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Glycosylation of Genistin into Soluble Inclusion Complex Form of Cyclic Glucans by Enzymatic Modification

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The enzymatic modification of genistin to enhance its water solubility was studied using two glycosyltransferases, cyclodextrin glucanotransferase from alkalophilic *Bacillus* sp. I-5 and 4-α-glucanotransferase from *Thermus scotoductus*. Two different catalytic reactions, the transglycosylation and cyclization activities, were observed when the reaction was performed with soluble starch as a donor and genistin as an acceptor. The reaction products were isolated and identified as [Glc(α1−4)]$_{1-22}$-Glc(β1−7)-genisteins and cycloamylose with DP 8−12 by HPLC and MALDI-TOF MS. A β-amylase treatment revealed inclusion complexes composed of Glc(α1−4)-Glc(β1−7)-genistein/Glc(α1−4)-Glc(α1−4)-Glc(β1−7)-genistein and cycloamylose with DP 8−12. The results indicated that the cycloamylose formed by the cyclization reaction of the enzyme included Glc(α1−4)-Glc-(β1−7)-genistein/Glc(α1−4)-Glc(α1−4)-Glc(β1−7)-genistein. The presence of cycloamylopectin, in which the Glc(α1−4)-Glc(β1−7)-genistein/Glc(α1−4)-Glc(α1−4)-Glc(β1−7)-genistein was enclosed, was also observed with HPLC, HPSEC-MALLS, and MALDI-TOF MS analyses. The solubility of genistin was highly improved, and the solution containing glycosylated genistin and the inclusion complex demonstrated excellent properties of transparency and stability during storage at 4 °C.

KEYWORDS: CGTase; cyclization; cycloamylose; genistin; 4-α-glucanotransferase; isoflavone; solubility; transglycosylation

INTRODUCTION

Isoflavonoids occur mostly in the subfamily Papilionoideae of the Leguminosae in the plant kingdom. Among them, genistein (4′,5,7-trihydroxyisoflavone) is predominantly present as a glycoside (genistin) in the soybean *Glycine max*. Epidemiological studies indicate that the consumption of soy-based foods may be associated with the low frequency of breast cancer in Asian women. Aside from the potential cancer prevention effect, isoflavones have been found to have other potential health benefits, including the prevention of heart disease, the increase of bone mass density to prevent osteoporosis, and the reduction of postmenopausal syndrome in women (*1*−*3*). However, the low water solubility of genistein and genistin limits their application as dietary supplements in the food industry (*4*). Transglycosylation reactions performed by various enzymes have successfully increased the water solubility of various food materials (*5*, *6*).

Certain hydrolytic enzymes, including cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19), 4-α-glucanotransferase (EC 2.4.1.25), β-glucosidases (EC 3.2.1.120), and maltogenic amylases (MAases; EC 3.2.1.133), can catalyze transglycosylation reactions as well as hydrolysis reactions under defined conditions (*7*−*13*). β-Glucosidase can kinetically control transglycosylation activity in the presence of an appropriate acceptor molecule, whereas it typically catalyzes the hydrolysis of β-glycosidic bonds linking carbohydrate residues in aryl, amino, or alkyl β-D-glucosides, cyanogenic glucosides, and oligo- or disaccharides (*8*, *9*). Thermostable β-glucosidases from the hyperthermophilic archaea *Sulfolobus solfataricus* and *Pyrococcus furiosus* have a marked preference for making new β-(1→3)- and β-(1→6)-glycosidic bonds via intermolecular and intramolecular transfer reactions. Recently, β-glucosidases from cassava, Thai rosewood, and almond were reported to transfer glucose from *p*-nitrophenyl β-glucoside to secondary alcohol acceptors, synthesizing alkyl glucosides (*8*). MAases are usually capable of hydrolyzing starch, cyclodextrins (CDs), and pullulan as well as acarbose, a potent α-amylase inhibitor (*12*). In the presence

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of various acceptor molecules, however, MAases can transfer maltose units of the donor molecule to the acceptor molecule by forming α(1,6), α(1,3), and α(1,4)-glycosidic linkages (*13*).

Similar to these enzymes, cyclodextrin glucanotransferases (CGTase; EC 3.2.1.19) demonstrate various enzymatic activities (*14*). They catalyze the conversion of α(1,4)-D-glucans such as starch and glycogen to CDs by intramolecular transglycosylation (cyclization reaction). In addition, in the presence of a suitable acceptor, the enzyme performs an intermolecular transglycosylation (transglycosylation reaction) in which the nonreducing ends of glycosyl residues that are formed by cleaving an α-(1,4)-D-glucan or a CD are transferred to the acceptor molecule (*15*, *16*). In addition, the enzyme can catalyze the disproportionation reaction between two molecules of oligosaccharide and the hydrolysis of α(1,4)-D-glucans or a CD (*14*). Recently, other types of enzymes have been shown to carry out both intramolecular and intermolecular transglycosylations similarly to CGTase. Glucan-branching enzyme (BE, EC 2.4.1.18) was originally known to introduce the α(1−6)-glucosidic linkages of amylopectin or glycogen and therefore is important for determining the structures and properties of amylopectin and glycogen (*17*). In addition to the typical branching reaction, BE has been shown to catalyze the cyclization reaction of amylose and amylopectin to form cycloamyloses, which are glucoamylase-resistant cyclic glucan molecules having cyclic α(1,4)-D-glucans with a degree of polymerization ranging from 17 to a few hundred (*17*−*19*). Amylomaltases (EC 3.2.1.25) are intracellular 4-α-glucanotransferases that can transfer a segment of an α(1,4)-D-glucan to a new 4-position on an acceptor, which may be glucose or another α(1,4)-D-glucan, resulting in the production of cycloamylose or a thermoreversible starch gel (*20*, *21*).

In the present study, the transglycosylation and cyclization activities of two different enzymes, CGTase from alkalphilic *Bacillus* I-5 (BSCGTase) and thermostable 4-α-glucanotransferase from *Thermus scotoductus* (TS4αGTase), were investigated in the presence of genistin, a major isoflavone in soybeans.

MATERIALS AND METHODS

Chemicals. The soybean isoflavone mixture, containing 7.72% daidzin, 0.95% glycitin, and 9.48% genistin, was generously provided by Pulmuone Co., Ltd. (Seoul, Korea). Genistin and soluble starch were purchased from Sigma Chemical Co. (St. Louis, MO) and Showa Chemical Co., Ltd. (Tokyo, Japan), respectively. HPLC grade methanol was supplied by Fisher Scientific Co. (Pittsburgh, PA). Water was prepared with a Milli-Q plus Ultrapure Water System (Millipore, Billerica, MA). All other chemicals used were of reagent grade from Showa and Sigma.

