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Master's Thesis of Science in Agriculture

Synthesis and Characterization of Novel Stevioside Glucoside Using Dextransucrase from *Leuconostoc mesenteroides*

덱스트란수크라아제를 이용하여 합성한 신규 스테비오사이드 배당체의 특성 연구

February 2018

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Abstract

Stevioside (Ste) is approximately 143-fold as sweet as sucrose at a concentration of 0.025%, which is why Ste has been used as a low-caloric natural sweetener. However, its bitter aftertaste minimizes its use for human consumption and limits its application in other food or pharmaceutical products. In this study, novel Stevioside Glucoside (novel Ste-Gs) using dextransucrase from *Leuconostoc mesenteroides*. Using the response surface methodology (RSM), the conversion yields were evaluated and reached to 85%. Each purified Ste-Gs was obtained by using high-performance liquid chromatography (HPLC) with amino column. The structures of Ste-Gs and novel Ste-Gs were elucidated by nuclear magnetic resonance (NMR) to be 13-*O*-β-sophorosyl-19-*O*-β-isomaltosyl-steviol and 13-*O*-[β-D-glucosyl (1 → 2)-β-nigerosyl]-19-*O*-β-isomaltosyl-steviol, respectively. Improved intensities of sweetness were observed for Ste-Gs and novel Ste-Gs, which are respectively 1.10 (158) and 1.41 (201) times sweeter than Ste. The quality of those two compounds was also improved compared to that of Ste. The biochemical properties of novel Ste-Gs were also characterized. The stability of Ste, Ste-Gs and novel Ste-Gs under pH 2, at 60 °C after 48 h were evaluated as 75.47±0.17%, 84.13±0.03%, and 85.94±1.10%, respectively. The solubilization ability of curcuminoids with Ste, Ste-Gs and novel Ste-Gs was from 8.07 ± 0.12 to 9.40 ± 0.12 mg per 10 g and that of idebenone with Ste, Ste-Gs and novel Ste-Gs was decreased from 9.94. ± 0.04 to 4.40 ± 0.38 mg per 10 mg.

Key words: Alternative sweetener, Dextransucrase, Stevioside, Transglycosylation
Student number: 2016-20008

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Introduction

1. Steviol glycosides and stevioside

The interest in natural sweeteners have been increased due to the awareness of sugar consumption and concern over the safety of some non-nutritive artificial sweeteners. Steviol glycosides are *ent*-kaurene type diterpenoid glycosides extracted from leaves of *Stevia rebaudiana* Bertoni. They were the first approved natural sweeteners for consumption in the United States, European Union, Australia, and New Zealand [1]. In *S. rebaudiana*, there are more than 30 types of steviol glycosides are accumulated at various concentrations [2]. The *S. rebaudiana* plant is the origin of rebaudioside C–E, rubusoside, dulcoside A (1–2% in total), and stevioside (Ste) [3]. Steviol glucosides have been received great attention as low cost natural sweeteners with its inherent benefits of intense sweetness, low calorie, high stability, and restorative properties such as anti-diarrheal, anti-diuretic, anti-hyperglycemic, anti-hypertensive, anti-inflammatory, anti-tumor, and immunomodulatory effects [4-6].

Ste has been used as low-carloric natural sweetener of relative sweetness of 143 compared to sucrose at concentration of 0.025%.

Japan alone invests 40% in the international sweetener market. Total market value of Stevia sweetener in Japan has been estimated to be approximately 25-35 million US dollars per year [7]. In Japan, commercial stevioside has been produced primarily for seasoning, pickled and salted foods since 1970s [8].

Ste also has been known for its ability to enhance solubility of numbers of principle compounds in pharmaceutical and medical industries with poor water solubility [9, 10]. Bioavailability is the primary challenge for designing oral medication for any drug, which depends on some factors including aqueous solubility, drug permeability, dissolution rate, first pass metabolism, pre-system metabolism, and susceptibility to efflux mechanism. The most frequent cause of low oral bioavailability includes poor solubility and low permeability [11].

2. Glycosylation of stevioside

The bitter aftertaste of Ste has been limited its wide application in food, beverage and pharmaceutical products. Glycosylation at the 13-hydroxyl or the 19-carboxyl group of stevioside effectively improves its taste quality, hence the glycosylation has been considered as one solution for improving the taste or obtaining new products with new functions[12]. Many modification or biotransformation have been performed using various enzymes including alternansucrase, dextrin-dextranase, β-fructofuranosidase, β-glucosidase and CGTase [12-15] to overcome the bitterness of stevioside. Only CGTase is commercialized to produce stevioside derivatives due to its high production yield. Randomized excess glucosylation reactions using CGTase make high-calorie stevioside derivatives which are unfavorable in this health-concerning society. This calls for optimized process with new enzyme for simple and efficient production of stevioside derivatives.

3. *Leuconostoc mesenteroides*

The synthesis of various dextrans and dextran-like polymers from sucrose is catalyzed with extracellular glucosyltransferases (GTFs) produced from different strains of *Leuconostoc mesenteroides* [5]. The catalysis works by hydrolyzing sucrose and adding the glucosyl group to glucose or nascent glucan chain with by-product of fructose. *L. mesenteroides* NRRL B-512F produces a dextransucrase that is used for production of commercial dextran, which has 95% α (1->6) linkages in the main chains and 5% α (1->3) branch linkages.

Isolating and sequencing the dextransucrase producing gene of *L. mesenteroides* NRRL B-512F (Wilke-Douglas *et al*., 1989) and identifying the amino acid residues at the catalytic site, Asp-511 and Asp-551 (Monchois *et al*., 1997) were conducted [16-17]. A high homology with other glucosyltransferase was observed from the *L. mesenteroides* NRRL B-512F dextranusucrase. A mutant of *L. mesenteroides* B-512FMC, which is hyper-producing and constitutive for dextransucrase, was developed as the first constitutive dextransucrase mutant of *L. mesenteroides* (Kim *et al.*, 1994) [18]. *L. mesenteroides* B- 512FMCM, the constitutive photon irradiated mutant, produced 13 times higher activity and 1,000 times more dextransucrase protein than those of its parent strain, *L. mesenteroides* B- 512FMC [19].

4. Dextransucrase

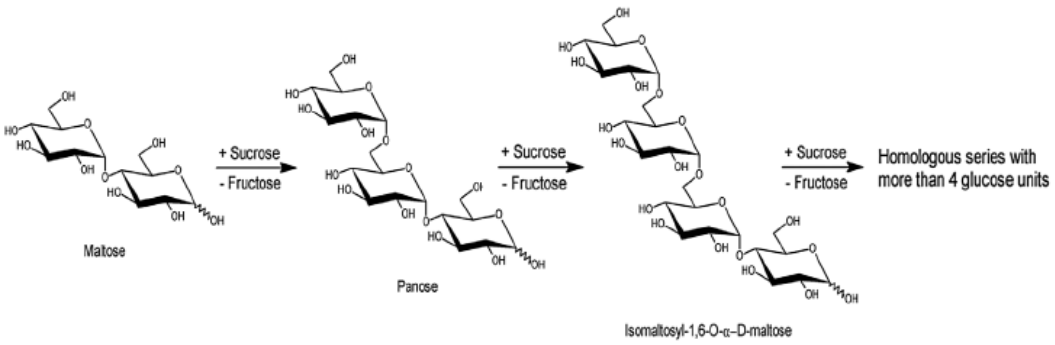
Dextransucrase is an emerging enzyme that transfers an α-D-glucosyl moiety from sucrose to hydroxyl group of mono- or di- saccharides or non-carbohydrate molecules (figure 1).

There are more than thirty different acceptors including monosaccharides, oligosaccharides, and polysaccharide for dextransucrase activity. The acceptor products of maltose and isomaltose themselves act as acceptors and homologous series of acceptor products are formed while D-fructose and lactose give only a single acceptor product [20, 21]. According to Robyt and Walseth, both glucosyl and dextranosyl groups were attached to the nonreducing end of the acceptors during the mechanism of acceptor reaction using sucrose and C-labeled acceptors. The acceptor reaction takes place by transferring glucosyl and dextranosyl groups from the covalent enzyme complex and the reaction terminates growth of dextran chain by displacing it from active site [20]. The results from Ebert and Schenk experiment showed some acceptors increase the rate of reaction and others decrease the rate of reaction with B-512F dextransucrase [22]. The most efficient acceptor are maltose and isomaltose [21]. The acceptor reaction of dextransucrase has been used in industry to produce leucrose, which is a noncariogenic disaccharide, and controlled molecular weight dextrans, which are used for medical purpose such as blood plasma substitute.

Dextransucrase previously has been applied for improving characteristics of various bioactive compounds such as inhibitor for α-amylase [23] solubility of epigallocatechin

gallate [24] and browning resistance of chlorogenic acid by forming glycoconjugates [25]. Stevioside glucoside production process and structure of final product were elucidated in this paper. Sensory evaluation was performed for the intensity and quality of its taste. The water solubility and stability of stevioside glucoside were also investigated to prove its applicability as an ingredient for food, beverage and pharmaceutical products.

