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The influence of mutanase and dextranase on the production and structure of glucans synthesized by streptococcal glucosyltransferases

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Abstract—Glucanohydrolases, especially mutanase [α-(1→3) glucanase; EC 3.2.1.59] and dextranase [α-(1→6) glucanase; EC 3.2.1.11], which are present in the biofilm known as dental plaque, may affect the synthesis and structure of glucans formed by glucosyltransferases (GTFs) from sucrose within dental plaque. We examined the production and the structure of glucans synthesized by GTFs B (synthesis of α-(1→3)-linked glucans) or C [synthesis of α-(1→6)- and α-(1→3)-linked glucans] in the presence of mutanase and dextranase, alone or in combination, in solution phase and on saliva-coated hydroxyapatite beads (surface phase). The ability of *Streptococcus sobrinus* 6715 to adhere to the glucan, which was formed in the presence of the glucanohydrolases was also explored. The presence of mutanase and/or dextranase during the synthesis of glucans by GTF B and C altered the proportions of soluble to insoluble glucan. The presence of either dextranase or mutanase alone had a modest effect on total amount of glucan formed, especially in the surface phase; the glucanohydrolases in combination reduced the total amount of glucan. The amount of (1→6)-linked glucan was reduced in presence of dextranase. In contrast, mutanase enhanced the formation of soluble glucan, and reduced the percentage of 3-linked glucose of GTF B and C glucans whereas dextranase was mostly without effect. Glucan formed in the presence of dextranase provided fewer binding sites for *S. sobrinus*; mutanase was devoid of any effect. We also noted that the GTFs bind to dextranase and mutanase. Glucanohydrolases, even in the presence of GTFs, influence glucan synthesis, linkage remodeling, and branching, which may have an impact on the formation, maturation, physical properties, and bacterial binding sites of the polysaccharide matrix in dental plaque. Our data have relevance for the formation of polysaccharide matrix of other biofilms.

Keywords: Mutanase; Dextranase; Glucosyltransferases; Glucans; Biofilm; Matrix

1. Introduction

Glucans, synthesized from dietary sucrose by glucosyltransferases (GTFs), are of central importance in adhesive interactions and in the expression of virulence by *Streptococcus mutans*, a cariogenic bacterium.[1–4] Glucans enhance the pathogenic potential of dental biofilm (plaque) by promoting the adherence and accumulation of cariogenic streptococci on the tooth surface, and by contributing to the bulk and structural integrity of dental plaque.[2,3,5–7] Furthermore, glucan-rich plaque matrix may increase the porosity,[8] and decrease its inorganic concentration.[9–12] *S. mutans* produces at least three GTFs: GTF B, which synthesizes mostly α-(1→3)-linked insoluble glucan; GTF C, which synthesizes a mixture of α-(1→3)-linked insoluble and α-(1→6)-linked soluble glucan; and GTF D, which synthesizes α-(1→6)-linked soluble glucan.[13–15] Among these enzymes, GTF B and C have been considered the most important GTFs related to dental caries.[4] Enzymatically active GTFs are present: (a) in the soluble

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fraction of human whole saliva, (b) in the salivary pellicle formed on the tooth surface, (c) on bacterial surfaces.[16–20] Furthermore, GTFs adsorbed on surfaces display distinct physical and kinetic properties compared to the same enzymes in solution.[19,21] The glucans synthesized by these enzymes in distinct loci play critical role in expression of virulence through promoting the adherence of the bacteria to tooth surface and to each other.

Glucanohydrolases, especially mutanase [α-(1→3) glucanase; EC 3.2.1.59] and dextranase [α-(1→6) glucanase; EC 3.2.1.11], have been found in dental plaque likely due to the ability of several oral microorganisms to produce these enzymes within the plaque matrix.[22–28] Clearly, the presence of mutanase and dextranase has the potential to influence the synthesis and structure of glucans within dental plaque. Therefore, a dynamic interaction of the enzymes responsible for glucans synthesis (GTFs) on one hand with those cleaving the glucosidic linkages (mutanase and dextranase) could be occurring concomitantly in the plaque matrix. Furthermore, the topical use of mutanase or dextranase as an approach to control dental plaque has been explored in vitro and in vivo with variable results.[29–34] However, the understanding of the influence of glucanohydrolases on both synthesis and chemical structure of different types of glucans remains unexplored.

By affecting the synthesis and/or structure of these polysaccharides, the bacterial adherence could be affected, thereby influencing the formation, development, and physical properties of dental plaque (also known as dental biofilm). Therefore, the purpose of this study was to evaluate the production and the structure of glucans synthesized by GTFs in the presence of mutanase and dextranase, alone or in combination, in an attempt to elucidate some of the interactions that may occur during the formation and maturation of dental plaque.

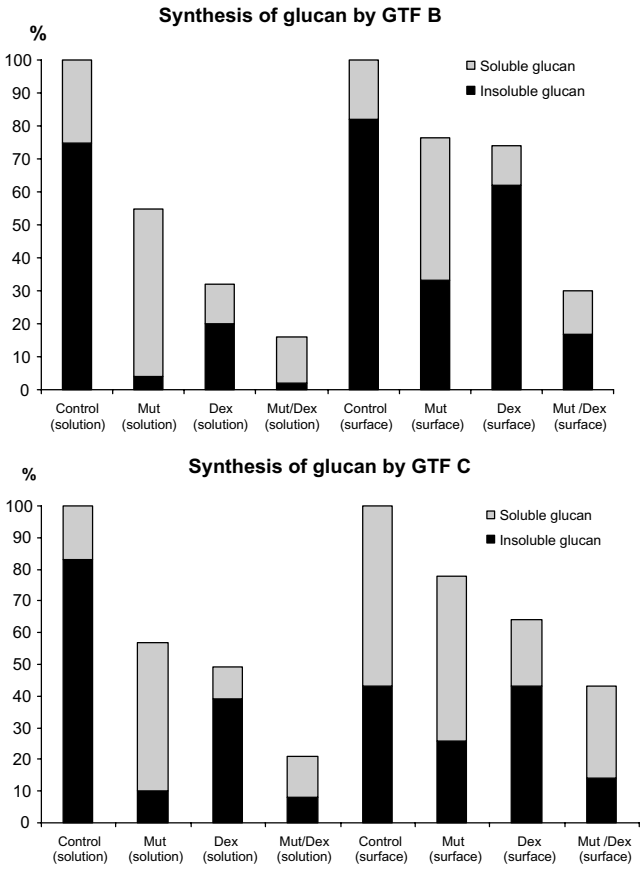

Figure 1. The effects of mutanase and/or dextranase on the synthesis of soluble and insoluble glucans by GTF B and C either in solution or on surface. The presence of mutanase and dextranase has an effect on total amount of and proportion of soluble and insoluble glucans. The amount of glucans synthesized by GTF B in the absence of mutanase and dextranase (control) was considered 100%. The values were expressed in percentage of glucans formed compared to control (100%).

