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Isolation and Biochemical Characterization of L-*myo*-Inositol-1-phosphate synthase from *Swertia bimaculata* Hook. f. & Thoms.

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ABSTRACT

The cellular level of myo-inositol is controlled by the enzyme L-myo- inositol-1-phosphate synthase (MIPS). The enzyme MIPS have been partially purified from the Swertia bimaculata, a temperate medicinal plant. The enzyme specifically utilized D-Glucose-6-phosphte and NAD⁺ as its substrate and co-factor respectively. It shows pH optima between 7.0 and 7.5 while the temperature maximum was at 30 °C. The enzyme activity was remarkably stimulated by NH_4^+ and highly inhibited by Cu^{2+}, Zn^{2+} and Hg^{2+}. The K_m values for D-glucose-6-phosphate and NAD⁺ was found to be as 0.32 mM and 0.16 mM respectively while the V_{max} values were 1.65 mM and 0.84 mM for D-glucose-6-phosphate and NAD⁺, respectively.

INTRODUCTION

Myo-inositol has long been considered as an important growth promoting factor. The importance of this compound as an essential component in lower plants (Shaktin and Tatum, 1961), plant cell and tissue in culture (Murashige and Skoog, 1962) mammalian cells and animals (Hegsted *et al.*, 1973; Kroes *et al.*, 1973). The biosynthesis of *myo*-Inositol from Glucose-6-phosphate has been documented from a number of biological systems (Loewus and Loewus; 1983).) L-*myo*-inositol-1-phosphate synthase (MIPS: EC: 5.5.1.4) catalyzes the conversion of D-glucose-6-phosphate (G-6-P) to L-*myo*-inositol-1-phosphate, the first and rate limiting step in the production of inositol which have been reported from evolutionarily diverse organisms (Chatterjee *et al.*, 2004).

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The product of this enzyme generates free *myo*-inositol on dephosphorylation. The inositol metabolism plays a vital role in growth regulation, signal transduction, membrane biogenesis, osmotolerance and other essential biochemical processes. The present study is concerned with the study on the occurrences of free *myo*-inositol in different *Swertia* species s and also the partial purification and characterization of MIPS from *Swertia bimaculata* Hook f. & Thoms. (Family-Gentianaceae) available in Darjeeling Hills.

MATERIALS AND METHODS

PLANT MATERIAL

Fresh specimens of Swertia species viz., *Swertia bimaculata* Hook f. & Thoms., *Swertia chirata* Buch.-Ham., *Swertia angustifolia* Buch.-Ham. ex D. Don and *Swertia purpurascens* Wall. were collected from their natural habitats in and around Darjeeling hills (ca 2134 m amsl) in the Eastern Himalayas.

ENZYME PREPARATION

Different samples (30g. each) were collected fresh in the

morning, washed twice with cold, sterile distilled water and homogenized in a chilled mortar and pestle in half the volume of 50 mM tris-acetate buffer (pH 7.5) containing 0.2 mM of ME. The crude homogenate was passed through four layers of muslin and the liquid was centrifuged at 1,000 x *g* for 5 minutes in a Plasto Crafts Superspin-R centrifuge. The supernatant was again centrifuged at 11,400 x *g* for 20 minutes and the resultant supernatant collected. The supernatant was dialyzed overnight against 50 mM tris-acetate (pH 7.5) buffer containing 0.2 mM ME and the clear supernatant was collected from the dialysis bag (11,400 *g* supernatant).

ENZYME ACTIVITY ASSAY

The MIPS activity was assayed by the procedure of Barnett *et al.*, (1970) with slight modifications. The assay mixture contained 50 mM tris-acetate (pH 7.5), 14 mM NH_4Cl, 0.8 mM NAD^+, 5 mM ME, 5 mM G-6-P and an appropriate aliquot (100-200 ig) of enzyme protein in a total volume of 500 il. The activity of the enzyme was determined by estimating the product-specific release of inorganic phosphate from *myo*-inositol-1-phosphate by MIPS reaction.

