INFECTION AND IMMUNITY, Oct. 1978, p. 266-274
0019-9567/78/0022-0266$02.00/0

Vol. 22, No. 1
Printed in U.S.A.

Degradation of Levan by *Actinomyces viscosus*

CHRIS H. MILLER* AND PENELOPE J. B. SOMERS†
Laboratory of Oral Microbiology, Indiana University School of Dentistry, Indianapolis, Indiana 46202

Received for publication 9 August 1978

Actinomyces viscosus ATCC 15987 was examined for its ability to hydrolyze its own levan. Washed whole cells and an ammonium sulfate fraction from cell-free culture fluids were shown to possess levan hydrolase activity. Analyses of reaction mixtures by gel filtration and thin-layer chromatography demonstrated that the product of levan hydrolysis was free fructose. The cell-associated and extracellular enzyme preparations also hydrolyzed inulin and the levans synthesized by *Aerobacter levanicum* and *Bacillus subtilis*. Growth of *A. viscosus* in media supplemented with 0.1% *A. viscosus* levan resulted in a 33-fold increase and a 7-fold increase in the specific activities of the respective extracellular and cell-associated enzymes when compared with those from 55 mM glucose cultures. Growth in the presence of 29.2 mM sucrose resulted in a 28-fold increase and a 5-fold increase in the specific activities of the respective enzymes when compared with those from the glucose cultures. The extracellular enzyme exhibited high activity over a wide pH range, with 87 and 89% of its pH 6.0 optimum activity at pH 5.0 and 7.0, respectively. The cell-associated enzyme also exhibited optimum activity at pH 6.0, but this was decreased to 10 and 20% at pH 5.0 and 7.0, respectively. Analysis for the presence of extracellular levan during growth of *A. viscosus* in sucrose broths demonstrated that peak levan concentrations occurred during the mid-exponential to late-exponential phase of growth followed by a rapid decline in extracellular levan as a result of levan hydrolase activity.

Several studies have demonstrated sucrose-dependent production of extracellular levan by oral microorganisms such as *Streptococcus salivarius* (12, 33, 39), *Actinomyces viscosus* (17, 25, 32, 34, 42), *Streptococcus mutans* (3, 4, 7, 47), and *Rothia dentocariosa* (27). The presence of levan in dental plaque also has been confirmed (5, 29, 46), and several studies have demonstrated or inferred that at least part of this levan can be degraded by plaque microbial enzymes (6, 14, 16, 30). This suggests that levan might serve as a fermentable plaque carbohydrate which could extend the period of acid production in plaque.

Several streptococcal strains isolated from human plaque have been shown to hydrolyze *S. salivarius* levan (6, 43). Although *S. salivarius* is primarily found in saliva (2, 13, 24, 44), it is possible that this organism could serve as a source of plaque levans. On the other hand, *S. mutans* and *A. viscosus* are important components of human plaque, and both synthesize levans from sucrose (2, 3, 9, 34). Although little is known about levan hydrolase activities toward specific levans produced by isolated strains of *S. mutans*, levan produced by *A. viscosus* has been shown to be hydrolyzed by in vitro plaque and washed whole cells of *Actinomyces naeslundii* (31). An additional report has suggested that *A. naeslundii* also elaborates an extracellular enzyme which hydrolyzes *A. viscosus* levan to free fructose (T. N. Warner and C. H. Miller, Int. Assoc. Dent. Res. Abstr., no. 115, 1975, p. 73). The levan hydrolase activities from either the streptococci or actinomycetes also might be active toward more than one type of levan.

Besides producing levan which might extend acid production in plaque, *A. viscosus* also has been shown to destroy periodontal tissues in experimental animals (18, 20, 21, 23). Several studies have shown that products, components, or metabolites of *A. viscosus* have the potential to induce a variety of immunopathogenic reactions, including delayed hypersensitivity (15), chemotaxis, and acute inflammatory response (11), and lymphocyte transformation (1, 10, 26, 36). The specific involvement of levan in the soft-tissue virulence of *A. viscosus* is not yet clear; however, it recently has been shown that this organism can produce a cell-associated levan in the form of a tenacious capsule (45). A preliminary report also has suggested that purified *A. viscosus* levan can activate the comple-

† Present address: Lilly Research Laboratories, Eli Lilly & Co., Indianapolis, IN 46206.

ment system (T. N. Warner and C. H. Miller, Am. Assoc. Dent. Res. Abstr., no. 410, 1977, p. B154). Thus, it is important to continue to investigate the properties, synthesis, and degradation of *A. viscosus* levan in relation to the virulence of this organism.

A preliminary report has suggested that culture filtrates of *Odontomyces viscosus* hydrolyzed levan synthesized from *Aerobacter levanicum*. (Z. Mesner, Int. Assoc. Dent. Res. Abstr., no. 50, 1971, p. 670). This, along with unpublished observations from our laboratory during studies on levan preparation (32, 45), suggests that *A. viscosus* ATCC 15987 may be able to degrade its own levan. Thus, the present study was undertaken with the following objectives: (i) to establish whether *A. viscosus* can hydrolyze its own levan and (ii) to characterize the end product of such hydrolysis in relation to its ability to serve as a fermentable carbohydrate.

