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Synthesis and biological evaluation of a novel baicalein glycoside as an anti-inflammatory agent

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ABSTRACT

Baicalein-6-α-glucoside (BG), a glycosylated derivative of baicalein, was synthesized by using sucrose and the amylosucrase of *Deinococcus geothermalis* and tested for its solubility, chemical stability, and anti-inflammatory activity. BG was 26.3 times more soluble than baicalein and highly stable in buffered solutions and Dulbecco's modified Eagle medium containing 10% fetal bovine serum. BG treatment decreased the production of nitric oxide in RAW 264.7 cells treated with lipopolysaccharide (LPS). Luciferase reporter assays, western blots, reverse transcription-polymerase chain reaction, and flow cytometric analyses indicated that BG activated nuclear factor erythroid 2-related factor 2 (Nrf2), an antioxidant transcription factor that confers protection from various inflammatory diseases, induced Nrf2-dependent gene expression, and suppressed the production of reactive oxygen species elicited by LPS more effectively than baicalein. Cellular uptake of BG assessed by confocal microscopy and HPLC analysis of the cell-free extracts of RAW 264.7 cells demonstrated that BG was gradually converted to baicalein inside the cells. These results explain that glycosylation increased the bioavailability of baicalein by helping to protect this vital molecule from chemical or enzymatic oxidation. Therefore, BG, a glycosylated derivative of baicalein, can be an alternative to baicalein as a therapeutic drug.

1. Introduction

Scutellaria baicalensis is one of the most widely used herbal medicine against bacterial infections of the respiratory and gastrointestinal tracts and various inflammatory diseases. Its roots have been prescribed for the treatment of fever, high blood pressure, and acute pneumonia in Korean traditional medicine (Gong and Sucher, 1999; Li et al., 2004; Kumagai et al., 2007). Baicalin (baicalein 7-*O*-glucuronide) is a major active ingredient of *S. baicalensis* root; acid hydrolysis of baicalin yields glucuronic acid and a flavone aglycone named baicalein (5,6,7-trihydroxyflavone). The anti-inflammatory effects of these constituents are well documented (Sekiya and Okuda, 1982; Kubo et al., 1984). Recently, baicalein has been shown to inhibit lipopolysaccharide (LPS)-induced nitric oxide (NO) production in macrophages (Wakabayashi, 1999). Although flavonoids such as baicalein and baicalin have been reported to show a variety of biological activities, they have limited pharmaceutical use due to their low water solubility, fast oxidative degradation, fast metabolism, and low absorption rate in the small intestine. Various methods have been used to overcome these issues of flavonoids. One is to use cyclodextrin, which is widely employed as an excipient to increase the solubility and stability of drugs (Oda et al., 2004; Bian et al., 2009). Zhang et al. (2011) reported that a hydroxypropyl-β-cyclodextrin-genipin complex increased water solubility 3.5-fold. Chemical modification of flavonoids with a pivaloxymethyl group is another method (Kim et al., 2010). However, these methods also have drawbacks, including compromised product purity, difficulty of preparation, and environmental pollution.

Addition of a sugar moiety to the compound by enzymatic glycosylation can be an alternative to overcome these liabilities. The glycosylated flavonoids, which are synthesized by bacterial glycosidases and glycosyltransferases, have been reported to have various merits: increased water solubility, oxidative stability, bioavailability, and decreased cytotoxicity. For example, the use of maltosyltransferase from *Caldicellulosiruptor bescii* DSM 6725 enhanced the water solubility of piceid glucosides by 1.86×10^3 times compared with a piceid (Park et al., 2012). Addition of glucose to puerarin by bioconversion using *Microbacterium oxydans* CGMCC 1788 improved water solubility and pharmacokinetic parameters, while maintaining its bioavailability (Jiang et al., 2008). Hijiya and Miyake (1991) synthesized glucosyl hesperidin (G-hesperidin) by transglycosylation with cyclodextrin glucanotransferase from *Bacillus stearothermophilus*. The solubility of

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G-hesperidin was approximately 10,000 times higher than that of hesperidin, and it possessed similar antioxidant properties as hesperidin in vitro (Yamada et al., 2003). Moreover, the biological activities of G-hesperidin were greater than those of hesperidin (Ohtsuki et al., 2003).

In this study, we tested whether the glycosylation of baicalein enhances the solubility and stability of baicalein. Glycosylation of baicalein was carried out by amylosucrase from *Deinococcus geothermalis*. The molecular structure of the transglycosylation product of baicalein was determined, and its water solubility and oxidative stability were examined. Since baicalein is used to treat inflammation, we examined whether glycosylation of baicalein affects inflammatory reaction by using LPS-treated RAW 264.7 cells.

2. Materials and methods

2.1. Chemicals and reagents

Baicalein, sucrose, baicalin, sulforaphane, fructose, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water and methanol [high-performance liquid chromatography (HPLC)-grade] were purchased from Burdick & Jackson (USA) for purification. All other chemicals were of reagent grade and were purchased from Sigma-Aldrich. TLR4-specific *Escherichia coli* LPS was purchased from Alexis Biochemical (San Diego, CA, USA). Antibodies against nuclear factor erythroid 2-related factor 2 (Nrf2) and Lamin A/C were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The recombinant amylosucrase from *D. geothermalis* (DGAS) was prepared in *E. coli* as previously described (Cho et al., 2011).

2.2. Animal cell culture

A murine macrophage cell line, RAW 264.7 cells, was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured in Dulbecco's Modified Eagle Medium supplemented with 10% (v/v) heat-inactivated FBS (cDMEM; Thermo, MA, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco; NY, USA). The cells were cultured at 37 °C under 5% CO_2 in fully humidified air. Nrf2 reporter cell lines were prepared as previously described (Choi et al., 2012).