INORGANIC PHOSPHATE DETERMINATION

Inorganic phosphate was determined by the method of Chen *et al.*, (1956) with slight modifications. A freshly prepared Pi-reagent (2.8 ml.) containing H_2SO_4 (6N), ascorbic acid (10%, w/v), chilled ammonium molybdate (2.5%, w/v) and H_2O mixed in 1:1: 1: 2 ratio was added to the reaction mixture and incubated at 37 °C for 1h. The absorbance was measured at 820 nm. and the inorganic phosphate released was quantified with a standard curve prepared using K_2HPO_4.

PROTEIN DETERMINATION

Protein was determined according to the method of Bradford (1976) with BSA as a standard. The protein content in fractions obtained from column chromatography was also determined by measuring absorbance at 280 nm.

PURIFICATION OF MIPS

The enzyme was purified from the leaves of *S. bimaculata* employing the techniques of low speed centrifugation, streptomycin sulphate precipitation, ammonium sulphate fractionation, anion exchange chromatography on DEAE-cellulose, and gel-filtration chromatography on *Sephadex-G-200* following the method of Chhetri *et al.* (2006 a) as outlined below:

The clear supernatant (11,400 *g* supernatant) was subjected to 2% (w/v) Streptomycin sulphate treatment which was kept in ice-bucket at 0 °C for 15 minutes and then centrifuged at 11,400 x *g* for 15 minutes. The supernatant (Streptomycin sulphate treated fraction) was collected, precipitated with 0-70% $(NH_4)_2SO_4$ and dialyzed. The dialyzed fraction (0-70% ammonium sulphate treated fraction) was chromatographed on DEAE-cellulose column. The effluent was collected and the adsorbed proteins were eluted from the column with a linear gradient of 0-5.0 M KCl. The active DEAE-cellulose fractions (DEAE-cellulose fraction) were pooled and loaded on top of a column of *Sephadex-G-200* pre-equilibrated with 50 mM tris-acetate buffer (pH 7.5) containing 02 mM ME. The proteins were eluted with the same buffer and the pooled active fractions (*Sephadex-G-200* fraction) was concentrated and used as the ultimate preparation in this experiment.

FREE MYO-INOSITOL DETERMINATION

Free *myo*-inositol was isolated by the method of Charalampous and Chen (1966) by chromatography through a mixed bed column of Dowex-1-Cl with Amberlite IR-120 and one dimensional descending chromatography through Whatman no.1 paper. Free *myo*-inositol was estimated according to the method of Gaitonde and Griffiths, (1966) using a standard curve prepared using known concentrations of pure *myo*-inositol.

Table 1. Free *myo*-inositol content [mg g^{-1}(FW)] in the different species of *Swertia*. FW=fresh weight.

Family	Plant species	Plant parts	Free myo-inositol mg^{-1} FW
Gentianaceae	*Swertia chirata* Buch.-Ham.	Leaves	2.69
	Swertia bimaculata Hook f. & Thoms.	Leaves	2.98
	Swertia angustifolia Buch.-Ham. Ex D. Don.	Leaves	2.41
	Swertia purpurascens Wall.	Leaves	1.19

RESULTS AND DISCUSSION

DETERMINATION OF FREE MYO-INOSITOL FROM DIFFERENT SWERTIA SPECIES

Appreciable quantity of free *myo*-inositol (the final product of *myo*-inositol biosynthesis) was detected from different *Swertia* species (Table 1). It has been revealed that the quantities of free *myo*-inositol in almost all plant species are moderately high. Maximum free *myo*-inositol content was detected in the leaves of *Swertia bimaculata*. However, the content of the same in the leaves of *Swertia chirata* is also comparable.

PURIFICATION OF L-MYO-INOSITOL-1-PHOSPHATE SYNTHASE

The enzyme, L-*myo*- inositol-1-phosphate synthase was isolated and purified from the leaves of freshly collected *Swertia bimaculata*. The summary on the purification of MIPS is given in Table 2. The ultimate chromatography on *Sephadex-G-200* column resulted in about four-fold purification over the penultimate purification step employing DEAE-cellulose column. Chromatography on DEAE column in turn resulted in nine-fold purification over the homogenate fraction. An overall purification of the enzyme to about 38 fold with about 23% recovery based on total activity could be achieved in the present study.

