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Novel Anti-cariogenic Characteristics of Rubusoside and Synthesis of Its Fructosides

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Novel Anti-cariogenic Characteristics of Rubusoside and Synthesis of Its Fructosides

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Abstract

Rubusoside (Ru, 13-O-*β*-glucosyl-19-O-*β*-D-glucosyl-steviol) is the main component of *Rubus suavissmimus S. Lee* (Roasaceae), which is known as Chinese sweet leaf. In this study, Ru was characterized as anti-cariogenic materials. Ru was produced from stevioside (Ste) using β-galactosidase from *Thermus thermophilus*, which was expressed in *E. coli* BL21 (DE3) pLysS through lactose induction. The enzyme was purified by heat-treatment at 70℃ for 15 min. The 73.3% of mesophilic proteins was eliminated and it showed 85.3% activity yield. Enzyme reaction was carried out with immobilized β-galactosidase and Ru was purified with medium performance liquid chromatography (MPLC) equipped with ESLD detector. Ru at 50 mM showed 97.1 ± 0.2% inhibition activity against 0.1 U/mL mutanscrase from *Streptococcus mutans*. It was shown competitive inhibition activity with IC_{50} of 2.3 mM and K_i value of 1.1 ± 0.2 mM. MIC and MBC of Ru against *S. mutans* growth were 7 mM and 10 mM, respectively. MBC was higher than MIC, that is, Ru inhibits *S. mutans* as a bacteriostatic agent. Additionally, fructosyl-rubusoside (Ru-Frcs) was synthesized using levansucrase from *Leuconostoc mesenteroides* to improve the taste of rubusoside. Optimal condition for synthesizing Ru-Frcs was 217.8 mM Ru, 723.2 mM sucrose and 22.8 U/mL levanuscrase with 33.5% conversion. Purified Ru-Frc was prepared with high-performance liquid chromatography (HPLC) equipped with NH_2

column at flow rate of 4 mL/min. The structure of Ru-Frc 1 and Ru-Frc 2 were confirmed with nuclear magnetic resonance (NMR) Spectrometer 850 MHz as 13-O-[*β*-fructofuranosyl-(2→6)-*β*-D-glucosyl]-19-O-*β*-D-glucosyl-steviol), 13-O-*β*-D-glucosyl-19-O-[*β*-fructofuranosyl-(2→6)-*β*-D-glucosyl]-steviol, respectively.

Key words: Rubusoside (Ru), *Streptococcus mutans*, Mutansucrase, Levansucrase, Acceptor reaction

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Contents

List of Tables

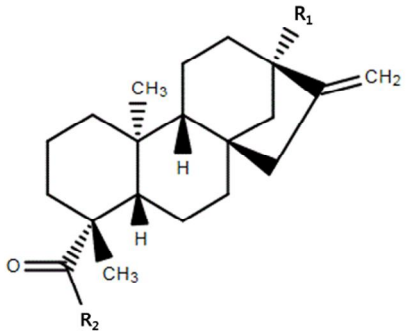
List of Figures

Review of literature

1. Steviol glycosides

The need for alternative sweeteners is expected to increase as obesity and type-II diabetes are becoming more prevalent. The natural sweeteners have been developed to provide alternative for sucrose that are non-cariogenic, non-calorific [1]. One of the most promising natural constitutes is a steviol glycosides, which is derived from the leaves of the South American plant called *Stevia rebaudiana* (Bertoni) and in other species, namely, the Chinese black berry *Rubus suavissimus S. Lee* (Roasceae), the Mexican *Stevia phlebophylla* A. Gray [2]. In the past, stevioside was regarded as the only steviol glycosides presents in the leaves of *S. rebaudiana* until researchers from the University of Hiroshima obtained rebaudiosides A and B from a methanol extraction of the leaves. By the early 21st century, nine steviol glycosides had been identified within *S. reabaudiana* leaves, namely, stevioside, rebaudiosides A, B, C, E and F, dulcoside A, and steviolbioside. Extensive selection procedures have yielded cultivars with large differences in total steviol glycoside content as well as percentage steviol glycoside compositions, but the most common composition of the wild variety, calculated on a dry weight basis, is often reported as follows: stevioside (5－10% w/w), rebaudiosides A (2－5%) and C (1%), dulcoside A (0.5%), rebaudiosides D, E, and F (0.2%), and steviolbioside (0.1%).

Table 1. Steviol glycosides from *Stevia rebaudiana*



	R_1	R_2
stevioside	Glcβ1-	Glcβ(1-2)Glcβ1-
Steviol	H	H
Rebaudioside A	Glcβ1-	Glcβ(1−2)[Glcβ(1−3)]Glcβ1−
Rebaudioside C (= dulcoside B)	Glcβ1-	Rhaα(1−2)[Glcβ(1−3)]Glcβ1−
Rebaudioside D	Glcβ (1−2)Glcβ1−	Glcβ(1−2)[Glcβ(1−3)]Glcβ1−
Rebaudioside E	Glcβ (1−2)Glcβ1−	Glcβ(1−2)Glcβ1−
Dulcoside A	Glcβ1−	Rhaα(1−2)Glcβ1−
Rubusoside	Glcβ1−	Glcβ1−

Glcβ = β-*D*-glucopyranosyl; Rhaα = α-*L*-rhamnopyranosyl

1.1. Stevioside

Stevioside is one of steviol glycosides, the most abundant compound in *stevia rebaudiana* (Bertoni). Stevioside has approximately 143-fold sweetness than sucrose at a concentration of 0.025%. The commercial stevioside has been released in the market since the 1970s in Japan. However, its bitter aftertaste restricts its use for human consumption and limits its applications in other food or pharmaceutical products. To overcome this problem, many researchers have tried to improve this weakness by enzyme based modification or biotransformation [2]. According to studies of the structure-sweetness relationship, taste quality of the derivatives produced by CGTase or b-fructofuranosidase was greatly improved when transglucosylation occurred at the 13-hydroxyl or the 19-carboxyl group. However, yields of these transfer products by other enzymes, except CGTase, have been too low for use in industrial applications. Process optimization for industrial scale and new enzyme are required to establish economical production procedures for stevioside derivatives [2].

1.2. Rubusoside

Rubusoside (Ru, 13-O-β-glucosyl-19-O-β-d-glucosyl-steviol) is the main component of the leaves of *Rubus suavissmimus S. Lee* (Rosaceae), which grows only in southern China with variable yearly yields depending on local climate. The leaves of *Rubus suavissmimus S. Lee* have been used to treat various diseases such as relieve coughs, hypertension, diabetes, atherosclerosis [38]. Recently, Thompson et al. have shown that rubusoside inhibits human GLUT1 and GLUT5 by transport activity assays (Fig. 2) [6]. In human, GLUT5 is upregulated in several disease states, including diabetes and some breast cancers, so it is an attractive target for therapeutic intervention. Protein interactions pinpointed a major difference in substrate cavity between these transporters, a residue that is a tryptophan in GLUT1 but an alanine in GLUT5 based on *in silico* analysis of rubusoside [6].

Additionally, Ru is about 110 times as sweet as sucrose at the concentration of 0.025% but it has a slightly bitter aftertaste. Darise et al. synthesized various Ru derivatives by transglycosylation by cyclomaltodextrin glucanotransferase (CGTase) and studied the relationship between the chemical structures and their tastes [4]. Evaluation of the sweetness and quality of the taste of these derivatives disclosed that sweetness and its quality of taste were greatly improved by the transglycosylation of the glucosyl residue at the 13-hydroxyl group, while a change of sweetness and quality of taste

for the worse was observed with transglycosylation to the glucosyl residue at the 19-carboxyl group [4].

Recently, Ru has been increasingly attracting attention for its solubilizing properties. The behavior of Lanmuir monolayers at interfaces could reveal the amphiphilic and self-assembled properties of amphiphile [3]. The different π-A isotherms of blank Ru particles showed that Ru molecular area was 25 nm^2 and the collapse pressure of the Ru Langmuir monolayer was merely 33 mN/m. It indicated that Ru had amphipathic features, which could be beneficial for micelle formation (Fig. 1) [3]. It was proved that Ru was self-assembled to form micelles. The Ru-based micelle system is a promising small molecule carrier that efficiently improved the solubility of insoluble drugs.

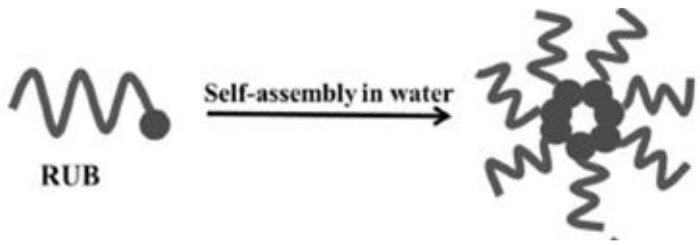


Figure 1. Micelle illustration of Ru in water

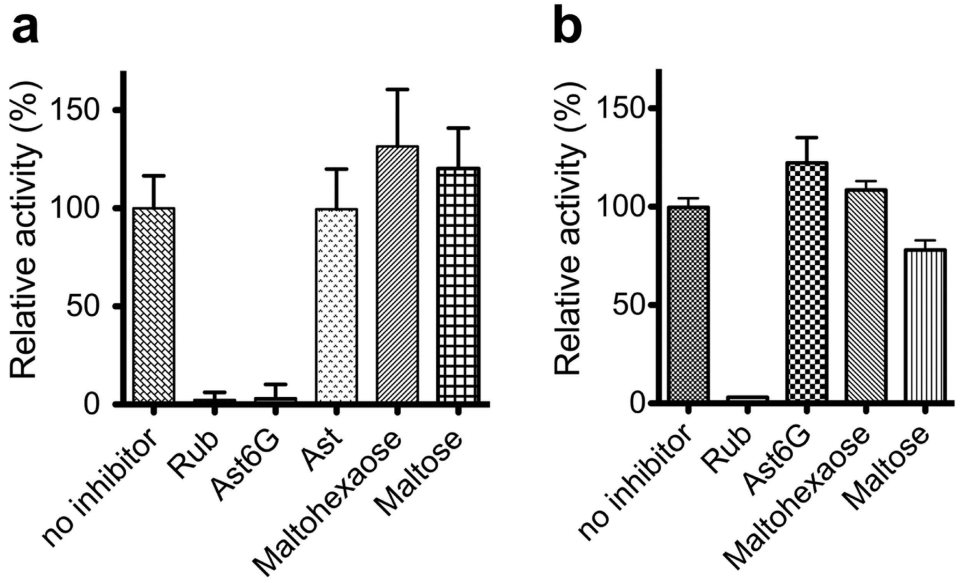


Figure 2. Relative transport activity of GLUTs in proteoliposomes in the presence of inhibitors, using the entrance counter-flow transport assay. Each point is an average of at least three measurements and error bars represent standard deviation. All inhibitors were added 20 mM. Rub, Ast and Ast6G are rubusoside, astragalin and astragalin-6-glucoside, respectively. (a) GLUT5-mediated fructose transport (b) GLUT1-mediated glucose transport [3]

