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Glucosylation of hydroxyflavones by glucosyltransferases from *Phytolacca americana*

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ABSTRACT

Cell suspension cultures of *Phytolacca americana* can glucosylate 6- and 7-hydroxyflavone, but not 5-hydroxyflavone. In order to identify the enzymes responsible for these transformations, glucosyltransferases (GTs) from *P. americana* were overexpressed in *Escherichia coli* and purified. The purified *Pa*GT3 enzyme could glucosylate 6- and 7-hydroxyflavone when incubated with UDP-glucose, a glucosyl donor molecule, but *Pa*GT2 could conjugate a glucose moiety only to 6-hydroxyflavone. *E. coli* cells expressing *Pa*GT2 and 3 could also be utilized for the glucosylation of hydroxyflavones. The glucoside products which had accumulated in the medium of overnight *E. coli* cell cultures were isolated using hydrophobic resins. This methodology might be suitable for the glucosylation of aglycones with important health-related properties.

1. Introduction

Glycosylation is one of the mechanisms for converting lipophilic molecules into hydrophilic compounds in order to regulate cellular homeostasis [1,2]. When the gene encoding ZOG1, a *Phaseolus* enzyme shown to *O*-glycosylate *trans*-zeatin, was overexpressed in tobacco callus, much higher levels of supplementary *trans*-zeatin were required for induction of shoot differentiation [3]. In *Arabidopsis* plants, the gene encoding UGT84B1, a glycosyltransferase for indol-3-acetic acid, was overexpressed, and the resulting transgenic plants displayed a phenotype resembling auxin deficiency [4]. The synthesis of glycosides is normally performed by glucosyltransferases (GTs) catalyzing the transfer of the glucose moiety of a nucleotide-activated sugar donor molecule (e.g., UDP-glucose) to an aglycone [5]. In vertebrates, the formation of water-soluble metabolites originating from the exposure to drugs and xenobiotics appears to be a conventional detoxification process [6]. In microorganisms, some GTs are involved in the production of peptidoglycan, which is the major component of the bacterial cell wall; therefore, GTs can be potential targets for drugs against infections by pathogenic bacteria [7]. It is also known that plants produce a variety of glycosylated metabolites. For example, glycoconjugates of aromatic volatiles have been reported in a number of fruits, including grape, apricot, mango, passion fruit, and kiwi [8–12]. Odorous aglycones might be released from the glycosylated volatiles during maturation. The production of anthocyanin is another example of a glucosylation reaction in plants [13]. The compound synthesized in red grape is a colorant that determines flower and fruit color [14,15]. Glucosylation leads to the transport of anthocyanin from the cytosol to the vacuole as the fruit ripens. More than 10,000 GT sequences from various organisms have now been collected and stored in the Carbohydrate-active Enzymes Database [16].

Phytolacca americana (*Pa*) is a perennial plant native to North America. Although the plant is toxic, it is used as a traditional herbal medicine in China [17,18]. The following compounds have been identified in the leaves of *P. americana*: kaempferol 3-*O*-β-D-glucopyranoside, kaempferol 3-*O*-β-D-xylopyranosyl (1→2)-β-D-glucopyranoside, kaempferol 3-*O*-α-L-rhamnopyranosyl (1→2)-β-D-glucopyranoside, kaempferol 3-*O*-diglucoside and quercetin 3-*O*-glucoside [19]. The glucosides of triterpene saponins (e.g., esculentoside B, phytolaccoside E, and esculentoside G) were extracted from the root of *P. americana*, and their structures were established on the basis of spectroscopic analysis [18]. Since these triterpene saponin glucosides were also obtained from cell cultures of *P. americana*, we examined if the cell suspensions could transform exogenous aglycones into their glucosides. Perillyl alcohol, 2,2,5,7,8-pentamethyl-6-chromanol,

