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Periplasmic production of the recombinant eugenol synthase from *Ocimum basilicum*

Neda Mazarei[1], Anahita Moradi[1], Seyyed Soheil Rahmatabadi[1], Shiva Hemmati[1,2,3,*]

[1]Department of Pharmaceutical Biotechnology, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.

[2]Pharmaceutical Sciences Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.

[3]Biotechnology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.

Abstract

Eugenol, the main bioactive compound of clove oil, is a simple molecule with considerable pharmacological properties, including analgesic, antimicrobial, and anti-inflammatory effects. Eugenol is synthesized in several plant species such as *Ocimum basilicum* (basil) through the activity of eugenol synthase enzyme. Regarding the valuable applications of eugenol as well as notable advantages of the secretory production of recombinant proteins, the excretory production of eugenol synthase has been targeted in this study. Eugenol synthase coding sequence was inserted after pelB peptide in the pET22b vector and expressed in *E. coli* to investigate if the secretory production of the recombinant protein is possible. Additionally, enzyme purification by Ni-NTA affinity chromatography, extraction of the enzyme from periplasmic fraction, and salting out of the enzyme secreted into the medium by ammonium sulfate precipitation were carried out. Our results showed the presence of eugenol synthase in the cytosol, periplasm, and culture medium with concentrations of about 208.8 μgml^{-1}, 212.5 μgml^{-1}, and 332.0 μgml^{-1}, respectively. The results of this study are useful for further studies to produce large-scale eugenol synthase as an unlimited source of the eugenol compound.

Keywords: Extracellular protein, Secretory protein, Signal peptide.

1. Introduction

Eugenol ($C_{10}H_{12}O_2$) (Figure 1), the main bioactive compound of clove, has considerable pharmacological and biological properties. This small molecule has variable applications in perfumery, as well as pharmaceutical, and dental preparations. Eugenol and its derivatives have been targeted in several studies due to antimicrobial, anti-inflammatory, analgesic, antioxidant, and anticancer activities (1-3).

Eugenol is mainly derived from *Eugenia caryophyllata* but has been identified in several aromatic plants such as *Ocimum* spp. Coniferyl alcohol acyltransferase (CFAT) and eugenol synthase (EGS) are the two key enzymes for eugenol biosynthesis in plants (Figure 1). Coniferyl alcohol is acetylated by CFAT activity and then EGS catalyzes eugenol formation from the acetylated coniferyl alcohol (coniferyl acetate). Successful expression of *Ocimum basilicum* (sweet basil) eugenol synthase 1 (ObEGS1) DNA sequence that encodes a 314-residue enzyme has been reported in other studies (4-6). Through further attempts, eugenol synthase in different tissues of five *Ocimum* species including *O. basilicum* (subtypes I, II, III and IV) has been described. However, based

Corresponding Author: Shiva Hemmati, Department of Pharmaceutical Biotechnology, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.
Email: hemmatish@sums.ac.ir

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on our knowledge there is not any report of evaluation or usage of signal peptides linked to the basil ObEGS1 for secretory production.

Considering several advantageous features, *E. coli* has been used more than any other microorganism in the cloning and expression of recombinant proteins. Nevertheless, there are several limitations using this organism, including intracellular host protease degradation of recombinant proteins, inclusion body formation, and the formation of insoluble aggregates of misfolded recombinant proteins (7-8). Target protein recovery from these aggregates needs extensive further processing (9). To confront these obstacles, extracellular production of recombinant proteins in periplasmic space or culture medium might be performed. Extracellular production is favorable due to simpler protein detection and purification of recombinant proteins wherein there is no need for cytoplasmic protein extraction processes. Additionally, the correct formation of disulfide bonds and less protease activity facilitates folding and improves protein biological activity. Reducing the effects of toxic proteins on cell physiology, increasing recombinant enzyme accessibility to extracellular substrates, removal of N-terminal methionine and higher product stability and solubility are the other advantages (10-12). Various strategies might be implemented to produce extracellular proteins (11). Signal peptides are short specific amino acid sequences using host natural secretory systems that mediate proteins to be exported outside the cytoplasm. By targeting the protein to a translocation system and then signal peptide cleavage by signal peptidase, mature protein can pass through the membrane and reach the periplasmic or extracellular space. PelB is among the popular signal peptides that have been used for the efficient secretory production of recombinant proteins in *E. coli* (10, 13). In this study, pelB was used for the secretory production of ObEGS1 in *E. coli*.