2. Results

Glucans were synthesized by GTF B and C in solution, and on the surface of saliva-coated hydroxyapatite in the presence of mutanase and dextranase to determine whether these glucanohydrolases could affect the formation and structure of glucans (Fig. 1; Tables 1–3).

2.1. Synthesis of glucan in the presence of glucanohydrolases

In general, mutanase and/or dextranase interfered with the amount, and proportion of soluble/insoluble glucans; the presence of the combination of glucanohydrolases resulted in the formation of the lowest amount of glucans. It is nevertheless important to note that the synthesis of glucans did proceed even in the presence of the glucanohydrolases.

Glucans formed by GTF B and C in the absence of the glucanohydrolases were considered as controls. GTF B synthesized primarily insoluble glucans irrespective of whether the enzyme was in solution or on surface (approximately 80% insoluble and 20% soluble, Fig. 1) confirming our previous observation.[42] In contrast, the glucans synthesized by GTF C in solution showed distinct percentage of insoluble and soluble glucans (80% insoluble and 20% soluble, Fig. 1) compared with those formed on the surface (45% insoluble and 55% soluble, Fig. 1) as we have shown previously.[42]

2.1.1. Solution phase. The presence of mutanase clearly affected the ability of GTF B and C to synthesize glucans. The predominant effect appeared to be on the synthesis of insoluble glucans by the GTFs. Mutanase

Table 1. Percentage of glycosyl linkages of soluble and insoluble glucans synthesized by GTF B, either in solution or on surface, in the presence of mutanase and/or dextranase

Glycosyl residue	Insoluble				Soluble			
	Control	Mut	Dex	Mut/Dex	Control	Mut	Dex	Mut/Dex
GTF B in solution								
Terminal-glucose	+	++	±	+	+	+	++	++
4-Linked xylose	–	±	±	–	–	–	–	+
3-Linked glucose	+++++	+	+++++	+++	+	+	+++	+
6-Linked glucose	++	+++	+	+	+++++	+++++	+	+
4-Linked glucose	±	++	+	++	+	±	++	+++
3,4-Linked glucose	±	–	+	+	–	–	–	–
3,6-Linked glucose	+	+	+	+	+	++	+	+
4,6-Linked glucose	–	–	–	+	+	+	+	+
GTF B in surface								
Terminal-glucose	±	+	+	+	+	+	+	+
4-Linked xylose	±	±	+	±	–	–	±	+
3-Linked glucose	+++	++	++++	++++	++	+	+++++	+
6-Linked glucose	++	++	+	+	++++	++++	+	+
4-Linked glucose	++	++	++	++	+	+	+	++++
3,4-Linked glucose	+	–	+	±	±	–	±	+
3,6-Linked glucose	+	+	+	±	++	++	+	+
4,6-Linked glucose	+	+	+	±	+	+	±	+

– 0%, ± 1–5%, + 6–15%, ++ 16–25%, +++ 26–35%, ++++ 36–45%, +++++ 46% or more.

Table 2. Percentage of glycosyl linkages of soluble and insoluble glucans synthesized by GTF C, either in solution or on surface, in the presence of mutanase and/or dextranase

Glycosyl residue	Insoluble				Soluble			
	Control	Mut	Dex	Mut/Dex	Control	Mut	Dex	Mut/Dex
GTF B in solution								
Terminal-glucose	+	+	±	+	+	+	++	++
4-Linked xylose	±	+	–	–	+	–	±	–
3-Linked glucose	++++	+	+++++	+	++++	+	++	+
6-Linked glucose	++	+	+	+	±	++++	+	++
4-Linked glucose	++	++++	+	+++++	–	+	++	++
3,4-Linked glucose	±	–	±	–	++	–	–	+
3,6-Linked glucose	+	+	+	–	+	++	+	+
4,6-Linked glucose	–	+	–	–	–	+	+	+
GTF B in surface								
Terminal-glucose (T-Glc)	+	+	+	+	+	+	+	+
4-Linked xylose	+	+	+	+	–	–	±	±
3-Linked glucose	++	+	+++	+	++	+	++++	+
6-Linked fructose	–	–	–	–	–	–	–	+
6-Linked glucose	+	+	±	+	++++	+++++	+	+
4-Linked glucose	++++	+++++	++++	+++++	+	±	+++	++
3,4-Linked glucose	+	–	±	+	±	–	±	–
3,6-Linked glucose	±	–	±	±	+	+	±	+
4,6-Linked glucose	±	+	±	+	+	+	±	–
1,6-Linked fructose	–	–	–	–	–	–	–	++

– 0%, ± 1–5%, + 6–15%, ++ 16–25%, +++ 26–35%, ++++ 36–45%, +++++ 46% or more.

clearly interfered with the synthesis of glucans in solution; 41–45% less total glucans were formed when compared with control (without addition of mutanase) (Fig. 1). Figure 1 also shows that mutanase reduced the amount of insoluble glucans synthesized by the GTFs (90% less glucans than control). In contrast, the total amount of soluble glucans produced by GTF B and C was increased by 2-fold compared with control. Furthermore, the proportion of insoluble to soluble glucans synthesized by the GTFs was significantly affected in the presence of mutanase (Fig. 1). The proportion of insoluble to soluble glucans formed by GTF B and C was 1 to 10 (*vs* 3 to 1 for control) and 1 to 5 (*vs* 4 to 1 for control).

Table 3. Products of the activity of glucanohydrolases during glucan synthesis by GTFs

	Mutanase	Dextranase	Mutanase/dextranase	Control
GTF B glucan (solution)				
Glucose	143.70 μg	89.74 μg	142.40 μg	72.47 μg
Fructose	300.10 μg	264.90 μg	286.90 μg	270.59 μg
Isomaltose	—	66.90 μg	81.57 μg	—
Maltose	—	11.50 μg	14.13 μg	—
Isomaltotriose	24.30 μg	31.20 μg	30.50 μg	18.29 μg
Total	468.10 μg	464.24 μg	555.50 μg	361.35 μg
GTF B glucan (surface)				
Glucose	198.03 μg	113.61 μg	160.60 μg	106.71 μg
Fructose	304.74 μg	220.62 μg	286.90 μg	201.28 μg
Isomaltose	—	48.43 μg	87.88 μg	—
Maltose	—	—	—	—
Isomaltotriose	—	26.99 μg	23.60 μg	—
Total	502.77 μg	409.65 μg	558.90 μg	307.99 μg
GTF C glucan (solution)				
Glucose	260.30 μg	170.84 μg	230.90 μg	145.60 μg
Fructose	459.80 μg	378.20 μg	460.87 μg	382.50 μg
Isomaltose	11.20 μg	69.30 μg	98.48 μg	—
Maltose	5.67 μg	35.74 μg	49.76 μg	2.39 μg
Isomaltotriose	8.65 μg	15.22 μg	15.36 μg	9.85 μg
Total	745.62 μg	669.3 μg	855.37 μg	540.34 μg
GTF C glucan (surface)				
Glucose	108.20 μg	78.70 μg	107.73 μg	64.19 μg
Fructose	176.10 μg	116.27 μg	152.82 μg	127.75 μg
Isomaltose	—	65.25 μg	70.79 μg	—
Maltose	—	—	—	—
Isomaltotriose	—	24.52 μg	21.82 μg	—
Total	284.30 μg	219.49 μg	353.16 μg	191.94 μg

The reaction mixture consisted of GTF B or C (1 unit; in solution or on surface), mutanase (10 units), and/or dextranase (50 units) and sucrose substrate (200.0 mmol/L sucrose, 40 μmol/L dextran 9000, 0.02% sodium azide in adsorption buffer, pH 6.5). The volume of the reaction mixture was 200 μL (solution phase) and 300 μL (surface phase).