Figure 1. Dextransucrase acceptor reaction with maltose [26]



Materials and Methods

1. Enzyme preparation

L. mesenteroides B-512FMCM, a constitutive mutant, was formed by vacuum ultraviolet radiation induced mutation of *L. mesenteroides* B-512FMC [12]. For dextransucrase production, *L. mesenteroides* B-512FMCM showed complete constitutivity, 13 times higher efficiency, and over 100 times more production yield than *L. mesenteroides* B-512F [12, 13]. Dextransucrase was obtained from glucose fermentation of *L. mesenteroides* B-512FMCM, and subsequently the enzyme was purified [19, 27]. One unit of dextransucrase was defined as the amount of enzyme required to liberate 1 μmol of fructose from 200 mM sucrose in 20 mM sodium acetate buffer (pH 5.2) at 28 °C. Thin layer chromatography (TLC) was used to measure the amount of fructose, and the AlphaEaseFc Image Program (Alpha Inotech, CA, USA) was used to quantify the amount.

2. The transglucosylation of stevioside

While incubating at 28 °C, there was the transglucosylation reaction. To halt the enzyme reaction, the reaction mixture was placed in a water bath for 5 min. The components of reaction mixture are as in the following: 500 mM sucrose (w/v), 50 mM stevioside (w/v), 20mM sodium acetate buffer (pH 5.2), dextransucrase (3U/mL) and water. A 1 μL aliquot of the reaction mixture was spotted on the aluminum TLC plate, silica gel coated with F_{254} (Merck, Darmstadt, Germany), and developed by using the solvent system nitromethane- *n*-propyl alcohol-water (2: 5: 1.5, v/v/v). Ste and Ste-Gs were visualized by developing the TLC plate in a solvent mixture of 0.5 (w/v) *N*-(1-naphthyl) ethylenediamine dihydrochloride (Sigma Aldrich, MO, USA), and 5% (w/v) sulfuric acid (Duksan Pure Chemicals, Gyunggido, South Korea) in methanol (Duksan Pure Chemicals, Gyunggido, South Korea) followed by heating at 125 °C for 5 min [28]. The conversion of Ste into Ste-Gs was quantified and expressed as an integrated density value (IDV) by employing the AlphaEaseFC 4.0 Image program (Alpha Inotech, CA, USA).

3. Separation of stevioside glucosides

The reaction mixture was added to two volumes of chilled ethanol and incubated at -20°C for 3 h for the polymer precipitation. The centrifugation follows to separate polymers from the ethanol mixture at 4°C for 20 min (12,000 g). The supernatant was concentrated using the rotary evaporator (EYELA, Tokyo, Japan) at 45°C under a vacuum. To remove monosaccharides from the resultant digests, they were loaded onto a 4 x 120 cm of the Diaion HP-20 column and eluted with water at a flow rate of 100 mL/min. For Ste-Gs collection, the Diaion HP-20 column was eluted with ethanol. The eluted solution was concentrated with a vacuum rotary evaporator (Heidolph, Germany) at 45°C, and it was freeze-dried with EYELA freeze dryer (Japan) for 24 h.

4. Purification of stevioside glucosides

The Ste-Gs mixtures were purified by HPLC (Waters e2695 system, MA, USA) with an amino column (Sunfire, 5μm, 150 x 4.6 mm), and detected with a refractive index (RI) detector. The mobile phase consisted of 75% acetonitrile at flow rate of 1 mL/min for 30 min. The purification and detection were operated at 40°C. A REVELERIS X2 flash chromatography system with an amino column (Grace, 40 μm, 120 g) was used for mass purification of Ste-Gs. The amino column was then eluted with gradient of 100% to 10% acetonitrile at speed of 80 mL/min for 60 min. After residual acetonitrile was removed from the fractions by using the rotary evaporator (Heidolph, Germany) at 45°C, the remaining fractions were lyophilized (Eyela, Japan) and stored at −20 °C for further study.

5. RSM

The central composite design (CCD) RSM software program (Design Expert 10.0.3, MN, USA) was used to optimize Ste conversion to Ste-Gs with the following three variables: Enzyme concentration (1U-5U), stevioside concentration (30–150 mM), and sucrose concentration (300-1100 mM). Seventeen runs of the experiment were carried out with Design Expert version 10.0.3 with four replications at the central point, which were utilized in the fitting of a second-order response surface [29] (Table 2). All statistical and mathematical analyses of the results were performed interactions using Design-Expert 10.0.3 to determine the effects of variables. Three-dimensional surface plots were drawn to determine the effects of independent variables on response. Correlation between variables and the response was determined using a quadratic model of a second-order polynomial as shown below:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta i Xi + \sum_{i=1}^{n} \beta ii Xi^2 + \sum_{i=1}^{n-1} \sum_{i=i+1}^{n} \beta ij Xi Xj$$

Where Y represented the predicted response; β_0, β_i, β_{ii}, and β_{ij} were the regression coefficients for intercept, linearity, square, and interaction, respectively, and X_i and X_j were the independent coded variables. The significance of the model was evaluated by determination of R^2 and adjusted R^2 coefficients. An experiment was also conducted to confirm the predicted optimum response using the selected optimum values of the three

variables.

6. MALDI-TOF analysis

Each Ste-Gs with the concentration of 3 mg/mL was diluted with deionized water, and the matrix assisted laser desorption/ionization (MALDI-TOF) mass spectrum of each Ste-Gs was acquired using the Applied Biosystems Voyager-DE STR (CA, USA). The obtained mass spectrum was in positive reflector mode with a delayed extraction method (average of 300 laser shots) at an acceleration voltage of 25 kV.

7. Nuclear Magnetic Resonance (NMR) analysis

10 mg of each purified Ste-Gs was dissolved in 600 μL of dimethyl sulfoxide-d6 (DMSO-d6), and placed into 5 mm of nuclear magnetic resonance (NMR) tubes for analysis. The NMR analysis was operated at 850 MHz for 1H and at 125 MHz for 13C at 25 °C. The spectra were recorded on AVANCEIII system (Bruker, Germany). The linkages between stevioside and glucose were evaluated using homonuclear correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), heteronuclear single-quantum coherence (HSQC), and heteronuclear multiple-bond correlation (HMBC).

8. Sensory evaluation

The sensory evaluation was performed as described by Ishikawa et al. (1990) with 12 individuals of panels [15]. Each samples of Ste, Ste-Gs, and novel Ste-Gs was dissolved in water to generate aqueous solutions whose intensity of sweetness corresponded to that of 3-6% (w/v) sucrose solutions. Standard sucrose solutions were prepared with intervals of 0.5% (w/v) at graduated concentration between 3 and 6% (w/v). Twelves of panelists were asked to taste and estimate total intensity of sucrose solutions compared to that of the sample solutions. Panelists repeated the sensory analysis of each sample five times. The assessment criteria were as follows: aftertaste, bitterness, and deliciousness of each sample solution at a concentration corresponding to sweetness of a 5% (w/v) sucrose solution.

9. Stability of stevioside glucosides in extreme pH and temperature condition

The degradations of Ste, Ste-Gs and novel Ste-Gs were analyzed at condition of pH 2.0 and 60°C. Samples were added to eppendorf tube which containing 4 mg/mL of Ste, Ste-Gs or novel Ste-Gs in water. The tubes were stored for up to 48 h and analyzed the stability by TLC. The degradation experiments were performed in duplicate for each Ste, Ste-Gs and novel Ste-Gs samples.

10. Solubilization ability of stevioside glucosides with insoluble Curcuminoids

The solutions of turmeric-stevioside (turmeric powder treated with Ste to solubilize curcuminoids; Tum-Ste), turmeric-stevioside glucoside (turmeric powder treated with Ste-Gs to solubilize curcuminoids; Tum-Ste-Gs), and turmeric-novel Stevioside Glucoside (turmeric powder treated with novel Ste-Gs to solubilize curcuminoids; Tum-novel Ste-Gs) were prepared as reported previously[10]. The effect of Ste and Ste-Gs to enhance the solubility of curcuminoids from turmeric powder was previously studied with various concentrations of Ste and Ste-Gs. In this study, the concentrations were chosen as follows: 10% (w/v) concentration of Ste or Ste-Gs mixing with 10% (w/v) of turmeric powder in 100% (v/v) ethanol [30]. The mixture in ethanol solution was vortexed for 15 min and centrifuged at 12,000 g for 10 min. The supernatant was transferred to a new tube and ethanol was evaporated[10]. The resulting powders were dissolved in water, centrifuged at 12,000 g for 10 min, and filtered through 0.20 μm membrane (Agilent, CA, USA). TLC with an ascent of acetonitrile/water 85:15 (v/v) for curcuminoids was used to determine curcuminoids. Curcuminoids on silica gel 60 F_{254}TLC plate (Merck, Darmstadt, Germany) were visualized under range of UV 254 nm. For visualization, the TLC plate was dipped in a solution mixture of 0.5 (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride and 5% (w/v) sulfuric acid in methanol followed by heating at 121 °C for 3 min.

11. Solubilization ability of stevioside glucosides with insoluble idebenone

The effect of Ste, Ste-Gs and novel Ste-Gs to enhance the solubility of idebenone were studied with 10% (w/v) concentration of each sample was mixed with 10% (w/v) of idebenone powder in 100% (v/v) ethanol. The mixture in ethanol solution was vortexed for 15 min and centrifuged at 12,000 g for 10 min. The supernatant was transferred to a new tube and ethanol was evaporated [10]. The resulting powders were dissolved in water, centrifuged at 12,000 g for 10 min, and filtered through 0.20 μm membrane (Agilent, CA, USA). TLC with an ascent of acetonitrile/water 85:15 (v/v) was used to determine idebenone. Idebenone on silica gel 60 F_{254}TLC plate (Merck, Darmstadt, Germany) were visualized under UV 254nm. They were also visualized by dipping the TLC plate into a solvent mixture of 0.5 (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride and 5% (w/v) sulfuric acid in methanol followed by heating at 121 ℃ for 3 min.

