CHAPTER I

INTRODUCTION

Enzymes are catalysts bearing some excellent properties (high activity, selectivity and specificity) that may permit to perform the most complex chemical processes under the most benign experimental and environmental condition (Wong and Whitesides, 1994; Koeller and Wong, 2001). Thus, the engineering of enzymes from biological entities to industrial reactors is very exciting goal. Fortunately, there are many techniques available that may permit to improve the enzyme features, involving many areas of science that have suffered impressive developments in the past years: microbiology, protein engineering, chemistry of proteins and etc. However, some apparently older fashioned techniques, as immobilization, have been revealed in the past as a very powerful tool to improve almost all enzyme properties (Mateo *et al.*, 2007).

1.1 Phenylalanine dehydrogenase

Phenylalanine dehydrogenase (L-phenylalanine: NAD^+ oxidoreductase, deaminating: EC 1.4.1.20) (PheDH) is one of the amino acid dehydrogenases, which catalyzes the reversible pyridine nucleotide-dependent oxidative deamination of L-phenylalanine to form ammonia, phenylpyruvate and NADH as shown in **Figure 1.1** (Brunhuber and Blanchard, 1994). Much attention has been paid to this enzyme because it is useful as an industrial catalyst in the asymmetric synthesis of L-phenylalanine and related L-amino acids from their keto analogs, and as a clinical reagent for the selective determination of L-phenylalanine and phenylpyruvate (Asano *et al.*, 1987a). It was first discovered in *Brevibacterium* sp. (Hummel *et al.*, 1984). Consequently, Asano and his colleagues screened the enzyme activity among a number of microorganisms from soil samples. It was found that the enzyme activity was very narrowly distributed in aerobic spore-forming, gram-positive bacteria, *Bacillus badius* (Asano *et al.*, 1987a), *Sporosarcina ureae* and *Bacillus sphaericus* (Asano *et al.*, 1987b). Later, the enzyme was found in *Rhodococcus* sp.

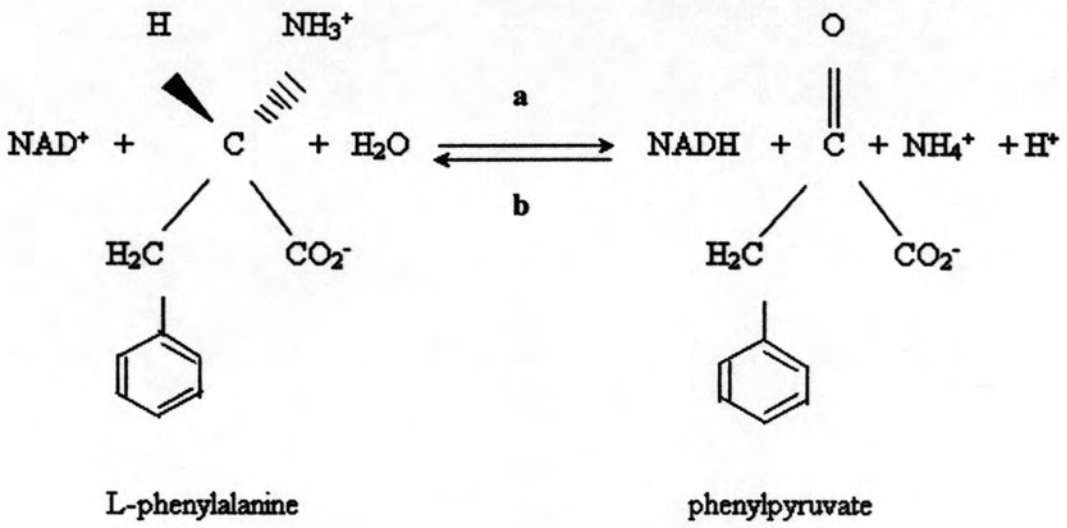


Figure 1.1 The reaction of L-phenylalanine dehydrogenase (Brunhuber and Blanchard, 1994).

(a) oxidative deamination

(b) reductive deamination

(Hummel *et al.*, 1987), *Rhodococcus maris* (Misono *et al.*, 1989) and *Norcardia* sp. (Boer *et al.*, 1989). The PheDH from several mesophiles are not stable enough for industrial and clinical applications. Therefore, thermostable enzyme have been focused in this field. The thermostable PheDH form *Thermoactinomyces intermedius* was studied by Ohshima *et al.* (1991). PheDH was later found in non-spore forming mesophilic bacteria, *Microbacterium* sp. (Asano and Tanetani, 1998). The occurrence of the stable PheDH in *Microbacterium* sp. was unique since the strain did not form spore. The distribution of PheDH is limited to some groups of gram-positive, spore-forming bacteria including actinomycetes. This may be owing to the fact that these enzymes being involved in microbial sporulation. Thus, connect the carbon and nitrogen metabolism of amino acids because of the reversible nature of the enzymes.

1.2 Purification of phenylalanine dehydrogenase

The study on physical and biochemical properties of PheDH requires separation techniques to purify enzyme. How pure enzymes should be used is dependent on the purpose of the enzyme application. PheDH has already been 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 interation chromatography (mostly by Butyl-Toyopearl column) and gel filtration chromatography. For instance, the affinity chromatography, Red-sepharose CL 4B column, was also applicable for the purification of PheDH from *R. maris* (Misono *et al.*, 1989) and *T. intermedius* (Ohshima *et al.*, 1991). FPLC, Mono Q column was used as one common step in the purification procedures of PheDH from *R.maris* and *Microbacterium* sp. (Misono *et al.*, 1989; Asano and Tanetani, 1998). Recently, Tynan *et al.* (2000) described a novel kinetic locking-on strategy for bioaffinity purification of NAD^+-dependent dehydrogenase including PheDH based on

immobilized cofactor derivatives through the use of enzyme-specific substrate analogues to promote selective and biospecific adsorption.

