CHAPTER II

MATERIALS AND METHODS

Materials

1) Equipments

Spectrophotometer DU 650 Beckman : U.S.A.
pH Meter PHM 83 Radiometer Copenhagen : Denmark
Autoclave Model HA-30 Hirayama Manufacturing Corporation : Japan
Laminar Flow Model BVT-124 International Scientific Supply CO. Ltd. : Thailand
Illumination Meter Topcorn MO-2
Rotary Shaker New Brunswick Scientific Edison, N.J. : U.S.A.
Electrophoresis unit 2050 MIDGET, LKB, Sweden and Mini-Protein, Bio-Rad : U.S.A.
Water Bath Buchi 461 : Switzerland
Fraction Collector Model 2211, Pharmacia LKB : Sweden
Peristaltic Pump Pharmacia LKB : Sweden
Refigerated Centrifuge Model J2-21, Beckman Instrument Inc : U.S.A.
^{1}H Nuclear Magnetic Resonance Spectroscopy JNM-A500 : Japan
Digital Lux Meter FT 710 : Taiwan
UV lamp
Micropipette

2) <u>Chemicals</u>

Acrylamide, Merck : U.S.A.

Glycine betaine, Sigma : U.S.A.

Betaine aldehyde chloride, Sigma : U.S.A.

Choline Chloride, Sigma : U.S.A.

Coomassie Brilliant Blue, BDH Laboratory Chemical Ltd.

DEAE-cellulose, Sigma : U.S.A.

Dialysis Tubing, Sigma : U.S.A.

Dowex-50W Resin (50-100 mesh, 2% cross-linkage), Sigma : U.S.A.

DSS (Sodium 2,2-Dimethyl-2-Silapentate-5-Sulphonic Acid), Sigma : U.S.A.

DTT (Dithiothreitol), Sigma : U.S.A.

EDTA (Ethylenediaminetetraacetic acid), Sigma : U.S.A.

Ethylene Dichloride (1,2-dichloroethane), Sigma : U.S.A.

Hydroxyapatite, Sigma : U.S.A.

Kit molecular weight marker for PAGE, Sigma : U.S.A.

- Phosphorylase B
- Bovine serum albumin
- Ovalbumin
- Carbonic anhydrase
- Soybean trypsin inhibitor
- Lysozyme

Lysozyme, Sigma : U.S.A.

Mercaptoethanol, Fluka : Switzerland

N,N′ -Methylene-Bis-Acrylamide, Sigma : U.S.A.

Nitro Blue Tetrazolium, Sigma : U.S.A.

p-cholromercuriphenyl sulfonic acid, Sigma:U.S.A.

Phenazine Methosulfate, Sigma: U.S.A.

TEMED (*N,N,N,N'* Tetramethylenediamine), Sigma:U.S.A.

3) Specimen

Aphanothece halophytica was isolated from solar lake in Israel. The organism was kindly provided by Dr. T. Takabe of Nagoya University, Japan.

Mathods

1. Growth of *A halophytica* in Turk Island Salt Solution plus modified BG_{11} medium.

10% Inoculum of *A. halophytica* was inoculated into a 250 ml flask containing 100 ml of Turk Island Salt Solution + modified BG_{11} medium (see Appendix 1) and grown on a rotary shaker with 160 rpm at 30 °C. Three 20-W fluorescent lamps placed overhead provided the flasks with 1,900 lux of continuous illumination.

For salt-stress experiment,10-day-old cells grown in medium containing 0.5 M NaCl were transferred to culture media that contained NaCl at 1.0 and 2.0 M. The growth of cells was determined by counting the number of the cells using the haemocytometer.

