Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 339 (2004) 2789–2797
Carbohydrate RESEARCH

In vitro enzymatic modification of puerarin to puerarin glycosides by maltogenic amylase

Dan Li,[a] Sung-Hoon Park,[a] Jae-Hoon Shim,[a] Hee-Seob Lee,[a] Shuang-Yan Tang,[a] Cheon-Seok Park[b] and Kwan-Hwa Park[a,*]

[a]*National Laboratory for Functional Food Carbohydrate and Center for Agricultural Biomaterials, School of Agricultural Biotechnology, Seoul National University, Shillim-dong, Kwanak-gu, Seoul 151-742, Korea*
[b]*Department of Food Science and Technology, Kyunghee University, Yongin 449-701, Korea*

Received 27 July 2004; accepted 22 September 2004

Abstract—Puerarin (daidzein 8-*C*-glucoside), the most abundant isoflavone in *Puerariae radix*, is prescribed to treat coronary heart disease, cardiac infarction, problems in ocular blood flow, sudden deafness, and alcoholism. However, puerarin cannot be given by injection due to its low solubility in water. To increase its solubility, puerarin was transglycosylated using various enzymes. *Bacillus stearothermophilus* maltogenic amylase (BSMA) was the most effective transferase used compared with *Thermotoga maritima* maltosyl transferase (TMMT), *Thermus scotoductus* 4-α-glucanotransferase (TS4αGTase), and *Bacillus* sp. I-5 cyclodextrin glucanotransferase (BSCGTase). TMMT and TS4αGTase lacked acceptor specificity for puerarin, which lacks an *O*-glucoside linkage between D-glucose and 7-OH-daidzein. The yield exceeded 70% when reacting 1% puerarin (acceptor), 3.0% soluble starch (donor), and 5 U/100 μL BSMA at 55 °C for 45 min. The two major transfer products of the BSMA reaction were purified using C_{18} and GPC chromatography. Their structures were identified as α-D-glucosyl-(1→6)-puerarin and α-D-maltosyl-(1→6)-puerarin using ESI^+ TOF MS–MS and ^{13}C NMR spectroscopy. The solubility of the transfer products was 14 and 168 times higher than that of puerarin, respectively.

Keywords: Isoflavone; Maltogenic amylase; *Pueraria lobata*; Puerarin; Solubility; Transglycosylation

1. Introduction

Pueraria lobata (Wild.) Ohwi, a perennial vine in the Leguminosae, occurs mainly in eastern Asia.[1] *Puerariae radix* (RP), the root of *Pueraria lobata*, was one of the first and most important crude plants used in traditional oriental medicine owing to its many profound pharmacological actions, including antipyretic, antidiarrheal, spasmolytic, diaphoretic, and *anti* emetic activities.[2,3] Although the main component of RP is starch, it contains fairly high amounts of isoflavones, including puerarin, daidzin, genistin, daidzein, and genistein.[4]

Puerarin (daidzein 8-*C*-glucoside), the most abundant isoflavone in RP, is prescribed to treat coronary heart disease,[5] angina pectoris,[6] cardiac infarction,[7,8] problems in ocular blood flow,[9,10] sudden deafness,[11] and alcoholism.[12,13] Studies show that puerarin may prevent cancer,[14,15] act as an antioxidant,[16] scavenge free radicals,[17] lower serum cholesterol,[18,19] and have antithrombotic and antiallergic activities.[20] As a result of these beneficial effects on human health, dietary supplements based on extracts from *Pueraria lobata* are available in many countries.[21]

Certain hydrolytic enzymes can kinetically control transglycosylation activity in the presence of an appropriate acceptor molecule.[22] Cyclodextrin glucanotransferase (CGTase), maltogenic amylase (MAase), and β-glucosidase are examples of such enzymes. CGTase can transfer

Abbreviations: CGTase, cyclodextrin glycosyltransferase; MAases, maltogenic amylases; ESI^+ TOF MS–MS, electrospray ionization time-of-flight tandem mass spectrometry; BSMA, *Bacillus stearothermophilus* maltogenic amylase

* Corresponding author. Tel.: +82 2 8804852; fax: +82 2 8735095; e-mail: parkkh@plaza.snu.ac.kr

0008-6215/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2004.09.017

the glycosyl unit to an acceptor molecule by forming an α-(1→4)- or α-(1→3)-glycosidic linkage.[23] 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. MAase conveys maltosyl or glycosyl residues to an acceptor molecule by primarily making α-(1→6)-glycosidic linkages.[24] This enzyme has a broad range of acceptor specificity, and not only transfers mono- or disaccharides but also many structurally diverse compounds, such as sugar alcohols, flavonoids,[25] and ascorbic acid.[26] Many biologically and industrially important molecules can be modified by the transglycosylation reaction, thereby changing their properties.[24–33]

This study used the glucosyl transfer activity of *Bacillus stearothermophilus* maltogenic amylase (BSMA) to synthesize new, highly soluble puerarin derivatives. The puerarin transglycosylation reaction with maltotriose and soluble starch was successful. The structures of the major transfer products of puerarin were determined using ESI^+ TOF MS–MS and ^{13}C NMR spectroscopy, and their solubilities in water were examined.

