CHAPTER IV

DISCUSSION

4.1 Amino acid sequence of phenylalanine dehydrogenase

Basic study in molecular genetic level was the nucleotide sequencing of interesting gene. PCR amplification was the popular method. This technique depends on two primers, which are specific to interesting gene. Although the specific primer could be designed from the conserve region of the gene from various sources, the similarity was low. Thus, deduced nucleotide sequence from amino acid sequence of PheDH was interesting. PheDH was partially digested with lysyl endopeptidase, the enzyme which cleavage peptide bond between lysine and its next amino acid residue. The digested peptides were further separated by reversed-phase high-performance liquid chromatography (HPLC). Then, the amino acid sequences of isolated peptides were determined by automated Edman degradation amino acid sequencer. The sequence at the N-terminus and 3 internal amino acid sequences of the enzyme were analyzed. Unfortunately, amino acid sequence at N-terminus was uncertain, while complete internal amino acid sequences were obtained. After alignments, the position of each peptide fragment which overlapped with amino acid sequences of the other PheDHs were indicated. From this data, the degenerated primers used for the PCR amplification of internal *phedh* gene were designed.

4.2 Nucleotide sequencing of phenylalanine dehydrogenase gene

Chromosomal DNA of *Bacillus lentus* was easily prepared by miniprep of bacterial genomic DNA method. Its molecular weight was found to be greater than 23.1 kb and A_{260}/A_{280} ratio was also greater than 1.8 indicating its high purity (Manchester, 1995). According to PCR amplification, it is well known that templates used in PCR reaction should be single strand in the purpose of annealing with primers. The heat denatured chromosomal DNA was previously used as templates. However, it gave poor PCR amplification due to effect of its viscosity. Thus,

the chromosomal DNA was digested by restriction enzyme before using. From *Bam*HI, *Eco*RI, *Kpn*I, *Spe*I, *Bgl*II, *Pst*I, and *Xba*I digestion, the digested chromosomal DNA fragment gave the smear pattern of DNA lower than 23.1 kb. It can be assumed that chromosomal DNA of *Bacillus lentus* has a number of restriction sites of those seven restriction enzymes.

For internal gene fragment amplification, those seven restriction enzyme digestion fragments were tested as template using primer F1 and R1 in PCR reaction. The result showed that all restriction enzyme digested fragments gave strong specific products having the size of about 700 bp as determined from relative mobility on agarose gel electrophoresis compared with λ/*Hin*dIII marker. Due to primers used for this step were degenerated primers, all PCR reactions gave a lot of non-specific bands. To confirm these specific products, the PCR solutions were used as source of templates for second PCR amplification using primer F1 and R2 which should give band about of 600 bp. Later on, the desired specific band was recovered from gel and sequenced. Once the nucleotide sequence of this amplified fragment had been identified, the inner part of gene fragment was obtained and the restriction sites on this part was also analyzed. It is widely known that the restriction enzymes used for template preparation must not have their sites on the gene since the amplification of desired gene fragment cannot be occurred. It was implied that *Bam*HI, *Bgl*II, *Eco*RI, *Kpn*I, *Pst*I, *Spe*I and *Xba*I restriction sites were not on this *phedh* gene fragment.

For the 5′-terminal and 3′-terminal gene fragments amplification for sequencing, inverse PCR was used. The inverse PCR is a rapid and reliable method for obtaining sequences flanking a known region of DNA. DNA containing the sequence of target gene is digested, using a suitable restriction enzyme to produce a nucleotide restriction fragment containing the known target sequence flanked by two regions of unknown. Consequently, this DNA is ligated as circularized DNA. The ligated DNA is amplified by PCR. Since polymerase works more efficiently with linear than circular DNA, the circular fragments should be linearized before amplification by digestion with a restriction enzyme which cuts only at a position between the two primer in the known target DNA sequence (Rosalind *et al.*, 1993). In this experiment, the inverse PCR was done for two times in which the PCR products from the first

PCR using outer pair of primers was then used as templates for the second PCR, and the inner pair of primers was used to produce more specific products. Nucleotide sequencing of this gene fragment was analyzed by using primer N2 and C1. After alignment, the nucleotide sequence of 5'-terminus of *phedh* gene was known. Unfortunately, the downstream sequence of this gene fragment was unclear though the nucleotide sequencing was done for several times. To obtain nucleotide sequence of 3'-terminal gene, inverse PCR was performed again and two new primers (N3 and N4) were designed from the obtained nucleotide sequence at 5'-terminus. For amplification, the PCR products from the first PCR were used as template using N3xC2 and N4xC2 to confirm specific product. Then, the desired PCR product was eluted and sequenced using primer C2 to achieve nucleotide sequence of 3'-terminal fragment. The obtained nucleotide sequence consisted of *phedh* gene flanking by short DNA fragment.

