CO-EXPRESSION OF FEEDBACK RESISTANT ENZYMES IN PHENYLALANINE BIOSYNTHESIS PATHWAY TO INCREASE PHENYLALANINE PRODUCTION

Miss Charintip Yenyuvadee

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biochemistry and Molecular Biology

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การแสดงออกร่วมของเอนไซม์ที่ดันทางการเกิดการยับยั้งแบบย้อนกลับในวิถีการสังเคราะห์ฟีนิลาน

น.ส.ชรินทร์ทิพย์ เย็นยุวดี

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By: Miss Charintip Yenyuvadee

Field of Study: Biochemistry and Molecular Biology

Thesis Advisor: Associate Professor KANOKTIP PACKDIBAMRUNG, Ph.D.

Accepted by the FACULTY OF SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

(Professor POLKIT SANGVANICH, Ph.D.)

THESIS COMMITTEE

Chairman: (Associate Professor TEERAPONG BUABOOCHA, Ph.D.)

Thesis Advisor: (Associate Professor KANOKTIP PACKDIBAMRUNG, Ph.D.)

Examiner: (Assistant Professor Rath Pichyangkura, Ph.D.)

External Examiner: (Assistant Professor Rattree Wongpanya, Ph.D.)
ABSTRACT (THAI)

ชรินทร์ทิพย์ เย็นยุวดี: การแสดงออกร่วมของเอนไซม์ที่ต้านทานการเกิดการยับยั้งแบบย้อนกลับในวิถีการสังเคราะห์ฟีนิลอะลานีนเพื่อเพิ่มการผลิตฟีนิลอะลานีน. (CO-EXPRESSION OF FEEDBACK RESISTANT ENZYMES IN PHENYLALANINE BIOSYNTHESIS PATHWAY TO INCREASE PHENYLALANINE PRODUCTION)

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แอล-ฟีนิลอะลานีน (L-Phe) เป็นกรดอะมิโนจำเป็นที่มีความสำคัญทางพาณิชย์ที่ถูกนำมาใช้ในอุตสาหกรรมอาหารและยา. ในปัจจุบันมีความต้องการของแอล-ฟีนิลอะลานีนสูงขึ้นตามความต้องการในการผลิตแอสปาแตมซึ่งเป็นสารให้ความหวานแทนน้ำตาลที่มีแคลอรี่ต่ำ. ใน Escherichia coli การสังเคราะห์แอล-ฟีนิลอะลานีนถูกควบคุมหลายลำดับขั้น เช่นไพรเมร์ AroG ของ 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHP synthase) และ chorismate mutase/prephenate dehydratase (PheA) เป็นเอนไซม์ที่สำคัญ 2 ชนิดที่ต้องการเขย่าแบบย้อนกลับได้โดย L-Phe การแสดงออกร่วมของ pheA ที่ดื่มด่ำการยับยั้งแบบย้อนกลับ (pheA<sup>L359D</sup>) กับยีนอื่น ๆ ที่สำคัญในวิธีการสังเคราะห์ L-Phe ได้แก่ aroB, aroL, phedh, tktA, aroG, pheA, yddG และยีนที่ใน pRSFDuet-1 (pPTFBLYA<sup>L359D</sup>) สามารถเพิ่มการผลิต L-Phe ได้เป็น 3.78 เท่า เมื่อเทียบกับโคลนที่มี pheA<sup>wt</sup> (pPTFBLYA<sup>wt</sup>) ในงานวิจัยนี้ยีน aroG<sup>wt</sup> และ aroG ที่ควบคุมแบบย้อนกลับ (aroG<sup>L175D</sup>, aroG<sup>Q151L</sup>, aroG<sup>Q151A</sup> และ aroG<sup>Q151N</sup>) ได้ถูกโคลนเข้า pRSFDuet-1 แล้วได้ผลิตเอนไซม์ DAHP synthase สูงสุดในภาวะที่มี L-Phe 20 มิลลิโมล แล้วจึงได้ทำการสร้างโคลนของ E. coli BL21(DE3) ที่มี pBLPT<sup>L359D,G<sup>Q151</sup>& pYF และ pBLPT<sup>L359D,G<sup>Q151N</sup>& pYF แล้วทำการตรวจวัดการผลิต L-Phe ใน minimum medium ที่มีกลีเซอรอลร้อยละ 6 เทียบกับโคลนที่มี pBLPT & pYF พบว่าโคลน pBLPTA<sup>L359D,G<sup>Q151</sup>& pYF มีการผลิต L-Phe สูงขึ้น 8.7 เท่า และโคลนที่มี AroG<sup>Q151N</sup> ผลิต L-Phe ได้มากกว่าโคลนที่มี AroG<sup>Q151</sup> 1.2 เท่า.
L-Phenylalanine (L-Phe) is an important commercial amino acid. It is widely used in food and pharmaceutical industries. Currently, the requirement of L-Phe is increased according to the great demand for the low-calorie sweetener, aspartame. In Escherichia coli, the synthesis of L-Phe is controlled by the multi-hierarchical regulations. AroG isoform of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHP synthase) and chorismate mutase/prephenate dehydratase (PheA), two important enzymes, are feedback inhibited by L-Phe. Co-expression of feedback-resistant pheA (pheA<sup>L359D</sup>) with other pivotal genes in L-Phe biosynthesis pathway: aroB, aroL, phedh, tktA, aroG, pheA, yddG, and glpF in pRSFDuet-1 (pPTFBLYA<sup>L359D</sup>) elevated L-Phe production of E. coli BL21(DE3) 3.78 fold in comparison to that of wildtype (pPTFBLYA<sup>WT</sup>). In this research, wildtype aroG (aroG<sup>WT</sup>) and feedback resistant aroG genes (aroG<sup>L175D</sup>, aroG<sup>Q151L</sup>, aroG<sup>Q151A</sup> and aroG<sup>Q151N</sup>) were cloned into pRSFDuet-1 and then transformed into E. coli BL21(DE3). AroG<sup>Q151N</sup> clone gave the highest specific activity of DAHP synthase in the presence of 20 mM L-Phe. Therefore, E. coli BL21(DE3) containing pBLPTA<sup>L359D</sup><sup>G</sup> & pYF and pBLPTA<sup>L359D</sup><sup>Q151N</sup> & pYF were constructed and their production of L-Phe in 6% glycerol medium were determined in comparison to pBLPT & pYF clone. The presence of PheA<sup>L359D</sup> and AroG<sup>Q151N</sup> elevated L-Phe production 8.7 fold while the clone containing AroG<sup>Q151N</sup> produced L-Phe 1.2 fold higher than AroG<sup>WT</sup> clone.
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Ala alanine
arOB 3-dehydroquinate synthase gene
AroG L-phenylalanine sensitive isoform of 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase
AroG\textsuperscript{fr} L-phenylalanine feedback resistant AroG
aroG L-phenylalanine sensitive isoform of 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase gene
aroG\textsuperscript{fr} L-phenylalanine feedback resistant 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase gene
aroL shikimate kinase II gene
Asn asparagine
Asp aspartate
bp base pairs
BSA bovine serum albumin
\textdegree C degree celsius
CMPD chorismate mutase/ prephenate dehydratase
DAHP 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase
dNTP 2’-deoxynucleoside 5’-triphosphate
EDTA ethylene diamine tetraacetic acid
E4P erythrose 4-phosphate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>fbr</td>
<td>feedback resistant</td>
</tr>
<tr>
<td>glpF</td>
<td>glycerol facilitator gene</td>
</tr>
<tr>
<td>Gln</td>
<td>glutamine</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-(\beta)-D-thiogalactoside</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
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<td>L</td>
<td>liter</td>
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<tr>
<td>L-Phe</td>
<td>L-phenylalanine</td>
</tr>
<tr>
<td>L-Trp</td>
<td>L-tryptophan</td>
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<tr>
<td>L-Tyr</td>
<td>L-tyrosine</td>
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<tr>
<td>LB</td>
<td>Luria-Bertani</td>
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<tr>
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<td>ultraviolet</td>
</tr>
<tr>
<td>Val</td>
<td>valine</td>
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</tbody>
</table>
v/v  volume by volume

wt  wildtype

yddG  aromatic amino acid exporter gene
CHAPTER I

Introductions

1.1 Aromatic amino acid

Aromatic amino acids are amino acids that have an aromatic ring in the side chain. Among the 20 standard amino acids, the following are aromatic: L-tryptophan (L-Trp), L-phenylalanine (L-Phe) and L-tyrosine (L-Tyr). Aromatic amino acids are the building blocks of proteins. Normally, they constitute less than 10% of the protein. L-tryptophan is the largest and the rarest of the amino acids in proteins [1].

The aromatic amino acids are produced from the shikimate pathway which is found in bacteria, fungi, plants, and some protists. In the three aromatic amino acids metabolism, L-Phe has the highest carbon flux because up to 30% of organic matter constitutes from L-Phe-derived compounds. Plant and microbe can synthesize their own aromatic amino acids to make proteins. In plants, the aromatic amino acids also use as precursors of plant natural products that play a key role in plant growth, development, reproduction, defense and environmental responses. L-Trp is used as a precursor of alkaloids, phytoalexins, indole glucosinolates and auxin. L-Tyr is used as a precursor of isoquinoline alkaloids, pigment betalains and quinones while L-Phe is also used as a precursor of numerous phenolic compounds including flavonoids, tannins, lignans, lignin, and phenylpropanoid/benzenoid volatiles (Figure 1) [2]. For animals, L-Phe and L-Trp are essential amino acids. However, animals have lost the metabolic pathway for aromatic amino acid. They must derive these amino acids through their food and L-Phe can be converted to L-Tyr by Phe hydroxylase. In animals and humans, the aromatic amino acids are used as precursors for the synthesis of biologically and neurologically active compounds that are essential for maintaining biological functions. Nowadays, the aromatic amino acids are important examples of chemical products that can be produced by renewable raw material in microorganisms such as glucose or glycerol [3].
Figure 1. The aromatic amino acid pathways support the formation of numerous natural products in plants. The shikimate pathway (shown in green) produces chorismate, a common precursor for the Trp pathway (blue), the Phe/Tyr pathways (red), and the pathways leading to folate, phyloquinone, and salicylate. Trp, Phe, and Tyr are further converted to a diverse array of plant natural products that play crucial roles in plant physiology, some of which are essential nutrients in human diets (bold). Abbreviations: ADCS, aminodeoxychorismate synthase; AS, anthranilate synthase; CM, chorismate mutase; CoA, coenzyme A; ICS, isochorismate synthase [2].
1.2 L-Phenylalanine

L-Phenylalanine (C₉H₁₁NO₂) is an essential hydrophobic aromatic amino acid. It has a benzyl group as a side chain as shown in Figure 2. It is classified as nonpolar and neutral because of the hydrophobic nature and inert of the side chain. L-Phe is also one of the most important commercial aromatic amino acid for humans and animals [4, 5]. It is provided from diet such as meat, cottage cheese, and wheat germ. In human brain, L-Phe is converted to L-Tyr which is used as a precursor to produce catecholamines such as tyramine, dopamine, epinephrine, and norepinephrine. The neurotransmitter catecholamines act like adrenalin substances that transmit signals between nerve cells and the brain for keeping us awake and alert, reduce hunger pains, function as an antidepressant and help improvement of memory [6-8].

In pharmaceutical industries, L-Phe is used for chemical synthesis of pharmaceutically active compounds like HIV protease inhibitor, anti-inflammatory drugs [9], phenylethylamine, catecholamines [10] and combination with UVA therapy for the treatment of vitiligo [11]. Moreover, L-Phe also used in several psychotropic drugs (mescaline, morphine, codeine, and papaverine). In food industries, L-Phe is used as a supplementary food and a precursor for low calorie sweetener, aspartame (L-aspartyl-L-phenylalanine methyl ester) which is 160–180 times sweeter than sucrose [12]. Currently, the requirement of L-Phe is increased according to the great demand for the low-calorie sweetener, aspartame which approximate the world market of US $1.5 billion.

![Figure 2. Structure of L-phenylalanine [7.](image)](image)
1.3 L-Phenylalanine production

The L-Phe can be produced by chemical synthesis and bioprocessing such as enzymatic transformation or microbial process [13]. In early industrial process of L-Phe, it was mainly produced by chemical process. However, the chemical synthesis of L-Phe has many disadvantages for example, chemical synthesis uses toxic raw materials that are nonrenewable and generate racemic mixtures of D and L Phe isomers which make it difficult for the purification processes. Furthermore, the process has a high cost and various problems. Therefore, the L-Phe biosynthesis is an attractive alternative since it is a clean technology and uses renewable simple carbohydrates that generates less environmental pollution [14, 15]. More recently, metabolic engineering in Escherichia coli (E. coli) has been focused because the main metabolisms of E. coli have enabled the introduction of such genetic modifications [16]. Furthermore, researchers can use a genetic engineering technique to enhance L-Phe yield [17].

1.4 L-Phenylalanine biosynthesis pathway in E. coli

When E. coli cell was cultured in medium containing carbon sources such as glucose or glycerol, the carbon sources can be converted to phosphoenolpyruvate (PEP) by glycolysis pathway and erythrose 4-phosphate (E4P) by pentose phosphate pathway. The biosynthesis of aromatic amino acids is started from the condensation of PEP and E4P to D-arabinoheptulosonate7-phosphate (DAHP) by DAHP synthase (EC 2.5.1.54). This step is tightly regulated by its final product, phenylalanine, tyrosine and tryptophan to convert the carbon flow into the shikimate pathway. There are three isozymes of DAHP synthase encoded by aroF (L-Tyr-sensitive), aroH (L-Trp-sensitive) and aroG (L-Phe-sensitive) [18-20].

In the second step of the pathway (shikimate pathway), there are 2 rate limiting enzymes including 3-dehydroquinate (DHQ) synthase encoded by aroB and
shikimate kinase II, which is encoded by *aroL*. At chorismite (CHA), the pathways of each aromatic amino acid are separated. To produce L-Phe, chorismate is converted to phenyl pyruvate (PPA) and then PPA is changed to phenylpyruvate (PPY) by bifunctional enzyme chorismate mutase/prephenate dehydratase (CMPD) encoded by *pheA*. This enzyme is feedback regulated by L-Phe. Finally, PPY is converted to L-Phe by amino transferase. The excess L-Phe is excreted by aromatic amino acid exporter encoded by *yddG*. The aromatic amino acid biosynthesis pathway is shown in Figure 3 [21].
Figure 3. Pathway of aromatic amino acid biosynthesis and its regulation in *E. coli*. To indicate the type of regulation, different types of lines are used: –––, transcriptional and allosteric control exerted by the aromatic amino acid end products; · · ·, allosteric control only; —, transcriptional control only. Abbreviations used: ANTA, anthranilate; aKG, α-ketoglutarate; CDRP, 1-(o-carboxyphenylamino)-1-deoxyribulose 5-phosphate; CHA, chorismate; DAHP, 3-deoxy-d-arabino-heptulosonate 7-phosphate; DHQ, 3-dehydroquinate; DHS, 3-dehydroshikimate; EPSP, 5 enolpyruvylshikimate 3-phosphate; E4P, erythrose 4-phosphate; GA3P, glyceraldehyde 3-phosphate; HPP, 4-hydroxyphenylpyruvate; I3GP, indole 3-glycerolphosphate; IND, indole; L-Gln, L-glutamine; L-Glu, L-glutamate; L-Phe, L-phenylalanine; L-Ser, L-serine; L-Trp, L-tryptophan; L-Tyr, L-tyrosine; PEP, phosphoenolpyruvate; PPA, prephenate; PPY, phenylpyruvate; PRAA, phosphoribosyl anthranilate; PRPP, 5-phosphoribosyl-1-pyrophosphate; Pyr, pyruvate; SHIK, shikimate; S3P, shikimate 3-phosphate [21].
1.5 L-Phe sensitive DAHP synthase

In *E. coli*, the three isozymes of DAHP synthase are L-Phe sensitive (AroG), L-Tyr sensitive (AroF) and L-Trp sensitive (AroH) which are encoded by *aroG*, *aroF* and *aroH*, respectively [22]. About 80% of total DHAPS activity is made up by AroG while AroF and AroH share 20% and 1% of total activity, respectively [20]. The structure of AroG is a homotetramer whereas AroH and AroF are homodimer. Specific activities of AroG varied widely with different metal ions as follows; Mn$^{2+} >$ Cd$^{2+} >$ Fe$^{2+} >$ Co$^{2+} >$ Ni$^{2+},$ Cu$^{2+},$ Zn$^{2+} >$ Ca$^{2+}$. Moreover, metal variation significantly affects the apparent affinity for the substrate, E4P, but not for the second substrate, PEP or for feedback inhibition, L-Phe [23].

