Master's Thesis of Science in Agriculture

Biochemical Properties of Rubusoside Glucoside Synthesized by Using B-512FMCM Dextransucrase

B-512FMCM 덱스트란수크라아제를 이용하여 합성한
루부소사이드 배당체의 생화학적 특성 연구

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Abstract

Rubusoside (Ru, 13-O-β-glucosyl-19-O-β-D-glucosyl-steviol) is the main component of *Rubus suavissminus S. Lee* (Roasaceae), which is known as Chinese sweet leaf. Ru is used as a food material as a sweetener and solubilizer. Because it has cell cytotoxicity and bitter aftertaste, dextransucrase acceptor reaction was conducted to improve this characteristics. Rubusoside glucoside (Ru-G, 13-O-(α-1→6-glucosyl)-β-glucosyl-19-O-β-D-glucosyl-steviol) was synthesized using dextransucrase from *Leuconostoc mesenteroides* and sucrose. The optimum condition of Ru-G synthesis was optimized by response surface methodology, purified by prep-HPLC and structure was determined by NMR. Ru-G would be used to enhance water solubilization of idebenone and curcumin. Also it showed 15% insoluble glucan formation inhibition by mutansucrase derived from *Streptococcus mutans*, which a one of strain to cause cavities. Cytotoxicity and anti-inflammation activity on mouse macrophage cell RAW264.7 was investigated. Cell toxicity of Ru-G was significantly reduced compared to Ru at 2 mM or higher, and anti-inflammatory effect of Ru-G was maintained as a similar level. The analysis of the sensory test machine showed that the bitter taste of Rub-G greatly improved compared to the stevioside, and similar or slightly improved compared to Ru.

Keywords: Rubusoside glucoside, glucansucrase, solubilization, mutansucrase, anti-inflammation

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Introduction

1. Rubusoside

1.1 Stevioside

1.1.1 Steviol glycosides

As global interest in sugar-free products increases, the expectation for natural sweeteners are also growing. Steviol glycosides are natural sweeteners extracted from the leaves of *Stevia rebaudiana* BERTONI. Steviol glucosides has the advantage of non-cariogenic and non-calorific characteristics compared with sucrose [37]. They are *ent*-kaurene-type diterpene glycosides. What exists at the major rate are stevioside and rebaudioside A, and following rebaudioside C-E, Ru, and dulcoside A [4]. Besides being sugar-free sweeteners, steviol glycosides have several product-value characteristics compared to sucrose. They have thermal stability, and pH stability [7]. Steviol glucosides has low calories compared to sucrose [13]. In the United States, high purity stevia glycoside extracts have been generally recognized as safe (GRAS) since 2008 and are permitted as food ingredient [14].

1.1.2 Stevisoide

Stevioside (Ste,13-O-β-sophorosyl-19-O-β-glucosyl-steviol) is one of the most widely used natural sweeteners, and although it has a sweet taste of more than 100 times that of sucrose, its calories are even lower [4]. As one of the steviol glucosides, it conserved the advantage of pH stability and thermal stability. Ste is a natural sweetener with no sugar added, and it also associated with the function of anti-diabetic effect [17]. Despite the many possibilities as a natural sweetener, Ste has the disadvantage of having a bitter aftertaste. At first there was a debate about genotoxicity or safety, but it was proved safe in the genetic/cariogenic part [18].

1.1 Rubusoside

Rubusoside (13-O-β-glucosyl-19-O-β-D-glucosyl-steviol) is one of steviol glycosides, which can be utilized as an alternative organic sweetener. It has a sweet taste 110 times stronger than sucrose at the concentration of 0.024% [4]. Ru is not easily metabolized by *S. mutans* which contributes to tooth decay, so it does not allow *S. mutans* to grow compared to other saccharide compounds [19]. One of the main characteristics of Ru is its ability to solubilize compounds that are not soluble in water. The behavior of Lanmuir monolayers as interfaces could reveal the amphiphilic and self-assembled properties of amphiphile. The pressure difference between hydrophilic and hydrophobic areas causes the micelle structure to form in water and dissolve in water by holding other molecules in it. Through this micelle, it is also confirmed that the molecules inside are absorbed into the cell [35]. Using this characteristic, several anti-cancer effects studies are under way. Nevertheless, because Ru has its own cytotoxicity [35] and has a bitter taste [36], Further improvement is still needed to be widely used in industry.

2. Dextransucrase from *Leuconotoc mesenteroides* B-512FMCM

Leuconostoc mesenteroides B-512FMCM is a dextransucrase constitutive and hyper-productive strain [22]. Dextransucrase is an enzyme that combines glucose to form glucan using sucrose as a substrate, which forms different structures depending on the strains derived. The dextran formed by using B-512FMCM dextransucrase has 95% of α-(1,6) bonds and 5% of α-(1,3) branch [23]. This enzyme reaction is used in transglycosylation and acceptor reaction to bind glucose to free –OH group of other substance [24]. Recently, it was used for formation of various types of dextran [39] or transglycosylation of flavonoid etc [40] using B-512 dextransucrase.

3. Transglycosylation

Transglycosylation is a enzymatic reaction for glycosidic bond formation, especially during polysaccharide synthesis; nucleoside phosphate derivative acts as activated donor compound in which the energy of their glycosidic bonds are partially conserved in the reaction products. Glycosides cannot be synthesized spontaneously from free monosaccharides owing to the high negative free energy ($-\Delta G$) of the hydrolysis reaction [15]. With these properties, transglycosylation was used to improve physical and chemical properties by combining sugars into materials using enzymes and changing biochemical structures. In this reaction, a small sugar, which also called ‘donor’, is broken by enzyme to glucose and its glycosyl residue is attached on acceptor [16].

4. Mutansucrase from *Streptococcus mutans*

S. mutans is a microorganism that forms insoluble glucan that cause cavities. The sticky polysaccharide glucan produced and released by *S. mutans* adheres to teeth surface to induce enamel damage [19]. There are two types of glucan produced by *S. mutans*. This is because the enzymes involved in forming glucan are different, one of which is dextransucrase. Dextransucrase of *S. mutans* synthesis soluble glucan with long glucose connection through α-(1,6) bonds. The other major enzyme, mutansucrase, forming insoluble glucan by mutansucrase. Mutansucrase reacts with sucrose to produce mutan, mainly through a glucosidic α-(1,3) bonds and fructan of β-(2,1), β-(2,6) bonds [20,21]. Glucan produced by *S. mutans* thus cause cavities to occur on the surface of the teeth.

