Characterization of Recombinant *Drosophila melanogaster* *Myo*-inositol-1-phosphate Synthase Expressed in *Escherichia coli*

Sang-Hee Park and Jong-Il Kim*

Department of Food and Microbial Technology, Seoul Women's University 126 Kongnung 2-dong, Nowon-Gu, Seoul 139-774, Korea

Cloned *myo*-inositol-1-phpsphate synthase (INOS) of *Drosophila melanogaster* was expressed in *Escherichia coli*, and purified using a His-affinity column. The purified INOS required NAD$^+$ for the conversion of glucose-6-phosphate to inositol-1-phosphate. The optimum pH for *myo*-inositol-1-phosphate synthase is 7.5, and the maximum activity was measured at 40°C. The molecular weight of the native enzyme, as determined by gel filtration, was approximately M_r 271,000±15,000. A single subunit of approximately M_r 62,000±5,000 was detected upon SDS-polyacrylamide gel electrophoresis. The Michaelis (K_m) and dissociation constants for glucose-6-phosphate were 3.5 and 3.7 mM, whereas for the cofactor NAD$^+$ these were 0.42 and 0.4 mM, respectively.

Key words: recombinant protein, inositol-1-phosphate synthase, multimeric protein, heat stability

Inositol is a precursor of essential macromolecules, such as membrane phospholipids. L-myo-inositol-1-phosphate synthase (INOS) catalyzes the conversion of D-glucose 6-phosphate to L-myo-inositol-1-phosphate, the first committed step in the production of all inositol-containing compounds, including phospholipids. The enzyme catalyzes the primary reaction for the synthesis of inositol, and exists in the cytoplasmic form in a wide range of plants, animals, and fungi (Majerus *et al.*, 1988). It has also been detected in several bacteria, and a chloroplast form is observed in alga and higher plants (Bachhawat and Mande, 2000). The enzyme has been purified from a wide range of organisms, with its active form being a multimer of identical subunits, with molecular weights ranging from 58,000 to 67,000 (Majumder *et al.*, 1997). The activity of the synthase is stimulated by NH$_4$Cl, and inhibited by glucitol 6-phosphate and 2-deoxyglucose 6-phosphate. The primary structure of this enzyme shows remarkable conservation across species. The structural gene (INO1), encoding the L-myo-inositol-1-phosphate synthase subunit, was first identified and cloned in yeast (Hirsch and Henry, 1986). Subsequently, homologues of this gene have been cloned from several prokaryotic (Bachhawat and Mande, 1999; 2000) and eukaryotic microorganisms (Ingavale and Bachhawat, 1999), and higher plants (Stein and Geiger, 2002). In baker's yeast, *Saccharomyces cerevisiae*, the transcriptional regulation of the *INO1* gene has been studied in detail, and its expression found to be sensitive to the availability of phospholipid precursors as well as the growth phase

(Graves and Henry, 2000).

The enzyme reaction for the conversion of glucose-6-phosphate to inositol requires NAD$^+$ (Fig. 1). The first step of the reaction mechanism is an oxidation. During this step, the NAD$^+$ serves as a hydrogen acceptor, followed by its condensation to a cyclic product and a subsequent reduction step, with NADH acting as the hydrogen donor. Therefore, NAD$^+$ is definitely required (Stein and Geiger, 2002). In this study, the INOS was cloned from *D. melanogaster*. The gene product, expressed in *Escherichia coli*, was purified by affinity column chromatography, and the enzyme was characterized using specific enzyme assays.

Materials and Methods

Expression of the INOS

The pET15b containing cDNA of INOS was transformed into *E. coli* BL21 (DE3) pLysS. The transformed cells were cultured in Luria-Bertani media (LB: 1% Trypton, 0.5% Yeast extract, 1% NaCl) with ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml), and cultured at 37°C, with vigorous shaking, until the OD$_{600}$ of the culture reached to 0.5. IPTG was then added to the culture to final concentration of 1 mM for induction of the INOS. The culture was then incubated for a further 3 h, at 28°C, with vigorous shaking.

