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Regioselective production of sulfated polyphenols using human cytosolic sulfotransferase-expressing *Escherichia coli* cells

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Dietary polyphenols present in fruits and vegetables have been reported to manifest beneficial health effects on humans. Polyphenol metabolites including their sulfated derivatives have been shown to be biologically active. Primarily due to the difficulty in preparing regiospecific sulfated polyphenols for detailed investigations, the exact functions of sulfated polyphenols, however, remain unclear. The current study aimed to develop a procedure for the regioselective production of sulfated polyphenols using *Escherichia coli* cells expressing human cytosolic sulfotransferases (SULTs). Two regioisomers of sulfated genistein were produced by *E. coli* cells expressing human SULT1A3, SULT1C4, or SULT1E1, and purified using Diaion HP20 resin, followed by high pressure liquid chromatography (HPLC). Structural analysis using mass spectrometry (MS) and nuclear magnetic resonance (NMR) revealed that *E. coli* cells expressing SULT1A3 preferentially produced genistein 4′-sulfate, whereas *E. coli* cells expressing SULT1C4 preferentially produced genistein 7-sulfate. To improve the bioproductivity, the effects of several factors including the concentrations of glucose and SO_4^{2-}, and growth temperature were investigated. The bioproduction procedure established in this study will be valuable for the production of regioselective sulfated polyphenols for use in future studies on their biological functions.

[**Key words:** Cytosolic sulfotransferase; Regioselective sulfation; Polyphenol metabolites; Sulfated genistein; Bioproduction]

Dietary polyphenols present in various fruits and vegetables have been reported to manifest beneficial health effects on humans (1,2). These conclusions were mostly based on *in vitro* studies using aglycone or glycoside forms of flavonoids (3,4). In human body, however, many phenolic compounds may be subjected to biotransformation via conjugation reactions such as sulfation and glucuronidation (5,6). Intriguingly, recent studies indicated that sulfated polyphenols may still be biologically active. For example, like unconjugated resveratrol, resveratrol 3-sulfate and resveratrol disulfate both were capable of inhibiting the *Escherichia coli* LPS-induced release of TNF-α by macrophages (7). In another study, daidzein 7-sulfate was shown to affect transcriptional and anti-proliferative activities of estrogen receptor-β in cancer cells, whereas daidzein 4′-sulfate and daidzein 7,4′-disulfate were inactive (8). While the biological activity of these polyphenol metabolites has been noted, the precise function of respective sulfated forms of flavonoids remains unclear due mostly to the difficulty in preparing regiospecific sulfated polyphenol compounds. Regioselective production of sulfated compounds by chemical synthesis is complicated and involves multiple reaction steps. On the other hand, enzymatic production, while allowing for regiospecifically producing sulfated polyphenols, is not suitable for their mass production. Recently, microbial bioconversion using genetically engineered bacteria, particularly *E. coli*, has been developed for the regioselective glucuronidation or methylation of polyphenol compounds (9,10). Similar procedures, however, have not yet been developed for the production of sulfated polyphenols.

Sulfate conjugation is known to be involved in the biotransformation and elimination of xenobiotics such as drugs and some dietary compounds, and for the homeostasis of key endogenous compounds such as steroid/thyroid hormones, catecholamines, and bile acids (11–13). The cytosolic sulfotransferases (SULTs) catalyze the transfer of a sulfonate group from the sulfate donor, 3′-phosphoadenosine 5′-phosphosulfate (PAPS), to various compounds containing hydroxyl and/or amino group(s) (14). In higher organisms, PAPS is synthesized in the cytosol by a bifunctional PAPS synthetase (PAPSS) with both the ATP sulfurylase and APS kinase activities (15–17). In bacteria, fungi, algae, and plants, these enzymatic activities are, however, associated with distinct enzymes encoded by separate genes (18,19). In humans, 13 SULTs that fall into four major gene families (SULT1, SULT2, SULT4 and SULT6) have been identified (20,21). Of the 13 human SULTs, the seven SULT1 family members, SULT1A1, SULT1A2, SULT1A3, SULT1B1, SULT1C2, SULT1C4 and SULT1E1, have been shown to be capable of catalyzing the sulfation of dietary polyphenols such as isoflavones (e.g., genistein, daidzein) and flavonols (e.g., quercetin, kaempherol) (22).

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Interestingly, the sulfation of polyphenols occurred in a regioselective manner and that the regiospecificity varied depending on the responsible SULT enzymes. For example, SULT1A3 catalyzes the 4’-sulfation of genistein and daidzein, whereas SULT1E1 catalyzes the sulfation at 7- and 4’-position of those polyphenols (23). A variety of regioisomers of sulfated polyphenols therefore may be produced in human body.

