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Biosynthesis of Two Flavones, Apigenin and Genkwanin, in *Escherichia coli*

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The flavonoid apigenin and its *O*-methyl derivative, genkwanin, have various biological activities and can be sourced from some vegetables and fruits. Microorganisms are an alternative for the synthesis of flavonoids. Here, to synthesize genkwanin from tyrosine, we first synthesized apigenin from *p*-coumaric acid using four genes (*4CL*, *CHS*, *CHI*, and *FNS*) in *Escherichia coli*. After optimization of different combinations of constructs, the yield of apigenin was increased from 13 mg/l to 30 mg/l. By introducing two additional genes (*TAL* and *POMT7*) into an apigenin-producing *E. coli* strain, we were able to synthesize 7-*O*-methyl apigenin (genkwanin) from tyrosine. In addition, the tyrosine content in *E. coli* was modulated by overexpressing *aroG* and *tyrA*. The engineered *E. coli* strain synthesized approximately 41 mg/l genkwanin.

Keywords: Apigenin, genkwanin, metabolic engineering

Introduction

Plants produce diverse secondary metabolites such as alkaloids, isoprenoids, and polyphenols [5]. Some of these chemicals have been used as medicines and/or for nutrition [18]. Many natural compounds originating from plants are considered as starting materials for the development of new medicines [1]. Microbial production of plant secondary metabolites has become an attractive topic. Biological pathways for the synthesis of several plant metabolites have been reconstructed in microorganisms, and hosts such as *Escherichia coli* and *Saccharomyces cerevisiae* have been engineered to supply precursors for the synthesis of final products [31].

Flavonoids form a class of phenolic compounds found in plants and can be classified into several groups, including flavanones, flavones, flavonols, anthocyanins, and isoflavones [34]. Among these groups, flavanones are starting compounds for the synthesis of other flavonoid groups. The flavanone naringenin can be synthesized from tyrosine by several enzymes, including tyrosine ammonia lyase (TAL), 4-coumaroyl coenzyme A ligase (4-CL), chalcone synthase (CHS), and chalcone isomerase (CHI) [36] (Fig. 1). Genes for flavanone biosynthesis have been cloned and characterized in various plants [37], making it possible to assemble these plants genes and reconstruct the flavonoid biosynthesis pathway in microorganisms for the synthesis of a target flavonoid [35]. The typical flavone apigenin is synthesized from naringenin (a flavanone) by flavone synthase (FNS). FNS has stereospecificity and uses only (*S*)-naringenin as a substrate [22]. Although apigenin itself has several biological activities, including anti-inflammatory [6], antidepressant [28], and anticancer activities [7], the regioselective *O*-methylation of apigenin (to generate genkwanin) confers new biological activities, including antibacterial [4, 23], antiplasmodial [13], radical scavenging [32], chemopreventive [8], and inhibiting 17β-hydroxysteroid dehydrogenase type 1 [3] activities. Although genkwanin has been shown to inhibit the development of cotton-pellet-induced granuloma in rat, the molecular mechanisms of this anti-inflammatory activity remain obscure [29]. Apigenin is found in various fruits and vegetables but the most

