CHAPTER IV

DISCUSSION

The use of biological systems to catalyze chemical reactions in commercial processes has recently become increasingly practical and affordable in many industries. Enzymes are natural catalysts that can speed up a chemical reaction. The enzymes are valuable tools in industrial processes as the reactions they catalyze generally proceed under mild conditions, high specificity, efficiency and their compatibility with the environment. Advances in screening technology, direct-evolution method and genetic engineering have greatly increased the availability of various enzymes for their application in food processing, pharmaceutical. Speciality enzymes are now increasingly used in the development of new drugs and antibiotic and analytical application (Gennari *et al.*, 1998). One of the most biocatalysts most used in industrial application is phenylalanine dehydrogenase (PheDH). It has been used for producing phenylalanine related amino acid from their keto analogues (Bruhuber and Blanchard, 1994 and Asano *et al.,* 1987c). Moreover, PheDH has been used in the diagnosis of phenylketonuria (PKU) and neonatal hyperphenylalaninaemia (Hummel *et al.*, 1984).

In the previous study, *phedh* gene from *Acinetobacter lwoffii* was successfully cloned and expressed in *E. coli* BL21(DE3) by Sitthai (2004). It was previously purified and some properties such as molecular mass, substrate specificity and production of amino acid were determined. Indeed, in the same year, Chumphukam (2004) successfully immobilized PheDH from *E. coli* JM 109 which contained *phedh* gene form *B. badius* BC1. Among various immobilization methods and supports, silica with glutaraldehyde as a cross-linking agent was found to be the most appropriate support and method. However, the immobilized activity of the immobilized enzyme was 0.14 U/g silica (1.05% of its original activity). Therefore, in order to improve the immobilization technique for PheDH, different immobilization methods and several carriers were investigated in this study and suitable carriers together with immobilization method were then selected. PheDH from recombinant *E.coli* BL 21(DE3) was partially purified and was used as an enzyme source for

immobilization. The optimization for immobilized enzyme was studied and some properties of the free and immobilized enzyme such as the optimum pH and temperature, pH stability, thermostability, storage stability and reusability were determined. Finally, the immobilized enzyme was applied to synthesize L-phenylalanine and various amino acids from their corresponding keto acids.

4.1 Partial purification of phenylalanine dehydrogenase from recombinant *E. coli*

In order to purify the PheDH, extraction procedure should be selected according to the source of enzyme. In this work, PheDH, an intracellular enzyme, was extracted from recombinant *E. coli* BL21(DE3) clone. Mechanical disruption methods are usually necessary to break down cell wall in order to release intracellular protein. The cell disintegration technique involved cell lysis by ultrasonication or high pressure sound waves, which causes cell breakage by cavitations and shear force, was used in this work. The extraction buffer consisted of phenylmethylsulfonyl fluoride (PMSF) and ethylenediamine tetraacetic acid (EDTA) as serine protease inhibitor and metalloprotease inhibitor, respectively. Because PMSF and EDTA could be used to retain desired protein that may be degraded by intrinsic catabolic enzyme when the cell is broken. 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. The enzyme activity may be lost during sonication, since mechanical cell disruption causes local overgesting with consequent denaturation of protein. To maximize recovery of the active enzyme, the extract and equipment were pre-chilled and several pauses of disruption was used instead of long continuous sonication because short interval of disruption can also minimizing denaturation (Harris and Angal, 1989 and Janson and Ryden, 1998).

Ammonium sulfate precipitation is usually used in the early step of purification procedure. 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). In preliminary experiment, ammonium sulfate precipitation was used. PheDH from *E. coli* BL21(DE3) was precipitated by 50-70% saturated ammonium sulfate. From this step of purification, about 68% of protein was removed while the activity remained 44%. When compared with the precipitation of recombinant *E. coli* from the study by Sitthai (2004), it was found that about 75% of protein were removed, however about half of enzyme activity was lost.

