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Hydroquinone, a control agent of agglutination and adherence of *Streptococcus mutans* induced by sucrose

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Abstract—Hydroquinone was found to alter agglutination of *Streptococcus mutans* induced by sucrose. The newly formed agglutination product produced by hydroquinone does not kill this cariogenic bacterium and the formation is reversible. The agglutination altering activity of hydroquinone seems to be specific for strains of *S. mutans*. As a result, hydroquinone inhibits sucrose-induced adherence of *S. mutans*.

1. Introduction

Streptococcus mutans has been implicated as one of the major cariogenic organisms present in dental plaque.[1,2] This cariogenic bacterium synthesizes extracellular gummy glucans from sucrose by glucosyltransferases and glucanases, and it has been postulated that such polymers are responsible for the ability of this streptococcic bacterium to form gelatinous plaques on the tooth surface. The gummy glucose polymers produced entrap other bacteria and materials until yellow tooth coating accelerates.[3] Hence, prevention of dental cavities can be accomplished by controlling *S. mutans* and/or its glucan formation. Numbers of antibacterial agents against *S. mutans* have been reported[4–7] but few as glucan control agents.

During our search for antimicrobial agents from plants, hydroquinone (**1**, Fig. 1) was unexpectedly found to increase sucrose-induce agglutination of *S. mutans* cells, although it did not exhibit any antibacterial activity against this cariogenic bacterium up to 800 μg/mL. Many high molecular weight materials that induced agglutination of *S. mutans* were reported.[3,8,9] However, low molecular weight agents that induce agglutination specific for *S. mutans* have not yet been reported. This prompted us to investigate the role of hydroquinone for sucrose-induced agglutination of *S. mutans* cells at a molecular basis. To facilitate it, hydroquinone interactions with sucrose-induced agglutination and adherence of *S. mutans* were studied. In addition, several hydroquinone related compounds were also tested for comparison. Accumulation of this kind of knowledge may provide a more rational and scientific approach to design safe and effective anticavity agents. This paper describes the effects of hydroquinone on agglutination of *S. mutans* in the presence of sucrose.

2. Results

In our continuing search for biologically active compounds from plants, hydroquinone *O*-β-D-glucopyranoside, otherwise known as arbutin (**2**), was previously characterized as the major bitter principle from the endosperm of the California buckeye *Aesculus californica* (Hippocaseanaceae).[10] Its congeners, hydroquinone (**1**) and benzoquinone (**3**), were also characterized from the same source, but subsequently found to be artifacts of arbutin during extraction procedure. Interestingly, hydroquinone and arbutin did not exhibit any antimicrobial activity against the sixteen selected microorganisms tested up to 800 μg/mL[5] while benzoquinone (**3**) showed a broad antimicrobial spectrum (data not listed).[11] During this antimicrobial assay, we became aware that hydroquinone increases the sucrose-induced agglutination of *S. mutans* cells. This preliminary observation was confirmed and the results are listed in Table 1. Among the microorganism tested, *S. mutans* is

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the only organism of which sucrose-induced agglutination was increased by hydroquinone. In addition, benzoquinone also enhanced the sucrose-induced agglutination of *S. mutans* but much weaker than that of hydroquinone.

The agglutination index (AI) was used for quantitation of agglutination activity.[12] If AI number is greater than 1 (>1) indicates that agglutination is enhanced. On the other hand, it should be noted that agglutination products with hydroquinone differs from without it. The gummy glucose polymer induced by sucrose without hydroquinone appears visible in the whole inner surface of test tube. The glucose polymer produced with hydroquinone appears as no longer a gummy polymer, but as a newly produced agglutination product induced by hydroquinone and appears to be a sticky gelatin like polymer but has a pebble-like form in the bottom of the test tube. Noticeably, in contrast to high molecular weight agglutinins, the pebble like agglutination precipitate was not formed if hydroquinone was added after growing *S. mutans* for 24 h even at a concentration of 800 μg/mL. Therefore, it appears that the above mentioned agglutination of *S. mutans* cells occurs only during cell growth in the presence of hydroquinone and the agglutinated products produced differs from that induced by high molecular weight agglutinins. On the other hand, when *S. mutans* cells agglutinated by hydroquinone were inoculated into the fresh brain heart infusion (BHI) medium that did not contain hydroquinone, this cariogenic bacterium growth became normal. This result indicated that *S. mutans* was not killed by agglutination induced by hydroquinone.

