CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave: Model HA-30, Hirayama Manufacturing Cooperation, Japan

Automated Edman degradation amino acid sequencer with Applied Biosystem model 610 A data analysis system for protein sequencing, Applied Biosystem, U.S.A.

Camera: Model K1000, Pentax, Asaki Opt.Co., Japan

Centrifuge: Model J2-21, Beckman Instrument Inc., U.S.A.

Conductivity meter: Model CD, Radiometer, Denmark

Electrophoresis setting: Model Minutesi VE Vertical Electrophoresis system, Pharmacia LKB, Sweden

Filter paper No. 1, Whatman, Japan

Fraction collector: Model 2070 LKB Redifrac, Pharmacia LKB, Sweden

Freeze-dryer: Model Flexi-Dry^{tm} µp, Stone Ridge, New York, U.S.A.

High Performance Liquid Chromatography (HPLC): Model 1050 series, Hewlett-Packard, U.S.A.

Reversed phase HPLC YMC-Pack ODS-AM column (100 x4.6 mm ID)
Pharmacia LKB, Sweden

Orbital incubator: Model Gallenkamp, Genway, England

pH meter: Model PHM 95, Radiometer, Pharmacia LKB, Sweden

Power supply: Model POWER PAC 300, Bio-rad Applied Biosystem company, U.S.A.

Psycrotherm: Model G 760, News brunswick scientific Co., U.S.A.

Shaking waterbath: Model G 76, News brunswick scientific Co., U.S.A.

Sonicator: Model W-375, Heat system-ultrasonic, U.S.A.

Spectrophotometer: Model 20 D, Beckman, U.S.A.

Spectrophotometer: Model DU series 650, Beckman, U.S.A.

Ultrafilter :Suprec $^{Tm-01}$, pore size 0.20 µm and 0.22 µm, Takara Shuzo Co,Ltd., Japan

2.2 Chemicals

Acetronitrile (HPLC grade): Merck, Germany

Acrylamide: Merck, Germany

N-acetylimidazole: Sigma, U.S.A.

Blue Sepharose (Cibacronblue 3G-A): Pharmacia LKB, Sweden

Chloramine T: Sigma, U.S.A.

DEAE-Toyopearl, Tosoh Co., Japan

Dialysis tubing 25 mm: Cut off 12,000 Da, Sigma, U.S.A.

Diethylpyrocarbonate: Sigma, U.S.A.

DL-dithiothreitol: Sigma, U.S.A.

5,5'-dithio(2-nitrobenzonic acid): Sigma, U.S.A.

N-ethylmaleimide: Sigma, U.S.A.

Hydroxyapatite biogel HTP: Bio-rad Applied Biosystem company, U.S.A.

Iodoacetamide: Sigma, U.S.A.

Nicotinamide adenine dinucleotide (NAD^+): Sigma, U.S.A.

Nicotinamide adenine dinucleotide reduced form (NADH): Sigma, U.S.A.

Pyruvic acid: Sigma, U.S.A.

L-alanine: Sigma, U.S.A.

Phenylglyoxal: Sigma, U.S.A.

Phenyl methyl sulfonyl fluoride (PMSF): Sigma, U.S.A.

Trifluoroacetic acid: BDH, England

2,4,6- trinitrobenzenesulfonic acid: Sigma, U.S.A.

other common chemical reagent , Sigma, U.S.A., BDH, England, Fluka, Switzerland and Merck: Germany

2.3 Bacteria

Aeromonas hydrophila, isolated from Bangkok soil, was screened for L-alanine dehydrogenase by Phungsangthum (1997).

2.4 Bacteria growth medium

Peptone medium in 1 liter consisted of 1% peptone, 0.2% dipotassium hydrogen phosphate, 0.2% potassium hydrogen phosphate, 0.2% sodium chloride, 0.01% magnesium sulfate and 0.01% yeast extract was prepared and adjusted pH to 7.2 with sodium hydroxide. For solid medium 1.5% of agar was added. Medium was steriled by autoclaving at 121°C for 20 minutes.