Purification of the INOS

The IPTG induced cell culture was harvested by centrifugation (6,000 rpm) at 4°C, for 10 min. The cell mass was resuspended in binding buffer [20 mM Tris-HCl (pH 7.9), 500 mM NaCl, and 5 mM imidazole] and disrupted by freezing-thawing. The lysate was sonicated until completely

* To whom correspondence should be addressed.
(Tel) 82-2-970-5638; (Fax) 82-2-970-5977
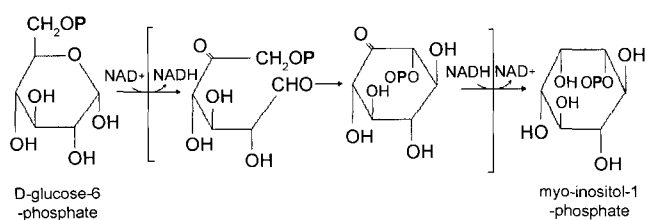(E-mail) jikim@swu.ac.kr

Fig. 1. Proposed chemical reaction mechanism of the conversion of glucose-6-P to inositol-1-P by INOS.

soluble. After centrifugation (12,000 rpm) at 4°C for 20 min, the supernatant was loaded onto the His-affinity column. The His-affinity column was charged with 0.1 M $NiCl_2$ and equilibrated with binding buffer. The column was washed with 5 volumes of binding buffer containing 0.1 M imidazole, and eluted with 5 volumes of binding buffer containing 0.2 M imidazole. The eluted fractions were dialyzed against 20 mM Tris-HCl (pH 7.0) and stored at -20°C.

Gel electrophoresis

The purified protein was analyzed on 10 and 5% SDS-polyacrylamide gel for separating and stacking, respectively. The sample was boiled in sample buffer (60 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue) for 5 min. The separating and stacking gels were run at 120 V and 15 mA. The gel was stained with Coomassie blue R250 for 1 h, and destained completely overnight.

Protein concentration determination

The protein concentration was determined by the Bradford assay, using bovine serum albumin as the standard.

Enzyme assay

The enzyme activity was assayed colorimetrically using the periodate oxidation method of Barnett et al. (1970), which was further corroborated by the inositol 1-phosphate phosphatase assay. The amount of Pi released from the INOS product on periodate oxidation, or inositol 1-phosphate hydrolysis, was estimated by the method of Eisenberg and Parthasarathy (1987), which measures the quantitative release of inorganic phosphate from inositol-1-phosphate. The standard reaction mixtures (0.3 ml) contained 5 mM glucose-6-phosphate, 1 mM NAD^+, 0.2 mM dithiothreitol, 2 mM NH_4Cl, 50 mM Tris-HCl (pH 7.5) and the enzyme. The enzyme activity was determined at 37°C, and the reaction stopped by the addition of 20% trichloroacetic acid (0.05 ml) and the mixture was then centrifuged (12,000 rpm) at 4°C, for 15 min, and supernatant (0.1 ml) was incubated with 0.1 ml of 0.2 M $NaIO_4$, at 37°C for 1 h. The excess periodate was destroyed by the addition of Na_2SO_3 (1 M, 0.1 ml), followed by the addition of 0.2 ml water. Ammonium molybdate-malachite green reagent (2.5 ml) was added, followed by 0.1 ml of 0.5% Tween 20. The absorbance was measured at 660 nm, and compared with a

KH_2PO_4 standard curve. The standard curve for released PO_4^{-3} was prepared using various concentrations of KH_2PO_4. The ammonium molybdate-malachite green reagent was prepared by dissolving 0.42 g of $(NH_4)_6 MoO_{24} \cdot 4H_2O$ to a final volume of 10 ml in 5 M HCl. The malachite green oxalate (0.015 g) was dissolved in 20 ml of water. The $(NH_4)_6 MoO_{24} \cdot 4H_2O$ solution was mixed with the malachite green oxalate solution, and after 30 min at room temperature was filtered through a 0.2 μm filter, and stored at room temperature in the dark.

