การทำให้บริสุทธิ์และลักษณะสมบัติของไพเพอริเดอีน-6-คาร์บอกซิเลตดีไฮโดรจิเนส

จาก *Pseudomonas putida*

นางสาวจุรีพร ศรีอินทร์



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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# PURIFICATION AND CHARACTERIZATION OF PIPERIDEINE-6-CARBOXYLATE DEHYDROGENASE FROM *Pseudomonas putida*

Miss Jureeporn Sri-in



A Thesis Submitted in Partial Fulfillment of the Requirements
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| Thesis Title | PURIFICATION AND CHARACTERIZATION OF PIPERIDEINE-6-CARBOXYLATE DEHYDROGENASE FROM *Pseudomonas putida* |
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จุรีพร ศรีอินทร์ : การทำให้บริสุทธิ์และลักษณะสมบัติของไพเพอริเดอีน-6-คาร์บอกซิเลตดีไฮโดรจิเนส จาก *Pseudomonas putida*. (PURIFICATION AND CHARACTERIZATION OF PIPERIDEINE-6-CARBOXYLATE DEHYDROGENASE FROM *Pseudomonas putida*) อ.ที่ปรึกษา: ผศ.ดร. กนกทิพย์ ภักดีบำรุง, 130 หน้า

การคัดเลือกแบคทีเรียในดินที่สามารถผลิตไพเพอริเดอีน-6-คาร์บอกซิเลตดีไฮโดรจิเนส (1.2.1.31) พบว่าไอโซเลทที่มีค่าแอกทิวิตีของเอนไซม์ชนิดนี้สูงสุดคือ *Pseudomonas putida* ภาวะที่เหมาะสมในการผลิตเอนไซม์ชนิดนี้คือ การเลี้ยงแบคทีเรียในอาหารอุดมเพปโทน 0.8 เปอร์เซ็นต์ pH 8.0 ที่อุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 15 ชั่วโมง จากการทำเอนไซม์ให้บริสุทธิ์โดยการตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟต และโดยเทคนิคโครมาโทกราฟีด้วยคอลัมน์ดีอีเออีโทโยเพิร์ล, คอลัมน์บิวทิลโทโยเพิร์ล, และคอลัมน์ไฮแทรปคิว พบว่าเอนไซม์มีแอกทิวิตีคงเหลือ 18.7 เปอร์เซ็นต์ และบริสุทธิ์ขึ้น 152 เท่า เอนไซม์มีน้ำหนักโมเลกุลประมาณ 301 กิโลดาลตัน ประกอบด้วย 6 หน่วยย่อยที่มีน้ำหนักโมเลกุลเท่ากันคือ ประมาณ 50 กิโลดาลตัน เอนไซม์มีความจำเพาะต่อไพเพอริเดอีน-6-คาร์บอกซิเลตสูงมาก และสามารถใช้แอล-พีพีโคลิคแอซิดเป็นซับสเตรตได้ เอนไซม์มีความจำเพาะต่อโคเอนไซม์นิโคทินาไมด์ไฮโปแซนทีนไดนิวคลีโอไทด์ มากกว่า $NAD^+$ 1.2 เท่า pH ที่เหมาะสมในการเร่งปฏิกิริยาคือ 8.7 อุณหภูมิที่เหมาะสมคือ 45 องศาเซลเซียส เอนไซม์มีความเสถียรต่อ pH ในช่วง 6.0 ถึง 12.0 และมีความเสถียรที่อุณหภูมิ 45 องศาเซลเซียสเป็นเวลา 3 ชั่วโมงโดยเอนไซม์ยังคงมีแอกทิวิตีเหลืออยู่ 50 เปอร์เซ็นต์ เมื่อบ่มที่อุณหภูมิเดียวกันนี้เป็นเวลา 3 วัน เอนไซม์ถูกยับยั้งอย่างสมบูรณ์ด้วย $CuSO_4$ and $FeSO_4$ ที่ความเข้มข้นสุดท้าย 1 มิลลิโมลาร์ เอนไซม์มีค่า $K_m$ ต่อ แอล-พีพีโคลิคแอซิดและ $NAD^+$ เท่ากับ 1.25 และ 0.18 มิลลิโมลาร์ ตามลำดับ



สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต จุรีพร ศรีอินทร์

ปีการศึกษา 2550 ลายมือชื่ออาจารย์ที่ปรึกษา กนกทิพย์ ภักดีบำรุง

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JUREEPORN SRI-IN: PURIFICATION AND CHARACTERIZATION OF PIPERIDEINE-6-CARBOXYLATE DEHYDROGENASE FROM *Pseudomonas putida*.
THESIS ADVISOR: ASST. PROF. KANOKTIP PACKDIBAMRUNG, Ph.D., 130 pp.

$NAD^+$-dependent piperideine-6-carboxylate dehydrogenase (EC 1.2.1.31) producing bacteria were screened from soil and the isolate which produced the highest enzyme activity was identified as *Pseudomonas putida*. The optimum condition for the enzyme production was 15 hours of cultivation in 0.8% peptone medium, pH 8.0 supplemented with 0.6% L-lysine at 37 °C. The enzyme was purified to homogeneity by 50-60% saturated ammonium sulfate, DEAE-Toyopearl, Butyl-Toyopearl and Hitrap Q column chromatographies with 18.7% yield and 152 purification fold. The enzyme had a molecular mass of about 301 kDa and consisted of 6 identical subunits. The enzyme showed high substrate specificity with piperideine-6-carboxylate. L-Pipecolic acid could act as a substrate. The $NAD^+$ analog nicotinamide hypoxanthine dinucleotide gave 1.2 times higher activity than its natural coenzyme, $NAD^+$. The optimum pH was 8.7 and optimum temperature was 45 °C. The enzyme was stable to a broad pH range of 6.0 to 12.0. No loss of the enzyme activity was observed upon incubation at 45 °C for 3 hours. The enzyme retained 50% of the activity after incubation at the same temperature for 3 days. The enzyme activity was inactivated completely by $CuSO_4$ and $FeSO_4$ at a final concentration of 1 mM. The apparent $K_m$ values for L-pipecolic acid and $NAD^+$ were 1.25 and 0.18 mM, respectively.



Field of study Biotechnology Student's signature Jureeporn Sri-in

Academic year 2007 Advisor's signature Kanoktip Packdibamrung

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# CONTENTS

**Page**

**Page**

**Page**

**Page**

## LIST OF TABLES

## LIST OF FIGURES

**Page**

# LIST OF ABBREVIATIONS

| | |
|---|---|
| A | absorbance, 2′-deoxyadenosine (in a DNA sequence) |
| bp | base pairs |
| BLAST | Basic Local Alignment Search Tool |
| BSA | bovine serum albumin |
| C | 2′-deoxycytidine (in a DNA sequence) |
| $^{o}$C | degree Celsius |
| Da | Dalton |
| DEAE | diethylaminoethyl |
| DNA | deoxyribonucleic acid |
| dNTP | 2′-deoxynucleoside 5′-triphosphate |
| DTT | dithiothreitol |
| EC | Enzyme Commission |
| EDTA | ethylene diamine tetraacetic acid |
| G | 2′-deoxyguanosine (in a DNA sequence) |
| HPLC | high-performance liquid chromatography |
| HCl | hydrochloric acid |
| IPTG | isopropyl-thiogalactoside |
| kb | kilobase pairs in duplex nucleic acid, kilobases in single-standed nucleic acid |
| KCl | potassium chloride |
| kDa | kiloDalton |
| $K_m$ | Michaelis constant |
| l | liter |
| LB | Luria-Bertani |
| *lysdh* | L-lysine-6-dehydrogenase gene |
| µg | microgram |
| µl | microliter |
| µM | micromolar |
| M | mole per liter (molar) |
| mA | milliampere |
| mg | milligram |
| min | minute |

| | |
|---|---|
| ml | milliliter |
| mM | millimolar |
| $M_r$ | relative molecular mass |
| MW | molecular weight |
| $NAD^+$ | nicotinamide adenine dinucleotide (oxidized) |
| NADH | nicotinamide adenine dinucleotide (reduced) |
| $NADP^+$ | nicotinamide adenine dinucleotide phosphate (oxidized) |
| nm | nanometer |
| nmole | nanomole |
| $NH_4Cl$ | ammonium chloride |
| $(NH_4)_2SO_4$ | ammonium sulfate |
| OD | optical density |
| P6C | piperideine-6-carboxylate |
| P6CDH | piperideine-6-carboxylate dehydrogenase |
| PAGE | polyacrylamide gel electrophoresis |
| *pcd* | piperideine-6-carboxylate dehydrogenase gene |
| PCR | polymerase chain reaction |
| PMSF | phenyl methyl sulfonyl fluoride |
| SDS | sodium dodecyl sulfate |
| T | 2′-deoxythymidine (in a DNA sequence) |
| TB | Tris-borate buffer |
| TE | Tris-EDTA buffer |
| TEMED | *N, N, N′, N′*-tetramethyl ethylene diamine |
| TLC | thin-layer liquid chromatography |
| $T_m$ | melting temperature, melting point |
| UV | ultraviolet |
| V | voltage |
| v/v | volume by volume |
| w/w | weight by weight |

# CHAPTER I

## INTRODUCTION

### 1.1 L-2-Aminoadipic acid

L-2-Aminoadipic acid is a nonprotein amino acid. It is usable as a valuable intermediate for medicines such as a methotrexate derivative effective as an antirheumatic drug, remedy for psoriasis and carcinostatic agent. This compound is also usable as a terminal-modifying agent for physiologically active peptides such as peptide antibiotics and peptide hormones and as a precursor in the fermentative production of β-lactam antibiotics typified by penicillins and cephalosprins (United States Patent 5906927).

L-2-Aminoadipic acid is found widely in the biological field such as *Cholera vibrio* which is a bacterium, vegetables typified by corns and frog embryos. Further, L-2-aminoadipic acid also holds a position of an intermediate in the biosynthesis of lysine with eukaryotic microorganisms or of a precursor in the biosynthesis of β-lactam antibiotics (United States Patent 5906927).

### 1.2 Lysine biosynthesis in fungi

L-lysine has two diverse pathways involve for its biosynthesis. In bacteria, some low fungi and green plants, L-lysine is synthesized *via* the diaminopimelate pathway (DAP pathway) starting from aspartate semialdehyde and pyruvate. In addition to lysine required for protein biosynthesis, this pathway provides diaminopimelate and lysine incorporated into bacterial cell wall peptidoglycan.

In higher fungi, *de novo* L-lysine biosynthesis proceeds through the intermediacy of L-2-aminoadipate in a series of transformations unrelated to the intermediate in DAP pathway. The L-2-aminoadipic acid pathway starts with the condensation of 2-ketoglutarate and acetyl-CoA catalyzed by homocitrate synthase. The resulting homocitric acid undergoes dehydration yielding *cis*-homoaconitric acid

which is converted to homoisocitric acid by homoaconitase. Homoisocitric acid is then oxidized by homoisocitrate dehydrogenase to form oxaloglutarate followed by loss of carbon dioxide to yield 2-ketoadipic acid. Then, glutamate-dependent transamination of 2-ketoadipate to form L-2-aminoadipic acid is catalyzed by aminoadipate amino transferase. This first half of the pathway takes place in mitochondria. The latter steps converting 2-aminoadipate to L-lysine are carried out in the cytoplasm. The side chain carboxy of L-2-aminoadipic acid is reduced to form L-2-aminoadipic acid-6-semialdehyde by aminoadipate reductase. Saccharopine reductase then catalyzed the condensation of L-2-aminoadipic acid-6-semialdehyde with L-glutamate and subsequent reduction of the imine to give L-saccharopine. The last step in the pathway is the cleavage of the carbon-nitrogen bond within the glutamate moiety of L-saccharopine by saccharopine dehydrogenase. The final products are L-lysine and 2-ketoglutarate (Zabriskie and Jackson, 2000).

## 1.3 Lysine catabolism

Lysine degradation is extremely varied in nature and the nine known catabolic fates are disclosed as shown in Figure 1.1 (Mark and Michael, 2000).

In mammals, lysine degradation is known to take place at least through two major pathways. The pathway which is catalyzed by lysine-ketoglutarate reductase (Figure 1.1; xii) and saccharopine dehydrogenase (Figure 1.1; xiii) is known as saccharopine pathway. The net process is effectively a transmination with 6-amino group of L-lysine being transferred to 2-ketoglutarate, producing 2-aminoadipate 6-semialdehyde and L-glutamate. In bovine and baboon liver and in human placenta, both of these enzyme activities are associated with aminoadipic semialdehyde syntase. Interestingly, saccharopine is not produced in central nervous system. In rat, monkey and human brain, L-lysine is specifically metabolized to L-pipecolate trough $\Delta^1$-piperideine-2-carboxylate reductase in pipecolic acid pathway. Both pathways eventually converge to form L - 2 - aminoadipic acid *via* L - 2 - aminoadipate 6-semialdehyde.

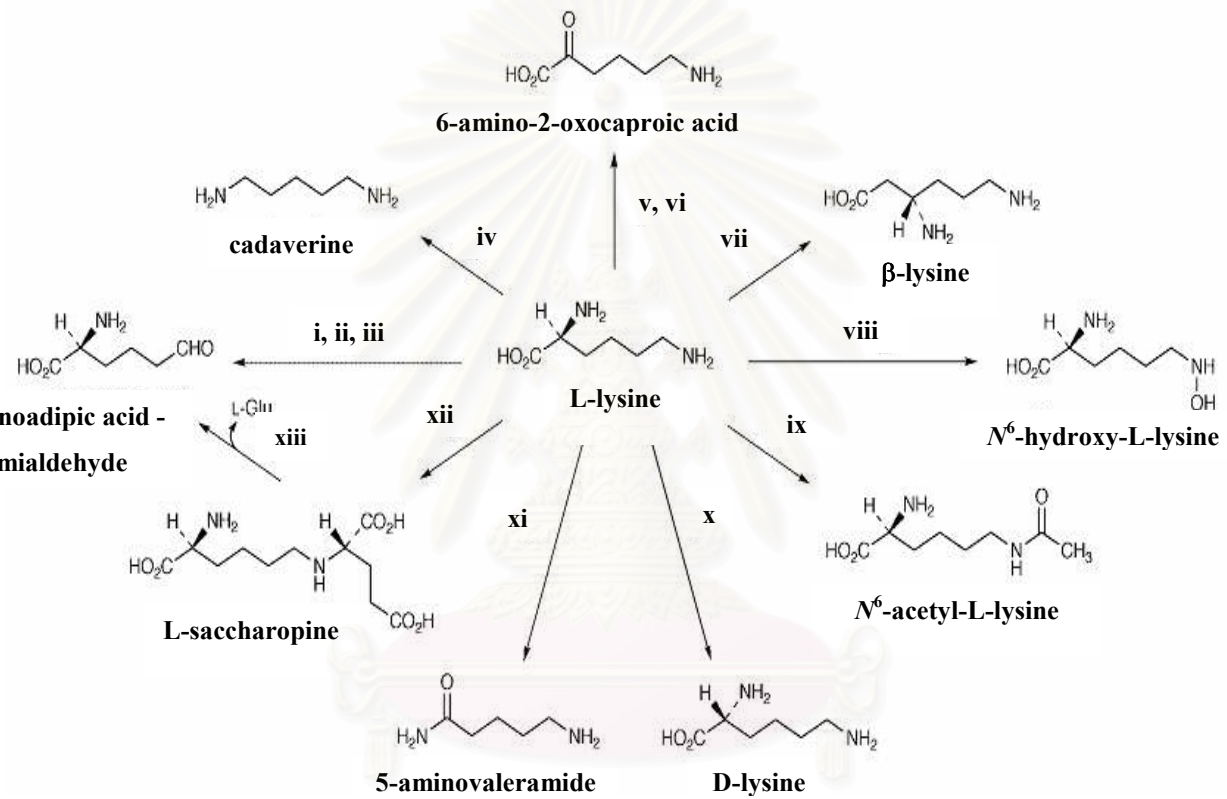



**Figure 1.1 Enzymes involved in the initial step of lysine degradation in various species**

i, lysine 6-dehydrogenase EC 1.4.1.18; ii, 2 -ketoglutarate 6-aminotransferase EC 2.6.1.36; iii, pyruvate 6-aminotransferase

EC 2.6.1.71; iv, lysine decarboxylase EC 4.1.1.18; v, lysine oxidase EC 1.4.3.14; vi, lysine dehydrogenase EC 1.4.1.15; vii, lysine 2,3-aminomutase EC 5.4.3.2; viii, lysine $N^6$-hydroxylase EC 1.14.13.59 (lysine 6-monooxygenase (NADPH)); ix, lysine $N^6$-acetyltransferase EC 2.3.1.32; x, lysine racemase EC 5.1.1.5; xi, lysine 2-monooxygenase EC 1.13.12.2 (lysine oxygenase); xii, saccharopine dehydrogenase ($NADP^+$, L-lysine forming) EC 1.5.1.8 (lysine-2-ketoglutarate reductase); xiii, saccharopine dehydrogenase ($NAD^+$, L-glutamate forming) EC 1.5.1.9.

Source Mark and Michael, 2000

In plants, L-lysine catabolism involves the action of a bifunctional enzyme with lysine: α-ketoglutarate reductase activity and saccharopine dehydrogenase (LKR/SDH) that catalyzes the first two steps in a catabolic pathway leading to 2-aminoadipate semialdehyde that is subsequently transformed to 2-aminoadipate (Karchi *et al*., 1994). Two documented functions of the 2-aminoadipate pathway in plants are to balance lysine levels and also to regulate carbon/nitrogen partition in response to abiotic stresses (Galili *et al*., 2001 and Galili, 2002).

The fungus *Trichoderma viride* produces a lysine 2-oxidase that generates 6-amino-2-oxocaproic acid from L-lysine (Figure 1.1; v) while in *Penicillium chrysogenum* lysine is catabolized to L-2-aminoadipic acid by two different pathways (Esmahan *et al*., 1994). The first catabolic pathway, L-lysine is converted to saccharopine and then to L-2-aminoadipic acid by saccharopine dehydrogenase and saccharopine reductase. The second catabolic pathway involves a lysine with L-lysine: 2-ketoglutarate 6-aminotransferase activity, which results in the direct formation of 2-aminoadipate semialdehyde. In actinomycetes, the 2-aminoadipic acid required for β-lactam biosynthesis is obtained by catabolism of lysine through the action of lysine 6-aminotransferase.

Two lysine degradative pathways are found in yeasts. The first pathway begins with formation of 2-aminoadipate-6-semialdehyde by either lysine 6-dehydrogenase (Figure 1.1; i) or lysine 6-aminotransferase. Aminotransferase utilizing either pyruvate (Figure 1.1; iii) or 2-ketoglutarate (Figure 1.1; ii) as amine acceptor have been found. A second degradative route proceeds through acetylated intermediates. In some species the first step is catalyzed by lysine $N^6$-acetyltransferase (Figure 1.1; ix) followed by loss of the 2-amine through transamination with 2-ketoglutarate.

Bacteria alter L-lysine in the greatest number of ways. *Clostridia* and several other bacteria are able to process 2-L-lysine to 3-L-lysine though the action of lysine 2, 3-aminomutase (Figure 1.1; vii). The oxidative decarboxylation of lysine to 5 -aminovaleramide has been observed in Pseudomonads possessing lysine 2-monooxygenase (Figure 1.1; xi). Some *Pseudomonas* species are able to decarboxylate lysine to yield cadaverine (Figure 1.1; iv).

## 1.4 Biosynthesis of β-lactam antibiotics

β-Lactam antibiotics, like many other secondary metabolites, have highly unusual chemical structure which is very different from those of classical primary metabolites. Three amino acids, L-2-aminoadipic acid, L-cysteine and L-valine are always the precursors of the basic structure of β-lactam antibiotics (Martin *et al*., 2006).

In β-lactam producing actinomycetes, lysine is converted into 2-aminoadipic acid semialdehyde by lysine-6-aminotransferase (LAT). This enzyme is found only in β-lactam producing microorganisms. The LAT reaction product 2-aminoadipic semialdehyde, cyclizes spontaneously to form piperideine-6-carboxylate (P6C) and later is oxidized to 2-aminoadipic acid by a piperideine-6-carboxylate dehydrogenase, encoded by the *pcd* gene (Fujii *et al*., 2000).

Two enzymatic steps are common to all β-lactam produces and result in the formation of isopenicillin N, the first compound in the pathway with antibiotic activity. The first enzyme is 6-(L-2-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase. This enzyme uses ATP to sequentially activate the three amino acid substrates to form aminoacyl-adenylates, then binds them to the enzyme as thioesters, epimerizes the L-valine to D-valine configuration, and finally links the three amino acids to form the peptide L-6-(L-2-aminoadipyl)-L-cysteinyl-D-valine (Figure 1.2). The second enzyme is isopenicillin N (IPN) synthase which removes four hydrogens from the ACV tripeptide, forming directly the bicyclic structure of isopenicillin N. Isopenicillin N is converted into the D-isomer, penicillin N by penicillin N epimerase. The next step is expansion of the five-membered thiazolidine ring of penicillin N to a six-membered dihydrothiazine ring. This step produces deacetylcephalosporin C (DAC). The final step is conversion of DAC to cephalosporin C, cephamycin C or cephabacin (Martin *et al*., 2006).

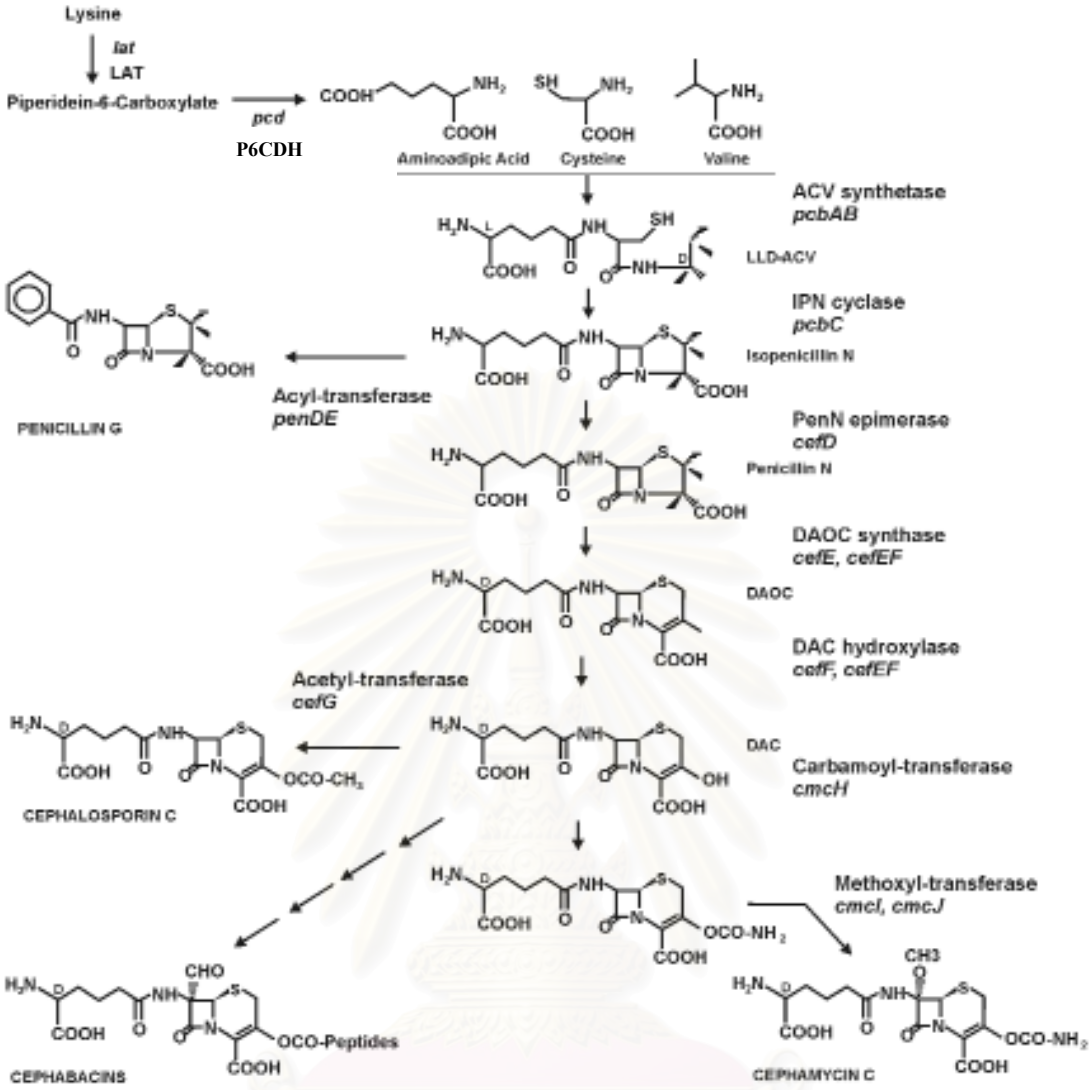



**Figure 1.2 Biosynthetic pathway of penicillins, cephalosporins, cephamycins, and cephabacins.**

Source Martin *et al*., 2006

## 1.5 Production of L-2-aminoadipic acid

Since L-2-aminoadipic acid is a precursor of various kinds of drug such as methotrexate derivative useful as an antirheumatic drug as well as carcinostatic agent and is one of three precursors of the basic structure of β-lactam antibiotics, many researchers attempt to synthesize it by both chemical and biological methods. However, the chemical synthesis of L-2-aminoadipic acid is not yet an effective means from the view point of the cost, since an optical resolution and multi-stage reactions are necessitated (United States Patent 5906927).

As for the production of L-2-aminoadipic acid with a microorganism in *Alcaligenes*, *Pseudomonas* or *Kurthia*, capable of converting L-pipecolic acid into L-2-aminoadipic acid, the microorganism is cultured in a liquid medium containing L-pipecolic acid at 30 ºC for 3 days. Then, the subject compound, L-2-aminoadipic acid, is collected from the culture mixture (Japanese Patent JP1098495). There is also known a process wherein it is produced from L-lysine with microorganism of *Agrobacterium*, *Klebsiella*, *Alcaligenes*, *Brevibacterium* or *Bacillus* (Japanese Patent JP6181787). A medium is inoculated with microorganism to conduct a shaking culture at 26 ºC for 48 hours, and the resultant culture fluid is centrifuged to gather the cell pellet. The microbes is suspended in 50 mM of a phosphate buffer solution, pH 9.0 containing L-lysine followed by shaking at 26 ºC for 120 hours to conduct a reaction followed by centrifugation of the reaction solution to eliminate the microbes. L-2-aminoadipic acid in the resulting solution is purified by basic anion exchange column chromatography. However, these two processes have problem when they are to be employed for the mass production. Namely, starting L-pipecolic acid is expensive in the former process and the reaction efficiency is usually low in the latter process. Therefore, a microorganism of the genus *Flavobacterium* is used (United States Patent 5906927). The bacteria is cultured in medium containing 2% L-lysine at 28 ºC for 96 hours. The supernatant was taken by centrifugation, and L-2-aminoadipic acid contained in the supernatant is purified using anion exchange column chromatography. This method is effective for the mass production. All of the above processes are microbial method.

Molecular biology is also applied for L-2-aminoadipic acid production. L-Lysine 6-aminotransferase (LAT) is an essential enzyme for the first step of L-2-aminoadipic acid biosynthesis in *Flavobacterium lutescens* IFO3084. The enzyme converts L-lysine into 2-aminoadipic semialdehyde, which is spontaneously cyclized to form $\Delta^1$-piperideine-6-carboxylate (P6C) (Figure 1.3; i) (Madduri *et al*., 1989). The gene encoding LAT (*lat*) from *F. lutescens* IFO3084 was cloned and sequenced. The expression in *Escherichia coli* revealed that *lat* encodes a single subunit protein with LAT activity (Fujii *et al*., 2000). In parallel, *pcd* gene encoding $\Delta^1$-piperideine-6-carboxylate dehydrogenase (P6CDH), catalyzed the conversion of P6C into L-2-aminoadipic acid, from *F. lutescens* IFO3084 was cloned. The *in vitro* analysis of L-2-aminoadipic acid production showed that L-2-aminoadipic acid is synthesized from L-lysine in two steps catalyzed by LAT and P6CDH (Figure 1.3; i, iii). It is noteworthy that the bioconversion of L-lysine to L-2-aminoadipic acid, unlike chemical synthesis, preserves chirality. Therefore, it would dramatically improve the L-2-aminoadipic acid manufacturing efficiency.

## 1.6 L-Lysine-6-dehydrogenase

From Figure 1.3, L-lysine-6-dehydrogenase can be used to convert L-lysine to P6C instead of lysine 6-aminotransferase. L-Lysine 6-dehydrogenase (LysDH: EC 1.4.1.18) catalyzes the oxidative deamination of the 6-amino group of L-lysine in the presence of $NAD^+$ to form L-2-aminoadipate-6-semialdehyde, which in turn nonenzymatically cyclized form piperideine-6-carboxylate.

The $NAD^+$ dependent LysDH was first discovered from plant pathogenic bacterium, *Agrobacterium tumefaciens* (Misono and Nagasaki, 1982). Consequently, Misono and colleagues screened the enzyme activity among microoganisms from soil samples and culture collections. It was found that the enzyme activity was very narrowly distributed only in *Agrobacterium tumefaciens, Alcaligenes faecalis, Bacillus spharicus, Klebsiella pneumoniae and Pseudomonas fragi* (Misono and Nagasaki,1982).

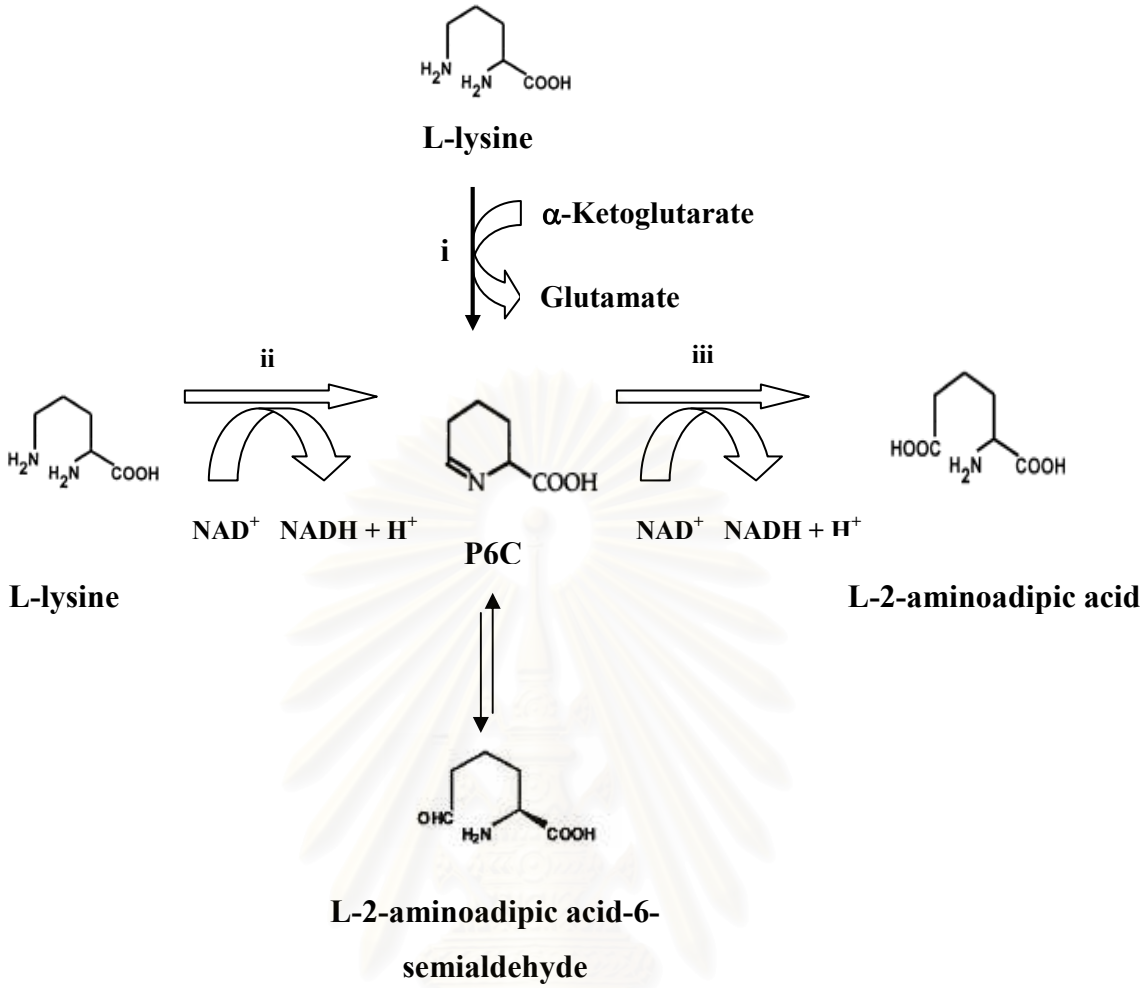



**Figure 1.3 Conversion of L-lysine into L-2-aminoadipic acid**

i: lysine 6-aminotransferase, ii: L-lysine-6-dehydrogenase,
iii: piperideine-6-carboxylate dehydrogenase

Source modified from Fuente, 1997 and Tadashi *et al*., 2001

In 1991, LysDH was found in a yeast *Cadida albicans* which is able to utilize L-lysine as the role nitrogen and carbon source accompanied by intracellular accumulation of 2-aminoadipate semialdehyde. The enzyme is strongly induced in cells grown on L-lysine as the role nitrogen source. Heydari *et al*., (2004) screened thermophilic and and hyperthermophilic microorganisms for a more stable form of LysDH and found one such enzyme in a moderately thermophilic bacterium, *Geobacillus stearothermophilus*, which was isolate from a Japanese hot spring.