The presence of dextranase also affected the synthesis of glucans by the GTFs; 68% (for GTF B) and 52% (for GTF C) less total glucans were formed in solution when compared with control (without addition of dextranase) (Fig. 1). It is also clear that dextranase interfered with the synthesis of soluble glucans by the GTFs (50–60% less than control) as shown in Figure 1. Interestingly, dextranase reduced the amount of insoluble glucans formed by GTF B and C by 4- and 2-fold compared with control. In addition, the proportion of insoluble to soluble glucans synthesized by GTF B was affected in the presence of dextranase (Fig. 1); the proportion of insoluble to soluble glucans formed was 2 to 1 in solution (*vs* 3 to 1 for control). The proportion of glucans produced by GTF C were unaffected by dextranase.

The combination of the glucanohydrolases remarkably affected the synthesis of glucans by GTF B and C resulting in more than 80% less total glucans compared with control (Fig. 1). The presence of both mutanase and dextranase effectively interfered with the synthesis of both insoluble (more than 90% less glucans than control) and to a lesser extent the soluble glucans (30–40% less glucans than control). The proportion of insoluble to soluble glucans synthesized by GTF B in the presence of dextranase and mutanase was 1 to 5 (*vs* 3 to 1 for control); the proportion of insoluble to soluble glucans synthesized by GTF C was 1 to 1.5 for GTF C (*vs* 4 to 1 for control) (Fig. 1). It is nevertheless important to point out that some glucan was indeed formed.

2.1.2. Surface phase. Mutanase also disrupted the synthesis of glucans by GTF B and C on surface, although its influence was not as pronounced as that observed in solution phase (Fig. 1); 22–25% less total glucans were formed on surface (*vs* 41–45% less in solution) when compared with control (without addition of mutanase). As shown in Figure 1, mutanase also reduced the amount of insoluble glucans synthesized by the surface-adsorbed GTFs (40–60% less glucans than control). In contrast, the total amount of soluble glucans formed on surface by GTF B was increased by 2-fold compared with control. Interestingly, the amount of soluble glucans formed by surface-GTF C was unaffected by the presence of mutanase. Furthermore, the proportion of insoluble to soluble glucans synthesized by GTF B on surface was significantly affected in the presence of

mutanase (Fig. 1). The proportion of insoluble to soluble glucans formed by GTF B was 1 to 1.25 (*vs* 4 to 1 for control); the proportion for GTF C glucans was unaffected by the presence of mutanase.

The presence of dextranase also influenced the synthesis of glucans by the surface-adsorbed GTFs; 25–35% less total glucans was formed on surface when compared to glucans formed in the absence of dextranase (Fig. 1). It is also clear that dextranase interfered with the synthesis of soluble glucans on the surface by the GTFs (40–60% less than control) as shown in Figure 1. The presence of dextranase had little effect on the amount of insoluble glucan formed on the surface, in contrast to that observed with mutanase. In addition, the proportion of insoluble to soluble glucans synthesized by the GTFs on surface was affected in the presence of dextranase (Fig. 1); the proportion of insoluble to soluble glucans formed by GTF B was 5.5 to 1 (*vs* 4 to 1 for control). For GTF C glucans, the proportion was 2 to 1 (*vs* 1 to 1.25 for control).

The presence of both glucanohydrolases clearly reduced the total amount of glucans synthesized by the surface-adsorbed GTFs resulting in more than 60% less total glucans compared with control (Fig. 1). The combination of mutanase and dextranase effectively interfered with the synthesis of both insoluble (more than 70% less on surface than control) and soluble glucans (30–50% less than control). Furthermore, the proportion of insoluble to soluble glucans was clearly affected in the presence of dextranase and mutanase (Fig. 1): 1.5 to 1 (*vs* 4 to 1 for control) for GTF B, and 1 to 2 (*vs* 1 to 1.25 for control) for GTF C. It is nevertheless important to point out that some glucan was indeed formed.

2.2. Linkage analysis

Linkage analysis of the insoluble and soluble glucans synthesized by GTFs, either in solution or on the surface, is a critical step towards identifying the structural changes of glucans formed in the presence of glucanohydrolases. Clearly the presence of mutanase and dextranase had profound effects on the structure of the synthesized polymers as shown in Tables 1 and 2.

2.2.1. GTF B glucans (solution phase). The insoluble glucans synthesized by GTF B in solution in the absence of glucanohydrolases were predominantly $(1\rightarrow3)$-linked glucans whereas the soluble glucans were comprised basically of $(1\rightarrow6)$-linked glucans (Table 1). The major branch points for insoluble glucans were 3,4- and 3,6-linked glucose, and 3,6- and 4,6-linked glucose for soluble glucans. However, the presence of mutanase and/or dextranase affected the proportion of linkages of the glucans. For example, the presence of mutanase resulted, as might be expected, in insoluble glucans with lower percentage of $(1\rightarrow3)$ linkages and higher percentage of $(1\rightarrow6)$ and $(1\rightarrow4)$ linkages compared to control; the effects on soluble glucans were negligible. The presence of dextranase decreased the percentage of $(1\rightarrow6)$ linkages and increased the percentage of $(1\rightarrow4)$ linkages of insoluble glucans compared to control. In addition, the presence of dextranase (alone) resulted in soluble glucans with higher percentage of $(1\rightarrow3)$ linkages. Glucans (both soluble and insoluble) synthesized in the presence of mutanase and dextranase resulted in higher percentage of $(1\rightarrow4)$-linked glucans and lower percentage of $(1\rightarrow6)$-linked glucans than control; the percentage of $(1\rightarrow3)$ linkages was lower in the insoluble glucans. The presence of the mixture of glucanohydrolases also resulted in insoluble glucans with an additional branch point 4,6-linked glucose. Interestingly, a 4-linked xylose appeared in the soluble glucan formed in the presence of mutanase and dextranase.