2. Determination of glycine betaine.

2.1 Extraction of glycine betaine from cells

30 ml of *A.halophytica* culture (10^7 cells/ml) was centrifuged at 8,000g for 20 min at room temperature to collect the cells. The cell pellet was suspended in 5 ml of 80%(v/v) ethanol and boiled for 5 min. The suspension was centrifuged at 1,000g for 10 min at room temperature and the supernatant was removed and stored at 4°C. The pellet was re-extracted with 80%(v/v) ethanol and incubated for 18 hr at 25°C. The suspension was again centrifuged and the supernatant was pooled. The pooled supernatant was dried by evaporation at 65°C. The residue was stored and used for the assay of glycine betaine by ^{1}H-NMR and spectrophotometric methods.

2.2 ^{1}H-NMR measurements

^{1}H-NMR Spectrum was measured on a JNM-A500 operating at a frequency of 500 MHz at a probe temperature of 29°C. The dried residue was dissolved in 1.5 ml D_2O and 0.6 ml of solution was transferred to a 5 mm NMR tube to which was added 5 µl of 1% DSS. Peak areas were used to quantitate the amount of the compound present. The quantitation was obtained by comparing integrated peak intensities against standard curves. Peak position was measured relative to DSS (Jones *et al.* 1986).

2.3 Spectrophotometric measurements

Dried residue was dissoved in 1.5 ml of distilled water and this solution was loaded onto a Dowex-50W column (1×3 cm, H^+ form). The column was washed with 10 ml of distilled water. Glycine betaine was eluted with 20 ml of 2M NH_4OH. The eluent was dried by lyophilization.

The dried pellet was dissolved in 250-600 μl of distilled water. Glycine betaine was determined by tri-iodide assay (Storey and Jones 1977). The acid potassium tri-iodide solution (0.2 ml) was added to a sample containing between 10-100μg of glycine betaine in distilled water. The mixture was shaken for at least 90 min in ice bath. The 2 ml cooled water was added rapidly to the mixture to reduce the absorbance of the blank. This was quickly followed by 5 ml of 1,2-dichloroethane and the 2 layers were mixed by stirring. The absorbance of the lower organic layer was measured at 365 nm. The quantitation was obtained by comparing OD at 365 nm against a standard curve.

2.4 The recovery of glycine betaine from extraction of cells and from ion-exchange chromatography as determined by ^{1}H-NMR and by spectrophotometric assay

2.4.1 The recovery of glycine betaine extracted from the cells as determined by ^{1}H-NMR assay

A.halophytica cells grown in the medium containing 0.5 M NaCl, at 10 days, were extracted twice by 5 ml of 80%(v/v) ethanol. The overall

recovery of glycine betaine from cells was evaluated by addition of known amount of glycine betaine to the cells prior to extraction. One sample represented the control, without added glycine betaine and the other was added with 3.5 mg commercial glycine betaine. The two samples were dried by evaporation at 65°C and glycine betaine was determined by ^{1}H-NMR method as described in section 2.2.

2.4.2 <u>The recovery of commercial glycine betaine from ion-exchange chromatography as determined by spectrophotometric assay</u>

The commercial glycine betaine (4.0 mg) was loaded onto the Dowex-50W column. After loading, the column was washed by 10 ml distilled water and glycine betaine was eluted by various volumes of 2M NH_4OH, i.e.,0-5, 5-10, 10-15 and 15-20 ml. All fractions were dried by lyophilization and glycine betaine was determined by tri-iodide assay as described in section 2.3.

2.4.3 <u>The recovery of glycine betaine from extraction of cells and from ion-exchange chromatography as determined by spectrophotometric method</u>

A.halophytica cells grown in the medium containing 0.5M NaCl, at 10 days, were extracted twice by 5ml of 80%(v/v) ethanol. The procedure of extraction was the same as that for ^{1}H-NMR method but 3.7 mg commercial glycine betaine was added to the cells prior to extraction and

no added commercial glycine betaine represented the control. The two samples were dried by evaporation at 65°C and the pellet was resuspended in distilled water. The suspension was loaded onto the ion-exchange chromatography column. The column was washed by 10 ml distilled water and glycine betaine was eluted by appropriate volume of 2M NH_4OH. The eluents were dried by lyophilization and glycine betaine was determined by tri-iodide assay.