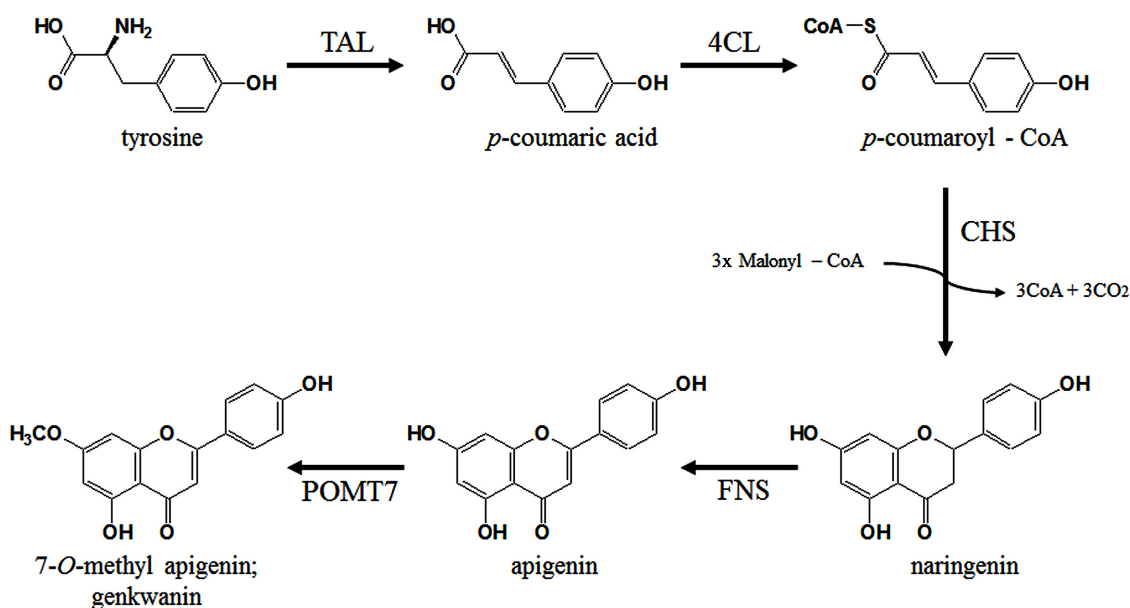

Fig. 1. Biosynthesis pathway of 7-*O*-methylapigenin (genkwanin) from tyrosine.
TAL, tyrosine ammonium lyase; 4CL, 4-coumarate CoA ligase; CHS, chalcone synthase; FNS, flavone synthase; POMT7, apigenin 7-*O*-methyltransferase.

common sources are parsley and celery [24]. Genkwanin has been identified only in *Daphne genkwa* [2]. Therefore, in order to explore novel biological functions of genkwanin, alternative approaches for genkwanin acquisition are necessary. Chemical synthesis is one alternative method to obtain genkwanin [21]. Conversion of apigenin into genkwanin using *E. coli* harboring *O*-methyltransferase was also successful [9]. By constructing flavonoid biosynthesis pathways in a microbe such as *E. coli*, diverse flavonoids have been synthesized from glucose [12, 25, 30]. However, most previous reports of flavonoid biosynthesis have focused on flavanones and their derivatives. Here, we synthesized the bioactive flavone derivative genkwanin from glucose using *E. coli*. By introducing six genes involved in genkwanin biosynthesis into an engineered *E. coli* strain, approximately 41 mg/l genkwanin was synthesized.

Materials and Methods

Constructs

The *TAL* gene from *Saccharothrix espanaensis* was cloned as previously described [12]. The *4CL* (*Os4CL*) and *CHS* (*PeCHS*) genes were also cloned as previously described [11, 15]. Both *Os4CL* and *PeCHS* were subcloned into the *Eco*RI/*Not*I sites and *Nde*I/*Kpn*I sites of the pCDFDuet vector (Novagene), respectively, and the resulting construct, in which both *Os4CL* and *PeCHS* are controlled by an independent *T7* promoter, was named pC-pPeCHS-pOs4CL. In this construct, both genes are controlled by different *T7* promoters. The construct in which both PeCHS and Os4CL are controlled by one *T7* promoter was named pC-pPeCHS-Os4CL. *CHI* from *Medicago truncatula* (GenBank No. XM_003592713) was cloned and inserted into the *Nde*I/*Kpn*I sites of pC-pPeCHS-pOs4CL and pC-pPeCHS-Os4CL. The resulting constructs were named pC-pPeCHS-pOs4CL-pMtCHI and pC-pPeCHS-Os4CL-pMtCHI. Flavone synthase (FNS; AY230247) was cloned from parsley [22] and apigenin 7-*O*-methyltransferase (POMT7; TC29789) was previously cloned [9]. FNS was subcloned into the *Nde*I/*Kpn*I site of the pET-Duet vector (pE-FNS), and POMT7 was then subcloned into the *Sal*I/*Not*I sites of pPET-Duet containing FNS (pE-POMT7-FNS). Plasmids pA-SeTAL, pA-aroG-SeTAL-TyrA, and pA-aroG^{fbr}-TAL-tyrA^{fbr} were constructed previously [11].

