Production of Genistein from Naringenin Using *Escherichia coli* Containing Isoflavone Synthase–Cytochrome P450 Reductase Fusion Protein

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Isoflavonoids are a class of phytoestrogens. Isoflavonone synthase (IFS) is responsible for the conversion of naringenin to genistein. IFS is a cytochrome P450 (CYP), and requires cytochrome P450 reductase (CPR) for its activity. Additionally, the majority of cytochrome P450s harbor a membrane binding domain, making them difficult to express in *Escherichia coli*. In order to resolve these issues, we constructed an in-frame fusion of the IFS from red clover (**RCIFS**) and CPR from rice (**RCPR**) after removing the membrane binding domain from **RCIFS** and **RCPR**. The resultant fusion gene, **RCIFS**–**RCPR**, was expressed in *E. coli*. The conversion of naringenin into genistein was confirmed using this *E. coli* transformant. Following the optimization of the medium and cell density for biotransformation, 60 µM of genistein could be generated from 80 µM of naringenin. This fusion protein approach may be applicable to the expression of other P450s in *E. coli*.

**Keywords:** Cytochrome P450, cytochrome P450 reductase, genistein, naringenin

Polyphenols, one group of plant secondary metabolites, include the (iso)flavonoids, cumarins, stilbenes, and lignins [2]. Among the polyphenols, more than 10,000 flavonoids have been identified thus far [18]. Flavonoids, like other phenylpropanoids, branch out from the primary metabolites (L-phenylalanine or L-tyrosine) via the phenylpropanoid pathway [6, 19]. The entry point of flavonoid biosynthesis is catalyzed by chalcone synthase, which mediates the condensation of three molecules of malonyl-CoA and one coumaryl-CoA. The resultant chalcone naringenin is converted to naringenin either spontaneously or via the action of chalcone isomerase. Naringenin functions as a substrate for the biosynthesis of other flavonoids [19].

Flavonoids are divided into seven major subgroups: chalcones, flavanones, (iso)flavones, flavonols, flavandiols, anthocyanins, and condensed tannins [19]. Among these subgroups, the isoflavone biosynthesis pathway is a branch of the flavone biosynthesis pathway, and is detected exclusively in leguminous plants. Isoflavonoids perform important roles in interactions with other organisms, such as in plant disease resistance or in nodulation [5, 19]. The impacts of isoflavonoids on humans (estrogenic, antioxidant, and anticancer activities) have been the focus of a great deal of attention in recent years [3, 4]. Isoflavone is synthesized via the migration reaction of an aryl ring of flavanone by IFS, cytochrome P450 enzyme CYP93C [1, 7, 11].

Despite the various biological activities of flavonoids, low productivity is a major obstacle to the use of flavonoids in medicinal food or drug products. Although chemical syntheses are available for some flavonoids, they typically involve toxic chemicals and extreme reaction conditions that render the entire process difficult to scale up. In order to circumvent these problems, microbial hosts such as *Escherichia coli* and yeast might be a useful tool for mass production. *E. coli* have been engineered to generate a variety of natural products from plants and other hard-to-culture organisms [8, 20, 21]. Using the *E. coli* system, curcuminoids, flavonoids, anthocyanins, and stilbenes have been manufactured [9, 10]. The biosynthesis of these compounds involves several genes. CYPs including IFS, flavone synthase II, flavonoid 3' hydroxylase, and flavonoid 3',5' hydroxylase are involved in the hydroxylation, double-bond formation, or ring migration of flavonoids. To execute these reactions, CYP must provide electrons from NADPH with the help of CPR [13, 14, 17]. Both CYP and CPR harbor hydrophobic membrane binding domains at the N-terminus, which makes the expression of these proteins difficult, owing to low solubility. Cytochrome P450 can be expressed in *E. coli* if the hydrophobic membrane binding domain is eliminated, and the CPR gene can then be fused with the P450 gene to transfer an electron from NADPH [13, 17].
IFS, with the help of CPR, converts naringenin into 2,5,7,4'-tetrahydroxyisoflavonone, which almost spontaneously becomes genistein [1]. To generate genistein from naringenin, we constructed an in-frame fusion of IFS from red clover and CPR from rice after removing the membrane binding domains from RCIFS and RCPR. We describe the production of genistein using an E. coli variant containing this fusion gene.

**Materials and Methods**

**DNA Manipulation**

Restriction enzymes and T4 DNA ligase were purchased from Takara Biochemicals. Hot-start and Proof Taq polymerases were purchased from Qiagen (Qiagen, Gaithersburg, MD, U.S.A.). Flavonoids were purchased from Indoline Chemicals (Hillsborough, NJ, U.S.A.).

**Construction of RCIFS–RCPR and RCIFS–CCPR**

To remove the membrane binding domain and stop codon of RCIFS, we designed two primers on the basis of a previously published PCR products of IFS and CPR from rice. We describe the production of genistein using an E. coli variant containing this fusion gene.

**HPLC Analysis of Reaction Products**

The reaction products were analyzed using a Varian high performance liquid chromatography apparatus (Waldorf Creek, CA, U.S.A.) equipped with a photo diode array (PDA) detector and a Varian C18 reversed-phase column (Varian, 4.60 × 250 mm, 3.5 μm particle size). For analytical scale, the mobile phase consisted of 0.1% formic acid (pH 3.0), programmed as follows: 20% acetonitrile at 0 min, 45% acetonitrile at 10 min, 60% acetonitrile at 20 min, 20% acetonitrile at 30 min, and 20% acetonitrile at 40 min. The flow rate was 1 ml/min and UV detection was dually monitored at 260 nm and 290 nm.

