CHAPTER I

INTRODUCTION

Amino acids, monomeric units of protein, are energy metabolites and precursors of many biologically important nitrogen-containing compounds, notably heme, physiologically active amines, glutathione, nucleotides, and nucleotide coenzymes. Excess dietary amino acids are neither stored for future use nor excreted. On the contrary, they are converted to common metabolic intermediates such as pyruvate, oxaloacetate and acetyl-CoA. Amino acids, therefore, are precursors of glucose, fatty acids, ketone bodies and metabolic fuels. Beyond their role in proteins, amino acids and their derivatives have so many biologically important functions such as chemical messengers in the communications between cells. For example, glycine, γ-aminobutyric acid (GABA) and dopamine are neurotransmitters (Voet, 2004).

Amino acids can be classified into two groups using the ability of rotation on the plane of polarized light. They are L-formed and D-formed amino acids. The first one plays important role in all life, while the later is rarely found in organism. Recently, the using of L-amino acids for many compounds synthesis are spread widely in animal nutrition, human medicine and the pharmaceutical industries. For example, L-leucine, L-valine, L-isoleucine are used as food and feed activities (Gu and Chang, 1990) whereas L-alanine is used as the precursor in drug production and can be also used as food additive due to its sweet taste (Suye *et al.*, 1992). L-phenylalanine, another interesting L-amino acid, is one of the essential raw material utilized in the manufacture of a dipeptide sweetener known as aspartame (L-aspartate-L-phenylalanine-1-methyl ester, or Nutrasweet) (Chao *et al.,* 2000). In addition, non-natural amino acids are increasingly in demand by the pharmaceutical industry for single-enantiomer drugs. They are in demand as precursor for asymmetric synthesis, however, they are very expensive (Busca *et al.,* 2004).

1.1 Amino acid dehydrogenase

The amino acid dehydrogenases (EC 1.4.1.-), a family of enzyme, are part of the oxidoreductase superfamily. They catalyze the reversible deamination of amino acids to their corresponding keto acids in the presence of the pyridine nucleotide coenzymes, NAD^+ and / or $NADP^+$. The general formula for this reaction can be written as shown in Figure 1.1. They are important enzymes that exist at the interface of nitrogen and carbon metabolism. The carbon skeleton of an amino acid can be metabolized for energy through the glycolytic or TCA cycle reactions by three enzymes. The first enzyme is pyridoxyl phosphate-dependent transaminases, which transfers the amino group of amino acid to keto acid and results in new amino acid such as glutamate. The second one is to employ deaminases in the way to remove amino group from amino acid in the form of ammonia. The last is to use an amino acid dehydrogenase. This enzyme has the advantages of removing the amino group as free ammonia which can then be used by the cell in diverse ways (Brunhuber and Blanchard, 1994). The amino acid dehydrogenases are categorized based on the specificity they display toward their amino acid substrate and more than ten kinds of them have been so far found in various kinds of organism as shown in Table 1.1 (Ohshima and Soda, 2000). The amino acid dehydrogenases have been studied intensively because of their ubiquitous distribution and a number of potential industrial applications. They have been used in the design of biosensor, in which they can monitor the levels of free amino acids in solution, especially in screening blood serum for elevated levels of amino acids in solution associated with certain diseases. The amino acid dehydrogenases have also been used in the industrial synthesis of amino acids. Since these enzymes act stereospecifically, they can produce the pure, natural L-isomer of the amino acids, which are important for pharmaceutical and dietary consumption purposes (Brunhuber and Blanchard, 1994).

