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INTER-SEROTYPE COMPARISON OF POLYSACCHARIDES PRODUCED BY EXTRACELLULAR ENZYMES FROM *Streptococcus mutans**

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ABSTRACT

The biochemical and morphological characteristics of polysaccharides synthesized from sucrose by extracellular enzymes from D-glucose-grown *Streptococcus mutans* representing serotypes *a–g* were compared. The polysaccharides synthesized by the enzymes from serotypes *a, d*, and *g* formed visible aggregates and firmly adhered to glass surfaces, whereas those formed by the enzymes from serotypes *b, c, e,* and *f* floated homogeneously and were poorly adherent. The enzymes of serotypes *a, d,* and *g* produced large amounts of water-insoluble polysaccharides (IPs, D-glucans), and those of serotypes *b, c, e,* and *f* water-soluble polysaccharides (SPs, D-glucans and D-fructans). As compared with the IPs of serotypes *b, c, e,* and *f*, the IPs of serotypes *a, d,* and *g* (a) contained a higher proportion of (1→3)-α-D-glucosidic linkages and α-D-(1→3,6) branch linkages; (b) showed higher susceptibility to (1→3)-α-D-glucanase (serotype *a* excepted) and lower (1→6)-α-D-glucanase sensitivity; (c) contained larger amounts of high-molecular-weight fractions; (d) showed higher intrinsic viscosities (serotype *b* excepted); and (e) had lower *S. mutans* cell-agglutination activities. On electron-microscope observation, the IPs of all serotypes showed two fibrillar components; a double-stranded fibril, with short, fluffy protrusions extending out of its periphery, and a fine, single-stranded fibril. Thus, the serotypes could be divided into two major groups: *a, d,* and *g*; and *b, c, e,* and *f*. No similar grouping of serotypes was indicated by the chemical and morphological properties of SPs.

INTRODUCTION

Streptococcus mutans is now considered to play the most important role in the etiology of dental caries. On the basis of cell-wall antigens, strains of the strep-

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tococcus are divided[1,2] into seven serotypes, *a–g*. An additional serotype (*h*) was found recently[3]. Serotypes may be grouped into several categories on the basis of serologic, genetic, biochemical, and phenotypic characteristics[4,5].

The streptococcus produces extracellular, water-insoluble (IPs) and water-soluble polysaccharides (SPs) from sucrose by the action of its constitutive enzymes, D-glucosyltransferase (EC 2.4.1.5) and D-fructosyltransferase (EC 2.4.1.10). Water-insoluble α-D-glucan (IG) is considered to be essential for the adherence of *S. mutans* cells to tooth surfaces, and water-soluble α-D-glucan (SG) and β-D-fructans may act as extracellular storage polysaccharides for this microorganism[4,6,7]. These polysaccharides have been studied[7,8] chemically, biologically, and morphologically. However, there have been few systematic comparisons of the polysaccharides of the *S. mutans* serotypes[9–11], the production from sucrose, by *S. mutans* serotypes *a–g*, of the extracellular polysaccharides by the cell-free enzymes produced by D-glucose-grown cells, and the biochemical and morphological characteristics.

EXPERIMENTAL

Bacterial strains. — Nineteen strains of *S. mutans* (serotypes *a–g*), listed in Table I, were selected from our stock-culture collection. Strains were routinely stored by adding sterile glycerol (5 mL) to an overnight culture grown in D-glucose broth (5 mL), and freezing these cell suspensions at −10 – −15°.

TABLE I

CELL-FREE ENZYME PREPARATIONS

Serotype	*Strain*	*Polysaccharide-synthesizing activity (mU/mL)*	*Protein (mU/mL)*	*Specific activity (mU/mg)*
a	HS1	13.0	1.70	7.6
	FIL	27.4	1.77	15.5
	HS6	54.9	1.90	28.9
d	OMZ176	32.6	2.77	11.8
	P1	32.8	1.38	23.8
g	AHT-k	84.1	1.26	66.7
	6715	29.2	3.72	8.0
	K1-R	40.4	1.59	25.4
	OMZ65	56.2	1.71	32.9
b	BHT	45.8	1.75	26.2
	FA1	64.7	1.77	36.6
c	OMZ70	18.1	1.95	9.3
	Ingbritt	92.0	1.23	74.8
	GS5	14.5	2.07	7.0
	PK1	48.3	1.62	29.8
e	B14	56.2	1.78	31.6
	P4	49.1	1.95	25.2
f	OMZ175	40.2	1.59	25.3
	MT3940	53.0	1.92	27.6

Preparation of cell-free enzymes. — The organisms were grown in Brain Heart Infusion broth (BBL Microbiology Systems, Cockeysville, MD 21030) for 18 h at 37°. Culture liquor was separated by centrifugation and filtered through a No. 101 filter paper (Toyo–Roshi, Tokyo). The filtrate was made neutral with 4M sodium hydroxide and then precipitated with ammonium sulfate at a saturation of 50% (w/v). The precipitate was collected by centrifugation, dissolved in a small volume (1/40 of the original) of 5mM sodium phosphate buffer (pH 6.0), and exhaustively dialyzed against the buffer.

