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# Biotechnological Production of Highly Soluble Daidzein Glycosides Using *Thermotoga maritima* Maltosyltransferase

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The use of soybean isoflavones in food products is limited due to their low hydrophilicity. To enhance its solubility, the isoflavone daidzin was transglycosylated as a model compound using *Thermotoga maritima* maltosyltransferase (MTase). Four novel transglycosylation products of daidzin were identified by TLC and MALDI-TOF MS: daidzein 7-*O*-triglucoside, daidzein 7-*O*-pentaglucoside, daidzein 7-*O*-heptaglucoside, and daidzein 7-*O*-nonaglucoside. The major product, daidzein 7-*O*-triglucoside, was purified by $C_{18}$ and gel filtration chromatography, and its molecular structure was determined using UV, IR, MALDI-TOF MS, and NMR. The solubility of daidzein 7-*O*-triglucoside was $7.5 \times 10^4$ times that of daidzin, suggesting that the transglycosylation greatly enhanced its water solubility.

**KEYWORDS: Daidzin; daidzein 7-*O*-triglucoside; isoflavone; maltosyltransferase; *Thermotoga maritima*; transglycosylation**

## INTRODUCTION

Most people include legumes in their diet. Among legumes, the soybean has gained much attention due to its beneficial effects and therapeutic potential, particularly in disease prevention. Soybean contains a high concentration of isoflavones, which are presumed to be involved in many of the health benefits of soybean. The isoflavones found mainly in the family Fabaceae are often referred to as phytoestrogens because of their estrogenic activity, which results from their interactions with estrogen receptors in cells (*1*, *2*). Many epidemiological and clinical investigations have shown that dietary intake of isoflavones can reduce the risks of hormone-dependent and -independent cancers and cardiovascular diseases, alleviate menopausal symptoms such as hot flashes and osteoporosis, and prevent hereditary chronic nose bleeds and autoimmune diseases (*3*−*5*). Recently, the presence of isoflavones in various plants other than soybean has been reported. Krishnan (*6*) identified genistin in the edible tubers of the American groundnut (*Apios americana* Medikus). Unlike soybeans, *A. americana* contains the diglucoside conjugate of genistein, which is more soluble and potentially more readily absorbed in the small intestine (*7*). In addition, it was found that the daidzin and genistin levels in kudzu root (*Puerariae radix*) were higher than those in soybean (*8*).

Transglycosylation has been used to modify the physiochemical properties of raw materials in foods and to improve their usefulness (*9*−*14*). Two types of enzymes are mainly used to transfer the glycosides to acceptor molecules. Kometani et al. (*15*) reported the transglycosylation of several flavonoids, including neohesperidin and naringin, by the reaction with cyclodextrin glucanotransferase (CGTase) from alkalophilic *Bacillus* species. It was found that glycosylneohesperidin and glycosylnaringin were less bitter or more soluble in water than the original compounds. Maltogenic amylases (MAases) from *Bacillus stearothermophilus* and *Thermus* sp. have also been used to modify bioactive compounds, such as ascorbic acid, naringin, and acarbose, by transferring maltosyl residues to the acceptor molecules to produce maltosyl-transfer products (*11*, *12*, *16*, *17*). Recently, a novel enzyme, which originates from the hyperthermophilic bacterium *Thermotoga maritima* MSB8 and acts on starch and maltooligosaccharides, was identified and designated maltosyltransferase (MTase) (*18*). MTase has a unique transfer activity confined to the transfer of maltosyl units and disproportionate maltooligosaccharides to form a set of maltosyl transfer products (e.g., maltose, maltotetraose, maltohexaose).

This study used the maltosyl-transfer activity of MTase from *T. maritima* to synthesize new highly soluble isoflavone derivatives. The transglycosylation reaction was successfully conducted with daidzin and maltotriose as the acceptor and donor, respectively. The structure of the major transglycosylation product of daidzin was determined and its water solubility examined.

## MATERIALS AND METHODS

**Chemicals.** An isoflavone mixture made from soybean (35% isoflavone; 7.72% daidzin, 0.95% glycitin, and 9.48% genistin) was

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kindly provided by Pulmuone Co., Ltd. (Seoul, Korea). Daidzin standard was obtained from LC Laboratories (Woburn, MA). Maltotriose was purchased from Sigma Chemical Co. (St. Louis, MO). Dimethyl sulfoxide (DMSO) was from Showa Chemical Co. (Tokyo, Japan). All other chemicals were of reagent grade and were purchased from Showa Chemical Co. or Sigma Chemical Co.

