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Glycosylation of daidzein by the *Eucalyptus* cell cultures

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ABSTRACT

The sequential glycosylation of a soybean isoflavone, daidzein, with cultured suspension cells of *Eucalyptus perriniana* and cyclodextrin glucanotransferase was studied. Daidzein was converted into two glycosylation products, daidzein 7-*O*-β-D-glucopyranoside (39%) and daidzein 7-*O*-[6-*O*-(β-D-glucopyranosyl)]-β-D-glucopyranoside (β-gentiobioside, 6%), by cultured *E. perriniana* cells. Further glycosylation of daidzein 7-*O*-β-glucoside with cyclodextrin glucanotransferase gave daidzein 7-*O*-[4-*O*-(α-D-glucopyranosyl)]-β-D-glucopyranoside (β-maltoside, 26%), daidzein 7-*O*-β-maltotrioside (15%), and daidzein 7-*O*-β-maltotetraoside (7%).

1. Introduction

Soybean is one of the most important legumes in the human diet. Daidzein (1) is a principal soy isoflavone and has been widely studied for its anti-inflammatory activity such as inhibitory effect on histamine release from mast cells (Dijsselbloem et al., 2004; Marotta et al., 2006; Chacko et al., 2007). However, pharmacological exploitation of daidzein (1) is limited due to its insolubility in aqueous solution.

Many kinds of secondary metabolites, such as saponins, are produced in the form of glycosides in plant cells (Biswas et al., 2005; Voutquenne et al., 2005; Eskander et al., 2006; Barile et al., 2007; Melek et al., 2007; Shao et al., 2007; Vincken et al., 2007; Zhang and Li, 2007). Glycosylation of organic compounds improves their bioavailability and pharmacological properties. Thus, daidzein glycosides are of pharmacological interest from the viewpoint of drug development of isoflavone. Glycosylation using cultured plant cells is useful for preparing water-soluble and stable glycosides from water-insoluble and unstable compounds. In addition, cyclodextrin glucanotransferase (Cyclodextrin glucanotransferase) is a convenient biocatalyst to synthesize glucooligosaccharides (Jeang et al., 2005).

We report here the sequential glycosylation of daidzein into the corresponding 7-*O*-β-glucoside (2), 7-*O*-β-gentiobioside (3), 7-*O*-β-maltoside (4), 7-*O*-β-maltotrioside (5), and 7-*O*-β-maltotetraoside (6) by cultured cells of *Eucalyptus perriniana* and Cyclodextrin glucanotransferase.

2. Results and discussion

2.1. Glycosylation of daidzein (1) by cultured cells of E. perriniana

Cultured cells of *E. perriniana* have been reported to have high potential to convert exogenous phenolic compounds into their glycoconjugates, and were used in this study (Shimoda et al., 2006). Biotransformation of daidzein (1) by cultured suspension cells of *E. perriniana* resulted in production of glycosides 2 (39%) and 3 (6%), the structures of which were determined to be daidzein 7-*O*-β-D-glucopyranoside (2) and daidzein 7-*O*-[6-*O*-(β-D-glucopyranosyl)]-β-D-glucopyranoside (β-gentiobioside, 3) based on their HRFABMS, ^{1}H and ^{13}C NMR (Table 1), H–H COSY, C–H COSY, NOE, and HMBC spectroscopic data. The β-gentiobioside product 3 has not been identified previously.

The molecular formula of 3 was established as $C_{27}H_{30}O_{14}$ based on its HRFABMS spectrum, which included a pseudomolecular ion $[M + Na]^+$ peak at *m/z* 601.1537 (calcd. 601.1533 for $C_{27}H_{30}O_{14}Na$). HRFABMS suggested that 3 was composed of one molecule of 1 and two hexoses. Its ^{1}H NMR spectrum showed two anomeric proton signals at δ 4.20 (1H, *d*, *J* = 8.0 Hz) and 5.11 (1H, *d*, *J* = 7.6 Hz). This suggested the presence of two β-anomers. The ^{13}C NMR spectrum included two anomeric carbon signals at δ 99.5 and 103.4. The sugar component of 3 was determined to be β-D-glucopyranose based on the chemical shifts of the carbon signals. The ^{13}C resonance of

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Table 1
^{13}C chemical shifts of the glycosylation products 2-6 in DMSO-d_6