The carbohydrate and some hydrophilic contaminants in the soybean isoflavone extract were removed using a Sep-Pak C_{18} cartridge (Waters, Milford, MA). The eluted soybean isoflavone in methanol was filtered through a GyroDisc Syr. CA-PC 30 mm 0.45-μm membrane (Orange Scientific, Braine-l'Alleud, Belgium) and then further purified with an LC-918 recycling preparative HPLC (JAI, Tokyo, Japan) equipped with an RI-50 refractive index detector and a W-251 polymeric gel filtration column (20 mm × 500 mm). Methanol was used as the mobile phase at 3 mL/min, and the injection volume was 3 mL. The fraction containing genistin was collected and concentrated on a rotary vacuum evaporator (Rikakikai Co. Ltd., Tokyo, Japan). The purified genistin was confirmed by comparison with standard genistin using HPLC. The yield of genistin isolated from soybean isoflavone extract was 24%. The sample was kept at 4 °C until it was used.

Enzyme Preparation. The genes corresponding to BSCGTase and TS4αGTase were cloned by PCR as described previously (*12*, *22*) and expressed in *Escherichia coli* MC1061 [*F*$^-$, araD139, recA13, Δ(*araABC-leu*)*7696*, galU, galK, Δ*lacX74*, *rpsL*, *thi*, *hsdR2*, *mcrB*].

The recombinant *E. coli* harboring the TS4αGTase gene was cultured in Luria−Bertani (LB) medium containing kanamycin (50 mg/mL) at 37 °C overnight with shaking. The cells were harvested from the culture broth by centrifugation (7000*g*) at 4 °C for 20 min. The pellet was resuspended in lysis buffer [50 mM Tris-HCl (pH 7.0), 300 mM NaCl, and 10 mM imidazole] and disrupted in an ice bath by sonication with VC-600 sonicator (Sonics & Materials Inc., Danbury, CT) at output 4, 5 min × 3 times, 60% duty. The crude cell extract was centrifuged (10000*g*) at 4 °C for 15 min. One milliliter of 50% nickel−nitrilotriacetic acid (Ni−NTA) slurry (Qiagen GmbH, Hilden, Germany) was added to 4 mL of cleared lysate and mixed gently by shaking at 4 °C for 60 min. The lysate−Ni−NTA resin mixture was loaded onto a 2 mL bed volume Poly-Prep chromatography column (Bio-Rad, Hercules, CA) and washed with 8 mL of washing buffer [50 mM Tris-HCl (pH 7.0), 300 mM NaCl, and 20 mM imidazole]. The recombinant TS4αGTase was eluted with 2 mL of elution buffer [50 mM Tris-HCl (pH 7.0), 300 mM NaCl, and 250 mM imidazole]. The eluants were concentrated by ultrafiltration with a YM 10 regenerated cellulose membrane (Millipore) at 4 °C and dialyzed against 50 mM Tris-HCl (pH 7.0) buffer.

The growth of *E. coli* MC1061 carrying the BSCGTase gene was similar to that of the recombinant *E. coli* harboring the TS4αGTase gene except the culture was grown in LB broth with ampicillin (50 mg/mL) at 37 °C. The enzyme in the crude cell extract was purified using a β-CD affinity column as previously described (*22*). Active fractions were collected, concentrated by ultrafiltration, and dialyzed against 50 mM sodium acetate buffer (pH 6.0). Protein concentration was determined according to the Bradford method (*23*) using bovine serum albumin as the standard. The purity and molecular weight of the purified enzymes were analyzed by 10% SDS-PAGE (*24*). The purified enzymes were preserved in 4 °C until they were used. The final yields of BSCGTase and TS4αGTase were 63 and 36%, respectively.

Determination of Enzyme Activity. TS4αGTase activity was determined as previously described (*25*). After 250 μL of 0.2% amylose in 90% DMSO, 50 μL of 1% maltose in 50 mM Tris-HCl (pH 7.5), and 600 μL 50 mM Tris-HCl (pH 7.5) had been preincubated for 10 min at 70 °C, 100 μL of a diluted enzyme was added, and the reaction was continued for 10 min. Then, a 100-μL aliquot was withdrawn and mixed with 1 mL of 0.02% I_2/KI solution at ambient temperature; the absorbance at 620 nm was measured immediately. One unit of TS4αGT activity was defined as the amount of enzyme that increased the absorbance by 1.0 in 1 min.

The activity of BSCGTase was assayed (*22*) using 1% β-CD in 50 mM sodium citrate buffer (pH 6.0). Thirty microliters of enzyme was reacted with 150 μL of 1% β-CD and 120 μL of reaction buffer at 55 °C for 10 min. The reaction was stopped by adding 900 μL of 3,5-dinitrosalicylic acid solution and was colorized by heating at 100 °C for 5 min. The absorbance of the mixture was measured with an Ultrospec III spectrophotometer (Pharmacia LKB, Uppsala, Sweden) at 575 nm. One unit of enzyme activity was defined as the amount of enzyme that produced the reducing sugar equivalent to a 1-unit change in absorbance at 575 nm. The specific activities of BSCGTase and TS4αGTase used in the experiment were 4.1 and 60.3 units/mg, respectively.

Transglycosylation and Cyclization Reaction. For the transglycosylation reaction, a solution containing 0.3 mg/mL of genistin and 20 mg/mL of soluble starch was prepared in sodium acetate buffer (50 mM, pH 6.0) or in Tris-HCl buffer (50 mM, pH 7.5) as a substrate. BSCGTase (1 unit/mL) or TS4αGT (2 units/mL) was added to a substrate solution, and then the reaction was conducted for 10 min at 60 °C or for 5 h at 75 °C, respectively. Placing the reaction tube in ice water stopped all reactions.

Purification of Reaction Products. A Sep-Pak Plus C_{18} cartridge (Waters), previously activated by ethyl acetate, methanol, and water, was used to absorb the isoflavone glycosides in the transglycosylation solutions and to remove the malto-oligosaccharide mixture and salt. The solution eluted by methanol was subjected to ultrafiltration to remove the enzyme. The major transfer product of genistin was finally purified with a polymeric gel filtration column (W-251) in recycling

preparative HPLC with 80% methanol as the mobile phase at a flow rate of 2 mL/min.

