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Isolation and Purification of *Flavobacterium* α-1,3-Glucanase-Hydrolyzing, Insoluble, Sticky Glucan of *Streptococcus mutans*

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Studies were made on the physical and chemical properties of polysaccharides synthesized by cell-free extracts of *Streptococcus mutans*, *Streptococcus sanguis*, and *Streptococcus* sp. and their susceptibilities to dextranases. Among the polysaccharides examined, insoluble glucans were rather resistant to available dextranase preparations, and the insoluble, sticky glucan produced by *S. mutans* OMZ 176, which could be important in formation of dental plaques, was the most resistant. By enrichment culture of soil specimens, using OMZ 176 glucans as the sole carbon source, an organism was isolated that produced colonies surrounded by a clear lytic zone on opaque agar plates containing the OMZ 176 glucan. The organism was identified as a strain of *Flavobacterium* and named the Ek-14 bacterium. EK-14 bacterium was grown in Trypticase soy broth, and an enzyme capable of hydrolyzing the OMZ 176 glucan was concentrated from the culture supernatant and purified by negative adsorption on a diethylaminoethyl-cellulose (DE-32) column and gradient elution chromatography with a carboxymethyl-cellulose (CM-32) column. The enzyme was a basic protein with an isoelectric point of pH 8.5 and molecular weight of 65,000. Its optimum pH was 6.3 and its optimal temperature was 42 C. The purified enzyme released 11% of the total glucose residues of the OMZ 176 glucan as reducing sugars and solubilized about half of the substrate glucan. The products were found to be isomaltose, nigerose, and nigerotriose, with some oligosaccharides. The purified enzyme split the α-1,3-glucan endolytically and was inactive toward glucans containing α-1,6, α-1,4, β-1,3, β-1,4, and/or β-1,6 bonds as the main linkages.

It has been suggested that there may be a close causal relation between extracellular polysaccharides produced by oral streptococci, especially *Streptococcus mutans*, and formation of dental plaques, which are believed to be critical in the pathogenesis of dental caries and periodontal diseases (9, 11, 14, 24). The chief properties of polysaccharides responsible for formation of a plaque matrix seems to be their insolubility and stickiness (10, 12, 16, 21, 22, 29). Consequently, measures to suppress the biosynthesis and/or to degrade these insoluble, sticky polysaccharides should be the most rational ways to prevent both dental caries and periodontal diseases.

However, the extracellular polysaccharides produced by oral microbes, even of the same species, have been shown to vary in chemical and physical properties. Moreover, little is known about the chemical and physicochemical properties of the sticky, insoluble glucan containing a high percentage of α-1,3-glucosidic linkages involved in dental plaque formation (3, 7, 8, 13).

In the present study, the polysaccharides formed by cell-free enzymes from a variety of oral streptococci, including different serotypes and strains of *S. mutans*, were compared with respect to their chemical compositions and susceptibilities to a variety of dextranases. The sticky, insoluble polyglucoses produced by some strains of *S. mutans* were found to be quite resistant to available dextranases. Furthermore, there have been a few reports on the α-1,3-glucanases (15, 18, 27, 32), of which, however, only one enzyme could solubilize the insoluble glucan of *S. mutans* (15). Therefore, an attempt was made to isolate soil bacteria

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capable of degrading the dextranase-resistant glucans by an enrichment culture method using the test glucan as the sole carbon source.

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MATERIALS AND METHODS

Bacterial strains. *S. mutans* strains AHT and BHT (D. D. Zinner, Division of Oral Biology, University of Miami, Miami, Fla.), FA-1 (R. J. Fitzgerald, The Veteran Administration Hospital, Miami, Fla.), Ingbritt (B. Krasse, Department of Cariology, University of Bothenburg, Sweden), JC-1 (J. Carlson, School of Dentistry, University of Umeå, Umeå, Sweden), and OMZ 176 and OMZ 65 (B. Guggenheim, Experimental Caries Research Laboratories, Dental Institute, University of Zürich, Switzerland), *Streptococcus sanguis* ATCC 10556, and *Streptococcus* species CHT (D. D. Zinner) were obtained through the courtesy of S. Hamada of this laboratory, to whom these strains had generously been given by the persons indicated in parentheses. Bacteria were maintained as stab cultures in anaerobic agar (Eiken Chemical Co., Toykyo, Japan) at room temperature and transferred monthly.

Dextranases. The following dextranases were used as references. Biken dextranase was a product of *Flavobacterium* sp. (K. Shimada, N. Ishikawa, F. Hojo, I. Tsunashima, K. Yamashita, K. Ukawa, T. Kubo, and K. Inoue, Jpn. J. Bacteriol. **28**:270, 1973) and was kindly supplied from the Kanonji Institute of the Research Foundation for Microbial Diseases of Osaka University, Kanonji, Kagawa, Japan. A partially purified preparation of *Spicaria* dextranase, from *Spicaria violaceae* (IFO 6120) (23), was a gift from Y. Murayama (Department of Oral Medicine, Osaka University Dental School). Two other preparations of *Penicillium* dextranase were purchased from Sigma Chemical Co. (St. Louis, Mo.) and Worthington Biochemical Co. (Freehold, N.J.).

Preparation of cell-free glucosyl and fructosyl transferases. *S. mutans* OMZ 176 and OMZ 65 were cultured in brain heart infusion broth (Difco Laboratories, Detroit, Mich.), and other streptococcal strains were grown in Trypticase soy broth (Baltimore Biological Laboratories, Cockeysville, Md.). After incubation at 37 C for 18 h, the cultures were centrifuged at 6,500 × *g* for 20 min and the supernatants were treated with half saturation of ammonium sulfate. The resulting precipitate was collected by centrifugation at 6,500 × *g* for 20 min and dissolved in deionized water, and the solution was dialyzed against water for 18 h. Then it was centrifuged to remove insoluble material and used as a crude preparation of glucosyl and fructosyl transferases.

Preparation of polysaccharides by use of the glycosyltransferases. The enzyme preparations obtained as described above were incubated with 10% sucrose in 0.1 M phosphate buffer, pH 6.8, at 37 C for 48 h. The reaction mixture became turbid due to the formation of insoluble polysaccharide, and this was collected by centrifugation at 45,000 × *g* for 30 min and washed several times with deionized water. Then it was incubated with crystalline trypsin (Trypsillin, Mochida Pharmaceutical Co., Tokyo, Japan) at 37 C for 2 h to remove contaminating protein. The purified insoluble polysaccharide was collected by centrifugation at 45,000 × *g* for 30 min, washed with deionized water, and lyophilized.