Results and discussion

1. Enzymatic synthesis of stevioside glucosides

The transglucosylation of Ste using dextransucrase was confirmed by TLC analysis with developing solution (figure 1). After removing saccharides (fructose, sucrose, glucooligosaccharides), Ste-Gs mixtures were analyzed by HPLC (Figure 2).

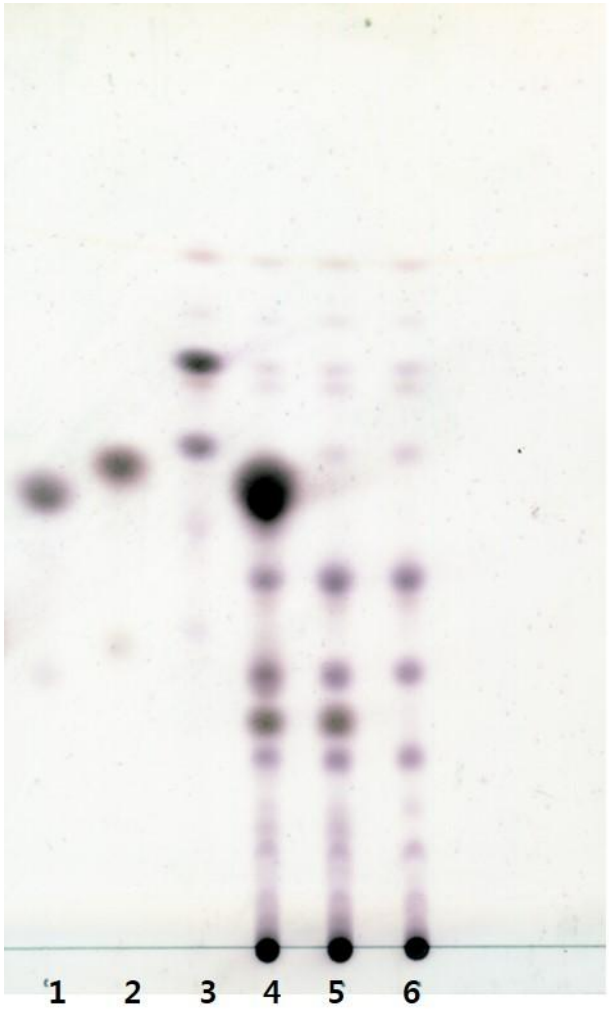


Figure 1. Thin layer chromatogram of stevioside glucosides synthesis by dextransucrase.

Lane 1, sucrose; Lane 2, glucose; Lane 3, stevioside; Lane 4, reaction mixture; Lane 5, reaction mixture without saccharides (glucose, fructose) ; Lane 6, reaction mixture without saccharides (glucose, fructose, glucooligosaccharides)

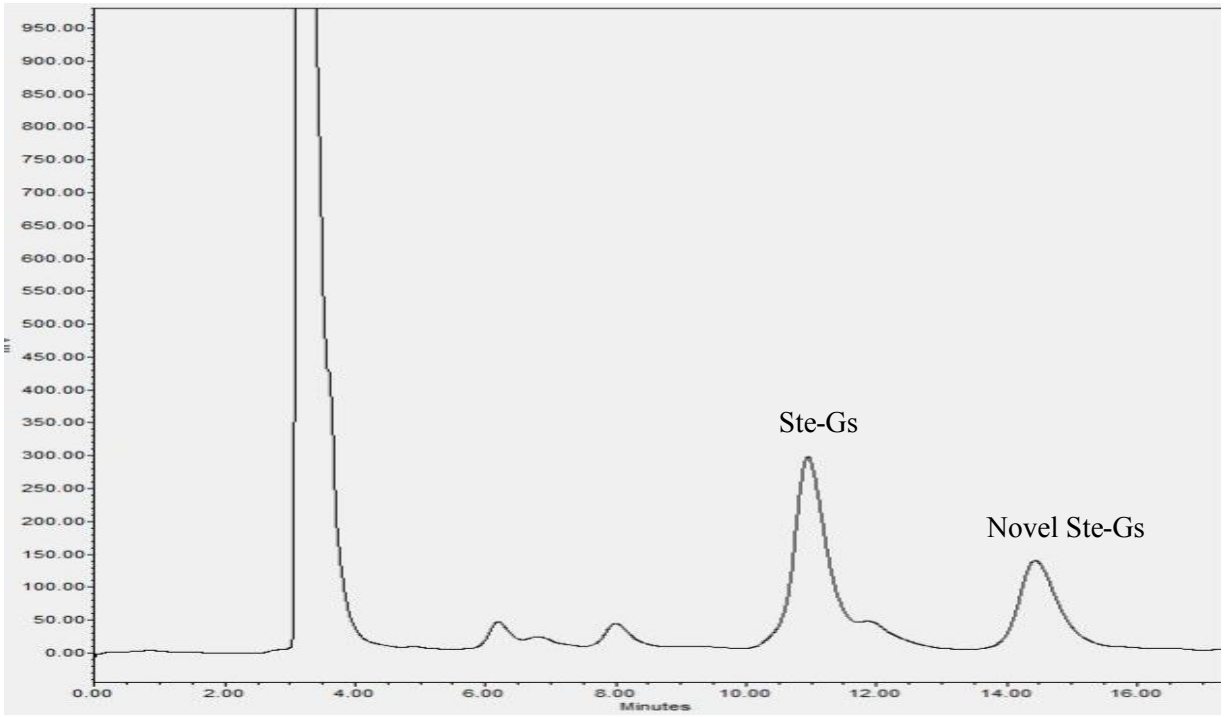


Figure 2. HPLC spectra of stevioside glucosides

2. Transglucosylation of stevioside by dextransucrase was optimized using response surface methodology.

In this study, RSM was used to optimize the conversion from Ste to Ste-Gs. The design matrix and corresponding results of RSM experiments used to determine the effect of the three independent factors [enzyme concentration (X_1), stevioside concentration (X_2), and sucrose concentration (X_3)]. RSM was used to study the interaction of these factors within a range from −1 to +1 in relation to Ste conversion to Ste-Gs. A total of 17 experiments were performed with different combinations of these factors (Table 1). The 3D response surface and 2D contour plots of independent variables with respect to the response are shown in Figure 3. Results of analysis of variance (ANOVA) of Ste conversion to Ste-Gs indicated that the experimental data had a determination coe□cient (R^2) of 0.9788 and adjusted coe□cient (R^2) of 0.9515 (Table 2), meaning that the calculated model was able to explain 97.88% and 95.15% of the results. The model used to fit the response variables was found to be significant ($p < 0.001$). The amount of Ste converted into Ste-Gs was expressed using the regression equation:

$$Y=+44.60528 +13.08224X_1 -0.20167X_2 +0.046905X_3 +0.026281X_1X_2 +7.95472E\text{-}003X_1X_3 -6.94907E\text{-}005X_2X_3 -2.15635X_1^2 +5.93429E\text{-}005X_2^2 -4.31376E\text{-}005X_3^2$$

Where Y is the percentage of Ste converted to Ste-Gs (%), X_1 is the concentration of enzyme dextransucrase (U/mL), X_2 is Ste concentration (mM), and X_3 is sucrose concentration (mM). The regression equation obtained from ANOVA was used to calculate R^2 (multiple correlation coe□cient). The predicted maximum rate of conversion from Ste to Ste-Gs was 85.55% with 4.5 U/mL dextransucrase, 97 mM Ste, and 972 mM sucrose. To validate the predicted Ste conversion rate to Ste -Gs, an experiment was conducted using the above conditions.

Table 1. Running condition for stevioside acceptor reaction and stevioside conversion.