**Cloning and Expression of the MTase Gene in *Escherichia coli*.** The gene corresponding to an MTase was cloned from *T. maritima* MSB8 genomic DNA by PCR. The two primers (MTF, 5′-TCAGGAGGGATGCATATGCTTTTGAGAGAG-3′, and MTR, 5′-CGAAAGCTTCGCGAGAAAAAG-3′) used for PCR contained *Nde*I and *Hin*dIII restriction enzyme sites, respectively. PCR was performed at an annealing temperature of 55 °C. The resulting PCR fragment was purified and digested with *Nde*I and *Hin*dIII. Then, it was ligated into the expression vector pGNX4 donated by Samyang Genex Corp. (Seoul, Korea), digested with the two restriction enzymes, to create pGNX4MT. The nucleotide sequence of the PCR-generated gene was determined using a BigDye Terminator Cycle Sequencing Kit for ABI3700 PRISM (Perkin-Elmer, Norwalk, CT). The expression vector was transformed into *E. coli* MC1061 [*F*$^-$, *araD139*, Δ(*araABC-leu*)*7696*, *galE15*, *galK16*, Δ*lacX74*, *rpsL*, *thi*, *hsdR2*, *mcrA*, *mcrB1*].

**Preparation of MTase.** Recombinant *E. coli* harboring the MTase gene was cultured in Luria−Bertani (LB; 1% bactopeptone, 0.5% yeast extract, and 0.5% NaCl) medium containing kanamycin (50 mg/L) at 37 °C overnight with shaking. The cells were harvested from the culture broth by centrifugation. The pellet was resuspended in lysis buffer (50 mM Tris-HCl buffer, pH 8.0) and disrupted with a VC-600 sonicator (Sonics and Materials Inc., Danbury, CT). After centrifugation, the supernatant was heated at 75 °C for 15 min to remove thermolabile proteins from the crude extract. The crude enzyme obtained by centrifugation was further purified using a 10 × 1 cm Q-Sepharose column (Pharmacia, Uppsala, Sweden) with the AKTA FPLC system (Pharmacia). The active fractions were concentrated by ultrafiltration through a YM10 regenerated cellulose membrane (Amicon 8010 UF kit; Millipore, Billerica, MA) at 4 °C and dialyzed against 50 mM Tris-HCl buffer (pH 8.0). MTase activity was determined using the method described by Meissner and Liebl (*18*). After 200 μL of 2% (w/v) maltooligosaccharide mixture (Sigma) and 100 μL of pH 6.5 McIlvaine buffer (prepared by titrating 0.1 M citric acid and 0.2 M $Na_2HPO_4$ at 75 °C) were preincubated for 10 min at 75 °C, 100 μL of appropriately diluted enzyme was added and the mixture was reacted for 10 min. Then, a 100-μL aliquot was withdrawn and mixed with 1 mL of 0.2% $I_2$/KI solution at ambient temperature. The absorbance was measured at 480 nm immediately. One unit of MTase activity was defined as the amount of enzyme required to increase the absorbance by 1.0 in 1 min. The protein concentration was determined using the Bradford method (*19*). Discontinuous SDS-PAGE was performed as described by Laemmli (*20*).

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

**Transglycosylation Reaction.** Daidzin was transglycosylated using MTase (0.005 unit/mg of maltotriose) in different solvents, including water, methanol, ethanol, and DMSO. The best yield of the transglycosylation products was obtained in the DMSO solution. To optimize the transglycosylation reaction, the yield of transglycosylation products was determined in DMSO solutions of several different concentrations (0−40%). As the optimal conditions, 10−15 mg/mL of daidzin and 20−30 mg/mL of maltotriose in 30% DMSO (in McIlvaine buffer, pH 6.5) were chosen. After MTase (0.005 unit/mg of maltotriose) was added to the substrate solution, the transglycosylation reaction was conducted at 85 °C for 10 min. The reaction was stopped by placing the reaction tube in ice water. The amounts of transfer products were determined by HPLC analysis.

**Purification of the Transfer Product.** A Sep-Pak Plus $C_{18}$ cartridge, previously activated using ethyl acetate, methanol, and water, was used to absorb the daidzein glycosides in the transglycosylation reaction mixture and to remove any maltooligosaccharides and salts. The enzyme in the eluted solution was eliminated by ultrafiltration. The main transfer product of daidzin was purified using a W-251 polymeric gel filtration column in the recycling preparative HPLC. The mobile phase was 80% (v/v) methanol at 2 mL/min.

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

**UV Analysis.** The purified transfer product was dissolved in methanol until its absorbance was 0.3−0.8 at 254 nm. Ultraviolet spectra (220∼360 nm) were obtained using a Ultrospec 4000 UV−vis spectrophotometer (Pharmacia Biotech, Uppsala, Sweden).