A series of chromatography steps that separate molecules according to their different molecule properties were applied to a purification of the PheDH from *E. coli* BL21(DE3). Ion exchange chromatography separates proteins with differences in charge to give a very high resolution with sample loading capacity. The separation is based on the reversible interaction between a charged molecule and an opposite charge of chromatographic medium. Molecules bind as they are loaded onto the column. Then, condition altered so that the bound substances are eluted differentially. Elution of bound proteins is usually performed by changes in salt concentration or pH of buffer with stepwise or continuous gradient. DEAE-Toyopearl is anion exchanger which has negatively charged counter-ions. It is widely used in the purification of PheDH from other sources such as PheDH from *B. badius, B. spaericus, S. ureae, T. intermedius* and *Microbacterium* sp. (Asano *et al.*, 1987a; Asano *et al.*, 1987b; Ohshima *et* al., 1991; Asaso and Tanetani, 1998). In this work, DEAE-Toyopearl 650M was used in the second step of purification. This column contributed greatly to the purification of PheDH from *E. coli* BL21(DE3), high % recovery and also high amount of protein removed. About 75% of other proteins were eliminated. The purity of enzyme was checked by native-PAGE. Three bands were observed with 1 major band corresponding to PheDH. In addition, SDS-PAGE showed relative molecular weight of PheDH subunit from recombinant clone which was determined to be about 44.5 kDa (**Figure 3.2 and 3.3**) and this was in agreement with the result of Sitthai (2004).

In conclusion, PheDH from recombinant *E. coli* was purified 2.6 fold with 20.2% yield from the cell free extract by a purification procedure involving ammonium sulfate precipitation and DEAE-Toyopearl column chromatography.

4.2 Effect of group specific reagents on phenylalanine dehydrogenase activity

As previously described in section 3.2, Chumphukam (2004), covalently immobilized PheDH by glutaraldehyde which is known to react with amino group of the enzyme, resulted in low immobilized activity yield. Consequently, to retain the catalytic activity of the enzyme in the immobilization state, the essential amino acid residues were determined.

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 analogues (Baker *et al.*, 1998), X-ray chromatography (Hirano *et al.*, 1991) or chemical modification (Bhattacharyya *et al.*, 1977) have been reported. Chemical modification using group-specific reagents is one of the most useful methods of identifying the functional group of a protein.

In this study, the screening of essential amino acid residues of enzyme, which was the initial phase of chemical modification study, was investigated. Chemical modification of nine amino acid residues, aspartic or glutamic, cysteine, histidine, lysine, methionine, serine, tryptophan and tyrosine have been selected because they are widely known as residues involved in enzyme catalysis (Ohshima and Soda, 2000). No inhibition of *Escherichai coli* BL 21 (DE3) PheDH activity was observed in the modification of tyrosine by *N*- acetylimidazole (NAI), aspartic or glutamic by ethylcarbodiimide hydrochloride (EDC) and cysteine by dithiothreitol (DTT). The result indicated that these amino acid residues were not important residues involved in enzyme catalytic activity. Very high inhibition was observed in the modification of serine by phenylmethylsulfonyl fluoride (PMSF). Moreover, modification with

N-bromosuccinimide (NBS), chloramine T (CT), diethylpyrocarbonate (DEPC) and 2,4,6-trinitrobenzene sulfonic acid (TNBS) which are known to react specifically with tryptophan, methionine, histidine and lysine residues, respectively, resulted in extensive inhibition of enzyme activity. This result concluded that OH group of serine, indole group of tryptophan, thioether group of methionine, imidazole group of histidine 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 was only the preliminary study of amino acid residues involved in enzyme catalysis. It cannot prove that these amino acid residues involved in PheDH activity are located within active site or not. The substrate protection experiments are necessary and should be performed for further studies because these group-specific compounds, covalently modify the accessible amino acid in general way. Thus, 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, it means that the amino acid residue is in the active site of the enzyme.