The supernatant of the reaction mixture from which the insoluble polysaccharide had been separated was dialyzed against deionized water and mixed with 2 volumes of absolute ethanol. The precipitate formed was collected by centrifugation at 6,500 × *g* for 20 min and dissolved in a minimum volume of water. It was found that part of the alcohol-precipitable fraction was insoluble in water. The insoluble material was collected by centrifugation at 6,500 × *g* for 20 min, washed thoroughly with water, and treated with crystalline trypsin, like the insoluble polysaccharide. The fraction thus obtained was termed soluble polysaccharide I, although it was insoluble in water after precipitation with alcohol, because it had been water soluble originally, in contrast with the insoluble polysaccharide described above. The supernatant obtained by centrifugation at 6,500 × *g* as described above was mixed with 2.5 volumes of absolute ethanol. The resulting precipitate was recovered and thoroughly washed with absolute ethanol by centrifugation at 6,500 × *g* for 20 min. This fraction was named soluble polysaccharide II.

Glucan polyalcohol. The insoluble glucan (200 mg) of *S. mutans* OMZ 176 was oxidized by treatment with 0.08 M sodium periodate (100 ml) at 20 C in the dark for 14 days. The insoluble oxidation product was recovered by centrifugation at 8,000 × *g* for 20 min. It was washed with water to remove excess periodate and then reduced with excess sodium borohydride for 18 h at room temperature. The residual borohydride was decomposed by addition of acetic acid to give a final pH of 6.5. The reaction mixture containing the insoluble glucan polyalcohol was dialyzed against water at 5 C and then lyophilized.

Aspergillus niger cell wall glucan (α-1,3-glucan). The growth medium used for cultivation of *A. niger* consisted of 5% glucose, 0.15% yeast extract (Difco), 0.0125% $MgCO_3 \cdot 7H_2O$, 0.0125% KH_2PO_4, and 0.0025% $ZnSO_4 \cdot 7H_2O$, adjusted to pH 5.0 with sodium hydroxide. Four 150-ml portions of the medium in 500-ml Erlenmeyer flasks were each inoculated with 1 ml of a 2-day culture of *A. niger* spores and incubated on a reciprocal shaker at 25 C for 4 days. They were used as a seed culture for 6 liters of medium in a fermenter. The medium was shaken at 200 to 300 rpm and stirred by aeration at a rate of 1.0 to 1.5 vol/vol per min at 25 C for 3 days.

The whole culture was then sterilized by autoclaving at 15 lb/in^2 for 20 min, and the mycelia were collected on cheesecloth by filtration. Filtration through the cloth was repeated once more.

The cell wall glucan was prepared from the mycelia by a slight modification of the method of Hasegawa et al. (18). The harvested mycelia were washed first with

a large amount of boiling water and then with cold water. They were then suspended in ice-cold 0.1 M sodium hydroxide and allowed to stand for 5 h at room temperature with occasional stirring. The suspension was then adjusted to 1 N with respect to sodium hydroxide, heated at 80 C for 4 h, and filtered through cheesecloth. The filtrate was left in a cold room for 3 days and then adjusted to pH 5.8 with diluted hydrochloric acid. The resulting turbid solution was centrifuged at 8,000 × *g* for 20 min, and insoluble material was collected, washed with water, and suspended in 1 N sodium hydroxide. The suspension was left at room temperature for 18 h and then centrifuged at 8,000 × *g* for 20 min. An equal volume of absolute ethanol was added to the supernatant, and the resulting precipitate of glucan was collected, washed, and suspended in 500 ml of 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.8) containing 200 mg of Pronase P (Kaken Chemical Co., Tokyo, Japan). The mixture was left at room temperature for 3 days, and then the Pronase-treated glucan was collected by centrifugation, washed, and lyophilized. The glucan of *A. niger* thus obtained was methylated by Hakomori's method (17) and hydrolyzed, and methylated sugars were analyzed by gas-liquid chromatography as their corresponding alditol acetates (5). The test glucan was proved to be α-1,3-glucan.

Other glucans. *Paracoccidioides brasiliensis* cell wall glucan (α-1,3), curdlan (β-1,3), and luteose (β-1,6) used as reference glucans were kindly given by F. Kanetsuna (Toneyama Hospital, National Sanatorium, Toyonaka, Osaka, Japan) and T. Harada and A. Amemura (The Institute of Scientific and Industrial Research, Osaka University, Yamada-Kami, Suita, Osaka, Japan), respectively.

Medium for isolation of bacteria decomposing the insoluble glucan of S. mutans. The medium was prepared as described by Baker and Whiteside (4) with some modifications. It contained 0.03% of *S. mutans* OMZ 176 insoluble glucan as the sole carbon source in a salt mixture consisting of 0.1% $(NH_4)_2SO_4$, 0.0005% $MgSO_4 \cdot 7H_2O$, and 0.0005% $FeCl_2 \cdot 6H_2O$, adjusted to pH 7.0 with 1% dipotassium hydrogen phosphate.

Determination of the susceptibility of test glucans to dextranases. The assay system consisted of 100 μl of a 0.2% solution of test glucan, 40 μl of 0.1 M acetate buffer, pH 5.8, and 10 μl of an appropriate concentration of dextranase solution. Ten-microliter samples were withdrawn before and after incubation for 30 min at 37 C. They were rapidly heated at 100 C for 3 min to stop the reaction and then assayed for reducing sugars. The amount of test dextranase in the assay system was adjusted to obtain liberation of 1,000 nmol of reducing sugars from the reference substrate, Dextran T-70 (Pharmacia, Uppsala, Sweden) containing 5,550 nmol of glucose residues.

Assay system for the EK-14 enzyme and definition of enzyme units. The assay system consisted of 100 μl of a 0.2% suspension of the insoluble polysaccharide of *S. mutans* OMZ 176, 50 μl of 0.2 M acetate buffer, pH 6.3, and 50 μl of a suitable dilution of a test enzyme solution. Samples for determination of reducing sugars were withdrawn before and after incubation for 20 min at 37 C and boiled at 100 C for 3 min to inactivate the enzyme. One unit of the EK-14 enzyme was defined as described by Hasegawa et al. (18) as the amount liberating 1.0 nmol of reducing sugar, equivalent to glucose, per min under the standard assay conditions.