2.5 Preparation of bacteria

2.5.1 Starter inoculum

A colony of *Aeromonas hydrophila*, from slant agar was growth in 60 ml of starter peptone medium pH 7.2, at 30°C with 250 rpm for 24 hours before inoculated into 600 ml of starter peptone medium, pH 7.2, at 30 °C with 250 rpm for 24 hours.

2.5.2 Cultivation of bacteria

The starter bacteria from 2.5.1 was inoculated to 6 liters of peptone medium, pH 4.5 and shaked at 250 rpm in psycrotherm at 30 °C. The bacteria was harvested after 24 hours by centrifugation at 8,500xg for 30 minutes at 4 °C. The collected cells were washed by 0.85% NaCl before rewashing with extraction buffer (0.1 mM potassium phosphate buffer (KPB), pH 7.4, 0.1 M phenyl methyl sulfonyl fluoride (PMSF), 1% M dithiothreitol (DTT), 1 mM ethylene diamine tetraacetic acid (EDTA). Harvested cells were storaged at –70 °C.

2.6 Preparation of crude enzyme solution

The collected cells 30 g from section 2.5.2 were resuspensed in 100 ml extraction buffer and then broken by discontinuously sonication for 10 minutes, stop 5 minutes with 3 cycle, output control is 7 and 50% duty cycle. Unbroken cells and cell debris were removed by centrifugation at 12,000xg for 45 minutes at 4 °C. The crude enzyme solution was collected for the determination of protein concentration and enzyme activity before purification.

2.6.1 Determination of enzyme activity

The standard reaction mixture for the deamination contained 20 µmol of L-alanine, 1 µmol of NAD^+, 200 µmol of glycine-potassium chloride-potassium hydroxide buffer, pH 10.5 and 5-10 µl of enzyme in a final volume 1.0 ml. The assay system for the amination reaction consisted of 10 µmol of pyruvate, 500 µmol of ammonium chloride, 0.2 µmol of NADH, 100 µmol of potassium phosphate buffer, pH 8.0 and 5-10 µl of enzyme in a final volume of 1.0 ml. The substrate was replaced by water in the blank. Incubation was done at 30°C in the cuvette with a 1-cm light path. The reaction was started by addition of NAD^+ or NADH and monitored by measuring the initial changes in the absorbance at 340 nm with a spectrophotometer: Model DU series 650, Beckman.

One unit of enzyme was defined as the amount that catalyzed the formation of 1 µmol of NADH per minute in the deamination of L-alanine. Specific activity was expressed as units per milligram of proteins.

2.6.2 Protein determination

The protein concentration was determined according to the method of Lowry *et al.*, (1951), using bovine serum albumin as standard.

The reaction mixture 5.6 ml contained 20-300 µg/µl of protein, 100 µl of solution A (0.5% copper sulfate, 1% potassium tartate, pH 7.0), 5 ml of solution B (2% sodium carbonate, 0.1 N sodium hydroxide) was mixed and incubated at 30°C for 10 minutes. After that, the solution mixture was incubated with 0.5 ml of solution C (phenol reagent) at room temperature for 20 minutes. The protein concentration was derived from the absorbance at 610 nm and calculated the concentration from the standard curve of protein standard (BSA).

2.7 Purification of L-alanine dehydrogenase

The crude enzyme from 2.6 was purified according to Figure 7(Phungsangthum, 1997). All procedures were done at 4°C and the buffer contained 10 mM potassium phosphate buffer (KPB), pH 7.4, 0.01% 2-mercaptoethanol and 1 mM EDTA was used.