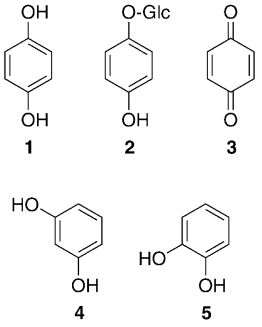

Figure 1. The structures of hydroquinone and related compounds.

Table 1. Agglutination activity of hydroquinone (**1**), arbutin (**2**) and benzoquinone (**3**)

Microorganisms tested	Agglutination Index (AI) of compounds tested[a]			
	None	**1**	**2**	**3**
Bacillus subtilis	0.86	0.82	0.90	0.94
Brevibacterium ammoniagenes	1.7	1.8	1.8	1.8
Staphylococcus aureus	1.1	1.1	1.1	1.1
Streptococcus mutans	1.0	2.4	1.1	1.5
Escherichia coli	0.94	0.95	0.94	0.97
Pseudomonas aeruginosa	1.1	1.0	1.0	1.0
Enterobacter aerogenes	0.90	0.92	0.89	1.0
Saccharomyces cerevisiae	1.0	1.0	1.0	ng[b]
Candida utilis	1.0	1.0	1.0	ng

[a] The concentration of the compounds tested was 100 μg/mL.
[b] No growth.

The influence of hydroquinone to agglutination of *S. mutans* was studied in the presence of several common saccharides such as glucose, galactose and fructose of monosaccharides, or sucrose, maltose and lactose of disaccharides. Since BHI medium contains 0.2% glucose, trypticase tryptase yeast (TTY) broth was used for this study. As shown in Table 2, the agglutination of *S. mutans* was induced only by sucrose (AI: 4.2) but not the other mono- and di-saccharides tested. At a level of 1% sugar, hydroquinone was less effective in the presence of other saccharides than sucrose. It appears that hydroquinone increased agglutination activity induced by sucrose (AI: 4.2→17). It should be noted that hydroquinone did not increase the sucrose-induced agglutination of *S. mutans* in the presence of galactose. In other words, the agglutination enhancing activity of hydroquinone was inhibited by galactose. Among the saccharides tested, galactose is the only sugar to show this inhibitory activity. All the other saccharides tested showed no effect on the agglutination.

Since hydroquinone enhanced the sucrose-induced agglutination of *S. mutans*, the influence of its concentration was examined. As shown in Table 3, higher concentration of hydroquinone increased the agglutination. For example, in the presence of 1% sucrose, 10 and 100 μg/mL of hydroquinone increased agglutinating activity of sucrose 2.9- and 4.1-fold, respectively (AI: 4.2→12 and 4.2→17, respectively), although 1 μg/mL of hydroquinone did not increased activity (AI: 4.2→4.3). Hydroquinone enhancing activity was found to be dose responsive with sucrose concentration. For example, in the presence of 0.01, 0.1 and 1% sucrose, 100 μg/mL of hydroquinone increased agglutinating activity 2.0-, 2.8- and 4.1-fold (AI: 1.2→2.4, 1.8→5.0 and 4.2→17), respectively. From the results observed, it

Table 2. Agglutination activity of saccharides against *S. mutans* ATCC 25175 with and without hydroquinone

Saccharides[a] tested	Hydroquinone (μg/mL)	
	100	0
Glucose	2.3	1.1
Galactose	1.2	1.1
Fructose	1.8	1.1
Sucrose	17	4.2
Maltose	1.6	1.1
Lactose	1.7	1.1

[a] The concentration of saccharides was 1%.

