

การโคลนและการแสดงออกของยีนฟีนอลอะลานีนคีไฮโดรจีเนสจาก *Bacillus lentus*



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CLONING AND EXPRESSION OF
PHENYLALANINE DEHYDROGENASE GENE
FROM *Bacillus lentus*

Miss Mayura Thongchuang

A Thesis Submitted in Partial Fulfillment of the Requirements
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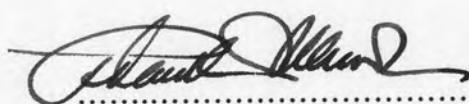
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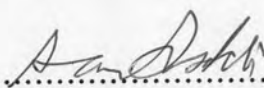
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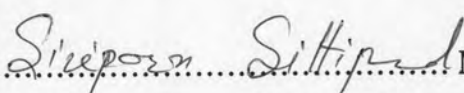
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มยุรา ทองช่วง : การโคลนและการแสดงออกของยีนฟีนิลอะลานินดีไฮโดรจิเนสจาก *Bacillus lentus*.
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ฟีนิลอะลานินดีไฮโดรจิเนส (EC 1.4.1.20) เร่งปฏิกิริยาการดึงหมู่อะมิโนจากแอล-ฟีนิลอะลานินให้ผลิตภัณฑ์เป็นฟีนิลไพรูเวทและแอมโมเนีย ซึ่งเป็นปฏิกิริยาที่ผันกลับได้และใช้ไพริดีนนิวคลีโอไทด์เป็นโคเอนไซม์ กลุ่มวิจัยของเราได้ศึกษาฟีนิลอะลานินดีไฮโดรจิเนสจาก *Bacillus lentus* และพบว่าเอนไซม์มีความจำเพาะสูงต่อแอล-ฟีนิลอะลานินซึ่งเป็นซับสเตรตในปฏิกิริยา oxidative deamination และฟีนิลไพรูเวทซึ่งเป็นซับสเตรตในปฏิกิริยา reductive amination นอกจากนี้ยังมีความเสถียรต่ออุณหภูมิสูงโดยไม่สูญเสียแอกติวิตีเมื่อบ่มเอนไซม์ที่ 50 องศาเซลเซียส เป็นเวลา 4 ชั่วโมงและเอนไซม์ยังคงมีแอกติวิตีเหลืออยู่ 50 เปอร์เซ็นต์เมื่อบ่มที่อุณหภูมิเดียวกันนี้เป็นเวลา 3 วัน ดังนั้นงานวิจัยนี้จึงนำลำดับกรดอะมิโนบางส่วนภายในสายเอนไซม์จำนวน 3 สายมาใช้เป็นข้อมูลในการออกแบบไพรเมอร์เพื่อเพิ่มชิ้นชิ้นส่วนของฟีนิลอะลานินดีไฮโดรจิเนสด้วยเทคนิคพีซีอาร์ และหาลำดับนิวคลีโอไทด์ของชิ้นชิ้นทั้งชิ้นด้วยเทคนิค inverse PCR จากนั้นโคลนยีนฟีนิลอะลานินดีไฮโดรจิเนสจาก *B. lentus* เข้าสู่ *E. coli* BL21(DE3) โดยใช้ expression vector (pET-17b) เมื่อวิเคราะห์สารละลายเอนไซม์หายบ่มที่ได้จากรีคอมบีแนนท์โคลนพบว่ามีความเสถียรสูงกว่าเอนไซม์จาก *B. lentus* 92.0 เท่า ภาวะที่เหมาะสมในการเหนี่ยวนำให้เกิดการแสดงออกของยีนฟีนิลอะลานินดีไฮโดรจิเนสคือ การเหนี่ยวนำด้วย IPTG 0.2 มิลลิโมลาร์เป็นเวลา 8 ชั่วโมง การทดสอบความเสถียรของการแสดงออกของยีนฟีนิลอะลานินดีไฮโดรจิเนสโดยการเพาะเชื้อต่อช่วงรีคอมบีแนนท์โคลน 50 ครั้ง พบว่าการแสดงออกของยีนฟีนิลอะลานินดีไฮโดรจิเนสยังคงเหลือถึง 57.7 เปอร์เซ็นต์ของเชื้อเริ่มต้นเมื่อทำเอนไซม์ให้บริสุทธิ์ด้วยการตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟต คอลัมน์ดีเอไอโทโยเฟิร์ล และคอลัมน์บิวทิลโทโยเฟิร์ล พบว่าเอนไซม์มีแอกติวิตีคงเหลือ 31.0 เปอร์เซ็นต์และมีความบริสุทธิ์ขึ้น 2.11 เท่า เอนไซม์มีน้ำหนักโมเลกุลประมาณ 340,000 คาลคันประกอบด้วย 8 หน่วยย่อยที่มีน้ำหนักโมเลกุลเท่ากัน pH ที่เหมาะสมในการเร่งปฏิกิริยา oxidative deamination และ reductive amination คือ 10.7 และ 7.8 ตามลำดับ และอุณหภูมิที่เหมาะสมในการเร่งปฏิกิริยาคือ 45 และ 50 องศาเซลเซียส เอนไซม์มีความเสถียรต่อ pH ในช่วง 7.0 ถึง 11.0 เอนไซม์มีค่า K_m ต่อแอล-ฟีนิลอะลานิน NAD^+ ฟีนิลไพรูเวท แอมโมเนีย และ NADH เท่ากับ 0.45, 0.40, 0.15, 48 และ 0.15 มิลลิโมลาร์ ตามลำดับ

