Transglycosylation of Naringin by *Bacillus stearothermophilus* Maltogenic Amylase To Give Glycosylated Naringin

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Naringin, a bitter compound in citrus fruits, was transglycosylated by *Bacillus stearothermophilus* maltogenic amylase reaction with maltotriose to give a series of mono-, di-, and triglycosylnaringins. Glycosylation products of naringin were observed by TLC and HPLC. The major glycosylation product was purified by using a Sephadex LH-20 column. The sturcture was determined by using MALDI-TOF MS, methylation analysis, and ^{1}H and ^{13}C NMR. The major transglycosylation product was maltosylnaringin, in which the maltose unit was attached by an α-1→6 glycosidic linkage to the D-glucose moiety of naringin. This product was 250 times more soluble in water and 10 times less bitter than naringin.

Keywords: *Naringin; maltogenic amylase from Bacillus stearothermophilus; maltosylnaringin; transglycosylation*

INTRODUCTION

Two main bitter compounds found in citrus juices are naringin and limonin (Shaw et al., 1991). Naringin (4,5,7−trihydroxyflavanone-7-rhamnoglucoside), a flavonone glycoside, is one of the predominant bitter components present in grapefruit (Soares and Hotchkiss, 1998). The reduction of the bitter taste of citrus juices by the removal or modification of naringin is highly desired by the citrus fruit industries.

Various approaches have been applied to reduce the amount of naringin in some citrus products (Puri et al., 1996a,b; Tsen, 1984; Olson et al., 1979; Tsen et al., 1989). Immobilized naringinase has been used to debitter grapefruit juices that contain high amounts of naringin (Soares and Hotchkiss, 1998; Tsen, 1984; Olson et al., 1979; Tsen et al., 1989; Puri et al., 1996b; Tsen and Yu, 1991). Naringinase is an enzyme possessing α-rhamnosidase and *β*-glucosidase activities. It hydrolyzes naringin to yield a nonbitter compound, naringenin (Puri et al., 1996a). Removal of naringin from clarified juice by adsorption on a divinylbenzene polymer has also been reported (Manlan et al., 1990).

Glycosylation has been used to modify the physicochemical properties of raw materials in foods to improve their use (Suzuki and Suzuki, 1991; Fukunaga et al., 1989; Ohtani et al., 1991). Kometani et al. (1996) reported the tranglycosylation of various flavonoids, including neohesperidin and naringin, by their reaction with alkalophilic *Bacillus* species cyclodextrin glucanotransferase and cyclomaltodextrin. It was found that glucosylneohesperidin and glycosylnaringin had improved properties, such as less bitterness or a higher water solubility. We have been studying a new type of α-amylase from a *Bacillus* species, a maltogenic amylase. It has remarkably unique properties that distinguish it from other α-amylases (Cha et al., 1998). Maltogenic amylases can hydrolyze various substrates having α-(1,4) and/or α-(1,6)-glycosidic linkages as found in starch, pullulan, and cyclomaltodextrins with different specificities. In addition to the hydrolytic activity, this enzyme also exhibits a high degree of transglycosylation activity. In the presence of various sugar molecules (acceptors) such as D-glucose, maltose, cellobiose, and sugar alcohols, maltogenic amylases transfer mono- or disaccharides to acceptor molecules by forming α-(1,3-), α-(1,4), and α-(1,6)-glycosidic linkages (Cha et al., 1998; Park et al., 1998).

In the present paper, we have studied the transglycosylation activity of *Bacillus stearothermophilus* maltogenic amylase (BSMA) to obtain the modification of naringin of the solubility in water. Transglycosylated naringin was purified and analyzed by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS), and methylation analysis. The structure of the major transglycosylation product was determined to be 6^{G}-α-maltosyl naringin. Its water solubility and degree of bitterness were examined.