2. Results and discussion

2.1. Transglycosylation properties and transfer products of maltogenic amylase and other glycosyltransferases

Various enzymes with glucosyl-transferring activity, such as *B. stearothermophilus* maltogenic amylase (BSMA; EC 3.2.1.133), *T. maritima* maltosyl-transferase (TMMT; EC 3.2.1.25), *T. scotoductus* 4-α-glucanotransferase (TS4αGTase; EC 3.2.1.25), and *Bacillus* sp. I-5 cyclodextrin glucanotransferase (BSCGTase; EC 3.2.1.19), were tested for their acceptor specificity toward puerarin. In order to elucidate the transglycosylation procedure, maltotriose was initially used as the donor molecule. The reactions were performed with 1% (w/v) puerarin and 5% (w/v) maltotriose. The enzymes TMMT and TS4GTase produced few puerarin transfer products (Fig. 1a: 3 and 5), even though these enzymes hydrolyzed and transglycosylated or disproportionated maltotriose (Fig. 1b: 3 and 5), as in the BSMA and BSCGTase reactions (Fig. 1b: 2 and 4). This implies that TMMT and TS4αGTase do not have acceptor specificity toward puerarin, which lacks an *O*-glucoside linkage between D-glucose and 7-OH-daidzein. By contrast, BSMA and BSCGTase produced significant amounts of puerarin transfer products (Fig. 1a: 2 and 4). A recent study also found that BSMA is capable of transferring the acarviosine-glucose residue from an acarbose donor onto glucopyranosylidene-*spiro*-thiohydantoin, in which the C-1 is modified with thiohydantoin.[33] Based on the crystal structure of maltogenic amylase, we proposed that an extra sugar binding site involves the alignment of the N-terminal domain of one subunit and the $(\beta/\alpha)_8$ barrel of the other subunit.[34,35] The molecules occupying this space are thought to serve as acceptors that attack the glycosyl–enzyme intermediate. The docking model of ThMA, a maltogenic amylase from *Thermus* sp. with maltose in this extra space, revealed that the C4-OH of the maltose

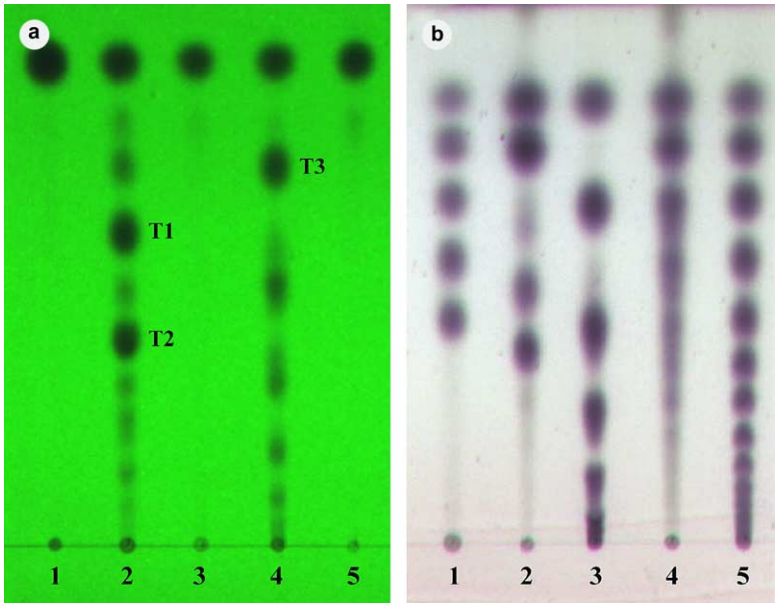

Figure 1. TLC analysis of the transglycosylation product involving puerarin and maltotriose catalyzed by four different glycosyltransferases. 1. Puerarin. G1 (glucose), G2 (maltose), G3 (maltotriose), G4 (maltotetraose), and G5 (maltopentaose) standards, 2. BSMA, 3. TMMT, 4. BSCGTase, and 5. TS4αGTase. Spots were visualized with a UV detector at 254 nm for isoflavones (a) and with a dipping solution at 110 °C for 10 min for carbohydrates (b). The detailed structures of the main spots are described in Figure 3.

at the nonreducing end is ~3.6 Å from Glu 357, one of the catalytic sites of maltogenic amylase. Consequently, the +1 subsite may not be involved in the transglycosylation of maltogenic amylase.

Compared with the major puerarin transfer product (T3) in the BSCGTase reaction, the major puerarin transfer products (T1 and T2) in the BSMA reaction should have greater water solubility based on their lower retardation factor (R_f) values on TLC. Thus, BSMA was determined to be the most efficient enzyme for the transglycosylation reaction with puerarin, and BSMA was used in the subsequent reactions studied.

TLC analysis revealed at least eight distinguishable spots, including two major spots, under the puerarin spot, implying that BSMA carried out an excellent transglycosylation reaction of puerarin (Fig. 1a: 2). BSMA preferentially transfers the maltose unit released by the hydrolyzing activity of the enzyme to an acceptor molecule by forming an α-(1→6)-glycosidic linkage between the donor and acceptor molecules.[36] Considering the broad range of transglycosylation products formed in the BSMA reaction, we postulate that both maltose and glucose generated from the donor molecule were transferred to an acceptor molecule by forming α-(1→3)- or α-(1→6)-glycosidic linkages. Two major products (T1 and T2) appeared in the middle of the TLC analysis with R_f values of 0.49 and 0.32, respectively.

The possible reaction mechanism of BSMA with puerarin and maltotriose is illustrated in Figure 2. BSMA attacks and hydrolyzes the donor, maltotriose, to form an intermediate complex between the hydrolyzed substrate (maltose or glucose) and the enzyme. Then, either water or an acceptor molecule (puerarin or maltooligosaccharide) attacks the intermediate complex immediately in the reaction mixture. Consequently, three BSMA reaction products are detected: hydrolysis products, maltooligosyl-transglycosylation products (Fig. 1b: 2), and puerarin transglycosylation products (Fig. 1a: 2). The major transfer products (T1 and T2 in Fig. 1a: 2) were purified for use in additional experiments to determine their structures.

