

Biological Synthesis of 7-*O*-Methyl Apigenin from Naringenin Using *Escherichia coli* Expressing Two Genes

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Within the secondary metabolite class of flavonoids, which consist of more than 10,000 known structures, flavones define one of the largest subgroups. The diverse function of flavones in plants as well as their various roles in the interaction with other organisms offers many potential applications including in human nutrition and pharmacology. We used two genes, flavone synthase (*PFNS-1*) that converts naringenin into apigenin and flavone 7-*O*-methyltransferase (*POMT-7*) that converts apigenin into 7-*O*-methyl apigenin, to synthesize 7-*O*-methyl apigenin from naringenin. The *PFNS-1* gene was subcloned into the *E. coli* expression vector pGEX and *POMT-7* was subcloned into the pRSF vector. Since both constructs contain different replication origins and selection markers, they were cotransformed into *E. coli*. Using *E. coli* transformants harboring both *PFNS-1* and *POMT-7*, naringenin could be converted into 7-*O*-methyl apigenin, genkwanin.

Keywords: Flavone synthase, genkwanin, *O*-methyltransferase

Plants synthesize various secondary metabolites that serve as valuable sources for development of nutraceuticals and new drugs [1]. In contrast to primary metabolites, these secondary metabolites with valuable biological activities are synthesized in limited species during a particular time in their growth periods. Thus, one of the drawbacks to utilize the plant secondary metabolites is the limited supplement. In addition, their structural complexity makes chemical synthesis impractical [4].

Flavonoids are a highly diverse class of secondary plant metabolites, and more than 10,000 structures have been elucidated up to now [15]. It is already well established that flavonoids have a significant impact on various aspects of plant biology including UV-protection, flower coloration, interspecies interaction, and plant defense [18]. Flavonoids

also displayed nutritional values and medicinal benefits to humans, such as antioxidant or putative anticancer activities [3, 13]. Like other secondary metabolites, total synthesis of flavonoids demands complicated procedures that are impractical for large-scale production. To circumvent these problems, biological modification of flavonoids using microorganisms harboring genes with interest has been successfully undertaken [5, 9]. In these approaches, and *Escherichia coli* transformant containing flavonoid modification enzyme(s) was employed and inexpensive substrate was supplied to the culture medium to produce a more valuable product [11].

Among the diverse flavanoids, naringenin is a common starting material for biosynthesis of more complicated flavonoids. Hydroxylation, formation of double bond, methylation, glycosylation, and manonylation are involved in further processes of naringenin [15]. With consideration of the biological impact of flavonoids on humans, simple extracts from plants might not satisfy the demand, and chemical synthesis needs complicated processes. We made an advantage of biosynthesis of flavonoids using an *E. coli* system containing cloned flavonoid biosynthetic genes. In this report, we designed two constructs to synthesize a 7-*O*-methyl apigenin (known as genkwanin) from naringenin. Genkwanin showed antibacterial activity against *Vibrio cholera* and *Enterococcus faecalis*. In addition, it contained anti-inflammatory activity [12]. The biological activity of genkwanin was tested with plant extract so only a limited amount of genkwanin could be supplied. Hence, we tested with two genes whether genkwanin was biologically synthesized from naringenin. Conversion of naringenin to genkwanin is catalyzed by two enzymes; flavone synthase (FNS) converts naringenin into apigenin, and 7-*O*-methyltransferase transfers a methyl group into the 7-hydroxy group of apigenin. The *FNSI* gene (*PFNS-1*), which is one of the dioxygenase family encoding flavone synthase, was cloned from poplar (*Populus deltoids* Marsh) using reverse transcription polymerase chain reaction and sequenced. The open reading frame of *PFNS-1* (TIGR Accession No. TC66305) was subcloned into *E. coli*

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expression vector pGEX 5X-2 (Amersham, U.S.A.). FNSI utilizes only the *S*-form of naringenin [17]. Naringenin found in nature is *S*-form because it is synthesized from *p*-coumaroyl-CoA and three molecules of malonyl-CoA with chalcone synthase and chalcone isomerase [2]. Naringenin contains a chiral center at carbon 2, and commercial naringenin is a mixture of two enantiomers. The second gene, *POMT-7* which catalyzes the reaction from apigenin into genkwanin, was previously reported [6] and subcloned into pRSF-Duet vector (Amersham, U.S.A.), which contains a different selection marker and replication origin from those of pGEX 5X-2. Both constructs were transformed into *E. coli* BL21.

