การตรึงฟีนิลอะลานีนดีไฮโดรจิเนส: การศึกษาเปรียบเทียบตัวค้ำจุนชนิดต่างๆ
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นางสาว อรดา ชุมภูคำ

IMMOBILIZATION OF PHENYLALANINE DEHYDROGENASE:

A COMPARATIVE STUDY OF VARIOUS SUPPORTS, OPTIMAL CONDITIONS

AND CHARACTERIZATION

Miss Orada Chumphukam

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อรดา ชุมภูคำ: การตรึงฟีนิลอะลานีนดีไฮโดรจิเนส: การศึกษาเปรียบเทียบตัวค้ำจุนชนิดต่างๆ ภาวะที่เหมาะสมและลักษณะสมบัติ. (IMMOBILIZATION OF PHENYLALANINE DEHYDROGENASE: A COMPARATIVE STUDY OF VARIOUS SUPPORTS, OPTIMAL CONDITIONS AND CHARACTERIZATION) อ.ที่ปรึกษา: อ.ดร.มัญชุมาส เพราะสุนทร, อ.ที่ปรึกษาร่วม: ผศ.ดร.กนกทิพย์ ภักดีบำรุง; 166 หน้า. ISBN 974-53-1380-7.

ฟีนิลอะลานีนดีไฮโดรจิเนส (PheDH) ที่ผลิตจากรีคอมบิแนนท์โคลนของ *Escherichia coli* ถูกนำมาทำให้บริสุทธิ์ด้วยการตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟตอิ่มตัว และแยกโดยโครมาโตกราฟีด้วยคอลัมน์ดีอีเออี-โทโยเพิร์ล และคอลัมน์บิวทิล-โทโยเพิร์ล พบว่ามีแอคติวิตีคงเหลือ 23.9 เปอร์เซ็นต์ และบริสุทธิ์เพิ่มขึ้น 10.1 เท่า หน่วยย่อยของเอนไซม์มีน้ำหนักโมเลกุล 45,000 ดาลตัน และมีความจำเพาะสูงมากต่อแอล-ฟีนิลอะลานีนและฟีนิลไพรูเวท ซึ่งเป็นสับสเตรทในปฏิกิริยาออกซิเดทีฟ-ดีอะมิเนชัน และรีดักทีฟอะมิเนชันตามลำดับ โดยใช้ NAD$^+$เป็นโคเอนไซม์ pH ที่เหมาะสมสำหรับปฏิกิริยาออกซิเดทีฟดีอะมิเนชันและรีดักทีฟอะมิเนชัน คือ 10.4 และ 8.7 ตามลำดับ อุณหภูมิที่เหมาะสม คือ 40 °ซ และ 45 °ซ ตามลำดับ เอนไซม์มีความเสถียรต่อ pH ในช่วง 6.5-12.5 และมีความเสถียรต่ออุณหภูมิเมื่อบ่มที่อุณหภูมิ 35 °ซ เป็นเวลา 10 นาที เมื่อตรึงเอนไซม์ด้วยวิธีโคเวเลนซ์และวิธีดูดซับบนวัสดุหลายชนิด ได้แก่ อะลูมินา ซิลิกา พอลิเมอร์พีวีเอชนิดเม็ด และ ไคโตแซน โดยใช้ 1,4-butanediol diglycidyl ether, tresyl chloride และกลูทารัลดีไฮด์เป็นตัวเชื่อมพบว่าเอนไซม์ที่ถูกตรึงบนซิลิกาโดยมีกลูทารัลดีไฮด์เป็นตัวเชื่อมมีความเหมาะสมต่อการนำไปใช้มากที่สุด ภาวะที่เหมาะสมในการตรึงคือกระตุ้นตัวค้ำด้วย สารละลายอะมิโนโพรพิลไตรเอทอกซีไซเลนเข้มข้น 6 เปอร์เซ็นต์โดยปริมาตร กลูทารัลดีไฮด์เข้มข้น 0.1 เปอร์เซ็นต์โดยปริมาตร และตรึงเอนไซม์ที่อุณหภูมิ 4 °ซ เป็นเวลา 6 ชั่วโมง โดยใช้เอนไซม์ 16 หน่วยต่อ ซิลิกา 1 กรัม จะสามารถตรึงเอนไซม์ได้ 0.14 หน่วย คิดเป็น 1.05 เปอร์เซ็นต์ของแอคติวิตีของเอนไซม์ตั้งต้น เมื่อเปรียบเทียบสมบัติของเอนไซม์ตรึงกับเอนไซม์อิสระโดยอาศัยปฏิกิริยาคู่ควบกับไดอะฟอเรส พบว่าเอนไซม์ตรึงและเอนไซม์อิสระมีค่า pH ที่เหมาะสมต่อการเร่งปฏิกิริยาที่ pH เท่ากับ 9.0 สำหรับอุณหภูมิที่เหมาะสมต่อการเร่งปฏิกิริยาของเอนไซม์ตรึงจะอยู่ในช่วงกว้างตั้งแต่ 30 °ซ ถึง 55 °ซ ความเสถียรต่อ pH และอุณหภูมิของเอนไซม์ตรึงมีความใกล้เคียงกับเอนไซม์อิสระ แต่เอนไซม์ตรึงมีความเสถียรมากกว่าเอนไซม์อิสระเมื่อเก็บในระยะยาว ในการผลิตกรดอะมิโนแบบไม่ต่อเนื่องของเอนไซม์ตรึง พบว่าภายใต้ภาวะที่กำหนดเอนไซม์ตรึงสามารถผลิตกรดอะมิโนชนิดต่างๆ จากสับสเตรทที่เป็นกรดคีโต ได้ผลผลิตในช่วง 63.7-100 เปอร์เซ็นต์

ภาควิชา.................ชีวเคมี....................ลายมือชื่อนิสิต...

สาขาวิชา.................ชีวเคมี....................ลายมือชื่ออาจารย์ที่ปรึกษา...............................

ปีการศึกษา..................2547.....................ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....................

ORADA CHUMPHUKAM: IMMOBILIZATION OF PHENYLALANINE DEHYDROGENASE: A COMPARATIVE STUDY OF VARIOUS SUPPORTS, OPTIMAL CONDITIONS AND CHARACTERIZATION. THESIS ADVISOR: MANCHUMAS PROUSOONTORN, Ph.D., THESIS COADVISOR: ASSIST. PROF. KANOKTIP PACKDIBAMRUNG, Ph.D. 166 pp. ISBN 974-53-1380-7.

Phenylalanine dehydrogenase (PheDH) produced by *Escherichia coli* transformant was partially purified by ammonium sulfate precipitation, DEAE-Toyopearl and Butyl-Toyopearl column chromatography with a 23.9% yield and 10.1 purification fold. The relative molecular weight of subunit was estimated to be 45,000 Da. The enzyme preferably acted on L-phenylalanine in oxidative deamination and phenylpyruvate in reductive amination and required NAD^+ as a natural coenzyme. The optimum pH for the oxidative deamination and reductive amination were 10.4 and 8.7, respectively whereas optimum temperatures were 45°C and 40°C, respectively. This enzyme was stable over the pH range of 6.5-12.5 and retained full activity when subjected to temperature at 35ºC for 10 minutes. PheDH was then covalently immobilized or adsorbed on various supports including alumina, silica, polyvinyl (alcohol) bead and chitosan. Different activating agents were used: 1, 4-butanediol diglycidyl ether, tresyl chloride and glutaraldehyde. Silica with glutaraldehyde as a cross-linking agent was found to be the most appropriate support and method. The optimum condition for enzyme immobilization was to activate silica with 6% (v/v) γ-aminopropyltriethoxysilane and 0.1 % (v/v) glutaraldehyde. PheDH was then added and incubated at 4°C for 6 hours, using an enzyme to support ratio of 16 units per gram silica. The activity of the immobilized enzyme was 0.14 U/g silica (1.05% of its original activity). When compared to the free enzyme, using coupled reaction with diaphorase, there was no change in pH optima (9.0) whereas the optimum temperature of the immobilized enzyme showed a broad range of 30ºC to 55ºC. The pH stability and thermostability of immobilized PheDH were closely similar to that of free enzyme. The immobilized PheDH exhibited good storage stability. For the batch production of amino acids, immobilized PheDH produced amino acids from their keto substrates with various % conversions from 63.7 to 100%.

Department/Program..........Biochemistry............Student's signature..

Field of study.................... Biochemistry............Advisor's signature.....................................

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ABBREVIATIONS

μg	microgram
μl	microliter
A	absorbance
BSA	bovine serum albumin
°C	degree Celsius
cm	centimeter
Da	dalton
DEAE	diethylaminoethyl
EDTA	ethylenediamine tetraacetic acid
g	gram
HCl	hydrochloric acid
K_m	Michaelis constant
KCl	potassium chloride
KOH	potassium hydroxide
l	liter
LeuDH	leucine dehydrogenase
LysDH	lysine dehydrogenase
M	molar
ml	milliliter
mol	mole
MW	molecular weight
N	normal
NAD^+	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate

NaOH	sodium hydroxide
nm	nanometer
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl chloride
SDS	sodium dodecyl sulfate
TEMED	N, N, N', N'-tetramethyl ethylene diamine
v/v	volume by volume
w/v	weight by volume
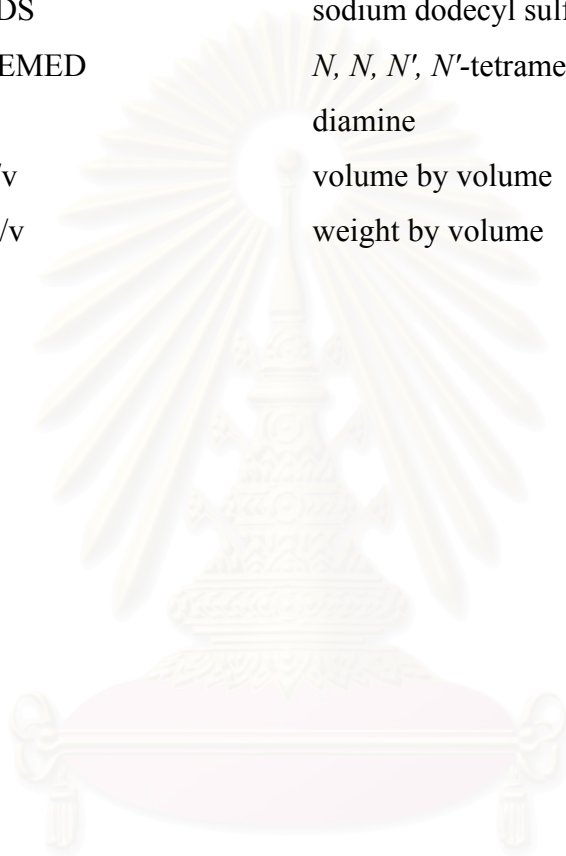
CHAPTER I

INTRODUCTION

Life is based on two fundamental conditions. Firstly, the living entity must be able to self-replicate; secondly, the organism must be able to catalyze chemical reactions efficiently and selectively. In the point of the second one, much attention has been focused on the reaction catalysts of biological systems: the enzymes, the most remarkable and highly specialized proteins. The outstanding characteristics of enzymes, for example a high degree of specificity for their substrates, a tremendous acceleration of chemical reactions and a function in aqueous solutions under very mild conditions of temperature and pH, put them apart from the conventional chemical catalysts (Nelson and Cox, 2000).

There are wide variety sources of enzymes both in eukaryote and prokaryote. All of them differ in their protein species. For commercial enzyme productions, microorganisms are the most popular employed. The exploitation of enzymes for analytical purpose, however, has been limited because of certain disadvantages, such as their instability and lack of availability. Moreover, aqueous solutions of enzymes often lose their catalytic ability fairly rapidly and the enzymes can neither be recovered from such solution, nor their activity regenerated (Guilbault and Sadar, 1979). In particular, the high cost of enzyme isolation and purification still discourages their use, especially in areas which currently have an established alternative procedure. The general unstable nature of enzymes, when removed from their natural environment, is also a major drawback to their more extensive use (Chaplin and Bucke, 1990). Nonetheless, many researches have been exploited the application of genetic engineering techniques to enzyme technology. Enzymes from dangerous and unapproved microorganisms and from slow growing of limited plant or animal tissue may be cloned into safe high-production microorganisms (Asano *et al*., 1987a; Ohshima and Soda, 1989; Galkin *et al*., 1997). Furthermore, various approaches are available to achieve immobilization of proteins. According to economical significance, these water-insoluble forms of enzymes can offer many

advantages over their soluble form because they can be separated from the reaction mixture containing the product and any residual reactant and can be reused in subsequent reactions (Chase and Yang, 1998; Yang *et al*., 2004). As a consequence, enzymes are being employed increasingly in many fields, not only in medical applications but also in the chemical industry, food processing, agriculture, environmental remediation and etc. (Seeger *et al*., 1997; Macek *et al*., 2000; Beilen and Li, 2002; Sogorb and Vilanova, 2002; Panke *et al*., 2004).

1.1 Phenylalanine dehydrogenase

One of the most attractive enzymes in a group of aromatic amino acid dehydrogenases is phenylalanine dehydrogenase (PheDH) [NAD^+-dependent oxidoreductase, deaminating; EC 1.4.1.20] which catalyzes the reversible oxidoreduction of L-phenylalanine to phenylpyruvate and ammonia in the presence of NAD^+ (**Figure 1.1**). It was first discovered in 1984 in *Brevibacterium* sp. (Hummel *et al*., 1984) and since then, it has been focused in numerous studies. Although its occurrence was completely unknown at that time, it appeared to be useful as an industrial catalyst. So much attention had been paid in the enantioselective synthesis of L-phenylalanine and related L-amino acids from their keto analogs (Asano *et al*., 1990). In 1985, Asano and Nakazawa screened the enzyme activity among a number of microorganisms from soil samples. It was discovered that the enzyme activity was very narrowly distributed in aerobic spore-forming gram-positive mesophiles, *Sporosarcina ureae* and *Bacillus sphaericus*. The enzyme from *Rhodococcus* sp. M4, *Bacillus badius*, *Rhodococcus maris* and *Norcardia* sp. 239 were later found in the same group of mesophiles. The thermostable PheDH has been studied by Ohshima *et al*. (1991) in *Thermoactinomyces intermedius* IFO1423. Moreover, further study by Asano and Tanetani in 1998, the unique strain showing PheDH occurred in *Microbacterium* sp. DM 86-1 which was non-spore forming bacteria was found. The distribution of PheDH which was limited to some groups of gram-positive, spore forming including actinomycetes may be due to this enzyme involves in microbial sporulation thereby connecting the carbon and nitrogen metabolism of amino acids.
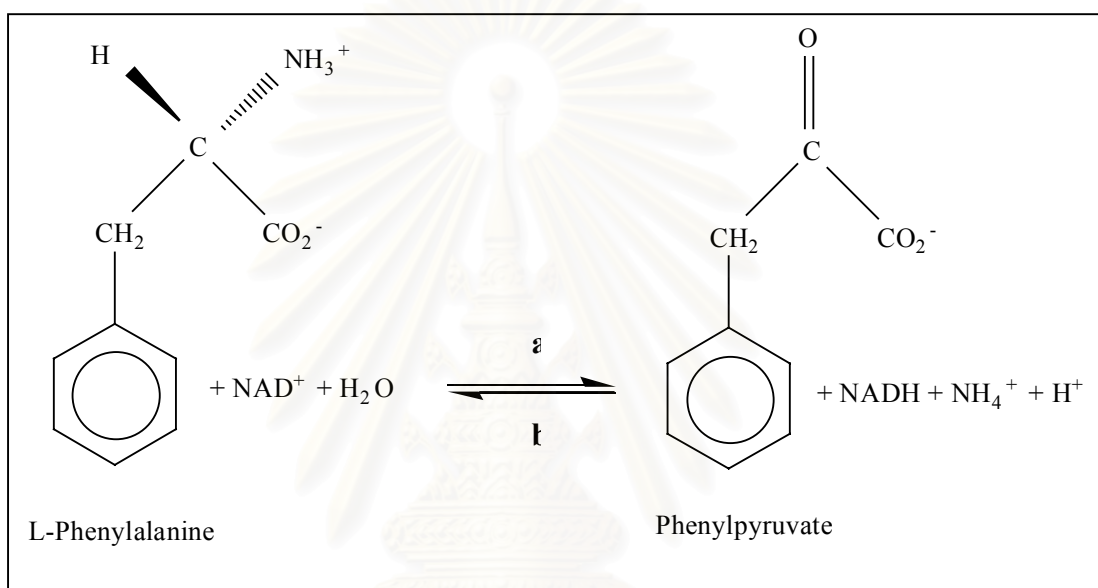
Figure 1.1 Reaction of phenylalanine dehydrogenase (Brunhuber and

Blanchard, 1994)

(a) oxidative deamination

(b) reductive amination

However, since *Microbacterium* strain does not form spores, the PheDH seems to be involved in the catabolism of L-phenylalanine and other amino acids (Asano and Tanetani, 1998). Thus, this enzyme is speculated to play an important role in the degradation of L-phenylalanine (Hummel *et al*., 1984; Hummel *et al*., 1987).

1.1.1 Purification of phenylalanine dehydrogenase

The study on physical and biochemical properties of PheDH requires separation techniques to purify enzyme. This enzyme has been already purified by means of multistage chromatography columns (Asano *et al*., 1987a; Asano *et al*., 1987b; Asano and Tanetani, 1998; Misono *et al*., 1989). Typical methods reported so far including heat treatment, protamine precipitation, ammonium sulfate precipitation, ion exchange chromatography (mostly by DEAE-Toyopearl column), adsorption chromatography (mostly by hydroxyapatite column), hydrophobic interaction chromatography (mostly by Butyl-Toyopearl column) and gel filtration chromatography. For instance, in 1988, Hummel *et al*. was successfully used an aqueous two-phase system composed of polyethylene glycol, potassium phosphate and sodium chloride in the industrial purification of PheDH from *Brevibacterium* sp. and later in 1989 applied the same procedures to purify enzyme from *Rhodococcus* sp. M4. The affinity chromatography, for example Red-sepharose CL 4B column, was also applicable to the PheDH from *R. maris* (Misono *et al*., 1989) and *T. intermedius* (Ohshima *et al*., 1991). Ion exchange high performance liquid chromatography or FPLC using mono Q column was also effectively used as one common step in the purification procedures (Asano *et al*., 1987a; Asano *et al*., 1987c; Misono *et al*., 1989; Asano and Tanetani, 1998). Tynan *et al*. (2000) described a novel kinetic locking-on strategy for bioaffinity purification of NAD^+-dependent dehydrogenases including PheDH based on immobilized cofactor derivatives through the use of enzyme-specific substrate analogues to promote selective and biospecific adsorption. To gain the straightforward and simple methods for purification of enzyme, Omidinia *et al*. (2002) also described new method using reactive blue 4 dye which was robusted affinity ligands promising for industrial-scale bioprocesses. This method provided a

facile and effective way for preparing the enzyme with a good yield that suitable for analytical purposes.

1.1.2. Basic molecular and catalytic properties of phenylalanine dehydrogenase

Some basic molecular and physicochemical properties of various microbial PheDH are summarized in **Table 1.1**. The PheDH from *S. ureae*, *B. sphaericus*, *B. badius* and *Microbacterium* sp. were characterized by their octameric structure, whereas *T. intermedius*, *Rhodococcus* sp. M4, *R. maris* and *Norcardia* sp. were found to be hexameric, dimeric, tetrameric and monomeric, respectively. The subunit molecular masses exhibit a narrow range between 36 and 42 kDa. PheDH is a member of a large family of amino acid dehydrogenases which includes glutamate dehydrogenase (GluDH), alanine dehydrogenase (AlaDH), leucine dehydrogenase (LeuDH) and the less common lysine ε-dehydrogenase (LysDH) and *meso*-α, ε-diaminopimelate D-dehydrogenase (DAPDH). Each of these enzymes has been identified, purified and physically characterized (Brunhuber and Blanchard, 1994). The stereospecificity of hydride transfer is conserved characteristic of amino acid dehydrogenases, with most enzymes reported so far catalyzing the transfer of the 4S hydrogen of the nicotinamide moiety of the reduced coenzyme, NAD(P)H, to the substrate amino acids as shown in **Figure 1.2** (Brunhuber *et al.*, 2000). The exceptions to this rule include AlaDH (Ohshima and Soda, 1989), LysDH (Hashimoto *et al.*, 1989) and DAPDH (Scapin *et al.*, 1996). The stereochemistry of hydride transfer from NAD(P)H was proved to be B-stereospecific or *pro-S* for PheDH from *B. sphaericus*, *T. intermedius* and *Rhodococcus* sp. M4. In all cases, the *pro-S* hydrogen of NADH was transferred to generate [2-^{2}H]-L-phenylalanine. The equilibrium constants (K_{eq}) of 4.5 x 10^{-14} M^2, 1.4 x 10^{-15} M^2 and 3.2 x 10^{-18} M^2, determined for the PheDH reaction from *Rhodococcus* sp. M4, *B. sphaericus* and *Norcardia* sp, respectively, were typical to other amino acid dehydrogenases (Brunhuber and Blanchard, 1994).

To extend the use of amino acid dehydrogenases as biocatalysts and to enable more detailed in enzymological studies, a more abundant and cheaper supply of stable

Table 1.1 Comparison of properties of phenylalanine dehydrogenase from various microorganisms [a]

Properties	*Brevibacterium* sp.	*Rhodococcus* sp. M4	*S. ureae*	*B. sphaericus*	*B. badius*	*R. maris*	*Norcardia* sp.	*T. inter-medius*	*Microbac-terium* sp.
Specific activity of final preparation (U/mg protein)	-	-	84	111	68	65	30	86	37
Native *Mr*									
-gel filtration	-	150,000	310,000	340,000	335,000	70,000	42,000	270,000	330,000
-deduced amino acid sequence	-	-	330,608	331,480	330,800	-	-	249,928	-
***Mr* of subunit**	-	39,500	41,326	41,435	41,350	36,000	42,000	40,488	41,000
Number of subunit	-	4	8	8	8	2	1	6	8
Isoelectric focusing point (pI)	-	5.6	5.3	4.3	3.5	-	-	-	5.8
pH optimum									
-oxidative deamination	10.5	10.1	10.5	11.3	10.4	10.8	-	11.0	12.0
-reductive amination	8.5	9.25	9.0	10.3	9.4	9.8	10.0	9.2	12.0
Thermostability (% remaining activity after incubation)	-	-	75 (40 ºC, pH 9, 10 min)	100 (55 ºC, pH 9, 10 min)	50 (55 ºC, pH 8, 10 min)	100 (35 ºC, pH 7.4, 10 min)	50 (53 ºC, pH 9.5-10, 2 h)	100 (70 ºC, pH 7.2, 1 h)	100 (55 ºC, pH 9, 10 min)
Substrate specificity [b]									
Oxidative deamination									
L-phenylalanine	100	100	100	100	100	100	100	100	100
L-tyrosine	-	12	5	72	9	2	-	0	4

Table 1.1 Comparison of properties of phenylalanine dehydrogenase from various microorganisms [a] (continued)

Properties	*Brevibacterium* sp.	*Rhodococcus* sp. M4	*S. ureae*	*B. sphaericus*	*B. badius*	*R. maris*	*Norcardia* sp.	*T. intermedius*	*Microbacterium* sp.
Substrate specificity [b]									
Reductive amination									
phenylpyruvate	100	100	100	100	100	100	100	100	100
p-hydroxyphynylpyruvate	96	5	24	136	53	91	28	0	21
indole-β-pyruvate	24	3	1	0	-	5	54	-	-
Apparent K_m (mM)									
L-phenylalanine	0.385	0.87	0.096	0.22	0.088	3.8	0.75	0.22	0.10
NAD^+	0.125	0.27	0.14	0.17	0.15	0.25	0.23	0.078	0.20
NADH	0.047	0.13	0.072	0.025	0.21	0.043	-	0.025	0.072
phenylpyruvate	0.177	0.13	0.16	0.4	0.106	0.5	0.06	0.045	0.02
ammonia	431	387	85	78	127	70	96	106	85
References	Hummel *et al*, 1984 ; Hummel *et al*., 1989	Ziehr *et al*, 1987; Vanhooke *et al*, 1999; Brunhuber *et al*, 1994 and 2000	Asano and Nakazawa, 1985; Asano *et al*, 1987b	Asano *et al*, 1987a	Asano *et al*, 1987b	Misono *et al*, 1989	de Boer *et al*, 1989	Ohshima *et al*, 1991	Asano and Tanetani, 1998

[a] *S.*; *Sporosarcina*, *B.*; *Bacillus*, *R.*; *Rhodococcus*, *T.*; *Thermoactinomyces*

[b] Substrate specificity expressed as relative activity (%)

- = data was not detected
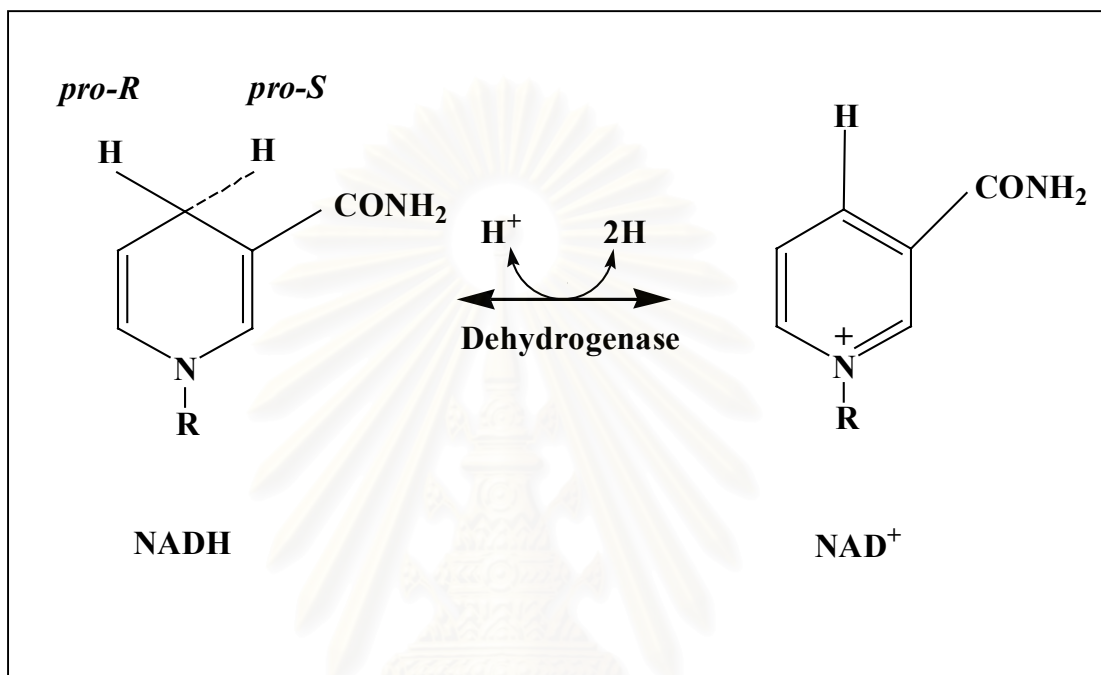
Figure 1.2 Stereospecificity of hydrogen transfer of NADH catalyzed with dehydrogenases (Ohshima and Soda, 1990)

R represents ADP-ribosyl

amino acid dehydrogenases were required (Ohshima and Soda, 1989). Extensive developments of the techniques in gene cloning and related fields have enabled rapid determination of the primary structures of amino acid dehydrogenases. Moreover, x-ray crystallographic analysis of several amino acids dehydrogenases has been under taken and revealed their ternary and quaternary structures in details (Ohshima and Soda, 2000). The three-dimentional x-ray crystallographic analysis on the hexameric GluDH from *Clostridium symbiosum* (Baker *et al*., 1992), the octameric LeuDH from *B. sphaericus* (Baker *et al*., 1995), the dimeric DAPDH from *Corybacterium glutamicum* (Scapin *et al*., 1996), the hexameric AlaDH from cyanobacterium, *Phormidium lapideum* (Baker *et al*., 1998) and the dimeric PheDH from *Rhodococcus* sp. (Vanhooke *et al*., 1999) were done.

For PheDH, there are few reports about cloning available. The gene encoding the enzyme was cloned and primary structures were so far sequenced from *B. badius* (Asano *et al*., 1987a and Yamada *et al*., 1995), *B. sphaericus* (Okazaki *et al*., 1988; Asano *et al*., 1990), *S. ureae* (Asano and Nakazawa, 1985), *T. intermedius* (Takada *et al*., 1991) and *Rhodococcus* sp. M4 (Brunhuber *et al*., 1994) by peptide and DNA sequencing methods. Although a computer-aided search of the protein sequence data base revealed rather low overall sequence similarities among amino acid dehydrogenases, a common partial sequence of about 30 residues in the nicotinamide coenzyme binding domain was observed (Takada *et al*., 1991). The coenzyme binding domain which binds the adenine nucleotide moiety shows a high degree of conservation of tertiary structures; it consists of a two-stranded parallel β-sheet and one α-helix with virtually identical arrangement (Ohshima and Soda, 1990).

In 1994, the structure of the dimeric PheDH from *Rhodococcus* sp. M4 that was the first example of the amino acid dehydrogenases with a ternary complex with enzyme•NAD$^+$•phenylpyruvate and enzyme•NAD$^+$•β-phenylpropionate was revealed by Brunhuber and Blanchard. It is found that the PheDH is a homodimeric and each monomer is composed of distinct globular N- and C-terminal domains which form a deep cleft containing the active site (**Figure 1.3**). The N-terminal domain binds the substrate and plays an important role in the reaction on the subunit-subunit interface. This region of high homology is thought to be the amino acid binding and catalytic
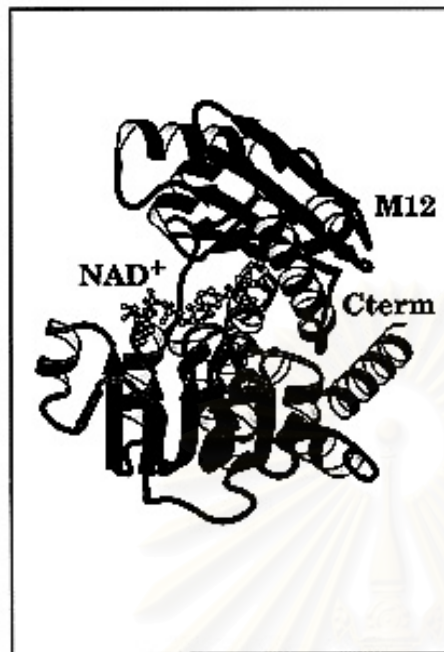
(a)
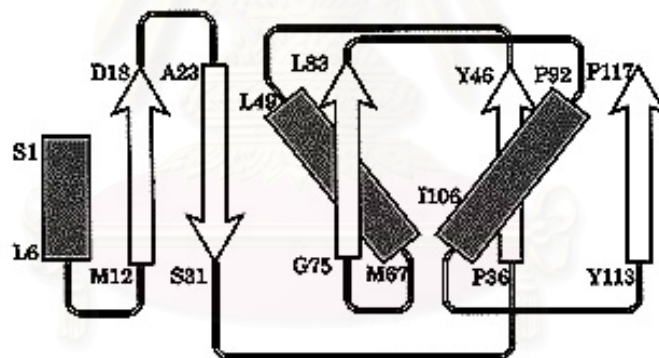
(b)

Figure 1.3 Structure of the *Rhodococcus* sp. M4 phenylalanine dehydrogenase
(Vanhooke *et al*., 1999)

a) Ribbon representation of one subunit of PheDH●NAD$^+$● phenylpyruvate ternary complex

b) The topology corresponding to the substrate binding domain. Rectangles and arrows represent α-helices and β-strands, respectively.

domain of this enzyme. The C-terminal domain forms a typical Rossmann fold, which consists of β–α–β–β–α–α–β–β–α–β structure, responsible for NAD^+ binding as found for GluDH and LeuDH. This shows that amino acid dehydrogenases are composed of structurally independent coenzyme and substrate binding domain. On the basis of the comparison of sequence homology, the highest homology was found between *Rhodococcus* PheDH and the LeuDH from *B. stearothermophilus* (39% sequence identity), followed by the PheDH from *B. sphaericus* and *T. intermedius* for 34% and 32%, respectively (Brunhuber and Blanchard, 1994).

To unravel the mechanism of substrate recognition, the chimeric enzyme have constructed by the two amino acid dehydrogenases, N-terminal domain of PheDH from *T. intermedius* containing the substrate binding region and a C-terminal domain of LeuDH from *Bacillus stearothermophilus* containing the NAD^+-binding region. The two enzymes share an overall sequence similarity for 47%. Although the catalytic efficiency of the chimeric enzyme on L-phenylalanine is 6% of the parental PheDH, the chimeric enzyme shows a similar K_m value for L-phenylalanine, pH optimum, and the same stereospecificity for hydrogen transfer at the C-4 position of the NADH. In contrast, the substrate specificity of the chimeric enzyme differs from PheDH: the chimeric enzyme shows lower substrate specificity than the parental PheDH (**Figure 1.4**). In addition, the chimeric enzyme acts on L-branched chain amino acids such as L-valine and L-isoleucine. The specificity of the chimeric enzyme in the reductive amination is an admixture of the specificities of the two parent enzymes (Kataoka *et al.*, 1994a). This suggested that amino acid dehydrogenases exhibit new substrate specificity. Indeed, in the same year, Kataoka and his colleagues (1994b) also reported the combination of chemical modification with a monoanionic acetylation reagent, methyl acetyl phosphate (MAP) and the site-directed mutagenesis of the *T. intermedius* PheDH which was useful not only for identification of active site lysyl residues (Lys-69 and Lys-81) but also to elucidate the electrostatic environment around the active site.

From the study by Seah *et al.* in 1995, PheDH from *B. sphaericus* also has been studied on substrate recognition mechanism. Gly-124 and Leu-307 were altered
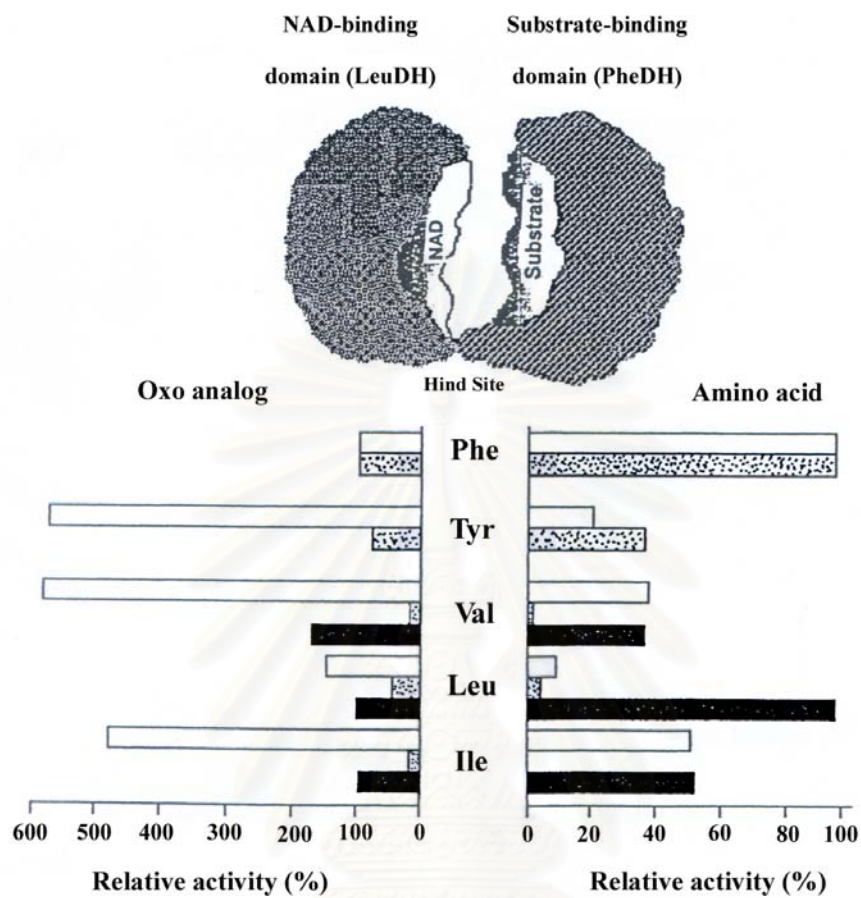
**Figure 1.4 Scheme of the chimeric enzyme consisting of an amino terminal
domain of phenylalanine dehydrogenase and carboxyl terminal
domain of leucine dehydrogenase** (Kataoka *et al*., 1994)

Comparison of substrate specificity of PheDH (□), chimeric enzyme
(▨), and LeuDH (■) on both amination and deamination

by site-specific mutagenesis to the corresponding residues in LeuDH: alanine and valine, respectively. These two residues have previously been implicated from molecular modeling as important in determining the substrate discrimination of the two enzymes. Single and double mutants displayed lower activities towards L-phenylalanine and enhanced activity towards almost all aliphatic amino acid substrates tested compared to the wild type.

1.2 Current methods for phenylalanine production

Amino acids gained commercial significance shortly after the turn of the century with the increased knowledge of the role amino acids play in the value of nutritional protein led to their being used to fortify animal feeds, as food supplements for humans, and to sustain seriously ill patients who had to be fed with intravenous solutions (www.foodsupplementwatch.com). Phenylalanine is an essential amino acid that is also one of the most commonly found aromatic amino acids. It plays a key role in the biosynthesis of other amino acids, tyrosine and subsequently to L-dopa which in turn is used to synthesize neurotransmitters, dopamine and norepinephrine. It is available in three different forms. L-phenylalanine is incorporated into the body's proteins. D-form acts as a painkiller. DL-form is a combination effect of the two (Russell and McCarty, 2000; www.doctoryourself.com). There have been reported that concurrent treatment with DL-phenylalanine (DLPA) often appears to potentiate chronic pain relief (Russell and McCarty, 2000). This nutrition could prove of benefit to people suffering from Parkinson's disease, tiredness, depression, painful condition such as arthritis. With all the researches that have been tried with new drugs to mimic phenylalanine's ability to enhance the body's natural endorphins, not one product has been discovered. Phenylalanine remains the safest and best all around pain fighter there is (Russell and McCarty, 2000).