2.2. Molecular weight of the main transfer products

The molecular weight of T1 was determined to be 578 Da in an ESI^+ TOF MS–MS analysis (Fig. 3a). The molecular ion peak appearing at *m/z* 579.51 corresponded to the molecular mass of the protonated ion of daidzein 8-*C*-maltoside (T1), in which a glucose molecule is attached to puerarin. The peak at *m/z* 417.36 matched T1 less a glucosyl unit, confirming that the

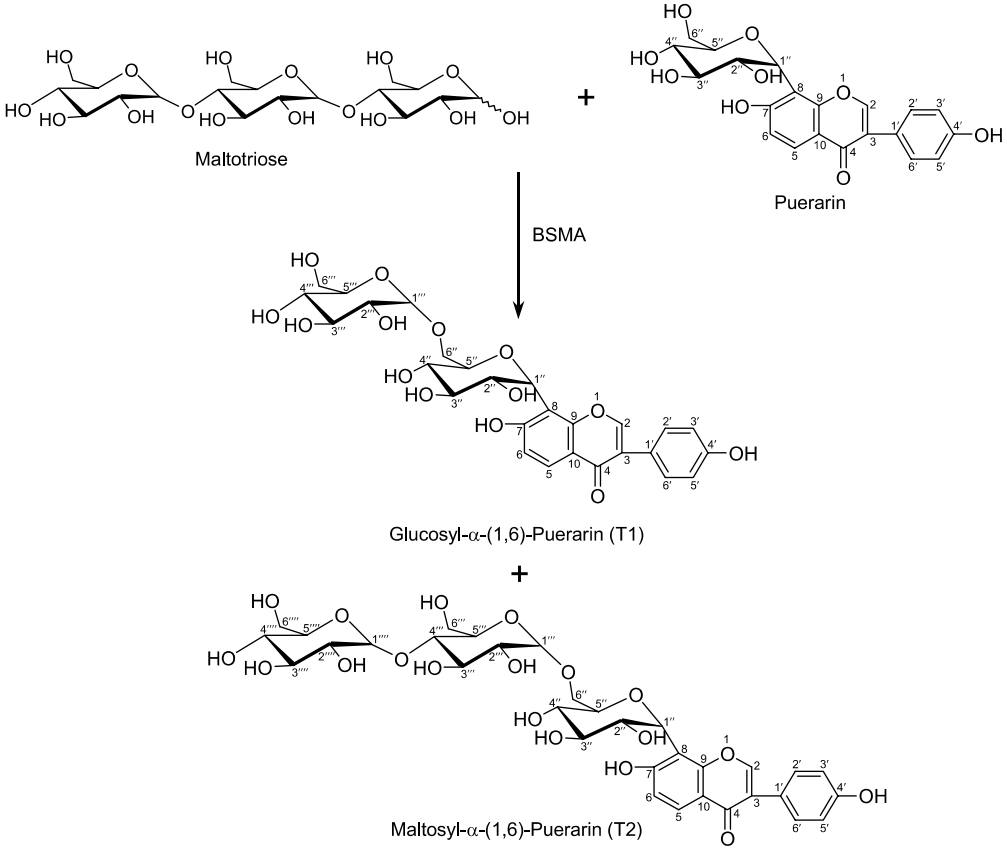

Figure 2. Proposed transglycosylation reaction mechanism for puerarin using *Bacillus stearothermophilus maltogenic amylase* (BSMA).

