jmb

Glycosylation Enhances the Physicochemical Properties of Caffeic Acid Phenethyl Ester

Keum-Ok Moon[1], Soyoon Park[1], Myungsoo Joo[2], Ki-Tae Ha[2], Nam-In Baek[3], Cheon-Seok Park[3], and Jaeho Cha[1,4]*

[1]*Department of Microbiology, Pusan National University, Busan 46241, Republic of Korea*

[2]*School of Korean Medicine, Pusan National University, Yangsan 50612, Republic of Korea*

[3]*Graduate School of Biotechnology and Institute of Life Sciences & Resources, Kyung Hee University, Yongin 17104, Republic of Korea*

[4]*Microbiological Resource Research Institute, Pusan National University, Busan 46241, Republic of Korea*

In this study, we synthesized a glycosylated derivative of caffeic acid phenethyl ester (CAPE) using the amylosucrase from *Deinococcus geothermalis* with sucrose as a substrate and examined its solubility, chemical stability, and anti-inflammatory activity. Nuclear magnetic resonance spectroscopy showed that the resulting glycosylated CAPE (G-CAPE) was the new compound caffeic acid phenethyl ester-4-*O*-α-D-glucopyranoside. G-CAPE was 770 times more soluble than CAPE and highly stable in Dulbecco's modified Eagle's medium and buffered solutions, as estimated by its half-life. The glycosylation of CAPE did not significantly affect its anti-inflammatory activity, which was assessed by examining lipopolysaccharide-induced nitric oxide production and using a nuclear factor erythroid 2-related factor 2 reporter assay. Furthermore, a cellular uptake experiment using high-performance liquid chromatography analysis of the cell-free extracts of RAW 264.7 cells demonstrated that G-CAPE was gradually converted to CAPE within the cells. These results demonstrate that the glycosylation of CAPE increases its bioavailability by helping to protect this vital molecule from chemical or enzymatic oxidation, indicating that G-CAPE is a promising candidate for prodrug therapy.

Keywords: Amylosucrase, anti-inflammation, bioavailability, caffeic acid phenethyl ester, glycoside, transglycosylation

Introduction

Propolis is a natural bee product that is used as a safe dietary supplement in traditional medicine for its therapeutic benefits. One of the natural active components in propolis is caffeic acid phenethyl ester (CAPE), which is an ester of caffeic acid and phenethyl alcohol [1]. Numerous studies have shown that CAPE possesses multifunctional biological properties across a broad range of systems, including antibacterial, anti-inflammatory, antioxidant, antiviral, anticarcinogenic, and immunomodulatory properties [2–9]. For example, CAPE exhibited inhibitory effects on the production of proinflammatory cytokines (interleukin-1β, tumor necrosis factor (TNF)-α, and monocyte chemoattractant protein-1) from lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages [10]. Song *et al.* [11] also found that CAPE protected human middle ear epithelial cells against H_2O_2-induced inflammatory and oxidative reactions via CAPE-mediated inhibition of expression of the proinflammatory cytokine TNF-α and the proinflammatory enzyme COX-2. Furthermore, CAPE has been shown to markedly reduce mRNA expression of the inflammatory cytokines TNF-α and interferon-gamma (IFN-γ) in streptozotocin diabetic rats; it has been suggested that CAPE could prevent the development of inflammation after irradiation, which is widely used for the treatment and management of cancer [12].

To benefit from these activities, CAPE needs to reach the target tissues at a specific concentration, which must then be maintained for a certain period of time. Therefore, since CAPE is usually administered orally as a component of propolis, it is crucial to assess its bioavailability following

oral ingestion. The solubility and stability of drugs are frequently improved using the solid dispersion/nanoparticle technique [13–17], chemical modification [18], and preparation of a complex with cyclodextrins [19]. However, all of these methods have drawbacks, including compromised product purity, difficulty of preparation, and environmental pollution.

Transglycosylation has been used to improve the physico-chemical properties (*e.g.*, water solubility and oxidative stability) of various compounds using the enzymes from various bacteria as catalysts. For example, the water solubility of piceid glucosides synthesized using maltosyltransferase from *Caldicellulosiruptor bescii* DSM 6725 was 1.86×10^3 times higher than that of the original piceid [20]. Furthermore, Kometani *et al.* [21] synthesized glucosyl hesperidin by transglycosylation with cyclodextrin glucanotransferase from alkalophilic *Bacillus*, which has been shown to have an approximately 10,000 times higher solubility and similar antioxidant properties to hesperidin in vitro [22], as well as greater biological activities [23]. Various phenolic compounds have been glycosylated using bacterial amylosucrases, such as rutin, baicalein, arbutin, (+)-catechin, and salicin, with the resulting glycosides having improved solubilities and stabilities than the original compounds [24–28].

Therefore, the aim of this study was to investigate the in vitro stability and solubility of glycosylated CAPE (G-CAPE) as a potential prodrug. CAPE was glycosylated by enzymatic transglycoslyation, and the effects of glycosylation on its bioavailability and anti-inflammatory activity were then assessed.

Materials and Methods

Chemicals and Reagents

CAPE, sulforaphane, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT), and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (USA). High-performance liquid chromatography (HPLC)-grade water, acetonitrile, and methanol were purchased from Burdick & Jackson (USA) for purification. All other chemicals were of reagent grade and purchased from Sigma-Aldrich. Toll-like receptor 4 (TLR4)-specific *Escherichia coli* LPS was purchased from Alexis Biochemical (USA). Antibodies against nuclear factor erythroid 2-related factor 2 (Nrf2) (H-300) and lamin A/C (H-110) were obtained from Santa Cruz Biotechnology (USA). Recombinant amylosucrase from *Deinococcus geothermalis* (DGAS) was prepared as previously described [26].

Animal Cell Culture

Cells derived from the murine macrophage cell line RAW 264.7 (ATCC, USA) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated FBS (cDMEM;

Thermo, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, USA) at 37°C under 5% CO_2 in a humidified culture chamber (Sanyo Electric Biochemical Co., Ltd, Japan). Nrf2 reporter cell lines were prepared as previously described [29].

