CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave / HA-30: Hirayama Manufacturing Co., Japan

Autopipette / Pipetman: Gilson, France

Camera / K1000: Pentax, Japan

Conductivity meter / CDM83: Radiometer, Denmark

Electrophoresis unit / Hoefer mini VE: Pharmacia LKB, Sweden

Fraction collector / 2070 LKB Redifrac: Pharmacia LKB, Sweden

Gyrotary water bath shaker / G76D: News Brunswick Scientific Co., USA

High intensity ultrasonic processor: Sonics & Materials Inc., USA

Incubator / OB-28L: Fisher Scientific Inc., USA

Microcentrifuge high speed / MC-15A: Tomy Seiko Co. Ltd., Japan

Orbital incubator / Gallenkamp: Genway, England

Orbital incubator / Psycotherm: News Brunswick Scientific Co., USA

pH meter / PHM 95: Pharmacia LKB, Sweden

Power supply / PAC 300: Bio-Rad Applied Biosystem Co., USA

Refrigerated centrifuge / J2-21: Beckman Instrument Inc., USA

Sonic dismembrator / M 300: Fisher Scientific Inc., USA

Spectrophotometer / 20 D: Beckman Instrument Inc., USA

Spectrophotometer / DU series 650: Beckman Instrument Inc., USA

Water bath: Charles Hearson Co. Ltd., England

2.2 Chemicals

N-Acetylimidazole: Sigma, USA

3-Acetylpyridine adenine dinucleotide: Sigma, USA

Acrylamide: Merck, Germany

α-Amino-β-phenylbutanoic acid (sodium salt) : Sigma, USA

Ammonium persulfate: Merck, Germany

Ammonium sulfate: Merck, Germany

Apoferritin: Sigma, USA

Aquasorb: BML, Thailand

Bis-acrylamide: Merck, Germany

Bovine serum albumin (BSA): Sigma, USA

Bromophenol blue: BDH, England

N-Bromosuccinimide: Sigma, USA

Butyl-Toyopearl 650M TSK gel: Tosoh, Japan

Catalase: Sigma, USA

Chloramine T: Sigma, USA

Coomassie brilliant blue G-250: Sigma, USA

Coomassie brilliant blue R-250: Sigma, USA

DEAE-Toyopearl 650M TSK gel: Tosoh, Japan

Diethylpyrocarbonate: Sigma, USA

DL-Dithiothreitol (DTT): Sigma, USA

Ethylenediamine tetraacetic acid (EDTA): Fluka, Switzerland

m-Fluoro-DL-phenylalanine: Sigma, USA

o-Fluoro-DL-phenylalanine: Sigma, USA

p-Fluoro-DL-phenylalanine: Sigma, USA

Hydrocinnamic acid (sodium salt): Sigma, USA

p-Hydroxyphenylacetic acid (sodium salt): Sigma, USA

p-Hydroxyphenylpyruvic acid (sodium salt): Sigma, USA

Indole-β-pyruvic acid (sodium salt): Sigma, USA

α-Keto-n-butyric acid (sodium salt): Sigma, USA

α-Ketocaproic acid (sodium salt): Sigma, USA

α-Ketoglutaric acid (sodium salt): Sigma, USA

α-Ketoisocaproic acid (sodium salt): Sigma, USA

α-Ketoisovaleric acid (sodium salt): Sigma, USA

α-Keto-γ-methyl-butyric acid (sodium salt): Sigma, USA

α-Keto-β-methyl-n-valeric acid (sodium salt): Sigma, USA

α-Ketovaleric acid (sodium salt): Sigma, USA

β-Mercaptoethanol: Fluka, Switzerland

α-Methyl-DL-phenylalanine: Sigma, USA

N- Methyl-L-phenylalanine: Sigma, USA

Myoglobin: Sigma, USA

β-Nicotinamide adenine dinucleotide (NAD): Sigma, USA

β-Nicotinamide adenine dinucleotide reduced form (NADH): Sigma, USA

β-Nicotinamide adenine dinucleotide phosphate (NADP): Sigma, USA

Nicotinamide 1, N^6-ethenoadenine dinucleotide: Sigma, USA

Nicotinamide guanine dinucleotide: Sigma, USA

Nicotinamide hypoxanthine dinucleotide: Sigma, USA

Nicotinic acid adenine dinucleotide: Sigma, USA

Ovalbumin: Sigma, USA

L-Phenylalanine: Nacalai, Japan

Phenylglyoxal: Sigma, USA

DL-β-Phenyllactic acid (sodium salt): Sigma, USA

Phenylmethylsulfonyl fluoride (PMSF): Sigma, USA

Protein molecular weight standards (MW 14,300-200,000): GibcoBRL, USA

Phenylpyruvic acid (sodium salt): Sigma, USA

3-Pyridinealdehyde adenine dinucleotide: Sigma, USA

Pyruvic acid (sodium salt): Sigma, USA

Sephadex G-200: Pharmacia, USA

Sodium laulyl sulfate (SDS): BDH, England

N,N,N',N'-Tetramethylene-ethylenediamine (TEMED): BDH, England

Thionicotinamide adenine dinucleotide: Sigma, USA

Thyroglobin: Sigma, USA

2,4,6-Trinitrobenzenesulfonic acid: Sigma, USA

The other L-amino acids were from Sigma, USA and D-amino acids were from Nacalai tesque and Wako, Japan. The other common chemicals were reagent grade from Aldrich; USA, BDH; England, Fluka; Switzerland, Merck; Germany, Serva; USA and Sigma; USA.