2. β-galactosidase from *Thermus thermophiles*

It has been reported that β-galactosidase from *Thermus thermophiles* hydrolyzes stevioside to rubusoside on the previous study [38]. Thirty commercial enzymes were investigated, having the mixed activities of pectinase, hemicellulases, cellulases, β-galactosidase β-galactosidase, and/or β-glucanase, along with a purified recombinant lactase. Crude pectinases from *Aspergillus niger* (Sumizyme SPC, sumilact L, validase AGS), naringinase from *Penicillium* spp. (Cellulase Kn), and recombinant lactase from *Thermus thermophilus* could all convert stevioside to rubusoside as a main product. Among these, the recombinant lactase from *T. thermophilu*s showed the highest rubusoside productivity [38].

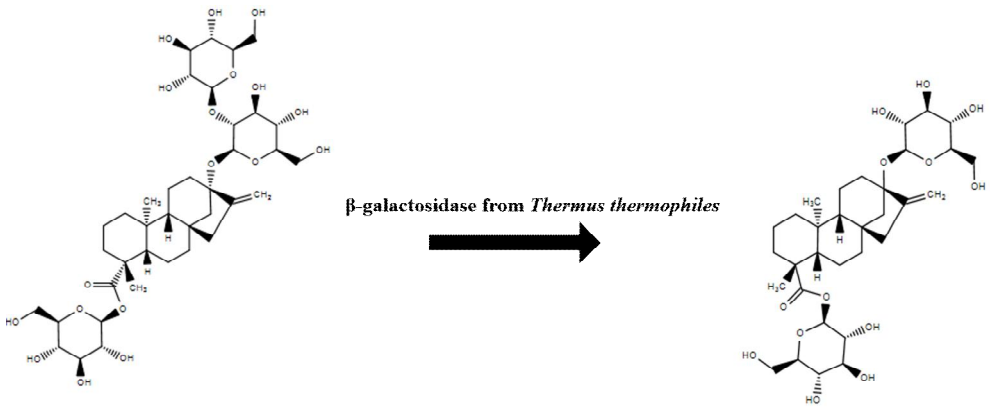


Figure 3. Hydrolysis activity of β-galactosidase from *Thermus thermophilus*

3. Mutansucrase from *Streptococcus mutans*

S. mutans is the main microbial etiological agent of dental caries, due to its ability to adhere to the tooth surface, by producing sticky extracellular polysaccharides from sucrose, and to ferment sucrose and other sugars to acids which attack the tooth enamel: cariogenicity [7]. Cariogenic *Streptococcus mutans* cells secrete two or more kinds of glycosyltransferases, resulting in the adherence of cells on smooth hard surfaces. Those are water-soluble glucan ('dextran', formed by dextransucrase) in which α-(1,6) glycosidic bonds predominate, insoluble glucan ('mutan', formed by mutansucrase) with a majority of α-(1,3) and a minority of α-(1,6) glycosidic bonds and fructan with either β-(2,1) or β-(2,6) linkages (formed by fructosyltransferase) [9, 10]. The synthesis of extracellular polysaccharides from sucrose is believed to be a major determinant in the induction of tooth decay by *Streptococcus mutans*.

4. Levansucrase from *Leuconostoc mesenteroides*

Levan (β-(2,6)-linked fructose resides, levansucrase, EC 2.4.1.10) and inulin (β-(2,1) linked fructose residues, inulosucrase, EC 2.4.1.9) were synthesized with fructosyltransferase enzymes, which belong to glycoside hydrolase family 68 (GH 68) [13]. Bacterial levansucrases hydrolyze sucrose into glucose and fructose and synthesize polymer of fructose called levan. It has been reported that levansucrase activity is involved in a variety of processes including survival of bacteria in soil (*B. subtilis*), phytopathogenesis (*Erwinia* and *Pseudomonas* species) and symbiosis (*Paenibacillus polymyxa*) of plant interactive bacteria [15].

5. Hypothesis and objectives

Rubusoside (Ru) has a variety of advantages as a solubilizer, pharmaceutical agent and natural sweetener in food industry. However, major drawback to industrialization is the high price due to small amount of Ru found in nature. Another drawback as a natural sweetener is its a bitter aftertaste. To overcome this problem, many researchers have tried to improve this weakness through biotransformation or enzyme based modification. In this study, various enzymes were screened in order to synthesize fructosyl-rubusoside and dextransucrase was inhibited under the presence of Ru. Dextransucrase catalyzes the polymerization of dextran from sucrose with α-(1,6) linkages and α-(1,3) linkages [10], which are similar features of mutansucrase from *Streptococcus mutans*. Based on this idea, we have hypothesized that Ru would also inhibit mutansucrase activity. The aim of this study, therefore, is (1) to mass-produce Ru with β-galactosidase from *Thermus thermophilus* through lactose induction. Secondly, it is (2) to improve the taste of Ru through synthesis of fructosyl-rubusoside with levansucrase from *Leuconostoc mesenteroids*. Lastly, (3) Ru will be studied for its anti-cariogenicity by investigating the inhibitory mechanism of Ru against mutansucrase and the growth of *S. mutans*.

Material and Methods

1. Preparation of rubusoside

1.1. Expression of β-galactosidase (β-*glypi* gene) in *E.coli*

In the previous study, pRSETB_β-*glypi* was constructed by inserting β -galactosidase (β-*glypi* gene) with XhoI/EcoRI digestion [38]. pRSETB_ β-*glypi* was transformed and expressed in *E. coli* BL21 (DE3) pLysS, *E. coli* BL21 (DE3) and *E. coli* Rosetta (DE3). Transformed *E. coli* with pRSETB_β-*glypi* were cultivated in LB media containing 1.0% (w/v) tryptone, 1.0% (w/v) NaCl, 0.5% (w/v) yeast extract and supplemented with 50 μg/ ml ampicillin at 37°C with 200 rpm shaking until the optical density (600 nm) reached 1.0. Then, induction was conducted with 5 mM lactose at 37°C for 12 h. The cell was harvested by centrifugation (8000 x *g* for 30 min at 4°C), resuspended in 50 mM Tris-HCl buffer (pH 7.0) with 4% volume (v/v) of total LB media. Sonication was conducted in order to disrupt the cell with Ultrasonic processor 250 (Sonics and Materials, Inc., USA; output 25, for 1 min, 4 repeat on ice). The cell debris was centrifuged for 30 min at 12,000 x *g* and the supernatant was heated at 70°C water bath to eliminate mesophilic proteins for 20 min. Heat-denatured proteins were removed by centrifugation at 8,000 x *g* for 15 min. Concentration of the purified enzyme was carried out with evaporator. Protein amount of the purified β-galactosidase was determined by Bradford method

with bovine serum albumin (Georgiachem, USA) as the standard. Also, size of the β-galactosidase was determined with SDS-PAGE.

1.2. β-galactosidase hydrolytic activity assay

The β-galactosidase activity was determined using 2.5mM pNPGlc as substrates and enzyme in 50 mM Tris-HCl buffer (pH 7.0) at 70°C for 20min and quenched with 250 mM Na_2CO_3. The absorbance at 420nm caused by release of p-nitrophenol was measured with in order to calculate β-galactosidase activity. One unit (U) of β-galactosidase activity was defined as the amount of enzyme required to release 1 μ M p-nitrophenol per minute.

1.3. Immobilization of β-galactosidase

Immobilization of β-galactosidase was carried out with the previous method with slight modification [38]. The β-galactosidase was mixed with 3% (w/v) sodium alginate solution to give final unit 200 unit/ml of alginate beads. To make even beads, vacuum degassing was carried out with Aspirator A-1000S pump (Eyela, Japan) until the gas was removed. The degassed enzyme solution with sodium alginate was extruded drop-wise through BT300-2J with YZ1515x (Longer Precision Pump Co., China) into 2% (w/v) $CaCl_2 \cdot 2H_2O$. It was lasted for 30

min in the ice with gentle stirring to obtain 2 mm sized bead. The alginate beads containing β-galactosidase were kept in 2% (w/v) $CaCl_2 \cdot 2H_2O$ to stabilize for 30 min following washing with distilled water, then kept at 4°C for 2 h for hardening.

1.4. Production and purification of rubusoside

Rubusoside (Ru) was produced with steviol glycosides as a substrate and immobilized β-galactosidase expressed in *Escherichia coli* BL21 (DE3) pLysS [16]. The alginate beads containing β-galactosidase was reacted with 2% (w/v) steviol glycosides (Daepyung, South Korea) in 50 mM Tris-HCl buffer (pH 7.0). The reaction temperature was controlled with a heat circulator NCB-1200 (EYELA, Japan) at 70°C. Ru was purified with medium performance liquid chromatography (MPLC) equipped Reveleris® Amino 120 g Flash Cartridge (GRACE Discovery Science, USA) at flow rate of 80 mL/min. The detection was achieved with ESLD detector.

