การทำให้บริสุทธิ์และลักษณะสมบัติของฟีนิลอะลานีนดีไฮโดรจิเนส จาก *Bacillus lentus*

นางสาวศิวพร อินเกื้อ

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

PURIFICATION AND CHARACTERIZATION OF PHENYLALANINE DEHYDROGENASE FROM *Bacillus lentus*

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Bacillus lentus เป็นแบคทีเรียที่สามารถผลิตฟีนิลอะลานีนดีไฮโดรจิเนส โดยใช้ NAD^+ เป็นโคเอนไซม์ ภาวะที่เหมาะสมในการผลิตฟีนิลอะลานีนดีไฮโดรจิเนสคือ การเลี้ยงในอาหารเหลวเปปโทน 1 เปอร์เซ็นต์ pH 7.0 ที่เสริมด้วยแอล-ฟีนิลอะลานีน 0.4 เปอร์เซ็นต์ ที่อุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 18 ชั่วโมง การทำเอนไซม์ให้บริสุทธิ์โดยคอลัมน์ดีอีเออีโทโยเพิร์ล คอลัมน์บิวทิลโทโยเพิร์ลและคอลัมน์เซฟาเด็กซ์ จี -200 พบว่าเอนไซม์มีแอคติวิตีคงเหลือ 18.4 เปอร์เซ็นต์และมีความบริสุทธิ์ขึ้น 166.3 เท่า เอนไซม์มีน้ำหนักโมเลกุลประมาณ 360,000 ดาลตันโดยวิธีของ Ferguson และประกอบด้วย 8 หน่วยย่อยน้ำหนักโมเลกุลเท่ากันคือ 41,000 ดาลตัน เอนไซม์มีความจำเพาะสูงมากต่อแอล-ฟีนิลอะลานีนซึ่งเป็นสับสเตรทในปฏิกิริยา oxidative deamination และฟีนิลไพรูเวทซึ่งเป็นสับสเตรทในปฏิกิริยา reductive amination เอนไซม์มีความจำเพาะต่อ 3-อะเซทิลไพริดีน ไดนิวคลีโอไทด์มากกว่า NAD^+ ประมาณ 1.74 เท่า pH ที่เหมาะสมในการเร่งปฏิกิริยา oxidative deamination และ reductive amination คือ 10.4 และ 8.5 ตามลำดับและอุณหภูมิที่เหมาะสมในการเร่งปฏิกิริยาคือ 50 และ 55 องศาเซลเซียส เอนไซม์มีความเสถียรต่อ pH ในช่วง 6.0 ถึง 12.0 และมีความเสถียรต่ออุณหภูมิค่อนข้างสูงโดยไม่สูญเสียแอคติวิตีเมื่อบ่มเอนไซม์ที่ 50 องศาเซลเซียส เป็นเวลา 4 ชั่วโมงและเอนไซม์ยังคงมีแอคติวิตีเหลืออยู่ 50 เปอร์เซ็นต์เมื่อบ่มที่อุณหภูมิเดียวกันนี้เป็นเวลา 3 วัน เอนไซม์ถูกยับยั้งอย่างสมบูรณ์ได้ด้วยซิลเวอร์ไนเตรทที่ความเข้มข้นสุดท้าย 1 มิลลิโมลาร์ ดี-ฟีนิลอะลานีน ดี-เมทไธโอนีน ดี-ลิวซีน ดี-ทริปโตเฟน ไฮโดรซินนาเมทและพารา-ไฮดรอกซีฟีนิลอะซิเตตสามารถยับยั้งปฏิกิริยา oxidative deamination เมื่อใช้แอล-ฟีนิลอะลานีนเป็นสับสเตรท เอนไซม์มีค่า K_m ต่อแอล-ฟีนิลอะลานีน NAD^+ ฟีนิลไพรูเวท แอมโมเนีย และ NADH เท่ากับ 0.59, 0.55, 0.18, 300 และ 0.09 มิลลิโมลาร์ ตามลำดับ จากการศึกษาจลนพลศาสตร์ของเอนไซม์ตลอดจนการยับยั้งโดยผลิตภัณฑ์ของปฏิกิริยาแสดงให้เห็นกลไกของปฏิกิริยา reductive amination เป็นแบบ sequential ordered ternary-binary mechanism ซึ่งมีลำดับของการจับของสับเตรทคือ NADH จับกับเอนไซม์ก่อนตามด้วย ฟีนิลไพรูเวทและแอมโมเนีย แล้วจึงปล่อยฟีนิลอะลานีนและ NAD^+ ตามลำดับ

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สาขาวิชา.....เทคโนโลยีชีวภาพ..... ลายมือชื่อนิสิต .....ศิวพร อินเกื้อ.....

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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....-.....

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KEY WORD : PHENYLALANINE DEHYDROGENASE / *Bacillus lentus* / PURIFICATION / CHARACTERIZATION

SIWAPORN INKURE: PURIFICATION AND CHARACTERIZATION OF PHENYLALANINE DEHYDROGENASE FROM *Bacillus lentus*. THESIS ADVISOR: ASST. PROF. KANOKTIP PACKDIBAMRUNG, Ph.D., 119 pp. ISBN 974-14-2326-8

Bacillus lentus is a NAD^+-dependent phenylalanine dehydrogenase (EC 1.4.1.20) producing bacteria. The optimum condition for the enzyme production was 18 hours of cultivation in 1% peptone medium, pH 7.0 supplemented with 0.4% L-phenylalanine at 37°C. The enzyme was purified to homogeneity by DEAE-Toyopearl, Butyl-Toyopearl and Sephadex G-200 column chromatography with 18.4% yield and 166.3 purification fold. The enzyme had a molecular mass of about 360,000 Da as determined by Ferguson plots and consisted of 8 identical subunits. The enzyme showed high substrate specificity in the oxidative deamination on L-phenylalanine and the reductive amination on phenylpyruvate. The NAD^+ analog 3-acetylpyridine-NAD^+, gave 1.74 times higher activity than its natural coenzyme, NAD^+. The optimum pH for the oxidative deamination and the reductive amination were 10.4 and 8.5, respectively and optimum temperature were 50°C and 55 °C, respectively. The enzyme was stable over a broad pH range of 6.0 and 12.0. No loss of the enzyme activity was observed upon incubation at 50°C for 4 hours. The enzyme retained 50% of the activity after incubation at the same temperature for 3 days. The enzyme activity was inactivated completely by $AgNO_3$ at a final concentration of 1 mM. D-Phenylalanine, D-methionine, D-leucine, D-tryptophan, hydrocinnamate and *p*-hydroxyphenylacetate inhibited the oxidative deamination of L-phenylalanine. The apparent K_m values for L- phenylalanine, NAD^+, phenylpyruvate, NH_4Cl and NADH were 0.59, 0.55, 0.18, 300 and 0.09 mM, respectively. Initial-velocity and product inhibition studies showed that the reductive amination proceeded through a sequential ordered ternary-binary mechanism. NADH bound first to the enzyme, followed by phenylpyruvate and then NH_4Cl, and the products were released in the order of L-phenylalanine and NAD^+.

Field of study.....Biotechnology.....
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Student's signature.....Siwaporn Inkure.....
Advisor's signature.....Kanoktip Packdibamrung.....
Co-advisor's signature .....

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ABBREVIATIONS

μg	microgram
μl	microliter
A	absorbance
BSA	bovine serum albumin
Da	Dalton
DEAE	diethylaminoethyl
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
h	hour
KCl	potassium chloride
kDa	kiloDalton
K_m	Michaelis constant
KCl	potassium chloride
KOH	potassium hydroxide
M	molar
MW	molecular weight
NAD^+	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
NH_4Cl	ammonium chloride
$(NH_4)_2SO_4$	ammonium sulfate
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PheDH	phenylalanine dehydrogenase
pI	isoelectric point
PMSF	phenyl methyl sulfonyl fluoride
SDS	sodium dodecyl sulfate
TEMED	*N, N, N′, N′*-tetramethyl ethylene diamine
v/v	volume by volume
w/w	weight by weight

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CHAPTER I

INTRODUCTION

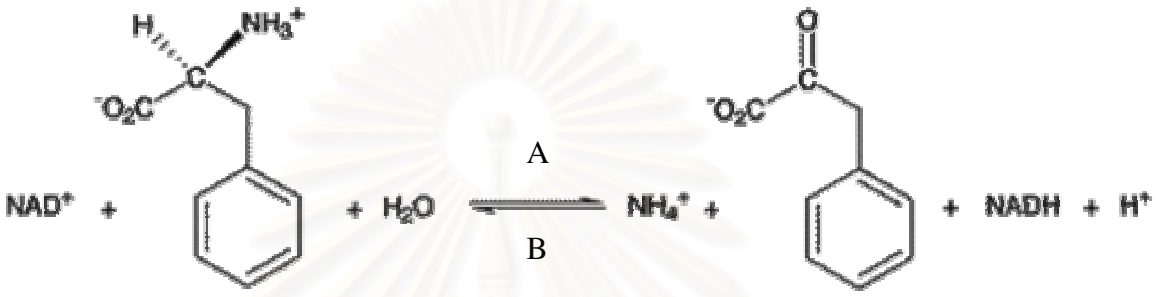
The amino acid dehydrogenases (EC 1.4.1.-) are a family of enzyme that is part of the oxidoreductase superfamily. They catalyze the reversible deamination of amino acids to their corresponding keto acids in the presence of the pyridine nucleotide coenzymes, NAD^+ and / or $NADP^+$. The amino acid dehydrogenases are categorized based on the specificity they display toward their amino acid substrate. About 17 different amino acid dehydrogenases have been so far found in various kinds of organisms as shown in Table 1.1 (Ohshima and Soda, 2000). The amino acid dehydrogenase has considerably commercial potential for the chiral synthesis of natural amino acids as well as novel nonproteogenic amino acid using in the pharmaceutical industry. It is also used as diagnostic reagents to monitor the serum level of amino acids which accumulate in a range of metabolic diseases (Baker *et al.,* 1997).

1.1 Phenylalanine dehydrogenase

Phenylalanine dehydrogenase (L-phenylalanine: NAD^+ oxidoreductase, deaminating: EC 1.4.1.20) (PheDH) is one of the amino acid dehydrogenases, which catalyzes the reversible pyridine nucleotide-dependent oxidative deamination of L-phenylalanine to form ammonia, phenylpyruvate, and NADH as shown in Figure 1.1 (Brunhuber and Blanchard, 1994). Much attention had been paid to this enzyme because it is useful as an industrial catalyst in the asymmetric synthesis of L-phenylalanine and related L-amino acids from their keto analogs, and also in the clinical and pharmaceutical applications.

1.2 Isolation of phenylalanine dehydrogenase

PheDH was first discovered in 1984. Hummel *et al*. screened for a PheDH producing strain from soil and isolated *Brevibacterium* sp. by an enrichment culture technique (Hummel *et al*., 1984). Subsequently, Asano *et al*. screened the enzyme



L-phenylalanine **Phenylpyruvate**

Figure 1.1 Reaction of phenylalanine dehydrogenase

A Oxidative deamination

B Reductive amination

Source : Brunhuber and Blanchard, 1994

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

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

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

Source: Ohshima and Soda, 2000

activity among a number of microorganisms from soil samples. It was discovered that the enzyme activity was very narrowly distributed in aerobic spore-forming, gram-positive mesophiles, *Bacillus badius* (Asano *et al*., 1987a), *Sporosarcina ureae* and *Bacillus sphaericus* (Asano *et al*., 1987b). Later, this enzyme was found in *Rhodococcus* sp. (Hummel *et al*., 1987), *Rhodococcus maris* (Misono *et al.*, 1989) and *Norcardia* sp. (De Boer *et al*., 1989). The PheDH from mesophilic bacteria are not stable enough for industrial and clinical application. Therefore, thermostable enzyme was focused in this field. The thermostable PheDH has been studied by Ohshima *et al*. (1991) in *Thermoactinomyces intermedius*. The last PheDH was found in non-spore forming mesophilic bacteria, *Microbacterium* sp. (Asano and Tanetani, 1998). The occurrence of the *Microbacterium* sp. PheDH was unique since the strain does not form spore. The distribution of phenylalanine dehydrogenase is limited to some groups of gram-positive, spore-forming bacteria including actinomycetes. This may be due to these enzymes being involved in microbial sporulation thereby connecting the carbon and nitrogen metabolism of amino acids because of the reversible nature of the enzymes.

Usually screening is carried out for the purpose of finding microorganisms that produce the enzyme effectively as well as medium and growth conditions that lead to abundant enzyme production are searched. The addition of L-phenylalanine, as an inducer, to the medium is usually very effective for promoting the enzyme production. Moreover, enzyme activity can also be induced by other amino acids such as L-histidine, L-tyrosine and L-methionine.

1.3 Purification and characteristics of phenylalanine dehydrogenase

1.3.1 Purification of phenylalanine dehydrogenase

The study on physical and biochemical properties of PheDH requires separation techniques to purify enzyme. Typical methods and the sequence of steps may be used to achieve effective purification with high yield in a minimal amount of time. Typical methods reported so far PheDH purification including heat denaturation, protamine precipitation, ammonium sulfate precipitation, are often excellent as a first purification step. The next step with ion exchange chromatography (mostly by

DEAE-Toyopearl column), adsorption chromatography (mostly by hydroxyapatite column), hydrophobic interaction chromatography (mostly by Butyl-Toyopearl column), gel filtration chromatography and others. For instance, the affinity chromatography, Red-sepharose CL 4B column, was also applicable to the PheDH from *R. maris* (Misono *et al*., 1989) and *T. intermedius* (Ohshima *et al*., 1991). FPLC, Mono Q column was used as one common step in the purification procedures of PheDH from *R. maris* and *Microbacterium* sp. (Misono *et al*., 1989; Asano and Tanetani, 1998).

The application of two enrichment steps after cell disintegration and extraction into the top phase of an aqueous two-phase system composed of polyethylene glycol, potassium phosphate and sodium chloride, followed by a second extraction to shift the enzyme into the bottom phase, was used in the industrial purification of PheDH from *Brevibacterium* sp. (Hummel *et al*., 1986) and *Rhodococcus* sp. M4. (Hummel *et al*., 1987). Recently, Tynan *et al*. (2000) described a novel kinetic locking-on strategy for bioaffinity purification of NAD^+-dependent dehydrogenases including PheDH based on immobilized cofactor derivatives through the use of enzyme-specific substrate analogues to promote selective and biospecific adsorption.

1.3.2. Properties of phenylalanine dehydrogenase

The enzyme was first discovered in *Brevibacterium* sp. in 1984 and since then, several other bacterial PheDHs were identified and characterized. Properties of PheDH from various source are summarized in Table 1.2. PheDHs exhibit a narrow range of subunit molecular masses between 36 and 46 kDa. There is considerable variation in quaternary structures of these enzymes. The enzymes from *S. ureae*, *B. sphaericus*, *B. badius* and *Microbacterium* sp. were reported to be octomers, the *T. intermedius* enzyme was reported to be hexamer, the *Rhodococcus* sp. M4 enzyme was reported to be tetramer, the *R. maris* enzyme was reported to be dimer, and *Nocardia* sp. enzyme was reported to be monomer. The enzyme has an isoelectric point in range 4.0 and 6.0, except enzyme from *B. badius* with pI 3.5. The pH optima for oxidation deamination are between 10.1 and 12.0 while for the reduction amination are between 8.5 and 10.3. The high

Table 1.2 Properties of phenylalanine dehydrogenase from various sources[a]

Properties	*Brevibacterium* sp.	*Rhodococcus* sp. M4	*S. ureae*	*B. sphaericus*	*B. badius*	*R. maris*	*Norcardia* sp.	*T. intermedius*	*Microbacterium* sp.
Specific activity of final preparation (U/mg protein)	-	-	84	111	68	65	30	86	37
Molecular mass of native									
- gel filtration	-	150,000	310,000	340,000	335,000	70,000	42,000	270,000	330,000
- deduced amino acid sequence	-	-	330,608	331,480	330,800	-	-	249,928	-
Molecular mass of subunit	-	39,500	41,326	41,435	41,350	36,000	42,000	40,488	41,000
Number of subunit	-	4	8	8	8	2	1	6	8
Isoelectric point (pI)	-	5.6	5.3	4.3	3.5	-	-	-	5.8
pH optimum									
-oxidative deamination	10.5	10.1	10.5	11.3	10.4	10.8	-	11.0	12.0
-reductive amination	8.5	9.25	9.0	10.3	9.4	9.8	10.0	9.2	12.0
Thermostability (% remaining activity after incubation)	-	-	75 (40 ºC, pH 9, 10 min)	100 (55 ºC, pH 9, 10 min)	50 (55 ºC, pH 8, 10 min)	100 (35 ºC, pH 7.4, 10 min)	50 (53 ºC, pH 9.5-10, 2 h)	100 (70 ºC, pH 7.2, 1 h)	100 (55 ºC, pH 9, 10 min)

Table 1.2 Properties of phenylalanine dehydrogenase from various sources (continue)

Properties	*Brevibacterium* sp.	*Rhodococcus* sp. M4	*S. ureae*	*B. sphaericus*	*B. badius*	*R. maris*	*Norcardia* sp.	*T. intermedius*	*Microbacterium* sp.
Equilibrium constant (M^2)	-	4.5×10^{-14}	2.0×10^{-14}	1.4×10^{-14}	-	-	3.2×10^{-18}	-	-
Apparent K_m (mM) for									
- L-phenylalanine	0.385	0.87	0.096	0.22	0.088	3.8	0.75	0.22	0.10
- phenylpyruvate	0.177	0.13	0.16	0.4	0.106	0.5	0.06	0.045	0.02
- NAD^+	0.125	0.27	0.14	0.17	0.15	0.25	0.23	0.078	0.20
- NADH	0.047	0.13	0.072	0.025	0.21	0.043	-	0.025	0.072
- ammonia	431	387	85	78	127	70	9.6	106	85
Substrate specificity [b]									
Oxidative deamination									
- L-phenylalanine	100	100	100	100	100	100	100	100	100
- L-tyrosine	-	12	5	72	9	2	2	0	4
- L-tryptophan	-	2	5	1	4	8	8	0	0
- L-methionine	-	4	4	3	8	5	5	0	**7**

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Table 1.2 Properties of phenylalanine dehydrogenase from various sources (continue)

Properties	*Brevibacterium* sp.	*Rhodococcus* sp. M4	*S. ureae*	*B. sphaericus*	*B. badius*	*R. maris*	*Norcardia* sp.	*T. intermedius*	*Microbacterium* sp.
- L-valine	-	-	3	1	4	0	0	0	5
- L-leucine	-	-	2	1	3	2	-	4	3
- L-isoleucine	-	-	1	0.5	0.2	3	-	0	0
- L-norvaline	-	-	6	1	5	0	-	-	6
- L-norleucine	-	-	15	4	19	16	-	-	16
- L-ethionine	-	-	7	3	7	13	-	-	-
- L-α-aminobutyrate	-	-	2	-	1	1	-	-	2
- L-phenylalaninamide	-	-	9	3	9	-	-	-	-
- L-phenylalaninol	-	-	9	0.6	9	-	-	-	-
- L-*p*-aminophenylalanine	-	-	-	-	-	-	-	7	-
- L-phenylalanine methyl ester	-	-	10	10	38	-	-	-	-
- L-tyrosine methyl ester	-	-	7	7	0.4	-	-	-	-
- *p*-fluoro-DL-phenylalanine	-	62	-	-	34	8	-	-	-
- *m*-fluoro-DL-phenylalanine	-	-	-	-	11	8	-	-	-
- *o*-fluoro-DL-phenylalanine	-	-	-	-	2	2	-	-	-
- D-phenylalanine	-	-	0	0	0	0	-	0	-

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Table 1.2 Properties of phenylalanine dehydrogenase from various sources (continue)

Properties	*Brevibacterium* sp.	*Rhodococcus* sp. M4	*S. ureae*	*B. sphaericus*	*B. badius*	*R. maris*	*Norcardia* sp.	*T. intermedius*	*Microbacterium* sp.
Substrate Specificity [b]									
Reductive amination									
- phenylpyruvate	100	100	100	100	100	100	100	100	100
- *p*-hydroxyphenylpyruvate	96	5	24	136	53	91	28	0	0
- indole-β-pyruvate	24	3	1	0	-	5	54	-	-
- α-ketovalerate	-	-	9	6	12	0	-	-	-
- α-ketocaproate	-	-	32	0	31	9	-	-	-
- α-ketoisovalerate	-	-	2	6	13	0	-	6	6
- α-ketoisocaproate	-	-	13	8	-	1	240	-	-
- α-ketobutyrate	-	-	-	-	3	0	-	1	1
- α-keto-γ-methylthiobutyrate	59	33	27	11	16	9	-	14	14
- α-keto-β-methylbutanoate	-	-	-	-	-	-	-	-	-
- α-keto-γ-methylpentanoate	-	-	-	-	13	-	-	6	6
- α-ketohexanoate	-	-	-	-	31	-	-	-	-

[a] *S.; Sporosarcina, B.; Bacillus, R.; Rhodococcus, and T.; Thermoactinomycete*

[b] Substrate specificity expressed as relative activity (%)

- = no data

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Source: *Brevibacterium* sp. (Hummel *et al*., 1984 and Hummel and Kula, 1989), *Rhodococcus sp*.M4 (Brunhuber and Blanchard, 1994, Vanhooke *et al*., 1999, and Brunhuber *et al.,* 2000), *Sporosarcina ureae* (Asano and Nakazawa, 1985, Asano *et al*., 1987b, and Asano and Nakazawa, 1987b), *Bacillus sphaericus* (Asano *et al.*, 1987a and b), *Bacillus badius* (Asano *et al.,* 1987c), *Rhodococcus maris* (Misono *et al*., 1989), *Nocardia* sp. (Boer *et al.,* 1989), *Thermoactinomyces intermedius* (Ohshima *et al*., 1991), and *Microbacterium* sp. (Asano and Tanetani, 1998)

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reactivity of the enzyme at rather high pHs is similar to those of other amino acids dehydrogenase such as alanine dehydrogenase (Keradjipoulos, D., and holldorf, A. W. 1979) and leucine dehydrogenase (Ohshima *et al.*, 1985). The *T. intermedius* enzyme was much more thermostable than other PheDHs from mesophiles. The equilibrium constant (K_{eq}) reported for the *Rhodococcus* sp. M4 enzyme was 4.5 x 10^{-14} M^2 and the *S. ureae* enzyme was 2.0 x 10^{-14} M^2 whereas the *Nocardia* sp. and *B. sphaerious* enzyme were reported to have K_{eq} of 3.2 x 10^{-18} M^2 and 1.4 x 10^{-15} M^2, respectively. PheDH from various sources have broad substrate specificities, the *B. sphaericus* enzyme acts on L-tyrosine as well as L- phenylalanine, whereas the *T. intermedius* enzyme was highly specific for L-phenylalanine. *p*-Hydroxyphenylpyruvate was a good substrate for reductive amination of the enzyme from *Brevibacterium* sp., *B. sphaericus* and *R. maris*.

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

Stereochemistry of hydrogen transfer of amino acid dehydrogenases showed either pro-*R* or pro-*S* stereospecificity for hydrogen transfer from the C-4 position of the nicotinamide moiety of NAD(P)H to the amino acid substrates as shown in Figure 1.2. For PheDH, the stereochemistry of hydride transfer was determined for the *B. sphaericus*, *T. intermedius* and *Rhodococcus* sp. M4 enzymes (Asano and Nakazawa, 1987b and Ohshima *et al.*, 1991). In all cases, the pro-*S* hydrogen of NADH was transferred to generate [2-^{2}H]-L-phenylalanine, placing the PheDH among the majority of amino acid dehydrogenases.

Steady-state kinetic analysis of oxidative deamination and reductive amination was performed to investigate several possible reactions proceed via the formation of a ternary complex with sequential or random substrate-binding mechanisms. Production inhibition patterns of the oxidative deamination or reductive amination reaction suggest the order of substrate addition and product release (Cleland, 1971). All studied amino acid dehydrogenases catalyze the reaction by a sequential ordered mechanism, with the exception of GluDH, which shows a random mechanism (Barton and Fisher, 1971). The kinetic mechanism on the PheDH from *R. maris*

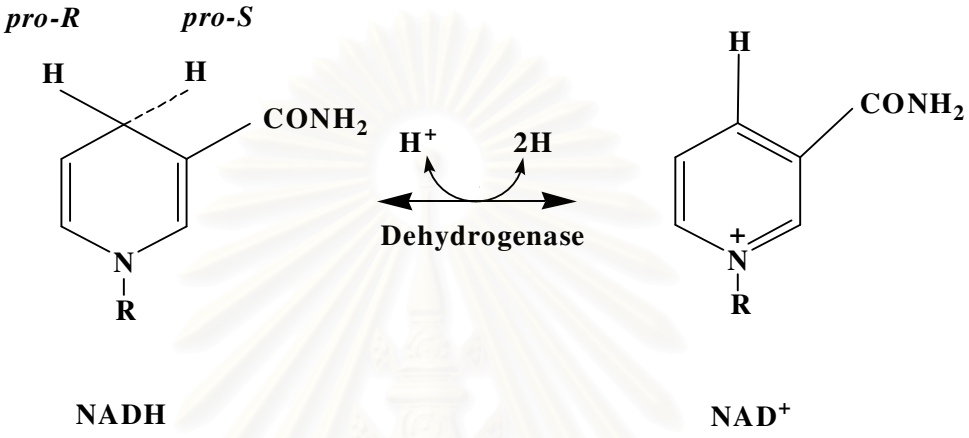

Figure 1.2 Stereospecificity of hydrogen transfer of NADH catalyzed with dehydrogenases

R represents ADP-ribosyl

Source: Ohshima and Soda, 1990

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showed that reductive amination proceeds through the sequential ordered ternary-binary mechanism in which NADH binds to the enzyme, followed by phenylpyruvate and then ammonia, and the products, L-phenylalanine and NAD^+, are released from the enzyme in that order after dehydrogenation (Misono *et al*., 1989).

Ohshima *et al*. (1991) reported that kinetic mechanism of the *T. intermedius* PheDH in oxidative deamination proceeds through a sequential ordered binary-ternary mechanism. In this case, the order of substrate binding is the same as the first one but the order of release was observed to be phenylpyruvate, ammonia and NADH. This conclusion was drawn from both initial velocity and product inhibition experiments. The kinetic mechanisms of PheDHs are summarized in Figure 1.3

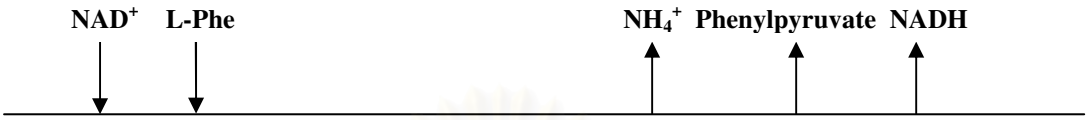
1.5 Structure of phenylalanine dehydrogenase

Extensive developments of the techniques in gene cloning have enabled rapid determination of the primary structures of amino acid dehydrogenases. In addition, x-ray crystallographic analysis of several amino acids dehydrogenases has been under taken and revealed their ternary and quaternary structures in details (Ohshima and Soda, 2000).

Primary structure of PheDH has been determined by peptide and DNA sequencing methods. The gene encoding the enzyme was cloned and sequenced from *B. badius*, *B. sphaericus*, *S. ureae*, *T. intermedius* and *Rhodococcus* sp. M4. Although a computer-aided search of the protein sequence data base revealed rather low overall sequence among amino acid dehydrogenases, a common partial sequence of about 30 residues in the nicotinamide coenzyme binding domain were observed in the entire enzyme. The coenzyme binding domain which binds the adenine nucleotide moiety shows a high degree of conservation of tertiary structures; it consists of a two-stranded parallel β-sheet and one ∝-helix with virtually identical arrangement (Ohshima and Soda, 1990).