Recently, our research group screened LysDH-producing bacteria from soil sample collected from various areas in Japan (Ruldeekulthamrong, 2007). Among them, an isolate giving a high activity of LysDH was selected and further identified as *Achromobacter denitrificans.* The low yield of enzyme in the wild-type strain triggered us to use recombinant DNA technology to obtain a sufficient amount of the LysDH. The enzyme gene, *lysdh*, was cloned and overexpressed into *E. coli* BL21 (DE3) using the expression vector, pET-17b. The activity of LysDH from 600 ml of cell culture was 1,664 units with specific activity of 1.89 unit/mg protein. After purification by DEAE-Toyopearl 650M column chromatography and DEAE-Sephadex A50 column chromatography, the enzyme was purified 2.8 fold with 47.43% recovery. The LysDH properties of all characterized enzymes are shown in Table 1.1. The promising yield of LysDH from *E. coli* clone and the enzyme properties show a high potentiality for application in L-2-aminoadipic acid production by coupling with piperideine-6-carboxylate dehydrogenase.

## 1.7 Piperideine-6-carboxylate dehydrogenase (P6CDH)

Piperideine-6-carboxylate dehydrogenase (EC 1.2.1.31) catalyzes the conversion of piperideine-6-carboxylate in the presence of $NAD^+$ into L-2-aminoadipic acid. There is very rare report on P6CDH. The enzyme was firstly studied in the cephamycic C producer *Streptomyces clavuligerus* (Fuente *et al*., 1997). P6CDH reach maximal activity later than other early enzymes of the cephamycin pathway.

**Table 1.1 Properties of L-lysine-6-dehydrogenase from various sources**

| **Properties** | ***Agrobacterium tumefaciens*** | ***Geobacillus stearothermophilus*** | ***Achromobacter denitrificans*** |
|---|---|---|---|
| Specific activity of final preparation (U/mg protein) | 4.54 | 6.2 | 5.31 |
| Molecular mass of native enzyme (Da)<br>- gel filtration<br>- deduced amino acid sequence<br>Molecular mass of subunit | 70,000<br>-<br>39,000 | -<br>42,239<br>- | 240,000<br>-<br>40,000 |
| Number of subunit | 2 | - | 6 |
| pH optimum for oxidative deamination | 9.7 | 10.0 | 9.3 |
| pH stability | 5.0-7.5 | 6.0-9.0 | 7.5-8.0 |
| Optimum temperature (ºC) | - | 70 | 50 |
| Thermostability (% remaining activity after incubation at 30 ºC for 10 minutes) | 40 | 60 | The enzyme was fully stable at 30 ºC for 3 days. |
| Apparent $K_m$ (mM) for<br>- L-lysine<br>- $NAD^+$<br>- NADH<br>- 3 acetylpyridine-$NAD^+$<br>- Deamino-$NAD^+$ | <br>1.5<br>0.059<br>-<br>3.13<br>1.85 | <br>0.73<br>0.088<br>0.48<br>-<br>- | <br>8.62<br>0.092<br>-<br>-<br>- |

Note: - = no data

Source *Agrobacterium tumefaciens* (Misono and Nagasaki, 1982)

*Geobacillus stearothermophilus* (Heydari *et al*., 2004)

*Achromobacter denitrificans* (Ruldeekulthamrong, 2007)

The P6CDH activity was decreased in ammonium supplemented cultures, as was that of lysine-6-aminotransferase. P6CDH was also found in other cephamycin C producers, *Streptomyces cattleya* and *Nocardia lactamdurans*, but not in actinomyces that do not produce β-lactam, suggesting that it is an enzyme specific for cephamycin biosynthesis, involved in the second stage of two steps conversion of lysine to L-2-aminoadipic acid. P6CDH from *S. clavuligerus* was purified and characterized. The enzyme was purified 124-fold to homogeneity with a 26% yield. The native enzyme is monomer of 56.2 kDa. The enzyme efficiently used P6C ($K_m$ 14 μM) and $NAD^+$ ($K_m$ 115 μM) but not $NAD^+$, as substrates. The enzyme activity was inhibited 66% by its end product NADH at 0.1 mM concentration. The *pcd* gene encoding P6CDH of *S. clavuligerus* was proved to be located in the cephamycin cluster by Llarena and Coworkers in 1998.

As mentioned in section 1.5, *pcd* gene from *Flavobacterium lutescens* IFO3084 was cloned and expressed in *E. coli*. The molecular mass of the recombinant P6CDH was estimated to be about 58 kDa by native PAGE, which is in good agreement with the value obtained by SDS-PAGE.

Our research group by Lertmongkolthum (2004) screened $NAD^+$ dependent P6CDH producing bacteria from 20 soil samples collected from various areas in Thailand. There were 58 isolates that could use L-lysine as carbon and nitrogen source. Their crude extracts were incubated with the purified LysDH from *Achromobacter denitrificans* in the presence of L-lysine as a substrate and then assayed for L-2-aminoadipic acid product by TLC analysis. Five isolates showed L-2-aminoadipic acid production. Thus these 5 isolates should possess P6CDH activity.

The objectives of this research are

1. To select the isolate that obtains the highest P6CDH activity
2. To determine the optimum condition for P6CDH production
3. To purify and characterize the biochemical properties of P6CDH

# CHAPTER II

## MATERIALS AND METHODS

### 2.1 Equipments and materials

Autoclave: MLS-2420, Sanyo Electric Co., Ltd., Japan

Autopipette: Pipetman, Gilson, France

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

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

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

Fraction collector: Frac-920, Pharmacia Biotech, Sweden

Heating box: Model RS232 Dri bath incubator, Taiwan

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

Magnetic stirrer: Model Fisherbrand, Fisher Scientific, USA

Microcentrifuge tubes 0.5 and 1.5 ml, Axygen Hayward, USA

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

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

Perista pump: Gradicon III AC-5900, Atta, Japan

pH meter: Model S20-K, Schwerzenbach, Switzerland

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

Shaking waterbath: Model G-76, New Brunswick Scientific Co., Inc., USA

Sonicator: Vibra cell$^{Tm}$, SONICS & MATERIALS, Inc., USA

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

Thin layer chromatography: DC-Plastikfolien cellulose, Merck, Germany

Ultrafilter: Suprec$^{Tm-01}$ molecular weight cut off 30,000
Takara Shuzo Co, Ltd., Japan

Vortex: Model K-550-GE, Scientific Industries, Inc., USA

## 2.2 Chemicals

Acrylamide: Merck, Germany

Agar: Merck, Germany

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

Ammonium persulphate: Sigma, USA

Ammonium sulphate: Carlo Erba Reagenti, Italy

Ampicillin: Sigma, USA

Aquasorb: BML, Thailand

Bis-acrylamide: Merck, Germany

Boric acid: Merck, Germany

Bovine serum albumin: Sigma, USA

*n*-Butanol: Carlo Erba Reagenti, Italy

Butyl-Toyopearl 650M TSK gel: Tosoh, Japan

Coomassie brilliant blue R-250: Sigma, USA

DEAE-Toyopearl 650M TSK gel: Tosoh, Japan

Dialysis tubing: Sigma, USA

DNA marker: Lamda (λ) DNA digested with *Hin*dIII, BioLabs, Inc., USA

100 base pair DNA ladder, Promega Co., USA

Ethanol: Lab-Scan, Thailand

Ethidium bromide: Sigma, USA

Ethylene diamine tetraacetic acid (EDTA): Merck, Germany

Ficoll type 400: Sigma, USA

Glacial acetic acid: Carlo Erba Reagenti, Italy

Glycerol: Merck, Germany

Glycine: Sigma, USA

Hydrochloric acid: Carlo Erba Reagenti, Italy

Isopropylthio-β-D-galactosidase (IPTG): Sigma, USA

β- mercaptoethanol: Fluka, Switzerland

Methanol: Lab-Scan, Thailand

Magnesium sulphate 7-hydrate: BDH, England

MOPS: Sigma, USA

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

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

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

Nicotinamide hypoxanthine dinucleotide: Sigma, USA

Nicotinic acid adenine dinucleotide: Sigma, USA

*N,N′*-methylene-bis-acrylamide: Sigma, USA

*N,N,N′,N′*-tetramethyl-1, 2-diaminoethane (TEMED): Carlo Erba Reagent, Italy

Ninhydrin: VWR Prolabo Range, France

Nitroblue tetrazolium: Koch-Light Laboratories Ltd., Japan

dNTP: Fermentas, USA

Peptone from meat pancreatically digested: Merck, Germany

Phenazine methosulfate: Nacalai Tesque, Inc., Japan

Phenol reagent: Carlo Erba Reagenti, Italy

Protein molecular weight marker (MW 14,400-116,000): Fermentas, USA

Potassium chloride: Merck, Germany

Potassium di-hydrogen phosphate: Carlo Erba Reagenti, Italy

di-Potassium hydrogen phosphate: Carlo Erba Reagenti, Italy

3-Pyridinealdehyde adenine dinucleotide: Sigma, USA

HiYield Gel/PCR DNA Fragments Extraction Kit: Real Biotech Corporation, Taiwan

Sodium acetate: Merck, Germany

Sodium chloride: Carlo Erba Reagenti, Italy

Sodium dodecyl sulfate: Sigma, USA

Sodium hydroxide: Merck, Germany

Standard protein marker for SDS-PAGE: Fermentas Inc., USA

*Taq* polymerase: Fermentas, USA

Thionicotinamide adenine dinucleotide: Sigma, USA

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

Yeast extract: Scharlau microbiology, European Union

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

## 2.3 Bacterial strains

*E. coli* BL21 (DE3) containing *lysdh* gene from *Achromobacter denitrificans* was used as a source of LysDH.

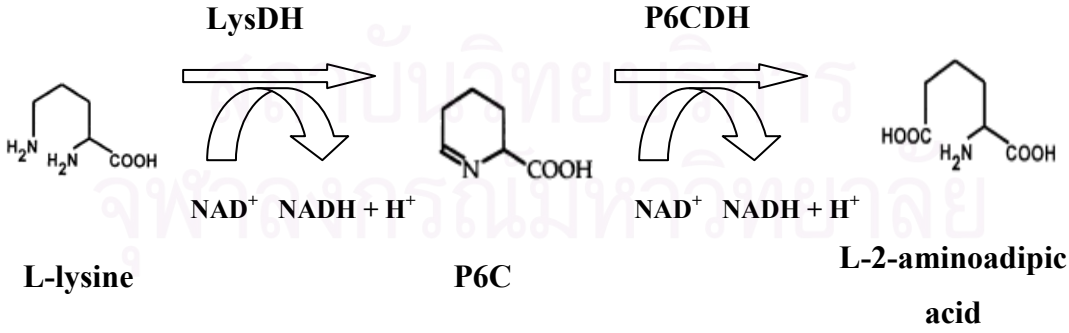
The five bacterial isolates, screened from soil samples in Thailand by Lertmongkolthum (2004), were used for selection of P6CDH producing bacterium.

## 2.4 Assay for L-lysine-6-dehydrogenase

The activity of LysDH for oxidative deamination of L-lysine was spectrophotometrically assayed. The 1 ml of reaction mixture contained 20 μmol of L-lysine, 200 μmol of glycine-KCl-KOH buffer, pH 9.5, 1 mM of $NAD^+$, and the enzyme. The reaction mixture without $NAD^+$ was incubated at 30 $^oC$ for 5 minutes in a cuvette of 1-cm light path. The reaction was started by addition of $NAD^+$ and monitored by measuring the initial change in absorbance of NADH at 340 nm.

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

## 2.5 Assay for piperideine-6-carboxylate dehydrogenase




The activity of P6CDH was spectrophotometrically assayed. The 1 ml of coupling reaction mixture contained 20 μmol of L-lysine, 200 μmol of MOPS-HCl buffer, pH 8.7, 1 μmol of $NAD^+$, 0.65 units of LysDH and P6CDH. Control reaction was carried out without P6CDH in the reaction mixture. The reaction mixture without

$NAD^+$ was incubated at 37 $^oC$ for 5 minutes in a cuvette of 1-cm light path. The reaction was started by addition of $NAD^+$ and monitored by measuring the initial change in absorbance of NADH at 340 nm.

The activity of P6CDH was calculated by subtraction activity of the coupling reaction with activity of the control reaction.

One unit of the enzyme is defined as the amount of enzyme that catalyzes the formation of 1 nmol of NADH in 1 minute. Specific activity is expressed as units per milligram of protein.

## 2.6 Protein determination

Protein concentration was determined by the method of Lowry *et al.,* (1951). The reaction mixture containing 20-100 μg of protein, 100 μl of solution A, 5 ml of solution B was mixed and incubated at 30 $^oC$ for 10 minutes. After that, the solution mixture was incubated with 0.5 ml of solution C at room temperature for 20 minutes. Then, the protein concentration was monitored by measuring the absorbance at 610 nm and calculated from the standard curve of protein standard (BSA). The preparation of all solutions was described in Appendix A.

## 2.7 Polyacrylamide gel electrophoresis

### 2.7.1 Non-denaturing gel electrophoresis

Discontinuous PAGE was performed on the slab gel of a 7.7% seperating gel and a 5% stacking gel. Tris-glycine buffer, pH 8.3 (25 mM Tris and 192 mM glycine) was used as electrode buffer. Preparation of solutions and polyacrylamide gel was described in Appendix E. The enzyme was mixed with 5x sample buffer (312.5 mM Tris-HCl, pH 6.8, 50% glycerol and 0.05% bromophenol blue) by ratio 5: 1 and loaded onto the gel. The electrophoresis was run from cathode towards anode at constant current (20 mA). For activity staining, the experiment was done at 4 $^oC$.

### 2.7.2 SDS-polyacrylamide gel electrophoresis

The SDS-PAGE system was performed according to the method of Bollag *et al*., (1996). The slab gel system consisted of 0.1% SDS (W/V) in 10% seperating gel and 5% stacking gel. Tris-glycine (25 mM Tris, 192 mM glycine and 0.1% SDS), pH 8.3 was used as electrode buffer. The gel preparartion was described in Appendix F. The enzyme was mixed with 5x sample buffer (60 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol blue and 14.4 mM β-mercaptoethanol) by ratio 5: 1 and boiled for 10 minutes before loading to the gel. The electrophoresis was run from cathode towards anode at constant current (20 mA) at room temperature. The molecular weight marker proteins were β-galactosidase (116,000 Da), bovine serum albumin (66,200 Da), ovalbumin (45,000 Da), lactate dehydrogenase (35,000 Da), restriction endonuclease Bsp98I (25,000 Da), β-lactoglobulin (18,400 Da) and lysozyme (14,400 Da).

### 2.7.3 Protein staining

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

## 2.8 Preparation of L-lysine-6-dehydrogenase

### 2.8.1 The culture medium

The LB medium containing 1% peptone, 0.5% NaCl and 0.5% yeast extract, pH 7.2 and supplement with 100 μg/ml ampicillin was used as a culture medium. For agar plate, the medium was supplemented with 1.5% agar. The medium was sterilized at 121 $^{o}$C for 15 minutes.

### 2.8.2 Starter inoculum

A single colony of *E. coli* BL21 (DE3) containing *lysdh* gene from *Achromobacter denitrificans* was picked from agar plate and cultured in 20 ml of LB medium, pH 7.2, containing 100 μg/ml ampicillin at 37 $^{o}$C with 250 rpm rotary shaking for 16 hours.

### 2.8.3 Bacterium cultivation and cell harvest

The starter inoculum from section 2.8.2 was inoculated into 1 liters of LB medium, pH 7.2, containing 100 μg/ml ampicillin and cultured at 37 $^{o}$C with 250 rpm shaking. When the turbidity of the culture at 600 nm had reached 0.5, IPTG was added to final concentration of 0.2 mM to induce *lysdh* gene expression and cutivation was continued at 37 $^{o}$C for 4 hours. The cells were harvested by centrifugation at 8,500xg for 10 minutes, then washed with cold 0.85% NaCl. After that, the cell pellet was washed once in cold extraction buffer (10 mM potassium phosphate buffer, pH 7.4 containing 0.01% $\beta$-mercaptoethanol and 1.0 mM EDTA) and centrifuged again. The cell pellet had been stored at -20$^{o}$C until it was sonicated.

### 2.8.4 Preparation of crude extract

Preparation of crude extract was performed by resuspending cell pellet in cold extraction buffer. Cell suspension was sonicated on ice. Unbroken cells and cell debris were removed by centrifugation at 10,000xg for 30 minutes. The LysDH activity and protein concentration were determined as described in section 2.4 and 2.6, respectively.

### 2.8.5 Purification of L-lysine-6-dehydrogenase from recombinant clone

Purification step was carried out at 4 $^{o}$C. The 10 mM potassium phosphate buffer, pH 7.4 containing 0.01% $\beta$-mercaptoethanol and 1 mM EDTA was used through the purification step. A procedure of LysDH purification was as follows.

### 2.8.5.1 DEAE-Toyopearl column chromatography

The activated DEAE-Toyopearl was prepared by washing with 0.5 N NaOH for 2-3 times, and rewashed by deionized water until the pH reached 8.0. The activated DEAE-Toyopearl was resuspened in the buffer, packed into 2.3 x 19.5 cm column and equilibrated with the same buffer for 5-10 column volume at flow rate 1 ml/min.

The crude enzyme solution from section 2.8.4 was applied into the DEAE-Toyopearl column. The unbound proteins were eluted from the column with the buffer until the absorbance at 280 nm was nearly zero. After that, the linear salt gradient of 0 to 0.5 M KCl in the same buffer was applied in order to elute the bound proteins from the column. The 3 ml fractions were collected using a fraction collector. The protein profile was determined by measuring the absorbance at 280 nm. LysDH activity was assayed using the method described in section 2.4. The KCl concentration was investigated by measuring its conductivity. The fractions containing LysDH activity was pooled and dialyzed against the buffer. The LysDH activity and protein concentration of pooled fraction were measured as described in section 2.4 and 2.6, respectively.

### 2.8.5.2 DEAE-Sephadex A50 column chromatography

The DEAE-Sephadex A50 was prepared by washing with deionized water for 2-3 times. The DEAE-Sephadex A50 was resuspened in the buffer, packed into 1.3 x 19.5 cm column and equilibrated with the same buffer for 5-10 column volume at flow rate 0.5 ml/min.

The pooled active fraction from section 2.8.5.1 was applied into the column. The unbound proteins were eluted from the column with the buffer until the absorbance at 280 nm was nearly zero. The bound protein was eluted from the column with linear salt gradient of 0 to 0.5 M KCl in the buffer. Three milliliter fractions were collected by using fraction collector and assayed for both protein concentration and

LysDH activity. Protein profile was determined by measuring an absorbance at 280 nm. LysDH activity was assayed by using the method as described in section 2.4. The fractions containing LysDH activity were pooled and dialyzed against the buffer. After desalting, the enzyme was concentrated with aquasorb. The LysDH activity and protein concentration were determined as described in section 2.4 and 2.6, respectively.

The protein from each active fraction was electrophoresed on non-denaturing and denaturing polyacrylamide gel as described in section 2.7.1 and 2.7.2, respectively. After that, the gels were stained with Coomassie blue as described in section 2.7.3.

The activity stain was performed by transferring the gel to a small box which consisted of 10 ml solution containing 40 μmol of L-lysine, 4.25 mmol of Tris-HCl buffer, pH 8.5, 50 μmol of $NAD^+$, 0.25 mg of phenazine methosulfate and 2.5 mg of nitroblue tetrazolium. The gel was gently shaked at room temperature for 20 min. After the brown band had appeared, the staining reaction was stopped by pouring off the staining solution. The gel was then quickly rinsed several times with deionized water.

The purified LysDH was used for the coupling reaction of P6CDH activity assay.

## 2.9 Selection of piperideine-6-carboxylate dehydrogenase producing bacterium

### 2.9.1 Bacterial cultivation

A single colony of each P6CDH producing isolate (5 isolates) from agar plate was inoculated into 5 ml of peptone medium, pH 7.2 (1% peptone, 0.2% sodium chloride, 0.2% di-potassium hydrogen phosphate, 0.2% potassium dihydrogen phosphate, 0.01% magnesium sulfate, 0.01% yeast extract and supplemented with 1% L-lysine) at 37 $^{o}$C with 250 rpm shaking for 16 hours. After that, the bacterial starter was transferred into 500 ml Erlenmeyer flask containing 200 ml of peptone medium,

pH 7.2 and cultured at 37 $^{o}$C with 250 rpm shaking. Every 3 hours (0, 3, 6, 9, 12, 15, 18, 21 and 24), the cell culture was taken and measured for optical density at 600 nm.

### 2.9.2 Cell harvest

Cells were harvested by centrifugation at 6,000xg for 10 minutes at 4 ºC. Then, the collected cells were washed twice with 0.85% NaCl and subsequently washed once with extraction buffer (10 mM Tris-HCl buffer, pH 8.0 containing 0.01% β-mercaptoethanol, 1 mM EDTA and 10% glycerol). The harvested cells had been stored at -20 ºC until used.

### 2.9.3 Preparation of crude extract

The collected cell from section 2.9.2 were resuspended in cold extraction buffer and then broken by discontinuously sonication on ice. Unbroken cells and cell debris were removed by centrifugation at 10,000xg for 30 minutes at 4 $^{o}$C. The crude extract was collected and assayed for the P6CDH activity and protein concentration as described in section 2.5 and 2.6, respectively. The enzyme had been kept at 4 $^{o}$C until used.

### 2.9.4 Piperideine-6-carboxylate preparation

The 0.5 ml of reaction mixture contained 20 μmol of L-lysine, 200 μmol of $Na_2CO_3$.$NaHCO_3$ buffer, pH 9.5, 10 μmol of $NAD^+$, and 30 U of LysDH. The reaction was incubated at 30 $^{o}$C for 16 hours. After that, the reaction mixture was heated at 100 $^{o}$C for 10 minutes and then centrifuged at 6,000xg for 10 minutes. The solution was used for L-2-aminoadipic acid production.

### 2.9.5 L-2-Aminoadipic acid preparation

The 0.5 ml of reaction mixture contained 100 μl of the solution from section 2.9.4, 200 μmol of MOP-HCl buffer, pH 8.7, 10 μmol of $NAD^+$, and crude enzyme from section 2.9.3. The reaction was incubated at 30 $^{o}$C for 16 hours. After that, the reaction mixture was filtrated by using Suprec$^{Tm-01}$ (molecular weight cut off 30,000). L-2-aminoadipic acid production was investigated by TLC analysis.

### 2.9.6 Determination of L-2-aminoadipic acid by TLC analysis

The 0.5 µl of the mixtures from section 2.9.5 were subjected to 10 cm x 10 cm cellulose TLC plastic sheets in parallel with standard L-lysine and L-2-aminoadipic acid. The TLC plates were developed with *n*-butanol:acetic acid: $H_2O$ (4:1:1). After that the plates were dried in hot air, developed in 0.5% ninhydrin in acetone and then dried at 100 $^oC$ for 5 minutes. The ratio of the distance a compound moved from the baseline to the distance of the solvent front moved from the baseline was defined as the retardation factor ($R_f$).

## 2.10 Identification of $NAD^+$ dependent piperideine-6-carboxylate dehydrogenase producing bacterium

The P6CDH producing bacterium was identified according to its morphological and biochemical properties such as fermentative production of acid from various carbon sources and activity of various enzymes by Department of Medical Sciences (DMSC), Thailand. The result was confirmed by 16S rRNA sequence.

### 2.10.1 16S rRNA gene amplification

The colony PCR was used for 16S rRNA gene amplification. The 25 µl of reaction mixture contained 1.5 U of *Taq* DNA polymerase, 1x buffer with $MgSO_4$, 0.2 mM each dNTP, one bacterial colony and 10 pmole of each primer. The sense pA and antisense pH′ primer sequences were 5′-ATTGAACGCTGGCGGCAGGC-3′ and 5′-GGGCCTTGTACACACCGCCC-3′, respectively. PCR was performed as described in the following procedure. Predenaturation at 94 $^oC$ for 5 minutes followed by 30 cycles of denaturation at 94 $^oC$ for 1 minute, annealing at 55 $^oC$ for 2 minutes and extension at 72 $^oC$ for 3 minutes. Then, 1 cycle of final extension at 72 $^oC$ for 5 minutes was done. After amplification, the PCR products were applied to agarose gel electrophoresis (Julian *et al*., 1998).

### 2.10.2 Agarose gel electrophoresis

Electrophoresis through agarose is the standard method used to separate, identify, and purify DNA fragments. The 0.8 g of agarose was added to 100 ml of electrophoresis buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0) in Erlenmeyer flask and heated until complete solubilization in a microwave oven. The agarose solution was left at room temperature to 50 $^{o}$C before pouring into an electrophoresis mould. When the gel was completely set, the DNA samples were mixed with gel loading buffer (0.025% bromphenol blue, 40% ficoll 400 and 0.5% SDS) and loaded into agarose gel. Electrophoresis had been performed at constant voltage of 100 volts until the bromphenol blue migrated to appropiate distance through the gel. The gel was stained with 2.5 μg/ml ethidium bromide solution for 5 minutes and destained to remove unbound ethidium bromide with distilled water for 10 minutes. DNA fragments on agarose gel were visualized under a long wavelength UV light. The concentration and molecular weight of DNA sample was determined by comparison of band intensity and relative mobility with those of the standard DNA markers (λ/*Hin*dIII and 100 bp ladder).

### 2.10.3 PCR product purification

After agarose gel electrophoresis, the DNA fragment was colleted from agarose gel by HiYield Gel/PCR DNA Fragments Extraction Kit. The DNA fragment from the agarose gel was excised with a clean and sharp scalpel. The gel slice was transferred into a microcentrifuge tube. Then, 500 μl of DF buffer was added to the sample and mixed by vortexing. The gel was incubated at 55 $^{o}$C for 10-15 minutes to completely dissolve the gel slice. During incubation, invert the tube every 2-3 minutes. After that, placed a DF column in a collection tube and 800 μl of the sample mixture from previous step was applied into the DF column and then centrifuged at 6,000xg for 1 minute. The flow-through was discarded and the DF column was placed back in the collection tube. If the sample mixture was more than 800 μl, repeat this DNA binding step. Five hundred μl of wash buffer (ethanol) was added into the DF column and centrifuged at 6,000xg for 1 minute. The flow-through was discarded and the DF column was placed back in the collection tube. The column matrix was dried by

centrifuged at 12,000xg for 2 minutes. The dried column was transferred in a new microcentrifuge tube. To elute DNA, 20 μl of elution buffer or water was added to the center of the column matrix, stand for 2 minutes until the elution buffer or water was absorbed by the matrix and centrifuged at 12,000xg for 2 minutes. The purified PCR product was used for nucleotide sequencing.

### 2.10.4 Nucleotide sequencing

After purification of PCR products from agarose gels by using HiYield Gel/PCR DNA Fragments Extraction Kit, the DNAs were sent to Ward Medic Co., Ltd., Part for determination of nucleotide sequence in both directions. The sequencing primers were the same as primers for PCR amplification.

### 2.10.5 Computer search for sequence similarities

Nucleotide sequence obtained from section 2.10.4 was compared with previously published nucleotide sequences from the EMBL-GenBank-DDBJ database. Multiple sequences were aligned by using the CLUSTAL W program.

## 2.11 Optimization for piperideine-6-carboxylate dehydrogenase production

### 2.11.1 Starter inoculation

Starter inoculum was prepared by inoculation of one single colony of *Pseudomonas putida* (the result from section 2.10) from agar plate to 5 ml of peptone medium, pH 7.2 and cultured for 16 hours with 250 rpm shaking at 37 $^{o}$C.

### 2.11.2 Optimum concentration of Bacto-peptone

The starter inoculum from section 2.11.1 was transferred to 500 ml Erlenmeyer flask containing 200 ml of peptone medium supplemented with 1% (w/v)

L-lysine. The concentrations of peptone were 0, 0.2, 0.4, 0.6, 0.8, 0.9, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0%. The cell cultures were cultivated with 250 rpm shaking at 37 $^{o}$C for 15 hours based on the result from section 2.9. The cell harvest, crude extract preparation, assay for P6CDH activity and protein measurement were performed as described in section 2.9.2, 2.9.3, 2.5 and 2.6 respectively.

### 2.11.3 Enzyme induction

The starter inoculum from section 2.11.1 was transferred to 500 ml Erlenmeyer flask containing 200 ml of peptone medium at suitable concentration of peptone based on the result from section 2.11.2 and 1.0% of various amino acids (L-lysine, L-pipecolic acid and L-ornithine). The control was peptone medium without amino acid. The cell culture was cultivated with 250 rpm shaking at 37 $^{o}$C for 15 hours. The cell harvest, crude extract preparation, assay for P6CDH activity and protein measurement were performed as described in section 2.9.2, 2.9.3, 2.5 and 2.6 respectively.

### 2.11.4 Optimum concentration of inducer

The starter inoculum from section 2.11.1 was transferred to 500 ml Erlenmeyer flask containing 200 ml of peptone medium at suitable concentration of peptone supplemented with the best inducer from section 2.11.3. The concentrations of inducer were 0, 0.2, 0.4, 0.6, 0.8, 0.9, 1.0, 1.2, 1.4, 1.6, and 1.8%. The cell cultures were cultivated with 250 rpm shaking at 37 $^{o}$C for 15 hours. The cell harvest, crude extract preparation, assay for P6CDH activity and protein measurement were performed as described in section 2.9.2, 2.9.3, 2.5 and 2.6 respectively.

### 2.11.5 Optimum pH of medium

The starter inoculum from section 2.11.1 was transferred to 500 ml Erlenmeyer flask containing 200 ml of the best medium obtained from section 2.11.4 at various pHs ranged from 4.0 to 11.0 (4.0, 5.0, 6.0, 7.0, 7.2, 8.0, 9.0, 10.0, and 11.0). The cell cultures were cultivated with 250 rpm shaking at 37 $^{o}$C for 15 hours. The cell

harvest, crude extract preparation, assay for P6CDH activity and protein measurement were performed as described in section 2.9.2, 2.9.3, 2.5 and 2.6 respectively.

### 2.11.6 Optimum cultivation temperature

The starter inoculum from section 2.11.1 was transferred to 500 ml Erlenmeyer flask containing 200 ml of the best medium obtained from section 2.11.5. The cell cultures were cultivated with 250 rpm shaking at various temperatures (28, 30, 37, 40, 45, and 50 $^{o}$C) for 15 hours. The cell harvest, crude extract preparation, assay for P6CDH activity and protein measurement were performed as described in section 2.9.2, 2.9.3, 2.5 and 2.6 respectively.

