Master's Thesis of Science in Agriculture

Biochemical Properties of Novel Steviol Glucosides Synthesized by Using Dextransucrase

덱스트란수크라아제를 이용한 신규 스테비올 배당체의

생화학적 특성 연구

February 2019

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Abstract

Steviol glycosides is nature, non-caloric sweet-tasting organic molecules, present in extracts of the scrub plant *Stevia reabaudiana*. Steviol glycosides are composed of various glycosides such as stevioiside, rebaudioside A-E, rubusoside, dulcoside A and have a sweetness of about 30 to 150 times that of sugar, and has thermal stability, pH stability, and non-fermentable characteristics and is therefore used as an alternative sweetener. However, the bitter aftertaste of steviol glycosides restricts its use in consumer foods and beverages. In this study, enzymatic transglucosylation using dextransucrase from *Leuconostoc mesenteroides* B-512FMCM was used in the biotransformation of steviol glycosides to partially reduce bitter aftertaste of steviol glycosides. Using RSM, the conversion of stevioside and rebaudioside A was estimated to be 86.8% and 73.6% at the optimal conditions of steviol glycosides 37.3 mg / mL, sucrose 381.3 mM and 2.7 U/mL. Each puried steviol glucosides was purified using HP-20 resin, HPLC-PDA equipped with NH_2, C18 column. The structures of steviol glucosides ware determined as 13-O-β-sophorosyl-19-O-β-isomaltosyl-steviol, 13-O-[β-(1→6)glucosyl]-β-glucosylsophorosyl-19-O-β-isomaltosyl-steviol, 13-O-β-sophorosyl-19-O-β-isomaltotriosyl-steviol, 13-O-[α-neohesperidosyl-(1→3)-β-glucosyl-19-O-β-isomaltosyl-steviol, 13-O-[β-sophorosyl-(1→3)-β-glucosyl]-19-O-β-isomaltosyl-steviol by MALDI-TOF and NMR. Steviol glucosides have higher stability under pH 2, at 60 ℃ condition and have the property of reducing the insoluble glucan formation of mutansucrase. In addition, Steviol glucosides have a very high level of water-solubility enhancement when dissolved in water with water-insoluble compounds such as pterostilbene, curcumin and idebenone. This

improvement in water solubility makes it possible to expect an improvement in bioavailability.

Keywords : Steviol glycosides, Transglucosylation, Dextransucrase

Student number : 2017-29821

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Introduction

1. Steviol glycosides

Steviol glycosides [1, 2] is nature, non-caloric sweet-tasting organic molecules, present in extracts of the scrub plant *Stevia reabaudiana*. These compounds are glycosides of steviol, a diterpene compound. Specifically, their molecules can be viewed as a steviol molecule, with its carboxyl hydrogen atom replaced by a glucose molecule to form an ester, and a hydroxyl hydrogen with the combinations of glucose and rhamnose to form an acetal. *Stevia reabaudiana* contains more than 30 steviol glycosides, such as stevioside, rebaudioside A-E, rubusoside and dulcoside A, at various concentrations [3]. The steviol glycoside has a sweetness of about 30 to 150 times that of sugar, and has thermal stability, pH stability [4], low calories [5, 6] and non-fermentable characteristics and is therefore used as an alternative sweetener. Most methods for the purification of steviol glycosides entailed initial extraction into an aqueous solvent, followed by refinement involving one or more of selective extraction into a polar organic solvent, decolorization, precipitation, coagulation, adsorption, ion exchange, and crystallization [7]. In the United States, high-purity stevia glycoside extracts are generally recognized as safe (GRAS) since 2008 and allowed as ingredients in food products [8], but stevia leaf and crude extracts do not have GRAS or Food and Drug Administration (FDA) approval for use in food.

1.1. Stevioside

Stevioside (Ste, 13-*O*-β-sophorosyl-19-*O*-β-glucosyl-steviol) is one of the steviol glycosides, the most abundant compound in *Stevia rebaudiana(Bertoni).* Stevioside is a diterpenoid glycoside, comprising an aglycone (steviol) and three molecules of glucose[9]. Stevioside was discovered in 1931 by French chemists who gave it its name and well known for 150-250 times sweeter than sugar and has thermal stability, pH stability, low-caloric, anti-diabetic [10] and non-fermentable characteristics and is therefore used as an alternative sweetener in several countries. However, the bitter aftertaste of stevioside restricts its use in consumer foods and beverages. Also pure stevioside is poor in water solubility because it crystallizes when dissolved in water [11]. To overcome this problem, many researchers have attempted to improve this weakness by using enzyme-based modification or biotransformation technique [1].

1.2. Rebaudioside A

Rebaudioside A (Reb A, 13-*O*-β-sophorosyl-(1-3)-β-glucosyl-19-*O*-β-glucosyl-steviol) [12, 13] is one of the steviol glycosides, the second abundant compound in *Stevia rebaudiana(Bertoni).* Generally, it has about 200-300 times the sweetness of sugar. Rebaudioside A has one more glucosyl residue than stevioside, so it is soluble in water even in its pure form [13], and it is more sweeter than stevioside and its bitter aftertaste is also eliminated or reduced. Like stevioside, it is used as an alternative sweetener with thermal stability, pH stability, low calorie and non-fermentable characteristics.

1.3. Rebaudioside C

Rebaudioside C (Reb C, 13-*O*-[α-neohesperidosyl-(1-3)-β-glucosyl]-19-*O*-β-glucosyl-steviol) [14, 15] , also known as dulcoside B, is one of the steviol glycosides and is present in *Stevia rebaudiana(Bertoni)* small amount. Rebaudioside C is comprising an aglycone (steviol) and two molecules of glucose and one molecule of rhamnose. it has about 30 times the sweetness of sugar. However, rebaudioside C has very low water solubility.

2. Transglycosylation and acceptor reaction

Transglycosylation catalyzed by enzymes has been used to improve the physicochemical properties (such as water solubility and oxidative stability) of various compounds to enter cells throughout sodium-glucose co-transporter 1 (SGLT1). Acceptor reaction is an enzymatic transglycosylation which is caused to aglycon attached glyco-oligomer. Aglycone, also called as an acceptor, could be broad range of compounds that have more than one hydroxyl group or carboxyl group including saccharides, polyphenols, flavonoids, protein, lipids, and other organic molecules. In this reaction, a small sugar, which also called ‘donor’, is broken by enzyme and its glycosyl residue is attached on acceptor[16].

3. Dextransucrase from *Leuconostoc mesenteroides* 512-FMCM

Dextransucrase is an enzyme that catalyzes a chemical reaction, using sucrose as a substrate to form fructose and glucosyl residues and then glucosyl residues are transferred to the reducing end of a growing glucanosyl chain, which is covalently linked to the active site of the enzyme [17]. *L. mesenteroides* B-512F [18] 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. *L. mesenteroides* B-512FMC [19] was developed as the first constitutive dextransucrase mutant of *L. mesenteroides* B-512F. *L. mesenteroides* B-512FMCM [20], 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.

4. Purpose of this study

Transglucosylation of stviol glycosides [21] could be a solution for not only sweetness improvement but also finding out new functional properties such as solubilization ability and stability improvement in water solution.

In this study, novel steviol glucosides were synthesized with sucrose as the glucose unit donor and dextransucrase from *L. mesenteroides* B-512FMCM. The effects of reaction factors based on the conversion yields of stevioside or rebaudioside A to steviol glucosides products were optimized using response surface methodology (RSM). After transglucosylation, function-improved materials were obtained from purification process. The structures and biochemical function of steviol glucosides were determined.

Materials and Methods

1. Enzyme preparation

L. mesenteroides B-512FMCM, a constitutive mutant, was developed by vaccum ultraviolet radiation induced mutation of *L. mesenteroides* B-512FMC for dextransucrase production, *L. mesenteroides* B-512FMCM showed complete constitutivity, 13 times higher efficiency, and over 100 times more production yiel than *L. mesenteroides* B-512F [18] . Dextransucrase was performed from glucose fermentation of *L. mesenteroides* B-512FMCM and subsequently the enzyme was purified as previously reported [20]. 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 Na-Ac buffer (pH 5.2) at 28℃. TLC was used to measure the amount of fructose, and the AlphaEaseFc Image Program (Alpha Inotech, USA) was used to quantify the amount.

2. Transglucosylation of steviol glycosides

Transglucosylation was conducted in reaction mixture containing 50 mg/mL steviol glycosides in water, 500 mM sucrose, and 5 U/mL dextransucrase and 20 mM sodium acetate buffer (pH 5.2). The raction mixture was incubated at 28℃ for 12h and then kept at 70℃ for 5 min to halt enzyme activation. One µL aliqout of reaction mixtures were spotted onto silica gel 60 F_{254} TLC plate (Merck Co, Darmstadt, Germany) and developed in nitromethane: n-propyl alcohol: water (2:5:1.5, v/v/v) solvent. steviol glycosides (Ste, RebC, RebA) were visualized by dipping the plate into a solvent mixture of 0.5% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride [22] and 5% (w/v) sulfuric acid in methanol followed by heating at 125℃ for 5 min.

3. Optimization of acceptor reaction using RSM

The central composite design (CCD) of RSM software program (Design Expert 11.1.0.1, USA) was used to optimize conversion of stevioside and rebaudioside A to steviol glucosides with following three variables; steviol glycosides concentration (20-40 mg/mL), sucrose concentration (250-750 mM), and enzyme concentration (1-3 U/mL). Twenty runs of the experiment were carried out with six replications at the central point, which were utilized in the fitting of a second-order response surface. Statistical and mathematical analyses of the results were performed interactions using Design-Expert 11.1.0.1 to determine the effects of independent 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.

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j$$

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.

4. Purification of steviol glucosides using HP-20

The reaction mixture was added to a final volume of 60% ethanol, followed by centrifugation at 8000 rpm for 10 minutes to precipitate the polymer, and the supernatant was separated. The ethanol present in the supernatant was removed under vacuum at 45℃ using a rotary evaporator (Heidolph, Schwabach, Germany). The concentrated reaction solution was loaded on a 4 x 120 mm open column packed with HP-20 resin, and monosaccharide and disaccharide were removed using water. And then steviol glucosides were eluted with ethanol. The eluted solution was concentrated using a rotary evaporator and lyophilized using a freeze dryer (EYELA, Japan).

5. Purification of steviol glucosides using HPLC

After removal of saccharides, lyophilized sample was prepared at 200 mg/mL. Sample was analyzed using HPLC (Waters Modular system with Waters PDA detector, Waters, Milford, MA, USA) with a Phenomenex Luna NH_2 (5 μm, 2.6 mm X 250 mm, Phenomenex, Torrance, USA) column and was detected at 210 nm. The mobile phase was acetonitrile (A) and water (B) with a gradient elution as follows: 17% to 23% B from 0 to 30 min, 23% to 20% B from 30 to 33 min, 20% to 25% B from 33 to 40 min, 25% to 40% B from 40 to 47 min, 40% to 90% B from 47 to 52 min, 90% B from 52 to 62 min, 90% to 17% B from 62 to 67 min, 17% B from 67 to 75 min at 1 mL/min flow rate.

In the above conditions, Phenomenex Luna NH_2 (5 μm, 21.2 mm X 250 mm, Phenomenex, Torrance, USA) was used for scale-up purification. The peaks that could not be separated were subjected to the second purification using Phenomenex Luna C18 column (5 μm, 21.2 mm X 150 mm, Phenomenex, Torrance, USA).

6. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis

Each purified steviol glucosides was diluted with DMSO-d_6 and mixed with 2,5-dihydroxy benzoic acid. The mass spectrum was obtained using a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, USA). Mass spectra were obtained in positive reflector mode with a delayed extraction method at an acceleration voltage of 25 kV.

7. Nuclear magnetic resonance (NMR) analysis

Each purified stevioside glucoside (10 mg) was dissolved in DMSO-d_6 and placed into 5 mm NMR tubes. NMR spectra was recorded on an AVANCEIII HD system (Bruker, Germany) operated at 850 MHz for ^{1}H and ^{13}C at 25℃. Linkage between steviol glycosides and glucose were evaluated using 1D (^{1}H, ^{13}C), 2D [COSY (homonuclear correlation spectroscopy), HSQC (heteronuclear single-quantum coherence), HMBC (heteronuclear multiple-bond correlation), TOCSY (Total correlated spectroscopy)] data.

8. Stability of stevioside steviol glucosides in extreme pH and temperature condition

The degradation of pure purified steviol glycoside (Ru, Ste, Ste-G1, Ste-G2, Ste-G2', RebC, RebC-G1, RebA or RebA-G1) was analyzed at pH 2.0 and 60℃. Each sample was dissolved in water of pH 2 at a concentration of 10 mg/mL in an eppendorf tube. Then samples were incubated in a 60 ℃ water bath and analyzed for stability [4] at 6 h, 12 h, 24 h, and 48 h. The degradation experiments were performed in triplicate for each sample.

9. Reduction of insoluble glucan formation

Mutansucrase from *S. mutans* was produced as previously reported [23, 24]. The efficiency of insoluble glucan formation by steviol glucosides was carried out in reaction mixture containing 0.1 U/mL mutansucrase, 100 mM sucrose, and 10 mg/mL samples in 20 mM Na-P buffer (pH 6.5) at 37ºC for 12 h. The reaction control contained the same reaction mixture except the same volume of water instead of sample solution. After 12 h, the reaction mixtures were centrifuged at 12,000 rpm for 20 min. The pellets were washed with distilled water for several times. The insoluble glucans in the reaction mixtures were dissolved in 1 M NaOH and spoted on TLC. TLC were developed in nitromethane: n-propyl alcohol: water (2:5:1.5, v/v/v) solvent and visualized by dipping the 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 125℃ for 5 min. The insoluble glucan formation was determined by measuring the amount of total carbohydrate as the insoluble glucan.

10. Solubilization ability of steviol glucosides for Insoluble compounds

Idebenone, pterostilbene and curcumin were purchased from TCI chemical, Sigma, respectively. Five mg of each insoluble compound (Idebenone, pterostilbene, or curcumin) was mixed with 50 mg of steviol glucosides (Ru, Ste, Ste-G1, Ste-G2, RebC, RebC-G1, RebA or RebA-G1) and then 500 μL ethanol was added to the mixture. The mixture solution was put on auto-shaker for 30 min at room temperature and centrifuged at 12,000 rpm for 15 min to remove the pellet. The ethanol was removed using speedvac (VISION, Korea). Then, 500 μL water was added, vortexed and centrifuged at 12,000 rpm for 15 min at 25 ℃. The concentration of each compound in solution was determined using UPLC-MS, PDA (Waters Acquity H-Class system with Waters QDA, PDA detector and Waters BEH C18 1.7 μm x 2.1 mm x 100 mm column, Waters, USA) with standard curves.

Results and Discussion

1. Synthesis of steviol glucosides using dextransucrase from *L. mesenteroides* B-512FMCM

The results of steviol glycosides acceptor reaction involving dextransucrase from *L. mesenteroides* B-512FMCM with steviol glycosides and sucrose are shown in **Figure 1**.

Steviol glycosides used in the reaction and saccharides (sucrose, glucoside, fructose or maltooligosaccharides) were removed and the components were analyzed by HPLC-PDA at 210 nm. The results are shown in **Figure 2**.

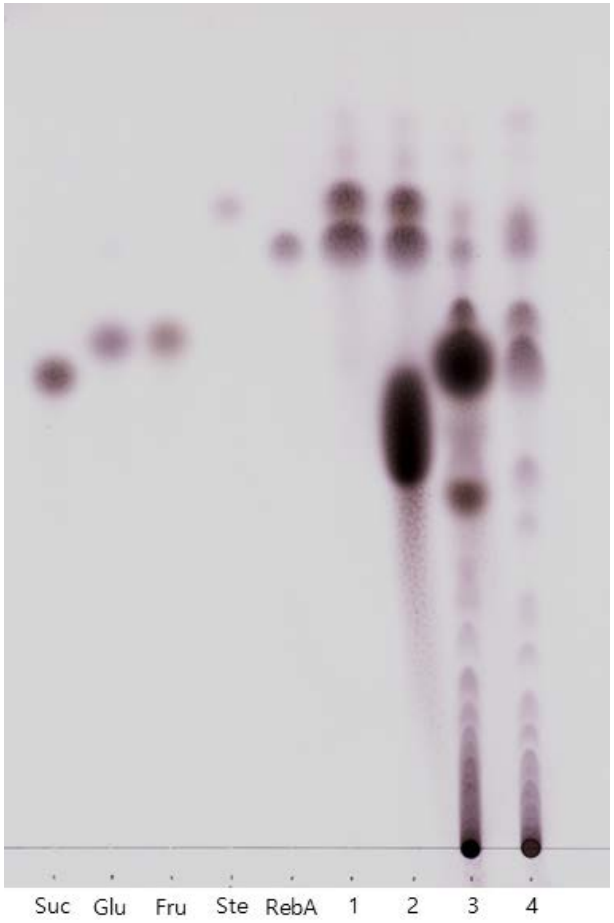
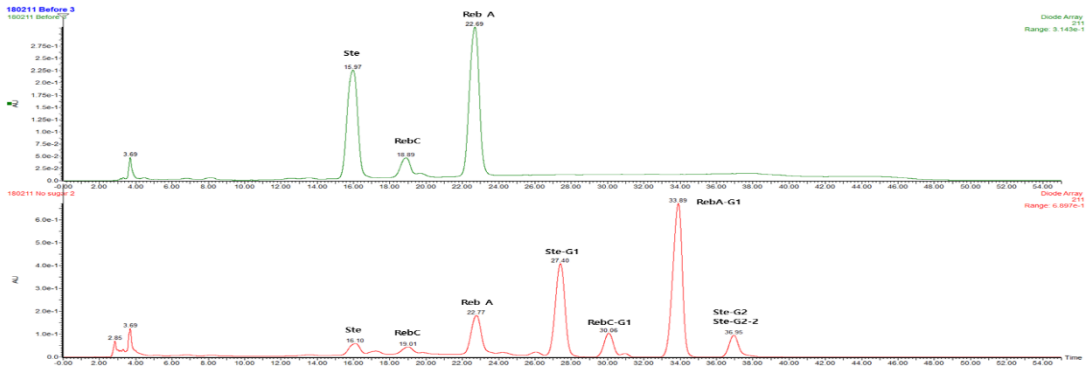


Figure 1. Thin layer chromatography of steviol glucosides synthesized using dextransucrase. Suc: 20 mM sucrose; Glu: 20 mM glucose; Fru; 20 mM fructose; Ste: stevioside; RebA: rebaudioside A; Lane 1: steviol glycosides; Lane 2: mixture before acceptor reaction; Lane 3: products of acceptor reaction; Lane 4: glucosylation products after removing saccharides using HP-20 resin.

(A)



(B)

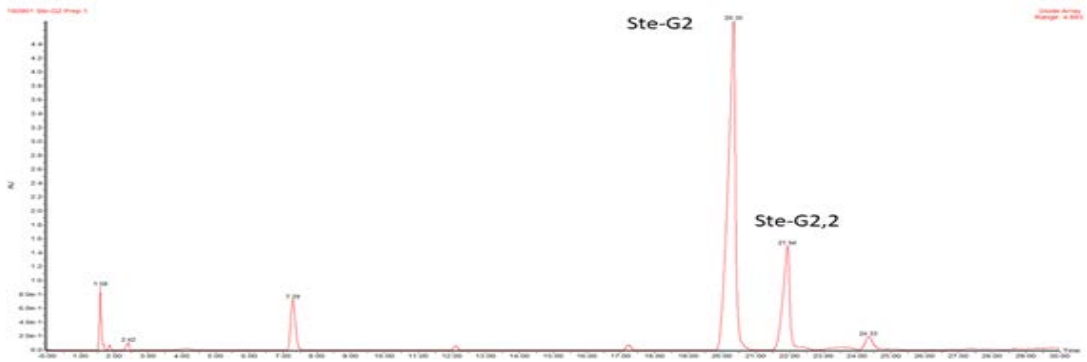


Figure 2. HPLC chromatogram of steviol glucosides. (A) using NH_2 column (B) using C18 column

2. Optimization of transglucosylation of steviol glucosides using RSM

Response surface methodology (RSM) is a statistical technique for modelling and optimizing with multiple variables. It can be used to determine the optimum process conditions by combining experimental designs with interpolation by first- or second-order polynomial equations in a sequential testing procedure [25]. In this study, RSM was progressed with following three independent variables: sucrose concentration (250-750 mM), steviol glycosides concentration (20-40 mg/mL), and enzyme concentration (1.0-3.0 U/mL). The predicted and actual conversion yields of stevioside and rebaudioside A are summarized in **Table 1**. The 3D response surface and 2D contour plots of independent variables with respect to the response are shown in Figure 3, 4 and results of ANOVA (Analysis of variance) are shown in **Table 2, 3**.

The experimental data had a determination coefficient (R^2) of 0.9784, 0.9779 and adjusted coefficient (Adj R^2) of 0.9590, 0.9579, meaning that the calculated model was able to explain 97.84%, 97.79% and 95.90%, 95.79% of results, respectively. This indicated that the model used to fit the independent response variables was significant ($p < 0.001$). The amount of steviol glycosides converted into steviol glucosides was expressed with the following regression equation:

$$Y = (68.94569 + 0.669318X_1 + 0.027144X_2 + 2.29313X_3 - 0.000165X_1X_2 - 0.003750X_2X_3 + 0.002050X_1X_3 - 0.009452X_1^2 - 0.000026X_2^2 - 0.0945237X_3^2)/X_2 \times 100$$

$$Y = (63.88875 + 0.374808X_1 + 0.015470X_2 + 1.75269X_3 + 0.000110X_1X_2 + 0.010000X_2X_3 + 0.001900X_1X_3 - 0.007423X_1^2 - 0.000022X_2^2 - 0.813115X_3^2)/X_2 \times 100$$

Where Y was steviol glycosides conversion to product (%), X_1 was steviol glycosides concentration for acceptor reaction (mM), X_2 was sucrose concentration (mM), and X_3 was reacted enzyme concentration (U/mL).

The predicted maximum both stevioside and rebaudioside A conversion to steviol glucosides was 86.8% and 73.6% at 37.3 mg/mL steviol glycosides, 381.3 mM sucrose, and 2.7 U/mL of enzyme. To validate the predicted steviol glycosides conversion yield, an experiment was conducted using the above conditions.

Table 1. Running condition for steviol glycosides acceptor reaction and stevioside and rebaudioside A conversion.