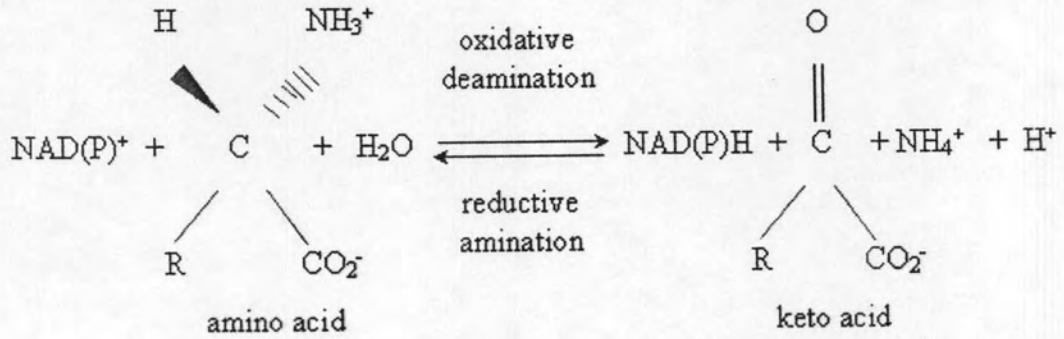

Figure 1.1 The general reaction of L-amino acid dehydrogenase

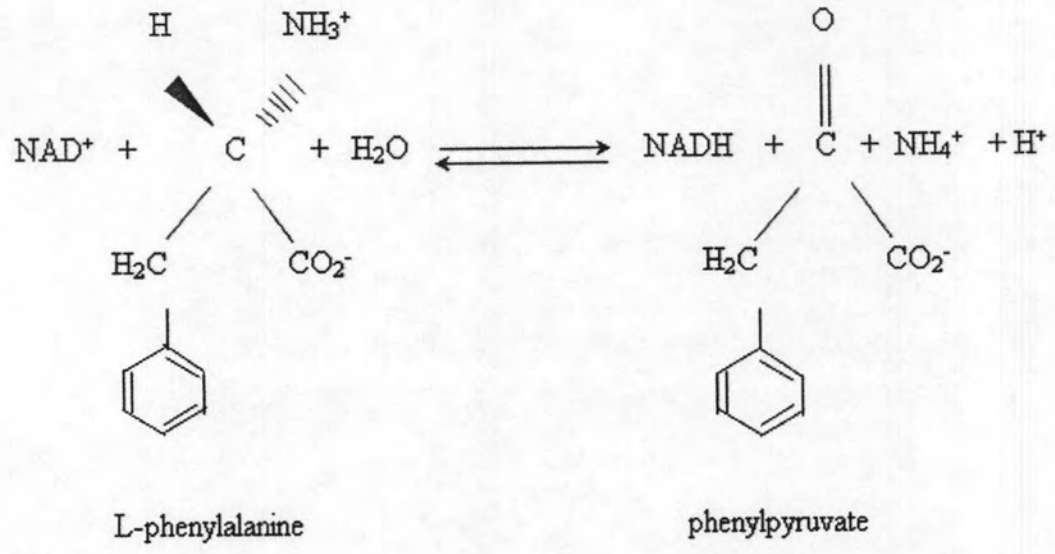

Figure 1.2 The reaction of L-phenylalanine dehydrogenase

Table 1.1 The group of NAD(P)$^+$-dependent amino acid dehydrogenase

EC number	Enzyme	Coenzymes	Major source
1.4.1.1	AlaDH	NAD	Bacteria (*Bacillus, Streptomyces, Anabena, Pseudomonas, Rhodobacter, Arthrobacter, Thermus, Enterobacter, Phormidium*), chrorella
1.4.1.2	GluDH	NAD	Plants, fungi, yeasts, bacteria
1.4.1.3	GluDH	NAD(P)	Animals (bovine liver, chicken liver), tetrahymena, bacteria (*Clostridium, Thiobacillus*)
1.4.1.4	GluDH	NADP	Plants, *Euglena gracilis, Chrorella sarokiniana*, fungi, yeasts, bacteria
1.4.1.5	L-Amino acidDH	NADP	Bacteria (*Clostridium sporogenes*)
1.4.1.7	SerDH	NAD	Plants (parsley)
1.4.1.8	ValDH	NAD,NADP	Bacteria (*Streptomyces, Alcaligenes faecalis, Planococcus*), plants (pea, wheat)
1.4.1.9	LeuDH	NAD	Bacteria (*Bacillus, Clostridium, Thermoactinomyces*)
1.4.1.10	GlyDH	NAD	Bacteria (*Mycobacterium tuberculosis*)
1.4.1.11	DAHDH	NAD,NADP	Bacteria (*Clostridium, Brevibacterium*)
1.4.1.12	DAPDH	NAD(P)	Bacteria (*Clostridium*)
1.4.1.15	LysDH (cyclizing)	NAD	Human liver
1.4.1.16	DAPMDH	NADP	Bacteria (*Corynebacterium glutamicum, Brevibacterium* sp., *Bacillus sphaericus*)
1.4.1.17	MethylalaDH	NADP	Bacteria (*Pseudomonas* sp.)
1.4.1.18	LysDH (Lys-6-DH)	NAD	Bacteria (*Agrobacterium tumefaciens, Klebsiella pneumoniae*)
1.4.1.19	TryDH	NAD(P)	Plants (*Nicotiana tabacum, Pisum sativum, Spinacia oleracea*)
1.4.1.20	PheDH	NAD	Bacteria (*Sporosarcina ureae, Bacillus sphaericus, Rhodococcus marinas, Thermoactinomyces ntermedius*)
1.4.1.-	AspDH	NADP	Bacteria (*Klebsiella pneumoniae*)

DH, dehydrogenase; NAD(P), NAD and NADP-nonspecific; DAHDH, L-*erythro*-3,5-diaminohexanoate dehydrogenase; DAPDH, 2,4-diaminopentanoate dehydrogenase; DAPMDH, *meso*-2,6-diaminopimelate dehydrogenase; MethylalaDH, *N*-methyl-L-alanine dehydrogenase.

Source: Ohshima and Soda, 2000

1.2 Phenylalanine dehydrogenase

Phenylalanine dehydrogenase (L-phenylalanine: NAD^+ oxidoreductase, deaminating: EC 1.4.1.20) (PheDH) is one of the most interesting 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 1.2 (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).

1.3 Isolation of phenylalanine dehydrogenase

In 1984, NAD^+-dependent PheDH was first discovered in *Brevibacterium* species, isolated from soil by Hummel and coworkers using an enrichment culture technique (Hummel *et al.*, 1984). Consequently, Asano and colleagues screened the enzyme activity among a number of microorganisms from soil samples and culture collections. It was found that the enzyme activity was very narrowly distributed in aerobic spore-forming, gram-positive bacteria, *Sporosarcina ureae* (Asano *et al.*, 1987a), *Bacillus sphaericus* (Asano *et al.*, 1987a) and *Bacillus badius* (Asano *et al.*, 1987b). Later, the enzyme was found in *Rhodococcus* sp. (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 application. Therefore, thermostable enzyme was focused in this field. The thermostable PheDH was studied by Ohshima *et al.* (1991) in *Thermoactinomyces intermedius*. The lastest PheDH was 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 does 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 these enzymes being

involved in microbial sporulation thereby connecting the carbon and nitrogen metabolism of amino acids because of the reversible nature of the enzymes.

Screening is usually carried out for the purpose of finding microorganisms that produce the enzyme effectively as well as search for medium and growth conditions that lead to abundant enzyme production. The addition of L-phenylalanine, as an inducer, to the medium is usually very effective for promoting the enzyme production. Furthermore, enzyme activity can also be induced by other amino acids such as L-histidine, L-tyrosine and L-methionine (Hummel *et al.*, 1987).

