Production of C-glucosides of flavonoids and related compounds by *Escherichia coli* expressing buckwheat C-glucosyltransferase

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**Abstract**  
C-Glucosides are glucose-containing glycosides that have carbon–carbon bonds between the anomeric carbon of the sugar moieties and aglycon, rendering the molecules remarkably stable against hydrolysis by enzymes or acids. In this work, we showed the production of C-glucosides of flavonoids and related compounds (i.e., 2-hydroxyflavanone, dihydrochalcone, and trihydroxyacetophenone) by *Escherichia coli* expressing buckwheat C-glucosyltransferase. The substrates in their respective cultures were taken up by the cells and C-glucosylated, and the products were released into the culture media. The bioconversion process was completed in 1–2 h, but products were already observed immediately after addition of the substrates (200 µM). The conversion rates of these substrates reached 80–95%. Without addition of glucose to the conversion media, almost no C-glucosides were produced. Although the amounts of the substrates fed to their respective cultures were limited by their solubility in water, repeated addition of the substrate to the culture at regular time intervals effectively increased the total amount of product obtained.

**Key words:** flavonoid C-glucoside, bioconversion in *E. coli*, C-glucoside production, C-glucosyltransferase, biocatalyst.

**Introduction**

Glycosylation is one of the predominant processes used by plants to modify their secondary metabolites. The process usually occurs at O-, N-, S-, or C-atoms of the metabolites, with the most common plant glycosides being O-glucosides (Jones and Vogt 2001). With this modification, the compounds possess increased water solubility and chemical stability, which regulate their biological activity and accumulation (Bowles et al. 2005; Gachon et al. 2005). Thus, glycosylation is important for the management of secondary metabolites in plants. With the exception of some anthocyanin glycosylations (Matsuba et al. 2010), glycosylations are usually catalyzed by uridine 5′-diphosphate (UDP)-sugar: glycosyltransferases (UGTs) (Bowles et al. 2005; Gachon et al. 2005).

C-Glycosides are characterized by the carbon–carbon bond that forms between the anomeric carbon of sugar moieties and the carbon atom of aglycon (Franz and Grün 1983). Because of this bond, C-glycosides are extremely stable against enzyme or acid hydrolysis compared with other glycosides that can be hydrolyzed. Many kinds of plant C-glycosides have been reported (Franz and Grün 1983), and the most common are the flavonoid C-glycosides that have been found in mosses, liverworts, ferns, gymnosperms, monocots, and dicots (Jay 1994; Talhi and Silva 2012). These C-glycosides are known to show various biological activities in planta, such as antioxidant, antifungal, insecticide, and allelochemical activities (Byrne et al. 1996; Hooper et al. 2010; Jay 1994; McNally et al. 2003). In animals, flavonoid C-glycosides have shown antioxidant, anti-inflammatory, antihyperglycemic, anti-obesity, and anti-ulcer activities (reviewed by Talhi and Silva 2012). Recently, many other medicinal effects were also reported, such as the abilities to potentiate pentobarbital-induced sleep (Wang et al. 2008) and to confer beneficial effects on memory loss (de Oliveira et al. 2014; Jung et al. 2014). Thus, flavonoid C-glycosides have received much attention for their human health and pharmaceutical applications.

Flavonoid C-glycosides are obtained by purification from natural resources. However, this is difficult and
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expensive in many cases, because these compounds are present at low concentrations and the process is complicated by the removal of other constituents. Chemical syntheses of flavonoid C-glucosides have been reported (reviewed by Talhi and Silva 2012), but the processes required many steps and resulted in relatively low total yields.

Brazier-Hicks et al. (2009) isolated a C-glucosyltransferase (CGT) gene from rice (OsCGT) and characterized it as belonging to the UGTs. The substrates of CGT were 2-hydroxyflavanones, and the products were then dehydrated to become C-glucosylflavones. Using this OsCGT enzyme, two groups have reported the production of flavone- and/or dihydrochalcone-C-glucosides via biotransformation in the yeast expression system (Brazier-Hicks and Edwards 2013) and enzymatic conversion coupled with sucrose synthase (Bungaruang et al. 2013).