The structure of PheDH from *Rhodococcus* sp. M4 has now been directly determined by high-resolution X-ray crystallographic analysis in two complexes with the enzyme•NAD^+•phenylpyruvate, and enzyme•NAD^+•*β*-phenylpropionate were recently reported by Vanhook *et al*. (1999). This is the first example of structures of

A



B

Figure 1.3 Kinetic mechanisms of phenylalanine dehydrogenase

A The *Rhodococcus maris* PheDH

B The *Thermoactinomyces intermedius* PheDH

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

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the amino acid dehydrogenase with a ternary complex. Both structures showed that PheDH is a homodimeric enzyme with each monomer composed of distinct globular N- and C-terminal domains separated by a deep cleft containing the active site (Figure1.4). The N-terminal domain binds the amino acid substrate and contributes to the interactions at the subunit, subunit interface. The C-terminal domain forms a typical Rossmann fold responsible for NAD binding as found for GluDH and LeuDH Moreover, they found that Lys78 and Asp118 act as the catalytic residues in the active site (Brunhuber *et al.,* 2000).

1.6 Application of phenylalanine dehydrogenase

The application of phenylalanine dehydrogenase can be categorized into two major fields: industrial and medical fields. Enzyme-catalyzed reductive amination is potentially useful for the production of optically pure amino acids such as L-phenylalanine, homophenylalanine and allysine ethylene acetal [(*S*)-2-amino-5-(1,3-dioxolan-2-yl)-pentanoic acid].

L- phenylalanine is an essential amino acid for human nutrition. It is used as a main intermediate utilized in the manufacture of a dipeptide artificial sweetener, aspartame (Hummel *et al*., 1987) and benzaldehyde which can be used as aromatic flavor compound in cheeses (Groot and de Bont, 1998). Several methods have also been produced of L-phenylalanine such as chemical synthesis, extraction form protein hydrolysates, fermentation or enzymatic method. In addition, fermentation and enzymatic processes in L-phenylalanine production have been proposed and developed over the last two decades in several ways (Choi and Tribe, 1982). These include production from *trans*-cinnamic acid to L-phenylalanine using *Rhodotorula glutinis* containing L-phenylalanine ammoniumlyase activity with 70% conversion yield (Yamade *et al*., 1981). However, the application of PheDH to industrial production of L-phenylalanine has been hampered by the cost of coenzyme because it is complex and rather labile organic chemical. A multienzyme reaction system for simultaneous coenzyme regeneration has been proposed to overcome this problem.

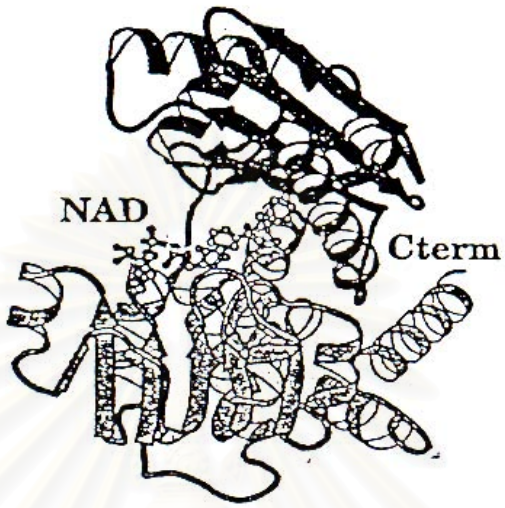

Figure 1.4 Structure of *Rhodococcus* sp. M4 phenylalanine dehydrogenase

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

Source: Vanhooke *et al*., 1999

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The continuous conversion of phenylpyruvate to L-phenylalanine was carried out by PheDH and formate dehydrogenase (FDH; EC 1.2.1.2) as shown figure 1.5. Reductive amination of phenylpyruvate by PheDH seems to be another promising way. The simultaneously oxidized NADH is regenerated by formate and FDH and therefore is required in catalytically amounts. The system contains PheDH, FDH and NAD^+, which binds with polyethyleneglycol by covalent bond (PEG-NAD^+), so the hybrid molecules cannot pass through the membrane. The reaction to form PEG-NADH is started by addition of formic acid and FDH. Then phenylpyruvate and ammonium formate are continuously passed through the reactor, the product of L-phenylalanine and carbon dioxide would be released (Hummel *et al.,* 1987).

Because of the instability of phenylpyruvate in aqueous solutions, two alternative routes have been studied. One starts from the racemic mixture of phenyllactate (Schmidt *et al*., 1987) while the other starts from acetamidocinnamic acid (Hummel and Kula, 1989). In both routes, phenylpyruvate is formed *in situ* and converted simultaneously by the action of PheDH to L-phenylalanine. The conversion of D, L-phenyllactate into the keto acid can be achieved utilizing the side reaction of two enzymes, D- and L-2-hydroxy-4-methyl-pentanoate dehydrogenase (2-hydroxy-caproate dehydrogenase). NADH is regenerated continuously by the substrate oxidation. The kinetic properties of the enzyme involved in the cyclic reaction make this approach unfavorable. Acetamidocinnamic acid is another stable precursor of phenylpyruvate. Deacetylation results in an unstable enamine-imine derivative, which hydrolysis spontaneously to yield phenylpyruvate. The deacetylation can be accomplished enzymatically by an acylase isolated from a strain of *Brevibacterium* sp. In this route, FDH is necessary for coenzyme regeneration.

PheDH from *Sporosarcina ureae* and FDH from *Candida boidinii* were used in the synthesis of L-phenylalanine and other L-amino acids. Amino acids such as L-phenylalanine, L-tyrosine, L-valine, L-methionine, and L-leucine were synthesized in yield as shown in Table 1.3 (Asano and Nakazawa, 1987).

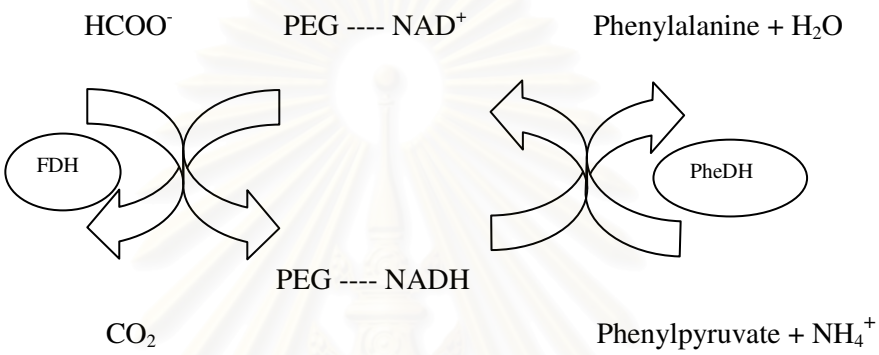

Figure 1.5 Enzymatic synthesis of L-phenylalanine with coenzyme regeneration

PheDH: phenylalanine dehydrogenase

FDH : formate dehydrogenase

PEG : polyethyleneglycol

Source: Hummel *et al*., 1987

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

Substrate	Product	%Yield
phenylpyruvate	L-phenylalanine	98
p-hydroxyphenylpyruvate	L-tyrosine	99
indolylpyruvate	L-tryptophan	11
α-keto-γ-methylthio- butyrate	L-methionine	87
α-ketoisovalerate	L-valine	97
α-ketoisocaproate	L-leucine	83
DL-α-keto-β-methyl-n-valerate	L-isoleucine allo-isoleucine	48 50

Source: Asano and Nakazawa, 1987

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

Enzyme-catalyzed reductive amination of phenylketobutyrate is potentially useful for the production of optically pure L-homophenylalanine, a component of an angiotension converting enzyme inhibitor used in the treatment of hypertension and heart failure (Patel, 2001).

PheDH is also being developed as a biosensor to screen for phenylketonuria (PKU). In 1989, Wendel *et al*. (1989) described a colorimetric method for the determination of plasma phenylalanine using PheDH coupled with an intermediate electron acceptor system as shown in Figure 1.6 and this method was adapted in microtiter-plate assay (Wendel *et al*., 1990). Further improvement of specificity with respect to cross reactivity towards tyrosine was achieved by elevation of pH 10.8 in the PheDH reaction (Wendel *et al*., 1991). Upon this basis, Porton Cambridge launched a commercial phenylalanine screening kit, the Quantase™, applicable for neonatal PKU screening (Newmarket, 1992). In 1996, Nakamura *et al*, found that the recycling assays involving the coupling of transaminases and dehydrogenases can be applicable to detect other amino acids that might be useful in the screening of human blood for abnormally high levels of these amino acids. In addition, assay of PheDH is also useful to the monitoring of the level of cells disrupted by shock wave since destruction of the spheroplast of recombinant cells leds to leaking of PheDH from the cells (Teshima *et al*., 1995 cited in Ohshima *et al*., 2000).

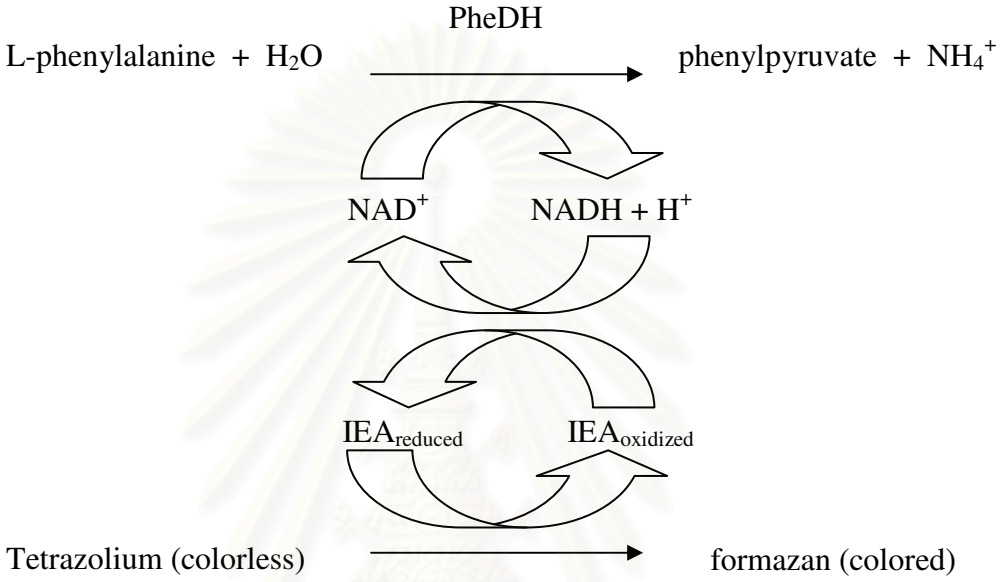

Figure 1.6 Reaction of the enzymatic phenylalanine determination

L-PheDH: L-phenylalanine dehydrogenase

IEA: intermediate electron acceptor

Source: Schulze *et al*., 2002

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1.7 Objective of this research

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

An initial step in understanding the relationship between the structure and function of PheDH from *Bacillus lentus*, the purification and characterization of the enzyme should be performed.

The objective of this thesis

1. To determine the optimal condition for PheDH production from *B. lentus*
2. To purify and characterize of PheDH
3. To determine the kinetic mechanism of PheDH

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CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave: Model H-88LL, Kokusan Ensinki Co., Ltd., Japan

Autopipette: Pipetman, Gilson, France

Electrophoresis apparatus: Model Mini-protein II Cell, BIO-RAD, USA

Fraction collector: Frac-100, Pharmacia Biotech, Sweden

Incubator shaker: Innova 4080, News Brunswick Scientific Co., USA

Incubator, waterbath: Model M20S, Lauda, Germany and Biochiller 2000, FOTODYNE Inc., USA

Magnetic stirrer: Fisherbrand, Fisher Scientific Inc., USA

Orbital incubator: Model 1H-100, Gallenkamp, England

pH meter: Model PHM95, Radiometer Copenhegen, Denmark

Peristaltic pump: pump p-1, Pharmacia Biotech, Sweden

Refrigerated centrifuge: Model J2-21, Beckman Instrument Inc., USA

Power supply: Model POWER PAC 300, Bio-Rad, USA

Sonicator: SONOPULS Ultrasonic homogenizers, BANDELIN, Germany

Spectrophotometer: DU Series 650, Beckman Instrument Inc., USA

2.2 Chemicals

Acrylamide: Merck, Germany

3-Acetylpyridine adenine dinucleotide: Sigma, USA

Agar: Merck, Germany

Ammonium persulphate: Sigma, USA

Ammonium sulphate: Carlo Erba Reagent, Italy

Aquasorb: BML, Thailand

Bis-acrylamide: Merck, Germany

Bovine serum albumin: Sigma, USA

Bromphenol blue: Merck, Germany

Butyl-Toyopearl 650M TSK gel: Tosoh, Japan

Coomassie brilliant blue R-250: Sigma, USA

DEAE-Toyopearl 650M TSK gel: Tosoh, Japan

Ethyl alcohol absolute: Carlo Erba Reagent, Italy

Ethylene diamine tetraacetic acid (EDTA): Merck, Germany

m-Fluoro-DL-phenylalanine: Sigma, USA

o-Fluoro-DL-phenylalanine: Sigma, USA

p-Fluoro-DL-phenylalanine: Sigma, USA

Glycine: Sigma, U.S.A.

Hydrochloric acid: Carlo Erba Reagenti, Italy

p-Hydroxyphenylactic acid (sodium salt): Sigma, USA

p-Hydroxyphenylpyruvic acid (sodium salt): Sigma, USA

Indole-β-pyruvic acid (sodium salt): Sigma, USA

α-Keto-n-butyric acid (sodium salt): Sigma, USA

α-Ketocaproic acid (sodium salt): Sigma, USA

α-Ketoglutaric acid (sodium salt): Sigma, USA

α-Ketoisocaproic acid (sodium salt): Sigma, USA

α-Ketoisovaleric acid (sodium salt): Sigma, USA

α-Keto-γ-methiol-butyric acid (sodium salt): Sigma, USA

α-Keto-β-methyl-n-valeric acid (sodium salt): Sigma, USA

α-Ketovaleric acid (sodium salt): Sigma, USA

β-Mercaptoethanol: Scharlau, Spain

Methanol: Lab-Scan, Thailand

β-Nicotinamide adenine dinucleotide (NAD^+): Sigma, USA

β-Nicotinamide adenine dinucleotide phosphate ($NADP^+$): Sigma, USA

β-Nicotinamide adenine dinucleotide reduced form (NADH): Sigma, USA

Nicotinamide 1, N^6-ethenoadenine dinucleotide: Sigma, USA

Nicotinamide guanine dinucleotide: Sigma, USA

Nicotinamide hypoxanthine dinucleotide: Sigma, USA

Nicotinic acid adenine dinucleotide: Sigma, USA

Nitroblue tetrazolium: Koch-Light Laboratories Ltd., Japan

Phenazine methosulfate: Nacalai Tesque, Inc., Japan

Phenol reagent: Carlo Erba Reagenti, Italy

L-Phenylalanine: Sigma, USA

Phenylmethylsulfonyl fluoride (PMSF): Sigma, USA

Protein molecular weight marker (MW 14,400 - 200,000): Fermentas, USA

Protein high molecular weight marker (MW 66,000 - 669,000): Amersham, USA

Phenylpyruvic acid (sodium salt): Sigma, USA

Potassium chloride: Merck, Germany

Potassium di-hydrogen phosphate: Carlo Erba Reagenti, Italy

di-Potassium hydrogen phosphate: Carlo Erba Reagenti, Italy

Potassium hydroxide: Scharlau, Spain

3-Pyridinealdehyde adenine dinucleotide: Sigma, USA

Pyruvic acid (sodium salt): Sigma, USA

Sephadex G-200: Pharmacia, USA

Sodium carbonate: Carlo Erba Reagenti, Italy

Sodium chloride: BDH, England

Sodium hydroxide: Merck, Germany

Sodium dodecyl sulfate (SDS): Sigma, USA

N, N, N′, N′- Tetramethyl-ethylenediamine (TEMED): BDH, England

Thionicotinamide adenine dinucleotide: Sigma, USA

Tris: USB, USA

Yeast extract: Scharlau Microbiology, Spain

L-amino acids were from Sigma, USA and D-amino acids were from Nacalai Tesque and Wako, Japan. Other common chemicals were reagent grade from Aldrich; USA, BDH; England, Fluka; Switzerland, Merck; Germany, Scharlau; Spain, Unilab; Australia and Sigma; USA.

2.3 Microorganism and growth medium

Bacillus lentus was aerobically cultivated in peptone medium consisted of 1% (w/v) peptone, 0.2% (w/v) sodium chloride, 0.01% (w/v) magnesium sulfate and 0.01% (w/v) yeast extract, pH 7.2 . For slant and plate, the medium was supplemented with 1.5% (w/v) agar. Medium was sterilized by autoclaving at 121°C for 20 minutes.

2.4 Assay of enzymatic activity

The enzyme activity can be determined in two manners, the oxidative deamination of L-phenylalanine (forward reaction) and the reductive amination of L-phenylpyruvate (reverse reaction).

$$\text{L-Phe} + NAD^+ + H_2O \rightleftharpoons \text{phenylpyruvate} + NH_3 + NADH + H^+$$

2.4.1 Oxidative deamination

The standard reaction mixture contained 20 μmol of L-phenylalanine, 1 μmol of NAD^+, 200 μmol of glycine-KCl-KOH buffer, pH 11.0 and the enzyme in a final volume of 1.0 ml. Incubation was carried out at 30°C in a cuvette of 1-cm light path. The reaction was started by addition of NAD^+ and was monitored by measuring the initial change in absorbance of NADH at 340 nm.

One unit of the enzyme is defined as the amount of enzyme which catalyzes the formation of 1 μmol of NADH per minute in the oxidative deamination. Specific activity is defined as units per milligram of protein.

2.4.2 Reductive amination

The reaction mixture 1 ml contained 10 μmol of L-phenylpyruvate, 500 μmol of ammonium chloride, 0.2 μmol of NADH, 100 μmol of glycine-KCl-KOH buffer, pH 9.0 and the enzyme in a final volume of 1.0 ml. Incubation was carried out at 30°C in a cuvette of 1-cm light path. The reaction was started by addition of NADH and was monitored by measuring the initial change in absorbance of NADH at 340 nm.

One unit of the enzyme is defined as the amount of enzyme which catalyzes the decrement of 1 μmol of NADH per minute in the reductive amination. Specific activity is defined as units per milligram of protein.

2.5 Protein determination

The protein concentration was determined according to the modified method of Lowry *et al.* (1951), using bovine serum albumin (BSA) as a standard. The reaction mixture of 5.1 ml containing 20-100 μg of protein, 100 μl of solution A and 5 ml of solution B was mixed and incubated at 30°C for 10 minutes. The solution mixture was incubated with 0.5 ml of solution C at room temperature for 20 minutes. Then, the absorbance at 610 nm. The protein concentration was calculated from a standard curve of BSA. Preparation of solution A, B and C were described in Appendix A.

2.6 Optimization for phenylalanine dehydrogenase production

2.6.1 Optimum concentration of L-phenylalanine as enzyme inducer

2.6.1.1 Starter inoculums

A single colony of *B. lentus* from agar slant was grown in 2 ml of peptone medium, pH 7.2 at 37°C with 250 rpm shaking for 24 hours before the starter was inoculated into 20 ml of the same medium and cultivated at the same condition.

2.6.1.2 Bacterium cultivation and cell harvest

The bacterial starter from 2.6.1.1 was transferred into 200 ml of peptone medium, pH 7.2 supplemented at various concentrations of L-phenylalanine. The cultivation was performed at 37°C with 250 rpm shaking for 24 hours. After that cells were harvested by centrifugation at 8,500 x g for 15 minutes at 4°C. The collected cells were washed with 0.85% NaCl before rewashing with extraction buffer (0.1 M potassium phosphate buffer, pH 7.4 containing 0.1 mM PMSF, 0.01% (v/v) of β-mercaptoethanol and 1 mM EDTA). The harvest cells were stored at 80°C until used.

2.6.1.3 Preparation of crude extract

The collected cells from section 2.6.1.2. were resuspened in cold extraction buffer and then disrupted by discontinuously sonication on ice. Cell debris was removed by centrifugation at 10,000 x g for 20 minutes at 4°C. Crude extract was collected for the determination of oxidative deamination activity and protein concentration as described in section 2.4.1 and 2.5, respectively in order to determine the effect of phenylalanine concentration.

2.6.2 Optimum pH of medium

Preparation of the starter inoculum cell and cultivation were carried out as described in section 2.6.1.1. Peptone medium supplemented with the suitable concentration of L- phenylalanine from section 2.6.1 at various pHs ranged from 4 to 10 were used. The bacterial cultivation and cells harvest, crude extract preparation, assay for oxidative deamination activity and protein determination were preformed as described in section 2.6.1.2, 2.6.1.3, 2.4.1 and 2.5, respectively.

2.6.3 Optimum cultivation temperature

Preparation of the starter inoculum was carried out as described in section 2.6.1.1. Peptone medium containing the suitable concentration of L-phenylalanine at optimum pH was used for bacterial cultivation at various temperatures ranged from 30 to 50^{o}C. The bacterial cultivation and cells harvest, crude extract preparation, assay for oxidative deamination activity and protein determination were preformed as described in section 2.6.1.2, 2.6.1.3, 2.4.1 and 2.5, respectively.

2.6.4 Optimum cultivation time

The starter inoculum was prepared as described in section 2.6.1.1. The bacterium was cultured in peptone medium at the optimum condition resulted from section 2.6.3 for various cultivation times (3, 6, 9, 12, 15, 18, 21 and 24 hours). The bacterial cultivation and cell harvest, crude extract preparation, assay for oxidative

deamination activity and protein determination were preformed as described in section 2.6.1.2, 2.6.1.3, 2.4.1 and 2.5, respectively.

2.7 Phenylalanine dehydrogenase production

2.7.1 Enzyme production

2.7.1.1 Starter inoculums

A single colony of B. *lentus* from agar slant was grown in 20 ml of starter peptone medium, pH 7.2 at 37°C with 250 rpm rotary shaking for 24 hours before 10% of cell culture was inoculated into 200 ml of the same medium and cultivated at the same condition.

2.7.1.2 Bacterial cultivation and cells harvested

The starter inoculums from 2.7.1.1 was transferred into 2 liters of peptone medium, pH 7.0 containing 0.4% L-phenylalanine and cultivated at 37°C with 250 rpm shaking for 18 hours. Cells were harvested by centrifugation at 8,500 x g for 15 minutes at 4°C. The collected cells were washed with 0.85% NaCl before rewashing with extraction buffer. Harvested cells were stored at -80°C.

2.7.2 Preparation of crude extract

The collected cells from section 2.7.1.2 were resuspened in cold extraction buffer and then disrupted by discontinuous sonication on ice for 2 minutes stop with 5 cycles. Cell debris was removed by centrifugation at 10,000 x g for 20 minutes at 4°C. Crude extract was collected for the oxidative deamination activity and protein concentration as described in section 2.4.1 and 2.5, respectively.

2.8 Purification procedure of phenylalanine dehydrogenase

Purification step was carried out at 4°C. The 10 mM potassium phosphate buffer, pH 7.4 containing 0.01% (v/v) β-mercaptoethanol and 1 mM EDTA was used through the purification step. A procedure of PheDH purification was as follows.

2.8.1 DEAE-Toyopearl column chromatography

The activated DEAE-Toyopearl was prepared by washing with 0.5 N NaOH for 2 - 3 times, and rewashed by deionizer water until the pH reached 8.0. The activated DEAE-Toyopearl was resuspened in the buffer and packed into 2.5 x 19.5 cm column and equilibrated with the same buffer for 5 - 10 column volume at flow rate 1 ml/min.

The crude enzyme solution from section 2.7.2 was applied to the DEAE-Toyopearl column. The unbound proteins were eluted from the column with the buffer until the absorbance at 280 nm was nearly zero. The bound proteins were eluted from the column with linear salt gradient of 0 to 0.5 M KCl in the same buffer. The 3 ml fractions were collected using a fraction collector. The protein profile was determined by measuring the absorbance at 280 nm. PheDH activity was assayed using the method described in section 2.4.1. The KCl concentration was investigated by measuring its conductivity. The fractions containing PheDH activity was pooled and dialyzed against the buffer. The PheDH activity and protein concentration of pooled fraction were measured as described in section 2.4.1 and 2.5, respectively.

2.8.2 Butyl-Toyopearl column chromatography

Butyl-Toyopearl was washed with deionized water for 2 - 3 times to remove impurities and then resuspended in the buffer containing 25% saturated ammonium sulfate and packed into 2.2 x 15 cm column followed by equilibrating with the same buffer for 5 - 10 column volume at flow rate 1 ml/min.

The pooled active fraction from section 2.8.1 was slowly adjusted to 25 % saturation with ammonium sulfate and stirred gently for at least 30 minutes. The protein solution was applied to the column at flow rate 1 ml/min. The unbound proteins were eluted from the column with the buffer containing 25% saturated ammonium sulfate until the absorbance to 280 nm was nearly zero. The bounded protein was eluted from the column with a negative salt gradient of 25% to 20% saturated ammonium sulfate in the buffer. Two milliliter fractions were collected using fraction collector and assayed for both protein concentration and PheDH

activity. Protein profile was determined by measuring an absorbance at 280 nm. PheDH activity was assayed using the method as described in section 2.4.1. The fractions containing PheDH activity were pooled and dialyzed against the buffer. After desalting, the enzyme was concentrated using aquasorb. The PheDH activity and protein concentration were determined as described in section 2.4.1 and 2.5, respectively.

2.8.3 Sephadex G-200 column chromatography

Sephadex G-200 column (1.6 x 63 cm) was equilibrated with the buffer containing 0.2 M KCl at 4°C for 5 column volume at flow rate 15 ml/hr to allow stabilization of the column. The enzyme solution from Butyl-Toyopearl column was applied to the column and the enzyme was eluted with the equilibration buffer. The 1 ml fractions were collected using fraction collector and assayed for both protein concentration and PheDH activity. The protein elution profile was monitored by measuring the absorbance at 280 nm. The PheDH activity was determined as described in section 2.4.1. The activity fractions were pooled and dialyzed against the buffer. The enzyme was concentrated using aquasorb. PheDH activity and protein concentration were measured as described in section 2.4.1 and 2.5, respectively.

2.8.4 Determination of enzyme purity by non-denaturing polyacrylamide gel electrophoresis (native-PAGE)

In order to determine the protein pattern and degree of purity, the enzyme from each step was subjected to native-PAGE according to the method of Bollag and Edelstein (1991). Electrophoresis conditions, protein and activity staining were described below.

The discontinuous PAGE was performed on the slab gel of a 7.7% (w/v) separating gel and a 5% (w/v) stacking gels. Tris-glycine buffer, pH 8.3 was used as electrode buffer. The non-denaturing polyacrylamide gel and solutions were prepared as described in Appendix B. The enzyme from each step was mixed with 5x sample buffer by ratio 5:1 and loaded onto the gel. The electrophoresis was carried out at constant current with 20 mA per slab gel at room temperature. For

determination of the enzyme activity, the experiment was done at 4°C. After electrophoresis, the protein bands were detected by protein and activity staining.

2.8.4.1 Protein staining

The proteins bands were visualized by Coomassie blue staining. The staining solution of 0.1%(w/v) Coomassie brilliant blue R-250 in 10% (v/v) acetic acid and 45% (v/v) methanol was used to stain the gel for at least 20 minutes. Destaining was performed by immersing the gel in destaining solution containing 10% (v/v) acetic acid and 10% (v/v) methanol followed by several changes of destaining solution until gel background was clear.