1.3 Basic molecular and catalytic properties of phenylalanine dehydrogenase

The enzyme was first found in *Brevibacterium* sp. in 1984 and since then, several other bacterial PheDHs were identified and characterized. The properties of PheDH from various sources are summarized in **Table 1.1**. The PheDHs exhibit a narrow range of subunit molecular masses between 36 and 46 kDa. There is considerable variation in quaternary structure of these enzymes. The enzymes from *S. ureae*, *B. sphaericus*, *B. badius* and *Microbacterium* sp. were shown to be octomers whereas monomeric, dimeric, tetrameric and hexameric are found in the *Nocardia* sp, *R. maris*, *Rhodococcus* sp. M4 and *T. intermedius* enzymes, respectively. PheDH has an isoelectric point in the range of 4.0 to 6.0 except enzyme from *B. badius* with pI of 3.5. The pH optima for oxidation deamination are between 10.1 and 12.0 whereas for the reduction amination are between 8.5 and 10.3. The high reactivity of the enzyme at rather high pH is similar to those of other amino acid dehydrogenases such as leucine dehydrogenase (Ohshima *et al.*, 1985) and alanine dehydrogenase (Keradjipoulos and Holldorf, 1979). Moreover, it was noteworthy that the *T. intermedius* enzyme was much more thermostable than PheDHs from other mesophiles. The equilibrium constant (K_{eq}) reported for the *Rhodococcus* sp. M4 enzyme was 4.5×10^{-14} M^2 and the *S. ureae* enzyme was 2.0×10^{-14} M^2 whereas the *Nocardia* sp. and *B. sphaericus* enzymes were reported to have K_{eq} of 3.2×10^{-18} M^2 and 1.4×10^{-15} M^2, respectively. PheDH from various sources have broad substrate specificities, the *B. sphaericus* enzyme acts on L-tyrosine as well as L-phenylalanine whereas the *T. intermedius* enzyme was highly specific to L-phenylalanine. In addition to phenylpyruvate, *p*-hydroxyphenylpyruvate was a good substrate for reductive amination of the enzyme from *Brevibacterium* sp., *B. sphaericus* and *R. maris*. Steriochemistry of hydrogen transfer of amino acid dehydrogenase showed

Table 1.1 Properties of phenylalanine dehydrogenase from various sources[a]

Properties	*Brevibacterium* sp.	*Rhodococcus* sp. M4	*S. ureae*	*B. sphaericus*	*B. badius*	*R. maris*	*Norcardia* sp.	*T. intermedius*	*Microbacterium* sp.	*B. lentus*	*A. iwoffii*
Specific activity of final preparation (U/mg protein)	-	-	84	111	68	65	30	86	37	171	79
Molecular mass of native enzyme											
- gel filtration	-	150,000	310,000	340,000	335,000	70,000	42,000	270,000	330,000	340,000	32,000
- deduced amino acid sequence	-	-	330,608	331,480	330,800	-	-	249,928	-	-	
Molecular mass of subunit	-	39,500	41,326	41,435	41,350	36,000	42,000	40,488	41,000	42,000	40,000
Number of subunit	-	4	8	8	8	2	1	6	8	8	8
Isoelectric 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	10.4	10.5
-reductive amination	8.5	9.25	9.0	10.3	9.4	9.8	10.0	9.2	12.0	8.5	9.2
Thermostability (%remaining activity after incubation)	-	-	75 (40 °C, pH 9, 10 min)	100 (55 °C, pH 9, 10 min)	50 (55 °C, pH 8, 10 min)	100 (35 °C, pH 7.4, 10 min)	50 (53 °C, pH 9.5-10, 2 h)	100 (70 °C, pH 7.2, 1 h)	100 (55 °C, pH 9, 10 min)	50 (50 °C, pH 10.4, 3 days)	55 (50°C, pH 7.4, 10 min)

Table 1.1 Properties of phenylalanine dehydrogenase from various sources (continued)

Properties	*Brevibacterium* sp.	*Rhodococcus* sp. M4	*S. ureae*	*B. sphaericus*	*B. badius*	*R. maris*	*Norcardia* sp.	*T. intermedius*	*Microbacterium* sp.	*B. letus*	*A. lwoffii*
Equilibrium constant (M^2)	-	4.5×10^{-14}	2.0×10^{-14}	1.4×10^{-14}	-	-	3.2×10^{-18}	-	-	-	-
Apparent K_m (mM) for											
- L-phenylalanine	0.385	0.87	0.096	0.22	0.088	3.8	0.75	0.22	0.10	0.59	4.5
- phenylpyruvate	0.177	0.13	0.16	0.4	0.106	0.5	0.06	0.045	0.02	0.18	0.56
- NAD^+	0.125	0.27	0.14	0.17	0.15	0.25	0.23	0.078	0.20	0.55	0.68
- NADH	0.047	0.13	0.072	0.025	0.21	0.043	-	0.025	0.072	0.09	0.12
- ammonia	431	387	85	78	127	70	9.6	106	85	50	149
Substrate specificity [b]											
Oxidative deamination											
- L-phenylalanine	100	100	100	100	100	100	100	100	100	100	100
- L-tyrosine	-	12	5	72	9	2	2	0	4	0	30
- L-tryptophan	-	2	5	1	4	8	8	0	0	2	3
- L-methionine	-	4	4	3	8	5	5	0	7	5	2

Table 1.1 Properties of phenylalanine dehydrogenase from various sources (continued)

Properties	*Brevibacterium* sp.	*Rhodococcus* sp. M4	*S. ureae*	*B. sphaericus*	*B. badius*	*R. maris*	*Norcardia* sp.	*T. intermedius*	*Microbacterium* sp.	*B. lentus*	*A. iwoffii*
- L-valine	-	-	3	1	4	0	0	0	5	2	-
- L-leucine	-	-	2	1	3	2	-	4	3	3	2
- L-isoleucine	-	-	1	0.5	0.2	3	-	0	0	1	0.7
- L-norvaline	-	-	6	1	5	0	-	-	6	2	-
- L-norleucine	-	-	15	4	19	16	-	-	16	10	7
- L-ethionine	-	-	7	3	7	13	-	-	-	4	2
- L-α-aminobutyrate	-	-	2	-	1	1	-	-	2	-	0.7
- L-phenylalaninamide	-	-	9	3	9	-	-	-	-	-	-
- L-phenylalaninol	-	-	9	0.6	9	-	-	-	-	-	-
- L-*p*-aminophenylalanine	-	-	-	-	-	-	-	7	-	-	-
- L-phenylalanine methyl ester	-	-	10	10	38	-	-	-	-	-	-
- L-tyrosine methyl ester	-	-	7	7	0.4	-	-	-	-	-	-
- *p*-fluoro-DL-phenylalanine	-	62	-	-	34	8	-	-	-	35	34
- *m*-fluoro-DL-phenylalanine	-	-	-	-	11	8	-	-	-	17	17
- *o*-fluoro-DL-phenylalanine	-	-	-	-	2	2	-	-	-	3	7
- D-phenylalanine	-	-	0	0	0	0	-	0	-	0	0