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and resveratrol were converted to the corresponding β-glucosides by *P. americana* cell cultures [20–22]. Interestingly, 4-[4-(β-D-glucopyranosyloxy)phenyl]-2-butanone, 4-[(3*S*)-3-hydroxybutyl]phenyl-β-D-glucopyranoside, (2*S*)-4-(4-hydroxyphenyl)but-2-yl-β-D-glucopyranoside, 2-hydroxy-4-[(3*S*)-3-hydroxybutyl]phenyl-β-D-glucopyranoside, and 2-hydroxy-5-[(3*S*)-3-hydroxybutyl]phenyl-β-D-glucopyranoside were produced from 4-(4-hydroxyphenyl)butan-2-one (raspberry ketone), suggesting that the cell suspension cultures of *P. americana* could glucosylate, reduce, and hydroxylate the exogenous substrate (Supplementary Fig. 1) [23].

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2013.01.016.

In order to investigate the glucosyltransferases of *P. americana*, we isolated three glucosyltransferase cDNAs (*Pa*GT1–3) from the cultured cells, heterologously overexpressed *Pa*GT3 in *Escherichia coli*, and purified the enzyme [24]. It was found that purified *Pa*GT3 enzyme could glucosylate resveratrol, capsaicin, kaempferol, quercetin, apigenin, genistein, and naringenin in the presence of UDP-glucose (Supplementary Fig. 2) [22,24]. When *Pa*GT3-expressing *E. coli* cells were cultured in the presence of resveratrol, the glucoside products of resveratrol were detected in the medium of overnight culture [22]. The addition of UDP-glucose was not required because glucosyl donor molecules were regenerated in *E. coli* cells.

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The amino acid sequence of *Pa*GT3 is homologous to that of flavonol 3-*O*-glucosyltransferase from *A. thaliana* (identity 56%) [25] and *Fragaria x ananassa* (identity 57%) [26], anthocyanin 3′-*O*-glucosyltransferase from *Gentiana triflora* (identity 56%) [27], and flavonol 7-*O*-glucosyltransferase from *A. thaliana* (identity 54%) [25]. Sequence identity between *Pa*GT3 and *Pa*GT2 is 27%, and *Pa*GT2 is related to hydroquinone glucosyltransferase from *Rauvolfia serpentine* (identity 58%) [28] and coniferyl alcohol 4-*O*-glucosyltransferase from *A. thaliana* (identity 39%) [29].

In this study, we explored the regiospecificity of glucosylation reaction by *Pa*GTs. Although apigenin (4′,5,7-trihydroxyflavone) was known to be glucosylated by *Pa*GT3 [24], the structure of glucoside product was not determined at the moment. Thus, we examined whether 5-, 6-, and 7-hydroxyflavone could be glucosylated to produce β-*O*-glucosides by cell suspension cultures of *P. americana*, *E. coli* expressing *Pa*GTs, and the purified enzymes. The *Pa*GT3 enzyme could glucosylate 6- and 7-hydroxyflavone, while the *Pa*GT2 enzyme could transfer only the glucosyl group to 6-hydroxyflavone. Neither *Pa*GT3 nor *Pa*GT2 could produce glucoside from 5-hydroxyflavone. Since prodrugs, procosmetics, and prosupplements have recently attracted a great deal of attention, there is a growing need for techniques that are capable of modifying functional compounds. Glucosylation is a useful chemical modification that can be used to stabilize biologically active compounds or to activate physiological functions.

2. Experimental

2.1. Materials

All chemicals and enzymes were purchased from Wako, Nakalai, Sigma–Aldrich, and Toyobo.

2.2. Glucosylation of hydroxyflavones with cultured plant cells

Callus tissue from *P. americana* was prepared as described previously [22]. Briefly, callus tissue (30 g) was cultured under light for 4 weeks, transferred to an Erlenmeyer flask (300 mL) containing 100 mL of MS liquid medium, and cultured with continuous shaking at 25 °C. 5-, 6-, or 7-hydroxyflavone (20 μmol) was dissolved in dimethyl sulfoxide (200 μL) and added to the flask. The cells were incubated for an additional 2 days at 25 °C. Following incubation, the cells were isolated by filtration, disrupted by sea sand, and extracted with methanol (200 mL) for 24 h. The methanol fraction was concentrated and partitioned between water (100 mL) and ethyl acetate (300 mL), and the ethyl acetate fraction was analyzed by high-performance liquid chromatography (HPLC). The yield was estimated on the basis of peak area of hydroxyflavone and its glucoside.

