Synthesis of Glycosides of α-Tocopherol, Daidzein, Resveratrol, Hesperetin, Naringenin, and Chrysin as Antiallergic Functional Foods and Cosmetics

Yuya Fujitaka¹, Hiroki Hamada¹ Ⓞ, Hatsuyuki Hamada², Takafumi Iwaki³, Kei Shimoda⁴ Ⓞ, Yuya Kiriake⁵, and Tomohiro Saikawa⁶

Glucosyltransferase from Phytolacca americana (Phytolaccaeae), glucosylated α-tocopherol, daidzein, resveratrol, hesperetin, naringenin, and chrysin to α-tocopherol 6-β-D-glucoside, daidzein 7-β-D-glucoside, resveratrol 3-β-D-glucoside, hesperetin 7-β-D-glucoside, naringenin 7-β-D-glucoside, and chrysin 7-β-D-glucoside, respectively. The antiallergic activity of the glycosides and their aglycons was examined by an in vivo immunoglobulin E (IgE) antibody formation-suppression bioassay using rat. It was found that α-tocopherol 6-β-D-glucoside showed much higher antiallergic activity against glutenin than the positive control, hydrocortisone. On the other hand, daidzein 7-β-D-glucoside had much higher antiallergic activity toward 7S-globulin than hydrocortisone. These glycosides inhibited O₂− generation from rat neutrophils, which leads to the suppression of histamine release from rat peritoneal mast cells, resulting in the decrease of IgE antibody formation in rat. Chrysin 7-β-D-glucoside had stronger antityrosinase activity than chrysin. Cultured P. americana cells regioselectively introduced methoxyl and glucosyl residues on exogenously administered chrysin to give 8-methoxychrysin and chrysin 7-β-D-glucoside. This is the first report on methoxylation of flavone compound at its eighth position by cultured plant cells.

Keywords
Glycosylation, methoxylation, anti-allergic activity, anti-tyrosinase activity, glucosyltransferase, cultured plant cells, P. americana

Plants produce secondary metabolites, such as terpenoids, cardenolides, coumarins, anthraquinones, flavonoids, glucosinolates, and alkaloids, which are used as drugs, flavors, pigments, and agrochemicals. Also, plants are the source of valuable products and some useful basic materials, including cellulose, wood, and rubber. Cultured plant cells have been reported to produce some secondary metabolites. Generally, cultured cells of higher plants do not accumulate secondary metabolites, and it has proven difficult to harness this potential to organic syntheses or industrial processes. So, biotransformation of various organic compounds has become a target in the biotechnological application of plant cell cultures and plant enzymes.³

Bioconversion of exogenously administrated substrates by cultured plant cells has been investigated so far.³ Cultured plant cells have the ability to specifically convert cheap and plentiful substrates into rare and expensive substances. There have been many studies on bioconversion of exogenous organic compounds, such as phenols, phenylpropanoids, terpenoids, flavones, and isoflavones.³ Cultured plant cells can be used to convert organic molecules to more useful compounds by catalyzing hydrolysis, oxidation, reduction, esterification, isomerization, and glycosylation reactions.⁴¹⁴ A few studies on hydroxylation and methylation of exogenous substrates by cultured plant cells have been reported.⁴¹⁴ Cultured plant cells of Phytolacca americana regioselectively introduced hydroxy, methyl, and glycosyl groups to exogenous stilbenoids, including resveratrol.⁵⁶ Cultured Phytolacca americana cells have been reported to possess high potential to biotransform exogenous compounds.¹² It has been reported that cultured plant cells can glycosylate α-tocopherol, daidzein, resveratrol, hesperetin, and naringenin.⁴ However, the
biotransformation of exogenous chrysin by cultured plant cells has not been reported.