Run No.	Independent variables			Stevioside conversion (%)		Rebaudioside A conversion (%)	
	X_1	X_2	X_3	Predicted	Actual	Predicted	Actual
1	30	500	0.32	86.1	86.0	73.1	73.0
2	40	250	3	84.5	84.2	71.1	71.2
3	20	250	3	83.0	83.0	71.4	71.3
4	30	500	2	88.2	87.8	75.0	74.8
5	30	500	2	88.2	88.9	75.0	75.6
6	30	500	2	88.2	88.3	75.0	75.0
7	30	500	2	88.2	88.0	75.0	75.0
8	30	80	2	83.5	83.7	71.0	70.9
9	30	500	3.68	84.9	85	72.3	72.4
10	13.2	500	2	85.0	85.2	72.8	72.6
11	46.8	500	2	85.9	85.8	71.7	73.2
12	20	750	1	84.7	84.9	72.7	71.6
13	40	750	3	84.7	85.0	72.7	72.4
14	40	250	1	86.1	86.3	72.4	72.2
15	30	920	2	83.6	83.4	71.1	71.2
16	40	750	1	84.3	84.3	72.1	72.2
17	20	250	1	84.8	84.5	73.1	73.4
18	30	500	2	88.15	87.9	75.0	74.8
19	20	750	3	84.92	84.7	71.9	72.1
20	30	500	2	88.15	88.0	75.0	74.8

X_1, the concentration of steviol glycosides;

X_2, the concentration of sucrose;

X_3, the concentration of enzyme.

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

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F Statistic	p-value Prob > F
Model	61.01	9	6.78	50.43	< 0.0001
A-Ste	1.01	1	1.01	7.50	0.0209
B-Sucrose	0.0115	1	0.0115	0.0855	0.7760
C-Enzyme	1.67	1	1.67	12.46	0.0055
AB	1.36	1	1.36	10.13	0.0098
AC	0.0112	1	0.0112	0.0837	0.7783
BC	2.10	1	2.10	15.63	0.0027
A^2	12.84	1	12.84	95.49	< 0.0001
B^2	38.46	1	38.46	286.09	< 0.0001
C^2	12.84	1	12.84	95.49	< 0.0001
Error	1.34	10	0.1344		
Total	62.35	19			

$R^2 = 0.9784$

Adj $R^2 = 0.9590$

Table 3. Results of two-way analysis of variance (ANOVA) of rebaudioside A.

Source of variation	Sum of vquares	Degrees of freedom	Mean squares	F Statistic	p-value Prob > F
Model	42.42	9	4.71	49.07	< 0.0001
A-Reb A	0.0271	1	0.0271	0.2820	0.6070
B-Sucrose	0.0363	1	0.0363	0.3781	0.5523
C-Enzyme	0.8512	1	0.8512	8.86	0.0139
AB	0.6050	1	0.6050	6.30	0.0309
AC	0.0800	1	0.0800	0.8328	0.3829
BC	1.81	1	1.81	18.79	0.0015
A^2	7.91	1	7.91	82.39	< 0.0001
B^2	28.06	1	28.06	292.15	< 0.0001
C^2	9.50	1	9.50	98.87	< 0.0001
Error	0.9606	10	0.961		
Total	43.39	19			

$R^2 = 0.9779$

Adj $R^2 = 0.9579$

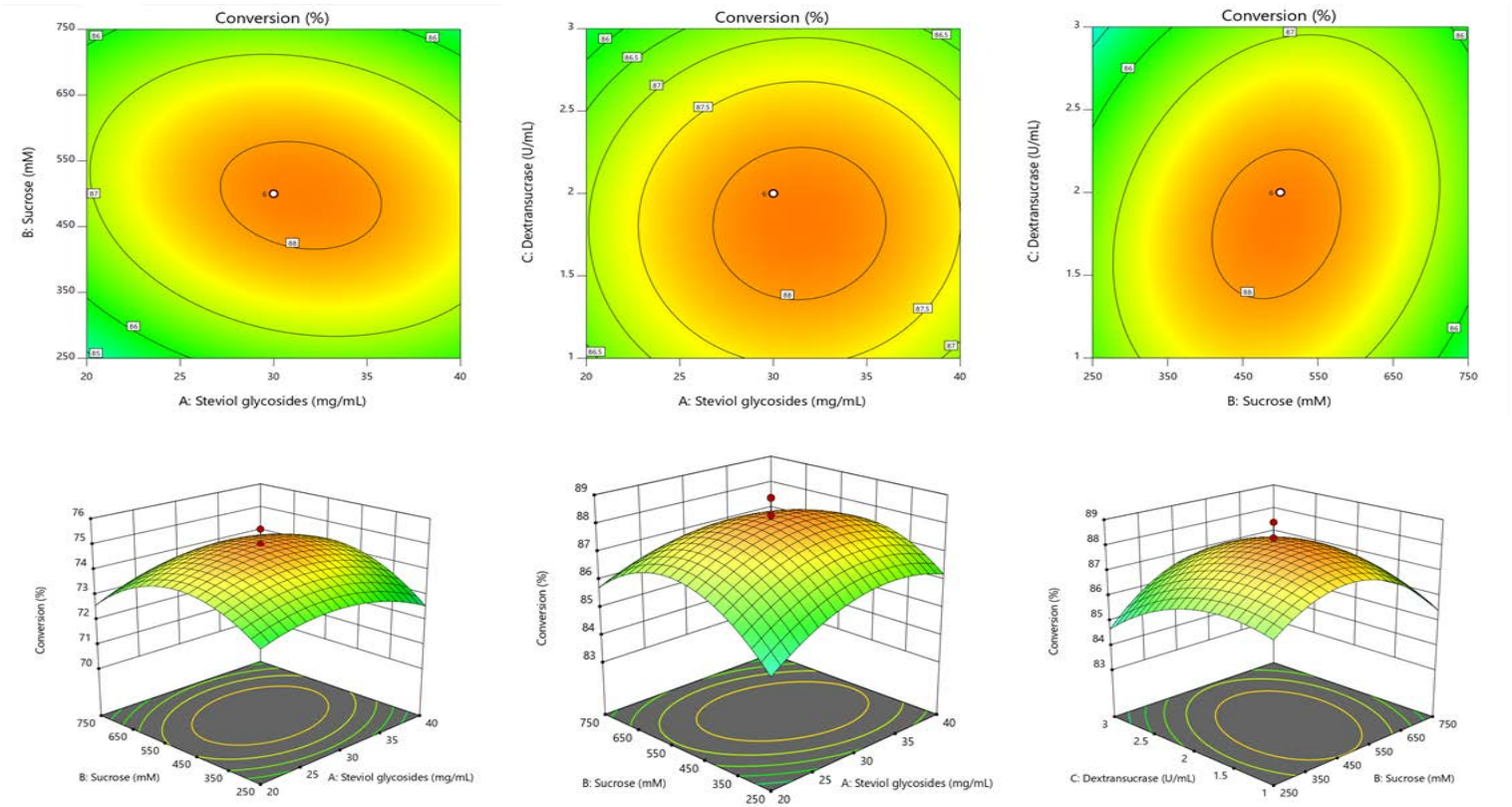


Figure 3. Response surface plot and contour plot of stevioside conversion.

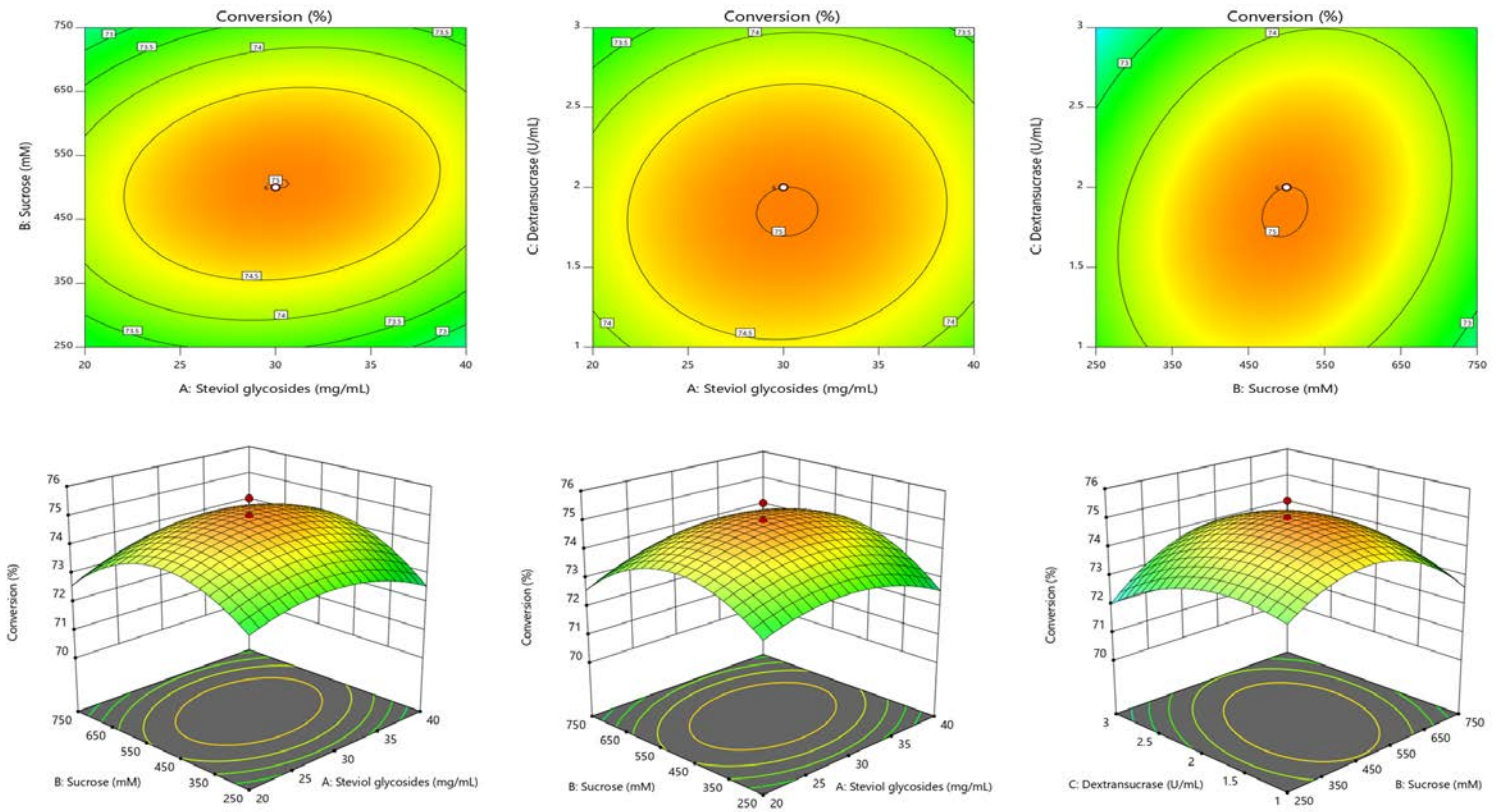


Figure 4. Response surface plot and contour plot of rebaudioside A conversion.

