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REDUCTION OF THE ADHERENCE OF *Streptococcus sobrinus* INSOLUBLE α-D-GLUCAN BY ENDO-(1→3)-α-D-GLUCANASE

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ABSTRACT

Insoluble α-D-glucan, previously formed on a glass surface from sucrose by the action of cell-free D-glucosyltransferases of *Streptococcus sobrinus* OMZ176, was significantly removed by a purified preparation of endo-(1→3)-α-D-glucanase (mutanase) from a strain of *Pseudomonas* sp. Almost complete dissociation of adherent glucan occurred at the highest enzyme concentration (40 mU/mL) tested. Synthesis and *de novo* adherence on glass of the glucan was markedly inhibited by the presence of mutanase, even at low concentrations (4 mU/mL or less). When compared to native glucan, the mutanase-modified glucan samples (a) contained lower proportion of D-(1→3) linkages; (b) showed lower susceptibility to mutanase and higher susceptibility to (1→6)-α-D-glucanase (dextranase); (c) contained larger amounts of low-molecular-weight fractions; (d) had lower intrinsic viscosities; (e) showed higher *S. sobrinus* cell-agglutinating activities; and (f) consisted of looser entwinement of coalescent single-stranded fibrils (a major component) and shorter double-stranded fibrils (a minor one).

INTRODUCTION

Cariogenic streptococci (so-called *mutans* streptococci) produce extracellularly water-insoluble (IG) and water-soluble (SG) α-D-glucans from sucrose by the action of D-glucosyltransferases (GTFs, EC 2.4.1.5). IG is highly adherent, and promotes the firm adherence and accumulation of this microorganism on tooth surfaces[1]. It possesses a branched structure containing a high proportion of α-D-(1→3), as well as -(1→6) linkages[2–4].

Several preparations of (1→6)-α-D-glucanase (dextranase, EC 3.2.1.11) and (1→3)-α-D-glucanase (mutanase, EC 3.2.1.59) have been tested for their ability to inhibit the formation of dental plaque, dental caries, or periodontitis in animals and in man[5]. Dextranase does not significantly degrade the IG produced by *S.*

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sobrinus (serotypes *d* and *g mutans* streptococcus), but remarkably inhibits the synthesis of IG from sucrose by *S. sobrinus* GTFs[6-9]. Mutanase also suppresses IG synthesis and significantly hydrolyzes IG[10], and its inability to dissociate adherent IG film previously formed on a glass surface by *S. sobrinus* GTFs has been demonstrated[10,11].

A highly active, purified preparation of (1→3)-α-D-glucanase from a *Pseudomonas* strain[12] was provided recently. No mutanase possessing such a mode of action as the α-D-glucanase had yet been examined to dissociate IG deposits once adhered to solid surfaces. We report herein the chemical and morphological changes of IG induced by the α-D-glucanase, in order to elucidate the mechanism of mutanase inhibition of the sucrose-dependent adherence of *S. sobrinus* to tooth surfaces.

EXPERIMENTAL

Cell-free GTF of S. sobrinus. — Cells of strain OMZ176 (serotype *d*) were anaerobically cultured for 16 h at 37° in BHI broth (Difco Lab., Detroit, MI, U.S.A.) as described previously[4]. The culture supernatant (pH 5.5) was obtained by centrifugation, filtered through a filter paper (No. 101, Toyo-Roshi, Tokyo), made neutral with 4M NaOH, and precipitated with $(NH_4)_2SO_4$ at 50% saturation. The precipitate was collected by centrifugation, dissolved in a small volume of 5mM sodium phosphate buffer (pH 6.0), and extensively dialyzed against the same buffer. The enzyme concentrate contained 32.6 mU of GTF activity[11] per mL.

Endo-(1→3)-α-D-glucanase. — A purified preparation of endo-(1→3)-α-D-glucanase (mutanase) from a *Pseudomonas* strain[12] was kindly provided by Dr. K. Shimada (Kanonji Institute of the Osaka University, Kagawa 768, Japan). The enzyme has been shown to hydrolyze *S. sobrinus* IG endolytically, and the end products are largely D-glucose[13]. The substrate, mutan, was the insoluble products derived from sucrose by cell-free GTFs of glucose-grown *S. sobrinus* OMZ176 (ref. 11).