We report here the bioconversion of α-tocopherol, daidzein, resveratrol, hesperetin, naringenin, and chrysin (Figure 1), by glucosyltransferase from P. americana. Glucosyltransferase from P. americana glucosylated α-tocopherol, daidzein, resveratrol, hesperetin, naringenin, and chrysin to α-tocopherol 6-β-glucosylated α-tocopherol, daidzein, resveratrol, hesperetin, naringenin, and chrysin (Figure 1), by glucosyltransferase expressed in cultured plant cells of P. americana (Figure 1). The glucosyltransferase expressed in both Bacillus subtilis and Escherichia coli transformed α-tocopherol, daidzein, resveratrol, hesperetin, naringenin, and chrysin to α-tocopherol 6-β-glucoside, daidzein 7-β-glucoside, resveratrol 3-β-glucoside, and chrysin 7-β-glucoside, naringenin 7-β-d-glucoside, and chrysin 7-β-d-glucoside, respectively. The effects of α-tocopherol 6-β-d-glucoside and α-tocopherol on immunoglobulin E (IgE) antibody formation were investigated by an in vivo bioassay using glutenin as an antigen. It was found that α-tocopherol 6-β-d-glucoside showed stronger antiallergic activity (IgE level 96) against glutenin than α-tocopherol (IgE level 192). Positive control of hydrocortisone exerted IgE level of 320. On the other hand, IgE levels of daidzein 7-β-d-glucoside and daidzein against 7S-globulin were 128 and 256. The IgE level of positive control, hydrocortisone, was 288. This demonstrates that daidzein 7-β-d-glucoside showed higher antiallergic activity against 7S-globulin than daidzein. It is known that glutenin and 7S-globulin are allergic compounds included in wheat flour and soybean, respectively. α-Tocopherol is a component in wheat and daidzein in soybean. α-Tocopherol 6-β-glucoside and daidzein 7-β-d-glucoside would be potent antiallergic food additives. The suppression activity of the glycosides was higher than that of the corresponding aglycons (Supplemental Table S1). The inhibitory activities of α-tocopherol, daidzein, α-tocopherol 6-β-glucoside, and daidzein 7-β-d-glucoside for O2− generation from rat neutrophils were 33%, 29%, 58%, and 50% inhibition, respectively (Supplemental Table S1). The positive control, mequitazine, exerted inhibitory activity of 60% inhibition. The antiallergic actions of the glycosides were caused by inhibition of histamine release with these compounds owing to their inhibitory aspects for O2− generation from rat neutrophils.

Flavones such as hesperetin have been used as cosmetics. So, the tyrosinase inhibitory activity of chrysins, a kind of flavone, and its glycoside was examined. The half-maximal inhibitory concentration (IC50) values of chrysin 7-β-d-glucoside and chrysin for tyrosinase inhibitory activity was 30 and 212 µM, respectively (Supplemental Table S1). Chrysin 7-β-d-glucoside showed higher antityrosinase activity than chrysin. Resveratrol 3-β-d-glucoside had the highest tyrosinase inhibitory activity among the compounds tested.

Chrysin was used as a substrate for the bioconversion system using cultured plant cells of P. americana. The substrate chrysin was administered to a 300 mL conical flask (10 mg/flask) containing suspension cell cultures of P. americana. The cultures were incubated at 25 °C for 2 days on a rotary shaker. Two compounds were purified from the extracts of the cells with methanol (MeOH) by high-performance liquid chromatography (HPLC). The chemical structure of the products was determined on the basis of electrospray ionization-mass spectrometry (MS), 1H and 13C nuclear magnetic resonance (NMR) spectra. The products were identified as 8-methoxychrysin and chrysin 7-β-d-glucoside (Figure 1).

Recently, the bioconversion, such as regioselective methylation and glycosylation, of stilbene compounds by cultured plant cells has been reported. It was found that cultured plant cells of P. americana regioselectively introduced methyl and glucosyl residues on a stilbene compound, that is, resveratrol.
Resveratrol was converted into 3,5-dimethylresveratrol 4'-β-D-glucoside (pterostilbene 4'-β-D-glucoside) by cultured *P. americana* cells.¹³ No studies have been reported on regioselective methoxylolation of flavones at their eighth position by cultured plant cells so far. The present study describes, for the first time, the regioselective methoxylolation of flavone, chrysin, at its eighth position using cultured plant cells of *P. americana* as biocatalysts. In the present study, it was demonstrated that cultured *P. americana* cells regioselectively introduced methoxyl and glycosyl residues to exogenous chrysin to give 8-methoxychrysin and chrysin 7-β-D-glucoside. The glycoside products showed potent antiallergic properties rather than substrates. The glycosides of substrates, such as α-tocopherol, products showed potent antiallergic properties rather than substrates. The glycosides of substrates, such as α-tocopherol, daidzein, resveratrol, hesperitin, and naringenin, had high anti-allergic activity toward NC/NgaSlc mice (data not shown). Further studies on the pharmacological activity of the glycosides are now in progress in our laboratory.