Previously, these crucial amino acid residues have also been demonstrated as essential residues for other PheDHs and amino acid dehydrogenase (Ohsima *et al.*, 2000; Onprasert, 2000; Leksakon, 2001). Kataoka *et al.* (1994) reported that lysine residues of PheDH from *T. intermedius* was modified by methyl acetyl phosphate and proposed to be involved in substrate binding and catalysis. In addition, Vanhooke *et al.* (1999) have been reported that enzymatic discrimination between imino and keto acids as substrates for reduction is a consequence of the hydrogen-bonding and electrostatic interations of the substrate and intermediates with lysine 78 and aspartate 118 in PheDH from *Rhodococcus* sp. M4. Thus, they concluded that lysine 78 and aspartate 118 function as the catalytic residues in the active site of this enzyme (Brunhuber *et al.*, 2000).

According to these results, lysine residues are involved in enzymatic catalysis of PheDH. Thus, for the immobilization of PheDH the amino acid residues used for the attachment of the enzyme to the support should be considered.

4.3 Selection of a suitable solid support and an appropriate method for surface activation

The key of the technique to immobilize enzyme is to prepare support material with high property and the physical chemical properties of support materials will decide the activity of immobilized enzyme to great extent. The support materials need to have binding power towards enzymes under mild conditions, either physical adsorption or chemical covalent bonding, so that enzymes can be immobilized (Gao *et al.*, 2006). Many organic and inorganic substances have been used as the support materials. PheDH was immobilized on a series of supports including alumina, silica, chitosan, epoxy-alumina and epoxy-chitosan. Three methods of immobilization, via its amino groups, carboxylic groups and ionic interaction, were examined.

In the present study, to confirm the result from the study by Chumphukam (2004), the optimum conditions for PheDH immobilization on silica by glutaraldehyde were re-investigated.

γ-Aminopropyltriethoxysilane (APTS) was chosen as silanization agent to introduce amino group onto the surface of silica and the resulting alkylamine derivative was activated by glutaraldehyde (Chibata, 1978). Silinization procedure is one of the most popular derivatisation methods of inorganic materials. In **Figure 3.6**, it showed that the suitable APTS concentration for silanization of silica was 1% (v/v). Change in APTS concentration did not have any effect on immobilized enzyme activity. Curiously, silanization with low APTS concentration (at 1%) presented a higher immobilized activity. The APTS concentration that has been reported was normally in the range 2-10% depending on the enzyme and support employed (Subramanian *et al.*, 1999; Park *et al.*, 2005; Wang and Jin, 2004; Blasi *et al.*, 2005).

Glutaraldehyde is most commonly employed as the functional reagent, and many enzymes have been immobilized by the formation of Schiff bases between the amino group of carriers and of enzyme protein (Chibata, 1978). In order to achieve

support with different numbers of aldehyde groups available to react with the enzyme (amine), the silica was initially saturated with silane. Therefore, the degree of activation of the support could be controlled with different glutaraldehyde concentrations, the enzyme activity was significantly altered. From **Figure 3.7**, it can be seen that when the amount of glutaraldehyde was 0.25% (v/v), the immobilized enzyme showed the highest activity. However, when the amount of glutaraldehyde concentration was over 0.25% (v/v), the activity of the immobilized enzyme decreased. This fact indicated that overfull covalent linking between the support and enzyme molecules resulted in the change in enzyme conformation and thus, enzyme activity was decreased (Gao *et al.*, 2005).

The coupling time is one of the immobilized parameters to be considered and is presented in **Figure 3.8**. It was found that the remaining activities of the immobilized enzyme were less significant in the range of 0.5-6 hours. The activity recovery of the immobilized PheDH increased with prolonged incubation time and the highest immobilized activity was obtained under immobilization allowed to proceed for 8 hours. However, immobilized activity decreased if the incubation time was more than 8 hours because enzyme denaturation or conformation change in tertiary structure may occur during the immobilization reaction (Liang *et al.*, 2005).

Different amount of PheDH was introduced to carry out the enzyme immobilization. The result is shown in **Figure 3.9**, indicating that the retaining activity of immobilized PheDH was highest at 18 units of PheDH with 0.102 u/g support of immobilized activity. The immobilized activity did not increase due to the reaction groups on the support appear to be saturated. This result is in agreement with the result obtained by Prousoontorn *et al.*,(2007) and Chumphukam (2004).