CHARACTERIZATION OF THE PURIFIED ENZYME

REQUIREMENTS FOR S. BIMACULATA MIPS ACTIVITY

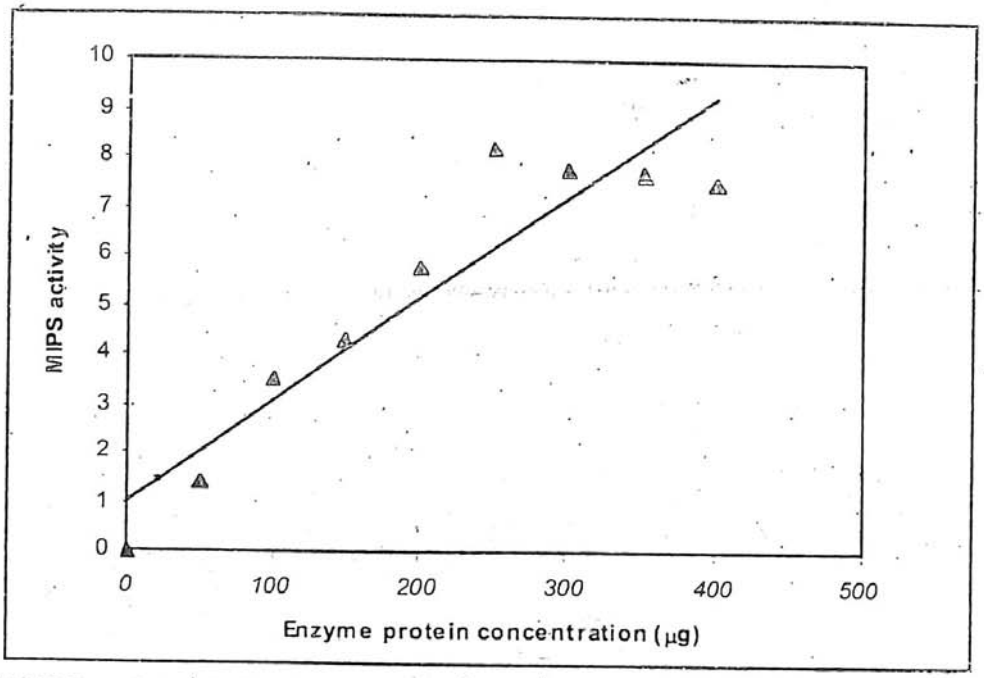
The *S. bimaculata* MIPS, when assayed in presence of 50 mM tris-acetate buffer (pH 7.5), 14 mM NH_4Cl, 0.8 mM NAD, 5 mM 2-mercaptoethanol (ME) and 5 mM glucose-6-phosphate (G-6-P) recorded maximal activity (Table-3). When the specific substrate (G-6-P) was not added in the incubation mixture, the enzymatic synthesis of L-*myo*-inositol-1-phosphate could not be detected. About 14% activity was lost when tris buffer was omitted from the reaction mixture. Deduction of NAD^+, NH_4Cl or ME resulted in the loss of enzyme activity by about 64%, 53% and 28% respectively. In comparison, the absence of NAD^+, NH_4Cl and ME decreased the activity of *Euglena gracilis* MIPS by 70%, 23% and 30% respectively (Dasgupta *et al.*, 1984).

SUBSTRATE SPECIFICITY

The *S. bimaculata* MIPS have been found to utilize G-6-P as the exclusive substrate for the production of L-*myo*-inositol-1-phosphate (MI-1-P). Among other hexose and pentose phosphates tested, such as D-fructose-6-phosphate, D-ribose-5-phosphate, D-fructose-1, 6-bisphosphate and D-glucose-1,6-bisphosphate used in place of G-6-P at identical concentrations (5 mM), all were ineffective. This result is in conformity with MIPS from other sources (RayChoudhuri *et al.*, 1997).