2.3 Bacteria

Bacteria strain BC1 and BC2, isolated from soil, were screened for NAD^+-dependent phenylalanine dehydrogenase activity by Suriyapanpong *et al.* [73] and later, strain BC1 was identified as *Bacillus badius* by Chareonpanich *et al.* [74] .

2.4 Bacteria growth medium

Peptone medium consisted of 1% peptone (meat source) , 0.2% dipotassium hydrogen phosphate, 0.2% potassium dihydrogen 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 Enzyme assay

The enzyme activity could be determined in two manners ; the oxidative deamination of L-phenylalanine (forward reaction) and the reductive amination of phenylpyruvate (reverse reaction) as shown in reactions below.

The standard reaction mixture for oxidative deamination contained 20 μmol of L-phenylalanine, 1 μmol of NAD^+, 200 μmol of glycine-potassium chloride-potassium hydroxide buffer, pH 11.5 and the enzyme in a final volume of 1.0 ml. The assay system for reductive amination consisted of 10 μmol of phenylpyruvate, 500 μmol of ammonium chloride, 0.2 μmol of NADH, 100 μmol of glycine-potassium chloride-potassium hydroxide buffer, pH 10 and the enzyme in a final volume of 1.0 ml. The substrate was replaced by water in a blank. The incubation was carried out at 30°C in a cuvette with a 1-cm light path. The reaction was started by addition of NAD^+ or NADH and monitored by measuring the initial change in the absorbance at 340 nm with a spectrophotometer: model DU series 650, Beckman.

One unit of the enzyme was defined as the amount of enzyme which catalyzed the formation of 1 μmol of NADH per minute in the oxidative deamination of L-phenylalanine. Specific activity was expressed as units per milligram of protein.

2.6 Protein determination

The protein concentration was determined according to the modified method of Lowry *et al.* [75], using bovine serum albumin (BSA) as the standard. The reaction mixture 6.1 ml containing 20-100 μg/μl of protein, 100 μl of solution A, 5 ml of solution B was mixed and incubated at 30°C for 10 minutes. After that, the solution mixture was incubated with 0.5 ml of solution C at room temperature for 20 minutes. Preparation of the solutions was described in Appendix A. 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 Optimization for phenylalanine dehydrogenase production of bacterial strain BC1 and BC2

2.7.1 Enzyme induction by amino acids

2.7.1.1) Starter inoculum

A colony of bacterial strain BC1 and BC2 from agar slant was grown in 2.5 ml of peptone medium, pH 7.2 (starter I), at 30°C with 250 rpm shaking for 20 hours before inoculated into 25 ml of peptone medium, pH 7.2 (starter II) , at 30°C with 250 rpm rotary shaking for 20 hours.

2.7.1.2) Enzyme production

The 0.2 % of various amino acids: L-phenylalanine, L-tryptophan and L-tyrosine, were supplemented to the peptone medium, pH 7.2. The peptone medium without amino acid was the control. The 25 ml of starter inoculum from 2.7.1.1 was transferred into 250 ml of those peptone medium in 500 ml erlenmeyer flask and cultivated at 37°C with 250 rpm rotary shaking for 24 hours in orbital incubator (psycotherm). The bacteria were harvested by centrifugation at 8,500 x g for 30 minutes at 4°C. The collected cells were washed by 0.85% sodium chloride before rewashing with extraction buffer (0.1 M potassium phosphate buffer, pH 7.4 containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.01% dithiothreitol (DTT) and 1 mM ethylenediamine tetraacetic acid (EDTA)). Harvested cells were stored at -70°C.

2.7.1.3) Preparation of crude enzyme solution

The collected cells from section 2.7.1.2 were resus-

pended in 20 ml of cold extraction buffer and then broken by discontinuously sonication on ice for 4 minutes, stop 4 minutes with 5 cycles by sonic dismembrator model M300. Unbroken cells and cell debris were removed by centrifugation at 10,000 x g for 30 minutes at 4°C. The crude enzyme solution was collected for the determination of oxidative deamination activity and protein concentration assays as described in section 2.5 and 2.6, respectively in order to determine the effect of amino acids as enzyme inducers.

2.7.2 Optimal concentration of inducer

Preparation of the starter inoculum and cultivation of bacteria was carried out in the same way as described in section 2.7.1.1 except the most suitable amino acid, which gave the highest enzyme activity based on the results from section 2.7.1, was added to peptone medium, pH 7.2 at varied concentration of 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 %. The cell harvest, crude enzyme preparation, assay for oxidative deamination activity and protein determination were performed as describe in section 2.7.1.2, 2.7.1.3, 2.5 and 2.6, respectively.

2.7.3 Optimal pH of medium

Preparation of the starter inoculum and cultivation of bacteria was carried out in the same way as described in section 2.7.1.1 in peptone medium, which contained the most suitable type and concentration of the inducer based on the results from section 2.7.2 except pHs were varied in the range of 3.5 to 8.5 (3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.2, 7.5, 8.0 and 8.5). The cell harvest, crude enzyme preparation, assay for oxidative deamination activity and protein determination were performed as describe in section 2.7.1.2, 2.7.1.3, 2.5 and 2.6, respectively.

