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

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(Received 24 November 2008; accepted 25 December 2008)

ABSTRACT

The cellular level of myo-inositol is controlled by the enzyme L-myoinositol-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-phosphate 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 NH4+ and highly inhibited by Cu2+, Zn2+ and Hg2+. The Km values for D-glucose-6-phosphate and NAD+ was found to be as 0.32 mM and 0.16 mM respectively while the Vmax 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, 1951), 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-myoinositol-1-phosphate synthase (MIPS; EC: 5.5.1.4) catalyzes the conversion of D-glucose-6-phosphate (G-6-P) to L-myoinositol-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).

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, osmoterlance and other essential biochemical processes. The present study is concerned with the study on the occurrences of free myo-inositol in different Swertia species 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 parviflora 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 NH₄Cl, 0.8
mM NAD⁺, 5 mM ME, 5 mM G-6-P and an appropriate
aliquot (100-200 mg) of enzyme protein in a total volume of
500 ll. 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.*, (1966) with slight modifications. A freshly
prepared Pi-reagent (2.8 ml) containing H₂SO₄ (6N),
ascorbic acid (10%, w/v), chilled ammonium molybdate
(2.5%, w/v) and H₂O mixed in 1:1:1:2 ratio was added to
the reaction mixture and incubated at 37 °C for 1 h. The
absorbance was measured at 820 nm, and the inorganic
phosphate released was quantified with a standard curve
prepared using K₂HPO₄.

**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% (NH₃)₂SO₄ and dialyzed.
The dialized fraction (0-70% ammonium sulphate treated
fraction) was chromatographed on DEAE-cellulose
column. The eluent 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 0.2 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-Cr 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>
<thead>
<tr>
<th>Family</th>
<th>Plant species</th>
<th>Plant parts</th>
<th>Free myo-inositol mg⁻¹ FW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentianaceae</td>
<td><em>S. chireta</em> Buch.-Ham.</td>
<td>Leaves</td>
<td>2.69</td>
</tr>
<tr>
<td></td>
<td><em>S. bimaculata</em> Hook f. &amp; Thoms.</td>
<td>Leaves</td>
<td>2.96</td>
</tr>
<tr>
<td></td>
<td><em>S. angustifolia</em> Buch.-Ham. Ex D. Don.</td>
<td>Leaves</td>
<td>2.41</td>
</tr>
<tr>
<td></td>
<td><em>S. purpurascens</em> Wall.</td>
<td>Leaves</td>
<td>1.19</td>
</tr>
</tbody>
</table>

Table 1. Free myo-inositol content [mg g⁻¹(FW)] in the different species of *S. chireta*. FW=fresh weight.
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.

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

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

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 NH₄Cl, 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⁺, NH₄Cl or ME resulted in the loss of enzyme activity by about 64%, 53% and 28% respectively. In comparison, the absence of NAD⁺, NH₄Cl 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).
(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 dithiothreitol (DTT) considerably increased the activity of the enzyme.

ENZYME AND THE TIME LINEARITY

The S. bimaculata MIPS exhibited enzyme linearity up to 250 lg of protein concentration under optimal assay conditions (Fig-1). The D. glaucum MIPS showed enzyme linearity up to 280 lg (Chhetri et al., 2006a). The rate of enzyme reaction proceeded linearily up to 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 up to 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 NH₄⁺ was an appreciable stimulator of the enzyme. NH₄⁺ 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 NH₄⁺ (Loewus and, Loewus, 1971). Using the similar concentrations of divalent cations it was revealed that Ca²⁺ and Mg²⁺ were slightly stimulatory, Mn²⁺ slightly inhibitory, Zn²⁺, Cu²⁺ and Hg²⁺ 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 up to a concentration of 4 mM. The Kₘ value for G-6-P calculated from Michaelis-Menten plot was 0.32 mM and the Vₘₐₓ value was calculated as 1.65 mM (Fig-3). The Kₘ value for G-6-P is identical with that of pine pollen (Kₘ=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 (Majeda and, Eisenberg, 1980) and 4.4 for rat brain enzyme (Adhikari and Majumder, 1988).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Specific activity [μ mol (L-myo-inositol-1-phosphate) mg⁻¹(protein) h⁻¹]</th>
<th>Percent activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete set</td>
<td>6.20</td>
<td>100</td>
</tr>
<tr>
<td>Minus substrate (G-R-P⁻)</td>
<td>0.0</td>
<td>0.00</td>
</tr>
<tr>
<td>Minus Tris buffer</td>
<td>5.31</td>
<td>85.64</td>
</tr>
<tr>
<td>Minus NAD⁺</td>
<td>2.25</td>
<td>36.29</td>
</tr>
<tr>
<td>Minus NH₄Cl</td>
<td>2.90</td>
<td>46.77</td>
</tr>
<tr>
<td>Minus ME</td>
<td>4.48</td>
<td>72.25</td>
</tr>
<tr>
<td>Heat Killed Enzyme</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>
(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

<table>
<thead>
<tr>
<th>Cation</th>
<th>Concentration (mM)</th>
<th>Specific activity [µmol (L-myo-inositol-1-phosphate) mg⁻¹(protein)h⁻¹]</th>
<th>Percent activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>7.10</td>
<td>100.00</td>
</tr>
<tr>
<td>K⁺</td>
<td>5</td>
<td>7.52</td>
<td>105.91</td>
</tr>
<tr>
<td>Na⁺</td>
<td>5</td>
<td>7.21</td>
<td>101.54</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>5</td>
<td>12.05</td>
<td>169.71</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>5</td>
<td>7.66</td>
<td>107.88</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>5</td>
<td>6.75</td>
<td>95.07</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>5</td>
<td>7.89</td>
<td>111.12</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>5</td>
<td>3.65</td>
<td>51.40</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>5</td>
<td>3.90</td>
<td>54.92</td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>5</td>
<td>1.46</td>
<td>20.56</td>
</tr>
</tbody>
</table>

**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 up to 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 Kᵱ value for NAD was 0.16 and the Vₐ₮₃ value was calculated as 0.84 mM from the Michaelis-Menten equation (Fig-4). The Kᵱ 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 mg concentrations inhibiting the MIPS activity by 50% and 100 mg 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; Voglmair 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.
REFERENCES


