglena gracilis*	Whole plant	Gupta *et al.*, (1984)
	Chlorella vulgaris	Whole plant	Gupta et al., (1984)
	Spirogyra maxima	Whole plant	Gupta *et al.*, (1984)
	Microspora willeana	Whole plant	Gupta *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	Escamilla *et al.*, (1982)
	Polyporus anthelminticus	Whole plant	Gupta *et al.*, (1984)
	Ganoderma lucidum	Whole plant	Gupta *et al*., (1984)
	Irpex flavus	Whole plant	Gupta *et al*., (1984)
	Agaricus compestris	Whole plant	Gupta *et al*., (1984)
	Schizophyllum commune	Whole plant	Gupta *et al*., (1984)
	Lentinus subnudus	Whole plant	Gupta *et al*., (1984)
	Scleroderma sp.	Whole plant	Gupta *et al*., (1984)

	Cryptococcus neoformans	Whole plant	Molina *et al*., (1999)
BRYOPHYTES	*Lunulariacruciata*	Whole plant,	Gupta *et al*., (1984)
	Lunularia cruciata	Reproductive	Chhetri *et al.,* (2009)
	Targigonia sp.	thallus	Dasgupta *et al*, (1984)
	Marchantia polymorpha	Whole plant	Gupta *et al*., (1984)
	Dumortiera sp.	Sex organs	Gupta *et al*., (1984)
	Marchantia nepalensis	Whole plant	Chhetri *et al*., (2006)
	Brachymenium bryoides	Reproductive thallus	
	Sphagnum	Whole plant	Yonzone *et al.,* (2018)
	junghuhnianum	Whole plant	Chhetri *et al*., (2023)
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 (1973)
	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	Leaves	Loewus & Loewus (1971)
	officinalis	Leaves	Loewus & Loewus (1971)
	Petroselinum crispum	Whole plant	Loewus & Loewus (1971)
	Lemna perpusilla	Leaves	Loewus & Loewus (1971)
	Salvia officinalis	Leaves	Chhetri *et al.,* 2008
	Swertia bimaculata		

2.7. Inositol isomers and its derivatives

MI have been reported from several medicinal plants and herbs (*Taraxacum officinale, Laurus nobilis, Sambucus nigra, Salvia officinalis, Chamomilla recutita, Hypericum perforatum, Mentha piperita*), spices *(Curcuma longa, Trigonella foenum-graecum, Zingiber officinale, Capsicuum annuum*) and vegetables (*Daucus carota*, *Lactuca sativa*, *Brassica oleracea, Solanum tuberosum*). *Allo*-inositol, ononitol and *scyllo*-inositols were also reported from different species (Ratiu *et al.,* 2019, Al-Suod *et al.,* 2018). D-Chiro-inositol, *scyllo*-inositol were detected in few vegetables belonging to the families of Asteraceae (artichoke, chicory, oak leaf lettuce, lettuce endive, escarole, iceberg lettuce), Amarantaceae (spinach and beetroot), Amarylidaceae (onion), Brassicaceae (radish and cabbage), Dioscoreaceae (purple yam), and Solanaceae (eggplant) (Hernández-Hernández, *et al.,* 2011).

Among different families, Fabceae was found to be particularly rich in inositols: free inositols (*myo*- and *chiro*-) and methyl esters of inositols have been detected in edible legume seeds, together with galactosyl-inositols (galactopinitols, galactosyl-ononitol, fagopyritols). MI has been isolated from some of the most common members of the Fabaceae family (*Vigna unguiculata*, *Ceratonia siliqua, Cicer arietinum L., Lathyrus sativus, Lens culinaris and Glycine max)* chiefly in *C. arietinum* the content was in considerable amounts (Ruiz-Aceituno. *et al.,* 2017). D-*Chiro*-inositol was revealed to exist at significantly low concentrations in nearly all the species investigated, with an exception of black-eyed and grass peas. Moreover, aside from carob pods, this particular metabolite was also detected in soybeans, chickpeas, and lentils. Also, black-eyed peas were found to contain another methyl-inositol that was identified as ononitol (Siracusa *et al.,* 2022).

Some apparently peculiar source of MI isomers and derivatives includes chamomile flowers for D-pinitol, *chiro*-inositol, *neo*-inositol, D-bornesitol, ononitol, *scyllo*-inositol, *myo*-inositol. (Al-Suod *et al.,* 2018). Needles of *Pinus* (Pinaceae) and *Juniperus* (Cupressaceae) for *myo*-inositol, D-pinitol, sequoyitol (Sarvin *et al.,* 2018), *Glycirrhiza glabra* (Fabaceae) leaves (Siracusa *et al.,* 2022; Molonia *et al.,* 2022) for different inositols, leaves from the medicinal plant *Hancornia speciosa* Gomes (Apocynaceae) (bornesitol) (Moreira *et al.,* 2019) and blue tansy (Boraginaceae) for *allo*-inositol, *scyllo*-inositol, *myo*-inositol (Al-Suod *et al.,* 2018)

The nutritional properties accounts for the presence of inositols in two different types of bean viz., *Vigna unguiculata* (cowpea) and *Phaseolus vulgaris* (common bean) that showed an increase in MI levels in both species when compared to quiescent seeds (Ribeiro *et al.,* 2011). Another member of the Fabaceae family, *Medicago sativa,* showed the presence of several inositols. Different morphological parts this plant (leaves, stems, flowers and roots) exhibited a particularly preferred accumulation of D-pinitol, over the other inositols. Their leaves were also found to be the only part of the plant containing *scyllo*-inositol. Nevertheless, quantitatively, the roots of *Medicago* were identified as the richest source of inositols of the whole plant (Siracusa *et al.,* 2022).

Table 2.2: Different Inositol isomer and their derivatives occurring in various plant species.

Family	**Species**	**Inositols**	**Refs.**
Adoxaceae	*Sambucus nigra*	D-pinitol,*allo*-inositol, D-*chiro*-inositol, ononitol, bornesitol,*scyllo*-inositol,*myo*-inositol	Ratiu *et. al.,* 2019
Agaricaceae	*Agaricus bisporus*	D-pinitol,*allo*-inositol, D-*chiro*-inositol,	Ratiu *et. al.,* 2019

		bornesitol,*scyllo*-inositol,*myo*-inositol	
Amaranthaceae	*Spinacia olearia*	*Myo*-inositol	Hernández *et.al.*,2011
	Beta vulgaris	D-*chiro*-inositol, bornesitol, *myo*-inositol	Hernández *et.al.*,2011
Amaryllidaceae	*Allium cepa*	D-*chiro*-inositol, bornesitol, *myo*-inositol	Hernández *et.al.*,2011
	Allium sativum	*Allo*-inositol, ononitol, *myo*-inositol	Ratiu *et. al.*, 2019
	Allium ursinum	D-pinitol, D-*chiro*-inositol, ononitol, *scyllo*-inositol,*myo*-inositol	Ratiu *et. al*., 2019
Apiaceae	*Anethum graveolens*	ononitol, bornesitol, *scyllo*-inositol, *myo*-inositol	Ratiu *et. al*., 2019
	Carum carvi	D-pinitol, D-*chiro*-inositol, bornesitol, *scyllo*-inosito, *myo*-inositol	Ratiu *et. al.*, 2019
	Daucus carota	D-*chiro*-inositol, bornesitol, *scyllo*-inositol, *myo*-inositol	Ratiu *et. al*., 2019
	Petroselinum crispum	D-pinitol, *allo*-inositol, D-*chiro*-inositol, bornesitol, *scyllo*-inositol, *myo*-inositol	Ratiu *et. al*., 2019
Apocynaceae	*Hancornia speciosa*	Bornesitol	Moreira. *et. al.*, 2019
Asteraceae	*Calendula anthodium*	D-pinitol, D-*chiro*-inositol, bornesitol,*scyllo*-inositol,*myo*-inositol	Ratiu *et. al*., 2019
	Cichorium intybus	D-*chiro*-inositol, *scyllo*-inositol,myo-inositol	Hernández *et al.*,2011
	Cichorium endivia	D-*chiro*-inositol, *scyllo*-inositol,*myo*-inositol	Hernández *et al.*,2011

	Cichorium endivia var. latifolia	D-*chiro*-inositol, *myo*-inositol	Hernández *et al.*,2011
	Cynara cardunculus	D-*chiro*-inositol, *scyllo*-inositol, *myo*-inositol	Hernández *et al.*,2011
	Lactuca sativa	D-*chiro*-inositol, *myo*-inositol	Hernández *et al.*,2011
	Matricaria chamomila	D-pinitol, *chiro*-inositol, bornesitol, *scyllo*-inositol, *myo*-inositol	Al-Suod, *et al.*, 2019.
	Solidago virgaurea	D-pinitol, D-*chiro*-inositol, ononitol, bornesitol, *scyllo*-inositol, *myo*-inositol	Ratiu *et al.*, 2019
	Tanacetum officinale	*Myo*-inositol	Ratiu *et al*., 2019
Boraginaceae	*Phacelia tanacetifolia*	*Allo*-inositol, *scyllo*-inositol, *myo*-inositol	Al-Suod *et al.*, 2018
Brassicaceae	*Brassica oleracea*	D-pinitol, D-*chiro*-inositol, bornesitol, *myo*-inositol, ononitol	Hernández *et al.*,2011
	Camelina sativa	D-pinitol, D-*chiro*-inositol, bornesitol, *myo*-inositol	Ratiu *et al.*, 2019
	Raphanus raphanistrum	*Myo*-inositol	Hernández *et al.*,2011
Convolvulaceae	*Ipomoea batatas*	D-pinitol, D-*chiro*-inositol, bornesitol, *scyllo*-inositol, *myo*-inositol	Ratiu *et al.*, 2019
Cupressaceae	*Juniperus communis*	D-pinitol, sequoytol	Sarvin, *et al.*, 2018
Dioscoreaceae	*Dioscorea alata*	*Scyllo*-inositol, *myo*-inositol	Hernández *et al.*,2011
Ericaceae	*Vaccinium myrtillus*	*Allo*-inositol, D-*chiro*-inositol, ononitol, bornesitol, *myo*-inositol	Ratiu *et al.*, 2019

Fabaceae	*Arachia hypogea*	D-pinitol, D-*chiro*-inositol, *myo*-inositol	Ratiu *et al*., 2019
	Ceratonia silique	D-pinitol, *allo*-inositol, D-*chiro*-inositol, bornesitol, *scyllo*-inositol, *myo*-inositol	Ratiu *et al.*, 2019; Ruiz-Aceituno *et al.*, 2013
	Cicer arietinum	D-pinitol, D-*chiro*-inositol, *myo*-inositol, galactosyl-inositol, galactosyl-pinitol, ciceritol	Ruiz-Aceituno *et al.*, 2013; Zuluaga *et al.* 2020
	Erythrina edulis	D-*chiro*-inositol, *myo*-inositol	Zuluaga *et al.* 2020
Polygonaceae	*Fagopyrum esculentum*	D-*chiro*-inositol, *myo*-inositol, galactosyl-inositol, fagopyritol	Ruiz-Aceituno et.al. 2013
Fabaceae	*Glycine max*	D-pinitol, D-*chiro*-inositol, *myo*-inositol, fagopyritol, galactosyl-pinitol	Ruiz-Aceituno *et al.*, 2013; Zuluaga *et al.* 2020; Streeter *et al.*, 2001
	Glycyrrhiza glabra	D-pinitol	Siracusa *et al.*, 2022
Fabaceae	*Latirus sativus*	Bornesitol, *myo*-inositol, galactosyl-inositol	Ruiz-Aceituno *et al.*, 2013
	Lens culinaris	D-pinitol, D-*chiro*-inositol, bornesitol, *myo*-inositol, galactosyl-inositol,	Ruiz-Aceituno *et al.*, 2013; Foti *et al.*, 2021
	Lupinus perennis	D-pinitol, *allo*-inositol, D-*chiro*-inositol, bornesitol, *myo*-inositol	Ratiu *et al.*, 2019
	Medicago sativa	D-pinitol, D-*chiro*-inositol, ononitol, *scyllo*-inositol,*myo*-inositol	Ratiu *et al.*, 2019

Fabaceae	*Phaseolus polyanthus*	*Myo*-inositol	Zuluaga *et al.* 2020
	Phaseolus vilgaris	*Myo*-inositol	Zuluaga *et al.* 2020
	Pisum sativum	*Myo*-inositol	Zuluaga *et al.* 2020
	Vigna unguiculata	ononitol, *myo*-inositol, galactosyl-inositol, galactosyl-ononitol	Ruiz-Aceituno *et al.*, 2013
Boraginaceae	*Phacelia tanacetifolia*	D-pinitol, allo-inositol, D-*chiro*-inositol, bornesitol, *Scyllo*-inositol,*myo*-inositol	Ratiu *et al.*, 2019
Hypericaceae	*Hypericum perforatum*	D-*chiro*-inositol, bornesitol, *scyllo*-inositol,*myo*-inositol	Ratiu *et al.*, 2019
Lamiaceae	*Mentha piperita*	D-pinitol, D-*chiro*-inositol, ononitol, bornesitol, *myo*-inositol	Ratiu *et al.*, 2019
	Salvia officinalis	D-pinitol, D-*chiro*-inositol, bornesitol, *scyllo*-inositol,*myo*-inositol	Ratiu *et al.*, 2019
Lauraceae	*Cinnamomum verum*	*Allo*-inositol, D-*chiro*-inositol, *scyllo*-inositol, *myo*-inositol	Ratiu *et al.*, 2019
	Laurus nobilis	D-pinitol, bornesitol, *scyllo*-inositol, *myo*-inositol	Ratiu *et al.*, 2019
Myristicaceae	*Myristica fragrans*	D-*chiro*-inositol, *myo*-inositol	Ratiu *et al.*, 2019
Myrtaceae	*Eugenia caryophyllus*	*Allo*-inositol, D-*chiro*-inositol, ononitol, bornesitol, *scyllo*-inositol, *myo*-inositol	Ratiu *et al.*, 2019
Pinaceae	*Abies sibirica*	D-pinitol, sequoytol	Sarvin *et al.*, 2018
	Larix gmelinii	D-pinitol, sequoytol	Sarvin *et al.*, 2018
	Picea abies	D-pinitol, sequoytol	Sarvin *et al.*, 2018

	Pinus sibirica	D-pinitol, sequoytol	Sarvin *et al.*, 2018
Plumbaginaceae	*Limonium perezii*	D-*chiro*-inositol, *myo*-inositol	Liu & Grieve, 2009
Plumbaginaceae	*Limonium sinvatum*	D-*chiro*-inositol, *myo*-inositol	Liu & Grieve, 2009
Poaceae	*Oryza sativa*	D-*chiro*-inositol, *myo*-inositol	Ratiu *et al.*, 2019
Rosaceae	*Rosa canina*	D-pinitol, D-*chiro*-inositol, bornesitol, *scyllo*-inositol, *myo*-inositol	Ratiu *et al.*, 2019
	Sorbus aucuparia	D-pinitol, *allo*-inositol, D-*chiro*-inositol, bornesitol, *scyllo*-inositol, *myo*-inositol	Ratiu *et al.*, 2019
Solanaceae	*Capsicum annuum*	*Allo*-inositol, D-*chiro*-inositol, bornesitol, *scyllo*-inositol, *myo*-inositol	Ratiu *et al.*, 2019
	Solanum melongena	*Scyllo*-inositol, *myo*-inositol	Hernández *et al.*,2011
	Solanum tuberosum	D-pinitol, D-*chiro*-inositol, bornesitol, *myo*-inositol	Ratiu *et al.*, 2019
Zingiberaceae	*Curcuma longa*	D-pinitol, D-*chiro*-inositol, bornesitol, *myo*-inositol	Ratiu *et al.*, 2019
	Elettaria cardamomum	D-pinitol, *allo*-inositol, D-*chiro*-inositol, ononitol, scyllo-inositol, *myo*-inositol	Ratiu *et al.*, 2019
	Zingiber officinalis	D-pinitol, D-*chiro*-inositol, bornesitol, *myo*-inositol	Ratiu *et al.*, 2019

2.8. Different roles of MI and its derivatives

MI is converted into other inositol stereoisomers and uronose and pentose sugars (Loewus, 1990; Loewus and Murthy, 2000). Inositol phosphates, phosphatidylinositides, and glycosylphosphatidylinositols constitutes a diverse group of inositol-containing compounds with a highly varied structural complexity and heterogeneity. They mediate a innumerable of biological processes and different research suggests that they might be actively involved in a great number of cellular processes (Bernfield *et al.,* 1999; Irvine and Schell, 2001; Low, 2000; Shears, 2001, 2004). The role of MI and its derivatives in plants are manyfold which are discussed in details below:

2.8. 1. *Myo*-inositol as an essential nutrient for cell growth and survival

A key plant nutrient, phosphate is stored in the plants in the form of inositol metabolites. Plant seeds store nutrients to aid the seedlings to grow. Specialized cellular organelles termed as globoids deposits inositol hexaphosphate (IP_6), which is converted into phosphorus and inositol during seed germination, catalyzed by the enzyme phytase (Loewus and Loewus, 1983). In other words, IP_6 can be entitled as the fully phosphorylated version of *myo*-inositol that functions to store phosphate. These phosphate storage are utilized during seed germination, where rapid hydrolysis of IP_6 by phytases takes place to provide nutrients to the developing seedling (Raboy and Gerbasi, 1996; Loewus and Murthy, 2000). Thus, phytic acid (IP_6) on hydrolysis supplies inorganic phosphate and carbon skeleton that is indispensable for the growth and development of germinating seeds (Maity and Loewus, 1978). Therefore, if the MI pathway is disturbed by MIPS gene mutation it not only disrupts the synthesis of phytic acid but also hinders several other biochemical pathways that are obligatory for

germination. In fact, level of phytic acid is seen to decline in a number of mutant MIPS lines of barley (Larson *et al*., 1998; Ockenden *et al.,* 2004) maize (Raboy and Cook, 2000), rice (Larson *et al.,* 2000; Suzuki *et al.,* 2007), and soybean (Wilcox *et al*., 2000; Hitz *et al.,* 2002).

The role of MI as an indispensable nutrient for growth and development can be comprehended in microorganisms grown in media or with auxotropic mutants. MI deficiency leading to lipid accumulation occurs in cells of yeast grown under MI-deficient media. Scarborough (1971) described the essentiality of MI for the role of the glucose active transport system in a MI needing the strain of *Neurospora crassa*. It has been seen in these organisms that the active transport system for glucose reduces as a consequence of the elimination of MI from the growth medium, that can rapidly be recovered by the introducing MI to the system. An analogous phenomenon was also noted by Marzluf (1973) in case of sulfate transport in *Neurospora* strains. Studies has suggested that foliar application of amalgamation of MI with Zn has amplified the absorption and mobility of Zn in the tissue and subsequently boosted Zn accumulation in grains (Amaral and Brown, 2022).

In majority of the plant species, MI is added in trivial quantities to stimulate cell growth. It plays a crucial role in cell division as it decomposes into ascorbic acid and pectin and integrates into phosphoinositides and phosphatidyl-inositol. In plant cell and tissue culture mediums, it is typically employed at doses of 50–5000 mg/l^{-1} (Vasil and Thorpe, 1998).

Quite a few metabolic disorders related with MI deficiency have been studied in diverse yeasts viz. *Saccharomyces cerevisiae*, *S. carlsbergensis*, *Schizo saccharomyces* pombe and *Kloeckera brevis.* The loss of viability was found to be maximum in MI requiring

mutants of *Saccharomyces cerevisiae* (Culbertson and Henry, 1975) *and Neurospora crassa* (Strauss, 1958) and the phenomenon has been specifically dubbed as "inositol-less-death". It was also observed in soybean callus cells that in suspension culture it necessitates a critical intracellular level of MI for its cell division. Elevating the intracellular level of inositol results in higher incorporation of inositol into pectin (Biffen and Hanke, 1990).

2.8.2. Plant cell wall biosynthesis

Inositol is also an essential molecule to produce the plant cell wall. MI forms an essential component of all cell membranes (Morre, 1975). Most plants have both primary and secondary cell walls, of which the constituents are polysaccharides, proteins and lignin. MI serves as a precursor for uronic acid of pentoses of cell wall polysaccharides (Loewus *et al.,* 1973). Inositol is oxidized to form urosine diphosphate (UDP)-D-glucuronic acid. This oxidized form is the most common and important nucleotide sugar synthesized through the inositol oxidation pathway that plays a role as both a precursor for other UDP-D-sugars (UDP-D-glucuronic acid, UDP-D-galacturonic acid, UDP-Dxylose, UDP-D-apiose and UDP-L-arabinose) and a donor for biosynthesis of polysaccharide (Loewus and Murthy, 2000). The formation pentosyl and galacturonosyl units of the cell wall was aided by the MI oxidation pathway that was observed in supension cultures of *Daucus carota* L. cells (Verma and Dougall, 1979). These nucleotide sugars other than cell wall biosynthesis, provide components for other pathways involved in transport, storage and development (Loewus and Loewus, 1983).

2.8.3. The Phosphatidylinositol (PI) signaling pathway

Plants also require to respond to their changing surroundings. Chemical signaling pathways are utilized by organisms to transmit the information between cells. In plants,

there exists a signaling mechanism known as the PI signaling pathway, which relies on the presence of *myo*-inositol (Munnik *et al.,* 1998). This pathway has been found to be involved in a number of plant responses, such as the capability of roots to grow downward in response to gravity (Perera, 1999) and the regulation of wilting through changes in pressure within leaf pores (Cote and Crain, 1993).

In the realm of plants, the PI signaling pathway forms a signal transduction cascade wherein inositol functions as a pivotal constituent (Munnik *et al.,* 1998). PI is considered the initial substrate for quite a number of phosphoinositide kinases that phosphorylate the hydroxyl groups along the inositol ring. Phosphorylation of PI at the 4-hydroxyl position produces phosphatidylinositol 4-phosphate (PI4P). Further, a subsequent phosphorylation at the 5-hydroxyl position generates phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2). Phosphorylation of PI can be at the 3-, 4-, and 5-hydroxyl positions to yield phosphatidylinositol mono-, bis-, or trisphosphate molecules (Shawn *et al.,* 2008). The inositol synthesized from the PI signaling pathway can be added to intracellular inositol pools or used again in the PI signaling pathway. The involvement of this pathway has been demonstrated in various plant reactions, including the phenomenon of gravitropism specifically in *Zea mays* (Perera *et al*., 1999) and turgor pressure alterations in stomatal guard cells (Cote and Crain, 1993). In the case of supplementation of PI with *S. cerevisiae* grown cells enhanced the concentration of phospholipids including PI. These PI-supplemented cells exhibited stimulated growth and total phospholipids contents (Behuria *et al.,* 2018).

2.9. MI in stress tolerance

Physiological, biochemical, metabolic and molecular changes occur in plants under various environmental stress. Increased activity of MIPS and a higher rate of inositol

production is one such mechanism in plants to resist stress conditions. The plant cell utilizes different mechanisms to tide over the various stress conditions. They may act by activating various genes and synthesizing a plethora of chemicals (Mukhia and Chhetri, 2022). Some of these genes and the molecules derived from MI responsible for abiotic stress tolerance is presented in Table 2.3 The genes encoding MIPS have been isolated from different plant species, such as *Arabidopsis* (Johnson, 1994), maize (Larson and Raboy, 1999), rice (Yoshida et al., 1999), soya bean (Hegeman et al., 2001), smooth cordgrass (Joshi *et al*., 2013), *Medicago falcata* (Tan *et al.,* 2013) and so on.

Table-2.3. Different genes coding for *myo*-inositol and its associated derivatives are potentially responsible for abiotic stress tolerance in plants.

Sl. No.	Type of Stress	Gene Responsible	Chemical/Molecular Response	Reference
1.	Osmotic	IMT 1	*Myo*-inositol o- methyl transferase	Rammesmayer *et al.*, 1995; Vernon &Bohnert, 1992
2.	Salinity	Imt 1, Inps 1 transcript	D-pinitol	Ishitani *et al.* 1996
3.	Cold	AtGolS3	RFO	Taji *et al.*, 2002
4.	Drought & salinity	AtGolS1 andAtGolS2	RFO	Taji *et al.*, 2002
5.	Drought, salinity, cold	DREB1A/CBF3 and DREB1B/CBF1	Raffinose	Jaglo-Ottosen *et al.*, 1998; Kasuga *et al.*, 1999; Liu *et al.*, 1998
6.	High temperature	AtGolS1	Raffinose	Panikulangara *et al.*, 2004)
7.	Salinity	Imt	*Myo*-inositol-O-methyl transferase	Nelson *et al.* 1998
8.	Salinity, high temperature	MIP2	inositol	Kaur *et al.*, 2008; Khurana *et al.*, 2012
9.	Drought	AKIN11	Up-regulation of stress-responsive genes	Umezawa *et al.*, 2004
10.	Drought and heat	DREB2A	Expression of downstream stress-inducible genes	Mizoi *et al.*, 2018
11	Heat Stress	Os02g0496100	Heat shock factor (HSF)-	Kikuchi *et al.*, 2003
12	Cold stress	WCOR410	Expression of the cold-Regulated genes	Danyluk *et al.*, 1994
13	Cold stress	WCS120	Expression of the cold-Regulated genes	Ganeshan *et al.*, 2008
14	Salinity stress	LOC543151	PM NADPH oxidase-dependent H(2)O(2) generation	Yang *et al.*, 2007
15	Salt stress	P5CS	Regulation of proline synthesis	Karthikeyan *et al.*, 2011
16	Salt stress	P5CSF129A	Proline accumulation	Kumar *et al.*, 2010
17	Salt stress	SAMDC	Increase in the level of spermidine and spermine	Roy and Wu, 2002
18	Salt stress	betA	Increase od glycine	He *et al.*, 2010

			betaine content	
19	Salt stress	mtlD	Biosynthesis of mannitol	Abebe *et al.*, 2003
20	Salt stress	SmCP	Increases in ion flux, germination rates, chlorophyll content and antioxidant enzyme activities	Zheng *et al.*, 2018
21	Salt stress	DHN-5	Increase in the level of proline biosynthesis enzyme (P5CS)	Saibi *et al.*, 2015
22	Salt stress and oxidative stress	PnF3H	Up-regulation of stress-related genes and increase in antioxidant enzyme activity	Li *et al.*, 2017
23	Salt stress	ADC	Increase in proline synthesis	Espasandin *et al.*, 2018

2.9.1. Inositol and ROS accumulation

One of the most common effects of stress in plants is the rise in reactive oxygen species (ROS) levels. ROS damages the photosynthetic pigments, cellular lipids, proteins and nucleic acids by oxidation and also stimulates apoptosis (Gadjev *et al.* 2008). An orderly up-regulation of ROS-scavenging genes was found in IbMIPS1-overexpressing sweet potato plants under salt and drought stresses (Zhai *et al.,* 2016). Methylated derivatives of inositol have been found to be efficient in stalling ROS, thereby shielding photosynthetic machinery (Patra, 2010). Various reports show that cyclitols, including inositol lessens the negative influence of osmotic stress, consequently increasing the tolerance to drought stress in plants (Cevik *et al.* 2014; Sambe *et al*. 2015). Superoxide dismutase (SOD) provides the first line of defence against the toxic impact of high levels of ROS (Gill and Tuteja 2010). Total SOD activities in pepper plants turned out to be a result of *myo*-inositol treatment in drought stress. Cu,Zn-SOD II isoform of SOD is enhanced by the treatment of *myo*-inositol in droughted plants (Yildizli *et al.,* 2018).

Mechri *et al.* (2015) reported that sugar alcohols may function as scavengers of activated oxygen species, by averting peroxidation of lipids and resulting cell damage. *Myo*-inositol has been observed to be effective as H_2O_2 scavenger during stress conditions in plants. Accumulation of ROS also affects the photosynthetic machinery and the total chlorophyll content of a plant (Sarkar *et al.,*2016). Chlorophyll reduction under abiotic stress signifies osmotic stress, which is a result of pigment photo-oxidation and chlorophyll degradation (Rai *et al.,* 2018, Farooq *et. al.,* 2016). Since photosynthesis is directly influenced by leaf chlorophyll content (Ravikumar *et. al*, 2014), chlorophyll retention capacity during salt stress was checked quantitatively. Transgenic 7PcINO1-plants retained the chlorophyll content, thus upholding the normal photosynthetic potential even under high concentration of salt, while the untransformed line showed maximum chlorophyll depletion (Mukherjee *et al*., 2019).

Malondialdehyde (MDA) is a commonly used marker of lipid injury inflicted by environmental stress. MDA content was observed to be higher in drought-stressed plants than well-watered plants. Fan *et al.,* (2021) reported an increase in MDA content in cucumber during drought stress. Low MI treatment significantly rised the level of MDA in both droughted and well-watered plants. In transgenic sweet potato plants, overexpression of IbMIPS1 significantly increased, while their MDA content significantly decreased compared to WT, indicating a noticeable enhancement of their salt tolerance (Fan *et al.,* 2015) under stress, as has been postulated for other polyols (Loewus and Loewus, 1983; Bohnert *et al.,* 1995). Further, inositols also has been reported to mimic the structure of water and uphold an artificial sphere of hydration around macromolecules (Mechri *et al.,* 2015). MI is known to accrue in a number of plants in response to stresses through an induced expression of MIPS (Nelson *et al.,* 1999, Majee *et al.,* 2004, Tan *et al.,* 2013). Therefore, an attempt to understand and determine the role

and mechanisms of *myo*-inositol in providing abiotic stress tolerance in higher plants has been made.

2.9.2. MI in salinity stress tolerance

Sugar alcohols like pinitol, mannitol, ononitol and inositol are identified to play a vital role as osmo protectants against salt stress in a great number of plants (Majee *et al.,* 2004, Ghosh *et al.,* 2006). In higher plants, the inositol that accumulates in response to salt stress may act as an osmolyte in addition to its critical roles associated with cellular machinery (Bohnert *et al.,* 1995). The extent to which the rise in levels of osmolyte accur is dependent on changes in the external osmotic potential (Hasegawa *et al.,* 2000, Chen and Murata, 2011). Compounds such as gums, cell wall-located carbohydrates, glycoproteins and mucilages, that are involved in protective functions during stress, are also synthesized from inositol and inositol-1-phosphate (Shen *et al*., 1997).

