การแสดงออกแบบเฮเทอโรโลกัสของยีนไพร์โรลีน-5-คาร์บอกซิเลตรีดักเทส และยีนแอล-ไลซีน-6-ดีไฮโดรจิเนสเพื่อการผลิตกรดแอล-พิพีโคลิก

นางสาวกษมา ศรีเมือง

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HETEROLOGOUS EXPRESSION OF *PYRROLINE-5-CARBOXYLATE REDUCTASE* AND *L-LYSINE-6-DEHYDROGENASE* GENES FOR L-PIPECOLIC ACID PRODUCTION

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กษมา ศรีเมือง : การแสดงออกแบบเฮเทอโรโลกัสของยีนไพร์โรลีน-5-คาร์บอกซิเลตรีดักเทส และยีนแอล-ไลซีน-6-ดีไฮโดรจิเนสเพื่อการผลิตกรดแอล-พิพีโคลิก. (HETEROLOGOUS EXPRESSION OF *PYRROLINE-5-CARBOXYLATE REDUCTASE* AND *L-LYSINE-6-DEHYDROGENASE* GENES FOR L-PIPECOLIC ACID PRODUCTION) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร. กนกทิพย์ ภักดีบำรุง, 137 หน้า.

กรดแอล-พิพีโคลิก (L-pipecolic acid: L-PA) เป็นกรดอะมิโนชนิดหนึ่งที่ไม่พบในโครงสร้างของโปรตีนและเป็นตัวกลางสำคัญในการผลิตสารเมแทบอไลท์ทุติยภูมิของพวกจุลชีพ เช่น rapamycin ซึ่งเป็น immunosuppressant งานวิจัยนี้ได้ศึกษาการผลิต L-PA ด้วยวิธีทางชีวภาพโดยอาศัยปฏิกิริยาคู่ควบของเอนไซม์สองชนิด คือ ไลซีน 6-ดีไฮโดรจิเนส (Lys 6-DH) ร่วมกับไพร์โรลีน-5-คาร์บอกซิเลต รีดักเทส (P5CR) โดยยีน *lys 6-dh* จาก *Acromobacter dinitrificans* ได้ถูกโคลนไว้แล้วใน *Escherichia coli* BL21(DE3) ดังนั้น งานวิจัยนี้ได้ทำการโคลนยีน *proC* ซึ่งเข้ารหัสให้ P5CR จาก *Bacillus cereus* ATCC 11778 เข้าสู่เวคเตอร์ pET-17b และถ่ายรีคอมบิแนนท์พลาสมิดเข้าสู่ *E. coli* BL21(DE3) รีคอมบิแนนท์โคลนมีการผลิต P5CR สูงสุดหลังจากการเหนี่ยวนำด้วย IPTG ความเข้มข้น 0.4 มิลลิโมลาร์เป็นเวลา 4 ชั่วโมง การทำเอนไซม์ให้บริสุทธิ์ด้วยคอลัมน์ดีอีเออี-โทโยเพิร์ล และคอลัมน์บิวทิล-โทโยเพิร์ล พบว่าเอนไซม์มีความบริสุทธิ์เพิ่มขึ้น 2.25 เท่า และมีแอคติวิตีคงเหลือ 14.9 เปอร์เซ็นต์ เอนไซม์มีน้ำหนักโมเลกุลของหน่วยย่อยประมาณ 28.9 กิโลดาลตัน ค่า K_m ต่อแอล-โพรลีน และ NAD^+ ของเอนไซม์เท่ากับ 1 และ 1.54 มิลลิโมลาร์ ตามลำดับ เมื่อนำยีน *proC* ที่ได้ไปโคลนร่วมกับยีน *lys 6-dh* เพื่อการผลิต L-PA โดย *E. coli* พบว่า เมื่อเหนี่ยวนำรีคอมบิแนนท์โคลนด้วย IPTG ความเข้มข้น 0.1 มิลลิโมลาร์เป็นเวลา 4 ชั่วโมง จากนั้นนำเซลล์เปียก 0.05 กรัมมาบ่มกับชุดปฏิกิริยาที่ประกอบด้วย แอล-ไลซีนความเข้มข้น 200 มิลลิโมลาร์ และบัฟเฟอร์ Tris-HCl ความเข้มข้น 200 มิลลิโมลาร์ เป็นเวลา 24 ชั่วโมง ได้ผลผลิต L-PA เท่ากับ 1.74 กรัมต่อลิตร

ภาควิชา.....ชีวเคมี..... ลายมือชื่อนิสิต.....

สาขาวิชา.....ชีวเคมี..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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KASAMA SRIMUANG : HETEROLOGOUS EXPRESSION *OF PYRROLINE-5-CARBOXYLATE REDUCTASE* AND *L-LYSINE-6-DEHYDROGENASE* GENES FOR L-PIPECOLIC ACID PRODUCTION. ADVISOR : ASSISTANT. PROFESSOR. KANOKTIP PACKDIBAMRUNG, Ph.D., 137 pp.

L-pipecolic acid (L-PA) is a non-protein amino acid. It is an important precursor of many useful microbial secondary metabolites such as an immunosuppressant rapamycin. This research aims to produce L-PA by coupling reaction of lysine 6-dehydrogenase (Lys 6-DH) and pyrroline-5-carboxylate reductase (P5CR). The *lys 6-dh* gene from *Acromobacter denitrificans* was already cloned into *Escherichia coli* BL21(DE3) in previous research. Therefore, in this study *proC* encoding P5CR from *Bacillus cereus* ATCC 11778 was cloned into an expression vector pET-17b and then transformed to *E. coli* BL21(DE3). The optimum condition for *proC* expression was induction with 0.4 mM IPTG for 4 hours. The enzyme was purified 2.25 fold by DEAE-Toyopearl and Butyl-Toyopearl column chromatographies with 14.9% yield. The molecular mass of enzyme subunit was about 28.9 kDa. The apparent K_m for L-proline and NAD^+ were 1 and 1.54 mM, respectively. The obtained *proC* was co-expressed with the *lys 6-dh* in *E. coli* BL21(DE3) in order to produce L-PA. The production of L-PA, approximately 1.74 g/l, was obtained by induction of the recombinant clone with 0.1 mM IPTG for 4 hours and then 0.05 g of cell wet weight was incubated in the reaction mixture consisted of 200 mM L-lysine in 200 mM Tris-HCl buffer, pH 9.0 for 24 hours.

Department : Biochemistry Student's Signature .....
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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
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
KOH	potassium hydroxide
kV	kilovolt
l	liter
LAT	L-lysine ε-aminotransferase
LB	Luria-Bertani
L-PA	L-pipecolic acid
lysdh	L-lysine-6-dehydrogenase gene
Lys 6-DH	L-lysine-6-dehydrogenase
μF	microfarad

μg	microgram
μl	microliter
μM	micromolar
M	mole per liter (molar)
mA	milliampere
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MOPS	3-(*N*-morpholino)propanesulfonic acid
M_r	relative molecular mass
MW	molecular weight
NAD^+	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
$NaHCO_3$	sodium carbonate-bicarbonate buffer
Na_2HPO_4	disodium hydrogen orthophosphate
ng	nanogram
nm	nanometer
NH_4Cl	ammonium chloride
$(NH_4)_2SO_4$	ammonium sulfate
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
P2C	Δ^1-piperideine-2-carboxylate acid
P5C	pyrroline-5-carboxylate
P5CR	pyrroline-5-carboxylate reductase
P6C	Δ^1-piperideine-6-carboxylate acid
pI	isoelectric point
pmol	picomole
PMSF	phenyl methyl sulfonyl fluoride

RNase	ribonuclease
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 Amino acids

Amino acids are small organic molecules that occur in nature in a multitude of biological forms, either free or conjugated to various types of compound, or as the building block of proteins (Papadoyannis and Theodoridis, 2005). In yeast, animals and plants, amino acids play fundamental roles in a multitude of processes, including protein synthesis, hormone metabolism, nerve transmission, cell growth, production of metabolic energy, nucleobase synthesis, nitrogen metabolism and urea biosynthesis. In multicellular organisms, many of the nitrogenous compounds are transported between cells (Wipf *et al.*, 2002). Generally, amino acids are important pharmaceutical compounds (Nishizawa, 2005). They are widely used in industry as intermediates for various organic syntheses, pharmacologically active compounds, food additives and agrochemicals (Bommarius, 1998).

Every amino acid, with the exception of glycine, can exist in two forms using the ability of rotation on the plane of polarized light, designated D and L, both of which are found in nature (Pasquale, 2008). The L-formed of amino acid are predominantly found in all living organisms while D-formed in general are also not uncommon in nature (Haniff, 2002). D-amino acids have been used as constituents for fermented food, peptidoglycan of bacterial cell walls and peptide antibiotics, as well as in certain animal (Dominguez, 1990). Nowadays, the using of L-amino acids for synthesis of many compounds are spread widely in animal nutrition, human medicine and the pharmaceutical industries. For example, L-leucine, L-valine, L-isoleucine are used as food and feed activities (Gu, 1990) while L-alanine is used as the precursor in drug production and can be also used as food additive due to its sweet taste (Suye *et al.*, 1992).

In a broad sense, amino acids can be put into two groups; the coded amino acid (primary protein amino acid) and the non coded amino acid (non-protein amino acid). The primary protein amino acids are about 20 amino acids that utilized in the synthesis of peptides and proteins under genetic control, which in turn are vital for life. The non-protein amino acids are found mostly in plants, microorganisms and arise as intermediates or as the end product of metabolic pathways. For example, *N*-acetyltyrosine, which supports brain function by improving the synthesis of the catecholamines norepinephrine and dopamine (neurotransmitters), is formed in the metabolism of tyrosine. Betaine, serve as organic osmolytes, substances synthesised or taken up from the environment by cells for protection against osmotic stress, drought, high salinity or high temperature, is involved in glycine biosynthesis and citrullin, normally contains in several proteins include myelin basic protein (MBP), filaggrin, and several histone proteins, participates in ornithine cycle (Wolinsky and Driskell, 2004). The non-protein amino acids are increasingly in demand by the pharmaceutical industry for single-enantiomer drugs. They are in demand as precursor for asymmetric synthesis, however, they are very expensive (Busca *et al.*, 2004). Even though more than 900 non-protein amino acids were isolated from plants, a detail biosynthetic pathway has not been worked out for many of them (Thayumanavan, 2003).

1.2 L-pipecolic acid

L-pipecolic acid (L-PA), which is homologous to proline, is a non-protein amino acid. L-PA was first isolated as a component of plants by Morrison *et al* (Morrison, 1953). It is widely distributed in microorganism as well as higher and lower plants. In higher plants, Leguminoseae species (Grobbelaar and Steward, 1953) and the genera *Acacia* (Kunii *et al.*, 1996) including *Bocoa* (Kite and Ireland, 2002), it has been reported that L-PA could be abundant during reproductive processes while it is absent during vegetative periods of plant life (Li *et al.*, 1996). It has been associated with flowering in *Lemna paucicostata* (Fijioka and Sakura, 1997). L-PA accumulation in higher plants seems to be related to metabolic responses induced by environmental stress. Therefore, it was shown to co-accumulate with proline in halophytic higher

plants and in certain sand dune plants (Goas *et al.*, 1970). Its function in bacteria has been related to osmoprotective properties in *Brevibacterium ammoniagenes* (Gouesbet *et al.*, 1992). It is used as an important precursor of many microbial and plant secondary metabolites, such as an immunosuppressant rapamycin, an antitumor agent swainsonine, a peptide antibiotic virginiamycin, and an anthelmintic agent marcfortine (Min, 2006) (Figure 1.1). In mammals, L-PA as long been recognized as a product of lysine metabolism (Rothstein, 1954). Moreover, it has been found in human physiological fluids (i.e. plasma, urine and cerebrospinal fluid) (Armstrong *et al.*, 1993). L-PA is also regarded as a possible neurotransmitter, however, its neurological and biological roles are unclear (Nomura *et al.*, 1981). L-PA is an important biomedical marker for peroxisomal disorder diagnosis, as its concentration in plasma is increased in neonatal adrenoleukodystrofy (Zellweger syndrome) and infantile Refsum disease (Trijjibels, 1979). It has been reported that L-PA can be prepared by the chemical method, hydrogenation, using platinum oxide. For chemical synthesis, the optical resolving agent used is expensive and a complicated procedure is required. Moreover, in the process using an enzyme for purposes of optical resolution, the use of a purified enzyme is also expensive (Fujii *et al.*, 2009). Therefore, many research groups attempt to synthesize L- PA by microbial method from catabolism of L-lysine (Fujii *et al.*, 2000).

1.3 L-pipecolic acid pathway

Biosynthesis of L-PA has been extensively investigated in animals and plants, because of its close relationship with lysine metabolism in animals and lysine degradation in plants (Fujita *et al.*, 1999). These studies have established two basic routes for converting lysine into PA, distinguishable at the loss of a specific amino group of lysine. One route is through the loss of the α-amino group of lysine and the incorporation of the ε-nitrogen into PA. The important intermediate in this pathway is Δ^1-piperideine-2-carboxylate acid (P2C). The alternative route is via the loss of ε-nitrogen and the incorporation of α-nitrogen into PA which Δ^1-piperideine-6-

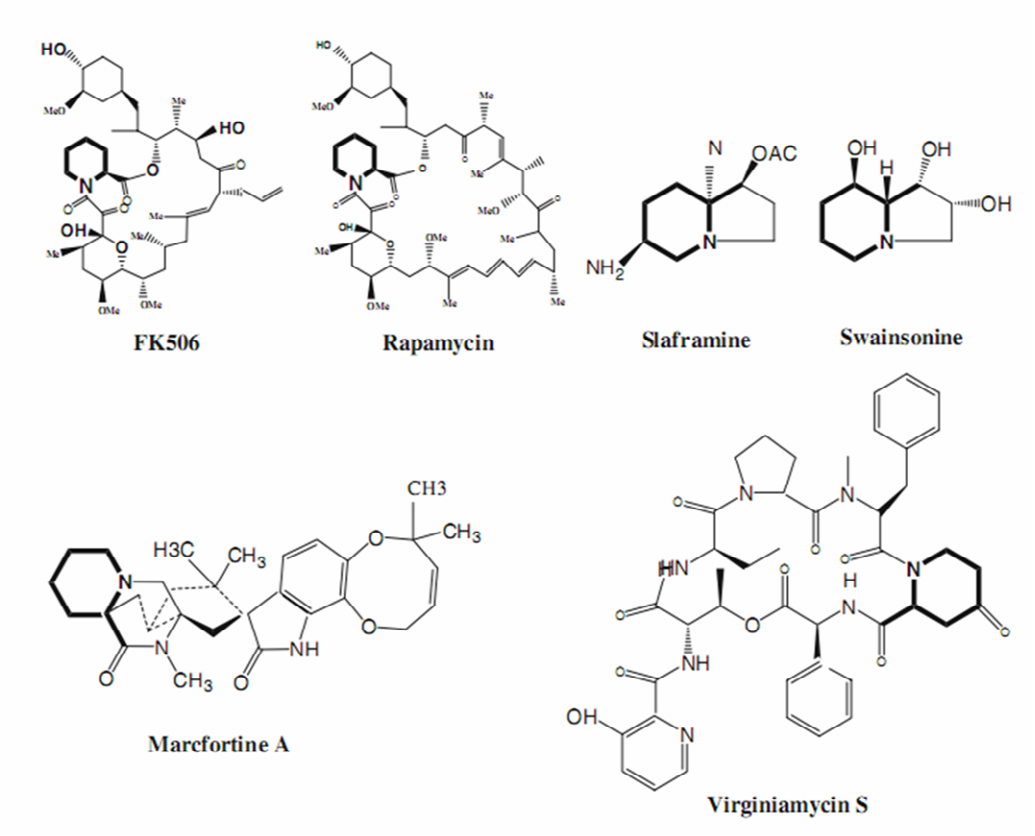

Figure 1.1 Microbial secondary metabolites containing pipecolic acid (bold-faced type) derived moieties

Source: Min, 2006

carboxylate acid (P6C) is an intermediate (shown in the Figure 1.2) (Min, 2006). In microorganisms, the biosynthesis of L-PA is related to lysine metabolism via three pathways.

1.3.1 P2C pathway

A L-lysine α-oxidase from *Trichoderma viride* Y244-2 and *T. harzianum* Rifai is a flavoprotein which can catalyse the oxidative deamination of the α-amino group of L-lysine. α-keto-ε-amino-caproic acid, product of the reaction, is spontaneously converted to P2C (reaction 1 in Figure 1.3). In *Streptomyces tendae* Tu901, α-amino group has been removed from L-lysine by L-lysine 2-aminotransferase (L2AT). α-keto-ε-amino-caproic acid and P2C are present as products of the reaction, respectively. In 2005, Muramatsu *et al.* found that an NADPH-dependent P2C reductase, the product of *dpk A* gene from *Pseudomonas putida* ATCC12633 involved in the conversion of P2C to L-PA (reaction 2 in Figure 1.3).

1.3.2 Lysine cyclodeamination pathway

Molnar *et al.* (1996) reported that L-lysine has been directly converted to L-PA by lysine cyclodeaminase in *S. hygroscopicus* and the experimentation was confirmed by Khaw *et al.* in 1998 (reaction 3 in Figure 1.3).

1.3.3 P6C pathway

In fungi parasite *Rhizoctonia leguminicola*, L-lysine was converted to saccharopine, which was in turn converted to P6C through oxidative cleavage by sacharopine oxidase and then readily reduced to L-PA (reaction 4, 5, and 6 in Figure 1.3). The reverse reactions of this pathway are investigated in aerobic red yeast *R. glutinis*, its showed that L-PA was converted to L-lysine via P6C, α-aminoadipic semialdehyde, and saccharopine. In β-lactam producing actinomycetes such as *S. clavuligerus* and *Nocardia lactamdurans*, P6C is synthesized from L-lysine by

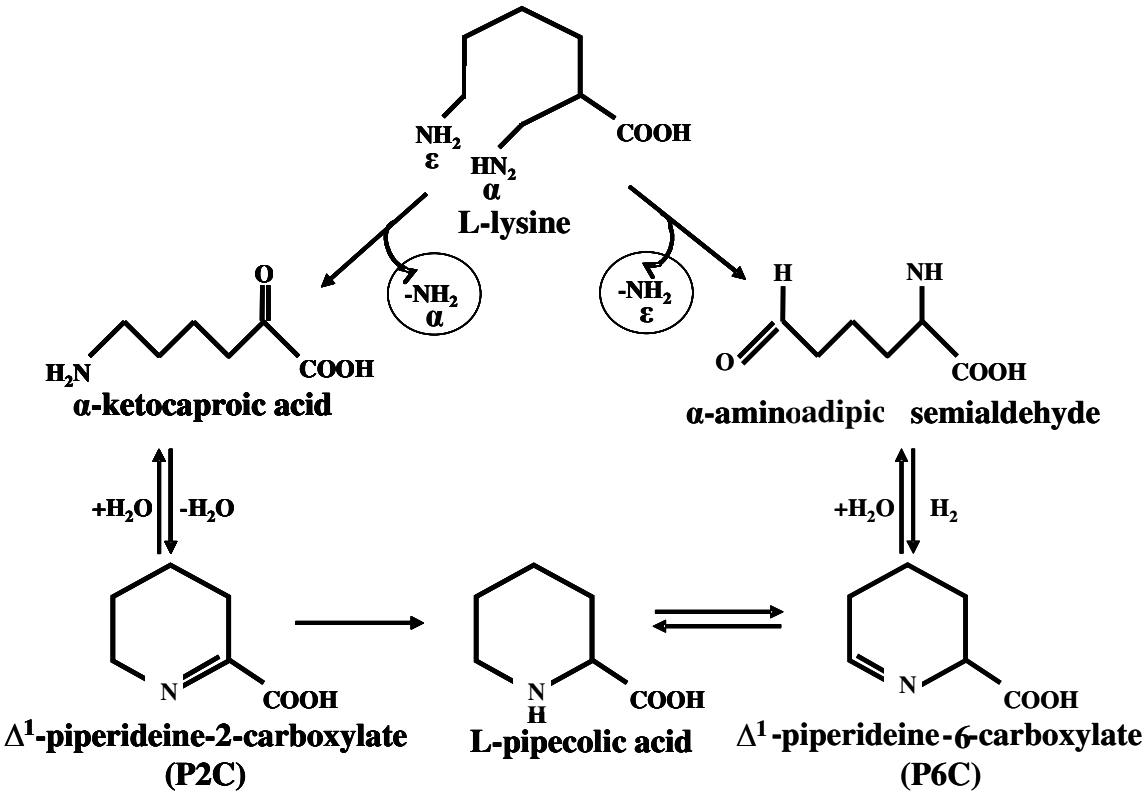

Figure 1.2 The two basic routes for converting lysine into pipecolic acid

Source: modified from Min, 2006

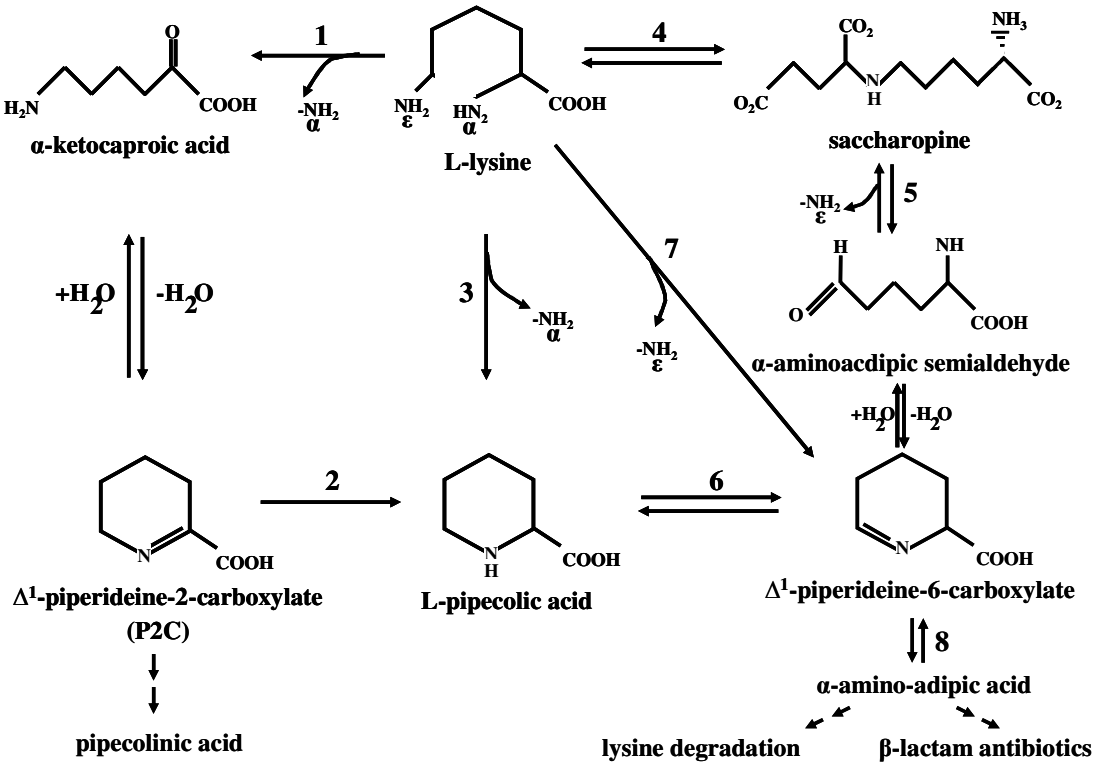

Figure 1.3 Various routes of pipecolic acid biosynthesis in microorganisms

Source: Min, 2006

L-lysine ε-aminotransferase (LAT) (reaction 7 in Figure 1.3). At the present time, no example can be given to show the direct involvement of LAT in L-PA biosynthesis in a naturally occurring microorganism, however, *lat* gene has been used to engineer recombinant *Escherichia coli* strains for L-PA production.

1.4 Biosynthesis of L-pipecolic acid

In 2002, Fujii *et al.* transformed the *lat* gene from *Flavobacterium lutescens* into *E. coli* JM109. They found that the transformant is able to produce L-PA by coupling reaction with pyrroline-5-carboxylate reductase (P5CR) in *E. coli* host cell which can also catalyse the reduction of P6C to L-PA because of the structural similarity of P5C with P6C. They, therefore, constructed high L-PA producing *E. coli* by co-expression of *proC*32, encoding P5CR from *E. coli* RK4904, with *lat* (Figure 1.4). After they cultivated the transformant for 159 hours, it showed the accumulation of L-PA about 3.9 g/l. The ee-value of the L-PA production was 100%.

To explain the ability of P5CR in L-PA production, Fujii and coworkers (2009) constructed four recombinant strains using *proC*-deficient *E. coli* RK4904 strain as the host, namely *E. coli* RK4904pUC19, *E. coli* RK4904pUClat, *E. coli* RK4904pUCproC and *E. coli* RK4904pUClatproC. *E. coli* RK4904pUClatproC only showed the accumulation of L-PA about 0.765 g/l. They suggested that the incorporation of L-lysine into cells limited the rate of L-PA production. Therefore, it may be desirable to provide a direct L-PA producing bacterium which could produce L-lysine and converted it into L-PA with no requirement of L-lysine in medium. They found that *Corynebacterium glutamicum* was able to produce L-lysine by itself and had *proC* gene in the genome. Then, they cloned *lat* gene from *F. lutescens* into the pC2 plasmid and transformed into *C. glutamicum*. The transformants showed accumulation of L-PA about 0.7 g/l after the addition of glycerol for 135 hours.

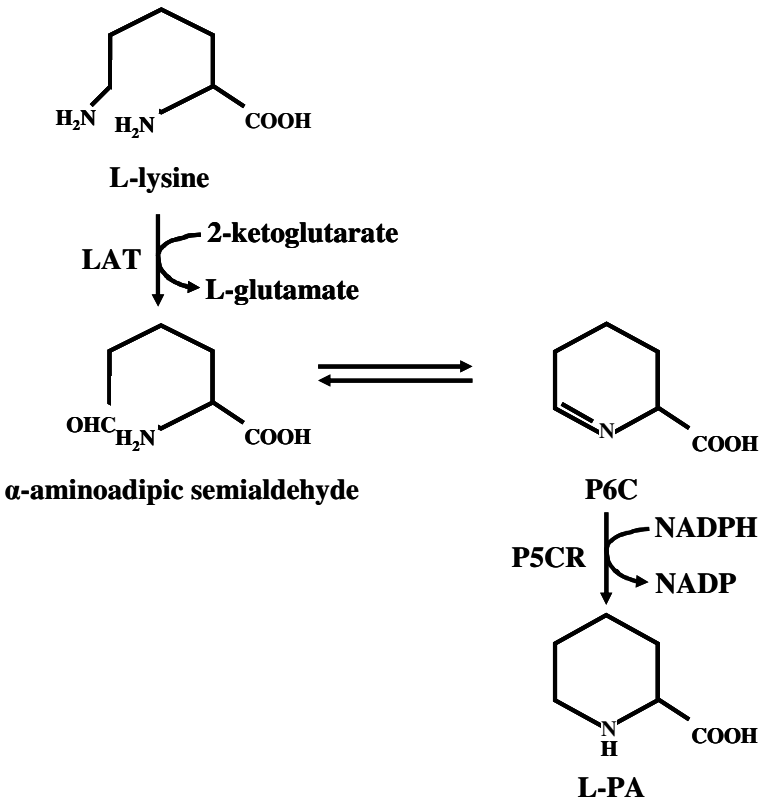

Figure 1.4 Conversion of L-lysine to L-PA by LAT and P5CR

Source: Fujii *et al.*, 2002

In addition to LAT, the enzyme that has the ability to catalyse the oxidative deamination of L-lysine to P6C was studied by Misono and Nagasaki in 1982. They suggested that lysine-6-dehydrogenase (Lys 6-DH) from *Achromobacter tumefaciens* is able to catalyse L-lysine in the presence of NAD^+. In 2004, Heydari *et al.* reported a second Lys 6-DH which was identified from *Geobacillus stearothermophilus*. In our laboratory, *lysdh* encoding Lys 6-DH from *Acromobacter denitrificans* K-1 was already cloned and expressed in *E. coli* BL21(DE3) (Ruldeekulthamrong *et al.*, 2008). In this thesis, the Lys 6-DH was used as the first enzyme for L-PA production via P6C pathway and P5CR, the second enzyme, was used to catalyze the reduction of P6C to L-PA.

1.5 Pyrroline-5-carboxylate reductase

Pyrroline-5-carboxylate reductase (P5CR, E.C. 1.5.1.2) is a member of a very large P5CR family, with over 400 family members and currently found in every domain of life, including eukarya, bacteria and archea. It catalyzes the final step in proline synthesis by NAD(P)H-dependent conversion of pyrroline-5-carboxylate (P5C) to proline (Figure 1.5). Nowadays, a large number of P5CR enzymes from different sources have been studied. They showed considerable diversity with respect to reaction rate with nicotinamide coenzyme (NADPH or NADH), molecular mass and affinity to substrate, as well as a product inhibition. It was proposed that this diversity may reflect the different role the enzyme plays in regulation and metabolism. Its physiological functions vary widely including regulation of intracellular redox potential, osmoregulation, enhancing nucleotide synthesis and energy production as well as regulation of apoptosis.

In bacteria, P5CR is involved in metabolism of proline-analog drugs such as thioproline and 3,4-dehydro-l-proline, drugs which have anti-tumor and anti-oxidant properties (Deutch *et al.* 2001 and Nocek *et al.* 2005). In insects like *Drosophila melanogaster*, the transcription of P5CR is increased in response to cold stress (Misener *et al.*, 2001). In cultered cells, P5C and proline cycle activates the pentose phosphate shunt and increases nucleotide synthesis by the salvage and *de novo*

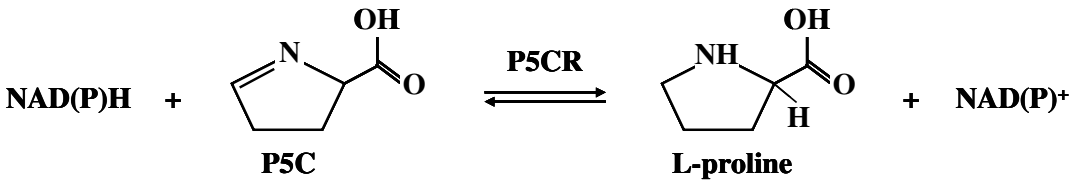

Figure 1.5 Reaction scheme for P5CR

Source: Yanping *et al.*, 2006

pathways (Phang *et al.*, 1982 and Kohl *et al.*, 1988). P5CR has been shown to play a role in the regulation of the purine precursor ribose 5-phosphate. In human erythrocytes, the level of P5CR activity is comparable with the activity levels of major erythrocyte enzymes. This is consistent with the interpretation that the function of the enzyme in human erythrocytes may be to generate an oxidizing potential in the form of $NADP^+$ (Yeh *et al.*, 1981). In some mammalian cells, the inter-conversion of proline and P5C provides a metabolic shuttle of redox equivalents between the cytosol and mitochondria. P5C can be transported into cells as a source of oxidizing potential where its reduction to proline generates $NADP^+$ (Hagedorn 1986 and Hagedorn *et al.*, 1986). The process is mediated by significant amounts of P5CR found in the cytosol and some membrane-containing fractions. To date, no human genetic disease caused by abnormalities in P5CR has been identified. However, overexpression of the mRNA for P5CR was reported by DNA microarray analysis in carcinoma (Ernst *et al.*, 2002, Febbo *et al.*, 2003 and Okabe *et al.,* 2001) and the raised activity of P5CR changes as a marker of pulmonary tumors and adult neoplastic colon (Greengard *et al.*, 1977 and Herzfeld *et al*., 1980). The decreased P5CR activity may be associated with retinal degeneration in mice (Matsuzawa 1982), and could be used to distinguish clearly between patients with and without non-hepatic cancer (Herzfeld and Greengard 1980). The expression of P5CR in LoVo cells, an epithelial cell lines, can be inhibited by adriamycin (Donald *et al.* 2001).

