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Levan and Levansucrase of *Actinomyces viscosus*

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A levansucrase was demonstrated in the growth medium and in association with the cell surface of *Actinomyces viscosus*. The amount of enzyme produced relative to cell density is not significantly affected by the growth conditions. Sugar alcohols inhibit growth of the cells. The levansucrase hydrolyzes sucrose to produce free glucose and levan; some free fructose is also formed. There is no requirement for cofactors. The K_m for sucrose is 12 mM. A variety of heavy metal ions and two disaccharides, lactose and cellobiose, inhibit the enzyme. The levansucrase was purified to homogeneity and has a specific activity of 90 μmol of glucose released per min per mg. The enzyme has a molecular weight of 220,000 and is composed of subunits of molecular weight 80,000. The levan product contains both β(2→1) and β(2→6) linkages. The enzyme remains tightly bound to the levan product, resulting in the formation of high-molecular-weight polymer on the order of 10^8 daltons. The possible role of the levan and levansucrase of *A. viscosus* in the pathogenesis of periodontal disease is discussed.

Human strains of actinomyces cause plaque, root-surface caries, and bone loss when monoinfected into germfree animals (8, 18, 29). *Actinomyces viscosus* produces extracellular levan (16, 24). This could be important in the pathogenesis of periodontal disease, because: (i) levan may be an important structural polysaccharide in plaque (7, 18), (ii) levan may provide a reservoir of carbohydrate for the growth of oral microbes (5, 15, 22), (iii) levan may trigger immunological injury, since levan from other organisms has been shown to be mitogenic for human B-lymphocytes (17) and to activate complement by the alternative pathway (6).

This report describes some of the properties of the levan produced by *A. viscosus*, and describes the purification and properties of levansucrase, the enzyme responsible for levan production. Recently, a report from vander Hoeven's laboratory appeared describing the properties of a partially purified levansucrase from another strain of *A. viscosus*. This enzyme appears to be similar to the one described here in that it has a similar isoelectric point, pH optimum and temperature optimum, and shows similar substrate and inhibitor specificity (14). Regarding levan, Miller and his colleagues made an estimate of the average molecular weight of levan produced by *A. viscosus* in culture and found it to be at least 5×10^7, in good agreement with the molecular weight estimate given here for levan produced by the purified levansucrase (24).

MATERIALS AND METHODS

Materials. *A. viscosus*, strain T-14V, virulent, isolated from a human oral cavity, was used in this work. The organism has been shown to form plaque and to cause periodontal disease in animals (S. Socransky, personal communication). B. F. Hammond (University of Pennsylvania, School of Dental Medicine and Center for Oral Health Research) has studied this organism and kindly supplied it to the author (12). Except where specified, cells were grown in Trypticase-yeast extract medium (TY) having the following composition: Trypticase (BBL), 17 g/liter; yeast extract (BBL), 4 g/liter; sodium chloride, 5 g/liter; and dipotassium phosphate, 2.5 g/liter.

[U-^{14}C]sucrose (394 mCi/mmol) was purchased from Amersham/Searle. [U-^{14}C-fructose]sucrose (275 mCi/mmol) and [U-^{14}C-glucose]sucrose (261 mCi/mmol) were purchased from New England Nuclear.

Sugars tested as inhibitors were obtained from Sigma Chemical Co. Descriptions of the di- and trisaccharides tested are given herewith: sucrose, α-D-glucopyranosyl-β-D-fructofuranoside; trehalose, α-D-glucopyranosyl-α-D-glucopyranoside; raffinose, α-D-galactopyranosyl(1→6)α-D-glucopyranosyl(1→2)β-D-fructofuranoside; melezitose, α-D-glucopyranosyl(1→3)β-D-fructofuranosyl(2→1)α-D-glucopyranoside; palatinose, 6-α-D-glucopyranosyl-D-fructose; turanose, 3-α-D-glucopyranosyl-D-fructose; thiodigalactoside, *S*-β-D-thiogalactopyranosyl-*S*-β-D-thiogalactopyranoside; cellobiose, 4-β-D-glucopyranosyl-D-glucose; lactose, 4-β-D-galactopyranosyl-D-glucose; maltose, 4-α-D-glucopyranosyl-D-glucose; melibiose, 6-α-D-galactopyranosyl-D-glucose.

Myeloma proteins. BALB/c mouse myeloma protein UPC 61, which is specific for β(2→1) fructan

linkages, and BALB/c myeloma protein UPC 10, which is specific for $\beta(2\rightarrow6)$ linkages, were generously provided by Michael Potter of the National Cancer Institute, Bethesda, Md. The amount of each myeloma protein present in sera was determined by quantitative precipitin reactions with *A. viscosus* levan (4).