In order to express both *PFNS-1* and *POMT-7* in one cell, the transformant harboring both constructs was grown for seed culture in LB medium containing 50 µg/ml ampicillin and 50 µg/ml kanamycin. The seed culture was used to inoculate LB medium containing the two antibiotics. The proteins were induced by addition of IPTG into the medium. After induction, cells were harvested, and expression of both proteins was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Fig. 1, PFNS-1 was expressed as an approximately 64-kDa protein, which was the sum of the molecular mass of PFNS-1 and that of fusion protein glutathione *S*-transferase (GST; 26-kDa). *POMT-7* was also expressed. We did not attempt to purify both proteins.

In order to optimize the condition of genkwanin production, the cell concentration for induction of both proteins was optimized. The cells were grown until absorbance at 600 nm reached to 0.4, 0.6, 0.7, or 0.8, respectively. Then, IPTG

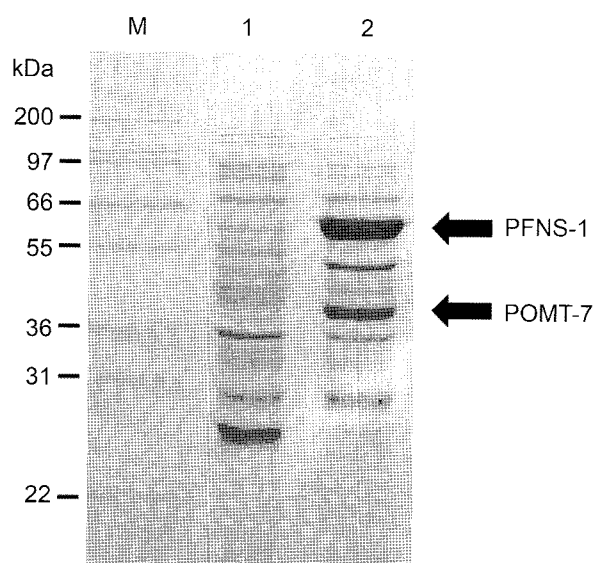


Fig. 1. Expression of PFNS-1 and POMT-7 in *E. coli*. PFNS-1 was fused with GST and POMT-7 was fused with His-tag. The soluble fraction of *E. coli* lysate expressing both proteins was analyzed using SDS-PAGE. The arrows indicate the expressed recombinant proteins.

was added at the final concentration of 1 mM and the culture was grown for 5 more hours at 28°C. Addition of naringenin and incubation were carried out as described below. The most effective production of genkwanin was done with the cell concentration at $A_{600}=0.6$. At this concentration, genkwanin was produced 83% more than at $A_{600}=0.8$.

In order to convert naringenin into genkwanin, the *E. coli* cells after induction of both proteins was washed with 10 mM potassium phosphate buffer containing 1% glucose. Three different pHs (6.0, 7.0, and 8.0) of phosphate buffer were tested and pH 6 was the best followed by 8.0 and 7.0. Thus, potassium phosphate buffer at pH 6.0 was used. The cells were finally resuspended in the same buffer and 100 µM of naringenin, 5 mM of ascorbate, and 10 µM of $FeSO_4$ were added. The resulting mixture was incubated at 28°C for 15 h. The reaction products were extracted with an equal volume of the ethylacetate, twice, and the extract was dried under vacuum and dissolved with methanol. The reaction products were also analyzed by HPLC on a Varian C18 reversed-phase column (Varian Polaris C18-A 250×4.6 mm) and a photodiodearray detector. The analysis condition of metabolites using HPLC was done as described in Lee *et al.* [8].