Most plants have required mechanisms which make them torolent to environmental stresses during their development. Water stresses caused by salinity and water deficiency in the soil severely hinder plant growth and agricultural productivity (Boyer, 1982). Proline accumulation is one of the major responses to water stresses (Handa *et al.*, 1983, Rhodes *et al.*, 1986 and Csonka, 1989). It has been suggested that the accumulated proline plays a role as an osmoregulator (Delauney and Verma, 1993), and an osmoprotectant (Venekamp *et al.*, 1989), and is also involved in redox regulation (Bellinger and Larher, 1987) as well as radical scavenging (Smirnoff and Cumbes, 1989). Some plants undergo alterations of photosynthetic functions under stress conditions (Cushman *et al.*, 1992 and Belkhodja *et al.*, 1994). Proline accumulation in *Mesembryanthemum crystallinum*, a facultative

halophyte, constituted an early event in the response to salinity stress (Sanada *et al.*, 1995). Two pathways of proline biosynthesis in higher plants have been demonstrated by Delauney and Verma (1993), one from glutamate, and the other from ornithine. Proline is synthesized from glutamate via P5C by two successive reactions, which are catalyzed by P5C synthetase and P5CR. The existence of these two enzyme activities was confirmed by molecular cloning of cDNA. P5CR cDNAs have been isolated from soybean (Delauney and Verma, 1990), pea (Williamson and Slocum, 1992), *Arabidopsis thaliana* (Verbruggen *et al.*, 1993), and kiwifruit (Walton *et al.*, 1998). Treichel 1986 and Andoh 1994 reported that the stimulation of proline synthesis in stressed plants is correlated with increased P5CR activity. On the contary it has also been reported that P5CR reaction was not rate-limiting and was not involved in osmotic stress-dependent regulation of proline synthesis (LaRosa *et al.* 1991 and Szoke *et al.* 1992). Furthermore, P5CR gene expression was not shown to be induced by osmotic stress (Yoshiba *et al.* 1995).

1.5.1 Properties and characterization of pyrroline-5-carboxylate reductase from various sources

In 1958, P5CR was partially purified first time in *Neurospora crassa* (Yura and Vogel, 1958). Subsequently, complete purification was performed in *Spinacia oleracea* in 2001 (Murahama *et al.,* 2001). Therefor, the properties of the enzyme from different sources have been elucidated. *E. coli* K-12 and spinach leave enzymes had a molecular weight of approximately 320,000 and 310,000, respectively (Rossi *et al.*, 1977 and Murahama *et al.*, 2001) while rat len enzyme had a molecular weight of 240,000 (Shiono *et al.*, 1986). The multimer architecture appears to be essential for the function of P5CRs from different organisms. The biological unit of the native enzyme is found to be a dimer in *Neisseria meningitides* (Nocek *et al.* 2005), a tetramer in yeast (Brandriss and Falvey 1992), an octomer in rat lens (Shiono *et al.* 1986), a decamer in *Streptococcus pyogenes* (Nocek *et al.* 2005) and a 10~12-mer in human (Merrill *et al.* 1989). P5C was used as substrate for the enzyme from *E. coli* (Rossi *et al.*, 1977) and plants (Kruegeretal *et al.,* 1985, Rena and Splittstoesser, 1974) at pH 6.5-7.5 whereas the enzyme from hyperthermophilic archeon *Sulfobus*

solfataricus and human used proline and proline analogues as substrate at high pH. The optimum temperature from two sources, *Mycobacterium tuberulosis* and human, were around 37 ℃. Meng *et al.* (2009) found that the enzyme from *S. solfataricus* was thermostable, since, its enzyme activity did not reduced when incubated at 70 ℃ for 30 minutes. However, human enzyme was 95% inactivated after incubation for 15 minutes at 68 ℃. Gates *et al.* (1983) reported that P5CR from all organisms could utilize either NADPH or NADH as a cofactor. The *Drosophila melanogaster* enzyme showed affinity for NADPH much grater than NADH. In contrast, *E. coli*, rat tissue and calf liver enzymes showed higher affinity for NADH than NADPH while *Clostridium sporogenes* enzyme used NADH exclusively. Moreover, kinetic analysis of rat len enzyme revealed that feedback enzyme inhibition was occurred from $NADP^+$ but not from NAD^+ and the increased proline concentration was slightly inhibited the reduction of P5C. Free ATP, heavy metal, sulfydryl reagent, and cation at high concentration were inhibitors to the enzyme activity of *Spinaia oleracea* (Murahama *et al.,* 2001). The summarization of the enzyme properties is shown in Table 1.1.

1.5.2 Cloning of pyrroline-5-carboxylate reductase

In 1992, Szoke *et al.* found that the expression of P5CR was higher in soybean root nodules than in leaves and roots, and its expression in roots appeared to be osmoregulated. They used the *E. coli* X342, harboring a pProCl plasmid expressing soybean P5CR and complementing the mutation in this strain to determine the enzyme properties. The purified enzyme has a subunit molecular weight of 29 kDa.

Yang *et al.* (2006) described that P5CR involved with intermediary metabolism and respiration in *Mycobacterium tuberculosis*. *proC*, the gene encoding P5CR, displayed the essential role in *M. tuberculosis* virulence and be required for mycobacterium growth. In order to look for a good drug-design target site for more effective agents against tuberculosis, the amplified *proC* was cloned into *E. coli* BL21(DE3) by using an expression vector pET30a. The gene consists of 888 bp.

(continued)

Table 1.1 Characteristics of pyrroline-5-carboxylate reductase from different sources

Properties	Sources							
	N. crassa[a]	***E. coli***[b]	***C. stricklandii***[c]	***M. tuberculosis***[d]	***S. solfataricus***[e]	**Yeast**[f]	**Rat lens**[g]	**Rat ratina**[h]
K_m (mM)								
- P5C[p]	0.53	0.14	1.49	0.04	-	0.08	0.62	0.13
- P5C[q]	0.45	0.15	0.23	0.05	-	-	0.05	-
- Proline[r]	-	-	-	0.12	-	-	-	-
- NADH	-	0.23	0.86	0.06	-	0.05	0.37	0.20
- NADPH	-	-	0.03	0.06	0.06	0.06	0.01	0.01
- $NADP^+$	-	-	-	0.09	0.17	-	-	-
- NAD^+	-	-	-	-	0.39	-	-	-
Optimum pH	6.0	6.9	7.4	10.3	10.0	-	6.5-7.1	7.0-7.5
Thermostability								
- Temp (℃)	-	67	100	60	85	-	67	-
- Time (min)	-	5	4-5	20	10	-	5	-
- Inactivation (%)	-	50	100	50	70	-	50	-
Native M_r (subunit M_r); kDa	-	300 (30x10)-350 (30x12)	230 (28.9x8)	356 (35.6x10)	300 (30.5x10)	125 (-)	240 (30x8)	-
Specific activity of purified enzyme (U/mg protein)	615	24.5	1,520	57	9.0	280	173.4	4.17

[a], Yura and Vogel, 1958; [b], Rossi *et al.*, 1977; [c], Kenllies *et al.,* 1999; [d], Yang *et al.,* 2006; [e], Meng *et al.,* 2009; [f], Mutsuzawa and Ishiguru, 1980; [g], Shiono *et al.,* 1986; [h], Mutsuzawa and Ishiguru, 1982; [i], Smith and Greenberg, 1956; [j], Peisach and Strecker, 1999; [k], Yeh and Phang, 1981; [l],Lorans and Phang, 1981; [m], human lymphoblastiod cell lines; [n], Kruegeretal *et al.,* 1985; [o], Rena and Splittstoesser, 1974; [p], NADH as cofactor; [q], NADPH ac cofactor; [r], $NADP^+$ as cofactor

Table 1.1 Characteristics of pyrroline-5-carboxylate reductase from different sources

Properties	Sources							
	Rat liver[i]	**Calf liver**[j]	**Human erythrocyte**[k]	**Human fibroblast**[k]	**LHN**[l,m]	**REN**[l,m]	**Barley**[n]	**pumpkin**[o]
K_m (mM)								
- P5C[p]	0.21	0.33	0.48	0.41	0.56	0.59	0.14	0.09
- P5C[q]	-	0.69	0.09	0.20	0.64	0.64	0.15	-
- Proline[r]	-	-	-	-	-	-	-	-
- NADH	0.25	0.84	0.39	0.12	0.15	0.23	-	0.06
- NADPH	-	0.02	0.02	0.09	0.10	0.03	-	-
- $NADP^+$	-	-	-	-	-	-	-	-
- NAD^+	-	-	-	-	-	-	-	-
Optimum pH	6.6-7.0	6.7-7.4	-	-	-	-	6.8-7.5	6.5
Thermostability								
- Temp (℃)	-	-	-	-	-	-	60	65
- Time (min)	-	-	-	-	-	-	10	15
- Inactivation (%)	-	-	-	-	-	-	100	0
Native M_r (subunit M_r); kDa	-	-	300 (30x10)	-	-	-	-	100 (-)
Specific activity of purified enzyme (U/mg protein)	37.6	14,600	70.1	-	-	-	720	2,070

[a], Yura and Vogel, 1958; [b], Rossi *et al.*, 1977; [c], Kenllies *et al.,* 1999; [d], Yang *et al.,* 2006; [e], Meng *et al.,* 2009; [f], Mutsuzawa and Ishiguru, 1980; [g], Shiono *et al.,* 1986; [h], Mutsuzawa and Ishiguru, 1982; [i], Smith and Greenberg, 1956; [j], Peisach and Strecker, 1999; [k], Yeh and Phang, 1981; [l],Lorans and Phang, 1981; [m], human lymphoblastiod cell lines; [n], Kruegeretal *et al.,* 1985; [o], Rena and Splittstoesser, 1974; [p], NADH as cofactor; [q], NADPH ac cofactor; [r], $NADP^+$ as cofactor

The level of P5CR was present about 25% of protein in soluble fraction.

In 2009, the *proC* (0.8 kb) from the hyperthermophilic archeon *S. solfataricus* was amplified and ligated into *Eco*RI and *Xho*I restriction sites of the pET-28a(+) vector. After that, the recombinant plasmid was transformed into *E. coli* BL21(DE3). The gene consists of 277 amino acid residues with a calculated molecular weight of 30.5 kDa per subunit, and shares significant sequence identity (22-30%) with its counterparts from eukarya and bacteria (Meng, 2009).

1.6 Objectives of this research

Our research group aims to produce L-PA by coupling reaction of two enzymes in microbial L-lysine catabolism. The first enzyme is Lys 6-DH which catalyzes L-lysine to P6C and the later is P5CR that catalyzes P6C to L-PA (Figure 1.6). Recently, *lysdh* encoding Lys 6-DH from *A. denitrificans* was already cloned and expressed in *E. coli* BL21(DE3) by Ruldeekulthamrong *et al.* (2008). We looked for a new source of P5CR in the EMBL-GenBank-DDBL database and the enzyme from *Bacillus cereus* showed high enzyme activity. Therefore, we selected *B. cereus* ATCC 11778 as a source of P5CR.

The objective of this thesis

1. Cloning of *proC* gene from *B. cereus* into *E. coli* BL21(DE3) using pET-17b as an expression vector
2. Optimization of *proC* gene expression
3. Purification and characterization of P5CR from recombinant clone
4. Cloning and expression of *proC* and *lysdh* genes into *E. coli* BL21(DE3) using pET-17b as an expression vector
5. Production of L-pipecolic using the recombinant clone

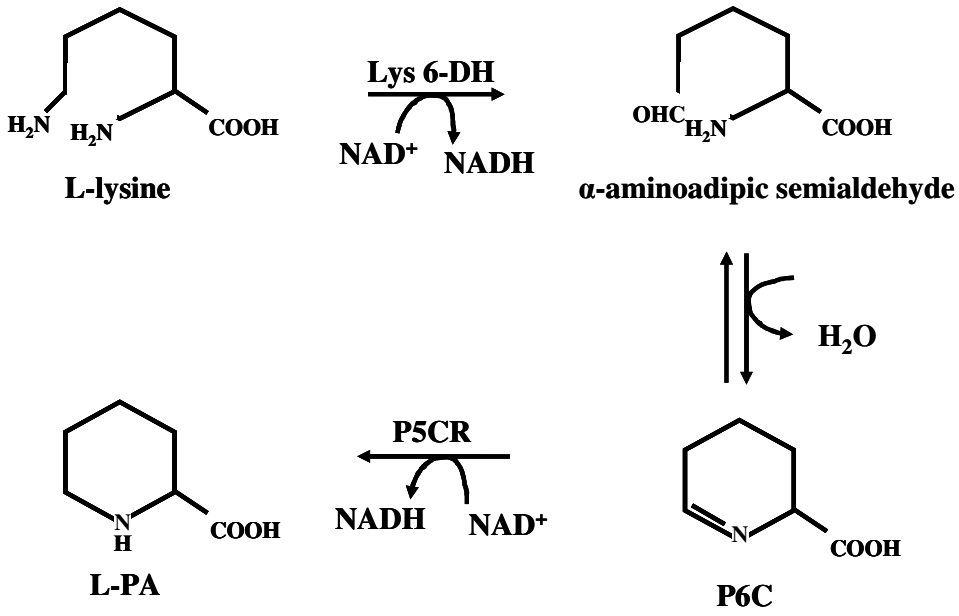

Figure 1.6 Reaction scheme of L-PA biosynthesis by Lys 6-DH and P5CR

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

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

Autopipette: Pipetman, Gilson, France

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

Centrifuge, microcentrifuge: Microfuge 22R, Beckman Instrument Inc., USA

Electrophoresis unit: Gelmate 2000, TOYOBO Co., LtD., Japan

Fraction collector: Frac-920, Amersham Biosciences, Sweden

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

Gel Documentation: BioDoc-It^{Tm} Imaging system, UVP, USA

Heating box: Model MD-01N Dry bath incubator, MS Major Science, USA

High Performance Liquid Chromatography (HPLC): Class 10ADVP, SHIMADZU, Kyoto, Japan

Incubator shaker: Innova^{TM} 4080, New Brunwick Scientific, USA

Incubator, shaker: Model E24R, New Brunswick Scientific, USA

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

Lamina flow: HT123, ISSCO, USA

Magnetic stirrer: Model Cerastir CH-1 series, Nickel-electro., Ltd., UK

Membrane filter: 0.2 µm VertiClean polytetrafluoroethylene (PTEE) syringe filter, Vertical Chromatography Co.,Ltd., Thailand

Membrane filter: nylon membrane filters, pore size 0.45 µm, Vertical Chromatography Co.,Ltd., Thailand

Microcentrifuge tubes 0.5 and 1.5 ml, Axygen Hayward, USA

MicroPulser™: Bio-Rad, USA

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

Orbital incubator: Model LD-427, Labinco BV, Netherlands

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

pH meter: Model S200, METTLER TOLEDO Co.,Ltd., Switzerland

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

Reversed phase HPLC Inertsil ODS-3, 250 mm x 4.6 mm x 5 µm column, GL Sciences Inc., Japan

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

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

Spectrophotometer: DU® 530, Beckman Instrument Inc., USA

Spin microtubes: Model microONE, Tomy Digital Biology Co., Ltd., Japan

Thermo cycler: Mastercycler gradient, eppendorf, Germany

Thin-wall microcentrifuge tubes 0.2 ml, Axygen Hayward, USA

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

UV transluminator: Model 2011 Macrovue, San Gabriel California, USA

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

Vortex:Top Mix FB15024, Fisher Scientific, Inc., USA

2.2 Chemicals

Acetone: Carlo ErbaReagenti, Italy

Acetonitrile (HPLC grade): RCI Labscan, Thailand

Acrylamide: Sigma, USA

Agar: Merck, Germany

Agarose: GenePure LE Agarose, ISC Bioexpress, USA

Ammonium persulphate: Sigma, USA

Ammonium sulphate: Carlo ErbaReagenti, Italy

Ampicillin: USBiological, USA

Aquacide I, Calbiochem, USA

Boric acid: Merck, Germany

Bovine serum albumin: Sigma, USA

Bromphenol blue: Merck, Germany

1-Butanol: Carlo ErbaReagenti, Italy

Butyl-Toyopearl 650M TSK gel: Tosoh, Japan

Chloroform: BDH, England

Coomassie brilliant blue R-250: Sigma, USA

DEAE-Toyopearl 650M TSK gel: Tosoh, Japan

Dialysis membrane: Cellu Sep T4, USA

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

100 base pair DNA ladder, Fermentas Inc., USA

Ethidium bromide: Sigma, USA

Ethyl alcohol absolute: Carlo ErbaReagenti, Italy

Ethylene diaminetetraacetic acid di-sodium salt (EDTA-di-sodium salt): Scharlau Chemie S.A., Spain

Ficoll type 400 : Sigma, USA

1-fluoro-2-4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey's Reagent) : Sigma, USA

Glacial acetic acid: Carlo ErbaReagenti, Italy

Glycerol: Analytical Univar Reagent, Australia

Glycine: BDH, England

High-Speed Plasmid Mini Kit: Geneaid, Taiwan

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

Hydrochloric acid: Carlo ErbaReagenti, Italy

Isopropanol: Merck, Germany

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

L-lysine: Sigma, USA

β- mercaptoethanol: Acros Organics, USA

Methanol (HPLC grade): RCI Labscan, Thailand

Magnesium sulphate 7-hydrate: BDH, England

3-morpholinopropane-1-sulfonic acid (MOPS, Sodium salt): BIO BASIC INC, Canada

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

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

Nitrobluetetrazolium: Koch-Light Laboratories Ltd., Japan

dNTP: Fermentas, USA

1-Pentanol: BDH, England

Peptone from meat pancreatically digested: Merck, Germany

Phenazinemethosulfate: NacalaiTesque, Inc., Japan

Phenol reagent: Carlo ErbaReagenti, Italy

Phenylmethylsulfonyl fluoride (PMSF): Sigma, USA

Phospheric acid: Mallinckrodt CHEMICALS, USA

L-pipecolinic acid: Wako, Japan

L-proline: Fluka, Switzerland

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

Potassium chloride: Merck, Germany

Potassium di-hydrogen phosphate: Carlo ErbaReagenti, Italy

di-Potassium hydrogen phosphate: Carlo ErbaReagenti, Italy

Sodium acetate: Merck, Germany

Sodium bicarbonate: Sigma, USA

Sodium carbonate anhydrous: Carlo ErbaReagenti, Italy

Sodium chloride: BDH, England

Sodium dodecyl sulfate: Sigma, USA

Sodium hydroxide: Carlo ErbaReagenti, Italy

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

Triethylamine: Merck, Germany

Trisma base: Riedel-de Haen, Germany

Yeast extract: Scharlau microbiology, European Union

2.3 Enzymes and Restriction enzymes

Lysozyme: Sigma, USA

Proteinase K: Sigma, USA

Restriction enzymes: New England BioLabs, Inc., USA

RNaseA: Sigma, USA

Pfu DNA polymerase: Promega, USA

T_4 DNA ligase: New England BioLabs, Inc., USA

2.4 Primers

Oligonucleotides: Bioservice Unit, Thailand

2.5 Bacterial strains and plasmid

Bacillus cereus ATCC 11778 was used as a source of *proC* gene encoding P5CR.

pET-17b was used as an expression vector for cloning of *proC* and *lysdh* gene (Appendix A).

Escherichia coli BL21(DE3), genotype: F^- *omp*T *hsdSB* (rB^- mB^-) *gal dcm* (DE3), was used as a host for cloning and expression.

2.6 Bacterial culture media

Luria-Bertani broth (LB medium)

LB medium containing 1% peptone, 0.5% NaCl and 0.5% yeast extract which was prepared and adjusted pH to 7.2 with NaOH (Sambrook *et al*. 1989). For agar plate, the medium was supplemented with 1.5% (w/v) agar. Medium was steriled for 20 minutes at 121 °C, 15 psi. If needed, selective antibiotic drug was then supplemented.

2.7 Preparation of *proC* gene

2.7.1 Chromosomal DNA extraction

Chromosomal DNA was isolated from *B. cereus* by the method of Frederick *et al.,* (1995). A single colony was inoculated into 5 ml of LB medium and incubated at 37 ^{o}C for 24 hours with shaking. Then each 1.5 ml of cell culture was centrifuged in microcentrifuge tube at 8,000xg for 2 minutes. The pellet was resuspended in 550 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) by repeated pipetting. The cell solution was then treated with 3 µl of 5 mg/ml lysozyme, 2 µl of 10 mg/ml RNaseA, 30 µl of 10% SDS followed by the addition of 3 µl of 20 mg/ml proteinase K and incubated for 1 hour at 37 ^{o}C. After incubation, the DNA was extracted with an equal volume of phenol-chloroform (1:1 V/V), mixed gently, and centrifuged at 12,000xg for 10 min. A viscous fluid formed at the aqueous layers was carefully transferred to a new microcentrifuge tube. DNA was precipitated by the addition of 5 M NaCl to the final concentration of 1 M and 2 volumes of absolute ethanol, standed at -20 ^{o}C at least 30 min. Afterwards, the DNA solution was centrifuged at 12,000xg for 10 min. DNA pellet was collected and washed with 70% ethanol. After drying, the pellet was dissolved in an appropriate volume of TE buffer. Finally, DNA concentration was estimated using submarine agarose gel electrophoresis by comparison the intensity of the fluorescence of ethidium bromide-DNA complex with known amount of λ/*Hin*dIII marker.

2.7.2 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 appropiately distance through the gel. The gel was stained with 2.5 μg/ml ethidium bromide solution for 5 minutes and destained to remove unbound ethidium bromide with distilled water for 10 minutes. DNA fragments on agarose gel were visualized under a long wavelength UV light. The concentration and molecular weight of DNA sample was determined by comparison of band intensity and relative mobility with those of the standard DNA markers (λ/*Hin*dIII and 100 bp ladder).

2.7.3 PCR amplification

2.7.3.1 Primers

The pair of primers that used for the PCR amplification of *proC* gene was designed by the data of nucleotide sequences obtained from the *proC* of *B. cereus* Q1 (CP000227.1), *B. cereus* AH187 (CP001177.1), *B. cereus* ATCC 10987 (AE017194.1) and *B. cereus* E33L (CP000001.1) in the EMBL-GenBank-DDBL database. The 5′-primer (p5cr-*Nde*I) consisted of *Nde*I restriction site, while the 3′-primer (p5cr-*Eco*RI) consisted of *Eco*RI restriction site. The sequence of all primers are shown in Table 2.1.

2.7.3.2 Templates for amplification of *proC* gene

The chromosomal DNA of *B. cereus* was prepared according to the method described in 2.7.1 and then was completely digested with various restriction enzymes: *Eco*RI, *Hin*dIII, *Kpn*I, *Sac*I and *Sal*I. The reaction mixture containing 5 μg of chromosomal DNA, 1 x reaction buffer suggested by the supplier and 10 U of each restriction enzyme in total volume of 50 μl was incubated at 37 ^{o}C for 3 hours. After incubation, the digested chromosomal DNA was examined on agarose gel electrophoresis. The DNA, which was digested by *Hin*dIII, was purified by HiYield

Table 2.1 Nucleotide sequence and *Tm* of all primers used in *proC* gene amplification

Primer	Sequence (5'⟶ 3')	*Tm* (^{o}C)	Remark
p5cr-*Nde*I	GGAATTCCATATGGATAAAC AAATTGGATTCATYGG	65.2	For the whole gene fragment amplification
p5cr-*Eco*RI	GGAATTCTTAYTTTTTCGTTT GACCAGATAGTTC	64.1	For the whole gene fragment amplification
T7-*Bam*HI	CGGGATCCGATCCCGCGAAA TTAATACG	60.0	For the *proC* gene fragment amplification used in the step of co-expression with *lysdh* gene

Note: Y = C, T

The underlined sequences are restriction sites.

Gel/PCR DNA Fragments Extraction Kit. The protocol of HiYield Gel/PCR DNA Fragments Extraction Kit was described in Appendix B. The DNA was dissolved in 50 µl of sterile distilled water. Three µl (about 500 ng) of the DNA solution was used as template in each reaction of PCR.

2.7.3.3 PCR condition

The *proC* gene was amplified by PCR in 50 µl of reaction mixture containing 1.5 U of *Pfu* DNA polymerase, 1x *Pfu* DNA polymerase buffer with $MgSO_4$, 0.2 mM each dNTPs, 2 µg of DNA template and 10 pmole of each primer. The thermocycle consisted of predenaturation at 95 °C for 10 minutes, and 34 cycles of denaturation at 95 °C for 10 minutes, annealing at 40.3 °C for 30 seconds, extension at 72 °C for 2 minutes following by final extension at 72 °C for 7 minutes. The PCR products were eletrophoresed through agarose gel. Finally, the putative *proC* gene fragment was recovered from agarose gel by HiYield Gel/PCR DNA Fragments Extraction Kit. The DNA solution was used for cloning in the next experiment.

2.8 Cloning of *proC* gene

2.8.1 Recombinant DNA preparation

2.8.1.1 Plasmid extraction

The *E. coli* BL21(DE3), which harboured pET-17b plasmid was grown in 5 ml LB medium containing 100 µg/ml ampicillin at 37 ^{o}C for 18 hours with shaking. The plasmid was extracted according to High-Speed Plasmid Mini Kit protocol as described in Appendix D

2.8.1.2 Vector DNA preparation

The expression vector pET-17b was linearized with *Nde*I and *Eco*RI. The reaction mixture containing of 2 μg pET-17b, 1x *Eco*RI reaction buffer, 20 U of *Nde*I and 20 U of *Eco*RI in total volume of 50 μl was incubated at 37 ^{o}C for 18 hours. The linear formed pET-17b was harvested from agarose gel by HiYield Gel/PCR DNA Fragments Extraction Kit.

2.8.1.3 The *proC* gene fragment preparation

After the gene fragment had been amplified, electrophoresed on agarose gel and then cleaned by HiYield Gel/PCR DNA Fragments Extraction Kit, the putative *proC* gene fragment was digested with *Nde*I and *Eco*RI. The reaction mixture containing of 2 μg of gene fragment, 1x *Eco*RI reaction buffer, 20 U of *Nde*I and 20 U of *Eco*RI in total volume of 50 μl was incubated at 37 ^{o}C for 18 hours. The DNA fragment was colleted from agarose gel by HiYield Gel/PCR DNA Fragments Extraction Kit.

2.8.1.4 Ligation of vector DNA and the gene fragment

The gene fragment (2.8.1.3) was ligated to the pET-17b vector (2.8.1.2) at vector: insert molar ratio of 1: 5. The 20 μl of ligation mixture contained 100 ng of vector DNA, 500 ng of the gene fragment, 1x ligation buffer and 10 U of T4 DNA ligase was incubated overnight at 22 ^{o}C. The recombinant plasmids (Figure 2.1), named pET-P5CR, were further used for transformation.

2.8.2 Transformation

2.8.2.1 Preparation of competent cells

A fresh overnight culture of *E. coli* BL21(DE3) was inoculated into 1 liter of LB broth with 1% inoculum size. Cells were grown to log phase at 37 ^{o}C with

vigorous shaking until OD_{600} was about 0.5 to 0.6. The culture was chilled on ice for 15 to 30 minutes and then centrifuged at 8,000 xg for 15 minutes at 4 ^{o}C. The cells were washed with 1 liter of cold water, spun down and washed again with 0.5 liter of cold water. After centrifugation, the cells were resuspended in approximately 20 ml of 10% glycerol in distilled water and centrifuged at 8,000xg for 15 minutes at 4 ^{o}C. Finally, the cell pellets were resuspended to a final volume of 2 to 3 ml in 10% glycerol. This suspension was divided into 40 µl aliquots and stored at -80 ^{o}C until used.

2.8.2.2 Transformation

The recombinant plasmids from 2.8.1.4 were transformed into competent cells of *E. coli* BL21(DE3) by electroporation. In the electroporation step, cuvette and sliding cuvette holder were chilled on ice. The Gene Pulser apparatus was set to the 25 µF capacitor, 2.5 kV, and the pulse controller unit was set to 200 Ω. Competent cells were gently thawed on ice. Two microliters of recombinant plasmid was mixed with 40 µl of the competent cells and then placed on ice for 1 minute. This mixture was transferred to a cold cuvette and the cuvette was applied one pulse at the above setting. Subsequently, 1 ml of LB medium was added immediately to the cuvette. The cells were quickly resuspended with a pasteur pipette. Then, the cell suspension was transferred to new tube, incubated at 37 ^{o}C for 1 hour with shaking and spun down to retain 200 µl. Finally, this suspension was spread onto the LB agar plates containing 100 µg/ml ampicillin and incubated at 37 ^{o}C overnight. Cells containing the recombinant plasmid which could grow on selective plate were picked and the plasmids were extracted.

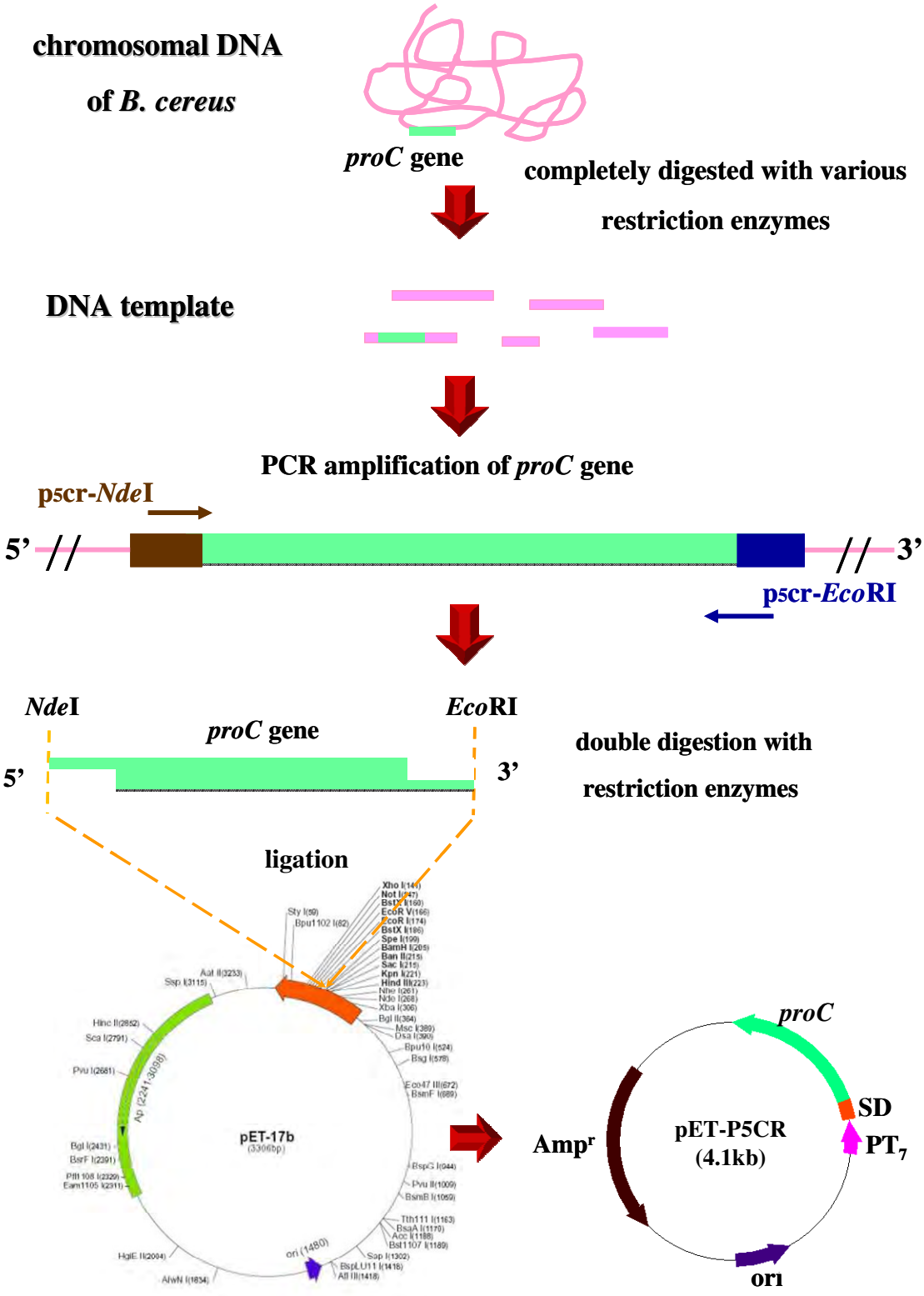

Figure 2.1 Construction of recombinant pET-P5CR

2.9 Expression of *proC* gene

2.9.1 Recombinant plasmid preparation

E. coli BL21(DE3) recombinant clones were grown in LB medium containing 100 μg/ml ampicillin. The growing condition was 37 °C for 18 hours with shaking. The cell cultures were collected in each 1.5 ml microcentrifuge tube by centrifugation at 8,000xg for 2 minutes. Then the plasmid from individual clone was extracted as described in 2.8.1.1. After that, the plasmids were completely digested with *Nde*I and *Eco*RI. The sizes of recombinant plasmids were estimated by agarose gel electrophoresis compared with λ/*Hin*dIII marker. Finally, the recombinant plasmids were confirmed for *proC* gene inserts by sequencing.