1.4 Characterization of phenylalanine dehydrogenase

Previously, 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 1.2. 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, the *T. intermedius* enzyme was hexamer. The *Rhodococcus* sp. M4 enzyme was pointed as tetramer while that of *R. maris* was dimeric enzyme. The *Nocardia* sp. enzyme was reported to be monomer. The enzyme usually has an isoelectric point in the range of 4.0 to 6.0, however, the pI of the enzyme from *B. badius* is pI 3.5. The enzyme showed maximal activity for oxidation deamination at pH between 10.1 and 12.0 whereas the pH optima 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. With regard to equilibrium constant, it was found that 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* enzyme were reported to have K_{eq} of 3.2×10^{-18} M^2 and 1.4×10^{-15}

Table 1.2 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*
Specific activity of final preparation (U/mg protein)	-	-	84	111	68	65	30	86	37	171
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
- 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
Number of subunit	-	4	8	8	8	2	1	6	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
-reductive amination	8.5	9.25	9.0	10.3	9.4	9.8	10.0	9.2	12.0	8.5
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)

Table 1.2 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*
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
- phenylpyruvate	0.177	0.13	0.16	0.4	0.106	0.5	0.06	0.045	0.02	0.18
- NAD^+	0.125	0.27	0.14	0.17	0.15	0.25	0.23	0.078	0.20	0.55
- NADH	0.047	0.13	0.072	0.025	0.21	0.043	-	0.025	0.072	0.09
- ammonia	431	387	85	78	127	70	9.6	106	85	50
Substrate specificity [b]										
Oxidative deamination										
- L-phenylalanine	100	100	100	100	100	100	100	100	100	100
- L-tyrosine	-	12	5	72	9	2	2	0	4	0
- L-tryptophan	-	2	5	1	4	8	8	0	0	2
- L-methionine	-	4	4	3	8	5	5	0	7	5

Table 1.2 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*
- L-valine	-	-	3	1	4	0	0	0	5	2
- L-leucine	-	-	2	1	3	2	-	4	3	3
- L-isoleucine	-	-	1	0.5	0.2	3	-	0	0	1
- L-norvaline	-	-	6	1	5	0	-	-	6	2
- L-norleucine	-	-	15	4	19	16	-	-	16	10
- L-ethionine	-	-	7	3	7	13	-	-	-	4
- L-α-aminobutyrate	-	-	2	-	1	1	-	-	2	-
- 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
- *m*-fluoro-DL-phenylalanine	-	-	-	-	11	8	-	-	-	17
- *o*-fluoro-DL-phenylalanine	-	-	-	-	2	2	-	-	-	3
- D-phenylalanine	-	-	0	0	0	0	-	0	-	0

Table 1.2 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*
Substrate specificity [b]										
Reductive amination										
- phenylpyruvate	100	100	100	100	100	100	100	100	100	100
- *p*-hydroxyphenylpyruvate	96	5	24	136	53	91	28	0	0	-
- indole-β-pyruvate	24	3	1	0	-	5	54	-	-	-
- α-ketovalerate	-	-	9	6	12	0	-	-	-	8
- α-ketocaproate	-	-	32	0	31	9	-	-	-	22
- α-ketoisovalerate	-	-	2	6	13	0	-	6	6	7
- α-ketoisocaproate	-	-	13	8	-	1	240	-	-	9
- α-ketobutyrate	-	-	-	-	3	0	-	1	1	-
- α-keto-γ-methylthiobutyrate	59	33	27	11	16	9	-	14	14	1
- 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)

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*.

1.5 Stereochemistry of hydrogen transfer of coenzyme and kinetic mechanism of phenylalanine dehydrogenase

The stereochemistry of hydrogen transfer of amino acid dehydrogenases showed either pro-*R* or pro-*S* stereospecificity for hydrogen removal from the C-4 position of the nicotinamide moiety of the reduced coenzyme (NAD(P)H) to the keto acid substrates as shown in Figure 1.3. 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 dehydrogenases.

To investigate several possible reactions proceeding via the formation of a ternary complex with sequential or random substrate-binding mechanisms, steady-state kinetic analysis of oxidative deamination and reductive amination was examined. Production inhibition patterns of the oxidative deamination or reductive amination reaction suggest the order of substrate addition and product release (Cleland, 1971). The amino acid dehydrogenases studied so far catalyze the reaction by a sequential ordered mechanism, with the exception of bovine liver glutamate dehydrogenase, which shows a random mechanism (Barton and Fisher, 1971 cited in Misono *et al.*, 1989). The sequences of substrate binding site, however, vary. The kinetic mechanism studies on the PheDH from *R. maris* showed that reductive amination proceeds through the sequential ordered ternary-binary mechanism in which NADH binds first to the enzyme, followed by phenylpyruvate and then

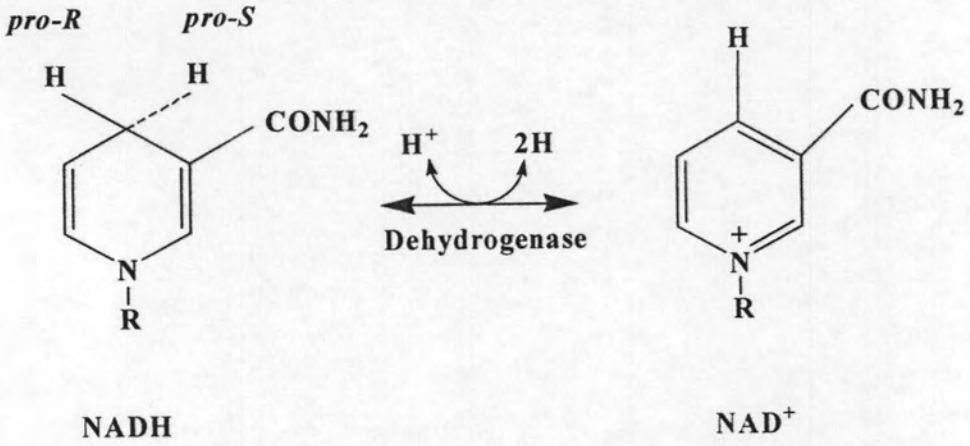

Figure 1.3 Stereospecificity of hydrogen transfer of NADH catalyzed with dehydrogenases

R represents ADP-ribosyl.

Source: Ohshima *et al.*, 1991

ammonia, and the products, L-phenylalanine and NAD^+, are released from the enzyme in that order after dehydrogenation (Misono *et al.*, 1989).