Run no.	Coded number			Stevioside conversion (%)	
	X_1 Unit/mL	X_2 Stevioside(mM)	X_3 Sucrose(mM)	Actual	Predicted
1	1	150	300	40.42	40.01
2	3	90	700	80.39	77.89
3	1	30	300	63.43	62.27
4	3	90	700	77.92	77.89
5	3	90	700	76.68	77.89
6	5	150	1100	77.85	78.59
7	5	30	300	78.27	75.55
8	5	90	700	84.72	85.42
9	1	30	1100	57.86	56.18
10	5	150	300	64.63	65.90
11	1	150	1100	24.94	27.24
12	1	90	700	52.16	53.11
13	3	150	700	72.36	68.46
14	3	90	700	79.90	77.89
15	3	90	1100	74.00	72.64
16	3	90	300	66.33	69.34
17	3	30	700	82.20	87.75

Table 2. Results of two-way analysis of variance (ANOVA)

Source of Variation	Sum of Squares	Degree of Freedom	Mean Square	F Statistic	p-value Prob > F
Model	4121.33	9	457.93	35.88	<0.0001
A-Enzyme	1756.93	1	1756.93	137.65	<0.0001
B-Stevioside	626.49	1	626.49	49.08	0.0002
C-Sucrose	18.30	1	18.30	1.43	0.2701
AB	49.52	1	49.52	3.88	0.0895
AC	201.61	1	201.61	15.80	0.0054
BC	13.85	1	13.85	1.08	0.3322
A^2	195.75	1	195.75	15.34	0.0058
B^2	0.12	1	0.12	0.01	0.9254
C^2	125.34	1	125.34	9.82	0.0165
Error	8.99	3	3.00		
Total	4210.68	16			

R^2=0.9788
Adj R^2=0.9515

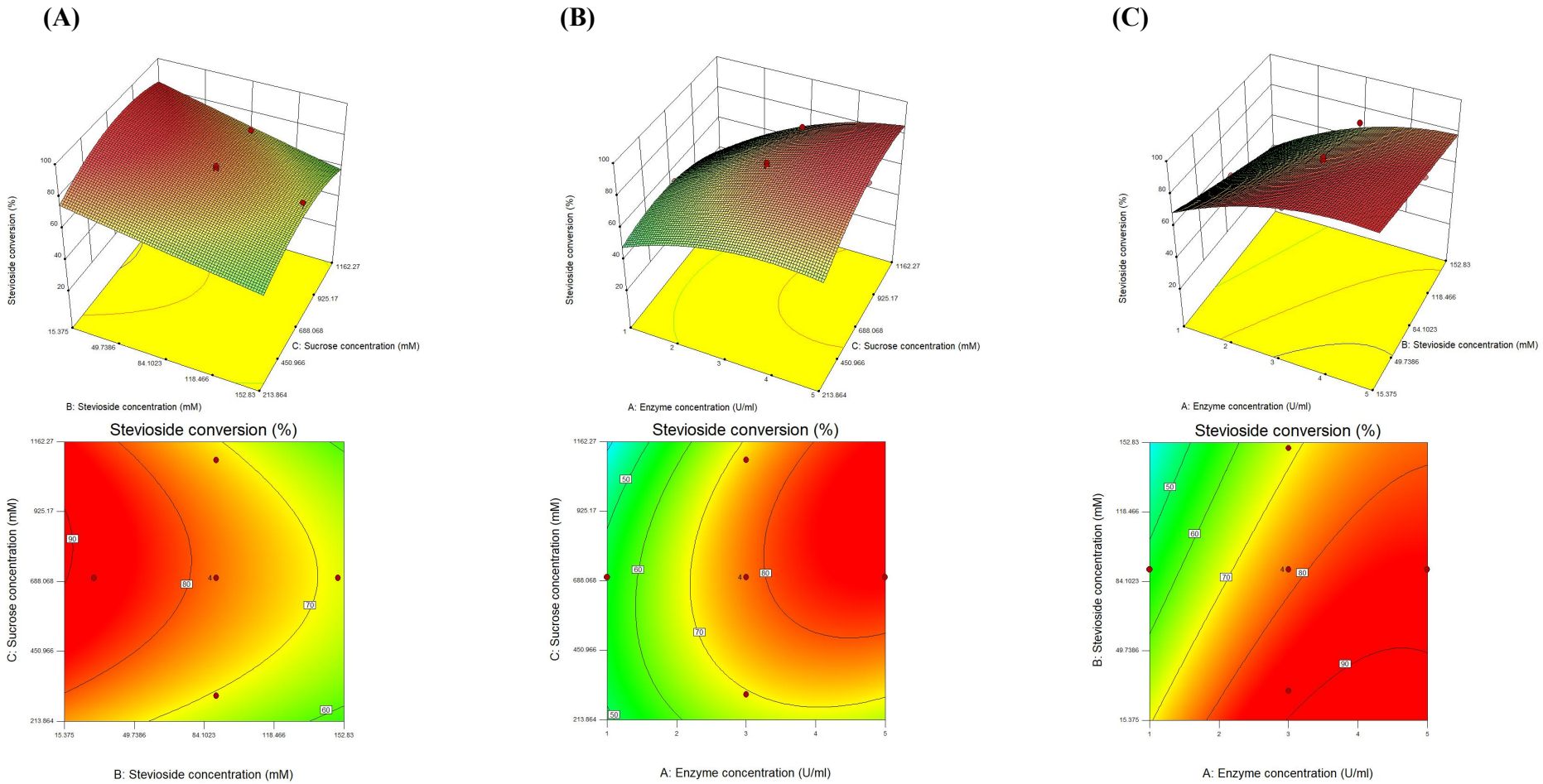


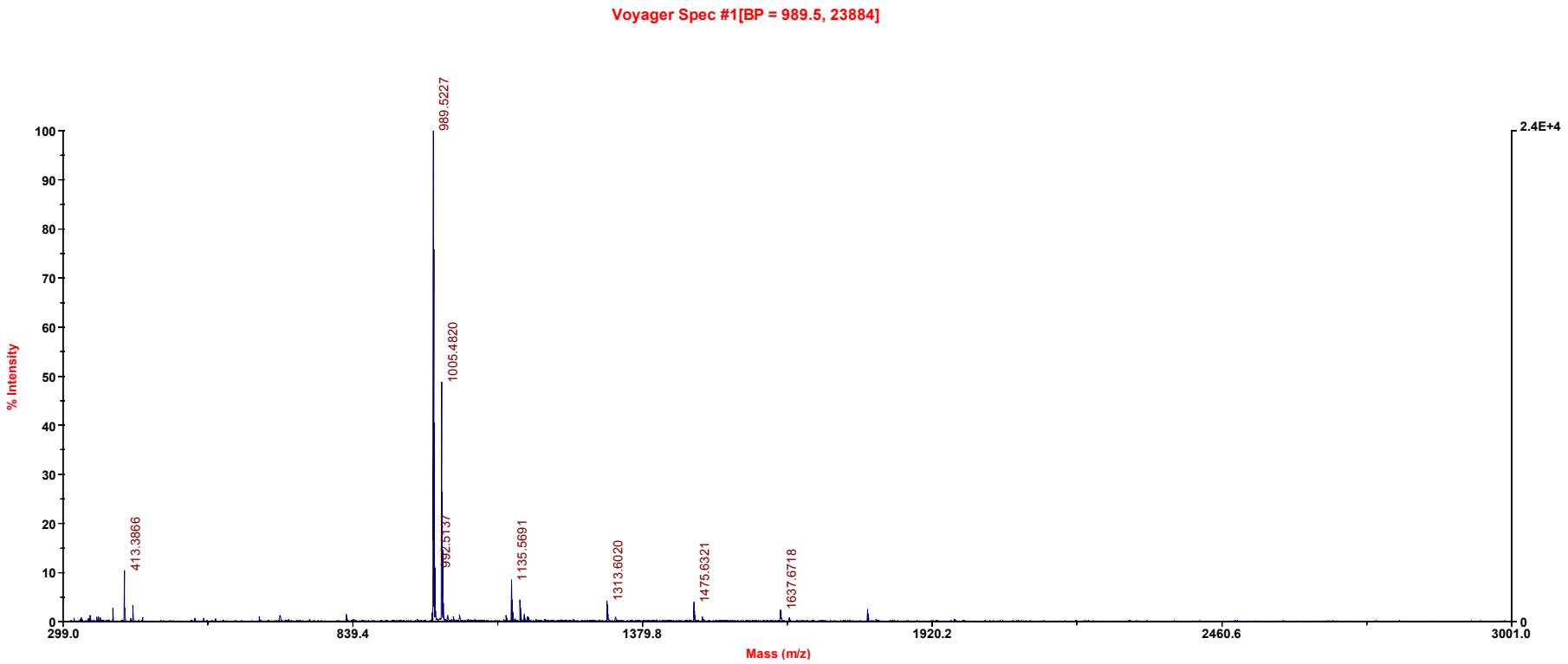
Figure 3. Response surface plot and contour plot.

(A), Sucrose concentration vs. Stevioside concentration; (B), Sucrose concentration vs. Enzyme concentration; (C), Stevioside concentration vs. Enzyme concentration

3. Structural elucidation by NMR analysis

The transglycosylation of stevioside using dextransucrase was confirmed by TLC analysis developing solution and HPLC-RI analysis. Two Ste-Gs were purified using HPLC. Through MALDI-TOF-MS analysis, the molecular weight of each purified Ste-Gs was determined. Compounds 1 was observed at m/z 989.5 $(M + Na)^+$ (Figure 4A), indicating that a single glucosyl residue was attached to stevioside. Compounds 2 was observed at m/z 1151.4 $(M + Na)^+$ (Figure 4B), indicating that two glucosyl residues were attached to stevioside. These Ste-Gs structures were identified by NMR analysis (1H, 13C, HSQC, HMBC, COSY and TOCSY).

