CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals Ascorbic acid, and N,N,N,N-Tetramethylene diamine (TEMED) were from BDH.

Dichloroindophenol (DCIP), Dithiothreitol, 5-Fluorodeoxyuridine, hydrogen peroxide (30% w/v), Paraquat chloride (1,1-dimethyl-4,4-bipyridinium dichloride), Polyethylene glycol PEG-6000 were from Fluka.

Acrylamide was from Merck.

Agarose (Type II), Bis (N,N-methylene bis acrylamide), Cesium chloride, Coomassie blue R, Cytochrome c, Erythrosine B, Ethidium bromide, 3(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU), Glacial acetic acid, Methyl-p-hydroxybenzoate, Nitro blue tetrazolium, Riboflavin, Sodium dodecyl sulfate (SDS), and Xanthine (sodium salt) were from Sigma.

Triton X-100 (Analyzed reagent) (Packard Instrument Company Inc.).

[methyl-^{14}C]paraquat dichloride specific radioactivity 111 mCi/m mol was from Amersham Laboratories.

Xanthine oxidase (Grade III from butter milk) and Pronase E (Protease type XIV) were from Sigma.

Restriction endonuclease, BamH I, Bgl II, EcoR I, Hind III,

Pst I, and Xho I were from BRL Co.

Electron microscopic reagents were supplied by Electron microscopy Section at the Scientific and Technological Research Equipment Centre, Chulalongkorn University and Biochemistry Unit at Central Laboratory and Greenhouse Complex, KURDI, Kasetsart University, Kamphaengsaen Campus.

Other chemicals were common in the laboratory.

2.1.2 Instruments Beckman Refrigerated Centrifuge, model J-21C (Beckman Instruments Inc., U.S.A.).

French Pressure Cell (American Instruments, U.S.A.).

Hitachi Ultracentrifuge, model 55P-72 (Hitachi, Japan).

LKB Vertical Electrophoresis System, model LKB 2001, and LKB UV Illuminator, model 2011 Macrovue Transilluminator (LKB Producter A, Sweden).

Nikon Microscope, model OPTIPHOT 145961, and Nikon Photomicrographic Attachments, model Microflex UFX-II (Nippon, Japan).

Packard PL-Tricarb Liquid Scintillation Counter, model 3320 (Packard Instrument Company Inc., U.S.A.).

Rotar meter (Keiso, Japan).

Shimadzu spectrophotometer, model UV-240 Graphicord (Shimadzu, Japan).

Scanning electron microscope, model JSM-35 CF and Transmission electron microscope, model JEM-200CX (JEOL, Japan).

2.1.3 Algal Strains: Chlamydomonas reinhardtii 137c mating type plus (mt+) strain was kindly supplied by Professor J.-D. Rochaix at the Department of Molecular Biology, University of Geneva, Switzerland.

Chlamydomonas reinhardtii SAG 11-31/b (mt-) and 11-32/c (mt+) strains were supplemented from the "Pflazenphysiologisches Institut der Universitat Gottingen", Germany.

2.2 Algal cultures

2.2.1 Culture Maintenance All algal strains were grown on Surzycki Tris minimum phosphate (TMP) medium (Appendix 1) solidified with 1.5% agar under light intensity of 4,000 lux generated from daylight fluorescent lamps at 25°C. Cells were maintained at 4°C and subcultured every month. In case of paraquat resistant cell lines, paraquat was also included in the medium as described hereafter. Strains could be stored by stab cultures at 25°C with shelf life more than one year.

2.2.2 Liquid Cultures "Seedling" cultures were prepared by transferring 3 loops of stock culture into TMP medium (100 ml in 250 ml flask) and shaking under continuous light at 4000 lux for one week.

Cultures of C. reinhardtii were initiated by inoculating seedling cultures in fresh TMP medium (either 750 ml in 1000 ml flask or 150 ml in 250 ml flask) at an inoculum size of 10^5 cells/ml media.

Cells were grown photoautotropically at 25°C according to the method of Surzycki (1971) with a period of 12 hour-12 hour light/dark cycle. Light sources from both sides of the cultures were automatically turned on at 5:00 a.m. and turned off at 5:00 p.m.. The algal suspensions were illuminated at 4000 lux and were constantly bubbled by sterile air containing 4-5% carbondioxide (v/v) which was monitored by two Rotar meters. The culture apparatus was shown in Fig. 2.1.

2.3 Growth Determination

Algal growth was determined by four parameters. At certain time intervals cell suspensions were withdrawn axenically to measure cell density, turbidity at 540 nm, chlorophyll a (CHL a) content, and protein content.