2.3. Time course experiments

Callus tissue (5 g) was cultured in an Erlenmeyer flask containing 50 mL of MS liquid medium with continuous shaking at 25 °C for 2 days. 5-, 6-, or 7-hydroxyflavone (10 μmol) was dissolved in dimethyl sulfoxide (100 μL) and added to the flask. After incubation for 2, 6, 24, and 48 h, the cells were isolated by filtration and disrupted by sea sand. The extraction and analysis procedures were as described in Section 2.2. Products yield was determined based on HPLC peak area and expressed as a relative percentage to the total amount of the whole reaction product extracted.

2.4. Glucosylation of hydroxyflavones by purified PaGT enzymes

*Pa*GT2 cDNA was previously cloned into pET32a, and the resultant plasmid was transformed into BL21(DE3) *E. coli* cells; however, the expression level was low [24]. In order to improve the expression level, we amplified the coding sequence of *Pa*GT2 from *Pa*GT2-pET32a by polymerase chain reaction (PCR) using the primers, 5′-CGGCATGCATGGAAATGGAAGCAC-3′ and 5′-GCGTCGACTTAGCTTTTGCATTGG-3′. The amplified fragment was digested with *Sal*I and *Sph*I and then ligated into pQE30 containing a 6×-His tag at the N-terminus. The resulting plasmid was transformed into M15 *E. coli* cells. *Pa*GT3-pQE30 constructed in our previous study [24] was also transformed into M15 *E. coli* cells.

The transformants were cultured with continuous shaking at 30 °C in Terrific broth (TB) medium containing 50 μg/mL ampicillin. TB medium contained tryptone 10 g, yeast extract 24 g, glycerol 4 g, KH_2PO_4 2.31 g, and K_2HPO_4 12.54 g per 1 L of deionized water. At OD_{600} = 1, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at 0.1 mM, followed by incubation at 30 °C for an additional 16 h. The harvested cells were resuspended in 15 mM potassium phosphate containing 1 mM EDTA and 2 mM 2-mercaptoethanol. The cells were lysed by sonication, and the *Pa*GT enzymes were purified from the supernatant using a His-Accept column (Nakalai). The enzyme solution eluted with the buffer supplemented with 200 mM imidazole was dialyzed with 10 mM Tris–HCl containing 5 mM dithiothreitol and NaCl 100 mM (pH 7.2), concentrated, and stored at −80 °C.

Glucosylation reactions were performed at 37 °C for 60 min in 0.5 mL of 50 mM potassium phosphate buffer (pH 7.2) supplemented with 50 μM hydroxyflavone, 200 μM UDP-glucose, and 5 μM enzyme. The incubation was stopped by adding 1.5% trifluoroacetic acid, and the reaction mixture was analyzed by HPLC to detect the glucoside products. When the K_m and k_{cat} values were determined, the concentration of hydroxyflavone was varied as follows: For glucosylation of 6-hydroxyflavone by *Pa*GT3, 50–150 μM; for glucosylation of 7-hydroxyflavone by *Pa*GT3, 50–200 μM; for glucosylation of 6-hydroxyflavone by *Pa*GT2, 10–120 μM. The average values from more than three independent experiments were used to construct Lineweaver–Burk plots.

To hydrolyze the glucosylated products, the reaction mixture (500 μl) containing β-glucosidase from sweet almond (1 unit) and the glucosides in 50 mM potassium phosphate buffer (pH 7.2) was