Tomato (*Lycopersicon esculentum* Mill. cv. New Yorker) plant exposed to NaCl stress displayed an increased in *myo*-inositol content which remained high throughout (Sacher and Staples, 1985). Plants alternated daily between salt and control solutions accumulated less *myo*-inositol and exhibited less growth as compared to the continuously salt-stressed plants. *Mesenbryanthemum crystallinum*, a facultative halophyte, has been seen to accumulate pinitol and ononitol when the plant is stressed (Bartels and Nelson, 1994). The free *myo*-inositol content in *Ulva lactuca* was found to increase proportionately with the increase of surface salinity of the Chilika Lagoon, Odisha, India. (Basu *et al.,* 2019). It was postulated that *myo*-inositol could serve not only as a substitute for the production of compatible solutes but also as a leaf-to-root signal to encourages sodium uptake (Nelson *et al.,* 1999). When subjected to salinity stress by NaCl treatment, the salt-tolerant varieties of rice (*Oryza sativa*) exhibited increased chloroplastic inositol synthase activity.

The rise in inositol synthase activity in the highly salt-tolerant varieties of rice as a consequence of salinity stress was analogous to that of *Porteresia coarctata*, also a halophytic wild rice species. This eventually suggested the role of inositol pathway in osmoregulation (Raychoudhury and Majumder, 1996). Upon stress imposition, rapid accumulation of *myo*-inositol occurs and equally rapid deterioration upon stress relief in *Actinidia deliciosa* and *A. arguta* was observed and it indicated that this is a primary response to salt stress, which is quickly adjustable to various alterations in environmental conditions (Klages *et al.,* 1999). Results of Nelson *et al.,* (1999) exhibited that MI plays a key role in metabolic responses leading to salt tolerance in *M. crystallinum*. Transgenic tobacco (*Nicotiana tabacum* L., cv SR1) plants transformed with IMT1 (*myo*-inositol O-methyltransferase) cDNA displayed improved tolerance to drought and salt stress when compared to control plants. An experimental work by Al-Mushhin *et al.,* (2021) exhibited, the influence of salinity stress in quinoa (*Chenopodium quinoa* L. var. Giza1) where it competently tackled by foliar application of MI. Salinity significantly reduced the water potential and water use potential in plants, however, exogenous application of MI was known to effectively decrease the hydrogen peroxide and superoxide accumulation. Additionally, it reduced lipid peroxidation, and electrolyte leakage related with an upsurge in the membrane stability index. The negative effects of salinity stress on quinoa were known to be further mitigated by amplified accumulation of osmolytes such as proline, glycine betaine, free amino acids, and soluble sugars in MI-treated seedlings (Al-Mushhin *et al.,* 2021).

MIPS from a wild halophytic rice *Porteresia coarctata*, introgressed into IR64 *indica* rice showed a role in conferring salt-tolerance. PcINO1 transformed transgenic rice lines revealed a significantly enhancement in tolerance at up to 200 mM or concentration of higher salt with insignificant refutation in their growth and other physiological parameters

when compared to the untransformed system grown without stress. Further the study of introgression of PcINO1 in *Brassica juncea* established that overexpression and maintenance of an undiminished pool of inositol in transgenic plants imparts a considerable tolerance to salt and oxidative stress and BjPcINO1 plants could be a prospective for salt-tolerant GM crop. Majee *et al.,* 2004 reported MIPS protein isolated from PINO1 transgenics exhibiting salt-tolerant properties in vitro asserting functional expression in plants of the PINO1 gene.

Efforts were made to produce salt-tolerant *indica* rice plants utilizing the transgenic functional co-introgression of both PcINO1 and PcIMT1 that comparatively showed a higher buildup of inositol in stress condition (Mukherjee *et al.,* 2019). Overexpression of MIPS in *B. juncea*, *Nicotiana tabacum*, *Arabidopsis* and rice improved the tolerance to salt, dehydration and chilling due to the rise in production of inositol (Das-Chatterjee *et al.,* 2006; Goswami *et al.,* 2014; Kaur *et al.,* 2013; Majee *et al.,* 2004; Tan *et al*., 2013).

DeWald *et al.,* (2001) reported a functional association between inositols and calcium content in plants. A change in inositol and calcium metabolism and gene expression through activation of calmodulin and a variety of protein kinases. Inositol derivatives have been shown to induce Ca^{2+} release from vacuole (Sanders *et al.,* 1999, 2002; Perera *et al.*, 2008). Inositol derivatives were also seen to participate in the regulation of calcium release in stoma guard cells and root cells (Engstrom et al. 2002; Munnik and Vermeer 2010). Ca^{2+} directly induces the expression of stress-response genes, following the enhancement of stress tolerance (Kanchiswamy *et al.,* 2014; Zhai *et al.,* 2016). Similarly, researchers have reported that stress caused by salinity and water results in the rise in the amount of cytosolic calcium in plants that in turn can induces the expression of resistance responsive genes, leading to improvement of resistance (Kanchiswamy *et al.,* 2014; Zhai *et al.,* 2016, Knight *et al.,* 1998; Sanders *et al.,* 1999).

MI has been implicated to salt tolerance in a large number of plants as a facilitator of uptake and long-distance transport of sodium (Nelson *et al.,* 1999). Enhanced tolerance to salinity was observed in transgenic *Arabidopsis* plants expressing SaINO1 (a gene from the halophyte grass *Spartina alterniflora* encoding MIPS) at germination level as well as during plant growth and development (Joshi *et. al.,* 2013). Normal growth was seen after a week of exposure to salt stress in transgenic plants while the WT plants struggled to survive and ultimately perished. Under salinity stress *Arabidopsis* transgenic plants were found to be less sensitive to photoinhibition as compared to WT plants. Increased MI resulting from the expression of SaINO1, aided as the substrate for accumulating metabolic end products and that in turn could facilitate sodium sequestration and protect photosynthesis (Nelson *et al.,* 1999, Bohnert *et al.,* 1995). These evidences indicated a substantial protection of photosystems particularly PSII in SaINO1-*Arabidopsi*s plants and the possible role of MI in protecting the chloroplast (Mukhia and Chhetri, 2022).

Several studies indicated that osmo protective compounds play a crucial role in mutants or transgenic plants with different potential, accumulating these metabolites for stress tolerance (Szabados *et al*. 2011). MI may act as a compatible solute for the defense against abiotic stress and it can be transformed to other compatible solutes as well (Taji *et al*., 2006). 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 injury by scavenging free radicals and lowering *Tm* value of nucleic acids (Crowe *et al.,* 1987; Nomura *et. al.,* 1995). Osmolytes not only help in osmotic adjustment in the cellular milieu but also functions as reactive oxygen species scavengers. The presence of D-ononitol and *myo*-inositol are the potential protectants of enzymes and membranes from damage caused by reactive oxygen species (Sheveleva *et. al.,* 1997). Administration of exogenous MI amplified the activities of antioxidant

enzymes and the levels of ascorbate and glutathione, that improved membrane stability and minimized oxidative damage (Al-Mushhin *et al.,* 2021).

Formation of O-methyl inositols through the isomerization and methylation of MI, alsowas found to directly participate in stress-related responses of plants (Loewus and Murthy, 2000). The plants, that are constantly exposed to saline conditions accumulate the cyclic sugar alcohols, pinitol and ononitol (Paul and Cockburn, 1989). Consequently, *Mesenbryanthemum crystallinum* has been seen to accumulate these compounds when subjected to such stress (Bartels and Nelson, 1994). The upregulation of inositol biosynthesis by subjecting plants to salinity stress may be used in increasing the production of *myo*-inositol from glucose-6-phosphate (Raychaudhuri and Majumder, 1996).

2.9.3. MI in drought tolerance

Plants develop composite and dynamic systems like various physiological, morphological, biochemical and metabolic approaches as a response to water deficit conditions and adapt to drought conditions (Vincent *et. al.,* 2007; Ahuja *et al.,* 2010; Saidi *et al*., 2011; Walbot, 2011). In plants the limitations in the accessibility of water incurs both hyperionic and hyperosmotic stress that has the potential to upset homeostasis both at the cellular level and whole-plant level. Molecular damages resulting from altered osmotic potential ultimately hinders growth and can even lead to death (Hasegawa *et al.,* 2000, Allen *et al.,* 1994). Water deficit or osmotic effects are potentially the major physiological mechanisms for growth reduction as both stresses lower the soil water potential (Hu and Schmidhalter, 2005). Some of the severe effects comprise of the production of reactive oxygen species, cell membranes disruption, drop in enzymatic and photosynthetic activities (Winicov, 1998).

Plants retort to drought stress by the production of diverse metabolites including polyols in ripe olive fruit (Martinelli, 2013), grape berry (Conde *et al.,* 2014) etc. Six polyols viz., mannitol, sorbitol, galactitol, *myo*-inositol, glycerol and dulcitol were significantly accumulated in the pulp of grape berries in response to water deficit. *Myo*-inositol was the amplest of the quantified polyols in mature leaves and tissues that aided the plant with water deficit, either directly as an osmolyte or indirectly as a precursor of galacinol and raffinose family oligosaccharides (Conde *et al.,* 2014). Despite being used as an osmolyte, it is already a established fact that *myo*-inositol like other sugar alcohols functions as a precursor of many metabolites involved in abiotic stress (Kaur *et al.,* 2013). Infact, *myo*-inositol is closely associated with the accumulation of RFOs and further stress tolerance (Elsayed *et al.,* 2014). There is a close knit relation between the metabolism of *myo*-inositol and RFO and the yield performance of maize under drought stress. Galactinol synthase (Go1S EC 2.4.1.123) is known to be the main enzyme that catalyzes to produce galactinol from *myo*-inositol. Therefore, galactinol synthase (Go1S) has been implicited to be the key regulatory factor in RFO biosynthesis (Taji *et al.,* 2002; Karner et al., 2004; Sengupta *et al.,* 2015).

Proline is known to accumulate in plants in the cytosol under water and salt stress (Filippou *et al.,* 2011; Hossain *et al.,* 2014). Yet, a rise in the level of MI decreased the proline content under stress. This deterioration in proline production was implicated to the increasing MI level in leaves under drought stress that directs to the fact that there may not be a need to increased the proline level as an osmo protectant (Yildizli *et. al.,* 2018). Exogenous MI decreased H_2O_2 and catalase activities, membrane damage and level of proline, whereas it induced the glutathione reductase activity and water status under drought stress (Yildizli *et. al*., 2018).

Myo-inositol buildup stimulated the expression of a stress responsive MIPS gene (SaINO1) in *Spartina alterniflora* for osmotic stress tolerance (Joshi *et al.,* 2013). The findings of Wei (2010) proposed that up-regulation in the level of the rcMIPS gene and their increased MIPS activity were involved in drought stress tolerance in *Ricinus communis*. The increase in activity by 19.8%, 37.4%, and 88.5% under 30% PEG stress conditions for 24, 48, and 72 h in the leaves were compared to the control, respectively. The activities in the stems and roots reached the peak at 24 and 48 h of stress, and the highest activity rise were by about 41.6% and 74.2%, respectively (Wei *et. al.,* 2010). In the leaves, the activities amplified by 88.5% under 30% PEG stress conditions for 72 h compared to the control (Wei *et. al.,* 2010). There are seven Go1S-related genes in the *Arabidopsis thaliana* named AtGo1S 1, 2, 3, 4, 5 6 and 7 respectively. Among these, AtGo1S1 is the drought responsive gene, that is mainly active during drought stress tolerance (Taji *et al.,* 2002). *Ajuga reptans*, a model plant was seen to express two distinct Go1S, ArGo1S1 and ArGo1S2 that regulate RFO metabolism (Sprenger and Keller, 2000). RFOs have long been implcated to function as anti-stress agents in both generative and vegetative tissues (Taji *et al.,* 2002; Pennecooke *et al.,* 2003). XvGo1S gene encoding galactinol synthase was also identified in *Xerophyta viscosa* leaves. This gene exhibited a negative correlation between RFO accumulation and depletion of *myo*-inositol which was overturned after rehydration. This establishment suggests that *myo*- inositol is channeled into synthesis of RFO during water shortage and channeled back to the metabolic pathway during rehydration to repair desiccation-induced injuries (Peter *et al.,* 2007). Additionally, in Go1S, MIPS also is known to control the galactinol and raffinose levels as it controls the synthesis of *myo*-inostiol, the galactinol precursor. Therefore, both Go1S and MIPS are confirmed to performance significant roles in drought stress tolerance (Taji *et al.,* 2002; Evers *et al.,* 2010).

2.9.4 MI in cold stress

At an optimum range of temperature, plants display a maximum rate of growth and development (Fitter and Hay, 2012). When ambient temperature diverges from the optimal, then physiological, metabolic, biochemical and molecular transformations occur within the plants. This is an effort of plants during adverse conditions to promote regular growth and developmental processes and to maintain cellular homeostasis. Stress tolerance can be induced by exposure to reduced temperature and is referred to as chilling tolerance and/or cold acclimation. The ability of a plant to tolerate low temperatures (0–15 °C) without injury is the chilling tolerance (Somerville, 1995), while cold acclimation is an enhanced tolerance to the physical and physiochemical vagaries of freezing stress (Thomashow, 1999). Both cold acclimation and chilling tolerance comprises of an array of biochemical, molecular and metabolic processes (Thomashow, 1999; Larkindale *et al*., 2005; Kotak *et al*., 2007; Zhu *et al.,* 2007).

Plants experience cold or chilling stress at temperatures from 0–15°C. Under such conditions, plants try to maintain homeostasis in order to acquire freezing tolerance and this includes an extensive reprogramming of gene expression and metabolism (Thomashow, 1999; Cook *et al.,* 2004). In plants, accumulation of sugars and altered gene expression are two important processes that confers freezing tolerance. Additionally, to withstand the cold temperature, plants have developed various antioxidation strategies combined with osmoprotection by sugars. In *Solanum tuberosum* elevated sugar levels showed an increase in levels of *myo*-inositol content has been established as a cold tolerance response strategy by the pants (Teper, 2023). Among cold-responsive genes, cDNA encoding MIPS had the highest abundance in the library, entailing that MIPS play a vital role in the cold tolerance in *Medicago sativa* subsp. *falcata* (Tan *et al.,* 2013). Cold tolerance was seen to be more effective in *M. falcata* than alfalfa (Riday *et al*., 2003,

Pennycooke *et al.,* 2008), that was associated with enhanced deposition of sucrose, MI, galactinol and RFOs during cold acclimation (Tan *et al*. 2013, Zhuo *et al.* 2013). Overexpression of MIPS in *B. juncea*, tobacco, *Arabidopsis* and rice has been known to increase the tolerance to salt, dehydration and chilling as a result of rise in production of inositol (Das-Chatterjee *et al.,* 2006; Goswami *et al.,* 2014; Kaur *et al.,* 2013; Majee *et al*., 2004; Tan *et al.,* 2013). Also, overexpression of Imt1gene in *Arabidopsis thaliana*, exhibitioned tolerance to low temperature stress which is suggestive of the accumulation of proline and total soluble sugars in leaves as a result of low temperature stress (Zhu *et al.,* 2012). Expression analysis, pharmacological experiments and overexpression studies of MfMIPS1 (*Medicago falcata* MIPS1) in tobacco lead to an enhanced tolerance to chilling stress by rising the levels of inositol, galactinol and raffinose sugars (Tan *et al.* 2013). Overexpression of MI-induced MfGOLS1 in tobacco resulted in rise in the levels of raffinose and stachyose further providing effective tolerance to cold stress (Zhuo *et al.,* 2013).

MI can be converted to galactinol, which in turn is converted to raffinose (Taji *et al.* 2002). The concentration of inositol in *Medicago falcata* (cold-tolerant) was higher when compared to *Medicago sativa* (cold-sensitive) at 5°C (Tan *et al.*, 2013). Some other authors demonstrated the role of inositol in alleviating the damage of cold stress on maize and rice seedlings (Guo *et al.*, 2014; Yao and Xia, 2014). MI induces galactinol synthase gene (GolS) expression and contribute to cold-induced MfGolS1 (galactinol synthase gene from *Medicago falcata*) expression (Zhuo *et al.,* 2013). A full-length cDNA encoding a MI transporter-like protein, MfINT-like, was cloned from *M. sativa* subsp. *falcata*, a species with better known for cold tolerance than *M. sativa* subsp. *sativa.* Advanced levels of MI were observed in leaves of transgenic tobacco plants overexpressing MfINT-like than the wild-type signifying that transgenic plants had more

MI transport activity than the wild-type. Transgenic plants had a improved tolerance to freezing temperature, indicating a lower ion leakage and higher maximal photochemical efficiency of photosystem II (Fv/Fm) after chilling treatment. Moreover, higher plant fresh weight was recorded in transgenic plants as compared with the wild-type when plants were grown under stress (Sambe *et al*., 2015).

The presence of cyclitol and low molecular weight compounds all together function as cryoprotective solutes under freezing conditions. Accumulation of these cryoprotectants avoids freeze induced shrinkage by balancing the concentration of cryotoxic substance during ice formation. Cyclitols like pinitol, quebrachitol, quercitol, O-methyl-muco-inositol are known to accumulate during low temperature conditions (Diamantoglou, 1974; Ericsson, 1979; Poop *et al.,* 1997). In mistletoe (*Viscum album*) more than 25% of its dry matter is constituted by the cyclitols during winter (Richter and Popp, 1992). Similarly, improved storage of cyclitols in the living bark tissue and buds has been found in a number of tree species during the onset of the cold season (Poop and Smirnoff, 1995; Poop *et al.,* 1997). The transcription of the enzyme *myo*-inositol-o-methyl-transferase, a key enzyme for the biosynthesis of ononitol and pinitol has been induced in the Mediterranean species *M. crystallium* when the plant was exposed to 4°C for 78hr. Comparable accumulation of pinitol in chickpea in the thylakoid membrane functions as cryoprotective solutes was observed (Orthen and Popp, 2000). Proline and raffinose also play a decisive role in compatible solutes in *Arabidopsis* in freezing tolerance (Hannah, *et al*., 2006; Korn *et al.,* 2010). MIPS-encoding gene cloned from yellow passion fruit (*Passiflora edulis* f. *flavicarpa*) called PeMIPS1 is differentially transcribed in cold and heat stress. This finding correlates with an ecological adaptation of yellow passion fruit, a typical species adapted to tropical and sub-tropical environments and has the capability to endures winter chills for short periods without injury (Abreu and Aragão, 2007).

Ability of the plant to withstand cold temperature is often associated with the calcium channels (Guo *et al.,* 2018). In Chinese cabbage and pepper seedlings, the mixture of different concentrations of Ca^{2+} and inositol significantly promoted the growth (Liu *et al.*, 2017; Yang *et al.,* 2017). In a study by Yan, 2022, 35 differently expressed genes encoding inositol enzymes were identified through RNA sequencing. Inositols were seen to positively regulate cold tolerance by elevating Ca^{2+} influx in *Brassica napus*. The results showed the expression of calcineurin B-like (*CBL1*) gene was repressed by inositol. Transgenic plants mediated the Ca^{2+} flux under cold stress indicating the vital role of inositol-Ca^{2+} pathway in cold tolerance. Further, the overexpression of *BnCBL1-2* in *Arabidopsis* depicted that those transgenic plants mediated the Ca^{2+} flux further stress the significance of the inositol-Ca^{2+} pathway in conferring cold stress.

2.8.5. MI in high temperature stress

High temperature results in photosynthetic acclimation and changes physiological processes directly and indirectly alters the pattern of development (Wahid *et al.,* 2007). It subsequently impacts the reproductive growth by increasing flower abortion and decreasing seed size (Talwar *et al.,* 1999). In response to heat stress (HS), plants are endowed with numerous mechanisms and regulatory networks, viz., regulating vital genes, managing various physiological and biochemical adaptations and so forth. One such mechanism to counter the effect of HS is by enhancing the level of inositol in plants.

Increase in the amount of cytoplasmic free Ca^{2+} under temperature stress could lessen the effect of stress and decrease lipid peroxidation (Wu *et al*. 2010). Overexpression of *Cicer arietinum* MIPS (CaMIPS2) is known to improve the tolerance of *S. pombe* cells under salinity and high temperature (Kaur *et al.,* 2008). Also, exogenous MI treatments increased the Ca^{2+} levels further contributing to the cell water use efficiency. While

inducing of HS, TaMIPS2, identified from a heat subtractive cDNA library from wheat was found to be expressed during various seed development stages. The transcript levels rised in unfertilized ovaries and a substantial amount was found during the recovery period signifying the pivotal role of MIPS during heat stress recovery and flower development.

Again Khurana *et al.,* 2017, studied the overexpression of TaMIPS2 in *Arabidopsis* that was examined physiologically and morphologically under different abiotic stress conditions. Their analysis of the plants under different stress conditions revealed that TaMIPS2 transgenics have reduced sensitivity to HS reflecting the role of MI during this stress. Enhanced levels of inositol upon TaMIPS2 overexpression in *Arabidopsis* transgenics and rise in *myo*-inositol level were correlated with enhanced tolerance towards HS and other abiotic stresses. TaMIPS2 transgenics when quantitatively analyzed was found to have a noteworthy modification in morphometric parameters upon stress treatment except for salt stress. TaMIPS2 overexpression transgenics demonstrated improved resilience to heat as well as cold and salt stress. Enhanced membrane stability index, FV/FM ratio along with total chlorophyll content in the transgenics were found when compared to wild type during stress. On assessment, TaMIPS2 overexpressing *Arabidopsis* transgenics during stress were found to have risen the levels of oligosaccharides. A remarkable increase in MI level was observed upon heat and ABA treatment that was associated with a decline in stress sensitivity of transgenics (Casaretto *et al.,* 2016). This may also be the cause of the elevated stachyose levels that boost stress tolerance.

3. MATERIALS AND METHODS

3.1. Materials

Fresh specimens of the plants were collected from different localities of Sikkim Himalaya, viz., alpine region- Kupup, East Sikkim (27.35876N, 88.83969 E), Yumthang, North Sikkim (27.815437 N, 88.701339 E); temperate- Gnathang Valley, East Sikkim (27.313593 N, 88.82799 E), Pangthang, East Sikkim (27.367181N, 88.566605); Lachung, North Sikkim (27.69068 N, 88.74589 E); tropical- Marchak, East Sikkim (27.288060 N, 88.590878 E), Namli, East Sikkim (27.270324 N, 88.584950E).

Fresh leaf specimens of the *Hippophae salicifolia* D. don and other alpine plants viz., *Primula denticulata* Sm. (Primulaceae), *Bistorta emodi* (Meisn.) H.Hara *(*Polygonaceae*)*, *Pedicularis siphonantha* D. don (Orobanchaceae), *Argentina lineata* (Trevir.) Soják. (Rosaceae) [synonym=*Potentilla lineata* Trevir.] (Plate-3.1) were collected from alpine areas of East and North Sikkim, *Koenigia mollis* (D. Don) T. M.Schust. & Reveal (Polygonaceae) [synonym=*Aconogonon molle* (D. Don) H. Hara], *Hellenia speciosa* (J. Koenig) S. R. Dutta (Costaceae) [synonym=*Costus speciosus* (J.Koenig) Sm]. were collected from from temperate areas of East Sikkim (Plate-3.2) and other plants *viz. Houttuynia cordata* Thunb. *(*Saururaceae), *Euphorbia hirta* L. (Euphorbiacaea), *Solanum viarum* Dunal (Solanaceae) were collected from different tropical regions of East Sikkim in the Sikkim Himalaya (Plate-3.3).

Table-3.1. Study sites across the altitudinal gradient in the Sikkim Himalayan region (with altitude in metres and latitude/longitude)

Sl. No.	**Altitudinal Zones**	**Location**	**Latitude and Longitude**	**Altitude (metres)**
1	**Tropical**	Namli	27.270324 N, 88.584950E	840

		Marchak	27.288060 N, 88.590878 E	950
2	**Temperate**	Panthang	27.367181 N, 88.566605 E	2025
		Lachung	27.69068 N, 88.74589 E	2659
3	**Alpine**	Yumthang Valley	27.8268° N, 88.6958° E	3564
		Gnathang Valley	27.313593 N, 88.82799 E	3727
		Kupup	27.35876 N, 88.83969 E	3979

Table-3.2 Plant samples and their collection sites in the different altitudinal zones of Sikkim Himalaya

Sl. No.	**Altitudinal Zones**	**Location**	**Plants collected**
1	**Tropical**	Namli	*Euphorbia hirta, Solanum viarum*
		Marchak	*Houttuynia cordata*
2	**Temperate**	Panthang	*Koenigia mollis, Hellenia speciosa*
		Lachung	*Hippophae salicifolia*
3	**Alpine**	Yumthang Valley	*Primula denticulata*
		Gnathang Valley	*Bistorta emodi*
		Kupup	*Pedicularis siphonantha, Argentina lineata*

Plate. 3.1-Experimenal plants from their natural habitat in alpine zone of Sikkim Himalaya. a) *Primula denticulate* b) *Pedicularis siphonantha* c) *Bistorta emodi* d) *Argentina lineata*

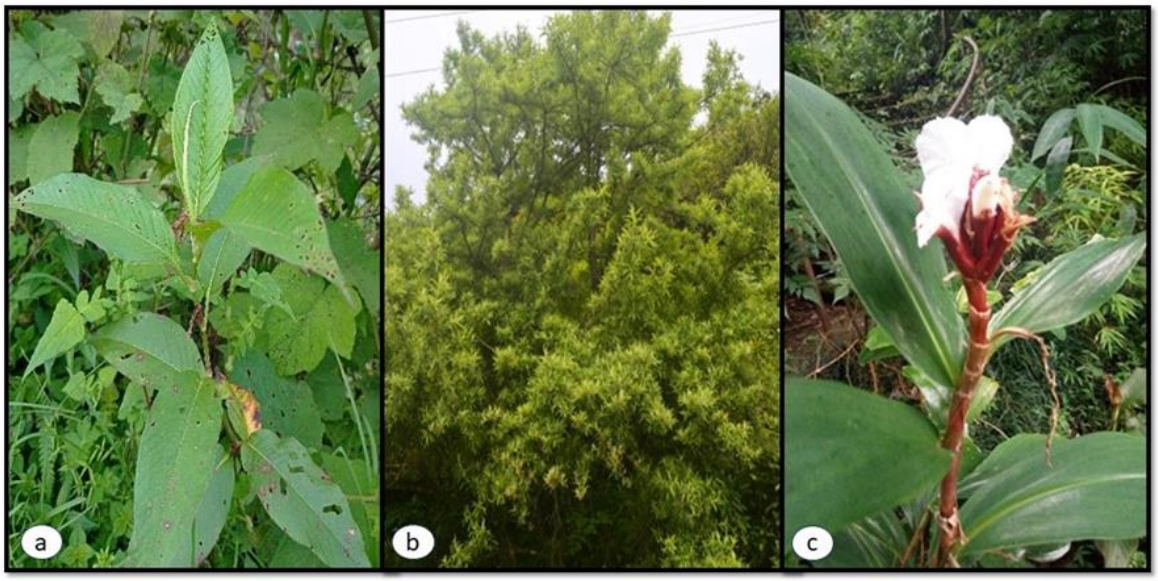


Plate. 3.2- Experimental plants from their natural habitat in temperate zone of Sikkim Himalaya. a). *Koenigia mollis* b) *Hippophae salicifolia* c) *Hellenia speciosa*

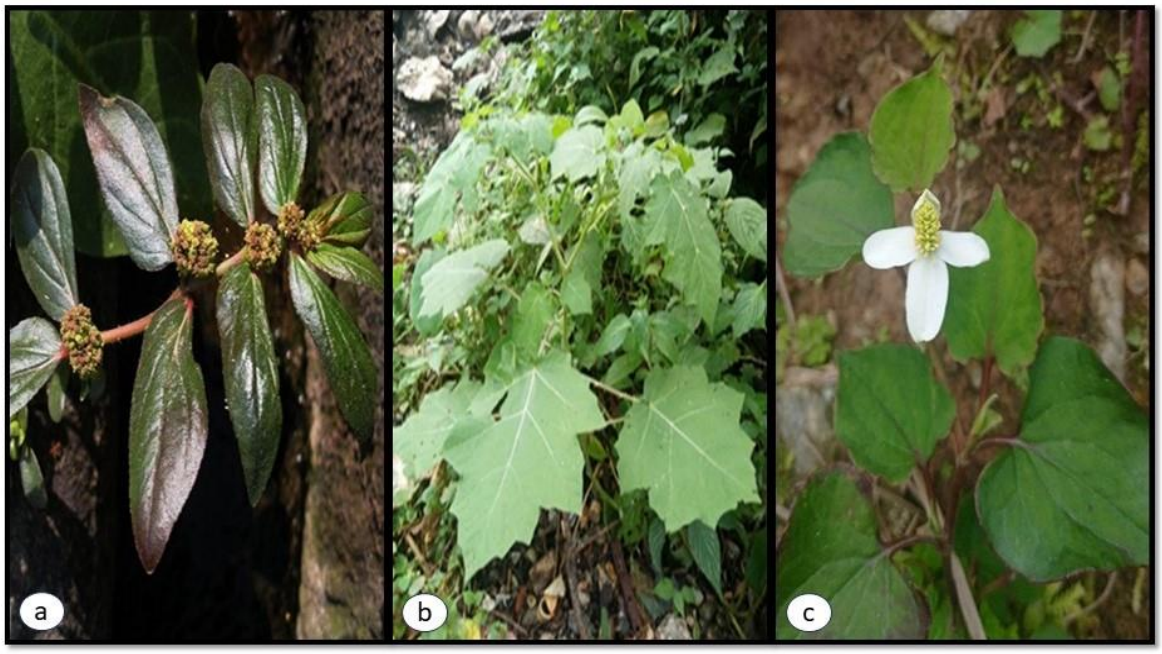


Plate. 3.3- Experimental plants from their natural habitat in tropical zone of Sikkim Himalaya. a) *Euphorbia hirta* c) *Solanum viarum* d) *Houttuynia cordata*

D-glucose-6-phosphate (G-6-P, di-sodium salt), β-NAD, D-galactose-6- phosphate (di-sodium salt), D-fructose-6P (di-sodium salt), D-mannose-6P (di-sodium salt), L-glucose-6P (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, Bisacrylamide papain, Coomassie brilliant blue (G-250), alcohol dehydrogenase (from bakers Yeast) and 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.

Hexylagarose was from Miles-Yeda, Israel. Bio-Gel A-0.5m was purchased from Bio-Rad, USA. Amberlite IR-120 (sodium form), Dowex-1-Cl^- (100-200 mesh), manganese chloride, catalase (from bovine liver), dialysis membrane, egg albumin was 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 a kind donation from the laboratory of Prof. Jukta Adhikari,

Chandernagore, Govt. College, Hooghly, West Bengal (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 cytosolic L-*myo*-inositol-1-phosphate synthase

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

Plant samples (20 g each) were collected, washed twice with 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 enabling the fine crushing. The crude homogenate was passed through 3 layers of muslin cloth and the liquid was centrifuged at 1,000×g for 5 min in a Hermle Labortechnik centrifuge. The pellet was discarded and the supernatant collected. The pellets were once again discarded after centrifugation of the supernatant at 10,000×g for 20 mininutes, dialysis was performed overnight on this preparation using 50 mM tris-acetate (pH 7.5) and 0.2 mM ME. The recovered fraction of 10,000×g supernatant was used as the enzyme source for the initial screening tests.