The first reported 3D structure of DAHP synthase is the crystal structure of AroG complexed with PEP and Pb$^{2+}$ [24]. The tetramer consists of two tight dimers. The monomers of the tight dimer are coupled by interactions including a pair of three stranded intersubunit β-sheets. The monomer is a ($\beta/\alpha$)$_8$ barrel with several additional β strands and α helices. The PEP and Pb$^{2+}$ are at the C-ends of the β strands of the barrel. Mutations that reduce feedback inhibition cluster 15 Å from the active site, indicating the location of a separate regulatory site (Figure 4).

In 2002, crystal structure of AroG in complex with it inhibitor, L-Phe, PEP and metal ion cofactor, Mn$^{2+}$ was determined to 2.8 Å resolution [25]. L-Phe binds in a cavity formed residues of two adjacent subunits and is located about 20 Å from the closet active site. The mechanism of allosteric mechanism was derived from conformational difference between Phe-bond and Phe-free structures. The inhibitory signal is transmitted from Phe-binding site to the active site of AroG by two interrelated paths. The first path is transmission within a single subunit of clone segments of the protein. The second involves alternative in the contact between subunits. On binding of Phe, AroG loses binding ability to E4P and binds PEP in a flipped orientation.
Figure 4. The inferred binding site of Phe in AroG. The proposed binding site of Phe in AroG is delineated by nine mutations—Pro19(α0), Asp146(β3–α3), Met147(β3–α3), Ile148(β3–α3), Pro150(α3), Gln151(α3), Ala154(α3), Gly178(α4–β5), and Ser180(α4–β5) (indicated in green, that eliminate or strongly reduce feedback inhibition. Elements that belong to subunit B are represented in darker colors and labeled with*. Residues forming the inferred pocket for binding Phe are labeled in magenta. (a) Ribbon diagram of the Phe-binding site of subunit A. (b) Stereoview of the Phe-binding site [24].
Hu and coworker (2005) investigated the feedback inhibition site of AroG using 3D structure of AroG co-crystallized with PEP. Phe141, Leu175, Leu179, Phe209, Trp215 and Val221 was replaced by using site-directed mutagenesis. The DAHP synthase activity in the presence of L-Phe from 0 mM to 3 mM showed that L175D was mostly resistant to feedback inhibition. The specific enzymatic activity of L175D at 0 mM of L-Phe increased significantly about 4.46 U/mg when compared with that of wild-type AroG and the relative enzymatic activity remained at 1 mM of L-Phe is about 83.5% [26].

Kikuchi and colleagues also studied the Phe-binding site of AroG. They performed hydroxamine mutagenesis on aroG. The mutant A220T, D146N and M147I were partially resistant to Phe inhibition [22]. From this result, Ding and coworker integrated different combinations of two mutation sites into the aroG, generating three double-site mutants. The E. coli clone possessing AroG\textsuperscript{A202T/D146N} showed the highest enzymatic activity and greatest resistant to feedback inhibition. The relative enzymatic activity of AroG\textsuperscript{A202T/D146N} remained at 20 mM of L-Phe was 96.66% [27].

1.6 Chorismate mutase and prephenate dehydratase (CM-PDT)

The chorismate mutase (CM) and prephenate dehydratase (PDT) (EC 5.4.99.5/4.2.1.51) is one of the allosteric enzymes in the aromatic amino acid biosynthesis pathway. CM-PDT which is encoded by pheA catalyzes the second committed step of L-Phe biosynthesis. It can be feedback inhibited by L-Phe. CM-PDT contains 386 amino acids with a molecular mass of 43 kDa. It is a bifunctional enzyme which contains two catalytic domains chorismate mutase domain (residues 1-109) and prephenate dehydratase domain (residues 101-285) and one regulatory-domain (residues 286-386) for L-Phe inhibitor binding site [28-30]. The feedback inhibition regulation of CM-PDT is mediated through allosteric binding of L-Phe which contributes a shift in the aggregation state of the enzyme from an active dimer to
less active tetrameric and octameric species [29]. Inhibition of the prephenate dehydratase activity at 1mM of L-Phe showed that almost total activity was inhibited (85\% inhibition). In contrast, chorismate mutase activity was inhibited only 55\% [31]. Overexpression of the CM-PDT domain of PheA could improve the metabolic influx to overproduce L-Phe and improve the survival ability under m-fluoro-DL-phenylalanine (an analog of Phe) stress [32].

To investigate L-Phe binding site, Nelms and coworker (1992) constructed four mutants which located within codons 304 to 310 of the pheA and measured the enzyme activity at various the L-Phe concentrations. They suggested that the recombinant E. coli harboring pheA\textsuperscript{W339P} displayed almost complete resistance to feedback inhibition of prephenate dehydratase by L-Phe concentrations up to 200 mM [29].

1.7 Glycerol as a carbon source

Biofuels, such as ethanol and biodiesel, are among the most promising source for the substitution of fossil. About 10\% (w/w) glycerol as a main by-product is generated in biodiesel production. The excess glycerol may become an environment problem. The market price of crude glycerol is low with the price of US$ 0.13-0.24 per kilogram [33]. Therefore, glycerol has been considered as a feedstock for new industrial fermentations [34]. Compared to the conventionally fermentation used glucose and sucrose, glycerol is efficient low-cost carbon source. The initial step of glycerol utilization in E. coli is the uptake of glycerol molecule into the cytoplasm via protein-mediated glycerol facilitator (GlpF) encoded by glpF. Glycerol is trapped by an ATP-dependent glycerol kinase (GlpK) to yield glycerol- 3-phosphate (G3P) which is then oxidized by a membrane-bound ubiquinone-8(UQ8)-dependent G3P dehydrogenase (GlpD) to dihydroxyacetone phosphate (DHAP) that enters glycolysis [35-37].
1.8 Our previous works

Thongchuang (2011) cloned glpF, tktA, aroG, aroB, aroL, yddG from E. coli and phenylalanine dehydrogenase gene (phedh) from Bacillus lentus. Each gene was regulated under T7 promoter in pRSFduet-1. The clone harboring pPTFBLY which contained phedh, tktA, glpF, aroB, aroL and yddG produced high level of L-Phe about 429 mg/L when it was cultured in minimum medium containing 3% glycerol for 240 h. The disadvantage of this clone were slowly growth rate and the colonies changed into flat shape when it was grown on the agar plate because of overexpression of membrane proteins under the regulation of T7 promoter [38]. To solve the problem, Ratchaneeladdajit (2014) applied the dual plasmid system. phedh, tktA, aroB, and aroL under T7 promoter was cloned into pRSFduet-1 (pBLPT) while glpF and yddG were expressed under ara promoter of pBad-33 (pYF). The ara promoter is tight regulated promoter and it uses arabinose as an inducer. After that, these two plasmids were co-transformed into E. coli BL21(DE3). The high level of L-Phe production at 746 mg/L was found when the medium was optimized (3.1% glycerol) [39].

Naksusuk (2015) improved the production of L-Phe by overexpression of phenylalanine feedback resistant PheA (PheA\textsuperscript{fbr}). Five Leu/Met residues in L-Phe binding pocket were selected for site-directed mutagenesis. The pheA\textsuperscript{fbr} was co-expressed with phedh, tktA, aroB, aroL, glpF and yddG in pRSFDuet-1. Among these mutated clones, pPTFBLYA\textsubscript{L359D} produced the highest concentration of L-Phe at 135 mg/L which was 3.8 fold of that of wildtype PheA clone (pPTFBLYA\textsubscript{wt}) [40] when the clones were cultured in glycerol medium formulated for pBLPT & pYF clone. All genes that were overexpressed in E. coli BL21(DE3) for L-Phe production are shown in Figure 5.

Kanoksinwuttipong analysed the amino acid residues that interact with L-Phe in the regulatory site of AroG using the crystal structure of AroG complex with Mn\textsuperscript{2+}, PEP and Phe (code 1KFL in protein Data Bank). The structure was displayed by UCSF.
chimera program (Figure 6). Q151 was selected for substitution by Leu, Ala and Asn. The these mutated genes were cloned into pRSFDuet-1 and transformed into *E. coli* BL21(DE3) on phenylalanine feedback inhibition L175D was used as a control [41].

Therefore, the objectives of this research are

1. To determine the expression of *PheA* and *AroG*.
2. To co-express *phe*\(^A_{359D}\) and *aro*\(^{fr}\) with *phedh*, *tktA*, *aroB*, *aroL*, *glpF* and *yddG* using dual plasmid system of pRSFDuet-1 and pBAD33 in order to produce high quantity of L-Phe.
Figure 5. All important genes in this study. Abbreviations used: F6P, fructose 6-phosphate; G3P, glycerol 3-phosphate; GA3P, glyceraldehyde 3-phosphate; glpF, glycerol facilitator; phedh, phenylalanine dehydrogenase, yddG, aromatic amino acid exporter

Figure 6. The 3D structure of AroG co-crystallized with inhibitor L-Phe [41].
CHAPTER II
MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments
Autoclave: MLS-3020, SANYO Electric Co, Ltd., Japan

Autopipette: Pipetman, Gilson, France

Analytical Balance: AB135-S/FACT, Mettler Toledo, Germany

Balance: GB1501-S, Mettler Toledo, Germany

Benchtop centrifuge: SorvallBiofuge Primo, Kendro Laboratory Products L.P., USA

Centrifuge, refrigerated centrifuge: Sorvall Legend XTR, Thermo Scientific, USA

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

Dry bath incubator: MD-01N, Major Science, USA

Electrophoresis unit: Minis-150, Major Science, USA

Electroporator: MicroPulser™ electrophoretor, Bio-Rad Laboratories Inc., USA

Electroporation cuvette: Gene Pulser®/E. coli Pulser® Cuvettes, Bio-Rad, USA

Gel documentation instrument: BioDoc-It™ Imaging system, UVP, USA

High Performance Liquid Chromatography (HPLC): UFLC, Shimadzu, Japan

HPLC column: Chirex® Chiral 3126 (D)-penicillamine, Phenomenex, USA

Incubator Shaker: Model E24R, New Brunswick Scientific, USA

Incubator Shaker: Innova™4000, New Brunswick Scientific, USA

Incubator oven: Series04067, Contherm Scientific., Ltd., New Zewland

Laminar flow: HT123, ISSCO, USA
Magnetic stirrer: Model Cerastir CH-1 series, Nickel-electro., Ltd., UK

Membrane filter: 0.45 μm Nylon Membrane Disc, Gs-Tek, USA

Microcentrifuge tube: 1.5 mL, Nest biotechnology, China

Microwave oven: GX-2021M, Galaxy, Korea

Mini personal centrifuge: Model microONE, Tomy Digital Biology Co., Ltd., Japan

PCR tube: thin-well dome-cap PCR tube, MCT-150, Axygen Inc., USA

pH meter: Model S200, Mettler Toledo Co., Ltd., Switzerland

Pipette: Labwarehouse, New Zealand

Pipette tip: Axygen Inc., USA

Sonicator: Vibra cellTm, SONICS & MATERIALS, Inc., USA

Spectrophotometer: BioSpectrometer® kinetic, Eppendorf, Germany

Syringe: 3 mL, 5 mL, 10mL, 20 mL latex free disposable syringe, Nipro Co., Ltd., Thailand

Syringe membrane filter: 0.2 μm Supor® Membrane Acrodisc®, PALL, USA

Thermal Cycler: T100™, Bio-Rad, USA

UV Transilluminator: MacroVueTM UV-25, Hoefer Inc., USA

Vacuum pump: Model number. WP6111560, Millipore Inc., USA

Vortex mixer: TopMix FP15024, Fisher Scientific, USA
2.1.2 Chemicals

Acrylamide: Sigma, USA

Agar: Himedia Laboratories Pvt. Ltd., India

Agarose: SERVA Electrophoresis GmbH, Germany

Agarose: ISC BioExpress, USA

Aluminium sulfate: Univar, USA

Ammonium sulphate: Carlo Erba Reagents, Italy

Boric acid: Merck, Germany

Bovine serum albumin: Sigma, USA

Bromophenol blue: Merck, Germany

Calcium chloride: Scharlau Chemie S.A., Spain

Cobalt sulphate: Sigma, USA

Copper sulfate: Carlo Erba, Italy

Coomassie brilliant blue R-250: Sigma, USA

D-Erythrose 4-phosphate sodium salt: Sigma, USA

Di-sodium hydrogen arsenate: Sigma, USA

6X DNA Loading Dye: Thermo Fisher Scientific Inc., USA

dNTP: Biotechrabbit, Germany

Ethanol (Absolute): RCI Labscan Limited, Thailand

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

Glacial acetic acid: Carlo Erba Reagents, Italy
Glycerol: Analytical Univar Reagent, Ajax finechem, Australia

Glycine: BDH, England

Hydrochloric acid: Carlo Erba Reagents, Italy

Isopropylthio-β-D-galactosidase (IPTG): Serva, Germany

Iron (II) sulfate heptahydrate: Sigma, USA

β-mercaptoethanol: Acros Organics, USA

Manganese sulfate heptahydrate: Carlo Erba Reagents, Italy

Magnesium sulfate heptahydrate: Carlo Erba Reagents, Italy

Methanol (HPLC grade): RCI Labscan, Thailand

N,N’-methylene-bis-acrylamide: Sigma, USA

N,N,N’,N’-tetramethyl-1, 2-diaminoethane (TEMED): Carlo Erba Reagents, Italy

Nickel(II) sulfate hexahydrate: Carlo Erba Reagents, Italy

Pancreatic digest of casein: Criterion, USA

10X pfu buffer with MgSO₄: Biolabs, England

Phenol reagent: Sigma, USA

L-Phenylalanine: Sigma, USA

Phosphoenol pyruvate: Sigma, USA

Potassium di-hydrogen phosphate: Carlo Erba Reagents, Italy

di-Potassium hydrogen phosphate: Carlo Erba Reagents, Italy

RedSafe™: Intron Biotechnology, Hongkong

Sodium (meta) arsenite: Sigma, USA
Sodium chloride: Univar, New Zealand
Sodium citrate: Carlo Erba Reagents, Italy
Sodium hydroxy: Carlo Erba Reagents, Italy
Sodium molybdate dihydrate: Carlo Erba Reagents, Italy
Sodium periodate: Sigma, USA
Thiamine hydrochloride: Sigma, USA
2-Thiobarbituric acid: Sigma, USA
Trichloroacetic acid: Sigma, USA
Tris(hydroxymethyl)-aminomethane: Carlo Erba Reagents, Italy
Yeast Extract: Scharlau Chemie S.A., Spain
Zinc sulfate heptahydrate: Carlo Erba Reagents, Italy

2.1.3 Antibiotics
Chloramphenicol: Sigma, USA
Kanamycin: Sigma, USA

2.1.4 Markers
100 base pair DNA ladder: Fermentas Inc., USA
GeneRuler 1 kb DNA Ladder: #SM0311, ThermoFisher Scientific, Inc., USA
Lamda (\(\lambda\)) DNA /HindIII: #SM0102, BioLabs, Inc., USA
TriColor protein ladder: Biotechrabbit, Germany

2.1.5 Kits
GenepHlow™ Gel/PCR Kit: Geneaid Biotech Ltd, Taiwan
Presto™ Mini Plasmid Kit: Geneaid Biotech Ltd, Taiwan
2.1.6 Enzymes and restriction enzymes

Pfu DNA polymerase: Biotechrabbit, Germany

Phusion High-Fidelity DNA Polymerase: Thermo Scientific, USA

Restriction enzymes: New England BioLabs, Inc., USA

T4 DNA ligase: Biotechrabbit, Germany

Taq DNA polymerase: Apsalagen, Thailand

2.1.7 Oligonucleotide primers

The oligonucleotide primers were synthesized by Integrated DNA Technologies, Singapore. The oligonucleotide primers used in this study are described in Table 1.