Synthesis of Caffeic Acid Phenethyl Ester Glucoside Using DGAS

To synthesize G-CAPE, a substrate solution containing 10 mM CAPE and 20 mM sucrose in 50 mM Tris-HCl buffer (pH 8.0) was preincubated at 30°C for 10 min. Dimethyl sulfoxide was then added (up to 20% (v/v)) to dissolve the CAPE. Following preincubation, DGAS (1 mg/ml) was added to the reaction mixture and the enzymatic synthesis was carried out at 30°C for 12 h [26]. The transfer reaction was stopped by heating the reaction mixture in boiling water for 10 min, following which the reaction mixture was centrifuged at 3,000 ×*g* for 20 min to remove any insoluble substances. The supernatant fraction was then filtered with a 0.45-μm syringe filter (Satorius, Germany).

Purification of G-CAPE

The reaction products were separated using a C_{18}-T cartridge and recycling preparative HPLC. The transglycosylation reaction mixture was filtered with a 0.45-μm syringe filter (Sartorius) and added to the Strata C_{18}-T cartridge (200 mg/3 ml; Phenomenex, USA), which had previously been activated with methanol and water, to absorb the G-CAPE. After washing twice, the transfer products were eluted with methanol. The main transfer product was then further purified using a JAIGEL W-252/W-251 column (2 × 50 cm; Japan Analytical Industry Co., Ltd (JAI), Korea) in the recycling preparative HPLC equipped with a refractive index detector (JAI) and eluted with methanol at a flow rate of 3.0 ml/min with 30 kgf/cm² column pressure. The purified product was lyophilized and the purity of G-CAPE was confirmed using thin-layer chromatography (TLC).

TLC Analysis

The purified transfer product was dissolved in *n*-butanol-ethanol-water (5:3:2 (v/v/v)) and analyzed using TLC with a Whatman K5F silica gel plate (Whatman, UK). After irrigating twice, the TLC plate was dried and visualized using an ultraviolet (UV) lamp in combination with a UV viewing box (Camag, Switzerland) at 254 nm. The transfer product was also visualized by dipping it into 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.

UPLC Analysis

CAPE and G-CAPE in cell lysates were analyzed by ultra-performance liquid chromatography (UPLC) using a previously published method [30] with slight modification. An Acquity UPLC H-Class system (Waters, Ireland) was used, which comprised a Model bioSample Manager-FTN, a Model bioQuaternary Solvent

Manager, and a Model Photodiode Array eλ detector set at 325 nm. Samples (1 μl) were injected with Model 701 syringes (Hamilton, Switzerland), separated at 35°C using a 1.7-μm Acquity UPLC BEH C18 column (2.1 mm × 50 mm), and eluted in gradient mode at a flow rate of 0.25 ml/min as follows: 75% A at 0−0.1 min; 75−70% A at 0.1−1.5 min; 70−60% A at 1.5−3.5 min; 60−50% A at 3.5−5.5 min; and 50% A at 5.5−6.5 min. The mobile phase was water containing 0.1% formic acid (solvent A) and acetonitrile (solvent B). The post-running time was 2.5 min. The identities of the CAPE and G-CAPE peaks were assigned by co-chromatography with authentic standards. Quantification was carried out by integrating the peak areas using the external standardization method.

Cellular Uptake of G-CAPE

The cellular uptakes of CAPE and G-CAPE, as well as chemical changes after cellular uptake, were monitored by UPLC. RAW 264.7 cells were seeded into 100-mm cell culture plates (1×10^6 cells/ml) and incubated overnight. CAPE or G-CAPE (100 μM) was then added, and the cells were incubated for 2 h at 37°C under 5% CO_2, following which the medium was removed and the cells were washed three times with 2 ml of ice-cold PBS and scraped from the wells into 2 ml of PBS. Following centrifugation, the cell pellet was resuspended in 0.1 ml of distilled water by vortexing, and lysed using three freeze-thaw cycles with liquid nitrogen. The solution was then re-centrifuged, and the supernatant was filtered with a 0.2-μm syringe filter and analyzed by UPLC.

Nuclear Magnetic Resonance (NMR) and Fast Atom Bombardment-Mass Spectrometry (FAB-MS) Analyses

Approximately 10 mg of CAPE or purified G-CAPE was dissolved in 0.5 ml of pure deuterated methanol (CD_3OD) and placed in 5-mm NMR tubes. NMR spectra were then obtained with an Inova AS 400 MHz FT-NMR spectrometer (Varian Inc., USA) operated at 400 MHz for ^1H and 100 MHz for ^{13}C at 25°C. The bond between CAPE and glucose in G-CAPE was confirmed by examining the heteronuclear multiple bond correlation spectrum. The sample was dissolved in CD_3OD with tetramethylsilane as an internal standard. The following peaks were obtained, whereby chemical shifts are reported as s (singlet), d (doublet), t (triplet), m (multiplet), or br s (broad singlet) in parts per million (δ) relative to the solvent peak and coupling constants are reported in Hz: ^1H-NMR (400 MHz, CD_3OD, δ_H) 7.52 (1H, d, $J = 16.0$ Hz, H-7), 7.27 (1H, d, $J = 8.4$ Hz, H-5), 7.30~7.25 (4H, overlapped, H-2′, 3′, 5′, 6′), 7.19 (1H, m, H-4′), 7.08 (1H, d, $J = 2.0$ Hz, H-2), 6.99 (1H, dd, $J = 8.4, 2.0$ Hz, H-6), 6.30 (1H, d, $J = 16.0$ Hz, H-8), 5.41 (1H, d, $J = 3.6$ Hz, H-1′′), 4.36 (1H, t, $J = 6.8$ Hz, H-8′), 3.90~3.41 (6H, m, H-2′′, 3′′, 4′′, 5′′, 6′′), 2.97 (1H, t, $J = 6.8$ Hz, H-7′); ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 168.76 (C-9), 148.92 (C-3), 148.54 (C-4), 146.15 (C-7), 139.35 (C-1′), 131.00 (C-1), 129.96 (C-2′, 6′), 129.49 (C-3′, 5′), 127.53 (C-4′), 122.11 (C-6), 118.47 (C-5), 117.21 (C-8), 116.09 (C-2), 100.80 (C-1′′), 74.78 (C-5′′), 74.66 (C-3′′), 73.34 (C-2′′), 71.28 (C-4′′), 66.22 (C-8′), 62.29 (C-6′′), 36.13 (C-7′). The negative FAB-MS was measured as previously described [27].