In conclusion the optimal condition for the immobilization of PheDH on silica using glutaraldehyde was to activate silica with 1% (v/v) of APTS, 0.25% (v/v) of glutaraldehyde and 18 units of PheDH was then added and incubated at 4°C for 8 hours. When compared with the study by Chumphukam (2004), the optimum condition to immobilize PheDH was to activate silica with 6% (v/v) APTS, 0.1%

(v/v) glutaraldehyde by using an enzyme to support ratio of 16 units per gram silica (1.05% yield) and incubated at 4°C for 6 hours. This may be due to the different sources of enzyme. In this study, enzyme was produced from *E. coli* BL21(DE3) bearing *phedh* gene of *Acinetobacter lwoffii* whereas Chumphukam (2004) used *E. coli* JM 109 which contained the *phedh* gene from *Bacillus badius* BC1.

To improve the immobilized activity yield, another support activation method that used 1,6-diaminohexane was carried out. In this study, 1,6-diaminohexane was used to immobilize of PheDH on epoxy-alumina and epoxy-chitosan. These epoxy-supports were synthesized from 1,4-butane dioldiglycidyl ether, and then was modified to contain amino groups using 1,6-diaminohexane (to form a six carbon spacer-arm). PheDH was covalently immobilized on aminated supports using glutaraldehyde via Schiff base formation. Very low immobilized activity was observed in both supports (**Figure 3.12**). Covalent binding of glutaraldehyde to the enzyme may result in the wrong orientation of the immobilized enzyme and glutaraldehyde may bind to the amino groups of lysine residues which involve in the enzyme activity, are the probable reasons. Unsatisfied result was also observed with other enzymes when immobilize using this activation method. Mohy Eldin *et al.* (2000) immobilized penicillin G acylase onto nylon particle. Only 12% of the immobilized activity was retained. Unlike the study of Arica *et al.* (2003), tyrosinase was covalently immobilized onto modified epoxide groups of the poly (methyl methcrylate-glycidyl methacrylate-divinyl benzene) (poly(MMA-GMA-DVB)) with 1,6-diaminohexane and glutaraldehyde. The activity yield of the immobilized tyrosinase was 68% and the immobilized enzyme had resistance to temperature inactivation as compared to that of the free form. In 2005, *Candida rugosa* lypase was covalently immobilized on poly (glycidylmethacrylate-2-hydroxyethylmethacrylate-ethylene glycoldimethacrylate) (poly (GMA-HEMA-EGDMA)) with or without spacer-arm attachment poly (GMA-HEMA-EGDMA) microspheres with glutaraldehyde (Bayramoğlu *et al.*, 2005). The 1,6-diaminohexane was used to attach onto epoxy groups carrying poly(GMA-HEMA-EGDMA) microspheres. The maximum lipase immobilization capacities of the poly (GMA -HEMA-EGDMA) and

poly (GMA-HEMA-EGDMA)-spacer-arm attachment microspheres were 16.1 and 28.3 mg/g, respectively. The attachment of the spacer-arm resulted in an increase in the apparent activity of the immobilized lipase with respect to the enzyme immobilized via the epoxy groups of the microspheres. The activity yield of the lipase immobilized on the spacer-arm attached microsphere was up to 45% and this was 9% for the enzyme directly immobilized through epoxy groups.

A part from immobilization via the enzyme' amino group, the introduction of cationic to the carrier using polyethyleneimine (PEI) was another approach. This carrier presents primary amino groups and fully reduces the hydrophobicity of the carrier. These carriers act as cationic exchangers and the presence of primary amino groups turn into a good option for the enzyme immobilization through glutaraldehyde techniques (López-Gallego *et al.*, 2005). On the other hand, PEI has been shown to be effective for immobilization of biocatalysts to solid support by adsorption (Anderson and Hatti-Kaul, 1999). The majority of amino groups on the molecular chains of PEI are in protonated state in aqueous solution of pH <10, so PEI is kind of cationic poly electrolyte (Gao *et al.*, 2006). PheDH has an isoelectric point (pI) in range 3.5-5.8 (**Table 1.1**), so PheDH is negatively charged under the pH 7.0 of phosphate buffer. Therefore, the support can produce strong physical adsorption towards enzyme molecules by right of electrostatic interaction.