5. RAW264.7 mouse macrophage cell

RAW264.7 cells are macrophages of mouse and have been described as an appropriate model cell line of macrophages because they can perform pinocytosis and phagocytosis [8,9] This murine macrophage cell line is frequently used as the model cell for inflammation-related research due to its reproducible response to lipopolysaccharide (LPS) or tumor necrosis factor (TNF)-α [10]. NO, stimulated by LPS, is a typical indicator of inflammatory reactions. In RAW264.7 cells, NO is produced mainly by inducible nitric oxide synthesis (iNOS), which is controlled by heme oxygenase-1 (HO-1), and an anti-inflammatory enzyme. HO-1 is mainly regulated by transcription factor Nrf2, which converts to the nucleus and activates the expression of HO-1 [11]. The macrophage secretes cytokines such as TNF-α and interleukin(IL)-1 or spreads a defense against cytotoxic molecules [12]. RAW264.7 cell line is also used in study to evaluate cell toxicity.

6. Purpose of this study

Based on the study that there is a relationship among the chemical structure, sweet and bitter taste in the Ru and its glycosides [6], I planned to improve the flavor of Ru through glucosylation reaction. Ru-G was newly synthesized with dextransucrase. I conducted experiments to ensure that the efficiency of other product-value characteristics such as mutansucrase inhibition, anti-inflammatory effect, solubilization ability, chemical stability and cell toxicity in Ru-G. Based on the function of Ru, solubilization ability test with water-insoluble compounds (idebenone and curcumin) was also conducted. Given there characteristics, Ru-G has the potential to be utilized in various products. Ru-G has sweet taste, low cytotoxicity and mutansucrase inhibition, which is expected to be used as an ingredient of dental care products. It also has solubilization ability which could be used in cosmetic products.

Materials and methods

1. Enzyme preparation

The constitutive dextransucrase was prepared from the *L. mesenteroides* B-512FMCM fermentation with glucose, which is an ultraviolet-treated mutant of *L. mesenteroides* B-512F that constantly produces dextransucrase enzyme [1]. The detail method is same progress as the one previously described [2]. 1 U of dextransucrase enzyme was defined as the amount of enzyme required to liberate 1 μmol of fructose from 200 mM of sucrose in 20 mM Na-Ac buffer (pH 5.2) at 28 ℃. The amount of fructose was measured and quantified using silica gel 60F254 TLC plate analysis (Merck, Germany) and AlphaEaseFc Image program (Alpha Innotech, USA).

2. Synthesis of rubusoside glucoside

2.1 Transglycosylation of rubusoside

The reaction mixture was prepared with 150 mM of Ru in water with dextransucrase 3 U/ml, 300 mM sucrose with 20 mM Na-Ac buffer (pH 5.2). The reaction mixture was incubated at 28 ℃ for 5h. After reaction, the reaction digest was incubated at 70 ℃ for 10 min to stop enzymatic activity. 1 μL of reactant was spotted onto TLC plate and developed in acetonitrile: water (85:15, v/v) solvent. Ru and Ru-G were visualized by dipping the plate into a solvent mixture of 0.5% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride [3] and 5% (w/v) sulfuric acid in methanol followed by heating at 125 ℃ for 5 min.

2.2 Optimization for acceptor reaction using response surface methodology

The central composite design (CCD) RSM software program Design Expert (Stat-ease, USA) was used to optimize conversion of Ru to Ru-G with the following three variables: Ru concentration, sucrose concentration, and enzyme concentration. Seventeen runs of the experiment were carried out with Design Expert with X replication at the central point, which were utilized in the fitting of a second-order response surface. All statistics and mathematical analysis of the results were performed with Design Expert to determine the effect of variables. Three dimensional variables on response and fitted through the response surface regression procedure using the second order polynomial equation.

2.3 Purification of rubusoside glucoside using HPLC

To detect the Ru-G, the reaction mixture of Ru and dextransucrase reactant loaded into HP-20 column after polymer elimination with 70% ethanol. After removed monosaccharides and small saccharides, fluent was eluted with 100% ethanol. With rotary evaporator, ethanol was separated from water. Compounds were confirmed with analytic HPLC at 210 nm with 2998 photodiode assay detector (Wasters, USA) equipped Luna 5 μm NH_2 100A (250 × 21.22 mm) (Phnomenex, USA) at flow rate 1 ml/min with water and acetonitrile.

2.4 Molecular weight measurement of rubusoside glucoside using Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

Purified Ru-G was solubilized with DMSO-d6. 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.

2.5 Structural elucidation of rubusoside glucoside using nuclear magnetic resonance

Purified Ru-G (10 mg) was dissolved in DMSO-d6 and placed into 5 mm NMR tube. NMR spectra was recorded on an AVANCEIII HD system (Bruker, USA) operated at 850 MHz for 1H and 13C at 25 ℃. Linkage between Ru-G and glucose were evaluated using 1D (1H, 13C), 2D [COSY (homonuclear correlation spectroscopy), HSQC (hetero nuclear single-quantum coherence), HMBC (hetero nuclear multiple-bond correlation), and TOCSY (total correlation spectroscopy)].

3. Study of characterization of rubusoside glucoside

3.1 Mutansucrase activity inhibition assay

Relative inhibition activity, against mutansucrase of Ru-G was conducted. First, Ru-G was dissolved in distilled water to obtain 50 mM. The reaction mixture contained 100 mM sucrose, 0.1 U/ml mutansucrase and 50 mM Ru-G in sodium phosphate buffer (pH 6.8). The inhibition reaction was carried out at 37 ℃ for 2 h. After reaction, centrifugation was carried out for 15 min at 12,000 rpm and the supernatant was removed. The mutan produced in the reaction mixture was dissolved in 1 M NaOH and a 1 μl aliquot of the mutan dissolved in NaOH was spotted on the TLC plate re-coated silica gel 60F254 (Merk, Germany). It was visualized by dipping in a solvent mixture of (85:15=acetonitrile:water) and hitting at 120 ℃ for 5 min. Relative inhibition activity was calculated with AlphaEase program. Inhibition activity was defined as a release of fructose compared with a reaction mixture containing mutansucrase without inhibitor.

3.2 Cell cytotoxicity test using RAW264.7 cell

RAW 264.7 mouse macrophage cell line was purchased from Korean Cell Line Bank and cultured. In Dulecco's modified Eagle's medium, DMEM (Genedepot, USA), supplemented with 10% (v/v) fetal bovine serum (FBS, Genedepot, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, USA) at 37 ℃ in 5% CO_2. RAW264.7 macrophage cell was seeded on 96 wells plate at 2X cells/well and cultured for 48 hr. Cells were rinsed with phosphate-buffered saline (PBS) and then treated with Ru and Ru-G in DMEM medium without FBS ranging from 0.0625 to 8.0 mM obtained by diluting Ru and Ru-G with culture medium. RAW264.7 cells cultured in a medium without adding samples were used as controls. After 24 hr at 37 ℃, 80 μL of medium was mixed with 10 μL of Ex-CyTox solution (Daeil Lab Service, Korea) and then incubated at 37 ℃ for 1 h. Absorbance was measured at 450 nm using spectraMas M3. Percent viability was calculated as cell viability relative to the control.

