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Biological Synthesis of Baicalein Derivatives Using *Escherichia coli*

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Two baicalein derivatives, baicalin and oroxylin A, were synthesized in this study. These derivatives exhibit diverse biological activities, such as anxiolytic and anticancer activities as well as memory enhancement. In order to synthesize baicalin from aglycon baicalein using *Escherichia coli*, we utilized a glycosyltransferase that regioselectively transfers glucuronic acid from UDP-glucuronic acid to the 7-hydroxy group of baicalein. To increase baicalin productivity, an *araA* deletion *E. coli* mutant, which accumulates UDP-glucuronic acid, was used, and *ugd*, which converts UDP-glucose to UDP-glucuronic acid, was overexpressed. Using these strategies, approximately 720.3 μM baicalin was synthesized from 1,000 μM baicalein. Oroxylin A was then synthesized from baicalein. Two *O*-methyltransferases (OMTs), ROMT-15 and POMT-9, were tested to examine the production of oroxylin A from baicalein. *E. coli* harboring *ROMT-15* and *E. coli* harboring *POMT-9* produced reaction products that had different retention times, indicating that they are methylated at different positions; the structure of the reaction product from POMT-9 was consistent with oroxylin A, whereas that from ROMT-15 was 7-*O*-methyl baicalein. Using *E. coli* harboring *POMT-9*, approximately 50.3 mg/l of oroxylin A (177 μM) was synthesized from 54 mg/l baicalein (200 μM).

Keywords: Baicalein, baicalin, biotransformation, oroxylin A

Introduction

Medicinal plants have been used to prevent or cure diseases throughout human history. Many studies have focused on the identification of phytochemicals exhibiting biological activities from these plants, some of which have demonstrated a similar effect on humans as produced by conventional synthetic drugs [15, 21]. In turn, phytochemicals have been used as the starting compounds to find new medicines. One popular medicinal herb used to treat anxiety, muscle tension, liver disease, and cancers comprises a plant of genus *Scutellaria* [5]. The biological activities of *Scutellaria* rely mainly on its flavonoids, including baicalein (5,6,7-trihydroxyflavone), wogonin (5,7-dihydroxy, 8-methoxyflavone), baicalin (baicalein 7-*O*-glucuronide), apigenin (2,5,7-trihydroxyflavone), and oroxylin A (5,7-dihydroxy, 6-methoxyflavone) [19, 24].

Many phytochemicals, including isoprenoids, phenylpropanoids, and alkaloids, have been synthesized using microorganisms [7]. Various flavonoids, which belong to phenylpropanoids, are synthesized in engineered *Escherichia coli*, wherein pathways for the synthesis of flavonoids from primary metabolites such as tyrosine and phenylalanine have been reconstructed. Flavanone, flavone, flavonol, and anthocyanin have been synthesized in *E. coli* using introduced flavonoid biosynthetic pathways [22]. Although some flavonoids synthesized using this approach are bioactive, the synthesis of flavonoids with greater complexity and enhanced activity has been limited owing to the difficulty of introducing and coordinating the expression of larger numbers of genes. Given that the flavonoid modification reactions such as *O*-methylation, glycosylation, hydroxylation, and prenylation lead to the synthesis of diverse flavonoids, and some flavonoids can be obtained commercially and cheaply, simple modifications of available flavonoids has instead been suggested as an advantageous method for the synthesis of diverse bioactive flavonoids [8, 11].

Two derivatives of baicalein, baicalin and oroxylin A, can be synthesized by either glycosylation or *O*-methylation, respectively. Baicalin and baicalein have anxiolytic effects

[17, 23]. Oroxylin A, one of the components of *Scutellaria*, has diverse activities, including anti-inflammatory, anti-allergy, and memory consolidation enhancing effects [3, 12, 20]. In this study, we aimed to synthesize these two bioactive baicalein derivatives.

Materials and Methods

Production of Baicalin

The uridine diphosphate (UDP)-dependent glycosyltransferase (UGT) gene from *Antirrhinum majus* (*AmUGT*) was cloned previously into the EcoRI/NotI sites of the pET-Duet1 vector (Novagen, Madison, WI, USA) and the UDP-glucose dehydrogenase gene from *E. coli* (*Ecugd*) was cloned into the NdeI/XhoI sites of the pET-Duet1 vector (Novagen) containing AmUGT [14]. To synthesize baicalin from baicalein using *E. coli* harboring *AmUGT* and *Ecugd*, *E. coli* was grown overnight at 37°C in Luria broth (LB) containing 50 μg/ml ampicillin. The culture was inoculated into fresh LB medium containing ampicillin and grown at 37°C until the OD_{600} reached 0.8. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to the medium at the final concentration of 1 mM and the culture was grown at 18°C for 20 h. The cells were harvested and the cell concentration was adjusted to 3 at OD_{600} with M9 containing 2% glucose, 50 μg/ml ampicillin, and 1 mM IPTG. Baicalein (50 μM) was added to the culture followed by incubation at 30°C. To analyze the reaction product, the culture was centrifuged and the supernatant was boiled for 5 min and then it was centrifuged at 12,000 rpm at 10 min to remove cell debris. The resulting supernatant was subject to high-performance liquid chromatography (HPLC) analysis [1].

