CHAPTER IV

DISCUSSION

Amino acid dehydrogenases are important enzymes that exist at the interface of nitrogen and carbon metabolism. In bacteria, amino acid can be metabolized for energy through the glycolytic or TCA cycle by three ways. A first way uses pyridoxyl phosphate-dependent transaminases, which transfer the amino group from one amino acid to another whereas the second method employs deaminases such as phenylalanine-ammonia lyase or aspartase. The third option is to use amino acid dehydrogenases. These enzymes have the advantage of removing the amino group as free ammonia, which can then be used by the cell in diverse ways and these enzymes also couple deamination to the production of a reduced nucleotide, which can be subsequently used in a variety of energy requiring processes [4]. Therefore, bacteria containing phenylalanine dehydrogenase (PheDH) can grow in medium containing L-phenylalanine as carbon and nitrogen sources because the amino group must first be removed by PheDH before the carbon skeleton of L-phenylalanine can be metabolized for energy through the TCA cycle reactions. Thermotolerant bacteria producing PheDH were screened from various places in Thailand and Japan and two strains, BC1 and BC2, were obtained [73]. In addition, strain BC1 was identified as *Bacillus badius* [74]. Much attention has been paid to this enzyme because it catalyzes the enantioselective synthesis of L-phenylalanine and related L-amino acids from their keto analogs [10-15]. In medical purposes, it has been used in the diagnosis of phenylketonuria (PKU) and the control of dietary therapy of PKU patients [60-69]. Extensive exploitation of enzymes from nature and wide knowledge of their physicochemical properties are required for successful applications [15]. The understanding in relationship between the structure and function of this enzyme for the advances in biotechnological applications are based on fundamental studies concerning characteristics of the enzyme and reaction mechanism. Therefore, the purification, characterization and kinetic mechanism study of this PheDH are necessary to investigate.

4.1 Optimization for phenylalanine dehydrogenase production of *Bacillus badius* BC1

As a first approach for PheDH applications, the screening was carried out for the purpose of finding microorganisms that produced this enzyme effectively [73-74] and the culture conditions that leaded to abundant enzyme production were examined in this work.

Enzyme induction is defined as a relative increase in the rate of synthesis of a specific enzyme resulting from exposure to a chemical substance – inducer [86]. The addition of enzyme inducers is usually very effective for maximize harvestable enzyme levels. Many amino acid dehydrogenases have been reported about their inducible properties such as the addition of L-alanine and L-branch chain amino acids to the medium promoted the production of alanine dehydrogenase (AlaDH) and leucine dehydrogenase (LeuDH), respectively, in *Bacillus* sp. [87-88]. In this work, the effect of various amino acids on the production of PheDH from bacterial strain BC1 and BC2 was studied. It was found that L-phenylalanine with the concentration of 0.8 % gave the optimal induction for PheDH production from the two strains. L-tryptophan and L-tyrosine were also found to support PheDH production, but there were not the notable effects. This observation, that enzyme levels are substantially increased in the presence of L-phenylalanine in the media, indicates that although amino acid synthesis is favored thermodynamically, which is typical of PheDHs [7-12], the physiological role of the enzyme in this soil bacterium is likely to be the degradation of L-phenylalanine for use as a carbon and nitrogen sources.

When compared to other sources of PheDH, L-phenylalanine has been reported using as inducer in PheDH productions from *S. ureae* [10], *B. sphaericus* [10], and *B. badius* [11] whereas L-tyrosine and L-methionine have been used in those from *T. intermedius* [14] and *Microbacterium* sp. [15], respectively. In *Brevibacterium* sp., L-phenylalanine as enzyme inducer can be substituted by several other amino acids such as D,L- phenylalanine, D-phenylalanine or L-histidine while L-phenylalanine cannot be substituted by these amino acids in *Rhodococcus* sp. M4 [7-8]. In addition, rather high concentrations of L-phenylalanine are observed for the induction of

PheDH from *Rhodococcus* sp. M4.

Bacterial growth rates and enzyme production are greatly influenced by pH of the environment, growth temperature and time. Most of bacteria producing PheDH, except *T. actinomycetes*, are mesophiles and have been reported to culture in the similar conditions as in bacterial strain BC1 and BC2. The pH of culturing medium, cultivation temperature and cultivation time used were in range of pH 7.0 -7.5, 18-30°C and 18-30 hours, respectively [7-13, 15].

4.2 Purification of phenylalanine dehydrogenase from *Bacillus badius* BC1

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 [89]. The principal properties of enzymes that can be exploited in separation methods are size, charge, solubility and the possession of specific binding sites [89-90]. Most purification protocols require 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 appropriate 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 [90].

Phenylalanine dehydrogenase was purified 160.7-fold with a 20% yield from the cell-free extract of *B. badius* BC1 by a procedure involving ammonium sulfate precipitation followed by column chromatographies: DEAE-Toyopearl, first Butyl-Toyopearl and second Butyl-Toyopearl, respectively.

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 [90-91]. In this work, PheDH, an intracellular enzyme, was extracted from *Bacillus* sp. which is gram-positive bacteria and the major component of the cell wall is peptidoglycan (40-90%) together with teichoic acids, teichuronic acids and other carbohydrates [91]. The cell wall is responsible for strength, rigidly as well as shape and is the major barrier to release of any intracellular proteins. Mechanical disruption methods are usually necessary to break down cell wall in order to release intracellular protein prior to purification [92-93]. Ultrasonication or high-pressure sound waves, which causes cell breakage by cavitation and shear forces, was used in this work. However, several potential problems may be consequent on disruption, due to the destruction of intracellular compartmentation and PheDH activity can be lost for a variety of reasons. It is therefore essential to consider strategies for protection of the enzyme activity. As 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, because the control of metabolic regulation mechanisms is lost when the cell is disrupted. Thus, the desired protein may be degraded by intrinsic catabolic enzymes such as proteolytic enzymes [90-94]. In addition, the protein will encounter an oxidizing environment after disruption that may cause inactivation, denaturation or aggregation [94]. Addition of a reagent containing a thiol group such as dithiothreitol (DTT) and also a chelating agent such as EDTA to chelate metal ions in the extraction buffer will minimize the oxidation damage. Furthermore, mechanical cell disruption may cause local overheating with consequent denaturation of protein. To maximize recovery of active enzyme, the extract and equipment, therefore, were 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, thereby minimizing denaturation [93-95].