2.8.4.2 Enzyme activity staining

The enzyme was stained for activity with a 10 ml solution containing 4.25 mmol of Tris-HCl buffer, pH 8.5, 40 μmol of L-phenylalanine, 50 μmol of NAD^+, 250 μg of phenazine methosulfate and 2.5 mg of nitroblue tetrazolium. The gel was gently shacked at room temperature. After the brown band was appeared, the staining reaction was stop by pouring off the staining solution The gel was then quickly rinsed several times with deionizer water until gel background was clear.

2.9 Characterization of phenylalanine dehydrogenase

2.9.1 Molecular weight determination of phenylalanine dehydrogenase

2.9.1.1 Molecular weight determination of phenylalanine dehydrogenase by native-PAGE

The native molecular weight was examined by non-denaturing polyacrylamide gel electrophoresis (native-PAGE) using of Ferguson plots analysis (Ferguson, 1964). The electrophoresis on a set of various polyacrylamide concentrations with 5, 6, 7, 8, 9, and 10% separating gels and 5% stacking gel. Tris-glycine buffer, pH 8.3 was used as electrode buffer. The enzyme and molecular weights marker proteins range 66 kDa to 669 kDa were load to the gel. The

electrophoresis was carried out at 20 mA constant current per slab gel. After electrophoresis, proteins in the gel were visualized by coomassie blue staining as described in section 2.8.4.1 and determine the electrophoresis mobility (R_f) of the proteins in each gel relative to the tracking dye and plotted against the percent gel concentration for each protein. The slope of such a plot was the retardation coefficient (K_R) and the log of negative slope was plotted against the log molecular weight of standard proteins. The K_R value of the PheDH was calculated in the same way and used to determine its native molecular weight from the calibration curve.

2.9.1.2 Molecular weight determination of phenylalanine dehydrogenase subunit by SDS-PAGE

The SDS- PAGE system was performed according to the method described by Bollag and Edelstein (1991). The enzyme from Sephadex G-200 column was treated with sample buffer and boiled for 10 minutes before loading on SDS-PAGE. The slab gel (10 x 10 x 0.75 cm) system consisted of 0.1% (w/v) SDS in 12.5% (w/v) separating gel and 5% (w/v) stacking gels. The gel preparation was performed as described in Appendix C. The electrophoresis was carried out at 20 mA constant current. The molecular weight marker proteins were β-galactosidase (MW 116,000), bovine serum albumin (MW 66,000), ovalbumin (MW 45,000), lactate dehydrogenase (MW 140,000), REase Bsp98I (MW 25,000), β-lactoglobulin (MW 18,400) and lysozyme (MW 14,400). After electrophoresis, the gel was gently shaked at room temperature in Coomassie blue staining as described in section 2.8.4.1.

2.9.2 Substrate specificity of phenylalanine dehydrogenase

The ability of the enzyme to catalyze the oxidative deamination of various amino acids and L-phenylalanine analogs was determined at a final substrate concentration of 20 mM except for L- tyrosine (1.25 mM) and L-tryptophan (12.5 mM). L-Phenylalanine was replaced by various amino acids and L-phenylalanine analogs as substrate for the oxidative deamination as described in section 2.4.1. In the same way, the ability of the enzyme to catalyze the reductive

amination of various keto acids and phenylpyruvate analogs was determined at a final concentration of 10 mM. Phenylpyruvate was replaced by various keto acids and phenylpyruvate analogs as substrate for the reaction. The reductive activities were determined as described in section 2.4.2. The results were expressed as a percentage of the relative activity.

2.9.3 Coenzyme specificity of phenylalanine dehydrogenase

The purified PheDH was used to study coenzyme specificity; NAD^+ was replaced by various NAD^+ analogs at final concentration of 2 mM for oxidative deamination. Assays with NAD^+ analogs were conducted by measuring the increase in absorbance at the following wavelengths: 3-acetylpyridine adenine dinucleotide, 363 nm (ε = 9.1 x 10^3 $M^{-1}.cm^{-1}$); β-nicotinamide adenine dinucleotide phosphate (NADP), 340 nm (ε = 6.2 x 10^3 $M^{-1}.cm^{-1}$); nicotinic acid adenine dinucleotide, 338 nm (ε = 6.2 x 10^3 $M^{-1}.cm^{-1}$); 3-pyridinealdehyde adenine dinucleotide, 358 nm (ε = 9.3 x 10^3 $M^{-1}.cm^{-1}$); nicotinamide 1, N^6- ethenoadenine dinucleotide, 334 nm (ε = 6.9 x 10^3 $M^{-1}.cm^{-1}$); nicotinamide hypoxanthine dinucleotide, 338 nm (ε = 6.2 x 10^3 $M^{-1}.cm^{-1}$) and thionicotinamide adenine dinucleotide, 395 nm (ε = 11.3 x 10^3 $M^{-1}.cm^{-1}$) (Misono *et al*., 1989). The reactions were carried out at pH 9.5 to avoid the degradation of NAD^+ analogs at a more alkaline pH. The result was expressed as a percentage of the relative activity.

2.9.4 Effect of pH on phenylalanine dehydrogenase activity

The effect of pH on the activity was determined under the standard assay conditions, for the oxidative deamination and reductive amination as described in section 2.4.1 and 2.4.2 at various pHs. The 200 mM of potassium phosphate for pH 6.0 to 8.5, Tris-HCl buffer for pH 7.0 to 9.0 and glycine-KCl-KOH buffer for pH 8.5 to 12.0 were used. The pH of each reaction mixture was measured with a pH meter at room temperature after the reaction. The percentage of relative activity was plotted against the final pH.

2.9.5 Effect of temperature on phenylalanine dehydrogenase activity

The effect of temperature on the activity was examined under the standard assay condition, for both of the oxidative deamination and reductive amination as described in section 2.4.1 and 2.4.2 at various temperatures from 25°C to 75°C. The percentage of relative activity was plotted against the temperature used for the assay of activity.

2.9.6 Effect of pH on phenylalanine dehydrogenase stability

The purified PheDH was used to study pH stability. After the enzyme was incubated at 30°C for 20 minutes in each of the 10 mM buffer at various pHs, an aliquot of the enzyme solution was withdrawn and the remaining activity of enzyme was measured under the standard assay condition for the oxidative deamination as described in section 2.4.1. The 10 mM buffers used were acetate buffer for pH 4.0 to 6.0, potassium phosphate for pH 6.0 to 8.5, Tris-HCl buffer for pH 7.0 to 9.0 and glycine-KCl-KOH buffer for pH 8.5 to 12.0. The percentage of PheDH relative activity was plotted against the incubated pH.

2.9.7 Effect of temperature on phenylalanine dehydrogenase stability

The effect of temperature on the stability of the enzyme was determined at 30°C to 75°C. The purified PheDH was incubated at various temperatures for 10 minutes before determination of enzyme activity under the standard assay condition in the oxidative deamination according to the method described in section 2.4.1. Afterward, the enzyme was incubated at the highest temperature which the enzyme activity still retained full activity and collected to assay as described previously for everyday. The result was expressed as a percentage of the relative activity.

2.9.8 Effect of metal ions and chemical substances on phenylalanine dehydrogenase activity

The enzyme activities were determined in the oxidative deamination as described in section 2.4.1 in the presence of various metal ions and chemical

substances at the final concentration of 10 mM, unless otherwise stated. The result was expressed as a percentage of the relative activity of the enzyme activity in the absence of reagents.

2.9.9 Inhibitory effect of various amino acids and keto acids on phenylalanine dehydrogenase activity

Inhibitory effect of various amino acids which the enzyme did not have the ability to catalyze as its substrates were determined at the final concentration of 20 mM, unless otherwise stated. The oxidative deamination of L-phenylalanine was performed as described in section 2.4.1 in the presence of various amino acids. The result was expressed as a percentage of the relative activity of the enzyme activity in the absence of reagents.

Inhibitory effect of various keto acids which the enzyme did not have the ability to catalyze as its substrates were determined at the final concentration of 10 mM, unless otherwise stated. The reductive amination of phenylpyruvate was performed as described in section 2.4.2 in the presence of various keto acids. The result was expressed as a percentage of the relative activity of the enzyme activity in the absence of reagents.

2.10 Kinetic mechanism studies of phenylalanine dehydrogenase

A series of steady-state kinetic analyzes were carried out in order to investigate the kinetic parameters and reaction mechanism as described below.

2.10.1 Initial velocity analysis for the oxidative deamination

Initial velocity studies for the oxidative deamination reactions were carried out under the standard reaction condition as described in section 2.4.1, except that various amounts of L-phenylalanine and NAD^+ were used. The concentrations of L-phenylalanine used were 0.2, 0.25, 0.3, 0.5 and 1.0 mM, and those of NAD^+ used were 0.2, 0.3, 0.4, 0.6 and 0.8 mM. The Lineweaver-Burke plots (double-reciprocal plots) of initial velocities against L-phenylalanine concentrations at a series of fixed concentrations of NAD^+ and the secondary plots of y intercepts against reciprocal

concentrations of NAD^+ were made from the data. K_m of L-phenylalanine and NAD^+ were calculated and determined from these two plots, respectively.

2.10.2 Initial velocity analysis for the reductive amination

Initial velocity studies for the reductive amination reactions were carried out under the standard reaction condition as described in section 2.4.2, except that various amounts of phenylpyruvate, NH_4Cl and NADH were used in the experiments as described below.

2.10.2.1 The enzyme was assayed in the reductive amination by using phenylpyruvate as a variable substrate (0.1, 0.15, 0.2 and 0.4 mM) at several fixed concentrations of NH_4Cl (40, 60, 80 and 100 mM) in the presence of a high and saturating concentration of NADH (0.2 mM). The double-reciprocal plots of initial velocities against phenylpyruvate concentrations at a series of fixed concentrations of NH_4Cl and the secondary plots of y intercepts against reciprocal concentrations of ammonium chloride were made for K_m determination of phenylpyruvate and NH_4Cl.

2.10.2.2 The enzyme was assayed in the reductive amination by using NH_4Cl as a variable substrate (40, 60, 80 and 120 mM) at several fixed concentrations of NADH (0.05, 0.06, 0.1 and 0.2 mM) in the presence of a high and saturating concentration of phenylpyruvate (10 mM). The double-reciprocal plots of initial velocities against NH_4Cl concentrations at a series of fixed concentrations of NADH and the secondary plots of y intercepts against reciprocal concentrations of NADH were made for K_m determination of NH_4Cl and NADH.

2.10.2.3 The enzyme was assayed in the reductive amination by using NADH as a variable substrate (0.05, 0.06, 0.08 and 0.1 mM) at several fixed concentrations of phenylpyruvate (0.06, 0.08, 0.1 and 0.2 mM) in the presence of a high and saturating concentration of NH_4Cl (500 mM). The double-reciprocal plots of initial velocities against NADH concentrations at a series of fixed concentrations of phenylpyruvate and the secondary plots of y intercepts against reciprocal

concentrations of phenylpyruvate were made for K_m determination of NADH and phenylpyruvate.

2.10.3 Product inhibition studies

Production inhibition studies were performed in the direction of the reductive amination reaction in different conditions as described below.

2.10.3.1 Product inhibition by NAD^+ with respect to NADH

Phenylpyruvate (10 mM) and NH_4Cl (500 mM) were held at a saturating and constant concentration. Inhibition by NAD^+ was analyzed with varied concentration of NADH. The NAD^+ concentrations were 0, 0.4, 1.0 and 2.0 mM, and concentrations of NADH were 0.06, 0.08, 0.1 and 0.2 mM.

2.10.3.2 Product inhibition by L-phenylalanine with respect to NADH

Phenylpyruvate (10 mM) and NH_4Cl (500 mM) were held at a saturating and constant concentration. Inhibition by L-phenylalanine was analyzed with varied concentration of NADH. The L-phenylalanine concentrations were 0, 2.0, 6.0 and 10.0 mM, and concentrations of NADH were 0.06, 0.08, 0.1 and 0.2 mM.

2.10.3.3 Product inhibition by NAD^+ with respect to phenylpyruvate

NADH (0.2 mM) and NH_4Cl (500 mM) were held at a saturating and constant concentration. Inhibition by NAD^+ was analyzed with varied concentration of phenylpyruvate. The NAD^+ concentrations were 0, 0.4, 1.0 and 2.0 mM, and concentrations of phenylpyruvate were 0.04, 0.06, 0.1 and 0.2 mM.

2.10.3.4 Product inhibition by L-phenylalanine with respect to phenylpyruvate

NADH (0.2 mM) and NH_4Cl (500 mM) were held at a saturating and constant concentration. Inhibition by L-phenylalanine was analyzed with varied concentration of phenylpyruvate. The L-phenylalanine concentrations

were 0, 1.0, 2.0 and 6.0 mM, mM and concentrations of phenylpyruvate were 0.04, 0.06, 0.1 and 0.2 mM.

2.10.3.5 Product inhibition by NAD^+ with respect to NH_4Cl

NADH (0.2 mM) and phenylpyruvate (10 mM) were held at a saturating and constant concentration. Inhibition by NAD^+ was analyzed with varied concentration of NH_4Cl. The NAD^+ concentration used were 0, 0.4, 1.0 and 2.0 mM, and concentrations of NH_4Cl were 20, 30, 40 and 60 mM.

2.10.3.5 Product inhibition by L-phenylalanine with respect to NH_4Cl

Phenylpyruvate (10 mM) and NADH (0.2 mM) were held at a saturating and constant concentration. Inhibition by L-phenylalanine was analyzed with varied concentration of NH_4Cl. The L-phenylalanine concentration used was 0, 2.0, 6.0 and 10.0 mM, and concentrations of NH_4Cl were 20, 30, 40 and 60 mM.

From the data, The plots of reciprocal initial velocities (1/V) against reciprocal substrate concentrations (1/[S]) at several concentrations of inhibitor were made for kinetics mechanism analysis. Velocity (V) was expressed as unit of enzyme which was defined as μmol of NADH decreased/min.

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CHAPTER III

RESULTS

3.1 Optimization for phenylalanine dehydrogenase production

3.1.1 Optimum concentration of L-phenylalanine as enzyme inducer

The optimum concentration of L-phenylalanine as an enzyme inducer was determined as described in section 2.6.1. As shown in Figure 3.1. The total activity and specific activity increased corresponding with increasing concentrations of the L-phenylalanine until the concentration reached 0.4% whereas the cell wet weight seemed to be constant. The results showed that addition of 0.4% L-phenylalanine to culture medium gave the highest total activity and specific activity of 168 units and 0.85 units/mg proteins, respectively. The specific activity could be induced to 17 fold. Thus, this concentration was used for further experiments.

3.1.2 Optimum pH of medium

The effect of pH of medium on phenylalanine dehydrogenase production was performed as described in section 2.6.2. As shown in Figure 3.2. The cell wet weight, total activity and specific activity increased rapidly corresponding with increasing pH of medium until the pH reached 7.0. At pH above 7.0, cell wet weight, total activity and specific activity were dramatically decreased. The culture medium, pH 7.0 which gave the highest total activity (180 units) and specific activity (0.89 units/mg protein) was used for further experiments.

3.1.3 Optimum cultivation temperature

The optimum cultivation temperature for enzyme production was determined as described in section 2.6.3. As shown in Figure 3.3, cell wet weight, total activity and specific activity increased corresponding with increasing of cultivation temperature from 30 to 37^{o}C. At 37^{o}C, the highest enzyme activities were obtained with total activity of 180 units and specific activity of 0.89 units/mg protein, respectively. At temperature above 37^{o}C, cell wet weight, total activity and specific

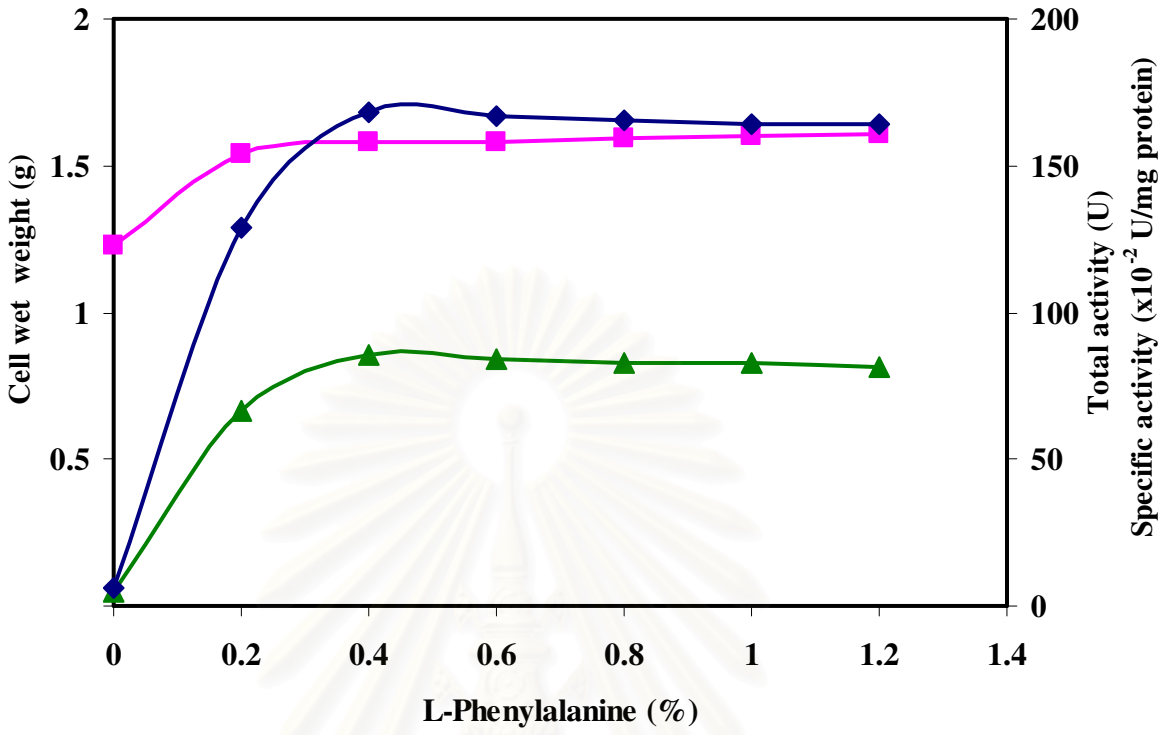

Figure 3.1 Effect of the concentration of L-phenylalanine on phenylalanine dehydrogenase production and cell growth

B. lentus was cultivated in 200 ml of peptone medium, pH 7.2 at 37°C for 24 hours at varied concentration of L- phenylalanine from 0 to 1.2%

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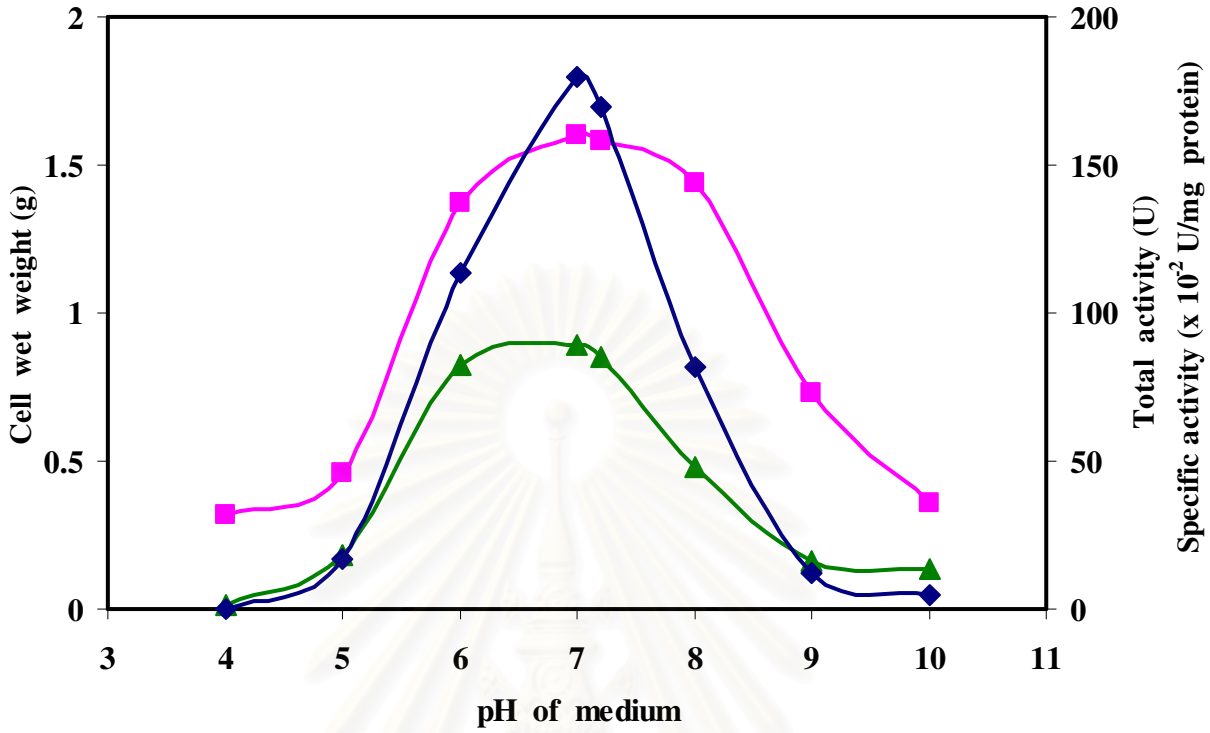

Figure 3.2 Effect of pH of medium on phenylalanine dehydrogenase production and cell growth

B. lentus was cultivated in 200 ml of peptone medium containing 0.4% L-phenylalanine at various pHs ranged from 4 to 10 at 37°C for 24 hours.

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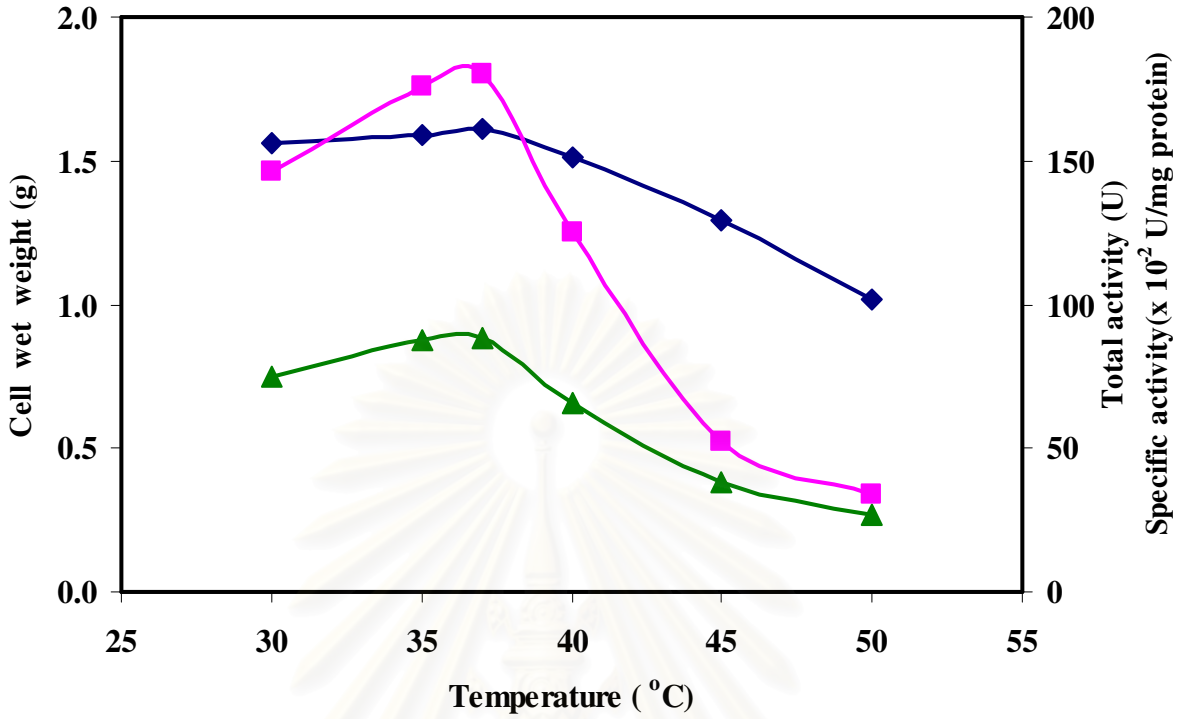
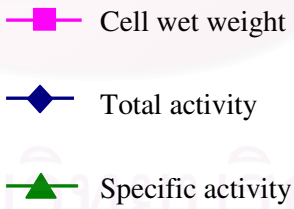


Figure 3.3 Effect of cultivation temperature on phenylalanine dehydrogenase production and cell growth

B. lentus was cultivated in 200 ml of peptone medium, pH 7.0 containing 0.4% L- phenylalanine at various temperatures ranged from 30 to 50 °C for 24 hours.



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activity was rapidly decreased. According to the result, cultivation at 37°C was used for further experiments.

3.1.4 Optimum cultivation time

The optimum cultivation time for enzyme production was performed as described in section 2.6.4. As shown in Figure 3.4, cell wet weight, total activity and specific activity increased rapidly from 0 to 15. After 18 hours of cultivation, they were quite constant. The highest enzyme activity obtained by cultivation for 18 hours. Total activity and specific activity were 185 units and 0.93 units/mg protein, respectively.

The optimum condition for phenylalanine dehydrogenase production from *B. lentus* was cultivation in 1% peptone medium, pH 7.0 containing 0.4% L-phenylalanine at 37°C for 18 hours.

3.2 Purification of phenylalanine dehydrogenase

3.2.1 Preparation of crude extract

Crude PheDH was prepared from 16 g of *B. lentus*, which was cultivated from 2 liter of medium, as described in section 2.7.1 Crude extract solution contained 2,170 mg proteins and 2,230 units of PheDH activity. Thus, the specific activity was 1.0 unit/mg protein (Table 3.1).

3.2.2 DEAE-Toyopearl column chromatography

At first step of purification, crude extract was purified by DEAE-Toyopearl column as described in section 2.8.1. The chromatography profile is shown in Figure 3.5 The unbound proteins were eluted from column with the phosphate buffer and then the bound proteins were eluted with linear salt gradient of 0 to 0.5 M KCl dissolved in the buffer. The enzyme was eluted with the buffer containing 0.25 M KCl. Pooled fraction containing PheDH activity was dialyzed against the buffer and concentrated by aquasorb to reduce enzyme volume. The proteins remained from this step was 202 mg with 1,490 units of PheDH activity.