**IR Analysis.** A sample containing the purified transfer product was prepared in a KBr pellet. The infrared spectrum (400∼4000 nm) was obtained with a Nicolet Magna 550 series II FT-IR spectrophotometer (Midac Co., Costa Mesa, CA).

**Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) Analysis.** The molecular weight of the transfer products in the reaction solution and the purified major transfer product were determined using a Voyager-DE MALDI-TOF MS (Perseptive Biosystem, Framingham, MA) in linear mode. A sample in methanol was mixed with the matrix, 10 mg/mL (w/v) of 2,5-dihydroxybenzoic acid (*21*) in 10% methanol, at a ratio of 1:1. To each sample well was added 1.5 μL of the mixture, which was allowed to dry.

***β*-Amylase Hydrolysis.** The purified transfer product was dissolved in 50 mM sodium acetate buffer (pH 4.8). β-Amylase (EC 3.2.1.2 type 1-B; from sweet potato) (Sigma) at a concentration of 0.5 unit/mg of transfer product was added to the substrate solution, and the reaction was carried out overnight at 20 °C. The reaction mixture was analyzed using TLC analysis.

**NMR Analysis.** $^1H$ and $^{13}C$ NMR spectra were recorded at 399.65 and 100.40 MHz, respectively, with a JEOL JNM LA-400 MHz NMR spectrometer (Tokyo, Japan). The sample was dissolved in DMSO-$d_6$ at 24 °C with tetramethylsilane (TMS) as the chemical shift reference. The multiplicities of the $^{13}C$ resonances were determined in DEPT experiments. $^1H-^1H$ COSY, $^1H-^{13}C$ HMQC, and $^1H-^{13}C$ HMBC experiments were performed using a pulse sequence supplied from JEOL.

**Solubility Determination.** Excess daidzin or daidzein 7-*O*-triglucoside was mixed with 1 mL of solvent in an Eppendorf tube at room temperature. An ultrasonic cleaner was used to maximize the solubility of the isoflavones. After sonication at room temperature for 1 h with intermittent pauses, the sample was diluted and filtered through a PVDF 0.45-μm membrane (Millipore, Billerica, MA). The daidzin or daidzin derivatives were quantified by HPLC, and the absolute solubilities were calculated.

**HPLC Analysis.** A Waters 600E HPLC system with a 150 × 3.9 mm i.d. Nova-Pak $C_{18}$ column (Waters, Milford, MA) and an SLC 200 UV−vis detector (Samsung, Seoul, Korea) at 254 nm were used to quantify the amounts of isoflavones. A gradient solvent system consisting of solvents A (water/formic acid, 100:0.1, v/v) and B (methanol/water/formic acid, 50:50:0.1, v/v/v) was used; solvent B was increased gradually from 20 to 100% over 20 min at a flow rate of 1.2 mL/min. The concentrations of isoflavone glycosides were calculated from the daidzin standard curve. The peaks were identified using a JEOL LC mate Mass Spectrum (Tokyo, Japan) in $ESI^+$ mode.

