3: MATERIALS AND METHODS

3:1 Materials:

Fresh specimens of bryophytes viz., *Asterella khasiana* (Griff.) Grolle, *Marchantia polymorpha* Linn., *Riccia cruciata* Kash, *Anthoceros angustus* Steph., *Bryum argenteum* Linn., *Brachymenium bryoides* Hook. ex Schwaegr., *Lyellia crispa* R. Br., *Funaria wallichii* (Mitt.) Broth. and *Sphagnum junghuhnianum* Doz. & Molk. were collected from the localities in and around Darjeeling hills (circa 2134 m amsl.) situated between 87°59' - 88°53' E and 26°31' - 27°13' N in the Eastern Himalayas of India.

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

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

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

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

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

3.2 Methods

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

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

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

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

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

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

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

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

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

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

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

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

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

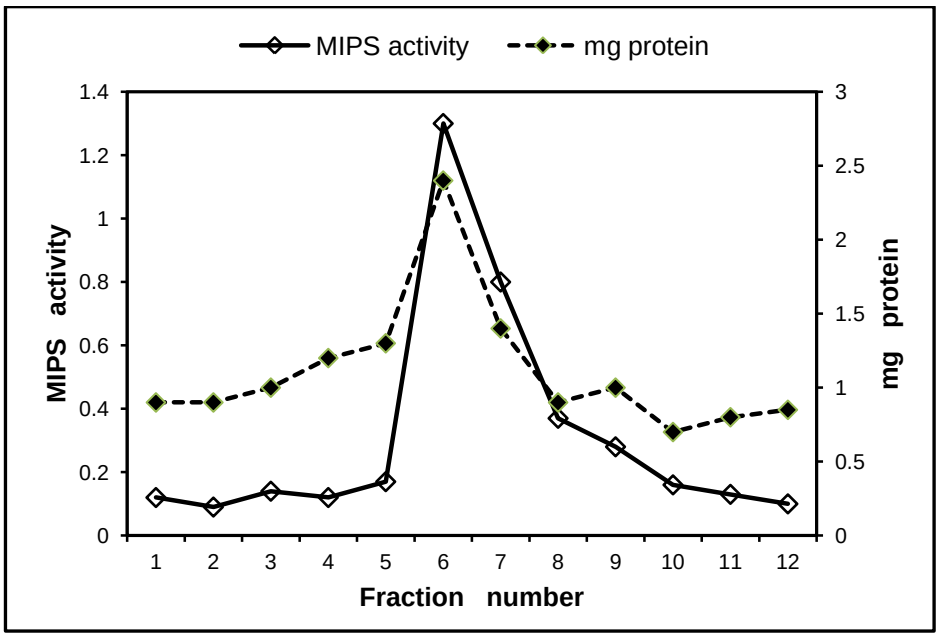

Fig-3.1 Elution profile of cytosolic MIPS from *A. khasiana* in DEAE cellulose column (MIPS activity is expressed as µM I-1-P produced/2ml fraction/hr)