Table 3. Agglutination activity of hydroquinone against *S. mutans* ATCC 25175 in the presence of sucrose

Hydroquinone (μg/mL)	Sucrose (%)		
	1	0.1	0
100	17	5.0	2.4
10	12	3.0	1.8
1	4.3	2.0	1.4
0	4.2	1.8	1.2

confirmed that hydroquinone increased agglutinating activity of sucrose. The agglutination enhancing activity of hydroquinone depended on the concentration of both sucrose and hydroquinone.

It was previously reported that agglutination of *S. mutans* is due to cells that are bound together by dextran molecules.[13,14] The binding site of dextran was determined by using radioactive dextran/glucan.[15] Similar to these glucose polymers, hydroxyl groups of hydroquinone likely form hydrogen bonds with dextran/glucan during the cell growth. Agglutination of *S. mutans* cells may be altered by hydroquinone bridge as shown in Figure 2.

The influence of the position of hydroxyl groups on the aromatic ring, which seemed to play an important role to the agglutination of *S. mutans*, was examined. BHI medium was used for this experiment. AI values of hydroquinone, resorcinol (**4**) and catechol (**5**) against *S. mutans* at the concentration of 100 μg/mL are shown in Table 4. As aforementioned, hydroquinone altered the sucrose-induced agglutination of *S. mutans* (AI: 2.4) by forming the hydroquinone bridge, while neither resorcinol nor catechol exhibited any activity, presumably because of their steric hindrance.[16] The result clearly indicates that the *para*-dihydroxy substitution is essential in making bridges between *S. mutans* cells.

The mechanism of cell-to-teeth surface adherence of *S. mutans* in vitro has been previously reported.[3] Adherence requires glucan synthesis mediated by cell associated glucosyltransferases and glucanases. Therefore, the influence of hydroquinone on adherence of *S. mutans* to the test tube in the presence of several saccharides was investigated and the result is shown in Table 5. Hydroquinone did not induce adherence in the presence of any saccharides, although it induced agglutination except in the presence of galactose. Interestingly, *S. mutans* failed to adhere to the surface of the test tube in the presence of hydroquinone. In other words, hydroquinone inhibited adherence of *S. mutans* induced by sucrose. The inhibition mechanism of adherence by hydroquinone may not be inhibition of glucosyltransferase activity, since hydroquinone increases the agglutination of *S. mutans* in the presence of sucrose. This inhibition may be due to strong agglutination fostered by the hydroquinone bridge. It can be concluded, hydroquinone alters cell-to-cell agglutination and inhibits cell-to-teeth surface adherence of *S. mutans* induced by sucrose, although it did not exhibit any antibacterial activity against this bacterium up to 800 μg/mL.

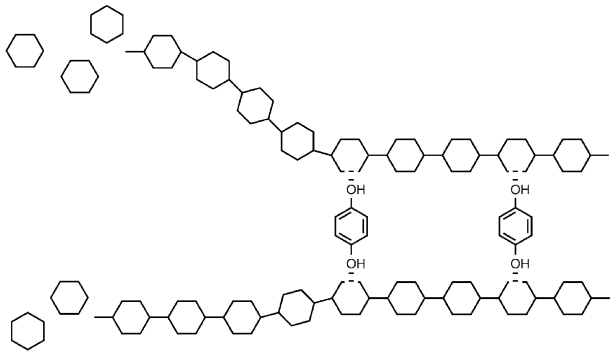

Figure 2. Possible hydroquinone bridged structure with dextran/glucan in part.

Table 4. Agglutination activity of hydroquinone, resorcinol and catechol against *S. mutans* ATCC 25175

Compounds[a] tested	Agglutination Index (AI)
None	1.0
Hydroquinone	2.4
Resorcinol	1.1
Catechol	1.2

[a] The concentration of compound was 100 μg/mL.