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 [76]. In the ammonium sulfate precipitation step, about half of the proteins were

removed but about one thirds of the enzyme activity was lost. The loss of significant portion of PheDH activity may be caused by the removal of some factors important for stabilizing the enzyme activity. Although serine proteases and metalloproteases were inhibited since the extraction step, however, other proteases such as acid proteases and thiol proteases were not inhibited at all. Acid proteases may not affect PheDH activity because their reactions occurred only in low pH environment [94], which was far from pH used in this work (pH 7.4) while thiol proteases may involve in the loss of enzyme activity. This group of proteases can be inactivated by *p*-chloromercuribenzoate, a thiol-specific reagent [94]. But this inhibitor was not used in this work because many scientific reports have indicated that *p*-chloromercuribenzoate also completely inhibit PheDH activity [9-12, 15]. In addition, the speed at which salt was added to an enzyme solution and the efficiency of stirring are important factors. For many proteins, salt addition must be quite gradual. Stirring must be regular and gentle because stirring too rapidly will cause protein denaturation as evidenced by foaming [91]. Moreover, care must be taken to monitor the pH because ammonium sulfate slightly acidify the extract, therefore, an appropriate buffer should be used to maintain a pH between 6.0-7.5 [93]. In this work, phosphate buffer pH 7.4 was used as the working buffer and also contained glycerol to stabilize proteins, a reducing agent (β-mercaptoethanol) to prevent protein oxidation and a chelating agent (EDTA) to remove possible traces of heavy metal cations in the ammonium sulfate which may be detrimental to the enzyme.

In some case, it is advantageous to carry out a selective heat denaturation as an early step in purification if the enzyme of interest is more stable than the other proteins under the conditions of heat treatment. In the purification of PheDHs from *B. sphaericus* [10], *B. badius* [11] and *T. intermedius* [14], heat treatment step has been successfully used as another promising way to get rid of other bulk proteins from crude enzyme solution before ammonium sulfate precipitation step because impurity proteins in the crude extract are mostly denatured during the treatment. Preliminary experiment on heat treatment was also done in this work, nevertheless, it was found that there was great loss of PheDH activity with less removal of other proteins. Thus, it was decided that this procedure was not the suitable step for purification of PheDH from *B. badius* BC1 and was not further used in this work.

Most purification schemes involve some forms of chromatography, which has become an essential tool in every laboratory where protein purification is needed. Ion exchange chromatography separates proteins with differences in charge to give a very high resolution with high sample loading capacity [90]. DEAE-Toyopearl is anion exchanger and widely used in the purification of PheDHs from other sources [10-11, 14-15]. Its popularity stems from the possibility of high resolving power, versatility reproducibility and ease of performance [90-92]. 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 88% of the other bulk proteins were eliminated. Moreover, the result from chromatogram also indicates that net charge of the enzyme in working buffer, 10 mM potassium phosphate buffer pH 7.4, was negative because our enzyme could bind to the column in this purification system (net charge of DEAE ligand was positive). This can be implied that pI of our enzyme was less than pH 7.4.

In the purification of PheDH, several purification methods have been employed including hydrophobic interaction chromatography (HIC) [10,12,15]. 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 [89, 91] Butyl-Toyopearl, the chemically bonding butyl groups on the surface of non-porous spherical synthetic hydrophilic resin, was used as HIC in the last two steps of the purification in this work. Adsorption of proteins to a HIC adsorbent is favored by a high salt concentration in the mobile phase (the higher the salt concentration, the stronger the interaction) because the ionic strength of solution controls the protein hydrophobicity and therefore its degree of adsorption. Midely hydrophobic proteins may not be adsorbed until the ionic strength is increased to just below that required for precipitation. Prior to HIC, the sample ionic strength should be adjusted with salt and buffered the required protein to enhance non-ionic (hydrophobic) adsorption [93, 95]. Thus, care must be taken in the aspect of the salt concentration used for adsorption. It should be lower than the concentration using in precipitation of our enzyme and 25% saturated salt solution was used for adsorption in this work. Elution, whether done stepwise or with a gradient, can generally be achieved in three different ways [95]. In this work, the elution of solutes, in the order of increasing

hydrophobicity, is accomplished by decreasing the salt concentration. Furthermore, different salts give rise to differences in the strength between proteins and the HIC adsorbent. Ammonium sulfate was chosen in this work because its effective property in promoting hydrophobic interactions and it was widely used in HIC [89, 91, 93, 95].

In first Butyl-Toyopearl column, stepwise elution by decreasing salt was achieved for eliminating other bulk proteins as much as possible and it was found that even though some of PheDH activity was lost but about 92 % of the other proteins was removed. In this column, our enzyme was eluted by 20% saturated ammonium sulfate solution. This shows that our enzyme is midely hydrophotic protein (weak degree of the hydrophobic adsorption) when compared to other proteins eluted by lower percentage of saturated ammonium sulfate solution (these proteins have stronger degree of the hydrophobic adsorption). In second Butyl-Toyopearl column, a decreasing salt gradient elution was successfully used for specific separation of PheDH from unwanted proteins effectively. The chromatogram (Figure 3.8) shows that two peaks of proteins were eluted after introducing a negative salt gradient in the range of 25% to 18%. The first peak was unwanted protein while another one was PheDH peak. In consequent of the result, about 80% of PheDH activity was retained while about 70% of proteins were removed and moreover, the success in using this column was judged by the homogeneity of PheDH according to gel electrophoresis.