Cell countings were made with a Bright-Line hemacytometer after adding 5% glycerol to stop cell motility (Houtz et al, 1985).

Chlorophyll a content was assessed by the method of Hansmann (1975). GF/B Whatman glass filter paper (2.4 cm in diameter) was used to filter cells before applying to extraction by 10 ml of 80% acetone. The extracts were kept overnight in the dark at 4°C. After centrifugation to remove cell debris and the suspended paper, the supernatants were scanned for absorption spectrum in the range of 600-750 nm by Shimadzu spectrophotometer. The amount of chlorophyll a was calculated as,

$$\text{CHL a (mg/L)} = 11.6\ OD_{665} - 1.31\ OD_{645} - 0.14\ OD_{630}$$

Chlorophyll a content was reported in terms of μg per ml culture

Figure 2.1 Set up of an apparatus for Chlamydomonas reinhardtii Cultivation.

Illuminations are from both sides of the culture vessels. Two sets of Rotar meter can be seen on the left-handed side, one controls the flow rate of air generating from an air pump and the other controls the flow rate of carbon dioxide from the reservoir providing air with 4-5% carbon dioxide.

and μg per 10^7 cells.

The amount of cellular protein was determined by Lowry method (Lowry et al, 1951).

2.4 Determination of Cell Mortality

Erythrosine B staining was used for detection of dead cells. Dye solution was prepared by weighing erythrosine B 0.4 g, sodium chloride 0.81 g, potassium hydrogen phosphate 0.06 g, and methyl-p-hydroxybenzoate 0.05 g, and dissolving in distilled water to make 100 ml. The pH of dye solution was adjusted to 7.2 .

Cell suspension was mixed with 0.5 M Tris-HCl (pH 7.5) and the dye solution at a ratio of 2: 2.5 : 0.5 (by volume). The mixture was incubated at room temperature for 5 min before observing under a light microscope. Only dead cells were red stained by the dye pigment.

2.5 Evaluation of Paraquat Effect

2.5.1 Spot Test The procedure was modified from the method of Spreitzer and Mets (1981).

Cells from exponential culture were collected and resuspended in TMP medium at cell density of $2x10^6$ cells/ml. Four drops of 50 μl (10^5 cells) were individually spotted at a diameter of 0.5 cm on medium containing paraquat. Bleaching phenomena of the spots was followed within one week after exposure to light at 4000 lux given from the upper direction at 25 $\pm 1^{o}$C.

2.5.2 LD_{50} Determination The whole procedure was originally developed in this research project at the Department of Biochemistry , Faculty of Science, Chulalongkorn University.

Cells in the exponential growth stage was divided into separated small portions, each was added with a sterile solution of paraquat to make a certain concentration of the herbicide. All treatments were exposed to light/dark cycles as of the liquid growth conditions. The exposure time was started at 11:00 a.m. and lasted for a period of 24 hours, followed by washing with 100 folds of paraquat free medium. Cells from each treatment were plated (300-400 cells/plate) on TMP medium containing 10 mM sodium acetate. The chemotrophic growth in the dark was allowed for two weeks. The number of emerged colonies was scored and the results were expressed as percentage of the control (growth without paraquat treatment). Lethal dose-50 (LD_{50}) was defined as the concentration of paraquat that caused 50% growth inhibition.

2.6 Development of Paraquat Resistant Mutants

2.6.1 Selection under paraquat pressure Cells from exponential culture were plated, by top agar plating method, at 10^6 cells/plate on TMP medium containing 2 μM paraquat. Selection plates were incubated under 4,000 lux continuous illumination for two weeks. Mutants from this step were isolated and weekly passaged in liquid medium plus 1 μM paraquat for 10 subcultures. They were used for further stepwise selection of mutants having higher resistance to

the herbicide by repeating the procedures as described above, but increasing the herbicide concentration up to 10 μM.

2.6.2 Double Mutagenesis by 5-Fluorodeoxyuridine (FdUd) Wild type culture at early stationary phase was collected and resuspended in medium containing 1 mM FdUd (Wurtz et al, 1979) at a final cell density of 10^7 cells/ml. Mutagenesis was established by shaking the suspension cultures under continuous illumination for 24 hours. After washing with TMP medium, paraquat resistant mutants were enriched by paraquat treatment using 0.5 μM paraquat and exposed to light/dark cycle as described in section 2.5.2. Cells were plated at 10^6 cells/ml on selective medium consisting of 2 μM paraquat. A selected mutant from this step was subjected to a second mutagenesis by repeating the overall previous process except that higher herbicide concentrations were used in the enrichment and the selection steps.