Production of Apigenin and Genkwanin

To measure the production of apigenin from *p*-coumaric acid, the *E. coli* transformant was grown in Luria-Bertani (LB) broth containing 50 μg/ml of chloramphenicol and spectinomycin at 37°C for 18 h. The culture was inoculated to a fresh LB medium containing 50 μg/ml of chloramphenicol and spectinomycin and incubated with shaking at 37°C until an OD_{600} of 0.8 was attained. IPTG was added to the culture at a final concentration of 1 mM and the culture was allowed to incubate with shaking at 18°C for 18 h. The cells were harvested and resuspended with M9 medium containing 2% glucose, 0.2% yeast, 50 μg/ml of chloramphenicol, 50 μg/ml spectinomycin, 1 mM IPTG, and 300 μM of *p*-coumaric acid. The resulting culture was incubated at 30°C for 24 h with shaking at 180 rpm. The culture was analyzed by high-performance

liquid chromatography (HPLC) as described previously [9].

E. coli harboring pE-POMT7-FNS was used for the production of genkwanin from naringenin. After induction of the proteins as described above, cells were collected and resuspended in fresh LB containing 50 μg/ml ampicillin. Naringenin (100 μM) and IPTG (1 mM) were added to the culture, and the culture was incubated at 30ºC with shaking at 180 rpm for 15 h. The culture was analyzed by HPLC.

To synthesize genkwanin from glucose, an overnight culture of *E. coli* transformant was inoculated into fresh LB medium and grown until OD_{600} = 1.0. Cells were harvested and resupended in M9 medium containing 2% glucose, 2% yeast extract, 1 mM IPTG, and 50 μg/ml antibiotics. Cells were grown at 30ºC for 24 h with shaking. The reaction products were analyzed by HPLC using an Ultimate 3000 HPLC (Thermo Scientific, USA). The separation condition was as described previously [10].

ESI-MS analyses were performed, on a LCQ fleet instrument (Thermo Scientific, Waltham, MA, USA) coupled to an Ultimate 3000 HPLC system, in the negative-ion mode within the *m/z* range 100–500 and processed with Xcalibur software (Thermo Scientific). The operating parameters were as follows: spray voltage 4.5 kV, sheath gas 15 arbitrary units, auxiliary gas 10 arbitrary units, heated capillary temperature 275°C, capillary voltage −15 V, tube lens −110 V. Tandem (MS2) or triple (MS3) mass spectrometry analysis was conducted with scan-typeturbo data-dependent scanning (DDS), and the fragment spectra were produced using 35% of normalized collision energies.

Results and Discussion

Optimization of Apigenin Production

Genkwanin is synthesized from apigenin by 7-*O*-methylation of POMT7. The yield of apigenin is therefore critical to the subsequent yield of genkwanin. Apigenin is synthesized from *p*-coumaric acid by four enzymes (4CL, CHS, CHI, and FNS; Fig. 1). In *E. coli*, conversion of naringenin chalcone to naringenin occurs spontaneously and, therefore, the CHI that catalyzes this step is not required. We introduced three genes (*Os4CL*, *PeCHS*, and *FNS*) into *E. coli* (Strain B-AP1 in Table 1) and tested if B-AP1 synthesized apigenin from *p*-coumaric acid. Analysis of the culture filtrate by HPLC and mass spectrometry showed that apigenin was synthesized (data not shown).

It is known that FNS uses (*S*)-naringenin as a substrate [22] and that the reaction product of CHI from naringenin chalcone is (*S*)-naringenin. Therefore, the final yield of apigenin might be higher when CHI was used in the biosynthetic pathway of naringenin. We made two *E. coli* transformants (B-AP1 and B-AP2). The B-AP1 contained three genes (*CHS*, *4CL*, and *FNS*), in which naringenin chalcone is spontaneously converted into naringenin, with both (*R*)- and (*S*)-naringenins being generated. The other strain, B-AP2, harbored *CHI* as well as the three genes (*CHS*, *4CL*, and *FNS*). In B-AP2, it would be expected that naringenin chalcone is converted into (*S*)-naringenin, which would serve as a substrate for FNS. We tested the production of apigenin using B-AP1 and B-AP2 (Fig. 2A). B-AP2 produced more apigenin (23 mg/l) than B-AP1 (13 mg/l). This indicates that CHI converted naringenin chalcone into (*S*)-naringenin, which could then be used as a substrate by FNS.

