CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Analytical balance: Mettler-Toledo, Switzerland

Autoclave: Model H-88LL, Kokusan Ensinki Co., Ltd., Japan

Autopipette: Pipetman, Gilson, France

Centrifuge, refrigerated centrifuge: J-30I, Beckman Instrument Inc., USA

Centrifuge, microcentrifuge: MC-15A, Taiwan

Electrophoresis apparatus: Model Mini-protein II Cell, BIO-RAD, USA

Fraction collector: Frac-100, Pharmacia Biotech, Sweden

Heating box: Model RS232 Dri bath incubator, Taiwan

Incubator, waterbath: Model M20S, Lauda, Germany and BioChiller 2000, FOTODYNE Inc.,USA

Magnetic stirrer: Model Fisherbrand, Fisher Scientific, USA

Microcentrifuge tubes 1.5 ml, Axygen Hayward, USA

Peristaltic pump: pump p-1, Pharmacia Biotech, Sweden

pH meter: Mettler-Toledo, Switzerland

Power supply: Model POWER PAC 300, Bio-Rad, USA

Rotary shaker: Model TC-7, News Brunswick Scientific Co., USA

Sonicator: Vibra cell^{Tm}, SONICS & MATERIALS, Inc., USA

Shaking waterbath: Model G-76, New Brunswick Scientific Co., Inc., USA

Spectrophotometer: DU Series 650, Beckman Instrument Inc., USA

Ultrafilter: Suprec$^{Tm-01,Tm-02}$, pore size 0.20 μm and 0.22 μm, Takara Shuzo Co, Ltd., Japan

