Enzymatic Synthesis of Apigenin Glucosides by Glucosyltransferase (YjIC) from *Bacillus licheniformis* DSM 13

Rit Bahadur Gurung, Eun-Hee Kim¹, Tae-Jin Oh, and Jae Kyung Sohng*

Apigenin, a member of the flavone subclass of flavonoids, has long been considered to have various biological activities. Its glucosides, in particular, have been reported to have higher water solubility, increased chemical stability, and enhanced biological activities. Here, the synthesis of apigenin glucosides by the *in vitro* glucosylation reaction was successfully performed using a UDP-glucosyltransferase YjIC, from *Bacillus licheniformis* DSM 13. The glucosylation has been confirmed at the phenolic groups of C-4' and C-7 positions ensuing apigenin 4'-O-glucoside, apigenin 7-O-glucoside and apigenin 4',7-O-diglucoside as the products leaving the C-5 position unglucosylated. The position of glucosylation and the chemical structures of glucosides were elucidated by liquid chromatography/mass spectroscopy and nuclear magnetic resonance spectroscopy. The parameters such as pH, UDP glucose concentration and time of incubation were also analyzed during this study.

INTRODUCTION

Flavonoids are polyphenolic compounds that are ubiquitous in nature and are categorized according to chemical structure into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins, and chalcones (Rice-Evans et al., 1996; Ross and Kasum, 2002). Over 9,000 flavonoids have been identified, many of which occur in fruits, vegetables, and beverages (tea, coffee, beer, wine and fruit drinks) (Middleton, 1998). Flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health, as they have antiviral, anti-allergic, antiplatelet, anti-inflammatory, antitumor, and antioxidant activities (Havsteen, 1983; Middleton, 1998; Ielpo et al., 2000; Toker et al., 2004; Wang et al., 1998). Regardless of their diverse pharmacological and physiological activities, their use as drugs and food additives has been restricted because of their water-insolubility and low absorbability (Desmet et al., 2012). Such compounds convert to water soluble and stable compounds when glycosylated and show improved bio-

availability and pharmacological properties (Jones and Vogt, 2001; Makino et al., 2009; Shimoda et al., 2007; Weymouth-Wilson, 1997).

Apigenin (4', 5, 7 - trihydroxyflavone) (Fig. 1) is a member of the flavone subclass of flavonoids present in fruits and vegetables such as onions, oranges, parsley (Shukla and Gupta, 2010), and chamomile (Manach et al., 2004; Surh, 2003). It is also an active ingredient in the memory herb *Ginkgo biloba* (Patel et al., 2007). Apigenin has long been considered to have various biological activities such as antioxidant (Singh et al., 2004), anti-inflammatory (Ha et al., 2008), anti-mutagenic (Myhrstad et al., 2002), and anti-tumorigenic (Wei et al., 1990) properties in various cell types. Current research trials indicate that apigenin may reduce DNA oxidative damage, inhibit the growth of human leukemia cells and induce these cells to differentiate. Furthermore, this compound inhibits cancer cell signal transduction and induce apoptosis, as well as acts as an anti-inflammatory, anti-spasmodic or spasmolytic agent (Gupta et al., 2002; Kobayashi et al., 2002; Lin et al., 1997; Segaert et al., 2000). Apigenin-rich plants such as passion flower have been used to treat insomniac, as an anti-spasmodic in Parkinson's disease and asthma, to reduce nerve pain in neuralgia, and to treat shingles. Apigenin and apigenin 7-O- β -glucoside penetrate deep into skin layers when applied topically as a antiphlogistic agent to treat inflammation in deep tissues (Merfort et al., 1994). Some of the naturally occurring apigenin glucosides (Fig. 1) have been isolated from various plant sources and their activities have been proven (Akingbala, 1991; Choo et al., 2012; Essokne et al., 2012; Hattori and Matsuda, 1952; Meyer et al., 2006; Nakazaki et al., 2013; Tsolmon et al., 2011; Zhang et al., 2008). Apigenin is the subject of intensive research for its biological properties. The most widespread research has been for its potential to fight cancer (Patel et al., 2007).