Immobilization of PheDH via ionic adsorption on PEI coated support (cationic support) showed a higher immobilized activity in all supports (silica, alumina, chitosan and epoxy-alumina). However, when treated with glutaraldehyde the immobilized activity was decreased (**Figure 3.14**). In this case, all the primary amino group (lysine moiety) of the enzyme and the support will be interacted with glutaraldehyde, inappropriate orientation of enzyme may be obtained and subsequently affect the immobilized enzyme activity. In addition, glutaraldehyde may be reacted with lysine amino groups in the active site of the enzyme and caused a decrease in the enzyme activity.

Other reports on the use of PEI in conjunction with glutaraldehyde have been reported. In the study of Atia (2005), cyclohexanone monooxygenase (CHMO) and glucose-6-phosphate dehydrogenase (G6PDH) were co-immobilized onto PEI-porous agarose polymeric composites. The activity of the co-immobilized enzymes was 87.2% with a CHMO/G6PDH ratio 2:1. In the same year López-Gallego *et al.* (2005), immobilized D-amino acid oxidase (DAAO) on sepabeads EA using ionic adsorption on the cationic sepabeads support and covalent attachment using glutaraldehyde. The result showed that the immobilization on sepabeads EA activated with glutaraldehyde led to a decrement in enzyme activity (the expressed activity was only 23%), while the adsorption on sepabeads EA did not have any effect on the activity of enzyme. However, the glutaraldehyde treatment of DAAO adsorbed on sepabeads EA showed a higher stability than the adsorbed DAAO. In the year 2005, the same researchers reported the immobilization and the stabilization of glutaryl acylase (GAC) on sepabeads EA by the glutaraldehyde method. The immobilization yield of GAC adsorbed on the support was 100% and the expressed immobilized activity was 90%. Similarly, the treatment with glutaraldehyde presented a low impact on the immobilized activity (activity recoveries were over 80%) and greatly improved the enzyme stability. A similar treatment using GAC adsorbed on sepabeads EA did not give rise to stabilization, suggesting that this stabilization was due to a reaction between the enzyme and the support through the glutaraldehyde chemistry (Alonson *et al.*, 2005).

Immobilization of enzyme via their carboxylic groups may be an excellent method for preparation industrial derivatives (e.g., industrial enzymes, immobilized antibodies, and so on). In fact, the sum of Asp and Glu residues usually constitutes the major fraction of surface groups and thus, could be used for immobilization of enzyme via very simple coupling method (Fernandez-Lafuente *et al.*, 1993). In this work, a water-soluble carbodiimide was employed to catalyze the formation of amine bonds between –COOH and $-NH_2$ by activating the carboxylate to form an *O*-acylurea (Kang et al., 2006).

Immobilization of PheDH via carboxylic groups which was activated with carbodiimide (EDC) resulted in the great amount of enzyme activity when immobilized on aminated silica and chitosan but very low activity was observed when immobilized on aminated alumina support (**Figure 3.16**). These results corresponding with the study of amino acid residues involved in enzyme activity in that, no inhibition of PheDH activity was observed when Asp and Glu were modified with EDC. However, in our experiment, the suitable support selected was silica. Although, chitosan offered a unique set of characteristics, it was difficult to perform the immobilization steps due to its light feature. Silica was then chosen among various kinds of carriers. Silica supports are commonly used in enzyme immobilization (Martin *et al.*, 2000). They are inexpensive and can be satisfactorily employed in continuous reactors (Abraham, 1995). Silica has been used previously for the immobilization of other enzymes, for example GluDH (Cordek *et al.*, 1999), CGTase (Martín *et al.*, 2002; Blasi *et al.*, 2005, Prousoontorn *et al.*,(2007), α-amylase and glucoamylase (Park *et al.*, 2005).

In conclusion, silica was selected for immobilization of PheDH from recombinant *E. coli*. The method used was to immobilize via its carboxylic groups.