2.9.2 Crude extract preparation

The *E. coli* BL21(DE3) transformants were grown overnight at 37 °C for 18 hours in 5 ml of LB medium, pH 7.2, containing 100 μg/ml ampicillin. After that, 2.5% of the cell culture was inoculated into 200 ml of the same medium and was cultured at 37 °C with shaking. When the turbidity of the culture at 600 nm had reached 0.6, IPTG was added to final concentration of 0.4 mM to induce *proC* gene expression, and cutivation was continued at 37 °C for 4 hours. The cells were harvested by centrifugation at 10,000xg for 10 minutes, then washed with cold 0.85% NaCl. After that, the cell pellet was washed once in cold extraction buffer (0.1 M potassium phosphate buffer, pH 7.4 containing 0.1 mM PMSF, 0.01% *β*-mercaptoethanol and 1.0 mM EDTA) and centrifuged again. The cell pellet had been stored at -80 °C until it was sonicated.

Preparation of crude extract was performed by resuspending cell pellet in 5 ml of cold extraction buffer (0.1 M potassium phosphate buffer, pH 7.4 containing 0.1 mM PMSF, 0.01% *β*-mercaptoethanol and 1.0 mM EDTA). Cell suspension was sonicated on ice. Unbroken cells and cell debris were removed by

centrifugation at 17,500xg for 30 minutes. The enzyme had been kept at 4 ^{o}C until it was used to assay activity and protein concentration.

2.9.3 Enzyme activity assay

2.9.3.1 Determination of P5CR activity

The activity of P5CR for oxidative deamination was spectrophotometrically assayed. One milliliter of reaction mixture contained 200 µmol of glycine-KCl-KOH buffer, pH 10.0, 20 µmol of proline, 1 µmole of NAD^+ and the enzyme. The reaction mixture without NAD^+ was incubated at 30 ^{o}C 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 increment of 1 µmol of NADH in 1 minute. Specific activity is defined as units per milligram of protein.

2.9.3.2 Determination of Lys 6-DH activity

The activity of Lys 6-DH for oxidative deamination was spectrophotometrically assayed. One milliliter of reaction mixture contained 200 µmol of glycine-KCl-KOH buffer, pH 9.5, 20 µmol of L-lysine, 1 µmole of NAD^+ and the enzyme. The reaction mixture without NAD^+ was incubated at 30 ^{o}C 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 increment of 1 µmol of NADH in 1 minute. Specific activity is defined as units per milligram of protein.

2.9.4 Protein measurement

Protein concentration was determined by the modified method of Lowry *et al.,* (1956). The reaction mixture 3.05 ml containing 20-100 μg of protein, 50 μl of solution A, 2.5 ml of solution B was mixed and incubated at 30 ^{o}C for 10 minutes. After that, the solution mixture was incubated with 250 μl 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 preparations of all solutions were described in Appendix E

2.10 Optimization for *proC* gene expression

The *E. coli* BL21(DE3) transformant which showed the highest P5CR activity from 2.9 was grown overnight at 37 ^{o}C in 5 ml of LB medium containing 100 μg/ml ampicillin. After that, 2.5% of the cell culture was inoculated into 200 ml of the same medium and was cultured at 37 ^{o}C with shaking. When the turbidity of the culture at 600 nm had reached 0.6, the *proC* gene was induced by IPTG at final concentration of 0-1.0 mM at various induction times: 0, 4, 8, 12, 16, 20 and 24 hours. The cells were harvested by centrifugation at 10,000xg for 10 minutes, then washed with cold 0.85% NaCl. After that, the cell pellet was washed once in cold extraction buffer and centrifuged again. The cell pellet was stored at -80 ^{o}C until the next step. In crude extract preparation, the cell pellet was resuspended in 5 ml of cold extraction buffer and then broken by sonication on ice. Unbroken cell and cell debris were removed by centrifugation at 17,500xg for 30 minutes. The supernatant was stored at 4 ^{o}C for enzyme and protein assays as described in 2.9.3.1 and 2.9.4, respectively.

2.11 Purification of pyrroline-5-carboxylate reductase

2.11.1 Preparation of crude extract

The transformant was grown in 1.6 liter of LB medium, pH 7.2, containing 100 μg/ml ampicillin at the optimum condition from 2.10. The cell cultivation, crude extract preparation, assay for activity and protein determination were performed as described in 2.9.2, 2.9.3.1 and 2.9.4.

2.11.2 Enzyme purification procedures

The crude extract from 2.11.1 was purified by the following steps. All operations were done at 4 ^{o}C. The buffer used in all steps was 10 mM potassium phosphate buffer, pH 7.4 containing 0.01% β-mercaptoethanol and 1 mM EDTA.

2.11.2.1 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 2.11.1 was applied to the DEAE-Toyopearl column. The unbound proteins were eluted from the column with the buffer until the absorbance at 280 nm was nearly zero. After that, the buffer was changed by making linear salt gradient of 0 to 0.5 M KCl 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. P5CR activity was assayed using the method described in section 2.9.3.1. The KCl concentration was investigated by measuring its conductivity. The fractions

containing P5CR activity was pooled and dialyzed against the buffer. The P5CR activity and protein concentration of pooled fraction were measured as described in section 2.9.3.1 and 2.9.4, respectively.

2.11.2.2 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 2.11.2.1 was slowly adjusted to 25% saturation with fine ammonium sulfate and stirred gently at least 30 minutes. The protein solution was applied to the column at flow rate 1 ml/min. The unbound proteins were eluted from the column with the buffer containing 25% saturated ammonium sulfate until the absorbance to 280 nm was nearly zero. The bounded protein was eluted from the column with a step wise of ammonium sulfate (20% to 0% saturation) in the buffer. Two milliliter fractions were collected by using fraction collector and assayed for both protein profile and P5CR activity. Protein profile was determined by measuring an absorbance at 280 nm. P5CR activity was assayed by using the method as described in section 2.9.3.1. The fractions containing P5CR activity were pooled and dialyzed against the buffer. After desalting, the enzyme was concentrated with aquasorb. The P5CR activity and protein concentration were determined as described in section 2.9.3.1 and 2.9.4, respectively.

2.12 Polyacrylamide gel electrophoresis

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

2.12.1 Non-denaturing gel electrophoresis

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

2.12.1.1 Protein staining

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

2.12.1.2 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, 40 μmol of L-proline, 50 μmol of NAD^{+}, 250 μg of phenazine methosulfate and 2.5 mg of nitroblue tetrazolium. The gel was gently shaked at room temperature for 5 minutes. 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 until gel background was clear.

2.12.2 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 prepararion was described in Appendix G**.** The enzyme was mixed with 5x sample buffer (60 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol blue and 14.4 mM β-mercaptoethanol) by ratio 5: 1 and boiled for 10 minutes before loading to the gel. The electrophoresis was run from cathode towards anode at constant current (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). After electrophoresis, the gel was stained with Coomassie blue as described in section 2.12.1.1.

2.13 Characterization of pyrroline-5-carboxylate reductase

2.13.1 Molecular weight determination of pyrroline-5-carboxylate reductase

The molecular mass of P5CR was determined by SDS-polyacrylamide gel eletrophoresis (SDS-PAGE). The electrophoretic migration of P5CR was compared to marker proteins; β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), REase Bsp98I (25 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.4 kDa).

2.13.2 Coenzyme specificity of pyrroline-5-carboxylate reductase

The purified P5CR was used to study coenzyme specificity. NAD^+ was replaced by NAD^+ analog, β-nicotinamide adenine dinucleotide phosphate ($NADP^+$),

340 nm ($\varepsilon = 6.2x10^3\ M^{-1}.cm^{-1}$) (Misono *et al*., 1989) at final concentration of 2 mM for oxidative deamination. The result was expressed as a percentage of the residual activity.

2.13.3 Effect of pH on pyrroline-5-carboxylate reductase activity

The effect of pH on the activity was determined under the standard assay conditions for the oxidative deamination as described in section 2.9.3.1 at various pHs. The 200 mM of acetate buffer for pH 4.0 to 6.0, potassium phosphate for pH 6.0 to 8.0, Tris-HCl buffer for pH 7.0 to 9.0 and glycine-KCl-KOH buffer for pH 8.5 to 13.0 were used. The pH of each reaction mixture was measured with a pH meter at room temperature after the reaction. To compare the effect of pH on P5CR activity, the percentage of residual activity was plotted against the final pH.

2.13.4 Effect of temperature on pyrroline-5-carboxylate reductase activity

The effect of temperature on the activity was examined under the standard assay condition of the oxidative deamination as described in section 2.9.3.1 at various temperatures from 20 ^{o}C to 80 ^{o}C. The percentage of residual activity was plotted against the temperature used for the activity assay.

2.13.5 Effect of pH on pyrroline-5-carboxylate reductase stability

The purified P5CR was used to study pH stability. After the enzyme had been incubated at 30 ^{o}C for 20 minutes in each of the 10 mM buffer at various pHs, an aliquot of the enzyme solution was withdrawn and the remaining activity of enzyme was measured under the standard assay condition for the oxidative deamination as described in section 2.9.3.1. The 10 mM buffers used were acetate buffer for pH 4.0 to 6.0, potassium phosphate for pH 6.0 to 8.0, Tris-HCl buffer for pH 7.5 to 9.0 and glycine-KCl-KOH buffer for pH 8.5 to 13.0. The percentage of P5CR residual activity was plotted against the incubated pH.

2.13.6 Effect of temperature on pyrroline-5-caboxylate reductase stability

The effect of temperature on the stability of the enzyme was determined from 35 ^{o}C to 60 ^{o}C. The purified P5CR was incubated at various temperatures for 10, 30, 60, 90 and 120 minutes, respectively before determination of enzyme activity under the standard assay condition in the oxidative deamination according to the method described in section 2.9.3.1. The result was shown as a percentage of the residual activity.

2.14 Kinetic studies of pyrroline-5-carboxylate reductase

Since P5C is unstable, we used L-proline as a substrate for kinetic studies. A series of steady-state kinetic analyzes were carried out in order to investigate the kinetic parameters. Initial velocity studies for the oxidative deamination were carried out under the standard reaction condition as described in section 2.9.3.1, except that various amounts of L-proline and NAD^+ were used. The concentrations of L-proline used were 0.1, 0.15, 0.2, 0.25, 0.3, 0.5, 0.75 and 1.0 mM, and those of NAD^+ used were 0.2, 0.3, 0.4, 0.6 and 0.8 mM. The Lineweaver-Burke plots (double-reciprocal plots) of initial velocities against L-proline 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-proline and NAD^+ were determined from these two plots, respectively.

2.15 Cloning and expression of *lysdh* and *proC* heterologous gene in *E. coli* BL21(DE3) using pET-17b

For construction pET-ADK-P5CR (Figure 2.2), the whole *proC* gene with T7 promoter and Shine-Dargano sequence of pET-vector was amplified by the PCR method and inserted to downstream of *lysdh* gene of pET-ADK. The primers were designed upon the sequence of pET-P5CR. The 5′-primer consisted of *Bam*HI restriction site following by T7 promoter and Shine-Dargano sequence of pET-17b

(T7-*Bam*HI), while the 3′-primer consisted of *Eco*RI restriction site and the sequence at 3′-end of *proC* gene (p5cr-*Eco*RI).

PCR was performed in 20 µl reaction mixture containing 1.5 U of *Pfu* DNA polymerase, 1x *Pfu* DNA Polymerase buffer with $MgSO_4$, 0.2 mM each dNTPs, 2 ng DNA template and 10 pmole of each primer. The thermocycle was consisted of predenaturation at 95 °C for 10 minutes, and 34 cycles of denaturation at 95 °C for 10 minute, annealing at 40.3 °C for 30 seconds, extension at 72 °C for 2 minutes following by final extension at 72 °C for 7 minutes.

The PCR fragment was digested with *Bam*HI and *Eco*RI and then ligated with *Bam*HI and *Eco*RI site of pET-ADK. The ligation products were transformed into *E. coli* BL21(DE3) by eletroporation. The transformed cells were grown on LB agar plates containing 100 µl/ml ampicillin and were incubated at 37 °C for 16 hours. Cells containing the recombinant plasmids, which had *lysdh* and *proC* gene were picked, and the plasmids were isolated and checked by agarose gel electrophoresis. Sequence of the heterologous genes in the recombinant plasmid was confirmed by sequencing. Crude enzymes were prepared. The activity and protein concentration of P5CR and Lys 6-DH were measured as described in 2.9.3.1, 2.9.3.2 and 2.9.4, respectively.

2.16 Determination of L-pipecolic acid production

2.16.1 L-pipecolic acid production in *E. coli* containing pET-ADK-P5CR

The *E. coli* harboring pET-ADK-P5CR was determined for its ability to biotransform L-lysine into L-pipecolic acid. Twelve transformants and four cell controls (*E. coli* BL21(DE3), *E. coli* harboring pET-17b, *E. coli* harboring pET-ADK and *E. coli* harboring pET-P5CR) were prepared in standard reaction to primary screening of L-PA production. The cells, with the exception of *E. coli* BL21(DE3),

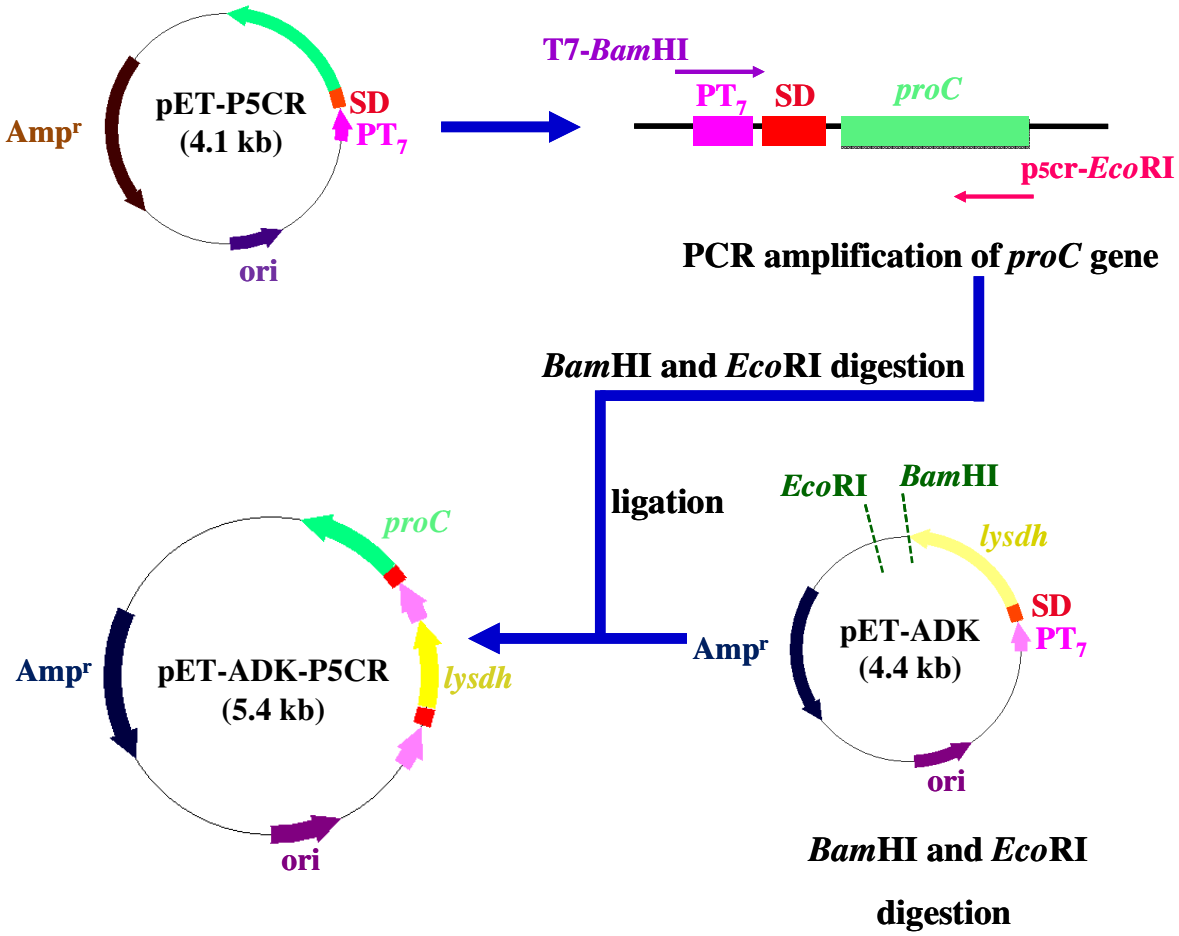

Figure 2.2 Construction of recombinant pET-ADK-P5CR

were grown overnight at 37 ^{o}C with shaking in 5 ml of LB medium containing 100 μg/ml ampicillin. For *E. coli* BL21(DE3), the cells were grown overnight at 37 ^{o}C with shaking in 5 ml of LB medium. After that, the cell culture was inoculated into 200 ml of the same medium and was cultured at 37 ^{o}C with shaking until OD_{600} reached 0.6. *lys 6-dh* and *proC* were induced by IPTG at final concentration of 0.4 mM and cultivated for 4 hours. Cells were harvested by centrifugation at 8,000xg for 10 minutes, twice washed and resuspended in 0.1 M potassium phosphate buffer, pH 7.4. The cell pellets were used in the next experiment.

2.16.2 Standard reaction for L-pipecolic acid production

The 500 μl of the reaction mixture consisted of 0.05 g of cell pellet, 200 mM L-lysine, 200 mM Tris-HCl buffer, pH 8.5 and distilled water was made. The reactions were performed at 30 ^{o}C with shaking at 250 rpm for 24 hours. The supernatants were separated from the cells by centrifugation at 8,000xg for 2 minutes. After that, the supernatants were analyzed by TLC and/or HPLC to estimate the amounts of L-lysine and L-PA.

2.16.3 Optimization of the *lysdh* and *proC* gene expression for L-pipecolic acid production

E. coli BL21(DE3) harboring pET-ADK-P5CR was grown overnight at 37 ^{o}C with shaking in 100 ml of LB medium containing 100 μg/ml ampicillin. After that, 5 ml of the cell culture was inoculated into 200 ml of the same medium and was cultured at 37 ^{o}C with shaking until OD_{600} reached 0.6. *lys 6-dh* and *proC* were induced by IPTG at final concentration of 0, 0.1, 0.2, 0.3, 0.4, and 0.6 mM and then 10 ml of each culture was collected at various times: 0, 4, 8, 12, 16, 20 and 24 hours. Cells were harvested by centrifugation at 8,000xg for 10 minutes, washed and resuspended in 0.1 M potassium phosphate buffer pH 7.4 and then the reaction was carried out as described in 2.16.2.

2.16.4 Effect of pH on production of L-pipecolic acid

E. coli BL21(DE3) containing pET-ADK-P5CR was determined for the L-PA production in various pHs. The *E. coli* transformant was cultured at its optimum condition for the enzyme induction. The reaction mixture was carried out as described in 2.17.2 but with various buffers (Tris-HCl buffer for pH 7.0 to 9.0, potassium phosphate buffer for pH 6.0 to 8.5, MOPS buffer for pH 7.5 to 8.7, borate buffer for pH 7.5 to 9.0, sodium carbonate-bicarbonate buffer for pH 9.5 to 11.0, Na_2HPO_4 buffer for pH 11.0 to 12.0 and potassium chloride buffer pH 12.0 to 13.0) at the same concentration (200 mM).

2.16.5 Effect of L-lysine concentration on production of L-pipecolic acid

The production of L-PA by the recombinant *E. coli* containing pET-ADK-P5CR was carried out as described in 2.17.2 but with various L-lysine concentrations (10-200 mM) at the optimum pH.

2.17 Determination of L-lysine and L-pipecolic acid

2.17.1 Analysis of L-lysine and L-pipecolic acid by TLC (Nelson and Cox, 2000)

The biotransformation mixtures were preliminarily analyzed by TLC. The 2 µl of the mixtures and commercial L-lysine and L-PA standards were subjected to 10 X 10 cm cellulose TLC plastic sheets (MEKCK, Germany) in parallel with standard. The TLC plates were developed with *n*-butanol: acetic acid: H_2O (4:1:1) and after that the plates were dried in hot air. The detection was carried out by dipping the plate in 0.5% ninhydrin in ethanol-acetone solution at volume ration of 30:70 and then drying at 110 °C for 5 minutes. Each spot of samples and standards, which gave high intensity in time course study, were quantified by Gel Document. The ratio of the distance a compound moves from the baseline to the distance of the solvent front from the baseline is defined as the retardation factor (R_f).

2.17.2 Quantitative determination of L-lysine and L-pipecolic acid by HPLC (Marfey, 1984)

Sample preparation: One hundred µl of a 5 µmol aqueous solution of amino acids are mixed with 40 µl of 1.0 M sodium bicarbonate and then 200 µl of 1% FDAA in acetone. The solution was vortexed and incubated at 40 °C for 1 hour. After the reaction was quenched by the addition of 20 µl of 2 M HCl, the 20 µl of this solution was analyzed by HPLC.

HPLC conditions: HPLC was performed using a Inertsil ODS-3 (250 x 4.6 mm) column maintained at 25 °C. Acetonitrile (solvent A) and 0.05 M triethylamine phosphate pH 3.0 (solvent B) were used as the mobile phase in this system (35:65). The flow rate was 1 ml/min with UV detection at 340 nm.

The yield of the enzyme reaction is defined as mole of L-PA per mole of L-lysine.

CHAPTER III

RESULTS

3.1 Cloning of *proC* gene using pET-17b vector

3.1.1 Chromosomal DNA extraction

The chromosomal DNA was extracted from *B. cereus* ATCC 11778 according to modified method of Frederick *et al* (1995). After chromosomal has been achieved, it was run on agarose gel eletrophoresis to examine its quality and quantity. It was found that extracted DNA had molecular weight over 23.1 kb and showed high purity which corresponded with its A_{260}/A_{280} ratio of 1.8–2.0 (Figure 3.1). The DNA concentration was about 40 ng/µl. Thus, the quality of obtained DNA was appropriate for digestion with restriction endonuclease for PCR amplification, nucleotide sequencing and further cloning.

3.1.2 PCR amplification of the full length *proC* gene

The chromosomal DNA of *B. cereus* ATCC 11778 was completely digested with various restriction enzymes: *Hin*dIII, *Kpn*I, *Sal*I, *Eco*RI and *Sac*I. The agarose gel eletrophoresis analysis of digested DNA in Figure 3.2 showed that the pattern resulting from all five digestions gave the smear pattern of DNA lower than 9.4 kb.

To express the *proC* gene in *E. coli* BL21(DE3) under T7 promoter of expression vector, pET-17b, a specific primer pair used for full length amplification of the *proC* gene was designed as described in 2.7.3.1. The 5' – primer, p5cr*Nde*I, comprised *Nde*I restriction site and 5' – end of *proC* gene. The 3' – primer, p5cr*Eco*RI, consisted of *Eco*RI site, the TAA translational termination signal and 3' – end of *proC* gene. Figure 3.3 showed the 0.8 kb PCR product of the *proC* gene

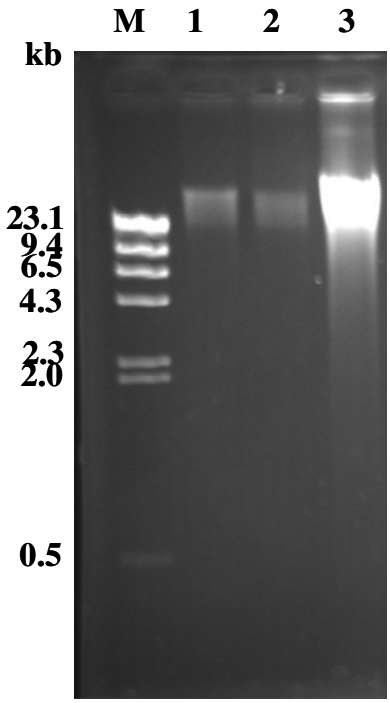

Figure 3.1 Chromosomal DNA of *B. cereus* ATCC 11778

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

Lane 1-3 = chromosomal DNA

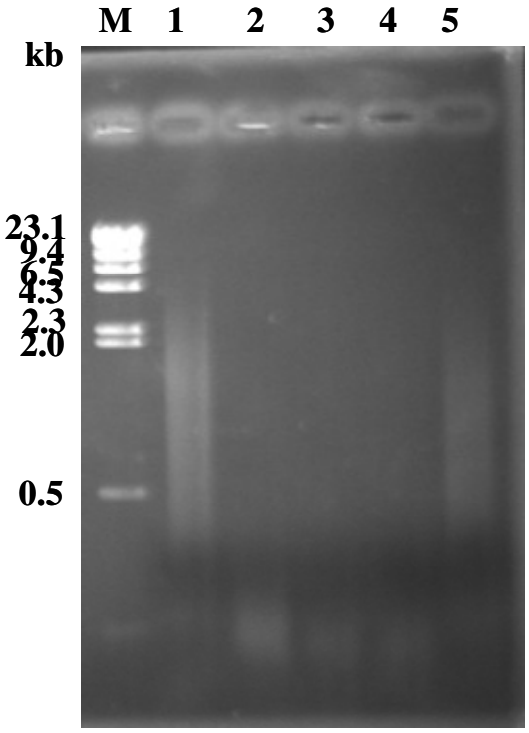

Figure 3.2 Restriction enzyme digested chromosomal DNA of *B. cereus*

Lane M = λ/*Hin*dIII standard DNA marker
Lane 1 = chromosomal DNA digested with *Hin*dIII
Lane 2 = chromosomal DNA digested with *Kpn*I
Lane 3 = chromosomal DNA digested with *Sal*I
Lane 4 = chromosomal DNA digested with *Eco*RI
Lane 5 = chromosomal DNA digested with *Sac*I

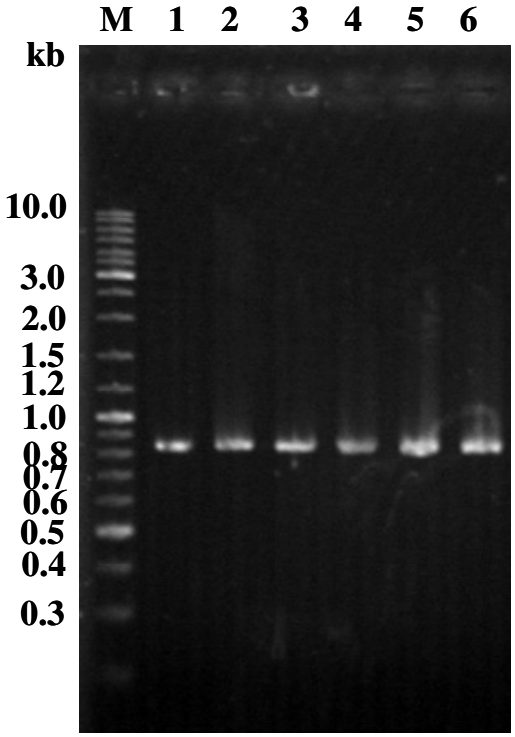

Figure 3.3 Whole *proC* gene amplication at various annealing temperatures using *Hin*dIII digested DNA as template

Lane M = 100 bp ladder DNA marker
Lane 1 = PCR product using annealing temperature at 40.3 ℃
Lane 2 = PCR product using annealing temperature at 42.5 ℃
Lane 3 = PCR product using annealing temperature at 44.7 ℃
Lane 4 = PCR product using annealing temperature at 46.8 ℃
Lane 5 = PCR product using annealing temperature at 48.5 ℃
Lane 6 = PCR product using annealing temperature at 49.8 ℃

fragment amplified from *Hin*dIII digested DNA template at various annealing temperatures. All of annealing temperatures gave strong band of *proC* gene without non–specific DNA fragment, so these PCR products were recovered and used for further cloning.

3.1.3 Transformation

The 0.8 kb amplified gene fragment was digested with *Nde*I and *Eco*RI, and then ligated with *Nde*I-*Eco*RI digested pET-17b vector. The recombinant plasmid was transformed into *E. coli* BL21(DE3) by eletroporation as described in 2.8.2.2. The sixty-two clones which could grow on LB plate containing 100 µl/ml ampicillin were randomly picked for plasmid extraction and digestion with *Nde*I-*Eco*RI as described in 2.8.1.1 and 2.8.1.2, respectively.

The recombinant plasmids (pET-P5CR) in *E. coli* BL21(DE3) gave two bands, relaxed and supercoiled bands, on agarose gel eletrophoresis in Figure 3.4. After digestion, a linear pET-17b with 3.3 kb and 0.8 kb of inserted *proC* gene fragment were obtained as shown in Figure 3.5. From sixty-two clones, twelve clones harboured recombinant plasmid (4.1 kb) which contain *proC* gene. Therefore, these transformants were assayed for P5CR activity.

3.1.4 Enzyme activity of *E. coli* BL21(DE3) harbouring pET-P5CR

The twelve recombinant clones were grown as described in 2.9.2 to prepare crude extract for determination of the enzyme activity. *E. coli* BL21(DE3) with and without the plasmid pET-17b were used as control. The transformants showed total activities varied from 9.79 to 2,565 U and specific activity 0.19 to 28.9 U/mg protein as shown in Table 3.1. The transformant No. 6 showed the highest total activity and specific activity of 2,565 U and 28.9 U/mg protein, respectively, which was 578 fold higher than that of the wild type *B. cereus*. Thus, this recombinant clone was used in further experiment.