MATERIALS AND METHODS

Microorganisms. *A. viscosus* ATCC 15987, *A. levanicum* ATCC 15552, and *B. subtilis* IUOM B-9 were maintained in the frozen state in Trypticase soy broth (Baltimore Biological Laboratory, Cockeysville, Md.) containing excess $CaCO_3$ and 10% glycerine. For enzyme production a Trypticase (Baltimore Biological Laboratories)-yeast extract (Difco Laboratories, Detroit, Mich.)-salts medium (19) supplemented with various carbohydrates was used. One-liter amounts of this medium contained in 2-liter flasks were inoculated with a 48-h culture of *A. viscosus* in the unsupplemented complex medium previously transferred once from stock. All cultures were incubated at 37°C in air.

Preparation of culture fractions. Cultures of *A. viscosus* as described above were centrifuged at 15,000 × *g* for 15 min at 4°C. Cells used for enzyme assay were washed three times by centrifugation with 0.85% NaCl and resuspended in saline to an absorbance at 520 nm reading of 0.9. This was equivalent to 0.58 mg (dry weight) of cells per ml from 29.2 mM sucrose cultures, 0.64 mg/ml from 55 mM glucose cultures, and 0.46 mg/ml from 55 mM fructose cultures. The extracellular enzyme fraction from the original culture supernatants was prepared by slow addition of ammonium sulfate to 90% saturation with subsequent stirring for 45 min at 4°C. Filtering the culture supernatants through a 0.45-μm membrane filter (Millipore Corp.) before addition of the ammonium sulfate did not alter the protein content or enzyme activity of the final extracellular preparation. The ammonium sulfate-precipitated protein was collected by centrifugation at 15,000 × *g* for 15 min at 4° C and dissolved in a minimal amount of 50 mM sodium acetate buffer, pH 6.0. The protein solution was dialyzed with stirring against 200 volumes of the same buffer for 48 h with two changes of the buffer. In experiments designed to determine the pH optimum of the enzyme, the protein solution was dialyzed against deionized water. This did not alter enzyme activity if the preparation was used within 24 h after dialysis. The dialyzed protein solution was then filtered through a 0.45-μm Millipore membrane filter and analyzed for protein content (28).

Levan preparation. Levan synthesized by washed whole cells of *A. viscosus, A. levanicum*, and *B. subtilis* was prepared as previously described (32, 45). The ethanol-precipitated levan was washed twice with ethanol, dissolved in water, and deproteinized by the method of Sevag et al. (38). The levan solution was dialyzed against deionized water, lyophilized, and stored at −20°C until used. Concentrations of working solutions were based upon dry weight and ketohexose determinations (37). Analyses of acid-hydrolyzed *A. viscosus* levan by thin-layer and paper chromatography has resulted in the detection of only fructose (32). Gel filtration of unhydrolyzed preparations through Sephadex G-75 and G-150 and subsequent analysis of all fractions for hexose, ketohexose, glucose, reducing sugar, and protein have shown that *A. viscosus* levan prepared as described is a pure fructan (32). Passive hemagglutination and immunodiffusion analyses with the antilevan myeloma proteins UPC 10 and UPC 61 have also demonstrated that *A. viscosus* levan contains both $\beta(2\rightarrow6)$ and $\beta(2\rightarrow1)$ linkages (45).

Enzyme assay. Levan-hydrolyzing activity of washed whole cells or of the extracellular preparations was measured in standard reaction mixtures containing 50 mM sodium acetate buffer, pH 6.0, *A. viscosus* levan at 1.0 mg/ml, and enzyme source in a total volume of 3.0 ml. Reaction mixtures and appropriate controls were incubated at 37°C, and the reaction was terminated by placing the marble-covered tubes in a boiling-water bath for 3 min. The reaction mixtures were passed through a 0.45-μm Millipore membrane filter and assayed for total reducing sugar (41). As a precaution, reaction mixtures were also routinely measured for free glucose with the glucose oxidase reagents to detect any contaminating glucose in the enzyme preparations. Such contamination was not observed. Controls consisted of reactions without levan or without enzyme, and complete reaction mixtures which had been heat inactivated at zero time. One unit of levan-hydrolyzing activity is defined as that amount of enzyme preparation which produces 1 μmol of reducing sugar (fructose) per min under the assay conditions. Specific activities are levan-hydrolyzing units per milligram of extracellular protein or per milligram (dry weight) of washed whole cells.

Thin-layer chromatography. Reactants and products from degradation of levan and inulin were separated by thin-layer chromatography. Fifty microliters of reaction mixtures, controls, and standard levan, inulin, fructose, or glucose solutions were spotted on silica gel thin-layer chromatogram sheets (20 by 20 cm, no. 13179, Eastman Kodak Co.) 2 cm from the bottom and at 2-cm intervals. The sheets were developed for 5 h at room temperature in a chloroform–acetic acid–water (3.0:3.5:0.5, volume ratio) solvent system. Sheets were removed from the glass chromatocab, air dried, and sprayed with a mixture of diphenylamine, aniline, phosphoric acid, and acetone (8). Carbohydrate-positive spots were made visible by heating the sheets in an oven for 15 min at 100°C. R_f values of each spot were calculated as the ratio of the distance between the origin and the center of the spot to the distance between the origin and the solvent

front. Inulin and levan appeared as brown spots, whereas fructose and glucose gave pink and blue spots, respectively. Unhydrolyzed levan and inulin remained at the origin as expected, and free fructose and glucose exhibited R_f values of 0.48 and 0.40, respectively.