EXPERIMENTAL PROCEDURES

Enzyme. BSMA was obtained from recombinant *Escherichia coli* DH5α [*supE44, ΔlacU69(ϕ80lacZΔM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1*], which harbors plasmid pSG12 (Cha et al., 1998). This plasmid was constructed by inserting the BSMA gene into the *Hin*dIII site of pUC18. Cultivation of the recombinant *E. coli* and purification of BSMA were previously reported (Cha et al., 1998). The hydrolysis activity of BSMA was assayed with 1% *β*-cyclodextrin (substrate) in 50 mM sodium citrate buffer (pH 6.0) at 55

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°C, using 3,5-dinitrosalicylic acid (DNS), according to the method described by Miller (1959). One unit of enzyme activity (CU) was defined as the amount of enzyme increasing 1 absorbance unit at 575 nm when the reaction was carried out at 55 °C for 30 min.

BSMA Transglycosylation Reaction with Naringin. Naringin solution (2.5%, w/v) was prepared by dissolution of naringin in 50 mM sodium acetate buffer (pH 6.0) preequilibrated at 90 °C and immediately brought to the reaction temperature of 55 °C.

The transglycosylation reaction mixture (20 mL) contained 2.5% (w/v) naringin as an acceptor, 2.5% (w/v) maltotriose as a donor, and 10000 CU of BSMA in 50 mM sodium citrate buffer (pH 6.0). After incubation at 55 °C for 20 h, the reaction mixture was boiled for 5 min to stop the reaction, and insoluble naringin was removed by centrifugation at 6000*g* for 5 min.

Detection of Transglycosylation Products by TLC. TLC analysis was performed with Whatman K5F TLC plates using the lower layer of chloroform/ methanol/water (65:35:10 v/v/v). After irrigation, the plate was thoroughly dried and the compounds visualized by dipping the plate into 0.3% (w/v) *N*-(1-naphthyl)ethylenediamine/5% (v/v) H_2SO_4 in methanol, followed by heating at 120 °C for 10 min or by a UV detector at 254 nm (CAMAG reprostar 3, Muttenz, Switzerland).

Detection of Reaction Products by HPLC. HPLC analysis of naringin and transglycosylated naringin was carried out with a Nova-Pak C_{18} reverse phase analytical column (3.9 mm i.d. × 150 mm), using an isocratic solvent system at 1 mL/min methanol/0.01 M phosphoric acid (1:2 v/v) as a mobile phase with detection by UV at 283 nm (SLC 200, Samsung, Korea).

Purification and Quantification of Transfer Product. The transglycosylation reaction mixture was concentrated 10-fold by freeze-drying. The concentrate was loaded onto a Sephadex LH-20 column and eluted with 50% ethanol at a flow rate of 1 mL/min at 20 °C. After the fractions containing transfer products had been determined by TLC analysis, those fractions were collected and lyophilized. The amount of transfer products was measured by UV spectrophotometry (Ultraspec III, Pharmacia LKB, Uppsala, Sweden) at 283 nm. Naringin was used as a standard to calculate the approximate amount of the transfer products.

MALDI-TOF Analysis. A Voyager-DE (PerSeptive Biosystem, Framingham,, MA) system with α-cyano-4-hydroxycinnamic acid (α-CHCA) as matrix and des-Arg^1-bradykinin for calibration was used to measure the molecular weight of the glycosylated naringin.

Methylation Analysis. Freeze-dried transfer product (10 mg) was dissolved in dimethyl sulfoxide (0.6 mL) by shaking until a clear solution was obtained. Hakomori's reagent (0.4 mL) was added to the solution, and the reaction mixture was left overnight at 20 °C. Methyl iodide (0.2 mL) was added, and reaction was allowed to take place for 2 h when the color of the reaction mixture had changed to brown. The mixture was extracted three times with 2 mL of chloroform. The chloroform extracts were combined and dried as previously described (Mukerjea et al., 1996). One milliliter of 4 M trifluoroacetic acid was added to the sample in an ampule, which was sealed and autoclaved for 2 h at 120 °C. The sample was completely dried using a SpeedVac (ISS100, Savant Instruments) at 45 °C for 4−6 h, and then 1 mL of methanol was added. To identify the methylated compounds, appropriate aliquots (1−3 μL) of the solution were spotted onto a Whatman K6F TLC plate, irrigated with two ascents of acetonitrile/chloroform/methanol (3:9:1 v/v/v). The methylated compounds were detected on the plate by dipping as previously described (Mukerjea et al., 1996).