To examine the role of the *ugd* gene and and the *arnA* deletion mutant in the production of baicalin, each strain was prepared as described above and the cell density was adjusted to OD_{600} = 3. Baicalein (500 μM) was added to the culture and the mixture was incubated at 30°C for 0.5 h. To examine the optimal initial concentration of baicalein for the production of baicalin, baicalin was added to the culture at the concentrations of 200, 400, 600, 800, and 1,000 μM, respectively and the production of baicalin was investigated after 12 h.

To monitor the production of baicalin from baicalein, 1 mM baicalein was added to the culture and the production of baicalin was monitored periodically for 13 h.

Molecular Modeling

The three-dimensional structures of the *O*-methyltransferases (OMTs), POMT-9 and ROMT-15 were constructed using the "First Approach Mode" at the Swiss-Model protein structure homology modeling server (swissmodel.expasy.org//SWISS-MODEL.html). The crystal structures of caffeic acid/5-hydroxyferulic acid 3/5 *O*-methyltransferase (PDB ID: 1kyz, 85.3% sequence identity) and caffeoyl CoA 3-*O*-methyltransferase from alfalfa (PDB ID: 1sui, 56.8% sequence identity) were used as templates for POMT-9 and ROMT-15, respectively. After superposition of the template (1kyz) with the corresponding modeled structure of POMT-9, *S*-adenosylhomocysteine (SAH) and ferulic acid in the template structure were merged into the modeled one by using the "Protein Structure Alignment" module in the molecular modeling suite Maestro (http://www.schrodinger.com). In the same manner, SAH, feruloyl CoA, and calcium ion in the template (1sui) were merged into the modeled structure of ROMT-15. The modeled structures of POMT-9 and ROMT-15 were optimized via conjugate gradient minimization according to the OPLS-AA force field and GB/SA continuum water model (0.05 convergence criteria) by using the "Macromodel" module incorporated into Maestro. On the other hand, the 3D structure of baicalein was generated by using the "Build" module, which was also optimized by conjugate gradient minimization using the OPLS-AA force field and GB/SA continuum water model (0.05 convergence criteria). Based on the modeled structures of POMT-9 and ROMT-15 complexed with their cofactors (SAH, Mg^{2+}) and ligand (ferulic acid), docking studies of baicalein were carried out using the "GLIDE" module incorporated into Maestro. The default setting of the standard precision mode of GLIDE was employed for the docking. Up to 10 poses were saved for analysis, and the top scored pose was chosen for binding mode analysis.

Production of Oroxylin A

POMT-9 was cloned from *Populus deltoides* [9] and *ROMT-15* and *ROMT-17* were cloned from *Oryza sativa* [16]. These genes were subcloned into the *E. coli* expression vector pGEX 5X-1 (GE Healthcare, Little Chalfont, UK) and the resulting construct was transformed into *E. coli* BL21 (DE3).

E. coli transformants were grown as described above and the protein was induced by adding IPTG at the final concentration of 0.1 mM. The cells were harvested and resuspended with LB containing 50 μg/ml ampicillin at OD_{600} = 1.0. Baicalein was added at the final concentration of 100 μM to the culture followed by incubation at 30°C. The culture was extracted with two volumes of ethylacetate and the organic layer was dried. Then, it was dissolved in dimethylsulfoxide (DMSO) and analyzed using HPLC [1]. To measure the conversion of baicalein into oroxylin A or 7-*O*-methylbaicalein, the cell density was adjusted to OD_{600} = 3 and 200 μM of baicalin was added.