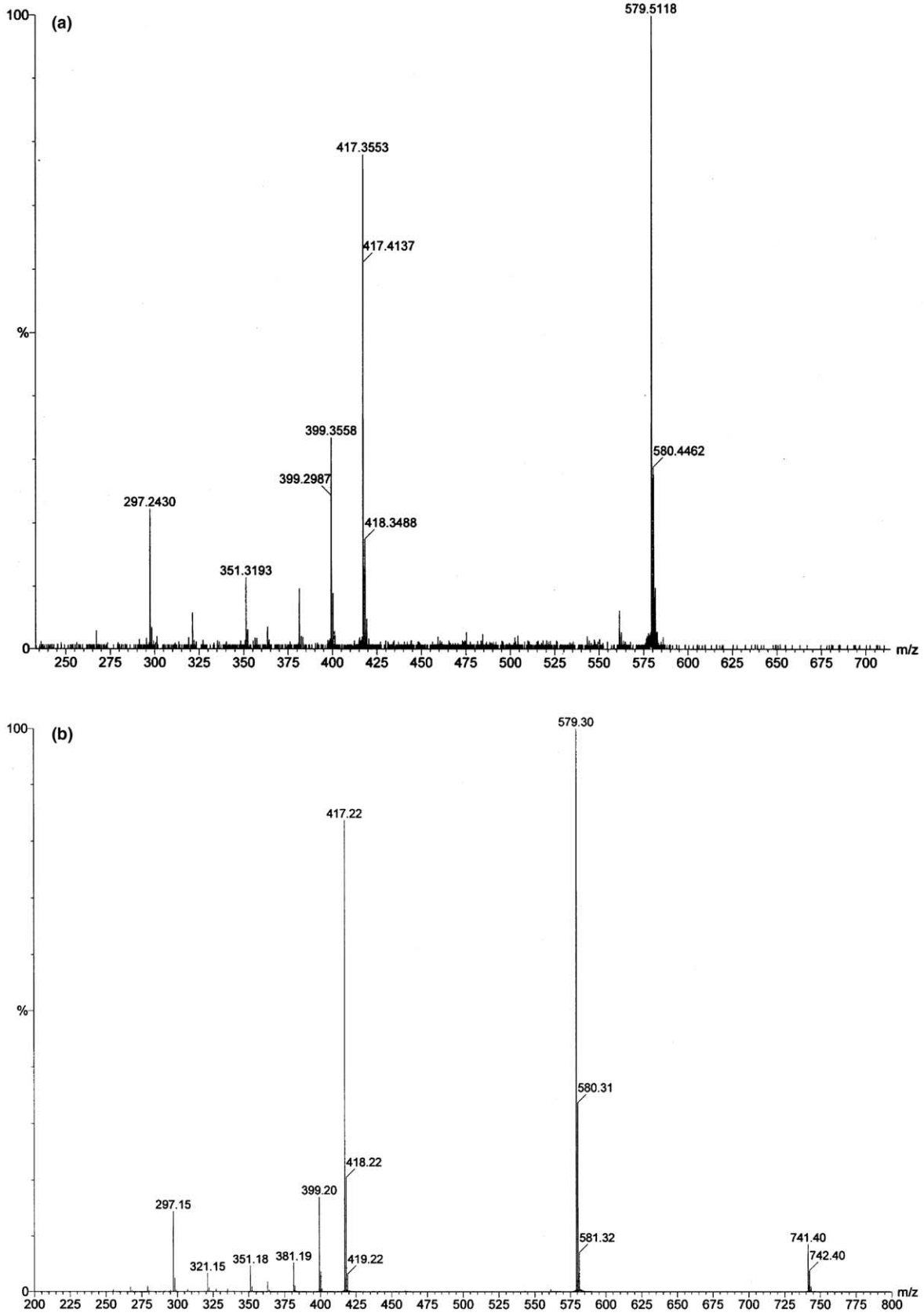

Figure 3. ESI$^+$ TOF MS–MS spectra of the major puerarin transfer products: (a) α-D-glucosyl-(1→6)-puerarin and (b) α-D-maltosyl-(1→6)-puerarin.

transfer product T1 was a glucosyl-puerarin derivative. The peaks at *m/z* 399.36, 381.19, 351.32, 321.15, and 297.24 are typical puerarin fragment ions.[21] The molecular weight of T2 was determined to be 740 Da from the molecular ion peak at *m/z* 741.40 (Fig. 3b). The other MS fragmentation peaks exactly matched those of T1. Combining these results, T2 was determined to be a maltosyl-puerarin derivative.

2.3. Molecular structures of the two main transfer products

The detailed molecular structures of the two main transfer products (T1 and T2) were determined using ^{13}C NMR spectroscopy. Chemical shifts in the ^{13}C NMR spectra of puerarin transfer product T1 were compared with those of puerarin, which were assigned previously.[37] As shown in Table 1, large downfield shifts (bold figures in Table 1) was observed at C-6″ in the glucose moiety of puerarin, from 61.4 to 66.2 ppm, implying that the transferred glucose unit was attached to C-6″ in the glucose moiety of puerarin. For transfer product T2, a distinct shift from 70.0 to 79.8 ppm occurred at C-4‴ in the second glucose moiety of transfer product T1, in addition to the chemical shift observed in transfer product T1. This led to the conclusion that T2 was formed by an α-(1→6)-glycosidic linkage between the transferred maltose and puerarin. Therefore, the molecular structures of the two major transglycosylation products of puerarin formed using BSMA (T1 and T2) were α-D-glucosyl-(1→6)-puerarin and α-maltosyl-(1→6)-puerarin, respectively.

2.4. Solubilities of the main transfer products

The solubilities of the transfer products in water were determined by comparing them with that of puerarin. The solubility of puerarin was found to be 12.5 mM, whereas the apparent solubilities of α-D-glucosyl-(1→6)-puerarin and α-maltosyl-(1→6)-puerarin in water were 0.18 and 2.09 M, respectively, or about 14 and 168 times that of natural puerarin. This suggests that the

Table 1. ^{13}C NMR data for puerarin and its transfer products (units: ppm)

Ring	Carbon atoms	Puerarin δ	α-D-Glucosyl-(1→6)-puerarin (δ1)	Difference (δ1−δ)	α-Maltosyl-(1→6)-puerarin (δ2)	Difference (δ2−δ1)
Daidzein	2	152.6	152.0	−0.6	152.2	0.2
	3	122.5	122.9	0.4	122.8	−0.1
	4	174.9	174.8	−0.1	174.8	0
	5	126.2	125.8	−0.4	126.0	0.2
	6	114.9	115.1	0.2	115.4	0.3
	7	161.2	163.8	2.6	163.3	−0.5
	8	112.6	112.0	−0.6	112.2	0.2
	9	157.1	157.2	0.1	156.6	−0.7
	10	114.9	117.0	2.1	115.6	−1.4
	1′	123.0	123.3	0.3	123.0	−0.3
	2′	130.0	130.2	0.2	130.0	−0.2
	3′	114.9	115.1	0.2	115.0	−0.1
	4′	157.1	157.2	0.1	157.1	−0.1
	5′	114.9	115.1	0.2	115.0	−0.1
	6′	130.0	130.2	0.2	130.0	−0.2
β-D-Glucosyl-(1→8)-	1″	73.4	73.4	0	73.8	0.4
	2″	70.7	70.9	0.2	70.8	−0.1
	3″	78.8	79.2	0.4	78.8	−0.4
	4″	70.5	70.2	−0.3	70.7	0.5
	5″	81.8	80.1	−1.7	80.0	−0.1
	6″	**61.4**	**66.2**	**4.8**	66.1	−0.1
α-D-Glucosyl-(1→6)-	1‴		98.7		98.4	−0.3
	2‴		72.3		71.6	−0.7
	3‴		74.3		73.0	−1.3
	4‴		**70.0**		**79.8**	**9.8**
	5‴		72.7		69.9	−2.8
	6‴		60.9		60.7	−0.2
α-D-Glucosyl-(1→4)-	1⁗				100.8	
	2⁗				73.3	
	3⁗				73.4	
	4⁗				69.7	
	5⁗				72.6	
	6⁗				60.1	