2.5 Effect of NaCl on glycine betaine accumulation in *A. halophytica*

A.halophytica cells were transferred from culture medium containing 0.5M NaCl to culture medium containing 0.5, 1.0 and 2.0M NaCl. 30 ml of the culture was centrifuged and the cell pellet was collected for glycine betaine extraction and quantitation by ^{1}H-NMR and spectrophotometric methods.

3. Protein determination

Protein concentration was determined by the dye-binding method according to Bradford (1976), using bovine serum albumin as a standard.

One hundred microlitres of sample was mixed with 1 ml of Coomassie blue reagent and left for 5 min before measuring the absorbance at 595 nm. One litre of Coomassie blue reagent was the mixture of 100 mg Coomassie blue, 50 ml of 90% ethanol, 100 ml of 85% H_3PO_4 and distilled water.

4. The BADH activity assay

The spectrophotometric method based on the determination of NADH at 340 nm was used (modified from Weretilnyk and Hanson 1989 and Pan *et al.*1981). The reaction mixture (final volume 1 ml) contained 50 mM HEPES-KOH buffer pH 7.5, 10 mM DTT, 1 mM EDTA, 0.5 mM NAD^+, 0.5 mM betaine aldehyde and appropriate amount of enzyme extract. The reaction was started by the final addition of betaine aldehyde. The slope of the linearly increased OD at 340 nm versus an incubation time 2 min at 25°C was the initial velocity of the enzyme and the enzyme activity was expressed in μmol NADH formed per min per mg protein.

5. Polyacrylamide gel electrophoresis (PAGE)

5.1 Non-denaturing PAGE

Discontinuous PAGE was performed on slab gels (10×8×0.75 cm), consisting of 7.5%(w/w) separating gel, and 5%(w/v) stacking gel. Tris-glycine buffer pH 8.8 was used as electrode buffer (see Appendix 2). The sample was treated with sample buffer (see Appendix 2) at a ratio of 4:1 (sample:sample buffer). The electrophoresis was run from cathode toward the anode at constant current of 20 mA per slab in a Midget LKB 2001 electrophoresis. The temperature was controlled at 4°C by LKB 2209 Multi-temperature thermostat water bath. The protein bands on the gel were stained with staining solution and destained with destaining solution.

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5.2 SDS-PAGE

The denaturing gel electrophoresis was performed according to Laemmli (1970). The gel consisted of 0.1%(w/v) SDS in 10%(w/v) separating gel and 5.0%(w/v) stacking gel, 25mM Tris-glycine buffer pH 8.8 containing 0.1% SDS was used as an electrode buffer (see Appendix 2). Sample to be analysed was treated with sample buffer for SDS-PAGE (see Appendix 2) and boiled for 5 min prior to application to the gel. The electrophorisis was performed at constant current of 20 mA per slab, on Midget LKB 2001 electrophoresis unit from cathode toward the anode, The gel was stained with coomassie blue. The protein molecular weight markers were phosphorylase B (97,400 dalton), bovine serum albumin (66,200 dalton), ovalbumin (45,000 dalton), carbonic anhydrase (31,000 dalton), soybean trypsin inhibitor (21,000 dalton) and lysozyme (14,400 dalton). Relative molecular weight of the protein sample was estimated from standard curve plotted on semilog scale between the molecular weight of protein marker and the relative electrophoretic mobility (R_f). The R_f was calculated by:

R_f= distance of protein migration / distance of tracking dye migration

5.3 Detection of proteins in the slab gel by coomassie blue staining

Affer electrophoresis, proteins in the gel were stained by coomassie blue. The slab was immersed in 0.2%(w/v) of coomassie

brilliant blue R-250 containing 50%(v/v) methanol and 7.5%(v/v) acetic acid for at least 2 hr. It was then destained with a solution of 25% methanol and 10%(v/v) acetic acid for 1-2 hr, followed by several changes of 5%(v/v) acetic acid solution until gel background was clear.

