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THE CELL WALL-ASSOCIATED LEVANSUCRASE OF *ACTINOMYCES VISCOSUS*

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Summary

Actinomyces viscosus produces both a soluble extracellular levansucrase and a cell wall-associated levansucrase. The enzyme from cell walls was solubilized by lysozyme digestion. The soluble extracellular and cell wall-associated forms of the enzyme were compared and appeared to be identical, based on molecular weight estimations, kinetic parameters, and reactions with antisera. The product of both forms of the enzyme was a high molecular weight, branched levan, as shown by its reactivity with myeloma proteins specific for $\beta(2 \rightarrow 1)$ and for $\beta(2 \rightarrow 6)$ linkages in fructosans. Although levansucrase remained tightly bound to the levan which it synthesized, the enzyme did not bind to exogeneously added levan.

Regarding the potential pathogenicity of the levan product, pure levan, produced using purified levansucrase, did weakly activate complement by the alternative pathway. However, the pure levan did not directly cause bone resorption in an in vitro bone resorption assay.

Introduction

Actinomyces viscosus, an organism which has been implicated in the etiology of periodontal disease [1—3], utilizes sucrose to produce an extracellular levan [4—6]. In a previous paper [7], one of us described a levansucrase found in the growth medium of *A. viscosus*. At that time, it was noted that the bulk of

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levansucrase activity was associated with the cell surface. The question arose whether the cell surface levansucrase was identical with the levansucrase found in the culture medium. In this paper, the association of levansucrase with the isolated bacterial cell wall fraction is described and the extracellular and cell wall-associated enzymes are demonstrated to be identical. These experiments also showed that a single enzyme produced both $\beta(2 \rightarrow 1)$ and $\beta(2 \rightarrow 6)$ linkages in the levan product, and that the levan product remained permanently attached to the enzyme.

We also wished to evaluate whether the levan produced could contribute to the pathogenicity of *A. viscosus*. Levan produced by the purified levansucrase activated complement by the alternative pathway, as has been shown with other preparations of levan [8—11]. However, it was not possible to demonstrate a direct involvement of levansucrase in either aggregation of *A. viscosus* or in bone resorption which are two properties of *A. viscosus* probably important in its pathogenicity.

Materials and Methods

Bacteria. The virulent (V) and avirulent (AV) strains of *A. viscosus* T14 were kindly provided by Dr. B.F. Hammond (University of Pennsylvania, School of Dental Medicine). Cells were grown in Trypticase-yeast extract as described previously [7].

Preparation of cell walls. Bacteria were harvested by centrifugation and washed several times with 50 mM Tris-HCl (pH 7.8). A 20% suspension (wet weight) of cells in Tris-HCl was disrupted by shaking for 4 min with glass beads (0.10—0.11 mm diameter) in a Braun MSK cell homogenizer. Liquid CO_2 was used for cooling. The disrupted cells were washed and suspended in 1 M NaCl, 0.05% NaN_3. Cell walls were separated from whole cells by allowing the cell suspension to settle in the cold over a period of several days. The supernatant, containing the cell walls, was collected by aspiration and was again allowed to settle. This operation was repeated 6 times. Cell walls were then washed several times with 1 M NaCl, 0.05% NaN_3, followed by several washes with water prior to lyophilization. Electron microscopy of thin sections prepared from cell wall preparations confirmed the absence of whole bacterial cells.

Lysozyme digestion of cell walls. 250 mg lyophilized *A. viscosus* T14AV cell walls were digested for 6 days at 37°C with 25 mg lysozyme in 15 ml 0.1 M ammonium acetate buffer (pH 6.25), 0.1% NaN_3. The cell wall-associated levansucrase was partially purified from this lysozyme digest. The T14AV strain was selected for use because its cell wall is essentially free of the virulence-associated antigen [12] which we have found to contaminate enzyme preparations obtained from *A. viscosus* T14V cell walls.

Enzymatic methods. The previously described spectrophotometric assay for levansucrase was used [7]. Specific activity is expressed as μmol of sucrose hydrolyzed/min per mg of protein. Protein was determined by the method of Lowry et al. [29] using bovine serum albumin as standard.

Partial purification of the cell wall-associated levansucrase. A lysozyme digest of cell walls from *A. viscosus* T14AV was dialyzed against 0.01 M Tris-HCl (pH 8.0), then applied to a column (1.3 × 28 cm) of DEAE-cellulose

(Whatman DE52), equilibrated with Tris-HCl. The column was rinsed with Tris-HCl to remove lysozyme and then eluted with a linear gradient of KCl (0.01—0.30 M) in Tris-HCl. A peak of enzyme activity was eluted toward the end of the gradient (7.3—9.3 mS at 25°C). The fractions were pooled, and dialyzed against 0.1 M ammonium acetate (pH 6.6) and then applied to a column (1.5 X 60 cm) of Bio-Gel Agarose A-0.5 m (Bio-Rad). The enzyme eluted at a position corresponding to 250 000 daltons for a globular protein, using standards previously described [7]. This peak of activity was pooled and used for further study.