In food industry, phenylalanine is used as a main intermediate utilized in the manufacture of a dipeptide artificial sweetener, aspartame (Hummel *et al*., 1987 and Schmidt *et al*., 1987). Moreover, it is a precursor of benzaldehyde, an important aromatic compound participating in flavor in the manufacture of cheese (Smit, 2004).

Various methods have been shown to achieve the production of phenylalanine. Traditionally, the syntheses of amino acids have been carried out by chemical methods (Nakamichi *et al*., 1984). However, the increasing demand for the optically active forms of amino acids has called for the investigation of the bioprocessing. Fermentative and enzymatic processes in L-phenylalanine production have been proposed and developed over the last two decades in several ways. In 1981, Yamada *et al*. studied the rapid conversion of *trans*-cinnamic acid to L-phenylalanine using *Rhodotorula glutinis* containing L-phenylalanine ammoniumlyase activity with 70% conversion yield. In addition, the production from acetamidocinnamic acid by using a specific acylase and transaminase found in *Alcaligenus faecalis* and *B. sphaericus*, respectively was proposed by Nakamichi *et al*. in 1984. The α-keto acids can also be converted to amino acids by amination. Chemically synthesized phenylpyruvate was converted to L-phenylalanine by *Coryebacterium glutamicum* which showed a high L-phenylalanine aminotransferase activity (Bulot and Cooney, 1985). As well as the study of Nakamichi *et al*. later in 1989, *Paracoccus denitrificans* also contained aminotransferase activity which was used both free and immobilized form without lost of activity. Moreover, Chao *et al*. (2000) successfully employed the immobilized cells which contained aminotransferase and aspartase activities entrapped in sodium alginate. Although the result illustrated a potential and attractive process to yield both L-aspartic and L-phenylalanine, the entrapping cells were not an appropriate approach due to the cracking and damaging surface of the immobilized particle.

Reductive amination offers another route for the conversion of corresponding α-keto acids with a specific NAD(H) amino acid dehydrogenase to chiral compounds. In this case, a method for enzymatic synthesis of L-phenylalanine with NAD^+-dependent PheDH has been investigated. However, the application of this enzyme to industrial production of L-phenylalanine has been hampered by the cost of coenzyme because it is complex and rather labile organic chemical. A multi-enzyme reaction system for simultaneous coenzyme regeneration has been proposed to overcome this problem. PheDH from *Brevibacterium* sp. (Hummel *et al*., 1986) and that from *Rhodococcus* sp. M4 (Hummel *et al*., 1987) have been successfully used for the production of L-phenylalanine in a membrane reactor exhibiting the behavior of a

continuously stirred tank reactor. The continuous conversion of phenylpyruvate was carried out by PheDH. The coenzyme, NADH which is required in stoichiometric amounts, was therefore regenerated by a second enzyme, formate dehydrogenase (FDH). In order to retain the coenzyme behind the ultrafiltration membrane, it was covalently bound to a polyethylene glycol 20,000. This way, the retention of the coenzyme by an ultrafiltration membrane was accomplished together with separation of the coenzyme from the product stream. The production scheme is shown in **Figure 1.5**. In comparison to *Brevibacterium* sp., *Rhodococcus* sp. M4 posses a high level of PheDH with lower substrate excess inhibition, obviously better stability and higher space time yield (Hummel *et al.*, 1987). PheDH from *B. sphaericus* was also quantitatively synthesized optically pure L-phenaylalanine, tyrosine and some other amino acids from their oxo analogs couple with FDH from *Candida boidinii*. Moreover, the enzyme overproduced in *Escherichia coli* JM109 was effectively used for the syntheses of (*S*)-amino acids from unnatural substrates. The continuously synthesized of L-phenylalanine was carried out with the enzyme packed in a dialysis tube (Asano *et al.*, 1990).

To lower the cost of commercial production of phenylalanine and to avoid the instability of phenylpyruvate in aqueous solutions, two alternative routes have been studied (Hummel and Kula, 1989). One started from the racemic mixture of phenyllactate, the other started from acetamidocinnamic acid. The conversion of DL-phenyllactate was achieved utilizing the side reaction of two enzymes as shown in **Figure 1.6a**. However, the kinetic properties of the enzyme involved in the cyclic reaction made this approach unfavorable. Another stable precursor, acetamido-cinnamic acid was used. Deacetylation resulted in an unstable enamine-imine derivative, which hydrolyzed spontaneously to yield phenylpyruvate (**Figure 1.6b**). This system has been later developed by Cho and Soda (1997). An aminoacylase, inducibly formed in *Bacillus thermoglucosidius* grown with a synthetic compound, acetamidocinnamic acid, and the *T. intermidius* PheDH were used for enzymatic synthesis of L-phenylalanine from low-priced chloroacetamidocinnamic acid. The reaction system consisted of the hydrolysis of chloroacetamidocinnamate to phenylpyruvate by aminoacylase and the reductive amination of phenylpyruvate to

a)

HCOO⁻

PEG-NAD⁺

L-Phenylalanine + H₂O

CO₂

PEG-NADH

Phenylpyruvate + NH₄⁺

b)

L-Phenylalanine ◇

CO₂ ■

Phenylpyruvate ◆

NH₄COOH □
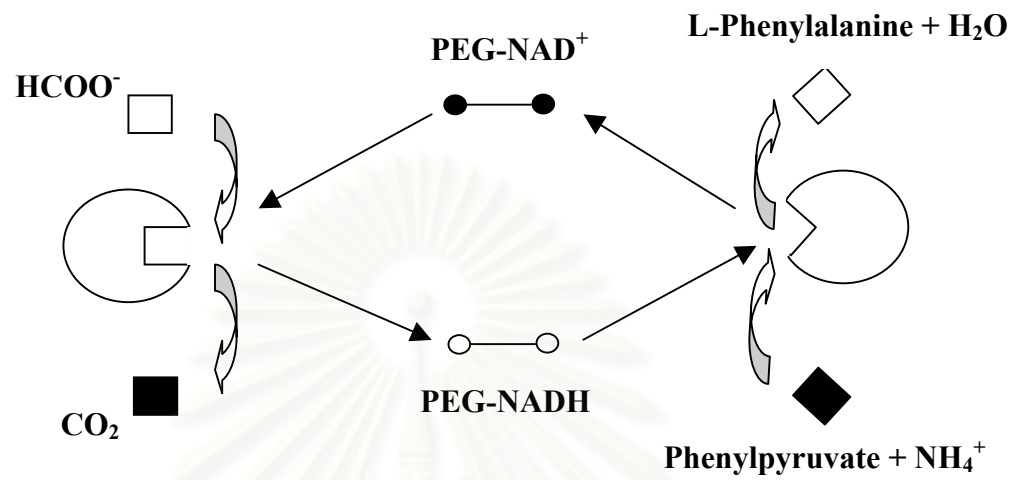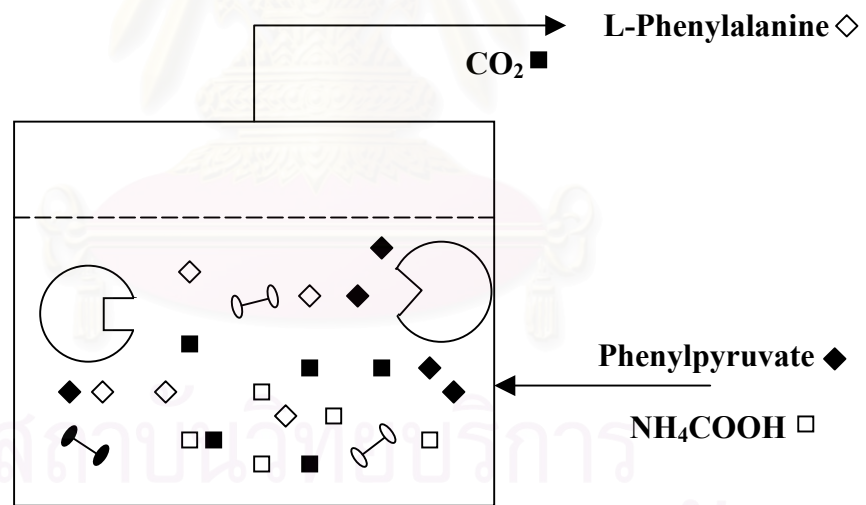
Figure 1.5 Enzymatic synthesis of L-phenylalanine with coenzyme regeneration

(Hummel *et al*., 1986)

a) Reaction scheme

b) Reactor scheme

Formate dehydrogenase   Phenylalanine dehydrogenase
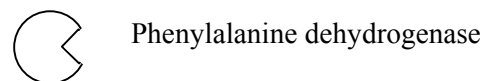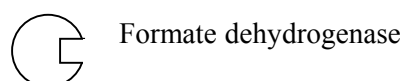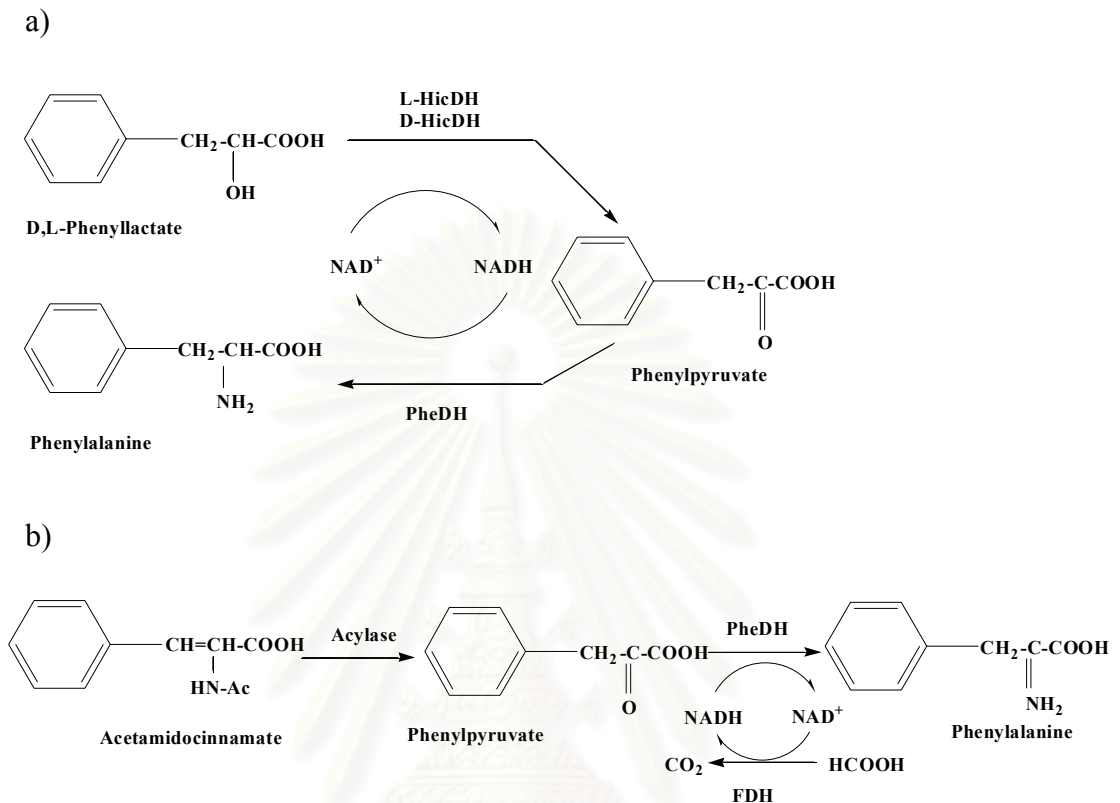
Figure 1.6 Enzymatic routes for the preparation of L-phenylalanine (Hummel
and Kula, 1989)

(a) Oxidation of DL-phenyllactate with D- and L-2-hydroxy-4-
methylpentanoate dehydrogenase (HicDH) and simultaneous reductive
amination of the *in situ* formed phenylpyruvate with PheDH. NADH is
substrate-coupled regenerated from phenyllactate.

(b) *In situ* formation of phenylpyruvate by enzymatic deacetylation of
acetamidocinnamic acid (acylase) followed by simultaneous reductive
amination with PheDH

L-phenylalanine by PheDH. The coenzyme consumed was effectively regenerated by a couple reactions with FDH shown in **Figure 1.7**. Under optimum condition, more than 98% of conversion was obtained without decomposition or racemization. This method may be promising as a commercial process.

1.3 Enzyme immobilization

An 'immobilized enzyme' can be defined as any enzyme which is not freely soluble and whose movement in space is restricted completely or partially to a small region with retention of their catalytic activity.

1.3.1 Advantages of immobilized enzyme

From the chemical engineering viewpoint, enzymes have characteristics to be ideal catalysts. However, soluble enzymes in general have little industrial applications other than as commercial biocatalysts. The disadvantages of using free soluble enzymes in solution hampered the wide application of the enzyme catalysts. Firstly, the cost of enzymes is very high for single use. Secondly, the contamination of the enzymes in the product streams is difficult to remove. In addition, soluble enzymes are usually inhibited by substrate and product. Finally, thermal instability of soluble enzymes in solution is usually occurred and thus, prevents the wide application of enzymes (Guilbault and Sadar, 1979; Macalta *et al.*, 1991; Schmid *et al.*, 2002; Krajewska, 2004; Yazbeck *et al*, 2004).

Some of the above mentioned drawbacks could be avoided or minimized by water insoluble forms of enzymes which offer many advantages over their soluble form. They usually provide, in addition to the desired reuse of the enzyme, unexcelled advantages such as ease in product separation and easy recovery. Other favorable considerations include a greater stability of enzyme activity over broad ranges of pH and temperature, their greater operational stability and the possibility for their use in continuous process. This last advantage would, in principle, facilitate the development of continuous, large-scale commercial processes (Yang and Chase, 1998; Albayrak and Yang, 2002).
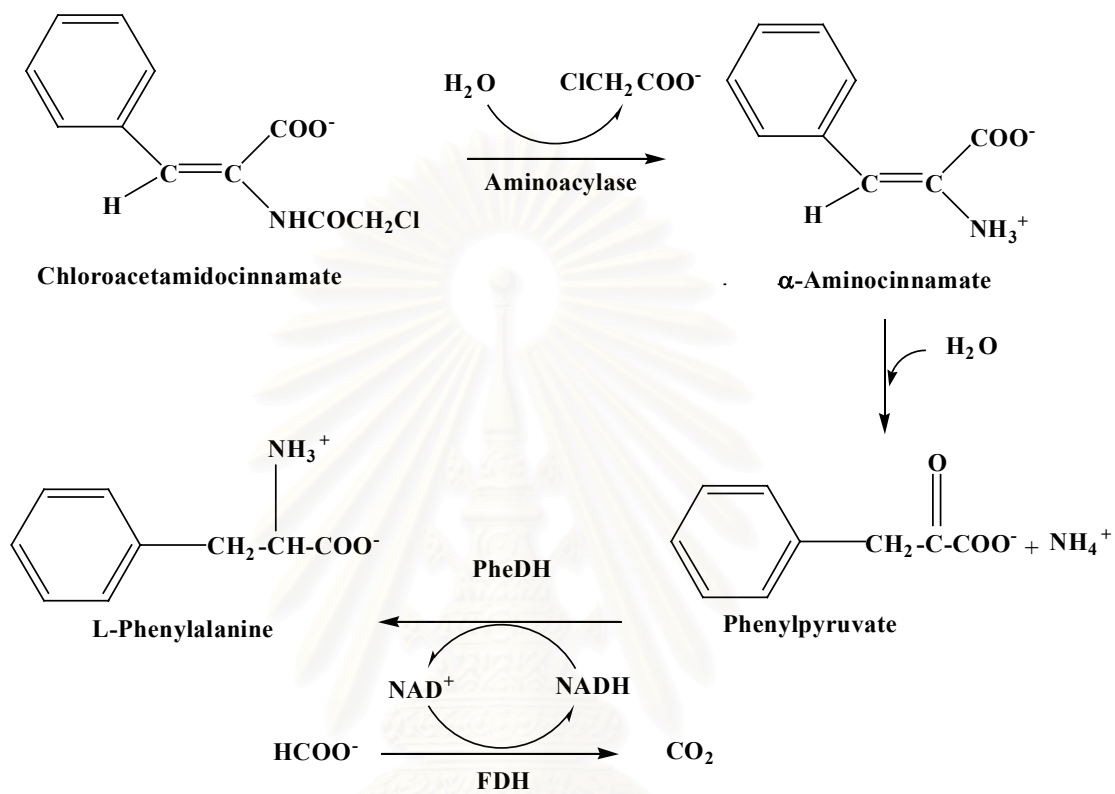
Figure 1.7 Coupled reaction systems for enzymatic synthesis of L-phenylalanine from chloroacetamidocinnamate and ammonium formate (Cho and Soda, 1997)

1.3.2 Immobilization methods

Immobilization of enzyme is basically undertaken either for the purpose of basic research or for the use in technical process of commercial interest. Studies on immobilized enzymes and their applications particularly in the biochemical, biomedical, pharmaceutical and food industries continue to expand. To obtain maximum possible service from the catalytic potential of the enzyme, it remains challenging to develop the most appropriate carrier together with the correct immobilization method (Chase and Yang, 1998). That is, in general, the protein structure can be altered by immobilization thereby changing the properties of the enzyme. Stearic hindrance due to immobilization may also interfere with the contact between the enzyme and substrate. The degree of change depends on the method of immobilization, the nature of the support and coupling agent and the specificity of the reactive groups. Hence, immobilization can be achieved in many ways, but it always affects enzyme activity to some extent (Chellapandian, 1998). The availability of large number of support materials and the mode of attaching the enzymes to the carrier leave virtually no bioactive species without a feasible rate of immobilization. It is, thus, important that the choice of support material and immobilization method over the free bioactive agent should be well justified (Arica *et al.*, 2004).

Numerous methods for achieving immobilization of enzymes are available (**Figure 1.8**), each involving a different degree of complexity and efficiency. Various methods used to date may be subdivided into two general classes. These methods open up possibilities of efficient industrial applications that are chemical methods, where covalent bonds are formed with the enzyme, and physical methods, where weaker interactions between support and enzyme exist (Kenedy, 1990; Chaplin and Bucke, 1990).

The detailed descriptions of different procedures along with applications were reported by various authors (Balcão *et al.*, 1996; Costa *et al.*, 2001; Yağar and Sağiroğlu, 2002; Park *et al.*, 2005). Adsorption and entrapment are included in
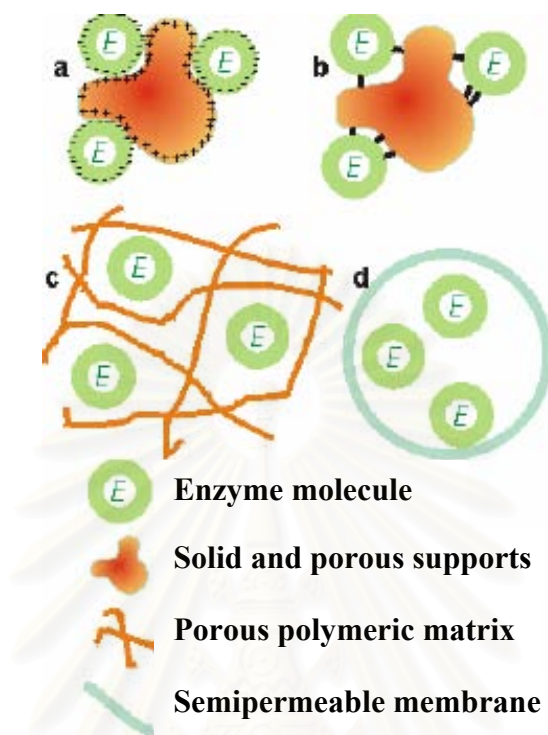
Figure 1.8   Immobilized enzyme systems (Chaplin and Bucke, 1990)

(a) enzyme non-covalently adsorbed to an insoluble particle; (b) enzyme covalently attached to an insoluble particle; (c) enzyme entrapped within an insoluble particle by a cross-linked polymer; (d) enzyme confined within a semipermeable membrane

physical immobilization. Covalent bonding and cross-linking are encountered in chemical immobilization. Characteristics of the immobilization of enzymes by the different approaches are briefly summarized below and presented in **Table 1.2**.

i) Adsorption, one example of physical interactions, is simple, inexpensive and easy to perform by the adhesion of an enzyme to the surface of the support which has not been specifically modified for covalent attachment. The physical adsorption usually depends on the intrinsic affinity of the carriers to adsorb the enzyme from the solutions. The binding forces involved ionic interaction, hydrogen bond, hydrophobic or even Van der Waals interactions (Gemeiner, 1992). The advantages of this method are its simplicity, large choice of differently charged and shaped of supports and since the operational conditions are often mild, they would not normally inactivate the enzyme. Regeneration is often possible after treatment with detergents, strong denaturants, or wetting agents. However, enzyme leakage by this method is highly dependent on pH, solvent and temperature. In other words, the change in pH, ionic strength or particular additives in the sample can easily cause desorption of the adsorbed proteins (Chaplin and Bucke, 1990). The limited half-life of the immobilized enzyme, which is a consequence of it being progressively released in the solution, is a drawback to its possible practical applications (Martino *et al*., 1996a).

ii) Entrapment of enzymes entails capture of the enzymes within a matrix of a crosslinkable resin or of a gel matrix. Various synthetic polymers like polyacrylamide and polyvinylalcohol have been used for the immobilization of enzymes by this technique. By this means of immobilization, the enzyme itself does not bind to the gel matrix which gives no change in the enzyme's intrinsic properties. However, the leakage of trapped enzyme from the matrix network can occur and another setback is the severe diffusional limitations in case of high molecular weight reactants are used (Chaplin and Bucke, 1990; Jia, 2002). Possible denaturation of the enzyme molecules as a result of free radicals can also occur (Scouten *et al*., 1995).

Table 1.2 Comparison of the attributes of different classes of immobilization techniques (Kenedy and Cabral, 1987)

Characteristics	Physical methods		Chemical methods	
	Adsorption	Entrapment	Covalent binding	Cross linking
Preparation	Easy	Difficult	Difficult	Difficult
Enzyme activity	Moderate	High	High	Moderate
Binding force	Weak	Strong	Strong	Strong
Regeneration	Possible	Impossible	Impossible	Impossible
General application	Low	High	Moderate	Low
Hydraulic properties	Good (in case of porous carriers)	Moderate	Good	Not good
Stability	Changeable (in pH)	Long life	Greater thermal stability	Greater thermal stability
Cost of immobilization	Low	Low	High	Moderate

iii) Covalent bonding of enzymes to solid matrices is the most widely employed technique in the development of enzyme engineering in order to stabilize enzymes bonding to support. Covalent attachment involves derivatization of the support to provide a chemical group capable of reacting with suitable coupling reagent and subsequently reacting with available functional groups of the proteinaceous backbone of enzyme. This method has the advantages of good retention of activity in use, high stable linkages thus inhibiting leakage and sometimes rendering excellent accessibility and thermal stability (Veilleux and Duran, 1996; Jia, 2002). Stability of enzyme might be achieved through the multiple point covalent attachment that the enzyme rigidity would be greatly increased. Hence, any conformational change on the enzyme structure becomes greatly prevented (Fernández-Lafuente *et al*., 1999). The functional groups which can take part in the covalent bond formation to the support are N-terminal groups, C-terminal groups, aspartate β and glutamate γ-carboxylate groups, cysteine thiol groups and tyrosine phenol groups and the most common reactive group, lysine ε-amino. In addition, the carbohydrate residues of glycoproteins can also provide centers for immobilization (Jurgensen *et al*., 1981; Gregorius *et al*., 1995; Nahar *et al*., 2001; Bílková *et al*., 2002; Martin *et al*., 2003; and Chiou and Wu, 2004).

One of the classical examples for covalent bonding is the treatment of porous glass (Weetall, 1976) or alumina (Dalvie and Baltus, 1992; Martino *et al*., 1996b) with γ-aminopropyltriethoxysilane (APTS) in organic solvent or aqueous solution, followed by reaction with the pretreated beads with glutaraldehyde solution. The silanization of glass and inorganic material method has been widely applied for immobilization of many enzymes (Dalvie and Baltus, 1992; Cho and Rhee, 1993). It has been reported that organic silanization of glass beads gave much higher loadings of alkylamine for covalent bonding of enzymes through glutaraldehyde (Jia, 2002). However, greater carrier durability with slightly lower enzymes loading is achieved by aqueous silanization (Weetall, 1976). Other methods of activation of inorganic materials are also available such as epoxy-, vinyl-, sulfhydryl-, alkylchloro-, and phenylsilanes. Cyanogen bromide activated Sepharose using ethyl dimethylamino-

propyl carbodiimide, agarose beads, cellulose, Sephadex and chitosan are also widely used instead of inorganic support (Jia, 2002).

The covalent immobilization may result in better biomolecular activity, reduced nonspecific adsorption and greater stability against temperature, denaturants, and organic solvents in several cases (Nahar *et al*., 2001; and Bayramoğlu *et al*., 2004). Since covalent attachment is a chemical method, whereby the immobilization is dependent on the formation of stable covalent bonds, the binding force is expected to be strong and thus leakage of the enzyme does not happen even when washed with salt solutions of high ionic strength (Chibata, 1978). This is also ideal for mass production and commercialization (Scouten *et al*., 1995). There are of course some disadvantages to this method that are complication, time consuming and expensive because of more chemical reagents involved in the preparation. Generally, covalent bonding lowers the immobilized enzyme activity compared to that from free soluble enzyme (Jia, 2002). It is also possible of activity loss due to the chemical modifying near the active site of enzymes (Braun *et al*., 1996). So it is highly desirable to have some knowledge of the amino acid residues that are essential for catalysis or binding so as to avoid complete inactivation of the enzyme upon immobilization (Gemeiner, 1992; Goel, 1994; Unlig, 1998). Furthermore, the carriers could not be easily regenerated after enzyme deactivation because of tight binding force between enzymes and carriers. However, in some cases, immobilized enzyme can show higher activity than that of free enzyme. Noda *et al*. (2001) proposed that immobilized β-amylase activity was higher than that of the free β-amylase on the alkaline side (pH 7.0-10.0).

iv) Crosslinking is, that can be said, the most suitable since a considerable enzyme load can be achieved on the support (Martino *et al*., 1996a). This method involves the formation of covalent bonds between enzyme molecules and the bifunctional reagent to give an intermolecularly crosslinked immobilized enzyme preparation. Low molecular weight bifunctional reagents are specialized reagents having reactive groups that will form a bond between two different groups, either on the same molecule or two different molecules. The bifunctional reagent glutaraldehyde has been extensively used as a crosslinker (Parrado *et al*., 1995; Ukeda

et al., 1996; Martino *et al*., 1996b; Gallifuoco *et al*., 1998; Çentinus and Öztop, 2000; and Arica *et al*., 2004).

The advantage of the crossliking method is that the procedure is simple. It is widely used in stabilizing physically adsorbed enzyme or proteins that are covalently bound onto a support. As a matter of fact, this method shows some disadvantages including the difficulty to control the pH of the reaction, concentration and other parameters which limit mechanical stability of the immobilized system (Unlig, 1998). Cross-linking an enzyme to itself is both expensive and insufficient, as some of the protein material will inevitably be acting mainly as a support. Cross-linking reactions are carried out under relatively severe conditions. These harsh conditions can change the conformation of active center of the enzyme; and so may lead to significant loss of activity (Goel, 1994). In addition, large amounts of enzyme are required and the enzyme may be inactivated which is caused by the chemical modification. The gelatinous nature of the protein layer, lacking of rigidity, relatively low enzyme activity are also observed (Scouten *et al*., 1995).

1.3.3 Characteristics of enzyme support materials

As far as the usefulness of immobilized enzymes have been applied worldwide, with the sole exception of the enzyme, another factor which is indeed the most important contributing component to the performance of an immobilized enzyme system is the carriers. A carrier imprudently chosen will adversely affect not only the half-life but also the performance of the immobilized enzyme. Factors that bearing upon the performance of the carrier are inevitably affected the performance of the enzyme. These factors included pH, temperature, ionic strength, pressure, agitation, cofactor delivery and substrate delivery with product removal (Messing, 1978). The physical structure and chemical composition of support can also influence the microenvironment of the immobilized enzymes and consequently their biological properties (Arica *et al*., 2000).

Both natural (organic and inorganic materials) and synthetic supports are used for the preparation of immobilized enzymes. Inorganic supports are widely used

mainly due to their physical properties, offer several advantages over their organic counterparts (Weetall, 1976). These include good flow through properties, high mechanical strength, thermal stability, resistance to microbial attack and organic solvents, easy handling, excellent shelf-life and easy regenerability by simple pyrolysis followed by acid treatment (Parrado *et al*., 1995). The commonly used inorganic supports are controlled pore glass, ceramics and alumina. Small quantity of such supports can immobilized large quantities of enzyme. However, these supports are relatively expensive. In case of porous materials, they have large surface area and hence give high enzyme activity. Porous structure could also protect the enzyme from external conditions. However, there is one limiting feature which is the low mass-transfer efficiency and thus less efficient utilization of immobilized biocatalyst. Some other carriers have been recommended with an expectation to gain ideally industrial applicable carriers of economical significance. In 1998, Chase and Yang proposed the use of α-amylase immobilized on highly promising support, solid poly (vinyl alcohol) perfluorocarbon by covalent coupling with *p*-β-sulfate-(ethyl sulphonide)-aniline, 2, 4, 6-trichloro-1, 3, 5-triazine, 1, 1′-carbonyldi-imidazole and 2, 2, 2-trifluoro-ethanesulphonyl chloride (tresyl chloride) activation procedures. This immobilized enzyme are greatly improved the thermostability and the resistance to inactivation by 6 M urea. It also showed great potential for the continuous hydrolysis of starch with only 20% loss of activity after use for 3 weeks at 72°C. Fibrous matrices are of interest due to their advantages which include high specific surface area, low pressure drop and negligible mass-transfer resistance. An immobilization of *Aspergillus oryzae* β-galactosidase on cotton cloth activated with *p*-toluenesulfonyl chloride was studied (Albayrak and Yang, 2002). This kind of support and activation procedure shows coupling efficiency of about 85% and enzyme activity yield of about 55%. The half-life of about 50 days at 50°C and more than one year at 40°C. The method to prepare tosylated cotton cloth for enzyme immobilization is simple, inexpensive and scaleable for industrial applications. Hydrocoated microtiter plates by tresyl-activated dextran molecules (Gregorius *et al*., 1995), novel porous gels of chitosan-SiO$_2$ (Yang *et al*., 2004), poly (hydroxyethylmethacrylate-glycidyl methacrylate) with epoxy groups (Bayramoğlu *et al*., 2004) was also studied.

1.3.4 Review of phenylalanine dehydrogenase immobilization

Most reports on PheDH immobilization have been related to medical application, for example the determination of serum levels of phenylalanine which accumulates in a range of metabolic diseases, phenylketonuria and hyper-phenylalaninemia. The effective screening of infants (normally within 7 days old neonates) encourages the effective treatment to prevent severe symptoms (National Institutes of Health Consensus Development Panel, 2001). Various methods have been used and developed for the determination of phenylalanine; those were microbiological technique (Guthrie and Susi, 1963), fluorimetric method (McCaman and Robins, 1962; Blau, 1983), HPLC, liquid chromatograhy and amino acid analysis methods (Necker *et al*., 1980; Robins and Reeds, 1984; Atherton and Green, 1988; Rudy *et al*., 1987). None of these methods offers the combination of rapid, accurate and technically straightforward analysis. Simple, rapid, accurate and precise enzymatic method were proposed using PheDH (Cooper *et al*., 1989; Wendel *et al*., 1989; Wendel *et al*., 1990; Wendel *et al*., 1991; Taylor *et al*., 1993; Nakamura *et al*., 1996). However, the enzyme cannot be reused after each assay.

In 1993, Girotti *et al*. developed a highly sensitive and rapid bioluminescent flow sensor with PheDH extracted from *B. badius*, *B. sphaericus* and *Rhodococcus* sp. M4 and luciferase, bacterial bioluminescent enzyme, separately immobilized on nylon coil for the determination of L-phenylalanine in serum. The nylon coils had been activated with triethyloxonium tetrafluoroborate, 1, 6-diaminohexane and glutaraldehyde. Considering both of activity and protein content, 60-70% of added PheDHs was bound to the matrix in all three cases. The apparent Michaelis constant values of the immobilized enzyme for substrates and coenzymes were not markedly different from those of the free enzymes. The activity recovery was 5% for *B. badius* and 4% for the other two enzyme sources. The detection limit of the assay was 0.5 μM. Although the lifetime and operation stability of immobilized enzymes were satisfactory, it was found that this continuous-flow system is unsuitable for routine work. So in 1997, Kiba and his co-workers proposed the flow injection system using PheDH from *Rhodococcus* sp. M4 immobilized on tresylated poly (vinyl alcohol) beads (PVA) and packed into a stainless-steel column to measure L-phenylalanine

level. With highly reactive activator, tresyl chloride, the PheDH was immobilized with a 95% yield. Also, epoxy-activated PVA bead and glutaraldehyde-activated PVA bead were used. Under the same preparation for the activated bead and coupling conditions with the study of Kiba *et al.* in 1995, the yields for epoxy-bead and glutaraldehyde-bead were 5% and 24%, respectively. In the year 2000, PheDH and LeuDH were co-immobilized on the tresylated PVA beads for simultaneous determination of L-phenylalanine and branched chain L-amino acids in plasma which are important in the diagnosis of inborn metabolism disorders by liquid chromatographic system. This method gave precise and reproducible results but slightly higher values presented compared with amino acid analyzer method due to the interference of the tyrosine in plasma (Kiba *et al.*, 2000).

A less expensive alternative that can provide a fast and simple quantitative measurement of phenylalanine is the biosensor method. The minimal sample preparation and fast analysis time makes the biosensor method an attractive option for the determination of L-phenylalanine. The schematic representation of the recycling system of an enzyme carbon paste electrode based on the enzymatic/electrochemical recycling of tyrosinase in combination with salicylate hydroxylase and PheDH is shown in **Figure 1.9** (Huang *et al.*, 1998).

According to the study of Hanson *et al.* (2000), PheDH was not only used to produce its related amino acids and their oxo analogs but also to prepare allysine ethylene acetal [(S)-2-amino-5-(1,3-dioxolan-2-yl)-pentanoic acid]. It is one of the building blocks used for an alternative synthesis of VANLEF, a vasopeptidase inhibitor, from keto acid by reductive amination of PheDH (**Figure 1.10**). It has been shown to be a useful method for synthesis of natural and unnatural amino acids (Galkin *et al.*, 1997). Hanson *et al.* (2000) reported the reductive amination of keto acid acetal to acetal amino acid using PheDH from *T. intermedius* and recombinant *E. coli* containing *T. intermedius* PheDH activity and using FDH from *Candida boidinii* in order to regenerate coenzyme. Wet cell, dry cell, extracted and immobilized enzymes were all useful for the reaction, but the heat-dried cell preparations were the simplest and most convenient to use and to scale-up.
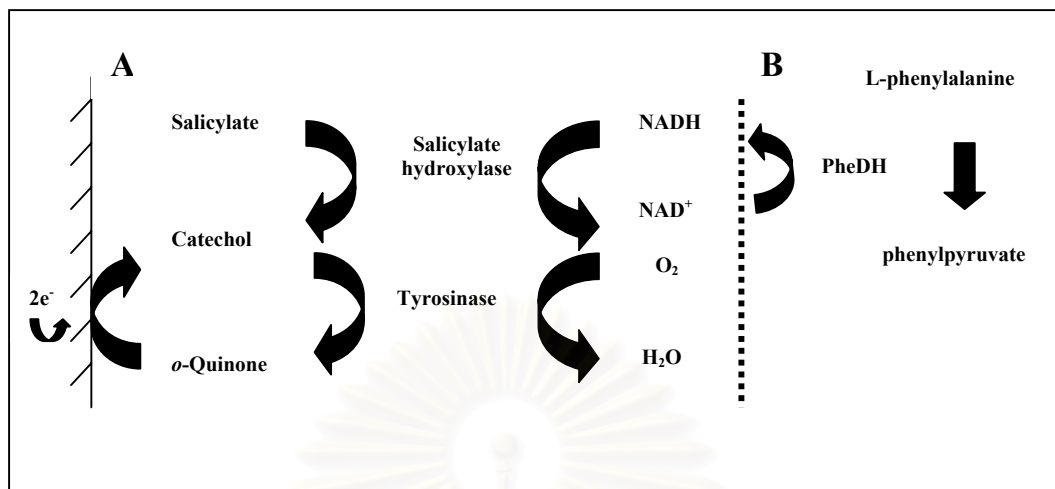
Figure 1.9 Schematic representation of an NADH-detecting biosensor (Huang *et al*., 1998)

    (A)   the salicylate hydroxylase/tyrosinase bioelectrocatalytic recycling system

    (B)   the coupling of PheDH to the bienzyme system
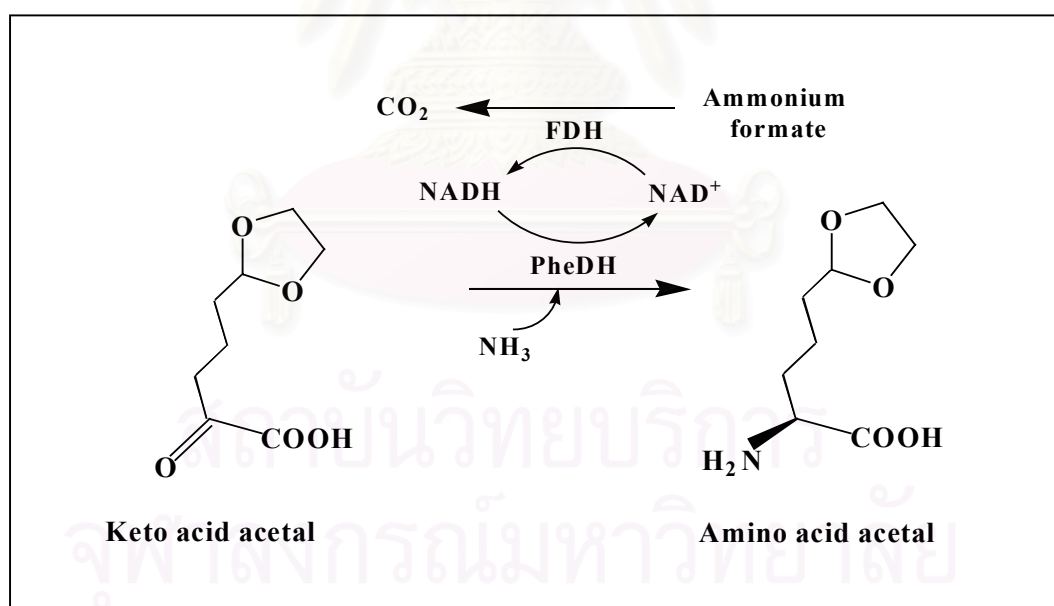
Figure 1.10 The synthesis of allysine ethylene acetal by phenylalanine dehydrogenase in pharmaceutical industry (Hanson *et al*., 2000; Patel, 2001) PheDH catalyzed the reductive amination of keto acid acetal to amino acid acetal (allysine ethylene acetal) and regenerated NADH was carried out using FDH.