Table 2. Solubility of puerarin and its transfer products

Isoflavones	Solubility in water (mM)[a]	Relative solubility
Puerarin	12.45 ± 0.11	1
α-D-Glucosyl-(1→6)-puerarin	178.59 ± 2.48	14
α-Maltosyl-(1→6)-puerarin	2094.56 ± 21.77	168

[a] Mean ± standard deviation (n = 3).

attachment of a glucosyl or maltosyl residue to puerarin by BSMA greatly enhanced the water solubility of the original compound (Table 2).

As we showed, enzymatic synthesis using BSMA is highly suited for producing puerarin derivatives with greatly increased water solubility. Furthermore, the new α-(1→4)- and α-(1→6)-glycosidic linkages formed by transglycosylation were easily hydrolyzed by various glycosyl hydrolases, such as α-glucosidase and β-glucosidase (data not shown), implying that the puerarin transglycosylation derivatives are metabolized in the same way as puerarin itself in the human body. Therefore, the bioavailability of puerarin transglycosylation derivatives is not changed much, implying that puerarin transglycosylation derivatives can be used as alternatives to puerarin.

2.5. The transfer products of different donor molecules

BSMA exhibited substrate preference toward cyclodextrins, maltotriose, and starch, in decreasing order. Under the same reaction conditions, that is, 1% (w/v) puerarin and 1 U/mg of a donor at 55 °C for 45 min, the yields of puerarin transfer products were 66.6% using 5% (w/v) maltotriose and 40.9% using 0.5% (w/v) soluble starch. Given the low cost of soluble starch, the reaction conditions were optimized further (Fig. 4). Increasing the soluble starch concentration to 5.0% increased the yield of puerarin transfer products to over 70%. Reactions with more than 5.0% soluble starch were not used owing to the high viscosity of the reaction mixture. When the concentration of soluble starch was less than 1.0%, more α-maltosyl-(1→6)-puerarin than α-D-glucosyl-(1→6)-puerarin was produced. However, more α-D-glucosyl-(1→6)-puerarin than α-maltosyl-(1→6)-puerarin was produced at soluble starch concentrations greater than 1.0%.

3. Experimental

3.1. Chemicals

Standard puerarin and maltotriose were purchased from Sigma Chemical Co. (St. Louis, MO). Soluble starch was obtained from Showa Chemical Co., Ltd. (Tokyo, Japan). HPLC-grade MeOH was obtained from Fisher Scientific Co. (Pittsburgh, PA). Water was prepared using the Milli-Q plus Ultra-Pure Water System (Millipore, Billerica, MA). All other chemicals used were of reagent grade and were purchased from Showa Chemical Co., Ltd.

3.2. Enzyme preparation

The preparations of the recombinant plasmid DNA constructs of *B. stearothermophilus* maltogenic amylase (BSMA), *T. maritima* maltosyl-transferase (TMMT), *T. scotoductus* 4-α-glucanotransferase (TS4αGTase),

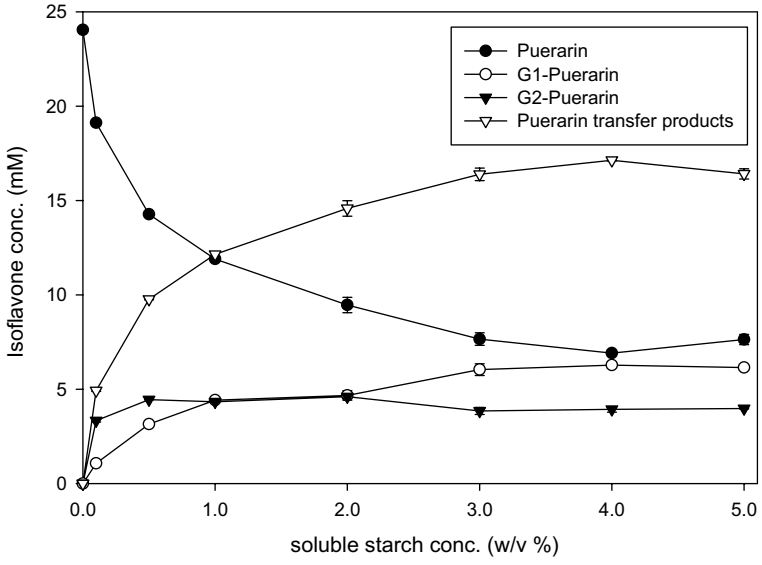

Figure 4. The yield of puerarin transfer products as a function of soluble starch concentration in the BSMA transglycosylation reaction.