Table 1.1 Properties of phenylalanine dehydrogenase from various sources (continued)

Properties	*Brevibacterium* sp.	*Rhodococcus* sp. M4	*S. ureae*	*B. sphaericus*	*B. badius*	*R. maris*	*Norcardia* sp.	*T. intermedius*	*Microbacterium* sp.	*B. lentus*	*A. iwoffii*
Substrate specificity [b]											
Reductive amination											
- phenylpyruvate	100	100	100	100	100	100	100	100	100	100	100
- *p*-hydroxyphenylpyruvate	96	5	24	136	53	91	28	0	0	-	120
- indole-β-pyruvate	24	3	1	0	-	5	54	-	-	-	-
- α-ketovalerate	-	-	9	6	12	0	-	-	-	8	0
- α-ketocaproate	-	-	32	0	31	9	-	-	-	22	43
- α-ketoisovalerate	-	-	2	6	13	0	-	6	6	7	0
- α-ketoisocaproate	-	-	13	8	-	1	240	-	-	9	0
- α-ketobutyrate	-	-	-	-	3	0	-	1	1	-	-
- α-keto-γ-methylthiobutyrate	59	33	27	11	16	9	-	14	14	1	0
- α-keto-β-methylbutanoate	-	-	-	-	-	-	-	-	-	-	-
- α-keto-γ-methylpentanoate	-	-	-	-	13	-	-	6	6	-	-
- α-ketohexanoate	-	-	-	-	31	-	-	-	-	-	-

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

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

- = no data

Source: *Brevibacterium* sp. (Hummel *et al.*, 1984 and Hummel and Kula, 1989), *Rhodococcus sp.*M4 (Brunhuber and Blanchard, 1994, Vanhooke *et al.*, 1999, and Brunhuber *et al.,* 2000), *Sporosarcina ureae* (Asano and Nakazawa, 1985, Asano *et al.*, 1987a, and Asano and Nakazawa, 1987), *Bacillus sphaericus* (Asano *et al.*, 1987a and c), *Bacillus badius* (Asano *et al.,* 1987b), *Rhodococcus maris* (Misono *et al.*, 1989), *Nocardia* sp. (Boer *et al.,* 1989), *Thermoactinomyces intermedius* (Ohshima *et al.*, 1991), *Microbacterium* sp. (Asano and Tanetani, 1998) and *Bacillus lentus* (Inkure, 2005)

either pro-*R* or pro-*S* stereospecificity for hydrogen transfer from the C-4 position of the nicotinamide moiety of NAD(P)H to the amino acid substrates as shown in **Figure 1.2**. For PheDH, the stereochemistry of hydride transfer was determined for the *B. sphaericus*, *T. intermedius* and *Rhodococcus* sp. M4 enzymes (Asano *et al.*, 1987b and Ohshima *et al.*, 1991). In all cases, the pro-*S* hydrogen of NADH was transferred to generate [2-^{2}H]-L-phenylalanine, placing the PheDH among the majority of amino acid dehydrogenase.

1.4 Structure of phenylalanine dehydrogenase

Extensive developments of the techniques in gene cloning have enabled rapid determination of the primary structures of PheDH. In addition, x-ray crystallography analysis of several amino acid dehydrogenases has been under taken and revealed their ternary and quaternary structures in details (Ohshima and Soda, 2000).

Among amino acid dehydrogenase, primary structures of GluDH, AlaDH, LeuDH, ValDH, DAPDH and PheDH have so far been determined by peptide and DNA sequencing methods (Ohshima and Soda, 2000). The gene encoding the enzyme was cloned and sequenced from *B.badius, B. spharicus, S. ureae, T.intermedius* and *Rhodococcus* sp. M4. Although a computer-aided search 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).

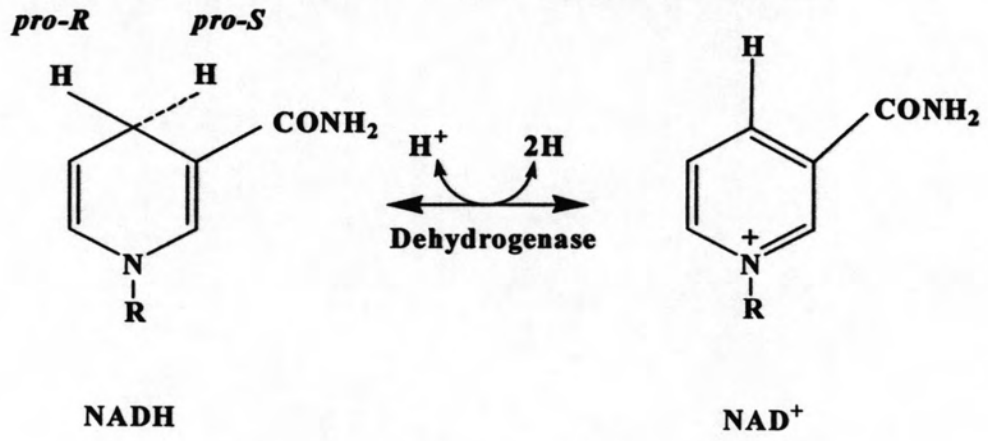


Figure 1.2 Stereospecificity of hydrogen transfer of NADH catalyzed with dehydrogenases (Ohshima *et al.*, 1991).
R represents ADP-ribosyl