2. Materials and Methods

2.1. Eugenol synthase sequence retrieval

The amino acid sequence of *O. basilicum* EGS1 (UniProt ID: Q15GI4) was retrieved in FASTA format using the UniProt protein database (http://www.uniprot.org).

2.2. Codon optimization of ObEGS1 gene

ObEGS1 sequence was submitted to wrangler server (http://www.mrc-lmb.cam.ac.uk/ms/methods/codon.html) to optimize the codons for heterologous expression in *E. coli* BL21 (DE3). The optimized coding sequence obtained from wrangler server was evaluated using GeneScript rare codon analysis server (https://www.genscript.com/tools/rare-codon-analysishttps://www.genscript.com/tools/rare-codon-analysis). The optimized ObEGS1 sequence was inserted in the pET22b expression vector in frame with pelB signal peptide between NcoI and XhoI restriction sites and the designated construct was sent to MWG Biotech (Germany) for synthesis.

2.3. PelB-ObEGS1 expression in E. coli

pET22b-ObEGS1 transformation into *E. coli* BL21 (DE3) was performed using InsTAclone PCR Cloning Kit (Thermo Fischer Scientific). The presence of ObEGS1 gene in the vector was con-

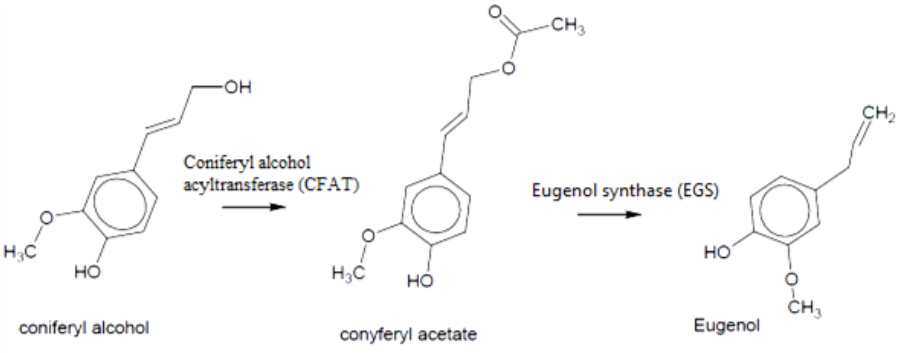

Figure 1. Biosynthetic pathway of eugenol. Coniferyl alcohol is acetylated by CFAT activity and then EGS catalyzes eugenol formation from acetylated coniferyl alcohol (coniferyl acetate).

firmed through plasmid extraction using plasmid extraction Kit (Qiagen, Germany). Transformed cells were inoculated into the Luria-Bertani (LB) medium supplemented with 100 µg/ml ampicillin and incubated at 37 °C overnight. To induce protein expression, a fresh culture of transformed cells was prepared by adding 1 ml of the overnight culture to every 50 ml of pre-warmed LB medium containing 100 µg/ml ampicillin. When an optical density of 0.6-0.8 at 600 nm was achieved in the fresh culture, protein expression was induced by adding a final concentration of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated at 30 °C for 5 h. One ml of culture before IPTG induction and 1 ml after IPTG induction were centrifuged (8000 rpm, 8 min). The pellet and supernatant were analyzed by SDS-PAGE.