Gel filtration

A Sephacryl S-300 column (1.5×60 cm) was equilibrated with 20 mM Tris-HCl (pH 7.0). Gel filtration column chromatography was performed with a flow rate of 15 ml/h, and 0.25 ml fractions collected. The void volume was determined with Blue dextran (2,000,000). The molecular weight standards used were bovine serum albumin (66,000), alcohol dehydrogenase (150,000), β-amylase (200,000), and apoferritin (443,000).

Results and Discussion

Purification of INOS

The cultured cells (2 L) were harvested by centrifugation at 6,000 rpm for 10 min, and resuspended in binding buffer. The cells were disrupted by sonication, and the clear lysate was obtained by centrifugation at 12,000 rpm for 20 min. The supernatant was applied to a His-affinity column. The column was washed with binding buffer containing 0.1 M imidazole and eluted with the binding buffer containing 0.2 M imidazole. The apparent yield (8 mg) for the preparation of INOS was obtained by a single chromatographic procedure. Examination of the enzyme by SDS-PAGE revealed a highly purified preparation containing only a single protein band (Fig. 2).

Molecular weight determination and subunit composition of native enzyme

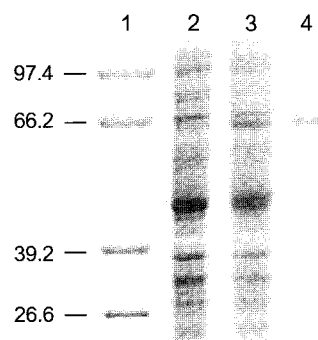The molecular weight of the purified enzyme was deter-

Fig. 2. SDS-PAGE of the purification steps. The electrophoresis was performed using 10% gel. Lane 1, molecular weight markers, Lane 2, total protein before induction, Lane 3, total protein after induction, Lane 4, purified recombinant protein.

mined on a 10% SDS-polyacrylamide gel. The calculated relative mobility of the INOS indicated that this protein had an approximate M_r of 62,000±5,000 Da (Fig. 3). The native molecular weight of the purified enzyme was determined on a calibrated column of Sephacryl S-300. Compared with a standard mixture of proteins of known molecular weight (albumin, alcohol dehydrogenase, amylase and apoferritin), the molecular weight, derived from gel filtration of the native *D. melanogaster* INOS for
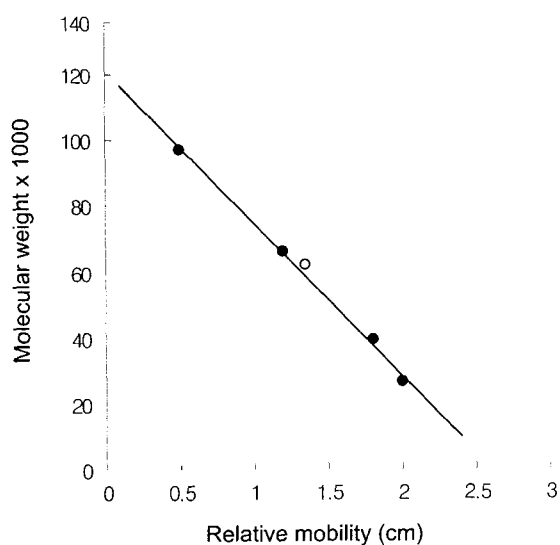