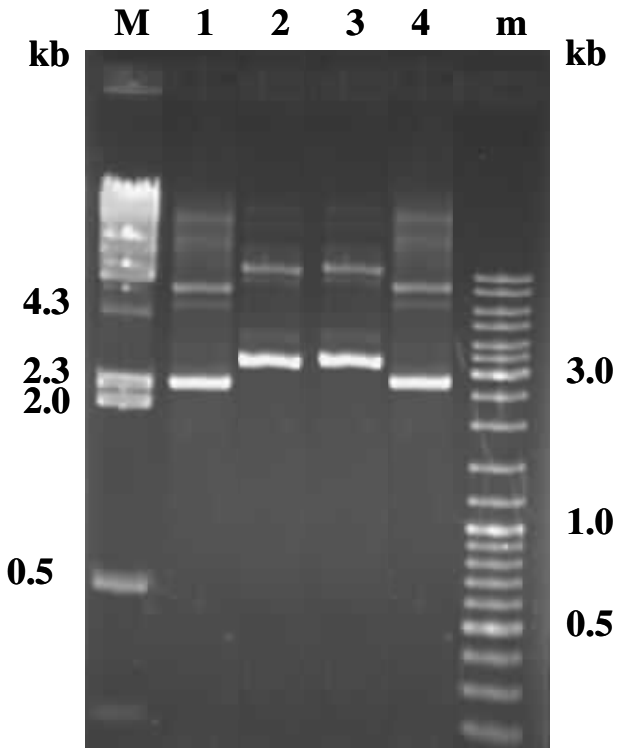

Figure 3.4 Extracted plasmid pattern

Lane M = λ/*Hin*dIII standard DNA marker
Lane 1,4 = undigested pET-17b
Lane 2-3 = extracted recombinant plasmid
Lane m = 100 bp ladder DNA marker

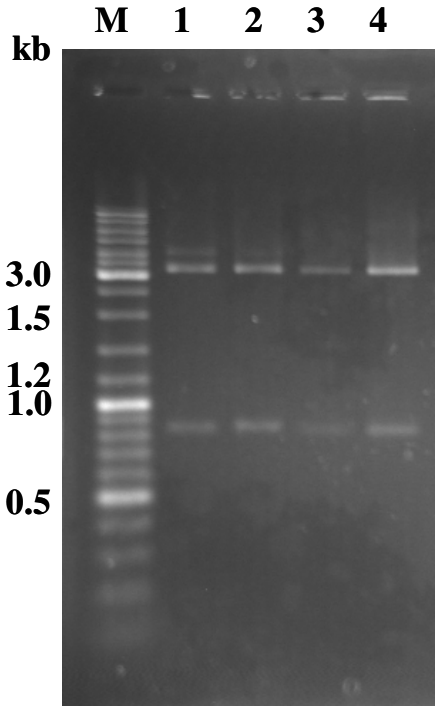

Figure 3.5 Restriction pattern of pET-P5CR from selected recombinant clones

Lane M = 100 bp ladder DNA marker

Lane 1-4 = *Eco*RI-*Nde*I digested plasmid

Table 3.1 P5CR activity from the crude extract of 12 selected *E. coli* BL21(DE3) transformants harbouring pET-P5CR[a]

Sources	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)
B. cereus ATCC 11778	6.00	116	0.05
E. coli BL21(DE3)	0	103	0
E. coli BL21(DE3) harbouring pET-17b	0	68.0	0
No.1	166	42.4	3.92
No.2	144	52.7	2.73
No.3	137	30.4	4.51
No.4	49	32.4	1.51
No.5	2,232	115	19.4
No.6	**2,565**	**88.7**	**28.9**
No.7	1,960	99.4	19.7
No.8	197	40.9	4.82
No.9	136	40.8	3.33
No.10	9.79	50.7	0.19
No.11	2,052	90.2	22.7
No.12	173	48.7	3.55

[a] Crude extract were prepared from 200 ml of cell culture by induction with 0.4 mM IPTG for 4 hours.

3.2 Nucleotide sequence and deduced amino acid sequence of *proC* gene

DNA insert of recombinant plasmid (pET-P5CR) was sequenced. The obtained complete nucleotide sequence was shown in Figure 3.6. The structural gene contained 819 bp open reading frame, which encoded a polypeptide of 272 amino acids. Molecular weight of the enzyme subunit calculated from the deduced amino acid sequence by compute pI/MW program was 28.9 kDa.

When the percentage of similarity of nucleotide sequences of the whole *proC* gene was compared to those in the EMBL-GenBank-DDBL database, the percentage of sequence similarity between *proC* from *B. cereus* ATCC 11778 and those from *B. cereus* Q1, *B. cereus* ATCC 10987, *B. cereus* AH187 and *B. cereus* E33L were 92, 92, 91 and 91%, respectively (Figure 3.6).

Moreover, the percentage of similarity between the deduced amino acid sequence of P5CR from *B. cereus* ATCC 11778 and those from *B. cereus* Q1, *B. cereus* ATCC 10987, *B. cereus* AH187 and *B. cereus* E33L were 98, 98, 98 and 98%, respectively (Figure 3.7).

3.3 Optimization of *proC* gene expression

3.3.1 Optimization of *proC* gene expression

The transformant No.6 giving the highest P5CR activity had been grown and induced by IPTG to final concentration of 0, 0.2, 0.4, 0.6, 0.8 and 1 mM at various times (0, 4, 8, 12, 16, 20 and 24 hours) before the cells were harvested as described in 2.10. The results were displayed in Figure 3.8. When recombinant clone was cultured without IPTG induction, the expression of *proC* gene was slightly increased until 8 hours with the specific activity of 26.3 U/mg protein after that

```
CLUSTAL 2.0.10 multiple sequence alignment

Q1       ATGGATAAACAAATTGGATTCATCGGATGCGGAAATATGGGGATGGCTATAATTGGCGGG 60
AH187    ATGGATAAACAAATTGGATTCATCGGATGCGGAAATATGGGGATGGCTATAATTGGCGGG 60
E33L     ATGGATAAACAAATTGGATTCATTGGATGCGGAAATATGGGGATGGCTATAGTTGGTGGG 60
10987    ATGGATAAACAAATTGGATTCATCGGATGCGGAAATATGGGGATGGCTATAATTGGCGGG 60
11778    ATGGATAAACAAATTGGATTCATCGGTTGCGGAAATATGGGAATGGCTATGATTGGCGGA 60
         ************************ ** ************** ********  **** **

Q1       ATGCTAAATAAACATATAGTGTCTTCAAATCATATCATTTGTTCAGATTTAAACACGACA 120
AH187    ATGCTAAATAAACATATAGTGTCTTCAAATCATATCATTTGTTCAGATTTAAACACGACA 120
E33L     ATGCTAAATAAAAAGATAGTGTCTTCAAATAAAATCATGTGTTCAGATTTAAACACGACG 120
10987    ATGCTAAACAAAAAGATAGTGTCTTCAAATAAAATCATGTGTTCAGATTTAAACACGACG 120
11778    ATGATAAACAAAAATATAGTGTCTTCAAATCAAATTATTTGTTCAGATTTAAACACTGCT 120
         *** **** *** * *************** * ** ** *****************  *

Q1       AATTTAGAAAATGCTAGTGAAAAGTACGGAATAACTATAACTACTGATAACAATGAAGTC 180
AH187    AATTTAGAAAATGCTAGTGAAAAGTACGGAATAACTATAACTACTGATAACAATGAAGTC 180
E33L     AATTTAGAAAATGCTAGTGAAAAGTACGGAATATCTATAACTACTGACAACAAGAAAGTC 180
10987    AATTTAGAAAATGCTAGTGAAAAGTACGGAATAACTATAACTACCGACAACAATGAAGTC 180
11778    AATTTAAAAAATGCTAGTGAAAAATATGGACTAACTACAACTACCGACAACAATGAAGTA 180
         ****** ***************** ** *** ** *** ****** ** *****  ****

Q1       GCTAAAAATGCTGATATTTTAATTTTATCAATTAAACCAGACTTATACCCATTAGTAATT 240
AH187    GCGAAAAATGCTGATATTTTAATTTTATCAATTAAACCAGACTTATACTCATTAGTAATT 240
E33L     GCTAAAAATGCTGATATTTTAATTTTATCAATTAAGCCAGACTTATACACCGCAGTAATT 240
10987    GCTAAAAATGCTGATATTTTAATTTTATCAATTAAACCAGACTTATACCCATTAGTAATT 240
11778    GCTAAAAATGCTGATATTTTAATTTTATCAATCAAACCAGACCTATACGCGTCGATAATT 240
         ** ****************************** ** ****** ***** *     *****

Q1       AACGAAATTAAAGAAGTGATAAAAAACGATGCTATCATCGTTACGATCGCTGCTGGTAAA 300
AH187    AACGAAATTAAAGAAGTGATAAAAAACGATGCTATCATCGTTACGATCGCTGCTGGTAAA 300
E33L     AACGAAATTAAAGAAGTGATAAAAAACGATGCTATCATCGTTACGATCGCTGCTGGTAAA 300
10987    AACGAAATTAAAGAAGTAATAAAAAACGATGCTATCATCGTTACGATCGCTGCTGGTAAA 300
11778    AATGAAATAAAAGAAGTAATCAAAAACGATGCTGTCATCGTAACGATCGCTGCTGGGAAA 300
         ** ***** ******** ** ************ ******* ************** ***

Q1       AGTATTGAAAGTACTGAAAATGCCTTTAATAAAAAATTAAAAGTTGTAAGAGTAATGCCT 360
AH187    AGTATTGAAAGTACTGAAAATGCCTTTAATAAAAAATTAAAAGTTGTACGAGTAATGCCT 360
E33L     AGTATTGAAAGTACTGAAAATGCCTTTAATCAAAAATTAAAAGTTGTAAGAGTAATGCCT 360
10987    AGTATTGAAAGTACTGAAAATGCCTTTAATAAAAAATTAAAAGTTGTACGAGTAATGCCT 360
11778    AGTATTGAAAGTACTGAAAATGCCTTTAATAAAAAAGTAAAGGTTGTAAGAGTAATGCCT 360
         ****************************** ***** **** ****** ***********

Q1       AATACTCCTGCTCTTGTTGGAGAAGGAATGTCAGCATTATGCCCGAATGAAATGGTGACA 420
AH187    AATACTCCTGCTCTTGTTGGAGAAGGAATGTCAGCATTATGCCCGAATGAAATGGTGACA 420
E33L     AATACTCCTGCTCTTGTTGGAGAAGGAATGTCTGCATTATGCCCGAATGAAATGGTGACA 420
10987    AATACTCCTGCTCTTGTTGGAGAAGGAATGTCTGCATTATGCCCAAATGAAATGGTGACA 420
11778    AATACTCCTGCTCTTGTTGGAGAGGGAATGTCTGCATTATGCCCTAATGAAATGGTGACA 420
         ********************** ******** *********** ***************
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(continued)

Figure 3.6 Linear alignment of the nucleotide sequence of *proC* gene from various sources. The linear alignment was made by clustalW program. Q1 = *B. cereus* Q1 (CP000227.1), AH187 = *B. cereus* AH187 (CP001177.1), E33L = *B. cereus* E33L (CP000001.1), 10987 = *B. cereus* ATCC 10987 (AE017194.1) and 11778 = *B. cereus* 117778. Conserve residues are indicated by asterisks (*).

```
CLUSTAL 2.0.10 multiple sequence alignment

Q1       GAAAAAGATTTAGAAGATGTGCTAAACATTTTCAATAGTTTTGGTCAATCAGAGATTGTA 480
AH187    GAAAAAGATTTAGAAGATGTGCTAAACATTTTCAATAGTTTTGGTCAATCAGAGATTGTA 480
E33L     GAAAAAGATTTAGAAGAAGTGCTAAACATTTTCAATAGTTTTGGTCAATCAGAGATTGTA 480
10987    GAAAAGGATTTAGAAGATGTGCTAAACATTTTCAATAGTTTTGGTCAATCAGAGATCGTA 480
11778    GAAAAAGATTTAGAAGATGTACTAAACATTTTCAATAGTTTTGGTCAAACAGAGATCGTA 480
         ***** *********** ** ************************** ******* ***

Q1       AGTGAAAAGTTAATGGATGTTGTAACATCTGTAAGTGGTTCTTCACCAGCATACGTATAT 540
AH187    AGTGAAAAGTTAATGGATATTGTAACATCTGTAAGTGGTTCTTCACCAGCATACGTATAT 540
E33L     AGTGAAAAGTTAATGGATGTTGTAACATCTGTAAGTGGTTCTTCACCAGCATACGTATAT 540
10987    AGTGAAAAGTTAATGGATGTTGTAACATCTGTAAGTGGCTCTTCACCAGCATACGTATAT 540
11778    AGTGAAAAATTAATGGATGTTGTAACATCTGTAAGTGGTTCTTCACCAGCTTATGTATAT 540
         ******** ********* ****************** *********** ** ******

Q1       ATGATTATAGAAGCGATGGCAGATGCTGCTGTACTAGATGGCATGCCAAGAAATCAAGCA 600
AH187    ATGATTATAGAAGCGATGGCAGATGCTGCTGTACTAGATGGCATGCCAAGAAATCAAGCA 600
E33L     ATGATTATAGAAGCGATGGCAGATGCTGCTGTACTAGATGGTATGCCAAGAAATCAAGCA 600
10987    ATGATTATAGAAGCGATGGCAGATGCTGCTGTACTAGATGGCATGCCAAGAAATCAAGCA 600
11778    ATGATTATAGAAGCGATGGCAGATGCTGCTGTACTAGATGGTATGCCAAGAAATCAAGCA 600
         ************************************** ******************

Q1       TATAAATTTGCTGCTCAGGCAGTATTAGGCTCTGCAAAAATGGTACTAGAAACAGGAATA 660
AH187    TATAAATTTGCTGCTCAGGCAGTATTAGGATCTGCAAAAATGGTACTAGAAACAGGCATA 660
E33L     TATAAATTCGCTGCTCAAGCAGTATTAGGCTCTGCAAAAATGGTACTAGAAACAGGAATA 660
10987    TATAAATTCGCTGCTCAAGCAGTATTAGGCTCTGCAAAAATGGTACTAGAAACAGGAATA 660
11778    TATAAATCCGCTGCTCAAGCTGTGTTAGGCTCTGCAAAAATGGTACTAGAAACAGGAATA 660
         *******  ******** ** ** ***** ************************** ***

Q1       CATCCAGGTGAATTGAAAGATATGGTTTGTTCTCCTGGCGGAACAACGATAGAAGCTGTA 720
AH187    CATCCAGGTGAATTGAAAGATATGGTTTGTTCTCCTGGCGGAACAACGATAGAAGCTGTA 720
E33L     CATCCAGGTGAATTGAAAGATATGGTTTGTTCTCCTGGCGGAACAACGATAGAAGCTGTA 720
10987    CATCCAGGTGAATTGAAAGATATGGTTTGTTCTCCTGGCGGAACAACGATAGAAGCTGTA 720
11778    CATCCAGGTGAATTGAAAGATATGGTTTGTTCTCCTGGCGGAACGACAATAGAAGCTGTA 720
         ****************************************** ** ************

Q1       GCAACTTTAGAGGAAAAAGGCTTACGAACAGCCATCATTTCAGCTATGCAACGTTGTACA 780
AH187    GCAACTTTAGAGGAAAAAGGCTTACGAACAGCCATCATTTCAGCTATGCAACGTTGTACA 780
E33L     GCAACTTTAGAGGAAAAAGGCTTACGAACAGCCATCATTTCAGCTATGCAACGTTGTACA 780
10987    GCAACTTTAGAGGAAAAAGGCTTACGAACAGCCATCATTTCAGCTATGCAACGTTGTACA 780
11778    GCAACATTAGAGGAAAAAGGACTGAGAATAGCGATTATTTCAGCTATGCAGCGTTGTACG 780
         ***** **************  *  *** *** ** ************** ********

Q1       CAAAAGTCTGTCGAACTATCTGGTCAAACGAAAAAATAA 819
AH187    CAAAAGTCTGTCGAACTATCTGGTCAAACGAAAAAATAA 819
E33L     CAAAAGTCTGTCGAACTATCTGGTCAAACGAAAAAGTAA 819
10987    CAAAAGTCTGTTGAACTATCTGGTCAAACGAAAAAGTAA 819
11778    CAAAAGTCTGTTGAACTATCTGGTCAAACGAAAAAGTAA 819
         *********** ********************** ***
```

Figure 3.6 Linear alignment of the nucleotide sequence of *proC* gene from various sources. The linear alignment was made by clustalW program. Q1 = *B. cereus* Q1 (CP000227.1), AH187 = *B. cereus* AH187 (CP001177.1), E33L = *B. cereus* E33L (CP000001.1), 10987 = *B. cereus* ATCC 10987 (AE017194.1) and 11778 = *B. cereus* 117778. Conserve residues are indicated by asterisks (*).

```
CLUSTAL 2.1 multiple sequence alignment


Q1       MDKQIGFIGCGNMGMAIIGGMLNKHIVSSNHIICSDLNTTNLENASEKYGITITTDNNEV 60
AH187    MDKQIGFIGCGNMGMAIIGGMLNKHIVSSNHIICSDLNTTNLENASEKYGITITTDNNEV 60
E33L     MDKQIGFIGCGNMGMAIVGGMLNKKIVSSNKIMCSDLNTTNLENASEKYGISITTDNKKV 60
10987    MDKQIGFIGCGNMGMAIIGGMLNKKIVSSNKIMCSDLNTTNLENASEKYGITITTDNNEV 60
11778    MDKQIGFIGCGNMGMAMIGGMINKNIVSSNQIICSDLNTANLKNASEKYGLTTTTDNNEV 60
         ****************::***:**:*****:*:******:**:*******:: ****::*

Q1       AKNADILILSIKPDLYPLVINEIKEVIKNDAIIVTIAAGKSIESTENAFNKKLKVVRVMP 120
AH187    AKNADILILSIKPDLYSLVINEIKEVIKNDAIIVTIAAGKSIESTENAFNKKLKVVRVMP 120
E33L     AKNADILILSIKPDLYTAVINEIKEVIKNDAIIVTIAAGKSIESTENAFNQKLKVVRVMP 120
10987    AKNADILILSIKPDLYPLVINEIKEVIKNDAIIVTIAAGKSIESTENAFNKKLKVVRVMP 120
11778    AKNADILILSIKPDLYASIINEIKEVIKNDAVIVTIAAGKSIESTENAFNKKVKVVRVMP 120
         ****************. :************:******************:*:*******

Q1       NTPALVGEGMSALCPNEMVTEKDLEDVLNIFNSFGQSEIVSEKLMDVVTSVSGSSPAYVY 180
AH187    NTPALVGEGMSALCPNEMVTEKDLEDVLNIFNSFGQSEIVSEKLMDIVTSVSGSSPAYVY 180
E33L     NTPALVGEGMSALCPNEMVTEKDLEEVLNIFNSFGQSEIVSEKLMDVVTSVSGSSPAYVY 180
10987    NTPALVGEGMSALCPNEMVTEKDLEDVLNIFNSFGQSEIVSEKLMDVVTSVSGSSPAYVY 180
11778    NTPALVGEGMSALCPNEMVTEKDLEDVLNIFNSFGQTEIVSEKLMDVVTSVSGSSPAYVY 180
         *************************:**********:*********:*************

Q1       MIIEAMADAAVLDGMPRNQAYKFAAQAVLGSAKMVLETGIHPGELKDMVCSPGGTTIEAV 240
AH187    MIIEAMADAAVLDGMPRNQAYKFAAQAVLGSAKMVLETGIHPGELKDMVCSPGGTTIEAV 240
E33L     MIIEAMADAAVLDGMPRNQAYKFAAQAVLGSAKMVLETGIHPGELKDMVCSPGGTTIEAV 240
10987    MIIEAMADAAVLDGMPRNQAYKFAAQAVLGSAKMVLETGIHPGELKDMVCSPGGTTIEAV 240
11778    MIIEAMADAAVLDGMPRNQAYKSAAQAVLGSAKMVLETGIHPGELKDMVCSPGGTTIEAV 240
         ********************** ************************************