Solubility Determination

Excess amounts of CAPE or G-CAPE were suspended in 200 μl of distilled water in a microcentrifuge tube at 25°C. A JAC-4020 ultrasonic cleaner (Kodo, Korea) was then used to maximize the solubility of each compound. Following sonication at room temperature for 1 h with intermittent pauses, each sample was centrifuged at 12,000 ×g for 20 min. The supernatant of each sample was then filtered through a 0.45-μm membrane filter and the concentration of the compound in the supernatant, which is defined as the water-soluble component, was estimated by measuring its absorbance at 325 nm using a GeneQuant *pro* UV/Vis spectrophotometer (Amersham Biosciences, UK) and its absolute solubility was calculated.

Stability against Oxidative Degradation

CAPE or G-CAPE was dissolved in buffer solutions of various pH values (pH 2.0, pH 7.4 (PBS), and pH 9.0) to reach a final concentration of 10 mM. The solutions were then incubated at 37°C, and an aliquot (300 μl) of the reaction mixture was extracted at different time points, and filtered and injected into an HPLC system equipped with a C_{18} reverse phase column. CAPE and G-CAPE were also dissolved in cDMEM and DMEM to reach final concentrations of 10 mM, and incubated in a humidified incubator (37°C and 5% CO_2). The medium was then harvested at different time points up to 72 h, vortexed, and analyzed by HPLC under the same conditions as outlined above.

Cell Viability Assay

Cell viability was determined using an MTT-based colorimetric assay, as described previously [27].

Nitrite Assay

RAW 264.7 cells were plated into 24-well plates at a density of 5×10^5 cells/well for 24 h, and were then treated with LPS (100 ng/ml) and 5, 10, 15, and 20 μM concentrations of CAPE or G-CAPE for an additional 12 h. The amount of nitrite (as an estimate of NO production) was measured using the Griess reaction, as described previously [27].

Luciferase Assay

Nrf2 reporter cells (5×10^5 cells/well) in the presence of G418 (100 μg/ml) were incubated with various concentrations of CAPE or G-CAPE for 6 h. Total cell lysate was then prepared and the luciferase activity was measured with a luciferase assay kit (Promega, USA) following the manufacturer's instructions. The luciferase activity was normalized to the amount of total protein in the cell extract.

Statistical Analysis

Statistical analysis of the data ($n = 3$ independent experiments) was performed using SPSS ver. 14.0. Student's *t*-test and one-way analysis of variance followed by Tukey's post hoc test were used to compare the means. All values are expressed as the means ±

Results

CAPE Is Glycosylated by DGAS

Although CAPE is a well-known anti-inflammatory agent, there are some restrictions to its use due to its low solubility and cytotoxicity. Therefore, since glycosylation has been shown to enhance the physiocochemial properties of baicalein and aesculin, we tested the possibility that it would also increases the bioavailability of CAPE without affecting its anti-inflammatory activity. CAPE was successfully transglycosylated by DGAS (Fig. 1), which has been used to synthesize various other glycoconjugates through the transfer of the glycosyl unit of sucrose [31]. TLC analysis of the reaction products identified a major product. The spot that corresponded to CAPE was invisible, but another spot, which possibly represented the newly produced G-CAPE, appeared after dipping the TLC plate into the sulfuric acid solution (Fig. 1, inset). A spot was also observed in the upper position under UV light (data not shown), which is likely to have originated from CAPE, since this was the only chromophore included in the reaction mixture. G-CAPE was isolated using preparative recycling HPLC, whereby CAPE and G-CAPE were eluted as single peaks at 180 min and 131 min, respectively (Fig. 1).
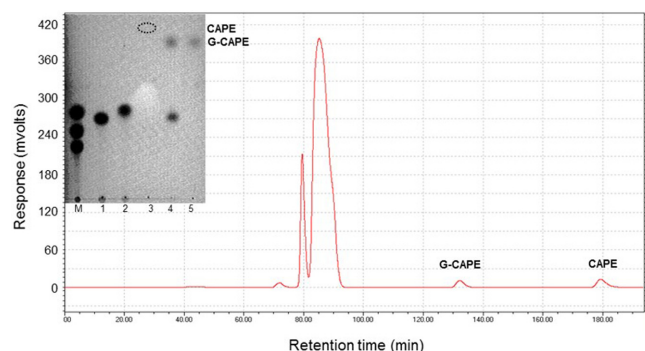

Fig. 1. Thin-layer chromatography (TLC) and recycling preparative high-performance liquid chromatography (HPLC) analyses of the transglycosylation reaction by recombinant amylosucrase from *Deinococcus geothermalis* (DGAS) with caffeic acid phenethyl ester (CAPE) and sucrose as the acceptor and donor, respectively.

TLC analysis: lane M, standard markers from glucose to maltotriose; lane 1, sucrose; lane 2, fructose; lane 3, CAPE; lane 4, reaction products of the CAPE transglycosylation reaction by DGAS; lane 5, purified glycosylated CAPE (G-CAPE). In the recycling preparative HPLC, CAPE and G-CAPE were eluted at 180 min and 131 min, respectively.

The molecular mass of the newly synthesized G-CAPE was determined to be 446 Da from the molecular ion peak at m/z 445 [M-H]$^-$, indicating that it consisted of one glucose unit and CAPE.

The ^{1}H-NMR (400 MHz, CD$_3$OD) spectrum of G-CAPE showed 10 downfield olefinic methine proton signals, which included three signals (δ_H 7.27 (1H, d, J = 8.4 Hz, H-5), 7.08 (1H, d, J = 2.0 Hz, H-2), 6.99 (1H, dd, J = 8.4, 2.0 Hz, H-6)) that resulted from a 1,2,4-trisubstituted benzene moiety, four overlapped signals (δ_H 7.30~7.25, 4H, overlapped, H-2′, 3′, 5′, 6′), and one signal (δ_H 7.19 (1H, m, H-4′)) that resulted from a monosubstituted benzene moiety, as well as two signals (δ_H 7.52 and 6.30) that arose from a double bond with the coupling constant (J = 16.0 Hz), indicating that the double bond had a *trans*-configuration. An oxygenated methylene proton signal (δ_H 4.36, 1H, t, J = 6.8 Hz, H-8′) and a methylene proton signal (δ_H 2.97, 1H, t, J = 6.8 Hz, H-7′) were also observed. These data suggest that the G-CAPE molecule contained phenylpropanoid and phenyl ethanol moieties. In addition, proton signals from a hexose were also observed: a hemiacetal (δ_H 5.41, 1H), four oxygenated methines, and an oxygenated methylene (δ_H 3.90~3.41, 6H). The stereostructure of the anomer hydroxyl was determined to be α from the coupling constant (J = 3.6 Hz) of the anomer proton signal.