The gene contains 1143 nucleotides open reading frame, which is capable of encoding a polypeptide of 380 amino acids which was similar to other PheDHs. The molecular weight of enzyme subunit was calculated from deduced amino acid sequence to be 41.4 kDa.

The nucleotide sequence of *phedh* gene from *Bacillus lentus* was compared with those of *phedh* gene from various sources in the EMBL-GenBank-DDBL database. It showed 99, 65, 62, 57 and 10% similarity to those of *Bacillus badius*, *Sporosarcina ureae*, *Bacillus sphaericus*, *Thermoactinomyces intermedius* and *Rhodococcus* sp. M4, respectively.

The deduced amino acid sequence was matched well with 3 amino acid sequences obtained by lysyl endopeptidase digestion. Using the CLUSTAL W (1.83) aligned amino acid sequences of all PheDHs, the overall similarity scores of PheDH from *Bacillus lentus* compared with *Bacillus badius*, *Bacillus sphaericus*, *Sporosarcina ureae*, *Bacillus halodurans*, *Thermoactinomyces intermedius*, and *Rhodococcus sp.* were calculated to be 99, 67, 66, 62, 53 and 32%, respectively. It was found that *Bacillus lentus* exhibited the highest overall level of similarity to

the enzyme from *Bacillus badius* since their differences only two amino acid residues which resulted from three different nucleotides. Ser-218 and Gln-287 in *Bacillus lentus* enzyme were replaced with Thr and His, repectively in *Bacillus badius* enzyme. However, substituted amino acid still had the same group of side chains and similar size. According to Brunhuber *et al.* (1994), Lys-78 and Asp-118 were necessary for stabilizing and binding of phenylalanine in the active site of the *Rhodococcus* enzyme. Both of residues were identical in all the PheDH sequences including *Bacillus lentus* enzyme. In addition, conserved residues in the catalytic domains G-G-(G or S or A)-K-X-(V or G)-X-X-X-(D or N)-(P or L) at the N-terminus (amino acid position 87 to 97 in Figure 3.16), and the glycine-rich nucleotide binding domain G-X-G-X-X-(G or A) at the C-terminus (amino acid position 190 to 195 in Figure 3.16) were also found (Yamada *et al.*, 1995).

4.3 Cloning and expression of phenylalanine dehydrogenase gene

A number of researchers attempted to clone *phedh* gene for the large amount of enzyme production in order to study properties of enzyme or use as catalyst for the synthesis of L-phenylalanine and related amino acids. Asano and coworkers (1987b) cloned *phedh* gene from *Bacillus badius* IAM 11059 using pUC19 into *E. coli* RRI. The total activity of the enzyme detected in crude enzyme was 6,890 units/liter of cell culture that was about 24 fold of that of the *B. badius* IAM 11059. In addition, the amount of the enzyme in the crude extract comprised 9.6% of the total extractable cellular protein. Consequently, research group of Takada cloned *phedh* gene from *Thermoactinomyces intermedius* into *E. coli* JM109 using a plasmid pKK223-3 (Takada *et al.*, 1991). Total activity of the enzyme in crude extract was reported to be 530 units/liter of cell culture. Moreover, *phedh* gene from *Bacillus badius* BC1 was cloned and expressed in *E. coli* JM109 using pUC18 plasmid (Chareonpanich, 2001). Crude extract gave 1,659 units of PheDH/liter of cell culture. Lately, Sitthai (2004) cloned *phedh* gene from *Acinetobacter lwoffii* into *E. coli* BL21(DE3) using pET-17b and studied the expression of gene in recombinant clone. The total activity of PheDH was 822 units/200 ml of cell culture. 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 55.6 fold higher than

that of the enzyme from *A. lwoffii*. Therefore, the basic requirements for the successful production of recombinant enzyme are the isolation of the gene encoding that enzyme and selection of a suitable expression system for the gene. In this research, the *phedh* gene from *Bacillus lentus* was cloned into *E. coli* BL21(DE3) using plasmid pET-17b. By pET system, *phedh* gene was expressed under T7 promoter; moreover, the upstream region of the inserted gene contained highly efficient ribosome binding site from the phage T7 major capsid protein (Novagen, 2002).