***β*-Amylase Hydrolysis.** The reaction mixture or fractionated solution was treated with β-amylase from *Bacillus cereus* using a 0.26 unit/mL concentration of the reaction mixture for 1 h at 40 °C. The assay buffer for β-amylase was 50 mM Tris-HCl (pH 7.0). Boiling stopped the reaction.

TLC Analysis. The reaction products were spotted on K5F silica gel plates (Whatman, Kent, U.K.) that had been activated by heating at 110 °C for 30 min (*4*, *12*). A solution of *n*-butanol/acetic acid/water (5:3:1; v/v/v) was used to develop isoflavones, and 2-propanol/ethyl acetate/water (5:3:1; v/v/v) was used for carbohydrates. The developed TLC plate was dried completely at room temperature. The isoflavones on the TLC plate were visualized using a CAMAG Reprostar 3 UV detector (Muttenz, Switzerland) at 254 nm. The carbohydrates were visualized by dipping the TLC plate into a methanol solution containing 3 g of *N*-(1-naphthyl)ethylenediamine and 50 mL of concentrated sulfuric acid per liter, drying the plate at room temperature, and then heating the plate at 110 °C for 10 min.

HPLC Analysis. A Waters 600E HPLC system with a 150 × 3.9 mm i.d. Nova-Pak C_{18} column and an SLC 200 UV−vis detector (Samsung, Seoul, Korea) set at 254 nm was used to quantify the amount of isoflavones. For the gradient solvent system, solvent A (water/formic acid, 100:0.1, v/v) and solvent B (methanol/water/formic acid, 50:50:0.1, v/v/v) were used, with solvent B increasing gradually from 20 to 100% during a 20-min interval at a flow rate of 1.2 mL/min. Twenty microliters of sample was injected into the analytical HPLC. The concentrations of genistin and its derivatives were calculated from genistin standard curves.

Preparative HPLC Analysis. The preparative HPLC system (Waters Delta Prep 4000) was equipped with a 300 × 19 mm i.d. Prep Nova-Pak C18 column and a model 486 detector set at 254 nm. For the gradient solvent system, solvent A (water/formic acid, 100:0.1, v/v) and solvent B (methanol/water/formic acid, 50:50:0.1, v/v/v) were used, with solvent B increasing gradually from 20 to 100% during a 50-min interval at a flow rate of 5.0 mL/min. One milliliter of samples was injected into the preparative HPLC.

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) Analysis. The molecular masses of the isoflavone glycosides in the reaction were determined using the MALDI-TOF MS (Voyager-DE Perceptive Biosystems, Framingham, MA) in linear mode (*26*). The sample in methanol was mixed with the matrix, 10 mg/mL 2,5-dihydroxybenzoic acid in 10% methanol, at a ratio of 1:1, and 1.5 μL of the mixture was applied to a MALDI-TOF MS probe and air-dried. The sample plate was then located in the Voyager-DE Biospectrometry workstation. The workstations were operated at an accelerating voltage of 25 kV.

NMR Analysis. The ^{13}C NMR spectra were recorded at 100.40 MHz on a Brucker 600 MHz NMR spectrometer. The sample was dissolved in DMSO-d_6 at 24 °C with tetramethylsilane (TMS) as the chemical shift reference. Zgpg30 was used as a pulse program.

Multiangle Laser Light Scattering (MALLS) Analysis. The transglycosylation reaction treated with TS4αGT was analyzed using size exclusion chromatography (SEC), MALLS, and a refractive index detector. A TSK high-pressure column (TOSOH, Japan) was used to analyze the molecular weight of amylopectin; the mobile phase was 50 mM sodium nitrate at a flow rate of 0.4 mL/min. The temperature of the RI detector was set at 30 °C. To calculate polysaccharide molecular weights and radii of gyration, a d*n*/d*c* (change in refractive index with change in polymer concentration) value of −0.146 was used for starch.

Solubility Determination. Excess genistin or its derivatives produced by transglycosylation were mixed with 200 μL of distilled water in an Eppendorf tube at room temperature. After sonication at room temperature for 1 h to maximize solubility, the sample was diluted and filtered through a 0.45-μm PVDF membrane (Millipore) for HPLC analysis of the sample solution concentration in the supernatant.

RESULTS AND DISCUSSION

Transglycosylation Reaction of Genistin by BSCGTase and TS4αGTase. Transglycosylation activities of BSCGTase and TS4αGTase were used to increase the solubility of genistin. The mixture of soluble starch and genistin was reacted with BSCGTase and TS4αGTase, respectively. Both enzymes are known to transfer a glucosyl unit to appropriate acceptor molecules (*27*, *28*). A TLC analysis of the reaction products indicated that both enzymes readily transferred glucosyl units to genistin and produced a series of other transglycosylated genistins. As shown in **Figure 1**, four transferred genistin products appeared as spots in both reactions, indicating that the glucosyl units generated from the hydrolysis of starch were transferred to the acceptor molecule (genistin) in serial mode. The newly formed isoflavone transglycosylation products were detectable at 254 nm; therefore, their formation could be easily recognized by HPLC. An HPLC analysis of the reaction mixtures revealed a more complicated transglycosylation pattern than that observed in TLC (**Figure 2A**,**C**).

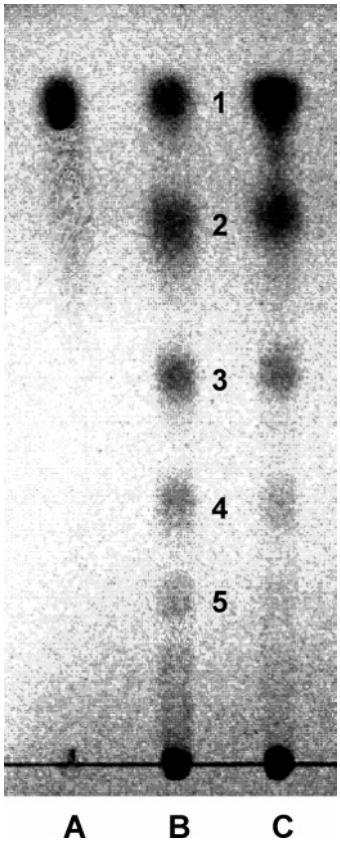

Figure 1. TLC analysis of the genistin transglycosylation reactions by TS4αGTase and BSCGTase: lane A, genistin standard; lane B, reaction of genistin and soluble starch by TS4αGTase; lane C, reaction of genistin and soluble starch by BSCGTase. Ultraviolet radiation was used to visualize the spots.