**Experimental**

**General**

HPLC was carried out with a column (YMC-Pack R&D ODS column: 150 × 4.6 mm; detection: ultraviolet [UV, 280 nm]; flow rate: 1.0 mL/min). The cultured plant cells were subcultured at 4-week intervals for 2 months on solid MS medium containing 2% glucose, 1 ppm 2,4-dichlorophenoxyacetic acid, and 1% agar (adjusted to pH 5.7). A suspension culture was started by transferring 20 g of the cultured cells to 100 mL of liquid MS medium in a 300 mL conical flask.

**Glycosylation by Glucosyltransferase From *P. americana***

*Bacillus subtilis* transformed with plasmid (pBSX1.101), which contains gene from *P. americana* encoding glucosyltransferase activity, was used as the source of the enzyme *PaGT3*. *Bacillus subtilis* was grown at 37 °C in Luria-Bertani broth. The medium was sterilized in 1 L aliquots in 2 L conical flasks, supplemented with 5 µg mL⁻¹ chloramphenicol, and inoculated with 0.5% fresh culture. Growth was carried out in an incubator with agitation (200 rpm) for 26 hours. Cells were lysed by sonication, and cellular debris was removed by centrifugation. The supernatant (20 mL) was applied to a His-accept column (1.6 × 30 cm) equilibrated with buffer A (50 mM potassium phosphate, 1 mM ethylenediaminetetraacetic acid, and 2 mM 2-mercaptopethanol). The column was washed with buffer A (50 mL) to remove impurities, and the bound enzyme was eluted with buffer A (100 mL) supplemented with 200 mM imidazole. The purified enzyme solution was dialyzed with 50 mM Tris-HCl (pH 7.2) containing 5 mM dithiothreitol and stored at −80 °C. Glucosylation reactions were performed at 35 °C for 24 hours in 5 mL of 50 mM potassium phosphate buffer (pH 7.2) supplemented with 50 mM substrate, 100 mM uridine diposphate-glucose, and 5 mM enzyme. The incubation was stopped by adding 1.5% trifluoroacetic acid, and the reaction mixture was analyzed by HPLC. The reaction mixture was extracted with n-butanol (n-BuOH). The n-BuOH fraction was concentrated by evaporation, and the residue was dissolved in water. The water fraction was applied to Diaion HP-20, and the column was washed with water (H₂O) followed by elution with MeOH. The MeOH solution was subjected to preparative HPLC. Large-scale experiments have been carried out to prepare enough amount of products for NMR analyses.

**Biotransformation Procedure**

The suspension plant cells were incubated in 300 mL conical flasks for 3 weeks. The cultured cells in the stationary growth phase have been used for experiments. After the cultivation period, 0.1 mmol of the substrate was added to the flask containing 70 g of suspension cell cultures. Total amount of 1 mmol of the substrate was administered to 10 flasks. The transformation was performed by incubating the mixture at 25 °C on a rotary shaker (70 rpm) for 2 days. The culture medium was extracted with ethylacetate (EtOAc). The cells were extracted (×3) by homogenization with MeOH. In the experiments of product analyses, the EtOAc and MeOH layers were combined, concentrated, and analyzed by HPLC. In the experiments of preparation of products, the MeOH fraction was concentrated and partitioned between H₂O and EtOAc. The EtOAc fractions were combined, concentrated, and analyzed by HPLC. The H₂O fraction was applied to a Diaion HP-20 column, and the column was washed with H₂O followed by elution with MeOH. The MeOH eluate was subjected to HPLC to give glycosylated product. The yield of the product was determined by HPLC analyses using calibration curves prepared with authentic samples and expressed as a percentage to the amount of the administered substrate. Large-scale experiments have been carried out to prepare enough amount of products for NMR analyses.

**Antiallergic Activity**

The inhibitory action of samples on IgE antibody formation was examined as follows. Glutenin or 7S-globulin (1 mg/rat) was used as the antigen, and aluminum hydroxide and pertussis toxin were used as the adjuvants (20 mg and 0.6 mL/rat, respectively). Sensitization was made by injection of a mixture (0.6 mL) of the antigen and the adjuvant into the paws of each rat (male, ca. 200 g). Paw edema was measured 24 hours after injection, and the treated rats were divided into groups with an equal average swelling volume. Each sample was dissolved in physiological saline containing 10% Nikkoli, and the solution containing 0.4 mg of sample was injected.
daily into each rat for 11 days starting on the day of grouping. Hydrocortisone was used as the positive control. The amount of IgE was measured on the 15th day by the passive cutaneous anaphylaxis method. The results were expressed as plasma IgE levels.