Vortex: Model K-550-GE, Scientific Industries, Inc., USA

2.2 Chemicals

Acetone: Scharlau, Spain

Acrylamide: Merck, Germany

N-Acetylimidazole: Sigma, USA

Alumina: sigma, USA

Aminopropyl triethoxysilane: Sigma, USA

Ammonium sulphate: Carlo Erba Reagenti, Italy

Ampicillin: Sigma, USA

Aquasorb: BML, Thailand

Bis-acrylamide: Merck, Germany

Boric acid: Merck, Germany

Bovine serum albumin: Sigma, USA

Bromophenol blue: Merck, Germany

N-Bromosuccinimide: Sigma, USA

1,4-Butanediol diglycidyl ether: Fluka, Switzerland

n-Butanol: Ajax Chemicals, Australia

Chitosan bead: a gift from Dr. R. Pichyangkura, Chulalongkorn University

Chloramine T: Sigma, USA

Coomassie brilliant blue R-250: Sigma, USA

DEAE-Toyopearl 650M TSK gel: Tosoh, Japan

Dialysis tubing: Sigma, USA

Diaphorase: Sigma, USA

Diethylpyrocarbonate: Sigma, USA

DL-Dithiothreitol (DTT): Sigma, USA

Ethylene diamine tetraacetic acid (EDTA): Merck, Germany

Glacial acetic acid: Carlo Erba Reagenti, Italy

Glutaraldehyde: Sigma, USA

Glycerol: Merck, Germany

Glycine: Sigma, USA

Hydrochloric acid: Carlo Erba Reagenti, Italy

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

2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1): Dojindo, Janpan

Isopropanol: Merck, Germany

Isopropylthio-β-D-galactosidase (IPTG): Sigma, USA

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

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

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

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

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

β- mercaptoethanol: Fluka, Switzerland

Methanol: Lab-Scan, Thailand

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

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

N,N'-methylene-bis-acrylamide: Sigma, USA

N,N,N',N'-tetramethyl-1, 2-diaminoethane (TEMED): Carlo Erba Reagenti, Italy

Nitroblue tetrazolium: Koch-Light Laboratories Ltd., Japan

Ninhydrin: BDH, England

Nitric acid: Mallinckrodt, France

L-phenylalanine: Koch-Light Laboratories Ltd., Japan

Peptone from casein pancreatically digested: Merck, Germany

Phenazine methosulfate: Nacalai Tesque, Inc., Japan

Phenylmethylsulfonyl fluoride (PMSF): Sigma, USA

Protein molecular weight marker (MW 14,400-116,000): Fermentas, USA

Phenylmethylsulfonyl fluoride (PMSF): Sigma, USA

Phenylpyruvic acid (sodium salt): Sigma, USA

Potassium acetate: Merck, Germany

Potassium chloride: Merck, Germany

Potassium di-hydrogen phosphate: Carlo Erba Reagenti, Italy

di-Potassium hydrogen phosphate: Carlo Erba Reagenti, Italy

Potassium hydroxide: Scharlau, Spain

Pyruvic acid (sodium salt): Sigma, USA
Potassium tartrate: BDH, England
Sodium acetate: Merck, Germany
Sodium carbonate anhydrous: Carlo Erba Reagenti, Italy
Sodium citrate: Carlo Erba Reagenti, Italy
Sodium chloride: Carlo Erba Reagenti, Italy
Sodium dodecyl sulfate: Sigma, USA
Sodium hydroxide: Merck, Germany
Thin layer chromatography (TLC): DC-Plastikfolien cellulose, Merck, Germany
Trifluoroacetic acid: BDH, England
Tris (hydroxymethyl)-aminomethane: Carlo Erba Reagenti, Italy
2,4,6-Trinitrobenzenesulfonic acid: Sigma,USA
Yeast extract: Scharlau microbiology, European Union

2.3 Bacterial strains

Escherichai coli BL21(DE3), which contained pET-17b plasmid vector has baring phenylalanine dehydrogenase gene of *Acinetobacter lwoffii* (Sitthai *et al.*, 2004), was used for the production of PheDH.

2. 4 Bacteria growth medium

The bacterial stain was grown under aerobic condition at 37 °C in LB medium which contained the following components: 1% (w/v) tryptone, 1% (w/v) NaCl and 0.5% (w/v) yeast extract and the pH was adjusted to 7.2. The LB medium contained 100 μg/ml ampicillin in order to select positive colonies. PheDH production was induced by IPTG at final concentration of 0.4 mM. For agar plate, the LB medium was supplemented with 2% (w/v) of agar. Medium was sterilized by autoclaving at 121°C for 15 minutes.

2.5 Free enzyme assay

Phenylalanine dehydrogenase catalyzes the reversible reaction. Thus, the enzyme activity can be determined in two directions according to the methods described by Asano *et al.* (1987). The forward reaction is oxidative deamination of L-phenylalanine, while the reverse reaction is the reductive amination of L-phenylpyruvate as shown in the reaction below.

$$\text{L-Phe} + NAD^+ + H_2O \rightleftharpoons \text{phenylpyruvate} + NH_3 + NADH + H^+$$

The PheDH activity was measured by oxidative deamination using L-phenylalanine as a substrate. The standard reaction mixture contained 200 μmol of glycine-KCl-KOH buffer, pH 10.5, 20 μmol of L-phenylalanine, 1 μmol of NAD^+, and the enzyme in a final volume of 1 ml. Incubation was carried out at 30°C for 5 minutes in a cuvette of 1-cm light path. The reaction was started by the addition of NAD^+ and monitored by measuring the initial change in absorbance of NADH at 340 nm. For reductive deamination, the activity of PheDH was assayed by using phenylpyruvate as a substrate. The reaction mixture of 1 ml contained 100 μmol of glycine-KCl-KOH buffer, pH 9.0, 500 μmol of NH_4Cl, 10 μmol of L-phenylpyruvate, 0.2 μmol of NADH and the enzyme. Incubation was carried out at 30°C for 5 minutes. The reaction was started by the addition of NADH and was monitored by measuring the initial change in absorbance of NADH at 340 nm.

One unit of the enzyme is defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH per 1 minute. Specific activity is defined as units per milligram of protein.

2.6 Protein measurement

Protein concentration was determined by the modified method of Lowry (Lowry *et al.*, 1956). The reaction mixture of 6.1 ml containing 20-100 μg 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 500 µl of solution C at room temperature for 20 minutes. The protein concentration was monitored by measuring the absorbance at 610 nm and calculated from the standard curve of protein standard (BSA). The preparations of all solutions were described in Appendix A and B, respectively.