2. Study for Anti-cariogenicity of Rubusoside

2.1. Preparation of mutansucrase from *Streptococcus mutans*

Streptococuccus mutans KCTC 3065 was obtained from Korean Collection for Type Cultures (KCTC). *S. mutans* KCTC 3065 was cultured in brain heart infusion media (BHI; BD Difco, USA) with 2% sucrose for seed at 37°C for 12 h. For main culture, 3% of the seed culture was inoculated into BHI with 2% glucose media and incubated at 37°C until glucose was all consumed. The cells was centrifuged with 8,000 x *g* for 15 min and the supernatant was obtained for crude enzyme. The crude enzyme was concentrated with Centriprep Centrifugal Filter Unit with Ultracel-YM10 (Merck, Germany). The concentrated enzyme was kept at –20°C for the further study.

2.2. Purification of mutansucrase

Crude mutansucrase from *Streptococcus mutans* was loaded onto a DEAE-Sepharose ion exchange chromatography column (1×1×60 cm) equilibrated with 20 mM sodium phosphate buffer (pH 6.8). Mutansucrase was eluted with the same buffer 0–1 M NaCl in 20 mM sodium phosphate buffer (pH 6.8). Fractions with mutansucrase activity were pooled and dialyzed with 20 mM sodium phosphate buffer (pH 6.8) at 4°C. The dialyzed fractions were concentrated with Centriprep Centrifugal Filter Unit with Ultracel-YM10 (Merck, Germany). The

purified enzyme was kept at –20°C for further study.

2.3. Characterization of mutansucrase

Amount of protein in mutansucrase was determined with the Bradford assay using crystalline bovine serum albumin as standard [39]. Mutansucrase activity was determined with a release of fructose when substrate was sucrose. The released fructose was determined with D-fructose kit (Megazyme, Ireland). The increase in absorbance at 340 nm was measured to calculate mutansucrase activity. One unit (U) of mutansucrase activity was defined as the amount of enzyme required to release 1 μM fructose per min under the above reaction condition. K_m of mutansucrase for Ru was calculated with the double reciprocal Lineweaver-Burk plot and Michaelis-Menten kinetic.

2.4. Inhibition activity of rubusoside against mutansucrase

For relative inhibition activity, mutansucrase inhibition activity depending on existence of Ru was calculated. First, Ru was dissolved in distilled water to obtain 50 mM stock solution. The reaction mixture contained 500 mM sucrose, 0.1 U/mL of mutansucrase and 50 mM Ru in 50 mM sodium phosphate buffer (pH 6.8). The reaction was carried out at 37°C for 12 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 precoated silica gel 60 F_{254} (Merck, Darmstadt, Germany). It was visualized by dipping in a solvent mixture of 0.5% (w/v) *N*–(1-naphtyl) ethylenediamine dihydrochloride (Sigma Aldrich, USA) and 5% (w/v) sulfuric acid (Duksan Chemicals, South Korea) in methanol (Duksan Chemicals, South Korea) and heating at 120°C for 5 min. Relative inhibition activity was calculated with AlphaEase 4.0 program (Alpha Inotech, CA, USA). Inhibition activity was defined as a release of fructose compared with a reaction mixture containing mutansucrase without inhibitor. The released fructose was determined with D-fructose kit (Megazyme, Ireland). Also, Inhibition activity was calculated with the following equation (1):

$$\text{Inhibition activity (\%)} = 100 - [(S - S_0) / (C - C_0)] \times 100 \quad (1)$$

Where C was absorbance of control (the reaction mixture except inhibitor) after reaction, C_0 was absorbance of control at zero time, S was absorbance of sample (the reaction mixture with inhibitor) after reaction and S_0 was absorbance of sample at zero time. The 50% inhibitory concentration (IC_{50}) was defined as concentration of Ru to reduce mutansucrase activity by 50% relative to a reaction mixture containing mutansucrase without any inhibitor. Inhibitor kinetic study for Ru was performed with various concentrations of inhibitor (0–10

mM) and substrate (35–175 mM). Lineweaver-Burk and Dixon plot (1/v as a function of inhibitor concentration [I]) was used for determining the inhibition type of Ru and inhibition constant (K_i). All experiments were carried out three times.

2.5 Antimicrobial susceptibility test for *S. mutans*

The antibacterial activity of Ru against *S. mutans* was assayed by disk diffusion test. For the disk diffusion test, *S. mutans* was aerobically cultured in brain-heart infusion (BHI) agar in 37°C incubator until OD_{600} reached approximately 1.0. Distilled water and disk papers were sterilized at 121℃ for 15 min. Rubusoside solution (250 mM) was filtered with Minisart® syringe filter 0.2 μm (Satorius, Germany). After spreading *S. mutans* (1.5 x 10^7 CFU/mL) on the BHI agar plate, sterile paper disks were impregnated with 20 μL with 250 mM Ru, 250 mM EGCG (positive control) [33] and distilled water, respectively.

2.6. Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) test for *S. mutans*

Minimum inhibitory concentraion (MIC) and minimum bacterial concentration (MBC) were determined with a modification of the

diluted method [34-37]. Rubusoside was diluted to various concentrations, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 mM, into strerile BHI broth in the 96-well plate. A 5 μL of fresh *S. mutans* culture (OD_{600} = 1.0, 1.5 x 10^7 CFU/mL) was inoculated to the BHI media in 96-well plate and cultivated at 37℃ for 24 h. Thereafter the 96-well plate was observed for growth and turbidity with SpectraMax M3 (Moleculardevices, USA) at A600 nm. Subsequently, 100 μL of broth from each well not showing growth, was inoculated into BHI agar plate and cultivated at 37℃ for 24 h to determine MBC. Then, the agar plate was examined for turbidity using SpectraMax M3 (Moleculardevices, USA) at absorbance 600 nm. All experiments were carried out three times.

3. Synthesis and characterization of fructosyl-rubusoside (Ru-Frcs)

3.1. Expression of levansucrase (*m1ft* gene) in *E. coli*

Escherichia coli BL21 (DE3) (Invitrogen, USA) was used as a host strain for expression of levansucrase coding gene (*m1ft*) from *L. mesenteroides* B-512 FMC as described in the previous study [13]. *E. coli* BL21 (DE3) was grown in LB media consist of 0.5% (w/v) yeast extract, 1% (w/v) tryptone, and 0.5% (w/v) NaCl supplemented with 50 μg/ml ampicillin at 37°C until the OD_{600} reached approximately 0.5. The cell was induced with 1 mM lactose for 15 h at 28°C and collected by centrifugation (8,000 x *g* for 20 min at 4°C), resuspended in 50 mM potassium phosphate buffer (pH 6.0) with 25% volume (v/v) of total LB media. Sonication was conducted in order to disrupt the cell with Ultrasonic processor 250 (Sonics and Materials, Inc., USA; output 25, for 30 s, 5 repeat on ice). The cell lysate was centrifuged for 30 min at 12,000 x *g*. The supernatant was kept at –20℃ for the further study as crude enzyme.

3.2. Levansucrase hydrolytic activity assay

Levansucrase activity was determined using 400 mM sucrose and enzyme in 50 mM potassium phosphate buffer (pH 6.0). The reaction was conducted at 37°C for 30 min. One μL aliquot of the enzyme reacted sample was spotted on TLC plate coated with silica gel 60

F_{254} plate (Merck, Germany) and developed with three ascents of acetonitrile-water [85:15 (v/v)]. The plate was visualized by heating at 125°C for 5 min followed by dipping into methanol containing 0.5% (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride (Sigma Aldrich, USA) and 5% (w/v) sulfuric acid (Duksan, South Korea). The fructose concentration liberated from sucrose was determined using integrated density values (IDV) by employing the AlphaEase 4.0 program (Alpha Inotech, USA). One unit of levansucrase activity was defined as the amount of enzyme that catalyzed the release of 1 μM fructose per min under the reaction condition.

3.3. Synthesis of Ru-Frcs using levansucrase

Ru acceptor reaction was carried out with 6 U/ml levansucrase, 500 mM sucrose, 50 mM Ru as an acceptor and 50 mM potassium phosphate buffer (pH 6.0). The reaction was incubated at 37°C for 12 h. One μL of reaction mixtures was spotted on silica gel 60 F_{254}TLC plate (Merck, Germany) and developed in acetonitrile-water [85:15 (v/v)]. The products on the TLC plate were shown by heating at 125℃ for 5 min after dipping into a solvent system of 5% (w/v) sulfuric acid (Duksan, Korea) and 0.5% (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride (Sigma Aldrich, USA) in methanol (Duksan, Korea). Molecular weights of the products were determined with

MALDI-TOF-MS analysis. The amount of Ru converted to Ru-Frcs was calculated with high performance liquid chromatography Waters e2695 system (Waters, USA) equipped Kromasil® 100-10-NH_2 (Azkonobel, Sweden) at flow rate of 4 mL/min. The detection was achieved with Wyatt T-rex (Wyatt, USA).

3.4. Optimization for acceptor reaction using response surface methodology (RSM)

The central composite design (CCD) RSM software program (Design Expert 10.0.3, USA) was used to optimize conversion of Ru to Ru-Frcs with the following three variables: Ru concentration (10–400 mM), sucrose concentration (100–1000 mM) and enzyme concentration (2–20 U/mL). Twenty runs of the experiment were carried out with Design Expert ver. 10.0.3 with six replications at the central point, which were utilized in the fitting of a second-order response surface. All statistical and mathematical analyses of the results were performed with 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 and fitted through the response surface regression procedure using the following second order polynomial equation:

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

where Y represented the predicted response; βo, βi, βii and βij were the regression coefficients for intercept, linearity, square and interaction, respectively. *Xi* and *Xj* were the independent coded variables. The significance of the model was evalutated 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.

3.5. Purification of Ru-Frcs

Acceptor reaction mixture containing 500 mM sucrose, 50 mM Ru, and 6 U/ml levansucrase and 50 mM potassium phosphate buffer (pH 6.0) was analyzed by TLC method as described above. Chilled Ethanol was used to precipitate polymers in the mixture with 90% final concentration and the mixture was centrifuged (8,000 x *g* for 20 min). The supernatant was loaded into HP-20 column (Iontech, South Korea). After washing the column with distilled water to remove monosaccharides, Ru-Frcs were eluted with 100% ethanol. The elutes were concentrated and lyophilizated with freeze dryer (Eyela, Japan). The dried Ru-Frcs were dissolved into 200 μg/mL of DMSO and

purified with high performance liquid chromatography Waters 2545 binary gradient module (pump), 2767 sample manager (injector). The compounds were monitored at 210 nm with 2998 photodiode array detector (Waters, USA) equipped Luna® 5 μm NH_2 100 Å (250 × 21.22 mm) (Phenomenex, USA) at flow rate of 20 mL/min with water and acetonitrile. Water was flew at 10% for 40 min in an isocratic manner and gradually increased to 90% for 5 min and then, sustained isocratically for 15 min. The purified Ru-Frc 1 and Ru-Frc 2 were lyophilized for the further study.