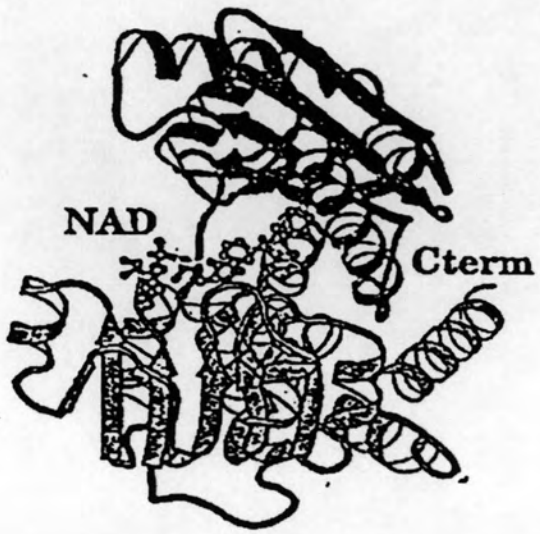
The structures of PheDH from *Rhodococcus.* sp. M4 has been directly determined by high-resolution X-ray crystallographic analysis in the two complexes with enzyme•NAD^+• phenylpyruvate, and enzyme•NAD^+•*β*-phenylpropionate were recently reported by Vanhook *et al.*, 1999. This was the first example of structures of the amino acid dehydrogenase with a ternary complex. Both structures showed that PheDH is a homodimeric enzyme and each monomer composed of distinct globular N- and C-terminal domains separated by a deep cleft containing the active site (**Figure1.3**). A ribbon representation of one subunit of the enzyme•NAD^+• phenylpyruvate abortive complex is displayed in **Figure 1.3A**. The N-terminal domain binds the amino acid substrate and contributes to the interactions at the subunit: subunit interface. Its motif contains five β-strands that form a mixed β-sheet with the overall topology shown in **Figure 1.3B**. The C-terminal domain forms a typical Rossmann fold responsible for NAD binding as found for GluDH and LeuDH (Vanhooke *et al.*, 1999). Moreover, they found that Lys78 and Asp118 act as the catalytic residues in the active site.

1.5 Current method for phenylalanine production

L-Phenylalanine is one of the most important commercially produced amino acids (Bongaerts *et al.*, 2001). In 1998, 11,000 tons of L-phenylalanine were consumed worldwide and world consumption in 2002 was estimated to be 14,000 tons (Budzinski, 2001), achieving approximately 850 M US$ phenylalanine sales in 2004 (Műller, 2001).

L-Phenylalanine is widely used in human nutrition, pharmaceutical and food industries. It is a precursor of some anti-cancer drugs. In addition, it is a principle ingredient in the dipeptide artificial sweetener aspartame (Hummel *et al.*, 1987) and benzaldehyde which can be used as aromatic flavor compound in cheese (Groot and de Bont, 1998). Therefore, the commercial demand on L-phenylalanine has increased

a)



b)

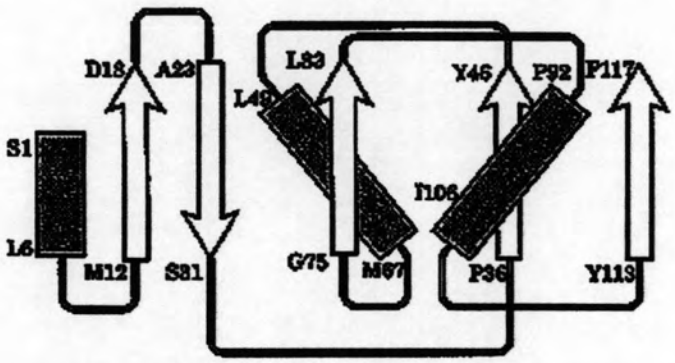


Figure 1.3 Structure of *Rhodococcus* sp. M4 phenylalanine dehydrogenase (Vanhooke *et al.*, 1999).

(a) Ribbon represents one subunit of PheDH•NAD^+•phenylpyruvate ternary complex.

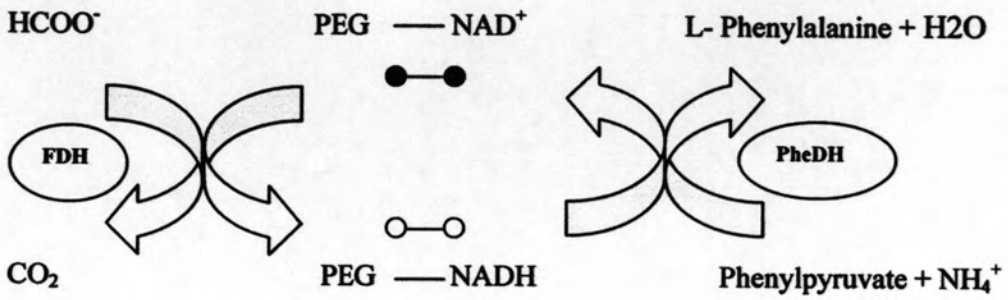
(b) The topology corresponding to the substrate binding domain. Rectangles and arrows represent α-helices and β–strands, respectively.

dramatically with the advent of this popular sweetener. A large scale production process of L-phenylalanine has been established in USA, Japan and Germany (Hamilton, Hsiao, Swann and Anderson, 1985). However a great demand of L-phenylalanine still is expected in the world markets (Klausner, 1985).

In general, there are four methods, including extraction from the natural protein, chemical synthesis, fermentation and enzymatic reaction, for the preparation of L-phenylalanine. However, an optically pure product with a high yield has led to the investigation of the microbial routes for L-phenylalanine production. The stereoselective synthesis of chiral active amino acids can be achieved by fermentation or enzyme-catalyzed processes (Deboer and Dijkuizen, 1990). In recent years, researchers are more interested in biotransformation method rather than the fermentation method to produce L-phenylalanine. 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 acid and L-phenylalanine, the entrapping cells were not an appropriate approach due to the cracking and damaging surface of the immobilized particle.

