การทำให้บริสุทธิ์และลักษณะสมบัติของลูซีนดีไฮโดรจิเนส จาก *Alcaligenes faecalis*

นางสาวสุภัจรี เรืองสมวงศ์

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PURIFICATION AND CHARACTERIZATION OF LEUCINE DEHYDROGENASE FROM *Alcaligenes faecalis*

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สุภัจรี เรืองสมวงศ์ : การทำให้บริสุทธิ์และลักษณะสมบัติของลูซีนดีไฮโดรจีเนสจาก *Alcaligenes faecalis* (PUREIFICATION AND CHARACTERIZATION OF LEUCINE DEHYDROGENASE FROM *Alcaligenes faecalis*) อ.ที่ปรึกษา: ผศ.ดร.กนกทิพย์ ภักดีบำรุง, 145 หน้า ISBN 974-14-32-66.

การคัดเลือกแบคทีเรียในดินที่สามารถผลิตลูซีนดีไฮโดรจิเนส พบว่าไอโซเลทที่มีค่าแอคติวิตีของเอนไซม์ชนิดนี้สูงสุด คือ *Alcaligenes faecalis* ภาวะที่เหมาะสมในการผลิตเอนไซม์ชนิดนี้ คือ การเลี้ยงแบคทีเรียในอาหารอุดมเปปโตน 1 เปอร์เซ็นต์ pH 7.2 ที่อุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 24 ชั่วโมง จากการทำเอนไซม์ให้บริสุทธิ์โดยเทคนิคโครมาโตกราฟฟีด้วยคอลัมน์ดีอีเออีโทโยเพิร์ล คอลัมน์บิวทิลโทโยเพิร์ล และคอลัมน์ไฮแทรปคิว พบว่าเอนไซม์มีแอคติวิตีคงเหลือ 16.4 เปอร์เซ็นต์ และบริสุทธิ์ขึ้น 66.4 เท่า เอนไซม์มีน้ำหนักโมเลกุลประมาณ 536,000 ดาลตัน และประกอบด้วย 10 หน่วยย่อยที่มีน้ำหนักโมเลกุลเท่ากันคือประมาณ 52,000 ดาลตัน ในปฏิกิริยา oxidative deamination เอนไซม์มีความจำเพาะต่อสับสเตรทแอล-วาลีนและแอล-ไอโซลูซีนมากกว่าแอล-ลูซีน 1.33 และ 1.13 เท่า ตามลำดับ และจำเพาะต่อแอล-แอลฟา-คีโตไอโซวาลีเรต แอลฟา-คีโตวาลีเรต และ แอลฟา-คีโต-บีตา-เมทิลวาลีเรตมากกว่าแอลฟา-คีโตไอโซคาโปรเอต 2.08, 1.35 และ 1.23 เท่า ตามลำดับ ในปฏิกิริยา reductive amination เอนไซม์มีความจำเพาะต่อโคเอนไซม์ 3-อะเซทิลไพริดีน-อะดีนีนไดนิวคลีโอไทด์มากกว่า NAD^+ 4.78 เท่า ในการเร่งปฏิกิริยา oxidative deamination และ reductive amination pH ที่เหมาะสม คือ 10.8 และ 8.8 อุณหภูมิที่เหมาะสม คือ 45 องศาเซลเซียส และ 55 องศาเซลเซียส ตามลำดับ เอนไซม์มีความเสถียรต่อ pH ในช่วง 6.0 ถึง 12.0 และมีความเสถียรที่อุณหภูมิ 45 องศาเซลเซียส นาน 8 ชั่วโมง เอนไซม์ถูกยับยั้งอย่างสมบูรณ์ด้วยเมอร์คิวริกคลอไรด์ที่ความเข้มข้นสุดท้าย 1 มิลลิโมลาร์ เมื่อดัดแปรกรดอะมิโน ทริปโตเฟน เมทไธโอนีน และไลซีนด้วย *N*-bromosuccinimide, chloramine T และ 2,4,6-trinitrobenzene sulfonic acid ตามลำดับที่ความเข้มข้นสุดท้าย 10 มิลลิโมลาร์ เป็นเวลา 20 นาที พบว่ามีผลทำให้เอนไซม์สูญเสียแอคติวิตีทั้งหมด ค่า K_m ของแอล-ลูซีน แอล-ไอโซลูซีน แอล-วาลีน NAD^+ NADH แอลฟา-คีโตไอโซคาโปรเอต และแอมโมเนีย เท่ากับ 4.2, 4.3, 13.67, 0.44, 0.02, 3.33 และ 100 มิลลิโมลาร์ ตามลำดับ การศึกษาจลนพลศาสตร์และการยับยั้งโดยผลิตภัณฑ์ของปฏิกิริยาแสดงให้เห็นว่ากลไกของปฏิกิริยา oxidative deamination เป็นแบบ sequential ordered binary-ternary mechanism ซึ่งมีลำดับของการจับกับสับสเตรท คือ NAD^+ จับกับเอนไซม์ก่อน ตามด้วยแอล-ลูซีนแล้วจึงปล่อยแอมโมเนีย แอลฟา-คีโตไอโซคาโปรเอต และ NADH ออกมาตามลำดับ ลำดับของกรดอะมิโนทางปลายอะมิโนคือ MEIFNYMEQADYEQLVIXQD และส่วนภายในสายโปรตีน 3 สาย คือ PGPXGPAGSKG (หรือ V) EPGPAGPXG, T (หรือ L,Y,V) LPGLAGTXG และ RDNIPSYVAADRLAEERIRVA

ภาควิชา.....ชีวเคมี..... ลายมือชื่อนิสิต.....สุภัจรี เรืองสมวงศ์.....

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

ปีการศึกษา.....2548..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....-.....

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KEY WORD : L- LEUCINE DEHYDROGENASE / *Alcaligenes faecalis* / PURIFICATION / CHARACTERIZATION

SUPATJAREE RUENGSOMWONG: PURIFICATION AND CHARACTERIZATION OF LEUCINE DEHYDROGENASE FROM *Alcaligenes faecalis*. THESIS ADVERSOR: ASST. PROF. KANOKTIP PACKDIBUMRUNG. Ph.D. 145 pp. ISBN 974-14-3266-6.

Leucine dehydrogenase producing bacteria were screened from soil sample and the isolate which produced the highest enzyme activity was identified as *Alcaligenes faecalis*. Optimum condition for L-leucine dehydrogenase production was cultivation in 1% peptone medium pH 7.2 at 37°C for 24 hours. After the enzyme was purified to homogeneity by DEAE-Toyopearl, Butyl-Toyopearl and Hitrap Q chromatography columns, % recovery and purification fold were 16.4 and 66.4, respectively. The enzyme had the molecular weight of 536,000 daltons and consisted of 10 identical subunits of 52,000 daltons. Substrate specificity of the enzyme on L-valine and L-isoleucine were 1.33 and 1.13 fold of that on L-leucine in oxidative deamination. In reductive amination, substrate specificity on α-ketoisovalerate, α-ketovalerate and α-keto-β-methylvalerate were 2.0, 1.35 and 1.23 fold of that on α-ketoisocaprorate. The coenzyme specificity on 3-acetylepyridine adinine dinucleotide was about 4.78 fold higher than NAD^+. The optimum pH for oxidative deamination and reductive amination were 10.8 and 8.8 where as optimum temperatures were 45°C and 55°C, respectively. The enzyme retained its full activity upon the incubation at 45°C for 8 hours. The enzyme activity was completely inhibited by $HgCl_2$ at final concentration of 1 mM. Chemical modification of the enzyme at tryptophan, methionine and lysine residues by incubation with 10 mM of group-specific reagents: *N*-bromosuccinimide, chloramine T and 2,4,6-trinitrobenzenesulfonic acid, respectively, led to completely loss of enzyme activity. The apparent K_m values for L-leucine, L-isoleucine, L-valine, NAD^+, NADH, α-ketoisocaproate and ammonia were 4.2, 4.3, 14.0, 0.44, 0.02, 3.33 and 100 mM, respectively. The steady state kinetic studies including product inhibition on the enzyme reaction indicated that the oxidative deamination proceeds through a sequential ordered binary-ternary mechanism in which NAD^+ binds first to the enzyme followed by L-leucine and products are released in the order of ammonia, α-ketoisocaproate and NADH, respectively. N-terminal sequence was M E I F N Y M E Q A D Y E Q L V I X Q D and internal amino acid sequences of the enzyme were, PGPXGPAGSKG (or V) EPGPAGPXG, T (or L,Y,V) LPGLAGTXG and RDNIPSYVAADRLAEERIRVA.

Department.....Biochemistry..... Student's signature.....
Field of study.....Biochemistry..... Advisor's signature.....
Academic year .....2005..... Co-advisor's signature.....

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CONTENTS

Page

Page

Page

Page

LIST OF TABLES

Page

LIST OF FIGUERS

Page

CHAPTER I

CHAPTER III

Page

Page

CHAPTER IV

LIST OF ABBREVIATIONS

A	Absorbance
BSA	bovine serum albumin
C	degree Celsius
Cm	centimeter
CT	chloramine T
Da	Dalton
DEAE	diethylaminoethyl
DEPC	deithylpyrocarbonate
DTT	dithiothereitol
EDTA	ethylene diamine tetraacetic acid
g	gram
GluDH	glutamate dehydrogenase
GlyDH	glycine dehydrogenase
HPLC	high- performance liquid chlomatography
HCl	hydrochloric acid
IAM	iodoacetamide
Kb	kilobase pairs
KCl	potassium chloride
K_m	Michaelis constant
KOH	potassium hydroxide
KPB	potassium phosphate buffer
l	liter
LeuDH	leucine dehydrogenase
LysDH	lysine dehydrogenase
μg	microgram
μl	microliter
M	molar, mole per liter
mA	milliampere
mg	milligram
min	minute

mM	millimolar
mol	mole
MW	molecular weight
NAD^+	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NADP	nicotinamide adenine dinucleotide phosphate
NEM	*N*-ethylmaleimide
ng	nanogram
NAI	acetylimidazole
NaOH	sodium hydroxide
NBS	*N*-bromosuccinimide
$(NH_4)_2\ SO_4$	aminonium sulfate
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PG	phenylglyoxal
PheDH	phenylmethylsulfonyl fluoride
rpm	revolution per minute
SDS	sodium dodecyl sulfate
TEMED	*N*,*N*,*N*′,*N*′- tetramethyl ethylene diamine
TNBS	2,4,6- trinitrobenzene sulfonic acid
UV	Ultraviolet
V	Volt
ValDH	valine dehydrogenase
V/V	volume by volume
W/V	weight by volume

CHAPTER I

INTRODUCTION

All proteins, whether from the most ancient lines of bacteria or from the most complex forms of life, are constructed from the same ubiquitous set of 20 amino acids, covalently linked in characteristic linear sequences. For all the standard amino acids except glycine, the α carbon is bonded to four different groups: a carboxyl group, an amino group, an R group and a hydrogen atom. The α carbon atom is thus a chiral center. Nearly all biological compounds with a chiral center occur naturally in only one stereoisomeric form, either D or L. The amino acid residues in protein molecules are exclusively L stereoisomers. D-Amino acid residues have been found only in a few, generally small peptides, including some peptide of bacterical cell walls and certain peptide antibiotics. Cell are able to specifically synthesize the L isomers of amino acid because the active site of enzyme are asymmetric, causing the reaction they catalyze to be stereospecific. Amino acids can be simplified by grouping the amino acids into 5 main classes based on the properties of their R group, inparticular, their polarity; nonpolar / aliphatic, aromatic, polar/uncharged, positively charged and negatively charged R groups (Lehninger, 2000).

Amino acids are also precursors of glucose, fatty acid, and ketone bodies and 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).

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-isoleucine, and L-valine 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). 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

Amino acid dehydrogenases (EC class 1.4.1) catalyze the pyridine nucleotide coenzyme-dependent reversible deamination of amino acids to the corresponding keto acids, and provide an important metabolic route for the interconversion between inorganic and organic nitrogen compounds as shown in figure 1.1 (Oikawa *et al*., 2001). The amino acid dehydrogenases are categorized based on the specificity they display toward their amino acid substrates and more than ten kinds of them has been so far found in various kind of organisms as shown in Table 1.1. 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 enzyme acting on the substrate L-amino acids, which are important for pharmaceutical and dietary consumption purposes (Ohshima and Soda, 2000).

Leucine dehydrogenase (L-leucine: NAD^+ oxidoreductase, deaminating, EC 1.4.19) is one of the most interesting amino acid dehydrogenase that catalyzes the reversible deamination of L-leucine and several other aliphatic L-amino acids to their keto analogues, ammonia and NADH as shown in figure 1.2 (Katoh *et al*., 2003).

1.2 Isolation and purification of leucine dehydrogenase

In 1970, Hermier *et al.* studied leucine dehydrogenase (LeuDH) from sporulating cells of *Bacillus subtitis.* Seven years later, Ohshima *et al.* screened several bacteria to find out bacterial strains that would produce a high activity of leucine dehydrogenase. They found the high enzyme activity occurred mainly in *Bacillus* species, especially in *Bacillus sphaericus, B. cereus, B. megaterium* and *B. subtilis* and slight activity was found in *Corynebacterium sepedonicum*,

C. pseudodiphtheiticum and *Alcaligenes faecalis.* *B. sphaericus* (IFO 3525) in which LeuDH occurred most abundantly was chosen for the purpose of purification of the enzyme (Ohshima *et al*., 1978).

Furthermore, LeuDHs were purified from *Bacillus stearothermophilus* (Ohshima *et al*., 1978), *Clostridium thermoaceticum* (Shimoi *et al*., 1987), *Corynebacterium pseudodiphtheriticum* (Misono *et. al*., 1990), *Thermoactinomyces intermedius* (Ohshima *et al*., 1994) and *Natronobacterium magadii* MS-3 (Katoh *et al*., 2003).

General purification methods reported for leucine dehydrogenase were ammonium sulfate precipitation, protamine sulfate treatment, heat treatment, ion exchange chromatography (DEAE-cellulose, DEAE-sephacel and DEAE-Toyopearl 650 M column) adsorption chromatography (hydroxyapatite and gigapite column), gel filtration chromatography (Sephadex G-150, Sephadex G-200, Sephacryl S-200 and Superfine column) and affinity chromatography (Sepharose CL-4B). For example, Ohshima *et al*. (1978) purified LeuDH from *Bacillus sphaericus* using protamine sulfate treatment, DEAE-cellulose, hydroxyapatite and twice Sephadex G-150 column. Recently, Katoh *et al*. (2003) purified the enzyme from *Natronabacterium magadii* MS-3 with Whatman HB-1, Sepharose CL-4B and gigapite column.

1.3 Catalytic properties of leucine dehydrogenase

The summarized catalytic properties and basic molecule of leucine dehydrogenase from various microorganisms were shown in Table 1.2. LeuDHs do not share a common quaternary structure because the enzyme from *B. sphaericus, B. stearothermophilus , N. magadii* MS-3 are hexameric enzymes while the enzyme *B. lichemisformis* TSN9 and *B. cereus* are octameric enzymes and *Corynebacterium pseudodiphtheriticum* ICR 2210 enzyme is consisted of a single polypeptide (Misono *et al*., 1990). Since all amino acid dehydrogenases from including leucine dehydrogenase are NAD specific enzymes, LeuDH from *B. sphaericus* was tested for alternate nucleotide coenzymes. It was found that 3-acetylpyridine-NAD^+, an analog of NAD^+, was much better coenzyme than NAD^+ while 3-acetylepyridine-deamino-

NAD^+ and deamino-NAD^+ were similar to NAD^+ in coenzyme specificity (Ohshima *et al*., 1978). On the whole, the substrate specificity for forward and reverse reaction of the enzyme from various microoganisms were different. For example, L-α-aminobutyrate and *S*-methyl-L-cysteine were moderate substrates for LeuDH from *N. magadii* MS-3 (Katoh *et al*., 2003) while *B. sphaericus* (Ohshima *et al*., 1978) enzyme nearly could not catalyze on them. All LeuDHs act on α-ketoisovalerate better than α-ketoisocaproate, a reductive amination substrate. pH optima of amino acid dehydrogenases from various microorganisms are at quiet high alkaline pH range. Moreover, the enzyme was inhibited with sulfhydryl-modifying agents, especially $HgCl_2$ and *p*-chloromercuribenzoate, whereas metal chelating reagents did not inhibit enzyme activity. In addition, the enzyme of *Bacillus* could stand at over 50°C for a long times.

1.4 Catalytic mechanism and structure of leucine dehydrogenase

1.4.1 Catalytic mechanism

The reaction mechanism was expressed by product inhibition and kinetic studies that carried out to determine the order of substrate binding and product release. The kinetic analysis of LeuDH from *Bacillus sphaericus* (Ohshima *et al*., 1978), *Corynebacterium pseudodiphtheriticum* (Misono *et al*., 1990) and *Bacillus licheniformis* TSN9 (Nagata *et al*., 1995) identicated that NAD^+ binds first to the enzyme, followed by L-leucine, then ammonia, α-ketoisocaproate and NADH are released in this order from the enzyme, respectively, as shown in figure 1.3.

1.4.2 Structure

The X-ray crystallographic studies of several amino acid dehydrogenases expressed that the enzymes are composed of structurally independent coenzyme and substrate binding domains like other $NAD(P)^+$-dependent dehydrogenases such as lactatate and alcohol dehydrogenases. The three dimensional structures and amino

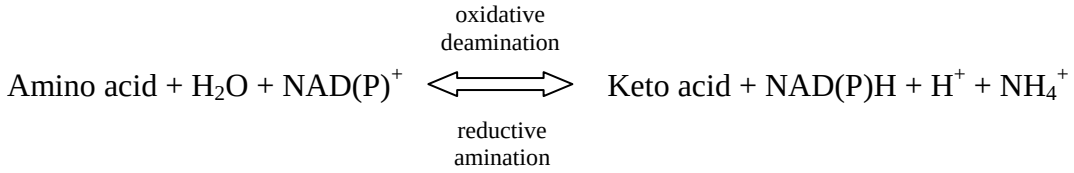


Figure 1.1 The typical reaction of L-amino acid dehydrogenase

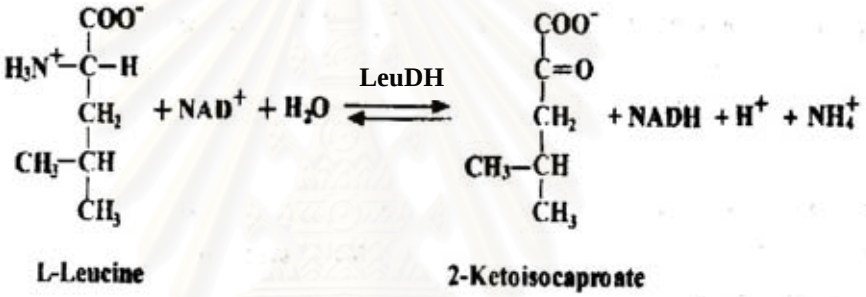


Figure 1.2 Leucine dehydrogenase reaction

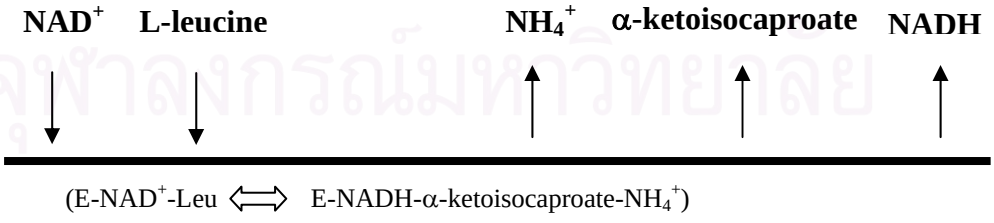


Figure 1.3 Kinetic mechanism of leucine dehydrogenase in *B. sphaericus*, *B. licheniformis* and *C. pseudodiphtheriticum*

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

EC number	Enzyme	Coenzyme	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 acid DH	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

Table 1.2 Leucine dehydrogenase properties from various microorganisms

Property	***Bacillus sphaericus***	***Bacillus licheniformis* TNS9**	***Bacillus cereus***	***Natronobacterium magadii* MS-3**	***Corynebacterium pseudodiphtheriticum***
Native M_r	245,000	360,000	310,000	330,000	34,000
Subunit M_r	41,000	44,000	39,000	55,000	34,000
No.of subunit	hexamer	octamer	octamer	hexamer	monomer
Optimum pH					
oxidative deamination	10.7	10.3	11.5	9.5	10.9
reductive amination	9.5	9.28	9.0	9.5	10.0
Optimum temperature					
oxidative amination	57°C	NR	60°C	65°C	NR
Thermostability (% remaining activity after incubation)	100%, 60°C, 5 mins	100%, 65°C, 60 mins in present of 2.5 M NaCl	98% , 50°C, 30 mins	100%,50°C, 60 mins in present of 2.5 M NaCl	100% , 35°C , 10 mins
Inhibitors	*p*-CMB [a] $HgCl_2$ Na_2S	NR	*p*-MB [b] $HgCl_2$ KCN	$HgCl_2$, $AgNO_3$	*p*-CMB [a], $HgCl_2$, 4,4-dithiopyridine
Substrate specificity					
oxidative deamination					
L-leucine	100	100	100	100	NR
L-isoleucine	58	72	61	72	NR
L-valine	74	59	61	59	NR
L-norvaline	41	NR	28	7	NR
L-norleucine	10	7	6	NR	NR
S-methyl-L-cysteine	1.4	19	0	19	NR
L-α-aminobutyrate	14	32	24	32	NR
L-alanine	0	10	NR	0	NR
L-serine	100	NR	NR	0	NR
L-proline	100	NR	NR	0	NR
reductive amination					
α-ketoisocaproate	100	100	100	100	NR
α-ketoisovalerate	126	140	154	150	NR
α-ketovalerate	76	96	51	96	NR
α-ketocaproate	46	69	51	69	NR
α-ketobutyrate	57	70	51	70	NR
phenylpyruvate	0	0	NR	NR	NR

Table 1.2 Leucine dehydrogenase properties from various microorganisms (continued)

Properties	***Bacillus sphaericus***	***Bacillus licheniformis* TNS9**	***Bacillus cereus***	***Natronobacterium magadii* MS-3**	***Corynebacterium pseudodiphtheriticum***
Apparent K_m (mM)					
oxidative deamination					
L-leucine	1.0	2.1	1.5	0.69	0.30
L-isoleucine	1.8	3.3	1.0	NR	0.81
L-valine	1.7	12.5	2.5	NR	0.57
NAD^+	0.39	0.31	0.34	0.48	0.10
reductive amination					
α-ketoisocaproate	0.31	1.0	0.45	4.0	0.40
α-ketoisovalerate	1.7	2.4	0.40	NR	0.56
α-ketovalerate	1.4	3.8	2.1	NR	0.45
ammonia	0.2	330	0.22	220	25
NADH	0.035	0.25	0.034	0.02	0.081

L-Glutamate, L-serine, L-threonine, L-aspatste, L-phenylalanine, L-lysine, L-tryptophan, L-arginine, D-leucine, D-isoleucine, D-valine, D-norvaline, D-norisoleucine, D-α-aminobutyrate, α-ketoglutarate, oxaloacetate and glyoxylarte were inert for leucine dehydrogenase from *Bacillus sphaericus*, *Bacillus licheniformis* TNS9, *Bacillus cereus* and *Natronobacterium magadii* MS-3.

[a] *p*-CMB = *p*-chloromercuribenzoate

[b] *p*-MB = *p*-mercuribenzoate

NR = no report

Sources: *Bacillus sphaericus* (Ohshima *et al*., 1978), *Bacillus licheniformis* TNS9 (Nagata *et al*., 1995), *Bacillus cereus* (SchÜtte *et al*., 1985), *Natronobacterium magadii* MS-3 (Katoh *et al*., 2003), *Corynebacterium pseudodiphtheriticum* (Misono *et al*., 1990)

acid sequences of leucine and phenylalanine dehydrogenases suggest that the N-terminal domain recognizes the substrate amino acid and the C-terminal domain binds the pyridine nucleotide cofactor (Oikawa *et al*., 2000).

The leucine dehydorgenases from *Bacillus stearothermophilus* and *Thermoactinomyces intermidius* were cloned and sequenced. They contain 429 and 366 amino acids, respectively. A comparison of their amino acid sequences showed that the additional residues in the *Bacillus stearothermophilus* enzyme form an extension at the C terminus. Sequence alignment studies have expressed the existence of strong sequence similarly between LeuDH and phenylalanine dehydrogenase (PheDH; 50% indentity) and a somewhat more remote similarity to glutamate dehydrogenase (GluDH; 20% identity), indicating the existence of an enzyme superfamily with differential substrate specificity (Kataoka and Tanizawa, 2003).

Baker and his colleagues (1995) solved the X-ray structure of the LeuDH from *B. sphaericus* and found that the LeuDH subunit is constructed from 14 α-helices and 12 β-strands, which comprise some 75% of the polypeptide chain. The secondary structure addsiments are given in figure 1.4. The secondary structure elements fold into two domains, separated by a deep cleft. DomainI consists of residues 1-136 and 332-364 and it composed of a mixed parallel / antiparallel β-sheet of six strands (βa-βf), flanked on each side by two α-helices (α1, α2 and α3, α4). A short helix (α5) joins the last strand of domainI (βf) with the long α–helix (α6) which leads into domainII. This domain (residues 137-331) resembles the classical dinucleotide binding domain and fold into a central six-stranded parallel β–sheet (βg-βl) flanked on each side by two helices (α7, α8 and α10, α11). A short helix (α9) is present between strands βi and βj. The last strand domainII (βl) is followed by helix α12 and a long helix, α13, which together with α6 dominate the domain interface and provide most of the interactions between the two domains. The subunit is completed by a further long α–helix (α14), part of which packs against the surface of the N-terminal domain, and an extended loop formed by the C-terminal 15 residues. The end of α14 and part of the C-terminal loop (residues 343-360) protrude somewhat

from the surface of domainI, before folding back into the domain with the C terminus lying close to the end of α12. Two of 14 α–helices (α12 and α13) contain 3_{10} –turns, formed by residues 287-289 and 311-313, respectively. Residue implicated in the catalytic mechanism of GluDH include Lys113, which binds the 1-carboxyl group of the glutamate substrate, Asp165, which has been proposed to be involved in proton transfer to and from the glutamate during catalysis, and Lys125, which has a low pK_a and is thought to enhance the nucleophilicity of an essential water molecule involved in the mechanism. These three residues are conserved in LeuDH, Lys68, Asp115 and Lys80, respectively. The model of the active site has enabled to compare the mode of binding of the side-chain atoms of the amino acid substrates of GluDH and LeuDH. The respective substrates lie in a pocket on the enzyme surface, which has a different character in the two enzymes. In GluDH, the aliphatic 2-,3- and 4-methylene groups of the glutamate side chain form interactions with Gly90, Ala163 and Val377, which are conserved in LeuDH, Gly41, Ala113 and Val291, respectively. However, the glutamate 5-carboxyl group makes hydrogen bonds to Lys89 and Ser 380, which in LeuDH are Leu40 and Val294, two crucial substitutions that have been recognized from the earlier homology–based modeling. The discrimination of the amino acid substrate between these two enzymes is achieved by a combination of point mutations and subtle changes in shape in the substrate side-chain-binding pocket. Lys89 and Ser380 provide a hydrophilic pocket in GluDH, whereas in LeuDH the equivalent residues are Leu40 and Val294, which make the pocket more hydrophobic (Baker *et al*., 1995 and Baker *et al*., 1997). Figure 1.5 shows stereo diagrams of a single subunit and figure 1.6 shows stereo diagrams of dimer and space-filling representations of the LeuDH octamer from *B. sphaericus*.

In the alteration of substrate specificity of the enzyme from *Bacilus stearothermophilus* by site-directed mutagenesis study indicated that A113, V291, L40 and V294 in LeuDH are the key residues involved in recognition of the substrate side chain. A113 in LeuDH and the corresponding G114 in PheDH control the volume of side-chain binding pocket and play a critical role in discrimination of the bulkiness of the side chain. Emergence of GluDH and LysDH activities in the

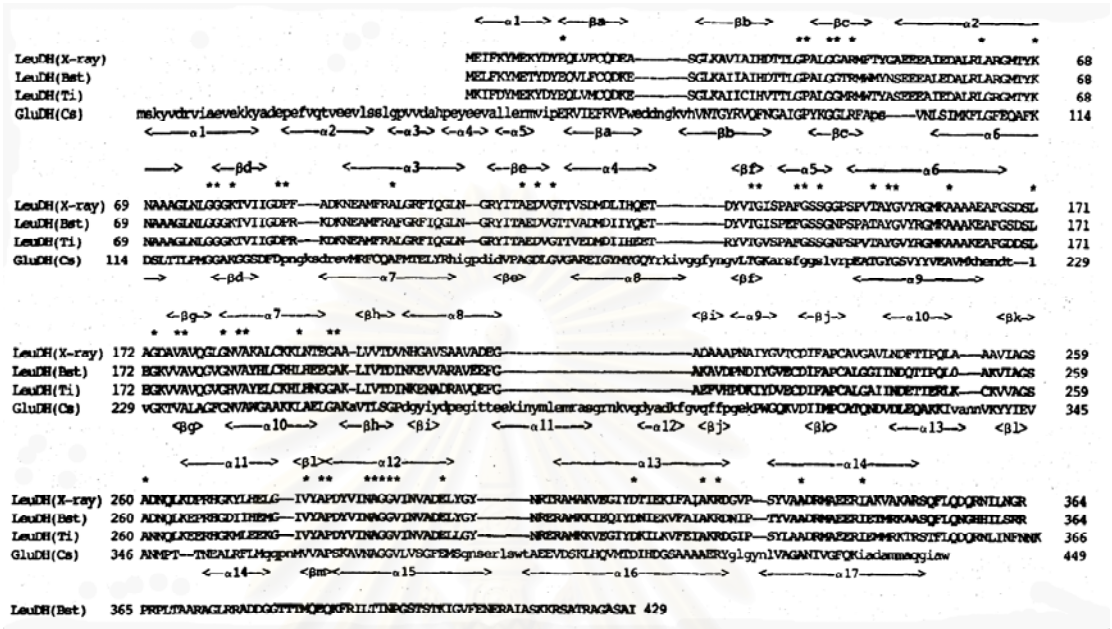


Figure 1.4 Structure-based sequence alignment of the X-ray sequence of the LeuDH from *B. sphaericus* (X-ray), together with sequences of the LeuDHs from *B. stearothermophilus* (Bst) and *T. intermedius* (Ti) and the GluDH from *C. symbiosum* (Cs).