Sephadex gel filtration. Sephadex G-75 gel filtration was also used to separate reactants and products in enzyme reaction mixtures. The G-75 beads were swelled at 4°C in 0.02% sodium azide, and the slurry was poured into a glass column (2 by 40 cm) and allowed to settle to a height of 32 cm. The column was washed and eluted at 23°C with 0.02% sodium azide at a hydrostatic head pressure of 65 cm. Sample volumes of 5.0 ml were uniformly applied to the surface of the gel, and 5.0 ml fractions were collected with the aid of an automatic fraction collector (Buchler Instruments, Inc.). The column exhibited a void volume of 30 ml with blue dextran and standard *A. viscosus* levan and had a flow rate of 1.25 ml/min. A small column of Sephadex G-75 (1 by 25 cm) was also used to analyze for the production of high-molecular-weight, ketohexose-positive material (levan) in culture supernatants of *A. viscosus*. One-milliliter samples of cell-free culture fluids were applied to the small column and eluted with 0.02% sodium azide at room temperature. The 1-ml fractions collected were then analyzed for ketohexose (37). *A. viscosus* levan standards eluted immediately after the void volume of 7.0 ml from the small column, and fructose, sucrose, and glucose standards eluted after 18.0 ml of effluent had passed through the column. The levan and sucrose peaks from culture filtrates were clearly distinguishable and were separated by seven sugar-negative fractions.

RESULTS

Extracellular levan recovery during growth. The organism was cultured in the complex medium supplemented with 29.2 mM sucrose. At various time periods samples were analyzed for pH and absorbancy at 520 nm and then centrifuged. The supernatants were passed through 0.45-μm Millipore membrane filters, and the cell-free culture fluid was analyzed for high-molecular-weight ketohexose material on Sephadex G-75 as described above. Control samples of uninoculated sucrose medium were also analyzed, and the trace amount of the background high-molecular-weight ketohexose detected was subtracted from all experimental analyses. Figure 1 shows that high-molecular-weight ketohexose material (presumed to be levan) accumulated in 29.2 mM sucrose cultures during the early phases of growth. However, during the late-exponential and stationary phases of growth, the extracellular levan concentration decreased to almost undetectable levels. Similar results (data not shown) were obtained in 50, 87, 116, and 146 mM sucrose cultures. In all cases, peak levan concentrations occurred during the mid-exponential to late-exponential phase of growth followed by a rapid decline in extracellular levan concentration.

Identification of levan degradation products. The standard reaction mixtures contained 1.0 mg of *A. viscosus* levan per ml, 50 mM sodium acetate (pH 6.0), and 0.62 mg of sucrose-cultured washed whole cells per ml or 0.62 mg of extracellular protein per ml. These reaction mixtures and controls in which either levan or enzyme had been eliminated were incubated at 37°C for 0, 3, and 8 h. The reaction was stopped

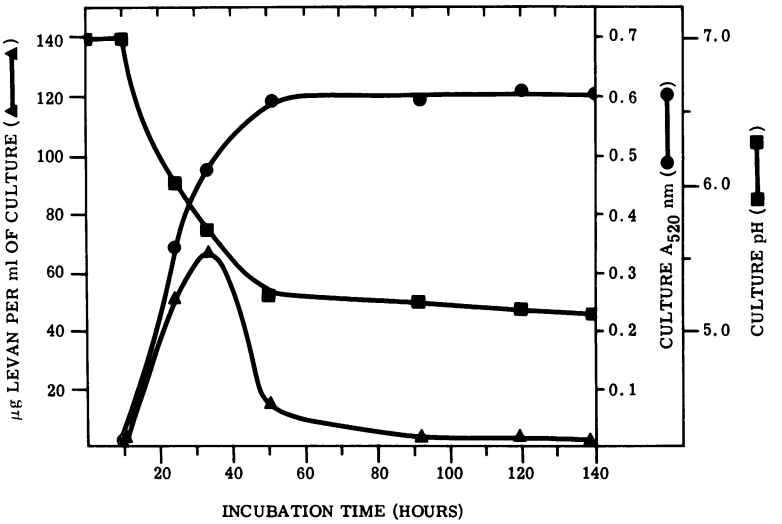

FIG. 1. *Relationship between incubation time and presence of extracellular levan during growth of A. viscosus in sucrose medium. Levan was measured by analyzing for high-molecular-weight, ketohexose-positive material in portions of the cell-free culture fluid after gel filtration through Sephadex G-75. A_{520}, Absorbance at 520 nm.*

by placing the tubes in a boiling-water bath for 3 min and passed through a 0.45-μm Millipore membrane filter. Equal amounts of the filtrates were chromatographed on thin-layer silica gel sheets as described above. Figure 2 shows the chromatogram of reaction components in mixtures containing the extracellular enzyme preparation. A complete reaction mixture in which the enzyme was heat inactivated at zero time exhibited a dark spot at the origin indicative of levan and a very faint spot with an R_f value of 0.40. The 3-h reaction mixture produced three carbohydrate-positive spots: a faint spot at the origin, one with an R_f value identical to that of fructose (0.48), and one with an R_f value of 0.40 identical to that present in the zero-time control. As the incubation time increased, intensities of the fructose and levan spots increased and decreased, respectively. Similar results were obtained from thin-layer chromatography of standard levan degradation reaction mixtures containing washed whole cells as the enzyme source.

