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Formation of Flavone Di-*O*-Glucosides Using a Glycosyltransferase from *Bacillus cereus*

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Microbial UDP-glycosyltransferases can convert many small lipophilic compounds into glycons using uridine-diphosphate-activated sugars. The glycosylation of flavonoids affects solubility, stability, and bioavailability. The gene encoding the UDP-glycosyltransferase from *Bacillus cereus*, *BcGT-3*, was cloned by PCR and sequenced. *BcGT-3* was expressed in *Escherichia coli* BL21 (DE3) with a glutathione *S*-transferase tag and purified using a glutathione *S*-transferase affinity column. BcGT-3 was tested for activity on several substrates including genistein, kaempferol, luteolin, naringenin, and quercetin. Flavonols were the best substrates for BcGT-3. The enzyme dominantly glycosylated the 3-hydroxyl group, but the 7-hydroxyl group was glycosylated when the 3-hydroxyl group was not available. The kaempferol reaction products were identified as kaempferol-3-*O*-glucoside and kaempferol-3,7-*O*-diglucoside. Kaempferol was the most effective substrate tested. Based on HPLC, LC/MS, and NMR analyses of the reaction products, we conclude that BcGT-3 can be used for the synthesis of kaempferol 3,7-*O*-diglucose.

Keywords: Glycosylation, UDP-glycosyltransferases, kaempferol 3,7-*O*-diglucoside

Genome projects from an increasing number of organisms have been completed or are in progress (http://www.genomesonline.org/). One useful application of this vast number of information is the modification of simple chemicals using microorganisms or proteins expressed in heterologous expression systems such as *Escherichia coli* or yeast. The use of biological systems for modifying chemicals could potentially create regioselective compounds more efficiently than chemical synthesis [14].

Secondary metabolites from plants and microorganisms are diverse and promising candidates for the development of new medicines [5]. Structural modifications of secondary metabolites are an emerging research area. Among the various secondary metabolites, flavonoids are found ubiquitously in nature and have a backbone of C6-C3-C6 [13]. Flavonoids are a good target for biological modification because they contain many hydroxyl groups, which increases their reactivity [3]. In addition, flavonoids have biological functions in humans, including enzyme inhibition and anti-inflammatory, estrogenic, antimicrobial, antiallergic, antioxidant, vascular, and cytotoxic antitumor activities [2]. The different activities of flavonoids can be attributed to their structural diversity. Accordingly, structural modification of flavonoids may be an important way to extend the pools of natural products.

Glycosylation is an essential step for storing and locating small compounds, and it also plays a role in the detoxification of xenobiotics and the alteration of biological activity [4, 10, 17]. Glycosyltransferases (GTs) constitute a superfamily of enzymes involved in synthesizing the carbohydrate moieties of many biological compounds including proteins, lipids, steroids, and other small molecules [4]. According to the current carbohydrate active enzyme (CAZy) database, GTs have been classified into 86 families on the basis of sequence, signature motif, stereochemistry of the glucoside linkage, and known target specificity. One of these families, the Family 1 GT enzymes, UDP glycosyltransferases (UGTs), transfer UDP-activated sugar moieties to specific small acceptor molecules including flavonoids and alkaloids [1].

Various forms of flavonoid *O*-glycosides are found in nature. They contain different kinds and/or different numbers of sugars. Even though flavonoid *O*-monoglycosides are prevalent, di- or triglucosides are also present [16]. We wanted to know if flavonoid di-*O*-glucosides could be synthesized using enzymes from microorganisms. Here, we report the di-*O*-glucosylation of flavonoids using a recombinant UGT, BcGT-3 from *Bacillus cereus*. Polymerase chain reaction (PCR) was carried out to clone the BcGT-3 using gene *B. cereus* 10987 genomic DNA as a template. A forward

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primer 5'-GTGGCACGTGTTTTATTCATTA-3' and reverse primer 5'-TTACTGACCTATAAATGTAAAAATC-3' were designed based on the published gene sequence (GenBank Accession No. AAS41737.1). The PCR product was subcloned into the pGEMT-easy vector (Promega, WI USA) and the resulting plasmid was sequenced. The open reading frame of *BcGT-3* consisted of 1,194 bp encoding a 44.7-kDa protein, and contained a conserved histidine residue (His14) that serves as a base for catalysis [12]. The UDP-sugar binding domain is located at the C-terminal as in other UGTs.