Polysaccharide (α-D-glucan and β-D-fructan)-synthesizing activity of the enzyme preparations was assayed by allowing adequate amounts of the enzymes to react with [U-^{14}C]sucrose (0.05 μCi, 4.7 Ci/mol; New England Nuclear, Boston, MA 02118) in 0.1M phosphate buffer (pH 6.0, 20 μL) for 60 min at 37°. Radioactivity incorporated into polysaccharides was measured by the filter paper method described previously[12]. One unit of enzyme activity was defined as the amount of enzyme that transformed 1 μmol of sucrose into polysaccharide per min under the conditions described. Protein content was determined by the method of Lowry *et al.*[13]. The enzyme activities and protein contents of the enzyme preparations used are summarized in Table I.

Adherence of polysaccharides produced by cell-free enzymes. — The reaction mixture consisted of cell-free enzymes (1.2–30 mU) and sucrose (150 mg) in 0.1M phosphate buffer (pH 6.0, 3 mL) containing 0.02% of merthiolate. The mixture was incubated at 37° in a glass test-tube (12 × 105 mm) kept at an angle of 30°. The polysaccharides adherent to the glass surface after a 16-h incubation were washed by hand with three changes of 50mM phosphate buffer (pH 6.0, 3 mL) and dissolved in M sodium hydroxide (3 mL). The amount of polysaccharides was estimated by the anthrone method[14] with D-glucose as a standard.

Synthesis and fractionation of polysaccharides. — Sucrose (200 mg) and cell-free enzyme (8 mU) in 0.1M phosphate buffer (pH 6.0, 4 mL) containing 0.02% of merthiolate were incubated for 16 h at 37°. The mixture was briefly mixed by sonication, and the turbidity was measured at 420 nm with a Spectronic 20 photometer (Shimadzu Works, Kyoto). The mixture was centrifuged (20 000*g*, 15 min, 4°) to harvest the water-insoluble products (IPs). The water-soluble products (SPs) were precipitated from the supernatant solution by addition of ethanol (2.5 vol.), followed by storage for 2 h in a freezer. The IP obtained was washed three times with 50mM phosphate buffer (pH 6.0) by centrifugation, and the SP was washed with 70% ethanol. Washed polysaccharides were exhaustively dialyzed against distilled water. Differential quantitative determination of α-D-glucan and β-D-fructan in the fractions was performed by the anthrone method, as described by Halhoul and Kleinberg[15], with D-glucose and D-fructose as standards.

Methylation analysis. — Polysaccharides (5 mg) were methylated by the method of Hakomori[16]. Some polysaccharides were methylated first by the procedure of Haworth[17], followed by the Hakomori method, as described by Lindberg[18]. The methylated polysaccharide was treated with 90% (v/v) formic acid for 6 h at

100°, and then hydrolyzed in 2M trifluoroacetic acid for 6 h at 100° under nitrogen. Alditol acetate derivatives of partially methylated hexoses were analyzed by g.l.c. with an instrument model GC-4CPF (Shimadzu Works) as described by Hisamatsu *et al.*[19].

Hydrolysis of polysaccharides by D-*glucanases.* — Endo-(1→6)-α-D-glucanase (dextranase, EC 3.2.1.11) was obtained from Calbiochem (San Diego, CA 92103). Endo-(1→3)-α-D-glucanase (EC 3.2.1.59) was purified from the culture supernatant of *Streptomyces chartreuses* F2 as described by Takehara *et al.*[20]. One unit of glucanase activity is the amount that releases 1 μmol of reducing sugar, as D-glucose, per min under the conditions described previously[20,21]. The susceptibility of polysaccharides to the action of the α-D-glucanases was determined by incubating the polysaccharide sample (0.2 mg) with (1→6)-α-D-glucanase (1.48 mU) or (1→3)-α-D-glucanase (1.57 mU) in 0.1M acetate buffer (pH 6.0, 1.2 mL) containing 5mM sodium fluoride as a preservative. After incubation for 0–3 h at 37°, the reducing sugars released were estimated by the Park and Johnson method[22] with D-glucose as a standard.