4CL and CHS catalyze the first two steps of apigenin biosynthesis. Therefore, coordinated expression of *4CL* and *CHS* in *E. coli* would be critical to the final yield of apigenin. We made a construct in which *4CL* and *CHS* were cloned in an operon (*i.e.*, one promoter controls the expression of both *4CL* and *CHS*; pC-pPeCHS-Os4CL in Table 1), which was named B-AP3. The production of apigenin in B-AP3 was compared with that of B-AP2 (*4CL* and *CHS* are controlled by independent *T7* promoter; pC-pPeCHS-pOs4CL). As shown in Fig. 2A, the yield of apigenin was greater in B-AP3 (30 mg/l) than in B-AP2 (23 mg/l).

Production of Genkwanin from Glucose in *E. coli*

We showed that the four genes (*Os4CL*, *PeCHS*, *MtCHI*, and *FNS*) worked properly to synthesize apigenin from *p*-coumaric acid. In order to synthesize genkwain from glucose, two additional genes (*TAL* and apigenin 7-*O*-methyltransferase (*POMT7*)) were needed. TAL uses tyrosine to make *p*-coumaric acid, and POMT7 catalyzes the conversion of apigenin to genkwanin. The six genes of the genkwanin biosynthetic pathway were introduced into *E. coli* (B-AP4) and the production of genkwanin was examined. As shown in Fig. 3B, HPLC spectra generated from *E. coli* transformants harboring the six genes showed several peaks. One of these peaks (at 12.5 min) had the same retention time as a genkwanin standard. The molecular mass of the peak at 12.5 min was 284 Da (Fig. 3E), which corresponded to that of a standard genkwanin (Fig. 3D). Besides the molecular ion peak of $[M-H]^-$, the fragmentation patterns at *m/z* 283 and the MS2 (*m/z* 268) and MS3 (*m/z* 240) of P5 (Fig. 3E) were indistinguishable to those of the standard genkwanin (Fig. 3D). In addition, P5 had a similar UV-spectrum with standard genkwanin (Fig. 3C). These results suggested that genkwanin was synthesized from glucose in the *E. coli* transformant.

The content of tyrosine in *E. coli* is critical, because *p*-coumaric acid is synthesized from tyrosine. The *aroG* (3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase) that condenses phosphoenolpyruvate (PEP) and

Table 1. Plasmids and strains used in the present study.