Analytical methods. Reducing sugar was determined by the method of Park and Johnson (26), and total hexose was determined by the anthrone method (1), using glucose as a standard. For determination of the total glucose content, the test polysaccharide was hydrolyzed in 4 N sulfuric acid at 100 C for 40 to 60 min. The hydrolysate was neutralized by addition of 4 N sodium hydroxide, diluted with 20 volumes of 0.1 M phosphate buffer, pH 7.0 (25), and assayed on a microscale with a Glucostat (Worthington Biochemical Co.). Protein was determined with Folin-Ciocalteau phenol reagent by the method of Lowry et al. (20) with crystalline bovine serum albumin (Sigma Chemical Co.) as reference.

Paper chromatography was done on Whatman no. 1 paper by the ascending technique in solvent A (*n*-butanol/acetic acid/water, 2:1:1, by volume) or B(*n*-propanol/ethanol/water, 6:1:3, by volume) at room temperature. Sugars on the paper chromatogram were detected with alkaline silver nitrate by the method of Trevelyan et al. (30).

Polyacrylamide gel electrophoresis. Gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed as described by Weber and Osborn (31). Gels were 8 cm long in glass tubes of 6-mm inside diameter. Eighty microliters of the test sample, containing about 40 μg of protein, was added to 40 μl of a sample buffer, consisting of 3% SDS in 0.1 M phosphate buffer (pH 7.2), 30% glycerol, and 0.006% bromophenol blue, and applied on the top of the 7.5% polyacrylamide gel. Electrophoresis was conducted for 5 h at a constant current of 8 mA per tube. Protein was detected by staining with Coomassie brilliant blue.

Isoelectric focusing. Isoelectric focusing was carried out with a density gradient of glycerol in a 110-ml column (LKB, Rockville, Md.). Ampholine (LKB) with a pH range of pH 3 to 10 was used as the carrier ampholyte. The sample was focused for 39 h with a terminal voltage of 500 to 700. Then 1-ml fractions were collected from the bottom of the column for determination of pH and optical density at 280 nm. The enzyme activity of fractions was determined under the standard assay conditions after being dialyzed.

RESULTS

Chemical composition of the extracellular polysaccharides produced by various oral streptococci. The contents of total hexoses, glucose, and protein in the insoluble and soluble polysaccharides produced by cell-free enzymes of nine streptococcal strains were determined. Most preparations contained more than 80% hexose (Table 1). The soluble polysaccharides II

TABLE 1. *Sugar composition and purity of the insoluble and soluble polysaccharides formed by cell-free enzymes of various strains of Streptococcus mutans, S. sanguis ATCC 10556, and Streptococcus species strain CHT*

Origin of polysaccharide	Serotype[a]	Total hexose		Glucose		Fructose	Protein (%)	Yield[b] (mg)
		μmol/mg	%	μmol/mg	%			
Insoluble polysaccharide								
S. mutans								
AHT	*a*	4.56	82	2.08	38	+[c]	tr[d]	353
BHT	*b*	5.53	100	0	0	+	tr	194
FA-1	*b*	5.56	100	5.12	92	–	tr	314
Ingbritt	*c*	5.29	95	5.18	93	–	tr	384
JC-1	*c*	5.57	100	0	0	+	tr	451
OMZ 176	*d*	5.91	107	5.52	99	–	tr	1,996
OMZ 65	*d*	5.52	99	5.41	97	–	tr	1,720
S. sanguis ATCC 10556		5.76	104	5.29	95	–	tr	623
Streptococcus sp. CHT		4.62	83	2.58	47	+	tr	222
Soluble polysaccharide I								
S. mutans								
AHT	*a*	5.48	99	5.46	98	–	tr	114
BHT	*b*	5.51	99	5.34	96	–	tr	436
FA-1	*b*	5.48	99	5.29	95	–	tr	298
Ingbritt	*c*	4.97	90	3.93	71	–	tr	726
JC-1	*c*	5.25	95	2.48	45	+	tr	1,023
OMZ 176	*d*	3.86	69	4.05	73	–	1.7	15
OMZ 65	*d*	ND[e]		ND		ND	ND	tr
S. sanguis ATCC 10556		4.63	83	4.61	83	–	1.5	348
Streptococcus sp. CHT		5.34	96	3.28	59	+	tr	460
Soluble polysaccharide II								
S. mutans								
AHT	*a*	5.29	95	5.53	99	–	1.5	1,363
BHT	*b*	5.10	92	5.36	96	–	1.5	1,472
FA-1	*b*	5.64	102	5.61	101	–	tr	1,985
Ingbritt	*c*	5.54	100	5.36	97	–	tr	3,083
JC-1	*c*	4.34	78	4.27	77	–	1.0	24
OMZ 176	*d*	2.18	39	1.40	25	–	17.15	895
OMZ 65	*d*	2.34	42	2.32	42	–	18.33	285
S. sanguis ATCC 10556		ND		ND		ND	ND	tr
Streptococcus sp. CHT		5.34	96	5.24	94	–	tr	2,179

[a] Serotype by Bratthall's classification (6).
[b] Amount of polysaccharide formed by crude enzyme obtained from 10 liters of culture.
[c] + and – indicated the presence and absence of fructose, respectively.
[d] tr, Trace.
[e] ND, Not determined because the yield was very low.

and I of *S. mutans* OMZ 176 and OMZ 65 were exceptions. The low contents of hexoses in these preparations may have been due to contamination of the test specimens with non-sugar constituents derived from the brain heart infusion broth used for cultivation of these two strains. Other strains were grown in Trypticase soy broth. In support of this, the protein contents of these preparations were exceptionally high. The glucose content, determined by the Glucostat method, varied with different strains. The insoluble polysaccharides of strains BHT and JC-1 contained no glucose, and those from strains AHT and CHT contained glucose amounting to less than 60% of the total hexose residues. Lower contents of glucose were also found in the soluble polysaccharide I's of Ingbritt (71%), JC-1 (45%), OMZ 176 (73%), and CHT (59%).