Source: Baker *et al*., 1995

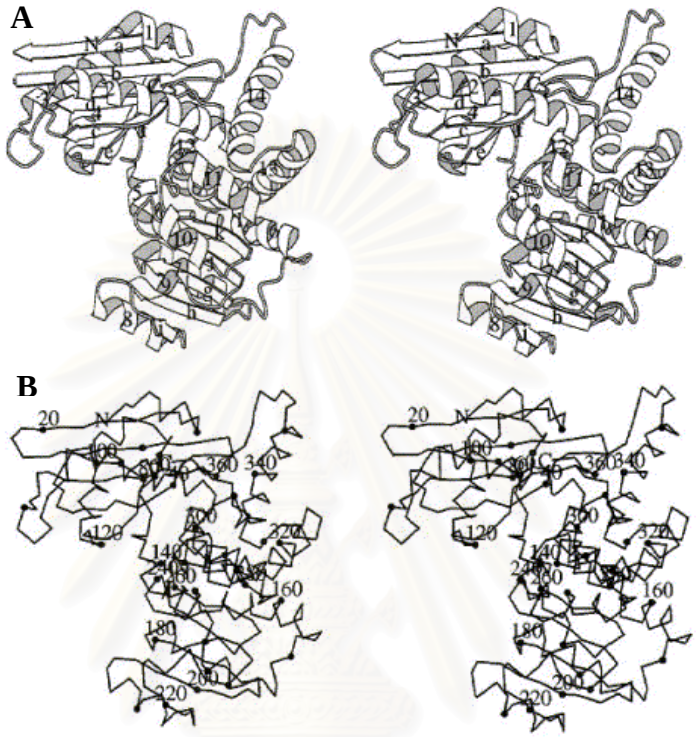


Figure 1.5 Stereo diagrams of a single subunit of the LeuDH from *B. sphaericus*

A. Schematic representation with the strands and helices numbered.

B. Cα trace with every tenth residue indicated by a block dot.

Source: Baker *et al.*, 1995

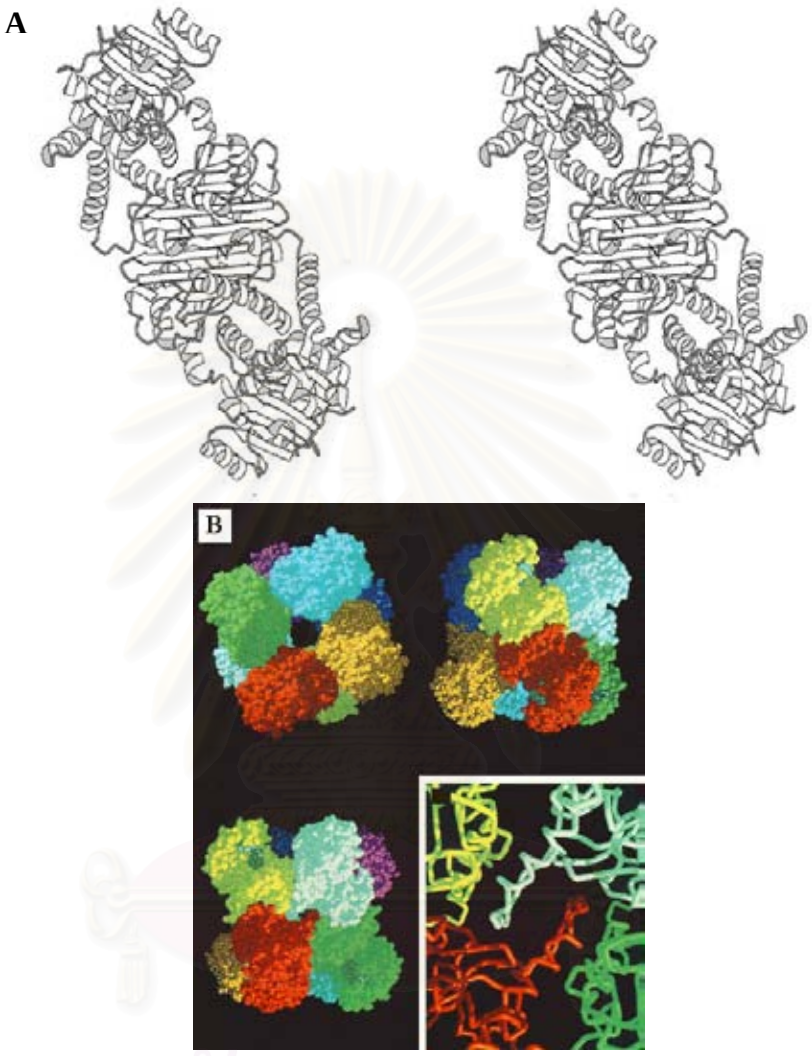


Figure 1.6 Stereo and space-filling representations of the LeuDH from *B. sphaericus*

A. Stereo representations of the dimers of LeuDH from *B. sphaericus*

B. Space-filling representations of the LeuDH octamer with each subunit individually coloured.

Source: Baker *et al.*, 1995

L40K/V294S and L40D/V294S mutants, indicates that the two residues in the active sites of the amino acid dehydrogenase are important for discrimination of the hydrophobicity / polarity / charge of aliphatic substrate side chains (Kataoka and Tanizawa, 2003). The alignment of amino acid dehydrogenases is shown in figure 1.7.

1.5 Leucine dehydrogenase in applications

Leucine dehydrogenase is applicable to two major fields: industrial and medical fields.

1.5.1 Medical and pharmaceutical applications

The amino acid dehydrogenase has considerable commercial potential for the production of novel non-proteinous amino acids in pharmaceutical industries and for the diagnosis of genetic diseases of amino acid metabolism including phenylketonuria, maple syrup urine disease, hypervalinaemia, hyperleucine-isoleucinaemia and homocystinuria.

Leucine amino peptidase (LAP) is an enzyme normally found in liver cells (hepatocytes) of various organisms. It is also released into the blood after damage of liver cells, such as from drugs or infection for example, hepatitis. In addition, LAP can also be released into blood by tumors that arise in the liver, so it may also serve as a tumor maker or indicator. So, measurement of leucine aminopeptidase in serum may serve as an indicator of lever damage but testing serum LAP is generally not as sensitive or as convenient as testing other liver enzymes to detect some liver problems cause of drugs affection to LAP measurement. Since, LAP reacts most rapidly with leucine at N-terminal of protein and peptides to a free leucine. Many aliphatic amides are also hydrolyzed as well as thioesters. So, leucine dehydrogenase was used to assayed leucine amino peptidase serum on the basis of the coupled reactions by a simple spectrophotometric method as the formular in figure 1.8 (Takamiya *et al*., 1983).

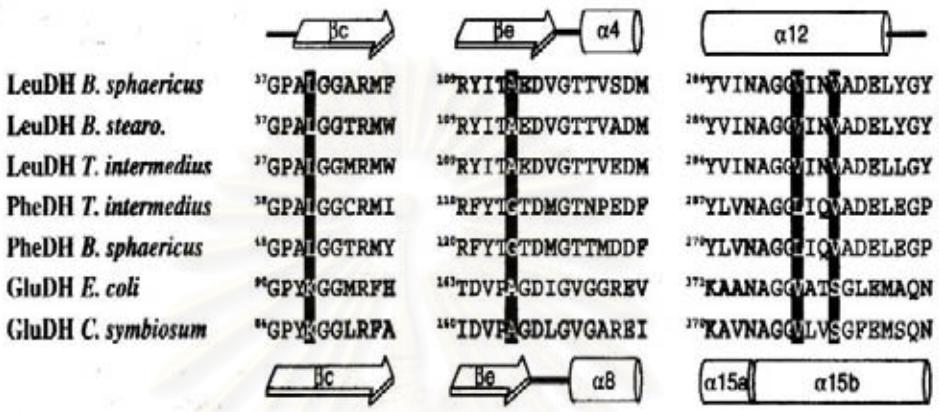


Figure 1.7 Sequence alignment of amino acid dehydrogenases using structural information from *B. sphaericus* LeuDH and *C. symbiosum* GluDH
Residues L40, A113, V291, and V294 of *B. stearothemophilus* LeuDH and their corresponding residues are shown with white letters in black background.

Source: Kataoka and Tanizawa, 2003

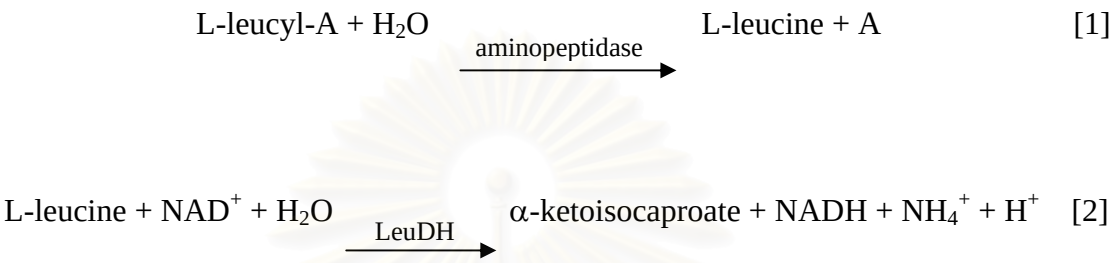


Figure 1.8 A simple spectrophotometric method for the assay of human serum aminopeptidase with leucine dehydrogenase on the basis of coupled reaction

Source: Takamiya *et al*., 1982

Maple syrup urine disease (MSUD) is an inborn error of metabolism caused by severe deficiency of the branched chain α-keto acid dehydrogenase complex (BCKAD, E.C.1.2.4.4) activity. The inability of this enzyme complex to oxidize α-ketoisoceproic acid, α-keto-β-methylvaleric acid and α-ketoisovaleric acid leads to tissue accumulation of these metabolites and their precursor amino acids leucine, isoleucine and valine, respectively, in the affected individuals. Patients with MSUD present poor feeding, apnoea, ketoacidosis, convulsions, coma and psychomotor delay. Central nervous system imaging reveals low density of white matter corresponding to hypomyelination / dimyelination and cerebral strophy. The disease is severe enough to cause a fatal outcome in a significant number of patients if not diagnosed and treated promptly. Therefore, elevated levels of serum branched chain α-keto acids have been associated with the occurance of matabdic disorders, the high-performance liquid chromatography (HPLC) has been widely used for the determination of branched chain α-keto acids in the physiological samples. Many fluorescent labeling reagents have been used for the pre-column derivatization of branched chain α-keto acids. The methods are sensitive but require a long time for the derivatization. Other HPLC method for the determination branched chain α-keto acids by Walser *et al*. (1987). Branched chain α-keto acids were separated by reverse-phase HPLC and detected by UV-absorbance at 214 nm. However, these methods require clean-up steps prior to the analysis to remove highly UV-absorbing substances that may coelute with branched chain α-keto acids. There by, Kiba *et al*. (1989) established a specific HPLC method with fluorescence detection for the determination of branched chain α-keto acids in serum using immobilized leucine dehydrogenase as post-column reactor. Serum sample is deproteinized by ultrafiltration. Branched chain α–keto acids are separated on an ODS column with a mobile phase containing ammonium salt buffer and NADH. The magnitude of decrease in intensity for fluorescence based on the NADH is dependent on the amount of branched chain α-keto acids in the eluate.

Moreover, the measurement of branched chain amino acid (L-leucine, L-isoleucine and L-valine) is clinical importance in the diagnosis of inborn errors of metabolism. Enzymatic methods have been developed for branched chain amino

acids in neonatal blood samples. The methods are unsuitable for monitoring patients under therapy and for the diagnosis of several disorders of metabolism such as maple syrup urine disease, hypervalinaemia and hyper leucine-isoleucinaemia because the individual branched chain amino acids cannot be measured separately. In routine analysis, the amino acids are separated and determined by amino acid analysers, which employ HPLC on the basis of post-column derivatization with ninhydrin of *o*-phthalaldehyde (Dresher *et al*., 1978 and Radhai *et al*., 1980). However, it produced an excessive amount of information that is usually not required for practical use. For the selective determination of individual branched chain amino acid, an HPLC system with fluorescence detection was used a reactor containing immobilized leucine dehydrogenase as mentioned above. The method is moderately sensitive and requires a tedious deproteinization procedure. Kiba *et al*. improved the method again in 1995. The new improvement was an HPLC system with co-immobilized LeuDH-NADH oxidase (NAOD) in a post-column reactor for the sensitive detection at branched chain amino acids in plasma. Branched chain amino acids were separated on a reversed-phase ODS column. In the reactor, the LeuDH catalysed the deamination of the branched chain amino acids in the presence of NAD^+ and the resulting reduced form NAD^+ (NADH) form was removed by the NADH oxidase with concomitant formation of hydrogen peroxide. The hydrogen peroxide produced in the reactor was detected by measuring the chemiluminescence emitted when mixed with luminal and potassium hexacyamoferrate (III) (Kiba *et al*.,1996).

Furthermore, LeuDH was used for determination of plasma amino acid concentration. In 1996, Beckett *et al*. developed a spectrophometric assay which determines the total concentration of all three branched chain amino acids in plasma within 1 minute because plasma amino acid concentration falls during insulin infusion. Amino acid concentration can be maintained using an infusion of amino acids if their plasma concentration can be determined within a few minutes. The leucine dehydrogenase oxidatively deaminates leucine, isoleucine and valine with stoichiometric reduction of NAD^+ that is measured using a spectrophotometer. The assay was developed in both a kinetic and end-point format. For the kinetic assay, the buffer conditions were formulated to obtain equivalent rates with all three amino

acids so that it could be used in sample containing unknown mixture. For the end-point assay, additional enzyme was added so that an end-point could be reached within 1 minute (Beckett *et al*., 1996).

Morishita and colleagues (1997) investigated a new enzymatic kinetic assay of serum or urine urea nitrogen by using leucine dehydrogenase and urease cause of some obstacle. Urea nitrogen in serum or urine has been measured base on enzymatic reaction with urease (EC 3.5.1.5) and glutamate dehydrogenase (EC 1.4. 1.4). Endogenous NH_4^+ in serum and urine is a potential interference, because NH_4^+ produced from urea by urease in the reaction system. Although the NH_4^+ in serum and urine has been dominated with the glutamate dehydrogenase and α-ketoglutaric acid before addition of urease but in some urine that has a large amount of endogenous NH_4^+. The elimination of the NH_4^+ may not be complete, even in samples diluted 10–20 fold. To eliminate much of the NH_4^+ in urine, a recycling system (NADH ⟷ NAD^+) with isocitrate dehydrogenase (EC 1.1.1.42) has been used (Mauri *et al*., 1988). Alternatively, ADP that produced from ATP by urease can be determined instead of NH_4^+ (Fossati, 1986). However, the cost of urea determination in these methods in very high. Moreover, the former method was linear only to urea concentrations of ~10g/L and the latter method was carried out at 376 nm, a wavelength unavailable on many automated analyzers. So they investigated a new enzymatic kinetic assay by using leucine dehydrogenase and urease. Interference from endogenous NH_4^+ in serum and urine is avoided by subtracting the endogenous NH_4^+ value assayed in sample blank (Morishita *et al*., 1997) The assay principle was shown in figure 1.9.

1.5.2 Industrial application

Branched chain amino acids are considered essential amino acids because human being cannot survive unless these amino acids are present in the diet. In addition, branched chain amino acids are needed for the maintenance of muscle tissue and appear to preserve muscle stores of glycogen and also help prevent muscle protein breakdown during exercise. Branched chain amino acid supplementation may

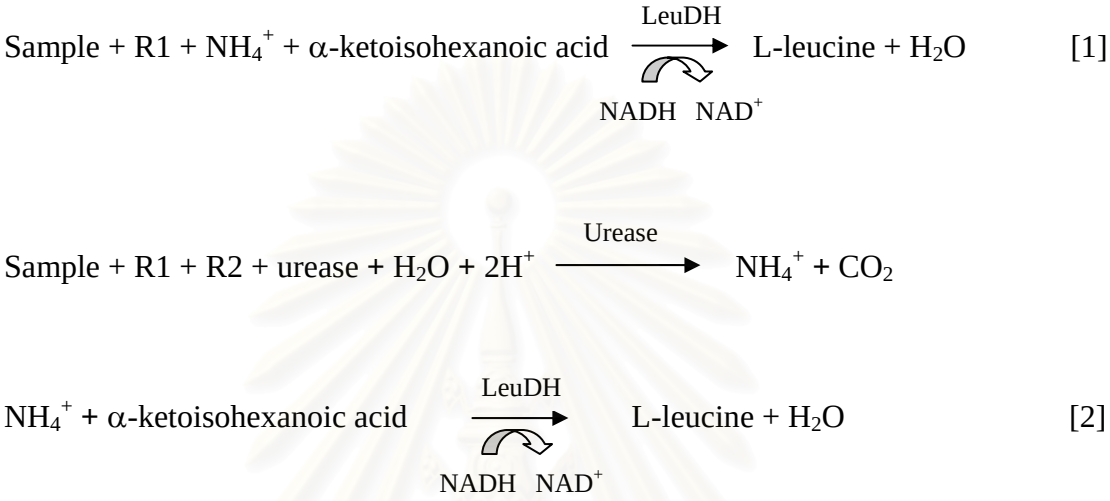


Figure 1.9 Enzymetic kinetic assay of serum or urine urea nitrogen by using leucine dehydrogenase and urease; Endogenous NH_4^+ in serum or urine is allowed to reaction with α-ketoisohexanoic acid, NADH, and LeuDH. The reaction rate at which NADH is oxidized to NAD^+ depends on the amount of the endogenous NH_4^+ measures [1]. Urease is added to the reaction system, and the oxidation rate of NADH to NAD^+ by both the NH_4^+ produced from urea and endogenous NH_4^+ is measured [2]. Urea nitrogen in the sample is calculated from the differences of oxidation rate between [1]and [2].

Source: Morishita *et al*., 1997

be useful in special situations, such as preventing muscle loss at high attitudes and prolonging endurance performance in the heat. Patients with liver diseases that lead to coma, called hepatic encephalopathy, have low concentrations of branched chain amino acids and excess levels of certain other amino acids. People with this condition should be given branched chain amino acid. People with chronic kidney failure may also benefit from branched chain amino acid supplementation. It was found that improved breathing and sleep quality in people given intravenous branched chain amino acids during kidney dialysis were observed. Moreover, branched chain amino acids are essential amino acid for animal nutrition. Leucine, isoleucine and valine affect chick growth rate. When only one or two of these three were reduced, growth rate was lower (Nakhata, 1975). The commercial values of essential amino acids come from their wide applicability in both pharmaceutical and animal food industries but they are very expensive.

As the above reason, multienzyme reaction systems with a simultaneous coenzyme regeneration have been developed for the production of L-leucine from α-ketoisocaproate (2-oxo-4-methylpentanoic acid), using leucine dehydrogenase from *Bacillus sphaericus*, formate dehydrogenase from *Candida boidinii* and NADH which was covalently bound to water-soluble polyethyleneglycol (PEG-NADH) (Rolf *et al*., 1981). In 1985, L-leucine production was improved by using leucine dehydrogenase from *Bacillus strearothermophilus*, formate dehydrogenase from *Candida boidinii* and NADH (PEG-NADH). This system was successfully scaled up and improved by using purified leucine dehydrogenase from *Bacillus strearothermophilus* was stable for longer time, at higher temperatures, and at wider pH range compared with the enzyme from mesophilic bacterium *B. sphaericus* (Ohshima *et al*., 1985).

Moreover, leucine dehydrogenase has been used for production of L-*tert*-leucine which is a component of an antibiotic. The L-tert-leucine synthesis was performed continuously in series of two enzyme-membrance reactors by reductive amination of trimethylpyruvate with leucine dehydrogenase. The necessary "native" cofactor NADH is generated with the formic acid from a second enzyme, formate dehydrogenase (Kragl *et al*., 1996) as shown in figure 1.10.

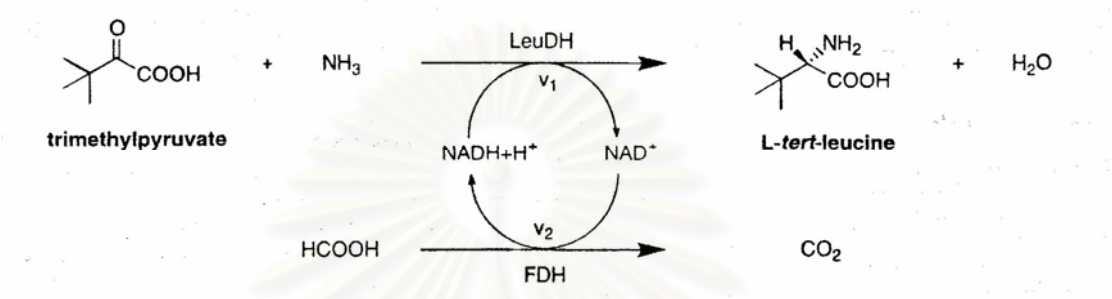


Figure 1.10 The L-*tert*-leucine synthesis in series of two enzyme-membrane reactors

LeuDH; leucine dehydrogenase

FDH; formate dehydrogenase

Source: Kragl *et al*., 1996

1.6 Objectives of this research

From all of the applications of leucine dehydrogenases, it is interesting to find out new source of enzyme and study it property.

The objectives of this research are

1. To screen for NAD^+ dependent LeuDH producing bacteria
2. To determine the optimum condition for LeuDH production
3. To purify and characterize the biochemical properties of LeuDH
4. To study kinetic mechanism of LeuDH

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave: Model LS-2D “REXALL”, Sanyo Co., Ltd., Japan

Autopipette: Pipetteman, Gilson, France

Centrifuge, refrigerated centrifuge: J-30I, Beckman Instrument Inc., USA

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

Conductivity meter: CDM 83, Radiometer, Denmark

Fraction collector: FRAC-100, Phamacia Biotech, USA

-20°C Freezer: medical freezer, Sanyo CO., Ltd., Japan

-70°C Freezer: REVCO, BARA LABORATORY Co., Ltd., USA

Electrophoresis unit: mini protein, Bio-Rad, USA

Hot air oven: Mermert, USA

Hot plate: Fisher Scientific, USA

Incubator shaker: Model IOC400.XX2.C (GALLENKAMP PLC)

Incubator water bath: M20S, Lauda, Germany and Biochiller 2000, FOTODYNE Tnc., USA

Laminar flow: International Scientific Supply Co., Ltd., Thailand

Magnetic stirrer: Fisher Scientific, USA

Microcentrifuge tube, Axygen Hayward, USA

Orbital incubator: 1H-100, Gallenkamp, England; Psycotherm, News Brunswick

pH meter: Scientific Co., USA

Peristatic pump: Pump P-1, Phamacia Biotech, USA

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

Sonicator: SONOPULS Ultrasonic homogenizer, BANDELIN, Germany

Spectrophotometer: DU series 650, Beckman, USA

Vortex mixer: K-550-GE, Scientific Industries, Inc, USA

2.2 Chemicals

3-Acetylpyridine adenine dinucleotide: Sigma, USA

Acrylamide: Merck, Germany

Agar: Merck, Germany

Amonium chloride: Merck, Germany

Ammonium persulfate: Merck, Germany

Ammonium sulphate: Merck, Germany

Aqua sorb: BML, Thailand

Bis-acrylamid: Merck, Germany

Bovine serum albumin (BSA): Sigma, USA

Bromphenal blue: BDH, England

Butyl-Toyopearl 650M TSK gel: Tosoh, Japan

Chloramine T: Sigma, USA

Coomassie brilliant blue R-250: Sigma, USA

DEAE- Toyopearl 650M TSK gel: Tosoh, Japan

Dithiothreitol (DTT): Sigma, USA

Ethylenediamine tetraacetic acid (EDTA): Scharlau Microbiology, European Union

Glacial acetic acid: Carlo Erba Reagenti, Italy

Glycerol: Merck, Germany

Glycine: Sigma, USA

L-Isoleucine: Sigma, USA

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

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

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

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

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

L-Leucine: Sigma, USA

Magnesium sulphate 7-hydrate: Carlo Erba Reagenti, Italy

β-Mercaptoethanol: Fluka, Switzerland

Methyl alcohol: Merck, Germany

β-Nicotinamide adenine dinucleotide (NAD): Kohjin Co. Ltd., Japan

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

β-Nicotinamide adenine dinucleotide phosphate (NADP) : Kohjin Co., Ltd. , Japan

Nitroblue tetrazolium: Koch-Light Laboratories Co., Ltd., Japan

Peptone from casein: Scharlau microbiology, European Union

Phenazine methosulfated: Nacalai Tesqu, Inc., Japan

Phenylmethyl sulfonyl fluoride (PMSF): Sigma, USA

Potassium chloride: Merck, Germany

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

Potassium phosphate monobasic: Carlo Erba Reagenti, Italy

Hitrap Q column: Phamacia, USA

Sodium chloride: Carlo Erba Reagenti, Italy

Sodium laulyl sulfate (SDS): Sigma, USA

Standard protein marker: Amercham Pharmacia Biotech Inc., USA

N,N,N′,N′-tertramethylethylene diamine (TEMED) : BDH , England

2, 4, 6-Trinitrobenzenesulfonic acid: Sigma, USA

Tris (hydroxymethyl)-amionomethane, Usb Corporation, USA

L-Valine: Sigma, USA

Yeast extract: Scharlau Microbiology, European Union

The other common chemicals (reagent grade) were from Aldrich; USA, BDH; England, Fluka; Switzerland, Merck; Germany and Sigma; USA. The other L-amino acids were from Sigma, USA and D-amino acids were from Nacalai tesque and Wako, Japan.

2.3 Bacteria growth medium

2.3.1 Screening medium (1% amino acid medium)

The medium consisted of 0.2% di-potassium hydrogen phosphate, 0.2% potassium dihydrogen phosphate, 0.2% sodium chloride, 0.01% magnesium sulfate and 1% each amino acid (lysine, leucine, isoleucine, phenylalanine, serine, arginine, tryptophan). The medium was adjusted pH to 7.2 with sodium hydroxide and steriled by autoclaving at 121˚C for 15 minutes.

2.3.2 Peptone medium

The medium consisted of 1% peptone (from casein), 0.2% di-potassium hydrogen phosphate, 0.2% potassium dihydrogen phosphate, 0.2% sodium chloride, 0.01% magnesium sulfate and 1% yeast extract was prepared. The pH of medium was adjusted to 7.2 with sodium hydroxide. For solid medium1.5% agar was added. The medium was steriled by autoclaving at 121˚C for 15 minutes.

2.4 Leucine dehydrogenase activity assay

2.4.1 Dye-forming method

In this method, tetrazolium salts are used for the detection of dehydrogenase activity. The water-soluble tetrazolium salts are yellowish dye precursors which can form deeply coloured pigments known as formazans upon the reduction. So if the crude enzyme contains dehydrogenase activity, the testing solution will change the colour solution from yellow to purple or dark blue (positive test). On the other hand, if the testing solution is still yellow, it means negative test.

Assay mixture (1.2 ml) containing of 4 μmol NAD^+, 4 μmol $NADP^+$, 80 μmol Tris buffer pH 9.2, 5 mg/ml phenazine methosulfate 8 μl, 10 mg/ml nitroblue tetrazolium salt 40 μl and distrilled water 272 μl. Assay mixture 60 μl and 40 μl of

0.1 M L-leucine were pipetted to a well of microtiter plate. Then, 0.1 ml of crude extract was added. Changing of colour was observed and compared to the control (without L-leucine). The positive samples were confirmed for their leucine dehydrogenase activity by spectrophotometric method.

2.4.2 Oxidative deamination

The reaction mixture for oxidative deamination of leucine dehydrogenase contained 20 μmol of L-leucine, 1 μmol of NAD^+, 200 μmol of glycine-potassium chloride-potassium hydroxide buffer, pH 12.0 and enzyme, in final volume of 1 ml. The mixture was incubated for 5 minutes at 30˚C in a cuvette of 1 cm light path. After 5 minutes of incubation, the reaction was started by addition of NAD^+ and was followed by measurement of the initial change of absorbance at 340 nm for a few minutes with a DU series 650 spectrophotometer, Backman. In blank, L-leucine was replaced by water.

One unit definition of enzyme activity is defined as the amount of enzymes that catalyzes the formation of 1 μmol of NADH per minute. The molar absorption coefficient is 6.2×10^3 $M^{-1}cm^{-1}$. Specific activity is expressed as units per milligram of protein.

2.4.3 Reductive amination

The reaction mixture for reduction amination of leucine dehydrogenase contained 10 μmol of α-ketoisocaproate, 500 μmol of ammonium chloride, 0.2 μmol of NADH, 100 μmol of glycine-potassium chloride-potassium hydroxide buffer (pH 10.5) and enzyme, in final volume of 1 ml. The mixture was incubated for 5 minutes at 30˚C in a cuvette of 1 cm light path. After 5 minutes of incubation, the reaction was started by addition of NADH and was followed by measurement of the initial change of absorbance at 340 nm for a few minutes with a DU series 650 spectrophotometer, Backman. In a blank, α-ketoisocaproate was replaced by water.

One unit definition of enzyme activity is defined as the amount of enzymes that consume 1 μmol of NADH per minute. Specific activity is expressed as units per milligram of protein.

2.5 Protein assay

The amount of protein was determined by the modified method of Lowry *et al.* (1951) with bovine serum albumin (BSA) as a standard. The mixture of 3.05 ml contained 20-100 μg of protein, 50 μl of solution A, 2.5 ml of solution B. The mixture was mixed and incubated for 10 minutes at 30˚C. After that, 250 μl of solution C was added to the mixture and incubated for 20 minutes at room temperature. After incubation, the absorption of the sample at 610 nm was detected by spectrophotometer. Protein concentration was calculated by comparison with standard curve of protein standard (BSA). Preparation of the solutions were described in Appendix A.

2.6 Screening for NAD^+dependent leucine dehydrogenase producing bacteria

Ten soil samples in Bangkok area were collected. Then, 0.5 g of each sample was suspended in 5 ml distilled water. One loop of soil solution was added to 1 ml screening medium (1% amino acid medium). The bacteria were grown at 37˚C with 250 rpm shaking for 4 days. After that, one loop of soil solution was streaked on peptone agar and incubated at 37˚C for 24 hours. Single colonies were collected on peptone plate (master plate). For leucine dehydrogenase assay, single colonies from the master plate were cultured in 10 ml of peptone medium, pH 7.2 at 37˚C with 250 rpm shaking for 24 hours before they were inoculated into 100 ml of peptone medium, pH 7.2 at the same condition. The bacteria were harvested by centrifugation at 9,800×g for 20 minutes at 4˚C. The collected cells were washed by 0.85% sodium chloride for 2 times and extraction buffer (0.1 M potassium phosphate buffer, pH 7.4, containing 0.1 mM phenylmethylsulfonyl fluofide (PMSF), 0.01% β-mercaptoethanol and 1 mM ethylenediamine tetraacetic acid (EDTA)) for 1 time. The collected cells were stored at -70˚C.

The collected cells were suspended in 2 ml of extraction buffer then broken by discontinuously sonication on ice 2 minutes, stop 2 minutes with 5 cycles, 10 rounds, power 10% by sonicator model SONOPULS Ultrasonic homogenizer (KE 76). After that, the supernatants were collected by centrifugation at 10,000×g for 30 minutes at 4˚C. The crude enzymes were assayed by dye forming method as described in section 2.4.1. The crude enzymes which gave positive result on dye forming assay was determined for oxidative deamination activity of leucine dehydrogenase and protein concentration as described in section 2.4.2 and 2.5, respectively.

2.7 Identification of NAD^+ dependent leucine dehydrogenase producing bacteria by morphological and biochemical properties

The leucine dehydrogenase producing bacteria was identified by Union Hitech Co., Ltd., Osaka, Japan according to its morphological and biochemical properties such as Gram reaction, fermentative production of acid from various carbon sources and activity of various enzymes.

2.8 Optimization for leucine dehydrogenase production

2.8.1 Starter inoculation

Starter inoculum was prepared by inoculation of one single colony of *Alcaligenes faecalis* from agar plate to 25 ml of peptone medium, pH 7.2 and cultured for 18 hours with 250 rpm shaking at 37˚C. The 25 ml of cell culture was transferred to 250 ml of peptone medium, pH 7.2 and cultured at the same condition as described previously.

2.8.2 Cell collection and preparation of cell free extract

The bacteria were collected by centrifugation at 9,800×g for 20 minutes at 4˚C. Then, the collected cells were washed twice with 0.85% NaCl and subsequently

once with extraction buffer (0.1 M potassium phosphate buffer, pH 7.4 containing 0.1 mM phenylmethylsulfonyl fluofide (PMSF), 0.01% β-mercaptoethanol and 1 mM ethylenediamine tetraacetic acid (EDTA)). The cells were suspended in cold extraction buffer (1 ml per 0.35 g cell wet weight) and then broken by discontinuously sonication on ice 2 minutes, stop 2 minutes with 5 cycles, 10 rounds power 10% by sonicator model SONOPULS Ultrasonic homogenizers (KE 76). After that, the supernatant was collected by centrifugation at 10,000×g for 30 minutes at 4˚C. The crude enzyme was determined for leucine dehydrogenase activity and protein concentration as described in section 2.4.2 and 2.5, respectively.