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

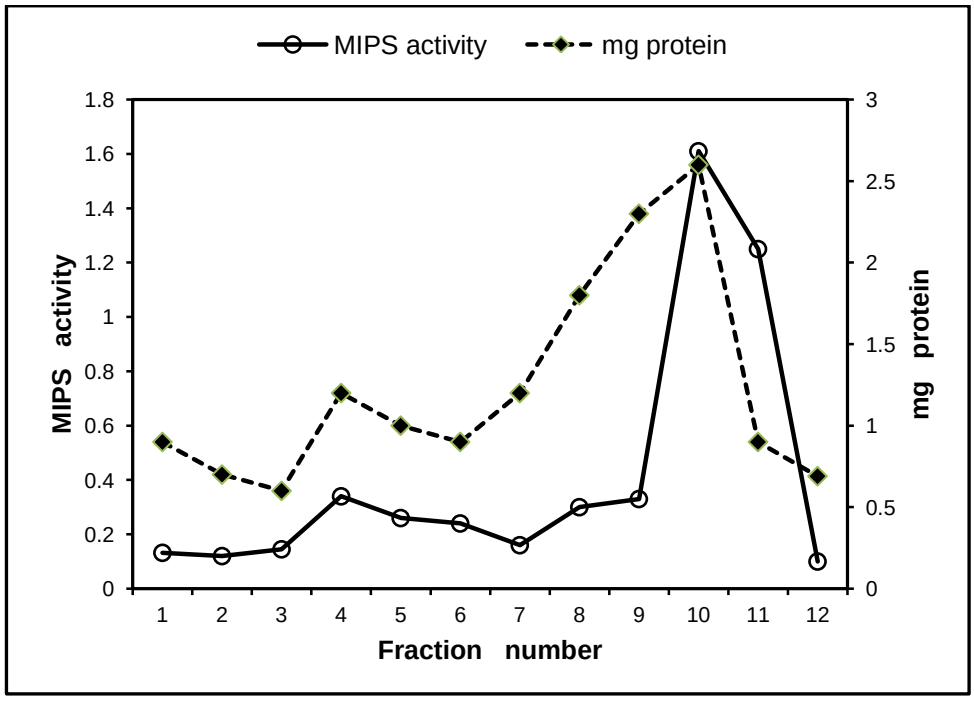

Fig-3.2 Elution profile of cytosolic MIPS from *S. junghuhnianum* in DEAE cellulose column (MIPS activity is expressed as µM I-1-P produced/2ml fraction/hr)

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

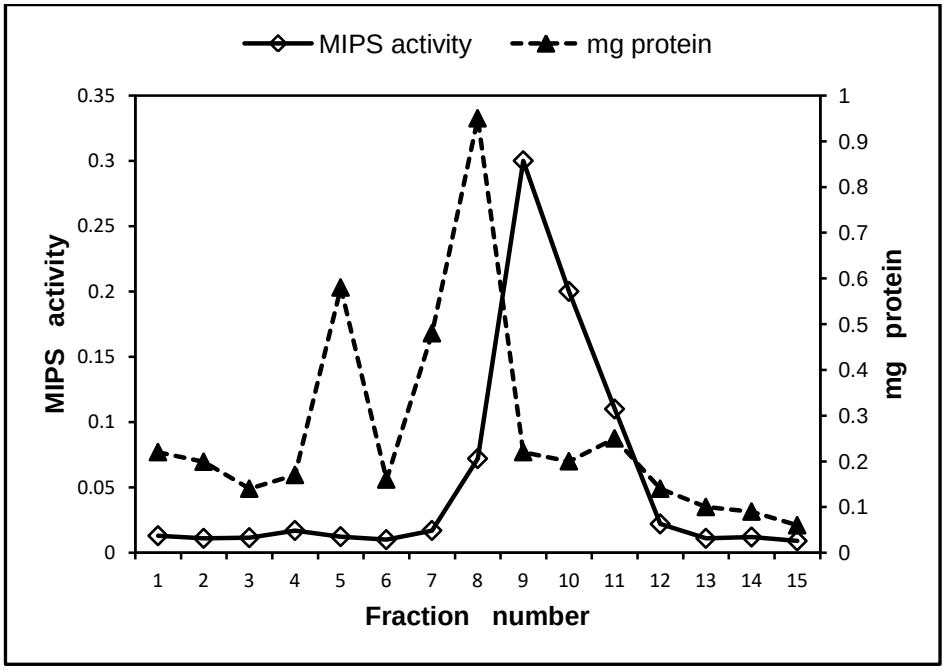

Fig-3.3 Elution profile of cytosolic MIPS from *A. khasiana* in Hexylagarose column (MIPS activity is expressed as µM I-1-P produced/1.25ml fraction/hr)