4.4 Optimization of immobilization of phenylalanine dehydrogenase

Since the efficiency of PheDH immobilization depends on various parameters such as activation time, a mount of functional groups created on the support, EDC concentration, coupling time and the concentration of enzyme per support ratio, these parameter were then optimized.

The variation of incubation time for EDC activation of surface carboxylic group of PheDH was investigated in the range of 1-10 hours. The results can be seen in **Figure 3.18**. The desirable time for activation that gave the highest immobilized activity was 6 hours. However, immobilized activity decreased if the activation time

was longer. This could be due to the increasing process of deactivation and carboxylic group of the enzyme may be fully modified with EDC.

The immobilized efficiency was also affected to some extent by the coupling time between the enzyme and support. It was found that the retained immobilized activity of PheDH increased with prolonged reaction time and the highest immobilized activity was obtained when immobilization was allowed to proceed for 21 hours (**Figure 3.19**). The shorter incubation time may result in less contact between enzyme and activated support. In some cases, at longer period of incubation may cause multipoint attachment of the enzyme molecule to the support and overcrowding the immobilized enzyme in the pore space of the support. As a result, substrate diffusion limitation could occur (Bayramoğlu *et al.*, 2008).

γ-Aminopropyltriethoxysilane (APTS), the common silanization reagent can be quickly chemisorbed onto the surface of silica containing –OH group via strong covalent bonds of Si-O-Si. APTS also introduced amino group onto the surface of silica support. From the results obtained (**Figure 3.20**), when increasing APTS concentration the immobilized enzyme activity was decreased. The suitable APTS concentration for silanization of silica was 2% (v/v).

Carbodiimide (EDC) was used to activate carboxylic groups of PheDH. **Figure 3.21**, showed the effect of EDC concentration on the immobilized enzyme activity. The results indicated that, the optimum EDC concentration for PheDH activation was 10 mM which was the same concentration used in the chemical modification experiment. The immobilized activity decreased with the enhancement of EDC concentration. The lost of activity could be due to the COOH groups of Asp and Glu residues were modified with EDC, resulted in an almost complete lost of enzyme activity. This also agrees with a report published by Fernandez-Latuente *et al.* 1993 and Matrin *et al.* 2002.

A major influence on the efficiency of PheDH immobilization on aminated silica was PheDH concentration as shown in **Figure 3.22.** The retained immobilized activity was highest at 25 units of PheDH per 500 mg support with relative immobilization yield of 5.17 %, the immobilized enzyme activity did not seem to increase in its activity when the amount of enzyme increased. This could be due to the reactive groups on the support appear to be saturated.

Through various single factor experiments, the appropriate immobilization conditions to immobilize PheDH on silica have been determined, as follows: 10 mM of EDC was used to activate PheDH for 6 hours. Then 25 units of the activated PheDH were incubated for 21 hours with silica (500 mg) which was previously been activated with 2% (v/v) APTS. Under these optimized condition the immobilized activity yield was 5.17%.

4.5 Properties of the immobilized phenylalanine dehydrogenase on silica

Information on the changes of enzymatic properties caused by the immobilization of enzymes is useful not only for the application of immobilized system but also for the elucidation of structure function relationships and the mechanism of enzyme reaction. Changes of enzymatic properties are considered to be caused by the following two factors. One is change of enzyme itself, and the other is due to the physical and chemical properties of the carriers used for immobilization. The former involves the modification of amino acid residues in the active center of the enzyme, the conformational changes of the enzyme protein, and the changes of charge on enzyme, while the latter involves the formation of diffusion layers around immobilized enzyme (Chibata, 1978).