3.3 Nitric oxide production inhibition assay using RAW264.7 cell

RAW264.7 cells were seeded in 96 wells plate at 2×10^3 cells/well and cultured at 37 ℃ for 48 hr. The sample treated with 1 μg LPS/ ml and 100 μM indomethacin was used as positive control. Cells were then treated with Ru and Ru-G in water ranging from 0.0625 to 8 mM without effects in cytotoxicity under testing, and cultured at 37 ℃ for 24 hr. Then, 80 μL of culture supernatant was mixed with 80 μL of Griess reagent containing 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid, and 0.1% (w/v) naphthylethlenediamine for 20 min, and absorbance was measured at 540 nm using SpectraMax M3. The amount of nitrite in the sample was evaluated from a standard curve generated with a sodium nitrite standard curve (0-500 μM in cell culture medium).

3.4 Stability of rubusoside glucoside in extreme pH and temperature condition

The degradation of pure purified Ru-G was analyzed at pH 2.0 and 60 ℃. Each sample was dissolved in water of pH 2 at the concentration of 10 mg/ml in an eppendorf tube. Then samples were incubated in a 60 ℃ water bath and analyzed for stability at 6 hr. 12, 24 and 48 hr. The degradation experiments were performed in triplicated for each sample.

3.5 Solubilization ability with insoluble compounds

Idebenone and curcumin were purchased from TCI chemical, and Sigma, respectively. 5 mg of each compound was mixed with 50 mg of Ru-G and then 500 μL ethnol was added to the mixture. The mixture solution was put on auto-shaker for 30 min and centrifuged at 12,000 rpm for 15 min at 25 ℃. The concentration of each compound in solution was determined using ultra performance liquid chromatography mass, UPLC-MS (Waters, USA) analysis.

3.6 Sensory test machine

The taste was measured four times using 100 ml of the prepared Ste, Ru, Ru-G solution at a concentration of 10 mg/ml, and the average was obtained from the rest of the measurements except for the first one. After analysis of each compounds, the sensor was cleaned with a standard solution (30 mM KCl + 0.3 mM targetatic acid) and a 10 mM KCl solution was used as a calibration solution. The taste sensor device used TS-5000Z, and the sensor was repeated four times by installing 5 types of food tap sensor (CT0, C00, CA0 and AE1), and the measurement results were analyzed by using the analysis software (Taste analysis application, Insight, Japan). Taste information unit, which is a unit of display, is a unit proposed by Kobayasi [25], which indicates a change in the concentration of flavor ingredients and specifies a minimum difference in unit in which humans can distinguish differences in taste.

Results and discussion

1. Synthesis of rubusoside glucoside

1.1 Transglycosylation of rubusoside

With sucrose and Ru, it was confirmed that Ru-G was produced through transglycosylation reaction of B-512FMCM dextransucrase. This result was verified using the analysis of the TLC plate (Fig. 1**)**. A new spot that does not appear in the (-) control was found to be produced of the Ru-G.

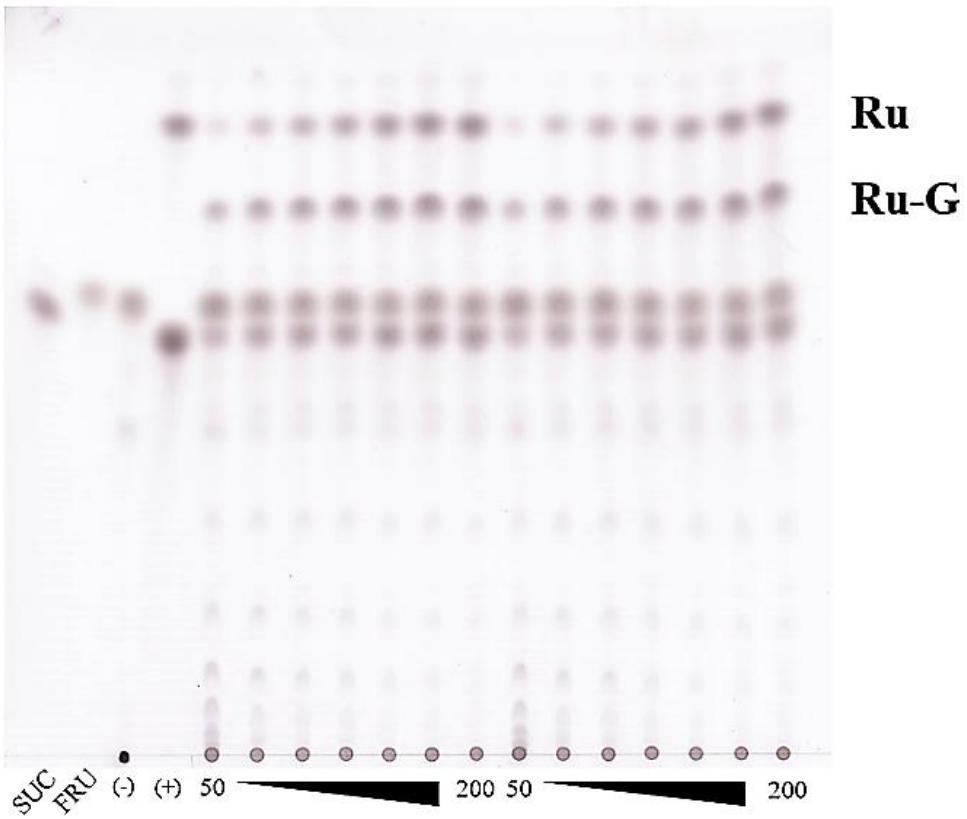


Fig. 1. Synthesis of Ru-G using dextransucrase from *L. mesenteroides* B-512FMCM.

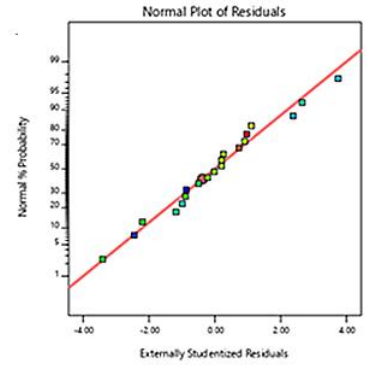
Ru concentration is increased (mg/ml). (-): reactant of dextransucrase and sucrose. (+): reactant of Ru and sucrose.

1.2 Optimization for acceptor reaction using Response surface methodology

Response surface methodology (RSM) was used as a way to optimize dextransucrase enzyme reaction for the three factors: sucrose concentration, Ru concentration, enzyme unit (Fig. 2**)**. The slightly tilted optimization slope associated Ru is expected to be due to the Ru itself having a inhibition to dextransucrase enzyme activity. The optimized conditions are followed as Table 1. Under this condition, 120 mM Ru was converted 48.9%, or 58.6 mM to Ru-G.

Table 1. Optimization condition of rubusoside glucosylation acceptor reaction.