2.7.4 Optimal cultivation temperature

Preparation of the starter inoculum was carried out in the same way as described in section 2.7.1.1 except that bacteria was cultivated in peptone medium, which contained the most suitable type and concentration of the inducer as well as pH of medium, based on the results from section 2.7.3, at various ranges of

temperatures (30, 35, 37, 40, 42 and 45°C). The cell harvest, crude enzyme preparation, assay for oxidative deamination activity and protein determination were performed as describe in section 2.7.1.2, 2.7.1.3, 2.5 and 2.6, respectively.

2.7.5 Optimal cultivation time

Preparation of the starter inoculum was carried out in the same way as described in section 2.7.1.1 except that bacteria was cultivated in peptone medium, which contained the most suitable type and concentration of the inducer and pH of medium at suitable cultivation temperature based on the results from section 2.7.4, for the various incubation times (0, 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 hours). The cell culture was taken and measured for optical density at 610 nm every 3 hours. The cell harvest, crude enzyme preparation, assay for oxidative deamination activity and protein determination were performed as describe in section 2.7.1.2, 2.7.1.3, 2.5 and 2.6, respectively.

Based on the results, the best of two strains (BC1 and BC2) was chosen for further step in purification, characterization and kinetic mechanism studies.

2.8 Purification of phenylalanine dehydrogenase from *Bacillus badius* BC1

2.8.1 Bacterium cultivation

2.8.1.1) Starter inoculum

One colony of bacterial strain BC1, identified as *Bacillus badius* BC1, from agar slant was grown in 60 ml of starter peptone medium, pH 6.5 , at 37°C with 250 rpm shaking for 24 hours before inoculated into 600 ml of starter peptone medium, pH 6.5 and grown at 37°C with 250 rpm rotary shaking for 24 hours.

2.8.1.2) Enzyme production

The starter inoculum from 2.8.1.1 was transferred into 6 liters of peptone medium, pH 6.5 containing 0.8 % L-phenylalanine and cultivated at 37°C with 250 rpm orbital shaking. The bacteria was harvested after 24 hours by centrifugation at 8,500 x g for 30 minutes at 4°C. The collected cells were washed with 0.85% sodium chloride before rewashing with extraction buffer (0.1 M potassium phosphate buffer, pH 7.4 containing 0.1 mM PMSF, 0.01 % DTT and 1 mM EDTA). Harvested cells were stored at -70 °C before the purification.

2.8.2 Preparation of crude enzyme solution

The collected cells 45 grams from section 2.8.1.2 were resuspended in 100 ml of cold extraction buffer and then broken by discontinuously sonication on ice for 10 minutes, stop 10 minutes with 8 cycles by high intensity ultrasonic processor (3 mm diameter-stepped microtip, 40 % amplitude). Unbroken cells and cell debris were removed by centrifugation at 10,000 x g for 30 minutes at 4°C. The crude enzyme solution was collected for the determination of oxidative deamination activity and protein concentration as described in section 2.5 and 2.6, respectively.

2.8.3 Purification procedures of the enzyme

The crude enzyme from section 2.8.2 was purified according to Figure 2.1. All operations were done at 4°C. The buffer used in all steps was 10 mM potassium phosphate buffer, pH 7.4 containing 0.01% 2-mercaptoethanol, 1 mM EDTA and 10 % glycerol. A typical purification procedure for phenylalanine dehydrogenase was as follows.

2.8.3.1) Ammonium sulfate precipitation

The precipitation of crude enzyme was done by slowly added solid ammonium sulfate to 40 % saturation with gentle stirring by magnetic