2.7 Preparation of Autolysin

Autolysin, a specific enzyme for cell wall hydrolysis of Chlamydomonas, was prepared in the laboratory from mating reaction of high mating efficiency strains of C. reinhardtii, 11-32/b and 11-32/c.

Preparations of culture of C. reinhardtii 11-32/b and 11-32/c were achieved under a light/dark controlled regime as described previously (section 2.2.2), but the TMP medium was replaced by the

minimal high magnesium (MM) medium (Surzycki, 1971) (Appendix 1). Gametogenesis was induced at the late exponential growth stage of the cultures by using the method described by Snell (1982) with some modification. Two hours after the onset of illumination (shortly after complete cell division), cells were centrifuged at 3,000xg, 20°C for 10 min, washed once in an excess volume of nitrogen free (N-free) medium (Appendix 2), and then resuspended with fresh N-free medium (1/2 volume of the original culture volume). The gamete induction culture (300 ml) was transferred to a 500 ml flask. Aeration was thoroughly provided from a glass tube extended to the bottom of the culture. Illuminations at 4,000 lux were supplied from two opposite sides of the vessels, usually for 14 hours at 25°C. The cultures were then kept in the dark for 8 hours. Cells were harvested and resuspended in fresh N-free medium (1/2 of the previous volume). 100 ml of cell suspension in a 1,000 ml beaker was illuminated at 25°C with continuous shaking for another 3 hours under light intensity of 4,000 lux.

Equal number of matured gametes of each mating type so obtained were mixed at final cell density of 10^8 cells/ml, mating was allowed under 4,000 lux of light for 90 min. Clear supernatant was collected as crude autolysin preparation which was then concentrated by "AMICON" membrane filter PM-10 (MW cut off 10,000) resulting protein concentration of 100 µg/ml, when determined by Lowry method (Lowry et al, 1951).

2.8 Preparation of protoplast

2.8.1 Reagents Autolysin solution (100 µg protein/ml), stock solution of 1 mg/ml cycloheximide, and stock solution of 0.04% Triton X-100 (5 mM EDTA, pH 8.0).

2.8.2 Method for Preparation The algal cells were harvested, washed once in N-free medium and then resuspended in the same N-free medium. C. reinhardtii protoplasts were prepared in the reaction mixture containing $6x10^6$ cells/ml, 10 µg/ml cycloheximide and autolysin (100 µg protein/ml). The reaction was performed by shaking at 34°C for 90 min. Protoplasts were indirectly identified by treatment with Triton X-100 as modified from Snell (1982). A portion of 0.6 ml of the protoplast preparations was incubated in 1.0 ml of cold 0.04% Triton X-100 (5 mM EDTA, pH 8.0) with gently shaking at 34°C for 5 min and counting the remaining intact cells. Replicate experiment was performed as control autolysin non-treated cells.

$$\% \text{ Protoplast} = \{ (X-Y)/X \} x100$$

where, X = total number of cells before autolysin treatment

Y = number of remaining cells after autolysin and Triton X-100 treatment

2.9 Isolation of Chloroplast

2.9.1 Reagents Solution A (5 mM potassium phosphate buffer, pH 6.5, 6% by weight of polyethylene glycol (PEG-6000), and 0.004% digitonin).

Solution B (20 mM Tris-glycine-NaOH buffer, pH 7.7, 2 mM EDTA, 0.15 M mannitol, and 1 mM magnesium chloride).

2.9.2 Method for Isolation A method was established in this Department of Biochemistry on the basis of digitonin lysis as published in different details by Kein et al (1983). Protoplasts generated by autolysin treatment as previously described were harvested and resuspended in ice-cold solution A (4°C) (approximately 10^7 cells/ml). The temperature of the protoplast suspension was increased rapidly to 30-32°C in 15-45 sec by gently shaking in hot water bath (80-85°C), and this temperature was maintained for 2 min. The reaction mixture was then transferred to an ice bath and the action of digitonin was immediately reduced by adding 10 volumes of chilled 5 mM phosphate buffer (pH 6.5) containing 6% PEG to the mixture. The detergent treated protoplasts were pelleted down by centrifugation at 800xg for 5 min at 4°C and washed with ice-cold solution B to give a suspension of about 40-80 µg chlorophyll a/ml. After removing solution B by centrifugation as above, the pellet was resuspended in a small volume of ice-cold solution B to obtain approximately 400-800 µg chlorophyll a/ml. The suspension was gently mixed by repeated pipetting through a plastic micropipette tip (1 mm in diameter) for 20 times. Chloroplasts were observed by a light microscope under phase contrast mode.