The UDP-glucosyltransferases (UGTs) are a group of enzymes that transfer a sugar residue from an activated donor to an acceptor molecule (Richman et al., 2005). There are more than 87,000 glucosyltransferases (GTs) in the protein data bank, which have been classified into 94 families according to their sequences, signature, motifs, stereochemistry of the gly-

Department of Pharmaceutical Engineering, Institute of Biomolecule Reconstruction, Sun Moon University, Asan 336-708, Korea, ¹Division of Magnetic Resonance, Korea Basic Science Institute, Ochang 363-883, Korea

*Correspondence: sohng@sunmoon.ac.kr

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coside linkage, and known target specificity (CAZy database; <http://www.cazy.org/>) (Breton et al., 2012; Campbell et al., 1997; Cantarel et al., 2009; Erb et al., 2009). The family 1 GTs, which include UGTs common in plants, animals, fungi, and bacteria, predominantly recognize low molecular weight small molecules such as flavonoids, alkaloids, and antibiotics as sugar acceptors with high regioselectivity and UDP-activated sugar moieties as donor molecules (Mackenzie et al., 1997). Attaching sugars to flavonoids using microbial GTs enhances their solubility, which makes the compounds more readily absorbed in the small intestine (Ko et al., 2006). In our present work, YjiC, a UGT from *Bacillus licheniformis* DSM 13 which is a member of the GT1 family, has been used to synthesize apigenin glucosides by an *in vitro* method. Recent studies with this UGT have reported successful glucosylation of geldanamycin analogs (Wu et al., 2012) and phloretin (Pandey et al., 2013). Both the aromatic and aliphatic hydroxyl groups in these substrates have been glucosylated. Besides, UGTs from other *Bacillus* species which are homologous to YjiC have been found to have more flexibility (Ko et al., 2006).

MATERIALS AND METHODS

Chemicals and reagents

Most of the chemicals used in this study were purchased from Sigma-Aldrich Chemical Co. (USA). High performance liquid chromatography (HPLC) grade trifluoroacetic acid (TFA) and acetonitrile were purchased from Mallinckrodt Baker (USA). UDP-D-glucose (UDPGlc) was obtained from GeneChem (Korea). The restriction enzymes were from Takara (Japan). All other chemicals were high-grade products obtained from commercially available sources.

Bacterial strains, vectors, media, and growth conditions

B. licheniformis (DSM 13) was used to isolate genomic DNA, and the *Escherichia coli* BL21 (DE3) host (Stratagene, USA) was used to express the protein. All *E. coli* strains were grown at 37°C in Luria Bertani (LB) liquid or plates supplemented with ampicillin (100 µg ml⁻¹) when required. *E. coli* BL21 (DE3) was grown at 20°C after induction with isopropylthio-β-D-galactoside (IPTG) to express the recombinant protein. The pET302/NT-His vector (Invitrogen, USA) was used as the expression vector.

Cloning of glucosyltransferase

The *yjiC* was amplified from genomic DNA of *B. licheniformis* by using primer pairs: YjiC-F; 5'-GAACTCGAGATGGGACATAAACATATCGCG-3' (*Xho*I) and YjiC-R; 5'-CCGGATCCTTATTTACTCCTGCGGGTG-3' (*Bam*HI), using 2U *Taq* polymerase (Solgent) and 1× *Taq* DNA polymerase buffer (MgCl₂). The PCR was performed in a total volume of 20 µl with 100 ng genomic DNA, 0.2 µM primer, 200 nM dNTP under the following conditions: denaturation at 94°C for 3 min, 25 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 1 min. The PCR product of *yjiC* (1,191 bp) was purified and cloned separately into pGEM-T easy vector for sequencing to confirm that no mutation had occurred during PCR amplification. The *Xho*I/*Bam*HI fragment of *yjiC* was excised from pGEM-T vector and cloned into the same sites of pET302/NT-His to generate recombinant expression vector pET302-YjiC.