3. Purification of steviol glucosides

Saccharides were removed steviol glucosides mixture using HP-20 resin and purified each steviol glucosides were obtained using HPLC (**Figure 5**). Each compound was purified by HPLC-PDA equipped with NH_2 column (**Figure 2**). Ste-G1, RebC-G1, RebA-G1 were confirmed as a single peak with high purity. However, Ste-G2 and Ste-G2' were performed a second purification using C18 column (**Figure 2**) because the peaks overlapped each other. High purification rate of pure purified STV was confirmed by thin layer chromatography.

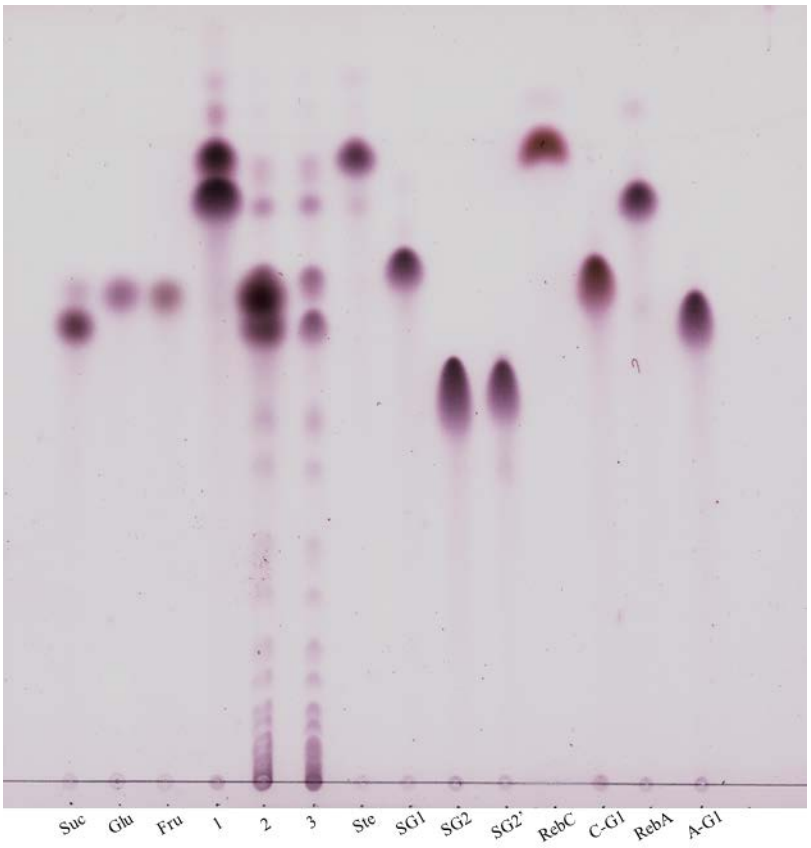


Figure 5. Thin layer chromatography of purified steviol glucosides.

Suc: 20 mM sucrose; Glu: 20 mM glucose; Fru; 20 mM fructose; Lane 1: steviol glycosides; Lane 2: acceptor reaction products; Lane 3: glucosylation products after removing saccharides using HP-20 resin; Ste: stevioside; SG1: stevioside glucoside 1; SG2: stevioside glucoside 2; SG2': stevioside glucoside 2'; RebC: rebaudioside C; C-G1: rebaudioside C glucoside 1; RebA: rebaudioside A; A-G1: rebaudioside A glucoside 1.

4. Structure determination by NMR analysis

Structures of steviol glucosdes were determined by MALDI-TOF-MS and NMR (^{1}H, ^{13}C, COSY, HSQC, and HMBC, TOCSY). Results are summarized in **Figure 6** and **Table 4**.

Ste-G1

The molecular ions of Ste-G1 were observed at m/z 989 $(M + Na)^{+}$, indicating that one glucosyl residue was attached to stevoside. A doublet signal at 4.63 ppm (J = 3.32 Hz) was assigned to the anomeric proton, indicating that one glucose unit was α-linked with stevioside. There are some carbon signals identical to those of stevioside except for the following signals: at 72.21 ppm to C-2', at 73.01 ppm to C-5', and at 66.11 ppm to C-6' (**Table 4**). According to these results, the structure of Ste-G1 could be assigned as 13-*O*-β-sophorosyl-19-*O*-β-isomaltosyl-steviol (**Figure 7A**).

Ste-G2

The molecular ions of Ste-G2 were observed at m/z 1151 $(M + Na)^{+}$, indicating that one glucosyl residues were attached to Ste-G1. Two doublet signals at 4.63 ppm (J = 3.57 Hz) and 4.67 ppm (J = 5.27 Hz) were assigned to anomeric proton, indicating that the both glucosyl units were α-linked stevioside. In Table 4, Ste-G2 showed the significant chemical shift change at C-6' indicating that the first glucose (4.63 ppm) was attached to C-6' of stevioside same as the Ste-G1 structure. Additionally, the other anomeric proton (4.67 ppm) of the second glucose was correlated with C-6''' (60.65) of the glucose in HMBC data. From these results, the structure of Ste-G2 could

be assigned as 13-*O*-[β-(1→6)glucosyl]-β-glucosylsophorosyl-19-*O*-β-isomaltosyl-steviol [25] (**Figure 7B**). This substance has been identified as novel steviol glucosides.

Ste-G2'

The molecular ions of Ste-G2 were observed at m/z 1151 $(M + Na)^+$, indicating that one glucosyl residues were attached to Ste-G1. Two doublet signals at 4.67 ppm (J = 3.57 Hz) and 4.62 ppm (J = 5.27 Hz) were assigned to anomeric proton, indicating that both glucosyl units were α-linked with stevioside. In Table 4, Ste-G2' showed the significant chemical shift change at C-6' (65.68 ppm) indicating that the first glucose (4.67 ppm) was attached to C-6' of stevioside same as the Ste-G1 structure. Additionally, the other anomeric proton (4.62 ppm) of the second glucose was correlated with C-6"" (61) of the first glucose in HMBC data. From these results, the structure of Ste-G2 could be assigned as 13-*O*-β-sophorosyl-19-*O*-β-isomaltotriosyl-steviol (**Figure 7C**). This substance has been identified as novel steviol glucosides.

RebC-G1

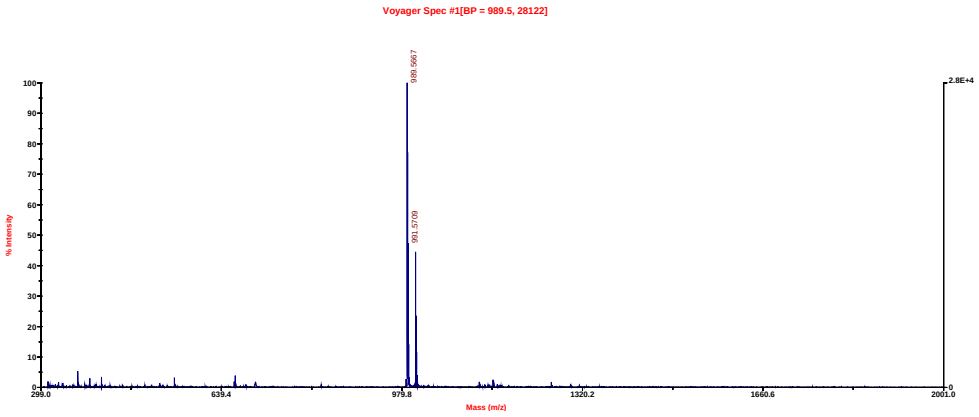
The molecular ions of RebC-G1 were observed at m/z 1135 $(M + Na)^+$, indicating that one glucosyl residues were attached to rebaudioside C. A doublet signal at 4.63 ppm (J = 3.7 Hz) was assigned to the anomeric proton, indicating that one glucose unit was α-linked with rebaudioside C. There are some carbon signals identical to those of rebaudioside C except for the following signals: at 72.21 ppm to C-2', at 76.92 ppm to C-5', and at 62.84 ppm to C-6' (**Table 3**). According to these results,

the structure of RebC-G1 could be assigned as 13-*O*-[α-neohesperidosyl-(1→3)-β-glucosyl-19-*O*-β-isomaltosyl-steviol (**Figure 7D**). This substance has been identified as novel steviol glucosides.

RebA-G1

The molecular ions of RebA-G1 were observed at m/z 1151 $(M + Na)^+$, indicating that one glucosyl residues were attached to rebaudioside A. A doublet signal at 4.64 ppm (J = 4.76 Hz) was assigned to the anomeric proton, indicating that one glucose unit was α-linked with rebaudioside A. There are some carbon signals identical to those of stevioside except for the following signals: at 72.23 ppm to C-2', at 73.14 ppm to C-5', and at 66.15 ppm to C-6' (**Table 4**). According to these results, the structure of RebA-G1 could be assigned as 13-*O*-[β-sophorosyl-(1→3)-β-glucosyl]-19-*O*-β-isomaltosyl-steviol (**Figure 7E**).

(A)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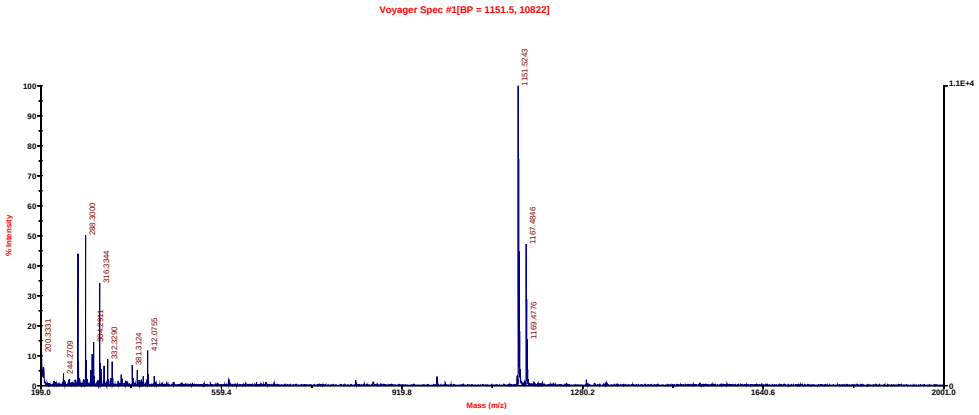
(B)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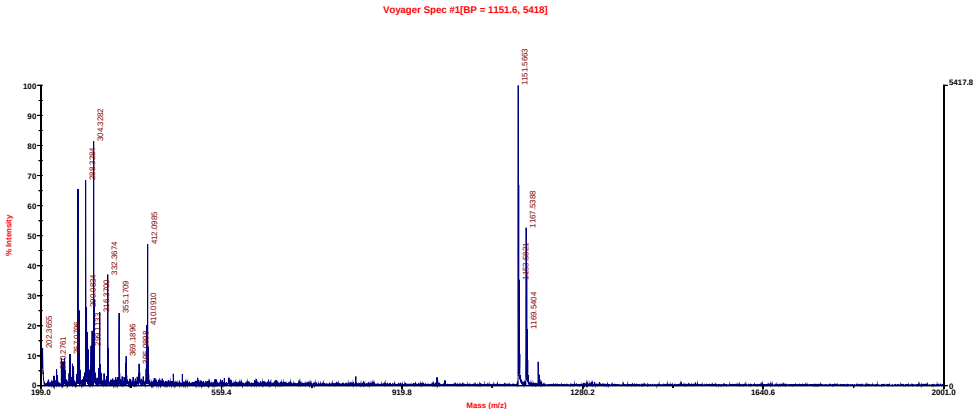
919.8
1280.2
1640.6
2001.0