Yield of the isolated chloroplasts was determined by either direct counting on the haemacytometer or lysing with distilled water.

In the latter method, number of chloroplasts could be indirectly estimated from a standard curve which was established between the known amount of chloroplasts and absorbancy at 435 nm.

2.10 Electron Microscopic Analysis

2.10.1 Cell Fixation Cells were harvested by centrifugation at 3,000xg for 10 min at 4°C. The packed cells were washed once in cold 10 mM sodium phosphate buffer pH 7.0. After treatment with 2.5% buffered glutaraldehyde (pH 7.0) at 4°C for 90 min, the fixed cells were washed 3 times with the same buffer.

2.10.2 Specimen Preparations

2.10.2.1 Scanning Electron Microscopy Glutaraldehyde fixed cells were necessarily dispersed on a filter paper Whatman #1 and immediately dipped in 10% ethanol to prevent air drying. Further stepwise dehydration was continued in 20, 30, 50, 70, 80, 90, 95% ethanol and followed by two times repeatedly dipping in 100% ethanol with 10 min incubation at room temperature for each step. Cells were finally dried by using critical point drying method. After the whole specimen was adhered on the surface of a brass stub, it was coated with gold vapor. Electron microscopic analysis was conducted by using a JSM-35 CF Scanning Electron Microscope.

2.10.2.2 Transmission Electron Microscopy Glutaraldehyde fixed cells were mixed with 1% agarose, cut into 2x5 mm pieces and

then post-fixed with 1% osmium tetra-oxide at 4°C for 60 min. Dehydration was conducted by serially soaking the materials for 10 min in 30, 50, 70, 80, 90, and 100% ethanol. The dehydrated specimens were embeded in Spurr's epoxy medium and baked at 60°C for 24 hours. Sections were cut on a LKB ultramicrotome to make very thin films of the specimens which were stained with 2% uranyl acetate. Fine structures of the alga were examined by using a JEM -200 CX Transmission Electron Microscope.

2.11 Measurement of PS I Activity

2.11.1 Reagents Methyl red, directly prepared in the reaction buffer (50 mM Tris-HCl, pH 7.2) at a concentration of 66 µM.

Stock solution of 20 mM dichloroindophenol (DCIP).

Stock solution of 2 mM 3(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) in 50% ethanol.

Stock solution of 120 mM ascorbic acid, freshly prepared for each experiment and kept in the dark.

2.11.2 Method for Measurement A simple set up of instrument was built and used for measurement of the photoreduction by PS I. As shown in Fig. 2 the instrument is composed of three parts as follows (1) light source from a 500 watt tungsten lamp, (2) a 5 inches thick tank of 0.45% cupric sulfate to absorb heat, and (3) a sample chamber which was designed to locate cuvette holder at the distance to obtain light intensity of 17,000 lux at the sample cuvette.

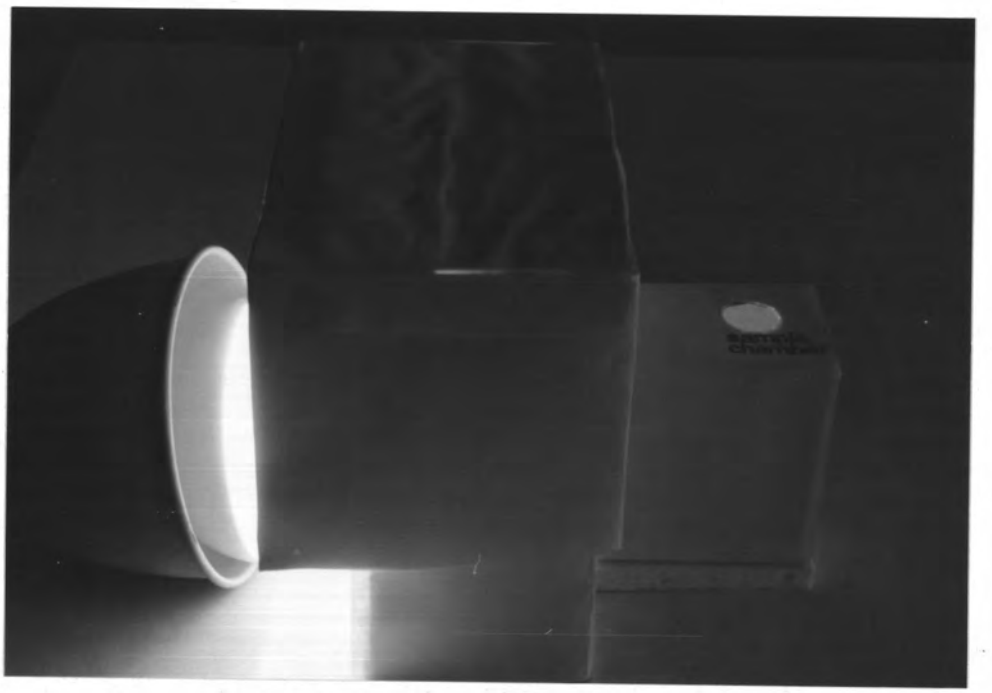

Figure 2.2 Set up for PS I activity determination.