Enzyme activity was assayed by incubating appropriate amounts of the enzyme with the mutan (12 mg) in 0.1M acetate buffer (pH 5.7, 0.5 mL) for 10 min at 40°. Reducing sugars released were estimated by the Somogyi–Nelson method[14] using D-glucose as a standard. One unit of enzyme activity was defined as the amount that could release 1 μmol of reducing sugar per min under the defined conditions. Specific activity of the mutanase preparation used was 7 U/mg protein. The enzyme preparation contained a trace of hydrolyzing activity against laminaran and carboxymethylcellulose, but neither dextranase nor protease activity was detected.

Synthesis and adherence of α-D-glucans and degradation of adherent IGs on glass. — A mixture (3 mL) of mutanase (0–4 mU), GTF (2 μL, 0.065 mU), sucrose (50 mg) in 0.1M sodium phosphate buffer (pH 6.0, 1 mL) and water was incubated for 18 h at 37° in a polypropylene centrifugation tube. IG was centrifuged off

(25 000*g*, 15 min, 4°), and SG was precipitated from the supernatant solution by addition of ethanol (3 vol.), followed by storage for 30 min at 4°. The IG and SG fractions were washed twice by centrifugation and by 75% ethanol precipitation, respectively. Finally, IG gave an homogeneous suspension by slight sonic oscillation.

To assay the effects of mutanase on the formation of adherent IG film on glass, the reaction mixture (3 mL) was incubated for 18 h at 37° in a glass test-tube (12 × 100 mm) kept at an angle of 30°. To examine the removal of adherent IG from glass with mutanase, an IG film preformed in the absence of mutanase was gently rinsed twice with 0.1M phosphate buffer (pH 6.0, 3 mL each), and then incubated with various amounts of mutanase (0–180 mU) in the same buffer (3 mL) for 24 h at 37°. These IG films formed or remaining on glass were rinsed as described above, and then suspended uniformly by sonic oscillation.

The amounts of α-D-glucans were estimated by the anthrone method[15] using D-glucose as a standard.

Biochemical and morphological analyses of IGs. — All the following experiments were repeated or performed in duplicate or triplicate. Quantitations were done usually in triplicate for each experimental sample.

Cell-agglutinating activity. As reported previously[9], resting cells of D-glucose-grown *S. sobrinus* OMZ176 and IG of various concentrations were incubated for 18 h at 37°, and cell agglutination was macroscopically scored from − (no agglutination) to 4+ (marked agglutination).

Hydrolysis by α-D-glucanases. IG (0.3 mg) and dextranase CB (4.8 mU; Calbiochem–Behring Corp., San Diego, CA, U.S.A.) or mutanase (2.4 mU) were incubated at 37° in 0.1M acetate buffer (pH 6.0, 0.96 mL). Aliquots (180 μL) were removed at 0, 1, 2, and 3 h, and immediately heated (100°, 20 min) to stop the enzymic reaction. Reducing sugars released were estimated by the Park and Johnson method[16] with D-glucose as a standard.

Viscosity and molecular-size distribution. The intrinsic viscosity and the molecular-size distribution of IG samples in M NaOH were determined as reported previously[4].

Methylation analysis. IG samples (2–5 mg) were methylated by the method of Hakomori[17], and the alditol acetate derivatives were analyzed by g.l.c.[4].

Electron microscope observation. IGs were first precipitated by centrifugation. In some cases, particularly with mutanase-modified ones, precipitation was promoted by the coexistence of D-glucose-grown OMZ176 cells. As described previously[18], IGs with or without cells were fixed with partially reduced OsO_4, embedded in epoxy resin, and sectioned[19]. The ultra-thin sections were counterstained[20] with alkaline-chelated $BiONO_3$ and examined under TEM (JEM-200CX, JEOL Ltd, Tokyo) at 100 kV.

RESULTS

Degradation of the adherent IG film, and inhibition of the IG synthesis and adherence by mutanase. — Adherent IG, previously formed on glass surface by cell-free GTFs of *S. sobrinus* OMZ176, was significantly degraded by the treatment with mutanase (Fig. 1). The degree of the degradation increased with increasing amounts of mutanase, and almost all of the adherent IGs was removed at the highest concentration (40 mU/mL) tested.

De novo formation of adherent IG film on a glass surface by cell-free GTFs was also markedly inhibited by the presence of mutanase at very low concentrations (Fig. 2). Coincidently, the total amount of IG synthesized decreased with increasing amounts of mutanase present (Fig. 3), although the extent of the inhibition was less when compared to that of the adherent IG synthesis at given concentrations of mutanase. Production of SG increased with the increase of α-D-glucanase concentration (Fig. 3). The aforementioned results were reproducible.