^{1}H and ^{13}C NMR Analysis. ^{1}H and ^{13}C NMR spectra were obtained with a JEOL LA 400 MHz NMR instrument on DMSO-d_6 at 26.3 °C.

Water Solubility. Purified glycosylated naringin transfer product (90 mg) was dissolved in distilled water (150 μL) at 25 °C and centrifuged at 9000*g* to remove insoluble material. The amounts of naringin and transfer product were determined by UV spectrophotometry (Ultraspec III, Pharmacia LKB) at 283 nm.

Taste Evaluation. Threshold values of naringin and glycosylated naringin transfer product were estimated using the methods of Patton and Josephson (1957). A series of samples (from 0.01 to 5 mM) were prepared by dissolving the compounds in distilled water at room temperature, and six individuals on a taste panel determined the taste of the samples. After evaluation of the bitterness of each sample, the panelists indicated their judgments as positive or negative with regard to the presence or absence of the bitterness of the compounds. Data were plotted, and the average threshold value of each compound was determined as the 50% level of positive responses. The results were collected and analyzed by using ANOVA and the *t* test in SigmaStat for Windows 1.0 (Jandel Corp., San Rafael, CA, 1993).

RESULTS AND DISCUSSION

Glycosylation of Naringin by the Action of BSMA. BSMA belongs to the maltogenic amylase family, a new type of amylolytic enzyme primarily elaborated by *Bacillus* species (Cha et al., 1998; Park et al., 1998). BSMA has been reported to have two catalytic activities, hydrolysis and transglycosylation. This enzyme is not only able to hydrolyze various substrates such as cyclodextrins, starch, and pullulan to produce maltose, glucose, and/or panose, but it is also able to transfer mono- or disaccharides from a donor to numerous acceptors, including D-glucose, maltose, cellobiose, and sugar alcohols (Park et al., 1998). Because glycosylation is one of the well-known processes used to improve properties of food components (Suzuki and Suzuki, 1991; Fukunaga et al., 1989; Ohtani et al., 1991), the transglycosylation activity of BSMA was employed in this study to modify naringin, a bitter compound present in various citrus juices.

As shown in Figure 1, several new compounds (Figure 1, a−d in lane 1) appeared in the lane of reaction of the chromatographed reaction mixture. To verify whether these new compounds had originated from the transglycosylation reaction of BSMA to naringin, each compound was purified using a Sephadex LH-20 gel column. Purified compounds were analyzed using MALDI-TOF MS to determine the molecular masses. The molecular masses of the transfer products corresponded to mono-, di-, and triglucose saccharides of naringin (data not shown), indicating that the new spots were naringin transglycosylation products. Park et al. (1998) studied the transfer products obtained by the transglycosylation reactions of BSMA with acarbose as a donor and various carbohydrate acceptors. They observed that BSMA cleaved the first glycosidic bond of acarbose to produce glucose and a pseudotrisaccharide (PTS), which was transferred to various carbohydrate acceptors forming α-(1,3), α-(1,4), α-(1,5), and α-(1,6)-glycosidic linkages, depending on the structures of the acceptor molecules. These results implied that BSMA transglycosylation products of naringin were most probably naringin mono-, di-, and triglucosides in which glucose, maltose, and maltotriose molecules were attached to naringin by the transglycosylation. The molecular structure of the major transfer product was determined in this study (see below).