Composition	Concentration
Sucrose	300 mM
Rubusoside	120 mM
B-512FMCM Dextransucrase	3 U/ml



Sequential Model Sum of Squares [Type I]

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	56216.69	1	56216.69			
Linear vs Mean	1029.61	3	343.20	8.25	0.0015	
2FI vs Linear	121.43	3	40.48	0.9667	0.4379	
Quadratic vs 2FI	**442.23**	**3**	**147.41**	**14.44**	**0.0006**	**Suggested**
Cubic vs Quadratic	82.23	4	20.56	6.21	0.0252	Aliased
Residual	19.88	6	3.31			
Total	57912.08	20	2895.60			

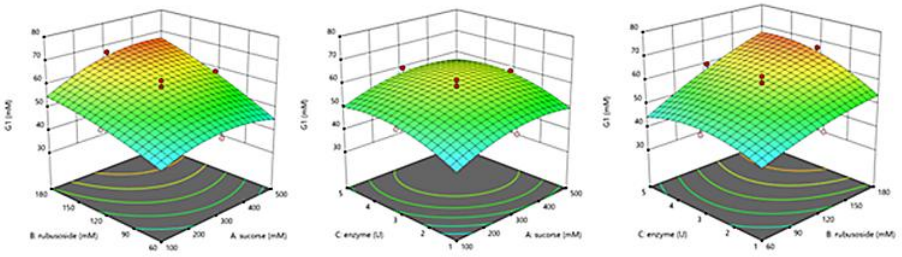


Fig. 2. Response surface plot and contour plot of Ru conversion. 3-dimensional optimization graph in RSM program.

1.3 Purification of rubusoside glucoside using HPLC

After removal of other saccharides (glucoside, sucrose, fructose) and polysaccharides, the Ru-G was detected using HPLC-PDA at 210 nm (Fig. 3). In the graph, there are rubusoside gucosides, which is assumed to have transglycosylation in other combination.

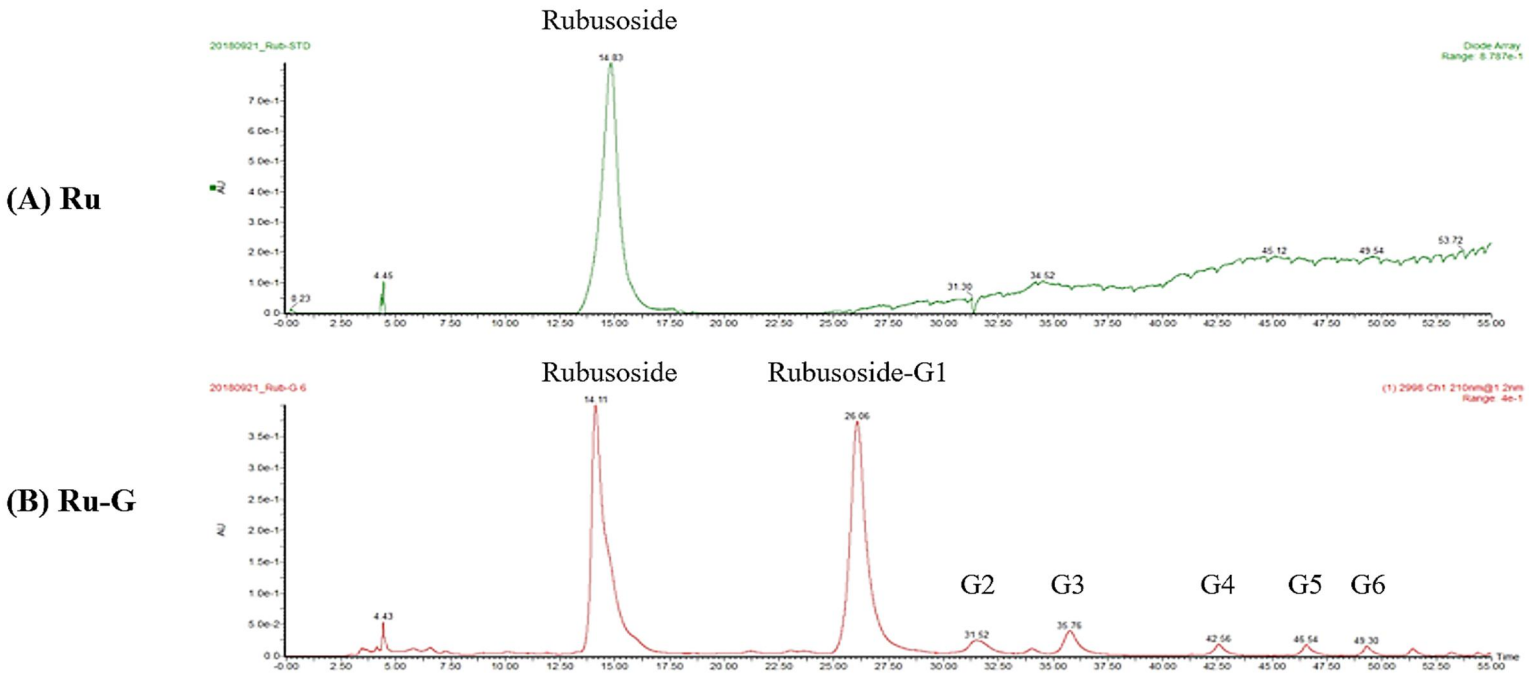


Fig. 3. The HPLC chromatogram of Ru-G. A: Ru standard (10 mM), B: Ru-Gs.

1.4 Molecular weight measurement of rubusoside gucoside

MALDI-TOF-MS analysis was used to measure molecular weight and purity of Ru-G (Fig. 4). Molecular weight of Ru was 642.74 g/mol and glucose was 180.16 g/mol. A sodium ion was combined during the analysis, and the weight of a single sodium ion was added to 827 g/mol. Since molecular weight of a sodium ion is 22.99 g/mol, the molecular weight of the Ru-G was 804.01 g/mol. Given that there are not many matrices on the analysis graph, the purity was also sufficient.