Plasmids / *E. coli* strain	Relevant properties or genetic marker	Source or reference
Plasmids		
pACYCDuet	P15A ori, Cm^r	Novagen
pCDFDuet	CloDE13 ori, Str^r	Novagen
pETDuet	f1 ori, Amp^r	Novagen
pC-pOs4CL-pPeCHS	pCDFDuet harboring *4CL* from *Oryza sativa* and *CHS* from *Populus euramericana*. Each gene is controlled by an independent *T7* promoter.	[11]
pC-pPeCHS-Os4CL	pCDFDuet harboring *CHS* from *Populus euramericana* and *4CL* from *Oryza sativa*. Both genes are controlled by one *T7* promoter.	[11]
pC-pPeCHS-pOs4CL-pMtCHI	pCDFDuet harboring *4CL* from *Oryza sativa*, *CHS* from *Populus euramericana*, and *CHI* from *Medicago truncatula*. Each gene is controlled by an independent *T7* promoter.	This study
pC-pPeCHS-Os4CL-pMtCHI	pCDFDuet harboring *CHS* from *Populus euramericana*, *4CL* from *Oryza sativa*, and *CHI* from *Medicago truncatula*. Both *CHS* and *4CL* are controlled by one *T7* promoter. *CHI* is controlled by an independent *T7* promoter.	This study
pE-FNS	pETDuet harboring *FNS* from *Petroselinum crispum*	This study
pE-POMT7-FNS	pETDuet harboring *POMT7* from *Populus deltoids* and *FNS* from *Petroselinum crispum*	This study
pA-SeTAL	pACYCDuet carrying *TAL* from *Saccharothrix espanaensis*	[12]
pA-aroG-SeTAL-tyrA	pACYCDuet carrying *TAL* from *S. espanaensis, aroG,* and *tyrA* from *E. coli*	[12]
pA-$aroG^{fbr}$-SeTAL-$tyrA^{fbr}$	pACYCDuet carrying *TAL* from *S. espanaensis*, $aroG^{fbr}$, and $tyrA^{fbr}$ from *E. coli*	This study
Strains		
E. coli BL21 (DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm lon* (DE3)	Novagen
B-AP1	BL21 harboring pC-pOs4CL-pPeCHS and pE-FNS	This study
B-AP2	BL21 harboring pC-pPeCHS-pOs4CL-pMtCHI and pE-FNS	This study
B-AP3	BL21 harboring pC-pPeCHS-Os4CL-pMtCHI and pE-FNS	This study
B-AP4	BL21 harboring pA-SeTAL, pC-pPeCHS-Os4CL-pMtCHI and pE-FNS	This study
B-AP5	BL21 harboring pA-aroG-SeTAL-tyrA, pC-pPeCHS-Os4CL-pMtCHI and pE-FNS	This study
B-AP6	BL21 harboring pA-$aroG^{fbr}$-SeTAL-$tyrA^{fbr}$, pC-pPeCHS-Os4CL-pMtCHI and pE-FNS	This study
B-GK	BL21 harboring pA-$aroG^{fbr}$-SeTAL-$tyrA^{fbr}$, pC-pPeCHS-Os4CL-pMtCHI and pE-POMT7-FNS	This study

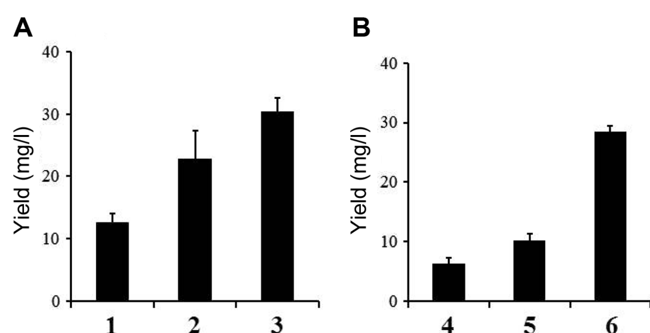

Fig. 2. (**A**) Production of apigenin by different *Escherichia coli* strains and (**B**) effect of tyrosine on the production of genkwanin.

erythrose 4-phosphate (E4P) to form DAHP, and *tyrA* (chorismate mutase/prephenate dehydrogenase) that converts prephenate into 4-hydroxy-phenylpyruvate are the rate-limiting steps of tyrosine biosynthesis [19]. Therefore, overexpression of these two genes in *E. coli* increases the tyrosine content. In addition, cells carrying feedback inhibition resistance versions of these two genes ($aroG^{fbr}$ and $tyrA^{fbr}$) were able to produce more tyrosine than with wild-type *aroG* and *tyrA* [20]. Three *E. coli* transformants (B-AP4 ~ B-AP6 in Table 1) were made and the production of apigenin was examined. As expected, B-AP6 (23 mg/l) produced more genkwanin than *E. coli* harboring either B-AP4 (7 mg/l) or B-AP5 (10 mg/l) (Fig. 2B). HPLC profiles