To elucidate the cause of the difference between the amounts of hexose and glucose, polysaccharide specimens with very different amounts were hydrolyzed in 0.5 N sulfuric acid at 60 C for 4 h for fructose detection and in 2 N sulfuric acid at 100 C for 3 h for glucose detection. The hydrolysates were neutralized with solid barium carbonate and passed through filter paper to remove excess barium carbonate

and the resulting barium sulfate. The filtrates were then concentrated to dryness in a rotary evaporator, and the sugar compositions of the residues were examined by paper chromatography in solvent A. It was found that the insoluble polysaccharide fractions of strains BHT and JC-1 contained only fructose with no glucose, whereas those of strains AHT and CHT contained both glucose and fructose. The other insoluble polysaccharides contained only glucose.

Among the soluble polysaccharides I's, those from CHT and JC-1 contained both glucose and fructose. No sugars other than glucose were found in other specimens. The soluble polysaccharide II's examined, including that of strain OMZ 176, contained only glucose.

Susceptibilities of the extracellular polysaccharides of oral streptococci to dextranases. The susceptibilities of the polysaccharides of the oral streptococci listed in Table 1 to four dextranase specimens were examined by measuring liberation of reducing sugars on incubation for 30 min at 37 C.

Judged by liberation of reducing sugars, the soluble polysaccharide II's were highly susceptible, the soluble polysaccharide I's were moderately susceptible, and the insoluble polysaccharides, especially those produced by strains OMZ 176 and OMZ 65, were scarcely susceptible (Table 2). The insoluble polysaccharides (fructans) of strains BHT and JC-1 were, of course, quite resistant to all dextranases.

Isolation of bacteria capable of degrading the insoluble glucan produced by S. mutans. Attempts were made by the enrichment culture method to isolate a microorganism from soil that degraded dextranase-resistant glucan. Three-milliliter portions of medium containing the insoluble glucan formed by *S. mutans* OMZ 176 as the sole carbon source were inoculated with 100 mg each of 16 soil specimens collected from various areas and incubated at 28 C for 10 days. Then 1 drop of each culture was transferred to fresh medium and incubated as described above. Transfers were repeated 12 times during 4 months.

One loopful of an appropriate subculture was spread on an agar plate, consisting of the salt mixture described under Materials and Methods with 1.2% agar overlayered with the above basal agar supplemented with 0.2% OMZ 176 insoluble glucan. (The ratio of the volumes of the top and basal layers was about 1:1.) After inoculation, the plates were incubated at 28 C for several days. Colonies surrounded by a clear zone due to degradation of the insoluble glucan were selected to obtain pure cultures. One of the pure cultures (Fig. 1) thus isolated was named the EK-14 bacterium. This strain was subcultured on Trypticase soy agar and stored in the lyophilized state as stock.

Characteristics of the EK-14 bacterium. The EK-14 bacterium was a nonsporeforming, nonmotile, gram-negative bacterium. It usually appeared as short rods, 0.5 to 0.7 μm wide and 1.5 to 4.0 μm long, but was fairly pleomorphic. It was a facultative anaerobe and grew well on nutrient medium at 30 C. Colonies were spherical, semispherical or drop-shaped, and were glistening, opaque, slimy, and yellow. The yellow pigment was nondiffusible. The organism grew luxuriantly on potato medium and produced yellow colonies.

The organism showed the following biochemical characteristics. Indole was not produced, hydrogen sulfide was not formed, urea was not hydrolyzed, nitrate was not reduced, catalase was positive, oxidase was positive, the methyl red reaction was negative, the Voges-Proskauer test was positive, citrate was not utilized, arginine was not hydrolyzed, glucuronic acid was not oxidized, and NaCl tolerance was 2.0% (wt/vol). Its utilizations of sugars and sugar alcohols were as follows: glucose, galactose, arabinose, xylose, mannose, fructose, maltose, sucrose, and lactose—acid (+), gas (−); and trehalose, sorbitol, mannitol, inositol, rhamnose, salicin, adonitol, and dulcitol—acid (−), gas (−).

The organism EK-14 was identified as a member of the genus *Flavobacterium* from the criteria in *Bergey's Manual of Determinative Bacteriology* (eighth edition).

Production and purification of the EK-14 enzyme. A stock culture of the EK-14 bacterium was inoculated into 10 ml of Trypticase soy broth and incubated at 28 C for 24 h. The whole culture was then transferred to 100 ml of Trypticase soy broth and cultured at 25 C for 16 h with mechanical shaking. After overnight culture, a 110-ml portion was transferred to 7 liters of Trypticase soy broth in a 10-liter fermenter. Cultivation was carried out at 25 C for 24 h with agitation at 350 rpm at an air flow rate of 2,000 ml/min. The growth curve of the EK-14 bacterium and the time course of enzyme production in the pilot culture are shown in Fig. 2.

The 7-liter culture of the EK-14 was centrifuged at 10,000 $\times$ *g* in a continuous centrifuge. All of the following steps were performed at 4 C. The active principle(s) in 30 liters of culture supernatant, obtained from five large-

TABLE 2. *Susceptibilities of the insoluble and soluble polysarccharides produced by oral streptococci to four dextranases*

Test polysaccharide	Serotype[a]	Dextranase[b]			
		Biken	*Spicaria*	*Penicillium* (Sigma)	*Penicillium* (Worthington)
Insoluble polysaccharide					
S. mutans					
AHT[c]	*a*	176	261	235	224
BHT[d]	*b*	0	0	0	0
FA-1	*b*	164	192	176	184
Ingbritt	*c*	167	191	177	201
JC-1[d]	*c*	0	0	0	0
OMZ 176	*d*	39	64	47	76
OMZ 65	*d*	49	66	59	83
S. sanguis ATCC 10556		320	313	290	292
Streptococcus sp. CHT[c]		343	375	339	362
Soluble polysaccharide I					
S. mutans					
AHT	*a*	251	322	292	301
BHT	*b*	316	289	271	284
FA-1	*b*	353	350	349	335
Ingbritt	*c*	327	339	329	323
JC-1[c]	*c*	336	312	317	332
OMZ 176	*d*	ND[e]	ND	ND	ND
OMZ 65	*d*	ND	ND	ND	ND
S. sanguis ATCC 10556		490	446	416	434
Streptococcus sp. CHT[c]		431	487	420	451
Soluble polysaccharide II					
S. mutans					
AHT	*a*	671	602	583	534
BHT	*b*	629	584	577	527
FA-1	*b*	683	572	625	569
Ingbritt	*c*	759	629	621	579
JC-1	*c*	ND	ND	ND	ND
OMZ 176	*d*	768	662	777	636
OMZ 65	*d*	632	603	606	592
S. sanguis ATCC 10556		ND	ND	ND	ND
Streptococcus sp. CHT		691	619	630	559

[a] See Table 1.