and *Bacillus* sp. I-5 cyclodextrin glucanotransferase (BSCGTase) were reported previously.[38–41] *E. coli* MC1061 [*F*⁻, *araD139*, Δ*araABC-leu 7696*, *galE15*, *galK16*, Δ*lacX74*, *rpsL*, *thi*, *hsdR2*, *mcrA*, *mcrB1*] was used as the host for producing each enzyme. The recombinant *E. coli* was cultured in Luria–Bertani (LB) medium containing 1.0% Bactotryptone, 0.5% yeast extract, and 0.5% NaCl with 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 (VC-600 Sonicator, Sonics & Materials Inc., Danbury, CT; output 4, 5 min × 3 times, 60% duty). The crude cell extract was centrifuged (10,000*g*) at 4 °C for 15 min. One milliliter of 50% 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 into a Poly-Prep chromatography column (bed volume: 2 mL, reservoir volume: 10 mL) (Bio-Rad, Hercules, CA) and washed with 8 mL of wash buffer [50 mM Tris–HCl (pH 7.0), 300 mM NaCl, and 20 mM imidazole]. The recombinant enzyme was eluted with 2 mL of elution buffer [50 mM Tris–HCl (pH 7.0), 300 mM NaCl, and 250 mM imidazole]. Finally, the eluant was concentrated by ultrafiltration (Ultrafiltration Membrane, regenerated cellulose YM 10; Amicon 8010 UF kit, Millipore, Billerica, MA) at 4 °C and dialyzed against 50 mM Tris–HCl (pH 7.0) buffer.

3.3. Determination of enzyme activity

The activities of BSMA and BSCGTase were assayed using 1% β-cyclodextrin (β-CD) and soluble starch in 50 mM sodium-citrate buffer (pH 6.0) and sodium-acetate buffer (pH 6.0). Thirty microliters of enzyme was reacted with 150 μL of 1% β-CD and 120 μL of the reaction buffer at 55 °C for 10 min. The reaction was stopped by adding 900 μL of 3,5-dinitrosalicylic acid (DNS) solution and was colorized by heating at 100 °C for 5 min. The absorbance of the mixture was measured with a spectrophotometer (Ultrospec III, 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 one-unit change in the absorbance at 575 nm.

TMMT activity was determined using the method described by Meissner and Liebl.[38] After 200 μL of 2% (w/v) maltooligosaccharide mixture (Sigma) and 100 μL McIlvaine buffer, pH 6.5, (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, approximately 100 μL of 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 0.2% I_2/KI solution at ambient temperature; the absorbance at 480 nm was measured immediately. One unit of TMMT activity was defined as the amount of enzyme that increased the absorbance by 1.0 in 1 min.

TS4αGT activity was determined as described previously.[39] After 250 μL of 0.2% amylose in 90% Me_2SO, 50 μL of 1% maltose in 50 mM Tris–HCl (pH 7.5), and 600 μL 50 mM Tris–HCl (pH 7.5) were preincubated for 10 min at 70 °C, approximately 100 μL of 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.

3.4. Preparation of puerarin

Fresh *Puerariae radix* (RP) of *Pueraria lobata* (Wild.) Ohwi, obtained from South Korea, was washed and cut into small slices. The isoflavones including puerarin were extracted with two volumes of MeOH at room temperature for 72 h and then passed through filter paper (Whatman, Maidstone, UK). The crude extract was concentrated using a rotary vacuum evaporator (Rikakikai Co. Ltd., Tokyo, Japan) to remove the MeOH. In order to remove carbohydrates and hydrophilic contaminants, the concentrate was passed through a Sep-Pak C_{18} cartridge (Waters, Milford, MA) that had been previously activated using ethyl acetate, MeOH, and water. The isoflavones were eluted from the Sep-Pak using MeOH, filtered through a 0.45-μm membrane (GyroDisc Syr. CA-PC 30 mm, Orange Scientific, Belgium), and further purified using recycling preparative HPLC (LC-918, JAI Co. Ltd., Tokyo, Japan) equipped with a refractive index detector (RI-50) and a polymeric gel-filtration column (W-251). For HPLC, the mobile phase was 80% MeOH at 2 mL/min, and the injection volume was 3 mL. The fraction containing puerarin was collected and concentrated using a rotary vacuum evaporator. The purified puerarin was confirmed by ESI^+ TOF MS–MS and ^{13}C NMR. The sample was kept at 4 °C until used.

3.5. Transglycosylation reaction

The specificity of four enzymatic acceptors toward puerarin was tested using 1% (w/v) puerarin and 5% (w/v) maltotriose in the optimum buffer. The solution containing these substrates was preincubated at the optimum temperature for 10 min. Then, BSMA (50 U/mL), TMMT (12 U/mL), BSCGTase (2.6 U/mL), or TS4αGTase (0.5 U/mL) was added. The reaction was stopped by boiling for 10 min after a 45-min incubation and then

filtered through a 0.45-μm membrane (PVDF, Millipore, Billerica, MA) before performing the following purification steps.

Puerarin transglycosylation catalyzed by BSMA was optimized using 1% (w/v) puerarin and 1–5.0% (w/v) soluble starch in order to obtain a high yield of puerarin transfer products as calculated from the peak area ratio on the HPLC chromatogram of the reaction mix.

3.6. Purification of puerarin transglycosylation products

A Sep-Pak Plus C_{18} cartridge, previously activated with ethyl acetate, MeOH, and water, was used to absorb the puerarin glycosides in the transglycosylation solution and to remove the remaining maltooligosaccharide mixture and salt. The solution that eluted with MeOH was purified using a polymeric gel-filtration column (W-251) with recycling preparative HPLC to obtain the final transglycosylation products. The mobile phase was 50% (v/v) MeOH at 2 mL/min.

