MATERIALS AND METHODS

2.4. MATERIALS AND METHODS

2.4.1. *Materials* :

The experimental plant *Sechium edule* Sw. was collected freshly from different spots in and around Darjeeling.

D-fructose-1, 6-bisphosphate (trisodium salt), D-fructose-6-phosphate (disodium salt), D-glucose-6-phosphate (Disodium salt), D-ribulose-1, 5-bisphosphate (tetrasodium salt), bovine serum albumin (BSA), Tris (hydroxymethyl) amino methane (Tris), beta-nicotinamide adenine dinucleotide (beta NAD), DEAE-cellulose (D-8382) were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A. 2-Mercaptoethanol and beta-glycerophosphate (disodium salt) were purchased from E. Merck, Germany. Ammonium molybdate was purchased from BDH, England. All other chemicals used were the analytical and guaranted grade available, purchased from E. Merck, BDH, Ranboxy and Loba Chemical Company, India.

2.4.2. *Methods* :

2.4.2.1. Isolation of Alkaline Phosphatase :

In order to search for the activity of alkaline phosphatase in different plant parts of *Sechium edule*, the enzyme was isolated following the method outlined below :

All preparations of the enzyme from different samples were carried out at 4°C.

The different samples were collected freshly, washed with cold distilled water and homogenized with a mortar and pestle in two volumes of a buffer of 20 mM Tris-HCl (pH 8.0) containing 0.2 mM 2-mercaptoethanol in presence of quartz sand.

The crude homogenate was centrifuged at 5,000g for 15 minutes in a Remi R8C centrifuge. The pellet was discarded and the supernatant fraction was collected.

The supernatant fraction was dialyzed against 500 volumes of the same buffer used for homogenization for 3 to 4 hours.

On completion of dialysis, the fraction which was recovered from the dialysis bag was used as the enzyme source.

2.4.2.2. Assay Procedure for Alkaline Phosphatase :

The colorimetric assay procedure devised by Banerjee and Adhikari (1986) was used to assay alkaline phosphatase activity.

Enzyme Incubation :

The incubation mixture contained, in a total volume of 1.0 ml, the following components : 50 mM Tris-HCl (pH 8.0), 2 mM 2-mercaptoethanol, 5 mM beta-glycerophosphate and an appropriate protein aliquot (200-300 ug). The reaction was started by addition of substrate (beta-glycerophosphate) immediately after the enzyme with proper mixing. Duplicate tubes were run along with an appropriate blank (without enzyme) and a zero minute control in which 0.25 ml of 20% chilled trichloroacetic acid (TCA) was added prior to the addition of the substrate. The enzyme incubation was carried out for one hour at 37°C. After one hour, the reaction was terminated according to the procedure applied for zero minute control. The denatured proteins were precipitated out by low speed centrifugation and the resulted supernatant was collected from each tube. Thus, in an assay set, there were four tubes i.e. one blank, one zero minute control and two experimental tubes. Now, the quantity of inorganic phosphate released from the substrate by the enzymic action was estimated according to the following procedure.

Estimation of Inorganic Phosphate :

Inorganic phosphate was measured by the method of Chen *et al.* (1956). Phosphate release was quantitative and an inorganic phosphate standard curve was used. Then the enzyme dependent release of inorganic phosphate was measured by substracting the zero minute value from the average value of the experimental sets.

As one mole of beta-glycerophosphate contains one mole of inorganic phosphate, mole number of inorganic phosphate estimated was equal to the number of beta-glycerophosphate hydrolyzed.

Estimation of Protein :

Protein was estimated by the method of Lowry *et al.* (1951)with slight modifications using bovine serum albumin (BSA) as standard.

Specific Activity of Alkaline Phosphatase :

Specific activity of alkaline phosphatase is defined as n mole inorganic phosphate (Pi) released per hour per milligram protein, i.e., nmol Pi released (mg protein)$^{-1}$ h^{-1}.

2.4.2.3. Isolation of Acid Phosphatase :

In order to trace the activity of acid phosphatase in different plant parts of *Sechium edule*, the enzyme was isolated according to the following method.