We recently characterized CGTs from buckwheat (FeCGTa and FeCGTb; Nagatomo et al. 2014). These CGTs showed significant activity against flavonoid substrates possessing a basic skeleton of 2′,4′,6′-trihydroxyacetophenone (THAP), such as 2-hydroxyflavanones and dihydroxychalcone. They also reacted significantly with non-flavonoid substrates possessing THAP-like skeleton, such as THAP and 2-phenyl-2′,4′,6′-trihydroxyacetophenone (PhTHAP). Compare to these flavonoid substrates, THAP has a structure lacking B-ring of flavonoids, and PhTHAP has a truncated methylene chain between A- and B-ring (Figure 1). OsCGT showed only faint activities against THAP, suggesting the broader substrate specificity of the FeCGTs. Therefore, FeCGTs were expected to be good tools for the biological production of C-glucosides.

In this study, we applied an Escherichia coli expression system for the production of C-glucosides, as an easy and useful biocatalyst. Lim and colleagues (Lim 2005; Lim et al. 2004) proposed E. coli expressing the plant UGT genes as novel biocatalysts for the bioconversion of aglycons to their glycosides, and other authors (Kim et al. 2012, 2013) have achieved high production yields of glycosides with these expression systems.

Materials and methods

Reagents
The reagents used were as follows: phloretin and THAP (Tokyo Chemical Industries, Tokyo, Japan), PhTHAP (Sigma-Aldrich, St. Louis, MO, USA), apigenin (Indofine Chemicals, Hillsborough, NJ, USA), chrysin and DMSO-d_6 (Across Organics, Geel, Belgium), vitexin and isovitexin (Extrasynthèse, Genay, France). 2-Hydroxypinocembrin and 2-hydroxynaringenin were synthesized from chrysin and apigenin, respectively, as previously described (Nagatomo et al. 2014). All other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), and Kanto Chemical Co., Inc. (Tokyo, Japan), unless otherwise specified.

Bioconversion of phenolic substrates by E. coli expressing recombinant FeCGTa

FeCGTa (UGT708C1; GenBank Accession No. AB909375) cDNA subcloned into pET28a(+) (Merck, Darmstadt, Germany) was transformed into E. coli Rosetta™ 2(DE3) (Merck), as described previously (Nagatomo et al. 2014). An aliquot (1 ml) of overnight culture was added to 200 ml of LB medium (Nacalai Tesque) containing kanamycin (50 mg l^-1) and chloramphenicol (34 mg l^-1), and this culture was incubated at 37°C with shaking at 150–200 rpm until the OD_600 reached 0.6. Isopropyl-β-D-1-thiogalactopyranoside (final concentration, 0.4 mM) was added and the culture was further incubated at 22°C for 24 h with shaking at 150–200 rpm. The bioconversion was performed according to the method described by Kim et al. (2012) with some modifications. The bacterial cells expressing FeCGTs were precipitated at 4000 × g for 10 min, and the pellet was suspended in M9 basal medium (Green and Sambrook 2012) containing kanamycin (50 mg l^-1) and chloramphenicol (34 mg l^-1), and this culture was incubated at 37°C with shaking at 150–200 rpm until the OD_600 reached 0.6