Physico-chemical properties of mutanase-modified IGs. — The IG samples synthesized in the presence of mutanase at high concentrations were degraded to a greater extent by dextranase, but to a lesser extent by mutanase, than those produced at low mutanase concentrations (Table I). Methylation analysis (Table II) revealed that the content of (1→3)-linked α-D-glucosyl residues in the mutanase-modified IGs decreased as mutanase concentrations increased, while the α-D-(1→6)-linkage content increased. The IGs modified at high concentrations contained a higher proportion of nonreducing terminal residue and α-D-(1→3,6)-branching residue. Molecular size and viscosity of the mutanase-modified IGs were markedly lower than those of the original, although no remarkable difference was

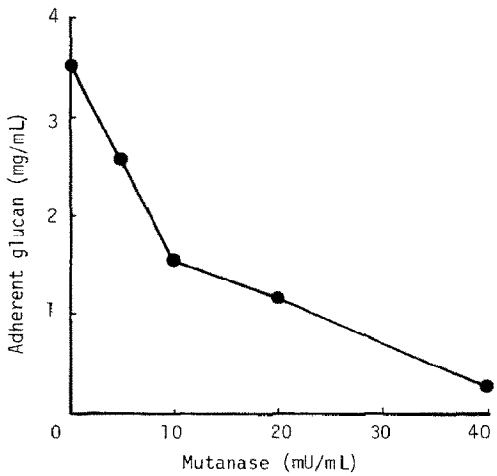

Fig. 1. Removal of the preformed IG deposits by mutanase treatment. The IG film preformed on a glass surface from sucrose by cell-free *S. sobrinus* GTFs was incubated with mutanase at the concentrations indicated in 0.1M phosphate buffer (pH 6.0, 3 mL) for 24 h at 37°.

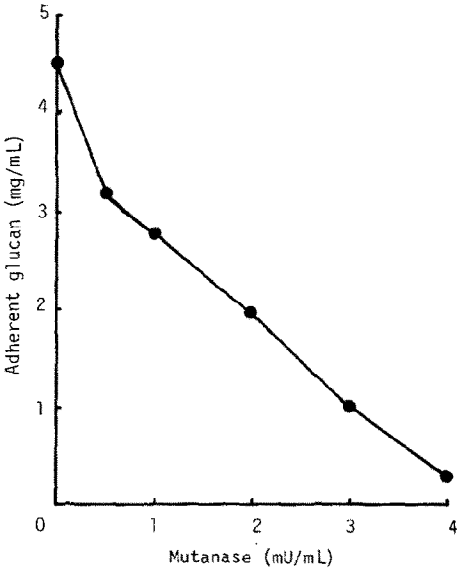

Fig. 2. Inhibition of the adherent IG deposition on glass by the presence of mutanase. Aliquot (6 μL) of cell-free GTFs was incubated with sucrose (150 mg) in the presence of mutanase at the concentrations indicated in 0.1M phosphate buffer (pH 6.0, 3 mL) for 18 h at 37°.

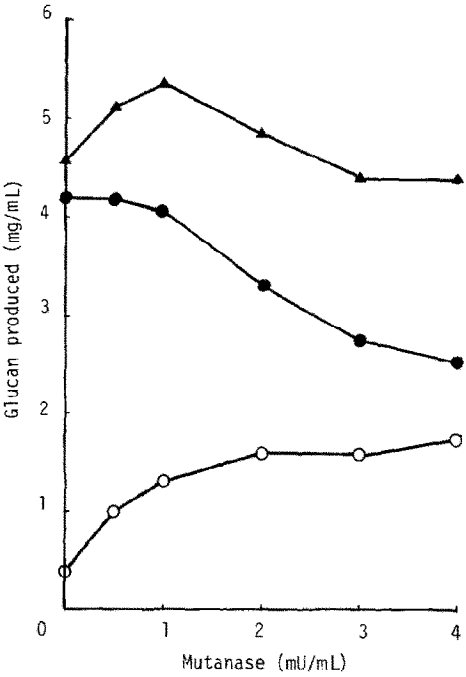

Fig. 3. Effect of mutanase on the production of α-D-glucans by cell-free GTFs. α-D-Glucans were produced under the conditions described for Fig. 2. The IG (●) and SG (○) fractions were separated by centrifugation and quantitated. The amounts of total α-D-glucans (▲) were obtained as the sum of IG and SG.