Light source is generated from a 500 W tungsten lamp at the distance of 7 inches from the sample dark controlled chamber to obtain light intensity at 17,000 lux at the sample cuvette.

Cells from exponential phase culture were harvested, washed in 10 mM Tris-HCl (pH 7.2) and resuspended in the same buffer to give a cell suspension having a final chlorophyll a concentration of approximately 300 µg/ml. The washed cells were disrupted by a French Pressure Cell at 5,000 psi. All washing and breaking procedures were conducted at 4°C. The crude homogenate obtained was kept at 4°C in a light-protected tube and PS I activity measurement was performed within 3 hours.

PS I activity was assayed in the presence of DCMU which was essential according to Vernon et al (1966). The procedure used was slightly modified from Schor et al (1970). Each 1 ml reaction mixture contained methyl red 0.060 mM in 0.9 ml of Tris-HCl buffer , pH 7.2 (45 mM), 0.2 mM DCIP, 0.02 mM DCMU, 1.2 mM ascorbic acid, and crude homogenate (3-5 µg chlorophyll a). The ascorbic acid and crude homogenate were added just prior to illumination. Cuvette containing the reaction mixture was exposed to light at a chamber temperature of 26 ±1°C. The remaining unreduced methyl red was measured at 30 sec time intervals at 430 nm, using a Shimadzu UV-240 spectrophotometer. The values of optical density change in the dark conditions were used as a control in each measurement. Quantitative calculation of methyl red was based on its molar extinction coefficient ($E_{430} = 14.1 \times 10^3$).

PS I activity was reported as the amount of methyl red reduced per mg chlorophyll a under the standard conditions.

2.12 Measurement of [^{14}C]Paraquat Uptake

2.12.1 Reagents [methyl-^{14}C]Paraquat dichloride.

10% Trichloroacetic acid (TCA) containing 8 mM paraquat.

Toluene-based Triton X-100 scintillation fluid (Appendix 3).

2.12.2 Method of measurement The procedures for the measurement of [^{14}C]paraquat uptake were essentially developed in this research project. Experiments were necessarily conducted in plastic tubes. Reaction mixture (2.5 ml) contained 10^7 cells or 10^7 protoplasts in TMP medium and [^{14}C]paraquat in a range of 0.03-0.35 µM. The mixture was incubated by shaking at 30°C for a period of time as indicated in the results (section 5.2.3). The reaction was stopped by placing the tube in an ice bath and centrifuged at 4°C (2,000xg for 5 min). The pellet was washed repeatedly by centrifugation for 3 times in cold TMP medium and then extracted with 500 µl of 10% TCA containing 8 mM paraquat. Addition of 500 µl distilled water was performed to reduce the quenching effect of TCA. After vigorously mixing on a vortex mixer, the lysate was centrifuged to obtain a clear supernatant. Five hundred microlitre aliquots were counted for radioactivity in 7 ml of Toluene-based Triton X-100 scintillation fluid in a glass vial, by using a LKB Liquid Scintillation Counter.

The amount of radioactive paraquat uptake was calculated from its specific activity and reported in terms of pmol/10^7 cells or pmol/10^7 protoplasts. The reactions were undertaken in triplicated.

For investigation of [^{14}C]paraquat distribution into chloroplast, the uptake reaction was performed in protoplasts (section 2.8). After the washing steps were completed, the [^{14}C]paraquat taken up protoplasts were pooled (n = 5) and subjected to digitonin lysis (section 2.9) to isolate chloroplasts. Free chloroplasts were lysed by adding 500 µl of distilled water. The remaining unlysed protoplasts were removed by centrifugation. Green supernatant (400 µl) was mixed with an equal volume of 10% TCA containing 8 mM paraquat as above. After subsequent centrifugation to separate protein precipitates, 500 µl portion of the clear supernatant was counted for the radioactivity. The amount of [^{14}C]Paraquat in free chloroplasts was calculated per 10^7 chloroplasts.