2.7 Partial purification of phenylalanine dehydrogenase

2.7.1 Enzyme production

The single colony of the *E. coli* BL21(DE3) transformant from LB agar plate was grown overnight at 37°C in 5 ml of starter LB medium containing 100 µg/ml ampicillin. After that, 2.5% (v/v) of the cell culture was inoculated into 2 liter of LB medium with the same supplement and was cultured at 37°C with shaking. When the turbidity of the culture at 600 nm had reached 0.6, IPTG was added to the final concentration of 0.4 mM to induce enzyme production. The cells were harvested after the induction for 8 hours by centrifugation at 10,000 x g for 10 minutes at 4°C, and then washed with cold 0.85% (w/v) NaCl. After that, the cell pellet was washed once in cold extraction buffer (0.1 M potassium phosphate buffer, pH 7.4 containing 0.1 mM PMSF, 0.01% (v/v) β-mercaptoethanol and 1.0 mM EDTA) and centrifuged again. The cell pellet was stored at -80°C until further used.

2.7.2 Crude extract preparation

The collected cell from section 2.7.1 was resuspended in 30 ml of cold extraction buffer (0.1 M potassium phosphate buffer, pH 7.4 containing 0.1 mM PMSF, 0.01% (v/v) β-mercaptoethanol and 1.0 mM EDTA) and then sonicated on ice. Unbroken cells and cell debris were removed by centrifugation at 17,500xg for 30 minutes at 4°C. The crude enzyme solution was collected for the determination of

oxidative deamination activity and protein concentration assayed as described in sections 2.5 and 2.6, respectively.

2.7.3 Purification procedures of phenylalanine dehydrogenase

The crude extract from 2.7.2 was purified by the following steps. 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% (v/v) β-mercaptoethanol and 1 mM EDTA.

2.7.3.1 Ammonium sulfate precipitation

The precipitation of crude extract was done by slowly adding solid ammonium sulfate to 50% saturation with gentle stirring. After 1 hour, the supernatant was collected by centrifugation at 17,500xg for 30 minutes and then adjusted to final concentration of 70% saturation with solid ammonium sulfate. The solution was left for 1 hour on ice with continuous stirring and centrifuged again. The precipitate was dissolved in the buffer. The protein solution was dialyzed against 100 volumes of the buffer at least 4 hours for 3 times before determination of the enzyme activity and protein concentration as described in 2.5 and 2.6, respectively.

2.7.3.2 DEAE-Toyopearl column chromatography

DEAE-Toyopearl was first activated by washing twice with 0.5 N NaOH, and rewashed by deionized water until the pH reached 8.0. The activated DEAE-Toyopearl was resuspened in the buffer (see section 2.7.3) and packed into the column and equilibrated with the same buffer for 5-10 column volumes at a flow rate of 1 ml/min.

The protein solution from section 2.7.3.1 was applied to the DEAE-Toyopearl column. The unbound proteins were eluted from the column with the buffer until the absorbance at 280 nm of eluent was nearly zero. After that, the bound proteins were eluted from the column with linear salt gradient of 0 to 0.5 M KCl in the buffer. The 3 ml fractions were collected using a fraction collector and protein

content was measured by reading the absorbance at 280 nm. The enzyme activity was determined as described in section 2.5 The KCl concentration was investigated by measuring the conductivity. Fractions containing PheDH activity was pooled and dialyzed against 100 volumes of the same buffer. The enzyme activity and protein concentration of pooled fraction were measured as described in section 2.5 and 2.6, respectively.

2.7.3.3 Polyacrylamide gel electrophoresis

The enzyme from each step of purification was analyzed by native PAGE and SDS-PAGE to determine the native protein and denatured protein pattern, respectively.

2.7.3.3.1 Non-denaturing gel electrophoresis

Discontinuous PAGE was performed on the slab gel of a 7.7% (w/v) seperating gel and a 5% (w/v) stacking gel. Tris-glycine buffer, pH 8.3 (25 mM Tris and 192 mM glycine) was used as electrode buffer. Preparation of solution and polyacrylamide gels was described in Appendix D. The enzyme was mixed with 5x sample buffer (312.5 mM Tris-HCl, pH 6.8, 50% (v/v) glycerol and 0.05% (w/v) bromophenol blue) by the ratio of 5: 1 and loaded onto the gel. The electrophoresis was run from cathode towards anode at constant current of 20 mA per slab gel. For activity staining, the experiment was done at 4°C. After electrophoresis, the gel was developed by protein and activity staining.

A. Protein staining

The gel was stained with Coomassie staining solution (1% (w/v) Coomassie Blue R-250, 45% (v/v) methanol, and 10% (v/v) glacial acetic acid) for 30 minutes on the shaker. The stain solution was poured out and the Coomassie destaining solution (10% (v/v) methanol and 10% (v/v) glacial acetic acid)

was added. The gel was gently destained for several times until gel background was clear.