Analysis of the reaction product using HPLC showed three peaks (Fig. 2A). The first peak at 15 min was determined to be a substrate naringenin by comparing the retention time of it with that of authentic naringenin (Fig. 2D). The second peak at 16 min had the same retention time with apigenin and the fraction containing the second peak was subjected to mass spectrometry that revealed its molecular mass as 270 Da, which corresponds with the molecular mass of apigenin. It indicated that PFNS-1 successfully converted naringenin into apigenin. The molecular mass of

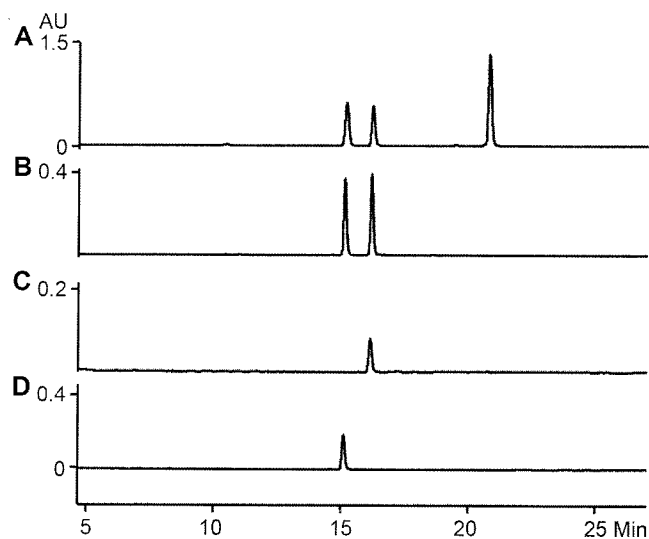


Fig. 2. HPLC analysis of reaction products. **A.** Reaction product of naringenin with PFNS-1 and POMT-7; **B.** Product of PFNS-1; **C.** Authentic apigenin; **D.** Authentic naringenin.