4.5.1 Effect of pH on activity and stability of free and immobilized phenylalanine dehydrogense

The enzymatic activity is markedly affected by environmental condition, especially the pH of the aqueous media, due to the protein nature of the enzymes. When enzymes are immobilized, the optimum pH of the enzyme can be changed or unchanged. In general, immobilization would result in the shift of optimal pH of the enzyme (Hong et al., 2002). However, this phenomenon was not observed in the present case. The pH profiles for free and immobilized enzyme were almost the same. The optimum pH was at 9.5 for free and immobilized PheDH (**Figure 3.23**). This suggested that the optimum pH value for enzyme activity had no marked changes after being bound the support (Hong *et al.*, 2007). Both of the optimum pH and pH profile of immobilized enzyme were the same as those of native enzyme in the following cases: immobilized chymotrypsin, trypsin and papain prepared by diazo binding with amino acid copolymer. Immobilized ribonuclease (Ryu *et al.*, 1972) and thrombokinase (Kamogashira *et al.*, 1972) prepared with CNBr-activated Sepharose.

One of the properties that have been generally considered to be improved via immobilization was enzyme pH stability. The pH stability of the immobilized PheDH on silica by covalent coupling was studied and compared with that of the native enzyme. The result is shown in **Figure 3.25**. The pH profile of the immobilized PheDH was broader than that of the free enzyme in the pH range 5 to 12, which means that the immobilization methods preserved the stability of PheDH in the alkaline region. According to the study of Tien-Chieh and Chiou (2003 and 2004), the pH stability of immobilized lipase on chitosan by activating its hydroxyl group with carbodiimide was also enhanced in a wider pH range.

4.5.2 Effect of temperature on activity and stability of free and immobilized phenylalanine dehydrogense

The catalytic activity of enzymes increases with elevation of temperature, as in the case of usual chemical catalysis. However, the enzyme reaction cannot be practically carried out at high temperature (Chibata, 1978). Effect of temperature on the activity of the free and immobilized PheDH was studied in the temperature range of 25 to 75°C. Typical results are shown in **Figure 3.24**. The optimum reaction temperature (40°C) of the PheDH was not altered by immobilization. However, the temperature profile for immobilized PheDH was broader indicating that the enzyme activity become less dependent on the temperature after immobilization.

The thermal stability of an immobilized enzyme is one of the most important criteria of their application. The thermal stabilities of the free and immobilized PheDH in term of the residual activities are compared in **Figure 3.26**. The activity of both enzymes decreased when increasing temperature. The immobilized PheDH was inactivated at a much slower rate that than of the native enzyme. At 45°C, the immobilized enzyme retained their activity about 54% whereas free enzyme retained only 20% of its activity. In general, the activity of the immobilized enzyme, especially in the covalently bound system, is more resistant than that of soluble form against heat (Arica *et al.*, 2003). The thermal stability of many immobilized enzyme has been studied, and there are examples showing increases (Tien-chieh *et al.*, 2003; Villalonga *et al.*, 2005; Bayramoğlu *et al.*, 2005; Hong *et al.*, 2007) , no change (Tosa *et al.*, 1969; Barth and Maskova, 1971) and decreases (Fujishima and Yoshino, 1967) of thermal stability after immobilization.

4.5.3 Storage stability

The storage stability of immobilized enzyme is one of the most important parameters to be considered for the application of immobilized enzyme. The storage stability of free and immobilized enzyme at 4°C and room temperature are depicted in **Figure 3.27**. At room temperature, the activity of free and immobilized PheDH decreased considerably in 5 and 10 days, respectively, but the immobilized enzyme decreased slower than that of the free enzyme. The covalent immobilization definitely holds the enzyme in a stable position in comparison to the free counterpart (Canofeni *et al.*, 1994). When both enzymes were stored at 4°C, it was found that the lost of their activity was slower. On the other hand, decrease of storage stability on immobilization has been reported. The remaining activity of naringinase immobilized by covalent binding with styrene-maleic anhydride copolymer was 50% of the initial activity after storage for a week at 4°C (Cohen *et al.*, 1974).