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MAYURA THONGCHUANG : CLONING AND EXPRESSION OF PHENYLALANINE
DEHYDROGENASE GENE FROM *Bacillus lentus*. THESIS ADVISOR : ASST. PROF.
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NAD⁺-dependent phenylalanine dehydrogenase (EC 1.4.1.20) catalyzes the reversible oxidative deamination of L-phenylalanine to form ammonia, phenylpyruvate and NADH. Our research group has studied phenylalanine dehydrogenase from *Bacillus lentus* and found that the enzyme had high substrate specificity in the oxidative deamination on L-phenylalanine and the reductive amination on phenylpyruvate. No loss of the enzyme activity was observed upon incubation at 50°C for 4 hours. The enzyme retained 50% of the activity after incubation at the same temperature for 3 days. Therefore, in this research, the amino acid sequence of 3 internal peptide fragments of phenylalanine dehydrogenase were determined and used for degenerated primer design. The nucleotide sequencing of phenylalanine dehydrogenase gene (*phedh*) was investigated by inverse PCR and then the whole gene was cloned and expressed in *E. coli* BL21(DE3) using expression vector, pET-17b. The specific activity of crude recombinant enzyme was 92.0 fold higher than that of the enzyme from *B. lentus*. The optimum condition for *phedh* gene expression was induction with 0.2 mM IPTG for 8 hours. In spite of daily subculture for 50 days, the *phedh* gene expression in *E. coli* BL21(DE3) remained 57.7% of that of the parent. Recombinant enzyme was purified 2.11 fold with a 31.0% yield by procedure involving 40-50% ammonium sulfate precipitation, DEAE-Toyopearl and Butyl-Toyopearl column chromatography. The enzyme had a molecular mass about 340 kDa and consisted of 8 identical subunits. The optimum pHs for the oxidative deamination and the reductive amination were 10.7 and 7.8, respectively and optimum temperatures were 45°C and 50°C, respectively. The enzyme was stable over a broad pH range from 7.0 to 11.0. The apparent K_m values for L-phenylalanine, NAD⁺, phenylpyruvate, NH₄Cl and NADH were 0.45, 0.40, 0.15, 48 and 0.15 mM, respectively.

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ABBREVIATIONS

A	absorbance, 2'-deoxyadenosine (in a DNA sequence)
ADP	adenine dinucleotide phosphate
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)
°C	degree Celsius
Da	Dalton
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
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)
NADP^+	nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
ng	nanogram
nm	nanometer
NH_4Cl	ammonium chloride
$(\text{NH}_4)_2\text{SO}_4$	ammonium sulfate
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
<i>phedh</i>	phenylalanine dehydrogenase gene
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	<i>N, N, N', N'</i> -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