Standard reaction mixtures containing either extracellular enzyme preparation (specific activity, 0.048) or washed whole cells (specific activity, 0.02) were also analyzed by gel filtration through a column of Sephadex G-75 (2 by 32 cm) as previously described. The reaction mixtures were incubated at 37°C for 3 h, heat inactivated, passed through a 0.45-μm Millipore membrane filter, and applied to the Sephadex column. Complete reaction mixtures (with either enzyme source) which were heat inactivated at zero time exhibited one ketohexose-positive peak which appeared immediately after the column void volume of 30 ml. This peak corresponded to the levan standard and was completely eluted after a total of 50 ml of effluent had passed through the column. Complete reaction mixtures incubated for 3 h exhibited two ketohexose-positive peaks, one smaller peak immediately after the void volume which repre-

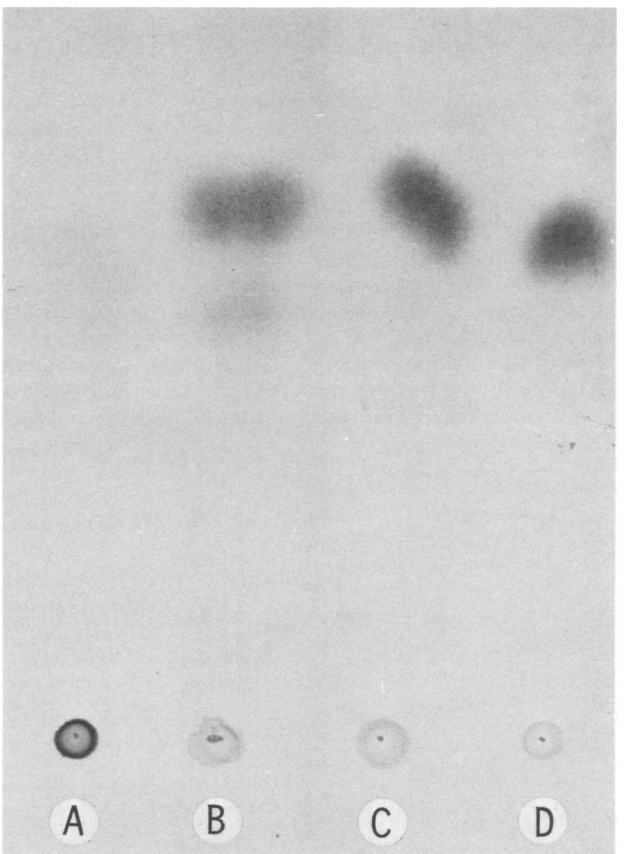

FIG. 2. *Thin-layer chromatogram of levan hydrolase reaction mixtures containing a 90% ammonium sulfate-precipitated extracellular enzyme preparation. (A) Complete reaction mixture (levan, enzyme, and buffer) heat inactivated at zero time. (B) Complete reaction mixture incubated at 37°C for 3 h. (C) Complete reaction mixture incubated at 37°C for 8 h. (D) Fructose standard.*

sented the remaining substrate and a second larger peak which appeared after a total volume of 100 ml had passed through the column. The latter exhibited elution properties identical to those of free fructose.

Standard reaction mixtures and controls containing either extracellular or washed whole-cell enzyme preparations were incubated at 37°C for 4 h and assayed for the production of reducing sugars at various intervals. With both enzyme sources, the micromoles of reducing sugar produced from levan per milliliter of reaction mixture increased proportionally with time up to at least 4 h of incubation. Increases in reducing sugar production after 3 h of incubation were also directly proportional with increases in washed whole cell concentration (up to at least 0.60 mg of cell [dry weight] per ml of reaction mixture) and in extracellular enzyme concentration (up to at least 700 μg of protein per ml).

Extracellular levan hydrolase activity as a function of growth. Identical 200-ml volumes of the complex medium supplemented with 29.2 mM sucrose received identical inoculations of cells grown for 48 h in the unsupplemented complex medium. At different times during aerobic incubation at 37°C, a single 200-ml culture was analyzed for absorbancy at 520 nm, and the extracellular ammonium sulfate enzyme preparation from the cell-free culture supernatant was assayed for levan hydrolase specific activity (Fig. 3). Maximum specific activity was detected during the early stationary phase of growth. The activity remained high during the stationary phase at least up to 100 h of incubation. About 70% of the maximum specific activity was detected at the mid-exponential phase of growth. Cell-associated specific activity was 0.08 after 30 h and decreased to 0.02 at 50 and 100 h of incubation.