All steps for isolation of the enzyme from different samples were carried out at 0° to 10°C.

The plant materials were collected freshly, thoroughly washed with chilled distilled water and then homogenized with a mortar and pestle in double volumes of a buffer of 20 mM Na-acetate (pH 6.5) having 0.2 mM 2-mercaptoethanol with the help of washed sand.

The crude homogenate was measured and centrifuged at 5,000g for 15 minutes in a Remi R-24 centrifuge. The pellet was discarded and the supernatant portion was collected.

The 5 K supernatant fraction was dialyzed against 500 volumes of 20 mM Na-acetate (pH 6.5) containing 0.2 mM 2-mercaptoethanol for 3 hours. On completion of dialysis, the dialyzed fraction was recovered from the dialysis bag and used as the enzyme source for the preliminary experiment.

2.4.2.4. Assay Procedure for Acid Phosphatase : Enzyme Incubation :

The colorimetric assay procedure designed by Banerjee and Adhikari (1986) was used to assay acid phosphatase activity.

The enzyme incubation mixture contained, in a total volume of 1.0 ml, the following components : 50 mM Na-acetate (pH 6.0), 2 mM 2-mercaptoethanol, 5 mM beta-glycerophosphate and an appropriate protein aliquot (200-300 ug). The reaction was started by addition of beta-glycerophosphate (substrate) immediately after the enzyme with proper mixing with the help of a cyclomixer (Remi). Duplicate tubes were run along with an appropriate blank (minus enzyme) and a zero minute control in which 0.25 ml of 20% chilled trichloroacetic acid (TCA) was added before the addition of substrate. The enzyme incubation was carried out for one hour at 37°C. After one hour of incubation the reaction was stopped following the procedure applied for zero minute control. The denatured proteins were separated out by low-speed centrifugation and the resultant supernatant was collected from each assay tube to respective larger test tubes. Therefore, in an assay set, there were four tubes, i.e., one blank, one zero minute control, and two duplicate experimental tubes. Later on, the quantity of inorganic phosphate released on hydrolysis from the substrate by the enzymic action was estimated according to the following procedure :

Estimation of Inorganic Phosphate :

Inorganic phosphate was estimated by the method of Chen *et al.* (1956). Phosphate release was quantitative and an inorganic phosphate standard curve was used. Then the enzyme dependent release of inorganic phosphate was calculated by subtracting the zero minute value from the average value of the experimental sets.

As one mole of beta-glycerophosphate contains one mole of inorganic phosphate, mole number of inorganic phosphate estimated was equal to the number of beta-glycerophosphate hydrolized.

Estimation of Protein :

Protein content was colorimetrically determined by the method of Lowry *et al.* (1951) with slight modification using BSA as standard.

Specific Activity of Acid Phosphatase :

Specific activity of acid phosphatase was calculated and defined as nanomole inorganic phosphate (Pi) released per hour per milligram protein, i.e., nmol Pi released/ hr/mg protein.

2.4.2.5. Isolation of L-*myo*-Inositol-1-phosphate synthase :

In order to find out the activity of L-*myo*-inositol-1-phosphate synthase in different plant parts of *Sechium edule* Sw., the enzyme was isolated following the method outlined below :

All preparations of the enzyme from different samples were carried out at 4°C.

The different samples were collected freshly, washed with chilled distilled water and homogenized with a mortar and pestle in two volumes of a buffer of 20 mM Tris-HCl (pH 7.0) containing 0.2 mM 2-mercaptoethanol in presence of quartz sand.

The crude homogenate was centrifuged at 10,000g for 20 minutes in a Remi R-24 centrifuge. The pellet was discarded and the supernatant fraction was collected.

The supernatant fraction was dialyzed against 500 volumes of the same buffer used for homogenization for 3 to 4 hours.

On completion of dialysis, the fraction which was recovered from the dialysis bag was used as the enzyme source.