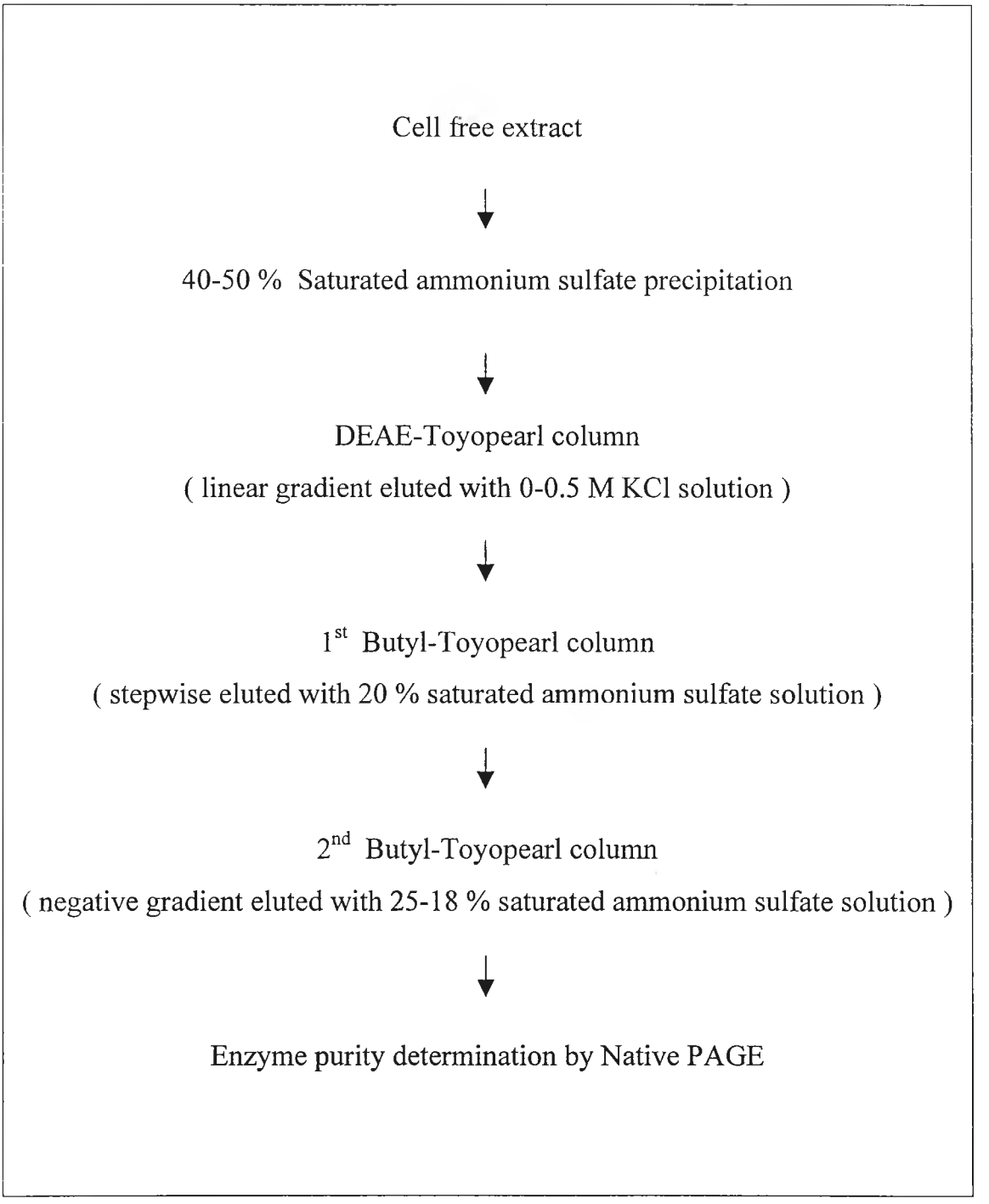

Figure 2.1 **Flow chart of purification process of phenylalanine dehydrogenase from *B. badius* BC1**

stirrer. After 30 minutes, the supernatant was collected by centrifugation at 10,000 x g for 30 minutes and then brought to final concentration at 50% saturation with solid ammonium sulfate. The solution was left for 30 minutes on ice with continuous stirring and was subsequently centrifuged at 10,000 x g for 30 minutes. The precipitate was dissolved in the buffer. The protein solution was dialyzed against the same buffer at least 4 hours for 3 times before determination of oxidative deamination activity and protein concentration as described in section 2.5 and 2.6, respectively.

2.8.3.2) DEAE-Toyopearl column chromatography

DEAE-Toyopearl was activated by washing with 0.5 N sodium hydroxide for 2-3 times before rewashing by deionized water until the pH reached 8.0. The activated DEAE-Toyopearl was resuspended in the buffer and packed into 2.5 x 22 cm column followed by equilibrating with the same buffer for 5-10 column volume at flow rate 1 ml/min.

The dialyzed protein solution from section 2.8.3.1 was applied to the DEAE-Toyopearl column. The unbound proteins were eluted from the column with the buffer. Normally, keep washing until the absorbance at 280 nm of eluant decreased to a low value. After the column was washed thoroughly with the buffer, the bounded proteins were eluted from the column with linear salt gradient of 0 to 0.5 M potassium chloride in the same buffer. The fractions of 5 ml were continuously collected using fraction collector. The elution profile was monitored for protein by measuring the absorbance at 280 nm. The enzyme activity was determined as described in section 2.5. The potassium chloride concentration was investigated by measuring the conductivity. The active fractions were pooled, concentrated using aquasorb to reduce volume for further purification steps and then dialyzed against the buffer at least 4 hours for 3 times. The enzyme activity and protein concentration were measured as described in section 2.5 and 2.6, respectively.

2.8.3.3) First Butyl-Toyopearl column chromatography

Butyl-Toyopearl was washed with deionized water for

2-3 times and then resuspended in the buffer containing 25 % saturated ammonium sulfate and packed into 2.2 x 20 cm column followed by equilibrating with the same buffer for 5-10 column volume at flow rate 1 ml/min.

The pooled active fractions from section 2.8.3.2 was slowly adjusted to 25% saturation with ammonium sulfate and stirred gently for at least 30 minutes. The protein solution were then applied to the equilibrated column at flow rate 1 ml/min. The proteins were eluted with a decreasing salt stepwise (25%, 20%, 15%, 10%, 5% and 0% saturated ammonium sulfate in the buffer, respectively). The fractions of 3 ml were collected using fraction collector. The protein elution profile was monitored by measuring the absorbance at 280 nm. The enzyme activity was determined as described in section 2.5. The active fractions were pooled and concentrated using aquasorb and dialyzed against the buffer at least 4 hours for 3 times. The enzyme activity and protein concentration were measured as described in section 2.5 and 2.6, respectively.

2.8.3.4) Second Butyl-Toyopearl column chromatography

Butyl-Toyopearl was washed with deionized water for 2-3 times and then resuspended in the buffer containing 25% saturated ammonium sulfate and packed into 2.2 x 9.5 cm column followed by equilibrating with the same buffer for 5-10 column volume at flow rate 1 ml/min.