3.2.2. Partial purification of cytosolic L-*myo*-inositol-1-phosphate synthase

The enzyme was partially purified from the leaves of *Hippophae salicifolia* following the procedure mentioned below:

3.2.2.1. Preparation of crude extract

About 50g 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 mixture was centrifuged at 1,000×g for 5 min in a centrifuge. The pellet was discarded and the supernatant was taken as crude extract (homogenate). The crude extract was spun again at 10,000 ×g for 20 min and the supernatant was collected (10K supernatant).

3.2.2.2. Streptomycin sulphate precipitation

To eliminate precipitated nucleic acids, streptomycin sulphate powder was gently added to the 10K supernatant. The powder was added slowly with constant stirring (using a REMI 10 MLH plus 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 10,000 ×g for 15 min. The supernatant was collected (SS fraction) after discarding the pellet.

3.2.2.3. Ammonium sulphate fractionation

The SS-fraction was then made 0-70 % saturated by adding the required quantity of the ammonium sulphate (43.6 g/100 ml of SS-fraction) with constant stirring (using a REMI 10MLH plus cyclomixer). The mixture was kept at 0ºC in an ice bucket for 15 min and then centrifuged at 10,000 ×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) comprising of 0.2 mM ME, that was poured into a dialysis sac and dialyzed overnight against 500 volume 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 prior by boiling the membrane for 10 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.

The ammonium sulphate fraction (A_2S-fraction) was retrieved from the dialysis sac after the procedure was completed.

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.

After adsorption of the plant material (A_2S fraction) 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 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 (Fig.3.1). The active fractions (DE) were pooled and used for the next purification step.

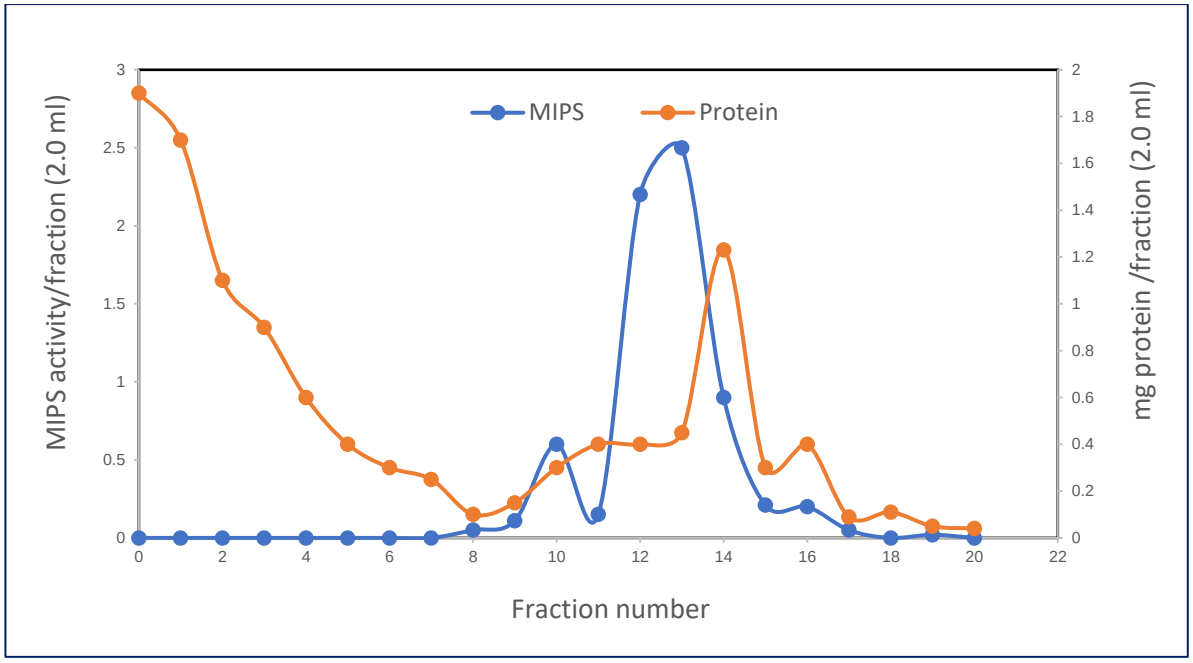


Fig. 3.1- Elution profile of cytosolic MIPS on DEAE cellulose column. MIPS activity presented as µ mol I-1-P produced fraction^{-1} h^{-1}

3.2.2.5. Chromatography on Hexylagarose

The pool of active DEAE-cellulose fraction, obtained from the previous step, was loaded on top of a column (0.8 cm × 7.5 cm) of hexylagarose. Before this step, the commercially available hexylagarose was soaked overnight in 200 volumes of distilled water, the water was decanted off and the matrix was equilibrated with 50 mM Imidazole-HCl buffer (pH 7.5) containing 0.2 mM ME. After collection of effluents, the column was eluted with the equilibration buffer and 15 fractions of 1.25 ml each were collected at a flow rate of 6-8 min per fraction (Fig.3.2). Each fraction along with the effluent was assayed for the enzyme activity and protein content and the active fractions were pooled and marked as the penultimate preparation (Hxl) of the partially purified enzyme.

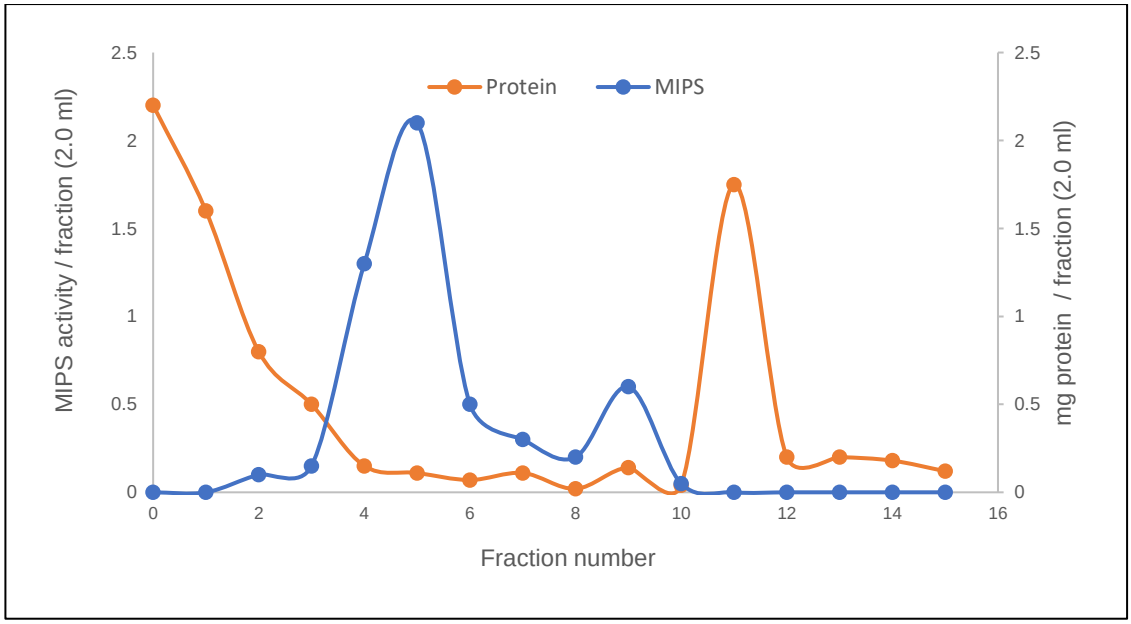


Figure. 3.2. Elution profile of cytosolic MIPS in Hexylagarose column. MIPS activity presented as μ mol I-1-P produced fraction^{-1} h^{-1}

3.2.2.6. Molecular sieve chromatography through BioGel A-0.5m

The enzymatically active fractions from the above preparation were pooled together and loaded in a column 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.

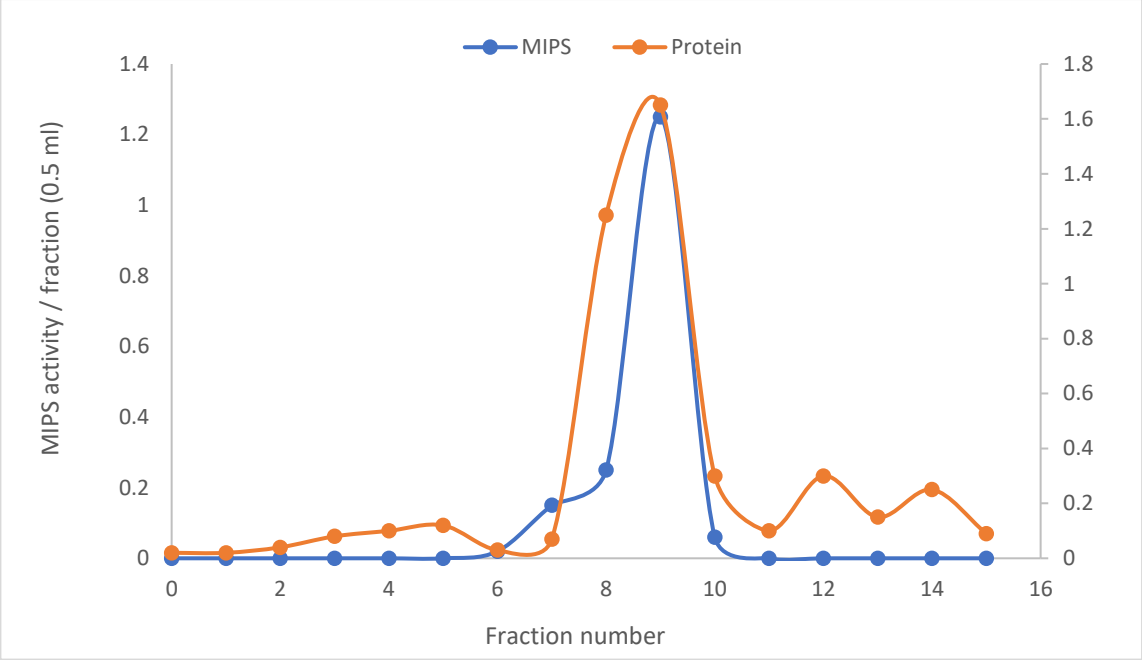


Figure. 3.3. Elution profile of cytosolic MIPS in BioGel A-0.5m column. MIPS activity presented as µ mol I-1-P produced fraction^{-1} h^{-1}

3.2.3. 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 1.5M 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 3g of Tris-base, 13.4 g of glycine and sufficient quantity of distilled water to adjust the volume to 1L) was poured in the 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 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.

3.2.4. Partial purification of chloroplastic MIPS from the leaves of *Hippophae salicifolia*

3.2.4.1. Isolation procedure of chloroplast

Chloroplasts of *H. salicifolia* were isolated following the method of Hachtel (1976) with some modifications according to Adhikari *et al*. (1987).

Juvenile leaves of *H. salicifolia* were collected fresh and washed several times with distilled water, allowed to drain off and placed in a plastic bag in refrigerator for few hours until these were turgid. Thereafter, all operations were carried out at 0 ^{0}C to 4 ^{0}C. The leaf samples (50g) were homogenized in a mortar and pestle with double volume of 20 mM Tris–acetate (pH 7.0) containing 0.35 M sucrose, 10 mM $MgCl_2$, 10 mM KCl, 1 mM ME, 10 mM sodium ascorbate and 2.0 ml (per 100 ml of buffer) of chicken egg albumin. Homogenization was carried out in presence of equilibrated neutral sand (in 20 mM Tris-acetate, pH 7.0). The crude homogenate was centrifuged at 435 ×g for 5 min in a Plasto Crafts Superspin-R centrifuge. The pellet containing the unbroken cells, nuclei, other debris and sand was discarded. The supernatant obtained was spun at 2,850 ×g for 15 min. The resultant chloroplast pellet was washed at least three times with the homogenizing medium. The final pellet was collected as chloroplasts and kept at 0˚C until further use.

3.2.4.2. Identification and purity of chloroplasts

The purity of isolated chloroplasts from *H. salicifolia* was identified and characterized biochemically by the ability of the preparation to carry out characteristic Hill reaction using DCPIP as the electron acceptor along with the determination of the total chlorophyll

content from the same batch of isolated chloroplasts. Identical experiments were also done with the suspension of 435 ×g pellet and with the 2,850 ×g supernatant.

3.2.4.3. The production of reducing equivalents by isolated chloroplasts (Hill reaction)

The production of carbohydrate from H_2O and CO_2 involves two stages, known as the light and dark reactions of photosynthesis. The light reaction takes place in the visible radiation, which is absorbed by the chlorophyll pigment present in the chloroplasts. Electrons in the chlorophyll are raised to a high energy level and return to the initial state through a series of radiations. ATP is generated during this electron transport. During this time, reducing equivalents in the form of $NADPH + H^+$ are also generated and O_2 evolves. The photolysis of water by isolated chloroplasts was first demonstrated by Hill and is often known as the Hill reaction. In this experiment, illuminated chloroplasts from *H. salicifolia* were shown to produce reducing equivalents with the introduction of an artificial hydrogen acceptor, DCPIP in the mixture. Reduction of the blue dye did not take place in the dark.

The isolated chloroplasts were suspended in 20 mM Tris-acetate (pH 7.0) to a considerable volume and 0.1 ml of this suspension was added to 9.9 ml of the DCPIP (0.1 M L^{-1}) solution. Three such sets were designed. The initial absorbances of these three tubes were measured at 520 nm in a Beckman DU-64 spectrophotometer. Then, one of these three tubes was kept in the dark for the duration of the experiment. To the second tube, several crystals of sodium dithionate was added to completely decolorize the blue dye. The third tube was placed under illumination of a fluorescent light for 2 h. The final absorbances (at 520 nm) of these tubes were recorded after the stipulated experimental period. In an experimental set, in addition to these three tubes, another control (without

chloroplast suspension) tube was also maintained. The mode of experimentation was identical where 435 ×g pellet and 2,850 ×g supernatant was used.

3.2.4.4. Estimation of chlorophyll content

The total content of chlorophyll was determined by the method of Arnon (1949) to account for its quantity in isolated chloroplasts of *H. salicifolia* and other experimental fractions of the same species.

10 ml of 80% (v/v) acetone was added to 1 ml of the isolated chloroplast suspension (and also to other fractions under experimentation, separately), shaken thoroughly and filtered through Whatman No. 1 filter paper into a volumetric flask. Rinsed out the test tube with a further 5 ml of acetone and used this to wash the filter paper. The washing was repeated once more and made up to 25 ml with 80% (v/v) acetone. The extinction was read at 652 nm against a solvent blank. The chlorophyll content was then calculated as:

Chrorophyll concentration (mg/ml) = Extinction at 652 nm × 5.8

3.2.4.5. Steps involved in partial purification of chloroplastic L-*myo*-inositol-1-phosphate synthase

The isolated chloroplasts were washed with chilled 50 mM Tris-acetate (pH 7.0) buffer containing 0.2 mM ME and the partial purification of MIPS from isolated chloroplasts was done following the method outlined below:

All the steps were carried out at 0 ^{0}C to 4 ^{0}C

3.2.4.5.1. Homogenate: Buffer-washed isolated chloroplasts obtained from 115 ×g of *H. salicifolia* leaves was homogenized in a mortar and pestle with 3 vol of 50 mM Tris-acetate (pH 7.0) containing 0.2 mM ME in presence of a little amount of neutral sand.

3.2.4.5.2. Low speed supernatant: The crude plastidial homogenate was centrifuged at 11,400 ×g for 20 min in a Plasto Crafts Superspin-R centrifuge. The pellet was discarded and the supernatant fraction was recovered from the centrifuge tubes.

3.2.4.5.3. Streptomycin sulphate precipitation: In order to remove chloroplastic nucleic acids (if any) in the form of precipitate, streptomycin sulphate powder was added gently 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 15 to 20 min followed by another spin at 11,400 ×g for 15 min. The pellet was discarded.

3.2.4.5.4. Ammonium sulphate fractionation: The streptomycin sulphate treated fraction obtained from the previous step was then made 0-80% saturated with $(NH_4)_2SO_4$ by adding requisite quantity (51.6 g /100 ml) of salt slowly with constant stirring. It was kept at 0 ^{0}C for 15 min and then centrifuged at 11,400 ×g for 20 min. The pellet thus obtained from 0-80% saturated $(NH_4)_2SO_4$ fraction was dissolved in minimal volume of 50 mM Tris-acetate (pH 7.0) buffer having 0.2 mM ME and dialyzed for a considerable period against the same buffer (at least 500 vol) for complete removal of $(NH_4)_2SO_4$. On completion of dialysis, the 0-80% $(NH_4)_2SO_4$ fraction was recovered from the dialysis bag.

3.2.4.5.5. Anion exchange chromatography with DEAE cellulose: The dialysed 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 6.0-7.0. The material was washed twice with distilled water and subsequently soaked in 0.5 N NaOH for another 30 min. The NaOH solution was decanted out and repeatedly washed with tap water till the pH became 7.0-7.5 after which it was washed twice with sterile distilled water. Finally, the

DEAE was soaked for 4 h in 100 vol of 50 mM Tris–acetate containing 0.2 mM ME. Now the DEAE became ready for adsorption of proteins. After adsorption for two hours the preparation was loaded in a glass column (1.2 × 6.0 cm) and the effluent was collected. After this, 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 (pH 7.0) containing 0.2 mM ME. Fractions of 1.5 ml/7 min were collected. Sixteen such fractions were collected and assayed for inositol synthase activity (Fig.3.4). The active fractions (DE) were pooled and used for the next purification step.

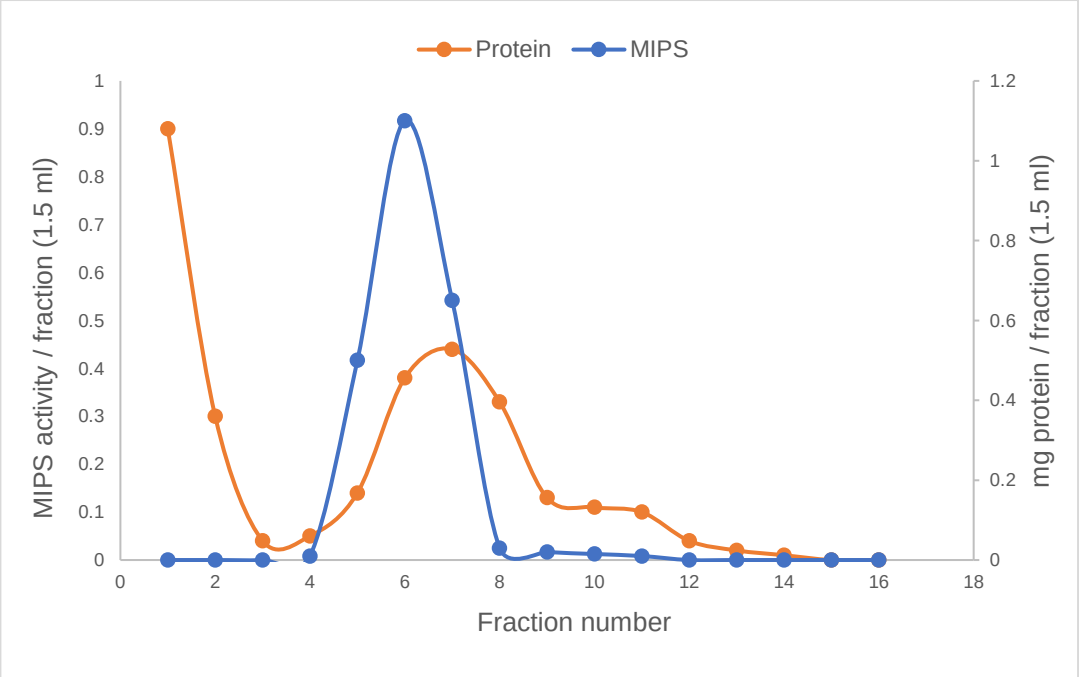


Figure. 3.4. Elution profile of chloroplastic MIPS on DEAE cellulose column. MIPS activity presented as µ mol I-1-P produced fraction^{-1} h^{-1}

3.2.4.5.6. Chromatography on Hexylagarose: The pool of active DEAE-cellulose fraction, obtained from the previous step, was loaded on top of a column (0.6 cm × 6.5 cm) of hexylagarose. Before this step, the commercially available hexylagarose was

soaked overnight in 200 vol of distilled water, decanted off the water and equilibrated with 50 mM Imidazole-HCl buffer (pH 7.5) containing 0.2 mM ME. After collection of effluent, the column was eluted with the equilibration buffer and 15 fractions of 0.75 ml each were collected at a flow rate of 5 to 6 minutes per fraction (Fig.3.5). Each fraction along with the effluent was assayed for the enzyme activity and the active fractions were pooled and marked as penultimate preparation (Hxl) of the partially purified enzyme.

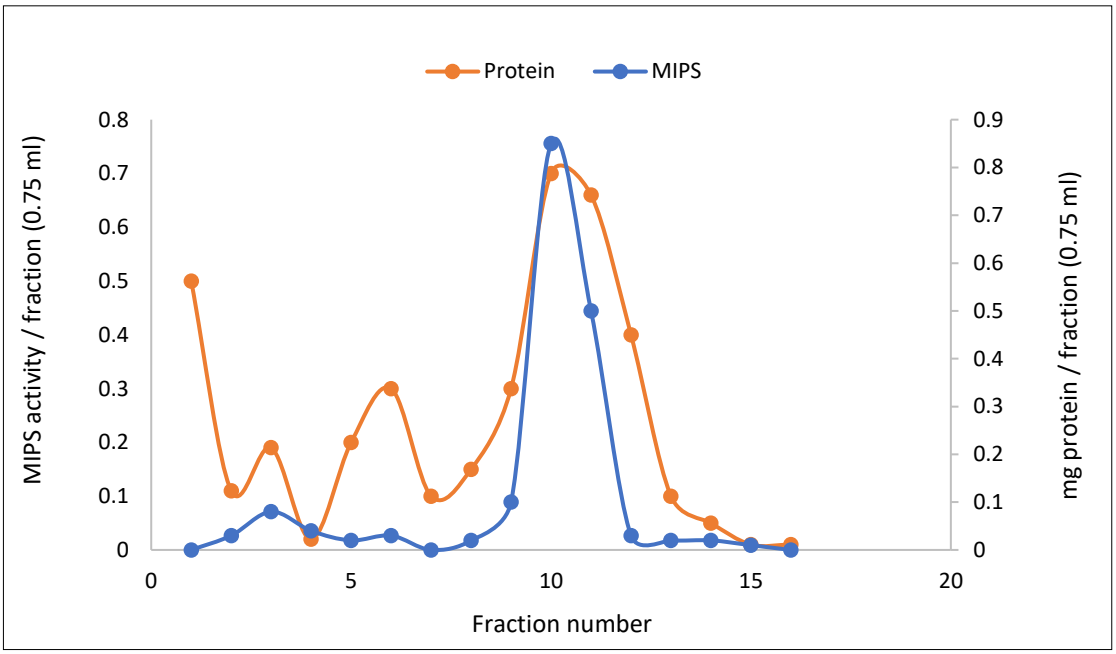


Figure 3.5. Elution profile of chloroplastic MIPS from *H. salicifolia* in Hexylagarose column. MIPS activity presented as µ mol I-1-P produced fraction^{-1} h^{-1}

3.2.4.5.7. Molecular sieve chromatography through BioGel A-0.5m: The enzymatically active fractions of hexylagarose were pooled together and loaded in a column (0.6 × 7.0 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 min (Fig.3.6). Fractions containing MIPS activity were pooled and dialyzed against 1L of 50 mM Tris-acetate buffer (pH 7.5) containing 0.2 mM ME. This preparation was concentrated and used as the enzyme source for the characterization regarding thermal stability.

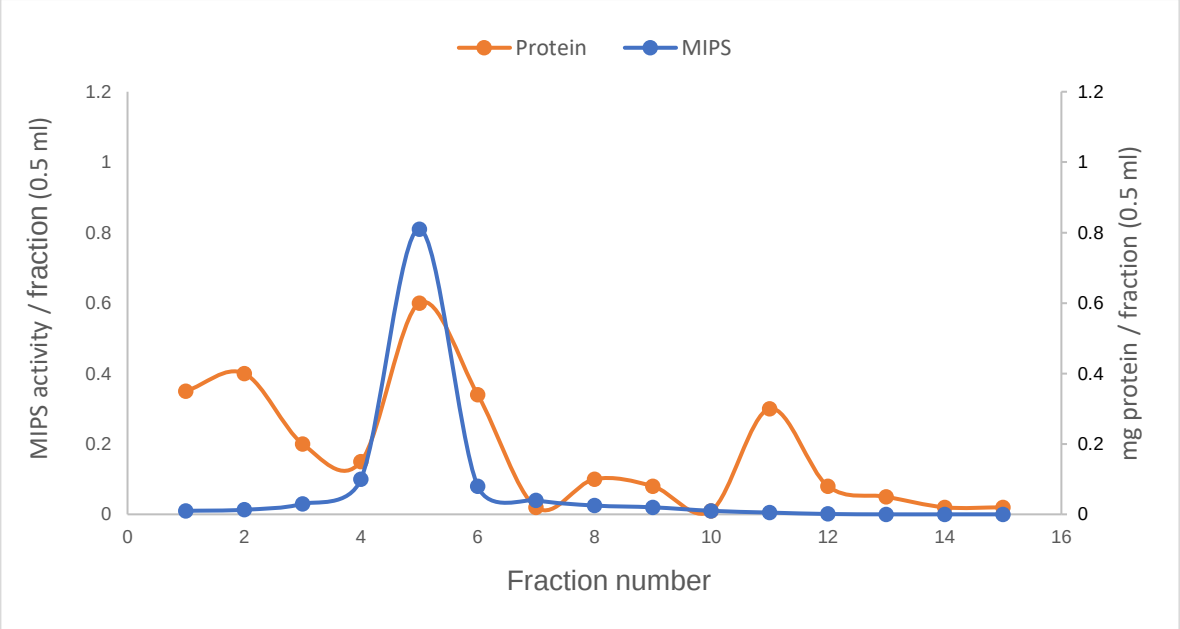


Figure 3.6. Elution profile of chloroplastic MIPS in BioGel A-0.5m column. MIPS activity presented as µ mol I-1-P produced fraction^{-1} h^{-1}

3.2.5. 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:

3.2.5.1 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 set 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.

3.2.5.2. 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 (freshly prepared) 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 Pi / 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.

3.2.6. Estimation of inorganic phosphate

Inorganic phosphate was estimated by the method of Chen *et al.*, (1956) with minor modifications. Pi-reagent (2.8 ml) was added to the reaction mixture (total volume now became 5.6 ml) and incubated at 37ºC for 60 minutes. The Pi 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 Thermo Scientific Evolution 201 UV-Visible 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.

3.2.7. Estimation of protein

Protein was estimated by the method of Bradford (1976) with minor modifications. BSA was used as standard. Dilutions of known amount of BSA were made with distilled water within 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), 5ml of Bradford reagent was added, mixed thoroughly and the absorbance was measured at 595 nm with a Thermo Scientific Evolution 201 UV-Visible spectrophotometer after 5 min of incubation. A blank reagent was also prepared with 100 µL of distilled water.

3.2.8. 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.

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.

3.2.9. Determination of molecular weight of L-*myo*-inositol-1-phosphate synthase

Approximate molecular weight of the native inositol synthase obtained from the leaves of *H. salicifolia* was determined by gel-filtration through Sephadex G-200. The Sephadex G-200 was suspended in 50 mM Tris- acetate (pH 7.5) and packed in a column of suitable size and calibrated with 1 ml each of marker proteins e.g., 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 (0.75 ml) were collected at a flow rate of 0.75 ml / 6 min. Each individual protein peak was located by spectrophotometric scanning at 280 nm in a Beckman DU-64 Spectrophotometer. A standard curve was prepared by plotting relative elution volume of proteins against their respective log molecular weights.

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. 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.

3.2.10. Extraction and estimation of free *myo*-inositol

3.2.10.1. Isolation of free *myo*-inositol

Free *myo*-inositol was estimated by the method of Charalampous and Chen (1966) with minor modifications. One gram of sample 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 run through a mixed bed column 0.3 × 1.5 cm each of Dowex-1-Cl- (100-200 mesh) and Amberlite IR-120 (sodium form). 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 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 then carefully washed and dissolved in 200 µL of water. From this solution inositol and sugars were separated with the help of paper chromatography.

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 H2O). The chromatography was carried out at room temperature for 2-4h

using acetone:water (85:15 v/v) as the solvent. On completion of the solvent run, the chromatography paper was taken out from the set and dried using a blower. 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 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 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 patched area turned out to be stained. In order to isolate the free *myo*-inositol from the unstained chromatogram, the paper strip corresponding to the inositol location was progressively eluted with 1 ml H2O inside a tiny chamber.

3.2.10.2. Estimation of free *myo*-inositol

Free *myo*-inositol was estimated spectrophotometrically according to the method of Gaitonde and Griffiths (1966) where 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, 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 Thermo-Scientific Evolution 201 UV- visible spectrophotometer after 5 min of incubation.

Inositol concentration was determined by extrapolation from a standard curve that was prepared using known concentrations of *myo*-inositol (0-100 µg).

3.2.11. Characterization of L-*myo*-inositol-1-phosphate synthase

3.2.11.1. Determination of essential requirements for the enzyme activity

For determining the essential assay components in the reaction mixture, the experiment was designed such that out of the standard assay mixture containing 50 mM Tris-acetate buffer (pH 7.5), 14 mM NH_4Cl, 0.8 mM NAD, 5mM ME and 5mM G-6-P and a protein aliquot, one component was not added in a set. In the next set, another component was absent and so on. The results in terms of specific activity were compared with the complete set.

3.2.11.2. Relation of enzyme activity with the duration of incubation

To determine the progress of the enzyme reaction with relation to incubation time, the standard assay mixtures as mentioned above was allowed to react for a period of 150 minutes. The specific activity of the enzyme was measured at the starting point considered as '0' minute with each interval of 30 minutes.

3.2.11.3. Progress of enzyme activity with increasing protein concentration

The enzyme activity was determined with different enzyme protein concentration to the tune starting from 0 to 400µg. Different set of experiments were conducted with gradually increasing protein concentration at each interval of 50 µg reaching upto 400µg and the specific activity of the enzyme was calculated for each set.