Some purification guidebooks suggest that HIC is ideal for use immediately after salt precipitation where the ionic strength of the sample will enhance hydrophobic interaction and also for avoiding the desalting step [89-90, 95]. However, this does not mean that these are always the most suitable methods for all enzymes. Preliminary experiment was performed by direct application of the enzyme solution from ammonium sulfate precipitation step to Butyl-Toyopearl column. It was found that even though great removal of other proteins was observed but elution time was too long which caused the unacceptable great loss of PheDH activity. Moreover, efficient purification of PheDHs from *S. ureae* [10] and *Microbacterium* sp. [15] had been previously reported using Butyl-Toyopearl column as the purification procedure after DEAE-Toyopearl column.

In addition, purification method based on affinity chromatography has also been described for PheDH [14, 16] and other amino acid dehydrogenases [96]. In preliminary experiment, Red-Sepharose CL-6B column which consists of Procion Red HE-3B as a covalently bound ligand for binding hydrogenases and most other enzymes requiring adenyl-containing cofactors such as $NADP^+$ and Blue-Sepharose 6 fast flow column containing Cibacron Blue F3G-A as the covalently bound ligand which is more selective for NAD^+-dependent enzymes were tested. It was found that our PheDH did not bind to these columns and the resolution of PheDH and other proteins was not good. Some unknown factor may be involved in the binding reaction between the enzyme and the ligand of matrix.

From many preliminary experiments on purification, it is concluded that ammonium sulfate precipitation followed by DEAE-Toyopearl column, first Butyl-Toyopearl column (stepwise elution) and second Butyl-Toyopearl column (gradient elution) are appropriate in the purification procedure of PheDH from *B. badius* BC1 with the acceptable yield and purification fold.

4.3 Characterization of phenylalanine dehydrogenase from *Bacillus badius* BC1

4.3.1 Molecular weight determination of phenylalanine dehydrogenase

The molecular sieving and SDS - polyacrylamide gel electrophoresis technique are used to determine the molecular weight and subunit existence of enzymes. The relative molecular weight of PheDH from *B. badius* BC1 was determined to be about 358,000 consisting of eight subunits with equal molecular weight of 44,500. Our native enzyme appears to have an octameric structure which is similar to PheDH from *S. ureae* [10], *B. sphaericus* [10], *B. badius* [11] and *Microbacterium* sp. [15]. The quaternary structure of PheDHs from various sources is diverse, with most being octamer, but hexamer, tetramer, dimer and monomer have also been detected [12-14, 16] as shown in Table 1.2. PheDHs exhibit a narrow range of subunit molecular weight between 36,000 and 42,000, so that the subunit of

our enzyme appears to be the biggest enzyme among these PheDHs.

When compared to other amino acid dehydrogenases, the octameric structure is not common in amino acid dehydrogenases. Only AlaDH of *Streptomyces aureofaciens* and LeuDH of *B. caldolyticus, B. licheniformis* and *B. cereus* are reported to be octameric, while most of the other amino acid dehydrogenases are hexameric, tetrameric and dimeric structures [1, 4, 6].

4.3.2 Substrate specificity of phenylalanine dehydrogenase

In general, a substrate-binding site consists of an indentation or cleft on the surface of an enzyme molecule that is complementary in shape to the substrate (geometrical complementary). Moreover, the amino acid residues that form the binding site are arranged to interact specifically with the substrate in an attractive manner (electronic complementary). Molecules that differ in shape or functional group distribution from the substrate cannot productively bind to the enzyme, that means they cannot form enzyme-substrate complexes that lead to the formation of products [98].

For the *B. badius* BC1 PheDH, the high substrate specificity is favorable for the selective determination of L-phenylalanine and phenylpyruvate. Of the various amino acids examined as substrates in the oxidative deamination, our enzyme exhibits a significant specificity on L-phenylalanine. No activity was detected with acidic or basic L-amino acids or those with uncharged polar groups. Thus, the structure and properties of the side chain of these amino acids may not fit on the active site pocket of the enzyme. All of hydrophobic, aliphatic or aromatic amino acids tested were inert. In particular, L-tyrosine, the active substrate for PheDHs from some mesophiles [8, 10, 13], was inert for our enzyme which is similar to the *R. maris* [12] and *T. intermedius* [14] enzyme. Therefore, it will be very especially useful in medical application to use this enzyme for the quantitative analysis of L-phenylalanine level in blood, serum or tissue extracts without any interference by L-tyrosine.

In addition, no activity is observed with D-amino acids, as reported for other PheDHs [10-17]. Palmer described that enzyme could exhibit stereochemical specificity if a substrate can exist in two stereochemical forms, chemically identical but with a different arrangement of atoms in three-dimensional space, then only one of the isomers will undergo reaction [99]. This supports the result about substrate specificity of our enzyme on D-amino acids.