## 2.12 Piperideine-6-carboxylate dehydrogenase production

### 2.12.1 Starter inoculum

A single colony of *Pseudomonas putida* from agar plate was inoculated in 150 ml of peptone medium, pH 7.2 at 37 $^{o}$C with 250 rpm rotary shaking for 16 hours.

### 2.12.2 Bacterial cultivation

The starter inoculums from section 2.12.1 was transferred into 6 liter of peptone medium, pH 8.0 containing 0.8% peptone, 0.6% L-lysine and cultivated at 37 $^{o}$C with 250 rpm shaking for 15 hours (the result from section 2.11.6). Cells were harvested by centrifugation at 6,000xg for 10 minutes at 4 $^{o}$C. The collected cells were washed with 0.85% NaCl before rewashing with extraction buffer. Harvested cells were stored at -20 $^{o}$C.

### 2.12.3 Preparation of crude extract

The collected cells from section 2.12.2 were resuspened in cold extraction buffer and then disrupted by discontinuous sonication on ice. Cell debris was

removed by centrifugation at 10,000xg for 30 minutes at 4 $^{o}$C. Crude extract was assayed for the P6CDH activity and protein concentration as described in section 2.5 and 2.6 respectively.

### 2.12.4 Purification procedures of piperideine-6-carboxylate dehydrogenase

The crude extract from section 2.12.3 was purified by the following steps. All operations were done at 4 $^{o}$C. The buffer used in all steps was 10 mM Tris-HCl buffer, pH 8.0 containing 0.01% $\beta$-mercaptoethanol, 1 mM EDTA, and 10% glycerol.

#### 2.12.4.1 Ammonium sulfate precipitation

The precipitation of crude extract was done by slowly adding fine solid ammonium sulfate to 50% saturation with gentle stirring by magnetic stirrer. After that, the supernatant was collected by centrifugation at 10,000xg for 30 minutes and then adjusted to final concentration of 60% saturation with solid ammonium sulfate. The solution was left for 1 hour on ice with continuous stirring and centrifuged again. The precipitate was dissolved in the buffer. The protein solution was dialyzed against 100 volumes of the buffer at least 4 hours for 3 times before determination of the enzyme activity and protein concentration as described in 2.5 and 2.6 respectively.

#### 2.12.4.2 DEAE-Toyopearl column chromatography

The activated DEAE-Toyopearl was prepared by washing with 0.5 N NaOH for 2-3 times, and rewashed by deionized water until the pH reached 8.0. The activated DEAE-Toyopearl was resuspened in the buffer, packed into 3.7 x 20.0 cm column and equilibrated with the same buffer for 5-10 column volume at flow rate 1 ml/min.

The enzyme solution from section 2.12.4.1 was applied into the DEAE-Toyopearl 650M column. The unbound proteins were eluted from the column with the

buffer until the absorbance at 280 nm was nearly zero. After that, the buffer was changed by making stepwise of 0.1, 0.2, and 0.3 M NaCl in the same buffer in order to elute the bound proteins from the column. The 3 ml fractions were collected using a fraction collector. The protein profile was determined by measuring the absorbance at 280 nm. P6CDH activity was assayed using the method described in section 2.5. The fractions containing P6CDH activity were pooled and dialyzed against the buffer. After desalting, the enzyme solution was concentrated with aquasorb. The P6CDH activity and protein concentration of pooled fraction were measured as described in section 2.5 and 2.6 respectively.

#### 2.12.4.3 Butyl-Toyopearl column chromatography

Butyl-Toyopearl was washed with deionized water for 2-3 times, and then resuspended in the buffer containing 25% saturated ammonium sulfate and packed into 2.3 x 18.5 cm column followed by equilibrating with the same buffer for 5-10 column volume at flow rate 1 ml/min.

The pooled active fraction from section 2.12.4.2 was slowly adjusted to 25% saturation with fine ammonium sulfate and stirred gently at least 30 minutes. The protein solution was applied into the column at flow rate 1 ml/min. The unbound proteins were eluted from the column with the buffer containing 25% saturated ammonium sulfate until the absorbance to 280 nm was nearly zero. The bound proteins were eluted from the column with stepwise of 20, 15, 10, and 5 % saturated ammonium sulfate in the buffer. Three milliliter fractions were collected by using fraction collector and assayed for both protein concentration and P6CDH activity. Protein profile was determined by measuring an absorbance at 280 nm. P6CDH activity was assayed by using the method as described in section 2.5. The fractions containing P6CDH activity were pooled and dialyzed against the buffer. After desalting, the enzyme was concentrated with aquasorb. The P6CDH activity and protein concentration were determined as described in section 2.5 and 2.6 respectively.

### 2.12.4.4 Hitrap Q column chromatography

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

After column equilibration, the concentrated enzyme was applied into the column. The unbound proteins were eluted from the column with the buffer until the absorbance at 280 nm of eluent reached baseline level. After that, the bound proteins were eluted from the column with the 20 column volume of linear salt gradient, 0-0.5 M NaCl in the buffer. The 2 ml fractions were collected. The protein profile was monitored by measuring the absorbance at 280 nm. The P6CDH activity was assayed as described in section 2.5. The fractions containing P6CDH activity were pooled, concentrated using aquasorb and dialysed against the buffer. The enzyme activity and protein concentration were determined as described in section 2.5 and 2.6 respectively.

The protein from each active fraction was electrophoresed on non-denaturing and denaturing polyacrylamide gel as described in section 2.7.1 and 2.7.2, respectively. After that, the gels were stained with Coomassie blue as described in section 2.7.3.

For activity staining, after electrophoresis at 4 $^{o}$C, the gel was moved to a small box which consisted of 10 ml solution containing 4.25 mmol of Tris-HCl buffer, pH 8.5, 50 μmol of $NAD^{+}$, 0.25 mg of phenazine methosulfate, 2.5 mg of nitroblue tetrazolium and 10 ml of P6C solution from section 2.9.4. The gel was gently shaked at room temperature for 20 min. After the brown band had appeared, the staining reaction was stopped by pouring off the staining solution. The gel was then quickly rinsed several times with deionized water.

## 2.13 Characterization of piperideine-6-carboxylate dehydrogenase

### 2.13.1 Molecular weight determination of piperideine-6-carboxylate dehydrogenase

The molecular weight of purified P6CDH was determined by gel filtration on TSK Gel G3000 SW column (0.75 x 60 cm ID) with 0.1 M potassium phosphate buffer, pH 7.0 containing 0.15 M NaCl at flow rate 1 ml/min. The molecular weight marker protein consisted of alcohol dehydrogenase (669,000 Da), bovine serum albumin (150,000 Da) and thyroglobulin (66,000 Da) was used.

### 2.13.2 Substrate specificity of piperideine-6-carboxylate dehydrogenase

The purified P6CDH was used to study substrate specificity. L-lysine was replaced by various amino acids as substrate for the P6CDH activity assay at a final substrate concentration of 20 mM as described in section 2.5. The results were expressed as a percentage of the relative activity.

Since L-pipecolic acid could act as the substrate of P6CDH, it was used in the assay reaction for further experiments. The 1 ml of reaction mixture contained 20 μmol of L-pipecolic acid, 200 μmol of MOPS-HCl buffer, pH 8.7, 1 μmol of $NAD^+$, and P6CDH. The reaction mixture without $NAD^+$ was incubated at 37 $^oC$ for 5 minutes in a cuvette of 1-cm light path. The reaction was started by addition of $NAD^+$ and monitored by measuring the initial change in absorbance of NADH at 340 nm.

### 2.13.3 Coenzyme specificity of piperideine-6-carboxylate dehydrogenase

The purified P6CDH was used to study coenzyme specificity as described in section 2.5. $NAD^+$ was replaced by various $NAD^+$ analogs at final concentration of 1 mM. Assays with $NAD^+$ analogs were conducted by measuring the increase in absorbance at the following wavelengths: β-nicotinamide adenine

dinucleotide phosphate (NADP), 340 nm ($\varepsilon = 6.2x10^3\ M^{-1}cm^{-1}$); nicotinic acid adenine dinucleotide, 338 nm ($\varepsilon = 6.2x10^3\ M^{-1}cm^{-1}$); 3-pyridinealdehyde adenine dinucleotide, 358 nm ($\varepsilon = 9.3x10^3\ M^{-1}cm^{-1}$); nicotinamide hypoxanthine dinucleotide, 338 nm ($\varepsilon = 6.2x10^3\ M^{-1}cm^{-1}$) and thionicotinamide adenine dinucleotide, 395 nm ($\varepsilon = 11.3x10^3\ M^{-1}cm^{-1}$) (Misono *et al*., 1989). The result was expressed as a percentage of the relative activity.

### 2.13.4 Effect of pH on piperideine-6-carboxylate dehydrogenase activity

The effect of pH on the activity was determined under the coupling reaction and P6CDH assay conditions as described in section 2.5 and 2.13.2, respectively at various pHs. The 200 mM of acetate buffer for pH 4.0 to 6.0, potassium phosphate for pH 6.0 to 8.5, MOP-HCl buffer for pH 7.0 to 9.0, Tris-HCl buffer for pH 7.0 to 9.0 and glycine-KCl-KOH buffer for pH 8.5 to 12.5 were used. The pH of each reaction mixture was measured with a pH meter at room temperature after the reaction. The percentage of relative activity was plotted against the final pH.

### 2.13.5 Effect of temperature on piperideine-6-carboxylate dehydrogenase activity

The effect of temperature on the activity was examined under the coupling reaction and P6CDH assay conditions as described in section 2.5 and 2.13.2, respectively at various temperatures from 25 °C to 75 °C. The percentage of relative activity was plotted against the temperature used for the activity assay.

### 2.13.6 Effect of glycerol on piperideine-6-carboxylate dehydrogenase stability

The purified P6CDH was used to study effect of glycerol on P6CDH stability at 4 $^oC$. P6CDH assay conditions as described in section 2.13.2. The concentrations of glycerol were 0, 5, 10, and 20%. P6CDH activity was assayed every week. The result was shown as a percentage of the relative activity.

### 2.13.7 Effect of pH on piperideine-6-carboxylate dehydrogenase stability

The purified P6CDH was used to study pH stability. After the enzyme had been incubated at 30 °C for 20 minutes in each of the 10 mM buffer at various pHs, an aliquot of the enzyme solution was withdrawn and the remaining activity of enzyme was measured under the coupling reaction and P6CDH assay conditions as described in section 2.5 and 2.13.2, respectively. The 10 mM buffers used were acetate buffer for pH 4.0 to 6.0, potassium phosphate for pH 6.0 to 8.5, Tris-HCl buffer for pH 7.0 to 9.0 and glycine-KCl-KOH buffer for pH 8.5 to 12.5. The percentage of P6CDH relative activity was plotted against the incubated pH.

### 2.13.8 Effect of temperature on piperideine-6-carboxylate dehydrogenase stability

The effect of temperature on the stability of the enzyme was determined from 30 °C to 75 °C. The purified P6CDH was incubated at various temperatures for 10 minutes before determination of enzyme activity under the coupling reaction and P6CDH assay conditions as described in section 2.5 and 2.13.2, respectively. Afterward, the enzyme was incubated at the highest temperature which the enzyme activity still remained full activity and daily collected for enzyme activity assay as described previously for 10 days. The result was shown as a percentage of the relative activity.

### 2.13.9 Effect of metal ions on piperideine-6-carboxylate dehydrogenase activity

The effect of metal ions on the activity was examined under the coupling reaction and P6CDH assay conditions as described in section 2.5 and 2.13.2, respectively in the presence of various metal ions and chemical substances at the final concentration of 10 mM, unless otherwise stated. The result was expressed as a percentage of the relative activity of the enzyme activity in the absence of reagents.

### 2.13.10 Inhibitory effect of various amino acids on piperideine-6-carboxylate dehydrogenase activity

Inhibitory effect of various amino acids which the enzyme did not have the ability to catalyze as its substrates were determination at the final concentration of 20 mM. The activity was examined under the coupling reaction and P6CDH assay conditions as described in section 2.5 and 2.13.2, respectively in the presence of various amino acids. The result was expressed as a percentage of the relative activity of the enzyme activity in the absence of reagents.

## 2.14 Kinetic studies of piperideine-6-carboxylate dehydrogenase

A series of steady-state kinetic analysis was carried out under the assay reaction condition as described in section 2.13.2, except that various amounts of L-pipecolic acid and $NAD^+$ were used. The concentrations of L-pipecolic acid used were 0.2, 0.25, 0.3, 0.5 and 1 mM, and those of $NAD^+$ used were 0.2, 0.3, 0.4, 0.6 and 0.8 mM. The Lineweaver-Burke plots (double-reciprocal plots) of initial velocities against L-pipecolic acid concentrations at a series of fixed concentrations of $NAD^+$ and the secondary plots of y intercepts against reciprocal concentrations of $NAD^+$ were made from the data. $K_m$ of L-pipecolic acid and $NAD^+$ were determined from these two plots, respectively.

## 2.15 L-2-Aminoadipic acid production

The 1.0 ml of reaction mixture contained 500 µl of the solution from section 2.9.4 using 150 units of LysDH, 200 µmol of MOP-HCl buffer, pH 8.7, 10 µmol of $NAD^+$, and the purified P6CDH (2,000 units) from section 2.12.4. The reaction was incubated at 30 $^oC$ for 24 hours. After that, the reaction mixture was filtrated using Suprec$^{Tm-01}$ (molecular weight cut off 30,000). The 0.2 µl of the mixture was subjected to 10 cm x 20 cm cellulose TLC plastic sheets in parallel with standard L-lysine and L-2-aminoadipic acid. The TLC plates were developed with *n*-butanol:acetic acid: $H_2O$ (4:1:1). After that the plates were dried in hot air, developed in 0.5% ninhydrin in acetone and then dried at 100 $^oC$ for 5 minutes. The ratio of the distance a compound

moved from the baseline to the distance of the solvent front moved from the baseline was defined as the retardation factor ($R_f$). Spot intensity of reaction product was determined by Image Master 2D platinum V.6.0 compared with standard L-2-aminoadipic acid.

# CHAPTER III

## RESULTS

## 3.1 Purification of L-lysine-6-dehydrogenase

### 3.1.1 Preparation of crude extract

Crude recombinant LysDH was prepared from 6.83 g of *E. coli* BL21 (DE3) harbouring *lysdh* gene from *Achromobacter denitrificans* which was cultivated in 1,000 ml of medium as described in section 2.8.4. Crude extract contained 780 mg proteins and 1,650 units of LysDH activity. Thus, the specific activity of the enzyme in the crude preparation was 2.12 units/mg protein.

### 3.1.2 DEAE-Toyopearl column chromatography

The recombinant crude enzyme was applied to DEAE-Toyopearl column as described in section 2.8.5.1. The chromatographic profile was shown in Figure 3.1. The unbound proteins were eluted from column by the phosphate buffer, whereas the bound proteins were eluted by linear salt gradient of 0 to 0.5 M potassium chloride in the buffer. LysDH was eluted at about 0.15 M potassium chloride as indicated in the profile. LysDH fractions were pooled, dialysed against the buffer, concentrated by aquasorb to reduce enzyme volume. The enzyme with 360 mg protein and 1,520 LysDH units was obtained by this operation. The specific activity of the enzyme from this step was 4.22 units/mg protein. The enzyme was purified 1.99 fold with 92.1% recovery.

### 3.1.3 DEAE-Sephadex A-50 column chromatography

The pooled enzyme from DEAE-Toyopearl was applied to DEAE-Sephadex A-50 column as described in section 2.8.5.2. The chromatographic profile was shown in Figure 3.2. The unbound proteins were eluted from column by the phosphate buffer, whereas the bound proteins were eluted by linear salt gradient of 0 to 0.5 M potassium chloride in the buffer. LysDH was eluted at about 0.2 M potassium chloride as

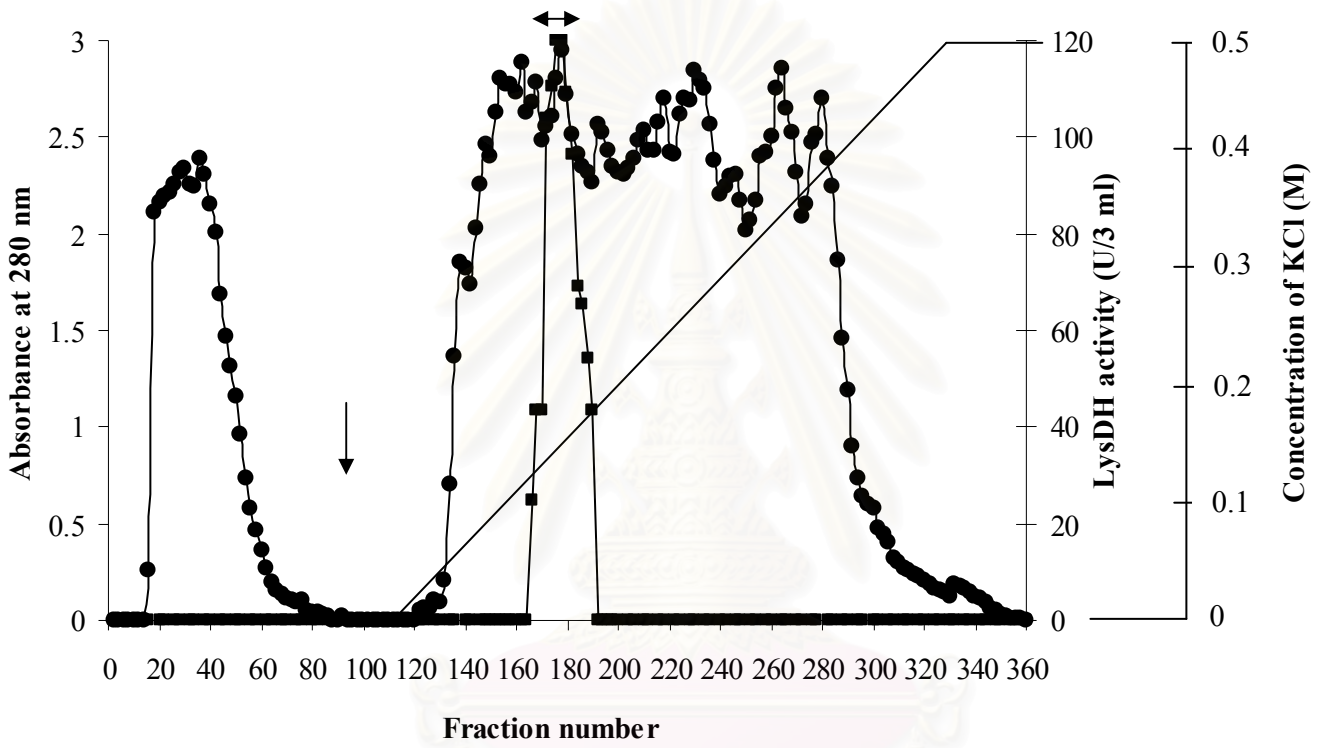



**Figure 3.1 Purification of L-lysine-6-dehydrogenase from recombinant clone by DEAE - Toyopearl column**

The enzyme solution was applied to DEAE -Toyopearl column and washed with 10 mM potassium phosphate buffer, pH 7.4 containing 0.01% (v/v) β-mercaptoethanol and 1 mM EDTA until $A_{280}$ decreased to base line. The bound proteins were eluted by 0-0.5 M KCl in the same buffer at the flow rate of 1 ml/min. The fractions of 3 ml were collected. The arrow indicates where gradient started. The protein peak from fraction number 170 to 184 was pooled ( ⟷ ). ● $A_{280}$, ■ LysDH activity, — [KCl]

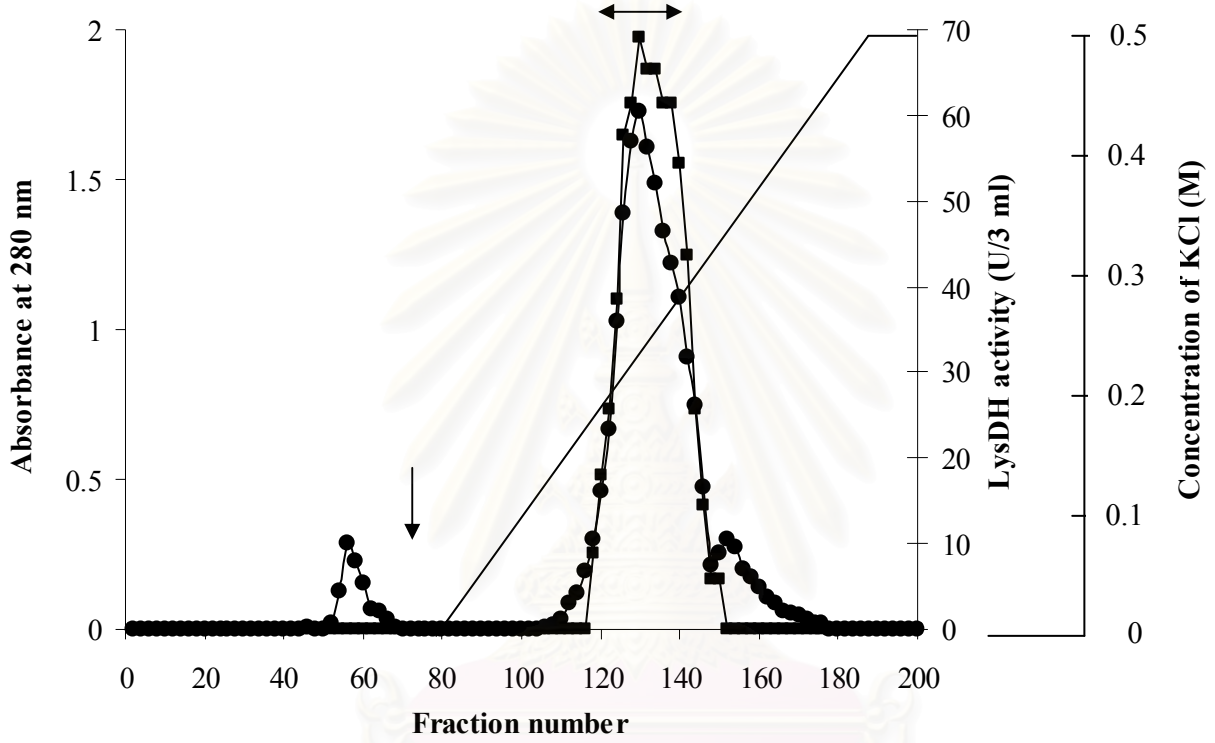



**Figure 3.2 Purification of L-lysine-6-dehydrogenase from recombinant clone by DEAE–Sephadex A-50 column**

The enzyme solution was applied to DEAE -Toyopearl column and washed with 10 mM potassium phosphate buffer, pH 7.4 containing 0.01% (v/v) β-mercaptoethanol and 1 mM EDTA until $A_{280}$ decreased to base line. The bound proteins were eluted by 0-0.5 M KCl in the same buffer at the flow rate of 1 ml/min. The fractions of 3 ml were collected. The arrow indicates where gradient started. The protein peak from fraction number 118 to 144 was pooled (⟷). ● $A_{280}$, ■ LysDH activity, — [KCl]

indicated in the profile. LysDH fractions were pooled, dialysed against the buffer, concentrated by aquasorb to reduce enzyme volume. The enzyme solution from this step contained 270 mg protein and 1,280 units of LysDH activity. The specificity activity of the enzyme from this step was 4.74 units/mg protein. The enzyme was purified 2.24 fold with 77.6% recovery.

The summary of purification of LysDH was shown in Table 3.1.

### 3.1.4 Determination of enzyme purity on non-denaturing polyacrylamide gel electrophoresis (native-PAGE) and SDS-polyacrylamide gel electrophoresis

The enzyme purity from each purification steps and native protein pattern were investigated by non-denaturing polyacrylamide gel electrophoresis as described in section 2.7.1. The results of protein staining and activity staining were shown in Figure 3.3. The single band from DEAE-Sephadex A-50 column in lane 3A and 3B indicated that the enzyme was highly purified, may be homogeneous.

Then, the molecular weight of the enzyme subunit and the enzyme purity were confirmed by denaturing polyacrylamide gel electrophoresis as described in section 2.7.2. The single band from DEAE-Sephadex A-50 column indicated that the enzyme was purified. The molecular weight of the enzyme subunit was calculated to be 40.0 kDa by its mobility in SDS-PAGE compared with those of standard proteins as shown in Figure 3.4. The molecular weight calibration curve as shown in Figure 3.5.

## 3.2 Selection of piperideine-6-carboxylate dehydrogenase producing strain

Production of L-2-aminoadipic acid by each isolate strain was confirmed as described in section 2.9. As shown in Figure 3.6, the result indicated that all of the five bacterial isolates could produce L-2-aminoadipic acid. The relative mobility ($R_f$) value of

**Table 3.1 Purification of L-lysine-6-dehydrogenase**

| Purification steps | Total activity (unit) | Total protein (mg) | Specific activity (units/mg protein) | % Recovery | Purification fold |
|---|---|---|---|---|---|
| **Crude enzyme** | 1,650 | 780 | 2.12 | 100.0 | 1.00 |
| **DEAE-Toyopearl** | 1,520 | 360 | 4.22 | 92.1 | 1.99 |
| **DEAE-Sephadex A-50** | 1,280 | 270 | 4.74 | 77.6 | 2.24 |

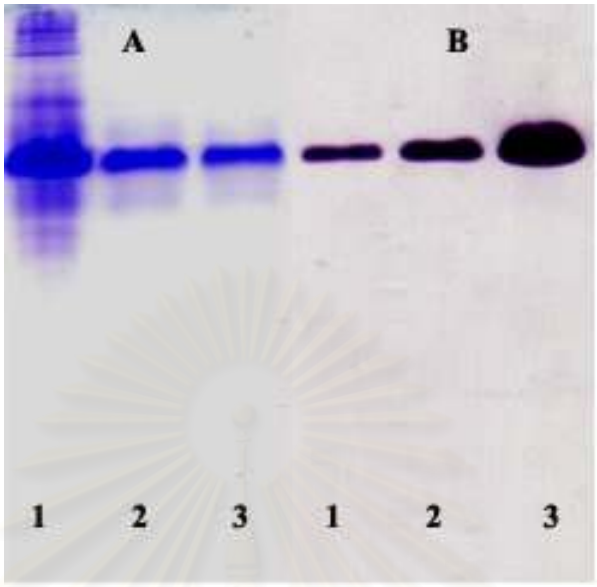



**Figure 3.3 Protein pattern from each step of purification investigated by native-PAGE**

**[A] Protein staining** **[B] Activity staining**

| | | | |
|---|---|---|---|
| Lane 1 | = | crude extract | 100 µg |
| Lane 2 | = | DEAE-Toyopearl column | 20 µg |
| Lane 3 | = | DEAE-Sephadex A-50 column | 15 µg |

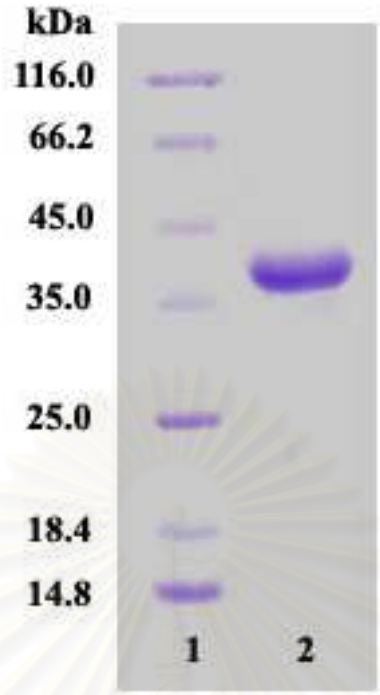



**Figure 3.4 The purified L-lysine-6-dehydrogenase at last step examined by SDS-PAGE**

Lane 1 = protein marker

| | |
|---|---|
| β-galactosidase | (MW 116,000) |
| bovine serum albumin | (MW 66,000) |
| ovalbumin | (MW 45,000) |
| lactate dehydrogenase | (MW 35,000) |
| restriction endonuclease *Bsp* 98I | (MW 25,000) |
| β-lactoglobulin | (MW 18,400) |
| lysozyme | (MW 14,800) |

Lane 2 = after DEAE-Sephadex A-50 column

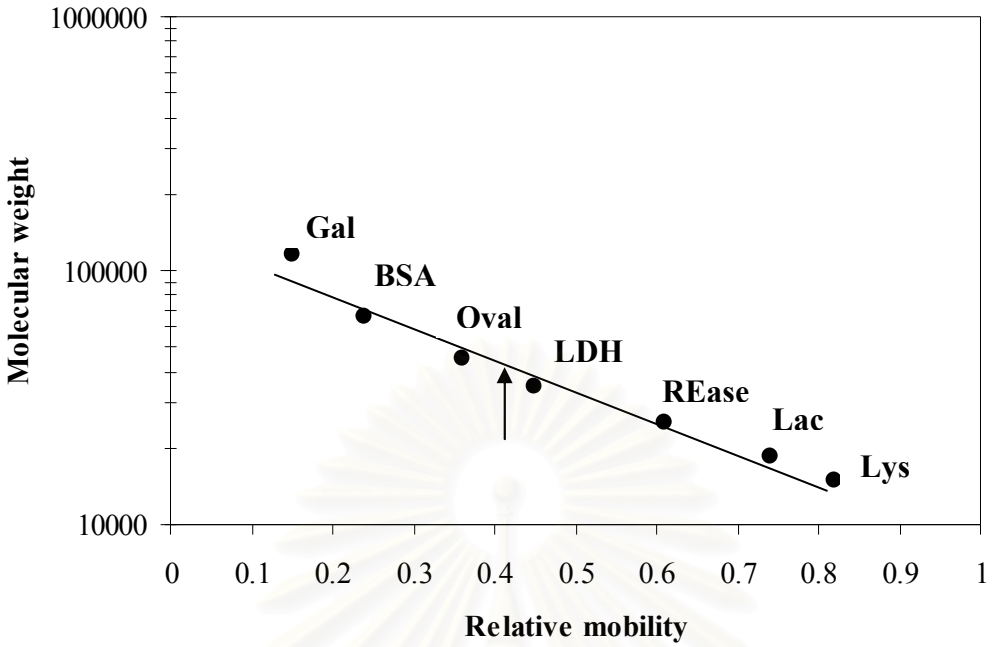



**Figure 3.5 Calibration curve for molecular weight of L-lysine-6-dehydrogenase subunit on SDS-polyacrylamide gel electrophoresis**

| | | | |
|---|---|---|---|
| Gal | = | β-galactosidase | (MW 116,000) |
| BSA | = | bovine serum albumin | (MW 66,000) |
| Oval | = | ovalbumin | (MW 45,000) |
| LDH | = | lactate dehydrogenase | (MW 35,000) |
| REase | = | restriction endonuclease *Bsp* 98I | (MW 25,000) |
| Lac | = | β-lactoglobulin | (MW 18,400) |
| Lys | = | lysozyme | (MW 14,800) |

Arrow indicates the $K_{av}$ of LysDH.

