การโคลนและการแสดงออกของยีนฟีนิลอะลานีนดีไฮโดรจิเนส จาก *Acinetobacter lwoffii* และความเป็นไปได้ในการผลิตกรดอะมิโน

นายภาคภูมิ สิตไทย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเคมี ภาควิชาชีวเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2547

ISBN 974-53-1058-1

CLONING AND EXPRESSION OF PHENYLALANINE DEHYDROGENASE GENE FROM *Acinetobacter lwoffii* AND THE POSSIBILITY FOR AMINO ACID PRODUCTION

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Biochemistry
Department of Biochemistry
Faculty of Science
Chulalongkorn University
Academic Year 2004
ISBN 974-53-1058-1

Thesis Title	Cloning and expression of phenylalanine dehydrogenase gene from *Acinetobacter lwoffii* and the possibility for amino acid production
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Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree.

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ภาคภูมิ สิตไทย: การโคลนและการแสดงออกของยีนฟีนิลอะลานีนดีไฮโดรจิเนสจาก *Acinetobacter lwoffii* และความเป็นไปได้ในการผลิตกรดอะมิโน (CLONING AND EXPRESSION OF PHENYLALANINE DEHYDROGENASE GENE FROM *Acinetobacter lwoffii* AND THE POSSIBILITY FOR AMINO ACID PRODUCTION) อาจารย์ที่ปรึกษา: ผศ.ดร.กนกทิพย์ ภักดีบำรุง, อาจารย์ที่ปรึกษาร่วม: รศ.ดร.ศิริพร สิทธิประณีต, 134 หน้า, ISBN 974-53-1058-1

ฟีนิลอะลานีนดีไฮโดรจิเนส (EC 1.4.1.20) เป็นเอนไซม์ในกลุ่มอะมิโนแอซิดดีไฮโดรจิเนส เร่งปฏิกิริยาการดึงหมู่อะมิโนจากแอล-ฟีนิลอะลานีนให้ผลิตภัณฑ์เป็นฟีนิลไพรูเวทและแอมโมเนีย โดยเป็นปฏิกิริยาที่ผันกลับได้ที่มีไพริดีนนิวคลีโอไทด์เป็นโคเอนไซม์ กลุ่มวิจัยของเราได้ศึกษาฟีนิลอะลานีนดีไฮโดร-จิเนสจาก *Acinetobacter lwoffii* และพบว่านอกจากใช้แอล-ฟีนิลอะลานีนเป็นสับสเตรทแล้ว ยังสามารถใช้แอล-เมไธโอนีน แอล-ทริปโตเฟน และแอล-นอร์ลูซีนเป็นสับสเตรทสำหรับปฏิกิริยา oxidative deamination ได้ด้วย นอกจากนั้นยังพบว่าเอนไซม์นี้ไม่สูญเสียแอคติวิตีเมื่อบ่มที่ 55 องศาเซลเซียสที่ pH 7.4 เป็นเวลา 10 นาที ด้วยเหตุนี้จึงอาจเป็นไปได้ที่จะใช้ฟีนิลอะลานีนดีไฮโดรจิเนสจาก *A. lwoffii* เพื่อผลิตกรดอะมิโนรูปแบบแอลชนิดต่างๆ เนื่องจากมีการศึกษานิวคลีโอไทด์ของยีนฟีนิลอะลานีนดีไฮโดรจิเนส จาก *A. lwoffii* ไว้แล้ว ดังนั้นงานวิจัยครั้งนี้จึงทำการโคลนยีนฟีนิลอะลานีนดีไฮโดรจิเนส จาก *A. lwoffii* เข้าสู่ *E. coli* BL21(DE3) และ *E. coli* BL21(DE3)pLysS โดยใช้ expression vector (pET-17b) เมื่อวิเคราะห์สารละลายเอนไซม์หยาบที่ได้จากรีคอมบีแนนท์โคลน พบว่าเอนไซม์มีค่าแอคติวิตีจำเพาะอยู่ในช่วง 0.81 - 4.46 หน่วยต่อมิลลิกรัมโปรตีน แอคติวิตีจำเพาะสูงสุดของโคลนที่ได้มากกว่าแอคติวิตีจำเพาะจาก *A. lwoffii* 55.75 เท่า ภาวะที่เหมาะสมในการเหนี่ยวนำให้เกิดการแสดงออกของยีนฟีนิลอะลานีนดีไฮโดรจิเนสคือ เหนี่ยวนำด้วย IPTG 0.4 มิลลิโมลาร์ เป็นเวลา 8 ชั่วโมง การทดสอบความเสถียรของการแสดงออกของยีนฟีนิลอะลานีนดีไฮโดรจิเนสในเซลล์เจ้าบ้าน *E. coli* BL21(DE3) โดยเพาะเชื้อต่อช่วงโคลนที่มีแอคติวิตีของเอนไซม์สูงสุดวันต่อวันเป็นเวลา 20 วัน พบว่าการเพาะเชื้อต่อช่วงมีผลให้การแสดงออกของยีนฟีนิลอะลานีนดีไฮโดรจิเนสลดลง เมื่อนำเอนไซม์มาทำให้บริสุทธิ์โดยการตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟต และแยกโดยโครโมโตกราฟฟีคอลัมน์ดีอีเออี-โทโยเพิร์ล พบว่ามีแอคติวิตีคงเหลือ 29.45 เปอร์เซ็นต์ และบริสุทธิ์ขึ้น 5.19 เท่า ในปฏิกิริยา oxidative deamination เอนไซม์มีความจำเพาะสูงมากต่อแอล-ฟีนิลอะลานีน ส่วน reductive amination เอนไซม์มีความจำเพาะต่อ α-ketocaproate, α-keto-γ-methiol-n-butyrate, α-ketovalerate และ α-ketoisocaproate สูงกว่าฟีนิลไพรูเวท 5.96, 4.12, 3.84 และ 3.15 เท่า ตามลำดับ เอนไซม์ไม่สูญเสียแอคติวิตีเมื่อบ่มที่ 30 องศาเซลเซียส pH 9.5 เป็นเวลา 4 ชั่วโมง และยังคงมีแอคติวิตีเหลืออยู่ 50 เปอร์เซ็นต์ เมื่อบ่มเป็นเวลา 12 ชั่วโมง เมื่อนำเอนไซม์ไปใช้ผลิตกรดอะมิโนรูปแบบแอลชนิดต่างๆจากสับสเตรทที่เป็นกรดคีโต พบว่าได้ผลผลิตในช่วง 36.0-72.2 เปอร์เซนต์

ภาควิชา ……ชีวเคมี…… ลายมือชื่อนิสิต ……

สาขาวิชา……ชีวเคมี……... ลายมือชื่ออาจารย์ที่ปรึกษา ……

ปีการศึกษา……..2547…… ลายมือชื่ออาจารย์ที่ปรึกษาร่วม ……

4572618323 : MAJOR BIOCHEMISTRY
KEY WORD : phenylalanine dehydrogenase/ cloning/ expression
PARKPOOM SITTHAI: CLONING AND EXPRESSION OF PHENYLALANINE DEHYDROGENASE GENE FROM *Acinetobacter lwoffii* AND THE POSSIBILITY FOR AMINO ACID PRODUCTION. THESIS ADVISOR: ASST. PROF. KANOKTIP PACKDIBAMRUNG, Ph.D., THESIS CO-ADVISOR: ASSOC. PROF. SIRIPORN SITTIPRANEED, Ph.D., 134 pp. ISBN 974-53-1058-1

Phenylalanine dehydrogenase (EC 1.4.1.20), one of amino acid dehydrogenases, catalyzes the reversible pyridine nucleotide - dependent oxidative deamination of L-phenylalanine to form ammonia, phenylpyruvate, and NADH. Our research group has studied phenylalanine dehydrogenase from *Acinetobacter lwoffii* and found that L-methionine, L-tryptophan and L-norleucine could act as substrate in the oxidative deamination of the enzyme. No loss of the enzyme activity was observed upon incubation at 55 ^{o}C, pH 7.4 for 10 minutes. From these properties, the enzyme seems to have more potential in the synthesis of various amino acids. Moreover, nucleotide sequence of phenylalanine dehydrogenase gene from *A. lwoffii* was already determined. Thus, in this research the phenylalanine dehydrogenase gene was cloned and expressed in *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS host cells using expression vector, pET-17b. The specific activity from crude extract of recombinant clones were found in the range of 0.81 – 4.46 units/mg protein. The highest specific activity was 55.75 fold higher than that of the enzyme from *A. lwoffii*. The optimum condition for phenylalanine dehydrogenase gene expression was induction with 0.4 mM IPTG for 8 hours. Stability of phenylalanine dehydrogenase gene expression in *E. coli* BL21(DE3) was studied. After daily subculture the recombinant clone showed the highest phenylalanine dehydrogenase activity for 20 days, it was found that phenylalanine dehydrogenase activity decreased with the increasing number of subculture. The enzyme was purified to homogeneity by 50-70 % saturated ammonium sulfate precipitation and DEAE-Toyopearl column chromatography with 29.45 % yield and 5.19 purification fold. The enzyme showed high substrate specificity in the oxidative deamination on L-phenylalanine while it acted on α-ketocaproate, α-keto-γ-methiol-n-butyrate, α-ketovalerate and α-ketoisocaproate with 5.96, 4.12, 3.84 and 3.15 fold of its natural substrate, phenylpyruvate, respectively in reductive amination. No loss of enzyme activity was observed upon incubation at 30 ^{o}C, pH 9.5 for 4 hours and 50 % of the activity was retained after incubation at this temperature for 12 hours. When phenylalanine dehydrogenase was used for production of amino acids using their corresponding keto acids as substrates, the product yield was in the range between 36.0-72.2 %.

Department ……Biochemistry…….….. Student's signature……..
Field of study…..Biochemistry……... Advisor's signature……..
Academic year……..2004……. Co-advisor's signature ….……...

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor, Assist. Prof. Dr. Kanoktip Packdibamrung, for her generous advice, skillful assistance, technical helps, guidance, encouragement, supporting, fruitful and stimulating discussions through the period of my study.

It is also a great honor and pleasure to acknowledge Assoc. Prof. Dr. Siriporn Sittipraneed for her valuable comments, new ideas and insights concerning.

My appreciation is also expressed to the laboratory of Applied Microbiology, Department of Bioresources Science and Research Institute of Molecular Genetics, Kochi University, Japan for many chemical reagents and amino acid analysis.

Sincere thanks and appreciation are due to Assoc. Prof. Dr. Aran Incharoensakdi, Assoc. Prof. Dr. Pairoh Pinphanichakarn and Dr. Rath Pichyangkura, who serve as the members of the master committees, for their helpful suggestions and comments.

My thanks also go to all friends of the Biochemistry and Biotechnology department for their assistance and friendships that make me enjoy and happy so much.

Last but not least, the greatest gratitude is expressed to my mother, Miss Kanlaya Sitthai for understanding, helping, supporting, unfinite inspiration and warmhearted love. I love you so much.

The financial support of research scholarship, Chulalongkorn university, is also gratefully acknowledged.

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ABBREVIATIONS

A	absorbance, 2′-deoxyadenosine (in a DNA sequence)
AlaDH	alanine dehydrogenase
AspDH	aspartic dehydrogenase
bp	base pairs
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
C	2′-deoxycytidine (in a DNA sequence)
^{O}C	degree Celsius
cm	centrimeter
Da	Dalton
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
dNTP	2′-deoxynucleoside 5′-triphosphate
DOPA	dihydroxyphenylalanine
DTT	dithiothreitol
EC	Enzyme Commission
EDTA	ethylene diamine tetraacetic acid
G	2′-deoxyguanosine (in a DNA sequence)
GluDH	glutamate dehydrogenase
GlyDH	glycine dehydrogenase
HPLC	high-performance liquid chromatography
HCl	hydrochloric acid
IPTG	isopropyl-thiogalactoside
kb	kilobase pairs in duplex nucleic acid, kilobases in single-standed nucleic acid
KCl	potassium chloride
kDa	kiloDalton
K_m	Michaelis constant
KOH	potassium hydroxide

l	liter
LB	Luria-Bertani
LeuDH	leucine dehydrogenase
μg	microgram
μl	microliter
μM	micromolar
M	mole per liter (molar)
mA	milliampere
mg	milligram
min	minute
ml	milliliter
mM	millimolar
M_r	relative molecular mass
MW	molecular weight
N	normal
NAD^+	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
ng	nanogram
NH_4Cl	ammonium chloride
NH_4OH	ammonium hydroxide
nm	nanometer
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PheDH	phenylalanine dehydrogenase
pI	isoelectric point
pmol	picomole
PMSF	phenyl methyl sulfonyl fluoride
RNase	ribonuclease
SDS	sodium dodecyl sulfate
SerDH	serine dehydrogenase
T	2′-deoxythymidine (in a DNA sequence)

TB	Tris-borate buffer
TE	Tris-EDTA buffer
TEMED	*N, N, N′, N′*-tetramethyl ethylene diamine
TLC	thin-layer liquid chromatography
T_m	melting temperature, melting point
TrpDH	tryptophan dehydrogenase
UV	ultraviolet
V	voltage
ValDH	valine dehydrogenase
v/v	volume by volume
w/w	weight by weight

CHAPTER I

INTRODUCTION

Amino acids, in addition to their role as protein monomeric units, are energy metabolites and precursors of many biologically important nitrogen-containing compounds, notably heme, physiologically active amines, glutathione, nucleotides, and nucleotide coenzymes. Excess dietary amino acids are neither stored for future use nor excreted. Rather, they are converted to common metabolic intermediates such as pyruvate, oxaloacetate and acetyl-CoA. Consequently, amino acids are also precursors of glucose, fatty acids, and ketone bodies and are therefore metabolic fuels. Besides their role in proteins, amino acids and their derivatives have many biologically important function. They often function as chemical messengers in the communications between cells. For example, glycine, γ-aminobutyric acid (GABA) and dopamine are neurotransmitters (Voet, 2004).

Amino acids can be divided into two groups by the ability of rotation the plane of polarized light. They are L-formed and D-formed amino acids. The amino acid in L-formed plays an important role in all life while D-formed is rarely found in organisms. Recently, the using of L-amino acids for many compounds synthesis are spread widely in animal nutrition, human medicine and the pharmaceutical industries. For example, L-leucine, L-valine, L-isoleucine are used as food and feed activities (Gu and Chang, 1990) while L-alanine is used as the precursor in drug production and can be also used as food additive due to its sweet taste (Suye *et al*., 1992). L-phenylalanine, another interesting L-amino acid, is one of the essential starting material for an artificial sweetener, aspartame (L-aspartate-L-phenylalanine-1-methyl ester, or Nutrasweet) (Chao *et al.,* 2000). In addition, non-natural amino acids are increasingly in demand by the pharmaceutical industry for single-enantiomer drugs. They are in demand as precursor to ligands for synthesis, however, they are very expensive (Busca *et al.*, 2004).

1.1 Amino acid dehydrogenase

The amino acid dehydrogenases (EC 1.4.1.-) are a family of enzyme that are 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 general formula for this reaction can be written as shown in Figure 1.1 (Brunhuber and Blanchard, 1994). They are important enzymes that exist at the interface of nitrogen and carbon metabolism and provide a route for interconversion of inorganic nitrogen with organic nitrogen, and, in other words, serve as a connecting link between amino acid and organic acid metabolism. The amino acid dehydrogenases are categorized based on the specificity they display toward their amino acid substrate and more than ten kinds of them has been so far found in various kind of organisms as shown in Table 1.1 (Ohshima and Soda, 2000). The metabolic role of amino acid dehydrogenases consists of regulation of the synthesis of amino acids and keto acids. In spite of their metabolic roles, the equilibrium of amino acid dehydrogenase reactions lies far to the amination of keto acid since the K_{eq} values are about 10^{-14}-10^{-18}. Therefore, the reactions are favorable for asymmetric synthesis of amino acids from their keto analogs and ammonia (Asano *et al.*, 1987a). The amino acid dehydrogenases have been studied intensively because of their ubiquitous distribution and a number of potential industrial applications. In addition, they have been used for analysis of amino acids and keto acids as well as assay of some enzymes acting on the substrate L-amino acids.

One of the most interesting amino acid dehydrogenases is phenylalanine dehydrogenase (L-phenylalanine: NAD^+ oxidoreductase, deaminating: EC 1.4.1.20) (PheDH), which catalyzes the reversible pyridine nucleotide-dependent oxidative deamination of L-phenylalanine to form ammonia, phenylpyruvate, and NADH as shown in Figure 1.2 (Brunhuber and Blanchard, 1994). The enzyme appears to be useful as an industrial catalyst in the asymmetric synthesis of L– phenylalanine and related L-amino acids from their keto analogs (Asano *et al*., 1987b).

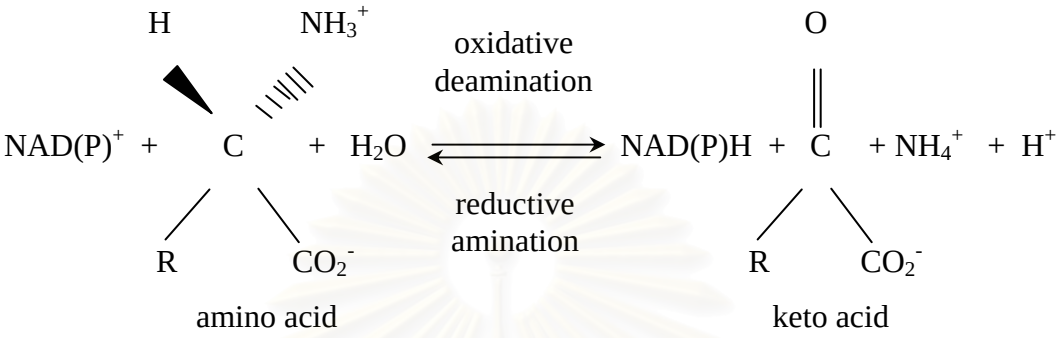


Figure 1.1 The general reaction of L-amino acid dehydrogenase

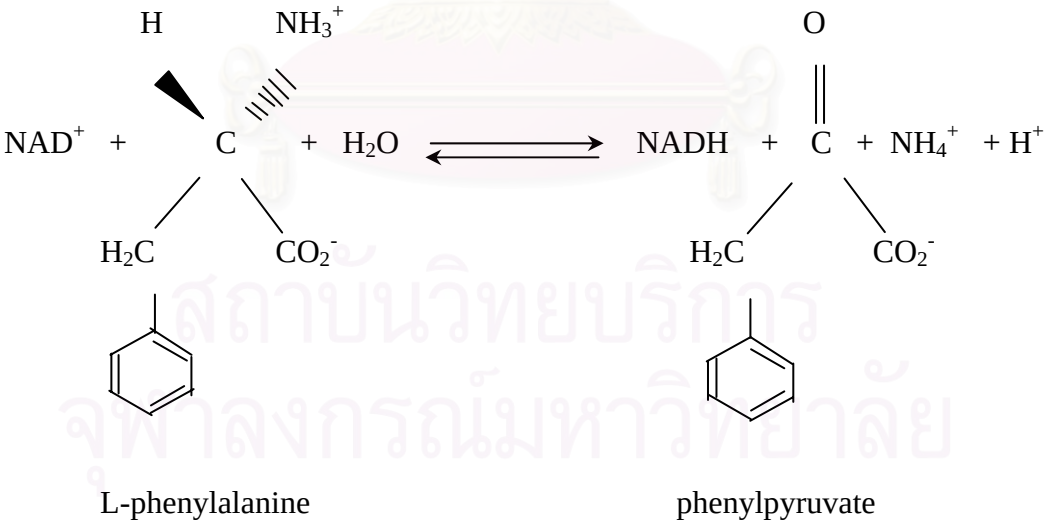


Figure 1.2 The reaction of L-phenylalanine dehydrogenase

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

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

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

Source: Ohshima and Soda, 2000

1.2 Characterization of phenylalanine dehydrogenase

NAD^+-dependent PheDH was firstly found in *Brevibacterium* species (Hummel *et al*., 1984). The distribution of this enzyme is limited to some groups of gram-positive, spore-forming bacteria including Actinomycetes. This may due to the involvement of this enzyme in microbial sporulation thereby connecting the carbon and nitrogen metabolism of amino acids (Asano *et al*., 1998). In some cases, addition of L-phenylalanine to the medium can induce enzyme activity. Moreover, it can also be induced by other amino acids, such as D-phenylalanine and L-histidine (Hummel *et al*., 1984). Subsequently, Asano and Nakazawa (1985) screened for the enzyme activity in cell-free extracts of various soil microorganisms and found that *Sporosarcina ureae* showed high NAD^+-dependent PheDH activity. In addition, Asano and coworkers found the enzyme activity in *Bacillus sphaericus* and *Bacillus badius*. The enzymes from these three bacteria were composed of eight identical subunits with the molecular masses of 39,000 to 42,000 daltons. The *Sporosarcina ureae* and *Bacillus badius* enzymes showed high substrate specificity in the oxidative deamination acting on L-phenylalanine, while that of *Bacillus sphaericus* acted on L-phenylalanine and L-tyrosine. All of them had lower substrate specificity in the reductive amination acting on α-keto acids such as phenylpyruvate and *p*-hydroxyphenylpyruvate (Asano *et al.*, 1987a, b and c).

In 1989, Misono *et al*. found a dimeric NAD^+-dependent PheDH in a soil bacterium identified as *Rhodococcus maris* K-18. The enzyme was purified to homogeneity to compare its properties with those of the octameric enzymes. The enzyme had a molecular mass of about 70,000 daltons and consisted of two identical subunits. It catalyzed the oxidative deamination of L-phenylalanine as well as other L-amino acids such as L-norleucine and L-ethionine, and the reductive amination of phenylpyruvate and *p*-hydroxyphenylpyruvate. The enzyme required NAD^+ as a natural coenzyme. 3-acetylpyridine-NAD^+, the NAD^+ analog, showed much greater coenzyme activity than NAD^+. D-Phenylalanine, D-tyrosine, and phenylethylamine inhibited the oxidative deamination of L-phenylalanine (Misono *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 enzyme from *Thermoactinomyces intermedius* was purified and characterized**.** This enzyme showed higher thermostability since it had not been inactivated by incubation at 70^{o}C, pH 7.2 for at least 60 minutes. The relative molecular weight of the native enzyme was 270,000 and consisted of six subunits identical in molecular weight (41,000). The enzyme preferably acts on L-phenylalanine and its keto analog, phenylpyruvate, in the presence of NAD and NADH, respectively (Ohshima *et al*., 1991).

Bacteria that produced NAD^{+}-dependent PheDH were screened among L-methionine utilizes isolated from soil. A bacterial strain showing PheDH activity was chosen and classified in the genus *Microbacterium*. PheDH was purified from the crude extract of mesophilic *Microbacterium* sp. strain DM 86-1. Its enzyme composed of eight identical subunits with a molecular weight of approximately 41,000. No loss of enzyme activity was observed upon incubation at 55^{o}C for 10 minutes (Asano and Tanetani, 1998). In addition, PheDH from *Rhodococcus* sp. M4 and *Nocardia* sp. 239 were characterized. The results indicated that PheDH isolated from *Rhodococcus* sp. M4 was a tetramer with molecular weight of 39,500 (Hummel *et al*., 1987) while the enzyme from *Nocardia* sp. 239 was active as a monomer with molecular weight of 42,000 (de Boer *et al*., 1989).

Recently, thermotolerant bacteria producing NAD^{+}-dependent PheDH were screened by formazan forming and spectrophotometric method from various places in Thailand and Japan (Suriyapanpong *et al.*, 2000). After that, the strain with showed the highest PheDH activity was identified as *Bacillus badius* BC1 (Chareonpanich., 2001). Its enzyme was purified to homogeneity with 20 % yield and 145.2 purification fold. This enzyme had a relative molecular weight of about 358,000 and consisted of 8 identical subunits with molecular weight of 44,500 (Leksakorn, 2001). Properties of PheDH from various sources were compared as shown in Table 1.2.

Table 1.2 Properties of phenylalanine dehydrogenase from various sources

Properties[a]	*Brevibacterium* sp.	*Rhodococcus.* sp. M4	*S. ureae*	*B. sphaericus*	*B. badius*	*R. maris*	*Nocardia* sp.	*T. intermedius*	*Microbacterium* sp.	*B. badius* BC1
- Molecular mass of native enzyme (kDa)	-	150	310	340	335	70	42	270	330	358
- Molecular mass of subunit (kDa)	-	39	41	41	41	36	42	41	41	44.5
- Number of subunit	-	4	8	8	8	2	1	6	8	8
pH optimum										
- Oxidative deamination	10.5	10.1	10.5	11.3	10.4	10.8	-	11.0	12.0	10.7
- Reductive amination	8.5	9.25	9.0	10.3	9.4	9.8	10.0	9.2	12.0	8.3
Isoelectric focusing point (pI)	-	5.6	5.3	4.3	3.5	-	-	-	5.8	-
Thermostability (remaining activity after incubation for 10 min)	-	-	75 % (40 ^{o}C, pH 9)	100 % (55 ^{o}C, pH 9)	50 % (55 ^{o}C, pH 8)	100 % (35 ^{o}C, pH 7.4)	50 % (53 ^{o}C, pH 9.5-10, 2 hours)	100 % (70 ^{o}C, pH 7.2, 1 hour)	100 % (55 ^{o}C, pH 9)	100 % (40 ^{o}C, pH 7.4, 2 hours)

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

Properties[a]	*Brevibacterium* sp.	*Rhodococcus.* sp. M4	*S. ureae*	*B. sphaericus*	*B. badius*	*R. maris*	*Nocardia* sp.	*T. intermedius*	*Microbacterium* sp.	*B. badius* BC1
Equilibrium constant (M^2)	-	4.5×10^{-14}	2.0×10^{-14}	1.4×10^{-15}	-	-	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	0.59
- phenylpyruvate	0.177	0.13	0.16	0.4	0.106	0.5	0.06	0.045	0.02	0.33
- NAD^+	0.125	0.27	0.14	0.17	0.15	0.25	0.23	0.078	0.20	0.28
- NADH	0.047	0.13	0.072	0.025	0.21	0.043	-	0.025	0.072	0.07
- ammonia	431	387	85	78	127	70	9.6	106	85	200
Substrate specificity[b]										
Oxidative deamination										
- L-phenylalanine	100	100	100	100	100	100	100	100	100	100
- L-tyrosine	-	12	5	72	9	2	2	0	4	0
- L-tryptophan	-	2	5	1	4	8	8	0	0	3
- L-methionine	-	4	4	3	8	5	5	0	7	4

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

Properties[a]	*Brevibacte-rium* sp.	*Rhodococ-cus.* sp. M4	*S. ureae*	*B. sphaericus*	*B. badius*	*R. maris*	*Nocardia* sp.	*T. interme-dius*	*Microbacte -rium* sp.	*B. badius* BC1
- L-valine	-	-	3	1	4	0	0	0	5	2
- L-leucine	-	-	2	1	3	2	-	4	3	0
- L-isoleucine	-	-	1	0.5	0.2	3	-	0	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	-	-	-	11
- *m*-fluoro-DL-phenylalanine	-	-	-	-	11	8	-	-	-	5
- *o*-fluoro-DL-phenylalanine	-	-	-	-	2	2	-	-	-	0
- D-phenylalanine	-	-	0	0	0	0	-	0	-	0

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

Properties[a]	*Brevibacterium* sp.	*Rhodococcus* sp. M4	*S. ureae*	*B. sphaericus*	*B. badius*	*R. maris*	*Nocardia* sp.	*T. intermedius*	*Microbacterium* sp.	*B. badius* BC1
Substrate Specificity [b]										
Reductive amination										
- phenylpyruvate	100	100	100	100	100	100	100	100	100	100
- *p*-hydroxyphenylpyruvate	96	5	24	136	53	91	28	0	0	0
- indole-β-pyruvate	24	3	1	0	-	5	54	-	-	0
- α-ketovalerate	-	-	9	6	12	0	-	-	-	3
- α-ketocaproate	-	-	32	0	31	9	-	-	-	12
- α-ketoisovalerate	-	-	2	6	13	0	-	6	6	5
- α-ketoisocaproate	-	-	13	8	-	1	240	-	-	4
- α-ketobutyrate	-	-	-	-	3	0	-	1	1	0
- α-keto-γ-methylthiobutyrate	59	33	27	11	16	9	-	14	14	0
- α-keto-β-methylbutanoate	-	-	-	-	-	-	-	-	-	0
- α-keto-γ-methylpentanoate	-	-	-	-	13	-	-	6	6	0
- α-ketohexanoate	-	-	-	-	31	-	-	-	-	-

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

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

- = no data

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, 1987), *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), *Microbacterium* sp. (Asano and Tanetani, 1998) and *Bacillus badius* BC1 (Leksakorn, 2001)

1.3 Catalytic mechanism and structure of phenylalanine dehydrogenase

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. The stereospecificity is an inherent characteristic of individual NAD(P) dehydrogenases and depends on the catalytic reaction and enzyme sources. GluDH, LeuDH, ValDH and DAPDH are pro-*S*-specific enzymes whereas AlaDH and LysDH are pro-*R*-specific enzymes (Brunhuber and Blanchard, 1994, and Ohshima and Soda, 2000). 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.

A series of steady-state kinetic analyzes provides information about the reaction mechanism. The oxidative deamination catalyzed by an amino acid dehydrogenase proceeds via the formation of a ternary complex with sequential or random substrate-binding mechanism. For PheDH, two steady-state kinetic mechanism were studied. In 1989, Misono *et al.* studied the initial-velocity and product inhibition of *R. maris* K-18 enzyme. They found that the sequence ordered mechanism in which NAD^{+} and L-phenylalanine bind to the enzyme in that order and three products, ammonia, phenylpyruvate and NADH, are released from the enzyme in that order after dehydrogenation (Brunhuber and Blanchard, 1994). The second kinetic mechanism was determined for PheDH from *T. intermedius* and found that it was slightly different from the other mechanisms. In this case the order of release was observed to be phenylpyruvate, ammonia, and NADH (Ohshima *et al.*, 1991).

Extensive developments of the techniques in gene cloning have enabled rapid determination of the primary structures of PheDH. The kind of sequence alignment information was used in attempts to change the substrate specificity of PheDH from L-phenylalanine to others amino acids such as L-leucine. From sequence alignment, Kataoka and coworkers (1993) found that 16 amino acid residues of LeuDH was

different from the corresponding amino acid residues which are conserved in three PheDH (Figure 1.3). They speculated that some of these residues involved in substrate recognition of the enzyme. So, they replaced the hexapeptide segment (^{124}F-V-H-A-A-^{129}R) of the PheDH from *T. intermedius* by M-D-I-I-Y-Q, which is the corresponding sequence of the LeuDH from *B. stearothermophilus*. The catalytic efficiencies (k_{cat}/K_m) of the mutant enzyme with aliphatic amino acids and aliphatic keto acids as substrates were 0.5 to 2 % of those of the wild-type enzyme. In contrast, the efficiencies for L-phenylalanine and phenylpyruvate decreased to 0.008 and 0.035 % of those of the wild-type enzyme, respectively. The results suggested that the hexapeptide segment plays a significant role in substrate recognition because this is the largest non-homologous segment in the substrate-binding domains of these two enzymes. In 1994, the same research group reported the genetic construction of a chimeric enzyme from two functionally related proteins sharing extensive sequence similarity and assessment of its catalytic properties. A chimeric enzyme consisting of an N-terminal domain of PheDH containing the substrate binding region and a C-terminal domain of LeuDH containing the NAD binding region were constructed by genetic engineering and characterized (Kataoka *et al.*, 1994). Although the catalytic efficiency of the chimeric enzyme on L-phenylalanine was 6 % of that of the parental PheDH, the chimeric enzyme showed a similar K_m value for L-phenylalanine, pH optimum, and the same stereospecificity for hydrogen transfer at the C-4 position of the NADH. In contrast, the chimeric enzyme showed a lower substrate specificity than the parental PheDH (Figure1.4). In addition to phenylalanine and derivatives, it acted on poor substrates of both parent enzymes such as L-methionine, L-tryptophan, and L-phenylglycine in the oxidative deamination. Furthermore, the chimeric enzyme acted on L-branched chain amino acids such as L-valine and L-isoleucine. The specificities of the chimeric enzyme in the reductive amination was an admixture of the specificities of the two parent enzymes. By this way, amino acid dehydrogenase that exhibited new substrate specificity was created.