In this communication, we report the establishment of a procedure for the regioselective production of sulfated polyphenols using *E. coli* cells expressing different human SULTs. Genistein was chosen as a model polyphenol in this study because genistein has been shown to be regioselectively sulfated by human SULTs (23). To our knowledge, this is the first study using genetically engineered bacteria for the regioselective production of sulfated polyphenols.

MATERIALS AND METHODS

Materials Genistein was purchased from Wako Pure Chemical Industries (Osaka, Japan). Trifluoroacetic acid (TFA) and sulfatase (from *Helix pomatia*) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Isopropyl β-D-thiogalactopyranoside (IPTG) was a product of Takara Bio (Shiga, Japan). BL21 *E. coli* host strain was from Stratagene (La Jolla, CA, USA). pGEX-2TK prokaryotic GST fusion vector was from GE Healthcare Biosciences (Amersham Place, Little Chalfont, UK). M9 medium and LB medium were prepared as reported previously (24). All other chemicals were of the highest grade commercially available.

Bioconversion of genistein using human SULT-expressing *E. coli* cells By employing the reverse transcription-polymerase chain reaction technique, we had previously cloned the cDNAs encoding human SULT1A3 (gene ID: NM_177552), SULT1C4 (gene ID: NM_006588), and SULT1E1 (gene ID: NM_005420) (22). These three human SULT cDNAs were individually ligated into the pGEX-2TK prokaryotic expression vector. pGEX-2TK vector harboring SULT1A3, SULT1C4, or SULT1E1 cDNA was transformed into competent *E. coli* BL21 cells. Transformed cells were grown to $OD_{600\ nm} = 0.6–1.0$ in 200 mL LB medium containing 100 μg/mL of ampicillin, and the expression of the recombinant SULT enzyme was induced using 0.25 mM IPTG. Expression of respective SULT was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed on 10% polyacrylamide gels based on Laemmli’s method (25). Following a 9-h induction at 24°C, the cells were collected by centrifugation and resuspended in 100 mL of M9 medium supplemented with 0.5 mM genistein, 5% dimethyl sulfoxide (DMSO), 50 mM glucose, 2 mM $MgSO_4$, 100 μg/mL ampicillin, and 0.25 mM IPTG, and the cell density was adjusted to $OD_{600\ nm} = 2.5–3.0$. This cell density was empirically chosen based on tolerance to potential toxicity of 5% DMSO needed to dissolve genistein to a 0.5 mM concentration and efficiency in the production of sulfated metabolite. The cell suspension thus prepared was incubated at 24°C or 37°C for 24 h. Afterwards, the cells were spun down and the supernatant was collected for use as described below.

Preparation of sulfated genistein The cultured supernatant prepared as described above was fractionated using 20 mL Diaion HP20 resin (Nippon Rensui, Tokyo, Japan). After fractionation, the resin was washed with H_2O, and genistein and its metabolites were eluted using 100 mL methanol. Ten mL H_2O was added to the eluate, and the mixture was evaporated and the remaining residue was lyophilized. The freeze-dried powder was dissolved in 2 mL DMSO, and the solubilized genistein and its metabolites were separated by HPLC using a Shimadzu Prominence HPLC system (Shimadzu, Kyoto, Japan) fitted with a photodiode array detector (258 nm). A 5 μm Capcell PAK C18 MGII column (4.6 × 250 mm; Shiseido, Tokyo, Japan) maintained at 40°C was used, and a gradient elution using 0.05 % TFA in H_2O and methanol at a flow rate of 1 mL/min for 55 min was applied. The methanol concentrations used were as follows: 0% (0–5 min), 0–70% (5–40 min), 70–100% (40–45 min), 100% (45–50 min), 100-0% (50–55 min), and 0% (55–60 min). The fractionated sample was evaporated. After evaporation, the sample was re-fractionated with 20 mL Diaion HP20 and eluted with methanol, and the eluate was again evaporated. The sample thus purified was dissolved in methanol and analyzed by mass spectrometer. For nuclear magnetic resonance (NMR) analysis, the purified sample was dissolved in DMSO-d6 (Aldrich, Chicago, IL, USA).