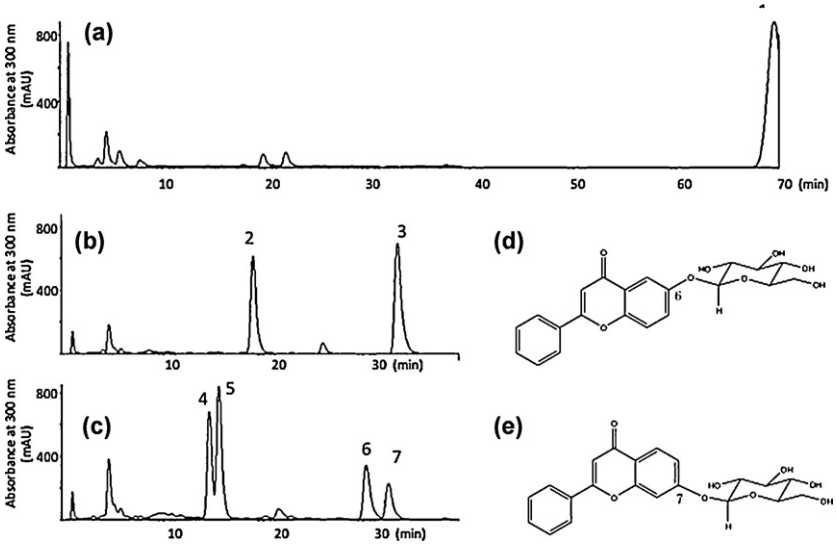

Fig. 1. HPLC profiles of the products obtained from (a) 5-hydroxyflavone, (b) 6-hydroxyflavone, and (c) 7-hydroxyflavone following incubation with *P. americana* cells. Peak 1: 5-hydroxyflavone; Peak 2: 6-hydroxyflavone monoglucoside; Peak 3: 6-hydroxyflavone; Peak 4: 7-hydroxyflavone monoglucoside; Peak 5: product X (a monoglucoside of product Y); Peak 6: 7-hydroxyflavone; and Peak 7: product Y. The structures of glucosylated products from 6-hydroxyflavone and 7-hydroxyflavone are shown in (d) and (e), respectively.

incubated for 90 min at 37 °C. The reaction mixture was analyzed by HPLC to examine the conversion of glucoside products to the corresponding hydroxyflavones.

2.5. Glucosylation of hydroxyflavones in E. coli cultures

E. coli M15 cells bearing pQE-*Pa*GT2 or pQE-*Pa*GT3 were cultured in 10 mL LB medium containing 1 mg hydroxyflavone and 0.5 mg ampicillin. Hydroxyflavone (1 mg) was previously dissolved in 0.5 mL of dimethyl sulfoxide and added to the medium. At 16 h after induction with 0.1 mM IPTG, the cells and medium were separated. Isolation of the accumulated glucosylated products in the medium was easily achieved by adsorption with Diaion HP20, a polyaromatic adsorbent resin for hydrophobic compounds. Diaion HP20 (1 g) was added to the medium and mixed gently by shaking at room temperature for 60 min. The medium-HP20 mixture was then loaded onto the column. HP20 was washed twice with 5 mL of water, and the glucoside products were eluted with 7.5 mL of methanol. The methanol solution was analyzed by HPLC to examine the production of glucosides. The yield was estimated based on the peak area of hydoxyflavone and its glucoside.

2.6. LC and LC–MS analysis

The substrates and glucosylated products were separated using a Crestpak C18S (4.6 mm × 150 mm) or an eco-ODS (4.6 mm × 150 mm). The ratio of water and acetonitrile was linearly increased from 85:15 (v/v) at $t = 0$ min to 60:40 (v/v) at $t = 40$ min. The flow rate was 1 mL/min, and the UV–visible detector was set at 300 nm. A Unison UK-C18 (2.0 mm × 150 mm) was used for liquid chromatography–mass spectrometry (LC–MS) analysis with a flow rate of 0.2 mL/min. The mass was performed in positive ion mode on an AB Sciex 3200 Q TRAP. The ESI conditions were as follows: declustering potential (DP), 30 V; entrance potential EP), 10 V; curtain gas, 40 (arbitrary units); collision gas, high; ion spray voltage, 5.5 kV; source temperature, 350 °C; ion source gas 1, 50 (arbitrary units); ion source gas 2, 80 (arbitrary units); and MS full scan range: m/z 100–450.