Structure of the Major BSMA Naringin Transglycosylation Product. To determine the structure of the major naringin transglycosylation product (compound **c** in Figure 1), the reaction mixture was loaded onto a Sephadex LH-20 column that was eluted with

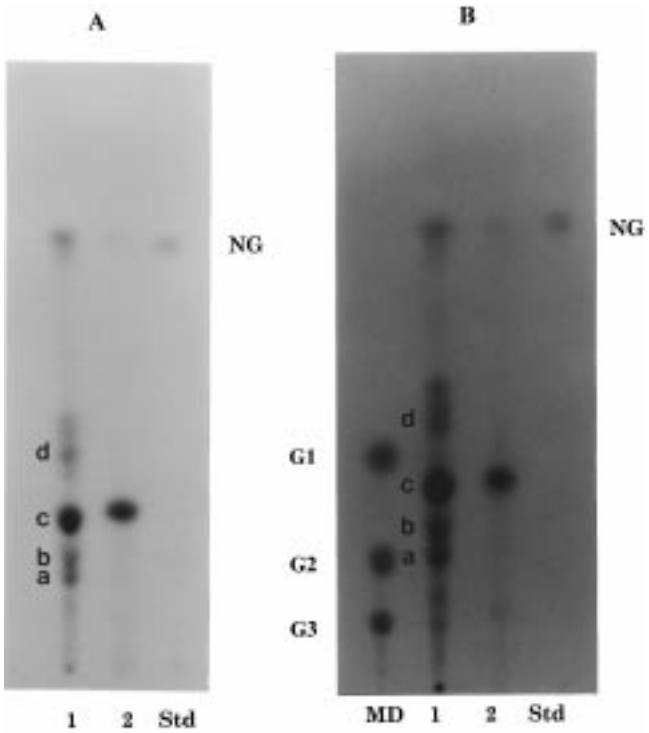

Figure 1. TLC analysis of naringin transglycosylation products: (A) UV detection at 254 nm; (B) detection by the sulfuric acid dipping reagent. Lane 1, naringin transglycosylation products after gel chromatography; lane 2, purified 6^G-α-maltosylnaringin (6-G2-naringin), a major transglycosylation product. Maltodextrins (MD) of G1−G3 and naringin (NG) were used as standards. Molecular weights determined by MALDI-TOF MS suggested the minor transglycosylation products, a, b, and d, were naringin tri-, di-, and monoglucosides, respectively.

50% ethanol. The eluents were analyzed by TLC, and the fractions containing the major naringin transglycosylation product were combined and lyophilized for further study. TLC and HPLC analyses showed that the purified compound was homogeneous (Figures 1 and 2).

To examine the molecular mass of the purified compound, MALDI-TOF MS analysis was performed (Figure 3). Two peaks appeared at *m/z* 927 ($[M + Na]^+$) and *m/z* 943 ($[M + K]^+$), which corresponded to the calculated molecular masses of sodium and potassium adducts of diglucosyl naringin (904 Da), respectively. This result indicated that the major transglycosylation product of naringin by BSMA was maltosylnaringin (G2-naringin). Methylation analysis was used to determine the position of the linkage between the maltosyl unit and naringin (Figure 4). The analysis of G2-naringin gave 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-glucose, and 3,4-di-*O*-methyl-D-glucose, indicating that the maltose was attached at C-6 of the glucose moiety of naringin by α-(1,6) glycosidic linkage.