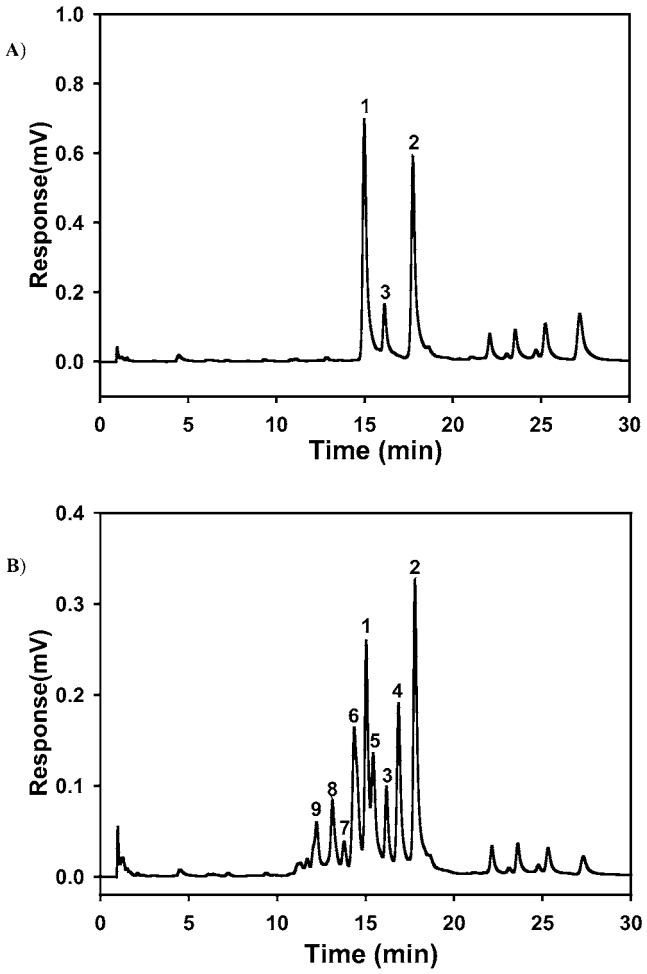


**Figure 1.** HPLC chromatogram of (**A**) soybean isoflavones and (**B**) transglycosylated soybean isoflavone products. Peaks: 1, daidzin; 2, genistin; 3, glycitin; 4, genistein 7-*O*-triglucoside; 5, genistein 7-*O*-pentaglucoside; 6, daidzein 7-*O*-triglucoside; 7, genistein 7-*O*-heptaglucoside; 8, daidzein 7-*O*-pentaglucoside; 9, daidzein 7-*O*-heptaglucoside.

## RESULTS AND DISCUSSION

**Transglycosylation of Soybean Isoflavones by MTase.** Soybean isoflavone extract was reacted with MTase to investigate the possible transglycosylation activity of MTase on soybean isoflavone. HPLC analysis showed that the soybean isoflavone extract had a high isoflavone content: 7.7% daidzin, 9.5% genistin, and 1.0% glycitin (**Figure 1**). MTase was reacted with soybean isoflavone extract in the presence of maltotriose as a donor molecule. The peaks corresponding to authentic soybean isoflavones were diminished, whereas other peaks, possibly newly produced isoflavone glycosides, appeared after the MTase reaction (**Figure 1**). It is known that MTase has transferase activity with a unique specificity strictly confined to the transfer of maltosyl units (*18*). Incubating maltosyltransferase with starch led to the formation of products with repeated maltose units. The result shown in **Figure 1** suggested that the maltosyl-transfer activity of MTase was successfully used to produce the isoflavone glycoside. This is the first reported application of MTase to modify a natural product to modulate its properties. To investigate the transglycosylation of soybean isoflavone by MTase in detail, daidzin was chosen as a model isoflavone compound, and the MTase reaction was conducted in different solvent systems.

**Optimization of the Transglycosylation Reaction.** The solubility of daidzin in water was examined and was maximal at a concentration of ~24.71 $\mu$M. In fact, the low solubility of daidzin is disadvantageous not only for its practical application as a food supplement but also for enzymatic reactions. Relatively high concentrations of soybean isoflavones can be obtained in various organic solvents, such as pyridine, dimethylformamide, and DMSO. Moreover, methanol and ethanol are often used to extract the isoflavones in soybean. With these solvents, the daidzin concentrations can exceed 1 mM, which is much higher than the concentration in water. Although MTase has a very high heat stability in water and shows the highest transglycosylation activity at 85−90 °C, the transglycosylation reaction in water is not efficient due to the low solubility of the acceptor daidzin in water. The solubility of daidzin increases only slightly at elevated temperature. This implies that both the volume of the reaction solution and the amounts of maltotriose and MTase needed can be reduced by performing the transglycosylation reaction in organic solvents, which greatly improves the reaction efficiency.

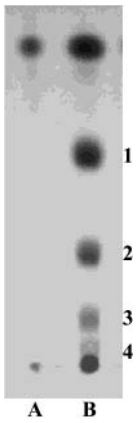


**Figure 2.** TLC analysis of transglycosylation products: (lane A) daidzin standard; (lane B) transglycosylation products. Spots were visualized by UV detector at 254 nm.

Transglycosylation reactions between daidzin and maltotriose using MTase were performed in hydrated organic solvents (**Figure 2**). Although daidzin dissolves well in pyridine, dimethylformamide, and methanol, these solvents can harm human health and are unsuitable for industrial production. Therefore, we used DMSO for the transglycosylation reaction. When the transglycosylation products were examined by TLC analysis, at least four transfer products of daidzin were detected by absorbance at 254 nm UV (**1**−**4** in **Figure 2**). The main transfer product (**1**) appeared as a large spot at an $R_f$ value of 0.56. The three other products were located at much lower $R_f$ values, implying that these compounds were larger than **1**.