Viscosity and molecular-size distribution. — The intrinsic viscosity of a solution of the polysaccharide in M sodium hydroxide was determined with an Ostwald viscometer, and the molecular-size distribution estimated by ultrafiltration[21] on Diaflo membranes, XM100A and XM300 (Amicon Corp., Lexington, MA 02173).

Cell-agglutinating activity. — The ability of the polysaccharide fractions to agglutinate *S. mutans* cells was determined as described previously[21]. Resting cells were prepared from a D-glucose-grown culture of *S. mutans* AHT-k (serotype *g*).

Electron-microscope procedures. — An IP suspension in distilled water (0.5 mg/mL) was centrifuged at 1700*g* for 10 min to give a pellet. The pellets of SP were obtained by incubating the SP sample (0.1 mg) for 18 h at 37° with resting *S. mutans* AHT-k cells, and then harvesting cells and SP by centrifugation. The pellet was fixed in a solution of 1% osmium tetraoxide–1.5% potassium ferrocyanide[23]. The fixed samples were dehydrated, embedded in Epok 812 (Nagase Industry Ltd, Tokyo), sectioned[24], stained with a 0.04% alkaline bismuth reagent[25], and examined under a transmission-electron microscope (model JEM-100B, JEOL Ltd, Tokyo) at 80 kV.

RESULTS

Synthesis and adherence of polysaccharides by cell-free enzyme. — Polysaccharide synthesized by extracellular enzymes from the serotypes *a*, *d*, and *g* (except strain HS-6) formed large aggregates that tended to precipitate, whereas those of serotypes *b*, *c*, *e*, and *f* (except strain PK1) were homogeneously dispersed (Table II). Typical macroscopic appearances of the polysaccharides produced by representative strains of serotype *a*–*g* are shown in Fig. 1. Sonicated polysaccharides suspensions of serotypes *a*, *d*, and *g* gave higher turbidity values than did those of serotypes *b*, *c*, *e*, and *f* (Table II).

TABLE II

TURBIDITY AND AGGREGATION OF POLYSACCHARIDES PRODUCED BY A CELL-FREE ENZYME

Serotype	*Strain*	*Turbidity*[a] (A_{420mn})	*Aggregation*[b]
a	HS1	0.75	+
	FIL	0.65	+
	HS6	0.33	−
d	OMZ176	0.70	+
	P1	0.65	+
g	AHT-k	0.82	+
	6715	0.65	+
	K1-R	0.80	+
	OMZ65	0.70	+
b	BHT	0.10	−
	FA1	0.20	−
c	OMZ70	0.30	−
	Ingbritt	0.16	−
	GS5	0.16	−
	PK1	0.75	+
e	B14	0.40	−
	P4	0.38	−
f	OMZ175	0.40	−
	MT3940	0.15	−

[a]The cell-free enzyme (8 mU) was allowed to react with sucrose (200 mg) for 16 h at 37° in 0.1M phosphate buffer, pH 6.0 (4 mL). [b](+) Aggregate formation; and (−) no aggregate formation. Refer to Fig. 1.

Polysaccharides synthesized *de novo* by the enzymes from serotypes *a, d,* and *g* preponderantly adhered to glass surfaces, whereas those synthesized by the enzymes from serotypes *b, c, e,* and *f* were only slightly adherent (Table III).

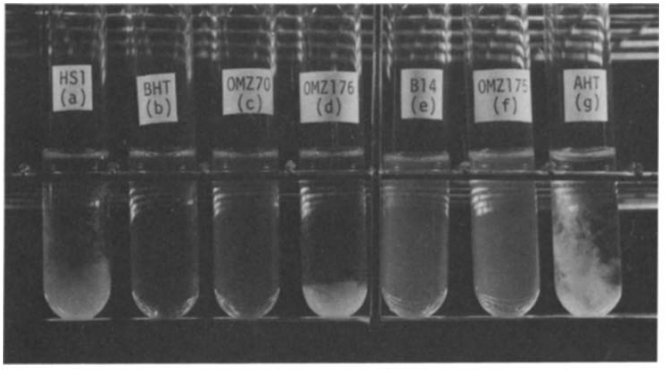

Fig. 1. Macroscopic appearance of polysaccharides produced by cell-free enzyme from representative serotype *a–g S. mutans* strains. The polysaccharides were synthesized by incubating cell-free enzymes (8 mU) and sucrose (200 mg) in 0.1M phosphate buffer (pH 6.0, 4 mL) containing 0.02% of merthiolate for 16 h at 37°.

