CHAPTER I

INTRODUCTION

Amino acids are known as biomolecules typically found in all organisms. They can be classified into two groups by ability to rotate the plane of polarized light: L-formed and D-formed amino acids. The first one plays important role in all life while the later is rarely found in organism. L-amino acids serve as building block of enzyme, hormone, antibody and protein. Furthermore, its can also balance buffering capacity in blood (Holum, 1982) and often function as chemical messengers in cell communication (Bender, 1975).

Recently, L-amino acids are widely used in various compounds synthesis by industry. For example, L-glutamic acid, L-lysine and L-leucine are used as food and feed additives while 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 starting material for an artificial sweetener, aspartame (L-aspartate-L-phenylalanine-1-methyl ester, or NutraSweet) (Ohshima and Soda, 1989).

The increasing demand for L-amino acids has stimulated the research on various methods for its synthesis such as chemical reaction, extraction from protein hydrolysates and fermentative (Hummel *et al.*, 1987), however, the products contain equal amount of D-formed and L-formed amino acid (Takai *et al.*, 1992). Therefore, some researchers have attempted to produce L-amino acid by enzymatic method e.g. L-amino acid transaminase, amino acid racemase (Berberich *et al.*, 1968), L-aminopeptidase (Kamphuis *et al.*, 1992), and L-amino acid-β-decarboxylase (Yamamoto *et al.*, 1980). For L-amino acid dehydrogenases, they are also in broad range of study. These enzymes, prepared from microorganism, are widely used for synthesis and measurement of L-amino acid in the samples (Ohshima and Soda, 1979).

There are several enzymes in amino acid dehydrogenase family (EC 1.4.1.-) which are part of the oxidoreductase superfamily as shown in Table 1.1. They catalyze the removal of the amino group, generally from L-amino acid, with the formation of

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 (cylizing)	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 intermedius*)
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

the corresponding keto acid in the presence of pyridine nucleotide coenzymes, $NAD(P)^+$. The general equation for this reaction can be illustrated as shown in Figure 1.1 (Brunhuber and Blanchard, 1994). These enzymes are found generally in an extensive number of diverse prokaryotic and eukaryotic organisms. They are known as important enzymes, which provide a route for interconversion of inorganic nitrogen with organic nitrogen. In other words, they serve as a connecting link between amino acid and organic acid metabolism. Their metabolic function can be described as the balance of both amino acid and keto acid synthesis. The amino group is firstly removed as free ammonia before the carbon skeleton of an amino acid can be metabolized for energy through glycolysis and/or TCA cycle. The participation of $NAD(P)^+$ makes these enzyme systems a valuable tool for the analysis of L-amino acids or their corresponding keto acids. By reductive amination of the keto acid, L-amino acid can be obtained in a lot of yield because the equilibrium of the reaction favors amino acid formation.

At the beginning of the 1980s, a wide screening of aromatic amino acid dehydrogenases led to the discovery of phenylalanine dehydrogenase (EC 1.4.1.20) in *Brevibacterium* species (Hummel *et al.*, 1984). The enzyme catalyzes the reversible pyridine nucleotide-dependent oxidative deamination of L-phenylalanine to form ammonia, phenylpyruvate, and NADH as shown in Figure 1.2. The distribution of this enzyme is limited to some groups of gram-positive, spore-forming bacteria including actinomycetes. This may due to involvement of this enzyme in microbial sporulation thereby connecting the carbon and nitrogen metabolism of amino acids (Asano *et al.*, 1998). In some cases, addition of L-phenylalanine to the medium can induce enzyme activity. Moreover, it can also be induced by other amino acids, such as D-phenylalanine and L-histidine (Hummel *et al.*, 1984 and 1987). Thus, this enzyme is speculated to play an important role in the degradation of L-phenylalanine (Hummel *et al.*, 1984 and Asano *et al.*, 1987).

In 1985, Asano and Nakazawa screened the enzyme activity in cell-free extracts of various soil microorganisms and found that *Sporosarcina ureae* showed high NAD^+-dependent phenylalanine dehydrogenase activity. Moreover, they also found enzyme activity in *Bacillus sphaericus*. The enzyme from *Sporosarcina ureae* had a molecular weight of 305,000, while that of *Bacillus sphaericus* had a molecular weight of 340,000. Each enzyme was composed of eight subunits identical in molecular weight.