Expression and purification of UDP-glucosyltransferase (YjiC)

For expression of *yjiC*, pET302-YjiC was transformed into *E. coli* BL21 (DE3) by heat shock transformation method. A single transformant of pET302-YjiC was inoculated in 4 ml LB medium supplemented with 100 µg ml⁻¹ ampicillin and grown at 37°C overnight. A 50 µl seed culture was inoculated into 50 ml LB medium with 100 µg ml⁻¹ of ampicillin and was grown at 37°C until the absorbance at 600 nm (OD₆₀₀) was 0.5-0.7. Then, expression was induced by adding 0.5 mM IPTG followed by continued growth at 20°C with shaking for 12 h. The cells were harvested by centrifugation at 3,000 rpm for 15 min, washed, and resuspended in 50 mM Tris-HCl buffer, pH 7.4, followed by sonication. The lysate was cleared by centrifugation at 12,000 rpm for 30 min at 4°C. The lysate was mixed with 1 ml Ni-nitrilotriacetic acid agarose (Qiagen, USA), shaken gently for 30 min in ice, and then packed into a His-TALON Gravity Column (Clontech, USA). The resin-bound protein was washed stepwise with increasing concentrations of imidazole using gravity flow (10, 50, 100, and 200 mM in buffer containing 300 mM NaCl and 50 mM Tris-HCl, pH 7.4). The protein was eluted in buffer (300 mM NaCl and 50 mM Tris-HCl, pH 7.4) supplemented with 200 mM imidazole, and 1 ml fractions were collected. Fractions containing purified protein were analyzed via 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, concentrated using an Amicon Ultra-15 (Millipore, USA; 10K NMWL device), and then dialyzed overnight at 4°C against buffer containing 50 mM Tris-HCl (pH 7.4) and 10% (v/v) glycerol. The concentration of protein was calculated by Bradford protein assay. The purified protein was stored in buffer containing 50 mM Tris-HCl, pH 7.4, and 10% glycerol until use.

Glucosylation of apigenin

The glucosylation was carried out using purified glucosyltransferase, YjiC. In a total volume of 1 ml reaction mixture, the final concentration of 100 mM Tris-Cl (pH 8.8), 1 mM MgCl₂ · 6H₂O, 3 mM apigenin, and 3 mM UDPGlc was maintained along with 35 µg/ml of appropriately diluted enzyme (GT). The reaction mixture without GT served as a control. The reaction mixture was incubated at 30°C for 3 h and then quenched by heating in boiling water for 5 min. Then, the mixture was centrifuged to remove protein. However, the concentration of UDPGlc and the incubation time for different reactions were varied according to our interest for analyzing different parameters. To elucidate the structure of the products, the reaction was performed at 30°C in a 10 ml volume containing 3 mM acceptor substrate, 6 mM UDPGlc, 35 µg/ml of appropriately diluted purified enzyme, and other components in the proportion as mentioned above for the 1 ml reaction. The reaction was quenched and centrifuged as stated before. The products were further purified by preparative HPLC, dried in a rotary evaporator, and reduced *in vacuo* with a freeze dryer. Thus purified products were analyzed by nuclear magnetic resonance spectroscopy.

Analyses of glucosylation products

The reaction products were analyzed by HPLC (SCL-10 AVP; Shimadzu, Japan) using a reverse-phase C₁₈ column (Mightysil RP-18 GP, 150 × 4.6 mm, Kanto Chemical, Japan) at 320 nm. The isocratic mobile phases were composed of solvent A [0.05% TFA in water, (v/v)] and solvent B (100% acetonitrile). Flow rate was 1.0 ml min⁻¹. Detection was carried out with a UV detector (Shimadzu, SPD-20A, UV/VIS Detector) at 320 nm. The masses of the *in vitro* glucosylation reaction products were confirmed by high resolution liquid chromatography-electro-

Fig. 1. Structures of apigenin and some of its naturally occurring glycosides isolated from various plant sources.

Fig. 2. HPLC analysis of *in-vitro* glucosylation reaction mixture, monitored at 320 nm. APG, apigenin; APG1, apigenin 4'-O-glucoside; APG2, apigenin 7-O-glucoside; APG3, apigenin 4',7-O-diglucoside.

spray ionization-quadrupole-time-of-flight-mass spectrometry (HPLC-ESI-Q-TOF-MS/MS) analysis using the ACQUITY UPLC® (Waters Corp., USA) coupled with SYNAPT G2-S (Waters Corp., USA). The products were purified by prep-HPLC with a C18 column [YMC-Pack ODS-AQ (150 × 20 mm I. D., 10 µm)] connected to a UV detector (320 nm). Nuclear magnetic resonance (NMR) analyses were performed on Varian Unity Inova 300 MHz spectrometer (Varian, USA) or by 800 and 900 MHz Bruker Biospin nuclear magnetic resonance (NMR) (USA).