Fig. 3. Molecular weight determination of the enzyme subunit by SDS-PAGE. The relative mobility of the purified enzyme was compared to known proteins. Purified enzyme was resolved on a slab 10% polyacrylamide gel containing SDS under fully dissociating conditions. Molecular weight marker, phosphorylase B (97,400 Da), bovine serum albumin (66,200 Da), aldolase (39,200 Da), and triose phosphate isomerase (26,600 Da), and recombinant INOS (○) were co-electrophoresed.
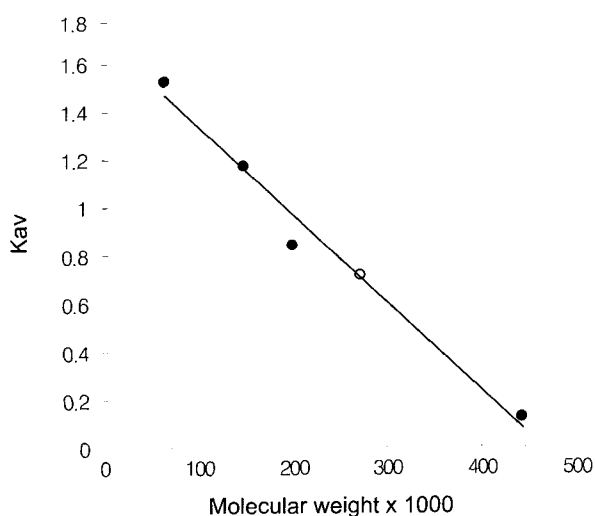

Fig. 4. Determination of molecular weight of the native enzyme. An estimate of the molecular weight of the native enzyme was performed by gel filtration with Sephacryl S-300 HR. The molecular weight standard proteins used were bovine serum albumin (66,000), alcohol dehydrogenase (150,000), amylase (200,000), apoferritin (443,000) and recombinant INOS (○).

recombinant protein, was 271,000±15,000 Da (Fig. 4). Taking the subunit molecular weight into account, this suggests a tetrameric protein structure, as with most other chloroplastic INOS similar to the fungal enzymes. The cytosolic form of the enzyme reportedly has a trimeric association, with the exception of *S. cerevisiae*, while the chloroplastic one has a tetrameric structure similar to that of fungal enzymes (Majumder *et al.*, 1997). The other preparation of inositol-1-phosphate synthase, purified to varying degrees from a variety of sources, suggests a tetrameric or trimeric structure, with a molecular weight in excess of 200,000 Da. The INOS of *S. cerevisiae*. (Donahue and Henry, 1981) has a tetrameric structure. The purified inositol-1-phosphate synthase from *Neurospora* (Escamilla *et al.*, 1982), *Glycine max* (Hegeman *et al.*, 2001), and bovine testes (Mauck *et al*, 1980) also have tetrameric structures. Conversely, the INOS isolated from rat testes (Meada and Eisenberg, 1980) has a trimeric structure. The INOS from *Spirodela polyrrhiza*, *Entamoeba histolyca*, *Oryza sativa*, *Lemna gibba*, and *Lilium longifolium* have trimeric structures.

Effect of pH on enzyme activity

The pH dependence of the purified recombinant enzyme was measured by assaying at various pH values, ranging from 5 to 11, with 50 mM buffers of MES-NaOH (pH 5 and 6), Tris-HCl (pH 7, 8 and 9), and CAPS-NaOH (pH 10 and 11). The enzyme was active over the pH range 5 to 10, with maximal activity between pH 7 and 8. Approximately 34% of the maximal activity was observed at pH 9, but little activity was seen at, or below pH 5, with no activity observed over pH 11 (Fig. 5). The optimal pH of the purified recombinant enzyme was 7.5, which was comparable to the enzymes purified from different sources, that operate within a broad pH range from 7.0 to 8.4 (Majumder *et al.*, 1997).