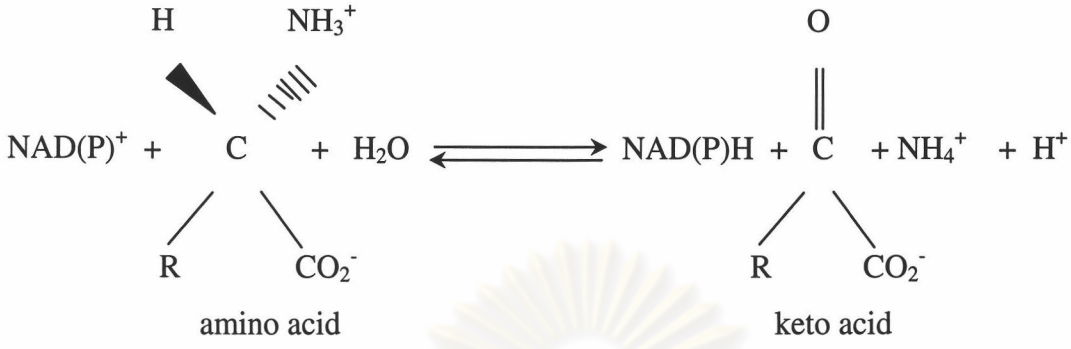


Figure 1.1 The general reaction of L-amino acid dehydrogenases

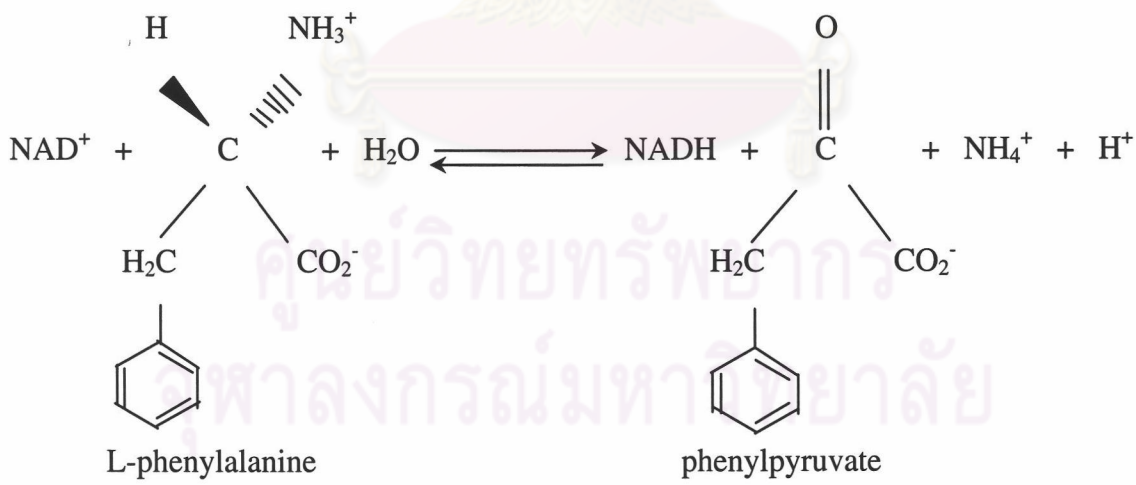


Figure 1.2 The reaction of L-phenylalanine dehydrogenase

The *Sporosarcina ureae* enzyme showed high substrate specificity in the oxidative deamination acting on L-phenylalanine, while that of *Bacillus sphaericus* acted on L-phenylalanine and L-tyrosine. Both of them had lower substrate specificity in the reductive amination acting on α-keto acids such as phenylpyruvate, *p*-hydroxyphenylpyruvate, and α-keto-γ-methylthiobutyrate. Two years later, Asano and Nakazawa found that *Bacillus badius* exhibited higher specific phenylalanine dehydrogenase activity than those of the previously reported strains. In their report, the enzyme had a molecular weight of 310,000-360,000 and composed of identical subunits with a molecular weight of 41,000-42,000. The substrate specificity of the enzyme in the oxidative deamination was high for L-phenylalanine but it was rather low in the reductive amination with phenylpyruvate, *p*-hydroxyphenylpyruvate, and 2-oxohexanoate.

Previous studies established that the enzymes from these bacteria composed of eight identical subunits with molecular weight of 39,000 to 42,000. In 1989, during the course of a study on microbial degradation of L-phenylalanine, Misono *et al.* found a dimeric NAD^+-dependent phenylalanine dehydrogenase in a soil bacterium. It was identified as *Rhodococcus maris* K-18 and its enzyme was purified to homogeneity in order to compare its properties with those of the octameric enzymes. The enzyme had a molecular weight about 70,000 and consisted of 2 identical subunits. It also catalyzed the oxidative deamination of L-phenylalanine including several other L-amino acids and the reductive amination of phenylpyruvate and *p*-hydroxyphenylpyruvate. The enzyme required NAD^+ as a natural coenzyme. 3-Acetylpyridine-NAD^+, the NAD^+ analog, showed much greater coenzyme activity than NAD^+. D-Phenylalanine, D-tyrosine, and phenylethylamine inhibited the oxidative deamination of L-phenylalanine. Moreover, the enzyme reaction was inhibited by *p*-chloromercuribenzoate and $HgCl_2$. Initial-velocity and product inhibition studies showed that the reductive amination proceeded through a sequential ordered ternary-binary mechanism. NADH bound first to enzyme, followed by phenylpyruvate and then ammonia, and the products were released in the order L-phenylalanine and NAD^+.