For the sake of enzyme immobilization, FDH was immobilized on Eupergit C and PheDH on Eupergit C250L. The immobilized enzymes were tested for reusability in a jacketed reactor maintained at 40ºC and were used six times. After six times, it took 9 days to reach the same amount of amino acid produced as the first time of use. For the seventh batch, soluble FDH was added to restore the original rate of reaction. Eupergit® beads bind protein via its oxirane groups which react at neutral and alkaline pH with the amino groups of the protein molecules and can also bind enzyme molecule via their sulfhydryl groups. Eupergit C250L has the same chemical structure as Eupergit C, but has larger pores (Katchalski-Katzir and Kraemer, 2000).

1.4 Objectives of this research

Due to the fact that enzymes from mesophilic bacteria which have thermotolerant property have been proposed to be an interesting alternative, much attention is being paid to this group of enzymes. The dehydrogenase research group at the department of Biochemistry, Faculty of Science, Chulalongkorn University has been working on PheDH from *B. badius* BC1. This thermotolerant bacterium isolated from soil samples collected from temperate and hot spring areas was screened by fomazan forming method (Suriyapanpong *et al.*, 2000). The enzyme was subsequently purified and characterized for some properties such as molecular weight, substrate specificity and optimum pH and temperature (Leksakorn, 2001). Later Chareonpanich (2001) used the cassette-ligation mediated PCR gene cloning technique to prepare full length of PheDH and the gene fragment was amplified. The whole nucleotide sequence of the structural gene consisted of 1,140 nucleotides encoding a polypeptide of 380 amino acids. The PheDH gene was successfully cloned and overexpressed in *E. coli* JM109. However, the enzyme has not yet been purified and characterized.

Since the commercial values of essential amino acids have wide applicability in pharmaceutical and food industries, immobilized enzyme technology would facilitate the development of continuous, large-scale commercial process and a corresponding high rate of return of capital costs fulfilling the industrial requirements. One of the recombinant clones was then selected for further study.

The objectives of this research are:

i) To partially purify PheDH from recombinant *E. coli*.

ii) To characterize some of the biochemical properties of partial purified PheDH including the kinetic parameters compared with those of the wild type.

iii) To determine and select the appropriate carrier together with the immobilization method.

iv) To determine the optimum immobilization condition

v) To characterize some catalytic properties and compare them with the soluble enzyme.

vi) To preliminarily evaluate the use of immobilized enzyme for the production of L-phenylalanine
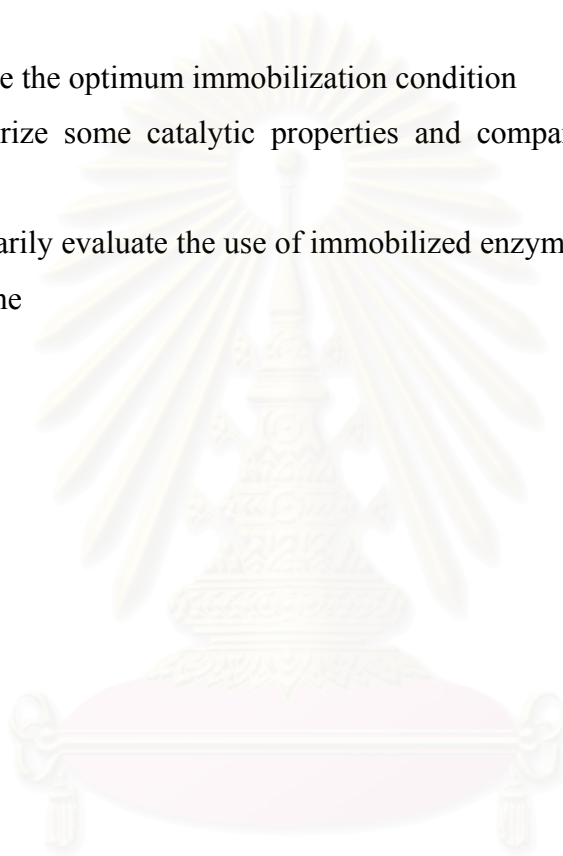
CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Analytical balance: Sartorius BP 310S, Scientific Promotion Co., Ltd., Germany

Mettler AB 204-S, Mettler-Toledo, Switzerland

Autopipette: Pipetman, Gilson, France

HT, High Tech Lab, Poland

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

Centrifuge: Refrigerated Centrifuge, Model J-21C, Beckman Instrument Inc., USA

Benchtop Centrifuge, Model H11n, Kokusan Enzinki Co., Japan

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

Fraction collector: Frac-100, Pharmacia Biotech, Sweden

Gel document: SYNGENE, England

Incubator: Gallenkamp, Genway, England

Incubator shaker: Innova 4080, News Brunswick Scientific Co., USA

Magnetic stirrer: Fisherbrand, Fisher Scientific Inc., USA

Orbital incubator: Model 1H-100, Gallenkamp, England

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

pH meter: Mettler MP220, Mettler-Toledo, Switzerland

Power supply: Model 1000/500, BIO-RAD, USA

Rocker platform: Bellco Biotechnology, USA

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

Sonopulse ultrasonic homoginizer: HD 2200, Bandelin, Germany

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

Vortex mixer: Geine model K-550-GE, Scientific Industries, USA

Water bath: Isotemp 210, Fisher Scientific Inc., USA

2.2 Chemicals

Acetone: Scharlau, Spain

3-Acetylpyridine adenine dinucleotide: Sigma, USA

Acrylamide: Merck, Germany

Alumina: Sigma, USA

Aminopropyl triethoxysilane: Sigma, USA

Ammonium chloride: M&B, England

Ammonium persulfate: Merck, Germany

Ammonium sulfate: Carlo Erba Reagenti, Italy

Ampicillin: Sigma, USA

Aquasorb: BML, Thailand

Bacto agar: Merck, Germany

Bacto tryptone: Merck, Germany

Bis-acrylamide: Merck, Germany

Bovine serum albumin (BSA): Sigma, USA

Bromophenol blue: BDH, England

1, 4-Butanediol diglycidyl ether: Fluka, Switzerland

n-Butanol: Ajax Chemicals, Australia

Butyl-Toyopearl 650M TSK gel: Tosoh, Japan

Citric acid: Fluka, Switzerland

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

Copper sulfate: Carlo Erba Reagenti, Italy

Coomassie brilliant blue R-250: Sigma, USA

DEAE-Toyopearl 650M TSK gel: Tosoh, Japan

Diaphorase: Fluka, Switzerland

Ethyl alcohol absolute: Carlo Erba Reagenti, Italy

Ethylenediaminetetraacetic acid (EDTA): Fluka, Switzerland

m-Fluoro-DL-phenylalanine: Sigma, USA

o-Fluoro-DL-phenylalanine: Sigma, USA

p-Fluoro-DL-phenylalanine: Sigma, USA

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

Glacial acetic acid: BDH, England

Glutaraldehyde: Sigma, USA

Glycerol: J.T. Baker, USA

Glycine: Scharlau, Spain

Hydrochloric acid: Lab-Scan, Thailand

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

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

Isopropyl-β-D-thiogalactoside (IPTG): Nacalai Tesque, Japan

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

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

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

Low molecular weight protein standard: Amersham Pharmacia Biotech, UK

β-Mercaptoethanol: Scharlau, Spain

Methanol: Lab-Scan, Thailand

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

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

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

Nicotinic acid adenine dinucleotide: Sigma, USA

Ninhydrin: BDH, England

Nitric acid: Mallinckrodt, France

Nitroblue tetrazolium: Koch-Light Laboratories Ltd., Japan

Phenazine methosulfate: Nacalai Tesque, Inc., Japan

Phenol reagent: Carlo Erba Reagenti, Italy

L-Phenylalanine: Sigma, USA

Phenylmethylsulfonyl fluoride (PMSF): Sigma, USA

Phenylpyruvic acid (sodium salt): Sigma, USA

Poly (vinyl alcohol) bead: Showa Denko, Japan

Potassium chloride: Merck, Germany

Potassium di-hydrogen phosphate: Carlo Erba Reagenti, Italy

di-Potassium hydrogen phosphate: Carlo Erba Reagenti, Italy

Potassium hydroxide: Scharlau, Spain

Potassium tartrate: BDH, England

Pyridine: Fluka, Switzerland

3-Pyridinealdehyde adenine dinucleotide: Sigma, USA

Pyruvic acid (sodium salt): Sigma, USA

Silica gel G-6: BDH, England

Sodium carbonate: Carlo Erba Reagenti, Italy

Sodium chloride: BDH, England

Tri-sodium citrate: Carlo Erba Reagenti, Italy

Sodium hydroxide: Merck, Germany

Sodium dodecyl sulfate (SDS): Sigma, USA

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

Thionicotinamide adenine dinucleotide: Sigma, USA

2, 2, 2-Trifluoroethanesulfonyl chloride (tresyl chloride): Sigma, USA

TLC cellulose plate: Merck, Germany

Tris: USB, USA

Yeast extract: Scharlau Microbiology, Spain

L-amino acids were from Sigma, USA and D-amino acids were from Nacalai Tesque and WaKo, Japan. Other common chemicals were reagent grade from Aldrich; USA, BDH; England, Fluka; Switzerland, Merck; Germany, Scharlau; Spain, Unilab; Australia and Sigma; USA.

2.3 Bacterial strain

Recombinant plasmid, pUC18 which contained the gene encoding phenylalanine dehydrogenase (PheDH) of a thermotolerant *Bacillus badius* BC1 (Suriyapongpan *et al.*, 2000) was previously cloned into *Escherichia coli* JM109 (Chareonpanich, 2001). This transformant was then used for the production of PheDH.

2.4 Bacteria growth medium

The bacterial stain was cultured aerobically in LB medium comprising 1% (w/v) bacto tryptone, 0.5% (w/v) NaCl and 0.5% (w/v) yeast extract, pH 7.4. The LB medium also contained 100 μg/ml ampicillin in order to select positive colonies and IPTG at final concentration of 0.05 mM was added to induce the PheDH production. For agar plates, the media was supplemented with 2% (w/v) agar. Medium was sterilized by autoclaving at 121ºC for 20 minutes.

2.5 Free enzyme assay

Activity of NAD^+-dependent PheDH was assayed at 30ºC according to the reactions below;

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

The oxidative deamination of L-phenylalanine (forward reaction) was carried out by measuring the reduction of NAD^+ at 340 nm in a cuvette placed in the beam of 1 cm light path. The reaction mixture contained 20 μmol of L-phenylalanine, 1 μmol of NAD^+, 200 μmol of glycine-KCl-KOH buffer, pH 11.5 and the enzyme in a final volume of 1.0 ml. Reductive amination offered another route for conversion of phenylpyruvate (reversible reaction) by means of measuring the disappearance of NADH at 340 nm in the reaction mixture consisting of 10 μmol of phenylpyruvate,

500 µmol of ammonium chloride, 0.2 µmol of NADH, 100 µmol of glycine-KCl-KOH buffer, pH 10.5 and the enzyme in the final volume of 1.0 ml. The substrate was replaced by water in a blank. The initial change in the absorbance was started by addition of NAD^+ or NADH and monitored with a spectrophotometer: model DU series 650, Beckman.

Enzyme activity was expressed in units in which one unit corresponded to the amount of enzyme which catalyzed the formation of 1 µmol of NADH per minute in the oxidative deamination. Specific activity was defined as units per milligram of protein.

2.6 Protein content determination by Lowry's method

The quantitative assay for determining protein content in a solution was carried out according to the modified method of Lowry *et al.* (1951) with bovine serum albumin (BSA) as a standard. The reaction mixture of 5.1 ml containing 20-100 µg of protein, 100 µl of solution A and 5 ml of solution B was mixed and incubated at 30ºC for 10 minutes. The solution mixture was subsequently incubated with 0.5 ml of solution C for 30 minutes at room temperature. The absorbance at 610 nm was then measured. The protein concentration was calculated from standard curve of BSA. Preparation of the solution and the protein standard curve were presented in Appendix A and B, respectively.

2.7 Partial purification of phenylalanine dehydrogenase

2.7.1 Enzyme Production

A single colony of the *E. coli* JM109 transformant from LB agar plate was grown in 40 ml of starter LB medium supplemented with 100 µg/ml ampicillin at 37ºC with 250 rpm shaking overnight. Then, 10% of cell culture was inoculated into 400 ml of the same medium and conditions for 16-18 hours. The starter bacteria was subsequently transferred into 4 liters medium with the same supplement and was allowed to grow at the same conditions until the turbidity of the growing culture at

600 nm reached 0.6. After that, IPTG was added to final concentration of 0.5 mM in order to induce enzyme production. Cells were harvested after the induction for 5 hours by centrifugation at 9,820 x g for 15 minutes at 4ºC. The collected cells were washed twice with cold 0.85% NaCl prior to rewash with cold extraction buffer (0.1 M potassium phosphate buffer, pH 7.4 containing 0.1 mM PMSF, 0.01% (v/v) of 2-mercaptoethanol and 1 mM EDTA). The wet cell mass was stored at -80ºC until used.

2.7.2 Crude extract preparation

The 13 grams of cell pellets from section 2.7.1 were resuspended in 50 ml of cold extraction buffer (see section 2.7.1) and then disrupted by discontinuous sonication on ice for 5 minutes and 5 minutes stop interval with 5 cycles by high intensity ultrasonic processor. The intact cells and cell debris were removed by centrifugation at 12,100 x g for 30 minutes at 4ºC. Crude extract was collected for the determination of oxidative deamination activity and protein concentration as described in section 2.5 and 2.6, respectively.

2.7.3 Purification procedures of phenylalanine dehydrogenase

The crude extract from previous section was purified according to **Figure 2.1**. All procedures were carried out at 4ºC. The buffer used throughout the purification procedure was 10 mM potassium phosphate buffer, pH 7.4 containing 0.01% (v/v) 2-mercaptoethanol, 1 mM EDTA and 10% (v/v) glycerol. A typical step of PheDH purification was as follows.

2.7.3.1 Ammonium sulfate precipitation

To treat the cell free extract, solid ammonium sulfate was added to crude extract to 30% saturation with gentle stirring. After 30 minutes, the precipitate was removed by centrifugation at 12,100 x g for 30 minutes. After that, ammonium sulfate was then added stepwise to supernatant at 10% increment from 30% to 60%. At each step of the increment, the solution was left for 30 minutes on ice with continuous

Cell free extract

↓

30-50% Saturated ammonium sulfate precipitation

↓

DEAE-Toyopearl column

(Linear gradient eluted with 0-0.5 M KCl solution)

↓

Butyl-Toyopearl column

(Stepwise eluted with saturated ammonium sulfate solution)

↓

Determination of enzyme purity by native-PAGE
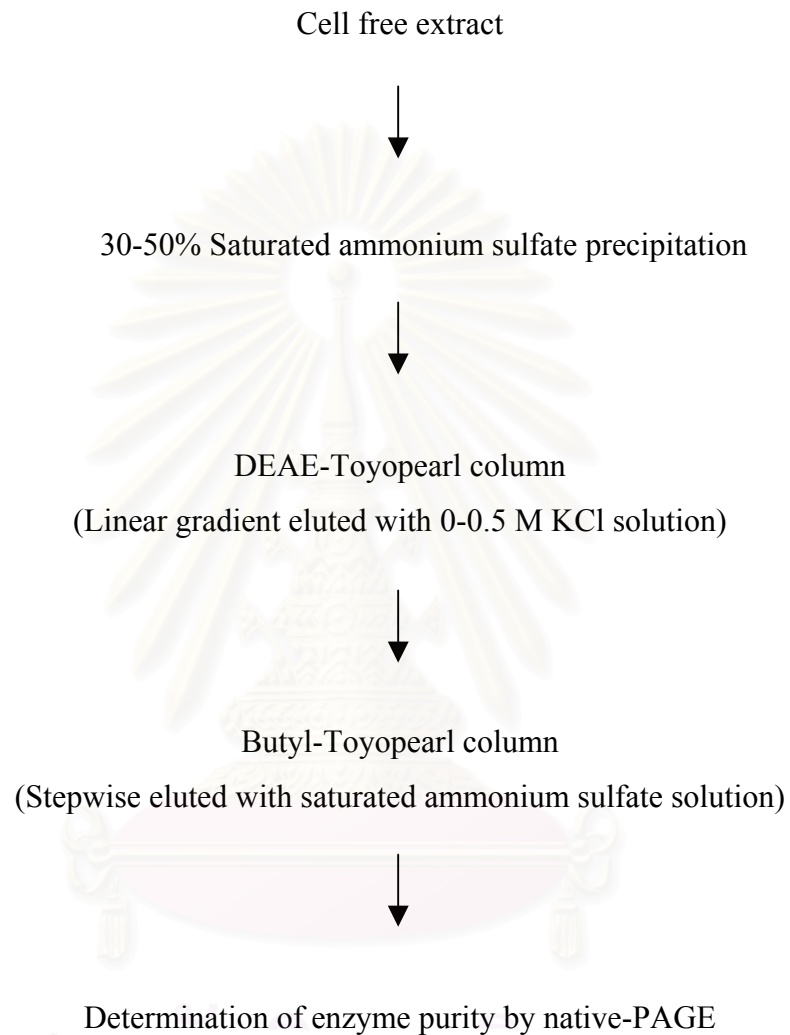
Figure 2.1 Schematic diagrams illustrating the partial purification process of
phenylalanine dehydrogenase

stirring and was subsequently centrifuged at 12,100 x g for 30 minutes. The precipitates were dissolved in the buffer (see section 2.7.3). The protein solution was dialyzed against 100 volumes of the same buffer at least 4 hours for 3 times. Each fraction was determined for their oxidative deamination activity (section 2.5). The fraction which gave the highest activity was subsequently determined for the protein concentration as described in section 2.6.

2.7.3.2 DEAE-Toyopearl column chromatography

To use DEAE-Toyopearl, the bead was first activated by washing sequentially with excess volume of 0.5 M sodium hydroxide for a couple of times, then rewashed by deionized water until the pH reached neutral. The activated DEAE-Toyopearl was resuspened in the buffer (see section 2.7.3) and packed into 1.9 x 16.5 cm column then equilibrated with the same buffer for 5-10 column volumes at flow rate of 1 ml/minute.

The enzyme from section 2.7.3.1 was applied to the DEAE-Toyopearl column. The unbound proteins were eluted from the column with the buffer as described in 2.7.3. A 5-ml fraction was collected and the absorbance at 280 nm was read. After the unbound proteins were washed thoroughly with the buffer or until the absorbance at 280 nm of eluent was negligible, the bound proteins were eluted from the column with linear salt gradient of 0 to 0.5 M potassium chloride in the same buffer. Fractions of 3 ml were collected using 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 potassium chloride concentration was investigated by measuring the conductivity. The active fractions were combined, and then dialyzed against 100 volumes of the same buffer at least 4 hours for 3 times. After the salt was removed, the enzyme activity was then checked again as described in section 2.5 and protein concentrations were measured by Lowry's method as described in section 2.6.

2.7.3.3 Butyl-Toyopearl column chromatography

Butyl-Toyopearl was washed with deionized water for 2-3 times to remove impurities. The settled bead was then resuspended in buffer (see section 2.7.3) containing 25% saturated ammonium sulfate and packed into glass column (1.9 x 17 cm). The column was equilibrated with 5-10 column volumes with the same buffer at flow rate of 1 ml/minute.

The pooled active fractions from section 2.7.3.2 was slowly adjusted to 25% saturation with ammonium sulfate and stirred gently for at least 30 minutes. The protein solution was then applied to the equilibrated column at flow rate of 1 ml/minute. The proteins were eluted with a decreasing salt stepwise of 25%, 20%, 15% and 0% saturated ammonium sulfate in the buffer. 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 dialyzed against the 100 volumes of buffer at least 4 hours for 3 times. After desalting, the enzyme activity was rechecked according to section 2.5 and protein concentrations were measured as described in section 2.6.

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

In order to determine the protein pattern and degree of purity, the enzyme from each step was subjected to native-PAGE according to the method of Bollag and Edelstein (1991). Electrophoresis conditions, protein and activity staining were described below.

2.7.3.4.1 Non-denaturing gel electrophoresis

Non-denaturing polyacrylamide gel was prepared as described in Appendix C. The discontinuous PAGE was performed on a slab gel (10 x 7.3 x 0.75 cm) of 7.7% (w/v) separating gel and a 5% (w/v) stacking gel. Tris-glycine buffer, pH 8.3 was used as electrode buffer. The enzyme from each step was mixed

with 5x sample buffer by ratio of 5:1 and then loaded onto the gel. The electrophoresis was run from cathode towards anode, at constant current of 20 mA per slab gel on a Mini-Gel electrophroresis unit. For the determination of the enzyme activity, the electrophoresis was done at 4ºC.

2.7.3.4.2 Detection of protein band

Eventually, after electrophoresis, proteins bands were visualized by Coomassie blue staining and activity staining as described in detail below.

A. Coomassie blue staining

The staining solution of 0.1% (w/v) Coomassie brilliant blue R-250 in 10% (v/v) acetic acid and 45% (v/v) methanol was utilized to stain the gel for at least 20 minutes. Destaining was performed by immersing the gel in destaining solution containing 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 enzyme was stained for activity with a 10 ml 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. The gel was allowed to soak in the solution at room temperature for 15-30 minutes or until the brown band of active enzyme developed. The gel was then quickly rinsed several times with deionized water until gel background was clear.

2.8 Characterization of phenylalanine dehydrogenase

2.8.1 Molecular weight determination of phenylalanine dehydrogenase subunit on SDS-PAGE

The denaturing gel which was carried out with 0.1% (w/v) SDS in 12.5% (w/v) separating gel and 5% (w/v) stacking gel was performed on slab gel (10 x 7.3 x 0.75 cm) system according to the method described by Bollag and Edelstein (1991).

Tris-glycine buffer, pH 8.3 containing 0.1% (w/v) SDS was used as electrode buffer. The gel preparation was described in Appendix D. The enzyme sample from each step was treated with sample buffer and boiled for 10 minutes before applying to the gel. The electrophoresis was performed at constant current of 20 mA per slab gel at room temperature on an electrophoresis unit 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 2.7.3.4.2 A.

2.8.2 Substrate specificity of phenylalanine dehydrogenase

The enzyme activity towards various substrates in both oxidative deamination and reductive amination reaction was studied for substrate specificity. The ability of the enzyme to catalyze the oxidative deamination of various amino acids and L-phenylalanine analogs was examined at a final substrate concentration of 20 mM except for L-tyrosine, 1.2 mM was used instead. The reaction was carried out under the standard reaction condition (section 2.5) by replacing L-phenylalanine with other substrates. The result was expressed as a percentage of the relative activity. Subsequent experiment was done on the ability of the enzyme to catalyze the reductive amination of various keto acids and phenylpyruvate analogs at a final substrate concentration of 10 mM except for p-hydroxyphenylpyruvate (2.5 mM) and indole-β-pyruvate (1 mM). Phenylpyruvate in the standard reaction as described in section 2.5 was replaced by various keto acids and phenylpyruvate analogs as substrates. The result was expressed as a percentage of the relative activity.

2.8.3 Coenzyme specificity of phenylalanine dehydrogenase

As the enzyme required natural coenzyme, NAD^+, for oxidative deamination, there are many other NAD^+ analogs that could replace NAD^+. Assays with NAD^+ analogs at final concentration of 2 mM were conducted by measuring the increase in absorbance at the following wavelengths: 3-acetylpyridine adenine dinucleotide, 363

nm (ε = 9.1 x 10^3); β-nicotinamide adenine dinucleotide phosphate (NADP), 340 nm (ε = 6.2 x 10^3); nicotinic acid adenine dinucleotide, 338 nm (ε = 6.2 x 10^3); 3-pyridinealdehyde adenine dinucleotide, 358 nm (ε = 9.3 x 10^3); and thionicotinamide adenine dinucleotide, 395 nm (ε = 11.3 x 10^3) (Misono *et al.*, 1989). The reaction was carried out at pH 9.5 to avoid the degradation of NAD^+ analogs at a more alkaline pH. The result was expressed as a percentage of the relative activity.

2.8.4 Effect of pH on phenylalanine dehydrogenase activity

The effect of pH on PheDH activity was studied by assaying its activity at various pH's ranging from 7.5-13.0 through the standard reaction condition as described in section 2.5. Two kinds of buffer were used: Tris-HCl (200 mM, pH 7.5-9.0) and glycine-KCl-KOH (200 mM, 8.5 – 13.0) for both 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%.

2.8.5 Effect of temperature on phenylalanine dehydrogenase activity

The purified PheDH was used to investigate the effect of temperature on its activity. Temperature was varied from 25ºC to 60ºC. The enzyme activity was assayed by the method described in section 2.5 for both oxidative deamination and reductive amination. The result was expressed as a percentage of the relative activity. The maximum activity which was observed for each reaction was set as 100%.

2.8.6 Effect of pH on phenylalanine dehydrogenase stability

The purified PheDH was used to study its stability over variable pH's of buffer. The enzyme was preincubated in 10 mM buffer at different pH values for 20 minutes at 30ºC. Then an aliquot of the enzyme solution was withdrawn and the residual activity was determined for the oxidative deamination of L-phenylalanine as described in section 2.5. The 10 mM buffers used were 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 result was expressed relatively to the original activity assayed under standard condition (section 2.5) when the enzyme was kept in 10 mM phosphate buffer, pH 7.4. The original activity was defined as 100%.

2.8.7 Effect of temperature on phenylalanine dehydrogenase stability

The purified PheDH was used to study thermostability which was investigated over the range of 30ºC to 70ºC. Prior to the determination of its activity in oxidative deamination (section 2.5), the enzyme was preincubated at various temperature for 10 minutes. The residual activity was measured under standard condition (section 2.5). The result was expressed as a percentage of the relative activity which was relative to the original activity assayed under standard condition at 30ºC (section 2.5) when the enzyme was not subjected to heat. The original activity was defined as 100%.

2.9 Initial velocity studies of phenylalanine dehydrogenase

The initial velocity studies were carried out on PheDH to determine the kinetic parameters as described below.

2.9.1 Initial velocity analysis 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 0.625, 1.25, 2.5, and 5 mM and those of NAD^+ used were 0.125, 0.25, 0.5 and 1 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.

2.9.2 Initial velocity analysis 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.

      2.9.2.1 The enzyme was assayed in the reductive amination by using phenylpyruvate as a variable substrate (0.0625, 0.125, 0.25 and 0.5 mM) at several fixed concentrations of ammonium chloride (62.5, 125 and 500 mM) in the presence of a high 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 of phenylpyruvate and ammonium chloride.

      2.9.2.2 The enzyme was assayed in the reductive amination by using ammonium chloride as a variable substrate (31.25, 125, 250 and 500 mM) at several fixed concentrations of NADH (0.025, 0.5, 0.1 and 0.2 mM) in the presence of a high 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 of ammonium chloride and NADH.

      2.9.2.3 The enzyme was assayed in the reductive amination by using NADH as a variable substrate (0.02, 0.04 and 0.08 mM) at several fixed concentrations of phenylpyruvate (0.0125, 0.025 and 0.1 mM) in the presence of a high 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 of NADH and ammonium chloride.

2.10 Immobilization of phenylalanine dehydrogenase

An investigation of different methods for the covalent immobilization of PheDH on several organic and inorganic supports including alumina, silica, poly (vinyl alcohol) (PVA) and chitosan was studied. The immobilized techniques used were based on the method previously described by several researchers as shown below.

2.10.1 Preparation of inorganic carrier

No matter which method was employed, cleaning of the inorganic support to generate reactive hydroxyl groups was critical for effective immobilization of biomaterials. Hence, alumina and silica should be cleaned to remove adsorbed organic contaminants from the surface (Weetall, 1993). The supports were soaked in 5% (v/v) nitric acid solution at 100ºC for 45 minutes followed by exhaustive washing with distilled water to remove any residual acid and dried at 110ºC.

2.10.2 Activation of solid supports material

2.10.2.1 Epoxy method with 1, 4-butanediol diglycidyl ether (Chase and Yang, 1998)

Fifty milligram of supports were washed with 1 M NaOH and then suspended in 0.5 ml of 1 M NaOH. After that 5 μl of 1, 4-butanediol diglycidyl ether was added. The mixture was stirred at room temperature for 5 hours or 60ºC for 2 hours. The product was then washed thoroughly with distilled water.

2.10.2.2 Glutaraldehyde method (Pantatan, 2002)

Fifty milligram of cleaned alumina, silica and PVA bead were silanized with 1 ml of 2% (v/v) γ-aminopropyltriethoxysilane (APTS). Chitosan was not subjected to silanization process because it already contains amino groups on the side chain. The mixture was gently stirred for 3 hours at room temperature. The silanized

carriers (alkylamine derivative) were removed by centrifugation at 1,380 x g for 10 minutes at room temperature. After being thoroughly washed with distilled water, the product was then further reacted with 1 ml of 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 10 minutes at room temperature, and then washed with deionized water several times to remove residual glutaraldehyde.

2.10.2.3 Tresyl chloride method (Chase and Yang, 1998; Kiba *et al.*, 1997; Kiba *et al.*, 2000)

Fifty milligram of alumina, silica, PVA and chitosan were washed with acetone (2 ml) before suspended in 1 ml of a mixture of acetone and pyridine (1:1 v/v). With vigorous magnetic stirring, 50 μl of 2, 2, 2-trifluoroethanesulfonyl chloride (tresyl chloride) was added dropwise to the suspension during 5 minutes. The reaction was continued for 15 minutes at room temperature with continuous stirring. The beads were washed successively with acetone (5 ml) and 1 mM HCl (5 ml). Before use, washing the beads with deionized water was required.

2.10.3 Enzyme coupling

One unit of PheDH (assayed under standard condition of colorimetric method described in section 2.11) was added into 50 mg of activated carriers, from section 2.12. For adsorption, the enzyme was added directly to non-activated supports. The volume was adjusted to 5 ml with 50 mM potassium phosphate buffer pH 7.0. The mixture was gently stirred at 4ºC for 12 hours. Then the immobilized enzyme was separated from the supernatant by centrifugation at 1,380 x g for 10 minutes at 4ºC. Next, the carriers were washed 3 times with 1 M NaCl in 10 mM potassium phosphate buffer pH 7.0, followed by successive wash with the same buffer without salt for 4 times until no activity or soluble protein was observed in the washing solutions. All eluates were collected and analyzed for the PheDH activities as described in sections 2.11. The covalently bound enzyme was stored in 10 mM potassium phosphate buffer pH 7.0 at 4ºC until further use.

2.11 Colorimetric method for the determination of phenylalanine dehydrogenase activity

Departing from the enzymatic assay as described in section 2.5, there is a spectrophotometric method that couples simultaneously 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. This 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.

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

$$NADH + WST\text{-}1 + H^+ \xrightarrow{\text{diaphorase}} NAD^+ + \text{water soluble formazan} \quad (2)$$
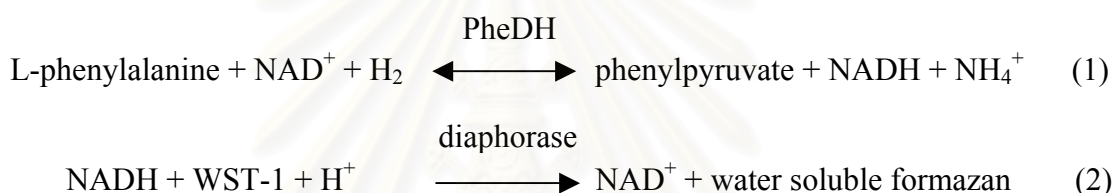
This coupled reaction is not only used to measure the L-phenylalanine level by soluble form of PheDH but also applied to determine the immobilized PheDH activity.

The optimal condition for the coupled reaction was first investigated with PheDH in soluble form by varying the concentration of WST-1 (0.1 μmol – 2 μmol), the amount of diaphorase (0.001 - 1 U), buffer pH (1 M Tris-HCl, pH 8.5 and pH 9.0 and 1 M Gly-KCl-KOH pH 8.5 and pH 9.0) and incubation time. The first parameter was optimized and was used to determine the optimal condition for the next parameter. After optimization, the reaction mixture contained 20 μmol of L-phenylalanine, 1 μmol of NAD^+, 200 μmol of glycine-KCl-KOH buffer, pH 9.0, 1 μmol WST-1, 0.01 U of diaphorase from *Clostridium kluyveri* and the enzyme (soluble form) in a final volume of 1.0 ml. The reaction was started by the addition of phenylalanine and was incubated at room temperature for 10 minutes. For immobilized enzyme, 10 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. The reaction ratio of NADH to WST-1 was equivalent to 1:1 (Sarker *et al*., 2001).

One unit of PheDH determined by this method was defined as the amount of enzyme which catalyzes the formation of 1 μmol of formazan per minute.

2.12 Optimization of phenylalanine dehydrogenase immobilization

The most suitable carrier and the best immobilization method from section 2.10 were selected by considering the color development of formazan at 438 nm which can be implied for the highest immobilized enzyme activity. Covalent coupling of PheDH on silica support with glutaraldehyde was selected for further study. The optimum conditions for covalent immobilization of PheDH were investigated by varying some of the conditions described in section 2.10.2.2 and 2.10.3. The APTS concentration was varied from 1-10% (v/v) and glutaraldehyde concentration was varied from 0.1-2.5% (v/v). Various amounts of PheDH applied to the support were investigated in the range of 0.2-1 U per 50 mg support. The coupling time between the enzyme and support was also examined during 0.5-16 hours. The immobilized enzyme activity was determined under standard condition described in section 2.11. The best condition yielding maximum activity retention of the immobilized PheDH was selected.

2.13 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:

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

2.14 Characterization of the catalytic properties of the immobilized phenylalanine dehydrogenase

2.14.1 Effect of pH on the immobilized phenylalanine dehydrogenase activity

Because the coupled reaction was used to determine the activity of PheDH; the optimum pH was then studied to obtain the appropriate condition for both PheDH and diaphorase. Free and immobilized enzyme activities were determined under standard condition as described in section 2.11. One molar glycine-KCl-KOH, pH range of 8.5-13.0 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, according to formazan formation, as a percentage of the relative activity. The highest value was assigned to 100% activity.

2.14.2 Effect of temperature on the immobilized phenylalanine dehydrogenase activity

Free and immobilized enzyme samples were assayed under standard condition as described in section 2.11, except that they were incubated at different temperature ranging from 25ºC to 60ºC. The result is presented in the same way as above.

2.14.3 Effect of pH on the immobilized phenylalanine dehydrogenase stability

The effect of pH on the stability of the free and immobilized enzyme was examined after preincubating enzyme samples with various buffers (the same buffer solution as in section 2.8.6) for 20 minutes at 30ºC. After that the immobilized PheDH was separated by centrifugation and the residual activity of enzyme in both forms was measured under standard condition described in section 2.11. 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%.

2.14.4 Effect of temperature on the immobililized phenylalanine dehydrogenase stability

Thermal stability of free and immobilized enzyme preparation was carried out by incubating the enzyme in 10 mM phosphate buffer pH 7.4 and then exposed to different temperature (25ºC - 90ºC) for 20 minutes. After incubation, 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 2.11. 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%.

2.14.5 Storage stability

One of the most important parameters to be considered in enzyme immobilization was storage stability. Both free and immobilized PheDH 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 4ºC and at room temperature for 1 month. The residual activity was measured with the experimental conditions given in section 2.11 and compared with the value at time zero.

2.15 Synthesis of amino acids from their keto acids using immobilized enzyme

2.15.1 Phenylalanine and other amino acids production

The enzyme reaction was performed by using various kinds of keto acids which were phenylpyruvate, α-ketocaproate, α-ketoisocaproate, α-ketovalerate, α-ketoisovalerate and α-keto-γ-methiol-butyrate as substrates. The reaction mixtures contained 5 μmol of substrate, 200 μmol of NH_4Cl-NH_4OH buffer, pH 9.5 and 5 μmol of NADH in a final volume of 0.5 ml in separated vial for each substrate. The reaction was carried out at 30ºC with gentle shaking for 20 hours. The solution was

then separated from the immobilized enzyme by centrifugation and kept at 4ºC until use.

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

TLC was used to determine amino acids produced in the reaction mixtures as described above. The samples were treated with 0.1 M HCl until pH reached neutral. Then, the sample solution was spotted in a pair with known amino acid on a TLC cellulose plate (9 x 10 cm for 3 samples) and developed with n-butanol/ acetic acid/ water (4: 1: 1). The glass chamber was equilibrated at 25ºC for 2 hours before used. When the ascending solvent front was near the top margin (about 1 cm), the plate was removed from the chamber and dried for 30 minutes at room temperature. After that 0.5% ninhydrin in acetone/ ethanol mixture was sprayed. The plate was initially air-dried for 15 minutes, then oven-dried for 15 minutes at 100ºC to develop color of ninhydrin-amino acid complex.

2.15.3 Semiquantitative determination of phenylalanine production

The amount of amino acids produced was semiquantitated by using TLC technique and Gel document. Standard L-phenylalanine with various concentrations (5-40 nmol) was 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 E) and the amount of L-phenylalanine in the sample was calculated.

CHAPTER III

RESULTS

3.1 Partial purification of phenylalanine dehydrogenase

3.1.1 Crude enzyme solution preparation

Crude extract was obtained from 13 g wet weight of *Escherichia coli* JM109 transformant cultivated in 4 liters of LB medium supplemented with 100 μg/ml ampicillin and 0.5 mM IPTG as described in section 2.3 and 2.4. Crude extract contained 931 mg proteins with total activity of 6,639 units of enzyme. Thus, the specific activity of the enzyme in the crude preparation was 7.1 units/mg protein.