3.6. Structural elucidation of Ru-Frcs

Ten mg of each purified Ru-Frc 1 was dissolved in deuterium oxide (Sigma, USA) and Ru-Frc 2 was dissolved in DMSO-d_6 and placed into 5 mm TXI (1H/13C/15N). Nuclear magnetic resonance (NMR) spectra were obtained with AVANCE III HD (Bruker, German) operated at 850 MH_Z in the National Center for Inter-University Research Faculties (NCIRF) of Seoul National University (Seoul, Korea). The structure of purified Ru-Frc 1 and Ru-Frc 2 were confirmed with ^{1}H, ^{13}C, heteronuclear multiple bond correlation (HMBC), heteronuclear single quantum coherence (HSQC), homonuclear correlation spectroscopy (COSY) and rotating frame overhause effect spectroscopy (ROESY).

Results and Discussion

1. Preparation of rubusoside

1.1. Expression and partial purification of β-galactosidase

The crude β-galactosidase activity from *T. thermophilus* expressed in *E. coli* BL21 (DE3) pLysS was shown 32 ± 0.5 U/mL (Table 1). Also, total protein in the crude β-galactosidase was 2339.8 mg. After heat-treatment, the activity of β-galactosidase was shown 30 ± 0.3 U/mL and the amount of total protein in the heat-treated β -galactosidase was 625.9 mg, which means 73.2% mesophilic proteins were eliminated with 85.3% total activity yield. Specific activity of partially purified β-galactosidase was increased with 3.2 fold purification. The size of purified β-galactosidase was analyzed with SDS-PAGE and it was approximately 48 kD (Fig. 4). Purified β -galactosidase was immobilized with 3% sodium alginate (Fig. 5).

Table 2. Partial purification of β-galactosidase

	Total volume (mL)	**Total protein (mg)**	**Total unit (U)**	**Specific activity (U/mg)**	**Purification (fold)**	**Yield (%)**
Crude enzyme	500	2339.8	16000	6.8	1	100
Heat treated enzyme	455	625.9	13650	21.8	3.2	85.3

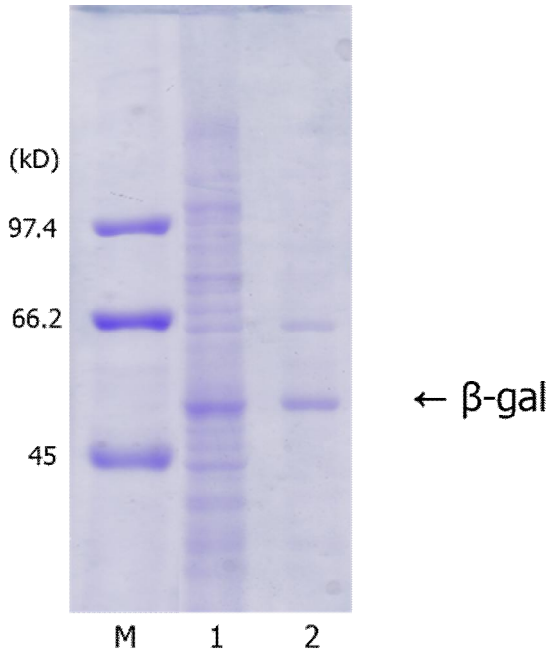


Figure 4. SDS-PAGE analysis for protein patterns of crude β -galactosidase and purified β-galactosidase; M, marker; 1, crude enzyme; 2, purifed β-galactosidase

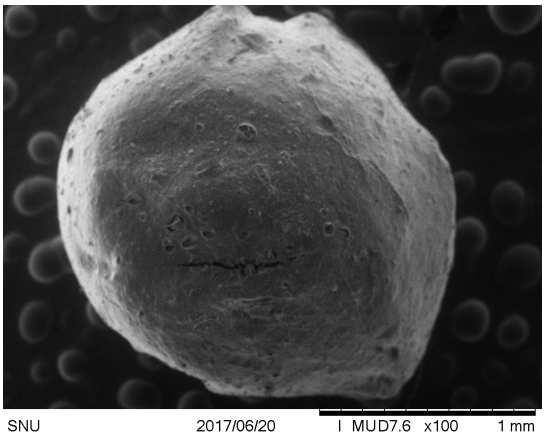


Figure 5. SEM images of immobilized β-galactosidase with 3% sodium alginate (x100)

1.2. Production of rubusoside

Immobilized β-galactosidase was mixed with 2% (w/v) steviol glycosides (Daepyung, Kroea) in 50 mM Tris-HCl (pH 7.0) at 70°C in a heat circulator NCB-1200 (EYELA, Japan) for 12 h. After reaction, Ru released from stevioside was checked with TLC analysis (Fig. 6A). Purification of Ru by using MPLC was carried out and purity of Ru was shown as ≥ 95.0% (TLC) (Fig. 6B).

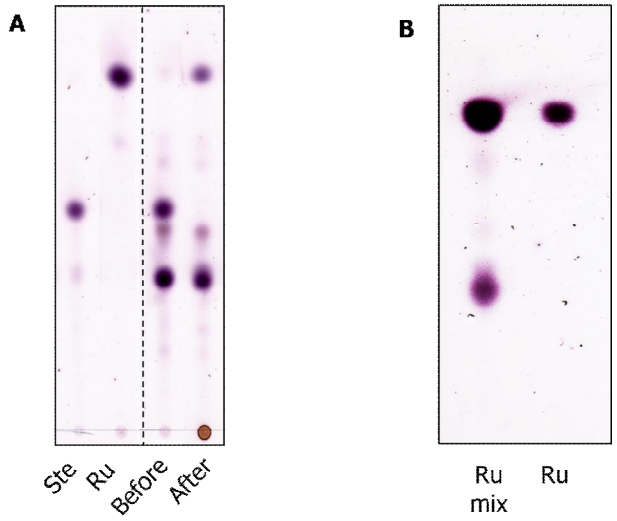


Figure 6. TLC analysis of immobilized β-galactosidase reaction (A) and purity of Ru (B) was shown as ≥95.0% (TLC); Ste, stevioside; Ru, rubusoside; Before, before reaction; After, after reaction; Ru mix, rubusoside mixture after reaction

2. Study for anti-cariogenicity of rubusoside

2.1. Characterization of mutansucrase from *S. mutans*

Fractions with mutansucrase activity were pooled and dialyzed with 20 mM sodium phosphate buffer (pH 6.8) at 4°C. The dialyzed fractions were concentrated with Centriprep Centrifugal Filter Unit with Ultracel-YM10 (Merck, Germany). Michaelis-Menten kinetic and Lineweaver-Burk plot for determination of K_m were shown in (Fig. 7). K_m of mutansucrase calculated from the double reciprocal Lineweaver-Burk plot was 34.5 ± 4.6 mM.

2.2. Inhibition activity of rubusoside against mutansucrase

The relative inhibition activity of Ru was shown in the Fig. 8 and it shows 97.1 ± 0.2 % relative inhibition activity. Also, 50% inhibitory concentration (IC_{50}) was defined as concentration of Ru to decrease mutanuscrase activity by 50%. IC_{50} of Ru against 0.1 U/mL of mutansucrase was 2.3 ± 0.0 mM. Lineweaver-Burk plot and Dixon plot were used to analyze the inhibition type of Ru (Fig. 9). The plots confirmed that Ru is a competitive inhibitor against mutansucrase. Based on linear regression analysis of the Dixon plot, K_i of Ru was determined to 1.1 ± 0.2 mM.

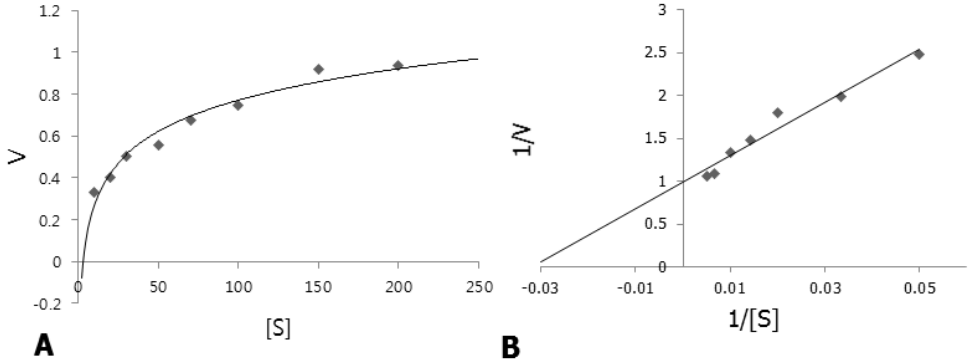


Figure 7. Michaelis-Menten kinetic (A) and Lineweaver-Burk (B) plot of the purified mutansucrase

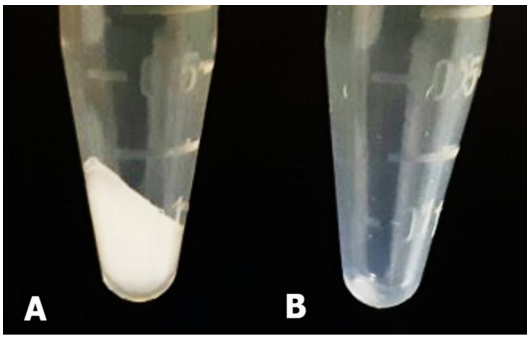


Figure 8. Relative amount of insoluble glucan (mutan) as a result of reaction with mutansucrase (A) and mutansucrase with Ru (B)