(C)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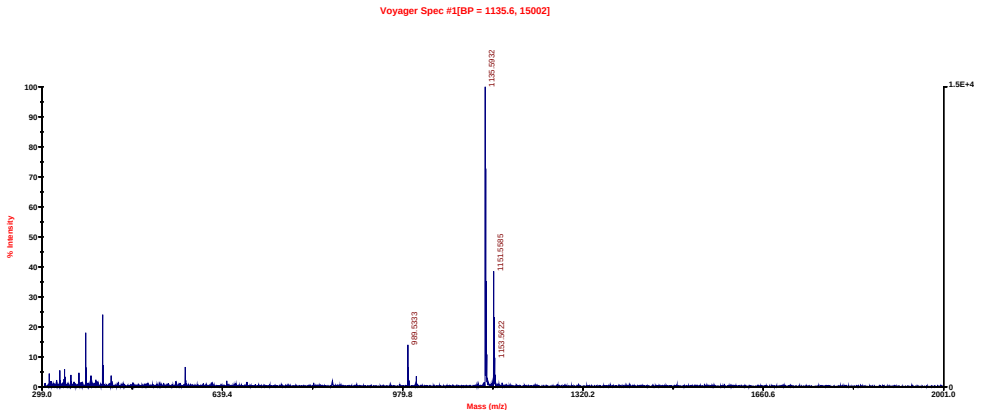
(D)



(E)

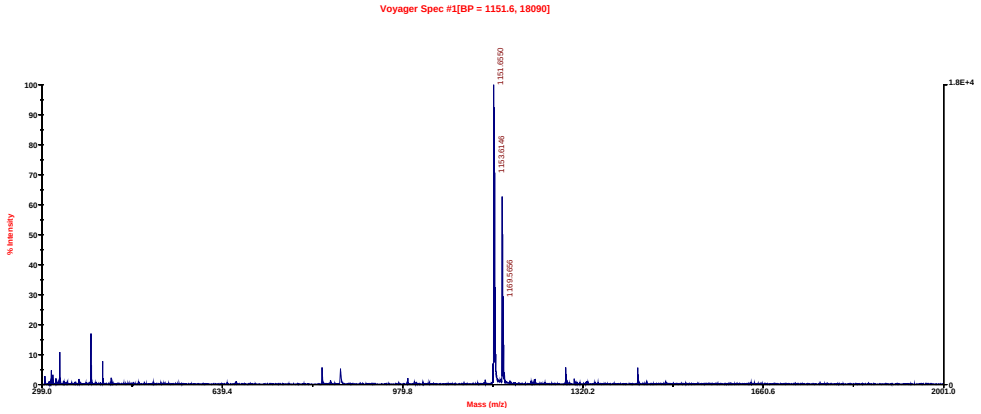


Figure 6. MALDI-TOF-MS spectra of steviol glucosides.

(A) MALDI-TOF-MS spectra of Ste-G1

(B) MALDI-TOF-MS spectra of Ste-G2

(C) MALDI-TOF-MS spectra of Ste-G2’

(D) MALDI-TOF-MS spectra of RebC-G1

(E) MALDI-TOF-MS spectra of RebA-G1

Table 4. ^{13}C and ^{1}H NMR data of steviol glucosides (ppm)

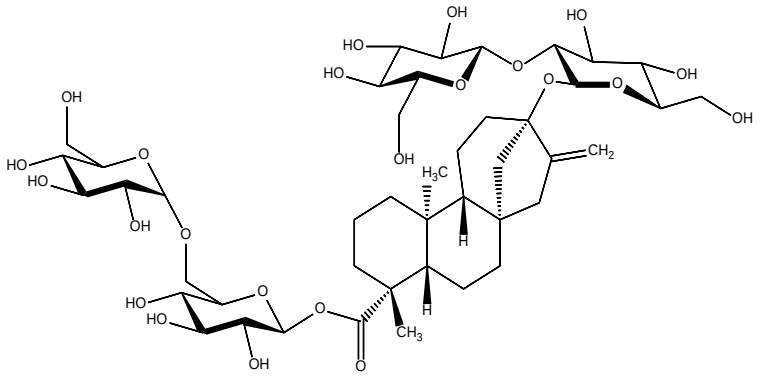
position	Ste-G1		Ste-G2		Ste-G2’		Reb C-G1		Reb A-G1	
Steviol	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	0.77 1.77	40.1	0.76 1.78	40.42	0.76 1.78	40.42	0.76 1.77	40.42	0.77 1.77	40.07
2	1.35 1.76	18.63	1.34 1.79	18.68	1.34 1.79	18.68	1.34 1.78	18.58	1.35 1.75	18.61
3	0.90 2.06	37.4	0.96 2.07	37.42	0.96 2.07	37.42	0.96 2.05	37.37	0.90 2.06	37.42
4		42.06		41.66		41.66		41.69		41.84
5	1.04	56.39	1.03	56.47	1.03	56.47	1.03	56.37	1.04	56.37
6	1.71 1.92	21.24	1.72 1.9	21.19	1.72 1.9	21.19	1.97	21.15	1.70 1.97	21.22
7	1.33 1.46	41.01	1.32 1.54	41.04	1.32 1.54	41.04	1.33 1.47	41.01	1.34 1.44	40.07
8		42.1		42.32		42.32		42.26		41.84

9	**0.90**	**53.17**	**0.9**	**53.45**	**0.9**	**53.45**	**0.90**	**53.14**	**0.90**	**53.23**
10		**38.95**		**38.94**		**38.94**		**38.94**		**38.95**
11	**1.50** **1.69**	**19.98**	**1.47** **1.68**	**19.84**	**1.47** **1.68**	**19.84**	**1.49** **1.69**	**19.96**	**1.48** **1.68**	**19.87**
12	**1.47** **1.87**	**35.54**	**1.47** **1.8**	**35.5**	**1.47** **1.87**	**37.3**	**1.43** **1.84**	**36.65**	**1.48** **1.87**	**36.21**
13		**84.62**		**84.61**		**85.42**		**85.56**		**85.2**
14	**1.38** **2.15**	**43.52**	**1.41** **2.04**	**43.43**	**1.38** **2.15**	**43.2**	**1.58** **2.02**	**43.14**	**1.35** **2.09**	**43.18**
15	**1.98** **2.02**	**46.89**	**1.98** **2.08**	**46.87**	**1.98** **2.02**	**47.25**	**1.97** **2.06**	**47.46**	**1.99** **2.03**	**47.01**
16		**153.64**		**153.63**		**152.79**		**152.76**		**153.23**
17	**4.71** **5.04**	**103.88**	**4.74** **5.03**	**103.87**	**4.71** **5.03**	**104.24**	**4.76** **5.01**	**104.34**	**4.73** **5.04**	**103.9**
18	**1.13**	**27.89**	**1.12**	**28.02**	**1.10**	**28.02**	**1.13** **1.23**	**27.84**	**1.13**	**27.93**
19		**175.69**		**175.74**		**175.55**		**175.69**		**175.65**
20	**0.86**	**15.09**	**0.85**	**15.12**	**0.85**	**15.12**	**0.86**	**14.89**	**0.86**	**14.98**

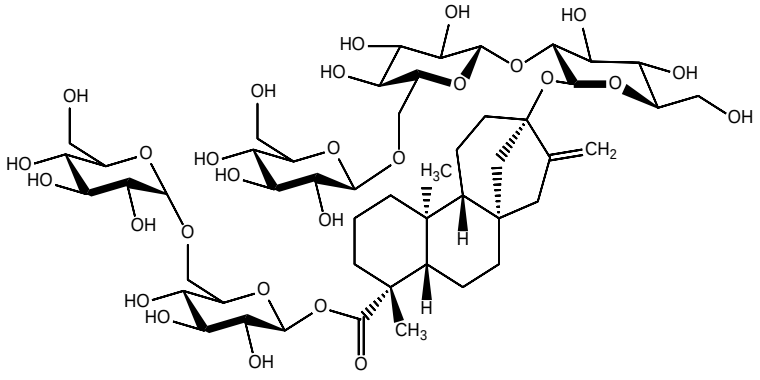
	Glucose-I		Glucose-I		Glucose-I		Glucose-I		Glucose-I	
1’	5.26 (d, *J*=7.91)	93.97	5.29 (d, *J*=8.16)	93.86	5.26 (d, *J*=8.16)	93.98	5.24 (d, *J*=8.21)	94.04	5.27 (d, *J*=8.16)	93.97
2’	3.18	72.21	3.21	71.94	3.18	72.00	3.17	72.21	3.18	72.23
3’	3.25	76.86	3.17	75.93	3.25	76.87	3.25	71.81	3.26	76.89
4’	3.20	69.8	3.28	69.81	3.21	69.57	3.05 3.43	70.17	3.20	69.96
5’	3.40	73.01	3.4	72.68	3.39	72.88	3.27 3.22	76.92	3.39	73.14
6’	3.54 3.69	66.11	3.54 3.70	66.1	3.53 3.74	65.68	4.65	62.84	3.54 3.70	66.15
	Glucose-II		**Glucose-II**		**Glucose-II**		**Glucose-II**		**Glucose-II**	
1”	4.45 (d, *J*=7.65)	96.37	4.44	95.99	4.46 (d, *J*=7.7)	96.27	4.46 (d, *J*=7.91)	96.62	4.46 (d, *J*=7.74)	96.64
2”	3.22	82.59	3.23	82.13	3.22	82.57	3.37 3.58	74.45	3.56	86.14
3”	3.37	75.86	3.39	75.58	3.38	76.00	3.58	88.84	3.49	78.87
4”	3.07	70.22	3.1	70.06	3.07	70.07	3.09	69.67	3.16	68.89

5”	**3.08**	**76.12**	**3.04**	**77.01**	**3.08**	**76.08**	**3.13**	**75.77**	**3.13**	**75.99**
6”	**3.49** **3.56**	**60.55**	**3.54**	**60.4**	**3.49**	**60.63**	**3.43**	**69.83**	**3.69** **3.54**	**60.85**
	Glucose-III		**Glucose-III**		**Glucose-III**		**Glucose-III**		**Glucose-III**	
1”’	**4.35** **(d, *J*=7.65)**	**104.63**	**4.37** **(d, *J*=7.8)**	**104.38**	**4.36** **(d, *J*=7.8)**	**104.53**	**4.32** **(d, *J*=7.86)**	**103.02**	**4.42** **(d, *J*=7.91)**	**103.04**
2”’	**3.01**	**75.24**	**3.05**	**75.2**	**3.02**	**75.22**	**3.02** **3.44**	**73.35**	**3.03**	**73.67**
3”’	**3.16**	**75.91**	**3.21**	**76.2**	**3.16**	**75.85**	**3.09**	**69.91**	**3.19**	**76.93**
4”’	**3.16**	**69.58**	**3.11**	**69.86**	**3.16**	**69.59**	**3.55** **3.7**	**68.93**	**3.16**	**69.83**
5”’	**3.05**	**76.96**	**3.03**	**76.91**	**3.04**	**76.94**	**3.04**	**69.74**	**3.05**	**76.64**
6”’	**3.45** **3.52**	**60.66**	**3.59**	**60.65**	**3.56**	**60.57**	**3.45** **3.52**	**60.56**	**3.45** **3.52**	**60.68**
	Glucose-IV		**Glucose-IV**		**Glucose-IV**		**Rhamnose-I**		**Glucose-IV**	
1””	**4.63** **(d, *J*=7.65)**	**98.32**	**4.63** **(d, *J*=3.57)**	**98.1**	**4.67** **(d, *J*=3.57)**	**98.01**	**5.27**	**100.21**	**4.63**	**98.33**
2””	**3.40**	**72.29**	**3.17**	**72.56**	**3.16**	**72.00**	**3.2**	**68.84**	**3.41**	**72.40**