2.1.8 Bacterial strains

E. coli Top10, genotype: F\(^{-}\) mcrA \(\Delta(mrr-hsdRMS-mcrBC)\Phi80lacZ\DeltaM15\)

\(\Delta lacX74\) recA1 araD139 \(\Delta(ara-leu)7697\) galU galK \(\lambda^-\) rpsL(Str\(^R\)) endA1 nupG, was used for cloning and plasmid preparation.

E. coli BL21(DE3), genotype: F\(^{-}\) ompT hsdS\(_B\) (r\(^{-}\), m\(_B\)\(^{-}\)) gal dcm (DE3), was used as an expression host for the overexpression of all genes.

2.1.9 Plasmids

pRSFDuet-1 was used for cloning and expression of aroB, aroL, tktA, phedh, aroG and pheA under T7 promoter (Appendix A).

pBAD33 was used for cloning and expression of glpF and yddG under arabinose pBAD promoter (Appendix B).

All plasmids used in this study are shown in Table 2.
Table 1. The oligonucleotide primers for PCR amplification and DNA sequencing used in this study.

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<td>5′-ATAAGAATGCCGGCGCCGATCCCGCGAAATTAA-3′</td>
<td>61.4</td>
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<tr>
<td>PheA_R</td>
<td>5′-TGATGTACAGGTTGGAATACAGGCA-3′</td>
<td>67.2</td>
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<td><strong>For DNA sequencing</strong></td>
<td></td>
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<tr>
<td>ACYCDuetUP1</td>
<td>5′-GGATCTCGACGCTCTCCCT-3′</td>
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<tr>
<td>DuetDown1</td>
<td>5′-GATTATGGCCGCGGTACAA-3′</td>
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<td>F_tktA_aroG_Int</td>
<td>5′-GCTATCGTGTTGATGACCAAACCTTTGCTGAAT-3′</td>
<td>63.9</td>
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<tr>
<td>T7 terminator</td>
<td>5′-GCTAGTTTATGCTAGGGCGG-3′</td>
<td>57.0</td>
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<tr>
<td>Seqduet_R</td>
<td>5′-CGCTTATGCTGCTGTTACCGG-3′</td>
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<td>pAroG</td>
<td>prRSFDuet-1 inserted with aroG&lt;sup&gt;wt&lt;/sup&gt; at NcoI and HindIII sites.</td>
<td>This study</td>
</tr>
<tr>
<td>pAroG&lt;sub&gt;fr&lt;/sub&gt;</td>
<td>prRSFDuet-1 inserted with aroG&lt;sup&gt;fr&lt;/sup&gt; (L175D, Q151L, Q151A and Q151N) at NcoI and HindIII sites.</td>
<td>This study</td>
</tr>
<tr>
<td>pBLPT</td>
<td>prRSFDuet-1 inserted with aroB, aroL, phedh and tkta.</td>
<td>Ratchaneeladdajit, 2014</td>
</tr>
<tr>
<td>pBLPT&lt;sub&gt;G&lt;/sub&gt;&lt;sup&gt;Q151N&lt;/sup&gt;</td>
<td>prRSFDuet-1 inserted with aroB, aroL, phedh, tkta, pheA&lt;sup&gt;L359D&lt;/sup&gt; and aroG&lt;sup&gt;Q151N&lt;/sup&gt;.</td>
<td>This study</td>
</tr>
<tr>
<td>pBLPT&lt;sub&gt;G&lt;/sub&gt;&lt;sup&gt;Q151L&lt;/sup&gt;</td>
<td>prRSFDuet-1 inserted with aroB, aroL, phedh, tkta and aroG&lt;sup&gt;Q151L&lt;/sup&gt;.</td>
<td>Ulfah, 2018</td>
</tr>
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<td>pBLPT&lt;sub&gt;G&lt;/sub&gt;&lt;sup&gt;Q151L&lt;/sup&gt;&lt;sub&gt;L359D&lt;/sub&gt;</td>
<td>prRSFDuet-1 inserted with aroB, aroL, phedh, tkta, aroG&lt;sup&gt;Q151L&lt;/sup&gt; and pheA&lt;sup&gt;L359D&lt;/sup&gt;.</td>
<td>This study</td>
</tr>
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<td>pDuet_AroG</td>
<td>prRSFDuet-1 inserted with aroG&lt;sup&gt;wt&lt;/sup&gt; at BamHI and HindIII sites.</td>
<td>Kanoksinwuttipong, 2015</td>
</tr>
<tr>
<td>pDuet_AroG&lt;sub&gt;fr&lt;/sub&gt;</td>
<td>prRSFDuet-1 inserted with aroG&lt;sup&gt;fr&lt;/sup&gt; (L175D, Q151L, Q151A and Q151N) at BamHI and HindIII sites.</td>
<td>Kanoksinwuttipong, 2015</td>
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<td>pDuet_pheA&lt;sub&gt;wt&lt;/sub&gt;</td>
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<td>This study</td>
</tr>
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<td>pDuet_pheA&lt;sub&gt;L359D&lt;/sub&gt;</td>
<td>prRSFDuet-1 inserted with pheA&lt;sup&gt;L359D&lt;/sup&gt; at HindIII and AflII sites.</td>
<td>This study</td>
</tr>
<tr>
<td>pPheA&lt;sub&gt;wt&lt;/sub&gt;</td>
<td>prRSFDuet-1 inserted with pheA&lt;sup&gt;wt&lt;/sup&gt; at NotI and BsrGI sites.</td>
<td>This study</td>
</tr>
<tr>
<td>pPheA&lt;sub&gt;L359D&lt;/sub&gt;</td>
<td>prRSFDuet-1 inserted with pheA&lt;sup&gt;L359D&lt;/sup&gt; at NotI and BsrGI sites.</td>
<td>This study</td>
</tr>
<tr>
<td>pYF</td>
<td>pBAD33 inserted with yddG and glpF.</td>
<td>Ratchaneeladdajit, 2014</td>
</tr>
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2.2 Methods

2.2.1 Construction of pPheA\textsuperscript{wt} and pPheA\textsubscript{L359D}

To clone pheA from pDuet_pheA into pBLPTG\textsuperscript{Q151L}, the restriction sites at 5’ and 3’ ends of pheA have to change from HindIII and AflII to NotI and BsrGI because there are no HindIII and AflII sites on pBLPTG\textsuperscript{Q151L}.

2.2.1.1 Plasmid preparation

The single colony of each E. coli BL21(DE3) clones harboring pDuet_pheA\textsuperscript{wt} and pDuet_pheA\textsubscript{L359D} was cultured in 5 mL LB medium (Appendix C) containing 30 mg/mL of kanamycin and incubated at 37 °C, 250 rpm for 16-18 h. After that, the cell pellet was harvested by centrifugation at 5,000xg, 3 min. The plasmid was extracted using Presto™ Mini Plasmid Kit as described in Appendix E.

2.2.1.2 Agarose gel electrophoresis

pDuet_pheA\textsuperscript{wt} and pDuet_pheA\textsubscript{L359D} were analyzed by agarose gel electrophoresis. To prepare agarose gel, 0.8% (w/v) agarose in 1x TBE buffer containing 89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0 was melted in microwave oven. After the gel solution was cooled to 50 - 60 °C, RedSafe\textsuperscript{TM} was added to make 5% (w/v) of final concentration and then poured into the tray. The DNA samples were mixed with 6x DNA loading dye and then loaded into the well of gel. The electrophoresis was set at 100 volts for 40 min. The DNA bands were detected by UV light of gel documentation instrument. The intensity and size of DNA samples were compared with DNA marker (GeneRuler 1 kb DNA ladder and λ DNA /HindIII).

2.2.1.3 PCR amplification of pheA\textsuperscript{wt} and pheA\textsubscript{L359D}

pheA\textsuperscript{wt} and pheA\textsubscript{L359D} (1,161 bp) were amplified from pDuet_pheA\textsuperscript{wt} and pDuet_pheA\textsubscript{L359D}, respectively, using PheA_F2 forward primer containing NotI site and PheA_R reverse primer containing BsrGI site as listed in Table 2. To increase the level of gene expression, the PheA_F2 primer was designed from 5’ end of T7 promoter. The 50 µL of PCR reaction mixture contained 1x Pfu reaction
buffer with MgSO$_4$, 1x PCR enhancer, 200 µM of dNTP mix, 1 µM of forward and reverse primer, 1.25 U of PfU polymerase, 1 ng of DNA template and nuclease free water. The PCR condition consisted of initial activation at 95 ºC for 2 min, 32 cycles of denaturation at 95 ºC for 30 s, annealing at 62 ºC for 45s, extension at 72 ºC for 2 min and final extension at 72 ºC for 5 min. The PCR products were cleaned by using GenepHlow™ Gel/PCR Kit (Appendix F). After that, the PCR fragments were separated by agarose gel electrophoresis.

### 2.2.1.4 Cloning of \textit{pheA}^{wt} and \textit{pheA}^{L359D}

#### 2.2.1.4.1 Preparation of inserts

Each \textit{pheA}^{wt} and \textit{pheA}^{L359D} fragments from section 2.2.1.3 was digested with BsrGI. The 25 µL of digestion mixture contained 1x NEBuffer™ 2.1, 10 U of BsrGI and 200 ng of DNA fragment. The digestion mixture was incubated at 37 ºC for 16 h. After cleaning by GenepHlow™ Gel/PCR Kit, each DNA fragment was checked by agarose gel electrophoresis. Next step, BsrGI digested \textit{pheA}^{wt} and \textit{pheA}^{L359D} fragments were used as DNA templates for digestion with NotI in 1x CutSmart® Buffer. After incubation at 37 ºC for 16 h, purified DNA fragments were confirmed by agarose gel electrophoresis.

#### 2.2.1.4.2 Preparation of vector

\textit{pRSFDuet-1} was extracted by using the method described in section 2.2.1.1. The single digestions with BsrGI and NotI of \textit{pRSFDuet-1} were performed as described in section 2.2.1.4.1. After incubation, linear form of \textit{pRSFDuet-1} was confirmed by agarose gel electrophoresis.

#### 2.2.1.4.3 Ligation of inserts and vector

Each purified DNA fragment (section 2.2.1.4.1) was ligated into \textit{pRSFDuet-1} (2.2.1.4.2) with vector to insert ratio of 3:1. The 20 µL of ligation mixture contained 89 ng of DNA fragment, 100 ng of vector DNA, 1x T4 DNA ligase buffer and 30 U of T4 DNA ligase. The ligation reaction was incubated at 16 ºC for 16 h. After incubation, the ligation reaction was purified by GenepHlow™ Gel/PCR
Kit. The recombinant plasmids from ligation reaction were called pPheA<sup>wt</sup> and pPheA<sup>L359D</sup>.

### 2.2.1.5 Transformation of recombinant plasmid

pPheA<sup>wt</sup> and pPheA<sup>L359D</sup> obtained from section 2.2.1.4.3 were transformed into *E. coli* Top10 by electroporation. In electroporation step, each 5 µL (5 ng) of pPheA<sup>wt</sup> and pPheA<sup>L359D</sup> was mixed with 50 µL of competent *E. coli* Top10 cells (Appendix D) and chilled on ice. Each of reaction mixture was transferred to a cold electroporation cuvette. After that, the electroporation cuvette was placed in the chamber and applied for one pulse by electroporator. Five hundred µL of LB medium was added in cuvette to resuspend the transformant cell and transferred to 1.5 mL microcentrifuge tube. After that, the transformant was incubated at 37 °C, 250 rpm for 1 h. Two hundred µL of transformant was spread on LB agar plate that contained 30 mg/mL of kanamycin and then incubated at 37 °C for 16-18 h. The construction of pPheA<sup>wt</sup> and pPheA<sup>L359D</sup> are shown in Figure 7.

### 2.2.1.6 Confirmation of recombinant plasmid

The plasmids of transformants from section 2.2.1.5 were identified by double digestion with *Not*<sup>I</sup> and *Bsr*<sup>GI</sup>. Each single colony of pPheA<sup>wt</sup> and pPheA<sup>L359D</sup> clones was picked up to culture in 5 mL of LB broth containing 30 mg/mL of kanamycin and incubated at 37 °C with shaking at 250 rpm for 16-18 h. Then recombinant plasmids were extracted by Presto™ Mini Plasmid Kit. Each recombinant plasmid was digested with *Not*<sup>I</sup> and *Bsr*<sup>GI</sup> by the method described in section 2.2.1.4.1. The DNA fragments were identified by agarose gel electrophoresis. The recombinant plasmids with correct size were sent to perform DNA sequencing.
Figure 7. Construction of pPheA<sup>wt</sup> and pPheA<sup>L359D</sup>
2.2.1.7 Nucleotide sequencing

The DNA sequencing of recombinant plasmids were performed by Bioneer, Korean Korean using ACYCDuetUP1 as a forward primer and T7 terminator as a reverse primer. The obtained DNA sequences were compared with wild type pheA in NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and translated to protein sequence using Genetyx-Win program.

2.2.1.8 Expression of PheA<sub>wt</sub>

The single colony of pPheA<sub>wt</sub> clone was cultured in 5 mL LB medium containing 30 mg/mL of kanamycin and incubated at 37 °C, 250 rpm for 16-18 h. The cell culture was inoculated into 100 mL of the same medium and cultured in the same conditions. When OD<sub>600</sub> reached log phase (OD<sub>600</sub> = 0.6), the expression was induced with 1 mM IPTG for 1-6 h. 1.5 mL of whole cells were harvested at 10,000xg for 3 min and kept for SDS-PAGE analysis.

2.2.1.9 SDS-PAGE analysis

The expression of PheA was confirmed by SDS-PAGE analysis. The SDS-PAGE analysis was performed by the method of Bollag et al., 1996 [42] (Appendix G). The slab gel solution consisted of 12.5% separating gel and 5% stacking gel. Tris-glycine buffer (Appendix G) at pH 8.3 was used for running buffer. For protein loading preparation, the whole cells from section 2.2.1.8 were mixed with 5x sample buffer (Appendix G) and boiled for 15 min. After that, the cell pellets were eliminated by centrifugation at 10,000xg for 15 min. The supernatants were loaded into the gel. Tri-color protein color was used for protein molecular weight marker. The electrophoresis was set at 20 mA per slab gel for 40 min. After running the gel, the SDS gel was stained by the staining solution (Appendix H) with shaking for 30-45 min and then was destained by the destaining solution (Appendix H) with shaking for 30 min. After that, the destaining solution in SDS gel was changed and shaken for overnight. The molecular weight of each protein was analyzed by comparing the band with the protein marker.
2.2.2 Construction of pBLPTG\textsuperscript{Q151L}A\textsuperscript{wt} and pBLPTG\textsuperscript{Q151L}A\textsuperscript{L359D}

2.2.2.1 pBLPTG\textsuperscript{Q151L} preparation

The pBLPTG\textsuperscript{Q151L} containing \textit{aroB}, \textit{aroL}, \textit{phedh}, \textit{tktA} and \textit{aroG}\textsuperscript{Q151L} was extracted using the method in section 2.2.1.1. The single digestions with \textit{NotI} and \textit{BsrGI} of pBLPTG\textsuperscript{Q151L} were performed, respectively as described in section 2.2.1.4.1. After incubation, purified linear form of pBLPTG\textsuperscript{Q151L} was confirmed by agarose gel electrophoresis.