827=Ru+glc+Na^+

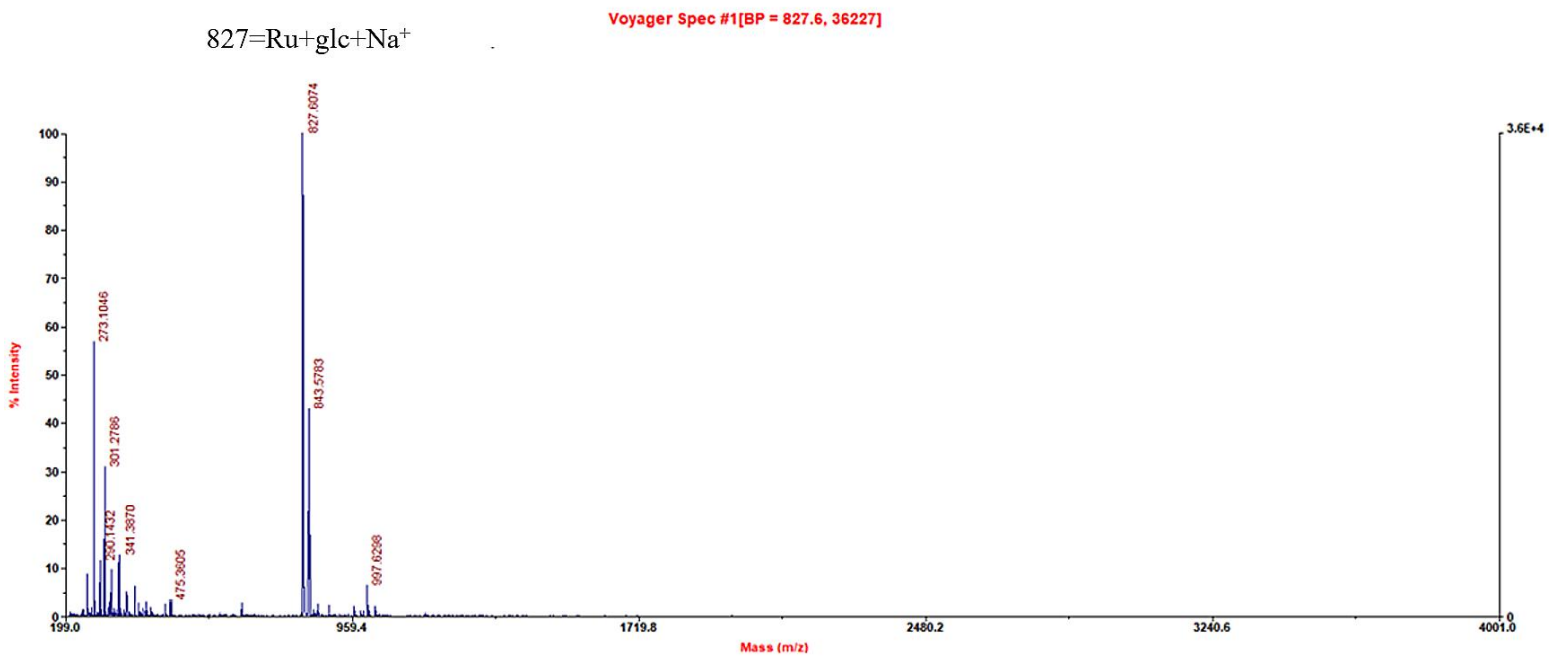
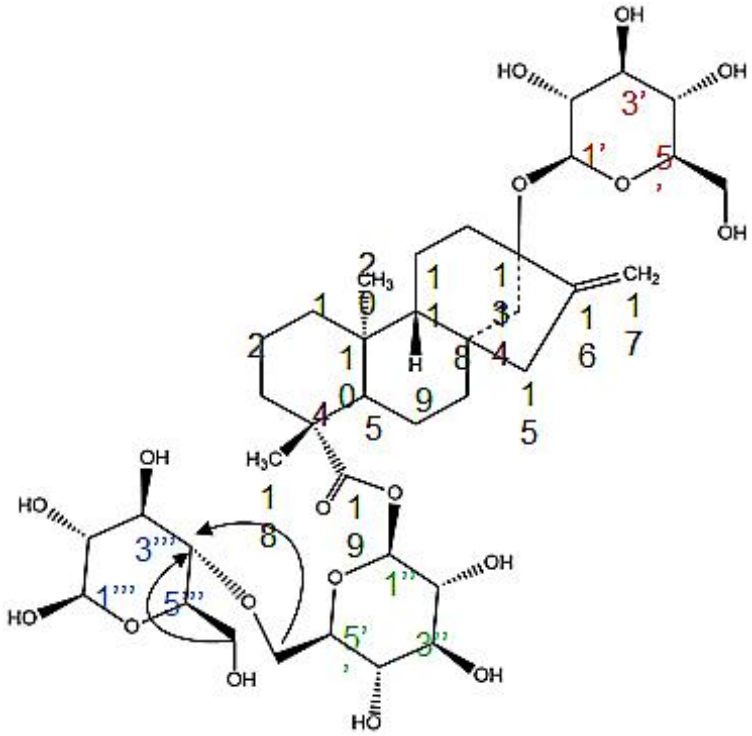


Fig. 4. MALDI-TOF-MS spectra of Ru-G.

1.5 Structure determination of rubusoside glucoside

Structure of Ru-G was determined by NMR (1H, 13C, COSY, HSQC, and HMBC). As a result, it as one glucose was added into the glucose of Ru in α(1→6) bond (Fig. 5**)**. Because 512FMCM dextransucrase induced α(1→6) bonds with 95% probability, Ru-G having α(1→6) structure was obtained at a high yield.



(13-O-(α-1→6-glucosyl)-β-glucosyl-19-O-β-D-glucosyl-steviol)

Fig. 5. Structures of Ru-G.

2. Inhibition for mutansucrase activity

After mutansucrase inhibition reaction of Ru-G, the insoluble glucan production decreased 35% in Ru and 15% with Ru-G (Fig. 6). In previous research of laboratory, Ru is a competitive inhibitor against mutansucrase. It is understood that the glucose binding pattern also affects mutansucrase inhibitory fuction.

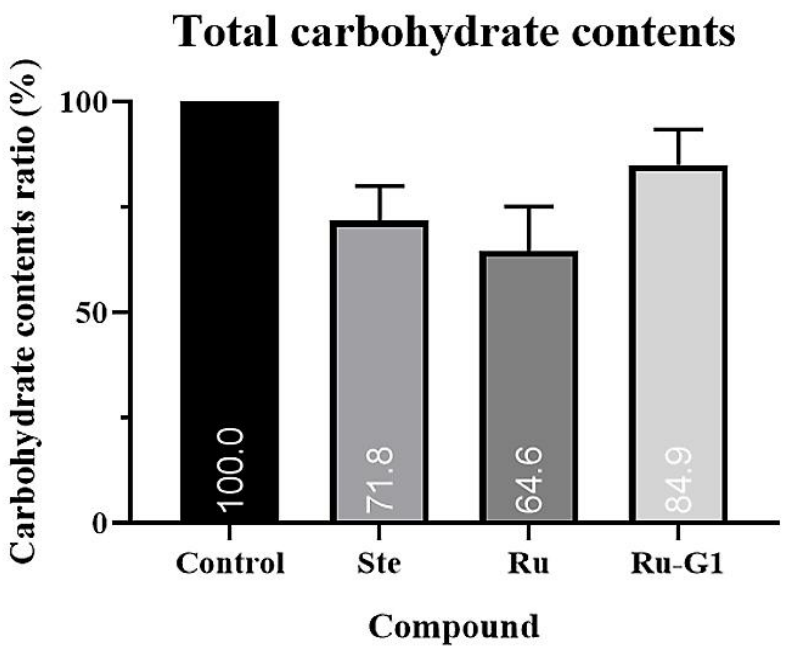


Fig. 6. Total carbohydrate contents assay.

Control: reaction with sucrose and mutansucrase, Ste: Stevioside, Ru: Rubusoside, Ru-G1: Rubusoside glucoside.