(A)


Voyager Spec #1[BP = 989.5, 23884]
989.5227
1005.4820
992.5137
413.3866
1135.5691
1313.6020
1475.6321
1637.6718
% Intensity
Mass (m/z)
2.4E+4
0
100
90
80
70
60
50
40
30
20
10
0
299.0
839.4
1379.8
1920.2
2460.6
3001.0

(B)

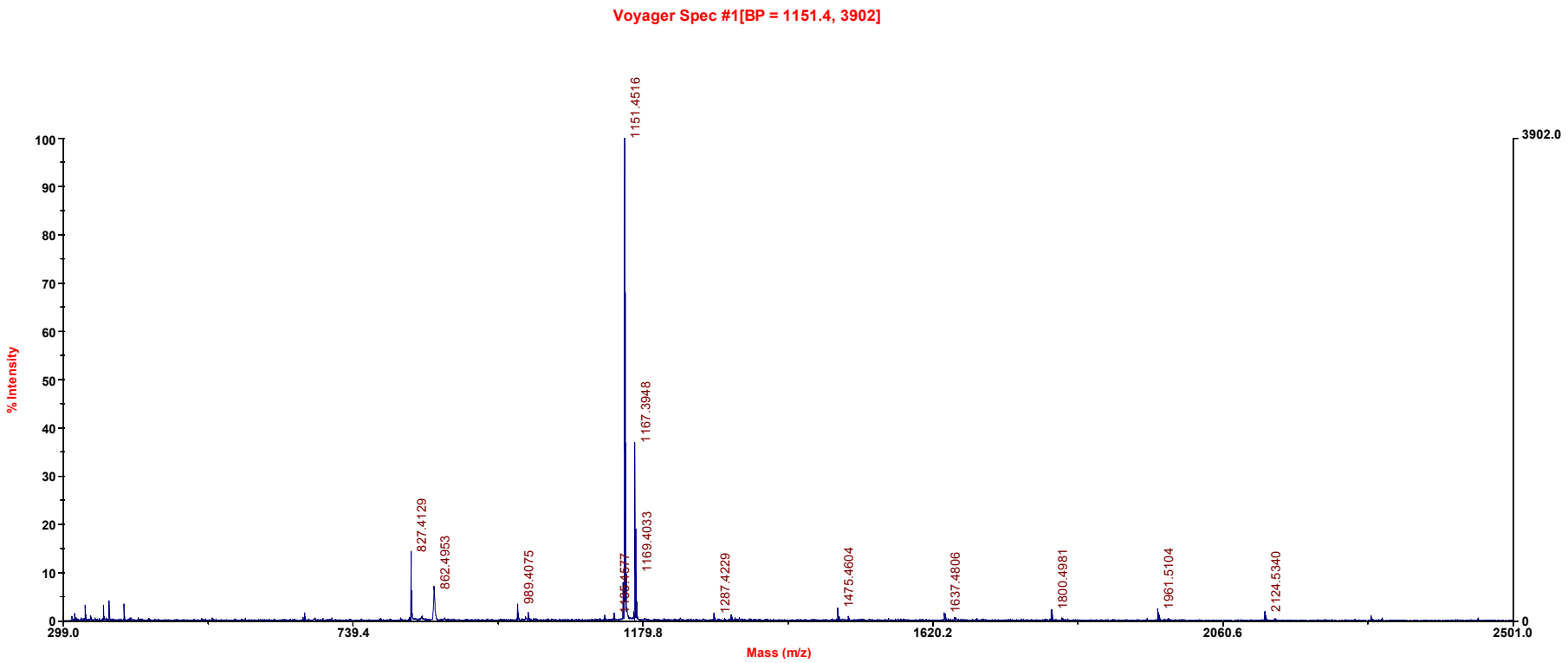


Figure 4. MALDI-TOF-MS spectra of Stevioside glucosides

(A), MALDI-TOF-MS spectra of Ste-Gs; (B), MALDI-TOF-MS spectra of novel Ste-Gs

Ste-Gs.

Compare to stevioside, new doublet signal at 4.63 ppm (J = 3.32 Hz) was assigned to anomeric proton, indicating that one glucosyl residue was α-linked with stevioside. Most of its spectra was very similar to stevioside except for signals at 73.01 ppm (corresponding to C-19) and 66.11 ppm (corresponding to C-19) with a shift of Δδ -5.62−3.98 compared to stevioside, indicating the occurrence of glucosylation in 19-COO-β-Glucoside residues of stevioside in the form of α-1, 6 linkage. This specific position of glucosylated hydroxyl group was confirmed by HMBC data (4.63 ppm corresponding to H-6‴). The structure of Ste-Gs 1 was identified as 13-*O*-β-sophorosyl-19-*O*-β-isomaltosyl-steviol (Table 3, Figure 5A).

Novel Ste-Gs.

Compare to Stevioside, two new doublet signals at 4.63 ppm (J = 3.32 Hz) and 4. 64(J = 4.76 Hz) were assigned to anomeric proton, indicating that two glucosyl residues were α-linked with stevioside. Most of its spectra were very similar to stevioside except for signals at 73.01 ppm and 66.11 ppm which were corresponding to 19-COO-β-Glucose, 86.14 ppm, 78.87 ppm, 68.89 ppm which were corresponding to 13-O-β-Glucoside), 104.56 ppm, 75.24 ppm and 76.23 ppm which were corresponding to 13-*O*-β-glucoside-β-glucosyl with a shift of Δδ -3.59−1.59 compared to stevioside, indicating the occurrence of glucosylation in 19-O-β-Glucoside and 13-O-β-Glucoside residues of stevioside in the form of α-1, 6 and α-1, 3 linkages. These specific positions of glucosylated hydroxyl group were confirmed by HMBC data (4.63 ppm corresponding to H-6‴ and 4.64 ppm corresponding to H-3′). The structure of this compound was identified as 13-*O*-[β-D-glucosyl (1 → 2)-β-nigerosyl]-19-*O*-β-isomaltosyl-steviol (Table 3, Figure 5B). This structure was discovered as novel compound which had not been identified before.

Table 3. ^{13}C and ^{1}H NMR data of stevioside and stevioside glucosides (ppm).

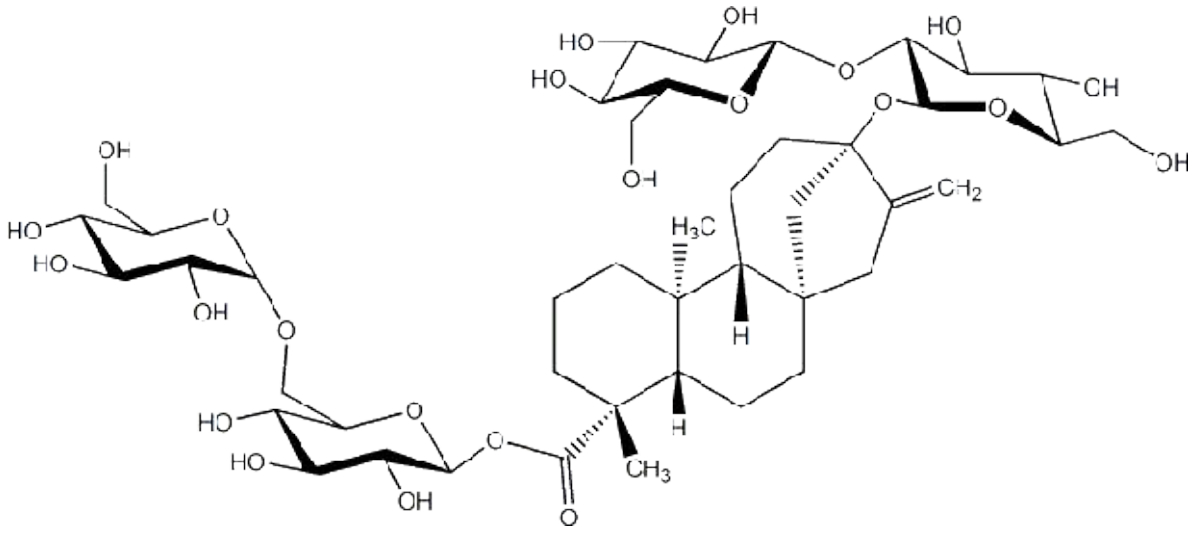
Position	Ste		Ste-Gs		novel Ste-Gs	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	0.77 (m) 1.77 (m)	40.09	0.77 (m) 1.77 (m)	40.1	0.77 (m) 1.77 (m)	40.07
2	1.35 (m) 1.76 (m)	18.68	1.35 (m) 1.76 (m)	18.63	1.35 (m) 1.75 (m)	18.61
3	0.90 (m) 2.06 (m)	37.38	0.90 (m) 2.06 (m)	37.4	0.90 (m) 2.06 (m)	37.42
4		42.05		42.06		41.84
5	1.04 (d, J=12.8)	56.46	1.04 (d, J=12.4)	56.39	1.04 (d, J=13.0)	56.37
6	1.71 (m) 1.98 (m)	21.19	1.71 (m) 1.92 (m)	21.24	1.70 (m) 1.92 (m)	21.22
7	1.34 (m) 1.46 (m)	40.98	1.33 (m) 1.46 (m)	41.01	1.34 (m) 1.44 (m)	40.07
8		42.05		42.1		41.84
9	0.91 (m)	53.12	0.90 (m)	53.17	0.90 (m)	53.23
10		38.93		38.95		38.95
11	1.50 (m) 1.69 (m)	19.98	1.50 (m) 1.69 (m)	19.98	1.48 (m) 1.68 (m)	19.87
12	1.48 (m) 1.85 (m)	35.57	1.47 (m) 1.87 (m)	35.54	1.48 (m) 1.87 (m)	36.21
13		84.63		84.62		85.2
14	1.37 (m) 2.14 (d, J=11.5)	43.5	1.38 (m) 2.15 (d, J=11.4)	43.52	1.35 (m) 2.09 (d, J=11.1)	43.18
15	1.98 (m)	46.89	1.98 (m)	46.89	1.99 (m)	47.01