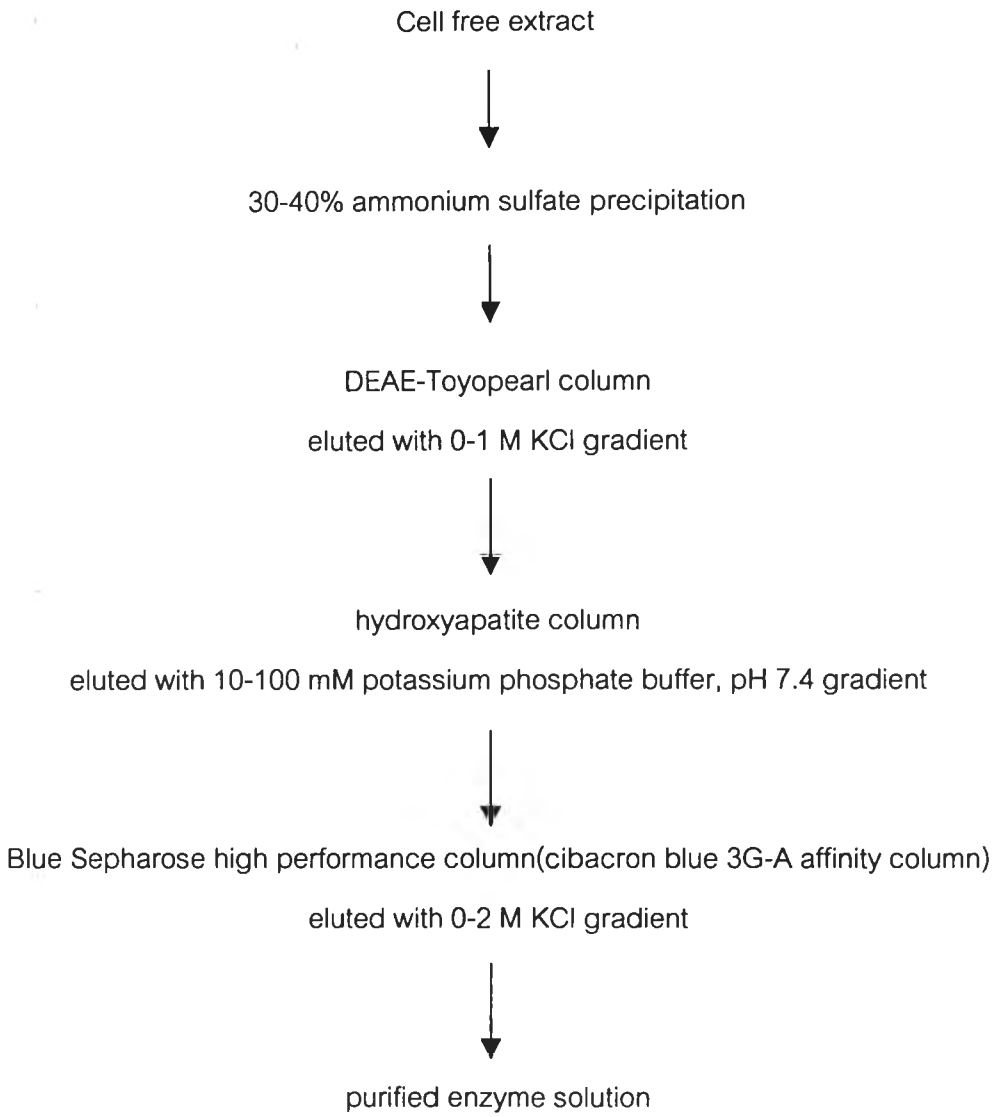

Figure 7 The purification of alanine dehydrogenase from *Aeromonas hydrophila*

2.7.1 Ammonium sulfate precipitation

The precipitation of crude enzyme was done by slowly added the initial concentration 0-30% of solid ammonium sulfate into enzyme solution with gentle stirred by magnetic stirrer. After 30 minutes, the supernatant was removed by centrifugation at 12,000xg for 30 minutes then brought to final concentration at 40% saturated with solid ammonium sulfate and left for 30 minutes. The precipitate was collected by centrifugation at 12,000xg for 30 minutes and dissolved in 10 mM KPB, pH 7.4. The protein solution was dialyzed against the same buffer, at least 4 hours with 3 changes of buffer, at 4°C, before determination of the activity and protein concentration as described in section 2.6.1 and 2.6.2, respectively.

2.7.2 Purification of enzyme by DEAE-Toyopearl column

2.7.2.1 Preparation of DEAE-Toyopearl column

DEAE-Toyopearl gel was activated by washing with 0.5 N NaOH for 2-3 times before rewashing by deionized water. The activated DEAE-Toyopearl gel was resuspended in 10 mM KPB, pH 7.4 and packed into 2x15 cm column followed by equilibration with the buffer for 5-10 column volume with flow rate 1 ml/min.

