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Research Article

SUBCLONING OF GENES ENCODING CYTOCHROME P450 MONOOXYGENASE INTO EXPRESSION VECTOR IN *ESCHERICHIA COLI*

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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_*cyp* into pETDUET1 through restriction site *Nde*I and *Xho*I 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 *Xho*I 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.

Keywords: Cytochrome P450 monooxygenase, pETDUET1, pETDUET1_cyp, *Eshcerichia coli.*

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INTRODUCTION

Artemisinin is a sesquiterpene lactone antimalarial drugs with endoperoxide structure isolated from *Artemisia annua* L. Artemisinin has reported to have high effectivity 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 artemisinin using microbes, cell culture, tissue, or organs of plants. One potential approach to get artemisinin in high amount by adding biosynthesis pathway of artemisinic acid as a precursor of artemisinin using microbial cells such as *Escherichia coli*.

E. coli provides farnesyl pyrophosphate that needs amorpha-4,11-diene synthase (ADS) and cytochrome p450 monooxygenase (CYP71AV1) to produce artemisinic acid. ADS and CYP71AV1 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 [7-10]. Optimization gene encoding CYP71AVI has been conducted to make its expression more optimum in *E. coli*. The gene was constructed into a pJexpress401 vector (pJexpress401_*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 *Nde*I (Roche), restriction enzyme *Xho*I (Roche), restriction 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 CACGGC CGCAT AATC-3'), pETDUET-1 plasmid, T4 ligase enzyme, and ligation buffer ×10 (Promega).

Methods

Preparation for DNA insert

Isolation of pJexpress401 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 *Nde*I and *Xho*I 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 *Nde*I and *Xho*I restriction enzymes. The result of 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 5420 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. The migration analysis (Fig. 2a) showed that pETDUET1_*cyp* migrate slower than pETDUET1 because of the size difference. The restriction analysis, pETDUET1, and pETDUET1_*cyp* were cut using *Xho*I 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.

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 pJc wells and between 5000 and 6000 bp at pD wells. Experimental calculation showed that DNA band at pJc 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

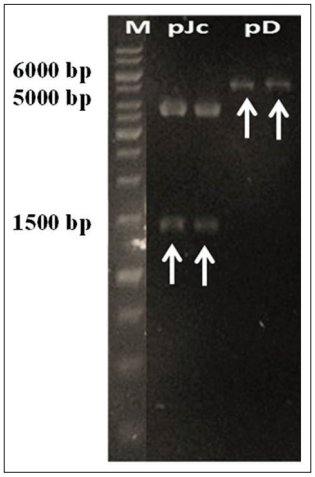

Fig. 1: Electrophoregram of preparation *CYP71AVI* gene for DNA insert. M - 1 kb DNA marker; pJc - pJexpress401_*cyp*; pD - pETDUET1

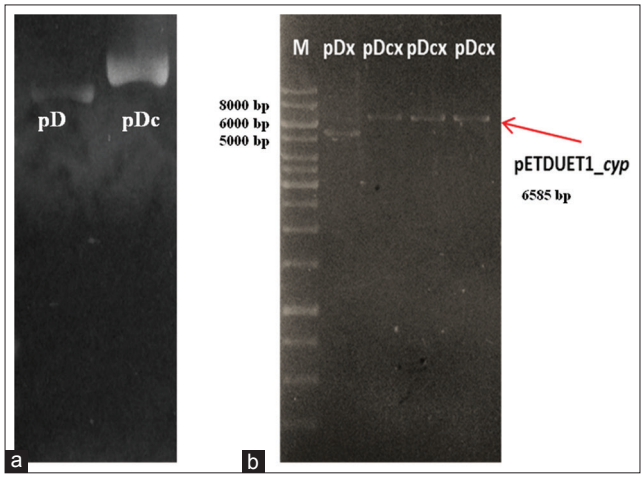

Fig. 2: Electrophoregram of pETDUET1_*cyp* characterization (a) migration analysis. pD - pETDUET1; pDc - pETDUET1_*cyp*, (b) restriction analysis with *Xho*I enzyme. M - 1 kb DNA marker; pDx - pETDUET1; pDcx - pETDUET1_*cyp*

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 (5420 bp) because its greater size (Fig. 2a). While the restriction analysis with *Xho*I restriction enzyme showed one DNA band at pDx well with experimental size 5401 bp and one DNA band at pDcx with experimental size 6585 bp. Theoretically, *CYP71AVI* gene size 1472 bp, pETDUET1 5420 bp, and pETDUET1_*cyp* 6892 bp. This result indicates that *CYP71AVI* gene has successfully cloned on pETDUET1 vector.