3.2.11.4. Relationship of increasing incubation temperature with enzyme activity

To determine the enzymatic responses of L-*myo*-inositol-1-phosphate synthase from *H. salicifolia*, the reaction mixtures under standard assay conditions were incubated

separately at different temperatures starting from 0°C to 60°C with an interval of 100°C, i.e., different sets were incubated separately at 0, 10, 20, 30, 40, 50 and 60°C respectively. Specific activity was calculated for each set of different temperatures.

3.2.11.5. Determination of substrate specificity

To determine whether, Glucose-6-phosphate is the exclusive substrate for the enzyme activity or other hexose phosphates or bisphosphates may also be the substrate of the MIPS from *H. salicifolia*, an experiment was designed as follows:

Under standard assay conditions, the enzyme was incubated separately with similar concentrations (10 mM) of different substrates or potential substrates viz., D-Glucose-6-phosphate, D-Fructose-6-phosphate, D-Galactose-6-phosphate, D-Mannose-6-phosphate, D-Fructose-1,6-bisphosphate etc. After incubation for the designated time (60 min) as per the standard assay condition, the specific activity of each of the above set was estimated.

3.2.11.6. Effect of substrate concentration and determination of V_{max} and K_m values

The BioGel A-0.5m purified L-*myo*-inositol-1-phosphate synthase from *H. salicifolia* was assayed with the different concentrations of D-glucose-6-phosphate in different sets. Varying concentration of the substrate, G-6-P between 0 to 10 mM was added with all the standard assay mixture and allowed to react with the enzyme extract for the designated time period after which the specific activity was determined. The maximum specific activity at any particular G-6-P concentration denotes the V_{max} value. The average K_m for G-6-P for *H. salicifolia* MIPS was determined in accordance with the rate equation of Michaelis Menten for enzyme kinetics as follows:

$V/V_{max} = [S]/Km + [S]$

where, [S] = substrate concentration

V = Specific activity of the enzyme

V_{max} = Maximum specific activity of the enzyme

The average Km value was calculated from the mean of three values (other than that of the V_{max}) determined as above.

3.2.11.7. Effect of co-enzyme concentration and determination of V_{max} and K_m values

The activity of BioGel A-0.5m purified L-*myo*-inositol-1-phosphate synthase from *H. salicifolia* as determined with respect to the concentration of the co-enzyme (NAD^+) between concetration ranges of 0 to1.0 mM mM as per the method mentioned above for the determination of the effect of substrate concentration. The maximum specific activity at any particular NAD^+ concentration denotes the *Vmax* value. The average *Km* for G-6-P for *H. salicifolia* MIPS was determined in accordance with the rate equation of Michaelis Menten tor enzyme kinetics as follows.

$V/V_{max} = [S]/Km + [S]$

where, [S] = substrate concentration

V = Specific activity of the enzyme

V_{max} = Maximum specific activity of the enzyme

The average K_m value was calculated from the mean of three values (other than that of the V_{max}) determined as above.

3.2.11.8. Effect of replacement of coenzyme NAD^+ with $NADP^+$

To determine if the coenzyme NAD^+ is specific for the enzyme *myo*-inositol synthase from *H. salicifolia* or if its requirement can be satisfied by similar type of coenzymes, an assay was performed in which NAD^+ was replaced with $NADP^+$ in the assay mixture. The

enzyme activity was determined in presence of 0 to 1.0 mM $NADP^+$ whose results were compared to that of the enzyme activity in the presence of 0-1.0mM NAD^+.

Specific activity was calculated for each set.**3.2.11.9. Effect of different pH on enzyme activity**

The activity of L-*myo*-inositol-1-phosphate synthase from *H. salicifolia*, was assayed under standard conditions by using 50 mM Tris-acetate buffer between the pH ranges of 6.0 to 9.0 with an interval of 0.5 pH.

Specific activity was calculated for each set with that were incubated with different pH.

3.2.11.10. Effect of different salts

To estimate the effect of different salts on the *H. salicifolia* MIPS activity. Different concentrations (0-100mM at an interval of each 20 mM) of $MgCl_2$, NH_4Cl and EDTA were added separately in the reaction mixture in place of 14 mM NH_4Cl and incubated for the designated time before calculating the specific activity of each set.

3.2.11.11. Estimation of the influence of monovalent and divalent cations on enzyme activity

In the standard reaction mixture 14 mM NH4Cl was replaced separately by 0-10mM (at an interval of 2 mM each) the chloride salts of K^+, Na^+, Li^+, Ca^{+2}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+} and Hg^{2+} and the reaction was run for standard time. MIPS activity was calculated for each of the set.

3.2.11.12. Determination of the effects of sugar-alcohols

To determine the effect of the of sugar alcohols, the 14mM NH_4Cl was replaced separately by different concentrations (0-10 mM at an interval of 2mM each) of sugar

alcohols like galactitol, *myo*-inositol and mannitol and the enzyme activity was calculated for each set.

4. RESULTS

4.1 Estimation of free *myo*-inositol content in different plant species

Free *myo*-inositol was estimated from fresh leaf specimens of *Hippophae Salicifolia* (Elaeagnaceae), *Primula denticulata* (Primulaceae), *Bistorta emodi (*Polygonaceae*)*, *Pedicularis siphonantha* (Orobanchaceae), *Argentina lineata* (Rosaceae), *Koenigia mollis* (Polygonaceae), *Hellenia speciosa* (Costaceae)*, Houttuynia cordata (*Saururaceae), *Euphorbia hirta* (Euphorbiacaea) and *Solanum viarum* (Solanaceae) collected from different altitudes of the Sikkim Himalaya. Collection of the specimen was done according to section 3.1. Isolation and estimation of free *myo*-inositol was carried out according to the method described in section 3.2.10. Appreciable quantity was detected in these species with minor variations.

The results have been tabulated in Table 4.1 (Summer) and Table 4.2 (Monsoon) from which the quantity of free *myo*-inositol in almost all the species tested were observed to be quantitatively appreciable. Maximum free *myo*-inositol content was detected in *P. siphonantha* i.e.,1.82mg/g FW, followed by *H. salicifolia* in summer while in monsoon the maximum detected was 1.64mg/g FW in *H. salicifolia*. In the samples collected in monsoon, the highest free *myo*-inositol content was found in *H. salicifolia* (1.22mg/g FW). Similarly, seasonal variation of free *myo*-inositol content was observed across different species from different altitudinal zones.

Table-4.1 Distribution of free *myo*-inositol in different plant species collected during summer (values are mean ±SE), FW=fresh weight.

Altitudinal range	**Family**	**Plant species**	**Plant part**	**Free *myo*-inositol content(mg/g)**
Alpine	Orobanchaceae	*Pedicularis siphonantha* D. don	leaf	**1.82 ± 0.20**
	Primulaceae	*Primula denticulata* Wight	leaf	1.18 ± 0.33
	Polygonaceae	*Bistorta emodi* Petrov	leaf	1.45 ± 0.08
	Rosaceae	*Argentina lineata* (Trevir.) Soják.	leaf	1.56 ± 0.11
Temperate	Elaeagnaceae	*Hippophae salicifolia* D. don	leaf	1. 64 ± 0.11
	Polygonaceae	*Koenigia mollis* (D.Don) T.M.Schust. & Reveal	leaf	1.29 ± 0.12
	Costaceae	*Hellenia speciosa* (J.Koenig) S.R.Dutta	leaf	0.86 ± 0.05
Tropical	Saururaceae	*Houttuynia cordata* Thunb.	leaf	1.27 ± 0.31
	Euphorbiacaea	*Euphorbia hirta* L.	leaf	0.96 ± 0.27
	Solanaceae	*Solanum viarum* Dunal	leaf	1.48 ± 0.32

Table-4.2 Distribution of free *myo*-inositol in different plant species during monsoon (values are mean ±SE), FW=fresh weight.

Altitudinal range	**Family**	**Plant species**	**Plant part**	**Free *myo*-inositol content (mg/g)**
Alpine	Orobanchaceae	*Pedicularis siphonantha* D. don	leaf	0.60 ± 0.04
	Primulaceae	*Primula denticulata* Wight	leaf	1.00 ± 0.01
	Polygonaceae	*Bistorta emodi* Petrov	leaf	0.97 ± 0.06
	Rosaceae	*Argentina lineata* (Trevir.) Soják.	leaf	0.45 ± 0.09
Temperate	Elaeagnaceae	*Hippophae salicifolia* D. don	leaf	**1.22 ± 0.02**
	Polygonaceae	*Koenigia mollis* (D.Don) T.M.Schust. & Reveal	leaf	0.87 ± 0.11
	Costaceae	*Hellenia speciosa* (J.Koenig) S.R.Dutta	leaf	0.42 ± 0.02
Tropical	Saururaceae	*Houttuynia cordata* Thunb.	leaf	0.92 ± 0.06
	Euphorbiacaea	*Euphorbia hirta* L.	leaf	0.51 ± 0.06
	Solanaceae	*Solanum viarum* Dunal	leaf	0.58 ± 0.11

4.2. Distribution of L-*myo*-inositol-1-phosphate synthase in different plant species:

Plant samples of *Hippophae salicifolia* (Elaeagnaceae), *Primula denticulata* (Primulaceae), *Bistorta emodi (*Polygonaceae*)*, *Pedicularis siphonantha* (Orobanchaceae), *Argentina lineata (*Rosaceae*), Koenigia mollis (*Polygonaceae*), Hellenia speciosa (*Costaceae*), Houttuynia cordata (*Saururaceae), *Euphorbia hirta* (Euphorbiacaea), *Solanum viarum* (Solanaceae) collected from different altitudes of Sikkim Himalaya for two seasons *viz*., summer (June) and monsoon (August). Collection of the specimen was done according to section 3.1. L-*myo*-inositol-1-phosphate synthase

was assayed according to the method described in section 3.2.5. Appreciable activity of MIPS was detected in most of these species.

The results presented in Table 4.3 and Table 4.4 show that the enzyme is active in all the plants species collected from different altitudinal zones belonging to different families. It has also been observed that the enzyme activity was the highest in *Hippophae salicifolia* in summer [0.397 µmol I-1-P produced $(mg)^{-1}$ protein h^{-1}] and in monsoon [0.288 µmol I-1-P produced $(mg)^{-1}$ protein h^{-1}]. Whereas the lowest enzyme activity was seen in *Houttuynia cordata* in June and *Hellenia speciosa* in the month of August.

Table-4.3. Distribution of L-*myo*-inositol-1-phosphate synthase in different plant species from different altitudinal range collected during summer. (Specific activity in µ mol I-1-P produced (mg protein)-1 h-1. FW = Fresh Weight)

Sl. No.	**Plant**	**Family**	**Plant part**	**Specific activity [µmol I-1-P produced $(mg)^{-1}$ protein h^{-1}]**
1	*Hippophae salicifolia* D. don	Elaeagnaceae	leaf	**0.397 ± 0.016**
2	*Primula denticulata* Wight	Primulaceae	leaf	0.378 ± 0.022
3	*Bistorta emodi* Petrov	Polygonaceae	leaf	0.207 ± 0.018
4	*Pedicularis siphonantha* D. Don	Orobanchaceae	leaf	0.329 ± 0.001
5	*Argentina lineata (Trevir.) Soják. (Rosaceae),)*	Rosaceae	leaf	0.219 ± 0.041
6	*Koenigia mollis (D.Don) T.M.Schust. & Reveal*	Polygonaceae	leaf	0.293 ± 0.002
7	*Hellenia speciosa (J.Koenig) S.R.Dutta*	Costaceae	leaf	0.209 ± 0.023
8	*Houttuynia cordata* Thunb.	Saururaceae	leaf	**0.163 ± 0.005**
9	*Euphorbia hirta* L.	Euphorbiacaea	leaf	0.198 ± 0.005
10	*Solanum viarum* Dunal	Solanaceae	leaf	0.243 ± 0.046

Table-4.4. Distribution of L-*myo*-inositol-1-phosphate synthase in different plant species from different altitudinal range collected during monsoon. (Specific activity in µ mol I-1-P produced (mg protein)-1 h-1. FW = Fresh Weight)

Sl. No.	**Plant**	**Family**	**Plant part**	**Specific activity [µmol I-1-P produced (mg)$^{-1}$ protein h^{-1}]**
1	*Hippophae salicifolia* D. don	Elaeagnaceae	leaf	**0.288 ± 0.100**
2	*Primula denticulata* Wight	Primulaceae	leaf	0.267 ± 0.121
3	*Bistorta emodi* Petrov	Polygonaceae	leaf	0.211 ± 0.080
4	*Pedicularis siphonantha* D. don	Orobanchaceae	leaf	0.223 ± 0.007
5	*Argentina lineata (Trevir.) Soják. (Rosaceae),)*	Rosaceae	leaf	0.198 ± 0.030
6	*Koenigia mollis (D.Don) T.M.Schust. & Reveal*	Polygonaceae	leaf	0.222 ± 0.008
7	*Hellenia speciosa (J.Koenig) S.R.Dutta*	Costaceae	leaf	**0.162 ± 0.007**
8	*Houttuynia cordata* Thunb.	Saururaceae	leaf	0.163 ± 0.015
9	*Euphorbia hirta* L.	Euphorbiacaea	leaf	0.175 ± 0.008
10	*Solanum viarum* Dunal	Solanaceae	leaf	0.200 ± 0.062

4.3. Probable occurrence of two forms of L-*myo*-inositol-1-phosphate synthase in *H. salicifolia*

In some plants, L-*myo*-inositol-1-phosphate synthase has been detected in two forms, both soluble and particulate (chloroplastic). Whether such isoforms are present in *H. salicifolia* or not, has still not been reported. However, such probability cannot be ruled out, as this is also a green plant. In order to answer this question, a preliminary experiment was designed where the activity of the enzyme was assayed in dialyzed homogenate and low-speed supernatant fractions obtained from the juvenile leaves (chloroplast dominating) of this plant species following the methods described in sections 3.2.5. The results are depicted in Table 4.8. The most striking observation is with the higher activity of this enzyme in homogenate than in low-speed supernatant fraction. This indicates a probability of occurrence of a particulate form of this enzyme. The probable occurrence of chloroplastic enzyme will be dealt in details later in this section.

4.4. Partial purification of cytosolic L-*myo*-inositol-1-phosphate synthase from *Hippophae salicifolia*

For the partial purification of L-*myo*-inositol-1-phosphate synthase from the leaves of freshly collected *H. salicifolia,* in the month of August was used. Isolation and purification were carried out by the standard procedure described in Sections 3.2.2. Table 4.5 shows the results procured after partial purification of the enzyme from the cytoplasm of *H. salicifolia*. The table show that the enzyme from cytoplasm could be purified approximately 64.46 times over the homogenate fraction, and the enzyme recovery percentage based on total activity was discovered to be about 21%.

Table-4.5. Partial purification of cytosolic MIPS from *H. salicifolia* [Specific activity defined as μ mol I-1-P produced (mg protein)-1 h-1] *(Values are mean ± SE, n=3)*

Purification step	**Protein content (mg)**	**Specific activity**	**Total activity**	**Recovery (%)**	**Purification (fold)**
Homogenate	12.05 ± 0.31	0.100 ± 0.02	157.3 ±1.02	100	1
10K supernatant	8.46 ± 0.17	0.161 ± 0.01	147.97 ± 1.19	94.07	1.61
SS-fraction	6 ± 0.06	0.26 ± 0.022	135.96 ± 0.30	86.43	2.58
A2S fraction	3.03 ± 0.13	0.56 ±0.065	119.05 ± 0.47	75.68	5.58
DE-fraction	1.023 ± 0.02	1.99 ± 0.081	112.42 ± 0.16	71.47	19.85
Hxl- fraction	0.834 ± 0.04	3.35 ± 0.19	101.92 ± 0.11	64.79	33.43
BioGel- fraction	0.482 ± 0.02	6.47 ± 0.92	33.38 ±0.04	21.22	64.46

4.5. Characterization of cytosolic MIPS from *H. salicifolia*

4.5.1. PAGE profile and corresponding enzymic activity

PAGE was carried out under native condition as described in section 3.2.3. Fig. 4.1 shows the PAGE profile of the protein as well as the MIPS activity of the protein bands determined from 5mm gel slice cut of the BioGel A 0.5m fraction of *H. salicifolia* leaves. Only one band exhibited enzymic activity and Fig.4.1 shows the matching protein band that accordingly showcased the MIPS activity.

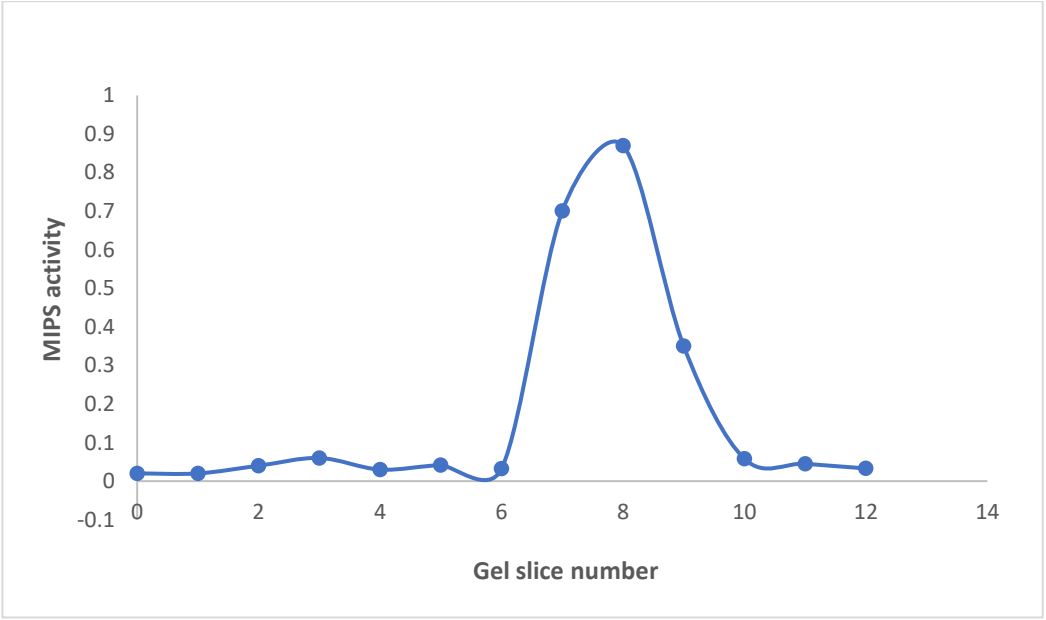


Fig. 4.1 PAGE profile showing cytosolic MIPS activity from *H. salicifolia.*

4.5.2. Molecular weight of the enzyme L-*myo*-inositol-1-phosphate synthase from *H. salicifolia*

The procedure outlined in Section 3.2.9. was employed to determine the apparent molecular weight of L-*myo*-inositol-1-phosphate synthase from *H. salicifolia.*

The molecular weight of the enzyme was found to be approximately 158.6kDa (Fig. 4.2).

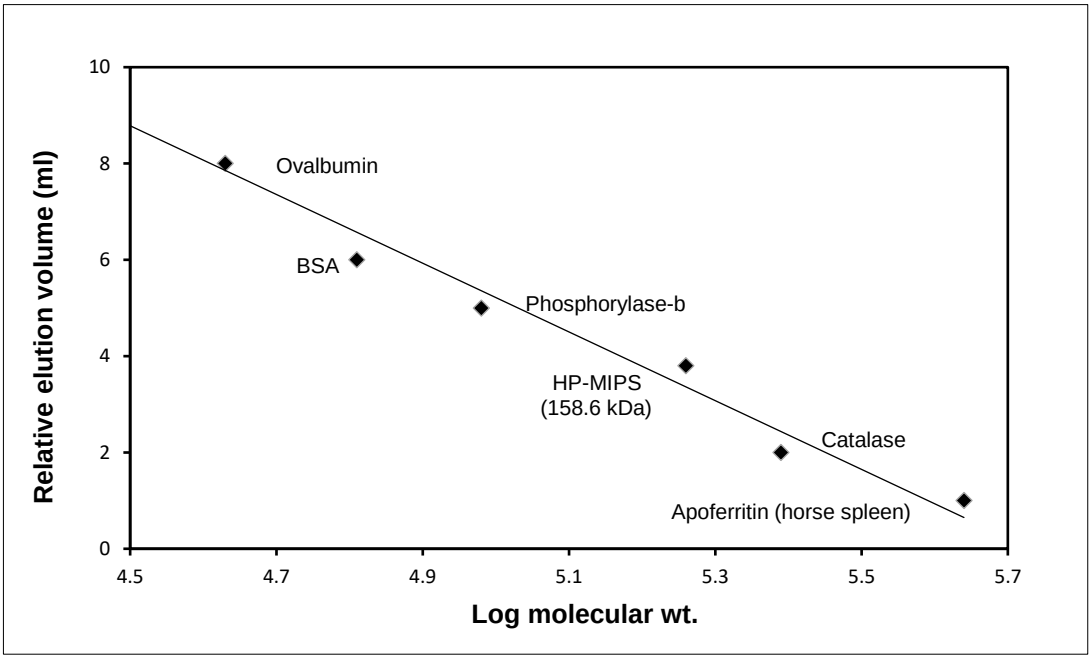


Fig.4.2. Determination of molecular weight of MIPS from *H. salicifolia*.

Figure in parenthesis denotes molecular weight in kilo Daltons. (HP-MIPS: *Myo* inositol-1-phophate synthase from *H. salicifolia*= 158.6 kDa)

4.5.3. Effect of different assay components on cytosolic MIPS activity from *H. salicifolia*

Cytosolic MIPS activity, when assayed in the complete set containing 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 with an appropriate protein aliquot showed maximum activity which is presented in Table 4.6.

The enzymatic synthesis of L-*myo*-inositol-1-phosphate could not be detected when the specific substrate of the enzyme, G-6-P was not added in the incubation mixture and also when the enzymes were destroyed using heat. Almost 20% activity was lost when Tris buffer was excluded from the complete set of reaction mixture. Likewise, exclusion of NAD^+ caused 67.15% loss of activity. Similarly, NH_4Cl and ME omission caused 39.83% and 45.41% decrease in MIPS activity respectively.

Table-4.6 Effect of composition of incubation medium on cytosolic MIPS activity in *H. salicifolia.* [Specific activity defined as μ mol I-1-P produced (mg protein)-1 h-1] *(Values are mean ± SE, n=3)*

Conditions	Specific activity	Percent activity
Complete set	2.118 ± 0.013	100 ± 1.88
Without substrate (G-6-P)	0.00	0
Without co-enzyme (NAD^+)	0.456 ± 0.022	32.85 ± 0.21
Without NH_4Cl	0.845 ± 0.003	60.17 ± 0.65
Without ME	0.803 ± 0.001	56.59 ± 0.44
Without buffer (tris- acetate)	0.936 ± 0.021	80.98 ± 0.58
Heat killed enzyme	0.00	0

4.5.4. Progress of the enzyme reaction with time

L-*myo*-inositol-1-phosphate synthase of *H. salicifolia* was incubated at different time periods between 0 and 150 min at an interval of 30 min each that was carried out under standard assay conditions. The reaction was observed to progress linearly with time upto 90 min (Fig. 4.3) after which the activity of the enzyme did not show much variation in the last three incubation interval in the MIPS extracted from cytoplasm.

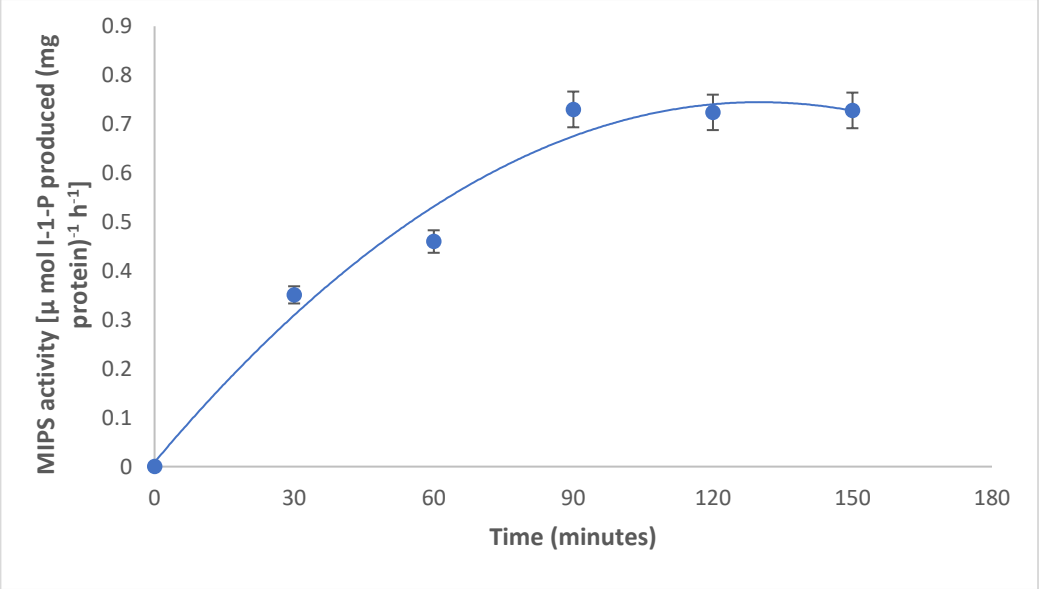


Fig. 4.3 Effect of incubation time on cytosolic MIPS activity from *H. salicifoia*

4.5.5. Progress of enzyme activity with increasing protein concentration

Cytosolic MIPS assay from *H. salicifolia* was carried out under optimal conditions using different concentrations of enzyme protein (0-400µg).

Fig. 4.4. shows the results where the enzyme activity was seen to increase suddenly up to a protein concentration of 100µg. And then the enzyme activity beyond this concentration seemed to increase slowly up until 300 µg and beyond this point the activity remained constant. Increase in protein concentration beyond this optimum concentration did not influence the enzyme activity.

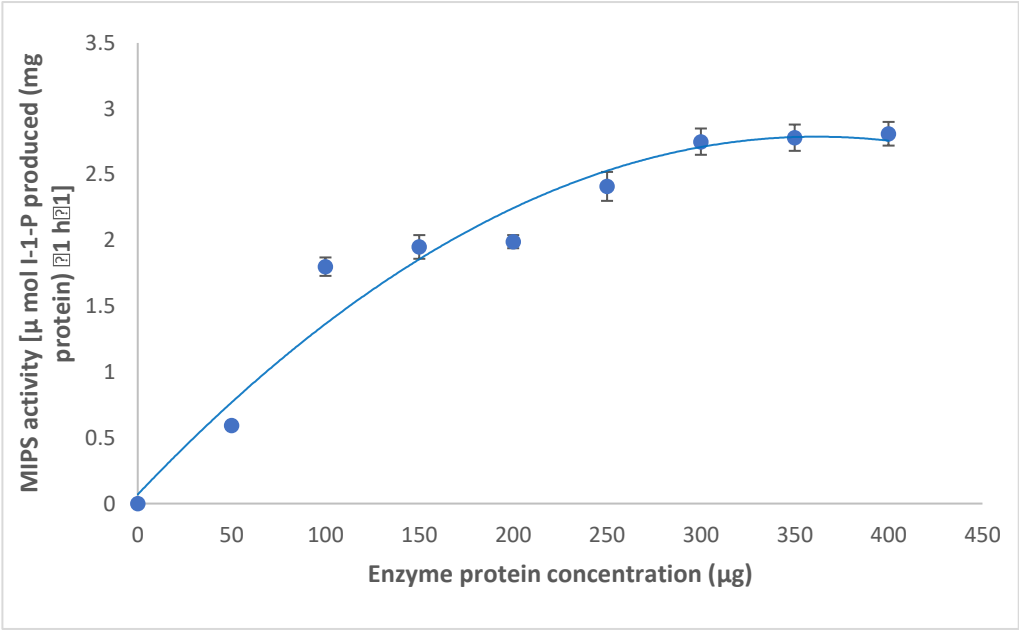


Fig. 4.4 Effect of different enzyme protein concentration on cytosolic MIPS activity from *H. salicifolia*

4.5.6. Progress of enzyme activity with increasing incubation temperature

Cytosolic MIPS activity from *H. salicifolia* was observed by incubating separately for one hour at temperature ranges of 0ºC to 60ºC at an interval of 10°C in presence of standard assay mixture.

Results depicted in Fig. 4.5, shows that the activity of the enzyme from cytoplasm was observed to be highest between 20ºC to 40ºC. It was observed that the enzyme activity was similar at this wide range of temperature. This is a unique observation in terms of its optimum functionality across a broad spectrum of incubation temperature showing temperature maximum at 30 ºC.

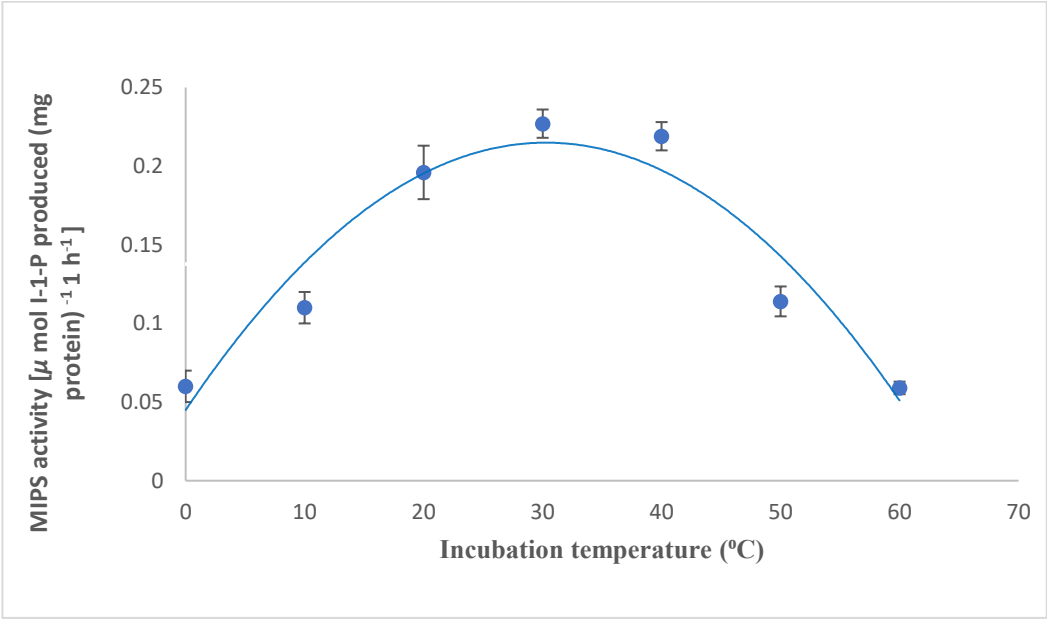


Fig-4.5. Effect of various incubation temperatures on cytosolic MIPS activity from *H. salicifolia*

4.5.7. Effect of Substrate specificity

D-glucose-6-phosphate has been discovered to be the exclusive substrate utilized by the enzyme L-*myo*-inositol-1-phosphate synthase in order to produce L-*myo*-inositol-1-phosphate. Hence, to confirm its substrate specificity, hexose phosphates, like, D-fructose-6-phosphate, D-galactose-6-phosphate, D-mannose-6-phosphate and hexose bisphosphate like D-fructose-1,6-bisphosphate were switched in place of D-glucose-6-phosphate at the identical concentration (10 mM).