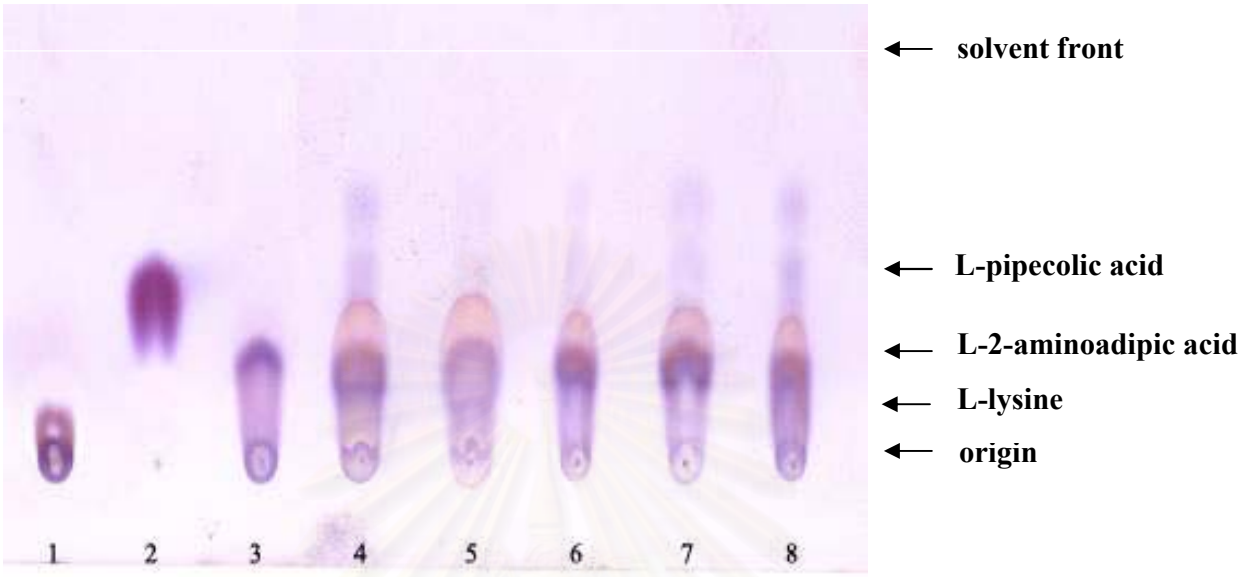



**Figure 3.6 TLC analysis of L-2-aminoadipic acid production of the five bacterial isolates**

Lane 1 = standard L-lysine
Lane 2 = standard L-pipecolic acid
Lane 3 = standard L-2-aminoadipic acid
Lane 4-8 = isolates No. 1-5

L-lysine, L-pipecolic acid and L-2-aminoadipic acid were 0.15, 0.40 and 0.28, respectively.

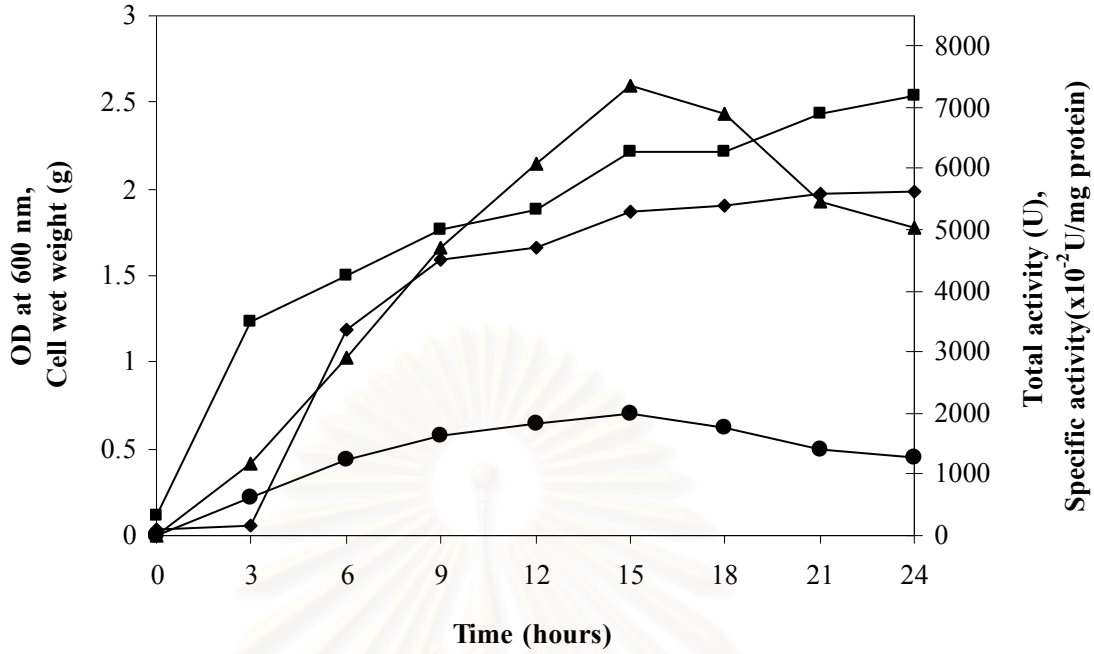
The highest P6CDH producing strain was determined as described in section 2.9 as shown in Figure 3.7 and 3.8. The results indicated that the isolates No. 1, 3, 4, and 5 had the similar pattern of total activity and specific activity. The total activity and specific activity of isolates No. 1, 3, 4, and 5 increased rapidly from 0-15 hours. After 15 hours, the total activity and specific activity were dramatically decreased. While, the total activity and specific activity of the isolate No. 2 increased rapidly from 0-12 hours. After 12 hours, the total activity and specific activity were dramatically decreased. The isolate No. 3 gave the highest total activity and specific activity with 7,920 units and 20.9 units/mg protein, respectively, which obtained by cultivation for 15 hours. Thus, the isolate No. 3 was selected and the cultivation time at 15 hours was used for further experiments.

## 3.3 Identification of $NAD^+$ dependent piperideine-6-carboxylate dehydrogenase producing bacterium

The bacterial isolate No. 3 was sent to Department of Medical Sciences, Ministry of Health, Thailand in order to characterize its physiological and biochemical properties. The important properties of isolate No.3 were shown in Table 3.2. From these properties, the strain was identified as *Pseudomonas putida*.

Moreover, identification of this bacterium was confirmed by 16S rRNA gene sequence. The 16S rRNA gene amplification was determined as described in section 2.10. A single band of PCR amplified product of 1.3 kb was obtained as shown in Figure 3.9. DNA sequence analysis showed that the amplified fragment was 1,372 bp. The sequence was compared with the DNA sequences deposited in the EMBL-GenBank-DDBL database. The highest similarity was found with 16S rRNA sequence of *Pseudomonas putida* (BCNU106) with 98% nucleotide sequence comparison between 16S rRNA of isolate No. 3 and that of *P. putida* was shown in Figure 3.10.

**[A]**




**[B]**

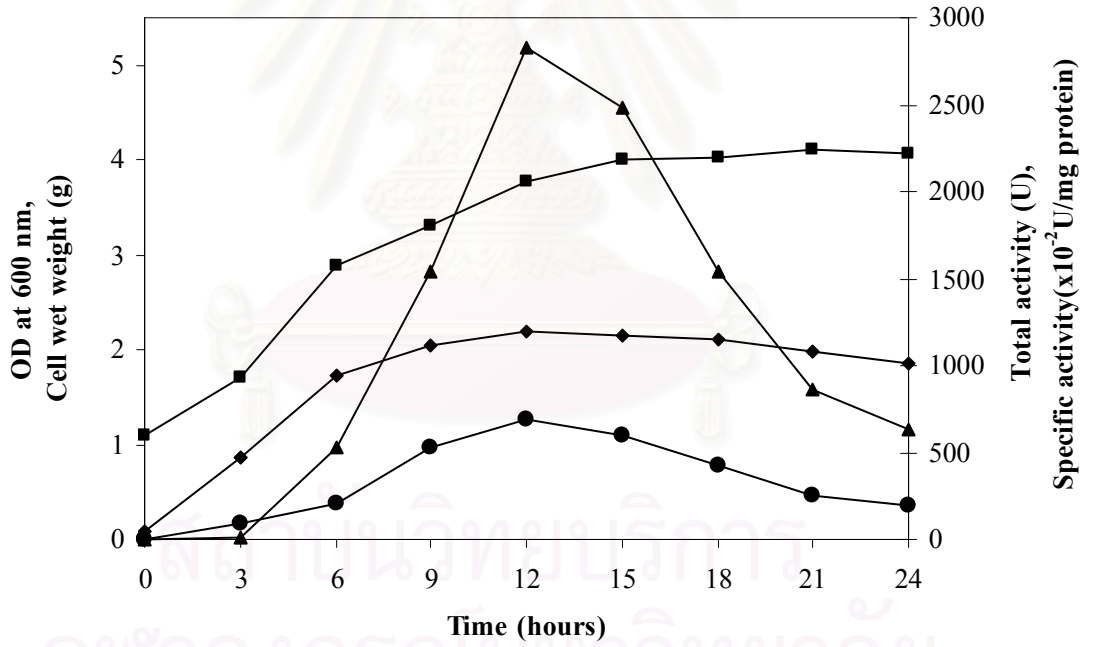



**Figure 3.7 Growth and piperideine-6-carboxylate dehydrogenase production of the 5 isolate strains**

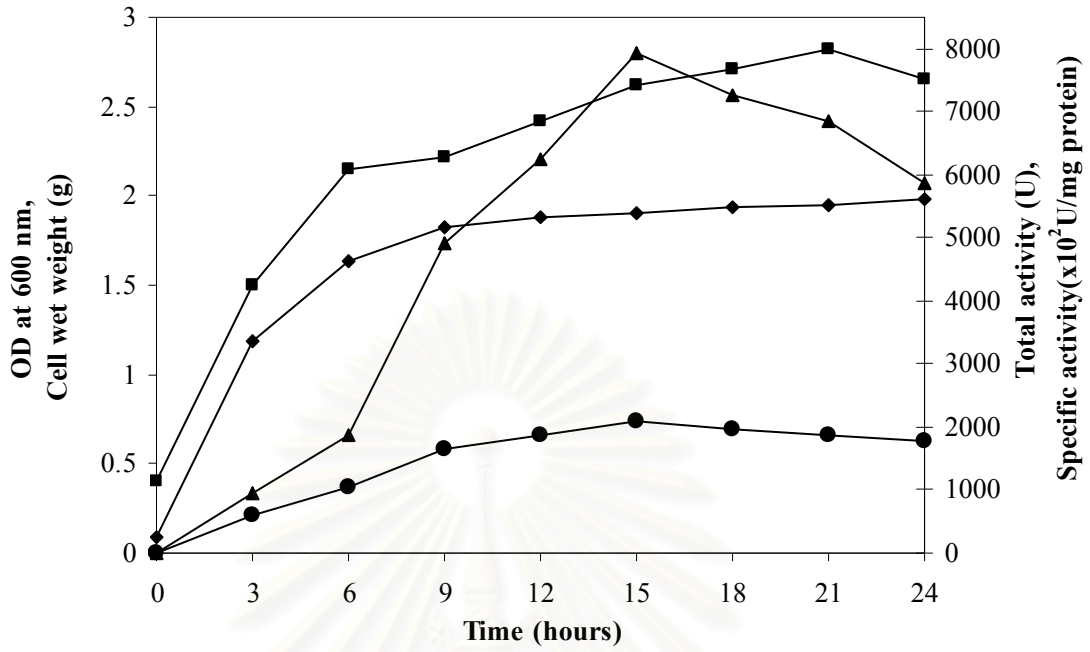
◆ $OD_{600}$ ■ cell wet weight

▲ total activity ● specific activity

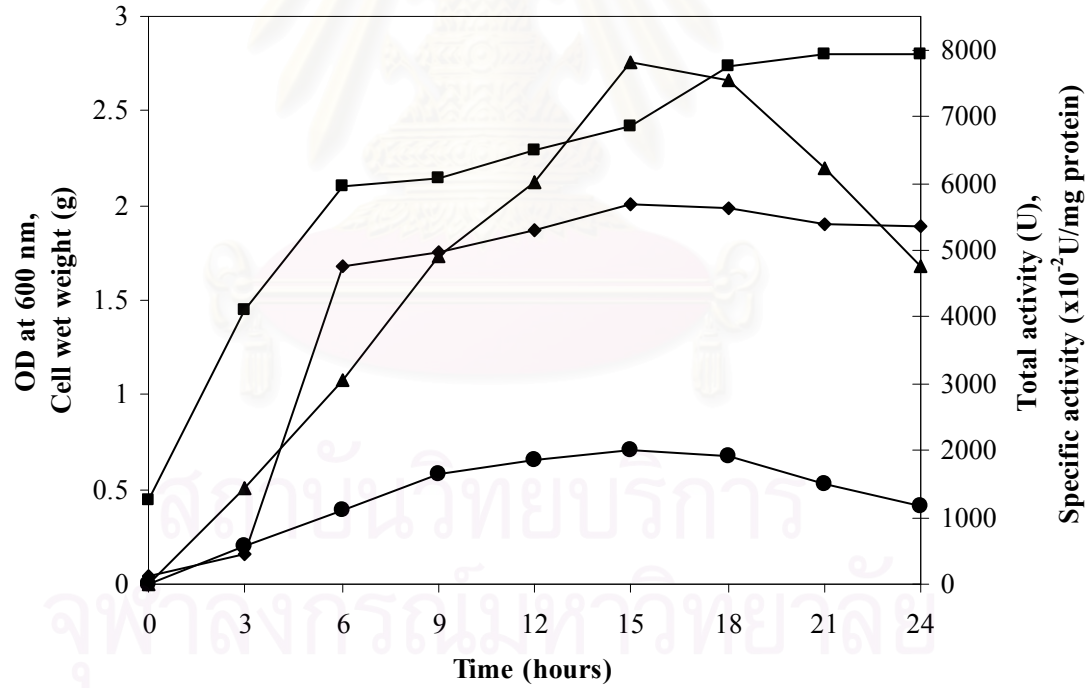
[A] isolate No. 1

[B] isolate No. 2

**[C]**




**[D]**




**(continued)**

**Figure 3.7 Growth and piperideine-6-carboxylate dehydrogenase production of the 5 isolate strains**

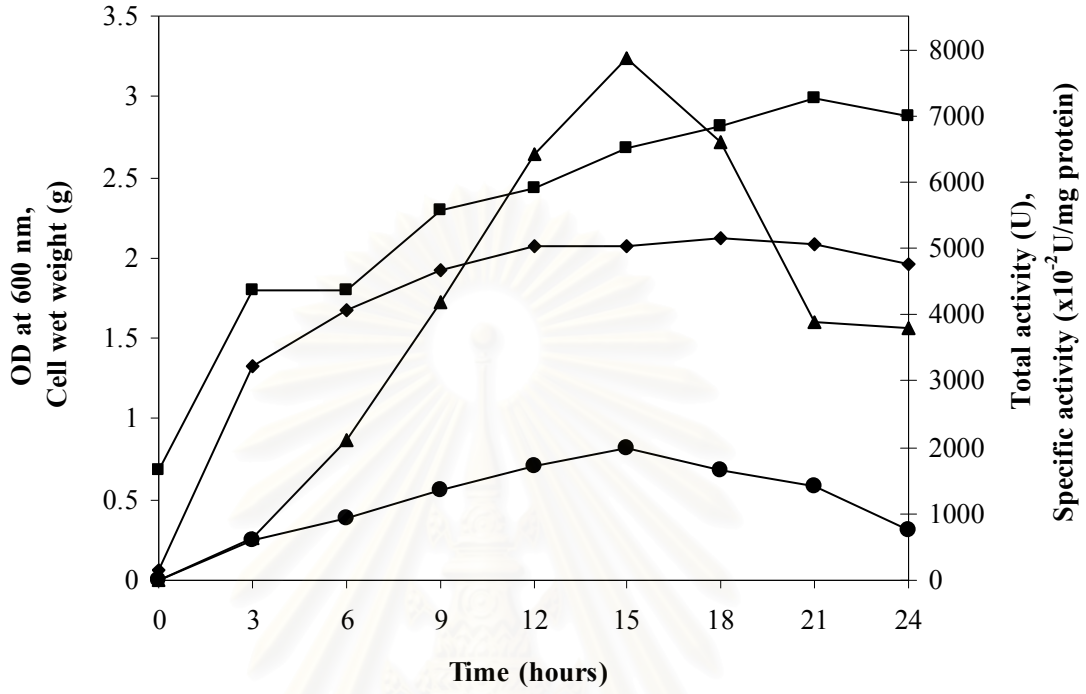
◆ $OD_{600}$ ■ cell wet weight

▲ total activity ● specific activity

[C] isolate No. 3

[D] isolate No. 4

**[E]**




**(continued)**

**Figure 3.7 Growth and piperideine-6-carboxylate dehydrogenase production of the 5 isolate strains**

◆ $OD_{600}$ ■ cell wet weight

▲ total activity ● specific activity

[E] isolate No. 5

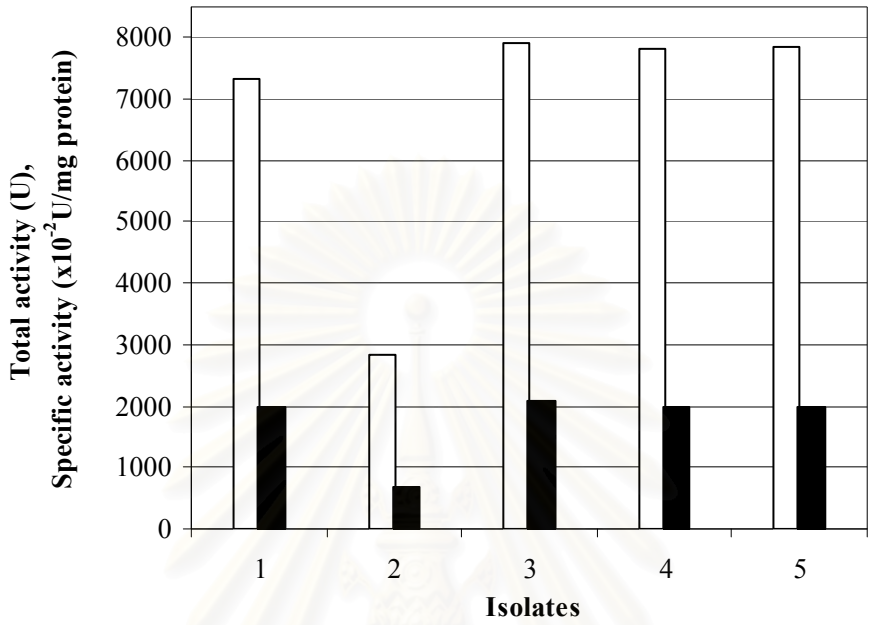



**Figure 3.8 Maximum piperideine-6-carboxylate dehydrogenase total activity and specific activity of the 5 isolate strains**

**Table 3.2 Characteristics of isolate No.3**

| Characteristics | Results |
|---|---|
| Oxidase | + |
| TSI/$H_2S$ | K/K |
| SIM ($H_2S$/indole/motile) | -/-/+ |
| Simmon's citrate | + |
| Urease | + |
| Nitrate/$N_2$ gas | +/- |
| Esulin | - |
| Acetate | + |
| Gelatinase | - |
| Glucose | + |
| Maltose | - |
| Lactose | - |
| Mannitol | - |
| D-Xylose | + |
| Sucrose | - |
| Adonitol | - |
| Fructose | + |
| Lysine decarboxylase | - |
| Arginine dihydrolase | + |
| Ornithine decarboxylase | - |
| Growth at 42 ºC | + |

Remark: (+) = positive reaction

(-) = negative reaction

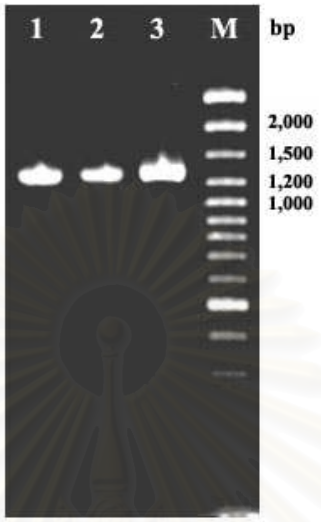



**Figure 3.9 16S rRNA gene amplified product of isolate No. 3**

Lane 1-3 = PCR products of 16S rRNA gene

Lane M = 100 bp marker

CLUSTAL W (1.83) multiple sequence alignment

```
Isolate No. 3 --------------------ATTGAACGCTGGCGGCAGGCCCGAGACTTGCAAATCGTCG 40
P. putida     AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCCGACACTTGCAAATCGTCG 60
                                  ************************************

Isolate No. 3 CCTTGGTGGGAGCTTGCTTTTTGATTCAGCGGGCTACGGGGGAGTAATGCCTAGGAATCT 100
P. putida     GGTTGGCGGGAGCTTGCTTTTTGATTCAGCGGGCTACGGGTGAGTAATGCCTAGGAATCT 120
              ****** ******************************** ******************

Isolate No. 3 GGCTGGGAGTGGGGGACAACGTTTCGAAAGGCACGCTAGTACCGCATACGTCCTACGGGA 160
P. putida     GGCTGGGAGTGGGGGACAACGTTTCGAAAGGAACGCTAGTACCGCATACGTCCTACGGGA 180
              ****************************** **************************

Isolate No. 3 GAAAGCAGGGGACCTTCGGGCCTTGGGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTT 220
P. putida     GAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTT 240
              ************************ **********************************

Isolate No. 3 GGTGAGGGAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCA 280
P. putida     GGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCA 300
              ******* **************************************************

Isolate No. 3 CACTGGAACTGAGACACGGTCCACACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA 340
P. putida     CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA 360
              ********************** **********************************

Isolate No. 3 ATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAG 400
P. putida     ATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAG 420
              ************************************************************

Isolate No. 3 CACTTTAAGTTGGGAGGAAGGGCAGTAAGCTAATACCTTGCTGTTTTGACGTTACCGACA 460
P. putida     CACTTTAAGTTGGGAGGAAGGGCAGTAAGCTAATACCTTGCTGTTTTGACGTTACCGACA 480
              ************************************************************

Isolate No. 3 GAATAAGCACCGGCTAACTCTGTGCCATTCAGCCGCGGTAATACAGAGGGTGCAAGCGTTA 520
P. putida     GAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTA 540
              ************************** ******************************
```

**Figure 3.10 Nucleotide sequence alignment of 16S rRNA gene of isolate No. 3 compared with *Pseudomonas putida***

Black colour indicated the different or missing nucleotides.

```
Isolate No. 3 ATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAAGCCC 580
P. putida     ATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAAGCCC 600
              ************************************************************

Isolate No. 3 CGGGCTCAACCTGGGAACTGAATCCAAAACTGGCGAGCTAGAGTACGGTAGAGGGTGGTG 640
P. putida     CGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTACGGTAGAGGGTGGTG 660
              ******************* ************************************

Isolate No. 3 GAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCG 700
P. putida     GAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCG 720
              ************************************************************

Isolate No. 3 ACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT 760
P. putida     ACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT 780
              ************************************************************

Isolate No. 3 ACCCTGGTAGTCCACGCCGTAAACGATGACAACTAGCCGTTGGAATCCTTGAGATTTTAG 820
P. putida     ACCCTGGTAGTCCACGCCGTAAACGATACCAACTAGCCGTTGGAATCCTTGAGATTTTAG 840
              **************************  ******************************

Isolate No. 3 TGGCGCAGCTTACGCATTAAGTTGACCGCCTGGGGAGTCCGGCCGCAAGGTTAAAACTCA 880
P. putida     TGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA 900
              ********** ************************** ********************

Isolate No. 3 AATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG 940
P. putida     AATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG 960
              ************************************************************

Isolate No. 3 AAGAACCTTACCAGGCCTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGG 1000
P. putida     AAGAACCTTACCAGGCCTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGG 1020
              ************************************************************

Isolate No. 3 GAACTCTGACACAGGTGCTGCAGGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA 1060
P. putida     GAACTCTGACACAGGTGCTGCAGGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA 1080
              ************************************************************

Isolate No. 3 GTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAA 1120
P. putida     GTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAA 1140
              ************************************************************
```

**(continued)**

**Figure 3.10 Nucleotide sequence alignment of 16S rRNA gene of isolate No. 3 compared with *Pseudomonas putida***

Black colour indicated the different or missing nucleotides.

```
Isolate No. 3 GGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTT 1180
P. putida     GGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTT 1200
              ************************************************************

Isolate No. 3 ACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGT 1240
P. putida     ACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGT 1260
              ************************************************************

Isolate No. 3 GGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGA 1300
P. putida     GGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGA 1320
              ************************************************************

Isolate No. 3 AGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTG 1360
P. putida     AGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTG 1380
              ************************************************************

Isolate No. 3 TACACACCGCCC----------------------------                    1372
P. putida     TACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAA                    1422
              ************
```

**(continued)**

**Figure 3.10 Nucleotide sequence alignment of 16S rRNA gene of isolate No. 3 compared with *Pseudomonas putida***

Black colour indicated the different or missing nucleotides.

## 3.4 Optimization for piperideine-6-carboxylate dehydrogenase production

### 3.4.1 Optimum concentration of Bacto-peptone

The effect of the concentration of Bacto-peptone on P6CDH production of *P. putida* was performed as described in section 2.11.2. As shown in Figure 3.11, cell wet weight rapidly increased with increasing of Bacto-peptone concentration. Total activity and specific activity increased rapidly corresponding with increasing concentration of Bacto-peptone until concentration of Bacto-peptone reached 0.8%. At concentration of Bacto-peptone above 1.0% total activity and specific activity were slowly decreased. Thus, the concentration of Bacto-peptone at 0.8% which gave the highest total activity (7,950 units) and specific activity (20.9 units/mg protein) was used for further experiments.

### 3.4.2 Enzyme induction

The effect of various inducers was studied for P6CDH induction as mentioned in section 2.11.3. As shown in Figure 3.12, total activity of P6CDH could be induced to 10.0, 8.2 and 6.7 fold while specific activity of P6CDH could be induced to 7.7, 6.3 and 5.0 fold by 1.0% L-lysine, L-pipecolic acid and L-ornithine in peptone medium pH 7.2, respectively. The highest total activity and specific activity obtained by addition of 1.0% L-lysine were 7,970 units and 21.02 units/mg protein, respectively. Thus, L-lysine was used as an enzyme inducer for the next experiments.

### 3.4.3 Optimum concentration of L-lysine

The effect of the concentration of inducer on P6CDH production was performed as described in section 2.11.4. Various concentrations of L-lysine from 0 to 1.8 % were added to peptone medium, pH 7.2. As shown in Figure 3.13, total activity and specific activity increased with increasing concentration of the L-lysine until the concentration reached 0.6%. At concentration of L-lysine above 1.0%, total activity and

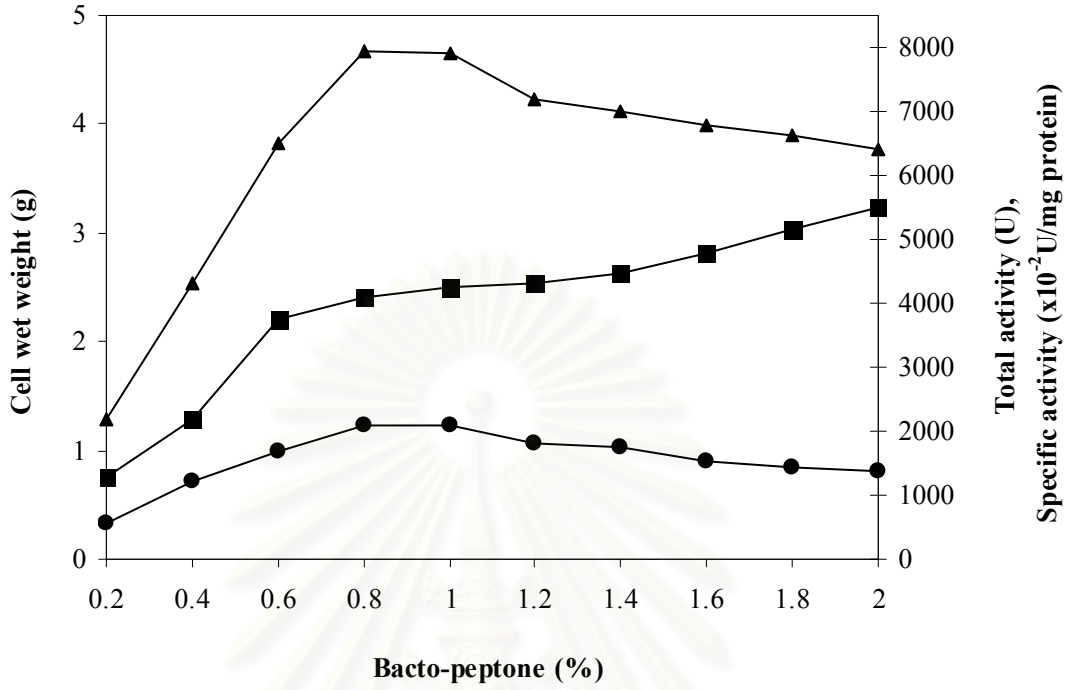



**Figure 3.11 Effect of the concentration of Bacto-peptone on piperideine-6-carboxylate dehydrogenase production**

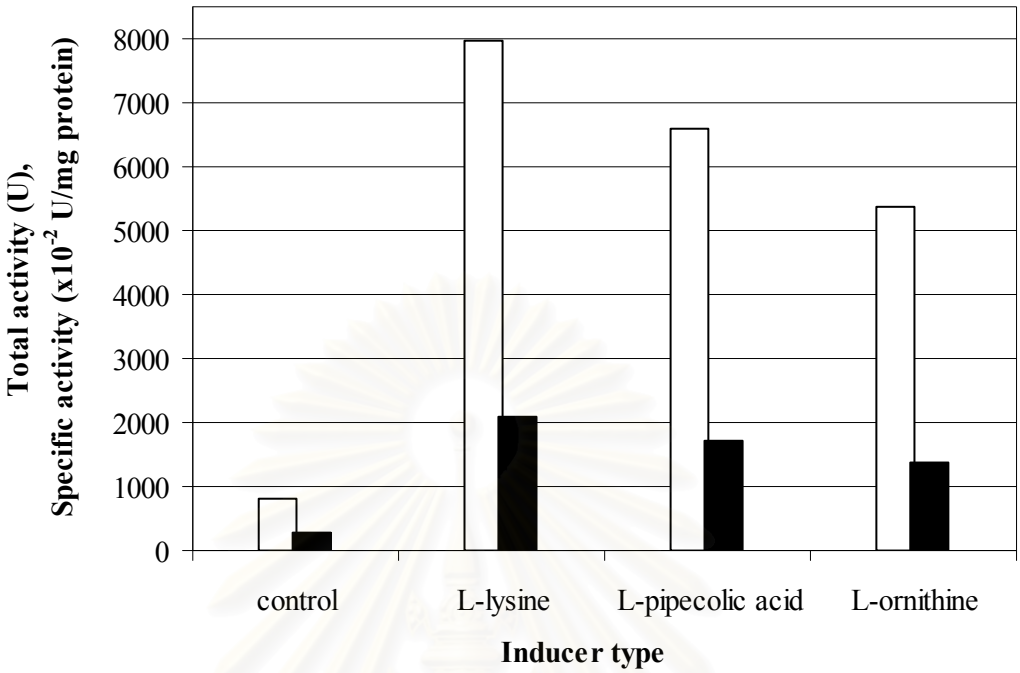



**Figure 3.12 Effect of inducer on piperideine-6-carboxylate dehydrogenase production**

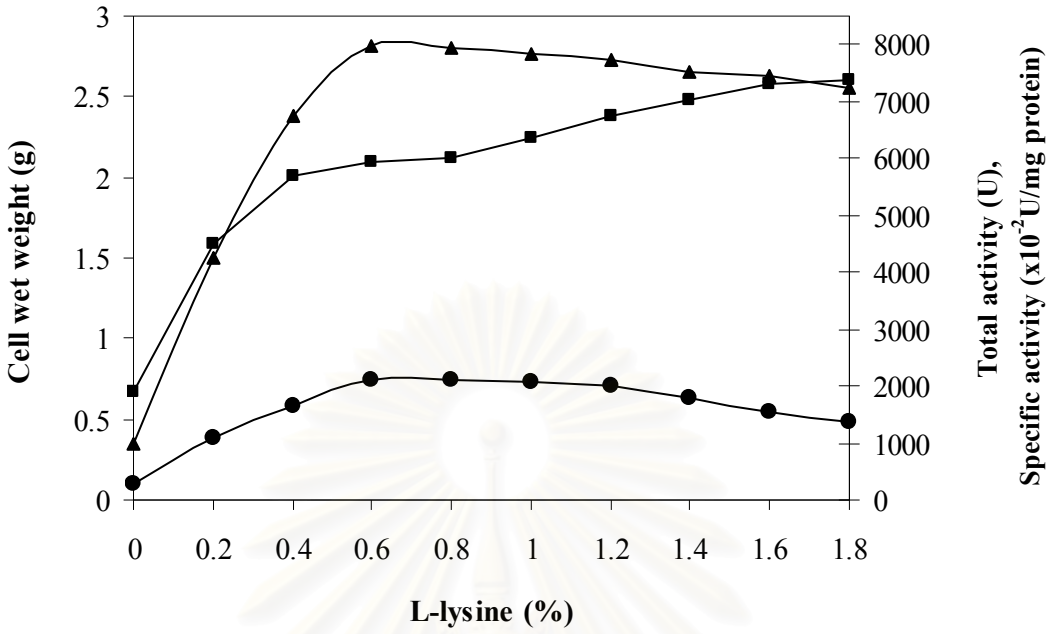



**Figure 3.13 Effect of the concentration of L-lysine on piperideine-6-carboxylate dehydrogenase production**

specific activity were slowly decreased while cell wet weight was dramatically increased with increasing concentration of the L-lysine until the concentration reached 0.4%. At concentration of L-lysine above 0.4%, cell weight was slowly increased. The results showed that addition of 0.6% L-lysine to culture medium gave the highest total activity and specific activity with 7,990 units and 21.0 units/mg protein, respectively. Thus, this concentration was used for further experiments.