Ohshima *et al.* (1991) studied on initial velocity and product inhibition of the thermophile, *T. intermedius* PheDH. It was known that kinetic mechanism in oxidative deamination also proceeds through a sequential ordered binary-ternary mechanism. In this case, the order of substrate binding is the same as the first one but the order of release was observed to be phenylpyruvate, ammonia and NADH. This conclusion was drawn from both initial velocity and product inhibition experiments. The kinetic mechanisms of PheDHs are summarized in Figure 1.4.

1.6 Cloning of phenylalanine dehydrogenase gene

A number of researchers attempted to clone *phedh* genes not only to study the evolutionary relationship among the $NAD(P)^+$-dependent amino acid dehydrogenase, but also to produce the large amount of enzyme which catalyzes asymmetric synthesis of L-phenylalanine and related amino acids. In 1987, Asano and coworkers recovered putative *phedh* gene fragment (2-9 kbs) from *Eco*RI digested chromosomal DNA from *B. badius* by electroelution, and then ligated the gene fragments with *Eco*RI-digested pBR322. After that, recombinant plasmids were transformed into *E. coli* RR1 (Asano *et al.*, 1987b).

Later on, Okazaki *et al.* (1988) partially digested chromosomal DNA from *B. sphaericus* with *Hin*dIII, ligated it into the *Hin*dIII site of pUC9 plasmid and transformed the recombinant plasmid into *E. coli* JM103. It was reported that *phedh* gene consisted of 1,143-bp open reading frame encoding for 381 amino acids residues.

In 1991, research group of Takada cloned gene encoding thermostable PheDH of a thermophile *T. intermedius* into *E. coli* MV1184, using plasmid pUC18. The *phedh* gene consisted of 1,098 bp and encoded 366 amino acid residues

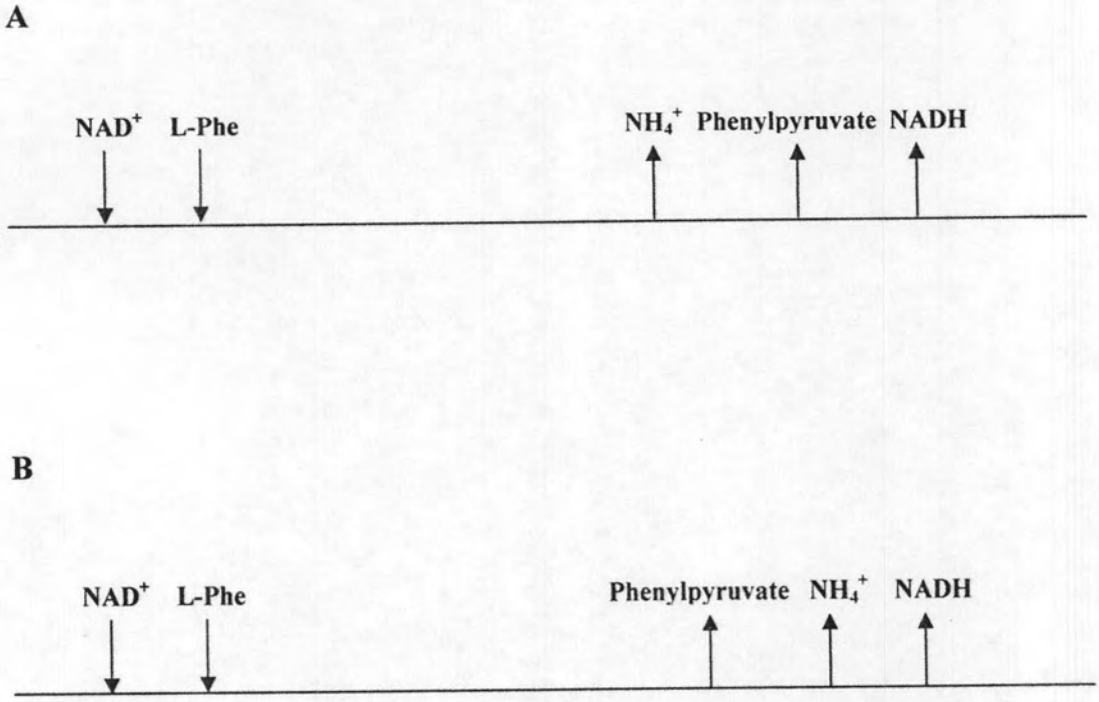

Figure 1.4 Kinetic mechanisms of phenylalanine dehydrogenase

A The *Rhodococcus maris* PheDH

B The *Thermoactinomyces intermedius* PheDH

Source: Misono *et al.*, 1989 and Ohshima *et al.*, 1991

corresponding to the 41,000 kDa subunit of the hexameric enzyme. The expression level of *phedh* gene in *E. coli* MV1184 was very low, about 0.35% of the total soluble protein. To increase the enzyme productivity, the structural gene of *phedh* of *T. intermedius* was amplified by PCR. The amplified 1.1 Kb fragment was ligated with plasmid pKK223-3. The *E. coli* JM109 was used as host cell for transformation. The enzyme, produced by the transformant, corresponded to about 8.3% of the total soluble proteins.

Moreover, *phedh* gene from *B. badius* BC1 was cloned and expressed in *E. coli* JM109, using plasmid vector pUC18. The PheDH activity of recombinant *E. coli* clone was about 60 times higher than that of wild type strain (Chareonpanich, 2001). In 2002, cloning and expression of *phedh* gene from *B. sphaericus* in *E. coli* was performed. The gene was cloned in the vector pET16-b and transformed into *E. coli* BL21(DE3). Expression of *phedh* gene under T7 promoter was over 140 times greater than that of the wild type *B. sphaericus* (Omidinia *et al.*, 2002a). Also, the same research team ligated this gene into the pHY300PLK shuttle vector. The resulting plasmid, pHYDH encoding polypeptide molecular weight of 340 kDa, was then transformed into *B. subtillis* ISW1214 and *E. coli* JM109 competent cells for expression. *B. subtillis* ISW1214/pHYDH exhibited PheDH activity (4,700 units/l) that was over 100 times greater than that of wild type *B. sphaericus* while *E. coli* JM109/pHYDH showed no activity of PheDH (Omidinia *et al.*, 2002b).

