ABSTRACT

Objective: Cytochrome P450 monooxygenase (CYP71AVI) is a key enzyme involved in the artemisinin biosynthesis pathway. In this research, subcloning gene encoding CYP71AVI into pETDUET1 vector in Escherichia coli has been done and then the expression products characterized with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Materials and Methods: Gene construction started with subcloning of cyp71avi gene from pJexpress401_L_cyp into pETDUET1 through restriction site NdeI and XhoI to get pETDUET1_cyp. Overproduction of CYP71AVI at temperature 37°C has conducted by isopropyl-β-D-thiogalactopyranoside induction.

Results: Confirmation of the recombinant vector pETDUET1_cyp was done by migration, restriction site, and sequencing analysis. The result of pETDUET1_cyp restriction analysis with XhoI restriction enzyme showed one DNA band with experimental size 6585 base pair. The CYP71AVI protein has been produced and characterized with SDS-PAGE method. Based on experimental calculation from SDS-PAGE analysis obtained molecular weight of CYP71AVI band was 57.55 kDa.

Conclusion: Construction of gene encoding CYP71AVI into pETDUET1 as the co-expression vector in E. coli has been successfully and confirmed by migration, restriction site, and sequencing analysis. The result of overproduction showed protein bands on SDS-PAGE analysis indicated as CYP71AVI.

INTRODUCTION

Artemisinin is a sesquiterpene lactone antimalarial drugs with endoperoxide structure isolated from Artemisia annua L. Artemisinin has reported to have high effectiveness to overcome Plasmodium falciparum resistance against chloroquine and other formerly used antimalarial drugs [1,2]. Artemisinin provides the basis for an effective treatment for malaria, particularly in the form of artemisinin-based combination therapies (ACTs). World Health Organization has recommended ACTs since 2001 as a malaria best treatment for overcoming the resistance of P. falciparum [3,4].

The low artemisinin content in A. annua ranging from 0.001% to 1% of the plant dry weight make the treatment using artemisinin, and their combinations become expensive and cannot be reached by people who are on the endemic of malaria. The price of artemisinin in 2006 ranged about 900-1600 USD/kg [5,6].

Many experiments have been conducted to gain artemisinin in high amount. One of the methods being developed to improve levels of artemisinin is biosynthesis pathway engineering through genetic engineering against the enzymes that play a role in the production of artemisinic acid. This involves providing farnesyl pyrophosphate that needs amorpha-4,11-diene synthase (ADS) and cytochrome p450 monooxygenase (CYP71AVI) to produce artemisinic acid. ADS and CYP71AVI are two enzymes involved in the final stages of artemisinic acid formation [6]. CYP71AVI belongs to the cytochrome P450 family. It is a multifunctional sesquiterpene oxidase with a key role in the biosynthesis of sesquiterpene lactone artemisinin which involves the conversion of amorpha-4,11-diene to artemisinic acid. Optimization gene encoding CYP71AVI has been conducted to make its expression more optimum in E. coli. The gene was constructed into a plexpress401 vector (plexpress401_cyp).

In this research, subcloning of gene encoding CYP71AVI into pETDUET1 vector and characterization of its co-expression resulted in E. coli BL21 using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method will be done.

MATERIALS AND METHODS

Materials

Materials used in this research have the quality grade for the use of analysis (pro analysis or p.a) or pro molecular biology, including materials for Luria-Bertani (LB) medium, materials for electrophoresis gel agarose, 1 kb DNA marker (Fermentas), restriction enzyme NdeI (Roche), restriction enzyme Xhol (Roche), restriction enzyme buffer B and H (Roche), High-Speed Plasmid Mini Kit, ethidium bromide (Promega), isopropyl-β-D-thiogalactoside (IPTG, Sigma), primers DUET_UP2 for sequencing (5’-TTGTA CAAGGC CGCAT AATC-3’), pETDUET-1 plasmid, T4 ligase enzyme, and ligase buffer x 10 (Promega).

Methods

Preparation for DNA insert

Isolation of plexpress401 plasmid containing a gene encoding CYP71AVI with High-Speed Plasmid Mini Kit.
Subcloning gene encoding CYP71AVI into pETDUET1 in E. coli TOP10

pJexpress401_cyp and pETDUET1 were restricted using NdeI and XhoI restriction enzymes and cloned to give recombinant pETDUET1_cyp. The recombinant plasmid was then transformed into E. coli TOP10 using heat shock method [11]. The recombinant pETDUET1_cyp was characterized by migration, restriction, and sequencing analysis.