To optimize the reaction conditions, the solubility of daidzin and the amount and yield of the transglycosylation products obtained using MTase in various concentrations of DMSO (0−40%) were determined (**Table 1**). The solubility of daidzin increased with the DMSO content. Whereas the yields of the transglycosylation products ranged from 40 to 45% in up to 30% DMSO, the yield dropped to 10% in 40% DMSO. Therefore, 30% DMSO was used for the transglycosylation reaction to obtain the highest amount of transfer products. In enzymatic reactions involving organic solvents, higher reaction rates are generally obtained by decreasing the viscosity and increasing the diffusion coefficient, which occur at elevated temperatures (*16*). *T. maritima* MTase has maximal transferase activity at pH 6.5 and 85−90 °C. In addition, the enzyme is extraordinarily resistant to thermal inactivation and has a half-life of 21 days at 70 °C. Therefore, because isoflavone has an

**Table 1.** Transglycosylation of Daidzin and Maltotriose in Various Concentrations of DMSO[a]

| DMSO (%) | daidzin (mM) | product concn (mM) G2-daidzin | G4-daidzin | G6-daidzin | G8-daidzin | total product (mM) | total yield (%) |
|---|---|---|---|---|---|---|---|
| 0 | 2.43 | 0.47 | 0.28 | 0.14 | 0.02 | 0.95 | 39.0 |
| 5 | 5.34 | 1.12 | 0.63 | 0.33 | 0.05 | 2.20 | 41.2 |
| 10 | 10.94 | 2.34 | 1.46 | 0.77 | 0.13 | 4.86 | 44.4 |
| 20 | 21.85 | 4.55 | 3.25 | 1.58 | 0.20 | 9.77 | 44.7 |
| 30 | 37.38 | 7.56 | 4.40 | 2.10 | 0.32 | 14.75 | 39.5 |
| 40 | 160.26 | 12.18 | 3.79 | 0.41 | 0.11 | 16.49 | 10.3 |

[a] Transglycosylation reactions were done at 85 °C for 10 min.

extremely low solubility in water but a relatively high solubility in organic solvents, MTase is an ideal enzyme for the transglycosylation reaction of isoflavone. We showed that MTase had a high level of transferase activity when organic solvents were used to dissolve daidzin.

**Identification of Daidzin Transfer Products.** The presence of four daidzin transglycosylation products was confirmed by MALDI-TOF MS analysis (**Figure 3**). Several peaks were seen when the transglycosylation mixture of daidzin was analyzed using MALDI-TOF MS, and the peaks corresponded to the calculated molecular masses of transglycosylated daidzin derivatives, including the daidzin substrate itself. The three peaks at *m*/*z* 417.7 ($[M + H]^+$), *m*/*z* 440.7 ($[M + Na]^+$), and *m*/*z* 457.3 ($[M + K]^+$) corresponded to the calculated molecular masses of the protonated ion and the sodium and potassium adducts of daidzin, respectively. Likewise, the molecular weight of **1** was calculated to be 740 Da from the three peaks at *m*/*z* 740.9 ($[M + H]^+$), *m*/*z* 763.1 ($[M + Na]^+$), and *m*/*z* 779.2 ($[M + K]^+$), which matched the expected molecular masses of the protonated ion and the sodium and potassium adducts of maltosyl-daidzin (daidzein 7-*O*-triglucoside). The second transfer product (**2**) produced two clear peaks: *m*/*z* 1087.1 ($[M + Na]^+$) and *m*/*z* 1103.9 ($[M + K]^+$); these correspond to the expected molecular masses of the sodium and potassium adducts of maltotetraosyl-daidzin (daidzein 7-*O*-pentaglucoside), respectively. The third derivative (**3**) had peaks at *m*/*z* 1411.5 ($[M + Na]^+$) and *m*/*z* 1428.4 ($[M + K]^+$), which are compatible with the molecular masses of the sodium and potassium adducts of maltohexaosyl-daidzin (daidzein 7-*O*-heptaglucoside). The fourth transfer product (**4**) had one peak at *m*/*z* 1735.0 ($[M + Na]^+$). These results, combined with the TLC analysis, demonstrate that at least four transglycosylation products of daidzin were produced in the MTase reaction.

The reaction mode of MTase with daidzin is illustrated in **Figure 4**. The donor, maltotriose, was first attached to MTase. This formed an intermediate complex, which was immediately attacked by an acceptor molecule in the reaction mixture, daidzin or a maltooligosaccharide. The transfer products included daidzein multiglucosides and maltooligosaccharides, as daidzin transfer products could be the acceptor in the reaction. Nevertheless, the resulting maltooligosaccharides could act as donor molecules by providing a maltose unit for the acceptor compounds.