The phenylalanine dehydrogenases have been purified and characterizd from some mesophilic bacteria. However, they are not stable enough for industrial and clinical application. In 1991, Ohshima *et al.* purified and characterized the enzyme from *Thermoactinomyces intermedius* and found that this enzyme was thermostable, not

inactivated on incubation at 70°C, pH 7.2 for 60 min. The enzyme consisted of six subunits identical in molecular weight (41,000). The enzyme preferably acts on L-phenylalanine and its keto analog, phenylpyruvate, in the presence of NAD and NADH, respectively. Initial velocity and product inhibition studies showed that the oxidative deamination proceeded through a sequential ordered binary-ternary mechanism.

Bacteria that produced NAD^+-dependent phenylalanine dehydrogenase were also screened among L-methionine utilizes isolated from soil by Asano and Tanetani in 1998. A bacterial strain showing phenylalanine denydrogenase activity was chosen and classified in the genus *Microbacterium*. Its enzyme is composed of eight identical subunits with a molecular weight of approximately 41,000. No loss of enzyme activity was observed upon incubation at 55°C for 10 min. In addition, phenylalanine dehydrogenases from *Rhodococcus* sp. M4 and *Nocardia* sp. 239 were characterized. The results indicated that phenylalanine dehydrogenase isolated from *Rhodococcus* sp. M4 was shown to be a tetramer of molecular weight 39,500 (Hummel *et al.*, 1987) while the enzyme from *Nocardia* sp. 239 was active as a monomer of molecular weight 42,000 (de Boer *et al.*, 1989, cited in Pasquo *et al.*, 1998). Comparison of properties of published various microbial phenylalanine dehydrogenase as shown in Table 2.

In a study of the stereoselectivity on hydride transfer from the C-4 of nicotinamide ring, Asano *et al.* and Ohshima *et al.* determined for the *B. sphaericus* and *Thermoactinomyces* enzymes in 1987 and 1991, respectively. In both cases, the *pro*-S hydrogen of NADH was transferred to generate [2-^{2}H]-L-phenylalanine. The result suggested that the phenylalanine dehydrogenase was in the majority group of amino acid dehydrogenases.

More recently, Vanhooke *et al.* presented the first model for amino acid dehydrogenase in a ternary complex, namely the enzyme•NAD^+•phenylpyruvate, and enzyme•NAD^+•β-phenylpropionate species, by X-ray crystallographic analyses. Both structures showed that *Rhodococcus* L-phenylalanine dehydrogenase was a homodimeric enzyme with each monomer composed of distinct globular N- and C-terminal domains separated by a deep cleft containing the active site. The N-terminal domain binds the amino acid substrate and contributes to the interactions at the subunit: subunit interface. The C-terminal domain contains a typical Rossmann fold and orients the dinucleotide (Vanhooke *et al.*, 1999). In addition, Brunhuber *et al.* found that

Table 1.2 Comparison of properties of microbial phenylalanine dehydrogenases

Properties	Sources			
	B. sphaericus	*S. ureae*	*B. badius*	*R. maris*
Native M_r (subunit M_r), kDa	340 (41 x 8)	310 (41 x 8)	335 (41 x 8)	70 (36 x 2)
Specific activity of final preparation (U/mg protein):	111	84	68	65
Optimum pH:				
Deamination	11.3	10.5	10.4	10.8
Amination	10.3	9.0	9.4	9.8
Thermostability (°C)	55	<40	<55	35
K_m (mM)				
NAD	0.17	0.14	0.15	0.25
L-Phe	0.22	0.096	0.088	3.80
NADH	0.025	0.072	0.21	0.043
Phenylpyruvate	0.40	0.16	0.106	0.50
Ammonia	78	85	127	70

	Nocardia sp.	*Thermoactinomyces intermedius*	*Microbacterium* sp.
Native M_r (subunit M_r), kDa	42 (42 x 1)	270 (41 x 6)	330 (41 x 8)
Specific activity of final preparation (U/mg protein):	30	86	37
Optimum pH:			
Deamination	10	11	12
Amination	-	9.2	12
Thermostability (°C)	<53	70	55
K_m (mM)			
NAD	0.23	0.07	0.20
L-Phe	0.75	0.22	0.10
NADH	-	0.025	0.07
Phenylpyruvate	0.06	0.045	0.30
Ammonia	96	106	85

Source: Ohshima and Soda, 2000

the kinetic mechanism of this enzyme is ordered as NAD^+ binding prior to phenylalanine and the products were released in the order of ammonia, phenylpyruvate, and NADH in a similar manner as previously reported. The enzyme shows no activity with NADH analogues. Interestingly, initial structural analyses of the enzyme•NAD^+•phenylpyruvate and enzyme•NAD^+•β-phenylpropionate complexes established that Lys78 and Asp118 function as the catalytic residues in the active site (Brunhuber *et al.*, 2000).