3. Discussion

S. mutans produces glucans, which are sticky glucose polymers, formed by glucosyltransferases and glucanases. Glucans entrap other bacteria and materials known as agglutination. High molecular weight compounds such as dextran,[17] lectin[18] and glycoprotein[8,9] are known to induce agglutination of *S. mutans* cells. Agglutination is defined as clumping of cells caused by the reaction between antigens on their surfaces and antibodies in their external environment. For instance, blood agglutination test (identification of blood) is a most well known example. In chemical terms, antibody usually has a very high binding affinity for specific antigen. Agglutination and adherence of *S. mutans* are known to be induced in the presence of sucrose.[19] Agglutination enhanced by hydroquinone appears to be a characteristic of *S. mutans*, since we did not observed it among the other bacterial species tested. In addition, hydroquinone seems to have a specific binding affinity for the sucrose-induced glucans of *S. mutans* and form a larger visible complex, similar to large molecule weight agglutinins. Hydroquinone forms this visible complex only when the cells grow in the presence of sucrose and, more importantly, this complex can be reversibly separated presumably to glucans and hydroquinone by ultrasonication. The properties observed with hydroquinone

Table 5. Agglutination and adherence of hydroquinone against *S. mutans* ATCC 25175

Saccharides[a] tested	Hydroquinone (μg/mL)			
	Agglutination		Adherence	
	100	0	100	0
Glucose	+[b]	–	–	–
Galactose	–[c]	–	–	–
Fructose	+	–	–	–
Sucrose	+	–	–	+
Maltose	+	–	–	–
Lactose	+	–	–	–

[a] The concentration of the compounds tested was 100 μg/mL.
[b] + Induced.
[c] – Not induced.

are similar to those of high molecular weight compounds in many aspects but differ from the defined antigen and antibody concept to some extends. The agglutination enhancing activity of hydroquinone is very likely resulted in a chemical reaction as described above and hence hydroquinone is unlikely termed as an agglutinin. It can be concluded that hydroquinone does not induce but alters the sucrose-induced agglutination of *S. mutans*. As a result, the sticky gelatin like polymers deposited on the inner surfaces of test tube is converted to a pebble-like precipitate in the bottom.

In the case of agglutination induced by high molecular weight agglutinins, they attach to the binding site on the surface of *S. mutans*.[8] On the other hand, the ability of *S. mutans* to agglutinate in the presence of hydroquinone appears to be due to formation of intermolecular hydrogen bonds with high molecular weight molecules but not present of specific receptor site of hydroquinone on the surface of *S. mutans*. More specifically, hydroxyl groups of hydroquinone form hydrogen bonds with extracellular dextran/glucan synthesized only during the cell growth and play a role in the bridge between cells. It should be emphasized that hydroquinone does not directly affect the sucrose-induced agglutination of *S. mutans* as an agglutinin. On the basis of the data obtained, it can be concluded that the sucrose-induced agglutination of *S. mutans* is altered by forming hydroquinone bridge.

The binding site of dextran was determined by using radioactive dextran/glucan.[15] It was reported that agglutination of *S. mutans* is due to cells that are bound together by dextran molecules.[14,17] Similarly, hydroxyl groups of hydroquinone likely form hydrogen bonds with dextran/glucan during the cell growth. If this is so, the question how galactose inhibits this agglutination enhancing activity of hydroquinone needs to be answered. Since galactose does not inhibit the sucrose-induced agglutination of *S. mutans* and is not incorporated into glucans, it should disrupt forming hydroquinone bridge during glucan synthesis. It seems that glucosyltransferases first hydrolyze sucrose and the freed glucose is simultaneously transferred to glucanases, and then glucans are produced. Further evidence of this postulate was obtained in experiments that glucose acts neither as a substrate nor an inhibitor of glucan formation. In the newly synthesized glucans, the principal chain is likely bonded with 1,4-glycosidic linkages and branches off the chain from 1,6-glycosidic linkages, similar to other glucans. Hence, galactose may preferentially form hydrogen bonding with hydroquinone and result in prevention of the agglutination enhancing activity of hydroquinone by terminating glycosidic linkages. Thus, galactose appears to interfere with the binding site for hydroquinone but not with the enzymatic synthesis of glucans.