The ^{13}C-NMR (100 MHz, CD$_3$OD) spectrum showed 23 carbon signals (Fig. 2A). The aglycone signals included one carbonyl carbon signal (δ_C 168.76, C-9), two oxygenated olefinic quaternary carbon signals (δ_C 148.92 (C-3), 148.54 (C-4)), two olefinic quaternary carbon signals (δ_C 139.35 (C-1′), 131.00 (C-1)), 10 olefinic methine carbon signals (δ_C 146.15 (C-7), 129.96 (C-2′, C-6′), 129.49 (C-3′, C-5′), 127.53 (C-4′), 122.11 (C-6), 118.47 (C-5), 117.21 (C-8), 116.09 (C-2)), one oxygenated methylene carbon signal (δ_C 66.22, C-8′), and one methylene carbon signal (δ_C 36.13, C-7′). The carbon chemical shifts combined with the proton data indicated that the aglycone was a conjugated compound produced by attaching a caffeic acid to a phenylethanol through an ester bond. The sugar was identified as an α-D-glucopyranose based on the carbon chemical shifts, which included an anomer carbon (δ_C 100.80), four oxygenated methine carbons (δ_C 74.78 (C-5′′), 74.66 (C-3′′), 73.34 (C-2′′), 71.28 (C-4′′)), and an oxygenated methylene carbon (δ_C 62.29, C-6′′). The position of the glucose was estimated to be HO-4 of the caffeic acid from comparison of the chemical shift of C-6 (δ_C 122.11) with that reported in the literature [32]. When the sugar was attached to HO-3, C-6 was usually observed at δ_C 126.70. This position was confirmed by the correlation between the anomer proton
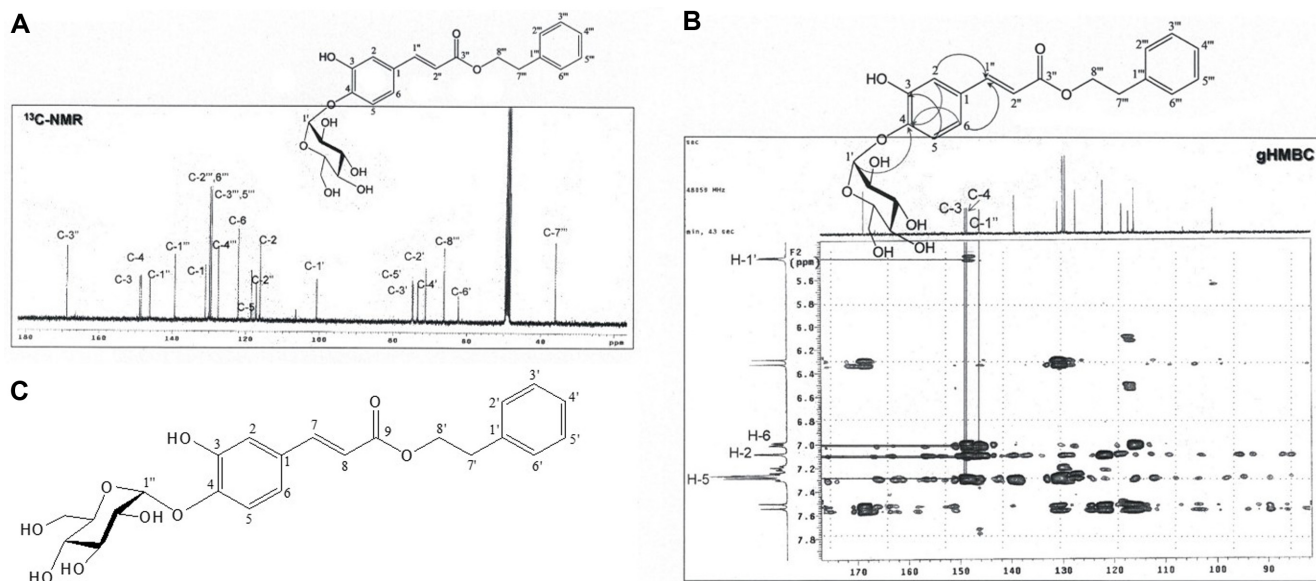
Fig. 2. Nuclear magnetic resonance (NMR) analysis of glycosylated caffeic acid phenethyl ester (G-CAPE).
(**A**) ^{13}C-NMR, (**B**) heteronuclear multiple bond connectivity (gHMBC) spectrum, and (**C**) the chemical structure of G-CAPE.

signal (δ_H 5.41, H-1'') and the oxygenated olefinic quaternary carbon signal (δ_C 148.54, C-4) in the heteronuclear multiple bond connectivity (gHMBC) spectrum (Fig. 2B). Thus, G-CAPE was identified as the new compound caffeic acid phenethyl ester-4-*O*-α-D-glucopyranoside (Fig. 2C).

Glycosylation Enhances the Water Solubility and Oxidative Stability of CAPE

Phenolic compounds such as CAPE require specific modification in pharmaceutical or cosmetic preparations owing to their low solubility and high sensitivity to oxidation. The water solubility of CAPE was determined to be 6.5 mg/l, whereas that of G-CAPE was 5×10^3 mg/l, which is 770 times higher. This implies that the attachment of a glucosyl residue to CAPE enhances its water solubility.

In general, phenolic compounds have short half-lives of <10 h in buffered aqueous solutions because they undergo fast autooxidative degradation [33, 34]. However, transient protection of the oxidative group with a promoiety would stabilize the phenolic compounds against oxidative degradation. The stabilities of CAPE and G-CAPE were determined by their half-lives ($t_{1/2}$ (h)) in various PBS solutions (pH 7.4, 2.0, or 9.0) as well as in a cell culture medium. We found that CAPE underwent rapid degradation at a high pH, whereas it showed high stability at a neutral pH (PBS) and under acidic conditions, with a half-life of >72 h. By contrast, G-CAPE showed greater stability in each of the buffered aqueous solutions over the varying pH

values. The half-life in cDMEM was >72 h for CAPE but only 3.08 h for G-CAPE, whereas that in DMEM was 6.18 h for CAPE and >72 h for G-CAPE (Table 1). The improved stability of G-CAPE would be due to the glycosylation of the oxidative group of CAPE, which prevents its oxidative decomposition even after 72 h of incubation at an alkaline pH [27]. These opposite tendencies depending on the presence of FBS suggest that G-CAPE is deglycosylated from glycone to aglycone in cDMEM by the specific reaction of the undefined glycosidases in FBS as previously observed in glycosylated baicalein [27].