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 multienzyme reaction system for simultaneous coenzyme regeneration has been proposed to overcome this problem. The continuous conversion of phenylpyruvate to L-phenylalanine in the stirred tank membrane reactor was carried out by PheDH from *Brevibacterium* sp. (Hummel *et al.*, 1986) and formate dehydrogenase (FDH; EC 1.2.1.2) from *Rhodococcus* sp. M4 (Hummel *et al.*, 1987) as shown **Figure 1.4**. The coenzyme, NADH which is required in stoichiometric amounts, was therefore regenerated by a second enzyme, formate dehydrogenase. In order to retain the coenzyme behind the ulrafiltration membrane, it was covalently bound to a polyethylene glycol 20,000. This way the retention of

a)



b)

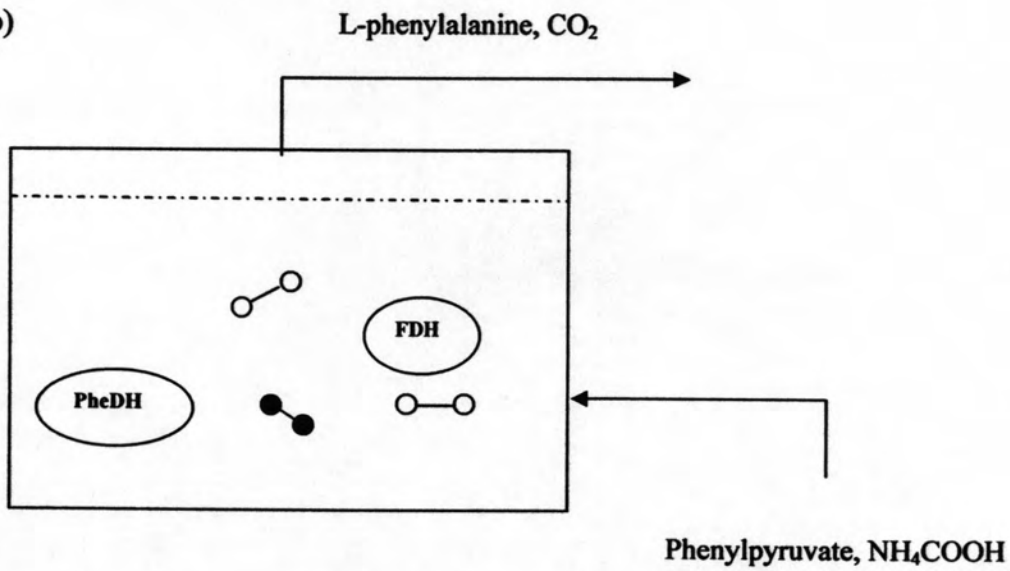


Figure 1.4 Enzymatic synthesis of L-phenylalanine with coenzyme regeneration (Hummel *et al.*, 1986).

a) Reaction scheme

b) Reactor scheme

coenzyme by an ultrafiltration membrane could be accomplished together with separation of the enzyme from the product stream.

The lower cost of commercial production of phenylalanine and to avoid the instability of phenylpyruvate in aqueous solution, two alternative routes have been studied (Hummel and Kula, 1989). One started from the racemic mixture of phenyllactate while the other started from acetamidocinnamic acid. The conversion of DL-phenyllactate was achieved utilizing the side reaction of two enzymes as shown in **Figure 1.5a**. The kinetic properties of the enzyme involved in the cyclic reaction make this approach unfavorable. Acetamidocinnamic acid is another stable precursor of phenylpyruvate. Deacetylation results in an unstable enamine-imine derivative, which hydrolyze spontaneously to yield phenylpyruvate (**Figure 1.5b**). The deacetlyation can be accomplished enzymatically by an acylase isolated from a strain of *Brevibacterium* sp. In this route, FDH is necessary for coenzyme regeneration.

PheDH from *Sporosarcina ureae* and FDH from *Candida boidinii* were used in the synthesis of L-phenylalanine and other L-amino acids. Amino acids such as L-phenylalanine, L-tyrosine, L-valine, L-methionine, and L-leucine were synthesized in % yield as shown in Table 1.2 (Asano and Nakazawa, 1987). In addition, genetic improvements through metabolic engineering are investigated. Galkin *et al.* (1997) recently reported a simple method for enzymatic synthesis of D and L-amino acid from α–keto acids with the recombinant *Escherichia coli* TG1 cells which contained plasmid with heterologous genes necessary for biotransformation. L-amino acid were produced by thermostable L-amino acid dehydrogenase and FDH from α–keto acids and ammonium formate with only an intracellular pool of NAD^+ for the regeneration of NADH. By this method, plasmid containing FDH and PheDH genes was constructed (pFDHPheDH) and L-phenylalanine and L-tyrosine were synthesized from phenylpyruvate and *p*–hydroxyphenylpyruvate with high yield of 95% and 92%, respectively, and the optical purity (enantiomeric excess) of them was 100%.