^1H and ^{13}C NMR analyses confirmed that the glycosidic linkage between the maltosyl unit and naringin was α-(1,6). Chemical shifts of G2-naringin were compared with those of naringin in the ^{13}C NMR spectrum. As shown in Table 1, there were an additional six carbon signals that presumably resulted from the transfer of maltose to naringin. In addition, there was a large chemical shift from 60.44 to 66.07 ppm of C-6 in the D-glucose carbons of naringin, confirming that the transferred maltosyl group was connected to C-6 of the D-glucose moiety of naringin. From these results, the

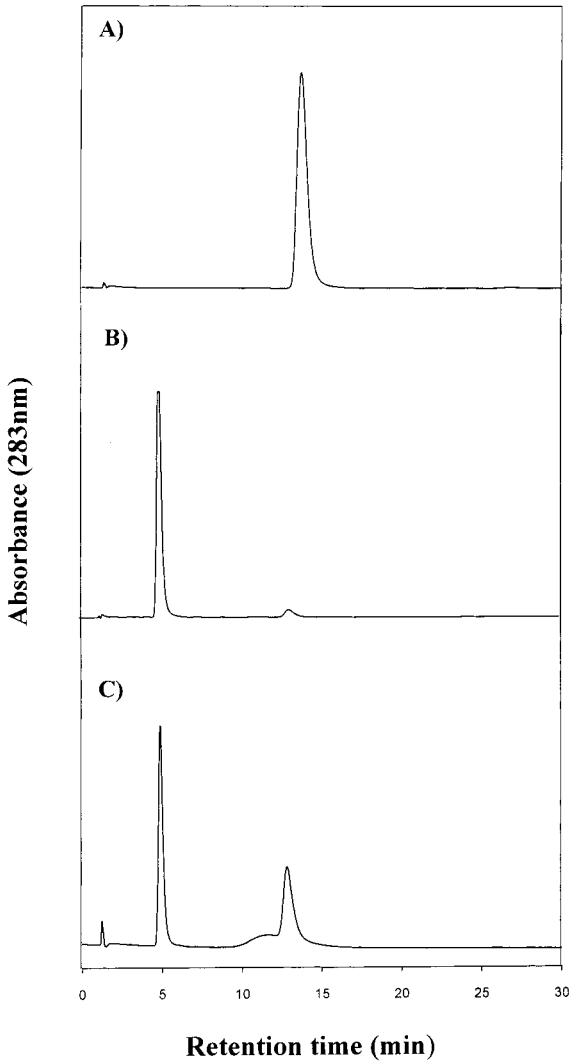

Figure 2. HPLC analysis of naringin and 6^G-α-maltosylnaringin (6-G2-naringin): (A) naringin; (B) purified 6-G2-naringin; (C) mixture of naringin and 6-G2-naringin. A Nova-Pak C_{18} analytical column was used for analysis by eluting with MeOH/0.01 M phosphoric acid (1:2 v/v) at 1 mL/min flow rate. The eluent was monitored with a UV detector at 283 nm.

major transglycosylation product was defined as 6^G-α-maltosylnaringin (6-G2-naringin).

Kometani et al. (1994, 1996) have used cyclodextrin glucanotransferase (CGTase) from alkalophilic *Bacillus* sp. to modify hesperidin, neohesperidin, and naringin to glycoside derivatives. The resulting transglycosylation products were monoglucosyl derivatives in all cases. However, although the structure of the monoglucosylhesperidin was identified as 4^G-α-D-glucopyranosylhesperidin in which transferred D-glucose was attached to C-4 of the glucose moiety of hesperidin by an α-(1,4) linkage, the structures of monoglucosylneohesperidin and monoglucosylnaringin were determined to be 3^G-α-D-glucopyranosylneohesperidin and 3^G-α-D-glucopyranosylnaringin in which the transferred glucose was connected to C-3 of the glucose moieties of neohesperidin and naringin by α-(1,3) linkage. In contrast, we found that the BSMA transglycosylation product of naringin was α-maltosylnaringin, in which the maltose unit was attached to the glucose moiety of naringin at C-6 rather than at C-4 or C-3 as found for the CGTase reaction. The BSMA transglycosylation reaction of naringin is shown in Figure 5.