G-CAPE Has Reduced Cytotoxicity toward RAW 264.7 Cells

To determine the concentration of G-CAPE that can be used without any cytotoxicity, MTT assays were carried out on the murine macrophage cell line RAW 264.7. The cells were treated with various amounts (5 to 20 μM) of

Table 1. Stability of CAPE and G-CAPE in buffer solutions and cell culture medium.

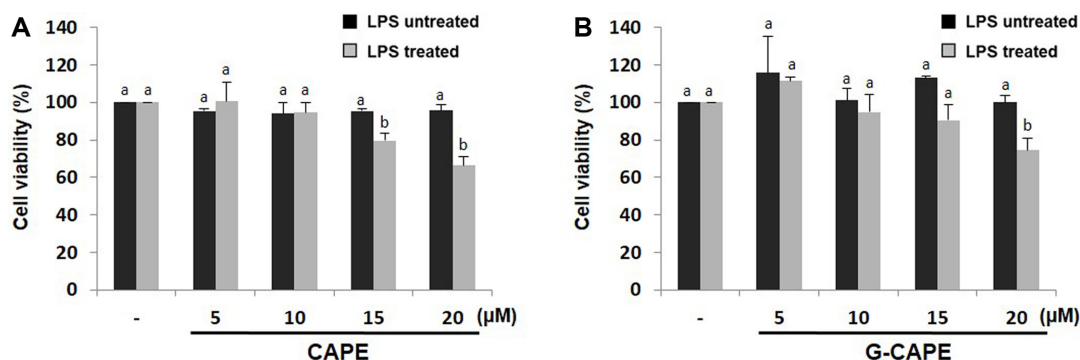|		Half-life, $t_{1/2}$ (h)	
		CAPE	G-CAPE
Buffer	pH 2.0	> 72	> 72
	PBS (pH 7.4)	> 72	> 72
	pH 9.0	2.3	64
cDMEM (with FBS)		> 72	3.1
DMEM (without FBS)		6.2	> 72

Fig. 3. Effects of caffeic acid phenethyl ester (CAPE; **A**) and glycosylated CAPE (G-CAPE; **B**) on the viability of lipopolysaccharide (LPS)-treated (100 ng/ml) and untreated RAW 264.7 cells.

An MTT assay was performed to determine the cytotoxicity of each compound. Cells were treated with the indicated amounts of each compound and incubated for 18 h. Data were derived from three independent experiments and are expressed as the means ± SEM. Values sharing the same superscript are not significantly different at $p < 0.05$.

CAPE or G-CAPE for 18 h. There was no significant difference in the cytotoxicity of CAPE or G-CAPE toward cells in cDMEM (Fig. 3). However, in the case of LPS-treated cells, CAPE slightly diminished cellular respiration at concentrations >10 μM, whereas G-CAPE showed cytotoxicity at concentrations >15 μM.

Glycosylation Does Not Affect NO Production and Nrf2 Activation in CAPE

CAPE is a well-known specific inhibitor of NF-κB activation [3, 35], and the treatment of LPS-stimulated RAW 264.7 cells with CAPE has been shown to significantly inhibit NO production [4]. Therefore, the difference in NO production between LPS-treated RAW 264.7 cells in the presence of CAPE and G-CAPE was evaluated, using NO levels in the culture supernatant as an index of the levels of inflammatory mediators. The administration of G-CAPE significantly inhibited NO production by approximately 20–80% in a dose-dependent manner, but this inhibitory effect appeared to be slightly lower than that of CAPE (Fig. 4A).

It has also been reported that CAPE activates the Nrf2 pathway by directly binding its catechol moiety to thiols in Kelch-like ECH-associated protein 1 (Keap1). Thus, the catechol moiety in CAPE is essential for binding to Keap1 [36]. To examine the effect of G-CAPE on Nrf2 activation, we took RAW 264.7-derived reporter cells that harbored a 1-kb-long *NQO1* proximal promoter fused with the firefly luciferase gene, and treated them with either CAPE or G-CAPE (5–15 μM) for 6 h. A luciferase reporter assay of the total cell lysate showed that both CAPE and G-CAPE increased the luciferase activity by 1.8- to 2.0-fold in

cDMEM (Fig. 4B). However, interestingly, only G-CAPE increased the luciferase activity by 1.5- to 1.7-fold in DMEM, whereas CAPE did not activate Nrf2 in this medium. This result is consistent with the decreased stability of CAPE in DMEM, as shown in Table 1. Therefore, it is believed that CAPE decomposes into caffeic acid or other compounds in DMEM.

G-CAPE Is Deglycosylated into CAPE within the Cells

The cellular uptake of each compound was investigated by HPLC of the cell lysates. CAPE or G-CAPE was added to RAW 264.7 cells, and the concentrations of each compound that were present in the cDMEM and had been taken up by the RAW 264.7 cells were then determined after 2 h. In the case of the CAPE treatment, intact CAPE was observed in the cell lysates. However, when G-CAPE was used, the intracellular CAPE content was somewhat higher than that of G-CAPE, indicating that a significant amount of G-CAPE had been converted into CAPE within the cells (Fig. 5). Since G-CAPE also disappeared rapidly in cDMEM (Table 1), this indicates that G-CAPE undergoes deglycosylation to release the parent compound molecule during its cellular transit or within cells.