No activity was seen in case of D-fructose-6-phosphate, D-fructose-1,6-bisphosphate, and D-glucose-1-phosphate as substrate for the MIPS activity in *H. salicifolia*. The enzyme could, however, only partially make use of D-galactose-6-phosphate and D-mannose-6-phosphate as substrate with about 7.5 % and 4.16 % efficiency, as compared to the activity where D-glucose-6-phosphate was utilized as substrate (Table 4.7).

Table-4.7. Substrate specificity of L-*myo*-inositol-1-phosphate synthase from *H. salicifolia* (cytoplasm) [Specific activity defined as μ lmo I-1-P produced (mg protein)-1 h-1] *(Values are mean ± SE, n=3)*

Compound	Conc. (mM)	Specific activity	Percent activity
D-Glucose-6-phosphate	10	0.898	100
D-Fructose-1,6-bisphosphate	10	0	0
Galactose-6-phosphate	10	0.066	7.5
D-Fructose-6-phosphate	10	0	0
D-Glucose-1-phosphate	10	0	0
D-mannose-6-phosphate	10	0.03	4.16

4.5.8. Effect of D-glucose-6-phosphate (substrate) concentration on cytosolic MIPS from *H salicifolia*: Determination of *K*m and *V*max values

4.5.8.1 Effect of substrate concentration

The activity of BioGel A0.5m purified *H. salicifolia* MIPS was found to increase with respect to the concentration of D-glucose-6-phosphate upto 8 mM when tried between concentration ranges of 0 to 10 mM of the substrate. Thereafter, the enzymatic activity did not undergo any noteworthy alteration (Fig. 4.6).

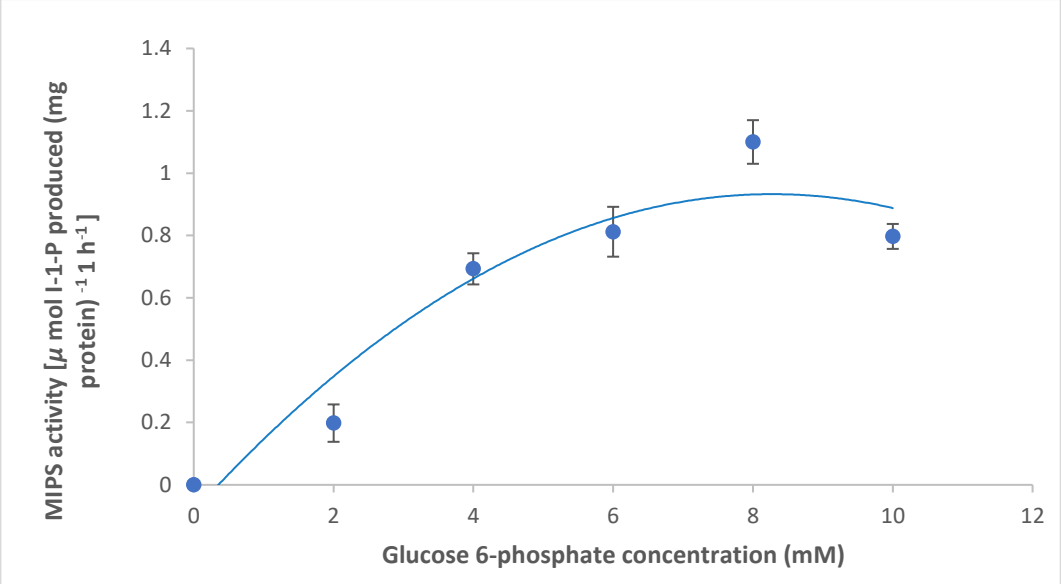


Fig. 4.6 Effect of substrate (G-6-P) concentration on cytosolic MIPS activity from *H. salicifolia*

4.5.8.2 Determination of K_m and V_{max} values (cytosolic MIPS)

The average K_m for D-Glucose-6-Phosphate for *H. salicifolia* MIPS extracted from cytoplasm was calculated to be approximately 0.55 mM in accordance with the rate equation of Michaelis-Menten for enzyme kinetics. The V_{max} value was calculated as 1.1 mM.

4.5.9. Effect of NAD^+ (co-enzyme) concentration on cytosolic MIPS activity from *H. salicifolia*: Determination of K_m and V_{max} values

4.5.9.1. Effect of coenzyme concentration

The activity of cytosolic MIPS from *H. salicifolia* was assayed according to standard procedure with varying co-factor concentration ranging from 0 to 1.0 mM NAD^+. The catalytic activity of the enzyme was found to increase linearly with respect to the concentration of NAD^+ up to 0.6 mM, beyond which the graph formed a plateau (Fig 4.7).

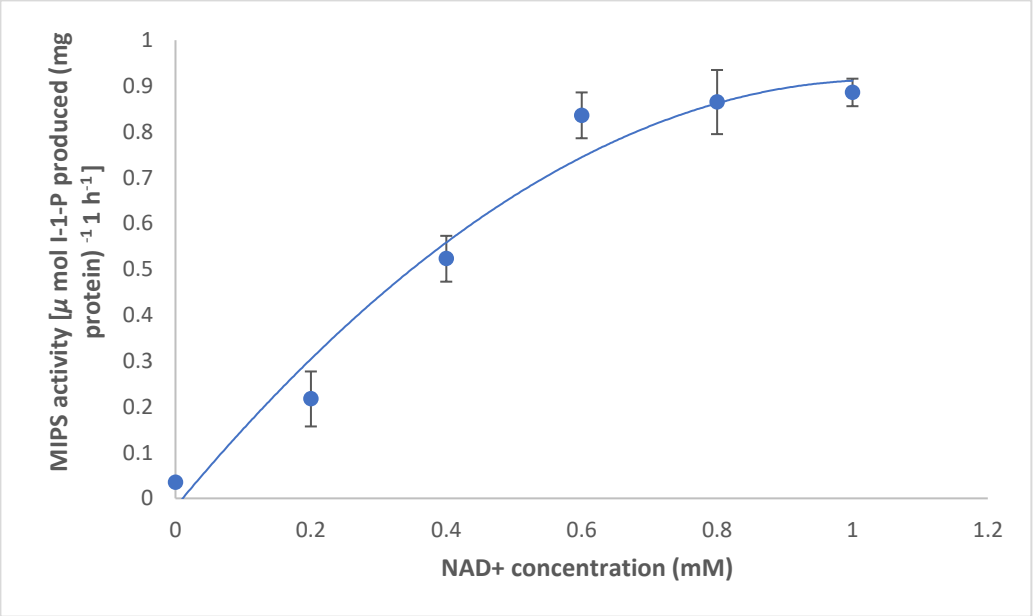


Fig. 4.7 Effect of different co-enzyme (NAD^+) concentration on cytosolic MIPS activity from *H. salicifolia*

4.5.9.2. Determination of K_m and V_{max} values

The average K_m for NAD^+ of *H. salicifolia* L-*myo*-inositol-1-phosphate synthase for cytoplasmic MIPS was calculated to be approximately 0.21 mM in accordance with the rate equation of Michaelis-Menten for enzyme kinetics. In the mean time the V_{max} was calculated as 0.83 mM for cytosolic MIPS activity from *H. salicifolia*.

4.5.10. Effect of replacement of NAD^+ with $NADP^+$

To check if the coenzyme NAD^+ is specific for the enzyme MIPS from *H. salicifolia* or if its requirement can be satisfied by similar type of coenzymes, an assay was performed in which NAD^+ was replaced with $NADP^+$ in the assay mixture. The enzyme activity was assessed in the presence of 0 to 1.0 mM $NADP^+$ and the obtained outcomes were compared with those of the enzyme activity in the presence of 0-1.0mM NAD^+ as depicted in Figure 4.8.

The findings indicated that NAD^+, which serves as the coenzyme for the enzyme MIPS from *H. salicifolia*, exhibited an inability to be replaced by $NADP^+$ at any given concentration. The highest peak of enzyme activity was noted when the concentration of NAD^+ in the cytoplasmic enzyme reached 0.6mM and 0.8mM. Nevertheless, a basal enzyme activity was detected in the experimental group across various concentrations of $NADP^+$.

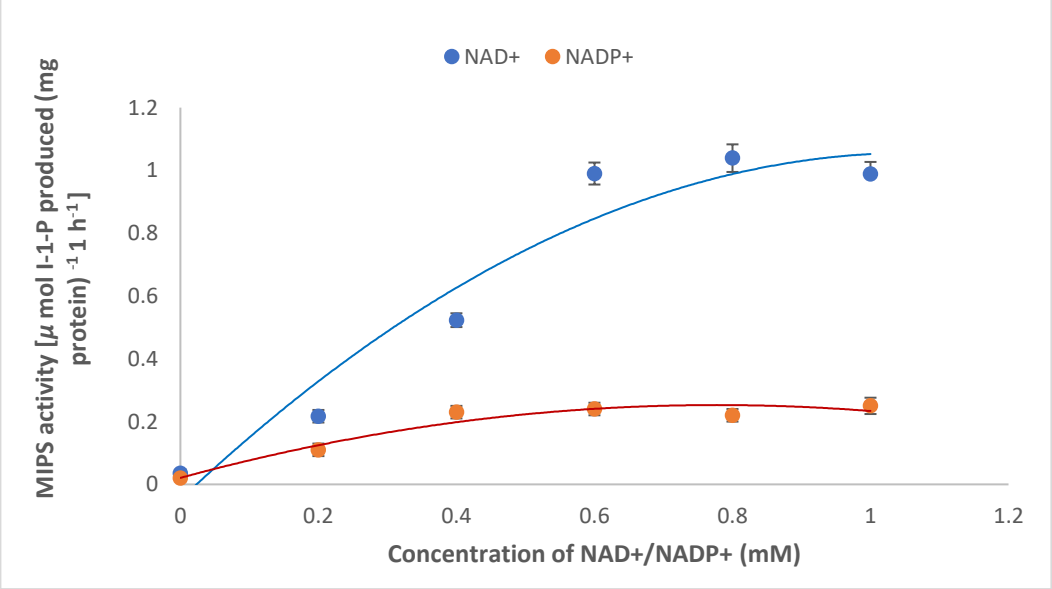


Fig. 4.8 Effect of replacement of co-enzyme NAD^+ with $NADP^+$ in cytosolic MIPS from *H. salicifolia*.

4.5.11. Effect of different pH on MIPS activity

The cytosolic enzyme MIPS from *H. salicifolia*, was assayed under standard conditions by using 50 mM Tris-acetate buffer between the pH ranges of 6.0 to 9.0. The influence of pH variation on its activity was found to be remarkably evident.

Fig. 4.9 illustrates the findings that demonstrate the heightened activity of the cytosolic MIPS from *H. salicifolia* enzyme within the pH range of 6.5 to 7.5. The enzyme exhibited its optimal activity at a pH of 7.0-7.5. However, any pH below 6.5 or above 7.5 resulted in a significant decline in the enzyme's catalytic activity.

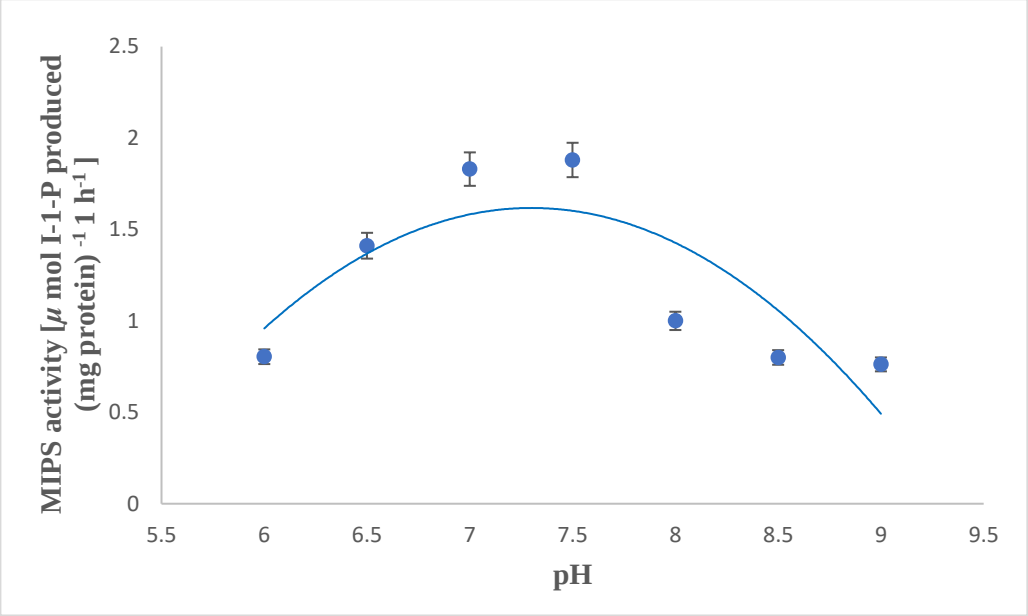


Fig. 4.9. Effect of pH on cytosolic MIPS from *H. salicifolia*

4.5.12. Effect of different salts, $MgCl_2$, NH_4Cl and EDTA

To find out the effect of varied concentration of different salts viz., $MgCl_2$, NH_4Cl and EDTA on *H. salicifolia* 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.

Like in most instances the reports were found to have a positive effect in case of $MgCl_2$ and NH_4Cl and negative in case of EDTA. The enzyme activity was amplified up to 6.12-fold and 3.14-fold by the influence of NH_4Cl and $MgCl_2$ respectively at its maximum concentration (100mM) as depicted in the Fig. 4.10. However, in the case of $MgCl_2$ it is noteworthy that the stimulation of the enzyme activity was minimal up to a concentration of 80mM after which the activity spikes. While in case of NH_4Cl the vertical spike happens after 60mM concentration. On the other hand, the influence of EDTA at its highest concentration of 100mM decreased the enzyme activity to roughly 30.63%. These results were observed in the MIPS extracted from cytoplasm.

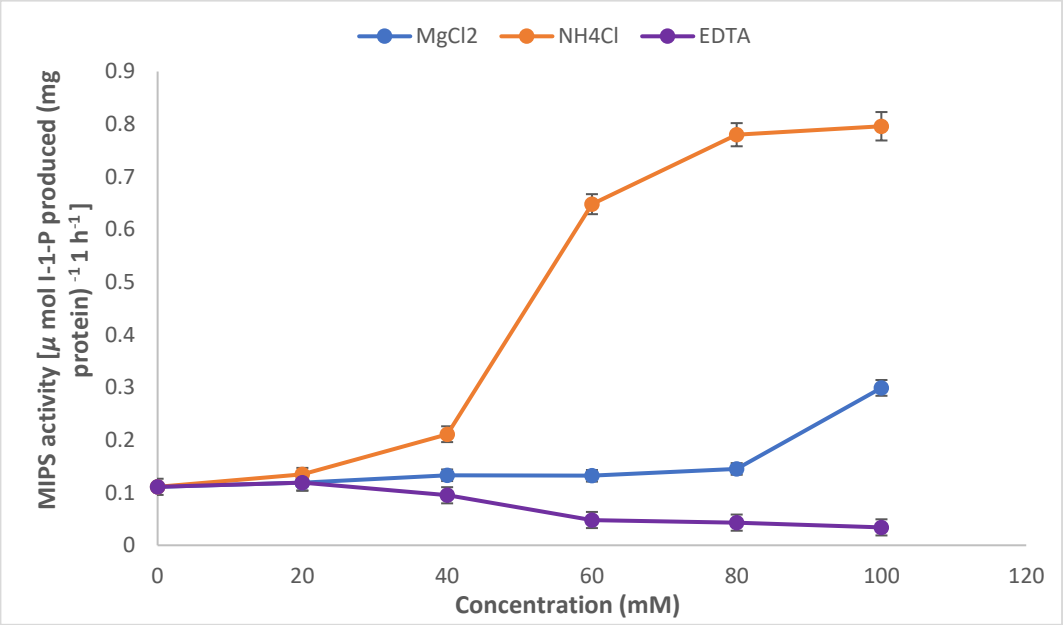


Fig. 4.10 Effect of different concentrations of salts on cytosolic MIPS from *H. salicifolia*

4.5.13. Effect of monovalent cations

Effects of some monovalent cations on cytosolic MIPS activity from *H. salicifolia* 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 was estimated keeping one control set without adding any such cation.

Results of the experiment is presented in Fig. 4.11. It is evident that the K^+ had a stimulatory effect on this enzyme activity and increased the activity by 2.55%. While the Na^+ played a remarkable inhibitory role decreasing the enzyme activity by approximately 68.75% at 10 mM concentration. Furthermore, Li^+ was a strong inhibitor of this enzyme, which could reduce the specific activity by about 85% at 10 mM concentration in cytosolic MIPS.

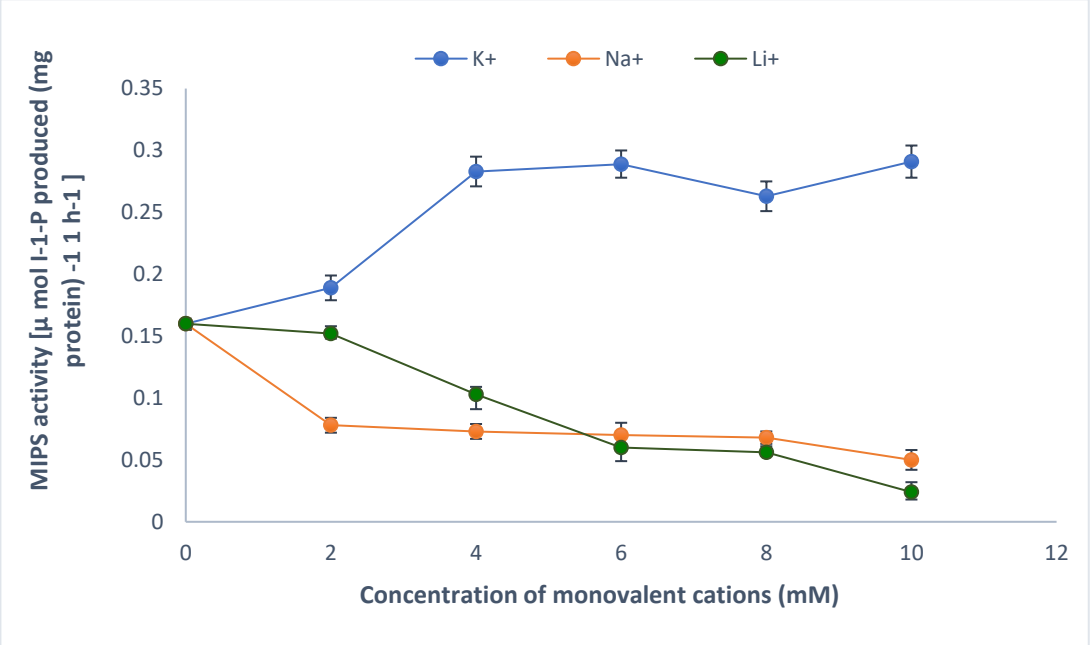


Fig. 4.11. Effect of monovalent cations on cytosolic MIPS from *H. salicifolia*

4.5.14 Effect of divalent cations

Effects of different divalent cations on cytosolic MIPS activity from *H. salicifolia* were studied using variable concentrations (1.0 to 10.0 mM) of chloride salts of Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Hg^{2+} and Zn^{2+}.

The 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 the experiment have been shown in Fig. 4.12 from which different types of effects of divalent cations were recorded. During the experiment, Ca^{2+} and Mg^{2+} showed a mild stimulatory effect of 2.05 and 1.65-fold respectively. Mn^{2+} and Cu^{2+} exhibited slightly inhibitory effect while in case of Zn^{2+} the inhibitory effect was more pronounced effect of 56.25%. On the other hand, Hg^{2+} exhibited a strong inhibition of the enzyme activity restricting it to about 84.37% in cytosolic enzyme at 10mM concentration. The inhibitory effect of different divalent cations proceeded in the order of $Cu^{2+} > Mn^{2+} > Zn^{2+} > Hg^{2+}$.

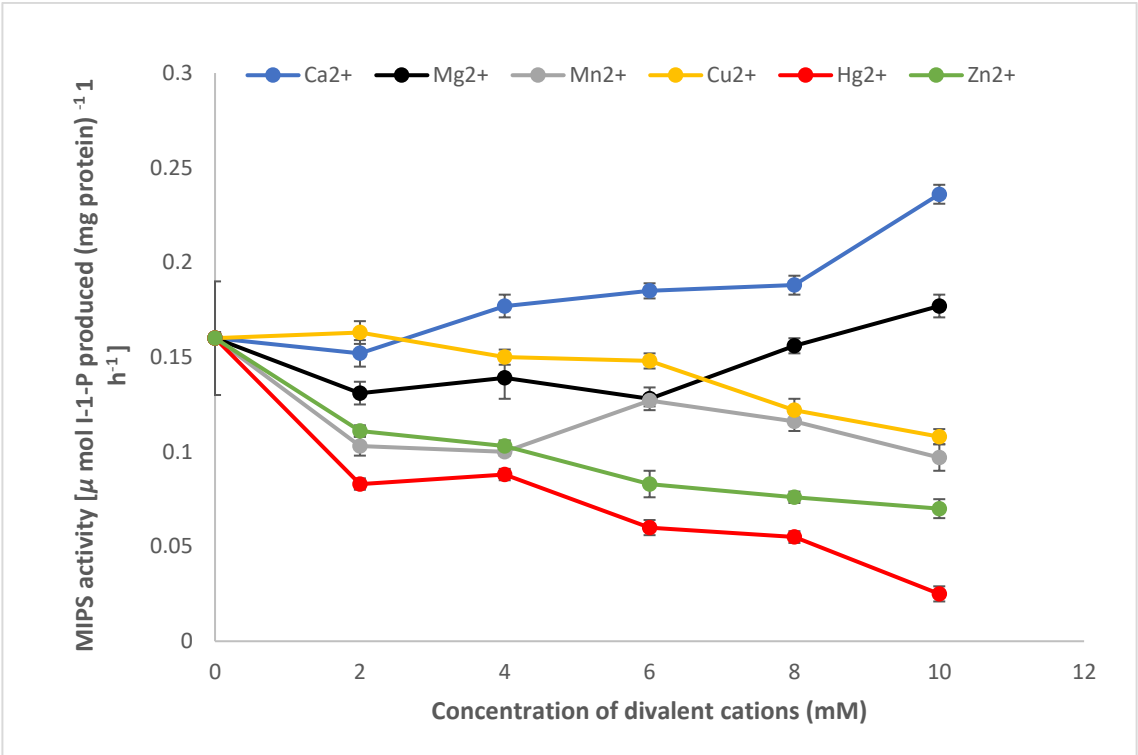


Fig. 4.12. Effect of divalent cations on cytosolic MIPS from *H. salicifolia*

4.5.15. Effect of Sugar-alcohols

Influence of different quantities of sugar alcohols like galactitol, mannitol, and *myo*-inositol have been discovered to have either no effect or to slightly alter the *myo*-inositol-1-phosphate synthase activity from *H. salicifolia.*

Fig. 4.13 shows results where it has been observed with galactitol, the synthase activity first increased mildly up to a concentration of 4mM and then the activity gradually declines. Similarly, the effect of *myo*-inositol itself was observed to stimulate the enzyme activity at the beginning slightly, after which a decline was seen. Also, not very significant increase or decrease was found in the case of *myo*-inositol. On the contrary, mannitol showed a sharp upsurge in the activity with increasing concentration up to 8mM after which it showed a decline.

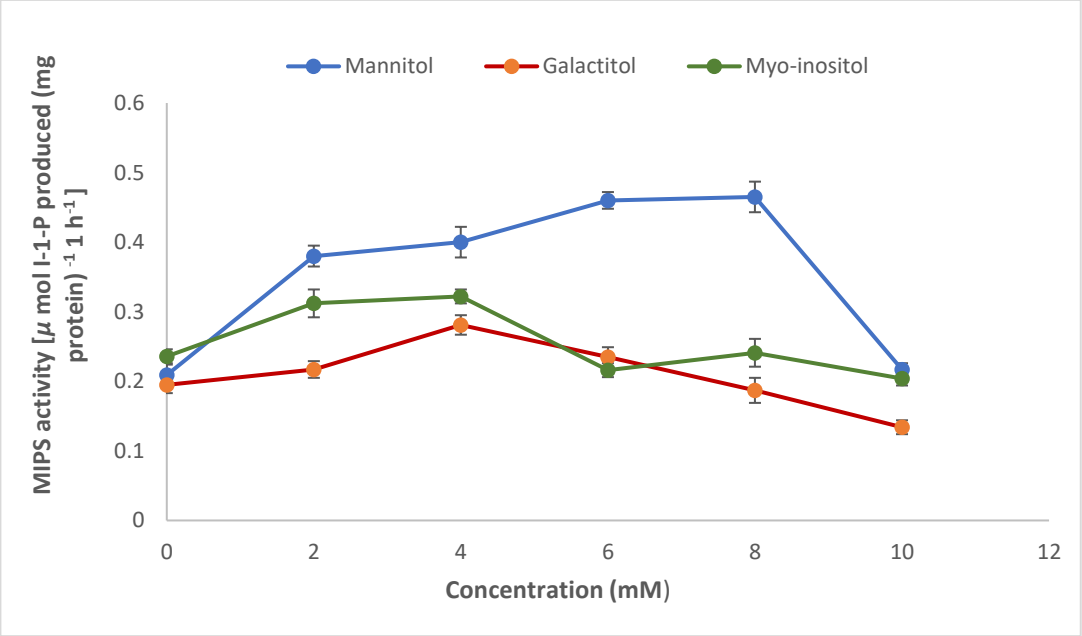


Fig. 4.13. Effect of varied concentration of mannitol, galactitol and *myo*-inositol on cytosolic MIPS from *H. salicifolia*

4.6. Chloroplastic L-*myo*- inositol-1 –phosphate synthase from *H. salicifolia*

It has been revealed from the literature that the enzymatic biosynthesis of *myo*-inositol via the L-*myo*-inositol-1-phosphate synthase has been documented among varied members of the biological kingdom. It has also been found that the inositol synthesizing capability in *H. salicifolia* was appreciable in terms of the activity of this enzyme. Earlier reports also suggest that, in plants, in addition to the cytosolic MIPS, a particulate form of the enzyme, chloroplastic MIPS may be present (Adhikar *et al.*, 1987; Chhetri *et al.*, 2006). Therefore, confirmation of whether such particulate enzyme is also present in *H. salicifolia* became essential at the onset of the work.

Experimental plant leaves from *H. salicifolia* were collected fresh as described in section 4.1. Using dialyzed supernatant and low speed supernatant, from juvenile leaves as the enzyme source, L-*myo*-inositol-1-phosphate synthase was assayed (Section 4.2.5). The results presented in Table 4.8 showed that the enzyme was functional in homogenate as well as in low-speed supernatant of *H. salicifolia*. It was also clear from the same Table that the homogenate fraction exhibited about 1.6-fold higher activity in comparison with the low speed supernatant fraction indicating the probability of existence of a membrane–bound or particulate form of this enzyme in addition to its conventional locale in cytosol.

Table 4.8. L-*myo*-inositol-1-phosphate synthase activity in the leaves of *H. salicifolia*. Variation in the enzyme activity between the homogenate fraction and low-speed supernatant of the sample. Specific activity defined as µmol of I-1-P produced (mg protein)$^{-1}$ h^{-1}. *Values are mean ±. SE, n=3; FW= fresh weight.*

Plant tissue	**Enzyme source**	**Total protein extracted**	**Specific activity [**µmol of I-1-P produced (mg protein)$^{-1}$ h^{-1}]
Leaves	Homogenate	1.485 ± 0.095	0.108 ± 0.007

(collected in May)	Low speed supernatant	0.938 ± 0.060	0.065 ± 0.002

4.6.1 Identification of chloroplasts isolated from the leaves of *H. salicifolia*:

The purity of the isolated chloroplasts was tested on the basis of the biochemical identification of this preparation. This was principally made by the ability of the preparation to carry out the characteristic Hill reaction along with the determination of the total chlorophyll content from the same batch of isolated chloroplasts as described.

In a set of experiments the detection of Hill activity was separately carried out with the isolated chloroplast suspension (suspension of 435 ×g pellet and 2,850 ×g supernatant) in order to look into the reality of principal confinement of such activity in isolated chloroplasts. The results of such activity are presented in Table 4.9, which also shows the total content of chlorophyll expressed in terms of mg chlorophyll per 0.1 ml suspension of experimental fraction.

It can easily be assessed from Table 4.9 that appreciable Hill activity was only confined to isolated chloroplast fraction as evident from the reduction in dye intensity under illuminated condition only. However, the other two fractions also exhibited a negligible amount of Hill activity, which might be contributed by some plastidial pigment contamination in these fractions incorporated during the isolation.

The variation in degree of the total chlorophyll content in respective fractions obviously did not stand in the way of such experiment. Therefore, the Hill activity and the total chlorophyll content of a fraction are directly proportional.

Table-4.9. Determination of Hill activity and total chlorophyll content in isolated chloroplast factions, 435 x g (pellet) and 2850 x g supernatant fractions of *H. salicifolia* leaves. FW=fresh weight.

Fraction	Observation No.	Experimental suspension (ml)	DCPIP soln. (ml)	H_2O (ml)	Na-dithionate crystals	Experimental condition	Initial O.D. (520 nm)	Final O.D. (520 nm)	Corrected O.D.	Total chlorophyll content (mg Chl g^{-1} FW)
Chloroplast suspension	1.	0.0	9.9	0.1	-	Light	0.51	0.51	0.00	2.97
	2.	0.1	9.9	0.0	-	Dark	0.47	0.45	0.02	
	3.	0.1	9.9	0.0	-	Light	0.60	0.31	0.29	
	4.	0.1	9.9	0.0	+	Light	0.18	0.17	0.01	
435 x g pellet suspension	1.	0.0	9.9	0.1	-	Light	0.53	0.52	0.01	0.35
	2.	0.1	9.9	0.0	-	Dark	0.57	0.54	0.03	
	3.	0.1	9.9	0.0	-	Light	0.58	0.49	0.09	
	4.	0.1	9.9	0.0	+	Light	0.12	0.13	0.00	
2850 x g supernatant	1.	0.0	9.9	0.1	-	Light	0.57	0.57	0.00	0.28
	2.	0.1	9.9	0.0	-	Dark	0.61	0.59	0.02	
	3.	0.1	9.9	0.0	-	Light	0.63	0.57	0.06	
	4.	0.1	9.9	0.0	+	Light	0.15	0.15	0.00	

4.6.2. Partial purification of chloroplastic MIPS activity from *H. salicifolia*

Chloroplastic MIPS was isolated and partially purified from the juvenile leaves of freshly collected *H. salicifolia,* in the month of May. This process was carried out according to the standard procedure described in Sections 3.2.4.5.