B. 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 5 minutes at room temperature or until the brown band had appeared. The gel was then quickly rinsed several times with deionized water until gel background was clear.

2.7.3.3.2 SDS-polyacrylamide gel electrophoresis

The SDS-PAGE system was performed according to the method of Bollag *et al.*, 1996. The slab gel system consisted of 0.1% (w/v) SDS in 10% (w/v) seperating gel and 5% (w/v) stacking gel. Tris-glycine (25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS), pH 8.3 was used as electrode buffer. The gel prepararion was described in Appendix E. The enzyme was mixed with 5x sample buffer (60 mM Tris-HCl pH 6.8, 25% (v/v) glycerol, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue and 14.4 mM β-mercaptoethanol) by the ratio of 5: 1 and boiled for 10 minutes before loading to the gel. The electrophoresis was run from cathode towards anode at constant current (20 mA) at room temperature. The molecular weight marker proteins were β-galactosidase (116,000 Da), bovine serum albumin (66,200 Da), ovalbumin (45,000 Da), lactate dehydrogenase (35,000 Da), restriction endronuclease (25,000 Da), β-lactoglobulin (18,400 Da) and lysozyme (14,400 Da). After electrophoresis, the gel was stained with Coomassie blue as described in section 2.7.3.3.1 A.

2.8 Effect of group-specific reagents on phenylalanine dehydrogenase activity

2.8.1 Effect of *N*- Acetylimidazole (NAI) on tyrosine residues

Modification of tyrosine residues was carried out according to the method of Means and Feeney (1971). The enzyme (5-10 µg/µl) was incubated with 10 mM of NAI in 50 mM phosphate buffer, pH 7.5 at 30°C for 20 minutes. 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.

2.8.2 Effect of *N*-Bromosuccinimide (NBS) on tryptophan residues

Modification of tryptophan residues was carried out according to the method of Means and Feeney (1971). The enzyme (5-10 µg/µl) was incubated with 10 mM of NBS in 50 mM phosphate buffer, pH 7.0 at 30°C for 20 minutes. 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.

2.8.3 Effect of *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) on aspartic and glutamic

Modification of aspartic and glutamic residues was carried out according to the method of Hoare and Koshland (1967). The enzyme (5-10 µg/µl) was incubated with EDC in 50 mM phosphate buffer, pH 6.0 at 30°C for 20 minutes. EDC was reacted 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.

2.8.4 Effect of chloramines T (CT) on methionine residues

Modification of methionine residues was carried out according to the method of Miles and Smith (1993). 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 was reacted 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.

2.8.5 Effect of diethylpyrocarbonate (DEPC) on histidine residues

Modification of histidine residues was carried out according to the method of Wakayama *et al.* (1996). The enzyme (5-10 µg/µl) was incubated with DEPC in 50 mM phosphate buffer, pH 7.0 at 30°C for 20 minutes. DEPC was reacted 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.

2.8.6 Effect of dithiothreitol (DTT) on cysteine residues

Modification of cysteine residues was carried out according to the method of Glazer *et al.* (1976). The enzyme (5-10 µg/µl) was incubated with DTT in 50 mM phosphate buffer, pH 7.5 at 30°C for 20 minutes. DTT was reacted 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.

2.8.7 Effect of phenylmethylsulfonyl fluoride (PMSF) on serine residues

Modification of serine residues was carried out according to the method of Wakayama *et al.* (1966). The enzyme (5-10 µg/µl) was incubated with 10 mM of

FMSF in 50 mM phosphate buffer, pH 7.0 at 30°C for 20 minutes. 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.

2.8.8 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 (1972). The enzyme (5-10 µg/µl) was incubated with TNBS in 50 mM phosphate buffer, pH 8.0 at 30°C for 20 minutes. TNBS was reacted 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 Immobilization of phenylalanine dehydrogenase

To investigate for an appropriate immobilization method, various immobilization processes on several organic and inorganic supports were studied. Supports including alumina, silica, chitosan and epoxy activated support were used for covalent coupling of the enzymes and a suitable support was selected for PheDH immobilization. The immobilization methods used were based on the method described by several researchers as shown below.