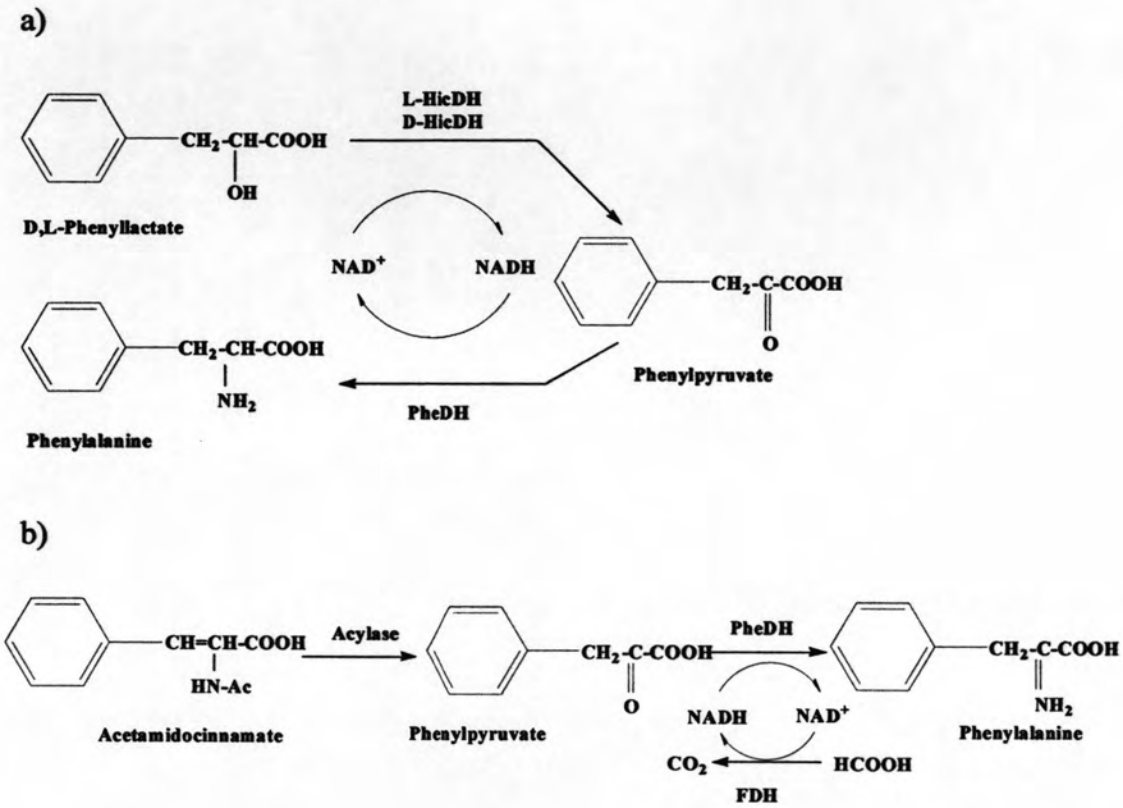


Figure 1.5 Enzymatic routes for the preparation of L-phenylalanine (Hummel and Kula, 1989).

(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

Table 1.2 Synthesis of L-amino acids from keto acids by *S. ureae* PheDH and *C. boidinii* FDH (Asano and Nakazawa, 1987)

Substrate	Product	%Yield
phenylpyruvate	L-phenylalanine	98
p-hydroxyphenylpyruvate	L-tyrosine	99
indolylpyruvate	L-tryptophan	11
α-keto-γ-methylthio- butyrate	L-methionine	87
α-ketoisovalerate	L-valine	97
α-ketoisocaproate	L-leucine	83
DL-α-keto-β-methyl-n-valerate	L-isoleucine allo-isoleucine	48 50

1.6 Enzyme immobilization

Immobilization of enzyme has been developed greatly in the past decades, this biotechnology overcomes the drawback of separating the free enzyme catalyst difficultly from the reaction mixture, it can permit the enzyme catalyst to be recovered and reused, and the efficiency and stability of the enzyme catalysis are greatly enhanced (Gao *et al.*, 2005).

Immobilized enzymes are defined as "enzyme physically confined or localized in a certain defined region of space with retention of their catalytic activities". Thus, as compared to free enzymes in solution immobilized enzymes are more robust and more resistant to environmental change. More importantly, the heterogeneity of the immobilized enzyme systems allows easy recovery of enzyme and product, multiple uses of enzymes, continuous operation of enzymatic process, rapid termination of reactions and greater variety of bioreactor designs (Krajewska, 2004).

1.6.1 Methods of enzyme immobilization

Since the late 1960s a variety of techniques have been developed for immobilization biocatalysts. Several schemes have been suggested for classifying immobilization techniques. The classification that is used here is based on that of Chibata (1978). The techniques available for the immobilization of enzymes and other biologically active protein and can be grouped in to four main classes. **Figure 1.6** showed a numerous methods for achieving immobilization of enzyme, each involving a different degree of complexity and efficiency.

1.6.1.2 Adsorption method

Adsorption of an enzyme can be achieved by simply bringing an enzyme solution contact with the support surface by physical or ionic interaction.

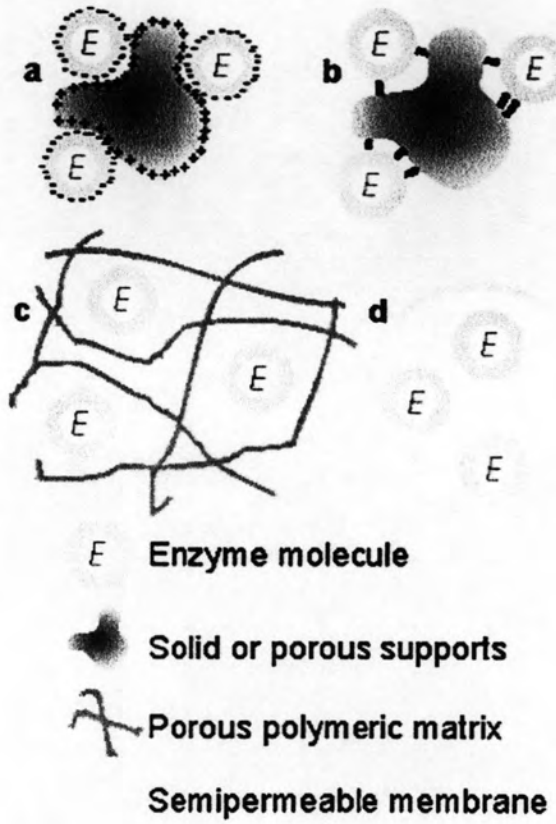


Figure 1.6 Immobilized enzyme system (Chaplin and Bucke, 1990).

(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 s cross-linked polymer

(d) enzyme confined within a semipermeable membrane

Physical adsorption Biocatalysts often bind to carrier by a physical interaction such as hydrogen bonding, hydrophobic interaction, van der Waal force, or their combined action. This method is simple and effective and often causes little or no conformation change of the enzyme protein, or destruction of its active center. However, this method has the disadvantage that the adsorbed enzyme may release from the carrier during utilization, because the binding force between the enzyme and carrier is weak. Both inorganic materials such as activated carbon, porous glass, alumina etc. and natural polymers such as starch, chitin and gluten etc. have been employed as carrier for this method.

Ionic binding The binding forces are ion-ion interactions and are stronger than simple physical adsorption. Binding enzyme on a support is affected by a kind of buffer used, pH, ionic strength and temperature. Several derivatives of cellulose and sephadex, as well as various ion-exchange resins, can be utilized for immobilization. This method has been applied for the immobilization of many enzymes because the procedure is varying simple, the supports are renewable and the enzymes are not modified.