Table-4.10. shows the results procured by the partial purification from the chloroplasts of *H. salicifolia*. The tables show that the enzymes from chloroplast were 69.59 times over the homogenate fraction, and the enzyme recovery percentage based on total activity was discovered to be about 19%.

Table-4.10. Partial purification of chloroplastic MIPS from *H. salicifolia* [Specific activity defined as μ mol I-1-P produced (mg protein)-1 h-1] *(Values are mean ± SE, n=3)*

Purification step	Protein content (mg)	Specific activity	Total activity	Recovery (%)	Purification (fold)
Homogenate	17.07 ± 0.11	0.091 ± 0.01	179.43 ±1.13	100	1
10K supernatant	12.13 ± 0.05	0.147 ± 0.06	179.19 ± 1.16	99.86	1.61
SS-fraction	11.04 ± 0.03	0.166 ± 0.03	171.13 ± 0.51	95.37	1.82
A_2S fraction	05.41 ± 0.02	0.367 ± 0.07	133.62 ± 0.27	74.47	4.02
DE-fraction	02.01 ± 0.02	1.174 ± 0.12	118.28 ± 0.29	65.92	12.85
Hxl- fraction	0.524 ± 0.03	5.744 ± 0.09	105.47 ± 0.17	58.78	62.85
BioGel- fraction	0.488 ± 0.01	6.359 ± 0.15	34.36 ± 0.08	19.15	69.59

4.6.3. Characterization of chloroplastic MIPS from *H. salicifolia*

4.6.3.1. Effect of different assay components on chloroplastic MIPS activity from *H. salicifolia*

Enzyme activity, 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 with the complete set, was checked and tabulated as shown in Table-4.11. The detection of the chloroplastic enzyme activity of L-*myo*-inositol-1-phosphate synthase was not feasible in the absence of the specific substrate, G-6-P, within the incubation mixture. Furthermore, the enzymatic synthesis was also unobservable when the enzymes were inactivated through the application of heat. Approximately 35% of the activity pertaining to the MIPS enzyme from the chloroplast was forfeited when the Tris buffer was omitted from the complete set reaction components. Likewise, elimination of NAD^+ caused 53% loss, NH_4Cl and ME omission caused 51% and 45% decrease in the activity in case of MIPS from chloroplast.

Table-4.11 Effect of different incubation conditions on chloroplastic MIPS activity from *H. salicifolia* (chloroplast). [Specific activity defined as μ mol I-1-P produced (mg protein)-1 h-1] *(Values are mean ± SE, n=3)*

Sl. No.	Conditions	Specific activity	Percent activity
1	Complete set	3.222 ± 0.15	100 ± 1.55
2	Without substrate (G-6-P)	0	0
3	Without co-enzyme (NAD^+)	0.862 ± 0.072	47.22 ± 0.65
4	Without NH_4Cl	0.750 ± 0.003	49.43 ± 0.21
5	Without ME	1.163 ± 0.019	54.60 ± 0.58
6	Without buffer (tris- acetate)	1.037 ± 0.027	64.93 ± 0.44
7	Heat killed enzyme	0	0

4.6.3.2. Progress of the enzyme reaction with time

L-*myo*-inositol-1-phosphate synthase of *H. salicifolia* extracted from chloroplast was incubated at different time periods between 0 and 150 minutes at an interval of 30 min each which was carried out under standard assay conditions. The reaction was observed to progress linearly with time up to 90 min (Fig. 4.14) after which the activity of the enzyme did not show much variation in the last three incubation interval.

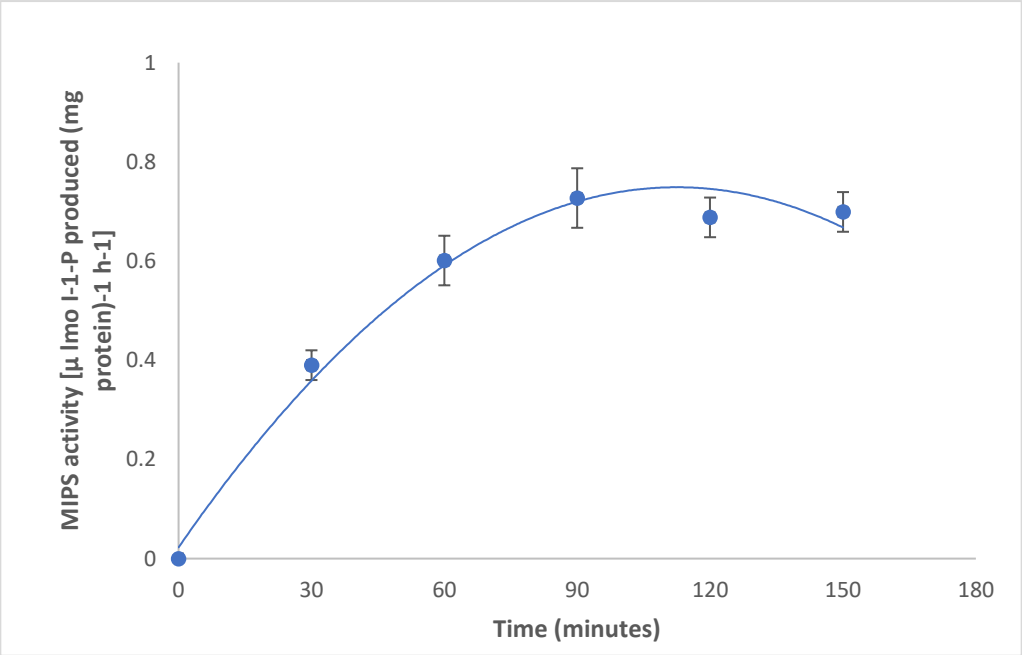


Fig. 4.14 Effect of incubation time on chloroplastic MIPS activity from *H. salicifolia*

4.6.3.3. Progress of enzyme activity with increasing protein concentration

Chloroplastic MIPS assay of *Hippophae salicifolia* was carried out under optimal conditions using different concentrations of enzyme protein (0-400µg).

Fig. 4.15. shows the result where the enzyme activity was seen to increase abruptly up to a protein concentration of 300 µg. Enzyme activity beyond this concentration seemed to remain

almost the same. Increase in protein concentration beyond this point did not influence the enzyme activity much.

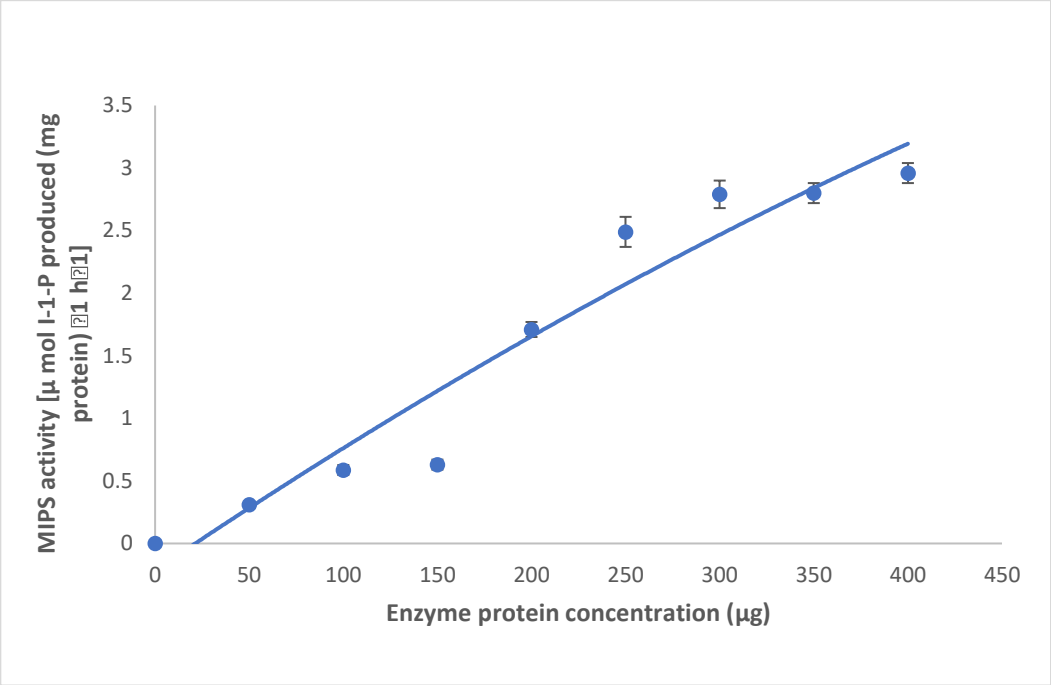


Fig. 4.15. Effect of different enzyme protein concentration on chloroplastic MIPS activity from *H. salicifolia*

4.6.3.4. Progress of enzyme activity with increasing incubation temperature

Chloroplastic MIPS activity from *H. salicifolia* was observed by incubating separately for one hour at temperature between the ranges of 0ºC to 60ºC with an interval of 10°C in presence of standard assay mixture.

Results depicted in Fig. 4.16 shows that activity of the enzyme from chloroplast was observed to have a temperature maximum at 20ºC. Thereafter only slight decline was seen at 30ºC after which the decline was rapid. This observation stands out due to its exceptional activity at a lower temperature between 10ºC to about 25ºC showing temperature maximum at about 20ºC. The narrow spectrum of temperature for the optimum activity of the chloroplastic enzyme was also unique.

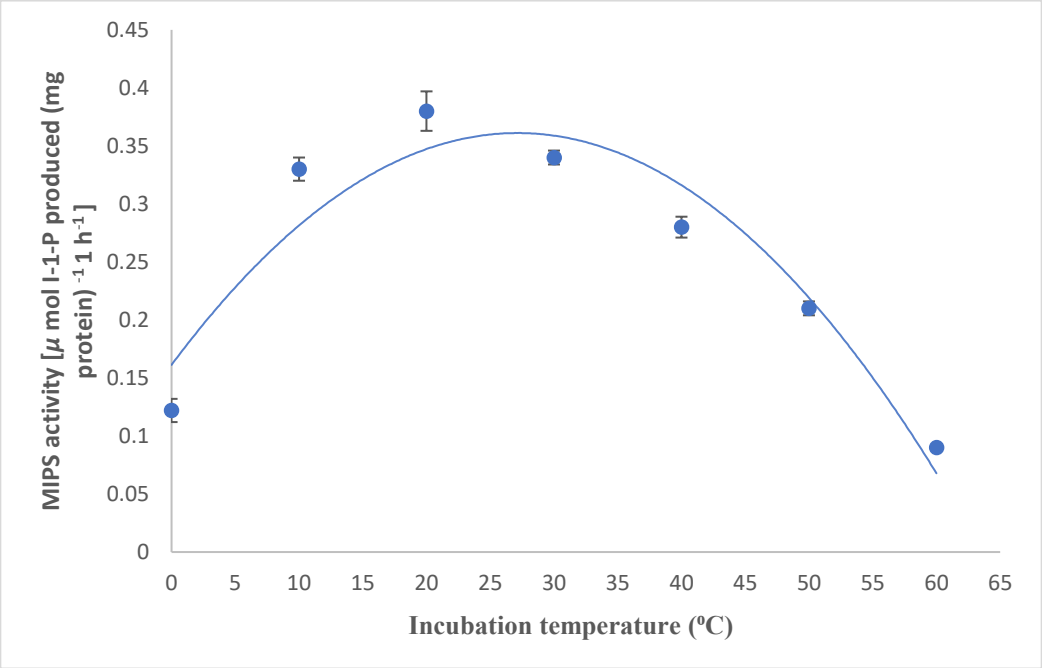


Fig. 4.16 Effect of various incubation temperatures on chloroplastic MIPS activity from *H. salicifolia*

4.6.3.5 Effect of Substrate specificity

D-glucose-6-phosphate has been identified as the sole substrate employed by the enzyme L-*myo*-inositol-1-phosphate synthase for the synthesis of L-*myo*-inositol-1-phosphate. In order to

validate its substrate specificity, hexose phosphates such as D-fructose-6-phosphate, D-galactose-6-phosphate, D-mannose-6-phosphate, and hexose bisphosphate such as D-fructose-1,6-bisphosphate were substituted for D-glucose-6-phosphate at the same concentration of 10 mM, and the reaction was permitted to proceed.

No enzymatic reaction was observed when the substrates employed were D-fructose-6-phosphate, D-fructose-1,6-bisphosphate, and D-glucose-1-phosphate for the synthesis of L-*myo*-inositol-1-phosphate by the enzyme L-*myo*-inositol-1-phosphate synthase in *H. salicifolia* originating from the chloroplast. Despite this, the enzyme was able to utilize D-galactose-6-phosphate and D-mannose-6-phosphate as substrate to a certain extent, with approximately 10% and 4.25% efficiency (Table-4.12) in the case of the chloroplast-extracted enzyme, as compared to the activity when D-glucose-6-phosphate was used as substrate.

Table-4.12. Substrate specificity of chloroplastic MIPS from *H. salicifolia* [Specific activity defined as μ lmo I-1-P produced (mg protein)-1 h-1] *(Values are mean ± SE, n=3)*

Sl. No.	Compound	Conc. (mM)	Specific activity	Percent activity
1	D-Glucose-6-phosphate	10	0.902 ± 0.065	100
2	D-Fructose-1,6-bisphosphate	10	0	0
3	Galactose-6-phosphate	10	0.11 ± 0.013	10
4	D-Fructose-6-phosphate	10	0	0
5	D-Glucose-1-phosphate	10	0	0
6	D-mannose-6-phosphate	10	0.033 ± 0.002	4.25

4.6.3.6. Effect of D-glucose-6-phosphate (substrate) concentration on chloroplastic MIPS activity from *H. salicifolia*: Determination of K_m and V_{max} values

4.6.3.6.1. Effect of substrate concentration

The chloroplastic MIPS activity from *H. salicifolia*, purified by BioGel A 0.5m, demonstrated an increase in activity in relation to the concentration of D-glucose-6-phosphate. This increase was observed up to a concentration of 6 mM that was also the maximum, within the substrate concentration range of 0 to 10 mM. Subsequently, no significant alteration in enzymatic activity was observed (Fig. 4.17).

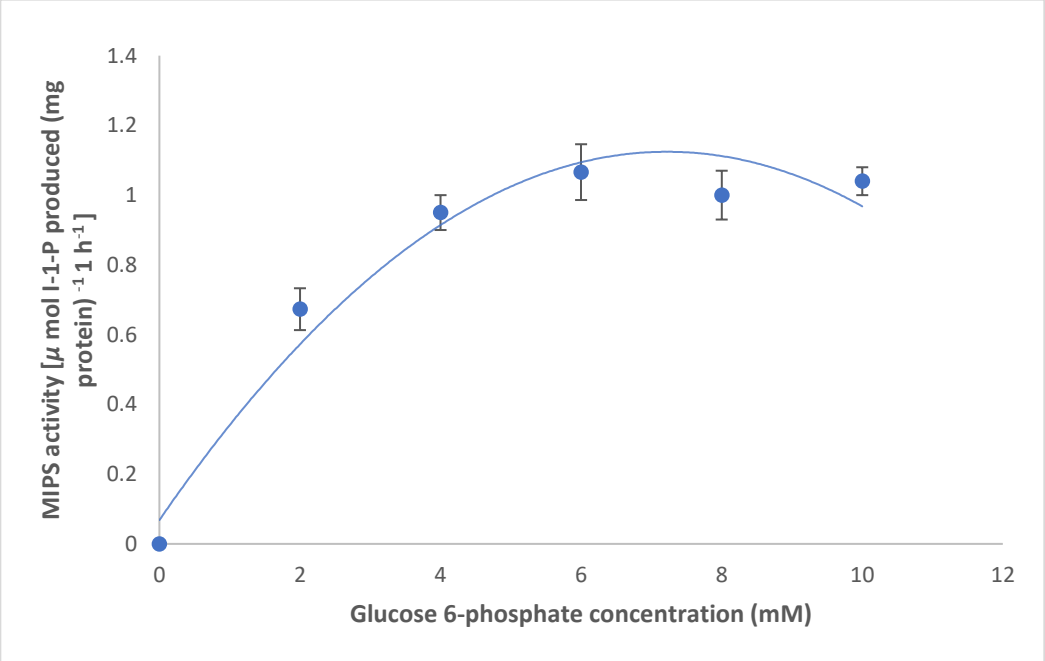


Fig. 4.17. Effect of substrate (G-6-P) concentration on chloroplastic MIPS from *H. salicifolia*

4.6.3.6.2. Determination of K_m and V_{max} values:

The average *K*m for D-Glucose-6-Phosphate for *H. salicifolia* L-*myo*-inositol-1-phosphate synthase extracted from chloroplast was calculated to be approximately 0.69 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 1.06 mM for the chloroplastic MIPS.

4.6.3.7. Effect of NAD^+ (co-enzyme) concentration on chloroplastic MIPS activity from *H. salicifolia*: Determination of K_m and V_{max} values

4.6.3.7.1. Effect of coenzyme concentration

The activity of chloroplastic MIPS from *H. salicifolia* was assayed according to standard procedure with varying co-factor concentration ranging from 0 to 1.0 mM NAD^+. The catalytic activity of the enzyme was found to increase linearly with respect to the concentration of NAD^+ up to 0.8 mM (Fig. 4.18), beyond which the activity remained stagnant.

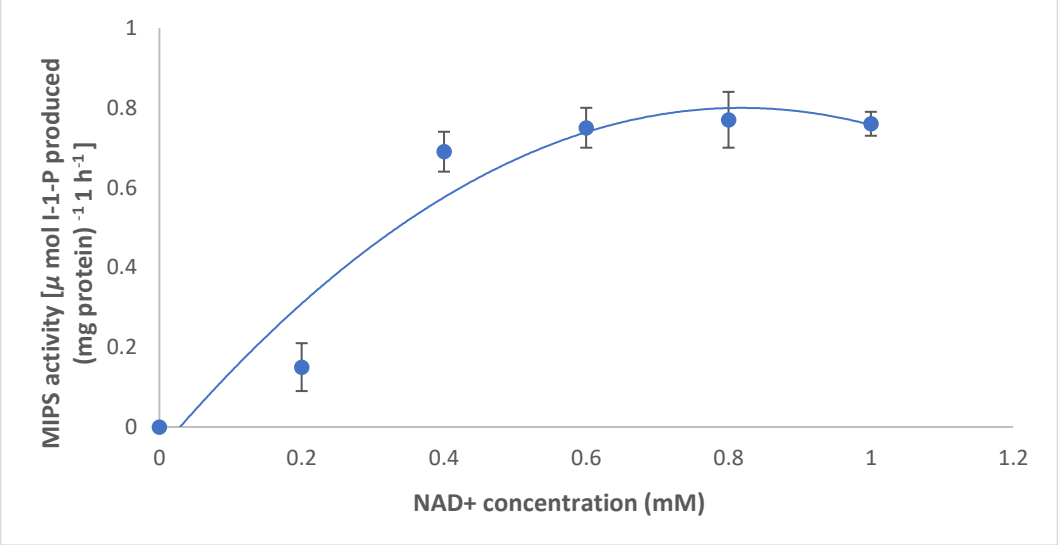


Fig. 4.18. Effect of different co-enzyme (NAD^+) concentration on chloroplastic MIPS from *H. salicifolia*

4.6.3.7.2. Determination of K_m and V_{max} values

The average K_m for NAD^+ of *H. salicifolia* L-*myo*-inositol-1-phosphate synthase for chloroplastic MIPS was calculated to be approximately 0.25 mM in accordance with the rate equation of Michaelis-Menten and by means of Lineweaver-Burk plot for enzyme kinetics. In the meantime, V_{max} was calculated to be 0.77mM for chloroplastic MIPS.

4. 6.3.8. Effect of replacement of NAD^+ with $NADP^+$

To check whether the coenzyme NAD^+ is specific for the enzyme *myo*-inositol synthase from *H. salicifolia* or if its requirement could be satisfied by similar other coenzymes, an assay was performed in which NAD^+ was replaced with $NADP^+$ in the assay mixture. The chloroplastic enzyme activity was determined in presence of 0 to 1.0 mM $NADP^+$ whose results were compared to that of the enzyme activity in the presence of 0-1.0mM NAD^+ as presented in Fig. 4.19.

The results revealed that NAD^+ which is the co-factor for the enzyme *myo*-inositol synthase from *H. salicifolia* could not be substituted with $NADP^+$ at any concentration. The highest enzyme activity peaked at 0.6 mM in chloroplastic enzyme. However, minimal enzyme activity was observed in the experimental set at different $NADP^+$ concentrations.

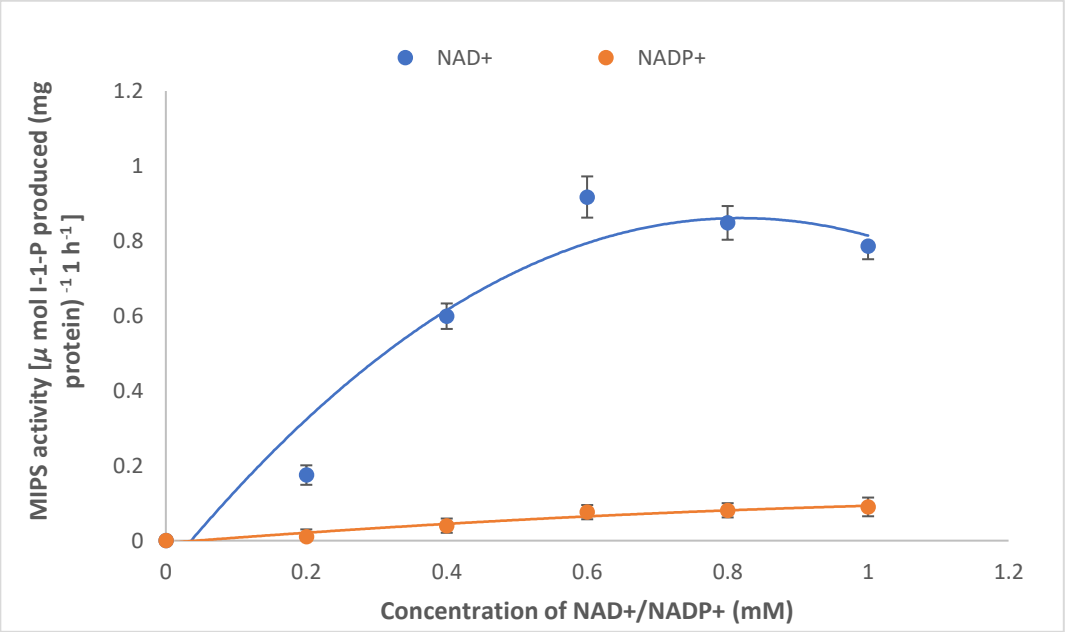


Fig-4.19 Effect of replacement of co-enzyme NAD^+ with $NADP^+$ in chloroplastic MIPS activity from *H. salicifolia*

4.6.3.9. Effect of different pH on MIPS activity

The chloroplastic MIPS activity from *H. salicifolia*, was assayed under standard conditions by using 50 mM Tris-acetate buffer between the pH ranges of 6.0 to 9.0. Its activity was observed to be remarkably influenced by the variation in pH.

Fig. 4.20 tend to portray that the chloroplastic MIPS from *H. salicifolia* became highly active at a wide pH range. The activity was observed to be highest at pH 7.0-7.5. However, below pH 6.5 or above pH 7.5 there was continuous decline in the catalytic activity of the enzyme.

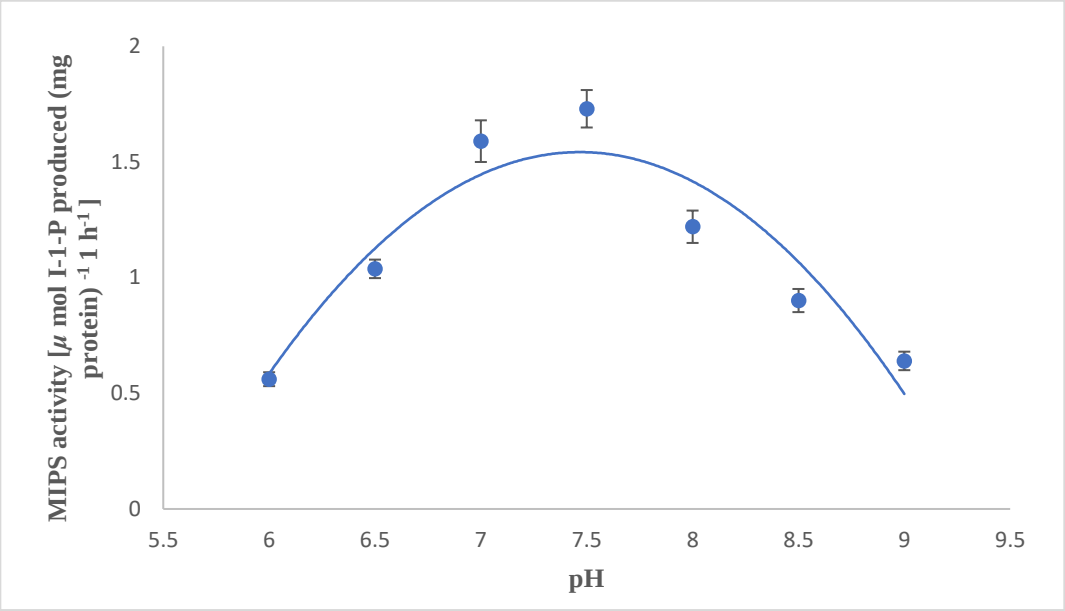


Fig.4.20. Effect of pH on chloroplastic MIPS activity from *H. salicifolia*

4.6.3.10. Effect of various concentration of salts

To find out the effects of varied concentration of different salts viz., $MgCl_2$, NH_4Cl and EDTA on chloroplastic MIPS from *H. salicifolia*, 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.

The overall result in case of chloroplastic MIPS activity (Fig. 4. 21) in presence of $MgCl_2$ and NH_4Cl was observed to have an increase in the enzyme activity and decrease in the activity in case of EDTA. The enzyme activity was observed to increased up to 4.75-fold and 2.76-fold by the influence of NH_4Cl and $MgCl_2$ respectively at its maximum concentration (100mM) which was slightly lesser than in the case of cytoplasmic MIPS activity at the same concentration. On

the contrary the influence of EDTA at its highest concentration of 100mM decreased the enzyme activity to roughly 30.63%.

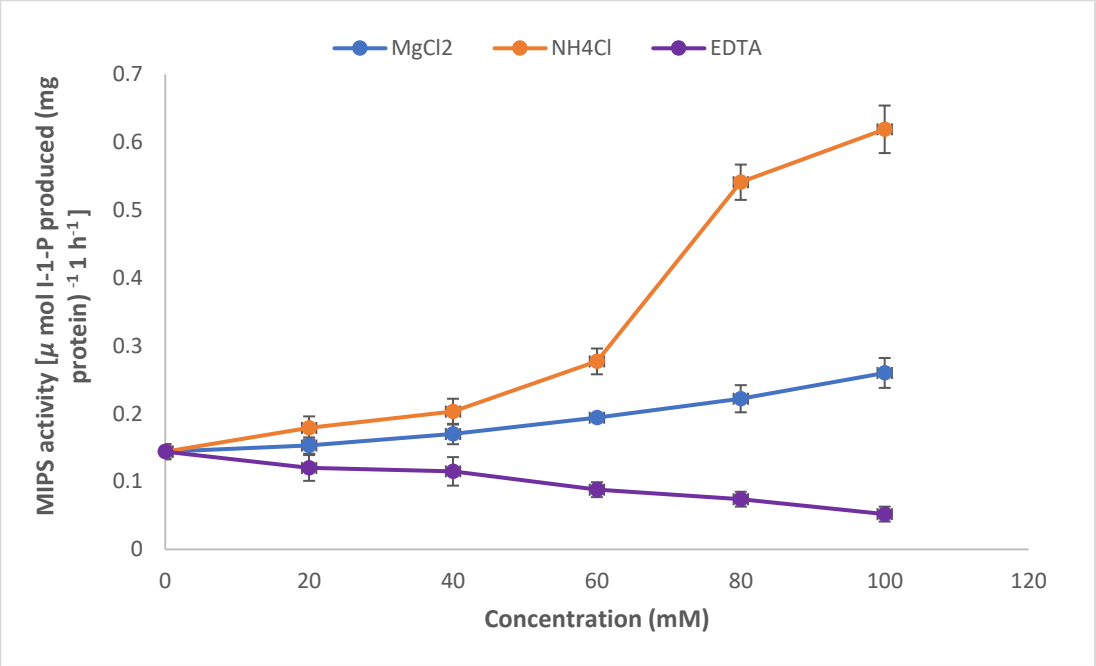


Fig. 4.21. Effect of different concentrations of salts on chloroplastic MIPS activity from *H. salicifolia*

4.6.3.11. Effect of monovalent cations

Effects of some monovalent cations on *H. salicifolia* MIPS activity extracted from the chloroplast 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 was estimated keeping one control set without adding any such cation.

Results of the experiment is presented in Fig. 4.22. It is evident, that the K^+ had a stimulatory effect on this enzyme activity and increased the activity 2.55-fold. While Na^+ played a remarkable inhibitory role decreasing the enzyme activity by specifically by 62.5% at 10 mM concentration. Furthermore, Li^+ was the strongest inhibitor of this enzyme, which could reduce the specific activity by about 80% enzyme activity at 10 mM concentration.