Primers DUET_UP2 and T7 terminator used for *CYP71AVI* gene sequencing. Alignment result of *CYP71AVI* gene from pETDUET1_*cyp* with *CYP71AVI* synthetic gene showed that not many changes occur in nucleotide base sequence. The mistake of proofreading in *CYP71AVI* sequencing may occur due to the concentration of DNA used as the sample was overly diluted, so the sequencing analysis of *CYP71AVI* gene still needs to be done with a higher concentration of the DNA.

The recombinant pETDUET1_*cyp* from *E. coli* TOP10 was isolated and then transformed in *E. coli* BL21(DE3) for overproduction of

Fig. 3: Sequencing analysis of pETDUET1_*cyp.* (a) forward primer DUET_UP2, (b) reverse primer T7 terminator

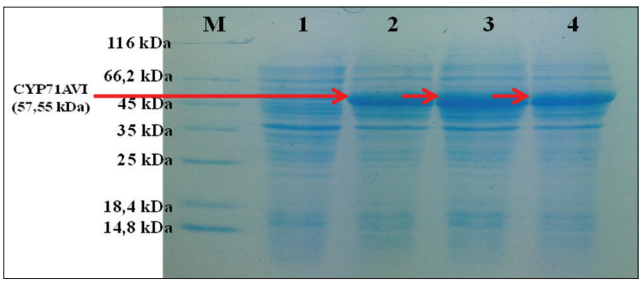

Fig. 4: Electrophoregram of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cell lysis extraction. M - Unstained protein marker; 1 - Non isopropyl-β-D-thiogalactopyranoside (IPTG) induction; 2 - With 0.1 mM IPTG induction; 3 - With 0.5 mM IPTG induction; 4 - With 1 mM IPTG induction

CYP71AVI. Based on an experimental calculation from SDS-PAGE analysis after cell lysis extraction obtained molecular weight of CYP71AVI band was 57.55 kDa that is similar with the theoretical size of CYP71AVI 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 CYP71AVI into pETDUET1 as the expression vector in *E. coli* has been successfully done and confirmed by migration, restriction site, and sequencing analysis. Overproduction of CYP71AVI was done at temperature 37°C and characterized by SDS-PAGE after cell lysis extraction.

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REFERENCES

1. Li Y, Huang H, Wu YL. Qinghaosu (artemisinin) - A fantastic antimalarial drug from a traditional chinese medicine. In: Liang XT, Fang WS, editors. Medicinal Chemistry of Bioactive Natural Products. New Jersey: John Wiley & Sons, Inc., Publication; 2006. p. 183-256.
2. Rathore D, McCutchan TF, Sullivan M, Kumar S. Antimalarial drugs: Current status and new developments. Expert Opin Investig Drugs 2005;14(7):871-83.
3. Duffy PE, Mutabingwa TK. Artemisinin combination therapies. Lancet 2006;367 Suppl 9528:2037-9.
4. White NJ. Qinghaosu (artemisinin): The price of success. Science 2008;320 Suppl 5874:330-4.
5. Mutabingwa TK. Artemisinin-based combination therapies (ACTs): Best hope for malaria treatment but inaccessible to the needy! Acta Trop 2005;95 Suppl 3:305-15.
6. Hale V, Keasling JD, Renninger N, Diagana NN. Microbially derived artemisinin: A biotechnology solution to the global problem of access to affordable antimalarial drugs. Am J Trop Med Hyg 2007;77 Suppl 6:198-202.
7. Teoh KH, Polichuk DR, Reed DW, Nowak G. *Artemisia annua* L. (*Asteraceae*) trichome-specific cDNAs reveal CYP71AV1, a cytochrome P450 with a key role in the biosynthesis of the antimalarial sesquiterpene lactone artemisinin. FEBS Lett 2006;580(5):1411-6.
8. Zeng QP, Zeng XM, Yin LL, Yang RY. Quantification of three key enzymes involved in artemisinin biogenesis in *Artemisia annua* by polyclonal antisera-based ELISA. Plant Mol Biol Rep 2009;27 Suppl 1:50-7.
9. Arsenault PR, Vail D, Wobbe KK, Erickson K. Reproductive development modulates gene expression and metabolite levels with possible feedback inhibition of artemisinin in *Artemisia annua*. Plant Physiol 2010;154 Suppl 2:958-68.
10. Maes L, Van Nieuwerburgh FC, Zhang Y, Reed DW, Pollier J, Vande Casteele SR, *et al.* Dissection of the phytohormonal regulation of trichome formation and biosynthesis of the antimalarial compound artemisinin in *Artemisia annua* plants. New Phytol 2011;189(1):176-89.
11. Chung CT, Niemela SL, Miller RH. One-step preparation of competent *Escherichia coli*: Transformation and storage of bacterial cells in the same solution. Proc Natl Acad Sci USA 1989;86:2172-5.