The effects of test compounds on compound 48/80-induced histamine release from rat peritoneal mast cells were examined as follows. Peritoneal mast cells were collected from the abdominal cavities of rats (Male Wistar rats, Nippon SLC) and purified to a level higher than 95%. The purified mast cells were suspended in a physiological buffered solution containing 145 mM sodium chloride, 2.7 mM potassium chloride, 1.0 mM calcium chloride, 5.6 mM glucose, and 20 mM 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid (pH 7.4) to give approximately 10^5 mast cells/mL. Cell viability was always greater than 90% as judged by the trypan blue exclusion test. Mast cells were preincubated with the test compound (1 µM) for 15 minutes at 37 °C and subsequently exposed to compound 48/80 at 0.35 µg/mL. Histamine release was determined by a fluorometric assay.

Effects of compounds on \( \text{O}_2^- \) generation from rat neutrophils were examined as follows. Male Wistar rats, each weighing 250 g, were used. Under ether anesthesia, whole blood was collected from the carotid artery and diluted twice with Hanks’ balanced salt solution (HBSS) (pH 7.4). Neutrophils were purified by Percoll density gradient centrifugation. Neutrophils were examined as follows. Male Wistar rats, each weighing approximately 104 mast cells/mL. Cell viability was always greater than 90% as judged by the trypan blue exclusion test. Mast cells were preincubated with the test compound (1 µM) for 15 minutes at 37°C and subsequently exposed to compound 48/80 at 0.35 µg/mL. Histamine release was determined by a fluorometric assay.

Effects of compounds on \( \text{O}_2^- \) generation from rat neutrophils were examined as follows. Male Wistar rats, each weighing 250 g, were used. Under ether anesthesia, whole blood was collected from the carotid artery and diluted twice with Hanks’ balanced salt solution (HBSS) (pH 7.4). Neutrophils were purified by Percoll density gradient centrifugation. Neutrophils were measured by the cypridina luciferin analog-dependent chemiluminescence. Neutrophil suspensions (10^6 cells/mL) were incubated for 3 minutes in HBSS containing 0.4 mM of cypridina luciferin analog and sample at 37°C in the dark. Five seconds later, \( \text{N}^-\text{formyl- methionyl-leucyl-phenylalanine (fMLP, 2.5 mM) was added into the assay mixture. Cypridina luciferin analog-dependent chemiluminescence was monitored. The results are expressed in terms of the percentage reduction of the \( \text{O}_2^- \) generation from rat neutrophils at 5 minutes after the administration of fMLP by test compounds.}

Tyrosinase Assay

Mushroom tyrosinase (EC 1.14.18.1; Sigma Chemical Co.) was used for the tyrosinase assay, with either \( l^-3,4^-\text{dihydroxyphenylalanine (l-DOPA)} \) or \( l^-\text{tyrosine} \) as a substrate. In spectrophotometric experiments, enzyme activity was taken as the initial velocity (Vi) monitored by observing dopachrome formation at 475 nm with a UV spectrophotometer at 30°C. All samples were dissolved in ethanol at 10 mM. First, 200 mL of a 2.7 mM \( l^-\text{tyrosine} \) or 5.4 mM \( l^-\text{DOPA} \) aqueous solution was mixed with 2687 mL of 0.25 M phosphate buffer (pH 6.8). Next, 100 mL of the sample solution and 13 mL of the same phosphate buffer solution containing mushroom tyrosinase (144 units) were added to the mixture. The inhibitor concentration that gave a 50% loss of activity (IC50) was obtained by fitting the experimental data to the logistic curve.

Acknowledgments

This research was supported by The Tojuro Iijima Foundation for Food Science and Technology.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

ORCID IDs

Hiroki Hamada https://orcid.org/0000-0002-7877-3367
Kei Shimoda https://orcid.org/0000-0001-6592-4990

Supplemental Material

Supplemental material for this article is available online.