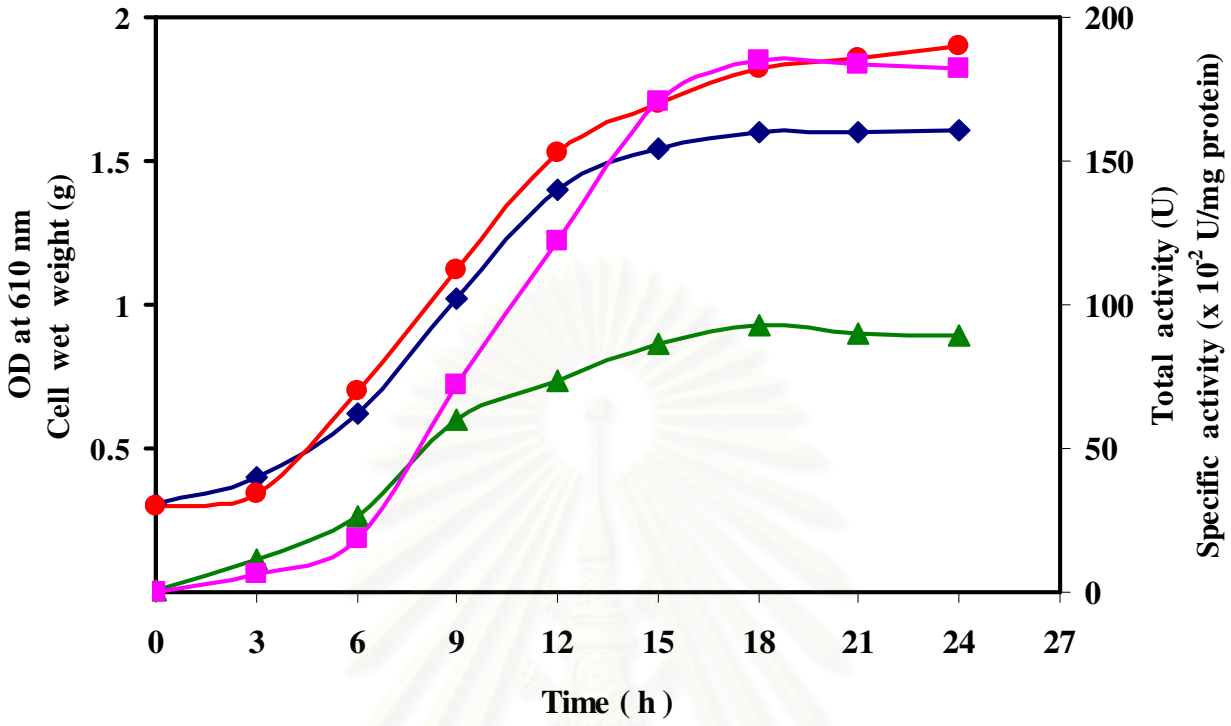

Figure 3.4 Effect of cultivation time on phenylalanine dehydrogenase production and cell growth

B. lentus was cultivated in 200 ml of peptone medium, pH 7.0 containing 0.4 % L-phenylalanine and various cultivation times for 0 to 24 hours at 37°C.

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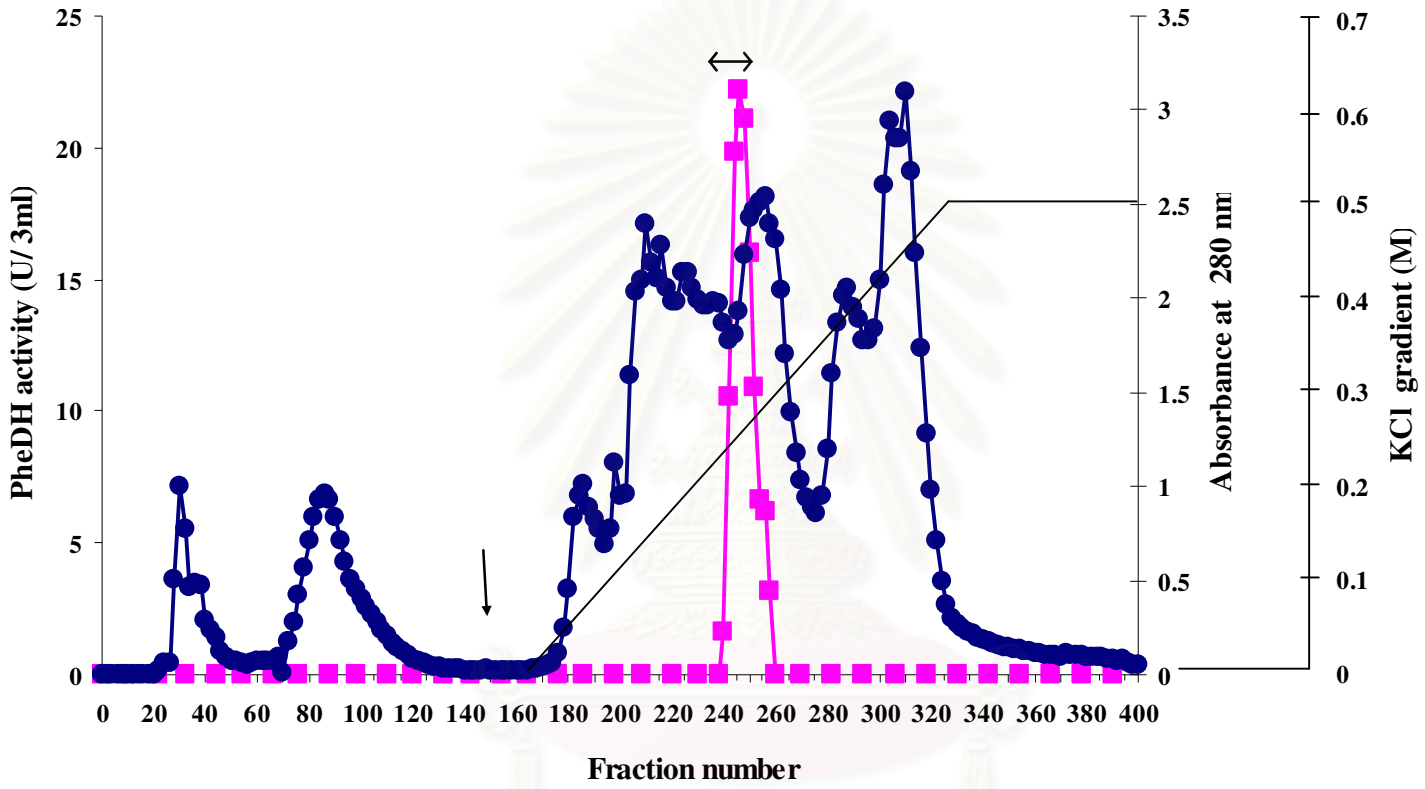

Figure 3.5 Purification of phenylalanine dehydrogenase from *B. lentus* by DEAE - Toyopearl column

The enzyme solution was applied to DEAE -Toyopearl column and washed with 10 mM potassium phosphate buffer, pH 7.4 containing 0.01%(v/v) β-mercaptoethanol and 1 mM EDTA until A_{280} decreased to base line. The bound proteins were eluted by 0-0.5 M KCl in the same buffer at the flow rate of 1 ml/min. The fractions of 3 ml were collected. The arrow indicates where gradient started. The protein peak from fraction numbers 241 to 251 was pooled (⟷). ● A_{280}, ■ PheDH activity, — KCl gradient

The specific activity of the enzyme was 7.4 units/mg protein. The PheDH was purified to 7.2 fold with 66.8% recovery (Table 3.1).

3.2.3 Butyl-Toyopearl column chromatography

The pooled active fraction from DEAE-Toyopearl column was applied to the Butyl-Toyopearl column as described in section 2.8.2. The chromatography profile is shown in Figure 3.6. The unbound proteins were eluted from column with buffer containing 25% saturated ammonium sulfate and then the bound proteins were eluted with negative salt gradient of 25 to 20% saturated ammonium sulfate in the buffer. The enzyme was eluted with the buffer containing 22% salt saturation. The fractions containing PheDH activity were pooled, dialyzed against the buffer and concentrated by aquasorb. The proteins remained from this step was 10.0 mg with 538 units of PheDH activity. The specific activity of the enzyme was 53.8 units/mg protein. The PheDH was purified to 52.4 fold with about 24% recovery (Table 3.1).

3.2.4 Sephadex G-200 column chromatography

The pooled active fraction from Butyl-Toyopearl column was applied to the Sephadex G-200 column as described in section 2.8.3. The chromatography profile is shown in Figure 3.7. The fractions containing PheDH activity were pooled, dialyzed against buffer and concentrated by aquasorb. The proteins remained from this step was 2.4 mg with 410 units of PheDH activity. The specific activity of the enzyme was 170.8 units/mg protein. The PheDH was purified to 166.3 fold with 18.4% recovery (Table 3.1). The enzyme from this step was kept at $4^{o}C$ for characterization experiments.

The summary of purification of *B. lentus* PheDH is shown in Table 3.1.

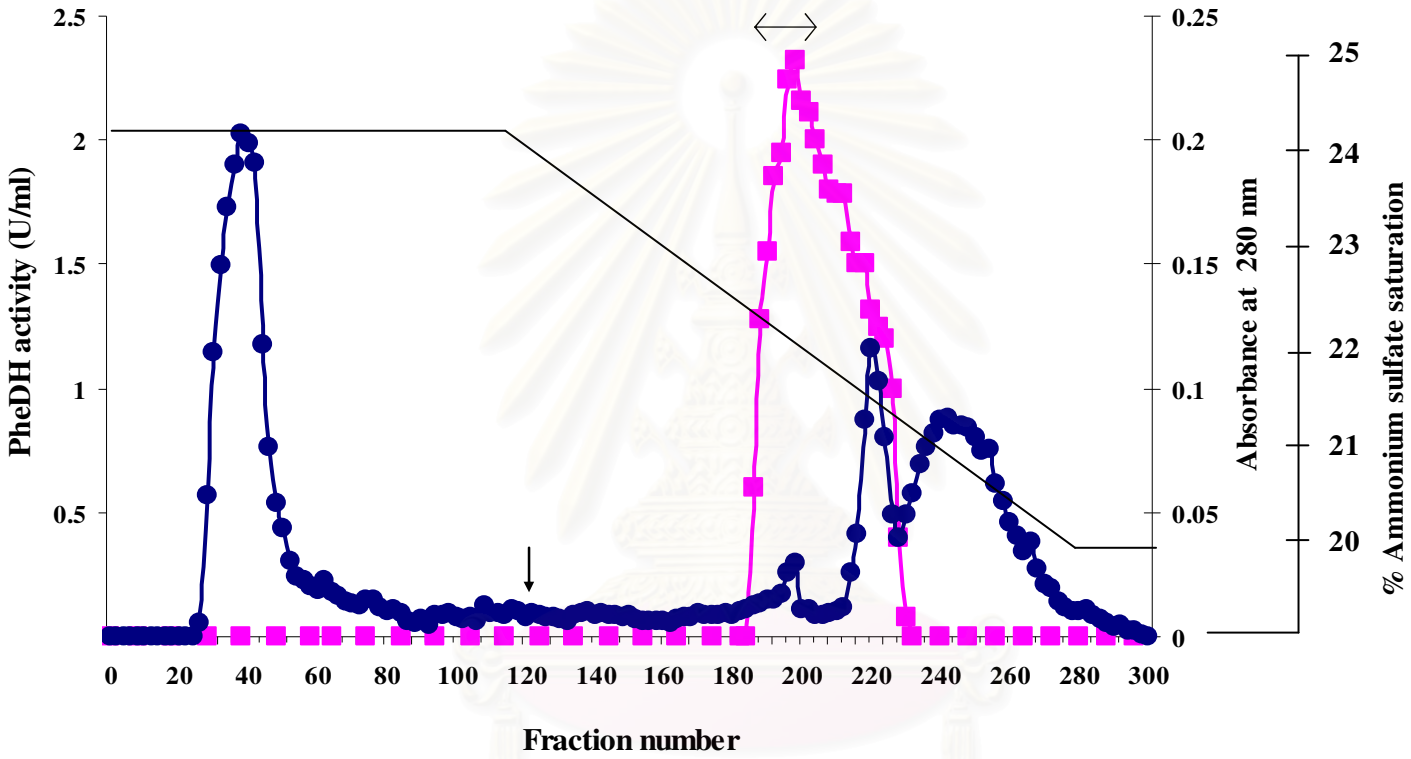

Figure 3.6 Purification of phenylalanine dehydrogenase from *B. lentus* by Butyl-Toyopearl column

The enzyme solution was applied to Butyl-Toyopearl column and washed with 25% saturated ammonium sulfate in 10 mM potassium phosphate buffer, pH 7.4 containing 0.01% (v/v) β-mercaptoethanol and 1 mM EDTA until A_{280} decreased to base line. The enzyme was eluted by 22% saturated ammonium sulfate in the same buffer at the flow rate of 1 ml/ min. The fractions of 1 ml were collected. The arrow indicates where gradient started. The protein peak from fraction numbers 187 to 221 was pooled (⟷).

● A_{280}, ■ PheDH activity, ⎯[ammonium sulfate]

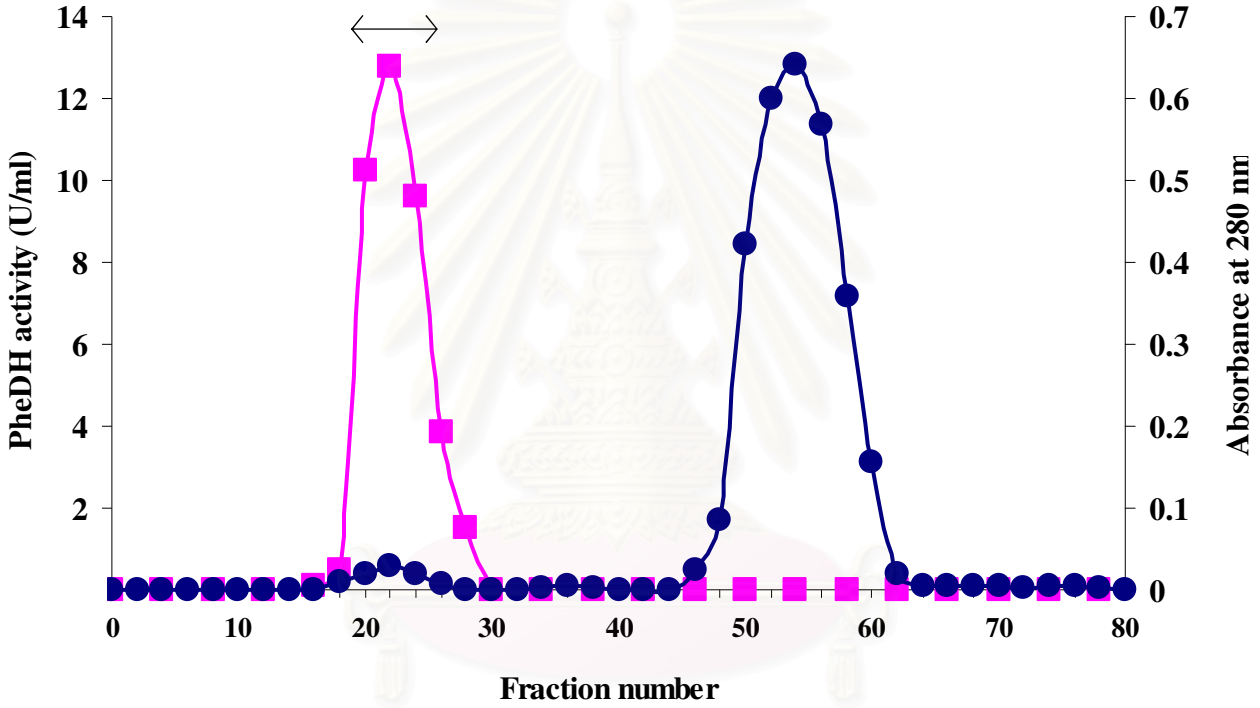

Figure 3.7 Purification of phenylalanine dehydrogenase from *B. lentus* by Sephadex G-200 column

The enzyme solution was applied to Sephadex G-200 column and the enzyme was eluted with 0.2 M KCl in 10 mM potassium phosphate buffer, pH 7.4 containing 0.01% (v/v) β-mercaptoethanol and 1 mM EDTA at the flow rate of 15 ml/hr. The fractions of 1 ml were collected. The protein peak from fraction numbers 17 to 27 was pooled (⟷) ● A_{280}, ■ PheDH activity

Table 3.1 Summary of phenylalanine dehydrogenase purification

Purification steps	Total activity (unit)	Total protein (mg)	Specific activity (units/mg protein)	% Recovery	Purification fold
Crude enzyme	2,230	2,170.0	1.0	100.0	1.0
DEAE-Toyopearl column	1,490.0	202.0	7.4	66.8	7.2
Butyl-Toyopearl column	538.0	10.0	53.8	24.1	52.4
Sephadex G-200 column	410.0	2.4	170.8	18.4	166.3

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3.2.5 Determination of enzyme purity by non-denaturing polyacrylamide gel electrophoresis (native-PAGE)

The enzyme from each step of purification was analyzed for purity and protein pattern by non-denaturing polyacrylamide gel electrophoresis as described in section 2.8.4. The activity staining was performed to compare with protein staining. As shown in Figure 3.8, The enzyme purity was increased from in each step of purification as the band of other proteins decreased. The purified enzyme after Sephadex G-200 column in lane 4 A showed a single protein band. Activity staining showed a single band with same mobility as that of protein band.

3.3 Characterization of phenylalanine dehydrogenase

3.3.1 Molecular weight determination of phenylalanine dehydrogenase

The native molecular weight of PheDH was determined from high molecular weight calibration curve (Figure 3.9) obtained from non-denaturing polyacrylamide gel electrophoresis as mentioned in section 2.9.1. The molecular weight of the native enzyme was estimated to be about 360,000 Da. The subunit molecular weight of the enzyme was estimated to be about 42,000 Da by SDS-PAGE, as previously described in section 2.9.2. The result is shown in Figure 3.10 and molecular weight calibration curve is shown in Figure 3.11.

3.3.2 Substrate specificity of phenylalanine dehydrogenase

Substrate specificity of PheDH in the oxidative deamination and amination directions were studied as mentioned in section 2.9.3. Various amino acids and their derivatives were used as substrates in the direction of oxidative deamination at a concentration of 20 mM as shown in Table 3.2. Among the substrates tested, the highest activity was observed with L-phenylalanine. Ten to thirty percent of the activity was observed with *p*-fluoro-DL-phenylalanine, *m*-fluoro-DL-phenylalanine and L-norleucine. While *o*-fluoro-DL-phenylpyruvate, *S*-methyl-L-cysteine, L-valine, L-ethionine, L-methionine, L-histidine, L-leucine, L-tryptophan, L-alloisoleucine

Figure 3.8 Non-denaturing polyacrylamide gel electrophoresis of phenylalanine dehydrogenase from each step of purification

A. Protein staining

Lane 1	crude enzyme	36	μg protein
Lane 2	DEAE-Toyopearl column	18	μg protein
Lane 3	Butyl-Toyopearl column	8.4	μg protein
Lane 4	Sephadex G-200 column	2.7	μg protein

B. Activity staining

Lane 1	crude enzyme	36	μg protein
Lane 2	DEAE-Toyopearl column	18	μg protein
Lane 3	Butyl-Toyopearl column	8.4	μg protein
Lane 4	Sephadex G-200 column	2.7	μg protein

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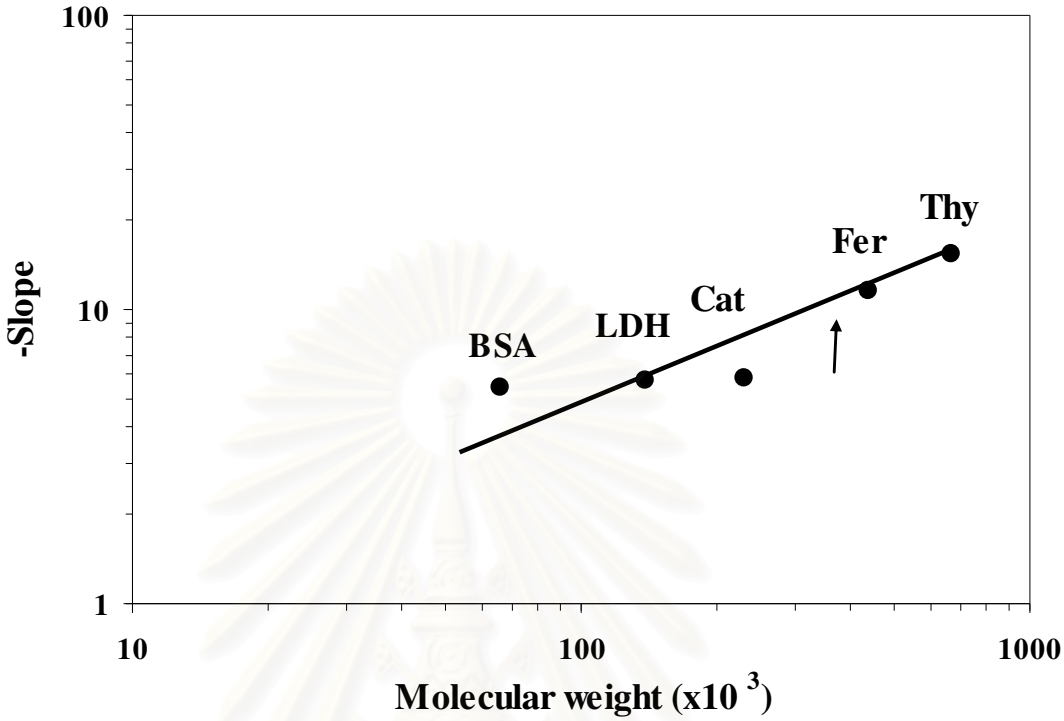

Figure 3.9 Calibration curve for native molecular weight of phenylalanine dehydrogenase on non-denaturing polyacrylamide gel electrophoresis

Thy	=	thyroglobulin	(MW 669,000)
Fer	=	ferritin	(MW 440,000)
Cat	=	catalase	(MW 232,000)
LDH	=	lactate dehydrogenase	(MW 140,000)
BSA	=	bovine serum albumin	(MW 66,000)

Arrow indicates a determined molecular weight of PheDH

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Figure 3.10 SDS-polyacrylamide gel electrophoresis of phenylalanine dehydrogenase

Lane 1:	PheDH from Sephadex G-200 column	
Lane 2:	β-galactosidase	(MW 116,000)
	bovine serum albumin	(MW 66,000)
	ovalbumin	(MW 45,000)
	lactate dehydrogenase	(MW 35,000)
	REase Bsp98I	(MW 25,000)
	β-lactoglobulin	(MW 18,400)
	lysozyme	(MW 14,400)

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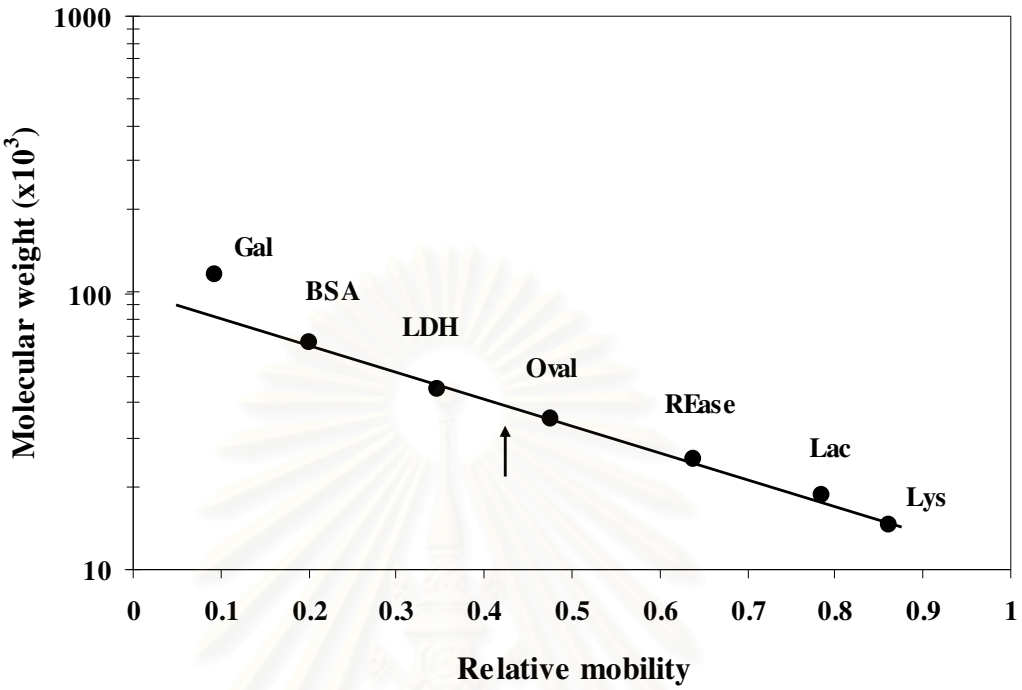

Figure 3.11 Calibration curve for molecular weight of phenylalanine dehydrogenase subunit on SDS -polyacrylamide gel electrophoresis

Gal	=	β-galactosidase	(MW 116,000)
BSA	=	bovine serum albumin	(MW 66,000)
Oval	=	ovalbumin	(MW 45,000)
LDH	=	lactate dehydrogenase	(MW 35,000)
REase	=	REase Bsp98I	(MW 25,000)
Lac	=	β-lactoglobulin	(MW 18,400)
Lys	=	lysozyme	(MW 14,400)

Arrow indicates the K_{av} of PheDH

L-isoleucine and L-norvaline gave less than 10% of the activity. Other amino acids and their derivatives listed below Table 3.2 were inert.

The reductive amination of the keto acids (10 mM) is shown in Table 3.3. The enzyme reacted on phenylpyruvate as the best good substrate. The relative activities for α-ketocaproate, α-ketoisocaproate, α-ketovalerate, α-ketoisovalerate, α-keto-β-methyl-n-valerate, α-keto-γ-methiol-butyrate were 22, 9, 8, 7, 6 and 1% relative activity, respectively, compared with the activity for phenylpyruvate. The following keto acids were not substrates for the reductive amination: α-keto-n-butyrate, indole- β-pyruvate (1.2 mM), *p*-hydroxyphenylpyruvate (2.5 mM), α-ketoglutarate and pyruvate.

3.3.3 Coenzyme specificity of phenylalanine dehydrogenase

Coenzyme specificity of PheDH was investigated as described in section 2.9.4. PheDH required NAD^+ as a natural coenzyme for oxidative deamination. Some analogs of NAD^+ could serve as a coenzyme as shown in Table 3.4. 3-Acetylpyridine-NAD^+ was much better coenzyme than NAD^+. Nicotinamide-1,N^6-ethenoadenine dinucleotide, nicotinamide hypoxanthine dinucleotide, nicotinamide guanine dinucleotide and thionicotinamide adenine dinucleotide showed 81, 79, 55 and 36% relative activity to that of NAD^+, while $NADP^+$, nicotinic acid adenine dinucleotide and 3-pyridinealdehyde adenine dinucleotide showed no activity.

3.3.4 Effect of pH on phenylalanine dehydrogenase activity

The effect of pH on the enzyme activity in both of the oxidation deamination and reduction amination was performed at various pHs ranging from 6.0 to 12.5 as mentioned in section 2.9.5. The result is shown in Figure 3.12 A. The enzyme exhibited maximal activity at pH 10.4 for oxidative deamination while the maximal activity for reductive amination was at pH 8.5 as shown in Figure 3.12 B.

Table 3.2 Substrate specificity of phenylalanine dehydrogenase in oxidative deamination

Substrate[a]	Relative activity (%)
L-phenylalanine	100
p-fluoro-DL-phenylalanine	35
m-fluoro-DL-phenylalanine	17
o-fluoro-DL-phenylpyruvate	3
S-methyl-L-cysteine	6
L-norleucine	10
L-ethionine	4
L-methionine	5
L-histidine	1
L-leucine	3
L-tryptophan	2
L-valine	2
L-alloisoleucine	5
L-isoleucine	1
L-norvaline	2

[a] Final concentration of each substrate was 20 mM.

The followings were inert: *p*-hydroxyphenylacetate, α-methyl-DL-phenylalanine, *N*-methyl-L-phenylalanine, α-amino-β-phenylbutanotate, hydrocinnamate, L-β-phenyllactate, D-alanine, D-aspartate, D-arginine, D-glutamate, D-leucine, D-threonine, D-tryptophan, D-phenylalanine, D-methionine, D-valine, D-serine, L-alanine, L-arginine, L-asparagine, L-cysteine(10 mM), L-dopa, L-glutamine, L-glycine, L-proline, L-tyrosine (1 mM), L-threonine, L-lysine and L-serine.