2.2.2.2 pheA\textsuperscript{wt} and pheA\textsuperscript{L359D} preparation

The pPheA\textsuperscript{wt} and pPheA\textsuperscript{L359D} from section 2.2.1.6 were digested with \textit{NotI} and \textit{BsrGI} as described in section 2.2.1.4.1. After incubation, the \textit{pheA}\textsuperscript{wt} and \textit{pheA}\textsuperscript{L359D} fragments were collected from agarose gel using GenepHlow\textsuperscript{TM} Gel/PCR Kit.

2.2.2.3 Cloning of \textit{pheA}\textsuperscript{wt} and \textit{pheA}\textsuperscript{L359D} into pBLPTG\textsuperscript{Q151L}

After digestion with \textit{NotI} and \textit{BsrGI}, each \textit{pheA}\textsuperscript{wt} and \textit{pheA}\textsuperscript{L359D} fragment was ligated with pBLPTG\textsuperscript{Q151L} using the method described in section 2.2.1.4.3. After that, the ligation reactions were transformed into \textit{E. coli} BL21(DE3) by electroporation method as described in section 2.2.1.5. The construction of pBLPTG\textsuperscript{Q151L}A\textsuperscript{wt} and pBLPTG\textsuperscript{Q151L}A\textsuperscript{L359D} are shown in Figure 8.

2.2.2.4 Confirmation of pBLPTG\textsuperscript{Q151L}A\textsuperscript{wt} and pBLPTG\textsuperscript{Q151L}A\textsuperscript{L359D}

The single colonies of pBLPTG\textsuperscript{Q151L}A\textsuperscript{wt} and pBLPTG\textsuperscript{Q151L}A\textsuperscript{L359D} from section 2.2.2.3 were picked up to culture in 5 mL of LB broth containing 30 mg/mL of kanamycin and incubated at 37 \textdegree C with shaking at 250 rpm for 16-18 h. Each of recombinant plasmid was extracted by Presto\textsuperscript{TM} Mini Plasmid Kit. The recombinant plasmids were digested with \textit{XhoI} by the method described in section 2.2.1.4.1. The recombinant plasmids with correct size were identified by agarose gel electrophoresis.
Figure 8. Construction of pBLPTG$^{Q151L}_{\text{A}^{\text{wt}}}$ and pBLPTG$^{Q151L}_{\text{A}^{\text{L359D}}}$
2.2.3 Expression of AroG

2.2.3.1 Thiobarbiturate assay

DAHP synthase activity was determined by the thiobarbiturate assay method modified from Schoner, 1976 [43] and Liu, 2008 [44]. Each single colony of pAroG\textsuperscript{wt} and pAroG\textsuperscript{fbr} (pAroG\textsuperscript{L175D}, pAroG\textsuperscript{Q151L}, pAroG\textsuperscript{Q151A} and pAroG\textsuperscript{Q151N}) was cultured in 5 mL LB medium containing 30 mg/mL of kanamycin and then incubated with shaking at 250 rpm, 37 °C for 16-18 h. 5% (v/v) of each culture was inoculated into 50 mL of the same medium and cultured in the conditions. For shake flask cultivation, the 5% (v/v) of starters were cultured into 200 mL of LB medium and incubated with shaking at 250 rpm, 37 °C. After the OD\textsubscript{600} reached 0.6 (log phase), the expression was induced with 1 mM IPTG for 2 h. The cells of each clone were collected by centrifugation at 8,000xg for 10 min and washed with resuspend buffer (0.1 mM KPB, pH 6.5, 200 µM PEP, 0.5 mM DTT, 0.1 mM PMSF and 10 mM EDTA) The cell pellet of each clone was dissolved in resuspend buffer and then broken by ultrasonic cell disruption. The crude extracts were centrifuged at 10,000xg for 20 min to collect the supernatants for dialysis. After dialysis, 1.5 mL of each crude extract was centrifuged at 10,000xg for 20 min to collect the supernatants for assay of enzyme activity.

In thiobarbiturate assay method, the reaction mixture contained 50 mM potassium phosphate, pH 6.5, 5 mM PEP, 2 mM E4P, 0-20 mM L-Phe, 30 µM MnCl\textsubscript{2}, crude enzyme and H\textsubscript{2}O in a total volume of 33.75 µL. The mixture was incubated at 30 °C for 10 min. The reaction was initiated when the enzyme was added and stopped by addition of 180 µL of 10% (w/v) trichloroacetic acid. After that, the 45 µL of mL of 25 mM NaIO\textsubscript{4} in 62.5 mM H\textsubscript{2}SO\textsubscript{4} was added in the mixture and incubated at 37 °C for 30 min. Then, the 45 µL of 2% (w/v) Na\textsubscript{2}SO\textsubscript{4} in 0.5 M HCl was rapidly mixed for stopped the reaction and 450 µL of 0.36% (w/v) thiobarbituric acid was added and mixed. The reaction mixture was boiled for 20 min
and then cooled in room temperature. The absorbance at a wavelength 549 nm was measured by spectrophotometer.

### 2.2.3.2 Protein measurement

The protein concentration of crude extracts were measured using Lowry’s method [45]. The 250 µL of crude extract was mixed with 50 µL of solution A and 2.5 mL of solution B and incubated at 30 °C for 10 min. After incubation, 250 µL of solution C was added and rapidly mixed and then incubated at room temperature for 20 min. The absorbance of protein was detected by spectrophotometer at a wavelength 610 nm. The protein concentration was calculated using the standard curve of BSA. All solutions were prepared as described in Appendix I. The expression of AroG<sup>wt</sup> and AroG<sup>fbr</sup> (L175D, Q151L, Q151A and Q151N) under T7 promoter were detected by SDS-PAGE analysis using the method described in section 2.2.1.9.

### 2.2.4 Reconstruction of pAroG and pAroG<sup>fbr</sup>

#### 2.2.4.1 Plasmid extraction

The single colonies of <i>E. coli</i> Top10 clones harboring pDuet_AroG<sup>wt</sup> and pDuet_AroG<sup>fbr</sup> from Kanoksinwutthipong were cultured in 5 mL LB medium containing 30 mg/mL of kanamycin and incubated at 37 °C, with shaking for 16-18 h. After that, the cell pellets were harvested by centrifugation at 5,000xg for 3 min. The plasmids were extracted using Presto™ Mini Plasmid Kit.

#### 2.2.4.2 Forward primer design

The forward primer containing Ncol site at 5’ end of aroG (5’ F_AroG_Ncol) was designed. The ATG of Ncol site was used as a start codon of aroG.

#### 2.2.4.3 PCR amplification of aroG<sup>wt</sup> and aroG<sup>fbr</sup>

To construct the pBLPTA<sup>L359D,G</sup>, restriction sites at 5’ and 3’ end of inserted aroG<sup>wt</sup> and aroG<sup>fbr</sup> from pDuet_AroG<sup>wt</sup> and pDuet_AroG<sup>fbr</sup> have to change to Ncol and HindIII, respectively. For PCR amplification of aroG<sup>wt</sup> and aroG<sup>fbr</sup> (1,053 bp), pDuet_AroG<sup>wt</sup> and pDuet_AroG<sup>fbr</sup> were used as DNA templates, respectively
Forward primer (F_AroG_NcoI) containing Ncol site and reverse primer (R_AroG_HindIII) containing HindIII site as listed in Table 2 were used. The 50 µL of PCR reaction mixture contained 1x Phusion HF buffer, 1 U Phusion DNA polymerase, 3% DMSO, 200 µM of dNTP mix, 1 µM of forward and reverse primers, 1 ng of DNA template and nuclease free water. The PCR condition consisted of initial activation at 98 ºC for 30 s, 32 cycles of denaturation at 98 ºC for 10 s, annealing at 62 ºC for 10 s, extension at 72 ºC for 1 min and final extension at 72 ºC for 10 min. The PCR products were cleaned by using GenepHlow™ Gel/PCR Kit. After that, the PCR fragments were separated by agarose gel electrophoresis.

2.2.4.4 Preparation of inserts and vector

The aroG<sup>wt</sup> and aroG<sup>flr</sup> fragments from section 2.2.1.3 and pRSFDuet-1 were digested with HindIII. The 25 µL of digestion mixture contained 1x NEBuffer™ 2.1, 10 U of HindIII and 200 ng of DNA template. The digestion mixture was incubated at 37 ºC for 16 h. After cleaning by GenepHlow™ Gel/PCR Kit, each DNA fragment was checked by agarose gel electrophoresis. Next step, aroG<sup>wt</sup>, aroG<sup>flr</sup> and pRSFDuet-1 fragments digested with HindIII were used as DNA template for digestion with Ncol using 1x CutSmart® Buffer. After digestion, DNA fragments were confirmed by agarose gel electrophoresis.

2.2.4.5 Ligation and transformation

After digestion with Ncol and HindIII, the aroG<sup>wt</sup> and aroG<sup>flr</sup> fragments were ligated with pRSFDuet-1 using the method described in section 2.2.1.4.3. After that, the ligation reactions were used for transformation into E. coli BL21(DE3) by electroporation method as described in section 2.2.1.5. The construction of pAroG<sup>wt</sup> and pAroG<sup>flr</sup> are shown in Figure 9.

2.2.4.6 Confirmation of recombinant plasmid

The plasmids of transformants from section 2.2.4.5 were identified by double digestion with Ncol and HindIII. Each single colony of pAroG<sup>wt</sup> and pAroG<sup>flr</sup> clones was picked up to culture in 5 mL of LB broth containing
Figure 9. Construction of pAroG<sup>wt</sup> and pAroG<sup>Δ</sup>
30 mg/mL of kanamycin and incubated at 37 ºC with shaking at 250 rpm for 16-18 h. Then recombinant plasmids were extracted by Presto™ Mini Plasmid Kit. Each of recombinant plasmid was digested with NcoI and HindIII by the method described in section 2.2.1.4.1. The DNA fragments were identified by agarose gel electrophoresis. The recombinant plasmids with the correct size was sent to perform DNA sequencing by Bioneer, Korean using ACYCDuetUP1 as a forward primer and DuetDown1 as a reverse primer as described in section 2.2.1.7.

2.2.5 Construction of pBLPTA<sup>L359D</sup><sub>G<sup>wt</sup></sub> and pBLPTA<sup>L359D</sup><sub>G<sup>Q151N</sup></sub>

2.2.5.1 pBLPTA<sup>L359D</sup> preparation
The recombinant pBLPTGA<sup>L359D</sup> containing aroB, aroL, pheD, tkTA, aroG and pheA from section 2.2.2.4 was double digested with PacI and AvrII. The 25 µL of digestion mixture contained 1x CutSmart® Buffer, 10 U of PacI and AvrII and 200 ng of DNA template. The digestion mixture was incubated at 37 ºC for 16 h. After incubation, linear fragment of pBLPTA<sup>L359D</sup> was separated by agarose gel electrophoresis. The linear form of pBLPTA<sup>L359D</sup> was purified by GenepHlow™ Gel/PCR Kit. The size of pBLPTA<sup>L359D</sup> was confirmed by agarose gel electrophoresis.

2.2.5.2 T7_<sub>aroG</sub><sup>wt</sup> and T7_<sub>aroG</sub><sup>Q151N</sup> preparation

2.2.5.2.1 PCR amplification of T7_<sub>aroG</sub><sup>wt</sup> and T7_<sub>aroG</sub><sup>Q151N</sup>
To construct pBLPTA<sup>L359D</sup><sub>G<sup>wt</sup></sub>, T7_<sub>aroG</sub> fragments were amplified using forward primer containing PacI and reverse primer containing AvrII site at their 5’ end. For PCR amplifications, aroG<sup>wt</sup> and aroG<sup>Q151N</sup> (1,053 bp) were amplified from pAroG<sup>wt</sup> and pAroG<sup>Q151N</sup>, respectively, using forward primer (aroG_Paci_F) and reverse primer (aroG_AvrII_R). To increase the level of gene expression, the aroG_Paci_F primer was designed from 5’ end of T7 promoter. The 50 µL of PCR reaction mixture contained 1x Pfu reaction buffer with MgSO<sub>4</sub>, 1x PCR enhancer, 200 µM of dNTP mix, 1 µM of forward and reverse primers, 1.25 U of Pfu polymerase, 1 ng of DNA template and nuclease free water. The PCR condition consisted of initial activation at 95 ºC for 2 min, 32 cycles of denaturation at 95 ºC
for 30 s, annealing at 62 °C for 45s, extension at 72 °C for 2 min and the last step of final extension at 72 °C for 5 min. The PCR products were cleaned by using GenepHlow™ Gel/PCR Kit. After that, the PCR fragments were separated by agarose gel electrophoresis.

2.2.5.2.2 Double digestion with restriction enzymes

Each aroG PCR fragment was double digested with Pactal and AvrII using the method described in section 2.2.5.1.

2.2.5.3 Ligation and transformation

After digestion with Pactal and AvrII, each aroG<sup>wt</sup> and aroG<sup>Q151N</sup> fragments was ligated with pBLPTA<sup>L359D</sup> vector using the method described in section 2.2.1.4.3. After that, each ligation reaction was transformed into E. coli BL21(DE3) by electroporation method as described in section 2.2.1.5. The construction of pBLPTA<sup>L359D-G<sup>wt</sup></sup> and pBLPTA<sup>L359D-G<sup>Q151N</sup></sup> are shown in Figure 10.

2.2.5.4 Confirmation of pBLPTA<sup>L359D-G<sup>wt</sup></sup> and pBLPTA<sup>L359D-G<sup>Q151N</sup></sup>

The transformants from section 2.2.5.3 were identified by digestion of their plasmid with XhoI. The single colonies of pBLPTA<sup>L359D-G<sup>wt</sup></sup> and pBLPTA<sup>L359D-G<sup>Q151N</sup></sup> were picked up to culture in 5 mL of LB broth containing 30 mg/mL of kanamycin and incubated at 37 °C with shaking at 250 rpm for 16-18 h. Then recombinant plasmids were extracted by Presto™ Mini Plasmid Kit. Each of the recombinant plasmid was digested with XhoI by the method in section 2.2.5.1. The DNA fragments were identified by agarose gel electrophoresis. The recombinant plasmids with correct size were sent to sequence by Bioneer, Korean using F_tktA_aroG_Int as a forward primer and Seqduet_R as a reverse primer as described in section 2.2.1.7.
Figure 10. Construction of pBLPTA\textsuperscript{L359D;wt} and pBLPTA\textsuperscript{L359D;Q151N}
2.2.6 Co-transformation of pBLPTA\textsuperscript{L359D,G\textit{wt}} and pBLPTA\textsuperscript{L359D,GQ151N} with pYF into \textit{E. coli} BL21(DE3)

The pBLPTA\textsuperscript{L359D,G\textit{wt}} and pBLPTA\textsuperscript{L359D,GQ151N} were co-transformed with pYF into \textit{E. coli} BL21(DE3) competent cell using method described in section 2.2.1.5. The transformants were spreaded on LB agar plate containing 30 mg/mL of kanamycin and 10 mg/mL of chloramphenicol and then incubated at 37 °C for 16-18 h. The growing colonies of pBLPTA\textsuperscript{L359D,G\textit{wt}} & pYF and pBLPTA\textsuperscript{L359D,GQ151N} & pYF were picked up to culture in 5 mL LB broth that contained 30 mg/mL of kanamycin and 10 mg/mL of chloramphenicol and incubated at 37 °C with shaking at 250 for 16-18 h. The pBLPTA\textsuperscript{L359D,G\textit{wt}} & pYF and pBLPTA\textsuperscript{L359D,GQ151N} & pYF were extracted by Presto\textsuperscript{TM} Mini Plasmid Kit. The recombinant plasmids were confirmed by restriction enzyme digestion. Each of recombinant plasmid was digested with \textit{BamH}I using method in section 2.2.5.1. The size of pBLPTA\textsuperscript{L359D,G\textit{wt}} & pYF and pBLPTA\textsuperscript{L359D,GQ151N} & pYF were identified by agarose gel electrophoresis. The colonies containing pBLPTA\textsuperscript{L359D,G\textit{wt}} & pYF and pBLPTA\textsuperscript{L359D,GQ151N} & pYF were collected to determine of L-Phe production. The construction of pBLPTA\textsuperscript{L359D,G\textit{wt}} & pYF and pBLPTA\textsuperscript{L359D,GQ151N} & pYF are shown in Figure 11.