2.8.3 Optimum pH of medium

The 10% of starter inoculum from section 2.8.1 was transferred into 250 ml erlenmeyer flask containing 100 ml of peptone medium at various pHs ranged from 4.5 to 8.5 (4.5, 5.0, 5.5, 6.0, 6.5, 7.2, 7.5, 8.0 and 8.5) and cultivated with 250 rpm shaking at 37˚C for 24 hours. The cell collection, cell free extraction, assay for oxidative deamination activity and protein measurement were performed as described in section 2.8.2, 2.4.2 and 2.5, respectively.

2.8.4 Optimum cultivation temperature

The 10% of starter inoculum from section 2.8.1 was transferred into 250 ml erlenmeyer flask containing 100 ml of peptone medium at proper pH which based on the result from section 2.8.3. The bacteria were cultivated with 250 rpm shaking for 24 hours at various temperatures (30, 34, 37 and 40˚C). The cell collection, cell free extraction, assay for oxidative deamination activity and protein measurement were performed as described in section 2.8.2, 2.4.2 and 2.5, respectively.

2.8.5 Optimum cultivation time

The 10% of starter inoculum from section 2.8.1 was transferred into 500 ml erlenmeyer flask containing 250 ml of peptone medium at pH based on the result from section 2.9.3. The bacteria were cultivated with 250 rpm shaking for 0, 4, 8, 12, 16, 20, 24, 28 and 32 hours at suitable cultivation temperature which based on the result from section 2.8.4. Every 4 hours, the cell culture was taken and measured for optical density at 610 nm. The cell collection, cell free extraction, assay for oxidative deamination activity and protein measurement were performed as described in section 2.8.2, 2.4.2 and 2.5, respectively.

2.8.6 Enzyme induction

The 10% of starter inoculum from section 2.8.1 was transferred into 250 ml erlenmeyer flask containing 100 ml peptone medium at suitable pH based on the result from section 2.8.3, and 0.2% of various amino acids (L-valine, L-isoleucine and L-leucine). The control was peptone medium without amino acid. The cell culture was cultivated with 250 rpm shaking at a suitable cultivation temperature as well as cultivation time. The cell collection, cell free extraction, assay for oxidative deamination activity and protein measurement were performed as described in section 2.8.2, 2.4.2 and 2.5, respectively.

2.8.7 Optimum concentration of inducer

The 10% of starter inoculum from section 2.9.1 was transferred into 250 ml erlenmeyer flask containing 100 ml of the most type of inducer as well as medium and proper cultivation time with 250 rpm shaking at suitable temperature which based on the result section 2.8.3, 2.8.4, 2.8.5 and 2.8.6. The concentrations of inducer varied in 0, 0.2, 0.4, 0.6, 0.8, and 1.0 %. The cell collection, cell free extraction, assay for oxidative deamination activity and protein measurement were performed as described in section 2.8.2, 2.4.2 and 2.5 respectively.

2.9 Purification of leucine dehydrogenase from *Alcaligenes faecalis*

2.9.1 Bacterial cultivation

One single colony of *Alcaligenes faecalis* from agar plate was inoculated into 40 ml of peptone medium, pH 7.2 at 37˚C with 250 rpm shaking for 18 hours and then was transferred into 400 ml of peptone medium, pH 7.2 at 37°C with 250 rpm shaking for 18 hours.

2.9.2 Enzyme production

The starter inoculum from section 2.9.1, was transferred into 4 liters of peptone medium, pH 7.2 and shaken at 250 rpm, 37˚C for 24 hours. The bacteria cells (28 g wet weight) were collected and broken following by crude enzyme preparation as described in section 2.8.2. The crude enzyme solution was dialysed against dialysis buffer (10 mM potassium phosphate buffer, pH 7.4 containing 0.01% β-mercaptoethanol and 1 mM ethylenediamine tetraacetic acid (EDTA) before determined oxidative deamination activity and protein concentration as described in section 2.4.2 and 2.5, respectively.

2.9.3 Enzyme purification steps

All steps of purification were done at 4˚C using 10 mM potassium phosphate buffer, pH 7.4 containing 0.01% β-mercaptoethanol and 1 mM EDTA.

2.9.3.1 DEAE-Toyopearl column chromatography

DEAE-Toyopearl beads were washed with 1 time of beads volume of 0.5 M sodium hydroxide for protein elimination and were washed with deionized water until the pH reached 7.0-8.0. The washed beads were resuspended in the buffer, packed into 1.7×28 cm column and equilibrated with the phosphate buffer at flow rate 1 ml/min for 5-10 column volume.

After column equilibration, the dialysed crude enzyme solution was applied to the DEAE-Toyopearl 650M column. The unbound proteins were eluted from the column with the phosphate buffer until the absorbance at 280 nm of eluant reached baseline level. After that, the bound proteins were eluted from the column by stepwise of potassium chloride in the buffer started from 0.1 M to 0.2, 0.3, 0.4 and 0.5 M. The fractions of 3 ml were collected. The protein profile was monitored by measuring the absorbance at 280 nm. The leucine dehydrogenase activity was assayed as described in section 2.4.2. The fractions containing leucine dehydrogenase were pooled and dialysed against the phosphate buffer. The enzyme activity and protein concentration were determined as described in section 2.4.2 and 2.5, respectively.

2.9.3.2 Butyl-Toyopearl column chromatography

Butyl-Toyopearl beads were washed with deionized water to eliminate the bead binding proteins. The washed beads were packed into 1.7x10 cm column and were equilibrated with 0.8 M ammonium sulfate in the phosphate buffer for 5-10 column volume at flow rate 1 ml/min.

The pooled LeuDH activity fraction from section 2.9.3.1 was softly added with ammonium sulfate to 0.8 M and gently stirred about 30 minutes. The solution was applied to the column. The unbound proteins were eluted from the column with 0.8 M ammonium sulfate in the phosphate buffer until the absorbance at 280 nm reached baseline level. Then, the bound proteins were eluted with 10 column volume of linear salt gradient of 0.8-0.5 M ammonium sulfate in the buffer followed by the phosphate buffer. The 2 ml fractions were collected. The protein profile was monitored by measuring the absorbance at 280 nm. The LeuDH activity was assayed as described in section 2.4.2. The activity fractions were pooled, concentrated using aquasorb and dialysed against the phosphate buffer. The enzyme activity and protein concentration were determined as described in section 2.4.2 and 2.5, respectively.

2.9.3.3 Hitrap Q column chromatography

Hitrap Q column (5 ml) was washed with deionized water for 5 column volume to remove ethyl alcohol. After that, the column was equilibrated with phosphate buffer for 5-10 column volume at flow rate 0.5 ml/min.

After column equilibration, the concentrated enzyme was applied to the column. The unbound proteins were eluted from the column with the phosphate buffer until the absorbance at 280 nm of eluant reached baseline level. After that, the bound proteins were eluted from the column with 20 column volume of linear salt gradient, 0-0.5 M potassium chloride in the buffer. The 2 ml fractions were collected. The protein profile was monitored by measuring the absorbance at 280 nm. The LeuDH activity was assayed as described in section 2.4.2. The activity fractions were pooled, concentrated using aquasorb and dialysed against the phosphate buffer. The enzyme activity and protein concentration were determined as described in section 2.4.2 and 2.5, respectively. The protein from each active fraction was electrophoresed on non-denaturing polyacrylamide gel.

2.9.4 Determination of enzyme purity on non-denaturing polyacrylamide gel eletrophoresis (native-PAGE)

The enzyme from each purification step was determined for its purity and native protein pattern on native-PAGE. The discontinuous polyacrylamide gel eletrophoresis was performed on the 7.7% separating gel and 5% stacking gel. The electrode buffer was Tris-glycine buffer, pH 8.3 (25 mM Tris and 192 mM glycine). Preparation of solutions, and polyacrylamide gels were described in Appendix E. The enzyme were mixed with 5×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 on ice from cathode toward anode at 20 mA constant current. After electrophoresis, the protein bands were developed by protein and activity staining.

2.9.4.1 Protein staining

The gel was submerged in staining solution (1% Coomassie Blue R-250, 45% methanol and 10% glacial acetic acid) and shaken on rocking shaker at least 30 minutes. Then, the staining solution was decanted and the destaining solution was replaced. The gel was softly destained for several times until gel background was clear.

2.9.4.2 Activity staining

After electrophoresis, the gel was submerged in activity staining solution (4.25 mmole of Tris-HCl pH 8.5, 40 μmole of L-leucine, 50 μmole of NAD^+, 25 μg/ml of phenazine methosulfate and 250 μg/ml of nitroblue tetrazolium) at room temperature until the purple band appeared. The staining solution was quickly decanted. The gel was washed with deionized water several times until gel background was clear.

2.10 Characterization of leucine dehydrogenase from *Alcaligenes faecalis*

2.10.1 Molecular weight determination of leucine dehydrogenase

2.10.1.1 Ferguson plot analysis

The purified enzyme was determined for its molecular weight using Ferguson plot. The enzyme and standard molecular weight proteins were electrophoresed on set of various concentrations of non-denaturing polyacrylamide gel (5-10% gel). The relative mobility (R_f) of protein in each gel was determined relative to the tracking dye. Then, 100[Log (R_f x 100)] was plotted against the percent gel concentration for each protein. The log of -slope value was plotted against the log molecular weight of each protein. The molecular weight of enzyme was determined from calibration curve of standard proteins. The molecular weight standard proteins were thyroglobulin (MW 669,000 Da), ferritin (MW 440,000 Da),

catalase (MW 232,000 Da), lactate dehydrogenase (MW 140,000 Da), and albumin (MW 66,000 Da).

2.10.1.2 SDS-polyacrylamide gel electrophoresis

The discontinuous SDS-polyacrylamide gel eletrophoresis was performed on the 10% separating slab gel and 5% stacking gel. The electrode buffer was Tris-glycine buffer, pH 8.3 (25 mM Tris, 192 mM glycine and 0.1 % SDS). The gel and buffer preparations were described in Appendix F. The enzyme was mixed with 5×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 5 minutes before loading to the gel. The electrophoresis was run at room temperature from cathode toward anode at 20 mM constant current. The standard molecular weight protein markers were β-galactosidase (MW 116,000 Da), bovine serum albumin (MW 66,200 Da), ovalbumin (MW 45,000 Da), lactate dehydrogenase (MW 35,000 Da) and RE *Bsp*98I (MW 25,000 Da). After electrophoresis, protein pattern was visualized by coomassie blue staining as described in 2.9.4.1.

2.10.2 Effect of pH on leucine dehydrogenase activity

The purified enzyme was assayed as described in section 2.4.2 at various pHs for oxidative deamination and reductive amination. The buffers in this experiment were 200 mM potassium phosphate buffer (pH 7.0-8.5), glycine-KCl-KOH buffer (pH 8.5-12.0), $NaHCO_3$-NaOH buffer (pH 9.5-12.0), and $NaHPO_4$-NaOH buffer (pH 10.0-11.5). After reaction, the pH of each reaction mixture was measured at room temperature. The highest activity observed from each reaction was set as 100% relative activity and the result was displayed as the percentage of the relative activity.

2.10.3 Effect of pH on leucine dehydrogenase stability

The purified enzyme was incubated in 10 mM buffer which various pHs at 30°C for 20 minutes. Then, an enzyme aliquot was assayed for the remaining activity in oxidative deamination at 30°C as described in section 2.4.2. The buffer used in this section were acetate buffer (pH 4.0-6.0), potassium phosphate buffer (pH 6.0-8.5), Tris-HCl (pH 7.0-9.0), glycine-KCl-KOH buffer (pH 8.5-13.0), and $NaHCO_3$-NaOH buffer (pH 9.5-12.0). The result was displayed as the percentage relative of the activity which the highest activity was set as 100%.

2.10.4 Effect of temperature on leucine dehydrogenase activity

The purified enzyme was assayed as described in section 2.5 at various temperatures (25, 30, 35, 40, 45, 50, 55, 60, 65 and 70°C) for oxidative deamination and reductive amination. The result was displayed as the percentage of the relative activity. The highest activity was set as 100% relative activity.

2.10.5 Effect of temperature on leucine dehydrogenase stability

The purified enzyme was incubated at various temperatures (30, 35, 40, 45, 50, 60, and 65°C) for 10 minutes before enzyme activity was assayed as described in section 2.4.2. The highest temperature that the enzyme activity was still fully remained was selected for further assay. The enzyme was incubated at the highest temperature for 0-15 days. The oxidative deamination assay was performed every 12 hours. The result was displayed as the percentage of the relative activity. The highest activity was set as 100%.

2.10.6 Substrate specificity of leucine dehydrogenase

To determine substrate specificity of the enzyme, the purified enzyme was assayed for oxidative deamination using various amino acids and leucine analogs as substrates at final concentration of 20 mM except L-cysteine (10 mM) and

L-tyrosine (5 mM) as described in section 2.4.2. In the same manner, the enzyme was assayed as described in section 2.4.3 for reductive amination of various keto acids as substrates at 10 mM final concentration.

The results in both of oxidative deamination and reductive amination were displayed as the percentage of the relative activity related to L-leucine and α-ketoisocaproate, respectively.

2.10.7 Coenzyme specificity of leucine dehydrogenase

Various coenzymes including nicotinamide guanine dinucleotide (340 nm, ε = 6.2 x 10^3 $M^{-1}cm^{-1}$), β-nicotinamide adenine dinucleotide phosphste ($NADP^+$, 340 nm, ε = 6.2 x 10^3 $M^{-1}cm^{-1}$), nicotinamide hypoxanthine dinucleotide (338 nm, ε = 6.2 x 10^3 $M^{-1}cm^{-1}$), nicotinic acid adenine dinucleotide (340 nm, ε = 6.2 x 10^3 $M^{-1}cm^{-1}$), nicotinamide 1,N^6-ethenoadenine dinucleotide (334 nm, ε = 6.9 x 10^3 $M^{-1}cm^{-1}$), 3-acetylpyridine adenine dinucleotide (363 nm, ε = 9 x 10^3 $M^{-1}cm^{-1}$), 3-acetylpyridine nicotinic acid dinucleotide (361 nm, ε = 9 x 10^3 $M^{-1}cm^{-1}$), and thionicotinamide adenine dinucleotide (395 nm, ε = 11.3 x 10^3 $M^{-1}cm^{-1}$) were replaced nicotinamide adenine dinucleotide for oxidative deamination. The reaction was carried out at pH 9.5 using glycine-KCl-KOH buffer, pH 9.5 to avoid degradation of coenzyme at quite alkaline pH. The result was displayed as the percentage of relative activity. The activity using nicotinamide adenine dinucleotide as coenzyme was set as 100%.

2.10.8 Inhibitory effect of various amino acids and keto acids on leucine dehydrogenase activity

In this study, the enzyme was determined for oxidative deamination activity as described in section 2.4.2 in presence of amino acids which the enzyme could not catalyze as its substrates at final concentration of 20 mM. In reductive amination as described in section 2.4.3, the enzyme was determined in the presence of

keto acids which the enzyme could not catalyze as its substrates, at final concentration of 10 mM.

The results in both of oxidative deamination and reductive amination were displayed as the percentage of the relative activity related to the enzyme activity without non substrate amino acids or keto acids.

2.10.9 Effect of metal ions and chemical substrances on leucine dehydrogenase activity

The purified enzyme was determined for its LeuDH activity as described in section 2.4.2 in presence of various metal ions and chemical substrances at final concentration of 10 mM, unless otherwise stated. The result was displayed as the percentage of relative activity related to the enzyme activity without metal ions and chemical substrances.

2.10.10 Effect of group-specific reagents on leucine dehydrogenase activity

All modified reagents were incubated with the enzyme at final concentration of 10 mM or unless otherwise stated. The total volume of the mixture was 50 µl.

2.10.10.1 Effect of *N*-acetylimidazole (NAI) on tyrosine residues

The tyrosine residues modification was performed using the method of Means and Feeney, 1971.

The enzyme was incubated with NAI in 50 mM potassium phosphate buffer, pH 7.5 at 30°C for 20 minutes. After that, the residual enzyme activity was assayed as described in section 2.4.2.

2.10.10.2 Effect of *N*-bromosuccinimide (NBS) on tryptophan residues

The tryptophan residues modification was performed using the method of Means and Feeney, 1971.

The enzyme was incubated with NBS in 50 mM potassium phosphate buffer, pH 7.0 at 30°C for 20 minutes. After that, the residual enzyme activity was assayed as described in section 2.4.2.

2.10.10.3 Effect of chloromine T (CT) on methionine residues

The methionine residues modification was performed using the method of Miles and Smith, 1993.

The enzyme was incubated with CT in 50 mM Tris-HCl buffer, pH 8.3 at 30°C for 20 minutes. After that, the residual enzyme activity was assayed as described in section 2.4.2.

2.10.10.4 Effect of diethylpyrocarbonate (DEPC) on histidine residues

The histidine residues modification was performed using the method of Wakayama *et al.*, 1996.

The enzyme was incubated with DEPC in 50 mM potassium phosphate buffer, pH 7.0 at 30°C for 20 minutes. After that, the residual enzyme activity was assayed as described in section 2.4.2.

2.10.10.5 Effect of dithiothreitol (DTT) on cysteine residues

The cysteine residues modification was performed using the method of Glazer *et al.*, 1976.

The enzyme was incubated with DTT in 50 mM potassium phosphate buffer, pH 7.5 at 30°C for 20 minutes. After that, the residual enzyme activity was assayed as described in section 2.4.2.

2.10.10.6 Effect of phenylmethylsulfonyl fluoride (PMSF) on serine residues

The serine residues modification was performed using the method of Wakayama *et al.*, 1996.

The enzyme was incubated with PMSF in 50 mM potassium phosphate buffer, pH 7.0 at 30°C for 20 minutes. After that, the residual enzyme activity was assayed as described in section 2.4.2.

2.10.10.7 Effect of 2,4,6-trinitrobenzene sulfonic acid (TNBS) on lysine residues

The lysine residues modification was performed using the method of Field, 1972.

The enzyme was incubated with TNBS in 50 mM potassium phosphate buffer, pH 8.0 at 30°C for 20 minutes. After that, the residual enzyme activity was assayed as described in section 2.4.2.

2.10.10.8 Effect of phenylglyoxal (PG) on arginine residues

The arginine residues modification was performed using the method of Dong *et al.*, 1991.

The enzyme was incubated with PG in 50 mM potassium phosphate buffer, pH 8.0 at 30°C for 20 minutes. After that, the residual enzyme activity was assayed as described in section 2.4.2.

2.10.10.9 Effect of iodoacetamide (IAM) on cysteine residues

The cysteine residues modification was performed using the method of Means and Feeney, 1971.

The enzyme was incubated with IAM in 50 mM potassium phosphate buffer, pH 7.5 at 30°C for 20 minutes. After that, the residual enzyme activity was assayed as described in section 2.4.2.

2.10.10.10 Effect of *N*-ethylmaleimide (NEM) on cysteine residues

The cysteine residues modification was performed using the method of Means and Feeney, 1971.

The enzyme was incubated with NEM in 50 mM potassium phosphate buffer, pH 7.5 at 30°C for 20 minutes. After that, the residual enzyme activity was assayed as described in section 2.4.2.

2.11 Kinetic mechanism studies of leucine dehydrogenase from *Alcaligenes faecalis*

2.11.1 Initial velocity studies for the oxidative deamination

The initial velocity studies oxidative deamination was carried out as previous described in section 2.4.2 except final concentration of L-leucine, L-isoleucine, L-valine and NAD^+ were varied. Final concentrations of L-leucine were 1, 2.5, and 10 mM. Final concentrations of L-isoleucine were 2, 2.5, 4, and 10 mM and final concentrations of L-valine were 6, 8, 10, and 15 mM while final

concentrations of NAD^+ were 0.5, 0.6, 0.8, 1.0, 1.2, 1.5 and 1.8 mM. The results were brought to draw the Lineweaver-Burke plot (double-reciprocal plot) of initial velocity against NAD^+ concentrations at a series of fixed concentration of L-leucine, L-isoleucine or L-valine**.** The secondary plot of y intercepts against reciprocal concentration of L-leucine, L-isoleucine or L-valine were drawn. K_m of L-leucine, L-isoleucine, L-valine and NAD^+ were determined from these plots. The velocity (v) was expressed as unit of the enzyme.

2.11.2 Initial velocity studies for the reductive amination

The initial velocity studies for reductive amination investigation of was performed as previous described in section 2.4.3 except final concentration of α-ketoisocaproate, ammonium chloride and NADH were varied.

2.11.2.1 The initial velocity for the reductive deamination of the enzyme was studied using various concentrations of α-ketoisocaproate and fixed concentrations of NADH in presence of saturating and constant concentration of ammonium chloride (500 mM). Final concentrations of α-ketoisocaproate were 1, 2, 4 and 8 mM and final concentrations of NADH were 0.8, 1.0, 0.12 and 0.15 mM. The Lineweaver-Burke plot of initial velocity against NADH concentrations at a series of fixed concentration of α-ketoisocaproate and the secondary plot of y intercepts against reciprocal concentration of α-ketoisocaproate were drawn for K_m of determination. The velocity (v) was expressed as unit of the enzyme.

2.11.2.2 The initial velocity for the reductive deamination of the enzyme was studied using various concentrations of α-ketoisocaproate and fixed concentrations of ammonium chloride in presence of saturating and constant concentration of NADH (0.2 mM). Final concentrations of α-ketoisocaproate were 1, 2, 4 and 8 mM and final concentrations of ammonium chloride were 60, 100, 150 and 250 mM. The Lineweaver-Burke plot of initial velocity against α-ketoisocaproate concentrations at a series of fixed concentration of ammonium chloride and the

secondary plot of y intercepts against reciprocal concentration of ammonium chloride were made for K_m determination. The velocity (v) was expressed as unit of the enzyme.

2.11.2.3 The initial velocity for the reductive deamination of the enzyme was studied using various concentrations of NADH and fixed concentrations of ammonium chloride in presence of saturating and constant concentration of α-ketoisocaproate (15 mM). Final concentrations of NADH were 0.08, 0.1, 0.12 and 0.15 mM and final concentrations of ammonium chloride were 60, 100, 150 and 250 mM. The Lineweaver-Burke plot of initial velocity against NADH concentrations at a series of fixed concentration of ammonium chloride and the secondary plot of y intercepts against reciprocal concentration of NADH were made for K_m determination. The velocity (v) was expressed as unit of the enzyme.

2.11.3 Product inhibition studies

2.11.3.1 Product inhibition by NADH with respect to L-leucine

The inhibition was investigated by varying concentration of L-leucine in the present of saturating and constant concentration of NAD^+ (2 mM). The L-leucine concentrations were 2, 5, 7 and 10 mM and NADH concentrations were 0, 0.025, 0.04, and 0.08 mM.

2.11.3.2 Product inhibition by NADH with respect to NAD^+

The inhibition was investigated by varying concentration of NAD^+ in the present of saturating and constant concentration of L-leucine (20 mM). The NAD^+ concentrations were 0.5, 0.8, 1.0 and 1.2 mM and NADH concentrations were 0, 0.025, 0.05, and 0.1 mM.

2.11.3.3 Product inhibition by α-ketoisocaproate with respect to L-leucine

The inhibition was investigated by varying concentration of L-leucine in the present of saturating and constant concentration of NAD^+ (2 mM). The L-leucine concentrations were 2, 5, 7 and 10 mM and α-ketoisocaproate concentrations were 0, 1, 2, and 5 mM.

2.11.3.4 Product inhibition by α-ketoisocaproate with respect to NAD^+

The inhibition was investigated by varying concentration of NAD^+ in the present of saturating and constant concentration of L-leucine (20 mM). The NAD^+ concentrations were 0.8, 1.0, 1.2 and 1.5 mM and α-ketoisocaproate concentrations were 0, 1, 2, and 5 mM.

2.11.3.5 Product inhibition by ammonium chloride with respect to L-leucine

The inhibition was investigated by varying concentration of L-leucine in the present of saturating and constant concentration of NAD^+ (2 mM). The L-leucine concentrations were 2, 5, 7 and 10 mM and ammonium chloride concentrations were 0, 20, 100, and 200 mM.

2.11.3.6 Product inhibition by ammonium chloride with respect to NAD^+

The inhibition was investigated by varying concentration of NAD^+ in the present of saturating and constant concentration of L-leucine (20 mM). The NAD^+ concentrations were 0.8, 1.0, 1.2 and 1.5 mM and ammonium chloride concentrations were 0, 20, 100, and 200 mM.

2.12 Amino acid sequence analysis

Two nanomoles of purified enzyme was dialysed against distilled water and dried up by lyophylization. For peptide digestion, the enzyme was dissolved in 40 μl of 8 M urea and was incubated at 37°C for 1 hour, followed by addition of 120 μl of 0.2 M Tris-HCl buffer, pH 9.0 and 10 μl (10 pmol) of lysyl endopeptidase. The mixture was incubated at 30°C for 20 hours. After that, the peptide separation was performed by reversed phase HPLC technique. The sample (40 μl) was applied to an ODS-3 column (110 x 4.6 mm, GL Science Ltd.). The sample was eluted with 0.1% trifluoroacetic acid for 5 minutes, and then with a 60 minutes linear gradient (0-60%B) of 0.1% trifluoroacetic acid aqueous solution (A) and acetonitrile containing 0.1% trifluoroacetic acid (B) at flow rate of 1 ml/min. Isolated peptide peaks were collected and evaporated to dryness. The sample was dissolved in mixture of 24 μl of 0.1% trifluoroacetic acid and 6 μl of acetonitrile, and then sequenced by automated Edman degradation with an Applied Biosystem model 610A data analysis system for protein sequencing.

CHAPTER III

RESULTS

3.1 Screening for NAD^+ dependent leucine dehydrogenase

Bacteria that can produce leucine dehydrogenase were screened from soil as described in section 2.6. From 10 soil samples, there were 5 isolates that showed leucine dehydrogenase activity when their crude extracts were assayed by dye-forming method. These 5 isolates were determined for leucine dehydrogenase activity by spectrophotometric method. Only isolate No.1 and No.2 showed the enzyme activity with 7.5 and 6.3 unit/g of cell wet weight, respectively. Thus, the isolate no.1 was further identified by morphological and biochemical properties.

3.2 Identification of NAD^+ dependent leucine dehydrogenase producing bacteria

Morphological and biochemical properties of isolated No.1, reported by Union Hitech Co., Ltd., Osaka, Japan, showed important properties that isolate No.1 is a gram negative, rod shape bacteria that can move by peritrichous fragella. This bacteria could grow at 42°C. The oxidase test and catalase test were positive. Acids from carbohydrates were negative. Nitrate reduction and urease test were negative. Hydrolysis of indole and gelatin were negative. Decarboxylation of lysine, arginine, and ornithine were negative. From these properties, the strain was identified as *Alcaligenes faecalis*. This organism distributes in soil, water and clinical material such as blood, urine, feces, purulent ear discharges, spinal fluid, wounds, etc.

3.3 Optimization for leucine dehydrogenase production from *Alcaligenes faecalis*

Alcaligenes faecalis screened from soil sample for leucine dehydrogenase activity was studied in the viewpoint of optimum condition for enzyme production.

3.3.1 Optimum pH of medium

The optimum pH of culture medium was studied in order to encourage leucine dehydrogenase production as mentioned in section 2.8.3. The effect of pH on enzyme activity is displayed in figure 3.1. The cell weight, total activity and specific activity speedily increased from pH 6.5 to 7.2 which the highest cell wet weight (0.79 g), total activity (17.02 units) and specific activity (0.17 units/mg protein) were obtained. At pH over 7.2, cell wet weight, total activity and specific activity were rapidly decreased. The bacteria could not grow at pH lower than 6.0. So, the culture medium pH 7.2 was used for further experiments.

3.3.2 Optimum cultivation temperature

The optimum cultivation temperature was performed as described in section 2.8.4. The result is shown in figure 3.2, cell wet weight gradually increased when temperature was increased from 30° to 37°C. Between 37° to 40°C, growth was significantly decreased. The maximum LeuDH activity could be obtained by cultivation in the temperature range from 30° to 37°C (at 37°C, total activity was 12.8 units and specific activity was 0.17 units/mg protein). This cultivation temperature of 37°C was used for further experiments.

3.3.3 Optimum cultivation time

This experiment was performed as described in section 2.8.5. The effect of cultivation time is shown in figure 3.3. Cell wet weight and OD. at 610 nm. tended to be raised corresponding with increasing growth period which rapidly increased in the first 8 hours and slowly increased after 8 hours cultivation. Total activity and specific activity were increased from 0 to 24 hours but after 24 hours of cultivation, values were rapidly decreased. The proper cultivation time was 24 hours that gave the highest total activity (14.63 units) and specific activity (0.17 units/mg protein).