Levansucrase purification. Enzyme was obtained from 18 liters of culture supernatant. The enzyme was precipitated by adding ammonium sulfate to 70% final concentration. The precipitated material was collected by centrifugation, suspended in water, and dialyzed against 0.01 M tris(hydroxymethyl)aminomethane (Tris)-chloride (pH 8.0). The material was then applied to a column (5 cm diameter by 32 cm) of diethylaminoethyl (DEAE)-cellulose (Whatman DE-52) equilibrated with the Tris buffer. The column was rinsed with buffer, and then levansucrase was eluted with a 1-liter linear gradient of 0 to 0.15 M KCl in buffer. The active fractions were pooled, dialyzed against 0.01 M potassium phosphate (pH 7.0), and then applied to a column (2.5 cm diameter by 40 cm) of hydroxylapatite (Bio-Rad) equilibrated with the phosphate buffer. The column was rinsed with buffer; the enzyme was then eluted with a 400-ml linear gradient of 0.01 to 0.20 M potassium phosphate (pH 7.0). The active fractions were pooled, dialyzed against 0.1 M potassium phosphate (pH 7.0), and then applied to a column (2.5 cm diameter by 90 cm) of Agarose A-0.5m (Bio-Rad) equilibrated with the 0.1 M phosphate buffer. The active fractions were pooled, dialyzed against 0.002 M potassium phosphate (pH 7.2), and then applied to a second column (0.9 cm diameter by 9 cm) of hydroxylapatite. The enzyme was eluted with a 150-ml linear gradient of 0.002 to 0.025 M potassium phosphate. Active fractions were analyzed by gel isoelectric focusing. Those fractions which were free from contamination were pooled and concentrated by ultrafiltration using an Amicon PM-10 membrane.

Gel isoelectric focusing. Gel isoelectric focusing was perfomed as described in Bio-Rad Technical Bulletin 1030 (1). For narrow-range focusing, pH 3 to 5, 40% of pH 3 to 10 range ampholytes and 60% of pH 3 to 5 range ampholytes were used. The gels were 0.6 cm in diameter by 10 cm in length. Focusing was carried out at a maximum of 200 V and 1 mA per tube for 7 h. Gels were stained with Coomassie blue by the procedure of Righetti and Drysdale (26).

SDS gel electrophoresis. Sodium dodecyl sulfate (SDS) gel electrophoresis was performed by the procedure of Weber and Osborn (30), except that the composition of the gel was altered to allow further migration of the high-molecular-weight subunits into the gel. The gel used was 7.5% total acrylamide with only 1% of the total acrylamide being the methylene bisacrylamide cross-linker.

Spectrophotometric levansucrase assay. The spectrophotometric levansucrase assay is based upon Sigma's ultraviolet glucose assay kit no. 15. This assay kit measures glucose enzymatically, using hexokinase and glucose-6-phosphate dehydrogenase. The amount of glucose in the sample is proportional to the amount of reduced nicotinamide adenine dinucleotide phosphate (NADPH) generated. To convert this assay kit into a continuous spectrophotometric assay for levansucrase, sucrose is added. The sucrose is hydrolyzed by levansucrase to produce glucose. Levansucrase assays were performed in a Cary 118C recording spectrophotometer at 340 nm. Glucose assay (0.75 ml), prepared as directed, was put in a cuvette; 0.05 ml of 1 M sucrose was then added. The assay was started by addition of enzyme sample plus water to yield a total volume in the cuvette of 1.0 ml. The rate of appearance of NADPH was directly proportional to levansucrase concentration up to 0.5 μmol of glucose formed per min, corresponding to an absorbance change of 0.08/min.

Radioassay of levansucrase. The radioassay was adapted from an assay used for dextransucrase (10). The total volume was 0.1 ml. The assay contained 0.1 M potassium phosphate (pH 6.5), 50 mM sucrose, and 0.19 μCi of [U-^{14}C]sucrose. At time 0, levansucrase was added and the mixture was incubated for exactly 5 min at 30°C. At 5 min, a 20-μl aliquot of the assay mix was withdrawn and spotted on a 1-cm square of Whatman 3MM paper. The square was immediately placed in a beaker containing absolute methanol. The squares of paper were washed extensively with methanol and then counted by liquid scintillation.

Fructose assay. Phosphoglucose isomerase, type X, was obtained from Sigma Chemical Co. as a lyophilized powder. To convert the Sigma glucose assay kit to an assay which measures both glucose and fructose, a few micrograms of the dry phosphoglucose isomerase was added to the cuvette containing the standard glucose assay.