3.7. TLC analysis

The reaction products were spotted on Whatman K5F silica gel plates (Whatman, Kent, UK) activated at 110 °C for 30 min. As developing solutions, *n*-butanol–acetic acid–water (5:3:1; v/v/v) was used for 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 UV detector (CAMAG Reprostar 3, Muttenz, Switzerland) at 254 nm. The carbohydrates were visualized by dipping the TLC plate into MeOH 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.

3.8. ESI^+ TOF MS–MS analysis

The molecular weights of the transfer products were determined using a TOF mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ion source. Spectra were obtained in the positive-ion mode. Product ion spectra were obtained by selecting protonated or deprotonated ions for collision (energy = 32 eV) using argon as the collision gas.[21]

3.9. NMR analysis

^{13}C NMR spectra were recorded at 100.40 MHz with a JEOL JNM LA-400 MHz NMR spectrometer (Tokyo, Japan). Samples were dissolved in DMSO-d_6 at 24 °C with tetramethylsilane (Me_4Si) as the chemical shift reference.

3.10. Solubility determination

Excess puerarin or its glycosyl derivatives produced by transglycosylation were mixed with 200 μL of solvent in an Eppendorf tube at room temperature. An ultrasonic cleaner (5510R-DTH, Branson, Danbury, CT) was used to maximize the solubility of each component. After sonication at room temperature for 1 h, the sample was diluted and filtered through a 0.45-μm membrane (PVDF, Millipore, Billerica, MA) for HPLC analysis of the sample solution concentration.

3.11. HPLC analysis

A Waters 600E HPLC system connected with a Nova-Pak C_{18} column (150 × 3.9 mm i.d., Waters, Milford, MA) and a UV detector (SLC 200, Samsung, Seoul, Korea) at 250 nm was used to quantify the amounts of puerarin and puerarin derivatives.[40] The gradient mobile phases were composed of solvent A (water–formic acid, 100:0.1, v/v) and solvent B (MeOH–water–formic acid, 50:50:0.1, v/v/v). The gradient started at 95% solvent A and 5% of solvent B, and solvent B was increased gradually from 5% to 100% over 20 min. The flow rate was 1.2 mL/min. The concentrations of puerarin and its transfer products were calculated from puerarin standard curves.

Acknowledgements

This work was supported by the Biogreen 21 project of the Rural Development Administration. We are also grateful for the financial support provided by the Brain Korea 21 project through a fellowship to D.Li.

References

1. Harrington, T. B.; Rader-Dixon, L. T.; Taylor, J. W., Jr. *Weed Sci.* **2003**, *51*, 965–974.
2. Wang, X.; Wu, J.; Chiba, H.; Umegaki, K.; Yamada, K.; Ishimi, Y. *J. Bone Miner. Metab.* **2003**, *21*, 268–275.
3. Keung, W. M.; Vallee, B. L. *Phytochemistry* **1998**, *47*, 499–506.
4. Kim, H. Y.; Hong, J. H. *Food Sci. Biotechnol.* **2003**, *12*, 29–35.
5. Xie, R. Q.; Du, J.; Hao, Y. M. *Zhongguo Zhong Xi Yi Jie He Za Zhi* **2003**, *23*, 895–897.
6. Zhao, Z.; Yang, X.; Zhang, Y. *Zhongguo Zhong Xi Yi Jie He Za Zhi* **1998**, *18*, 282–284.
7. Fan, L. L.; Sun, L. H.; Li, J.; Yue, X. H.; Yu, H. X.; Wang, S. Y. *Chinese Med. J.* **1992**, *105*, 11–17.
8. Meng, P.; Zhou, D.; Hu, X. *Zhongguo Zhong Xi Yi Jie He Za Zhi* **1999**, *19*, 647–648.
9. Ren, P.; Hu, H.; Zhang, R. *Zhongguo Zhong Xi Yi Jie He Za Zhi* **2000**, *20*, 574–576.
10. Xuan, B.; Zhou, Y. H.; Yang, R. L.; Li, N.; Min, Z. D.; Chiou, G. C. *J. Ocul. Pharmacol. Ther.* **1999**, *15*, 207–216.