2.7.2.2 Application of sample into DEAE-Toyopearl column

The dialyzed enzyme from 2.7.1 was applied to the DEAE-Toyopearl column by pumping. The unbound proteins were eluted from the column with 10 mM KPB, pH 7.4. Normally, washing continued until the absorbance at 280 nm of eluate decreased to a low value. The bounded proteins were eluted from the column by linear salt gradient 0-1 M potassium chloride buffer. The fractions of 5 ml were continuously collected by fraction collector, measured at A_{280} or the protien profile. The enzyme activity was determined as described in section 2.6.1. The potassium chloride concentration was investigated by measuring the conductivity. The active fractions were pooled and concentrated with an aquacide and dialyzed 10 mM KPB, pH 7.4 for at least 4 hours, at 4°C with 3 changes of buffer. The protein concentration and enzyme activity were measured as described in section 2.6.1 and 2.6.2

2.7.3 Purification the enzyme by hydroxyapatite column

2.7.3.1 Preparation hydroxyapatite column

The hydroxyapatite gel was generated by dispersed in 10 mM KPB, pH 7.4 with ratio 1:6 (w/v). The solution was stirred gently two times in order to remove fines. Then, the activated gel was packed in 2x10 cm column and equilibrated with the buffer for 3-5 fold of column volume with flow rate 0.25 ml per min.

2.7.3.2 Application of sample into hydroxyapatite column

The enzyme solution from 2.7.2.2 was applied to the equilibrated column by pumping with flow rate 0.25 ml per min. The unbound proteins were eluted by 10 mM KPB, pH7.4 and continuously collected for measuring the protein concentration at absorbance at 280 nm, until eluate absorbance were reduced to low value. The bound proteins were eluted with linear gradient of 10-100 mM potassium phosphate buffer, pH 7.4, containing 0.01% 2-mercaptoethanol, 1 mM EDTA, for 100 ml approximately. Fractions of 2 ml were collected, determination of the enzyme activity, protein and conductivity of potassium phosphate were perform to that described in section 2.6.2. The active fraction were pooled and concentrated with aquacide before dialyzed against 10 mM KPB, pH 7.4 for 4 hours, at 4°C with 3 times changes buffer. The protein concentration and enzyme activity were determined as described in section 2.6.1 and 2.6.2.

2.7.4 Purification the enzyme by Blue Sepharose column

2.7.4.1 Preparation of Blue Sepharose column

Blue Sepharose column was used to separated the pyridine nucleotide-dependent enzyme. It contained with cibacron blue 3G-A which is specific for $NAD(P)^+$-dependent enzyme. The other proteins such as albumin are bound to the column with weak force, hydrophobic interaction and/or electrostatic force, than $NAD(P)^+$-dependent dehydrogenase so separation of $NAD(P)^+$-dependent dehydrogenase from other protein was occurred by eluted with the lower concentration of coenzyme or increasing the ionic strength of eluate.

In this experiment, the Blue Sepharose column (Pharmacia) with void volume 5 ml was used. The column was equilibrated by 10 mM KPB, pH 7.4 for 5 column volume (25 ml) at flow rate 1 ml per min.

2.7.4.2 Application of sample in Blue Sepharose column

The dialyzed enzyme from 2.7.3.2 was applied into Blue Sepharose column by pumping with flow rate 1 ml per min. The 10 mM KPB, pH 7.4 were used to eluted the unbound proteins, fractions of 1 ml were continuously collected and measuring the protein containing at absorbance 280 nm until the absorbance decreased to low value. The bound proteins were eluted with 0-2 M potassium chloride linear salt gradient elution. Fractions of 0.5 ml were collected. The determination of the enzyme activity, protein and conductivity of potassium chloride were similar to section 2.6.2. The active fractions were pooled before concentrating with an aquacide, then dialyzed against 10 mM KPB, pH 7.4 for 4 hours, at 4 °C with 3 time changes. The protein concentration and enzyme activity were determined as described in section 2.6.1 and 2.6.2.