**Structure of the Main Transfer Product.** The main transfer product (**1**) showed maximum UV absorbances at both 260 and 306 nm, as seen with soybean isoflavone. In addition, the infrared spectra of **1** showed that the transfer product contained −OH (3350−3400), −C═O (1640), $-C_6H_5$ (1470, 1520, 1600), and −C−O−C− (1080, 1268) groups (data not shown). These results implied that **1** was an isoflavone derivative. In addition, the molecular weight of the major transglycosylation product (**1**) was 740 Da, as determined by MALDI-TOF MS. The three molecular ion peaks in the MALDI-TOF MS spectrum exactly matched the protonated ion and the sodium and potassium adducts of daidzein 7-*O*-triglucoside, respectively. Combining these results, we concluded that **1** was daidzein 7-*O*-triglucoside produced by the transmaltosylation of MTase to daidzin.

The molecular structure of the main transfer product of daidzin was confirmed by enzymatic digestion and $^{13}C$ NMR analysis. *β*-Amylase is an exoenzyme, which releases successive maltose units from the nonreducing end of a polysaccharide chain by the hydrolysis of α-1,4-glucosidic linkages (*22*). When the main transfer product (**1**) was treated with *β*-amylase, **1** was broken down into daidzin and maltose primarily, according to the TLC analysis (**Figure 5**). This proved that the maltose unit was attached to the glucose moiety of daidzin and the new linkage formed between the maltose and glucose of daidzin was an α-1,4-glucosidic bond, because it was cleaved by *β*-amylase.