CYP71AVI overproduction

For CYP71AVI overproduction purposes, pETDUET1_cyp was transformed into E. coli BL21 (DE3) using heat shock method [11]. Overproduction of CYP71AVI at temperature 37°C using 0.5 mM IPTG induction. The protein produced was characterized using SDS-PAGE analysis.

RESULTS

Preparation for DNA insert

Gene encoding CYP71AVI from pJexpress401_cyp was constructed for subcloning into pETDUET1 vector. pJexpress401_cyp and pETDUET1 were restricted using NdeI and XhoI restriction enzymes. The result of 1% (w/v) agarose gel electrophoresis against cyp71avi gene for DNA insert showed a two DNA bands located at plc wells have approached size around 1500 base pair (bp) and 3000 bp of DNA marker, while at the pd wells contained one band that has a size up to 5000 bp (pETDUET1) of DNA marker (Fig. 1). Theoretically, CYP71AVI gene size is 1472 bp, and pETDUET1 size is 5240 bp.

Subcloning gene encoding CYP71AVI into pETDUET1 in E. coli TOP10

The subcloning result examined by analysis of migration, restriction site analysis, and sequencing. The migration and restriction analysis performed on 1% (w/v) agarose gel electrophoresis against cyp71avi gene for DNA insert showed a two DNA bands located at pJc wells have approached size around 1500 base pair (bp) and 3000 bp of DNA marker, while at the pD wells contained one band that has a size up to 5000 bp (pETDUET1) of DNA marker (Fig. 1). Theoretically, CYP71AVI gene size is 1472 bp, and pETDUET1 size is 5240 bp.

Sequencing analysis of nucleotides was performed for CYP71AVI. Based on sequencing analysis result (Fig. 3), CYP71AVI gene was successfully cloned into pETDUET1. Sequencing analysis showed the designed components of CYP71AVI gene such as codon start, codon stop, and restriction site.

Overproduction of CYP71AVI

Overproduction of CYP71AVI 5 ml scale was done in E. coli BL21(DE3) that carries pETDUET1_cyp. For optimization of overproduction condition, cell cultures were differentiated with and without the addition of 0.5 mM IPTG as the inducer at temperature 37°C. The result of overproduction showed protein bands on SDS-PAGE analysis after cell lysis extraction indicated as CYP71AVI (Fig. 4). Theoretically, CYP71AVI protein size is 53.57 kDa.

DISCUSSION

The result of preparation DNA insert (Fig. 1) showed DNA band located around 1500 bp at plc wells and between 5000 and 6000 bp at pd wells. Experimental calculation showed that DNA band at plc wells was 1438 bp with the theoretical size of CYP71AVI gene is 1472 bp, that DNA band was suspected as CYP71AVI gene. Whereas in pd wells, experimental calculation showed that DNA band was 5383 bp with the theoretical size of 5420 bp, that DNA band suspected as pETDUET1.

Ligation process was done in 1:3 molar ratio between pETDUET1 as vector and CYP71AVI as DNA insert to make the DNA become ligated together to give pETDUET1_cyp. The ligation product was transformed into E. coli TOP10 competent cells and grown in solid LB medium containing 100 µg/ml of ampicillin.

The subcloning result examined by analysis of migration and restriction site. The migration analysis showed that pETDUET1_cyp (6892 bp) migrate slower than pETDUET1 because of the size difference. The restriction analysis, pETDUET1, and pETDUET1_cyp were cut using XhoI restriction enzyme. The result of 1% (w/v) agarose gel electrophoresis showed two DNA band with a size between 5000 to 6000 bp and 6000 to 8000 bp of DNA marker (Fig. 2b). Theoretically, pETDUET1_cyp size is 6892 bp, and pETDUET1 size is 5420 bp.

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CYP71AV1. Based on an experimental calculation from SDS-PAGE analysis after cell lysis extraction obtained molecular weight of CYP71AV1 band was 57.55 kDa that is similar with the theoretical size of CYP71AV1 band was 53.57 kDa. Cell culture with IPTG induction produces thicker protein band compared to the cell culture without IPTG induction (Fig. 4).

CONCLUSION

Subcloning of gene encoding CYP71AV1 into pETDUET1 as the expression vector in E. coli has been successfully done and confirmed by migration, restriction site, and sequencing analysis. Overproduction of CYP71AV1 was done at temperature 37°C and characterized by SDS-PAGE after cell lysis extraction.

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