3. Cell cytotoxicity of rubusoside glucoside

To evaluate cytotoxicity, survival rates were measured by treatment the compound (Ru, Ru-G, Ste) in RAW264.7 cells (Fig. 7). Test have shown that at concentration of 4mM (3.2 mg/ml), Ru-G does not show cell toxicity. This was significantly reduced compared to the toxicity of Ru (2 mM, 1.29 mg/ml). This, too, appears to have ease the area of attacking cells due to structural deformation.

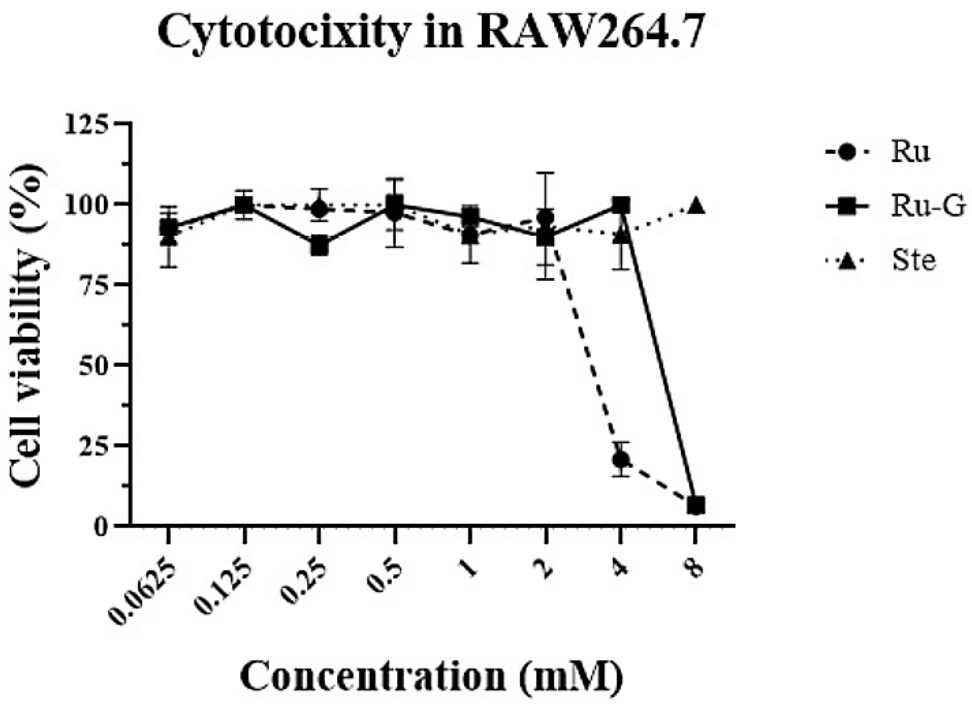


Fig. 7. Cell viability on RAW 264.7 mouse macrophage cell. Ru: rubusoside, Ru-G: Rubusoside glucoside, Ste: stevioside

4. Nitric oxide production inhibition of rubusoside glucoside

Production of nitric oxide was investigated by lipopolysaccharide (LPS) stimulation. The anti-inflammatory activities of Ste, Ru, Ru-G increase as the concentration went higher (Fig. 8). Since nitric oxide production is caused by inflammation, Ru, Ru-G has effect for anti-inflammatory activity by reducing nitric oxide production.

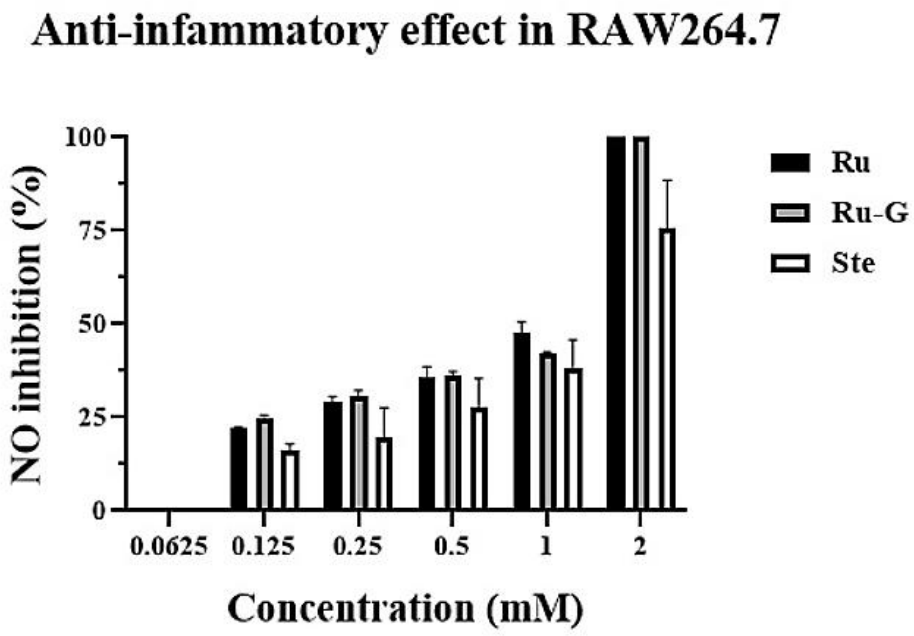


Fig. 8. Nitric oxide production inhibition on RAW264.7 mouse macrophage cell.

Ru: rubusoside, Ru-G: rubusoside glucoside, Ste: stevioside.

5. Stability of rubusoside glucoside in extreme condition

In order to confirm the stability of Ru-G in water, it was confirmed in extreme environment (Table 2) After 48 hours, Ru-G showed higher stability compared to Ste and Ru. A slight low stability after 24 hr seems to be easily disassembled by transglycosylation.

Table 2. Stability of Ru-G under extreme condition.

Stability of rubusoside glucoside			
Time	Compounds		
	Stevioside	Rubusoside	Rubusoside glucoside
0 hr	100.0	100	100
6 hr	95.5 ± 3.9	96.7 ± 5.6	97.1 ± 2.9
12 hr	95.5 ± 5.1	94.8 ± 1.7	95.3 ± 1.7
24 hr	95.5 ± 7.1	94.8 ± 6.6	77.4 ± 0.4
48 hr	50.3 ± 2.4	45.3 ± 5.9	77.3 ± 6.0

6. Solubilization insoluble compounds with rubusoside glucoside

6.1 Curcumin

Curcumin [26] is a polyphenols that is mainly present in tumeric (*Curcuma longa L*). Curcumin is not only known to have an excellent effect in anti-oxidant [27,28], it is also known to have anti-cancer effect [29] and good effect related to diabetes [30]. Still, it has water-insolubility, making it difficult to be used as a food material for which organic solvents cannot be used.

The results of the solubilization test using Ru and Ru-G confirmed by UPLC (Fig. 9) that the water-solubility increased 612 times (2.45 mg/ml) with Ru and 517 times (2.11 mg/ml) with Ru-G compared to curcumin only (0.004 mg/ml) (Table 3).

Table 3. The UPLC quantification of curcumin for water solubilization.