Discussion

In this study, we explored the potential of G-CAPE as a prodrug. Prodrugs are precursors of drug molecules that must undergo an enzymatic and/or chemical bioconversion by metabolic processes to release the active parent drug, which can then exert the desired pharmacological effect [37, 38]. CAPE is one of the active components in the
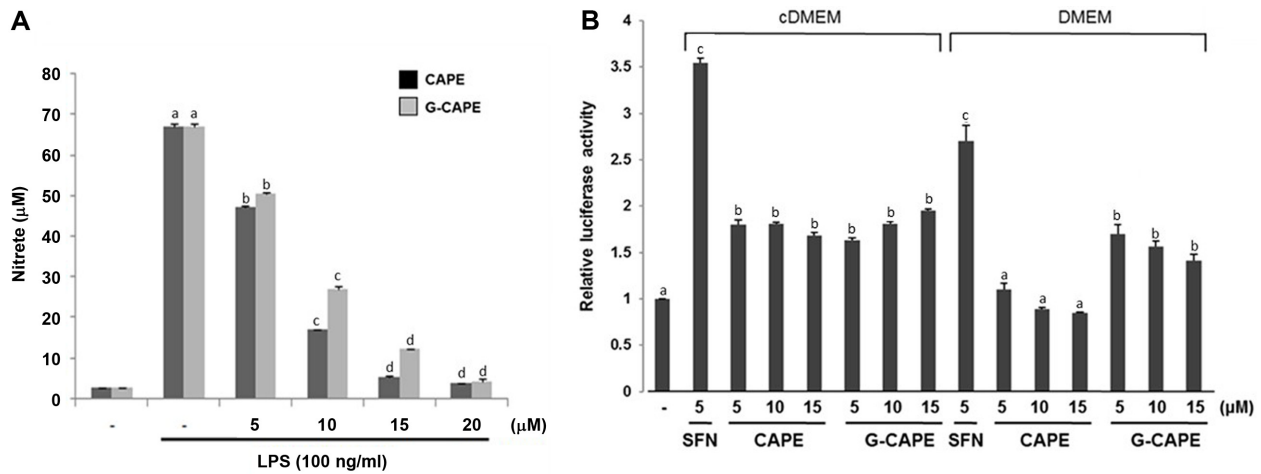
A

B

Fig. 4. Effects of caffeic acid phenethyl ester (CAPE) and glycosylated CAPE (G-CAPE) on nitrite production (**A**) and Nrf2-dependent gene expression (**B**).

For the nitrite analysis, RAW 264.7 cells were treated with the indicated amounts of each compound and incubated for 18 h in the presence of lipopolysaccharide (LPS). Values sharing the same superscript are not significantly different at $p < 0.05$ compared with the LPS-treated group. For the luciferase analysis of the Nrf2-dependent *NQO1* gene, Nrf2 reporter cells were treated with 5 µM sulforaphane (SFN) for 18 h or the indicated amounts of CAPE or G-CAPE for 6 h. For both analyses, data were derived from three independent experiments and are expressed as the means ± SEM. Values sharing the same superscript are not significantly different at $p < 0.05$ compared with the untreated group.
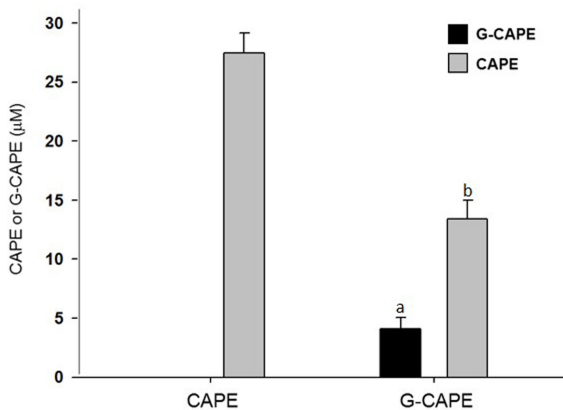

Fig. 5. Examination of caffeic acid phenethyl ester (CAPE) and glycosylated CAPE (G-CAPE) uptake in RAW 264.7 cells by ultra-performance liquid chromatography (UPLC).

RAW 264.7 cells were incubated with each compound at a concentration of 100 µM for 2 h and then harvested. The cells were lysed using the freezing and thawing method, and the concentration of each compound in the cell lysates was then determined by UPLC. Data were derived from three independent experiments and are expressed as the means ± SEM. Values sharing the same superscript are not significantly different at $p < 0.05$.

natural bee product propolis and has several beneficial effects. However, its bioavailability greatly limits its clinical application owing to it being highly insoluble in water and unstable in alkaline pH buffer conditions and rat plasma [39]. Furthermore, it shows cytotoxicity at high concentrations.

To examine the effects of glycosylation on the physical properties and biological functions of CAPE, we synthesized G-CAPE using amylosucrase from *D. geothermalis*, and analyzed the water solubility and oxidative stability of the product, as well as its effects on the modulation of inflammatory activity via NO production and the Nrf2 signaling pathway. We found that glycosylation of CAPE enhanced its water solubility, which is consistent with the findings for other phenolic compounds, such as ampelopsin, epigallocatechin gallate, naringin, and puerarin [40–43]. The stability of natural compounds at different pHs and in plasma is also important for their bioactivity because they are easily degraded in biological fluids during absorption in the gut. Here, we found that G-CAPE was more stable in buffered solutions of varying pH values and in serum-free DMEM than unglycosylated CAPE. However, G-CAPE was not stable in cDMEM, possibly as a result of it being deglycosylated into CAPE by the glycosidases present in FBS, which is a component of cDMEM. The cellular uptake of baicalein and glycosylated baicalein by RAW 264.7 cells has previously been investigated by confocal microscopy and HPLC analysis, which showed that glycosylated baicalein was taken up in an intact form and then gradually converted into nonglycosylated baicalein during metabolism [27]. Therefore, we also examined whether G-CAPE was

converted into CAPE after absorption. As shown in Fig. 5, G-CAPE was rapidly converted into CAPE within 2 h of treatment, indicating that it undergoes deglycosylation to release the aglycon form under intracellular conditions. These properties indicate that G-CAPE may be used as a prodrug for the treatment of various inflammatory diseases.

CAPE is known to exhibit anti-inflammatory activity in vitro [3, 4, 36]. Therefore, we compared the anti-inflammatory activity of G-CAPE with that of its aglycone CAPE by measuring the levels of the proinflammatory mediator NO, large amounts of which are generated by inducible nitric oxide synthase when exposed to inflammatory stimulants. This showed that G-CAPE inhibited LPS-induced NO production at a similar level to CAPE. We also investigated the effects of G-CAPE on the Nrf2 signaling pathway, as CAPE is known to activate Nrf2 by directly binding its catechol moiety to thiol(s) in Keap1, which is the cytosolic repressor of Nrf2 [36]. We found that glycosylation did not affect the anti-inflammatory activity of CAPE in cDMEM and that G-CAPE exhibited even better activity than CAPE in DMEM (Fig. 4B), which can be explained by its higher stability as estimated by its half-life. Celli *et al*. [39] previously reported that CAPE is hydrolyzed into caffeic acid after 6 h in rat plasma in vitro and is also hydrolyzed into caffeic acid as the major metabolite in rats. These pharmacokinetic data support our observations in this study, but additional knowledge is still required about the bioavailability and distribution of CAPE and its derivatives.