Determination of Product Structure

To determine the structure of the reaction product, nuclear magnetic resonance spectroscopy was used [13]. Samples were prepared as described in An *et al*. [1]. The NMR data were as follows; baicalin: ^{1}H NMR: δ 6.78 (1H, s, H-3), 7.01 (1H, s, H-8), 8.0 (2H, dd, 7.8, 1.6Hz, H-2′/6′), 7.55 (2H, m, H-3′/5′), 7.56 (1H, m, H-4′), 5.18 (1H, d, 7.4Hz, H-1′′), 3.54-3.69 (3H, m, H-2′′, H-3′′, H-4′′), 4.13 (1H, d, 9.65Hz, H-5′′); oroxylin A: ^{1}H NMR (DMSO-d_6) δ: 3.93 (3H, s), 6.98 (1H, s), 7.00 (1H, s), 7.59 (3H, m), 8.11 (2H, d, *J* = 8.3Hz),

12.49 (1H, s); and 7-*O*-methyl baicalein: ^{1}H NMR δ (ppm): 6.92 (1H, s, H-3), 6.61 (1H, s, H-8), 8.04 (2H, dd, J = 8.3, 1.7Hz, H-2′), 7.59 (3H, H-3′ and H-4′), 3.74 (3H, s, OMe), 12.89 (br s).

Results and Discussion

Biological Synthesis of Baicalin from Baicalein Using Engineered *E. coli*

Baicalin is a baicalein 7-*O*-glucuronide. To synthesize baicalin from baicalein, regioselective glycosylation is required. AmUGT, which was shown to utilize flavonoids as a sugar acceptor and to transfer glucuronic acid from UDP-glucuronic acid to the 7-OH group of flavonoid [18], was employed to synthesize baicalin from baicalein in the current study. As shown in Fig. 1, the culture filtrate from *E. coli* harboring AmUGT exhibited a new peak of molecular mass 446.3 Da, which is the predicted molecular mass of the baicalin. In addition, the structure of this reaction product was determined to be baicalin using NMR.

AmUGT uses UDP-glucuronic acid as a sugar donor, which is synthesized from UDP-glucose by UGD. Overexpression of *ugd* would accelerate the conversion of UDP-glucose into UDP-glucuronic acid. In *E. coli*, UDP-glucuronic acid serves as a substrate of UDP-L-Ara4*N* formyltransferase/UDP-GlcA C-4′′-decarboxylase (*arnA*) for the synthesis of UDP-4′′-ketose, which is one of the components of the cell wall [2]. Therefore, deletion of *arnA* leads to the accumulation of UDP-glucuronide, which could be used as a sugar donor of AmUGT to synthesize baicalin. Therefore, we utilized four *E. coli* strains (wild-type *E. coli*; wild-type *E. coli* overexpressing *ugd*; *arnA* deletion mutant; and *arnA* deletion mutant overexpressing *ugd*) and tested their ability to synthesize baicalin. As expected, overexpression of *ugd* increased the production of baicalin in both wild types (76.0 mg/l) and the *arnA* mutant (80.1 mg/l). In addition, the *arnA* mutant (38.9 mg/l) produced more baicalin than wild type (19.6 mg/l) (Fig. 2). The *arnA* mutant overexpressing *ugd* produced more baicalin than the other strains. Therefore, this indicated that the supply of UDP-glucuronic acid is important for the final yield of baicalin.

Using the *arnA* mutant overexpressing *ugd*, the optimal initial concentration of baicalein for the production of baicalin was examined. The initial concentration of baicalein correlated with the formation of baicalin: at 1,000 μM, approximately 720 μM of baicalin was produced. Regardless of the initial baicalein concentration, 20–30% of the baicalein was degraded without conversion into baicalin. The first step of the phenolic compound degradation pathway in bacteria, including *E. coli*, is the formation of a catechol structure in the phenolic compounds, which then begins to be degraded [4]. Because baicalein contains the catechol structure, some of them went through the degradation pathway. However, once sugar was attached to the baicalein, it was stable and no longer degraded. Higher

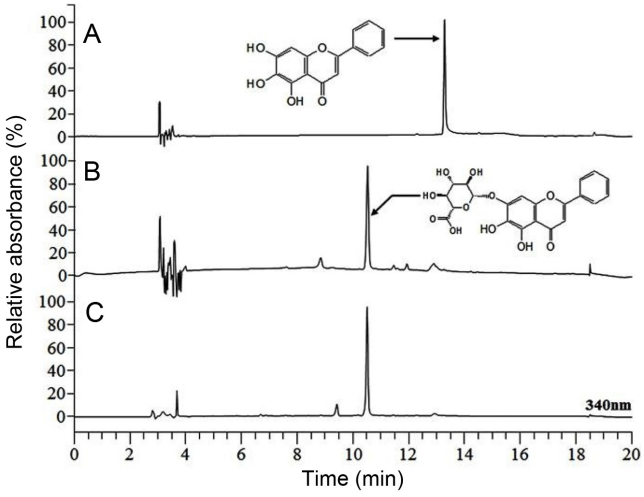

Fig. 1. Analysis of the reaction product of *E. coli* harboring *AmUGT*.
A, Baicalein standard; B, baicalin standard; C, reaction product by *E. coli* harboring *AmUGT*.