PheDH, *T. intermedius*	58	D V L R – L S K G M T Y K C S L A D V D F G G G K M V I I G D P K K D K S P
PheDH, *B. sphaericus*	68	D V L R – L S E G M T Y K C A A A D I D F G G G K A V I I G D P E K D K S P
PheDH, *S. ureae*	69	D V L R – L S K G M T Y K C A A A D V D F G G G K S V I I G D P L K D K T P
LeuDH, *B. stearothermophilus*	57	D A L R – L A R G M T Y K N A A A G L N L G G G K T V I I G D P R K D K N E
GluDH (NAD^+), *C. symbiosum*	101	S I M K F L G F E Q A F K D S L T T L P M G G A K G G S D F D P – N G K S –
GluDH ($NADP^+$), *E. coli*	104	S I L K F L G F E Q T F K N A L T T L P M G G G K G G S D F D P – K G K S -

PheDH, *T. intermedius*	95	E L F R V I G R F V G G L N G R F Y T G T D M G T N P E D F V H A A R E
PheDH, *B. sphaericus*	105	A L F R A F G Q F V E S L N G R F Y T G T D M G T T M D D F V H A Q K E
PheDH, *S. ureae*	106	E K F R A F G Q F I E S L N G R F Y T G T D M G T T L E D F V H A M K E
LeuDH, *B. stearothermophilus*	94	A M F R A F G R F I Q G L N G R Y I T A E D V G T T V A D M D I I Y Q E
GluDH (NAD^+), *C. symbiosum*	137	D R E V M R F C Q A F M T E L Y R H I G P D I D V P A G D L G V G A R E
GluDH ($NADP^+$), *E. coli*	140	E G E V M R F C Q A L M T E L Y R H L G A D T D V P A G D I G V G G R E

Figure 1.3 Sequence comparison of the conserved regions around the Lys residue in Gly-rich regions of several amino acid dehydrogenases The residues conserved in three PheDHs but not in LeuDH are indicated by shading.

Source: Kataoka *et al*., 1993

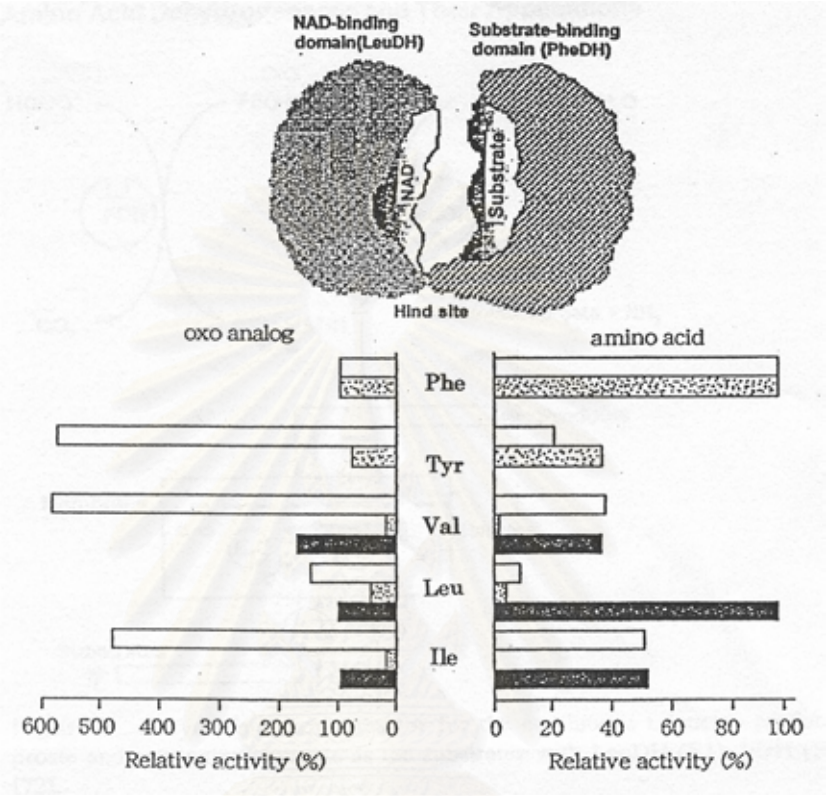


Figure 1.4 Scheme of the chimeric enzyme consisting of an amino terminal domain of phenylalanine dehydrogenase and a carboxy terminal domain of leucine dehydrogenase

Comparison of substrate specificity of PheDH (□), chimeric enzyme (▨), and LeuDH (■) on both amination and deamination

Source: Kataoka *et al.*, 1994

In 1995, the nucleotide sequence of phenylalanine dehydrogenase (*phedh*) gene coding for PheDH from *B. badius* IAM 11059 was analyzed. The gene consisted of an ORF of 1,140 nucleotides encoding 380 amino acid residues. From amino acid sequence comparison of *B. badius* PheDH with leucine, phenylalanine and glutamate dehydrogenases, the catalytic domain of *B. badius* enzyme appeared to be G-G-(G or S or A)-K-X-(V or G)-X-X-X-(D or N)-(P or L) (Yamada *et al*., 1995).

Seah *et al*. (1995) undertook site-directed mutagenesis to allowed alteration of amino acid residues surrounding substrate–binding pocket of PheDH. Glycine-124 and leucine-307 of PheDH from *B. sphaericus* were replaced by alanine and valine, respectively. They reported that the resulting enzyme displayed reduced activity for L-phenylalanine compared to the wild type enzyme and enhanced activity towards aliphatic amino acid substrates. This result indicated that the substrate profile of the enzyme varied significantly by the mutations. On this basis, Busca and coworkers (2004) envisaged that engineered PheDH mutants might prove to be biocatalyst for the asymmetric synthesis of non-natural amino acids, especially phenylalanine analogs which used as precursors to ligand for drugs synthesis. Asparagine-145 of PheDH from *B. sphaericus* was replaced by the less polar alanine, leucine and valine. It was found that they could better accommodate substituted aromatic derivatives of phenylpyruvate, such as 2-F-phenylpyruvate and 2-Cl-phenylpyruvate, than wild-type enzyme.

In 1994, Brunhuber and coworkers cloned and sequenced *phedh* gene from *Rhodococcus.* sp. M4. They found that PheDH composes of two domains: the amino-terminal portion contains residues involved in general amino acid binding and catalysis while the carboxyl-terminal portion contains the presumptive dinucleotide-binding domain (Brunhuber *et al*., 1994). In 1999, Vanhooke *et al*. determined structure, namely the enzyme•NAD^+•phenylpyruvate, and enzyme•NAD^+•*β*-phenylpropionate species, of PheDH from *Rhodococcus* sp. M4 by X-ray crystallographic analyses. This was the first example of structures of 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 (Figure1.5). 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 (Vanhooke *et al*., 1999). Moreover, they found that Lys78 and Asp118 act as the catalytic residues in the active site (Brunhuber *et al.,* 2000).

1.4 Cloning of phenylalanine dehydrogenase gene

Many researchers attempted to clone *phedh* genes not only to study the evolutionary relationship among the $NAD(P)^+$-dependent amino acid dehydrogenase, but also to produce the enzyme which catalyzes for the asymmetric synthesis of L-phenylalanine and related amino acids. In 1987, Asano and coworkers recovered putative *phedh* gene fragment (2-9 kbs) from *B. badius* digested chromosomal DNA from agarose gel by electroelution, and then ligated the gene fragments with *Eco*RI-digested pBR322. After that, recombinant plasmids were transformed into *E. coli* RR1 (Asano *et al*., 1987c). Moreover, Okazaki *et al*. (1988) used the same method to prepare gene fragments from *B. sphaericus*, ligated it into the *Hin*dIII site of pUC9 and transformed the recombinant plasmid into *E. coli* JM103. They reported that *phedh* gene consisted of 1,143-bp open reading frame encoding for 381 amino acids residues. In 1991, research group of Takada cloned gene encoding PheDH of a thermophile, *T. intermedius* into *E. coli* MV1184, using plasmid pUC18. The *phedh* gene consisted of 1,098 bp and encoded 366 amino acid residues corresponding to the 41,000 KDa subunit of the hexameric enzyme. The expression level of *phedh* gene in *E. coli* MV1184 was very low, about 0.35 % of the total soluble protein. In overexpression of *phedh* gene, the structural gene of PheDH of *T. intermedius* was amplified by PCR. The amplified 1.1 Kb fragment was ligated with plasmid pKK223-3. The *E. coli* JM109 was used as host cell. The enzyme produced by the transformant, corresponded to about 8.3 % of the total soluble protein. Moreover, *phedh* gene from *B. badius* BC1 was cloned and expression in *E. coli* JM109, using plasmid vector pUC18. The PheDH activity of *E. coli* clone was about 60 times

Figure 1.5 Structure of *Rhodococcus* sp. M4 phenylalanine dehydrogenase

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

Source: Vanhooke *et al*., 1999

higher than that of wild-type strain (Chareonpanich, 2001). Recently, cloning and expression of *phedh* gene from *B. sphaericus* in *E. coli* was performed. The gene was cloned in the vector pET16-b and transformed into *E. coli* BL21(DE3). Expression of *phedh* gene under T7 promoter was over 140 times greater than that of the wild type *B. sphaericus* (Omidinia, 2002).

1.5 Application of phenylalanine dehydrogenase

Amino acid dehydrogenases have been used for the stereospecific synthesis of amino acids from chiral substrates, keto acids and ammonia, as well as for analysis of L-amino acids, keto acids and assay of enzymes of which amino acids and keto acids are their substrates or products.

PheDH is also being developed as a biosensor to screen for phenylketonuria (PKU) which is an inborn metabolic disorder where is a result from impaired activity of phenylalanine hydroxylase (EC 1.14.16.1). This enzyme catalyze conversion of the essential amino acid phenylalanine to tyrosine in liver. PKU causes an excess of phenylalanine accumulated in the blood and spinal fluid (Guthrie and Susi, 1963). Although several methods have been reported for the quantitative determination of L-phenylalanine in physiological fluids such as spectrofluorometric methods or by column chromatography using amino acid analyzers, they are not routinely applied since spectrofluorometric method requires deproteinization of samples, a large sample size (>1 ml blood) and also lack specificity while the use of amino acid analysis or high-performance liquid chromatography requires highly sophisticated instrumentation and deproteinization of samples. Enzymatic assay is particularly suitable method for clinical routine because this method has many advantages such as rapid, simple as well as specific, and requires only a drop of blood for the simultaneous determination of L-phenylalanine. This method couples simultaneously the reaction of an NAD(H)-dependent PheDH with an intermediate electron acceptor system as shown in Figure 1.6 (Wendel *et al.*, 1989 and Schulze *et al.*, 2002). Moreover, Nakamura *et al*. (1996) found that the recycling assays involving the coupling of transaminases and dehydrogenases can be applicable to detect other

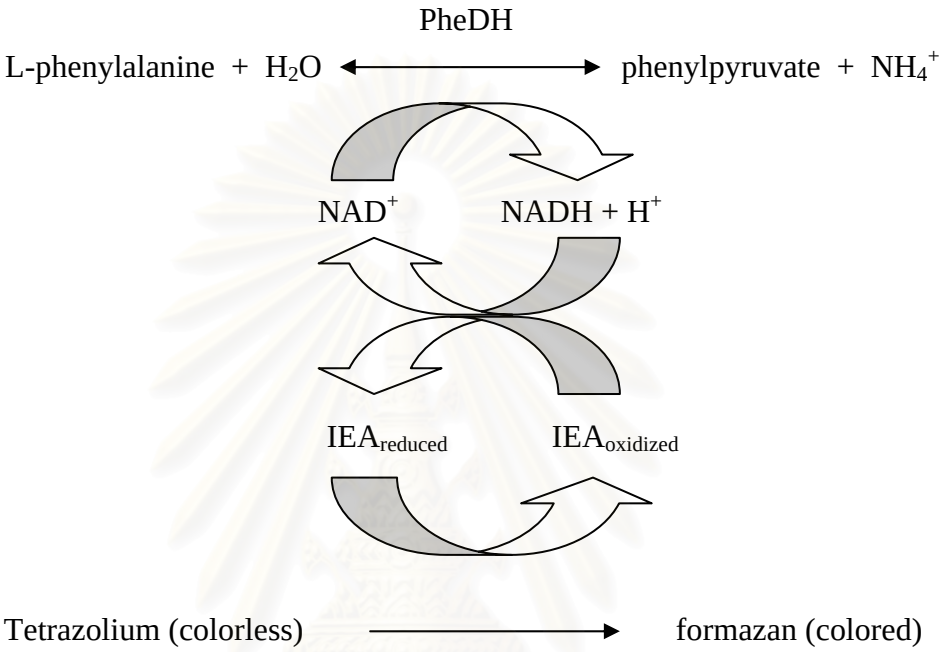


Figure 1.6 Reaction of the enzymatic phenylalanine determination

L-PheDH: L-phenylalanine dehydrogenase

IEA: intermediate electron acceptor

Source: Schulze *et al*., 2002

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).

One of the most important strategies for asymmetric synthesis is biocatalysts. The application of biological species such as microbial cells or enzymes derive therefrom to catalyze organic reagents. Many biocatalysts exhibit high stereo-selectivity making them superior to chemical catalysts for asymmetric synthesis. Furthermore, biocatalysts are ideal energy-efficient, environmentally acceptable reagents, as virtually all reactions proceed under mild conditions and avoid the use of toxic reagents and disposal of byproducts. Thus biocatalysts offer a good opportunity to prepare industrially useful chiral compounds (Busca *et al*., 2004).

PheDH has been used for the production of optically pure L-phenylalanine, a component of the artificial sweetener aspartame and benzaldehyde which can be used as aromatic flavor compound in cheeses (Groot and de Bont, 1998). Moreover, the enzyme also important for the synthesis of its related natural and unnatural amino acids from the corresponding keto analogs and ammonia (Asano *et al.*, 1987b and Hummel, 1987).

Continuous conversion of phenylpyruvate to L-phenylalanine was carried out by PheDH and formate dehydrogenase (FDH; EC 1.2.1.2) as shown figure 1.7. 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 catalytical 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).

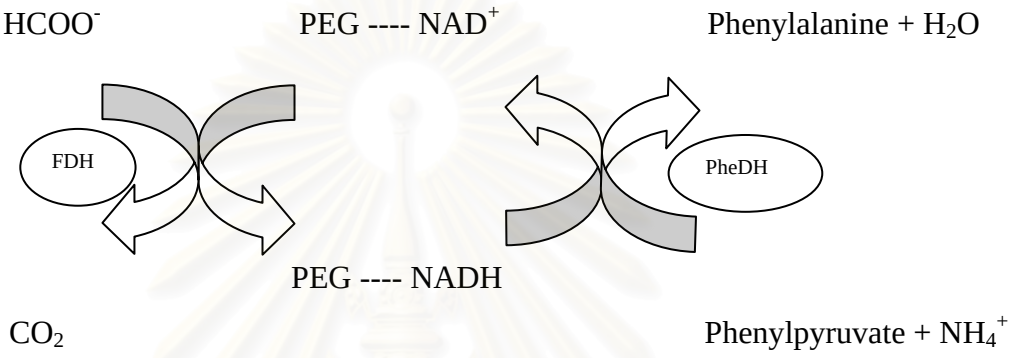


Figure 1.7 Enzymatic synthesis of L-phenylalanine with coenzyme regeneration.

PheDH: phenylalanine dehydrogenase

FDH : formate dehydrogenase

PEG : polyethyleneglycol

Source: 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.

Equilibrium constant of PheDH from various sources are vary from 3.2×10^{-18} to 2.0×10^{-14}. This showed that the equilibrium of the enzyme reaction strongly favors synthesis of amino acid. PheDH from *Sporosarcina ureae* and FDH from *Candida boidinii* was 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). In addition, (*S*)-amino acids such as (*S*)-tyrosine were synthesized from their keto analogs with the coupling reaction of PheDH and FDH as shown in Table 1.4 and Figure 1.8 (Asano *et al*., 1990). Moreover, allysine ethylene acetal [(*S*)-2-amino-5-(1,3-dioxolan-2-yl)-pentanoic acid], that is one of three building blocks used for an alternative synthesis of VANLEV, a vasopeptidase inhibitor, was prepared from the corresponding keto acid by reductive amination 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).

Table 1.3 Synthesis of L-amino acids from keto acids

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

Table 1.4 Synthesis of (*S*)-amino acids from α-keto acids by using phenylalanine dehydrogenase and formate dehydrogenase

Substrate	Product	% Yield
phenylpyruvate	(*S*)-phenylalanine	>99
p-hydroxyphenylpyruvate	(*S*)-tyrosine	>99
p-fluorophenylpyruvate	(*S*)-*p*-fluorophenylalanine	>99
α-keto-*γ*-phenylbutyrate	(*S*)-α-amino-*γ*-phenylbutyrate	99
α-keto-*δ*-phenylvalerate	(*S*)-α-amino-*δ*-phenylvalerate	98
α-keto-*β*-methylphenylpropionate	(*S*)-α-amino-*β*-methylphenylpropionate	98
α-ketononanoate	(*S*)-α-aminononanoate	99

Source: Asano *et al*., 1990

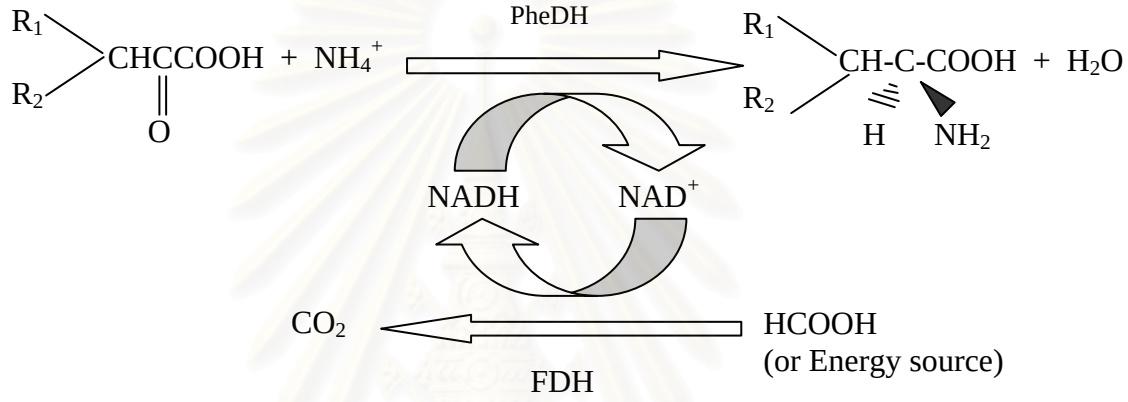


Figure 1.8 Synthesis of (*S*)-amino acid from its α-keto analogue by phenylalanine dehydrogenase with a regeneration of NADH by formate dehydrogenase

Source: Asano *et al*., 1990

In pharmaceutical studies, 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).

1.6 Objectives of this research

From our previous study, PheDH from *Acinetobacter lwoffii* was purified by Butyl-Toyopearl, DEAE-Toyopearl, Sephadex G-150, Mono Q, Phenyl-Superose and Sephadex G-200 column chromatography with 20.0 % yield and 463 purification fold. This enzyme had molecular mass of about 320 kDa, which consisted of 8 identical subunits. The pH optimum of oxidative deamination and reductive amination were 10.5 and 9.2, respectively, and *K*m for L-phenylalanine, phenylpyruvate, NAD^+, NADH and ammonia were 4.50, 0.56, 0.68, 0.12 and 149 mM, respectively. Furthermore, various amino acid such as L-methionine, L-tryptophan and L-norleucine could act as substrate of the enzyme. No loss of the enzyme activity was observed upon incubation at 55 ^oC, pH 7.4 for 10 mins. From these properties, the enzyme is of interest for use in the synthesis of various amino acids from their corresponding keto acids by reductive amination reaction. The low yield of enzyme in the wild-type strain triggered us to use recombinant DNA technology to obtain a sufficient amount of the PheDH. From the nucleotide sequence of *phedh* gene of *A. lwoffii*, the structural gene of PheDH will be amplified and cloned into *Escherichia coli* BL21(DE3) and BL21(DE3)pLysS using the expression vector, pET-17b. Then optimal condition of gene expression will be studied for high PheDH production. After that, the enzyme will be purified and preliminarily tested for production of various amino acids from their corresponding keto acids.

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Amino acid analyzer: L8500A, Hitachi, Japan

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

Autopipette: Pipetman, Gilson, France

Centrifuge, refrigerated centrifuge: J-30I, Beckman Instrument Inc., U.S.A.

Centrifuge, microcentrifuge: MC-15A, Tomy Seiko Co., Ltd., Japan

Electrophoresis unit: Hoefer^{TM} miniVE, Amersham Pharmacia Biotech., U.S.A.; 2050 MIDGET, LKB, Sweden; Mini protein, Bio-Rad, U.S.A. and submarine agarose gel electrophoresis unit

Gene Pulser^{R}/*E. coli* Pulser^{TM}Cuvettes: Bio-Rad, U.S.A.

High Performance Liquid Chromatography (HPLC): SHIMADZU, Japan

Incubator, waterbath: M20S, Lauda, Germany and Biochiller 2000, FOTODYNE Inc., U.S.A.

Light box: 2859 SHANDON, Shandon Scientific Co., Ltd., England

Magnetic stirrer: Fisherbrand, Fisher Scientific, U.S.A.

Membrane filter: cellulose nitrate, pore size 0.45 μm, Whatman, England

Microcentrifuge tubes 0.5 and 1.5 ml, Axygen Hayward, U.S.A.

Microwave oven: TRX1500, Turbora International Co., Ltd., Korea

Orbital incubator: 1H-100, Gallenkamp, England

pH meter: PHM95, Radiometer Copenhegen, Denmark

Power supply: POWER PAC 300, Bio-Rad, U.S.A.

Sonicator: SONOPULS Ultrasonic homogenizers, BANDELIN, Germany

Spectrophotometer: Spectronic 2000, Bausch & Lomb, U.S.A.; UV-240, Shimadzu, Japan, and DU Series 650, Beckman, U.S.A.

Thermo cycler: Mastercycler gradient, eppendorf, Germany

Thin-wall microcentrifuge tubes 0.2 ml, Axygen Hayward, U.S.A.

Thin layer chromatography (TLC): DC-Plastikfolien cellulose, Merck, Germany

Ultrafilter: Suprec^{Tm}-01,^{Tm}-02, pore size 0.20 μm and 0.22 μm, Takara Shuzo Co., Ltd., Japan

UV transluminator: 2011 Macrovue, San Gabriel California, U.S.A.

Vortex: K-550-GE, Scientific Industries, Inc., U.S.A.

2.2 Chemicals

Acetone: Lab-Scan Ltd, Ireland

Acrylamide: Merck, Germany

Agar: Merck, Germany

Agarose: SEKEM LE Agarose, FMC Bioproducts, U.S.A.

Ammonium hydroxide: BDH, England

Ammonium persulphate: Sigma, U.S.A.

Ammonium sulphate: Carlo Erba Reagenti, Italy

Ampicillin: Sigma, U.S.A.

β-Mercaptoethanol: Fluka, Switzerland

Boric acid: Merck, Germany

Bovine serum albumin: Sigma, U.S.A.

Bromphenol blue: Merck, Germany

Chloramphenicol: Nacalai tesque, Inc., Japan

Chloroform: BDH, England

Coomassie brilliant blue R-250: Sigma, U.S.A.

di-Potassium hydrogen phosphate anhydrous: Carlo Erba Reagenti, Italy

di-Sodium ethylene diamine tetraacetic acid: M&B, England

DNA marker: Lambda (λ) DNA , BioLabs, Inc., U.S.A.; 100 base pair DNA ladder, Promega Co., U.S.A.

Ethidium bromide: Sigma, U.S.A.

Ethyl alcohol absolute: Carlo Erba Reagenti, Italy

Ethylene diamine tetraacetic acid (EDTA): Merck, Germany

Ficoll type 400: Sigma, U.S.A.

Glacial acetic acid: Carlo Erba Reagenti, Italy

Glycerol: Merck, Germany

Glycine: Sigma, U.S.A.

Glucose: BDH, England

Hydrochloric acid: Carlo Erba Reagenti, Italy

Isoamyl alcohol: Merck, Germany

Isopropanol: Merck, Germany

Isopropylthio-β-D-galactoside (IPTG): Sigma, U.S.A.

L-Phenylalanine: Sigma, U.S.A.

Magnesium sulphate 7-hydrate: BDH, England

Methylalcohol: Merck, Germany

N-acetyl-*N*,*N*,*N*-trimethylammonium bromide (CTAB): Sigma, U.S.A.

N,*N*-dimethyl-formamide: Fluka, Switzerland

N,*N'*-methylene-bis-acrylamide: Sigma, U.S.A.

N,*N*,*N'*,*N'*-tetramethyl-1,2-diaminoethane (TEMED): Carlo Erba Reagenti, Italy

Nicotinamide adenine dinucleotide (oxidized form) (NAD^+): Kohjin Co. Ltd., Japan

Nicotinamide adenine dinucleotide (reduced form) (NADH): Kohjin Co. Ltd., Japan

Ninhydrin: VWR Prolabo RANGE, France

Nitroblue tetrazolium: Koch-Light Laboratories Ltd., Japan

Peptone from casein pancreatically digested: Merck, Germany

Perchloric acid: BDH, England

Phenazine methosulfate: Nacalai Tesque, Inc., Japan

Phenol: BDH, England

Phenylmethylsulfonyl fluoride (PMSF): Sigma, U.S.A.

Potassium acetate: Merck, Germany

Potassium chloride: Merck, Germany

Potassium hydroxide: Carlo Erba Reagenti, Italy

Potassium phosphate monobasic: Carlo Erba Reagenti, Italy

QIA quick Gel Extraction Kit: QIAGEN, Germany

Sodium acetate: Merck, Germany

Sodium carbonate anhydrous: Carlo Erba Reagenti, Italy

Sodium citrate: Carlo Erba Reagenti, Italy

Sodium chloride: Carlo Erba Reagenti, Italy

Sodium dodecyl sulfate: Sigma, U.S.A.

Sodium hydroxide: Carlo Erba Reagenti, Italy

Standard protein marker: Amersham Pharmacia Biotech Inc., U.S.A.

Sucrose: Sigma, U.S.A.

Trifluoroacetic acid: BDH, England

Tris (hydroxymethyl)-aminomethane: Carlo Erba Reagenti, Italy

Yeast extract: Scharlau microbiology, European Union

2.3 Enzymes and Restriction enzymes

Lysozyme: Sigma, U.S.A.

Proteinase K: Sigma, U.S.A.

Restriction enzymes: New England BioLabs, Inc., U.S.A.

RNaseA: Sigma, U.S.A.

Taq DNA Polymerase: TAKARA SHUZO Co., Ltd., Japan

T_4 DNA ligase: New England BioLabs, Inc., U.S.A.

2.4 Primers

Oligonucleotides: Bioservice Unit, Thailand

2.5 Bacterial strains and plasmid

Acinetobacter lwoffii was used as a source of phenylalanine dehydrogenase gene

pET-17b was used as an expression vector for cloning of phenylalanine dehydrogenase gene (Appendix A).

Escherichia coli BL21(DE3), genotype: F^- *omp*T *hsd*S_B (r_B^- m_B^-) *gal dcm* (DE3), was used as a host for expression.

Escherichia coli BL21(DE3)pLysS, genotype: F^- *ompT* $hsdS_B$ (r_B^- m_B^-) *gal dcm* (DE3) pLysS (Cm^R) , was used as a host for expression.

2.6 Cloning of phenylalanine dehydrogenase gene

2.6.1 Chromosomal DNA Extraction

Chromosomal DNA was isolated from *Acinetobacter lwoffii* by the method of Frederick *et al.,* (1995). A single colony was inoculated into 10 ml of peptone medium (1.5 % peptone, 0.2 % K_2HPO_4, 0.2 % KH_2PO_4, 0.2 % NaCl, 0.015 % $MgSO_4.7H_2O$ and 0.015 % yeast extract, pH 7.2) and incubated at 30 ^oC for 24 hours with shaking. Then each 1.5 ml of cell culture was centrifuged in microcentrifuge tube at 8,000xg for 2 minutes. The pellet was resuspended in 550 μl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) by repeated pipetting. The cell solution was then treated with 30 μl of 10 % SDS followed by the addition of 3 μl of 20 mg/ml proteinase K and incubated for 1 hour at 37 ^oC. After incubation, 100 μl of 5 M NaCl and 50 μl of CTAB-NaCl solution (10 % CTAB and 0.7 M NaCl) were added and incubated for 10 minutes at 65 ^oC. The DNA was extracted with an addition of an equal volume of chloroform-isoamyl alcohol (24: 1 V/V), mixed gently, and centrifuged at 10,000xg for 10 minutes. A viscous fluid formed at the aqueous layers was carefully transferred to a new microcentrifuge tube and extracted with equal volume of phenol-chloroform-isoamyl alcohol (25: 24: 1 V/V) to ensure the complete extraction of DNA. DNA was precipitated by the addition of 0.6 volume of isopropanol to the aqueous phase and collected by centrifugation at 10,000xg for 10 minutes. The DNA was washed with 70 % ethanol. After drying, the pellet was dissolved in an appropiate volume of TE buffer. Finally, DNA concentration was estimated by submarine agarose gel electrophresis compared with known amount of λ/*Hin*dIII marker.

2.6.2 Agarose gel electrophoresis

Electrophoresis through agarose is the standard method used to separate, identify, and purify DNA fragments. The 0.7 g of agarose was added to 100 ml electrophoresis buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0) in Erlenmeyer flask and heated until complete solubilization. The agarose solution was left at room temperature to 50 ^{o}C before pouring into an electrophoresis mould. When the gel was completely set, the DNA samples were mixed with gel loading buffer (0.025 % bromphenol blue, 40 % ficoll 400 and 0.5 % SDS) and loaded into agarose gel. Electrophoresis had been performed at constant voltage of 10 volt/cm until the bromphenol blue migrated to appropiately distance through the gel. The gel was stained with 2.5 μg/ml ethidium bromide solution for 5 minutes and destained to remove unbound ethidium bromide with distilled water for 10 minutes. DNA fragments on agarose gel were visualized under a long wavelength UV light. The concentration and molecular weight of DNA sample was compared with the intensity and relative mobility of the standard DNA markers (λ/*Hin*dIII and 100 bp ladder).