For the whole gene fragment amplification, the new pair of primers was designed. The sense primer contained *Nde*I site, while the antisense primer consisted of *Bam*HI site for further cloning purpose. The amplified whole gene fragment was purified, ligated with *Nde*I-*Bam*HI site of pET-17b and then transformed into *E. coli* BL21(DE3). The sixteen from eighty-two recombinant clones were found to contain recombinant plasmid. They were grown in LB medium, pH 7.2 containing 100 µg/ml ampicillin. When OD_{600} reached 0.6, IPTG was added in the medium at final concentration of 0.4 mM. After that, the culture was continued for 4 hours before harvest. The crude extract of each recombinant clone was assayed for PheDH activity. Their PheDH specific activities were found between 9.34-92.0 units/mg protein. The variation of PheDH activity from each recombinant clone may cause by point mutation occurring in PCR amplification step. The highest specific activity of recombinant clone was higher than that of the enzyme from *Bacillus lentus* with 92.0 fold.

Because of construction of *phedh* gene under T7 promoter, expression of *phedh* gene can be induced by IPTG. Therefore, the studies of induction time and final concentration of IPTG were required for maximum expression. pBLPheDH clone which showed the highest PheDH activity was grown at various conditions. Even in absence of IPTG, the expression of *phedh* gene was occurred because there is some expression of T7 RNA polymerase from the *lacUV5* promoter in the DE3 lysogen from *E. coli* genome (Novagen, 2002). At 0.2 mM final concentration of IPTG which was the least concentration to be enough for induction, expression of the gene reached the maximum at 8 hours. For expression of pET plasmid carrying

the T7 promoter, a final concentration of 0.4 mM IPTG is recommended for full induction (Novagen, 2002). However, different optimum conditions have been reported for some inserted genes of pET plasmids. For example, 3 hours of induction with 1 mM IPTG maximized the expression of *phedh* gene from *Rhodococcus* sp. M4 which was cloned into *E. coli* BL21(DE3) using pET-3d plasmid (Brunhuber *et al.*, 1994). Moreover, alanine dehydrogenase gene from *Aeromonas hydrophila*, expressed under T7 promoter of pET-17b in *E. coli* BL21(DE3), showed the highest expression at 4 hours of induction with 0.4 mM IPTG (Hatrongjitt, 2004). In addition, Kim *et al*, (2002) cloned and expressed bovine brain glutamate dehydrogenase gene using pET-15b in *E. coli* BL21(DE3). Induction of 1 mM IPTG for 3 hours was reported for maximum the enzyme production. Therefore, final concentrations of IPTG and induction time seem to influence the optimization of individual gene expression. The variation of expression level of recombinant PheDH from various bacteria may be obtained from the promoters, mRNA construction, and condition of expression e.g. optimization of IPTG induction, type of cell line, media, and incubation circumstances.

Stability of *phedh* gene from recombinant clones that showed the highest enzyme activity was studied by daily subculturing for 50 days. The crude extract of the 1st, 5th, 10th, 15th, 20th, 25th, 30th, 35th, 40th, 45th and 50th subcultured colonies were prepared and enzyme activities were determined in order to compare with the activity of their parent. The result indicated that expression of *phedh* gene had still remained although it was subcultured daily for a long time. Barely was the copy number of recombinant plasmid lost during susbculture step.

4.4 Purification of phenylalanine dehydrogenase

The development of techniques and methods for the separation and purification of proteins has been an essential pre-requisite for many of the recent advancements in bioscience and biotechnology research. The global aim of a protein purification process is not only the removal of unwanted contaminants, but also the concentration of the desired protein and its transfer to an environment where it is stable and in

a form ready for the intended application. The principal properties of enzymes that can be exploited in separation methods are size, charge, solubility, density and the possession of specific binding sites. Most purification protocols required more than one step to achieve the desired level of product purity. This includes any conditioning steps necessary to transfer the product from one technique into conditions suitable to perform the next technique. Each step in the process will cause some loss of product. Consequently, the key to successful and efficient protein purification is to select the most appropriately techniques, optimize their performance to suit the requirements and combine them in a logical way to maximize yield and minimize the number of steps required (Amersham pharmacia biotech, 1999).