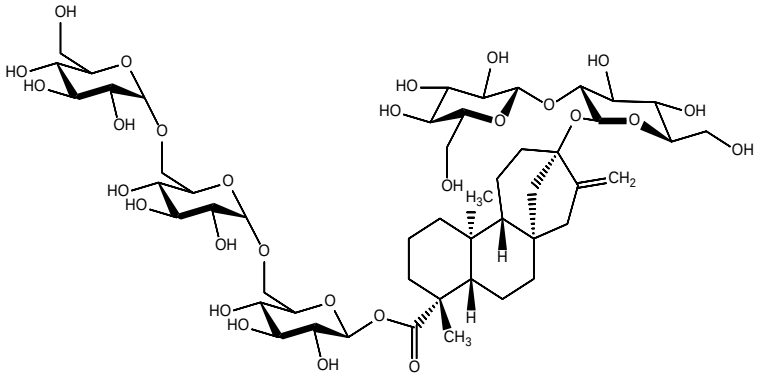
3’’’’	**3.39**	**75.61**	**3.41**	**75.61**	**3.26**	**75.58**	**3.95**	**68.05**	**3.39**	**75.58**
4’’’’	**3.08**	**69.9**	**3.09**	**69.77**	**3.17**	**69.59**	**3.66**	**60.81**	**3.05**	**70.06**
5’’’’	**3.16**	**75.91**	**3.28**	**72.25**	**3.53**	**75.71**	**3.74**	**72.01**	**3.17**	**76.55**
6’’’’	**3.42** **3.67**	**60.95**	**3.46** **3.67**	**60.54**	**3.46** **3.68**	**61**	**1.07**	**18.16**	**3.42** **3.61**	**60.96**
	Glucose-V		**Glucose-V**		**Glucose-V**		**Glucose-IV**		**Glucose-V**	
1’’’’’			**4.67** **(d,*J*=5.27)**	**98.25**	**4.62** **(d,*J*=5.27)**	**97.92**	**4.63** **(d, *J*=3.7)**	**98.32**	**4.64** **(d, *J*=4.76)**	**102.46**
2’’’’’			**3.43**	**72.43**	**3.2**	**71.8**	**3.41**	**72.39**	**2.98**	**74.53**
3’’’’’			**3.21**	**69.6**	**3.4**	**72.88**	**3.7** **3.54**	**66.09**	**3.14**	**76.41**
4’’’’’			**3.39**	**75.61**	**3.17**	**69.59**	**3.39**	**75.66**	**3.04**	**70.23**
5’’’’’			**3.05**	**74.9**	**3.64**	**71.97**	**3.69**	**60.92**	**3.12**	**75.99**
6’’’’’			**3.67**	**66.14**	**3.49**	**60.52**	**3.39**	**60.68**	**3.41** **3.69**	**61.17**



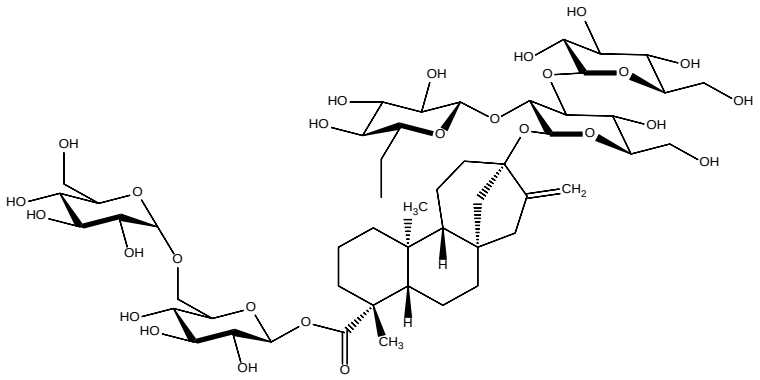
(A) Ste-G1 (13-*O*-β-sophorosyl-19-*O*-β-isomaltosyl-steviol)



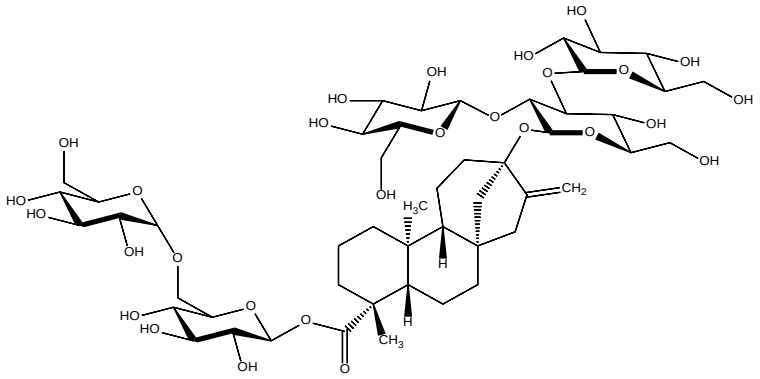
(B) Ste-G2 (13-*O*-[β-(1→6)glucosyl]-β-glucosylsophorosyl-19-*O*-β-isomaltosyl-steviol)



(C) Ste-G2’ (13-*O*-β-sophorosyl-19-*O*-β-isomaltotriosyl-steviol)



(D) RebC-G1 (13-*O*-[α-neohesperidosyl-(1→3)-β-glucosyl-19-*O*-β-isomaltosyl-steviol)



(E) RebA-G1 (13-*O*-[β-sophorosyl-(1→3)-β-glucosyl]-19-*O*-β-isomaltosyl-steviol)

Figure 7. Structures of steviol glucosides.

5. Stability of steviol glucosides under extreme condition

In aqueous solutions, steviol glycosides were remarkably stable at a wide range of temperatures and pH. However, chemical degradation of sweeteners inevitably occurs at extreme temperature and pH conditions. Therefore, in order to confirm the stability of steviol glucosides in water, it was confirmed by accelerated stability experiment in extreme environment [4].

After 48 hr, Ste-G1 showed about 3% higher stability compared to stevioside, Ste-G2 about 15% higher stability compared to stevioside, and Ste-G2' about 9% higher stability compared to stevioside. RebA-G1 also showed about 3% higher stability than rebaudioside A **(Table 5)**.

The changes of steviol glucosides were observed for 48 hr, It was confirmed that the steviol glucosides produced by the acceptor reaction has higher stability than the steviol glycosides before the reaction.

As a result, it was confirmed that most steviol glucosides showed higher stability as the glucosyl residue increased.

Table 5. Stability of steviol glucosides at extreme condition.

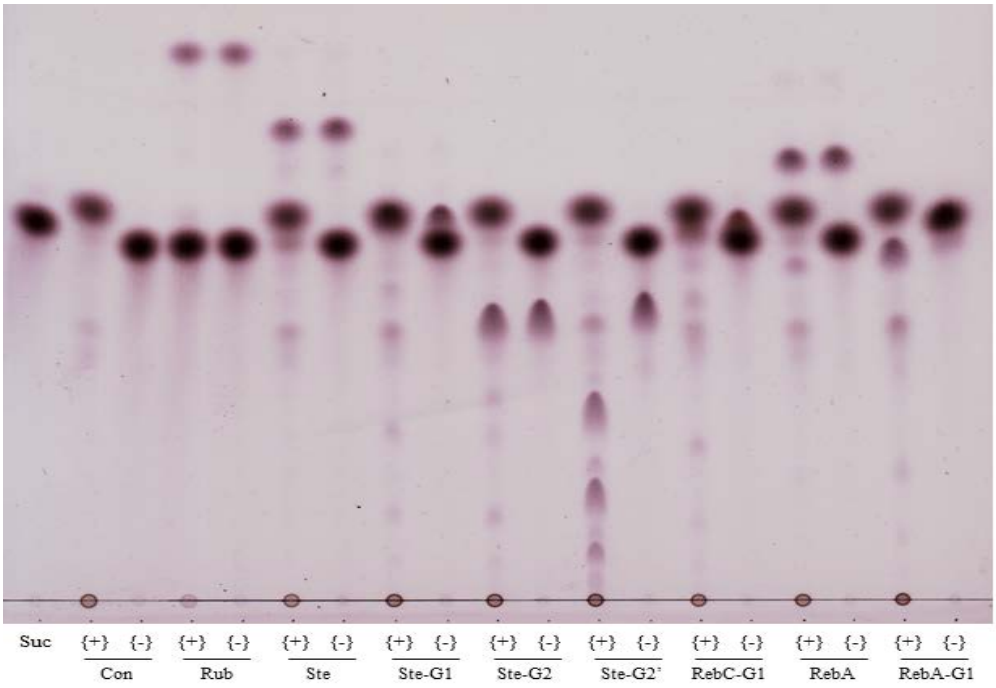
Stability of steviol glucosides							
Time	**Steviol glucosides concentration (%)**						
	Ste	**Ste-G1**	**Ste-G2**	**Ste-G2'**	**RebC-G1**	**RebA**	**RebA-G1**
0 h	100	100	100	100	100	100	100
6 h	96.36±1.29	100	98.78±1.06	100	96.70±2.80	95.65±1.18	100
12 h	95.45±2.23	96.71±1.14	97.25±0.83	97.06±4.16	96.70±1.55	92.27±1.81	99.26±1.82
24 h	91.82±2.57	91.51±1.23	92.89±3.82	91.73±2.11	96.70±3.11	90.34±2.46	95.91±1.58
48 h	77.54±0.39	77.37±0.39	92.89±4.12	86.76±1.27	96.15±1.55	86.47±1.18	89.96±2.29

6. Reduction effect of steviol glucosides for insoluble glucan production

Mutansucrase derived from *S. mutans*. produces a mutant which is well known as a substance inducing tooth decay using sucrose [24]. Rubusoside has been reported to be a steviol glycoside with antimutagenicity that inhibits mutansucrase [26]. The effect of other steviol glucosides on the production of mutans was examined. The activity of the mutansucrase in the supernatant of the reaction mixture was not observed in the sample containing rubusoside. Enzyme activity was observed in the mixture containing steviol glycoside except rubusoside. It was confirmed that the mixtures containing the steviol glycosides resulted in a glycosylation reaction and a new stevol glucosides was formed. A high level of conversion was observed in the mixture containing Ste-G2' and a low conversion was observed in the other steviol glucosides. **(Figure 8A)**.

In the experiment confirming the insoluble glucan production, the reduction of insoluble glucan was the highest in Ste-G2' at 18% reduction compared to the control. **(Figure 8B, Table 6)** In addition, the decrease in insoluble glucan formation was also observed in steviol glucosides in which other glycosylation reactions occurred. Therefore, it was confirmed that insoluble glucan was reduced by transglycosylation.