TABLE III

ADHERENCE[a] TO GLASS OF POLYSACCHARIDES PRODUCED BY VARIOUS CONCENTRATIONS OF CELL-FREE ENZYMES

Serotype	*Strain*	*Enzyme concentration (mU/mL)*		
		0.4	*2.0*	*10.0*
a	HS1	1.80	4.86	5.00
d	OMZ176	2.14	5.11	3.58
g	AHT-k	1.84	5.97	8.18
b	BHT	0.01	0.02	0.05
c	OMZ70	0.01	0.01	0.04
e	B14	0.01	0.04	0.12
f	OMZ175	0.01	0.02	0.07

[a]In mg/mL.

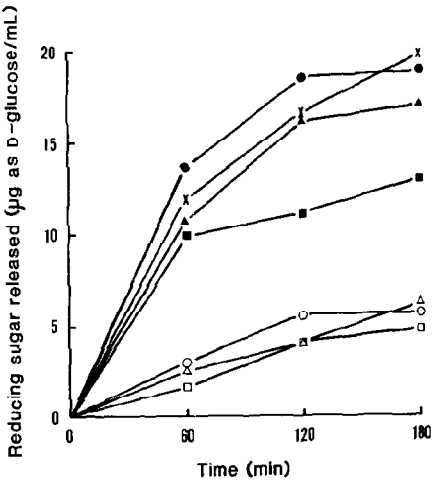

Fig. 2. Hydrolysis of water-insoluble polysaccharide (IP) by (1→6)-α-D-glucanase: IP (10 μg) was treated with (1→6)-α-D-glucanase (0.07 mU) at 37° in 0.1M acetate buffer (pH 6.0, 60 μL): ○, HS1(*a*); ●, BHT(*b*); ▲, OMZ70(*c*); △, OMZ176(*d*); ■, B14(*e*); ×, OMZ175(*f*); and □, AHT-k(*g*).

Polysaccharides synthesized by the enzymes from serotypes *a*, *d*, and *g* (except strain HS-6) contained higher proportions of IP, but the bulk of the polysaccharides produced by the enzymes from serotypes *b*, *c*, *e*, and *f* (except strain PK1) was SP (Table IV). The IPs of most strains tested consisted of only α-D-glucan (IG), whereas SPs comprised both α-D-glucan (a major component) and β-D-fructan. The ratio of β-D-fructan to α-D-glucan in the total polysaccharides (IPs + SPs) of serotypes *b*, *c*, *e*, and *f* appeared to be higher, in general, than that in the polysaccharides of serotypes *a*, *d*, and *g*.

Properties of polysaccharides. — The IPs of serotypes *b*, *c*, *e*, and *f* strains

TABLE IV

AMOUNTS OF WATER-INSOLUBLE AND -SOLUBLE POLYSACCHARIDES PRODUCED BY CELL-FREE ENZYME

Serotype	*Strain*	*Water-insoluble polysaccharide*			*Water-soluble polysaccharide*			*Ratio of insoluble to soluble polysaccharide*	*Ratio of total D-fructan to total D-glucan*
		D-Glucan (mg/mL)	*D-Fructan (mg/mL)*	*Ratio of D-fructan to D-glucan*	*D-Glucan (mg/mL)*	*D-Fructan (mg/mL)*	*Ratio of D-fructan to D-glucan*		
a	HS1	2.24	0	0	0.38	0.08	0.21	4.87	0.03
	FIL	1.60	0	0	0.46	0.11	0.24	2.81	0.05
	HS6	0.71	0	0	1.26	0.14	0.11	0.51	0.07
d	OMZ176	0.93	0	0	0.67	0.12	0.18	1.18	0.08
	P1	1.33	0	0	0.48	0.10	0.21	2.29	0.06
g	AHT-k	1.46	0	0	0.32	0.10	0.31	3.48	0.06
	6715	0.53	0	0	0.36	0.10	0.28	1.15	0.11
	K1-R	0.88	0	0	0.64	0.14	0.22	1.13	0.09
	OMZ65	1.08	0	0	0.61	0.11	0.18	1.50	0.07
b	BHT	0.09	0	0	0.23	0.28	1.22	0.18	0.88
	FA1	0.10	0	0	1.19	0.25	0.21	0.07	0.19
c	OMZ70	0.35	0	0	0.75	0.27	0.36	0.34	0.25
	Ingbritt	0.13	0	0	0.95	0.23	0.24	0.11	0.21
	GS5	0.01	0.09	9.0	2.01	0.73	0.36	0.04	0.41
	PK1	2.38	0	0	0.76	0.12	0.16	2.70	0.04
e	B14	0.82	0	0	3.96	0.31	0.08	0.19	0.06
	P4	0.33	0.06	0.18	3.72	0.54	0.15	0.09	0.15
f	OMZ175	0.77	0.07	0.09	0.89	0.39	0.44	0.66	0.28
	MT3940	0.13	0	0	1.54	0.28	0.18	0.07	0.17

