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Extracellular Polysaccharides of *Actinomyces viscosus*

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Polysaccharide(s) have been isolated from supernatants of broth cultures of *Actinomyces viscosus*, strain T6. The organism produces significant quantities (2 mg/mg of cell [dry weight], 108 mg/liter) of this polysaccharide in the absence of sucrose. Chemical analysis indicated that *N*-acetylglucosamine (62%) was the major component; galactose (7%), glucose (4%), uronic acid (3%), and smaller amounts of glycerol, rhamnose, arabinose, and xylose were also present. The polysaccharide(s) is only slightly soluble in aqueous solutions. Although further purification of the polysaccharides will be necessary before definitive chemical and structural studies, the formation of extracellular polysaccharides is significant because it offers a possible explanation for plaque formation by these organisms.

Both in vivo and in vitro experiments indicated that strains of *Actinomyces* are capable of dental plaque formation (10, 13). The plaque which accumulates has been shown to be related to both root surface caries and periodontal disease (12, 23).

Although many of these studies employed sucrose as a substrate, Socransky et al. (23) indicated that the initiation of periodontal disease in gnotobiotic rats infected with *A. naeslundii* did not require a high-sucrose diet; similar observations were reported for implantation of *A. viscosus* strain T6 in hamsters (12). Since levan or dextran production requires a sucrose substrate, it was apparent that these polysaccharides were not required for plaque formation or the production of periodontal disease in these experiments.

Cell wall polymers could also function as cohesive agents to bind bacteria to tooth surface (6). Studies of the cell wall of *A. viscosus* indicated that rhamnose was the major neutral sugar in the non-peptidoglycan portion of the cell wall (18); this fraction also contained glucosamine and phospholipid. Hammond et al. (8) have shown that 6-deoxytalose, glucose, and galactose were also found in the cell walls of *Actinomyces* species. Although it is tempting to speculate that either the phospholipid or the deoxytalose is somehow related to the cohesiveness and slimy consistency of broth cultures of *A. viscosus*, there is no evidence to support this hypothesis. Indeed, bacterial slimes are usually associated with extracellular polysaccharides. In studies of levan production by *A. viscosus*, it was observed that polysaccharides were produced in the absence of sucrose (15); a study of extracellular polysaccharides produced by a strain of *A. viscosus* has been published (27). The objectives of our studies were to determine if extracellular polysaccharides were produced by *A. viscosus*, strain T6, in a medium which did not contain sucrose and to determine the nature of these polysaccharides.

MATERIALS AND METHODS

Preparation of the polysaccharide. A plaque-forming strain of *A. viscosus*, strain T6, (ATCC #15987) was provided by S. Socransky (Forsyth Dental Center, Boston, Mass.). The cells were grown in 10 liters of Trypticase soy broth containing 0.25% glucose (pH 7.3) (BBL). The cultures were grown at 37 C for 24 h and were separated from the supernatant broth by centrifugation in a Sharples continuous-flow centrifuge. The polysaccharide was obtained from the broth supernatant by procedures described in Results.

Chemical analyses. The total hexoses were determined by the anthrone procedure (14), and uronic acid was determined by the carbazole method (4). Reducing sugars were determined by the method of Thompson and Shockman (25); *N*-acetyl groups were determined by the method of Lüdowieg and Dorfman (16). Rhamnose and pentoses were determined by the methods of Dische and Shettles (5) and the orcinol reaction (4), respectively. Total phosphate was determined by the method of Chen et al. (3); sulfate was determined spectrophotometrically (19). A Bausch and Lomb 505 scanning spectrophotometer was used to detect the presence of nucleic acid and proteins. Analyses for amino acids and hexosamines were

performed by the method of Spackman et al. (24) as modified by Rosan (20). The optimal conditions for release of amino acids and hexosamines was determined to be 6 N HCl in vacuo at 100 C for 8 h.

In addition to specific colorimetric tests for monosaccharides, gas liquid chromatography was employed for carbohydrate analysis. The method of Sawardeker et al. (22), which involves acetylation of reduced sugar alcohols, was employed. Inositol was used as an internal standard and individual sugars were identified by co-chromatography with known samples of the acetylated carbohydrate (Regis Chemical Co., Chicago, Ill.). Prior to chromatography, the optimal conditions for release of reducing sugars was determined to be 1 N HCl in vacuo at 100 C for 4 h.

A Hewlett Packard model 5000 gas chromatograph containing stainless steel columns (⅛ inch by 6 feet; ca. 0.32 by 183 cm) packed with Chromsorb 100/120 mesh coated with OV225 (Applied Science Laboratory, State College, Pa.) was used for chromatography (7). The conditions for analysis were: (i) injection port temperature, 250 C; and (ii) flame ionization detector, 250 C; the oven was temperature programed from 160 to 200 C at 2 C/min. Nitrogen was used as the carrier gas at a flow rate of 60 ml/min.

Infrared spectrophotometry was performed on a Beckman IR18AX spectrophotometer using KB disks containing 1 to 2 mg of dried polysaccharide.