The first step in the purification of a protein is the preparation of an extract containing the protein in a soluble form and extraction procedures should be selected according to the source of the protein. In this work, PheDH, an intracellular enzyme, was extracted from pBLPheDH clone. Mechanical disruption methods are usually necessary to break down cell wall in order to release intracellular protein prior to purification. The cell disintegration technique involved cell lysis by ultrasonication or high pressure sound waves, which causes cell breakage by cavitations and shear forces, so in this work, cell wall was disrupted by ultrasonication. However, several potential problems may be consequent on disruption, due to the destruction of intracellular compartmentation. PheDH activity can be lost by various reasons. It is essential to consider strategies for protection of the enzyme activity. First of all, since the disruption of cells results in the release of proteases from subcellular compartment (Cooper, 1977), the effect of the free proteases must be eliminated. In this work, phenylmethylsulfonyl fluoride (PMSF), and ethylenediamine tetraacetic acid (EDTA) were used in the extraction buffer as serine protease inhibitor, and metalloprotease inhibitor, respectively. As a result, the control of metabolic regulation mechanism is lost when the cell is disrupted. These reagents protect the desired protein from the degradation of proteolytic enzyme. Addition of a reagent containing a thiol group such as β-mercaptoethanol and also a chelating agent such as EDTA to chelate metal ions in the extraction buffer will minimize the oxidation damage (Bollag et al., 1996). However, acid proteases were not inhibited at all. Acid proteases may not affect PheDH activity because their reactions occurred only in low pH

environment. Furthermore, mechanical cell disruption may cause local overheating with consequent denaturation of protein. To maximize recovery of active enzyme, the extract and equipment were, therefore, pre-chilled and several pauses of disruption used instead of one long continuous sonication because short interval of disruption will also minimize foaming and shearing (Harris and Angal, 1989 and Janson and Ryden, 1998).

Solubility differences in salt are frequently exploited to separate proteins in the early stages of purification protocols. Ammonium sulfate was the salt of choice and was used in this work because it combined many useful features such as salting out effectiveness, pH versatility, high solubility, low heat of solution and low price (Bollag et al., 1996 and Creighton, 1993). Preliminary experiment on ammonium sulfate precipitation was also done in this work. PheDH from B. lentus was precipitated by 40-50% saturated ammonium sulfate. In this step, although about 51% of protein was removed, about half of enzyme activity was lost.

Most purification protocols involve some forms of chromatography, which has become an essential tool in protein purification. Ion exchange chromatography separates proteins with differences in charge to give a very high resolution with high sample loading capacity. The difference in charge properties of protein is often considerable. Ion exchange chromatography is capable of separating species with very minor differences in properties, such as two proteins differing by only one amino acid. It is a very powerful separation technique indeed (Amersham pharmacia biotech, 1999). DEAE-Toyopearl is anion exchanger which has negatively charged counter-ions. It widely used in the purification of PheDH from other sources such as PheDH from B. badius (Asano et al., 1987b), B. sphaericus, S. ureae (Asano et al., 1987a) T. intermedius (Ohshima et al., 1991) and Microbacterium sp. (Asano and Tanetani, 1998). Its popularity comes from the possibility of high resolving power, versatility, reproducibility and ease of performance. Consequent upon the result, this column contributed greatly to the purification procedures, with less loss of PheDH activity compared to the amount of proteins removed. About 56% of the protein in the step of 40-50% saturated ammonium sulfate precipitation was eliminated.

The next step of purification was the hydrophobic interaction chromatography (HIC). HIC takes advantage of the hydrophobicity of proteins promoting its separation on the basis of reversible hydrophobic interaction between immobilized hydrophobic ligands on chromatographic medium and non-polar regions on the surface of proteins (Queiroz *et al.*, 2001). Butyl-Toyopearl, which butyl groups are chemically bonded on the surface of hydrophilic resin was used. Adsorption of proteins to a HIC adsorbent is favored by a high salt concentration in the mobile phase. The elution of solute is accomplished by decreasing the salt concentration with increasing hydrophobic. In this work, the enzyme was eluted from Butyl-Toyopearl column using 22.5% saturated ammonium sulfate. From this step, the unwanted protein about 32% of the protein obtained in DEAE-Toyopearl step was removed.