MALDI-TOF MS Analysis of Transglycosylation Products. To analyze the molecular structures of the transglycosylation products, the reaction mixtures were analyzed by MALDI-TOF MS (**Figure 3**). Various reaction products produced byTS4αGTase appeared in the HPLC chromatogram; the fractions corresponding to peaks 1−18 (**Figure 2**) were isolated and subjected to MALDI-TOF MS analysis. As shown in **Figure 3A**, peaks with fixed intervals (molecular mass of 162) were detected at *m*/*z* 617, 779, 941, 1103, 1265, 1427, 1589, 1751, 1913, 2075, 2237, 2399, 2561, 2723, 2885, 3047, 3209, and 3371. These peaks matched the sodium adducts of genistin and $[Glc(\alpha1-4)]_n$-Glc(β1−7)-genisteins ($n = 1-18$), suggesting that TS4αGTase could transfer glycosyl residues to the acceptor molecule genistin in a continuous manner. Interestingly, there

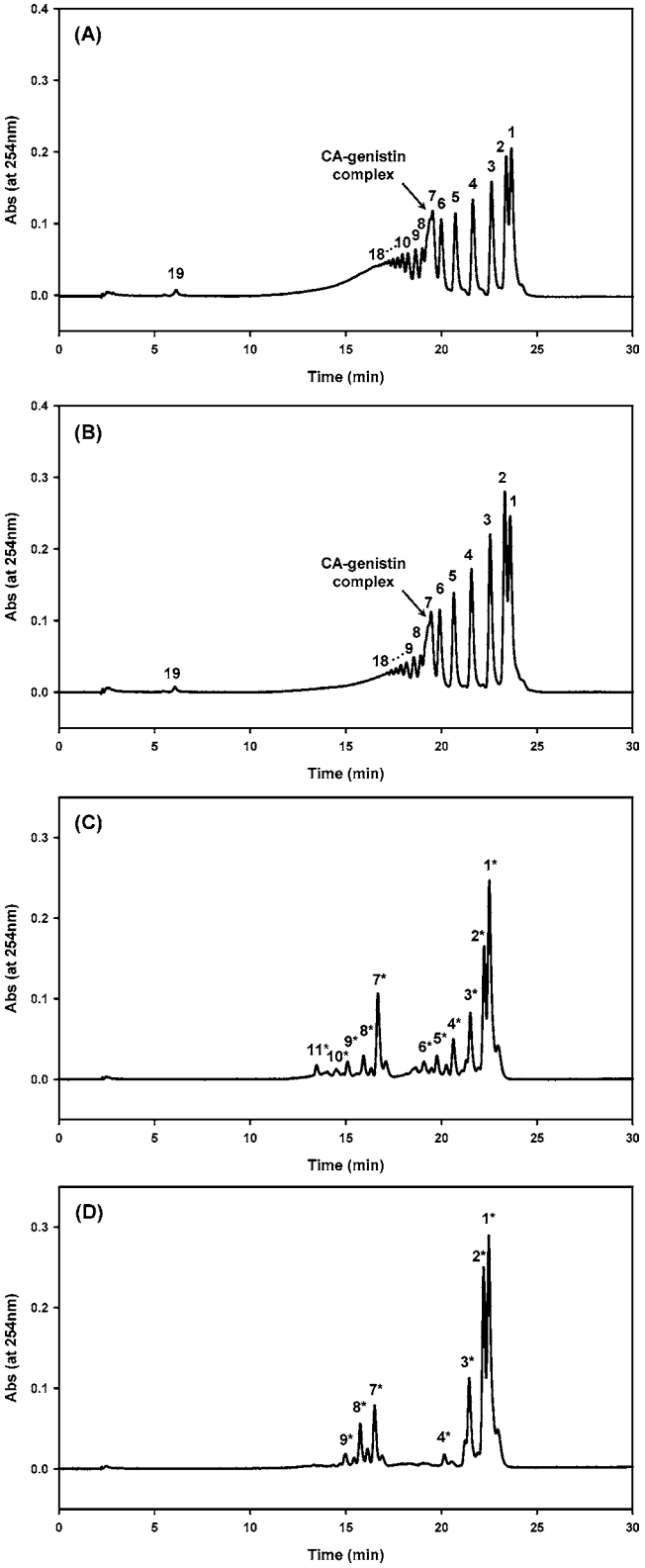

Figure 2. HPLC analysis of the genistin transglycosylation reactions by TS4αGTase (**A**) before and (**B**) after β-amylase treatment and by BSCGTase (**C**) before and (**D**) after β-amylase treatment. β-Amylase treatment was performed for 5 h. CA–genistin complex is the molecule between cycloamylose and genistin transglycosylation products. Peaks in the BSCGTase reaction are distinguished from peaks in the TS4αGTase reaction by asterisks (*).

were peaks at *m*/*z* 996, 1158, 1320, 1482, 1644, and 1806, equivalent to the sodium adducts of cyclic glucan composed of 6, 7, 8, 9, 10, and 11 glucose units, respectively. Previously, it was known that 4-α-glucanotransferase from the hyperthermophilic archaeon *Thermococcus litoralis* catalyzed not only intermolecular transglycosylation to produce linear α-(1,4)-D-glucan but also intramolecular transglycosylation to produce cyclic α-(1,4)-D-glucan (cycloamylose) (*28*). The results implied that TS4αGTase could catalyze cyclization reactions as well as transglycosylation reactions by forming α-, β-, γ-CD and other cycloamyloses (DP 9−11 or greater).

BSCGTase formed various glucosyl genistins as shown by peaks at *m*/*z* 617, 779, 941, 1103, 1265, 1427, 1589, and 1751, corresponding to the sodium adducts of genistin and [Glc(α1−4)]$_n$-Glc(β1−7)-genisteins (n = 1−8) (**Figure 3B**). Different cycloamyloses appeared owing to the cyclization reaction of BSCGTase. Unexpectedly, cycloamyloses with DPs >8 were produced by BSCGTase, which generally produces cycloamyloses with DPs of 6−8. The time course analysis of the CGTase reaction product demonstrated that larger cyclic α(1,4)-D-glucans were produced in the initial stage of the reaction and were subsequently converted into smaller cyclic α(1,4)-D-glucans, such as α-CD and β-CD (*29*). On the basis of this observation, the cycloamyloses with DPs >8 could not be converted into smaller cyclodextrins in the presence of genistin.