TABLE I

HYDROLYSIS OF THE MUTANASE-MODIFIED IGS BY α-D-GLUCANASES

Concentration of mutanase present during α-D-glucan synthesis (mU/mL)	*Reducing sugar released (μg as α-D-glucose/mL)*							
	Mutanase				*Dextranase*			
	30 min	*60 min*	*120 min*	*180 min*	*30 min*	*60 min*	*120 min*	*180 min*
0	13.4	21.9	24.7	32.5	2.5	3.1	5.7	8.1
0.5	2.7	8.3	13.7	17.3	3.9	5.0	11.0	16.3
2.0	2.1	6.7	8.1	11.0	6.0	14.8	16.6	18.5
4.0	1.6	3.2	3.3	3.8	12.3	18.4	30.3	31.3

observed among the mutanase-modified IGs (Table III). The ability of IGs to agglutinate *S. sobrinus* cells increased with increasing concentrations of mutanase (Table IV).

Morphological changes of mutanase-modified IGs. — Native adherent IG consisted of two fibrillar components (Fig. 4, a and b), and electron-dense, double-stranded fibril with peripheral protrusions, and an electron-lucent, single-stranded fibril. The single-strands often coalesced to form heavily electron-dense, homogeneous globular masses which were usually surrounded by layers of double strands. These components and constructions were densely intertwined to form large aggregates.

Mutanase-modified IGs (Fig. 4, c, d, and e) lost a large portion of the double-stranded fibrils, even the layered ones surrounding the globular masses, and only a few shorter fragmented ones remained. The globular masses became smaller and properly round. They appeared stellate, extending single-stranded fibrils from their periphery. The fibrillar and globular components were loosely intertwined. These morphological changes became more distinct as mutanase concentration was increased.

When native IG was digested with mutanase (40 mU/mL) for 24 h, basically

TABLE II

METHYLATION ANALYSIS OF THE MUTANASE-MODIFIED IGS

Concentration of mutanase present during α-D-glucan synthesis (mU/mL)	*O-Methyl-D-glucose*				
	2,3,4,6-Tetra-[a]	*2,4,6-Tri-*[b]	*2,3,4-Tri-*[c]	*2,4-Di-*[d]	*Ratio of 2,4,6- to 2,3,4-*
0	15.3	52.8	22.7	9.4	2.33
0.5	10.3	60.1	25.0	4.1	2.40
2.0	24.7	26.3	36.3	12.7	0.72
4.0	31.8	11.7	39.1	17.1	0.30

[a]Nonreducing terminal group. [b]α-D-(1→3)-linked residue. [c]α-D-(1→6)-linked residue. [d]Branched residue.

TABLE III

INTRINSIC VISCOSITY AND MOLECULAR SIZE DISTRIBUTION OF THE MUTANASE-MODIFIED IGS

Concentration of mutanase present during α-D-glucan synthesis (mU/mL)	*Intrinsic viscosity, η (100 cm³/g)*	*Retention rate (%)*		
		PM10	*XM100A*	*XM300*
0	1.61	98.2	93.9	89.0
0.5	0.63	73.9	68.5	60.3
2.0	0.65	78.5	70.4	69.0
4.0	0.83	81.5	77.7	70.4

TABLE IV

S. sobrinus CELL-AGGLUTINATING ACTIVITY OF THE MUTANASE-MODIFIED IGS[a]

Concentration of mutanase present during α-D-glucan synthesis (mU/mL)	*Reciprocals of dilution of IG (100 μg/mL)*									
	2^0	2^1	2^2	2^3	2^4	2^5	2^6	2^7	2^8	2^9
0	2+	2+	1+	1+	±	±	−	−	−	−
0.5	2+	2+	2+	1+	1+	±	−	−	−	−
2.0	3+	3+	2+	2+	2+	2+	1+	1+	−	−
4.0	3+	3+	4+	4+	2+	2+	1+	1+	1+	±

[a]Cell agglutination was scored as − (no agglutination) to 4+ (marked agglutination).

similar morphological changes occurred (Fig. 4, f). The digested IG consisted of two loosely intertwined fibrillar components, and double-stranded fibrils with peripheral protrusions became shorter, some of which still surrounded globular masses.