2.3. Synthesis of baicalein glycoside (BG) using DGAS

To synthesize baicalein glycoside (BG), a substrate solution containing 20 mM baicalein and 40 mM sucrose in 50 mM Tris–HCl buffer (pH 8) was preincubated at 30 °C for 30 min. After preincubation, DGAS (1 mg/ml) was added to the reaction mixture and the enzymatic synthesis was carried out at 30 °C for 12 h (Cho et al., 2011). The transfer reaction was stopped by heating in boiling water for 30 min and placing the mixture tube in ice. The reaction mixture was centrifuged at 3000*g* for 20 min to get rid of the insoluble substances. The supernatant fraction was filtered using a 0.22-μm syringe filter (Satorius; Goettingen, Germany).

2.4. Purification of BG

The transfer products were separated using a C_{18}-T cartridge (100 mg/ml; Strata) and recycling preparative HPLC equipped with an ultraviolet (UV) detector (JAI; Tokyo, Japan). A C_{18}-T cartridge, which was previously activated using methanol and water, was used to absorb the BG from the transglycosylation reaction mixture and to remove any sugars and salts. The transglycosylation reaction mixture was filtered using a 0.45-μm syringe filter (Sartorius) and added to the C_{18}-T cartridge. After washing twice, elution of the transfer products was carried out with methanol. The main transfer product in methanol was purified using a combination W-252/W-251 polymeric gel filtration column (2×50 cm^2; JAI) in the recycling preparative HPLC system. The mobile phase was 100% methanol at a flow rate of 3 ml/min. The fractions corresponding to the detected peaks were collected and freeze-dried. The purity of each sample was confirmed using thin layer chromatography (TLC) analysis.

2.5. TLC analysis

The purified transfer products were spotted on Whatman K5F silica gel plates (Whatman; Maidstone, UK) activated at 110 °C for 30 min. The plates were developed in developing solution composed of *n*-butanol:ethanol:water (5:3:2, v/v/v) for BG. The developed TLC plates were dried completely at room temperature after irrigating once or twice, and visualized using a UV lamp in combination with a UV viewing box (Camag; Muttenz, Switzerland) at 254 nm. The transfer products were also visualized by dipping in a solution containing 0.3% (w/v) *N*-(1-naphthyl)-ethylenediamine and 5% (v/v) H_2SO_4 in methanol followed by heating at 110 °C for 10 min.

2.6. HPLC analysis

Cellular uptake was investigated by using HPLC. RAW 264.7 cells were seeded into 60 mm cell culture plates (2×10^6 cells/ml). After overnight incubation, 100 μM baicalein or BG was added, and the cells were incubated for up to 4 h at 37 °C under 5% CO_2; then the medium was removed and the cells were washed with 2 ml of ice-cold PBS three times and scrapped with 2 ml of PBS. After centrifugation, the cell pellet was resuspended with 0.1 ml of distilled water by vortexing and lysed by freezing and thawing using the liquid nitrogen three times. After centrifugation, the supernatant was filtered by a 0.2 μm syringe filter and analyzed by UPLC. UPLC analysis was carried out using an Acquity UPLC H Class system (Waters, Ireland) comprised a Model bioSample Manager-FTN, a Model bioQuaternary Solvent Manager, and a PDA eλ detector. Identification of baicalein and BG was carried out by reverse-phase HPLC using a C_{18} (4.6×250 mm^2) column (Shodex). The column temperature was 35 °C, the mobile phase was 59% methanol and 41% water containing 0.2% phosphoric acid. The flow rate was 0.8 ml/min and the injection volume was 10 μl. Baicalein and BG were observed at 276 nm. Three injections were performed for each sample and standard.

2.7. Nuclear magnetic resonance (NMR) and fast atom bombardment-mass spectrometer (FAB-MS) analysis

Approximately 5.5 mg of baicalein and purified BG were dissolved in 0.5 ml of pure CD_3OD and placed in 5 mm NMR tubes. The ^1H and ^{13}C NMR spectra of baicalein and purified BG were obtained with a Varian Inova AS 600 MHz NMR spectrometer (Varian; Palo Alto, CA). The sample was dissolved in CD_3OD at 24 °C with tetramethylsilane (TMS) as an internal standard. Chemical shifts are reported as s (singlet), d (doublet), t (triplet), m (multiplet), or br s (broad singlet). Coupling constants are reported in Hz. The chemical shifts are reported as parts per million (δ) relative to the solvent peak. ^1H NMR (600 MHz, CD_3OD, δ) 7.96 (2H, br s, J=7.2 Hz, H-2′,6′), 7.55 (3H, m, H-3′,4′,5′), 6.70 (1H, s, H-3), 6.53 (1H, s, H-8), 5.19 (1H, d, J=3.6 Hz, H-1″), 4.29 (1H, dt, J=9.6, 3.0 Hz, H-5″), 3.92 (1H, dd, J=9.6, 9.6 Hz, H-3″), 3.82 (2H, d, J=3.0 Hz, H-6″), 3.56 (1H, dd, J=9.6, 3.6 Hz, H-2″), 3.49 (1H, dd, J=9.6, 9.6 Hz, H-4″); ^{13}C NMR (150 MHz, CD_3OD, δ) 184.1 (C-4), 165.6 (C-2), 162.2 (C-8a), 155.9 (C-7), 154.6 (C-5), 133.1 (C-4′), 132.8 (C-1′), 131.9 (C-6), 130.4 (C-3′,5′), 127.6 (C-2′,6′), 105.7