Inclusion Complex between Cycloamylose and Genistin Transglycosylation Products. The reaction mixture was treatedwith β-amylase for 5 h, and then the HPLC chromatogram was monitored to confirm the presence of cyclic glucan in the reaction of TS4αGTase (**Figure 2B**,**D**). During the reaction, peaks from 8 to 18 gradually decreased, whereas peaks 2 and 3, corresponding to glycosyl-/maltosyl-genistin, increased. The result demonstrated that the peaks in the HPLC chromatogram represent the transfer products composed of different (glucosyl)$_n$-genistins, with which β-amylase reacted, and gradually hydrolyzed longer maltosyl units of the (glucosyl)$_n$-genistins to produce glycosyl-/maltosyl-genistin. However, peak 7 remained almost constant during the β-amylase treatment (**Figure 2A**,**B**). Peak 7 was isolated by preparative HPLC and subjected to MALDI-TOF MS analysis. As shown in **Figure 4**, the MALDI-TOF MS spectrum of peak 7 unexpectedly yielded a variety of peaks at *m*/*z* 780, 996, 1158, 1320, 1482, and 1644. The peaks at *m*/*z* 617 and 780 corresponded to glucosyl-genistin and maltosyl-genistin, whereas the peaks at *m*/*z* 996, 1158, 1320, 1482, and 1644 were cyclic glucans with DPs of 6−10, respectively. These results suggest that peak 7 was a mixture of compounds, in which the maltosyl-genistin was entrapped by cycloamylose (DP 6−10). Therefore, we assumed that the transfer products such as Glc(α1−4)-Glc(β1−7)-genistein/Glc-(α1−4)-Glc(α1−4)-Glc(β1−7)-genisteins were included in the hydrophobic cavity of the cyclic glucan, which suggests that the cyclic glucans were produced from starch and formed complexes with the newly formed transfer product of genistin. The reaction mode of TS4αGTase for the production of cycloamylose and a Glc(α1−4)-Glc(β1−7)-genistein/Glc(α1−4)-Glc(α1−4)-Glc(β1−7)-genisteins complex is described in **Figure 5**.

Similar to TS4αGTase, BSCGTase yielded cycloamylose and a Glc(α1−4)-Glc(β1−7)-genistein/Glc(α1−4)-Glc(α1−4)-Glc-(β1−7)-genisteins complex (**Figures 3** and **4**). The β-amylase-resistant compounds were composed of Glc(α1−4)-Glc(β1−7)-genistein/Glc(α1−4)-Glc(α1−4)-Glc(β1−7)-genistein and cycloamyloses with DPs of 8−11. Typically, BSCGTase produces cycloamyloses with DPs of 6−8 (α-CD, β-CD, and γ-CD). It is interesting that the ratio of the CDs produced by BSCGTase can be changed in the presence of the transfer product; for example, the γ-CD production can be enhanced

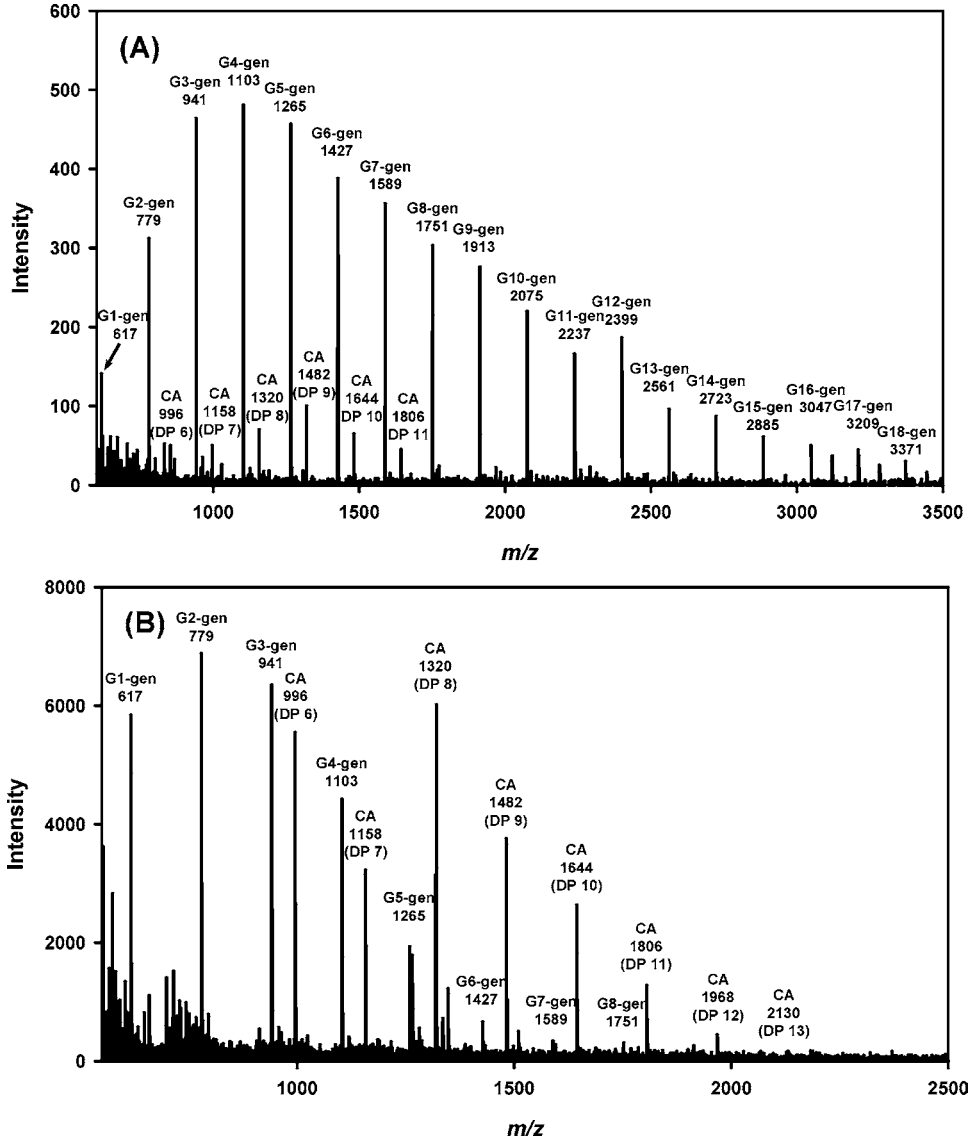

Figure 3. MALDI-TOF MS analysis of the genistin transglycosylation reactions by (**A**) TS4αGTase and (**B**) BSCGTase. G1-gen to G18-gen are [Glc-(α1–4)]$_n$-Glc(β1–7)-genisteins (where n = 1–18).

by the addition of bromobenzene as the complexing agent (*30*, *31*). The transfer product Glc(α1−4)-Glc(β1−7)-genistein/Glc-(α1−4)-Glc(α1−4)-Glc(β1−7)-genistein may act as a complexing agent for a special cyclic glucan such as γ-CD.