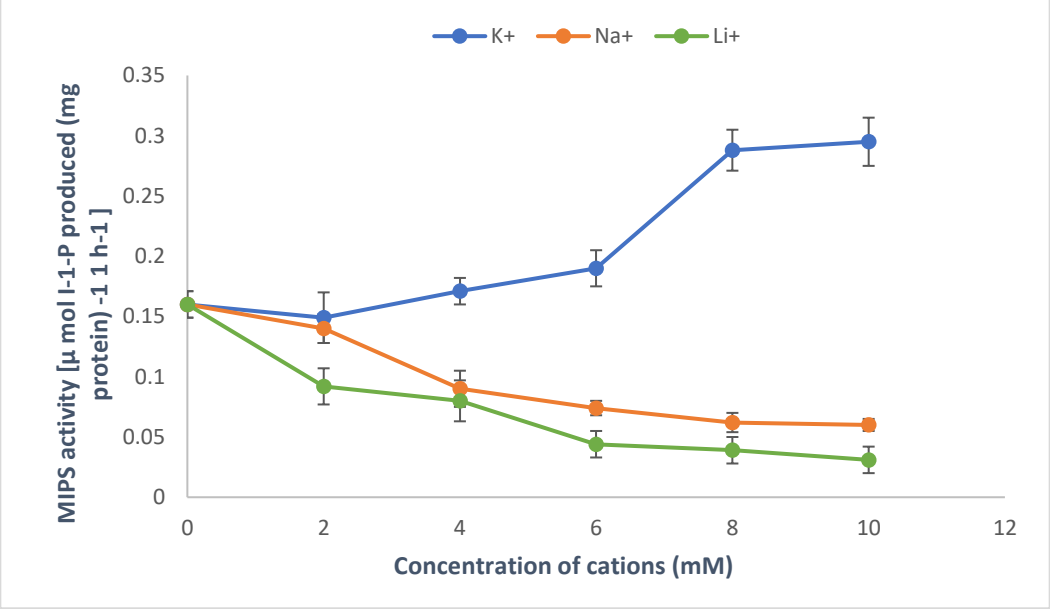


Fig- 4. 22. Effect of monovalent cations on chloroplastic MIPS activity from *H. salicifolia*

4.6.3.12. Effect of divalent cations

Effects of different divalent cations on the chloroplastic MIPS activity from *H. salicifolia* was studied using variable concentrations (1.0 to 10.0 mM) of chloride salts of Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Hg^{2+} and Zn^{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 the experiment have been shown in Fig. 4.23, from which different types of effects of divalent cations were recorded. Different divalent cations reacted in a relatively similar mode in both the enzyme extracted from cytoplasm and chloroplast.

1.95 % and 1.80 % increase in the activity was seen in case of Ca^{2+} and Mg^{2+} respectively. Mn^{2+} and Cu^{2+} exhibited slightly inhibitory effect showing 29.72% and 13.51% reduction in the enzyme activity while in case of Zn^{2+} the inhibitory effect was more pronounced (43.75%). On the other hand, Hg^{2+} exhibited a strong inhibition of the enzyme activity restricting it by about 72.07% in chloroplastic enzyme at 10mM concentration. The inhibitory effect of different divalent cations proceeded in the order of $Cu^{2+} > Mn^{2+} > Zn^{2+} > Hg^{2+}$.

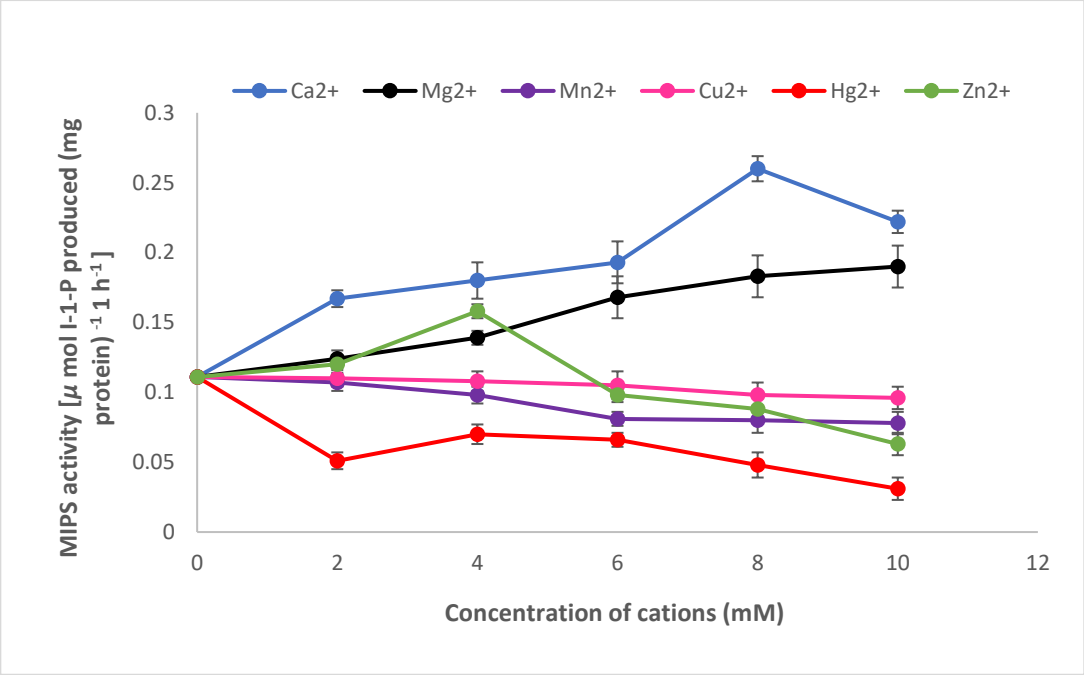


Fig-4.23. Effect of divalent cations on chloroplastic MIPS from *H. salicifolia*

4.6.3.13. Effect of sugar-alcohols

Influence of different quantities of sugar alcohols like galactitol, mannitol, and *myo*-inositol have been discovered to erratically alter the chloroplastic MIPS activity from *H. salicifolia.*

Fig. 4.24 showed that mannitol increased the chloroplastic enzyme activity linearly up to a concentration of 8mM after which a decline was seen. *Myo*-inositol seemed to have a similar effect with a slightly lesser effect whereas galactinol had a very mild effect and slightly increased the activity only up to 6 mM after which it declined.

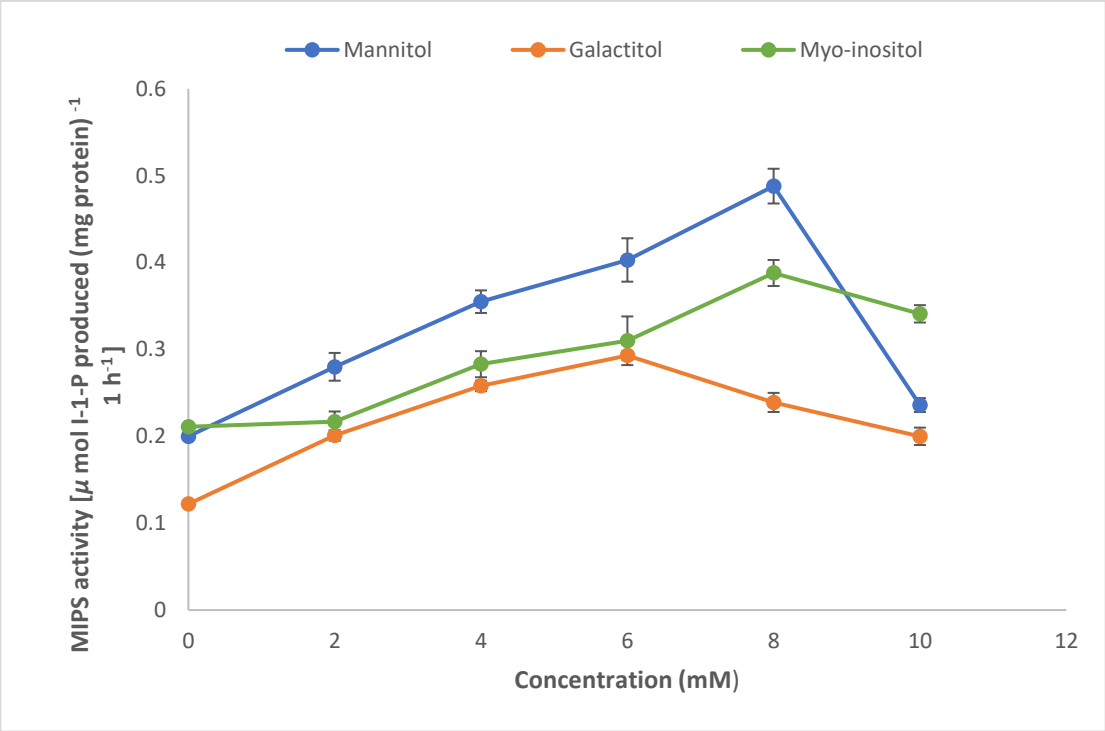


Fig. 4.24. Effect of varied concentration of mannitol, galactitol and *myo*-inositol on chloroplastic MIPS activity from *H. salicifolia*

5. DISCUSSION

L-*myo*-inositol phosphate synthase (MIPS; EC 5.5.1.4) is the rate limiting enzyme for *myo*-inositol synthesis, a molecule of paramount importance due to its multiple functionalities. This enzyme is one such biological compound that has been able to attract considerable interest among enzymologists due to its role in the biosynthesis of m*yo*-inositol (MI) which is a natural compound widely known to have numerous crucial biological functions and its essentiality in several biochemical pathways is well known. MIPS is an enzyme that caters to various tissues in accordance with their specific needs (Seelan *et al.,* 2009). Its metabolism is vital for the normal functioning and development of plants, animals, and microorganisms (Lackey *et al.*, 2003). Furthermore, occurrence of MIPS in prokaryotes and eukaryotes, provides evidence for its early presence during the process of evolution (Bachhawat and Mande, 2000).

Inositol and its derivatives have been known to be functional in growth regulation, carbohydrate and nutritional metabolism, hormone regulation, pathogen resistance, membrane biosynthesis, membrane protection, cell signalling, as a precursor in the wall biogenesis, gums, mucilage and stress adaptation in higher plants (Loewus and Loewus, 1983; Bohnert *et al.,* 1995; Loewus and Murthy, 2000; Stevenson *et al.,* 2000; Michell, 2008; Ravi & Wim, 2011). This cyclohexanehexol's role in both abiotic and biotic stress responses in plants are widely known (Shinozaki *et al.,* 2006). It has been observed that *myo*-inositol derivatives regulate stress response by serving as compatible solutes and signalling molecules (Kido *et al.,* 2013). According to the biosynthetic pathway of *myo*-inositol, the first key step is formation of *myo*-inositol-1-phosphate from glucose-6-phosphate (Loewus and Kelly, 1962; Majumder *et al*.,

1997) catalysed by the enzyme, *myo*-inositol-1-phosphate synthase (EC 5.5.1.4, MIPS) which was our focus of interest.

The role of MIPS enzyme and *myo*-inositol in combating stress in plants is well known. The role of MIPS in enhancing tolerance towards cold, drought and salt stress in several plants has already been well established (Joshi *et al.,* 2013; Kusuda *et al*., 2015; Majee *et al.,* 2004; Patra *et al.,* 2010; Tan *et al*., 2013; Wang *et al*., 2016; Zhai *et. al*., 2016). However, their purification and biochemical characterization from plant species of Sikkim Himalaya especially the cold stressed mountain plants has not hitherto been carried out. Therefore, this study is an effort to fill the lacuna. This research work details the preliminary and baseline work regarding the presence of the enzyme MIPS in ten different plant species belonging to different families. Further it enumerates the basic biochemical properties of the enzyme isolated from *H. salicifolia* of Sikkim Himalaya, belonging to the family Elaegnaceae.

This work reckons the importance of the biochemical properties of the enzyme, MIPS isolated from cold tolerant species, *H. salicifolia* found only in the Lachen and Lachung Valleys of Sikkim Himalaya. Table 4.3 and Table 4.4 contains the crucial information regarding the presence of the enzyme *myo*-inositol-1-phosphate synthase in various plant species from different altitudinal zones of Sikkim Himalaya, which fulfils the preliminary response regarding the presence of the enzyme MIPS in various plant species.

Furthermore, the finished product in the MI biosynthesis pathway i.e., MI itself has also been isolated and estimated during this study from high altitude flora of Sikkim. Free MI is biologically important in all organisms and especially it may have a role in stress tolerance including cold stress (Kamenov *et.* al., 2015). The recovery of free MI from other metabolic

MI-containing products serves as the foundation for all other sources. Free MI is often considered to be a ubiquitous component of plant tissues. Therefore, free *myo*-inositol was screened in all the target plant species in the current research whereby significant levels of free *myo*-inositol were found in all the species examined in two different seasons as seen in Table 4.1 (Summer) and Table 4.2 (Monsoon). Maximum free *myo*-inositol content was detected in *Pedicularis siphonantha* followed by *H. salicifolia* in May while in the next season maximum was detected in *H. salicifolia* while the least was detected in *Hellenia speciosa* in both the seasons. MI was found to be the chief sugar content almost 60-65% in *Actinidia arguta* (Boldhing *et al.*, 2000). One striking feature observed during the experiment was that both free MI content and the enzyme MIPS activity was decidedly higher in the samples collected during May (summer). This may not necessarily be the effect of seasonality *per-se*, but rather the sample leaves were developing and juvenile during summer, naturally more biosynthetic activity was expected from a developing organ during the time. Moreover, in overwintering ladybird beetles (*Creatomegilla undecimnotata*), free MI acted as a potential cryoprotectant and increases more than fourfold (from 2.5 to 11mg: mg wet weight (Kostal *et al.,* 1997). Mg-dependent phosphatase was found to hydrolyze Ins (1)P1 to produce free MI from lily pollen (Dickinson, 1968). The presence of total amount of free MI is also indicative of its role in different biosynthetic activity in the species which in turn reflects the role of the enzyme MIPS in its biosynthesis.

The results displayed in Table 4.3 and Table 4.4 depicts that the enzyme is active in almost all the plants species collected from different altitudinal zones belonging to different families with a slight variation. This signifies that the presence of the enzyme in all the species of the plant irrespective of their families. It has also been observed that the enzyme activity was highest in

H. salicifolia in both the seasons hence their utilization for further purification and characterization experiments were carried out in this particular plant species. Among all the species, the lowest enzyme activity was seen in case of *Houttuynia cordata*. Although the variation in the amount of enzyme has been portrayed in this experiment, the unambiguous presence of the enzyme across different plant groups ranging from algae (Dasgupta *et al*., 1984; RayChaudhuri *et al*., 1997); fungi (Escamilla *et al.*, 1982; Dasgupta *et al*., 1984; Suliman *et al.*, 2021); bryophytes (Chhetri *et al*., 2009, 2023); pteridophytes (Chhetri *et al.*, 2006, Basak *et al.*, 2012); gymnosperm (Gumber *et al.*, 1984; Chhetri and Chiu, 2004) and angiosperm (Loewus and Loewus, 1971; Johnson and Sussex, 1995; Johnson and Wang, 1996; Ray Chaudhuri *et al*., 1997; Kusuda *et al.*, 2015), throws light on its universal prevalence and justifies the distribution of MIPS among various randomly chosen families of different species from three different altitudinal zones.

Understanding the basic process of metabolic control of inositol biosynthesis fundamentally requires the biochemical characterization. Consequently, biochemical analysis of the enzyme, is an essential requirement for comprehending the underlying metabolic regulation pertaining to the synthesis of inositol in the designated species. Therefore, the same was carried out in the leaf samples of *H. salicifolia.* The enzyme, L-*myo*-inositol-1-phosphate synthase from the aforementioned plant species, was isolated and partial purification of the enzyme was carried out of which the results are detailed in section 4.2. Juvenile leaves were chosen for the partial purification of the enzyme from the species concerned. Consequent upon the isolation and partial purification of the enzyme concerned from the target samples, study of the different parameters of the enzyme and its behaviour under the influence of varying internal and external environment was undertaken. A successful enzyme purification process aims to achieve a high

purification fold that implies to a significant reduction in impurities and a high recovery rate further indicating a high yield of the target enzyme (Gonzalo and Lvandera, 2021). Henceforth, by using low-speed centrifugation, streptomycin sulphate precipitation, ammonium sulphate fractionation, successive chromatography on DEAE cellulose, hexylagarose, and one final molecular sieve chromatography through BioGel A 0.5m, the enzyme, MIPS from *H. salicifolia*, was purified up to approximately 64.46-fold for cytosolic MIPS (Table-4.5) and 69.59-fold for chloroplastic MIPS (Table-4.10) over the homogenate fraction respectively. At this level of purification, the enzyme had a recovery rate based on total activity of about 21.22% and 19.15% respectively for cytosolic and chloroplastic MIPS respectively. A comparable yield of 60.83-fold purification with 53.69% of the enzyme protein recovery has been recorded from *Lycopodium clavatum* while approximately 75-fold purification with 45% recovery has been recorded from *Selaginella monospora* (Basak *et al.,* 2012). Similarly, a purification of 22.16-fold with 10.67% recovery from *Brachymenium bryoides* has also been reported (Yonzone *et al*, 2018). A high recovery rate indicates that the purification process is efficient in isolating and concentrating the target enzyme without significant loss. These parameters are crucial for obtaining a pure and active enzyme preparation for further analysing the enzyme characteristic. Purification and characterization of the enzyme MIPS has so far been through conventional purification techniques such as ammonium sulphate precipitation, gel filtration chromatography, ion-exchange chromatography, etc. The native PAGE profiles of the cytosolic MIPS from *H. salicifolia* preparation exhibited one band which was not very distinct (Fig-4.1) which suggested that the preparations lacked homogeneity. Further, the apparent molecular weight of the cytosolic MIPS from the plant species in our current investigations was determined to be approximately 158.6 kDa. So far various ranges of molecular weight has been

found for this enzyme in different plants. As per earlier reports, the apparent molecular weight of the enzyme from plant sources have been found to be 135 kDa in *Lemna gibba* (Ogunyemi *et al.,* 1978), 150 kDa in *Acer pseudoplatanus* (Loewus and Loewus, 1971), 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), 260 kDa in *Neurospora crassa* (Escamilla *et al.,* 1982). However, the MIPS enzyme purified by employing the techniques of gel-filtration chromatography through Ultrogel AcA-34, anion exchange chromatography through DEAE-Sephacel and 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 and *Spirulina platensis* was 200.32 kDa (RayChaudhuri *et al.,* 1997). In the pteridophyte, *D. glaucum*, it was found to be 170.8 (Chhetri *et al.,* 2006b), while in case of *Sphagnum*. *junghuhnianum* it was determined to be of approximately 174 kDa (Chhetri *et. al.,* 2023). Donahue and Henry (1981) purified the enzyme from yeast and determined the molecular weight to be about 240 kDa of which a single sub-unit was of about 62 kDa. Thus, our enzyme from *H. salicifolia* seems to have three subunits as in many plants.

Considering the effect on L-*myo*-inositol-1-phosphate synthase activity of *H. salicifolia* at different incubation conditions, it did not exhibit any activity in the absence of its substrate glucose-6-phosphate (G-6-P) in cytosolic (Table 4.6) as well as chloroplastic MIPS (Table 4.11). This consistency in the behaviour of the enzyme where no activity was seen in the absence of its substrate have been similar in results reported from bryophyte, pteridophytes, gymnosperms, angiosperms in plants and even human foetus (Gumber *et al*., 1984; Adhikari and Majumder, 1988; Johnson and Wang, 1996; Basak *et al.,* 2012; Chhetri *et al*., 2012; Kusuda *et al*., 2015; Yonzone *et al*., 2018; Chhetri *et al.,* 2023). Therefore, this makes it evident that

G-6-P is the specific substrate of this enzyme. Also, the enzymatic synthesis of L-*myo*-inositol-1-phosphate could not be detected when the enzymes were destroyed using heat which is known since time immemorial that heat exposure has the capacity to destroy enzyme activity (Kumar *et al*, 2022; Spitzer and Taylor, 1924).

Several enzymes require organic groups which are vital to enzymes for their catalytic activity (Donald and Judith, 2010). Hence, the elimination of NAD^+ which is the co-enzyme in the synthesis of MIPS caused almost 67% and 52% reduction in the activity of the cytosolic and chloroplastic enzyme as shown in table 4.6 and 4.11 respectively. This result is in consonance with the result in case of deduction of NAD^+ causing a decrease in MIPS activity by about 68.41 % as reported by Chhetri *et al*. in *Sphagnum junghuhnianum* (2023). Therefore, the co-enzyme NAD^+ was determined to be essential for the enzyme to express its full potential. Similar quantity of loss in the enzyme activity was observed in case of *D. glaucum* when NAD^+ was omitted from the reaction set (Chhetri *et al.*, 2006). Likewise, 55% of loss in the enzyme activity in case of rice was reported by Funkhouser and Loewus (1975).

However, various prior investigations have demonstrated that a significant level of MIPS activity may be observed even in the event of the co-enzyme NAD^+ being absent. This exhibition of the discernible degree of NAD^+ independent activity in the reaction mixture has been known to be due to the presence of endogenous NAD^+ in the enzyme system and due to the existence of some bound NAD^+ in the molecular construct of this enzyme (Pittner and Hoffmann, 1976; Barnet *et al*., 1970; DasGupta *et al*., 1984, Adhikari and Majumder, 1988). This explains the apparent 32.85% (cytosolic MIPS) and 47.22% (chloroplastic MIPS) activity seen in case of *H. salicifolia* even in the absence of NAD^+. Previous research has also revealed

that the NAD^+ coupled to the enzyme is responsible for the thermal stability of MIPS (Adhikari and Majumdar, 1983).

Kinetic studies of MIPS from *H. salicifolia* exhibited an increase of the enzyme activity in relation to its specific substrate (G-6-P) concentration up to 8 mM in case of the cytosolic enzyme while it increased upto a concentration of 6 mM in the chloroplastic one (Fig-4.6 and Fig-4.17). The Michaelis-Menten constant (K_m) is an indispensable parameter in the fields of enzymology and provides valuable understandings of the affinity and efficacy of an enzyme towards its substrate ((Yun and Han, 2020). The *Km* value for G-6-P of this enzyme was calculated to be 0.55 mM for cytosolic MIPS and 0.69 mM for the chloroplastic MIPS. The closest range of K_m value to our result was found to be 0.83 mM in the pteridophytic MIPS from *D. glaucum* for glucose-6-phosphate (Chhetri *et al.*, 2006b), MIPS from the gymnosperm *Taxus baccata* had a K_m of roughly 1.05 mM (Chhetri and Chiu, 2004) and in *S. junghuhnianum* MIPS was calculated to be approximately 1.81 mM (Chhetri *et al*., 2023). Apart from these, the K_m value found in other cases were slightly higher than our result where the K_m in case of *Euglena gracilis* enzyme was about 2.1 mM (DasGupta *et al*., 1984) and in case of rat testis enzyme it was found to be about 3.89 (Maeda and Eisenberg Jr., 1980). A low K_m value indicates that an enzyme possesses a heightened affinity for its substrate, thereby allowing the enzyme to effectively create a bond with the substrate even at low concentrations (Yun and Han, 2020). Further the V_{max} values for the substrate, G-6-P have been calculated to be 1.1 mM for cytosolic MIPS and 1.06 mM for the chloroplastic MIPS. Accordingly, the V_{max} for G-6-P in case of MIPS obtained from *S. junghuhnianum* MIPS was found to be 1.42 mM (Chhetri *et al.*, 2023), in yeast enzyme it was calculated as 1.6 mM (Donahue and Henry, 1981), in *Euglena*

gracilis it was 4.0, in *Oryza sativa* 4.42 and in *Spirulina platensis* the V_{max} was 5.05 (RayChaudhury *et al.,* 1997).

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 up to a concentration of 0.6 mM. (Fig-4.7 and Fig-4.18). The K_m for the coenzyme, NAD^+ was found to be about 0.21 mM for cytosolic MIPS and 0.25 mM for chloroplastic MIPS. While, the K_m for NAD^+ for MIPS from many other plants were found to be different where, 8 mM was calculated for the yeast enzyme (Donahue and Henry, 1981), 0.11mM for the *Spirulina platensis*, 0.16 mM for *Euglena gracilis*, 0.13mM for *Oryza sativa* (RayChaudhuri *et al*., 1997). The V_{max} for NAD^+ was about 0.83 mM for cytosolic MIPS and 0.77 mM for chloroplastic MIPS which were comparatively less than those from other plant species like *Marchantia nepalensis* (1.11mM), *Lunularia cruciata* (1.21mM), *D. glaucum* (1.8 mM), *Euglena gracilis* (3.98 mM), *Spirulina platensis* (4.24mM) and *Oryza sativa* (5.08 mM) (RayChaudhuri *et al.,* 1997; Chhetri, 2004; Chhetri *et al.*, 2009). The V_{max} was found to be 1.14 mM for the yeast enzyme (Donahue and Henry, 1981).

In a biological system, the purpose of the buffer is to keep intracellular and extracellular pH levels within a relatively small range and to withstand pH fluctuations brought on by both internal and external factors (Gueffroy, 1975). Consequently, the exclusion of Tris buffer from the complete set of the reaction mixture appeared to decrease the enzyme activity by 20% (cytosolic MIPS) and 35% (chloroplastic MIPS). Chhetri *et. al*., 2023 reported a loss of about 18% in MIPS activity when Tris buffer was eliminated from the reaction mixture in *S. junghuhnianum.* Likewise, when ME and NH_4Cl was eliminated from the reaction mixture, the

MIPS activity was decreased by 43.41% and 39.89% respectively in cytosolic MIPS. By the elimination of same components, ME and NH_4Cl (Table 4.6), the loss shown by chloroplastic MIPS was to the tune of 45.39% and 50.56% respectively (Table 4.11). Deduction of these two components, NH_4Cl and ME caused a decrease in MIPS activity by about 40.01% and 33.35% respectively in *S. junghuhnianum* (Chhetri *et al.,* 2023).

During incubation of the reaction mixture for various times between 0 and 150 minutes at an interval of 30 mins exhibited that MIPS activity from *H. salicifolia* was observed to progress linearly with time up to 90 min in both cytosolic (Fig. 4.3) and chloroplastic MIPS (Fig. 4.14), after which the activity of the enzyme did not show much variation in the last three incubation intervals in both the MIPS extracts. This progression with time has been observed to be quite similar in case of pteridophytic MIPS from *D. glaucum* (Chhetri *et al.,* 2006). However, slightly different results have been obtained in other species. The rate of enzyme reaction proceeded linearly up to 60 minutes in a moss, *B. bryoides* (Yonzone *et al*., 2018). The same from *Lycopodium clavatum* and *Selaginella monospora* showed time linearity between 75 and 90 minutes (Basak, 2013). While the MIPS from *Acer pseudoplatanus* cell culture exhibited time linearity up to 150 minutes (Loewus and Loewus, 1971).

When cytosolic MIPS activity was checked with respect to the concentration of enzyme protein in the assay mixture, it increased steadily up to a concentration of 100 µg and then gradually up to 300 µg after which the graph formed a plateau (Fig.4.4). Similar increase in enzyme activity up to 300 µg was seen in chloroplastic MIPS activity (Fig. 4.15). The MIPS activity with respect to enzyme protein linearity was identical to results found in *Lunularia cruciata,* which was 300µg (Chhetri *et al*., 2009). Similarly, the values for the same was found to be 250

μg and 350 μg for human fetal liver and *B. bryoides* respectively under optimal assay conditions (Chhetri *et al*., 2012; Yonzone *et al.,* 2018). However, the value for the same from algae was divergent in having its optimal activity at approximately 120μg (Basak, 2013).

When cytosolic and chloroplastic MIPS activity of *H. salicifolia* was checked for its thermal stability as show cased in Fig.4.5 and Fig. 4.16 respectively, remarkable activity of the enzyme was observed between 10 °C-40 °C with the maximum activity recorded at 30 °C (in case of the cytosolic enzyme). Below 10 °C and above 50 °C the enzyme showed least activity in case of cytoplasmic sample (Fig.4.5). Strangely enough, in case of chloroplastic enzyme (Fig. 4.16) the temperature maxima was at 20 °C while appreciable activity was recorded between a temperature band of 10 °C to 25 °C. This temperature maxima found in *H. salicifolia* was remarkably lower than that reported from other sources. The temperature maxima of MIPS in the enzyme, isolated from a number of other sources were found to be, 35^{o}C for *Entamoeba histolytica* (Lohia *et al*., 1999), 35^{o}C-37^{o}C for *Spirulina platensis, Euglena gracilis, Oryza sativa and Vigna radiata* (RayChaudhuri *et al.*, 1997), 40^{o}C for *B. bryoides* and human fetal liver enzyme (Yonzone et al., 2018; Chhetri *et al*., 2012). However, the unusual behaviour exhibited by MIPS from *H. salicifolia* was that an appreciable enzyme activity was observed even at a low temperature of 10^{o}C. The XlN01 proteins produced by bacteria cloned from *Xerophyta viscosa* proved to be functional even at lower temperatures of 10 ^{o}C (Majee *et al*., 2005). This enzyme's potential to function even at such low temperature could be related to MIPS potential characteristic tolerance to cold temperature. Maybe, this is one of the reasons why the alpine plants could tolerate extremely cold temperature and still survive.

In a study by Chhetri *et al*. (2006), it was discovered that the added co-factor NAD^{+} for MIPS enzyme provided the chloroplastic *D. glaucum* enzyme with heat stability. This thermal

stability in the case of cytosolic *D. glaucum* MIPS, was found due to the NAD^+ bound to the enzyme because when the endogenous NAD^+ was removed from the MIPS by activated charcoal treatment, preincubation temperature maxima dropped from 35°C to 25°C. It may be possible that, in the case of the cytosolic enzyme, at least a portion of the area of the enzyme molecule may be stabilised by full occupancy of NAD^+ given the molecular construct of the enzyme, MIPS (Stein and Greiger, 2002). NAD^+ and inorganic phosphate may be present at the active sites of the subunit of archeal MIPS, which may be the reason for its increased thermostability (Stieglitz *et al.,* 2005). Also, the INO1 gene from the thermotolerant *Archaeoglobus fulgidus* (Chen *et al.,* 2000) has demonstrated to code for the sole thermotolerant MIPS out of more than 65 INO1 genes identified so far that code for MIPS (Majee *et al.,* 2004).