2.6.3 PCR Amplification of phenylalanine dehydrogenase gene

2.6.3.1 Primers

Primers used for the PCR amplification of *phedh* gene was designed by using the nucleotide sequence from phenylalanine dehydrogenase gene of *Acinetobacter lwoffii.* The sequences of 5′-primer (PheDHpETF) was 5′-GGAATTCCATATGGCAAAACAGCTTGAAAAGT-3′ which contained *Nde*I site. The sequence of 3′-primer (PheDHpETR) was 5′-CGGGATCCCTATTCTT GTTTATTCCAT-3′ which contained *Bam*HI site. The melting temperature of primers were 49 and 40 ^{o}C, respectively.

2.6.3.2 Template

Chromosomal DNA of *A. lwoffii* was prepared according to the method described in 2.6.1 and then was completely digested with each restriction enzyme: *Bam*HI, *Hind*III and *Kpn*I. The 20 µl reaction mixture contained 1 µg of chromosomal DNA, 1x reaction buffer, 1x BSA solution (supply from the manufacture) and 10 U of each restriction enzyme. The reaction mixture was incubated at 37 ^{o}C for 18 hours. One microliter (about 50 ng) of the DNA solution was used as template in each reaction of PCR.

2.6.3.3 PCR condition

The *phedh* gene was amplified using gradient PCR method. Twenty five microliters reaction mixture contained 2.5 U of *Taq* DNA polymerase, 0.3 mM dNTPs, 1x PCR buffer (100 mM Tris-HCl, pH 8.8 , 500 mM KCl and 1 % Triton X-100), 2 mM $MgCl_2$, 50 ng DNA template and 10 pmole of each primer. The PCR condition was predenaturation at 94 ^{o}C for 10 minutes, and 30 cycles of denaturation at 94^{o}C for 1 minute, annealing at 37.6, 41.7 and 44.9 ^{o}C for 1 minute, extension at 72 ^{o}C for 2 minutes following by final extension at 72 ^{o}C for 7 minutes. The PCR products were electrophoresed through agarose gel. Finally, the putative *phedh* gene fragment was harvested from agarose gel by QIA Quick gel extraction kit (Appendix B).

2.6.4 Recombinant DNA preparation

2.6.4.1 Vector DNA preparation

The *E. coli* BL21(DE3), which harboured pET-17b plasmid was grown in 5 ml LB medium containing 100 µg/ml ampicillin at 37 ^{o}C for 16 hours with shaking. The cell culture was collected in each 1.5 ml microcentrifuge tube by centrifugation at 8,000xg for 1 minute. Then 100 µl of solution I (50 mM glucose, 25 mM Tris-HCl and 10 mM EDTA, pH 8.0) was added and the cell pellet was

resuspended by repeated pipetting. After that, the 200 μl of freshly prepared solution II (0.2 N NaOH and 1 % SDS) was added, gently mixed by inverting the tube and placed on ice for 5 minutes. Then 150 μl of cooled solution III (3 M sodium acetate, pH 4.8) was added and the tube was placed on ice for 5 minutes. The mixture was centrifuged at 10,000xg for 10 minutes and the supernatant was transferred to a new microcentrifuge tube. Then DNA solution was extracted with equal volume of phenol-chloroform-isoamyl alcohol (25: 24: 1 V/V). The plasmid DNA was precipitated by the addition of 2 volume of absolute ethanol to the aqueous phase, collected by centrifugation at 10,000xg for 10 minutes and washed with 70 % ethanol. After drying, the pellet was dissolved in an appropiate volume of TE buffer, pH 8.0 containing 20 μg/ml DNase-free pancreatic RNase.

The expression vector pET-17b was linearized with *Nde*I and *Bam*HI. The reaction mixture containing of 1 μg pET-17b, 1x *Bam*HI reaction buffer, 1x BSA solution, 2 U of *Nde*I and 2 U of *Bam*HI in total volume of 20 μl was incubated at 37 ^{o}C for 18 hours. The linear-formed pET-17b was harvested from agarose gel by QIA Quick gel extraction kit.

2.6.4.2 The phenylalanine dehydrogenase gene fragment preparation

The putative *phedh* gene fragment from 2.6.3.3 was linearized with *Nde*I and *Bam*HI. The reaction mixture containing of 1 μg of gene fragment, 1x *Bam*HI reaction buffer, 1x BSA solution, 2 U of *Nde*I and 2 U of *Bam*HI in total volume of 20 μl was incubated at 37 ^{o}C for 18 hours. The DNA fragment was harvested from agarose gel by QIA Quick gel extraction kit.

2.6.4.3 Ligation of vector DNA and the gene fragment

The gene fragment (2.6.4.2) was ligated to the pET-17b vector (2.6.4.1) at vector: insert molar ratio of 1: 5. The ligation mixture of 20 μl contained 50 ng of vector DNA, 250 ng of the gene fragment, 1x ligation buffer (50 mM Tris-

HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT and 5 % (W/V) polyethylene glycol - 8000) and 10 U of T_4 DNA ligase was incubated overnight at 16 ^oC. The recombinant plasmids in this reaction mixture were further used for transformation.

2.6.5 Transformation

2.6.5.1 Preparation of competent cells

A fresh overnight culture of *E. coli* BL21(DE3) or *E. coli* BL21(DE3)pLysS were inoculated into 1 liter of LB broth (1 % tryptone, 1 % NaCl and 0.5 % yeast extract, pH 7.2) with 1 % inoculum size. Cells were grown to log phase at 37 ^oC with vigorous shaking until OD_{600} was about 0.5 to 0.8. The culture was chilled on ice for 15 to 30 minutes and then centrifuged at 8,000 xg for 15 minutes at 4 ^oC. The cells were washed with 1 liter of cold water, spun down and washed again with 0.5 liter of cold water. After centrifugation, the cells were resuspended in approximately 20 ml of 10 % glycerol in distilled water and centrifuged at 8,000xg for 15 minutes at 4 ^oC. Finally, the cell pellets were resuspended to a final volume of 2 to 3 ml in 10 % glycerol. This suspension was divided into 40 µl aliquots and stored at – 80 ^oC until used.

2.6.5.2 Transformation

The recombinant plasmids from 2.6.4.3 were transformed into competent cells of *E. coli* BL21(DE3) or *E. coli* BL21(DE3)pLysS by electroporation. In the electroporation step, cuvette and sliding cuvette holder were chilled on ice. The Gene Pulser apparatus was set to the 25 µF capacitor, 2.5 kV, and the pulse controller unit was set to 200 Ω. Competent cells were gently thawed on ice. One microliter of recombinant plasmid was mixed with 40 µl of the competent cells and then placed on ice for 1 minute. This mixture was transferred to a cold cuvette and the cuvette was applied one pulse at the above setting. Subsequently, LB medium was added immediately to the cuvette. The cells were quickly resuspended with a pasteur pipette. Then the cell suspension was transferred to new tube and incubated at

37 ^{o}C for 1 hour with shaking. Finally, this suspension was spread onto the LB agar plates containing 100 μg/ml ampicillin (when *E. coli* BL21(DE3)pLysS was used as host, 34 μg/ml chloramphenicol was also included.) and incubated at 37 ^{o}C for 10 hours. Cells containing the recombinant plasmids which could grow on selective plate were picked and the plasmids were isolated.

2.7 Expression of phenylalanine dehydrogenase gene

2.7.1 Recombinant plasmid preparation

E. coli BL21(DE3) recombinant clones were grown in LB medium containing 100 μg/ml ampicillin (whereas the recombinant clones of *E. coli* BL21(DE3)pLysS were grown in LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol.). The growing condition was 37 ^{o}C for 16 hours with shaking. The cell cultures were collected in each 1.5 ml microcentrifuge tube by centrifugation at 8,000xg for 2 minutes. Then the plasmid from individual clone was extracted as described in 2.6.4.1. After that, the plasmids DNA were completely digested with *Nde*I and *Bam*HI. The size of recombinant plasmids were estimated by submarine agarose gel electrophoresis compared with λ/*Hin*dIII marker. Finally, the recombinant plasmids were confirmed to contain *phedh* gene inserts by sequencing.

2.7.2 Crude extract preparation

The *E. coli* BL21(DE3) transformants were grown overnight at 37 ^{o}C in 5 ml of LB medium containing 100 μg/ml ampicillin (whereas the recombinant clones of *E. coli* BL21(DE3)pLysS were grown in LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol). After that, 2.5 % of the cell culture was inoculated into 100 ml of the same medium and was cultured at 37 ^{o}C with shaking. When the turbidity of the culture at 600 nm had reached 0.6, IPTG was added to final concentration of 0.4 mM to induce *phedh* gene expression, and cutivation was continued at 37 ^{o}C for 4 hours. The cells were harvested by centrifugation at 10,000xg for 10 minutes, then washed with cold 0.85 % NaCl. After that, the cell pellet was

washed once in cold extraction buffer (0.1 M potassium phosphate buffer, pH 7.4 containing 0.1 mM PMSF, 0.01 % β-mercaptoethanol and 1.0 mM EDTA) and centrifuged again. The cell pellet was stored at – 80 ^{o}C until the next step.

Preparation of crude extract was performed by resuspended cell pellet in 5 ml of cold extraction buffer (0.1 M potassium phosphate buffer, pH 7.4 containing 0.1 mM PMSF, 0.01 % β-mercaptoethanol and 1.0 mM EDTA) and then sonicated on ice. Unbroken cells and cell debris were removed by centrifugation at 17,500xg for 30 minutes. The supernatant was assayed for enzyme activity and protein concentration.

2.7.3 Enzyme activity assay

2.7.3.1 Oxidative deamination

The activity of PheDH for oxidative deamination of phenylalanine was spectrophotometrically assayed. Reaction mixture of 1 ml containing 200 μmol of glycine–KCl-KOH buffer, pH 11.0, 20 μmol of L-phenylalanine, 1 μmol of NAD^+, and the enzyme. Incubation was carried out at 30 ^{o}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 that catalyzes the formation of 1 μmol of NADH in 1 minute. Specific activity is expressed as units per milligram of protein.

2.7.3.2 Reductive amination

The activity of PheDH for reductive amination of phenylpyruvate was spectrophotometrically assayed. Reaction mixture of 1 ml comprised of 200 μmol of NH_4Cl-NH_4OH buffer, pH 9.0, 10 μmol of L-phenylpyruvate, 0.2 μmol of NADH, and the enzyme. Incubation was carried out at 30 ^{o}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.

2.7.4 Protein measurement

Protein concentration was determined by the method of Lowry *et al* (1956). The reaction mixture 6.1 ml containing 20-300 μg of protein, 100 μl of solution A , 5 ml of solution B was mixed and incubated at 30 ^{o}C for 10 minutes. After that, the solution mixture was incubated with 0.5 ml of solution C at room temperature for 20 minutes. The preparation of all solutions were described in Appendix C. The protein concentration was monitored by measuring the absorbance at 610 nm and calculated from the standard curve of protein standard (BSA).

2.8 Optimization for phenylalanine dehydrogenase gene expression

The transformants of *E. coli* BL21(DE3)pLysS were grown overnight at 37 ^{o}C in 5 ml of LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol (whereas in the medium for *E. coli* BL21(DE3) 34 μg/ml chloramphenicol was omitted). After that, 2.5 % of the cell culture was inoculated into 100 ml of the same medium and was cultured at 37 ^{o}C with shaking. When the turbidity of the culture at 600 nm had reached 0.6, the transformant was induced by IPTG at final concentration of 0 - 1.0 mM at various induction time : 0, 1, 2, 4, 8, 16, and 24 hours. The cells were harvested by centrifugation at 10,000xg for 10 minutes, then was washed with cold 0.85 % NaCl. After that, The cell pellet was washed once in cold extraction extraction buffer and centrifuged again. The cell pellet was stored at – 80 ^{o}C until the next step. In crude extract preparation, the cell pellet was resuspended in 5 ml of cold extraction buffer and then broken by sonication on ice. Unbroken cell and cell debris were removed by centrifugation at 17,500xg for 30 minutes. The supernatant was stored at 4 ^{o}C for enzyme and protein assays as described in 2.7.3 and 2.7.4, respectively.

2.9 Stability of phenylalanine dehydrogenase gene expression

The transformant was daily subcultured by streaking on LB plate contained 100 μg/ml ampicillin for 20 days. Then the 1^{st}, 5^{th}, 10^{th}, 15^{th}, and 20^{th} subcultured colonies were picked up to culture at the optimum condition obtained from 2.8 and assayed for enzyme activity and protein as described in 2.7.3 and 2.7.4, respectively.

2.10 Purification of phenylalanine dehydrogenase

2.10.1 Preparation of crude extract solution

The transformant was grown in 1 liter of LB medium at the optimum condition from 2.8. The cell cultivation, crude extract preparation, assay for activity and protein determination were performed as described in 2.7.2, 2.7.3, and 2.7.4, respectively.

2.10.2 Enzyme purification procedures

The crude extract from 2.10.1 was purified by the following steps. All operations were done at 4 ^{o}C. The buffer used in all steps was 10 mM potassium phosphate buffer, pH 7.4 containing 0.01 % β-mercaptoethanol and 1 mM EDTA.

2.10.2.1 Ammonium sulfate precipitation

The precipitation of crude extract was done by slowly adding solid ammonium sulfate to 50 % saturation with gentle stirring by magnetic stirrer. After 1 hour, the supernatant was collected by centrifugation at 17,500xg for 30 minutes and then adjusted to final concentration of 80 % saturation with solid ammonium sulfate. The solution was left for 1 hour on ice with continuous stirring and centrifuged again. The precipitate was dissolved in the buffer. The protein solution was dialyzed against 100 volumes of the buffer at least 4 hours for 3 times before determination of the

enzyme activity and protein concentration as described in 2.7.3 and 2.7.4, respectively.

2.10.2.2 DEAE-Toyopearl colum chromatography

DEAE-Toyopearl was activated by washing with 0.5 N NaOH for 2 times before rewashing by deionized water until pH was 8.0. The 50 ml of activated DEAE-Toyopearl was resuspended in the buffer and packed into column followed by equilibrating with the same buffer for 5 - 10 column volume at flow rate 1 ml/min. The protein solution from 2.10.2.1 was applied to DEAE-Toyopearl column. The unbound proteins were eluted from the column with the buffer. Normally, keep washing until the absorbance at 280 nm of eluent decreased to base line value. After that, the bounded proteins were eluted from the column with linear salt gradient of 0 to 0.5 M KCl in the buffer. The fraction of 3 ml were collected using fraction collector. The elution profile was monitored for protein by measuring the absorbance at 280 nm and the enzyme activity was determined as described in 2.7.3.1. The KCl concentration was investigated by measuring the conductivity. The active fractions were pooled. The protein solution was dialyzed against the buffer before determination of the enzyme activity and protein concentration as described in 2.7.3 and 2.7.4, respectively.

2.11 Polyacrylamide gel electrophoresis

The enzyme from each step of purification was analyzed by native PAGE and SDS-PAGE to determine the native protein and denature protein pattern, respectively.

2.11.1 Non-denaturing gel electrophoresis

Discontinuous PAGE was performed on the slab gel of a 7.7 % seperating gel and a 5 % stacking gel. Tris-glycine buffer, pH 8.3 (25 mM Tris and 192 mM glycine) was used as electrode buffer. Preparation of solution and polyacrylamide gels was described in Appendix F The enzyme was mixed with 5x sample buffer (312.5

mM Tris-HCl pH 6.8, 50 % glycerol and 0.05 % bromophenol blue) by ratio 5: 1 and loaded onto the gel. The electrophoresis was run from cathode towards anode at constant current (30mA). For activity staining, the experiment was done at 4 ^{o}C. After electrophoresis, the gel was developed by protein and activity staining.

2.11.1.1 Protein staining

The gel was transferred to a small box containing Coomassie staining solution (1 % Coomassie Blue R-250, 45 % methanol, and 10 % glacial acetic acid). The gel was agitated for 30 minutes on the shaker. The stain solution was poured out and the Coomassie destaining solution (10 % methanol and 10 % glacial acetic acid) was added. The gel was gently destained for several times until gel background was clear.

2.11.1.2 Activity staining

After electrophoresis at 4 ^{o}C, the gel was transferred to a small box containing activity staining solution (4.25 mmol of Tris-HCl, pH 8.5, 40 μmol of L-phenylalanine, 50 μmol of NAD^{+}, 25 μg/ml of phenazine methosulfate and 250 μg/ml of nitroblue tetrazolium) for 5 minutes at room temperature and then quickly rinsed several times with water until gel background was clear.

2.11.2 SDS-polyacrylamide gel electrophoresis

The SDS-PAGE system was performed according to the method of Bollag *et al.*, 1996. The slab gel system consisted of 0.1 % SDS (W/V) in 10 % seperating gel and 5 % stacking gel. Tris-glycine (25 mM Tris, 192 mM glycine and 0.1% SDS), pH 8.3 was used as electrode buffer. The gel preparartion was described in Appendix G**.** The enzyme was mixed with 5x sample buffer (60 mM Tris-HCl pH 6.8, 25 % glycerol, 2 % SDS, 0.1 % bromophenol blue and 14.4 mM β-mercaptoethanol) by ratio 5: 1 and boiled for 10 minutes before loading to the gel. The electrophoresis was run from cathode towards anode at constant current (30 mA)

at room temperature. The standard molecular weight markers were phosphorylase B (MW 97,000), bovine serum albumin (MW 66,000), ovalbumin (MW 45,000), carbonic anhydrase (MW 31,000), trypsin inhibitor (MW 20,100) and α-lactalbumin (MW 14,400). After electrophoresis, proteins in the gel were visualized by comassie blue staining.

2.12 Characterization of phenylalanine dehydrogenase

2.12.1 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). Substrate, L-phenylalanine was replaced by various amino acids and L-phenylalanine analogs for the assay reaction. In the same way, The ability of the enzyme to catalyze the reductive amination of various keto acids and phenylpyruvate analogs were determined at a final concentration of 10 mM. Substrate, phenylpyruvate was replaced by various keto acids and L-phenylpyruvate analogs for the assay reaction. The enzyme activities of oxidative deamination and reductive amination were determined as described in section 2.7.3.1 and 2.7.3.2, respectively. The result was expressed as a percentage of the relative activity. The highest activity was defined as 100 %.

2.12.2 Effect of temperature on phenylalanine dehydrogenase stability

The purified enzyme was preincubated in NH_4OH-NH_4Cl buffer, pH 9.5 at 30 ^oC before determining its activity as described in section 2.7.3. The enzyme was collected to assay for every 4 hours. The result was expressed a percentage of the relative activity. The highest activity was defined as 100 %.

2.13 Preliminary production of L-amino acids

Five hundreds microliter of reaction mixture comprised of 10 mM keto acids, 400 mM NH_4Cl-NH_4OH buffer (pH 9.5), 10 mM NADH and 5 U of PheDH. The keto acid substrates of each reaction are phenylpyruvate, α-ketocaproate, α-keto-γ-methiol-n-butyrate, α-ketovalerate, α-ketoisocaproate, α-ketoisovalerate, α-keto-n-butyrate, 3-methyl-2-oxovalerate and 4-methyl-2-oxovalerate. The reaction was initiated by the addition of the enzyme and allowed to proceed for 16 hours at 30 ^{o}C. After that, samples were neutralized to pH ~7.0 with 3.5 N HCl.

The products of enzyme reactions were firstly analyzed by the cellulose thin-layer chromatography (TLC) using n-butanol: glacial acetic acid: water = 4: 1: 1 as solvent and developed with 0.5 % ninhydrin solution (0.5 % ninhydrin in acetone: ethanol = 70: 30). After that, L-amino acids produced from the enzyme reactions were identified and their quantities were determined by using amino acid analyzer Model L8500A, Hitachi. Commercial L-phenylalanine, norleucine, methionine, norvaline, leucine, valine, α-aminobutyrate, and isoleucine were used as standards for identification and quantification. The yield of the enzyme reaction is defined as mole of product per mole of substrate.

CHAPTER III

RESULTS

3.1 Cloning of phenylalanine dehydrogenase gene

3.1.1 Chromosomal DNA template preparation

The chromosomal DNA was extracted from *Acinetobacter lwoffii*. It was determined for quality and quantity by agarose gel electrophoresis. High molecular weight DNA larger than 23.1 kb was obtained (Figure 3.1, lane 1). The DNA concentration was about 15 μg/1.5 ml cell culture. A_{260}/A_{280} ratio was in the range between 1.8-2.5 indicated high purity. Thus the quality of obtained DNA was suitable for molecular procedure such as restriction endonuclease digestion and template of PCR amplification. Figure 3.1, lane 2-4 shows the digested chromosomal DNA of *A. lwoffii.*

3.1.2 PCR amplification of phenylalanine dehydrogenase gene

To express *phedh* gene in *E. coli* under T7 promoter of expression vector, pET-17b. The *phedh* gene was amplified by using a pair of primers as described in section 2.6.3.1. The 5′-primer (PheDHpETF) comprised of *Nde*I restriction site and 5′-end of *phedh* gene. The 3′-primer (PheDHpETR) comprised of *Bam*HI site, 3′-end of *phedh* gene and the TAG translational termination signal. Figure 3.2 shows the 1.1 kb PCR product of the putative *phedh* gene fragment amplified from the various templates and annealing temperatures. *Bam*HI and *Hin*dIII digested DNA templates gave high amount of PCR product more than those of *Kpn*I digested DNA template at all of annealing temperature while the PCR product could not be detected when using undigested DNA template. Moreover, PCR product of *Bam*HI digested DNA template at 37.6 ^{o}C gave non-specific band (lane 4). In all digested DNA templates, the amount of PCR product decreased with the increasing of

Figure 3.1 Restriction enzyme digested chromosomal DNA of *Acinetobacter lwoffii*

Lane M = λ/*Hin*dIII standard DNA marker

Lane 1 = undigested chromosomal DNA

Lane 2 = chromosomal DNA digested with *Bam*HI

Lane 3 = chromosomal DNA digested with *Hind*III

Lane 4 = chromosomal DNA digested with *Kpn*I

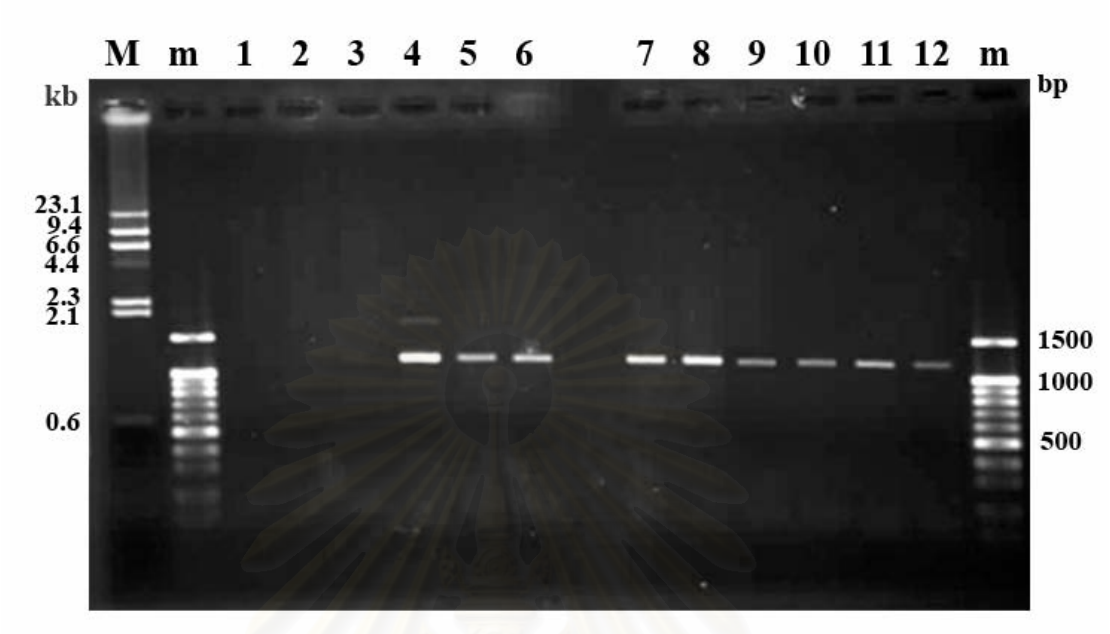


Figure 3.2 PCR product using various DNA templates and annealing temperatures

Lane M = λ/*Hin*dIII standard DNA marker

Lane m = 100 bp standard DNA marker

Lane 1* = PCR product using undigested DNA as template

Lane 2** = PCR product using undigested DNA as template

Lane 3*** = PCR product using undigested DNA as template

Lane 4* = PCR product using *Bam*HI digested DNA as template

Lane 5** = PCR product using *Bam*HI digested DNA as template

Lane 6*** = PCR product using *Bam*HI digested DNA as template

Lane 7* = PCR product using *Hin*dIII digested DNA as template

Lane 8** = PCR product using *Hin*dIII digested DNA as template

Lane 9*** = PCR product using *Hin*dIII digested DNA as template

Lane 10* = PCR product using *Kpn*I digested DNA as template

Lane 11** = PCR product using *Kpn*I digested DNA as template

Lane 12*** = PCR product using *Kpn*I digested DNA as template

* = PCR product using annealing temperature of 37.6 ^{o}C

** = PCR product using annealing temperature of 41.7 ^{o}C

*** = PCR product using annealing temperature of 44.9 ^{o}C

annealing temperature. Therefore, the PCR product of *Hin*dIII digested DNA template at 37.6 ^{o}C annealing temperature was used for further cloning because we could obtained high amount of putative *phedh* gene fragments without non-specific amplified DNA**.**

3.1.3 Transformation

The 1.1 kb amplified gene fragment was digested with *Nde*I and *Bam*HI, ligated with *Nde*I-*Bam*HI digested pET-17b vector, and then transformed into *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS by electroporation as described in 2.6.4.1, 2.6.4.2, 2.6.4.3 and 2.6.5.2, respectively. The recombinant plasmids were ramdomly picked for plasmid extraction and digestion with *Nde*I-*Bam*HI as described in 2.7.1. The recombinant plasmids (pALPheDH) in *E. coli* BL21(DE3) gave two bands, relaxed and supercoiled bands, on agarose gel electrophoresis. After digestion, a linear pET-17b with 3.3 kb and 1.1 kb of inserted *phedh* gene fragment were shown in Figure 3.3. The recombinant plasmids in *E. coli* BL21(DE3)pLysS gave three bands, relaxed and supercoiled of pET-17b as well as pLysS bands, on agarose gel. After digestion with *Nde*I and *Bam*HI, a linear pET-17b of 3.3 kb and 1.1 kb of inserted putative *phedh* gene fragment were obtained as shown in Figure 3.4**.** The inserted fragment size was the same as the PCR product.

3.2 Phenylalanine dehydrogenase activity of transformants

Six recombinant clones from each host (*E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS) were grown for enzyme assay as described in 2.7.2.. *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS with and without plasmid pET-17b were used as references. The result is shown in Table 3.1 and 3.2. The clones showed various levels of the specific activity from 0.81 – 4.46 units/mg protein. The highest specific activity with 55.75 fold higher than that of *A. lwoffii* was produced by *E. coli* BL21(DE3) transformant No. 4. Thus, this recombinant clone would be used for further studies.

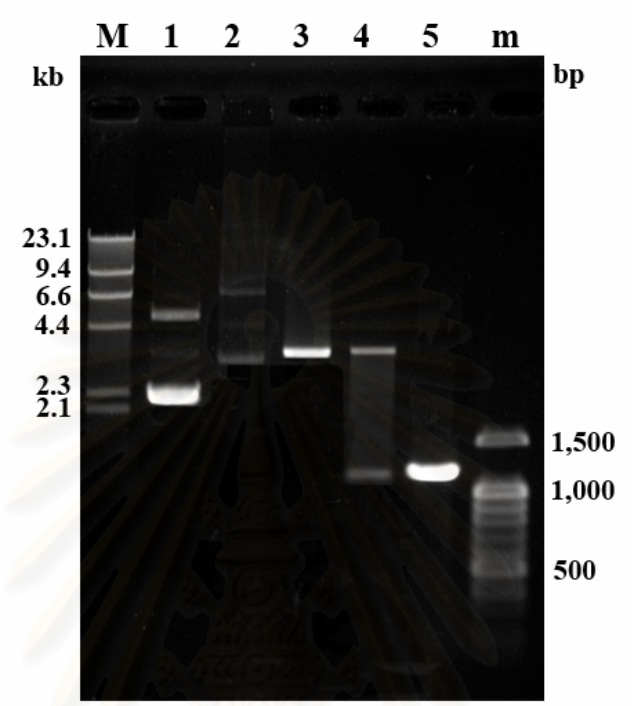


Figure 3.3 Restriction pattern of recombinant plasmid (pALPheDH) in *E. coli* BL21(DE3)

Lane M = λ/*Hin*dIII standard DNA marker

Lane 1 = undigested pET-17b

Lane 2 = undigested pALPheDH

Lane 3 = *Nde*I-*Bam*HI digested pET-17b

Lane 4 = *Nde*I-*Bam*HI digested pALPheDH

Lane 5 = PCR product of putative *phedh* gene

Lane m = 100 bp standard DNA marker

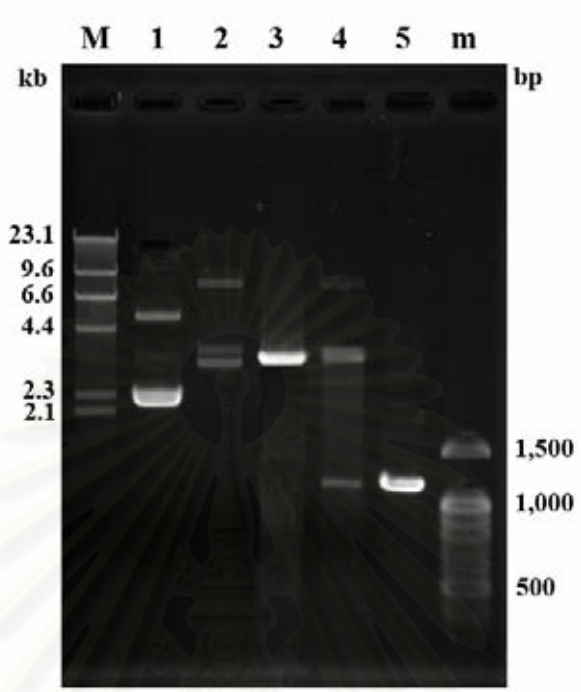


Figure 3.4 Restriction pattern of recombinant plasmid (pALPheDH) in *E. coli* BL21(DE3)pLysS

Lane M = λ/*Hin*dIII standard DNA marker

Lane 1 = undigested pET-17b

Lane 2 = undigested pALPheDH

Lane 3 = *Nde*I-*Bam*HI digested pET-17b

Lane 4 = *Nde*I-*Bam*HI digested pALPheDH

Lane 5 = PCR product of putative *pheDH* gene

Lane m = 100 bp standard DNA marker

Table 3.1 Phenylalanine dehydrogenase activity from crude extract of *E. coli* BL21(DE3) transformants[a]

Sources	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)
A. lwoffii	6.5	79.8	0.08
E. coli BL21(DE3)	0	87.9	0
E. coli BL21(DE3) harbouring pET-17b	0	101.0	0
Transformant No.1	346.5	145.0	2.39
Transformant No.2	282.0	144.2	1.96
Transformant No.3	507.5	165.5	3.07
Transformant No.4	822.0	184.3	4.46
Transformant No.5	358.0	152.9	2.34
Transformant No.6	205.0	142.9	1.43

[a] Crude extracts were prepared from 200 ml of cell culture.