2.7.5 Determination of alanine dehydrogenase activity by non-denaturing polyacylamide gel electrophoresis (PAGE)

2.7.5.1 Preparation of native polyacrylamide gel

The non-denaturing PAGE was performed according to Davis (1964). The solution mixture 9 ml of the resolving gel (7.7%) contained 2 ml of solution A (0.23% TEMED, 1.5 Tris-HCl, pH 8.9), 2 ml of solution C (30.8% acrylamide), 5 ml of distilled water and 11.2 mg of ammonium persulfate, stirred with gentle and released gas by vacuum before loaded into slap gel for 6.5 cm height. The total volume 1.75 ml of stacking gel was prepared by the polymerization of 0.5 ml of solution D (12.5% acrylamide), 1 ml of solution G (40% sucrose), 0.25 ml of solution E (0.004% riboflavin), stirred with gentle and applied to the top of the separating gel. The stacking gel was polymerized under the fluorescence light for 1 hour.

2.7.5.2 Sample preparation

The protein sample were mixed with dye marker with the ratio 5:1 (v/v)

2.7.5.3 Application of sample to Disc-PAGE electrophoresis

The polymerized gel was replaced to chamber which contained 1 liter of solution F (0.05 M Tris, 0.384 M glycine). The electrophoresis was run from cathode towards anode at constant voltage of 100 V per slap at room temperature. For determination of the enzyme activity, the electrophoresis was done at 4°C.

2.7.5.4 Protein staining

The proteins in slap gel from 2.7.5.3 were visualized by staining with the protein staining solution (0.04% Comassie Billaint Blue G-250 and 3.5% perchloric acid) for 15 minutes. The slap gels were destained with a destaining solution (7% acetic acid) followed by several changes of destaining solution until gel background was clear.

2.7.5.5 Enzyme activity staining

The gel was soaked in 10 ml of activity staining solution containing 4.25 mM of Tris-HCl, pH 8.5, 40 µM of L-alanine, 50 µM of NAD^+, 250 µg of phenazine methosulfate and 2.5 mg of nitroblue tetrazolium for 15 minutes, at room temperature. It quickly rinse several times with distilled water until gel background was clear.

2.8 Determination of group-specific reagents affect on alanine dehydrogenase activity

2.8.1 Effect of *N*-acetylimidazole (NAI) on tyrosine residue

The modification of enzyme by NAI was followed by method of Means and Feeney (1971). The reaction mixture 20 µl contained alanine dehydrogenase (5-10 µg/µl) and 10 mM of NAI in 0.1 M potassium phosphate buffer, pH 7.5. The reaction was incubated at 30°C for 20 minutes and determined the residual activity as described in section 2.6.1.

2.8.2 Effect of chloramine T (CT) on methionine residue

The method was carried out according to Miles and Smith (1993). The reaction mixture 20 µl contained 5-10 µg/µl of alanine dehydrogenase and 10 mM of CT in 0.1 M

Tris-HCl buffer, pH 8.3. The reaction was incubated at 30°C for 20 minutes and determined the residual activity as described in section 2.6.1.

2.8.3 Effect of diethylpyrocarbonate (DEPC) on histidine residue

The modification of DEPC was carried out according to Miles (1977). The reaction mixture 20 µl contained with alanine dehydrogenase (5-10 µg/µl) and 10 mM of DEPC in 0.1 M potassium phosphate buffer, pH 6.5. The reaction mixture was incubated at 30°C for 20 minutes and determined the residual activity as described in section 2.6.1.

2.8.4 Effect of dithiothreitol (DTT) on cysteine residue

The modification of DTT was followed the method of Means and Feeney (1971). The reaction mixture 20 µl contained with alanine dehydrogenase (5-10 µg/µl) and 10 mM of DTT in 0.1 M potassium phosphate buffer, pH 7.5. The reaction mixture was incubated at 30°C for 20 minutes and determined the residual activity as described in section 2.6.1.

2.8.5 Effect of phenylglyoxal (PG) on arginine residue

The modification of phenylglyoxal (PG) was followed the method of Robinson and Barnes (1991). The reaction mixture 20 µl contained alanine dehydrogenase (5-10 µg/µl) and 10 mM of PG in 0.1 M carbonate-bicarbonate buffer, pH 8.5. The reaction mixture was incubated at 30°C for 20 minutes and determined the residual activity as described in section 2.6.1.