RESULTS

Localization of levansucrase activity. To determine whether levansucrase is located primarily extracellularly, on the cell surface, or intracellularly in *A. viscosus*, a culture was grown in 50 ml of TY in a 250-ml Erlenmeyer flask in a shaking incubator for 60 h at 37°C. A portion of the culture (20 ml) was homogenized in a Braun cell homogenizer. The culture was kept cold during the agitation by a stream of liquid carbon dioxide. Breakage of the cells was confirmed by phase-contrast microscopy. A second portion of the culture was centrifuged for 30 min at 45,000 $\times$ *g* to obtain a cell-free culture supernatant.

The levansucrase activity of the whole intact culture was 95 nmol/min per ml, using the spectrophotometric assay. The homogenized culture had less activity—50 nmol/min per ml. The activity of the cell-free culture supernatant was 18 nmol/min per ml. Therefore, the bulk of the levansucrase activity is associated with the surface of the cells, although a considerable amount can be found in the culture supernatant. No additional activity was released by

homogenizing the cells; in fact, some activity was lost. An attempt was made to disrupt the cells ultrasonically to see if a more gentle disruption would preserve the activity. However, the cells proved resistant to sonic oscillation.

To simplify the purification and characterization of the protein, the cell-free supernatant was used as the source for further study. However, there is no evidence that rules out the possibility that the cell-bound enzyme and the free enzyme may be different.

Nature of the sucrose hydrolyzing activity. To be sure that the activity being measured was that of levansucrase, and not invertase or some other enzyme, assays were performed which measured the incorporation of radioactive sucrose into methanol-insoluble polysaccharide. Various ^{14}C-labeled sucrose preparations were tested for their ability to incorporate ^{14}C into polymer. Uniformly labeled sucrose was used routinely to assay levansucrase activity (see Materials and Methods). In addition, in this experiment, sucrose preparations specifically labeled in either the glucose or fructose moiety were used (see Materials and Methods). Cell-free supernatant (100 μl) was added last to each of three assay mixtures containing 135 μl of potassium phosphate (0.1 M, pH 7.0), 5 μl of 1.0 M cold sucrose, plus water to a total volume of 250 μl. The first assay mixture also contained 0.47 μCi of uniformly labeled sucrose. The second assay contained 0.25 μCi of sucrose specifically labeled in the glucose moiety, and the third assay contained 0.20 μCi of sucrose specifically labeled in the fructose moiety. At various times after the addition of culture supernatant, 20-μl aliquots of the assay mixture were removed and spotted on a 1-cm square of Whatman 3 MM paper. The squares were thoroughly rinsed in methanol and then counted. When the uniformly labeled sucrose was used, the activity found was 23 nmol/min per ml of culture supernatant. When sucrose specifically labeled in the fructose moiety was used, the activity was 20 nmol/min per ml. When sucrose specifically labeled in the glucose moiety was used, there was no activity detectable. (The assay could have detected less than 1 nmol/min per ml of glucose incorporated.) Therefore, despite inaccuracies in the assays inherent in assaying large volumes of crude material having low activity, all of the sucrose hydrolyzing activity detected in the culture supernatant, using the spectrophotometric assay, could be accounted for by the production of levan. There was no detectable production of glucans.

Effect of growth conditions on the concentration of levansucrase in the culture. *A. viscosus* was grown at 37°C for different lengths of time in 10 ml of TY, containing a variety of sugars, each at a concentration of 0.1% by weight. All of the cultures were inoculated with 0.1 ml of a 2-day-old culture of *A. viscosus* grown in TY without any sugar. The results of one experiment are summarized in Table 1. Two phenomena were observed repeatedly. First, although sucrose, fructose, and glucose are apparently metabolized by *A. viscosus*, and these sugars allow the organism to grow more rapidly than it would in the absence of sugar or in the presence of the other sugars listed, the amount of levansucrase activity present in

TABLE 1. *Effect of sugars in the medium on the production of levansucrase*[a]

Sugar	Levansucrase activity (nmol/min/ml)					
	24 h of growth			48 h of growth		
	Culture density (A_{660})	Supernatant	Whole culture	Culture density (A_{660})	Supernatant	Whole culture
None	0.25	2.7	16	0.70	24	140
Fructose	0.75	5.1	17	1.20	26	140
Sucrose	0.72	8.8	21	1.25	32	160
Raffinose	0.22	4.3	13	0.90	61	240
Lactose	0.26	3.2	16	0.63	37	280
Cellobiose	0.24	3.5	13	0.59	22	150
Melezitose	0.20	4.0	20	0.50	22	120
Glucose	0.87	6.0	23	1.20	39	170
Palatinose	0.33	4.0	18	0.83	40	200
Mannose	0.27	10.5	48	0.55	40	120
Xylitol	0.14	2.7	13	0.48	21	100
Sorbitol	0.17	3.5	8	0.45	19	70

[a] *A. viscosus* was grown in 10 ml of TY with the addition of 0.1% of the indicated sugars. Culture supernatants were prepared by centrifuging a portion of the whole culture at 45,000 × *g* for 30 min. Levansucrase was assayed spectrophotometrically.

these more rapidly growing cultures is not significantly greater than that found in the culture containing no added sugar. Second, the sugar alcohols, xylitol and sorbitol, apparently inhibit growth. Accompanying this inhibition is a decrease in levansucrase activity.