Mass spectrometry (MS) analysis Samples prepared as described above were analyzed using a Q-Exactive hybrid quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific, Rockford, IL, USA) with a heated electrospray ionization source generated by direct infusion injection using a syringe pump. Data were acquired via Target-MS^2 scan. Typical mass spectrometric analysis conditions used were as follows: polarity, negative ionization mode; spray voltage 2.0 kV; sheath gas flow rate, 10; auxiliary gas, 0; sweep gas, 0; heated capillary temperature, 270°C. The resolution was set at 140,000, and the AGC target was 2E5. The maximum injection time was 100 ms, and the normalized collision energy was 20%. The raw data files were analyzed using the Qual browser software in Xcalibur (Thermo Fisher Scientific).

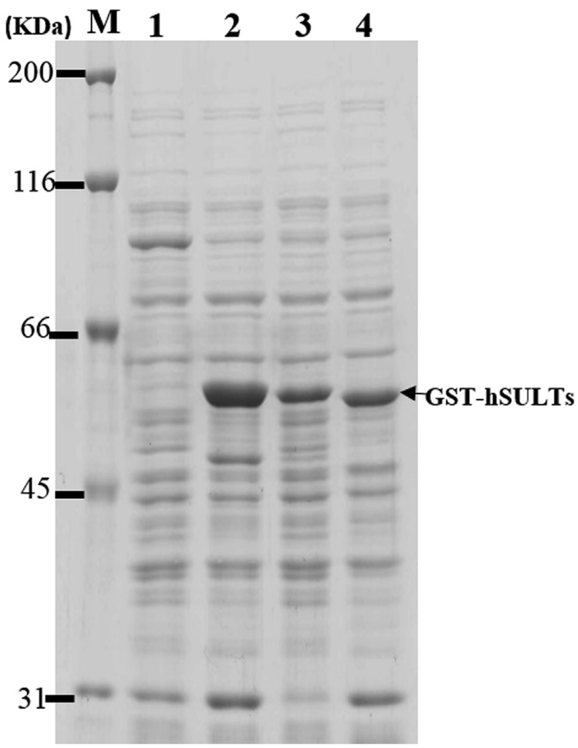

FIG. 1. Expression of GST-SULT in *E. coli* BL21 cells. *E. coli* BL21 cells were transformed with pGEX-2TK or pGEX-2TK harboring cDNA encoding human SULT, grown to 0.4 $OD_{600\ nm}$, and induced with IPTG for the expression of GST-SULT (see Materials and methods). Recombinant GST-SULT expression was verified by SDS-PAGE. Lane M, molecular maker; lane 1, Mock *E. coli* BL21 cells; lane 2, *E. coli* BL21 cells expressing GST-SULT1A3; lane 3, *E. coli* BL21 cells expressing GST-SULT1C4; lane 4, *E. coli* BL21 cells expressing GST-SULT1E1.

NMR analysis The sample dissolved in DMSO-d6 was analyzed using a Bruker Advance 400 instrument (400 MHz, 9.4 T) (Bruker, Karlsruhe, Germany). The chemical shifts (δ) for proton were given in parts per million (ppm) relative to tetramethylsilane (TMS) as an internal standard. For the ^1H NMR experiment, 32 transients were acquired with a spectral width of 8000 Hz. All NMR data were processed using XWINNMR (Bruker). The chemical structures of two sulfated forms of genistein were determined based on the NMR data.

Quantitative analysis of sulfated genistein To quantitatively determine the portion of genistein that was present in sulfated form, the sample was treated with type H-1 sulfatase from *H. pomatia*, and the sulfatase-treated sample was compared with untreated control with the same amount of unconjugated genistein. Treatment with sulfatase was performed according to the manufacturer’s instructions with a slight modification. The reaction mixture, with a final volume of 100 μL, contained sulfatase (0.1 unit), fractionated genistein sulfate preparation, and 150 mM of Tris–HCl buffer (pH 8.0). Upon incubation for 4 h at 37°C, 50 μL of methanol was added and the mixture heated at 98°C for 3 min to stop the reaction. The heated sample was centrifuged at 15,000 ×g for 10 min, and the supernatant collected was analyzed by HPLC for unconjugated genistein, compared with a standard curve prepared using commercial genistein.

RESULTS AND DISCUSSION

Production of sulfated genistein by *E. coli* cells expressing human SULTs In this study, genistein was chosen as a model compound for investigating the production of regioselectively sulfated product(s) using *E. coli* BL21 cells expressing human SULT1A3, SULT1C4, or SULT1E1. A previous study demonstrated that SULT1A3 catalyzed exclusively 4’-sulfation of genistein, whereas SULT1E1 catalyzed both 4’-sulfation and 7-sulfation (23). In the case of SULT1C4, while it has been shown to display strong sulfating activity toward genistein, its regioselectivity in mediating the sulfation of genistein remained unclear (22). It is noted that three other human SULT1 enzymes, SULT1A1,

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