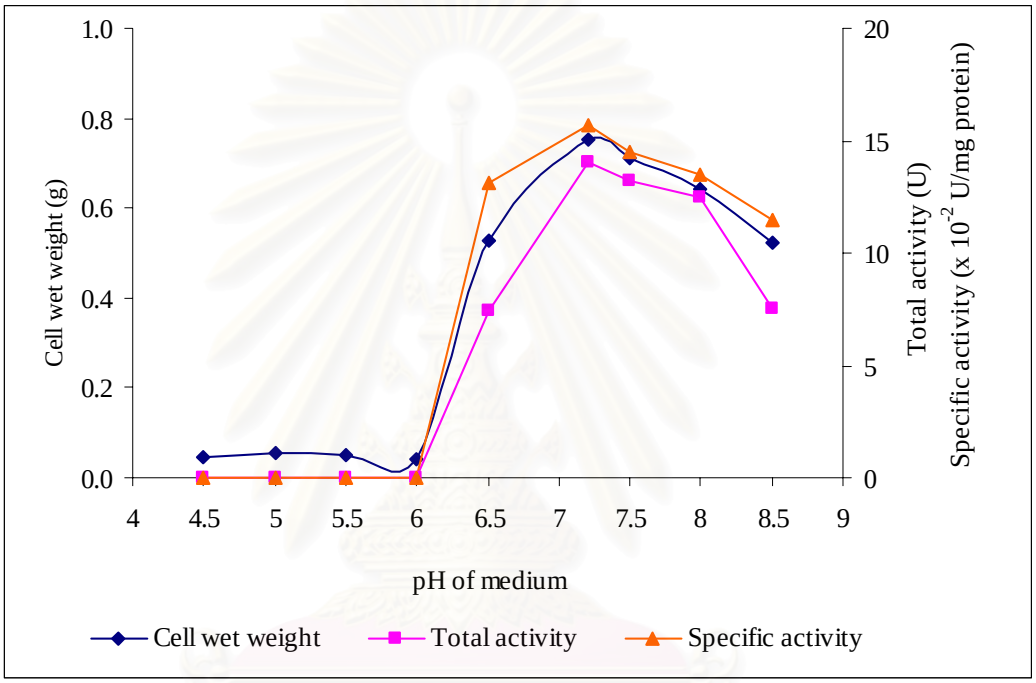


Figure 3.1 Effect of pH of medium on leucine dehydrogenase production and growth of *Alcaligenes faecalis*

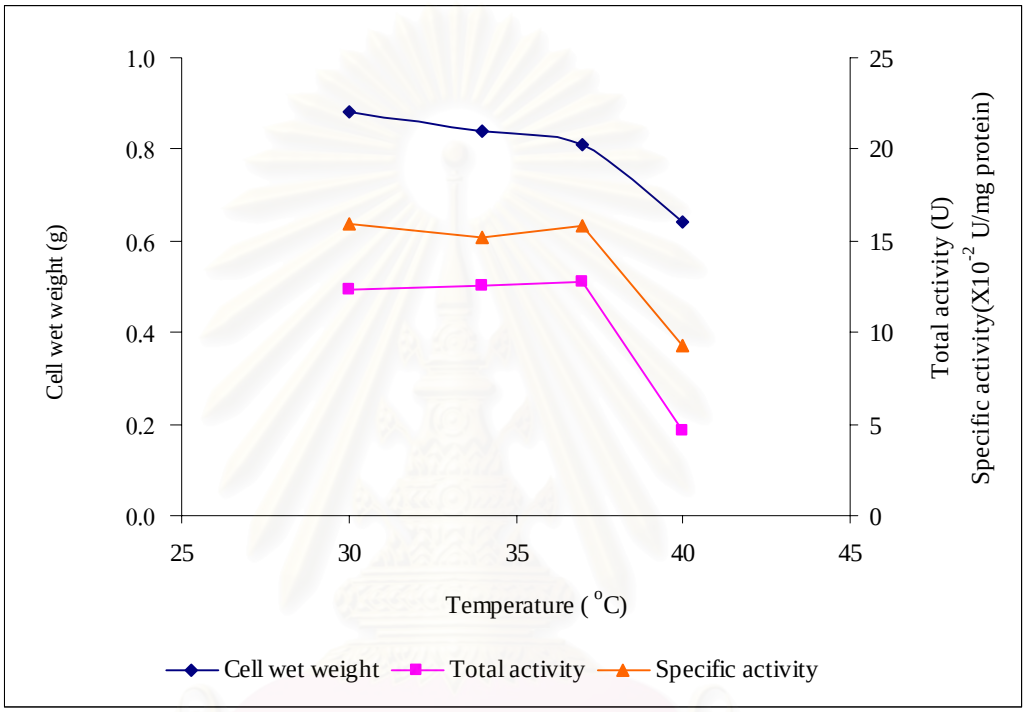


Figure 3.2 Effect of cultivation temperature on leucine dehydrogenase production and growth of *Alcaligenes faecalis*

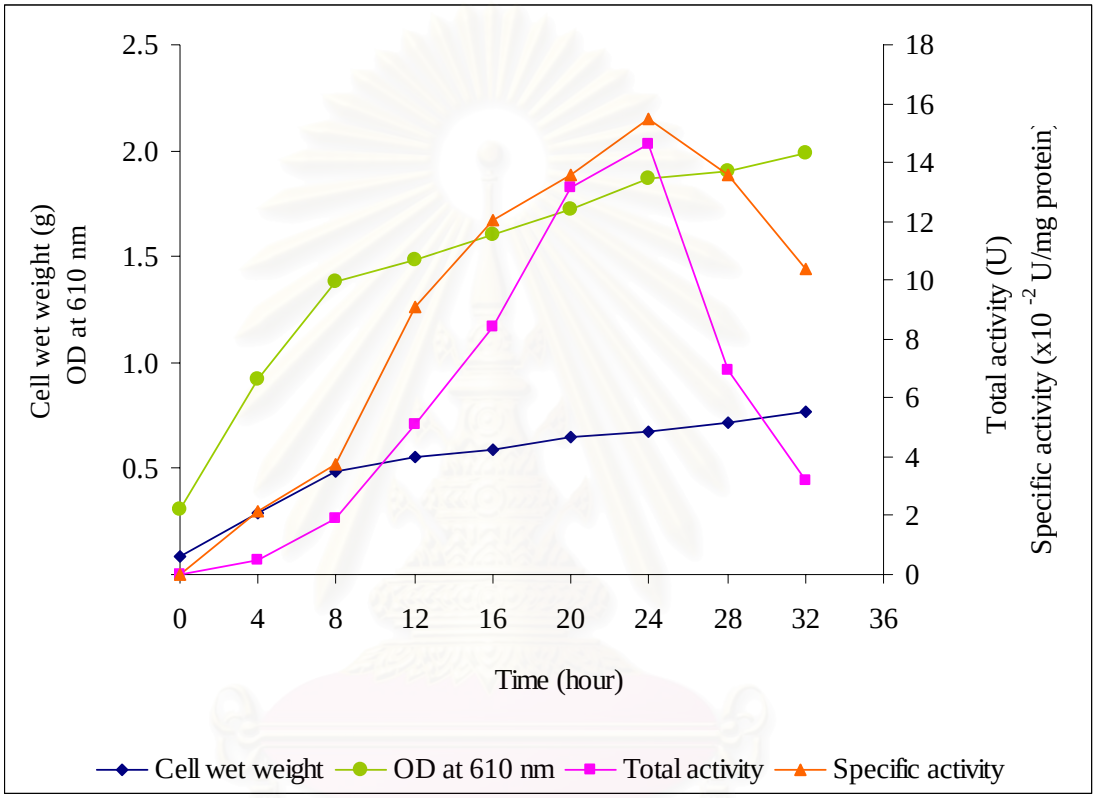


Figure 3.3 Effect of cultivation time on leucine dehydrogenase production and growth of *Alcaligenes faecalis*

3.3.4 Enzyme induction

The enzyme inducer was studied for LeuDH production as mentioned in section 2.8.6. As shown in figure 3.4, LeuDH could be induced to 1.3, 1.6, 1.9 and 1.1 fold by 0.2% D-Leucine, L-Leucine, L-isoleucine and L-valine, in peptone medium pH 7.2, respectively. The highest total activity and specific activity were obtained by addition of 0.2% L-isoleucine with 39.25 units and 0.25 units/mg protein, respectively. Thus, L-isoleucine was used as an enzyme inducer for the next experiment.

3.3.5 Optimum concentration of inducer

To study the influence of inducer concentration on LeuDH yield and specific activity as described in section 2.8.7, various L-isoleucine concentrations from 0 to 1.0% were added to peptone medium, pH 7.2. Total activity speedly increased corresponding with increasing inducer concentration while cell wet weight and specific activity slightly increased. Since, the specific activity increased to 1.6 times at 0.2% L-isoleucine, 0.4% to 1.0% L-isoleucine showed no marked different and L-isoleucine is quite expensive. Therefore, L-isoleucine was omitted for *A. faecalis* cultivation in the next experiment (Figure 3.5).

In conclusion, the optimum condition for leucine dehydrogenase production from *Alcaligenes faecalis* was 24 hours of cultivation in 1% peptone medium, pH 7.2 at 37°C.

3.4 Purification of leucine dehydrogenase from *Alcaligenes faecalis*

3.4.1 Enzyme production

The 28 g cell wet weight of *Alcaligenes faecalis*, obtained from 4 liters of cell culture as described in section 2.9.1, was disrupted as mentioned in section 2.9.2. Cell free extract contained 1,820 mg proteins with 270 units of LeuDH activity.

Therefore, the specific activity of the enzyme in cell free extract preparation was 0.15 units/mg protein.

3.4.2 Enzyme purification steps

3.4.2.1 DEAE-Toyopearl column chromatography

The crude enzyme solution was applied on DEAE-Toyopearl 650M column as described in section 2.9.3.1. From chromatographic profile (Figure 3.6), the unbound proteins were eluted from the column with 10 mM phosphate buffer and the bound proteins were eluted with stepwise of 0.1, 0.2, 0.3, 0.4 and 0.5 M KCl in the phosphate buffer, respectively. The enzyme was eluted with the buffer containing 0.3 M potassium chloride. The activity fractions were pooled, concentrated by aquasorb, and dialysed against phosphate buffer to remove potassium chloride. This step removed 1,547 g of contaminated proteins. The LeuDH activity was increased to 333.0 units after dialysis and specific activity was 1.22 units / mg as shown in Table 3.1. The enzyme was about 8 fold purified with 121.7% recovery.

3.4.2.2 Butyl-Toyopearl column chromatography

The pooled LeuDH active fractions from DEAE-Toyopearl column was further purified as described in section 2.9.3.2 on the Butyl-Toyopearl column. The chromatographic profile is given in figure 3.7. The unbound proteins were eluted with the phosphate buffer and the bound proteins were eluted with linear salt gradient of 0.8 to 0.5 M ammonium sulfate. The enzyme was eluted at 0.7 M ammonium sulfate.

The LeuDH active fractions were pooled, reduced the enzyme volume with aquasorb and dialysed against the phosphate buffer. The remaining protein was 22.2 mg. Total activity and specific activity were 59.7 units and 2.69 units/mg protein. The LeuDH was purified for 17.9 folds with 121.7% recovery.

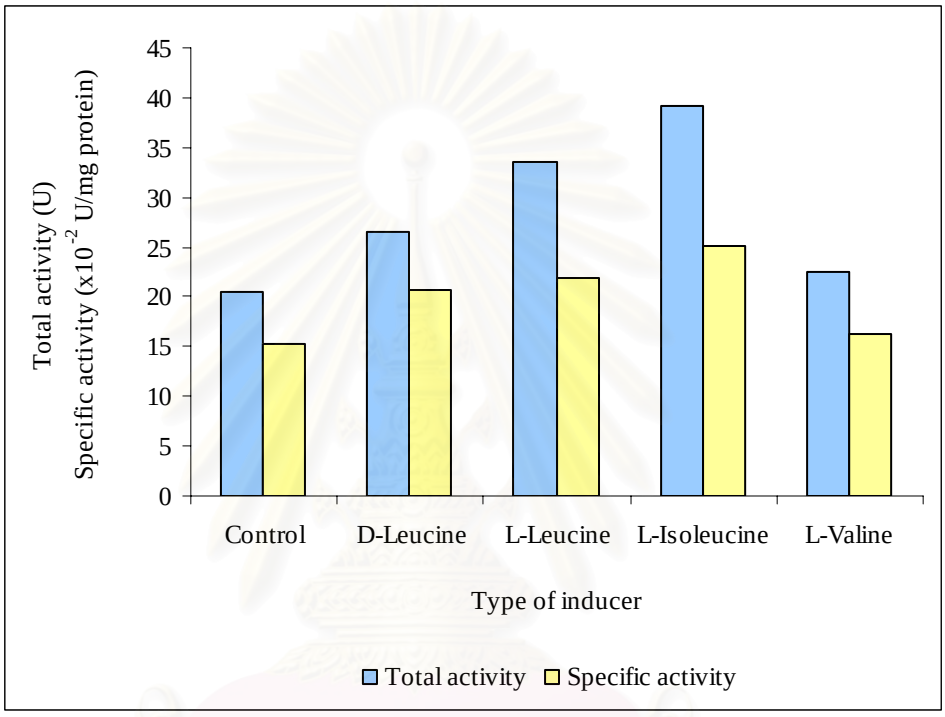


Figure 3.4 Effect of various amino acids as enzyme inducers on leucine dehydrogenase production of *Alcaligenes faecalis*

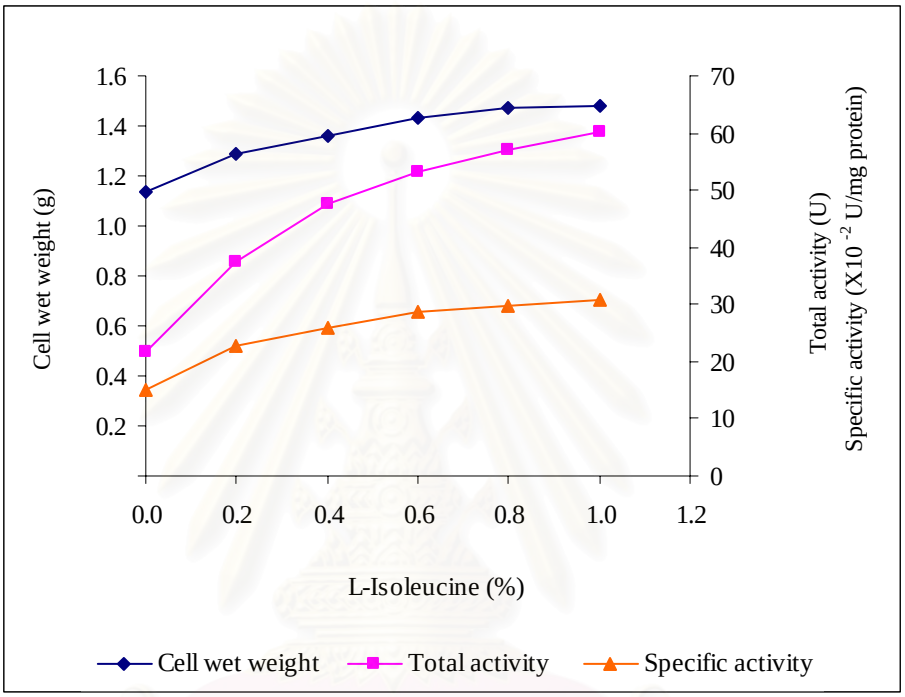


Figure 3.5 Effect of concentration of L-Isoleucine as enzyme inducer on leucine dehydrogenase production and growth of *Alcaligenes faecalis*

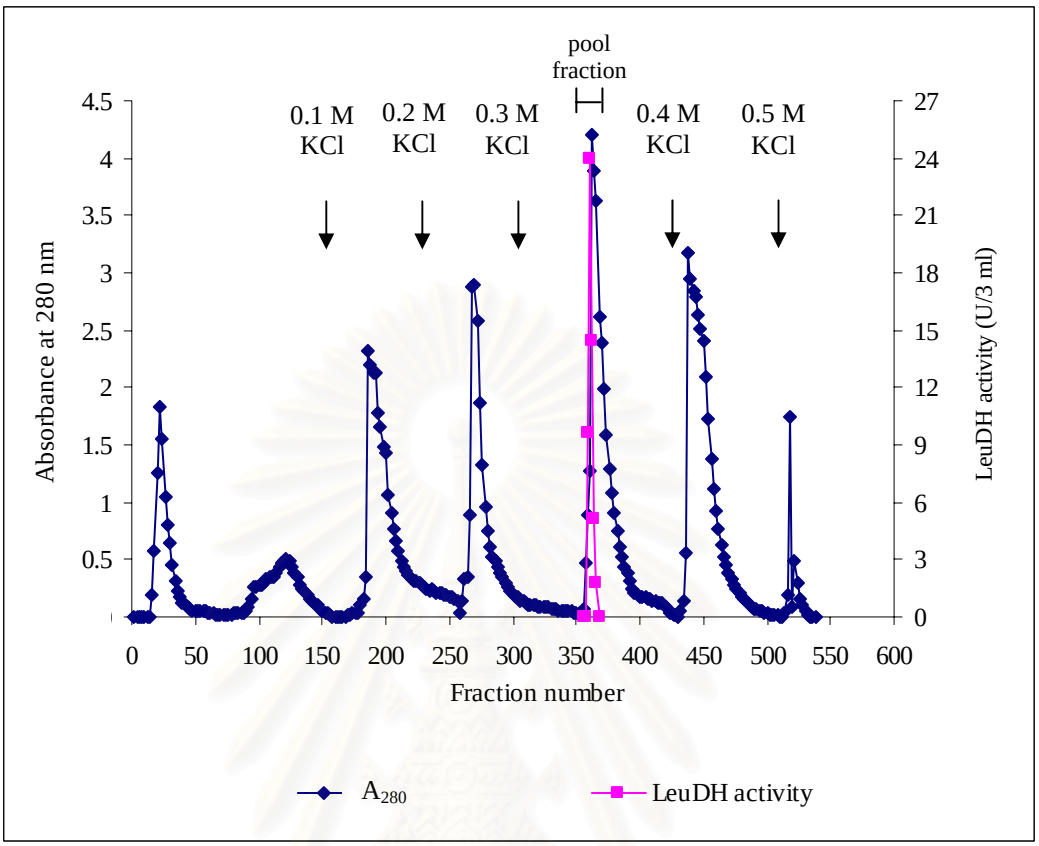


Figure 3.6 Purification of leucine dehydrogenase from *Alcaligenes faecalis* by DEAE-Toyopearl 650M column

The enzyme was applied to DEAE-Toyopearl 650M column (1.7x 28 cm) and washed with 10 mM phosphate buffer, pH 7.4 containing 0.01% β-mercaptoethanol and 1 mM EDTA until A_{280} decreased to base line. The bound protein elution was made by stepwise of 0.1, 0.2, 0.3, 0.4 and 0.5 M KCl in the same buffer at the flow rate 1 ml/min. Fractions of 3 ml were collected. The arrows indicate where each stepwise started. The protein peak from fraction numbers 359-365 were pooled.

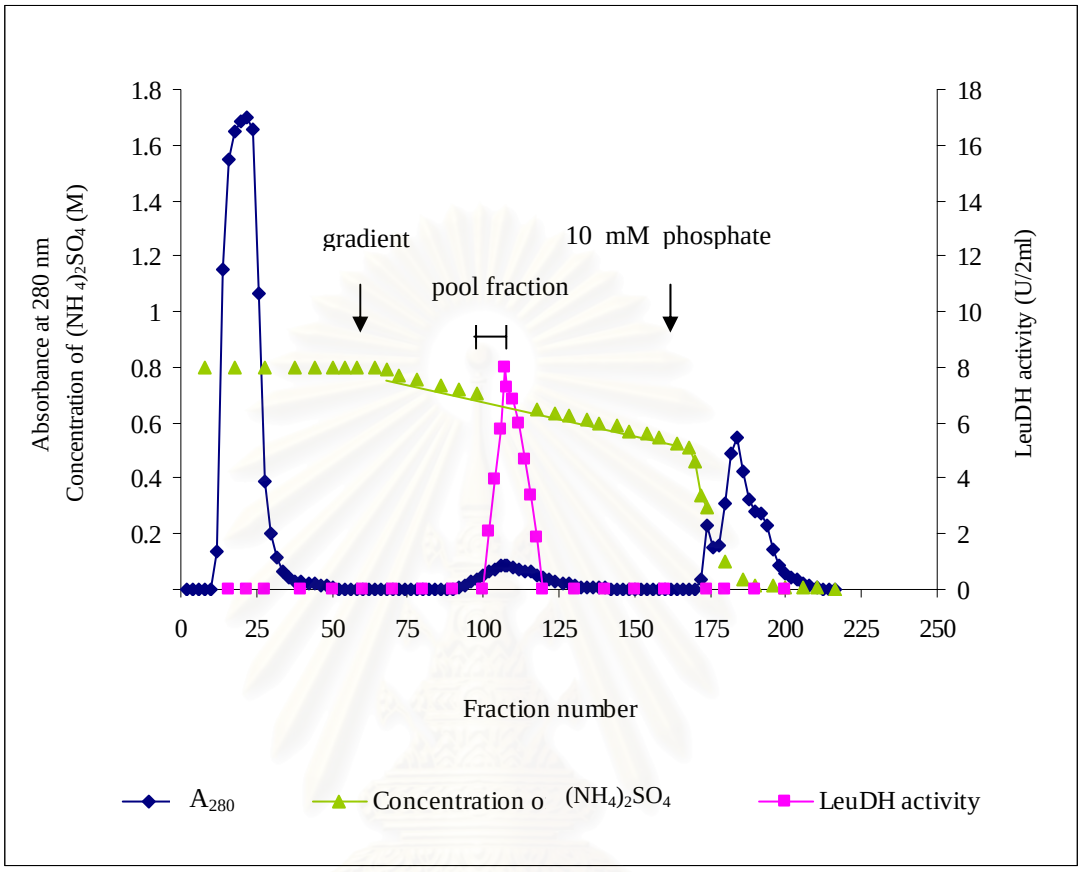


Figure 3.7 Purification of leucine dehydrogenase from *Alcaligenes faecalis* by Butyl-Toyopearl column

The enzyme was applied to Butyl-Toyopearl column (1.7x 10 cm) and washed with 0.8 M ammonium sulfate in 10 mM phosphate buffer, pH 7.4 containing 0.01% β-mercaptoethanol and 1 mM EDTA until A_{280} decreased to base line. The bound protein elution was made by gradient of 0.8-0.5 M ammonium sulfate in the same buffer followed by the phosphate buffer at the flow rate 1 ml/min. The fractions of 2 ml were collected. The arrows indicate gradient started and stopped. The protein peak from fraction numbers 102-118 were pooled.

3.4.2.3 Hitrap Q column chromatography

The pooled LeuDH active fraction from Butyl-Toyopearl column was further purified as described in section 2.9.3.3 by Hitrap Q column. The chromatographic profile is shown in figure 3.8. The unbound proteins were eluted with the phosphate buffer and the bound proteins were eluted with linear gradient of 0 to 0.5 M potassium chloride in the buffer. The enzyme was eluted at 0.32 M potassium chloride. The LeuDH activity fractions were pooled, reduced the enzyme volume with aquasorb and dialysed against the phosphate buffer. The remaining protein after Hitrap Q column was 4.5 mg with enzyme activity 44.8 units and the specific activity of 9.96 units / mg. The enzyme was purified to 66.4 fold with 16.4% yield. The purified enzyme was kept at 4°C for characterization and kinetic experiment. The summary of leucine dehydrogenase purification is given in Table 3.1.

3.4.2.4 Determination of enzyme purity on non-denaturing polyacrylamide get electrophoresis (native-PAGE)

The enzyme purity from each purification step and native protein pattern were investigated by non-denaturing polyacrylamide gel electrophoresis as described in section 2.9.4 The results of protein staining and activity staining are shown in figure 3.9. The single band from Hitrap Q column in lane 4A and 4B indicates that the enzyme was purified to homogeneity.

3.5 Characterization of leucine dehydrogenase from *Alcaligenes faecalis*

3.5.1 Molecular weught determination of leucine dehydrogenase

3.5.1.1 Ferguson plot analysis

The native molecular weight of LeuDH was determined from molecular weight calibration curve of standard proteins obtain from 5-10% non-denaturing polyacrylamide gel as described in section 2.10.1.1. From the calibration curve, the native molecular weight of the enzyme was calibrated was calculated to be 536 kDa.

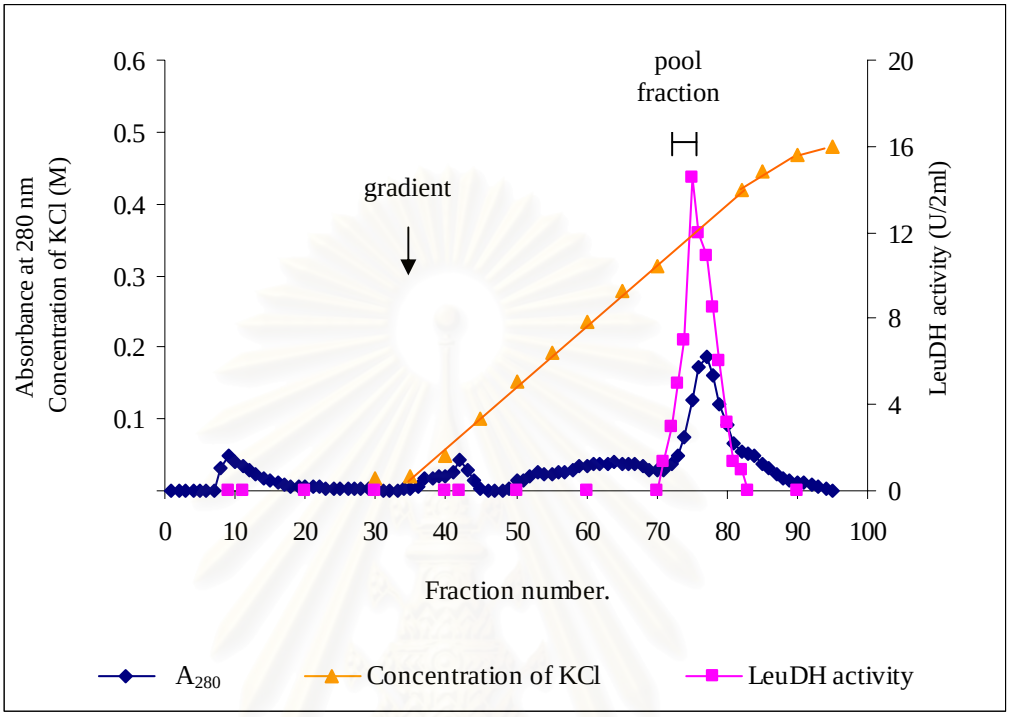


Figure 3.8 Purification of leucine dehydrogenase from *Alcaligenes faecalis* by Hitrap Q column

The enzyme was applied to Hitrap Q column and washed with 10 mM phosphate buffer, pH 7.4 containing 0.01% β-mercaptoethanol and 1 mM EDTA until A_{280} decreased to base line. The bound protein elution was made by gradient of 0-0.5 M potassium chloride in the same buffer at the flow rate 0.5 ml/min. The fractions of 2 ml were collected. The arrow indicates where gradient started. The protein peak from fraction numbers 72-76 were pooled.

Table 3.1 Purification of leucine dehydrogenase

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Purification fold	%Recovery
Crude extract	1,820.0	273.0	0.15	1.0	100
DEAE-Toyopearl column	273.0	333.0	1.22	8.1	121.7
Butyl-Toyopearl column	22.2	59.7	2.69	17.9	21.8
Hitrap Q column	4.5	44.8	9.96	66.4	16.4

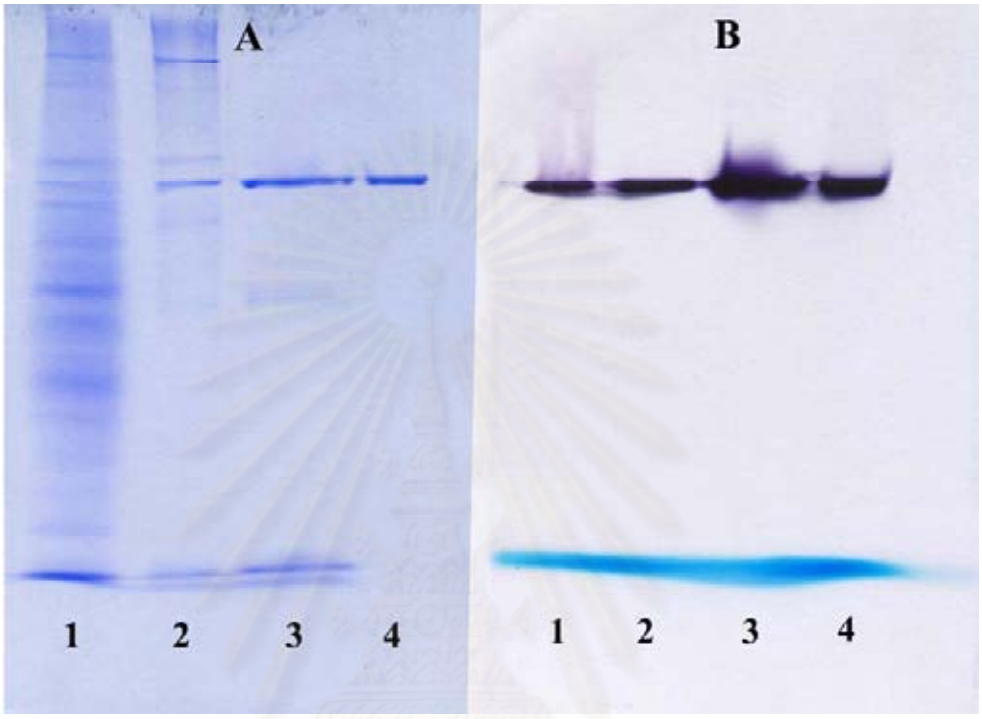


Figure 3.9 Non-denaturing PAGE of the *Alcaligenes faecalis* leucine dehydrogenase from each purification step

A. Protein staining B. Activity staining

Lane 1	Crude extract	114	µg
Lane 2	DEAE-Toyopearl column	20	µg
Lane 3	Butyl-Toyopearl column	15	µg
Lane 4	Hitrap Q column	6	µg

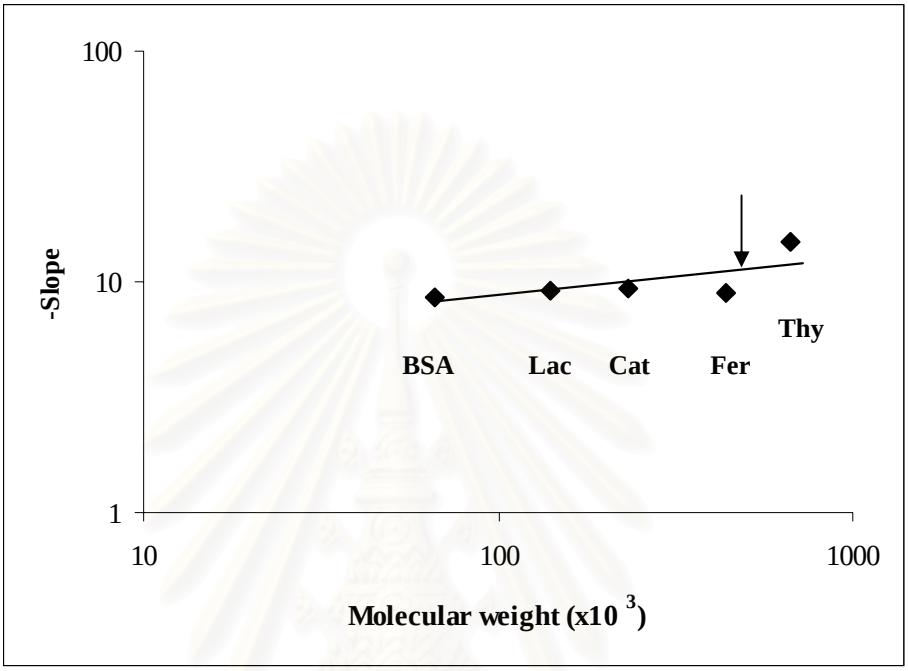


Figure 3.10 Calibration curve for native molecular weight of leucine dehydrogenase from *Alcaligenes faecalis* determined by Ferguson plot

Thy	=	Thyroglobulin	(MW 669,000)
Fer	=	Ferritin	(MW 440,000)
Cat	=	Catalase	(MW 232,000)
Lac	=	Lactate dehydrogenase	(MW 140,000)
BSA	=	Bovine serum albumin	(MW 66,000)

Arrow indicates molecular weight of LeuDH

3.5.1.2 SDS-polyacrylamide gel electrophoresi

As described in section 2.10.1.2, the molecular weight of the enzyme subunit was also determined by SDS-polyacrylamide gel electrophoresis (Figure 3.11). The molecular weight of enzyme subunit was calculated to be 52,000 Dalton from molecular weight calibration curve as shown in figure 3.12. This suggest that the *Alcaligenes faecalis* LeuDH was a decamer.

3.5.2 Effect of pH on leucine dehydrogenase activity

The LeuDH optimum pH was assayed at various pHs as described in section 2.10.2. The result is shown in figure 3.13. The LeuDH activity when using $NaHCO_3$-NaOH and $NAHPO_4$-NaOH buffer were not significantly different from glycine–KCI–KOH buffer (data were not showed). When potassium phosphate buffer was used, it showed lower LeuDH activity than glycine–KCI–KOH buffer. Thus, glycine–KCI–KOH buffer at 200 mM final concentration were used. The enzyme exhibited the maximum activity for oxidative deamination and reductive amination at pH 10.8 and 8.8, respectively.

3.5.3 Effect of pH on leucine dehydrogenase stability

The pH stability of the enzyme was investigated by incubating the enzyme in 10 mM buffer at various pHs for 20 minutes at 30°C as mentioned in section 2.10.3. The enzyme activity was high stability on pH range of 6.0 to 12.5 as shown in figure 3.14.

3.5.4 Effect of temperature on leucine dehydrogenase activity

The LeuDH optimum temperature was investigated at various temperatures as described in section 2.10.4. The enzyme showed the highest activity at 45°C for oxidative deamination and 55°C for reductive animation as shown in figure 3.15.