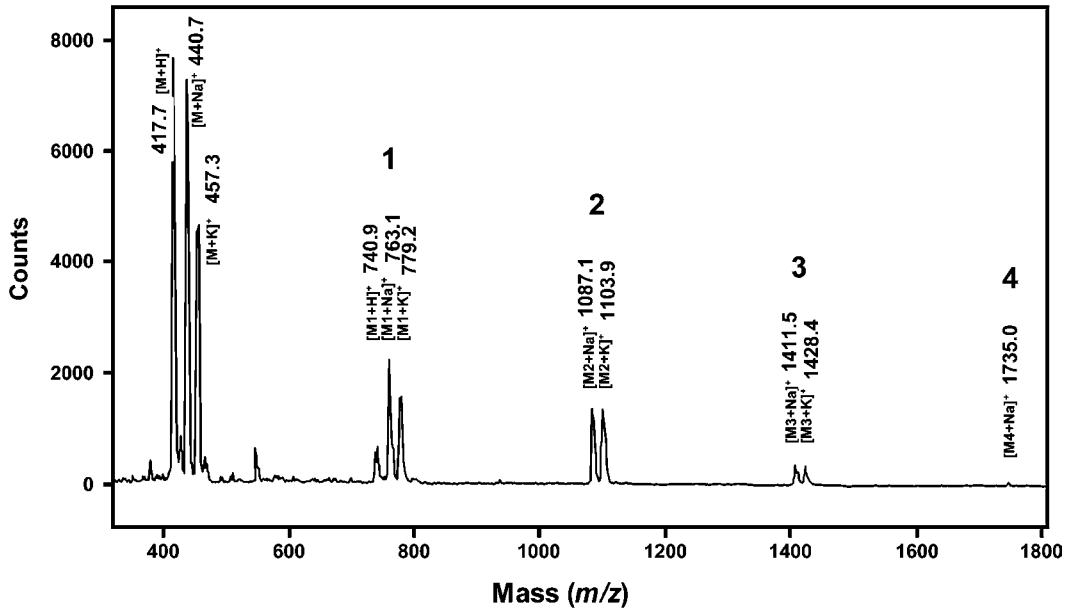


**Figure 3.** MALDI-TOF MS spectra of daidzin transglycosylation products.

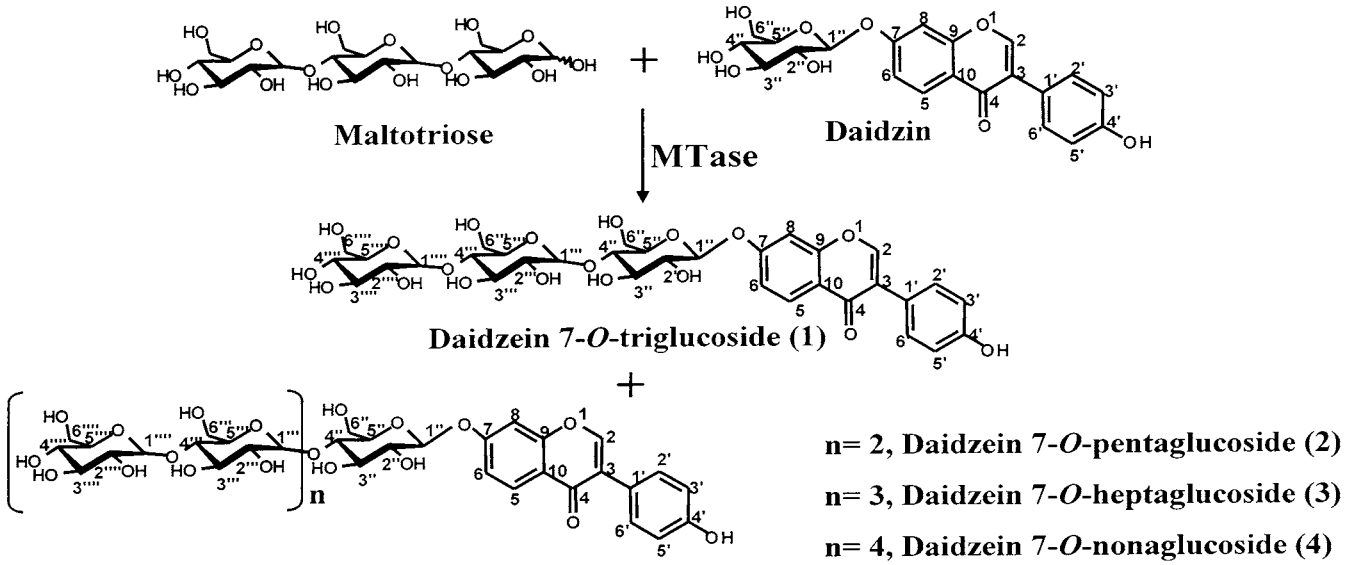


**Figure 4.** Transglycosylation reaction mode of daidzin with *T. maritima* maltosyltransferase.

**Table 2.** $^{1}H$ and $^{13}C$ NMR Data of Daidzein 7-*O*-Triglucoside

| | $^{1}H$ | $^{13}C$ | dept | COSY | HMQC | HMBC |
|---|---|---|---|---|---|---|
| 2 | 8.39 s | 153.35 | CH | | 153.35 | $C_3$ (122.28), $C_4$ (174.73), $C_9$ (157.26) |
| 3 | | 122.28 | CH | | | |
| 4 | | 174.73 | C | | | |
| 5 | 8.04 d, $J_{5,6}$ = 8.8 Hz | 127.01 | CH | H6 (8.04) | 127.01 | $C_4$ (174.73), $C_7$ (161.25), $C_9$ (157.26) |
| 6 | 7.13 dd, $J_{5,6}$ = 8.8 Hz, $J_{6,8}$ = 2.4 Hz | 114.97 | CH | H5,8 (7.13) | 114.97 | $C_7$ (161.25), $C_8C_{10}$ (118.53) |
| 7 | | 161.25 | C | | | |
| 8 | 7.22 m, $J_{6,8}$ = 2.4 Hz | 103.34 | CH | H6 (7.22) | 103.34 | $C_6$ (114.97), $C_7$ (161.25), $C_9$ (157.26), $C_{10}$ (118.53) |
| 9 | | 157.26 | C | | | |
| 10 | | 118.53 | C | | | |
| 1′ | | 123.70 | C | | | |
| 2′ | 7.40 d, $J_{2',3'}$ = 6.6 Hz, $J_{5',6'}$ = 2.1 Hz | 130.07 | CH | H3′ (7.40) | 130.07 | $C_{1'}$ (123.70), $C_{4'}$ (157.00) |
| 3′ | 6.80 d, $J_{2',3'}$ = 6.6 Hz, $J_{5',6'}$ = 2.1 Hz | 115.54 | CH | H2′ (6.80) | 115.54 | $C_{1'}$ (123.70), $C_{4'}$ (157.00) |
| 4′ | 9.52 br s | 157.00 | C | | | |
| 5′ | 6.80 d, $J_{2',3'}$ = 6.6 Hz, $J_{5',6'}$ = 2.1 Hz | 115.54 | CH | H6′ (6.80) | 115.54 | |
| 6′ | 7.40 d, $J_{2',3'}$ = 6.6 Hz, $J_{5',6'}$ = 2.1 Hz | 123.70 | CH | H5′ (7.40) | 123.70 | $C_{1'}$ (123.70), $C_{4'}$ (157.00) |
| 1″ | 5.17 d, $J_{1'',2''}$ = 7.6 Hz | 99.63 | CH | | 99.63 | $C_7$ (161.25) |
| 2″ | | 73.51 | CH | | | |
| 3″ | | 76.03 | CH | | | |
| 4″ | | 79.50 | CH | | | |
| 5″ | | 75.39 | CH | | | |
| 6″ | | 60.79 | $CH_2$ | | | |
| 1‴ | 5.08 d, $J_{1''',2'''}$ = 3.4 Hz | 100.44 | CH | | 100.44 | $C_{4''}$ (79.50) |
| 2‴ | | 71.97 | CH | | | |
| 3‴ | | 73.30 | CH | | | |
| 4‴ | | 79.08 | CH | | | |
| 5‴ | | 71.80 | CH | | | |
| 6‴ | | 60.25 | $CH_2$ | | | |
| 1⁗ | 5.00 d, $J_{1'''',2''''}$ = 3.4 Hz | 100.84 | CH | | 100.84 | $C_{4'''}$ (79.08) |
| 2⁗ | | 72.53 | CH | | | |
| 3⁗ | | 73.17 | CH | | | |
| 4⁗ | | 69.90 | CH | | | |
| 5⁗ | | 72.67 | CH | | | |
| 6⁗ | | 60.14 | $CH_2$ | | | |