Figure 1. Substrates used in this study.
at 30°C with shaking at 150–200 rpm, and an aliquot of the medium was collected at appropriate intervals for product determination. To confirm an effect of sugar concentration on the conversion efficiency, glucose (0.4–2%) or maltose (2%) was added into M9 basal medium, and phloretin was used as a substrate. For production from the substrates phloretin and PhTHAP, the substrate was added at six different times to 200 ml of culture, at 1.5–2.5 h intervals, for a total of 12 h. The culture medium containing the compounds was adsorbed to a Sep-Pak Plus C18 column (Waters, Milford, MA, USA) to remove residual glucose, and was then eluted with methanol. The eluates were diluted with water and adsorbed to an ODS column (Wako-gel 50C18, 15 mm i.d. X 120 mm; Wako Pure Chemical Industries, Ltd.), equilibrated with 20% methanol, for purification. The compounds were eluted with a linear gradient of 20–60% methanol (300 ml), and the fraction containing the product was concentrated and crystallized. NMR spectra were recorded on a Bruker Avance-400 spectrometer (Bruker Biospin, Yokohama, Japan), and compared with reported NMR spectra of related compounds (Ogawa et al. 2001; Yerepyan et al. 2010). Phloretin C-glucoside (nothofagin): 1H NMR (400 MHz, DMSO-d6): δ 13.77 (1H, s, Ar-OH), 11.03 (1H, s, Ar-OH), 10.05 (1H, s, Ar-OH), 9.12 (1H, s, Ar-OH), 7.02 (2H, d, J = 8.4 Hz, H-2 and H-6), 6.66 (2H, d, J = 8.4 Hz, H-3 and H-5), 5.94 (1H, s, H-5″), 4.82 (1H, m, glucose-OH), 4.79 (1H, m, glucose-OH), 4.51 (1H, d, J = 9.8 Hz, H-1″), 4.47 (2H, m, glucose-OH), 4.36 (2H, s, H-α), 3.89 (1H, m, H-2″), 3.65 (1H, d, J = 11.5 Hz, H-6″), 3.42 (1H, d, J = 10.9 Hz, H-6″), 3.16–3.12 (3H, m, H-3″−H-5″); 13C NMR (100 MHz, DMSO-d6): δ 202.9 (C=O), 165.4 (C-2′), 164.4 (C-6′), 161.9 (C-4′), 136.4 (C-1′), 130.0 (C-2′ and C-6′), 128.5 (C-3′ and C-5′), 126.6 (C-4′), 104.5 (C-3′), 103.9 (C-1′), 95.0 (C-5′), 81.7 (C-3′), 79.3 (C-1′), 71.0 (C-2′), 70.9 (C-4′), 61.7 (C-6′), 49.4 (C-α).

**LC-MS analysis**

LC-MS analysis was performed using a Waters UPLC ACQUITY SQD system (Waters) with an electron-spray ionization probe, as described previously (Nagamoto et al. 2014).

**Results and discussion**

C-Glycosides have received much attention for their human health and pharmaceutical applications, owing

![Figure 2](image-url)
to their outstanding stability against enzyme and acid hydrolyses. We attempted to produce the C-glucosides of flavonoids and related compounds, using an E. coli strain expressing FeCGTa (Ec-FeCGT). The FeCGTa enzyme catalyzes the C-glucosylation of the open-circular form of 2-hydroxylflavanone and compounds possessing a THAP-like structure, shown in Figure 1 (Nagatomo et al. 2014). The substrate dihydrochalcone phloretin (200 µM) was added to the Ec-FeCGT culture, and the medium contents were monitored at appropriate intervals. HPLC analysis revealed that most of the phloretin (Figure 2B Peak 1) had disappeared after its addition to the culture, and a more hydrophilic compound appeared in the medium (Peak 2). LC-MS analysis identified the compound as the C-glucoside of phloretin (nothofagin) (Figure 2C), showing both the [M–H]− ion at m/z 435 and the fragment ion [M–H–120]− at m/z 315 that is typical of C-glucosides (Jay 1994). Other substrates such as 2-hydroxyflavanones and THAP-like compounds (shown in Figure 1) were also added to the Ec-FeCGT system, and were converted to their C-glucosides and released into the media (Figure 2D). The conversions were fast, such that we detected the products even in the 0h samples, because it took 1–2 min for the cells and the medium to separate after addition of the substrate. The conversion was mostly completed in 1–2 h for all the compounds tested at this condition, and the conversion rate of these compounds reached 80–95% (Figure 2). In the conversion of phloretin, when the incubation was continued even after the substrate had been expended, a small amount of nothofagin was sometimes modified further to produce an unknown compound (Figure 2B peak 3). The compound exhibited the [M–H]− ion at m/z 507, which was bigger than that of nothofagin at m/z 72. This modification was also observed in the conversions of THAP and PhTHAP. We have no idea of the structure of this modification at present. The effect of glucose on the conversion efficiency was tested using phloretin as a substrate (Figure 3). At the glucose concentration of 0.4–2%, most of the phloretin had converted to nothofagin within 1.5 h. In contrast, in the absence of glucose, almost no C-glucosides were found in the culture media. When maltose (a dimer of glucose that can be used as a sugar source by E. coli) was added at the concentration of 2%, the conversion rate was significantly low. These results showed that the addition of glucose was important for the bioconversion, and the conversion rate of these compounds reached 80–95% (Figure 2). In the conversion of phloretin, when the incubation was continued even after the substrate had been expended, a small amount of nothofagin was sometimes modified further to produce an unknown compound (Figure 2B peak 3). The compound exhibited the [M–H]− ion at m/z 507, which was bigger than that of nothofagin at m/z 72. This modification was also observed in the conversions of THAP and PhTHAP. We have no idea of the structure of this modification at present. The effect of glucose on the conversion efficiency was tested using phloretin as a substrate (Figure 3). At the glucose concentration of 0.4–2%, most of the phloretin had converted to nothofagin within 1.5 h. In contrast, in the absence of glucose, almost no C-glucosides were found in the culture media. When maltose (a dimer of glucose that can be used as a sugar source by E. coli) was added at the concentration of 2%, the conversion rate was significantly low. These results showed that the addition of glucose was important for the bioconversion, and 0.4% of glucose was enough for the conversion, with a substrate concentration at 200 µM. The glucose could work as a source of the glucose moiety of UDP-glucose, and as an energy source for the production of UDP-glucose.