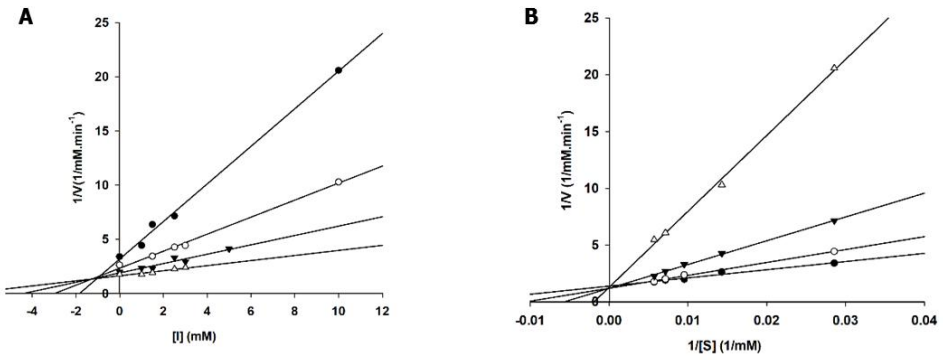


Figure 9. Dixon plot (A) and Lineweaver-Burk plot (B) for mutansucrase inhibition activity of Ru

2.3. Antimicrobial susceptibility test for *S. mutans*

As a result of disk diffusion test, Ru and EGCG (positive control) showed an inhibition halo on the BHI agar plate and control (distilled water) did not show an inhibition activity (Fig. 10). The diameters of halo on 250 mM Ru and EGCG were 1.13 ± 0.1 cm and 1.9 ± 0.1 cm, respectively. The concentration of Ru in the 96-well plate, showed no bacterial growth or turbidity after 24 h incubation at 37℃, was considered as minimum inhibitory concentration (MIC) [32]. While minimum bactericidal concentration (MBC) was regarded as the concentration of Ru that showed no growth after further 24 h incubation at 37℃ on BHI agar plate devoid of Ru. MIC was obtained for Ru against *S. mutans* growth with 7 mM and MBC of Ru was 10 mM (Table 6, 7). MBC was higher than MIC, that is, Ru inhibits *S. mutans* as a bacteriostatic agent.

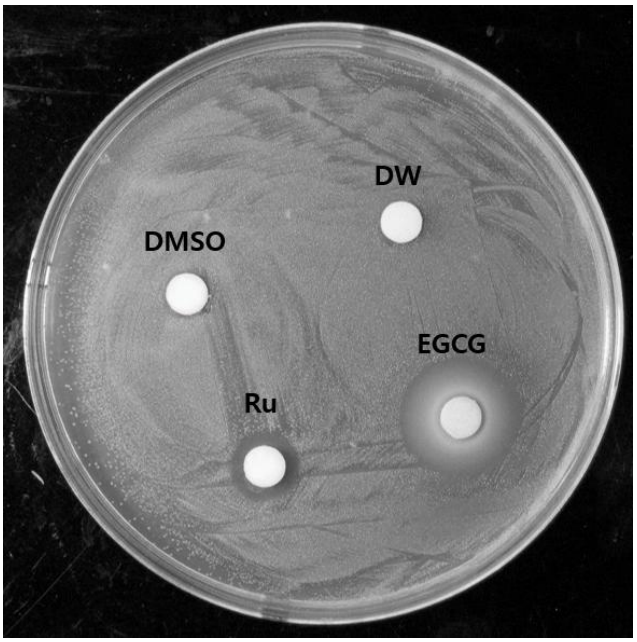


Figure 10. Antimicrobial susceptibility test of Ru for *Streptococcus mutans*; DW, distilled water; DMSO, dimethyl sulfoxide; Ru, 250 mM rubusoside; EGCG, 250 mM epigallocatechin gallate (positive control)

Table 3. Inhibition pattern of *S. mutans* using different concentrations of rubusoside in broth after 24 h incubation at 37℃

Rubusosid conc. (mM)	**Turbidity in broth**												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**Ctrl**
Streptococcus mutans	+	+	+	+	+	+	–	–	–	–	–	–	+

Table 4. Growth pattern of *S. mutans* 24 h after inoculation of broth from different concentrations of rubusoside that inhibited bacteria onto BHI agar plates

Rubusosid conc. (mM)	**Growth on agar**												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**Ctrl**
Streptococcus mutans	+	+	+	+	+	+	+	+	+	–	–	–	+

3. Synthesis and chracterization of fructosyl-rubusoside (Ru-Frcs)

3.1. Synthesis and optimization of Ru-Frcs using levansucrase

The results of acceptor reaction using levanuscrase with Ru and sucrose are shown (Fig. 11A). After removing polymers and monosaccharides (glucose) with 90% ethanol and HP-20 column, respectively, molecular weights of Ru-Frcs in the reaction products were analyzed via MALDI-TOF-MS (Fig. 11B). Based on MALDI-TOF-MS analysis (Fig. 12), in the Ru-Frcs products, Ru-Frc 1 and Ru-Frc 2 containing one fructosyl unit was observed at *m/z* 827 $(M + Na)^+$, Ru-Frc 3 and Ru-Frc 4 containing two attached fructosyl units was observed at *m/z* 989 $(M + Na)^+$. When it was considered yield of the products, Ru-Frc 1 and Ru-Frc 2 were focused on this study.

Response surface methodology (RSM) is a statistical technique for modelling and optimization of 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. In this study, RSM was progressed with three independent variables: Ru concentration (10–400 mM), sucrose concentration (100–1000 mM) and enzyme concentration (2–20 U/mL). The predicted and actual Ru-Frcs conversion yields are summarized. (Table 5). The 3D response surface and 2D contour plots

of independent variables with respect to the response are shown in (Fig. 13). Results of ANOVA (Analysis of variance) are shown in (Table 6). Ru-Frc products were mostly affected ($p<0.05$) by Ru concentration (B), followed by sucrose concentration (C, $p=0.033$) and enzyme concentration (A, $p=0.077$). In this case, B, C, BC, A^2, B^2, C^2 are significant model terms. The experimental data had a determination coefficient (R^2) of 0.9126, meaning the calculated model was able to explain 91.26% of results. This indicated that the model used to fit the response variables was significant ($p=0.0003$). The amount of converted Ru-Frc was expressed with the following regression equation:

$$Y = -5.44531 + 1.19180X_1 + 0.16779X_2 + 0.016757X_3$$
$$-1.23889E\text{-}003X_1X_2 - 1.87555E\text{-}004X_1X_3 + 1.25087E\text{-}004X_2X_3 -$$
$$0.019653X_1^2 - 5.03417E\text{-}004X_2^2 - 2.54586E\text{-}005X_3^2$$

Where Y was the amount of converted Ru-Frc (mM), X_1 was unit concentration (U/mL), X_2 was Ru concentration (mM) and X_3 was reacted sucrose concentration (mM). The predicted maximum Ru-Frc conversion was 33.5% at 217.8 mM Ru, 723.2 mM sucrose and 22.8 U/mL enzyme.

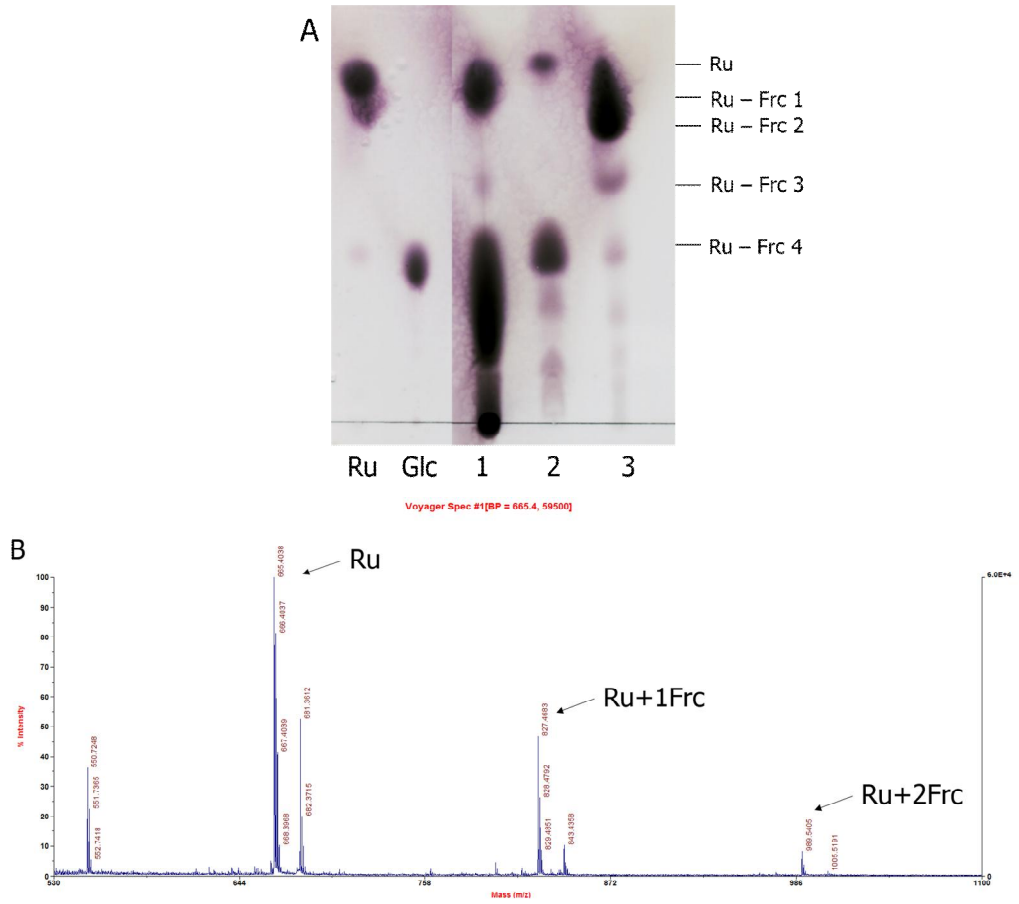


Figure 11. Analysis of levansucrase acceptor reaction with Ru using thin layer chromatography (A) and MALDI-TOF-MS (B) ; Ru, rubusoside; Glc, glucose; 1, After reactioin; 2, After removal of polyemers; 3, After removal of saccharides; Ru+1Frc, Ru containing one fructosyl unit was observed at *m/z* 827 $(M + Na)^+$; Ru+2Frc, Ru containing two attached fructosyl units was observed at *m/z* 989 $(M + Na)^+$