### 3.4.4 Optimum pH of medium

The effect of pH of medium on P6CDH production was performed as described in section 2.11.5. As shown in Figure 3.14, cell wet weight, total activity, and specific activity rapidly increased with increasing of pH of medium until the pH reached 8.0. At pH above 8.0, activity was dramatically decreased while cell wet weight and specific activity was slowly decreased. Therefore, culture medium, pH 8.0 which gave the highest total activity (8,000 units) and specific activity (21.1 units/mg protein) was used for further experiments.

### 3.4.5 Optimum cultivation temperature

The optimum cultivation temperature for enzyme production was determined as described in section 2.11.6. Cell wet weight, total activity, and specific activity increased with increasing of cultivation temperature from 28 to 37 ºC as shown in Figure 3.15. The highest total activity and specific activity were detected at 37 ºC with 8,050 units and 21.2 units/mg protein, respectively. At temperature above 37 ºC, cell wet weight, total activity, and specific activity was rapidly decreased. According to the result, cultivation at 37 ºC was used for further experiments.

From the above results, the optimum condition for P6CDH production of *Pseudomonas putida* was cultivation in peptone medium, pH 8.0 containing 0.8% peptone, 0.6% L-lysine at 37 ºC for 15 hours.

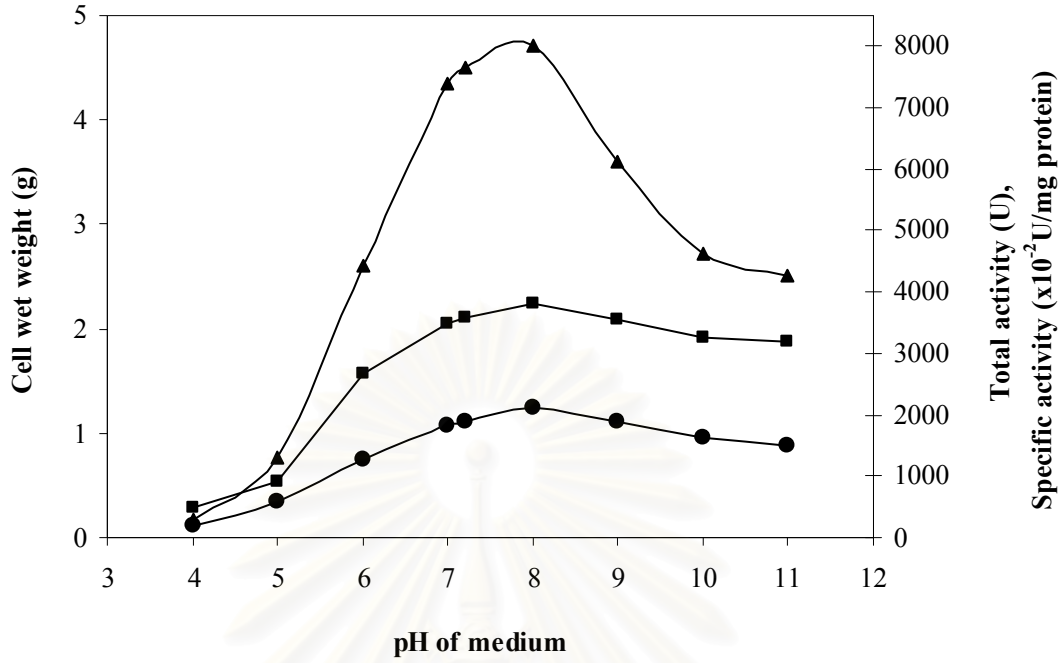



**Figure 3.14 Effect of the pH of medium on piperideine-6-carboxylate dehydrogenase production**

■ cell wet weight

▲ total activity

● specific activity

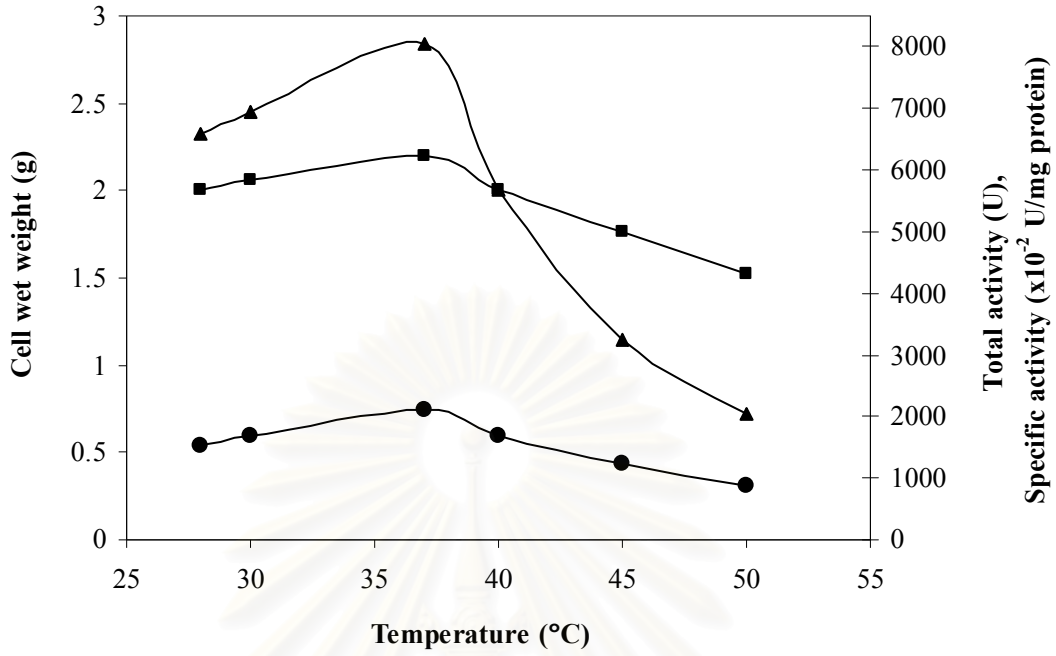



**Figure 3.15 Effect of the cultivated temperature on piperideine-6-carboxylate dehydrogenase production**

■ cell wet weight

▲ total activity

● specific activity

## 3.5 Purification of piperideine-6-carboxylate dehydrogenase

### 3.5.1 Preparation of crude extract

Crude P6CDH was prepared from 60.8 g of *Pseudomonas putida*, which was cultivated in 6 liters of medium, as described in section 2.12.3. Crude extract solution contained 3,250 mg protein and 68,300 units of P6CDH activity. Thus, the specific activity was 21.0 units/mg protein.

### 3.5.2 Enzyme purification steps

#### 3.5.2.1 Ammonium sulfate precipitation

First step of purification, crude extract was purified by ammonium sulfate precipitation as described in section 2.12.4.1. To determine the proper ammonium sulfate concentration for enzyme precipitation, the precipitation using 0-30%, 0-40%, 40-50%, 50-60% and 60-70% saturated ammonium sulfate were performed in preliminary experiment. The result showed that most of enzyme activity was found in the 50-60% fraction. Therefore, protein from 50-60% saturated ammonium sulfate fraction was collected and dialysed against the buffer. The recovered protein and enzyme activity were 1,420 mg and 40,900 units, respectively. The specificity activity of the enzyme from this step was 28.8 units/mg protein. The enzyme was purified 1.4 fold with 59.9% recovery.

#### 3.5.2.2 DEAE-Toyopearl column chromatography

The enzyme from 50-60% saturated ammonium sulfate precipitation was loaded into DEAE-Toyopearl column as described in section 2.12.4.2. The chromatographic profile was shown in Figure 3.16. The unbound proteins were eluted from column by the 10 mM Tris-HCl buffer, pH 8.0 containing 0.01% β-mercaptoethanol, 1 mM EDTA and 10% glycerol, whereas the bound proteins were eluted by stepwise of 0.1, 0.2, and 0.3 M sodium chloride in the buffer. P6CDH was eluted at 0.1 M sodium chloride as indicated in the profile. P6CDH fractions were pooled, dialysed against the buffer, concentrated by aquasorb to reduce enzyme volume.

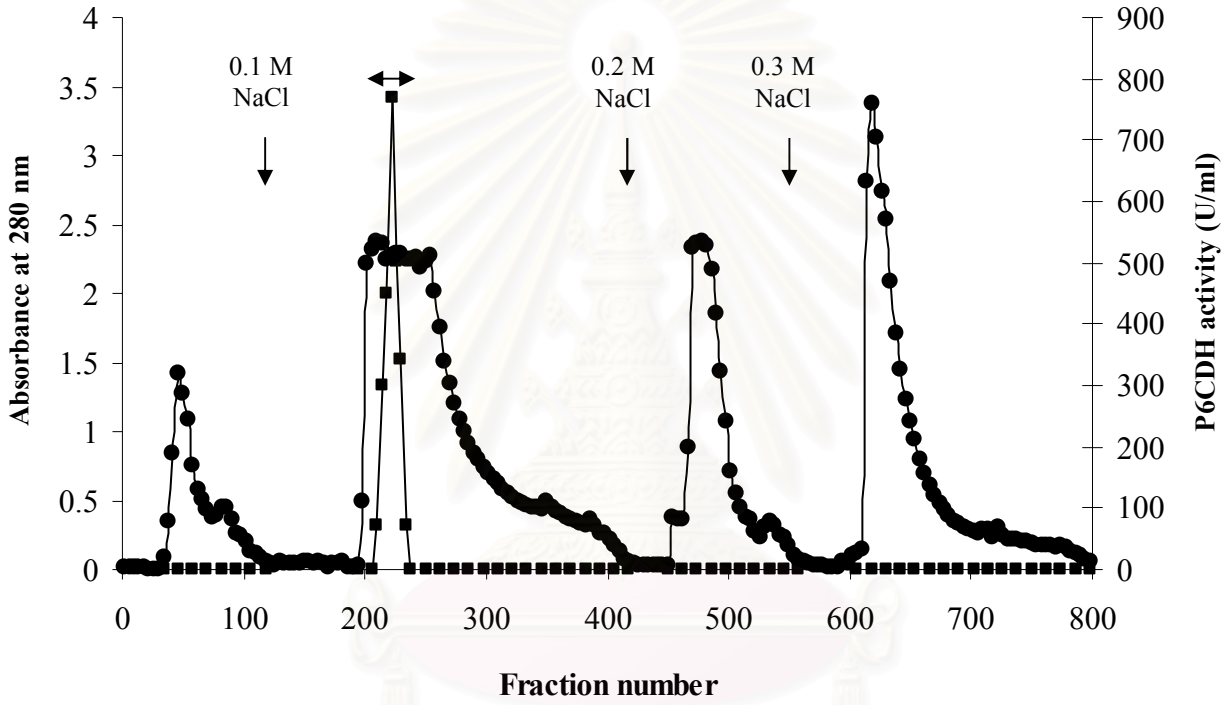



**Figure 3.16 Purification of piperideine-6-carboxylate dehydrogenase from *Pseudomonas putida* by DEAE-Toyopearl 650M column**

The enzyme solution was applied to DEAE -Toyopearl 650M column and washed with 10 mM Tris-HCl buffer, pH 8.0 containing 0.01% (v/v) β-mercaptoethanol, 1 mM EDTA, and 10% glycerol until $A_{280}$ decreased to base line. The bound proteins were eluted by stepwise of 0.1, 0.2, and 0.3 M NaCl in the same buffer at the flow rate of 1 ml/min. The fractions of 3 ml were collected. The arrow indicate where each stepwise started. The protein peak from fraction number 216 to 230 was pooled (⟷). ● $A_{280}$, ■ P6CDH activity

This operation obtained the enzyme with 206 mg proteins and 35,100 activity units. The specificity activity of the enzyme from this step was 170.4 units/mg protein. The enzyme was purified 8.1 fold with 51.4 % recovery.

### 3.5.2.3 Butyl-Toyopearl column chromatography

The pooled active fraction from DEAE-Toyopearl column was applied to the Butyl-Toyopearl column as described in section 2.12.4.3. The chromatographic profile was shown in Figure 3.17. The unbound proteins were eluted from column with the buffer containing 25% saturated ammonium sulfate and then the bound proteins were eluted with stepwise of 20, 15, 10, and 5% saturated ammonium sulfate in the buffer. The enzyme was eluted immediately with the buffer containing 15% salt saturation. The pooled fraction containing P6CDH activity was dialyzed against the buffer and concentrated by aquasorb. The proteins remained from this step was 10.0 mg with 18,000 units of P6CDH activity. The specific activity of the enzyme was 1,800 units/mg protein. The P6CDH was purified 85.7 fold with about 26.4% recovery.

### 3.5.2.4 Hitrap Q column chromatography

The pooled active fraction from Butyl-Toyopearl column was applied to the Hitrap Q column as described in section 2.12.4.4. The chromatographic profile was shown in Figure 3.18. The unbound proteins were eluted from column with the buffer and then the bound proteins were eluted with linear salt gradient of 0-0.5 M NaCl in the buffer. The enzyme was eluted with 0.1 M NaCl. The pooled fraction containing P6CDH activity was dialyzed against the buffer and concentrated by aquasorb. The proteins remained from this step was 4.0 mg with 12,800 units of P6CDH activity. The specific activity of the enzyme was 3,200 units/mg protein. The P6CDH was purified 152.4 fold with about 18.7% recovery.

The summary of purification of P6CDH was shown in Table 3.3

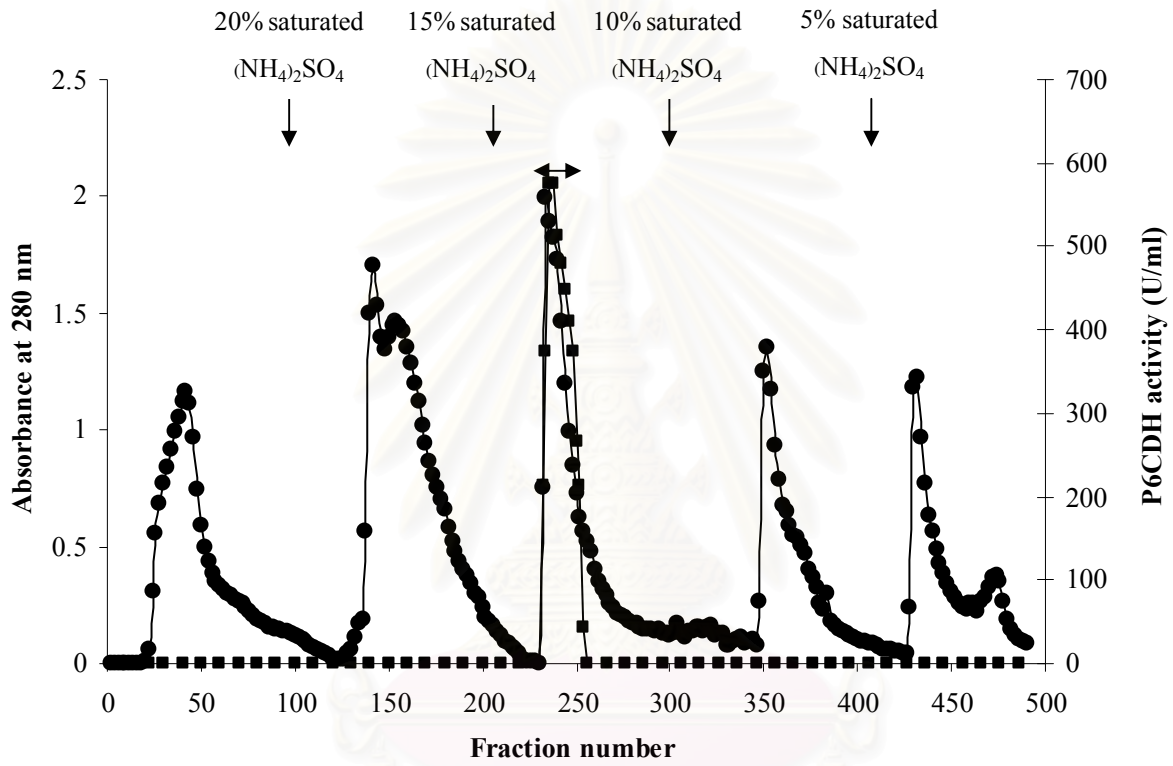



**Figure 3.17 Purification of piperideine-6-carboxylate dehydrogenase from *Pseudomonas putida* by Butyl-Toyopearl 650M column**

The enzyme solution was applied to Butyl-Toyopearl column and washed with 25% saturated ammonium sulfate in 10 mM Tris-HCl buffer, pH 8.0 containing 0.01% (v/v) β-mercaptoethanol, 1 mM EDTA, and 10% glycerol until $A_{280}$ decreased to base line. The enzyme was eluted by 15% saturated ammonium sulfate in the same buffer at the flow rate of 1 ml/ min. The fractions of 3 ml were collected. The arrows indicate where each stepwise started. The protein peak from fraction number 234 to 248 was pooled ( ⟷ ).

● $A_{280}$, ■ P6CDH activity

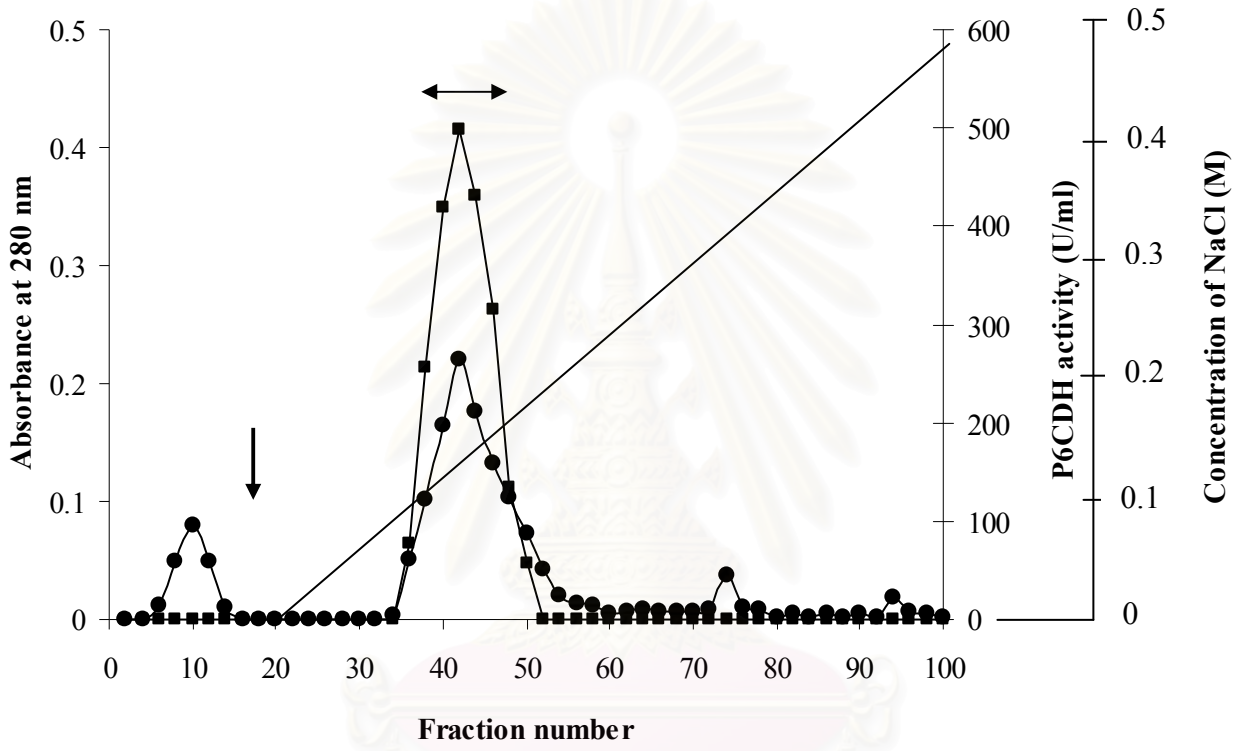



**Figure 3.18 Purification of piperideine-6-carboxylate dehydrogenase from *Pseudomonas putida* by Hitrap Q column**

The enzyme solution was applied to Hitrap Q column and washed with 10 mM Tris-HCl buffer, pH 8.0 containing 0.01% (v/v) β-mercaptoethanol, 1 mM EDTA, and 10% glycerol until $A_{280}$ decreased to base line. The enzyme was eluted by gradient of 0-0.5 M NaCl in the same buffer at the flow rate of 0.5 ml/ min. The fractions of 2 ml were collected. The arrow indicates where gradient started. The protein peak from fraction number 36 to 49 was pooled ( ⟷ ). ● $A_{280}$, ■ P6CDH activity, ── [NaCl]

**Table 3.3 Purification of piperideine-6-carboxylate dehydrogenase**

| Purification steps | Total activity (unit) | Total protein (mg) | Specific activity (units/mg protein) | %Recovery | Purification fold |
|---|---|---|---|---|---|
| **Crude enzyme** | 68,300 | 3,250 | 21.0 | 100.0 | 1.0 |
| **50-60% Saturated ammonium sulfate precipitation** | 40,900 | 1,420 | 28.8 | 59.9 | 1.4 |
| **DEAE-Toyopearl** | 35,100 | 206 | 170 | 51.4 | 8.1 |
| **Butyl-Toyopearl** | 18,000 | 10.0 | 1,800 | 26.4 | 85.7 |
| **Hitrap Q** | 12,800 | 4.0 | 3,200 | 18.7 | 152 |

### 3.5.3 Determination of enzyme purity on non-denaturing polyacrylamide gel electrophoresis (native-PAGE) and SDS-polyacrylamide gel electrophoresis

The enzyme purity from each purification steps and native protein pattern were investigated by non-denaturing polyacrylamide gel electrophoresis as described in section 2.7.1. The results of protein staining and activity staining were shown in Figure 3.19. The single band from Hitrap Q column in lane 5A and 5B indicated that the enzyme was purified to homogeneity.

After that, the molecular weight of the enzyme subunit and the enzyme purity were determined by denaturing polyacrylamide gel electrophoresis as described in section 2.7.2. The single band from Hitrap Q column chromatography confirmed that the enzyme was purified to homogeneity and the molecular weight of the enzyme subunit was calculated to be 50.2 kDa by its mobility in SDS-PAGE compared with those of standard proteins as shown in Figure 3.20. The molecular weight calibration curve was shown in Figure 3.21.

## 3.6 Characterization of piperideine-6-carboxylate dehydrogenase

### 3.6.1 Molecular weight determination of piperideine-6-carboxylate dehydrogenase

The native molecular weight of P6CDH was determined from high molecular weight calibration curve obtained by gel filtration on HPLC by TSK gel G3000SW column as mentioned in section 2.13.1 (Figure 3.22). The molecular weight of the native enzyme was estimated to be about 301,000 Da. The subunit molecular weight of the enzyme was estimated to be about 50,200 Da by SDS-PAGE, as previously described in section 3.5.3. The result indicated that the enzyme was consisted of six identical subunits.

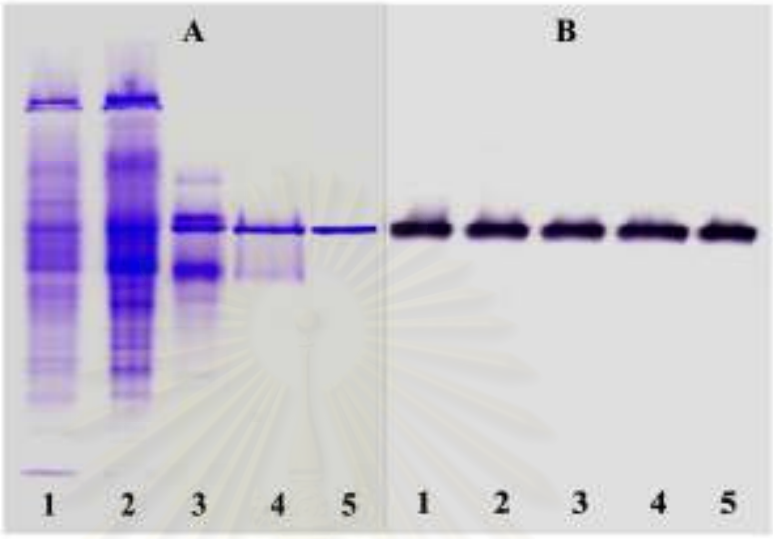



**Figure 3.19 Protein pattern from each step of purification investigated by native-PAGE**

**[A] Protein staining** **[B] Activity staining**

| | | |
|---|---|---|
| Lane 1 | = crude extract | 115 µg |
| Lane 2 | = 50-60% saturated ammonium sulfate precipitation | 115 µg |
| Lane 3 | = DEAE-Toyopearl column | 20 µg |
| Lane 4 | = Butyl-Toyopearl column | 12 µg |
| Lane 5 | = Hitrap Q column | 8 µg |

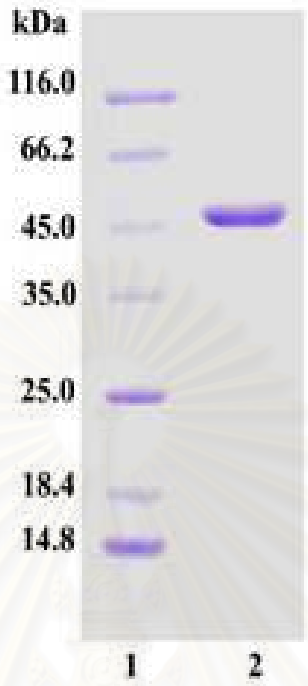



**Figure 3.20 SDS-PAGE of piperideine-6-carboxylate dehydrogenase**

| | | | |
|---|---|---|---|
| Lane 1 | = | protein marker | |
| | | β-galactosidase | (MW 116,000) |
| | | bovine serum albumin | (MW 66,000) |
| | | ovalbumin | (MW 45,000) |
| | | lactate dehydrogenase | (MW 35,000) |
| | | restriction endonuclease *Bsp* 98I | (MW 25,000) |
| | | β-lactoglobulin | (MW 18,400) |
| | | lysozyme | (MW 14,800) |
| Lane 2 | = | protein from Hitrap Q column | 8 μg |

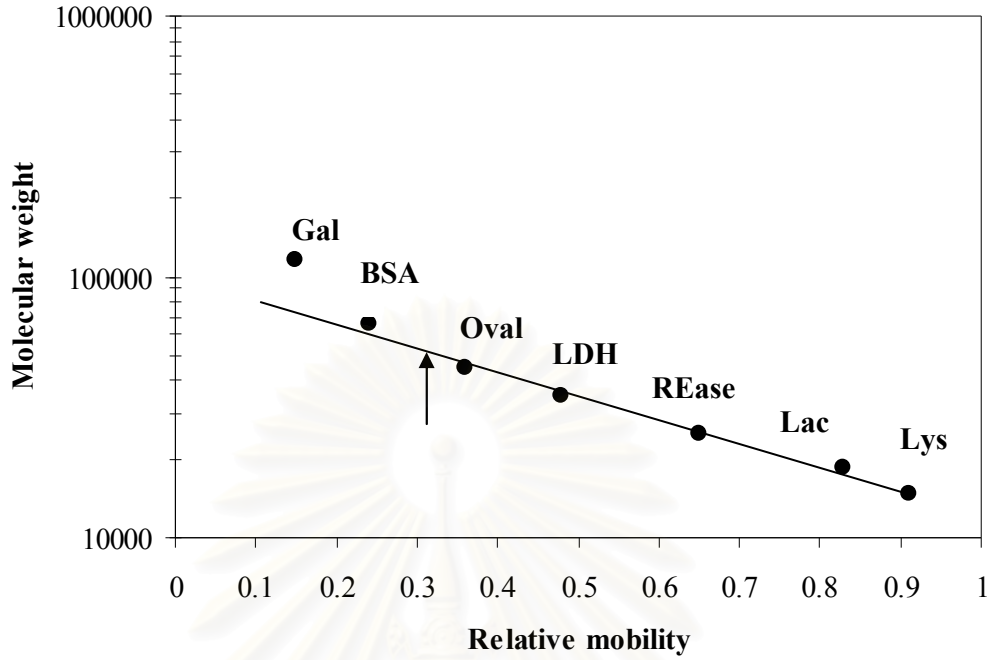



**Figure 3.21 Calibration curve for molecular weight of piperideine-6-carboxylate dehydrogenase subunit on SDS-polyacrylamide gel electrophoresis**

| | | | |
|---|---|---|---|
| Gal | = | β-galactosidase | (MW 116,000) |
| BSA | = | bovine serum albumin | (MW 66,000) |
| Oval | = | ovalbumin | (MW 45,000) |
| LDH | = | lactate dehydrogenase | (MW 35,000) |
| REase | = | restriction endonuclease *Bsp* 98I | (MW 25,000) |
| Lac | = | β-lactoglobulin | (MW 18,400) |
| Lys | = | lysozyme | (MW 14,800) |

Arrow indicates the $K_{av}$ of P6CDH.

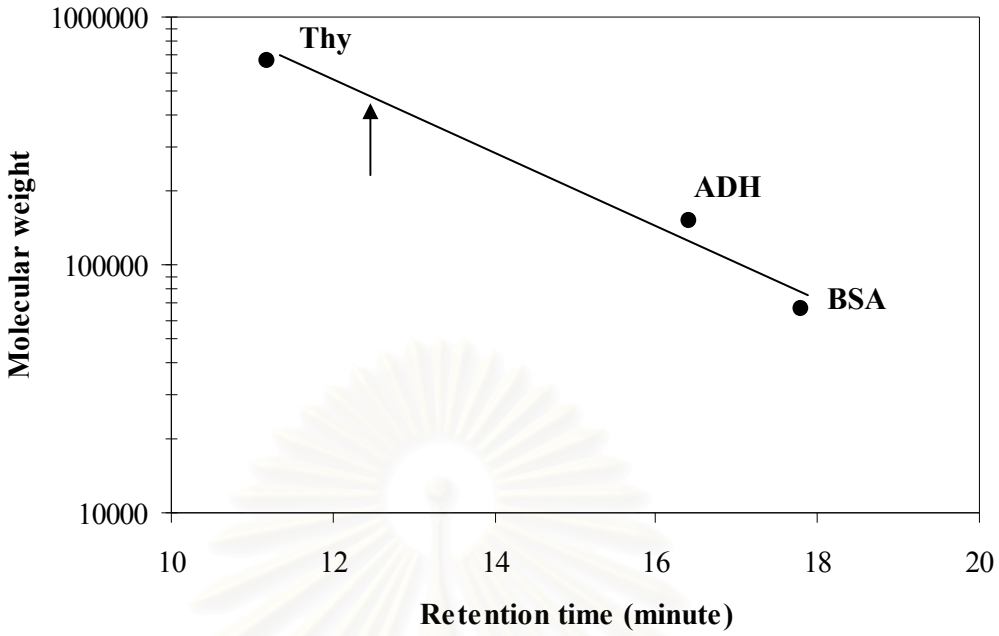



**Figure 3.22 Calibration curve for molecular weight of piperideine-6-carboxylate dehydrogenase by gel filtration on HPLC**

| | | | |
|---|---|---|---|
| Thy | = | thyroglobulin | (669,000 Da) |
| ADH | = | alcohol dehydrogenase | (150,000 Da) |
| BSA | = | bovine serum albumin | (66,000 Da) |

Arrow indicated a determined molecular weight of P6CDH.