Among the phenylalanine analogs tested, only α-amino-β-phenyl butanoate and *p*- and *m*- fluoro-DL-phenylalanine, which have additional methyl group and fluoro group in the aromatic side chain of phenylalanine, are slightly oxidized by the enzyme. The reaction rate with *p*-fluorophenylalanine of the *Rhodococcus* sp. M4 [8] and *B. badius* [11] enzyme were 62% and 34% of the maximal reaction rate obtained with phenylalanine, respectively, while that of our enzyme was only 11% of the maximal reaction rate. Hydrocinnamate, DL-β-phenyllactate and *N*-methyl-L-phenylalanine, which their amino groups are substituted by H, OH and $NHCH_3$ groups, respectively, and α-methylphenylalanine, which its H atom of the chiral carbon is substituted by methyl group, were inert for our enzyme. These observations indicate that the amino group and H atom of the chiral carbon are essential on the substrate specificity of the enzyme. However, *o*-fluoro-DL-phenylalanine was not the substrate of the enzyme which may explain that the position of the additional fluoro group on the aromatic ring of phenylalanine is also important for the substrate specificity in which the steric effect of this additional group may involve in the reaction. The structures of these analogs are shown in Appendix G.

Although PheDHs from various sources have broad substrate specificities in the reductive amination reaction [9-13, 15], our enzyme is different in substrate specificity in this respect. The enzyme showed high substrate specificity acting on phenylpyruvate. Only α-ketocaproate (the keto analog of norleucine) was slightly reduced by the enzyme and α-ketovalerate (the keto analog of norvaline), α-ketoisocaproate (the keto analog of leucine) as well as α-ketoisovalerate (the keto analog of valine) were very poor substrates. A marked difference between the *B. badius* BC1 PheDH and other PheDHs [9-13, 15], is that the later were considerably

active towards *p*-hydroxyphenylpyruvate, which is the keto analog of tyrosine. Furthermore, the *Nocardia* sp. PheDH [13] has been reported to be active toward indole-β-pyruvate (the keto analog of tryptophan) with 54% relative activity and α-ketoisocaproate with 240% relative activity whereas our enzyme did not act on indole-β-pyruvate and very slightly worked on α-ketoisocaproate (4% relative activity). In addition, PheDHs from *S. ureae* [10] and *B. badius* [11] have been reported to be active toward α-ketocaproate with 32% and 31% relative activity, respectively. The substrate specificity in oxidative deamination and reductive amination of PheDHs are summarized as shown in Table 1.2.

By the way, according to the high substrate specificity on L-phenylalanine and phenylpyruvate of the *B. badius* BC1 PheDH, it may be useful and interesting for the technical use of this enzyme in the production of L-phenylalanine or in medical diagnostics for the assays of disease, especially with phenylketonuria.

4.3.3 Coenzyme specificity of phenylalanine dehydrogenase

Many biochemical reactions involve oxidation and reduction. One of the most important coenzymes used in the transfer of hydrogen atom are nicotinamide adenine dinucleotide (NAD^+). All PheDHs are NAD^+-specific and require NAD^+ as a natural cofactor. The coenzyme binding domains of amino acid dehydrogenases have been shown to display a high degree of conservation of tertiary structure as shown in Figure 1.5. The presence of conserved hydrophobic residues, the G-X-G-X-X-(G/A) fingerprint sequence and the conserved aspartate residue involved in ribose hydroxyl hydrogen bonding suggest that the amino acid dehydrogenases bind dinucleotides in a manner analogous to other structurally characterized dehydrogenases [24].

In this research, NAD^+ is replaced by some of the NAD^+ analogs as coenzymes for the *B. badius* BC1 PheDH. The NAD^+ analogs used in this work can be divided into three groups based on their modified structures (Appendix H). According to the result, $NADP^+$, which differs from NAD^+ only by the addition of a

phosphoric group at C-2 position of its adenosyl ribose, is inert for our PheDH and other PheDHs [7-17]. The enzyme exhibits a strict specificity against 2'-phosphorylated dinucleotide but broad specificity for nonphosphorylated dinucleotides. In addition, alternate nucleotide study of the *Rhodococcus* 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 crystal structures and those hydrogen bonds would be disrupted by the presence of a 2'-phosphate [17].

Nicotinamide hypoxanthine dinucleotide (deamino-NAD^+), nicotinamide guanine dinucleotide and nicotinamide 1, N^6-ethenoadenine dinucleotide, which are the NAD^+ analogs modified at the amino group in the adenine moiety, can substitute for NAD^+ as coenzyme for our enzyme. Moreover, deamino-NAD^+ and NAD^+ have closely similar reactivities. These observations have also been reported for other PheDHs [8,12] and amino acid dehydrogenases [100-101]. Accordingly, it is concluded that the amino group in the adenine moiety of NAD^+ is not crucial importance for the coenzyme activity.

There is notable observation about the NAD^+ analogs modified at the amino group of the nicotinamide moiety. 3-Acetylpyridine adenine dinucleotide and thionicotinamide adenine dinucleotide were utilized by our enzyme while 3-pyridinealdehyde adenine dinucleotide and nicotinic acid adenine dinucleotide (deamido-NAD^+) were not. This suggests that the amino group of the nicotinamide moiety is essential for the enzyme activity and the type of the substituted groups of NAD^+ analogs also has the influence on enzyme activity. 3-Acetylpyridine adenine dinucleotide is reduced by the enzyme more rapidly than is NAD^+, as has been reported for PheDH from *R. maris* [12] and LeuDHs from *B. sphaericus* [103] and *Corynebacterium pseudodiphtheriticum* [104]. This suggests that the replacement of the amino group of the nicotinamide moiety by methyl group results in the enhancement of the reactivity. However, the enzyme cannot use deamido-NAD^+ and 3-pyridinealdehyde-NAD^+, of which their amino groups in the nicotinamide moiety are substituted by OH group and H atom, respectively. This may involve polarity of the substituents which may affect the binding mechanism in the area of the conserved hydrophobic residues of the enzyme.