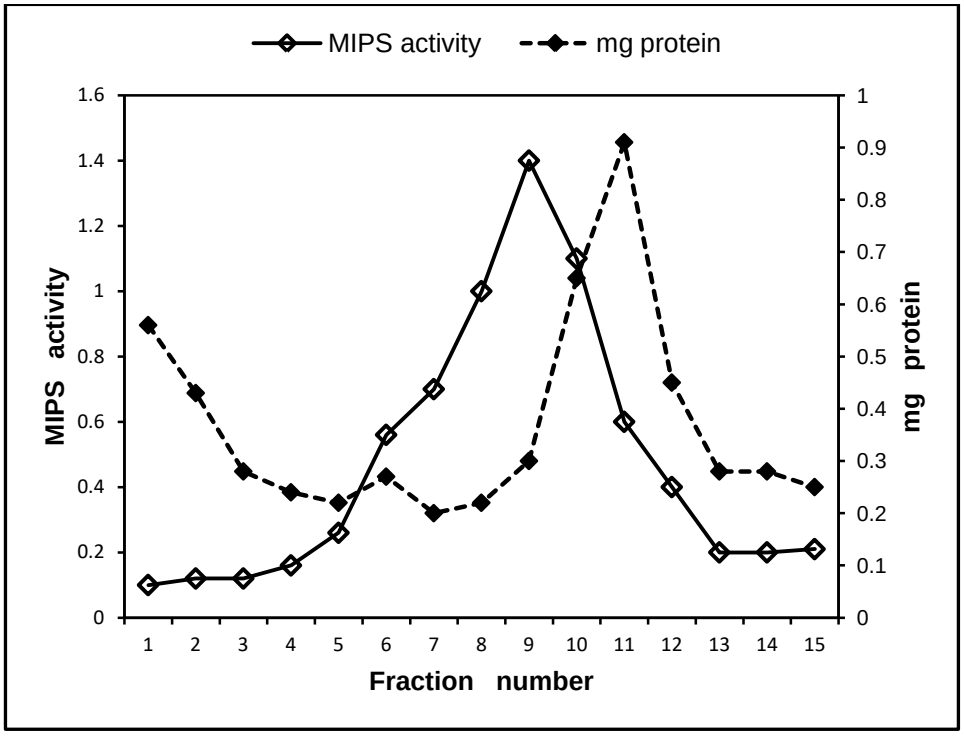

Fig-3.4 Elution profile of cytosolic MIPS from *S. junghuhnianum* in Hexylagarose column (MIPS activity is expressed as µM I-1-P produced/1.25ml fraction/hr)

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

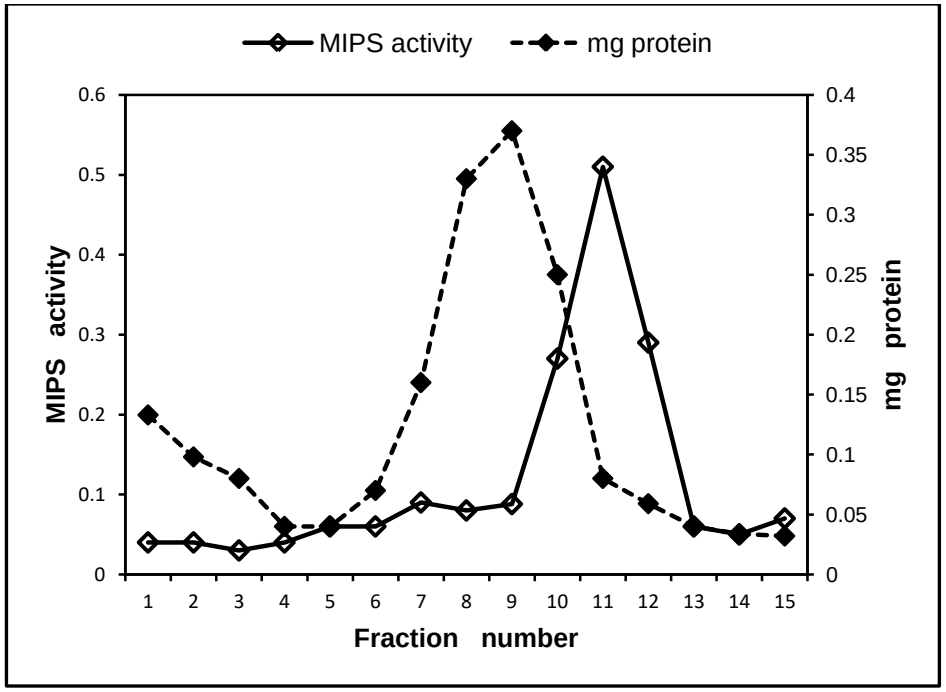

Fig-3.5 Elution profile of cytosolic MIPS from *A. khasiana* in BioGel A-0.5m column (MIPS activity is expressed as µM I-1-P produced/0.5ml fraction/hr)