Table 3.2 Phenylalanine dehydrogenase activity from crude extract of *E. coli* BL21(DE3)pLysS transformants[a]

Sources	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)
A. lwoffii	6.5	79.8	0.08
E. coli BL21(DE3) pLysS	0	81.0	0
E. coli BL21(DE3) pLysS harbouring pET-17b	0	94.0	0
Transformant No.1	111.8	112.5	0.99
Transformant No.2	105.0	128.9	0.81
Transformant No.3	105.0	114.2	0.92
Transformant No.4	145.0	115.4	1.26
Transformant No.5	96.0	111.0	0.86
Transformant No.6	120.0	125.2	0.96

[a] Crude extracts were prepared from 200 ml of cell culture.

3.3 Nucleotide sequence and deduced amino acid sequence of phenylalanine dehydrogenase gene

The inserted fragment in recombinant plasmid pALPheDH of *E. coli* BL21(DE3) transformant No 4 was sequenced. The result showed that it contained 1,143 bp open reading frame which encoded the polypeptide of 380 amino acid residues as shown in figure 3.5. The molecular weight of enzyme subunit was calculated from deduced amino acid sequence to be 41.5 kDa. The nucleotide sequence was compared with those in the EMBL-GenBank-DDBL database. It showed 82, 66, 64, 53, and 9% homology to *phedh* gene of *Bacillus sphaericus, Bacillus badius, Sporosarcina ureae, Thermoactinomyces intermedius, and Rhodococcus* sp. M4, respectively (figure 3.6). The percentage of identical amino acids of the enzyme compared with PheDH from *B. sphaericus, B. badius, S. ureae, T. intermedius, and Rhodococcus sp*. M4, were 88, 68, 62, 51, and 32%, respectively (Figure 3.7).

3.4 Optimization of phenylalanine dehydrogenase gene expression

3.4.1 Optimization of phenylalanine dehydrogenase gene expression

The *E. coli* BL21(DE3) transformant No. 4 which showed the highest PheDH activity had been grown and induced by IPTG at final concentration of 0, 0.2, 0.4, 0.6, 0.8 and 1 mM at various times (0, 1, 2, 4, 8, 16 and 24 hours) before cells were harvested as described in 2.8.1. The results are shown in Figure 3.8. When recombinant clone was cultured without IPTG induction, the expression of *phedh* gene was slightly increased until 16 hours with total activity of 220 U/100 ml culture after that expression of gene was decreased. In the case of transformant was induced by various final concentrations of IPTG, the expression was highest at induction time of 8 hours for all final concentration of IPTG after that activity of PheDH was decreased. The optimum condition for induction of *phedh* gene was 0.4 mM IPTG at 8 hours of induction, the obtained total activity of PheDH was about 560 U/100 ml culture.

```
atggcaaaacagcttgaaaagtcatcagtcggcaatgaggatatttttcaaaaaatagcg
 M  A  K  Q  L  E  K  S  S  V  G  N  E  D  I  F  Q  K  I  A
aatcacgaacaagtcgtcttctgcaatgatccggcgtctggtctgcaggcaatcattgct
 N  H  E  Q  V  V  F  C  N  D  P  A  S  G  L  Q  A  I  I  A
attcacgatacaacacttggtcctgcactgggcgggactcggatgtatccatataaaaat
 I  H  D  T  T  L  G  P  A  L  G  G  T  R  M  Y  P  Y  K  N
gtgaatgaggcacttgaagatgtgcttcgcctgtcagaaggaatgacatataaatgtgct
 V  N  E  A  L  E  D  V  L  R  L  S  E  G  M  T  Y  K  C  A
gctgctgatatcgatttcggcggcgggaaagctgtcattatcggcgatccggagaaggat
 A  A  D  I  D  F  G  G  G  K  A  V  I  I  G  D  P  E  K  D
aaatccccggcattattccgtgcatttggacaattcgtggactctctcaacggaaggttc
 K  S  P  A  L  F  R  A  F  G  Q  F  V  D  S  L  N  G  R  F
tatacaggtactgatatggggacaacaatggatgattttgtccatgcacagaaagagaca
 Y  T  G  T  D  M  G  T  T  M  D  D  F  V  H  A  Q  K  E  T
aaatttatcaacggtatcccagagcagtacggaggaagcggagactcctccattcctacg
 K  F  I  N  G  I  P  E  Q  Y  G  G  S  G  D  S  S  I  P  T
tccaaaggggtcgtctatgcgcttaaagcgacaaatcagtatttgtttggcagcgacagc
 S  K  G  V  V  Y  A  L  K  A  T  N  Q  Y  L  F  G  S  D  S
ctttcaggaaaaacatacgccatccaagggatgggcaaagtggggtataaggttgcggaa
 L  S  G  K  T  Y  A  I  Q  G  M  G  K  V  G  Y  K  V  A  E
cagctcctggaagcaggtgccgaattatttgtgaccgatatacatgaagatgtcctgaat
 Q  L  L  E  A  G  A  E  L  F  V  T  D  I  H  E  D  V  L  N
tcaatcaaggaaaaatcaaaagagatcggcggttcagtaaccgttgtaaaaagcgatgag
 S  I  K  E  K  S  K  E  I  G  G  S  V  T  V  V  K  S  D  E
atctatagtgtggaagcggatgtatttgttccttgtgcgatgggaggcgtgatcaatgat
 I  Y  S  V  E  A  D  V  F  V  P  C  A  M  G  G  V  I  N  D
gaaacgatcccaagattgaaagtgaaggccgtcgtcggatcagctaataatcagctcaaa
 E  T  I  P  R  L  K  V  K  A  V  V  G  S  A  N  N  Q  L  K
aatctctcccatgctgacgtactgaatgaaaaagggattctgtatgcacctgattacatc
 N  L  S  H  A  D  V  L  N  E  K  G  I  L  Y  A  P  D  Y  I
gtcaatgcaggaggattgatccaggttgccgacgaattatacggtccgaataaggagcgg
 V  N  A  G  G  L  I  Q  V  A  D  E  L  Y  G  P  N  K  E  R
gtattgctcaagacaaaaggaatttaccattctcttctggaaatttttcaacaggcagaa
 V  L  L  K  T  K  G  I  Y  H  S  L  L  E  I  F  Q  Q  A  E
cttgattgcgttactacggtggaagcggcaaacagaaaatgtcagaagacaattgaagat
 L  D  C  V  T  T  V  E  A  A  N  R  K  C  Q  K  T  I  E  D
cagcggaaccggaatagtttcttttctagaggccgcaggccgaaatggaataaacaagaa
 Q  R  N  R  N  S  F  F  S  R  G  R  R  P  K  W  N  K  Q  E
tag
```

Figure 3.5 Nucleotide sequence and the deduced amino acid sequence of phenylalanine dehydrogenase gene from *Acinetobacter lwoffii*

```
CLUSTAL W (1.82) multiple sequence alignment

A.lwo           ---ATGGCAAAACAGCTTGAAAAGTCATCAG---TCGGCAATGAGGATATTTTTCAAAAA 54
B.sph           ---ATGGCAAAACAGCTTGAAAAGTCATCAAAAATTGGTAATGAGGACGTTTTTCAAAAA 57
S.ure           ATGATTTTGGTAACTTTAGAACAGACTTTACAAGACGACAAGGCAAGTGTTTTGGATAAA 60
B.bad           ------ATGAGCTTAGTAGAAAAAACATCCATCATAAAAGATTTCACTCTTTTTGAAAAA 54
T.int           ---------------------------------ATGCGCGACGTGTTTGAAATGATGGAC 27
R.sp            ----------------------------------------ATGAGTATCGACAGCGCACT 20
                                                       *

A.lwo           ATAGCGAATCACGAACA-AGTCGTCTTCTGCAATGATCCGGCGTCTGGTCTGCAGGCAAT 113
B.sph           ATAGCGAATCACGAGCA-GATTGTGTTCTGTAATGATCCGGTATCCGGCCTGCAAGCTAT 116
S.ure           ATGGTCGAGCATGAACA-AATTCTATTTTGTCATGATAAAGCAACCGGTCTTCAAGCCAT 119
B.bad           ATGTCTGAACATGAACA-AGTTGTTTTTTGCAACGATCCGGCGACAGGACTAAGGGCCAT 113
T.int           CGCTATGGCCACGAGCA-GGTCATTTTTTGCCGTCATCCGCAAACCGGTCTCAAAGCGAT 86
R.sp            GAACTGGGACGGGGAAATGACGGTCACCCGATTCGACCGGGAGACTGGTGCCCATTTCGT 80
                       *  *   *     *     *     *       * **           *

A.lwo           CATTGCTATTCACGATACAACACTTGGTCCTGCACTGGGCGGGACTCGGATGTATCCATA 173
B.sph           CATTGCTATCCACGATACAACCCTAGGCCCCGCTTTAGGTGGAACTCGCATGTATCCCTA 176
S.ure           CATTGCAGTCCACGATACGACTATGGGACCTGCACTCGGTGGATGTCGCATGGCGCCTTA 179
B.bad           TATCGCTATTCATGACACCACACTCGGACCTGCGCTCGGCGGCTGCCGCATGCAGCCTTA 173
T.int           CATCGCCTTGCATAATACAACCGCGGGGCCGGCTTTGGGTGGATGCCGCATGATCCCGTA 146
R.sp            CATTCGACTCGATTCGACCCAACTCGGACCGGCGGCCGGAGGCACCAGAGCCGCACAGTA 140
                 **     *  *   **       ** ** **    ** **     *       *  **

A.lwo           TAAAAATGTGAATGAGGCACTTGAAGATGTGCTTCGCCTGTCAGAAGGAATGACATATAA 233
B.sph           TAAAAATGTGGATGAAGCTCTGGAAGATGTGCTTCGCCTGTCAGAAGGAATGACGTATAA 236
S.ure           TAAAACGATGGATCTCGCATTAAAAGATGTTCTTCGCCTTTCAAAAGGGATGACATATAA 239
B.bad           TAACAGTGTGGAAGAAGCATTGGAAGATGCTCTTCGCCTTTCCAAAGGAATGACTTACAA 233
T.int           TGCTTCGACGGACGAAGCCTTGGAGGATGTTTTGCGGTTGTCCAAAGGCATGACCTATAA 206
R.sp            CTCACAGCTGGCGGACGCCCTCACCGACGCCGGCAAATTGGCGGGGGCGATGACGTTGAA 200
                       *    **  *    ** *       *  *    *  ***** *  **
```

(continue)

Figure 3.6 Linear alignment of the nucleotide sequence of phenylalanine dehydrogenase gene from various sources

A.lwo = *Acinetobacter lwoffii* , B.sph = *Bacillus sphaericus*, S.ure = *Sporosarcina* ureae, B.bad = *Bacillus badius*, T.int = *Thermoactinomyces intermedius* and R.sp = *Rhodococcus* sp.

```
A.lwo          ATGTGCTGCTGCTGATATCGATTTCGGCGGCGGGAAAGCTGTCATTAT-----CGGCGAT 288
B.sph          ATGCGCAGCCGCCGATATCGATTTCGGCGGCGGGAAGGCGGTCATTAT-----CGGAGAT 291
S.ure          ATGTGCGGCAGCTGATGTAGACTTTGGCGGCGGAAAATCCGTCATCAT-----CGGAGAC 294
B.bad          ATGCGCGGCGTCCGATGTCGACTTTGGCGGCGGAAAAGCAGTCATTAT-----CGGTGAT 288
T.int          ATGCAGTCTGGCGGATGTGGACTTTGGCGGGGGAAAAATGGTTATCAT-----CGGCGAT 261
R.sp           GATGGCAGTGAGCAACCTTCCGATGGGCGGGGGCAAATCCGTCATTGCGCTTCCTGCGCC 260
                             *  *     * ***** ** **    ** **        * * *

A.lwo          CCGGAGAAGGATAAATC-------CCCGGCAT---TATTCCGTGCATTTGGACA-TTCGT 337
B.sph          CCAGAAAAGGATAAATC-------TCCGGCAT---TGTTCCGTGCATTTGGTCAATTTGT 341
S.ure          CCGCTAAAAGATAAAAC-------GCCTGAGA---AATTCCGTGCTTTCGGTCAATTCAT 344
B.bad          CCGCAGAAAGATAAATC-------TCCAGAAC---TGTTCCGCGCGTTTGGCCAATTTGT 338
T.int          CCGAAAAAAGATAAATC-------GCCGGAGT---TGTTTCGCGTGATCGGCCGTTTTGT 311
R.sp           GCGTCATTCGATCGATCCGAGCACGTGGGCACGCATCCTCCGAATCCACGCCGAGAACAT 320
                *       ***  * *          *        * **       *         *

A.lwo          GGACTCTCTCAACGGAAGGTTCTATACAGGTACTGATATGGGGACAACAATGGATGATTT 397
B.sph          GGAATCACTGAATGGACGATTTTACACAGGTACTGACATGGGGACCACGATGGATGATTT 401
S.ure          CGAATCATTGAACGGACGCTTCTATACAGGTACAGACATGGGCACAACGCTTGAAGACTT 404
B.bad          TGATTCGCTTGGCGGCCGTTTCTATACAGGTACTGATATGGGAACGAATATGGAAGATTT 398
T.int          GGGCGGGTTAAACGGCCGTTTCTATACCGGAACCGACATGGGAACCAATCCGGAAGATTT 371
R.sp           CGACAAGTTGTCCGGCAACTACTGGACCGGACCGGACGTCAACACCAATTCGGCAGACAT 380
                *      *    **    *  *  ** **  * **  *    ** *     *  **  *

A.lwo          T-TCCATGCACAGAAAGAGACAAAATTTATCAACGGTATCCCAGAGCAGTACGGAGGAAG 456
B.sph          TGTCCATGCACAGAAAGAGACGAATTTCATTAACGGAATTCCTGAGCAGTATGGTGGAAG 461
S.ure          TGTGCATGCCATGAAAGAAACAAACTACATCGTGGGCAAGCCGGTCGAATATGGTGGCGG 464
B.bad          CATTCACGCCATGAAAGAAACAAACTGCATTGTTGGGGTGCCGGAAGCTTACGGCGGCGG 458
T.int          TGTCCATGCCGCCAGGGAATCGAAATCTTTTGCCGGATTGCCGAAATCGTACGGCGGAAA 431
R.sp           GGATACTCTGAACGACACCACCGAGTTCGTGTTCGGACGGTCGCTCGAACGCGGCGGCGC 440
                              *  * *   *    **     *          ** **
```

(continue)

Figure 3.6 Linear alignment of the nucleotide sequence of phenylalanine dehydrogenase gene from various sources

A.lwo = *Acinetobacter lwoffii*, B.sph = *Bacillus sphaericus*, S.ure = *Sporosarcina* ureae, B.bad = *Bacillus badius*, T.int = *Thermoactinomyces intermedius* and R.sp = *Rhodococcus* sp.

```
A.lwo          CGGAGACTCCTCCATTCCTACGTCCAAAGGGGTCGTCTATGCGCTTAAAGCGACAAATCA 516
B.sph          CGGCGACTCGTCGATTCCGACCGCCCAGGGAGTCATTTATGCACTGAAGGCTACAAACCA 521
S.ure          TGGAGACTCATCGATCCCTACTGCACTCGGAGTCTTCTATGGCATTAAAGCGACAAACCA 524
B.bad          CGGAGATTCCTCTATTCCAACTGCCATGGGTGTCCTGTACGGCATTAAAGCAACCAACAA 518
T.int          GGGGGACACATCCATTCCCACCGCGCTCGGGGTGTTTCACGGAATGCGGGCCACCGCCCG 491
R.sp           GGGTTCGAGCGCGTTCACCACCGCCGTTGGCGTGTTCGAGGCGATGAAGGCGACCGTCGC 500
                **        *  *  * **  *    ** **  *  * *   *    ** **

A.lwo          GTATTTGTTTGGCAGCGACAGCCTTTCAGGAAAAACATACGCCATCCAAGGGATGGGCAA 576
B.sph          GTATTTATTTGGAAGCGATAGCCTTTCAGGTAAAACATATGCTATTCAAGGGCTGGGAAA 581
S.ure          GAATCTGTTTGGCGACGACAAAGTAGAAGGCCGAAAATACAGTATCCAAGGTCTTGGGAA 584
B.bad          AATGTTGTTTGGCAAGGACGATCTTGGCGGCGTCACTTATGCCATTCAAGGACTTGGCAA 578
T.int          GTTTTTATGGGGGACGGATCAGCTGAAAGGGCGTGTGGTTGCCATCCAAGGAGTCGGCAA 551
R.sp           GCACC---GTGGGCTGGGCTCACTCGACGGTTTGACGGTCCTGGTCCAAGGACTGGGGGC 557
                      **    *     *    **             * *****  * **

A.lwo          AGTGGGGTATAAGGTTGCGGAACAGCTCCTGGAAGCAGGTGCCGAATTATTTGTGACCGA 636
B.sph          AGTAGGGTATAAAGTAGCGGAACAGCTCTTAAAAGCCGGCGCCGATTTATTTGTAACGGA 641
S.ure          AGTAGGTTACAAAGTAGCTGAACATATTATCAACGAAGGTGGAAACGTGATCGTCACAGA 644
B.bad          AGTAGGCTACAAAGTAGCGGAAGGGCTGCTCGAAGAAGGTGCTCATTTATTTGTAACGGA 638
T.int          GGTGGGAGAGCGCTTGTTGCAGCTTTTGGTCGAAGTGGGGGCTTACTGCAAAATTGCCGA 611
R.sp           AGTCGGAGGATCATTGGCATCCCTGGCCGCCGAAGCGGGTGCGCAACTCCTGGTGGCAGA 617
                ** **       *                * *  ** *   *        *  * **

A.lwo          TATACAT---GAAGATGTCCTGAATTCAATCAAGGAAAAATCAAAAGAGATCGGCGGTTC 693
B.sph          TATACAT---GAAAATGTCCTCAATTCCATTAAGCAAAAATCAGAAGAGCTTGGCGGTTC 698
S.ure          TATTAAT---GAGCAAGC---GATTGCAGATATTCAGAAGCTCGG------TGGAAGCGC 692
B.bad          TATTAAC---GAGCAAACGTTGGAGGCTATCCAGGAAAAAGCAAAAACAACATCCGGTTC 695
T.int          CATCGAT---TCG-GTGCGATGCGAACAGCTGAAAGAAAAGTA--------TGGCGACAA 659
R.sp           CACCGACACCGAGCGAGTAGCGCACGCTGTTGCGTTG----------------GGCCA 659
                *   *                   *
```

(continue)

Figure 3.6 Linear alignment of the nucleotide sequence of phenylalanine dehydrogenase gene from various sources

A.lwo = *Acinetobacter lwoffii*, B.sph = *Bacillus sphaericus*, S.ure = *Sporosarcina* ureae, B.bad = *Bacillus badius*, T.int = *Thermoactinomyces intermedius* and R.sp = *Rhodococcus* sp.

```
A.lwo          AGTAACCGTTGTAAAAAGCGATGAGATCTATAGTGTGGAAGCGGATGTATTTGTTCCTTG 753
B.sph          AGTGACCATTGTAAAAAGTGACGATATTTACAGCGTACAAGCGGATATATTTGTTCCGTG 758
S.ure          TGTCAGGGTCGTATCAAGTGAGGAGATTTACAGTCAGCAAGCAGATGTTTTTGTTCCTTG 752
B.bad          TGTCACGGTAGTAGCGAGCGATGAAATTTATTCCCAGGAAGCCGATGTGTTCGTTCCGTG 755
T.int          GGTCCAATTGGTGGATGTGAACCGGATTCACAAGGAGAGTTGCGATATTTTCTCGCCTTG 719
R.sp           CACAGCGGTTGCCCTCGAGGACGTTCTGTCCACCCCGTGT---GATGTCTTCGCACCCTG 716
                       * *         *     *               *** * **    ** **

A.lwo          TGCGATGGGAGGCGTGATCAATGATGAAACGATCCCAAGATTGAAAGTGAAGGCCGTCGT 813
B.sph          TGCGATGGGTGGTATTATCAATGATAAAACCATTCCTAAGTTAAAGGTGAAGGCTGTTGT 818
S.ure          TGCATTTGGTGGCGTGATCAATGACGACACGCTAAAGGTGCTGAAAGTACGAGGAATCTC 812
B.bad          TGCATTTGGCGGCGTTGTTAATGATGAAACGATGAAGCAGTTCAAGGTGAAAGCAATCGC 815
T.int          CGCCAAAGGCGGCGTGGTCAATGATGACACCATTGACGAGTTCCGTTGCCTGGCCATTGT 779
R.sp           CGCAATGGGCGGCGTCATCACCACCGAGGTGGCGCGAACACTCGACTGTTCCGTCGTGGC 776
                **    ** **  *  * *     *               *          *   *

A.lwo          CGGATCAGCTAATAATCAGCTCAAAAATCTCTCCCATGCTGACGTACTGAATGAAAAAGG 873
B.sph          GGGATCAGCCAATAACCAGCTCAAAGACCTCCGCCATGCAAATGTACTAAACGAAAAGGG 878
S.ure          CGGTTCAGCAAACAATCAGCTCGCGGAAAGCCGCCATGGAGAGCTACTACGTGAAAAGGG 872
B.bad          CGGTTCAGCCAACAATCAGCTGCTTACGGAGGATCACGGCAGACACCTTGCAGACAAAGG 875
T.int          CGGATCCGCCAACAACCAACTGGTGGAAGACCGGCATGGGGCACTGCTTCAAAAACGGAG 839
R.sp           CGGTGCCGCCAACAACGTCATCGCCGACGAGGCCGCCTCGGACATCCTGCACGCACGCGG 836
                **  * ** ** **     *                     **          *

A.lwo          GATTCTGTATGCACCTGATTACATCGTCAATGCAGGAGGATTGATCCAGGTTGCCGAC-- 931
B.sph          AATTCTATATGCACCCGATTATATCGTCAATGCCGGCGGCTTGATCCAGGTTGCTGAC-- 936
S.ure          TATTTTGTACGCACCAGACTATATCGTCAACGGCGGCGGTTTAATCCAAGTGGCGGAT-- 930
B.bad          CATTCTGTATGCTCCGGATTATATTGTTAACTCTGGCGGTCTGATCCAAGTAGCCGAC-- 933
T.int          CATTTGTTATGCACCCGATTATCTGGTGAATGCCGGCGGGCTGATTCAAGTGGCTGAT-- 897
R.sp           AATTCTGTACGCTCCCGACTTCGTGGCCAACGCCGGCGGTGCCATCCACCTCGTAGGCCG 896
                ***   ** ** ** ** *   * *  **    ** **    ** **  * *  *
```

(continue)

Figure 3.6 Linear alignment of the nucleotide sequence of phenylalanine dehydrogenase gene from various sources

A.lwo = *Acinetobacter lwoffii*, B.sph = *Bacillus sphaericus*, S.ure = *Sporosarcina* ureae, B.bad = *Bacillus badius*, T.int = *Thermoactinomyces intermedius* and R.sp = *Rhodococcus* sp.

```
A.lwo          -GAATTATACGGTCCGAATAAGGAGCGGGTATTGCTCAAGACAAAAGGAATTTACCATTC 990
B.sph          -GAACTTTATGGGCCGAATAAAGAGCGGGTCTTGCTCAAAACGAAAGAAATTTACCGTTC 995
S.ure          -GAATTGTACGGAACGAATCCTGCACGTGTACTCGCTAAAACTGAAAACATCTATACCTC 989
B.bad          -GAATTGTATGAGGTGAACAAAGAACGCGTGCTTGCGAAGACGAAGCATATTTACGACGC 992
T.int          -GAACTGGAAGGCTTCCATGAAGAGAGAGTGCTCGCCAAAACCGAAGCGATTTATGACAT 956
R.sp           GGAGGTTCTCGGTTGGTCCGAGTCGGTTGTCCACGAACGAGCAGTTGCCATAGGCGACAC 956
                **  *    *                **          *       **

A.lwo          TCTTCTGGAAATTTTTCAACAGGCAGAACTTGATTGCGTTACTACGGTGGAAGCGGCAAA 1050
B.sph          TCTGCTTGAAATTTTTAATCAGGCAGCCCTTGACTGCATCACAACAGTGGAGGCCGCAAA 1055
S.ure          ACTGCTTGAAGTATTCCATCAGGCAGAACAGGATCATATGACAACTGCCACTGCCGCAGA 1049
B.bad          AATTCTTGAAGTGTACCAGCAAGCGGAATTAGATCAAATCACCACAATGGAAGCAGCCAA 1052
T.int          GGTCCTGGATATTTTTCACCGGGCGAAAAATGAGAATATTACCACTTGTGAGGCAGCGGA 1016
R.sp           CCTGAATCAGGTCTTCGAGATCTCCGACAACGACGGCGTCACCCCGGACGAGGCCGCCCG 1016
                 *     *  * *   *     *       **     * **  *       ** **

A.lwo          CAGAAAATGTCAGAAG-ACAATTGAAGATCAGCGGAACCGGAATAGTTTCTTTTCTAGAG 1109
B.sph          TAGGAAGTGTCAAAAG-ACGATTGAGGGCCAGCAAACCCGTAATAGTTTCTTTTCTAGGG 1114
S.ure          CCGTATGTGTGAAAAG-CGTATTGCGGATGCCAAGAATCGCAACAGCTTCTTCACACAGT 1108
B.bad          CAGAATGTGTGAGCAA-AGAATGGCGGCAAGAGGCCGACGCAACAGCTTCTTTACTTCTT 1111
T.int          CCGGAT-CGTGATGGA-GCGTTTGAAAAAGTTAACCGATATTCGCCGGATCTTGTTGGAG 1074
R.sp           CACTCTCGCTGGACGGCGCGCCCGCGAGGCCTCGACAACGACAGCGACTGCCTAG----- 1071
                        *           *

A.lwo          GCCGCAGGCCGAAATGGAATAAACAAGAATAG 1141
B.sph          GACGCAGGCCGAAGTGGAACATAAAAGAGTAA 1146
S.ure          CAAACCGACCGAAATGGAATTTTCATCAGTAA 1140
B.bad          CTGTTAAGCCAAAATGGGATATTCGCAACTAA 1143
T.int          GATCCCCGCAACAGCGCAA-----GGAGGTAA 1101
R.sp           --------------------------------
```

Figure 3.6 Linear alignment of the nucleotide sequence of phenylalanine dehydrogenase gene from various sources

A.lwo = *Acinetobacter lwoffii*, B.sph = *Bacillus sphaericus*, S.ure = *Sporosarcina* ureae, B.bad = *Bacillus badius*, T.int = *Thermoactinomyces intermedius* and R.sp = *Rhodococcus* sp.

```
CLUSTAL W (1.82) multiple sequence alignment

A.lwo           -MAKQLEKSS-VGNEDIFQKIANHEQVVFCNDPASGLQAIIAIHDTTLGPALGGTRMYPY 58
B.sph           -MAKQLEKSSKIGNEDVFQKIANHEQIVFCNDPVSGLQAIIAIHDTTLGPALGGTRMYPY 59
B.bad           --MSLVEKTSIIKDFTLFEKMSEHEQVVFCNDPATGLRAIIAIHDTTLGPALGGCRMQPY 58
S.ure           MILVTLEQTLQDDKASVLDKMVEHEQILFCHDKATGLQAIIAVHDTTMGPALGGCRMAPY 60
T.int           ----------MRDVFEMMDRYG-HEQVIFCRHPQTGLKAIIALHNTTAGPALGGCRMIPY 49
R.sp            -----------MSIDSALNWDG--EMTVTRFDRETGAHFVIRLDSTQLGPAAGGTRAAQY 47
                                ::     *  :   .  :* : :* :..*  *** ** *   *

A.lwo           KNVNEALEDVLRLSEGMTYKCAAADIDFGGGKAVIIG-DPEKDKSP----ALFRAFGQFV 113
B.sph           KNVDEALEDVLRLSEGMTYKCAAADIDFGGGKAVIIG-DPEKDKSP----ALFRAFGQFV 114
B.bad           NSVEEALEDALRLSKGMTYKCAASDVDFGGGKAVIIG-DPQKDKSP----ELFRAFGQFV 113
S.ure           KTMDLALKDVLRLSKGMTYKCAAADVDFGGGKSVIIG-DPLKDKTP----EKFRAFGQFI 115
T.int           ASTDEALEDVLRLSKGMTYKCSLADVDFGGGKMVIIG-DPKKDKSP----ELFRVIGRFV 104
R.sp            SQLADALTDAGKLAGAMTLKMAVSNLPMGGGKSVIALPAPRHSIDPSTWARILRIHAENI 107
                     ** *. :*: .** * : ::: :**** **    * :.  *      :*  .. :

A.lwo           DSLNGRFYTGTDMGTTMDDFVHAQKETKFINGIPEQYGGSGDSSIPTSKGVVYALKATNQ 173
B.sph           ESLNGRFYTGTDMGTTMDDFVHAQKETNFINGIPEQYGGSGDSSIPTAQGVIYALKATNQ 174
B.bad           DSLGGRFYTGTDMGTNMEDFIHAMKETNCIVGVPEAYGGGGDSSIPTAMGVLYGIKATNK 173
S.ure           ESLNGRFYTGTDMGTTLEDFVHAMKETNYIVGKPVEYGGGGDSSIPTALGVFYGIKATNQ 175
T.int           GGLNGRFYTGTDMGTNPEDFVHAARESKSFAGLPKSYGGKGDTSIPTALGVFHGMRATAR 164
R.sp            DKLSGNYWTGPDVNTNSADMDTLNDTTEFVFGRSLERGGAGSSAFTTAVGVFEAMKATVA 167
                  *.*.::**.*:.*.  *:      :: . * .   ** *.::::.*: **. .::**

A.lwo           YLFGSDSLSGKTYAIQGMGKVGYKVAEQLLEAGAELFVTDIHEDVLNSIKEKSKEIGGSV 233
B.sph           YLFGSDSLSGKTYAIQGLGKVGYKVAEQLLKAGADLFVTDIHENVLNSIKQKSEELGGSV 234
B.bad           MLFGKDDLGGVTYAIQGLGKVGYKVAEGLLEEGAHLFVTDINEQTLEAIQEKAKTTSGSV 233
S.ure           NLFGDDKVEGRKYSIQGLGKVGYKVAEHIINEGGNVIVTDINEQAIADIQKLG---GSAV 232
T.int           FLWGTDQLKGRVVAIQGVGKVGERLLQLLVEVGAYCKIADIDSVRCEQLKEKY---GDKV 221
R.sp            HR-GLGSLDGLTVLVQGLGAVGGSLASLAAEAGAQLLVADTDTERVAHAVALG------H 220
                   * ..: *    :**:* **  : .   : *.   ::* .
```