Table 3.3 Substrate specificity of phenylalanine dehydrogenase in reductive amination

Substrate [a]	Relative activity (%)
phenylpyruvate	100
α-ketocaproate	22
α-ketoisocaproate	9
α-ketovalerate	8
α-ketoisovalerate	7
α-keto-β-methyl-n-valerate	6
α-keto-γ-methiol-butyrate	1

[a] Final concentration of keto acids was 10 mM.

The followings were inert: α-keto-n-butyrate, *p*-hydroxyphenylpyruvate (2.5 mM), indole- β-pyruvate (1.2 mM), α-ketoglutarate and pyruvate.

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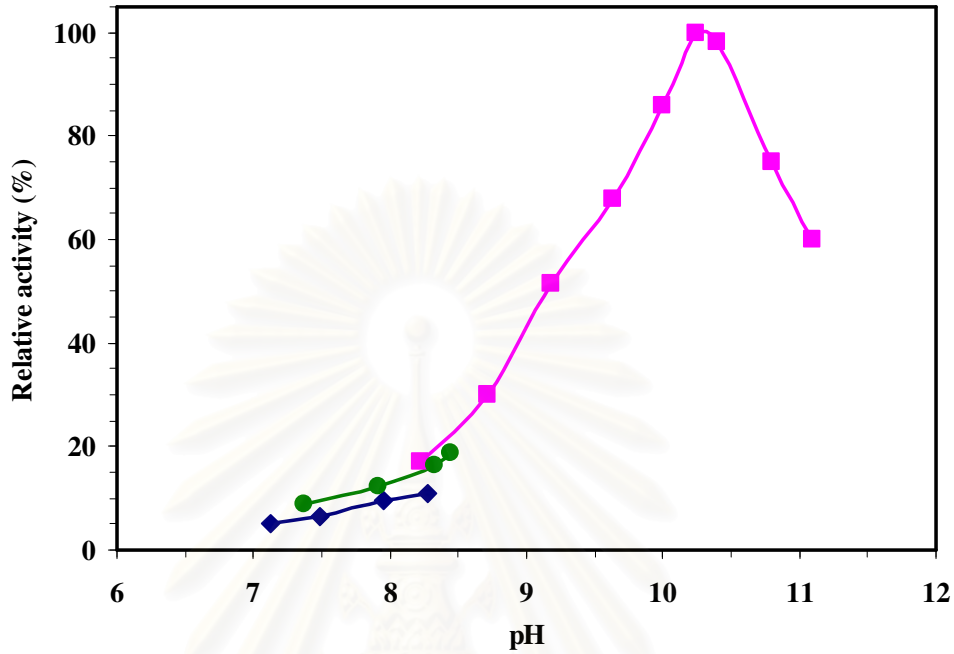
Table 3.4 Coenzyme specificity of phenylalanine dehydrogenase [a]

Coenzyme [b]	Relative activity (%)
β-nicotinamide adenine dinucleotide	100
3-acetylpyridine adenine dinucleotide	174
nicotinamide -1, N^6-ethenoadenine dinucleotide	81
nicotinamide hypoxanthine dinucleotide	79
nicotinamide guanine dinucleotide	55
thionicotinamide adenine dinucleotide	36
nicotinic acid adenine dinucleotide	0
β-nicotinamide adenine dinucleotide phosphate	0
3-pyridinealdehyde adenine dinucleotide	0

[a] The data represent the mean values of three independent experiments.

[b] Final concentration of each coenzyme analog was 2.0 mM. The assay was conducted at the following wavelengths: 3-acetylpyridine adenine dinucleotide, 363 nm ($\varepsilon = 9.1 \times 10^3$ $M^{-1}.cm^{-1}$); β-nicotinamide adenine dinucleotide phosphate ($NADP^+$), 340 nm ($\varepsilon = 6.2 \times 10^3$ $M^{-1}.cm^{-1}$); nicotinamide-1, N^6-ethenoadenine dinucleotide, 334 nm ($\varepsilon = 6.9 \times 10^3$ $M^{-1}.cm^{-1}$); nicotinamide hypoxanthine dinucleotide (deamino-NAD^+), 338 nm ($\varepsilon = 6.2 \times 10^3$ $M^{-1}.cm^{-1}$); nicotinamide guanine dinucleotide, 340 nm ($\varepsilon = 6.2 \times 10^3$ $M^{-1}.cm^{-1}$); nicotinic acid adenine dinucleotide (deamido-NAD^+), 338 nm ($\varepsilon = 6.2 \times 10^3$ $M^{-1}.cm^{-1}$); 3-pyridinealdehyde adenine dinucleotide, 358 nm ($\varepsilon = 9.3 \times 10^3$ $M^{-1}.cm^{-1}$); and thionicotinamide adenine dinucleotide, 395 nm ($\varepsilon = 11.3 \times 10^3$ $M^{-1}.cm^{-1}$). The reaction was carried out at pH 9.5 to avoid the degradation of NAD^+ analogs at a more alkaline pH.

A



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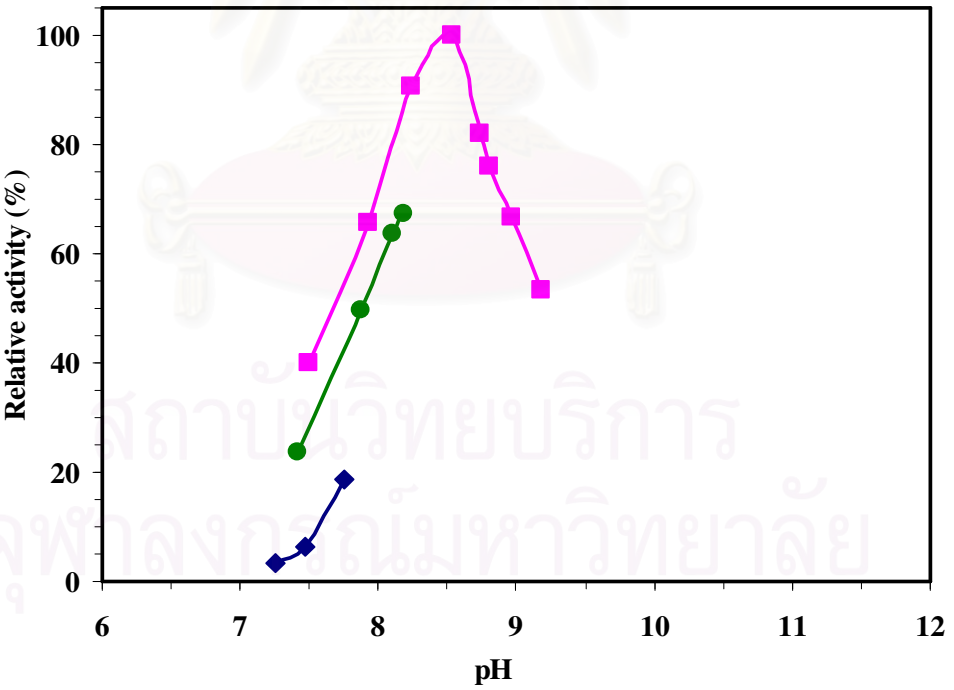

Figure 3.12 Effect of pH on phenylalanine dehydrogenase activity

The PheDH activities for oxidative deamination (A) and reductive amination (B) were measured at different pHs with 200 mM potassium phosphate buffer (●), Tris-HCl buffer (◆) and glycine-KCl-KOH buffer (■).

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3.3.5 Effect of temperature on phenylalanine dehydrogenase activity

The effect of temperature on enzyme activity was investigated as described in section 2.9.6. The temperature was varied from 25°C to 75°C. The result is shown in Figure 3.13**.** The enzyme showed the highest activity at 50ºC for oxidative deamination and at 55ºC for reductive amination.

3.3.6 Effect of pH on phenylalanine dehydrogenase stability

The pH stability of PheDH was studied as described in section 2.9.7. The enzyme was preincubated at 30ºC for 20 minutes in various 10 mM buffers at various pHs ranging from 4.0 to 12.5. The result is shown in Figure 3.14. The enzyme was stable over the pH ranged from 6.0 to 12.5.

3.3.7 Effect of temperature on phenylalanine dehydrogenase stability

The thermostability of PheDH was studied as described in section 2.9.8. The enzyme was preincubated at various temperatures ranged from 30ºC to 70ºC for 10 min. The enzyme activity of non-preincubated enzyme was defined as 100% relative activity. The result is shown in Figure 3.15 A. The enzyme retained its full activity at temperature up to 50ºC and lost about half of its activity at about 60ºC. At 65ºC, PheDH completely lost its activity. The enzyme stability was tested at 50ºC by incubated for 0 to 12 days and checked for its activity everyday. The remained deamination activities were expressed as the percentage of the original activity. The result is shown in Figure 13.5 B. The enzyme was fully stable at 50ºC for 2 hours and retained 50% of its activity after treatment for 3 days. The enzyme activity was relatively decreased with increasing of incubation time and the activity was completely lost after incubation for 11 days.

3.3.8 Effect of metal ions and chemical reagents on phenylalanine dehydrogenase activity

The effect of various metal ions and chemical reagents on the PheDH activity was tested in section 2.9.9. The result is shown in Table 3.5. Fe^{2+}, Fe^{3+} and

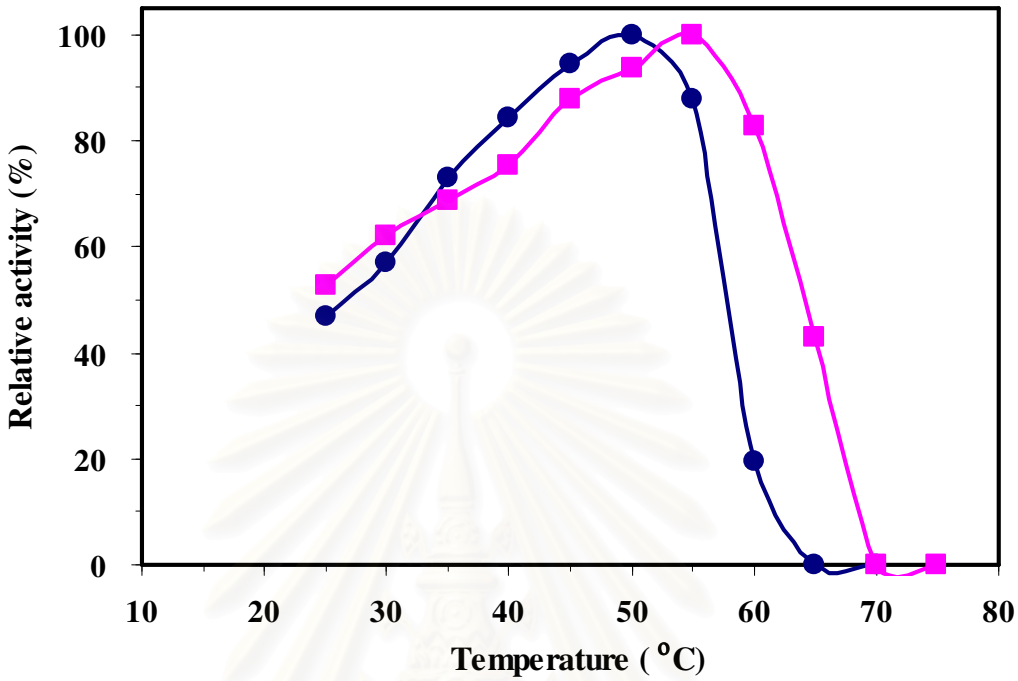

Figure 3.13 Effect of temperature on phenylalanine dehydrogenase activity

The PheDH activities for oxidative deamination(●)and reductive amination (■) reactions were measured at various temperatures varying from 25°C to 75°C.

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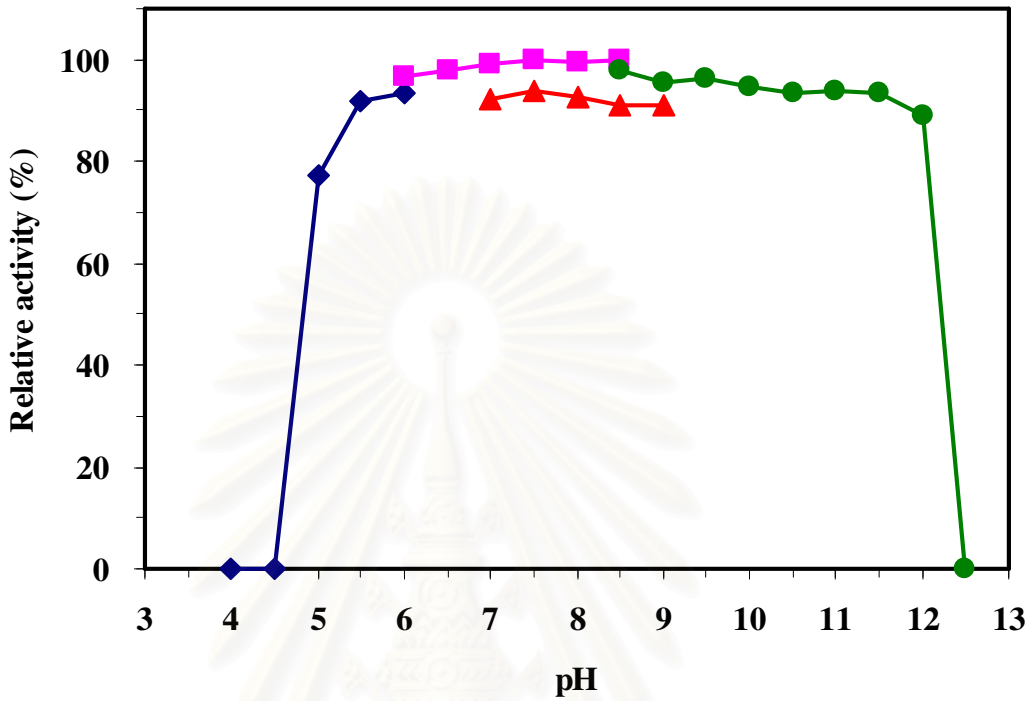

Figure 3.14 Effect of pH on phenylalanine dehydrogenase stability

The enzymes in buffers at various pHs were incubated at 30ºC for 20 minutes and then the relative activities were assayed for the oxidative deamination. The pHs stability was determined at pH 4.0 to 12.0. The 10 mM buffers used were acetate buffer (pH 4.0 - 6.0; ♦), potassium phosphate buffer (pH 6.0 - 8.5;■), Tris-HCl buffer (pH 7.0 - 9.0;▲) and glycine-KCl-KOH buffer (pH 8.5 -12.5; ●).

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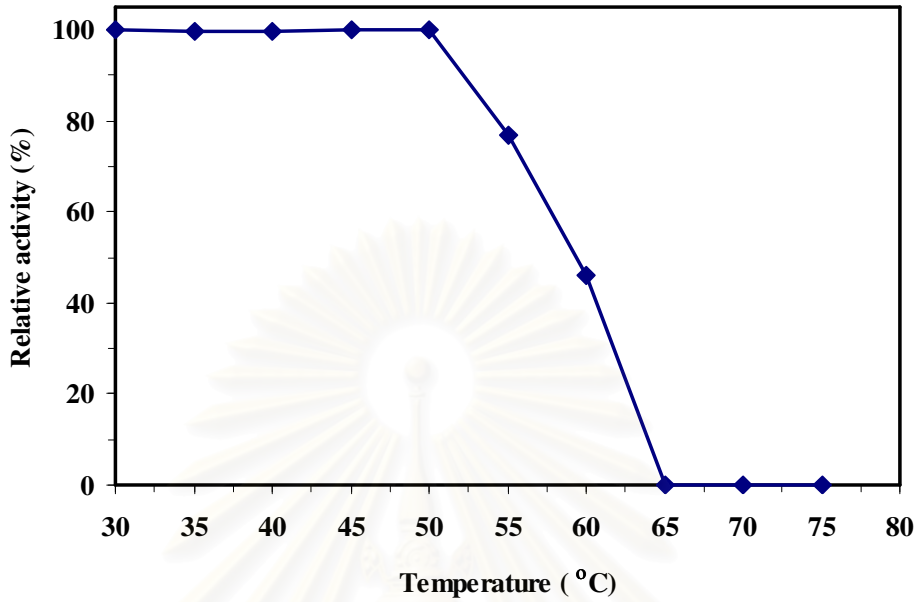
A



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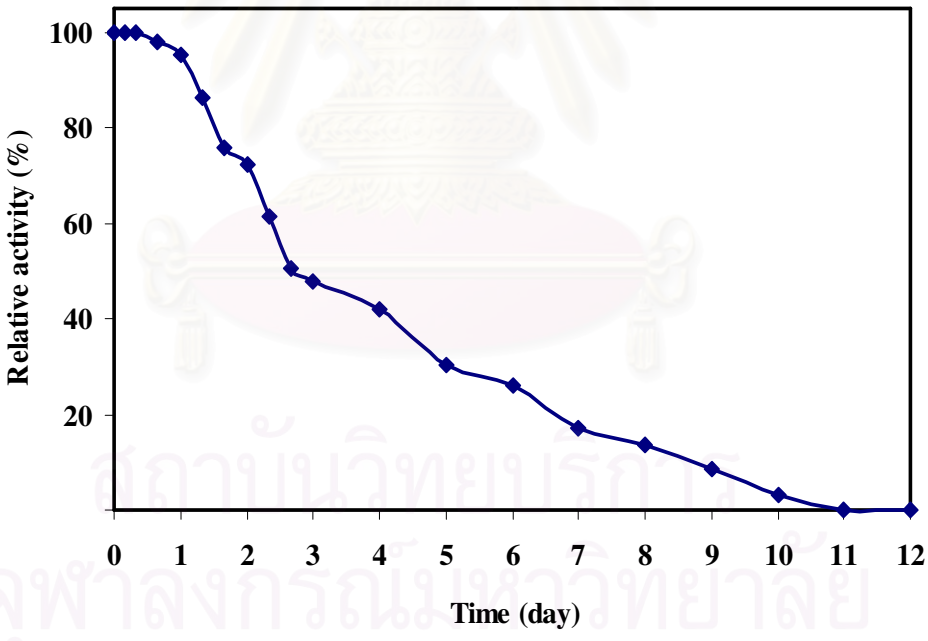

Figure 3.15 Effect of temperature on phenylalanine dehydrogenase stability

A The effect of temperature on stability of the enzyme activity was performed at 30 to 75ºC for 10 minutes before the residual oxidative deamination activity was determined under standard condition at 30ºC.

B The enzyme stability was tested at 50ºC and the residual oxidative deamination activity was determined under standard condition at 30ºC.

sulfhydryl reagent ($AgNO_3$) strongly inhibited the enzyme. Reducing agent (DTT) and chelating or carbonyl reagents (EDTA, sodium azide and hydroxylamine) had almost no effect on the enzyme activity. No marked inhibition was observed with Na^+, K^+, Li^+, Mg^{2+} and Mn^{2+}.

3.3.9 Inhibitory effect of various amino acids and keto acids on phenylalanine dehydrogenase activity

Inhibitory effect of various amino acids and keto acids, which were not the substrates of PheDH, on the oxidative deamination and reductive amination were investigated as described in section 2.9.10. The result is shown in Table 3.6. Among various non substrate amino acids examined for inhibitory effects on the direction of oxidative deamination, D-forms of amino acids with non-polar side chain, except D-alanine and D-valine, exhibited significant inhibition against L-phenylalanine. In addition, *p*-hydroxyphenylacetate and hydrocinnamate also inhibited enzyme activity. For the inhibitory effect of reductive amination, no significant inhibition was found upon the addition of various keto acids.

3.4 Kinetic mechanism studies of phenylalanine dehydrogenase

3.4.1 Initial velocity studies for oxidative deamination

A series of steady-state kinetic analyses was carried out to investigate the reaction mechanism. First, initial velocity studies for oxidative deamination were performed. The concentration of L-phenylalanine was varied in the presence of several fixed concentration of NAD^+. Double-reciprocal plots of initial velocity against reciprocals of L-phenylalanine concentrations gave a family of straight lines, which intersected in the upper left quadrant as shown in Figure 3.16 A. These results show that the reaction proceeds via the formation of a ternary complex of the enzyme with NAD^+ and L-phenylalanine (Cleland, 1971). The apparent K_m value for L-phenylalanine was calculated to be 0.59 mM. From the secondary plots of intercept at the ordinate versus reciprocal concentrations of NAD^+ as shown in Figure 3.16 B, The apparent K_m value for NAD^+ was calculated to be 0.55 mM.

Table 3.5 Effect of metal ions and chemical reagents on phenylalanine dehydrogenase activity

Compound [a]	Final concentration (mM)	Relative activity (%)
None	-	100
NaCl	10	100
KCl	10	100
LiCl	10	100
$MgCl_2.6H_2O$	10	100
$CaCl_2.2H_2O$	10	92
$MnCl_2.7H_2O$	10	100
$MgSO_4.7H_2O$	10	100
$MnSO_4.H_2O$	10	100
$CoCl_2.6H_2O$	10	90
EDTA	10	97
Hydroxylamine	10	94
Sodium azide	10	100
DTT	10	100
$FeCl_3$	1	0
$AgNO_3$	1	0
$FeSO_4.7H_2O$	1	0

[a] Abbreviation: EDTA, ethylenediamine tetraacetic acid; DTT, dithiothreitol

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Table 3.6 Inhibitory effect of various amino acids and keto acids on phenylalanine dehydrogenase activity

Process and amino acids / keto acids	Relative activity (%)
Oxidative deamination [a]	
none	100
D-phenylalanine	28
D-methionine	72
D-leucine	80
D-tryptophan	60
p-hydroxyphenylacetate	80
hydrocinnamate	84
Reductive amination [b]	
none	100
pyruvate	98

[a] Amino acid concentration was 10 mM. *N*-Methyl-L-phenylalanine, α-methyl-DL-phenylalanine, α-amino-β-phenylbutanotate, L-β-phenyllactate, D-alanine, D-aspartate, D-arginine, D-glutamate, D-threonine, D-serine, D-valine, L-alanine, L-arginine, L-asparagine, L-cysteine (10 mM), L-dopa, L-glutamine, L-glycine, L-tyrosine (1 mM), L-threonine and L-lysine did not inhibit the reaction.

[a] Keto acids concentration was 10 mM. *p*-Hydroxyphenylpyruvate (2.5 mM), indole-β-pyruvate (1.2 mM), α-keto-n-butyrate and α-ketoglutarate did not inhibit the reaction.

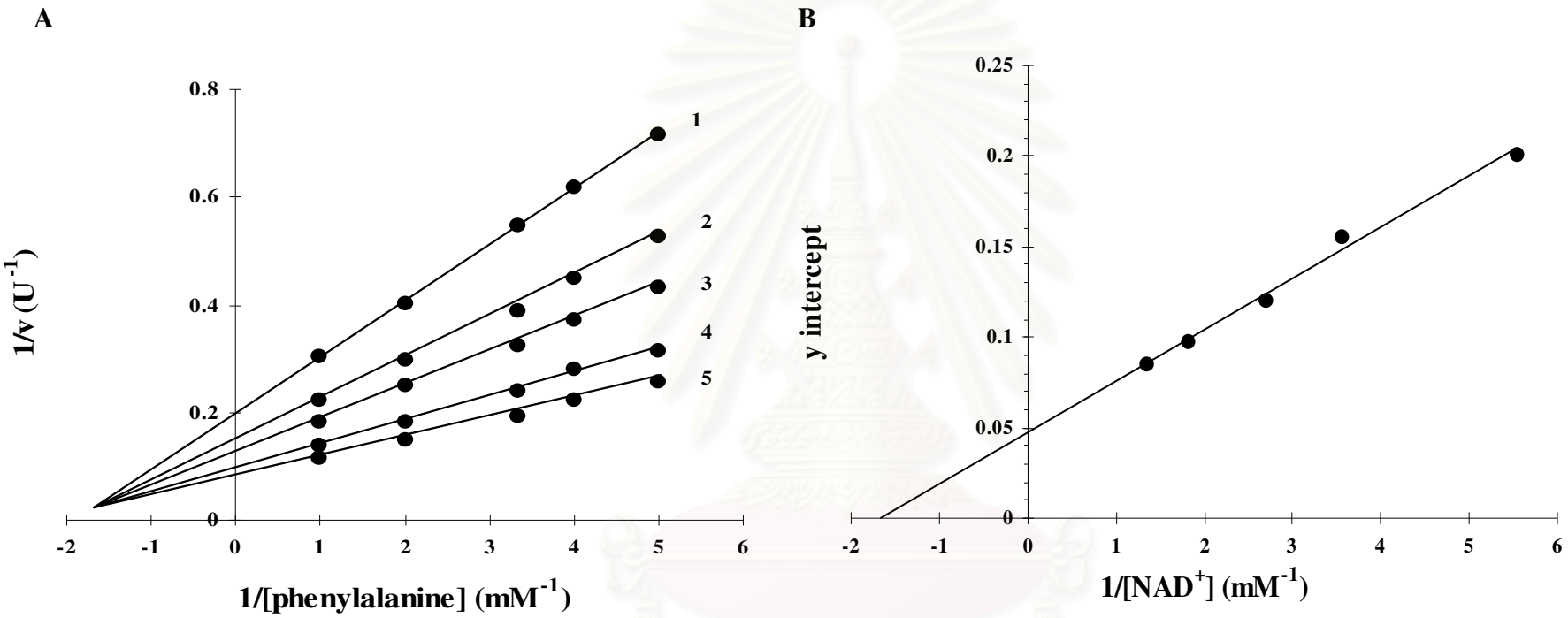

Figure 3.16 Initial velocity patterns for oxidative deamination

A. Double-reciprocal plots of initial velocities versus L-phenylalanine concentrations at a series of fixed concentrations of NAD^+. Concentrations of NAD^+ were: 1, 0.2 mM; 2, 0.3 mM; 3, 0.4 mM; 4, 0.6 mM; and 5, 0.8 mM.

B Secondary plots of y intercepts versus reciprocal NAD^+ concentrations.

3.4.2 Initial velocity studies for reductive amination

A kinetic analysis of reductive amination was performed to investigate several possible reaction mechanisms.

3.4.2.1 At a saturating concentration of NADH (0.2 mM), the double reciprocal plots of initial velocities versus phenylpyruvate concentrations at several fixed concentrations of NH_4Cl gave straight intersecting lines as shown in Figure 3.17 A. The apparent K_m value for phenylpyruvate was calculated to be 0.18 mM from this figure. The apparent K_m value for NH_4Cl was calculated to be 300 mM from the secondary plots of intercept at the ordinate versus reciprocal concentrations of NH_4Cl as shown in Figure 3.17 B.

3.4.2.2 At a saturating concentration of phenylpyruvate (10 mM), the double-reciprocal plots of initial velocities versus NH_4Cl concentration at several fixed concentrations of NADH gave parallel straight lines as shown in Figure 3.18.

3.4.2.3 At a saturating concentration of NH_4Cl (500 mM), the double reciprocal plots of initial velocities versus NADH concentration at several fixed concentrations of phenylpyruvate gave straight intersecting lines as shown in Figure 3.19 A. The apparent K_m of NADH was calculated to be 0.09 mM. The secondary plots of intercept at the ordinate versus reciprocal concentrations of phenylpyruvate, as shown in Figure 3.19 B, gave the same apparent K_m of 300 mM with that in Figure 3.17 A.

The apparent K_m values of the substrates of PheDH were summarized in Table 3.7. The observed kinetic patterns from Figure 3.17, 3.18 and 3.19 rules out the possibility of random addition of substrates and indicate a sequential ordered mechanism in which phenylpyruvate binds to the enzyme between NADH and NH_4Cl (Cleland, 1971).