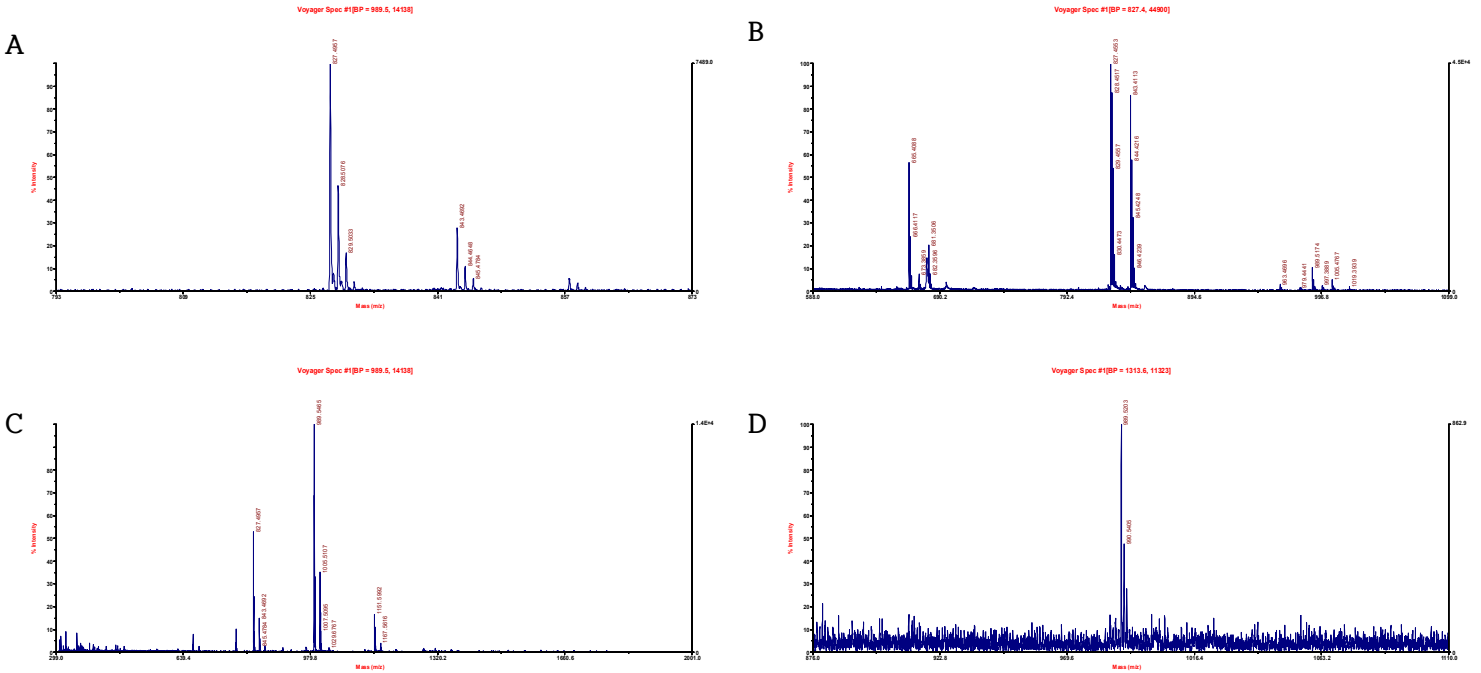


Figure 12. Analysis of rubusoside acceptor reaction products using MALDI-TOF-MS; Ru-Frc 1 (A) and Ru-Frc 2 (B) containing one fructosyl unit was observed at *m/z* 827 $(M + Na)^+$, Ru-Frc 3 (C) and Ru-Frc 4 (D) containing two attached fructosyl units was observed at *m/z* 989 $(M + Na)^+$

Table 5. Running condition for rubusoside acceptor reaction and Ru-Frcs conversion (%)

Run No.	Independent variables			Ru-Frcs conversion (%)	
	X_1	X_2	X_3	Actual	Predicted
1	10	70	750	19.2	16.3
2	10	70	250	18.3	17.2
3	20	150	500	30.1	30.2
4	20	150	500	31.3	30.2
5	30	230	250	22.2	24.4
6	20	150	500	31.2	30.2
7	20	15.6	500	11.3	13.8
8	30	70	750	18.2	19.9
9	36.8	150	500	28.2	26.7
10	20	150	500	29.2	30.2
11	3.2	150	500	20.2	22.5
12	20	150	500	30.2	30.2
13	10	230	250	25.2	22.9
14	20	150	80	21.3	23.0
15	20	150	500	29.3	30.2
16	20	284.4	500	30.0	28.4
17	30	70	250	25.1	22.7
18	30	230	750	31.2	31.6
19	10	230	750	30.2	32.0
20	20	150	920	29.2	28.3

X_1, The concentration of levansucrase; X_2, The concentration of Ru; X_3, The concentration of sucrose

Table 6. Results of two-way analysis of various (ANOVA)

Source	Sum of Squares	DF	Mean Square	F Value	p-value Prob > F
Model	582.09	9	64.68	11.60	0.0003
A-unit	21.68	1	21.68	3.89	0.0768
B-Ru	259.65	1	259.65	46.59	< 0.0001
C-sucrose	33.96	1	33.96	6.09	0.0332
AB	7.86	1	7.86	1.41	0.2625
AC	1.76	1	1.76	0.32	0.5867
BC	50.07	1	50.07	8.98	0.0134
A^2	55.49	1	55.49	9.96	0.0102
B^2	149.12	1	149.12	26.75	0.0004
C^2	36.37	1	36.37	6.53	0.0286
Residual	55.74	10	5.57		
Cor Total	637.83	19			

R^2 = 0.9126; Adj R^2 = 0.8340; DF = Degrees of Freedom

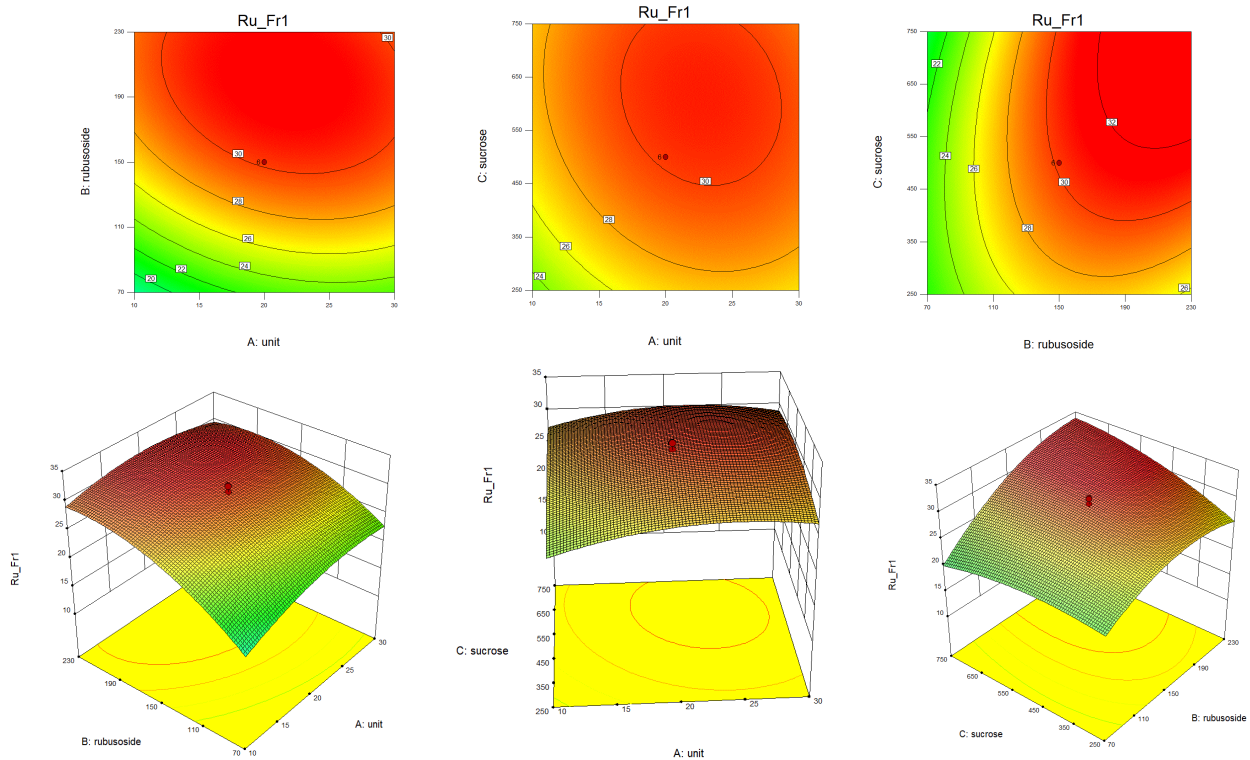


Figure 13. Response surface plot and contour plot of rubusoside concentration vs. enzyme concetration; sucrose concentration vs. enzyme concentration; sucrose concentration vs. rubusoside concentration

3.2. Purification and structural elucidation of Ru-Frcs

The molecular weight of each purified Ru-Frcs (Fig. 14) was determined throughout MALDI-TOF-MS analysis. These structures of Ru-Frcs were identified with 850 HM_Z NMR. The result of NMR analysis is summarized (Table 7). The molecular ions of Ru-Frc 1 and Ru-Frc 2 containing one fructosyl unit was observed at *m/z* 827 $(M + Na)^+$. There are some carbon signals identical to those of Ru except for the following signals: at 103.19 ppm to Frc-2 on Ru-Frc 1, which interacts with Glc-6 proton (3.72 ppm) on 13-*O*-Glc; at 104.12 ppm to Frc-2 on Ru-Frc 2, which interacts with Glc-6 proton (3.89 ppm) on 19-*O*-Glc. The signal indicated the occurrence of fructosylation on 13-*O*-Glc δ 3.73 (dt, J = 12.4, 3.4 Hz, 1H) and 19-*O*-Glc residues of Ru in the form of β-2,6 linkage, respectively. This specific position of fructoslyted hydroxyl group was confirmed with HMBC data (Fig. 15). The structure of Ru-Frc 1 and Ru-Frc 2 were elucidated to 13-O-[*β*-fructofuranosyl-(2→6)-*β*-D-glucosyl]-19-O-*β*-D-glucosyl-steviol), 13-O-*β*-D-glucosyl-19-O-[*β*-fructofuranosyl-(2→6)-*β*-D-glucosyl]-steviol, respectively (Fig. 16).