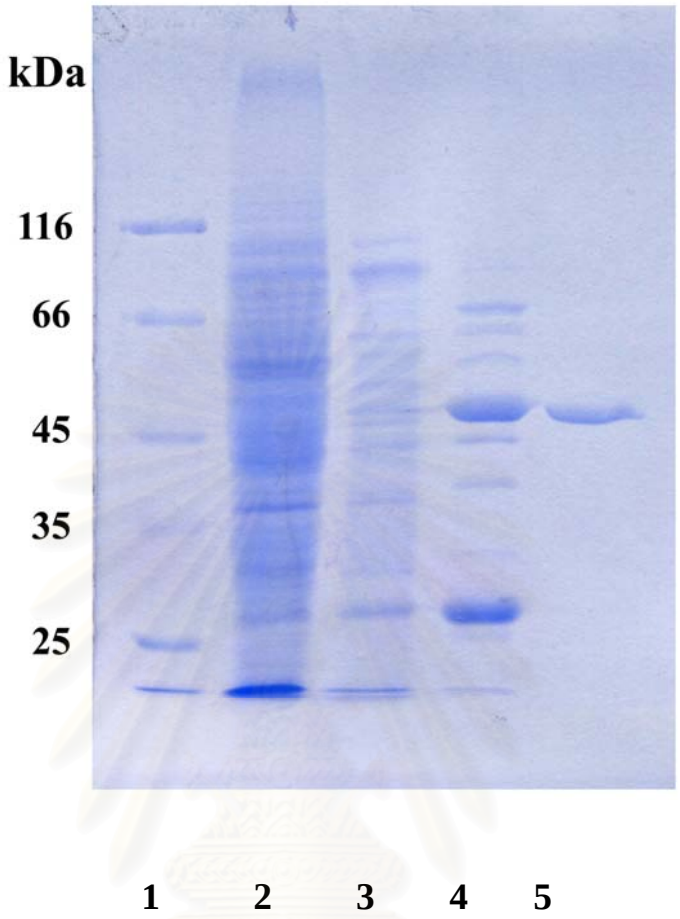


Figure 3.11 SDS-PAGE of the *Alcaligenes faecalis* leucine dehydrogenase from each purification step

Lane 1	Protein marker		
	β-galactosidase	(MW	116,000)
	Bovine serum albumin	(MW	66,000)
	Ovalbumin	(MW	45,000)
	Lactate dehydrogenase	(MW	35,000)
	RE *Bsp* 98I	(MW	25,000)
Lane 2	Crude extract	114	μg
Lane 3	DEAE-Toyopearl column	20	μg
Lane 4	Butyl-Toyopearl column	15	μg
Lane 5	Hitrap Q column	6	μg

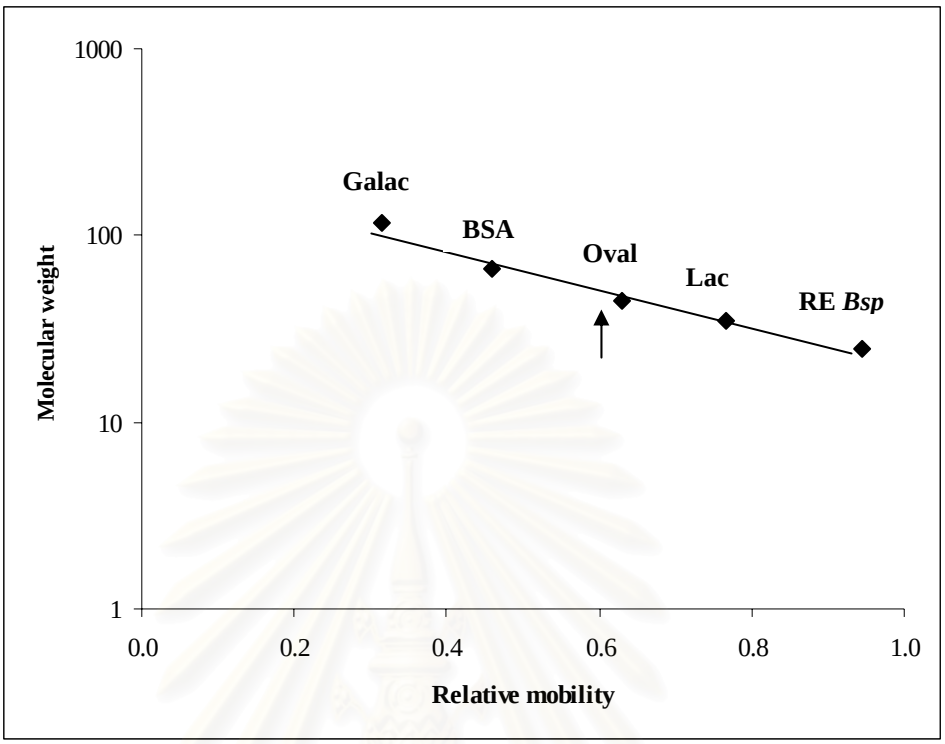


Figure 3.12 Calibration curve for molecular weight of leucine dehydrogenase subunit on SDS-polyacrylamide gel electrophoresis

Galac	=	β-Galactosidase	(MW 116,000)
BSA	=	Bovine serum albumin	(MW 66,200)
Oval	=	Ovalbumin	(MW 45,000)
Lac	=	Lactate dehydrogenase	(MW 35,000)
RE *Bsp*	=	RE *Bsp* 98I	(MW 25,000)

Arrow indicates the relative mobility of LeuDH

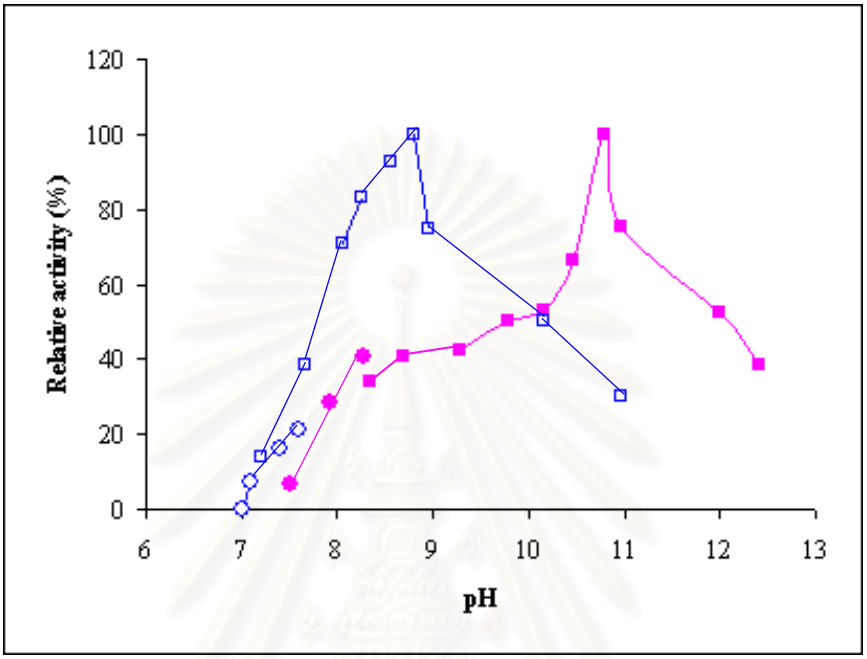


Figure 3.13 Effect of pH on leucine dehydrogenase activity

The oxidative deamination: (●) Potassium phosphate buffer, (■) Glycine-KCl-KOH buffer. The reductive amination: (o) Potassium phosphate buffer, (□) Glycine-KCl-KOH buffer.

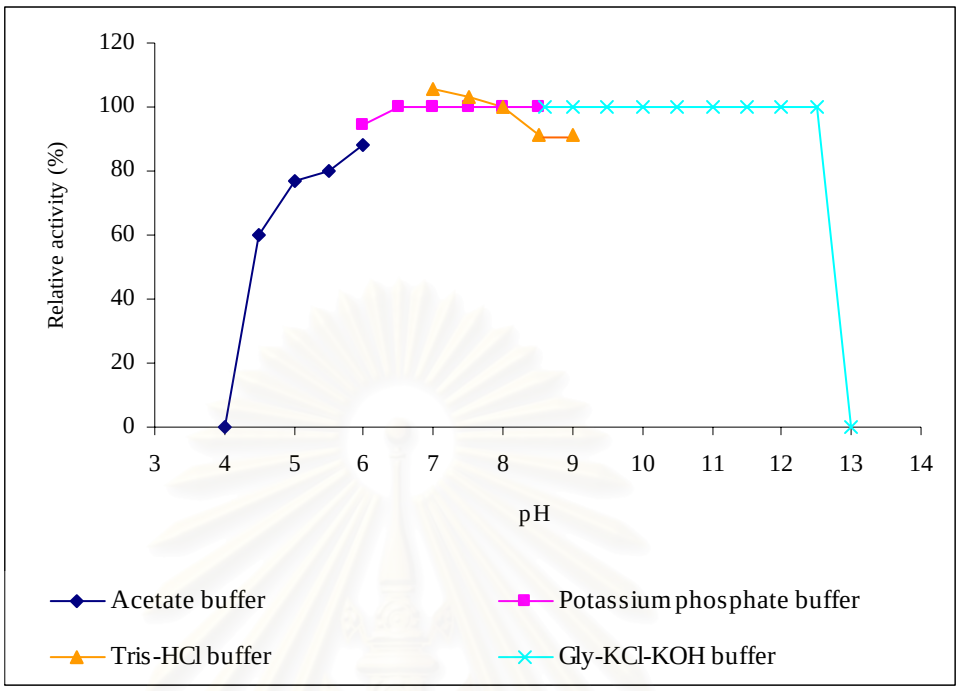


Figure 3.14 Effect of pH on leucine dehydrogenase stability

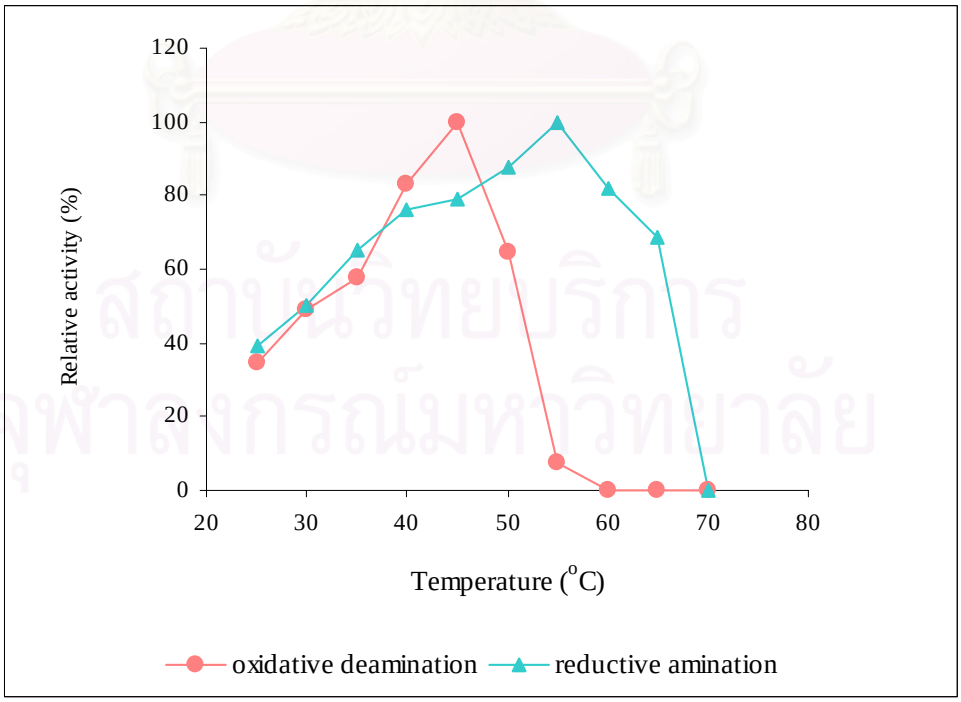


Figure 3.15 Effect of temperature on leucine dehydrogenase activity

3.5.5 Effect of temperature on leucine dehydrogenase stability

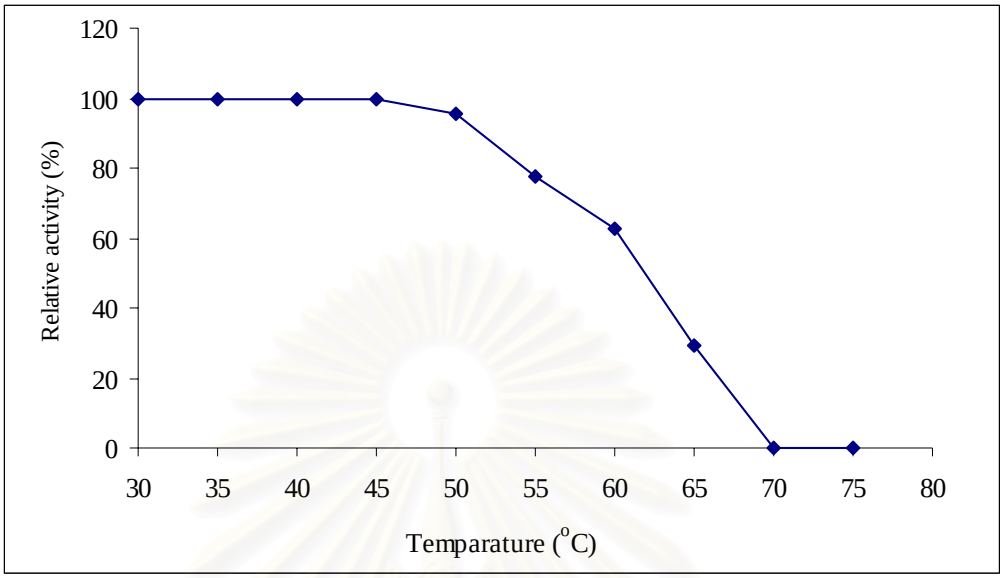
The effect of temperature on the enzyme stability was determined as described in section 2.10.5. The result is shown in figure 3.16A. The enzyme remained its full activity up to 45°C and lost all activity at 70°C after incubation for 10 minutes. From this data, the enzyme was incubated at 45°C and taken for assay of its remaining activity every 6 hours. The result was displayed in figure 3.16B. The enzyme retained its full activity for 8 hours. At the 8^{th} day, half of the activity was lost and its activity was completely lost after 15 days of incubation.

3.5.6 Substrate specificity of leucine dehydrogenase

The ability of LeuDH to catalyse its substrates in deamination and amination was determined as described in section 2.10.6. 7-Aminoheptanoate, L-penicillamine, L-phenylalanine, L-arginine, L-asparagine, L-theronine, L-glutamate, L-alanine, glycine, L-histidine, L-proline, L-serine, L-lysine, L-methionine, L-tryptophan, L-aspartate, L-cysteine, L-tyrosine L-alloisoleucine, D-norvaline, D-norleucine, D-alloisoleucine, D-leucine and D-valine could not be used as substrates. The result is shown in Table 3.2. L-Valine and L-isoleucine were better substrate than L-leucine with 133 and 113% relative activity, respectively. The enzyme poorly catalysed L-norleucine, L-γ-methylleucine, *S*-methyl–L-cysteine and 4-azaleucine for oxidative deamination.

The enzyme activities for α-ketoisovalerate, α-keto-β-methylvalerate and α-ketovalerate were higher than that of α-ketoisocaproate for reductive amination. The enzyme had 30% activity when α-keto-γ-methiol-n-butyrarate was used as a substrate. The LeuDH used DL-keto-β-methyl-n-valerate, α-ketocaproate, α-keto-n-butyrate as substrates. On the other hand, α-ketoglutarate and phenylpyruvate could not be used as substrate.

A



B

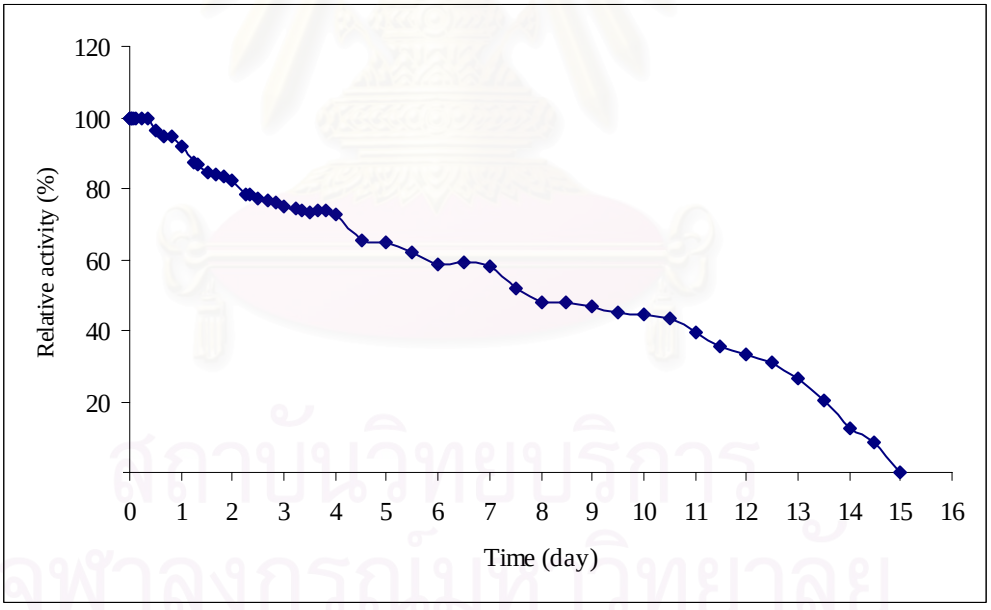


Figure 3.16 Effect of temperature on leucine dehydrogenase stability

(A) Thermostability of enzyme (B) Enzyme stability at 45°C

The leucine dehydrogenase activity for oxidative deamination was assayed at 30°C.

Table 3.2 Substrate specificity of *Alcaligenes faecalis* leucine dehydrogenase

Process / substrate	Relative activity (%)
Oxidative deamination [a]	
L-Leucine	100
L-Isoleucine	113
L-Valine	133
DL-Isoleucine	88
L-Norvaline	86
L-Norleucine	9
L-α-Aminobutyrate	35
DL-Allylglycine	11
4-Azaleucine	6
L-γ-Methylleucine	5
S-Methyl-L-cysteine	5
Reductive amination [b]	
α-Ketoisocaproate	100
α-Ketoisovalerate	208
α-Ketovalerate	135
α-Keto-β-methylvalerate	123
DL-Keto-β-methyl-n-valerate	96
α-Ketocaproate	58
α-Keto-n-butyrate	58
α-Keto-γ-methiol-n-butyrate	33

[a] Final concentration of each substrate was 20 mM. The followings were inert: 7 aminoheptanoate, L-penicillamine, Lphenylalanine, L-arginine, L-asparagine, L-threonine, L-glutamate, L-alanine, glycine, L-histidine, L-proline, L-serine, L-lysine, L-methionine, L-tryptophan, L-aspatate, L-cysteine (10mM), L-tyrosine (5 mM), L-alloisoleucine, D-norvaline, D-norleucine, D-leucine, D-isoleucine and D-valine.

[b] Final concentration of each substrate was 20 mM. The followings were inert: α-ketoglutarate and phenylpyruvate.

3.5.7 Coenzyme specificity of leucine dehydrogenase

Coenzyme specificity was investigated as mentioned in section 2.11.7. The enzyme required NAD^+ as a natural coenzyme for the oxidative deamination of L-leucine as shown in Table 3.3. In addition, analogues of NAD^+ could serve as coenzymes. 3-Acetylpyridine-adenine dinucleotide showed the activity about 5 fold of that of NAD^+. Nicotinamide hypoxanthine dinucleotide (deamino-NAD^+) showed a similar relative activity to NAD^+ while β-nicotinamide adenine dinucleotidephosphate (NADP), nicotinic acid adenine dinucleotide (deamido-NAD^+), nicotinamide 1,N^6-ethenoadenine dinuclectide (ε-NAD^+) and 3-pyridinealdehyde adenine dinucleotide were inert. The enzyme activities of nicotinamide guanine dinucleotide and thionicotinamide adenine dinuclectide were lower than that of NAD^+.

3.5.8 Inhibitory effect of various amino acids and keto acids on leucine dehydragenase activity

This experiment was done as described in section 2.11.8 to focus on effect of various amino acids and keto acids, which were nonsubstrate of LeuDH, on oxidative deamination and reductive amination. The result is shown in Table 3.4. D-Leucine and L-γ-methylleucine moderately inhibited the enzyme activity while D-norvaline, L-lysine, 4-azaleucine, L-norleucine and L-methionine showed slight inhibition. Other nonsubstrate amino acids had no effect. For inhibitory effect of keto acids, α-ketoglutarate slightly inhibited the enzyme activity while phenylpyruvate did not affect.

3.5.9 Effect of metal ions and chemical substrances on leucine dehydrogenase activity

The summary of effect of metal ions and some chemical reagents on LeuDH activity is given in Table 3.5. The enzyme was incubated in 10 mM final concentration of metal ions or chemical reagents, unless otherwise stated. All

Table 3.3 Coenzyme specificity of leucine dehydrogenase *from Alcaligenes faecalis*

Coenzyme [a]	Relative activity (%)
β-Nicotinamide adenine dinucleotide (NAD^+)	100
Nicotinamide hypoxanthine dinucleotide (Deamino-NAD^+)	100
3-Acetylpyridine adenine dinucleotide	478
Nicotinamide guanine dinucleotide	45
Thionicotinamide adenine dinucleotide	18
β-Nicotinamide adenine dinucleotidephosphate ($NADP^+$)	0
Nicotinic acid adenine dinucleotide (Deamido-NAD^+)	0
Nicotinamide 1,N^6-ethenoadenine dinuclectide (ε-NAD^+)	0
3-Pyridinealdehyde adenine dinucleotide	0

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

Table 3.4 Inhibitory effect of various amino acids and keto acids on leucine dehydrogenase activity

Process and non substrate	Relative activity (%)
Oxidative deamination [a]	
None	100
D-Leucine	64
D-Norvaline	78
L-Lysine	88
4-Azaleucine	88
L-Norleucine	83
L-Methionine	83
L-γ-Metylleucine	69
Reductive amination [b]	
None	100
α-Ketoglutarate	88

[a] Amino acid concentration was 20 mM except for L-tyrosine (5 mM). D-Isoleucine, D-valine, D-norleucine, D-norvaline, L-alanine, L-aspatate, L-serine, L-threonine, L-phenylalanine, L-arginine, L-penicillamine, L-tyrosine and L-glutamate did not inhibit the reaction.

[b] Keto acid concentration was 10 mM. Phenylpyruvate did not inhibit the reaction.

Table 3.5 Effect of metal ions and chemical substances on leucine dehydrogenase activity

Compound [a]	Final concentration (mM)	Relative activity (%)
None	-	100
NaCl	10	97
KCl	10	100
$MgCl_2$	10	100
$MnCl_2$	10	100
$CoCl_2$	10	91
$ZnCl_2$	10	91
$MgSO_4$	10	100
$CuSO_4$	10	100
Na_2CO_3	10	100
NaBr	10	97
$HgCl_2$	1	0
β-Mercaptoethanol	10	88
EDTA	10	90
NaN_3	10	96
Hydroxylamine	10	99

[a] Abbreviations: EDTA, ethylenediamine tetraacetic acid.

reagents tested had no significant effect on the enzyme activity except $HgCl_2$. Strong inhibition of $HgCl_2$ may be cause of enzyme denature which this heavy metal inhibits all of proteins.

3.5.10 Effect of group-specific reagents on leucine dehydrogenase activity

The effect of group-specific reagents on enzyme activity was determined by incubating the enzyme with each chemical reagent at a final concentration of 10 mM as mentioned in section 2.11.10.1 to 2.11.10.10. The residual activity for oxidative deamination were determined and compared with that of untreated enzyme as shown in Table 3.6. The enzyme activity was completely inhibited by *N*-bromosuccinimide (NBS), chloramine T (CT), and 2,4,6-trinitrobenzene sulfonic acid (TNBS). This result indicated that tryptophan, methionine and lysine residues probably were essential residues for enzyme activity and played important roles in the active site of LeuDH. The significant loss of activity was observed when the enzyme was modified with diethylpyrocarbonate (DEPC) and phenylglyoxal (PG) which were specific for histidine and arginine, respectively. The significant loss of activity was observed when the enzyme was modified with diethylpyrocarbonate (DEPC) and phenylglyoxal (PG) which were specific for histidine On the other hand, *N*-acetylimidazole, (NAI), dithiothreitol (DTT), iodoacetamide (IAM)**,** *N*-ethylmaleimide (NEM) and phenylmethylsulfonyl fluoride (PMSF) which modify tyrosine, cysteine and serine, respectively, had no effect on the enzyme activity.

3.6 Kinetic mechanism studies of leucine dehydrogenase from *Alcaligenes faecalis*

A series of steady state kinetic analyses was conducted to investigate the reaction mechanism of LeuDH.

Table 3.6 Effect of various group-specific reagents on leucine dehydrogenase from *Alcaligenes faecalis*

Group-specific reagent	Amino acid involved	Residual activity (%)		
		10 mM[a]	**1 mM[a]**	**0.1mM[a]**
None	-	100	100	100
N-Acetylimidazole (NAI)	Tyrosine	85	-	-
N-Bromosuccinimide (NBS)	Tryptophan	0	0	0
Chloramine T (CT)	Methionine	0	0	54
Diethylpyrocarbonate (DEPC)	Histidine	16	87	-
Dithiothreitol (DTT)	Cysteine	95	-	-
Phenylglyoxal (PG)	Arginine	70	-	-
Iodoacetamide (IAM)	Cysteine	65	100	-
N-Ethylmaleimide (NEM)	Cysteine	71	100	-
Phenylmethylsulfonyl fluoride (PMSF)	Serine	100	-	-
2,4,6-Trinitrobenzene sulfonic acid (TNBS)	Lysine	0	18	75

[a] = Concentration of group-specific reagent

- = Did not observed

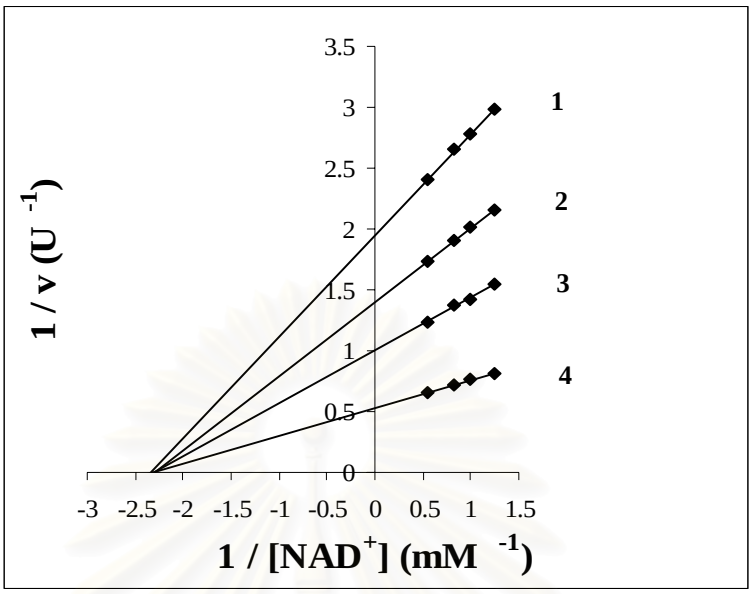
3.6.1 Initial velocity studies for oxidative deamination

The initial velocity studies for oxidative deamination were first performed with NAD^+ as a variable substrate in the presence of several fixed concentration of L-leucine. Reciprocals plots of initial velocities against reciprocal of NAD^+ concentrations gave a family of straight lines which intersect in the 2nd quadrant as shown in figure 3.17-3.19. The apparent Michaelis constant (K_m) for NAD^+ and L-leucine were calculated to be 0.44 and 4.2 mM from figure 3.17A and 3.17B, respectively. The result indicated that the reaction proceeds via the formation of ternary complex of the enzyme with NAD^+ and L-leucine. In the same way, reciprocals plots of initial velocities against reciprocals of NAD^+ concentrations in the presence of several fixed concentrations of L-isoleucine or L-valine were performed. The Michaelis constant (K_m) for L-isoleucine and L-valine were calculated to be 4.3 and 14.0 mM as shown in figure 3.18 and 3.19, respectively.

3.6.2 Initial velocity studies for the reductive amination

A kinetic analysis in the reductive amination was performed to investigate the reaction mechanism. As shown in figure 3.20, double reciprocal plots of initial velocities against α-ketoisocaproate concentrations at several fixed concentrations of NADH and saturating and constant concentration of ammonium chloride gave straight intersecting. At saturating and constant concentration of NADH, the double reciprocal plots of velocities against α-ketoisocaprorate concentrations at several fixed concentrations of ammonium chloride also gave straight lines intersecting as shown in figure 3.21. In addition, with α-ketoioscaproate at a saturating and constant concentration, the double reciprocal plots of velocities against NADH concentrations at several different ammonium chloride concentrations gave paralle lines as shown in figure 3.22. The apparent Michaelis constant (K_m) for NADH, α-ketoisocroproate, and ammonium chloride were calculated to be 0.02, 3.3 and 100 mM, respectively.

A



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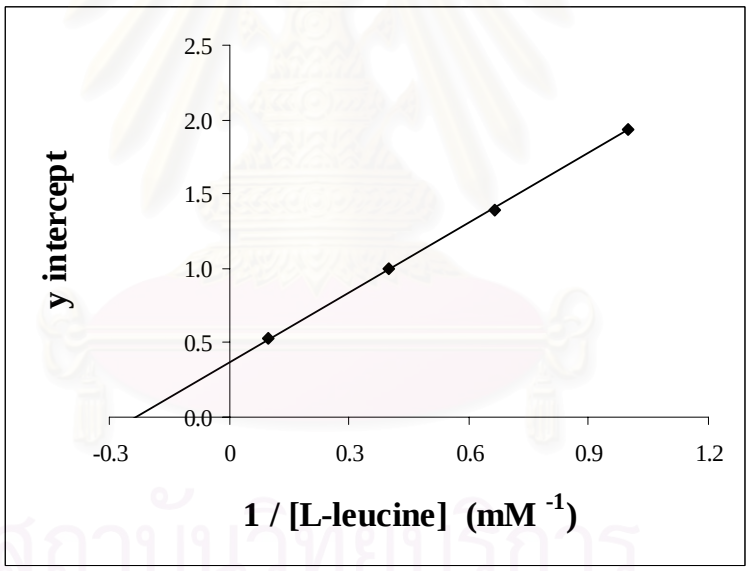
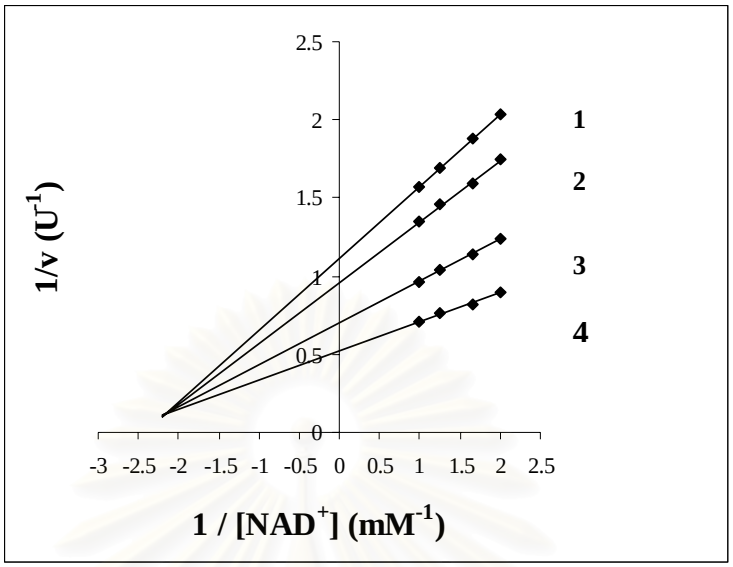


Figure 3.17 Initial velocity for oxidative deamination using L-leucine as a substrate

A. Double-reciprocal plots of initial velocities versus NAD^+ concentrations at a series of fixed concentration of L-leucine. L-Leucine concentrations were 1.0 mM (1), 1.5 mM (2), 2.5 mM (3), and 10 mM (4). NAD^+ concerntrations were 0.8, 1.0, 1.2, and 1.8 mM.