![Figure 11. Construction of pBLPTA\textsuperscript{L359D,G\textit{wt}} & pYF and pBLPTA\textsuperscript{L359D,GQ151N} & pYF](image-url)
2.2.7 Determination of L-Phe production by HPLC

Each single colony of pBLPT & pYF, pBLPTA$^{L359G}$-wt & pYF and pBLPTA$^{L359G}$-Q151N & pYF clones from section 2.2.6 was cultured in 5 mL LB medium containing 30 mg/mL of kanamycin and 10 mg/mL of chloramphenicol and then incubated at 37 ºC, 250 rpm for 16-18 h. 5% (v/v) of each culture was inoculated into 50 mL of the same medium and cultured of the same conditions. For shake flask cultivation, the 5% (v/v) of starters were separately cultured into 200 mL of minimum medium containing (g/L): 60 glycerol, 42.5 (NH$_4$)$_2$SO$_4$, 0.3 MgSO$_4$-7H$_2$O, 0.075 FeSO$_4$-7H$_2$O, 0.015 CaCl$_2$-2H$_2$O, 12 K$_2$HPO$_4$, 3 KH$_2$PO$_4$, 1 NaCl, 1 Na-citrate, 0.0075 thiamine-HCl and 1.5 mL of trace elements solution contained (g/L): 2.0 Al$_2$(SO$_4$)$_3$-18H$_2$O, 3.0 Na$_2$MoO$_4$-2H$_2$O, 0.75 CoSO$_4$-7H$_2$O, 15 ZnSO$_4$-7H$_2$O, 2.5 CuSO$_4$-5H$_2$O, 0.5 H$_3$BO$_3$, 24 MnSO$_4$-7H$_2$O and 2.5 NiSO$_4$.6H$_2$O at pH 7.0. The cultures were shaken at 37 ºC, 250 rpm. After the OD$_{600}$ reached 0.6 (log phase), the expression of yddG and glpF under ara promoter of pBAD33 were induced with 0.02% arabinose. The 1.5 mL of each sample were collected every 24 h for 8 days to measure cell density (OD$_{600}$) and L-Phe production. The supernatants of each sample were filtrated through 0.22 µm nylon syringe filter. The L-Phe production was measured by HPLC method using Chirex 3126 (D)-penicillamine column. The ratio of 75:25 of 2 mM copper sulfate and methanol was used as a mobile phase and the flow rate was 0.7 mL/min. The peak of L-Phe was detected at wavelength 254 nm. The concentrations of L-Phe were estimated from the standard curve of L-Phe.
CHAPTER III
RESULTS AND DISCUSSIONS

3.1 Construction of pPheA\textsuperscript{wt} and pPheA\textsuperscript{L359D}

3.1.1 Plasmid extraction

pDuet\_pheA\textsuperscript{wt} and pDuet\_pheA\textsuperscript{L359D} (5,078 bp) were extracted from \textit{E. coli} Top10. The agarose gel electrophoresis was shown in Figure 12. Then, the pDuet\_pheA\textsuperscript{wt} and pDuet\_pheA\textsuperscript{L359D} were confirmed by digested with \textit{Afl}II and \textit{Bam}HI. From the result of agarose gel electrophoresis in Figure 13, the DNA fragments were observed around 3.4 kb and 1.2 kb. Therefore, pDuet\_pheA\textsuperscript{wt} No.1 and pDuet\_pheA\textsuperscript{L359D} No.1 were used for pheA amplification.

3.1.2 Amplification of pheA\textsuperscript{wt} and pheA\textsuperscript{L359D}

The pheA\textsuperscript{wt} and pheA\textsuperscript{L359D} were amplified from pDuet\_pheA\textsuperscript{wt} and pDuet\_pheA\textsuperscript{L359D}, respectively, using forward primer containing \textit{Not}I site and reverse primer containing \textit{Bsr}GI site. After cleaning by GenepHlow\textsuperscript{TM} Gel/PCR Kit, the PCR fragments were separated by agarose gel electrophoresis. The size of pheA\textsuperscript{wt} and pheA\textsuperscript{L359D} fragments were detected around 1.2 kb as shown in Figure 14.

3.1.3 Cloning of pheA\textsuperscript{wt} and pheA\textsuperscript{L359D}

The PCR products of pheA\textsuperscript{wt} and pheA\textsuperscript{L359D} from section 3.1.2 and pRSFDuet-1 vector were digested with \textit{Not}I and \textit{Bsr}GI. The results of pheA\textsuperscript{wt} and pheA\textsuperscript{L359D} fragments (1.2 kb) and pRSFDuet-1 linear vector (3.9 kb) after digestion are shown in Figure 15. After that, pheA\textsuperscript{wt} and pheA\textsuperscript{L359D} fragments were ligated with pRSFDuet-1 linear vector and then transformed into \textit{E. coli} Top10 by electroporation. Four colonies of pPheA\textsuperscript{wt} and five colonies of pPheA\textsuperscript{L359D} transformants were randomly picked and cultured in 5 mL of LB broth containing 30 mg/mL of kanamycin. Each recombinant plasmid was extracted and then detected by agarose gel electrophoresis. From Figure 16, the plasmid from pPheA\textsuperscript{wt} transformant No.1, 3 and 4 and pPheA\textsuperscript{L359D} transformant No.1, 2 and 4 moved slower than pRSFDuet-1. Thus, the pPheA\textsuperscript{wt} No.1, 3 and 4 and pPheA\textsuperscript{L359D} No.1, 2 and 4 might harbor the inserted genes. These six plasmids were confirmed by digestion with \textit{Not}I and \textit{Bsr}GI.
From digestion pattern, each recombinant plasmid gave two bands of DNA fragments around 3.9 kb and 1.2 kb as shown in Figure 17. This result confirmed that $\text{pheA}^{\text{wt}}$ and $\text{pheA}^{L359D}$ were successfully inserted into pRSFDuet-1. After that, the nucleotide sequences of the inserts were checked by Bioneer Inc. (Korea).

### 3.1.4 Nucleotide sequencing

To verify the nucleotide sequence of $\text{pheA}^{\text{wt}}$ and $\text{pheA}^{L359D}$ genes, the DNA sequencing of recombinant plasmids were performed by Bioneer, Korean using ACYCDuetUP1 as a forward primer and T7 terminator as a reverse primer. The obtained DNA sequences were compared with wild type $\text{pheA}$ reported by Naksusuk in 2015 using nucleotide blast tools in NCBI and then translated to protein sequence using Genetyx-Win program.

From Figure 18 - 19, nucleotide sequences of $\text{pheA}^{\text{wt}}$ and $\text{pheA}^{L359D}$ are similar to that of reference nucleotides [40]. Only nucleotide sequence at restriction sites were changed from $\text{AflII}$ to $\text{NotI}$ and $\text{BamHI}$ to $\text{BsrGI}$. The correct $\text{pheA}^{\text{wt}}$ and $\text{pheA}^{L359D}$ were used in the further experiment.

### 3.1.5 Expression of PheA$^{\text{wt}}$

The expression of PheA$^{\text{wt}}$ under T7 promoter was evaluated by SDS-PAGE analysis. The $E. \text{coli BL21(DE3)}$ harboring pPheA$^{\text{wt}}$ from section 3.1.4 was cultured in LB medium containing 30 mg/mL of kanamycin. After cell culture reached log phase, IPTG was added to 1 mM to induce $\text{pheA}$ expression. The whole cells of $E. \text{coli BL21(DE3)}$ harboring pPheA$^{\text{wt}}$ were mixed with 5x sample buffer. The supernatants were loaded into the gel. Tri-color protein color was used as protein molecular weight marker. The protein of $E. \text{coli BL21(DE3)}$ and $E. \text{coli BL21(DE3)}$ harboring pRSFDuet-1 were used as a control in lane 1 and 2, respectively. The protein band of chorismite mutase/prephenate dehydratase was detected after 1-6 h induction for 1-6 h in lane 3-8, respectively. The size of recombinant protein was approximately 43 kDa as shown in Figure 20.
Figure 12. Electrophoretic patterns of pPheA<sup>wt</sup> and pPheA<sup>L359D</sup>

Lane M1: λ DNA /HindIII marker
Lane 1: pRSFDuet-1
Lane 2-4: pPheA<sup>wt</sup> No.1-3, respectively
Lane 5-7: pPheA<sup>L359D</sup> No.1-3, respectively
Lane M2: Gene Ruler 1 kb DNA ladder
Figure 13. *Afl*II and *Hind*III digestion patterns of pPheA<sup>wt</sup> and pPheA<sup>L359D</sup>.

Lane M1: Gene Ruler 1 kb DNA ladder
Lane 1: uncut pRSFDuet-1
Lane 2: *Afl*II/*Hind*III digested pRSFDuet-1
Lane 3, 5, 7: uncut pPheA<sup>wt</sup> No.1-3, respectively
Lane 4, 6, 8: *Afl*II/*Hind*III digested pPheA<sup>wt</sup> No.1-3, respectively
Lane 9, 11, 13: uncut pPheA<sup>L359D</sup> No.1-3, respectively
Lane 10, 12, 14: *Afl*II/*Hind*III digested pPheA<sup>L359D</sup> No.1-3, respectively
Lane M2: λ DNA /*Hind*III marker
Figure 14. PCR products of $phea^{wt}$ and $phea^{L359D}$

Lane M : Gene Ruler 1 kb DNA ladder
Lane 1 : PCR product of $phea^{wt}$
Lane 2 : PCR product of $phea^{L359D}$
Figure 15. NotI and BsrGI digestion patterns of PCR products and pRSFDuet-1.

Lane M : Gene Ruler 1 kb DNA ladder
Lane 1 : uncut pRSFDuet-1
Lane 2 : NotI and BsrGI digested pRSFDuet-1
Lane 3 : uncut PCR product of pheA<sup>wt</sup>
Lane 4 : NotI and BsrGI digested PCR product of pheA<sup>wt</sup>
Lane 5 : NotI and BsrGI digested PCR product of pheA<sup>L359D</sup>
Figure 16. Electrophoretic pattern of plasmid from pPheA transformants.

Lane M : Gene Ruler 1 kb DNA ladder
Lane 1  : pRSFDuet-1
Lane 2-5 : pPheA\textsuperscript{wt} from transformant No.1-4, respectively
Lane 6-10 : pPheA\textsuperscript{L359D} from transformant No.1-5, respectively
Figure 17. NotI and BsrGI digestion patterns of pPheA\textsuperscript{wt} and pPheA\textsuperscript{L359D}.

Lane M: Gene Ruler 1 kb DNA ladder

Lane 1: uncut pRSFDuet-1

Lane 2: pRSFDuet-1 digested with NotI and BsrGI

Lane 3: uncut pPheA\textsuperscript{wt} transformant No.1

Lane 4: NotI and BsrGI digested pPheA\textsuperscript{wt} from transformant No.1

Lane 5: NotI and BsrGI digested pPheA\textsuperscript{wt} from transformant No.3

Lane 6: NotI and BsrGI digested pPheA\textsuperscript{wt} from transformant No.4

Lane 7: uncut pPheA\textsuperscript{L359D} transformant No.1

Lane 8: NotI and BsrGI digested pPheA\textsuperscript{L359D} from transformant No.1

Lane 9: NotI and BsrGI digested pPheA\textsuperscript{L359D} from transformant No.2

Lane 10: NotI and BsrGI digested pPheA\textsuperscript{L359D} from transformant No.4
Figure 18. Nucleotide sequence of pheA<sup>wt</sup>

Query represents the nucleotide sequence of pheA<sup>wt</sup> in this work.

Sbjct represents the nucleotide sequence of pheA<sup>wt</sup> reference [40].

The chromatogram of pheA<sup>wt</sup> is shown in Appendix N.
Figure 19. Nucleotide sequence of pheA<sup>L359D</sup>

Query represents the nucleotide sequence of pheA<sup>L359D</sup> in this work.
Sbjct represents the nucleotide sequence of pheA<sup>L359D</sup> reference [40].
Red box represents the mutation of Leu359Asp.
The chromatogram of pheA<sup>L359D</sup> is shown in Appendix O.
Figure 20. SDS-PAGE of whole cell extracts of *E. coli* BL21(DE3) harboring pheA<sup>wt</sup> after induction with 1 mM IPTG.

Lane M : TriColor Protein Ladder (10-180 kDa)
Lane 1 : *E. coli* BL21(DE3) after IPTG induction for 1 h
Lane 2 : *E. coli* BL21(DE3) harboring pRSFDuet-1 after IPTG induction for 1 h
Lane 3 : *E. coli* BL21(DE3) harboring pPheA<sup>wt</sup> after IPTG induction for 1 h
Lane 4 : *E. coli* BL21(DE3) harboring pPheA<sup>wt</sup> after IPTG induction for 2 h
Lane 5 : *E. coli* BL21(DE3) harboring pPheA<sup>wt</sup> after IPTG induction for 3 h
Lane 6 : *E. coli* BL21(DE3) harboring pPheA<sup>wt</sup> after IPTG induction for 4 h
Lane 7 : *E. coli* BL21(DE3) harboring pPheA<sup>wt</sup> after IPTG induction for 5 h
Lane 8 : *E. coli* BL21(DE3) harboring pPheA<sup>wt</sup> after IPTG induction for 6 h
3.2 Construction of pBLPTG\textsubscript{Q151L}A\textsuperscript{wt} and pBLPTG\textsubscript{Q151L}A\textsuperscript{L359D}

The pPheA\textsuperscript{wt} and pPheA\textsuperscript{L359D} from section 3.1.4. were digested with \textit{BsrGI} and \textit{NotI}. After that, the \textit{pheA}\textsuperscript{wt} and \textit{pheA}\textsuperscript{L359D} fragments were purified from agarose gel using GenepHlow\textsuperscript{TM} Gel/PCR Kit. The pBLPTG\textsubscript{Q151L} was digested with the same restriction enzymes and purified using same method. From Figure 21, the linear form of pBLPTG\textsubscript{Q151L} was shown at size around 9.9 kb in lane 3. For \textit{pheA}\textsuperscript{wt} and \textit{pheA}\textsuperscript{L359D} fragments, the DNA bands were obtained at size around 1.2 kb as shown in Figure 22 in lane 3 and 6, respectively. Then, \textit{pheA}\textsuperscript{wt} and \textit{pheA}\textsuperscript{L359D} fragments were ligated with linear pBLPTG\textsubscript{Q151L} vector and transformed into \textit{E. coli} BL21(DE3). The single colonies of transformants were randomly picked up and cultured in LB broth containing 30 mg/mL of kanamycin. After extraction, the recombinant plasmids were digested with X\textit{hoI} to confirm the positive plasmids of pBLPTG\textsubscript{Q151L}A\textsuperscript{wt} and pBLPTG\textsubscript{Q151L}A\textsuperscript{L359D} as shown in Figure 23. The digestion of pBLPTG\textsubscript{Q151L}A\textsuperscript{wt} and pBLPTG\textsubscript{Q151L}A\textsuperscript{L359D} with X\textit{hoI} gave two DNA bands around 9.9 kb and 1.2 kb as shown in lane 2, 4, 6, 8 and 10. This result showed that the pBLPTG\textsubscript{Q151L}A\textsuperscript{wt} and pBLPTG\textsubscript{Q151L}A\textsuperscript{L359D} were successfully constructed.