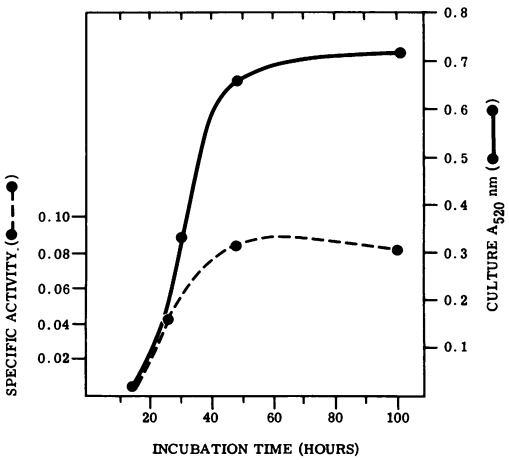

FIG. 3. *Extracellular levan hydrolase activity as a function of growth. A_{520}, Absorbance at 520 nm.*

Effect of growth carbohydrate on enzyme activity. *A. viscosus* was cultured for 30 h at 37°C (mid-exponential phase in sucrose culture) in the medium supplemented with 1.0% sucrose, glucose, fructose or 0.1% *A. viscosus* levan. Each 200-ml culture was inoculated with equivalent amounts of cells previously cultured for 24 h at 37°C in the unsupplemented complex medium. Specific activities for levan degradation by washed whole cells and by the extracellular enzyme preparations were determined from each culture (Table 1). Obvious growth occurred in all cases, and all final culture pH values were below 5.6. Identical inoculation and incubations of the organisms in the unsupplemented medium resulted in poor growth with final culture pH values of 6.7 to 6.9. High specific activities from both cell-associated and extracellular preparations occurred only when sucrose or levan was present in the growth medium. Levan cultures exhibited the highest specific activities (0.165 U/mg of extracellular protein; 0.034 U/mg [dry weight] of whole cells). Glucose cultures exhibited only 18 and 4% of the respective cell-associated and extracellular specific activities of sucrose cultures. Fructose cultures exhibited 33 and 16% of the respective specific activities of sucrose cultures.

Parameters of levan degradation. Variation of the pH of 50 mM acetate buffer from 5.0 to 7.5 in otherwise standard reaction mixtures resulted in the identification of a pH optimum of 6.0 for levan hydrolysis. Both the cell-associated and the extracellular activities exhibited the same optimum; however, deviation from the optimum pH more severely affected the cell-associated activity than the extracellular activity. At pH 5.0 and 7.0 the extracellular activities were 87 and 89%, respectively, of the maximum activity at pH 6.0, whereas those of the cell-

TABLE 1. *Effect of growth medium carbohydrate on levan hydrolase activity*

Medium supplement		Dry wt of cells (mg/ml of culture)	Sp act (% of control)[a]	
			Cell associated	Extracellular
Sucrose	(1.0%)[a]	0.58	100[b]	100[c]
Glucose	(1.0%)	0.64	18	4
Fructose	(1.0%)	0.68	33	16
Levan	(0.1%)	0.46	126	117

[a] Specific activities of the whole-cell and extracellular preparations from the sucrose culture served as the 100% control because this was the standard culture system used throughout the study.

[b] 0.141 U/mg of protein.

[c] 0.027 U/mg (dry weight) of whole cells.

associated activity were only 10 and 20%, respectively, of the maximum activity at pH 6.0.

Both the cellular and extracellular enzyme preparations exhibited maximal levan degradation activity at 37°C when standard reaction mixtures were incubated at temperatures ranging from 25 to 53°C.

The concentration of *A. viscosus* levan was varied from 12.5 to 1,500 μg/ml in otherwise standard reaction mixtures. A Lineweaver-Burk double-reciprocal plot of the washed whole-cell activity versus levan concentration revealed a calculated K_m of 5×10^{-4} M levan based on fructose equivalents. Saturation of the standard amount of the extracellular enzyme (620 μg of protein per ml) was not reached under identical assay conditions. The increase in activity of the latter was linear over the range of 12.5 to 1,500 μg of levan per ml.

Substrate specificity. Both extracellular and cell-associated enzyme preparations were incubated with *A. levanicum* levan, *B. subtilis* levan, inulin, and sucrose in otherwise standard reaction mixtures. Table 2 compares activities toward these substrates with that for *A. viscosus* levan. Both enzyme sources catalyzed the production of reducing sugars from all substrates tested. To partially characterize the nature of the reducing sugars, 8-h reaction mixtures and controls without enzyme or without substrate were chromatographically analyzed on silica gel as previously described. The results with *A. levanicum* levan, *B. subtilis* levan, and inulin were identical to those shown in Fig. 2 for *A. viscosus* levan. The levan or inulin reaction mixtures each produced two spots: a faint spot of unhydrolyzed substrate at the origin and a dark spot with an R_f value identical to that of free fructose. A separate spot corresponding to free glucose from the complete reaction mixtures containing inulin was not detected. This may have been due to the relative low percentage of glucose in the inulin molecule or a retention of the glucose moiety with any unhydrolyzed portion of the inulin polymers remaining at the origin. As expected (31) resolution of sucrose and fructose from the sucrose reaction mixtures was poor on the one-dimensional silica gel system used. Other studies have described degradation of sucrose by *A. viscosus* (22, 34, 35, 42).

TABLE 2. *Substrate specificity*

Substrate[a]	Sp Act[b]	
	Cell associated	Extracellular
A viscosus levan	0.029	0.104
A. levanicum levan	0.035	0.115
B. subtilis levan	0.033	0.090
Inulin	0.012	0.024
Sucrose	0.051	0.026

[a] Final substrate concentrations were 1.0 mg/ml in standard reaction mixtures.

[b] Enzyme sources were obtained from the standard 29.2 mM sucrose cultures incubated for 48 h.