The pooled active fraction from section 2.8.3.3 was adjusted to 25% saturation with ammonium sulfate and stirred gently for at least 30 minutes. The protein solution were then applied to the equilibrated column at flow rate 1 ml/min. The unbound proteins were eluted from the column with the buffer containing 25% saturated ammonium sulfate. Normally, keep washing until the absorbance at 280 nm of eluant decreased to a low value. After the column was washed thoroughly with the buffer, the bounded proteins were eluted from the column with a negative salt gradient, from 25% to 18% saturated ammonium sulfate in the buffer. The fractions of 2 ml were collected using fraction collector. The protein elution profile was monitored for protein by measuring the absorbance at 280 nm. The enzyme activity was determined as described in section 2.5. The ammonium

sulfate concentration (percentage) was investigated by measuring the conductivity. The active fractions were pooled and concentrated using aquasorb and dialyzed against the buffer for at least 4 hours for 3 times. The enzyme activity and protein concentration were measured as described in section 2.5 and 2.6, respectively.

2.8.3.5) Determination of enzyme purity by non-denaturing polyacrylamide gel electrophoresis (Native PAGE)

The enzyme from each step of purification was analyzed by native PAGE to determine the native protein pattern and its purity according to the method of Bollag *et al.* [76] Electrophoresis conditions, protein and activity staining were described below.

2.8.3.5.1) Non-denaturing gel electrophoresis

Discontinuous PAGE was perform on the slab gel (10 x 10 x 0.75 cm) of a 7.7% (W/V) seperating gel and a 5% (W/V) stacking gel. Tris-glycine buffer, pH 8.3 was used as electrode buffer. Preparation of solutions and polyacrylamide gels was described in Appendix B. The enzyme from each step was mixed with 5x sample buffer by ratio 5:1 and loaded onto the gel. The electrophoresis was run from cathode towards anode at constant voltage of 100 volts per slab at room temperature. For determination of the enzyme activity, the electrophoresis was done at 4°C.

2.8.3.5.2) Detection of protein bands

After electrophoresis, proteins in the gel were visualized by coomassie blue staining and activity staining.

A. Coomassie blue staining

The gel was stained with staining solution (0.1% (W/V) coomassie brilliant blue R-250 in 10% (V/V) acetic acid and 45% (V/V) methanol) for at least 20 minutes. Destaining was performed by immersing the gel

in destaining solution (10% (V/V) acetic acid and 10% (V/V) methanol) followed by several changes of destaining solution until gel background was clear.

B. Enzyme activity staining

The gel was soaked in 10 ml of activity staining solution containing 4.25 mmol of Tris-HCl buffer, pH 8.5, 40 μmol of L-phenylalanine, 50 μmol of NAD^+, 250 μg of phenazine methosulfate and 2.5 mg of nitroblue tetrazolium for 15-30 minutes, at room temperature and then quickly rinse several times with deionized water until gel background was clear.

2.9 Characterization of phenylalanine dehydrogenase from *Bacillus badius* BC1

2.9.1 Molecular weight determination of phenylalanine dehydrogenase

2.9.1.1) Sephadex G-200 column chromatography

Sephadex G-200 column (2.0 x 75 cm) was equilibrated with the buffer containing 0.2 M potassium chloride at 4°C for 5 column volumes at flow rate of 15 ml/hr to allow stabilization of bed volume of the column. An aliquot of the concentrated enzyme solution from second Butyl-Toyopearl column was applied to the column and the enzyme was then eluted with the buffer containing 0.2 M potassium chloride. Fractions of 2 ml were collected using fraction collector. The elution profile was monitored for protein and enzyme activity as previously described.

The elution volume (V_e) of enzyme was compared with standard molecular weight markers proteins; thyroglobin (669 kDa), apoferritin (443 kDa), catalase (232 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa) and myoglobin (17.2 kDa). Blue dextran 2000 and potassium dichromate were used to determine the position of the void volume (V_o) and the total bed volume (V_t),

respectively. The partition coefficient (K_{av}) values for each standard marker protein calculated from $(V_e-V_o) / (V_t-V_o)$ were plotted against log molecular weight of each protein on semi-logarithmic graph paper to obtain a calibration curve. The K_{av} of the enzyme was calculated in the same way and used to determine its native molecular weight from the calibration curve.

2.9.1.2) SDS-polyacrylamide gel electrophoresis

The SDS-PAGE system was performed according to the method of Bollag *et al.* [76]. The slab gel (10 x 10 x 0.75 cm) system consisted of 0.1 % (W/V) SDS in 12.5 % (W/V) seperating gel and 5 % (W/V) stacking gel. Tris-glycine buffer, pH 8.3 containing 0.1% (W/V) SDS was used as electrode buffer. The gel preparation was described in Appendix C. The concentrated enzyme from second Butyl-Toyopearl column was treated with sample buffer and boiled for 10 minutes before loading to the gel. The electrophoresis was performed at constant voltage of 100 volts per slab at room temperature from cathode towards anode. The standard molecular weight markers used were phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa). After electrophoresis, proteins in the gel were visualized by coomassie blue staining as described in section 2.8.3.5.2.A