3.3 Expression of AroG\textsuperscript{wt}

The expression of AroG\textsuperscript{wt} under T7 promoter was evaluated by SDS-PAGE analysis. The \textit{E. coli} BL21(DE3) harboring pDuet_AroG\textsuperscript{wt} from Kanoksinwutthipong was cultured in LB medium containing 30 mg/mL of kanamycin. After cell culture reached log phase, IPTG was added to 1 mM to induce \textit{aroG} expression. The whole cells of \textit{E. coli} BL21(DE3) harboring pDuet_AroG\textsuperscript{wt} were mixed with 5x sample buffer. The supernatants were loaded into the gel. Tri-color protein color was used as protein molecular weight marker. The whole cell extract of \textit{E. coli} BL21(DE3) harboring pRSFDuet-1 was used as a control in lane 1. The protein bands of \textit{E. coli} BL21(DE3) harboring pDuet_AroG\textsuperscript{wt} after 1 mM IPTG induction for 1-6 h are shown in lane 3-8, respectively (Figure 24).
Figure 21. *Not*I and *Bsr*GI digestion pattern of pBLPTG<sub>Q151L</sub>.

Lane M: Gene Ruler 1 kb DNA ladder
Lane 1: uncut pBLPTG<sub>Q151L</sub>
Lane 2: *Not*I digested pBLPTG<sub>Q151L</sub>
Lane 3: *Not*I and *Bsr*GI digested pBLPTG<sub>Q151L</sub>
Figure 22. *Not*I and *Bsr*GI digestion patterns of pPheA<sup>wt</sup> and pPheA<sup>L359D</sup>.

Lane M: Gene Ruler 1 kb DNA ladder
Lane 1: uncut pPheA<sup>wt</sup>
Lane 2: *Not*I digested pPheA<sup>wt</sup>
Lane 3: *Not*I and *Bsr*GI digested pPheA<sup>wt</sup>
Lane 4: uncut pPheA<sup>L359D</sup>
Lane 5: *Not*I digested pPheA<sup>L359D</sup>
Lane 6: *Not*I and *Bsr*GI digested pPheA<sup>L359D</sup>
Figure 23. XhoI digestion patterns of pBLPTG$^{Q151L_{A^{wt}}}$ and pBLPTG$^{Q151L_{A^{L359D}}}$.

Lane M : Gene Ruler 1 kb DNA ladder
Lane 1 : uncut pBLPTG$^{Q151L_{A^{wt}}}$ from transformant No.1
Lane 2 : XhoI digested pBLPTG$^{Q151L_{A^{wt}}}$ from transformant No.1
Lane 3 : uncut pBLPTG$^{Q151L_{A^{wt}}}$ from transformant No.2
Lane 4 : XhoI digested pBLPTG$^{Q151L_{A^{wt}}}$ from transformant No.2
Lane 5 : uncut pBLPTG$^{Q151L_{A^{L359D}}}$ from transformant No.1
Lane 6 : XhoI digested pBLPTG$^{Q151L_{A^{L359D}}}$ from transformant No.1
Lane 7 : uncut pBLPTG$^{Q151L_{A^{L359D}}}$ from transformant No.2
Lane 8 : XhoI digested pBLPTG$^{Q151L_{A^{L359D}}}$ from transformant No.2
Lane 9 : uncut pBLPTG$^{Q151L_{A^{L359D}}}$ from transformant No.3
Lane 10 : XhoI digested pBLPTG$^{Q151L_{A^{L359D}}}$ from transformant No.3
Figure 24 SDS-PAGE of whole cell extracts of *E. coli* BL21(DE3) harboring *aroG<sup>wt</sup> after induction with 1 mM IPTG.

Lane M: TriColor Protein Ladder (10-180 kDa)
Lane 1: *E. coli* BL21(DE3) harboring pRSFDuet-1 after IPTG induction for 1 h
Lane 2: *E. coli* BL21(DE3) harboring pDuet_AroG<sup>wt</sup> after IPTG induction for 1 h
Lane 3: *E. coli* BL21(DE3) harboring pDuet_AroG<sup>wt</sup> after IPTG induction for 2 h
Lane 4: *E. coli* BL21(DE3) harboring pDuet_AroG<sup>wt</sup> after IPTG induction for 3 h
Lane 5: *E. coli* BL21(DE3) harboring pDuet_AroG<sup>wt</sup> after IPTG induction for 4 h
Lane 6: *E. coli* BL21(DE3) harboring pDuet_AroG<sup>wt</sup> after IPTG induction for 5 h
Lane 7: *E. coli* BL21(DE3) harboring pDuet_AroG<sup>wt</sup> after IPTG induction for 6 h
In our previous work, we paid a lot of attempt to determine the expression of AroG by SDS-PAGE analysis and DAHP synthase activity assay. The AroG band could not be observed on SDS-PAGE. Moreover, DAHP synthase activity of the recombinant clone was not differ from that of *E. coli* host cell. At first, we suspected that there were some defects on T7 promoter of pRSFDuet-1 vector used in our laboratory, so *aroG* was subcloned under T7 promoter of pET-28b and again the protein band and activity of AroG could not be detected.

In this research, the sequence of pDuet_AroG was rechecked. The *aroG* was cloned into pRSFDuet-1 between BamHI and HindIII sites. As shown in Figure 25, the sequence of forward primer was 5’-CGGGATCCATGAATTATCAGACGACGATTACGC-3’. BamHI site is shown in blue and start codon of *aroG* is shown in red. Translation of the gene inserted under T7 promoter-1 is started from Met of His-tag (shown in red box). When *aroG* was inserted, the translation frame of *aroG* was one base shifted and translation was stopped at TGA (shown in green box). Thus, AroG could not be synthesized.

### 3.4 Reconstruction of pAroG and pAroGfr

In this part, new forward primer was designed and used for cloning *aroG* into pRSFDuet-1.

#### 3.4.1 Plasmid extraction

pDuet_AroGwt and pDuet_AroGfr (5,039 bp) were extracted from *E. coli* Top10. The agarose gel electrophoresis is shown in Figure 26. Each recombinant plasmid was used as a template for *aroG* amplification.
Figure 25. Frameshift mutation of aroG

A. pRSFDuet-1 vector, red box shows start codon of the recombinant protein.

B. Sequence of inserted aroG, BamHI site – blue letter

\[ \text{start codon of aroG} - \text{red letter} \]

\[ \text{stop codon} - \text{green box} \]
Figure 26. Electrophoretic patterns of pDuet_AroG<sub>wt</sub> and pDuet_AroG<sup>Thr</sup>

Lane M1 : Gene Ruler 1 kb DNA ladder
Lane 1 : pRSFDuet-1
Lane 2 : pDuet_AroG<sub>wt</sub>
Lane 3 : pDuet_AroG<sub>L175D</sub>
Lane 4 : pDuet_AroG<sub>Q151L</sub>
Lane 5 : pDuet_AroG<sub>Q151A</sub>
Lane 6 : pDuet_AroG<sub>Q151N</sub>
Lane M2 : λ DNA / HindIII marker
3.4.2 Primer design at 5’end of aroG

The new forward primer containing Ncol at 5’end was designed. The sequence of new forward primer F_AroG_Ncol is

5’-CATGCCCATGGTGTATCAGAACGACGATTTACGCATCAAAGAAATC-3’

Blue letter indicates Ncol sites while blue underline shows start codon of aroG.

3.4.3 PCR amplification of aroG<sub>wt</sub> and aroG<sub>fbr</sub>

The aroG<sub>wt</sub> and aroG<sub>fbr</sub> were amplified from pDuet_AroG<sub>wt</sub> and pDuet_AroG<sub>fbr</sub> using forward primer containing Ncol site and reverse primer containing HindIII site. After cleaning by GenepHlow™ Gel/PCR Kit, the PCR fragments were separated by agarose gel electrophoresis. From the result shown in Figure 27, the size of aroG<sub>wt</sub> and aroG<sub>fbr</sub> fragments were detected around 1.1 kb.

3.4.4 Digestion of aroG<sub>wt</sub> and aroG<sub>fbr</sub> fragments and pRSFDuet-1

The aroG<sub>wt</sub> and aroG<sub>fbr</sub> fragments from section 3.4.3 and pRSFDuet-1 vector were digested with Ncol and HindIII. The linear pRSFDuet-1 (3.9 kb) and aroG<sub>wt</sub> and aroG<sub>fbr</sub> fragments (1.1 kb) after digestion are shown in Figure 28-29, respectively. After that, aroG<sub>wt</sub> and aroG<sub>fbr</sub> fragments were ligated to pRSFDuet-1 linear vector and then transformed into E. coli Top10 by electroporation. The pAroG<sub>wt</sub> and pAroG<sub>fbr</sub> transformants were randomly picked and cultured in 5 mL of LB broth containing 30 mg/mL of kanamycin. Each recombinant plasmid was extracted and then detected by agarose gel electrophoresis. The pAroG<sub>wt</sub> from transformant No.1, 3, 4, 5 and 6, pAroG<sub>L175D</sub> from transformant No.2 - 6 (Figure 30), pAroG<sub>Q151L</sub> from transformant No.3 and pAroG<sub>Q151A</sub> from transformant No.4 (Figure 31) as well as pAroG<sub>Q151N</sub> from transformant No.6 (Figure 32) moved slower than that of pRSFDuet-1.
**Figure 27.** PCR products of *aroG<sup>wt</sup>* and *aroG<sup>fr</sup>*

Lane M: Gene Ruler 1 kb DNA ladder
Lane 1: PCR product of *aroG<sup>wt</sup>*
Lane 2: PCR product of *aroG<sup>L175D</sup>*
Lane 3: PCR product of *aroG<sup>Q151L</sup>*
Lane 4: PCR product of *aroG<sup>Q151A</sup>*
Lane 5: PCR product of *aroG<sup>Q151N</sup>*
Figure 28. *NcoI* and *HindIII* digestion pattern of pRSFDuet-1.

Lane M : Gene Ruler 1 kb DNA ladder
Lane 1 : uncut pRSFDuet-1
Lane 2 : *NcoI* and *HindIII* digested pRSFDuet-1
Figure 29. *Ncol* and *HindIII* digestion pattern of PCR products.

Lane M : Gene Ruler 1 kb DNA ladder
Lane 1 : uncut PCR product of aroG<sup>wt</sup>
Lane 2 : *Ncol* and *HindIII* digested PCR product of aroG<sup>wt</sup>
Lane 3 : uncut PCR product of aroG<sup>L175D</sup>
Lane 4 : *Ncol* and *HindIII* digested PCR product of aroG<sup>L175D</sup>
Lane 5 : uncut PCR product of aroG<sup>Q151L</sup>
Lane 6 : *Ncol* and *HindIII* digested PCR product of aroG<sup>Q151L</sup>
Lane 7 : uncut PCR product of aroG<sup>Q151A</sup>
Lane 8 : *Ncol* and *HindIII* digested PCR product of aroG<sup>Q151A</sup>
Lane 9 : uncut PCR product of aroG<sup>Q151N</sup>
Lane 10 : *Ncol* and *HindIII* digested PCR product of aroG<sup>Q151N</sup>
**Figure 30.** Electrophoretic pattern of plasmid from pAro\textsuperscript{wt} and pAro\textsuperscript{L175D} transformants.

- **Lane M**: Gene Ruler 1 kb DNA ladder
- **Lane 1**: pRSFDuet-1
- **Lane 2-7**: pAro\textsuperscript{wt} from transformant No.1-6, respectively
- **Lane 8-13**: pAro\textsuperscript{L175D} from transformant No.1-6, respectively
Figure 31. Electrophoretic pattern of plasmid from pAroG^{Q151L} and pAroG^{Q151A} transformants.

Lane M : Gene Ruler 1 kb DNA ladder
Lane 1 : pRSFDuet-1
Lane 2-6 : pAroG^{Q151L} from transformant No.1-5, respectively
Lane 6-11 : pAroG^{Q151A} from transformant No.1-5, respectively
Figure 32. Electrophoretic pattern of plasmid from pAroG\textsuperscript{Q151N} transformants.

Lane M : Gene Ruler 1 kb DNA ladder
Lane 1 : pRSFDuet-1
Lane 2-7 : pAroG\textsuperscript{Q151N} from transformant No.1-6, respectively
Thus, the transformant No.1 from pAroG\textit{wt}, transformant No.2 from pAroG\textit{L17SD}, transformant No.3 from pAroG\textit{Q15IL}, transformant No.4 from pAroG\textit{Q15LA} and transformant No.6 from pAroG\textit{Q15IL} were confirmed by digested with \textit{NcoI} and \textit{HindIII}. From digestion pattern, each transformant gave two bands of DNA fragments around 3.9 kb and 1.1 kb as shown in Figure 33. This result confirmed that \textit{aroG} genes were inserted into pRSFDuet-1. After that, the nucleotide sequences of the inserts were checked by Bioneer Inc. (Korea).

### 3.4.5 Nucleotide sequence of \textit{aroG} genes

To verify the nucleotide sequences of \textit{aroG}\textit{wt} and \textit{aroG}\textit{fbr}, the DNA sequencing of recombinant plasmids were performed by Bioneer, Korean using \texttt{F_tktA_aroG_Int} as a forward primer and \texttt{Seqduet_R} as a reverse primer. The obtained DNA sequences were compared with wild type \textit{aroG} reported by Kanoksinwutthipong in 2014. Genetyx-Win program was used to translate protein sequence [41].

The all of nucleotide sequence of \textit{aroG}\textit{wt} and \textit{aroG}\textit{fbr} were changed only at the mutated sites (Figure 34 - 38). The \textit{aroG}\textit{wt} and \textit{aroG}\textit{fbr} were used in the further experiment.

### 3.4.6 Expression of \textit{AroG}\textit{wt} and \textit{AroG}\textit{fbr}

#### 3.4.6.1 Protein expression

The expression of \textit{aroG}\textit{wt} and \textit{aroG}\textit{fbr} under T7 promoter were evaluated by SDS-PAGE analysis. The crude enzyme of AroG\textit{wt} and AroG\textit{fbr} were mixed with 5x sample buffer. The supernatants were loaded into the gel. Tri-color protein color was used for protein molecular weight marker. Crude extracts of \textit{E. coli} BL21(DE3) and \textit{E. coli} BL21(DE3) harboring pRSFDuet-1 were used as controls in lane 1 and 2. The protein bands of AroG\textit{wt} and AroG\textit{fbr} were detected after 1 mM IPTG induction for 2 h in lane 3-7, respectively. The sizes of recombinant proteins were approximately 38 kDa as shown in Figure 39.
Figure 33. *NcoI* and *HindIII* digestion patterns of pAroG<sup>wt</sup> and pAroG<sup>for</sup>.

Lane M1: Gene Ruler 1 kb DNA ladder

Lane 1: uncut pRSFDuet-1

Lane 2: *NcoI* and *HindIII* digested pRSFDuet-1

Lane 3: uncut PCR product of aroG<sup>wt</sup>

Lane 4: *NcoI* and *HindIII* digested PCR product of aroG<sup>wt</sup>

Lane 5: uncut pAroG<sup>wt</sup> from transformant No.1

Lane 6: *NcoI* and *HindIII* digested pAroG<sup>wt</sup> from transformant No.1

Lane 7: uncut pAroG<sup>L175D</sup> from transformant No.2

Lane 8: *NcoI* and *HindIII* digested pAroG<sup>L175D</sup> from transformant No.2

Lane 9: uncut pAroG<sup>Q151L</sup> from transformant No.3

Lane 10: *NcoI* and *HindIII* digested pAroG<sup>Q151L</sup> from transformant No.3

Lane 11: uncut pAroG<sup>Q151A</sup> from transformant No.4

Lane 12: *NcoI* and *HindIII* digested pAroG<sup>Q151A</sup> from transformant No.4

Lane 13: uncut pAroG<sup>Q151N</sup> from transformant No.6

Lane 14: *NcoI* and *HindIII* digested pAroG<sup>Q151N</sup> from transformant No.6
Figure 34. Nucleotide sequence of \textit{aroG}_{wt}

Orange and blue underlines represent the restriction site of \textit{NcoI} and \textit{HindIII}, respectively.