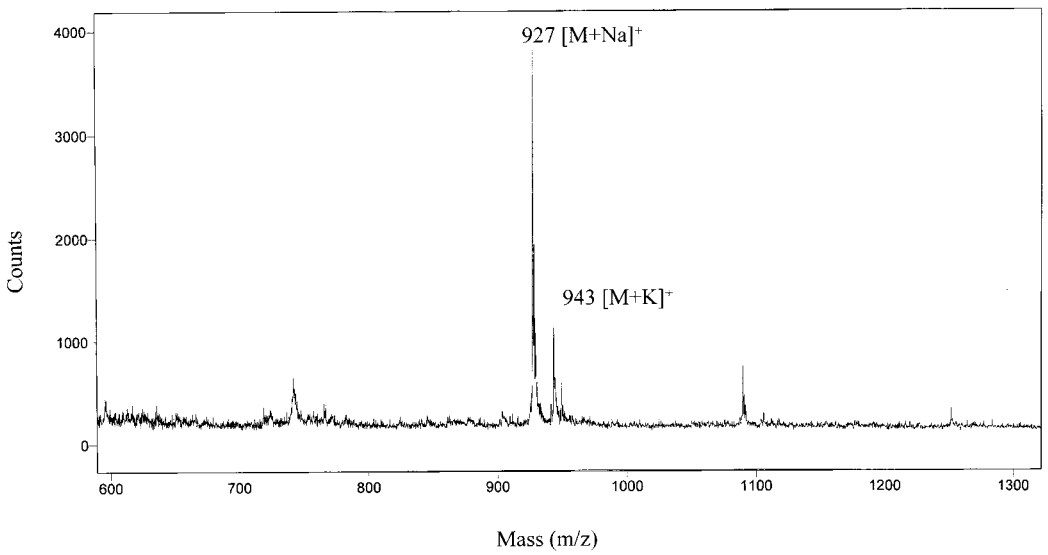

Figure 3. MALDI-TOF MS analysis for determining the molecular mass of purified G2-naringin.

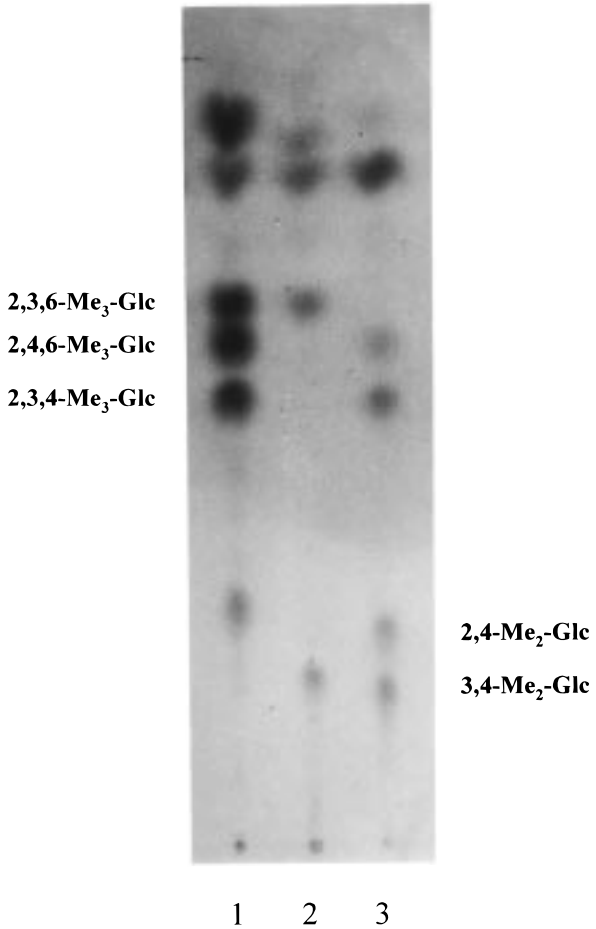

Figure 4. TLC methylation analysis of the major naringin transfer product (6-G2-naringin). Lane 1, standards of *O*-methyl-D-glucoses; lane 2, methylated naringin transfer product; lane 3, standards of di-*O*-methyl-D-glucoses.