Product	2	3	4	5	6
Aglycone					
2	153.0	153.1	153.1	153.0	153.1
3	122.1	122.1	122.1	122.2	122.1
4	174.6	174.6	174.6	174.7	174.6
5	127.0	127.0	126.9	127.0	127.0
6	115.5	115.5	115.4	115.5	115.4
7	161.3	161.2	161.2	161.2	161.2
8	103.3	103.3	103.3	103.3	103.3
9	157.2	157.1	157.2	157.2	157.1
10	118.4	118.4	118.4	118.4	118.4
11	123.6	123.6	123.6	123.6	123.6
12	130.0	130.0	130.0	130.0	130.0
13	114.9	114.9	114.9	114.9	114.9
14	156.9	156.9	156.9	156.9	156.9
15	114.9	114.9	114.9	114.9	114.9
16	130.0	130.0	130.0	130.0	130.0
Glc					
1′	99.9	99.5	98.9	99.5	98.8
2′	73.0	73.3	73.3	73.4	73.1
3′	77.1	77.0	76.0	75.9	76.0
4′	69.5	69.8	78.9	79.4	79.3
5′	76.4	76.1	75.3	75.3	75.3
6′	60.5	68.7	61.0	60.7	60.7
1″		103.4	100.6	100.5	100.5
2″		72.9	72.0	71.9	72.0
3″		76.5	73.2	73.2	73.1
4″		69.2	69.6	79.0	79.3
5″		75.9	72.6	71.7	72.0
6″		60.8	60.2	60.2	60.2
1‴				100.7	100.6
2‴				72.4	72.0
3‴				73.1	73.1
4‴				69.8	78.9
5‴				72.6	72.0
6‴				60.2	60.2
1⁗					100.7
2⁗					72.6
3⁗					73.1
4⁗					69.7
5⁗					72.6
6⁗					60.2

C-6′ was shifted downfield to δ 68.7. Correlations were observed between the anomeric proton signal at δ 5.11 (H-1′) and the carbon resonance at δ 161.2 (C-7), and between the anomeric proton signal at δ 4.20 (H-1″) and the carbon resonance at δ 68.7 (C-6′) in the HMBC spectrum. These findings confirmed that the inner glucopyranosyl residue was attached to the phenolic hydroxyl group at C-7 of daidzein (1), and that the pair of β-D-glucopyranosyl residues was 1,6-linked. Thus, 3 was identified as daidzein 7-*O*-[6-*O*-(β-D-glucopyranosyl)]-β-D-glucopyranoside.

A time-course experiment was carried out to investigate the ability of cultured *E. perriniana* cells to biotransform daidzein (1). As shown in Fig. 1, 1 was readily glucosylated to 2 at an early stage of incubation, whereas the yield of product 3 was not so high throughout the incubation period. *E. perriniana* cell culture was a good biocatalyst for producing daidzein 7-*O*-β-glucoside (2) rather than daidzein 7-*O*-β-gentiobioside (3).

2.2. Glycosylation of daidzein 7-*O*-β-D-glucoside (2) by Cyclodextrin glucanotransferase

Daidzein 7-*O*-β-D-glucoside (2), which had been produced by the biotransformation of daidzein (1) with cultured *E. perriniana* cells, was subjected to further glycosylation with Cyclodextrin glucanotransferase. Incubation of 2 with Cyclodextrin glucanotransferase in the presence of starch gave three products, 4 (26%), 5 (15%), and 6 (7%), which were identified as daidzein 7-*O*-[4-*O*-(α-D-glucopyranosyl)]-β-D-glucopyranoside (β-maltoside, 4), daidzein 7-*O*-β-maltotrioside (5), and daidzein 7-*O*-β-maltotetraoside (6). The di- and tetrasaccharide products 4 and 6 have not been identified previously.