Although there are some differences in Table 1, there is, on the whole, no evidence of any significant induction or repression of levansucrase activity.

The data in Table 1 were obtained with cells grown aerobically. *A. viscosus* grows well anaerobically when provided with sucrose, glucose, or fructose. Levansucrase activity in the culture is comparable to that obtained with aerobic cells of the same density. Growth is poor and levansucrase activity is correspondingly low if the organism is grown anaerobically in the absence of fermentable sugar.

Purification of levansucrase. Levansucrase was purified from *A. viscosus* cultures grown in TY. No sugar was added to the medium, since addition of sugar does not increase the yield of enzyme, whereas addition of sugar could complicate the purification. Twelve cultures, each 1.5 liter in a 2-liter flask were grown aerobically with shaking for 40 h at 37°C. The 18 liters of culture was centrifuged and the enzyme was purified from the supernatant (see Materials and Methods). A summary of the purification is presented in Table 2. The enzyme was purified 37,000-fold to a specific activity of 90 μmol/min per mg of protein, with an $\epsilon^{1\%}_{280\ nm} = 17$. Levansucrase was the only enzyme encountered in the course of the purification capable of hydrolyzing sucrose.

To assess the purity of the final preparation, 15 μg of purified protein was subjected to gel isoelectric focusing, using narrow-range, pH 3 to 5, ampholytes. When stained with Coomassie blue, two bands less than 1-mm apart appeared. When the gels were sliced, activity could be detected in three 1-mm slices in the position corresponding to the two bands. The pH of the active fractions was pH 4.2, indicating the isoelectric point of the levansucrase. One gel was placed into a 25% sucrose solution. After 2 h, turbidity was observed in the gel at a position corresponding to the stained bands. However, after 2 h of diffusion, it was not possible to say with certainty whether both bands were active.

Since this microheterogeneity in narrow-range isoelectric focusing was the only indication of non-homogeneity that was observed in characterizing the enzyme, further work proceeded on the assumption that the enzyme was pure by normal standards but that it might exist in two forms differing slightly in charge.

The enzyme remained stable over the course of 6 months when stored frozen in 10 mM potassium phosphate (pH 7) at −15°C. The activity was not affected by repeated freezing and thawing.

Molecular weight and subunit composition. An estimate of molecular weight was made using a standardized agarose gel filtration column. When chromatographed in Agarose A-0.5m (Bio-Rad), the enzyme eluted in a single symmetrical peak at a position corresponding to a molecular weight of 220,000. Beef liver catalase (247,000), rabbit muscle aldolase (180,000), and bovine serum albumin (65,400) were used, along with blue dextran, as standards.

SDS gel electrophoresis was performed to estimate the molecular weight of the subunits. Levansucrase displayed a single sharp band, corresponding to a subunit molecular weight of 80,000. *Escherichia coli* β-galactosidase (130,000), rabbit muscle phosphorylase a (94,000), and bovine serum albumin (68,000) were used as standards.

The above results suggest that levansucrase is composed of three subunits of equal molecular weight. Alternatively, if the enzyme deviated substantially from a spherical shape, so that the molecular weight estimated by gel filtration was too high, a two-subunit model of 160,000 molecular weight might be possible.

TABLE 2. *Purification of levansucrase*[a]

Step	Volume (ml)	Activity (μmol/min/ml)	Protein (mg/ml)	Sp act (μmol/min/mg)	Purification (fold)	Yield (%)
Crude culture supernatant	18,000	0.022	9.0	0.0024	1	100
Dialyzed $(NH_4)_2SO_4$ concentrate	1,350	0.22	4.0	0.059	25	76
DEAE-cellulose pooled peak	140	1.46	3.2	0.46	192	52
Hydroxylapatite pooled peak	9.5	16.2	1.7	9.5	3970	39
Agarose A-0.5m pooled peak	7.4	19.8	0.25	79	33,000	37
Hydroxylapatite pooled peak	1.4	13.4	0.15	90	37,000	5

[a] Activity was determined by the spectrophotometric assay; protein was determined by the Lowry method.

Effect of pH and temperature on levansucrase activity. Using the radioassay, a study of the effect of pH on activity showed that levansucrase is active over a broad range of pH. Maximum activity was observed at pH 6.0. At the pH extremes of 4.0 and 9.0, 40% of the activity remained. At pH 7.5, the enzyme showed 82% of the maximum activity. Acetate, phosphate, and glycylglycine buffer (0.1 M) were used in their appropriate ranges. Tris was found to be an inhibitor.