For cloning, there are few reports about phenylalanine dehydrogenase available. The genes encoding the enzymes were cloned and sequenced from *Bacillus badius* (Asano *et al.*, 1987 and Yamada *et al.*, 1995), *Bacillus sphaericus* (Okazaki *et al.*, 1988), *Sporosarcina ureae* (Hibino *et al.*, 1990), *Thermoactinomyces intermedius* (Takada *et al.*, 1991), and *Rhodococcus* sp. M4 (Brunhuber *et al.*, 1994). The papers showed that all of phenylalanine dehydrogenase enzymes were composed of two domains: the N-terminal domain responsible for amino acid binding and C-terminal domain responsible for nucleotide binding. The results of conserved residues in the amino acid substrate binding domain of phenylalanine, leucine, glutamate, and alanine dehydrogenases suggested that the N-terminal region may be involved in general amino acid binding, and in amino acid discrimination (Brunhuber *et al.*, 1994).

Although the phenylalanine dehydrogenase from various sources have been cloned and sequenced by the conventional method, they were consumed a lot of time and difficult to obtained the target gene. The polymerase chain reaction (PCR) is a powerful technique that has been developed recently in the area of recombinant DNA research and is having an impact on many areas of molecular cloning and genetics. For cloning and sequencing of amino acid dehydrogenase gene, PCR technique has been also applied. Since only two short peptide sequences of the enzyme are required for design the initial primers. It makes more convenience and takes less time when compared with genomic library cloning method. That is, with this technique, a target sequence of DNA can be amplified a billion fold in few hours.

Due to the selectivity and specificity of the PCR rest on two primers specific for the gene, the requirement of two specific primers itself restricts the application of conventional PCR to amplify sequences that lie outside the boundary of known sequences. A modification of PCR, which is known as "inverse PCR", has been invented to amplify an unknown sequence which juxtaposes a known region (Ochman *et al.*,

1988). However, this technique rests on rather unreliable self-circularization reaction, which often results in concatemerization (Rosenthal and Jones, 1990).

In 1990, Rosenthal and Jones described a modification of PCR that allows walking and sequencing in any direction along genomic DNA starting from a known sequence. This method, referred as "cassette-ligation mediated polymerase chain reaction", comprises the following three steps: (i) digestion of target DNA with multiple restriction enzymes, (ii) ligation of cleavage products to double-stranded DNA cassettes possessing a corresponding restriction site and (iii) amplification of cassette-ligated restriction fragments containing a short known sequence by PCR using the specific primer which anneals to the known sequence of the DNA (Primer S) and the cassette primer which anneals to one strand of the cassette (Primer C) (Figure 1.3). Walking along the DNA can be taken by repeating the cassette-ligation mediated PCR using a new primer synthesized according to the sequence information from the cassette primer. Since the direct PCR sequencing method can determine only up to 300 bp from the ends, smaller DNA fragments usually need to be prepared from a larger DNA fragment by repeating cassette-ligation mediated PCR exploiting different restriction sites and different sets of cassette primers (Rosenthal and Jones, 1990). Hence, PCR cloning and nucleotide sequencing by using these method are interesting for this research.

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, ammonia, and assay of enzymes of which amino acids and keto acids are their substrates or products. Phenylalanine dehydrogenase is used for the continuous production of L-phenylalanine, which is important as a starting material for an artificial sweetener, aspartame and also important for the synthesis of its related amino acids from the corresponding keto analogs and ammonia (Asano, 1987 and Hummel, 1987).

Several enzymatic processes of L-phenylalanine synthesis have been reported previously: L-specific hydrolysis of benzylhydantoin (Yokozeki *et al.*, 1987), amination of *trans*-cinnamic acid (Yamada *et al.*, 1981), transamination from an amino donor to phenylpyruvate (Wood and Calton, 1985 cited in Schmidt et al., 1987), and two-step conversion starting from acetamidocinnamic acid via phenylpyruvate