4.3.4 Effect of pH on phenylalanine dehydrogenase activity and stability

Each enzyme has an optimum pH at which the rate of the reaction that it catalyzes is at its maximum. Slight shifts in the pH from the optimum value lead to a decrease in the reaction rate, due to changes in the ionization of charged amino acid residues that function in the active site of the enzyme such as lysine 78 and aspartate 118 in the *Rhodococcus* sp. PheDH [17]. Large shifts in pH lead to denaturation of the enzyme, due to interference with the many weak noncovalent bonds maintaining its three-dimensional structure [105-106].

PheDH from *B. badius* BC1 showed the optimum pH at the fairly alkaline range for the oxidative deamination and reductive amination which were 10.7 and 8.3, respectively. For the majority of PheDHs, the pH optima for oxidative deamination lie between 10.1 and 12.0 while the corresponding optima for reductive amination are between 8.5 and 10.3, except for the enzyme of *Microbacterium* sp. [15] which has very high optimum pH for reductive amination (Table 1.2). The high reactivity of the enzyme at rather high pHs is similar to those of other amino acid dehydrogenases [1, 4, 6, 97]. In addition, according to the results, it was found that Tris-HCl buffer is not suitable for the *B. badius* BC1 PheDH due to the low enzyme activity observed with this buffer. Tris-HCl buffer may interfere the reaction of this enzyme while glycine-KCl-KOH buffer is an appropriate buffer for this enzyme and, moreover, has been chosen to use widely as an assay buffer for almost PheDH assay systems [9-15].

PheDH from *B. badius* BC1 was most stable over a pH range of 6.0 to 11.0 upon incubation at 30°C for 20 minutes. The *R. maris* PheDH has been reported to be stable over the pH range 6.7 to 10.0 upon incubation at 35°C for 10 minutes whereas the *Microbacterium* sp. enzyme has been reported to be stable over the pH range of 8.0 to 12.5 upon incubation at 30°C for 30 minutes [12, 15]. In addition, the studies of pH stability in the *Bacillus* sp. LeuDHs showed that they were stable over the pH range of 5.5 to 10.5 [88, 107]. The pH stability of an enzyme also depends on many factors including temperature, ionic strength, chemical nature of the buffer, concentration of various preservations, concentration of substrates or cofactors of the enzyme and enzyme concentration [106].

4.3.5 Effect of temperature on phenylalanine dehydrogenase activity and stability

Temperature affects the rate of an enzyme - catalyzed reaction by increasing the thermal energy of the substrate molecules. It 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 chances of breaking the multiple weak noncovalent interactions holding the three-dimensional structure [105-106].

The optimum temperature of PheDH from *B. badius* BC1 for the oxidation deamination and reductive amination were 50°C and 45°C, respectively. For other PheDHs, such as *S. ureae* [10], *B. sphaericus* [10], *Nocardia* sp. [13] and *B. badius* [11], the optimum temperatures for oxidative deamination have been reported to be 40, 50, 53 and 65°C, respectively. Besides, the range of optimum temperature of other amino acid dehydrogenases is about 40-65°C which is within the same range with our findings [97].

Temperature stability of the *B. badius* BC1 PheDH was investigated. When the enzyme was incubated at various temperatures for 10 minutes, 100% activity was retained at 40°C and dropped about 50% at 45°C. When incubated at 40°C, it was found that although full activity was maintained for 2 hours, 80% of remaining activity was observed for 20 hours of the incubation. The range of temperature stability of other PheDHs from mesophilic bacteria has been reported in this similar manner as our enzyme as shown in Table 1.2.

4.3.6 Effects of metal ions and chemical substances on phenylalanine dehydrogenase activity

PheDH of *B. badius* BC1 was tested for the effect of metal ions and chemical reagents on enzyme activity. The enzyme was not inactivated by metal chelating or carbonyl reagents and reducing agents but by sulfhydryl reagents such as

$HgCl_2$ and $AgNO_3$. This inhibition suggests that the enzyme contains a reactive sulfhydryl group(s). Some amino acid dehydrogenases have been reported that Li^+, Na^+ or K^+ are needed for enzyme activation [108-110]. But in this case, monovalent and divalent cations had no effect on our PheDH activity. In addition, Fe^{2+} and Fe^{3+} had notable effects on the enzyme activity which similar to those of AlaDH from *Halobacterium cutirubrum* [109]. Mn^{2+} has been reported to be an activator cation of some amino acid dehydrogenases [97]. However, it showed inhibitory effect for our enzyme which was similar to that of AlaDH and LeuDH from *Bacillus subtilis* [97]

4.3.7 Inhibitory effects of various amino acids and keto acids on phenylalanine dehydrogenase activity

Many types of molecule can interfere with the activity of an individual enzyme. Substrate analog is one of inhibitor of the enzyme. Inhibitory effects of D- and L- amino acids and keto acids, which were not substrates of the enzyme, on the oxidative deamination of L-phenylalanine and the reductive amination of phenylpyruvate, respectively, were investigated for the *B. badius* BC1 PheDH. In this work, nonsubstrate D-amino acids tested can be divided into four groups based on characteristic of their side chains; (i) non-polar side chain group, (ii) polar uncharged side chain group, (iii) acidic side chain group and (iv) basic side chain group. The summary of nonsubstrate and noninhibitor amino acids observed in this work is shown in Appendix I. It was found that D-amino acids with non-polar aliphatic and aromatic side chain, except for D-glycine and D-alanine, and those with acidic side chain exhibited significant inhibition against L-phenylalanine. D-amino acids with polar uncharged side chain and those with basic side chain had no effect on the enzyme activity. Asano *et al.* reported about the inhibitory effects of D-amino acids on the *S. ureae* and *B. sphaericus* PheDH that they were inhibited by D-amino acids with non-polar side chain such as D-valine, D-isoleucine, D-leucine, D-methionine, D-tryptophan and D-phenylalanine. In addition, it is noted that D-phenylalanine is the strong inhibitor for our PheDH (6% remaining activity) which is similar to the *R. maris* enzyme (7% remaining activity) while D-phenylalanine slightly inhibited the *S. ureae* (77% remaining activity) and *B. sphaericus* (89% remaining activity) enzyme [10,12]. Inhibition by D-enantiomer of the substrates has

also been reported for LeuDH of *B. sphaericus* [103]. The degree of inhibition, however, was stronger for PheDH than for LeuDH.