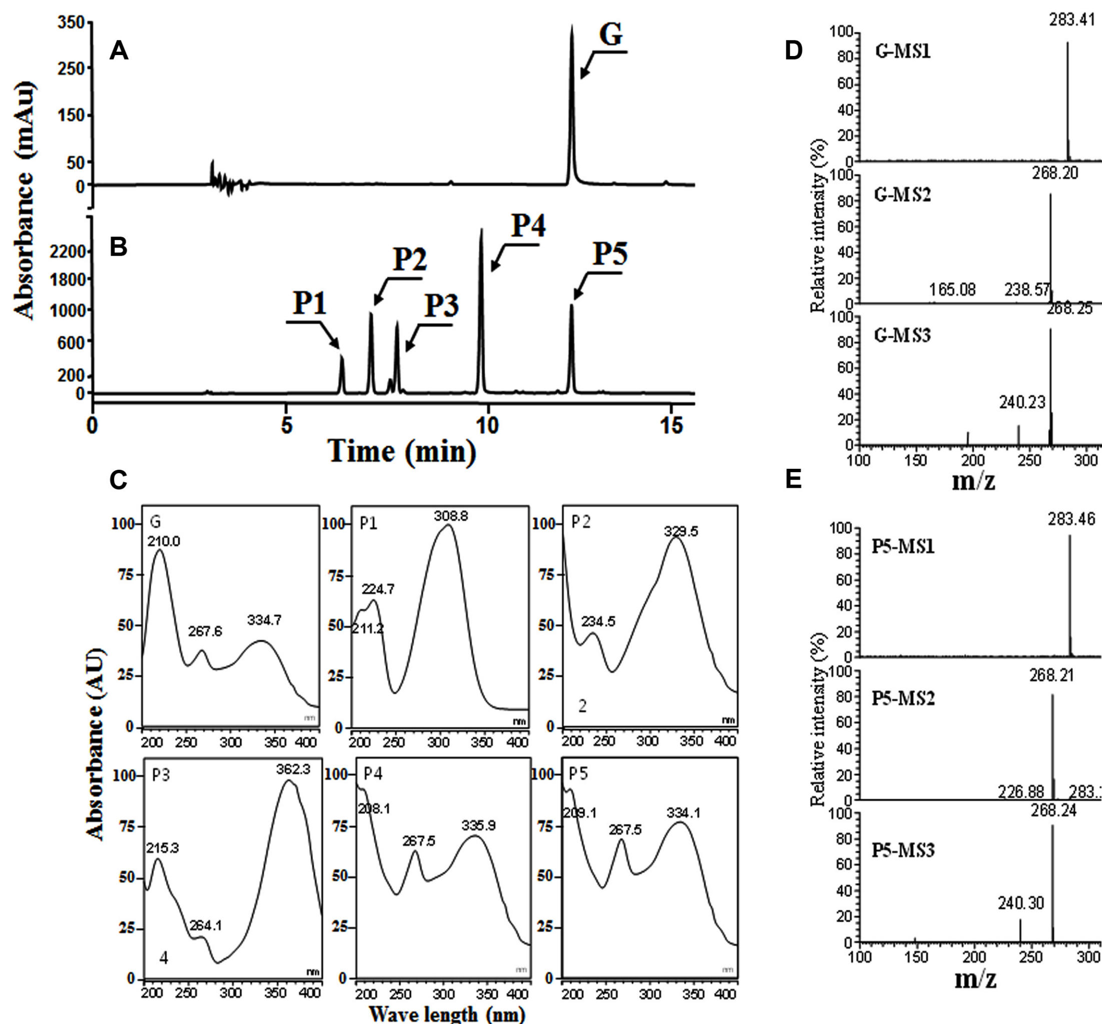

Fig. 3. HPLC and MS spectra of reaction products from B-AP4.

(**A**) HPLC profile of standard genkwanin; (**B**) HPLC profile of reaction products. P1 was identified to be *p*-coumaric acid by comparing with authentic *p*-coumaric acid. P2 and P3 were likely to be bis-noryangonin (BNY) and naringenin chalcone, respectively. P4 was identified to be apigenin by comparing with authentic apigenin. P5 was identified to be genkwanin. (**C**) UV spectra of authentic genkwanin and reaction products. (**D**) MS1, MS2, and MS3 spectra of standard genkwanin (G). (**E**) MS1, MS2, and MS3 spectra of reaction product (P5).

of the reaction product from *E. coli* harboring pA-aroG^{fbr}-TAL-tyrA^{fbr} still contained apigenin (data not shown). However, lower amounts of apigenin were observed in B-AP4 and B-AP5. These results revealed that introducing the feedback-insensitive *aroG* and the *tyrA* genes increases the production of flavones. Therefore, introducing the *P7OMT* gene in the *E. coli* harboring pA-aroG^{fbr}-TAL-tyrA^{fbr} would likely increase genkwanin production.