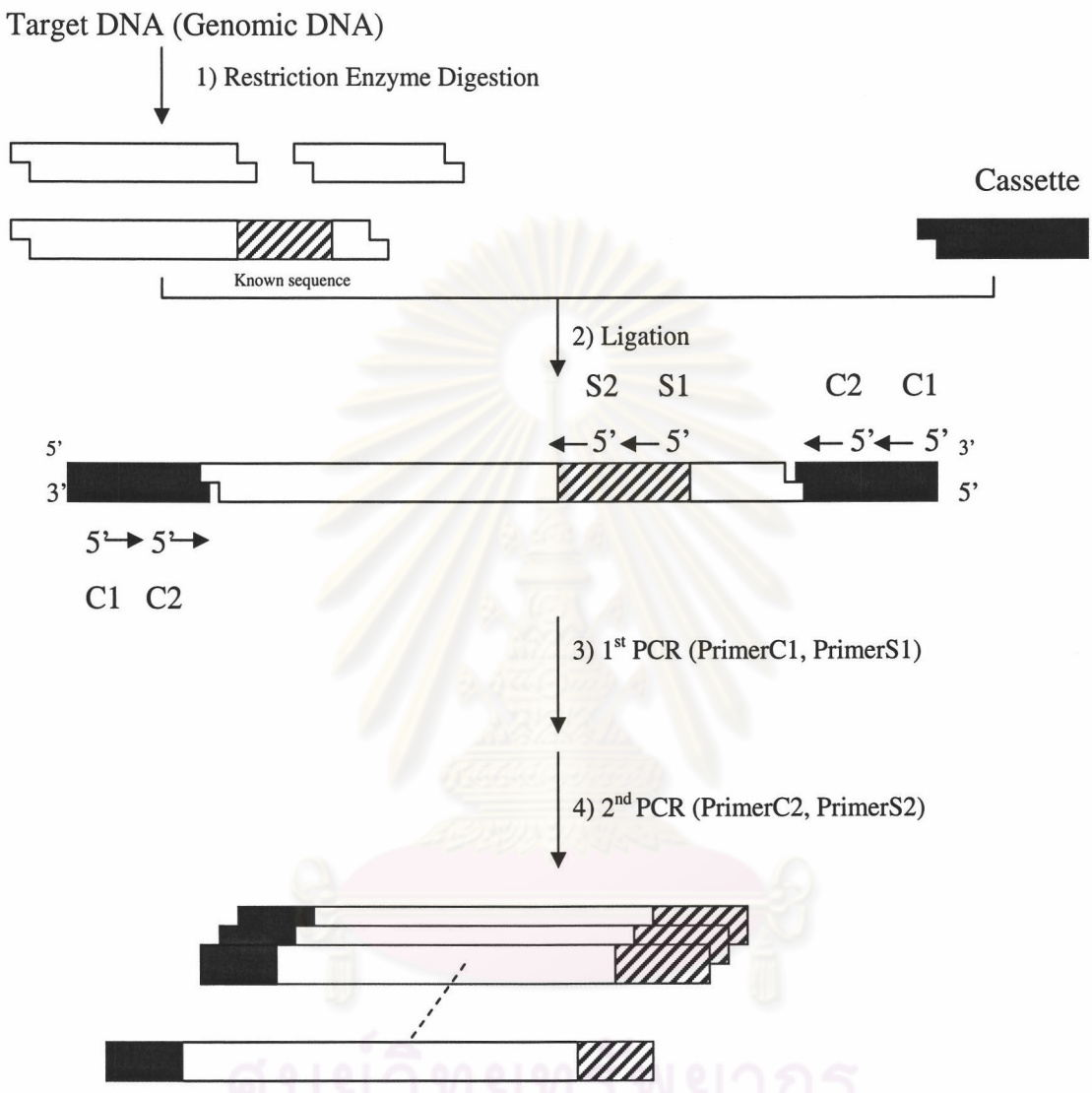


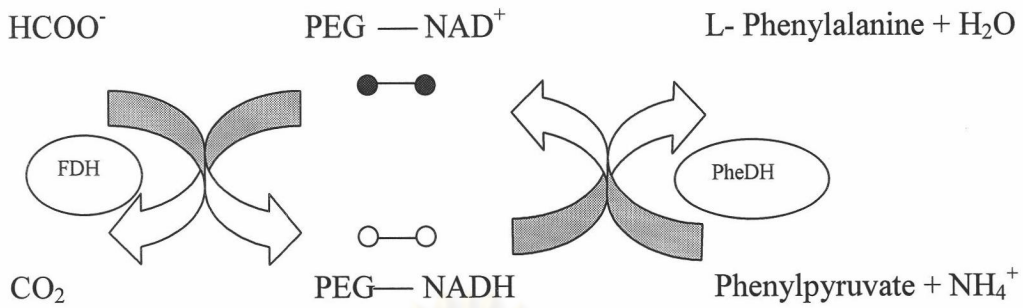
Figure 1.3 Strategy of cassette-ligation mediated polymerase chain reaction.

(Nakamichi *et al.*, 1984). However, these methods are not practicable in a large-scale production. Reductive amination of phenylpyruvate by the action of phenylalanine dehydrogenase seems to be another promising way. Since this reaction requires just only the coenzyme, NADH, which was regenerated by a second enzyme, formate dehydrogenase (EC 1.2.1.2) (Wichmann *et al.*, 1981). The modified coenzyme is retained behind an ultrafiltration membrane as shown in Figure 1.4. The system contains L-phenylalanine dehydrogenase, formate dehydrogenase 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 was started by addition of formic acid and formate dehydrogenase. Then passing phenylpyruvate and ammonium formate (NH_4COOH) continues the reaction through reactor and the products, L-phenylalanine and carbon dioxide, were released. In addition, optically pure three-substituted pyruvates with bulky substituents, such as S-2-amino-4-phenylbutyrate and S-2-amino-5-phenylvalerate, were synthesized from their keto analogs in a similar way (Asano *et al.*, 1990) (Table 1.3 and Figure 1.5).