5.4 BADH activity staining

After non-denaturing polyacrylamine gel electrophoresis, the gel was activity stained for 1 to 3 hr in the following solution : 150 mM Tris-HCl pH 8.0, 5 mM betaine aldehyde, 1 mM NAD^+, 300 μg/ml nitro blue tetrazolium and 20 μg/ml phenazine methosulfate. The presence of BADH activity was confirmed by the appearance of brown colour band.

6 The purification of BADH

6.1 Cells extraction

Approximately 10g of *A. halophytica* cell pellet was suspended in 25 ml of 50mM HEPES-KOH buffer pH 7.5 containing 2 mg/ml lysozyme and allowed to incubate at 37°C for 60 min to lyse the cells. The suspension was centrifuged at 14,000g for 20 min. The supernatant was subjected to ammonium sulfate precipitation.

6.2 Ammonium sulfate precipitation

Solid ammonium sulfate was gradually added to the supernatant to 35% saturation with gentle stirring for 1 hr. The supernatant obtained after centrifugation at 14,000g for 20 min was made to 70% saturation of ammonium sulfate and centrifuged at 14,000g for 20 min.The final pellet was suspended in a small volume of 10mM Tris-HCl buffer pH 7.5 containing 1 mM DTT and 10%(v/v) glycerol before dialysis against the same buffer.

6.3 Column chromatography for BADH purification

6.3.1 DEAE-cellulose column chromatography

Approximately 10 g of DEAE-cellulose was swollen in 1 liter of distilled water and then washed several times at room temperature to remove the fine particles. The resin was activated by washing sequentially with excess volume of 0.5 M HCl for 30 min followed by distilled water until the pH was 7.0. The resin was then treated with 0.5M NaOH for 30 min followed by distilled water until pH was 7.0. The activated resin was equilibrated with 10mM Tris-HCl buffer pH 7.5 containing 10%(v/v) glycerol and 1mM DTT overnight. The prepared DEAE-cellulose was packed into a column (2.5×20 cm) at the height of 17 cm. The column was equilibrated with the buffer for at least 4 to 6hr at the flow rate of 30 ml/hr. Three ml of the protein solution containing about 100 mg protein was loaded and allowed to be absorbed. Elution was carried out by a

continuous linear gradient of 0 to 1.0 M NaCl prepared in the buffer. A flow rate of 30 ml/hr was maintained and 5 ml fractions were collected. The concentration of NaCl in the fraction was estimated by using a conductivity meter. The protein content of each fraction was monitored by measuring the OD at 280 nm. The BADH activity was assayed as described in section 4. The activity was expressed in μmol NADH formed per min per ml.

6.3.2 Hydroxyapatite column chromatography

Approximately 15 g of the commercial hydroxyapatite was swollen in 1mM potassium phosphate buffer pH 7.5 and equilibrated overnight with 10mM potassium phosphate buffer pH 7.5 containing 10%(v/v) glycerol and 1 mM DTT. The prepared hydroxyapatite was packed into a column (2.0×12 cm) at the height of 6.5 cm. The column was equilibrated with 10mM potassium phosphate buffer pH 7.5 containing 10%(v/v) glycerol and 1 mM DTT for at least 4 to 6 hr at the flow rate of 3 ml/hr. Two ml of the protein solution containing about 5 to 10 mg was loaded and allowed to be absorbed. Elution was carried out by a continuous linear gradient of 10 to 200 mM potassium phosphate buffer pH 7.5. A flow rate of 3 ml/hr was maintained and 2 ml fractions were collected. The protein content of each fraction was monitored by measuring the OD at 280 nm. The BADH activity was detected by determination of NADH by measuring the OD at 340 nm. The BADH activity was assayed as described in section 4. The activity was expressed in μmol NADH formed per min per ml.

7 The properties of BADH

The partially purified BADH was characterized with respect to pH, temperature, coenzyme requirements, substrate analogs inhibition and effect of cations.