2.2.2. GTF B glucans (surface phase). Linkage analysis of the insoluble glucans made by GTF B on the surface displayed mostly $(1\rightarrow3)$ linkages and, in a lesser extent, $(1\rightarrow6)$ and $(1\rightarrow4)$ linkages; the branch points consisted of 3,4-, 3,6-, and 4,6-linked glucose (Table 1). In addition, 4-linked xylose was also present. The soluble glucans were comprised of predominantly $(1\rightarrow6)$-linked glucans as well as $(1\rightarrow3)$ linkages, and some $(1\rightarrow4)$ linkages; the same branch points observed for the insoluble glucans were found in the soluble glucan. The presence of mutanase did not affect dramatically the percentages of linkages in the insoluble glucans compared to control; the proportion of $(1\rightarrow3)$, $(1\rightarrow6)$, and $(1\rightarrow4)$ linkages was equally distributed, and the branch point 3,4-linked glucose was not found. The effects of the presence of mutanase on the structure of the soluble glucans were also minimal; a decrease of the $(1\rightarrow3)$ linkages was observed, and the branch point of 3,4-linked glucose was absent. Dextranase had little effect on the percentage of linkages in the insoluble glucans compared to control, except a lower percentage of $(1\rightarrow6)$ linkages and a higher percentage of $(1\rightarrow3)$ linkages were observed. The soluble glucans formed in the presence of dextranase displayed predominantly $(1\rightarrow3)$-linked glucans. In addition, the percentage of $(1\rightarrow6)$ linkages was more than three times lower compared to control. The mixture of mutanase and dextranase caused significant changes in the soluble glucans, showing lower percentage of $(1\rightarrow3)$ and $(1\rightarrow6)$ linkages and higher percentage of $(1\rightarrow4)$-linked glucans than control.

2.2.3. GTF C glucans (solution phase). Linkage analysis of GTF C derived insoluble glucans in solution showed predominantly $(1\rightarrow3)$ linkages as well as significant amounts of $(1\rightarrow6)$- and $(1\rightarrow4)$-linked glucans (Table 2). The soluble glucan was rich in $(1\rightarrow3)$ linkages, and

also some (1→6)-linked glucans; both insoluble and soluble glucans harbored 4-linked xylose. The major branch points were 3,4- and 3,6-linked glucose. The presence of mutanase during the glucan formation resulted in higher percentage of (1→4) linkages (in the insoluble glucan) and (1→6) linkages (in the soluble glucan), and lower percentage of (1→3) linkages (in both insoluble and soluble glucans) compared to control. In addition, no branch point 3,4-linked glucose was found but instead 4,6-linked-glucose was present. Insoluble glucans synthesized in the presence of dextranase displayed predominantly (1→3) linkages whereas the soluble glucans consisted of equal proportions of (1→3) and (1→4) linkages, and also some (1→6) linkages. The branch points of the insoluble glucans were not affected by dextranase compared to control. However, soluble glucans formed in the presence of dextranase displayed 4,6-linked glucose instead of 3,4-linked glucose. The presence of mutanase and dextranase during glucan synthesis resulted insoluble glucans with predominantly (1→4) linkages with no branch points; the soluble glucans showed a similar pattern of that formed in the presence of dextranase with the presence of an additional branch point of 3,4-linked glucose.

2.2.4. GTF C glucans (surface phase). Table 2 also shows that the insoluble glucans synthesized by surface-adsorbed GTF C displayed basically (1→4) and (1→3) linkages, and smaller percentage of (1→6) linkages. The soluble glucans was comprised predominantly of (1→6) and (1→3) linkages, and smaller percentages of (1→4) linkages. The branch points 3,4-, 3,6-, 4,6-linked glucose were present in both insoluble and soluble glucans. The presence of mutanase during the insoluble glucan synthesis on the surface resulted in higher percentage of (1→4) linkages and lower percentage of (1→3) linkages compared to control. The soluble glucans formed in the presence of mutanase displayed predominantly (1→6) linkages. Mutanase also affected the branch points in both glucans compared to control; 3,4- and 3,6-linked glucose were not found in the insoluble glucan whereas 3,4-linked glucose was absent in the soluble glucan. The presence of dextranase had little effect on the percentage of linkages of insoluble glucans; a slightly lower percentage of (1→6) linkages and a higher percentage of (1→3) linkages were observed. The effects of the presence of dextranase were more evident in the soluble glucans, where an increase of the percentages of (1→3) and (1→4) linkages and a decrease of the percentage of (1→6) linkages compared to control were observed. The mixture of mutanase and dextranase remarkably affected the percentage of linkages of surface GTF C derived glucans. The insoluble glucans displayed predominantly (1→4)-linked glucans and lower percentage of (1→3)-linked glucans than control. The soluble glucans showed 6-linked fructose, and a branch point of (1→6)-linked fructose; in addition, the mixture of glucanohydrolases increased the percentage of (1→4)-linked glucans and decreased the percentage of (1→3) and (1→6) linkages compared to control.

2.3. End products of the action of glucanohydrolases during glucan synthesis

Investigation of the end products following mutanase activity on the forming GTF B and C glucans, made either in solution or on the surface, indicated that the major products were glucose and fructose (Table 3). A small amount of isomaltotriose was also detected from GTF B glucans formed in solution whereas isomaltose, maltose, and isomaltotriose were detected from GTF C glucans made in solution. Interestingly, isomaltose, maltose, and isomaltotriose were not detected from either GTF B or C glucans formed on the surface.

In contrast, glucose, fructose, and isomaltose were the major products when dextranase was present during the synthesis of GTF B and C glucans made either in solution or on the surface; a trace of isomaltotriose was also detected. A trace of maltose was found from GTF B and C glucans made in solution, but not when they were formed on the surface.

The end products of the action of the combination of mutanase and dextranase during the synthesis of GTF B and C glucans either in solution or on the surface were comprised mostly of glucose, fructose, and isomaltose, as well as small amounts of isomaltotriose. A trace of maltose was found from GTF B and C glucans made in solution, but not when they were formed on the surface.

In the absence of glucanohydrolases, the end products during glucan synthesis by either GTF B or C were mostly fructose and, to a lesser amount, glucose.

2.4. Bacterial adherence

Streptococcus sobrinus, an important cariogenic bacterium, produces glucan-binding proteins and binds avidly to glucan-coated HA.[7,21,48] Since mutanase and dextranase may potentially affect the structure of glucan, and consequently the bacterial binding sites, we explored the ability of *S. sobrinus* 6715 to adhere to the glucan, which was formed in the presence of mutanase and/or dextranase.

The results of *S. sobrinus* 6715 adherence to saliva-coated hydroxyapatite with GTF B and C glucans made in the presence of mutanase and or dextranase are shown in Figure 2. GTF B and C glucans formed in the presence of mutanase provided as many binding sites for *S. sobrinus* 6715 as control group, as shown in Figure 2. In contrast, fewer bacteria adhered to glucan formed in the presence of dextranase or the combination of mutanase and dextranase.