The open reading frame of *BcGT-3* was subcloned into *E. coli* expression vector pGEX 5X-3 (Amersham Biotech, USA) and the resulting construct was transformed into *E. coli* BL21 (DE3). The induction and purification of the recombinant BcGT-3 was carried out as described previously [8]. The purity of the expressed protein was analyzed using SDS-PAGE. The molecular mass of the recombinant BcGT-3 was about 72-kDa, which agreed with the predicted molecular mass of BcGT-3 plus that of the glutathione *S*-transferase (Fig. 1).

To evaluate whether BcGT-3 could utilize flavonoids as a substrate, we tested several flavonoids including isoflavone (genistein), flavanone (naringenin), flavone (luteolin), and flavonol (kaempferol and quercetin). The reaction mixture contained 5 μg of the purified recombinant BcGT-3, 10 mM KH_2PO_4 (pH 7.4), 5 mM $MgCl_2$, 1 mM UDP-glucose, and 100 μM flavonoid. The flavonoids were purchased from Indofine Chemicals (NJ, U.S.A.) or came from our laboratory's collection. The reaction mixture was incubated at 37°C for 10 min and then stopped

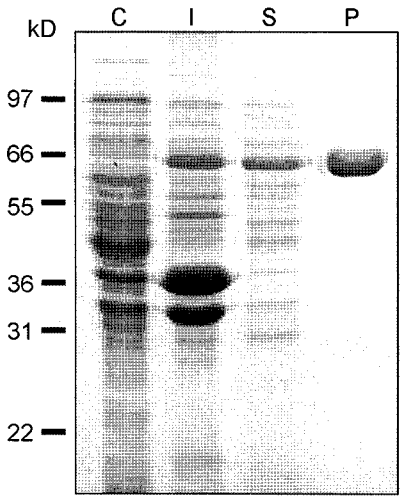


Fig. 1. Expression and purification of BcGT-3 recombinant protein.
C, *E. coli* transformant containing BcGT-3 expression vector lysate before induction; P, *E. coli* transformant lysate after induction; S, Soluble fraction from *E. coli* transformant after induction; P, the purified recombinant BcGT-3.

by adding a trichloroacetic acid/acetonitrile mixture (3.5:50). The reaction products were analyzed using high performance liquid chromatography (HPLC; Varian, CA, U.S.A.) on a C18 reversed-phase column (Varian, CA, USA; 4.60×250 mm, 0.5 μm) with a photodiode array detector. For the analytical scale, the mobile phase consisted of 0.1% formic acid buffer (pH 3.0) and was programmed as follows: 10% acetonitrile at 0 min, 40% acetonitrile at 10 min, 70% acetonitrile at 20 min, and 90% acetonitrile at 25 min. The flow rate was 1 ml/min and UV detection was performed at 340 nm.

Genistein and naringenin yielded one reaction product each whose molecular mass was increased by 162 kDa, indicating that one glucose molecule was attached. Flavone (luteolin) and flavonol (kaempferol and quercetin) produced more than one reaction product. Analysis of these compounds using a mass spectrometer revealed that both mono- and di-*O*-glucosides were produced. For example, HPLC analysis of the kaempferol reaction product displayed two peaks. LC/MS analysis of these products showed that the molecular mass of the first peak at 7.8 min was 324 Da greater than that of kaempferol, indicating that two glucose residues were attached, and the molecular mass of the second peak was increased by 162 Da (Fig. 2A). These results indicate that kaempferol produced both mono- and di-*O*-glucosides. As the reaction time increased, the mono-*O*-glucoside was converted into di-*O*-glucoside. Overall, the double bond between carbons 2 and 3 was important for the formation of di-*O*-glucoside; flavones and flavonols containing a double bond between carbons 2 and 3 produced di-*O*-glucosides, whereas flavanone lacking a double bond did not produce di-*O*-glucosides.

The glucosylation position of the naringenin reaction product was determined by comparing the HPLC retention time and UV-spectrum of the reaction product with those of authentic compounds. The HPLC retention time and UV-spectra of the naringenin reaction product with BcGT-3 were indistinguishable from those of the authentic naringenin 7-*O*-glucoside, indicating that the reaction product was naringenin 7-*O*-glucoside (Fig. 2B). The genistein reaction product was confirmed as genistein 7-*O*-glucoside (Fig. 2C).