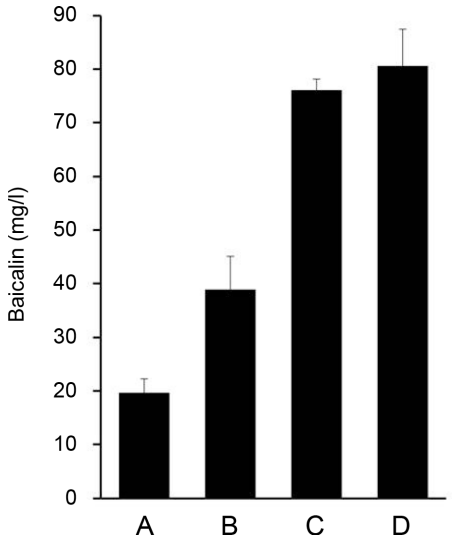

Fig. 2. Production of baicalin by different *E. coli* strains.
A, *E. coli* BL21 harboring *AmUGT*; B, *E. coli araA* deletion mutant harboring *AmUGT*; C, *E. coli* BL21 harboring *AmUGT* and *ugd*; D, *E. coli araA* deletion mutant harboring *AmUGT* and *ugd*. Three independent experiments were performed. The error bars indicate standard deviation.

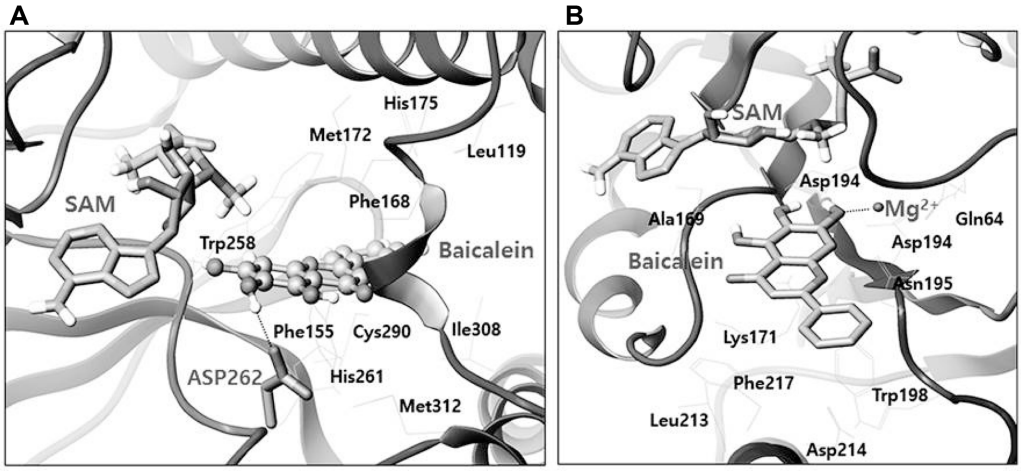

Fig. 3. Docking of baicalein and *S*-adenosylmethionine (SAM) into POMT-9 (**A**) and ROMT-15 (**B**).

concentrations of baicalein were not tested owing to its limited solubility. Thus, we were able to synthesize 720 μM baicalin from 1,000 μM baicalein using the *E. coli* transformant.

Biological Synthesis of Oroxylin A from Baicalein Using *E. coli* Harboring OMT

Oroxylin A is a 6-*O*-methyl baicalein. Regioselective *O*-methylation of baicalein by OMT theoretically could synthesize oroxylin A. However, to date, no OMT that methylates baicalein has been identified. Baicalein is a 5,6,7-trihydroxy flavone and carries adjacent hydroxyl groups. As some OMTs have been shown to utilize flavones having adjacent hydroxyl groups [6, 8, 16], we used molecular modeling approaches to identify an OMT that could methylate baicalein. We constructed the three-dimensional structures of two OMTs (POMT-9 and ROMT-15) that could methylate the hydroxyl group of flavonoids having adjacent hydroxyl groups. Docking of *S*-adenosylmethionine (SAM) and baicalein into either POMT-9 or ROMT-15 showed that these two substrates fit into POMT-9 and ROMT-15. Asp262 of POMT-9 appeared likely to deprotonate from the 6-hydroxy group of baicalein, which might serve as a driving force to transfer the methyl group from SAM to the 6-hydroxy group of baicalein. The 7-hydroxy group of baicalein formed a hydrogen bond with the carboxyl group of Trp258. In addition, the hydrophobic B-ring of baicalein fitted into the hydrophobic pocket formed by Met172, Phe168, His175, Leu119, Phe155, Cys290, His261, Ile308, and Met312 of POMT-9 (Fig. 3A). Overall, POMT-9 was predicted to transfer a methyl group to the 6-hydroxy group of baicalein. However, ROMT-15 was considered likely to transfer a methyl group to the 7-hydroxy group of baicalein (Fig. 3B). A metal ion (Mg^{+2}) appeared to be involved in the deprotonation of the 7-hydroxy group of baicalein. This metal ion was coordinated by Asp169, Gln64, and Asp194. The hydrophobic B-ring of baicalein fitted into a hydrophobic pocket formed by Phe217, Leu213, Asp214, and Trp198.