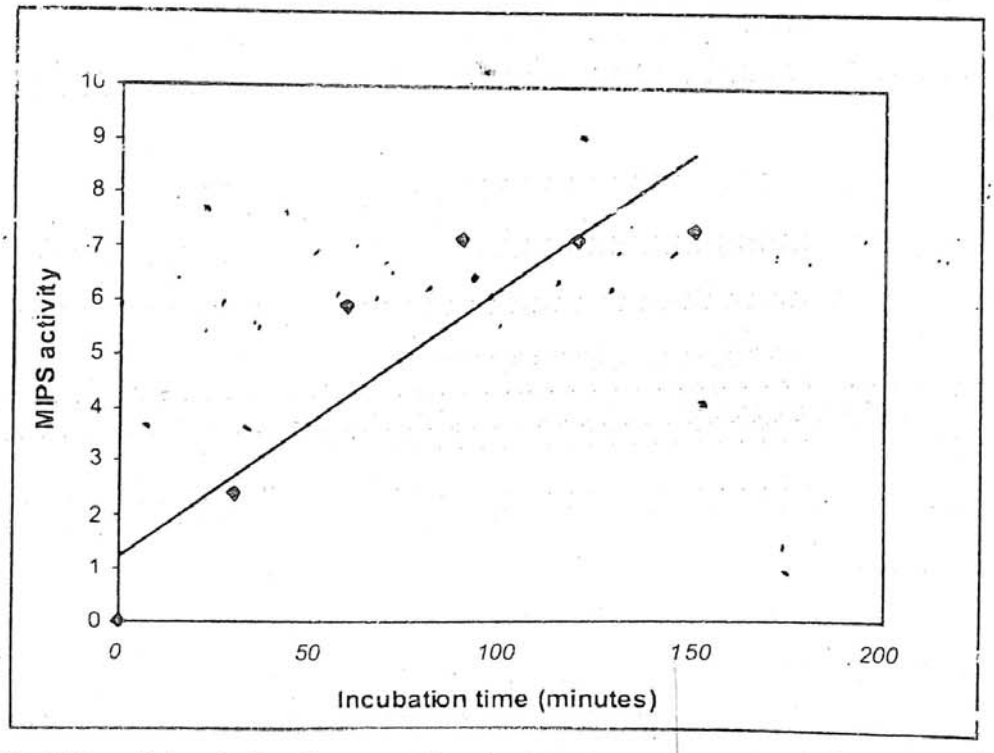
Table 2. Summary of partial purification of L-*myo*-inositol-1-phosphate synthase from *Swertia bimaculata*

Fraction	Protein (mg/ml)	Total Protein (mg)	Specific activity [μ mol (L-*myo*-inositol-1-phosphate) mg^{-1}(protein)h^{-1}]	Total activity [μ mol (L-*myo*-inositol-1-phosphate) h^{-1}]	Recovery [%]	Purification [fold]
Homogenate	0.57	45.60	0.13	5.93	100	1.00
11,400 *g* supernatant	0.56	40.70	0.14	5.69	96.08	1.07
Streptomycin sulfate treated fraction	0.36	9.36	0.46	4.30	72.51	3.53
0-70 % ammonium sulfate fraction	3.43	6.86	0.53	5.45	61.31	4.07
DEAE-cellulose fraction	0.28	1.40	1.17	1.63	27.62	9.00
Sephadex G-200 fraction	0.14	0.28	4.97	1.39	23.46	38.23



(MIPS activity defined as µ mol inositol-1-phosphate produced (mg protein)$^{-1}$ h^{-1})

Fig. 1. Effect of varied enzyme concentration on *Swertia bimaculata* MIPS activity.



(MIPS activity defined as µ mol inositol-1-phosphate produced (mg protein)$^{-1}$ h^{-1})

Fig. 2. Progress of MIPS reaction with incubation time on MIPS activity from *Swertia bimaculata*.

STABILITY OF THE ENZYME

An important feature of *S. bimaculata* MIPS is the moderate stability of its catalytic activity. Stability varies with the enzyme at different stages of purification. While the low speed supernatant remained active for 14-15 days when stored at "20 °C, the *Sephadex-G-200* purified fractions maintained its activity only up to 7-10 days when stored at identical temperature. However, repeated freezing and thawing resulted in remarkable loss of activity. Addition of enzyme stabilizer, 2-mercaptoethanol (ME) or dithiothritol (DTT) considerably increased the activity of the enzyme.