TABLE V

METHYLATION ANALYSIS OF WATER-INSOLUBLE AND -SOLUBLE GLUCANS PRODUCED BY CELL-FREE ENZYME

Serotype	*Strain*	O-*Methyl*-D-*glucose*									
		Water-insoluble* D-*glucan					***Water-soluble* D-*glucan***				
		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-*	*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-*
a	HS1	22.8	41.0	14.5	21.7	2.83	25.9[e]	1.0[e]	51.0[e]	22.1[e]	0.02
	FIL	30.4[e]	25.7[e]	23.9[e]	20.0[e]	1.06	22.1[e]	2.3[e]	60.2[e]	15.4[e]	0.04
d	OMZ176	11.6	61.2	15.2	12.0	4.03	13.4	4.1	75.6	6.9	0.05
	P1	20.0	48.0	16.1	15.9	2.98	18.7	17.4	48.2	15.7	0.36
g	AHT-k	15.7	54.6	15.4	14.3	3.55	18.7[e]	8.7[e]	57.7[e]	14.9[e]	0.15
	6715	11.2	66.2	14.6	8.0	4.53	32.9	14.6	34.1	18.4	0.43
b	BHT	9.9	41.4	39.6	9.1	1.05	17.6	9.7	57.7	15.0	0.17
	FA1	7.8	55.8	34.3	2.1	1.63	20.5[e]	0.9[e]	62.6[e]	16.0[e]	0.01
c	OMZ70	7.7	56.5	31.0	4.8	1.82	20.2[e]	3.4[e]	63.2[e]	13.2[e]	0.05
	Ingbritt	12.0	45.9	36.2	5.9	1.27	19.6	5.6	58.3	16.5	0.10
e	B14	10.5	27.2	47.0	15.3	0.58	21.6[e]	4.8[e]	57.4[e]	16.2[e]	0.08
	P4	22.2[e]	25.8[e]	41.2[e]	10.8[e]	0.63	17.1	0.7	54.2	18.0	0.20
f	OMZ175	14.4	35.2	41.2	9.2	0.85	18.5[e]	16.6[e]	52.6[e]	12.3[e]	0.32
	MT3940	7.2	60.4	26.0	6.4	2.32	22.6	18.6	48.9	9.9	0.38

[a]Nonreducing terminal group. [b]α-D-(1→3)-linked residue. [c]α-D-(1→6)-linked residue. [d]Branched residue. [e]Methylation was performed by the procedure of Haworth[17], followed by the method of Hakomori[16].

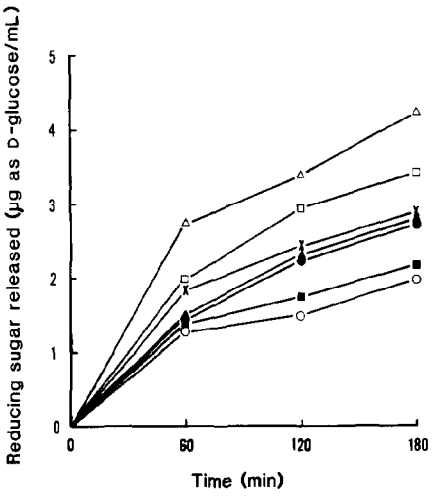

Fig. 3. Hydrolysis of water-insoluble polysaccharide (IP) by (1→3)-α-D-glucanase: IP (20 μg) was treated with (1→3)-α-D-glucanase (0.16 mU) at 37° in 0.1M acetate buffer (pH 6.0, 120 μL); for symbols, see legend to Fig. 2.

TABLE VI

INTRINSIC VISCOSITY AND MOLECULAR-SIZE DISTRIBUTION OF WATER-INSOLUBLE AND -SOLUBLE POLYSACCHARIDES

Serotype	*Strain*	*Insoluble polysaccharide*			*Soluble polysaccharide*		
		Intrinsic viscosity (100 cm/g)	*Retention rate (%)*		*Intrinsic viscosity (100 cm/g)*	*Retention rate (%)*	
			XM100A	*XM300*		*XM100A*	*XM300*
a	HS1	1.93	93.6	77.6	0.70	53.9	22.2
d	OMZ176	2.12	92.0	85.7	0.08	11.8	3.6
g	AHT-k	3.88	98.0	96.2	1.10	37.3	30.5
b	BHT	2.33	97.0	82.1	0.54	84.6	66.5
c	OMZ70	1.42	89.3	67.6	0.27	29.2	22.6
e	B14	1.38	77.4	60.2	0.50	49.3	34.7
f	OMZ175	1.42	86.3	49.4	0.85	76.8	49.9