In conclusion, this study provides evidence that glycosylation enhances the water solubility and stability of CAPE. Therefore, we propose that the use of G-CAPE would increase the bioavailability of CAPE by helping to protect it from oxidative degradation, thereby extending its half-life in cells to be able to undergo bioconversion, allowing its beneficial biological properties to be exerted.

Acknowledgments

References

1. Viuda-Martos M, Ruiz-Navajas Y, Fernandez-Lopez J, Perez-Alvarez JA. 2008. Functional properties of honey, propolis, and royal jelly. *J. Food Sci.* **73**: R117-R124.

2. Chiao C, Carothers AM, Grunberger D, Solomon G, Preston GA, Barrett JC. 1995. Apoptosis and altered redox state induced by caffeic acid phenethyl ester (CAPE) in transformed rat fibroblast cells. *Cancer Res.* **55**: 3576-3583.

3. Natarajan K, Singh S, Burke TR Jr, Grunberger D, Aggarwal BB. 1996. Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-kappa B. *Proc. Natl. Acad. Sci. USA* **93**: 9090-9095.

4. Song YS, Park EH, Hur GM, Ryu YS, Lee YS, Lee JY, *et al.* 2002. Caffeic acid phenethyl ester inhibits nitric oxide synthase gene expression and enzyme activity. *Cancer Lett.* **175**: 53-61.

5. Toyoda T, Tsukamoto T, Takasu S, Shi L, Hirano N, Ban H, *et al.* 2009. Anti-inflammatory effects of caffeic acid phenethyl ester (CAPE), a nuclear factor-kappaB inhibitor, on *Helicobacter pylori*-induced gastritis in Mongolian gerbils. *Int. J. Cancer* **125**: 1786-1795.

6. Orsolic N, Terzic S, Mihaljevic Z, Sver L, Basic I. 2005. Effects of local administration of propolis and its polyphenolic compounds on tumor formation and growth. *Biol. Pharm. Bull.* **28**: 1928-1933.

7. Tolba MF, Azab SS, Khalifa AE, Abdel-Rahman SZ, Abdel-Naim AB. 2013. Caffeic acid phenethyl ester, a promising component of propolis with a plethora of biological activities: a review on its anti-inflammatory, neuroprotective, hepato-protective, and cardioprotective effects. *IUBMB Life* **65**: 699-709.

8. Armutcu F, Akyol S, Ustunsoy S, Turan FF. 2015. Therapeutic potential of caffeic acid phenethyl ester and its anti-inflammatory and immunomodulatory effects (Review). *Exp. Ther. Med.* **9**: 1582-1588.

9. Erdemli HK, Akyol S, Armutcu F, Akyol O. 2015. Antiviral properties of caffeic acid phenethyl ester and its potential application. *J. Intercult. Ethnopharmacol.* **4**: 344-347.

10. Juman S, Yasui N, Ikeda K, Ueda A, Sakanaka M, Negishi H, *et al.* 2012. Caffeic acid phenethyl ester suppresses the production of pro-inflammatory cytokines in hypertrophic adipocytes through lipopolysaccharide-stimulated macrophages. *Biol. Pharm. Bull.* **35**: 1941-1946.

11. Song JJ, Lim HW, Kim K, Kim KM, Cho S, Chae SW. 2012. Effect of caffeic acid phenethyl ester (CAPE) on H_2O_2 induced oxidative and inflammatory responses in human middle ear epithelial cells. *Int. J. Pediatr. Otorhinolaryngol.* **76**: 675-679.

12. Akyol S, Ginis Z, Armutcu F, Ozturk G, Yigitoglu MR, Akyol O. 2012. The potential usage of caffeic acid phenethyl ester (CAPE) against chemotherapy-induced and radiotherapy-induced toxicity. *Cell Biochem. Funct.* **30**: 438-443.

13. Craig DQ. 2002. The mechanisms of drug release from solid dispersions in water-soluble polymers. *Int. J. Pharm.* **231**: 131-144.

14. Leuner C, Dressman J. 2000. Improving drug solubility for oral delivery using solid dispersions. *Eur. J. Pharm. Biopharm.* **50**: 47-60.

15. Couvreur P. 2012. Nanotechnologies for designing new medicines. *Biol. Aujourdhui* **206**: 237-248.

16. Dinarvand R, Sepehri N, Manoochehri S, Rouhani H, Atyabi F. 2011. Polylactide-co-glycolide nanoparticles for controlled delivery of anticancer agents. *Int. J. Nanomedicine* **6**: 877-895.

17. Steichen SD, Caldorera-Moore M, Peppas NA. 2013. A

review of current nanoparticle and targeting moieties for the delivery of cancer therapeutics. *Eur. J. Pharm. Sci.* **48:** 416-427.

18. Kim MK, Park KS, Lee C, Park HR, Choo H, Chong Y. 2010. Enhanced stability and intracellular accumulation of quercetin by protection of the chemically or metabolically susceptible hydroxyl groups with a pivaloxymethyl (POM) promoiety. *J. Med. Chem.* **53:** 8597-8607.

19. Szente L, Szejtli J. 1999. Highly soluble cyclodextrin derivatives: chemistry, properties, and trends in development. *Adv. Drug Deliv. Rev.* **36:** 17-28.

20. Park H, Kim J, Choi KH, Hwang S, Yang SJ, Baek NI, *et al.* 2012. Enzymatic synthesis of piceid glucosides using maltosyltransferase from *Caldicellulosiruptor bescii* DSM 6725. *J. Agric. Food Chem.* **60:** 8183-8189.

21. Kometani T, Terada Y, Nishimura T, Takii H, Okada S. 1994. Transglycosylation to hesperidin by cyclodextrin glucanotransferase from an alkalophilic *Bacillus* species in alkaline pH and properties of hesperidin glycosides. *Biosci. Biotechnol. Biochem.* **58:** 1990-1994.