[b] The amount of test dextranase was adjusted to release about 1,000 nmol of reducing sugars from Dextran T-70 under the standard assay conditions. Data are expressed as nanomoles of reducing sugars released per 5,550 nmol of glucose in the test polysaccharide on incubation for 30 min at 37 C.

[c] A mixture of glucan and fructan.

[d] Fructan.

[e] ND, Not determined because of low yield.

scale cultures, was salted out with 70% saturation with ammonium sulfate. The precipitate obtained by centrifugation at 6,500 × *g* for 20 min was dissolved in deionized water and dialyzed against water. The nondialyzable portion was concentrated to 109 ml by ultrafiltration using a Diafilter G-10 T (cutting limit of molecular weight, 10,000; Bio-Engineering Co., Ltd., Tokyo, Japan) with stirring under pressure from a nitrogen atmosphere of 2 kg/cm^2.

The concentrated enzyme(s) was equilibrated with 0.05 M phosphate buffer, pH 8.0, and was applied to a DE-32 (microgranular form of diethylaminoethyl-cellulose, 1.0 meq/g; Whatman Biochemicals Ltd., Kent, England) column (3.5 by 40 cm) that had been equilibrated with 0.05 M phosphate buffer, pH 8.0. The column was washed with the same buffer. The flow rate was adjusted to 1 ml/min, and 10-ml fractions were collected. Aliquots of each fraction were assayed for enzyme activity, and their protein content was measured in terms of their ultraviolet absorption at 280 nm.

Almost all of the dark-brown material in the

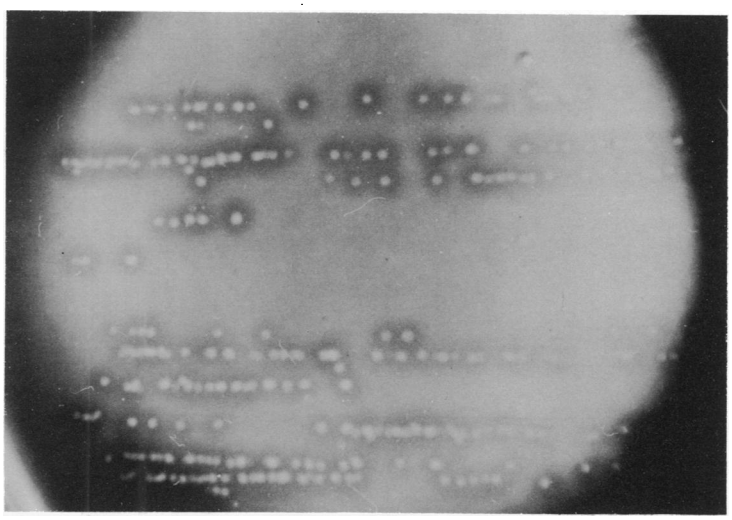

FIG. 1. *Colonies of the EK-14 bacterium grown on an agar plate containing the insoluble glucan of S. mutans OMZ 176.*

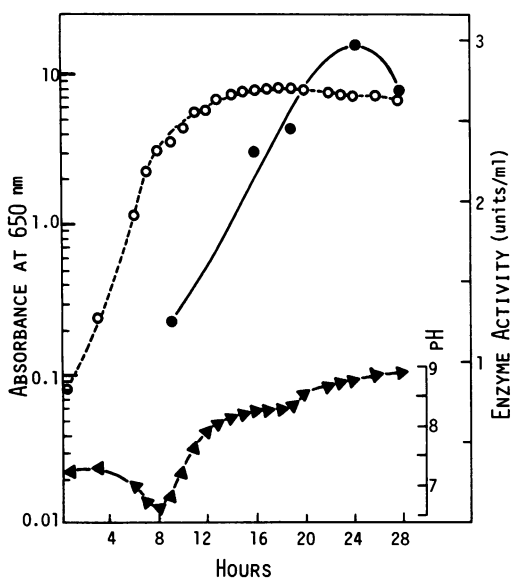

FIG. 2. *Time course of growth, enzyme production, and pH change of the EK-14 culture in Trypticase soy broth in a fermenter. The aeration rate was 2,000 ml/min and the extent of agitation was 350 rpm. Symbols:* ○, *Growth curve (absorbance at 650 nm);* ●, *enzyme activity;* ▲, *pH of the culture.*

concentrated enzyme preparation was retained on the DE-32 column, and a practically colorless active fraction was obtained in the effluent. The efficiency of purification was high in this step (Table 3). Fractions with activity were combined (156 ml), dialyzed against 0.05 M potassium dihydrogen phosphate, pH 4.3, and concentrated by ultrafiltration through a Diafilter G-10 T.

The concentrated DE-32 fraction (50 ml) was applied to a CM-32 (microgranular form of carboxymethyl-cellulose, 1.0 meq/g; Whatman Biochemicals Ltd., Kent, England) column (3 by 15 cm) that had been equilibrated with 0.05 M potassium dihydrogen phosphate, pH 4.3. The column was washed first with 200 ml of 0.05 M potassium dihydrogen phosphate, pH 4.3, and then eluted with a linear gradient of 0.05 M phosphate buffer, pH 4.3, to 0.5 M buffer, pH 8.0 (900 ml). The flow rate was adjusted to 1 ml/min. An elution profile is shown in Fig. 3. The enzyme activity was adsorbed on the CM-32 column and eluted as a single peak on increasing the salt concentration and pH of the elution buffer. The enzyme activity was mainly found in fractions eluted with 0.2 M buffer, pH 6.0.

Data on the concentration and purification of the EK-14 enzyme are summarized in Table 3. About a 160-fold increase in the specific activity was achieved (units per milligram of protein) with a yield of 38%.