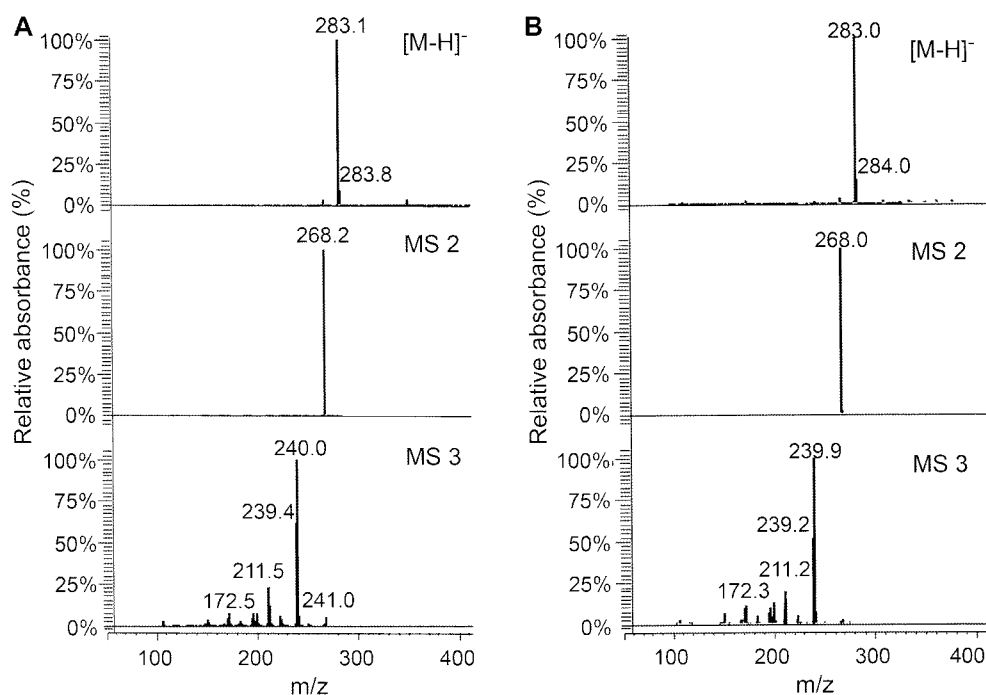


Fig. 3. Identification of reaction product using tandem mass spectrometry. A. Authentic genkwainin; B. Reaction product of naringenin with *E. coli* expressing *PFNS-1* and *POMT-7*.

the third peak at 20 min was determined to be 284 Da, indicating the one methyl group was attached to apigenin, an indication it is a reaction product of *POMT-7*. In order to verify the structure of the third peak, MS/MS analysis was carried out [7] and its fragmentation pattern was compared with that of authentic genkwainin. As shown in Fig. 3, the fragmentation pattern of these two compounds was indistinguishable, suggesting that the reaction product is genkwainin. These results showed that conversion of

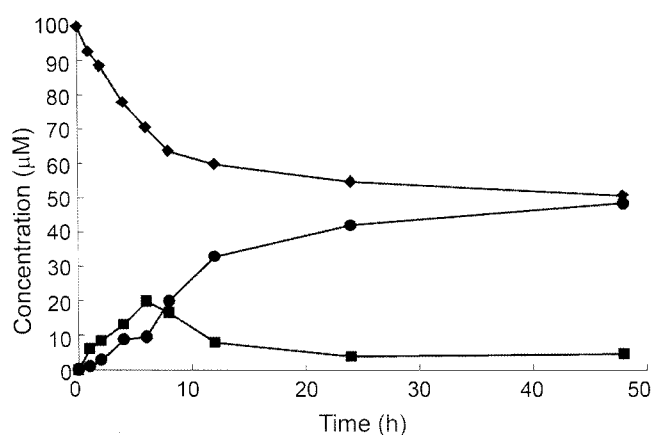


Fig. 4. Conversion of naringenin to apigenin and then into 7-*O*-methyl apigenin (genkwainin) with *E. coli* expressing *PFNS-1* and *POMT-7*
 ◆: Naringenin; ■: apigenin; ●: genkwainin.

naringenin to genkwainin is feasible using *E. coli* expressing *PFNS-1* and *POMT-7*.

The production of genkwainin using *E. coli* transformant containing both *PFNS-1* and *POMT-7* was monitored. As a control, *E. coli* transformant harboring empty vectors was used. The amount of naringenin with *E. coli* transformant harboring empty vectors was decreased approximately 3 µM, which seems to be a result of spontaneous degradation. One hundred µM of naringenin was added to the culture as described above. The reactant was collected periodically and analyzed using HPLC (Fig. 3). Production of apigenin was observed after 1 h of incubation and it continued to increase until 6 h. Genkwainin was observed from 1 h and continued to increase to 53 µM. The amount of naringenin gradually decreased to 40 µM. The remaining naringenin is likely to be 2-*(R)*-naringenin because FNS only converts (*S*)-naringenin but does not have activity for (*R*) form.

The utilization of microorganisms for the production of metabolites has been carried out. The combination of several genes from different organisms resulted in the production of complicated secondary metabolites of interest, which were not difficult to be synthesized chemically [4]. However, reconstruction of the whole biosynthetic pathway sometimes demands technical challenges and the yield of the final product using the whole biosynthetic pathway is often low. As an alternative, the supplement of cheap reaction intermediates to the reaction medium could be a way to circumvent these problems. Hence, biotransformation using one or two key genes might be a more valuable tool for the

production of biologically active compounds like genkwanin. This approach was carried out to produce genistein from naringenin using isoflavone synthase and cytochrome P450 reductase in *E. coli* [10]. The key element for efficient production of metabolites would be to find an optimal gene. Genome projects from a myriad of organisms will serve as the tool box for the selection of genes.

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