B. Secondary plots of y intercepts versus reciprocal L-leucine concentrations.

A



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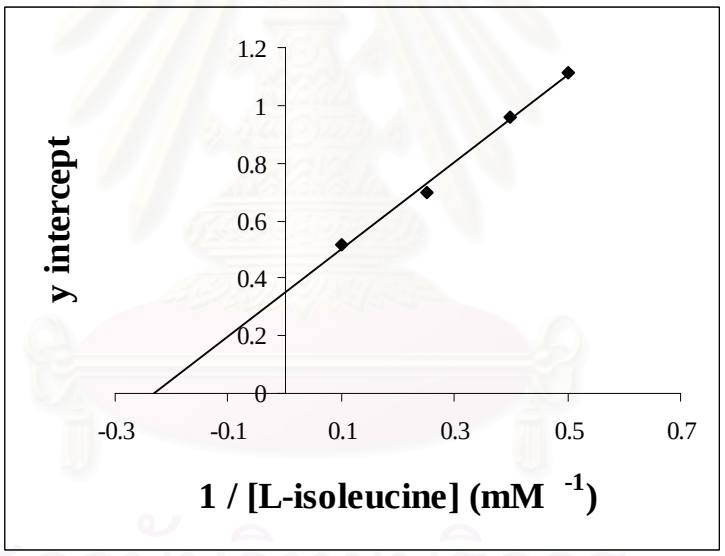
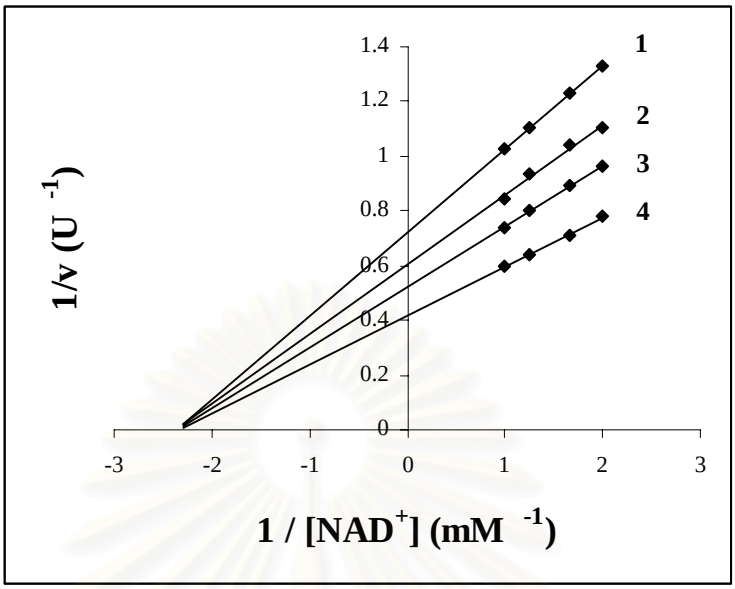


Figure 3.18 Initial velocity for oxidative deamination using L-isoleucine as a substrate

A. Double-reciprocal plots of initial velocities versus NAD^+ concentrations at a series of fixed concentration of L-isoleucine. L-Isoleucine concentrations were 2.0 mM (1), 2.5 mM (2), 4.0 mM (3), and 10 mM (4). NAD^+ concerntrations were 0.5, 0.6, 0.8, and 1 mM.

B. Secondary plots of y intercepts versus reciprocal L-isoleucine concentrations.

A



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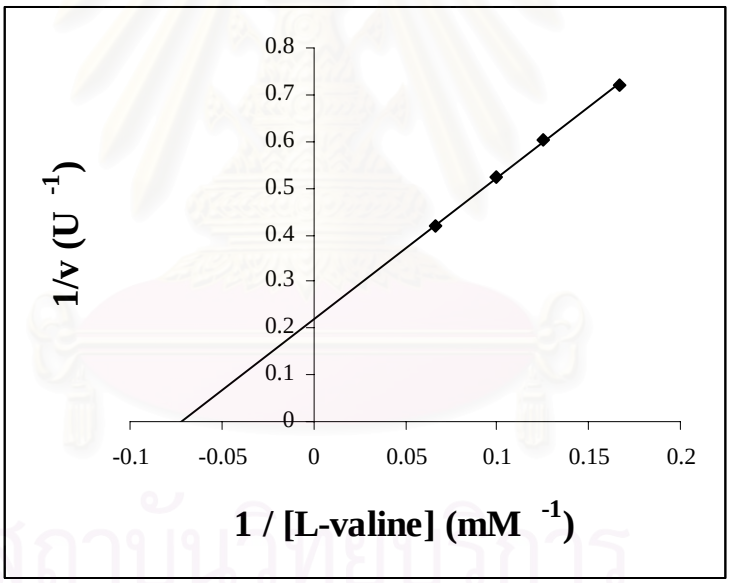
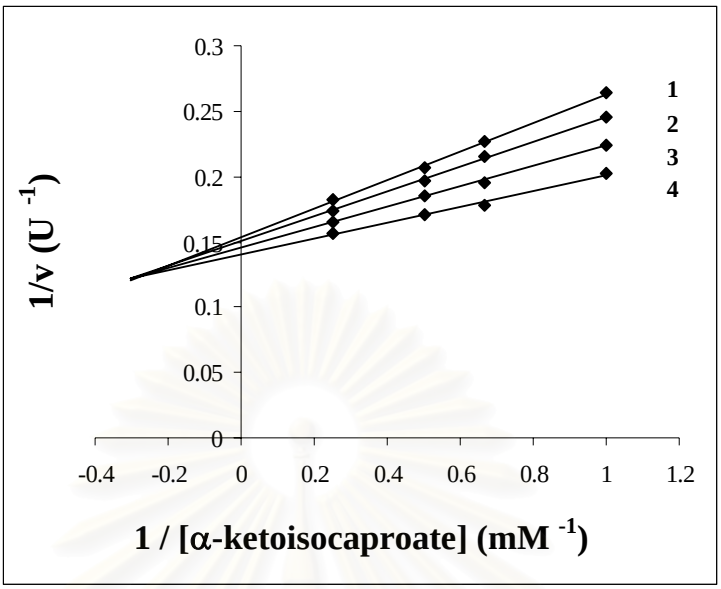


Figure 3.19 Initial velocity for oxidative deamination using L-valine as a substrate

A. Double-reciprocal plots of initial velocities versus NAD^+ concentrations at a series of fixed concentration of L-valine. L-valine concentrations were 6.0 mM (1), 8.0 mM (2), 10.0 mM (3), and 15.0 mM (4). NAD^+ concerntrations were 0.5, 0.6, 0.8, and 1 mM.

B. Secondary plots of y intercepts versus reciprocal L-valine concentrations.

A



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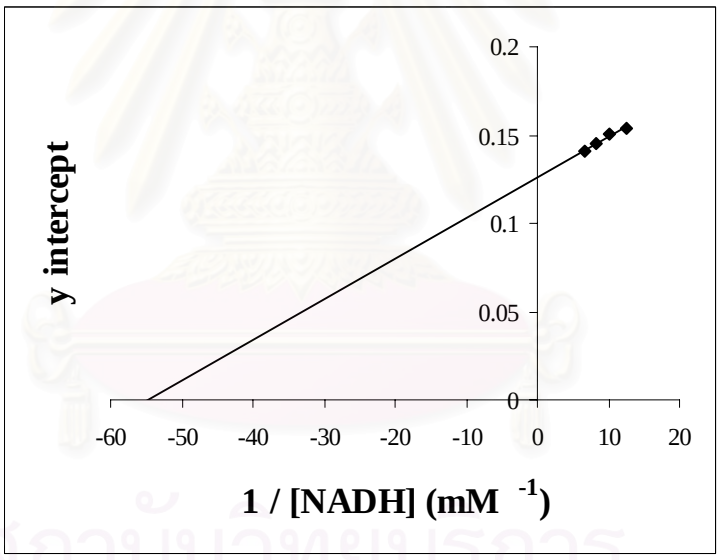
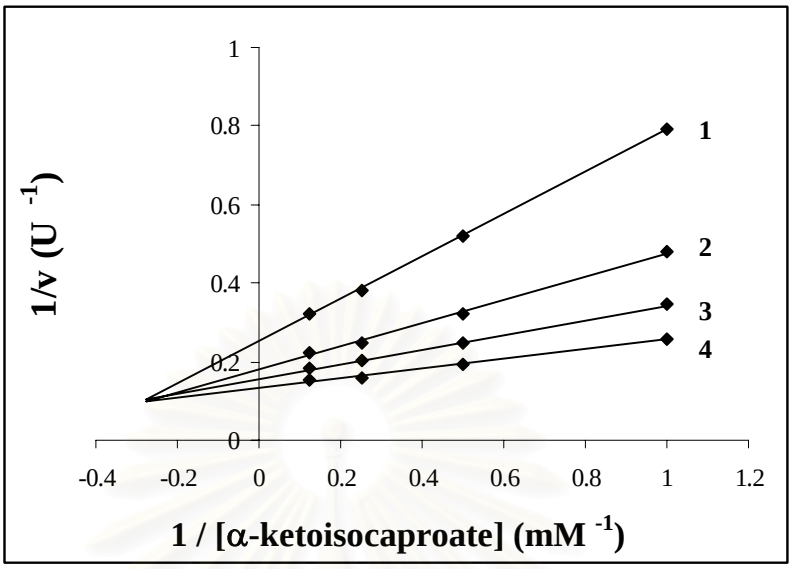


Figure 3.20 Initial velocity pattern for reductive amination (α-ketoisocaproate vs NADH)

A. Double-reciprocal plots of initial velocities versus α-ketoisocaproate concentrations at several fixed concentrations of NADH in the presence of saturating and constant concentration of NH_4Cl (500 mM). Concentrations of NADH were 0.08 mM (1), 0.1 mM (2), 0.12 mM (3) and 0.15 mM (4).

B. Secondary plots of y intercepts versus reciprocal concentrations of α-ketoisocaproate

A



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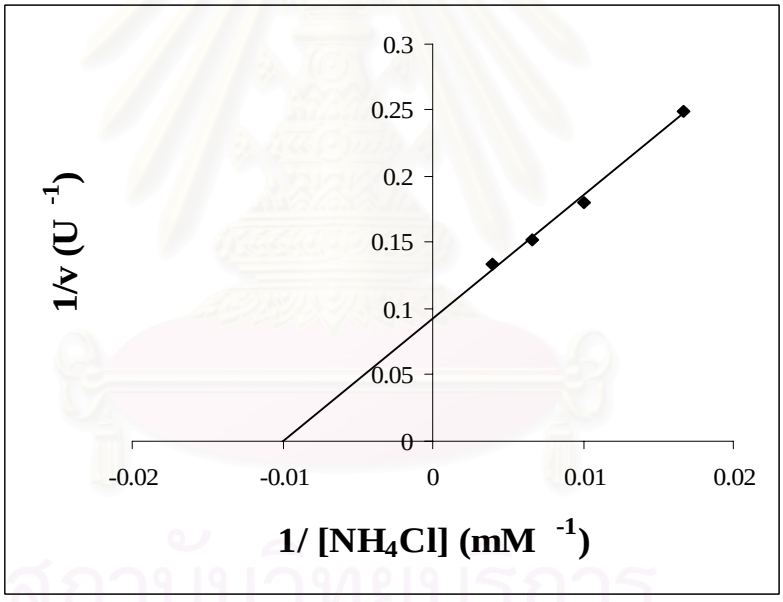


Figure 3.21 Initial velocity pattern for reductive amination (α-ketoisocaproate vs NH_4Cl)

A. Double-reciprocal plots of initial velocities versus α-ketoisocaproate concentrations at several fixed concentrations of NH_4Cl in the presence of saturating and constant concentration of NADH (0.2 mM). Concentrations of NH_4Cl were 60 mM (1), 100 mM (2), 150 mM (3) and 250 mM (4).

B. Secondary plots of y intercepts versus reciprocal concentrations of NH_4Cl

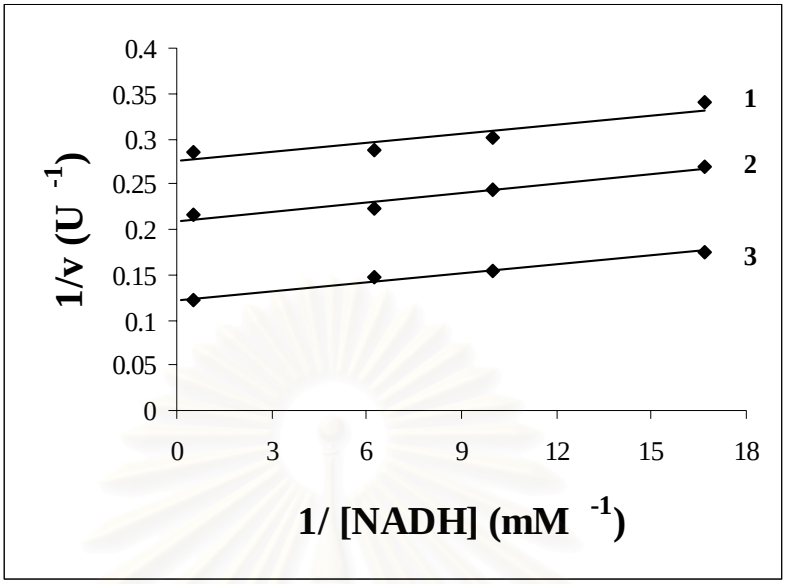


Figure 3.22 Initial velocity pattern for reductive amination (NADH vs NH_4Cl)

Double-reciprocal plots of initial velocities versus NADH concentrations at several fixed concentrations of NH_4Cl in the presence of saturating and constant concentration α-ketoisocaproate (15 mM). Concentrations of NH_4Cl were 60 mM (1), 80 mM (2) and 200 mM (3).

These observed kinetic patterns rule out the possibility of random addition of substrates and represent a sequential ordered mechanism in which α-ketoisocaoprate binds to the enzyme between NADH and ammonia. The apparent K_m of substrates of both oxidative and reductive reactions were summarized in Table 3.7.

3.6.3 Product inhibition studies

The product inhibition studies in the oxidative deamination were carried out to determine the order of substrate addition and product release according to the method of Cleland (Cleland, 1971) as mentioned in section 2.12.3. With NADH as an inhibitor, the double reciprocal plots of velocities against NAD^+ concentrations at saturating and constant concentration of L-leucine showed a competitive inhibition pattern as shown in figure 3.23A. This finding suggests that NAD^+ and NADH can bind to the free form of the enzyme and thus NAD^+ binds first to the enzyme followed by L-leucine and that NADH is the last product that is released from the enzyme. The product inhibition by NH_4Cl with respect to L-leucine was uncompetitive as shown in figure 3.24B. The other product inhibition patterns observed with ammonium chloride and α-ketoisocaproate as inhibitors were identical with the predicted patterns for the sequential ordered binary ternary kinetic mechanism as shown in figure 3.24 and 3.25, respectively. The observation of uncompetitive inhibition by α-ketoisocaproate with respect to L-leucine (Figure 3.25B) ruled out a mechanism of Theorell-Chance type (Theorell and Chance, 1951). These results showed that the sequence of addition of the substrate in the oxidative deamination is NAD^+ and L-leucine and that of release of products is ammonium chloride, α-ketoisocaproate and NADH as shown in figure 3.26. The observed product inhibition patterns of oxidative deamination were summarized in Table 3.8.

3.7 Amino acid sequence analysis

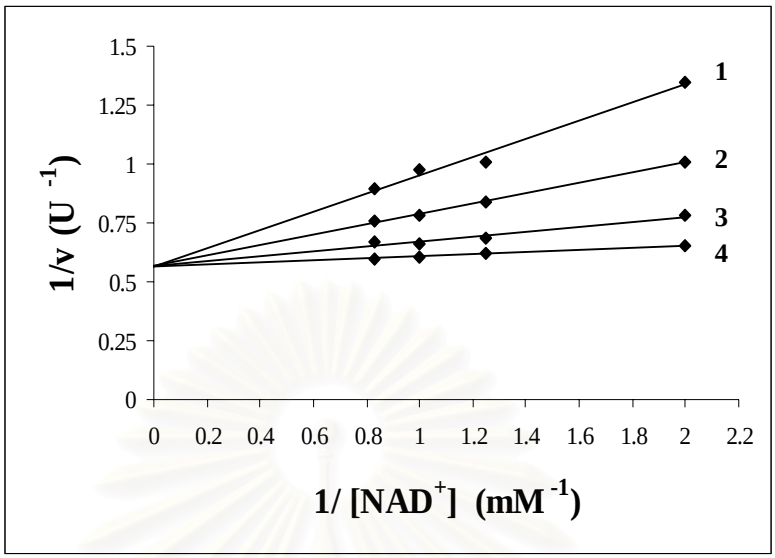
Amino acid sequence analysis of leucine dehydrogenase from *Alcaligenes faecalis* was performed as mentioned in section 2.12. After the enzyme was digested by lysyl endopeptidase, the isolated peptide fragment ware sequenced. The peptide

at the N-terminus of LeuDH identified as MEIFNYMEQADYEQLVIX QD while amino acid sequence of 3 internal peptide fragments were PGPXGPAGS KG (or V) EPGPAGPXG, T (or L,Y,V) LPGLAGTXG, and RDNIPSYVAADRL AEERIRVA.

Table 3.7 The apparent K_m value of substrates of leucine dehydrogenase from *Alcaligenes faecalis*

Substrate	K_m (mM)
L-Leucine	4.2
L-Isoleucine	4.3
L-Valine	14.0
NAD^+	0.44
α-Ketoisocaproate	3.3
NH_4Cl	100
NADH	0.02

A



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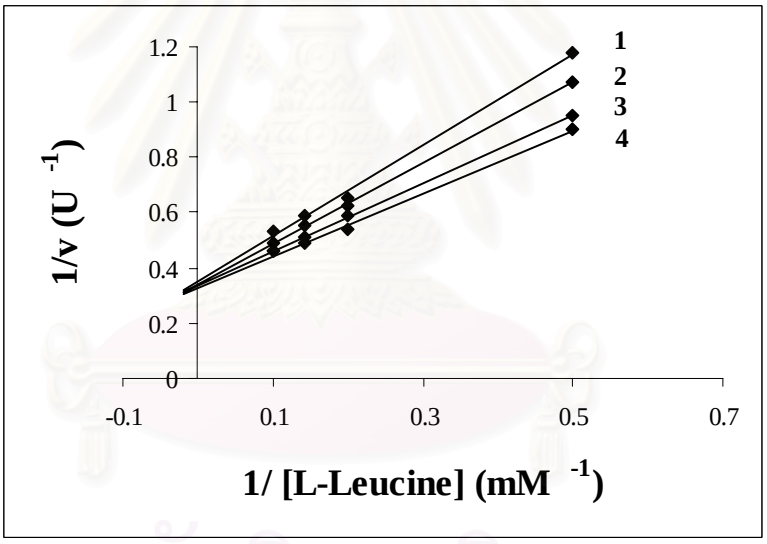
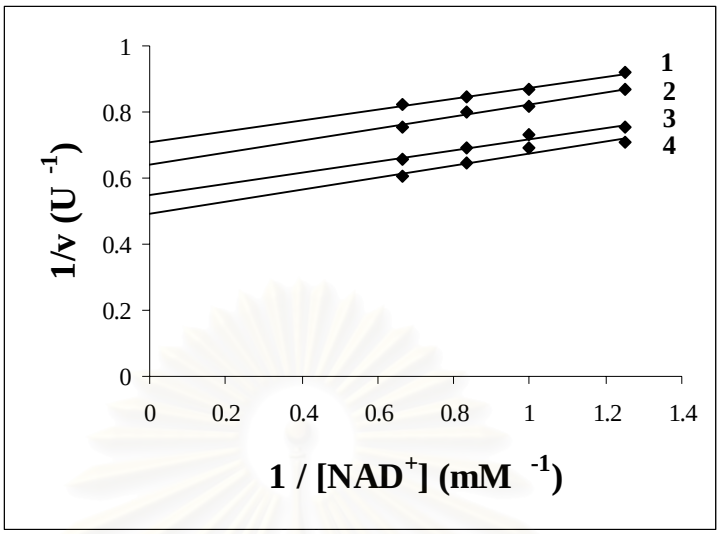


Figure 3.23 Product inhibition pattern of oxidative deamination by NADH

A. Product inhibition pattern by NADH with respect to NAD^+ as the varied substrate. L-Leucine was held at saturating and constant concentraton (20 mM). Concentrations of NADH were 0 mM (1), 0.025 mM (2), 0.05 mM (3) and 0.1 mM (4).

B. Product inhibition pattern by NADH with respect to L-leucine as the varied substrate. NAD^+ was held at saturating and constant concentraton (2 mM). Concentrations of NADH were 0 mM (1), 0.025 mM (2), 0.05 mM (3) and 0.1 mM (4).

A



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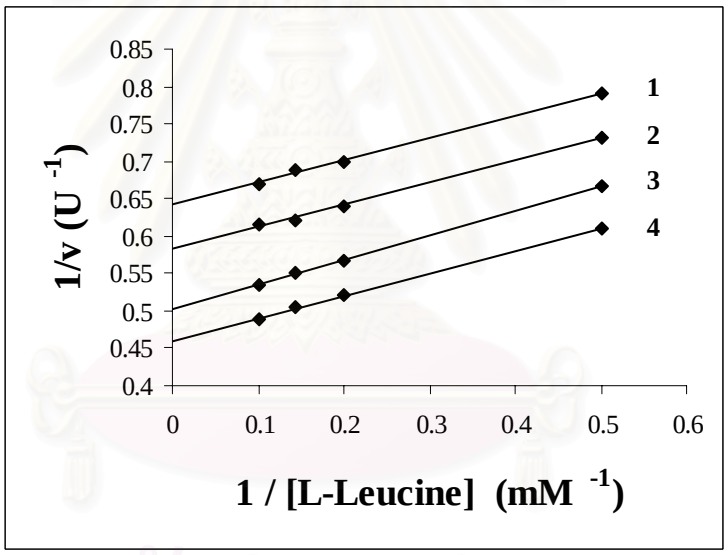

Figure 3.24 Product inhibition pattern of oxidative deamination by NH_4Cl

A. Product inhibition pattern by NH_4Cl with respect to NAD^+ as the varied substrate. L-Leucine was held at saturating and constant concentraton (20 mM). Concentrations of NH_4Cl were 0 mM (1), 20 mM (2), 100 mM (3) and 200 mM (4).

B. Product inhibition pattern by NH_4Cl with respect to L-leucine as the varied substrate. NAD^+ was held at saturating and constant concentraton (2 mM). Concentrations of NH_4Cl were 0 mM (1), 20 mM (2), 100 mM (3) and 200 mM (4).

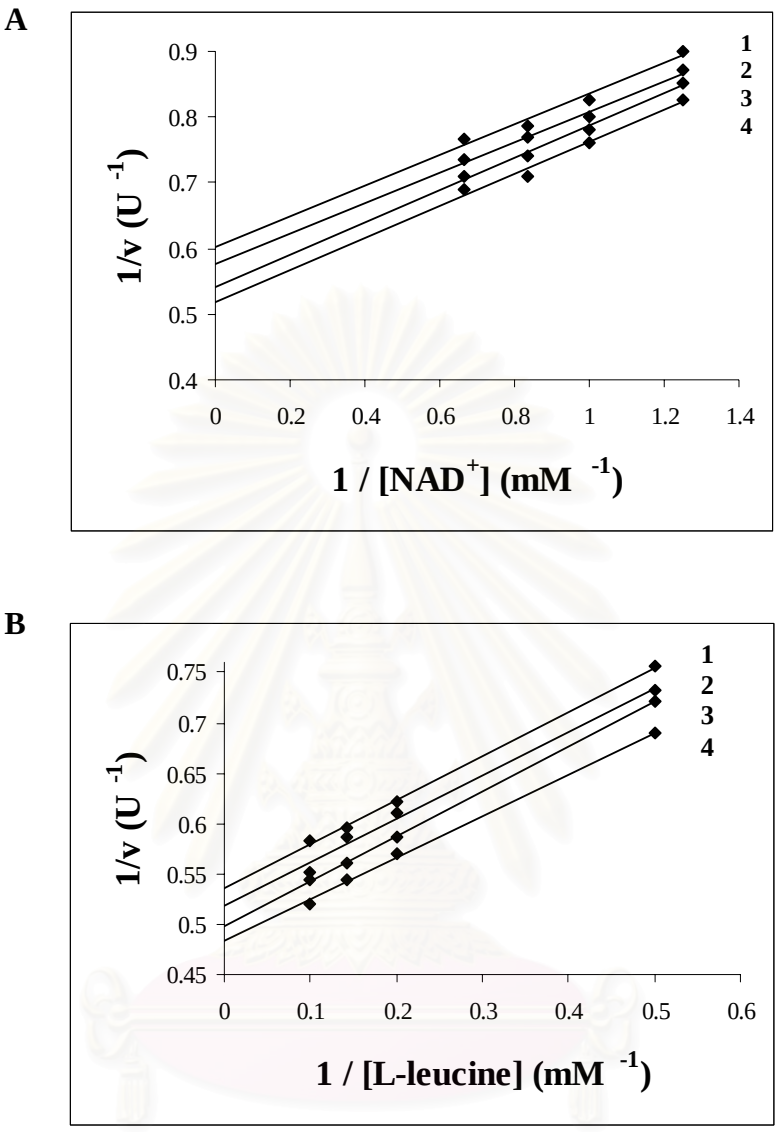

Figure 3.25 Product inhibition pattern of oxidative deamination by α-ketoisocaproate

A. Product inhibition pattern by α-ketoisocaproate with respect to NAD^+ as the varied substrate. L-Leucine was held at saturating and constant concentraton (20 mM). Concentrations of α-ketoisocaproate were 0 mM (1), 1 mM (2), 2 mM (3) and 5 mM (4).

B. Product inhibition pattern by α-ketoisocaproate with respect to L-leucine as the varied substrate. NAD^+ was held at saturating and constant concentraton (2 mM). Concentrations of α-ketoisocaproate were 0 mM (1), 1 mM (2), 2 mM (3) and 5 mM (4).

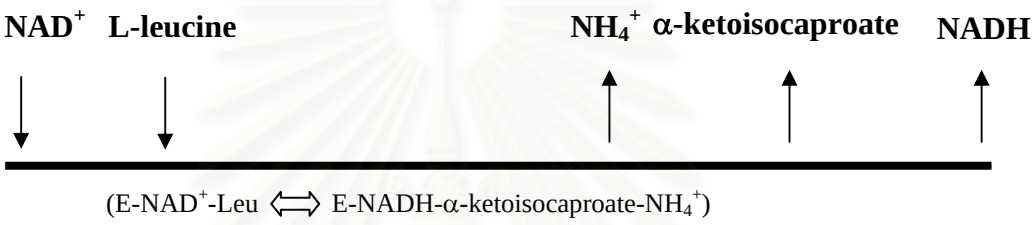

Figure 3.26 Kinetic mechanism of leucine dehydrogenase from *Alcaligenes faecalis*

Table 3.8 Product inhibition patterns of oxidative deamination of leucine dehydrogenase from *Alcaligenes faecalis*

Substrate	Product inhibition pattern by		
	NADH	**α-ketoisocaproate**	**NH_4Cl**
NAD^+	competitive	uncompetitive	uncompetitive
L-leucine	noncompetitive	uncompetitive	uncompetitive

CHAPTER IV

DISCUSSION

4.1 Screening and identification for NAD^+ dependent leucine dehydrogenase producing bacteria

In bacteria, amino acid can be metabolized for their energy through the glycolytic or TCA cycle in three ways. The first way, they use pyridoxyl phosphate-dependent transaminases which transfer the amino group from one amino acid to another one. On the second way, they employ deaminases such as aspartase to transfer amino group from amino acid to obtain free ammonia. The third way is to use amino acid dehydrogenases. These enzymes have an advantage of removing the amino group as free ammonia which can be used in the cell and these enzymes also couple deamination to the production of a reduced nucleotide which can be subsequencely used in a variety of energy requiring processes (Brunhuber and Blanchard, 1994).

From the screening of leucine dehydrogenase producing bacteria, isolation No. 1 which showed the highest enzyme activity was selected. It was identified as *Alcaligenes faecalis*.

LeuDH from *Bacillus* was reported to have a catabolic function and is important for spore germination (Misono *et al*., 1990). However, physiological role of the enzyme in *Alcaligenes faecalis* has not been established. The enzyme may function in the catabolism of branched chain L-amino acids since this bacterium can utilize amino acid as a carbon source (Kersters and Ley, 1985). Formation of NADH and branched chain α-keto acids is important for energy production and for the synthesis of branched chain fatty acids which are needed for membrane phospholipids.

4.2 Optimization for leucine dehydrogenase production of *Alcaligenes faecalis*

In general, enzyme inducer is very effective for maximize harvestable enzyme levels. Many amino acid dehydrogenases have been reported about their inducible properties such as the addition of L-alanine and L-branched chain amino acids to the medium could promote the production of alanine dehydrogenase (AlaDH) and leucine dehydrogenase (LeuDH), respectively, in *Bacillus* sp. (Oshima *et al*., 1985 and Schutle *et al.*, 1985). In this research, the effect of various amino acids on leucine dehydrogenase production from *Alcaligenes faecalis* was investigated. It was found that 0.2% L-isoleucine could increase enzyme activity and cell wet weight. However, addition of 1% L-isoleucine could induce just only 2.7 fold of LeuDH activity. L-isoleucine was omitted for LeuDH production from *Alcaligenes faecalis* since branched chain amino acids are very expensive.

4.3 Purification of leucine dehydreogenase from *Alcaligenes faecalis*

The protein purification process was performed to remove contaminants or unwanted proteins and to concentrate the desired protein. Most purification protocols require more than one step to achieve the desired level of protein purity. This includes any conditioning steps necessary to transfer the target protein from on technique into suitable condition to perform the next technique. Each step in the process will cause some loss of product. Consequently, the key to successful a efficient protein purification is to select the most appropriate techniques, optimize their performance to suit the requirements and combine them in a logical way to maximize yield and minimize the number of steps required (Amersham Phamacia Biotech, 1999).

The LeuDH was purified to 66.4 fold with 16.4% yield from the cell free extract of *Alcaligenes faecalis* by a purification procedure involving DEAE-Toyopearl column chromatography followed by Butyl-Toyopearl and Hitrap Q column chromatography, respectively.

LeuDH from *Alcaligenes faecalis* is an intracellular enzyme. Therefore, disruption method is necessary to break down cell wall in order to release intracellular protein. In this work, ultrasonication was used which causes cell breakage by cavitation and shear forces. The extraction buffer consisted of phenylmethysulfonyl fluoride (PMSF) and ethylenediamine tetraacetic acid (EDTA) as serine protease inhibitor and metalloprotease inhibitor, respectively, because PMSF and EDTA could be used to remain metabolic regulation mechanism control which is lost when the cell is broken. Therefore, desired protein may be degraded by intrinsic catabolic enzyme. The enzyme activity may be lost during sonication, since mechanical cell disruption causes local overgesting with consequent denaturation of protein. To maximize recovery of the active enzyme, the extract and equipment were pre-chilled and several pauses of disruption was used instead of one long continuous sonication because short interval of disruption can also minimize foaming and shearing, thereby minimizing denaturation (Harris and Angal, 1989 and Chambers and Rickwood, 1993).

Solubility differences in salt are frequently exploited to separate protein in early stages of purification protocols. Ammonium sulfate was the salt of choice 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). In preliminary experiment, ammonium sulfate precipitation step was used, the highest enzyme activity was obtained in 50-60% saturated ammonium sulfate precipitation fraction. This procedure removed more than 80% undesired proteins, however, only two fifth of the enzyme activity was remained. This may be caused by the removal of some factors which were important for stabilizing the enzyme activity. Although PMSF and EDTA were used since the extraction step for inhibition of serine proteases and metalloproteases. The thiol proteases may involve in the loss of enzyme activity. Although, this group ofprotease can be inactivated by *p*-chloromercuribenzoate (Chambers and Rickwood, 1993) but *p*-chloromercuribenzoate could not be used in this work because many scientific reports indicated that it completely inhibited LeuDH activity (Table 1.2).

A wide variety of protein purification techniques are available today, however, different types of chromatography have become dominant due to their high resolving power. In gel filtration chromatography, ion exchange chromatography, affinity chromatography and hydrophobic interaction chromatography (HIC), the protein separation is dependent on their biological and physico-chemical properties: molecular size, net charge, biospecific characteristic and hydrophobicity, respectively (Queiroz *et al*., 2001).