The synthesis of various D-amino acids by a multienzyme system has been developed. In this system, D-amino acids are produced from the corresponding keto acids and ammonia by coupling four enzyme reactions catalyzed by D-amino acid aminotransferase, phenylalanine racemase, phenylalanine dehydrogenase, and formate dehydrogenase as shown in Figure 1.6. This is based on the high substrate specificity of phenylalanine racemase and the strict enantioselectivity and low structural specificity for the substrates of D-amino acid aminotransferase. D-phenylalanine and NADH are regenerated with phenylalanine dehydrogenase and formate dehydrogenase, respectively (Galkin *et al.*, 1997a, 1997b).

Recently, Hanson *et al.* found that allysine ethylene acetal [(S)-2-amino-5-(1,3-dioxolan-2-yl)-pentanoic acid], that is one of three building blocks used for an alternative synthesis of VANLEV, a vasopeptidase inhibitor (Figure 1.7), was prepared from the corresponding keto acid reductive amination using phenylalanine dehydrogenase from *Thermoactinomyces intermedius*. The reaction requires ammonia and NADH as shown in Figure 1.8. NAD produced during the reaction was recycled to NADH by the oxidation of formate to carbon dioxide using formate dehydrogenase (Hanson *et al.*, 2000).

a)



b)

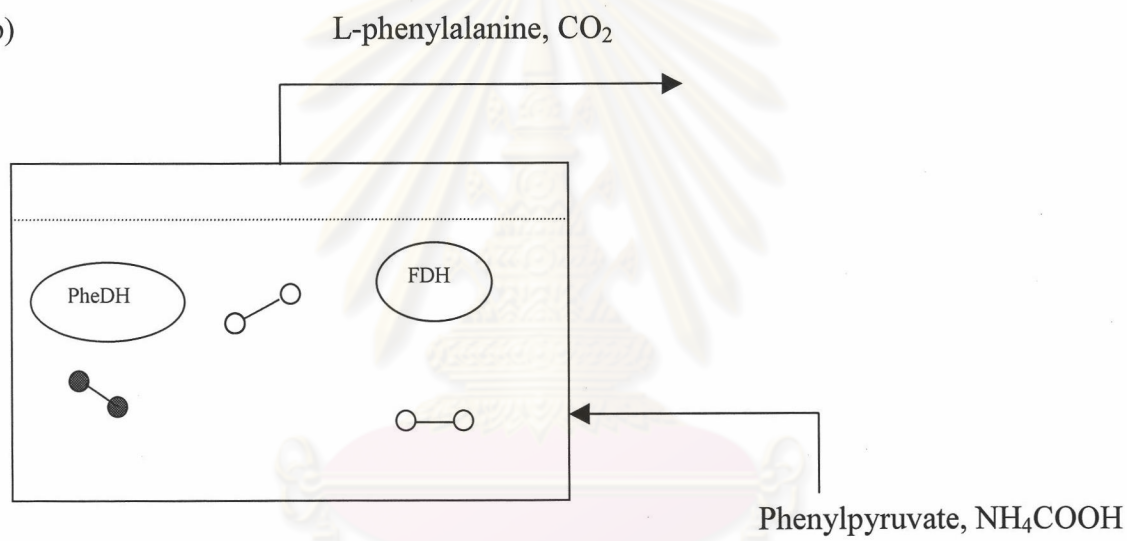


Figure 1.4 Enzymatic synthesis of L-phenylalanine with coenzyme regeneration.
(a) Reaction scheme, (b) Reactor scheme
PheDH: phenylalanine dehydrogenase
FDH : formate dehydrogenase
PEG : polyethyleneglycol

Source: Hummel *et al.*, 1987

Table 1.3 Synthesis of (S)-amino acids from 2-keto acids by using phenylalanine dehydrogenase and formate dehydrogenase

Substrate	Product	Yield, %
Phenylpyruvate	(S)-phenylalanine	>99
4-(hydroxyphenyl)-pyruvate	(S)-tyrosine	>99
4-(fluorophenyl)-pyruvate	(S)-(4-fluorophenyl)alanine	>99
2-oxo-4-phenylbutyrate	(S)-2-amino-4-phenylbutyric acid	99
2-oxo-5-phenylvalerate	(S)-2-amino-5-phenylvaleric acid	98
2-oxo-3-methyl-3-phenylpropionate	(S)-2-amino-3(RS)-methyl-3-phenylpropionate	98
2-oxononanoate	(S)-2-aminononanoic acid	99