4.5.4 Batch reusability of immobilized phenylalanine dehydrogenase

Besides the storage stability, the reusability of immobilized enzyme is one of the most important factors affecting the success of industrialization of immobilized system. To evaluate the operational stability, the immobilized enzyme was washed with 10 mM potassium phosphate buffer, pH 7.0 after each cycle and reintroduced into a fresh reaction mixture for the production of L-phenylalanine. The immobilized PheDH was used to produce L-phenylalanine 3 times with the incubation time of 6 hours in each cycle. The relative activity and the production yield are shown in **Figure 3.28**. The immobilized PheDH retained 84% of its original activity after 3 repeated uses with the production yield in the range of 58.0-62.7%. The leakage of protein from support's surface during stirring and denaturation or conformation changes caused by the surrounding conditions such as buffer solution may be a main reason for the loss of activity. Activity retention of 11% after 3 repeated uses (Fadiluglu *et al.*, 1998), 27% after seven reuses (Mojovic *et al.*, 1998) also has been reported.

4.6 Synthesis of amino acid from their keto acids using immobilized enzyme

Utilization of L-amino acid in medicine, food product has been developed rapidly in recent year, and the economical production of optically active amino acids has been investigated extensively (Chibata, 1987).

Immobilized PheDH from *E. coli* BL21(DE3) can catalyze reductive amination of many keto acids with high relative activity to phenylpyruvate, the possibility to use these keto acids as substrate for synthesis of their corresponding amino acids was performed. (Appendix H). In this experiment, the identification and semiquatitation of amino acids production could be performed by TLC techniques. Amino acids samples were subjected to TLC plate. As the solvent font passes the sample spots the compounds were moved up the plate dependent on characteristics of their functional groups, size and interaction with the cellulose matrix. The ratio of the distance a compound moves from the baseline to the distance of the solvent front from the baseline is defined as the retardation factor (R_f). Different amino acids usually have different R_f under suitable conditions (Nelson and Cox, 2000). After that, spots were developed with 0.5 % ninhydrin solution, which reacted with amino group of amino acid to give purple. The result indicated that the R_f value of product from each enzyme reaction was not different from its corresponding amino acid standard, L-phenylalanine, L-norleucine, L-leucine, L-norvaline and L-methionine **(Figure 3.30 and Table 3.3)**.

The immobilized enzyme showed the production yield of 70.9% conversion of phenylpyruvate to L-phenylalanine. α-ketocaproate, α-ketoisocaproate, α-ketovalerate, and α-keto-γ-methiol-butyrate were converted to their amino acids with 96.7%, 80.4%, 62.5% and 100% conversion, respectively. When compared to the study by Chumphukam (2004), the immobilized PheDH form *E. coli* JM109 showed the production of amino acids from their keto substrates with various % conversions from 63.7 to 100%. Sitthai (2004) used PheDH from *A. lwoffii* for the synthesis of L-phenylalanine and related amino acids such as valine, norleucine, methionine,

norvaline, leucine, α-aminobutyrate and isoleucine, the production yield was in the range of 36.0-72.2%. Asano and Nakayama (1987) used PheDH from *Sporosarcina ureae* coupling with formate dehydrogenase from *Candida boidinii* for the synthesis of various amino acids. At the optimal condition for amino acid synthesis, yield of L-phenylalanine, L-tyrosine, L-tryptophan, L-methionine, L-valine, L-leucine and L-isoleucine were 98, 99, 11, 87, 97, 83 and 48 %, respectively. Later, in the same year, Hummel *et al*, used *Rhodococcus* sp M4 in enzyme membrane reactor for phenylalanine production with 95% conversion. The different degree of L-phenylalanine production could be attributed to different sources of enzymes and different production condition used. It can be seen that PheDH exhibited specificity for oxidative deamination more than reductive amination. However, it has to mention that the condition for the synthesis of the amino acids in this study has not yet been optimized. In addition, substrate specificity of PheDH from *A. lwoffii* in the free form was also studied (Sitthai, 2004). In the reductive amination, the enzyme greatly acted on α-ketocaproate, α-keto-γ-methiol-n-butyrate, α-ketovalerate and α-keto isocaproate, respectively when compared with phenylpyruvate. This result showed that aliphatic keto acids were preferable substrates for reductive amination of the enzyme and this was in good agreement with our study.

The result obtained from this research indicated that the immobilized PheDH showed high possibility to be used for the synthesis of L-phenylalanine and related amino acids. Although the condition for L-phenylalanine production using immobilized enzyme have yet to be further optimized.