Solubilization	Compounds		
	Water	Rubusoside	Rubusoside glucoside
	mg/ml	mg/ml	mg/ml
Curcumin concentration	0.004 ± 0.00	2.45 ± 0.06*	2.11 ± 0.03*
Relative solubility (fold)	1	612.5	517.5

* Significant difference ($p<0.01$)

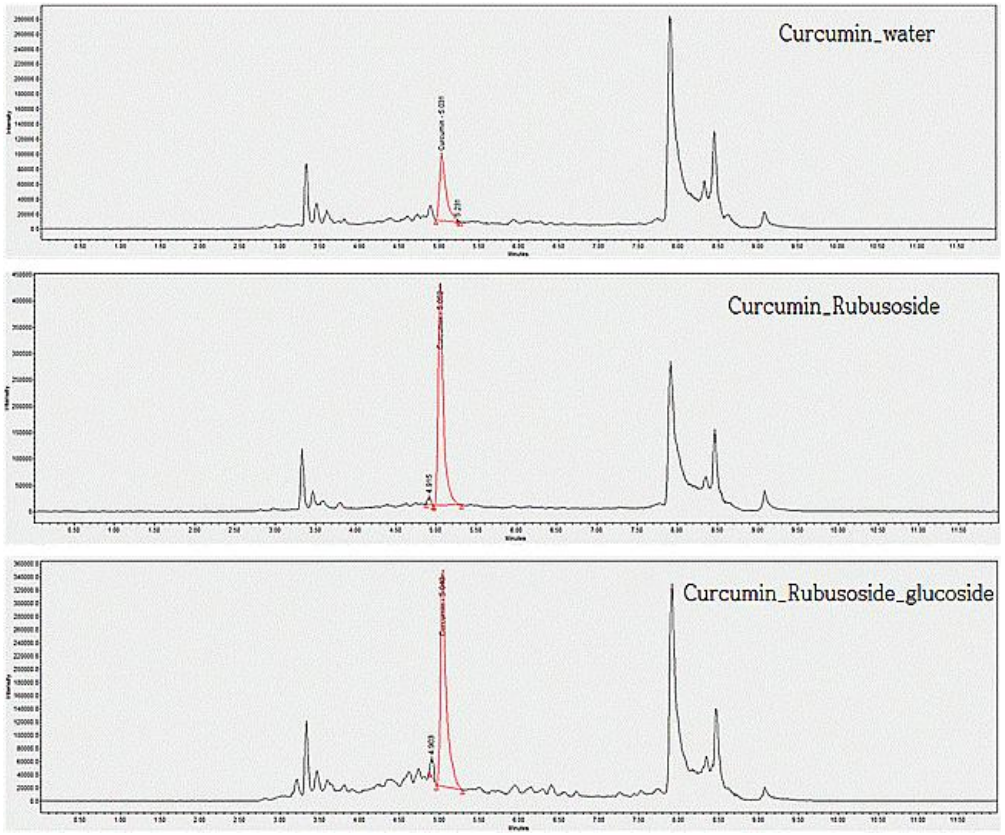


Fig. 9. The UPLC chromatogram of curcumin for water solubilization.

6.1 Idebenone

Idebenone [31] is an organic compound of the quinone family. It is known that idebenone has a good effect on brain-related disease [32, 33], as well as a decrease in cardiovascular disease [34]. Recently, the cosmetic industry is paying attention to idebenone, but it having difficulty in using it due to poor water solubility [38].

The results of the solubilization test using Ru and Ru-G confirmed by UPLC (Fig. 10) that the water-solubility increased 435 times (6.09 mg/ml) with Ru and 430.7 times (6.03 mg/ml) with Ru-G compared to curcumin only (0.014 mg/ml) (Table 4). It is confirmed that Ru-G's ability to solubilize idebenone is maintained at level similar to that of Ru. Considering that the molecular weight of Ru-G is heavier than that of Ru, there is a possibility that the solubilization ability of Ru-G has increased compared to Ru.

Table 4. The UPLC quantification of idebenone for water solubilization.

Solubilization	Compounds		
	Water	Rubusoside	Rubusoside glucoside
	mg/ml	mg/ml	mg/ml
Idebenone concentration	0.014 ± 0.0001	6.09 ± 0.03*	6.03 ± 0.003*
Relative solubility (fold)	1	435	430.7

* Significant difference ($p<0.01$)

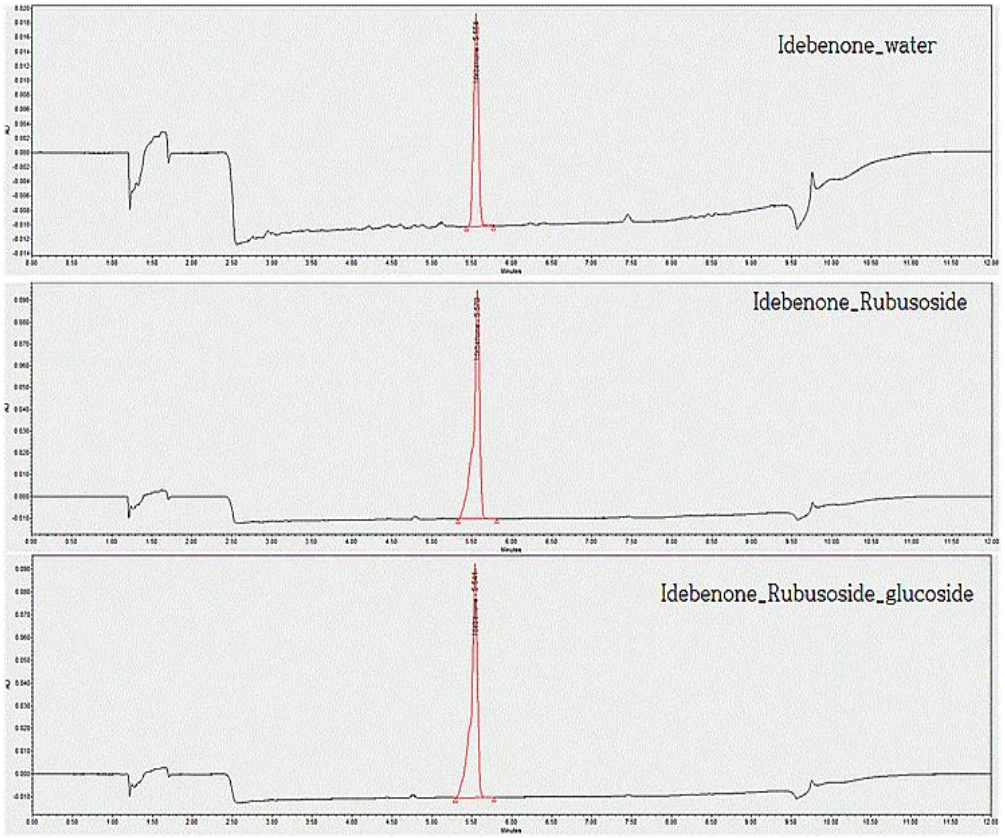


Fig. 10. The UPLC chromatogram of idebenone for water solubilization.