3.1.2 Ammonium sulfate precipitation

Crude extract was further purified by ammonium sulfate precipitation as mentioned in section 2.7.3.1. The suitable ammonium sulfate concentration was determined. The increment of ammonium sulfate concentration was from 0-30%, 30-40%, 40-50% and 50-60%. The small amount of enzyme activity was found in the range of 0-30% and 50-60% fractions with the highest activity presence in 30-40% and 40-50% fractions. Thus, the enzyme was harvested in the range of 30-50% saturated ammonium sulfate precipitation to achieve most of the PheDH. The precipitated protein was then dissolved with 10 mM potassium phosphate buffer pH 7.4 containing 0.01% (v/v) 2-mercaptoethanol, 1 mM EDTA and 10% (v/v) glycerol (working buffer) and dialyzed against the same buffer. In this step, the remaining protein was 468 mg with the enzyme activity recovered at 5,655 units. The specific activity was 12.1 units/mg. The enzyme was purified 1.7 fold with 85.2% recovery compared with the crude extract.

3.1.3 DEAE-Toyopearl column chromatography

The enzyme precipitation from section 3.1.2 was applied onto DEAE-Toyopearl column as described in section 2.7.3.2. The chromatographic profile is shown in **Figure 3.1**. The unbound proteins were eluted from DEAE-Toyopearl
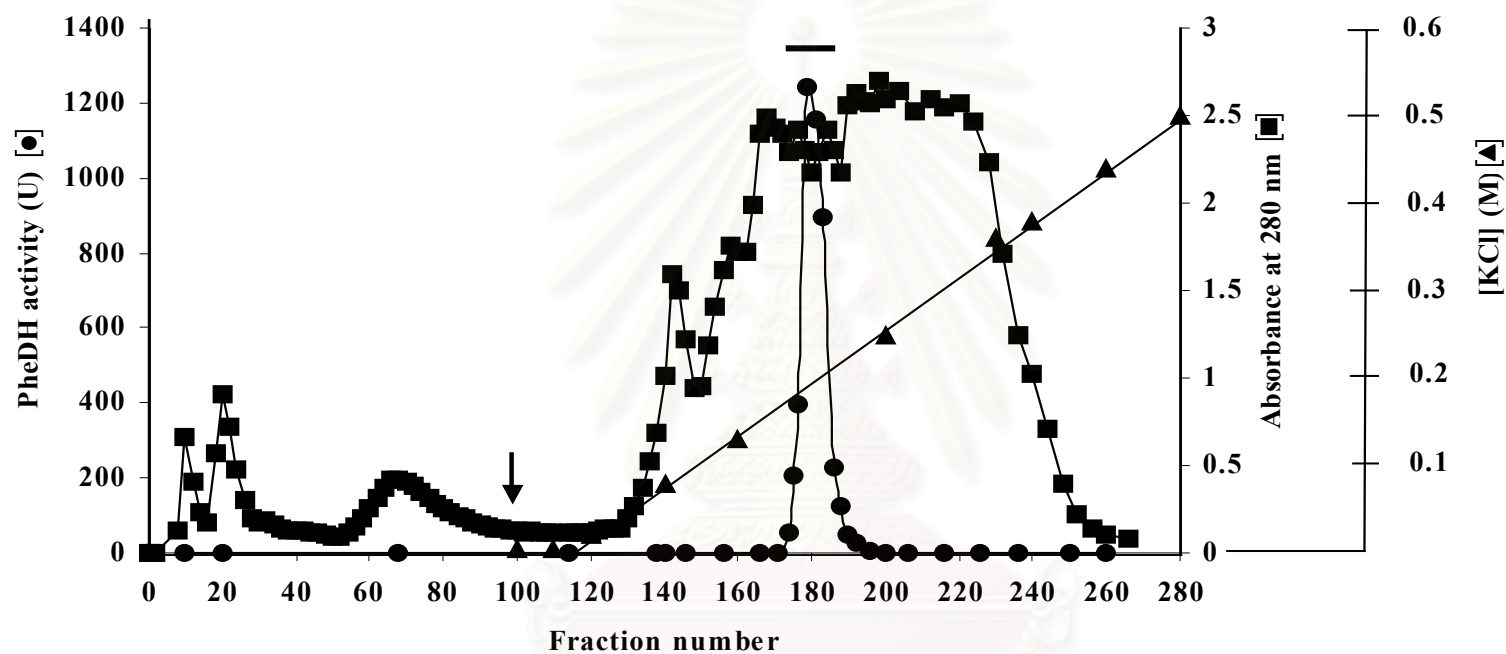
Figure 3.1 Chromatographic profile of phenylalanine dehydrogenase from DEAE-Toyopearl column

The enzyme solution from ammonium sulfate precipitation step was applied to DEAE-Toyopearl column and washed with 10 mM potassium phosphate buffer, pH 7.4 containing 0.01% (v/v) 2-mercaptoethanol, 1 mM EDTA and 10% (v/v) glycerol. The bound enzyme was then eluted with linear salt gradient of 0-0.5 M KCl at the flow rate of 1 ml/min. Fractions of 3 ml were collected. The arrow indicates the starting gradient point. The fractions of active protein were pooled from 176-185 (——).

column with working buffer until the absorbance at 280 nm was negligible. The bound proteins were then eluted with linear salt gradient of 0 to 0.5 M potassium chloride solution. The enzyme was eluted at 0.2 M potassium chloride solution as indicated in the profile. The fractions with PheDH activity were pooled. Aquasorb was used to concentrate the enzyme which was further dialyzed against the buffer mentioned above. After this purification step, the enzyme was purified for about 7.5 fold with 60.4% recovery compared to the crude extract. The remaining protein from this step was 75 mg with 4,011 total activity units and the specific activity was 53.4 units/mg protein (**Table 3.1**).

3.1.4 Butyl-Toyopearl column chromatography

The pooled active fraction from DEAE-Toyopearl column was further purified by Butyl-Toyopearl column as described in section 2.7.3.3. The chromatographic profile is shown in **Figure 3.2**. The enzyme solution was loaded into column then unbound proteins were eluted with working buffer containing 25% saturated ammonium sulfate. The proteins, which were bound to the column, were then eluted by negative salt stepwise method with the working buffer containing 25, 20, 15 and 0% saturated ammonium sulfate, respectively. The enzyme was eluted with the working buffer containing 20% salt saturation. The fractions containing PheDH activity were pooled, concentrated by aquasorb and dialyzed against working buffer. This operation obtained the enzyme with 22 mg remaining protein and 1,589 activity units. The specific activity was 71.9 units/mg proteins. From this final step, the enzyme was purified about 10.1 fold with 23.9% recovery compared with the crude extract as shown in **Table 3.1**.

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

The protein pattern of enzyme from each step of purification was analyzed by non-denaturing polyacrylamide gel electrophoresis as described in section 2.7.3.4. The activity staining was performed (section 2.7.3.4.2B) to compare

Table 3.1 Purification of recombinant phenylalanine dehydrogenase

Purification steps	Total activity (unit)	Total protein (mg)	Specific activity (units/mg protein)	% Recovery	Purification fold
Crude extract	6,639	931	7.1	100	1
30-50% Saturated ammonium sulfate precipitation	5,655	468	12	85	1.7
DEAE-Toyopearl	4,011	75	53	60	7.5
Butyl-Toyopearl	1,589	22	72	24	10
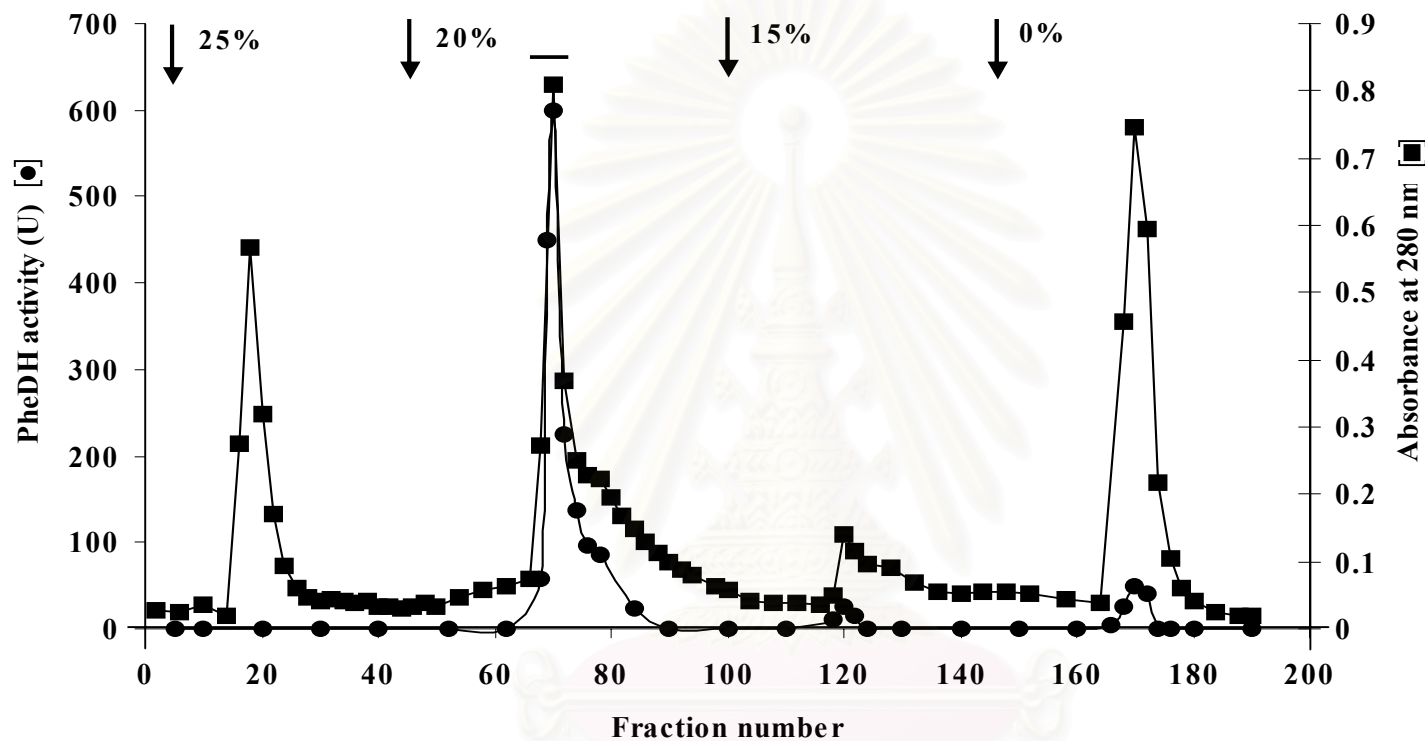
Figure 3.2 Purification of recombinant phenylalanine dehydrogenase by Butyl- Toyopearl column

The enzyme solution was applied to Butyl-Toyopearl column and washed with 10 mM potassium phosphate buffer, pH 7.4 containing 0.01% (v/v) 2-mercaptoethanol, 1 mM EDTA, 10% (v/v) glycerol and 25% saturated ammonium sulfate. Stepwise elution of bound protein was performed at 20%, 15% and 0% saturated ammonium sulfate in the same buffer at the flow rate of 1 ml/ min. Fractions of 3 ml were collected. The arrows indicate where each stepwise started. The protein peak from fraction number 69-75 (▬) was pooled.

with protein staining. The result is presented in **Figure 3.3**. The enzyme has more purity in each step of purification as the bands of other proteins decreased (gel A, lane 1-4). The enzyme in lane 4 showed three protein bands with 1 major band. The major band coincided with PheDH activity by activity staining (gel B, lane 1-4). This partially purified PheDH was then used as a source of enzyme for immobilization study.

3.2 Characterization of phenylalanine dehydrogenase

3.2.1 Molecular weight determination of phenylalanine dehydrogenase subunit on SDS-PAGE

The molecular weight and subunit existence of PheDH was determined from SDS-PAGE as described in section 2.8.1. PheDH from Butyl-Toyopearl column was subjected to 12.5% SDS polyacrylamide gel electrophoresis. Standard proteins were run in parallel and a standard curve was constructed from their molecular weight and relative mobilities (**Figure 3.4 and 3.5**). The molecular weight of the enzyme protein monomer was calculated to be about 45,000 Da.

3.2.2 Substrate specificity of phenylalanine dehydrogenase

The ability of enzyme to catalyze the oxidative deamination and amination directions was studied in this work as mentioned in section 2.8.2. Various amino acids and their derivatives were examined. The specificity of PheDH in the direction of oxidative deamination and reductive amination is illustrated in **Table 3.2**. The enzyme was highly active towards L-phenylalanine. No activity was observed with *p*-hydroxyphenylacetate, *N*-methyl-L-phenylalanine, α-methyl-DL-phenylalanine, DL-β-phenyllactate, D-alanine, D-aspartate, D-arginine, D-histidine, D-serine, D-threonine, D-phenylalanine, L-alanine, L-asparagine, L-arginine, L-glutamine, L-glycine, L-histidine, L-leucine, L-tyrosine, L-hydroxyproline.

For reductive amination, in addition to phenylpyruvate, α-ketocaproate was also a good substrate. *p*-Hydroxyphenylpyruvate, indole-β-phenylpyruvate, α-keto-n-butyrate, α-ketoglutarate and pyruvate were found to be inert.
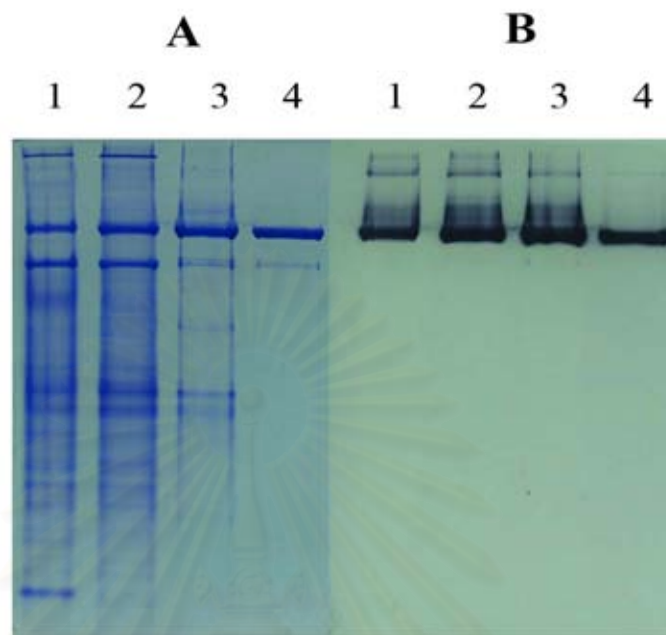
Figure 3.3 Non-denaturing PAGE of recombinant phenylalanine dehydrogenase expressed in *E. coli* JM109.

Each lane represents each step of purification.

A. Protein staining

Lane 1 Crude extract — 20 μg protein

Lane 2 30-50 % Saturated ammonium sulfate precipitation — 20 μg protein

Lane 3 DEAE-Toyopearl column — 16.7 μg protein

Lane 4 Butyl-Toyopearl column — 1.6 μg protein

B. Activity staining

Lane 1 Crude extract

Lane 2 30-50 % Saturated ammonium sulfate precipitation

Lane 3DEAE-Toyopearl column

Lane 4 Butyl-Toyopearl column
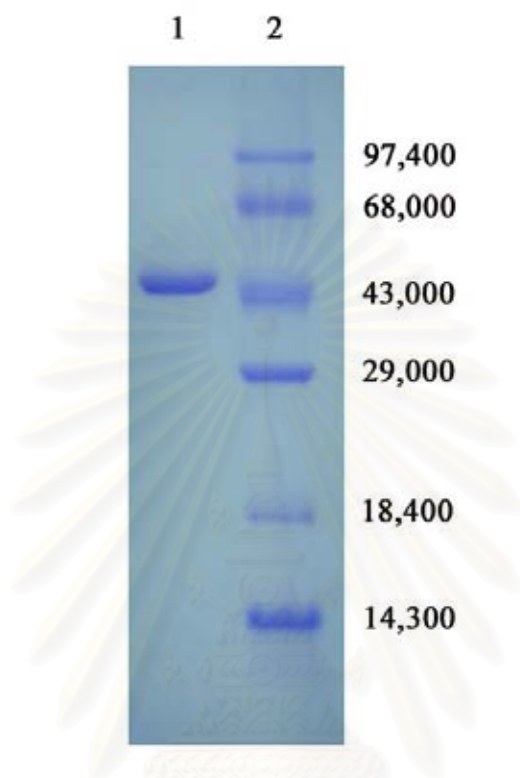
Figure 3.4 SDS polyacrylamide gel electrophoresis of phenylalanine dehydrogenase from recombinant *E. coli*

Lane 1:   PheDH from Butyl-Toyopearl column

Lane 2:   Phosphorylase B (MW 97,400)

Bovine serum albumin (MW 68,000)

Ovalbumin (MW 43,000)

Carbonic anhydrase (MW 29,000)

β-Lactoglobulin (MW 18,400)

Lysozyme (MW 14,300)
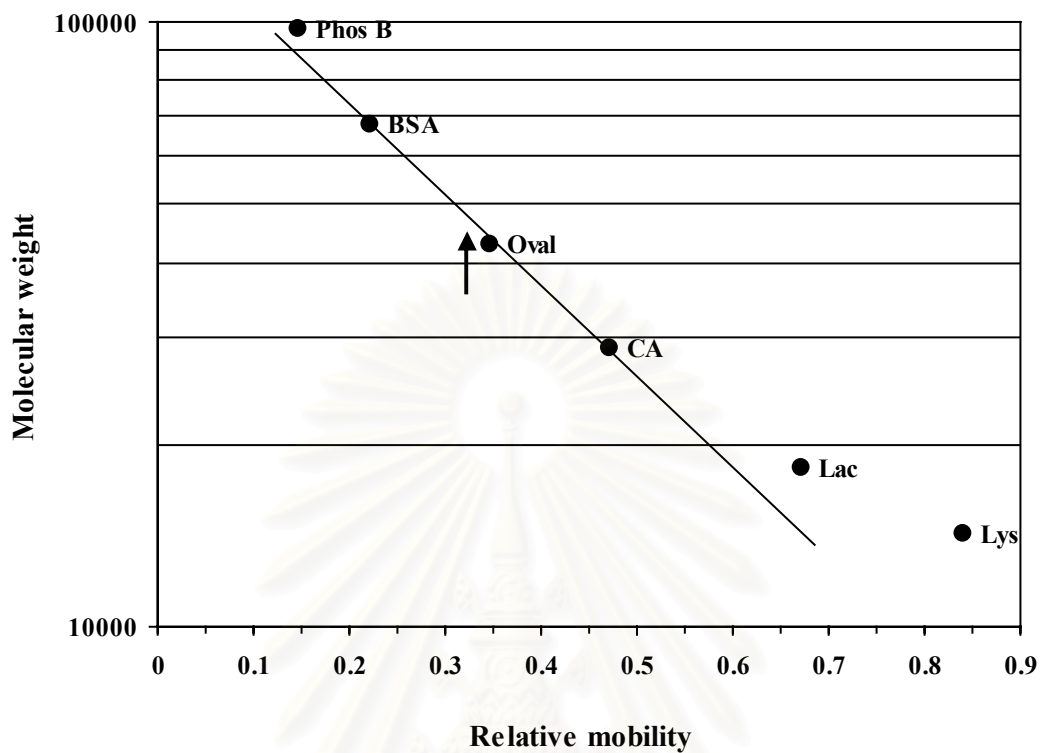
Figure 3.5 Calibration curve for molecular weight of phenylalanine
dehydrogenase subunit from recombinant *E. coli* on SDS
polyacrylamide gel electrophoresis

Phos B	= Phosphorylase B, MW 97,400	
BSA	= Bovine serum albumin, MW 68,000	
Oval	= Ovalbumin, MW 43,000	
CA	= Carbonic anhydrase, MW 29,000	
Lac	= β-Lactoglobulin, MW 18,400	
Lys	= Lysozyme, MW 14,300	

Arrow indicates the K_{av} of PheDH

Table 3.2 Substrate specificity of phenylalanine dehydrogenase from *E. coli* JM109 transformant [a]

Process and substrate	Relative activity (%)
Oxidative deamination [b]	
L-Phenylalanine	100.0
p-Fluoro-DL-phenylalanine	43.9
m-Fluoro-DL-phenylalanine	9.3
o-Fluoro-DL-phenylpyruvate	4.9
α-Amino-β-phenylbutanoate	6.7
L-Methionine	6.8
L-Valine	3.3
L-Tryptophan	4.6
Reductive amination [c]	
Phenylpyruvate	100.0
α-Ketocaproate	57.5
α-Ketoisocaproate	23.5
α-Ketovalerate	18.7
α-Ketoisovalerate	11.4
α-Keto-γ-methiol-butyrate	22.0
α-Keto-β-methyl-n-valerate	8.6

[a] The data represent the mean values of three independent experiments.

[b] Final concentration of each substrate was 20 mM. The following substrate were inert: *p*-hydroxyphenylacetate, *N*-methyl-L-phenylalanine, α-methyl-DL-phenylalanine, DL-β-phenyllactate, D-alanine, D-aspartate, D-arginine, D-histidine, D-serine, D-threonine, D-phenylalanine, L-alanine, L-asparagine, L-arginine, L-glutamine, L-glycine, L-histidine, L-leucine, L-tyrosine (1.2 mM) and L-hydroxyproline.

[c] Final concentration of keto acids was 10 mM. The following were inert: *p*-hydroxyphenylpyruvate (2.5 mM), indole-β-phenylpyruvate (1 mM), α-keto-n-butyrate, α-ketoglutarate and pyruvate.

3.2.3 Coenzyme specificity of phenylalanine dehydrogenase

Coenzyme specificity of PheDH was investigated as described in section 2.8.3. NAD^+, NAD^+ analogs and $NADP^+$ were assayed by measuring the increase in absorbance at various wavelengths. While $NADP^+$ showed no activity, NAD^+ was required as a natural coenzyme for PheDH in oxidative deamination. Moreover, some NAD^+ analogs could serve as a coenzyme (**Table 3.3**). Nicotinamide guanine dinucleotide and nicotinamide -1, N^6 -ethenoadenine dinucleotide were similar to NAD^+ in cofactor activity. 3-Acetylpyridine-NAD^+ was twice as much better coenzyme than NAD^+.

3.2.4 Effect of pH on phenylalanine dehydrogenase activity

The effect of pH on the activity of the enzyme was performed at various pH's ranging from 7.5 to 13.0 as mentioned in section 2.8.4. Tris-HCl buffer (pH range 7.5-9.0) and glycine-KCl-KOH buffer (pH range 8.5-13.0) at final concentration of 200 mM were used. The pH of each reaction mixture was measured with a pH meter after the reaction. Preliminary study of other buffers (potassium phosphate buffer, sodium phosphate buffer and citric acid/ sodium citrate buffer) was also investigated. However, there were no activities observed. The rate of reaction was calculated for the relative activity expressed in percentage. The enzyme exhibited maximal activity at pH 10.4 for oxidative deamination while the enzyme showed maximal activity for reductive amination at pH 8.7. The result is shown in **Figure 3.6**.

3.2.5 Effect of temperature on phenylalanine dehydrogenase activity

The enzyme was used to investigate the effect of temperature on its activity as described in section 2.8.5. The enzyme was incubated with reaction mixture contained L-phenylalanine, and glycine-KCl-KOH buffer at a final concentration of 200 mM, pH 11.5 at various temperatures ranging from 25ºC to 70ºC for 10 minutes. After that the reaction was started by the addition of NAD^+ to the solution with rapid mixing and the amount of NADH was measured. As shown in **Figure 3.7**, the enzyme showed the highest activity at 45ºC for oxidative deamination and at 40ºC for reductive amination.

Table 3.3 Coenzyme specificity of recombinant phenylalanine dehydrogenase
expressed in *E. coli* JM109 transformant [a]

Coenzyme[b]	Relative activity (%)
β-Nicotinamide adenine dinucleotide	100
3-Acetylpyridine adenine dinucleotide	200.5
3-Pyridinealdehyde adenine dinucleotide	22.4
Thionicotinamide adenine dinucleotide	50.7
Nicotinamide hypoxanthine dinucleotide	84.4
Nicotinamide guanine dinucleotide	98.7
Nicotinamide -1, N^6-ethenoadenine dinucleotide	105.7
β-Nicotinamide adenine dinucleotide phosphate	0

[a] The data represent the mean values of three independent experiments.

[b] Final concentration of each coenzyme analog was 2.0 mM. The assay was conducted at the following wavelengths: 3-acetylpyridine adenine dinucleotide, 363 nm ($\varepsilon = 9.1 \times 10^3$); 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 ($\varepsilon = 6.2 \times 10^3$); 3-pyridinealdehyde adenine dinucleotide, 358 nm ($\varepsilon = 9.3 \times 10^3$); and thionicotinamide adenine dinucleotide, 395 nm ($\varepsilon = 11.3 \times 10^3$). The reaction was carried out at pH 9.5 to avoid the degradation of NAD^+ analogs at a more alkaline pH.
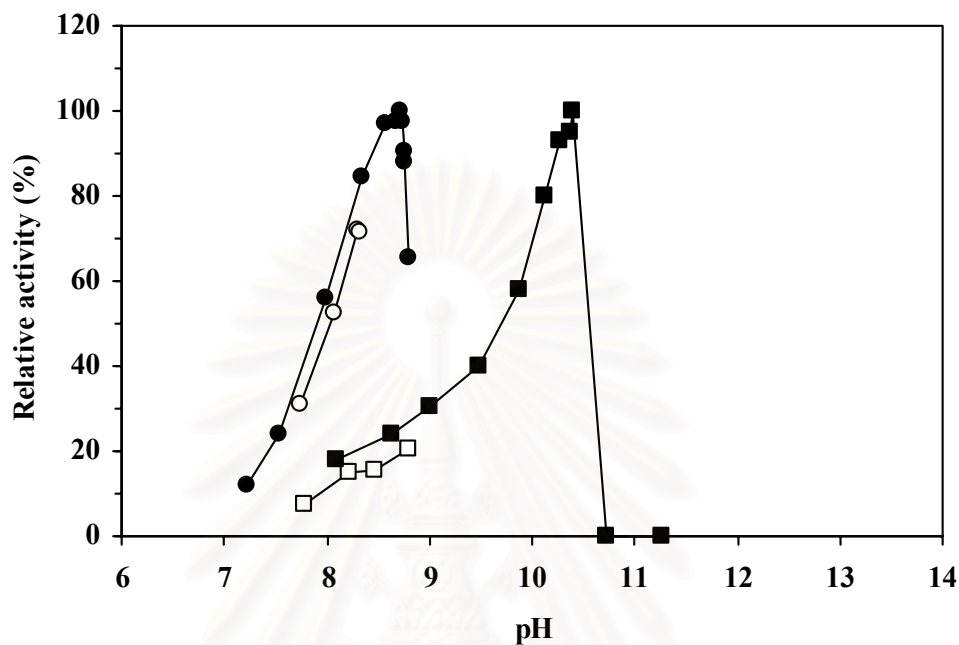
Figure 3.6 Effect of pH on phenylalanine dehydrogenase activity

The comparative study of phenylalanine dehydrogenase activity for both oxidative deamination and reductive amination was determined by using two kinds of buffer (200 mM, Tris-HCl and glycine-KCl-KOH). The pH of each reaction mixture was measured at room temperature. The relative activity of three independent experiments was averaged.

The oxidative deamination (■) Glycine-KCl-KOH buffer

(□) Tris-HCl buffer

The reductive amination (●) Glycine-KCl-KOH buffer

(○) Tris-HCl buffer
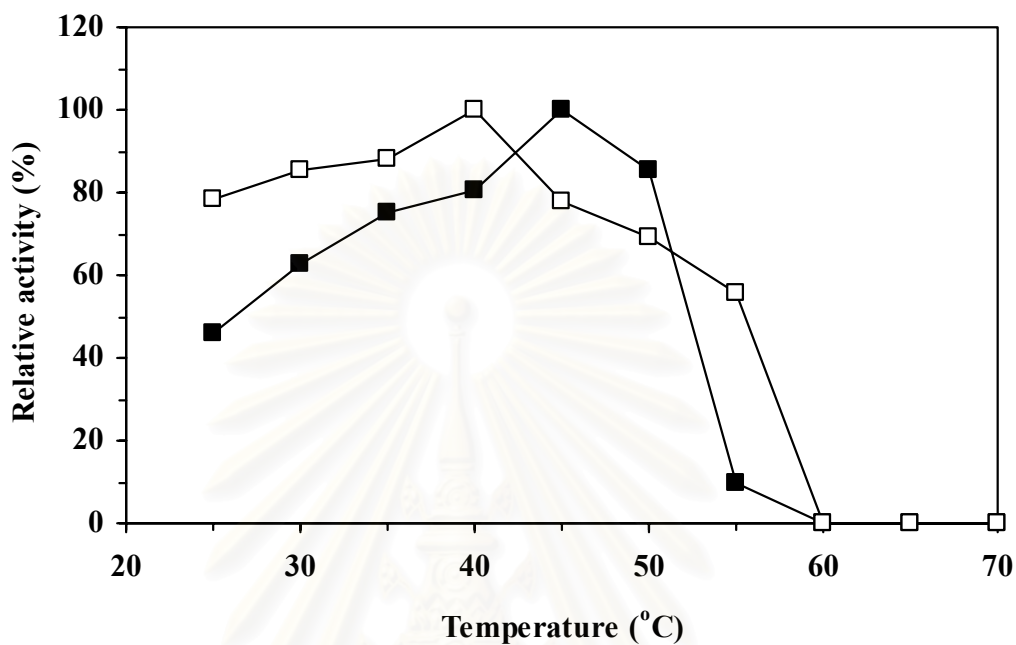
Figure 3.7 Effect of temperature on phenylalanine dehydrogenase activity

    The activity of enzyme for both oxidative deamination (■) and reductive amination (□) reactions was measured at various temperatures. Results are presented as a percentage of relative activity averaged from three independent experiments.

3.2.6 Effect of pH on phenylalanine dehydrogenase stability

The stability of PheDH against pH was studied as described in section 2.8.6. The enzyme was preincubated in 10 mM buffers at 30ºC for 20 minutes before the oxidative deamination activity was assayed under standard condition (glycine-KCl-KOH, pH 11.5 at 30ºC; section 2.5). Buffers of various pH's ranging from 3.0-13.0 were used. The residual activity was expressed relatively to the original activity which was defined as 100% when the enzyme was kept in 10 mM phosphate buffer, pH 7.4. As shown in **Figure 3.8**, this enzyme was stable over the wide range of pH values (6.5-12.5).

3.2.7 Effect of temperature on phenylalanine dehydrogenase stability

The thermostability of PheDH was studied as described in section 2.8.7. The enzyme was preincubated at various temperatures ranging from 30ºC to 70ºC for 10 minutes before the remaining oxidative deamination activity was determined. The original enzyme activity when the enzyme was not subjected to heat was assayed under standard condition at 30ºC (section 2.5) and was set as 100%. The residual activity was expressed relatively to the original activity. The result is shown in **Figure 3.9**. The enzyme retained its full activity at temperature up to 35ºC and lost about half of its activity at about 47.5ºC - 48ºC. At 55ºC, PheDH completely lost its activity.

3.3 Initial velocity study of phenylalanine dehydrogenase

3.3.1 Initial velocity analysis for oxidative deamination

Initial velocity studies for oxidative deamination were performed. The concentration of L-phenylalanine was varied in the presence of several fixed concentration of NAD^+. Double-reciprocal plots of initial velocity against reciprocals of L-phenylalanine concentrations gave a family of straight lines, which intersected in the upper left quadrant as shown in **Figure 3.10A**. The Michaelis constant for L-phenylalanine was calculated to be 2.27 mM. The apparent K_m value for NAD^+ was calculated to be 0.156 mM from the secondary plots of the intercepts at the ordinate versus reciprocal concentrations of NAD^+ (**Figure 3.10B**).
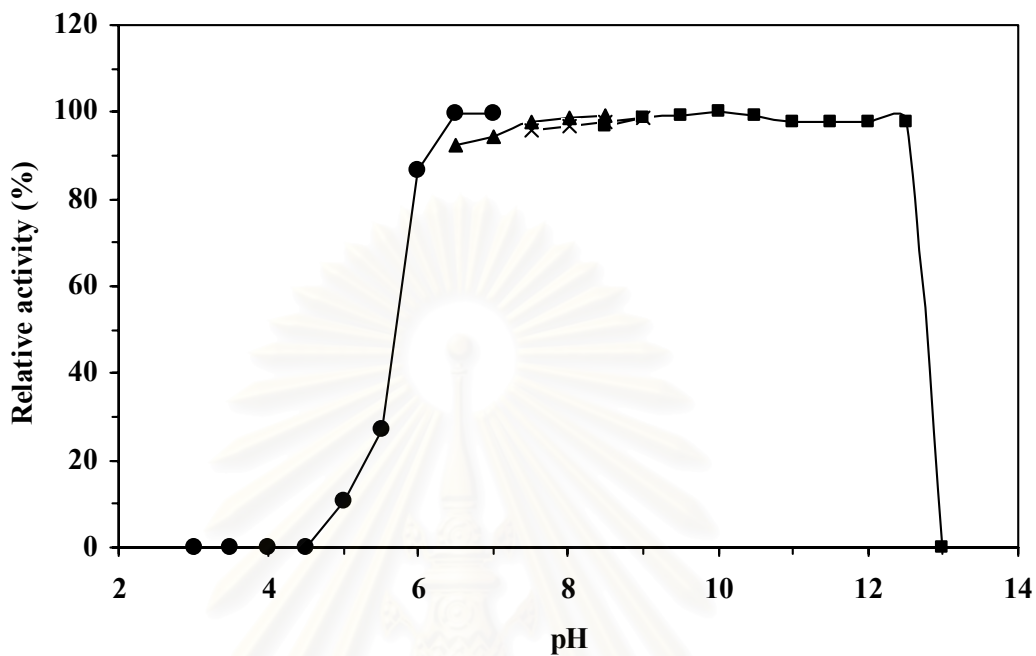
Figure 3.8 Effect of pH on phenylalanine dehydrogenase stability

       The enzyme was incubated with buffer at various pH's. The incubation was carried out at 30ºC for 20 minutes before the oxidative deamination activity was determined under standard condition. The 10 mM buffers used were Tris-HCl (pH 7.5-9.0; ×), glycine-KCl-KOH (pH 8.5-13.0; ■), potassium phosphate buffer (pH 6.5-8.5; ▲) and citrate buffer (pH 3.0-7.0; ●). Results shown were average values of triplicate experiments.
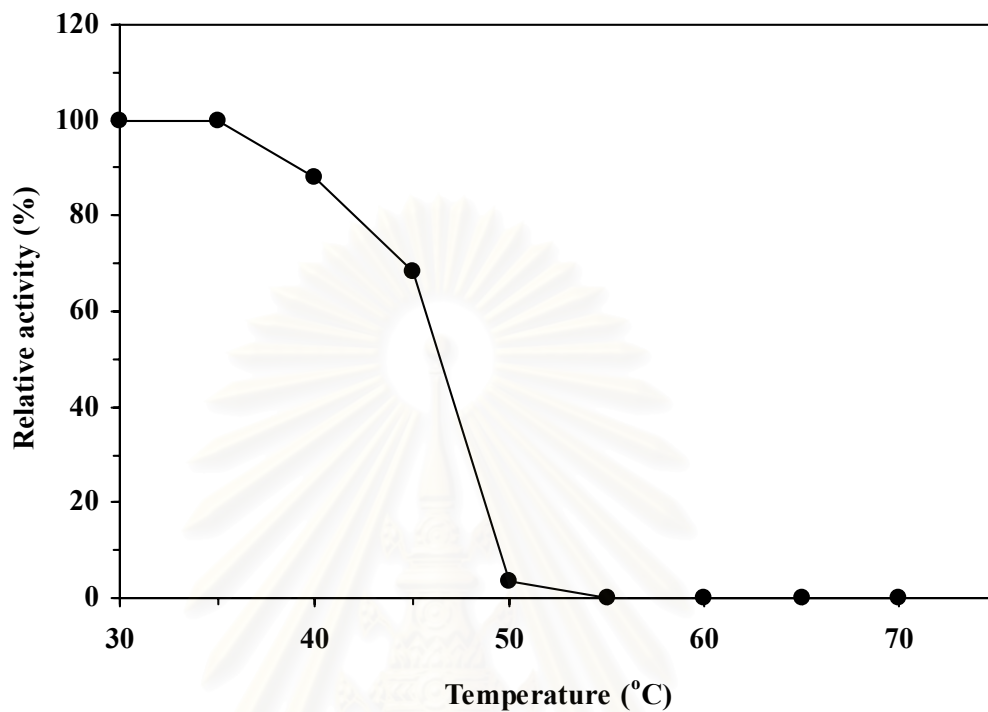
Figure 3.9 Effect of temperature on the stability of the phenylalanine

dehydrogenase. The enzyme was treated at various temperatures for 10

minutes before the residual oxidative deamination activity under standard

condition was determined at 30ºC. Results shown were average values of

triplicate experiments.
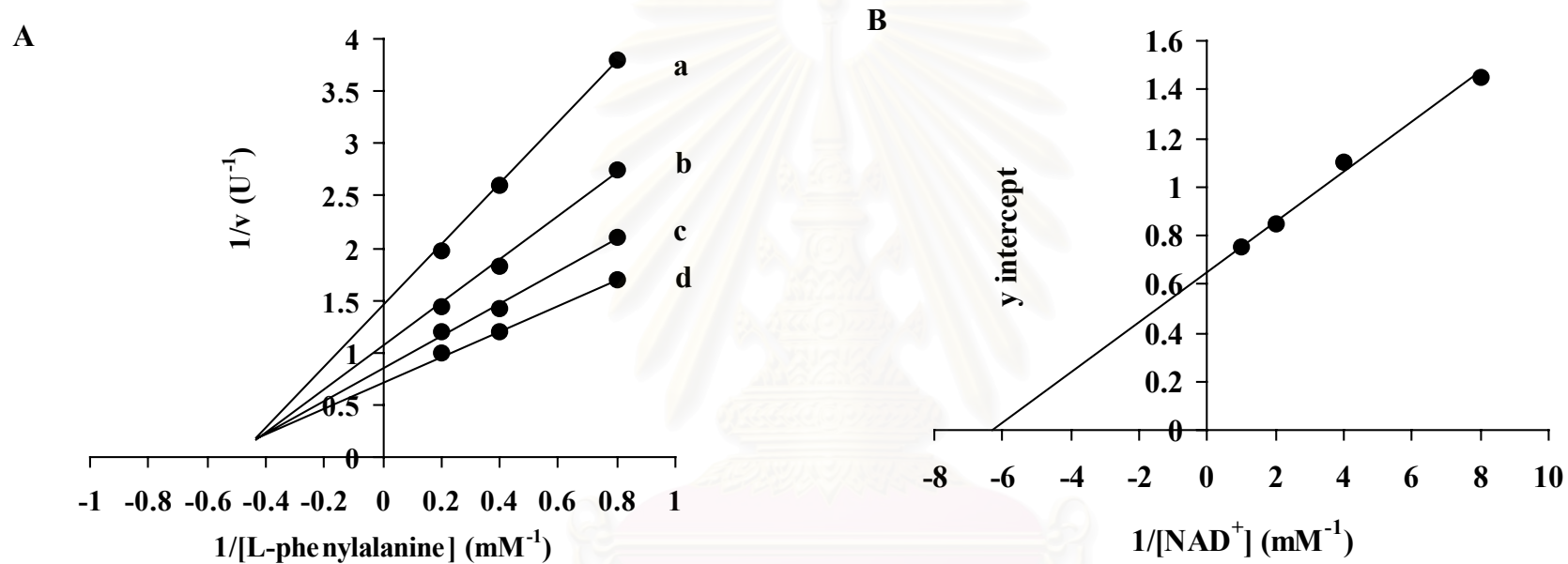
Figure 3.10   Initial velocity patterns of oxidative deamination

(A)   Double-reciprocal plots of initial velocities against L-phenylalanine concentrations at a series of fixed concentrations of NAD$^+$. The oxidative deamination reactions were carried out under the standard reaction conditions as described in section 2.5. The concentrations of NAD$^+$ used were 0.125 mM (a), 0.25 mM (b), 0.5 mM (c) and 1 mM (d). The concentrations of L-phenylalanine used were 1.25, 2.5 and 5 mM.