2.9.1 Preparation of supports

2.9.1.1 Preparation of inorganic carrier

The inorganic supports (alumina and silica) were cleaned to remove any organic matter from the surface (Weetall, 1993). The supports were first refluxed in

5% (v/v) nitric acid for 45 min at 100°C. The acid treatment was followed by extensive distilled water rinses to remove any residual acid and dried at 110°C.

2.9.1.2 Preparation of chitosan bead

Chitosan beads were prepared using a modified version of the method of Chiou and Wu (2003). Chitosan powder of 2% (w/v) (having a degree of deacetylation of 80%) was dissolved in 200 ml of 1% (v/v) acetic acid solution with stirring for 12 hours. The obtained solution was dropped into a gently stirred 1 M NaOH solution at room temperature. Afterward, the chitosan beads were removed by filtration and washed with distilled water until neutrality. The beads were stored in distilled water at 4°C.

2.9.2 Surface modification of the carriers

2.9.2.1 Introduction of epoxy group to the carriers using 1,4-butanediol diglycidyl ether (Chase and Yang. 1998)

Five hundred milligrams of alumina, silica and chitosan were washed with 1 M NaOH and then suspended in 3 ml of 1 M NaOH, after which 50 µl of 1,4-butanedioldiglycidyl ether was added. The mixture was stirred at room temperature for 5 hours. The product was then washed thoroughly with distilled water.

2.9.2.2 Introduction of amino group to the carriers

A: Silanization of inorganic support using γ-aminopropyl triethoxysilane (Pantatan, 2002)

Five hundred milligrams of cleaned alumina and silica were reacted with fresh solution of 2% (v/v) γ-aminopropyltriethoxysilane (APTS). The suspension

was gently stirred for 3 hours at room temperature. Then the silanized carriers were removed by centrifugation at 1,380 x g for 5 minutes at room temperature, followed by rinsing with distilled water several times.

B: 1, 6-Diaminohexane (Arica *et al.*, 2003, Bayramoğlu *et al.*, 2005)

Five hundred milligrams of epoxy-activated alumina and epoxy-activated chitosan (prepared as described in section 2.9.2.1) were aminated with 5 ml of 1% (w/v) 1,6-diaminohexane at 65°C for 6 hours with stirring. The long chain aminated support was extensively washed with distilled water and then resuspended in 5 ml of 50 mM potassium phosphate buffer, pH 7.0 for 12 hours before subjected to enzyme immobilization.

2.9.2.3 Introduction of cationic to the carriers using polyethyleneimine (PEI)

A: For coating alumina, silica and chitosan (Atia, 2005)

Five hundred milligrams of supports were added to 10% (v/v) of polyethyleneimine (PEI) in 0.2 M borate buffer, pH 8.5. The suspension was gently stirred for 3 hours at room temperature and then washed with excess distilled water to eliminate any molecules of PEI adsorbed on the support.

B: For coating epoxy supports (Alonso *et al.*, 2005)

Five hundred milligrams of epoxy supports including epoxy-activated alumina and epoxy-activated chitosan were incubated in 5 ml of 2 M HCl for 10 minutes, in order to open epoxy ring to diol group. Then the support was washed with excessive volume of distilled water and incubated in 11 mM sodium periodate for 2 hours to oxidize all the diol groups to glyoxyl groups. The support activated with glyoxyl groups was washed with an excess volume of distilled water. Next the support

was added to 10% (v/v) PEI in 0.1 M borate buffer, pH 8.5. The suspension was kept under mild stirring at 25°C for 3 hours. Then, the suspensions were reduced by the addition of solid sodium borohydride to a final concentration to 10 mg/ml and were left under mild stirring for 2 hours. Finally, the reduced suspension were withdrawn and washed with 0.1 M sodium acetate buffer, pH 4.0, 0.1 M sodium borate buffer, pH 9.0, and with an excess volume of distilled water.

2.9.3 Enzyme coupling

Three different immobilization procedures of PheDH were studied: immobilization via its amino groups, immobilization via ionic interaction and immobilization via its carboxyl group. All experiments were performed at 4°C and 18 units of PheDH were used.

2.9.3.1 PheDH immobilization via its amino groups

In this study, glutaraldehyde was used as a crosslinker to covalently immobilize of PheDH on to supports, which were aminated with γ-aminopropyl triethoxy silane (APTS) and 1,6-diaminohexane.