PheDH from pBLPheDH clone was purified 2.11 fold with a 31.0% yield by procedure involving ammonium sulfate precipitation, DEAE-Toyopearl column chromatography and Butyl-Toyopearl. When compared with purification of PheDH from wild type strain, *Bacillus lentus*, purified by DEAE-Toyopearl, Butyl-Toyopearl and Sephadex G-200 column chromatography, the purification step of cloned enzyme was easier and more convenience.

4.5 Characterization of phenylalanine dehydrogenase

The identity of the *Bacillus lentus* enzyme and the recombinant enzyme from pBLpheDHs was confirmed with respect to the molecular weight of the purified enzyme, the specificity on substrate, effect of pH on activity etc. Hence, to compare these properties with wide type enzyme, studies about certain characterization and kinetic mechanism should be elucidated.

4.5.1 Molecular weight determination of phenylalanine dehydrogenase

The molecular weight of the native recombinant enzyme was calculated to be approximately 340,000 Da by gel filtration on TSK Gel G3000 SW column.

The molecular weight of subunits was calculated to be 42,000 Da by comparing the mobility on SDS-polyacrylamide electrophoresis to that of standard proteins. This value was good agreement with that calculated from the amino acid sequence of the enzyme (41,330 Da). Thus, the enzyme probably consisted of eight subunits identical in molecular weight which was similar to PheDH from *B. badius B. sphaericus*, *S. urea* and *Microbacterium sp.* (Asano *et al.*, 1987b; Asano *et al.*, 1987a; Asano and Tanetani, 1998). The enzyme from various sources is considered variable in the quaternary structures: octamer, hexamer, tetramer, dimer and monomer as shown in Table 1.2.

4.5.2 Substrate specificity of phenylalanine dehydrogenase

PheDH catalyzes the reversible NAD$^+$-dependent oxidative deamination of a broad range of hydrophobic amino acid substrates, with particular preference for aromatic side chains (Seah *et al.*, 2002). Of the various amino acids examined as possible substrates, the recombinant enzyme from pBLpheDH exhibited a significant specificity on L-phenylalanine and showed slight activity with aliphatic non-polar amino acids, L-leucine, L-isoleucine, L-norleucine, L-valine, L-norvaline, L-methionine, L-ethionine and *S*-methyl-L-cysteine. These results were the same as those of wild type enzyme. Size of aliphatic side chain seemed to affect the specificity of enzyme since relative activity was decreased with increasing size of amino acid in the order L-ethionine > L-methionine > *S*-methyl-L-cysteine. For L-leucine, L-isoleucine and L-norleucine, the relative activity were L-norleucine > L-leucine > L-isoleucine, respectively.

From all of aromatic amino acids tested, only L-tryptophan showed slightly relative activity. Nonetheless, no activity on tyrosine and L-dopa, which had additional one and two hydroxyl group on the aromatic side chain of phenylalanine, were observed. In addition, no activity was detected with acidic or basic L-amino acid or those with uncharged polar groups. Thus, the side chain of these amino acids may not fit on the active site of the enzyme. No activity was observed with D-amino acids as reported for other PheDHs. Palmer (1995) described that enzyme can exhibit stereochemical specificity if a substrate can exist in two stereochemical forms,

chemically identical but with a different arrangement of atoms in three-dimensional space, then only one of the isomers will undergo reaction.

During the phenylalanine analogs tested, The *p-*, *m-* and *o*-fluoro-DL-phenylalanine, which have additional fluoro group at the aromatic side chain of phenylalanine, were slightly oxidized by the enzyme. The reaction rate with *p-*, *m-* and *o*-fluoro-DL-phenylalanine were 30%, 16% and 5% of relative activity, respectively, while those of wild type enzyme were 35%, 17% and 3% of relative activity, respectively. Moreover, the reaction rate of wild type *B. badius* enzyme on *p-*, *m-* and *o*-fluoro-DL-phenylalanine were 34%, 11% and 2% relative activity, respectively (Asano *et al.,* 1987b). The structure of amino acids and analogs are shown in Appendix K.