2.12.3 Reliability of the Measurement Method for [^{14}C]Paraquat Uptake The reliability of the measurement method could be determined by incubating various concentration of [^{14}C]paraquat with 10^7 cells in the total volume of 100 µl at 30°C for 30 min. After that, cells were lysed and proteins were precipitated by 10% TCA solution (section 2.12.2). Radioactivity was counted in Toluene-based Triton X-100 scintillation fluid. Recovery of [^{14}C]paraquat was determined as percentage of the total [^{14}C]paraquat added.

At any amount of [^{14}C]paraquat added, precision within assay of the extraction method was also measured from the coefficient of variation (c.v.) as followings ;

$$\% \text{ c.v.} = (\text{S.D.}/\bar{X}) \times 100$$

where , $\bar{X}$ = average value of the radioactivity.

S.D. = standard deviation

2.13 Enzyme Assay

2.13.1 Enzyme Preparation Crude extracts of paraquat susceptible and resistant C. reinhardtii were prepared from exponential phase cell cultures. Cells were harvested and resuspended (0.8 g wet weight per 5 ml) in extraction buffer (50 mM potassium phosphate buffer, pH 7.8, 0.1 mM EDTA for superoxide dismutase, 50 mM potassium phosphate buffer, pH 7.8 for catalase, and 5 mM potassium phosphate buffer, pH 7.8, 1 mM ascorbate, 10 µM ferrous sulfate for ascorbate peroxidase). Following the disruption of cells with a French Pressure Cell at 8,000 psi, the extracts were centrifuged at 20,000xg, 4°C for 45 min, and the clear supernatants were collected and stored on ice for use as the crude enzymes.

2.13.2 Superoxide Dismutase (SOD) Assay

2.13.2.1 Reagents 50 mM Potassium phosphate buffer, 1 mM EDTA (pH 7.0) as an assay buffer.

Xanthine oxidase, suspended in 2 M ammonium sulfate, 1 mM EDTA (pH 7.8).

4 mM xanthine, 0.8 mM cytochrome c, and 2 mM potassium cyanide.

2.13.2.2 Method for SOD Assay SOD activity was determined which was based on SOD inhibition of superoxide-mediated cytochrome c reduction as modified from Furusawa et al (1984). A standard assay mixture in 1 ml cuvette consisted of assay buffer (oxygen-equilibrated by an air pump at least 1 hour before used), 100 µM xanthine, 20 µM cytochrome c, and 20 µM potassium cyanide. The reaction was performed at 30°C. The control rate was monitored by adding xanthine oxidase (6.25 µg protein) to the reaction mixture and the cytochrome c reduced was measured by the decrease of absorbancy at 550 nm which was consistently at 0.025 absorbance unit change per min. When SOD was to be assessed, crude extract was included in the reaction.

One unit of SOD activity was expressed as the amount of enzyme that caused 50% inhibition of cytochrome c reduction under standard assay conditions (Asada et al, 1974).

2.13.3 Catalase Assay

2.13.3.1 Reagents. 50 mM Tris-HCl buffer, pH 8.5 containing 44 mM hydrogen peroxide as an assay buffer.

2.13.3.2 Method for Catalase Assay Catalase was assayed at 30°C by following the reduction of hydrogen peroxide at 240 nm (Beers and Sizer, 1952 ; Nelson and Kiesow, 1972 ; Powles and Cornic, 1987)). Crude enzymes were added to 1 ml of assay buffer. The change in the amount of hydrogen peroxide was deter-

mined from a standard curve of hydrogen peroxide concentrations versus absorbancy at 240 nm. Catalase activity was expressed as nmol hydrogen peroxide reduced per min.

One unit of catalase was defined as the amount of enzyme that reduced 1 nmol of hydrogen peroxide per min under standard conditions.

2.13.4 Ascorbate Peroxidase Assay

2.13.4.1 Reagents

50 mM Tris-HCl buffer, pH 7.0 containing 0.1 mM hydrogen peroxide as an assay buffer.

10 mM Ascorbic acid.

2.13.4.2 Method for Ascorbate Peroxidase Assay

Ascorbate peroxidase was assayed at 30°C by measuring the change in absorbancy at 290 nm of ascorbate according to Nakano and Asada (1987). Crude extracts were added to a 1 ml of assay buffer containing 0.5 mM ascorbate. Ascorbate peroxidase activity was expressed as nmol ascorbate oxidized per min. Molar extinction coefficient of ascorbate at 290 nm was 2.8×10^{-3} .

One unit of ascorbate peroxidase was defined as the amount of enzyme that oxidized ascorbate 1 nmol per min under standard conditions.