was easily dispersed by agitation, and was more susceptible to hydrolysis by endo-(1→6)-α-D-glucanase than those of serotypes *a*, *d*, and *g* (Fig. 2). Generally, a higher content of (1→6)-linked α-D-glucosyl residues was observed for the IG of serotypes *b*, *c*, *e*, and *f*, as compared to the D-glucans of serotypes *a*, *d*, and *g* (Table V). Although the α-D-(1→3) linkage content of IG differed considerably among the strains, the ratio of α-D-(1→3) to α-D-(1→6) linkages in IGs was clearly higher for serotypes *a*, *d*, and *g* than for serotypes *b*, *c*, *e*, and *f* (Table V). The IP of serotype *a* contained the highest proportion of α-D-(1→3,6) branch linkages. Among the other serotype, the degree of branching of IG seemed to be higher for serotype *d*, *g*, and *e* than for type *b*, *c*, and *f*. The IPs of serotype *d* and *g* appeared

to be more readily degraded by (1→3)-α-D-glucanase, as compared to other serotypes, whereas the IP of type *a* showed the least susceptibility to (1→3)-α-D-glucanase (Fig. 3).

Most D-glucose residues of SPs were α-D-(1→6)-linked (Table V). The content of α-D-(1→6) and α-D-(1→3) linkages differed greatly among the strains tested, but the degree of branching was ~15% for most strains. The extent of hydrolysis of SPs by (1→6)-α-D-glucanase, however, was lowest for serotypes *d* and *g*, and highest for serotype *a* (data not shown). The SP of all the strains tested was little hydrolyzed by (1→3)-α-D-glucanase (data not shown).

The intrinsic viscosities of the IPs of serotypes *a*, *b*, *d*, and *g* were higher than those of the IPs of serotypes *c*, *e*, and *f* (Table VI). This grouping of serotypes was also demonstrated for molecular-size distribution of the polysaccharides. IPs of serotypes *a*, *b*, *d*, and *g* contained a larger proportion of higher-molecular-weight fractions than IPs of serotypes *c*, *e*, and *f* (Table VI).

The intrinsic viscosity and the retention rate on Amicon membranes of SPs were lower than those of IPs (Table VI). Among the strains tested, the SP of serotype *g* possessed the highest intrinsic viscosity, and the SP of serotype *b* had the highest molecular size, whereas the SP of serotype *d* possessed the lowest viscosity and size.

The ability of SPs to agglutinate *S. mutans* cells was distinctly higher for serotypes *b*, *c*, *e*, and *f*, as compared to serotypes *a*, *d*, and *g* (Table VII). The IP of serotype *a* as well as those of types *b*, *c*, *e*, and *f*, however, possessed higher agglutinating abilities than those of types *d* and *g*.

Morphology of polysaccharides. — The IPs of all serotypes consisted of two fibrillar components. One component was a heavily electron-dense, double-stranded fibril possessing short fluffy protrusions. The other component was a less electron-dense, single-stranded fibril. The double-stranded fibrils of the IPs of

TABLE VII

S. mutans CELL-AGGLUTINATING ACTIVITY OF WATER-INSOLUBLE (IPs) AND -SOLUBLE (SPs) POLYSACCHARIDES

Serotype	*Strain*	*Agglutination titer*[a]	
		Insoluble	*Soluble*
a	HS1	1024	64
d	OMZ176	64	32
g	AHT-k	128	128
b	BHT	1024	1024
c	OMZ70	1024	1024
e	B14	1024	1024
f	OMZ175	1024	1024

[a]Reciprocal of the highest dilution of IP suspension or SP solution (0.5 mg/mL) to give an apparent agglutination of *S. mutans* AHT-k (*g*) cells.