(continue)

Figure 3.7 Linear alignment of the deduced amino acid sequence of phenylalanine dehydrogenase gene from various sources

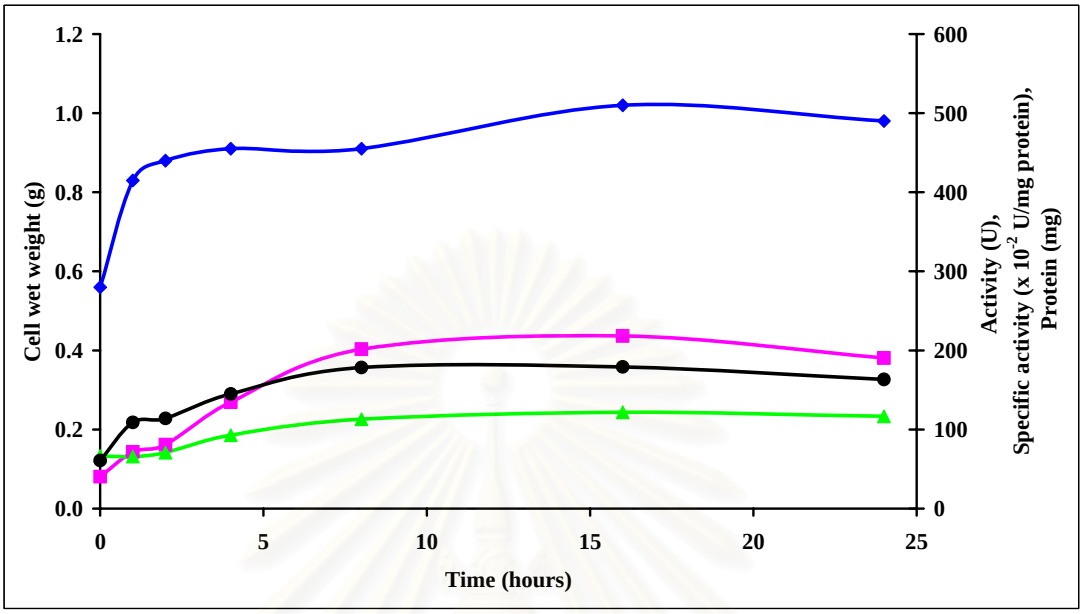
A.lwo = *Acinetobacter lwoffii*, B.sph = *Bacillus sphaericus*, B.bad = *Bacillus badius*, S.ure = *Sporosarcina* ureae, T.int = *Thermoactinomyces intermedius* and R.sp = *Rhodococcus* sp.

```
A.lwo           TVVKSDEIYSVEADVFVPCAMGGVINDETIPRLKVKAVVGSANNQLKNLSHADVLNEKGI 293
B.sph           TIVKSDDIYSVQADIFVPCAMGGIINDKTIPKLKVKAVVGSANNQLKDLRHANVLNEKGI 294
B.bad           TVVASDEIYSQEADVFVPCAFGGVVNDETMKQFKVKAIAGSANNQLLTEDHGRHLADKGI 293
S.ure           RVVSSEEIYSQQADVFVPCAFGGVINDDTLKVLKVRGISGSANNQLAESRHGELLREKGI 292
T.int           QLVDVNRIHKESCDIFSPCAKGGVVNDDTIDEFRCLAIVGSANNQLVEDRHGALLQKRSI 281
R.sp            TAVALEDVLSTPCDVFAPCAMGGVITTEVARTLDCSVVAGAANNVIADEAASDILHARGI 280
                  *  : : .  .*:* *** **::. ..   :    : *:*** :     .  *  :.*

A.lwo           LYAPDYIVNAGGLIQVAD-ELYGPNKERVLLKTKGIYHSLLEIFQQAELDCVTTVEAANR 352
B.sph           LYAPDYIVNAGGLIQVAD-ELYGPNKERVLLKTKEIYRSLLEIFNQAALDCITTVEAANR 353
B.bad           LYAPDYIVNSGGLIQVAD-ELYEVNKERVLAKTKHIYDAILEVYQQAELDQITTMEAANR 352
S.ure           LYAPDYIVNGGGLIQVAD-ELYGTNPARVLAKTENIYTSLLEVFHQAEQDHMTTATAADR 351
T.int           CYAPDYLVNAGGLIQVAD-ELEGFHEERVLAKTEAIYDMVLDIFHRAKNENITTCEAADR 340
R.sp            LYAPDFVANAGGAIHLVGREVLGWSESVVHERAVAIGDTLNQVFEISDNDGVTPDEAART 340
                 ****::.*.** *::.. *:       *  ::  *   : :::. :  : :*.  **

A.lwo           KCQKTIEDQRNRNSFFSRGRRPKWNKQE 380
B.sph           KCQKTIEGQQTRNSFFSRGRRPKWNIKE 381
B.bad           MCEQRMAARGRRNSFFTSSVKPKWDIRN 380
S.ure           MCEKRIADAKNRNSFFTQSNRPKWNFHQ 379
T.int           IVMERLKKLTDIRRILLED--PRNSARR 366
R.sp            LAGRRAREASTTTATA------------ 356
```

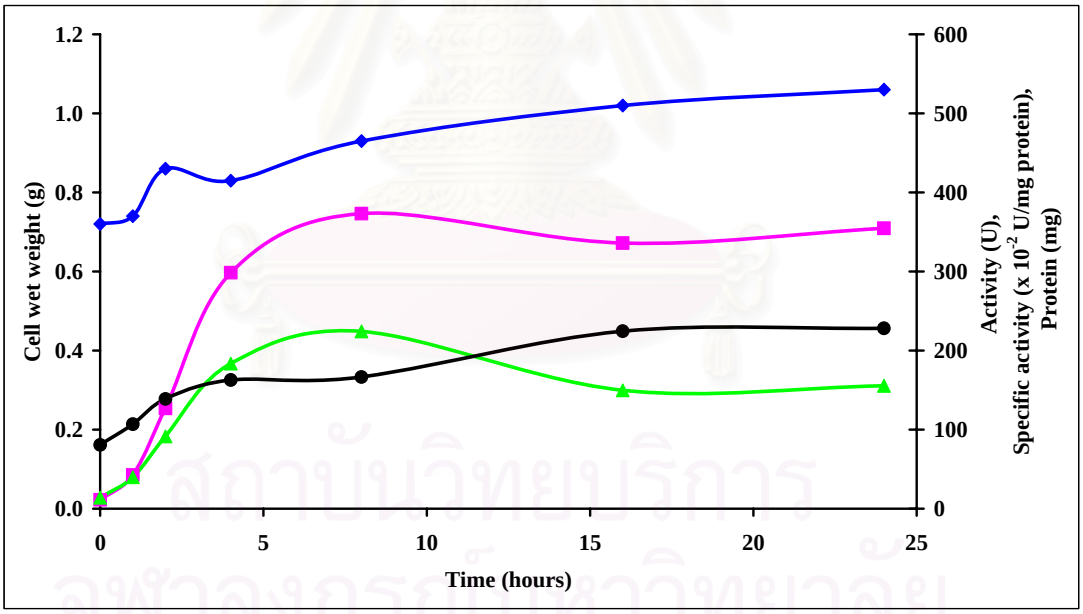
Figure 3.7 Linear alignment of the deduced amino acid sequence of phenylalanine dehydrogenase gene from various sources

A.lwo = *Acinetobacter lwoffii*, B.sph = *Bacillus sphaericus*, B.bad = *Bacillus badius*, S.ure = *Sporosarcina* ureae, T.int = *Thermoactinomyces intermedius* and R.sp = *Rhodococcus* sp.

0 mM IPTG



0.2 mM IPTG



(continue)

Figure 3.8 Expression of phenylalanine dehydrogenase gene in *E. coli* BL21(DE3) at various final concentrations of IPTG

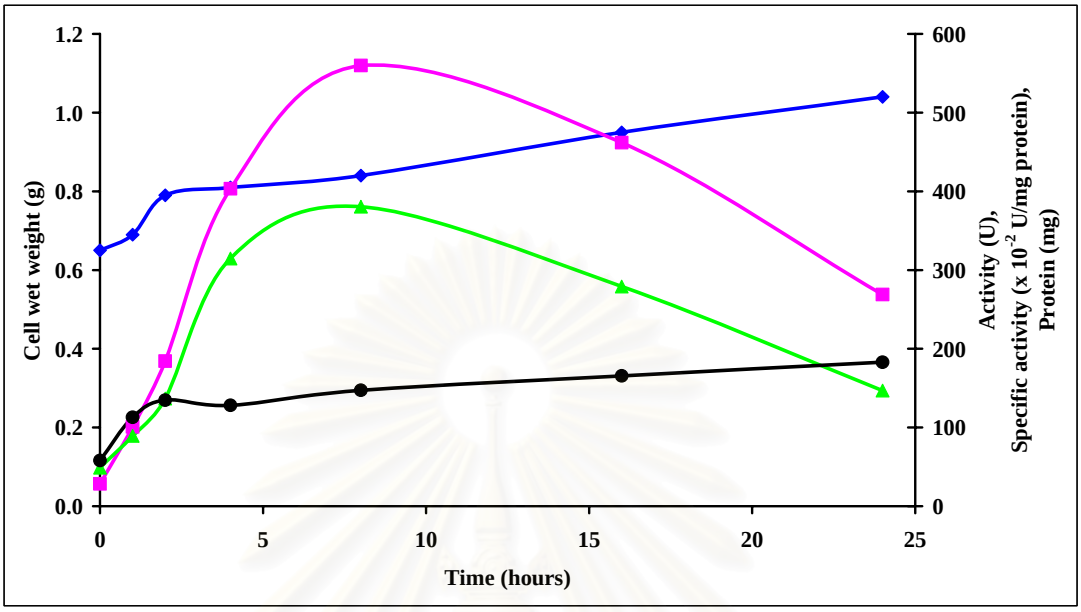
◆ = cell wet weight

■ = total acitivity

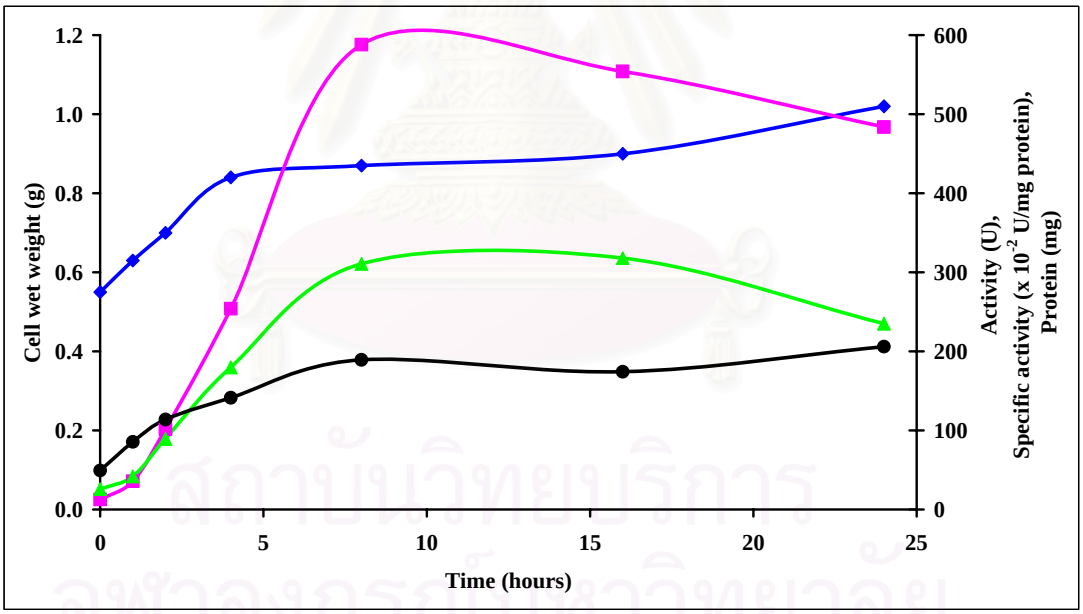
▲ = specific activity

● = protein

0.4 mM IPTG



0.6 mM IPTG



(continue)

Figure 3.8 Expression of phenylalanine dehydrogenase gene in *E. coli* BL21(DE3) at various final concentrations of IPTG

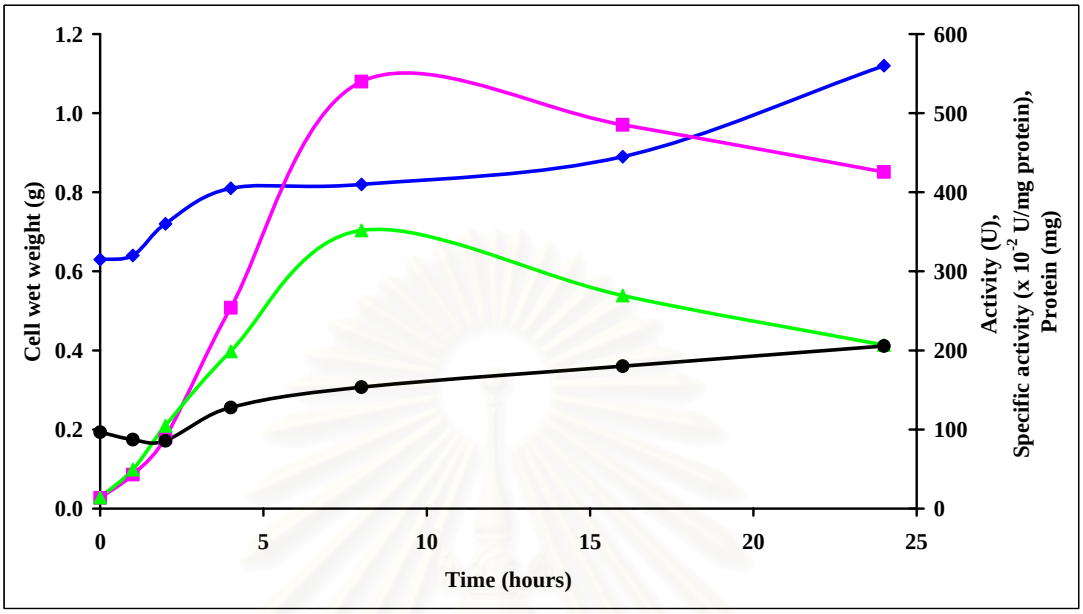
◆ = cell wet weight

■ = total acitivity

▲ = specific activity

● = protein

0.8 mM IPTG



1.0 mM IPTG

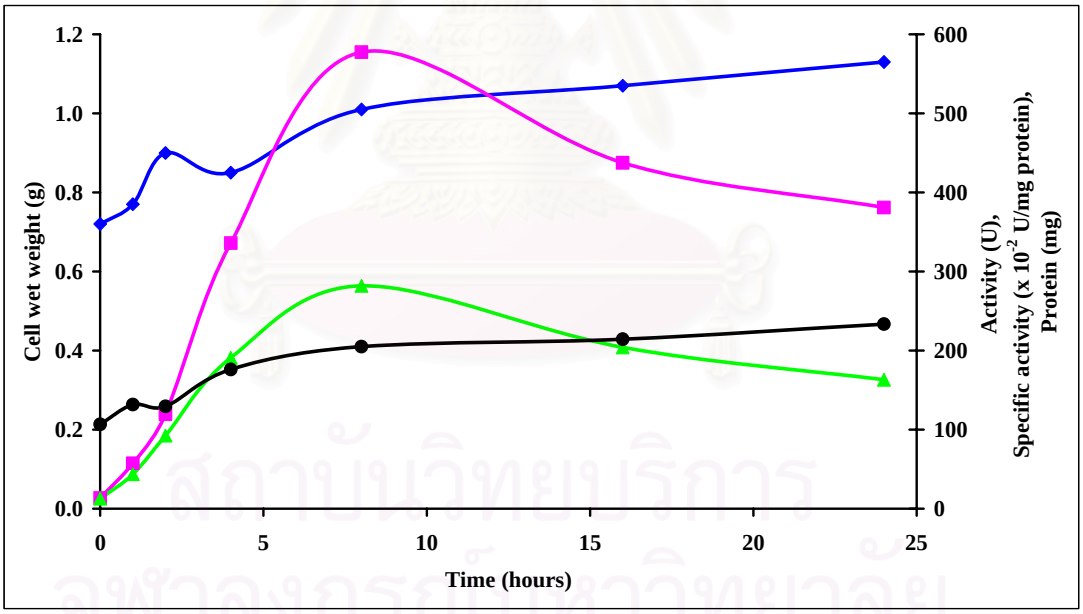


Figure 3.8 Expression of phenylalanine dehydrogenase gene in *E. coli* BL21(DE3) at various final concentrations of IPTG

◆ = cell wet weight

■ = total acitivity

▲ = specific activity

● = protein

3.4.2 Protein pattern of cells and crude extracts

The 1.0 ml of transformant No.4 cultures which had grown at various concentrations of IPTG and various times as described in 2.8 were harvested and centrifuged in microcentrifuge tube. The cell pellets were resuspended in 50 µl of 5x sample buffer. Ten microliters of cell samples or 15-20 µg protein of crude extracts were subjected to electrophoresis on 10 % SDS-polyacrylamide gel. The results in Figure 3.9-3.14 showed that the intensity of major protein band at 44.5 kDa of cell and crude extracts at each induction time was corresponded to the level of enzyme activity from its crude extract.

3.5 Stability of phenylalanine dehydrogenase gene expression in *E. coli* BL21(DE3)

The 1^{st}, 5^{th}, 10^{th}, 15^{th}, and 20^{th} subcultured colonies were picked up to culture and assay for oxidative deamination activity. The result shown in Table 3.3 indicated that each generation showed varied expression level of *phedh* gene. The 1^{st} pALPheDH clone subcultured gave the highest expression after that expression of *phedh* gene was decreased with increasing number of generation increased.

3.6 Purification of phenylalanine dehydrogenase

3.6.1 Preparation of crude extract

Crude PheDH was prepared from 5 g of transformant which was cultivated from 1 liter of medium as described in section 2.10.1. Crude extract contained 621.0 mg proteins and 1,925 units of PheDH activity. Thus, the specific activity of the enzyme in the crude preparation was 3.10 units/mg protein.

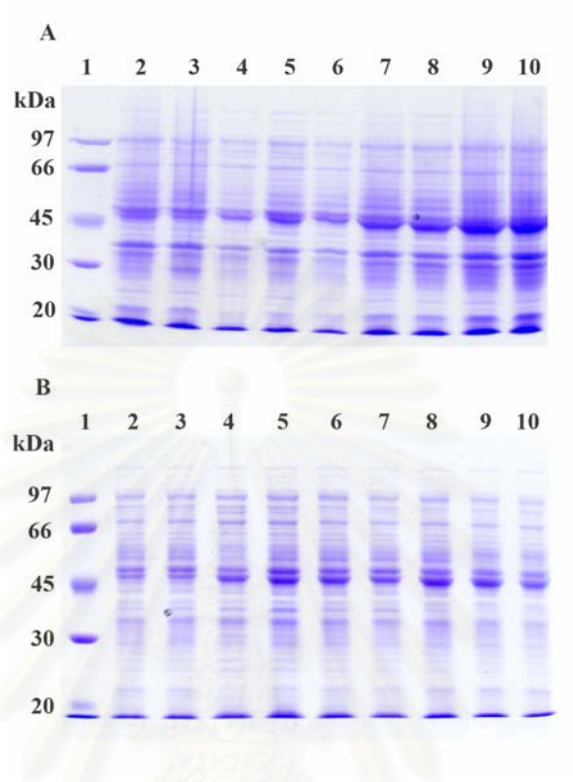


Figure 3.9 SDS-PAGE of whole cell and crude extract of pALPheDH clone induced by 0 mM IPTG at various times

A: whole cell

B: crude extract

Lane 1 = protein marker

Lane 2 = cell and crude extract of *E. coli* BL21(DE3)

Lane 3 = cell and crude extract of *E. coli* BL21(DE3) harbouring pET-17b

Lane 4-10 = cell and crude extract of pALPheDH clone at various induction times: 0, 1, 2, 4, 8, 16 and 24 hours, respectively

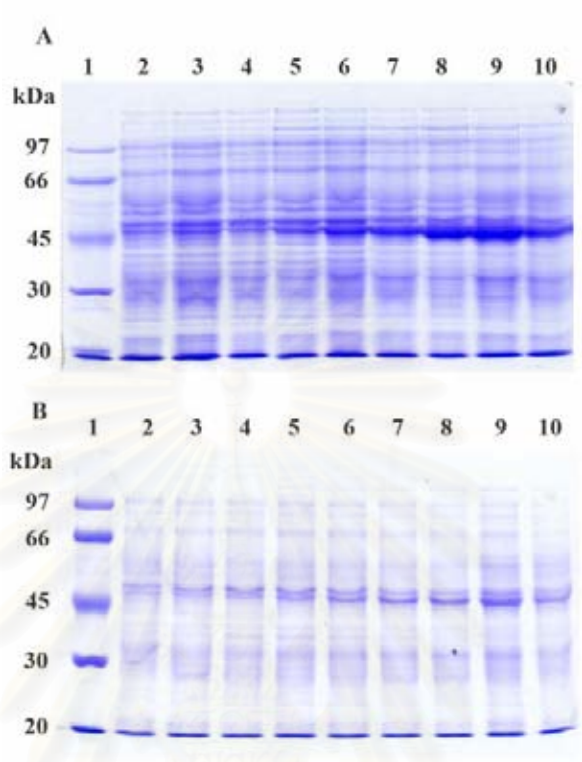


Figure 3.10 SDS-PAGE of whole cell and crude extract of pALPheDH clone induced by 0.2 mM IPTG at various times

A: whole cell

B: crude extract

Lane 1 = protein marker

Lane 2 = cell and crude extract of *E. coli* BL21(DE3)

Lane 3 = cell and crude extract of *E. coli* BL21(DE3) harbouring pET-17b

Lane 4-10 = cell and crude extract of pALPheDH clone at various induction times: 0, 1, 2, 4, 8, 16 and 24 hours, respectively

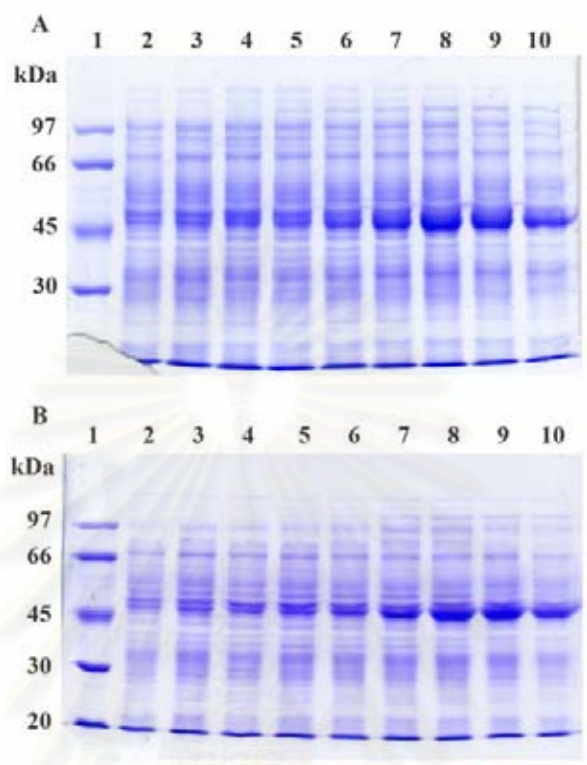


Figure 3.11 SDS-PAGE of whole cell and crude extract of pALPheDH clone induced by 0.4 mM IPTG at various times

A: whole cell

B: crude extract

Lane 1 = protein marker

Lane 2 = cell and crude extract of *E. coli* BL21(DE3)

Lane 3 = cell and crude extract of *E. coli* BL21(DE3) harbouring pET-17b

Lane 4-10 = cell and crude extract of pALPheDH clone at various induction times: 0, 1, 2, 4, 8, 16 and 24 hours, respectively

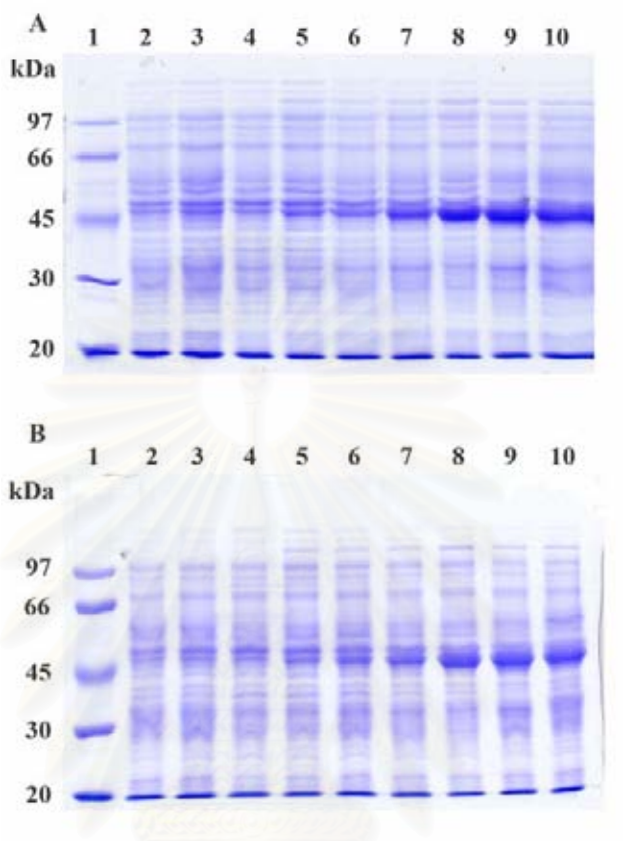


Figure 3.12 SDS-PAGE of whole cell and crude extract of pALPheDH clone induced by 0.6 mM IPTG at various times

A: whole cell

B: crude extract

Lane 1 = protein marker

Lane 2 = cell and crude extract of *E. coli* BL21(DE3)

Lane 3 = cell and crude extract of *E. coli* BL21(DE3) harbouring pET-17b

Lane 4-10 = cell and crude extract of pALPheDH clone at various induction times: 0, 1, 2, 4, 8, 16 and 24 hours, respectively

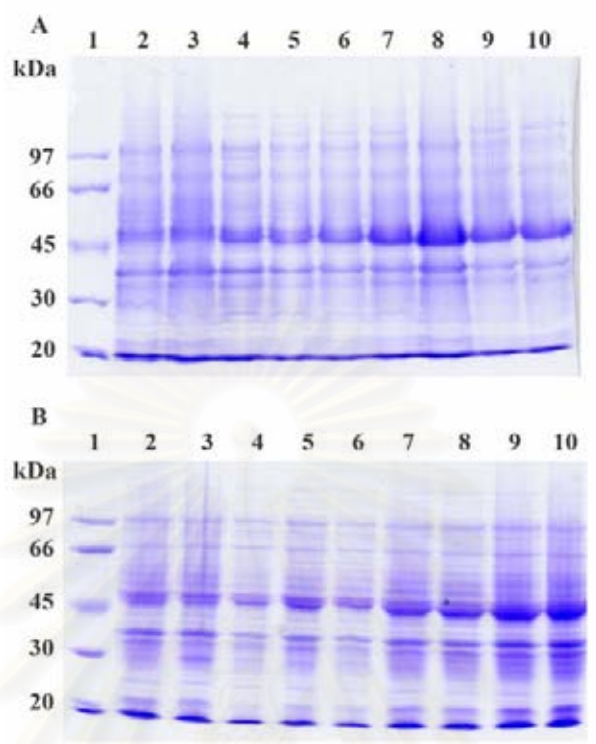


Figure 3.13 SDS-PAGE of whole cell and crude extract of pALPheDH clone induced by 0.8 mM IPTG at various times

A: whole cell

B: crude extract

Lane 1 = protein marker

Lane 2 = cell and crude extract of *E. coli* BL21(DE3)

Lane 3 = cell and crude extract of *E. coli* BL21(DE3) harbouring pET-17b

Lane 4-10 = cell and crude extract of pALPheDH clone at various induction times: 0, 1, 2, 4, 8, 16 and 24 hours, respectively

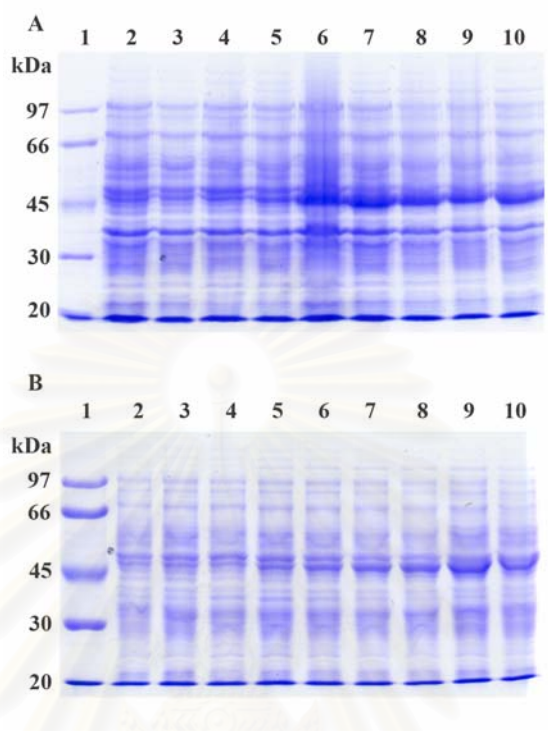


Figure 3.14 SDS-PAGE of whole cell and crude extract of pALPheDH clone induced by 1.0 mM IPTG at various times

A: whole cell

B: crude extract

Lane 1 = protein marker

Lane 2 = cell and crude extract of *E. coli* BL21(DE3)

Lane 3 = cell and crude extract of *E. coli* BL21(DE3) harbouring pET-17b

Lane 4-10 = cell and crude extract of pALPheDH clone at various induction times: 0, 1, 2, 4, 8, 16 and 24 hours, respectively

Table 3.3 Stability of phenylalanine dehydrogenase gene expression in pALPheDH clone[a]

Number of subculture	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)
1	415.5	140.6	3.0
5	303.0	154.5	2.0
10	318.0	118.7	2.7
15	303.0	118.5	2.6
20	270.0	148.0	1.8

[a] Crude extracts were prepared from 100 ml of cell culture.

3.6.2 Ammonium sulfate precipitation

First step of purification, crude extract was purified by ammonium sulfate precipitation as described in section 2.10.2.1. To determine the suitable ammonium sulfate concentration for precipitation of enzyme, preliminary experiment was performed by using 10 % step-wise increment of ammonium sulfate from 0 % to 80 %. The result showed that most of enzyme activity was determined in the 50-60 % and 60-70 % fractions. Therefore, protein fraction of the range of 50-70 % saturated ammonium sulfate precipitation was collected and dialysed against the buffer. The protein and enzyme activity were recovered at 155.0 mg and 921 units (47.84 % recovery from crude extract activity), respectively. The specificity activity of the enzyme from this step was 5.94 units/mg protein.