(B)   Secondary plots of y intercepts versus reciprocal plots concentrations of NAD$^+$.

3.3.2 Initial velocity analysis for reductive amination

3.3.2.1) At a high concentration of NADH (0.2 mM), the concentration of phenylpyruvate was varied in the presence of several fixed concentration of ammonium chloride. Double-reciprocal plots in **Figure 3.11A** showed straight intersecting lines from which the apparent K_m of phenylpyruvate was calculated to be 0.385 mM. The secondary plots of intercept versus reciprocal concentrations of ammonium chloride (**Figure 3.11B**) gave the apparent K_m of this substrate to be 83.3 mM.

3.3.2.2) At a saturating concentration of phenylpyruvate (10 mM), the double-reciprocal plots of velocities versus ammonium chloride concentration at several fixed concentrations of NADH gave parallel straight lines (**Figure 3.12**).

3.3.2.3) At a high concentration of ammonium chloride (500 mM), the double reciprocal plots of velocities versus NADH concentration at several fixed concentration of phenylpyruvate were studied. The straight intersecting lines were observed (**Figure 3.13A**) and the apparent K_m of NADH was calculated to be 0.056 mM. The secondary plots of the intercepts at the ordinate versus reciprocal concentrations of phenylpyruvate (**Figure 3.13B**) gave the same apparent K_m of 0.385 mM as in **Figure 3.11A**.

The apparent K_m values of the substrates of PheDH were summarized in **Table 3.4**.

3.4   Immobilization of phenylalanine dehydrogenase

The main focus of this topic was to covalently immobilize the recombinant PheDH on various solid supports including alumina, silica, poly (vinyl alcohol) bead (PVA) and chitosan bead. Three activating agents, 1, 4- butanediol diglycidyl ether, glutaraldehyde and 2, 2, 2-trifluoroethanesulfonyl chloride (tresyl chloride) were investigated in order to achieve maximum immobilization efficiency.
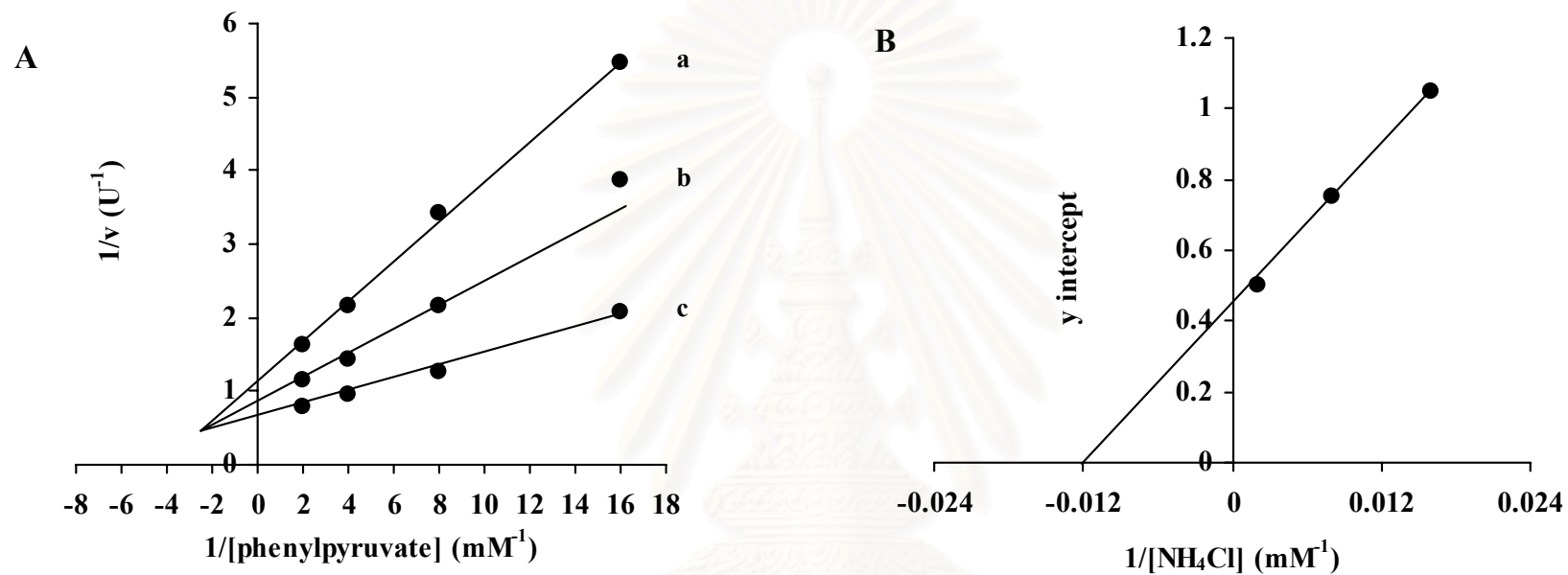
Figure 3.11   Initial velocity patterns for reductive amination (phenylpyruvate vs NH₄Cl)

(A)   Double-reciprocal plots of initial velocities against phenylpyruvate concentrations at several fixed concentrations of NH₄Cl in the presence of a saturating and constant concentration (0.2 mM) of NADH. The concentrations of NH₄Cl used were 62.5 mM (a), 125 (b) and 500 mM. The concentrations of phenylpyruvate were 0.0625, 0.125, 0.25 and 0.5 mM.

(B)   Secondary plots of y intercepts versus reciprocal concentrations of NH₄Cl
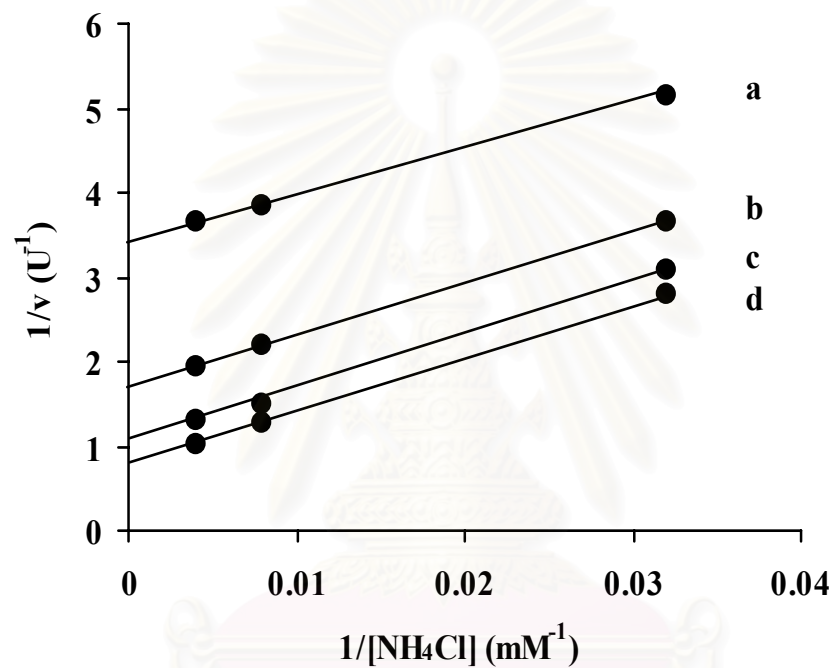
Figure 3.12 Initial velocity pattern for reductive amination (NH₄Cl vs NADH)

Double-reciprocal plots of initial velocities versus NH_4Cl concentration at several fixed concentration of NADH in the presence of a saturation and constant concentration (10 mM) of phenylpyruvate. The concentrations of NADH were 0.025 mM (a), 0.05 mM (b), 0.1 mM (c) and 0.2 mM (d). The concentrations of NH_4Cl used were 31.25, 125 and 250 mM.
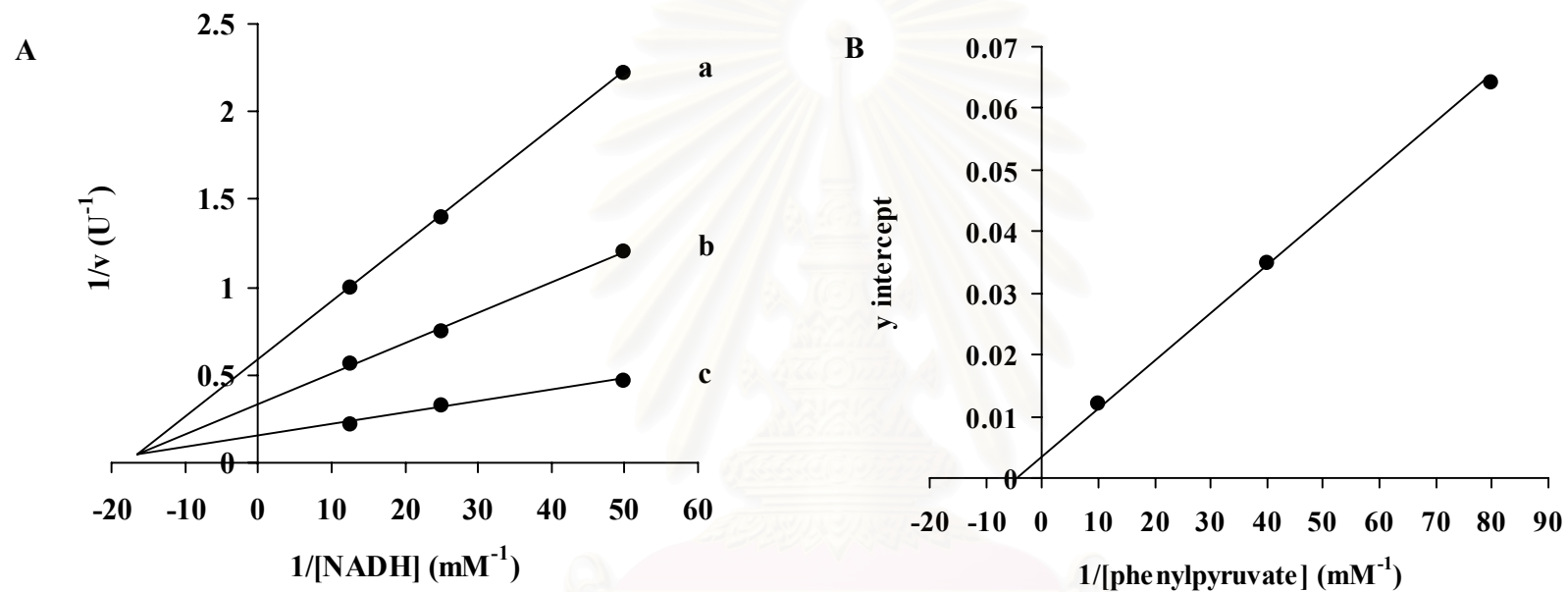
Figure 3.13 Initial velocity patterns for reductive amination (NADH vs phenylpyruvate)

(A)   Double-reciprocal plots of initial velocities against NADH concentration at several fixed concentrations of phenylpyruvate in the presence of a saturating and constant concentration (500 mM) of NH_4Cl. The concentrations of phenylpyruvate were 0.0125 mM (a), 0.025 mM (b) and 0.1 mM (c). The concentrations of NADH were 0.02, 0.04 and 0.08 mM.

(B)   Secondary plots of y intercepts versus reciprocal concentrations of phenylpyruvate

Table 3.4 The apparent K_m values of substrates of recombinant phenylalanine dehydrogenase from *E. coli* JM109 transformant

Substrate	K_m (mM)
L-Phenylanine	2.27
NAD^+	0.156
Phenylpyruvate	0.385
NH_4Cl	83.3
NADH	0.056

3.4.1 Optimization of the coupled reaction for phenylalanine dehydrogenase activity assay

Several reaction parameters involved in the coupled reaction as described in section 2.11 including WST-1 concentration, the amount of diaphorase, pH of buffer and incubation time were optimized. The enzyme used was free PheDH.

3.4.1.1 Optimal concentration of WST-1

PheDH oxidized phenylalanine to phenylpyruvate in the presence of NAD^+. The cofactor NAD^+ was converted to its reduced form (NADH) by accepting the electrons generated in the enzymatic reaction. WST-1 was then reduced to WST-1 formazan, which was determined spectrophotometrically. The effect of WST-1 concentration on this coupled reaction is shown in **Figure 3.14**. The concentration of WST-1 was varied from 1 mM to 20 mM. The assay was carried out under standard condition as described in section 2.11 except that various WST-1 concentrations were used. A linear range was observed to all WST-1 concentration. The excessive amount of WST-1 is necessary. From the result shown, 10 mM was selected to set as a coupled reaction assay condition.

3.4.1.2 Appropriate amount of diaphorase

The redox reaction takes place between NADH and WST-1. In this reaction WST-1 was reduced, forming WST-1 formazan with a simultaneous oxidation of NADH to NAD^+. Diaphorase from *Clostridium kluyveri* acted as an NADH-dye oxidoreductase (Sarker *et al.*, 2001). The amount of diaphorase was varied from 0.001 to 1 U. The assay was carried out under the condition from section 3.4.1.1 except that various amount of diaphorase was used as described in section 2.11. The result is shown in **Figure 3.15**. The absorbance at 438 nm was increase when the amount of diaphorase was increase. There was no significant difference in absorbance shown between 0.1 U and 1 U of diaphorase. So 0.1 U of diaphorase was selected to be an appropriate amount of coupling enzyme in this coupled reaction system.
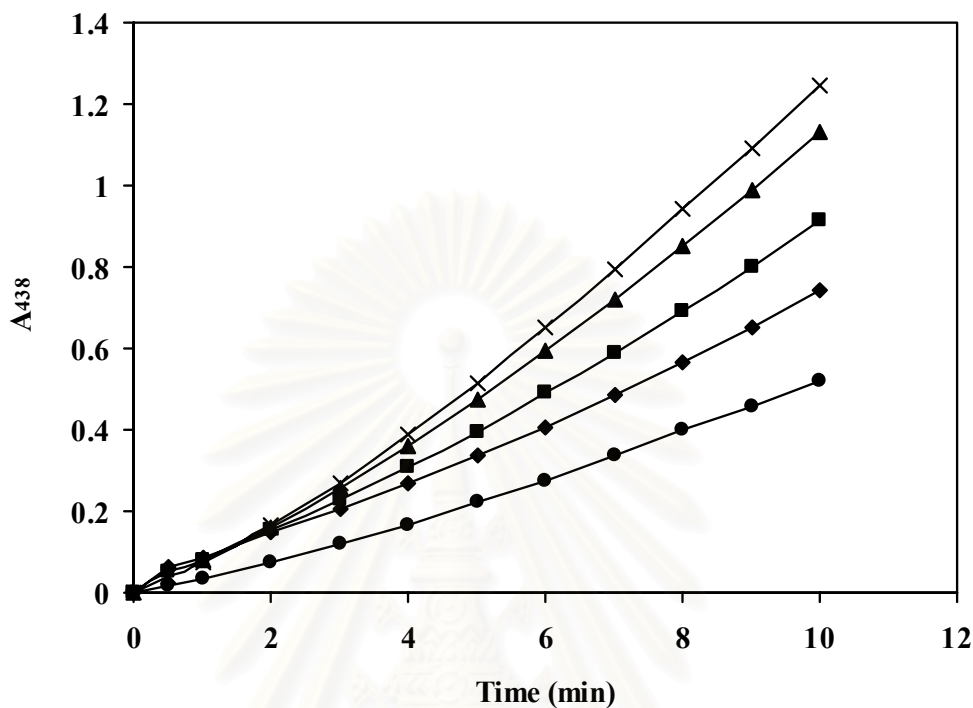
Figure 3.14 Effect of WST-1 concentration on the coupled reaction for phenylalanine dehydrogenase activity assay. The reaction mixture contained 0.4 ml of 20 mM of L-phenylalanine, 0.1 ml of 1 mM of NAD^+, 0.2 ml of 200 mM of glycine-KCl-KOH buffer, pH 9.0, 0.01 U of diaphorase. The concentration of WST-1 was varied, 1mM (●), 5 mM (x), 10 mM (▲), 15 mM (■) and 20 mM (♦). Results shown were average values of triplicate experiments.
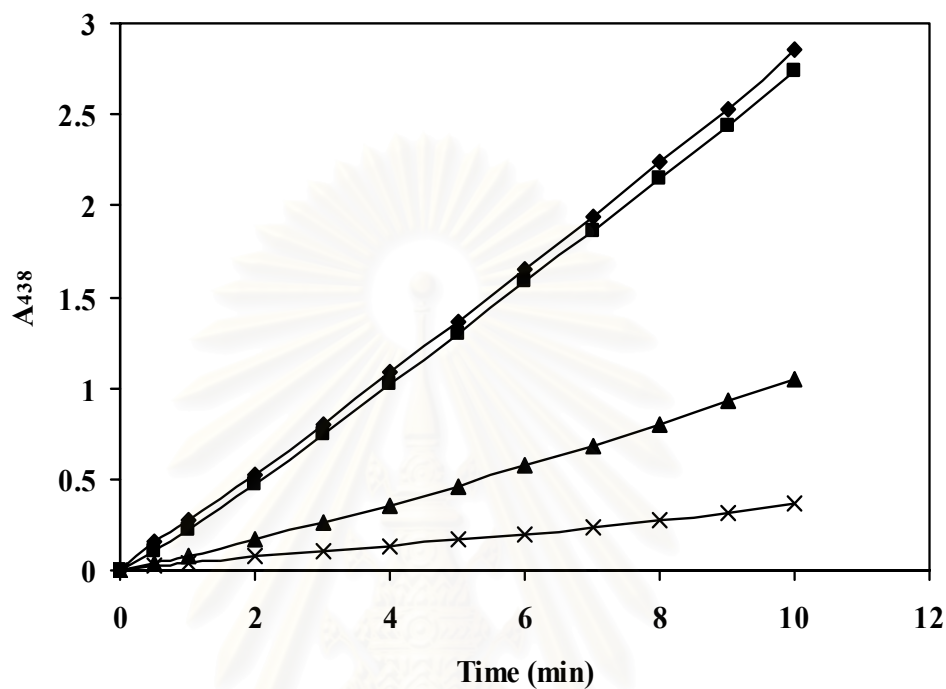
Figure 3.15 Effect of the amount of diaphorase unit on the coupled reaction for phenylalanine dehydrogenase assay. The reaction mixture contained 0.4 ml of 20 mM of L-phenylalanine, 0.1 ml of 1 mM of NAD^+, 0.2 ml of 200 mM of glycine-KCl-KOH buffer, pH 9.0, and 0.1 ml of 10 mM WST-1. The amount of diaphorase was varied, 0.001 U (**x**), 0.01 U (**▲**), 0.1 U (**■**), 1 U (**♦**). Result shown were average values of triplicate experiments.

3.4.1.3 Buffer type and pH

To compromise the pH of the coupled reaction for both enzymes, the buffers used were Tris-HCl buffer (pH 8.5 and 9.0) and glycine-KCl-KOH buffer (pH 8.5 and 9.0). The result is shown in **Figure 3.16**. Glycine-KCl-KOH buffer, pH 9.0 gave highest absorbance among the selected buffers and then was selected to an appropriate buffer.

The suitable incubation time can be observed from the experiments above. When the coupling enzyme is used, it is important to remember that its activity must be greater than the primary enzyme rate and that the observed activity is not equal to the rate of the primary enzyme until a defined lag period has elapsed (Brooks and Suelter, 1989). As can be seen from **Figure 3.14 - 3.16**, the lag period was at the first 5 minutes of incubation, so that a precise measurement of the initial enzyme's activity could be obtained after this lag time. Ten minutes was selected, because initial activity of the enzyme was still linear.

3.4.2 Selection of a suitable solid support and an appropriate method of activation

The partially purified PheDH was immobilized on various solid supports activated with various reagents except for adsorption which did not need any activation. The activation procedures were described in section 2.10.2. Enzyme coupling was carried out when 20 U of PheDH was incubated with 1 g of alumina, silica, PVA or chitosan for 12 hours at 4ºC (section 2.10.3). The enzyme activity was assayed under condition described in section 2.11.

3.4.2.1 Epoxy activated supports

As described in section 2.10.2.1, to give an epoxy- functional group to the support, 1, 4-butanediol diglycidyl ether was used. One of the oxirane side chains was reacted with the hydroxyl group of alumina, silica and PVA and with the amino group of chitosan while the other end with the similar oxirane ring was reacted with amino, hydroxyl and thiol group of the enzyme. The schematic diagram showing this approach is shown in **Figure 3.17**. Alumina, silica and PVA showed very low
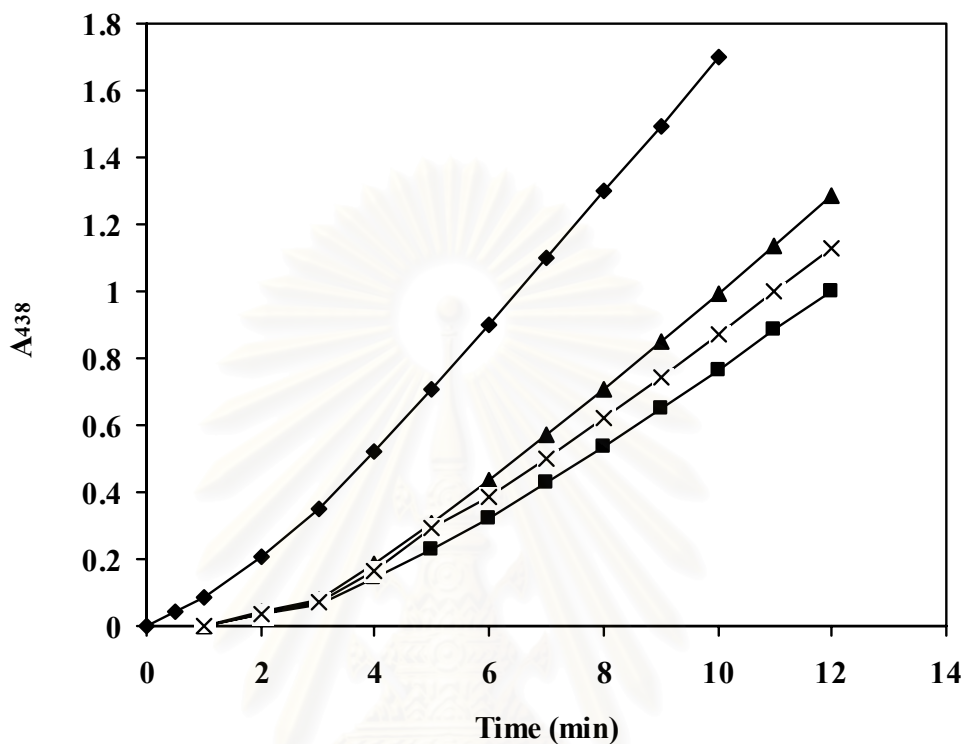
Figure 3.16 **pH dependence of phenylalanine dehydrogenase and diaphorase coupled reaction on WST-1 reduction.** The reaction mixture contained 0.4 ml of 20 mM of L-phenylalanine, 0.1 ml of 1 mM of NAD$^+$, 0.1 ml of 10 mM WST-1, and 0.01 U of diaphorase. Two kinds of 0.2 ml of 200 mM buffer were determined; Tris-HCl, pH 8.5 (**x**), Tris-HCl, pH 9.0 (▲), glycine-KCl-KOH, pH 8.5 (■) and glycine-KCl-KOH, pH 9.0 (♦). Results shown were average values of triplicate experiments.
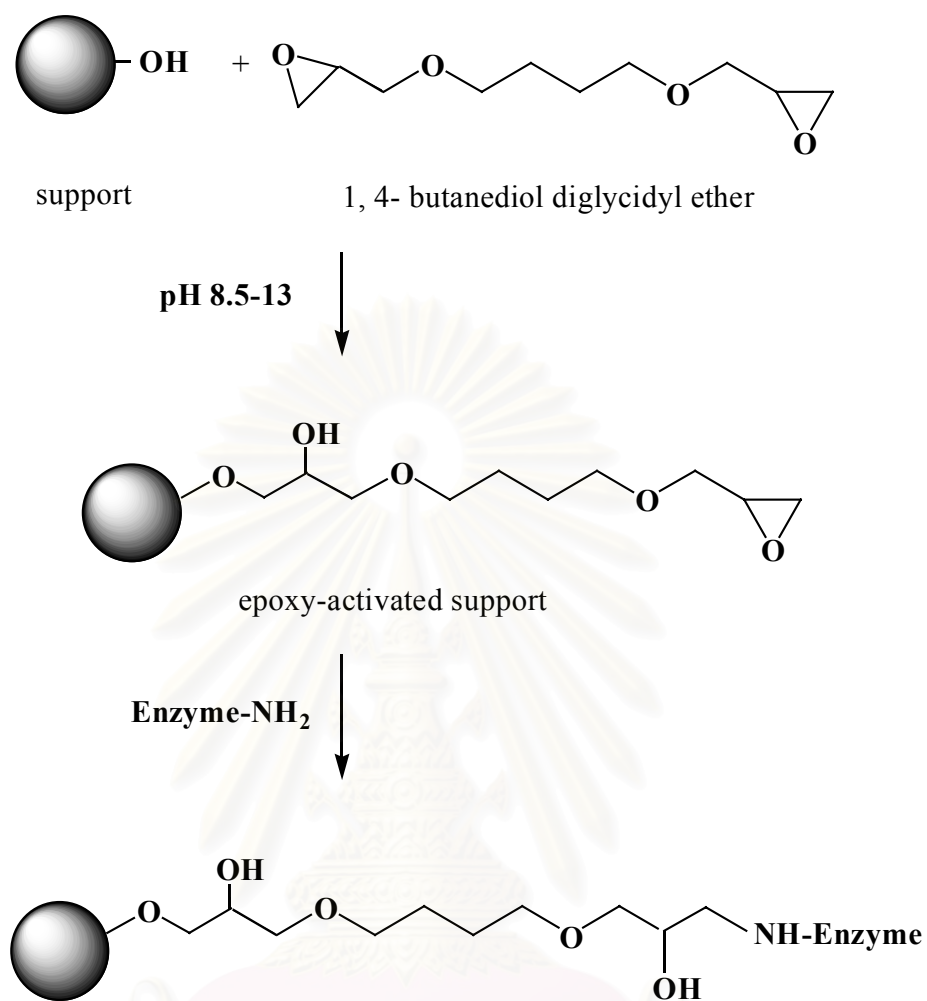
Figure 3.17 Reaction scheme for coupling a primary amine (R-NH₂) to epoxy-activated support (Ragnitz *et al*., 2001; Martín *et al*., 2003).

immobilized activity and thus, were not good supports activated with this method (**Figure 3.18**). Silica was not an appropriate support because it dissolved when incubated with 1, 4-butanediol diglycidyl ether solution. Chitosan gave the best result among these four supports activated with epoxide reagent.

3.4.2.2 Glutaraldehyde method

Glutaraldehyde was selected as one of the coupling agents applied to the supports which already contained amino functional group. As described in section 2.10.2.2, 2% (v/v) APTS was added to alumina, silica, PVA except chitosan since it already contains the amino groups on the surface followed by the addition of 0.25% (v/v) glutaraldehyde. The aldehyde group can react with amino group presented on activated bead and amino lysine of enzyme. The schematic diagram is shown in **Figure 3.19**. As illustrated in **Figure 3.18**, alumina and PVA showed very low immobilized activity while silica and chitosan showed high immobilized activity compared with other methods. The coupling of enzyme to a glutaraldehyde activated support is considered to be due to the formation of Schiff base of the amino group on an enzyme molecule with the aldehyde group.

3.4.2.3 Tresyl chloride method

Tresyl chloride was used to convert hydroxyl groups into good leaving group to yield sulfonated supports. This activated supports were shown to couple to primary amines and/or to the sulfhydryl groups of protein (Nilsson and Mosbach, 1984). The method was described in section 2.10.4.3. The schematic diagram is shown in **Figure 3.20**. No activity was observed on alumina, while silica and PVA gave slightly better immobilized activities. The feature of this technique was observed when chitosan was used as a support (**Figure 3.18**).
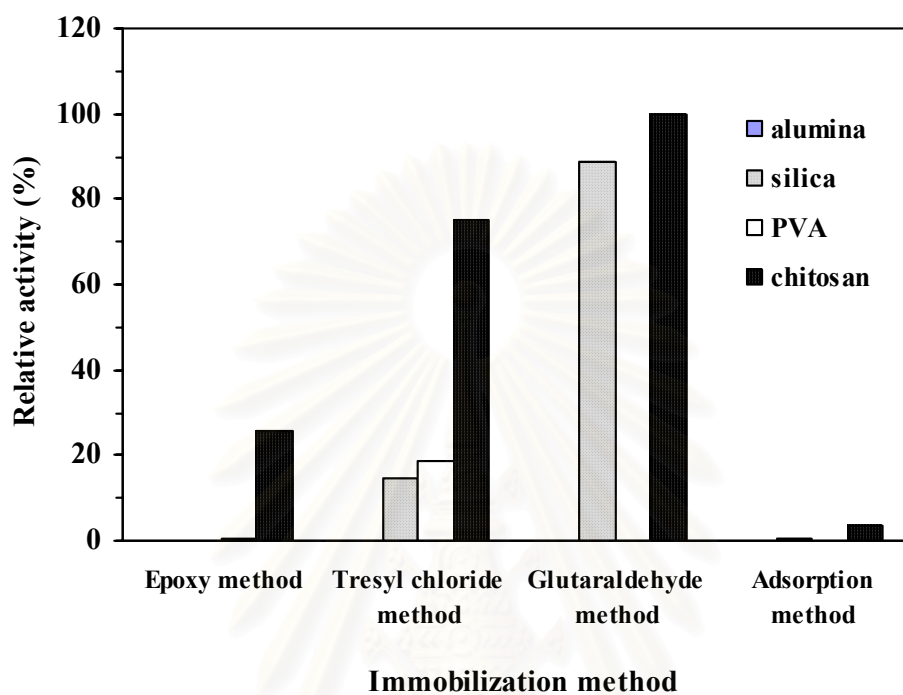
**Figure 3.18 Immobilization of phenylalanine dehydrogenase on various
support materials and different activation methods.**
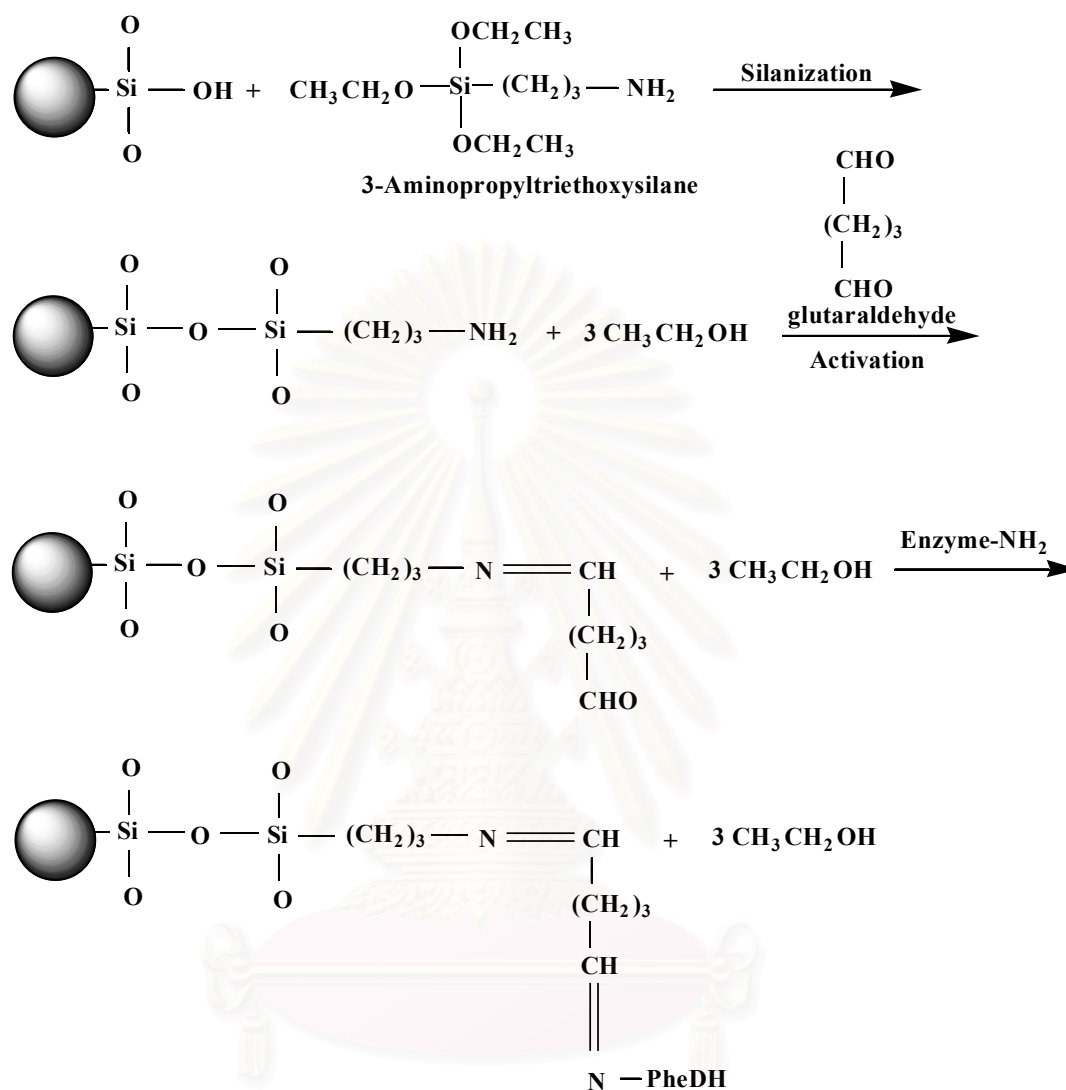
Figure 3.19 Schematic diagram showing an approach of covalent attachment of enzyme through their amino groups using glutaraldehyde as a coupling agent (Ikediobi *et al*., 1997; Jia, 2002; Martín *et al*., 2002).

Activation step:
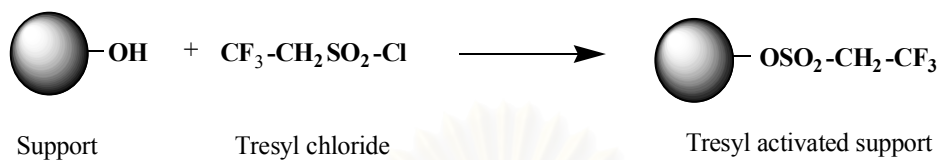
Coupling step:
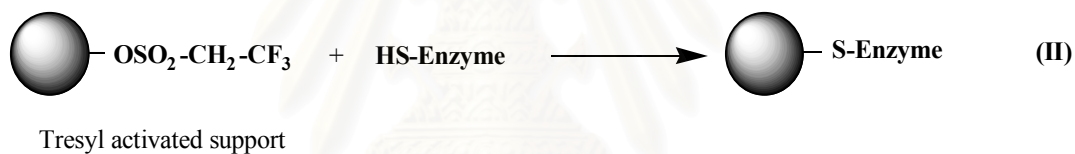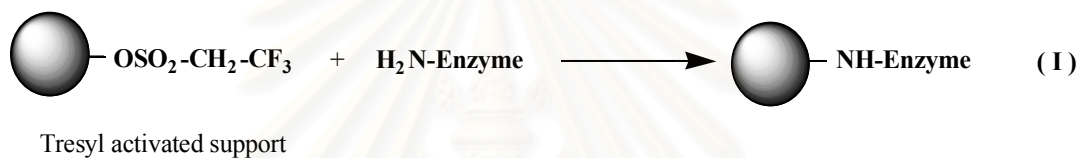
Figure 3.20 **The coupling of the ligand to the support activated with 2, 2, 2-trifluoroethanesulfonyl chloride (tresyl chloride)**

3.4.2.4 Adsorption method

In order to confirm that the enzyme was immobilized through covalent linkage, physical adsorption was also investigated. The enzyme was directly added and incubated with the supports. Multiple types of bonds were formed. Due to weak bond formation, all types of supports showed very low activity after washing with high ionic strength buffer (**Figure 3.18**).

After the preparation of immobilized enzyme, its activity was measured with the colorimetric method as mentioned in section 2.11. The activity was expressed in the unit of enzyme per gram of support. Among these methods of activation which afforded the covalent linkage, glutaraldehyde method gave the highest activity of PheDH immobilized on both silica and chitosan. The result is summarized in the form of bar graph in **Figure 3.18**. Although chitosan offers a unique set of characteristics, it was difficult to perform the immobilization steps due to its light feature. Hence, the immobilized enzyme was lost in each step of separation. Silica was then chosen among various kinds of carriers with the most promising coupling agent, glutaraldehyde.