Physicochemical properties of the EK-14 enzyme. The purity or homogeneity of the final enzyme preparation was examined by polyacrylamide gel electrophoresis in the presence of SDS. A sample (40 μg of protein) of the eluate from CM-32 was examined, and only one major band was detected on staining with Coomassie brilliant blue (Fig. 4).

The molecular weight of the enzyme protein was estimated to be 65,000 by electrophoresis

TABLE 3. *Summary of concentration and purification of the EK-14 enzyme*

Fraction	Vol (ml)	Enzyme activity (U/ml)	Total activity (U)	Protein (mg/ml)	Sp act (U/mg of protein)	Yield (%)	Purification (fold)
Culture filtrate	30,000	3	90,000	0.53	5	100	1
$(NH_4)_2SO_4$ precipitate	500	158	79,000	8.01	20	88	4
Concentrate of $(NH_4)_2SO_4$ precipitate	109	513	56,019	31.11	17	62	3
DE-32 fraction	156	272	43,432	1.15	237	48	47
Concentrate of DE-32 fraction	50	799	39,950	3.26	245	44	49
CM-32 fraction	110	308	33,880	0.38	813	38	162

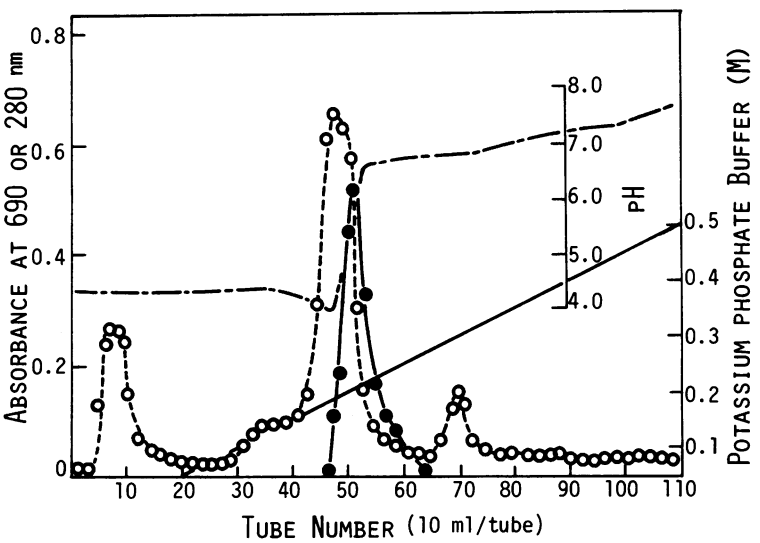

FIG. 3. *Chromatography of the DE-32 fraction on a CM-32 column. The column (3 by 15 cm) was equilibrated with 0.05 M KH_2PO_4, pH 4.3. Symbols: O, absorbance at 280 nm; ●, enzyme activity (absorbance at 690 nm); -·-·, pH of effluent; —, relative salt concentration.*

(Fig. 5) of the CM-32 fraction according to the method of Weber and Osborn (31). The isoelectric point of the DE-32 fraction, as estimated by isoelectric focusing, was determined to be 8.5. The pH of the fraction that exhibited maximal enzyme activity was taken as the isoelectric point of the EK-14 enzyme. The recovery of enzyme activity on isoelectric focusing was very low, so the enzyme could not be purified in this way.

General characteristics of the enzyme. Under standard conditions, the optimum pH of the enzyme was around 6.3 (Fig. 6). It is interesting that the enzyme had considerable activity at pH 8, because the α-1,3-glucanases of *Trichoderma viride* (18), *Trichoderma harizianum* (15), and *Cladosporium resinae* (27) have no activity at pH 8.

The effect of ionic strength on the enzyme activity was investigated by addition of increasing amounts of sodium chloride to the assay system, containing acetate buffer, pH 6.3, of 0.004 ionic strength. The enzyme activity was scarcely affected by a change in the ionic strength between 0.004 to 0.254.

The enzyme was most active at around 40 C and was almost completely inactivated by heating at 60 C for 30 min.

The effects of various metal ions, disodium ethylenediaminetetraacetate, and iodoacetamide on the activity of the CM-32 fraction (2 units) were studied under the standard assay conditions. The reagents used were added as chlorides, except lead acetate and silver nitrate. None of the metal ions tested potentiated the enzyme activity of the EK-14 enzyme, and

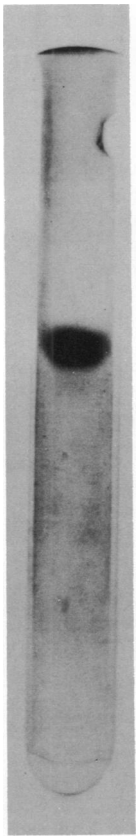

FIG. 4. *Gel electrophoretic pattern of the CM-32 fraction on an SDS-polyacrylamide column. The column was stained with Coomassie brilliant blue for detection of proteins.*

Ag^{+}, Hg^{2+}, and Pb^{2+} were strongly inhibitory (Table 4).

Substrate specificity and mode of action of the EK-14 enzyme. The substrate specificity of the EK-14 enzyme was studied by measuring the amounts of reducing sugar released from various test substrates under standard assay conditions. The extents of hydrolysis of the substrates tested relative to that of the reference OMZ 176 insoluble polysaccharide are shown in Table 5.

The purified enzyme (CM-32 fraction) attacked test glucans containing α-1,3-glucosidic

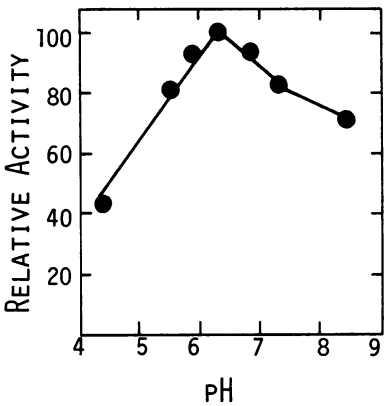

FIG. 6. *Effect of pH on the enzyme activity. Each tube contained 200 μg of the substrate (insoluble glucan of S. mutans OMZ 176) and 3 units of the CM-32 enzyme in 200 μl of 0.05 M acetate or phosphate buffer, adjusted to the indicated pH value. After 20 min of incubation, liberated reducing sugar was determined.*