We tested these predictions using POMT-9 and ROMT-15. HPLC analysis from transformant harboring either *POMT-9* or *ROMT-15* showed that a new peak, which had a different retention time from that of baicalein, was observed in the culture filtrate from *E. coli* harboring either *POMT-9* or *ROMT-15*. The HPLC retention time of the

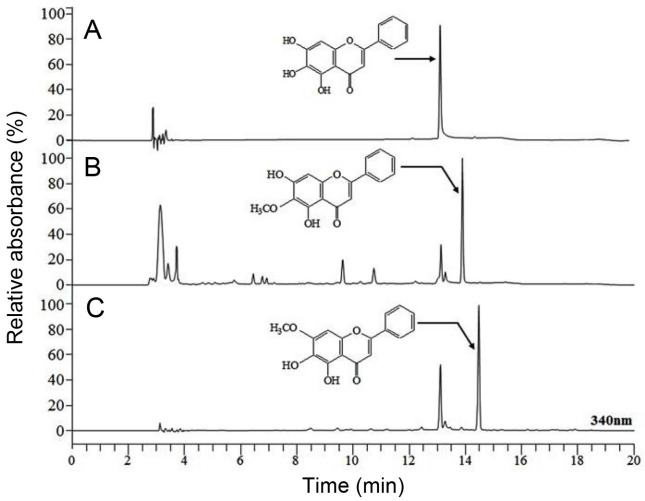

Fig. 4. HPLC analysis of the product obtained from the biotransformation of baicalein.
A, Baicalein standard; B, reaction product of *E. coli* harboring *POMT-9*; C, reaction product of *E. coli* harboring *ROMT-15*.

product from ROMT-15 also differed from that of the product from POMT-9 (Fig. 4). The molecular masses of both products were 284.4 kDa, indicating that a single methyl group was attached. These results suggested that the methylation position of product from the ROMT-15 differed from that of POMT-9. The methylation position of each product was determined using NMR. ROMT-15 was shown to have transferred a methyl group to the 7-OH group of baicalein; thus, POMT-9 transferred a methyl group to the 6-OH group; POMT-9 synthesized oroxylin A from baicalein, consistent with the result predicted from the molecular docking study.

Production of oroxylin A and 7-*O*-methylbaicalein using *E. coli* harboring POMT-9 or ROMT-15 was monitored for 8 h after the addition of 200 μM baicalein. Both compounds reached the maximum production after 3 h, at which 50.3 mg/l (176.9 μM) of oroxylin A and 43.5 mg/l (153.0 μM) 7-*O*-methylbaicalein were produced. POMT-9 converted baicalein into oroxylin A with a conversion rate of 88.5% and ROMT-16 converted baicalein into 7-*O*-methylbaicalein with a conversion rate of 76.5%.

In this study, we synthesized two *O*-methyl baicaleins (oroxylin A and 7-*O*-methylbaicalein) and one baicalein glycoside (baicalin) from baicalein with a more than 70% conversion rate. *O*-Methylation lowered the solubility of the substrate and we did not test high initial concentrations. In contrast, glycosylation was found to increase the solubility of the substrate and, therefore, the final yield of baicalin was higher than the two methylated baicaleins.

Through molecular docking analysis, we identified two regioselective OMTs: POMT-9 and ROMT-15. The experimental data agreed with the predicted results of this analysis. Although genome information from many organisms is available, the identification of suitable enzymes for regioselective reactions has been an obstacle for the bacterial production of modified flavonoids, as characterization of individual genes after expression in a heterologous system is time-consuming. Thus, the approach used in this study might provide an alternative approach to determine optimal enzymes for a regioselective reaction.

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