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RESEARCH PAPER

# Anti-cariogenic Characteristics of Rubusoside

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**Abstract** *Streptococcus mutans* plays an important role in the development of dental caries in humans by synthesizing adhesive insoluble glucans from sucrose by mutansucrase activity. To explore the anti-cariogenic characteristics of rubusoside (Ru), a natural sweetener component in *Rubus suavissimus S. Lee* (Rosaceae), we investigated the inhibitory effect of Ru against the activity of mutansucrase and the growth of *Streptococcus mutans*. Ru (50 mM) showed 97% inhibitory activity against 0.1 U/mL mutansucrase of *S. mutans* with 500 mM sucrose. It showed competitive inhibition with a $K_i$ value of 1.1 ± 0.2 mM and $IC_{50}$ of 2.3 mM. Its inhibition activity was due to hydrophobic and hydrogen bonding interactions based on molecular docking analysis. Ru inhibited the growth of *S. mutans* as a bacteriostatic agent, with MIC and MBC values of 6 mM and 8 mM, respectively. In addition, Ru showed synergistic anti-bacterial activity when it was combined with curcumin. Therefore, Ru is a natural anti-cariogenic agent with anti-mutansucrase activity and antimicrobial activity against *S. mutans*.

**Keywords:** Anti-cariogenicity, mutansucrase, natural sweetener, rubusoside, *Streptococcus mutans*

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## 1. Introduction

Dental caries affects 80% of the world's population. It is costly to health care. Dental caries is initiated by interaction between acidogenic bacteria and sucrose in susceptible hosts. *Streptococcus mutans* is an acidogenic and acid-tolerant bacterial species. It has been recognized as the primary pathogen of dental caries because of its ability to produce glucansucrase synthesizing water insoluble glucan called mutan [1]. Mutan can mediate the adherence of *S. mutans* and other oral bacterial species to tooth surfaces, contributing to the formation of dental plaque biofilms [2,3]. Glucansucrases are a member of glycoside hydrolase (GH) family 70 that catalyze the formation of glucan with various types of glucosidic linkages such as α(1-3), α(1-4), or α(1-6) bonds, from sucrose via transglucosylation reactions. Glucansucrases are classified at mutansucrase, dextransucrase, alternansucrase, and reuternansucrase, which catalyzed α(1-3), α(1-6), α(1-3 and 1-6), and α(1-4 and 1-6) bond formation, respectively [4]. *S. mutans* produces three extracellular glycosyltransferases (GTFs), GTF-I, GTF-SI and GTF-S, which are encoded by *gtf*B, *gtf*C, and *gtf*D, respectively. GTF-I and GTF-SI are mutansucrases that synthesize mainly insoluble glucan with α(1-3) glucosidic linkages [5,6]. In addition, other crucial virulence factors of *S. mutans* include a membrane-bound F1Fo-ATPase system, agmatine deiminase system, enolase, and lactate dehydrogenase [7]. Diet, especially high sucrose intake, elevates the risk for dental caries. The cariogenic nature of sucrose has led food and beverage industries to develop alternative sweeteners without having carcinogenicity.

Rubusoside (13-O-β-glucosyl-19-O-β-D-glucosyl-steviol; Ru) is one of the steviol glycosides present in the leaves of *Rubus suavissimus S. Lee* (Rosaceae). It is a well-known natural nontoxic sweetening agent. It has been used in various food and beverage products [8]. In addition, Ru has been used to increase the solubility of several pharmaceutically and medically important compounds with poor solubility in water [9]. It can decrease the growth and glass-surface adherence of *S. mutans* [10] when Ru and sucrose are incubated together and then the growth, adherence, and acid production of *S. mutans* are measured. The mechanism involved in the inhibition of Ru against mutansucrase or its reduction of the growth of *S. mutans* is currently unknown. Therefore, the objective of this study was to investigate the mechanism of action of Ru against mutansucrase and *S. mutans* growth. Kinetic characterization of Ru against mutansucrase activity and molecular docking analysis were carried out. The growth inhibition type of Ru against *S. mutans* was also studied.