ENZYME AND TIME LINEARITY

The *S. bimaculata* MIPS exhibited enzyme linearity upto 250 ig of protein concentration under optimal assay conditions (Fig-1). The *D. glaucum* MIPS showed enzyme linearity upto 280 ig (Chhetri *et al.*, 2006a). The rate of enzyme reaction proceeded linearly upto 90 minutes with G-6-P as the substrate (Fig-2). This is quite different from MIPS from that of the *Acer pseudoplatanus* cell culture that shows time linearity upto 150 minutes (Loewus and Loewus, 1971)

PH – ACTIVITY RELATIONSHIP

The *S. bimaculata* MIPS exhibited optimum activity at a pH range of 7.0 – 7.5 when 50 mM tris-acetate buffer at a pH range of 6.0 – 8.5 were employed. This value is a little less in comparison to the pH optima for MIPS from other species like *Spirulina platensis* -7.8, *Euglena gracilis*- 8 2 (RayChoudhuri *et al.*, 1997) and rice cell culture-8.4 (Funkhouser and Loewus, 1975).

EFFECT OF TEMPERATURE

The effect of temperature was studied in the temperature range of 0' - 50 °C at 10 °C intervals. The activity of the enzyme was least both at 10 °C and 50 °C. However, it was remarkably active between the temperature ranges of 20-40 °C with maxima at 30 °C.

EFFECT OF METAL IONS

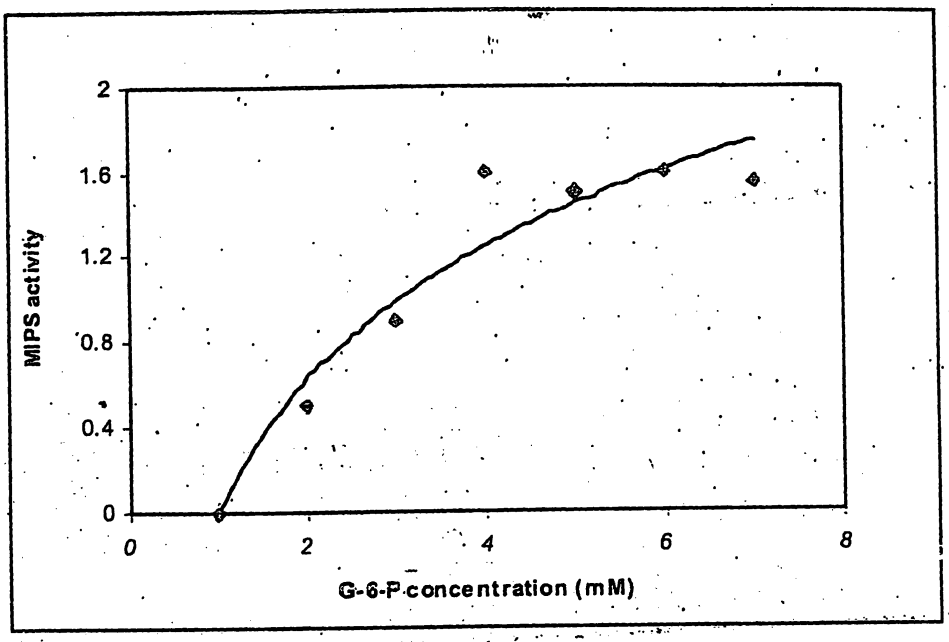
Effect of different metal ions was tested in 5 mM concentrations using chloride salts of metals. Of the monovalent cations tested K^+ and Na^+ had little effect, while NH_4^+ was an appreciable stimulator of the enzyme NH_4^+stimulation of *S. bimaculata* MIPS was to the tune of 1.7 times in contrast to the *Acer pseudoplatanus* MIPS which is stimulated 2.3 times with NH_4^+ (Loewus and Loewus, 1971). Using the similar concentrations of divalent cations it was revealed that Ca^{2+} and Mg^{2+} were slightly stimulatory, Mn^{2+} slightly inhibitory, Zn^{2+}, Cu^{2+} and Hg^{2+} strongly inhibitory (49%, 45% and 81% respectively) to the enzyme activity (Table-4). In general, the effects of monovalent and divalent cations are similar to those obtained from other sources.