3. Results and discussion

3.1. Glucosylation of hydroxyflavones by cell suspension cultures of P. americana

Cell suspension cultures of *P. americana* did not glucosylate 5-hydroxyflavone (Fig. 1(a)), but transformed 6-hydroxyflavone into the corresponding glucoside with a yield of approximately 40% (Fig. 1(b)). Under reverse-phase HPLC conditions, the glucoside product (Fig. 1(b), peak 2) eluted faster than the substrate (Fig. 1(b), peak 3), and the mass spectrum of the isolated product showed a molecular ion $[M+H]^+$ peak at m/z 400.8 (162(gluc) + 238(6-hydroxyflavone)), suggesting the monoglucosylation of 6-hydroxyflavone. Furthermore, upon treatment of the glucosylated product with β-glucosidase, the glucoside peak in the HPLC profile completely disappeared, and the aglycone was regenerated in the reaction mixture. These results clearly indicate that the cell suspension cultures of *P. americana* produced the β-*O*-glucoside of 6-hydroxyflavone, 6-(β-D-glucopyranosyloxy)-2-phenyl-4*H*-1-benzopyran-4-one (Fig. 1(d)).

Somewhat interestingly, we obtained the following three products during the incubation of 7-hydroxyflavone: 7-hydroxyflavone

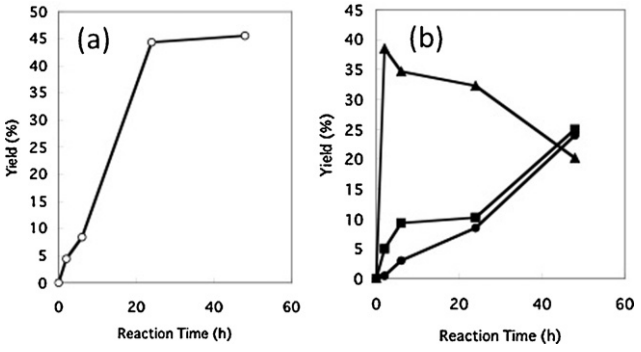

Fig. 2. Time course of the biotransformation of (a) 6-hydroxyflavone and (b) 7-hydroxyflavone by cultured cells of *P. americana*. Yield is expressed as a percentage of the total amount of reaction products. Yields are shown by symbols as follows: 6-hydroxyflavone monoglucoside (○); 7-hydroxyflavone monoglucoside (●); product X (■); and product Y (▲).

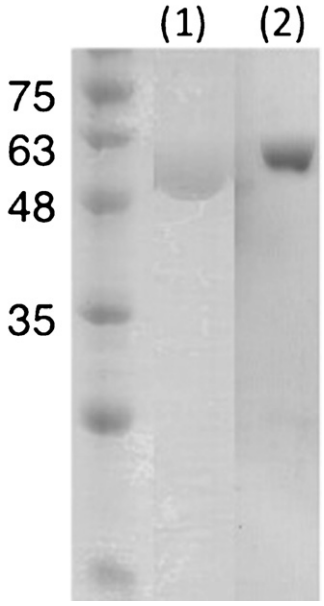

Fig. 3. Coomassie Brilliant Blue (CBB) staining of purified (1) *Pa*GT2 and (2) *Pa*GT3 in SDS-PAGE.

monoglucoside, product X, and product Y (Fig. 1(c), peaks 4, 5, 7). LC–MS analysis indicated the production of 7-hydroxyflavone monoglucoside (Fig. 1(c), peak 4, a molecular ion $[M+H]^+$ at *m*/*z* 400.9, 162(gluc) + 238(7-hydroxyflavone)). We also confirmed that the glucoside product was hydrolyzed by β-glucosidase. The molecular ions $[M+H]^+$ associated with product X (Fig. 1(c), peak 5) and product Y (Fig. 1(c), peak 7) were detected at *m*/*z* 430.9 (162(gluc) + 238(7-hydroxyflavone) + 30) and 268.9 (238(7-hydroxyflavone) + 30), respectively, in the mass spectra. Product X appears to be a monoglucosylated adduct of product Y. It was previously reported that cell suspension cultures of *P. americana* could reduce the carbonyl group and the hydroxylate phenyl ring of 4-(4-hydroxyphenyl)butan-2-one [23]. The increase of *m*/*z* by 30 might be related to these activities, but the exact structures of products X and Y remain unknown.