DISCUSSION

There have been many reports of the plaque-inhibitory and caries-preventive properties of mutanase preparations from various origins[5], but the inability of mutanase to degrade IG film preformed by *S. sobrinus* GTFs has been demonstrated for the *Flavobacterium* endo-(1→3)-α-D-glucanase[11]. The results shown in Fig. 1 clearly indicated that the high activity of the purified *Pseudomonas* mutanase significantly removed adherent IG film from a glass surface. The discrepancy appears due primarily to different modes of action existing between the α-D-glucanases; enzymic end products of *S. sobrinus* IGs are shown to be oligosaccharides for the *Flavobacterium* enzyme[10], but largely D-glucose for the *Pseudomonas* one[13].

The removal of preformed IG deposits (Fig. 1) occurred linearly with increasing enzyme concentrations, and a vast amount of IG film was removed at the

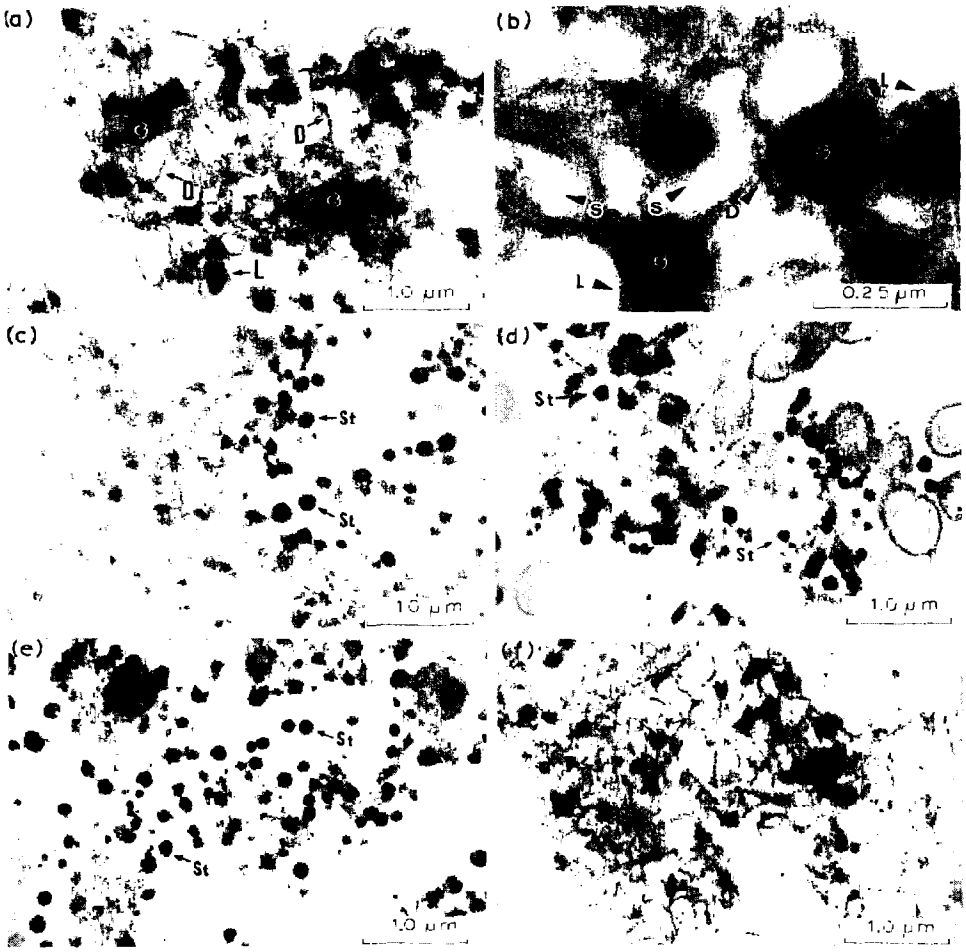

Fig. 4. IGs produced from sucrose by cell-free GTFs in the absence (a and b) or presence of mutanase [(c) 0.5 mU/mL, (d) 2.0 mU/mL, and (e) 4.0 mU/mL]; and (f) the native IG treated with mutanase (40 mU/mL). Abbreviation: (D) an electron-dense, double-stranded fibril with peripheral protrusions. (S) A moderately electron-dense, single-stranded fibril. (G) Structureless globular mass which is presumed to be a coalescence of single-stranded fibrils. (L) Layers of double-stranded fibrils. (St) Stellate appearance of the globules losing surrounding double-stranded layers. Large ovals seen in some of the photos are D-glucose-grown OMZ176 cells used to coprecipitate IGs by centrifugation.

highest concentration tested. Considering its efficiency to inhibit synthesis and adherence of IG, as well as to degrade IG deposits (Figs. 2 and 3), the *Pseudomonas* mutanase tested here preferably suppresses sucrose-induced adherence of *S. sobrinus* on tooth surfaces[21] and subsequent induction of tooth decay in animals[12,21].