Source: Asano *et al.*, 1990

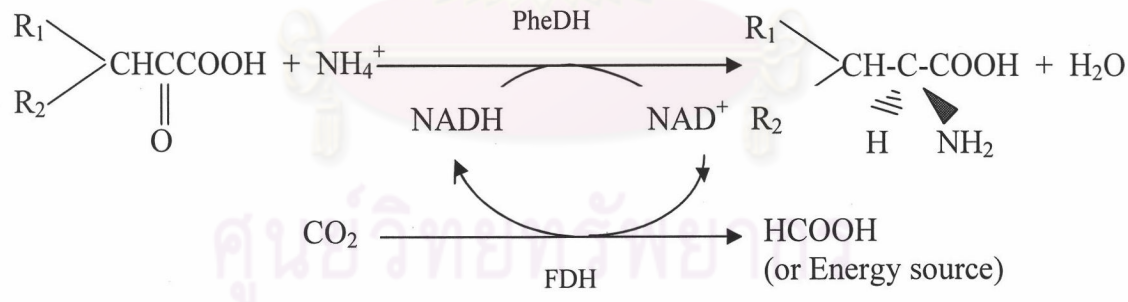


Figure 1.5 Synthesis of (S)-amino acid from their 2-keto analogues by phenylalanine dehydrogenase (PheDH) with a regeneration of NADH by formate dehydrogenase (FDH).

Source: Asano *et al.*, 1990

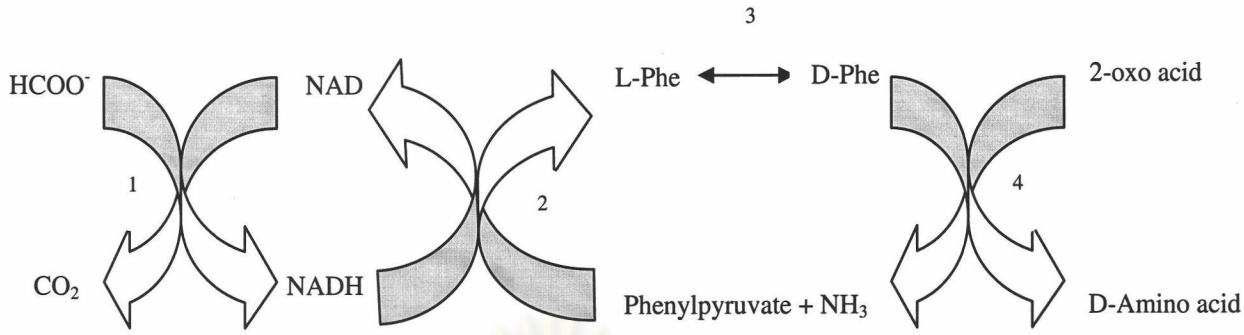


Figure 1.6 Enzymatic synthesis of D-amino acids by a multienzyme system consisting of the coupling reaction of four enzymes.