2.4. Purification, periplasmic extraction, and medium concentration with ammonium sulfate precipitation

After IPTG induction of cells harboring pelB-ObEGS1, *E. coli* cells were harvested by centrifugation and re-suspended in 4 ml lysis buffer (50 mM NaH_2PO4, 300 mM NaCl, 10 mM imidazole). Then, 300 µl of lysozyme and 50 µl of PMSF were added and incubated on ice for 30 min. Cells were destroyed through sonication for 7×30 s (TOMY, Japan) and centrifuged for 23 min at 4000 rpm at 4 °C. One ml Ni-NTA resin (Qiagen, Germany) was added to every 4 ml of supernatant for 1 h at 4 °C. One ml of the supernatant-Ni-NTA mixture was centrifuged at 700 rpm for 1 min at 4 °C, then 2 ml lysis buffer was added and centrifuged at 700 rpm for 1 min at 4 °C. The remaining amount of the supernatant-Ni-NTA mixture was added to the pellet and stirred gently for 1 h at 4 °C. The mixture was transferred onto a PD10 column and washed two times by the wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole). Finally, His-tagged-pelB-ObEGS1 was eluted using the elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole) and the purified protein was subjected to SDS-PAGE analysis. Bradford assay was used to determine protein concentration.

To extract the protein secreted into the periplasmic space, expression induction and centrifugation was carried out for 50 ml of LB medium. About 80 ml of the extraction buffer containing sucrose 20%, Tris-HCl 30 mM (pH=7.6), and Na_2-EDTA (500 mM) was added to each 1 g cell lysate after centrifugation and incubated at room temperature for 15 min with gentle agitation and then centrifuged at 4 °C (13000 rpm, 15 min). SDS-PAGE analysis was performed on the supernatant containing periplasmic proteins.

In addition to periplasmic extraction, the secreted ObEGS1 into the culture medium was precipitated by supersaturated ammonium sulfate to a final concentration of 80% (V/V) at 4 °C with gentle stirring. After centrifugation at 8000 g for 20 min, the pellet containing precipitated proteins was re-suspended in Tris buffer (pH=7.5) and subjected to SDS-PAGE analysis.

3. Results and Discussion

3.1. Codon optimization of ObEGS1 gene

Since different codon usage between protein's native organism and host organism like *E. coli* impairs the recombinant protein expression significantly (14), the substitution of more frequent used codons in *E. coli* that code the same amino acid increases recombinant protein expression (15). We employed *in silico* techniques to improve codon usage of basil EGS1 gene for recombinant production in *E. coli*. Optimized sequence obtained from wrangler server was analyzed compared with the non-optimized coding sequence and results are shown in Table 1.

Codon Adaptation Index (CAI) improved from 0.58 to 1.00 after optimization. The lower CAI indicates a higher chance of poor protein expression. CAI>0.8 is considered to be good and a CAI of 1 is ideal. Results showed the presence of 14% rare codons in the non-optimized gene that potentially reduces protein expression. This amount reduced to 0% after optimization. The GC content of ObEGS1 gene was reported to be 47.69% after optimization that is in the optimal range (30-70%). According to the results, the optimized coding sequence potentially increases ObEGS1 expression in *E. coli* after expression and purification.

3.2. PelB-ObEGS1 expression in E. coli

IPTG induction of fresh media containing pelB-ObEGS1 transformed *E. coli* cells resulted

Table 1. Codon characteristics of ObEGS1 gene before and after optimization.

Index	Actual value		Ideal value
	Non-optimized	Optimized	
CAI (Codon Adaptation Index)	0.58	1.0	0.8-1.0
GC Content	44.40%	47.69%	30-70%
CFD (Codon Frequency Distribution)	14%	0%	<30%

in pelB-ObEGS1 expression on the SDS-PAGE (Figure 2). ObEGS1 band is observed with a molecular mass of about 37 kDa. This finding is in agreement with previous studies reporting the successful ObEGS1 expression in *E. coli* (4-5). A significant band with approximately the same mass could be detected in the culture lane after IPTG induction indicating ObEGS1 extracellular secretion using pelB signal peptide as a promising result toward ObEGS1 production in *E. coli*.

3.3. Recombinant ObEGS1 purification, periplasmic extraction, and concentration with ammonium sulfate

Figure 3 illustrates the SDS-PAGE analysis of purified EGS1, the periplasmic fraction of recombinant EGS1, and the concentrated medium with ammonium sulfate precipitation. The His-tag at the C-terminal of the recombinant ObEGS1 made its purification possible through affinity column chromatography (16-17). *E. coli* cells harboring ObEGS1 were harvested and purified with Ni-NTA affinity column chromatography. The result is shown at about 37 kDa (Figure 3, lane 8). The concentration of the purified ObEGS1 was 208.8 μgml^{-1} using Bradford protein assay.