The cariogenicity of *S. mutans* relates to its ability to form extracellular glucans, which contribute to plaque formation by mediating cell-to-teeth surface adherence and cell-to-cell agglutination. Since glucan formation by *S. mutans* appears to participate in the initial step of tooth decay, the unique activity of hydroquinone to control glucan formation may provide a novel idea to prevent cavity formation, although hydroquinone itself is unlikely suitable as an anticavity agent since safety is the first priority for oral care products.[20] It also can be practical in certain ways. For example, hydroquinone may be used for the isolation of *S. mutans* from oral flora since it selectively alters the sucrose-induced agglutination of *S. mutans* and glucan formation which is a characteristic property of this bacterium when grown with sucrose.

4. Experimental

4.1. Chemicals

Arbutin was available from our previous work.[10] Hydroquinone, benzoquinone, resorcinol, catechol, glucose, fructose, galactose, sucrose, maltose and lactose were purchased from Sigma Chemical Co. (St. Louis, MO). *N,N*-Dimethylformamide (DMF) was obtained from EM Science (Gibbstown, NJ).

4.2. Test strain and medium

The microorganisms, *S. mutans* ATCC 25175, *Bacillus subtilis* ATCC 9372, *Brevibacterium ammoniagenes* ATCC 6872, *Staphylococcus aureus* ATCC 12598, *Escherichia coli* ATCC 9637, *Pseudomonas aeruginosa* ATCC 10145, *Enterobacter aerogenes* ATCC 13048, *Saccharomyces cerevisiae* ATCC 7754 and *Candida utilis* ATCC 9226 used for the experiment were purchased from American Type Culture Collection (Manassas, VA).

The culture media for the bacteria consisted of 0.8% nutrient broth (BBL), 0.5% yeast extract (DIFCO), and 0.1% glucose except for the case of *S. mutans*. For the culture of *S. mutans*, BHI consisting of 3.7% brain heart infusion (DIFCO) was used. For the culture of fungi, 2.5% malt extract (BBL) was used. For the agglutination study of hydroquinone with sugars, TTY broth, 1.5% trypticase (BBL), 0.8% tryptase (DIFCO), 0.4% yeast, 0.2% K_2HPO_4, 0.5% KH_2PO_4, 0.2% Na_2CO_3, 0.2% NaCl, and a saccharide, was used.[21] Saccharides were autoclaved separately and mixed with the broth medium aseptically.

The freeze-dried microorganisms were reactivated before the assay in the following manner. *B. subtilis*, *S. cerevisiae* and *C. utilis* were cultured with shaking at 30 °C and *B. ammoniagenes* of *E. aerogenes* were cultured without shaking at 30 °C, for 2 days. The remaining microorganisms were cultured without shaking at 37 °C for 2 days.

4.3. Agglutination Index (AI)

The test compound was first dissolved in DMF and 1% of the sample solution was added to the appropriate broth medium. Microorganisms were cultured without shaking in the broth medium that contained test compounds. The agglutination index (AI) was measured after 48 h.[12] After mixing the culture, the test tube was

left without shaking for one min, and turbidity A_1 was measured (absorbance at 660 nm). If cells adhered to the surface of the test tube, they were removed with a spatula, and turbidity A_1 was measured. After the cells were suspended by ultrasonication to maximize turbidity, the test tube was left without shaking for one min, and turbidity A_2 was measured (absorbance at 660 nm). The ultrasonication was carried out by using Cole Parmer ultrasonic. AI was calculated as turbidity A_1 divided turbidity A_2.

4.4. Antimicrobial assay

The antimicrobial assay was performed by a two-fold serial broth dilution method.[22] for the antimicrobial assay, *S. mutans* was cultured without shaking. After 2 days, the growth of *S. mutans* was examined by observing the turbidity (absorbance at 660 nm). The lowest concentration of the test compounds in which no growth occurred was defined as the minimum inhibitory concentration (MIC). The assays were performed in triplicate on separate occasions.

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