3.6.3 DEAE-Toyopearl column chromatography

The enzyme from 50-70 % saturated ammoium sulfate precipitation was loaded into DEAE-Toyopearl column as described in section 2.10.2.2. The chromatography profile is shown in Figure 3.15. Unbound proteins were eluted from column by the buffer. The bound proteins were eluted by linear salt gradient of 0 to 0.5 M potassium chloride in the buffer. PheDH was eluted at about 0.2 M potassium chloride as indicated in the profile. PheDH fractions were pooled, dialysed against the buffer, concentrated by aquasorb and centricon to reduce enzyme volume. This operation obtained the enzyme with 35.2 mg proteins and 567 activity units. The specificity activity of the enzyme from this step was 16.10 units/mg protein. The enzyme was purified 5.19 fold with 29.45 % recovery. The enzyme from this step was kept at 4 ^{o}C for further experiments.

The summary of purification of PheDH is shown in Table 3.4.

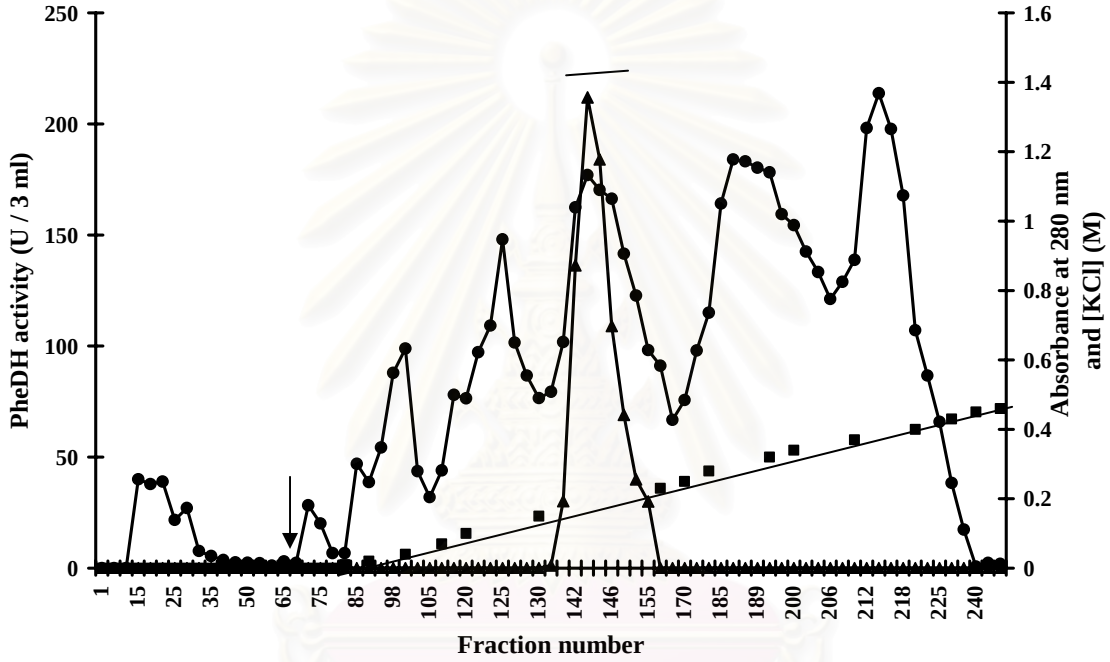


Figure 3.15 Purification of phenylalanine dehydrogenase from pALPheDH clone by DEAE – Toyopearl

The enzyme solution was applied to DEAE – Toyopearl column and washed with 10 mM potassium phosphate buffer, pH 7.4 containing 0.01 % β-mercaptoethanol, 1 mM EDTA until A_{280} decreased to base line. Bound proteins was 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 using fraction collector. The arrow indicates where gradient started. The protein peak from fraction number 141 to 148 were pooled.

● A_{280} ▲ PheDH activity ■ [KCl] — the pool fraction (No 141-148)

Table 3.4 Purification of phenylalanine dehydrogenase from pALPheDH clone[a]

Purification step	Total activity (unit)	Total protein (mg)	Specific activity (unit / mg protein)	% Recovery	Purification fold
Crude extract	1,925	621.00	3.10	100.00	1.00
50 – 70 % saturated ammonium sulfate precipitation	921	155.00	5.94	47.84	1.82
DEAE – Toyopearl column	567	35.20	16.10	29.45	5.19

[a] Crude extract was prepared from 1 liter of cell culture.

3.6.4 Determination of enzyme purity and protein pattern on non-denaturing polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis

The enzyme from each step of purification was analyzed for purity and protein pattern by and SDS-PAGE as described in section 2.11.2. In addition, purified enzyme from the last step of purification was electrophoresed on nondenaturing PAGE followed by protein and activity staining as described in 2.11.1.1 and 2.11.1.2, respectively. The results are shown in Figure 3.16. The purified enzyme in lane 4A on SDS-PAGE showed a single band which corresponded with a single protein band in lane 1B an its activity staining in lane 2B on native-PAGE. It indicated that PheDH from DEAE-Toyopearl column was a pure enzyme. The molecular weight of PheDH subunit was calculated to be 44.5 kDa by its mobility in SDS-PAGE compared with those of standard proteins.

3.7 Characterization of phenylalanine dehydrogenase

3.7.1 Substrate specificity of phenylalanine dehydrogenase

The oxidative deamination and reductive amination of recombinant PheDH were studied as described in section 2.7.3. Substrate specificity of the enzyme in the direction of oxidative deamination is shown in Table 3.5. The highest activity was monitored with L-phenylalanine as substrate. No activity was measured when α-methyl-DL-phenylalanine, L-3-phenylactate and D-phenylalanine were used as substrate.

In the reductive amination (Table 3.5), the enzyme greatly acted on α-ketocaproate, α-keto-γ-methiol-n-butyrate, α-ketovalerate and α-ketoisocaproate with 596, 412, 384 and 315 % relative activity compared to its natural substrate, phenylpyruvate, respectively. In addition, α-Ketoglutarate could not be used as substrate for reductive amination of PheDH.

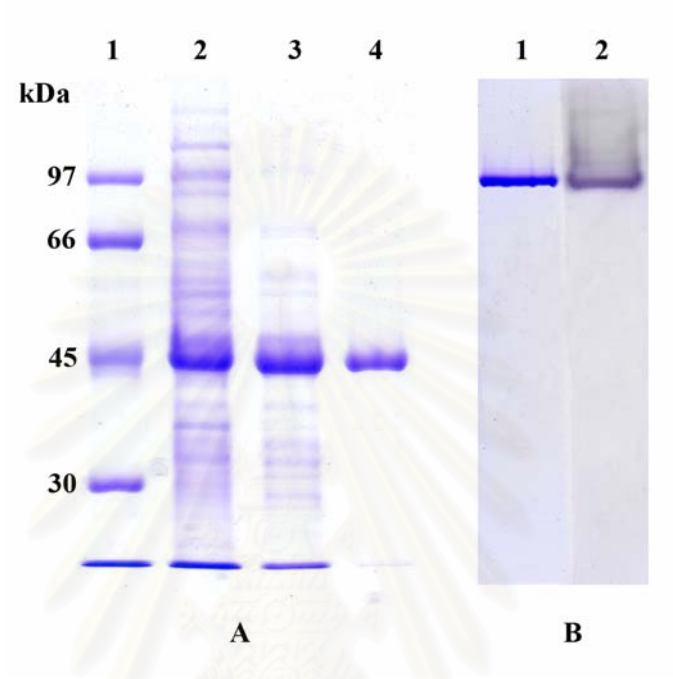


Figure 3.16 Protein pattern from each step of purification detected by SDS-PAGE and native-PAGE

A: SDS-PAGE

Lane 1 = protein marker

Lane 2 = crude extract

Lane 3 = 50 - 70 % saturated ammonium sulfate precipitation

Lane 4 = DEAE-Toyopearl column

B: native-PAGE

Lane 1 = DEAE-Toyopearl column (protein staining)

Lane 2 = DEAE-Toyopearl column (activity staining)

Table 3.5 Substrate specificity of phenylalanine dehydrogenase

Process and substrate	Relative activity (%)
Oxidative deamination*	
L-phenylalanine	100.0
L-tyrosine	6.8
L-tryptophan	4.0
L-methionine	8.6
L-ethionine	11.3
S-methyl-L-cysteine	5.1
L-leucine	2.0
L-isoleucine	3.2
L-allo-isoleucine	1.2
L-norleucine	13.1
DL-allylglycine	2.2
o-fluoro-DL-phenylalanine	5.0
m-fluoro-DL-phenylalanine	17.0
p-fluoro-DL-phenylalanine	33.3
L-DOPA	10.0
α-methyl-DL-phenylalanine	0.0
L-3-phenylactate	0.0
D-phenylalanine	0.0
L-valine	4.1
L-norvaline	8.1
Reductive amination*	
β-phenylpyruvate	100.0
α-ketocaproate	596.0
α-keto-γ-methiol-n-butyrate	412.0
α-ketovalerate	384.0
α-ketoisocaproate	315.0
α-ketoisovalerate	60.0
α-keto-n-butyrate	17.0
α-keto-*β*-methylvalerate	73.0
α-ketoglutarate	0.0

* Final concentration of each substrate was 20 mM except L-tyrosine and L-tryptophan are 1.25 and 12.5 mM, respectively.

** Final concentration of each substrate was 10 mM.

3.7.2 Effect of temperature on phenylalanine dehydrogenase stability

Production of various amino acids using recombinant PheDH would be performed at 30 ^{o}C. Therefore, the stability of the enzyme at this temperature had to be studied. The enzyme was preincubated in NH_4OH-NH_4Cl buffer, pH 9.5 at 30 ^{o}C for 4 hours interval from 0-48 hours before its activity was assayed as described in 2.7.3.1. The remaining activities were expressed as the percentage of the original activity. The result is shown in Figure 3.17. The enzyme activity was relatively decreased with increasing incubation time. For approximately half of the enzyme activity was lost after incubation for 12 hours and completely abolished after incubation at 30 ^{o}C for 40 hours.

3.8 Production of amino acids

Three microliter of product of each enzyme reactions from 2.13 using their keto acids as substrate were applied on cellulose thin-layer chromatography. The tested keto acids were phenylpyruvate, α-ketocaproate, α-keto-γ-methiol-n-butyrate, α-ketovalerate, α-ketoisocaproate, α-ketoisovalerate, α-keto-n-butyrate and α-keto-β-methylvalerate. The products were determined using by TLC 25 μmol of their corresponding amino acids, L-phenylalanine, norleucine, methionine, norvaline, leucine, valine, α-aminobutyrate and isoleucine as standards, respectively. After developing the TLC chromatogram with 0.5 % ninhydrin solution, it was found that the R_f value of product from each enzyme reaction was the same with its expected amino acid standard. The result of TLC profile and R_f value of product from each enzyme reaction are shown in Figure 3.18 and Table 3.6, respectively.

After that, products from the enzyme reactions were confirmed and their quantity were determined using amino acid analyzer, Model L8500A. Ten microliters of their corresponding amino acids, L-phenylalanine, norleucine, methionine, norvaline, leucine, valine, α-aminobutyrate, and isoleucine were used as standards.

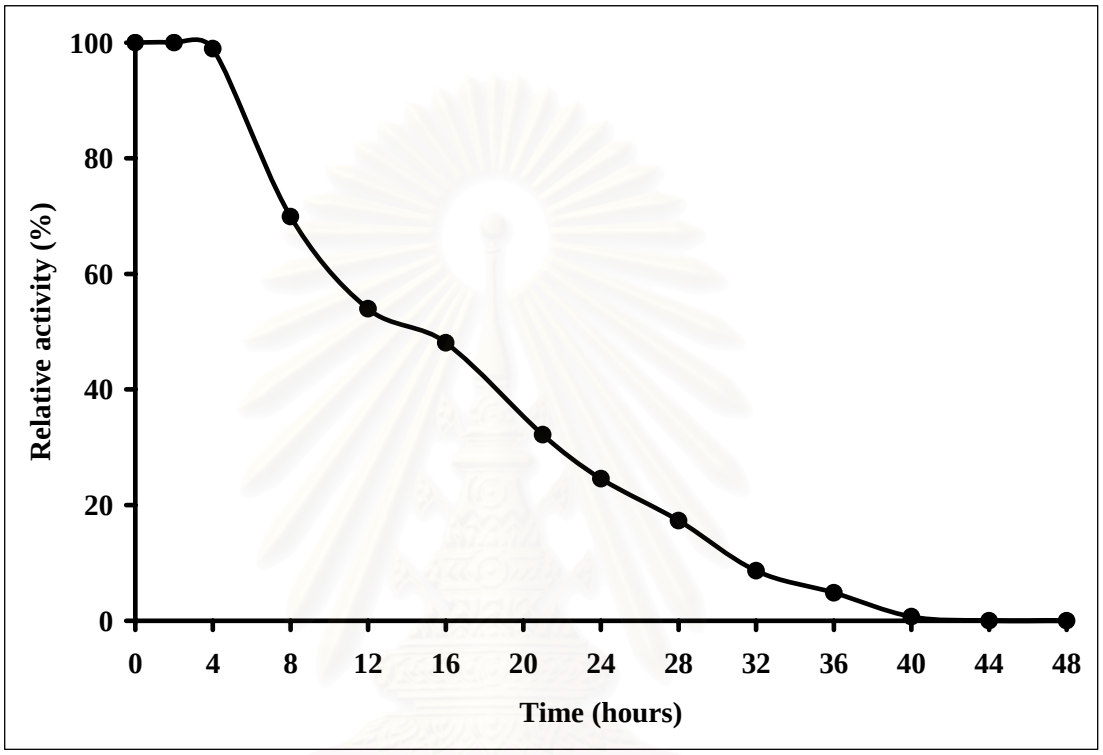


Figure 3.17 Stability of phenylalanine dehydrogenase at 30 ^{o}C

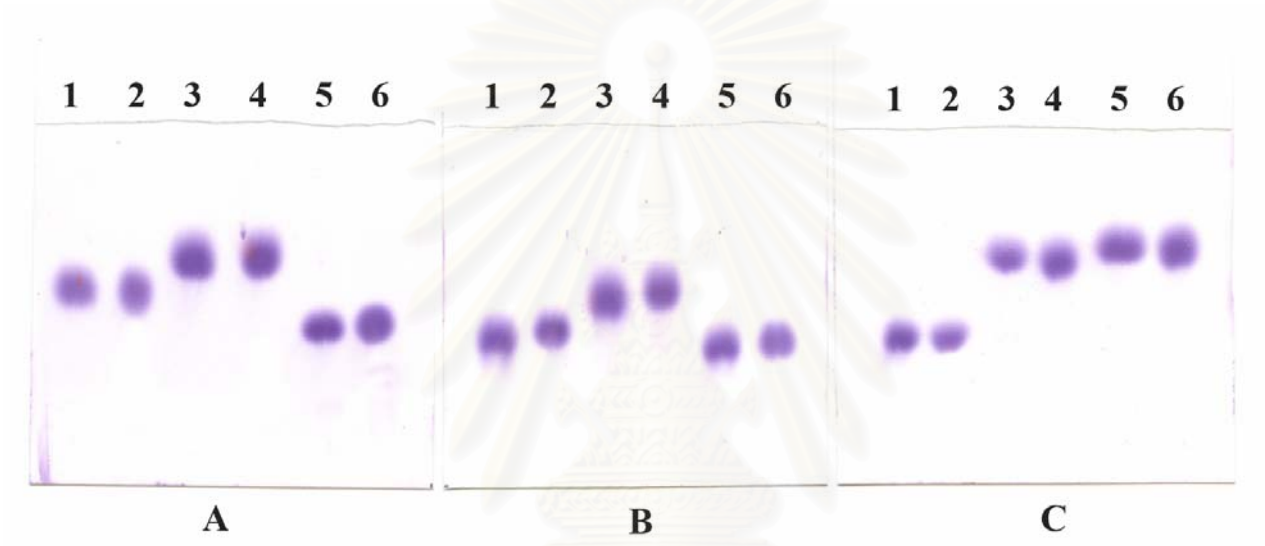


Figure 3.18 TLC analysis of the reaction products catalyzed by the phenylalanine dehydrogenase

A: Lane 1 = L-phenylalanine standard
Lane 2 = phenylpyruvate reaction
Lane 3 = L-norleucine standard
Lane 4 = α-ketocaproate reaction
Lane 5 = L-methionine standard
Lane 6 = α-keto-γ-methiol-n-butyrate reaction

B: Lane 1 = L-norvaline standard
Lane 2 = α-ketovalerate reaction
Lane 3 = L-leucine standard
Lane 4 = α-ketoisocaproate reaction
Lane 5 = L-valine standard
Lane 6 = α-ketoisovalerate reaction

C: Lane 1 = α-aminobutyrate standard
Lane 2 = α-keto-n-butyrate reaction
Lane 3 = L-isoleucine standard
Lane 4 = α-keto-*β*-methylvalerate reaction
Lane 5 = L-leucine standard
Lane 6 = α-ketoisocaproate reaction

Amount of standard amino acid in each spot was 25 nmole

Table 3.6 R_f value of product from each enzyme reaction separated by TLC

TLC plate	Lane	Reaction	R_f value
A	1	L-phenylalanine standard	0.47
	2	product from phenylpyruvate reaction	0.47
	3	L-norleucine standard	0.57
	4	product from α-ketocaproate reaction	0.57
	5	L-methionine standard	0.36
	6	product from α-keto-γ-methiol-n-butyrate reaction	0.36
B	1	L-norvaline standard	0.32
	2	product from α-ketovalerate reaction	0.32
	3	L-leucine standard	0.45
	4	product from α-ketoisocaproate reaction	0.45
	5	L-valine standard	0.30
	6	product from α-ketoisovalerate reaction	0.30
C	1	α-aminobutyrate standard	0.32
	2	product from α-keto-n-butyrate reaction	0.32
	3	L-isoleucine standard	0.56
	4	product from α-keto-*β*-methylvalerate reaction	0.56
	5	L-leucine standard	0.61
	6	product α-ketoisocaproate reaction	0.61

The retention time of reaction products were determined from amino acid profile. For example, the retention time of product from phenylpyruvate reaction and standard phenylalanine were 53.97 and 53.86, respectively (Figure 3.19). Retention time of all products and standard amino acids are summarized in Table 3.7. In addition, amount of reaction products were calculated from their peak area compared with those of their corresponding amino acids. As shown in Table 3.7 and Figure 3.20, yield of amino acid production was in the range between 36.0-72.2 %.

A

B

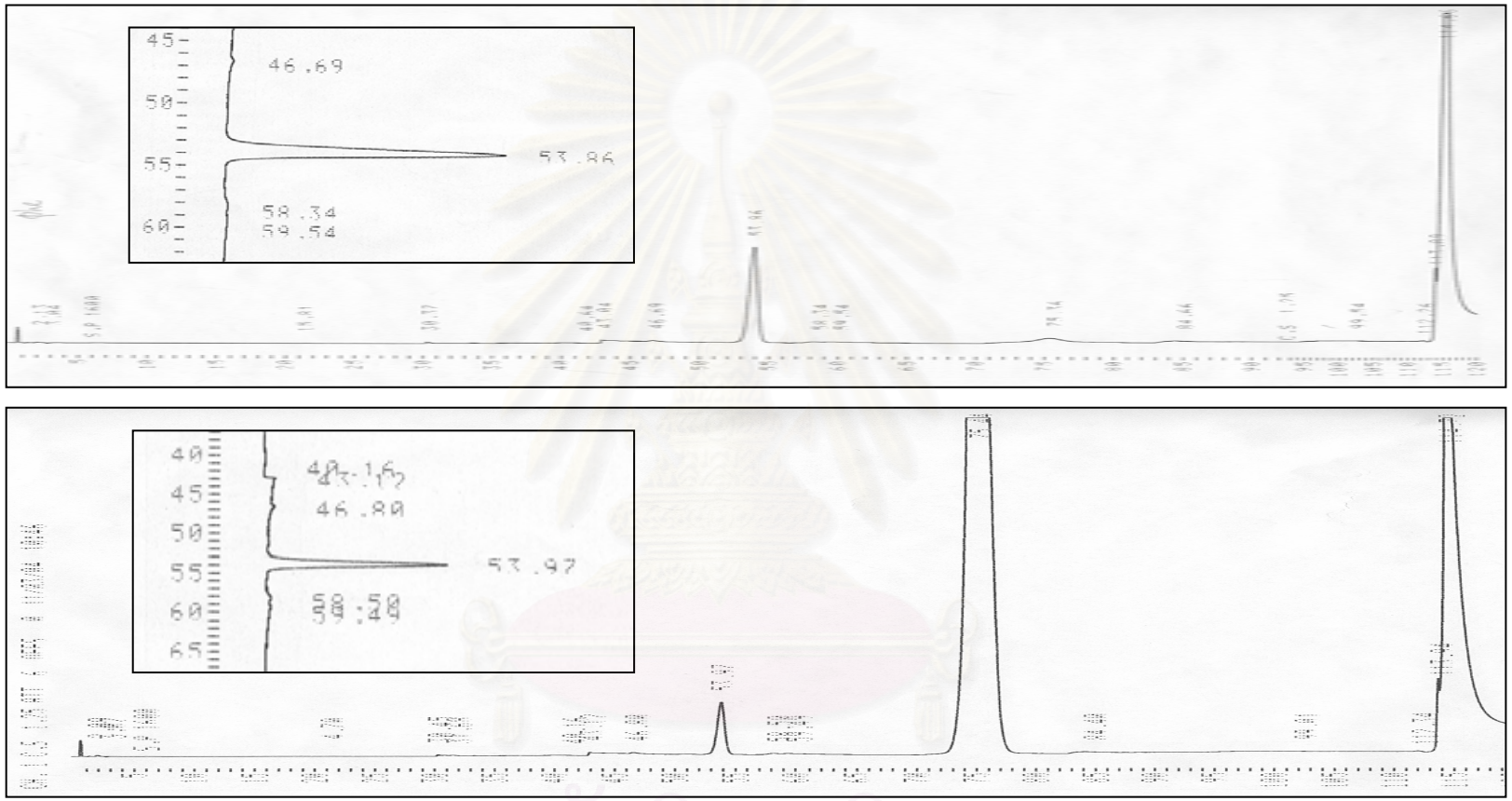


Figure 3.19 Chromatogram of phenylalanine detected by amino acid analyzer

A: L-phenylalanine standard

B: product from enzyme reaction using phenylpyruvate as substrate

Table 3.7 Amino acid production by reductive amination of phenylalanine dehydrogenase

Reaction	Retention time (R_t)	% yield
L-phenylalanine standard	53.86	
product from phenylpyruvate reaction	53.97	58.8
L-norleucine standard	50.51	
product from α-ketocaproate reaction	50.58	57.7
L-methionine standard	43.81	
product from α-keto-γ-methiol-n-butyrate reaction	43.89	72.2
L-norvaline standard	44.80	
product from α-ketovalerate reaction	44.77	67.9
L-leucine standard	48.26	
product from α-ketoisocaproate reaction	48.37	56.3
L-valine standard	38.85	
product from α-ketoisovalerate reaction	38.88	70.2
α-aminobutyrate standard	36.34	
product from α-keto-n-butyrate reaction	36.37	47.6
L-isoleucine standard	47.04	
product from α-keto-*β*-methylvalerate reaction	46.98	36.0

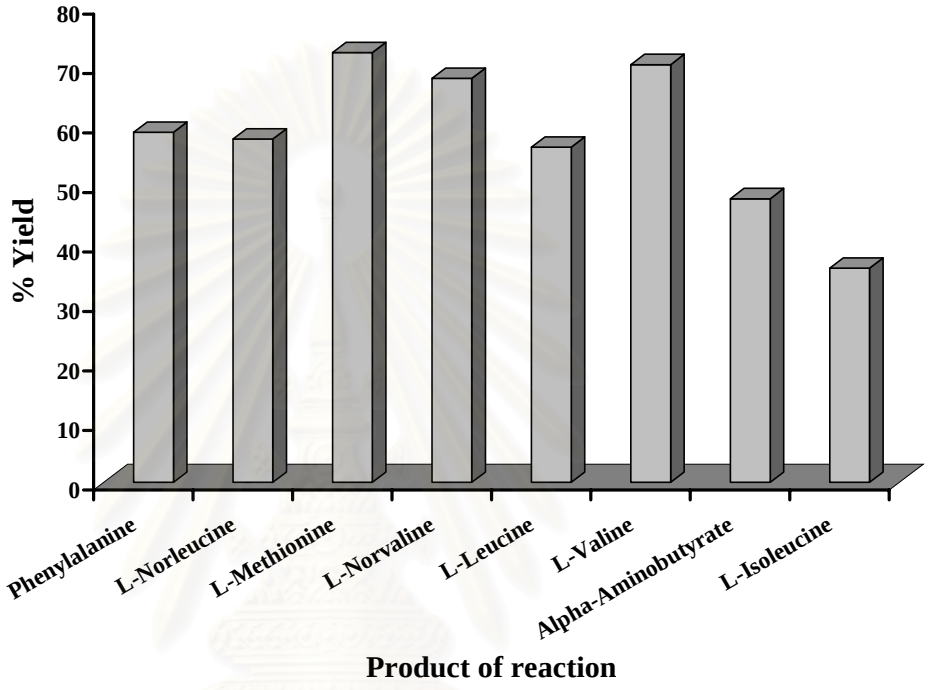


Figure 3.20 Production of amino acids by phenylalanine dehydrogenase

CHAPTER IV

DISCUSSION

Amino acids, in addition to their role as protein monomeric units, are energy metabolites and precursors of many biologically important nitrogen-containing compounds, notably heme, physiologically active amines, glutathione, nucleotides, and nucleotide coenzymes. Bacteria can metabolize amino acid as the energy sources through the glycolytic or TCA cycle reactions by using three enzymes. The first enzyme is pyridoxyl phosphate-dependent transaminases, which transfers the amino group from one amino acid to another, usually glutamate, a predominant nitrogen storage molecule of the cell. The second is to employ deaminases in the way to remove amino group from amino acid in the form of ammonia such as phenylalanine –ammonia lyase, or aspartase. The third is to use an amino acid dehydrogenase. This enzyme has the advantages of producing the amino group as free ammonia and other important metabolize molecules such as pyruvate and α-ketoglutarate. Phenylalanine dehydrogenase is one of the enzymes used for producing phenylalanine and related amino acids from their keto analogs, which are of industrial importances (Brunhuber and Blanchard, 1994 and Asano *et al.*, 1987c). Moreover, this enzyme can be applied for diagnosis of neonatal hyperphenylalaninaemia and phenylketonuria (Hummel *et al*., 1984).

4.1 Cloning and expression of phenylalanine dehydrogenase gene

Many researchers attempted to clone *phedh* gene for the large amount of enzyme production in order to study properties of enzyme or used as catalyst for the synthesis of L-phenylalanine and related amino acids. Asano and coworkers (1987c) cloned *phedh* gene from *Bacillus badius* IAM 11059 using pUC19 into *E. coli* RRI. The total activity of the enzyme detected in crude enzyme was 6,890 units/liter of cell culture which was evaluated 24 fold of the *B. badius* IAM 11059. In addition, the

amount of the enzyme in the crude extract comprised 9.6 % of the total extractable cellular protein. Subsequently, research group of Takada cloned *phedh* gene from *Thermoactinomyces intermedius* into *E. coli* JM109 using a plasmid pKK223-3 (Takada *et al.*, 1991). Total activity of the enzyme in crude extract was reported to be 530 units/liter of cell culture. Moreover, *phedh* gene from *Bacillus badius* BC1 was cloned and expressed in *E. coli* JM109 using pUC18 plasmid (Chareonpanich, 2001). Crude extract of the enzyme gave 1,659 units of PheDH/liter of cell culture. Therefore, the basic requirements for the successful production of recombinant enzyme are the isolation of the gene encoding that enzyme and the development of a suitable expression system for the gene. In this research, the *phedh* gene from *Acinetobacter lwoffii* was cloned into *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS using plasmid pET-17b. By pET system, *phedh* gene was expressed under T7 promoter, moreover, the upstream region of the inserted gene contained highly efficient ribosome binding site from the phage T7 major capsid protein (Novagen, 2002).

Polymerase chain reaction (PCR) is the popular method for *in vitro* synthesis the interesting gene for cloning. This method requires a pairs of primer which is specific to the target gene. In this study, the *phedh* gene fragment was amplified by using two primers which designed by using the nucleotide sequence from *phedh* gene of *A. lwoffii*. The 5′ primer comprised of *Nde*I site while 3′ primer comprised of *Bam*HI site. Templates used in PCR reaction should be single strand in the purpose of annealing with primers. The heat denatured chromosomal DNA was previously used as templates, however, it did not gave PCR amplification due to effect of its viscosity. So, the *Bam*HI, *Hin*dIII and *Kpn*I digested chromosomal was used as template in amplification. Moreover, various annealing temperature were used in amplification. Size PCR products were determined by their relative mobility on agarose gel electrophoresis compared with standard marker. Due to complementary binding of DNA template and primer was not appropriate at high annealing temperature, the amount of PCR product decreased when annealing temperature was increased in all digested DNA template. After that, the gene fragment was purified before the digestion *Nde*I and *Bam*HI. Then, the *phedh* gene fragment was ligated to

*Nde*I-*Bam*HI site of pET-17b and transformed into *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS. Six recombinant clones from each host, *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS, were grown in LB medium, pH 7.2 containing 100 μg/ml ampicillin (when *E. coli* BL21(DE3)pLysS was used as host, 34 μg/ml chloramphenicol was also included). When OD_{600} reached 0.6, IPTG was added in the medium at final concentration of 0.4 mM. After that, the cultures were continued for 4 hours before cultivation. Crude extract of each recombinant clone was assayed for PheDH activity. Their PheDH specific activities were found between 0.81 – 4.46 units/mg protein. The variation of PheDH activity from each recombinant clone may be due to point mutation occurred in PCR amplification step. The highest specific activity of *E.coli* BL21(DE3) recombinant clone was higher than that of *A. lwoffii* with 55.75 fold. Furthermore, the enzyme activity of *E.coli* BL21(DE3) recombinant clone was higher than that of *E. coli* BL21(DE3)pLysS because pLysS plasmid encodes T7 lysozyme, which is a natural inhibitor of T7 RNA polymerase. Thus, transcription level of *phedh* gene in *E. coli* BL21(DE3)pLysS was reduced that led to lower expression of pALPheDH. However, the presence of pLysS has the further advantage of facilitating the preparation of crude extract because T7 lysozyme cuts a specific bond in the peptidoglycan layer of the *E. coli* cell wall (Mierendorf *et al.*, 1994) (Appendix J).

The nucleotide sequence of *phedh* gene from *A. lwoffii* was compared with those of *phedh* gene from various sources in the EMBL-GenBank-DDBL database. It showed 82, 66, 64, 53, and 9% homology to those of *Bacillus sphaericus, Bacillus badius, Sporosarcina ureae, Thermoactinomyces intermedius,* and *Rhodococcus* sp. M4, respectively. The deduced amino acid sequence of all phenylalanine dehydrogenases were aligned by using the CLUSTAL X (1.82). The percentage of identical amino acid of the enzyme compared with PheDH from *B. sphaericus, B. badius, S. ureae, T. intermedius, and Rhodococcus* sp. M4, was 88, 68, 62, 51, and 32%, respectively. According to Brunhuber *et al.* (1999), Lys-78 and Asp-118 are necessary for stabilizing and binding of phenylalanine in the active site of the *Rhodococcus* enzyme. Both residues were conserved in all the phenylalanine dehydrogenase sequences including *A. lwoffii* enzyme. In addition, conserved residues

in the catalytic domains G-G-(G or S or A)-K-X-(V or G)-X-X-X-(D or N)-(P or L) at the N-terminus and the glycine-rich nucleotide binding domain G-X-G-X-X-(G or A) at the C-terminus (Yamada *et al*., 1995) were also found.