Lately, the *phedh* gene from *Acinetobacter lwoffii* was cloned and expressed in *E. coli* BL21(DE3) host cell using expression vector, pET-17b. The specific activities from crude extract of recombinant clones were 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 *A. lwoffii* (Sitthai, 2004).

1.7 Structure and enzyme engineering of phenylalanine dehydrogenase

Extensive developments of the techniques in gene cloning have enabled rapid determination of the primary structures of PheDH. The sequence alignment information was used in attempts to change the substrate specificity of PheDH from L-phenylalanine (aromatic amino acid) to others amino acids such as L-leucine (aliphatic amino acid). From sequence alignment, Kataoka and coworkers (1993) found that 16 amino acid residues of leucine dehydrogenase (LeuDH) were different from the corresponding amino acid residues which were conserved in three PheDHs (Figure 1.5). They speculated that some of these residues involved in substrate recognition of the enzyme, so they prepared a PheDH mutant enzyme from *T. intermedius* whose inherent hexapeptide segment (^{124}F-V-H-A-A-^{129}R) in the substrate-binding domain was replaced by the corresponding part of LeuDH (M-D-I-I-Y-Q) in order to investigate the machanism of substrate recognition by PheDH. The relative activities of mutant enzyme toward aliphatic amino acids such as L-leucine, L-isoleucine, and L-norleucine were higher than those of the wild type enzyme. In contrast, its relative activities toward aromatic amino acids, especially L-tyrosine were lower than those of wild type enzyme. The results suggested that the segment plays a significant role in substrate recognition because this is the largest non-homologous segment in the substrate-binding domain of these two enzymes.

In 1994, the same research group (Kataoka *et al.*) reported the genetic construction of a chimeric enzyme from two functionally related proteins sharing extensive sequence similarity and assessment of its catalytic properties to provide valuable information on the structure-function relationship of the parent protein. An active chimeric enzyme consisting of an N-terminal domain of PheDH containing the substrate-binding region and a C-terminal domain of LeuDH containing the NAD^+ binding region were constructed by genetic engineering and characterized. The catalytic efficiency of the chimeric enzyme on L-phenylalanine was 6% of that of the parental PheDH and showed a broad substrate specificity in the oxidative

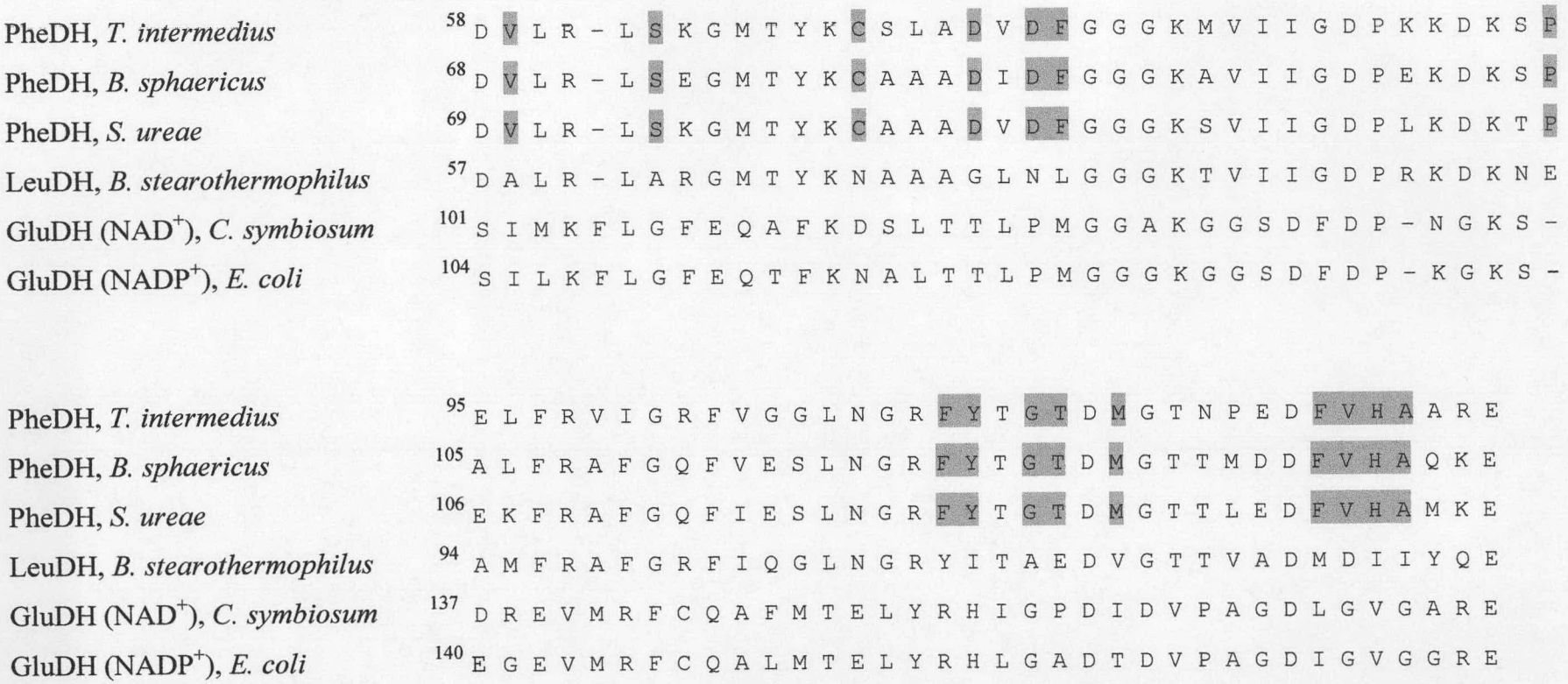

Figure 1.5 Sequence comparison of the conserved regions around the Lys residue in Gly-rich regions of several amino acid dehydrogenases

The residues conserved in three PheDHs but not in LeuDH were indicated by shading.