References

1. Asada Y, Saito H, Yoshikawa T, Sakamoto K, Furuya T. Biotransformation of 18 beta-glycyrrhetinic acid by ginseng hairy root culture. Phytochemistry. 1993;34(4):1049-1052. doi:10.1016/S0031-9422(00)90711-8
2. Wang C-J, Staba EJ. Peppermint and spearmint tissue culture. II. DUAL-CARBOY culture of spearmint tissues. J Pharm Sci. 1963;52(11):1058-1062. doi:10.1002/jps.2600521108
3. Suga T, Hirata T. Biotransformation of exogenous substrates by plant cell cultures. Phytochemistry. 1990;29(8):2393-2406. doi:10.1016/0031-9422(90)85155-9
4. Ishihara K, Hamada H, Hirata T, Nakajima N. Biotransformation using plant cultured cells. J Mol Catal B Enzym. 2003;23(2-6):145-170. doi:10.1016/S1381-1177(03)00080-8
5. Shimoda K, Kubota N, Uesugi D, et al. Synthesis of glycosides of resveratrol, pinostilbene, and piceatannol by bioconversion with Phytolacca americana. Nat Prod Commun. 2019;14(8):1-3. doi:10.1177/1934578X19868396
6. Uesugi D, Hamada H, Shimoda K, Kubota N, Ozaki S-I, Nagatani N. Synthesis, oxygen radical absorbance capacity, and tyrosinase inhibitory activity of glycosides of resveratrol, pinostilbene, and piceatannol by plant cell cultures. J Pharm Sci. 2017;106(5):1080-1088. doi:10.1016/j.xphs.2016.12.003
7. Tabata M, Ikeda F, Hiraoka N, Konoshima M. Glucosylation of phenolic compounds by Plasmodium falciparum. J Mol Catal B Enzym. 1997;10(1):1-10. doi:10.1016/S1381-1177(97)00045-2
8. Tsuchiya M, Terao T, Miura H, Ohashi H. Glucosylation of salicyl alcohol in cultured plant cells. Phytochemistry. 1992;32(8):679-680. doi:10.1016/0031-9422(90)86961-7
9. Tabata M, Umetani Y, Ooya M, Tanaka S. Glucosylation of phenolic compounds by plant cell cultures. Phytochemistry. 1992;8(10):809-813. doi:10.1016/0031-9422(92)00605-C
10. Mizukami H, Terao T, Miura H, Ohashi H. Glucosylation of salicyl alcohol in cultured plant cells. Phytochemistry. 1992;8(10):809-813. doi:10.1016/0031-9422(92)00605-C
11. Mizukami H, Terao T, Miura H, Ohashi H. Glucosylation of salicyl alcohol in cultured plant cells. Phytochemistry. 1992;8(10):809-813. doi:10.1016/0031-9422(92)00605-C
10. Ushiyama M, Furuya T. Glycosylation of phenolic compounds by root culture of Panax ginseng. *Phytochemistry*. 1989;28(11):3009-3013. doi:10.1016/0031-9422(89)80269-9

11. Lewinsohn E, Berman E, Mazur Y, Gressel J. Glucosylation of exogenous flavanones by grapefruit (*Citrus paradisi*) cell cultures. *Phytochemistry*. 1986;25(11):2531-2535. doi:10.1016/S0031-9422(00)84502-1

12. Sato D, Shimizu N, Shimizu Y, et al. Synthesis of glycosides of resveratrol, pterostilbene, and piceatannol, and their anti-oxidant, anti-allergic, and neuroprotective activities. *Biosci Biotechnol Biochem*. 2014;78(7):1123-1128. doi:10.1080/09168451.2014.921551

13. Iwakiri T, Imai H, Hamada H, Nakayama T, Ozaki S-ichi, Ozaki S. Synthesis of 3,5,3',4'-tetrahydroxy-trans-stilbene-4'-O-beta-D-glucopyranoside by glucosyltransferases from *Phytolacca americana*. *Nat Prod Commun*. 2013;8(1):119-120.

14. Uesugi D, Hamada H, Shimoda K, Kubota N, Ozaki S-I, Nagatani N. Synthesis, oxygen radical absorbance capacity, and tyrosinase inhibitory activity of glycosides of resveratrol, pterostilbene, and pinostilbene. *Biosci Biotechnol Biochem*. 2017;81(2):226-230. doi:10.1080/09168451.2016.1240606

15. Koda A, Miura T, Inagaki N, et al. A method for evaluating anti-allergic drugs by simultaneously induced passive cutaneous anaphylaxis and mediator cutaneous reactions. *Int Arch Allergy Appl Immunol*. 1990;92(3):209-216. doi:10.1159/000235179