2.9.2 Substrate specificity of phenylalanine dehydrogenase

The ability of the enzyme to catalyze the oxidative deamination of various amino acids and L-phenylalanine analogs was determined at a final substrate concentration of 20 mM except for L-tyrosine (1.2 mM) and L-cysteine (5 mM). L-phenylalanine was replaced by various amino acids L-phenylalanine analogs as substrates for the reaction and enzyme activities were determined as described in section 2.5. The result was expressed as a percentage of the relative activity. In the same way, the ability of the enzyme to catalyze the reductive amination of various keto acids and phenylpyruvate analogs was determined at a final substrate concentration of 10 mM except for *p*-hydroxyphenylpyruvate (2.5 mM) and indole-β-

pyruvate (1 mM). Phenylpyruvate was replaced by various keto acids and phenylpyruvate analogs as substrates for the reaction and enzyme activities were determined as described in section 2.5. The result was expressed as a percentage of the relative activity.

2.9 3 Coenzyme specificity of phenylalanine dehydrogenase

NAD^+ was replaced by various coenzymes for the oxidative deamination at a final concentration of 2 mM. Assays with NAD^+ analogs were conducted by measuring the increase in absorbance at the following wavelenghts: 3-acetylpyridine adenine dinucleotide, 363 nm (molar absorption coefficient (ε = 9.1x 10^3); β-nicotinamide adenine dinucleotide phosphate (NADP), 340 nm ($\varepsilon = 6.2 \times 10^3$); Nicotinamide 1, N^6-ethenoadenine dinucleotide, 334 nm ($\varepsilon = 6.9 \times 10^3$); Nicotinamide guanine dinucleotide, 340 nm ($\varepsilon = 6.2 \times 10^3$); Nicotinamide hypoxanthine dinucleotide, 338 nm ($\varepsilon = 6.2 \times 10^3$); Nicotinic acid adenine dinucleotide, 338 nm ($\varepsilon = 6.2 \times 10^3$); 3-pyridinealdehyde adenine dinucleotide, 358 nm ($\varepsilon = 9.3 \times 10^3$) and thionicotinamide adenine dinucleotide, 395 nm ($\varepsilon = 11.3 \times 10^3$) [12, 77-78]. The reaction was carried out at pH 9.5 to avoid degradation of NAD^+ analogs at a more alkaline pH. The result was expressed as a percentage of the relative activity.

2.9.4 Effect of pH on phenylalanine dehydrogenase activity

The purified PheDH was used to study the effect of pHs on its activity. The enzyme was assayed as described in section 2.5 at various pHs. The 200 mM of Tris-HCl and glycine-KCl-KOH were used as reaction buffers for pH 7.5-9.0 and 8.5-12.5 , respectively, in both of the oxidative deamination and reductive amination. The pH of each reaction mixture was measured with a pH meter at room temperature after the reaction. The result was expressed as a percentage of the relative activity. The pH at which maximum activity was observed for each reaction was set as 100 %.

2.9.5 Effect of temperature on phenylalanine dehydrogenase activity

The purified PheDH was used to investigate the effect of temperature on its activity. The enzyme was assayed by the method as described in section 2.5 at various temperatures from 25 to 60°C (25, 30, 35, 40, 45, 50 ,55 and 60°C), in both of the oxidative deamination and reductive amination. The result was expressed as a percentage of the relative activity. The temperature at which maximum activity was observed for each reaction was set as 100 %.

2.9.6 Effect of pH on phenylalanine dehydrogenase stability

The purified PheDH was used to study pH stability. After the enzyme in 10 mM buffer at various pHs was incubated at 30°C for 20 minutes, an aliquot of the enzyme solution was withdrawn and the remaining activity was assayed for the oxidative deamination of L-phenylalanine at 30°C as described in section 2.5. The buffers (10 mM) used were citrate buffer (pH 3.0 to 7.0), potassium phosphate buffer (pH 6.0 to 8.0), Tris-HCl buffer (pH 7.5 to 9.0) and glycine-KCl-KOH buffer (pH 9.5 to 12.0). The result was expressed as a percentage of the relative activity. The highest activity was defined as 100 %.

2.9.7 Effect of temperature on phenylalanine dehydrogenase stability

The thermostability of the enzyme was investigated over the range of 30 to 70°C (30, 35, 40, 45, 50, 55, 60, 65 and 70°C). The purified enzyme was preincubated at various tempertures for 10 minutes before determining enzyme activity in oxidative deamination as described in section 2.5. Afterward, the enzyme was incubated at the highest temperature, which the enzyme activity still retained full activity, from 0 to 60 hours and collected to assay as described previously for every 3 hours. The result was expressed as a percentage of the relative activity. The highest activity was defined as 100 %.

2.9.8 Effect of metal ions and chemical substances on phenylalanine dehydrogenase activity

The purified PheDH was used to study the effects of metal ions and chemical substances on enzyme activity. The enzyme was assayed in the oxidative deamination as described in section 2.5 in the presence of various metal ions and chemical substances at the final concentration of 10 mM, unless otherwise stated. The result was expressed as a percentage of the relative activity of the enzyme activity in the absence of reagents.

2.9.9 Inhibitory effect of various amino acids and keto acids on phenylalanine dehydrogenase activity

Inhibitory effect of various amino acids on the oxidative deamination of L-phenylalanine were studied by determining the enzyme activity as described in section 2.5 in the presence of various amino acids, which the enzyme did not have the ability to catalyze as its substrates, at the final concentration of 20 mM, unless otherwise stated. The result was expressed as a percentage of the relative activity of the enzyme activity in the absence of reagents.