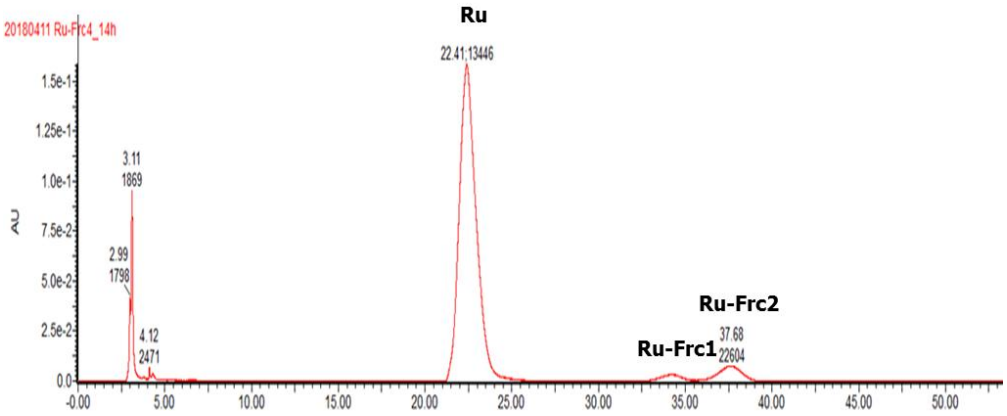


Figure 14. The HPLC chromatogram of Ru-Frcs

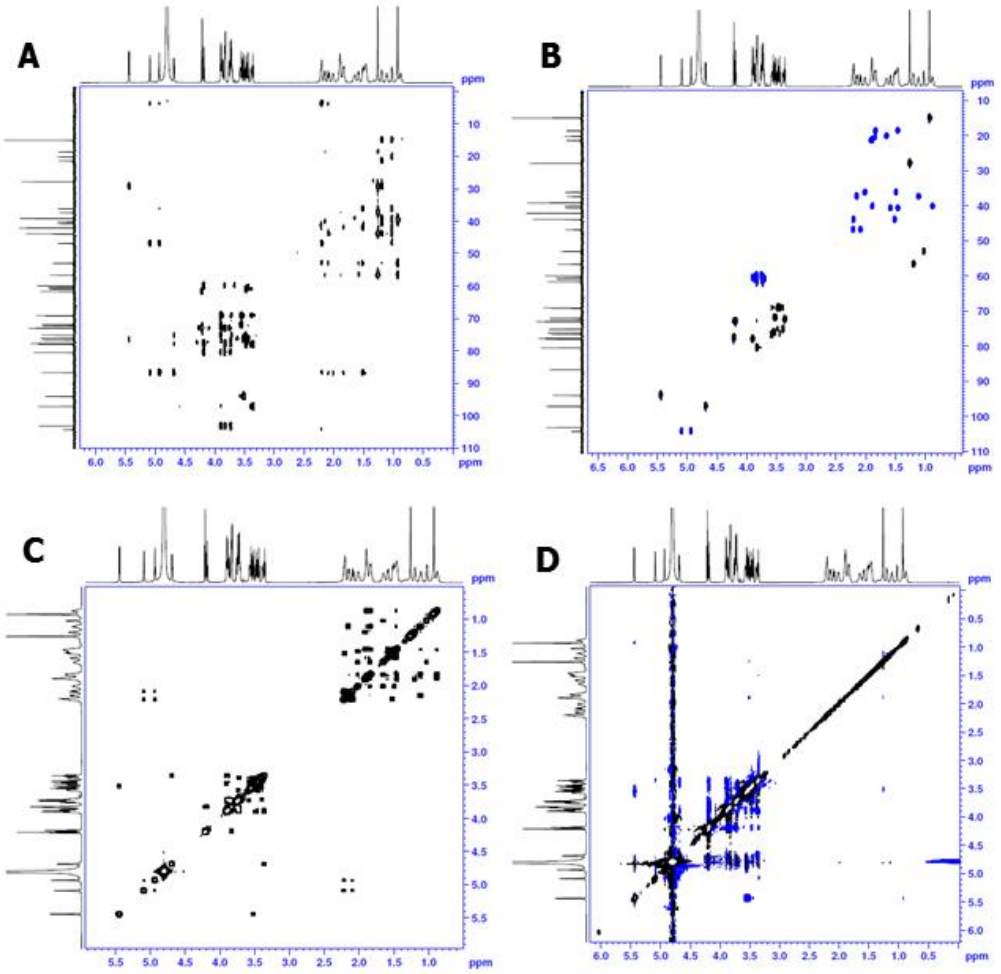


Figure 15. NMR spectra of Ru-Frc 1; A, HMBC; B, HSQC; C, COSY and D, ROESY

Table 7. ^{13}C and ^{1}H NMR data of rubusoside and fructosyl-rubusoside (ppm)

Carbon position	Rubusoside (δ)			Fructosyl rubusoside (δ_1)			Fructosyl rubusoside (δ_2)		
	δ_C	δ_H		δ_C	δ_H		δ_C	δ_H	
Steviol									
1	40.12	0.78	1.78	40.11	0.87	1.89	40.42	0.78	1.77
2	18.69	1.78	1.34	18.61	1.46	1.83	18.7	1.77	1.35
3	37.33	2.08	0.99	37.33	1.11	2.14	37.45	0.99	2.07
4	41.54			42.07			41.5		
5	56.42	1.05		56.59	1.19		56.36	1.05	
6	21.19	1.92	1.72	21.33	1.89		21.16	1.86	1.88
7	41.02	1.47	1.34	40.66	1.46	1.58	40.98	1.35	1.48
8	43.24			43.84			42.94		
9	53.06	0.91		52.98	1.02		53.14	0.92	
10	38.92			39.15			38.9		
11	19.93	1.49	1.68	20.15	1.84	1.65	19.94	1.5	1.69
12	36.86	1.85	1.37	36.15	1.48	2.01	37.36	1.39	2.07
13	85.01			86.69			85.25		
14	43.42	2.07	1.43	43.93	2.2	1.5	43.23	2.04	1.46
15	47.26	2.05	2.03	46.84	2.21	2.07	47.4	1.98	2.05
16	153.02			153.67			152.88		
17	104.18	4.75	5.1	104.29	5.09	4.93	104.27	5.1	4.76
18	28.13	1.14		27.82	1.26		28.13	1.13	

19	175.59			179.24			175.4		
20	15.23	0.86		14.97	0.92		15.18	0.85	
13-*O*-Glc									
1	97.96	4.29		97.14	4.68		98.05	4.28	
2	73.68	2.92		72.38	3.36		76.89	3.12	
3	76.48	3		76.15	3.52		73.58	2.92	
4	76.86	3.22		75.17	3.39		76.38	3	
5	77.66	3.18		69.07	3.48		70.02	3.05	
6	60.46	3.61	3.45	60.95	3.72		61.03	3.42	
19-*O*-Glc									
1	94.09	5.26		94	5.44		93.83	5.35	
2	72.5	3.15		71.91	3.51		77.11	3.27	
3	63.31	3.22		76.75	3.57		71.46	3.19	
4	70.38	3		69.17	3.44		68.38	3.34	
5	69.51	3.13		77.97	3.89		60.39	3.44	3.62
6	61.1	3.63	3.4	60.47	3.87		77.9	3.89	
Fructose									
1				59.91	3.82	3.75	59.94	3.66	3.53
2				103.19			104.12		
3				80.47	3.82		81.52	3.54	
4				72.98	4.19		73.33	3.98	
5				61.63	3.82		62.5	3.44	
6				77.71	4.21	4.19	76.7	4.07	

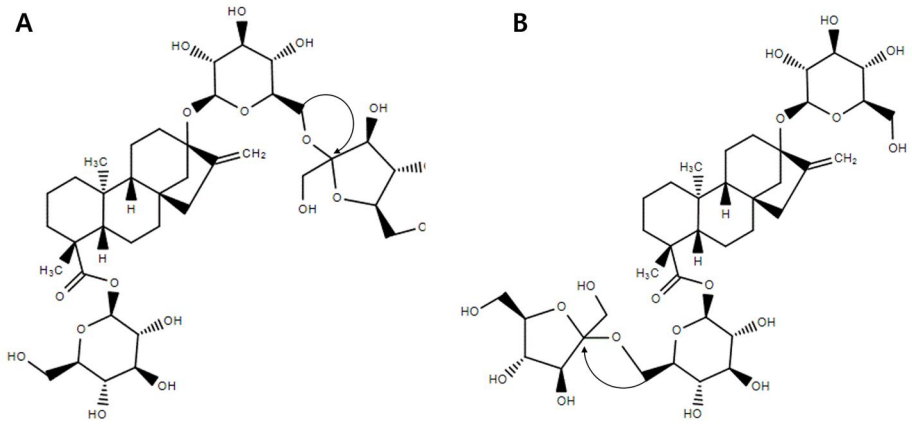


Figure 16. Structures and HMBC correlation of Ru-Frc 1 (A) and Ru-Frc 2 (B)

Conclusion

Rubusoside (Ru, 13-O-β-glucosyl-19-O-β-d-glucosyl-steviol) is the main component of the leaves of *Rubus suavissmimus S. Lee* (Rosaceae), known as tiancha in China. However, the tea plant grows only in Southern China with variable yearly yields depending on local climate. In order to overcome the quantitative limitation of Ru found in nature, Ru was mass-produced using β-galactosidase from *T. thermophilus* through lactose induction. Considering the activity yields, partial purification was carried out by heat-treatment at 70℃. Consequently, purified β-galactosidase showed 85.3% activity yield. Because the enzyme is heat-stable until 90℃, it is applicable for production procedure of industrial scale in enzyme engineering industry. Secondly, Ru showed competitive inhibition activity against mutansucrase with IC_{50} of 2.3 mM and K_i value of 1.1 ± 0.2 mM. Additionally, Ru inhibited *S. mutans* growth as a bacteriostatic agent as well as mutansucrase activity. With these features, Ru has a potential to be applied as an anti-cariogenic materials. With these features, Ru has a potential to be applied as an anti-cariogenic materials.