1.6.1.2 Entrapment method

In principles, all entrapment method are based on the coupling enzyme to the lattice of a polymer matrix or enclosing them in semipermeable membranes, tight enough to prevent the protein from diffusing into the surrounding medium, while still allowing penetration of substrate. Free enzymes are entrapped within the interstitial space of a crosslinked, water-insoluble polymeric gel. Gel forming materials such as polysaccharides, protein or synthetic polymers can be employed. The advantages of entrapping methods are that not only single enzymes but also several different enzymes, cellular organelles and cell can be immobilized with essentially the same procedure.

1.6.1.3 Cross-linking method

This immobilization method is based on the production of three-dimensional cross-linked enzyme aggregates, which are completely insoluble in water, by means of bi- or multifunctional reagents, which link covalently to the enzyme molecules. Cross-linking reagent such as glutaraldehyde (Schiff base), isocyanate derivatives (peptide pond), bisdiazobenzidine (diazo coupling) have been employed. The primary disadvantages of this method are the relatively severe reaction conditions and difficulties in controlling the reaction. Hence, activities of such enzymes are generally low.

1.6.1.4 Covalent binding method

Covalent coupling for the immobilization of enzymes is based upon the formation of a covalent binding between the enzyme molecules and the support material. The functional groups that take part in the covalent binding of enzyme to the carrier are as follows: (1) amino group, (2) carboxyl group, (3) sulfhydryl group, (4) hydroxyl group, (5) imidazole group and (6) phenolic group. This method can be further classified into 6 types according to the mode of linkage. The selection of conditions for immobilization by covalent binding is more difficult than in the other immobilization method. The reaction conditions required are relatively complicated and not usually mild. Therefore, in some cases, covalent binding alters the conformational structure and active center of the enzyme, resulting in major loss of activity and / or change of substrate specificity. However, the binding force between enzyme and carrier is strong and the leakage of the enzyme hardly occurs even in the presence of substrate or salt solution of high ionic strength.

Enzymes immobilized by covalent binding have the following advantages: (1) because of the tight binding, they do not detach from supports during utilization; (2) immobilized enzymes can easily come into contact with substrates because the enzymes are localized on the surface of support; and (3) an increase in heat stability is often observed because of the strong interaction between

enzyme molecules and supports. On the other hand, disadvantages of covalent binding are: (1) active structures of enzyme molecules are liable to be destroyed by partial modification; (2) strong interaction between enzyme molecules and support often hinders the free movement of enzymes molecules, resulting in decreased enzyme activity; (3) optimal conditions of immobilization are difficult to find; (4) this method is not suitable for immobilization of cells; and (5) supports, in general, are not renewable. Hence, this principle is well-suited for expensive enzymes whose stability is significantly improved by covalent binding.

1.6.2 Comparison of immobilization techniques (Kenedy and Cabral, 1987)

Although a number of immobilization techniques have been applied to many enzymes, it is recognized that no one particular procedure can be considered an ideal universal method for enzyme immobilization, because of the widely different composition and chemical characteristics of the enzymes and their substrate and product properties. Therefore, each method of immobilization has specific limitations, and for any particular application it is necessary to find an immobilization procedure that is simple and inexpensive that yields an immobilized enzyme with a good retention of activity and suitable operational stability. Also sometimes efficient immobilization of enzyme is achieved by combination of methods, such as enzyme adsorption followed by chemical crosslinking. However, a general comparison of the different enzyme immobilization processes can be made based on the main characteristics of these methods and on the support matrix. **Table 1.3** summarizes some of the relative advantages and disadvantages of the different techniques of enzyme immobilization.

1.6.3 Review of phenylalanine dehydrogenase immobilization

PheDH has considerable commercial potential both for the chiral synthesis of novel nonprotogenic amino acids for the use the pharmaceutical industry and also for the use as diagnostic reagents to monitor the serum levels of L-phenylalanine which

Table 1.3 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

accumulate in a range of metabolic diseases, phenylketonuria (PKU) and hyperphenylalaninemia. Most reports on PheDH immobilization have been related to medical application. The effective screening of infants (normally within 7 days old neonates) encourages the effective treatment to prevent severe symptoms (National Institutes of Health Consenus Development Panel, 2001). Various methods have been used and developed for determination of phenylalanine. Microbiological method (Guthrie and Susi, 1963), which is used worldwide as a semiquantitative test, is low precision and cannot be applied in infants on antibiotic treatment. Fluorimetric method can be automated, but is susceptible to interference; especially for blood spots (Mccaman *et al.*, 1962). HPLC and amino acid analysis methods are accurate and precise but require expensive equipment, special expertise and are time consuming (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.

Almost all of the PheDH immobilization methods have been for a biosensor construction to screen of phenylketonuria (PKU). From the study by *Girotti et al* (1993), a highly sensitive and rapid bioluminescent flow sensor was developed for the determination of the content of L-phenylalanine in serum produced by immobilized PheDH with luciferase, bacterial bioluminescent enzyme immobilized on a separate nylon coil. PheDH was extracted from *B.badius*, *B. sphaericus* and *Rhodococcus* sp. M4. The nylon coil had been activated with triethyloxonium tetrafluoroborate, 1,6-diaminohexane and glutaraldehyde. Considering both of activity and protein content, 60-70% of added PheDH were 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 enzyme source. 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 unsuitable for routine work.

In the year of 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 beads and glutaraldehyde-activated PVA bead were used for the coupling PheDH. Under the same preparation for the activated bead and coupling conditions with the study of Kiba *et al.*, 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 recently described by Huang *et al.* (1998) by using an enzyme carbon paste electrode containing three different enzymes. This sensor was based on the enzymatic/electrochemical recycling of tyrosinase in combination with salicylate hydroxylase and the *Rhodococcus* sp.M4 PheDH. As shown in **Figure 1.7**, the minimal sample preparation and fast analysis time makes the biosensor method an attractive option for the determination of L-phenylalanine.