DISCUSSION

Both washed whole cells and cell-free culture supernatant preparations of *A. viscosus* ATCC 15987 hydrolyzed levan. Chemical analysis of complete reaction mixtures containing either enzyme source revealed an increase in the production of reducing sugars which was proportional with increases in incubation time and with enzyme and substrate concentrations. Analysis of the reaction products generated by both the cell-associated and extracellular preparations suggested that the levan was hydrolzyed to free fructose. The results from gel filtration of zero-time and incubated reaction mixtures demonstrated degradation of the levan to a single peak of low-molecular-weight, ketohexose-positive material with an elution pattern identical to that of free fructose. Additional evidence for production of free fructose was revealed by thin-layer chromatographic analysis of complete reaction mixtures incubated for various times. Increased incubation time increased the intensities of carbohydrate-containing spots with chromatographic properties identical to those of free fructose (Fig. 2).

A. viscosus grew when the standard medium used throughout the study was supplemented with levan previously synthesized by strain 15987. Growth in the unsupplemented medium was poor. The total cell yield from growth on levan was 72, 67, and 79% of the cell yields from respective glucose, fructose, and sucrose cultures inoculated and incubated under identical conditions (Table 1). Growth on levan reduced the culture pH from 7.0 to 5.5 after 30 h of incubation. The specific activities of both the cell-associated and extracellular levan hydrolase were also the highest in levan and sucrose cultures when compared with those associated with glucose and fructose cultures (Table 1). The glucose and fructose cultures even exhibited the highest cell yields. These data suggest that *A. viscosus* not only utilizes levan as a fermentable carbohydrate, but also may be induced to synthesize levan hydrolase when cultured in the presence of levan or sucrose.

Measurement of extracellular levan levels during growth of the organism in the presence of sucrose revealed that during the late-exponential and stationary phases of growth the extra-

cellular levan concentration decreased to very low levels (Fig. 1). This decrease in extracellular levan concentration coincided with that portion of the growth phase in which extracellular levan hydrolase specific activity was at its maximum (Fig. 3). The culture pH during the period of levan degradation was also at a level (5.5 to 5.2) which would allow for extracellular levan hydrolase activity. At pH 5.0 the enzyme exhibited 87% of its maximum activity expressed at pH 6.0. Thus, it appears that the decrease in extracellular levan during the stationary phase of growth resulted from hydrolysis. However, this does not preclude the possibility that a small portion of the extracellular levan may have become associated with the cell surface and, thus, would not have been detected in the cell-free culture fluid. This latter possibility seems plausible in light of recent findings which demonstrated that sucrose-grown *A. viscosus* cells possess a low level of cell-associated levan (45). On the other hand, the cell-associated levan of sucrose-grown cells was also shown to partially exist as a loose slime rather than as a tenacious capsule which is present on glucose-grown cells subsequently incubated with sucrose (45). This previous study also shows that the maximum amount of levan found to be associated with the surface of *A. viscosus* cells is about 2.0 $\mu g/10^9$ cells or about 0.02 to 0.03% of the cellular dry weight (45). Assuming that this relationship is reasonably accurate, cell binding of extracellular levan during the stationary phase of growth could not have accounted for the total decrease in extracellular levan observed in the 500-ml sample culture analyzed as described in Fig. 1. There were not sufficient cells present to bind the total 32 mg of levan produced in this culture during the exponential phase of growth. Thus, it appears that the decrease in extracellular levan concentration primarily resulted from the action of levan hydrolase.

The relationship between levan synthesis and degradation during growth of strain 15987 in the presence of sucrose is not exactly clear. The rate of degradation may have simply exceeded that of synthesis during the stationary phase of growth when the hydrolase appeared to be at its maximum activity. On the other hand, the levansucrase may have been inactivated during the stationary phase as suggested by Van der Hoeven et al. (42) for strain Ny 1 cultures. If such inhibitors were present in strain 15987 cultures, the levan synthesized during the early phases of growth should have remained at a relatively constant level throughout the stationary phase in the absence of a levan hydrolase. In the presence of a levan hydrolase, a rapid decline in extracellular levan concentration would be expected, as observed in the present study.

The possibility exists that the observed hydrolysis of levan was actually a reversal of levan synthesis catalyzed by levansucrase. Although it was not the intent of this investigation to purify and fully characterize the enzyme involved, another study which included such an objective has shown that purified levansucrase from *A. viscosus* Ny 1 does not catalyze levan hydrolysis (42).

Previous investigations of levan synthesis by *A. viscosus* have not concentrated on experiments designed to study levan degradation. However, a report by Krichevsky et al. (25), which described levan formation by strain 15987 as a function of the level and type of carbohydrate in the growth medium, presented data which showed an increase in extracellular levan concentration which decreased after 100 h of growth in 5.0% (146 mM) sucrose. However, no such decrease was noted in 0.25% (7.3 mM) or 0.1% (2.9 mM) sucrose cultures under identical aerobic conditions of incubation. A 30% decrease from the exponential-phase concentration of extracellular levan was noted when these investigators analyzed for levan present in 0.5% (14.6 mM) sucrose cultures incubated under an argon gas phase (25).