### 3.6.2 Substrate specificity of piperideine-6-carboxylate dehydrogenase

Substrate specificity of P6CDH was studied as mentioned in section 2.13.2. The ability of the enzyme to catalyze the oxidative deamination of various amino acids and their derivatives was determined at a concentration of 20 mM as shown in Table 3.4. In addition to P6C (100% relative activity) which was the preferred substrate, various L-amino acids served as substrates (relative activities given in parentheses): L-pipecolic acid (22), L-proline (18), L-lysine (12), L-ornithine (10), L-norleucine (6), and L-valine (4). The tested amino acids, which were not substrate were D-alanine, D-arginine, D-aspatic acid, D-glutamic acid, D-leucine, D-methionine, D-phenylalanine, D-serine, D-threonine, D-tryptophan, D-valine, *m*-fluoro-DL-phenylalanine**,** *o*-fluoro-DL-phenylpyruvate, *p*-fluoro-DL-phenylalanine**,** *S*-methyl-L-cysteine, L-isoleucine, L-leucine, L- methionine, L-norvaline, L-phenylalanine.

### 3.6.3 Coenzyme specificity of piperideine-6-carboxylate dehydrogenase

Coenzyme specificity of P6CDH was investigated as described in section 2.13.3. P6CDH required $NAD^+$ as a natural coenzyme for oxidative deamination. Some analogs of $NAD^+$ could serve as coenzymes as shown in Table 3.5. Nicotinamide hypoxanthine dinucleotide (120%) was much better coenzyme than $NAD^+$ while β-nicotinamide adenine dinucleotide phosphate ($NADP^+$), nicotinic acid adenine dinucleotide, thionicotinamide adenine dinucleotide and 3-pyridinealdehyde adenine dinucleotide showed no activity.

### 3.6.4 Effect of pH on piperideine-6-carboxylate dehydrogenase activity

The effect of pH on the enzyme activity in the oxidative deamination was examined at various pHs ranged from 6.5 to 12.5 as mentioned in section 2.13.4. The result was shown in Figure 3.23. The enzyme exhibited maximal activity at pH 9.2 and 8.7 for coupling reaction and P6CDH reaction, respectively.

**Table 3.4 Substrate specificity of piperideine-6-carboxylate dehydrogenase**

| Substrate [a] | Relative activity (%) |
|---|---|
| P6C | 100 |
| L-Pipecolic acid | 22 |
| L-Proline | 18 |
| L-Lysine | 12 |
| L-Ornithine | 10 |
| L-Norleucine | 6 |
| L-Valine | 4 |

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

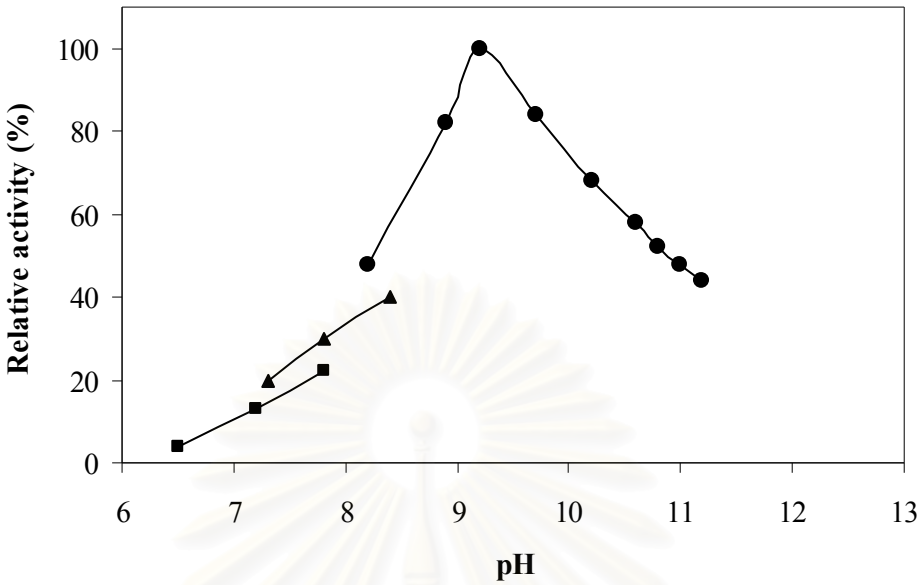
The followings were inert: D-alanine, D-arginine, D-aspatic acid, D-glutamic acid, D-leucine, D-methionine, D-phenylalanine, D-serine, D-threonine, D-tryptophan, D-valine, *m*-fluoro-DL-phenylalanine**,** *o*-fluoro-DL-phenylpyruvate, *p*-fluoro-DL-phenylalanine**,** *S*-methyl-L-cysteine, L-isoleucine, L-leucine, L-methionine, L-norvaline, L-phenylalanine.

**Table 3.5 Coenzyme specificity of piperideine-6-carboxylate dehydrogenase**

| Coenzyme [a] | Relative activity (%) |
|---|---|
| β-Nicotinamide adenine dinucleotide | 100 |
| Nicotinamide hypoxanthine dinucleotide | 120 |
| β-Nicotinamide adenine dinucleotide phosphate | 0 |
| Nicotinic acid adenine dinucleotide | 0 |
| Thionicotinamide adenine dinucleotide | 0 |
| 3-Pyridinealdehyde adenine dinucleotide | 0 |

[a] Final concentration of each coenzyme analog was 2.0 mM. The assay was performed at the following wavelengths: nicotinamide hypoxanthine dinucleotide, 338 nm ($\varepsilon = 6.2 \times 10^3$ $M^{-1}cm^{-1}$); β-nicotinamide adenine dinucleotide phosphate (NADP), 340 nm ($\varepsilon = 6.2 \times 10^3$ $M^{-1}cm^{-1}$); nicotinic acid adenine dinucleotide, 338 nm ($\varepsilon = 6.2 \times 10^3$ $M^{-1}cm^{-1}$); thionicotinamide adenine dinucleotide, 395 nm ($\varepsilon = 11.3 \times 10^3$ $M^{-1}cm^{-1}$) and 3-pyridinealdehyde adenine dinucleotide, 358 nm ($\varepsilon = 9.3 \times 10^3$ $M^{-1}cm^{-1}$).

**[A]**




**[B]**

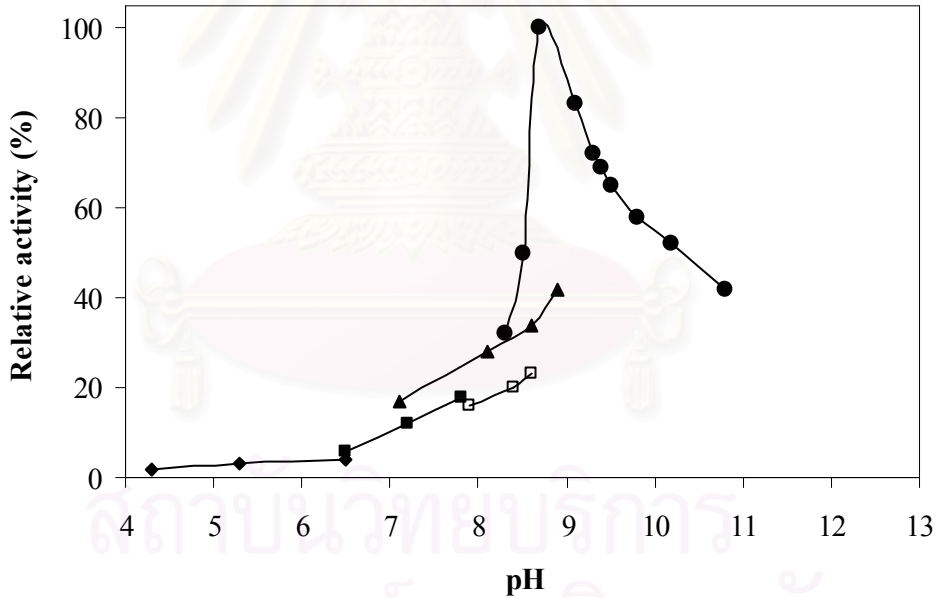



**Figure 3.23 Effect of pH on piperideine-6-carboxylate dehydrogenase activity**

Results shown were average values of duplicate experiment.

[A] coupling reaction [B] P6CDH reaction

◆ acetate buffer ▲ Tris-HCl buffer

■ potassium phosphate buffer ● glycine-KCl-KOH buffer

□ MOPS-HCl

### 3.6.5 Effect of temperature on piperideine-6-carboxylate dehydrogenase activity

The effect of temperature on enzyme activity was investigated as described in section 2.13.5. The temperature was varied from 25 °C to 80 °C. The result was shown in Figure 3.24. The enzyme performed the highest activity at 45 ºC for both coupling reaction and P6CDH reaction.

### 3.6.6 Effect of glycerol on piperideine-6-carboxylate dehydrogenase stability

Effect of glycerol on P6CDH stability was investigated as described in section 2.13.6. The concentrations of glycerol were 0, 5, 10, and 20%. The enzyme retained 50% of its activity after 8, 12, 23, and 29 days for 0, 5, 10, and 20% glycerol, respectively. The result was shown in Figure 3.25.

### 3.6.7 Effect of pH on piperideine-6-carboxylate dehydrogenase stability

The pH stability of P6CDH was studied as described in section 2.13.7. The enzyme was preincubated at 30 ºC for 20 minutes in various 10 mM buffers at various pHs ranging from 4.0 to 12.5. The result was shown in Figure 3.26. The enzyme was stable over the pH ranged from 6.5 to 11.5 and 7 to 11 for coupling reaction and P6CDH reaction, respectively.

### 3.6.8 Effect of temperature on piperideine-6-carboxylate dehydrogenase stability

The thermostability of P6CDH was studied as described in section 2.13.8. The enzyme was preincubated at various temperatures ranged from 30 ºC to 75 ºC for 10 min. The enzyme activity of non-preincubated enzyme was defined as 100% relative activity. The result was shown in Figure 3.27 A. The enzyme retained its full

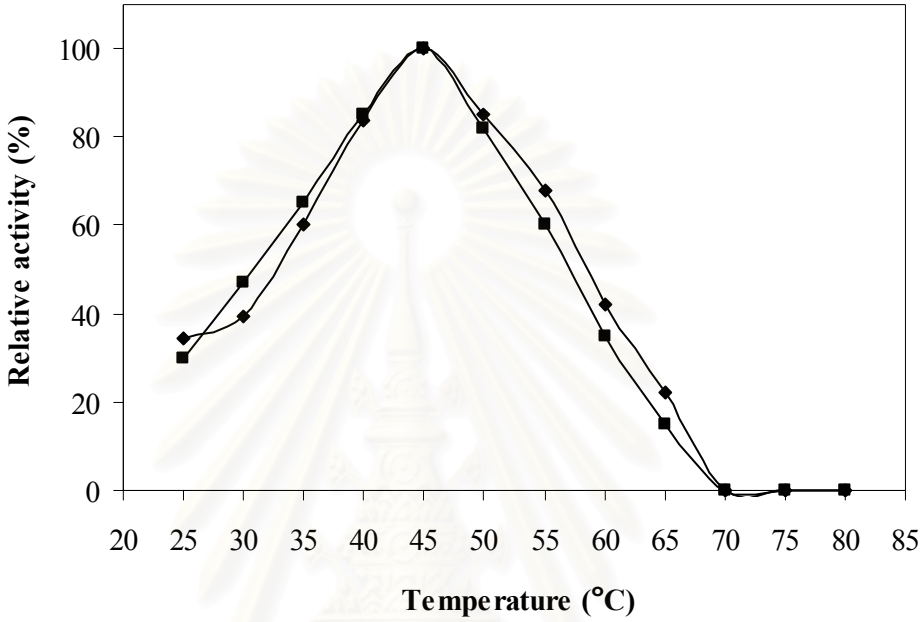



**Figure 3.24 Effect of temperature on piperideine-6-carboxylate dehydrogenase activity**

Results shown were average values of duplicate experiment.

◆ coupling reaction ■ P6CDH reaction

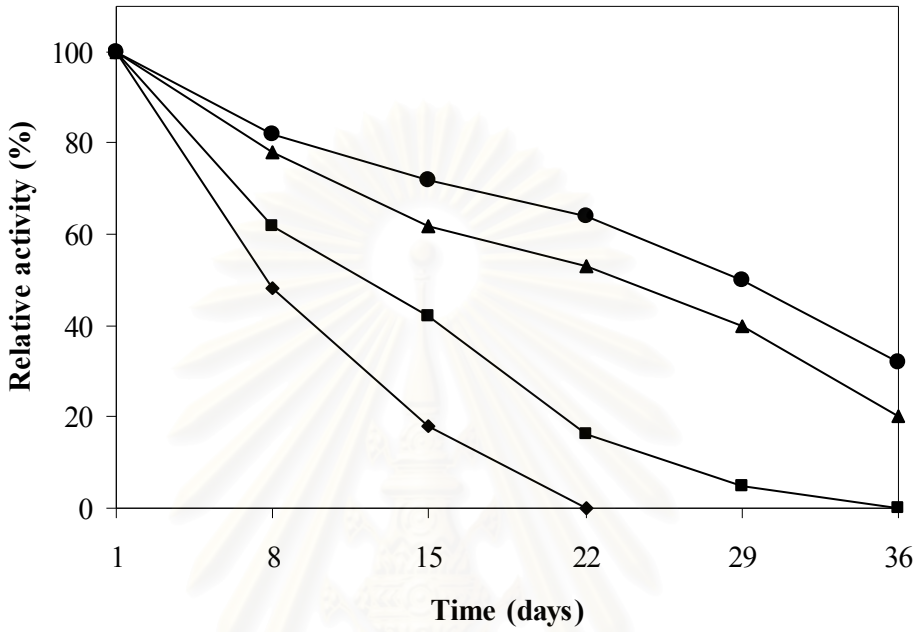
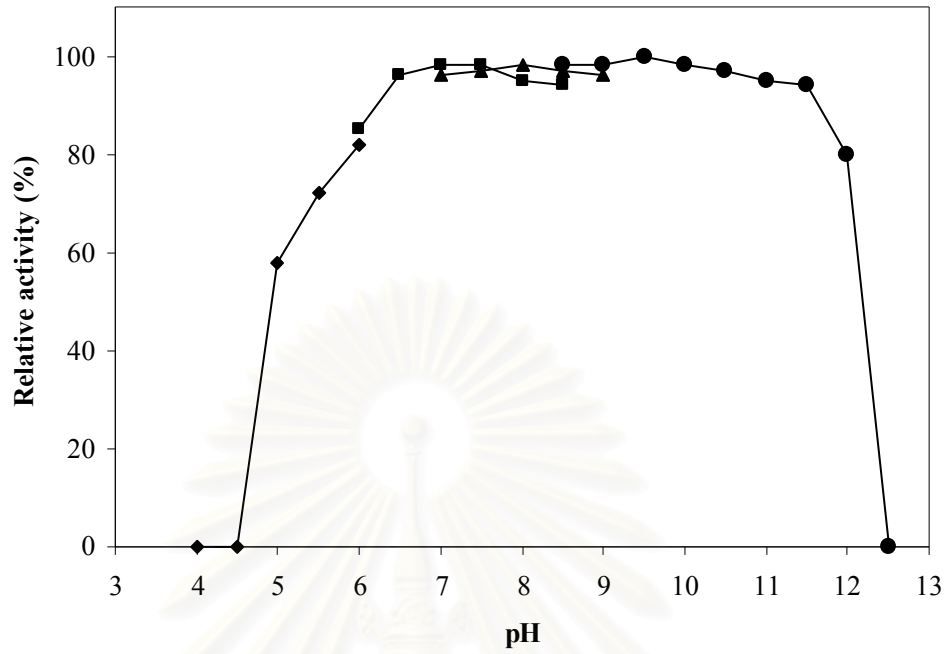



**Figure 3.25 Effect of glycerol on piperideine-6-carboxylate dehydrogenase stability**

**[A]**




**[B]**

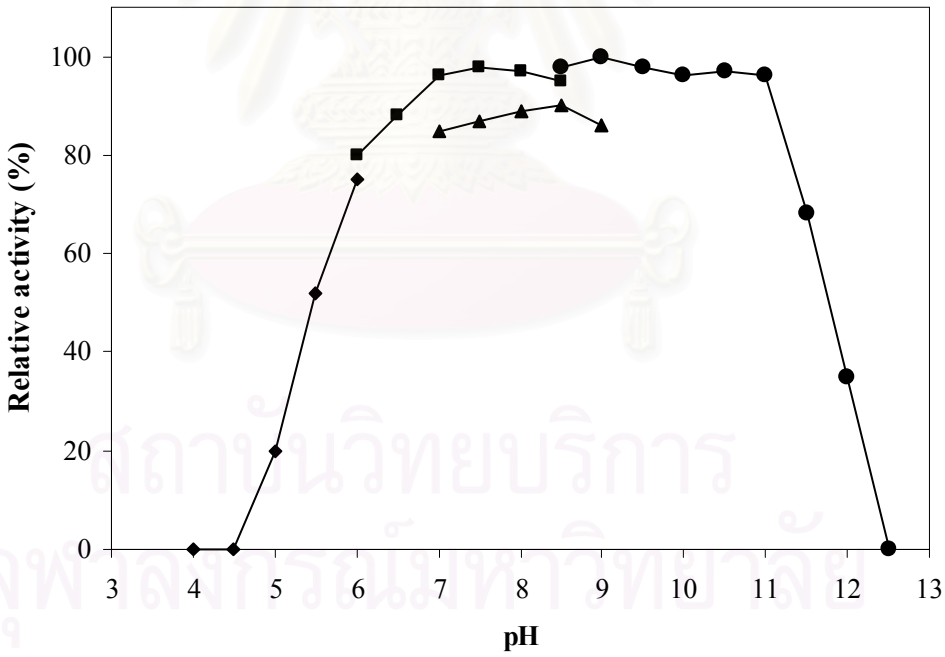



**Figure 3.26 Effect of pH on piperideine-6-carboxylate dehydrogenase stability**

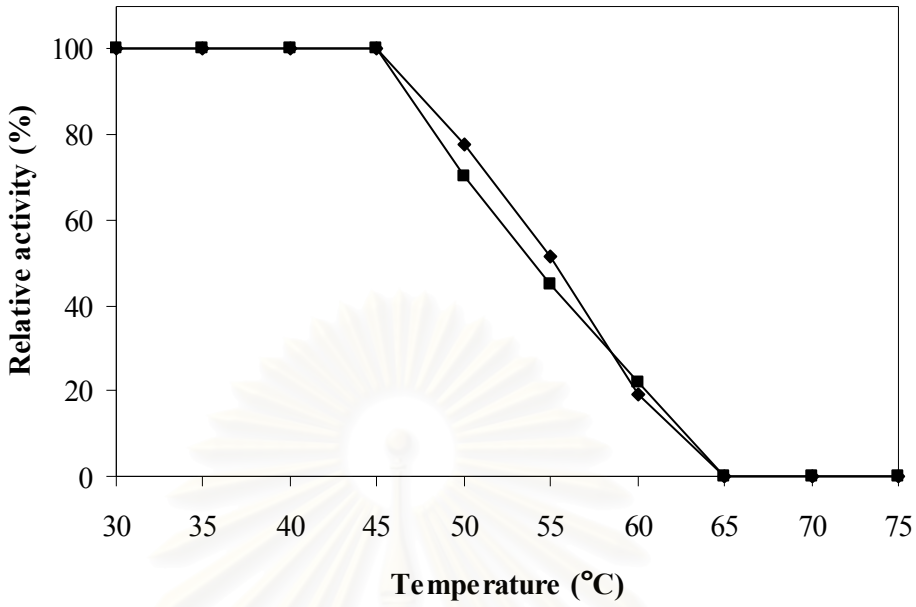
Results shown were average values of duplicate experiment.

[A] coupling reaction [B] P6CDH reaction

◆ acetate buffer ▲ Tris-HCl buffer

■ potassium phosphate buffer ● glycine-KCl-KOH buffer

**[A]**




**[B]**

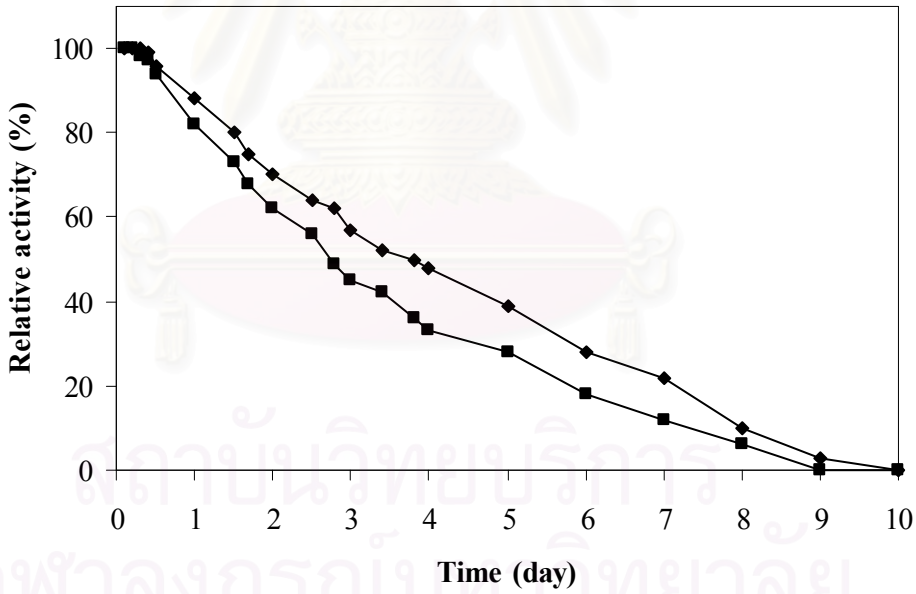



**Figure 3.27 Effect of temperature on piperideine-6-carboxylate dehydrogenase stability**

Results shown were average values of duplicate experiment.

[A] thermostability of enzyme [B] enzyme stability at 45 ºC

◆ coupling reaction ■ P6CDH reaction

activity at temperature up to 45 ºC and lost about half of its activity at 55 ºC. At 65 ºC, P6CDH absolutely lost its activity. The enzyme stability was tested at 45 ºC by incubation for 0 to 10 days and investigated its activity everyday. The remained deamination activities were expressed as the percentage of the original activity. The result was shown in Figure 3.27B. The enzyme was fully stable at 45 ºC only 3 and 2 hours for coupling reaction and P6CDH reaction, respectively. The enzyme activity was relatively decreased with increasing of incubation time. It retained 50% of activity after treatment for 3 and 2 days for coupling reaction and P6CDH reaction, respectively, and the activity was completely lost after incubation for 9 and 8 days for coupling reaction and P6CDH reaction, respectively.

### 3.6.9 Effect of metal ions on piperideine-6-carboxylate dehydrogenase

This experiment was done as described in section 2.13.9. The summary of effect of metal ions and some chemical reagents on P6CDH activity was shown in Table 3.6. The enzyme was incubated in 10 mM of metal ions or chemical reagents, except 1 mM for $CuSO_4$, $FeCl_3$ and $FeSO_4$. $CuSO_4$ and $FeSO_4$ strongly inhibited the enzyme activity while $CoCl_2$ showed mild inhibition. No inhibition was observed with NaCl, KCl, $MgCl_2$, $MgSO_4$, $CaCl_2$, $MnSO_4$, and $MnCl_2$.

### 3.6.10 Inhibitory effect of various amino acids on piperideine-6-carboxylate dehydrogenase

This experiment was done as described in section 2.13.10 to focus on effect of various amino acids, which were nonsubstrate of P6CDH. The result was shown in Table 3.7. *m*-Fluoro-DL-phenylalanine, *o*-fluoro-DL-phenylpyruvate, *p*-fluoro-DL-phenylalanine, D-leucine, D-phenylalanine, D-tryptophan**,** D-valine**,** L-leucine, L-methionine and L-norvaline showed slight inhibition**.** Other nonsubstrate amino acids had no effect.

**Table 3.6 Effect of metal ions on piperideine-6-carboxylate dehydrogenase activity**

| Compound | Final concentration (mM) | Relative activity (%) | |
|---|---|---|---|
| | | Coupling reaction | P6CDH reaction |
| None | - | 100 | 100 |
| NaCl | 10 | 100 | 100 |
| KCl | 10 | 100 | 100 |
| $MgCl_2$ | 10 | 100 | 100 |
| $MgSO_4$ | 10 | 100 | 100 |
| $CaCl_2$ | 10 | 100 | 100 |
| $MnSO_4$ | 10 | 100 | 100 |
| $MnCl_2$ | 10 | 100 | 100 |
| $CoCl_2$ | 10 | 94 | 90 |
| $FeCl_3$ | 1 | 40 | 42 |
| $FeSO_4$ | 1 | 0 | 0 |
| $CuSO_4$ | 1 | 0 | 0 |

**Table 3.7 Inhibitory effect of various amino acids on piperideine-6-carboxylate dehydrogenase activity**

| Amino acids [a] | Relative activity (%) | |
|---|---|---|
| | Coupling reaction | P6CDH reaction |
| None | 100 | 100 |
| *m*-Fluoro-DL-phenylalanine | 81 | 84 |
| *o*-Fluoro-DL-phenylpyruvate | 84 | 87 |
| *p*-Fluoro-DL-phenylalanine | 82 | 85 |
| D-Leucine | 82 | 80 |
| D-Phenylalanine | 84 | 85 |
| D-Tryptophan | 84 | 86 |
| D-Valine | 84 | 81 |
| L-Leucine | 87 | 90 |
| L-Methionine | 85 | 82 |
| L-Norvaline | 90 | 92 |

[a] Amino acid concentration was 20 mM. *S*-Methyl-L-cysteine, D-alanine, D-arginine, D-aspatic acid, D-glutamic acid, D-methionine, D-serine, D-threonine, L-isoleucine and L-phenylalanine did not inhibit the reaction.

## 3.7 Kinetic studies of piperideine-6-carboxylate dehydrogenase

A series of steady-state kinetic analysis was carried out to investigate the kinetic parameters. Initial velocity studies for oxidative deamination were performed by varying concentration of each substrate. Double-reciprocal plots of initial velocity against reciprocals of L-pipecolic acid concentrations gave a family of straight lines, which intersected in the upper left quadrant as shown in Figure 3.28 A. The apparent $K_m$ value for L-pipecolic acid was calculated to be 1.25 mM. From the secondary plots of intercept at the ordinate versus reciprocal concentrations of $NAD^+$, the apparent $K_m$ value for $NAD^+$ was calculated to be 0.18 mM as shown in Figure 3.28 B.

## 3.8 Production of L-2-aminoadipic acid

Production of L-2-aminoadipic acid by the purified LysDH and the purified P6CDH was performed as described in section 2.15. As shown in Figure 3.29, the relative mobility ($R_f$) value of standard L-pipecolic acid, standard L-lysine, standard L-aminoadipic acid, P6C solution and L-2-aminoadipic acid solution were 0.42, 0.10, 0.28, 0.18 and 0.28, respectively. After that, product from the enzyme reaction was quantified by its intensity on TLC plate using Image Master 2D platinum V.6.0, compared with standard L-2-aminoadipic acid. The standard curve was shown in Appendix I. The product yield was calculated to be 98%.

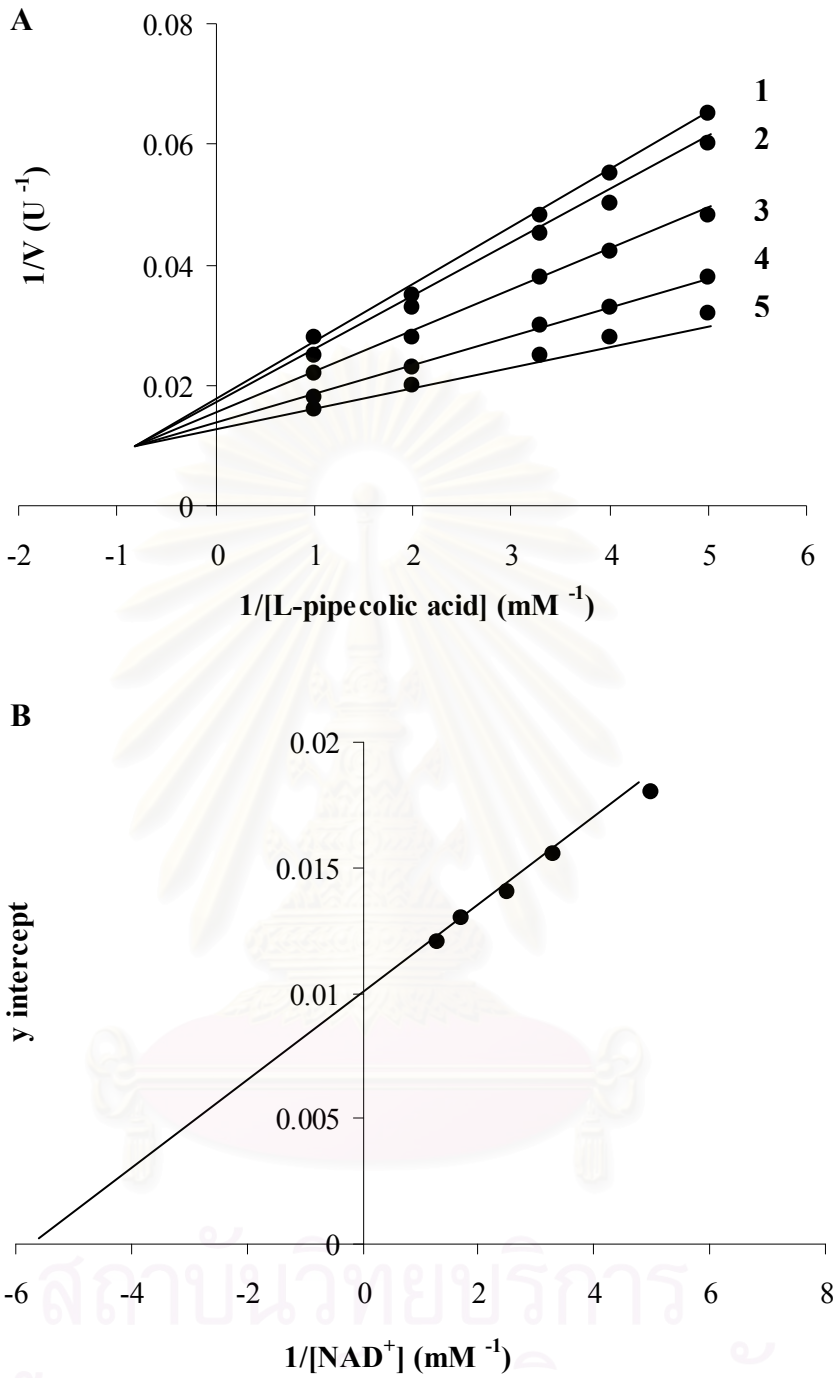



**Figure 3.28 Initial velocity patterns for P6CDH reaction**

**A** Double-reciprocal plots of initial velocities versus L-pipecolic acid concentrations at a series of fixed concentrations of $NAD^+$. Concentrations of $NAD^+$ were 0.2, 0.3, 0.4, 0.6 and 0.8 mM, respectively.