Source: Kataoka *et al.*, 1993

deamination, like PheDH. However, it acted much more effectively than PheDH on isoleucine and valine. Its K_m values for L-phenylalanine and L-leucine were similar to those of PheDH. The substrate specificity of the chimeric enzyme in the reductive amination was an admixture of those of the two parent enzymes. These results suggested that the two domains of PheDH and LeuDH were probably fold independently. However, the two domains of chimeric enzyme interact and communicate with each other to form new active site and consequenctly show the new substrate specificity (Kataoka *et al.*, 1994).

In 1995, the nucleotide sequence of *phedh* gene encoding for *B. badius* IAM 11059 PheDH was analyzed. The gene consisted of an ORF of 1,140 nucleotides encoding 380 amino acid residues. From amino acid sequence comparison of *B. badius* PheDH with leucine, phenylalanine and glutamate dehydrogenases, the catalytic domain of *B. badius* enzyme appeared to be G-G-(G or S or A)-K-X-(V or G)-X-X-X-(D or N)-(P or L) (Yamada *et al.*, 1995).

Seah *et al.* (1995) undertook site-directed mutagenesis to allow alteration of amino acid residues surrounding substrate–binding pocket of PheDH to alter the size, shape and polarity of the pocket. Glycine-124 and leucine-307 of PheDH from *B. sphaericus* were replaced by the corresponding residues in LeuDH: alanine and valine, respectively. When compared to the wild type enzyme, the mutants showed decreasing activity towards L-phenylalanine and increasing activity towards almost tested aliphatic amino acid substrates. This result implied that the substrate profile of the enzyme varied significantly by the mutations. On this basis, Busca and coworkers (2004) envisaged that engineered PheDH mutants might prove useful as biocatalysts for the asymmetric synthesis of non-natural amino acids, especially phenylalanine analogs which used as precursors for drug synthesis. Therefore, asparagine-145 of PheDH from *B. sphaericus* was replaced by the less polar alanine, leucine and valine. It was found that they could better accommodate substituted aromatic derivatives of phenylpyruvate, such as 2-F-phenylpyruvate and 2-Cl-phenylpyruvate, than wild-type enzyme.

In 1994, Brunhuber and coworkers cloned and sequenced *phedh* gene from *Rhodococcus.* sp. M4. They found that PheDH composes of two domains: the amino-terminal portion contains residues involved in general amino acid binding and catalysis while the carboxyl-terminal portion contains the presumptive dinucleotide-binding domain (Brunhuber *et al.*, 1994). In 1999, Vanhooke *et al.* determined structure, namely the enzyme•NAD^+•phenylpyruvate, and enzyme•NAD^+•*β*-phenylpropionate species, of PheDH from *Rhodococcus* sp. M4 by X-ray crystallographic analyses. 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 with each monomer composed of distinct globular N- and C-terminal domains separated by a deep cleft containing the active site (Figure1.6). The N-terminal domain binds the amino acid substrate and contributes to the interactions at the subunit: subunit interface. 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.

1.8 Application of phenylalanine dehydrogenase

Amino acid dehydrogenases have been used for the stereospecific synthesis of amino acids from chiral substrates, keto acids, and ammonia, as well as for analysis of L-amino acids, keto acids and assay of enzymes of which amino acids and keto acids are their substrates or products. The application of PheDH can be categorized into two major fields: industrial and medical fields.

Protein catalysts, or enzyme, are useful for organic synthesis in view of their high catalytic efficiency, high stereo-selectivity and high product yield. They are also environmentally friendly compare to chemical catalysts. Enzyme-catalyzed reductive amination is potentially useful for the production of optically pure amino acids such as L-phenylalanine, homophenylalanine and allysine ethylene acetal [(*S*)-2-amino-5-(1,3-dioxolan-2-yl)-pentanoic acid].

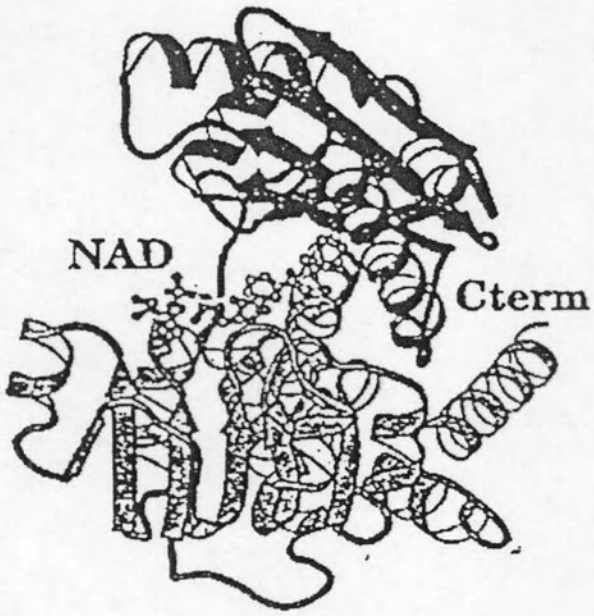

Figure 1.6 Structure of *Rhodococcus* sp. M4 phenylalanine dehydrogenase

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

Source: Vanhooke *et al*., 1999

L-phenylalanine is an essential amino acid for human nutrition. It is used as a main intermediate utilized in the manufacture of a dipeptide artificial sweetener, aspartame (Hummel *et al.*, 1987) and benzaldehyde which can be used as aromatic flavor compound in cheeses (Groot and de Bont, 1998). Several methods have been used for the production of L-phenylalanine such as chemical synthesis, extraction form protein hydrolysates, fermentation or enzymatic method. In addition, fermentation and enzymatic processes in L-phenylalanine production have been proposed and developed in several ways (Choi and Tribe, 1982). These include production from *trans*-cinnamic acid to L-phenylalanine using *Rhodotorula glutinis* containing L-phenylalanine ammoniumlyase activity with 70% conversion yield (Yamada *et al.*, 1981). However, the application of PheDH 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 was carried out by PheDH and formate dehydrogenase (FDH; EC 1.2.1.2) as shown Figure 1.7. Reductive amination of phenylpyruvate by PheDH seems to be another promising way. The simultaneously oxidized NADH is regenerated by formate and FDH and therefore is required in catalytically amounts. The system contains PheDH, FDH and NAD^+, which binds with polyethyleneglycol by covalent bond (PEG-NAD^+), so the hybrid molecules cannot pass through the membrane. The reaction to form PEG-NADH is started by addition of formic acid and FDH. Then phenylpyruvate and ammonium formate are continuously passed through the reactor. The product of L-phenylalanine and carbon dioxide would be released (Hummel *et al.*, 1987).