For inhibitory effect of nonsubstrate L-amino acids on the *B. badius* BC1 PheDH activity, the result was in the same manner as the result of D-amino acid effects. L-amino acids with non-polar side chain, except for L-glycine and L-alanine, and those with acidic side chain showed notable inhibition against L-phenylalanine while L-amino acids with polar uncharged side chain and those with basic side chain had no effect on the enzyme activity. In addition, L-isoleucine and L-leucine have been reported to have inhibitory effect on the *S. ureae* and *B. sphaericus* PheDHs [9-10].

According to these result, It should be explained that glycine and alanine, which are small hydrophobic amino acids, have no potential and are not involved in enzymatic catalysis of the *B. badius* BC1 PheDH because D- and L-forms of these two amino acids are neither substrates nor inhibitors for the deamination of L-phenylalanine. In the same manner, either the polar uncharged or positively charged group in the side chain probably prevents the amino acids from binding with the enzyme because D- and L- forms of serine, threonine, arginine, histidine and lysine are neither substrates nor inhibitors for the deamination of L-phenylalanine. On the other hand, D- and L- forms of aspartate and glutamate, acidic amino acids, are not substrates of the enzyme but are inhibitors of the deamination of L-phenylalanine. This shows that the negatively charged group in their side chain may be affected as inhibitors leading to decrease the rate of an enzyme-catalyzed reaction.

For the inhibitory effect of nonsubstrate phenylalanine analogs on our PheDH activity, it was found that only *o*-fluoro-DL-phenylalanine served as inhibitor of the L-phenylalanine deamination (Appendix I). This indicates that the similarity of structure of this analog and the fluoro group at the *ortho* position on the aromatic ring of phenylalanine may be involved in this inhibition. In addition, 3-phenylpropionate (hydrocinnamate) has been reported to be a competitive inhibitor for the *Rhodococcus* sp. PheDH [12, 17]. But for our PheDH, it had no effect on the

enzyme activity. The differences observed about this may be physiologically important.

For the inhibitory effect of reductive amination of phenylpyruvate, no significant inhibition was found and there was no any report about this effect on activity of other amino acid dehydrogenases.

4.3.8 Effect of group-specific reagents on phenylalanine dehydrogenase activity

Several methods for investigation of amino acid residues which are essential for function or structure of protein such as affinity labeling with their substrate or substrate analog, X-ray crystallography, site-directed mutagenesis or chemical modification have been reported [79, 82]. Chemical modification using group-specific reagent is one of the most useful method for identifying the functional groups of a protein. The principle is that if an amino acid side chain involved in the catalytic activity is chemically modified, the enzyme will be inactivated [79]. The specificity of reagents used to chemically modify proteins is shown in Appendix J and the modification reaction of these reagents is shown in Appendix K.

In this work, the screening of essential amino acid residues of the enzyme, which was the initial phase of chemical modification study, was investigated. Eight different amino acid residues tested were arginine, cysteine, histidine, lysine, methionine, serine, tryptophan and tyrosine. These amino acid residues have been selected because they are widely known as residues involved in enzyme catalysis[6]. Incubation of purified enzyme with a series of modifying agents of amino acid residues at 10 mM concentration resulted in variable changes in the catalytic ability of this enzyme. No inhibition of the *B. badius* BC1 PheDH activity was observed in the modification of cysteine by dithiothreitol (DTT), tyrosine by *N*-acetylimidazole (NAI) and serine by phenylmethylsulfonyl fluoride (PMSF). It may be assumed that cysteine, tyrosine and serine are not the important residues involved in enzyme catalytic activity. Very high inhibition was observed in the modification of arginine by phenylglyoxal (PG). Moreover, the modification with chloramine T (CT),

diethylpyrocarbonate (DEPC), *N*-bromosuccinimide (NBS) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) which were known to react specifically with methionine, histidine, tryptophan and lysine residues, respectively, resulted in extensive inhibition of PheDH activity. It is concluded that guanidino group of arginine, thioether group of methionine, imidazole of histidine, indole group of tryptophan and amino group of lysine are all likely involved in PheDH activity as the essential residues for enzyme biological function. As mentioned above that this experiment is only the initial phase of amino acid selective chemical modification. To prove that the amino acid residues involved in PheDH activity are located within active site or not, the substrate protection experiments are necessary and should perform for the further studies because these group-specific compounds covalently modify the accessible amino acids in a general way, so that treatment of an enzyme with such reagents will lead to modification of both catalytically critical residues and nonessential residues as well. If an interesting amino acid residue can be protected by substrate, the loss of activity will be less than that of without substrate protection. It means that the amino acid residue is in the active site of the enzyme [96].