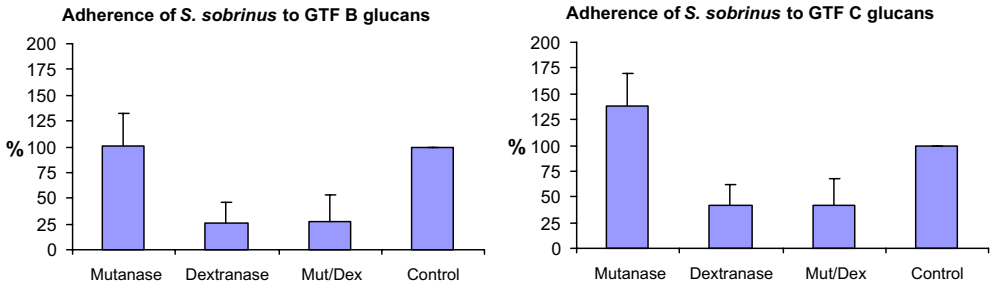

Figure 2. Adherence of *S. sobrinus* 6715 to HA coated with GTF B or C glucans made in the presence of mutanase and/or dextranase. The number of bacteria binding to HA coated with GTF B or C glucans formed in the absence of mutanase and dextranase (control) was considered 100% adherence. The values were expressed as percentage of adherence compared to the control (100% adherence).

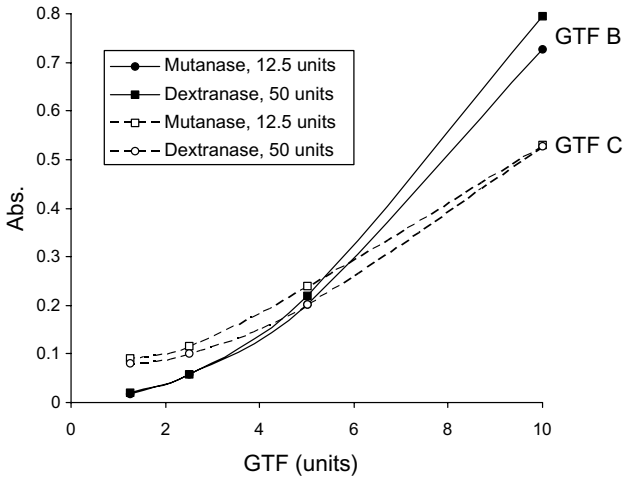

Figure 3. GTF B and C binding to mutanase and dextranase. The negative controls (samples that contained no mutanase or dextranase, or no GTFs enzymes) displayed negligible absorbance at 405 nm (<0.09).

2.5. Interactions of GTFs to glucanohyrolases

To investigate whether the mutanase and dextranase were digesting the GTF B and C enzymes, GTF enzymes were incubated with the glucanohydrolases, and the protein profile analyzed by SDS-PAGE; the data (not shown) revealed that the GTF enzymes were not digested by mutanase and dextranase.

We also explore the possibility that the GTFs may bind to mutanase and dextranase. Enzyme-linked immunosorbent assay (ELISA) using anti-GTF B and anti-GTF C rabbit sera indicate that GTF B and C bound to mutanase and dextranase (Fig. 3).

3. Discussion

All the available evidence shows very clearly that the matrix of biofilm plays a critical role in its adherence to surfaces and biological properties. The majority of biofilm matrices are composed of polysaccharide and dental plaque is no exception;[2,3,5–7] we and others have shown that the formation of extracellular polysaccharides by mutans streptococci is essential for their expression of virulence in rat caries model.[3,4] Despite the importance of extracellular polysaccharide in the etiology and pathogenesis of dental caries, information on structure and formation of the matrix of dental plaque is indeed sparse. The published data on the structure of polysaccharide found in dental plaque[6] does not completely correspond to the known structures of dextran and mutan produced by glucosyltransferases (GTFs) from oral streptococci such as *S. mutans* and *S. sanguis*, using sucrose as substrate. Some of the disparities can be explained in part by our observations that starch hydrolyzates may serve as acceptors for GTF B in the presence of sucrose;[49] furthermore, GTF C adsorbed to a surface forms a structurally distinct glucan compared with that formed by the same enzyme in solution.

There are a myriad of biological enzymatic interactions occurring within dental plaque including dextranase and mutanase produced by oral microorganisms. The structure of the polysaccharide matrix of plaque is clearly a reflection of a combination of synthesis and degradative processes. Use of both enzymes has been advocated as therapeutic approaches for plaque reduction and prevention of dental caries, with less than spectacular success, possibly because streptococcal glucans are relatively resistant to digestion by those enzymes.[33,42] Results presented here have shown that the presence of dextranase and/or mutanase during the synthesis of glucans by GTF B and C has a profound effect on the physical and structural properties of resulting glucan. The proportions of soluble to insoluble glucan were affected. It is noteworthy that the presence of dextranase or mutanase alone has relatively modest effect on the total amount of glucan formed, especially on surface. However, the glucanohydrolases in combination were effective in reducing the amount of total glucan. The presence of dextranase clearly reduced, as expected, the amount of $(1 \rightarrow 6)$-linked glucan as this in turn leads to the formation of a less soluble glucan. This

observation may explain the relative lack of therapeutic effect of dextranase (even though our results showed that fewer *S. sobrinus* adhered to GTF glucans formed in the presence of dextranase).[33] In contrast, the presence of mutanase in the synthesizing mixture tends to promote a more soluble glucan. However it is essential to note that the linkage analyses reveal glucans of quite distinct structure from those found in absence of mutanase and/or dextranase. In line with our previous reports GTF adsorbed to surfaces appears less susceptible to external influences.[35,36,42,43] One of the explanation for the enhanced resistance is related to conformational changes that the enzyme undergoes during the adsorption process, which could affect the catalytic site at structural level; our studies have shown that surface-adsorbed GTFs exhibit distinct physical and kinetic properties compared to the same enzymes in solution.[19,21]

Mutanase reduced the percentage of $(1\rightarrow3)$ linkages of GTF B and C glucans whereas dextranase was without effect (although dextranase increased the percentage of $(1\rightarrow3)$ linkages of GTF glucans formed in the surface phase); the combinations of mutanase and dextranase had a smaller effect than mutanase alone, an observation consistent with previous results, which showed that combination of dextranase and mutanase had less effect on glucose release from pre-formed glucan or plaque matrix. It appears probable that steric hindrance is occurring on the susceptible substrate.

Release of fructose is well recognized as a by-product of the synthesis of glucan from sucrose by GTF. Oligosaccharides are less frequently observed. However, in the presence of dextranase and mutanase the amount of oligosaccharides present was greatly enhanced. It is unclear whether they arise from breakdown of formed glucan or they represent aborted stages in the assembly of branches in complex glucans. Certainly if they occurred in plaque they could be readily metabolized and lead to protracted acid formation; persons who have active carious generally display low fasting pH values.[50]

The presence of free glucose in control incubation mixture runs contrary to the generally accepted reaction in which all glucose from sucrose is incorporated into polymer and fructose is the only reducing sugar liberated. Our observations support those of who also noted the liberation of glucose during GTF–sucrose interactions and have suggested that determination of free glucose might be used as a measure of GTF activity.[51] It is clear that the sequence of synthesis and assembly of complex glucans are poorly understood.