Molecular Structures of Transfer Products. The detailed molecular structures of the two major transglycosylation products, **2** and **3** (**Figure 1**), formed by TS4αGTase and BSCGTase were determined using ^{13}C NMR spectroscopy. The chemical shifts in the ^{13}C NMR spectra of transglycosylation products **2** and **3** were compared with those of genistin. Large downfield shifts (**Table 1**) were observed at C-4″ in the glucose moiety of genistin, from 69.62 to 79.03 ppm, implying that the transferred glucose unit was attached to C-4″ of the glucose moiety of genistin. For transglycosylation product **3**, a distinct shift from 69.91 to 78.49 ppm occurred at C-4″ in the second glucose moiety of **2**, in addition to the chemical shift observed in transglycosylation product **2**. This led to the conclusion that product **3** was formed by an α(1→4)-glycosidic linkage between the transferred maltose and genistin. In addition, the treatment of β-amylase, which specifically cleaves maltosyl units of the α(1,4)-glycosidic linkages, on the transglycosylation reaction product led to the accumulation of genistin and glucosyl-α-(1→4)-genistin (data not shown), implying the linkage between transferred glucosyl or maltosyl units and genistin is an α(1→4)-glycosidic linkage. Therefore, the molecular structures of the two major transglycosylation products, **2** and **3**, of genistin formed by TS4αGTase and BSCGTase were glucosyl-α(1→4)-genistin and maltosyl-α(1→4)-genistin, respectively. These results corresponded to the reaction modes of TS4αGTase and BSCGTase, in which the transferred glycosyl moiety was linked to the acceptor molecule by forming an α(1→4)-glycosyl linkage (*5*, *28*).

Analysis of the Cycloamylopectin and Glc(α1−4)-Glc(β1−7)-genistein/Glc(α1−4)-Glc(α1−4)-Glc(β1−7)-genisteins Complex. An HPLC chromatogram of the TS4αGTase reaction with starch and genistin showed small peaks (peak 19) at early elution times of 6−7 min (**Figure 2A**). These peaks were collected and analyzed by MALLS and MALDI-TOF MS. As shown in **Figure 6**, the peaks at *m*/*z* 617 and 779 represent the sodium adducts of Glc(α1−4)-Glc(β1−7)-genistein/Glc(α1−4)-Glc-(α1−4)-Glc(β1−7)-genistein, respectively, suggesting that these peaks contained Glc(α1−4)-Glc(β1−7)-genistein/Glc(α1−4)-Glc(α1−4)-Glc(β1−7)-genistein transglycosylation products. On the basis of HPLC retention times, it seemed that there was also a high molecular weight amylopectin. High-pressure SEC (HPSEC) equipped with MALLS and an RI detector was applied

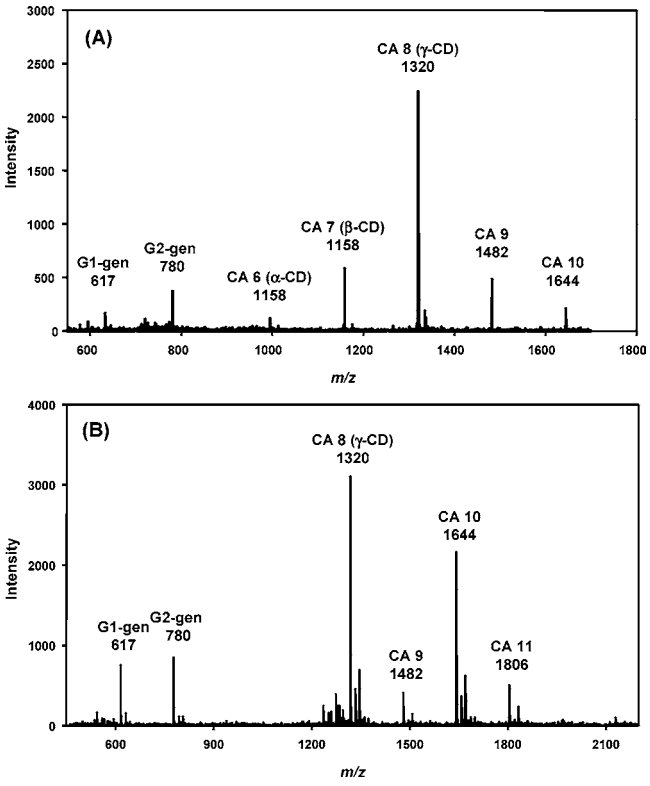

Figure 4. MALDI-TOF MS analysis of cycloamylose and Glc(α1–4)-Glc(β1–7)-genistein/Glc(α1–4)-Glc(α1–4)-Glc(β1–7)-genistein transglycosylation product complexes produced by (**A**) TS4αGTase and (**B**) BSCGTase. Peaks 7 and 7* in **Figure 2** were subjected to MALDI-TOF MS analysis.

to determine the molecular weight of amylopectin (**Figure 7**). Although two peaks appeared with the laser scattering detector, only one broad peak was observed with the RI detector. This single peak corresponded to the main peak with the laser scattering detector. The measured molecular mass of the cycloamylopectin complex (**Figure 7**) was 9.62×10^3 Da, which is similar to the molecular mass of the amylopectin cluster formed by the endo-attack of α-GTase at the internal chain segments between units of clusters (*32*). The data from the MALDI-TOF MS and MALLS demonstrated that the cycloamylopectin made a complex with the newly formed Glc(α1−4)-Glc(β1−7)-genistein/Glc(α1−4)-Glc(α1−4)-Glc(β1−7)-genisteins. The reaction mode of TS4αGTase in the production of cycloamylopectin and the Glc(α1−4)-Glc(β1−7)-genistein/Glc(α1−4)-Glc(α1−4)-Glc(β1−7)-genisteins complex is described in **Figure 5** and **Table 2**. TS4αGTase could carry out two different reactions, intermolecular transglycosylation to produce genistin transglycosylation products and intramolecular transglycosylation to produce both cycloamylose and cycloamylopectin.