Q1       ATLEEKGLRTAIISAMQRCTQKSVELSGQTKK 272
AH187    ATLEEKGLRTAIISAMQRCTQKSVELSGQTKK 272
E33L     ATLEEKGLRTAIISAMQRCTQKSVELSGQTKK 272
10987    ATLEEKGLRTAIISAMQRCTQKSVELSGQTKK 272
11778    ATLEEKGLRIAIISAMQRCTQKSVELSGQTKK 272
         ********* ***********************
```

Figure 3.7 Linear alignment of the deduced amino acid sequence of P5CR from various sources. The linear alignment was made by clustalW program. Q1 = *B. cereus* Q1 (CP000227.1), AH187 = *B. cereus* AH187 (CP001177.1), E33L = *B. cereus* E33L (CP000001.1), 10987 = *B. cereus* ATCC 10987 (AE017194.1) and 11778 = *B. cereus* 117778. Conserve residues are indicated by asterisks (*). : means amino acids which have the same group of side chains and similar size while . means amino acids which have the same group of side chains but different size.

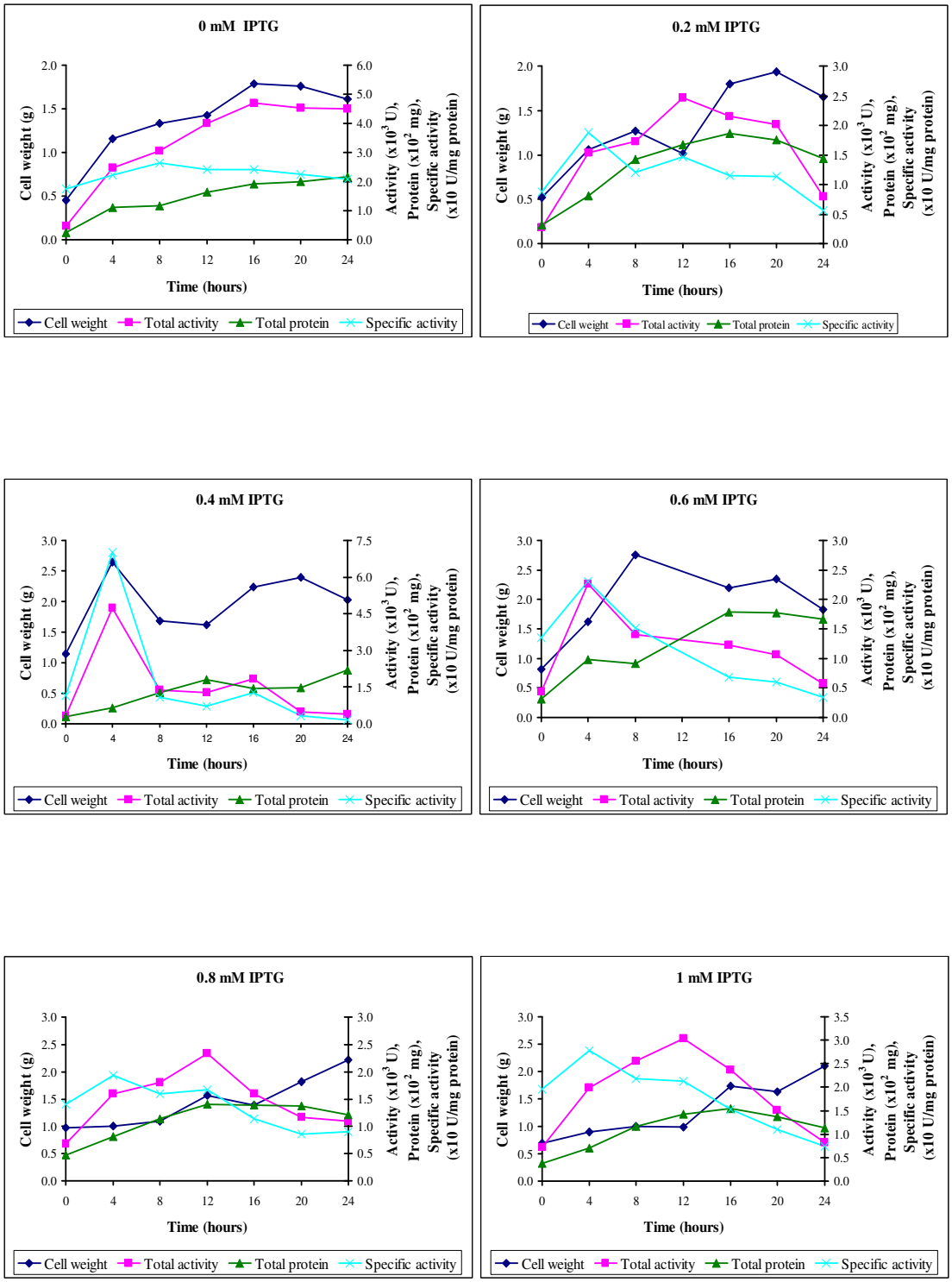


Figure 3.8 The P5CR activity of *E. coli* BL21(DE3) transformant No.6 containing pET-P5CR induced by various concentrations of IPTG

expression of the gene trended to decrease. After induction with 0.4 mM IPTG for 4 hours, the maximal specific activity was 1.3 times (33.3 U/mg protein) higher than that of absence of IPTG for 8 hours. Even though, the highest activity was also found at 16 hours without IPTG induction, it was still lower than of induction with 0.4 mM IPTG at 4 hours. Hence, the optimum condition for induction of *proC* gene was 0.4 mM IPTG at 4 hours after induction.

3.3.2 Protein pattern of cells and crude extacts

The 1.5 ml of transformant No.6 cultures which had been grown at various concentrations of IPTG and various times as described in 2.10 were harvested and then centrifuged in microcentrifuge tube. The cell pellets were resuspened in 100 µl of 5x sample buffer. Seven microliters of cell samples or 20 µg protein of crude extracts were subjected to electrophoresis on 12.5% SDS-PAGE. The results in Figure 3.10-3.15 showed that the intensity of major protein band at 28.9 kDa of crude extracts at each induction time was quite corresponded to the level of enzyme activity from its crude extract.

3.4 Purification of pyrroline-5-carboxylate reductase from recombinant clone

3.4.1 Preparation of crude extract

Crude recombinant P5CR was prepared from 4.71 g of transformant No.6 which was cultivated in 1.6 liters of medium as described in 2.11.1. Crude extract contained 564 mg proteins and 13,200 units of P5CR activity. Thus, the specific activity of the enzyme in the crude preparation was 23.4 units/mg proteins.

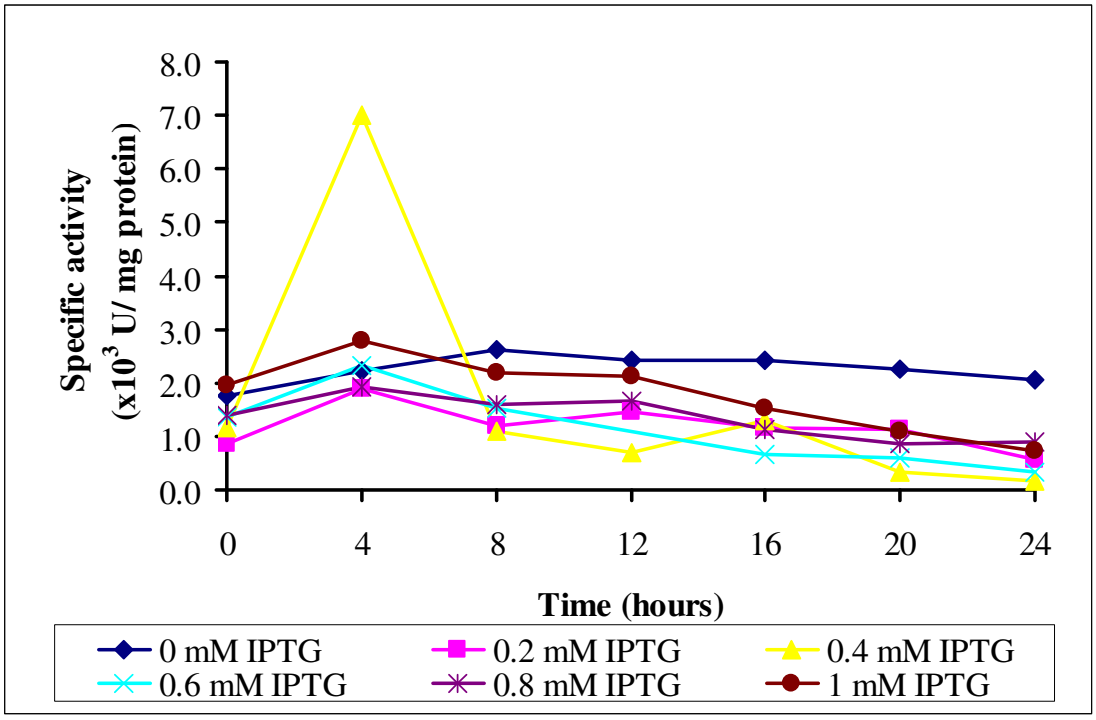


Figure 3.9 The P5CR specific activity of *E. coli* BL21(DE3) transformant No.6 containing pET-P5CR induced by various concentrations of IPTG

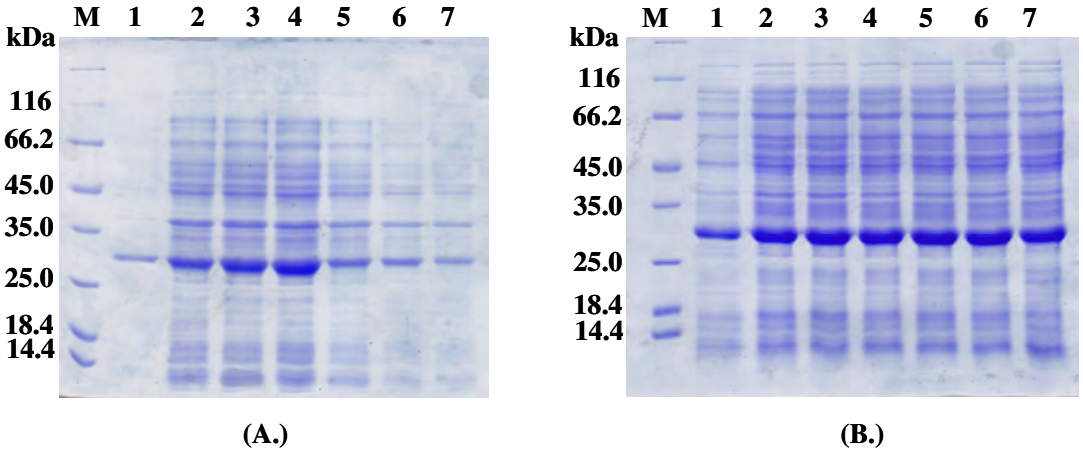


Figure 3.10 SDS-PAGE of whole cell and crude extract of the *E. coli* BL21(DE3) transformant No.6 containing pET-P5CR induced by 0 mM IPTG at various times

(A.): whole cell

(B.): crude extract (20 µg)

Lane M = protein marker

Lane 1-7 = cell and crude extract of pET-P5CR clone at 0, 4, 8, 12, 16, 20 and 24 hours after induction, respectively

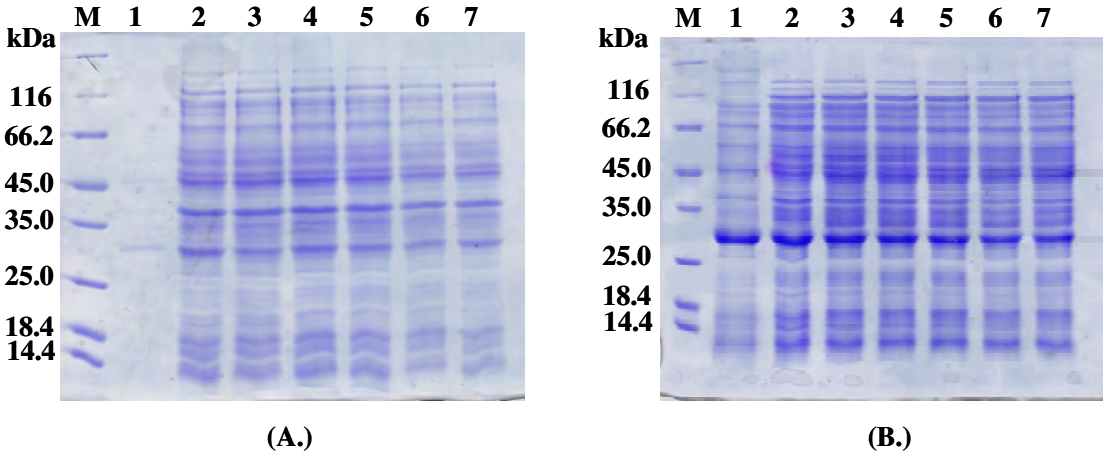


Figure 3.11 SDS-PAGE of whole cell and crude extract of the *E. coli* BL21(DE3) transformant No.6 containing pET-P5CR induced by 0.2 mM IPTG at various times

(A.): whole cell
(B.): crude extract (20 µg)

Lane M = protein marker
Lane 1-7 = cell and crude extract of pET-P5CR clone at 0, 4, 8, 12, 16, 20 and 24 hours after induction, respectively

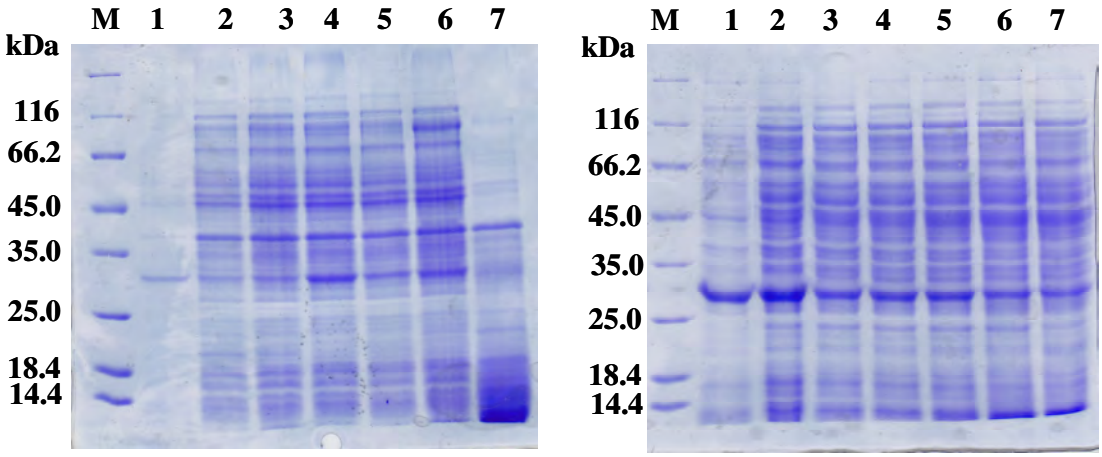


Figure 3.12 SDS-PAGE of whole cell and crude extract of the *E. coli* BL21(DE3) transformant No.6 containing pET-P5CR induced by 0.4 mM IPTG at various times

(A.): whole cell

(B.): crude extract (20 µg)

Lane M = protein marker

Lane 1-7 = cell and crude extract of pET-P5CR clone at 0, 4, 8, 12, 16, 20 and 24 hours after induction, respectively

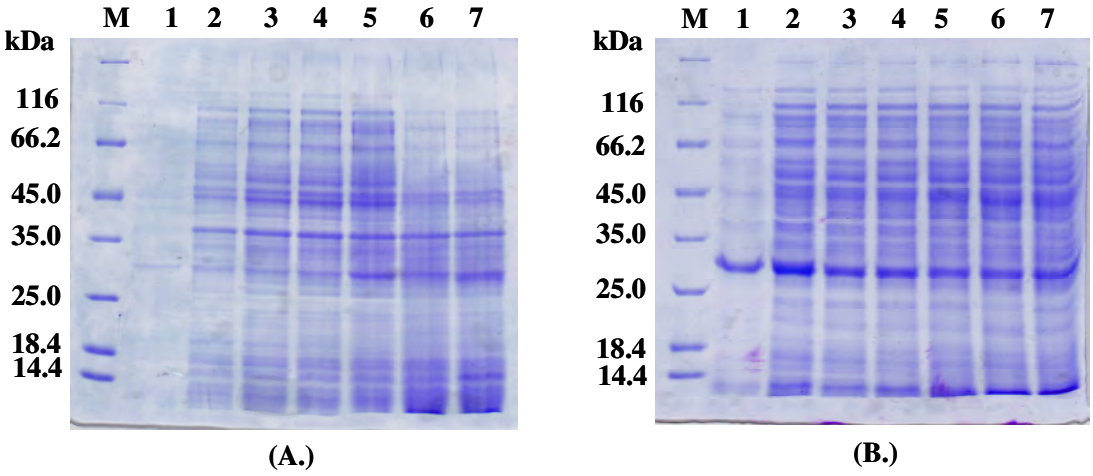


Figure 3.13 SDS-PAGE of whole cell and crude extract of the *E. coli* BL21(DE3) transformant No.6 containing pET-P5CR induced by 0.6 mM IPTG at various times

(A.): whole cell

(B.): crude extract (20 µg)

Lane M = protein marker

Lane 1-7 = cell and crude extract of pET-P5CR clone at 0, 4, 8, 12, 16, 20 and 24 hours after induction, respectively

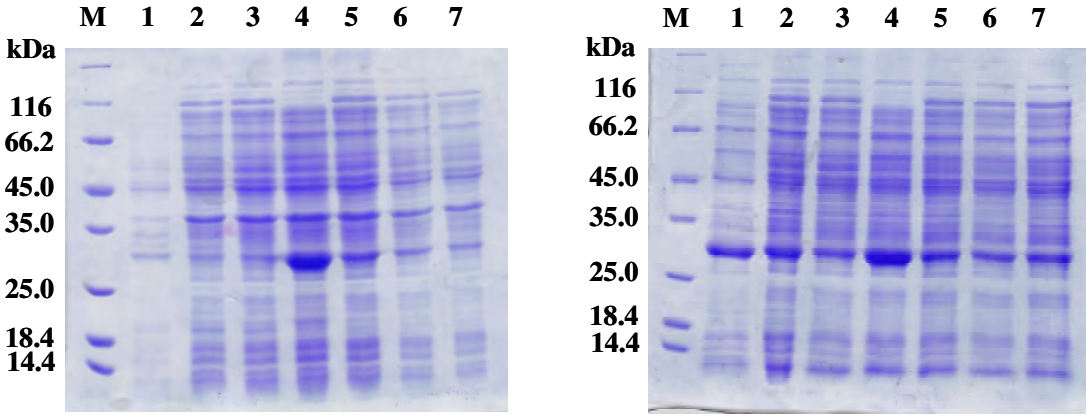


Figure 3.14 SDS-PAGE of whole cell and crude extract of the *E. coli* BL21(DE3) transformant No.6 containing pET-P5CR induced by 0.8 mM IPTG at various times

(A.): whole cell
(B.): crude extract (20 µg)

Lane M = protein marker
Lane 1-7 = cell and crude extract of pET-P5CR clone at 0, 4, 8, 12, 16, 20 and 24 hours after induction, respectively

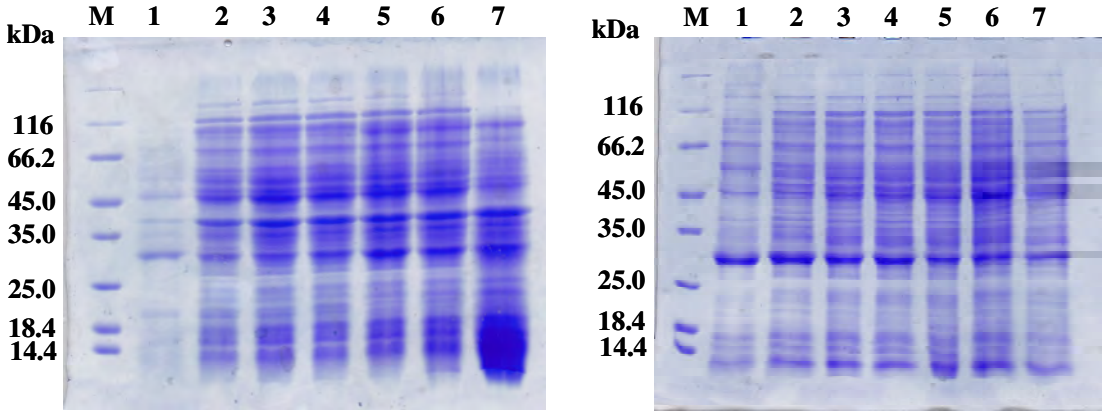


Figure 3.15 SDS-PAGE of whole cell and crude extract of the *E. coli* BL21(DE3) transformant No.6 containing pET-P5CR induced by 1 mM IPTG at various times

(A.): whole cell
(B.): crude extract (20 µg)

Lane M = protein marker
Lane 1-7 = cell and crude extract of pET-P5CR clone at 0, 4, 8, 12, 16, 20 and 24 hours after induction, respectively

3.4.2 DEAE-Toyopearl column chromatography

Crude extract was dialyzed against the buffer. The enzyme solution was applied onto DEAE-Toyopearl column as described in 2.11.2.1. 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 same buffer. P5CR was eluted at about 0.04 M potassium chlorides as indicated in the profile (Figure 3.16). P5CR fractions were pooled, dialyzed against the buffer and concentrated by aquasorb to reduce enzyme volume. The protein remained from this step was 139 mg proteins with enzyme activity recovered at 4,650 units. The specific activity of the enzyme from this step was 33.5 units/mg proteins. The enzyme was purified 1.43 fold with 35.2% recovery (Table 3.2).

3.4.3 Butyl-Toyopearl column chromatography

The pooled active fraction from DEAE-Toyopearl column was applied to the Butyl-Toyopearl column as described in 2.11.2.2. The chromatographic profile was shown in Figure 3.17. The unbound proteins were eluted from column with 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 20% salt saturation. The pooled fraction containing P5CR activity was dialyzed against the buffer and concentrated by aquasorb. This operation obtained the enzyme with 37.4 mg proteins and 1,970 activity units. The specific activity of the enzyme was 52.7 units/mg proteins. The P5CR was purified 2.25 fold with 14.9% recovery (Table 3.2). The purified enzyme from this step was kept at 4 ℃ for further experiment.

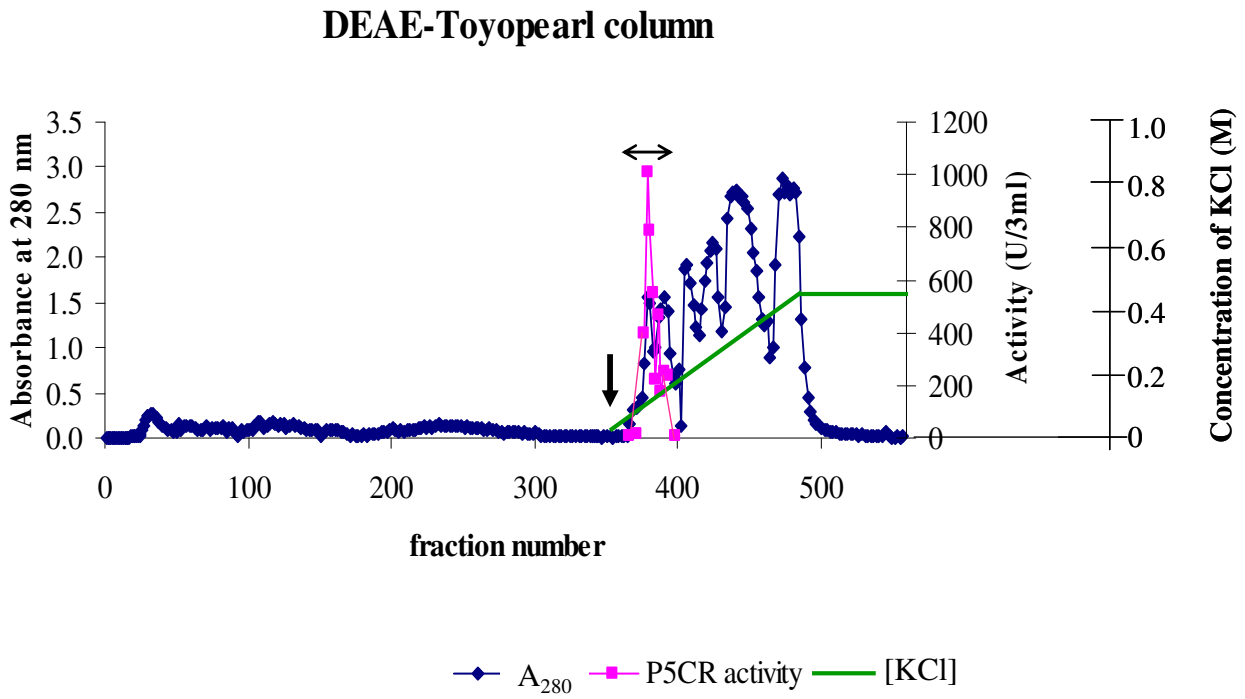


Figure 3.16 Purification of pyrroline-5-carboxylate reductase from pET-P5CR clone by DEAE-Toyopearl column

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 1ml/min. The fractions of 3 ml were collected. The arrow indicates where gradient started. The protein peak from fraction number 367 to 399 was pooled (⟷).

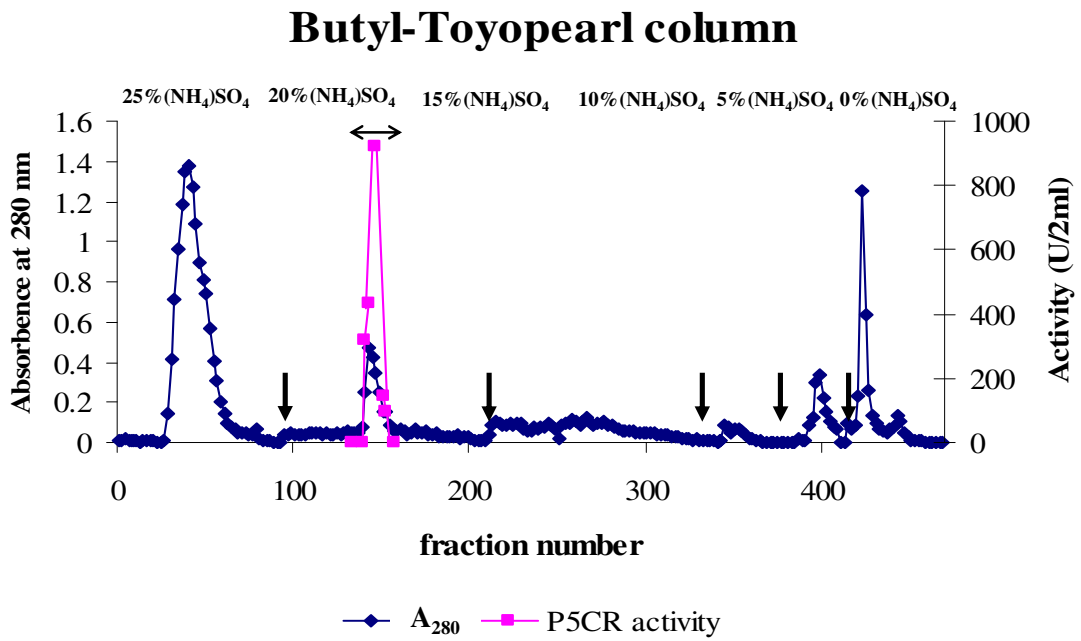


Figure 3.17 Purification of pyrroline-5-carboxylate reductase from pET-P5CR clone by Butyl-Toyopearl column

The enzyme solution was applied to Butyl-Toyopearl column and washed with 25% saturated ammonium sulfate in 10 mM potassium phosphate buffer, pH 7.4 containing 0.01%(v/v) β-mercaptoethanol and 1 mM EDTA until A_{280} decreased to base line. The enzyme was eluted by 20% saturated ammonium sulfate in the same buffer at the flow rate of 1ml/min. The fractions of 2 ml were collected. The arrows indicate where 20%, 15%, 10%, 5% and 0% saturated ammonium sulfate in the same buffer started. The protein peak from fraction number 139 to 157 was pooled (⟷).

Table 3.2 Purification of pyrroline-5-carboxylate reductase from pET-P5CR clone[a]

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	13,200	564	23.4	100	1.0
DEAE-Toyopearl column	4,650	139	33.5	35.2	1.43
Butyl-Toyopearl column	1,970	37.4	52.7	14.9	2.25

[a] Crude extract was prepared from 1.6 liters (4.71 g wet weight) of cell culture.

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

The enzyme from each step of purification was examined for purity and protein pattern by SDS-PAGE as described in 2.12.2. In addition, purified enzyme from the last step of purification was run on non-denaturing PAGE followed by protein and activity staining as described in 2.12.1.1 and 2.12.1.2, respectively. The results are shown in Figure 3.18. The purified enzyme in Lane 3A on SDS-PAGE showed a single band which corresponded with a single protein band in Lane 3B and its activity staining in Lane 3C on native-PAGE. It indicated that P5CR from Butyl-Toyopearl column was purified to homogeneity.

3.5 Characterization of pyrroline-5-carboxylate reductase

3.5.1 Molecular weight determination of pyrroline-5-carboxylate reductase

The molecular weight of recombinant P5CR subunit was determined from molecular weight calibration curve obtained by SDS-PAGE as described in 2.13.1. The molecular weight of P5CR subunit was estimated to be about 28,960 Da (Figure 3.19).

3.5.2 Coenzyme specificity of pyrroline-5-carboxylate reductase

Coenzyme specificity of P5CR was investigated as described in 2.13.2. P5CR is capable of utilizing either NAD^+ or $NADP^+$ as a natural coenzyme for oxidative deamination. The purified enzyme expressed a preferential affinity for NAD^+ (100% residual activity) over $NADP^+$ (3.5% residual activity) (Table 3.3).

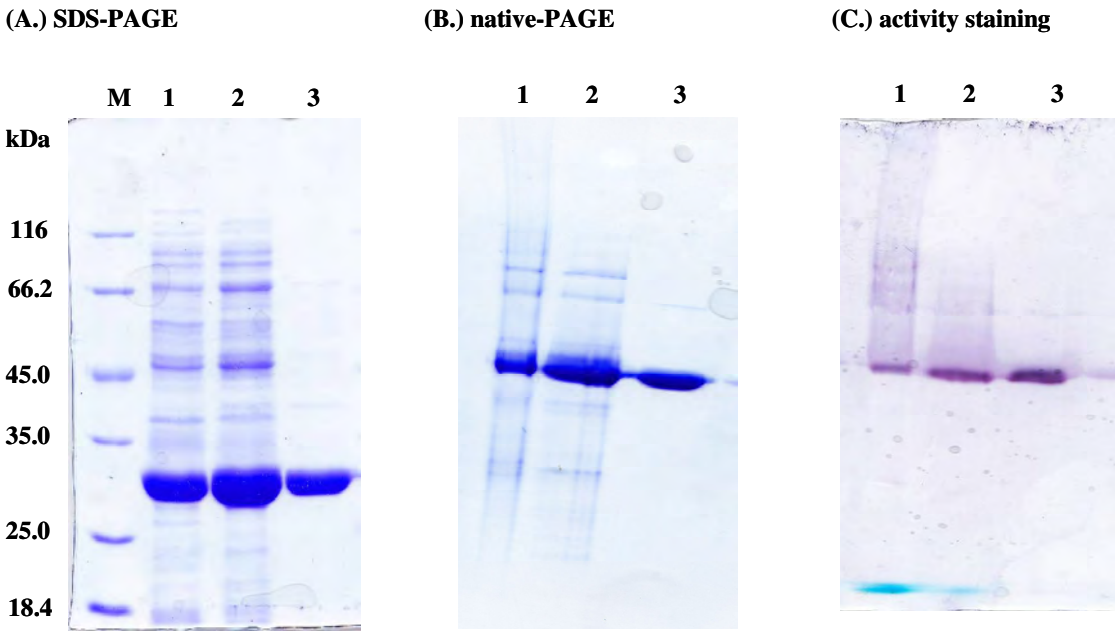


Figure 3.18 Protein pattern from each step of purification investigated by SDS-PAGE and native-PAGE

(A.) SDS-PAGE

Lane M = protein marker

Lane 1 = crude extract (20 µg)

Lane 2 = DEAE-Toyopearl column (20 µg)

Lane 3 = Butyl-Toyopearl column (20 µg)

(B.) native-PAGE

Lane 1 = crude extract (20 µg)

Lane 2 = DEAE-Toyopearl column (20 µg)

Lane 3 = Butyl-Toyopearl column (20 µg)

(C.) activity staining

Lane 1 = crude extract (20 µg)

Lane 2 = DEAE-Toyopearl column (20 µg)

Lane 3 = Butyl-Toyopearl column (20 µg)

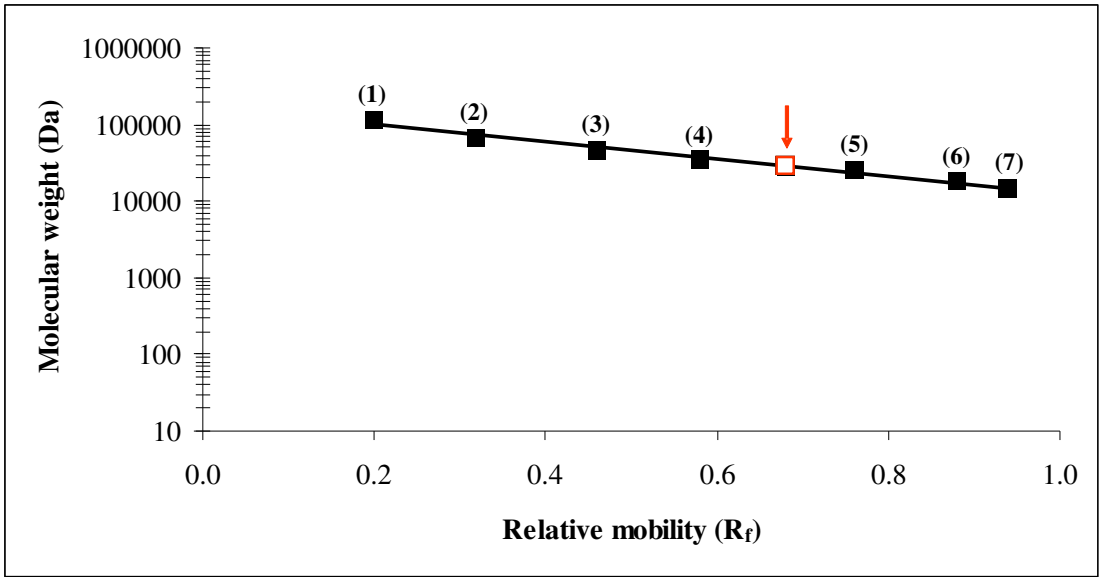


Figure 3.19 Calibration curve for molecular weight estimation of P5CR by SDS-PAGE

(1) = β-galactosidase (MW 116,000 Da)
(2) = Bovine serum albumin (MW 66,200 Da)
(3) = Ovalbumin (MW 45,000 Da)
(4) = Lactate dehydrogenase (MW 35,000 Da)
(5) = REase Bsp981 (MW 25,000 Da)
(6) = β-lactoglobulin (MW 18,400 Da)
(7) = Lysozyme (MW 14,400 Da)

Arrow indicated the estimated molecular weight of P5CR

Table 3.3 Coenzyme specificity of pyrroline-5-carboxylate reductase[a]

Coenzyme[b]	Residual activity (%)
β-nicotinamide adenine dinucleotide	100
β-nicotinamide adenine dinucleotide phosphate	3.50

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

[b] Final concentration of coenzymes were 2.0 mM.

The assay was conducted at 340 nm ($\varepsilon = 6.2 \times 10^3\ M^{-1}.cm^{-1}$).

3.5.3 Effect of pH on pyrroline-5-carboxylate reductase activity

Effect of pH on the enzyme activity for oxidative deamination was examined at various pHs of buffer ranged from 4.0 to 13.0 as mentioned in 2.13.3. The result was shown in Figure 3.20 (A.). The enzyme exhibited maximal activity at pH 9.4. At pH 8.3 to 10.0, the enzyme activities were more than 50% residual activity while the enzyme activities were loss at the other pH that less than 4.8 and more than 10.5.

3.5.4 Effect of temperature on pyrroline-5-carboxylate reductase activity

The effect of temperature on enzyme activity was investigated as described in 2.13.4. The temperature was varied from 20 ℃ to 80 ℃. The result was shown in Figure 3.20 (B.). The enzyme performed the highest activity at 30 ℃ for oxidative deamination.

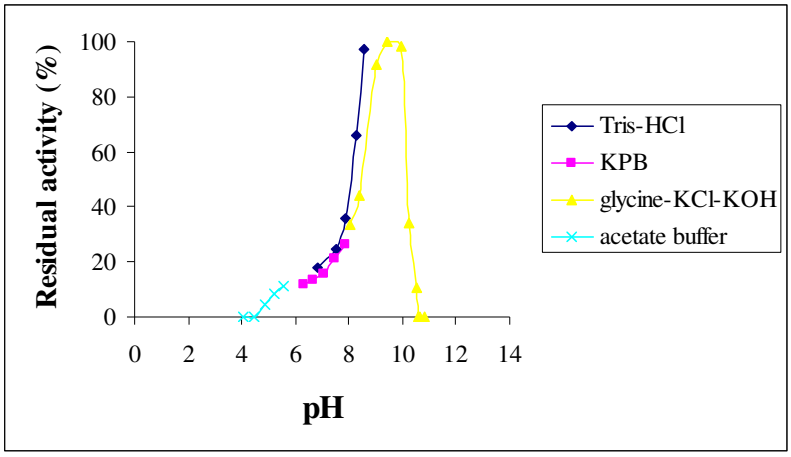
3.5.5 Effect of pH on pyrroline-5-carboxylate reductase stability

The pH stability of P5CR was studied as described in 2.13.5. The enzyme was preincubated at 30 ℃ for 20 minutes in various 10 mM buffers at various pHs ranging from 4.0 to 13.0. The result was shown in Figure 3.21 (A.). The enzyme was stable over the pH ranged from 9.0 to 10.0.

3.5.6 Effect of temperature on pyrroline-5-carboxylate reductase stability

The thermostability of P5CR was studied as described in 2.13.6. The enzyme was preincubated at various temperature ranged from 35 ℃ to 60 ℃ for 10, 30, 60, 90 and 120 minutes, respectively. The enzyme activity of non-preincubated enzyme was defined as 100% residual activity. The result was shown in Figure 3.21 (B.). The enzyme lost about half of its activity upon the incubation at 40 ℃ for 30 minutes. At 0 to 30 minutes of incubation time, the enzyme activity was relatively

(A.)



(B.)

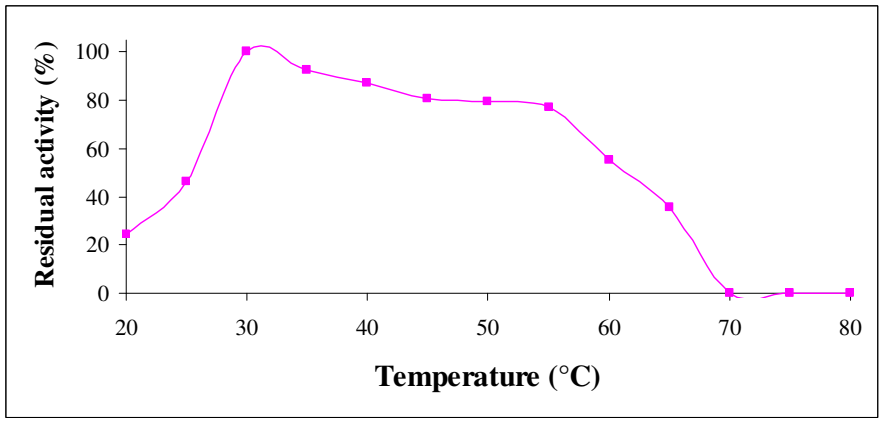


Figure 3.20 Effect of pH and temperature on pyrroline-5-carboxylate reductase activity

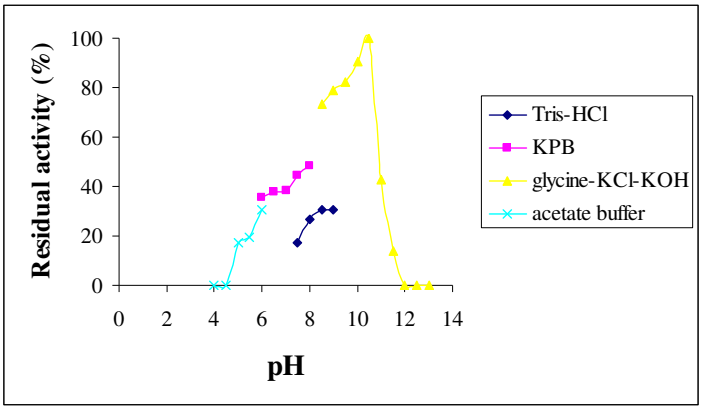
(A.) Effect of pH on P5CR activity

The P5CR activities for oxidative deamination was measured at difference pHs by the standard enzyme assayed condition.

(B.) Effect of temperature on P5CR activity

The P5CR activities for oxidative deamination was measured at various temperatures from 20 °C to 80 °C under the standard assay condition.

(A.)



(B.)

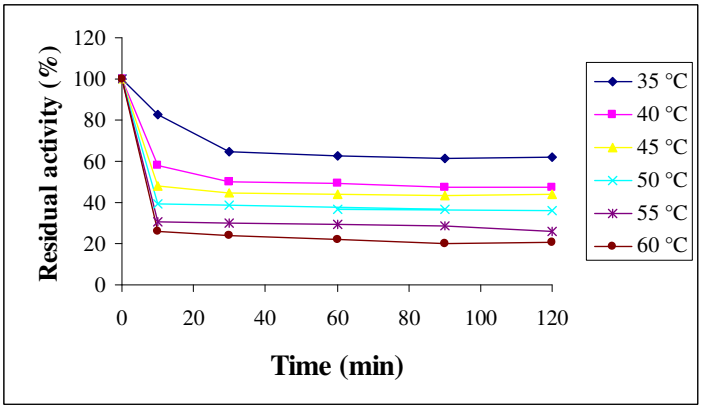


Figure 3.21 Effect of pH and temperature on pyrroline-5-carboxylate reductase stability

(A.) Effect of pH on P5CR stability

The enzymes in 10 mM buffers at various pHs ranged from 4.0 to 13.0 were incubated at 30 °C for 20 minutes and then the residual activities were assayed for oxidative deamination.

(B.) Effect of temperature on P5CR stability

The effect of temperature on stability of the enzyme activity was performed at 35 °C to 60 °C for 10, 30, 60, 90 and 120 minutes, respectively before the residual oxidative deamination activity was determined under standard condition at 30 °C

decreased with increasing of incubation temperature. After that, the enzyme activity was quite constant.

3.6 Kinetic mechanism studies of pyrroline-5-carboxylate reductase

A series of steady-state kinetic analysis was carried out to investigate the reaction mechanism. Initial velocity studies for oxidative deamination were performed. The concentration of L-proline was varied in the presence of several fixed concentration of NAD^+. Double-reciprocal plots of initial velocity against reciprocals of L-proline concentrations gave a family of straight lines, which intersected in the upper left quadrant as shown in Figure 3.22 (A.). The apparent K_m value for L-proline was calculated to be 1.0 mM. From secondary plots of intercept at the ordinate versus reciprocal concentrations of NAD^+, the apparent K_m value for NAD^+ was calculated to be 1.54 mM as shown in Figure 3.22 (B.).

3.7 Cloning and expression of heterologous genes of *lys 6-dh* and *proC* in *E. coli* BL21(DE3) using pET-17b vector

3.7.1 PCR amplification of *proC* gene

The 915 bp of *proC* gene fragment was amplified using a pET-P5CR containing *proC* gene as a template. The gene was amplified by PCR using the following primers, T7-*Bam*HI which contained *Bam*HI restriction site and T7 promoter of expression vector of pET series as a forward primer and p5cr-*Eco*RI, a reverse primer, containing 3'-end of *proC* gene, the TAA translational termination signal followed by the restriction site for *Eco*RI as described in 2.16.1. Figure 3.22 showed the 915 bp PCR product of the *proC* gene. The optimum annealing temperature which gave strong specific PCR product was 44.7 ℃. The PCR product was purified by agarose gel electrophoresis before using for further cloning.

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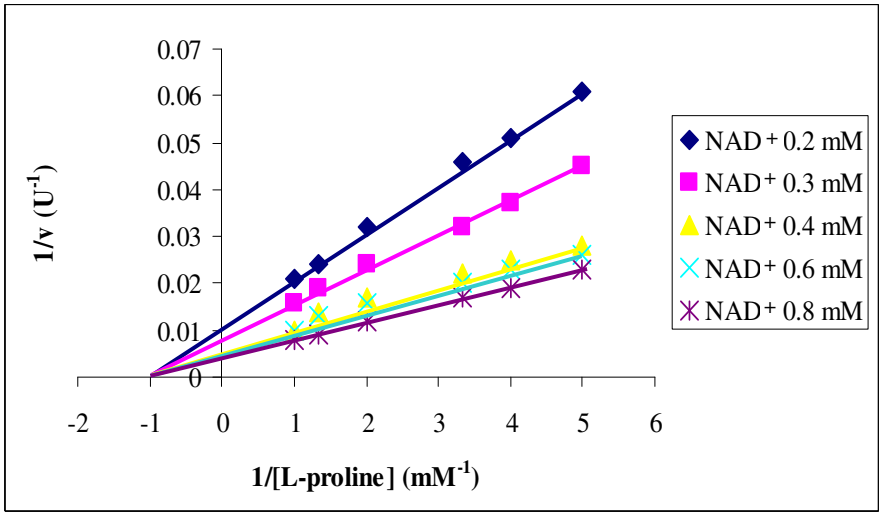
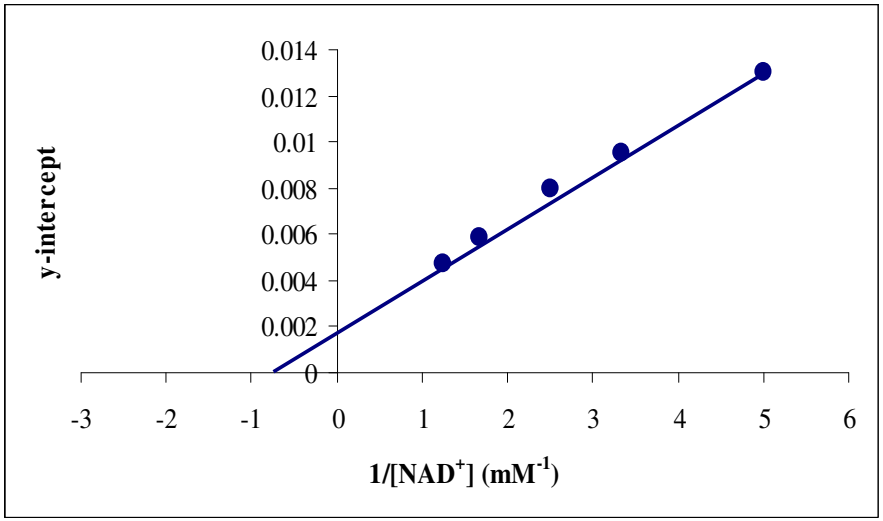


(A.)



(B.)

Figure 3.22 Initial velocity pattern for oxidative deamination (A.) Double-reciprocal plot of initial velocities versus L-proline 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 plot of y intercepts versus reciprocal NAD^+ concentrations.

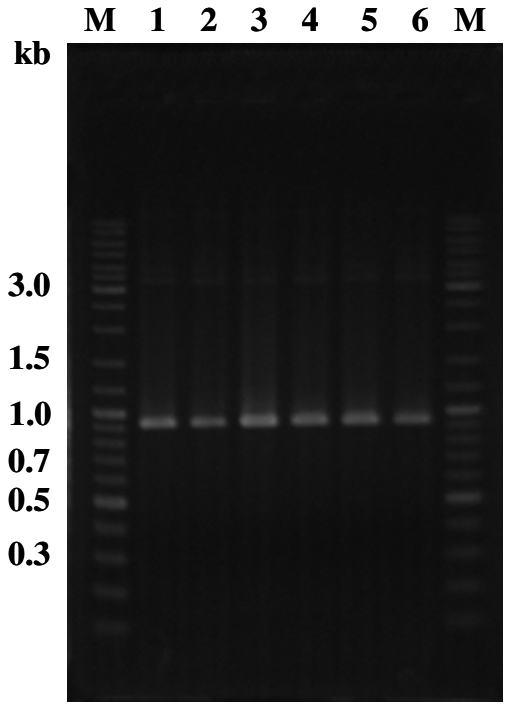


Figure 3.23 *proC* gene amplication at various annealing temperatures using pET-P5CR containing *proC* gene as a template

Lane M = 100 bp ladder DNA marker
Lane 1 = PCR product using annealing temperature at 40.3 ℃
Lane 2 = PCR product using annealing temperature at 42.5 ℃
Lane 3 = PCR product using annealing temperature at 44.7 ℃
Lane 4 = PCR product using annealing temperature at 46.8 ℃
Lane 5 = PCR product using annealing temperature at 48.5 ℃
Lane 6 = PCR product using annealing temperature at 49.8 ℃

3.7.2 Construction of pET-ADK-P5CR

To express the *lys 6-dh* and *proC* genes in *E. coli* BL21(DE3) under separated T7 promoters of expression vector pET-17b, the pET-ADK (containing the whole *lys 6-dh* gene with T7 promoter and Shine-Dalgarno sequence of pET-17b) was digested with *Bam*HI and *Eco*RI. The 4.4 kb of linear pET-ADK containing the *lys 6-dh* gene was recovered from agarose gel eletrophoresis. As described in 2.15, the 915 bp PCR product of *proC* gene, which containing T7 promoter and Shine-Dalgarno sequence of expression vector pET-17b, was digested with *Bam*HI and *Eco*RI. The *Bam*HI-*Eco*RI fragment of *proC* gene was ligated to the *Bam*HI-*Eco*RI site of 4.4 kb pET-ADK vector. Then the obtain recombinant plasmid with a size of 5.4 kb was transformed into *E. coli* BL21(DE3) by electroporation as shown in Figure 3.24 Lane 1. The recombinant clone was confirmed by *Bam*HI and *Nde*I digestion. Figure 3.24 Lane 2 showed two digested product bands, upper band was a 4.3 kb of linear pET-17b vector and *proC* gene which containing T7 promoter sequence and Shine-Dalgarno sequence and lower band was a 1.1 kb of *lys 6-dh*. The product of *Bam*HI-*Eco*RI digestion was showed in Figure 3.24 Lane 3. Upper band was 4.4 of linear pET-ADK and lower band was a 915 bp of *proC* gene which containing T7 promoter sequence and Shine-Dalgarno sequence.