The time course experiments indicated that approximately 40% of 6-hydroxyflavone was converted into the glucoside product after incubation for 24 h (Fig. 2(a)). On the other hand, 7-hydroxyflavone was transformed into product Y after incubation for 2 h (Fig. 2(b)). Then, 7-hydroxyflavone glucoside and product X accumulated with a concomitant decrease in the amount of 7-hydroxyflavone and product Y. These results indicate that product Y was formed first and that further glucosylation yielded product X. Finally, it should be noted that the use of cell suspension cultures is advantageous for the synthesis of glucosides because they do not require expensive UDP-glucose. *P. americana* cells can regenerate nucleotide-activated sugars as glucosyl donor molecules.

3.2. Glucosylation of hydroxyflavone by purified PaGT2 and PaGT3 enzymes

To identify the enzymes responsible for the glucosylation of hydroxyflavone in cell suspension cultures, we performed an in vitro assay using the purified enzymes. Previously, the three glycosyltransferase genes encoding *Pa*GT1, 2, and 3 were isolated from *P. americana*, and *Pa*GT3 expressed in *E. coli* was purified [24]. This procedure, with some modifications, allowed us to obtain purified *Pa*GT2 as well as *Pa*GT3 (Fig. 3). The typical yields were 5 mg/L of *E. coli* culture.

Purified *Pa*GT3 enzyme could catalyze the glucosylation of 6- and 7-hydroxyflavone when UDP-glucose was used as the glucosyl donor. The extracts from the reaction mixtures were analyzed by LC–MS, and mass ions corresponding to the respective glucosides of hydroxyflavones were confirmed (Fig. 4). Since the glucosides were hydrolyzed upon reaction with β-glucosidase, we concluded that the products contained β-*O*-glucosidic bonds. The K_m and k_{cat} values for 6-hydroxyflavone were $70 \pm 7.7\ \mu M$ and $(1.4 \pm 0.2) \times 10^{-2}\ s^{-1}$, respectively. When 7-hydroxyflavone was used as an acceptor (i.e., an aglycone), we observed a 2-fold increase in the K_m value and a 1.5-fold decrease in the k_{cat} value (k_{cat} $(9.4 \pm 0.8) \times 10^{-3}\ s^{-1}$, K_m $140 \pm 10\ \mu M$). These results indicate that 6-hydroxyflavone is a better substrate than 7-hydroxyflavone for *Pa*GT3. The purified *Pa*GT2 enzyme could also catalyze the glucosylation of 6-hydroxyflavone, albeit inefficiently; the K_m and k_{cat} values were $46 \pm 6.4\ \mu M$ and $(1.7 \pm 0.2) \times 10^{-4}\ s^{-1}$, respectively. The K_m value did not differ significantly from that of *Pa*GT3, but the k_{cat} value decreased by approximately 100-fold. When 7-hydroxyflavone and UDP-glucose were incubated with *Pa*GT2, the corresponding glucoside product was not detected. Furthermore, neither *Pa*GT2 nor *Pa*GT3 was able to conjugate glucose to 5-hydroxyflavone.