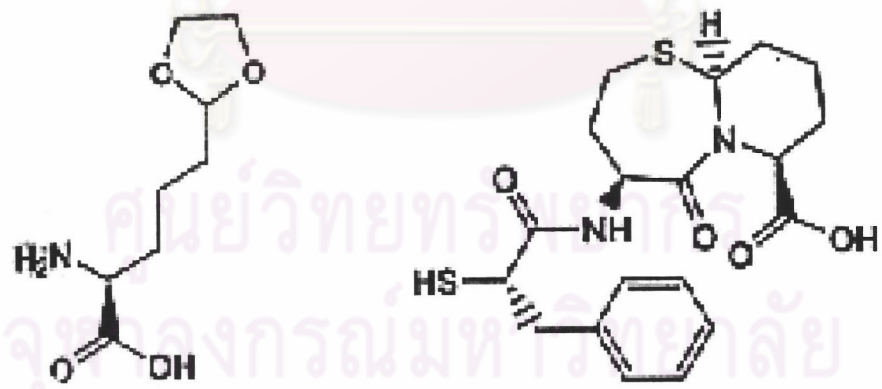
1 : Formate dehydrogenase

2 : Phenylalanine dehydrogenase

3 : Phenylalanine racemase

4 : D-Amino acid aminotransferase

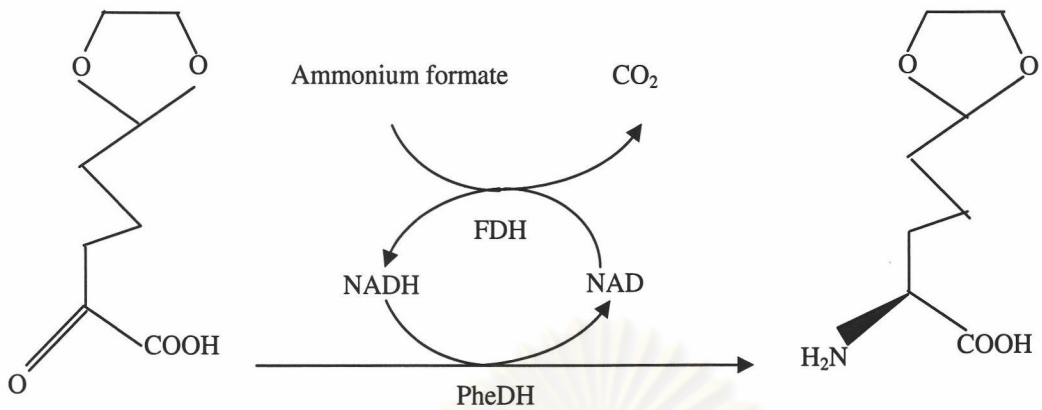
Source: Galkin *et al.*, 1997 a



allysine ethylene acetal VANLEV

Figure 1.7 Structures of allysine ethylene acetal and VANLEV

Source: Hanson *et al.*, 2000



keto acid acetal acetal amino acid

Figure 1.8 Scheme for conversion of the corresponding keto acid acetal to acetal amino acid. Phenylalanine dehydrogenase (PheDH) catalyzes reductive amination of the keto acid. Formate dehydrogenase (FDH) is used for regeneration of NADH.

Source: Hanson *et al.*, 2000

$$\text{L-phenylalanine} + NAD^+ + H_2O \xleftarrow{\text{PheDH}} \text{phenylpyruvate} + NADH + NH_4^+$$

$$NADH + \text{INT} + H^+ \xrightarrow{\text{diaphorase}} NAD^+ + \text{formazan}$$

Figure 1.9 The detection system of L-phenylalanine
PheDH: phenylalanine dehydrogenase
INT: iodonitro tetrazolium chloride

Source: Wendel *et al.*, 1989

For clinical analysis, phenylalanine dehydrogenase is therefore applicable to diagnosis of phenylketonuria, which are transmitted by an autosomal recessive gene. This deficiency is a result from impaired activity of phenylalanine hydroxylase (EC 1.14.16.1), the enzyme catalyzing conversion of the essential amino acid phenylalanine to tyrosine in liver. It causes an excess of phenylalanine accumulated in the blood and spinal fluid (Guthrie and Susi, 1963).

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 require 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 phenylalanine dehydrogenase by using a second reaction in which initially formed NADH and diaphorase convert INT to a formazan. This product is measured in the visible range at 492 nm. The catalyzed reactions are shown in Figure 1.9 (Wendel *et al.*, 1989).

Moreover, Nakamura *et al.* found that the recycling assays involving the coupling of transaminases and dehydrogenases can be applicable to detect other amino acids that might be useful in the screening of human blood for abnormally high levels of these amino acids (Nakamura *et al.*, 1996). In addition, assay of phenylalanine dehydrogenase is also useful to the monitoring of the level of cells disrupted by shock wave since destruction of the spheroplast of recombinant cells is monitored sensitively by measuring phenylalanine dehydrogenase activity leaked from the cells (Teshima *et al.*, 1995 cited in Ohshima *et al.*, 2000).

Thermotolerant bacteria, isolated from 20 and 22 soil samples collected from temperate and hot spring areas, were screened in medium containing 1% of each amino acid. They were further screened for various amino acid dehydrogenase activities using formazan forming method. The results showed that strain BC1 was one of bacteria, which

showed high activity of phenylalanine dehydrogenase (Suriyapanpong *et al.*, 2000). Furthermore, enzyme activity can be induced by L-phenylalanine and phenylalanine analogs such as tyrosine and tryptophan. Subsequently, Leksakorn purified and characterized phenylalanine dehydrogenases from the thermotolerant bacterial strain BC1 and found that this enzyme had molecular weight of about 358,000, which consisted of 8 identical subunits. The enzyme showed high substrate specificity in the oxidative deamination on L-phenylalanine while that of the reductive amination was on phenylpyruvate. No loss of the enzyme activity was observed upon incubation at 40 °C for 2 hours and 50 % of the activity was retained after incubation at the same temperature for 30 hours (Leksakorn, 2001). From these properties, the enzyme is suitably used in drug and food industries for investigation of L-phenylalanine in physiological fluids and also used for synthesis of L-phenylalanine and its derivatives.

In this study identification of thermotolerant bacterial BC1 strain will be performed. Cassette-ligation mediated PCR will be used to prepare full length of phenylalanine dehydrogenase gene for cloning. In detail, the N-terminal amino acid sequence and one part of internal amino acid sequences will be analyzed and used as the data for designing the degenerated primers for PCR amplification of one region of phenylalanine dehydrogenase gene from this organism. After that, amplified fragment will be used as template for DNA sequencing and its result will be further used as a primary data for the next amplification, which led to cloning and overexpression this gene in the final step.