Due to construction of *phedh* gene under T7 promoter, expression of *phedh* gene can be induced by IPTG. Hence, the study of induction time and final concentration of IPTG were required for maximum expression. pALPheDH clone which showed the highest PheDH activity was grown at various condition. Without induction by IPTG, the expression of *phedh* gene was occurred because there is some expression of T7 RNA polymerase from the *lacUV5* promoter in the DE3 lysogen from *E. coli* genome (Novagen, 2002). Expression of the gene reached the highest point at 8 hours in every final concentration of IPTG, after that, activity of PheDH was decreased. This may be occurred by 1) PheDH, which is an unnatural protein in *E. coli*, was degraded by an ATP-dependent protease (Nelson and Cox, 2000) or 2) low stability of mRNA transcribed from *phedh* gene (Savvas, 1996). The result showed that induction by 0.4 mM IPTG for 8 hours gave the optimum condition for *phedh* gene expression. For expression of pET plasmid carrying the T7 promoter, a final concentration of 0.4 mM IPTG is recommended for full induction (pET system manual, 2002). However, different optimum conditions have been reported for some inserted genes of pET plasmids. For example, 3 hours of induction with 1 mM IPTG maximized the expression of *phedh* gene from *Rhodococcus* sp. M4 which was cloned into *E. coli* BL21(DE3) using pET-3d plasmid (Brunhuber *et al*., 1994). Moreover, alanine dehydrogenase gene from *Aeromonas hydrophila*, expressed under T7 promoter of pET-17b in *E. coli* BL21(DE3), showed the highest expression at 4 hours of induction with 0.4 mM IPTG (Hatrongjitt, 2004). In addition, Kim *et al*, (2003) cloned and expressed bovine brain glutamate dehydrogenase gene using pET-15b in *E. coli* BL21(DE3). Induction of 1 mM IPTG for 3 hours was reported for maximum enzyme production. Therefore, final concentration of IPTG and induction time seem to influence the optimization of individual gene expression. The variation of expression level of recombinant PheDH from various bacteria may be obtained from the promoters, mRNA construction and its topology, and condition of expression e.g.

optimization of IPTG induction, type of cell line, media, and incubation circumstances.

Stability of *phedh* gene from recombinant clones that showed the highest enzyme activity was studied by daily subculturing for 20 days. The crude extract of the 5^{th}, 10^{th}, 15^{th} and 20^{th} subcultured clone were prepared and enzyme activities were determined and compared with the activity of their parents. The result indicated that expression of *phedh* gene decreased when the number of subculture was increased. Variation of PheDH activity in each subculture clone may be caused from losing of plasmid copy number during subculture step.

4.2 Purification of phenylalanine dehydrogenase

The development of techniques and methods for the separation and purification of proteins has been an essential pre-requisite for many of the recent advancements in bioscience and biotechnology research. The global aim of a protein purification process is not only the removal of unwanted contaminants, but also the concentration of the desired protein and its transfer to an environment where it is stable and in a form ready for the intended application. The principal properties of enzymes that can be exploited in separation methods are size, charge, solubility, density and the possession of specific binding sites. Most purification protocols required more than one step to achieve the desired level of product purity. This includes any conditioning steps necessary to transfer the product from one technique into conditions suitable to perform the next technique. Each step in the process will cause some loss of product. Consequently, the key to successful and efficient protein purification is to select the most appropriately techniques, optimize their performance to suit the requirements and combine them in a logical way to maximize yield and minimize the number of steps required (Amersham pharmacia biotech, 1999).

The first step in the purification of a protein is the preparation of an extract containing the protein in a soluble form and extraction procedures should be selected

according to the source of the protein. In this work, PheDH, an intracellular enzyme, was extracted from pALPheDH clone. Mechanical disruption methods are usually necessary to break down cell wall in order to release intracellualr protein prior to purification. Ultrasonication or high pressure sound waves, which causes cell breakage by cavitation and shear forces, was used in this work. However, several potential problems may be consequent on disruption, due to the destruction of intracellular compartmentation and PheDH activity can be lost for a variety of reasons. It is essential to consider strategies for protection of the enzyme activity. In this work, phenylmethylsulfonyl fluoride (PMSF), and ethylenediamine tetraacetic acid (EDTA) were used in the extraction buffer as serine and thiol protease inhibitor, and metalloprotease inhibitor, respectively, because the control of metabolic regulation mechanism is lost when the cell is disrupted. Thus, the desired protein may be degraded by intrinsic catabolic enzymes such as proteolytic enzymes. In addition, the protein will encounter an oxidizing environment after disruption that may cause inactivation, denaturation or aggregation (Scopes, 1987). 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 was not inhibited at all. Acid proteases may not effect to PheDH activity because their reactions occured only in low pH environment. Furthermore, mechanical cell disruption may cause local overheating with consequent denaturation of protein. To maximize recovery of active enzyme, the extract and equipment, therefore, were pre-chilled and several pauses of disruption used instead of one long continuous sonication because short interval of disruption will also minimize foaming and shearing , thereby minimizing denaturation (Harris and Angal, 1989 and Janson and Ryden, 1998).

Solubility differences in salt are frequently exploited to separate proteins in the early stages of purification protocols. Ammonium sulfate was the salt of choice and was used in this work because it combined many useful features such as salting out effectiveness, pH versatility, high solubility, low heat of solution and low price (Bollag *et al*., 1996 and Creighton, 1993). In the ammonium sulfate precipitation step, about 75 % of proteins were removed, however about half of enzyme activity was

lost. The solution to which salt is to be added should be provided with a stirring system which must be regular and gentle because it may be cause protein denaturation as evidenced by foaming. pH may be important in precipitation. It is best to operate at a neutral value (6-7.5). Ammonium sulfate has a slight acidifying action, so around 50 mM buffer should be present (Scopes, 1987). In this work, ammonium sulfate precipitation was operated by using extraction buffer which contained 0.1 M potassium phosphate buffer, pH 7.4. Therefore, this buffer is appropriately used for ammonium sulfate precipitation.

Most purification protocols involve some forms of chromatography, which has become an essential tool in protein purification. Ion exchange chromatography separates proteins with differences in charge to give a very high resolution with high sample loading capacity. The difference in charge properties of protein are often considerable. Ion exchange chromatography is capable of separating species with very minor differences in properties, such as two proteins differing by only one amino acid. It is a very powerful separation technique indeed (Amersham pharmacia biotech, 1999). DEAE-Toyopearl is anion exchanger which has negatively charged counter-ions. It widely used in the purification of PheDH from other sources such as PheDH from *B. badius* (Asano *et al.*, 1987c), *B. sphaericus, S. ureae* (Asano *et al.*, 1987b) and *T. intermedius* (Ohshima *et al.*, 1991) and *Microbacterium* sp.(Asano and Tanetani, 1998). Its popularity stems from the possibility of high resolving power, versatility, reproducibility and ease of performance. Consequent upon the result, this column contributed greatly to the purification procedures, with less loss of PheDH activity compared to the amount of proteins removed. About 80 % of the other bulk proteins were eliminated.

PheDH from pALPheDH clone was purified 5.19 fold with a 29.45 % yield by procedure involving ammonium sulfate precipitation and DEAE-Toyopearl column chromatography. When compared to purification of PheDH from wild type strain, *A. lwoffii,* which was purified by Butyl-Toyopearl, DEAE-Toyopearl, Sephadex G-150, Mono Q, Phenyl-Superose and Sephadex G-200 column chromatography, the purification step of cloned enzyme is faster and more convenience. The purification of

other PheDH cloned enzymes were also reported. *T. intermedius* PheDH from *E. coli* transformant RR1/pBB19 was purified by heat treatment, ammonium sulfate precipitation, DEAE-Toyopearl, Sephadex G-200 column chromatography with 27 % yield and 10 purification fold (Asano *et al.,* 1987c). In addition, Omidinia and coworkers (2002) purified *B. sphaericus* PheDH from *E. coli*BL21pETDH by 5 steps, 30 % saturated ammonium sulfate precipitation, the first 60 % saturated ammonium sulfate precipitation, the first Reactive Blue 4 dye column chromatography, the second 60 % saturated ammonium sulfate precipitation, and the second Reactive Blue 4 dye with a 28 % yield and 88.8 purification fold. The different results may be obtained from type of host cell, enzyme property and purification procedure.

4.3 Characterization of phenylalanine dehydrogenase

4.3.1 Substrate specificity of phenylalanine dehydrogenase

In general, a substrate binding site consists of an indentation or cleft on the surface of an enzyme molecule that is complementary in shape to the substrate. Morever, the amino acids residues that form the binding site are arranged to interact specifically with the substrate in an attractive manner. Molecules that differ in shape or functional group distribution from the substrate can not productively bind to the enzyme, that means they can not form enzyme-substrate complexes that lead to the formation of products.

According to PheDH from *A. lwoffii* was previously reported to use broad non-polar side chain amino acid substrate such as L-methionine, L-tryptophan and L-norleucine in the oxidative deamination, in this study various kinds of non-polar side chain amino acid as well as analog of L-phenylalanine were tested. The structures of amino acids are shown in Appendix K.

The enzyme can use all tested aliphatic non-polar amino acid, L-valine, L-norvaline, L-leucine, L-isoleucine, L-norleucine, L-methionine, L-ethionine and *S*-methyl-L-cysteine as substrate with slight relative activity to L-phenylalanine. Size

of aliphatic side chain seemed to affect the specificity of enzyme since relative activity was increased with increasing size of amino acid in the order *S*-methyl-L-cysteine < L-methionine < L-ethionine and L-norvaline < L-norleucine. Moreover, relative activity of straight chain L-norvaline and L-norleucine were higher than those of branched chain L-valine and L-leucine as well as L-isoleucine, respectively. Seah *et al*. (2002) also reported that relative activity of PheDH from *B. sphaericus* with valine was lower than norvaline.

Oxidative deamination of enzyme on tyrosine and L-DOPA, which had additional one and two hydroxyl group on the aromatic side chain of phenylalanine, was 6.8 and 10 % relative activity, respectively. PheDHs from *Rhodococcus* sp. M4, *B. badius, S. ureae, Microbacterium* sp. and *R. maris* slightly acted toward L-tyrosine with 12, 9, 5, 4 and 2 % relative activity, respectively while no activity was obtained PheDHs from *T. intermedius* and *B. badius* BC1 (Table 1.2). Surprisingly, PheDH from *A. lwoffii* is the first enzyme which was reported to act on L-DOPA.

The *m*- and *o*-fluoro-DL-phenylalanine, which have additional fluoro group at the aromatic side chain of phenylalanine, were slightly oxidized by the enzyme while *p*-analog gave higher reaction rate with 33 % of reaction rate obtained from phenylalanine. The reaction rate with *p*-fluoro-DL-phenylalanine of *Rhodococcus* sp. M4 and *B. badius* enzyme were 62 and 34 % relative activity, respectively (Table 1.2).

The above results show that the position of the addition group, size and polarity of R-group are important for the substrate specificity in which the steric effect may involve in the reaction.

For pALPheDH enzyme, no activity was observed with α-methyl-DL-phenylalanine which its H atom of the chiral carbon is substituted by methyl group and L-3-phenyllactate, which its amino group is substituted by OH group. These observations indicate that the amino group and H atom of the chiral carbon are essential on the substrate specificity of the enzyme.

In addition, D-phenylalanine was inert for pALPheDH enzyme as reported for other PheDHs such as PheDH from *B. sphaericus* and *S. ureae* (Asano *et al.*,1987b). Palmer described that enzyme could exhibit stereochemical specificity if a substrate can exist in two stereochemical forms, chemically identical but with a different arrangement of atoms in three-dimensional space, then only one of the isomers will undergo reaction. (Palmer, 1995).

Most of PheDH from various sources have boarder substrate specificity in the reductive amination than those of their oxidative deamination. For example, PheDHs from *B. sphaericus, Brevibacterium* sp., *R. maris, B. badius, S. ureae* and *Nocardia* sp. actived toward *p*-hydroxyphenylpyruvate with 136, 96, 91, 53, 24 and 28 % relative activity, respectively (Table 1.2). Except PheDH from *Nocardia* sp. which had 240 % relative activity for α-ketoisocaproate (Boer *et al.*, 1989), all of reported PheDH acted on aromatic keto acid better than aliphatic one. In contrast with other PheDHs, pALPheDH enzyme showed 596, 412, 384 and 315 % relative activity for α-ketocaproate, α-keto-γ-methiol-n-butyrate, α-ketovalerate and α-ketoisocaproate, respectively when compared with phenylpyruvate. This result showed that aliphatic keto acids were preferable substrates for reductive amination of the enzyme.

In addition, α-ketoglutarate (Appendix L) was inert with pALpheDH enzyme since it contained two carboxyl groups. The similar results were also reported for PheDH from *S.* ureae, *B. sphaericus* and *B. badius* (Asano *et al*., 1987b and c).

4.3.2 Temperature stability of phenylalanine dehydrogenase

Temperature affects the rate of an enzyme-catalyzed reaction by increasing the thermal energy of the substrate molecules. It increases the proportion of molecules with sufficient energy to overcome the activation barrier and hence increases the rate of the reaction. In addition, increasing of the thermal energy of the molecules which make up the protein structure of the enzyme itself will increase the

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

In a previous report, PheDH from *Acinetobacter lwoffii* was incubated at various temperatures for 10 minutes, 100 % activity was retained at 55 ^{o}C and dropped about 50 % at 60 ^{o}C. The investigation was extended to determine thermostability of enzyme at 30 ^{o}C, pH 9.5. It was found that the activity was not lost upon incubation for 4 hours and half of enzyme activity was lost at incubation for 12 hours. Therefore, the enzyme is applicable at 30 ^{o}C.

4.4 Production of amino acids

Since PheDH from *A. lwoffii* can catalyze reductive amination of many keto acids with high relative activity to phenylpyruvate, the possibility to use these keto acids as substrate for synthesis of their corresponding amino acids was performed (Appendix M). The reaction products were analyzed by cellulose thin-layer chromatography. Amino analysis by this method usually involves two distinct processes, first separation of the individual amino acids from each other and from other contaminants, and then detection of the separated components. Samples were subjected to cellulose thin-layer chromatography. As the solvent front passes the sample spots. The compound in each sample were carried along at the rate which is characteristic of their functionality, size and interaction with the cellulose matrix. Some compounds move rapidly up the plate, while others may scarcely move at all. The ratio of the distance a compound moves from the baseline to the distance of the solvent front from the baseline is defined as the retardation factor (R_f). Different amino acids usually have different R_f under suitable conditions (Nelson and Cox, 2000). After that, developing with 0.5 % ninhydrin solution, which react with amino group of amino acid to give purple color was done. A common application of the ninhydrin test is the visualization of amino acids in thin-layer chromatography.The result indicated that the R_f value of product from each enzyme reaction was not different from its corresponding amino acid standard, L-phenylalanine, L-valine,

L-norleucine, L-methionine, L-norvaline, L-leucine, α-aminobutyrate and L-isoleucine.

Product from the enzyme reaction were identified and quantified by amino acid analyzer, which uses ion-exchange chromatography to separate amino acids followed by a "post-column" ninhydrin reaction detection system. The system can quantify individual amino acids down to the 50 pmole level (http://msf.ucdavis.edu/aaa.html). The product yield was range between 36.0-72.2 %. The highest productivity was obtained in the reductive amination of α-keto-γ-methiol-butyrate to methionine with 72 % yield through the synthesis was not optimized. Asano and Nakazawa (1987) used PheDH from *Sporosarcina ureae* coupling with formate dehydrogenase from *Candida boidinii* for synthesis of various amino acids. At the optimal condition for amino acid synthesis, yield of L-phenylalanine, L-tyrosine, L-tryptophan, L-methionine, L-valine, L-leucine and L-isoleucine were 98, 99, 11, 87, 97, 83 and 48 %, respectively. The result obtained from this research indicated that PheDH from *A. lwoffii* shows high possibility to be used for synthesis of L-phenylalanine and related amino acids such as valine, norleucine, methionine, norvaline, leucine, α-aminobutyrate and isoleucine. These amino acids were used in animal nutrition, human medicine and the pharmaceutical industries. For example, L-leucine, L-valine, L-isoleucine are used as food and feed activities (Gu and Chang, 1990).

CHAPTER V

CONCLUSION

1. The *phedh* gene from *Acinetobacter lwoffii* was cloned into *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS using the expression vector, pET-17b. The recombinant plasmid (pALPheDH) contained the inserted fragment of *phedh* gene with open reading frame of 1,143 bp which encoded the polypeptide of 380 amino acid residues.

2. Comparison of the nucleotide sequence compared with those in the EMBL-GenBank-DDBL database showed 82, 66, 64, 53, and 9% homology with *phedh* gene of *Bacillus sphaericus, Bacillus badius, Sporosarcina ureae, Thermoactinomyces intermedius, and Rhodococcus* sp. M4, respectively.

3. The percentage of identical deduced amino acids of the enzyme compared with PheDH from *B. sphaericus, B. badius, S. ureae, T. intermedius, and Rhodococcus* sp. M4, were 88, 68, 62, 51, and 32 %, respectively.

4. The recombinant clones showed various levels of the specific activity from 0.81 – 4.46 units/mg protein. The highest specific activity of pALPheDH was 55.75 fold higher than that of *A. lwoffii*.

5. The optimum condition of *phedh* gene expression was induction with 0.4 mM IPTG for 8 hours.

6. Stability of *phedh* gene expression of recombinant clone that showed the high PheDH activity was studied by daily subculturing for 20 days. Each subculture showed varied expression level of *phedh* gene. Expression of *phedh* gene was decreased, with increasing number of subculture.

7. PheDH from pALPheDH clone was purified by 50-70 % saturated ammonium sulfate precipitation and DEAE-Toyopearl column chromatography with 29.45 % yield and 5.19 purification fold.

8. The enzyme showed the highest substrate specificity in the oxidative deamination on L-phenylalanine. In the reductive amination, the enzyme acted on α-ketocaproate, α-keto-γ-methiol-n-butyrate, α-ketovalerate and α-ketoisocaproate with 5.96, 4.12, 3.84 and 3.15 fold of its natural substrate, phenylpyruvate, respectively.

9. The stability of PheDH from pALPheDH clone was relatively decreased with increasing of incubation time and approximately half of the enzyme activity was lost after incubation for 12 hours and completely abolished after incubation at 30 ^{o}C for 40 hours. Therefore, the enzyme was appropriate for the amino acids production at 30 ^{o}C.

10. When PheDH from pALPheDH clone was used for the amino acids production using their corresponding keto acids as substrate. The product yield was in the range of 36.0 - 72.2 %.

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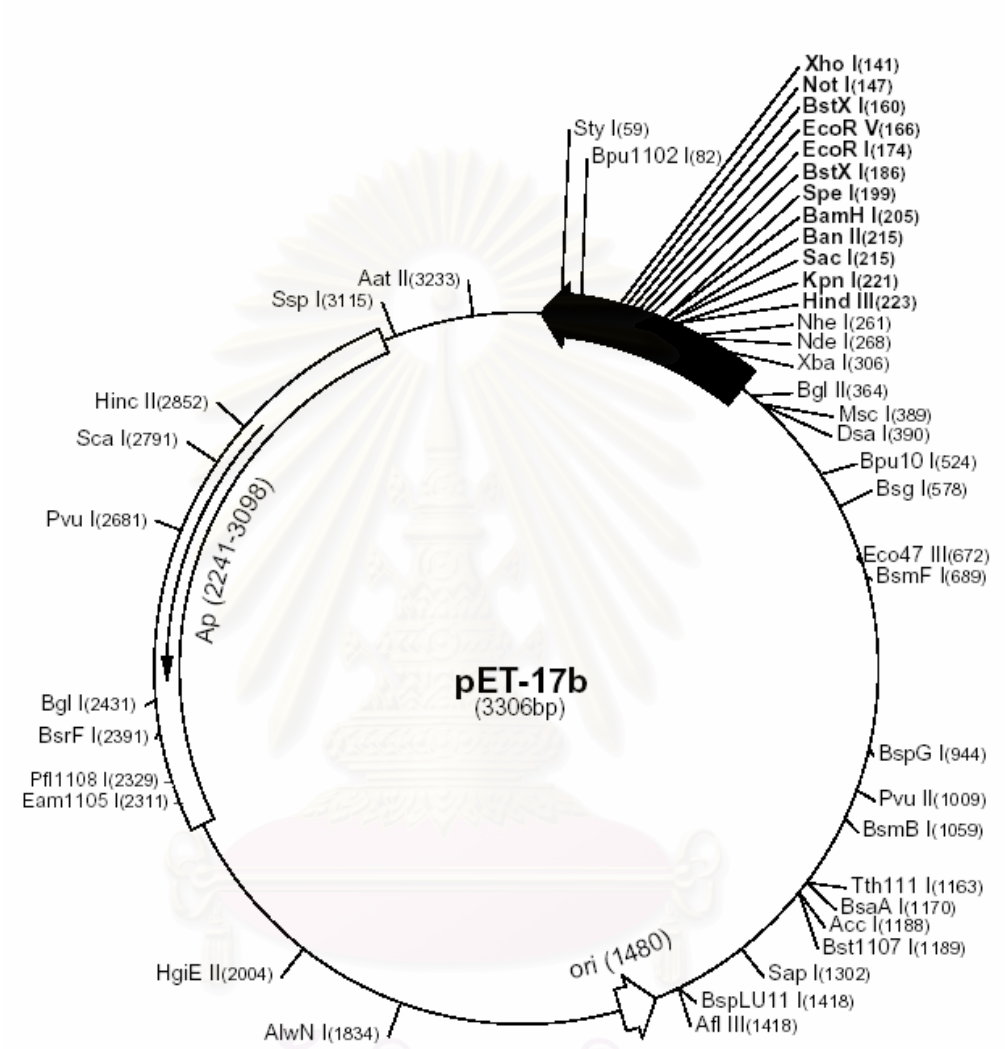
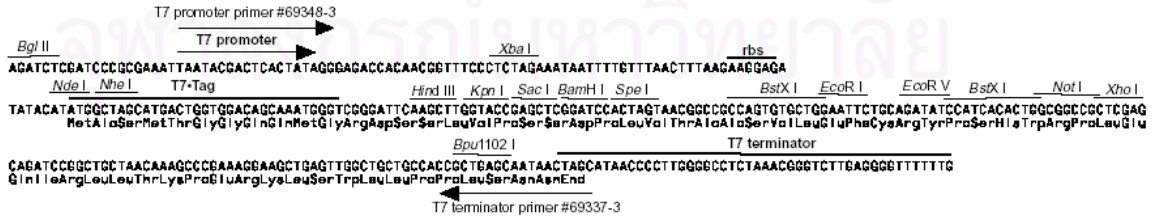
APPENDICES

APPENDIX A

Restriction map of pET-17b





pET-17b cloning/expression region

APPENDIX B

QIAquick® gel extraction kit protocol

1. The DNA fragment from the agarose gel was excised with a clean and sharp scalpel.
2. The gel slice was weighed in a colorless tube. Then, 3 volumes of buffer QG was added to 1 volume of gel (100 mg ~ 100 μl).
3. The gel was incubated at 50 ^{o}C for 10 min (or until the gel slice has completely dissolved) and mixed by vortexing the tube every 2-3 minutes during the incubation.
4. After the gel slice has dissolved completely, 1 gel volume of isopropanol was added to the sample and mixed.
5. QIAquick spin column was placed in a provided 2-ml collection tube.
6. To bind DNA, the sample was applied to the QIAquick column and centrifuged at 10,000xg for 1 minute.
7. The flow-through was discarded and QIAquick column was placed back in the same collection tube.
8. Then, 0.5 ml of buffer QG was added to QIAquick column and centrifuged at 10,000xg for1 minute.
9. Buffer PE 0.75 ml was added to QIAquick column to wash and further centrifuged at 10,000xg for 1 minute.
10. The flow-through was discarded and QIAquick column was centrifuged at 12,000xg for an additional 1 minute.
11. To elute DNA, 50 μl of buffer EB (10 mM Tris-Cl, pH 8.5) or H_2O was added to the center of QIAquick membrane and centrifuged at 10,000xg for 1 minute.

APPENDIX C

Preparation for protein determination

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

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

Potassium tartate	1.0	g
Copper sulfate	0.5	g

Dissolved in distilled water to 100 ml and adjust pH to 7.0

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

Sodium carbonate	20.0	g
Sodium hydroxide	4.0	g

Dissolved in distilled water to 1 liter

Solution C (phenol reagent)

Sodium tungstate	50.0	g
Sodium molybdate	12.5	g
85% phosphoric acid	25.0	g
Distilled water	350	ml
Hydrochlolic acid	50	ml

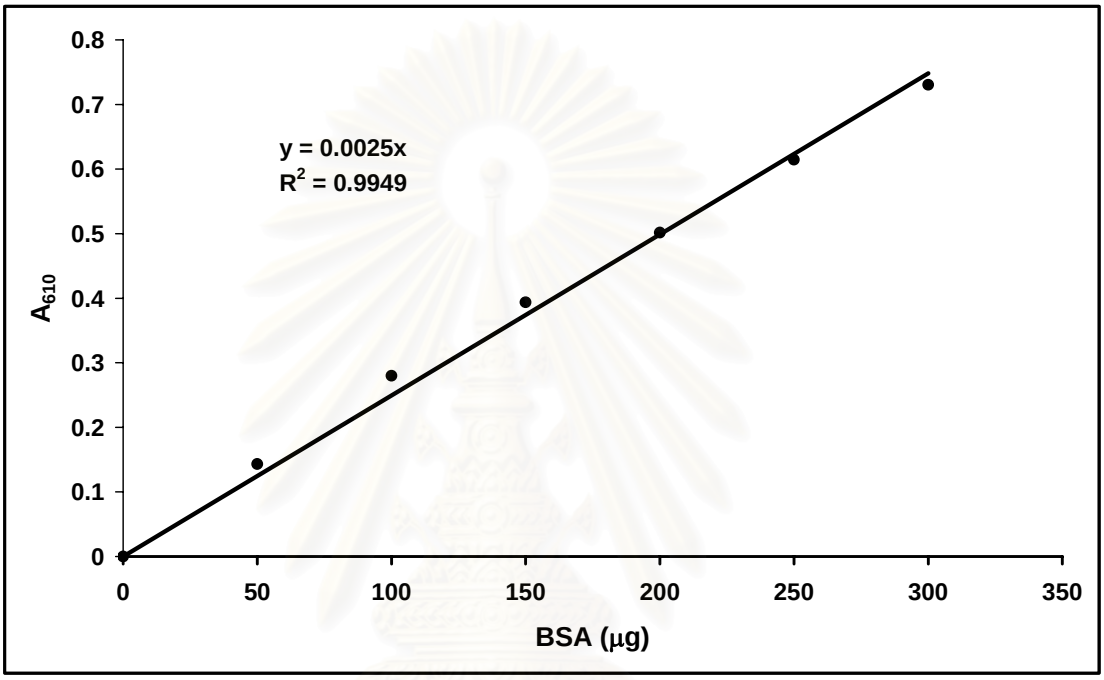
Reflex for 10 hours

Lithium sulphate	75.0	g
Distilled water	25	ml
Bromine solution	2-3	drops

The solution was boiled to reduce excess bromine for 15 minutes, then adjusted volume to 500 ml with distilled water and stored at 4 ^{o}C. The stock solution was diluted with distilled water in ration 1: 1 (V/V) before using.