We then determined whether repeated substrate additions to the culture medium would result in the production of large amounts of the C-glucosides of each substrate tested. The result of repeated phloretin feeding (total 102 mg) is shown in Figure 4. At every 1.5–2.5 h, the fed phloretin (retention time at 3.35 min) had mostly disappeared from the media, and the peak of nothofagin (retention time at 2.6 min) increased instead. The total conversion efficiency was more than 90%. Repeated feeding until 9 h was effective for the bioconversion of the substrate. After purification by column chromatography, 110 mg of nothofagin was obtained (67% yield from phloretin). In the case of PhTHAP, repeated feeding resulted in an approximately 70% conversion to its C-glucosides. Both of the products were purified and analyzed by NMR in DMSO-d_6. The correlation between H-1 of glucose (δ 4.52 and 4.51) and C-3′ of the compounds (δ 104.0 and 103.9) was observed by HMBC.
analysis, which proved that these products were indeed C-glucosides.

The production of C-glucosides by means of bioconversion using OsCGT has been reported by two groups. Brazier-Hicks and Edwards (2013) reported an 80% yield of nothofagin from 500 µM phloretin in 24 h, in a yeast expressing OsCGT. They also reported a high yield of C-glucosyl-2-hydroxynaringenin from naringenin that was converted by a yeast polyprotein system co-expressing a flavanone 2-hydroxylase from rice and an OsCGT. Compared with the yeast system, the conversion rate by our Ec-FeCGT system was significantly faster, converting more than 90% of phloretin (200 µM) in 1–2 h (Figure 2A). The fed substrates are supposed to enter into E. coli cells by simple diffusion, so that an incorporation rate would be affected by a difference of their concentrations between inside and outside of the cells. Therefore, a high intracellular glucosyltransferase activity is favorable to keep the incorporation rate constant, because the incorporated substrates are efficiently glucosylated and successively excreted out of the cells. In general, a yield of recombinant proteins in E. coli is higher than that in budding yeast, which would have resulted in a fast conversion rate of the substrate in Ec-FeCGT system. It should be noted that a suitable concentration of the fed substrates is important for an efficient conversion. When phloretin was added to the medium at an initial concentration of 2 mM, a precipitation of the substrate occurred, and a conversion to nothofagin was scarcely observed (data not shown). Our Ec-FeCGT system was effective as a biocatalyst of C-glucosylation, not only for C-glucosylflavonoids and THAP-like compounds shown in Figure 1. FeCGTs has a relatively broad substrate preference and high enzyme activity toward these substrates (Nagatomo et al. 2014), which would have resulted in this efficient conversion of these compounds. Enzymatic conversion of phloretin to nothofagin was achieved, with a high yield, using a recombinant OsCGT coupled with sucrose synthase that could reuse the expensive UDP-glucose and enzyme (Bungaruang et al. 2013). Compared with that co-enzymatic conversion process, however, Ec-FeCGT is simpler and inexpensive because it does not require the preparation of the enzymes and the addition of the expensive UDP substrate.

In this work, we have demonstrated the production of C-glucosides of flavonoids and THAP-like compounds, using E. coli expressing buckwheat CGT. Similar to the concept proposed by Lim (2005) and previous works using E. coli cells expressing UGT genes (Kim et al. 2012, 2013), the glucosides produced were released into the media and were easily recovered without the need for extraction from the cells. We conclude that Ec-FeCGT is a simple and useful C-glucoside production system.

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