3.4.3   Optimization of the immobilization procedure

Covalent immobilization of PheDH onto silica gel was conducted to improve the enzyme efficiency. There were, basically, several reaction parameters involved in support activation and enzyme coupling steps. To gain high immobilization efficiency, four different factors which may influence the immobilized activity including APTS and glutaraldehyde concentration, coupling time and enzyme loading were optimized as described previously in section 2.12.

3.4.3.1 Effect of APTS concentration

The preparation of silanized inorganic carriers was given earlier by Weetall (1976). In order to investigate the influence of APTS concentration on the amount of PheDH immobilized on silica, the carrier was treated with different concentration of APTS varying from 1 to 10% (v/v). Twenty units of enzyme were used per gram support. The incubation was carried out for 2 hours at room

temperature before activation with 0.25% (v/v) glutaraldehyde. As can be seen from **Figure 3.21**, at 6% (v/v) of APTS, the activity of PheDH immobilized on silica gel exhibited the maximum with 0.24 U/g support. The immobilized activity was slightly decreased at other concentration of APTS in the range of 0.16-0.22 U/g support. Therefore, the suitable APTS concentration for silanization of silica gels was 6% (v/v) and was set as a standard condition for the next step.

3.4.3.2 Effect of glutaraldehyde concentration

The next factor to be concerned was glutaraldehyde concentration. After functionalized the silica bead with 6% (v/v) APTS, the bead was then activated with different concentrations of glutaraldehyde in the range of 0.1 to 2.5% (v/v), in 0.1 M phosphate buffer, pH 7.0 for 2 hours at room temperature. The excessive amount of glutaraldehyde was washed to prevent self-crosslink reaction and the unwanted linkage between enzyme and excess glutaraldehyde. The 20 U of enzyme was subsequently added to 1 g of supports and incubated for 12 hours at 4ºC. The effect of the activation condition with glutaraldehyde on the activity of the immobilized PheDH is depicted in **Figure 3.22**. The highest activity resulted from the activation by 0.1% (v/v) glutaraldehyde. The higher glutaraldehyde concentration was used, the lower immobilized PheDH activity was detected. Hence, 0.1% (v/v) of glutaraldehyde concentration was selected for the activation of solid support.

3.4.3.3 Effect of enzyme concentration

To obtain a greater degree of immobilized activity, different amounts of PheDH were also tested. **Figure 3.23** shows the immobilized activity increased when increasing the amount of enzyme applied to the carrier from 4 U to 16 U per gram silica support. The immobilization yield was calculated as described in section 2.13. Further increase of enzyme concentration gave poorer immobilization yield, probably due to saturation of the given quantity of support. The immobilization yield, however, was inversely proportional to the immobilized activity. From the result, the activity
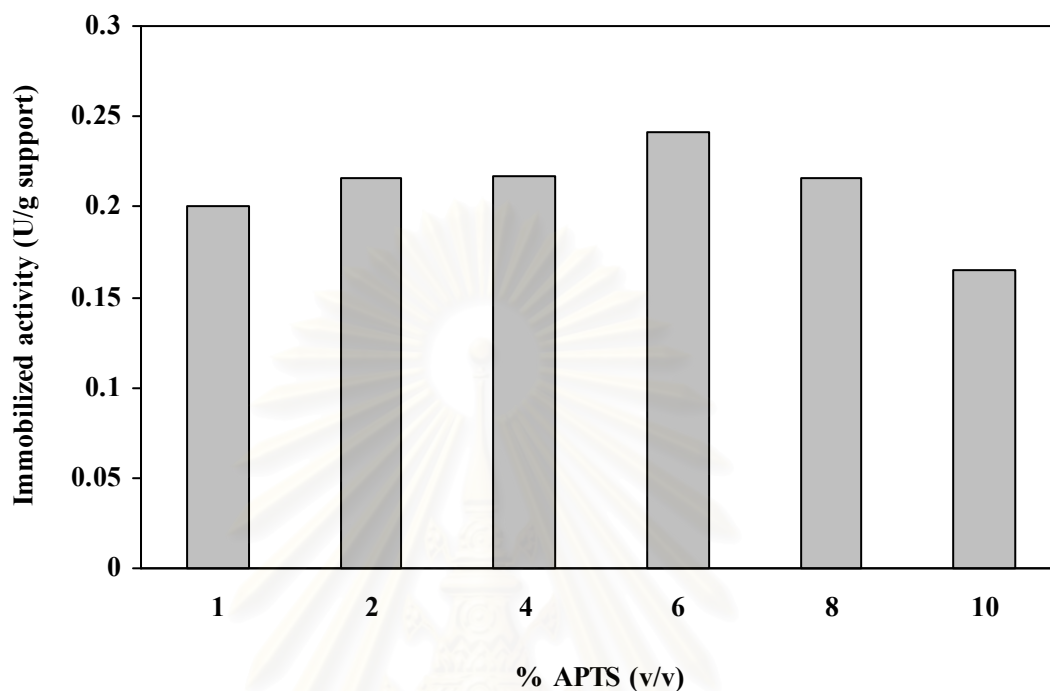
Figure 3.21 Influence of APTS concentration on the amount of phenylalanine dehydrogenase immobilized on silica gel. The silica support was silanized with different concentration of APTS, activated with 0.25% (v/v) glutaraldehyde and incubated with 20 U of enzyme at 4ºC for 12 hours. Results shown were mean values of duplicate experiments.
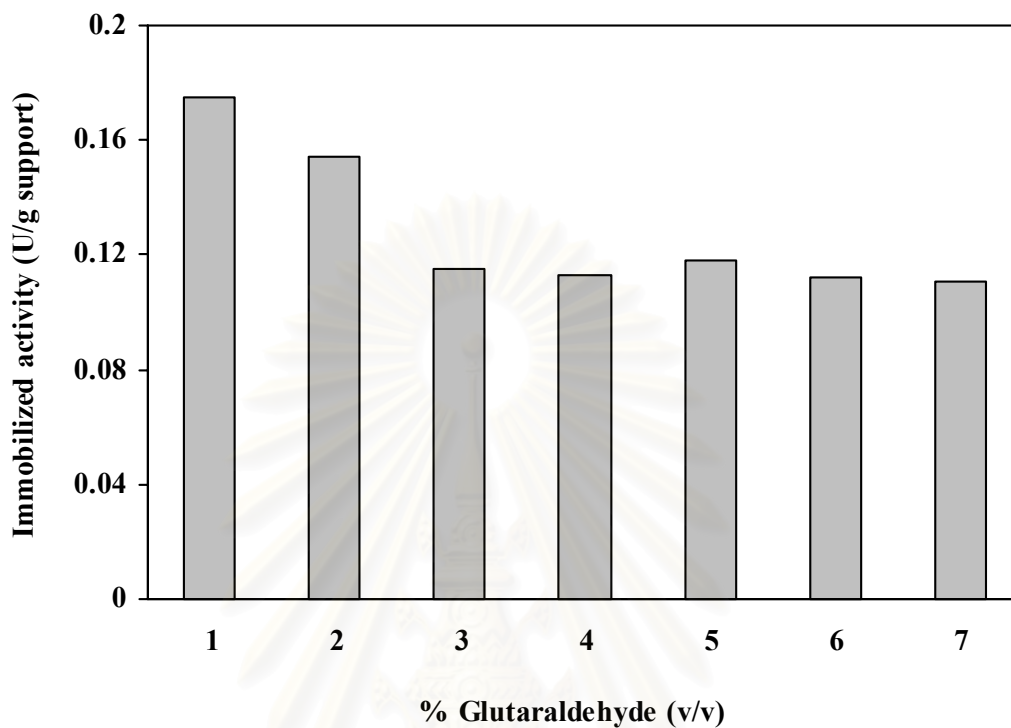
Figure 3.22 Influence of glutaraldehyde concentration on the amount of phenylalanine dehydrogenase immobilized on silica gel. The silica support was silanized with 6% (v/v) APTS, activated with different concentration of glutaraldehyde in 0.1 M phosphate buffer (pH 7.0). The incubation was carried out at 4ºC for 12 hours. Results shown were average values of duplicate experiments.

Figure 3.23 Effect of phenylalanine dehydrogenase unit applied on the exhibited activity and immobilization yield. 4-20 U of enzyme was subjected to the silica support silanized with 6% (v/v) APTS and activated with 0.1% (v/v) glutaraldehyde. The incubation proceeded at 4°C for 12 hours. Results shown were mean values of duplicate experiments.

reached equilibrium when the 16 unit of enzyme was used and the immobilization yield at this point was 1.05% yield.

3.4.3.4 Effect of coupling time

The results of the influence of the incubation time between PheDH and activated silica support are presented in **Figure 3.24**. The coupling time was varied from 0.5 to 16 hours at 4ºC. It was found that the most efficient incubation time between the enzyme and the support was 6 hours. After that, the immobilized activity gradually decreased. The recovered activity of the immobilized enzyme was notably dependent on the coupling time. Therefore, a coupling time of 6 hours was selected to be used in subsequent studies of PheDH immobilization.

3.5 Characterization of the free and immobilized phenylalanine dehydrogenase

Immobilized enzymes differ from theirs soluble counter part, not only in their physical properties but also in many other characteristics (Gemeiner, 1992). In this way, the enzymatic properties such as pH and temperature optimum, pH and temperature stability, and storage stability of immobilized PheDH were compared with those of soluble PheDH.

3.5.1 Effect of pH on phenylalanine dehydrogenase activity

The effect of pH on the free and immobilized PheDH activities was investigated as described in section 2.14.1. The reaction rate of both forms of enzyme was measured at various pH values ranging from 8.5 – 13.0. After the reaction, the pH of each reaction mixture was measured with a pH meter due to the presence of proton from the reaction. The pH-activity curves of soluble and immobilized PheDH are depicted in **Figure 3.25**. Both biocatalysts showed an optimum pH of 9 in the presence of glycine-KCl-KOH, pH 9.5. The soluble enzyme has a slightly broader pH range. Immobilized enzyme was slightly more sensitive to pH changes than soluble enzyme at pH < 9 and at pH > 9.8.
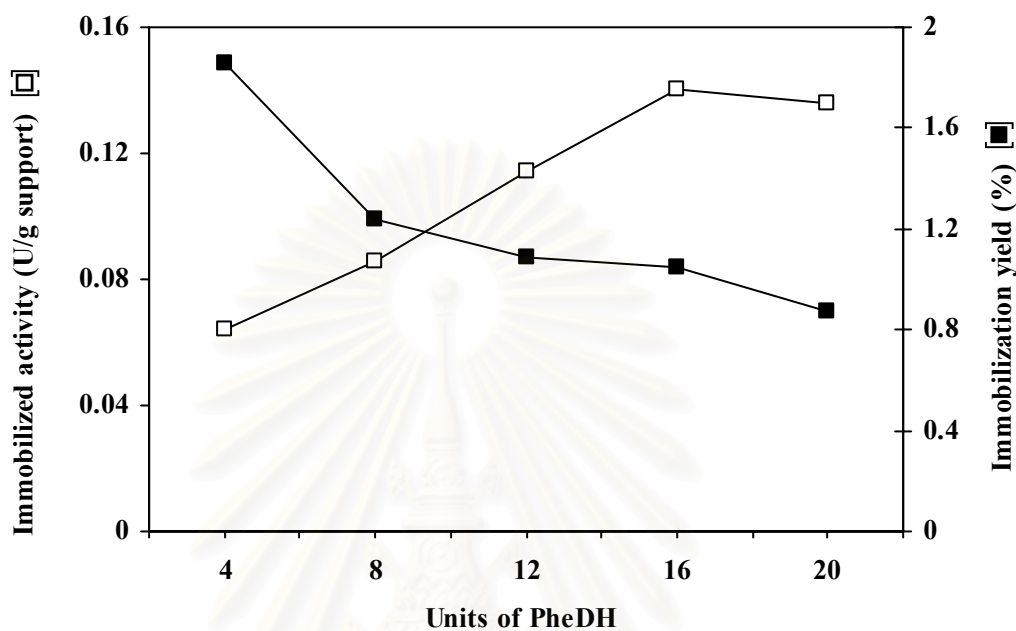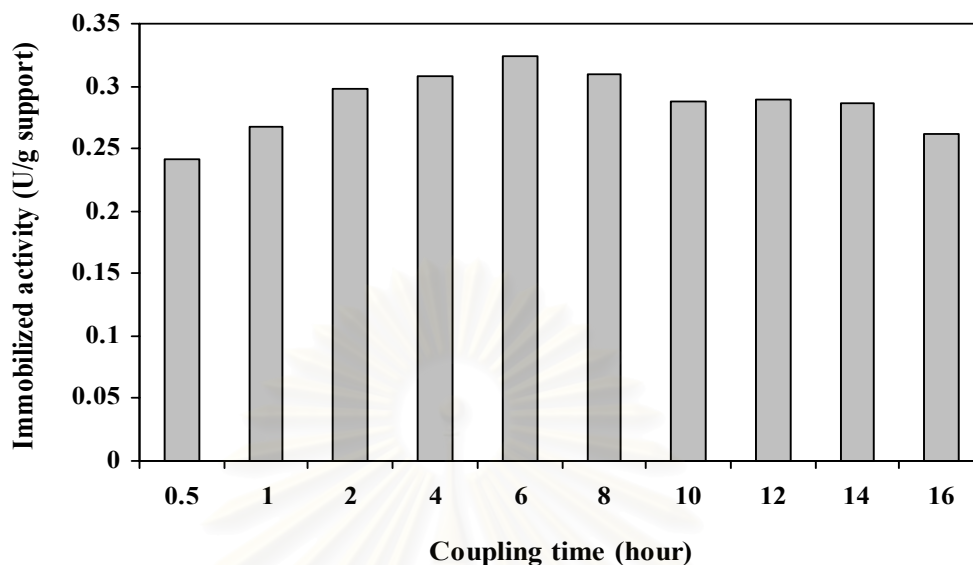
Figure 3.24 Effect of coupling time on the amount of phenylalanine

dehydrogenase immobilized on silica. The silica support was silanized
with 6% (v/v) APTS, activated with 0.1% (v/v) glutaraldehyde and incubated
with 16 U of enzyme at 4ºC at different incubation times. Results shown were
average values of duplicate experiments.
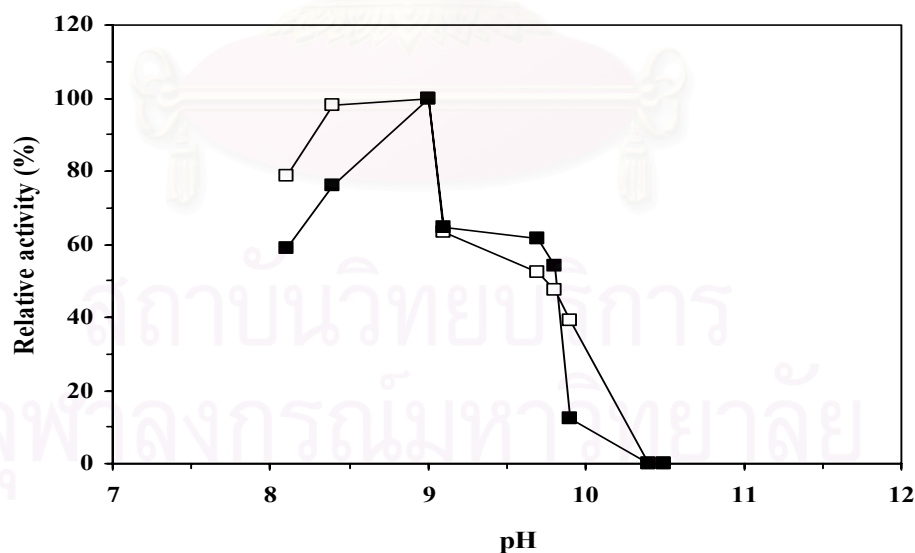
Figure 3.25 Effect of pH on the activity of free and immobilized phenylalanine

dehydrogenase. The activity of free (□) and immobilized (■) enzyme was
measured in glycine-KCl-KOH buffer (200 mM, pH range 8.5-13.0) at room
temperature. The highest activity was regarded as 100%. Results shown were
average values of duplicate experiments.

3.5.2 Effect of the temperature on phenylalanine dehydrogenase activity

The activity of soluble and immobilized PheDH was measured at various temperatures as described in section 2.14.2. The effect of temperature on enzyme activity is shown in **Figure 3.26**. The optimal temperature of free PheDH was 35ºC while immobilized enzyme showed a broad range of optimal the temperatures from 30ºC to 55ºC.

3.5.3 Effect of pH on phenylalanine dehydrogenase stability

The stability of the soluble and immobilized PheDH was also examined as mentioned in section 2.14.3. Both enzyme preparations were exposed to different pH values (3.0 - 13.0) at room temperature for 20 minutes then assayed under the condition described in section 2.11 and calculated for the relative activity expressed in percentage. Both biocatalysts were notably stable over the wide pH range of 6.0 – 11.5 when they were incubated at different pH values of 10 mM buffers. It can be said that immobilization did not affect the pH stability of the enzyme. The profile is shown in **Figure 3.27**.

3.5.4 Effect of the temperature on phenylalanine dehydrogenase stability

The thermal inactivation of soluble and immobilized PheDH was investigated at various temperatures (25ºC – 90ºC) as mentioned in section 2.14.4. Both enzymes were preincubated in 10 mM phosphate buffer pH 7.0 at various temperatures for 20 minutes. The residual activities were then assayed under standard condition at 25ºC (section 2.11). The free and the immobilized enzymes showed the same profile of thermostability (**Figure 3.28**). The activity of both enzymes decreased with increasing temperature but the relative activity of free enzyme was lower than that of immobilized one at temperature > 55ºC. At 70ºC, free and immobilized PheDH completely lost their activity.
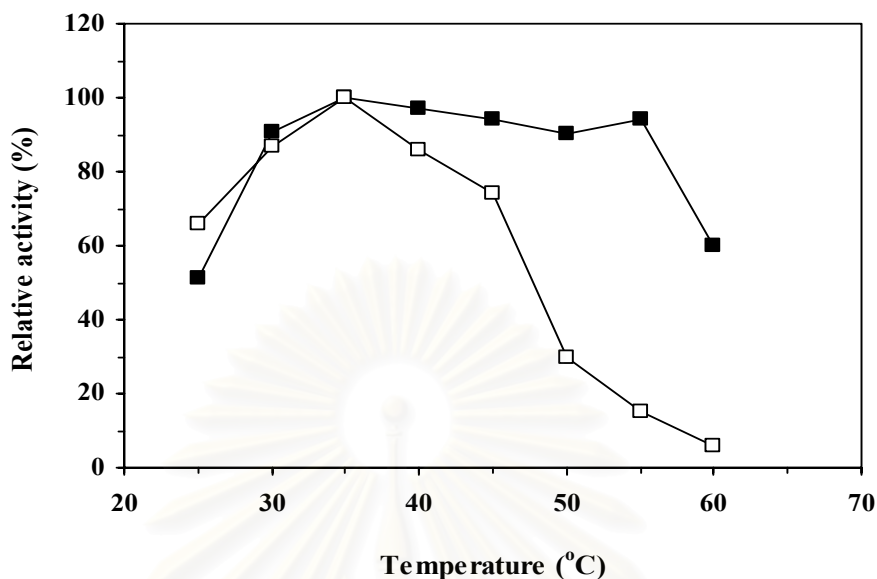
Figure 3.26 Effect of temperature on the activity of free and immobilized

phenylalanine dehydrogenase. The reaction of free (□) and immobilized
(■) enzyme was performed in glycine-KCl-KOH (200 mM, pH 9.0) at various
temperatures. Results shown were average values of duplicate experiments.
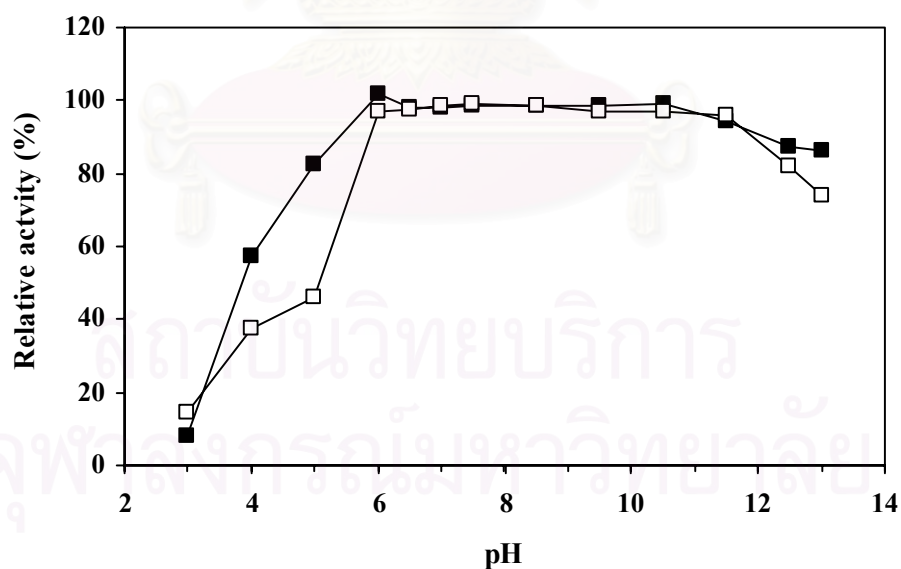
Figure 3.27 Effect of pH on the stability of free and immobilized

phenylalanine dehydrogenase. Free (□) and immobilized (■) enzymes
were treated in various buffers pH 3.0-13.0 for 20 minutes at room temperature.
The remaining activity was then assayed under standard condition. Results
shown were mean values of duplicate experiments.
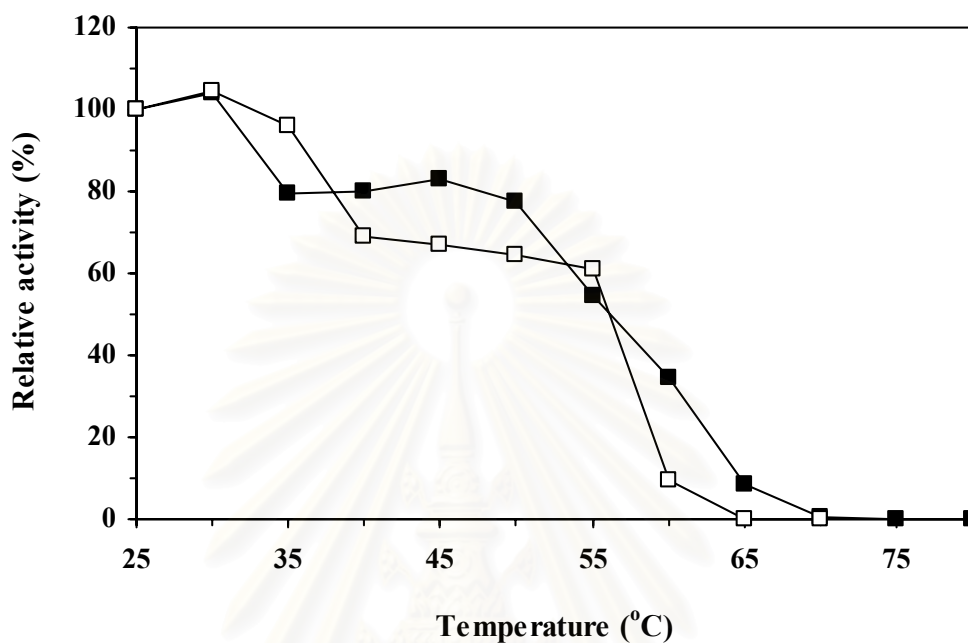
Figure 3.28 Effect of temperature on the stability of free and immobilized

phenylalanine dehydrogenase. Free (□) and immobilized (■) enzyme samples were preincubated in 10 mM phosphate buffer, pH 7.4 for 10 minutes at various temperatures and then were assayed for residual activities at room temperature. Results shown were mean values of duplicate experiments.

3.5.5 Storage stability

As previously described in section 2.14.5, the storage stability of both free and immobilized enzymes was studied. They were kept at 4ºC and room temperature. Immobilized enzyme samples stored in 10 mM phosphate buffer pH 7.4 were withdrawn every week to determine their residual activities for 1 month. In the case of free PheDH, it was stored in 10 mM phosphate buffer pH 7.4 with 2-mercaptoethanol and 10% (v/v) glycerol as stabilizer. It can clearly be seen from **Figure 3.29** that immobilized enzyme was still stable at both temperatures after 1 month of storage. For the free enzyme, the residual activities were gradually lost for the first week of storage even at 4ºC. After one month of storage, soluble PheDH retained its activity for only 69% at 4ºC while completely lost its activity at room temperature.

3.6 Synthesis of amino acids from their keto acids using immobilized enzyme

Attempts were made to synthesize amino acids from their keto acids using immobilized PheDH. Six kinds of keto acids were phenylpyruvate, α-ketocaproate, α-ketoisocaproate, α-ketovalerate, α-ketoisovalerate and α-keto-γ-methiol-butyrate which were converted to the products including phenylalanine, norvaline, valine, norleucine, leucine and methionine, respectively. The method was described in section 2.15. All of the substrates selected were based on the specificity of enzyme in the direction of reductive amination (section 3.2.2). After 10 mg (0.006 U) of immobilized PheDH incubated with 5 μmol of each substrate in reaction mixture containing 200μmol NH_4Cl-NH_4OH, pH 9.5, and 5 μmol NADH in the total volume of 0.5 ml for 20 hours, the products were tested by running the sample with standard amino acids in parallel. The results are shown in **Figure 3.30**. It can be concluded that immobilized PheDH can be used to produce amino acids from their keto acids.

(A)
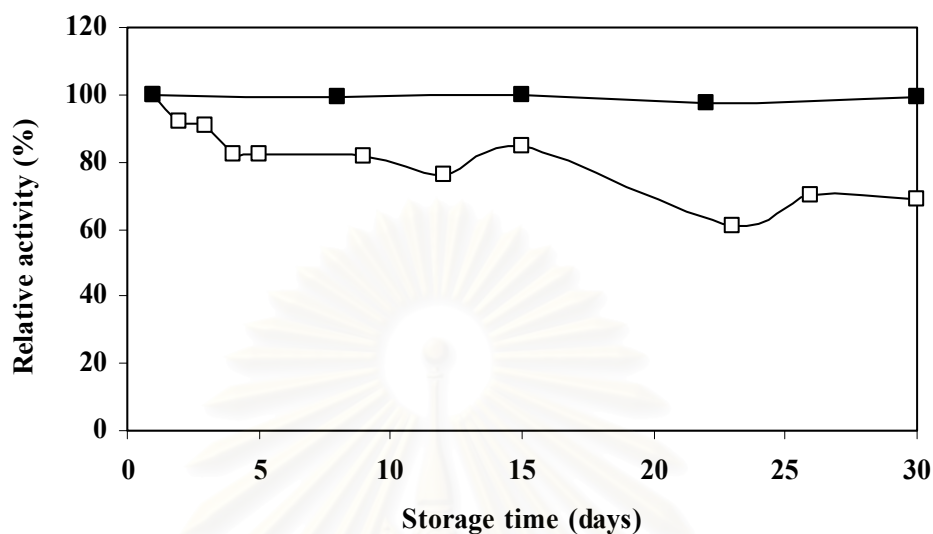
(B)
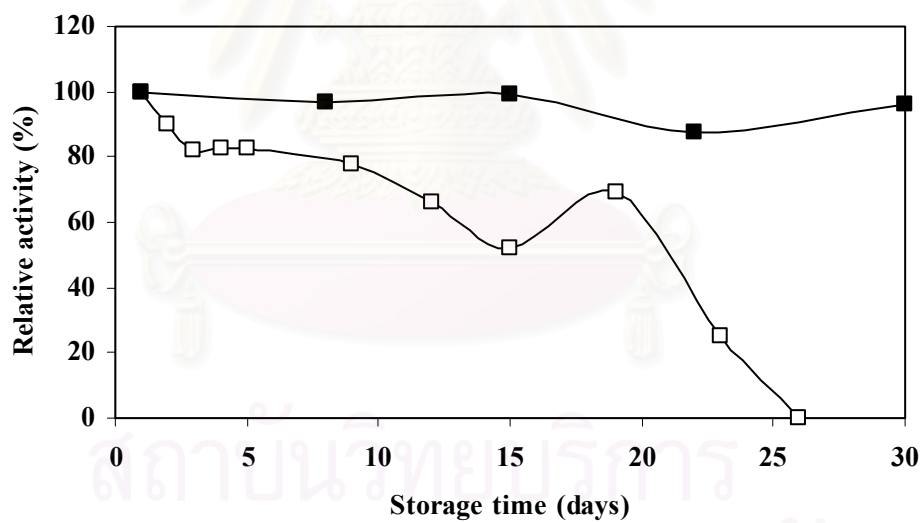
Figure 3.29 Storage stability at 4ºC (A), and room temperature (B) of the free and immobilized phenylalanine dehydrogenase on silica gel. Free (□) and immobilized (■) enzyme samples were stored in 10 mM phosphate buffer, pH 7.4. The buffer used for free enzyme contained 2-mercaptoethanol and glycerol as a stabilizer. Results shown were average values of duplicates experiments.

Figure 3.30 Illustrating produced amino acids from their keto acids using

immobilized phenylalanine dehydrogenase determined by TLC plate

Ten milligrams of immobilized PheDH on silica was incubated with the mixture containing 5 μmol of substrates, 200 μmol of NH_4Cl-NH_4OH, pH 9.5 and 5 μmol of NADH at 30ºC for 20 hours. The products were applied on TLC plate. After developed with solvent, the ninhydrin solution was sprayed to invisible spot of amino acids to form purple color.

Lane 1 = standard phenylalanine   Lane 2 = phenylalanine sample

Lane 3 = standard norvaline   Lane 4 = norvaline sample

Lane 5 = standard valine   Lane 6 = valine sample

Lane 7 = standard norleucine   Lane 8 = norvaline sample

Lane 9 = standard leucine   Lane 10 = leucine sample

Lane 11 = standard methionine   Lane 12 = methionine sample

A semiquantitative determination of amino acids produced by immobilized PheDH was established by TLC technique. The TLC-intensity calibration curve was linearly expressed as shown in Appendix E. Sample was run in parallel with 5-40 nmol of amino acid standards as described in section 2.15.3. L-phenylalanine content was calculated from the standard curve to be 4.2 μmol which was 84.4% conversion. Norvaline, valine, norleucine, leucine and methionine were produced with 79.1%, 63.7%, 100%, 67.4% and 75.3% conversion, respectively.

CHAPTER IV

DISCUSSION

Commercial interest in amino acids is an outgrowth of an understanding of many functions that these life-giving substances perform in humans and animals. As understanding of the functions and properties of amino acids increases, new commercial applications enter development while current commercial uses continue to expand their markets on a worldwide basis (www.neutraceuticalsworld.com; www. ajinomoto.com). For a nutritional supplement, the market growth will be a direct result of the trend towards healthier and active lifestyles and the proliferation of amino acids into multiple product areas. In 1999, the amino acids market posted sales of $178 million for the pharmaceutical and nutritional segments, which was an 8.1% increase from 1989. In 2004, the market was increased to $244 million and expected to $331 million by 2009 (www.neutraceuticalsworld .com). As previously described in section 1.3, PheDH involved in phenylalanine production. However, the use of such enzyme in free form was hampered by the sensitivity of enzyme towards the action of external factors (Bryjak and Kolarz, 1997). The difficulties of catalyst regeneration also vitally limit large-scale applications in industry. Thus, to extend the use of PheDH, a more abundant and cheaper supply of stable enzymes was required. One of the possible ways was to use the recombinant enzyme in insoluble form. Immobilized enzymes have now a proven track record of success, having prevailed in a 40-year campaign for their utilization in industry (Wiseman and Woods, 2004).

PheDH from *B. badius* BC1 was previously purified and some properties such as molecular mass, substrate specificity and kinetics were characterized (Leksakorn, 2001). *phedh* gene was then successfully cloned and overexpressed in *E. coli* JM109 by Chareonpanich (2001), but the recombinant enzyme properties have not yet been studied. Following this line, the present work described the partial purification of the recombinant PheDH from this strain and then characterized for some properties compared to those of the wild type. Since the use of immobilized enzyme is one of the most effective ways to facilitate the industrial production of compounds via enzyme-

catalyzed reaction. The appropriate support and the immobilization procedure for the immobilization of PheDH were selected. Some properties such as optimum pH and temperature, pH stability, thermostability and storage stability were determined. The attempt of this research was also extended to test the feasibility of the immobilized PheDH for the production of L-phenylalanine.

4.1 Partial purification of phenylalanine dehydrogenase from recombinant *E. coli*

B. badius BC1 has previously been reported to produce high PheDH activity (Suriyapanpong *et al*., 2000). Its gene was then cloned and overexpressed in *E. coli* (Chareonpanich, 2001). After the expression of recombinant PheDH, it is important to purify the enzyme rapidly in order to characterize its features and evaluate its applicability. Nevertheless, the enzyme produced by *E. coli* transformant has not yet been purified and characterized. Three approaches are usually applied for the purification of the enzyme: ammonium sulfate precipitation (Asano *et al*., 1987a; Asano *et al*., 1987b; Misono *et al*., 1989; Yamada *et al*., 1995; Asano *et al*., 1998), ion-exchange chromatography (Asano *et al*., 1987a; Asano *et al*., 1987b; Ohshima *et al*., 1991; Yamada *et al*., 1995; Asano *et al*., 1998) and hydrophobic interaction chromatography (Yamada *et al*., 1995; Asano *et al*., 1998). Leksakorn (2001) successfully purified PheDH from *B. badius* BC1 with a procedure involving: ammonium sulfate precipitation, DEAE-Toyopearl, Butyl-Toyopearl with stepwise elution and Butyl-Toyopearl with gradient elution. In this study, we then purified the recombinant PheDH according to Leksakorn (2001) as described above with minor adaptation.

4.1.1 Crude enzyme preparation

The mechanical disruption methods are usually necessary to break down cell wall in order to release intracellular protein prior to purification (Cooper, 1977; Harris and Angal, 1989). The intracellular PheDH produced in *E. coli* was prepared by disrupting the bacterial cell wall with ultrasonication. The cells were sonicated for 5

minutes (totally 25 minutes). Variation of the sonication time has been reported (Asano *et al*., 1987b). However, the long continuous sonication may cause foaming and shearing of protein. So it is necessary to use a short interval of disruption in ice cold buffer followed by a cooling period (Harris and Angal, 1989; Scopes, 1994). The buffer used is also an important factor in order to protect enzyme activity (Leksakorn, 2001). Therefore, to prepare crude extract, 100 mM phosphate buffer, pH 7.4 containing phenylmethylsulfonyl fluoride (PMSF), EDTA and 2-mercaptoethanol was then used. PMSF inhibits serine protease while EDTA serves as metalloprotease inhibitor. These reagents protect the desired protein from the degradation of proteolytic enzyme because the disruption of cells results in the release of proteases from subcellular compartments (Cooper, 1977; Bollag and Edelstein, 1991). 2-Mercaptoethanol which contains a thiol group and EDTA which also acts as a chelating agent were added in the buffer in order to protect the enzyme from oxidizing environment. This usually occurred after cells were disrupted (Leksakorn, 2001).

4.1.2 Protein separation by precipitation

After cell disintegration, the homogenate was then centrifuged to remove any insoluble materials. The desired protein remains dissolved in the solution because their charged surface residues interact with molecules of the solvent. When high concentrations of salt are present, proteins tend to aggregate and precipitate out of the solution (Bollag and Edelstein, 1991).

The most effective salt to bring about protein precipitation was ammonium sulfate. Because it combines many useful features such as salting out effectiveness, pH versatility, high solubility, low heat of solution and low price (Scopes, 1994). Our recombinant PheDH was precipitated at the presence of 30-50% saturated ammonium sulfate. From this step of purification, half of the proteins were removed while the activity remained over 85% (**Table 3.1**). When compared with the precipitation step of *B. badius* BC1, it was found that the active protein fraction was in the range of 40-50% saturated ammonium sulfate. Half of the proteins were also removed, but about one third of the enzyme activity was lost (Leksakorn, 2001). This may be due to the different surrounding proteins presented in the crude extract of both sources of

bacteria, thus affecting the salting out procedure (Scopes, 1994). The explanation for the different degree of PheDH activity could be the removal of some factors, during the salt precipitation, which are important to stabilize the enzyme activity and are different in both bacteria (Leksakorn, 2001). In addition, the speed at which salt was added to an enzyme solution and the efficiency of stirring were also important factors. For many proteins, small amount of salt was added and allowed to dissolve before making further additions. After the last bit of salt has dissolved, stirring should continue for 10-30 minutes to allow complete equilibrium between dissolved and aggregated proteins. Stirring must be regular and gentle because stirring too rapidly will cause protein denaturation as evidenced by foaming (Cooper, 1977; Leksakorn, 2001).

4.1.3 Column chromatography

Ion-exchange is probably the most frequently used chromatographic technique for the separation and purification of protein. The reasons for the success of ion exchange are its widespread applicability, its high resolving power, its high capacity, and the simplicity and controllability of the method (Amersham Pharmacia handbook, 1999). This technique is based on the interaction between charged solute molecules and oppositely charged moieties covalently linked to a chromatography matrix. From the result obtained as shown in section 3.1.3, 84% of proteins from the precipitation step were eliminated with 71% recovered activity **(Table 3.1)**. Leksakorn (2001) also used DEAE-Toyopearl as a first step of purification to purify PheDH from *B. badius* BC1 with less loss of enzyme activity while eliminated about 88% of other proteins. This can be concluded that the DEAE-column contributed greatly to the purification procedures. **Figure 3.1** also indicated the net charge of enzyme in phosphate buffer, pH 7.4. The net charge of our recombinant enzyme was negative because it can bind to the column. This can imply that the pI of this enzyme was less than 7.4 which corresponded to that of the wild type. The pI of PheDHs from various sources was presented in **Table 1.1**.