We have reported previously that glucan on surfaces may provide binding sites for a range of oral microorganisms,[7] and that binding of *S. mutans* and *S. sobrinus* to GTF C glucans on a surface may be prevented by dextranase but not by mutanase. Our present results confirm and extend these observations and show that glucan formed in presence of dextranase provides fewer binding sites than glucan formed in presence of mutanase. It appears that $(1\rightarrow6)$-linked glucan may provide sites of adherence for mutans streptococci and other oral microorganisms. Interestingly, mutanase alone actually increases the amount of soluble glucan and the percentage of $(1\rightarrow6)$-linked glucan, which may explain the negligible effects on bacterial adherence.

Based on the presence of α-$(1\rightarrow3)$-linked glucose, Holtz et al.[6] estimated that plaque contained 1.35% mutan. It is possible that this estimate is on the low side because it is likely that enzymatic restructuring of mutan by mutanase and dextranase could lead to significant changes in the determined linkage analysis. The presence of heteropolysaccharides derived from oral microorganisms such as *Actinomyces* sp.[52] cannot be discounted although given that sucrose and starch are the mostly commonly ingested carbohydrates, mutan, dextran (modified and unmodified), and fructan are likely to be dominant part of the polysaccharide matrix in dental plaque.

We have shown that the binding of antibody to GTF, even with minimum of inhibition of activity affects the structure of the formed glucans. In the present study, we noted that the GTFs bind to mutanase and dextranase and therefore could clearly cause conformational changes in the enzymes resulting in distinct products. It is also possible that the GTFs are affecting the activity of the glucanohydrolases. Clearly, additional studies are needed to elucidate the precise mechanisms involved in the interaction of these enzymes.

The precise mechanisms involved in the synthesis of glucans by GTFs are largely unresolved (for review see Monchois et al.[53]). It has been hypothesized that a separate active site of GTFs is involved in the formation of branches and that glucan can act as an acceptor. Our data are consistent with this hypothesis in that it is clear that the presence of our enzymes could remove or block acceptor sites leading in some instances alternative acceptor sites and the formation of an unconventional glucan.

In conclusion, it is evident that the formation and maturation of the polysaccharide matrix in dental plaque is not a simple process, and is an essential part of the development of this important biofilm. Our results may appear at variance to those of Tanzer et al.[3] who suggested that dextranase from *S. mutans* does not have a role in glucan synthesis or linkage remodeling or branching, while pointing out that many oral microorganisms present in the plaque display active dextranase.

Our observations have relevance to the development of matrices of biofilms beyond the mouth. Most attention is focused on the synthesis of matrix of biofilms and sparse attention is given to post-formation modification. Clearly, this is a deficit that should be explored.

4. Experimental

4.1. Bacterial strains

The bacterial strains used for the production of GTFs were: (1) *Streptococcus milleri* (now classified as *Streptococcus anginosus*) KSB8, which harbors the *gtfB* gene from *S. mutans* GS-5 (for GTF B production); (2) *S. mutans* WHB 410,[35] from which the genes for GTF B, D, and fructosyltransferase were deleted (for GTF C). *S. sobrinus* 6715 was used for the bacterial adherence assays. The cultures were stored at −80 °C in brain heart infusion (BHI) or tryptic soy broth (TSB) containing 20% glycerol. The *S. milleri* (now classified as *S. anginosus*) KSB8 was a kind gift from Dr. Howard K. Kuramitsu (SUNY, Buffalo, NY).

4.2. Preparation of enzymes

The GTFs B and C were obtained from *S. milleri* KSB8 and *S. mutans* WHB 410, and purified to near homogeneity by hydroxyapatite column chromatography as detailed by Venkitaraman et al.[21] and Koo et al.[36] All the purification procedures were carried out using buffers containing the protease inhibitor, phenylmethylsulfonyl fluoride (PMSF, 1 mM, final concentration), and NaN_3 (0.02%, final concentration) as preservative. Neither of the reagents had any effects on enzyme activity or stability. The purity of the enzyme preparations was analyzed by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a Hoefer Mighty Small SE245 system (Hoefer Scientific Instruments, San Francisco, CA, USA) and silver staining.[37] Pre-stained standards were purchased from BioRad Laboratories. Protein concentration was determined by the method of Lowry et al.,[38] with bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as a standard. Glucosyltransferase activity was measured by the incorporation of [^{14}C]glucose from labeled sucrose (NEN Research Products, Boston, Mass.) into glucans.[21] Briefly, GTF B or C was mixed with ([^{14}C]glucosyl)-sucrose substrate (0.2 μCi/mL; 200.0 mmol/L sucrose, 40 μmol/L dextran 9000, 0.02% sodium azide in adsorption buffer, pH 6.5) to reach a final concentration of 100 mmol/L sucrose (reaction volume of 200 μL), and incubated at 37 °C. After 4-h incubation, the total amount of glucans formed was measured by scintillation counting. One unit of activity of GTF was defined as the amount of enzyme required to incorporate 1 μmol of glucose over a 4-h reaction.

Mutanase [α-(1 → 3) glucanase; EC 3.2.1.59], produced from *Trichoderma harzianum*, and dextranase [α-(1 → 6) glucanase; EC 3.2.1.11], produced from *Penicillium aculeatum*, were gifts from Novo Nordisk, Denmark. The glucanohydrolases activity was determined as described by Kopec et al.[42] Briefly, one hundred micrograms of mutan [α-(1 → 3)-linked glucans] or dextran [α-(1 → 6)-linked glucans] was incubated for 1 h at 30 °C with mutanase or dextranase in 0.1 M sodium acetate buffer, pH 5.5 (reaction volume of 300 μL). One unit of activity of the mutanase or dextranase was defined as the amount of enzyme that releases 1 μmol of reducing sugar from pre-formed mutan or dextran per minute over a 1-h reaction as determined colorimetrically.[39]

4.3. Synthesis of glucan

A pilot study was performed to determine the optimum concentration of mutanase and dextranase to be mixed with GTFs, which would result in the formation of lowest amount of glucan. GTF B or C (1 unit) was mixed with either mutanase or dextranase (ranging from 1 to 1000 units), and incubated with ([^{14}C]glucosyl)-sucrose substrate (0.2 μCi/mL; 200.0 mmol/L sucrose, 40 μmol/L dextran 9000, 0.02% sodium azide in adsorption buffer, pH 6.5) to reach a final concentration of 100 mmol/L sucrose (200 μL). After 4 h of incubation (at 37 °C), the total amount of glucans formed was measured by scintillation counting. The saturation curve showed that the presence of 10 units of mutanase and 50 units of dextranase were sufficient to reach the plateau in reducing the total amount of glucans (synthesized by 1 unit of GTF B or C) formed over 4-h period, and these amounts were selected for use in further experiments.