When compared to the previous studies, these crucial amino acid residues for our PheDH have been demonstrated as essential residues for other PheDHs and amino acid dehydrogenases as well [6,96]. Sequence comparisons between the putative catalytic domains of amino acid dehydrogenases reveal patterns of homology that suggest conserved residues involved in L-amino acid binding and residues which are possibly involved in amino acid discrimination [24]. It has been suggested that the consensus G-G-G-K sequence of amino acid dehydrogenases constitutes the substrate-binding site and the lysine residue in this sequence participates in catalysis through formation of a Schiff base with a substrate keto acid [27]. For PheDH, Kataoka *et al.* reported that lysine residues of PheDH from *T. intermedius* were modified by methyl acetyl phosphate and were proposed to be involved in substrate binding and catalysis [28]. Recently, the same research group also suggested that the hexapeptide segment (^{124}F-V-H-A-A-R^{129}) of this enzyme plays a significant role in substrate recognition [27] and multiple lysyl residues are present at the active site of this enzyme especially with lysine 69 and lysine 81 which are involved in substrate binding and catalysis, respectively [28]. In addition, Vanhooke *et al.* has been reported that the enzymatic discrimination between imino and keto acids as substrates

for reduction is a consequence of the hydrogen-bonding and electrostatic interactions of the substrate and intermediates with lysine 78 and aspartate 118 in PheDH from *Rhodococcus* sp. M4 [16]. Thus, they concluded that lysine 78 and aspartate 118 function as the catalytic residues in the active site of this enzyme [17]. Our attention was also paid to the modification of carboxylic amino acid residue. Nevertheless, preliminary modification experiment on carboxylic residues by carbodiimide was unsuccessful because the buffer system used for carbodiimide is low pH (pH 4-5) which is not suitable for the enzyme system and, moreover, could denature our enzyme.

4.4 Kinetic mechanism studies of phenylalanine dehydrogenase from *Bacillus badius* BC1

The mechanism of the oxidative deamination and reductive amination was explored through initial - velocity studies and product-inhibition patterns. These experiments are carried out by varying the concentration of one substrate at several different fixed concentrations of the other substrate(s) or the product. The analysis of kinetic data is carried out according to the methods proposed by Cleland [85, 111-113].

Of the known amino acid dehydrogenases, all of them appear to proceed via the formation ternary complex with sequential mechanism and not with ping-pong mechanism. Diversity is found in the manner of substrate binding and product release. Most amino acid dehydrogenases appear to operate via a sequential ordered Bi-Ter mechanism with the cofactor binding before the amino acid [2,4]. However, some different kinetic mechanisms are also observed. The reaction of bovine liver NAD(P)-GluDH proceeds via a random ordered binding while AlaDH from a nitrogen-fixing microorganism such as *Bradyrhizobium japonicum* and soybean nodule bacteroids show the Ter-Bi Theorell-Chance mechanism [114].

The kinetic mechanism of the *B. badius* BC1 PheDH is sequential ordered Bi-Ter with NAD^+ and L-phenylalanine binding in that order to form a ternary complex, followed by the ordered released of NH_4^+, phenylpyruvate and NADH.

This ordered-binding sequence has been reported for other amino acid dehydrogenases, including the *R. maris* [12] and *Rhodococcus* sp. M4 [18] PheDHs. However, another ordered-releasing mechanism was reported for the *T. intermedius* PheDH which releases products in the order of phenylpyruvate, NH_4^+ and NADH [14]. The proposed kinetic mechanism of PheDH from *B. badius* BC1 is shown in Figure 4.1. This conclusion of the kinetic mechanism of our enzyme was drawn from both initial velocity and product inhibition experiments which are described below.

Initial velocity studies

A general method for prediction of the mechanism by initial velocity and product inhibition patterns is presented by Cleland [113]. Cleland has formulated a series of general rules for prediction of the effect of a compound on the slope and intercept of reciprocal plots for a given varied substrate [85, 111-113].

Initial velocity patterns are usually obtained by making reciprocal plots for one substrate (variable substrate) at different fixed concentrations of one of the others (changing fixed substrate) while keeping all other substrates, if there are any, at saturating and constant concentration. Thus, there are two possible initial velocity patterns: parallel lines when no reversible connection exists or lines intersecting to the left of the vertical axis when such a connection does exist [85, 115].

Cleland proposed and summarized the prediction of mechanism from bireactant initial velocity patterns (the oxidative deamination case) that ping pong mechanisms give the parallel initial velocity pattern and sequential mechanisms give the intersecting or occasionally the equilibrium ordered pattern [111]. In this work, the intersecting patterns obtained with the initial velocity experiments clearly indicated a sequential as opposed to a ping-pong mechanism (Figure 3.17). Thus, all of the substrates must be bound to the enzyme before any products are released. However, these data can indicate only that the addition of NAD^+ and L-phenylalanine is sequential but cannot clarify which substrate adds first or whether addition is in obligatory order.

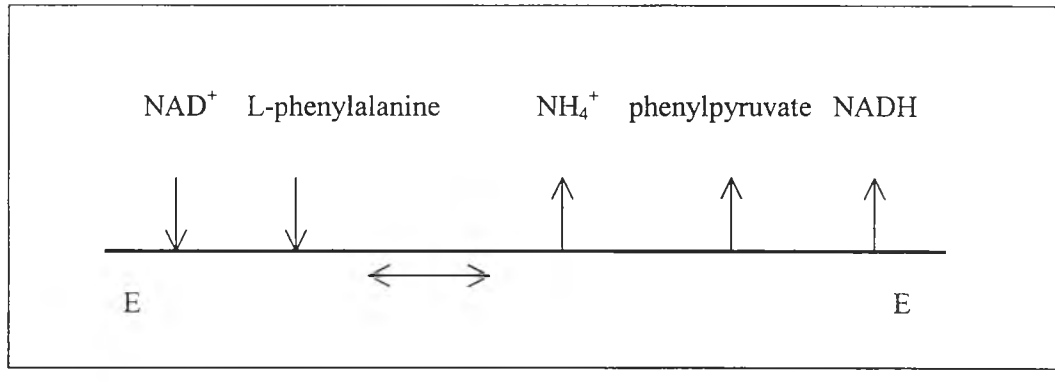

Figure 4.1 Proposed kinetic mechanism of phenylalanine dehydrogenase from *B. badius* BC 1