Table 1. ^{13}C NMR Spectra of Naringin and 6-G2-Naringin

	naringin	6-G2-naringin	difference
trihydroxyflaonone			
2	78.8	78.7	−0.1
3	42.0	42.06	+0.6
4	197.1		
5			
6	96.2	96.0	−0.2
7	164.7	164.53	−0.17
8	95.11	94.83	−0.28
9	162.742	162.74	−0.002
10	103.3	103.4	+0.1
1′		129.6	
2′, 6′	128.4	128.45	+0.05
3′, 5′	115.2	115.17	−0.03
4′	157.8	157.78	−0.02
glucose of naringin			
1	100.37	100.4	+0.03
2	78.616	78.49	−0.126
3	77.1	77.14	+0.04
4	69.58	71.59	+2.01
5	76.85		
***6**	**60.44**	**66.07**	**+5.63**
rhamnose of naringin			
1	97.29	97.25	−0.10
2	70.37	70.339	−0.03
3	70.46	70.43	−0.03
4	71.82	70.72	−1.1
5	68.27	68.27	0
6	18.02	180.3	+0.1
glucose 1			
4		72.53	
6		60.228	
glucose 2			
4		69.53	
6		60.728	

Properties of Naringin. The properties of 6^G-α-maltosylnaringin (6-G2-naringin) were studied by comparing the bitterness and the water solubility of 6-G2-naringin with those of naringin (Table 2). Eighty milligrams of purified 6-G2-naringin and 80 mg of naringin were dissolved in 150 μL of water at 25 °C to determine their water solubilities. The apparent solubility of 6-G2-naringin was 272.8 mg/mL in water (301.59 mM). The solubility of naringin was determined to be 0.69 mg/mL (1.19 mM). The water solubility of 6-G2-naringin was ~250 times greater than that of naringin.

The bitterness of 6-G2-naringin was compared with that of naringin by determining threshold values of the two compounds using the method of Patton and Josephson (1957). The mean threshold value of naringin was 0.07 mM, but that of 6-G2-naringin was 0.71 mM, indicating that the transglycosylated compound, 6-G2-naringin, was 10 times less bitter than naringin. These results clearly indicated that transglycosylation by BSMA could be used to significantly reduce the bitterness of citrus juices.