2.14 Enzyme Induction

C. reinhardtii cells grown in log phase were collected and

resuspended ($4x10^6$ cells/ml) in a fresh TMP medium containing paraquat at sublethal concentrations. Cells were exposed to the herbicide and light for 48 hours under the growth conditions (section 2.2.2). At the end of the induction, cells were harvested, crude extract was prepared and assayed for superoxide dismutase, catalase, and ascorbate peroxidase activities.

2.15 Isozyme of Superoxide Dismutase (SOD)

Electrophoresis was performed on a 10% polyacrylamide gel. Bands of SOD were visualized by staining with the photochemical nitro blue tetrazolium (NBT) as described by Beauchamp and Fridovich (1971) with a slight modification.

2.15.1 Polyacrylamide Gel Electrophoresis (PAGE)

2.15.1.1 Reagents Solution A : 100 ml contains Tris 36.6 g, TEMED 0.23 ml and 1 N hydrochloric acid 48 ml, adjusted to pH 8.9 .

Solution B: 100 ml contains Tris 5.98 g, TEMED 0.46 ml and 1 N hydrochloric acid 40 ml, adjusted to pH 6.7 .

Solution C: 100 ml contains acrylamide 28 g and Bis 0.74 g.

Solution D: 100 ml contains acrylamide 10 g and Bis 2.5 g .

Solution E: 0.004% riboflavin.

Electrode buffer (stock 10x): 1 Litre contains Tris

6 g, glycine 28.8 g, adjusted to pH 8.3 .

Tracking dye: 0.25% bromophenol blue in 0.5 M Tris-HCl, pH 6.7.

Resolving gel: 45 ml contains solution A, solution C, solution E and distilled water in the ratio of 5:16:5:19.

Stacking gel: 20 ml contains solution B, solution D, solution E and distilled water in the ratio of 2.5:5:2.5:10.

2.15.1.2 Method for PAGE Slab gel was set on a LKB slab gel electrophoresis apparatus according to the instruction mannual. The polymerization, which was accomplished within 30-45 min , was initiated by light from a fluorescent lamp .

Crude SOD (0.5-1.5 unit) was mixed with glycerol and tracking dye solution by the ratio of 400:100:20 (v/v). The enzyme solution was loaded on a polyacrylamide slab gel (13 cm) which was previously prepared. The electrophoresis was carried out by anodic system at a constant voltage of 30 V/slab gel at 4°C. After the tracking dye had reached the edge of the gel, the electric field was further provided until presumably the complete separation of enzymes was obtained on the total gel length of 18 cm.

2.15.2 Activity Staining of SOD

2.15.2.1 Reagents 0.036 M Potassium phosphate buffer, pH 7.8, as a staining buffer.

1 mM NBT, freshly prepared in staining buffer.

2.15.2.2 Method for SOD Activity Staining Gel was submerged in NBT solution at 30°C in a light protected box for 15 min. Then it was transferred into a reaction mixture consisting of 0.028 M TEMED and 0.03 M riboflavin in the assay buffer which was pre-equilibrated with air at least 1 hour before using. This step was carried out at 30°C for 20 min without light exposure. At the end of the incubation period, gel was placed under a fluorescent lamp. During this period, most part of the gel turned from yellow color of the water soluble NBT to a dark blue color of the water insoluble photoreduced NBT. This photoreduction of NBT was mediated by oxygen radicals (or superoxide) through the light excited riboflavin. Consequently, zones of SOD on the gel were gradually visualized as colorless bands on the blue background owing that the local oxygen radicals were scavenged by the SOD activity. R_f of each isozyme was calculated as follow,

R_f = distance of SOD band from the origin/ gel length (18 cm)

To distinguish the copper/zinc enzyme (Cu/Zn-SOD) from the iron enzyme (Fe-SOD) and the manganese enzyme (Mn-SOD) as well, triplicate samples were parallel treated with either 4 mM potassium cyanide or 10 mM hydrogen peroxide or both in all steps of the activity staining (modified from Rabinowitch et al, 1983).

2.16 Detection of Proteins in Cell Lysate by PAGE

2.16.1 Reagents Stock 20% sodium dodecyl sulfate.

Sample buffer: 0.5 M Tris-HCl pH 6.7.

Electrode buffer: 0.005 M Tris , 0.04 M glycine , pH 8.3 .

Protein staining solution: 0.25% coomassie blue R in 50% methanol and 10% glacial acetic acid.

Destaining solution : 5% methanol (7% glacial acetic acid).

2.16.2 Method for Separation of Proteins by PAGE The components of proteins in cell lysate were detected by breaking the algal cell (10^8 cells) with 2% sodium dodecyl sulfate in 1 ml of the sample buffer and boiled for 5 min. After centrifugation the clear lysate was loaded on a polyacrylamide slab gel (10% acrylamide and 13 cm in length) and electrophoresis was performed at 30 V for 3-4 hours.