Ion exchange chromatography is capable for separation of separating molecules that have only slight differences in charge to give a very high resolution. The technique is most suited for the capture or intermediate step purification. The separation is based on the reversible interaction between a charged molecule and an opposite charge of chromatographic medium. Molecules bind as they are loaded onto the column. Then, conditions are altered so that the bound substrances are eluted differentially. Elution of bound proteins on is usually performed by changes in salt concentration or pH of buffer with stepwise or continuous gradient.

In this work, DEAE-Toyopearl 650M, an anion exchanger was used in the first step of purification. The column matrix is a synthetic polymer containing bound cationic groups. The elution of LeuDH with 0.3 M KCl indicated that the net charge of the enzyme is negative (anion group) at pH 7.4. The column removed about 85% of other proteins. After DEAE-Toyopearl 650M column, % recovery increased to 121.7% which might be due to removal of some enzyme inhibitors.

Ion-exchange column was often used in early stage of purification be cause of its high capacity for proteins. There are reports that used ion exchange column for LeuDH purification such as LeuDH from *C. pseudodiphtheriticum* (DEAE-cellulose, Misono *et al*., 1990), *B. sphaerius* (DEAE-cellulose, Ohshima *et al*., 1978), *B. lichniformis* TSN9 (DEAE-Toyoreal 650M, Nagata *et al*., 1995) and *Bacillus* sp. DSM 730 (DEAE-Toyopearl 650M, Nagata *et al*., 1990).

Hydrophobic column chromatography (HIC) can separate protein on the basis of reversible interaction between hydrophobic ligands and non-polar regions on the surface of proteins. This kind of column is usually used after a precipitation step which often comes at the binding of the down-stream process, or in combination with gel filtration or with ion-exchange chromatography (Queiroz *et al*., 2001).

In this research, Butyl-Toyopearl, containing butyl groups on the surface of hydrophile resin, was used to purify the enzyme after DEAE-Toyopearl 650M column with linear gradient of ammonium sulfate (0.8-0.5 M). This step eliminated other bulk proteins about 90% while LeuDH activity was marked lost about 80%. Lost of LeuDH activity may be caused by the potassium phosphate buffer containing ammonium sulfate which correspond to preliminary experiment, ammonium sulfate precipitation step, as mentioned above.

The last step of purification used Hitrap Q column, a strong anion exchanger chromatography column, from Amersham Phamacia Biotech. After this column, about 80% of other proteins were removed and 75% LeuDH activity was still remained. The success of enzyme purification using Hitrap Q column was judged by the homogeneity of LeuDH on non-denaturing electrophoresis gel and SDS-PAGE gel.

In preliminary experiment, Red-Sepharose column, containing Procion Red HE-3B ligand which can bind with hydrogenase and most other enzymes requiring adenyl-containing cofacters such as $NADP^+$, and Blue-Sepharose 6 fast flow column, containing Cibacron Blue F3G-A as the covalently bound ligand which is more selective for NAD^+-dependent enzymes, were tested. It was found that LeuDH did not bind to both columns. Some unknown factors may involve in the binding reaction between the enzyme and the ligand matrices. Schütte *et al*. (1985) used 5′AMP-Sepharose 4B column and Katoh *et al*. (2003) used Sepharose CL-4B column for purification of LeuDH from *C. pseudodiphthriticum* and *Natronobacterium magadii* MS-3, respectively.

It can be concluded that DEAE-Toyopearl 650M column (KCl stepwise elution) followed by Butyl-Toyopearl column (Ammonium sulfate gradient elution) and HitrapQ column (gradient elution) are appropriate in the purification procedure of LeuDH from *Alcaligenes faecalis.*

4.4 Characterization of leucine dehydrogenase from *Alcaligenes faecalis*

4.4.1 Molecular weight determination of leucine dehydrogenase

The relative molecular weight of LeuDH from *Alcaligenes faecalis* was determined to be about 536,000 consisting of 10 subunits with equal molecular weight of 52,000. The enzyme appears to have a decameric structure which has never been reported for LeuDH. The quaternary structure of LeuDHs from various sources is diverse as shown in Table 1.2 while valine dehydrogenase (ValDH) from *Alcaligenes faecalis* (Ohshima and Soda, 1993), *Streptomyces aureofaciens* (Nguyen *et al*., 1988) and *Streptomyces fradiae* (Vancura *et al*., 1988) is a dimer, a hexamer and a dodecamer, respectively**.**

4.3.2 Effect of pH on leucine dehydrogenase activity and stability

The optimum pH for oxidative deamination and reductive amination of *Alcaligenes faecalis* LeuDH were 10.8 and 8.8, respectively. The pH optima for oxidative dermination of LeuDHs from others sources lie between 9.5 and 11.5 while the corresponding optima for reductive amination are between 9.0 and 10.0 (Table 1.2).

The *Alcaligenes faecalis* LeuDH was stable over a pH range of 6.0 to 12.0 upon incubation at 30°C for 20 minutes. The *Bacillus cereus* LeuDH was reported to be stable in the pH range between 6.0 to 8.0 on incubation at 20°C for 24 hours. The LeuDH activity of *Bacillus* sp. DSM 730 was not lost by incuabation between pH 5.4 to 10.3 at 55°C for 10 minutes (Nagata, S. *et al*., 1990) whereas the enzyme from *Bacillus stearothermophilus* stabled over the pH range of 6.5 to 8.0 upon incubation

at 30°C. Many factors including ionic strength, temperature, preservation conditions and chemical nature of the buffer involved in pH stability (Segal, 1976).

4.4.3 Effect of temperature on leucine dehydrogenase activity and stability

The optimum temperature of the enzyme for oxidative deamination and reductive amination were 45°C and 55°C, respectively. For LeuDH from *Bacillus cereus, Bacillus sphaericus, Bacillus strearothemophillus* and *Natronobacterium magadii* MS-3, the optimum temperatures for oxidative deamination were reported to be 60°, 57°, 70°, and 65°C, respectively. The range of optimum temperature of other amino acid dehydrogenases is 40°-65°C. The LeuDH of *Alcaligenes faecalis* showed full activity after heating at 45°C for 10 minutes and 60% remaining activity at 60°C. Upon incubation at 45°C, it was found that its full activity was maintained for 8 hours. The thermostability of other LeuDHs from various bacteria are shown in Table 1.2.

The temperature is important to the reaction or enzyme activity because it increases the sufficient energy of molecules to overcome the action barrier and hence increases the rate of reaction, however, high thermal energy of molecules can break non-covalent bonds that hold the dimensional structure of enzyme.

4.4.4 Substrate specificity of leucine dehydrogenase

Substrate specificity is the ability of enzyme to discriminate between a substrate and competing molecules. If an enzyme active site has functional groups arranged optimally to form a variety of weak interactions with a given substrate in the transition state, the enzyme will not be able to interact to some degree with any other molecules. In general, specificity is derived from the formation of multiple weak interactions between the enzyme and its specific substrate molecule (Lehninger, 2000).

The activity of the enzyme to catalyze the oxidative deamination of various amino acids is presented in Table 3.2 In addition to L-leucine, L-isoleucine and L-valine are the preferred substrates. Straight chain aliphatic L-amino acids such as L-norvaline and L-α-aminobutyrate (C4) were effective while L-norleucine (C6) was a poor substrate. On the other hand, other L-amino acids such as aromatic amino acids (L-phenylalanine, L-tyrosine, L-tryptophan), positively charged amino acids (L-lysine, L-arginine, L-histidine), negatively charged amino acids (L-aspartate, and L-glutamate), uncharged amino acids (L-serine, L-thereonine, L-cysteine, and L-asparagine), other aliphatic amino acids (glycine, L-alanine, L-proline, and L-methionine) could not act as substrates. The length of a straight chain of L-amino acids also significantly influences the susceptibility to the enzyme: C5>C4>C6. 4-Azaleucine is very poor substrate, indicating that the substitution of -CH of leucine with nitrogen or the presence of a double bond in the side chain leads to a decrease in the rate of deamination. Sulfur analogues of norvaline, (*S*-methyl-L-cysteine), and norleucine, (L-methionine) in which γ- and δ-CH_2 were replaced, were less reactive substrates than the parent compounds. Either the positively or negatively charged group in the side chain, e.g. hydroxyl, ε-amino, and γ-carboxyl groups, probably prevents the amino acids from binding with the enzyme, because L-lysine, L-arginine, L-histidine, L-aspartate and L-glutamate were neither substrates nor inhibitors for the deamination of L-leucine. This property of LeuDH of *Alcaligenes faecalis* is similar to other LeuDHs from other organisms as shown in Table 1.2 but it catalyst L-valine and L-isoleucine better than L-leucine.

For reductive amination, the enzyme showed higher substrate specificity on α-ketoisovalerate (keto analog of valine) and α-ketovalerate (keto analog of norvaline) than α-ketoisocaproate (keto analog of leucine) 2 and 1.35 fold, respectively. α-Ketoglutarate and phenylpyruvate were not substrate as the same as other LeuDHs as shown in Table 1.2.

According to the high substrate specificity on branched chain amino acids of *Alcaligenes faecalis* LeuDH, the enzyme may be useful in medical applications such as determination of its substrate.

4.4.5 Inhibitory effect of various amino acids and keto acids on leucine dehydrogenase activity

Many types of molecule can interfere with the activity of enzyme. Substrate analog is one of enzyme inhibitors. In this work, nonsubstrate amino acids were investigated. We found that D-leucine and D-norvaline inhibited the enzyme activity but D-valine, D-alloisoleucine and D-norleucine were not. Ohshima *et al*. (1978) reported about the inhibitory effects of nonsubstate amino acids on *B. sphaericus* LeuDH. The *B. sphaericus* enzyme was inhibited by D-leucine (69% relative activity), D-valine (85% relative activity), D-norvaline (88% relative activity), and L-alloisoleucine (89% relative activity). Amino acids which did not inhibit enzyme activity of LeuDH from *Alcaligenes faecalis* were L-alanine, L-aspartate, L-serine, L-theronine, L-phenylalanine, L-arginine, L-penicillamine, *S*-methyl-L-cysteine and 7-amino heptanoate. The similar result was also reported from *B. sphaericus* LeuDH. In addition, Hermier *et al*. (1970) reported that LeuDH from sporulating cells of *B. subtilis* could deaminate L-alanine. The *B. subtilis* enzyme was inhibited competitively by D-alanine and D-aminobutyrate and noncompetitively by D-leucine, D-valine and D-norvaline. Zink and Sanwal (1962) reported that the enzyme from *B. subtilis* was not inhibited by D-leucine and D-valine.

From the result, it should be explained that alanine which is a small hydrophobic amino acid, is not involved in enzyme catalysis of LeuDH from *Alcaligenes faecalis* because L-alanine is neither substrate nor inhibitor for deamination of L-leucine. Uncharged amino acids (L-serine and L-threonine), negatively charged amino acids (L-aspartate and L-glutamate) are neither substrate nor inhibitor for deamination of L-leucine. 7-Aminoheptanoate and L-penicillamine, derivatives of leucine, were neither substrate nor inhibitor for oxidative deamination of the enzyme. L-γ-Methylleucine and 4-azaleucine were poor substrates which inhibited the deamination of L-leucine.

For the inhibitory effect on reductive amination of α-ketoisocaproate, α-ketoglutarate was not a substrate but it could inhibit the reductive amination. There are no report about this effect on activity of other amino acid dehydrogenases.

4.4.6 Coenzyme specificity on leucine dehydrogenase

Some enzymes require no chemical groups for activity other than their amino acid residues. Others require an additional chemical component called a cofactor. Cofactors that are small organic molecules are called coenzymes.

In this work, NAD^+ is replaced by some of NAD^+ analogues as a cofactor for leucine dehydrogenase. According to the result, NAD^+ could not be replaced by $NADP^+$ which differs from NAD^+ only by the addition of a phosphoric group at C-2 position of its adenosyl ribose. $NADP^+$ is also inert for our enzyme and LeuDH from *B. sphaericus*, *B. cereus*, and *Bacillus* sp. DSM 730 (Ohshima *et al*., 1978, Schűtt *et al*., 1985 and Nagata *et al*., 1990).

Nicotinamide hypoxanthine dinucleotide (deamino-NAD^+) gave similar reactivity to NAD^+. These observations have also been reported for LeuDH of *B. sphearicus* and amino acid dehydrogenases (Vancura *et al*., 1988 and Vancurova, 1998). LeuDH from *C. pseudodiphtheriticum* could use deamino-NAD^+ much better than NAD^+ (Misono *et al*., 1990). Nicotinamide guanine dinucleotide (NGD^+) slightly influenced on the enzyme activity. Nicotinamide hypoxanthine dinucleotide (deamino-NAD^+), nicotinamide guanine dinucleotide, nicotinamide 1,N^6-ethenoadenine dinucleotide (ε-NAD^+) which are the NAD^+ analogs modified at amino group in the adenine moiety, were utilized by LeuDH from *Alcaligenes faecalis*, except ε-NAD^+. It can be concluded that the amino group in the adenine moiety of NAD^+ is not critical for the coenzyme activity.

3-Acetylpyridine adenine dinucleotide, thionicotinamide adenine dinucleotide (thionicotinamide-NAD^+), 3-pyridinealdehyde adenine dinucleotide and nicotinic acid adenine dinucleotide (deamido-NAD^+) are NAD^+ analogs modified at the amino group of the nicotinamide moiety were tested for coenzyme specificity.

Thionicotinamide-NAD^+ was utilized by *Alcaligenes faecalis* LeuDH while 3-pyridinealdehyde adenine dinucleotide and deamido-NAD^+ were not. This suggests that the amino group of the nicotinamide moiety is essential for the enzyme activity and the type of substituted groups of NAD^+ analogs also has the influence on enzyme activity. The enzyme could not use deamido-NAD^+ and 3-pyridinealdehyde adenine dinucleotide which their amino groups in the nicotinamide moiety are substituted by OH group and H atom, respectively. This may involve polarity of the substituents which may affect the binding mechanism in the area of the conserved hydrophobic residues of the enzyme. An appearance that 3-acetylpyridine adenine dinucleotide is reduced by the enzyme more rapidly than NAD^+ has been reported for LeuDH from *B. sphaericus* (Ohshima *et al*., 1978), LeuDH from *Corrynebacterium pseudodiphtheriticum* (Misono *et al*., 1990), PheDH from *B. badius* BC1 (Leksakorn, 2001) PheDH from *Rhodococus maris* K-18 (Misono *et al*., 1989) bovine liver GluDH and horse liver alcohol dehydrogenase (Kaplan *et al*., 1956).

4.4.7 Effect of metal ions and chemical substrates on leucine dehydrogenase activity

The enzyme was inactivated by $HgCl_2$ which is an inhibitor of all of proteins similar to other LeuDH (Table 1.2). Monovalent and divalent cations had no effect on the enzyme activity.

4.4.8 Effect of group-specific reagents on leucine dehydrogenase activity

Several methods for investigation of amino acid residues which are essential for function or structure of protein such as affinity labeling with their substrates or substrate analogues (Hirano *et al*, 1991), X-ray crystallography (Baker *et al*., 1998) or chemical modification (Bhattacharyya *et al*., 1977) have been reported.

Chemical modification is one of the most useful method of identifying the functional groups of protein. Various types of amino acid side chains which are involved in a protein function can be determined by chemical modification.

This requires only chemical reactions that are reasonably specific for each type of amino acid side chain (Means and Smith, 1993). Inactivation of enzyme with or without its substrate may suggest that the inactivation is due to a direct modification of essential amino acid residues at the enzyme active site or due to the modification of amino acid residues remote from the active site (Dong *et al*., 1991).

In this work, the screening of essential amino acid residues of the enzyme was performed by chemical modification with group-specific modifying reagents. Chemical modification of eight amino acid residues, tyrosine, tryptophan, serine, methionine, lysine, histidine, cysteine and arginine have been selected because they are widely known as residues involved in enzyme catalysis (Ohshima and Soda, 2000). No inhibition of LeuDH activity from *Alcaligenes faecalis* was observed in the modification of cysteine by dithiothreitol (DTT) and serine by phenylmethylsulfonyl fluoride (PMSF). The result indicated that these amino acid residues were not the important residues involved in enzyme catalytic activity. The modification with chloramine T (CT), diethylpyrocarbonate (DEPC), *N*-bromosuccinimide (NBS) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) which are known to react specifically with methionine, histidine, tryptophan and lysine residues, respectively, resulted in extensive inhibition of enzyme activity. Incubation of the enzyme with *N*-acetylimidazole and phenylglyoxal reduced enzyme activity. This result concluded that thioether group of methionine, imidazole group of histidine, indole group of tryptophan, amino group of lysine, OH group of tyrosine, and guanidine group of arginine are involved in LeuDH activity as the essential residues for enzyme biological function. This experiment is only at the initial phase of amino acid selective chemical modification. It cannot prove that these amino acid residues involved in LeuDH activity are located within active site or not. The substrate protection experiments are necessary and should be performrd for further studies because these group-specific compounds covalently modify the accessible amino acids in a general way, so that treatment of an enzyme with such reagents will lead to modification of both catalytically critical residues and nonessential residues as well. If an interesting amino acid residue can be protected by substrate, it likely that the amino acid residue is in the active site of enzyme.

For alteration of substrate specificity of leucine dehydrogenase from *B. stearothermophilus* by site-directed mutagenesis, Kataoda and Tanizawa reported that the kinetic parameters obtained with the mutants clearly showed that A113, V291, L40 and V294 in LeuDH are the key residues involved in recognition of substrate side chain. A113 in LeuDH and the corresponding G114 in *T. intermedius* PheDH control the volume of side chain binding pocket and play a critical role in discrimination of the bulkiness of the side chain (Kataoka and Tanizawa, 2003). The ε-amino group of Lys-80 of *B. stearothermophilus* LeuDH participates in catalysis as a general base, assisting the nucleophilic attack of a water molecule to the substrate α-carboxyl group of substrate through an ionic interaction (Sekimoto *et al*., 1994). Our attention was also paid to the modification by carbodiimide which interact with carboxylic residue, however, the experiment was unsuccessful because the system for carbodiimide used buffer pH 4-5 which is not proper for the enzyme system and could denature the enzyme.

4.5 Kinetic mechanism studies of leucine dehydrogenase from *Alcaligenes faecalis*

Multiple approaches are commonly used to study the mechanism of action of purified enzymes. One knownledge of the three-dimentional structure of the protein important information and the value of structural information is greatly enhanced by classical protein chemistry and modern methods of site-directed mutagenesis. The central approach to study the mechanism of an enzyme-catalyzed reaction is to determine the rate of the reaction and how it changes in response to changes in experimental parameters, a discipline known as enzyme kinetics. This is the oldest approach to understanding enzyme mechanism and remains the most important today. One simplifying approach in kinetics experiments is to measure the initial rate or initial velocity (Lehninger, 2000).

The mechanism of the oxidative deamination and reductive amination was explored through initial velocity studies and product inhibition patterns. These experiments were carried out by varying the concentration of one substrate at several

different fixed concentrations of the other substrate(s) or the product. The analysis of kinetic data was carried out according to the methods proposed by Cleland (Cleland, 1963 a, b, and c)

All of amino acid dehydrogenases appear to proceed via the formation of ternary complex with sequential mechanism and not with ping pong mechanism. Diversity is found in the manner of substrate binding and product released. Most amino acid dehydrogenases appear to operate via a sequential ordered Bi-Ter mechanism with the cofactor binding before the amino acid. Two different kinetic mechanisms are proposed for GluDH, bovine liver glutamate dehydrogenase catalyzes the reaction in a random ordered mechanism and the enzymes from microorganisms and plant show a sequential ordered mechanism (Ohshima *et al*., 1978).

A. Initial velocity studies

A general method for prediction of the mechanism by initial velocity and product inhibition patterns was presented by Cleland. Cleland formulated a series of general rules for prediction of the effect of a compound on the slope and intercept of reciprocal plots for a given varied substrate.

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

Cleland proposed and summarized the prediction of mechanism from bireactant initial velocity patterns (the oxidative deamination case) that ping pong mechanisms give the parallel initial velocity pattern and sequential mechanisms give the intersecting or occasionally the equilibrium ordered pattern. In this work, the

intersecting patterns obtained with the initial velocity experiments clearly indicated a sequential as opposed to a ping pong mechanism (Figure 3.17-3.19). Thus, all of the substrates must be bound to the enzyme before any products are released. However, these data can indicate only that the addition of NAD^+ and L-leucine is sequential but cannot clarify which substrate is added first or whether addition is in obligatory order.

For the terreactant initial velocity patterns (the reductive amination case), Cleland explained by given an example model of the ordered addition of three substrates (A, B and C are substrates in that order). If substrate B is truly saturating, however, the reversible sequence is broken and the A-C initial velocity pattern becomes a parallel one whereas A-B and B-C initial velocity pattern will always be intersecting, regardless of the level of the other substrate. That means the ordered terreactant mechanism will show two intersecting and one parallel initial velocity patterns, which is similar to the patterns observed in our enzyme. In this work, the parallel initial velocity pattern obtained by varying NH_4^+ and NADH at a fixed, saturating level of α-ketoisocaproate (Figure 3.23) indicated that α-ketoisocaproate binds to the enzyme between NH_4^+ and NADH. That means α-ketoisocaproate is the middle substrate, which has also been observed in *B. sphaericus* LeuDH. Although it is possible to conclude something about the order of addition of reactants in which the middle substrate can be identified, but the first cannot be distinguished from the third. Thus, while much useful information can be obtained from initial velocity experiments, more complex types of kinetic studies are needed to work out the details of the kinetic mechanism (Cleland, 1971a).

From initial velocity studies of the enzyme, we found that K_m value of L-leucine was about 3 fold lower than that of L-valine which coresponded to the result from section 3.3.4 (enzyme induction) that L-leucine could induce the enzyme production more than L-valine. It indicates that the purified enzyme from *Alcaligenes faecalis* was leucine dehydrogenase not valine dehydrogenase.

B. Product inhibition studies

A compound that is a product of a reaction, reacts as a substrate in the reverse reaction and can inhibit the reaction by combining only with the enzyme is called a product inhibitor. According to Cleland's fundamental rules, there are three basic types of product inhibition determined by the effect on the slope and intercept of the double-reciprocal plot. Competitive inhibition refers to the enzyme-substrate (ES) binary complex. This inhibition pattern is a series of intersecting lines on the 1/v-axis (slopes only vary). Uncompetitive inhibitor refers to the case of the inhibitor binding exclusively to the ES complex. This inhibition pattern is a series of parallel line at different product concentrations (intercepts only vary). Noncompetitive inhibition refers to the case in which an inhibitor displays binding affinity for both the free enzyme and the ES complex. This inhibition pattern is a series of intersecting lines on the left of the 1/v-axis, above, on, or below the horizontal axis (both slopes and intercepts vary). Inhibition patterns may be predicted in the same way as initial velocity patterns. Basically, the expected effects of the inhibitor on the slopes and intercepts of reciprocal plots are determined separately and then the results are combined to predict the inhibition pattern. Cleland suggested a set of rules to predict the type of inhibition expected for a steady-state mechanism. Applications of these rules are illustrated in the original article by Cleland (Cleland, 1971c).

A simple ordered mechanism gives only one competitive product inhibition pattern due to the two reactants, the first substrate to add and the last product to come off, binding to the free enzyme. The two competitive inhibitions are consistent with either a Theorell-Chance or a random mechanism with dead-end complexes forming. In the Theorell-Chance mechanism, an additional competitive inhibition pattern is obtained with the last substrate to add and the first product to be released. This is due to the rapid breakdown of the central complex to undetectable levels, so that the enzyme forms appear to interconvert directly. For a rapid-equilibrium random mechanism, all product inhibitions are competitive unless products are able to bind to enzyme forms as dead-inhibition patterns due to the combination of competitive product inhibition and uncompetitive dead-end inhibition (Cleland, 1971a).

As reported in Chapter 3, product inhibition by NADH with respect to NAD^+ gave competitive inhibition demonstrated by lines intersecting on the 1/v axis. This is consistent with NAD^+ and NADH adding first and coming off last, respectively, as occurs in many other dehydrogenases with ordered mechanisms. That means both the Theorell-Chance and the random mechanisms do not hold for the enzyme because only one competitive product inhibition pattern was obtained in this work. Therefore, the ordered mechanism fits best with the data. This inhibition pattern combining with the results from initial velocity patterns strongly support the ordered addition of the substrates in which NAD^+ binds the enzyme first and then follows by L-leucine binding. About the ordered releasing of the products, it is predicted by the result from the competitive inhibition pattern which indicated that the last product is NADH and the results from the terreactant initial velocity patterns which indicated that the middle product is α-ketoisocaproate. That means the rest of them, ammonium chloride, should be the first product to be released. Therefore, the ordered releasing of the product is: NH_4^+, α-ketoisocaproate and NADH, respectively,

However, it is noted that the unexpected noncompetitive inhbition displayed by α-ketoisocaproate as a product inhibitor against L-leucine suggests that α-ketoisocaproate could bind to the enzyme- NAD^+ complex and form the dead-end complex (abortive enzyme-NAD^+-α-ketoisocaproate complex). This same discrepancy has also been observed in kinetic mechanism of the *Streptomyces cinnamonesis* ValDH (Pristley and Robison, 1989).

4.6 Amino acid sequencing of N-terminal of leucine dehydrogenase

N-terminal amino acid sequence of *Alcaligenes faecalis* LeuDH was compared with the LeuDHs from various microorganisms and ValDH from *Streptomyces* (Figure 4.1). LeuDH from *Alcaligenes faecalis* showed hight similality to those of LeuDHs from endospore forming bacteria, *Bacillus* and *Clostridium*. On the other hand, low similarity was observed between the enzyme and ValDHs. These facts suggest that the *Alcaligenes faecalis* LeuDH is closely related to the other LeuDHs.

In addition, N-terminal and internal amino acid sequence of the enzyme will be used to design primers for sequencing and cloning of LeuDH gene.

	1 5 10 15 20
Bacillus cereus (LeuDH)	MTLEI FE YLEKYDYEQVVFCQDK
B. caldolyticus (LeuDH)	MELFQYMEKYDYEQVLFXQDK
B. stearothemophilus (LeuDH)	MELFKYMETYDYEQVLFCQDK
Clostridium thermoaceticum (LeuDH)	MELFKYMETYDYEQVLFXQDK
Alcaligenes faecalis	MEI FDYMEQADYEQLVI XQD
Streptomyces albus (ValDH)	MTDVTGAPADVLHTLFHSDQGGHEQVVLCQDR
S. cinnamonensis (ValDH)	VTEADNGVLHTLFHSDQGGHEQVVLCQDR
S. coelicolor (ValDH)	VTDVNGAPADVLHTLFHSDQGGHEQVVLCQDR
S. fradiae (ValDH)	MTDASHPTAADDLGALSTLFRSEQGGHE RVLLCQDR

Figure 4.1 Comparison of N-terminal amino acid sequence of *Alcaligenes faecalis* leucine dehydrogenase with various leucine dehydrogenases and valine dehydrogenases

Amino acid sequences identical to those of *Alcaligenes faecalis* LeuDHs and ValDHs are yellow hilight.

Source; *Bacillus cereus* (Hyun *et al*., 2000),
B. caldolyticus (Karst *et al*., 1989),
B. stearothemophilus (Shimoi et al., 1987 and Nagata *et al*., 1988),
Clostridium thermoaceticum (Shimoi *et al*., 1987),
Streptomyces albus (Hyun *et al*., 2000)
S. cinnamonensis, *S. coelicolor*, and *S. fradiae* (Turnbull *et al*., 1997)

CHAPTER V

CONCLUSION

1. The bacteria, screened from soil samples which produced leucine dehydrogenase, was identified as *Alcaligenes faecalis.*

2. The optimum condition for production of leucine dehydrogenase from *Alcaligenes faecalis* production was cultivation in 1% peptone medium, pH 7.2 at 37˚C for 24 hours.

3. The leucine dehydrogenase was purified by DEAE-Toyopearl, Butyl-Toyopearl and Hitrap Q columns with 66.4 purification fold and 16.4% recovery.

4. The enzyme had the molecular weight of 536,000 and consists of 10 subunits of 52,000

5. The enzyme showed higher substrate specificity on L-valine than L-leucine in oxidative dermination and showed higher substrate specificity on α-ketoisovalerate, α-ketovalerate and α-keto-β-methylvalerate than α- ketoisocaproate in reductive amination.

6. $NADP^+$ was inert coenzyme while 3-acetylpyridine adenine dinucleotide showed higher activity than NAD^+.

7. The optimum pH of the enzyme were 10.8 and 8.8 for oxidative deamination and reductive amination, respectively.

8. The optimum temperature of the enzyme were 45˚C and 55˚C for oxidative deamination and reductive amination, respectively.

9. The enzyme was stable over pH range of 6.0 to 12.0.

10. The enzyme retained its activity upon the incubation at 45˚C for 8 hours.

11. The enzyme activity was completely lost in the presence of 1 mM $HgCl_2$.

12. Aromatic acidic and basic amino acids showed no significant inhibition on oxidative deamination but D-leucine and its derivatives significantly inhibited the oxidative deamination.

13. Tryptophan, methionine and lysine may played important role in an active tive site of the LeuDH.

14. The apparent K_m value for L-leucine, L-isoleucine, L-valine, NAD^+, NADH, α- ketoisocaproate and ammonia were 4.2 , 4.3 , 14.0 , 0.44 , 0.02 , 3.3 and 100 mM, respectively.

15. The steady state kinetic studies including product inhibitions on the enzyme reaction indicated that the oxidative deamination proceeds through a sequential ordered binary-ternary mechanism in which NAD^+ bind first to the enzyme followed by L-leucine and products are released in the order of ammonia, α-ketoisocaproate and NADH, respectively.

16. N-terminal amino acid sequence of the enzyme was M E I F N Y M E Q A D Y E Q L V I X Q D while amino acid sequences of 3 internal peptides were PGPXGP AGSKG (or V) EPGPAGPXG, T (or L,Y,V) LPGLAGTXG and RDNIPSYVAA DRLAEERIRVA.

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APPENDICES

APPENDIX A

Preparation for protein determination

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

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

Potassium tartate	1 g
Cooper sulfate	0.5 g

Adjust pH to 7.0 then, adjust the solution volume to 100 ml

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

Sodium carbonate	20 g
Sodium hydroxide	4 g

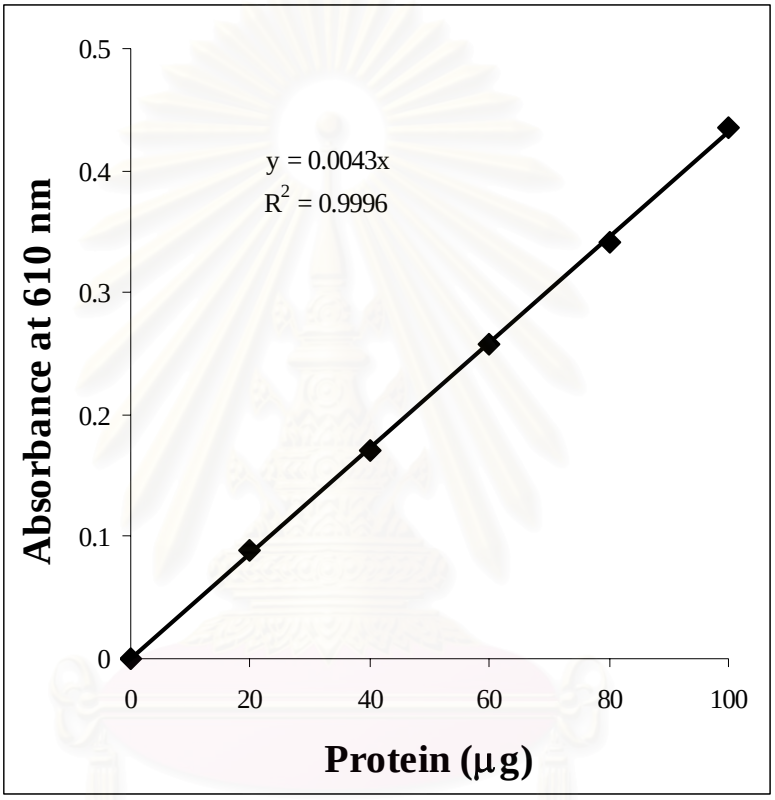
Dissolved in distilled water to 1 liter.