## 2. Materials and Methods

### 2.1. Preparation of rubusoside

Ru was prepared using stevioside as a substrate with immobilized β-galactosidase expressed in *Escherichia coli* BL21 (DE3) pLysS and was purified as described in our previous study [9].

### 2.2. Preparation of mutansucrase from *S. mutans*

*S. mutans* KCTC 3065 was obtained from Korean Collection for Type Cultures (KCTC, Korea). Mutansucrase was prepared by culturing *S. mutans* in brain heart infusion media (BHI; BD Difco, USA) supplemented with 2% (w/v) glucose at 37ºC with shaking (at 150 rpm) until all glucose was consumed. After fermentation, cells were separated from the broth by centrifugation at 8,000 × g for 15 min. The supernatant was concentrated using a Centriprep Centrifugal Filter Unit with Ultracel-YM10 (Merck, Germany) and 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 a salt gradient of 0-1,000 mM NaCl in 20 mM sodium phosphate buffer (pH 6.8). Fractions containing mutansucrase were pooled and dialyzed with 20 mM sodium phosphate buffer (pH 6.8) at 4°C. These dialyzed fractions were concentrated using a Centriprep Centrifugal Filter Unit with Ultracel-YM10 and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was measured by the Bradford method using crystalline bovine serum as standard [9]. The partially purified enzyme was kept at –20°C for further study. One unit (U) of mutansucrase activity was defined as the amount of enzyme required to release 1 μM fructose from sucrose per min at 37°C and pH 6.8. Fructose concentration in the reaction mixture was measured with a D-fructose kit (Megazyme, Ireland). The kinetic parameter of mutansucrase activity (0.1 U/mL) was measured using 0-200 mM sucrose as the substrate for 35 min. Reaction responses were linear within this time frame. The Michalis-Menten constant ($K_m$) was calculated from Lineweaver-Burk plot [11].

### 2.3. Inhibition activity of rubusoside against mutansucrase

Ru was dissolved in distilled water to prepare a 250 mM stock solution. The reaction mixture was composed of 0.1 U/mL mutansucrase, 500 mM sucrose, and 50 mM Ru in 20 mM sodium phosphate buffer (*p*H 6.8). After incubating at 37°C for 12 h, water-insoluble glucan was harvested by centrifugation at 12,000 × g for 15 min. The pellet was dissolved in 1 M NaOH. The amount of water-insoluble glucan was measured via thin-layer chromatography (TLC) [12]. One μl of sample was spotted onto a precoated TLC plate (Merck, Darmstadt, Germany) and developed with two ascents of acetonitrile:water (85:15, v/v). Carbohydrates were visualized by dipping the TLC plate into 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) followed by heating at 120°C for 5 min. The amount of carbohydrates was analyzed by conversion to the integrated density value using the AlphaEaseFC 4.0 Image Program (Alpha Inotech, CA, USA) [12].

The 50% inhibitory concentration ($IC_{50}$) was defined as the concentration of Ru necessary to reduce mutansucrase activity by 50% relative to a reaction mixture containing mutansucrase without inhibitor. $IC_{50}$ and $K_i$ value of Ru against mutansucrase were calculated based on the amount of fructose released into the reaction mixture using a fructose kit. Inhibition activity was calculated using the following formula:

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

where $C$ was the absorbance of control (enzyme, buffer, and substrate) after 35 min of reaction, $C_0$ was the absorbance of control at the zero-time point, $S$ was the absorbance of the sample (enzyme, Ru, buffer, or substrate) after 35 min of reaction, and $S_0$ was the absorbance of sample at the zero-time point. Inhibitor kinetic studies of Ru were done like those used in a kinetic study for mutansucrase, except that multiple concentrations (0-

10 mM) of Ru and various concentrations (35-175 mM sucrose) of substrate were used. The type of inhibition was identified using a Lineweaver-Burk and Dixon plot (1/v) as a function of inhibition concentration [I]. The kinetic parameter ($K_i$) was calculated using the SigmaPlot program.