7.1 Effect of pH on BADH activity

The partially purified BADH was assayed in the reaction mixture containing 10mM DTT, 1mM EDTA, 0.5mM NAD^+, 0.5mM betaine aldehyde and appropriate amount of enzyme . The reaction was started by the final addition of betaine aldehyde. In each assay the pH was adjusted with appropriate buffer (50mM potassium phosphate buffer pH 6.0 and 6.5, 50mM HEPES-KOH buffer pH 6.5, 7.0 and 7.5 and 50mM Tris-HCl buffer pH 7.5, 8.0 and 9.0). The reaction was run at 25°C and incubation time was 2 min. The initial velocity of enzyme was expressed in μmol NADH formed per min per mg protein.

7.2 Effect of incubation temperature on BADH activity

The partially purified BADH was assayed at optimal pH and the reaction mixture contained 10mM DTT, 1mM EDTA, 0.5mM NAD^+, 0.5mM betaine aldehyde and appropriate amount of enzyme. The reaction was started by the final addition of betaine aldehyde and the incubation time was 2 min. The reaction was incubated at various temperatures. The

initial velocity of enzyme was expressed in μmol NADH formed per min per mg protein.

7.3 The kinetic of BADH

7.3.1 The Michaelis constant (K_m) and maximum velocity (V_{max})

The kinetics of the BADH was studied by assaying the enzyme at pH 7.5 and 25°C. The reaction mixture contained 50mM HEPES-KOH buffer pH 7.5, 10mM DTT, 1mM EDTA, 0.5mM NAD^+, and appropriate amount of enzyme. The reaction was started by the addition of the varying substrate (betaine aldehyde) concentrations at 0.05, 0.1, 0.25 and 0.5 mM. The velocity of enzyme was expressed in μmol NADH formed per min per mg protein. The Michaelis constant (K_m) and maximum velocity (V_{max}) were calculated from x-axis and y-axis intercepts respectively of the double-reciprocal plot, i.e., 1/[s] vs 1/[v].

7.3.2 Coenzyme requirements

Two coenzymes NAD^+ and $NADP^+$ were tested by assaying the enzyme at pH 7.5 and 25°C. The reaction mixture contained 50mM HEPES-KOH buffer pH 7.5, 10mM DTT, 1mM EDTA,and appropriate amount of enzyme. For each assay the varying NAD^+ or $NADP^+$ concentrations at 0.05, 0.1, 0.25 and 0.5 mM were added to the reaction mixture. The reaction was started by the addition of 0.5mM

betaine aldehyde. The velocity of enzyme was expressed in μmol NADH formed per min per mg protein. The Michaelis constant (K_m) and maximum velicity (V_{max}) were calculated as described in section 7.3.1

7.4 The substrate analog inhibition

The partially purified BADH was assayed at pH 7.5 and 25°C. The reaction mixture contained 50mM HEPES-KOH buffer pH 7.5, 10mM DTT, 1mM EDTA, 0.5mM NAD^+, 0.5mM betaine aldehyde and appropriate amount of enzyme. The substrate analog (glycine betaine, choline, ethanolamine or acetaldehyde) was present in the reaction mixture. The reaction was started by the final addition of 0.5mM betaine aldehyde. BADH activity without substrate analog represented the control. The enzyme activity was expressed as the percentage of remaining activity compared to the control.

7.5 Effect of cation on BADH activity

The partially purified BADH was assayed at pH 7.5 and 25°C. The reaction mixture contained 50mM Tris-HCl buffer pH 7.5, 10mM DTT, 1mM EDTA, 0.5mM NAD^+, 0.5mM betaine aldehyde and appropriate amount of enzyme. Various salts (NaCl, KCl, $MgCl_2$ or $CaCl_2$) at the varying concentrations of 0.05, 0.1, 0.25, 0.5, 1.0 and 2.0M were added to the reaction mixture. The reaction was started by the final addition of 0.5mM betaine aldehyde. The velocity of enzyme was expressed in μmol NADH formed per min per mg protein.