2.4.2.6. Assay Procedure of *myo*-Inositol-1-phosphate synthase :

The colorimetric assay procedure devised by Barnett *et al.* (1970) was used to assay L-*myo*-inositol-1-phosphate synthase activity with slight modification (Adhikari *et al.* 1987).

Enzyme Incubation :

The incubation mixture contained, in a total volume of 0.5 ml, the following components : 50 mM Tris-acetate (pH 7.5), 14 mM NH_4Cl, 5 mM 2-mercaptoethanol, 0.8 mM NAD, 5 mM D-glucose-6-phosphate and an appropriate protein aliquot (100-250 ug). The reaction was started by addition of substrate immediately after the enzyme with proper mixing. Duplicte tubes were run along with an appropriate blank (without enzyme) and a zero minute control in which 0.2 ml of 20% chilled TCA was added prior to the addition of the enzyme. The enzymatic incubation was carried out for one hour at 37°C. After one hour the reaction was terminated according to the procedure applied for zero minute control. Two such sets (Set I-periodate; Set II nonperiodate) were done simultaneously i.e. each had one blank, one zero minute control and two experimental tubes.

The quantity of the enzymatic product was estimated by periodate oxidation followed by estimation of inorganic phosphate.

Oxidation with Sodium Metaperiodate :

After completion of enzyme incubation, the resultant supernatant was subjected to a treatment with 0.7 ml of 0.2 M $NaIO_4$ and incubated for one hour at 37°C. Then 1.4 ml of 1 M Na_2SO_3 (prepared immediately before use) was added to destroy excess $NaIO_4$ (in case of Set I). In Set II, instead of $NaIO_4$ and Na_2SO_3, H_2O was added to maintain the volume equal with Set I. Inorganic phosphate was liberated from *myo*-inositol-1-phosphate during oxidation while cleavage of glucose-6-phosphate was very much minimal (2m moles of Pi are released per mole of glucose-6-phosphate) which could be subtracted considering blank or zero minute control from the experimental value in Set I. Hydrolysis

of phosphate from glucose-6-phosphate by contaminating phosphatase (if any) was measured by subtracting 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 Set I.

Estimation of Inorganic Phosphate :

Inorganic phosphate was measured by the method of Chen *et al.* (1956). As one mole of *myo*-inositol-1-phosphate contains one mole of inorganic phosphate, mole number of inorganic phosphate(s) estimated was equal to the number of *myo*-inositol-1-phosphate produced.

Estimation of Protein :

Protein was estimated by the method of Lowry *et al.* (1951) with slight modifications using bovine serum albumin (BSA) as standard.

Specific Activity of L-*myo*-inositol-1-phosphate synthase :

Specific activity of L-*myo*-inositol-1-phosphate synthase is defined as nano mole L-*myo*-inositol-1-phosphate (I-I-P) produced per hour per milligram protein, i.e., n mole I-I-P produced (mg protein)$^{-1}$ h^{-1}.

2.4.2.7. Isolation of Fructose-1, 6-bisphosphatase from leaves of *Sechium edule*:

In order to trace the basic activity of Fructose-1, 6-bisphosphatase ($FruP_2ase$) in leaves from *Sechium edule* the enzyme was isolated according to the following method.

All steps for isolation of the enzyme were carried out at 0° to 10°C.

The plant materials were collected freshly (unless otherwise treated for some experiments), thoroughly washed with chilled distilled water and then homogenized with a mortar and pestle in double volumes of a buffer of 50 mM Tris-HCl (pH 7.0) having 0.2 mM 2-mercaptoethanol with the help of washed sand.

The volume of the crude homogenate was measured and centrifuged at 10,000g for 15 minutes in a Remi R-24 centrifuge (unless otherwise stated in some experiments). The pellet was discarded and the supernatant was collected.

The 10 K supernatant was dialyzed against 500 volumes of 50 mM Tris-HCl (pH 7.5.) containing 0.2 mM 2-mercaptoethanol for 2 hours in order to remove free contaminating inorganic phosphate and other small molecular weight contaminants. On completion of dialysis, the dialyzed fraction was recovered from the dialysis bag and would be used as the enzyme source for the preliminary experiments.