**2.4. Antibacterial effect assay by disc diffusion method**

*S. mutans* was cultured in brain-heart infusion (BHI) at 37°C until $OD_{600}$ reached approximately 1.0 [13,14]. The antibacterial effect of Ru solution (250 mM) against *S. mutans* was measured using epigallocatechin gallate (EGCG) (250 mM) as a control [15]. Both the Ru solution and EGCG (250 mM) were filtered with a Minisart® syringe filter (0.2 μm, Satorius, Germany). A disc-diffusion antimicrobial test was carried out by spreading 100 μL of suspension containing $10^8$ CFU/mL onto a BHI agar plate [15]. Then a 6-mm diameter disc containing 20 μL of 250 mM Ru, 250 mM EGCG, or dimethyl sulfoxide (DMSO) was processed in triplicate. These discs were impregnated into the inoculated agar plate. These plates were then incubated at 37°C for 12 h.

**2.5. Molecular docking of Ru against mutansucrase**

The three-dimensional structure of mutansucrase was retrieved from the Protein Data Bank (http://www.rcsb.org, accession code 3AIC). Alpha acrabose (ACR) is located in the active site of 3AIC [6]. All water molecules and co-crystal ligand ACR were removed. Structure information containing only amino-acid residues of mutansucrase enzyme was used for molecular docking. All docking experiments were carried out according to the previous published method [16].

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

Curcumin was purchased from Sigma. Water-soluble curcumin (Ru-CUR) was prepared as described previously [9]. A broth microdilution method was used to find the minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) [17]. To measure MIC, Ru or Ru-CUR was placed into each well of a 96-well-plate. Each well was then inoculated with 5 μL of *S. mutans* culture ($1.5 \times 10^7$ CFU/mL) and incubated at 37°C for 24 h. Thereafter, turbidity of each well was measured with a SpectraMax M3 (Molecular devices, USA) at a wavelength of 600 nm. The lowest concentration that inhibited bacterial growth was considered to be the MIC. MBC was measured by subculturing the medium from MIC on a BHI agar plate. These plates were incubated at 37°C for 24 h. MBC was defined as the consequent concentration that killed 99.9% of cells. All experiments were carried out in triplicate.

## 3. Results and Discussion

**3.1. Characterization of mutansucrase from *S. mutans***

Mutansucrase from *Streptococcus mutans* was purified using DEAE-Sepharose column (Fig. S1). The specific activity of mutansucrase after purification was 14.4 U/mg protein when using sucrose as a substrate. The Michaelis-Menten constant ($K_m$) of mutansucrase using sucrose as a substrate was 34.5 ± 4.6 mM (Fig. S2) based on the amount of fructose released in the reaction mixture. The $K_m$ value of mutansucrase in this study was higher than that of mutansucrase from *S. mutans* Ingbritt c ($K_m$ = 9.4 mM) [18] and that of *S. mutans* 6715 ($K_m$ = 2.4 mM)[19]. The $K_m$ values of *S. mutans* Ingbritt c and 6715 were determined by the phenol sulfuric acid assay, which measured measuring the total glucan formation in the reaction mixture containing sucrose and dextran T10 [19].

**3.2. Inhibition activity of rubusoside against mutansucrase**

Ru was tested for its ability to inhibit mutansucrase. At 50 mM, Ru showed 97% inhibition against mutansucrase (Fig. 1A) based on insoluble glucan formation. The $IC_{50}$ value of Ru against mutansucrase was 2.3 mM. Based on both Lineweaver-Burk and Dixon plots, Ru exhibited competitive inhibition toward mutansucrase, because the Lineweaver-Burk plot of 1/v versus 1/[S] resulted in a family of straight lines with the same *y*-axis intercept (Fig. 1B). The $K_i$ value of Ru was found to be 1.1 ± 0.2 mM (Fig. 1C). To our best knowledge, this is the first report about the inhibitory effect of Ru against mutansucrase.