A. Amination with γ-aminopropyltriethoxysilane (APTS)

Silica was selected for the immobilization of PheDH and the method used was through glutaraldehyde since it showed the highest immobilized activity in the previous report (Chumphukam, 2004). However, the optimum conditions were re-investigated to see the reproducibility. The optimum conditions for covalent immobilization of PheDH were investigated by varying the concentration of APTS, glutaraldehyde, coupling time and the amount of PheDH to fixed amount of support (500 mg). The first parameter was used to determine the optimal condition for the next parameter. The best condition yielding maximum activity retention of immobilized PheDH was selected for further immobilization studies.

Five hundred milligrams of activated carriers with APTS (1-10% (v/v)) (prepared as in section 2.9.2.2A) were reacted with 5 ml of 0.1-0.25% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.0 under mild agitation for 2 hours at room temperature. The excessive amount of glutaraldehyde was removed by centrifugation at 1,380 x g for 5 minutes at room temperature, and then washed with deionized water several times to remove residual glutaraldehyde. The amounts of PheDH of 2-40 units were then added. The mixture was stirred at 4°C for 0.5-12 hours. The immobilized PheDH was then removed by centrifugation at 1,380 x g for 5 minutes at 4°C. Next, the carriers were washed 3 times with 1 M NaCl in 10 mM potassium phosphate buffer, pH 7.4, followed by washing with the same buffer without NaCl 4 times. The immobilized enzyme was analyzed for PheDH activities as described in sections 2.10 and stored at 4°C for further used.

B. Amination with 1, 6-diaminohexane

Five hundred milligram of aminated alumina and chitosan (prepared as in section 2.9.2.2B) was reacted with 1% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.0 (5 ml), at 25°C for 12 hours, with stirring. After this, excess glutaraldehyde was removed by sequentially washing with distilled water. Five hundred milligrams of glutaraldehyde cross-linked support were added into PheDH solution (18 units). The volume was adjusted to 5 ml with 50 mM potassium phosphate buffer, pH 7.0. The suspension was kept under mild stirring at 4°C for 6 hours. The solution was then removed by centrifugation at 1,380 x g for 5 minutes at 4°C. Next, the carriers were washed 3 times with 1 M NaCl in 10 mM potassium phosphate buffer, pH 7.4, followed by washing with the same buffer without NaCl 4 times. The immobilized enzyme was analyzed for PheDH activities as described in sections 2.10 and stored at 4°C for further used.

2.9.3.2 PheDH immobilization via ionic interaction

A. Adsorption of PheDH onto PEI activated support

Eighteen units of PheDH were added into 500 mg of PEI activated support (see section 2.9.2.3) and the volume was adjusted to 5 ml with 50 mM potassium phosphate buffer, pH 7.0. This suspension was kept under mild stirring for 3 hours at 4°C. Finally, the immobilized PheDH was separated from the supernatant by centrifugation at 1,380 x g for 5 minutes at 4°C. The immobilized PheDH was washed 3 times with 1 M NaCl in 10 mM potassium phosphate buffer pH, 7.4, followed by washing with the same buffer without salt. The immobilized enzyme was analyzed for PheDH activities as described in section 2.10 and stored at 4°C until further used.

B. Crosslinking with glutaraldehyde

Five hundred milligrams of adsorbed PheDH on PEI support (prepared with the same procedure as described above) was added into 5 ml of 0.25% (v/v) glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.0. The mixture was gently stirred for 1 hour at 4°C. Then, the immobilized enzyme was separated from the solution by centrifugation at 1,380 x g for 5 minutes at 4°C. The immobilized PheDH was washed 3 times with 1 M NaCl in 10 mM potassium phosphate buffer, pH 7.4, followed by washing with the same buffer without NaCl 4 times. The enzyme activity was then analyzed as described in section 2.10 and stored at 4°C until further used.

2.9.3.3 PheDH immobilization via its carboxylic group

(Villalonga *et al.*, 2005)

Five milligrams of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was added to a reaction mixture containing 18 units of PheDH in the final volume of 3 ml of 50 mM potassium phosphate buffer, pH 6.0. The solution was stirred for 2 hours at 4°C. After that, five hundred milligrams of chitosan

(prepared as described in section 2.9.1.2), aminated alumina or aminated silica as described in section 2.9.2.2 was added to the reaction and the volume was adjusted to 5 ml with 50 mM potassium phosphate buffer, pH 6.0. The mixture was gently stirred for 4 hours at 4°C. After that the immobilized enzyme was removed by centrifugation at 1,380 x g for 5 minutes at 4°C and the immobilized PheDH was successively washed (3 times) with 1 M NaCl in 10 mM potassium phosphate buffer pH 7.4 followed by successive wash (4 times) with the same buffer without NaCl. The immobilized enzyme was analyzed for PheDH activities as described in section 2.10. When the immobilization was completed, the immobilized PheDH was stored at 4°C for further experiments.