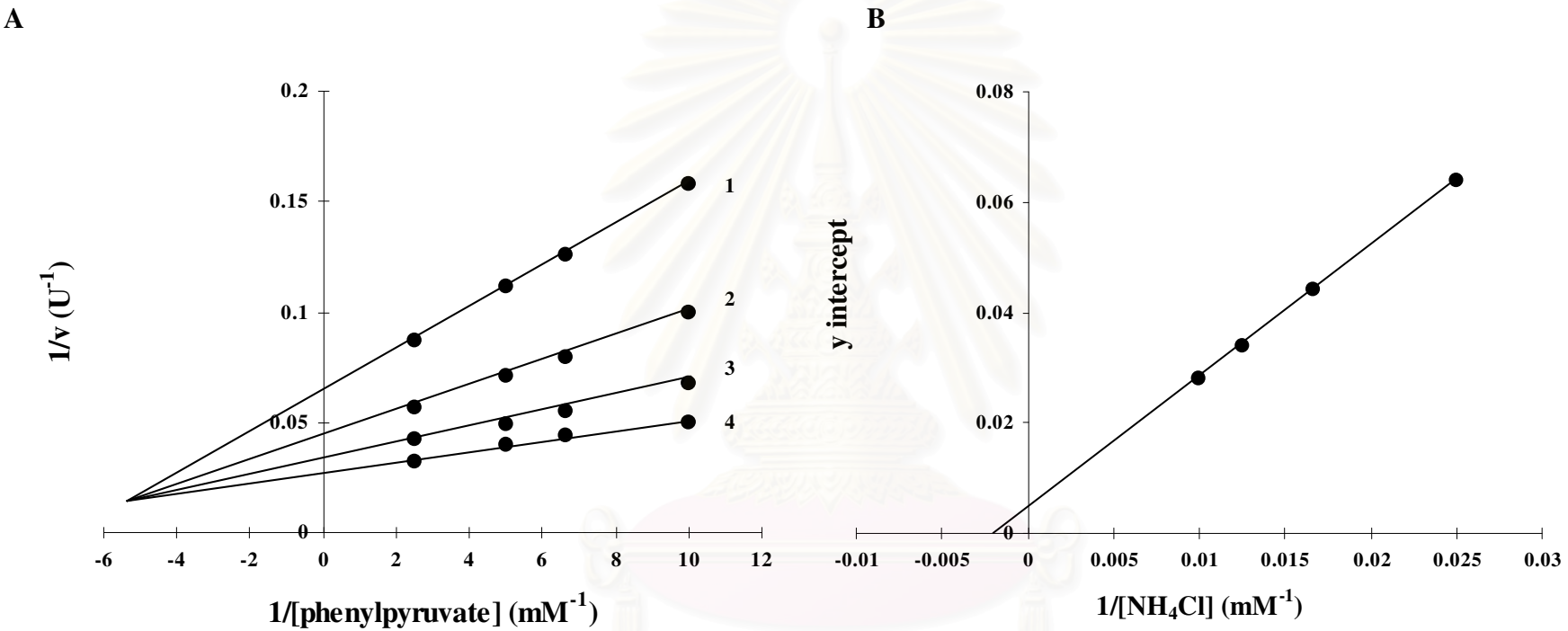

Figure 3.17 Initial velocity patterns for reductive amination (phenylpyruvate versus NH_4Cl)

A Double-reciprocal plots of initial velocities versus phenylpyruvate concentration at several fixed concentrations of NH_4Cl in the presence of a high concentration (0.2 mM) of NADH. The concentrations of NH_4Cl were: 1, 40 mM; 2, 60 mM; 3, 80 mM; and 4, 120 mM.

B Secondary plots of y intercepts versus reciprocal NH_4Cl concentrations.

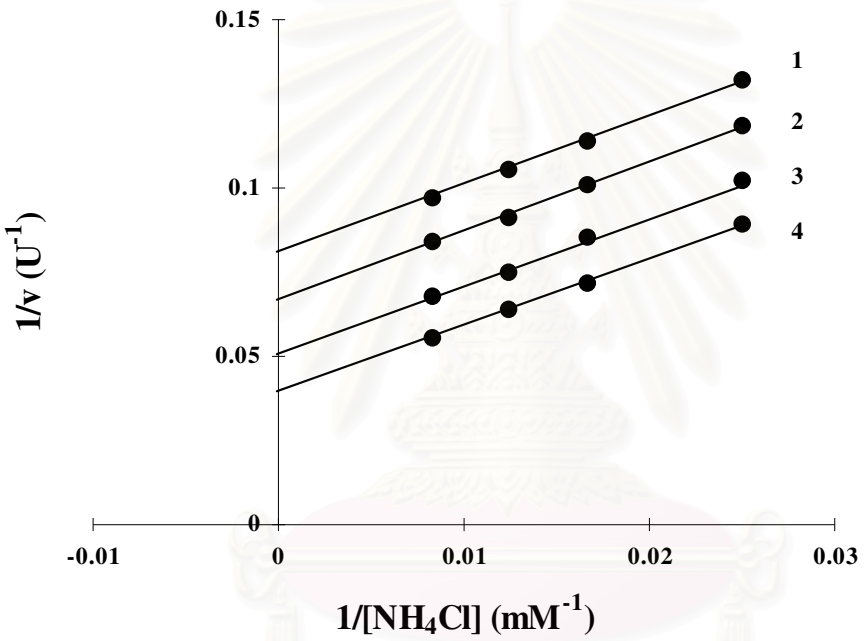

Figure 3.18 Initial velocity patterns for reductive amination (NH_4Cl versus NADH)

Double-reciprocal plots of initial velocities versus NH_4Cl concentration at several fixed concentrations of NADH in the presence of a high concentration (10 mM) of phenylpyruvate. The concentrations of NADH were: 1, 0.05 mM; 2, 0.06 mM; 3, 0.1 mM; and 4, 0.2 mM.

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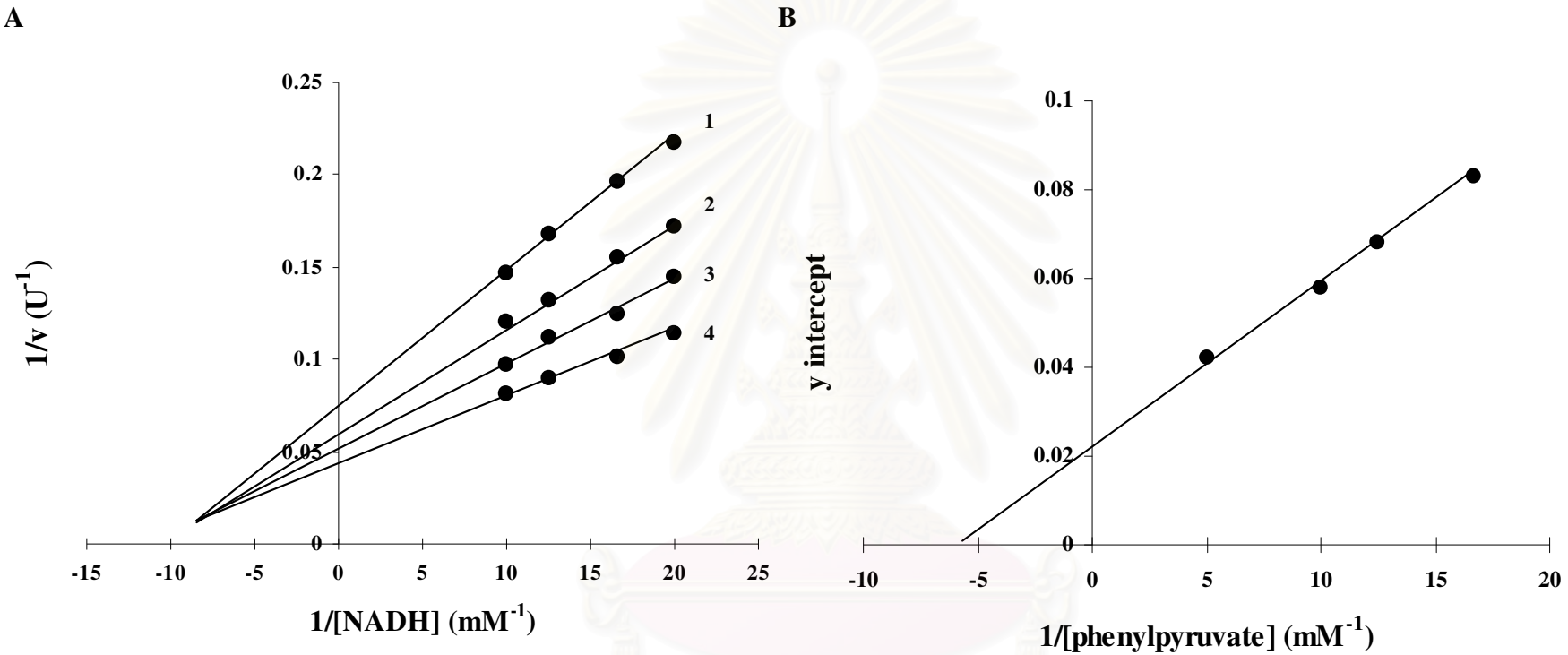

Figure 3.19 Initial velocity patterns for reductive amination (NADH versus phenylpyruvate)

A Double-reciprocal plots of initial velocities versus NADH concentration at several fixed concentrations of NH_4Cl in the presence of a high concentration (500 mM) of NH_4Cl . The concentrations of phenylpyruvate were: 1, 0.06 mM; 2, 0.08 mM; 3, 0.1 mM; and 4, 0.2 mM.

B Secondary plots of y intercepts versus reciprocal phenylpyruvate concentrations.

Table 3.7 The apparent K_m values of substrates of phenylalanine dehydrogenase from *B. lentus*

Substrate	K_m (mM)
L-phenylanine	0.59
NAD^+	0.55
phenylpyruvate	0.18
NH_4Cl	300
NADH	0.09

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3.4.3 Product inhibition studies

Product inhibition studies of the reductive amination reaction were performed according to the method of Cleland (1971) in order to determine the order of substrate addition and product release. With NAD^+ as an inhibitor, the double reciprocal plots of velocities versus NADH concentration at high and constant concentrations of phenylpyruvate and NH_4Cl showed competitive inhibition (Figure 13.20 A). This result suggests that NAD^+ and NADH can bind to the free form of the enzyme. L-Phenylalanine showed uncompetitive inhibition with respect to NADH (Figure 13.20 B). The other product inhibition patterns observed with NAD^+ and L-phenylalanine (Figure 13.21 and Figure 13.22) as inhibitors were identical to the predicted patterns for the sequential ordered ternary-binary mechanism except for the noncompetitive inhibition by L-phenylalanine with respect to phenylpyruvate (Figure 13.21 B). Noncompetitive inhibition by phenylalanine with respect to NH_4Cl rules out the Theorell-Chance mechanism. Observed product inhibition patterns of reductive amination are summarized in Table 3.8.

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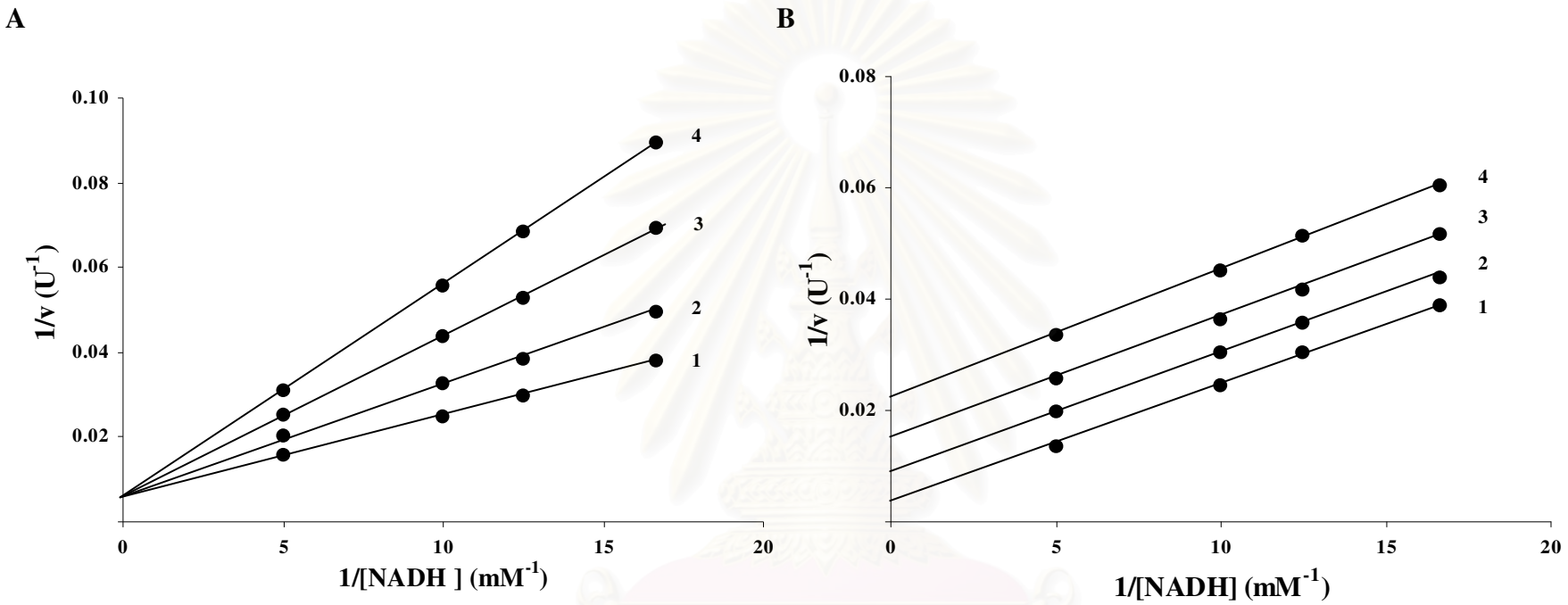

Figure 3.20 Product inhibition patterns of reductive amination by NAD^+ and phenylalanine with respect to NADH

A Product inhibition pattern by NAD^+ with NADH as the varied substrate. Phenylpyruvate (10 mM) and NH_4Cl (500 mM) were held at saturating and constant concentrations. The concentrations of NAD^+ were: 1, 0 mM; 2, 0.4 mM; 3, 1.0 mM; and 4, 2.0 mM.

B Product inhibition patterns by L-phenylalanine with NADH as the varied substrate. Phenylpyruvate (10 mM) and NH_4Cl (500 mM) were held at saturating and constant concentrations. The concentrations of L-phenylalanine were: 1, 0 mM; 2, 2.0 mM; 3, 6.0 mM; and 4, 10.0 mM.

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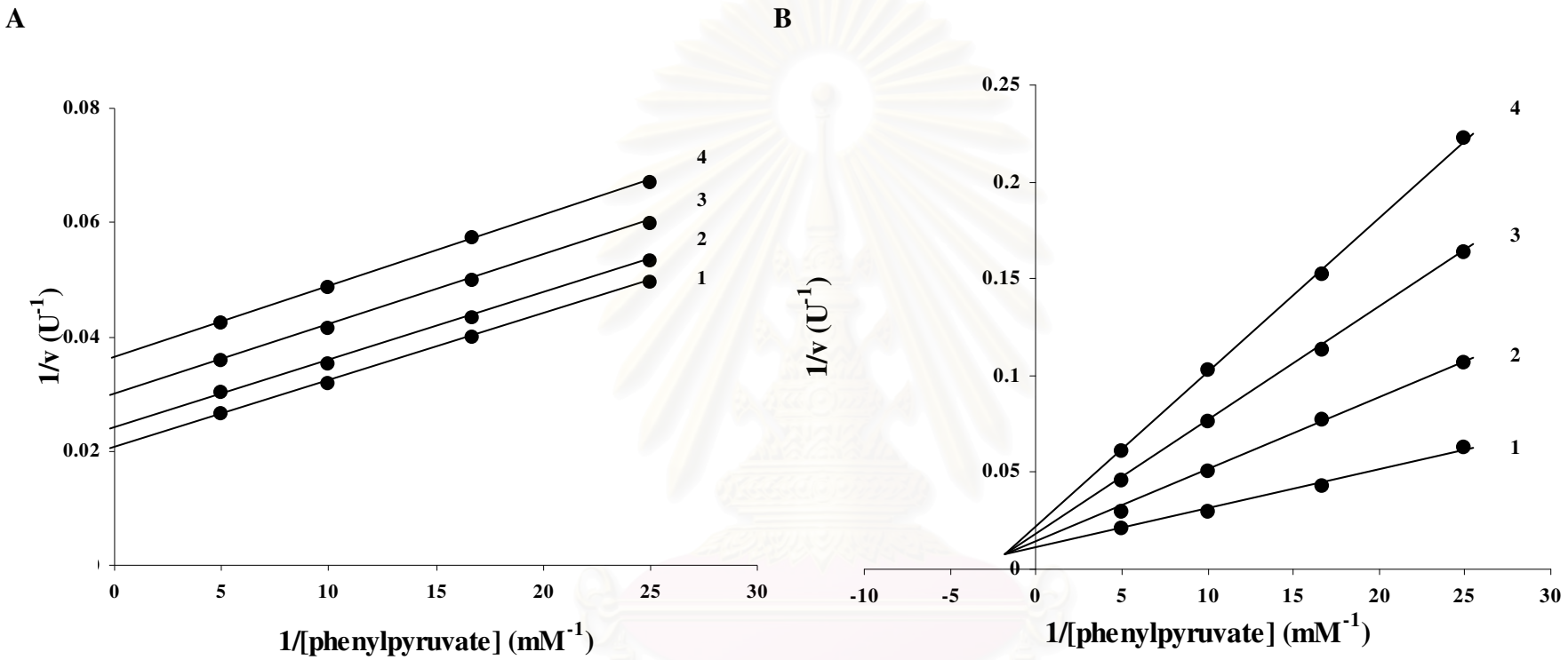

Figure 3.21 Product inhibition patterns of reductive amination by NAD^+ and phenylalanine with respect to phenylpyruvate

A Product inhibition pattern by NAD^+ with phenylpyruvate as the varied substrate. NADH (0.2 mM) and NH_4Cl (500 mM) were held at saturating and constant concentrations. The concentrations of NAD^+ were: 1, 0 mM; 2, 0.4 mM; 3, 1.0 mM; and 4, 2.0 mM.

B Product inhibition pattern by L-phenylalanine with phenylpyruvate as the varied substrate. NADH (0.2 mM) and NH_4Cl (500 mM) were held at saturating and constant concentrations. The concentrations of L-phenylalanine were: 1, 0 mM; 2, 2.0 mM; 3, 6.0 mM; and 4, 10.0 mM.

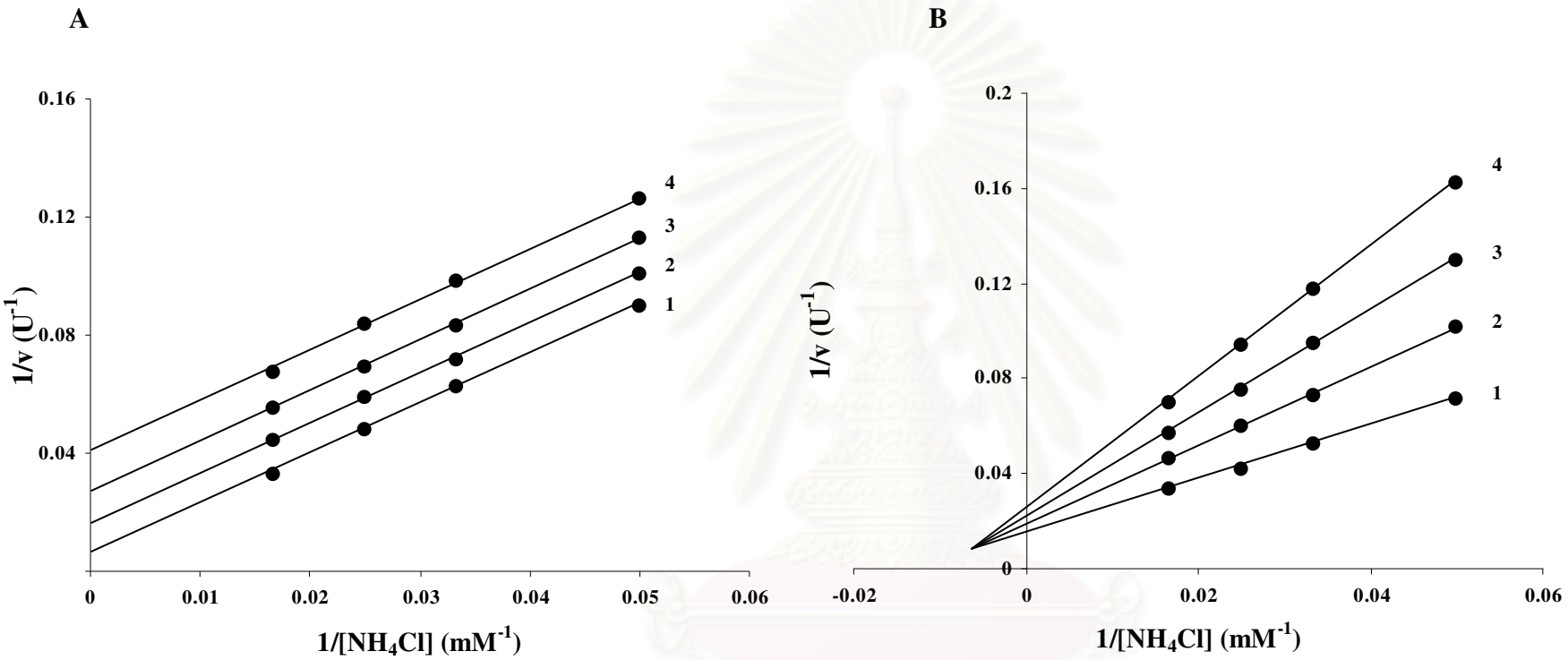

Figure 3.22 Product inhibition patterns of reductive amination by NAD^+ and phenylalanine with respect to NH_4Cl

A Product inhibition pattern by NAD^+ with NH_4Cl as the varied substrate. NADH (0.2 mM) and phenylpyruvate (10 mM) were held at saturating and constant concentrations. The concentrations of NAD^+ were: 1, 0 mM; 2, 0.4 mM; 3, 1.0 mM and 4, 2.0 mM.

B Product inhibition pattern by L-phenylalanine with NH_4Cl as the varied substrate. Phenylpyruvate (10 mM) and NADH (0.2 mM) were held at saturating and constant concentrations. The concentrations of L-phenylalanine were: 1, 0 mM; 2, 2.0 mM; 3, 6.0 mM and 4, 10.0 mM.

Table 3.8 Production inhibition patterns of the reductive amination of phenylalanine dehydrogenase from *B. lentus*

Substrate	**Product inhibition pattern by**	
	NAD^+	**L-phenylalanine**
NADH	competitive	uncompetitive
phenylpyruvate	uncompetitive	noncompetitive
NH_4Cl	uncompetitive	noncompetitive

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CHAPTER IV

DISCUSSION

4.1 Optimization of phenylalanine dehydrogenase production from *Bacillus lentus*

The production of PheDH by *B. lentus* was improved by optimization of medium and culture condition: concentration of inducer, pH of medium, cultivation temperature and cultivation time.

The addition of enzyme inducer is usually very effective for maximize harvestable enzyme levels. In this research, addition of phenylalanine at 0.4% final concentration to culture medium gave the optimal induction for PheDH production. The enzyme levels are substantially increased in the presence of L-phenylalanine in the media, indicates that although amino acid synthesis is thermodynamically favored in all of PheDHs with K_{eq} values ~ 10^{-14}-10^{-18}, the physiological role of the enzyme *B. lentus* is likely to be the degradation of L-phenylalanine for use as a carbon and nitrogen sources.

PheDH production from other sources have been reported about their inducers such as the addition of L-phenylalanine as enzyme inducer to the medium promotes the PheDH production from *S. urea* (Asano *et al*., 1987b), *B. sphaericus* (Asano *et al*., 1987b) and *B. badius* (Asano *et al*., 1987a) whereas L-tyrosine and L-methionine have been used in those from *T. intermedius* and *Microbacterium* sp., respectively (Ohshima *et al*., 1991; Asano and Tanetani, 1998). In *Brevibacterium* sp., L-phenylalanine as enzyme inducer can be substituted by L-histidine while L-phenylalanine cannot be substituted by these amino acids in *Rhodococcus* sp. M4 (Hummel *et al*., 1984). In addition, rather high concentrations of L-phenylalanine are observed for the induction of PheDH from *Rhodococcus* sp.M4 (Hummel *et al*., 1987)

In culture medium, pH values are controlled to achieve optimal growth rates. Most of bacteria producing PheDH grow well over a range of pH 7.0 to 7.5. Temperature is one of the most important factors that influence growth rate and enzyme production. PheDH from *B. lentus* showed highest rate of growth and enzyme

production at 37°C. All of PheDH producing bacteria, except *T. intermedius*, are mesophile which their optimum temperature for PheDH production is at moderate temperature (20°C to 40°C). Cultivation time for PheDH production used is in range of 18 to 30 hours. For *B. lentus*, PheDH production increases parallel with the cell growth and reaches the highest level at late log phase in approximately 18 hours. The growth-associated pattern of PheDH formation indicates that the enzyme is responsible for the catabolism of L-phenylalanine.

4.2 Purification of phenylalanine dehydrogenase

In order to purify the PheDH, extraction procedures should be selected according to the source of the protein. In this work, PheDH, an intracellular enzyme, was extracted from *B. lentus* which is a gram-positive bacteria and the major component of the cell wall is peptidoglycan together with teichoic acid, teichuronic acids and other carbohydrates (Eisenthal and Dansor, 1992). Mechanical technique are usually used to break down cell wall in order to release intercellular protein. The cell disintegration technique involved cell lysis by ultrasonication or high pressure sound waves, which causes cell breakage by cavitations and shear forces, was used in this work. Upon the sonication, PheDH activity can be lost by many reasons. Ultrasonication itself can also damage structure of a target protein. Moreover, several problems may be consequence of disruption, due to the destruction of intracellular compartmentation. 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; Bollag and Edelstein, 1991), 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. 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.

Ammonium sulfate precipitation is usually used in the early step of purification procedure. In the purification of PheDHs from *B. sphaericus*, *S. ureae*, *R. maris* and *Microbacterium* sp., ammonium sulfate precipitation step have been successfully used as another promising way to get rid of other bulk proteins from crude enzyme solution.(Asano *et al*., 1987b; Misono *et al*., 1989; Hummel *et al*., 1987) Preliminary experiment on ammonium sulfate precipitation was also done in this work, PheDH from *B. lentus* was precipitated by 40-50% saturated ammonium sulfate. Nevertheless, it was found that there was great loss of PheDH activity with low removal of other proteins. Thus, this procedure is omitted for purification of PheDH from *B. lentus*.

A series of chromatographic steps that separate molecules according to their different molecular properties was applied to an purification of PheDH from *B. lentus*. Ion exchange chromatography separates proteins with differences in charge to give a very high resolution with high sample loading capacity. DEAE-Toyopearl is an 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. sphaericus, S. ureae*, *T. intermedius* and *Microbacterium* sp. (Asano *et al*., 1987a; Asano *et al*., 1987b; Ohshima *et al*., 1991; Asano and Tanetani, 1998). Its popularity comes from the high resolving power, versatility, reproducibility and ease of performance. This column contributed greatly to the purification of PheDH from *B. lentus*, high % recovery and also high amount of proteins removed. About 90% of other bulk proteins were 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, Butyl-Toyopearl column were eluted with

a negative salt gradient in the range of 25% to 20%. More than 90% of unwanted proteins were removed.