Effect of temperature

To determine the effect of temperature on the enzyme activity, reactions were measured at various temperatures, ranging from 20°C to 60°C. The enzymatic activity increased with increasing temperature, between 20°C and 40°C. The
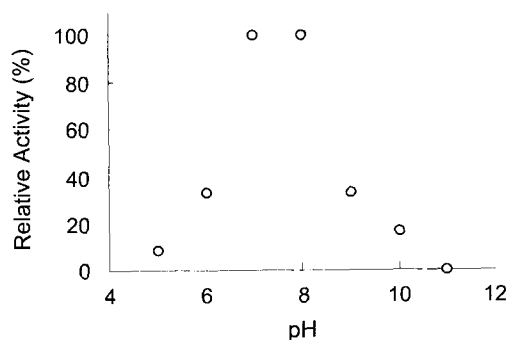

Fig. 5. Effect of pH on enzyme activity. Reaction mixtures were incubated at 37°C for 10 min in standard reaction mixtures at various pH values.

maximum enzyme activity was obtained at 40°C. The enzyme activity decreased at temperatures over 40°C, and was abruptly lost above 50°C due to thermal inactivation (Fig. 6).

Heat stability of INOS

To examine the thermal stability of enzyme, the enzyme was preincubated at 50 or 60°C, for the indicated times, and the remaining activities were assayed as described in the Materials and Methods section. The relative stabilities are shown in Fig. 7. The enzyme activity was stable when heated at 50°C for 10 min, but after 60 min incubation at 50°C, 60% of the activity was lost, and completely destroyed with further incubation at 50°C. The enzyme was sensitive to heat treatment at 60°C, with immediate decrease in the enzyme activity. More than 60% of the activity was lost by treatment at 60°C for 5 min. From the data for the heat stability, the recombinant enzyme was judged to exhibit low thermostability. This could be a gen-
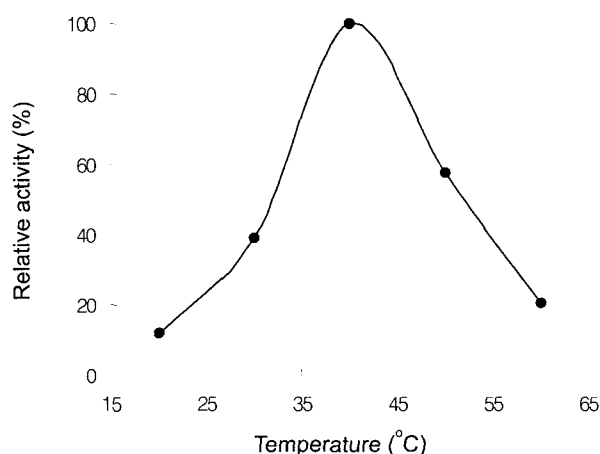
eral feature of the thermosensitivty of INOS. Eisenberg Jr. (1987) reported the stability of the INOS activity at 60°C. The biochemical characterization of the INOS from amoebae showed little difference between the native and recombinant proteins (Lohia et al., 1999). Both the proteins have optimum activity within the pH and temperature ranges 7.4 to 7.6 and 35°C to 37°C, respectively. Both the recombinant and native proteins were stabilized with 20% (v/v) glycerol, 2 mM PMSF and 1 mM E-64, throughout most of the purification procedure. The purified protein could be stored at -20°C, in 20% (v/v) glycerol for several weeks with no noticeable loss in activity. The specific activity of the native INOS from amoebae was approximately double that of the specific activity of the recombinant protein from E. coli.

Kinetic parameter

The determination of the kinetic characteristics of the INOS was carried out by measuring the initial velocity of the reaction at various concentrations of one substrate (glucose-6-phosphate), keeping the concentrations of the other substrate (NAD⁺) fixed. The kinetic parameters for the enzymatic conversion of glucose-6-phosphate to inositol were estimated by analysis of Lineweaver-Burk plots for each reaction performed with a fixed NAD⁺ concentration. The velocity increased with increasing NAD⁺ concentration. Replots of the slopes and the intercepts of the curves for each reaction, were linear, with finite intercepts (Fig. 8). To calculate the kinetic parameters the data were fitted, according to a Cleland analysis (Kuby, 1991), into the equation shown below:

Fig. 6. Effect of temperature on enzyme activity. The enzyme was assayed in standard reaction mixtures at various temperatures for 10 min.