We have reported that even very highly active dextranase does not remove IG film previously formed on a glass surface[9]. The difference between mutanase and dextranase in the ability to degrade preformed IG deposits can be explained by

the observation that susceptibility of native IG to these enzymes is greatly different; the degree of hydrolysis by mutanase is much higher than that by dextranase (Table I). Furthermore, hydrolysis of α-D-(1→3)- but not of α-D-(1→6)-linked chains may be essential for the ease of removal of α-D-glucan deposits. This assumption gained some support from the electron microscope observations that (a) in the mutanase-treated IGs, α-D-(1→3)-linked, double-stranded fibrils are lost or become fragmented and the constructive components are loosely intertwined (Fig. 4, e), and that (b), when treated with dextranase, native IG loses most of α-D-(1→6)-linked, single-stranded fibrils and their globular masses, but the remaining double-stranded components are still densely intertwined as in the adherent native IG[22].

The results shown in Figs. 2 and 3 confirm the previous findings that mutanase markedly suppresses production and adherence of IG from sucrose by *S. sobrinus* GTFs even at low concentrations[11]. Although the suppression of IG synthesis is not as great as that of IG adherence at all mutanase concentrations tested (Fig. 3), the mutanase-modified IGs were less viscous and of lower molecular weight than the original (Table III). These combined physico-chemical changes contribute to the marked suppression of adherent IG formation.

We have shown[11,18] that the adherent IG of *S. sobrinus* forms large aggregates of α-D-(1→3)-linked, double-stranded fibrils carrying α-D-(1→6)-linked peripheral protrusions, α-D-(1→6)-linkage-rich single-stranded fibrils, and their constructive components (Fig. 4, e). The presence of dextranase[9] or mutanase[11] (Fig. 2) inhibits synthesis of adherent IG by *S. sobrinus* GTFs, the enzymic products obtained in limited amounts contain large amounts of the low-molecular-size fractions, and the double-stranded fibrils become fragmented. These findings were confirmed and extended by the results shown here: in mutanase-modified, nonadherent IGs, the ratio of α-D-(1→3)- to α-D-(1→6)-linkages became lower (Table II); double-stranded fibrils became shorter (Fig. 4); and the amounts of low-molecular-size fractions increased (Table III). These previous and present results indicated that adherence of IG to surfaces requires the formation of high-molecular-size aggregates and the size of IG aggregates depends on the length of α-D-(1→3)-linked chains. The synthesis of fairly long α-D-(1→3)-linked, double-stranded backbone chain is critical for occurrence of the adherence and subsequent preservation of IG on surfaces.

A significant role of α-D-(1→6)-linkages in the adherence of *S. sobrinus* IGs to surfaces was also evident. Studies using the highly purified cell-free GTF isozymes from *S. sobrinus* strains[23,24] demonstrated that the adhesion of *S. sobrinus* IG cannot be evoked by formation of chains of α-(1→3)-linked D-glucose units only, but that the simultaneous formation of α-D-(1→6)-linked chain is also required. The long sequence of α-D-(1→3)-linkages is considered to contribute to water insolubility[2], and the high molecular size of IG (this study) and the presence of α-D-(1→6)-linked chain contribute to the adhesive properties[8,9]. Densely intertwined clumps of the two types of fibrils are formed in the adherent IG of *S. sobrinus* (see for example, Fig. 4, a). These findings collectively support the hypo-

thesis that *in situ* formation of densely gathered clumps of the two α-D-glucan components, which could be evoked by the simultaneous synthesis of the two glucan fibrils[22], results in the firm adherence and accumulation of *S. sobrinus* IG on solid surfaces[25].

It has been observed that glucans of *S. sobrinus* are sometimes very difficult to methylate. The molar proportion of 2,3,4,6-tetra-*O*-methyl-D-glucose to 2,4-di-*O*-methyl-D-glucose in the present IG samples seems not reasonably close to each other, even after successive methylations (Table II). Even though, the data clearly indicate that the ratio of α-D-(1→3) to α-D-(1→6)-linkages consistently decreased with increasing amount of mutanase.

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