2.8.6 Effect of 2,4,6-trinitrobenzene sulfonic acid (TNBS) on lysine residue

The modification of TNBS was performed accorfing to the method of Fields, (1972). The reaction mixture 20 µl contained alanine dehydrogenase (5-10 µg/µl) and 10 mM of TNBS in 0.1 M potassium phosphate buffer, pH 8.0. The reaction mixture was incubated at 30 °C for 20 minutes in the dark. The excess chemicals were then removed by ultraflitration with Suprec $^{Tm-01}$,0.20 µm, before determined the residual activity as described in section 2.6.1.

2.9 Determination of suitable concentration of group-specific reagents

Alanine dehydrogenase (5-10 µg/µl) was incubated with various concentrations of each group-specific reagent in 30 µl of reaction mixture at 30 °C for 20 minutes. After that, the enzyme was determined as described in section 2.6.1

2.10 Determination of suitable time for inactivation

In order to determine the suitable time, alanine dehydrogenase (5-10 µg) was incubated with suitable concentration of each group-specific reagent at 30 °C. At various times, the enzyme activity was determined as described in section 2.6.1.

2.11 Identification of amino acid residues involved in enzyme activation

The inactivation of each modifying agent was performed in the presence or absence of pyruvate and/or NADH. The alanine dehydrogenase (5-10 µg/µl) was preincubated with substrate at 30 °C for 5 minutes prior to the addition of suitable concentration of each modifying agent. The reaction was further incubated for each suitable time duration, after that the residual activity was determined as described in section 2.6.1

2.12 The kinetic study of enzyme modification

2.12.1 The kinetic study of histidine modification

The kinetic of modification of histidine residue by DEPC was examined by incubating the enzyme (5-10 µg/µl) with various concentrations of DEPC (0.01-0.08 mM) in the final volume 80 µl, at 30°C. The solution was determined for the residual activity at various interval times, 0-25 minutes, and compared with untreated enzyme.

2.12.2 The kinetic study of arginine modification

The kinetic study of modified arginine modification by PG was silmilar to section 2.12.1 except the concentration of PG ranged from 0.3- 6 mM were used.

2.13 Proteolysis of DEPC modification of alanine dehydrogenase

Proteolysis of modified alanine dehydrogenase was performed by preincubated alanine dehydrogenase (5.62 nmol) with 1.5 mM DEPC at 30°C for 30 minutes. After the

reaction, modified enzymes were separated from the chemical substances by passing through Hitrap desalting column and concentrated by lyophilization. The digestion of enzyme was achieved by incubating the lyophilyzed enzyme with 40 µl of 8 M urea at 37°C for 1 hour. The solution was then added with 120 µl of 0.2 M of potassium phosphate buffer, pH 7.2 and lysyl endopeptidase at ratio 1:50 (w/w) relative to the enzyme. The reaction mixture was carried out at 37°C, for 4 hours. The digested enzyme was then analyzed on a reverse phase HPLC. A control experiment with native alanine dehydrogenase was performed in parallel in exactly the same condition.

2.14 Separation and detection of peptide

The digested peptides from section 2.14.1 were separated by reverse phase HPLC on a YMC-Pack ODS-AM column (100 x 4.6 mm ID). The solvent system of 0.1% TFA in ultrapure water (solvent A) and acetonitrile contained with 0.07% TFA (solvent B) were used to elute the peptides. The peptide samples were dissolved with 20 µl of ultrapure water and removed fine particles by ultrafiltration with Suprec^{Tm-01}, 0.22 µm. The solution was injected to the column equilibrated with solvent A. The gradient used was 5-5% solvent B for 5 minutes, 5 to 50% solvent B for 95 minutes, followed by 50 to 80% solvent B for 10 minutes. Then, 80% solvent was used to wash the column for 10 minutes. The elution was carried out at a flow rate of 1.0 ml/minutes. The absorbance of the eluted peptide at 210 nm was continuously monitored. Eluted peptides were collected and lyophilized for further analysis.

2.15 N-terminal amino acid sequencing

The amino acid sequence of interesting peptides from section 2.14.2 were identified by automated Edman degradation with an Applied Biosystem model 610A data analysis system for protein sequencing.