The mono-*O*-glucoside of kaempferol was determined to be kaempferol 3-*O*-glucoside, based on the HPLC retention time and UV spectrum [15]. Glucosylation positions of the kaempferol di-*O*-glucoside were determined using a nuclear resonance spectrometer (NMR). The NMR samples were dissolved in 0.5 ml of DMSO-d_6. The NMR spectra of the reaction product and the corresponding authentic product were acquired on a Bruker Avance 400 spectrometer system (9.4T; Karlsruhe, Germany). Detailed experimental procedures followed those described by Kim *et al.* [6]. The ^{13}C NMR spectrum of kaempferol di-*O*-glucoside showed 25 peaks, where 13 peaks belong to kaempferol and 12 peaks to two glucoses. In the HMBC spectrum, we noted two

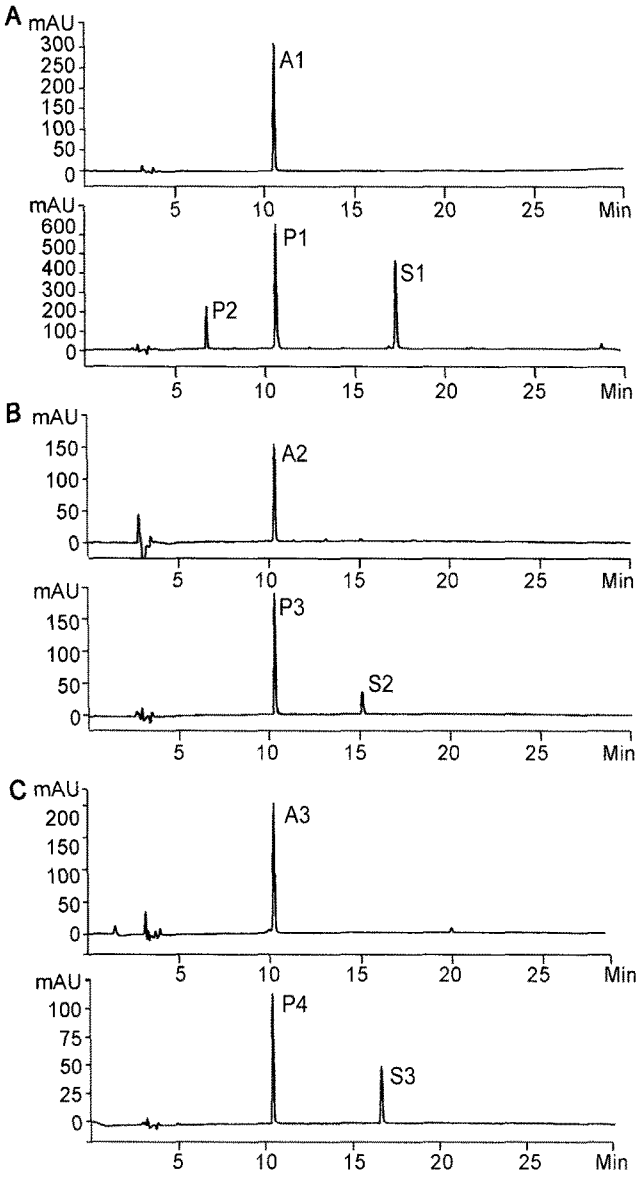


Fig. 2. HPLC analysis of kaempferol (**A**), naringenin (**B**), and genistein (**C**) BcGT-3 reaction products.
A1, authentic kaempferol 3-*O*-glucoside; A2, authentic naringenin 7-*O*-glucoside; A3, authentic genistein 7-*O*-glucoside; P1 and P2, kaempferol reaction product; P3, naringenin reaction product; P4, genistein reaction product; S1, kaempferol; S2, naringenin; S3, genistein.

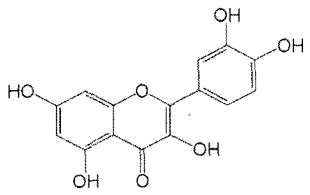
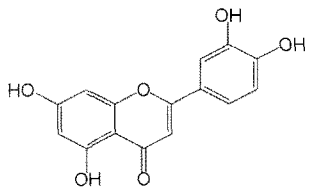
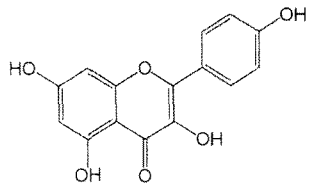
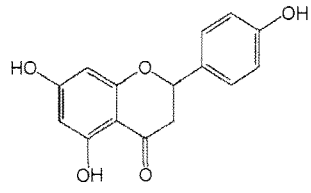
separated long-ranged couplings between C-3 (δ 133.4) of kaempferol and an anomeric proton (δ 5.47) of glucose, and C-7 (δ 162.8) of kaempferol and another anomeric proton (δ 5.07). These observations demonstrated that kaempferol was connected to two glucoses through C-3 and C-7.