**3.3. Molecular docking of Ru against mutansucrase**

To have a better comprehension of the molecular recognition process between mutansucrase and Ru, docking experiments were done using the crystal structure of mutansucrase (3AIC) [6] and Autodock 4.2 for docking simulations. The free binding energy of Ru was -8.27 kcal/mol (Fig. 2A). Ru inhibited mutansucrase from *S. mutans* potentially through hydrophobic and hydrogen bond (H-bond) interactions between amino-acid residues in the active site pocket of mutansucrase and Ru (Fig. 2B). Carbon atoms of Ru interacted hydrophobically with Leu 433, Leu434, Ala478, Asp480, Glu515, Trp517, and Tyr916. Ru formed three H-bonds with residues in the catalytic binding pocket of mutansucrase. The $O^7$ atom of the 2-hydroxyl group of the glucosyl group at C19 of Ru accepted an H-bond with the amino group of Lys977 at a distance of 2.61 Å. The O atom of the side chain carboxyl group of Asp909 formed

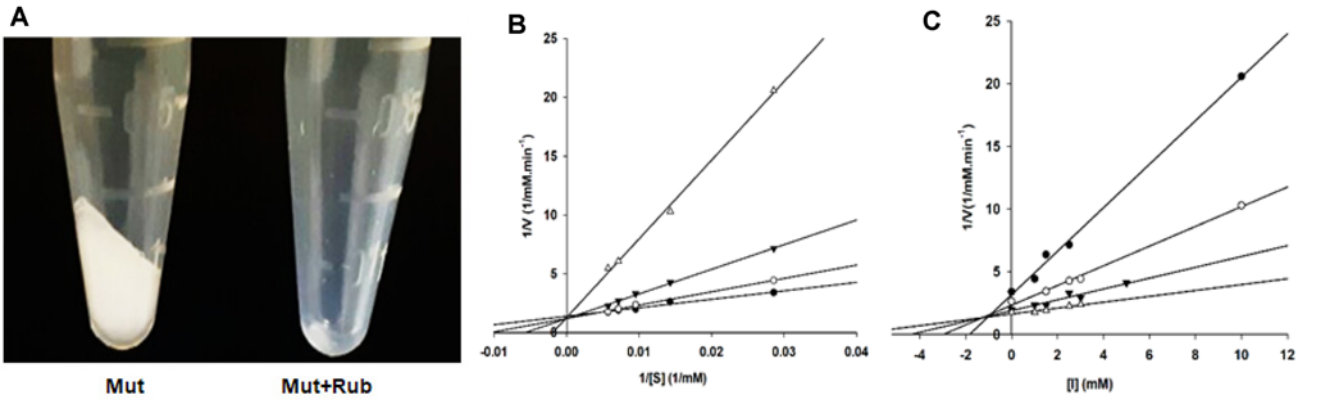


**Fig. 1.** Effect of Ru on reducing insoluble glucan formation using mutansucrase from *S. mutans* (A) and the plots of Lineweaver-Burk (B) and Dixon (C) analyzing Ru inhibition of mutansucrase activity. Kinetic constants $K_m$ and $K_i$ were calculated using linear regression analysis. In (A), Mut, mutansucrase reaction without Ru and Mut+Ru, mutansucrase reaction with 50 mM Ru. The while color material indicates the formation of insoluble glucan. The mutansucrase reaction mixture comprised of 0.1 U/mL mutansucrase, 500 mM sucrose, and 50 mM Ru in 20 mM sodium phosphate buffer (pH 6.8) and reacted at 37°C for 12 h. The amount of water-insoluble glucan was determined using thin-layer chromatography (TLC). The amount of carbohydrates was analyzed by the AlphaEaseFC 4.0 Image Program which measures the integrated density value. In (B), the Ru concentration was 0 mM (●), 1 mM (○), 2.5 mM (►), and 10 mM (△). In (C), the sucrose substrate concentration was 35 mM (●), 70 mM (○), 10 mM (▲), and 175 mM.