Gel-filtration chromatography is frequently used as a furnishing step in protein purification. It is also useful for separate proteins with different in size of molecules. Sephadex-G 200 consists of cross-linked dextran. It can be exploited for separation of molecules with molecular masses ranging from ~ 10^4 to ~ 6 x 10^5 Da (Scropes, 1994). It was used in the purification of PheDH from *S. ureae*, *B. sphaericus* and *Microbacterium* sp. (Asano *et al*., 1987b; Ohshima *et al*., 1991; Asano and Tanetani, 1998). PheDH from *B. lentus* could be completely separated from unwanted proteins by this column. The success in using this column was judged by the homogeneity of PheDH according to native-PAGE.

4.3 Characterization of phenylalanine dehydrogenase

4.3.1 Molecular weight determination of phenylalanine dehydrogenase

Non-denaturing polyacrylamide gel electrophoresis can separate molecular of proteins according to their size and charge. However, the influence of charge is eliminated by Ferguson plot analysis (Ferguson, 1964). Thus, by using this plot, the relative mobility of protein from native-PAGE can be used to determine its molecular weight. The native molecular weight of PheDH from *B. lentus* was estimated to be almost 360,000 Da. Molecular weight of subunit was calculated to be about 42,000 Da by SDS-polyacrylamide electrophoresis. Thus, the native enzyme probably has an octameric structure which is similar to PheDH from *B. badius, B. sphaericus, S. urea and Microbacterium sp.* (Asano *et al*., 1987a; Asano *et al*., 1987b; 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 1.1.

4.3.2 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 enzyme from *B. lentus* 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. 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 are L-norleucine > L-leucine > L-isoleucine, respectively.

From all of aromatic amino acids tested, only L-tryptophan showed slightly relative activity, while 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.

Among 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 35%, 17% and 3% of relative activity, respectively. The reaction rate of *B. badius* enzyme on *p*-, *m*- and *o*-fluoro-DL-phenylalanine were 34%, 11% and 2% relative activity, respectively. No activity was observed with hydrocinnamate, L-β-phenyllactate and *N*-methyl-L-phenylalanine, which their amino groups are substituted by -H, -OH and $-NHCH_3$ group, respectively while α-methyl-DL-phenylalanine, which H atom of the chiral carbon is substituted

by methyl group, were inert for *B. lentus* enzyme. These observations indicated that the amino group and H atom of the chiral carbon are essential for the substrate specificity of the enzyme. The structure of amino acids and analogs are shown in Appendix G.

The reductive amination reaction was observed with various keto acid analogs. The *B. lentus* 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. In contrast to PheDH from *B. lentus* and *Nocardia* sp., aromatic keto acid, *p*-hydroxylphenylpyruvate and indole-β-pyruvate were inert. The structures of these keto acid analogs are shown in Appendix H.

4.3.3 Coenzyme specificity of phenylalanine dehydrogenase

NAD^+-dependent phenylalanine dehydrogenase catalyzes the reversible oxidative demination of L-phenylalanine. In this research, NAD^+ is replaced by some of the NAD^+ analogs as coenzyme for the *B. lentus* PheDH. $NADP^+$, which differs from NAD^+ only by the addition of a phosphoric group at C-2 position of NAD^+ adenosyl ribose, is inert for *B. lentus* PheDH similar to other 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 disrupted by the presence of a 2-phosphate (Brunhuber *et al*., 2000).

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

The NAD^+ analogs which were 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^+, β-nicotinamide adenine dinucleotide phosphate 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 J.

4.3.4 Effect of pH on phenylalanine dehydrogenase activity and stability

The enzyme activity is often limited to a relatively narrow pH range. On either side of this range, the enzyme activity may decrease. A pH change may affect 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, however, 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 enzyme.

The pH which *B. lentus* PheDH showed maximum activity were pH 10.4 for the oxidative deamination of L-phenylalanine and pH 8.5 for the reductive amination. This was similar to PheDHs from various sources. The pH optima for oxidative deamination lay between 10.1 and 12.0 while the pH optima for reductive amination were between 8.5 and 10.3, except for the enzyme of *Microbacterium* sp. which had 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 *B. lentus* PheDH activity due to the low activity observed with these buffers.

The glycine-KCl-KOH buffer was an appropriate buffer for the *B. lentus* enzyme. This kind of buffer is widely used for the assay of amino acid dehydrogenase.

The pH stability of an enzyme depends on many factors including temperature, ionic strength, and chemical nature of the buffer, concentration of various preventions, concentration of substrates or cofactors of the enzyme and enzyme concentration (Segal, 1976). The *B. lentus* PheDH was stable over a pH range of 6.0 to 11.5 upon incubation at 30°C for 20 minutes. In previous report, 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).

4.3.5 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 chances of breaking the multiple weak noncovalent interactions holding the three-dimensional structure (Segal, 1976).

The optimum temperature of PheDH from *B. lentus* for the oxidative deamination and reductive amination 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, 100% activity was retained at 50^{o}C and dropped about 50% at 65^{o}C. So, time course of the enzyme stability was further performed at 50°C. It was found that the activity was not lost upon incubation for 4 hours and approximately 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.

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

The PheDH activity was measured in the presence to metal ions and chemical reagents. The enzyme was not inactivated by metal chelating or carbonyl reagents (hydroxylamine, EDTA and sodium azide) and reducing agent (DTT). In contrast, the enzyme was strongly inhibited by sulfhydryl reagents ($AgNO_3$). This inhibition suggests that the enzyme may contain a reactive sulfhydryl group. The monovalent and divalent cations, tested in this work, had a little or no effect on its activity, except Fe^{2+} and Fe^{3+} which completely inhibited the enzyme activity at 1 mM final concentration.

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

Inhibitory effect of nonsubstrate D- and L- amino acids on the oxidative deamination was determined. All L-amino acids tested, showed no effect on the *B. lentus* PheDH activity. The enzyme was inhibited by some D-amino acids with non-polar aliphatic and aromatic side chain. D-Methionine, D-leucine and D-tryptophan showed little or moderate inhibition while D-phenylalanine showed strong inhibition. Similar results were observed in PheDH from *S. ureae* and *B. sphaericus* except D-valine showed inhibitory effect on both enzyme activities (Asano *et al*., 1987b).

For the inhibitory effect of nonsubstrate phenylalanine analogs on the *B. lentus* enzyme, *p*-hydroxyphenylactate and hydrocinnamate inhibited the enzyme reaction. Among various keto acids and analogs, which were not substrates for the enzyme on the reductive amination, no significant inhibition against phenylpyruvate was found and there was no report of this effect on activity of other PheDHs. Misono *et al*.

(1989) reported that hydrocinnamate (3-phenylpropionate) competitively inhibited against L-phenylalanine for *Rhodococcus* PheDH.

4.4 Kinetic mechanism studies of phenylalanine dehydrogenase

The kinetic mechanism of *B. lentus* PheDH was determined by initial velocity studies and product inhibition 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 3.16) 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 3.18). In addition, the initial velocity pattern when fixed and saturated substrate was NH_4Cl and NADH gave the set of intersecting lines (Figure3.17 and 3.18). Cleland explained a model of the ordered addition of three substrate (A, B and C are substrates in that order). 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.

Cleland suggested a set of rules to predict the type of inhibition expected for a steady-state mechanism. 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 complex forming (Cleland, 1971). 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 center complexes to interconvert directly. For a rapid-equilibrium random mechanism, all product inhibitions are competitive unless product is able to bind to enzyme and forms as dead-end inhibitions. In that case, they would product apparently non-competitive

inhibition patterns due to the combination of product inhibition of competitive dead-end inhibition.

In this work, product inhibition by NAD^+ with respect to NADH showed competitive inhibition (Figure 3.20). This result indicates that NAD^+ and NADH can bind to the free form of the enzyme. The other product inhibition patterns were identical to the predicted patterns for the sequential ordered ternary-binary mechanism except for the noncompetitive inhibition by L-phenylalanine with respect to phenylpyruvate (Figure 3.21 B). This finding suggests that L-phenylalanine may also bind to the NADH-enzyme complex. Noncompetitive inhibition by phenylalanine with respect to NH_4Cl rules out the Theorell-Chance mechanism. The results obtained from these initial-velocity and product inhibition studies show that the sequence of addition of substrates in reductive amination is NADH, phenylpyruvate, and NH_4Cl and that the sequence of release of products is L-phenylalanine and NAD^+. The proposed kinetic mechanism of PheDH from *B. lentus* is shown in Figure 4.1

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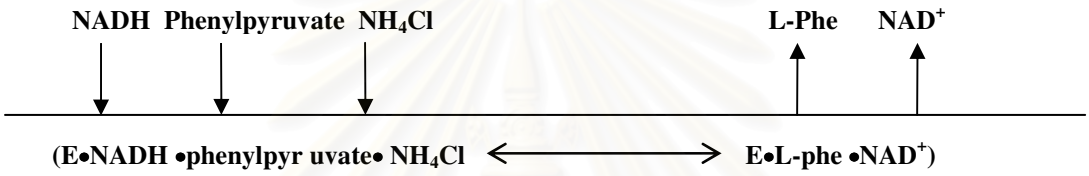

Figure 4.1 Proposed kinetic mechanism of phenylalanine dehydrogenase from *B. lentus*

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CHAPTER V

CONCLUSIONS

1. The optimal condition for PheDH production of *Bacillus lentus* was cultivation in 1% peptone medium, pH 7.0 containing 0.4% L-phenylalanine at 37°C for 18 hours.

2. The PheDH from *B. lentus* was purified to homogeneity from a crude enzyme by column chromatographies on DEAE-Toyopearl, Butyl-Toyopearl and Sephadex G-200. The enzyme was purified about 166.3 fold from the crude enzyme with a recovery of the activity of about 18.4%.

3. The molecular weight of the native was determined to be about 360,000 Da and consisted of 8 identical subunits (approximately 41,000 Da).

4. The enzyme had high substrate specificity on L-phenylalanine in the oxidative deamination and phenylpyruvate on the reductive amination.

5. The enzyme required NAD^+ as a natural coenzyme for oxidative deamination. $NADP^+$ was inert while the NAD^+ analog, 3-actylpyridine-NAD^+, showed 1.74 times higher activity than NAD^+.

6. The optimum pH of the enzyme for the oxidation deamination and reductive amination were 10.4 and 8.5, respectively.

7. The optimum temperature of the enzyme for the oxidation deamination and reductive amination were 50°C and 55°C, respectively.

8. The enzyme was stable over a pH range from 6.0 to 12.0.

9. The enzyme was fully stable at 50°C for 4 hours and retained 50% of its activity after incubation at same temperature for 3 days.

10. Sulfhydryl group was important to enzyme activity since the enzyme activity was completely lost in the presence of 1 mM final concentration of $AgNO_3$. Reducing agent (DTT) and chelating or carbonyl reagent (EDTA, hydroxylamine and sodium azide) had almost no effect on the enzyme activity.

11. The enzyme was inhibited by some D-amino acids with non-polar and aromatic side chain on the oxidative deamination of L-phenylalanine, while α-keto acids tested showed no significant inhibition on the reductive amination of phenylpyruvate.

12. The apparent K_m for L-phenylalanine, NAD^+, phenylpyruvate, ammonium and NADH were 0.59, 0.55, 0.18, 300 and 0.09 mM, respectively.

13. Initial velocity and product inhibition studies showed that the reductive amination proceeded through a sequential order ternary-binary mechanism. NADH bound first to the enzyme, followed by phenylpyruvate and then ammonium, and the products were released in the order L-phenylalanine and NAD^+.

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REFERENCES

Asano, Y., and Nakazawa, A. 1985. Crystallization of phenylalanine dehydrogenase from *Sporosarcina ureae*. *Agril. Biol. Chem*. 49: 3631-3632.

Asano, Y., Nakazawa, A., Endo, K., Hibino, Y., Ohnori, M., Numao, N., and Kondo, K. 1987a. Phenylalanine dehydrogenase of *Bacillus badius*: purification, characterization and gene cloning. *Eur. J. Biochem*. 168: 153-159.

Asano, Y., Nakazawa, A., and Endo, K. 1987b. Novel phenylalanine dehydrogenases from *Sporosarcina ureae* and *Bacillus sphaericus*: purification and characterization. *J. Biol. Chem*. 262: 10346-10354.

Asano, Y., Endo, K., Nakazawa, A., Hibino, Y., Okazaki, N., Ohmori, M., Numao, N., and Kondo, K. 1987c. *Bacillus* phenylalanine dehydrogenase produced in *E. coli* – its purification and application to L-phenylalanine synthesis. *Agric. Biol. Chem*. 51 (9): 2621-2623.

Asano, Y., and Tanetani, M. 1998. Thermostable phenylalanine dehydrogenase from mesophilic *Microbacterium* sp. strain DM 86-1. *Arch. Microbiol*. 169: 220-224.

Baker, P.J., Waugh, M.L., Wang, X-G., Stillman, T.J., Turnbull, A.P., Engel, P.C. and Rice, D.W. 1997. Determinants of substrate specificity in the superfamily of amino acid dehydrogenase. *Biochemistry*. 36: 16109-16115

Bollag, D. M., Rozycki, M. D., and Edelstein, S. J. 1996. *Protein methods.* 2nd ed. New York: Wiley-Liss, Inc. 230pp.

Boer, L. D., Rijssel, M. V, Euverink, G.J., and Dijkhuizen, L. 1989. Purification, characterization and regulation of a monomeric L-phenylalanine dehydrogenase from the facultive methyltroph *Nocardia* sp. 239. *Arch. Microbacbiol.* 153: 12-18.

Brunhuber, N. M. W., and Blanchard, J. S. 1994. The biochemistry and enzymology of amino acid dehydrogenases. *Critical Reviews in Biochemistry and Molecular Biology*. 29: 415-467.

Brunhuber, N. M. W., Thoden, J. B., Blanchard, J. S., and Vanhooke, J. L. 2000. *Rhodococcus* L-phenylalanine dehydrogenases: Kinetics, mechanism, and structural basis for catalytic specificity. *Biochemistry*. 39: 9174-9187.

Buchanan, R.E., and Gibbons, N.E. 1974. *Bergey's manual of determinative bacteriology*. 8th ed. U.S.A.: Waverly press.

Choi, Y.J., Tribe, D.E. 1982. Continuous production of L- phenylalanine using an *Escherichia coli* regulatory mutant. *Biotechnol Lett*. 4: 223-228.

Cleland, W. W. 1971. Steady state kinetics. In P. D. Boyer (ed.), *The enzymes*, 3rd ed., vol. 2, 1-43. New York: Academic Press.

Cooper, T. G. 1977. *The tools of biochemistry*. New York: John Wiley & Sons. 423 pp.

Eisenthal, R., and Danson, M. J. eds. 1992. *Enzyme assay*: *a practical approach*. New York:IRL press.

Ferguson, K. A. 1964. *Metabolism* 13, 985 pp.

Groot, M. N. N., and de Bont, J. A. M. 1998. Conversion of phenylalanine to benzaldehyde initiated by an aminotransferase in *Lactobacillus plantarum*. *Appl. Environ. Microbiol.* 64(8): 3009-3013.

Hanson, R. L., Howell, J. M., LaPorte, T. L., Donovan, M. J., Cazzulino, D. L., Zannella, V., Montana, M. A., Nanduri, V. B., Schwarz, S. R., Firing, R. F., Durand, S. C., Wasylyk, J. M., Parker, W. L., Lui, M. S., Okuniewicz, F. J., Chen, B., Harris, J. C., Natalie, K. J., Ramig, K., Swaminathan, S., Rosso, V. W., Pack, S. K., Lotz, B. T., Bernot, P. J., Rusowicz, A., Lust, D. A., Tse, K. S., Venit, J. J., Szarka, L. J., and Patel, R. N. 2000. Synthesis of allysine ethylene acetal using phenylalanine dehydrogenase from *Thermoactinomyces intermedius*. *Enzyme Microb. Technol*. 26: 348-358.

Hummel, W., Weiss, N., and Kula, M. R. 1984. Isolation and characterization of a bacterium possessing L-phenylalanine dehydrogenase activity. *Arch. Microbiol*. 137: 47-52.

Hummel, W., Schmidt, E., Wandrey, C., and Kula, M. R. 1986. L-phenylalanine dehydrogenase from *Brevivacterium* sp. for production of L-phenylalanine by reductive amination of phenylpyruvate. *Appl. Microbiol. Biotechnol*. 25:175-185.

Hummel, W., Schutte, H., Schmidt, E., Wandrey, C., and Kula, M. R. 1987. Isolation of L-phenylalanine degydrogenase from *Rhodococcus* sp. M4 and its application for the production of L-phenylalanine. *Appl. Microbiol. Biotechnol*. 26: 409-416.

Hummel, W., and Kula, M. R. 1989. Dehydrogenases for the synthesis of chiral compounds. *Eur. J. Biochem*. 184: 1-13.

Keradjipoulos, D., and holldorf, A. W. 1979. Purification and properties of alanine dehydrogenase from *Halobacterium salinarium. Biochem. Biophys. Acta.* 570: 1-10.

Leksakorn, A. 2001. Purification and characterization of phenylalanine dehydrogenase from thermotolerant *Bacillus* sp. BC1. Master's Thesis, Department of Biochemistry, Science, Chulalongkorn University.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.

Misono, H., Yonezawa, J., Nagata, S., and Nagasaki, S. 1989. Purification and characterization of a dimeric phenylalanine dehydrogenase from *Rhodococcus maris* K-18. *J. Bacteriol*. 171: 30-36.

Ohshima, T.,Nagata, S.,and Soda, K. 1985. Purification and characterization of thermostable leucine dehydrogenase from *Bacillus stearothermophilus*. *Arch. Microbiol*. 141: 407-411.

Ohshima, T., and Soda, K. 1990. Biochemistry and biotechnology of amino acid dehydrogenases. *Adv. Biochem. Eng. Biotechnol*. 42: 187-209.

Ohshima, T., Takada, H., Yoshimura, T., Esaki, N., and Soda, K. 1991. Distribution, purification and characterization of thermostable phenylalanine dehydrogenase from thermophilic actinomyces. *J. Bacteriol*. 173: 3943-3948.

Ohshima, T., and Soda, K. 2000. Stereoselective biocatalysis: Amino acid dehydrogenases and their applications. In R. N. Patel (ed.), *Stereoselective biocatalysis*, 877-902. New York: Marcel Dekker.

Palmer, T. 1995. *Understanding enzymes.* UK: Prentice Hall/Ellis Horwood. pp. 67-68.

Patel, R. N. 2001. Enzymatic synthesis of chiral intermediates for Omapatrilat, an antihypertensive drug. *Biomolecular Engineering*. 17: 167-182.

Queiroz, J. A., Tomaz, C. T., and Cabral, J. M. S. 2001. Hydrophobic interaction chromatography of proteins. *J. Biotechnol*. 87: 143-159.

Schmidt, E., Vasic-Racki, D., and Wandrey, C. 1987. Enzymatic production of L-phenylalanine out of the racemic mixture of D,L-phenyllactate. *Appl. Microbiol. Biotechnol*.

Schulze, A., Mayatepek, E., and Hoffmann, G. F. 2002. Evaluation of 6-year application of the enzymatic colorimetric phenylalanine assay in the setting of neonatal screening for phenylketonuria. *Clinica Chimica Acta* 317: 27-37.

Scopes, R. K. 1994. *Protein purification: principles and practice*. 2rd edition, New York: Springer-Verlag. 423 pp.

Seah, S. Y. K., Britton, K. L., Rice, D. W., Asano, Y., and Engel, P. C. 2002. Single amino acid substitution in *Bacillus sphaericus* phenylalanine dehydrogenase dramatically increases its discrimination between phenylalanine and tyrosine substrate. *Biochemistry*. 41: 11390-11397.

Segal, I. H. 1976. *Biochemical calculations*. New York: John Wiley & Sons. 207-278.

Newmarket, 1992. Quantase™ phenylalanine product information. Porton Cambridge Ltd., UK.

Tynan, J., Forde, J., McMahon, M., and Mulcahy, P. 2000. Synthesis of a highly substitured N^6-linked immobilized NAD^+ derivative using a rapid solid-phase modular approach: suitability for use with the kinetic locking-on tactic for bioaffinity purification of NAD^+-dependent dehydrogenases. *Protein Expression and Purification*. 20: 421-434.

Vanhooke, J. L., Thoden, J. B., Brunhuber, N. M. W., Blanchard, J. S., and Holden, H. M. 1999. Phynylalanine dehydrogenase from *Rhodococcus* sp. M4: high-resolution x-ray analyses of inhibitory ternary complexes reveal key features in the oxidative deamination mechanism. *Biochemistry*. 38: 2326-2339.

Voet, D., and Voet, J. G. 1995. *Biochemistry*. Canada: John Wiley & Sons. 333-337.

Wendel, U., Hummel, W., and Langenbeck, U. 1989. Monitoring of phenylketonuria: a colorimetric method for the determination of plasma phenylalanine using L-phenylalanine dehydrogenase. *Anal. Biochem*. 180: 91-94.

Wendel, U., Koppelkamm, M., Hummel, W., Sander, J., and Langenbeck, U. 1990. A new approach to the newborn screening for hyperphenylalaninemias: use of L-phenylalanine dehydrogenase and microtiter plates. *Clinica. Chimica. Acta*. 192: 165-170.

Wendel, U., Koppelkamm, M., and Hummel, W. 1991. Emzymatic phenylalanine estimation for the management of patients with phenylketonuria. *Clinica. Chimica. Acta.* 201: 95-98.

Yamada, S., Nabe, K., Izuo, N., Nakamichi, K., and Chibata, I. 1981. Production of L-phenylalanine from *trans*-cinnamic acid with *Rhodotorula glutinis* containing L-phenylalanine ammonialyase activity. *Appl. Environ. Microbiol*. 42: 773-778.

Ziehr, H. Schmidt, E., Stock, J., Wandrey, C., Klein, J., and Kula, M. R. 1987. Continuous production of L-phenylalanine by transamination. *Biotechnol. Bioeng*. 23: 2789-2802.

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APPENDICES

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APPENDIX A

Preparation for protein determination

Reagent for determination of protein concentration (modified from Lowry *et al*., 1951)

Solution A (0.5% copper sulfate and 1% potassium tartate, pH 7.0)

Potassium tartate	1	g
Copper sulfate	0.5	g

Adjusted pH to 7.0 and adjust the solution volume to 100 ml.

Solution B (2% sodium carbonate and 1 N sodium hydroxide)

Sodium carbonate	20	g
Sodium hydroxide	4	g

Dissolved in distilled water to 1 liter.

Solution C (phenol reagent)

Folin-Ciocalteu phenol reagent used in this work was reagent grade from Carlo Erba, Italy.

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APPENDIX B

Preparation for non-denaturing polyacrylamide gel electrophoresis (Native-PAGE)

1. Stock solutions

2 M Tris-HCl (pH 8.8)

Tris (hydroxymethyl)-aminomethane	24.2	g

Adjusted pH to 8.8 with 1 N HCl and adjusted volume to 100 ml with distilled water.

1 M Tris-HCl (pH 6.8)

Tris (hydroxymethyl)-aminomethane	12.1	g

Adjusted pH to 6.8 with 1 N HCl and adjusted volume to 100 ml with distilled water.

1 % (w/v) Bromophenol blue

Bromophenol blue	100	mg

Brought to 10 ml with distilled water and stirred until dissolved.

The filtration will remove aggregated dye.

2. Working solutions

Solution A (30 % (w/v) acrylamide, 0.8 % (w/v) bis-acrylamide)

Acrylamide	29.2	g
N, N′-methylene-bis-acrylamide	0.8	g

Adjusted volume to 100 ml with distilled water.

Solution B (1.5 M Tris-HCl, pH 8.8)

2 M Tris-HCl (pH 8.8)	75	ml
Distilled water	25	ml

Solution C (0.5 M Tris-HCl, pH 6.8)

1 M Tris-HCl (pH 6.8)	50	ml
Distilled water	50	ml

APPENDIX B (continued)

10 % (w/v) Ammonium persulfate

Ammonium persulfate	0.5	g
Distilled water	5.0	ml

Electrophoresis buffer (25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminomethane	3.0	g
Glycine	14.4	ml

Dissolved and adjusted to total volunm 1 liter with distilled water (final pH should be approximately 8.3)

5x Sample buffer (312.5 mM Tris-HCl pH 6.8, 50 % (v/v) glycerol, 1 % (v/v) bromophenol blue)

1 M Tris-HCl (pH 6.8)	0.6	ml
Glycerol	5.0	ml
1 % Bromophenol blue	0.5	ml
Distilled water	1.4	ml

3. Native-PAGE

7.7 % Separating gel

Solution A	2.6	ml
Solution B	2.5	ml
Distilled water	4.9	ml
10 % (w/v) Ammonium persulfate	50	µl
TEMED	5.0	µl

5.0 % Stacking gel

Solution A	0.67	ml
Solution C	1.0	ml
Distilled water	2.3	ml
10 % (w/v) Ammonium persulfate	30	µl
TEMED	5.0	µl

APPENDIX B (continued)

4. Protein staining solution

Staining solution, 1 liter

Coomassie brilliant blue R-250	1.0	g
Glacial acetic acid	100	ml
Methanol	450	ml
Distilled water	450	ml

Destaining solution, 1 liter

Methanol	100	ml
Glacial acetic acid	100	ml
Distilled water	800	ml

5. Enzyme activity staining solution

1 M Tris-HCl, pH 8.5

Tris (hydroxymethyl)-aminomethane 6.06 g

Adjusted to pH 8.5 with 1 N HCl and make 100 ml with distilled water

40 mM L-phenylalanine

L-phenylalanine 0.066 g

Dissolved with 10 ml distilled water

50 mM NAD^+

NAD^+ 0.359 g

Dissolved with 10 ml distilled water

0.25 mg/ml phenazine methosulfate

Phenazine methosulfate 0.0025 g

Dissolved with 10 ml distilled water

2.5 mg/ml nitroblue tetrazolium

Nitroblue tetrazolium 0.025 g

Dissolved with 10 ml distilled water

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Activity staining solution (4.25 mM Tris-HCl, pH 8.5, 40 μM L-phenylalanine 50 μM NAD^+, 250 μg phenazine methosulfate and 2.5 mg nitroblue tetrazolium)

1 M Tris-HCl, pH 8.5	4.25	ml
40 mM L-phenylalanine	1.0	ml
50 mM NAD^+	1.0	ml
0.25 mg/ml phenazine methosulfate	1.0	ml
2.5 mg/ml nitroblue tetrazolium	1.0	ml
Distilled water	1.75	ml

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APPENDIX C

Preparation for denaturing polyacrylamide gel electrophoresis

1. Stock solution

2 M Tris-HCl (pH 8.8)

Tris (hydroxymethyl)-aminomethane 24.2 g

Adjusted pH to 8.8 with 1 N HCl and adjusted volume to 100 ml with distilled water.

1 M Tris-HCl (pH 6.8)

Tris (hydroxymethyl)-aminomethane 12.1 g

Adjusted pH to 6.8 with 1 N HCl and adjusted volume to 100 ml with distilled water.

10 % (w/v) SDS

Sodium dodecyl sulfate (SDS) 10 g

Added distilled water to a total volume of 100 ml.

50 % (w/v) Glycerol

100 % Glycerol 50 ml

Added distilled water to a total volume of 100 ml.

1 % (w/v) Bromophenol blue

Bromophenol blue 100 mg

Brought to 10 ml with distilled water and stirred until dissolved.

The filtration will remove aggregated dye.

2. Working solutions

Solution A (30 % (w/v) acrylamide, 0.8 % (w/v) bis-acrylamide)

Acrylamide 29.2 g

N, N′-methylene-bis-acrylamide 0.8 g

Adjusted volume to 100 ml with distilled water.