Luteolin mono-*O*-glucoside was glucosylated at the 3'-hydroxyl group, which we identified by comparing the retention time with that of authentic luteolin 3'-*O*-glucoside. The glucosylation position of the luteolin di-*O*-glucosides was determined by analyzing the reaction products of a few *O*-methylated luteolins such as 3'-*O*-methyl luteolin, 4'-*O*-methyl luteolin, 3',4'-*O*-dimethyl luteolin, 7,3'-di-*O*-methyl luteolin, and 7,3',4' *O*-trimethyl luteolin. 3'-*O*-Methyl luteolin gave two peaks (mono- and di-*O*-glucosides; the di-*O*-glucoside was expected to have a glucose at the 7- and 4'-hydroxyl groups). 3',4'-*O*-Dimethyl luteolin also produced one peak, which was predicted to glucosylate at the 7-hydroxyl group. Furthermore, 7,3'-*O*-dimethyl luteolin also produced one peak that was predicted to glucosylate at the 4'-hydroxyl group. 7,3',4' *O*-trimethyl luteolin did not give any reaction product. The 5-hydroxyl group of flavonoids is seldom glucosylated [8, 10]. Taken together, these results indicate that the glucosylation position of luteolin di-*O*-glucosides was probably at the 7- and 3'-hydroxyl groups.

The reaction of quercetin with BcGT-3 also produced di-*O*-glucosides. As with kaempferol, BcGT-3 first converted quercetin into quercetin 3-*O*-glucoside, which was eventually converted into quercetin 3,7-*O*-diglucosides. It is generally known that the 3-hydroxyl group is glycosylated first followed by the 7-hydroxyl group, and finally either the 3' or 4'-hydroxyl group [7]. Based on the glucosylation positions observed with kaempferol, luteolin, and quercetin, the glycosylation of flavonoids using BcGT-3 also followed this rule.

We also examined the substrate preference of BcGT-3. Flavonols (quercetin and kaempferol) and flavones (luteolin)

Table 1. Relative flavonoid conversion rate by BcGT-3.

Flavonoid	Structure	Relative conversion rate (%)
Quercetin		100
Luteolin		99.7
Kaempferol		99
Naringenin		75.8

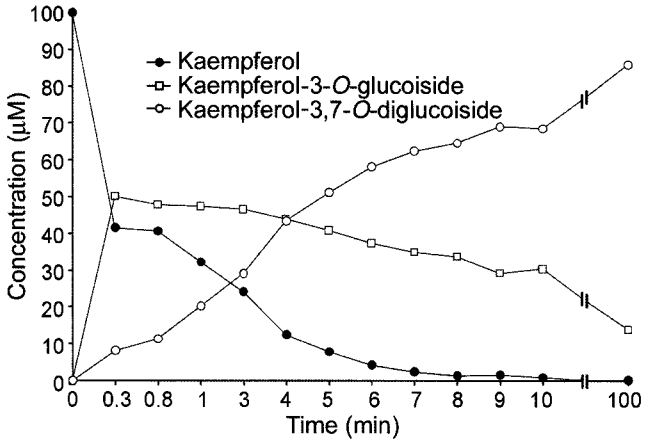


Fig. 3. Bioconversion of kaempferol using BsGT-3.

were better substrates than flavanones (naringenin), suggesting that the double bond between carbons 2 and 3 was important. There was not much difference among flavonols and flavones (Table 1).

The conversion of kaempferol into 3,7-*O*-diglucoside was monitored periodically. One hundred micromoles of kaempferol was incubated with 1 μg of the purified recombinant protein. The substrate was converted into 3-*O*-glucoside, which was then eventually converted into 3,7-*O*-diglucoside. After a 10 min incubation, kaempferol was completely used up and 35 μM of kaempferol 3-*O*-glucoside and 65 μM of kaempferol 3,7-*O*-diglucoside were formed. After a longer incubation (100 min), the final concentration of kaempferol 3-*O*-glucoside and kaempferol 3,7-*O*-diglucoside was 15 and 85 μM, respectively (Fig. 3).

In vitro enzymatic reaction products of plant flavonoid UGTs are generally mono-*O*-glycosides. Comparison with the modeled structure of BcGT-3 with the known molecular structure of flavonoid *O*-glycosyltransferase from plants [12, 13] revealed why BcGT-3 produced di-*O*-glycosides in our experiments. The two structures shared a high overall structural similarity (unpublished results). Furthermore, the active site of BcGT-3 has a large volume, allowing larger substrates like flavonoid di-*O*-glucoside to fit into it. Compared with plant UGTs, UGTs from microorganisms are promiscuous, in that they recognize diverse substrates. The non-promiscuous nature of plant UGTs might be the reason that plants contain many more UGTs than microorganisms.