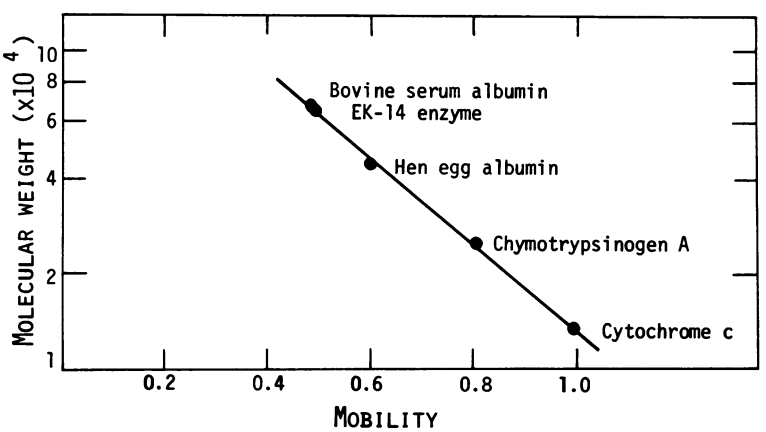

FIG. 5. *Molecular weight determination of EK-14 enzyme by SDS-polyacrylamide gel electrophoresis. Bovine serum albumin (molecular weight, 67,000; Combithek, C. F. Boehringer & Soehne GmbH, Mannheim, Germany), hen egg albumin (molecular weight, 45,000; Combithek), chymotrypsinogen A from bovine pancreas (molecular weight, 25,000; Combithek), and cytochrome c from horse heart (molecular weight, 12,500; Combithek) were used as references. The logarithms of the molecular weights of known proteins are plotted against their respective mobilities. The molecular weight of the EK-14 enzyme was determined from the standard curve thus obtained.*

TABLE 4. *Effects of metal ions, ethylenediaminetetraacetic acid (EDTA), and iodoacetamide on the activity of the EK-14 enzyme*[a]

Test compound	Conc ($M \times 10^{-2}$)	Relative enzyme activity (%)
None	0	100
Ba^{2+}	1.0	88
Ni^{2+}	1.0	63
Co^{2+}	1.0	62
Fe^{2+}	0.5	72
Fe^{3+}	0.5	41
Ag^{+}	1.0	19
Hg^{2+}	1.0	13
Pb^{2+}	1.0	11
EDTA	1.0	98
Iodoacetamide	1.0	62

[a] The reaction mixture consisted of 50 μl of an appropriate dilution of CM-32 enzyme solution (40 U/ml), 100 μl of 0.2% insoluble glucan of *S. mutans* OMZ 176, and 50 μl of test solution in acetate buffer, pH 6.3. The final concentrations of test compounds were adjusted to 0.005 to 0.01 M.

bonds as the major linkage, i.e., the insoluble polysaccharides of *S. mutans* strains OMZ 176 and OMZ 65 and the cell wall glucans of *P. brasiliensis* and *A. niger*. (An exception was NRRL 1355 dextran of *Leuconostoc mesenteroides*.) However, glucans that did not contain α-1,3 linkages were not hydrolyzed.

To determine whether the enzyme split α-1,3 linkages by exo- or endo-type degradation, the hydrolyses of native OMZ 176 insoluble glucan and its polyalcohol obtained by periodate oxidation and borohydrate reduction were compared under standard assay conditions, using the purified enzyme fraction (CM-32 fraction). Samples were withdrawn to follow the liberation of reducing sugar every 10 min during incubation for 1 h. The native glucan and the polyalcohol derivative were degraded at the same rates, indicating that modification on the terminal nonreducing glycosidic units and/or α-1,6-linked glycosidic units within the glucan chain did not affect the susceptibility of the OMZ 176 insoluble glucan to the EK-14 enzyme. This indicates that the enzyme acts on native glucan by an endo-type mechanism.

Fifty milligrams of the insoluble glucan formed by *S. mutans* OMZ 176 was incubated with 462 units of the CM-32 fraction in 10.5 ml of 0.05 M actetate buffer, pH 6.3, at 37 C for 24 h. During the incubation, 31.3 μmol (626 nmol/mg of glucan) of reducing sugar was liberated, amounting to about 11.3% of the total glucose residues in the substrate. The digest was centrifuged at 20,000 × *g* for 25 min. The precipitate was washed with a small amount of deionized water and lyophilized. It was found to weigh 25.5 mg, indicating that about half the test glucan became water soluble on treatment with the EK-14 enzyme. The supernatant obtained by centrifugation as described above was submitted to paper chromatography in solvent B by the ascending technique. The paper was developed in three cycles over 3 days (28), and sugars were detected with alkaline silver nitrate. The R_f values of the three main products were identical with those of authentic isomaltose, nigerose, and nigerotriose, respectively. In addition, faint but distinct spots were detected, presumably corresponding to α-1,3-oligosaccharides. The glycosidic linkages in the residual insoluble core after thorough digestion with the EK-14 enzyme were examined quantitatively by methylation. The results will be reported elsewhere (unpublished data).

TABLE 5. *Substrate specificity of the EK-14 enzyme*

Test glucan	Major linkage(s) contained	Relative hydrolysis (%)
Streptococcus mutans		
OMZ 176 insoluble polysaccharide	α-1,3; α-1,6	100
OMZ 65 insoluble polysaccharide	α-1,3; α-1,6	93
OMZ 176 soluble polysaccharide II	α-1,6	0
OMZ 65 soluble polysaccharide II	α-1,6	0
Leuconostoc mesenteroides		
Dextran T-70	α-1,6	0
Dextran T-2000	α-1,6	0
NRRL 1355 dextran	α-1,6; α-1,3	4
Paracoccidioides brasiliensis cell wall glucan	α-1,3	90
Aspergillus niger cell wall glucan	α-1,3	88
Soluble starch	α-1,4; α-1,6	0
Cellulose	β-1,4	0
Pachyman	β-1,3	0
Laminaran	β-1,3; β-1,6	0
Curdlan	β-1,3	0
Luteose	β-1,6	0

The reaction mixture, consisting of 50 μl of appropriately diluted CM-32 enzyme solution (60 units/ml), 100 μl of 0.2% test glucan and 50 μl of acetate buffer, pH 6.3, was incubated at 37 C for 20 min. Reducing sugars were measured in samples withdrawn before and after incubation for 20 min. The liberated reducing powers in incubation mixtures with insoluble glucan from *S. mutans* OMZ 176 were arbitarily taken as 100%.