**Results and Discussion**

RCIFS is a cytochrome P450. Cytochrome P450 harbors a membrane anchorage domain that is hydrophobic and renders the protein insoluble [15]. Secondary structure analysis of RCIFS using the MLRC secondary structure prediction program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_mlrc.html) revealed a helix motif at the first 21 amino acids of the N-terminal region, which has been identified as a membrane anchor sequence. The first 21 amino acids of the N-terminal region of the RCIFS were eliminated in order to achieve the functional expression of IFS in E. coli. The first codon of the truncated IFS gene was subsequently exchanged into ATG. Finally, the stop codon of RCIFS was removed and then a Gly-Ser-Thr linker sequence was introduced in order to fold the two proteins separately. Next, RCPR was fused to this modified gene. Similar to the case of RCIFS, the membrane binding domain of RCPR (the first 50 amino acids) was removed and a Gly-Ser-Thr linker sequence was introduced into the N-terminal region. These two modified genes were then fused via a polymerase chain reaction and subcloned into pGEX. The resultant construct was verified via DNA sequencing. The construction strategy of the fusion chimera is described in Fig. 1. Another CPR from C. roseus was also connected to the modified RCIFS via the same strategy. The final constructs were designated RCIFS–RCPR and RCIFS–CCPR. The two constructs were transformed separately into E. coli BL21 (DE3).
The biotransformation of naringenin with *E. coli* transformants was conducted. After 24 h, the reaction product from *E. coli* harboring RCIFS–RCPR, as well as that from *E. coli* harboring RCIFS–RCPR, yielded new peaks (P1 and P2 in Fig. 2C) when analyzed via HPLC. P2 evidenced a retention time identical to that of authentic genistein (Fig. 2C). Additionally, the UV spectrum of the reaction product was indistinguishable from that of authentic genistein. The molecular mass of P2 was 270 Da, which is identical to that of genistein. P1 was assumed to be a reaction intermediate, 2,5,7,4'-tetrahydroxyisoflavanone, which appeared during the conversion of naringenin to genistein [1]. These results showed that both of the *E. coli* transformants converted naringenin to genistein. However, *E. coli* harboring RCIFS–CCPR converted naringenin into genistein less effectively than *E. coli* harboring RCIFS–RCPR (data not shown). It appears likely that the CPR from *C. roseus* did not effectively transfer electrons from NADPH to RCIFS. It is known that the capacity of CPR to transfer the electron to the corresponding P450 is crucial for the catalytic efficiency of CPR [12, 16, 17].

In an effort to maximize the production of genistein in the *E. coli* transformant, we tested six culture media: LB (Luria–Bertani Broth), LB plus 1% glucose, M9, M9 plus 1% glucose, M9 plus 2% glucose, TB (Terrific Broth), and TB plus 2% glucose. After the induction of genes in the *E. coli* transformants, the cells were harvested and resuspended in the above-mentioned medium. Among the tested culture media, LB was determined to be the most efficient culture medium for genistein production (Fig. 3). The addition of glucose to the medium conspicuously inhibited genistein production. Genistein production in TB or M9 was measured at about 72% and 35% of that observed in LB. Although the initial amount of cell concentration was identical in six different media, the final cell density of each medium differed; TB medium was best and M9 was worst. However, different cell growth was not the only factor in the maximum production of genistein (Fig. 3C). Additionally, the UV spectrum of the reaction product was indistinguishable from that of authentic genistein. The molecular mass of P2 was 270 Da, which is identical to that of genistein. P1 was assumed to be a reaction intermediate, 2,5,7,4'-tetrahydroxyisoflavanone, which appeared during the conversion of naringenin to genistein [1]. These results showed that both of the *E. coli* transformants converted naringenin to genistein. However, *E. coli* harboring RCIFS–CCPR converted naringenin into genistein less effectively than *E. coli* harboring RCIFS–RCPR (data not shown). It appears likely that the CPR from *C. roseus* did not effectively transfer electrons from NADPH to RCIFS. It is known that the capacity of CPR to transfer the electron to the corresponding P450 is crucial for the catalytic efficiency of CPR [12, 16, 17].
production of genistein, as LB is not the optimal medium for the production of cell growth.

We also assessed the effects of initial cell density with regard to genistein production. After the induction of the fusion gene in E. coli, cell densities were adjusted to 1, 2, 4, 6, and 10 at 600 nm using LB containing 50 µg/ml of ampicillin. E. coli harboring either RCIFS-RCPR or RCIFS-CCPR increased genistein production when the cell density was increased. Although the most effective cell density at OD_{600} was 10, it was noted that E. coli metabolites were quite high at this cell density. Thus, we elected to utilize a cell density at OD_{600}=6, at which the purification of genistein proved easier owing to the low concentration of E. coli metabolites.

With the culture medium and under optimized cell density conditions, the production of genistein using E. coli IR or IC was monitored. Eighty µM of naringenin, which is a mixture of two enantiomers (R and S forms of naringenin), was added to the E. coli transformants after the induction of either gene. As shown in Fig. 4, RCIFS–RCPR was able to produce 56 µM of genistein. However, E. coli harboring only vector did not produce genistein, and the concentration of naringenin remained unchanged.

In the current study, the yield of genistein by transgenic E. coli was calculated as approximately 16 mg per liter. However, transgenic yeast harboring IFS was measured at 5.9 mg per liter [8]. The observed difference in yield might be the result of (1) different levels of IFS expression in E. coli and S. cerevisiae and/or (2) the partnership of IFS and CPR, because the previous study utilized S. cerevisiae cytochrome CPR rather than plants. The approach we utilized could be applicable to the expression of other P450s from plants for the definition of substrates or the modification of chemicals including flavonoids or antibiotics.

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