The influences of the presence of mutanase and/or dextranase on the synthesis of glucans by GTF B and C were determined in both solution phase and on saliva-coated hydroxyapatite beads (surface phase).[19,21] GTF B and C were selected for this study because they have been shown to be essential for the expression of virulence by mutans streptococci associated with the pathogenesis of dental caries.[4] For synthesis in the solution phase, each GTF (1 unit of the enzyme) was mixed with mutanase and/or dextranase, and incubated with ([^{14}C]glucosyl)-sucrose substrate as described above. The control was the same reaction mixture with adsorption buffer replacing mutanase and dextranase. The solutions were incubated at 37 °C with rocking for 4 h to allow glucan synthesis. Subsequently, insoluble glucans were collected by centrifugation and washed three times using dH_2O. Soluble glucan was precipitated with ice-cold ethanol (final concentration of 70%) for 18 h at 4 °C. The soluble and insoluble radiolabeled glucans were determined by scintillation counting.[21]

For surface phase assays, the GTFs were adsorbed onto hydroxyapatite (HA) beads (Macro-Prep Ceramic Hydroxyapatite Type 1, 80 μm, Bio-Rad), which had been coated with clarified whole saliva, which contained negligible levels of GTFs, as described by Schilling and Bowen.[7] Following adsorption of GTFs, the beads were washed three times with adsorption buffer and exposed to the glucanohydrolases, either alone or in

combination, or control (absorption buffer) for 30 min. Then, the beads were washed three times and exposed to 300 μL of ([^{14}C]glucosyl)-sucrose substrate for 4 h with rocking at 37 °C. The HA beads, with their adsorbed glucans, were collected by centrifugation and washed three times with dH_2O. The HA beads were dissolved by incubation at 4 °C for at least 24 h in 0.1 M EDTA, releasing the adsorbed glucans in solution. The insoluble glucans were collected by centrifugation, and the soluble glucans in the supernatant were precipitated with ice-cold EtOH (final concentration of 70%) for 18 h at 4 °C. The soluble and insoluble radiolabeled glucans were determined by scintillation counting. All assays were performed in triplicate in at least three separate experiments.

4.4. Linkage analysis

The glycosyl linkage analysis was conducted to investigate whether the presence of glucanohydrolases during synthesis affected the structure of the glucans. The samples were methylated by a modification method of Ciucanu and Kerek,[40] followed by combined gas chromatography/mass spectrometry (CG/MS) analysis as described by York et al.[41] The partially methylated aldital acetates were analyzed on a 30-m Supelco 2330 fused silica capillary column by GC/MS using a Hewlett–Packard 5890 GC with a 5970 MSD (mass selective detector, electron impact).

4.5. Products of glucanohydrolases activity during glucan synthesis

For this part of the investigation, glucans were synthesized in the presence of the glucanohydrolases as described in item 4.3 using nonradiolabeled sucrose. The reaction mixture consisted of GTF B or C (1 unit; in solution or on surface), mutanase (10 units), and/or dextranase (50 units), and sucrose substrate. The volume of the reaction mixture was 200 μL (solution phase) and 300 μL (surface phase). The soluble products of the combined reaction of GTF, sucrose, and mutanase and/or dextranase were quantified by anion-exchange chromatography with pulsed amperometric detection (HPLC-PAD) according to Kopec et al.[42,43] using a Waters 625LC gradient controller, and an ED 40 electrochemical detector. Monosaccharides were separated on a Dionex Carbopak PA 100 column (4 × 250 mm) equipped with a guard column (3 × 25 mm). Chromatographic data were integrated and plotted using a Waters 746 data module-integrator.

4.6. Bacterial adherence

Mutans streptococci, such as *S. sobrinus*, produce glucan-binding proteins, which play a significant role in the bacterial adherence on tooth surface.[7,44,45]

For adherence assays, *S. sobrinus* 6715 was grown in 2.5% tryptic soy broth, 1.5% yeast extract (Difco laboratories, Detroit, Mich., USA) supplemented with 1% glucose and 10 μCi of [^{3}H]thymidine (New England Nuclear) per mL as described elsewhere.[7] Briefly, saliva-coated hydroxyapatite (sHA) beads were exposed to either GTF B or GTF C, and glucans were synthesized as described above in the presence of either buffer, mutanase, dextranase, or both glucanohydrolases. The glucan-coated beads were then exposed to 1×10^9 cells [^{3}H]thymidine labeled *S. sobrinus* 6715 cells for 1 h, 37 °C. Unbound bacteria were removed by washing with buffer, and the number of radioactively labeled adherent bacteria was measured by scintillation counting. All the assays were performed in triplicate in at least three separate experiments.

4.7. Interactions of GTFs to glucanohydrolases

To investigate whether the mutanase and dextranase were digesting the GTF enzymes, GTF B or C was incubated with mutanase and dextranase, alone or in combination, for 4 h at 37 °C. Approximately 10 μg of protein was taken from each sample and performed SDS-PAGE according to Laemmli[46] and silver stained according to the method of Morrissey.[37]

We also explored whether the GTFs were binding to the glucanohydrolases. For this experiment, mutanase and dextranase (12.5 units of mutanase and 50 units of dextranase) were coated onto an ELISA plate. The plates were washed and blocked by means of a buffer provided in an ELISA kit (Kirkegaard and Perry, Gaithersburg, MD). Following washing, decreasing dilutions of GTF B and C (ranging from 10 to 1.25 units of GTFs) were added to separate wells of the plate and incubated. Controls included samples that contained no mutanase or dextranase, or no GTFs enzymes. After incubation the samples were probed with polyclonal anti-GTF B and anti-GTF C rabbit sera. Again, plates were incubated and washed and probed with a secondary antibody, goat anti-rabbit IgG whole molecule, alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, Mo). The plates were then developed using the kit substrate according to the manufacturer's instructions. Following color development, which reflected the amount of bound GTF enzymes, the absorbance of the solution was read at 405 nm ELISA reader.[47]