The chromatogram of \textit{aroG}_{wt} is shown in Appendix P.
Figure 35. Nucleotide sequence of *aroG<sup>L175D</sup>*

Orange and blue underlines represent the restriction site of *NcoI* and *HindIII*, respectively.

Red box represents the mutation of Leu175Asp (CTG to GAT).

The chromatogram of *aroG<sup>L175D</sup>* is shown in Appendix Q.
Figure 36. Nucleotide sequence of \textit{aroG}^{Q151L}.

Orange and blue underlines represent the restriction site of \textit{NcoI} and \textit{HindIII}, respectively.

Red box represents the point mutation of Gln151Leu (CCA to CTG).

The chromatogram of \textit{aroG}^{Q151L} is shown in Appendix R.
Figure 37. Nucleotide sequence of aroG<sup>Q151A</sup>

Orange and blue underlines represent the restriction site of Ncol and HindIII, respectively.

Red box represents the point mutation of Gln151Ala (CCA to GCC).

The chromatogram of aroG<sup>Q151A</sup> is shown in Appendix S.
Figure 38. Nucleotide sequence of aroG<sub>Q151N</sub>

Orange and blue underlines represent the restriction site of Ncol and HindIII, respectively.

Red box represents the point mutation of Gln151Asn (CCA to AAT).

The chromatogram of aroG<sub>Q151N</sub> was shown in Appendix T.
Figure 39. SDS-PAGE of crude extract of aroG clones.

lane M : TriColor Protein Ladder (10-180 kDa)

Lane 1 : E. coli BL21(DE3)

Lane 2 : E. coli BL21(DE3) harboring pRSFDuet-1

Lane 3 : E. coli BL21(DE3) harboring pAroG$^{\text{wt}}$

Lane 4 : E. coli BL21(DE3) harboring pAroG$^{L175D}$

Lane 5 : E. coli BL21(DE3) harboring pAroG$^{Q151L}$

Lane 6 : E. coli BL21(DE3) harboring pAroG$^{Q151A}$

Lane 7 : E. coli BL21(DE3) harboring pAroG$^{Q151N}$
3.4.6.2 DAHP synthase activity

In previous studies, Hu and coworkers investigated the feedback inhibition site of AroG using the 3D structure of AroG co-crystallized with PEP. The amino acids were replaced at Phe144, Pro150, Leu175, Leu179, Phe209, Trp215 and Val221. DAHP synthase activity in crude extract of each clone was measured in the presence of L-Phe from 0 mM to 3 mM. The results showed that the mutant at position L175D was mostly resistant to feedback inhibition. L175D enzyme elevated specific enzyme activity at 0 mM phenylalanine from 2.70 U/mg of wild type to 4.46 U/mg and increased of relative enzymatic activity at 1 mM phenylalanine from 8.2% to 83.5% [26]. In 2014, Ding and coworkers constructed three single-site mutant and combined to generate three double-site aroGฟร mutant alleles. They analyzed enzymatic activity in all of mutants. The results showed that AroG8/15 had high level of feedback resistance to L-Phe at 20 mM of L-Phe. The relative enzymatic activity of AroG8/15 remained at 20 mM of L-Phe was 96.66% [27].

The amino acid residues that interact with phenylalanine at the regulatory site of AroG are displayed in Figure 40 using Discovery Studio 2020 program. Van der Waal interaction was found between Leu175 and phenylalanine. Among Gln151 as well as Asp6 and Asp7 of the companion tight subunit which form one H-bonding with phenylalanine, we interested in Gln151 since the pocket accommodative the aromatic ring of phenylalanine is formed by hydrophobic side-chains including that of Gln151. To investigate this amino acid residue, structure of AroG when Gln151 was substituted by Ala, Asn and Leu were simulated. All replaced amino acid cannot form H-bonding with phenylalanine (Figure 40B-40D). Differ from Ala and Asn, hydrophobic interaction between Leu151 and phenylalanine was detected.
Figure 40. The amino acid residues that interact with phenylalanine at the regulatory site of AroG.

A. AroG<sup>wt</sup>  B. AroG<sup>Q151A</sup>  C. AroG<sup>Q151N</sup>  D. AroG<sup>Q151L</sup>
DAHP synthase activity of crude extract from AroG clones were assayed for sensitivity to feedback inhibition by L-Phe at a concentration from 0 mM to 20 mM. The results are shown in Figure 41. In the absent L-Phe, all mutated AroG at Gln151 exhibited higher specific activities than AroG<sup>wt</sup> (1.88 U/mg). In contradiction to the result of Hu and co-worker [26], the control AroG<sup>L175D</sup> mutant showed lower specific activity (0.82 U/mg) than AroG<sup>wt</sup> (1.88 U/mg). The activity of all recombinant enzymes were decreased in the same pattern when L-Phe was added. Moreover, all AroG mutants at Gln151 showed greater resistance to feedback inhibition when compared with AroG<sup>wt</sup> and AroG<sup>L175D</sup>. AroG<sup>Q151N</sup> gave the greatest inhibition pattern at the concentration of L-Phe at 0 - 20 mM. % inhibitions by 20 mM phenylalanine were decreased from 51% of wild type to 12, 16 and 27% for Q151L, Q151N and Q151A, respectively (Table 3). Destruction of two H-bonding between Ser180 and phenylalanine by substitution with Phe (S180F) was reported to decline % inhibition by phenylalanine at concentration of 20 mM from 58% to 7.4% [46]. The result indicated that H-bonding between Gln151 of AroG and the inhibitor, phenylalanine, had a high impact on phenylalanine feedback inhibition. Then, the aroG<sup>Q151N</sup> gene was used for combination with other genes.
Figure 41. Phenylalanine inhibition pattern of DAHP synthase activities. The data were received from three independent experiments. 

(opened diamonds: E.coli BL21(DE3), opened triangles: E.coli BL21(DE3) harboring pRSFDuet-1, closed diamonds: E.coli BL21(DE3) harboring pAroG<sup>wt</sup>, crosses: E.coli BL21(DE3) harboring pAroG<sup>L175D</sup>, closed boxes: E.coli BL21(DE3) harboring pAroG<sup>Q151L</sup>, closed triangles: E.coli BL21(DE3) harboring pAroG<sup>Q151A</sup> and closed circles: E.coli BL21(DE3) harboring pAroG<sup>Q151N</sup>)
Table 3 DAHP synthase activities and feedback inhibition of various AroG clones.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Specific activity (U/mg)</th>
<th>%inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mM L-Phe</td>
<td>20 mM L-Phe</td>
</tr>
<tr>
<td>E. coli BL21(DE3)</td>
<td>0.31 ± 0.04</td>
<td>0.18 ± 0.05</td>
</tr>
<tr>
<td>pRSFDuet-1</td>
<td>0.40 ± 0.07</td>
<td>0.24 ± 0.04</td>
</tr>
<tr>
<td>AroG&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>1.89 ± 0.39</td>
<td>0.93 ± 0.45</td>
</tr>
<tr>
<td>AroG&lt;sup&gt;L175D&lt;/sup&gt;</td>
<td>0.82 ± 0.40</td>
<td>0.25 ± 0.09</td>
</tr>
<tr>
<td>AroG&lt;sup&gt;Q151L&lt;/sup&gt;</td>
<td>2.15 ± 0.11</td>
<td>1.90 ± 0.05</td>
</tr>
<tr>
<td>AroG&lt;sup&gt;Q151A&lt;/sup&gt;</td>
<td>2.11 ± 0.75</td>
<td>1.55 ± 0.41</td>
</tr>
<tr>
<td>AroG&lt;sup&gt;Q151N&lt;/sup&gt;</td>
<td>2.26 ± 0.06</td>
<td>1.90 ± 0.06</td>
</tr>
</tbody>
</table>
3.5 Construction of pBLPTA<sup>L359D</sup><sup>wt</sup> and pBLPTA<sup>L359D</sup><sup>Q151N</sup>

3.5.1 pBLPTA<sup>L359D</sup> preparation

The pBLPTG<sup>Q151L</sup><sup>L359D</sup> containing aroB, aroL, phedh, tktA, aroG and pheA was double digested with PacI and AvrII. After digestion, linear fragment of pBLPTA<sup>L359D</sup> was separated by agarose gel electrophoresis and purified by GenepHlow™ Gel/PCR Kit. From Figure 42, the linear form of pBLPTA<sup>L359D</sup> vector around 9.9 kb was detected (lane 3).

3.5.2 Amplification of T7_<sup>aroG</sup><sup>wt</sup> and T7_<sup>aroG</sup><sup>Q151N</sup>

T7_<sup>aroG</sup><sup>wt</sup> and T7_<sup>aroG</sup><sup>Q151N</sup> fragments were amplified from pAroG<sup>wt</sup> and pAroG<sup>Q151N</sup> using forward primer containing PacI site and reverse primer containing AvrII site. After cleaning, the PCR fragments were separated by agarose gel electrophoresis. The size of T7_<sup>aroG</sup><sup>wt</sup> and T7_<sup>aroG</sup><sup>Q151N</sup> fragments were detected around 1.2 kb as shown in Figure 43. Then, PCR fragments were double digested with PacI and AvrII. After digestion, T7_<sup>aroG</sup><sup>wt</sup> and T7_<sup>aroG</sup><sup>Q151N</sup> fragments were purified by GenepHlow™ Gel/PCR Kit. The size of T7_<sup>aroG</sup><sup>wt</sup> and T7_<sup>aroG</sup><sup>Q151N</sup> fragments were confirmed by agarose gel electrophoresis. From Figure 44, size of T7_<sup>aroG</sup><sup>wt</sup> and T7_<sup>aroG</sup><sup>Q151N</sup> fragments were around 1.2 kb in lane 2 and 4, respectively.

3.5.3 Cloning of pBLPTA<sup>L359D</sup><sup>wt</sup> and pBLPTA<sup>L359D</sup><sup>Q151N</sup>

The _aroG<sup>wt</sup>_ and _aroG<sup>Q151N</sup>_ fragments were ligated into pBLPTA<sup>L359D</sup> linear vector and then transformed into _E. coli_ BL21(DE3) by electroporation. The single colonies of pBLPTA<sup>L359D</sup><sup>wt</sup> and pBLPTA<sup>L359D</sup><sup>Q151N</sup> transformants were randomly picked and cultured in 5 mL of LB broth containing 30 mg/mL of kanamycin. Each recombinant plasmid was extracted, digested with _XhoI_ and then detected by agarose gel electrophoresis. From digestion pattern, each recombinant plasmid gave two DNA bands around 10.0 kb and 1.1 kb as shown in Figure 45. This result confirmed that _aroG_ genes were inserted into pBLPTA<sup>L359D</sup>. After that, the nucleotide sequences of the inserts were checked by Bioneer Inc. (Korea).
Figure 42. PacI and AvrII digestion pattern of pBLPTA<sup>L359D</sup>.

Lane M1 : Gene Ruler 1 kb DNA ladder
Lane 1 : pBLPTA<sup>L359D</sup> uncut
Lane 2 : PacI and AvrII digested pBLPTA<sup>L359D</sup>

Figure 43. PCR products of T7_aroG<sup>wt</sup> and T7_aroG<sup>Q151N</sup>

Lane M : Gene Ruler 1 kb DNA ladder
Lane 1 : PCR product of T7_aroG<sup>wt</sup>
Lane 2 : PCR product of T7_aroG<sup>Q151N</sup>
Figure 44. Pacl and AvrII digestion patterns of PCR products of T7_aroG<sup>wt</sup> and T7_aroG<sup>Q151N</sup>.

Lane M: Gene Ruler 1 kb DNA ladder
Lane 1: uncut PCR product of T7_aroG<sup>wt</sup>
Lane 2: Pacl and AvrII digested PCR product of T7_aroG<sup>wt</sup>
Lane 3: uncut PCR product of T7_aroG<sup>Q151N</sup>
Lane 4: Pacl and AvrII digested PCR product of T7_aroG<sup>Q151N</sup>
Figure 45. XhoI digestion patterns of pBLPTA$^{L359D_G^{wt}}$ and pBLPTA$^{L359D_G^{Q151N}}$.

Lane M : Gene Ruler 1 kb DNA ladder
Lane 1 : uncut pBLPTA$^{L359D_G^{wt}}$
Lane 2 : XhoI digested pBLPTA$^{L359D_G^{wt}}$
Lane 3 : uncut pBLPTA$^{L359D_G^{Q151N}}$
Lane 4 : XhoI digested pBLPTA$^{L359D_G^{Q151N}}$
3.5.4 Nucleotide sequencing

To verify the nucleotide sequences of $aroG^{wt}$ and $aroG^{Q151N}$, the DNA sequencing of recombinant plasmids were performed by Bioneer, Korean using ACYCDuet1 as a forward primer and DuetDown1 as a reverse primer. The obtained DNA sequences were compared with $aroG$ sequence from section 3.4.4 by nucleotide blast tools in NCBI.

The nucleotide sequence of $aroG^{wt}$ and $aroG^{Q151N}$ showed 100% similarity to these of $aroG$ in pAroG (Figure 46 - 47). The correct $aroG^{wt}$ and $aroG^{Q151N}$ genes were used in the further experiment.

3.6 Co-transformation of pBLPTA$^{L359D,G^{wt}}$ and pBLPTA$^{L359D,G^{Q151N}}$ with pYF into E. coli BL21(DE3)

The correct pBLPTA$^{L359D,G^{wt}}$ and pBLPTA$^{L359D,G^{Q151N}}$ from section 3.2.3.4 were co-transformed with pYF into E. coli BL21(DE3) competent cell using electroporation. The growing transformants of pBLPTA$^{L359D,G^{wt}}$ & pYF and pBLPTA$^{L359D,G^{Q151N}}$ & pYF clones were picked up to culture in 5 mL LB broth that contained 30 mg/mL of kanamycin and 10 mg/mL of chloramphenicol. After extraction, the pBLPTA$^{L359D,G^{wt}}$ & pYF and pBLPTA$^{L359D,G^{Q151N}}$ & pYF were confirmed by digestion with BamHI. From Figure 48, pBLPTA$^{L359D,G^{wt}}$ & pYF digested with BamHI in lane 10 gave three bands at 10.0 kb, 6.0 kb and 1.1 kb which were same size as pBLPTA$^{L359D,G^{wt}}$ (10.0 kb) in lane 2 and pYF (6.0 and 1.1 kb) in lane 6. pBLPTA$^{L359D,G^{Q151N}}$ & pYF also gave the same result (lane 12). Then, pBLPTA$^{L359D,G^{wt}}$ & pYF in lane 8 and pBLPTA$^{L359D,G^{Q151N}}$ & pYF were used to determine L-Phe production.
<table>
<thead>
<tr>
<th>Score</th>
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<th>Identities</th>
<th>Gaps</th>
<th>Strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>1949 bits(1055)</td>
<td>0.0</td>
<td>1055/1055(100%)</td>
<td>0/1055(0%)</td>
<td>Plus/Plus</td>
</tr>
</tbody>
</table>
| Query | 123 | CCGCTGTTATGACGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT

Figure 46. Nucleotide sequence of aroG<sup>wt</sup> in pBLPTA<sub>1359D<sup>wt</sup></sub>G<sup>wt</sup>

Query represented the nucleotide sequence of aroG<sup>wt</sup> in pBLPTA<sub>1359D<sup>wt</sup></sub>G<sup>wt</sup>.