Inhibitory effect of various keto acids on the reductive amination of phenylpyruvate were studied by determining the enzyme activity as described in section 2.5 in the presence of various keto acids, which the enzyme did not have the ability to catalyze as its substrates, at the final concentration of 10 mM, unless otherwise stated. The result was expressed as a percentage of the relative activity of the enzyme activity in the absence of reagents.

2.9.10 Effect of group-specific reagents on phenylalanine dehydrogenase activity

2.9.10.1) Effect of *N*-acetylimidazole (NAI) on tyrosine residues

Modification of tyrosine residues was carried out according to the method of Means and Feeney [79]. The enzyme (5-10 μg/μl) was incubated with NAI in 50 mM potassium phosphate buffer, pH 7.5 at 30°C for 20 minutes. NAI

were tested in the final concentration of 10 mM. The total volume of the reaction mixture was 20 µl. After the incubation, the residual activity of enzyme was determined as described in section 2.5.

2.9.10.2) Effect of *N*-bromosuccinimide (NBS) on tryptophan residues

Modification of tryptophan residues was carried out according to the method of Means and Feeney [79]. The enzyme (5-10 µg/µl) was incubated with NBS in 50 mM potassium phosphate buffer, pH 7.0 at 30°C for 20 minutes. NBS were tested in the final concentration of 10 mM. The total volume of the reaction mixture was 20 µl. After the incubation, the residual activity of enzyme was determined as described in section 2.5.

2.9.10.3) Effect of chloramine T (CT) on methionine residues

Modification of methionine residues was carried out according to the method of Miles and Smith [80]. The enzyme (5-10 µg/µl) was incubated with CT in 50 mM Tris-HCl buffer, pH 8.3 at 30°C for 20 minutes. CT were tested in the final concentration of 10 mM. The total volume of the reaction mixture was 20 µl. After the incubation, the residual activity of enzyme was determined as described in section 2.5 .

2.9.10.4) Effect of diethylpyrocarbonate (DEPC) on histidine residues

Modification of histidine residues was carried out according to the method of Wakayama *et al.* [81]. The enzyme (5-10 µg/µl) was incubated with DEPC in 50 mM potassium phosphate buffer, pH 7.0 at 30°C for 20 minutes. DEPC were tested in the final concentration of 10 mM. The total volume of the reaction mixture was 20 µl. After the incubation, the residual activity of enzyme was determined as described in section 2.5.

2.9.10.5) Effect of dithiothreitol (DTT) on cysteine residues

Modification of cysteine residues was carried out according to the method of Glazer *et al.* [82]. The enzyme (5-10 µg/µl) was incubated with DTT in 50 mM potassium phosphate buffer, pH 7.5 at 30°C for 20 minutes. DTT were tested in the final concentration of 10 mM. The total volume of the reaction mixture was 20 µl. After the incubation, the residual activity of enzyme was determined as described in section 2.5.

2.9.10.6) Effect of phenylglyoxal (PG) on arginine residues

Modification of arginine residues was carried out according to the method of Dong *et al.* [83]. The enzyme (5-10 µg/µl) was incubated with PG in 50 mM potassium phosphate buffer, pH 8.0 at 30°C for 20 minutes. PG were tested in the final concentration of 10 mM. The total volume of the reaction mixture was 20 µl. After the incubation, the residual activity of enzyme was determined as described in section 2.5.

2.9.10.7) Effect of phenylmethylsulfonyl fluoride (PMSF) on serine residues

Modification of serine residues was carried out according to the method of Wakayama *et al.* [81]. The enzyme (5-10 µg/µl) was incubated with PMSF in 50 mM potassium phosphate buffer, pH 7.0 at 30°C for 20 minutes. PMSF were tested in the final concentration of 10 mM. The total volume of the reaction mixture was 20 µl. After the incubation, the residual activity of enzyme was determined as described in section 2.5.

2.9.10.8) Effect of 2,4,6-trinitrobenzene sulfonic acid (TNBS) on lysine residues

Modification of lysine residues was carried out according to

the method of Fields [84]. The enzyme (5-10 μg/μl) was incubated with TNBS in 50 mM potassium phosphate buffer, pH 8.0 at 30°C for 20 minutes. TNBS were tested in the final concentration of 10 mM. The total volume of the reaction mixture was 20 μl. After the incubation, the residual activity of enzyme was determined as described in section 2.5.

2.10 Kinetic mechanism studies of phenylalanine dehydrogenase from *Bacillus badius* BC1

A series of steady-state kinetic analyzes were carried out in order to investigate the kinetic parameters and reaction mechanism as described below.

2.10.1 Initial velocity studies for the oxidative deamination

Initial velocity studies for the oxidative deamination reactions were carried out under the standard reaction condition (as described in section 2.5) except that various amounts of L-phenylalanine and NAD^+ were used. The concentrations of L-phenylalanine used were 0.1, 0.2, 0.5 and 1.0 mM and those of NAD^+ used were 0.067, 0.1, 0.2, 0.4 and 1.0 mM. The Lineweaver-Burke plots (double-reciprocal plots) of initial velocities against L-phenylalanine concentrations at a series of fixed concentrations of NAD^+ and the secondary plots of y intercepts against reciprocal concentrations of NAD^+ were made from the data. K_m of L-phenylalanine and NAD^+ were calculated and determined from these two plots, respectively. Velocity (v) was expressed as unit of enzyme which was defined as μmol of NADH formed / min.