	2.02 (m)		2.02 (m)		2.03 (m)	
16		153.58		153.64		153.23
17	4.71 (s) 5.03 (s)	103.92	4.71 (s) 5.04 (s)	103.88	4.73 (s) 5.04 (s)	103.9
18	1.14 (s)	28.07	1.13 (s)	27.89	1.13 (s)	27.93
19		175.66		175.69		175.65
20	0.87 (s)	15.14	0.86 (s)	15.09	0.86 (s)	14.98
Glucose-I						
1'	4.45 (d, J=7.65)	96.38	4.45 (d, J=7.65)	96.37	4.46 (d, J=7.74)	96.64
2'		82.65	3.22 (m)	82.59	3.56 (m)	86.14
3'		76.23	3.37 (m)	75.86	3.49 (m)	78.87
4'		70.34	3.07 (m)	70.22	3.16 (m)	68.89
5'		76.21	3.08 (m)	76.12	3.13 (m)	75.99
6'		61.01	3.49 (m) 3.56 (m)	60.55	3.69 (m) 3.54 (m)	60.85
Glucose-II						
1"	4.35 (d, J=7.74)	104.65	4.35 (d, J=7.65)	104.63	4.42 (d, J= 7.91)	103.04
2"		75.33	3.01 (m)	75.24	3.03 (m)	73.67
3"		76.02	3.16 (m)	75.91	3.19 (m)	76.93
4"		69.63	3.16 (m)	69.58	3.16 (m)	69.83
5"		76.91	3.05 (m)	76.96	3.05 (m)	76.64

6"		60.66	3.45 (m) 3.52 (m)	60.66	3.45 (m) 3.52 (m)	60.68
Glucose-III						
1'''	5.25 (d, J=8.16)	94.15	5.26 (d, J=7.91)	93.97	5.27 (d, J=8.16)	93.97
2'''		72.55	3.18 (m)	72.21	3.18 (m)	72.23
3'''		77.66	3.25 (m)	76.86	3.26 (m)	76.89
4'''		69.51	3.20 (m)	69.8	3.20 (m)	69.96
5'''		76.99	3.40 (m)	73.01	3.39 (m)	73.14
6'''		60.49	3.54 (m) 3.69 (m)	66.11	3.54 (m) 3.70 (m)	66.15
Glucose-IV						
1''''			4.63 (d, J=3.32)	98.32	4.63 (d, J=3.4)	98.33
2''''			3.40 (m)	72.29	3.41 (m)	72.40
3''''			3.39 (m)	75.61	3.39 (m)	75.58
4''''			3.08 (m)	69.9	3.05 (m)	70.06
5''''			3.16 (m)	75.91	3.17 (m)	76.55
6''''			3.42 (m) 3.67 (m)	60.95	3.61 (m) 3.42 (m)	60.96
Glucose-V						
1'''''					4.64 (d, J=4.76)	102.46

2""					2.98 (m)	74.53
3""					3.14 (m)	76.41
4""					3.04 (m)	70.23
5""					3.12 (m)	75.99
6""					3.69 (m) 3.41 (m)	61.17

(A)



(B)

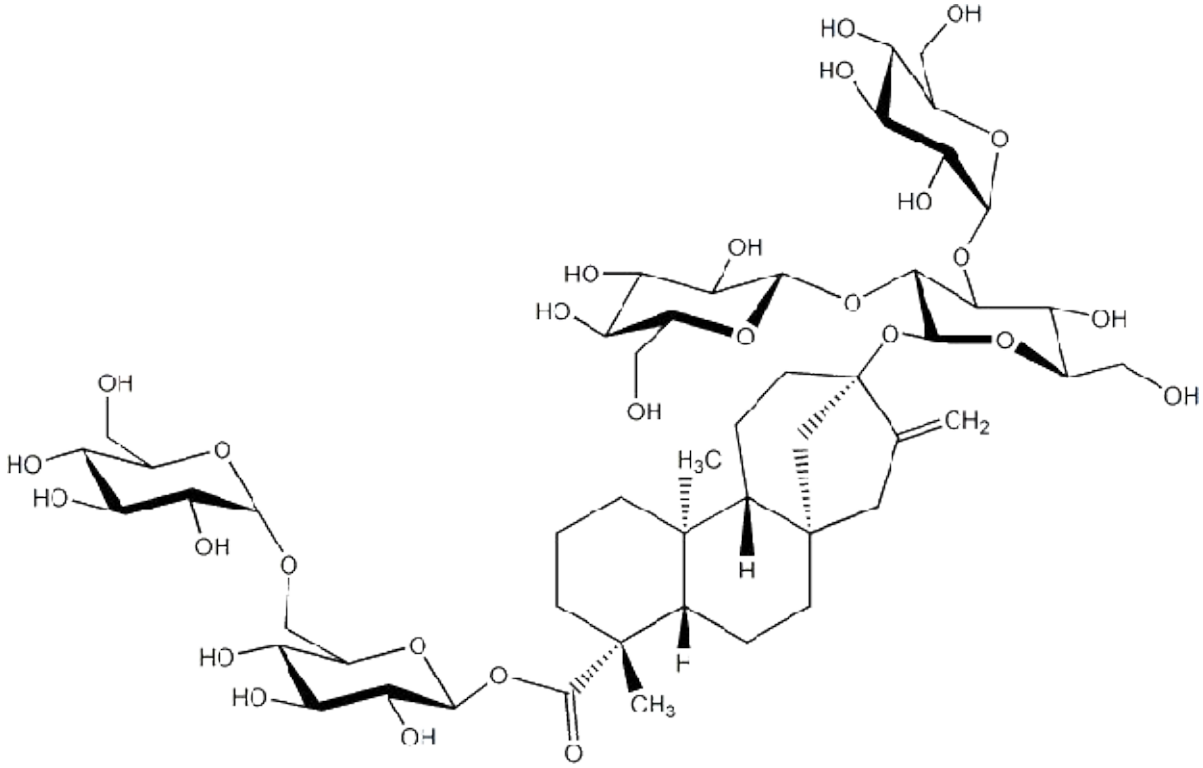


Figure 5. Structures of Stevioside glucosides

(A) Ste-Gs, 13-*O*-β-sophorosyl-19-*O*-β-isomaltosyl-steviol

(B) novel Ste-Gs, 13-*O*-[β-D-glucosyl (1 → 2)-β-nigerosyl]-19-*O*-β-isomaltosyl-steviol

4. Sensory evaluation

The relative sweetness of the synthesized Ste-Gs as well as the relative quality of the taste is listed in Table 4. The quality of taste was 2-fold higher for Ste-Gs and novel Ste-Gs than for stevioside. An improved intensity of sweetness was also observed for Ste-Gs, which exhibited a 1.10 (158)-fold sweetness than stevioside and especially novel Ste-Gs was showed highly increased 1.41 (201)-fold sweetness than stevioside (143). The quality of taste for stevioside glucoside at the same position by Pullulanase and pullulan[31] was also improved. In case of novel Ste-Gs, one glucosyl moiety was attached at the same position of Ste-Gs, the other is same with rebaudioside A. The modification of the transglucosyl residue at these position may play a critical role in the sweetness and quality of taste.

Table 4. Relative sweetness and quality of taste of stevioside glucosides and steviol glucosides.

compound	R1	R2	RS	Quality of taste	Glc ratio R1:R2	References
Rebaudioside D	β-Glc(2 → 1)β-Glc (3 → 1)β-Glc	β-Glc(2 → 1)β-Glc	221	+2	3:2	[32]
Rebaudioside E	β-Glc(2 → 1)β-Glc	β-Glc(2 → 1)β-Glc	174	+2	2:2	
Rubusoside	β-Glc	β-Glc	114	-2	1:1	
Stevioside	b-Glc(2 → 1)b-Glc	β-Glc	143	0	2:1	
Ste-Gs 1	b-Glc(2 → 1)b-Glc	β-Glc(6 → 1)α-Glc	158 ± 4	+2	2:2	This study
Ste-Gs 2	b-Glc(2 → 1)b-Glc (3 → 1) α -Glc	β-Glc(6 → 1)α-Glc	201 ± 4	+2	3:2	This study

RS: relative sweetness to sucrose (on a weight basis).

[a] Quality of taste relative to 3: +3 fairly better; +2, better; +1, slightly better; 0, almost the same.

5. Stability of stevioside glucosides under extreme condition

In aqueous solutions, stevioside is remarkably stable over a wide range of pH values and temperatures. However, under extreme temperature and pH conditions, chemical degradation of the sweetener inevitably occurs [33]. Therefore, we compared the stabilities of Ste, Ste-Gs and novel Ste-Gs were tested with condition of pH 2.0 and 60 °C for accelerated stability test.

After 24 h, the stevioside concentration decreased by nearly 18.87% (Table 5). The highest degradation (24.53%) was observed after 48 h. Ste-Gs was stable than stevioside until 24 h and the final degradation rates after 48 h were 15.87%. Among the tested compounds, the novel Ste-Gs showed the highest stability. After 24 h, the novel Ste-Gs concentration decreased by 5% and the final degradation rates after 48 h were 14.06%.