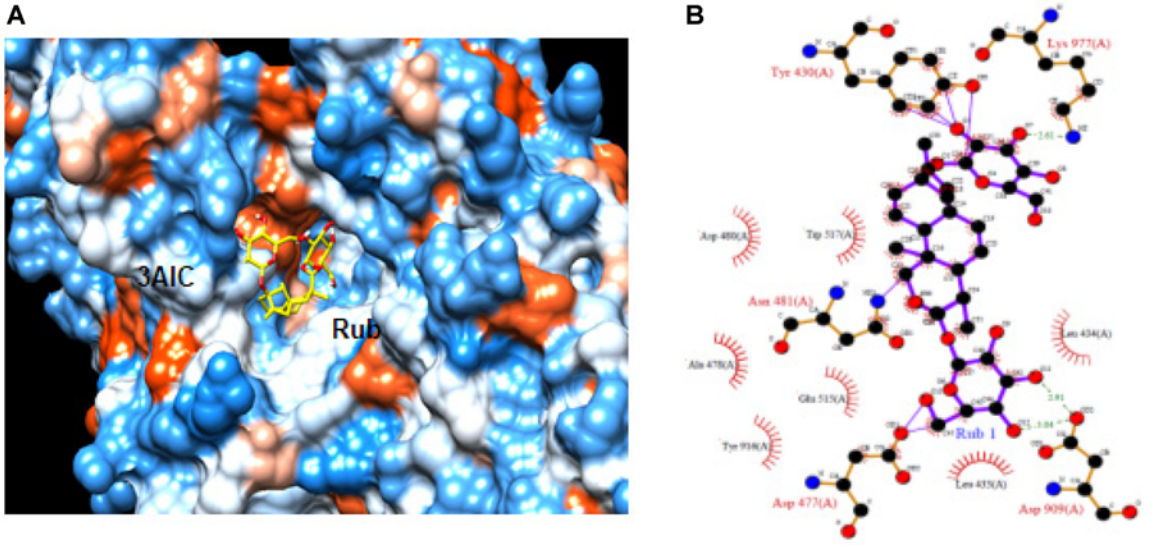


**Fig. 2.** Computation docking and hydrophobic and hydrogen bond interactions of Ru with amino-acid residues in the active site of mutansucrase. (A) Comparison of the binding mode of Ru (yellow) in the active site of mutansucrase. (B) Hydrophobic and H-bond interactions between Ru and amino-acid residues in the active site of mutansucrase. H-bond interactions are represented by green dashed lines (Red, oxygen; cornflower blue, nitrogen; black, carbon).

two H-bonds with O11 and O12 of the glucosyl group at C13 of Ru at a distance of 2.91 Å and 3.04 Å, respectively.

### 3.4. Antimicrobial susceptibility test of rubusoside and rubusoside-curcumin complex against *S. mutans* growth

The antimicrobial activities of Ru against *S. mutans* have been reported previously by Chu et al. (2016) after incubating Ru with *S. mutans* in the presence of sucrose. Our study confirmed the antibacterial activity of Ru by MIC and MBC studies. The antimicrobial activities of Ru against *S. mutans* growth were assessed by an inhibition zone assay and zone diameter measurement (Fig. S3). Ru inhibited the growth of *S. mutans*. The inhibition zone at 5 mM/disc was 11.3 ± 1 mm. EGCG was used as a positive control. Its inhibition zone at 5mM/ disc was 19 ± 1 mm. MIC and MBC values of Ru against *S. mutans* were 6 mM and 8 mM, respectively (Fig. 3A). MBC was higher than MIC, indicating that Ru could inhibit *S. mutans* as a bacteriostatic agent [20]. Ru is known to be less acidogenic than is glucose, maltose, fructose, or sucrose, which can be