(A)



(B)

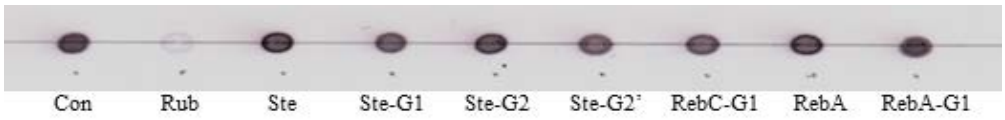


Figure 8. Thin layer chromatography of prevented mutansucrase formation using steviol glucosides. (A) TLC analysis of mutansucrase supernatant reaction mixture (B) TLC analysis of insoluble glucan formation (pellet) by mutansucrase reaction mixture. Con: Mutansucrase reaction control with mutansucrase (+) and without mutansucrase (-); Rub: mutansucrase reaction with rubusoside (+) and without mutansucrase (-), Ste: mutansucrase reaction with Stevioside (+) and without mutansucrase (-); Ste-G1: mutansucrase reaction with Ste-G1 (+) and without mutansucrase (-); Ste-G2: mutansucrase reaction with Ste-G2 (+) and without mutansucrase (-); Ste-G2': mutansucrase reaction with Ste-G2' (+) and without mutansucrase (-); RebC-G1: mutansucrase reaction with RebC-G1 (+) and without mutansucrase (-); RebA: mutansucrase reaction with Rebaudioside A (+) and without mutansucrase (-); RebA-G1: mutansucrase reaction with RebA-G1 (+) and without mutansucrase (-)

Table 6. The reduction of insoluble glucan formation by steviol glucosides

Insoluble glucan formation	
Sample	**The reduction of insoluble glucan formation(%)**
Control	0
Rub	82 ± 0.7*
Ste	0
Ste-G1	14 ± 1.5*
Ste-G2	15 ± 0.5*
Ste-G2’	18 ± 2.0*
RebC-G1	13 ± 1.3*
RebA	0
RebA-G1	4 ± 1.1

*Significant difference ($p<0.01$)

7. Solubilization ability of water-insoluble compounds with steviol glucosides

Pterostilbene

Pterostilbene (trans-3,5-dimethoxy-4-hydroxystilbene) [27] is a stilbenoid polyphenol that is present mainly in berries such as blueberries and mulberries. It has various functional activities such as anti-cancer [28, 29], anti-oxidant [30]. But, it has limited applications due to its low water solubility and bioavailability.

When pterostilbene powder alone is directly dissolved in water, it dissolved very little. However, when pterostilbene is co-dissolved in water with steviol glucosides [31], its water solubility was increased 4.63 mg/mL to 5.80 mg/mL, 72.1 to 90.3 times more soluble than water **(Figure 9, Table 7)**.

Except in the case of RebC-G1, it was confirmed that the water solubility was increased when glucoside group was attached to the C-19 position of steviol glycosides.

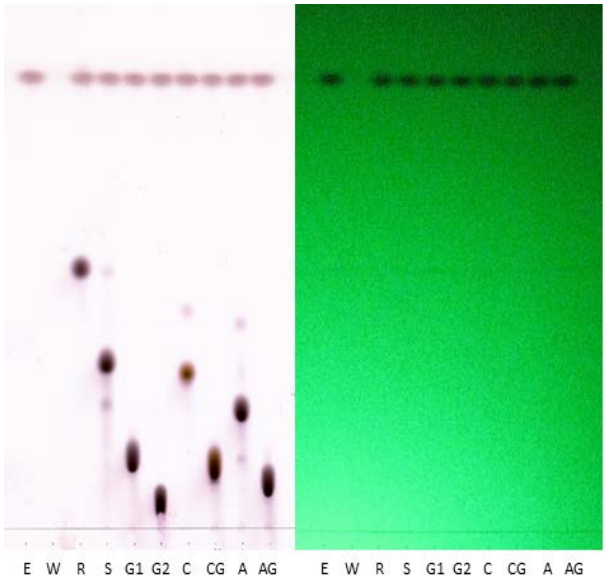


Figure 9. Thin layer chromatography of steviol glucosides with pterostilbene.

E: pterostilbene in ethanol; W: pterostilbene in water; R: pterostilbene in rubusoside solution; S: pterostilbene in stevioside solution; G1: pterostilbene in Ste-G1 solution; G2: pterostilbene in Ste-G2 solution; C: pterostilbene in rebaudioside C solution; CG: pterostilbene in RebC-G1 solution; A: pterostilbene in rebaudioside A solution; AG: pterostilbene in RebA-G1 solution.

Table 7. Water solubility of pterostilbene with steviol glucosides

Pterostilbene Solubility		
Sample	**Concentration (mg/mL)**	**Relative solubility (fold)**
Pterostilbene-Water	0.06 ± 0.00	1
Pterostilbene-Rub	5.80 ± 0.00*	90.3
Pterostilbene-Ste	5.55 ± 0.01*	86.4
Pterostilbene-Ste-G1	5.68 ± 0.02*	88.4
Pterostilbene-Ste-G2	5.53 ± 0.01*	86.1
Pterostilbene-RebC	5.71 ± 0.02*	89.0
Pterostilbene-RebC-G1	4.63 ± 0.01*	72.1
Pterostilbene-RebA	5.69 ± 0.01*	88.6
Pterostilbene-RebA-G1	5.80 ± 0.03*	90.3

*Significant difference ($p<0.01$)

Curcumin

Curcumin [32] is a polyphenol that is mainly present in turmeric (*Curcuma longa L*). Curcumin has been studied since it has properties that are effective for anti-oxidant [33, 34], anti-cancer [35], anti-diabetic characteristics. However, since curcumin has very low water solubility [36], it is very difficult to use it in food or medicine. Thus, many studies are under way to overcome these problems.

When curcumin powder alone is dissolved in water, it dissolved very little. However, when curcumin is co-dissolved in water with steviol glucosides [37], the water solubility was increased to 2.74 mg/mL to 4.55 mg/mL, 47.8 to 79.3 times more soluble than water **(Figure 10, Table 8)**.

As a result, it was confirmed that steviol glucosides showed lower curcumin solubilization ability as the glucosyl residues increased. However, it has been confirmed that it has a much higher level of curcumin solubilization ability than when it is dissolved in water.

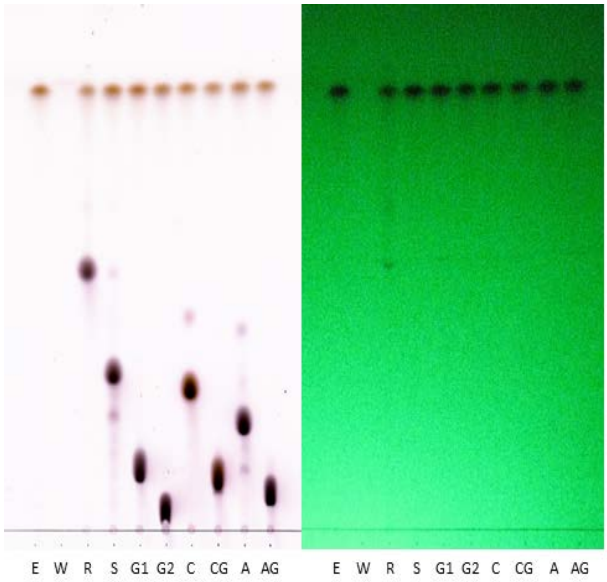


Figure 10. Thin layer chromatography of steviol glucosides with curcumin.

E: curcumin in ethanol; W: curcumin in water; R: curcumin in rubusoside solution; S: curcumin in stevioside solution; G1: curcumin in Ste-G1 solution; G2: curcumin in Ste-G2 solution; C: curcumin in rebaudioside C solution; CG: curcumin in RebC-G1 solution; A: curcumin in rebaudioside A solution; AG: curcumin in RebA-G1 solution.

Table 8. Water solubility of curcumin with steviol glucosides

Curcumin Solubility		
Sample	**Concentration (mg/mL)**	**Relative solubility (fold)**
Curcumin-Water	0.06 ± 0.00	1
Curcumin-Rub	3.36 ± 0.02*	58.6
Curcumin-Ste	4.55 ± 0.01*	79.3
Curcumin-Ste-G1	3.92 ± 0.01*	68.3
Curcumin-Ste-G2	3.21 ± 0.03*	55.9
Curcumin-RebC	3.66 ± 0.06*	63.8
Curcumin-RebC-G1	2.74 ± 0.05*	47.8
Curcumin-RebA	3.66 ± 0.04*	63.9
Curcumin-RebA-G1	3.35 ± 0.03*	58.5

*Significant difference ($p<0.01$)

Idebenone

Idebenone [38] is an organic compound of the quinone family. It is now being used as a drug because it is known to have an effect on brain-related diseases [39, 40], cardiovascular diseases [41]. However, due to its low water solubility, it has a very low efficiency in oral intake.

When idebenone powder alone is dissolved in water, it dissolved very little. When idebenone was co-dissolved in water with steviol glucosides, the initial idebenone solubility increased from 0.52 mg / mL to 5.72 mg / mL, 5.7 to 62.7 times more soluble than water. However, as time goes by, crystal-shape form was precipitated and the solubility of idebenone decreased. The solubility of idebenone was measured again after the precipitation, and it was confirmed that the solubility of idebenone was decreased overall. Ste and RebC were significantly decreased. the final idebenone solubility increased from 0.16 mg / mL to 4.13 mg / mL, 1.9 to 49 times more soluble than water. The final idebenone water solubility was confirmed to be significantly reduced when using Ste and RebC.

As a result, it was confirmed that newly synthesized steviol glycosidess had higher water solubility than steviol glucosides in the long term. **(Figure 11, Table 9)**.

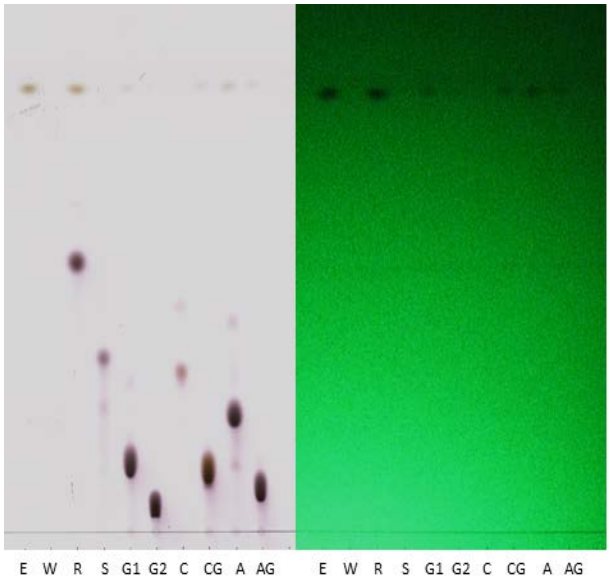


Figure 11. Thin layer chromatography of steviol glucosides with Idebenone.

E: idebenone in ethanol; W: idebenone in water; R: idebenone in rubusoside solution; S: idebenone in stevioside solution; G1: idebenone in Ste-G1 solution; G2: idebenone in Ste-G2 solution; C: idebenone in rebaudioside C solution; CG: idebenone in RebC-G1 solution; A: idebenone in rebaudioside A solution; AG: idebenone in RebA-G1 solution.