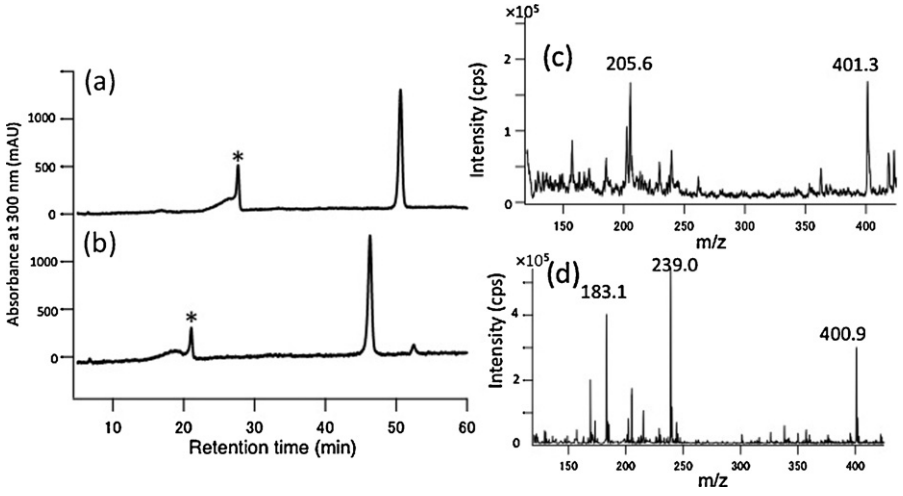

Fig. 4. HPLC profiles of the glucosylated products obtained from (a) 6-hydroxyflavone and (b) 7-hydroxyflavone following incubation with purified *Pa*GT3 enzyme. The glucoside products (*) eluted faster than the hydroxyflavones. The mass spectra of the glucosides from (c) 6-hydroxyflavone and (d) 7-hydroxyflavone. The spectra show molecular ions $[M+H]^+$ at *m*/*z* 401.3 (162(gluc) + 238(6-hydroxyflavone)) in (c) and 400.9 (162(gluc) + 238(7-hydroxyflavone)) in (d).

Our results on the synthesis of hydroxyflavone glucosides by the purified enzymes suggest that *Pa*GT2 and *Pa*GT3 are responsible for the biotransformation of 6-hydroxyflavone in *P. americana*. We also found that *Pa*GT3 can accept 7-hydroxyflavone as a glucose acceptor, although the conversion was less efficient.

3.3. Glucosylation of hydroxyflavones by E. coli expressing PaGTs

We attempted to use *E. coli* expressing *Pa*GTs to produce the glucosides because *E. coli* grows faster than *P. americana* and can regenerate UDP-glucose. When *Pa*GT3-expressing *E. coli* were cultured in the presence of hydroxyflavones, 6- and 7-hydroxyflavone glucosides were detected in the medium of overnight cultures (Supplementary Fig. 3(a) and (b)). In these incubations, endogenous glucosyl donor molecules were sufficient for the reaction, and we did not need to add UDP-glucose.

The conversion rates by *Pa*GT3-expressing *E. coli* were 15% and 20% for 6- and 7-hydroxyflavone, respectively. 6-Hydroxyflavone was also transformed by *Pa*GT2-expressing *E. coli* with a yield of 16% (Supplementary Fig. 3(c)). However, 5-hydorxyflavone glucoside was not synthesized by *Pa*GT-expressing *E. coli*. These results were consistent with those of the incubations using the purified enzymes.

4. Conclusions

Cell suspension cultures of *P. americana* could glucosylate 6- and 7-hydroxyflavone, but not 5-hydroxyflavone. Two glucosyltransferases, *Pa*GT2 and 3, were overexpressed in *E. coli* and purified successfully. Incubation with the purified enzymes clearly indicated that *Pa*GT3 could transform 6- and 7-hydroxyflavone into the corresponding glucoside and that 6-hydroxyflavone was a better glucose-acceptor molecule. In contrast, the *Pa*GT2 enzyme could only glucosylate 6-hydroxyflavone. We also proved that *E. coli* expressing the *Pa*GT enzymes could be utilized for the glucosylation of hydroxyflavones. The glucoside products could be easily isolated from the medium by adsorption on HP20 resin. An advantage of using *E. coli* cultures and *P. americana* cultures is that the addition of exogenous glucosyl donors is not required. *E. coli* and *P. americana* can use endogenous UDP-glucose to convert aglycone to glucosides. It is known that glycosylation of bioactive compounds can increase their water solubility and biological half-life. Since the biological effects of flavonoids, including their anti-tumor, anti-inflammatory, and antimicrobial activity, are now recognized [30–33], methods to endow flavonoids with health-related properties by conjugating a sugar moiety may lead to the development of new technology [34–36].

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