For the second step of purification, the hydrophobic interaction chromatography was employed. The neutral salts used must be highly soluble to avoid

salt precipitation when they were added in high concentration to the eluent to drive the hydrophobic interaction (Queiroz *et al.*, 2001). Salt concentration influences the selectivity in protein adsorption. Ammonium sulfate was chosen in this work because of its characteristics described above in the precipitation step. In this Butyl-Toyopearl column, our enzyme was eluted (stepwise) by 20% saturated ammonium sulfate solution (**Figure 3.2**). PheDH from *B. badius* BC1 was also eluted at the same concentration of salt whereas PheDH from *Microbacterium* sp. was eluted at the salt concentration lower than 15% (Asano *et al.*, 1998). This showed that PheDH from *B. badius* BC1 and its recombinant were mildly hydrophobic protein. As can be seen from **Table 3.1**, when Butyl-Toyopearl column was used, 71% of other proteins obtained from the DEAE-Toyopearl column were removed whereas 61% of activity was also lost. High protein removal and a loss of enzyme activity were also found at this purification step with PheDH from *B. badius* BC1 (Leksakorn, 2001) and *Microbacterium* sp. (Asano *et al.*, 1998). Leksakorn (2001) reported the use of a second Butyl-Toyopearl column with decreasing salt gradient elution to successfully get rid of other proteins. Recombinant PheDH was then further purified using a second Butyl-Toyopearl column. However, this column did not further remove unwanted protein and the loss of PheDH activity was still observed (data not shown). Thus, after expression of PheDH in *E. coli*, the enzyme was purified using salt precipitation, ion-exchange (DEAE-Toyopearl column) and hydrophobic interaction (Butyl-Toyopearl column) chromatography. The purity of the enzyme was checked by native-PAGE. Three bands were observed with 1 major band corresponding to PheDH. Although the enzyme was not 100% pure, this partial purified enzyme might compromise for its application in the industrial viewpoint. The more purification step is used, the higher cost it is and the greater loss of enzyme activity.

In conclusion, PheDH from recombinant *E. coli* was purified 10.1 fold with 23.9% yield from the cell free extract. The specific activity of this enzyme was 71.9 units/mg proteins. When compared to the PheDH from *B. badius* BC1 where a second Butyl-Toyopearl column (gradient elution) was also performed, it showed a single band of protein on native gel elctrophoresis. The purification fold was 160.7 with 20% yield. The specific activity was 48.2 units/mg proteins.

4.2 Characterization of phenylalanine dehydrogenase from recombinant *E. coli*

4.2.1 Molecular weight determination of phenylalanine dehydrogenase subunit

SDS polyacrylamide gel electrophoresis separates proteins based primarily on their molecular weights. SDS binds along polypeptide chain, and the length of the reduced SDS-protein complex is proportional to its molecular weight (Bollag and Edelstein, 1991). The relative molecular weight of PheDH subunit from recombinant clone was determined to be about 45,000 Da (**Figure 3.4** and **3.5**.) which was closely in agreement with PheDH from *B. badius* BC1 (44,500 Da per subunit) (Leksakorn, 2001). Other PheDHs, as can be seen in **Table 1.1**, exhibit a narrow range of subunit molecular weight between 39,500 Da and 42,000 Da. Hence, the subunit of PheDH from *B. badius* and its clone appears to be the biggest enzyme among these PheDHs.

4.2.2 Substrate specificity of phenylalanine dehydrogenase

The catalysis takes place in specific region of the enzyme referred as the active center or catalytic cavity. In general, the substrate 'fits' into the active site of the enzyme in a precise geometric alignment. Moreover, the amino acid residues that form the binding site are arranged to interact specifically with the substrate in an attractive manner. Molecules that differ in shape or functional group distribution from the substrate cannot productively bind to the enzyme which means they cannot form enzyme-substrate complexes that lead to the formation of products (Voet and Voet, 1995).

For oxidative deamination (**Table 3.2**), recombinant PheDH was highly active towards L-phenylalanine. Similar result was also found in other sources of PheDH (Asano *et al*., 1987a; Misono *et al*., 1989; Kataoka *et al*., 1994; Seah *et al*., 1995; Asano *et al*., 1998; Leksakorn, 2001). Among the phenylalanine analogs tested, our recombinant enzyme slightly oxidized *m*- and *o*-fluoro-DL-phenylalanine. However, the substitution of fluoro group in the aromatic side chain at *p*-position gave higher

reaction rate (44% of the maximal reaction rate obtained with phenylalanine). When compared to PheDH from *B. badius* BC1, similar results were obtained with *m*- fluoro substrates except for *p*-fluoro substrate. This enzyme gave only 11% of the maximal rate while *o*-fluoro-DL-phenylalanine was inert. DL-β-Phenyllactate and *N*-methyl-L-phenylalanine, which their amino groups are substituted by -OH and -NHCH$_3$, were inert for our recombinant enzyme. The substitution of -H atom of the chiral carbon by methyl group was also inert. For PheDH from *B. badius* BC1, these three substrates were found to be inert. These observations indicated that the amino group and H atom of the chiral carbon are essential for the substrate specificity of the enzyme.

The test was extended to other amino acids. Our recombinant PheDH showed no activity with acidic or basic L-amino acids or those with uncharged polar groups. The same results were detected from PheDH from *B. badius* BC1 (Leksakorn, 2001). This can be explained that the structure and properties of the side chain of these amino acids may not fit on the active site pocket of the enzyme. All of hydrophobic, aliphatic or aromatic amino acids tested were found to be inert. In addition, no activity was observed with D-amino acids as reported for other PheDHs (Asano *et al*., 1987a; Asano *et al*., 1987b; Misono *et al*., 1989; Leksakorn, 2001). Palmer (1995) described that enzyme could exhibit stereochemical specificity if a substrate can exist in two stereochemical forms, chemically identical but with a different arrangement of atoms in three-dimensional space, then only one of the isomers will undergo reaction. This supports the result about substrate specificity of PheDH for D-amino acids.

The reductive amination reaction was observed with various keto acid analogs as presented in **Table 3.2**. Recombinant PheDH showed high substrate specificity for phenylpyruvate. α-Ketocaproate, α-ketoisocaproate, α-ketovalrate and α-ketoisovalerate were substrates. This group of substrates was also active to PheDH from *B. badius* BC1 but the relative activity was lower than that from recombinant enzyme. In addition, α-keto-γ-methiol-butyrate and α-keto-β-methyl-n-valerate were substrate for our recombinant enzyme, yet they were inert for wild type enzyme. *p*-Hydroxyphenylpyruvate, which is the keto analog of tyrosine, was not active for our enzyme, nor PheDH from *B. badius* BC1.

From this experiment, substrate specificity of this enzyme was identified and they will further be used for the production of various amino acids.

4.3.3 Coenzyme specificity of phenylalanine dehydrogenase

Dehydrogenases are enzymes that catalyze the transfer of hydrogen from one substrate to another. This reversible system normally needs a coenzyme. All PheDHs are NAD$^+$-specific (Brunhuber and Blanchard, 1994).

The obtained results are shown in **Table 3.3**. The enzyme required NAD$^+$ as a natural coenzyme for oxidative deamination, but NADP$^+$ was not a substrate for our recombinant PheDH and other PheDHs (Hummel *et al*., 1984; Ziehr *et al*., 1987; Misono *et al*., 1989; Ohshima *et al*., 1991; Asano *et al*., 1998; Leksakorn, 2001). From the study of Brunhuber *et al*. (2000), they revealed that PheDH from *Rhodococcus* sp. formed strong hydrogen bonds between the 2′- and 3′-hydroxyl groups of the adenosyl ribose and the carboxylate of Asp 205. These hydrogen bonds would be disrupted by the presence of a 2′-phosphate.

When NAD$^+$ was modified at nicotinamide moiety, the analogs replaced the NAD$^+$ in oxidative deamination. Our recombinant enzyme exhibited twice as much better relative activity towards 3-acetylpyridine-NAD$^+$. This suggested that the replacement of the amino group of the nicotinamide moiety by methyl group results in the enhancement of the reactivity. The activity towards 3-pyridinealdehyde-NAD$^+$ and thio-NAD$^+$ showed 22.4% and 50.7% relative to NAD$^+$. PheDH from *B. badius* BC1 showed the same relative activity to thio-NAD$^+$, but 3-pyridinealdehyde- NAD$^+$ was inert for this enzyme (Leksakorn, 2001). Accordingly, it was concluded that the substitution at nicotinamide moiety may involve the change in polarity which may affect the binding mechanism in the area of the conserved hydrophobic residues of the enzyme (Leksakorn, 2001).

The NAD$^+$ analogs which were modified at an amino group in the adenine moiety then replaced the NAD$^+$. Nicotinamide -1, N^6-ethenoadenine dinucleotide, nicotinamide guanine dinucleotide, and nicotinamide hypoxanthine dinucleotide have closely similar reactivity compared to NAD$^+$. Equal amount of relative activity

towards nicotinamide hypoxanthine dinucleotide has also reported for other PheDHs (Misono *et al*., 1989; Leksakorn, 2001). Thus the amino group of the adenine moiety of NAD^+ was not of crucial importance for coenzyme activity (Misono *et al*., 1989).

4.3.4 Effect of pH on phenylalanine dehydrogenase activity and stability

pH influences the velocity of an enzyme-catalyzed reaction. Optimal pH of each enzyme is one of its characteristic to be concern. Because, slightly shift in the pH from the optimum value leads to a decrease in the reaction rate, also the large shifts may cause a severe damage in protein structures (Segal, 1976).

Our recombinant PheDH show maximum reactivity at pH 10.4 for the oxidative deamination of L-phenylalanine and at around 8.7 of for the reductive amination of phenylpyruvate (**Figure 3.6**). The optimum pH of *B. badius* BC1 showed the same characteristic (Leksakorn, 2001).

The stability curve can be obtained by preincubating the enzyme at the indicated pH for a time at least as long as the usual assay time (Segal, 1976). A pH stability study is shown in **Figure 3.8**. Our recombinant PheDH was stable over a pH range of 6.5-12.5 upon incubation at 30ºC for 20 minutes. PheDH from *B. badius* BC1 was most stable over a pH range of 6.0-11.0 (Leksakorn, 2001). The profile of broad pH stability was also found in other dehydrogenases (Ohshima *et al*., 1985; Ohshima and Soda, 1993; Koike *et al*., 1996).

4.3.5 Effect of temperature on phenylalanine dehydrogenase activity and stability

The optimum temperature depends on the assay time chosen. The true optimum temperature for an assay is the maximum temperature at which the enzyme exhibits a constant activity over a time period at least as long as the assay time (Segal, 1976). From **Figure 3.7**, the highest activity for our recombinant enzyme for deamination and amination reaction was observed around 45 and 40ºC, respectively. When compared with PheDH from *B. badius* BC1, this enzyme showed optimum

temperature at 50 and 45ºC for the oxidative deamination and reductive amination, respectively (Leksakorn, 2001).

The temperature stability depends on number of factors including the pH and ionic strength of the medium (Uruakpa and Arntfield, 2004) and the presence or absence of substrate (Mukherjee *et al.*, 2001). Our recombinant enzyme retained 100% relative activity at 35ºC (pH 7.4) when incubated for 10 minutes (**Figure 3.9**). The activity was decreased with increase in temperature and was abolished abruptly at 55ºC. Our recombinant enzyme showed lower thermostability than PheDH from *B. badius* BC1 which retained a 100% relative activity at 40ºC. The range of temperature stability of other PheDHs from mesophile has been reported in **Table 1.1**.

4.4 Initial velocity study of phenylalanine dehydrogenase

Initial velocity patterns are usually obtained by making reciprocal plots for one substrate (variable substrate) at different fixed concentrations of one of the others (changing fixed substrate) while keeping all other substrates at saturation and constant concentration. Thus, there are two possible initial velocity patterns: parallel lines when no reversible connection exists or lines intersecting to the left of the vertical axis when such a connection does exist (Cleland, 1971; Segal, 1976).

Kinetic studies were carried out to determine the Michaelis constant (K_m). The initial velocity of L-phenylalanine oxidation was studied with various concentrations of L-phenylalanine at fixed concentrations of NAD^+. As shown in **Figure 3.10a** in the double reciprocal plot, the apparent K_m value for L-phenylalanine was calculated to be 2.27 mM. The apparent K_m value for NAD^+ was calculated to be 0.156 mM from a secondary of the intercepts at the ordinate against reciprocal concentration of NAD^+ as shown in **Figure 3.10b**. When compared with PheDH from *B. badius* BC1, apparent K_m values were 0.59 mM for L-phenylalanine and 0.28 mM for NAD^+ (Leksakorn, 2001). There was highly different value of apparent K_m of L-phenylalanine between recombinant and wild type PheDH. Other PheDHs' K_m values are shown in **Table 1.1**.

The apparent K_m value of phenylpyruvate was calculated to be 0.385 mM in the double reciprocal plots as shown in **Figure 3.11a**. From the secondary plot of the intercepts at the ordinate against reciprocal concentration of NH$_4$Cl (**Figure 3.11b**), the apparent K_m of this substrate was calculated to be 83.3 mM. Double-reciprocal plots of velocity against NH$_4$Cl concentration at various fixed concentrations of NADH and a constant concentration of phenylpyruvate gave parallel straight line (**Figure 3.12**). The apparent K_m value for NADH was calculated to be 0.056 mM from **Figure 3.13a**. The K_m value of phenylpyruvate and NADH of our recombinant enzyme was closely similar to that of *B. badius* BC1 PheDH. However, the K_m of NH$_4$Cl of wild type showed a different value of 200 mM (Leksakorn, 2001).

Properties of PheDH from *B. badius* BC1 and enzyme from *E. coli* transformant was summarized in Appendix F.

4.5 Immobilization of phenylalanine dehydrogenase

Loss of catalytic activity is determined by changing protein molecular architecture. Some methods of stabilization are commonly employed to preserve enzyme activity including physical and/or chemical immobilization techniques, which open up possibilities of efficient industrial applications (Janecek, 1993).

4.5.1 Optimization of the coupled reaction for phenylalanine dehydrogenase activity assay

Due to the need of a rapid, sensitive, accurate and precise assay for L-phenylalanine in plasma samples, colorimetric method was developed to replace the enzymatic UV method. In 1989, Wendel *et al*. proposed a colorimetric assay for the determination of plasma phenylalanine which is based on the oxidative deamination reaction of PheDH. As shown in **Figure 4.1**, the method couples simultaneously the reaction of PheDH that catalyzes L-phenylalanine to phenylpyruvate in the presence of NAD$^+$ and a second reaction in which initially formed NADH and diaphorase convert iodonitrotetrazolium chloride (INT) to a formazan. This product is measured in the visible range at 492 nm. It was found that the sensitivity of the assay was

$$\text{L-phenylalanine} + \text{NAD}^+ + \text{H}_2\text{O} \xrightleftharpoons{\text{PheDH}} \text{phenylpyruvate} + \text{NADH} + \text{H}^+ + \text{NH}_3 \quad (1)$$

$$\text{NADH} + \text{INT} + \text{H}^+ \xrightarrow{\text{diaphorase}} \text{NAD}^+ + \text{formazan} \quad (2)$$
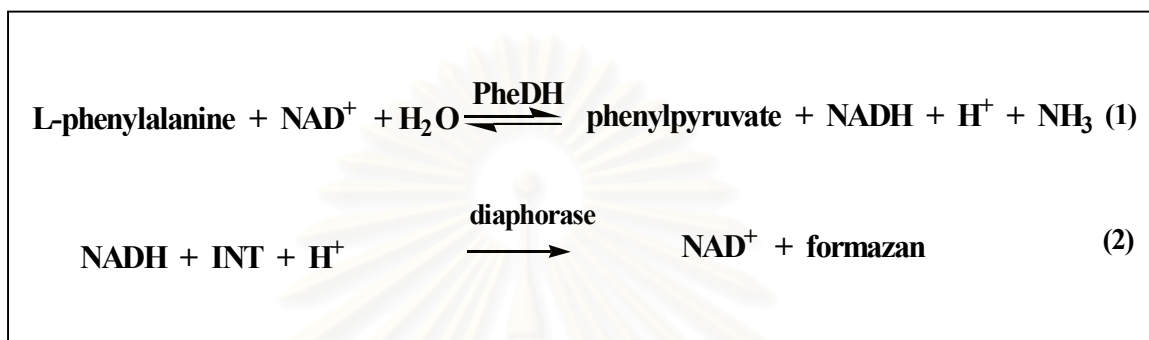
Figure 4.1 Coupling reactions of phenylalanine dehydrogenase and diaphorase
   for the determination of phenylalanine in plasma or serum (Wendel *et al.*, 1989) Phenylalanine dehydrogenase has been used in assays generating NADH from the NAD$^+$-dependent oxidative deamination of phenylalanine, and others employ the additional step of the reaction of a iodonitro tetrazolium salt (INT) to a formazan dye catalyzed by diaphorase.

increased by a factor of three because of high molar absorptivity (Wendel *et al*., 1989). Thus, this coupled reaction was appropriate in order to measure the activity of immobilized enzyme which normally has lower activity than its soluble counterpart. Initially, INT was used to determine the PheDH activity. However, the product produced adsorbed irreversibly to the solid supports. Water soluble tetrazolium chloride (WST-1) which produces a soluble formazan was then used instead (section 2.11). Sarker *et al*. (2001) proposed the use of WST-1 as a redox mediator for the determination of ethanol produced from the reaction of alcohol dehydrogenase. The use of WST-1 for the determination of phenylalanine by PheDH was then optimized. The effect of WST-1 concentration on NADH oxidation is shown in **Figure 3.14**. WST-1 concentration at 10 mM was selected. The excessive amount of WST-1 is necessary to obtain a quantitative linear range of NADH corresponding to WST-1 concentration. However, too high concentration of WST-1 was not used due to its high cost. For electron carrier, diaphorase catalyzed the oxidation of NADH in the presence of an electron acceptor. The appropriate amount of this enzyme was 0.1 U which still gave a linear relationship between the absorbance at 438 nm and incubation time in **Figure 3.15**. Incubation time at 10 minutes was chosen to avoid the lag period and the saturation of product.

In conclusion, the reaction mixture contained 20 µmol of L-phenylalanine, 1 µmol of NAD^+, 200 µmol of glycine-KCl-KOH buffer, pH 9.0, 1 µmol WST-1, 0.01 U of diaphorase from *Clostridium kluyveri* and the enzyme (soluble form) in a final volume of 1.0 ml were used. The reaction was started by the addition of phenylalanine and was incubated at room temperature for 10 minutes.

4.5.2 Pretreatment of inorganic carrier

The surfaces of inorganic supports, alumina and silica, have several types of Si-OH groups/ Al-OH groups. The surfaces of these supports should be cleaned from oil, dirt, detergent, etc., to generate reactive hydroxyl groups. If the dirty supports were to be employed for covalent coupling, the loading of the silane agents would be extremely low and perhaps loosely bonded (Messing, 1978; Shriver-Lake, 1998). Hence, the inorganic supports used in this study were soaked with 5% (v/v) HNO_3 for

45 minutes at 100ºC and thoroughly washed with water prior to the incubation with APTS (Weetall, 1993).

4.5.3 Selection of a suitable solid support and an appropriate method of activation

There is no general guidance on how enzymes can be most effectively immobilized on solid phases for use in practical applications. A somewhat random screening is frequently used and optimization is performed, in general, by trial and error (Chase and Yang, 1998). The immobilization of PheDH was carried out on both organic and inorganic support. Inorganic supports used were alumina and silica. Organic supports used were PVA and chitosan. Two methods of immobilization were selected, covalent binding and adsorption. **Figure 3.18** shows the comparison of the covalent immobilization and adsorption. For covalent binding, three activating methods were used. These methods involved epoxy, glutaraldehyde and tresylchloride method.

In this work, glutaraldehyde method, as illustrated in **Figure 3.18**, gave the best results. Glutaraldehyde is the most used coupling agent to couple protein to varieties of amine-containing supports (Kenedy and Cabral, 1987; Scouten *et al.*, 1995). The attachment of the enzyme molecule to glutaraldehyde occurs via Schiff's base linkage (**Figure 3.19**). The major problem in glutaraldehyde coupling is reproducibility (Scouten *et al.*, 1995). Even so, in many situations, glutaraldehyde coupling is by far the best method which has been selected to immobilize many enzymes. CGTase was immobilized on aminated polyvinylchloride (Abdel-Naby, 1999) and silica (Martín et al., 2002) by covalent binding with glutaraldehyde. Kerase, microbial serine protease which has been used in both food and non-food industry, was also immobilized on porous glass through glutaraldehyde coupling reaction (Parrado *et al.*, 1995). β-Glucosidase, wine-making and juice processing enzyme, was effectively immobilized on chitosan using glutaraldehyde as a coupling agent (Martino *et al.*, 1995).

For successful development and application of biocatalyst, the enzyme support is generally considered as the most important component (Yang *et al*., 2004). Non-toxic support and also acceptable cost of the support materials are the major conditions in order to make a selection of the profitable matrices for enzyme use (Sun *et al*., 1999; Yang *et al*., 2004). Both organic and inorganic matrices can be used to immobilize an enzyme through chemical binding. PheDH immobilized on silica and chitosan showed good relative immobilized activity. Silica gave 88.6% relative to that of chitosan. However, in our experiment, the suitable support selected was silica. Although, chitosan offered a unique set of characteristics, it was difficult to perform the immobilization steps due to its light feature. Hence, the immobilized enzyme was lost in each step of preparation. Silica was then chosen among various kinds of carrier with the most promising coupling agent, glutaraldehyde. Inorganic supports are widely used for immobilization of enzymes. This is mainly due to their good flow through properties, mechanical strength, regeneration and resistance to microbial attack (Guilbault and Sadar, 1980; Alcalde *et al*., 1999). Silica is also inexpensive and can be satisfactorily employed in continuous reactors (Abraham, 1995). This support has been used previously for the immobilization of other enzymes, for example acylase (Park *et al*., 2003), CGTase (Martín *et al*., 2002), dextransucrase (Alcalde *et al*., 1999), and alcohol dehydrogenase (Wu *et al*., 2004).

For the epoxy method in our study, the supports were activated with 1, 4-butanediol diglycidyl ether. Very small immobilized activity was observed in every support (**Figure 3.18**). This low coupling efficiency could be a result of the poor reactivity between epoxy groups on the support and the amino groups of the enzyme or the support surface was not efficiently activated (**Figure 3.17**). The PheDH was also immobilized with epoxy method by Hanson *et al*. (2000). The enzyme was immobilized on Eupergit C250L, a support containing oxirane groups to produce amino acid acetal. This immobilized enzyme can be reused but the result obtained was not as good as dry cell. Unsatisfied result was also observed with other enzymes when immobilized using this activation method. Chase and Yang (1998) immobilized trypsin and α-amylase on polyvinyl alcohol perfluorocarbon. The result showed small amounts of immobilized protein and low specific activities. Ragnitz *et al*. (2001)

immobilized cabamoylase and hydantoinase on Eupergit C and C250L, respectively. Both immobilized enzymes showed low yields of activity. Unlike the study of Bayramoğlu *et al*. (2004), α-amylase was immobilized onto the poly (hydroxyethylmethacrylate-glycidyl methacrylate) (poly (HEMA-GMA)) membrane by means of the amide linkage formation between the amino groups of α-amylase and the epoxy groups of the support. The immobilized amylase retained its activity over 76%. This synthetic support which contains controllable degree of reactive epoxy groups could enhance immobilized amylase activity.

The activation with tresyl chloride resulted in the great amount of enzyme activity when immobilized on chitosan but very low activity when immobilized on PVA and silica and no enzyme activity was detected on alumina support (**Figure 3.18**). Support activated with tresyl chloride could react with the thiol groups of cysteine residues or amino groups of lysine (**Figure 3.20**). Different result was reported in the study of Girotti *et al*. (1993) and Kiba *et al*. (1997). Girotti *et al*. immobilized PheDH from *Rhodococcus* sp. on nylon coil. The results showed the coupling of 60-70% of added PheDH considering both the immobilized activity and the amount of protein bound. Kiba *et al*. immobilized PheDH on PVA support. The yield obtained was 95%. The difference in activity yield could be attributed to different sources of enzyme and different carriers used. In addition, Kiba *et al*. did not identify whether the 95% yield obtained was either activity yield or the yield of protein bound.

Adsorption was not a good method to immobilize PheDH. From **Figure 3.18**, low immobilized activity was detected. This may be due to only weak bonds were formed during simple adsorption. This non covalent bond was easily broken by the high ionic strength of washing buffer, phosphate buffer saline.

In conclusion, silica was selected for immobilization of PheDH from recombinant *E. coli*. The method used was through glutaraldehyde coupling agent.

4.6 Optimization conditions for preparing immobilized phenylalanine dehydrogenase

The efficiency of PheDH immobilization on the silica was affected by various parameters such as the amount of reactive groups on support surface, the contact time and the concentration of enzyme per support ratio.

γ-Aminopropyltriethoxysilane (APTS) was used as silanization agent to introduce amino group onto the surface of silica support. From **Figure 3.21**, the suitable APTS concentration for silanization of silica is 6% (v/v). In other enzymes and supports, %APTS used was varied from 2% to 10% (v/v) (Martino *et al.*, 1995a; Martino *et al.*, 1995b; Parrado *et al.*, 1995; Ikediobi *et al.*, 1997; Pantatan, 2002). Caravajal *et al.* (1988) used NMR technique to study the APTS-modified silica gel surface. The structure complexity of APTS-modified silica gel was shown in **Figure 4.2**. This study provided useful information on the possible conformation of the amino groups on silica surface. Only the C conformation can form bond with glutaraldehyde. This may be one of the reasons to explain the low immobilization yield of PheDH onto silica support.

Glutaraldehyde concentration was optimized. The optimum concentration was at the level of 0.1 (v/v) as shown in **Figure 3.22**. The yield of immobilization decreased with the enhancement of glutaraldehyde concentration. This finding can be explained by assuming that the increase in glutaraldehyde concentration creates more bonds per enzyme molecule and, consequently, may cause molecular structure deformation (Martino *et al.*, 1995b). However, glutaraldehyde concentration has normally been reported in the range of 0.2-10% (v/v) depending on the enzyme and support employed (Martino *et al.*, 1995b; Parrado *et al.*, 1995; Ikediobi *et al.*, 1997; Bryjak and Kolarz, 1998; Abnel-Naby, 1999; Martín *et al.*, 2002; Pantatan, 2002).

Various PheDH concentrations attached to the carrier was studied by varying the amount of enzymes (in terms of unit by colorimetric method, section 2.11) from 4 U to 20 U per gram support. In **Figure 3.23**, the retained immobilized activity was highest at 16 U of PheDH per 1 g support with relative immobilization yield of 1.05%.
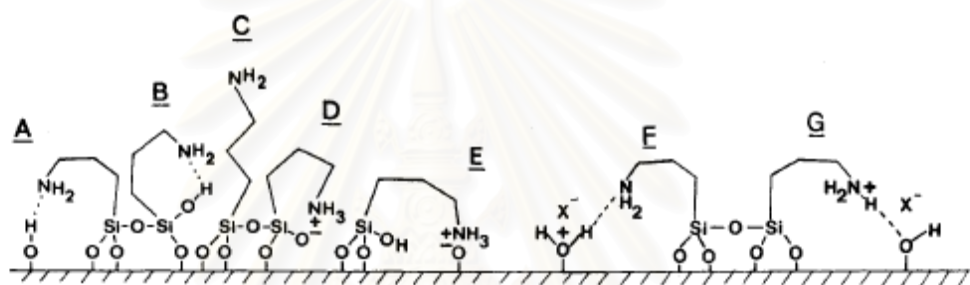
Figure 4.2 Structural possibilities anticipated for APTS-derivatized silica gel

(Caravajal *et al*., 1988) The potential structural complexity of APTS-modified

silica is to some degree represented by the variety of possible chemical structures

(A to G).

The immobilized enzyme activity did not seem to increase in its activity when the amount of enzyme increased. This could be due to the reactive groups on the support appearing to be saturated. To confirm that the low immobilized PheDH activity observed was not due to low enzyme attachment, the amount of protein bound to the support was determined. It was calculated by subtracting the protein recovered in the combined washing of the immobilized enzyme and in the filtrate from the amount of PheDH used for immobilization. It was found that most of the protein added was bound to the support since hardly any PheDH was detected in the filtrate or in the washings (data not shown). Moreover, high enzyme loading onto the non-porous support generally leads to low immobilization yield. This was due to the steric hindrance preventing access of the substrate to the active sites of the enzyme and/or diffusional effect of the substrate to the immobilized enzyme molecules (Abdel-Naby, 1999). Other enzymes were shown to be covalently linked to solid support by using identical procedure of immobilization, but the ratio of enzyme to support required and the amount of enzyme immobilized varied depending upon the enzyme and support used (Weetall, 1976). Martín *et al*. (2002) studied the immobilization of CGTase onto silica support using glutaraldehyde as a coupling agent. Two different weight ratios of enzyme/support were used: 29 mg or 14.5 mg protein per gram of support. Activity obtained was 58 and 21 U/g support, respectively. Optimum enzyme/support ratio of immobilized CGTase by Pantatan (2002) on alumina support was 14 U/g support.

The coupling time, additional parameter affected immobilization efficiency, was also investigated. On silica support, the retained immobilized activity was notably depending on the incubation time. As shown in **Figure 3.24**, the most appropriate incubation time was 6 hours which gave the highest immobilized activity of 0.32 U/g support. The shorter incubation time may result in less contact between enzyme and activated support. In some cases, at the longer period of incubation time, the immobilized enzyme may result in much higher operational stability. This may due to increased multipoint attachment (Martín *et al*., 2003).

The loss of enzyme activity after immobilization can be caused by the random covalent coupling of certain lysine residues which lie on the surface of PheDH. With random interaction between glutaraldehyde and lysine moiety, inappropriate

orientation of enzyme may be obtained and subsequently affect the immobilized enzyme activity.

4.7 Properties of the immobilized phenylalanine dehydrogenase on silica

A common finding of many studies on immobilization is that some properties of the immobilized enzyme, such as its catalytic activity or thermal stability, differ from those of its soluble counterpart (Martín *et al.*, 2003). The changes of enzymatic properties are considered to be caused by the following two factors. One is changes of the enzyme itself, and the other is physical and chemical properties of the carriers used for immobilization (Chibata, 1978). The procedure of enzyme immobilization on insoluble carriers has a variety of effects on the protein conformation as well as on the state of ionization and dissociation of the enzyme and its environment (Chibata, 1978; Gemeiner, 1992).

4.7.1 Effect of pH on activity and stability of free and immobilized phenylalanine dehydrogenase

The pH optimum of the enzyme after immobilization can be changed or remain the same as the free form (Chibata, 1978). In our case, the optimum pH of PheDH after immobilization was not altered from soluble enzyme. As can be seen in **Figure 3.25**, the optimum pH for both enzymes was at 9.0. This can be explained by the fact that the pH value at the liquid-matrix interface played an essential role in the immobilized enzyme preparation. This effect resulted mainly from the buffering properties of the bound proteins, the matrix, and the Schiff bases formed by the reaction between glutaraldehyde, proteins and carrier (Bryjak and Kolarz, 1998). According to the study of Bryjak and Kolarz (1998), the pH optimum of immobilized trypsin on acrylic copolymers remained the same as that of the free enzyme. The study of Abdel-Naby (1999) showed no shift of optimum pH in both free and immobilized CGTase.

The pH stability of the immobilized PheDH was investigated and compared with that of free form. It was noticed from **Figure 3.27** that at various pH's presented, the pH profile of both form of enzymes were almost the same. Immobilization was slightly enhanced the stability of PheDH in the pH range of 4.0-6.0 and 12.0-13.0.

4.7.2 Effect of temperature on activity and stability of free and immobilized phenylalanine dehydrogenase

The activity of soluble and immobilized PheDH was measured at various temperatures. The result is shown in **Figure 3.26**. The optimal temperature of free PheDH was at 35ºC while immobilized enzyme showed a broad range of optimum temperatures from 30ºC to 55ºC. At temperature higher than 35ºC, the activity of the soluble PheDH was drastically decreased. At 60ºC, the activity of immobilized PheDH retained over 60% while soluble enzyme showed its relative activity less than 10%. This could probably be explained that the activity is lost at temperature above a certain limit due to the predominance of thermal denaturation of the enzyme (Chibata, 1978; Parrado *et al.*, 1995). Therefore, the enzyme immobilization seems to be the attractive method for enzyme usage and stabilization.

The effect of temperature on stability is one of the most important criteria with respect to applications. As shown in **Figure 3.24**, the activity of both enzymes decreased when increasing temperature. At temperature over 55ºC, immobilized enzyme has higher retained activity than the soluble enzyme. Incubation at 70ºC resulted in the completely lost of enzyme activity in both forms. Enhancement of thermal stability has been observed in many enzymes (Parrado *et al.*, 1995; Martín *et al.*, 2003; Yang *et al.*, 2004). However, some examples showing decreases of heat stability have also been reported, for example the immobilization of invertase onto DEAE-cellulose (Chibata, 1978). In fact, no correlation between thermal stability and immobilization method has yet been established. This characteristic depends on source of enzyme and immobilization process applied (Chibata, 1978).

4.7.3 Storage stability

The storage stability is an important factor to be considered for the applications of immobilized enzyme. The stability of immobilized PheDH at 4ºC and at room temperature was studied and compared to that of the free enzyme. The results shown in **Figure 3.29** indicated that the free enzyme completely lost its activity after 1 month of storage at room temperature while the activity still remained 69% at 4ºC. The immobilized enzyme retained its full activity after 1 month of storage at both temperatures. When the storage time for the immobilized enzyme was extended for 1 month, no loss of enzyme activities was found (data not shown). This indicated that the immobilization of PheDH on silica increased the stability of the enzyme. This stabilization probably resulted from stiffening of the protein tertiary structure (Bryjak and Kolarz, 1997).

4.8 Synthesis of amino acids from their keto acids using immobilized enzyme

With a stricter demand on the quality of human life, many food products with emphasis on good taste as well as the benefit to health emerge enormously in the last two decades. The immobilized PheDH was then exploited for the production of various amino acids. The substrates were selected among keto acids presented in **Table 3.2**. As can be seen in **Figure 3.30**, semiquantitative study of amino acid production was carried out by TLC technique. Amino acids were tested by running the standard amino acids in parallel with the samples.

In a batch production, the condition used for the production of amino acids was 5 μmol of substrate, 200 μmol of NH_4Cl-NH_4OH buffer, pH 9.5 and 5 μmol of NADH. The immobilized enzyme showed 84.4% conversion of phenylpyruvate to L-phenylalanine. α-Ketovalerate, α-ketoisovalerate, α-ketocaproate, α-ketoisocaproate and α-keto-γ-methiobutyrate were converted to their amino acids with 79.1%, 63.7%, 100%, 67.4% and 75.3% conversion, respectively. In 1986, Hummel *et al*. used an enzyme membrane reactor to produce L-phenylalanine. The enzyme used was from

Brevibacterium sp. The coenzyme was regenerated by a second enzyme, formate dehydrogenase (FDH). The experiment was carried out over a period of 13 days in Tris-HCl buffer containing 20 mM phenylpyruvate and 2 M ammonium formate. The average conversion was about 93%. Later, in the same year, high yield synthesis of L-phenylalanine was reported by Asano and Nakazawa using PheDH from *Sporosarcina ureae* in combination with FDH. The reaction mixture contained 100 μmol of α-keto acid, 5 μmol NAD^+, 800 μmol ammonium formate, 250 μmol Tris-HCl, pH 8.5, 2.5 U of PheDH and 0.5 U of FDH. The incubation time was 24 hours. The product conversion was 98% yield. In 1987, Hummel *et al.* used *Rhodococcus* sp. M4, whose enzyme reported to have high stability, in enzyme-membrane-reactor for phenylalanine production. The mean conversion yield obtained was 95%. The different degree of L-phenylalanine production could be attributed to different sources of enzymes and different method used. Moreover, the condition for phenylalanine production using immobilized PheDH was not at the optimum condition.

Most industrial enzymic processes are carried out batch-wise with soluble enzymes, but these processes are very limited, mainly for economic reasons such as low efficiency and high cost. One approach to solve these problems is the use of immobilized enzymes (Parrado *et al.*, 1995). PheDH has a great value in various industries for production of optical pure L-phenylalanine. In this study, PheDH was covalently immobilized on silica. The storage stability of this immobilized enzyme was of great advantage to use in industry. This immobilized enzyme was successfully used to produce amino acid from their keto acids. However, the production yield was lower than the use of free enzyme in previous reports. For further study, continuous production with optimized condition of amino acid conversion should be conducted. Furthermore, the reusability, which is one of the most important criteria for evaluating the possibility of a practical application of immobilized enzyme, should be studied.

CHAPTER V

CONCLUSIONS

1. PheDH from recombinant *E. coli* was partially purified by 30-50% saturated ammonium sulfate precipitation, DEAE-Toyopearl and Butyl-Toyopearl column chromatography with 23.9% yield and 10.1 purification fold.

2. The relative molecular weight of PheDH subunit was estimated to be about 45,000 Da by SDS polyacrylamide gel electrophoresis.

3. The enzyme showed high substrate specificity for phenylalanine and phenylpyruvate in the oxidative deamination and reductive amination, respectively.

4. NAD^+ can be used as a natural coenzyme for oxidative deamination. $NADP^+$ was inert while 3-acetylpyridine-NAD^+ showed higher activity than NAD^+ for 2 times.

5. The optimum pH for the oxidative deamination and reductive amination were 10.4 and 8.7, respectively.

6. The optimum temperature for deamination and amination were 45ºC and 40ºC, respectively.

7. The stability was over a pH range of 6.5 to 12.5.

8. The apparent K_m values for L-phenylalanine, NAD^+, NADH, phenylpyruvate and ammonium were 2.27, 0.156, 0.056, 0.385, and 83.3 mM, respectively.

9. Covalent immobilization of PheDH from recombinant *E. coli* was investigated on various supports including alumina, silica, PVA and chitosan. Among these

supports, PheDH bound to chitosan was found to exhibit highest retained activity. However, the immobilized enzyme was easily lost during preparation steps. Silica which showed slightly less immobilized activity was then selected.

10. The best condition for immobilized enzyme preparation to achieve high immobilized activity was to activate the silica with 6% (v/v) APTS, 0.1% (v/v) glutaraldehyde. PheDH was then added and incubated at 4ºC for 6 hours.

11. Under optimum immobilization condition, 0.14 U of PheDH was immobilized on 1 g of silica with 1.05% of immobilization yields when 16 U of PheDH was applied.

12. Both free and immobilized enzyme showed the same optimum pH of 9. The optimum temperature of free PheDH was at 35ºC whereas the optimum temperature of the immobilized enzyme showed a broad range from 30ºC to 55ºC.