REACTION RATE-SUBSTRATE CONCENTRATION RELATIONSHIP

Kinetic studies were carried out using G-6-P (substrate) in the range of 0-10 mM. The reaction rate was found to increase with respect to G-6-P upto a concentration of 4 mM. The K_m value for G-6-P calculated from Michaelis-Menten plot was 0.32 mM and the V_{max} value was calculated as 1.65 mM (Fig-3). The K_m value for G-6-P is identical with that of pine pollen (K_m=0.33) (Gumber *et al.*, 1984), but completely different for that from animal sources e.g., 2.7 for bovine testis enzyme (Mauck *et al.*, 1980); 3.89 for rat testis enzyme(Maeda and Eisenberg, 1980) and 4.4 for rat brain enzyme (Adhikari and Majumder, 1988).

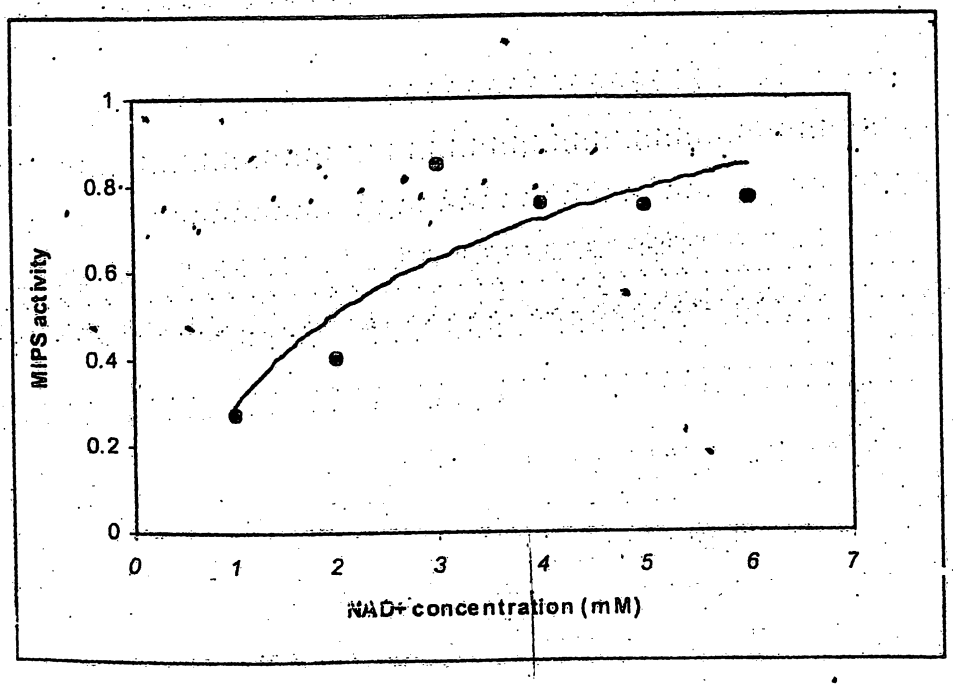
Table 3. Requirements for the L-*myo*-inositol-1-phosphate synthase activity from *Swertia bimaculata*

Condition	Specific activity [µ mol (L-*myo*-inositol-1-phosphate) mg^{-1} (protein)h^{-1}]	Percent activity
Complete set	6.20	100
Minus substrate (G-6-P)	0.0	0.00
Minus Tris buffer	5.31	85.64
Minus NAD^+	2.25	36.29
Minus NH_4Cl	2.90	46.77
Minus ME	4.48	72.25
Heat Killed Enzyme	0.0	0.0



(MIPS activity defined as μ mol inositol-1-phosphate produced (mg protein)$^{-1}$ h^{-1})

Fig. 3. Effect of various substrate (G-6-P) concentrations on *Swertia bimaculata* MIPS activity.



(MIPS activity defined as μ mol inositol-1-phosphate produced (mg protein)$^{-1}$ h^{-1})

Fig. 4. Effect of various co-enzyme (NAD^+) concentrations on *Swertia bimaculata* MIPS activity.