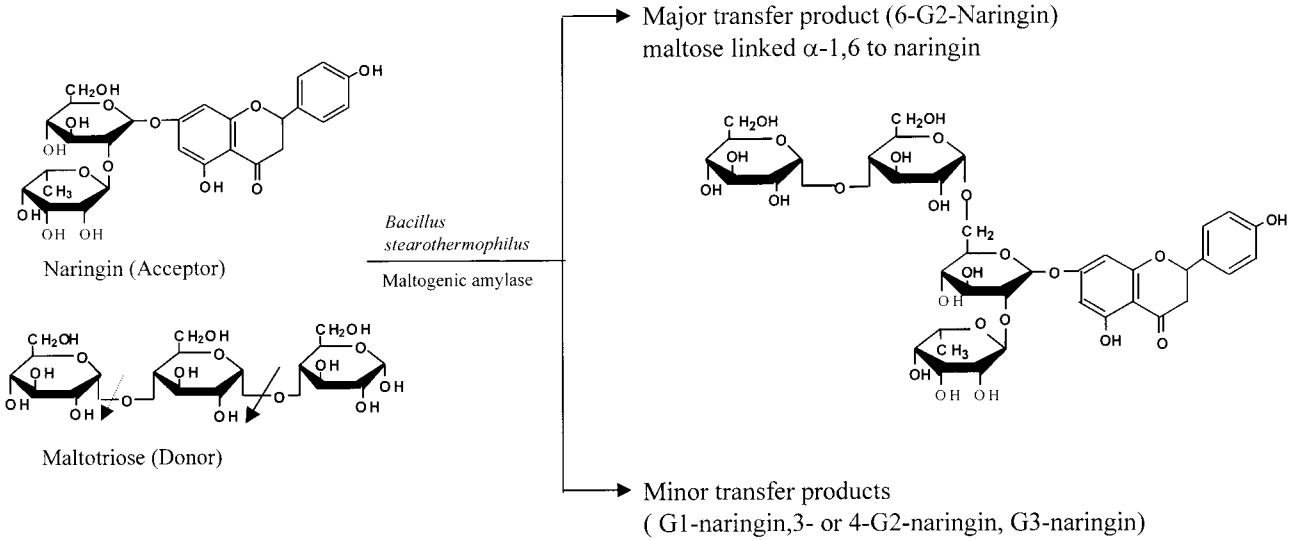

Figure 5. Transglycosylation of reaction of naringin by *B. stearothermophilus* maltogenic amylase.

Table 2. Solubility in Water and Threshold Value of Naringin and 6-G2-Naringin

compound	water solubility (mM, 25 °C)	relative solubility	threshold value (mM)	relative bitterness
naringin	1.12	1	0.0763	100
6-G2-narinigin	301.6	269.3	0.7102	10.75

It has been reported that the solubility of 3^G-α-D-glucopyranosylnaringin produced by CGTase was 1000 times higher than that of naringin, but the bitterness of the glucosyl compound was not altered (Kometani et al., 1996). Interestingly, however, 6-G2-naringin, obtained by BSMA transglycosylation reaction, had 250 times higher water solubility and $^1/_{10}$ the bitterness of naringin. It would be interesting to compare the water solubilities and degrees of bitterness of different transfer products of naringin in which D-glucose, maltose, and other sugars are added at different positions.

The coupled transglycosylation and hydrolysis activities of maltogenic α-amylases, including BSMA, have been used for the production of branched oligosaccharides from liquefied starch in more efficient ways than the traditional process (Kim et al., 1997). Branched oligosaccharide mixtures are used in the food industry as substitutes for sucrose and other saccharides because of their lower viscosity, lower sweet taste, and lower freezing point depression. In addition to these properties, this paper has shown that BSMA can be used to modify food components by transferring various glucosyl residues to many food compounds, thereby improving their properties and increasing their applications in the food industry.

The bitter compounds such as neohesperidin and naringin from grapefruits can be converted into sweet dihydrochalcone derivatives by hydrogenation. As their solubility in water is very low, dihydrochalcone can be glycosylated using BSMA to increase by 718 times the solubility in water for applications in the food industry (Cho et al., Supporting Information). Generally, a procedure for the preparation of an α-glycosylnaringin with α-(1,4)-glycosidic linkage has been known by which a saccharide-transferring enzyme, for example, α-glucosidase or cyclomaltodextrin glucanotransferase, is allowed to act on a solution at alkaline pH conditions (Kometani et al., 1996; Yoneyama and Miyake, 1991). As BSMA exhibited an optimum pH of 6−8, the enzyme cannot be applied to the process of debittering grapefruit juice, which has a pH <6.0. The above limitations warranted the search for biotechnological approaches in the modification of the enzyme using immobilization or enzyme engineering. Enzymatic transglycosylation of naringin can be applied to the production of bioflavonoids supplements for foods and beverages (Yoneyama and Miyake, 1991). For complete modification and degradation of bitter components research should be further focused on developing either enzymes acting at acidic pH or microbes or immobilized enzymes capable of metabolizing naringin.

Supporting Information Available: Unpublished data of J. S. Cho, M. J. Kim, T. W. Moon, and K. H. Park. This material is available free of charge via the Internet at http://pubs.acs.org.

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