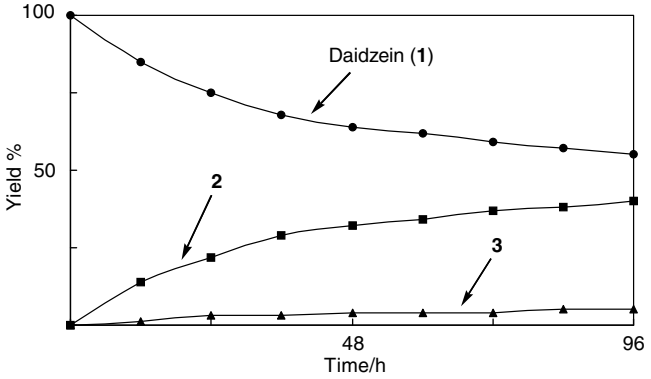


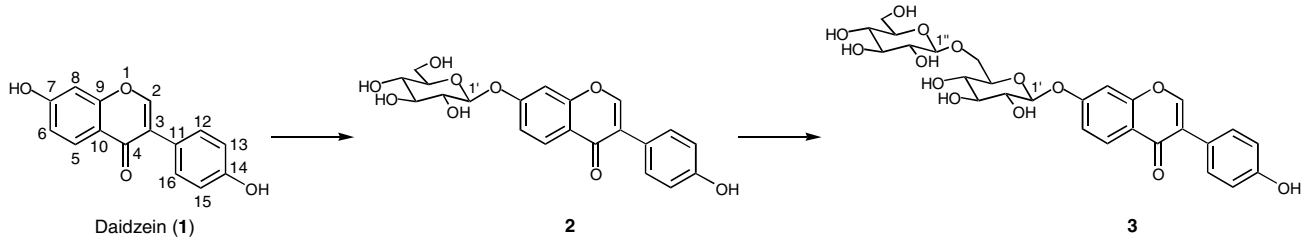
Fig. 1. Time-course of the glycosylation of daidzein (1) by cultured cells of *E. perriniana*. [a]Yield is expressed as a percentage relative to the total amount of all reaction products.

The HRFABMS spectrum of 4 with a pseudomolecular ion $[M + Na]^+$ peak at *m*/*z* 601.1538 indicated a molecular formula of $C_{27}H_{30}O_{14}$ (calcd. 601.1533 for $C_{27}H_{30}O_{14}Na$). The ^{1}H NMR spectrum of 4 included two anomeric proton signals at δ 5.07 (1H, *d*, *J* = 3.4 Hz) and 5.17 (1H, *d*, *J* = 7.6 Hz), indicating the presence of both the α- and β-anomers in the sugar moiety. The ^{13}C NMR spectroscopic data of 4 showed two anomeric carbon signals at δ 98.9 and 100.6. HMBC correlations were observed between the anomeric proton signal at δ 5.17 (H-1′) and the carbon resonance at δ 161.2 (C-7), and between the anomeric proton signal at δ 5.07 (H-1″) and the carbon resonance at δ 78.9 (C-4′). These data confirm that the inner β-D-glucopyranosyl residue was attached to the C-7 position of daidzein (1), and that the second α-D-glucopyranosyl residue and the inner β-D-glucopyranosyl residue were 1,4-linked. Thus, compound 4 was identified as daidzein 7-*O*-[4-*O*-(α-D-glucopyranosyl)]-β-D-glucopyranoside (β-maltoside).

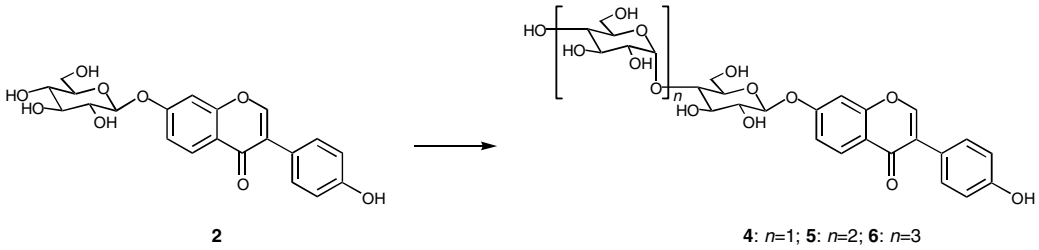
The HRFABMS spectrum of 6, which included a pseudomolecular ion $[M + Na]^+$ peak at *m*/*z* 925.2602, established that the molecular formula of 6 was $C_{39}H_{50}O_{24}$ (calcd. 925.2590 for $C_{39}H_{50}O_{24}Na$). The ^{1}H NMR spectrum of 6 showed four anomeric proton signals at δ 5.00 (2H, *d*, *J* = 3.4 Hz), 5.09 (1H, *d*, *J* = 3.0 Hz), and 5.17 (1H, *d*, *J* = 7.6 Hz), suggesting the presence of three α-anomers and one β-anomer in the sugar moiety. The ^{13}C NMR chemical shifts of C-4′ (δ 79.3), C-4″ (δ 79.3), and C-4‴ (δ 78.9) were shifted relatively downfield. The HMBC experiment established connections between H-1′ (δ 5.17) and C-7 (δ 161.2), between H-1″ (δ 5.09) and C-4′ (δ 79.3), between H-1‴ (δ 5.00) and C-4″ (δ 79.3), and between H-1⁗ (δ 5.00) and C-4‴ (δ 78.9). Thus, compound 6 was identified as daidzein 7-*O*-β-maltotetraoside.