The reductive amination reaction was observed with various keto acid analogs. The recombinant PheDH showed high substrate specificity for phenylpyruvate. The enzyme catalyzes the amination of several keto acids such as α-ketocaproate, α-ketoisocaproate, α-ketovalerate, α-ketoisovalerate, α-keto-β-methyl-n-valerate and α-keto-γ-methiol-butyrate. Also, these results were similar to those of wild type enzyme. The structures of these keto acid analogs are shown in Appendix L.

4.5.3 Coenzyme specificity of phenylalanine dehydrogenase

NAD^+-dependent PheDH catalyzes the reversible oxidative demination of L-phenylalanine. In this research, NAD^+ was replaced by some of the NAD^+ analogs as coenzyme for the PheDH. $NADP^+$, which differs from NAD^+ only by the addition of a phosphoric group at C-2 position of NAD^+-adenosyl ribose, was inert for recombinant PheDH similar to some PheDHs. X-crystallographic study of the *Rhodococus* sp. PheDH reveals that the 2- and 3-hydroxyl groups of the adenosyl ribose form strong hydrogen bonds with the carboxylate of aspartate 205 in the crytal structures and those hydrogen bonds would be disturbed by the presence of a 2-phosphate (Brunhuber *et al.,* 2000).

The NAD$^+$ analogs modified at an amino group in the adenine moiety, nicotinamide hypoxanthine dinucleotide (deamino-NAD$^+$), nicotinamide guanine dinucleotide and nicotinamide-1, N^6-ethenoadenine dinucleotide, could be used as coenzymes of the PheDH. This suggests that the amino group in the adenine moiety of NAD$^+$ is not important for the coenzyme specificity.

NAD$^+$ analog modified at the amino group of the nicotinamide moiety, 3-acetylpyridine adenine dinucleotide, exhibited higher activity than NAD$^+$, as has been reported for PheDH from *R. maris*. This suggests that replacement of the amino group of the nicotinamide moiety by methyl group can enhance the reactivity. In addition, the enzyme cannot use deamido-NAD$^+$ and 3-pyridinealdehyde adenine dinucleotide, which their amino groups in the nicotinamide moiety are substituted by OH group and H atom, respectively. The structure of NAD$^+$ and NAD$^+$ analogs are shown in Appendix N.

4.5.4 Effect of pH on phenylalanine dehydrogenase activity and stability

The enzyme activity is often limited to a relatively narrow pH range. Beyond this range, the enzyme activity is dramatically decreased. A pH change affects a reaction by altering the state of ionization of the enzyme, the substrate, or an intermediate or causing a change in the conformation of the enzyme or the substrate, or both. The enzyme forming an enzyme-substrate complex requires electrostatic attraction between oppositely charged groups of the enzyme and the substrate. Within a relatively narrow pH range, the charges of these groups remain essentially the same so that the rate, a function of substrate binding, does vary greatly. At a sufficiently low or high pH, some of these groups lose their charge, thereby impairing binding. Additionally, previously uncharged groups may acquire a charge as the pH increases or decreases. At some pH values, complementary regions of the enzyme and the substrate may carry charges, of the same sign and repel each other, leading to a drop in rate. Altering the charges of functional groups leads to altered ionic interactions involving these groups. These new interactions may produce a conformational change at the active site, affecting the site's capacity to bind

substrate. The enzyme may undergo conformational changes and lose their capacity to bind to the subtrate.

The pH which the PheDH showed maximum activity were 10.7 for the oxidative deamination of L-phenylalanine and 7.8 for the reductive amination, while the wild type enzyme showed the optimum pH for the oxidative deamination and the reductive amination at 10.4 and 8.5, respectively. This was similar to PheDHs from various sources. Between 10.1 and 12.0 lay the pH optima for oxidative deamination whereas the pH optima for reductive amination were between 8.5 and 10.3, except for the enzyme of *Microbacterium* sp. having the high optimum pH for reductive amination at 12.0 (Table 1.2). In addition, potassium phosphate buffer and Tris-HCl buffer were not suitable for the activity of PheDH from pBLpheDH clone, due to the low activity observed with these buffers. The glycine-KCl-KOH buffer was an appropriate buffer for the enzyme. This kind of buffer is widely used for the assay of amino acid dehydrogenases.