In the year 2006, Tachibana and his co-worker developed a new microquantification method of L-phenylalanine concentration in an extract from a dried blood spot by using the diaphorase-resazurin system. To miniaturize the fluorometric enzymatic microplate assay for the diagnosis of phenylketonuria, an enzyme with His-tag fused PheDH was developed. Moreover, His-tag fused PheDH-immobilizing enzyme chip could be used for other assays when immobilized with other NAD^+-dependent oxidoreductases.

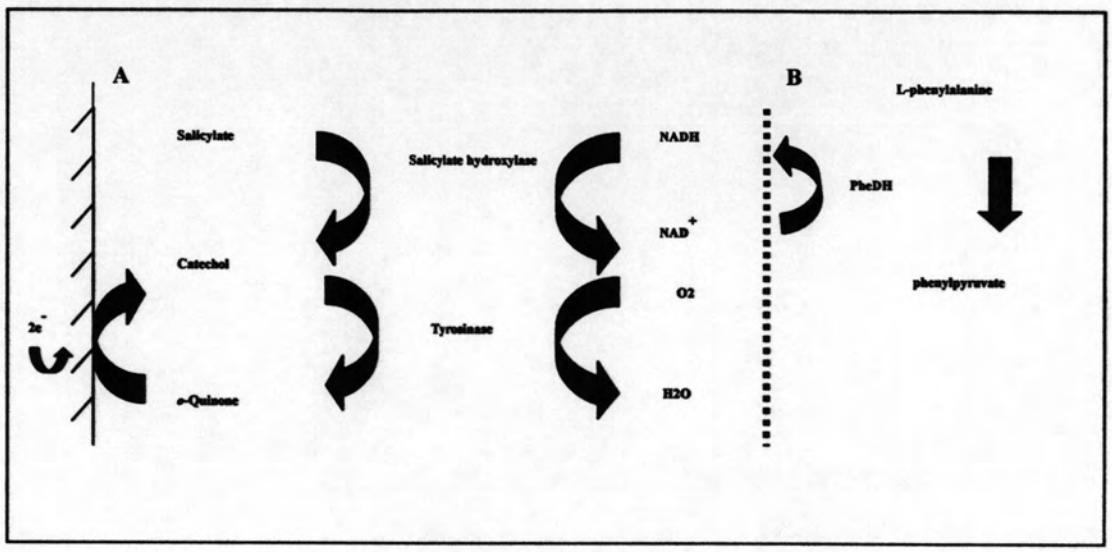


Figure 1.7 Schematic representation of an NADH-detecting biosensor

(Huang *et al.*, 1998).

(A) the salicylate hydroxylase/tyrosinase bioelectrocatalytic recycling system

(B) the coupling of PheDH to the bienzyme system

According to the study of Villalonga *et al.* (2008), *Bacillus badius* PheDH was covalent immobilized on amino-activated cellulose membrane by cross-linking with glutaraldehyde which was used for the construction of an amperometric biosensor device for L-phenylalanine quantification. The enzyme electrode showed a linear amperometric response up to 9.1 mM L-phenylalanine with a detection limit of 0.25 μM. The biosensor reached 95% of steady-state current in about 25s and its sensitivity was 177 μA/M cm^2.

There has been only one report on the immobilization of PheDH for the production of acetal amino acid (Hanson *et al.*, 2000). PheDH from *T. intermedius* and recombinant *E. coli* containing *T. intermedius* PheDH activity were used and FDH from *Candida boidinii* were also immobilized 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.

1.7 Objectives of this research

The dehydrogenase research group at the Department of Biochemistry, Faculty of sience, Chulalongkorn University has successfully cloned PheDH gene and overexpressed in *E.coli* BL23 (DE3) using the expression vector, pET-17b. The specific activity from crude extract of recombinant clones was found in the range of 0.81 – 4.46 units/mg protein. The highest specific activity was 55.75 fold higher than that of the enzyme from *Acinetobacter lwoffii*. The optimum condition for phenylalanine dehydrogenase gene expression was induction with 0.4 mM IPTG for 8 hours. The enzyme was purified to homogeneity by 50-70 % saturated ammonium sulfate precipitation and DEAE-Toyopearl column chromatography with 29.45 % yield and 5.19 purification fold. The enzyme showed high substrate specificity in the oxidative deamination on L-phenylalanine while it acted on α-ketocaproate, α-keto-γ-methiol-n-butyrate, α-ketovalerate and α-ketoisocaproate with 5.96, 4.12, 3.84 and 3.15 fold of its natural substrate, phenylpyruvate, respectively in reductive amination.

When phenylalanine dehydrogenase was used for production of amino acids using their corresponding keto acids as substrates, the product yield was in the range between 36.0-72.2 %.

Covalent immobilization of PheDH from recombinant *E. coli* JM 109 was studied by Chumphukam (2004). PheDH was immobilized on various supports including alumina, silica, PVA and chitosan and different activating agents were used: 1,4-butanedioldiglycidyl ether, tresyl chloride and glutaraldehyde. Silica with glutaraldehyde as a cross-linking agent was found to be the most appropriate support and method. In addition, immobilized enzyme was able to synthesize of amino acid from their keto substrates. However, the immobilized activity was only 1.05% of its original activity.

The enzyme is of interest to be used in the industrial synthesis of various amino acids from their corresponding keto acids by reductive amination. In addition, enzyme immobilization is one of the most attractive methods to avoid the problems inherent the use of free enzymes. Immobilization also facilitates 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 the production of PheDH. Furthermore, for PheDH immobilization, there have yet been no literature reports on the immobilization and characterization of the immobilized PheDH in comparison with the free enzyme. In addition, the production of L-phenylalanine by the immobilized PheDH has not either been reported. Therefore, the objectives of this thesis were

i) To partially purify PheDH from recombinant *E. coli*
ii) To improve the immobilization of PheDH method
iii) To determine and select the appropriate carrier together with the immobilization procedure
iv) To determine the optimum conditions for PheDH immobilization

v) To study the properties of immobilized PheDH and compare to those of the soluble enzyme

vi) To investigate the reusability of the immobilized enzyme for the production of L-phenylalnine

vii) To preliminarily evaluate the use of immobilized enzyme for the production of L-phenylalanine and various amino acid from their corresponding keto acids