Serological methods. Vaccines were prepared from whole washed cells suspended in 0.15 M saline containing 0.2% formalin; a 1:10 dilution of the cells gave a Klett reading of 150 using a no. 46 filter. Female albino rabbits were injected as described previously (21); pre-immune sera were used as controls. The immunoelectrophoretic techniques using borate buffers were also described previously (21). Supernatants of cells disrupted by sonication were used for control antigens.

RESULTS

The flow chart for preparation of the polysaccharide is shown in Fig. 1. We added 3 vol of acetone to the culture supernatant, and the precipitate which formed was allowed to settle for 4 days at 4 C. The precipitate was recovered by siphoning and then centrifugation at 39,100 × *g*. The precipitate was suspended in 10% trichloroacetic acid at 4 C for 24 h and again centrifuged. One volume of acetone was added to the trichloroacetic acid supernatant, and the precipitate which formed overnight was removed by centrifugation. Both the trichloroacetic acid precipitate and the acetone precipitate were dialyzed against distilled water and lyophilized. The same procedure was applied to sterile broth.

Except for obvious differences in the amounts of precipitate, both sterile broth and culture supernatant behaved similarly up to the second precipitation with acetone. At this step of the procedure no precipitate was obtained from sterile broth, whereas a viscous gel-like material was precipitated from the culture supernatant. Analysis of the trichloroacetic acid precipitates of the culture supernatant and the sterile broth indicated both were composed of peptides of similar composition. Thus, all further studies were limited to the viscous gel obtained from the second acetone precipitation.

Three batches of the viscous gel were prepared over a period of several months. The average yield was 2 mg of polysaccharide per mg of cell (dry weight); 108 mg ± 3 mg/liter of culture supernatant. Lyophilization of the gel after dialysis yielded a fibrous type of material which did not dissolve readily in water, 0.1 N HCl, 0.1 N NaOH, or acid.

The preliminary chemical analysis is shown in Table 1. No nucleic acid or protein was detected, the anthrone reaction indicated that 16% hexose was present, the major component appeared to be hexosamine, and uronic acid and pentoses were also detected. The polysaccharide contained phosphate but no sulfate.

Amino acid analysis of 1, 2, 4, and 6 N HCl hydrolysates of the polysaccharide indicated that glucosamine was the only hexosamine present. In the 6 N HCl hydrolysates, small amounts of aspartic, threonine, serine, glu-

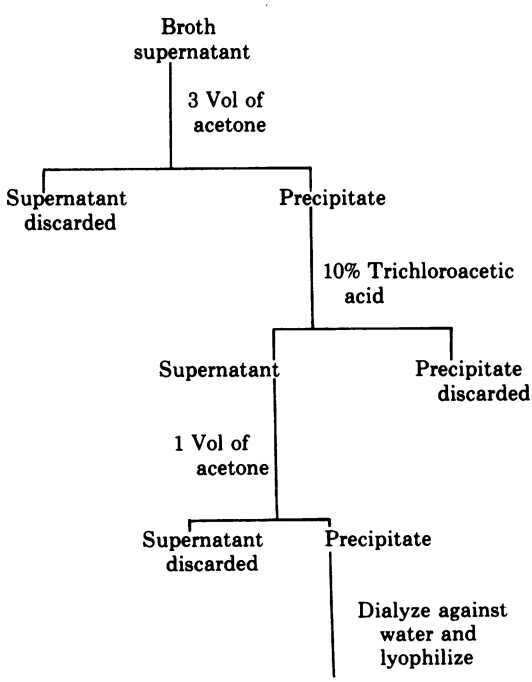

FIG. 1. *Flow sheet for the preparation of the extracellular polysaccharides of A. viscosus.*

tamic, alanine, valine, and leucine were detected. The total quantities of these amino acids was less than 1%. The glucosamine varied between 43 and 46%. Analysis for *N*-acetyl groups suggested that all of the glucosamine was acetylated (Table 2).

Gas chromatographic analysis of the polysaccharides is shown in Fig. 2. The individual sugars were identified by their retention time and by co-chromatography with known standards. In addition to small amounts of glycerol, the neutral sugars found in the polysaccharides were: galactose, glucose, rhamnose, arabinose, and xylose. The percentage of each is shown in Table 2.

An infrared spectrum of the polysaccharide is shown in Fig. 3. The absorption peaks at 1,648 cm^{-1} probably presents the acetyl groups of the hexosamines (1); the peak at 1,555 cm^{-1} also is associated with the presence of N-H groups; phosphate esters were detected at 1,310 cm^{-1}. The presence of an absorption peak at 885 cm^{-1} and the absence of such a peak at 840 cm^{-1} suggests that the β linkage is dominant in this polysaccharide (11).

The polysaccharide did not precipitate with antibody produced by immunization with whole

TABLE 1. *Preliminary chemical analysis of the extracellular polysaccharide of A. viscosus, T6*

Component	Percent detected
Nucleic acid[a]	ND[b]
Proteins[a]	ND
Hexose	16
Hexosamine	45
Uronic acid	3.4
Pentose	4.0
Phosphorus (PO_4)	5.3

[a] Polysaccharide was dissolved at concentration of 100 μg/ml and scanned from 200 to 300 nm.
[b] ND, Not detected.