3. Conclusion

The results obtained with cultured cells of *E. perriniana* showed that *E. perriniana* can glycosylate daidzein (1) to give the corresponding 7-*O*-β-glucoside (2) and 7-*O*-β-gentiobioside (3) (Scheme 1). This is the first description of daidzein 7-*O*-β-gentiobioside (3) formation using cultured plant cells. Recently, it has been reported that cultured *E. perriniana* cells converted exogenously added thymol, carvacrol, and eugenol into their mono-glucosides and gentiobiosides (Shimoda et al., 2006). The yield of daidzein 7-*O*-β-gentiobioside (3) obtained here is relatively low, probably due to the substrate specificity of glycosyltransferases which produce gentiobiosides.



Scheme 1. Glycosylation of daidzein (1) by cultured cells of *E. perriniana*.



Scheme 2. Glycosylation of daidzein 7-*O*-β-D-glucopyranoside (2) by Cyclodextrin glucanotransferase.

Oligomerization of sugar at the C-7 position of daidzein gave daidzein (1) 7-*O*-β-maltooligosaccharides, i.e., 7-*O*-β-maltoside (4), 7-*O*-β-maltotrioside (5), and 7-*O*-β-maltotetraoside (6), by the glycosylation of daidzein 7-*O*-β-glucoside (2) with Cyclodextrin glucanotransferase in the presence of starch (Scheme 2). Daidzein 7-*O*-β-maltoside (4) and daidzein 7-*O*-β-maltotetraoside were identified for the first time. Li et al. reported that daidzein 7-*O*-β-glucoside was glycosylated to daidzein 7-*O*-β-maltotrioside, daidzein 7-*O*-β-maltopentaoside, daidzein 7-*O*-β-maltoheptaoside, and daidzein 7-*O*-β-maltononaoside by maltosyltransferase from *Thermotoga maritime*, and daidzein 7-*O*-β-maltotrioside (5) was isolated and subjected to structure determination using spectroscopic methods (Li et al., 2004). The glycosylation system in the present study is useful for the practical preparation of a series of daidzein 7-*O*-β -maltooligosaccharides which differ by a glucose unit.

Sequential glycosylation with *E. perriniana* cells and Cyclodextrin glucanotransferase may be useful for the production of isoflavone maltooligosaccharides as food additives. This method is simple and environmentally friendly. Further studies on the physiological activities of daidzein glycosides are now in progress.

4. Experimental

4.1. General experimental details

Daidzein (1) was purchased from Aldrich Chemical Co. (St. Louis, MO). Cyclodextrin glucanotransferase from *Bacillus macerans* was purchased from Amano Pharmaceutical Co. Ltd. (Nagoya, Japan). HPLC was performed using a YMC-Pack R&D ODS column (150 × 30 mm, YMC Co. Ltd., Kyoto, Japan) (solvent: MeOH-H_2O (9:11, v/v); detection: UV (280 nm); flow rate: 1.0 ml/min). HRFABMS was performed using a JEOL MStation JMS-700 spectrometer (JEOL Ltd., Tokyo, Japan). ^1H and ^{13}C NMR, H–H COSY, C–H COSY, NOE, and HMBC spectra were measured using a Varian XL-400 spectrometer (Varian Technologies Japan Ltd., Tokyo, Japan) in DMSO-d_6 solution and the chemical shifts were expressed in δ (ppm) with reference to TMS.

4.2. Plant cell culture

A cell culture of *E. perriniana* was induced in our laboratory, and has been cultivated for over 20 years (Furuya et al., 1987). Cultured *E. perriniana* cells were subcultured at 4-week intervals on solid Murashige and Skoog (MS) medium (100 ml in a 300-ml conical flask) containing 3% sucrose, 10 mM 2,4-dichlorophenoxyacetic acid, and 1% agar (adjusted to pH 5.7) at 25 °C in the dark. A suspension culture was started by transferring the cultured cells to 100 ml of liquid medium in a 300-ml conical flask, and incubated on a rotary shaker (120 rpm) at 25 °C in the dark. Prior to use for this work, part of the callus tissues (fr. wt 40 g) was transplanted to freshly prepared MS medium (100 ml in a 300-ml conical flask) and grown with continuous shaking for 2 weeks on a rotary shaker (120 rpm).