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 1.3 (Asano and Nakazawa, 1987).

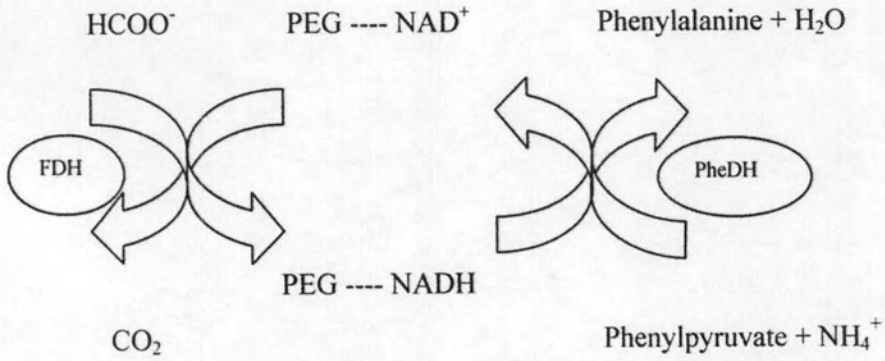

Figure 1.7 Enzymatic synthesis of L-phenylalanine with coenzyme regeneration

PheDH: phenylalanine dehydrogenase

FDH: formate dehydrogenase

PEG: polyethyleneglycol

Source: Hummel *et al.*, 1987

Table 1.3 Synthesis of L-amino acids from keto acids by *S. ureae* PheDH and *C. boidinii* FDH

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

Source: Asano and Nakazawa, 1987

Moreover, allysine ethylene acetal [(*S*)-2-amino-5-(1,3-dioxolan-2-yl)-pentanoic acid], is one of three building blocks used for an alternative synthesis of VANLEV, a vasopeptidase inhibitor which is now in clinical trial, was prepared from the reductive amination of the corresponding keto acid using PheDH from *T. intermedius*. NAD^+ produced during the reaction was recycled to NADH by the oxidation of formate to carbon dioxide using FDH (Hanson *et al.*, 2000).

In 2006, Chen and Engel studied on L307V, engineered PheDH mutant from *B. sphaericus*. The enzyme still had high activity on the oxidation of L-Phe and five non-natural *p*-substituted derivatives (4-F/Cl/CH_3/OCH_3/NO_2-L-Phe) in water-miscible organic solvents methanol, ethanol and acetronitrile. Besides, they reported on a practical application of L307V PheDH with baker's yeast alcohol dehydrogenase (ADH) for coenzyme recycling to synthesize a non-natural amino acid, *p*-OCH_3-L-phe. This illustrates the utility of the versatile biocatalyst for potential industrial uses.

Wild type PheDH from *B. sphaericus*, and three mutants N145A, N145V and N145L, were used with a coenzyme recycling system to synthesize L-phenylalanine and three non-natural amino acids (*p*-F-phenylalanine, *p*-MeO-phenylalanine and *p*-CF_3-phenylalanine). In these studies, stoichiometric consumption of costly NAD^+/NADH in dehydrogenase-catalysed syntheses was frequently avoided by recycling with ADH which offers advantages with non-natural substrates, whose insolubility can be a problem (Paradisi *et al.*, 2007).

Phenylketonuria (PKU) is a group of inborn metabolism errors in which the conversion of L-phenylalanine to L-tyrosine is impaired. This disease is due to an autosomal recessive inheritance that codes for a type of phenylylanine hydroxylase (EC 1.14.16.1) with reduced enzymatic activity, resulting in high levels of L-Phe in inborn fluid and phenylpyruvate in urine. Early quantitative determination of plasma L-Phe is essential for the diagnosis of PKU and control of dietary therapy of PKU patients. Neonatal mass-screening for PKU has been established in most countries. The Guthrie method utilizing a bacteria inhibtion assay is the universal diagnostic; however it requires empirical observation and a long incubation time and it is

sometimes difficult to record accurate diagnosic results. Furthermore, fluoromatric, spectrophotomatric, enzymatic, and chromatographic methods have been described for newborn screening diseases. Although several methods have been reported for the quantitative determination of L-phenylalanine in physiological fluids such as spectrofluorometric methods or by column chromatography using amino acid analyzers, they are not routinely applied since spectrofluorometric method requires deproteinization of samples, a large sample size (>1 ml blood) and also lack specificity while the use of amino acid analysis or high-performance liquid chromatography requires highly sophisticated instrumentation and deproteinization of samples. Enzymatic assay is particularly suitable method for clinical routine because this method has many advantages such as rapid, simple as well as specific, and requires only a drop of blood for the simultaneous determination of L-phenylalanine. This method couples simultaneously the reaction of an NAD(H)-dependent PheDH with an intermediate electron acceptor system as shown in Figure 1.8 (Wendel *et al.*, 1989 and Schulze *et al.*, 2002).