22. Yamada M, Tanabe F, Arai N, Mitsuzumi H, Miwa Y, Kubota M, *et al.* 2006. Bioavailability of glucosyl hesperidin in rats. *Biosci. Biotechnol. Biochem.* **70:** 1386-1394.

23. Ohtsuki K, Abe A, Mitsuzumi H, Kondo M, Uemura K, Iwasaki Y, *et al.* 2003. Glucosyl hesperidin improves serum cholesterol composition and inhibits hypertrophy in vasculature. *J. Nutr. Sci. Vitaminol. (Tokyo)* **49:** 447-450.

24. Seo DH, Jung JH, Ha SJ, Song MC, Cha J, Yoo SH, *et al.* 2009. Highly selective biotransformation of arbutin to arbutin-alpha-glucoside using amylosucrase from *Deinococcus geothermalis* DSM 11300. *J. Mol. Catal. B Enzym.* **60:** 113-118.

25. Jung JH, Seo DH, Ha SJ, Song MC, Cha J, Yoo SH, *et al.* 2009. Enzymatic synthesis of salicin glycosides through transglycosylation catalyzed by amylosucrases from *Deinococcus geothermalis* and *Neisseria polysaccharea*. *Carbohydr. Res.* **344:** 1612-1619.

26. Cho HK, Kim HH, Seo DH, Jung JH, Park JH, Baek NI, *et al.* 2011. Biosynthesis of (+)-catechin glycosides using recombinant amylosucrase from *Deinococcus geothermalis* DSM 11300. *Enzyme Microb. Technol.* **49:** 246-253.

27. Kim KH, Park YD, Park H, Moon KO, Ha KT, Baek NI, *et al.* 2014. Synthesis and biological evaluation of a novel baicalein glycoside as an anti-inflammatory agent. *Eur. J. Pharmacol.* **744:** 147-156.

28. Kim MD, Jung DH, Seo DH, Jung JH, Seo EJ, Baek NI, *et al.* 2016. Acceptor specificity of amylosucrase from *Deinococcus radiopugnans* and its application for synthesis of rutin derivatives. *J. Microbiol. Biotechnol.* **26:** 1845-1854.

29. Choi JY, Kwun MJ, Kim KH, Lyu JH, Han CW, Jeong HS, *et al.* 2012. Protective effect of the fruit hull of *Gleditsia sinensis* on LPS-induced acute lung injury is associated with Nrf2 activation. *Evid. Based Complement. Alternat. Med.* **2012:** 974713.

30. Kim KH, Park H, Park HJ, Choi KH, Sadikot RT, Cha J, *et al.* 2016. Glycosylation enables aesculin to activate Nrf2. *Sci. Rep.* **6:** 29956.

31. Emond S, Mondeil S, Jaziri K, Andre I, Monsan P, Remaud-Simeon M, *et al.* 2008. Cloning, purification and characterization of a thermostable amylosucrase from *Deinococcus geothermalis*. *FEMS Microbiol. Lett.* **285:** 25-32.

32. Nishimura T, Kometani T, Takii H, Terada Y, Okada S. 1995. Glucosylation of caffeic acid with *Bacillus subtilis* X-23 alpha-amylase and a description of the glucosides. *J. Ferment. Bioeng.* **80:** 18-23.

33. Meng W, Xiaoliang R, Xiumei G, Vincieri FF, Bilia AR. 2009. Stability of active ingredients of traditional Chinese medicine (TCM). *Nat. Prod. Commun.* **4:** 1761-1776.

34. Walle T, Wen X, Walle UK. 2007. Improving metabolic stability of cancer chemoprotective polyphenols. *Expert Opin. Drug Metab. Toxicol.* **3:** 379-388.

35. Jung WK, Choi I, Lee DY, Yea SS, Choi YH, Kim MM, *et al.* 2008. Caffeic acid phenethyl ester protects mice from lethal endotoxin shock and inhibits lipopolysaccharide-induced cyclooxygenase-2 and inducible nitric oxide synthase expression in RAW 264.7 macrophages via the p38/ERK and NF-kappa B pathways. *Int. J. Biochem. Cell Biol.* **40:** 2572-2582.

36. Kim H, Kim W, Yum S, Hong S, Oh JE, Lee JW, *et al.* 2013. Caffeic acid phenethyl ester activation of Nrf2 pathway is enhanced under oxidative state: structural analysis and potential as a pathologically targeted therapeutic agent in treatment of colonic inflammation. *Free Radic. Biol. Med.* **65:** 552-562.

37. Stella VJ, Nti-Addae KW. 2007. Prodrug strategies to overcome poor water solubility. *Adv. Drug Deliv. Rev.* **59:** 677-694.

38. Rautio J, Kumpulainen H, Heimbach T, Oliyai R, Oh D, Jarvinen T, *et al.* 2008. Prodrugs: design and clinical applications. *Nat. Rev. Drug Discov.* **7:** 255-270.

39. Celli N, Dragani LK, Murzilli S, Pagliani T, Poggi A. 2007. In vitro and in vivo stability of caffeic acid phenethyl ester, a bioactive compound of propolis. *J. Agric. Food Chem.* **55:** 3398-3407.

40. Woo HJ, Kang HK, Nguyen TT, Kim GE, Kim YM, Park JS, *et al.* 2012. Synthesis and characterization of ampelopsin glucosides using dextransucrase from *Leuconostoc mesenteroides* B-1299CB4: glucosylation enhancing physicochemical properties. *Enzyme Microb. Technol.* **51:** 311-318.

41. Moon YH, Lee JH, Ahn JS, Nam SH, Oh DK, Park DH, *et al.* 2006. Synthesis, structure analyses, and characterization of novel epigallocatechin gallate (EGCG) glycosides using the glucansucrase from *Leuconostoc mesenteroides* B-1299CB. *J. Agric. Food Chem.* **54:** 1230-1237.

42. Lee SJ, Kim JC, Kim MJ, Kitaoka M, Park CS, Lee SY, *et al.* 1999. Transglycosylation of naringin by *Bacillus stearothermophilus* maltogenic amylase to give glycosylated naringin. *J. Agric. Food Chem.* **47:** 3669-3674.

43. Ko JA, Ryu YB, Park TS, Jeong HJ, Kim JH, Park SJ, *et al.* 2012. Enzymatic synthesis of puerarin glucosides using *Leuconostoc* dextransucrase. *J. Microbiol. Biotechnol.* **22:** 1224-1229.