Both the cell-associated and extracellular enzyme preparations hydrolyzed levans from *A. viscosus*, *A. levanicum*, and *B. subtilis* along with inulin (Table 2). Thus, it appears that the activity expressed may be that of the nonspecific type of β-fructofuranosidase capable of attacking both $\beta(2\rightarrow1)$ and $\beta(2\rightarrow6)$ fructofuranoside linkages. Both of these types of linkages are present in *A. viscosus* levan (45), whereas inulin has only the $\beta(2\rightarrow1)$-linked fructofuranosides. A nonspecific fructan hydrolase from *Saccharomyces fragilis* has been characterized (40). On the other hand, the crude enzyme preparations used in the present study may have contained more than one enzyme, each with specificity toward a single type of fructofuranoside linkage.

Great care must be taken when discussing the potential importance of *A. viscosus* levan synthesis and degradation in the human oral cavity based upon data describing the activities of animal strains of this organism. Until recently, only animal strains such as 15987 from the hamster or Ny 1 from the rat have been used in studies on *A. viscosus* levan synthesis. The studies of Pabst (34) on levan synthesis by the human strain T-14 V and the preliminary report on levansucrases of fresh human isolates of *A. viscosus* and *A. naeslundii* (T. N. Warner and D.

Holland, Int. Assoc. Dent. Res. Abstr. no. 1131, 1978, p. 357) should greatly facilitate studies involving levan and its potential activities in the human oral cavity. If such studies indeed show that human strains of *A. viscosus* are also capable of degrading levan in a fashion similar to that reported here for strain 15987, then it will be clear that this species has the potential to extend acid production in human plaques containing *A. viscosus* levan.

ACKNOWLEDGMENT

A portion of this work was supported by Public Health Service grant DE 03537 from the National Institute of Dental Research.

LITERATURE CITED

1. **Baker, J. J., S. P. Chan, S. S. Socransky, J. J. Oppenheim, and S. E. Mergenhagen.** 1976. Importance of *Actinomyces* and certain gram-negative anaerobic organisms in the transformation of lymphocytes from patients with periodontal disease. Infect. Immun. **13**:1363-1368.
2. **Carlsson, J.** 1967. Presence of various types of non-haemolytic streptococci in dental plaque and in other sites of the oral cavity in man. Odontol. Revy **18**:55-74.
3. **Carlsson, J.** 1970. A levansucrase from *Streptococcus mutans*. Caries Res. **4**:97-113.
4. **Ceska, M.** 1971. Biosynthesis of levan and a new method for the assay of levansucrase activity. Biochem. J. **125**:209-211.
5. **Critchley, P., J. M. Wood, C. A. Saxton, and S. A. Leach.** 1967. The polymerization of dietary sugars by dental plaque. Caries Res. **1**:112-129.
6. **Da Costa, T., and R. J. Gibbons.** 1968. Hydrolysis of levan by human plaque streptococci. Arch. Oral Biol. **13**:609-617.
7. **Dahlquist, A., B. Krasse, J. Olsson, and S. Gardell.** 1967. Extracellular polysaccharides formed by caries-inducing streptococci. Helv. Ondontol. Acta **11**:15-21.
8. **DeStefanis, V. A., and J. G. Ponte, Jr.** 1968. Separation of sugars by thin-layer chromatography. J. Chromatogr. **34**:116-120.
9. **Ellen, R. P.** 1976. Establishment and distribution of *Actinomyces viscosus* and *Actinomyces naeslundii* in the human oral cavity. Infect. Immun. **14**:1119-1124.
10. **Engel, D., J. Clagett, and R. Page.** 1975. A possible role for bacterial mitogens in chronic inflammatory periodontal disease. J. Periodontol. **46**:630.
11. **Engel, D., D. Van Epps, and J. Clagett.** 1976. In vivo and vitro studies of possible pathogenic mechanisms of *Actinomyces viscosus*. Infect. Immun. **14**:548-554.
12. **Garszczynski, S. M., and J. R. Edwards.** 1973. Synthesis of a broth levan by a cell-bound levansucrase from *Streptococcus salivarius*. Arch. Oral Biol. **18**:239-251.
13. **Gibbons, R. J., B. Kapsimalis, and S. S. Socransky.** 1964. The source of salivary bacteria. Arch. Oral Biol. **9**:101-103.
14. **Gold, W., F. B. Preston, M. C. Lache, and H. Blechman.** 1974. Production of levan and dextran in plaque in vivo. J. Dent. Res. **53**:442-446.
15. **Guggenheim, B., and H. E. Schroeder.** 1974. Reactions in the periodontium to continuous antigenic stimulation in sensitized gnotobiotic rats. Infect. Immun. **10**:565-577.
16. **Higuchi, M., Y. Iwami, T. Yamada, and S. Araya.** 1970. Levan synthesis and accumulation by human dental plaque. Arch. Oral Biol. **15**:563-567.
17. **Howell, A., and H. V. Jordan.** 1967. Production of an extracellular levan by *Odontomyces viscosus*. Arch. Oral Biol. **12**:571-573.
18. **Irving, J. T., S. S. Socransky, and J. D. Heeley.** 1974. Histological changes in experimental periodontal disease in gnotobiotic rats and conventional hamsters. J. Periodont. Res. **9**:73-80.
19. **Jordan, H. V., R. J. Fitzgerald, and A. E. Bowler.** 1960. Inhibition of experimental caries by sodium metabisulfite and its effect on the growth and metabolism of selected bacteria. J. Dent. Res. **39**:116-123.
20. **Jordan, H. V., and P. H. Keyes.** 1961. Aerobic, gram-positive, filamentous bacteria as etiologic agents of experimental periodontal disease in hamsters. Arch. Oral Biol. **9**:401-414.
21. **Jordan, H. V., P. H. Keyes, and S. Bellack.** 1972. Periodontal lesions in hamsters and gnotobiotic rats infected with *Actinomyces* of human origin. J. Periodont. Res. **7**:21-28.
22. **Kiel, R. A., J. M. Tanzer, and F. N. Woodiel.** 1977. Identification, separation, and preliminary characterization of invertase and β-galactosidase in *Actinomyces viscosus*. Infect. Immun. **16**:81-87.
23. **Keyes, P. H., and H. V. Jordan.** 1964. Periodontal lesions in Syrian hamsters. III. Findings related to an infectious and transmissible component. Arch. Oral Biol. **9**:377-400.
24. **Krasse, B.** 1954. The proportional distribution of *Streptococcus salivarius* and other streptococci in various parts of the mouth. Odontol. Revy **5**:203-211.
25. **Krichevsky, M. I., A. Howell, and S. Lim.** 1969. Levan formation by *Odontomyces viscosus*. J. Dent. Res. **48**:938-942.
26. **Lehner, T., M. A. Wilton, S. J. Challacombe, and L. Ivanyi.** 1974. Sequential cell-mediated immune response in experimental gingivitis in man. Clin. Exp. Immunol. **16**:481-492.
27. **Lesher, R. J., and V. F. Gerencser.** 1977. Levan production by a strain of *Rothia*: activation of complement resulting in cytotoxicity for human gingival cells. J. Dent. Res. **56**:1097-1105.
28. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:365-375.
29. **McDougall, W. A.** 1964. Studies on the dental plaque. IV. Levan and the dental plaque. Aust. Dent. J. **9**:1-5.
30. **Manly, R. S., and D. T. Richardson.** 1968. Metabolism of levan by oral samples. J. Dent. Res. **47**:1080-1086.
31. **Miller, C. H.** 1974. Degradation of sucrose by whole cells and plaque of *Actinomyces naeslundii*. Infect. Immun. **10**:1280-1291.
32. **Miller, C. H., T. N. Warner, C. J. Palenik, and P. J. B. Somers.** 1975. Levan formation by whole cells of *Actinomyces viscosus* ATCC 15987. J. Dent. Res. **54**:906.
33. **Newbrun, E., and S. Baker.** 1968. Physicochemical characteristics of the levan produced by *Streptococcus salivarius*. Carbohydr. Res. **6**:165-170.
34. **Pabst, M. J.** 1977. Levan and levansucrase of *Actinomyces viscosus*. Infect. Immun. **15**:518-526.
35. **Palenik, C. J., and C. H. Miller.** 1975. Extracellular invertase activity from *Actinomyces viscosus*. J. Dent. Res. **54**:186.
36. **Patters, M. R., R. J. Genco, M. J. Reed, and P. A. Mashimo.** 1976. Blastogenic response of human lymphocytes to oral bacterial antigens: comparison of individuals with periodontal disease to normal and edentulous subjects. Infect. Immun. **14**:1213-1220.
37. **Roe, J. H., J. H. Epstein, and N. P. Goldstein.** 1949. A photometric method for the determination of inulin in plasma and urine. J. Biol. Chem. **178**:839-845.
38. **Sevag, M. G., D. B. Lackman, and J. Smolens.** 1938. The isolation of the components of streptococcal nucleoproteins in serologically active form. J. Biol. Chem.