3.7.3 Enzyme activities of *E. coli* BL21(DE3) harbouring pET-ADK-P5CR

Twelve of *E. coli* BL21(DE3) harbouring pET-ADK-P5CR were grown for enzyme assay of Lys 6-DH and P5CR activity as described in 2.9.3.2 and 2.9.3.1. The transformants were induced by IPTG at final concentration of 0.4 mM before cells were harvest at 4 hours. *E. coli* BL21(DE3), *E. coli* BL21(DE3) harbouring pET-17b, *E. coli* BL21(DE3) harbouring pET-ADK and *E. coli* BL21(DE3) harbouring pET-P5CR were used as control. The transformants showed various levels of the enzyme total activity and specific activity of Lys 6-DH from 5.35-1,550 units and 0.08-20.6 units/mg proteins as well as P5CR total activity and specific activity from 8.35-1,460 units and 0.12-19.4 units/mg proteins, respectively, as shown in Table 3.4. The transformant No.9 had the highest total activities and specific activity

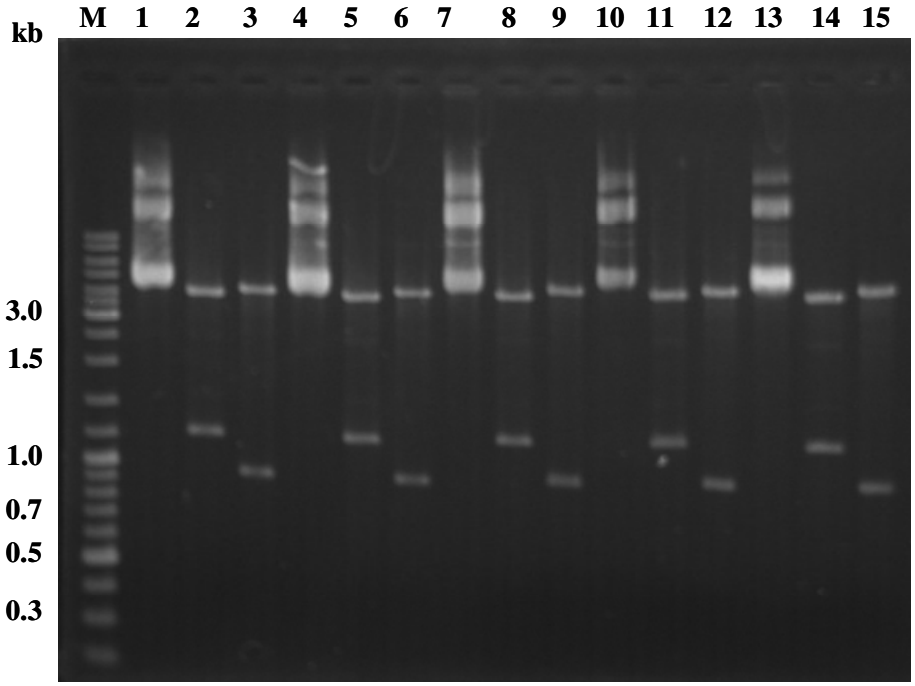


Figure 3.24 Restriction enzyme digested pET-ADK-P5CR from different clones

Lane M = 100 bp ladder DNA marker
Lane 1 = undigested pET-ADK-P5CR
Lane 2 = pET-ADK-P5CR digested with *Bam*HI and *Nde*I
Lane 3 = pET-ADK-P5CR digested with *Bam*HI and *Eco*RI
Lane 4 = undigested pET-ADK-P5CR
Lane 5 = pET-ADK-P5CR digested with *Bam*HI and *Nde*I
Lane 6 = pET-ADK-P5CR digested with *Bam*HI and *Eco*RI
Lane 7 = undigested pET-ADK-P5CR
Lane 8 = pET-ADK-P5CR digested with *Bam*HI and *Nde*I
Lane 9 = pET-ADK-P5CR digested with *Bam*HI and *Eco*RI
Lane 10 = undigested pET-ADK-P5CR
Lane 11 = pET-ADK-P5CR digested with *Bam*HI and *Nde*I
Lane 12 = pET-ADK-P5CR digested with *Bam*HI and *Eco*RI
Lane 13 = undigested pET-ADK-P5CR
Lane 14 = pET-ADK-P5CR digested with *Bam*HI and *Nde*I
Lane 15 = pET-ADK-P5CR digested with *Bam*HI and *Nde*I

Table 3.4 Lys 6-DH and P5CR activities from the crude extracts of 12 selected *E. coli* BL21(DE3) transformants harbouring pET-ADK-P5CR[a]

Sources	Total activity (U)		Total protein (mg)	Specific activity (U/mg protein)	
	Lys 6-DH	P5CR		Lys 6-DH	P5CR
E.coli BL21(DE3)	0	0	98.2	0	0
E.coli BL21(DE3) harbouring pET-17b	0	0	46.9	0	0
E.coli BL21(DE3) harbouring pET-ADK	1,760	0	116	15.1	0
E.coli BL21(DE3) harbouring pET-P5CR	0	2,050	40.6	0	50.6
No.1	32.8	38.9	75.1	0.44	0.52
No.2	5.35	8.35	68.9	0.08	0.12
No.3	88.9	112	76.9	1.16	1.46
No.4	1,170	1,230	81.9	14.3	15.0
No.5	958	1,330	88.4	10.8	15.0
No.6	1,170	1,270	88.5	13.2	14.4
No.7	800	638	98.5	8.12	6.48
No.8	200	365	109	1.83	3.34
No.9	**1,550**	**1,460**	**75.2**	**20.6**	**19.4**
No.10	112	286	88.5	1.27	3.23
No.11	46.7	100	93.4	0.50	1.07
No.12	1,110	1,290	104	10.7	12.4

[a] Crude extract were prepared from 200 ml of cell culture by induction with 0.4 mM IPTG for 4 hours.

of both Lys 6-DH and P5CR as 1,550 units, 20.6 units/mg proteins, 1,460 units and 19.4 units/mg proteins, respectively.

3.7.4 Protein patterns of cells and crude extracts

The protein patterns of the cells and crude extracts of twelve transformants and four controls were subjected to SDS-PAGE. Ten microliters of cell samples or 20 µg proteins from crude extracts were separated on 12.5% SDS-polyacrylamide gel. The results in Figure 3.25 and 3.26 showed that the intensity of a major protein band about 40 kDa in cell and crude extracts from each transformant was corresponded to the level of Lys 6-DH activity from its crude extract. Similary, the protein band of P5CR at the molecular size of 28.9 kDa was found as the major band in all analysis.

3.8 Production of L-pipecolic acid

3.8.1 TLC analysis of L-pipecolic acid production from recombinant clones

Twelve of *E. coli* BL21(DE3) harbouring pET-ADK-P5CR were used to produced L-PA. The 0.05 g of cell pellet was resuspended in 500 µl reaction mixture consisted of 200 mM L-lysine, 200 mM Tris-HCl, pH 8.5 and distilled water as described in 2.16.2. The reaction was performed at 30 ℃ for 24 hours. Then, L-lysine and L-PA from all samples were detected on TLC plate as described in 2.17.1. After developed with 0.05% ninhydrin in ethanol : acetone (30:70), the purple spot of amino acid could be seen. In order to elucidate the role of P5CR in L-PA production, the ability to produce L-PA was examined with respect to four recombinant *E.coli* BL21(DE3) clones harbouring each pET-17b, pET-ADK, pET-P5CR and pET-ADK-P5CR. Figure 3.27 showed the intensity of the sample spots in comparison with that of the standard. *E. coli* BL21(DE3) harbouring pET-ADK-P5CR only showed the accumulation of L-PA. The relative mobility (R_f) value of L-lysine and L-PA were

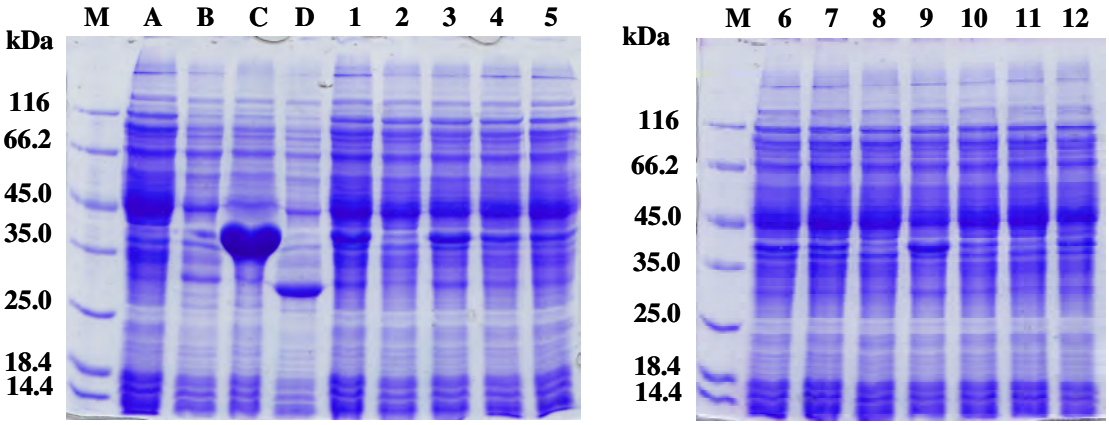


Figure 3.25 SDS-PAGE of whole cell of four controls and 12 selected *E. coli* BL21(DE3) transformants harbouring pET-ADK-P5CR induced by 0.4 mM IPTG at 4 hours

Lane M = protein marker
Lane A = whole cell of *E. coli* BL21(DE3)
Lane B = whole cell of *E. coli* BL21(DE3) harbouring pET-17b
Lane C = whole cell of *E. coli* BL21(DE3) harbouring pET-ADK
Lane D = whole cell of *E. coli* BL21(DE3) harbouring pET-P5CR
Lane 1-12 = whole cell of *E. coli* BL21(DE3) harbouring pET-ADK-P5CR No.1, No.2, No.3, No.4, No.5, No.6, No.7, No.8, No.9, No.10, No.11 and No.12, respectively

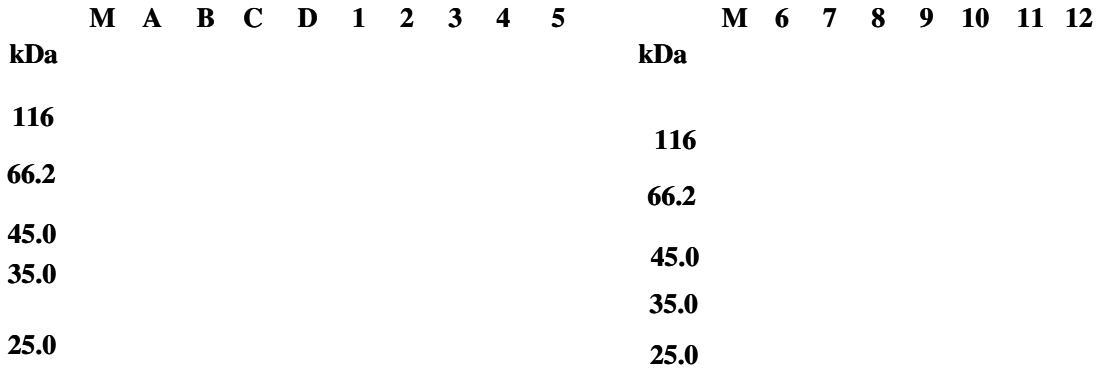


Figure 3.26 SDS-PAGE of crude extracts of four controls and 12 selected *E. coli* BL21(DE3) transformants harbouring pET-ADK-P5CR induced by 0.4 mM IPTG at 4 hours

Lane M = protein marker
Lane A = crude extracts of *E. coli* BL21(DE3)
Lane B = crude extracts of *E. coli* BL21(DE3) harbouring pET-17b
Lane C = crude extracts of *E. coli* BL21(DE3) harbouring pET-ADK
Lane D = crude extracts of *E. coli* BL21(DE3) harbouring pET-P5CR
Lane 1-12 = crude extracts of *E. coli* BL21(DE3) harbouring pET-ADK-P5CR No.1, No.2, No.3, No.4, No.5, No.6, No.7, No.8, No.9, No.10, No.11 and No.12, respectively

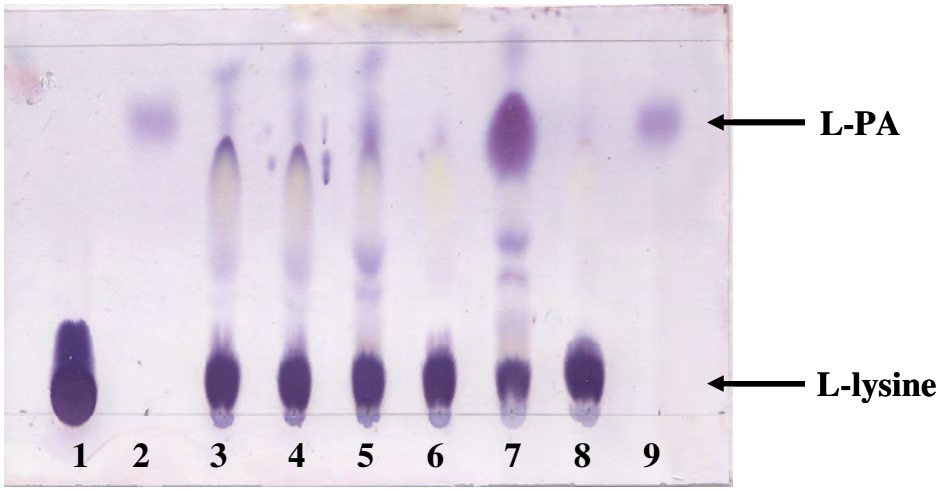


Figure 3.27 TLC analysis of the L-pipecolic acid production

Lane 1 = L-lysine (3 µg)
Lane 2,9 = L-pipecolic acid (0.5 µg)
Lane 3 = reaction product from *E. coli* BL21(DE3) cell
Lane 4 = reaction product from *E. coli* BL21(DE3) cell harbouring pET-17b
Lane 5 = reaction product from *E. coli* BL21(DE3) cell harbouring pET-ADK
Lane 6 = reaction product from *E. coli* BL21(DE3) cell harbouring pET-P5CR
Lane 7 = reaction product from *E. coli* BL21(DE3) cell harbouring pET-ADK-P5CR
Lane 8 = reaction product from *E. coli* BL21(DE3) cell harbouring pET-ADK-P5CR at 0 hour

0.09 and 0.69, respectively. The result in Figure 3.28 indicated that the transformant No.9 had the highest intensity of L-PA spot.

3.8.2 HPLC analysis of L-pipecolic acid production from recombinant clones

The amount of L-PA produced from each recombinant clones was determined by HPLC using Inertsil ODS-3 column as mentioned in 2.17.2. HPLC analysis showed that peak of L-lysine and L-PA were eluted at retention times around 3.8 and 18.9 minutes, respectively. The concentration of L-lysine and L-PA in recombinant clones was calculated by compared with L-lysine and L-PA standard curve (Appendix M). As a result in Figure 3.29, the transformant No. 9 showed the highest production of L-PA about 11.9 mM (1.53 g/l) with 5.9% of the conversion reaction of L-lysine to L-PA after 24 hours incubation. Thus, this recombinant clone was used in further experiment.

3.9 Optimization of L-pipecolic acid production

The optimum condition for high expression of heterologous genes of *lys 6-dh* and *proC* were investigated under standard condition as described in 2.16.3. The amount of L-PA produced from the recombinant *E. coli* without IPTG induction was slightly increased to the maximum at 8 hours while the cells induced with 0.1, 0.2, 0.3, 0.4, and 0.6 mM IPTG showed the highest production of L-PA at 4 hours after induction. After induction with 0.1 mM IPTG for 4 hours, the highest L-PA production was 2.28 times (12.2 mM or 1.57 g/l) higher than that of absence of IPTG for 8 hours. However, the amount of L-PA when induced for 4 hours with other final concentrations of IPTG was less than that of induction with 0.1 mM IPTG. Therefore, the optimum condition for induction of heterologous genes of *lys 6-dh* and *proC* was 0.1 mM IPTG at 4 hours after induction (Figure 3.30).

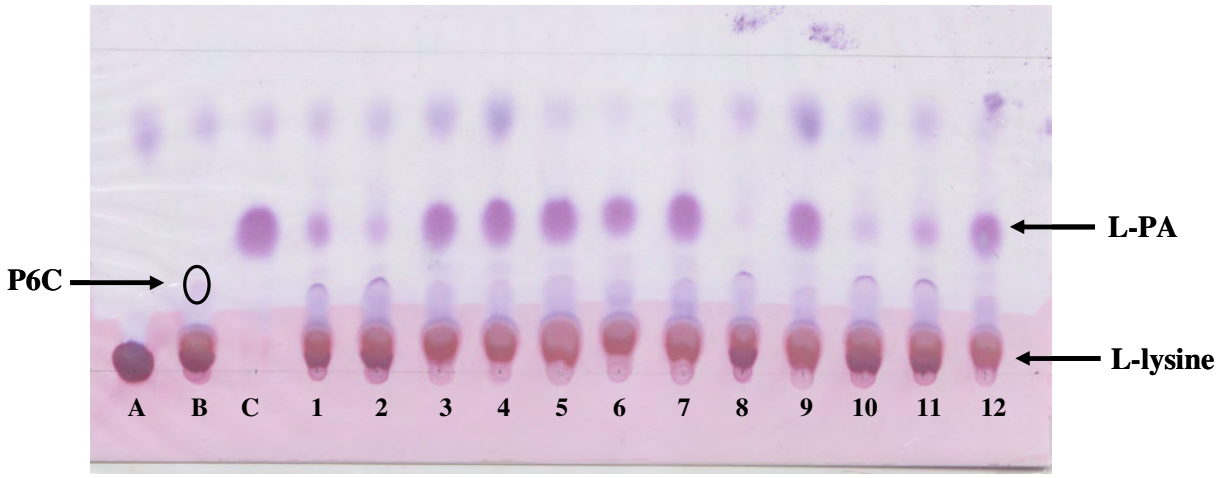


Figure 3.28 TLC analysis of the L-pipecolic acid production from *E. coli* BL21 (DE3) harboring pET-ADK-P5CR transformant

Lane A = L-lysine (3 µg)

Lane B = P6C from the reaction consisted of 200 mM L-lysine, 200 mM Tris-HCl buffer, pH 8.5,10 mM NAD^+ and Lys 6-DH and incubated at 37 ℃ for 1 hour

Lane C = L-pipecolic acid (1 µg)

Lane 1-12 = reaction products from *E. coli* BL21(DE3) cell harbouring pET-ADK-P5CR No.1, No.2, No.3, No.4, No.5, No.6, No.7, No.8, No.9, No.10, No.11 and No.12, respectively

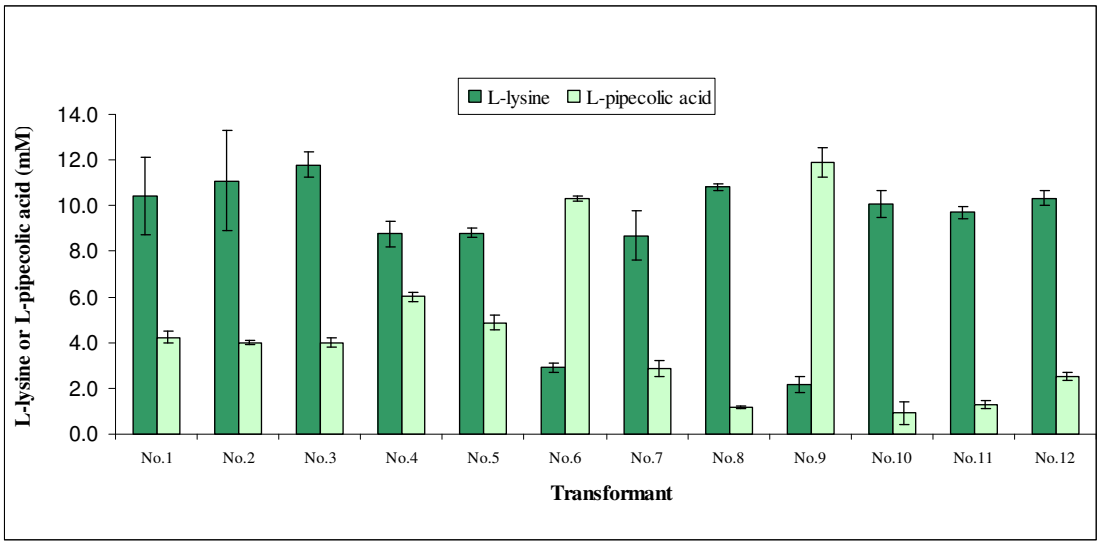


Figure 3.29 HPLC analysis of L-pipecolic acid production in *E. coli* BL21(DE3) transformants containing pET-ADK-P5CR (The data represent the mean values of three independent experiments.)

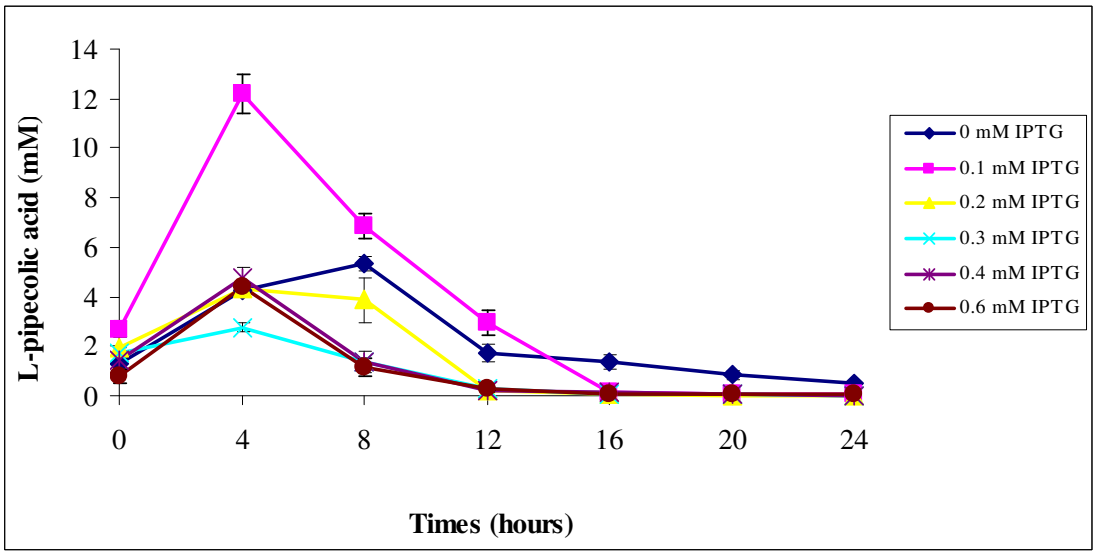


Figure 3.30 HPLC analysis of L-pipecolic acid production in *E. coli* BL21 (DE3) transformant No.9 containing pET-ADK-P5CR after induction with various final concentrations of IPTG (The data represent the mean values of three independent experiments.)

3.10 Effect of pH on L-pipecolic acid production

The optimum pH for the production of L-PA from L-lysine was investigated under strandard reaction as described in 2.16.4 using the recombinant *E. coli* BL21(DE3) induced with 0.1 mM IPTG for 4 hours. L-PA productions were determined by HPLC after incubation at 30 ºC using 200 mM Tris-HCl buffer (pH 7.0 to 9.0), potassium phosphate buffer (pH 6 to 8.5), MOPS buffer (pH 7.5 to 8.7), borate buffer (pH 7.5 to 9.0), sodium carbonate-bicarbonate buffer ($NaHPO_3$ buffer, pH 9.5 to 11.0), Na_2HPO_4 buffer (pH 11.0 to 12.0) and potassium chloride buffer (pH 12.0 to 13.0). The results in Figure 3.31 exhibited the maximum production of L-PA when 200 mM Tris-HCl buffer, pH 9.0 was used.

3.11 Effect of L-lysine concentration on L-pipecolic acid production

The effect of L-lysine concentration (10, 20, 25, 50, 75, 100, 120, 150,180 and 200 mM) was determined as described in 2.16.5. Figure 3.32 showed that the highest production of L-PA with 13.4 mM (1.74 g/l) was obtained when 200 mM L-lysine was used. Therefore, about 6.7% L-lysine at the initial concentration was converted to L-PA upon 24 hours incubation.

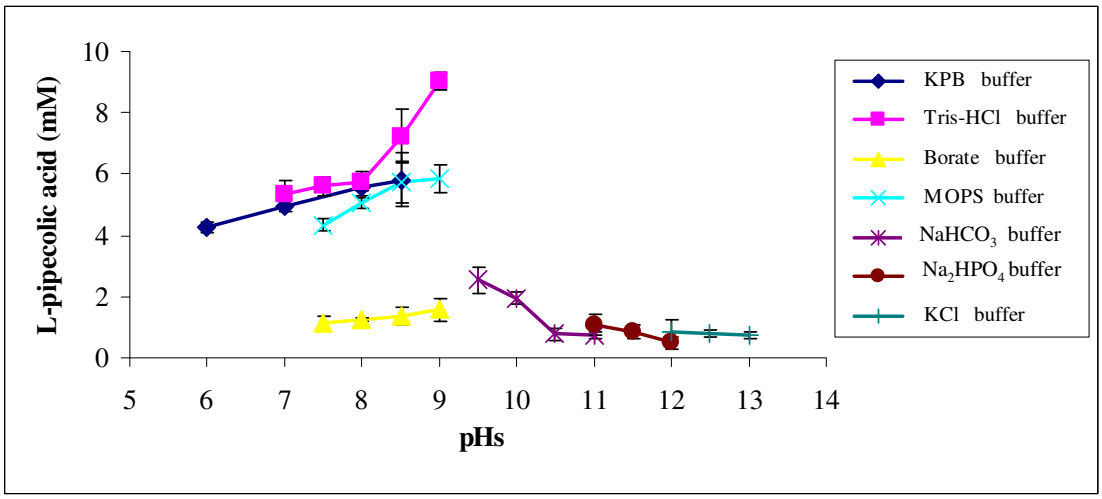


Figure 3.31 HPLC analysis of effect of pH on L-pipecolic acid production in *E. coli* BL21(DE3) transformants No.9 containing pET-ADK-P5CR (The data represent the mean values of three independent experiments.)

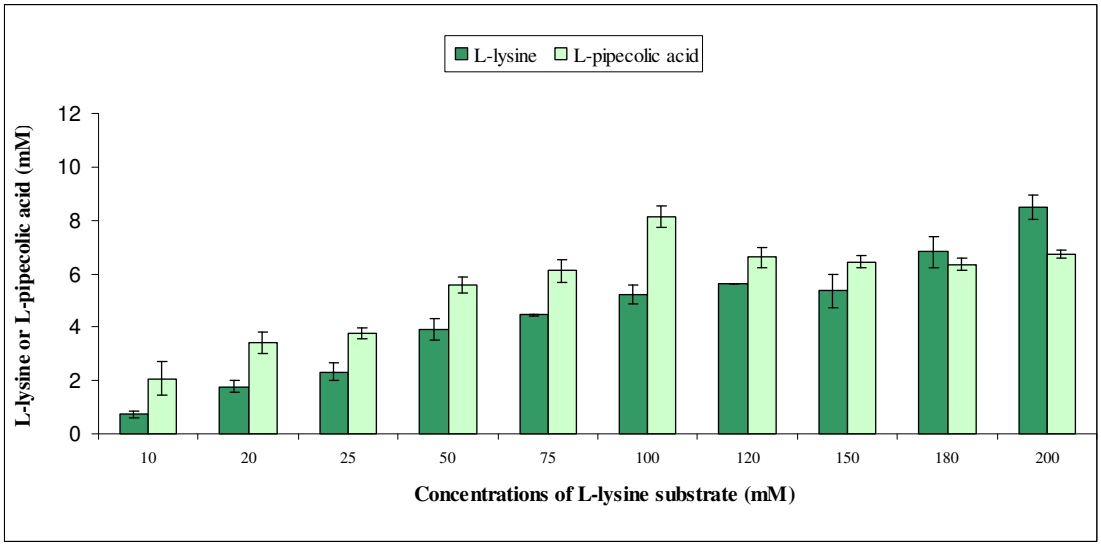


Figure 3.32 HPLC analysis of effect of L-lysine concentration on L-pipecolic acid production in *E. coli* BL21(DE3) transformants No.9 containing pET-ADK-P5CR (The data represent the mean values of three independent experiments.)

CHAPTER IV

DISCUSSION

4.1 Nucleotide sequencing of *proC* gene

Polymerase chain reaction (PCR) is the popular method for *in vitro* synthesis of the interesting gene for cloning. This technique depends on two primers, which are specific to an interesting gene. For whole gene amplification of *proC* gene from *B. cereus* ATCC 11778, *Hin*dIII restriction enzyme digestion fragments were used as a template while p5cr-*Nde*I and p5cr-*Eco*RI were used as forward primer and reverse primer, respectively in PCR reaction. The result showed that *Hin*dIII digested template gave strong specific products having size of about 800 bp. Later on, the desired specific band was recovered from gel and sequenced.

It is known that the restriction enzyme used for template preparation must not have its site on the gene otherwise the amplification of this desired gene fragment cannot be occurred. It was implied that *Hin*dIII restriction site was not on this *proC* gene fragment. We also found the other restriction enzymes including *Eco*RI, *Sal*I, *Kpn*I and *Sac*I that did not have their site on *proC* gene fragment by Genetyx version 6.0 program.

The nucleotide sequence of *proC* gene was determined as shown in Figure 3.6. The first ATG was presumed to be the translational start site as : (1) substantial similarity to the amino acid sequence (Met) of other P5CR (Kelly and Register, 1996); (2) the first ATG conformed to the 'AUG' initiator consensus motif (Kozak, 1981); (3) the first ATG is the translational start site of most genes (Kozak, 1984). The whole *proC* gene consisted of 819 bp which encode a polypeptide of 272 amino acids. The molecular weight of enzyme subunit calculated from deduced amino acid sequence using Compute pI/Mw program was 28.9 kDa.

The nucleotide sequence was compared with those of DNA sequences in the EMBL-GenBank-DDBL database. The percentages of sequence similarity between the nucleotide sequences of *proC* from *B. cereus* ATCC 11778 and those from *B. cereus* Q1, *B. cereus* ATCC 10987, *B. cereus* AH187 and *B. cereus* E33L were 92, 92, 91 and 91%, respectively. When the deduced amino acid sequence of the whole P5CR was compared to those in the EMBL-GenBank-DDBL database, it was found that the percentages of sequence similarity between the deduced amino acid sequences of P5CR from *B. cereus* ATCC 11778 and those from *B. cereus* Q1, *B. cereus* ATCC 10987, *B. cereus* AH187 and *B. cereus* E33L were 98, 98, 98 and 98%, respectively. P5CR from *B. cereus* ATCC 11778 exhibited the highest overall level of similarity to the enzyme from other strains of *B. cereus* due to their differences only in four amino acid residues which resulted from seven different nucleotides. Thr-53, Ser-203 and Ile-250 in *B. cereus* ATCC 11778 enzyme were replaced with Ile, Phe and Thr, respectively in the enzyme four strains of *B. cereus*. For Ser-78 in *B. cereus* ATCC 11778 enzyme. it was replaced with Ala in *B. cereus* E33L and Leu in the three rest of *B. cereus* enzyme.

4.2 Cloning and expression of *proC* gene

A number of researchers attempted to clone *proC* gene for the large amount of enzyme production in order to study properties of enzyme or use as catalyst for the synthesis of L-proline and related amino acids. Deutch and coworkers (1982) ligated the 1.2 kb of *E. coli* DNA fragment containing the *proC* gene into the expression plasmid pGW7, that derived from pBR322, and then transformed into *E. coli* strain BRL 1945 for examination of P5CR activity levels. The results showed that a relatively small increase (2.2-fold) was observed in the specific activity of P5CR. Savioz and coworkers (1990) cloned 2.2 kb of *proC* gene from *Pseudomonas aeruginosa* PAO1 into the broad-host-range vector pKT240. After that, the *proC* structural gene but not *proC* promoter (s) was subcloned into pKT240. The specific activity of this enzyme was about 1,250 units/mg protein corresponding to an overall 250-fold purification. In 2006, research group of Yang cloned *proC* gene from *M. tuberculosis* and transformed into *E. coli* using pUC18 vector. The recombinant clone

was selected and sequenced to confirm that *proC* was in the proper configuration for expression and no mutations had occurred during the PCR amplification. Then, the recombinant plasmid was extracted and isolated the DNA fragment containing *proC*. The DNA fragment was cloned into *E. coli* BL21(DE3) using pET30a vector. About 25% of the His-P5CR was present in soluble fraction after the supernatant was obtained by sonicator. The total activity of crude extract was 1,146 units/liter of culture medium. Moreover, the *proC* gene from *S. sulfataricus* was ligated into pET-28a(+) vector with a 6x His tag at the N-terminus and transformed into *E. coli* DH5α. After sequencing, the recombinant was transformed into *E. coli* BL21(DE3). The P5CR comprised about 30% of the total protein and most of the expressed P5CR was soluble. The total activity of P5CR was reported to be 1,100 units/45 milliliters (Yang *et al*., 2009). Therefore, the basic requirements for the successful production of recombinant enzyme are the isolation of the gene encoding that enzyme and selection of a suitable expression system for the gene. In this research, the *proC* gene from *B. cereus* ATCC 11778 was cloned into *E. coli* BL21(DE3) using plasmid pET-17b. By pET system, *proC* gene was expressed under T7 promoter; moreover, the upstream region of the inserted gene contained highly efficient ribosome binding site from the phage T7 major capsid protein (Novagen, 2002).