2.10.2 Initial velocity studies for the reductive amination

Initial velocity studies for the reductive amination reactions were carried out under the standard reaction condition (as described in section 2.5) except that various amounts of phenylpyruvate, ammonium chloride and NADH were used in the experiments as described below.

2.10.2.1) The enzyme was assayed in the reductive amination by using phenylpyruvate as a variable substrate (0.04, 0.05, 0.067, 0.1, 0.2, and 1 mM) at several fixed concentrations of ammonium chloride (10, 20, 40 and 80 mM) in the presence of a saturating and constant concentration of NADH (0.2 mM). The double-reciprocal plots of initial velocities against phenylpyruvate concentrations at a series of fixed concentrations of ammonium chloride and the secondary plots of y intercepts against reciprocal concentrations of ammonium chloride were made for K_m determination. Velocity (v) was expressed as unit of enzyme which was defined as μmol of NADH consumed / min.

2.10.2.2) The enzyme was assayed in the reductive amination by using ammonium chloride as a variable substrate (10, 20, 40 and 80 mM) at several fixed concentrations of NADH (0.0125, 0.025, 0.050 and 0.1 mM) in the presence of a saturating and constant concentration of phenylpyruvate (10 mM). The double-reciprocal plots of initial velocities against ammonium chloride concentrations at a series of fixed concentrations of NADH and the secondary plots of y intercepts against reciprocal concentrations of NADH were made for K_m determination. Velocity (v) was expressed as unit of enzyme which was defined as μmol of NADH consumed / min.

2.10.2.3) The enzyme was assayed in the reductive amination by using NADH as a variable substrate (0.0125, 0.025, 0.050 and 0.1 mM) at several fixed concentrations of phenylpyruvate (0.0125, 0.025, 0.050 and 0.1 mM) in the presence of a saturating and constant concentration of ammonium chloride (500 mM). The double-reciprocal plots of initial velocities against NADH concentrations at a series of fixed concentrations of phenylpyruvate and the secondary plots of y intercepts against reciprocal concentrations of phenylpyruvate were made for K_m determination. Velocity (v) was expressed as unit of enzyme which was defined as μmol of NADH consumed / min.

2.10.3 Product inhibition studies

Product inhibition studies were performed in the direction of the oxidative deamination reaction in different conditions as described below.

2.10.3.1) Product inhibition by NADH with respect to NAD^+

Inhibition by NADH was analyzed with varied concentrations of NAD^+ in the presence of a saturating and constant concentration of L-phenylalanine (20 mM). The NADH concentrations used were 0, 0.025, 0.05 and 0.1 mM and concentrations of NAD^+ were 0.067, 0.1, 0.2 and 1.0 mM.

2.10.3.2) Product inhibition by NADH with respect to L-phenylalanine

Inhibition by NADH was analyzed with varied concentrations of L-phenylalanine in the presence of a saturating and constant concentration of NAD^+ (2 mM). The NADH concentrations used were 0, 0.025, 0.05 and 0.1 mM and concentrations of L-phenylalanine were 0.1, 0.2, 0.5 and 1.0 mM.

2.10.3.3) Product inhibition by phenylpyruvate with respect to NAD^+

Inhibition by phenylpyruvate was analyzed with varied concentrations of NAD^+ in the presence of a saturating and constant concentration of L-phenylalanine (20 mM). The phenylpyruvate concentrations used were 0, 0.2, 0.4 and 1.0 mM and concentrations of NAD^+ were 0.067, 0.1, 0.2 and 1.0 mM.

2.10.3.4) Product inhibition by phenylpyruvate with respect to L-phenylalanine

Inhibition by phenylpyruvate was analyzed with varied

concentrations of L-phenylalanine in the presence of a saturating and constant concentration of NAD^+ (2 mM). The phenylpyruvate concentrations used were 0, 0.2, 0.4 and 1.0 mM and concentrations of L-phenylalanine were 0.1, 0.2, 0.5 and 1.0 mM.

2.10.3.5) Product inhibition by ammonium chloride with respect to NAD^+

Inhibition by ammonium chloride was analyzed with varied concentrations of NAD^+ in the presence of a saturating and constant concentration of L-phenylalanine (20 mM). The ammonium chloride concentrations used were 0, 20, 40 and 80 mM and concentrations of NAD^+ were 0.067, 0.1, 0.2 and 1.0 mM.

2.10.3.6) Product inhibition by ammonium chloride with respect to L-phenylalanine

Inhibition by ammonium chloride was analyzed with varied concentrations of L-phenylalanine in the presence of a saturating and constant concentration of NAD^+ (2 mM). The ammonium chloride concentrations used were 0, 20, 40 and 80 mM and concentrations of L-phenylalanine were 0.1, 0.2, 0.5 and 1.0 mM.

From the data, The plots of reciprocal initial velocities (1/v) against reciprocal substrate concentrations (1/ [s]) at several concentrations of inhibitors were made for kinetic mechanism analysis. Velocity (v) was expressed as unit of enzyme which was defined as μmol of NADH formed / min.