13. Free and immobilized PheDH were stable in the pH range of 6.0-11.5, and the immobilized enzyme exhibited slightly improved thermal stability.

14. The immobilized PheDH exhibited higher stability than the free enzyme when stored at both 4ºC and room temperature. No loss of activity was observed after 1 month. For soluble PheDH, it retained only 69% at 4ºC while completely lost its activity at room temperature.

15. The immobilized enzyme produced L-phenylalanine, norvaline, valine, norleucine, leucine and methionine with 84.4%, 79.1%, 63.7%, 100%, 67.4% and 75.3% conversion, respectively.

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APPENDICES

APPENDIX A

Preparation for protein determination

Reagent for determination of protein concentration (modified from Lowry *et al.*, 1951)

Solution A (0.5% copper sulfate and 1% potassium tartate, pH 7.0)

Potassium tartate	1 g
Copper sulfate	0.5 g

Adjusted pH to 7.0 and adjust the solution volume to 100 ml.

Solution B (2% sodium carbonate and 1 N sodium hydroxide)

Sodium carbonate	20 g
Sodium hydroxide	4 g

Dissolved in distilled water to 1 litre.

Solution C (phenol reagent)

Folin-Ciocalteu phenol reagent used in this work was reagent grade from Carlo Erba, Italy.

APPENDIX B

Calibration curve for protein determination by Lowry's method

$y = 0.0028x + 0.0126$

$R^2 = 0.9909$
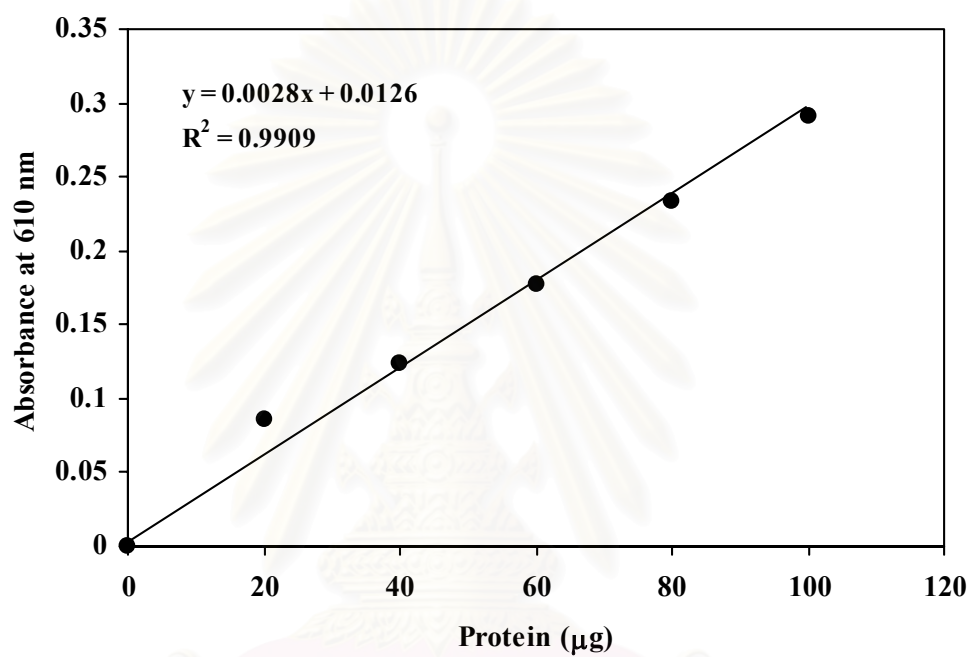
APPENDIX C

Preparation for non-denaturing polyacrylamide gel electrophoresis (Native-PAGE)

1. Stock solutions

2 M Tris-HCl (pH 8.8)

Tris (hydroxymethyl)-aminomethane	24.2 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

1 M Tris-HCl (pH 6.8)

Tris (hydroxymethyl)-aminomethane	12.1 g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

1 % (w/v) Bromophenol blue

Bromophenol blue	100 mg

Brought to 10 ml with distilled water and stirred until dissolved.

The filtration will remove aggregated dye.

2. Working solutions

Solution A (30 % (w/v) acrylamide, 0.8 % (w/v) bis-acrylamide)

Acrylamide	29.2 g
N, N′-methylene-bis-acrylamide	0.8 g

Adjusted volume to 100 ml with distilled water.

Solution B (1.5 M Tris-HCl, pH 8.8)

2 M Tris-HCl (pH 8.8)	75 ml
Distilled water	25 ml

Solution C (0.5 M Tris-HCl, pH 6.8)

1 M Tris-HCl (pH 6.8)	50 ml
Distilled water	50 ml

APPENDIX C (continued)

10 % (w/v) Ammonium persulfate

Ammonium persulfate	0.5 g
Distilled water	5.0 ml

Electrophoresis buffer (25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminomethane	3.0 g
Glycine	14.4 ml

Dissolved in distilled water to 1 litre without pH adjustment (final pH should be approximately 8.3)

5x Sample buffer (312.5 mM Tris-HCl pH 6.8, 50 % (v/v) glycerol, 1 % (v/v) bromophenol blue)

1 M Tris-HCl (pH 6.8)	0.6 ml
Glycerol	5.0 ml
1 % Bromophenol blue	0.5 ml
Distilled water	1.4 ml

3. Native-PAGE

7.7 % Separating gel

Solution A	2.6 ml
Solution B	2.5 ml
Distilled water	4.9 ml
10 % (w/v) Ammonium persulfate	50 µl
TEMED	5.0 µl

5.0 % Stacking gel

Solution A	0.67 ml
Solution B	1.0 ml
Distilled water	2.3 ml
10 % (w/v) Ammonium persulfate	30 µl
TEMED	5.0 µl

APPENDIX D

Preparation for denaturing polyacrylamide gel electrophoresis

1. Stock solution

2 M Tris-HCl (pH 8.8)

Tris (hydroxymethyl)-aminomethane   24.2   g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

1 M Tris-HCl (pH 6.8)

Tris (hydroxymethyl)-aminomethane   12.1   g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

10 % (w/v) SDS

Sodium dodecyl sulfate (SDS)   10   g

Added distilled water to a total volume of 100 ml.

50 % (w/v) Glycerol

100 % Glycerol   50   ml

Added distilled water to a total volume of 100 ml.

1 % (w/v) Bromophenol blue

Bromophenol blue   100   mg

Brought to 10 ml with distilled water and stirred until dissolved.

The filtration will remove aggregated dye.

2. Working solutions

Solution A (30 % (w/v) acrylamide, 0.8 % (w/v) bis-acrylamide)

Acrylamide   29.2   g

N, N'-methylene-bis-acrylamide   0.8   g

Adjusted volume to 100 ml with distilled water.

APPENDIX D (continued)

Solution B (1.5 M Tris-HCl, pH 8.8 and 0.4 % SDS)

2 M Tris-HCl (pH 8.8)	75 ml
10 % (w/v) SDS	4 ml
Distilled water	21 ml

Solution C (0.5 M Tris-HCl, pH 6.8, 0.4 % SDS)

1 M Tris-HCl (pH 6.8)	50 ml
10% (w/v) SDS	4 ml
Distilled water	46 ml

10 % (w/v) Ammonium persulfate

Ammonium persulfate	0.5 g
Distilled water	5.0 ml

Electrophoresis buffer (25 mM Tris, 192 mM glycine and 0.1 % (w/v) SDS)

Tris (hydroxymethyl)-aminomethane	3.0 g
Glycine	14.4 ml
SDS	1 g

Dissolved in distilled water to 1 litre without pH adjustment (final pH should be approximately 8.3)

5x Sample buffer (312.5 mM Tris-HCl pH 6.8, 50 % (v/v)glycerol, 1 % (w/v) bromophenol blue)

1 M Tris-HCl (pH 6.8)	0.6 ml
50 % (v/v) Glycerol	5.0 ml
10 % (w/v) SDS	2 ml
1 % (w/v) Bromophenol blue	1 ml
2-Mercaptoethanol	0.5 ml
Distilled water	1.4 ml

APPENDIX D (continued)

3. SDS-PAGE

12.5 % Separating gel

Solution A	4.2 ml
Solution B	2.5 ml
Distilled water	3.3 ml
10 % (w/v) Ammonium persulfate	50 µl
TEMED	5 µl

5.0 % Stacking gel

Solution A	0.67 ml
Solution B	1.0 ml
Distilled water	2.3 ml
10% (w/v) Ammonium persulfate	30 µl
TEMED	5 µl

APPENDIX E

Calibration curve for the determination of amino acids by measuring the intensity of standard amino acids on TLC plate

(1)

$y = 5.4576x + 76.751$

(2)

$y = 4.9837x + 128.22$

APPENDIX E (continued)

(3)

$y = 4.011x + 145.97$

(4)

$y = 3.139x + 80.28$

APPENDIX E (continued)

(5)

$$y = 5.6171x + 135.84$$

(6)

$$y = 4.5915x + 132.58$$
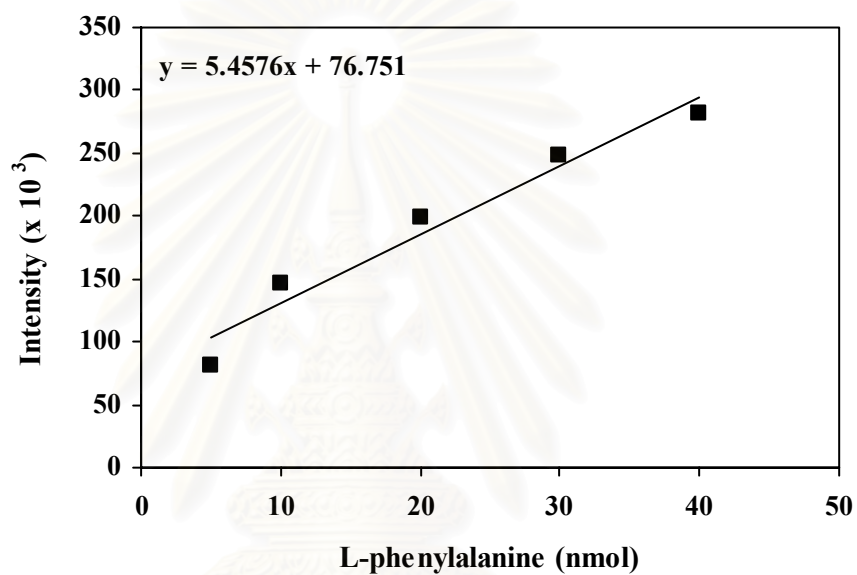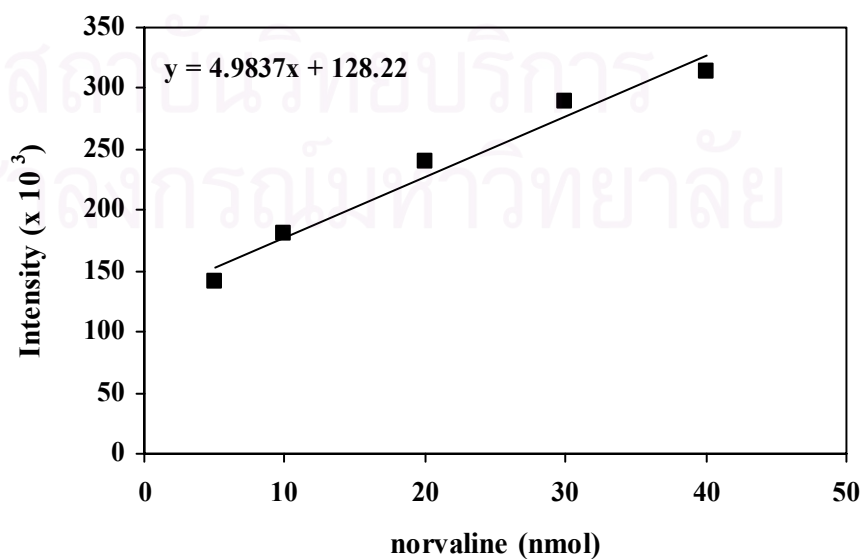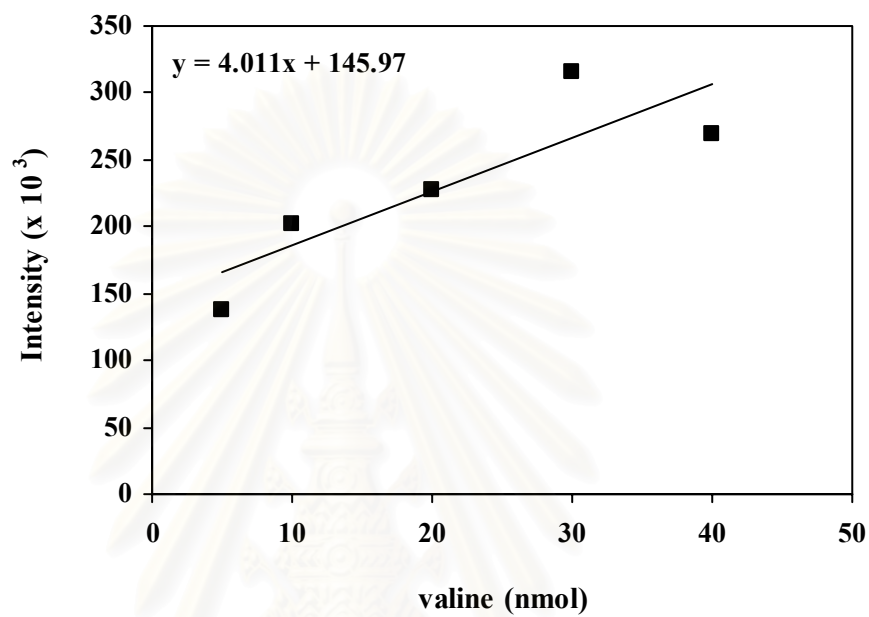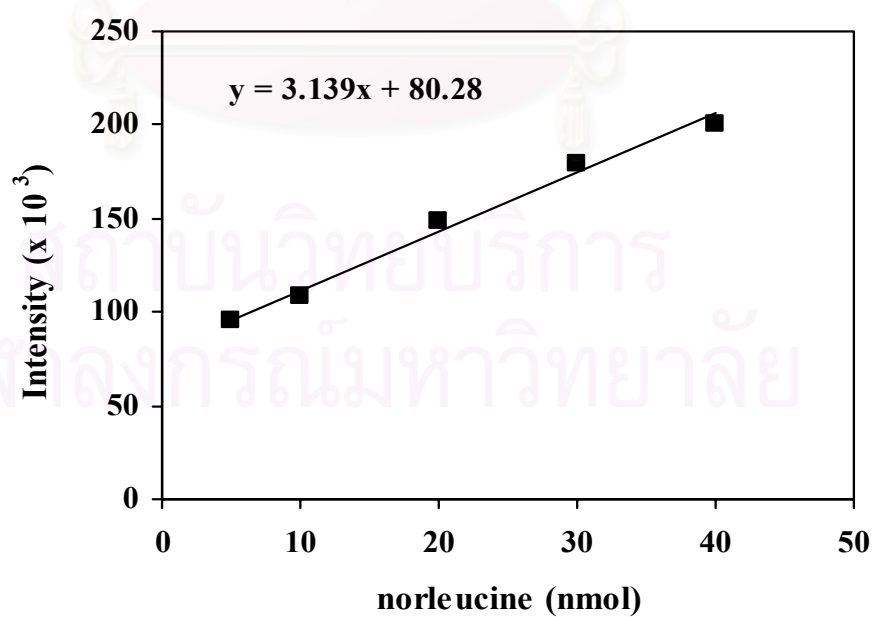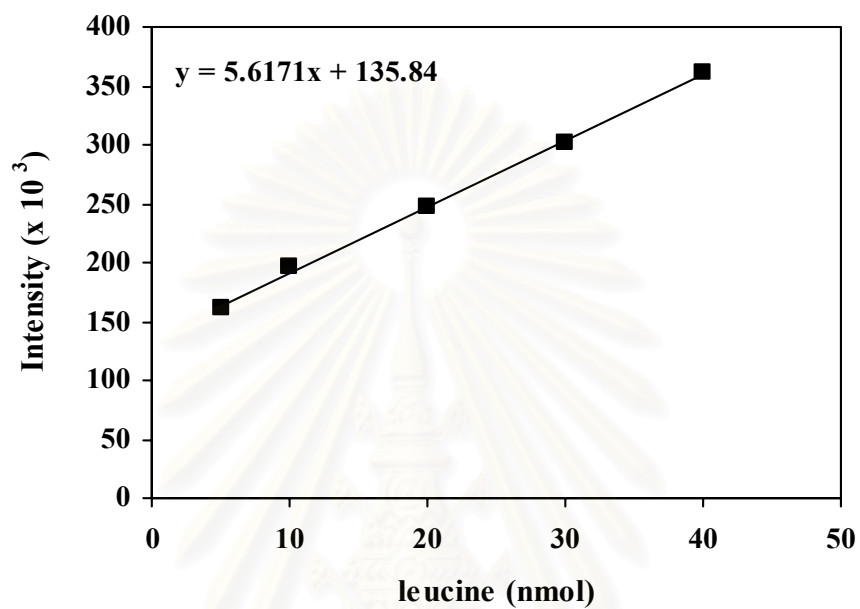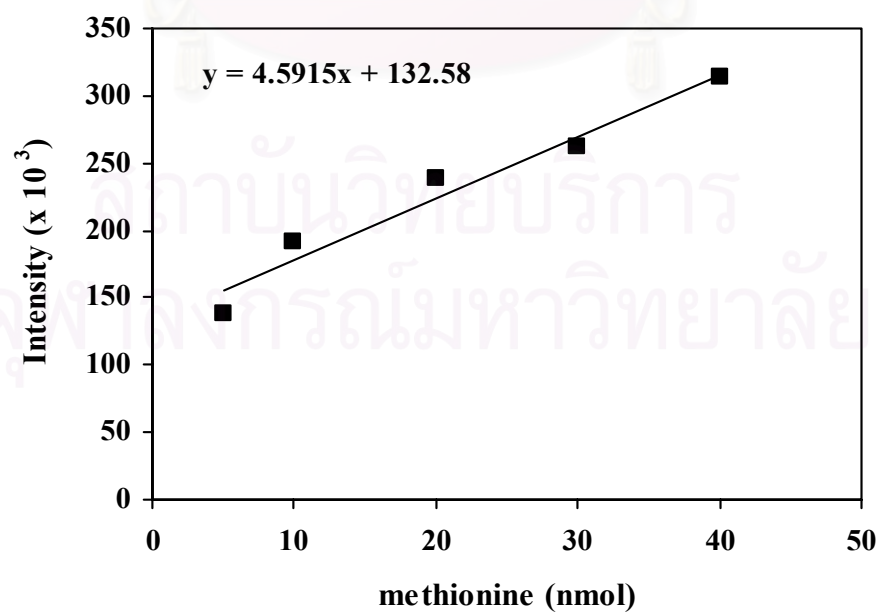
APPENDIX F

Comparison of properties of phenylalanine dehydrogenases from *Bacillus badius* BC1 and recombinant enzyme from *Escherichia coli*

Properties	*B. badius* BC1[a]	*E. coli* transformant
Specific activity of final preparation (U/mg protein)	48.2	71.9
Native molecular weight	358,000	ND
Subunit molecular weight	44,500	45,000
Structure	octamer	ND
pH optimum		
Oxidative deamination	10.7	10.4
Reductive amination	8.3	8.7
Substrate specificity (% relative activity)		
Oxidative deamination		
L-phenylalanine	100	100
L-tyrosine	0	0
L-tryptophan	3	5
L-methionine	4	7
L-valine	2	3
p-fluoro-DL-phenylalanine	11	44
m-fluoro-DL-phenylalanine	5	9
o-fluoro-DL-phenylalanine	0	5
α-amino-β-phynylbutanoate	8	7
D-amino acids	0	0

APPENDIX F (continued)

Properties	B. badius BC1[a]	E. coli transformant
Reductive amination		
Phenylpyruvate	100	100
α-ketovalerate	3	19
α-ketocaproate	12	58
α-ketoisovalerate	5	11
α-ketoisocaproate	4	24
α-keto-γ-methylthiobutyrate	0	22
Coenzyme specificity (% relative activity)[b]		
NAD^+	100	100
NADP	0	0
3-Acetylpyridine-NAD^+	166	200.5
Thio-NAD^+	43	50.7
NGD^+	70	98.7
NHD^+	96	84.4
Nicotinamide-1,N^6-ethenoadenine dinucleotide	51	105.7
pH stability (pH range)	6.0-11.0	6.5-12.5
Optimum temperature (^{o}C)		
Oxidative deamination	50	45
Reductive amination	45	50

APPENDIX F (continued)

Properties	*B. badius* BC1[a]	*E. coli* transformant
Apparent K_m (mM)		
L-phenylalanine	0.59	2.27
NAD^+	0.28	0.156
NADH	0.067	0.056
Phenylpyruvate	0.33	0.385
Ammonia	200	83.3

[a] *Bacillus badius* BC1

[b] Abbreviation: 3-Acetylpyridine-NAD^+; 3-acetylpyridine adenine dinucleotide, Thio-NAD^+; thionicotinamide adenine dinucleotide, NGD^+; nicotinamide guanine dinucleotide, NHD^+; nicotinamide hypoxanthine dinucleotide
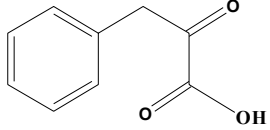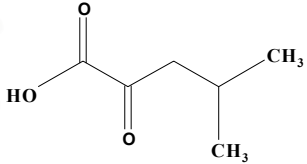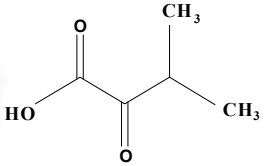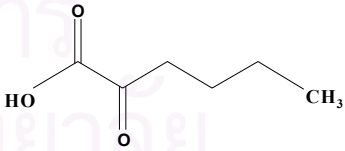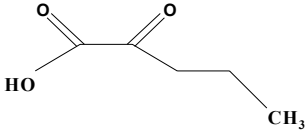
Leksakorn, 2001. Purification and characterization of phenylalanine dehydrogenase from thermotolerant *Bacillus badius* BC1. Master's Thesis, Department of Biochemistry, Faculty of Science, Chulalongkorn University.

ND = Not determined
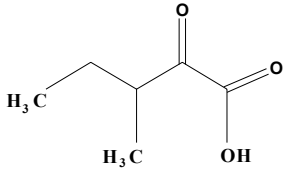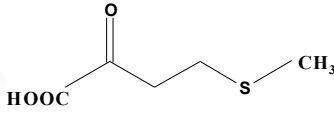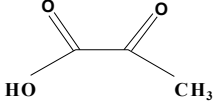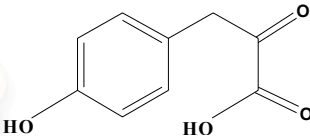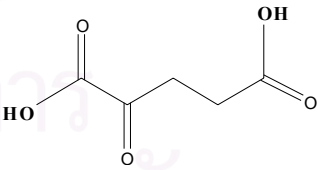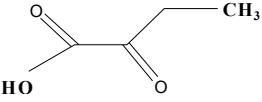
APPENDIX G
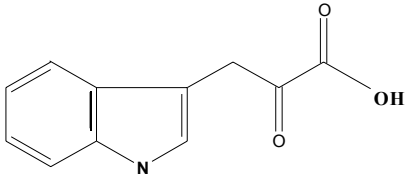
α-Keto acids and their analogs: structure and synonyms

α-Keto acid	Synonyms	Structure
Phenylpyruvic acid	phenylpyruvate, 3-phenylpyruvate	
α-Ketoisocaproic acid (leucine keto analog)	4-methyl-2-oxopentanoic acid, 4-methyl-2-oxopentanoate, 4-methyl-2-oxovalerate, 2-keto-4-methylvalerate, 2-ketoisocaproate, 2-oxo-4-methylvaleric acid, 2-oxoisocaproic acid, α-ketoisocaproate, α-oxoisocaproate, ketoleucine	
α-Ketoisovaleric acid (valine keto analog)	3-methyl-2-oxobutanoate, 3-methyl-2-oxobutyrate, 2-ketoisovalerate, 2-oxoisovalerate, α-keto-isovaleric acid, α-ketoisopentanoic acid, α-ketoisovalerate, α-ketovaline, α-oxoisovalerate, ketovaline	
α-Ketocaproic acid (norleucine keto analog)	2-oxohexanoic acid, 2-ketocaproate, 2-oxohexanoate	
α–Ketovaleric acid (norvaline keto analog)	2-oxopentanoic acid, 2-ketopentanoic acid, 2-ketovalerate	

APPENDIX G (continued)

α-Keto acid	Synonyms	Structure
α-Keto-β-methylvaleric acid	2-ketomethylvalerate, 2-keto-3-methylvaleric acid, 2-oxo-3-methylvalerate, 3-methyl-2-oxopentanoate, α-keto-β-methyl-n-valeric acid, α-keto-β-methylvalerate, α-keto-β-methylvaleric acid	
α-Keto-γ-methiolbutyric acid	4-methylthio-2-oxobutanoic acid	
Pyruvic acid[a]	pyruvate	
p-Hydroxyphenylpyruvic acid[b] (tyrosine keto analog)	4-hydroxy-α-oxobenzenepropanoic acid, 4-hydroxyphenylpyruvate, 4-hydroxyphenylpyruvic acid, (*p*-hydroxyphenyl) pyruvic acid, *p*-hydroxyphenylpyruvate	
α-Ketoglutaric acid[c]	2-ketoglutarate, 2-ketoglutaric acid, 2-oxoglutarate, 2-oxoglutaric acid, α-oxoglutarate	
α-Ketobutyrate[d]	α-ketobutyric acid, 2-oxobutanoic acid, 2-ketobutyrate, propionylformic acid, α-oxobutyric acid	

APPENDIX G (continued)

α-Keto acid	Synonyms	Structure
Indole-β-pyruvic acid[e]	α-oxo-indole-3-propanoic acid, indole-3-pyruvic acid, indole-3-(2-oxo)-propanoic acid, 5-iodophthalide	

[a-e] This keto acids are inert.
Sources: CTD: The Comparative Toxicogenomics Database (cited from http://ctd.mdibl.org)
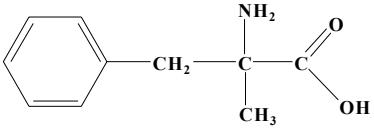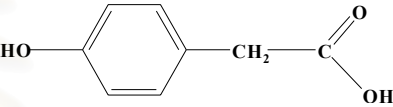
APPENDIX H

Phenylalanine and their analogs: Structures

Phenylalanine and analogs	Synonyms	Structure
L-Phenylalanine	α-amino-β-phenylpropionate	
α-Amino-β-phenylbutanoate		
p-Fluoro-DL-phenylalanine		
m-Fluoro-DL-phenylalanine		
o-Fluoro-DL-phenylalanine		
DL-β-Phenyllactate	DL-2-hydroxy-3-phenylpropionate, DL-α-hydroxyhydrocinnamate	
N-Methyl-L-phenylalanine		

APPENDIX H (continued)

Phenylalanine and analogs	Synonyms	Structure
α-Methyl-DL-phenylalanine	α-amino-α-methyl-β-phenylpropionate	
p-Hydroxyphenylacetate		

APPENDIX I

NAD$^+$ analogs

Nicotinamide moiety →

Adenine moiety
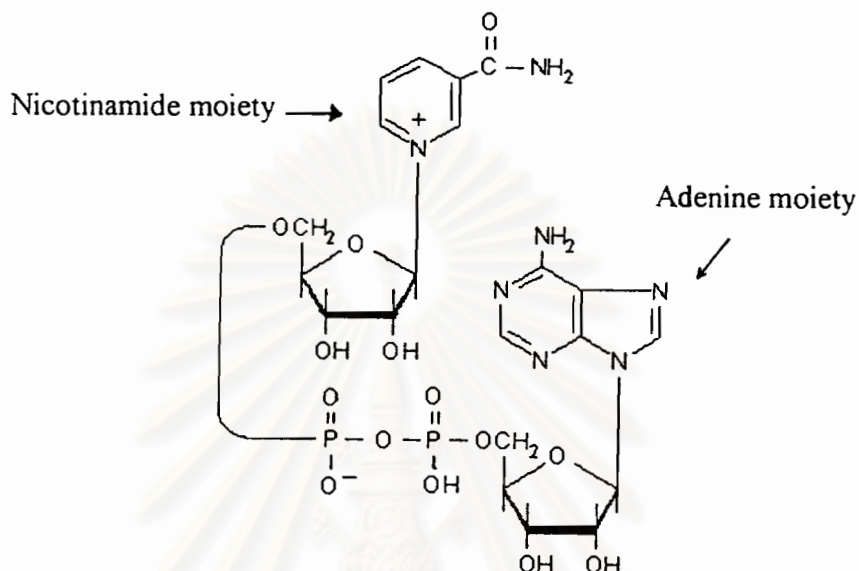
Nicotinamide adenine dinucleotide (NAD$^+$)

The NAD+ analogs used in this work can be divided into 3 groups based on their modified structure.
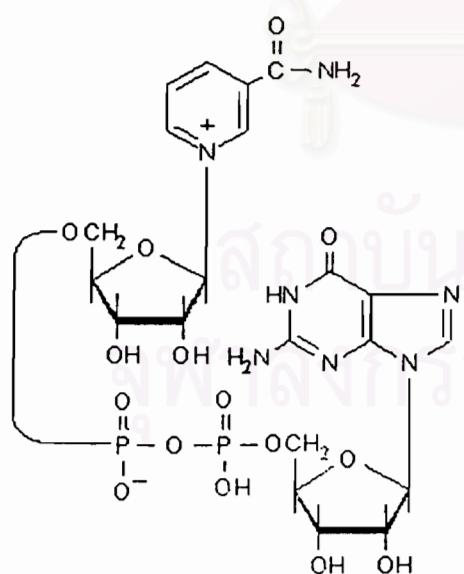
1. **Coenzyme analog modified at C-2 position of the adenosyl ribose**
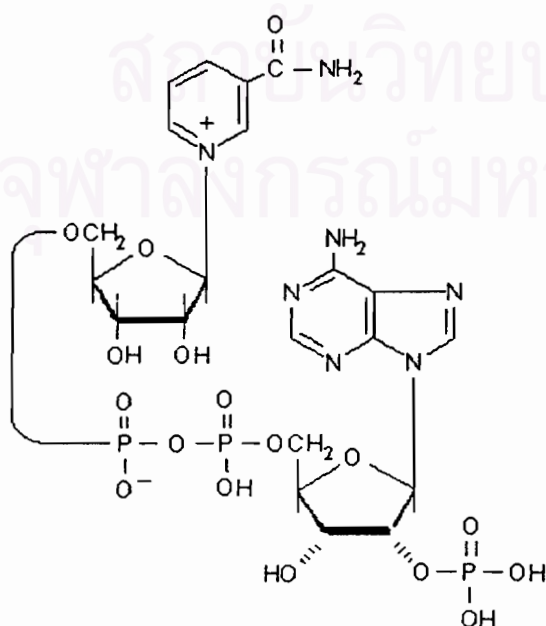
Nicotinamide adenine dinucleotide phosphate (NADP$^+$)

APPENDIX I (continued)

2. Coenzyme analog modified at the amino group in the adenine moiety
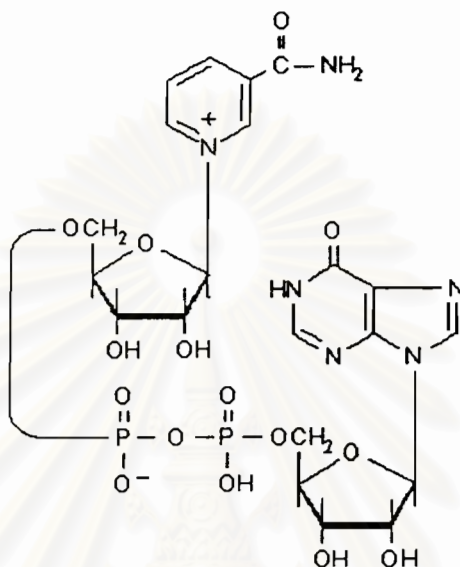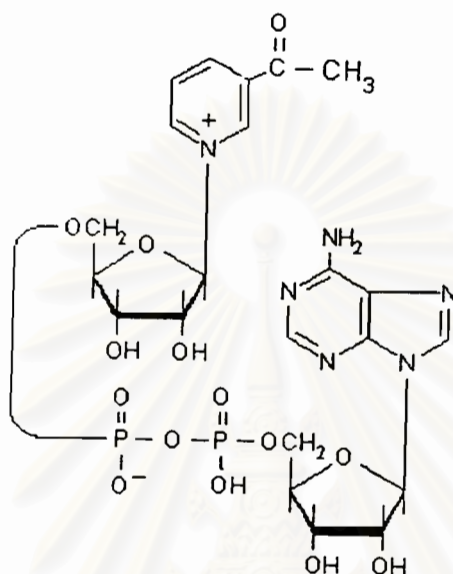

Nicotinamide hypoxanthine dinucleotide (Deamino-NAD$^+$)
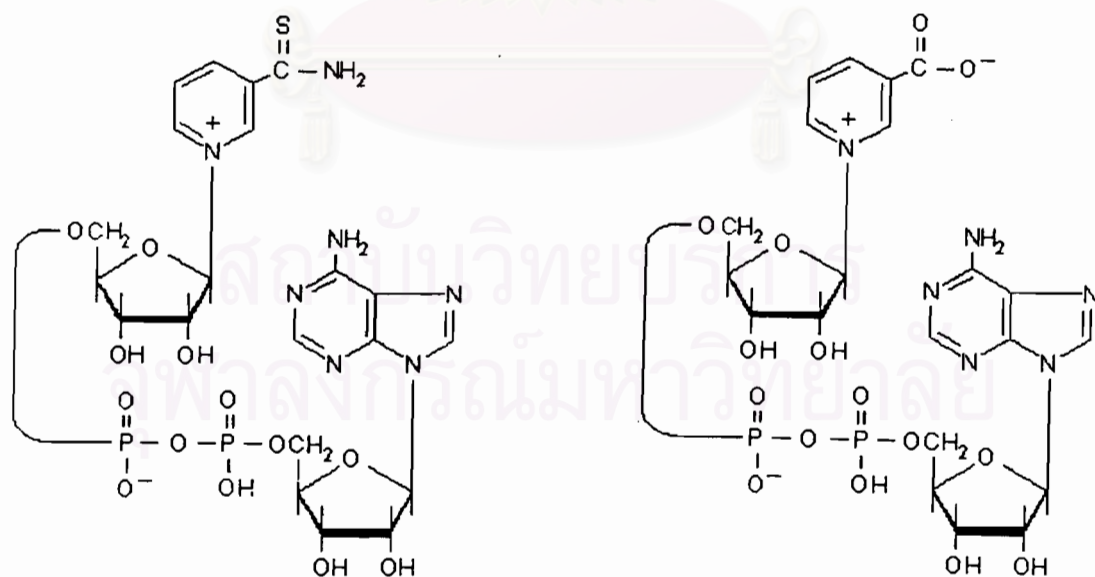

Nicotinamide guanine dinucleotide
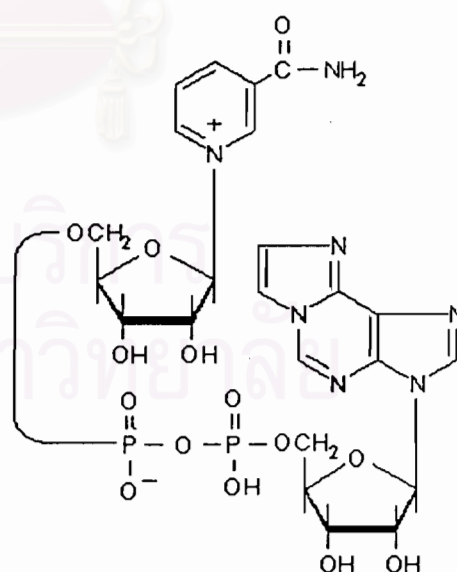
Nicotinamide 1, N^6-ethenoadenine dinucleotide

APPENDIX I (continued)

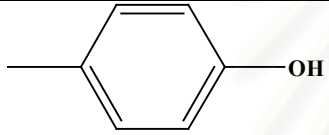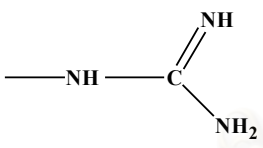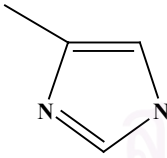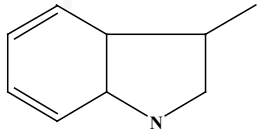## 3. Coenzyme analog modified at the nicotinamide moiety

3-Acetylpyridine adenine dinucleotide

3-Pyridinealdehyde adenine dinuleotide

Thionicotinamide adenine dinucleotide

APPENDIX J

Reactive functional groups of protein

Reactive functional group		pK_a
Formula	Originating from amino acid	
-NH$_2$	Lysine (Lys, ε-NH$_2$), N-terminal amino groups (α-NH$_2$)	10.53; 9.0-9.9
-SH	Sulfhydryl of cysteine (Cys)	8.27
-COOH	Carboxyl of aspartate (Asp), glutamate (Glu), C-terminal carboxyl groups	3.86; 4.07; 1.8-2.4
	Phenolic of tyrosine (Tyr)	10.07
	Guanidino of arginine (Arg)	12.48
	Imidazole of histidine (His)	6.10
-S-S-	Disulfide of cystine	-
	Indole of tryptophan (Trp)	-
CH$_3$-S-	Thioester of methionine (Met)	-
-CH$_2$-OH	Hydroxyl of serine (Ser) and threonine (Thr)	-

(Sources: Gemeiner, 1992)

APPENDIX K

Abbreviations of amino acid residues (Nelson and Cox, 2000)

Amino acid	3-Letter-abbreviation	1-Letter-abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Unknown	-	X

BIOGRAPHY

Miss Orada Chumphukam was born on April 6, 1980 in Chiangmai. She finished high school at Wattanothaipayap School, Chiangmai and enrolled in the Faculty of Science, Chiangmai University. She graduated with the B. Sc. in Biochemistry and Biochemical Technology in 2001 and continued studying for M. Sc. in Biochemistry Program, Faculty of Science, Chulalongkorn University in that year.