Although this enzyme constitutes a valuable analytical tool for the colorimetric screening of PKU, and has been also proposed as biocatalyst for the enantioselective synthesis of phenylalanine and related L-amino acids from their keto analogs, these applications could be limited by the lower thermal stability showed by this enzyme. In fact, PheDH was rapidly inactivated at high temperature through a mechanism in which protein aggregation processes were involved, so in 2006 Villalonga and coworkers reported that the polysaccharide *O*-carboxymethyl poly-β-cyclodextrin was employed as glycosidation for the *B. sphaericus* PheDH. The neoglycoenzyme retained 63% of its initial activity. The optimum temperature for the enzyme was increased by 15°C and its thermostability was improved by about 6°C over 10 minutes incubation (Villalonga *et al.*, 2006a). Moreover, two different monoactivated β-cyclodextrin (β-CD) derivatives, named mono-6-amino-6-deoxy-β-CD (CD1) and mono-6-amino-(-5-carboxypetane)-6-deoxy-β-CD (CD2) were evaluated as modifying agents for the same PheDH. The enzyme glycosidated with CD1 and CD2 retained 60% and 80% of the initial activity, respectively. The optimum temperature for catalytic activity of PheDH was increased in 10°C after

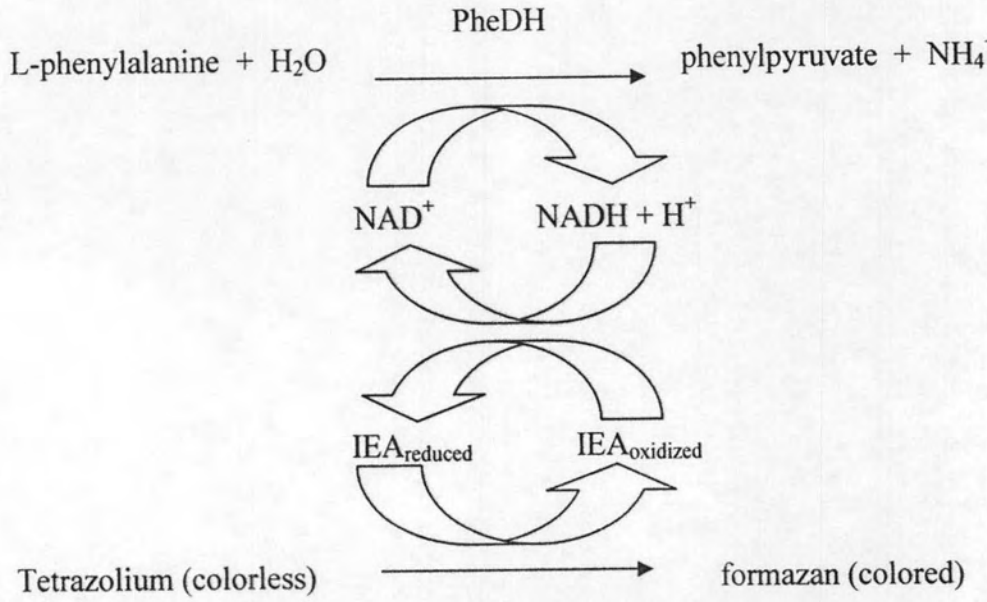

Figure 1.8 Reaction of the enzymatic phenylalanine determination

L-PheDH: L-phenylalanine dehydrogenase

IEA: intermediate electron acceptor

Source: Schulze *et al.*, 2002

attaching the CDs residues. The enzyme thermostability profile was improved, and its resistance to thermal inactivation at different temperature ranging from 40°C to 60°C was noticeably increased after glycosidation (Villalonga *et al.*, 2006b).

1.9 Objectives of this research

Our research group screened for thermotolerant PheDH-producing bacteria from soil samples collected from various areas in Thailand and Japan. Among them, an isolate giving a high activity of PheDH was selected and further identified as *Bacillus lentus*.

Recently, Inkure (2005) investigated the optimal condition for PheDH from *Bacillus lentus*. Later on, the enzyme was purified as well as characterized its properties and kinetic mechanism. The results were shown as the follow.

The optimum condition for the PheDH production from *Bacillus lentus* was 18 hours of cultivation in 1% peptone medium, pH 7.0 supplemented with 0.4% L-phenylalanine at 37°C. The enzyme was purified to homogeneity by DEAE-Toyopearl, Butyl-Toyopearl and Sephadex G-200 column chromatography with 18.4% yield and 166.3 purification fold. The enzyme had a molecular mass of about 340,000 Da and consisted of 8 identical subunits. The enzyme showed high substrate specificity in the oxidative deamination on L-phenylalanine and the reductive amination on phenylpyruvate. The NAD^+ analog 3-acetylpyridine-NAD^+, gave 1.74 times higher activity than its natural coenzyme, NAD^+. The optimum pHs for the oxidative deamination and the reductive amination were 10.4 and 8.5, respectively and optimum temperatures were 50°C and 55°C, respectively. The enzyme was stable over a broad pH range of 6.0 and 12.0. No loss of the enzyme activity was observed upon incubation at 50°C for 4 hours. The enzyme retained 50% of the activity after incubation at the same temperature for 3 days. The enzyme activity was inactivated completely by $AgNO_3$ at a final concentration of 1 mM. D-Phenylalanine, D-methionine, D-leucine, D-tryptophan, hydrocinnamate and *p*-hydroxyphenylacetate inhibited the oxidative deamination of L-phenylalanine.

The apparent K_m values for L-phenylalanine, NAD^+, phenylpyruvate, NH_4Cl and NADH were 0.59, 0.55, 0.18, 50 and 0.09 mM, respectively. Initial-velocity and product inhibition studies showed that the reductive amination proceeded through a sequential ordered ternary-binary mechanism. NADH bound first to the enzyme, followed by phenylpyruvate and then NH_4Cl, and the products were released in the order of L-phenylalanine and NAD^+.

From these properties, the enzyme is of interest to use in the industrial synthesis of various amino acids from their corresponding keto acids by reductive amination, because its noteworthy property is a high thermostabillity. The low yield of enzyme in the wild-type strain triggered us to use recombinant DNA technology to obtain a sufficient amount of the PheDH.

The objective of this thesis

1. Nucleotide sequencing of *phedh* gene from *Bacillus lentus*
2. Cloning of *phedh* gene into *Escherichia coli* BL21(DE3) using the expression vector, pET-17b
3. Optimization of *phedh* gene expression