Table 9. Water solubility of idebenone with steviol glucosides

Idebenone Solubility				
Sample	**Initial conc. (mg/mL)**	**Final conc. (mg/mL)**	**Relative solubility**	
Idebenone-Water	0.09 ± 0.00	0.08 ± 0.00	1	1
Idebenone-Rub	4.28 ± 0.03*	4.13 ± 0.01*	46.9	49
Idebenone-Ste	2.63 ± 0.01*	0.16 ± 0.00*	28.8	1.9
Idebenone-Ste-G1	0.78 ± 0.00*	0.70 ± 0.00*	8.5	8.3
Idebenone-Ste-G2	0.52 ± 0.00*	0.40 ± 0.00*	5.7	4.8
Idebenone-RebC	5.72 ± 0.02*	0.28 ± 0.00*	62.7	3.3
Idebenone-RebC-G1	0.89 ± 0.00*	0.75 ± 0.00*	9.7	8.9
Idebenone-RebA	1.76 ± 0.01*	1.49 ± 0.00*	19.3	17.7
Idebenone-RebA-G1	0.81 ± 0.00*	0.68 ± 0.00*	8.8	8.0

*Significant difference ($p<0.01$)

Conclusion

In this study, I report the synthesis of novel steviol glucosides using dextransucrase from *L. mesenteroides* B-512FMCM. The optimum conditions for steviol glucosides synthesis were 37.3 mg/mL steviol glycosides, 381.3 mM sucrose, and 2.7 U/mL enzyme based on response surface methodology and central composite design. Structures of purified steviol glucosides were determined by and MALDI-TOF and nuclear magnetic resonance (NMR) [^{1}H, ^{13}C, HMBC, HSQC, COZY and TOCSY]. The water stability of newly synthesized steviol glucosides in extreme environments was much greater than that of steviol glycosides. Ste-G2' and several glycosides were showed to reduce the formation of insoluble glucan by the use of mutansucrase from *S. mutans*. Also, steviol glucosides could increase solubility of the water insoluble compounds such as pterostilbene, curcumin and idebenone. Overall, steviol glucosides have potentials as a natural sweetener and could be applied to the industry with their biochemical characterizations. Several early studies have shown that glycosylation of the carbohydrate moiety at the steviol glycosides gave a remarkable improvement in quality of taste. Therefore, it is expected that the newly synthesized Ste-G2, Ste-G2' and RebC-G1 were also improved in quality of taste.

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Abstract in Korean

스테비올 배당체는 관목 식물인 Stevia rebaudiana 추출물에 존재하는 달콤한 맛을 가진 저칼로리 천연 유기물이다. 스테비올 배당체는 스테비오사이드, 레바우디오사이드 A-E, 루부소사이드, 둘코사이드 A와 같은 다양한 스테비올 배당체들로 구성되어 있으며, 열 안정성, pH 안정성, 비 발효성의 특징을 가지며, 설탕의 약 30에서 150배 가량의 단맛을 가지고 있기 때문에 대체감미료로써 사용된다. 그러나 스테비올 배당체의 씁쓸한 후미가 스테비올 배당체의 식품과 음료로의 사용하기에 제한적이다. 이 연구에서는 류코노스톡속의 B-512FMCM 균주로부터 유래된 덱스트란수크라아제를 사용한 효소 당전이 반응을 사용하여여 스테비올 배당체의 쓴맛을 부분적으로 감소시켰다. RSM을 통하여 최적조건인 스테비올 배당체 37.3 mg/mL, 수크로오스 381.3 mM, 2.7 U/mL조건에서 스테비오사이드와 레바우디오사이드 A의 전환율을 평가하여 86.8%와 73.6%에 도달하였다. 각각의 정제된 스테비올 배당체를 HP-20수지, NH_2컬럼, C18컬럼이 장착된 HPLC-PDA를 사용하여 정제하였다. 스테비올 배당체의 구조는 NMR과 MALDI-TOF에 의해 13-O-β-sophorosyl-19-O-β-isomaltosyl-steviol, 13-O-[β-(1→6)glucosyl]-β-glucosylsophorosyl-19-O-β-isomaltosyl-steviol, 13-O-β-sophorosyl-19-O-β-isomaltotriosyl-steviol, 13-O-[α-neohesperidosyl-(1→3)-β-glucosyl-19-O-β-isomaltosyl-steviol, 13-O-[β-sophorosyl-(1→3)-β-glucosyl]-19-O-β-isomaltosyl-steviol로 구조결정되었다. 신규 스테비올 배당체들을 pH 2, 60 ℃의 조건에서 가속안정성 실험 결과 기존의 스테비올 배당체보다 더 높은 안정성을 가지는 것을 확인하였으며, 뮤탄수크레이즈에 의해 생성되는 불용성 글루칸 형성을 감소시키는 특성을 가지는 것을 확인하였다. 또한 스테비올 배당체를 프테로스틸벤, 커큐민, 이데베논과 같은 난수용성 화합물과 함께 물에 녹였을 때, 높은 수준으로 수용화를 증가시키는 특성을 가지는 것을 확인하였다. 이렇듯 수용화 능력의 향상과 더불어 생체 이용률의 향상 또한 기대할 수 있게 되었다.

Acknowledgement

유난히 춥고 눈도 많이 오던 2017년 1월부터 시작된 대학원 생활이 어느덧 2년이라는 지나 마무리가 되었습니다. 되짚어보면 여전히 부족하고, 아쉬운 점이 많다고 그래도 그 이상으로 많은 것들을 배웠던 것 같습니다. 이렇게 무사히 학위 논문을 작성할 수 있기까지 많은 분들의 도움이 있었습니다. 감사의 마음을 담아 한 분 한 분 이렇게 기록을 남깁니다.

먼저 부족한 저를 2년간 이끌어주시고 다양한 기회를 주신 김도만 교수님께 진심으로 감사의 인사를 드립니다. 때로는 엄하셨지만, 항상 믿고 따뜻하게 지도해주셨기 때문에 제가 여기까지 올 수 있었던 것 같습니다. 누구보다 먼저 실험실에 출근하셔서 연구에 임하시는 성실함과 열정적인 모습들은 지금으로 그렇지만 앞으로도 좋은 귀감으로 남아 있을 것 같습니다.

다음으로 바쁘신 와중에도 본 논문의 심사위원장을 맡아주시고, 검토해주신 정동화 교수님께 감사의 말씀을 드립니다. 항상 밝게 인사해주시고, 디펜스 때에도 시작 전에 교수님의 농담 덕분에 긴장을 많이 덜고 시작할 수 있었습니다. 그리고 날카로운 심사를 해주시고, 마지막까지 꼼꼼하게 신경써서 논문을 검토해주신 김효진교수님께도 감사의 말씀을 드립니다.

실험실 생활 시작부터 저의 연구방향을 잡아주시고, 빼질거리고 징징거리던 것도 늘상 웃으면서 받아주시던 탄한박사님. 아마 박사님 안계셨으면 제 실험들은 늘 엉망이였을 거에요. 항상 감사하고, 박사님의 누구보다도 열심히 하는 모습들을 보며 2년간 많이 배울 수 있었습니다. 앞으로 원하시는 일 다 잘 되시길 바랄게요. 그리고 목박사님. 그간 박사님께서 해주신 살아가는데 있어 필요한 많은 조언들은 앞으로도 많이 기억에 남을 것 같습니다. 늘 건강하시기 바랍니다.

다음은 실험실 생활을 같이 해주셨던 선배님들이 떠오르는데, 먼저 미국에서 박사과정으로 꿈을 이루어나가는 중이신 남현선배, 처음와서 제가 적응하는데 많은 도움을 주셨던 정민선배, 같이 실험하면서 맨날 투닥거리고 싸웠던 송희선배, 맨날 장난치고 빼질거려도 받아주던 희정선배, 브론

즈 동구선배, 츤데레였던 본철선배, 모든 일에 가장 열심히 하셨던 태경선배, 같이 장난을 주고받던 채리선배, 같이 졸업하기로 했던 시나선배, 치명적인 척하던 재원선배, 연애의 달인 창섭선배, 항상 묵묵히 자기일 하시는 소형선배, 언제나 나의 축구영웅 지수선배 덕분에 실험실에서 잘 적응하고 여기까지 왔습니다. 다들 감사합니다.

그리고 앞으로 뒤이어 실험실을 이끌어나갈 후배님들. 인도네시아에서 온 오다리 이스, 항상 어설프지만 착한 주희, 이상한 드립날려도 잘 받아주시는 유진씨, 식단표를 달달 외우던 햄찌 선민씨, 부족한 저를 잘 도와주던 항상 부지런한 병수씨, 그리고 요즘 맡은 일 많은 주호씨. 앞으로도 실험실 잘 이끌어 나가주시고, 학위 잘 마치고 사회에서 보도록 합시다.

나의 하나뿐인 동기 강희형. 형이 내 동기라서 다행이에요. 형이 있었던 덕에 이 2년간 탈선안하고 버틸 수 있었던 것 같아요. 2년간 매일같이 붙어있는동안 맨날 잔소리듣고, 일도 같이하고, 다투기도 하고, 늙어서 라면물 못 맞춘다고 놀리고, 같이 일본에도 갔다오는 등 많은 일이 있었네요. 형 취업하고 나가니까 실험실이 어색하고, 진짜 심심해서 미칠거 같아요. 이제 여기말고 나가서 봅시다요. 그리고 들어오는 순간부터 거의 개노답삼형제처럼 붙어다닌 채선생님. 2년이라는 시간이 흐르는동안 결혼도 하시고 아들 시원이도 곧 나오겠네요. 맨날 징징거리고, 이상한 스캔들 만들어주고, 구박도 많이 했지만 그동안 많이 챙겨줘서 감사했어요. 심심할때마다 가서 징징대는거 받아주느라 고생하셨습니다. 그래도 나 없으면 더 심심할텐데 잘 참고 버티도록 해요.

석사과정동안 고등학교때부터 항상 살아있나 안부 확인해주고, 놀러가는 것도 내 시간 맞춰 조절해주는 청수, 용일이, 종명이, 동균이, 스터디부터 시작해서 서울갈때마다 모여서 같이 놀아주는 우리 영민이, 슬기, 리아, 희대, 집에 내려갈때마다 놀러가면 놀아주는 숭숭이, 깐족거려도 받아주는 진희, 요즘들어 석사생활 힘들다고 징징거리는 지현이, 츤데레 경태, 늘상 못난동생이랑 놀아주는 정웅이형, 평창에서 만난 착한 동규, 내 삶의 멘토 큰형, 집안의 분위기 메이커 호기형, 그리고 내가 가장 좋아하는 규화형에게도 감사의 말을 전합니다. 항상 응원해줘서 고맙고, 감사합니다.

마지막으로 사랑하는 우리 가족들. 바쁘다는 이유로 2년간 연락도 잘 안하고, 까칠했던 저를 묵묵히 믿고 지원해주신 아버지, 어머니 항상 감사하고, 또 사랑합니다. 그리고 맨날 싸우다가 떨어져사니까 이제 보면 가끔씩은 애틋한 내 동생. 제발 내려가서는 싸우지 말자. 내가 잘 하도록 노력은 해볼게. 아무튼 우리 가족 앞으로도 다같이 힘내서 행복하게 살도록 합시다!

이렇듯 대학원 생활을 통해서 피가 되고, 살이 되는 경험들을 얻어서 서울대학교 국제농업기술대학원 생활을 마무리하게 되었습니다. 앞으로도 한사람의 사회인으로써 수많은 기로에 서게 되겠지만 이 같은 경험들을 발판삼아 잘 헤쳐나가도록 하겠습니다. 감사합니다.

2019년 2월

손 규 민