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

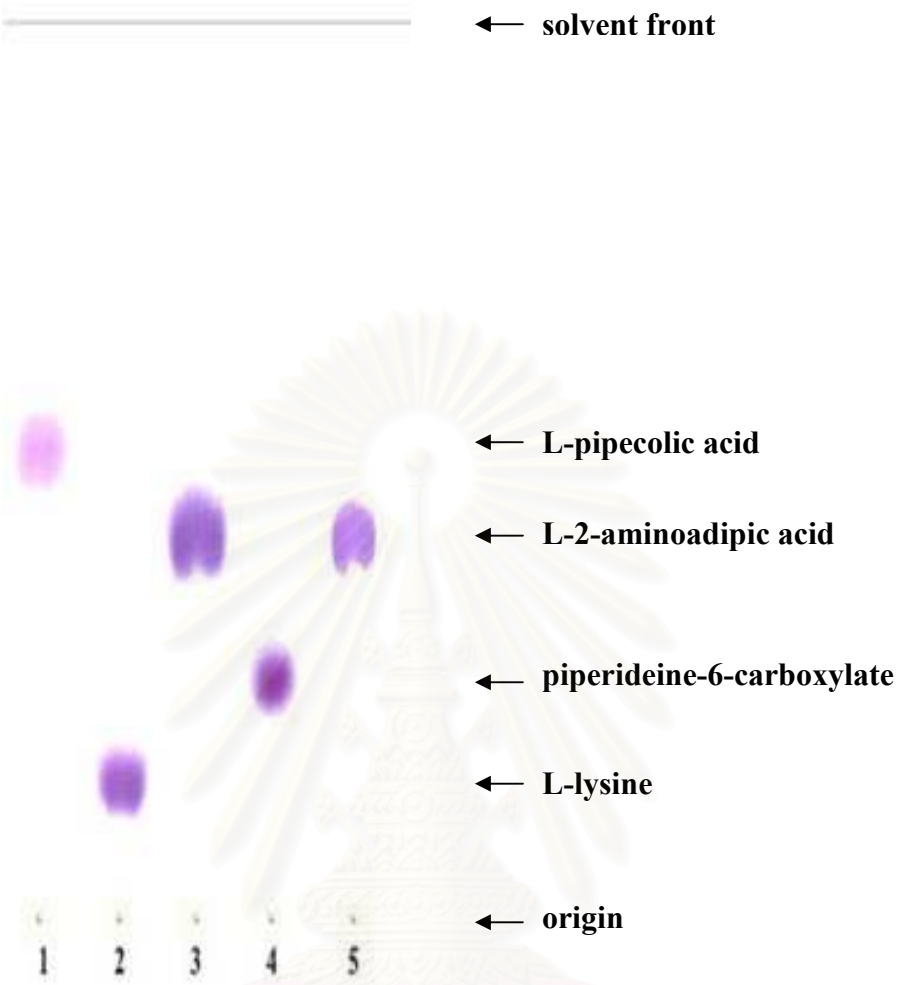



**Figure 3.29 TLC analysis of L-2-aminoadipic acid production**

Lane 1 = standard L-pipecolic acid
Lane 2 = standard L-lysine
Lane 3 = standard L-2-aminoadipic acid
Lane 4 = P6C solution obtained from LysDH reaction
Lane 5 = L-2-aminoadipic acid obtained from P6CDH reaction

# CHAPTER IV

## DISCUSSION

In lysine catabolic pathway, lysine can be converted into L-2-aminoadipic semialdehyde, which spontaneously forms piperideine-6-carboxylate, by lysine-6-aminotransferase or lysine-6-dehydrogenase. The piperideine-6-carboxylate is transformed to L-2-aminoadipic acid by piperideine-6-carboxylate dehydrogenase. To study of P6CDH, P6C is required as a substrate in assay reaction, however, there is no P6C supplied by chemical companies. Fuente *et al*., (1997) who firstly discovered piperideine-6-carboxylate dehydrogenase in *Streptomyces clavuligerus* freshly prepared P6C by chemical synthesis before each assay (Angel *et al*., 1995).

Ruldeekulthamrong (2007) cloned and expressed *lysdh* gene encoding lysine-6-dehydrogenase from *Achromobacter denitrificans* into *E. coli* BL21 (DE3) using pET-17b vector. The optimum condition for *lysdh* gene expression was induction with 0.2 mM IPTG for 4 hours. Recombinant enzyme was purified 2.8 fold with 47% yield by procedure involving DEAE-Toyopearl column chromatography and DEAE-Sephadex A50 column chromatography. In this research, *E. coli* BL21 (DE3) containing *lysdh* gene from *A. denitrificans* was used as a source of LysDH for coupling reaction with P6CDH.

### 4.1 Purification of L-lysine-6-dehydrogenase

The procedures for purification steps were followed the method of Ruldeekulthamrong (2007). The first step in the purification of an intracellular protein is the preparation of an extract containing the protein in a soluble form. Cell wall of *E. coli* clone containing *lysdh* gene was disrupted by ultrasonication. Cell free extract was applied into DEAE-Toyopearl, an anion exchanger which has negative charge counter-ion of diethylaminoethyl. Using this column, 53.8% of the other bulk proteins were eliminated with 92.1% recovery of LysDH activity. The purity of the enzyme was increased to 1.99 times.

DEAE-Sephadex A50 column chromatography was used in next purification step. This column is molecular sieve with weak anion exchanger which is a good choice for the high molecular weight proteins ranging from 30,000 to 100,000. High molecular weight molecules, which are excluded from the bead, may be adsorbed to some extent on the outer surface. From this column, the unwanted protein 25% of the protein obtained in DEAE-Toyopearl 650M step was removed and 84.2% of the enzyme activity was recovered. The overall result of LysDH purification was 77.6% recovery with 2.24 purification fold. In which % recovery was higher than that of Ruldeekulthamrong (47.4% recovery with 2.8 purification fold).

## 4.2 Selection and identification of $NAD^+$ dependent piperideine-6-carboxylate dehydrogenase producing bacteria

Lertmongkolthum (2004) screened $NAD^+$ dependent P6CDH producing bacteria from 20 soil samples collected from various areas in Thailand. Fifty eight isolates could grow in minimum medium containing L-lysine as carbon and nitrogen source. Among them, only 5 isolates showed P6CDH activity.

In this thesis, we used the coupling reaction of purified LysDH and crude extract from the five isolates for P6CDH activity assay. Isolate No. 1, 3, 4 and 5 showed similar pattern of enzyme activity and isolate No. 3 which showed the highest enzyme activity was selected and was identified as *Pseudomonas putida*.

Lysine catabolism in *Pseudomonas putida* occurs via two pathways, one in which the key metabolite is 6-aminovalerate called AMV pathway and another one in which the key metabolite are pipecolate and 2-aminoadipate called AMA pathway (Revelles *et al*., 2005). As shown in Figure 4.1, AMV pathway involves the following steps: L-lysine $\longrightarrow$ 6-aminovaleramide $\longrightarrow$ 6-aminovalerate (AMV) $\longrightarrow$ glutarate semialdehyde $\longrightarrow$ glutarate, which is then channeled to the Krebs cycle. The operation of the second pathway involves an early lysine racemase that converts L-lysine into D-lysine. The pathway involves the following steps: D-lysine $\longrightarrow$ $\Delta^1$-piperideine-2-carboxylate $\longrightarrow$ L-pipecolate $\longrightarrow$ $\Delta^1$-piperideine-6-carboxylate

→ 2-aminoadipate (AMA) → 2-ketoadipate → 2-ketoglutarate. Sequence similarity search of deduced amino acid sequence of protein PP5258 encoded by *amaA* gene revealed high identity (54 to 78 %) with a number of piperideine-6-carboxylate dehydrogenase. Therefore, protein PP5258 should be the P6CDH in our study.

## 4.3 Optimization for piperideine-6-carboxylate dehydrogenase production

The production of P6CDH by *P. putida* was improved by optimization of medium and culture condition: concentration of Bacto-peptone, type and concentration of inducer, pH of medium, cultivation temperature and cultivation time.

The addition of enzyme inducer is usually effective for maximize enzyme level. In this research, compounds related to P6C: L-lysine, L-ornithine and L-pipecolic acid were used as inducers. Specific activity of P6CDH could be induced 7.7, 6.3, and 5.0 fold by addition of 1% L-lysine, L-pipecolic acid and L-ornithine into peptone medium, respectively. Addition of L-lysine at 0.6% final concentration to culture medium gave the optimal induction for P6CDH production. In contrast, 10 mM of L-pipecolic acid repressed P6CDH in *Streptomyces clavuligerus* (48% of control activity) while 10 mM of L-lysine did not effect the enzyme production (Fuente *et al*., 1997).

In culture medium, pH should be controlled to achieve optimal growth rates. *P. putida* produced P6CDH and grew well at pH 8.0. Temperature is one of the most important factors that influence growth rate and enzyme production. *P. putida* showed the highest rate of growth and the enzyme production at 37 $^{o}$C. P6CDH production by *P. putida* which increased rapidly after 6 hours of cultivation and reached the highest level at stationary phase around 15 hours confirms the role of P6CDH in synthesis of secondary metabolite, β-lactam antibiotics.

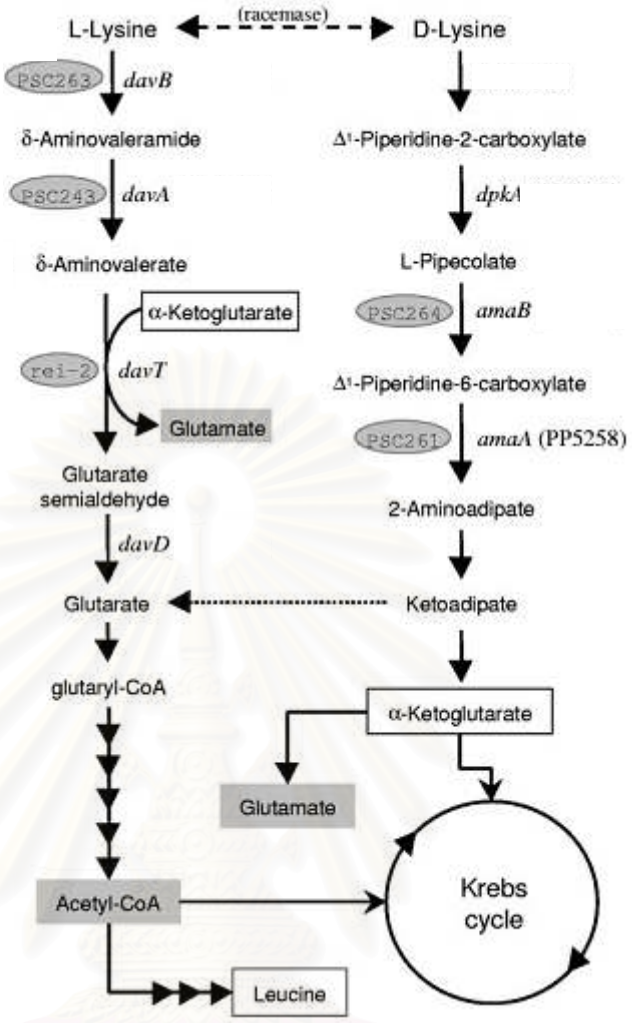



**Figure 4.1 Proposed catabolic pathways for the degradation of L-and D-lysine by bacteria of *Pseudomonas putida***

Reactions from D-lysine to 2-aminoadipate represent the AMA pathway and those from L-lysine to glutaric acid represent the AMV pathway. When they are known, the corresponding gene and the number of its translated product are given.

Source Revelles *et al*., 2005

## 4.4 Purification of piperideine-6-carboxylate dehydrogenase

P6CDH from *P. putida* is an intracellular enzyme. Therefore, disruption method is required to break down cell wall in order to release intracellular protein. In this work, ultrasonication was used which causes cell breakage by cavitations and shear forces. The extraction buffer consisted of phenylmethylsulfonyl fluoride (PMSF) and ethylenediamine tetraacetic acid (EDTA) as thiol protease inhibitor and metalloprotease inhibitor, respectively (Bollag *et al*., 1996). β-Mercaptoethanol which contains a thiol group was added in the buffer in order to protect the enzyme from oxidizing environment. Moreover, glycerol, a thickening agent, was added to increase enzyme stability (Harris and Angal, 1989 and Chambers and Rickwood, 1993).

Solubility differences in salt are frequently exploited to separate protein in early stage of purification protocols. Ammonium sulfate was the salt of choice because it combines many useful features such as salting out effectiveness, pH versatility, high solubility, low heat of solution and low price (Bollag *et al*., 1996). For P6CDH from *P. putida*, 50-60% saturated ammonium sulfate could precipitated most of the enzyme. In this step, 56% of protein was removed, however, 40% of enzyme activity was lost.

Most purification protocols involve some forms of chromatography, which has become an essential tool in protein purification. 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 charge 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. The column matrix is a synthetic polymer containing bound cationic groups. The elution of P6CDH with 0.1 M NaCl indicated that the net change of the enzyme is negative (anion group) at pH 8.0. Consequent upon the result, this column contributed greatly to the purification procedures, with less loss of P6CDH activity (14%) compared to the amount of proteins removed. About 85% of the protein in the step of 50-60% saturated ammonium sulfate precipitation was eliminated.

Hydrophobic interaction chromatography (HIC) can separate protein on the basis of reversible interaction between hydrophobic ligands and non-polar regions on the surface of protein (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 chromatography. Adsorption of proteins to a HIC adsorbent is favored by a high salt concentration in the mobile phase. The elution of solute is accomplished by decreasing the salt concentration with increasing hydrophobic. In this work, the enzyme was eluted from Butyl-Toyopearl column with stepwise of ammonium sulfate. From this step, the unwanted protein about 95% of the protein obtained in DEAE-Toyopearl step was removed.

The last step of purification used Hitrap Q column, a strong anion exchanger chromatography column. After this column, 60% of other proteins were removed and 71% P6CDH activity was still remained. The success of enzyme purification using Hitrap Q column was judged by the homogeneity of P6CDH on non-denaturing electrophoresis gel and SDS-PAGE gel.

It can be concluded that, P6CDH from *P. putida* was purified to 152.4 fold from the cell free extract by a purification procedure involving 50-60% ammonium sulfate precipitation followed by DEAE-Toyopearl column chromatography, Butyl-Toyopearl column chromatography and Hitrap Q column chromatography, respectively.

In 1997, Fuente and coworkers used different technique for purification of P6CDH from *S. clavuligerus*. Blue-Sepharose CL6B column is used in the capture step. This column contains Cibacron Blue F3G-A as the covalently bound ligand which is more selective for $NAD^+$-dependent enzymes. After that, DEAE-Sepharose column, molecular sieve with anion exchange, was used in intermediate step. Gel-filtration chromatography is frequently used as a furnishing step in protein purification. It is also useful for separate proteins with different in size of molecules. Sephadex-G75 consists of cross-linked dextran was used in the last step. The *S. clavuligerus* enzyme was purified 124 fold with 26.5% yield.

## 4.5 Characterization of piperideine-6-carboxylate dehydrogenase

### 4.5.1 Molecular weight determination of piperideine-6-carboxylate dehydrogenase

The molecular weight of the native recombinant enzyme was calculated to be approximately 301,000 Da by gel filtration on TSK Gel G3000 SW column. The molecular weight of subunits was calculated to be 50,200 Da by comparing the mobility on SDS-polyacrylamide electrophoresis to that of standard proteins. Thus, the enzyme consisted of six identical subunits while P6CDH from *S. clavuligerus* and *F. lutescens* were monomer with molecular weight 56,200 and 58,000 Da, respectively (Fuente *et al*., 1997 and Tadashi *et al*., 2000).

### 4.5.2 Substrate specificity of piperideine-6-carboxylate dehydrogenase

Substrate specificity is the ability of enzyme to discriminate between a substrate and competing molecules. Basically, a substrate binding site consists of a cleft on the surface of an enzyme molecule that is complementary in shape to the substrate. The amino acids residues that form the binding site are arranged to interact specifically with the substrate in an attractive manner. Molecules that differ in shape or functional group distribution from the substrate did not productively bind to the

enzyme that means they cannot form enzyme-substrate complexes that lead to the formation of products (Lehinnger, 2000) .

P6CDH from *P. putida* exhibited a significant specificity on P6C. Although the concentration of P6C substrate in the reaction could not be determined, most of lysine was converted to P6C by lysine-6-dehydrogenase. Thus, P6C concentration was near 20 mM. In addition to P6C, L-pipecolic acid, L-proline, L-lysine, and L-ornithine could act as substrates with 22, 18, 12 and 10% relative activity, respectively compared with that from P6C. All of these compounds are analogs of P6C or 2-aminoadipate-6-semialdehyde and they can induce P6C production. The structure of L-pipecolic acid, L-proline, L-lysine and L-ornithine are shown in Appendix G. Some L-aliphatic amino acids, L-norleucine and L-valine were very poor substrates. On the other hand, other aromatic amino acids: L-phenylalanine, *m*-fluoro-DL-phenylalanine**,** *o*-fluoro-DL-phenylpyruvate, and *p*-fluoro-DL-phenylalanine, L-aliphatic amino acids: L-alanine, L-isoleucine, L-leucine and L-threonine, sulfer-containing amino acid: L-methionine and D- amino acids could not act as substrates. In the next experiments, L-pipecolic acid which gave 22% relative activity was used as the substrate for P6CDH assay in parallel with coupling reaction assay.

### 4.5.3 Coenzyme specificity of piperideine-6-carboxylate dehydrogenase

In this research, $NAD^+$ was replaced by some of the $NAD^+$ analogs as coenzyme for the P6CDH. $NADP^+$, which differs from $NAD^+$ only by the addition of a phosphoric group at C-2 position of $NAD^+$-adenosyl ribose, was inert for P6CDH activity. The $NAD^+$ analogs modified at an amino group in the adenine moiety, nicotinamide hypoxanthine dinucleotide (deamino-$NAD^+$) could be used as coenzymes of the P6CDH from *P. putida*. This suggests that the amino group in the adenine moiety of $NAD^+$ is not important for the coenzyme specificity. In addition, the enzyme could not use thionicotinamide adenine dinucleotide and 3-pyridinealdehyde adenine dinucleotide, which their amino groups in the nicotinamide moiety are substituted by OH group and H atom, respectively. The result is similar to that of P6CDH from *S. clavuligerus* that used $NAD^+$ as coenzyme, but no $NADP^+$ or

FAD (Fuente *et al*., 1997). The structure of $NAD^+$ and $NAD^+$ analogs are shown in Appendix H.

### 4.5.4 Effect of pH on piperideine-6-carboxylate dehydrogenase activity and stability

The enzyme activity is often limited to a relatively narrow pH range. Beyond this range, the enzyme activity is dramatically decreased. A pH change affects a reaction by altering the state of ionization of the enzyme, the substrate, or an intermediate or causing a change in the conformation of the enzyme or the substrate, or both. The enzyme forming an enzyme-substrate complex requires electrostatic attraction between oppositely charged groups of the enzyme and the substrate. Within a relatively narrow pH range, the charges of these groups remain essentially the same so that the rate, a function of substrate binding, does vary greatly. At a sufficiently low or high pH, some of these groups lose their charge, thereby impairing binding. These new interactions may produce a conformational change at the active site, affecting the site's capacity to bind substrate. The enzyme may undergo conformational changes and lose their capacity to bind to the substrate.

The pH which P6CDH showed maximum activity was 9.2 and 8.7 for coupling reaction and P6CDH reaction, respectively. The optimum pH for coupling reaction was appropriated for both LysDH and P6CDH. This was similar to P6CDH from *S. clavuligerus* (pH 8.7). In addition, acetate buffer, potassium phosphate buffer and Tris-HCl buffer were not proper for the activity of P6CDH from *P. putida*, due to the low activity observed with these buffers. The glycine-KCl-KOH buffer was a suitable buffer for the enzyme. This kind of buffer is widely used for the assay of amino acid dehydrogenases.

The pH stability of an enzyme depends on many factors including temperature, ionic strength, and chemical nature of the buffer, concentration of various stabilizer, concentration of substrates or cofactors of the enzyme and enzyme concentration (Segal, 1976). P6CDH was stable over a pH range of 6.5 to 11.5 and 7.0

to 11.0 for coupling reaction and P6CDH reaction, respectively, upon incubation at 30°C for 20 minutes.

### 4.5.5 Effect of temperature on piperideine-6-carboxylate dehydrogenase activity and stability

The influence of temperature on an enzymatic reaction is resulted from two opposing effects, an increase in rate and an increase in denaturation. The optimum temperature of P6CDH from *P. putida* was 45 °C for both coupling reaction and P6CDH reaction. When the enzyme was incubated at various temperatures for 10 minutes, it kept 100% activity at 45 $^{o}$C and dropped about 50% at 55 $^{o}$C. Hence, time course of the enzyme stability was further performed at 45 °C. It was found that the activity was not lost upon incubation for 3 and 2 hours for coupling reaction and P6CDH reaction, respectively, and about half the enzyme activity was lost when incubated for 3 days. Due to its temperature stability, the enzyme seems to be a good candidate for industrial applications.

### 4.5.6 Effect of glycerol on piperideine-6-carboxylate dehydrogenase stability

In this research, preliminary purification procedures for P6CDH did not use glycerol in the buffer. The P6CDH activity was dramatically decreased in the first week. Purification of P6CDH from *S. clavuligerus* required 5% glycerol in the buffer to stabilize P6CDH. Therefore, the effect of glycerol on stability of P6CDH form *P. putida* was tested. The enzyme retained more activity when the concentration of glycerol was increased. The result indicated that thickening agent such as glycerol increased stability of P6CDH. The purified P6CDH from *P. putida* showed a half life of 8, 12, 23, and 29 days at 4 ºC in 0, 5, 10, and 20% glycerol, respectively, while the purified P6CDH from *S. clavuligerus* showed a half life of 3 days at 4 ºC in 5% glycerol (Fuente *et al*., 1997).

### 4.5.7 Effect of metal ions on piperideine-6-carboxylate dehydrogenase

The P6CDH activity was measured in the presence to metal ions. The monovalent and divalent cations, tested in this work, had a little or no effect on its activity, except $FeSO_4$ and $CuSO_4$ which completely inhibited the enzyme activity at 1 mM final concentration, for both coupling reaction and P6CDH reaction. In addition $FeCl_3$ showed strong inhibition with 40% retained activity.

### 4.5.8 Inhibitory effect of various amino acids on piperideine-6-carboxylate dehydrogenase

Many types of molecule can interfere the activity of enzyme. Substrate analog is one of enzyme inhibitors. In this work, nonsubstrate amino acids were investigated. Inhibitory effect of nonsubstrate D- and L- amino acids on the oxidative deamination was determined. The enzyme was mildly inhibited by some D- and L- amino acids with aromatic side chain (*m*-fluoro-DL-phenylalanine**,** *o*-fluoro-DL-phenylpyruvate, *p*-fluoro-DL-phenylalanine, D-phenylalanine, and D-tryptophan), aliphatic side chains (D-leucine, D-valine, L-leucine and L-norvaine), and non-polar side chains (L-methionine). Therefore, these amino acids should have their binding site on the enzyme.

## 4.6 Kinetic studies of piperideine-6-carboxylate dehydrogenase

Kinetic parameters of P6CDH from *P. putida* against its substrates were determined by initial velocity studies. Unfortunately, we could not prepare piperideine-6-carboxylate, a physiological substrate of P6CDH, in pure form. Thus, in this research we could determine only the apparent $K_m$ values for L-pipecolic acid and $NAD^+$.

Double-reciprocal plots of initial velocity versus L-pipecolic acid concentration at a series of fixed concentration of $NAD^+$ gave the lines intersecting to the left of the vertical axis. The apparent $K_m$ value for L- pipecolic was calculated to be 1.25 mM from this plot. The apparent $K_m$ value of $NAD^+$ was calculated to be 0.18 mM from the secondary plots of the intercepts at the ordinate versus reciprocal concentration of $NAD^+$. P6CDH from *S. clavuligerus* showed the apparent $K_m$ values against $NAD^+$ (0.115 mM) similar to that of *P. putida* while $K_m$ values against P6C was 0.014 mM. The properties of P6CDH from *P. putida* and *S. clavuligerus* are compared as shown in Table 4.1.

## 4.7 Production of L-2-aminoadipic acid

In this research, we used the coupling reaction of LysDH and P6CDH for L-2-aminoadipic acid production. It was the first studied on L-2-aminoadipic acid production by enzymatic method. Although the optimum condition was not investigated, the product yield was calculated to be 98% compared with standard L-2-aminoadipic acid. The results obtained from this research indicated that P6CDH from *Pseudomonas putida* shows high possibility to be used for synthesis of L-2-aminoadipic acid.

**Table 4.1 Properties of P6CDH from *Streptomyces clavuligerus* and *Pseudomonas putida***

| **Properties** | ***Streptomyces clavuligerus*** | ***Pseudomonas putida*** |
|---|---|---|
| Specific activity of final preparation (U/mg protein) | 3,307 | 3,200 |
| Molecular mass of native enzyme (kDa)<br>Molecular mass of subunit | 56,200<br>56,200 | 301,000<br>50,200 |
| Number of subunit | 1 | 6 |
| PH optimum for oxidative deamination | 8.7 | 8.7 |
| pH stability | - | 7.0-11.0 |
| Optimum temperature (ºC) | - | 45 |
| Thermostability (% remaining activity after incubation at 45 ºC for 10 minutes) | - | 2 hours |
| Apparent $K_m$ (mM) for<br>- P6C<br>- L-Pipecolic acid<br>- $NAD^+$ | <br>0.014<br>-<br>0.115 | <br>-<br>1.25<br>0.18 |

Source Fuente *et al*., 1997

# CHAPTER V

## CONCLUSIONS

1. The LysDH from *E. coli* BL21 (DE3) was purified to homogeneity from a crude enzyme by column chromatographies on DEAE-Toyopearl, and DEAE-Sephadex A50. The enzyme was purified about 2.24 fold from the crude enzyme with 77.6% recovery.

2. The molecular weight of the subunits was determined to be about 40,000 Da.

3. The bacteria, screened from soil samples which produced piperideine-6-carboxylate dehydrogenase, was identified as *Pseudomonas putida*.

4. The optimum condition for piperideine-6-carboxylate dehydrogenase production of *Pseudomonas putida* was cultivated in 0.8% peptone medium, pH 8.0 containing 0.6% L-lysine at 37 $^{o}$C with 250 rpm shaking for 15 hours.

5. The P6CDH from *P. putida* was purified to homogeneity from a crude enzyme by 50-60% ammonium sulfate precipitation, column chromatographies on DEAE-Toyopearl, Butyl-Toyopearl and Hitrap Q. The enzyme was purified 152 fold from the crude enzyme with 18.7% recovery.

6. The molecular weight of the native enzyme was determined to be about 301,000 Da and the enzyme consisted of 6 identical subunits (approximately 50,200 Da).

7. The enzyme had high substrate specificity on piperideine-6-carboxylate (P6C).

8. Piperideine-6-carboxylate dehydrogenase retained 50% of its activity after incubation at 4 $^{o}$C in the buffer containing 0, 5, 10, and 20% glycerol for 8, 15, 22, and 29 days, respectively.

9. The enzyme required $NAD^+$ as a natural coenzyme. $NADP^+$ was inert while nicotinamide hypoxanthine dinucleotide showed 1.2 times higher activity than $NAD^+$.

10. The optimum pH of the enzyme for couple reaction and P6CDH reaction were 9.2 and 8.7, respectively.

11. The optimum temperature of the enzyme was 45 $^oC$ in both couple reaction and P6CDH reaction.

12. The enzyme was stable over a pH range from 6.5 to 11.5 for couple reaction and from 7.0 to 11.0 for P6CDH reaction.

13. The enzyme was fully stable at 45 $^oC$ for 3 hours and retained 50% of its activity after incubation at same temperature for 3 days for couple reaction and fully stable at 45 $^oC$ for 2 hours and retained 50% of its activity after incubation at same temperature for 2 days for P6CDH reaction.

14. The enzyme activity was completely lost in the presence of 1 mM $CuSO_4$ and $FeSO_4$.

15. The apparent $K_m$ for L-pipecolic acid and $NAD^+$ were 1.25 and 0.18 mM, respectively.

16. When P6CDH from *P. putida* was used for the L-2-aminoadipic acid production using correspouding L-2-aminoadipic acid as substrate. The product yield was 98%.

# REFERENCES


Aharonowitz, Y., Cohen, G. and Martin, J.F., 1992. Penicillin and cephalosporin biosynthetic genes: structure, organization, regulation, and evolution. Annu. Rev. Microbiol. 46: 461-496.

Angel, R., Juan, F.M., Angeles, L., Paloma, L., and Cristina, E., 1995. Chemical synthesis of allysine ethylene acetal and conversion *in situ* into $\Delta^1$-piperideine-6-carboxylic acid: key intermediate of the 2-aminoadipic acid for β-lactam antibiotics biosynthesis. Medicinal Chem. 3: 1237-1240.

Calvert, A.F. and Rodwell, V.W., 1966. Metabolism of pipecolic acid in *Pseudomonas* species. J. Biochem. 241: 409 - 414.

Chang, Y.F. and Adams, E., 1974. D-Lysine catabolic pathway in *Pseudomonas putida*: Interrelations with L-lysine catabolism. J. Bacteriol. 117: 753-764.

Christina, E., Emilio, A., Eduardo, and Juan, F., 1994. Catabolism of lysine in *Penicillium chrysogenum* leads to formation of aminoadipic acid, a precursor of penicillin biosynthesis. J. Gen. Microbiol. 60: 1705-1710.

De La Fuente, J.L., Rumbero, A.,Martin, J.F.,and Liras, P., 1997. Δ-1-Piperideine-6-carboxylate dehydrogenase, a new enzyme that forms L-2-aminoadipate in *Streptomyces clavuligerus* and other cephamycin C-producing actinomycetes. J. Biochem. 327: 59-64.

Fothergill, J.C. and Guest, J.R., 1997. Catabolism of L-lysine by *Pseudomonas aeruginosa.* J. Gen. Microbiol. 99: 139 - 155.

Francisco, J.P., Antonio, R.G., Francisco, J.E., Juan, F.M., and Paloma, L., 1998. The *pcd* gene encoding piperideine-6-carboxylate dehydrogenase involved in biosynthesis of 2-aminoadipic acid is located in the cephamycin cluster of *Streptomyces clavuligerus*. J. Bacteriol. 180: 4753-4756.

Haruo, M., and Susumn, N., 1982. Occurrence of L-lysine 6-dehydrogenase in *Agrobacterium tumefaciens*. J. Bacteriol. 150: 398-401.

Haruo, M., Hiroyuki, H., Haruhiko, U., Shinji, N., and Susumu, N., 1989. Properties of L-lysine 6-dehydrogenase from *Agrobacterium tumefaciens*. J. Biochem. 105: 1002-1008.

Hiroyuki, H., Haruo, M., Shinji, N., and Susumu, N., 1989. Activation of L-lysine 6-dehydrogenase from *Agrobacterium tumefaciens* by several amino acids and monocarboxylates. J. Biochem. 106: 76-80.

Hui-Qiu, W., Urban, U., and Robert, S., 1995. L-2-Aminoadipic acid as a regulator of kynurenic acid production in the hippocampus: A microdialysis study in freely moving rats. J. Pharmacol. 281: 55-61.

Julian, R.M., Takuichi, S., Andrew, J.W., Tracey, A.M., John, C.F., Sarah, J.H., and William, G.W., 1998. Design and evaluation of useful bacterium specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl. Microbiol. 64: 795-799.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Leitao, A.L., Enguita, F.J., Martin, J.F., and Santos, J.F., 2001. Effect of exogenous lysine on the expression of early cephamycin C biosynthetic genes and antibiotic production in *Nocardia lactamdurans* MA4213. Appl. Microbiol. Biotechnol. 56: 670-675.

Leopoldo, N., Martin, V., Javier, C., Ricardo, V.U., Monica, L.M., Oscar, B., and Juan, F.M., 2004. Inactivation of the *lys*7 gene, encoding saccharopine reductase in *Penicillium chrysogenum*, lead to accumulation of the secondary metabolite precursors piperideine-6-carboxylic acid and pipecolic acid from 2-aminoadipic acid. Appl. Microbiol. 70: 1031-1039.

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

Lertmongkolthum, M., 2004. Screening of piperideine-6-carboxylate dehydrogenase producing bacteria. Senior Project. Department of Biochemistry, Science, Chulalongkorn University.

Madduri, K., Stuttard, C. and Vining, L.C., 1989. Lysine catabolism in *Streptomyces* spp. is primarily through cadaverine: β-lactams producers also make aminoadipate. J. Bacteriol. 171: 299-302.

Mark, Z., and Michael, D.J., 1999. Lysine biosynthesis and metabolism in fungi. Nat. Prod. Rep. 17: 85-97.

May, J.T., Yung-Feng, C., Robert, S., and Neville, B., 1996. Characterization of L-2-aminoadipic acid transport in cultured rat astrocytes. Brain Research. 741: 166-173.

Mehta, R.J., Speth, J.L., and Nash, C.H., 1979. Lysine stimulation of cephamycin C synthesis. J. Appl. Microbiol. 8: 177-182.

Misono, H., Hashimoto, H., Uehigashi, H., Nagata, S. and Nagasaki, S., 1989. Properties of L-lysine dehydrogenase from *Agrobacterium tumefaciens*. J. Biochem. 105: 1002-1008.