2.4.2.8. Assay Procedure for Fructose-1, 6-bisphosphatase :

Fructose-1, 6-bisphosphatase ($FruP_2$ase, EC 3.1.3.11) was assayed according to the procedure of Biswas *et al.* (1981).

Enzyme Incubation :

The standard incubation mixture contained 100 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, 0.2 mM fructose-1, 6-bisphosphate ($FruP_2$), 0.1 mM EDTA, and an appropriate protein aliquot (200-250 ug) in a total volume of 1.0 ml. 0.2 mM of fructose-6-phosphate (F-6-P) substituted $FruP_2$ in each set of assay which served as control against non-specific phosphatases. Zero minute controls for both $FruP_2$ and F-6-P where the protein of the incubation mixture was denatured by 0.25 ml of 20% ice-cold trichloroacetic acid (TCA) prior to the addition of substrate served as double check against interferring inorganic phosphate (if any). In addition to these sets, an appropriate blank (minus enzyme) was also maintained. The enzyme incubation was carrid out for one hour at 37°C. After one hour of incubation, the reaction was terminated following the procedure applied for zero minute control. The denatured proteins were separated out by low-speed centrifugation and the resultant supernatant was collected from each assay tube to respective larger test tube. Therefore, in an assay set, there were six tubes, i.e., one blank, one zero minute control and one experimental tube for each set. Later on, the quantity of inorganic phosphate released on hydrolysis from the substrate(s) by the enzymic action was estimated according to the following procedure.

Estimation of Inorganic Phosphate :

Inorganic phosphate was estimated by the method of Chen *et al.* (1956). A reagent (3.0 ml), prepared by adding (immediately before use) 1 volume of 6N H_2SO_4, 1 volume of 10% (w/v) ascorbic acid, 1 volume of 2.5% (w/v) ammonium molybdate and 2 volumes of H_2O, was added to the deproteinized supernatants with an additional volume of H_2O i.e., 1.25 ml of deproteinized supernatant and 3.0 ml Pi reagent plus 3.0 ml H_2O; T.V. of 7.25 ml. This mixture was subjected to incubate for one hour at 37°C. The blue colour was read at 660 nm in a Systronic-105 spectrophotometer. Phosphate release was quantitative and an inorganic phosphate standard curve was used. Then the $FruP_2$ase dependent release of inorganic phosphate was calculated by subtracting the second corrected value (experimental tube zero minus tube of F-6-P set) from the first corrected value (experimental tube - zero minute tube of $FruP_2$ set).

As one mole of inorganic phosphate was hydrolyzed from one mole of $FruP_2$, mole number of inorganic phosphate estimated was equal to the number of $FruP_2$ hydrolyzed at C-1.

Estimation of Protein :

Protein content was colorimetrically determined by the method of Lowry *et al.* (1951) with slight modifications using BSA as standard. A reagent (Reagent-A: 0.9 ml); prepared by adding (immediately before use) 100 volumes of 2% Na_2CO_3 in 0.4% NaOH, 1 volume of 1% $CuSO_4$ and 1 volume of 2% Na^+K^+ tartrate; was added to the protein aliquots (0.25 ml) and incubated at room temperature for 15 minutes. After the first incubation, another reagent (Reagent-B: 0.1 ml); prepared by commercially available Folin-phenol reagent with double dilution, was added to the previously incubated tubes. The tubes were kept at room temperature for 30 minutes. On completion of the second incubation, 5.0 ml H_2O was added to it (i.e. total volume = 6.0 ml), mixed thoroughly and the blue colour was read at 620 nm in a Systronics-105 spectrophotometer.

Specific Activity of Fructose-1, 6-bisphosphatase :

Specific activity of fructose-1, 6-bisphosphatase was calculated and defined as nano mole inorganic phosphate (Pi) released per hour per milligram protein, i.e. n mol Pi released /hr/mg protein.