For the whole gene fragment amplification, the pair of primers was designed. The sense primer contained *Nde*I site, while the antisense primer consisted of *Eco*RI site for further cloning purpose. The amplified whole gene fragment was purified, ligated with *Nde*I-*Eco*RI site of pET-17b and then transformed into *E. coli* BL21(DE3). The twelve from sixty-two recombinant clones were found to contain recombinant plasmid. They were grown in LB medium, pH 7.2 containing 100 μg/ml ampicillin. When OD_{600} had reached 0.6, IPTG was added in the medium at final concentration of 0.4 mM. After that, the culture was continued for 4 hours before harvest. The crude extract of each recombinant clone was assayed for P5CR activity. Their P5CR specific activities were found between 0.19 to 28.9 units/mg protein. The variation of P5CR activity from each recombinant clone may cause by the metabolic reactions of plasmids in *E. coli* host cell. Therefore, the recombinant clone which has high copy number of plasmid is able to show high P5CR activity. The

highest specific activity of recombinant clone was higher than that of the enzyme from *B. cereus* with 578 fold.

Because of construction of *proC* gene under T7 promoter, expression of *proC* gene can be induced by IPTG. Therefore, the studies of induction time and final concentration of IPTG were required for maximum expression. pET-P5CR clone which showed the highest P5CR activity was grown at various conditions. Even in absence of IPTG, the expression of *proC* gene was occurred because there is some expression of T7 RNA polymerase from the *lacUV5* promoter in the DE3 lysogen from *E. coli* genome (Novagen, 2002). At 0.2 mM final concentration of IPTG which was the least concentration to be enough for induction, expression of the gene reached the maximum at 4 hours. For expression of pET plasmid carrying the T7 promoter, a final concentration of 0.4 mM IPTG is recommended for full induction (Novagen, 2002). However, different optimum conditions have been reported for some inserted genes of pET plasmids. For example, *proC* gene from *M. tuberculosis,* expressed under T7 promoter of pET30a in *E. coli* BL21(DE3), showed the highest expression at 12 hours of induction with 0.5 mM IPTG (Yang *et al.*, 2004). Meng and coworkers (2006) reported that the over-expression of the protein from human hepatoma cell-line reached maximum when the cell was induced with 0.25 mM IPTG for 3 hours. Moreover, overnight of induction with 1 mM IPTG maximized the expression of *proC* gene from *S. solfatsricus* which was cloned into *E. coli* BL21(DE3) using pET-28a(+) plasmid (Meng *et al*., 2009). Therefore, final concentrations of IPTG and induction time seem to influence the optimization of individual gene expression. The variation of expression level of recombinant P5CR from various bacteria may be obtained from the promoters, mRNA construction, and condition of expression e.g. optimization of IPTG induction, type of cell line, media, and incubation circumstances.

4.3 Purification of pyrroline-5-carboxylate reductase from recombinant clone

The development of techniques and methods for the separation and purification of proteins has been an essential pre-requisite for many of the recent

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

The first step in the purification of a protein is the preparation of an extract containing the protein in a soluble form and extraction procedures should be selected according to the source of the protein. In this work, P5CR, an intracellular enzyme, was extracted from pET-P5CR clone. Mechanical disruption methods are usually necessary to break down cell wall in order to release intracellular protein prior to purification. The cell disintegration technique involved cell lysis by ultrasonication or high pressure sound waves, which causes cell breakage by cavitations and shear forces. So in this work, cell wall was disrupted by ultrasonication. However, several potential problems may be consequent on disruption, due to the destruction of intracellular compartmentation. P5CR activity can be lost by various reasons. It is essential to consider strategies for protection of the enzyme activity. First of all, since the disruption of cells results in the release of proteases from subcellular compartment (Cooper, 1977), the effect of the free proteases must be eliminated. In this work, phenylmethylsulfonyl fluoride (PMSF), and ethylenediamine tetraacetic acid (EDTA) were used in the extraction buffer as serine protease inhibitor, and metalloprotease inhibitor, respectively. As a result, the control of metabolic regulation mechanism is lost when the cell is disrupted. These reagents protect the desired protein from the degradation of proteolytic enzyme. Addition of a reagent containing a thiol group such as β-mercaptoethanol and also a chelating agent such as EDTA to chelate metal

ions in the extraction buffer will minimize the oxidation damage (Bollag *et al.,* 1996). Acid proteases may less affect P5CR activity because their reactions occurred in high pH environment. Furthermore, mechanical cell disruption may cause local overheating with consequent denaturation of protein. To maximize recovery of active enzyme, the extract and equipment were, therefore, pre-chilled and several pauses of disruption used instead of one long continuous sonication because short interval of disruption will also minimize foaming and shearing (Harris and Angal, 1989 and Janson and Ryden, 1998).

Solubility differences in salt are frequently exploited to separate proteins in the early stages of purification protocols (Bollag *et al*., 1996 and Creighton, 1993). Most purification protocols involve some forms of chromatography, which has become an essential tool in protein purification. Ion exchange chromatography separates proteins with differences in charge to give a very high resolution with high sample loading capacity. The difference in charge properties of protein is often considerable. Ion exchange chromatography is capable of separating species with very minor differences in properties, such as two proteins differing by only one amino acid. It is a very powerful separation technique indeed (Amersham pharmacia biotech, 1999). DEAE is anion exchanger which has negatively charged counter-ions. It widely used in the purification of P5CR from other sources such as P5CR from *E. coli* (Deutch *et al.,* 1982), pumpkin cotyledons (Rana and Splittstoesser, 1974), barley (Krueger *et al.,* 1985), and *Spinacia oleracea* L.(Murahama *et al*., 2001). Its popularity comes from the possibility of high resolving power, versatility, reproducibility and ease of performance. Consequent upon the result, this column contributed greatly to the purification procedures, with less loss of P5CR activity compared to the amount of proteins removed. About 75 % of the protein in the step of crude extract was eliminated.

The next step of purification was the hydrophobic interaction chromatography (HIC). HIC takes advantage of the hydrophobicity of proteins promoting its separation on the basis of reversible hydrophobic interaction between immobilized hydrophobic ligands on chromatographic medium and non-polar regions on

the surface of proteins (Queiroz *et al.,* 2001). Butyl-Toyopearl, which butyl groups are chemically bonded on the surface of hydrophilic resin was used. Adsorption of proteins to a HIC adsorbent is favored by a high salt concentration in the mobile phase. The elution of solute is accomplished by decreasing the salt concentration with increasing hydrophobic. In this work, the enzyme was eluted from Butyl-Toyopearl column using 20% saturated ammonium sulfate. From this step, the unwanted protein about 70% of the protein obtained in DEAE-Toyopearl step was removed.

P5CR from pET-P5CR clone was purified 2.25 fold with a 14.9% yield by procedure involving DEAE-Toyopearl and Butyl-Toyopearl. The purification step of cloned enzyme was easier and more convenience.

4.4 Characterization of pyrroline-5-carboxylate reductase

4.4.1 Molecular weight determination of pyrroline-5-carboxylate reductase

The molecular weight of subunits was calculated to be 28,960 Da by comparing the mobility on SDS-polyacrylamide electrophoresis to that of standard proteins. This value was good agreement with that calculated from the amino acid sequence of the enzyme (29,920 Da). However, the molecular weight of the native recombinant enzyme was not determined by gel filtration chromatography. Thus, the enzyme subunit was not considered. The enzyme from various sources is considered variable in the quaternary structures as shown in Table 1.1.

4.4.2 Coenzyme specificity of pyrroline-5-carboxylate reductase

NAD^+-dependent P5CR catalyzes the reversible oxidative demination of L-proline. In this research, NAD^+ was replaced by the $NADP^+$ as coenzyme for the P5CR. $NADP^+$, which differs from NAD^+ only by the addition of a phosphoric group at C-2 position of NAD^+-adenosyl ribose, was inert for recombinant P5CR.

The purified P5CR expressed a preferential affinity for NAD^+, similar to the enzyme from *S. solfataricus* (Meng *et al.,* 2009). The enzymes from the rat (Herzeld *et al.,* 1977) and calf (Peisach and Strecker, 1962) have higher affinity for NADH than NADPH, while the enzyme for *C. sporogenes* appears to use NADH exclusively (Costilow and Cooper, 1978). The enzyme from *D. melanogaster* can oxidize NADH as well as NADPH, although the affinity for NADPH is much greater. In this respect it is like the enzyme from *E. coli* (Rossi *et al.,* 1977). The previous study indicated that most of the P5CRs can utilize either NADPH or NADH cofactor, but generally there will be a preference for one or the other. Nocek and coworkers (2005) studied in the structure of the complex P5CR from *Streptococus pyogenes* with $NADP^+$. They identified that Ser31 and Arg35 hydrogen bond to 2'phosphomonoester of $NADP^+$. Because the interaction of an arginine residue with the monophosphate of the ribose moiety of $NADP^+$ is considered to be a determinant of the specificity of binding NADPH or NADH, this residue may be an important of the cofactor specificity in the P5CRs family. Among P5CRs family Arg35 is only moderately conserved, consistent with the data suggesting that preference for NAD^+ and $NADP^+$ vary for enzymes from different sources. The structure of NAD^+ and $NADP^+$ are shown in Appendix J.

4.4.3 Effect of pH on pyrroline-5-carboxylate reductase 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. Additionally, previously uncharged groups may acquire a charge

as the pH increases or decreases. At some pH values, complementary regions of the enzyme and the substrate may carry charges, of the same sign and repel each other, leading to a drop in rate. Altering the charges of functional groups leads to altered ionic interactions involving these groups. These new interactions may produce a conformational change at the active site, affecting the site's capacity to bind substrate. The enzyme may undergo conformational changes and lose their capacity to bind to the substrate.

The pH which the P5CR showed maximum activity were 9.4 for the oxidative deamination of L-proline, like the enzyme from *S. solfataricus* that worked well at high pH and the human enzyme which used proline as a substrate. In contrast, the optimum pH of P5CR from *M. tuberculosis* was 7.5 similar to the enzyme from rat lens and spinach leaves which appeared an optimum pH between 6.5 and 7.1 and 7.0, respectively (Table 1.1). In addition, acetate buffer was not suitable for the activity of P5CR from pET-P5CR clone, due to the low activity observed with this buffer. The Tris-HCl buffer and glycine-KCl-KOH buffer were appropriate buffers for the enzyme. These kind of buffers are widely used for the assay of amino acid reductases.

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). The P5CR from recombinant clone was preincubated at 30 °C for 20 minutes in various 10 mM buffers at various pHs of buffer ranging from 4.0-13.0. The enzyme was stable over the pH ranged 9.0-10.0.

4.4.4 Effect of temperature on pyrroline-5-carboxylate reductase activity and stability

The influence of temperature on an enzymatic reaction is resulted from two opposing effects, an increase in rate and an increase in denaturation. Increasing of thermal energy of the substrate molecules increases the proportion of molecules with

sufficient energy to overcome the activation barrier and hence increases the rate of the reaction. In addition, increasing of the thermal energy of the molecules which make up the protein structure of the enzyme itself will increase the chance of breaking the multiple weak noncovalent interactions holding the three-dimensional structure (Segal, 1976).

For optimum temperature, the P5CR from pET-P5CR performed the highest activity at 30 °C for the oxidative deamination, whereas, the maximum activity of P5CR from *M. tuberculosis* was around 37 °C (Yang *et al.,* 2005).

The thermostability of P5CR was studied. The enzyme was preincubated at various temperature ranged from 35-60 °C for 10, 30, 60, 90 and 120 min, respectively. The enzyme lost about half of its activity upon the incubation at 40 °C for 30 min, unlike the half-life of the *M. tuberculosis* enzyme that displayed at 60 °C for 20 min. The range of temperature stability of other P5CRs have been reported as shown in Table 1.1.

4.5 Kinetic studies of pyrroline-5-carboxylate reductase

The kinetic parameters of recombinant P5CR was determined by initial velocity studies. Double-reciprocal plots of initial velocity versus L-proline concentration at a series of fixed concentration of NAD^+ gave the lines intersecting to the left of the vertical axis (Figure 3.22a.) indicated a sequential mechanism which all of substrates must be bound to the enzyme before any products are released and ruled out a ping pong mechanism that possesses a parallel initial velocity pattern (Cleland, 1971 and Segal, 1976).

In initial velocity studies, that the apparent K_m value for L-proline and NAD^+ were 1.0 and 1.54 mM, respectively. When the kinetic constant was compared, the apparent K_m of P5CR from *M. tuberculosis* for L-proline (120 µM at pH 10.3 and 37 °C) was 10 fold lower than the K_m of P5CR from pET-P5CR. The apparent K_m of

NAD^+ of P5CR from recombinant clone was 4 fold higher than the apparent K_m (0.151 ± 0.023 mM) of human P5CR (Meng *et al.,* 2009).

Although cloning of the gene encoding P5CR has been widely performed, studies about characterization and kinetic mechanism such as effect of temperature, pH on activity, stability of enzyme have rarely been examined. To compare between those of wild type and recombinant enzyme, study about recombinant enzyme are mostly focus on specific activity, purification, molecular weight determination, and amino acid composition. Due to a purpose of cloning to accomplish the large amount of enzyme production for use as catalyst for the synthesis of L-proline and related amino acids, properties of recombinant enzyme should not be altered.

In this study, the sequence of *proC* gene from *B. cereus* was determined and the structural gene has been successfully cloned and overproduced by PCR method. The obtained information can be used as primary data for further development of P5CR production and useful for the biosynthesis of L-PA from L-lysine.

4.6 Cloning and expression of *lys 6-dh* and *proC* using pET-17b

Heterologous expression systems are tools to find out the L-PA production. In these system, the *proC* gene was cloned with T7 promoter into the expression vector, pET-17b, which already cloned *lys 6-dh* gene. *E. coli* was used for the heterologous expression system, the advantages of *E. coli* is able to include easy manipulation, rapid growth of the bacteria, and simple media requirements (Ziegler *et al.,* 1999).

To co-exist high expression of *lys 6-dh* gene and *proC* gene in *E. coli* host cell, cloning of heterologous gene of *lys 6-dh* gene and *proC* gene under separated T7 promoter of high expression vector pET-17b was performed. Twelve recombinant clones showed various levels of the specific activity of Lys 6-DH and P5CR from 0.08-20.6 and 0.12-19.4 units/mg proteins, respectively. For *E. coli* BL21(DE3) transformant No.9, the highest specific activity of Lys 6-DH and P5CR were 686 and

388 fold higher than that of *A. denitrificans* K-1 (Ruldeekulthamrong *et al.,* 2008) and *B. cereus* ATCC 11778 wild type. When compare the specific activity of Lys 6-DH and P5CR with those of *E. coli* BL21(DE3) harbouring pET-ADK and pET-P5CR, the activities were decreased 1.14 and 1.41 fold, respectively.

4.7 Production of L-pipecolic acid