$$v = \frac{V[A][B]}{K_{ia}K_b + K_a[B] + K_b[A] + [A][B]}$$

where v is the initial velocity, V is the maximum velocity, [A] and [B] are the concentrations of glucose-6-phosphate
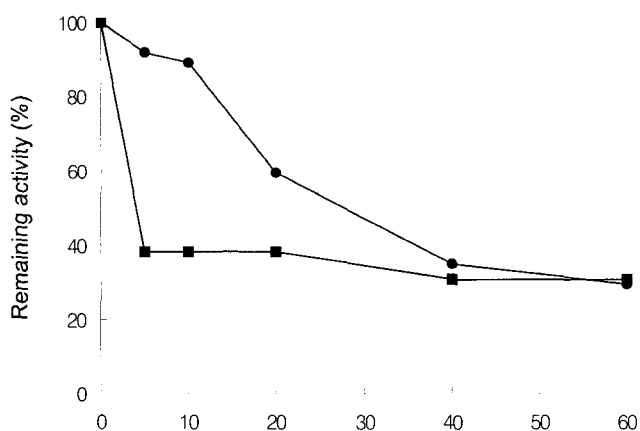

Fig. 7. Heat stability of INOS. The recombinant enzyme was preincubated at 50°C (●) or 60°C (■) and then assayed in standard reaction mixtures at 37°C for 10 min.
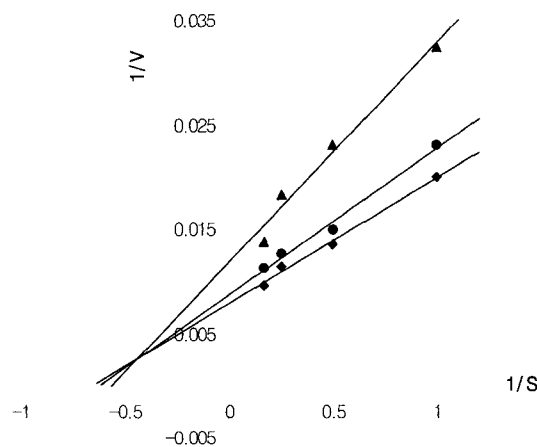

Fig. 8. Kinetics of INOS with glucose-6-phosphate at various NAD⁺ concentrations. Enzyme (1 µg) was incubated with 1 (▲), 2 (●), and 5 mM (◆) NAD⁺ and the indicated concentration of glucose-6-phosphate at 37°C for 10 min.

and NAD^+, respectively, K_a and K_b are the Michaelis constants for the substrates, glucose-6-phosphate and NAD^+, respectively, with K_{ia} (the dissociation constant for glucose-6-phosphate) and K_{ib} (the dissociation constant for NAD^+) being calculated from the symmetrical relationship, Kia $Kb=K_{ib}$ K_a. From this analysis, the K_ms for glucose-6-phosphate and NAD^+ were estimated to be 3.45 and 0.43 mM, respectively. The dissociation constant for glucose-6-phosphate and NAD^+ were 3.71 and 0.4 mM, respectively. Compared to the enzyme from other sources, Majumder et al. (1997) reported that the substrate affinities of the rat brain and human fetal brain synthase had very similar K_m (4.4 and 4.47 mM, respectively). Maeda and Eisenberg (1980) also reported a similar value (3.8 mM). Others have reported lower values, with the enzymes from rat and bovine testis having values of 0.05 (Barnett et al., 1973) and 0.012 mM (Pittner and Hoffmann-Ostenhof, 1976), respectively. There are significant differences between the K_m values for glucose 6-phosphate of native and recombinant INOS. The lower K_m value for glucose 6-phosphate for the native INOS suggests a higher substrate specificity compared to the recombinant protein. A four-fold stimulation of the synthase activity was observed on treating both the recombinant and native proteins with NH_4Cl (10 to 14 mM) (Lohia et al., 1999).