2.10 Colorimetric method for the determination of phenylalanine dehydrogenase activity

Enzymatic activity assay as described in section 2.5 could not apply to determine the immobilized PheDH activity. Therefore, colorimetric method that simultaneously couples the reaction of NAD(H)-dependent PheDH and a second reaction in which initially formed NADH and diaphorase convert water soluble tetrazolium (WST-1) to a water soluble formazan was used to measure the immobilized and free enzyme activities. The product is measured in the visible range at 438 nm. The catalyzed reactions adapted from the study of Wendel *et al.* (1991) are shown below.

$$\text{L-phenylalanine} + NAD^+ + H_2 \xleftrightarrow{\text{PheDH}} \text{phenylpyruvate} + NADH + NH_4^+ \quad (1)$$

$$NADH + \text{WST-1} + H^+ \xrightarrow{\text{diaphorase}} NAD^+ + \text{water soluble formazan} \quad (2)$$

The enzymatic activity of free and immobilized enzyme was determined at room temperature in 200 µmol of glycine-KCl-KOH buffer, pH 9.0 containing

20 μmol of L-phenylalanine, 1 μmol of NAD^+, 1 μmol WST-1, 0.01 U of diaphorase from *Clostridium kluyveri* and the free enzyme in a final volume of 1.0 ml. The reaction was started by the addition of phenylalanine and incubated for 5 minutes. For immobilized enzyme, 5 mg of immobilized enzyme was mixed with 1 ml of reaction mixture, incubated with shaking, and then the supernatant was separated by centrifugation and analyzed for the increasing absorbance at 438 nm.

2.11 Optimization of phenylalanine dehydrogenase immobilization

The immobilization method that gave the highest yield of immobilized enzyme activity and the most suitable support were selected from section 2.9.3 By considering the color intensity of formazan at 438 nm, the covalent coupling carbodiimide activated PheDH on silica support was selected for further study. The optimum conditions for covalent immobilization of PheDH were investigated by varying some of the conditions described in section 2.9.2.2 A and 2.9.3.3. The APTS concentrations in the range of 1-10% (v/v), and EDC concentration in the range of 1-100 mM, were tested. Different amounts of PheDH ranging from 3-35 units were applied to 500 mg support. The duration of immobilization was examined from 3-30 hours. The protein content and activities of immobilized enzyme were analyzed under standard condition described in sections 2.6 and 2.10.

2.12 Calculation of the immobilization yield

The efficiency of immobilized enzyme was usually expressed as the activity retention after immobilization. The immobilization yield of the immobilized PheDH was calculated by equation as follows:

$$\text{Immobilization yield (\%)} = \frac{\text{Immobilized enzyme activity (U)} \times 100}{\text{Free enzyme activity applied (U)} - \text{Unbound enzyme activity (U)}}$$

2.13 Characterization of the catalytic properties of the phenylalanine dehydrogenase

2.13.1 Effect of pH on the phenylalanine dehydrogenase activity

The effects of pH on free and immobilized enzyme activities were determined under standard condition as described in section 2.10 at various pHs. The 200 mM of glycine-KCl-KOH buffer, pH range of 8.5 to 13.0 was used. After the reaction, the immobilized enzyme was separated by centrifugation. The pH of each reaction mixture was measured with a pH meter at room temperature. The result was expressed as a percentage of the relative activity. The maximum activity which was observed was set as 100% relative activity.

2.13.2 Effect of temperature on the phenylalanine dehydrogenase activity

Free and immobilized enzymes were assayed under standard condition as described in section 2.10. Temperature was varied from 25°C to 60°C. The result was expressed as a percentage of the relative activity. The maximum activity which was observed was set as 100%.