For the terreactant initial velocity patterns (the reductive amination case), Cleland explained by given an example model of the ordered addition of three substrates (A, B and C are substrates in that order) [85]. If substrate B is truly saturating, however, the reversible sequence is broken and the A-C initial velocity pattern becomes a parallel one whereas A-B and B-C initial velocity patterns will always be intersecting, regardless of the level of the other substrate. That means the ordered terreactant mechanism will show two intersecting and one parallel initial velocity patterns, which is similar to the patterns observed in our enzyme. In this work, the parallel initial velocity pattern obtained by varying NH_4^+ and NADH at a fixed, saturated level of phenylpyruvate (Figure 3.19) indicated that phenylpyruvate binds to the enzyme between NH_4^+ and NADH. That means phenylpyruvate is the middle substrate, which has also been observed in *R. maris* and *Rhodococcus* sp. PheDHs. Although it is possible to conclude something about the order of addition of reactants in which the middle substrate can be identified, but the first cannot be distinguished from the third. Thus, while much useful information can be obtained from initial velocity experiments, more complex types of kinetic studies are needed to work out the details of the kinetic mechanism.

Product inhibition studies

A compound that is a product of a reaction, react as a substrate in the reverse reaction and can inhibit the reaction by combining only with the enzyme is called a product inhibitor [112]. According to Cleland's fundamental rules, there are three basic types of product inhibitions determined by the effect on the slope and intercept of the double-reciprocal plot. Competitive inhibition refers to the case of the inhibitor binding exclusively to the free enzyme and not at all to the enzyme-substrate (ES) binary complex. This inhibition pattern is a series of intersecting lines on the 1/v-axis (slopes only vary). Uncompetitive inhibitor refers to the case of the inhibitor binding exclusively to the ES complex. This inhibition pattern is a series of parallel lines at different product concentrations (intercepts only vary). Noncompetitive inhibition refers to the case in which an inhibitor displays binding affinity for both the free enzyme and the ES complex. This inhibition pattern is a series of intersecting

lines on the left of the 1/v-axis, above, on, or below the horizontal axis (both slopes and intercepts vary). Inhibition patterns may be predicted in the same way as initial velocity patterns. Basically, the expected effects of the inhibitor on the slopes and intercepts of reciprocal plots are determined separately and then the results are combined to predict the inhibition pattern [85]. Cleland has suggested a set of rules to predict the type of inhibition expected for a steady-state mechanism. Applications of these rules are illustrated in the original article by Cleland [85, 111-113].

A simple ordered mechanism gives only one competitive product inhibition pattern due to the two reactants, the first substrate to add and the last product to come off, binding to the free enzyme. The two competitive inhibitions are consistent with either a Theorell-Chance or a random mechanism with dead-end complexes forming [85]. In the Theorell-Chance mechanism, an additional competitive inhibition pattern is obtained with the last substrate to add and the first product to be released. This is due to the rapid breakdown of the central complexes to undetectable levels, so that the enzyme forms to which these reactants bind appear to interconvert directly. For a rapid-equilibrium random mechanism, all product inhibitions are competitive unless products are able to bind to enzyme forms as dead-end inhibitors. In that case, they would produce apparently non-competitive inhibition patterns due to the combination of competitive product inhibition and uncompetitive dead-end inhibition.

As reported in Chapter III, product inhibition by NADH with respect to NAD^+ gave competitive inhibition demonstrated by lines intersecting on the 1/v axis. This is consistent with NAD^+ and NADH adding first and coming off last, respectively, as occurs in many other dehydrogenases with ordered mechanisms [4]. That means both the Theorell-Chance and the random mechanisms do not hold for this enzyme because only one competitve product inhibition pattern was obtained in this work. Therefore, the ordered mechanism fits best with the data. This inhibition pattern combining with the results from initial velocity patterns strongly support the ordered addition of the substrates in which NAD^+ binds the enzyme first and then follows by L-phenylalanine binding. About the ordered releasing of the products, it is predicted by the result from the competitive inhibition pattern which indicated that the last product is NADH and the results from the terreactant initial

velocity patterns which indicated that the middle product is phenylpyruvate. That means the rest of them, ammonium chloride, should be the first product to release. Therefore, the ordered releasing of the product is: NH_4^+, phenylpyruvate and NADH, respectively,

However, it is noted that the unexpected noncompetitive inhibition displayed by phenylpyruvate as a product inhibitor against L-phenylalanine suggests that phenylpyruvate could bind to the enzyme-NAD^+ complex and form the dead-end complex (abortive enzyme-NAD^+-phenylpyruvate complex). This same discrepancy has also been observed in kinetic mechanism of the *R. maris* [12], *Rhodococcus* sp. M4 [18], and *T. intermedius* [14] PheDHs, the *B. sphaericus* AlaDH [116] and the *Streptomyces cinnamonesis* ValDH [117]. In case of the *B. sphaericus* AlaDH, Ohshima *et al.* suggested that the presence of an abortive ternary dead-end inhibition by the formation of an enzyme-NAD^+-pyruvate complex may play an important physiological role in preventing the enzyme functioning in the L-alanine synthesis. In addition, the comparison of product inhibition patterns in various PheDHs is shown in Appendix L.

However, Asano *et al.* has been previously reported bacteria producing PheDH identified as *B. badius* IAM 11059 [11]. Nevertheless, all the results obtained in this work showed differences in properties of these two isolates especially in substrate specifications as summarized in Appendix M. Therefore, the PheDH from *B. badius* BC1 should be the novel PheDH which is a new alternative enzyme to potentially use and apply in the industrial and medical fields in the future.