At present, L-PA is synthesized from L-lysine or by the optical resolution of DL-PA, prepared from picolinic acid (Rodwell, 1971). As methods for optical resolution, a diastereomer salt method using D-tartaric acid and an enzymatic method in which D-amino acid oxidase are used to decompose the D-isomer from L-isomer (Hasegawa, 1975). On the other hand, it is known that L-PA is produced in animals, plants and microorganisms. However, since the amount of L-PA accumulated therein is small, no process for the production of L-PA using these organisms has been put to practical use. From previous investigations on the metabolism of L-lysine, it is known that P6C is formed from L-lysine through a transamination reaction by lysine 6-aminotransferase (LAT) (Soda, 1968) or by the action of Lys 6-DH. It has been reported that P6C can be chemically converted into L-PA by hydrogenation using platinum oxide (Baginsky, 1968), but there is no report about the formation of L-PA by the biological or enzymatic reduction of P6C. Moreover, a metabolic pathway is supposed in which *Pseudomonas putida* produces L-PA from D-lysine via delta-1-piperideine-2-carboxylic acid (Payton *et al.,* 1982). It is also difficult to utilize such biological pathways for the mass production of L-PA.

In the above described process involving the optical resolution of L-PA prepared by chemical synthesis, the optical resolving agent used is expensive and a complicated procedure is required. Moreover, in the process using an enzyme for purposes of optical resolution, the use of a purified enzyme is also expensive. Because of these disadvantages, both processes are not efficient from an industrial point of view and cannot produce L-PA cheaply. Furthermore, conventional processes for the production of L-PA by fermentation of microorganisms have not been put to practical

use because the amount of L-PA accumulated is small. Hence, new and convenient methods for preparation of optically active L-PA are required.

The formation of L-PA from accumulated P6C by the action of P5CR is suggested as an alternative metabolic route. Fujii *et al*. (2002) overexpressed lysine amino transferase gene in *E. coli* and found that P6C product was converted to L-PA by P5CR encoded by *proC* gene which located in *E. coli* chromosome. The increase in *proC* gene copy number had no effected of L-PA production. Contrastingly, in our experiment, L-PA production was compared between *E. coli* BL21(DE3) harbouring pET-ADK-P5CR and those of *E. coli* BL21(DE3), *E. coli* BL21(DE3) harbouring pET-17b, *E. coli* BL21(DE3) harbouring pET-ADK and *E. coli* BL21(DE3) harbouring pET-P5CR. We found *E. coli* BL21(DE3) harbouring pET-ADK-P5CR only showed the accumulation of L-PA The transformant No. 9 showed the highest production of L-PA about 11.9 mM (1.53 g/l). The result indicated that the expression of both *lys 6-dh* and *proC* gene were essential and the increasing of *lys 6-dh* and *proC* gene copy number affected the L-PA production. Fujii and coworkers (2002) suggested that the rate-limiting step of L-PA production was neither the transamination step catalyzed by LAT nor the reduction step catalyzed by P5CR, but was the L-lysine uptake step. Since they found that increasing of L-lysine uptake into *E.coli* cell by cloning the *lysP* gene which was responsible to L-lysine transportation into cell, could promote L-PA production about 6 fold.

4.8 Optimization of L-pipecolic acid production

Since both *lys 6-dh* and *proC* gene were under T7 promoter, the variation of induction time between cloning of single gene and two genes expression system may differed because of sharing the materials required for transcription and translation steps. After induction with 0.1 mM IPTG for 4 hours, the highest L-PA production was increased 2.28 fold (1.57 g/l) when it was compared with non optimizing condition. Fujii and coworkers (2002) exhibited L-PA production by *E. coli* JM 109 harboring *lat* expression plasmid using L-lysine and glycerol as a starting material. The expression of LAT was induced by the addition of 1 mM IPTG when A_{660}

reached 1.0. After 4 hours of cultivation, glycerol and L-lysine were added to the culture, and the culture was cultivated for 15 hours more. The supernatants of these cultures were analyzed by TLC and HPLC. L-PA production was accumulated about 3.9 g/l. Their product was 2.48 fold higher than our product.

4.9 Effects of pH and L-lysine concentration on production of L-pipecolic acid

The optimum pH for the production of L-PA from L-lysine was investigated using the recombinant *E. coli* BL21(DE3) induced with 0.1 mM IPTG for 4 hours. L-PA productions were determined by HPLC after incubation at 30 ºC using various buffers. We found that borate buffer, sodium carbonate-bicarbonate buffer, Na_2HPO_4 and potassium chloride buffer were not suitable for L-PA production by recombinant *E. coli* due to their low productivities.

The effect of L-lysine concentration was determined and the highest production of L-PA with 1.74 g/l (13.4 mM) was obtained when 200 mM L-lysine was used. About 6.7% L-lysine at the initial concentration was converted to L-PA upon 24 hours incubation. Since P6C is chemically unstable, it is difficult to analyze quantitatively the enzymatic reaction of P6C. The instability of P6C may be the reason why the number of moles of produced L-PA was not equal to the number of moles of used L-lysine. The amount of L-PA production was compared with L-PA production by Fujii and coworkers (2002) that accumulated 3.9 g/l of the maximum production after 159 hours of cultivation. Although, the amount of L-PA production in this research was 2.24 fold lower than the L-PA production by Fujii and coworkers, the L-PA production in this research consumed less production time because the L-PA production was prepared within 24 hours. If we try to construct the recombinant *E. coli* with L-lysine transporter gene or increase cell permeabilization, the L-PA production may increase. Finally, the data obtained from this work will be useful for the industrial fields, if immobilize technique is applied to the *E. coli* recombinant cell.

CHAPTER V

CONCLUSION

1. The full length *proC* gene from *Bacillus cereus* ATCC 11778 was sequenced and cloned into *E. coli* BL21(DE3) using pET-17b, an expression vector. It has a single open reading frame of 819 nucleotides encoding the polypeptide of 272 amino acid residues.

2. The percentages of sequence similarity between the nucleotide sequences of *proC* from *B. cereus* ATCC 11778 and those from *B. cereus* Q1, *B. cereus* ATCC 10987, *B. cereus* AH187 and *B. cereus* E33L were 92, 92, 91 and 91%, respectively.

3. The deduced amino acid sequence of the whole P5CR from *B. cereus* ATCC 11778 was compared to those in the EMBL-GenBank-DDBL database. It was found that the percentages of sequence similarity between the deduced amino acid sequences of P5CR from *B. cereus* ATCC 11778 and those form *B. cereus* Q1, *B. cereus* ATCC 10987, *B. cereus* AH187 and *B. cereus* E33L were 98, 98, 98 and 98%, respectively.

4. The *E. coli* containing *proC* gene gave the highest specific activity of P5CR of 33.3 U/mg protein which was 660 fold higher than the wild type *B. cereus* when the cell was induced with 0.4 mM IPTG for 4 hours.

5. P5CR from the recombinant clone was purified from crude enzyme by DEAE-Toyopearl and Butyl-Toyopearl column chromatographies to homogeneity with 2.25 purification fold and 14.9% yield.

6. The molecular weight of P5CR subunit was calculated to be about 28.9 kDa by SDS-PAGE.

7. The P5CR enzyme was capable of utilizing NAD^+ as a natural coenzyme for oxidative deamination.

8. The enzyme showed the optimum pH and temperature for the oxidative deamination at 9.4 and 30 °C, respectively.

9. The enzyme was stable over the pH ranging from 9.0 to 10.0 and lost about half of its activity upon the incubation at 40 °C for 30 minutes.

10. The steady state kinetic studies indicated that the apparent K_m value for L-proline and NAD^+ were 1.0 and 1.54 mM, respectively.

11. The heterologous genes of *lys 6-dh* and *proC* were constructed using pET-17b and cloned into *E. coli* BL21(DE3). The transformants showed various levels of specific activity of Lys 6-DH and P5CR from 0.08 to 20.6 and 0.12-19.4 units/mg proteins, respectively. The highest specific activity of Lys 6-DH and P5CR were 686 and 388 fold higher than that of their wild type.

12. The highest production of L-PA, approximately 1.74 g/l, was obtained by induction of the recombinant clone with 0.1 mM IPTG for 4 hours and then 0.05 g of cell wet weight was incubated in the reaction mixture consisted of 200 mM L-lysine in 200 mM Tris-HCl buffer, pH 9.0 for 24 hours.

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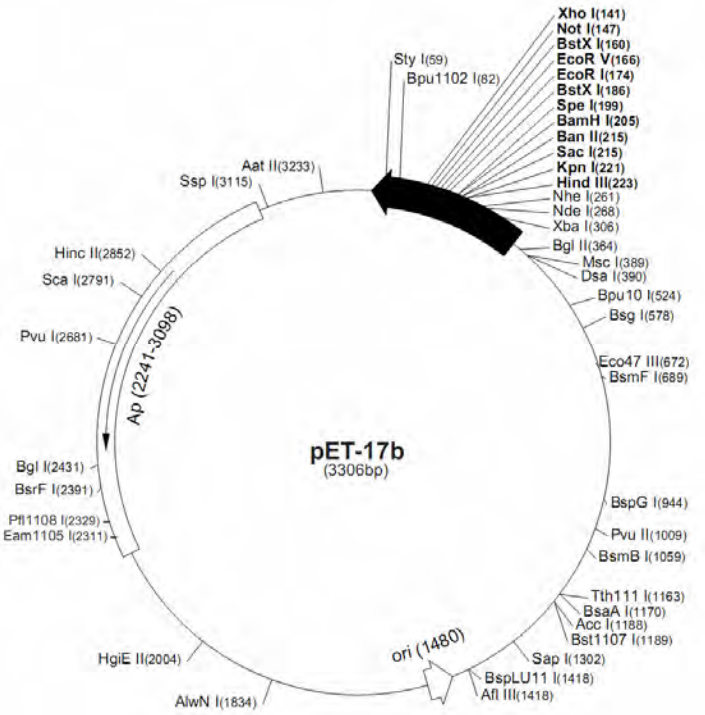
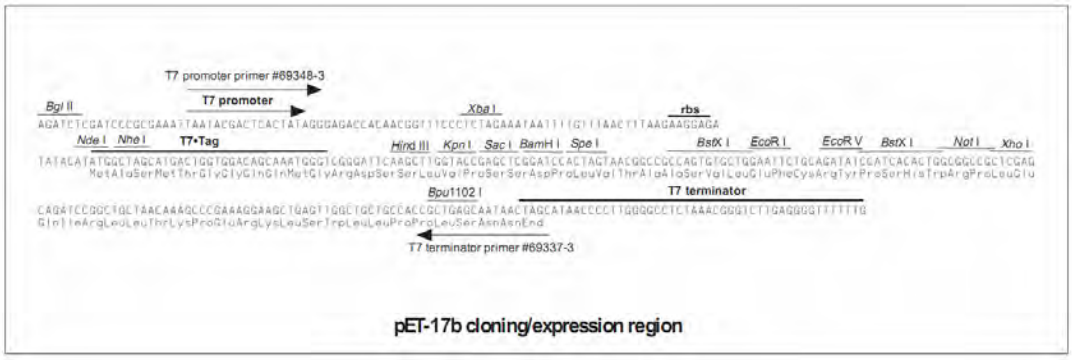
APPENDICES

APPENDIX A

Restriction map of pET-17b

pET-17b sequence landmarks	
T7 promoter	333-349
T7 transcription start	332
T7•Tag coding sequence	237-269
Multiple cloning sites	
(Hind III - Xho I)	141-228
T7 terminator	28-74
pBR322 origin	1480
bla coding sequence	2241-3098

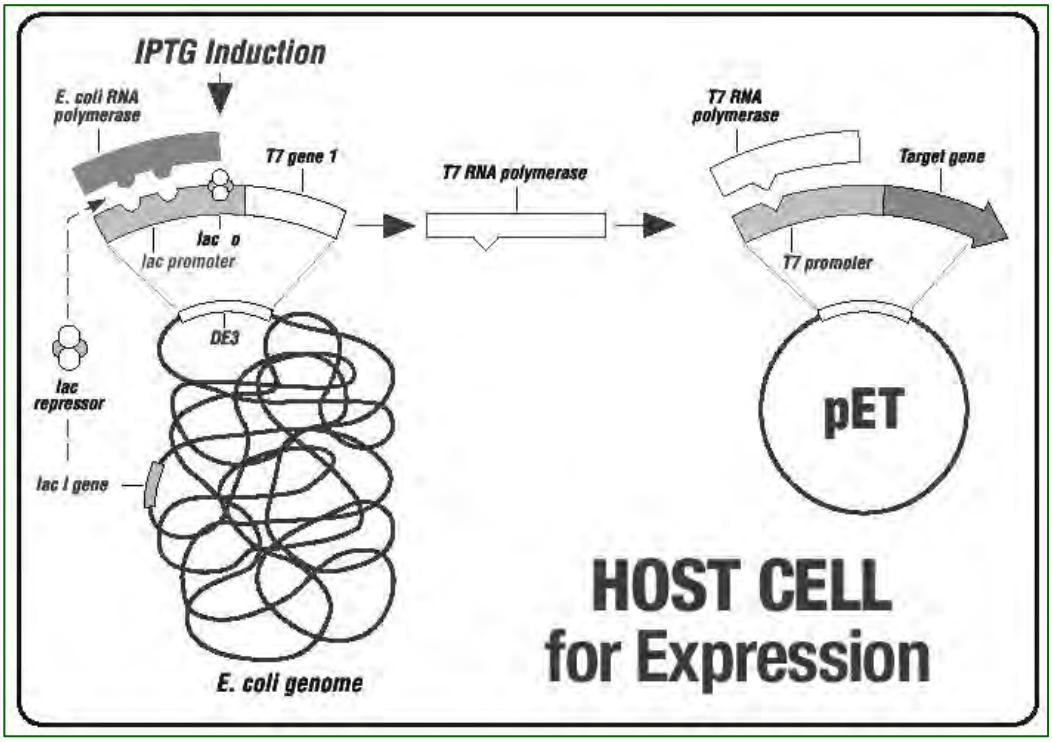




pET-17b cloning/expression region

APPENDIX B

Control element of the pET system



APPENDIX C

HiYield Gel/PCR DNA Fragments Extraction Kit

PCR Clean up Protocol

1. Transfer up to 100 µl reaction product to a microcentrifuge tube
2. Add 5 volumes of DF Buffer to 1 volume of the sample and mix by vortexing.
3. Place a DF Column in a Collection Tube.
4. Apply the sample mixture from previous step into the DF Column.
5. Centrifuge at 6,000xg (8,000 rpm) for 30 seconds.
6. Discard the flow-through and place the DF Column back in the Collection Tube.
7. Add 500 µl of Wash Buffer (ethanol added) in DF Column.
8. Centrifuge at 6,000xg (8,000 rpm) for 30 seconds.
9. Discard the flow-through and place the DF Column back in the Collection Tube.
10. Centrifuge again for two minutes at full speed (14,000 rpm) to dry the column matrix.
11. Transfer dried column to a new microcentrifuge tube.
12. Add 15 µl of Elution Buffer or water into the center of column matrix.
13. Stand for 2 minutes until Elution Buffer or water absorbed by the matrix.
14. Centrifuge for 2 minutes at full speed to elute purified DNA.

APPENDIX C (continued)

Gel Extraction Protocol

1. Excise the agarose gel slice containing relevant DNA fragments and remove extra agarose to minimize the size of the gel slice.
2. Transfer up to 300 mg of the gel slice into microcentrifuge tube.
3. Add 500 µl of DF Buffer to the sample and mix by votexing.
4. Incubate at 55°C for 10-15 minutes until the gel slice has been completely dissolved. During incubation, invert the tube every 2-3 min.
5. Place a DF Column in a Collection Tube.
6. Apply 800 µl of the sample mixture from previous step into the DF Column.
7. Centrifuge at 6,000xg (8,000 rpm) for 30 seconds.
8. Discard the flow-through and place the DF Column back in the Collection Tube.
9. If the sample mixture is more than 800 µl, repeat this DNA Binding Step.
10. Add 500 µl of Wash Buffer (ethanol added) into DF Column.
11. Centrifuge at 6,000xg (8,000 rpm) for 30 seconds.
12. Discard the flow-through and place the DF Column back in the Collection Tube.
13. Centrifuge again for 2 minutes at full speed (14,000 rpm) to dry the column matrix.
14. Transfer dried column in a new microcentrifuge tube.
15. Add 15 µl of Elution Buffer or water in the center of the column matrix.
16. Stand for 2 minutes until Elution Buffer or water absorbed by the matrix.
17. Centrifuge for 2 minutes at full speed to elute purified DNA.

APPENDIX D

High-Speed Plasmid Mini Kit Protocol

1. Transfer of 1.5 ml of culture bacterial cells to a microcentrifuge tube.
2. Centrifuge for 1 minute and discard the supernatant.
3. If more than 1.5 ml of cultured bacterial cells is used, repeat the Harvesting Step.
4. Add 200 µl of PD1 Buffer (RNase A added) to the tube and resuspend the cell pellet by vortex or pipetting.
5. Add 200 µl of PD2 Buffer and mix gently by inverting the tube 10 times. Do not vortex to avoid shearing the genomic DNA.
6. Let stand at room temperature for 2 minutes or until the lysate is homologous.
7. Add 300 µl of PD3 Buffer and mix immediately by inverting the tube 10 times. Do not vortex.
8. Ccentrifuge for 3 minutes and place a PD Column in a 2 ml Collection Tube.
9. Add the supernatant from previous step to the PD Column and centrifuge for 30 seconds.
10. Discard the flow-through and place the PD Column back in the Collection Tube.
11. Add 400 µl of W1 Buffer into the PD Column and centrifuge for 30 seconds.
12. Discard the flow-through and place the PD Column back in the Collection Tube.
13. Add 600 µl of Wash Buffer (ethanol added) into the PD Column and centrifuge for 30 seconds.
14. Discard the flow-through and place the PD Column back in the Collection Tube.
15. Centrifuge again for 3 minutes to dry the column matrix.
16. Transfer the dried PD Column to a new microcentrifuge tube.
17. Add 50 µl of Elution Buffer or TE into the center of the column matrix.
18. Stand for 2 minutes or until the Elution Buffer or TE is absorbed by the matrix.
19. Centrifuge for 2 minutes to elute the DNA.

APPENDIX E

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

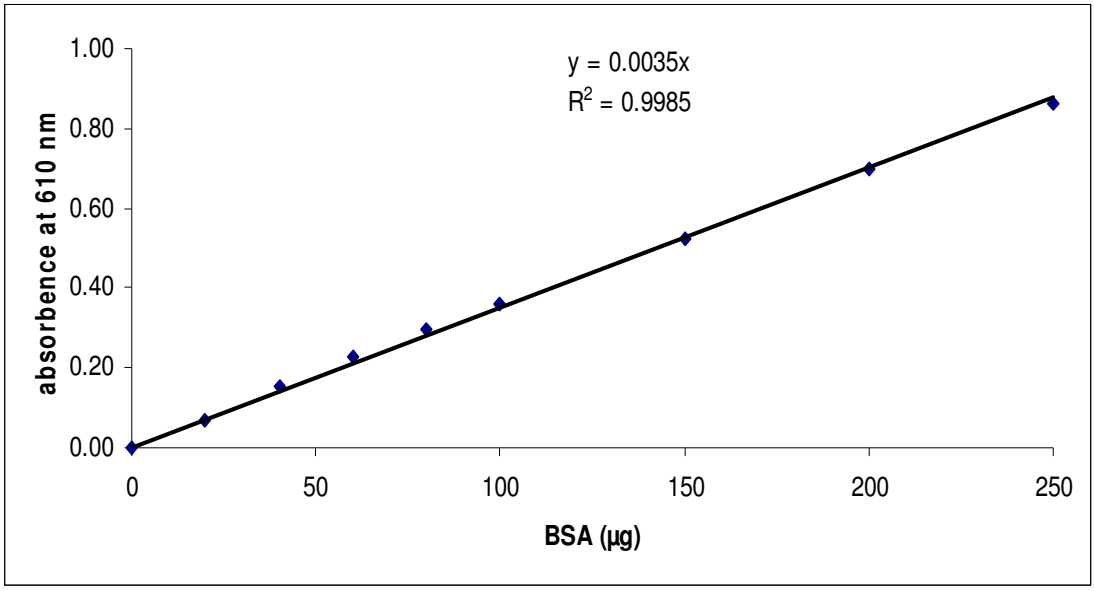
Dissolved in distilled water to 1 liter.

Solution C (phenol reagent)

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

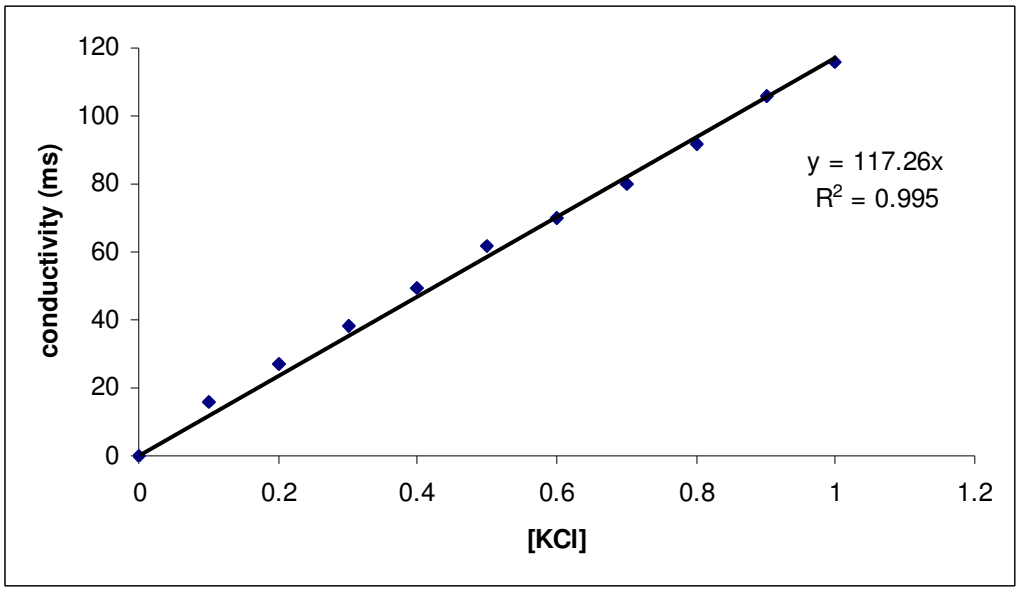
APPENDIX F

Standard curve for protein determination by Lowry's method



APPENDIX G

Calibration curve for conductivity of potassium chloride



APPENDIX H

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 H (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 H (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-proline

L-proline 0.066 g

Dissolved with 10 ml distilled water

50 mM NAD^+

NAD^+ 0.359 g

Dissolved with 10 ml distilled water

0.25 mg/ml phenazine methosulfate

Phenazine methosulfate 0.0025 g

Dissolved with 10 ml distilled water

2.5 mg/ml nitroblue tetrazolium

Nitroblue tetrazolium 0.025 g

Dissolved with 10 ml distilled water

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

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 I (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 I (continued)

3. SDS-PAGE

12.5% Separating gel

Solution A	4.2	ml
Solution B	2.5	ml
Distilled water	3.3	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

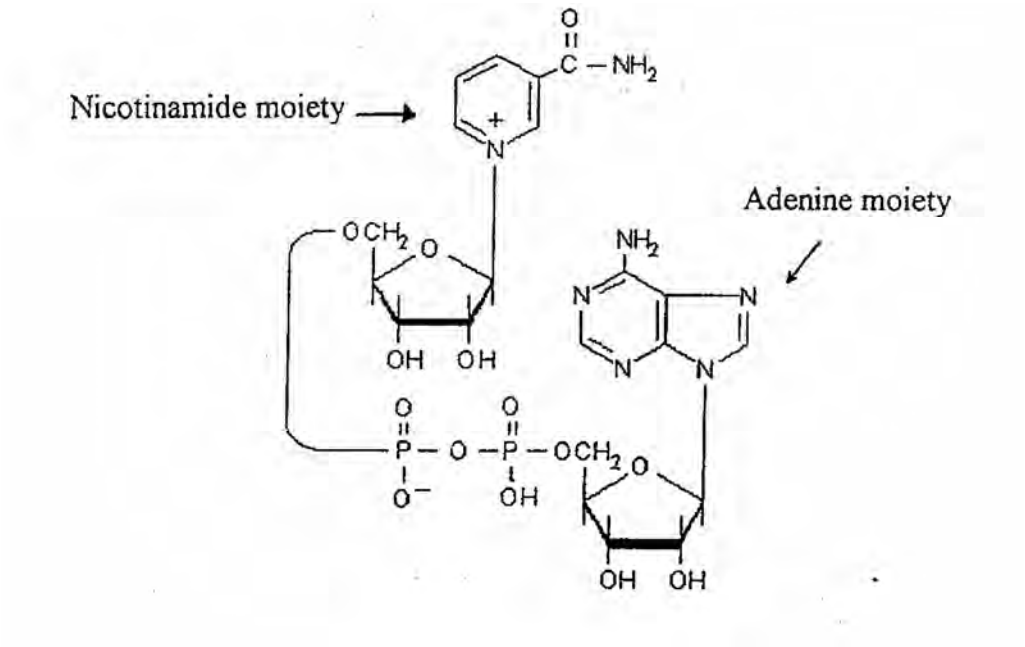
Coomassie brilliant blue R-250	1.0	ml
Methanol	450	ml
Distilled water	450	ml

Destaining solution, 1 liter

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

APPENDIX J

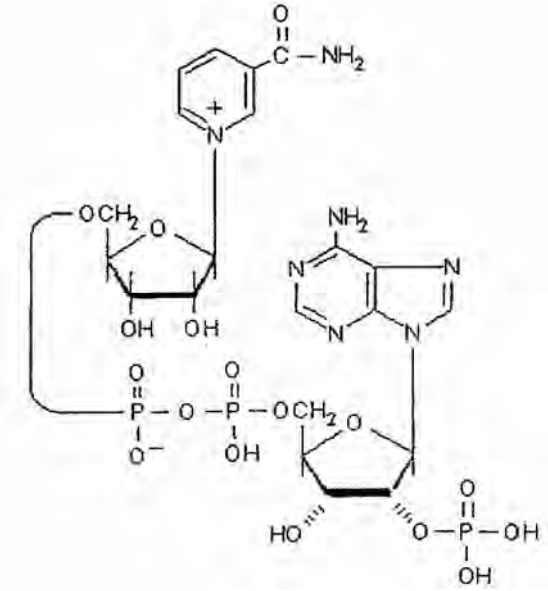
NAD^+ analogs



Nicotinamide adenine dinucleotide (NAD^+)

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

Coenzyme analog modified at C-2 position of the adenosylribose



Nicotinamide adenine dinucleotide phosphate ($NADP^+$)

APPENDIX K

Abbreviation for amino acid residues

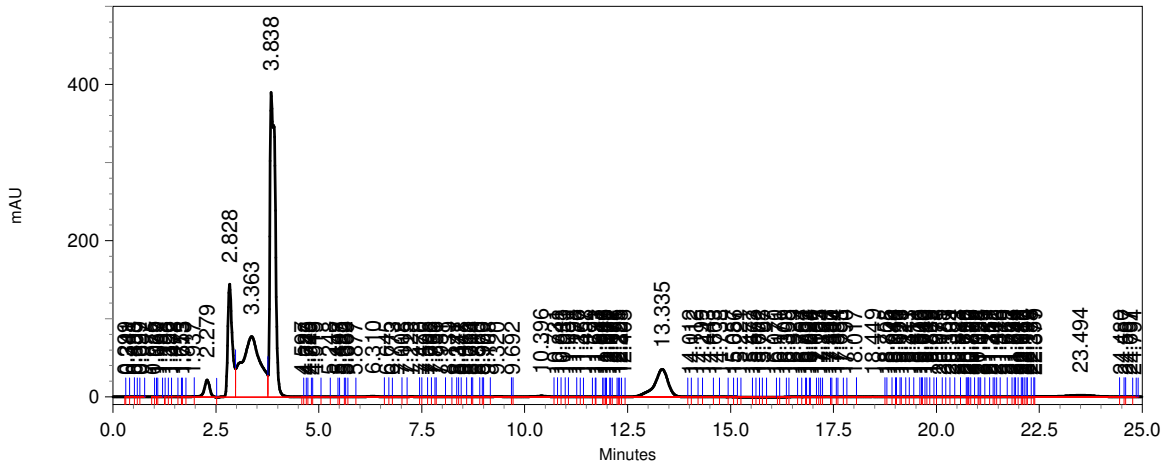
Amino acid	3 Letters-Abbreviation	1-Letter-Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine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

Source: Voet, 2004

APPENDIX L

HPLC profile of L-lysine and L-pipecolic acid

A.) L-lysine standard (5 µg/µl)



Condition :

Column : Inertsil ODS-3 (250 x 4.6 mm) column

Temperature : 25 °C

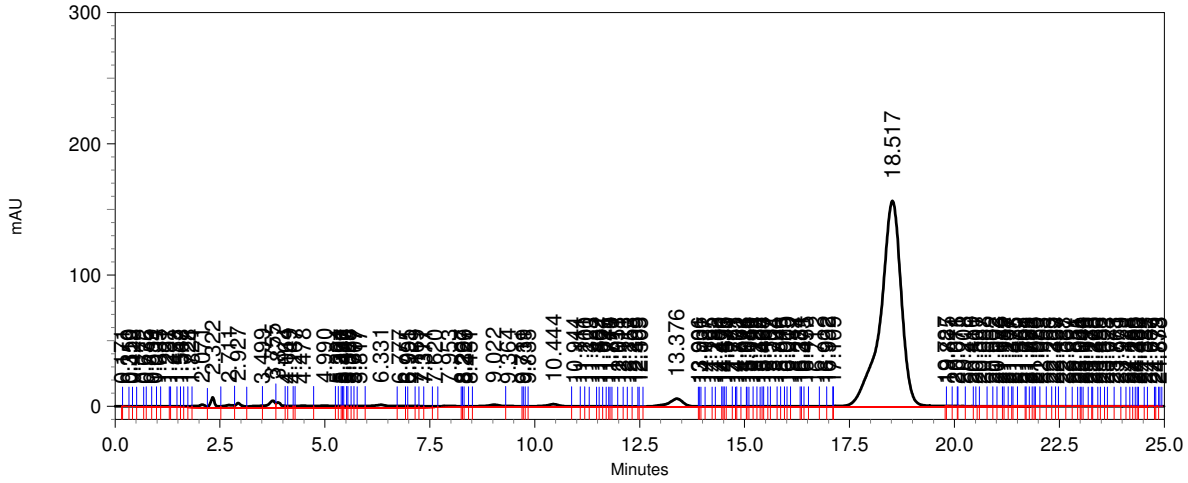
Flow rate : 1 ml/min

Mobile phase : acetonotrile : 0.05 M triethylamine phosphate pH 3.0 (35 : 65)

APPENDIX L (continued)

HPLC profile of L-lysine and L-pipecolic acid

B.) L-pipecolic acid standard (100 µg/µl)



Condition :

Column : Inertsil ODS-3 (250 x 4.6 mm) column

Temperature : 25 °C

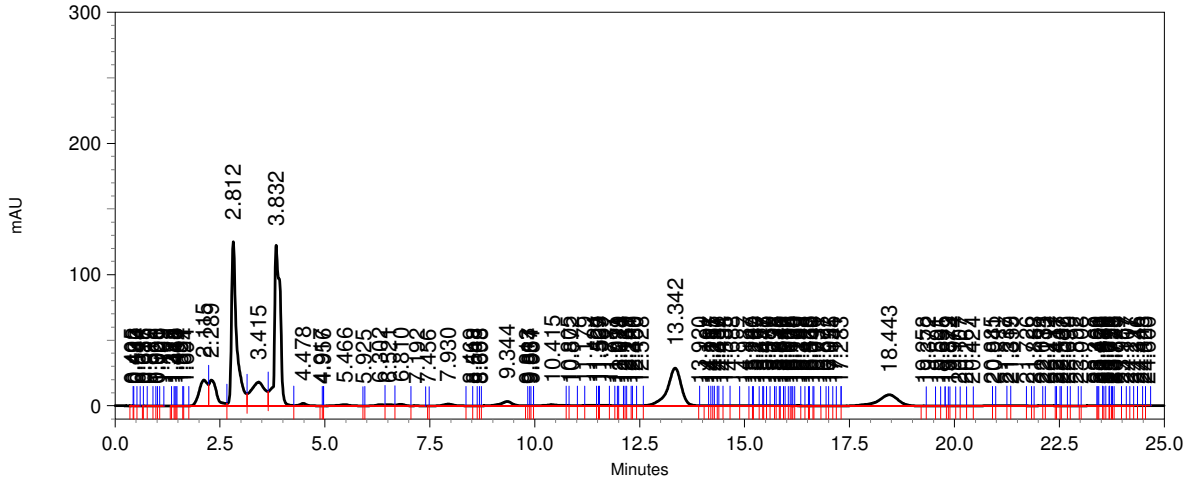
Flow rate : 1 ml/min

Mobile phase : acetonotrile : 0.05 M triethylamine phosphate pH 3.0 (35 : 65)

APPENDIX L (continued)

HPLC profile of L-lysine and L-pipecolic acid

C.) Sample at production time 24 hours



Condition :

Column : Inertsil ODS-3 (250 x 4.6 mm) column

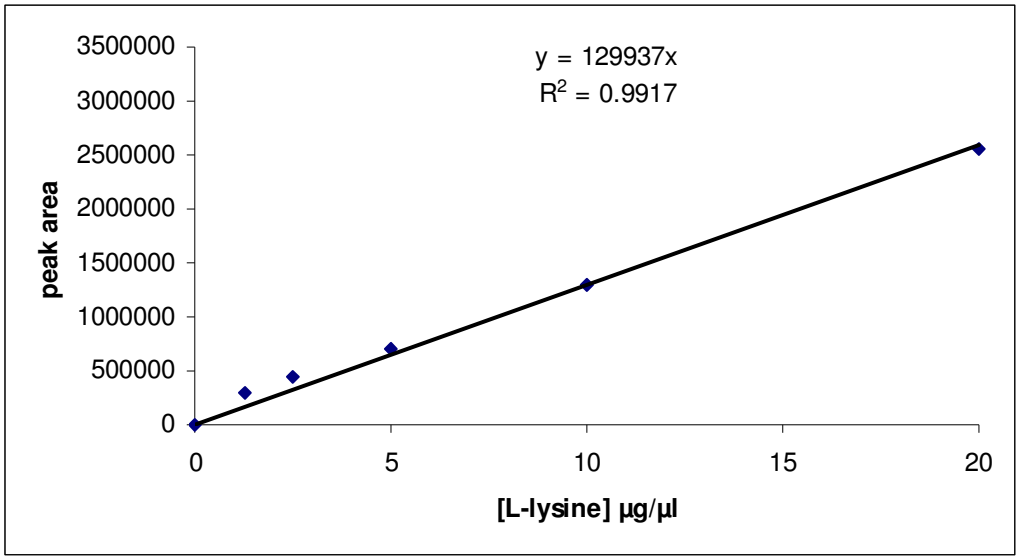
Temperature : 25 °C

Flow rate : 1 ml/min

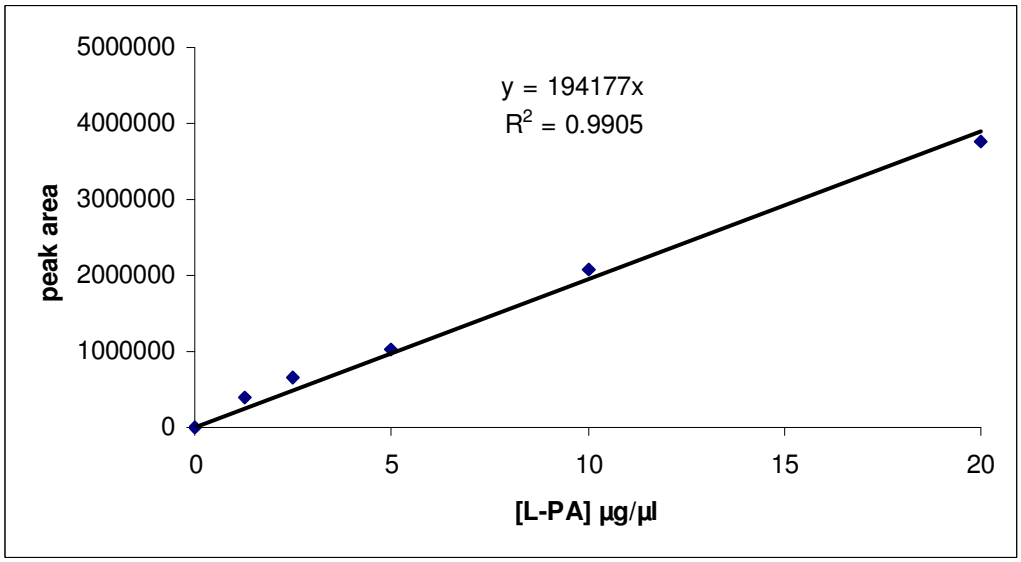
Mobile phase : acetonotrile : 0.05 M triethylamine phosphate pH 3.0 (35 : 65)

APPENDIX M

Standard curve for L-lysine concentration by HPLC



Standard curve for L-pipecolic acid concentration by HPLC



BIOGRAPHY

Miss Kasama Srimuang was born on May 18th, 1986 in Nakhonsrithammarat. After graduating with degree of Bachelor of Science from the Department of Medical Science at Naresuan University in 2007, she keeps on studying for Master of Science at the Biochemistry Program, Faculty of Science at Chulalongkorn University in that year. In her third year of research work, she presented a poster entitle "Purification and characterization of pyrroline-5-carboxylate reductase from *Bacillus cereus* ATCC 11778" at the 3rd Biochemistry and Molecular Biology Conference, organized by Chiang Mai University on April 6-8, 2011.