DISCUSSION

The polysaccharides produced by cell-free enzyme preparations of a variety of oral streptococci, especially *S. mutans*, were compared. Results showed that the sticky, insoluble polysaccharides, which may be important in the pathogenesis of periodontal diseases and dental caries by causing formation of dental plaque, are generally resistant to the action of available dextranase preparations.

The insoluble glucans produced by cell-free enzymes from different strains of oral streptococci varied somewhat in susceptibility to test dextranases. These insoluble glucans were obtained by centrifugation at 45,000 × *g* for 30 min, as described under Materials and Methods. However, the insoluble glucans of OMZ 176 and OMZ 65 could be precipitated by centrifugation at 4,000 × *g* for 20 min, whereas those from other strains of *S. mutans*, *S. sanguis*, and *Streptococcus* sp. could only be precipitated by centrifugation at 45,000 × *g* for 30 min. Thus the water solubilities of insoluble glucans of OMZ 176 and OMZ 65 differ from those of other insoluble glucans. This agrees with the results of structural studies of these glucans by methylation analysis: the glucans of OMZ 176 and OMZ 65 had higher contents of α-1,3 and lower contents of α-1,6-glucosidic linkages than other insoluble glucans (Ebisu et al., manuscript in preparation). In this connection, it is interesting that strains BHT and FA-1 were less active than strains OMZ 176 in cariogenicity tests on Sprague-Dawley rats fed on Diet 2000 (S. Hamada, personal communication). It seems that the cariogenicities of strains BHT and FA-1 are gradually lost during successive transfer on artificial media in the laboratory.

The chemical structure of the sticky, dextranase-resistant glucan formed by strain OMZ 176 (serotype *d*) of *S. mutans* was extensively studied by methylation analysis and Smith degradation, as reported elsewhere (8). Briefly, it was found that hydrolysis of a methylated derivative of this glucan ($[\alpha]_D^+$ 222°, in chloroform) gave 2,3,4,6-tetra-, 2,4,6-tri-, 2,3,4-tri- and 2,4-di-*O*-methyl-D-glucose in percentages of 14.6, 50.5, 20.9, and 14.0, respectively. This indicates that the OMZ 176 insoluble glucan has a highly branched structure with an average repeating unit of seven sugar residues. By controlled Smith degradation of the glucan, all the α-1,6-linked side chains were eliminated as glycerol. The resulting degraded glucan without side chain (yield, 63% of the starting material) was insoluble but not sticky. The glycosidic linkages of this insoluble degradation product were also analyzed by methylation analysis. After hydrolysis of the methylated material, only 2,4,6-tri-*O*-methyl-D-glucose was recovered, indicating that almost all the glycosidic linkages were α-1,3 linkages. Thus the susceptibility of the insoluble, sticky glucan of *S. mutans* OMZ 176 to available dextranases seems to be due to the fact that the main chain of this glucan consists of α-1,3-glucosidic linkages, and these are not hydrolyzed by so-called dextranases that hydrolyze α-1,6-glucosidic linkages.

During chemical analyses of polysaccharides, we detected fructans, other than glucan, in insoluble and soluble polysaccharides formed by cell-free enzymes of *S. mutans* strains AHT, BHT, and JC-1 and *Streptococcus* species CHT. It was found by methylation analysis and enzymatic examination that all these fructans consisted of β-2,1-D-fructofuranosidic linkages (inulin-type linkages). Details of this work have been reported separately (7a).

The EK-14 enzyme, α-1,3-glucanase, was purified 162-fold from the culture superntant of a soil organism obtained by the enrichment culture method. The purification procedure involved concentration by salting out with ammonium sulfate and column chromatographies on DE-32 and CM-32. Activity for hydrolysis of β-1,3 linkages in pachyman and laminaran was detected in the eluate from the DE-32 column, but this activity was completely eliminated by CM-32 column chromatography. Thus the principles responsible for the α-1,3-glucanase or β-1,3-glucanase activities of the crude EK-14 preparation were different entities. It is interesting in this connection that Guggenheim and Haller (15) reported that α-1,3-glucanase purified from *T. harizianum* still contained β-1,3-glucanase activity at the final stage of purification. These authors claimed that this could be explained by supposing that "the anomeric configuration of the substrate would not be a limiting factor for the enzyme activity," but this argument seems inconsistent with the present results.

The purified EK-14 enzyme (CM-32 fraction) attacked cell wall glucans of *P. brasiliensis* and *A. niger* that contain consecutive α-1,3 bonds as the major glucosidic linkages, as well as the insoluble α-D-glucans of *S. mutans* strain OMZ 176 and OMZ 65. It is interesting that it did not attack a water-soluble dextran of *L. mesenteroides* NRRL 1355 containing a relatively high percentage of α-1,3 linkages. Recent studies showed that 1355 dextran consists of single α-1,3-linked glucose residues flanked by α-1,6

linkages (Misaki et al., unpublished data). Thus from the above findings, it seems that consecutive α-1,3-linked glucose units along the main chain are required for formation of an enzyme-substrate complex and consequently for susceptibility to the EK-14 enzyme.

So far, α-1,3-glucanases from *T. viride* (18), *T. harizianum* (15), *C. resinae* (27), and *Aspergillus nidulans* (32) have been reported. However, these fungal α-1,3-glucanases were apparently different from the enzyme produced by EK-14 bacterium in their physicochemical properties, and both *T. viride* and *T. harizianum* required α-1,3-glucan as an inducer for the enzyme production. A partially purified enzyme from *A. nidulans* only split α-1,3-glucan exolytically, not endolytically. α-1,3-Glucanase activity has also been found in culture filtrates of *Streptomyces* sp. (2), *Penicillium funiculosum*, *Penicillium wortmanni*, *Penicillium vermiculatum*, *Spicaria violacea*, and *Chloridium viride* (27), *Cladosporium resinae* (M. D. Dewar and G. J. Walker, J. Dent. Res. **52:** 573, 1973), and *Streptomyces werraensis* and *chartreusis* (19). However, these enzymes were not purified and their properties are unknown. It should be noted that the α-1,3-glucanase produced by EK-14 bacterium seems to be unique in that α-1,3-glucan is not required as an inducer for enzyme production.

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