124:425-436.

39. **Snyder, M. L., H. M. Hackedorn, D. O. Martin, and D. D. Johnston.** 1955. The synthesis of mucinous polysaccharides from sucrose by oral bacteria. J. Dent. Res. **34**:368-379.
40. **Snyder, H. E., and H. J. Phaff.** 1960. Studies on a beta-fructosidase (inulase) produced by *Saccharomyces fragilis*. Antonie van Leewenhoeck J. Microbiol. Serol. **26**:433-452.
41. **Somogyi, M.** 1945. A new reagent for the determination of sugars. J. Biol. Chem. **160**:61-68.
42. **Van der Hoeven, J. S., G. D. Vogels, and M. F. J. Bekkers.** 1976. A levansucrase from *Actinomyces viscosus*. Caries Res. **10**:33-48.
43. **Van Houte, J., and H. M. Jansen.** 1968. Levan degradation by streptococci isolated from human dental plaque. Arch. Oral Biol. **13**:827-830.
44. **Van Houte, J., R. J. Gibbons, and S. B. Banghart.** 1970. Adherence as a determinant of the presence of *Streptococcus salivarius* and *Streptococcus sanguis* on the human tooth surface. Arch. Oral Biol. **15**:1025-1034.
45. **Warner, T. N., and C. H. Miller.** 1978. Cell-associated levan of *Actinomyces viscosus*. Infect. Immun. **19**:711-719.
46. **Wood, J. M.** 1967. The amount, distribution and metabolism of soluble polysaccharides in human dental plaque. Arch. Oral Biol. **12**:849-858.
47. **Wood, J. M., and P. Critchley.** 1966. The extracellular polysaccharide produced from sucrose by a cariogenic *Streptococcus*. Arch. Oral Biol. **11**:1039-1042.