The detailed molecular structure of the main transfer product (**1**) was identified using NMR (**Table 2**). The aglycon $^{1}H$ and $^{13}C$ in the main transfer product were assigned by COSY, HMQC, and HMBC experiments and compared with the reported NMR data for daidzein and daidzin (*23*, *24*). The $^{13}C$ assignments of the three glucosyls in the main transfer product were determined from the reported NMR data for daidzin, maltose, and maltotriose (*25*). The $^{1}H$ assignments of the three glucosyl residues were measured in HMQC experiments. The HMBC experiment proved that the glucose molecules were connected by α-1,4-glycosidic linkages.

**Solubility of the Main transfer Daidzin Product.** The solubility of daidzein 7-*O*-triglucoside (**1**) in water was studied by comparing the water solubility of **1** with that of daidzin. The solubility of daidzin was determined to be 24.71 $\mu$M, whereas the apparent solubility of the main transfer daidzin product in water was 1.86 M, which is $\sim 7.5 \times 10^4$ times that of natural daidzin. This implies that the attachment of a maltosyl residue to daidzin by MTase greatly enhanced the water solubility of the original compound.

The recommended isoflavone intake to benefit health based on various clinical results is 60−100 mg of aglycons/person/

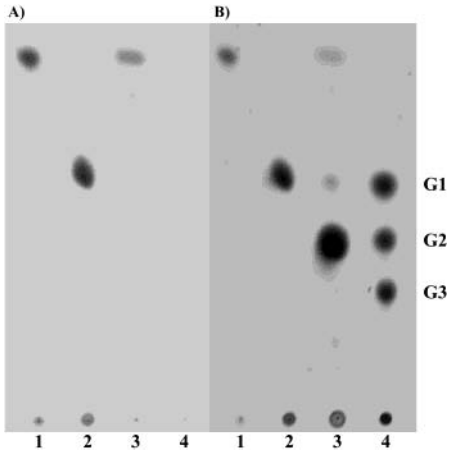


**Figure 5.** TLC analysis of the daidzin transglycosylation products after $\beta$-amylase treatment. The spots were visualized by (**A**) using UV at 254 nm and (B) dipping solution: (lane 1) daidzin; (lane 2) daidzein 7-*O*-triglucoside; (lane 3) daidzein 7-*O*-triglucoside after $\beta$-amylase treatment; (lane 4) glucose, maltose, and maltotriose.

day (*26*, *27*). Nevertheless, even in Japan, where large amounts of soybeans and soybean-based foods are consumed, the estimated daily intake of isoflavones is only 28 mg/day (*28*). The low solubility of isoflavones in water results in low concentrations in liquid soybean food. The isoflavone concentration of soybean milk is 1.26−21.13 mg/100 g (*29*, *30*). Some people do not like to eat soy foods due to their smell, taste, and texture. In this case, soybean isoflavones should be taken as dietary supplements. Unfortunately, soybean isoflavones and their derivatives are not available as dietary supplements because of their low solubility in water (*31*). Much effort has been made to increase the solubility of natural soybean isoflavone. An efficient, simple practice is to chelate isoflavone using $\beta$-cyclodextrin, which produces a complex that is 2−6 times more soluble and is much less bitter (*32*). However, the use of this complex is limited because the solutions are cloudy. As we showed here, enzymatic synthesis using MTase is highly applicable for producing isoflavone transglycosylation products with improved solubility. Furthermore, the α-1,4-glycosidic linkage formed in the transglycosylation reaction is easily hydrolyzed by various glycosylhydrolases, such as α-glucosidase and $\beta$-amylase, implying that the human body metabolizes isoflavone transglycosylation derivatives in the same way as isoflavone itself. It can be inferred that the bioavailability of isoflavone transglycosylation derivatives is not changed greatly.

## ABBREVIATIONS USED

CGTase, cyclodextrin glycosyltransferase; MAases, maltogenic amylases; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MTase, maltosyltransferase; TMS, tetramethylsilane.

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