Table 4. Effect of incubation with monovalent and divalent cations (5 mM) on L-*myo*-inositol-1-phosphate synthase activity from *Swertia bimaculata*

Cation	Concentration (mM)	Specific activity [μ mol (L-*myo*-inositol-1-phosphate) mg^{-1}(protein)h^{-1}]	Percent activity
Control	0	7.10	100.00
K^+	5	7.52	105.91
Na^+	5	7.21	101.54
NH_4^+	5	12.05	169.71
Mg^{2+}	5	7.66	107.88
Mn^{2+}	5	6.75	95.07
Ca^{2+}	5	7.89	111.12
Zn^{2+}	5	3.65	51.40
Cu^{2+}	5	3.90	54.92
Hg^{2+}	5	1.46	20.56

REACTION RATE-CO-ENZYME CONCENTRATION RELATIONSHIP

Between concentrations of 0-1.0 mM of NAD (co-enzyme) the activity of purified enzyme was found to increase upto 0.4 mM concentration. With more increase in co-enzyme concentration the activity could not be increased. The co-enzyme saturation curve is hyperbolic in nature. The K_m value for NAD was 0.16 and the V_{max} value was calculated as 0.84 mM from the Michaelis-Menten equation (Fig-4). The K_m value for NAD+ was comparable to that from *Euglena gracilis* cytosolic enzyme having values 0.16-0.20 (RayChoudhuri *et al.*, 1997).

REPLACEMENT OF NAD+ WITH NADP+

MIPS from most of the sources require NAD+ as an essential coenzyme for the oxidation reduction reaction. To ascertain whether the MIPS from *S. bimaculata* is specific for its coenzyme NAD+, experiments were performed in which the enzyme activity was determined in presence of 0 – 1.0 mM NAD+ and NADP+ in two parallel experiments. It was determined that NAD+ could not be substituted with NADP+ of any concentration as in other cases (Adhikari and Majumder, 1988). However, there was a basal activity of this enzyme even in the experimental set with NADP+.

EFFECT OF EDTA

Between concentration ranges of 0-100 mM, EDTA had very significant effect on MIPS activity in the light of enzyme inhibition. The extent of inhibition is dependent on the concentration of EDTA with 40 ig concentrations inhibiting the MIPS activity by 50% and 100 ig inhibiting the activity by more than 80%. In contrast, the MIPS from lily pollen is not inhibited by EDTA (Loewus *et al.*, 1984).

In the present communication we report the occurrence and partial purification of MIPS for the first time from *S. bimaculata*. Table-2 summarizes the partial purification of this enzyme from different *S. bimaculata*. The enzyme from *S. bimaculata* was highly specific for G-6-P. Though the enzyme exhibits its optimal activity in presence of co-enzyme NAD+, still it could maintain about one third of the total activity when NAD+ was not added externally. This indicates the presence of bound NAD+ in the molecular architecture of this enzyme which has also been reported earlier (Dasgupta *et al.*, 1984, Chhetri *et al.*, 2006b). MIPS is involved in the metabolic utilization of G-6-P and it also generates Ribulose-5-phosphate as a product. The activity of this enzyme also seems to be related to that of Fructose-1,6-bisphosphatase and coupled with this are myriad of biochemical activities of the phosphate esters of *myo*-inositol (Murthy, 1996; Voglmaier *et al.*, 1997; Ogawa, 1999). All these points towards a central role played by this enzyme in metabolism and make this study worthwhile in understanding the basic metabolism in plants (RayChoudhuri and Majumder, 1996). It was found in *Swertia bimaculata* a four fold increase in the specific activity of the enzyme over the homogenate fraction could be achieved at ammonium sulphate fraction stage. Ultimately chromatography on Sephadex-G-200 through DEAE-cellulose brought a 38 fold increase in the enzyme activity with 74% loss of enzyme.

The obvious next step regarding the present work would be the purification of the enzyme to homogeneity, looking for particulate form of this enzyme, if any and determination of molecular weight of the native enzyme and its sub-units. Ultimately, the isolation of the gene and its molecular characterization would culminate the study.

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