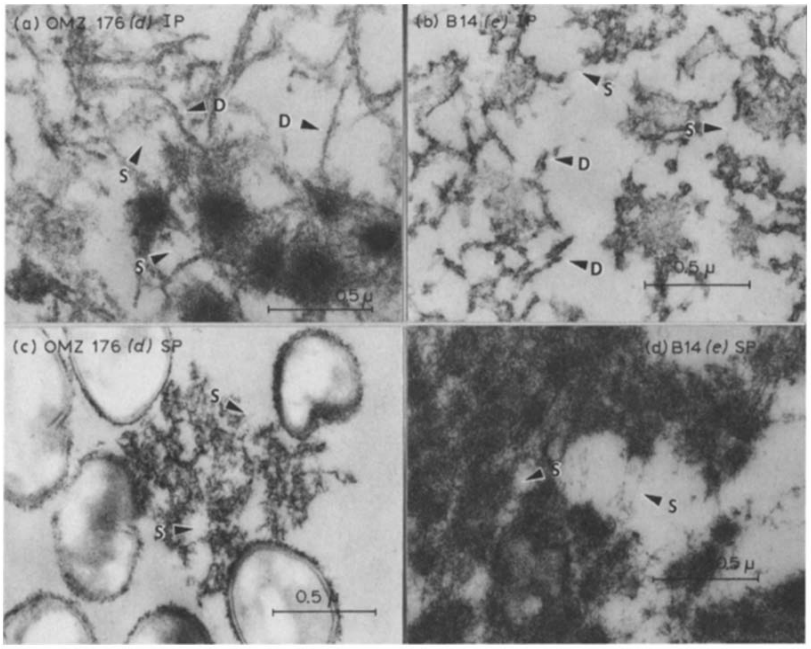

Fig. 4. Electron micrograph of insoluble (IPs) and soluble (SPs) polysaccharides produced by cell-free enzymes from representative *S. mutans* strains: IP(a) and SP(c) of strain OMZ176(*d*), and IP(b) and SP(d) of strain B14(*e*). Abbreviations: (D) a double-stranded fibril; (S) a single-stranded fibril. Bars indicate 0.5 μm. Empty shells of cocci appearing in one of the photos are *S. mutans* AHT-k cells used to coprecipitate the SP by centrifugation (see the Experimental section). Similarity and dissimilarity of the morphological features of IPs or SPs among the serotypes *a–g* are described in the text.

serotypes *d* and *g* were long and coalesced with the single-stranded fibrils to form vast networks, frequently containing very densely-aggregated, globular masses (Fig. 4,a). The IP of serotype *a* was morphologically similar to those of serotypes *d* and *g*, but seemed to contain slightly shorter double-stranded fibrils and fewer globular masses. Globular masses were absent in the IPs of serotypes *c*, *e*, and *f*; the double-stranded fibrils of this IP were markedly shorter than those of serotypes *a*, *d*, and *g*, and formed small, isolated aggregates with the single-stranded fibrils (Fig. 4,b). The morphology of the IP of serotype *b* was basically similar to that of the IPs of serotypes *c*, *e*, and *f*, but the small aggregated masses formed by two fibrillar components occurred occasionally.

The primary component of the SPs of all serotypes resembled the fine, single-stranded fibril observed in the IP. The bulk of the SP fibrils of serotypes *a* and *g* comprised electron-dense, thick, globular clumps assuming a stellate appearance. The fibrils of the SP of serotype *d* mainly formed loose meshworks (Fig. 4,c). The fibrils of serotypes *e* and *f* SP were densely gathered to form thick meshworks showing an indistinct globular appearance (Fig. 4,d). The SPs of serotypes *b* and *c* consisted of small, heavily electron-dense, amorphous particles, the thick clumps of single-stranded fibrils being absent.

DISCUSSION

Trautner, Birkhed, and Svensson[11] reported that the extracellular, water-soluble α-D-glucans (WS-II) and the alkali-soluble, cell-associated α-D-glucans (AS-I) produced in sucrose cultures of serotypes *b*, *c*, or *e* were easily distinguished from the same α-D-glucan fractions produced by sucrose cultures of serotypes *a* or *d*. The α-D-glucan fractions of serotypes *b*, *c*, and *e*, for instance, had larger proportions of α-D-(1→6) linkages and significantly lower proportions of α-D-(1→3,6)-linked branching sites than the α-D-glucan fractions of serotypes *a* and *d*. The electrophoretic patterns[26] and immunologic similarities[27–29] of the extracellular D-glucosyltransferases suggested that *S. mutans* serotypes could be separated into two groups: *a*, *d*, and *g*; and *c*, *e*, and *f*.

The results presented here indicate that, on the basis of the morphological and biochemical properties of the polysaccharides produced by cell-free enzymes, *S. mutans* serotypes form two groups: *a*, *d*, and *g*; and *b*, *c*, *e*, and *f*. Such a grouping of serotypes is supported by the marked differences of the proportions of IPs (IGs) and SPs synthesized (Table IV), the macroscopic appearance of the polysaccharides (Table II and Fig. 1), the proportions of α-D-(1→3)- and α-D-(1→6)-glucosidic linkages in IGs (Table V), susceptibility of IGs to (1→6)-α-D-glucanase (Fig. 2), the cell-agglutinating ability of IG (Table VII), and the ultrastructural characteristics of IGs (Fig. 4). There were, however, marked differences between the IGs within each of these two groups of serotypes. The susceptibilities of IG to (1→3)-α-D-glucanase (Fig. 3) and the proportions of α-D-(1→3,6)-linked branching sites in IG (Table V) varied in the subgroup comprising serotypes *a*, *d*, and *g*, whereas clear differences were observed in the cell-agglutinating abilities (Table VII) and the intrinsic viscosities and molecular sizes of IGs (Table VI) within the subgroup comprising serotypes *b*, *c*, *e*, and *f*. *S. mutans* serotypes, therefore, may comprise four groups: serotype *a*; serotypes *d* and *g*; serotype *b*; and serotypes *c*, *e*, and *f*. Thus, the results reported herein support and extend the grouping of serotypes already suggested by comparisons of other properties of these organisms[4,5].