Using the radioassay, the enzyme was found to be 32% more active at 37 than at 25°C, and 18% more active at 30 than at 25°C. Sucrose hydrolysis as measured by the spectrophotometric assay showed similar increases with temperature.

K_m for sucrose. A K_m for sucrose of 12 mM was determined by the spectrophotometric assay. The double reciprocal plot of velocity versus sucrose concentration was linear over a range of sucrose concentrations from 3 to 100 mM.

Substrate specificity. A number of di- and trisaccharides were tested to determine whether they could serve as substrates for the synthesis of polysaccharide by levansucrase. Sucrose and raffinose were substrates, producing visible turbidity when incubated at 30°C for several hours in the presence of levansucrase. The sugars were present at a concentration of 0.1 M, in 0.01 M potassium phosphate buffer (pH 6.5), to which 0.05% sodium azide was added. As expected, lactose and cellobiose were not substrates. However, neither was melezitose nor trehalose a substrate, both of which contain a "high energy" diglycosidic bond similar to that found in sucrose. Both raffinose and melezitose are trisaccharides composed of sucrose with a third sugar attached. In raffinose, a galactose is linked $\alpha(1\rightarrow6)$ to the glucose moiety of sucrose. In melezitose, a glucose is linked $\alpha(1\rightarrow3)$ to the fructose moiety of sucrose.

In a related experiment, a number of sugars were tested for their ability to be hydrolyzed by levansucrase, irrespective of polymer synthesis. Liberation of free glucose was measured by the standard spectrophotometric assay, substituting the sugar to be tested for sucrose. Only in the case of sucrose was free glucose liberated. Raffinose, of course, was also hydrolyzed; however, hydrolysis of the diglycosidic bond of raffinose yields melibiose plus the fructose which is incorporated into levan. The spectrophotometric assay is not sensitive to melibiose. Hydrolysis of melezitose, however, would yield glucose plus turanose. No glucose was detected, nor was glucose detected as a conseqeunce of hydrolysis of lactose, cellobiose, trehalose, turanose, or palatinose.

Stoichiometry of sucrose hydrolysis versus levan formation. In the course of experiments utilizing the purified levansucrase, several opportunities arose which allowed a careful comparison of the activity of this purified, standardized enzyme preparation in the two assay systems. It became apparant that the enzyme showed slightly less activity when assayed by the radioassay which measures polymer production than when assayed by the spectrophotometric assay, which measures only sucrose hydrolysis. These observations suggested that some proportion of the total fructose obtained by hydrolysis may be released as free fructose. To test for the production of free fructose, the standard spectrophotometric assay was modified to include an excess of phosphoglucose isomerase, which converts fructose-6-phosphate to glucose-6-phosphate. Using the standard spectrophotometric assay, the purified enzyme showed an activity of 13.5 μmol/min per ml. When the modified assay was used which included phosphoglucose isomerase, the activity observed was 17.4 μmol/min per ml. Thus, 3.9 μmol/min per ml of free fructose was released (17.4 − 13.5 = 3.9), and 9.6 μmol/min per ml of levan was formed (13.5 − 3.9 = 9.6). Therefore, at pH 7.5 and 25°C, under the conditions of the standard spectrophotometric assay, 71% of the fructose is converted into polymer and 29% is released into the medium. Similar assays were done at 30 and 37°C. The proportion of fructose released to fructose incorporated was not significantly affected by the change in temperature.

Inhibition of levansucrase. As previously noted, Tris (Sigma Chemical Co., reagent grade) is an inhibitor. When 0.1 M Tris was compared with 0.1 M potassium phosphate, at pH 7.5, Tris produced a 60% inhibition, and at pH 8.0 Tris produced an 80% inhibition of levan formation measured by the radioassay.

The effects of a variety of metal ions and chelators were tested in the radioassay, substituting 0.1 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer (pH 6.5) for the standard phosphate buffer. Ethylenediaminetetraacetic acid (EDTA) (10 mM) and diethyldithiocarbamate (10 mM) had no effect on the activity. Ca^{2+}, Mg^{2+}, Mn^{2+}, and Co^{2+}, each at a final concentration in the assay of 1 mM, had no significant inhibitory effect. However, a number of metal ions at the same concentration did produce inhibition: Zn^{2+}, 87%; Pb^{2+}, 84%; Cd^{2+}, 88%; Cu^{2+} (as tartrate), 63%.

A number of agents active against sulfhydryl

or disulfide groups were tested. The agents to be tested were added at a final concentration of 1 mM. Dithiothreitol, glutathione, *p*-hydroxymercuribenzoate, and iodoacetate had no inhibitory effect.

Sodium azide (0.05%) and sodium fluoride (10 mM) were found not to be inhibitors, making possible their use as bacteriostatic agents in long incubations.