Table 5. Stability of Ste, Ste-Gs and novel Ste-Gs

Concentraion (%) / Time	Ste	Ste-Gs	novel Ste-Gs
0	100.00	100.00	100.00
12	92.45±1.41	98.41±0.37	95.63±1.33
24	81.13±1.62	92.06±0.01	95.00±0.88
48	75.47±0.17	84.13±0.03	85.94±1.10

Stability was calculated based on the relative amount of remaining and initial steviol glucoside content.

6. Extraction of water soluble curcuminoids from turmeric powder

The curcuminoids powder prepared with ethanol directly from turmeric powder showed low or no solubility in water (Figure 6). When Ste, Ste-Gs or novel Ste-Gs were used to extract curcuminoids from turmeric powder, as attached glycoside residue increased, the amount of prepared water soluble curcuminoids was also increased from 8.07. ± 0.12 to 9.40 ± 0.12 mg per 10 g of turmeric powder (Figure 7). The highest water soluble curcuminoids yield (9.40. ± 0.12 mg/g turmeric powder) was obtained with novel Ste-Gs. As a result, Ste, Ste-Gs or novel Ste-Gs could be used to prepare water soluble curcuminoids directly from turmeric powder.

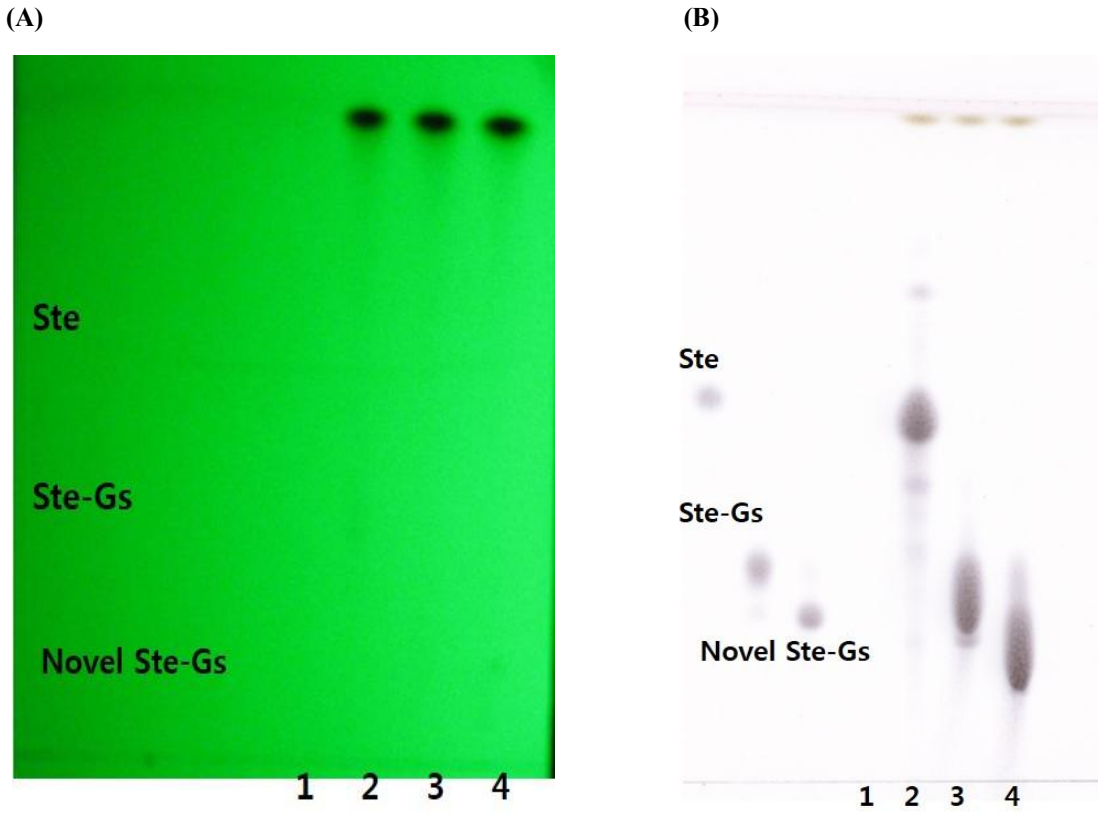


Figure 6. Thin layer chromatography of Ste, Ste-Gs, novel Ste-Gs with curcuminoids

Lane 1, curcuminoids with water; Lane 2-4, water soluble curcuminoids prepared using Ste, Ste-Gs or novel Ste-Gs. Thin layer chromatography analysis of curcuminoids with one ascent of acetonitrile/water 85:15 (v/v). They were detected under UV254 nm (A) and by dipping the TLC plate into a solvent mixture of 0.5 (w/v) N-(1-Naphthy) ethylenediamine dihydrochloride and 5% (w/v) sulfuric acid in methanol followed by heating at 90°C for 3min (B).

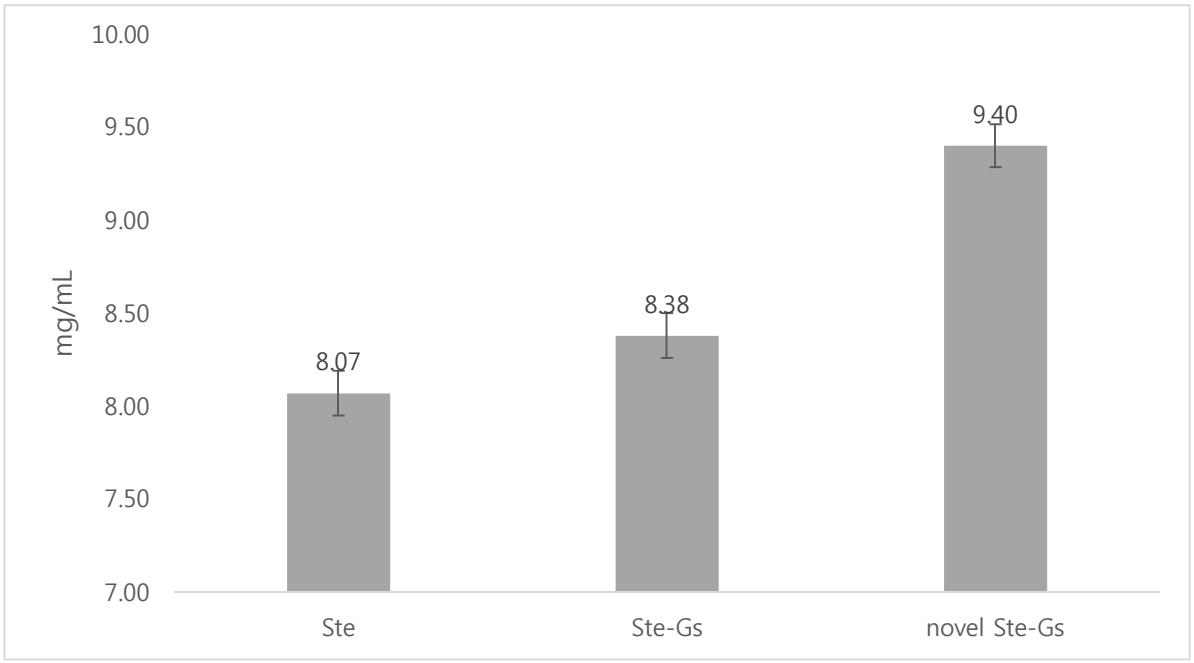


Figure 7. Solubilization ability of Ste, Ste-Gs, novel Ste-Gs with curcuminoids

7. Water soluble idebenone from idebenone powder

The idebenone powder prepared with ethanol showed low or no solubility in water (Figure 8). When Ste, Ste-Gs or novel Ste-Gs were used to solublilized idebenone, as attached glycoside residue increased, the amount of prepared water soluble idebenone was reduced from 9.94. ± 0.04 to 4.40 ± 0.38 mg per 10 mg of idebenone powder (Figure 9). As a result, Ste, Ste-Gs or novel Ste-Gs could be used to prepare water soluble idebenone directly from idebenone powder and applicability as pharmaceutical products.