Solubilities of the Main Transfer Products. The solubilities of the transfer products in water were compared with the solubility of genistin (**Table 3**). The solubility of genistin was 0.023 mM, whereas the apparent solubilities of **2**, Glc(α1−4)-Glc(β1−7)-genistein, and **3**, Glc(α1−4)-Glc(α1−4)-Glc(β1−7)-genistein, in water were 0.084 and 1.012 M, which are 3.7×10^3 and 4.4×10^4 times that of genistin. These findings imply that the attachment of a glucosyl residue or a maltosyl residue to genistin by TS4αGTase or BSCGTase greatly enhances the water solubility of the original compound. The turbidity of the

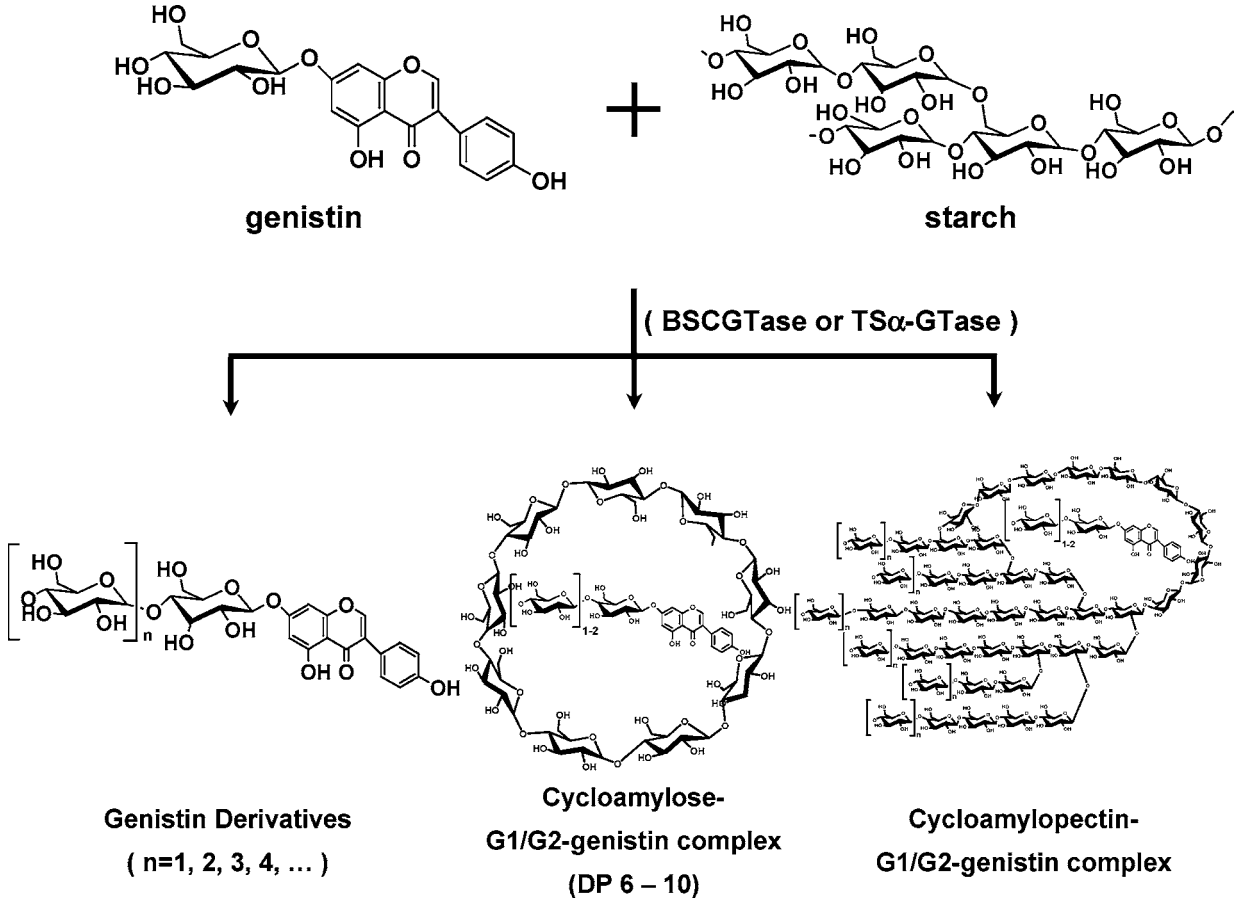

Figure 5. Reaction modes of TS4αGTase and BSCGTase.

Table 1. ^{13}C NMR Data (Parts per Million) of Genistin and Its Transfer Products

ring	carbon atom	genistin (δ)	Glc(α1–4)-Glc(β1–7)-genistein (δ1)	difference ($\delta1-\delta$)	Glc(α1–4)-Glc(α1–4)-Glc(β1–7)-genistein (δ2)	difference ($\delta2-\delta1$)
genistein	2	154.62	154.66	0.04	154.04	–0.62
	3	122.64	122.64	0	122.09	–0.55
	4	180.56	180.56	0	180.00	–0.56
	5	161.76	161.78	0.02	161.15	–0.63
	6	99.64	99.47	–0.17	98.96	–0.51
	7	163.05	162.91	–0.14	162.30	–0.61
	8	94.51	94.48	–0.03	93.97	–0.51
	9	157.3	157.64	0.34	156.99	–0.65
	10	106.17	106.21	0.04	105.66	–0.55
	1′	120.95	120.99	0.04	120.44	–0.55
	2′	130.23	130.24	0.01	129.67	–0.57
	3′	115.18	115.17	–0.01	114.63	–0.54
	4′	157.3	157.29	–0.01	156.71	–0.58
	5′	115.18	115.17	–0.01	114.63	–0.54
	6′	130.23	130.24	0.01	129.67	–0.57
Glc(β1–7)	1″	99.87	99.6	–0.27	100.27	0.67
	2″	73.12	72.68	–0.44	72.94	0.26
	3″	77.24	76.08	–1.16	75.39	–0.69
	4″	**69.62**	**79.03**	**9.41**	78.40	–0.63
	5″	76.45	75.40	–1.05	74.81	–0.59
	6″	60.65	60.83	0.18	60.29	–0.54
Glc(α1–4)	1‴		99.46		99.90	0.44
	2‴		72.48		71.40	–1.08
	3‴		73.60		72.75	–0.85
	4‴		**69.91**		**78.49**	**8.58**
	5‴		73.34		71.24	–2.1
	6‴		60.21		59.76	–0.45
Glc(α1–4)	1⁗				99.05	
	2⁗				71.97	
	3⁗				72.65	
	4⁗				69.36	
	5⁗				72.09	
	6⁗				59.66	