Acknowledgment

References

Bachhawat, N. and S.C. Mande. 1999. Identification of the INO1 gene of Mycobacterium tuberculosis H37Rv reveals a novel class of inositol-1-phosphate synthase enzyme. J. Mol. Biol. 291, 531-536.

Bachhawat, N. and S.C. Mande. 2000. Complex evolution of the inositol-1-phosphate synthase gene among archaea and eubacteria. Trends Genet. 16, 111-113.

Barnett. J.E.G., R.E. Brice, and D.L. Corina. 1970. A colorimetric determination of inositol monophosphates as an assay for D-glucose-6-phosphate-1L-myo inositol-1-phosphate cyclase. Biochem. J. 119, 183-186.

Communi, D., R. Lecocq, and C. Erneux. 1996. Arginine 343 and 350 are two site residues involved in substrate binding by human type I D-myo-inositol-1,4,5-triphosphate 5-phosphatase. J. Biol. Chem. 271, 11676-11683.

Dean-Johnson, M. and S.A. Henry. 1989. Biosynthesis of inositol in yeast. J. Biol. Chem. 264, 1274-1283.

Dean-Johnson, M. and I.M. Sussex. 1995. 1L-myo-inositol-1-phosphate synthase from Arabidopsis thaliana. Plant Physiol. 107, 613-619.

Dean-Johnson, M. and X. Wang. 1996. Differentially expressed forms of 1L-myo-Inositol-1-phosphate synthase in Phaseolus vulgaris. J. Biol. Chem. 271, 17215-172218.

Hoshizaki, D.K., J.E. Hill, and S.A. Henry. 1990. The Saccharomyces cerevisiae INO4 gene encodes a small highly basic protein required for derepression of phospholipid biosynthetic enzyme. J. Biol. Chem. 265, 4736-4745.

Eisenberg JR, F. and R. Parthasarathy. 1987. Measurement of Biosynthesis of myo-Inositol from glucose-6-phosphate. Methods Enzymol. 141, 127-143.

Funkhouser, E.A. and F.A. Loewus. 1975. Purification of myo-Inositol-1-phosphate synthase from rice cell culture by affinity chromatography. Plant Physiol. 56, 786-790.

Hegeman, C.E., L.L. Gold, and E.A. Grabau. 2001. Expression of D-myo-inositol-3-phosphate synthase in soybean. Implications for phytic acid biosynthesis. Plant Physiol. 125, 1941-1948

Ingavale, S.S. and A.K. Bachhawat. 1999. Restoration of inositol prototrophy in the fission yeast Schizosaccharomyces pombe. Microbiology. 145, 1903-1910.

Johnson, M.D. 1994. The arabidopsis thaliana myo-inositol-1-phosphate synthase. Plant Physiol. 105, 1023-1024.

Kuby, S.A. 1991. A Study of Enzymes. CRC Press. I, 106-119.

Lohia, A., N.C. Hait, and A.L. Majumder. 1999. L-myo-Inositol-1-phosphate synthase from Entamoeba histolytica. Mol. Biochem. Parasitol. 98, 67-79.

Maeda, T. and F. Eisenberg, Jr. 1980. Purification, structure and catalytic properties of L-myo-inositol-1-phosphate synthase from rat testis. J. Biol. Chem. 255, 8458-8464.

Majerus, P., T. Connolly, V. Bansal, R. Inhorn, T. Ross, and D. Lips. 1988. Inositol phosphate: Synthesis and degradation. J. Biol. Chem. 263, 3051-3054.

Majumder, A.L., M.D. Johnson, and S.A. Henry. 1997. 1L-myo-inositol-1-phosphate synthase. Biochim. Biophy. Acta. 1348, 245-256.

Mauck, L.A., Y-H. Wong, and W. R. Sherman. 1980. 1L-myo-inositol 1-phosphate synthase from bovine testis: Purification to homogeneity and partial characterization. Biochemistry 19, 3623-3629.