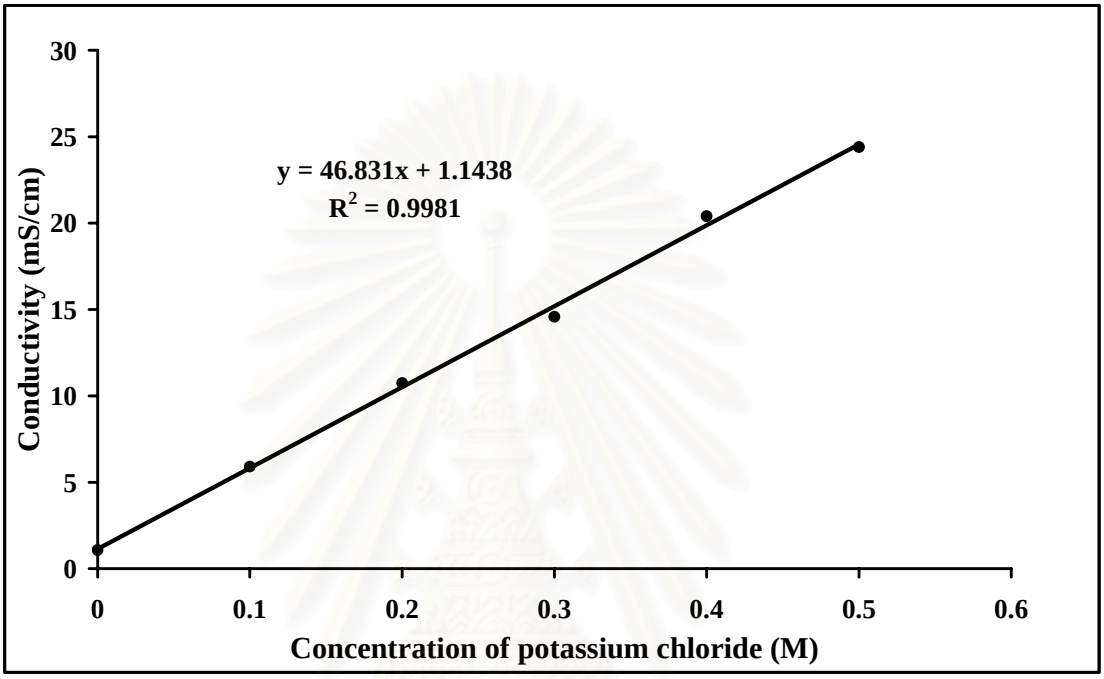
APPENDIX D

Standard curve for protein determination by Lowry' s method



APPENDIX E

Calibration curve for conductivity of potassium chloride



APPENDIX F

Preparation for nondenaturing gel electrophoresis

1. Working solutions

Solution A (30 % (W/V) acrylamide and 0.8 % (W/V) bis-acrylamide)

Acrylamide	29.2	g
Bis-acrylamide	0.8	g

Adjusted volume to 100 ml with distilled water and stirred until completely dissolved

Solution B (1.5 M Tris HCl pH 8.8)

Tris (hydroxymethyl)-aminomethane	18.2	g

Adjusted to pH 8.8 with HCl and make 100 ml with distilled water

Solution C (0.5 M Tris-HCl pH 6.8)

Tris (hudroxymethyl)-aminomethane	6	g

Adjusted to pH 6.8 with HCl and make 100 ml with distilled water

10 % Ammonium persulfate

Ammonium persulfate	0.5	g
Distilled water	5	ml

Electrophoresis buffer (25 mM Tris and 192 mM glycine)

Tris (hydroxymethyl)-aminomethane	3	g
Glycine	14.4	g

Dissolved and adjusted to total volume 1 litre with distilled water (pH should be ~ 8.3)

5 x Sample buffer (312.5 mM Tris-HCl pH 6.8, 50 % glycerol and 0.05 % bromophenol blue)

1M Tris-HCl (pH 6.8)	3.1	ml
100 % glycerol	5.0	ml
1 % Bromophenol blue	0.5	ml
Distilled water	1.4	ml

2. Native-PAGE

7.7 % Separating gel

Solution A	2.6	ml
Solution B	2.5	ml
Distilled water	4.9	ml
10% Ammonium persulfate	50	µl
TEMED	10	µl

5 % stacking gel

Solution A	0.67	ml
Solution C	1.0	ml
Distilled water	2.3	ml
10 % Ammonium persulfate	30	µl
TEMED	5	µl

3. 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

4. Enzyme activity staining solution

1 M Tris-HCl, pH 8.5

Tris (hydroxymethyl)-aminomethane	6.06	g

Adjusted to pH 8.5 with 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

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

Appendix G

Preparation for denaturing polyacrylamide gel electrophoresis

1. Stock solutions

2 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane 24.2 g

Adjusted to pH 8.8 with HCl and make 100 ml with distilled water

1 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane 12.1 g

Adjusted to pH 6.8 with HCl and make 10 ml with distilled water

10 % SDS (W/V)

Sodium dodecyl sulfate (SDS) 10 g

Added distilled water to a total volume of 100 ml

50 % Glycerol (W/V)

100 % glycerol 50 ml

Added distilled water to a totaol volume of 100 ml

1 % Bromophenol blue (W/V)

Bromophenol blue 100 mg

Added distilled water to a total volume of 10 ml

2. Working solutions

Solution A (30 % (W/V) acrylamide and 0.8 % (W/V) bis-acrylamide)

Acrylamide 29.2 g

Bis-acrylamide 0.8 g

Adjusted volume to 100 ml with distilled water and stirred until completely dissolved

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

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

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

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

10 % Ammonium persulfate

Ammonium persulfate	0.5	g
Distilled water	5	ml

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

Tris (hydroxymethyl)-aminomethane	3	g
Glycine	14.4	g
SDS	1.0	g

Dissolved and adjusted to total volume 1 litre with distilled water (pH should be ~ 8.3)

5 x Sample buffer (60 mM Tris-HCl pH 6.8, 25 % glycerol, 2 % SDS, 0.1 % bromophenol blue and 14.4 mM 2-mercaptoethanol)

1M Tris-HCl (pH 6.8)	0.6	ml
50 % glycerol	5.0	ml
10 % SDS	2.0	ml
1 % Bromophenol blue	1.0	ml
2-mercaptoethanol	0.5	ml
Distilled water	0.9	ml

3. SDS-PAGE

10 % Separating gel

Solution A	3.3	ml
Solution B	2.5	ml
Distilled water	4.2	ml
10 % Ammonium persulfate	50	µl
TEMED	5	µl

5 % stacking gel

Solution A	0.67	ml
Solution C	1.0	ml
Distilled water	2.3	ml
10 % Ammonium persulfate	30	µl
TEMED	5	µl

4. Protein staining solution

Staining solution, 1 liter

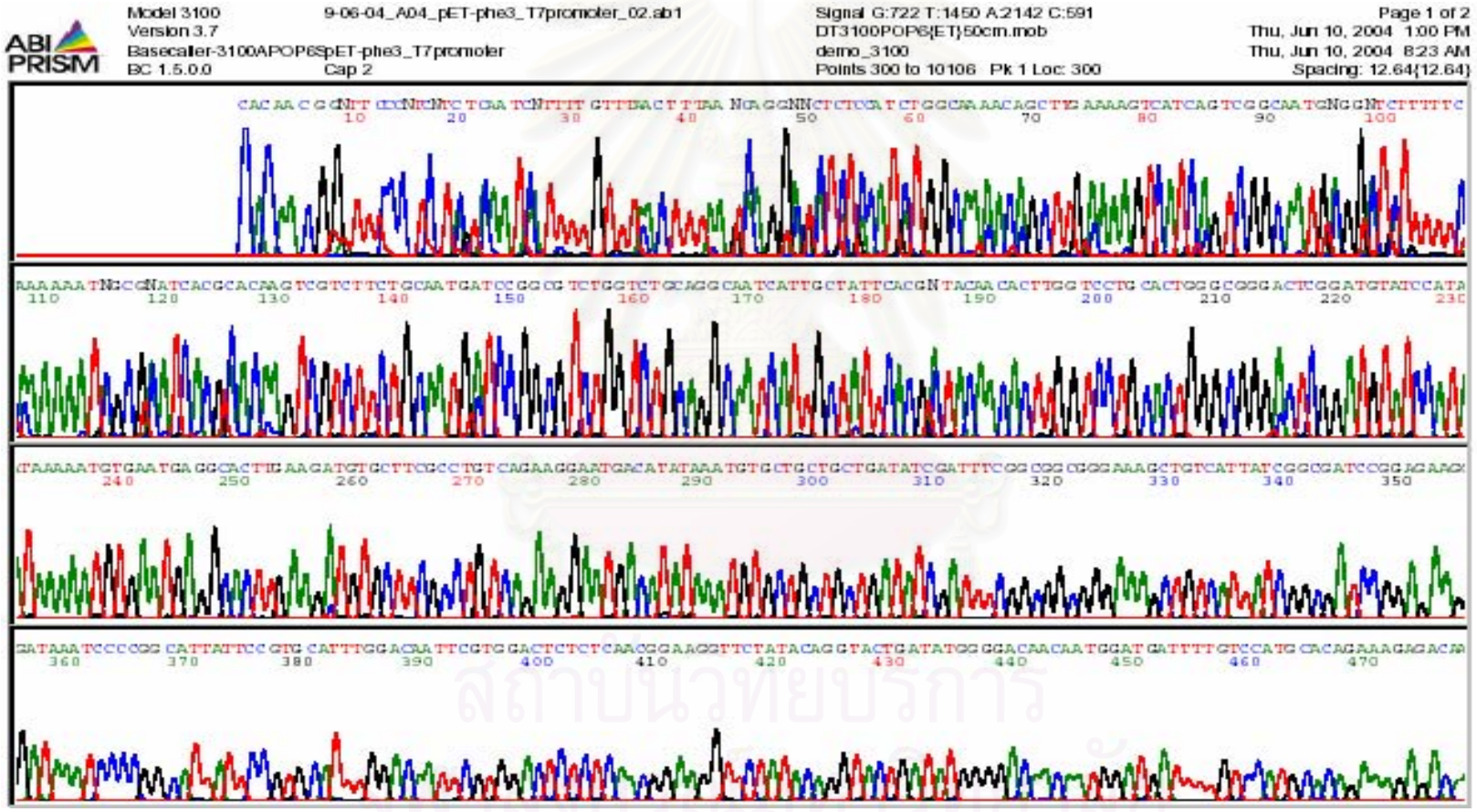
Coomassie brilliant blue R-250	1.0	g
Glacial acetic acid	100	ml
Methanol	450	ml
Distilled water	450	ml

Destaining solution, 1 liter

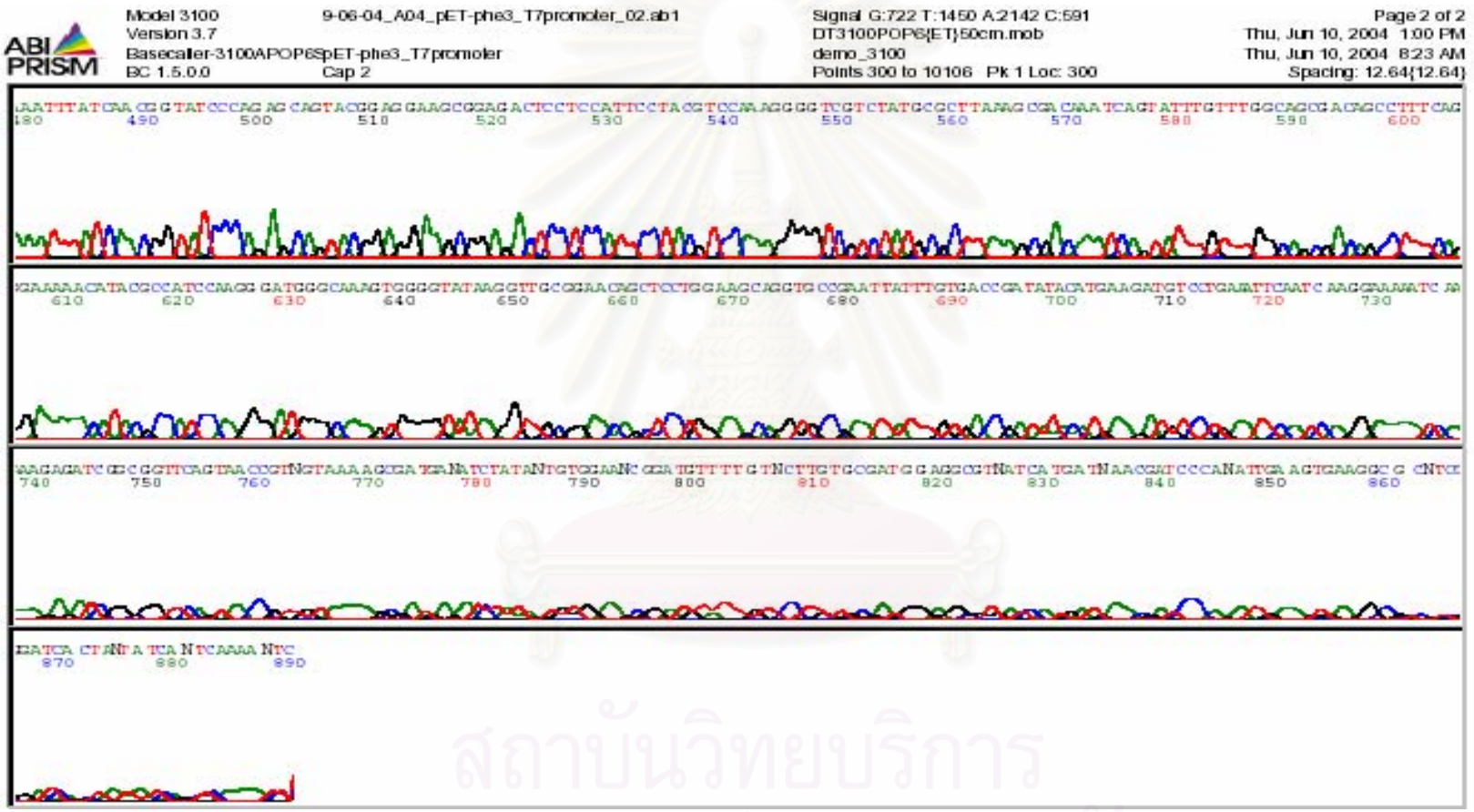
Methanol	100	ml
Glacial acetic acid	100	ml
Distilled water	800	ml

Appendix H

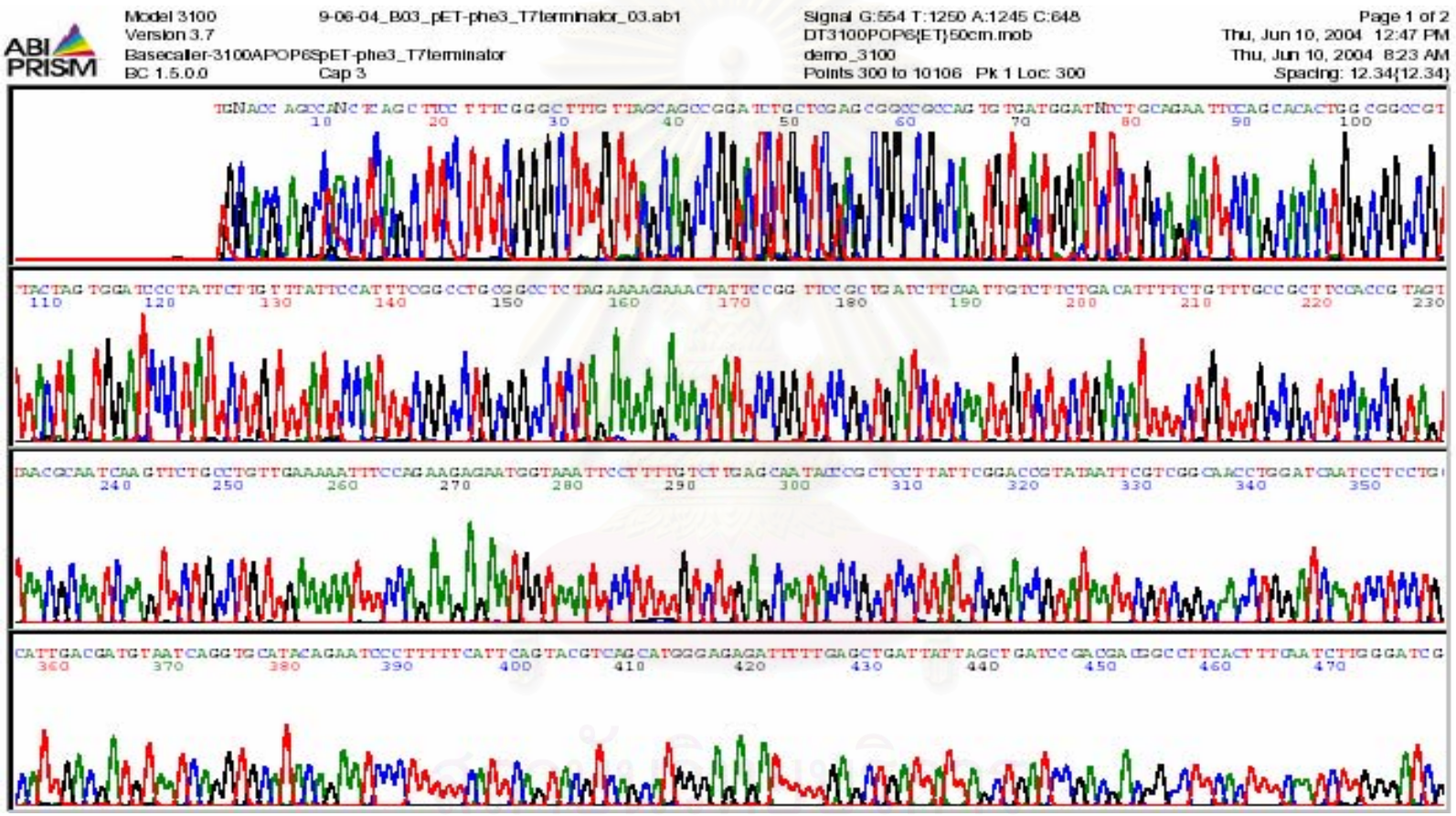
The DNA sequencing profiles of the phenylalanine dehydrogenase gene from *Acinetobacter lwoffii*



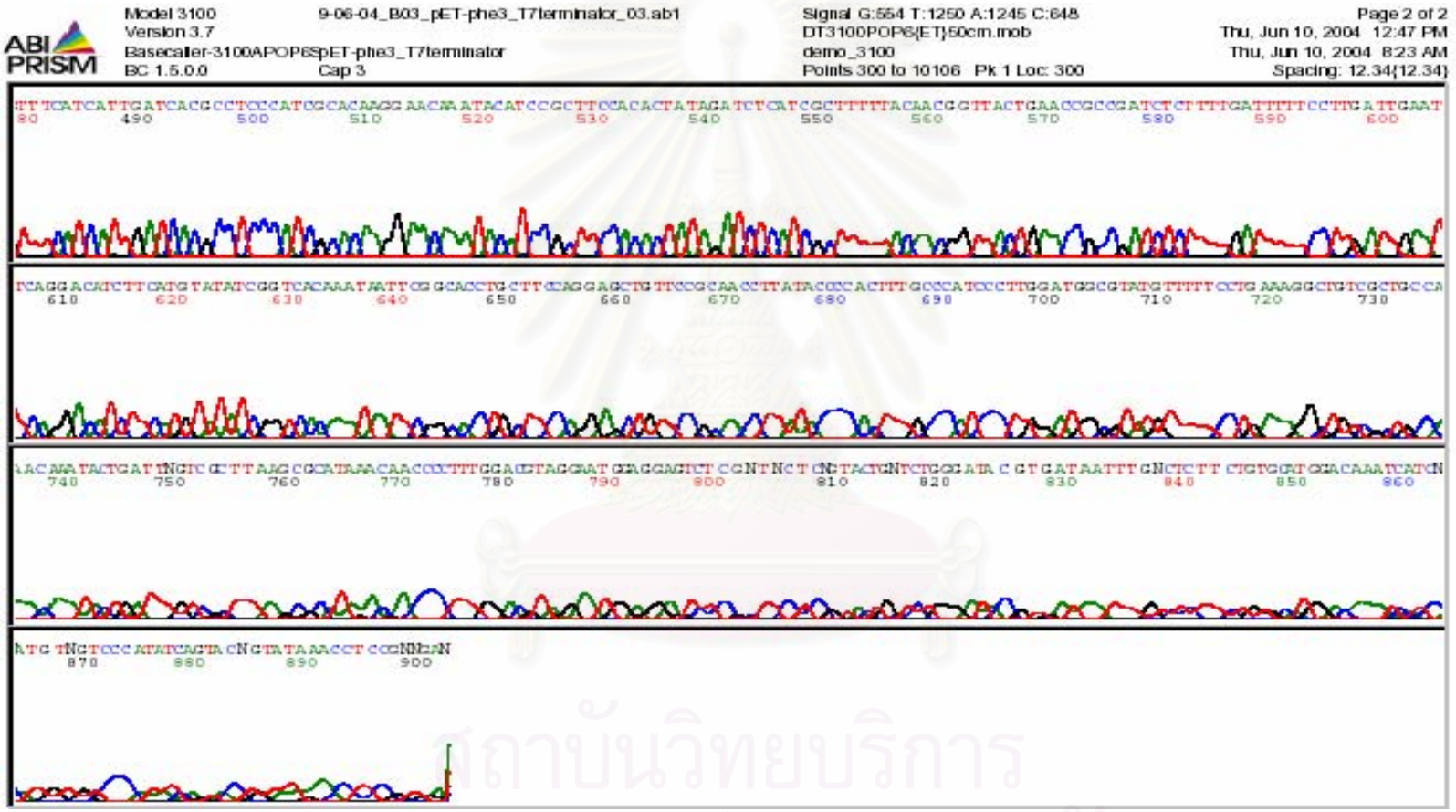
a) The DNA sequencing profile of the 5′-terminal gene fragment of pALPheDH: T7 promoter primer



a) The DNA sequencing profile of the 5′-terminal gene fragment of pALPheDH: T7 promoter primer (continue)



b) The DNA sequencing profile of the 3′-terminal gene fragment of pALPheDH: T7 terminator primer



b) The DNA sequencing profile of the 3′-terminal gene fragment of pALPheDH: T7 terminator primer (continue)

APPENDIX I

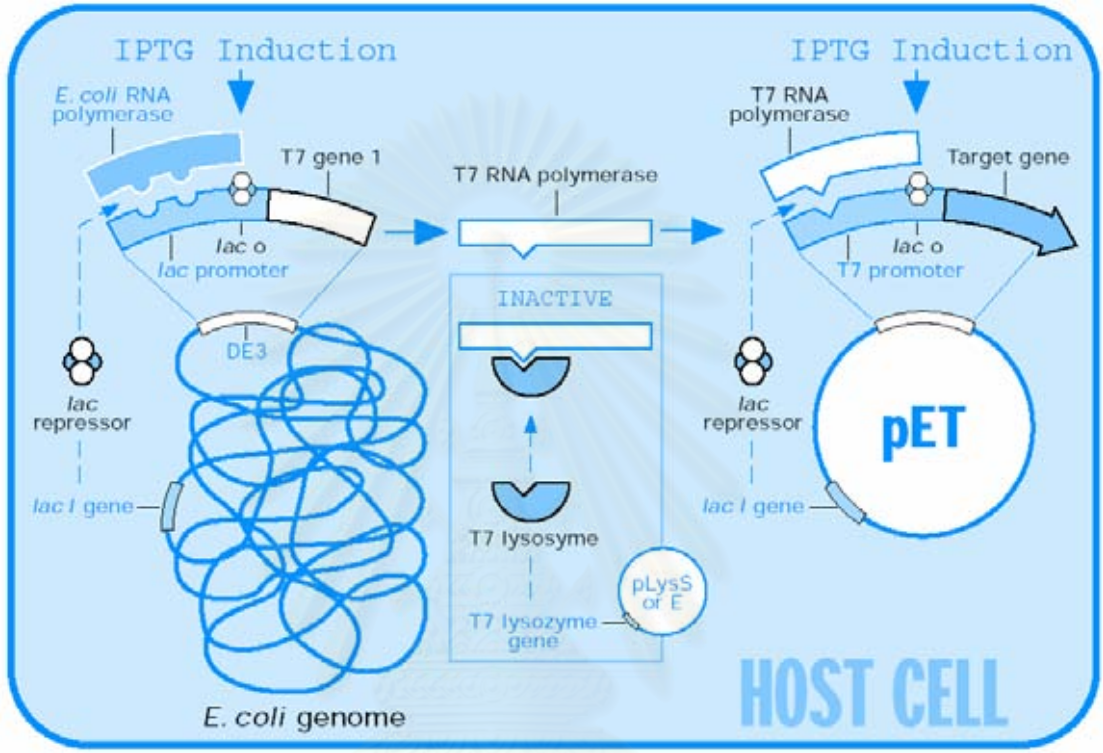
Abbreviation for amino acid residues

Amino acid	3 Letters-Abbreviation	1-Letter-Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Source: Voet, 2004

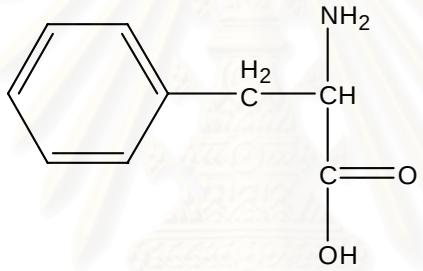
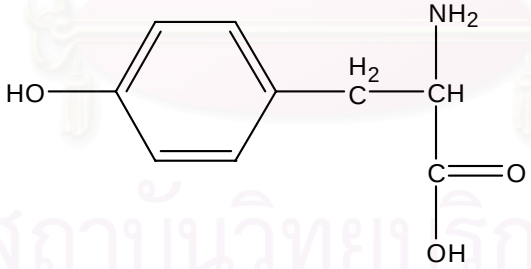
APPENDIX J

Control element of the pET system

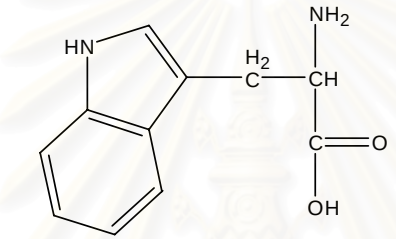
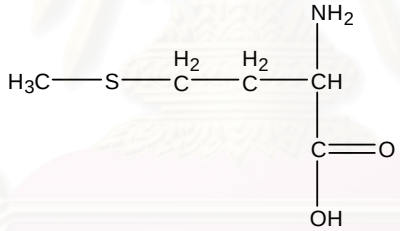
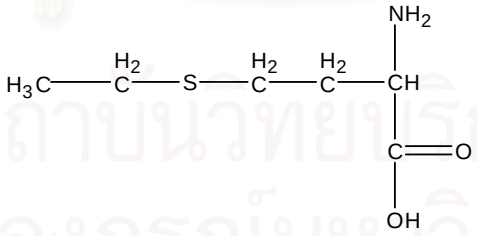


APPENDIX K

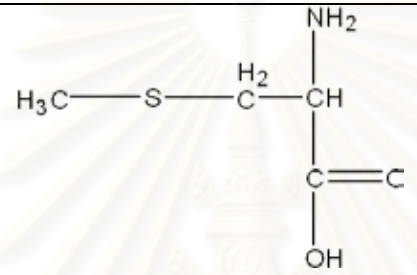
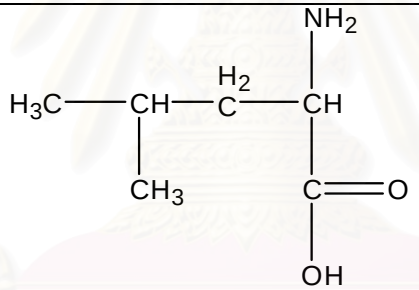
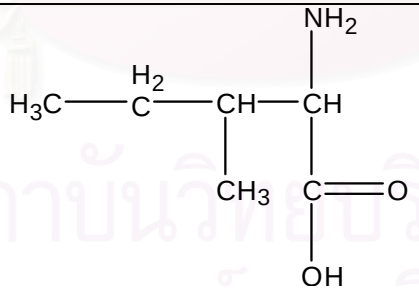
Amino acids and their effect as substrate

Chemical	Structure	Substrate[a]
L-phenylalanine		Y
L-tyrosine		Y

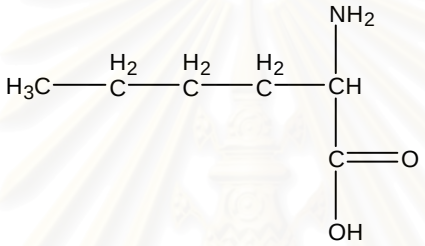
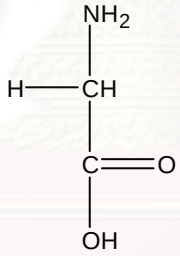
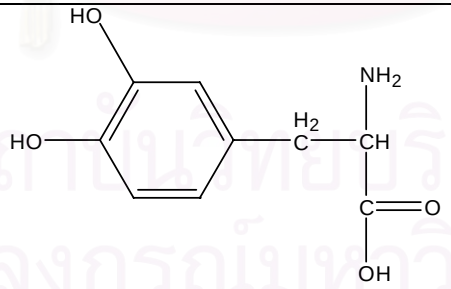
(continue)

Chemical	Structure	Substrate[a]
L-tryptophan		Y
L-methionine		Y
L-ethionine		Y

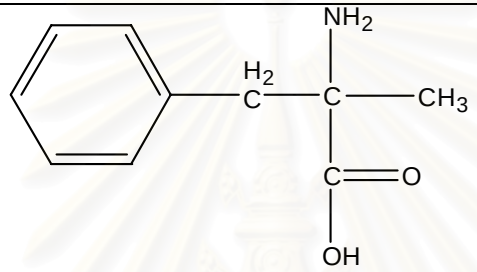
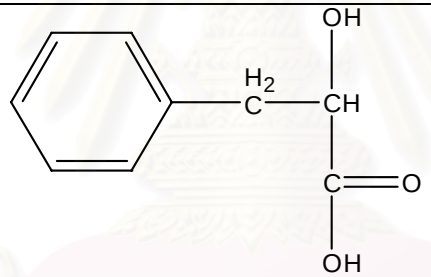
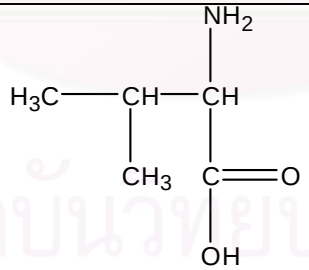
(continue)

Chemical	Structure	Substrate[a]
S-methyl-L-cysteine		Y
L-leucine		Y
L-isoleucine		Y

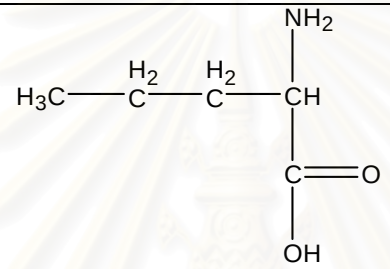
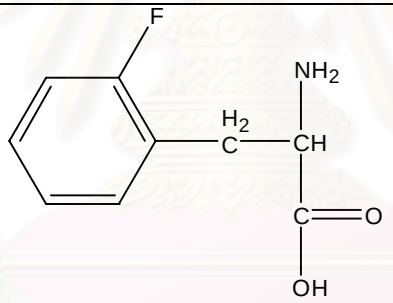
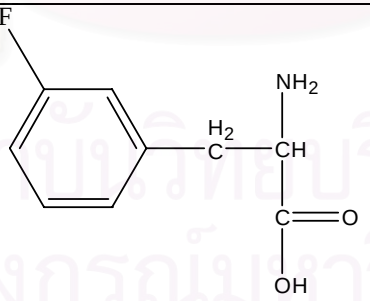
(continue)

Chemical	Structure	Substrate[a]
L-norleucine		Y
DL-allylglycine		Y
L-3, 4-dihydroxyphenylalanine (L-DOPA)		Y

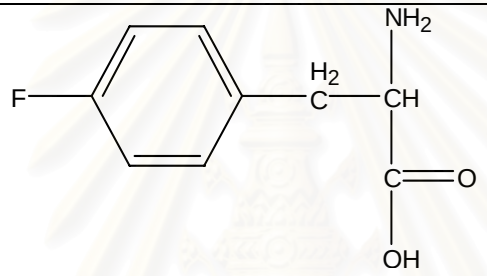
(continue)

Chemical	Structure	Substrate[a]
α-methyl-DL-phenylalanine		N
L-3-phenyllactate		N
L-valine		Y

(continue)

Chemical	**Structure**	**Substrate**[a]
L-norvaline		Y
o-fluoro-DL-phenylalanine		Y
m-fluoro-DL-phenylalanine		Y

(continue)

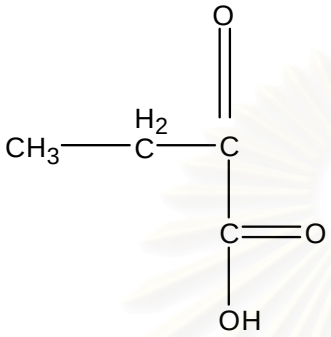
Chemical	**Structure**	**Substrate**[a]
p-fluoro-DL-phenylalanine		Y

[a] Substrate of the pALPheDH phenylalanine dehydrogenase. The result was obtained from substrate specificity on oxidative deamination experiment (section 3.7.1)

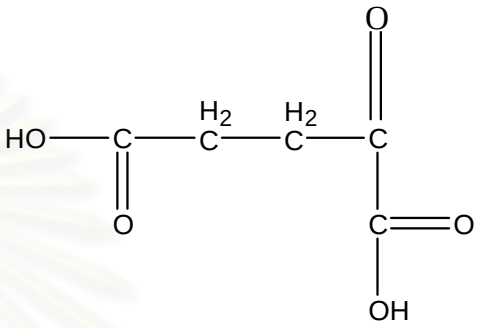
Y: yes and N: No

APPENDIX L

Keto acid structures



α- keto-n-butyrate



α- ketoglutarate

APPENDIX M

Amino acids and their corresponding keto acids

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

Source: Cooper *et al*., 1983

BIOGRAPHY

Mr Parkpoom Sitthai was born on July 31, 1979. He finished High school at Mahidol Wittayanusorn school, Nakhon Pathom. He graduated with the B. Sc. in Biotechnology from Faculty of Engineering and Industrial Technology, Silpakorn University in 2001. He has studied for Master degree in Biochemistry, Faculty of Science at Chulalongkorn University since 2002.