7. Analysis of sensory test machine

The taste was measured in a flavor tester that responds to eight flavors similar to the human tongue. As a result, the bitter taste of Ru-G was remarkably reduced compared to Ste (Fig. 11), and a 10% improvement was found when compared with Ru (Table 5). These differences in taste are expected to be caused by chemical structural deformation [6], it maybe considered that structural deformation of the compound and affected the binding with the taste-sensing receptors in the tongue.

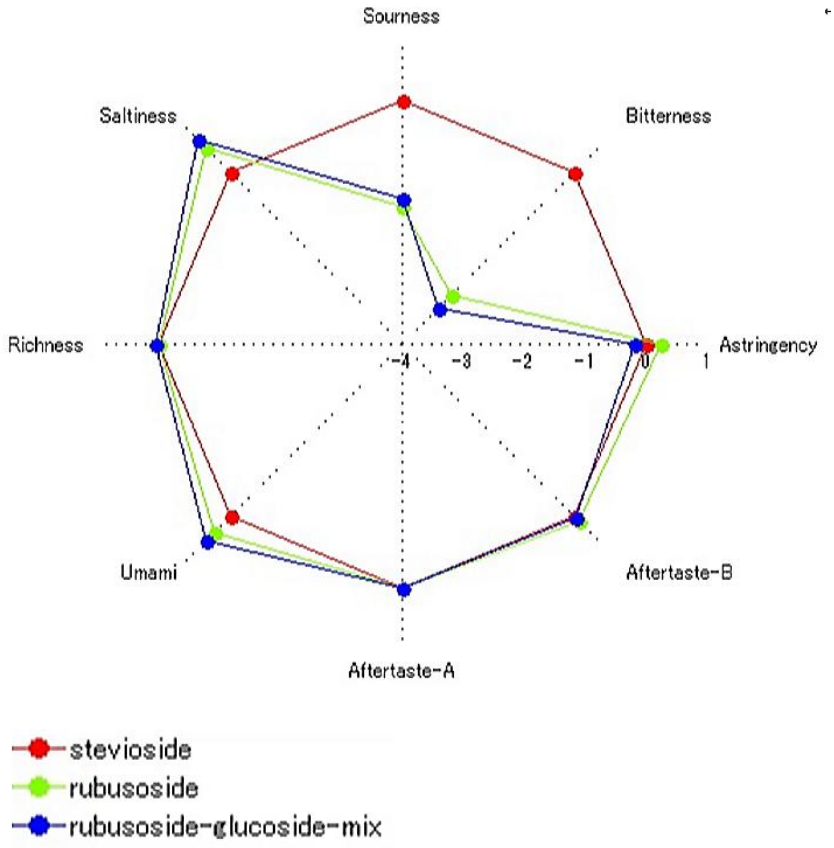


Fig. 11. The diagram of sensory test machine.

Aftertaste-A: after astringency taste, Aftertaste-B: after bitterness taste.

Table 5. The quantification of sensory test machine.

Compounds	Taste							
	Sourness	Bitterness	Astringency	Saltiness	Umami	Richness	Aftertaste-A	Aftertaste-B
Stevioside	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Rubusoside	-1.76 ± 0.12	-2.86 ± 0.09	0.24 ± 0.16	0.36 ± 0.16	0.57 ± 0.43	0.02 ± 0.14	0.01 ± 0.01	0.11 ± 0.01
Rubusoside glucoside	-1.62 ± 0.20	-3.18 ± 0.04	-0.19 ± 0.22	0.54 ± 0.09	0.77 ± 0.14	0.08 ± 0.27	0.00 ± 0.01	0.05 ± 0.04

Aftertaste-A: after astringency taste, Aftertaste-B: after bitterness taste.

Conclusion

In this study, a structural transformation was conducted through an enzyme reaction to improve the cell toxicity and bitter taste of Ru. With conducting transglycosylation using B-512FMCM dextransucrase, a novel Ru-G was synthesized having one more glucose combined in the Ru. Under the optimized condition, Ru was converted to Ru-G at a rate of 48.9%. Ru-G was analyzed and purified through HPLC, and MALD-TOF-MS analysis having a molecular weight of 804.01 g/mol. Ru-G has an anti-cariogenic effect of 15% compared to sugar only for mutansucrase by *S. mutans* and it has a stronger stability than Ru when exposed to more than 48 hr in extreme condition of pH 2 and 60 °C. In the experiment to solubilize curcumin and idebenone, Ru-G was found to be able to solubilize 517.5 and 430.7 times more the compounds compared to solubilized in water only, respectively. Ru-G has a level of anti-inflammatory effect similar to Ru, and the cytotoxicity decreased to less than half. In addition, the results of the analysis of the sensory test machine showed that the bitter taste decreased by 10% compared to Ru, and that it is possible to improve the after bitterness taste.

Based on these study, Ru-G has a potential to used in products such

as mouth wash and dental spay related to dental care, and cosmetic area. This study suggests the possibility that the original characteristics can be improved by applying enzyme reaction to organic sweeteners being used as food materials.

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Abstract in Korean

루부소사이드 (Ru, 13-O-β-glucosyl-19-O-β-D-glucosyl-steviol)는 중국 단풍으로 알려진 *Rubus suavissminus S. Lee* (Roasaceae)의 주요 성분이다. 루부소사이드는 감미료로서 음식 소재로 사용되며, 물에 녹지 않는 물질을 녹이는 기능을 가지고 있다. 그러나 루부소사이드는 세포 독성과 쓴맛을 가지고 있기 때문에 이러한 특성을 개선하기 위하여 당전이 반응을 연구하였다. 당전이 반응은 설탕과 *Leuconostoc mesenteroides* B-512FMCM 덱스트란수트라아제를 반응시켜 진행하였고, 생성된 루부소사이드 배당체 (Ru-G, 13-O-(α-1→6-glucosyl)-β-glucosyl-19-O-β-D-glucosyl-steviol)의 특성을 확인하였다. 루부소사이드 배당체 생성 최적 반응 조건은 RSM을 이용하여 확인하였고, HPLC를 이용하여 정제하였으며, NMR로 구조를 분석하였다. 기능성 연구 결과 배당체는 이데베논(Idebenone), 커큐민(Curcumin)의 수용성을 증가시켰다. 또한 충치의 원인이 되는 *S. mutans* 균에서 얻어진 뮤탄수크라아제가 불용성 글루칸을 형성하는 특성을 15%가량 억제하는 것을 확인하였다. 세포 독성과 항염증 작용은 쥐의 대식 세포인 RAW264.7 세포를 사용해 연구하였다. 배당체의 세포 독성은 4 mM 이하의 농도에서 루부소사이드에 비하여 크게 감소하였으며, 항염증 작용은 비슷하게 유지되었다. 맛테스터기 기계 분석 결과 배당체의 쓴맛은 스테비오사이드에 비하여 상당부분 개선되었으며, 루부소사이드와 비교하여 10% 감소하였다.