TABLE 2. *Chemical composition of extracellular polysaccharide of A. viscosus, strain T6*[a]

Component	μmol/mg	%
Glucosamine	2.61	46.7
N-Acetyl	2.60	15.3
Galactose	0.40	7.1
Glucose	0.23	4.1
Glycerol	0.18	1.6
Arabinose	0.06	1.0
Rhamnose	0.04	0.7
Xylose	0.03	0.5

[a] Including uronic acid and phosphate a total of 88.7% of dry weight is accounted for.

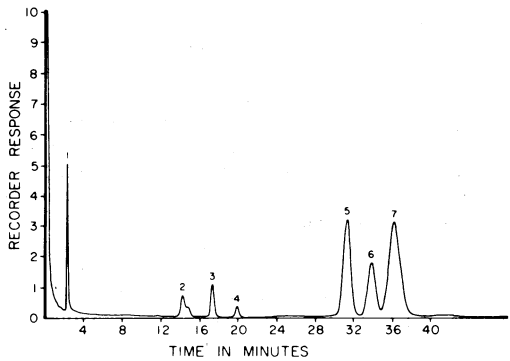

FIG. 2. *Gas chromatogram of acetylated derivatives of the A. viscosus polysaccharide. (1) Glycerol; (2) rhamnose; (3) arabinose; (4) xylose; (5) galactose; (6) glucose; (7) inositol (internal standard).*

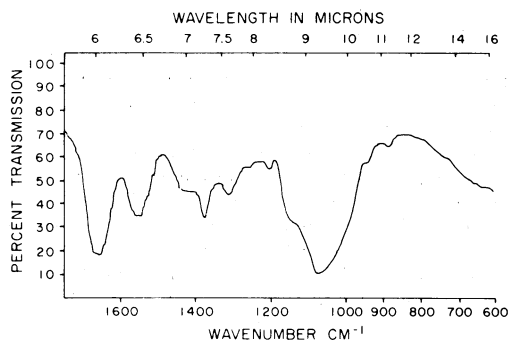

FIG. 3. *Infrared spectra: spectrum of A. viscosus polysaccharide.*

cells. However, bands were detected when supernatants of disrupted cells were tested against sera of immunized rabbits; no bands were observed with pre-immune sera. Negative staining with India ink did not detect a distinct capsular layer; a modified quellung reaction (9) also failed to detect a capsule. A small amount of an acetone-precipitable material could be stripped from the surface of the cells by passage through a needle valve at 10,000 lb/in^2. The infrared absorption spectra of this fraction indicated it was distinct from the extracellular slime.

DISCUSSION

The failure to detect either a capsule or a surface polysaccharide(s) similar to the slime suggests that this material is an extracellular product of *A. viscosus*. It has not as yet been determined if the small amounts of rhamnose and glycerol are components of the polysaccharide or whether they represent contaminants. Until further purification is carried out, it is

safer to assume that these substances represent either cell wall and/or cell membrane contaminants. In a sense this is fortuitous since it suggests the polysaccharide does have some association with the bacterial surface. Extracellular polysaccharide(s) also rich in glucosamine was isolated from another strain of *A. viscosus* (27); however, this polysaccharide also contained much less hexose (1.4%), larger amounts of protein (9%), and no uronic acids were detected. However, it is possible the differences between polysaccharides in the two studies reflect only the differences in isolation and preparative techniques. The large quantity of *N*-acetylglucosamine (ca. 60%) places the *A. viscosus* polymers in the class of mucopolysaccharides and probably accounts for the viscous quality of the polysaccharide (17). In addition to uronic acid, the polysaccharide(s) contained glycerol, arabinose and xylose, glucose, and galactose. A substantial quantity of phosphorus was present, suggesting the phosphoesters were important linkages in the polysaccharide; the infrared spectrum suggests the predominance of the β linkage. However, further purification of the polysaccharides are being undertaken prior to definitive chemical and structural analysis.

The failure to elicit precipitating antibodies against the polysaccharides using whole cells as an immunogen was not surprising since no substantial quantities of the extracellular polysaccharide was found on the cell surface. van der Hoeven (27) also found that the major portion of the extracellular slime was not immunogenic. However, antibodies against other cell components are produced, indicating the cells are immunogenic. The use of formalized cells for immunization was probably a poor choice since viable cells would be required for polysaccharide production. Currently, viable cells are being used to determine if antibodies against the polysaccharides can be produced.

The polysaccharide obtained by the methods described previously is only partially soluble in aqueous solutions, and even this process takes a long time. The relative insolubility of the *Streptococcus mutans* type dextrans is believed to be related to the plaque-forming potential of the latter bacteria (6). Since acidic polysaccharides are known to interact with proteins to yield aggregates under appropriate conditions (26), the interaction of the *Actinomyces* mucopolysaccharide with salivary proteins or surface antigens of other bacteria could provide an alternate mechanism by which *Actinomyces* species could form plaque. The significance of these investigations lies in detection and partial characterization of the slime(s) associated with *A. viscosus*. The polysaccharides could explain the ability of these organisms to form plaque in the absence of sucrose.

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