Recombinant ObEGS1 was successfully expressed and secreted into the periplasmic space and culture medium. Un-concentrated culture medium (Figure 3, lane 3) shows a band of about 37 kDa. Periplasmic extraction also showed the ObEGS1 secretion into periplasmic space (Figure 3, lane 7). The amounts of EGS1 secreted into the periplasmic space and culture medium were 212.5 μgml^{-1} and 332.0 μgml^{-1}, respectively. We have also concentrated *ObEGS1* secreted into the medium using ammonium sulfate precipitation (Figure 3, lane 4) (18). The concentrated protein has a more apparent band at about 37 kDa than non-concentrated medium. However, this was not highly specific due to the contaminant precipitation probability with the target protein. Thereby,

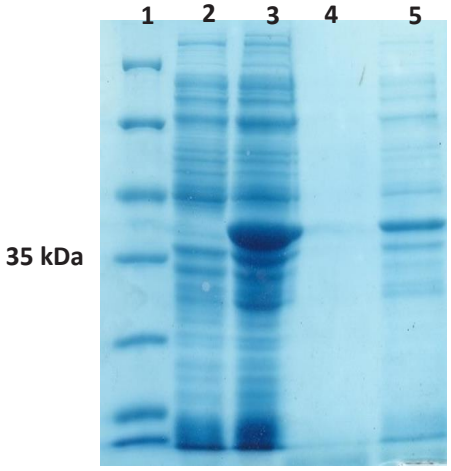

Figure 2. SDS-PAGE analysis of heterologous expression of pelB-ObEGS1 in *E. coli*. Lane 1 is the protein marker. Lanes 2 and 4 are *E. coli* cell lysate and culture medium, respectively, before induction. Lanes 3 and 5 are *E. coli* cell lysate and culture medium after IPTG induction. ObEGS1 band is at about 37 kDa.

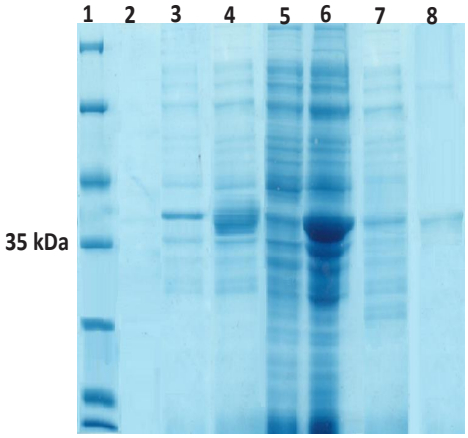

Figure 3. SDS-PAGE analysis of heterologous expression of pelB-ObEGS1 in *E. coli*. Lane 1: protein marker. Lane 2: control culture medium. Lane 3: non-concentrated culture medium. Lane 4: concentrated culture medium with supersaturated ammonium sulfate. Lane 5: cytoplasmic fraction. Lane 6: inclusion body, lane 7: periplasmic fraction, and lane 8: purified ObEGS1 with Ni-NTA. EGS1 band is at about 37 kDa.

additional purification processes such as ion-exchange chromatography and gel filtration chromatography might be needed if secreted ObEGS1 with higher purity is needed (19). Although considerable advantages for the extracellular production of recombinant enzymes have been reported previously, there was no report on the extracellular production of recombinant ObEGS1 until now. Thereby the results of this study are useful for the production of recombinant ObEGS1 in large-scale culture media.

4. Conclusion

In this study, the basil eugenol synthase 1 (ObEGS1), a key enzyme in the biosynthesis of eugenol, and a simple molecule of high biological value was expressed as a conjugated chimera to the pelB signal peptide. PelB-ObEGS1 was successfully expressed in E. coli cells. ObEGS1 was secreted both in the periplasmic space and culture medium with a considerable amount, which might facilitate the laboratory-scale as well as the large-scale production of ObEGS1 and also the biosynthesis of eugenol.

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Conflict of Interest

None declared.

5. References

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