RESULTS AND DISCUSSION

In vitro glucosylation of apigenin

The *in vitro* glucosylation reaction was carried out and the reaction products were analyzed by HPLC, which suggested the formation of three products (Fig. 2). The substrate molecule,

apigenin, has three phenolic hydroxyl groups at the C-5, C-7, and C-4' positions. Therefore, glucosylation at these three positions is possible. Mono-, di- and tri-glucosylated derivatives of apigenin have been previously reported in various plants as well as synthesized enzymatically (Moussaoui et al., 2010; Rajbhandari and Roberts, 1983; Švehlíková et al., 2004; Terasaka et al., 2012).

When the reaction products were analyzed by HPLC-ESI-Q-TOF-MS/MS, the first peak (APG3, retention time of 3.49 min) was confirmed to be a diglucoside with a molecular ion $[M + H]^+$ at m/z 595.1677. Similarly, the remaining two peaks (APG2 and APG1, retention times of 5.17 and 5.53 min respectively) produced molecular ion $[M + H]^+$ peaks at m/z of 433.1092 and 433.1112 respectively, which confirmed their identity as monoglucosides of apigenin, $[M + H]^+$, m/z 271.0613 (refer to Supplementary Figs. S2a-S2c). Although the substrate molecule con-

Fig. 3. Chemical structures of glucosylation products. APG1, apigenin 4'-O-glucoside; APG2, apigenin 7-O-glucoside; APG3, apigenin 4',7-O-diglucoside.

tained three phenolic hydroxyl groups, triglucoside was not detected. Furthermore, the chemical structures of APG1-APG3 (Fig. 3) were confirmed by comparison of the ^1H and ^{13}C NMR spectra with those reported previously. When the ^1H NMR of APG1 was analyzed, the presence of signals at δ_{H} 12.91 (1H, s, 5-OH) and δ_{H} 10.91 (1H, s, 7-OH) confirmed the glucosylation at C-4' position. The anomeric proton signal at δ_{H} 5.04 (1H, d, $J = 6.8$ Hz) was consistent with the presence of a β -glucoside unit in APG1. The structure of APG1 was, therefore, determined to be apigenin 4'-O-glucoside (Lin et al., 2000; She et al., 2009). The signals obtained in the ^1H -NMR and ^{13}C -NMR for APG2 were compared with previously published results (Ersoz et al., 2002; Moussaoui et al., 2010; Yassa et al., 2007) which confirmed the product to be apigenin 7-O-glucoside. Similarly, the ^1H -NMR and ^{13}C -NMR of APG3 confirmed its chemical structure as apigenin 4', 7-O-diglucoside when the spectra were compared (Agrawal and Bansal, 1989; Markham and Geiger, 1993; Terasaka et al., 2012). The NMR spectra of these products have been presented in the Supplementary Figs. S3-S6.

Effect of incubation time on glucosylation

Commercially available UDPGlc was used for apigenin glucosylation. The concentration of apigenin and UDPGlc was 3 mM. However, the incubation time was varied (1, 2, 3 and 10 h) for the four sets of reactions but maintaining the temperature (30°C) and other parameters constant. The HPLC analyses of the reaction mixture showed that the concentration of APG1 was the least at 1 h incubation time and increased gradually with increasing time of incubation. Even the two other products, APG2 and APG3 followed the same trend until 3 h of incubation. The concentration of all three products was the highest at 3 h time of incubation which decreased when the time of incubation was extended to 10 h. The product APG2 at 10 h was even lesser than that at 1 h (Fig. 4A). One reason for the decrease in product concentration when the incubation period was extended might be because of the deglucosylation as reported by Pandey et al. (2013). Time course experiments indicated that approximately 90% of the substrate was converted into the glucoside product after 24 h of incubation (data not shown).

Effect of UDP-D-glucose concentration on apigenin glucosylation

In a set of three reactions, 3 mM apigenin was used but the concentration of UDPGlc was varied. All three reactions were

incubated for 3 h at 30°C. HPLC analysis of the reaction products showed that APG1 favored low concentration of UDPGlc. The formation of APG1 was highest at a 3 mM concentration of UDPGlc. In contrast, the formation of APG2 was highest with a 6 mM concentration of UDPGlc, but it decreased slightly at 9 mM concentration. Similarly, APG3 formation was highest with 9 mM UDPGlc and lowest with 3 mM UDPGlc. Not much difference was observed in the amount of APG3 between the two reactions with 6 mM and 9 mM UDPGlc (Fig. 4B). These results show that the choice of suitable UDPGlc concentration is important for synthesizing a particular glucoside.