Filter nad store in dark (brown bottle) at 4 ^{o}C

APPENDIX C (continued)

Solution B (1.5 M Tris-HCl, pH 8.8 and 0.4 % SDS)

2 M Tris-HCl (pH 8.8)	75	ml
10 % (w/v) SDS	4	ml
Distilled water	21	ml

Solution C (0.5 M Tris-HCl, pH 6.8, 0.4 % SDS)

1 M Tris-HCl (pH 6.8)	50	ml
10% (w/v) SDS	4	ml
Distilled water	46	ml

10 % (w/v) Ammonium persulfate

Ammonium persulfate	0.5	g
Distilled water	5.0	ml

Electrophoresis buffer (25 mM Tris, 192 mM glycine and 0.1 % (w/v) SDS)

Tris (hydroxymethyl)-aminomethane	3.0	g
Glycine	14.4	ml
SDS	1	g

Dissolved and adjusted to total volunm 1 liter with distilled water
(final pH should be approximately 8.3)

5x Sample buffer (312.5 mM Tris-HCl pH 6.8, 50 % (v/v) glycerol, 1 % (w/v) bromophenol blue)

1 M Tris-HCl (pH 6.8)	0.6	ml
50 % (v/v) Glycerol	5.0	ml
10 % (w/v) SDS	2	ml
1 % (w/v) Bromophenol blue	1	ml
β-Mercaptoethanol	0.5	ml
Distilled water	1.4	ml

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APPENDIX C (continued)

3. SDS-PAGE

10 % Separating gel

Solution A	3.3	ml
Solution B	2.5	ml
Distilled water	4.2	ml
10 % (w/v) Ammonium persulfate	50	µl
TEMED	5	µl

5.0 % Stacking gel

Solution A	0.67	ml
Solution C	1.0	ml
Distilled water	2.3	ml
10% (w/v) Ammonium persulfate	30	µl
TEMED	5	µl

4. Protein staining solution

Staining solution, 1 liter

Coomassie brilliant blue R-250	1.0	ml
Methanol	450	ml
Distilled water	450	ml

Destaining solution, 1 liter

Methanol	100	ml
Glacial acetic acid	100	ml
Distilled water	800	ml

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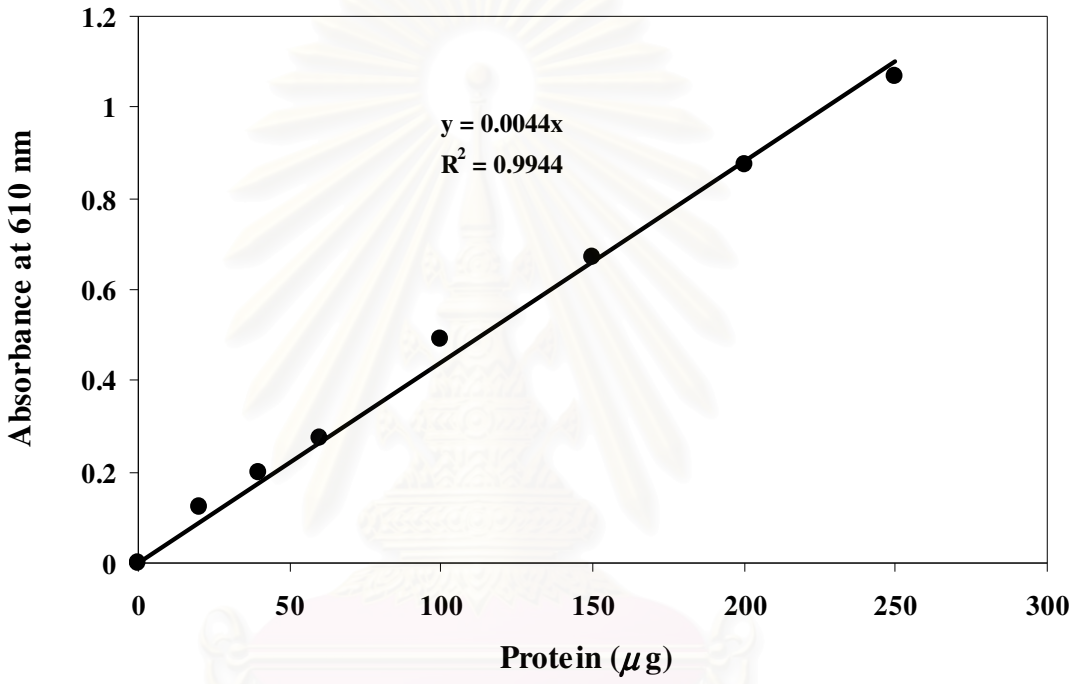
APPENDIX D

Calibration curve for protein determination by Lowry's method



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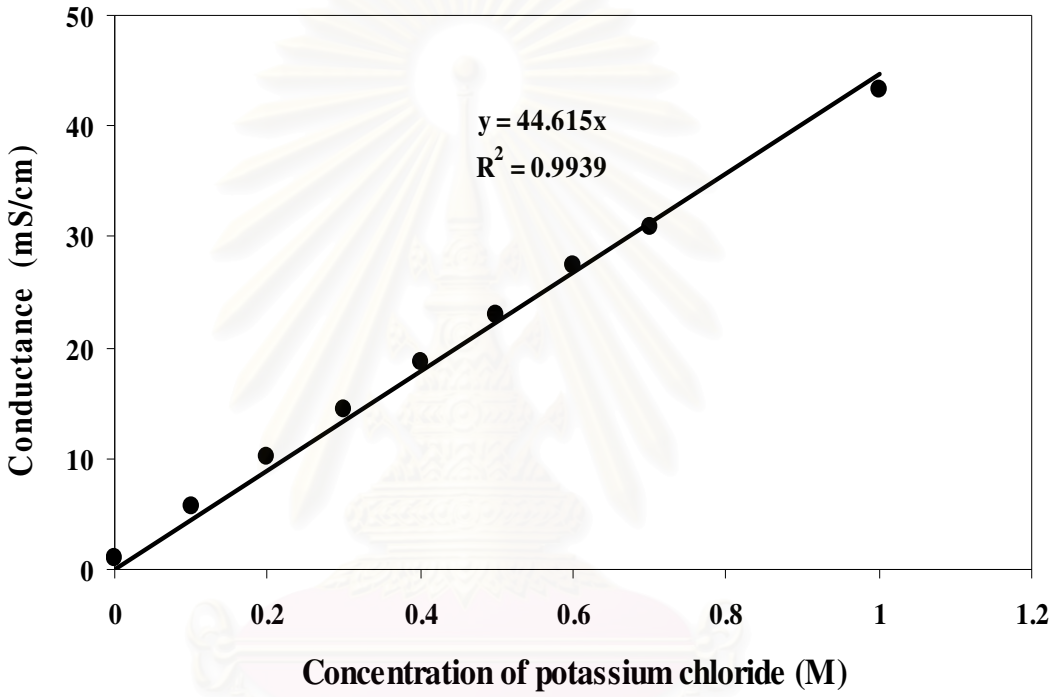
APPENDIX E

Calibration curve for conductivity of potassium chloride



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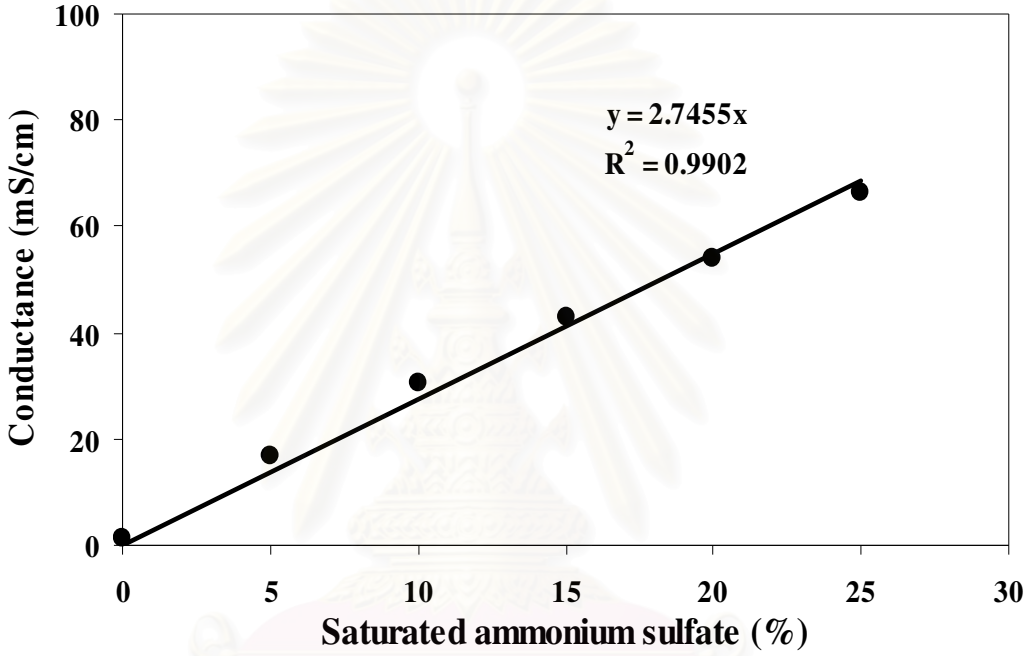
APPENDIX F

Calibration curve for conductivity of ammonium sulfate



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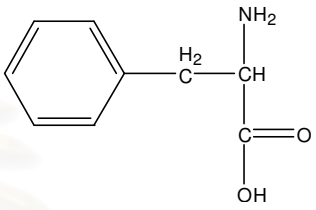
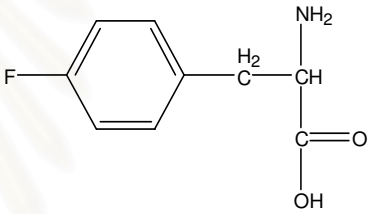
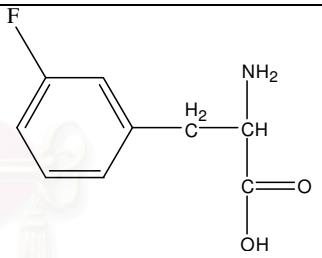
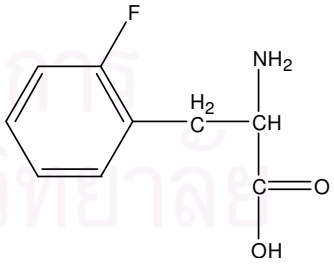
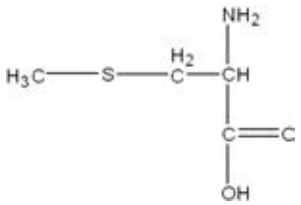
APPENDIX G

Amino acids and their analogs structures

Amino acids and analogs	Structure
L-phenylalanine	
p-fluoro-DL-phenylalanine	
m-fluoro-DL-phenylalanine	
o-fluoro-DL-phenylalanine	
S-methyl-L-cysteine	

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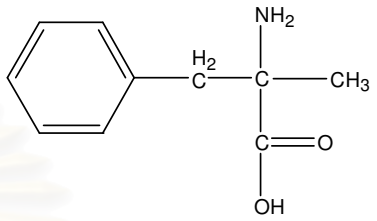
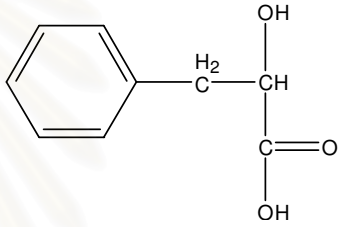
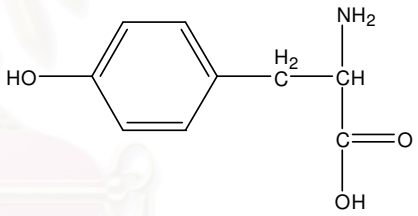
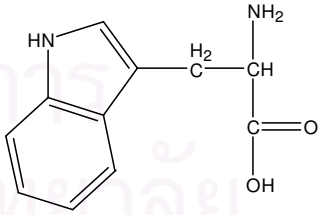
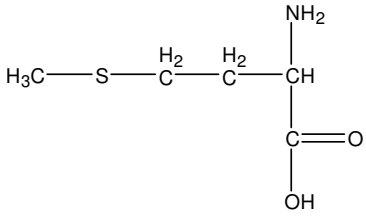
APPENDIX G (continued)

Amino acids and analogs	Structure
α-methyl-DL-phenylalanine	
L-β-phenyllactate	
L-tyrosine	
L-tryptophan	
L-methionine	

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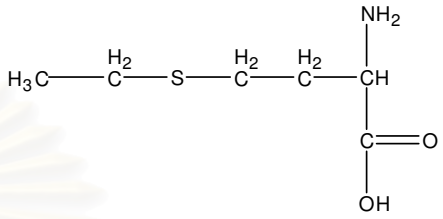
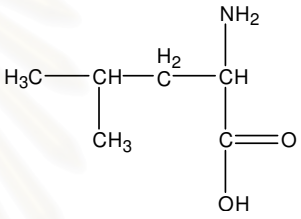
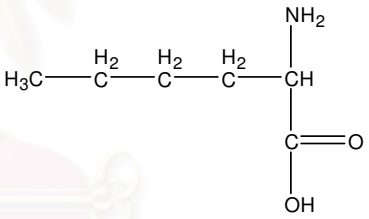
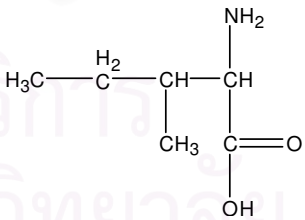
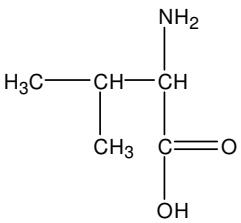
APPENDIX G (continued)

Amino acids and analogs	Structure
L-ethionine	
L-leucine	
L-norleucine	
L-isoleucine	
L-valine	

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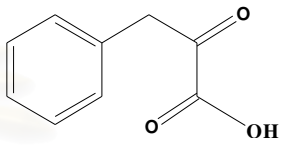
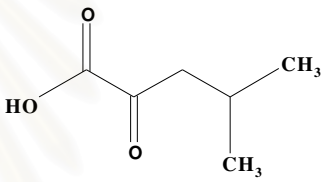
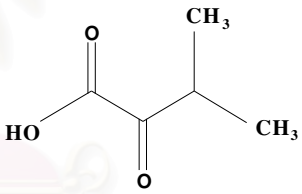
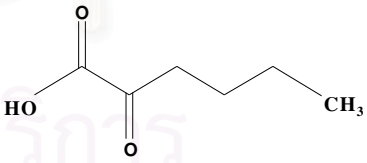
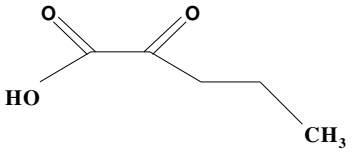
APPENDIX H

Keto acids and their analogs structure

Keto acid	Structure
phenylpyruvate	
α-ketoisocaproate	
α-ketoisovalerate	
α-ketocaproate	
α–ketovalerate	

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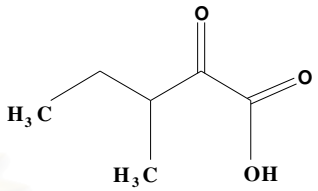
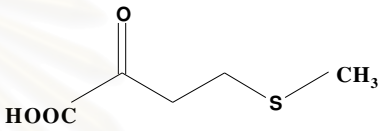
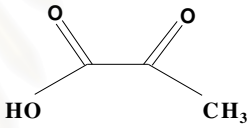
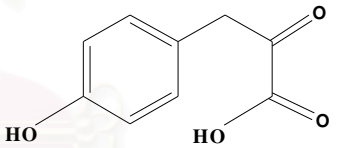
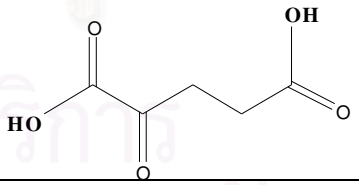
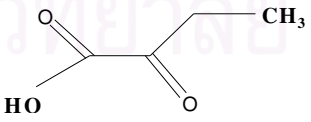
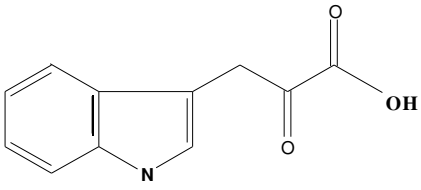
APPENDIX H (continued)

Keto acid	Structure
α-keto-β-methyl-n-valerate	
α-keto-γ-methiol-butyrate	
pyruvate	
p-hydroxyphenylpyruvate	
α-ketoglutarate	
α-keto-n-butyrate	
indole-β-pyruvic acid	

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APPENDIX I

Amino acids and their corresponding keto acids

Amino acid	Keto acid
L-phenylalanine	*β*-phenylpyruvate
L-norleucine	α-ketocaproate
L-leucine	α-ketoisocaproate
L-norvaline	α-ketovalerate
L-valine	α-ketoisovalerate
L-methionine	α-keto-γ-methiol-n-butyrate
α-aminobutyrate	α-keto-n-butyrate
L-isoleucine	α-keto-*β*-methylvalerate
L-glutamic acid	α-ketoglutarate

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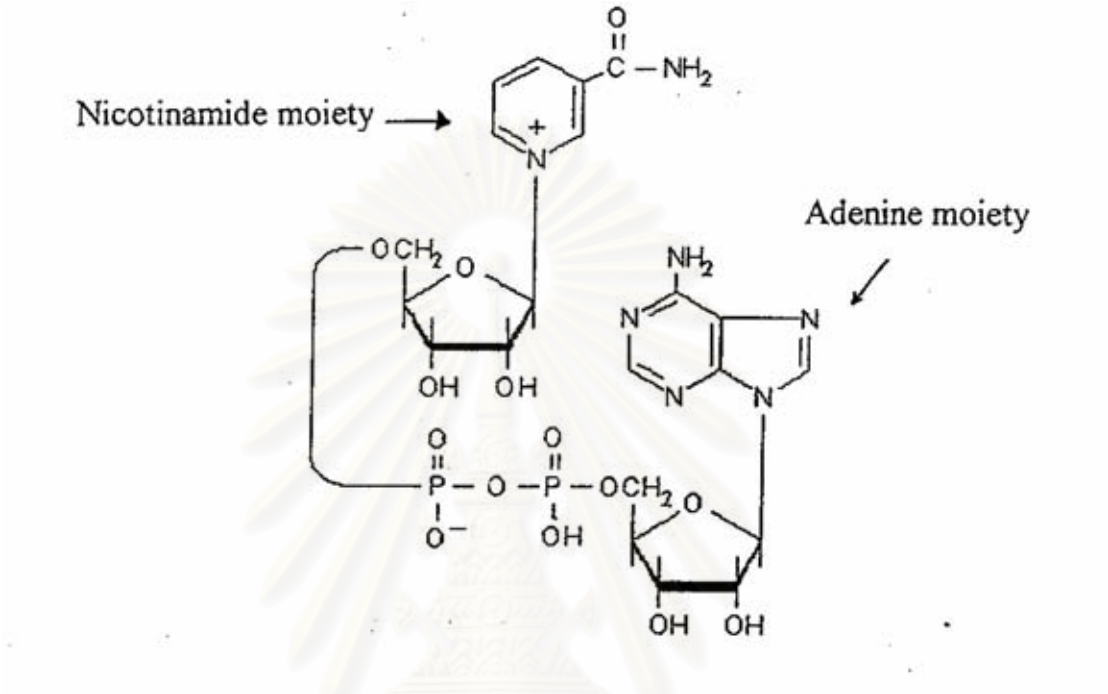
APPENDIX J

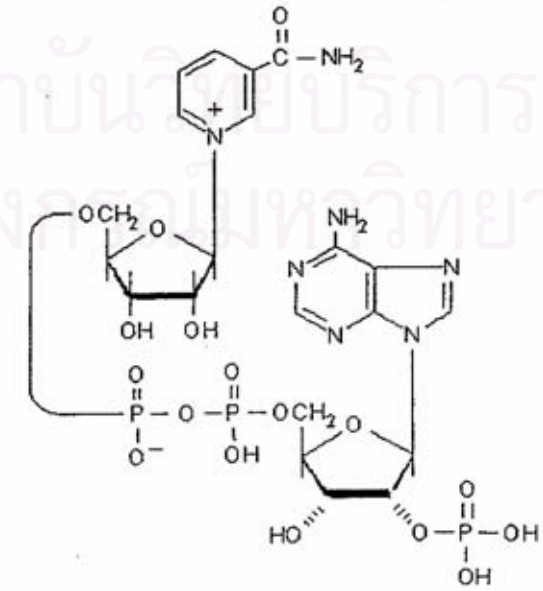
NAD^+ analogs



Nicotinamide adenine dinucleotide (NAD^+)

The NAD+ analogs used in this work can be divided into 3 groups based on their modified structure.

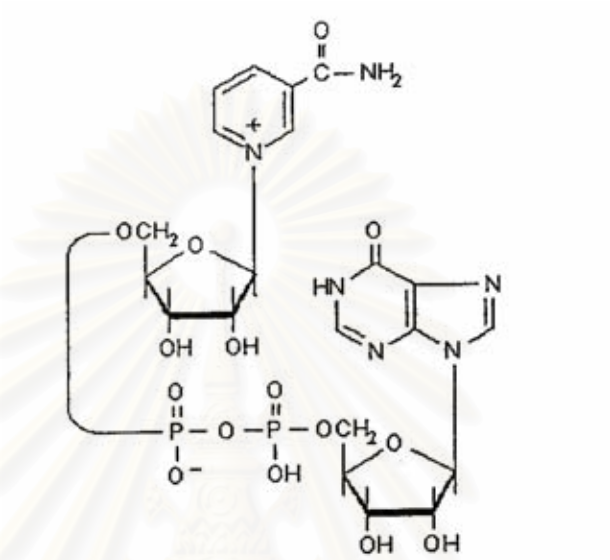
1. Coenzyme analog modified at C-2 position of the adenosylribose



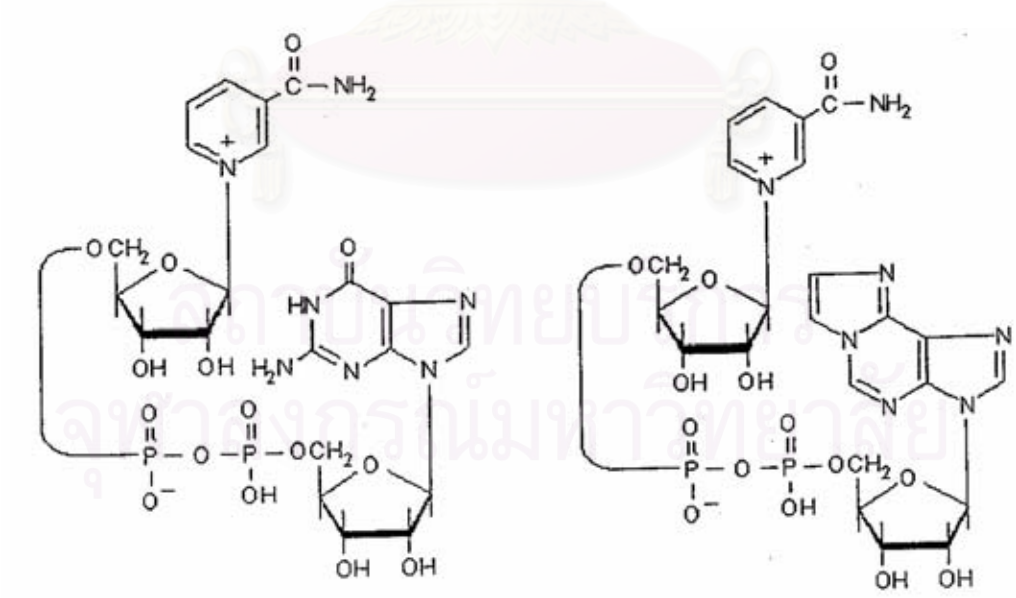
Nicotinamide adenine dinucleotide phosphate ($NADP^+$)

APPENDIX J (continued)

2. Coenzyme analog modified at the amino group in the adenine moiety



Nicotinamide hypoxanthine dinucleotide (Deamino-NAD$^+$)

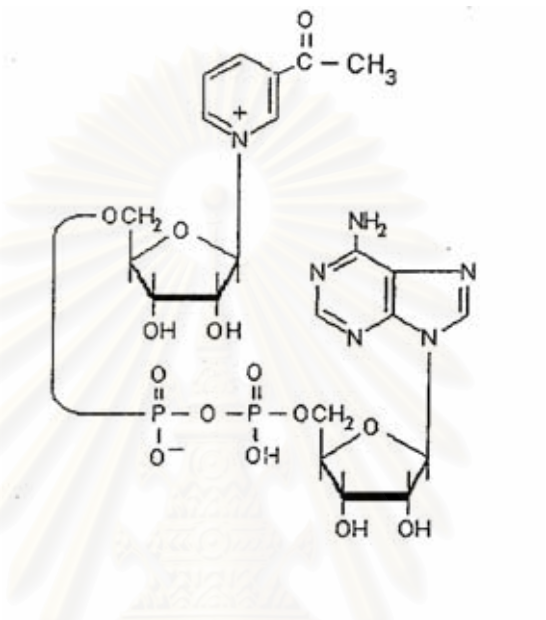


Nicotinamide guanine dinucleotide

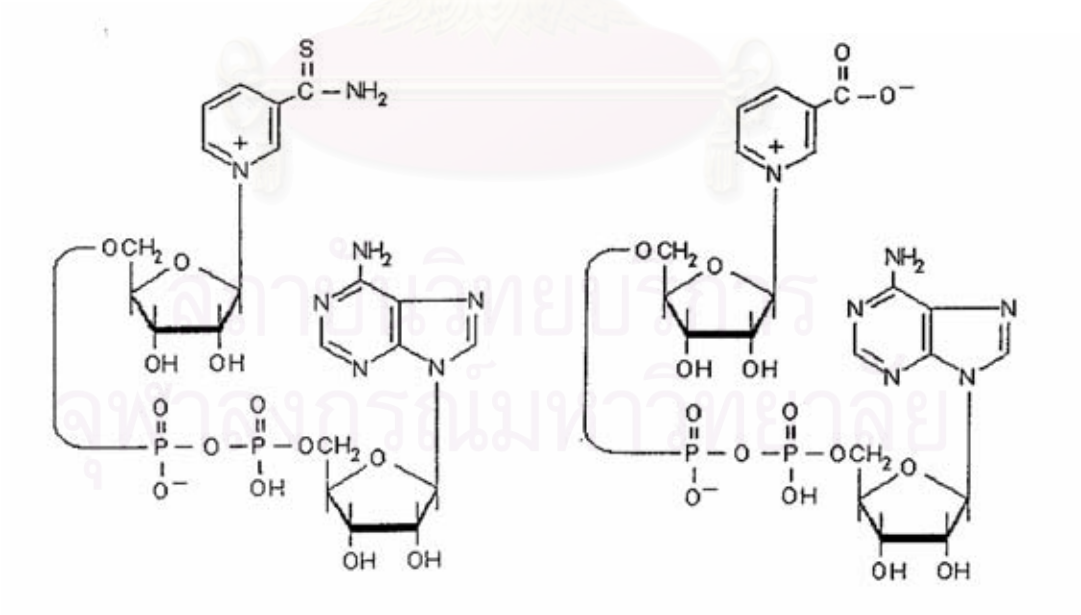
Nicotinamide 1, N^6-ethenoadenine dinucleotide

APPENDIX J (continued)

3. Coenzyme analog modified at the nicotinamide moiety



3-Acetylpyridine adenine dinucleotide



3-Pyridinealdehyde adenine dinuleotide **Thionicotinamide adenine dinucleotide**

BIOGRAPHY

Miss Siwaporn Inkure was born on January 7, 1981 in Suratthani. She graduated with the B.Sc. in Biotechnology Faculty of Science, King Mongkut's Institution of Technology Ladkabang in 2002 and continues studying for M.Sc. in Biotechnology Program Faculty of Science at Chulalongkorn University in that year.

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