Acknowledgments

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References

1. Bowles, D., J. Isayenkova, E.-K. Lim, and B. Poppenberger. 2005. Glycosyltransferases: Managers of small molecules. *Curr. Opin. Plant Biol.* **8:** 254–263.
2. Harvsteen, B. H. 2002. The biochemistry and medical significance of the flavonoids. *Pharmacol. Ther.* **96:** 67–202.
3. Ibrahim, R. K., A. Bruneau, and B. Bantignies. 1998. Plant *O*-methyltransferase: Molecular analysis, common signature and classification. *Plant Mol. Biol.* **36:** 1–10.
4. Jones, P. and T. Vogt. 2001. Glycosyltransferases in secondary plant metabolism; tranquilizers and stimulant controllers. *Planta* **213:** 164–174.
5. Julsing, M. K., A. Koulman, W. J. Woerdenbag, W. J. Quax, and K. O. Oliver. 2006. Combinatorial biosynthesis of medicinal plant secondary metabolites. *Biomol. Eng.* **23:** 265–279.
6. Kim, J. H., B. G. Kim, J. A. Kim, Y. Park, Y. J. Lee, Y. Lim, and J.-H. Ahn. 2007. Glucosylation of flavonoids with *E. coli* expressing glycosyltransferase from *Xanthomonas campestris*. *J. Microbiol. Biotechnol.* **17:** 539–542
7. Kroon, P. A., M. N. Clifford, A. Crozier, A. J. Day, J. L. Donovan, C. Manach, and G. Williamson. 2004. How should we assess the effects of exposure to dietary polyphenol *in vitro*? *Am. J. Clin. Nutr.* **80:** 15–21.
8. Lee, Y. J., B. G. Kim, Y. Park, Y. Lim, H.-G. Hur, and J.-H. Ahn. 2006. Biotransformation of flavonoids with *O*-methyltransferase from *Bacillus cereus*. *J. Microbiol. Biotechnol.* **16:** 1090–1096.
9. Masada, S., Y. Kawase, M. Nagatoshi, Y. Pguchi, K. Terasaka, and H. Mizukami. 2007. An efficient chemienzymatic production of small molecule glucosides with *in situ* UDP-glucose recycling. *FEBS Lett.* **581:** 2562–2566.
10. Méndez, C. and J. A. Salas. 2001. Altering the glycosylation pattern of bioactive compounds. *Trends Biotechnol.* **19:** 449–456.
11. Offen, W., C. Martinez-Fleites, M. Yang, E. Kiat-Lim, B. G. Davis, C. A. Tarling, C. M. Ford, D. J. Bowles, and G. J. Davies. 2006. Structure of a flavonoid glucosyltransferase reveals the basis for plant natural product modification. *EMBO J.* **25:** 1396–1405.
12. Shao, H. S., X. He, L. Achnine, J. W. Blount, R. A. Dixon, and X. Wang, 2005. Crystal structures of a multifunctional triterpene/flavonoid glycosyltransferase from *Medicago truncatula*. *Plant Cell* **17:** 3141–3154.
13. Tahara, S. 2007. A journey of twenty-five years through the ecological biochemistry of flavonoids. *Biosci. Biotechnol. Biochem.* **71:** 1387–1404.
14. Van Lanen, S. G. and B. Shen. 2006. Progress in combinatorial biosynthesis for drug discovery. *Drug Discov. Today Technol.* **3:** 285–292.
15. Vogt, T., E. Zimmermann, R. Grimm, M. Meyer, and D. Strack. 1997. Are the characteristics of betanidin glucosyltransferases from cell-suspension cultures of *Dorotheanthus bellidiformis* indicative of their phylogenetic relationship with flavonoid glucosyltransferases? *Planta* **203:** 349–361.
16. Williams, C. 2006. Flavone and flavonol *O*-glycosides, pp. 749–856. *In* Ö. M. Anderson and K. R. Markham (eds.). *Flavonoids; Chemisty, Biochemistry and Application.* CRC Press, Boca Raton.
17. Yang, J., D. Hoffmeister, L. Liu, X. Fu, and J. S. Thorson. 2004. Natural product glycorandomization. *Bioorg. Med. Chem.* **12:** 1577–1584.