Solution C (phenol reagent)

Folin-Ciocateu phenol reagent used in this work was reagent grade from Sigma, USA.

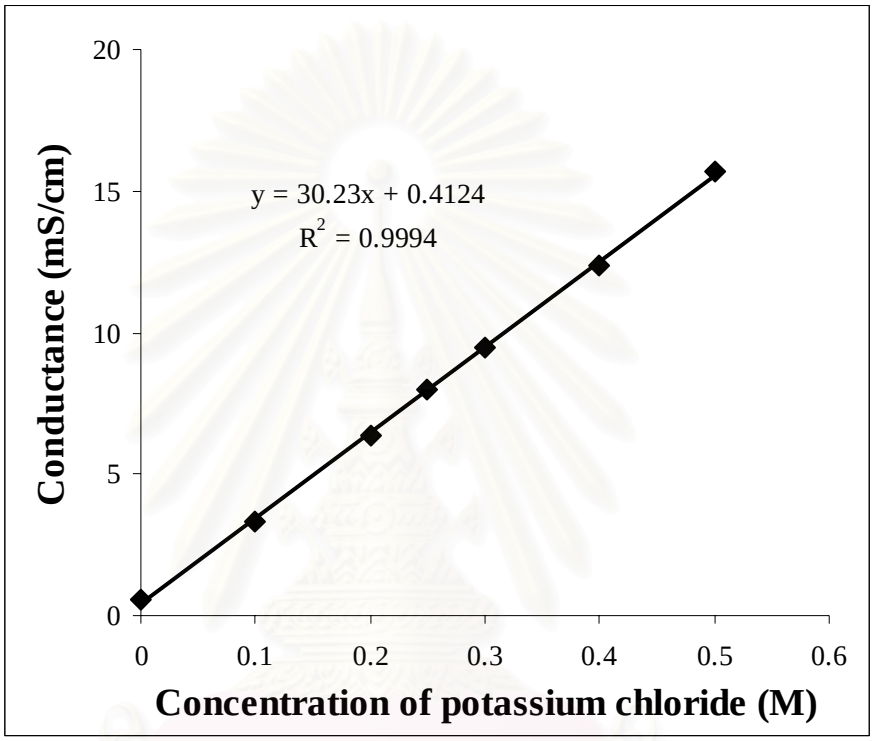
APPENDIX B

Calibration curve for protein determination by Lowry's method



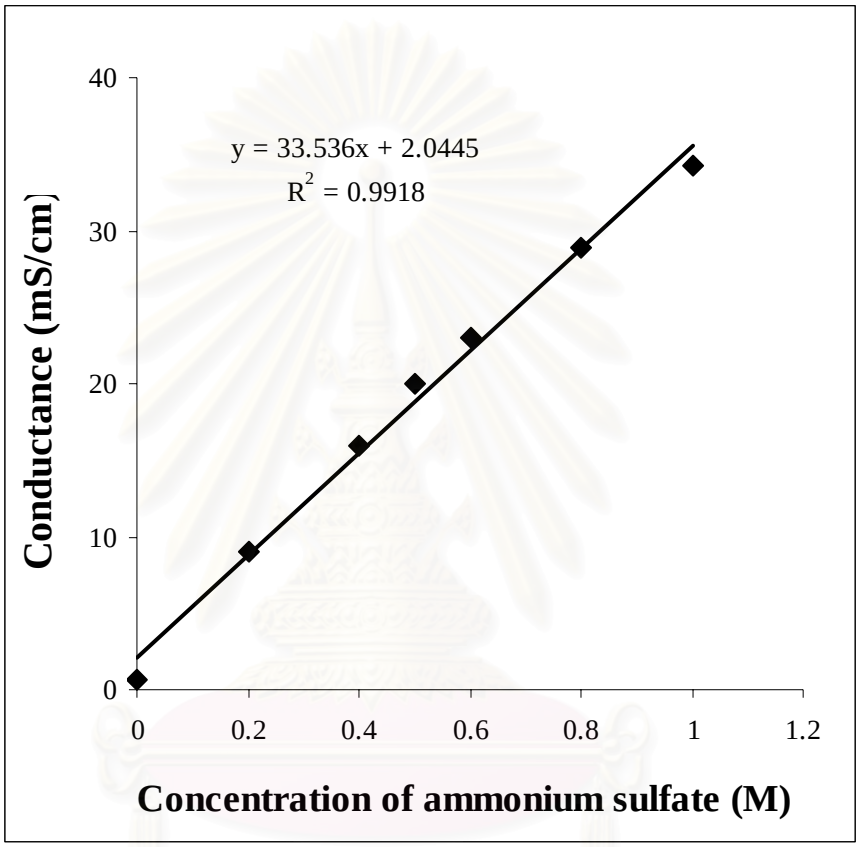
APPENDIX C

Calibration curve for conductivity of potassium chloride



APPENDIX D

Calibration curve for conductivity of ammonium sulfate



APPENDIX E

Preparation for non-denaturing polyacrylamide gel eletrophoresis (native-PAGE)

1. Stock solution

2 M Tris-HCl (pH 8.8)

Tris (hydroxymethyl)-aminomethane	24.2	g

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

1 M Tris-HCl (pH 6.8)

Tris (hydroxymethyl)-aminomethane	12.1	g

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

1 % Bromophenol blue (W/V)

Bromophenol	100	mg

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

Filtration will remove aggregated dye.

2. Working solutions

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

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

Adjusted volume to 100 ml with distilled water.

Solution B (1.5 M Tris-HCl pH 8.8)

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

Solution C (0.5 M Tris-HCl pH 6.8)

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

10% Ammonium persulfate

Ammonium persulfate	0.5	g
Distilled water	5.0	ml

Electrophoresis buffer (25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminomethane	3.0	g
Glycine	14.4	g

Dissolved in distilled water to 1 liter without pH adjustment
(final pH should be approximately 8.3)

5× Sample buffer
(312.5 mM Tris-HCl pH 6.8, 50% glycerol, 1% bromphenol blue)

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

3. Native-PAGE

7.7% Separating gel

Solution A	2.6	ml
Solution B	2.5	ml
Distilled water	4.9	ml
10 % Ammonium persulfate	50	µl
TEMED	5.0	µl

5.0 % Stacking gel

Solution A	0.67	ml
Solution C	1.0	ml
Distilled water	2.3	ml
10 % Ammonium persulfate	30	µl
TEMED	5.0	µl

APPENDIX F

Preparation for denaturing polyacrylamide gel eletrophoresis

1. Stock solution

2 M Tris-HCl (pH 8.8)

Tris (hydroxymethyl)-aminomethane 24.2 g

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

1 M Tris-HCl (pH 6.8)

Tris (hydroxymethyl)-aminomethane 12.1 g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 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 50 ml of distilled water

1 % Bromophenol blue (W/V)

Bromophenol 100 mg

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

Filtration will remove aggregated dye.

2. Working solutions

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

Acrylamide	29.2	g
N,*N*´-methylene-bis-acrylamide	0.8	g

Adjusted volume to 100 ml with distilled water.

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

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	5.0	ml

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

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

Dissolved in distilled water to 1 liter without pH adjustment

(final pH should be approximately 8.3)

5× Sample buffer

(60 mM Tris-HCl pH 6.8, 25% glycerol, 2 % SDS, 0.1% bromphenol blue, 14.4 mM β-mercaptoethanol)

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

3. SDS-PAGE

12.5 % Separating gel

Solution A	4.2	ml
Solution B	2.5	ml
Distilled water	3.3	ml
10 % Ammonium persulfate	50	µl
TEMED	5.0	µl

5.0 % Stacking gel

Solution A	0.67	ml
Solution C	1.0	ml
Distilled water	2.3	ml
10 % Ammonium persulfate	30	µl
TEMED	5.0	µl

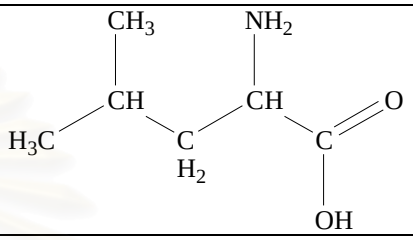
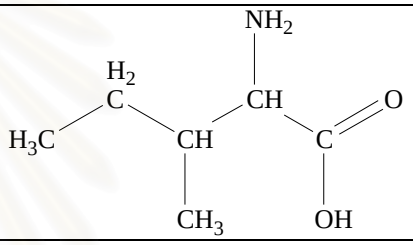
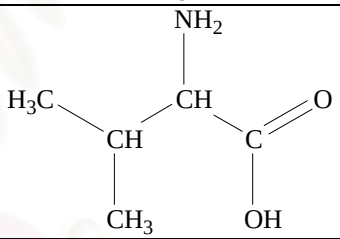
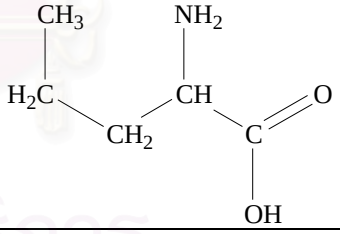
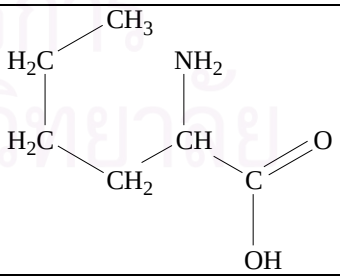
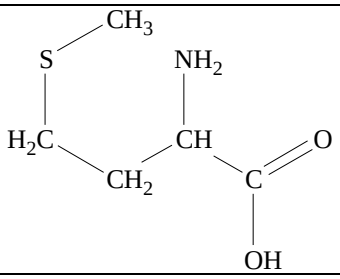
APPENDIX G

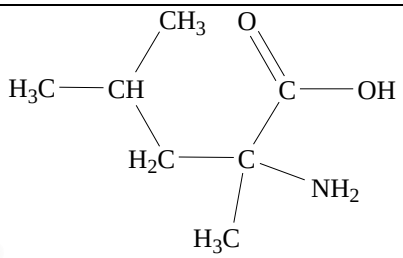
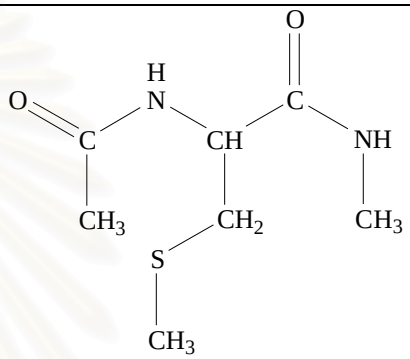
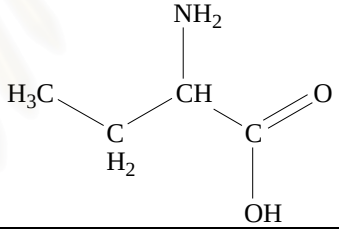
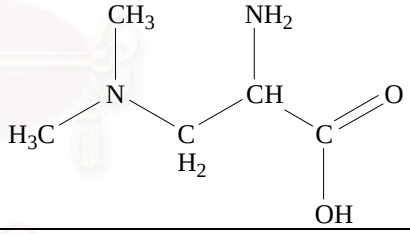
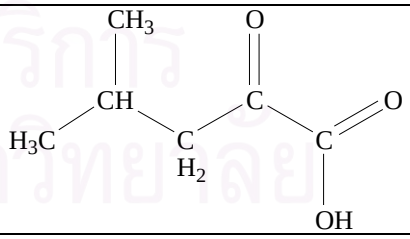
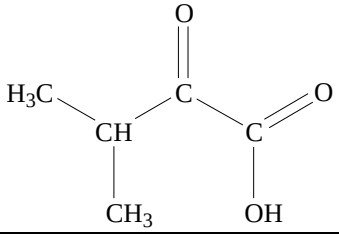
Abbreviation for amino acid residues

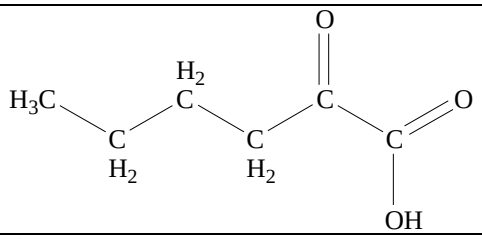
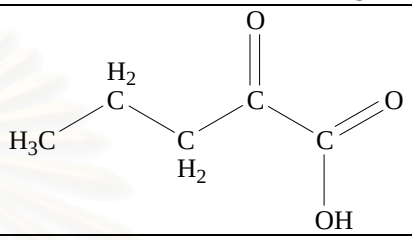
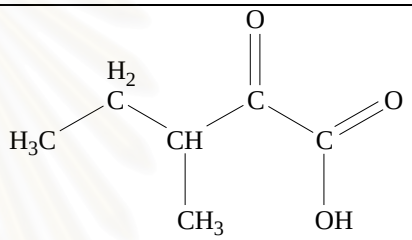
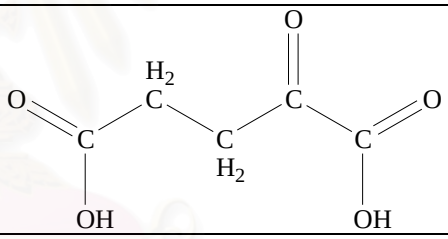
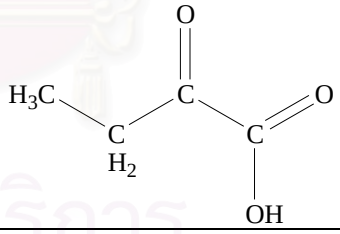
Amino acid	3 Letters- Abbreviation	1 Letter- Abbreviation
Alanine	**Ala**	**A**
Arginine	**Arg**	**R**
Asparagine	**Asn**	**N**
Aspartic acid	**Asp**	**D**
Cysteine	**Cys**	**C**
Glutamine	**Gln**	**Q**
Glutamic acid	**Glu**	**E**
Glycine	**Gly**	**G**
Histidine	**His**	**H**
Isoleucine	**Ile**	**I**
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**
Unknown	**-**	**X**

APPENDIX H

Amino acids and keto acids structure

Chemical	Structure
L-leucine	
L-isoleucine	
L-valine	
L-norvaline	
L-norleucine	
L-methionine	

Chemical	Structure
L-γ-methlylleucine	
S-methyl-L-cysteine	
L-aminobutyrate	
4-azaleucine	
α-ketoisocaproate	
α-ketoisovalerate	

Chemical	Structure
α-ketocaproate	
α-ketovalerate	
α-keto-β-methyl-n-valerate	
α-ketoglutarate	
α-keto-n-butyrate	

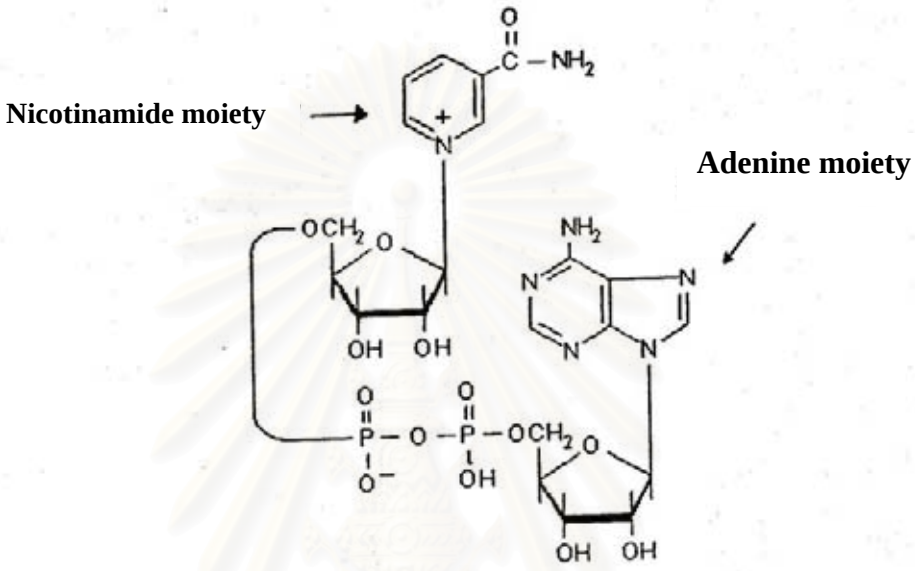
APPENDIX I

Amino acids and their corresponding keto acids

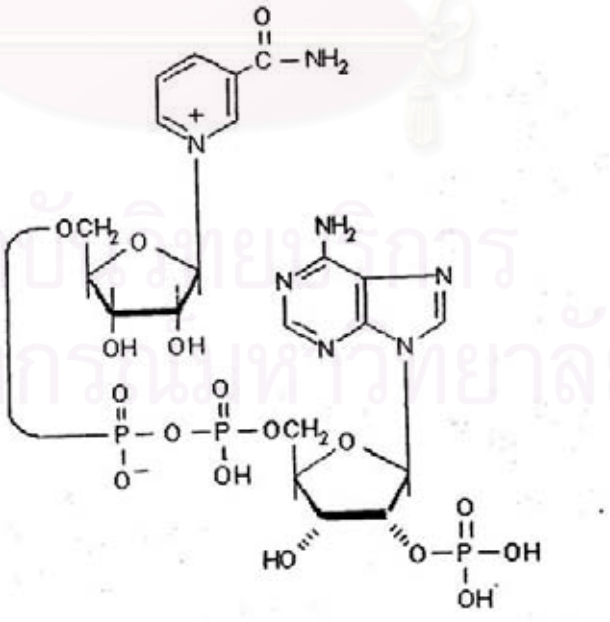
Amino acid	Keto acid
L-leucine	α-ketoisocaproate
L-valine	α-ketoisovalerate
L-isoleucine	α-keto-β-methlyvalerate
L-norleucine	α-ketocaproate
L-norvaline	α-ketovalerate
α-aminobutyrate	α-keto-n-butyrate
L-glutamic acid	α-ketoglutarate
L-phenylalanine	β-phenylpyruvate
L-methionine	α-keto-γ-methiol-n-butyrate

APPENDIX J

NAD^+ anlogs

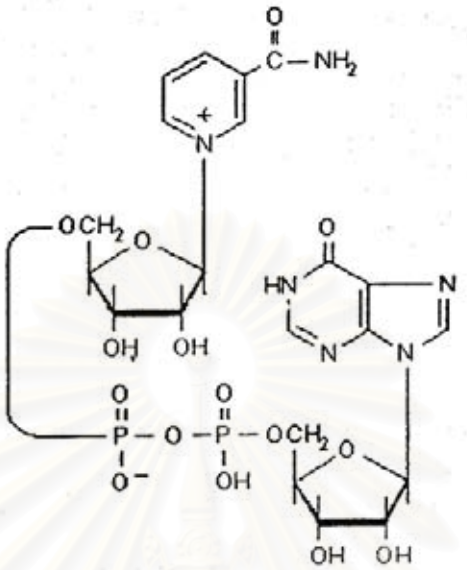


Nicotinamide adenine dinucleotide (NAD^+)



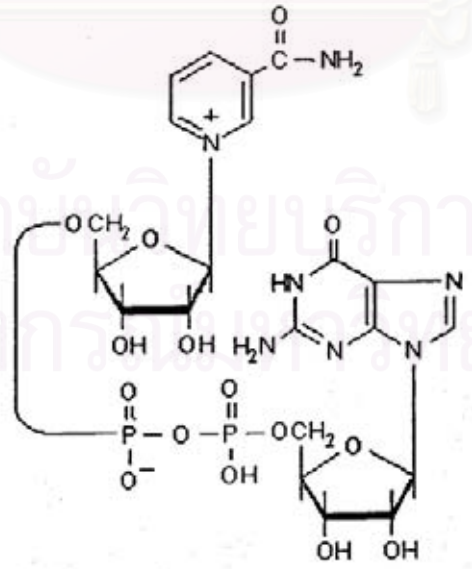
Nicotinamide adenine dinucleotide phosphate ($NADP^+$)

(Coenzyme analog modified at C-2 of the adenosyl ribose)



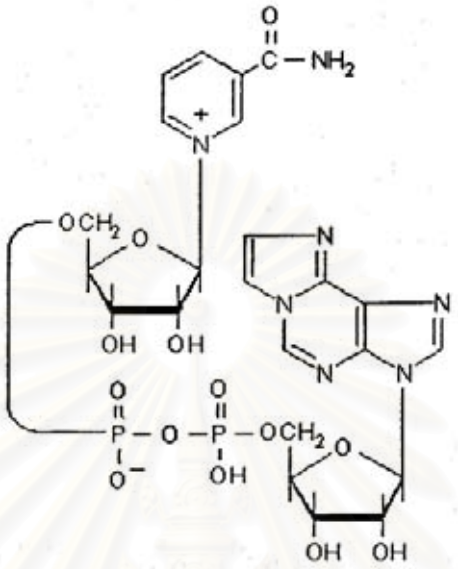
Nicotinamide hypoxanthine dinucleotide (Deamino-NAD^+)

(Coenzyme analog modified at the amino group of the adenine moiety)



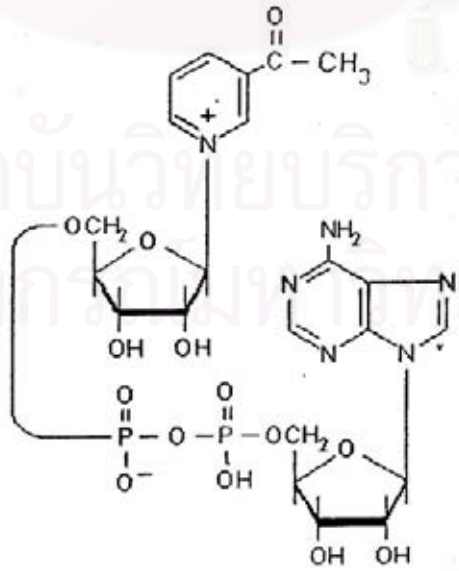
Nicotinamide guanine dinucleotide (NGD^+)

(Coenzyme analog modified at the amino group of the adenine moiety)



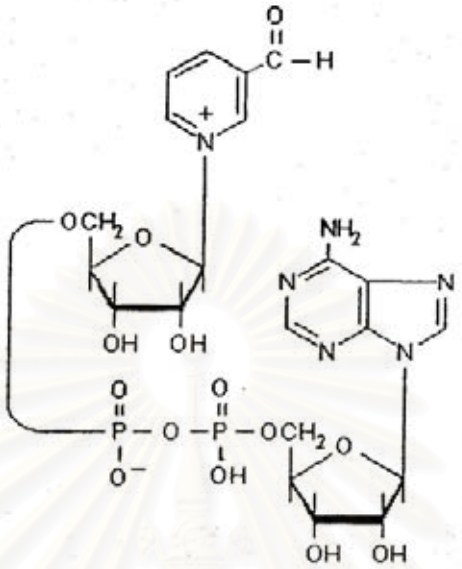
Nicotinamide 1,N^6-ethenoadenine dinucleotide (ε-NAD^+)

(Coenzyme analog modified at the amino group of the adenine moiety)



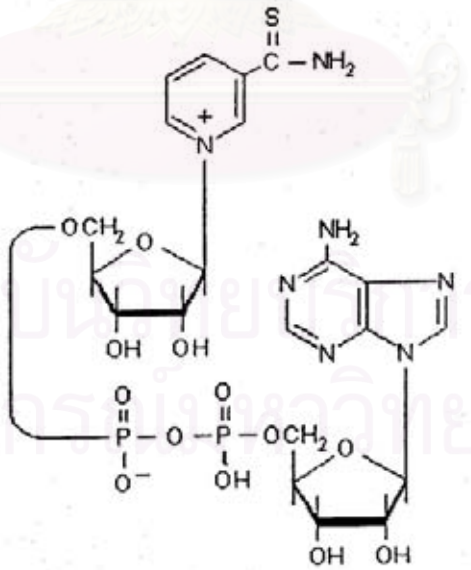
3-Acetylpyridine adenine dinucleotide

(Coenzyme analog modified at the nicotinamide moiety)



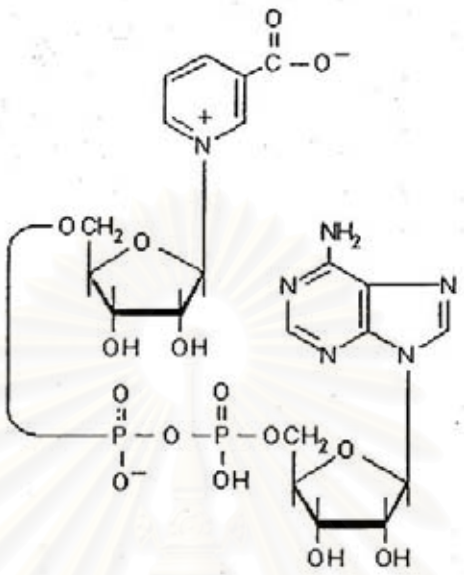
3-Pyridinealdehyde adenine dinucleotide

(Coenzyme analog modified at the nicotinamide moiety)



Thionicotinamide adenine dinucleotide

(Coenzyme analog modified at the nicotinamide moiety)



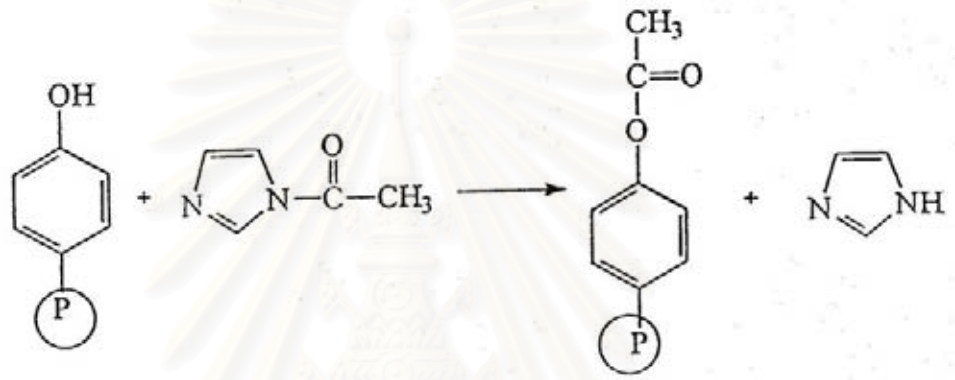
Nicotinic acid adenine dinucleotide (Deamido-NAD^+)

(Coenzyme analog modified at the nicotinamide moiety)

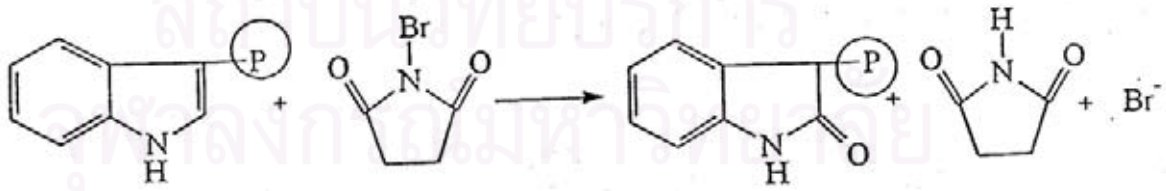
APPENDIX K

Modification reaction of group-specific reagent

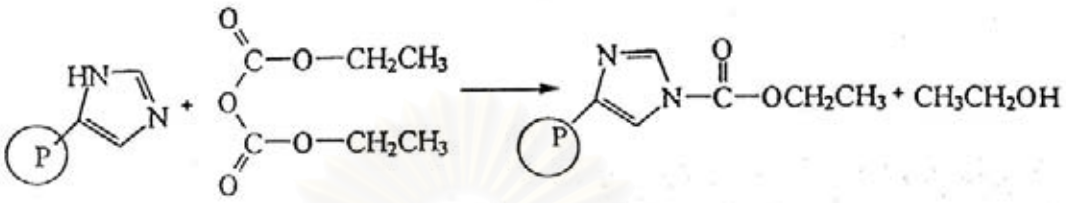
Modification reaction of *N*-acetylimidazole (NAI) with tyrosine residue in protein (P)



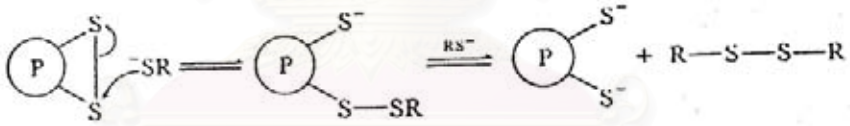
Modification reaction of *N*-bromosuccinimide (NBS) with tryptophan residue in protein (P)

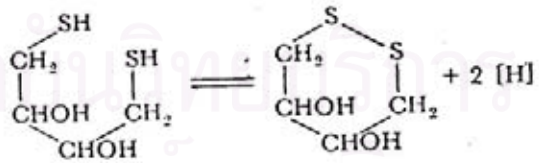


Modification reaction of diethylpyrocarbonate (DEPC) with histidine residue in protein (P)



Modification reaction of dithiothreitol (DTT) with cysteine residue in protein (P)



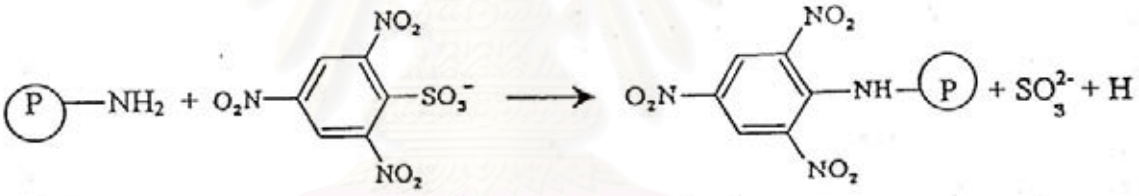


1,4-Dithiothreitol (reduced)

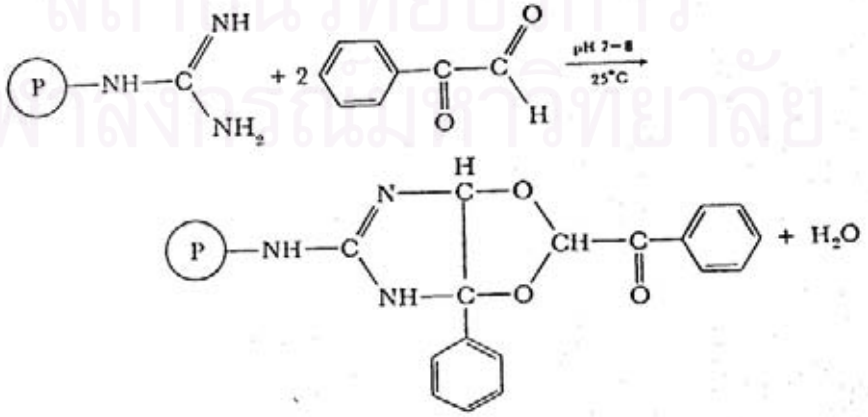
1,4-Dithiothreitol (oxidized)

Modification reaction of phenylmethylsulfonyl fluoride (PMSF) with serine residue in protein (P)

Modification reaction of 2,4,6-trinitrobenzene sulfonic acid (TNBS) with lysine residue in protein (P)



Modification reaction of phenylglyoxal (PG) with arginine residue in protein (P)



BIOGRAPHY

Miss Supatjaree Ruengsomwong was born on January 16, 1981 in Bangkok. She graduated high school from Horwang School, Bangkok and enrolled in the Faculty of Science, Kasetsart University. She graduated with the B.Sc. in Biochemistry in 2003 and was studing for M.Sc. in Biochemistry Program, Faculty of Science, Chulalongkorn University in that year.