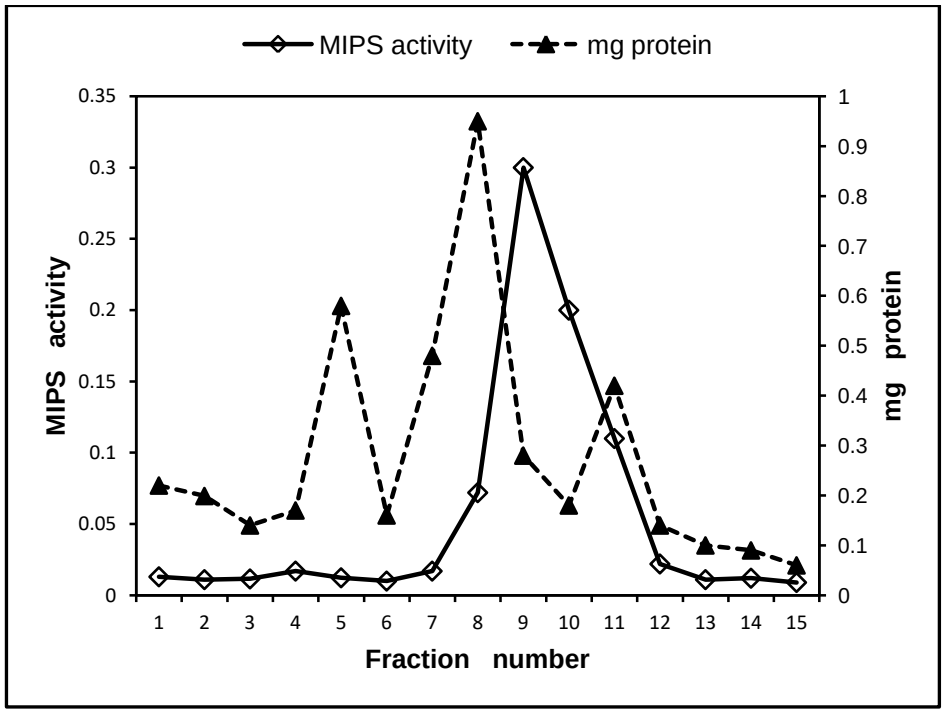

Fig-3.6 Elution profile of cytosolic MIPS from *S. junghuhnianum* in BioGel A-0.5m column (MIPS activity is expressed as µM I-1-P produced/0.5ml fraction/hr)

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

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

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

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

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

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

The following procedure was followed for the assay:

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

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

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

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

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

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

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

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

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

3.3.6 Determination of molecular weight of L-*myo*-inositol-1-phosphate synthase: Approximate molecular weight of the native L-*myo*-inositol-1-phosphate synthase obtained from the plant body of bryophytes *A. khasiana* and *S. junghuhnianum* bearing reproductive structures were determined by gel-filtration through Sephadex G-200 column. The Sephadex G-200 was suspended in 50 mM Tris- acetate (pH 7.5) and packed in a column of suitable size and calibrated with 1 ml each of marker proteins e.g., ovalbumin (43 kDa), BSA (66 kDa), phosphorylase-b (97.4 kDa), catalase (221.6 kDa) and apoferritin (443 kDa). The void volume was determined with blue dextran 2000 (1 mg / ml). All standards were loaded in the column separately and fractions (0.5 ml) were collected at a flow rate of 0.5 ml/5 min. Each individual protein peak was located by spectrophotometric scanning at 280 nm in a Spectrophotometer. A standard curve was prepared by plotting relative elution volume of proteins against their respective log molecular weights (sections 4.5.3 and 4.8.3). Partially purified enzyme (BioGel A-0.5 m) was loaded into the Sephadex G-200 column under identical conditions. Fractions were assayed for *myo*-inositol synthase activity by the procedure described in Section 3.3. Molecular weight was determined from the relative elution

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

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

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

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

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

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

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

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