2.4.2.9. Isolation of chloroplasts from leaves of *Sechium edule*:

2.4.2.9.1. Isolation procedure of chloroplasts :

Chloroplastids of *Sechium edule* were homogenized in a mortar and pestle with double volumes of 20 mM Tris-acetate (pH 7.0) containing 0.35 M sucrose, 10 mM $MgCl_2$, 100 mM KCl, 1 mM 2-mercaptoethanol, 10 mM Na-ascorbate and 2.0 ml of chicken egg albumin. Homogenization was carried out in presence of equilibrated (in 20 mM Tris-acetate, pH 7.0) sand. The crude homogenate was centriguged at 500g for 5 minutes in a Remi R-8C centrifuge. The pellet containing the unbroken cells, nuclei, other debris and sand was discarded. The supernatant obtained after centrifugation at 500g for 5 minutes, was spun at 2,500g for 15 minutes in a Remi R-8C centrifuge. The resultant chloroplast pellet was washed at least two times with the homogenizing medium. The final pellet was collected as chloroplasts and kept at 0°C until use.

2.4.2.9.2. Identification of the purity of the chloroplasts :

The purity of the isolated chloroplasts from *Sechium edule* could not be checked either by transmission electron microscopy or by radio-active enzyme assay of the marker chloroplastidial enzyme, ribulose-1, 5-bisphosphate carboxylage (EC 4.1.1.39) as this laboratory was not equipped with such kinds of sophisticated instrumental facilities. Therefore, the fundamental identification of the isolated chloroplasts was made under light-microscopic observation where almost uniform green-coloured bead-like structures could be seen. And the chloroplasts were further identified and characterized by the ability of the preparation to carry out characteristic Hill reaction using dichlorophenol-indophenol (DCIP) as the electron acceptor along with the determination of the total chlorophyll content from the same batch of isolated chloroplasts. Identical experiments were also made with the 500g pellet and with the 2,500g supernatant.

The productin of reducing equivalents by isolated chloroplasts (Hill reaction) :

The synthesis of carbohydrate from H_2O and CO_2 involves two stages, known as the light and dark reactions of photosynthsis. The light reaction takes place in the visible radiation, which is absorbed by the green pigment chlorophyll 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 reactions. ATP is generated during this electron transport. At the same time, reducing equivalents in the form of NADPH + H^+ are also generated and O_2 is evolved.

The photolysis of H_2O by isolated chloroplasts was first demonstrated by Hill and is often known as the Hill reaction. In this experiment, illuminated chloroplasts from *Sechium edule* are shown to produce reducing equivalents by including an artificial hydrogen acceptor, 2, 6-dichlorophenol-indophenol (DCIP) in the mixture. Reduction of the blue dye does not take place in the dark.

The isolated chloroplasts were suspended with the 20 mM Tris-acetate (pH 7.0) buffer to a considerable volume and 0.1 ml of this suspension was added to 9.9 ml of the DCIP (0.1 mol./litre) solution. Three such sets were prepared. The initial O.D. of these three tubes were recorded at 520 nm in a Systronics-105 colorimeter. Then, one of these three tubes was kept in the dark for the period of the experiment. The second tube to which several crystals of sodium dithionite had been added to completely decolorize the blue dye. And the third tube was placed under illumination of a fluorescent light for 2 hours. The final O.D. 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 500g pellet and 2,500g supernatant were used.

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 *Sechium edule* and other experimental fractions of the same species.

10.0 ml of 80% (v/v) acetone was added to 1.0 ml of the isolated chloroplast suspension (and also to the other fractions under experimentation, separately), shaked thoroughly and filtered through a Whatman No.1 filter paper into a volumetric flask. Rinsed out the test tube with a further 5 ml of aqueous acetone and used this to wash the filter paper. The washing was repeated once more, and made up to the 25 ml mark with 80% (v/v) acetone. The extinction was read at 652 nm in a Systronics-105 colorimeter against a solvent blank.

Chlorophyll concentration (mg/ml) = Extinction at 652 nm x 5.8.