In this study, fructosyl-rubusoside (Ru-Frcs) was synthesized using levansucrase from *L. mesenteroides* to improve the taste of Ru. Optimal condition for synthesizing Ru-Frcs was 217.8 mM Ru, 723.2 mM sucrose and 22.8 U/mL levanuscrase with 33.5% conversion. The structure of Ru-Frc 1 and Ru- Frc 2 were elucidated to 13-O-[*β*

-fructofuranosyl-(2→6)-*β*-D-glucosyl]-19-O-*β*-D-glucosyl-steviol), 13-O-*β*-D-glucosyl-19-O-[*β*-fructofuranosyl-(2→6)-*β*-D-glucosyl]-steviol, respectively. Several early studies have shown that glycosylation of the carbohydrate moiety at the steviol C-13 site gave a remarkable improvement in quality of sweetness. In the context of these results, Ru-Frcs synthesized using levansucrase has a potential to be a natural sweetener. For the further study, therefore, sensory evaluation and functional tests should be conducted to confirm the potential as a natural sweetener of Ru-Frcs.

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Abstract in Korean

루부소사이드는 *Rubus suavissmimus S. Lee* (Roasaceae)에 존재하는 주성분 중의 하나로, 예로부터 당뇨, 천식 치료 및 예방에 이용이 되어왔다. 하지만 자연계에 존재하는 루부소사이드의 양은 극미량으로, 이로 인해 가격이 비싼 단점이 있다. 따라서 본 연구에서는 호열성 균주 유래 베타 갈락토시데이즈를 이용하여 루부소사이드의 대량 생산을 진행하였다. 대장균에서 발현된 베타 갈락토시데이즈는 70℃에 15분간 열처리를 함으로서 중온성 단백질이 73% 제거되었으며 활성 수율은 85%로 나타났다. 이렇게 생산된 효소는 고정화 방법을 이용하여 스테비오사이드를 기질로 루부소사이드를 생산하였고, 생성된 루부소사이드는 중압 액체크로마토그래피를 이용하여 정제하였다.

또한 본 연구에서는 루부소사이드의 항 우식성 특성 연구를 진행하였으며, 뮤탄수크레이즈를 억제하는 활성을 처음으로 밝혀내었다. 뮤탄수크레이즈는 스트렙토코커스 뮤탄스가 분비하는 효소 중의 하나로, 충치나 치석의 원인이 되는 효소이다. 루부소사이드 존재 유무에 따른 뮤탄 생성 비교 실험을 통해 루부소사이드 존재 하에 뮤탄 생성이 97.1 ± 0.2%가 억제됨을 확인하였다. 또한, 루부소사이드는 뮤탄수크레이즈에 대해 2.3 ± 0.0 mM의 IC_{50}와 1.1 ± 0.2 mM의 *Ki* 값을 가지며, 경쟁적으로 억제하는 것을 밝혀내었다. 추가적으로 루부소사이드의 스트렙토코커스 뮤탄스에 대해 최소 저지농도와 최소 살균 농도를 확인하였으며, 각각 7 mM과 10 mM로 나타난 것으로 보아 루부소사이드는 스트렙토코커스 뮤탄스 균에 대

해 정균 작용을 하는 것을 확인하였다.

이러한 다양한 기능성을 보유한 루부소사이드는 설탕보다 약 115배 단맛을 가지고 있지만 후미가 쓰다라는 특성 때문에 감미 소재로서의 한계를 지닌다. 본 연구에서는 루부소사이드의 감미도를 높이기 위해 수크로오즈를 기질로 하고 류코노스톡 메센테로이즈 균주에서 유래한 러반수크레이즈를 활용하여 배당체를 합성하였다. 표면반응법을 이용하여 루부소사이드 배당체가 최대로 생산 될 수 있는 반응 조건을 확립하였으며, 이는 루부소사이드 217.8 mM, 수크로오즈 723.2 mM, 효소 22.8 U/mL일 때 최고 33.5%의 전환율을 보였다. 합성된 배당체는 고압 액체크로마토그래피를 이용하여 정제되었으며 핵자기 공명 분광법을 통해 구조를 분석하였다. 그 결과, 첫 번째와 두 번째 배당체는 각각 13-O-[β-fructofuranosyl-(2→6)-β-D-glucosyl]-19-O-β-D-glucosyl-steviol, 13-O-β-D-glucosyl-19-O-[β-fructofuranosyl-(2→6)-β-D-glucosyl]-steviol로 정의되었다.

본 연구의 내용은 (1) 호열성 효소를 이용하여 루부소사이드의 대량 생산 공정을 이용했다는 점, (2) 루부소사이드의 항 우식성 특성을 밝혀냈다는 점, (3) 루부소사이드 배당체 합성을 통해 감미 소재로서의 가능성을 높였다는 점에서 식품 공학 산업에 기여할 수 있다.

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먼저 제가 대학원을 입학하면서 볼 것도 없이 여기로 와야겠다고 결정하게 된 가장 큰 이유였던 김도만 교수님께 감사드립니다. 교수님으로서, 때론 인생 선배로서 쓴 소리로 참교육을 해주셨기에 철부지였던 제가 철이 들어 이제 사회로의 걸음마를 시작할 수 있게 되었습니다.

그리고 처음 대학원 생활을 하는 저에게 실험의 기초를 알려주신 유신혜 선배, 실험실의 엄마, 아빠와 같았던 희정선배와 송희선배, 나의 사수이자 친구로서 본받을 점이 많았던 태경. 태경이와 실험을 직접하게 되면서 실험을 대하는 태도에 많이 배울 수 있었습니다.

동기는 아니었지만 동기처럼 나를 잘 챙겨줬던 시나, 채리, 재원이 그리고 창섭이. 이들과 있었기에 힘들었던 시간도 웃을 수 있었습니다. 앞으로도 채리의 호탕한 웃음소리와 개그가 그리울 것이며, 우리의 야식과 식사를 담당했던 시나식당이 그리울 것입니다. 그리고 항상 차분하게 상대방 얘기를 잘 들어주는 재원이와 창섭이가 있어서 든든했습니다.

나의 유일한 동기, 소형언니. 항상 상대방을 먼저 배려해주고 생각해주는 언니가 있어서 좋았습니다. 우리 실험실의 두 기둥 규민씨, 강희씨. 두 분이 있어 든든했고 실험적으로, 업무적으로 많이 힘이 되었습니다. 감사합니다.

타지에서 고생하는 이스와 주희. 나한테 잔소리 듣느라 스트레스 많이 받았을 텐데 노력하는 모습 보니 앞으로도 지금처럼 열심히 하면 좋은 결과 있을 거라 믿습니다.

그리고 우리 신입생들. 현지씨, 연지씨, 유진씨, 우혁씨, 선민씨 그리고 병수씨까지. 역대로 가장 많은 신입생들이 입학하면서 박사님의 부재와 겹쳐 혹여나 분위기가 흐려지진 않을까 걱정했던 것이 무색할 정도로 잘 적응해주셔서 고맙고 앞으로도 열심히 하셔서 원하는 뜻 이루시길 바랍니다.

또한, 실험실의 초석을 마련해주신 은배선배, 지연선배, 남현선배, 본철선배, 동구상에게도 감사의 말씀 전해드리고 싶습니다.

투덜이지만 업무적으로는 프로페셔널한 우리의 채쌤. 학생들의 노조 위원장이 되어 주셔서 감사했습니다. 그리고 우리의 정신적 지주, 한 박사님과 늦게 실험실에 합류하시게 된 목박사님까지, 항상 많은 학생들의 실험 알려주시랴 실험하시랴 바쁘신 와중에도 디스커션 할 시간을 내어주셔서 감사했습니다.

그리고 철없는 학부시절 저를 지도해주신 이인형 교수님, 이도엽 교

수님, 박용철 교수님, 성문희 교수님, 오상택 교수님께도 감사의 인사를 전합니다.

본 논문의 심사위원장을 맡아주시고 검토해주신 정동화 교수님께도 감사의 말씀을 전해드리고 싶습니다. 교수님의 콜로이드학과 국제 농산물 가공학 수업을 듣고 배움의 기쁨을 얻을 수 있어 좋았습니다. 또한 바쁜 와중에 본 논문의 심사위원을 맡아주신 김효진 교수님께도 감사의 말씀을 전합니다.

마지막으로 저를 믿고 묵묵히 제 결정에 따라 물심양면으로 지원을 아끼지 않으시는 저희 부모님 그리고 오빠. 저를 항상 믿고 지지해주는 가족이 있기에 밖에 나가서도 잘 해낼 수 있었습니다.

나중에 제가 졸업논문을 쓰고 감사의 글을 쓸 날이 온다면 진짜 감개무량할 것 같다고 태경이와 얘기 했던 적이 있는데, 힘들었던 만큼 이 논문에 제 정성과 땀이 깃들어 있기에 이 글을 쓰는 순간이 더욱 벅차게 느껴지는 것 같습니다.

대학원 생활을 하시는 후배님들께 마지막으로 당부 드리고 싶은 말은 아무리 힘이 들고 포기하고 싶더라도, 우리가 이루고자 했던 그 뜻을 이루기 위해 이 악물고 도전하고 견뎌낸다면 꼭 빛을 보는 날이 있을 거라고 전하고 싶습니다. 부디 우리가 하는 일에 연구자로써 자긍심을 가지고 임하면 더욱 보람 있는 실험실 생활이 될 것이라고 생각합니다.

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모든 것이 저에게 피가 되고 살이 되어 어디서 구하지 못할 값진 경험이 되었습니다. 이러한 경험을 가지고 더욱 단단한 제가 되어 사회에 나가서도 인정받을 수 있는 사람이 되겠습니다.

감사합니다.

2018년 7월
평창을 떠나면서,
김 지 수 올림