A large assortment of sugars were tested for their ability to inhibit levansucrase. To optimize the susceptibility of the enzyme to inhibition, the concentration of sucrose substrate in the radioassay was reduced to 10 mM, i.e., approximately the K_m level. Each of the sugars to be tested was also present at 10 mM. EDTA (0.2 mM) was added to prevent any interference from metal ions which might be present. Results of the inhibition tests are shown in Table 3. For ease of discussion, the sugars have been grouped into categories. Of the monosaccharides, only D-glucose produced significant inhibition. Since D-glucose is a product of the reaction, the 38% inhibition is what might be expected for a product inhibitor. Interestingly, D-fructose, which might be called a side-product of the reaction, was not an inhibitor. Although they do depress the growth of *A. viscosus*, resulting in lower enzyme concentrations in cultures (Table 1), the sugar alcohols, xylitol and sorbitol, do not have any inhibitory effect on the enzyme itself. The two thio-sugars tested had no effect.

TABLE 3. *Inhibition of levansucrase by various sugars*[a]

Type of sugar	Sugar	Inhibition (%)
Monosaccharides	D-Glucose	38
	D-Fructose	2
	D-Mannose	4
	D-Galactose	13
	D-Xylose	14
	D-Ribose	8
Sugar alcohols	Xylitol	0
	D-Sorbitol	4
Thiol sugars	Thioglucose	0
	Thiodigalactoside	0
Disaccharides	Lactose	64
	Cellobiose	64
	Maltose	12
	Trehalose	0
	Palatinose	0
	Turanose	0
	Melibiose	10
Methyl glycosides	α-Methyl-D-glucoside	5
	β-Methyl-D-glucoside	0
	α-Methyl-D-galactoside	12
	β-Methyl-D-galactoside	12
Trisaccharides	Melezitose	8
	Raffinose	21

[a] The radioassay for levansucrase was used, modified to contain 10 mM of the sugar to be tested for inhibition.

The results with the disaccharides were surprising. Sugars which seem to be related to sucrose, such as trehalose (diglucoside), palatinose [α(1→6) glucosyl fructose], and turanose [α(1→3) glucosyl fructose], were not inhibitors. Maltose and melibiose also were ineffective. However, lactose and cellobiose were very good inhibitors, both causing 65% inhibition. These two sugars have in common a β(1→4) glycosidic linkage; but they are not in any obvious way related to sucrose, so that their effectiveness as inhibitors was unexpected. Neither lactose nor cellobiose is a substrate for the enzyme; they are not converted to polymer nor are they hydrolyzed.

Since two β(1→4)-linked disaccharides were effective inhibitors, α- and β-methyl glucosides and galactosides were tested to see if the β-linkage by itself was inhibitory. The methyl glycosides were not effective inhibitors.

Two trisaccharide analogues of sucrose were tested. Raffinose is an alternate substrate, and therefore was found to be an inhibitor, as expected. Melezitose, which is not a substrate, was not an effective inhibitor.

Synthesis of levan by levansucrase. A batch of levan was prepared by adding 4.5 μg of purified enzyme to 10 ml of 0.1 M sucrose containing 0.05% sodium azide in 0.01 M potassium phosphate buffer (pH 6.5). The mixture was incubated at 30°C for 24 h. Approximately 2 h after the start of the incubation, visible turbidity developed. After 24 h the mixture was centrifuged for 30 min at 45,000 × *g*. A transparent gelatinous-looking pellet beneath a clear supernatant resulted. (The supernatant was treated with 3 volumes of acetone, but no precipitate was formed.) The pellet was suspended in 0.5 ml of water and then lyophilized. A yield of 8.4 mg of levan was obtained, which is 5% of the theoretical yield. (The low yield may result from enzyme becoming trapped in levan product.) Complete sedimentation within 30 min at 20,000 rev/min in the Beckman JA-20 rotor indicated a sedimentation coefficient of the order of $S(\times 10^{13}) = 1{,}100$. Such an S value would correspond to a biological particle of molecular weight approximately 10^8.

Gel filtration of levan. A small column (0.9 cm diameter by 30 cm) was packed with Agarose A-50m (Bio-Rad) and equilibrated with 0.1 M potassium phosphate (pH 6.5). A small sample of radiolabeled levan was prepared by incubating 3 μg of levansucrase in 0.2 ml of the

phosphate buffer containing 50 mM sucrose and 0.38 μCi of uniformly labeled [^{14}C]sucrose for 1 h at 30°C. The reaction proceeded to 50% completion. The incubation mixture was then applied to the column. The elution profile is shown in Fig. 1 (blue dextran was used to mark the void volume of the column.) As shown by the elution profile, both the labeled levan and the levansucrase activity eluted in the void volume of the column. Agarose A-50m excludes globular particles of molecular weight greater than 5×10^7, confirming the estimate of the 10^8 molecular weight of the levan, based on sedimentation behavior. Note the virtual absence of levan of molecular weight low enough to be retained by the column. Very little radioactivity appears after the void volume until the total volume of the column is reached, at which point unused sucrose and the products, glucose and fructose, appear.