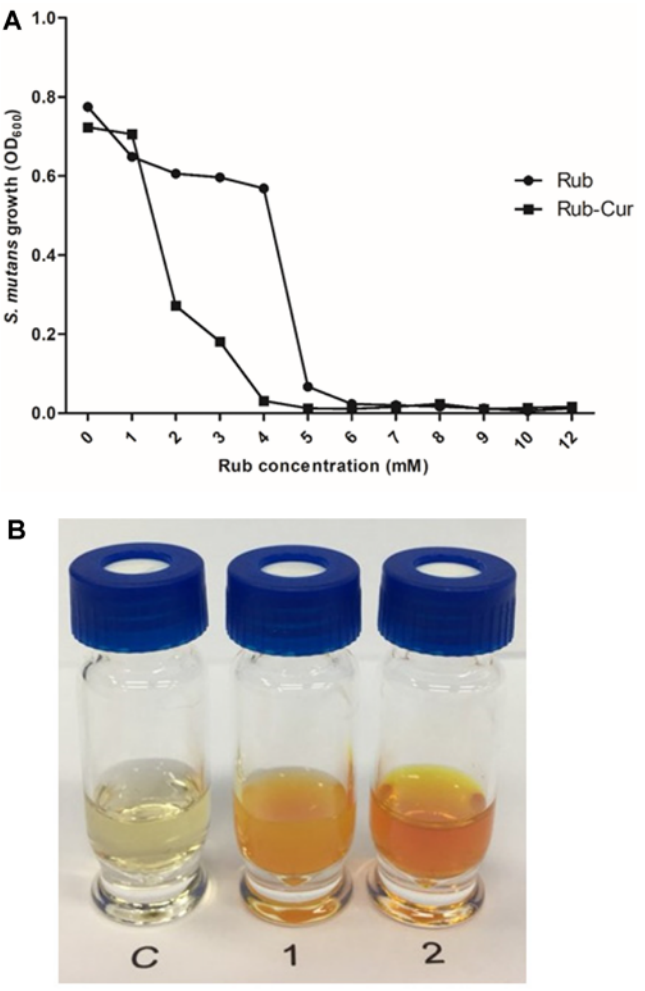


**Fig. 3.** Growth curve of *S. mutant* in BHI medium containing Ru/ or Ru-CUR (A) and solubilization of curcumin in DMSO and CUR-Ru complex in BHI medium (B). C, BHI medium; 1, CUR-DMSO in BHI medium; 2, CUR-Ru in BHI medium.

metabolized only very slowly or not at all by S. *mutans* [10].

Ru is well-known as a solubilizer for insoluble compounds [9,21]. Curcumin is a compound that has low solubility in water (Zhang et al. 2017). The MIC value of curcumin has been reported against *S. mutans* [22,23]. When trying to measure the MIC of curcumin against *S. mutans*, we faced difficulty in interpreting our results, possibly because of the insoluble and colored precipitant caused by its insolubility in aqueous phase and formation of a colored precipitate (Fig. 3B). Thus, curcumin was prepared to be soluble in water with a Ru solution. The MIC value of curcumin could not be found, because of its precipitation in solution. CUR-Ru complex with 0.9 mM curcumin and 5 mM Ru was used for both MIC and MBC measurement (Fig. 3A). By combining curcumin with Ru, the MIC value of Ru against *S. mutans* decreased from 6 mM by itself to 5 mM in the complex, and the MBC value of Ru decreased from 8 mM by itself to 5 mM in the complex. Such lower MIC and MBC values of the Ru-CUR complex compared to those of Ru by itself were due to the synergism between Ru and curcumin. This result suggests that Ru can inhibit *S. mutans* as a bacteriostatic agent, whereas the Ru-CUR complex can inhibit *S. mutans*.

## 4. Conclusion

Rubusoside (Ru, 13-O-β-glucosyl-19-O-β-d-glucosyl-steviol), a natural sweetener obtained from leaves of *R. suavissimus S. Lee* (Rosaceae), showed competitive inhibition against mutansucrase, with $IC_{50}$ of 2.3 mM and a $K_i$ value of 1.1 ± 0.2 mM. Such inhibition was through hydrophobic and hydrogen bonding interactions between amino-acid residues in the active site pocket of mutansucrase and Ru. Ru also inhibited *S. mutans* growth as a bacteriostatic agent. In addition, Ru-CUR showed synergy against *S. mutans*. With these features, Ru could well be applied as an anti-cariogenic material and a natural sweetener with low calories.

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