Effect of buffer pH on apigenin glucosylation

Buffer of different pHs were prepared at 30°C to study the effect of pH of Tris-Cl buffer on glucosylation, and the reactions were carried out under similar conditions using buffer of different pHs. When the reaction products were analyzed by HPLC, the Tris-Cl buffer of pH 8.8 provided a better result, as the proportion of glucosides formed was almost same in this case. Therefore, Tris-Cl buffer of pH 8.8 was selected to carry out all reactions during this study. But, the formation of APG2 was higher in more alkaline conditions, whereas APG3 preferred a pH range of 7.5-8.8 (Fig. 4C). The results showed that the buffer of lower pHs is less favored by this enzyme for glucosylation of apigenin.

DISCUSSION

Flavonoids are present in food and medicinal plants and are thus consumed by humans. Much attention has been received by flavonoids over the past decade and a variety of potential beneficial effects have been revealed. They are found in plants mainly as glycoside derivatives. Flavonoids undergo deglycosylation either by lactase phloridzin hydrolase or cytosolic β -glucosidase before oral absorption. The absorbed aglycone is then conjugated by methylation, sulfonation, or glucuronidation (Jäger and Saaby, 2011). Besides, several natural products used as antibiotics have glucose or other sugar moieties attached, and these sugars play a vital role in the water solubility and biological activity of those antibiotics (Harle and Bechthold, 2009). Thus, apigenin was glucosylated to synthesize its glucosides, which might increase water solubility and enhance biological activity of this compound since the glucosyl conjugation of such low molecular weight compounds help to enhance water solubility, improve stability which will ultimately help in increasing bioavailability and modify bioactivity (Lim and Bowles, 2004;

Fig. 4. (A) Effect of incubation time on glucosylation of apigenin. (B) Effect of UDPGlc concentration on the glucosylation of apigenin. (C) Effect of pH of Tris-HCl buffer used in the glucosylation of apigenin. The bar diagrams are marked with error bars with percentage (5% value).

Shimoda et al., 2007; Weymouth-Wilson, 1997).

The UDP-glucosyltransferase YjiC has been successfully used to glucosylate apigenin. Though apigenin consists of phenolic hydroxyl groups at C-5, C-7 and C-4' positions, glucosylation at C-5 position was not observed in this study. Most probably the hydroxyl group at this position undergoes intramolecular hydrogen bond formation with the carbonyl oxygen at the C-4 position and is not available under normal conditions. This may be one reason for making 5-*O*-glucosides of flavonoids rare in nature. However, the triglucoside of apigenin was reported previously (Terakasa et al., 2012). Although three different glucosides were reported during this study, each favored different sugar concentrations, pHs, and incubation times. When the glucosylation reactions were performed for different incubation times, 3 h of incubation time produced three products in almost equal proportion. The concentration of products were 74.3, 80.2, and 72.5 mg/L respectively for APG1, APG2 and APG3. However, the extended incubation time reduced the amount of

product (Fig. 4A). This reduction may be attributed to deglucosylation reaction by the enzyme (Pandey et al., 2013).

Similarly, when 6 mM UDPGlc was used in the reaction. The concentration of both APG1 and APG2 decreased in the reaction with 9 mM UDPGlc but the concentration of APG3 was maximum (136.1 mg/L) in the same (Fig. 4B). This showed that formation of diglucoside favored higher concentration of donor sugar in the reaction whereas the monoglucosides favored moderate to low concentration. Therefore, these parameters of the reaction can be varied to synthesize the glucoside of interest.

This presents an attractive alternative, as a GT that can catalyze the attachment of sugars to an aglycone. The GT can transfer various UDP-sugars to both aromatic and aliphatic nucleophiles (Pandey et al., 2013; Wu et al., 2012) with broad substrate specificity. In the case of the plant GTs, more than one glucosylated product has been reported for flavonoids when several glycosylation positions are available. Our results

indicate that even the bacterial GTs can glycosylate at multiple positions when such positions are available. Such GTs with substrate flexibility and less regioselectivity can be utilized for glycosylation of broad range of pharmacologically and industrially important compounds.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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