Gel was stained overnight with 0.25% coomassie blue R and then destained for several times with the destaining solution until a clear background was obtained.

2.17 Analysis of Chloroplast DNA

2.17.1 Isolation of chloroplast DNA

2.17.1.1 Reagents TEN buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA disodium salt, 150 mM sodium chloride).

TE buffer (10 mM Tris-HCl pH 8.0, 10 mM EDTA disodium salt).

30% Sodium dodecyl sulfate.

40 mg/ml Pronase E, pretreated at 37°C for 2 hours.

2.5 M Sodium acetate (pH 5.2).

1 mg/ml Ethidium bromide

cesium chloride

2.17.1.2 Method for Isolation of Chloroplast DNA

C. reinhardtii cells at the exponential growth phase were harvested, washed once in TEN buffer and resuspended at $4x10^8$ cells/ml. Stock solution of sodium dodecyl sulfate and the pretreated Pronase E were added to a final concentration of 1% and 2 mg/ml, respectively. The lysis mixture was incubated overnight at 25°C. Each lysate was then extracted with an equal volume of TEN-saturated phenol/chloroform/ isoamyl alcohol (25:24:1 v/v) and the lower organic phase was re-extracted once with 0.5 volume of TEN buffer. The upper phases were pooled and adjusted to 0.25 M sodium acetate (pH 5.2) before precipitation overnight at -20°C with 2 volumes of absolute ethanol. The total DNA was dissolved in 7 ml of TE buffer. Sample was added with 7.7 g of solid cesium chloride and ethidium bromide was also included to a final concentration of 20 ug/ml. The solution was centrifuged at 45,000 rpm in Polyaroma tubes in Hitachi 55P-72 Ultracentrifuge, Rotor type RP-55t. The upper UV fluorescing band containing chloroplast DNA (Grant et al ,1980) was collected by piercing the tubes with a 18 guage syringe needle. The resulting DNA solution was diluted with TE buffer to a volume of 10 ml and dialysed overnight against 2 changes of TE buffer (1000 ml each) at 4°C to remove cesium chloride. DNA was precipitated with absolute ethanol in a similar manner as described above.

The DNA so obtained was washed twice with 70% ethanol, once with 95% ethanol and finally dissolved in TE buffer.

2.17.2 Analysis of DNA Restriction Fragments by Agarose Gel electrophoresis

2.17.2.1 Reagents Medium salt buffer: 10 mM Tris-HCl, pH 7.5, 50 mM sodium chloride, 10 mM magnesium chloride, and 1 mM dithiothreitol.

High salt buffer: 50 mM Tris-HCl pH 7.5, 100 mM sodium chloride, 10 mM magnesium chloride, and 1 mM dithiotreitol.

0.5 M EDTA disodium salt pH 7.5.

Tracking dye solution: 0.25% bromophenol blue, 40% sucrose in distilled water.

E buffer (stock 50x): 1 Litre contains Tris 242.28 g , EDTA disodium salt 37.22 g and glacial acetic acid 57.1 ml.

2.17.2.2 Method for Analysis of DNA Restriction Fragments DNA digestions were performed at 37°C. Reaction buffers suitable for each restriction enzyme were as described in "Molecular Cloning" of Maniatis et al (1982), that was (a) medium salt buffer for Bam H1, Bgl II, Hind III, and Pst 1 (b) high salt buffer for Eco R1 , and Xho 1.

Reaction mixture (40 ul) contained 1-3 µg of DNA and 10-20 units of a restriction enzyme. The DNA was cut by two step digestions. During the first step, half amount of the restriction

enzymes were included in the reaction which was carried out for 3 hours. Thereafter, another half of the enzyme was then added and further digestion was continued for 6 hours. The reaction was stopped by addition of 0.5 M EDTA, pH 7.5 (1 µl). The resulting DNA fragments solution was mixed with 10 µl of the tracking dye and was subjected to agarose gel electrophoresis (0.7% agarose).

Agarose gel electrophoresis (0.8x0.5x16.0 cm for each lane) was carried out in E buffer with a constant voltage of 3 V/cm for about 13-15 hours at room temperature. DNA fragments were stained in ethidium bromide solution (2 µg/ml) for 30 min and destained in distilled water for 30-45 min at room temperature. The fluorescence pattern of DNA fragments were detected and photographed on a UV illuminator. Standard λ phage DNA (digested by Hind III) was used as a reference for fragment sizes.