Mochizuki, K., Yamazaki, Y., Maeda, H., and Suzuki, H., 1989. Production of L-2-aminoadipic acid using microorganism. Japanese patent JP1098495.

Morgan, H., Toshihisa, O., Naoki, N.K., and Haruhiko, S., 2004. Highly stable L-lysine 6-dehydrogenase from the thermophile *Geobacillus stearothermophilus* isolated from a Japanese hot spring: Characterization, gene cloning and sequencing, and expression. J. Microbiol. 70: 937-942.

Nakata, K., Narita, T., Tsenekawa, H. and Yoshioka, T., 1999. Process for producing L-2-aminoadipic acid. United States patent 5, 906,927.

Nakayama, K., 1994. Production of L-2-aminoadipic acid. Japanese patent 06-181787.

Olga, R., Manuel, E.U., Tobias, F., Uwe, S., and Juan, L.R., 2005. Multiple and interconnected pathways for L-lysine catabolism in *Pseudomonas putida* KT2440. J. Bacteriol. 187: 7500-7510.

Paloma, L., Antonio, R.G., and Juan, F.M., 1998. Evolution of the clusters of genes for β-lactam antibiotics: Combinatorial assembly of new β-lactam. J. Microbiol. 1: 271-278.

Paloma, L., and Juan, F.M., 2006. Gene clusters for β-lactam antibiotics and control of their expression: Why have clusters evolved, and from where did they originate. J. Microbiol. 9: 9-19.

Perez-Llarena, F.J., Rodriguez-Garcia, A., Enguita, F.J., Martin, J.F., and Liras, P., 1998. The *pcd* gene encoding piperideine-6-carboxylate dehydrogenase involved in biosynthesis of aminoadipic acid is located in the cephamycin cluster of *Streptomyces clavuligerus*. J. Bacteriol. 180: 4753-4756.

Ruldeekulthamrong, P., 2007. Gene cloning and characterization of L-lysine 6-dehydrogenase from Agromobacter denitrificans for L-pipecolic acid production. Doctor's Thesis. Biological Sciences Program, Chulalongkorn University.

Romero, J., Martin, J.F., Liras, P., Demain, A.L., and Rius, N., 1997. Partial purification, characterization and nitrogen regulation of the lysine 6-aminotransfrase of *Streptomyces clavuligerus*. J. Microbiol., and Biotechnol. 18: 241-246.

Soda, K., Misono, H. and Yamamoto, T., 1968. L-Lysine-alpha-ketoglutarate aminotransferase. Identification of a product, Δ-1-piperideine-6-carboxylic acid. J. Biochem. 7: 4102-4109.

Tadashi, F., Manabu, M., Hitori, A., and Hiroshi, T., 2002. Biotransformation of L-lysine to L-pipecolic acid catalyzed by L-lysine 6-aminotransferase and pyrroline-5-carboxylate reductase. Biosci. Biotechnol. Biochem. 66: 622-627.

Tadashi, F., Takao, N., Hitosi, A., Naoki, A., and Kunio, I., 2000. Cloning and characterization of *pcd* encoding $\Delta^1$-piperideine-6-carboxylate dehydrogenase from *Flavobacterium lutescens* IFO3084. J. Biochem. 128: 975-982.

Yung-Feng, C., and Elijah, A., 1974. D-Lysine catabolic pathway in *Pseudomonas putida*: Interrelations with L-lysine catabolism. J. Bacteriol. 117: 753-764.

Yung-Feng, C., Pradeep, G., and Vallabhaneni, V.R., 1990. L-Pipecolic acid metabolism in human liver: L-2-Aminoadipate 6-semialdehyde oxidoreductase. J. Biophys. 1038: 300-305.

# APPENDICES

# APPENDIX A

## Preparation for protein determination

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

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

| | | |
|---|---|---|
| Potassium tartate | 1 | g |
| Copper sulfate | 0.5 | g |

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

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

| | | |
|---|---|---|
| Sodium carbonate | 20 | g |
| Sodium hydroxide | 4 | g |

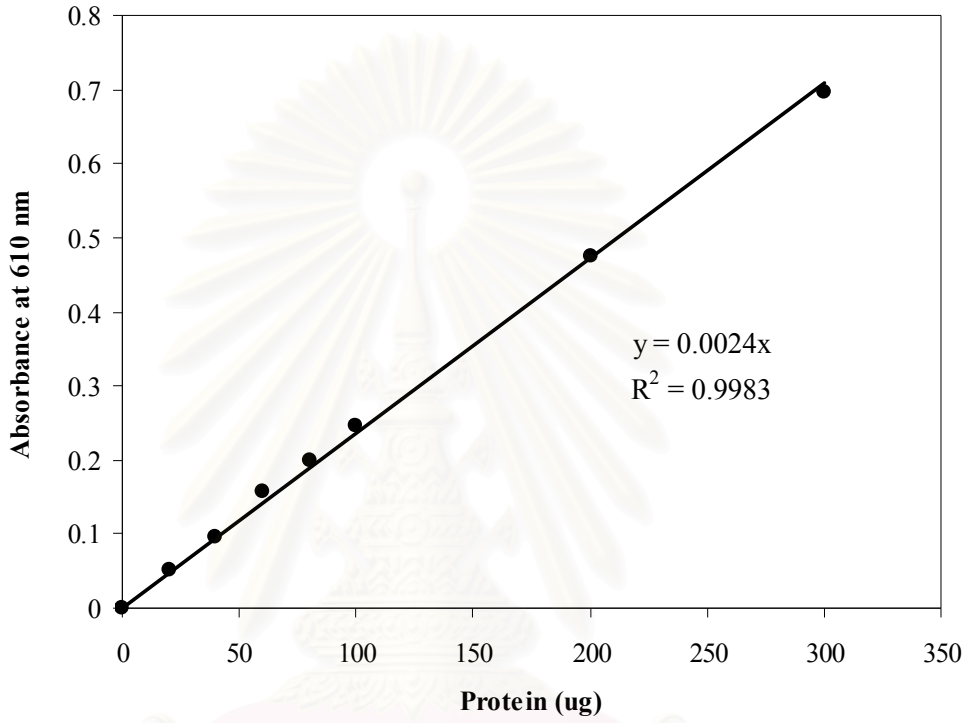
Dissolved in distilled water to 1 liter.

**Solution C (phenol reagent)**

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

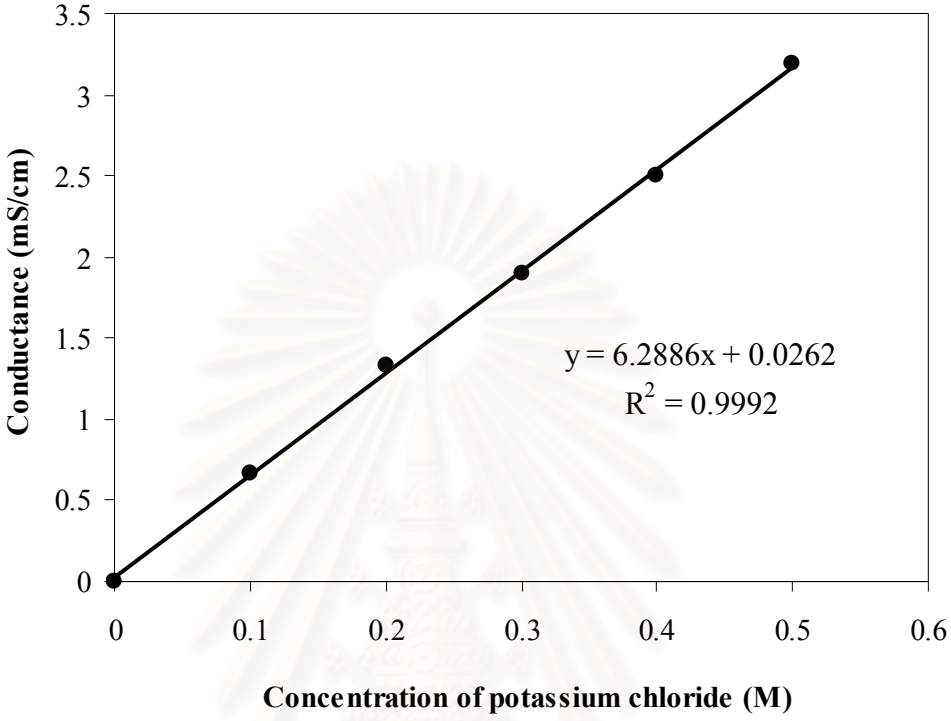
# APPENDIX B

## Standard curve for protein determination by Lowry's method



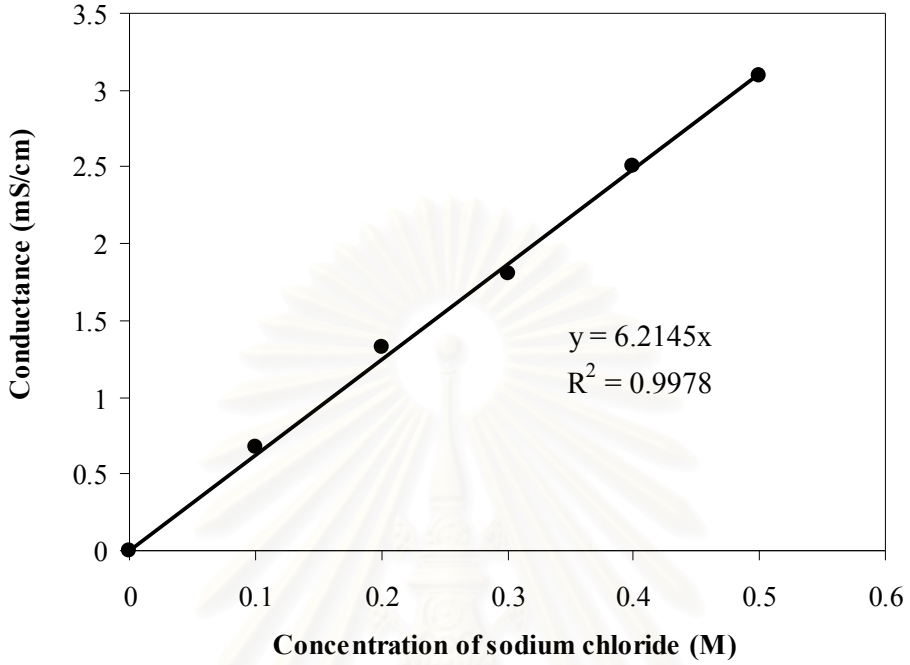
# APPENDIX C

## Calibration curve for conductivity of potassium chloride



# APPENDIX D

## Calibration curve for conductivity of sodium chloride



# APPENDIX E

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

### 1. Stock solutions

**2 M Tris-HCl (pH 8.8)**

| | | |
|---|---|---|
| Tris (hydroxymethyl)-aminomethane | 24.2 | g |

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

**1 M Tris-HCl (pH 6.8)**

| | | |
|---|---|---|
| Tris (hydroxymethyl)-aminomethane | 12.1 | g |

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

**1% (w/v) Bromophenol blue**

| | | |
|---|---|---|
| Bromophenol blue | 100 | mg |

Brought to 10 ml with distilled water and stirred until dissolved.
The aggregated dye was removed by filtration.

### 2. Working solutions

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

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

Adjusted volume to 100 ml with distilled water.

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

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

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

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

## APPENDIX E (continued)

**10% (w/v) Ammonium persulfate**

| | | |
|---|---|---|
| Ammonium persulfate | 0.5 | g |
| Distilled water | 5.0 | ml |

**Electrophoresis buffer (25 mM Tris, 192 mM glycine)**

| | | |
|---|---|---|
| Tris (hydroxymethyl)-aminomethane | 3.0 | g |
| Glycine | 14.4 | ml |

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

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

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

### 3. Native-PAGE

**7.7% Separating gel**

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

**5.0% Stacking gel**

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

# APPENDIX E (continued)

## 4. Protein staining solution

**Staining solution, 1 liter**

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

**Destaining solution, 1 liter**

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

## 5. Enzyme activity staining solution

**1 M Tris-HCl, pH 8.5**

Tris (hydroxymethyl)-aminomethane 6.06 g

Adjusted to pH 8.5 with 1 N HCl and made up volume to 100 ml with distilled water

**40 mM L-lysine**

L-lysine 0.066 g

Dissolved with 10 ml distilled water

**50 mM $NAD^+$**

$NAD^+$ 0.359 g

Dissolved with 10 ml distilled water

**0.25 mg/ml phenazine methosulfate**

Phenazine methosulfate 0.0025 g

Dissolved with 10 ml distilled water

**2.5 mg/ml nitroblue tetrazolium**

Nitroblue tetrazolium 0.025 g

Dissolved with 10 ml distilled water

# APPENDIX E (continued)

Activity staining solution (4.25 mM Tris-HCl, pH 8.5, 40 μM L-lysine

50 μM $NAD^+$, 250 μg phenazine methosulfate and 2.5 mg nitroblue tetrazolium)

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

# APPENDIX F

## Preparation for denaturing polyacrylamide gel electrophoresis

### 1. Stock solution

**2 M Tris-HCl (pH 8.8)**

Tris (hydroxymethyl)-aminomethane 24.2 g

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

**1 M Tris-HCl (pH 6.8)**

Tris (hydroxymethyl)-aminomethane 12.1 g

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

**10% (w/v) SDS**

Sodium dodecyl sulfate (SDS) 10 g

Added distilled water to a total volume of 100 ml.

**50% (w/v) Glycerol**

100% Glycerol 50 ml

Added distilled water to a total volume of 100 ml.

**1% (w/v) Bromophenol blue**

Bromophenol blue 100 mg

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

The aggregated dye was removed by filtration.

### 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.

Filtered and stored in dark (brown bottle) at $4^{o}C$

# APPENDIX F (continued)

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

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

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

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

**10% (w/v) Ammonium persulfate**

| | | |
|---|---|---|
| Ammonium persulfate | 0.5 | g |
| Distilled water | 5.0 | ml |

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

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

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

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

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

# APPENDIX F (continued)

## 3. SDS-PAGE

**10% Separating gel**

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

**5.0% Stacking gel**

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

## 4. Protein staining solution

**Staining solution, 1 liter**

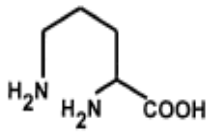
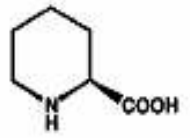
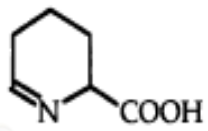
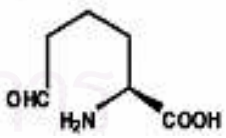
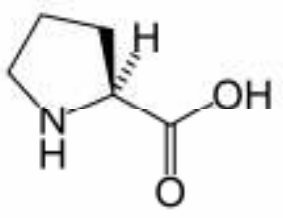
| | | |
|---|---|---|
| Coomassie brilliant blue R-250 | 1.0 | ml |
| Methanol | 450 | ml |
| Distilled water | 450 | ml |

**Destaining solution, 1 liter**

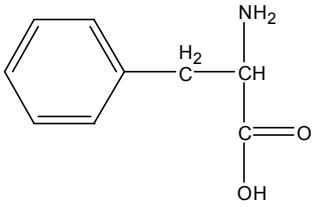
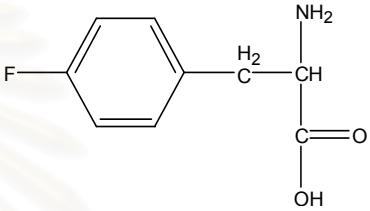
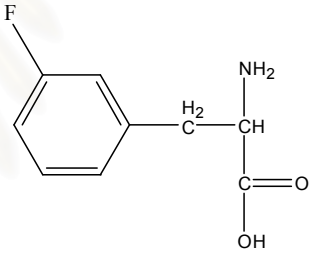
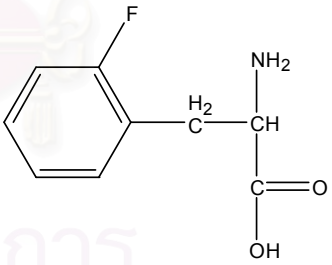
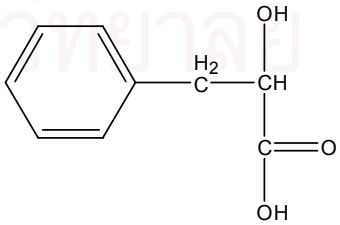
| | | |
|---|---|---|
| Methanol | 100 | ml |
| Glacial acetic acid | 100 | ml |
| Distilled water | 800 | ml |

# APPENDIX G

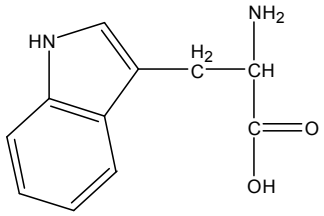
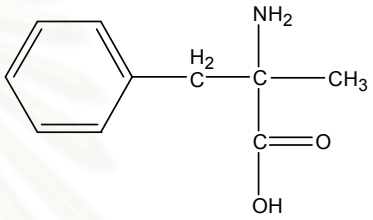
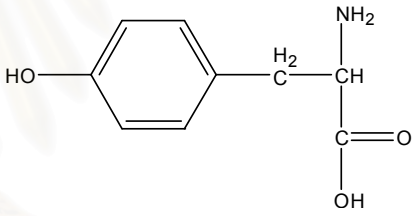
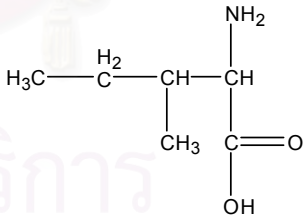
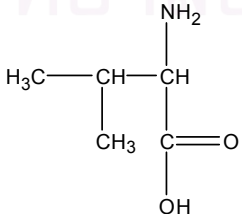
## Structure of amino acids and their analogs

| Amino acids and analogs | Structure |
|---|---|
| L-lysine |  |
| L-pipecolic acid |  |
| piperideine-6-carboxylate |  |
| L-2-aminoadipic semialdehyde |  |
| L-proline |  |

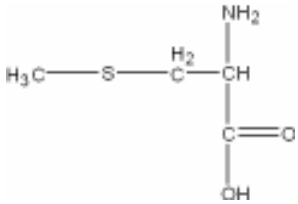
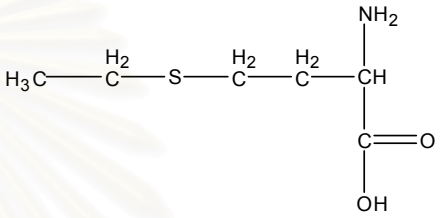
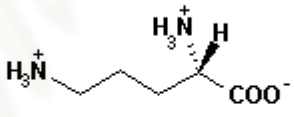
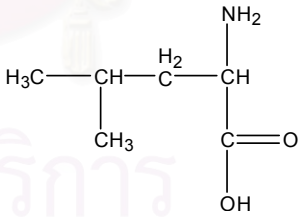
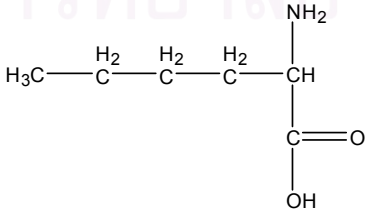
# APPENDIX G (continued)

| Amino acids and analogs | Structure |
|---|---|
| L-phenylalanine |  |
| *p*-fluoro-DL-phenylalanine |  |
| *m*-fluoro-DL-phenylalanine |  |
| *o*-fluoro-DL-phenylalanine |  |
| L-2-phenyllactate |  |

# APPENDIX G (continued)

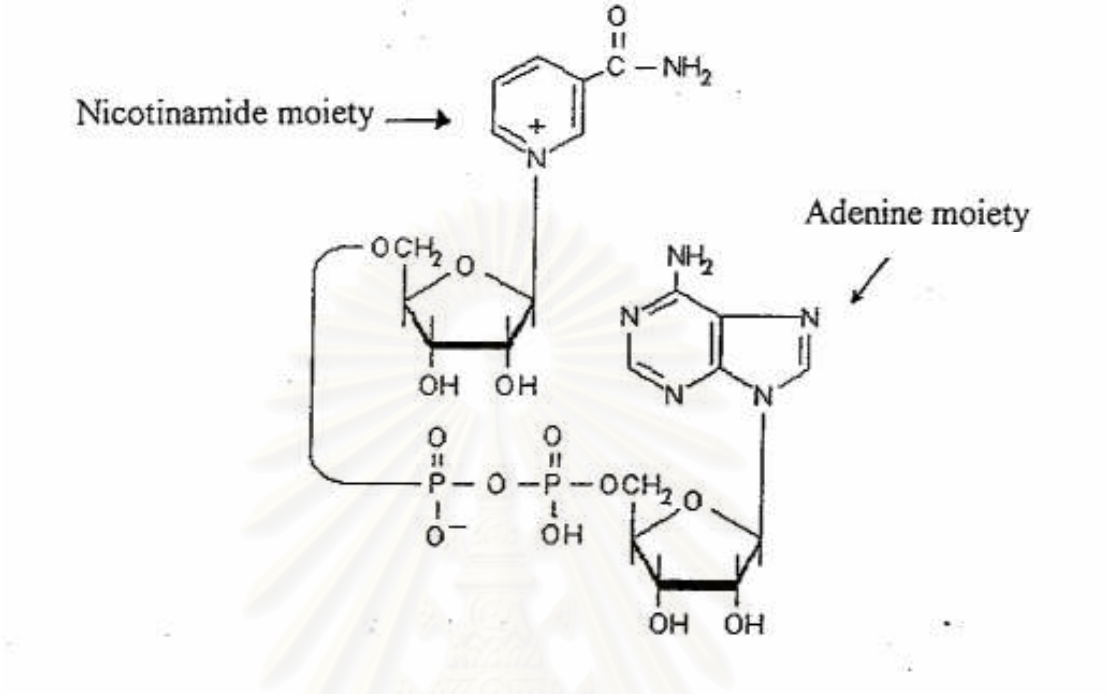
| Amino acids and analogs | Structure |
|---|---|
| L-tryptophan |  |
| 2-methyl-DL-phenylalanine |  |
| L-tyrosine |  |
| L-isoleucine |  |
| L-valine |  |

# APPENDIX G (continued)

| Amino acids and analogs | Structure |
|---|---|
| *S*-methyl-L-cysteine |  |
| L-ethionine |  |
| L-ornithine |  |
| L-leucine |  |
| L-norleucine |  |

# APPENDIX H

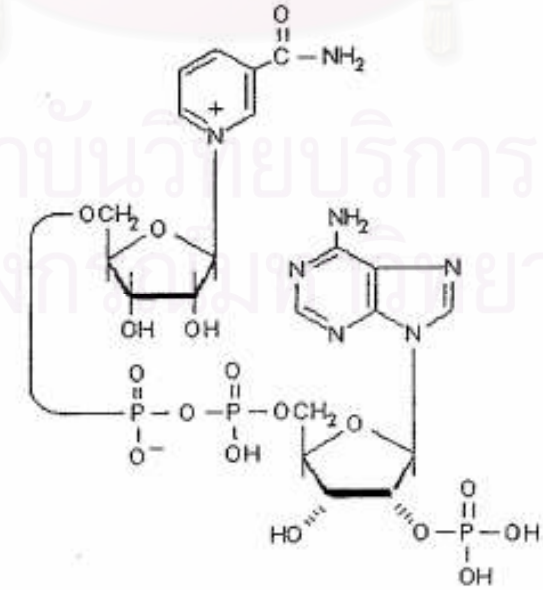
## $NAD^+$ analogs




**Nicotinamide adenine dinucleotide ($NAD^+$)**

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

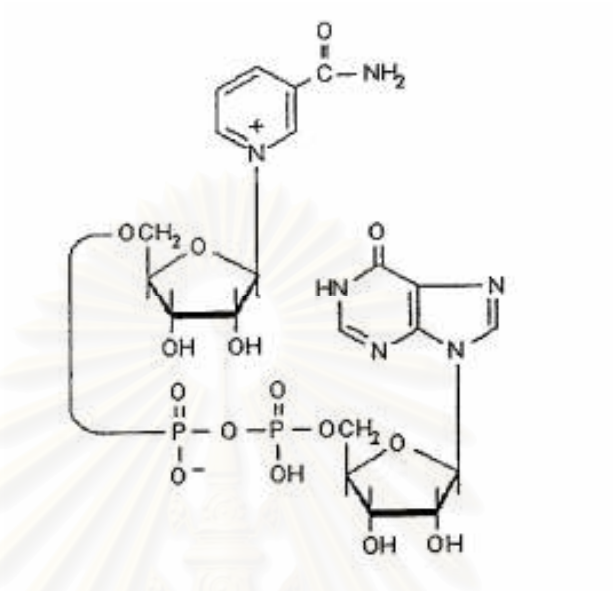
**1. Coenzyme analog modified at C-2 position of the adenosylribose**



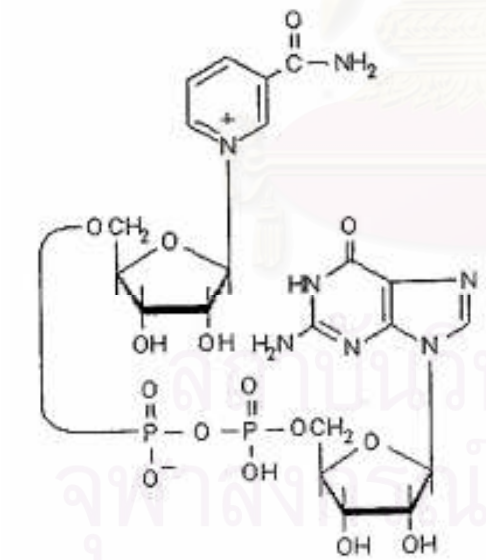
**Nicotinamide adenine dinucleotide phosphate ($NADP^+$)**

# APPENDIX H (continued)

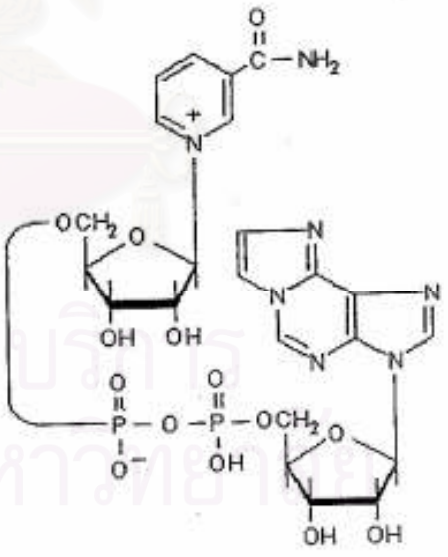
## 2. Coenzyme analog modified at the amino group in the adenine moiety



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



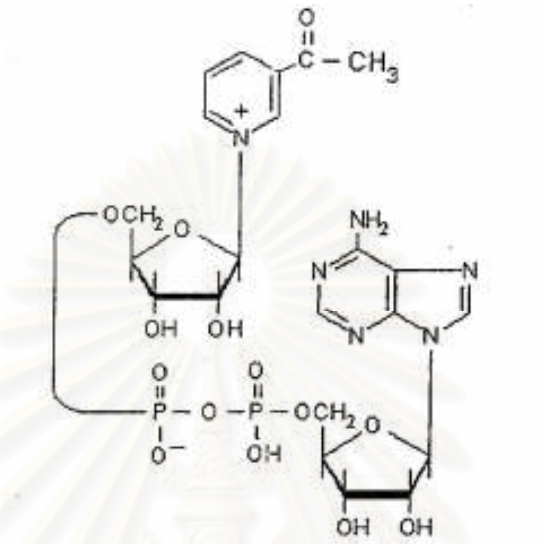
**Nicotinamide guanine dinucleotide**



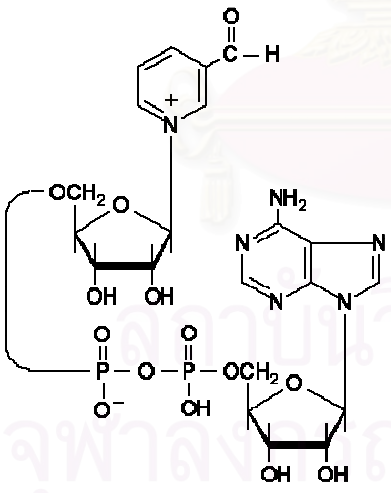
**Nicotinamide 1, $N^6$-ethenoadenine dinucleotide**

# APPENDIX H (continued)

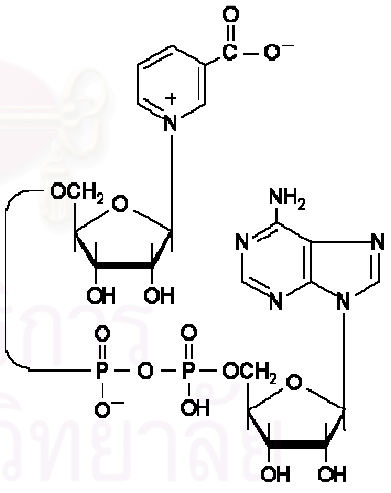
**3. Coenzyme analog modified at the nicotinamide moiety**



**3-Acetylpyridine adenine dinucleotide**



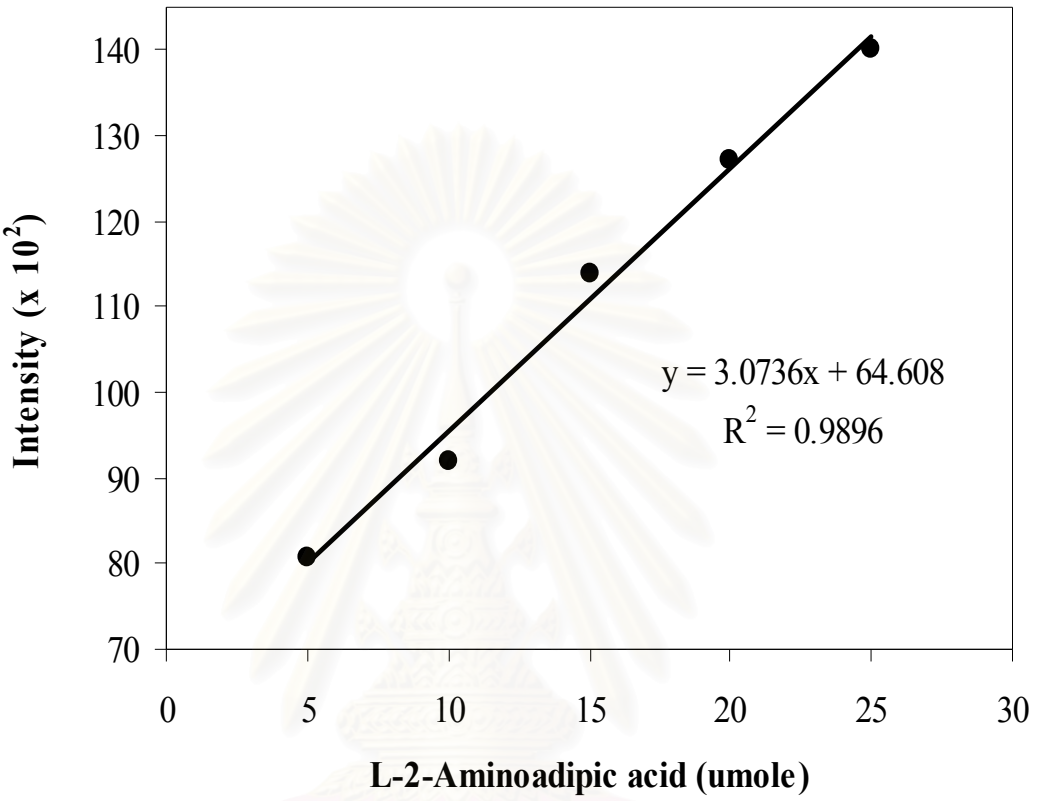
**3-Pyridinealdehyde adenine dinucleotide**



**Nicotinic acid adenine dinucleotide (deamido-$NAD^+$)**

# APPENDIX I

## Standard curve for L-2-aminoadipic acid



# BIOGRAPHY

Miss Jureeporn Sri-in was born on April 27th, 1982 in Nakornsrithammarat. After graduating with degree of Bachelor of Science from the Department of Biotechnology at Ramkhumhaeng University in 2004, she keeps on studying for Master of Science at the Biotechnology Program, Faculty of Science at Chulalongkorn University in that year.