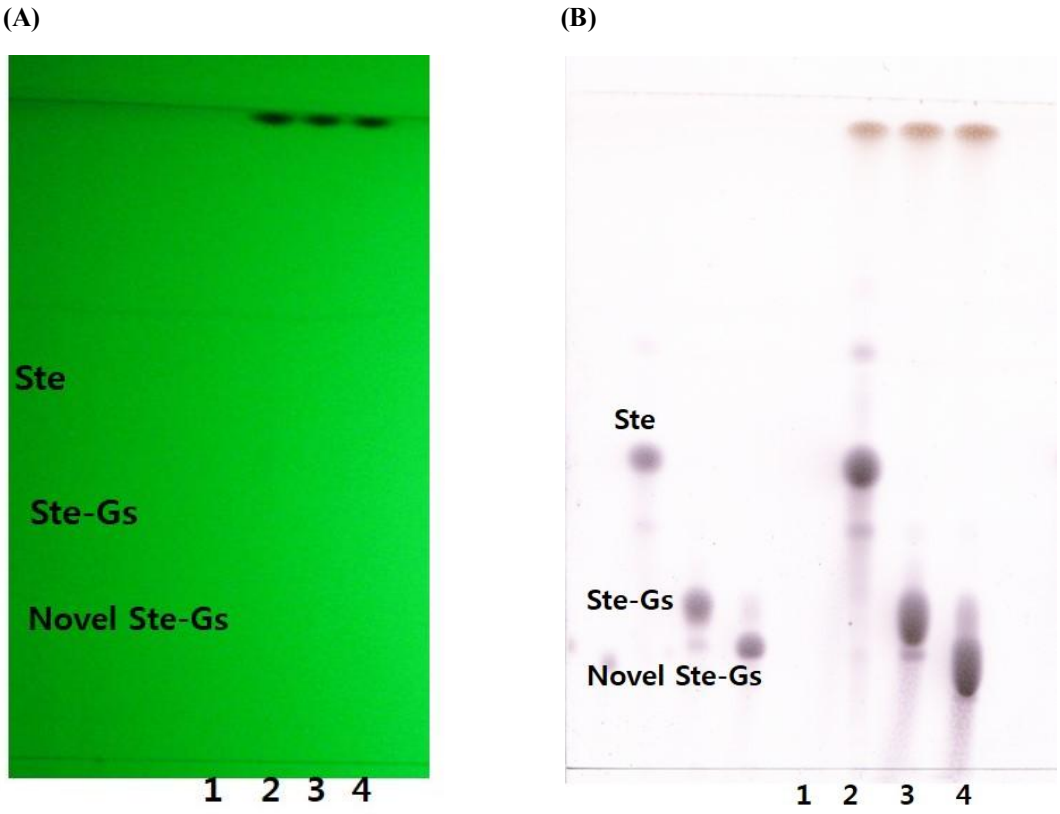


Figure 8. Thin layer chromatography of Ste, Ste-Gs, novel Ste-Gs with idebenone

Lane 1, idebenone with water; Lane 2-4, water soluble idebenone prepared using Ste, Ste-Gs or novel Ste-Gs Thin layer chromatography analysis of idebenone with one ascent of acetonitrile/water 85:15 (v/v). They were detected under UV254 nm (A) and by dipping the TLC plate into a solvent mixture of 0.5 (w/v) N-(1-Naphthy) ethylenediamine dihydrochloride and 5% (w/v) sulfuric acid in methanol followed by heating at 90°C for 3min (B)

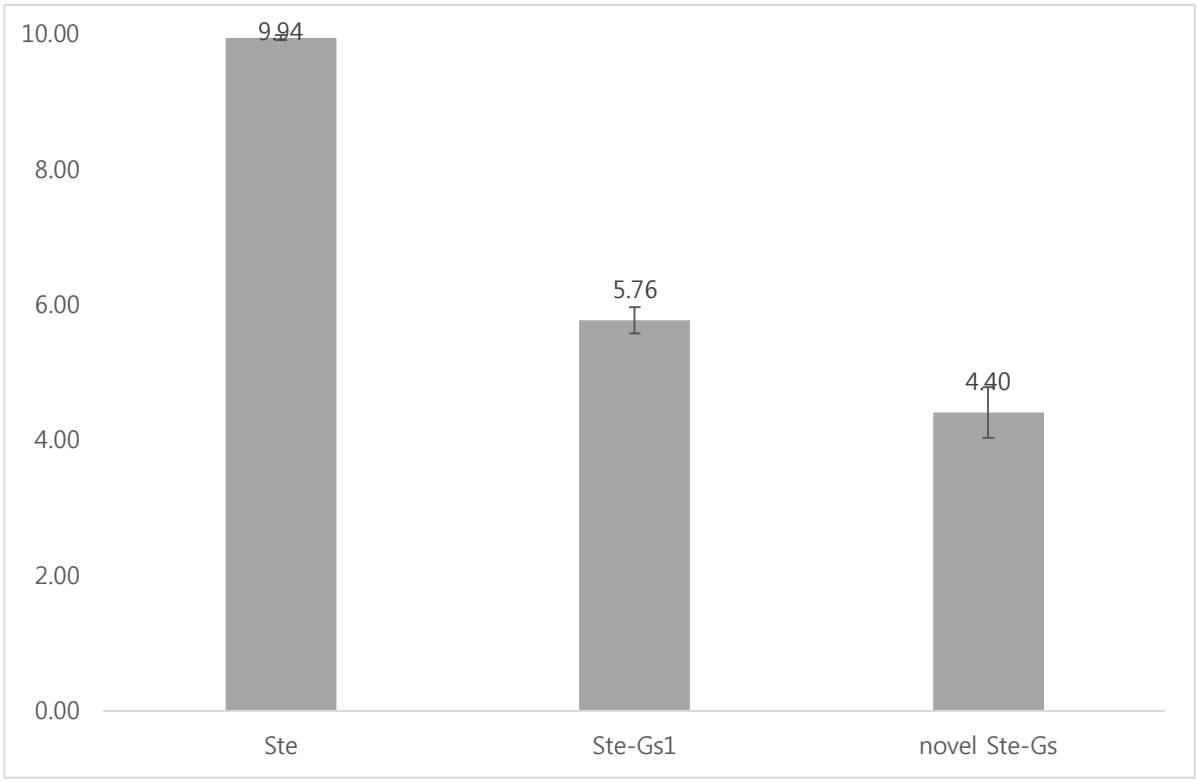


Figure 9. Solubilization ability of Ste, Ste-Gs, novel Ste-Gs with idebenone

CONCLUSION

The synthesis of novel Stevioside Glucoside using dextransucrase from *Leuconostoc mesenteroides* was successfully performed for the first time. The optimum conditions for Ste-Gs production were as follows: concentrations of 4.5 U/mL dextransucrase, 97 mM Ste, and 972 mM sucrose based on response surface methodology and central composite design in pH 5.2 at 28 ℃. Structures of Ste-Gs and novel Ste-Gs were elucidated with MADI-TOF MS and 1D (^{1}H, ^{13}C), 2D (HSQC, HMBC, COSY and TOCSY) NMR data. Glycosylated stevioside showed dramatic improvements in relative sweetness and quality of taste. The stability of Ste-Gs and novel Ste-Gs under extreme conditions was also increased than that of stevioside. Lastly, Ste, Ste-Gs and novel Ste-Gs could increase solubility of the water insoluble compounds such as curcuminoids and idebenone.

Overall, Ste-Gs and novel Ste-Gs have potentials as a novel natural sweetener and could be applied to the industry with their biological characterizations.

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Abstract in Korean 국문초록

덱스트란수크라아제를 이용하여 합성한 신규 스테비오사이드 배당체의 특성 연구

스테비오사이드는 설탕 대비 143 배의 감미도를 지니는 저칼로리 천연감미료로 주목받고 있다. 그러나, 스테비오사이드의 쓴 후미로 인해 식품 및 제약산업, 섭취에 제한적인 단점이 있다. 이 연구에서는 스테비오사이드 배당체를 설탕을 기질로 하고 *Leuconostoc mesenteroides* 균주에서 유래한 덱스트란수크라아제를 활용하여 효소적으로 합성하였다. 스테비오사이드 배당체가 최대로 생산될 수 있는 반응조건을 RSM 을 이용하여 확립하였으며, 스테비오사이드 배당체가 가장 효율적으로 생산될 수 있는 조건은 효소 4.5 U/mL, 스테비오사이드 97 mM, 설탕 972 mM 일때로 85.6%의 생산율을 보였다. 중압 및 고압크로마토그래피 (MPLC, HPLC)와 아미노 컬럼을 이용하여 Ste-Gs 와 novel Ste-Gs 를 순수하게 정제하였다. Ste-Gs 와 novel Ste-Gs 를 MALDI-TOF MS 분석과 핵자기공명분석법(NMR analysis)을 이용하여 분석한 결과, Ste-Gs 는 13-*O*-β-sophorosyl-19-*O*-β-isomaltosyl-steviol, novel Ste-Gs 는 13-*O*-[β-D-glucosyl (1 → 2)-β-nigerosyl]-19-*O*-β-isomaltosyl-steviol 로 정의하였다.

스테비오사이드, Ste-Gs 그리고 novel Ste-Gs 의 관능평가 결과 Ste-Gs 의 경우 감미도가 설탕 대비 158 배, novel Ste-Gs 의 경우 201 배 증가했음을 확인하였다. 특히, 감미도의 경우 두 시료 모두 스테비오사이드 대비 2 배 이상 증가했음을 밝혔다. 또한, 스테비오사이드, Ste-Gs 그리고 novel Ste-Gs 의 급속안정성연구(pH 2, 60 ℃, 48 시간) 결과 48 시간 이후, 초기 농도 대비 각각 75.47±0.17, 84.13±0.03, 85.94±1.10 의 잔존율을 보이는 것으로 확인되었으며, 특히 글루코사이드기가 증가함에 따라 안정성 또한 증가하는 특성이 나타났다. 마지막으로, 스테비오사이드, Ste-Gs 그리고 novel Ste-Gs 를 이용하여 불용성 물질인 커큐민노이드, 이데베논의 수용성 증가 실험을 연구한 결과 세 시료 모두 수용화

능력이 있음을 확인하였다. 커큐민노이드의 경우 글루코사이드기의 수가 증가함에 따라 수용화되는 커큐민노이드의 양도 8.07. ± 0.12 에서 9.40 ± 0.12 mg 와 같이 증가함을 확인하였다. 이데베논의 경우 글루코사이드기의 수가 증가함에 따라 수용화되는 이데베논의 양이 9.94. ± 0.04 에서 4.40 ± 0.38 mg 로 감소함을 알 수 있었다.

국문 주요어: 스테비오사이드, 대체 감미료, 수용체 반응, 덱스트란수크라아제

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