When the effect of different substrate isomers was checked, D-glucose-6-phosphate was universally discovered to be the exclusive substrate utilized by the enzyme L-*myo*-inositol-1-phosphate synthase in order to produce L-*myo*-inositol-1-phosphate. No activity was observed when D-glucose-6-phosphate was replaced with D-fructose-6-phosphate, D-fructose-1,6-bisphosphate and D-Glucose-1-phosphate as substrate for the cytosolic as well as chloroplastic MIPS activity in *H. salicifolia* (Table 4.7 and Table 4.12). The cytosolic MIPS enzyme (Table 4.7) could, however, partially make use of D-galactose-6-phosphate and D-mannose-6-phosphate as substrate with about 7.5% and 4.16 % activity, while the same substrate isomers exhibited about 10% and 4.25% activity respectively (Table 4.12) in case of chloroplastic MIPS as compared to the activity where D-glucose-6-phosphate was utilized as substrate. In validation with our results, 9.38% and 1.42% activity of the enzyme MIPS was logged in with galactose-6-phosphate and mannose-6-phosphate respectively as substrates in case of a

pteridophyte (Chhetri *et al*., 2006). 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 (Basak, 2013). But in the case of MIPS from human fetal liver it was found to be completely dependent upon G-6-P as a substrate and the other hexose phosphates viz., D-fructose-6-phosphate, D-mannose-6-phosphate and D-galactose-6-phosphate at identical concentration exhibited no effectiveness as the substrate for human fetal liver MIPS (Chhetri *et al*., 2012). It may be mentioned here than an oxido-reductase may act like MIPS and isomerize galactose-6-phosphate to *muco*-inositol. Mannos-6-phosphate may also be utilized to form such isomers in plants. Now, different isomers of inositols are readily interconvertible which justifies the discernible MIPS activity in presence of galactose-6-phosphate and mannose-6-phosphate (Ogawa, 1999).

The results as shown in Fig. 4.8 and Fig. 4.19. checked for specificity of the coenzyme NAD^+ for MIPS enzyme from *H. salicifolia* or if its requirement can be satisfied by similar type of coenzyme(s), made it evident that NAD^+ is the co-factor for the enzyme MIPS from *H. salicifolia* that which could not be substituted with $NADP^+$ at any concentration because only minimal enzyme activity was observed in the experimental set with different concentration of $NADP^+$. The enzyme activity was found to be highest at 0.8 mM NAD^+ concentration in cytosolic enzyme (Fig. 4.8) and at 0.6 mM in chloroplastic enzyme (Fig. 4.19)

The two nicotinamide coenzymes, nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate (NADP), differ only in the presence or absence of a phosphate group on the 2'-C of the adenosyl moiety. Both NAD^+ and $NADP^+$ are used in a variety of enzymatic processes and serve as reversible carriers of reducing equivalents in the

cell (Broderick, 2001). The evidence for enzyme-bound endogenous NAD^+, as already discussed, is interestingly strengthened by the observation that some basal enzyme activity was present even in the absence of the supplied NAD^+. The remaining enzyme activity when $NADP^+$ is present validates the result. In addition, similar results have been reported from MIPS from *D. glaucum* (Chhetri *et al.,* 2006b). Therefore, MIPS from most of the sources require NAD^+ as an essential coenzyme for the oxidation reduction reaction.

The influence of pH variation was found to have a remarkable impact on its activity. The MIPS activity from *H. salicifolia* showed remarkably significant and maximum activity at a pH of 7.5 in both cytosolic (Fig.4.9) and chloroplastic (Fig. 4.20) MIPS. The results portray that the *H. salicifolia* enzyme became highly active at a pH range of 6.5 to 7.5. The activity was observed to be highest at pH 7.5. The rate of activity of the enzyme at pH 7 was also very close to pH 7.5. However, below pH 6.5 or above pH 7.5 there was continuous decline in the catalytic activity of the enzyme. This was observed in case of cytosolic MIPS (Fig. 4.9). However, in case of chloroplastic MIPS (Fig. 4.20) high activity was observed from pH6.5-8 and the highest was marked at 7.5 pH. Similar results have been reported in case of *Swertia bimaculata* and *Brachymenium bryoides* where both showed pH optima at a pH range of 7.0 to 7.5 (Chhetri, 2008; Yonzone *et al.,* 2018). While pH optima for MIPS from other species has been reported to vary slightly, like pH 7.7 from *Lemna gibba* (Ogunyemi *et al*., 1978) and *Neurospora* (Escamilla *et al*., 1982), pH 7.8 from *Spirulina platensis* (RayChaudhuri *et al*., 1997). *Pinus ponderosa* pollen and *Lilium longiflorum* pollen exhibited pH optima between pH 7.2-7.7 and pH 7.8-8.5 respectively (Gumber *et al*., 1984; Loewus *et al*., 1984), pH 8.0 from *Acer pseudoplatanus* (Loewus and Loewus, 1971), pH 8.4 from *Oryza sativa* (Funkhouser and Loewus, 1975), *Euglena gracilis*-8.2 (RayChoudhury, 1997).

For checking the influence of varied concentration of different salts viz., $MgCl_2$, NH_4Cl and EDTA on the enzyme, MIPS from *H. salicifolia* were determined and found that the effects of the salts were found to be directly correlated with their corresponding levels of concentration (Fig. 4.10 and Fig. 4.21). $MgCl_2$ and NH_4Cl salts were found to have a positive influence whereas the results in case of EDTA, it was negative in both cytosolic as well as chloroplastic enzyme. In cytosolic MIPS, NH_4Cl was found to be a strong stimulator which amplified the enzyme activity to 6.12-fold and $MgCl_2$ functioned as a moderate stimulator and increased the enzyme activity by 3.14-fold at their maximum concentration of 100mM as depicted in the Fig. 4.10. However, in case of $MgCl_2$ it is noteworthy that the stimulation of the enzyme activity was minimal up to a concentration of 80mM after which the activity spikes. On the other hand, during the present experiment, the influence of EDTA at its highest concentration of 100mM decreased the enzyme activity to roughly 30.63%. These results were observed in the case of MIPS extracted from cytoplasm.

The overall result in case of chloroplastic MIPS activity (Fig. 4. 21) was similar to that of the results depicted in cytosolic MIPS activity, where $MgCl_2$ and NH_4Cl was observed to have an increase in the enzyme activity and EDTA decreased the enzyme activity. The enzyme activity was observed to increased up to 5.75-fold and 2.76-fold by the influence of NH_4Cl and $MgCl_2$ respectively at its maximum concentration (100mM) which was slightly lesser than the case of cytoplasmic MIPS activity at the same concentration. On the contrary the influence of EDTA at its highest concentration of 100mM decreased the enzyme activity to roughly 28.5%. Different levels of stimulation of the enzymatic activity in case of NH_4Cl has been reported from various sources. It was seen in case of MIPS from *Oryza sativa*, *Vigna radiata* and *Euglena gracilis* that NH_4Cl produced about 5.0-fold stimulation of activity (RayChaudhuri *et*

al., 1997). Likewise, 2.3-fold increase in the enzyme activity in case of *Acer pseudoplatanus* (Loewus and Loewus, 1971), 2.0-fold stimulation effect of NH_4Cl in the activity of MIPS of *Euglena gracilis* (Dasgupta *et al*., 1984), 1.86 times stimulation in case of *B. bryoides* (Yonzone *et al*., 2018).

Influence of EDTA was noticed to have only a slight inhibition at the beginning but when it reached its highest concentration of 100mM it was observed to strongly inhibit the enzyme activity to roughly 30% while at up to 40mM it showed negligible change in the activity in bot cytosolic as well as chloroplastic results. Similar concentration dependent EDTA effect on the enzyme activity has been established in many plant species (Loewus and Loewus, 1980; Dasgupta *et al*., 1984; Chhetri, 2004). While in case of *Lemna gibba,* MIPS activity was found to be inhibited by 30% at a concentration of 1mM EDTA and at 10mM EDTA it completely inhibited the enzyme activity (Ogunyemi *et al*., 1978). Similarly, enzyme MIPS isolated from *Streptomyces griseus* was found to be inhibit the activity completely at 60mM concentration of EDTA (Pittner *et al*., 1979).

Influence of monovalent cations like K^+, Na^+ and Li^+ were also investigated, with the assay mixture containing various chloride salts in concentrations ranging from 0 to 10 mM. Results of the experiment for cytosolic and chloroplastic MIPS activity are presented in Fig. 4.11 and Fig. 4.22 respectively. It is evident that K^+ had a stimulatory effect on this enzyme activity and enhanced the enzyme activity by 2.58-fold and 2.55-fold for cytosolic and chloroplastic MIPS activity respectively. While very negligible effect was seen in case of *Lunularia cruciata* and *B. bryoides* by K^+ cation (Chhetri *et al.,* 2009; Yonzone *et al.,* 2018). 2.0-fold stimulation of the enzyme activity was seen in presence of K^+ in MIPS from bovine testis (Mauck *et al*., 1980)

which is quite agreeable with our results. However, Na^+ played a remarkable inhibitory role and decreased the enzyme activity by approximately 68.75% and 62.5% for cytosolic and chloroplastic enzyme respectively at 10 mM concentration. In case of *Neurospora crassa,* high concentration of Na^+ inhibited the enzyme activity (Escamilla *et al*., 1982). Furthermore, Li^+ was a strong inhibitor of this enzyme, which had the ability to reduce the specific activity of cytosolic and chloroplastic enzyme by about 85.0% and 80.65% respectively at 10 mM concentration. This unique inhibition of Li^+ was seen in a concentration-dependent manner in *Oryza sativa, Vigna radiata,* and *Euglena gracilis,* and even its 5 mM concentration was reported detrimental to the enzyme activity (RayChaudhuri *et al.,* 1997).

Variable quantities (1.0 to 10.0 mM) of the chloride salts of divalent cation like, Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Hg^{2+}, and Zn^{2+} were used to examine the effects on the activity of MIPS in *H. salicifolia* as displayed in Figure. 4.12 (cytosolic MIPS) and Fig. 4.23 (chloroplastic MIPS). The enzyme derived from both the cytoplasm and chloroplast exhibited a comparable manner of reaction towards various divalent cations. During the experiment, Ca^{2+} and Mg^{2+} showed a mild stimulatory effect to the tune of 2.05-fold and 1.65-fold stimulation in the enzyme activity respectively in cytoplasmic enzyme (Fig. 4.12). Similarly, in chloroplastic MIP), 1.95 % and 1.80 % increase in the activity was seen in case of Ca^{2+} and Mg^{2+} respectively (in Fig. 4.23). Comparable to this, Ca^{2+} increased the stimulation of the enzyme in *B. bryoides* by 2.39 times, but Mg^{2+} only marginally stimulated the enzyme activity (Yonzone *et al.,* 2018).

On the other hand, inhibitory effect was demonstrated in cytoplasmic enzyme (Fig.4.12) by Cu^{2+}, Mn^{2+}, Zn^{2+} and Hg^{2+}. Of these, Mn^{2+} and Cu^{2+} exhibited inhibitory effect of 39.37% and 6.25% respectively while in case of Zn^{2+} the inhibitory effect was more pronounced which

reduced the enzyme activity by 56.25%. Likewise, in case of chloroplastic enzyme (Fig 4.23), Mn^{2+} and Cu^{2+} demonstrated an inhibition by 29.72% and 13.51% respectively while Zn^{2+} reduced the activity by 43.24%. On the contrary, Hg^{2+} exhibited a strong inhibition of the enzyme activity inhibiting the enzyme activity by about 84.37 % in cytosolic MIPS activity and 72.07 % in chloroplastic MIPS activity at 10mM concentration. The inhibitory effect of different divalent cations proceeded in the order of $Cu^{2+} > Mn^{2+} > Zn^{2+} > Hg^{2+}$ in both forms of the enzyme.

As per RayChaudhuri *et al*. (1997), the action of Ca^{2+}, Mg^{2+}, and Mn^{2+} were discovered to operate in certain other plants as inhibitors to varying degrees at 5mM (RayChaudhuri *et al*., 1997). Divalent cations most likely displayed behaviours that were comparable to those previously described from other sources (Wells *et al.,* 1974; Loewus and Loewus, 1980). This strong heavy metal-induced enzyme inhibition could be due to the presence of one or more free sulfhydryl groups in the active site of the enzyme (Nelson and Cox, 2000). The presence of these unbound sulfhydryl units within the active site renders the enzyme susceptible to interaction with heavy metals, conceivably resulting in the impeding of its functionality (Valko *et al.,* 2005).

Like in many other eukaryotes, NH_4^+ seems to have the best stimulatory effect on MIPS from *H. salicifolia* over other divalent cations for its optimal activity. On the contrary, divalent cation have been seen to increase the enzyme activity in prokaryotes (Majumder *et al.*, 2003). This could indicate that the MIPS from *H. salicifolia* belongs to type-III aldolase. Though, this categorization is still vague because the enzyme activity has also been appreciably exhibited in

the presence of divalent cations, Ca^{2+} and Mg^{2+}which makes it belong to Class-II aldolase. Therefore, the place of MIPS from *H. salicifolia* among the class of aldolase is still ambiguous.

During the investigation of the impact of addition of sugar alcohols, with different quantities of galactitol, mannitol, and *myo*-inositol it was found to variably alter the cytosolic MIPS activity from *H. salicifolia*. Results in Fig. 4.13 indicate that when galactitol is present, the synthase activity, first raised fairly up to a concentration of 4 mM, followed by a slow fall up to a concentration of 10 mM, culminating to loss in enzyme activity up to 31.28%. *Myo*-inositol itself seemed to act similarly like galactitol where, a drop in the enzyme activity was noticed up to a concentration of 10 mM before the activity increased slightly at 4mM. But, not very drastic increase or decrease was seen for *myo*-inositol. The loss in the enzyme activity here accounted for just 13%. These results agree with reports on the L-myo-inositol-1-phosphate synthase activity of *Diplopterygium glaucum* (Chhetri, 2004). However, very pronounced alteration amongst the sugar alcohol could be seen only in case of mannitol, where there was a rapid linear increase in activity with increasing concentration up to 8mM after which the enzyme activity began to fall.

Results in Fig. 4.24 (chloroplastic MIPS) showed that mannitol increased the activity linearly up to a concentration of 8mM after which the activity decreased abruptly. Galactitol seemed to have a similar effect with a slightly lesser effect whereas *myo*-inositol had a very mild effect and slightly increased the activity only up to 6 mM after which it declined. No major difference in the enzyme activity could be observed in this set. However, in most of the cases, sugar alcohols have been reported to show inhibitory behaviour. Enzyme activity in case of *Acer pseudoplatanus* cell culture and *Arabidopsis thaliana* were identified to be inhibited by the

presence of inositol (Loewus and Loewus, 1973; Johnson and Sussex, 1995). Reduced MIPS activity was seen in *Euglena gracilis* cells in culture even when sugar alcohol concentrations were considerably lower (RayChaudhuri *et al*., 1997). However, raffinose series of sugars are found in plants, synthesized by the combination of UDP-galactose and inositol are very often seen to be involved in enabling carbohydrate transportation and stress tolerance (Bhattacharya and Kundu, 2020).

Inositol and their derivatives are bioactive substances that control how cells metabolise sugar and defend them against oxidative, cytotoxic, and mutagenesis damage (Fontles *et al*., 2000; Kreft and Jetz, 2007; De Olinda *et al.,* 2008). In plants, during cold or water stress, inositol and their derivatives are reported to accumulate (Ford, 1984; Pattanagul and Madore, 1999; Orthen and Popp, 2000). These compounds might therefore function as cryoprotectants and osmotic regulators. Inositol and other sugar alcohols have frequently been linked to inadequate watering of the plants (Pattanagul and Madore, 1999; Pharr *et al.,* 1995).

Hippophae sp. has been known to be a hardy plant which thrives well under dry conditions. Chinese seabuckthorn has a comparatively high concentration of l-quebrachitol, which may represent a defence mechanism the plant evolved to combat the protracted drought in the growth area. For cells to transmit signals, inositols like inositol-1,4,5-triphosphate are a crucial component of mediator molecules (Lanner *et al.,* 1988; Alberts *et al.,* 2002). Identification of MIPS and their metabolism may offer fresh perspectives on the biologically active elements and mechanisms underlying the cold stress resistance in *H. salicifolia*.

Plants grow and develop at their fastest rate when the temperature is optimal or falls within a certain range throughout the day (Fitter and Hay, 1981). Consequently, its essential to consider

temperature stress while evaluating the overall functioning of the plant. Physiological, metabolic, biochemical, and molecular changes in plants take place when ambient temperature deviates from its ideal range. In order to uphold cellular homeostasis under such challenging circumstances and to promote regular growth and developmental processes, plants make this effort. Therefore, in order to adjust to the changing temperature variables, modification in an array of biochemical, molecular and metabolic processes are made by the plants (Thomashow, 1999; Larkindale *et al*., 2005; Kotak *et al*., 2007; Zhu *et al*., 2007). One such modification is the accumulation of sugars which is considered to be one of the chief developments that has been shown to confer cold tolerance in plants. Role of MIPS and inositol in cold tolerance has been reported in cases including *Medicago sativa, Brassica juncea, Nicotiana tabacum, Arabidopsis thaliana, Oryza sativa, Passiflora edulis* (Tan *et al.,* 2013; Zhuo *et al.,* 2013; (Das-Chatterjee *et al.,* 2006; Goswami *et al.,* 2014; Kaur *et al.,* 2013; Majee *et al.,* 2004; Tan *et al.,* 2013; Zhu *et al*. 2012, Abreu, 2007).

A remarkable find of this research work is that it showcases the behaviour of the enzyme MIPS to function and exhibit its activity in a wide range of temperature ranging from 0ºC to 50ºC. It was exhibited by both cytosolic as well as chloroplastic MIPS from *H. salicifolia* that appreciable enzyme activity was observed even at a low temperature of 10 ºC and up to as high as 50 ºC. Enzymes that are able to function in a wide range of temperatures indicate their adaptability and flexibility in different environmental conditions. These enzymes have been found to exhibit lower activation enthalpies and entropies, allowing them to maintain their activity at both high and low temperatures (Deeva *et al.,* 2022). The ability of enzymes to function over a wide temperature range suggests that they have mechanisms in place to adjust their ligand-binding enthalpies and volumes, enabling them to optimize their performance

under different temperature conditions (Burns, 2022). This adaptability may involve amino acid substitutions throughout the protein, even in regions that are not traditionally considered active sites (Fields *et al.*, 2015). The wide temperature range of enzyme activity is of particular interest in the field of synthetic chemistry, as enzymes can catalyze reactions under mild conditions that are difficult to achieve using conventional chemical methods (Roberts, 1995). Overall, the potential of the enzymes, MIPS to function in a wide range of temperatures highlights and reflects its versatility and potential applications in various fields

This study is also an attempt to provide details for the essential evidence for the occurrence of the enzyme L-*myo*-inositol-1-phosphate synthase from a cold tolerant plant, *Hippophae salicifolia* from Sikkim Himalayas. The distribution of this enzyme, its biochemical traits, physiological functioning, and metabolic regulation have long been known from studies on bacteria, algae, fungus, pteridophytes, bryophytes, gymnosperms, angiosperms, and mammals. But study of the enzyme and its product inositol in plants from Sikkim Himalayas has not been carried out so far. Examining the biochemical traits of this enzymes, provides an insight into the functional diversity of enzymes. It is important in understanding how enzymes function within an organism and helps unravel various physiological processes. Therefore, this research work details a study for comprehending how the plant respond to changes in their internal and external environments and also fulfill the lacuna that has been created on the information with regards to the biosynthesis and metabolism of the *myo*-inositol and their role and functions of the enzyme, MIPS from a cold resistant plant. This research could help in understanding the mechanism of regulation and metabolism of the enzyme and its product in plants during cold stress and further form a base for biotechnological applications.

6. CONCLUSION

The research presented here on partial purification and characterization on MIPS provides a fundamental understanding regarding the mechanism of the enzyme regulation from a cold tolerant plant, *Hippophae salicifolia* from Sikkim Himalaya. Preparatory to the investigation, MI have been isolated from different species of mountain plants examined from different altitudinal zones. Further, significant MIPS activity was also detected in the species examined with the highest activity recorded in *H. salicifolia* from which the enzyme was partially purified up to the extent of 64-69 fold over the homogenate fraction with a recovery rate of about 19-21% depending on the enzyme source being the cytosol or the chloroplast. The apparent molecular weight of the cytosolic form of the enzyme from *H. salicifolia* was determined to be approximately 158.6 kDa. In most plants, the enzyme is composed of subunits of equal size and the subunit molecular weight was found to be approximately 60 kDa. Thus, this enzyme may be hypothesised to be composed of three subunits. G-6-P was observed to be the specific substrate for MIPS from the *H. salicifolia*. A discernible activity of the enzyme in presence of the substrate isomer galactose-6-phosphate and mannose-6-phosphate even when the specific substrate, G-6-P was not present indicates the formation of the product isomers like *muco*-inositol which may be readily converted to MI. NAD^+ was found to be the co-factor of this enzyme. However, even in absence of NAD^+, quite some activity was shown by the enzyme, pointing towards the presence of endogenous NAD^+in the molecular architecture of the *H. salicifolia* MIPS.

The cytosolic MIPS from *H. salicifolia* showed temperature maximum at 30ºC while the same was 20ºC in case of chloroplastic MIPS. An exceedingly atypical behaviour was demonstrated

by MIPS from *H. salicifolia*, in which noticeable enzymatic function was detected even at a low temperature of 10 °C.This ability of the enzyme may be linked to the inherent tolerance of MIPS to cold temperatures. MIPS from different other plant sources have already been associated with other abiotic stress tolerance. This information could be extrapolated in understanding the adaptation of the plants in colder areas.

As per previous studies, the activity of the enzyme is positively influenced either by NH_4^+ or Mg^{2+} which are mutually exclusive. However, in case of *H. salicifolia*, it was found that enzyme activity was positively stimulated by the presence of NH_4^+ and to some extent by Mg^{2+} ions too. This attribute of the enzyme indicates the trait of Class-II aldolase that necessitates the presence of divalent metals as well as a Class-III aldolase that necessitates NH_4^+ for its optimum activity.

This study contributes to the knowledge of the regulation of metabolism of the enzyme MIPS in plants from cold stressed flora of Sikkim. Knowledge of biochemistry of this enzyme and its product, may lead to its potential applications in the field of biotechnology, whereby cold-tolerant transgenics of important crops may be produced and the scope of agriculture may be extended to the fallow mountain areas.

7. SUMMARY

1. The fundamental screening of L-*myo*-inositol-1-phosphate synthase (EC 5.5.1.4) activity from some randomly selected plants from Sikkim Himalaya representing tropical, temperate and alpine regions showed that the enzyme is distributed in all the selected species.

2. L-*myo*-inositol-1-phosphate synthase (MIPS) was partially purified from the cytosolic sources of *Hippophae salicifolia.* 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 purification was about 64.46 fold over the homogenate fraction and the recovery was about 21.22% at the final stage of purification in case of the cytosolic enzyme

3. In order to evaluate the probable occurrence of inositol synthase within the chloroplasts, chloroplasts were isolated and the authenticity of the isolated chloroplasts was identified by their ability to carry out Hill reaction along with the determination of total chlorophyll content from the isolated chloroplasts.

4. Appreciable specific activity of inositol synthase was found in the isolated chloroplasts of *H. salicifolia* indicating the presence of a membrane bound or particulate form of this enzyme in addition to the cytosolic form. The particulate form of the enzyme was isolated and partially purified by the method described above. It could be purified to approximately 69.59 fold over the homogenate fraction with 19.15% recovery in case of chloroplastic enzyme.

5. The *H. salicifolia* MIPS activity dropped to about 80% in absence of the Tris-acetate buffer, while the activity was reduced to about 32%, 60% and 56% in absence of NAD^+, NH_4Cl and 2-mercaptoethanol respectively in case of the cytosolic MIPS. Similarly, in case of the chloroplastic enzyme, the activity was reduced to about 64%, 47%, 49% and 54% in absence of Tris buffer, NAD^+, NH_4Cl and 2-mercaptoethanol respectively. No enzyme activity could be observed in absence of glucose-6-phosphate (substrate) and in case of heat-killed enzyme from either source.

6. The MIPS activity was directly proportional to the time of incubation up to 90 minutes in the enzymes from both cytosolic and chloroplastic sources. Similarly, the enzyme from both sources exhibited maximum activity with respect to protein concentration of up to 300 µg.

7. The cytosolic enzyme was found to be most active between the temperatures of 20°C-40 °C with temperature maximum at 30 °C. On the contrary, the chloroplastic enzyme was most active between 10 °C and 25 °C with temperature maximum at 20 °C.

8. D-glucose-6-phosphate was found to be the specific substrate for MIPS of *H. salicifolia* Among the other hexose phosphates, D-fructose-6-phosphate, D-fructose-1,6-bisphosphate, D-glucose-1-phosphate could not act as substrates for this enzyme. However, this enzyme could partially utilize Galactose-6-phosphate and D-mannose-6-phosphate by both the cytosolic and the chloroplastic forms of MIPS.

9. The activity of *H. salicifolia* MIPS increases with the increase in substrate (G-6-P) concentration from 0 to 8 mM in case of the enzyme from cytosolic source, and the

same increases with the increase in substrate concentration from 0 to 6 mM in case of the enzyme from chloroplastic source.

10. The Bio Gel A 0.5 m purified cytosolic MIPS from *H. salicifolia* showed a K_m value for G-6-P as 0.55 mM and the same showed a K_m value of 0.67 mM in case of chloroplastic MIPS. The V_{max} value for G-6-P for the cytosolic and the chloroplastic MIPS were 1.1 mM and 1.06 mM respectively

11. The enzyme activity increases with respect to the increase in NAD^+ (coenzyme) concentration from 0 to 0.6 mM the in case of cytosolic MIPS and the same increases with the increase in NAD^+ concentration from 0 to 0.8 mM in case of chloroplastic MIPS from *H. salicifolia.*

12. With respect to the co-enzyme NAD^+, the Bio Gel A 0.5 m purified cytosolic MIPS showed K_m value of 0.21 mM and for the chloroplastic MIPS the K_m for NAD^+ was calculated as 0.25 mM. The V_{max} value for NAD^+ were 0.83 mM and 0.77 mM for the cytosolic and the chloroplastic MIPS respectively.

13. The *H. salicifolia* MIPS required NAD^+ as essential cofactor and it could not be replaced by $NADP^+$.

14. The MIPS from *H. salicifolia* operates between a pH range of 6.5 to 7.5. However, the maximum activity was found at pH 7.5.

15. Ammonium chloride highly stimulated the MIPS activity in a concentration guided manner, $MgCl_2$ was slightly stimulatory and EDTA was distinctly inhibitory to the *H. salicifolia* MIPS.

16. Among the monovalent cations, K^+ had stimulatory effect; while Li^+ was markedly inhibitory causing the loss of 80-85% of the enzyme activity.

17. Among the divalent cations studied, Ca^{2+} and Mg^{2+} were slightly stimulatory, Cu^{+2} and Mn^{2+} were slightly inhibitory, while Zn^{2+}, and Hg^{2+} were strongly inhibitory to the MIPS from *H. salicifolia*. It may be mentioned here that in presence of Hg^{2+}, the chloroplastic MIPS showed about 28% activity, while the cytosolic MIPS showed only about 16% activity at 10 mM concentration.

18. The sugar alcohols, mannitol showed stimulatory effect up to a concentration of 8 mM after which the activity sharply declined in both the cytosolic and chloroplastic forms of MIPS. *Myo*-inositol and galactitol showed only marginal effect in the cytosolic MIPS. However, *myo*-inositol showed a stimulatory effect up to 8 mM concentration in case of chloroplastic MIPS.

19. The apparent molecular weight of *H. salicifolia* MIPS (cytosolic form) was determined as approximately 158.6 kDa.

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APPENDIX-A

List of Publications

- Mukhia, R., & Chhetri, D. R. (2022). *Myo*-inositol and its metabolites in abiotic stress tolerance in plants. *Journal of Stress Physiology & Biochemistry*, *18*(4), 48-63.
- Chhetri, D. R., Yonzone, S., & Mukhia, R. (2023). Bioprospecting for enzymes in bryophytes: Extraction of L-*Myo*-inositol-1-phosphate synthase from Sphagnum junghuhnianum Doz. et Molk. and its characterization. *South African Journal of Botany*, *163*, 692-702.

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REVIEW

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Myo- inositol and its metabolites in abiotic stresstolerance in plants

Raksha Mukhia and Dhani Raj Chhetri*

[1] *Department of Botany, Sikkim University, Tadong, Gangtok, Sikkim, 737102, India*

*E-Mail: ***drchhetri@cus.ac.in***

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Myo-inositol (MI) is a sugar-alcohol produced by most plants and animals. 1L-myo-Inositol- 1-phosphate synthase (MIPS) is the rate limiting enzyme that catalyzes the conversion of D-glucose 6- phosphate to 1L-myo-inositol-1-phosphate, the first step in the production of all inositol- containing compounds. The enzyme exists in a cytoplasmic form in a wide range of plants, animals, and fungi. In plants, a chloroplastic form of the enzyme is also widely known. The significance of MI and its direct and more downstream derivatives lies in their dual functions as signalling molecules as well as key metabolites under stress. The role of MI and its derivatives in aiding the plants to cope with various abiotic stress conditions through physiological and biochemical changes have been discussed in this paper.

Key words: abiotic stress, myo-inositol, myo-inositol-1-phosphate synthase, osmolytes, stress tolerance

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Bioprospecting for enzymes in bryophytes: Extraction of L-*Myo*-inositol-1-phosphate synthase from *Sphagnum junghuhnianum* Doz. et Molk. and its characterization

Dhani Raj Chhetri[a,*], Sachina Yonzone[b], Raksha Mukhia[a]

[a] *Biochemistry and Molecular Biology Laboratory, Department of Botany, Sikkim University, 6th Mile-Tadong, Gangtok, Sikkim 737102, India*
[b] *Plant Physiology and Biochemistry Laboratory, Post Graduate Department of Botany, Darjeeling Government College, Darjeeling 734 101, India*

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ABSTRACT

L-*myo*-inositol-1-phosphate synthase (MIPS) and its product, free *myo*-inositol have been isolated from different bryophytes. MIPS was purified for the first time from the moss, *Sphagnum junghuhnianum* to 58.67 fold over its homogenate fraction with about 32.86 % recovery. D-glucose-6-phosphate was the exclusive substrate of the enzyme without which there was no enzyme activity and the deduction of NH_4Cl, ME and NAD^+ substantially reduced the activity of the enzyme. The K_m for D-glucose-6-phosphate and NAD^+were 1.81 mM and 0.25 mM, while the V_{max} for the same were 1.42 mM and 1.12 mM respectively. The molecular weight of the enzyme was assessed to be approximately 174 kDa. The activity of the enzyme increased with the increase in the duration of incubation time for up to 90 min and with the increase in protein concentration for up to 300 mg. The pH and temperature maxima were pH 7.0 and at 30 °C, respectively, but a significant activity was observed at 10 °C also. NH_4Cl substantially stimulated the enzyme activity while K^+ and Ca^{2+} also raised the activity slightly . Li^+ greatly inhibited the activity. Inhibitory activity was also shown by Cd^{2+}, Mn^{2+}, $Zn^{2+}$and Hg^{2+} of which Hg^{2+} showed the maximum inhibition. Interestingly, the *Sphagnum junghuhnianum* MIPS showed the characteristics of both Class-II and Class-III aldolase.