The enzyme remains firmly bound to the levan product on passage through the column. The elution position of levansucrase in the absence of levan was determined. The free enzyme eluted at the expected point near the total volume of column as indicated on Fig. 1.

A second experiment was performed on this column, in which a sample of radiolabeled levan was prepared as before, except that the incubation was for 4 min instead of 1 h, and 1.5 μg of enzyme was used rather than 3 μg. The purpose of this second experiment was to determine whether the same results observed in the first experiment would be obtained if the formation of the levan were allowed to proceed to no more than 5% of completion. The results of this second experiment were identical to those of the first experiment. Very high-molecular-weight levan was formed, to which the enzyme adhered, even after only 4 min of incubation, during which time no more than 5% of the sucrose could have been utilized.

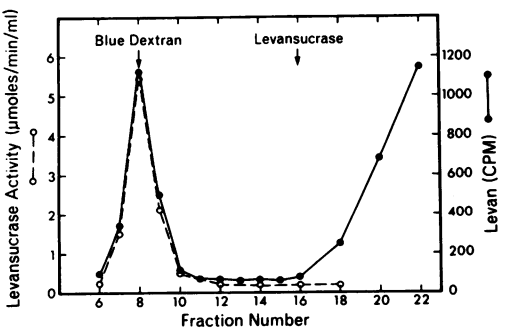

FIG. 1. *Agarose A-50m gel filtration chromatography of ^{14}C-labeled levan in the presence of levansucrase. The rise in radioactivity starting at fraction number 18 is due to [^{14}C]sucrose, [^{14}C]glucose, and [^{14}C]fructose eluting at the total volume of the column. The elution position of blue dextran (Pharmacia), which marks the void volume, and the elution position of levansucrase, when chromatographed in the absence of sucrose, are marked with arrows. Levansucrase was assayed spectrophotometrically.*

Linkages in the levan product. Two well-characterized myeloma proteins were tested for their ability to precipitate the levan produced by the purified levansucrase. One of the myeloma proteins (UPC 61) reacts specifically with $\beta(2\rightarrow1)$-linked fructans whereas the other (UPC 10) reacts specifically with $\beta(2\rightarrow6)$-linked fructans. Both myeloma proteins precipitated the levan, indicating that the product of the purified enzyme contains both $\beta(2\rightarrow1)$ and $\beta(2\rightarrow6)$ linkages (Fig. 2). In a similar gel diffusion experiment, concanavalin A also precipitated the levan, suggesting that the levan has a branched structure. Concanavalin A has been shown to bind to branched but not to linear dextrans (11).

DISCUSSION

A levansucrase from the culture medium of *A. viscosus* has been purified 37,000-fold to homogeneity by the criterion of gel isoelectric focusing. The enzyme is a polymeric protein of molecular weight 220,000, having three (or possibly two) apparently identical subunits of molecular weight 80,000. The enzyme catalyzes the production of a high-molecular-weight levan (approximately 10^8 daltons) from sucrose. The specific activity of the purified enzyme is 90

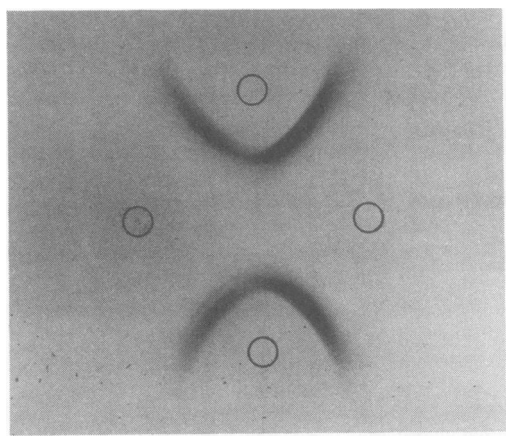

FIG. 2. *Precipitation of levan by myeloma proteins specific for $\beta(2\rightarrow1)$ and $\beta(2\rightarrow6)$ fructan linkages. The upper well contains levan prepared using the purified enzyme. The lower well contains levan prepared from the crude ammonium sulfate precipitate. The right-hand well contains protein UPC 10 specific for $\beta(2\rightarrow6)$ linkages, and the left-hand well contains protein UPC 61 specific for $\beta(2\rightarrow1)$ linkages.*

μmol of sucrose hydrolyzed per min per mg of protein. With a molecular weight of 220,000, this specific activity corresponds to a turnover number of 20,000 molecules of sucrose hydrolyzed per min per molecule of polymeric enzyme.