The pH stability of an enzyme depends on many factors including temperature, ionic strength, and chemical nature of the buffer, concentration of various stabilizer, concentration of substrates or cofactors of the enzyme and enzyme concentration (Segal, 1976). The recombinant PheDH was stable over a pH range of 7.0 to 11.0 upon incubation at 30°C for 20 minutes. Previously, pH stability for *R. maris* was in pH range 6.7 to 10.0 upon incubation at 35°C for 10 minutes (Misono *et al.*, 1989) whereas the *Microbacterium* sp. enzyme was stable over the pH range of 8.0 to 12.5 upon incubation at 30°C for 30 minutes (Asano and Tanetani, 1998).

4.5.5 Effect of temperature on phenylalanine dehydrogenase activity and stability

The influence of temperature on an enzymatic reaction is resulted from two opposing effects, an increase in rate and an increase in denaturation. Increasing of thermal energy of the substrate molecules increases the proportion of molecules with

sufficient energy to overcome the activation barrier and hence increases the rate of the reaction. In addition, increasing of the thermal energy of the molecules which make up the protein structure of the enzyme itself will increase the chance of breaking the multiple weak noncovalent interactions holding the three-dimensional structure (Segal, 1976).

The optimum temperature of PheDH from pBLpheDH for the oxidative deamination and reductive amination were 45°C and 50°C, respectively, while optimum temperature of wide type enzyme were 50°C and 55 °C, respectively. For other PheDHs, such as, *S. ureae*, *B. sphaericus*, *Nocardia* sp., *B. badius*, and *Microbacterium* sp., the optimum temperatures for the oxidative deamination have been reported to be 40, 50, 53, 65 and 70°C, respectively.

When the enzyme was incubated at various temperatures for 10 minutes, it kept 100% activity at 45°C and dropped about 50% at 55°C. Hence, time course of the enzyme stability was further performed at 45°C. It was found that the activity was not lost upon incubation for 1 hour and about half the enzyme activity was lost when incubated for 3 days. Due to its temperature stability, the enzyme seems to be a good candidate for industrial applications. The range of temperature stability of other PheDHs has been reported as shown in Table 1.2.

4.6 Kinetic studies of phenylalanine dehydrogenase

The kinetic parameters of recombinant PheDH was determined by initial velocity studies. Double-reciprocal plots of initial velocity versus L-phenylalanine concentration at a series of fixed concentration of NAD^+ gave the lines intersecting to the left of the vertical axis (Figure 3.33) indicated a sequential mechanism which all of substrates must be bound to the enzyme before any products are released and ruled out a ping pong mechanism that possesses a parallel initial velocity pattern (Cleland, 1971 and Segal, 1976).

For initial velocity patterns in the reductive amination, when varying NH_4Cl and NADH at a fixed and saturated level of phenylpyruvate, the parallel initial velocity pattern was obtained (Figure 3.35). In addition, the initial velocity patterns when fixed and saturated substrate were NH_4Cl and NADH gave the set of intersecting lines (Figure 3.34 and 3.35). Cleland explained a model of the ordered addition of three substrate (A, B and C are substrates in that order) that the A-B and B-C initial velocity patterns are intersecting while that of A-C is parallel. Thus, phenylpyruvate binds to the enzyme between NH_4Cl and NADH.

In initial velocity studies, the apparent K_m values for L- phenylalanine, NAD^+, phenylpyruvate, NH_4Cl and NADH were 0.45, 0.40, 0.15, 48 and 0.15 mM, respectively, while those of *Bacllilus lentus* enzyme were 0.59, 0.55, 0.18, 50 and 0.09 mM, respectively.

Although cloning of the gene encoding PheDH has been widely performed, studies about characterization and kinetic mechanism such as effect of temperature, pH on activity, stability of enzyme have hardly been examined. To compare between those of wild type and recombinant enzyme, study about recombinant enzyme are mostly focus on specific activity, purification, molecular weight determination, and amino acid composition. Due to a purpose of cloning to accomplish the large amount of enzyme production and study properties of enzyme or use as catalyst for the synthesis of L-phenylalanine and related amino acids, properties of recombinant enzyme should not be altered.

In this study, the sequence of *phedh* gene from *Bacillus lentus* was determined and the structural gene has been successfully cloned and overproduced by PCR method. The obtained information can be used as primary data for further development of PheDH production.