For the production of genkwanin from glucose, *POMT7* was transformed into B-AP6 and the resulting transfomant was named B-GK. Using B-GK, the production of genkwanin from glucose was examined. Initial cell density was first determined. The cell density was adjusted to OD_{600} = 0.5, 1.0, 1.5, and 2.0. The production of genkwanin was analyzed after 24 h incubation at 30°C. The cell density of OD_{600} = 1.0 showed the highest yield among the tested cell densities, followed by OD_{600} = 0.5, 1.5, and 2.0. Using B-GK at OD_{600} = 1.0, the effect of temperature on the production of genkwanin was evaluated at 25°C, 30°C, and 37°C. The incubation temperature at 30°C gave a higher yield than 25°C or 37°C. Using the optimized cell density and reaction temperature, the production of genkwanin from glucose using the strain B-GK was monitored for 36 h. Both apigenin and genkwanin were produced rapidly. However, as observed above, apigenin was synthesized more rapidly than genkwanin. The highest production was

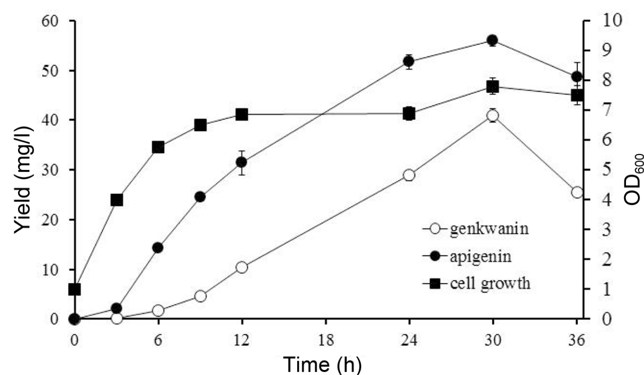

Fig. 4. Production of genkwanin by *Escherichia coli* strain B-GK. We monitored cell growth (filled square), apigenin production (filled circle), and genkwanin production (empty circle), periodically.

observed at 30 h, at which time approximately 41 mg/l genkwanin was produced, while approximately 55 mg/l apigenin was remaining (Fig. 4). At 36 h, the yields of both apigenin and genkwanin had declined. At the time, *E. coli* growth also started declining, which indicated that cells began to die. It seemed that some *E. coli* cell debris such as lipids and fatty acids inhibited the extraction of apigenin and genkwanin from the culture, which lowered the final yield after 36 h.

Apigenin has been synthesized from *p*-coumaric acid in *Saccharomyces cerevisiae* with a final yield of approximately 3.2 mg/l [17]. Apigenin was also synthesized from glucose in *E. coli* with a yield of 13 mg/l [25]. If it is assumed that there was no degradation during the biosynthesis of genkwanin, approximately 100 mg/l of apigenin was synthesized from glucose in the current study. The final apigenin yield would likely have been higher if apigenin was synthesized from *p*-coumaric acid. These differences in final yield could be a result of the host organism (*E. coli* and yeast) and/or different sources of flavone biosynthesis genes.

Apigenin was biotransformed into genkwanin by Kim *et al.* [9] with a final yield of approximately 17 mg/l, and some apigenin was not converted into genkwanin. The synthesis of genkwanin from glucose reported here was higher than that from apigenin. Therefore, the current approach could be applicable to the synthesis of apigenin derivatives from cheap or available precursors.

Until recently, flavonoid synthesis from glucose using *E. coli* was targeted to the synthesis of naringenin. Although naringenin contains several biological activities [14, 16, 26, 38], flavone and flavonol derivatives have novel activities that are not found in naringenin. Findings from the current study and others [27, 33, 38] show that flavone and flavonol derivatives can be synthesized using *E. coli* or yeast. Although it is still challenging, it should now be possible to synthesize a particular bioactive flavonoid using *E. coli*.

Acknowledgments

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