The levan produced contains both β(2→1) and β(2→6) linkages, as demonstrated by binding of the levan to myeloma proteins specific for these two linkages. This observation, together with the binding of levan by concanavalin A, suggests that the levan is branched. Since the enzyme appears to be homogeneous, a single levansucrase protein must be capable of producing both the β(2→1) and β(2→6) linkages.

The tight binding between levansucrase and its product, levan, as shown by the agarose gel filtration experiment, suggests an explanation for the uniformly high molecular weight of the levan. If the enzyme were free to dissociate from the product levan at each step of the polymerization, then one might expect to find a relatively large number of low-molecular-weight molecules, whereas if the enzyme tends to remain bound to a particular polymeric molecule, the result will be a few molecules of high molecular weight. Assuming a molecular weight of 220,000 for the enzyme and 10^8 for the levan, in the experiment where 4.5 μg of enzyme produced 8.4 mg of levan, the stoichiometry is such that 2.2×10^{-11} mol of enzyme produced 8.4×10^{-11} mol of levan. Therefore, one molecule of enzyme produces one, or at most a few, molecules of levan.

The tight binding of levansucrase to levan is also of interest when considered together with the observation that a large amount of levansucrase activity is found associated with the cell surface. On the assumption that the cell-associated enzyme and the free enzyme are similar or identical, the binding of the levan to the enzyme and of the enzyme to the cell surface suggests that the levan-levansucrase system may play a role in the aggregation of *A. viscosus* in plaque. Specific tests of the ability of levan to promote cell adherence to surfaces and to promote cell-to-cell aggregation should be undertaken to clarify the plaque-forming potential of the levan-levansucrase system.

Of course, levan-levansucrase is only one facet of the total mechanism of pathogenicity of *A. viscosus*. It is clear from animal studies that *A. viscosus* can cause periodontal disease when introduced into normal or germfree animals which are maintained on low-sucrose diets. However, the severity of the disease appears to be worse in animals maintained on high sucrose diets (19, 29).

Recently, *A. viscosus* has been shown to aggregate in the presence of high-molecular-weight dextrans, such as those produced by *Streptococcus sanguis* and *Streptococcus mutans* (21). In addition, dextran has been shown to mediate interbacterial aggregation between dextran-synthesizing streptococci and *A. viscosus* (2). Although dextran-induced aggregation is undoubtedly important in the mixed flora of normal plaque, the ability of *A. viscosus* to produce plaque and periodontal disease when monoinfected into germfree rats indicates that dextran is not an indispensable component of the pathogenic mechanism of *A. viscosus*.

In addition to levan, *A. viscosus* also produces an extracellular mucopolysaccharide in the absence of sucrose (13, 28). This mucopolysaccharide may be involved in plaque formation by *A. viscosus*. However, the polysaccharide is produced in large amounts by the avirulent strain of *A. viscosus* T-14, but its production has not been observed in the virulent strain (12), which calls into question the significance of the mucopolysaccharide in the etiology of periodontal disease.

Thus, although it appears that levan may not be an absolute requirement for periodontal disease induction by *A. viscosus*, it may be an important contributor to the disease. The levans which are produced by cariogenic streptococci, and which have been implicated in plaque formation by these microbes, are very similar to the levan of *A. viscosus* in having a high molecular weight and a branched structure (3, 9, 27). It would be surprising if the levan of *A. viscosus* did not also contribute to plaque formation.

In evaluating the role of levan in the pathogenicity of *A. viscosus*, the potential for levan to serve as a reservoir of carbohydrate for the microorganisms of plaque should also be considered. A number of common oral organisms, including *A. viscosus*, have been reported to possess levanase activity (5, 15, 22, 23). In addition, levan may interact with the immune system by activating lymphocytes or by activating complement (6). Levan from *Corynebacterium levanformis* has been shown to be mitogenic for human B-lymphocytes (17).

Should the levan-levansucrase system of *A. viscosus* prove to be pathologically significant, the results of this work suggest ways of minimizing the production of levan by *A. viscosus* in vivo. Obviously, minimizing sucrose in the diet would be helpful. Since complete elimination of dietary sucrose is an impractical goal, other measures could be taken. Xylitol, a sweetener shown to be effective in minimizing caries (25), also tends to depress the growth of *A. viscosus*, which, in turn, tends to limit the production of

levansucrase and levan. Sorbitol, though not as sweet as xylitol, is also effective at slowing the growth of *A. viscosus*. Finally, the observation that lactose and cellobiose are good inhibitors of levansucrase may be put to practical use. Diets could be arranged in such a way as to accompany or follow an intake of sucrose with an intake of lactose: milk with cookies, for example.

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