Sbjct represented the nucleotide sequence of in pAroG<sup>wt</sup>.
Figure 47. Nucleotide sequence of $aroG^{Q151N}$ in pBLPTA$^{L359D-Q151N}$.

Query represented the nucleotide sequence of $aroG^{Q151N}$ in pBLPTA$^{L359D-Q151N}$.

Sbjct represented the nucleotide sequence of $aroG^{Q151N}$ in pAroG$^{Q151N}$.
Figure 48. BamHI digestion patterns of pBLPTA<sup>L359D<sup>wt</sup></sup> & pYF and pBLPTA<sup>L359D<sup>G<sub>Q151N</sub></sup></sup> & pYF.

Lane M: Gene Ruler 1 kb DNA ladder
Lane 1: uncut pBLPTA<sup>L359D<sup>wt</sup></sup>
Lane 2: BamHI digested pBLPTA<sup>L359D<sup>wt</sup></sup>
Lane 3: uncut pBLPTA<sup>L359D<sup>G<sub>Q151N</sub></sup></sup>
Lane 4: BamHI digested pBLPTA<sup>L359D<sup>G<sub>Q151N</sub></sup></sup>
Lane 5: uncut pYF
Lane 6: BamHI digested pYF
Lane 7: uncut pBLPT & pYF
Lane 8: BamHI digested pBLPT & pYF
Lane 9: uncut pBLPTA<sup>L359D<sup>wt</sup></sup> & pYF
Lane 10: BamHI digested pBLPTA<sup>L359D<sup>wt</sup></sup> & pYF
Lane 11: uncut pBLPTA<sup>L359D<sup>G<sub>Q151N</sub></sup></sup> & pYF
Lane 12: BamHI digested pBLPTA<sup>L359D<sup>G<sub>Q151N</sub></sup></sup> & pYF
3.7 Production of L-Phe

Each recombinant clone was cultured in 200 mL of minimum medium containing 6% glycerol as a carbon source (Ulfah, 2018). Membrane protein genes (glpF and yddG) under the tight regulation of ara promoter were induced by 0.02% arabinose. The growth profile measured at a wavelength of 600 nm showed that growth of pBLPTA$^{L359D-G_{wt}}$ & pYF and pBLPTA$^{L359D-G_{Q151N}}$ & pYF were not significantly different and higher than that of pBLPT & pYF. Cell growth of each clone exhibited the exponential phase until 72 h (Figure 49). L-Phenylalanine production was correlated with phase of cell growth. At 192 h, the recombinant pBLPTA$^{L359D-G_{Q151N}}$ & pYF clone gave the highest L-Phe production at 1.95 g/L that was 8.7 and 1.2 fold of that obtained from pBLPT & pYF (0.224 g/L) and pBLPTA$^{L359D-G_{wt}}$ & pYF (1.61 g/L), respectively (Figure 50). Addition of recombinant phenylalanine feedback resistant PheA clearly showed to have more impact on L-Phe production than the addition of feedback resistant AroG. PheA (chorismate mutase/prephenate dehydratase) catalyzes a conversion of chorismate to phenylpyruvate through prephenate intermediate that is the key step in determining the L-Phe production while AroG, a main isoform of DAHP synthase, catalyzes the first step of aromatic amino acid biosynthesis pathway. DAHP, the product of AroG can be used not only in the biosynthesis of phenylalanine but also in the synthesis of tyrosine and tryptophan. The cultivating conditions and medium used in this experiment was optimized by Ulfah (2018) for pBLPT & pYF clone. Thus, the composition of glycerol medium as well as culture conditions should be adjusted to improve yield of L-Phe.
Figure 49. Growth curve of recombinant clones in minimum medium. The data were received from three independent experiments.

Figure 50. L-Phe production of recombinant clones in minimum medium. The data were received from three independent experiments.
CHAPTER IV
CONCLUSIONS

1. The expression of PheA in pRSFDuet-1 (pPheA) was confirmed by the appearance of a high intensity protein band around 43 kDa in SDS-gel.

2. Frame shift mutation of aroG inserted at BamHI site of pRSFDuet-1 was found. Therefore, pAroG\text{wt} and pAroG\text{fr} (pRSFDuet-1 harboring aroG\text{wt} and aroG\text{fr}, respectively) were reconstructed.

3. All obtained aroG clones gave the protein band approximately 38 kDa on SDS-gel. The mutated enzymes exhibited slightly higher DAHP synthase activity to the wildtype enzyme and %inhibitions by 20 mM L-Phe were decreased from 51% to 12 – 27%. Thus, H-bonding between Gln151 of AroG and the inhibitor, phenylalanine, has a high impact on phenylalanine feedback inhibition.

4. The recombinant E. coli BL21(DE3) clones containing aroB, aroL, phedh, tktA, aroG, glpF, yddG and pheA was successfully constructed. After 8 days of fermentation in 6% glycerol medium, pBLPTA\text{L359D-GQ151N} & pYF clone gave the highest L-phenylalanine production (1.95 g/L) that was 8.7 and 1.2 fold of that obtained from pBLPT & pYF and pBLPTA\text{L359D-GW} & pYF clones, respectively. The result revealed that feedback resistant PheA and AroG could elevate L-phenylalanine production.
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Appendix A

Map of pRSFDuet-1
Appendix B

Map of pBAD-33
Appendix C

Preparation of Luria-Bertani (LB) broth

Luria-Bertani (LB) broth is used for medium growth of *E. coli*. LB medium contained 1% pancreatic digestion of casein, 0.5% NaCl and 0.5% yeast extract. For agar plate, the LB medium is supplemented with 1.5% (w/v) agar. The medium was sterilized by autoclave for 15 minutes at 121 °C, 15 psi. Then, antibiotic drug (kanamycin, ampicillin or chloramphenicol) was added for selection.
Appendix D

Preparation of competent cell

In this study, we used *E. coli* Top10 and *E. coli* BL21(DE3) as competent cells. Each single colony of *E. coli* Top10 and *E. coli* BL21(DE3) was picked up to culture in 5 ml of LB broth and incubated at 37 ºC with shaking at 250 rpm for 16-18 h. 5% of starter was inoculated into 50 ml of LB broth. The cultures were incubated at 37 ºC with shaking at 250 rpm for 16-18 h. The 5% of each starter was inoculated into 200 ml of LB broth and grown at 37 ºC with shaking at 250 rpm until *OD*$_{600}$ reached 0.3-0.4. The cultures were chilled on ice and centrifuged at 4 ºC, 3,000xg for 10 min. The supernatants were removed and the cell pellets were washed with 2 volume of cool sterilized DI water for 2 times. After that, the cell pellets were washed with 20 ml of cool sterilized 10% glycerol and centrifuged at 4 ºC, 3,000xg for 10 min. Then, the cell pellets were resuspended with cool sterilized 10% glycerol to the final volume 2 ml. Finally, 50 µl of competent cell was aliquoted into microcentrifuge tube and stored at -80 ºC.
Appendix E

Protocol of Presto™ Mini Plasmid Kit

Plasmid extraction was performed using Presto™ Mini Plasmid Kit (Geneaid).

1. Harvesting

The 1.5 ml of cultured bacterial cells was transferred to a microcentrifuge tube and centrifuged at 5,000 x g for 2 min at room temperature. Then, the supernatant was discarded. The harvesting step was repeated using the same 1.5 ml microcentrifuge tube.

2. Resuspension

Two hundred µl of PD1 buffer contained RNaseA was added to the tube containing the cell pellet and then mixed by vortex.

3. Cell Lysis

Two hundred µl of PD2 buffer was added to lyse the cell, mixed gently by inverting the tube and incubated at room temperature for 2 min.

4. Neutralization

Three hundred µl of PD3 buffer was added to neutralize the reaction and then mixed immediately by inverting the tube. After that the supernatant was separated by centrifugation at 10,000 x g for 15 min at room temperature.

5. DNA Binding

All the supernatant was transferred to the PDH column and centrifuged at 10,000 x g for 2 min at room temperature and the flow-through was discarded.

6. Wash

Four hundred µl of W1 Buffer was added into the PDH column. The column was taken centrifuged at 10,000 x g for 2 min to discard the flow-through. After that, 600 µl of wash buffer containing absolute ethanol was added into the PDH column. Centrifugation was performed at 10,000 x g for 2 min at room temperature to discard the flow through follow by centrifugation at 10,000 x g for 3 min at room
temperature to dry the column matrix. The dried PDH column was transferred to a new microcentrifuge tube.

7. Elution

fifty µl of water was added into the center of the column matrix and stood for at least 2 min. Centrifugation was performed at 10,000 x g for 3 min at room temperature to elute the purified DNA.
Appendix F

Protocol of GenepHlow™ Gel/PCR Kit

Purification of DNA fragment using Gel/PCR DNA Fragments Extraction Kit (Geneaid) was performed following these steps:

1. Sample preparation

   Gel Dissociation

   The 300 mg of agarose gel slice containing relevant DNA fragments was cut and transferred into a microcentrifuge tube. Five hundred µl of Gel/PCR Buffer was added to the sample then mix by vortex. The sample was incubated at 55-60ºC for 10-15 min to completely dissolve the gel. After incubation, cool the dissolved sample mixture was cooled to room temperature.

   PCR reaction

   The 5 volumes of Gel/PCR Buffer was added to the PCR reaction and mixed.

2. DNA Binding

   The sample mixture was transferred to the DFH column and centrifuged at 10,000 x g for 2 min. The flow-through was discarded then place the DFH column back in the 2 ml collection tube.

3. Wash

   Four hundred µl of W1 buffer was added into the DFH column and centrifuged at 10,000 x g for 2 min and discarded the flow-through. After that, 600 µl of wash buffer contained absolute ethanol was added into the DFH column and stood for 1 min. Centrifugation at 10,000 x g for 2 min was performed to discard the flow-through followed by centrifugation at 10,000 x g for 3 min at room temperature to dry the column matrix. The dried DFH column was transferred to a new microcentrifuge tube.
4. Elution

The dried DFH column was transferred to a new microcentrifuge tube. 20-50 µl of water was added into the center of the column matrix and stood for at least 2 min. The column was centrifuged at 10,000 x g for 3 min at room temperature to elute the purified DNA.
Appendix G

Preparation for SDS-PAGE analysis

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

Dissolved in distilled water to a total volume of 100 ml.

50% (w/v) Glycerol

100% Glycerol 50 ml

Dissolved in 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.
Appendix G (continued)

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 and 0.4% SDS)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>2 M Tris-HCl (pH 8.8)</td>
<td>75 ml</td>
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<tr>
<td>10% (w/v) SDS</td>
<td>4 ml</td>
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<td>Distilled water</td>
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**Solution C (0.5 M Tris-HCl, pH 6.8, 0.4% SDS)**

<table>
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</thead>
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<td>1 M Tris-HCl (pH 6.8)</td>
<td>50 ml</td>
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<td>10% (w/v) SDS</td>
<td>4 ml</td>
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<td>Distilled water</td>
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**10% (w/v) Ammonium persulfate**

<table>
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<th>Component</th>
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<td>Ammonium persulfate</td>
<td>0.5 g</td>
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<tr>
<td>Distilled water</td>
<td>5.0 ml</td>
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Appendix G (continued)

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

<table>
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<th>Amount</th>
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<tbody>
<tr>
<td>Tris (hydroxymethyl)-aminomethane</td>
<td>3.0 g</td>
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<tr>
<td>Glycine</td>
<td>14.4 ml</td>
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<tr>
<td>SDS</td>
<td>1 g</td>
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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-Cl pH 6.8, 50% (v/v) glycerol, 1% (w/v) bromophenol blue)

<table>
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<tr>
<td>10% (w/v) SDS</td>
<td>2 ml</td>
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<tr>
<td>1% (w/v) Bromophenol blue</td>
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<tr>
<td>β-Mercaptoethanol</td>
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<tr>
<td>Distilled water</td>
<td>1.4 ml</td>
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</table>
Appendix G (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
Appendix H

Preparation for 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 I

Preparation for Lowry’s method solution

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

Copper sulfate  
0.5 g

Potassium tartate  
1 g

Adjusted pH to 7.0 and adjusted to total 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 a total volume of 1 liter.

Solution C (phenol reagent)

Folin-Ciocalteu phenol reagent : distilled water is 1:1
Appendix J

Preparation of 10X TBE for agarose gel electrophoresis

Electrophoresis buffer (10X TBE)

Tris (hydroxymethyl)-aminomethane 54 g
Boric acid 27.5 g
Ethylenediaminetetraacetic acid, disodium salt 9.3 g

Adjust volume to 1 liter with deionized water
Appendix K

Standard curve for protein determination by Lowry’s method

\[ y = 0.0034x \]

\[ R^2 = 0.9871 \]
### Appendix L

Standard curve for L-Phe determination by HPLC

--- Shimadzu LabSolutions Calibration Curve ---

<table>
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<tr>
<th>ID#</th>
<th>Name</th>
<th>Quantitative Method</th>
<th>Function</th>
<th>R²</th>
<th>RSS</th>
<th>MeanRF</th>
<th>RFSD</th>
<th>RFRSD</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>L-Phe</td>
<td>External Standard</td>
<td>( f(x) = 1.05647e+007 \times x + 45327.1 )</td>
<td>0.9941009</td>
<td>5.287072e+011</td>
<td>1.072561e+007</td>
<td>5.161007e+003</td>
<td>4.811853</td>
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<td></td>
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</table>

**Fit Type:** Linear  
**Zero Through:** Not Through  
**Weighted Regression:** None  
**Detector Name:** PDA-M20A

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<th>Area</th>
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<tr>
<td>2</td>
<td>2.76915</td>
<td>4.27831</td>
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<td>3</td>
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<tr>
<td>5</td>
<td>10.99274</td>
<td>10.99274</td>
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</table>

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Chulalongkorn University

ชุลalogkorn University
Appendix M

HPLC Chromatogram of L-Phenylalanine

1. L-Phe Standard

A) L-Phe at 0.2 g/L

B) L-Phe at 0.4 g/L
C) L-Phe at 0.6 g/L

D) L-Phe at 0.8 g/L

E) L-Phe at 1.0 g/L
Appendix N

The sequencing chromatogram of \textit{pheA} in pRSFDuet-1 using ACYCDuetUP1 (A) and T7terminator (B) primers
Appendix O

The sequencing chromatogram of $\text{pheA}^{L359D}$ in pRSFDuet-1 using ACYCDuetUP1 (A) and T7terminator (B) primers.
Appendix O (continued)
Appendix P

The sequencing chromatogram of aroG^st in pRSFDuet-1 using ACYCDuetUP1 (A) and DuetDown1 (B) primers
Appendix P (continued)
Appendix Q

The sequencing chromatogram of \textit{aroG}^{L175D} in pRSFDuet-1 using ACYCDuetUP1 (A) and DuetDown1 (B) primers
Appendix Q (continued)
Appendix R

The sequencing chromatogram of \textit{aroG}^{Q151L} in pRSFDuet-1 using ACYCDuetUP1 (A) and DuetDown1 (B) primers

(A)
Appendix R (continued)
Appendix S

The sequencing chromatogram of $aroG^{Q151A}$ in pRSFDuet-1 using ACYCDuetUP1 (A) and DuetDown1 (B) primers
Appendix S (continued)
Appendix T

The sequencing chromatogram of \textit{aroG}^{Q151N} in pRSFduet-1 using ACYCDuetUP1 (A) and DuetDown1 (B) primers
Appendix T (continued)
VITA

NAME
Charintip Yenyuvadee

DATE OF BIRTH
1 January 1995

PLACE OF BIRTH
Nakornpathom

INSTITUTIONS ATTENDED
Bachelor of Science (Biochemistry)

HOME ADDRESS
35/269 Supalia vista tiwanon condominium Krungthep-Nonthaburi Road, Taladkwan, Muang Nonthaburi, Nonthaburi, Thailand 11000

PUBLICATION