A clear distinction existed between the IP of serotype *a* and those of serotypes *d* and *g*. The high resistance to (1→3)-α-D-glucanase of the IP of serotype *a* (Fig. 3) may be due to the higher proportion of α-D-(1→3,6)-branch linkages occurring in this IP, as compared to the IPs of serotypes *d* and *g* (Table V). The higher cell-agglutinating capacity of the IP of serotype *a* (Table VII) may result from the combined effect of the higher proportions of branching and (1→6)-α-D linkages found in this IP (Table V), and the propensity of this polysaccharide to form somewhat smaller aggregates, as suggested by differences in intrinsic viscosity and molecular-size distribution (Table VI), and in morphology. Our previous studies indicated that only the α-D-glucan present in the peripheral regions of α-D-glucan aggregates was available to induce agglutination[30,31].

No clear grouping of serotypes was indicated by the SPs on the basis of the biochemical and morphological characteristics. However, it was shown that the SPs

of serotypes *a, d,* and *g* did not differ markedly in their ability to agglutinate *S. mutans* cells (Table VII), and that the SPs of serotypes *d* and *g* were far more resistant to (1→6)-α-D-glucanase (not shown). Trautner *et al.*[11] have shown certain similarities between the chemical structures of SPs, as well as of IPs (IGs), for serotypes *a* and *d*, and also for serotypes *b, c,* and *e*. Their findings, however, are not in agreement with our methylation results (Table V). Ellwood *et al.*[9] also have suggested variations in the α-D-glucosidic linkage content of SPs among serotypes *a–e*. These inconsistent results may be ascribed to the complex composition of SPs. The SPs of all *S. mutans* serotypes consists of both α-D-glucan and β-D-fructan (Table IV), and the soluble α-D-glucan (SG) fraction of several *S. mutans* strains consists of three subfractions having distinctly different molecular sizes and chemical structures[32]. The difference between results may also be ascribed to the different conditions of synthesis and fractionation of the polysaccharides[11].

The cause for the higher susceptibility of the SP of serotype *a* to (1→6)-α-D-glucanase (not shown) is unknown at present, because no great differences were found, among the serotypes, in either the relative amount of soluble α-D-glucan and β-D-fructan produced (Table IV) or the proportions of α-D-glucosidic linkages of SP (Table V).

We have reported previously the ultrastructural characteristics of the IG and SG produced by *S. mutans* AHT-k (serotype *g*)[23,30,31]; the IG formed coalescent clumps of two fibrillar components, *i.e.*, a single-stranded fibril and a double-stranded fibril with short fluffy protrusions from its sides, whereas the SG almost exclusively formed a single-stranded fibril which was morphologically similar to that observed in the IG. The present study indicates that this is also the case for the SPs and IPs (mostly α-D-glucans) of all the serotypes *a–g* (Fig. 4), although some characteristics of the polysaccharides (particularly IPs) are distinctly different between the serotypes *a, d,* and *g*; and *b, c, e,* and *f* (Fig. 4).

The formation of water-insoluble α-D-glucan films on glass surfaces by the cell-free D-glucosyltransferases of *S. mutans* has been used as an *in vitro* model of the sucrose-dependent adherence of *S. mutans* cells to smooth tooth-surfaces[33]. The IPs (IGs) of serotypes *a, d,* and *g* adhered firmly to glass in large amounts, but the D-glucans of serotypes *b, c, e,* and *f* adhered little (Table III). This difference may be attributed to the amount of IG produced (Table IV), the proportions of α-D-(1→3)- and α-D-(1→6)-glucosidic linkages, the levels of α-D-(1→3,6)-branch linkages (Table V), and the intrinsic viscosity and molecular size (Table VI) of the IGs. All of these properties were higher for serotypes *a, d,* and *g* than for serotypes *b, c, e,* and *f*. The two fibrillar components of IG also formed large, more dense clumps for serotypes *a, d,* and *g* than for serotypes *b, c, e,* and *f* (Fig. 4). The differences in IPs between the two groups of serotypes probably contributed to the characteristic macroscopic appearances of the total polysaccharides produced by cell-free enzymes (Fig. 1).

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