11. Liu, J. M.; Ma, L.; He, W. P. *Di Yi Jun Yi Da Xue Xue Bao* **2002**, *22*, 1044–1045.
12. Rezvani, A. H.; Overstreet, D. H.; Perfumi, M.; Massi, M. *Pharmacol. Biochem. Behav.* **2003**, *75*, 593–606.
13. Overstreet, D. H.; Keung, W. M.; Rezvani, A. H.; Massi, M.; Lee, D. Y. *Alcohol Clin. Exp. Res.* **2003**, *27*, 177–185.
14. Boue, S. M.; Wiese, T. E.; Nehls, S.; Burow, M. E.; Elliott, S.; Carter-Wientjes, C. H.; Shih, B. Y.; McLachlan, J. A.; Cleveland, T. E. *J. Agric. Food Chem.* **2003**, *51*, 2193–2199.
15. Jun, M.; Fu, H. Y.; Hong, J.; Wan, X.; Yang, C. S.; Ho, C. T. *J. Food Sci.* **2003**, *68*, 2117–2122.
16. Benlhabib, E.; Baker, J. I.; Keyler, D. E.; Singh, A. K. *J. Med. Food* **2002**, *5*, 109–123.
17. Cervellati, R.; Renzulli, C.; Guerra, M. C.; Speroni, E. *J. Agric. Food Chem.* **2002**, *50*, 7504–7509.
18. Hsu, F. L.; Liu, I. M.; Kuo, D. H.; Chen, W. C.; Su, H. C.; Cheng, J. T. *J. Nat. Prod.* **2003**, *66*, 788–792.
19. Chen, W. C.; Hayakawa, S.; Yamamoto, T.; Su, H. C.; Liu, I. M.; Cheng, J. T. *Planta Med.* **2004**, *70*, 113–116.
20. Choo, M. K.; Park, E. K.; Yoon, H. K.; Kim, D. H. *Biol. Pharm. Bull.* **2002**, *25*, 1328–1332.
21. Prasain, J. K.; Jones, K.; Kirk, M.; Wilson, L.; Smith-Johnson, M.; Weaver, C.; Barnes, S. *J. Agric. Food Chem.* **2003**, *51*, 4213–4218.
22. Rye, C. S.; Withers, S. G. *Curr. Opin. Chem. Biol.* **2000**, *4*, 573–580.
23. Kometani, T.; Nishimura, T.; Nakae, T.; Okada, S. *Biosci. Biotechnol. Biochem.* **1996**, *60*, 645–649.
24. Baek, J. S.; Kim, M. J.; Cha, H. J.; Lee, H. S.; Li, D.; Kim, J. W.; Kim, Y. R.; Moon, T. H.; Park, K. H. *Food Sci. Biotechnol.* **2003**, *12*, 639–643.
25. Lee, S. J.; Kim, J. C.; Kim, M. J.; Kitaoka, M.; Park, C. S.; Lee, S. Y.; Ra, M. J.; Moon, T. W.; Robyt, J. F.; Park, K. H. *J. Agric. Food Chem.* **1999**, *47*, 3669–3674.
26. Bae, H. K.; Lee, S. B.; Park, C. S.; Shim, J. H.; Lee, H. Y.; Kim, M. J.; Baek, J. S.; Roh, H. J.; Choi, J. H.; Choe, E. O.; Ahn, D. U.; Park, K. H. *J. Agric. Food Chem.* **2002**, *50*, 3309–3316.
27. Cho, J. S.; Yoo, S. S.; Cheong, T. K.; Kim, M. J.; Kim, Y.; Park, K. H. *J. Agric. Food Chem.* **2000**, *48*, 152–154.
28. Meulenbeld, G. H.; Hartmans, S. *Biotechnol. Bioeng.* **2000**, *70*, 363–369.
29. Kometani, T.; Terada, Y.; Nishimura, T.; Takii, H.; Okada, S. *Biosci. Biotechnol. Biochem.* **1994**, *58*, 1990–1994.
30. Fukunaga, Y.; Miyata, T.; Nakayasu, N.; Mizutani, K.; Kasai, S.; Tanaka, O. *Agric. Biol. Chem.* **1989**, *53*, 1603–1607.
31. Ohtani, K.; Aikawa, Y.; Ishikawa, R.; Kasai, R.; Kitahata, S.; Mizutani, S.; Doi, S.; Nakaura, M.; Tanaka, O. *Agric. Biol. Chem.* **1991**, *55*, 449–453.
32. Suzuki, Y.; Suzuki, K. *Agric. Biol. Chem.* **1991**, *55*, 181–187.
33. Kandra, L.; Remenyik, J.; Batta, G.; Somsák, L.; Gyémánt, G.; Park, K. H. *Org. Lett.*, in press.
34. Kim, T. J.; Park, C. S.; Cho, H. Y.; Cha, S. S.; Kim, J. S.; Lee, S. B.; Moon, T. W.; Kim, J. W.; Oh, B. H.; Park, K. H. *Biochemistry* **2000**, *39*, 6773–6780.
35. Kim, J. S.; Cha, S. S.; Kim, H. J.; Kim, T. J.; Ha, N. C.; Oh, S. T.; Cho, H. S.; Cho, M. J.; Kim, M. J.; Lee, H. S.; Kim, J. W.; Choi, K. Y.; Park, K. H.; Oh, B. H. *J. Biol. Chem.* **1999**, *274*, 26279–26286.
36. Cha, H. J.; Yoon, H. G.; Kim, Y. W.; Lee, H. S.; Kim, J. W.; Kweon, K. S.; Oh, B. H.; Park, K. H. *Eur. J. Biochem.* **1998**, *253*, 251–262.
37. Cheng, G.; Bai, Y. J.; Zhao, Y. Y.; Tao, J.; Liu, Y.; Tu, G. Z.; Ma, L. B.; Liao, N.; Xu, X. J. *Tetrahedron* **2000**, *56*, 8915–8920.
38. Meissner, H.; Liebl, W. *Eur. J. Biochem.* **1998**, *258*, 1050–1058.
39. Lee, H. S.; Auh, J. H.; Yoon, H. G.; Kim, M. J.; Park, J. H.; Hong, S. S.; Kang, M. H.; Kim, T. J.; Moon, T. W.; Kim, J. W.; Park, K. H. *J. Agric. Food Chem.* **2002**, *50*, 2812–2817.
40. Nobuyuki, O.; Hirotsugu, M.; Hiroshi, O.; Yasue, M.; Satie, Y.; Hiroshi, K.; Akira, Y. *J. Pharm. Biomed. Anal.* **1999**, *19*, 603–612.
41. Shim, J. H.; Kim, Y. W.; Kim, T. J.; Chae, H. Y.; Park, J. H.; Cha, H.; Kim, J. W.; Kim, Y. R.; Schaefer, T.; Spendler, T.; Moon, T. W.; Park, K. H. *Protein Eng. Des. Sel.* **2004**, *17*, 205–211.