7.6 Effect of DTT and *p*-chloromercuriphenyl sulfonic acid (PCMS) on BADH activity

The partially purified BADH from DEAE-cellulose column was dialyzed against 10 mM Tris-HCl pH 7.5 buffer containing 10%(v/v) glycerol(without DTT). Enzyme was assayed at pH 7.5 and 25°C. The reaction mixture contained 50 mM HEPES-KOH buffer pH 7.5, 1 mM EDTA, 0.5 mM NAD^+, 0.5 mM betaine aldehyde and appropriate amount of enzyme. The reaction was started by the final addition of betaine aldehyde. The initial velocity of enzyme was expressed in µmol NADH formed per min per mg protein. The specific activity of the enzyme without added DTT represented the control. Various assay conditions were as follows :

- 5 mM DTT with 30 min incubation time
- 0.1 mM PCMS with 10 min incubation time
- 5 mM DTT with 30 min incubation time, followed by 0.1 mM PCMS with 30 min incubation time
- 0.1 mM PCMS with 10 min incubation time, followed by 5 mM DTT with 30 min incubation time

7.7 The determination of molecular weight of partially purified BADH by Sephadex G-200 column chromatography

Approximately 10g of hydrated Sephadex G-200 was swollen in 500ml of 10mM Tris-HCl buffer pH 7.5 containing 1mM DTT by boiling for 5hr. The Sephadex G-200 suspension was poured into a 1.8×120 cm

column at a height of 105 cm. The column was equlibrated with 10mM Tris-HCl buffer pH 7.5 containing 1mM DTT at least for two times of the column volume. The column was tested by loading 3 mg/ml blue dextran solution and 0.3 mg/ml potassium dichromate solution ($K_2Cr_2O_7$). These two markers were detected by measuring the OD at 620nm and at 410nm respectively. The standard protein mixture containing thyroglobulin (669,000 dalton), alcohol dehydrogenase (150,000 dalton), acid phosphatase (95,000 dalton), bovine serum albumin (66,200 dalton), ovalbumin (45,000 dalton), chymotrypsinogen A (23,240 dalton) and cytochrome C (12,380 dalton) was layered on the column. Elution was carried out by the same buffer and the flow rate was maintained at 12-15 ml/hr. Two ml fractions were collected and the absorbance of each fraction was monitored at 280nm. The distribution coefficient(K_{av}) was calculated by:

$$K_{av} = (V_e - V_o) / (V_t - V_o)$$

V_e (elution volume) = The volume required to elute protein or enzyme from the column.

V_o (void volume) = The volume required to elute blue dextran from the column.

V_t (total bed volume) = The volume of space occupied by the gel beads which could be determined by the volume required to elute potassium dichromate from the column.

Three ml of dialyzed BADH from DEAE-cellulose column (about 1 mg protein) was loaded on the column. The protein was eluted by the same buffer at the flow rate of 12-15 ml/hr. Two ml fractions were collected and the absorbance of each fraction was measured at 280 nm. The molecular weight of the protein was estimated from standard curve plotted on semilog scale between the molecular weight of protein markers and the K_{av}.

8 Effect of external salinity on BADH activity of *A. halophytica*

A. halophytica cells (10 days) from 400 ml of culture with 0.5M NaCl and 2.0M NaCl were disrupted in 50mM HEPES-KOH buffer pH 7.5 containing 2.0 mg/ml lysozyme. The cell suspension was incubated at 37°C for 1 hr and centrifuged at 14,000g for 30 min. The supernatant was subjected to 35-70% ammonium sulfate precipitation. The final pellet was suspended in a small volume of 50 mM HEPES-KOH buffer pH 7.5 before desalting through a Sephedex G-25 column. The blue extract obtained was used for enzyme assay. The BADH activity was expressed in μmol NADH formed per min per mg protein.

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