2.13.3 Effect of pH on the phenylalanine dehydrogenase stability

The effect of pH on the stability of the free and immobilized enzyme was investigated. After the enzyme had been incubated at 30°C for 20 minutes in each of the 10 mM buffer at various pHs: citrate buffer (pH 3.0 - 7.0), potassium phosphate buffer (pH 6.0 – 8.0), Tris-HCl buffer (pH 7.5 – 9.0) and glycine-KCl-KOH buffer (pH 9.5 – 13.0), the immobilized enzyme was separated by centrifugation and the remaining activity of enzyme in both forms were measured under standard condition as described in section 2.10. The result was expressed relatively to the original activity when the enzyme was kept in 10 mM phosphate buffer, pH 7.4. The original activity was defined as 100%.

2.13.4 Effect of temperature on the phenylalanine dehydrogenase stability

The free and immobilized enzymes were used to study thermostability which was investigated over the range of 25°C-75°C. The enzyme in both form were incubated in 10 mM phosphate buffer pH, 7.4. After that, the immobilized enzyme was separated by centrifugation and then the residual activity for both forms of enzyme was measured under standard condition as described in section 2.10. The result was expressed as a percentage of the relative activity which was relative to the original activity when the enzyme was not subjected to heat. The original activity was defined as 100%.

2.13.5 Storage stability

The free and immobilized enzymes were stored in 10 mM potassium phosphate buffer, pH 7.4 containing 0.01% (v/v) 2-mercaptoethanol, 1 mM EDTA and 10% (v/v) glycerol at 4°C and at room temperature for a period of 45 days. The residual activity for free and immobilized PheDH was measured by the method described in section 2.10. The initial value of enzyme activity in each set was assigned the value of 100% activity.

2.13.6 Batch reusability of immobilized enzyme

Reusability is a crucial parameter for immobilized enzymes in practical application. PheDH immobilized by carbodiimide method was used repeatedly to produce L-phenylalanine. Reaction was performed for 6 hours at room temperature with 5 μmol phenylpyruvate, 200 μmol NH_4Cl-NH_4OH buffer (pH 8.5), 5 μmol NADH and 500 mg (wet weight) of immobilized PheDH. Immobilized enzyme was washed with 10 mM potassium phosphate buffer, pH 7.0 after every incubation and reintroduced into fresh reaction mixture. The residual activity of immobilized PheDH

after multiple uses was assayed as described in section 2.10 and the semiquantity of L-phenylalanine was determined by TLC technique.

2.14 Synthesis of amino acids from their keto acids using immobilized phenylalanine dehydrogenase

2.14.1 Phenylalanine and other amino acids production

The production of phenylalanine and other amino acids was carried out by various kinds of keto acids which were phenylpyruvate, α-ketocaproate, α-ketoisocaproate, α-ketovalerate, α-ketoisovalerate and α-keto-γ-methiol-butyrate as substrates. Five-hundred microliters of the reaction mixture comprised of 5 μmol of keto acids, 200 μmol of NH_4Cl-NH_4OH buffer (pH 9.5), 5 μmol of NADH and 5 mg (wet weight) of immobilized PheDH. The reaction mixture was incubated for 20 hours at 30°C with gentle shaking. After that, the supernatant was separated from the immobilized enzyme by centrifugation and were subjected to the analysis of amino acid production by TLC technique.

2.14.2 Determination of phenylalanine and other amino acids produced in the reaction by thin-layer chromatography (TLC)

Cellulose thin-layer chromatography (TLC) was used to determine amino acids produced in the reaction mixtures as described above. The samples were neutralized to pH ~7.0 with 2 N HCl. Then, the 2 μl of sample solutions were spotted in parallel with their standard amino acids on a TLC cellulose plate and developed with n-butanol: acetic acid: water (4: 1: 1). The plate was dried for 10 minutes under hot air. After that 0.5% (w/v) ninhydrin solution in acetone and ethanol (30:70) was sprayed on plate and then dried in the oven for 15 minutes to develop color of ninhydrin-amino acid complex.

2.14.3 Semiquantitative product analysis

The semiquantity of L-amino acids produced from the enzyme reactions were determined by using TLC and 2D gel electrophoresis. The commercial L-phenylalanine, norleucine, leucine, norvaline and methionine were used as standards for identification and semiquantification. Various concentrations of the standard (5 μmol-25 μmol) were spotted on TLC plate. The sample was also spotted on the same plate and processed under the same procedure as described above. The intensity of each spot was measured by gene tool program. The standard curve was plotted (Appendix G) and the amount of L-phenylalanine in the sample was calculated.