4.3. Glycosylation by cultured cells of E. perriniana

The substrate daidzein (1) was added to each of ten 300-ml flasks (1 mmol/l) containing 100 ml of MS medium and cultured suspension cells of *E. perriniana*, and the cultures were incubated at 25 °C for five days on a rotary shaker (120 rpm) in the dark. After incubation, the cells and medium were separated by filtration with suction. Extraction and purification of the biotransformation products were achieved as previously reported (Shimoda et al., 2006, 2007a, 2007b).

4.4. Glycosylation by Cyclodextrin glucanotransferase

Daidzein 7-*O*-β-D-glucoside (2, 50 mg) was incubated with 3 μk at of Cyclodextrin glucanotransferase in 10 ml of 25 mM sodium phosphate buffer (pH 7.0) containing 5 g of soluble starch at 40 °C for 24 h. After incubation, the reaction mixture was centrifuged at 3000 g for 10 min. The supernatant was applied to a Sephadex G-25 column equilibrated with water. Fractions that contained glycosides were lyophilized, re-dissolved with water, and purified by preparative HPLC using a YMC-Pack R&D ODS column (150 × 30 mm).

4.5. Identification of glycosylation products

The structures of the products were determined on the basis of their HRFABMS, ^{1}H and ^{13}C NMR, H–H COSY, C–H COSY, NOE, and HMBC spectra.

Daidzein 7-*O*-[6-*O*-(β-D-glucopyranosyl)]-β-D-glucopyranoside (3): HRFABMS: *m*/*z* 601.1537 $[M + Na]^+$; ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.18-3.71 (12H, *m*, H-2′, 2″, 3′, 3″, 4′, 4″, 5′, 5″, 6′, 6″), 4.20 (1H, *d*, *J* = 8.0 Hz, H-1″), 5.11 (1H, *d*, *J* = 7.6 Hz, H-1′), 6.82 (2H, *d*, *J* = 6.4 Hz, H-13, 15), 7.13 (1H, *dd*, *J* = 8.6, 2.0 Hz, H-6), 7.24 (1H, *d*, *J* = 2.0 Hz, H-8), 7.40 (2H, *d*, *J* = 6.4 Hz, H-12, 16), 8.04 (1H, *d*, *J* = 8.6 Hz, H-5), 8.39 (1H, *s*, H-2); for ^{13}C NMR (100 MHz, DMSO-d_6) spectroscopic data see Table 1.

Daidzein 7-*O*-[4-*O*-(α-D-glucopyranosyl)]-β-D-glucopyranoside (β-maltoside, 4): HRFABMS: *m*/*z* 601.1538 $[M + Na]^+$; ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.02–3.78 (12H, *m*, H-2′, 2″, 3′, 3″, 4′, 4″, 5′, 5″, 6′, 6″), 5.07 (1H, *d*, *J* = 3.4 Hz, H-1″), 5.17 (1H, *d*, *J* = 7.6 Hz, H-1′), 6.81 (2H, *d*, *J* = 6.4 Hz, H-13, 15), 7.13 (1H, *dd*, *J* = 8.6, 2.0 Hz, H-6), 7.25 (1H, *d*, *J* = 2.0 Hz, H-8), 7.41 (2H, *d*, *J* = 6.4 Hz, H-12, 16), 8.05 (1H, *d*, *J* = 8.6 Hz, H-5), 8.40 (1H, *s*, H-2); for ^{13}C NMR (100 MHz, DMSO-d_6) spectroscopic data see Table 1.

Daidzein 7-*O*-β-maltotetraoside (6): HRFABMS: *m*/*z* 925.2602 $[M + Na]^+$; ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.05-3.95 (24H, *m*, H-2′, 2″, 2‴, 2⁗, 3', 3″, 3‴, 3⁗, 4′, 4″, 4‴, 4⁗, 5′, 5″, 5‴, 5⁗, 6′, 6″, 6‴, 6⁗), 5.00 (2H, *d*, *J* = 3.4 Hz, H-1‴, 1⁗), 5.09 (1H, *d*, *J* = 3.0 Hz, H-1″), 5.17 (1H, *d*, *J* = 7.6 Hz, H-1′), 6.81 (2H, *d*, *J* = 6.4 Hz, H-13, 15), 7.14 (1H, *dd*, *J* = 8.6, 2.0 Hz, H-6), 7.24 (1H, *d*, *J* = 2.0 Hz, H-8), 7.40 (2H, *d*, *J* = 6.4 Hz, H-12, 16), 8.05 (1H, *d*, *J* = 8.6 Hz, H-5), 8.39 (1H, *s*, H-2); for ^{13}C NMR (100 MHz, DMSO-d_6) spectroscopic data see Table 1.

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