2.4.2.10. Partial Purification of Photosynthetic (Chlor- oplastidial) Fructose-1, 6-bisphosphatase from *Sechium edule* :

The isolated chloroplasts were washed with chilled 50 mM Tris-HCl (pH 7.0) buffer containing 0.2 mM 2-mercaptoethanol and the partial purification of Fructose-1, 6-bisphosphatase from these isolated chloroplasts was done following the method outlined below :

All steps were carried out at 0° to 10°C.

1. Homogenate :

Buffer-washed isolated chloroplasts obtained from 100 gms of *Sechium edule* leaves was homogenized with a mortar and pestle in 3-volumes of 50 mM Tris-HCl (pH 7.0) buffer containing 0.2 mM 2-mercaptoethanol in presence of a little amount of washed sand.

2. Low-speed supernatant :

The crude pastidial homogenate was centrifuged at 10,000g for 20 minutes in a Remi R-24 centrifuge. The pellet was discarded and the supernatant fraction was recovered from the centrifuge tubes.

3. Precipitation with Streptomycin Sulfate :

In order to remove chloroplastic nucleic acids, the 10 K supernatant was subjected to a treatment with streptomycin sulfate powder in a final concentration of 1% (w/v) with constnt stirring. It was kept at 0°C for 10 minutes under cold condition and then it was centrifuged at 10,000g for 20 minutes. The pellet was discarded.

4. Fractionation with Ammonium sulfate :

The supernatant obtained from the previous step was then made 0-80% saturated with $(NH_4)_2SO_4$ by adding requisite amount (51.6 gms. in 100 ml) of solid salt slowly with constant stirring (by using Remi magnetic stirrer). It was kept in an ice-bucket at 0°C for 15 to 20 minutes and then centrifuged at 10,000g for 15 minutes. The pellet obtained from 0-80% saturated $(NH_4)_2SO_4$ fraction was dissolved in minimal volume of 50 mM Tris-HCl (pH 7.5) buffer having 0.2 mM 2-mercaptoethanol and dialyzed for a considerable period against the same buffer (500 volumes) 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.

5. Anion-exchange Column Chromatography with DEAE-cellulose :

The dialyzed 0-80% $(NH_4)_2SO_4$ fraction was adsorbed in considerable quantity of DEAE-cellulose (commercially available ion-exchanger was immensely washed with 0.5N HCl, 0.5N NaOH water etc. accordingly and then equilibrated with 50 mM Tris-HCl, pH 7.5, containing 0.2 mM 2-mercaptoethanol) taking collectively in a small beaker and kept for 2 to 3 hours under refrigeration. Thereafter this preparation was loaded onto a column carefully with the help of a 5 ml pipette (column size ; 7.5 x 1.2 cm) and the effluent was collected. After collection of the effluent the column was washed with 2-bed volumes by the same buffer. Lastly, the adsorbed proteins were eluted from the column with a linear gradient of 0.0 to 0.5 M KCl prepared in 50 mM Tris-HCl (pH 7.5) having 0.2 mM 2-mercaptoethanol. Fractions of 1.5 ml were collected at a flow rate of 45 minutes per tube. 15 such fractions were collected and assayed for $FruP_2$ase activity.

6. Gel-filtration of Sephadex G-200 :

The pool of active DEAE-cellulose fraction, obtained from the previous step, was loaded on top of a column (column size : 8 x 1.2 cm) of Sephadex G-200 (before this experimentation, commercially available Sephadex G-200 was soaked in 300 volumes of glass-distilled water and kept in a water bath at 70°-80°C for 5 to 6 hours for complete swelling of gel-beads. The excess water was then decanted and the column material was equilibrated with 50 mM Tris-HCl, pH 7.5, containing 0.2 mM 2-mercaptoethanol). After collection of the effluent, the column was eluted with the same buffer, used for equilibration. Fractions of 0.75 ml were collected with a flow rate of 30 minutes per tube and 15 such fractions were collected. Each fraction and also the effluent were assayed for the $FruP_2$ase activity. Active fractions were pooled and marked as the exact preparation of the partially purified enzyme.