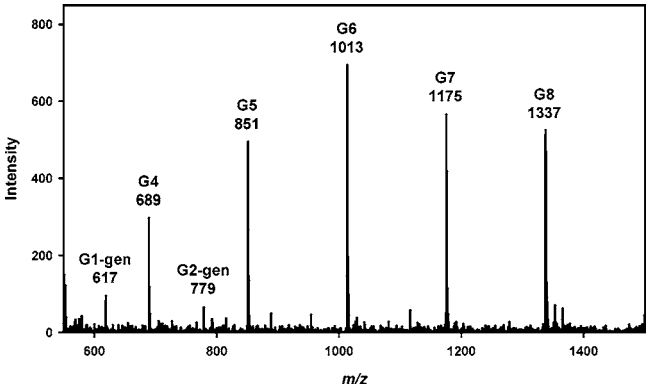

Figure 6. Analysis of cycloamylopectin and Glc(α1–4)-Glc(β1–7)-genistein/Glc(α1–4)-Glc(α1–4)-Glc(β1–7)-genistein complexes by MALDI-TOF MS. G4–G8 are maltotetraose to maltooctaose.

genistin solution was greatly decreased in the reaction mixture of TS4αGTase compared with that of the original genistin solution.

Owing to the many beneficial effects, enriched sources of genistin are marketed to consumers around the world in a wide variety of nutritional supplements. Many of the health benefits of soy products are ascribed to the presence of genistin (*33*, *34*). However, the limited water solubilities of genistin and other soybean isoflavones complicate their formulation into foodstuffs and cosmetics, as many of the processes for creating those products are aqueous-based. Low solubility also is an impediment to the efficient bioavailability of orally administered products and intravenous medications, which are most often

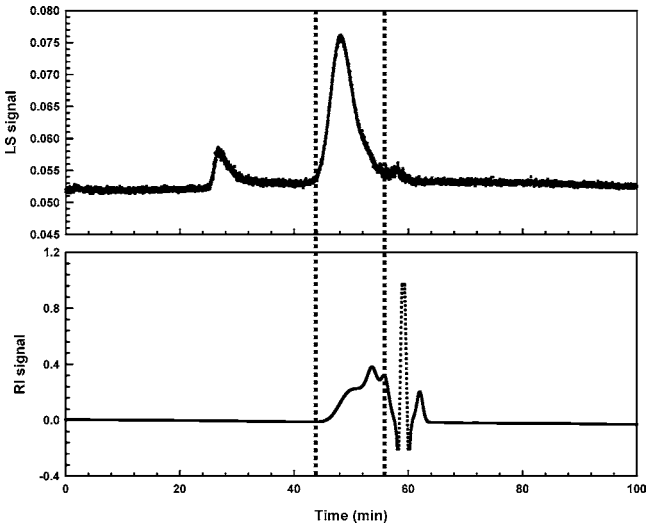

Figure 7. Molecular weight determination of amylopectin in the cycloamylopectin and Glc(α1–4)-Glc(β1–7)-genistein/Glc(α1–4)-Glc(α1–4)-Glc(β1–7)-genistein complex by HPSEC equipped with MALLS and RI detector. The peak in the upper panel at a retention time of 30 min was ignored because it did not make any signal in RI detector.

Table 2. Summary of Enzymatic Modification of Genistin and Starch by BSCGTase and TSαGTase

	BSCGTase	TSαGTase
modification of starch		
amylose	cyclic glucans DP 6–8	cyclic glucans DP 8–18
amylopectin		
hydrolysis product	$\sim1\times10^4$ (amylopectin cluster)	$\sim1\times10^4$ (amylopectin cluster)
side-chain length	no change	rearrangement increase of MW ($7.6\times10^7\rightarrow 6.4\times10^8$)
transglycosylation of genistin		
transfer product		
linkage	α-1,4	α-1,4
no. of glucosyl unit (n)	1–13	1–18
inclusion complex	cycloamylose$_{(DP8-11)}$-Glc(α1–4)$_{1-2}$-Glc(β1–7)-genistin	cycloamylose$_{(DP6-10)}$-Glc(α1–4)$_{1-2}$-Glc(α1–4)-Glc (β1–7)-genistin cycloamylopectin-Glc(α1–4)$_{1-2}$-Glc(α1–4)-Glc(β1–7)-genistin

Table 3. Solubility of Genistin and Its Transfer Products

isoflavone	water solubility[a] (mM, 25 °C)	relative solubility
genistin	0.023 ± 0.002	1
glucosyl-α(1–4)-genistin	83.7 ± 2.8	3.7×10^3
maltosyl-α(1–4)-genistin	1013.4 ± 31.7	4.4×10^4

[a] Mean $\pm$ SD ($n=3$).

delivered in aqueous media. Thus, there is a continuing demand for isoflavones in forms with increased bioavailability, especially enhanced aqueous solubility relative to the unmodified compounds that retain the active properties of the unmodified isoflavones.

As shown in this study, the transglycosylation reaction via glucosyltransferases is highly applicable to improving the water solubility of isoflavones. The major advantage of this method is that it maintains the bioavailability of isoflavones while increasing the water solubility. The newly formed α(1,4)-glycosidic linkage of the transglycosylation products was easily

hydrolyzed in the human body by various intestinal microorganisms with glycosyl hydrolases (*35*), such as α-glucosidase, implying that genistin transglycosylation derivatives are metabolized in the same way as genistin or genistein.

ABBREVIATIONS USED

BE, glucan-branching enzyme; BSCGTase, CGTase from alkalphilic *Bacillus* I-5; CA, cycloamylose; CDs, cyclodextrins; CGTase, cyclodextrin glucanotransferases; MAases, maltogenic amylases; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MALLS, multiangle laser light scattering; Ni−NTA, nickel−nitrilotriacetic acid; SEC, size exclusion chromatography; TMS, tetramethylsilane; TS4αGTase, 4-α-glucanotransferase from *Thermus scotoductus.*

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