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References

1. DeStoppelaar, J. D.; König, K. G.; Plasscharert, A. J. M.; van der Hoeven, J. S. *Arch. Oral Biol.* **1971**, *16*, 971–975.
2. Hamada, S.; Slade, H. D. *Microbiol. Rev.* **1980**, *44*, 331–384.
3. Tanzer, J. M.; Freedman, M. L.; Fitzgerald, R. J. Virulence of Mutants Defective in Glucosyltransferase, Dextran-Mediated Aggregation, or Dextranase Activity. In *Molecular Basis of Oral Microbial Adhesion*; Mergenhagen, S. E., Rosan, B., Eds.; American Society for Microbiology: Washington, DC, 1985; pp 204–211.
4. Yamashita, Y.; Bowen, W. H.; Burne, R. A.; Kuramitsu, H. D. *Infect. Immun.* **1993**, *61*, 3811–3817.
5. Critchley, P.; Wood, J. M.; Saxton, C. A.; Leach, S. A. *Caries Res.* **1967**, *1*, 112–129.
6. Hotz, P.; Guggenheim, B.; Schmid, R. *Caries Res.* **1972**, *6*, 103–121.
7. Schilling, K. M.; Bowen, W. H. *Infect. Immun.* **1992**, *60*, 284–295.
8. Dibdin, G. H.; Shellis, R. P. *J. Dent. Res.* **1988**, *67*, 890–895.
9. Bowen, W. H.; Cornick, D. E. *Helv. Odontol. Acta* **1967**, *11*, 27–31.
10. Emilson, C. G.; Nilsson, B.; Bowen, W. H. *J. Oral Pathol.* **1984**, *13*, 213–220.
11. Cury, J. A.; Rebello, M. A. B.; Del Bel Cury, A. A. *Caries Res.* **1997**, *31*, 356–360.
12. Cury, J. A.; Rebelo, M. A. B.; Del Bel Cury, A. A.; Derbyshire, M. T.; Tabchoury, C. P. *Caries Res.* **2000**, *34*, 491–497.
13. Aoki, H.; Shiroza, T.; Hayakawa, M.; Sato, S.; Kuramitsu, H. K. *Infect. Immun.* **1986**, *53*, 587–594.
14. Hanada, N.; Kuramitsu, H. K. *Infect. Immun.* **1988**, *56*, 1999–2005.
15. Hanada, N.; Kuramitsu, H. K. *Infect. Immun.* **1989**, *57*, 2079–2085.
16. McCabe, R. M.; Donkersloot, J. A. *Infect. Immun.* **1977**, *18*, 726–734.
17. Rölla, G.; Ciardi, J. E.; Eggen, K.; Bowen, W. H.; Afseth, J. Free Glucosyl- and Fructosyltransferase in Human Saliva and Adsorption of these Enzymes to Teeth In Vivo. In *Glucosyltransferases, Glucans Sucrose, and Dental Caries*; Doyle, R. J., Ciardi, J. E., Eds.; Clemical Senses IRL: Washington, DC, 1983; pp 21–30.
18. Scheie, A. A.; Eggen, K.; Rölla, G. *Scand. J. Dent. Res.* **1986**, *95*, 212–215.
19. Schilling, K. M.; Bowen, W. H. *J. Dent. Res.* **1988**, *67*, 2–8.
20. Vacca-Smith, A. M.; Bowen, W. H. *Arch. Oral Biol.* **1998**, *43*, 103–110.
21. Venkitaraman, A. R.; Vacca-Smith, A. M.; Kopec, L. K.; Bowen, W. H. *J. Dent. Res.* **1995**, *74*, 1695–1701.
22. Gold, W.; Preston, F. B.; Blechman, H. *J. Periodontol.* **1973**, *44*, 263–268.
23. Staat, R. H.; Gawronski, T. H.; Schachtele, C. F. *Infect. Immun.* **1973**, *8*, 1009–1016.
24. Guggenheim, B.; Burckhardt, J. J. *Helv. Odontol. Acta* **1974**, *18*, 101–113.
25. Walker, G. J.; Hare, M. D. *Carbohydr. Res.* **1977**, *58*, 415–432.
26. Holm-Pedersen, P.; Folke, L. E.; Gawronski, T. H. *J. Dent. Res.* **1980**, *59*, 771–776.
27. Johnson, I. H. *Microbios* **1991**, *65*, 155–167.
28. Igarashi, T.; Yamamoto, A.; Goto, N. *Oral Microbiol. Immunol.* **1998**, *13*, 382–386.
29. Fitzgerald, R. J.; Keyes, P. H.; Stoudt, T. H.; Spinell, D. M. *J. Am. Dent. Assoc.* **1968**, *76*, 301–304.
30. Block, P. L.; Dooley, C. L.; Howe, E. E. *J. Periodontol.* **1969**, *40*, 105–110.
31. Bowen, W. H. *Br. Dent. J.* **1971**, *131*, 445–449.
32. Guggenheim, B.; Regolati, B.; Schmid, R.; Muhlemann, H. R. *Caries Res.* **1980**, *14*, 128–135.
33. Simonson, L. G.; Lambert, B. L. Glucanhydrolases and the Control of Glucans. In *Glucosyltransferases, Glucans Sucrose, and Dental Caries*; Doyle, R. J, Ciardi, J. E., Eds.; Clemical Senses IRL: Washington, DC, 1983.
34. Inoue, M.; Yakushiji, T.; Mizuno, J.; Yamamoto, Y.; Tanii, S. *Clin. Prevent. Dent.* **1990**, *12*, 10–14.
35. Wunder, D.; Bowen, W. H. *Arch. Oral Biol.* **1999**, *44*, 203–214.
36. Koo, H.; Rosalen, P. L.; Cury, J. A.; Park, Y. K.; Bowen, W. H. *Antimicrob. Agents Chemother.* **2002**, *46*, 1302–1309.
37. Morrissey, J. J. *Anal. Biochem.* **1981**, *117*, 307–310.
38. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265–275.
39. Somogyi, M. *J. Biol. Chem.* **1945**, *160*, 61–73.
40. Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209–217.
41. York, W. S.; Darvill, A. G.; McNeil, M.; Stevenson, T. T.; Albersham, P. *Methods Enzymol.* **1985**, *118*, 3–40.
42. Kopec, L. K.; Vacca-Smith, A. M.; Bowen, W. H. *Glycobiology* **1997**, *7*, 929–934.
43. Kopec, L. K.; Vacca-Smith, A. M.; Wunder, D.; Ng-Evans, L.; Bowen, W. H. *Caries Res.* **2002**, *36*, 108–115.
44. Jenkinson, H. F.; Lamont, R. J. *Crit. Rev. Oral Biol. Med.* **1997**, *8*, 175–200.
45. Banas, J. A.; Vickerman, M. M. *Crit. Rev. Oral Biol. Med.* **2003**, *14*, 89–99.
46. Laemmli, U. K. *Nature* **1970**, *227*, 680–685.
47. Voller, A.; Bidwell, D. E.; Barlett, A. *The Enzyme-Linked Immunosorbent Assay (ELISA), a Guide with Abstracts of Microplate Applications*; Dynatech Laboratories: Alexandria, VA, 1979.
48. Gibbons, R. J.; Cohen, L.; Hay, D. I. *Infect. Immun.* **1986**, *52*, 555–561.
49. Vacca-Smith, A. M.; Venkitaraman, A. R.; Quivey, R. G., Jr.; Bowen, W. H. *Arch. Oral Biol.* **1996**, *41*, 291–298.
50. Stephan, R. M. *J. Am. Dent. Assoc.* **1940**, *27*, 718–723.
51. Herbort, K.; Wimalasena, R. L.; Goulbourne, E. A., Jr. *J. Dent. Res.* **1997**, *76*, 105.
52. Allen, P. Z.; Bowen, W. H. *Arch. Oral Biol.* **1990**, *35*, 55–62.
53. Monchois, V.; Willemot, R. M.; Monsan, P. *FEMS Microbiol. Rev.* **1999**, *23*, 131–151.