Antisera. 20 intravenous injections of live *A. viscosus* cells were used to immunize female New Zealand white rabbits. The cell suspension (A_{650} = 2.0) was administered on alternate days in increasing amounts starting with 0.1 ml/injection and ending with 2 ml/injection (last nine injections). Blood was collected 7 days after the last injection. Immunization with both the virulent and avirulent strains of *A. viscosus* T14 induced the formation of antibodies against several antigens including levansucrase. Antisera partially absorbed with bacterial cell walls were found to be specific for levansucrase in immunodiffusion experiments. Presumably the cell walls absorbed antibodies against the other cell wall antigens more efficiently than they absorbed antibodies against levansucrase. The particular antiserum (R14-2) used in this study was made using *A. viscosus* T14AV as the antigen. Absorption with T14AV cell walls (5 mg dry cell walls/ml serum) and T14V cell walls (4 mg dry cell walls/ml serum) rendered antiserum R14-2 specific for levansucrase as determined by gel diffusion tests. Absorption with T14V cell walls was necessary for efficient removal of antibodies against the virulence-associated antigen [12].

Fructosan-specific mouse IgA myeloma proteins. Protein UPC-10 which is specific for $\beta(2 \rightarrow 6)$ linkages and protein UPC-61 which is specific for $\beta(2 \rightarrow 1)$ linkages have been described [7,14].

Immunochemical methods. Gel diffusion experiments were performed by the plastic template method [15]. Visibility of precipitated levansucrase was enhanced by incubating the gel diffusion plate in a 2% sucrose solution. Quantitative precipitin assays were done by a microtechnique and the amount of precipitate was measured by the ninhydrin method for nitrogen [14]. Sera were dialyzed against saline to remove glucose which interferes with the levansucrase assay.

Bone resorption assay. The ability of levan to stimulate bone resorption was assayed in a previously described bone organ culture system [16]. Bone rudiments from 19-day fetal rats were explanted and cultured in BGJ medium with or without levan. Resorption was quantitated by the release of previously incorporated ^{45}Ca from the bones. Medium and bone ^{45}Ca were counted by liquid scintillation, and the results expressed as the percent of total bone ^{45}Ca released into the medium. A synthetic bovine parathyroid hormone (1—34 peptide; Beckman), a potent stimulator of ^{45}Ca release in this system, was used as a positive control. In addition to levan, the medium from an *A. viscosus* culture was tested for its effect on ^{45}Ca release. The T14V strain was grown in BGJ medium for 3 days; the medium was then passed through a 0.2 μm membrane, diluted 1 : 2 with fresh medium and assayed for stimulation of bone resorption.

Complement activation by levan. Activation of the alternative complement pathway by levan was assessed by incubating 1.2 ml of normal pooled human serum with 120, 12, or 1.2 μg levan or with 12 μg of lipopolysaccharide for 1 h at 37°C. The serum samples were put on ice; then aliquots were assayed for the amount of alternative complement activity remaining, using a kinetic assay which measured the rate of lysis of unsensitized rabbit erythrocytes by the disappearance of turbidity at 700 nm [13]. Under the conditions employed, the amount of complement activity present in the samples is inversely proportional to the $T_{1/2}$ or the time required to lyse 50% of the erythrocytes [13].

Results

Purification of cell-associated levansucrase from isolated cell walls

Both the virulent (V) and the avirulent (AV) strains of *A. viscosus* T14 synthesize a cell-associated levansucrase, as well as a soluble extracellular levansucrase [7] (Pabst, M.J., unpublished data). Cell walls were prepared from avirulent cells. Cell-associated levansucrase activity (0.78 μmol/min per mg) was found in the isolated cell wall preparation. The residual cellular material, which was divided by ultracentrifugation at 105 000 × *g* for 1 h into cytoplasmic and membrane fractions, had no detectable activity (less than 0.01 μmol/min per mg).

The cell-associated enzyme is attached firmly to the bacterial cell wall. This was shown by the inability of various salts in high concentration to extract the enzyme from cell walls. 8 M LiCl, which removed levansucrase from *Streptococcus salivarius* [17], inactivated the enzyme from *A. viscosus.* Other salts, 2 M KCl, 2.5 M $(NH_4)_2SO_4$, and 6 M CsCl, had no effect on enzyme activity, and also did not remove the enzyme from the cell walls.

Lysozyme digestion was found to solubilize greater than 90% of the *A. viscosus* cell wall including the associated levansucrase.

Levansucrase was partially purified from the lysozyme digest by ion exchange and gel filtration chromatography. The elution positions of the cell wall-associated levansucrase on DEAE-cellulose and on agarose chromatography (Bio-Gel A-0.5 m) were very similar to those observed for the extracellular enzyme [7]. Gel filtration of the cell wall enzyme indicated a molecular weight of approx. 250 000, whereas a molecular weight of 220 000 was estimated for the extracellular enzyme.

The enzyme was purified 64 fold from the isolated cell walls (which contained 0.78 μmol of sucrose hydrolyzing activity/mg of Lowry protein) to a final specific activity of 50 μmol/min per mg. The recovery was 36%. Only one sucrose-hydrolyzing enzyme was encountered in the course of the purification from the cell walls. The cell wall enzyme preparation did contain a contaminating cell wall antigen which was detected by antisera against whole *A. viscosus* cells.

Kinetic properties of the extracellular and cell wall-associated enzymes

Both forms of the enzyme displayed normal hyperbolic substrate saturation curves with the same K_m for sucrose: 12 mM. As expected, the specific activities of the two preparations were also similar, though not identical. At 50 mM

sucrose (standard assay conditions), the extracellular enzyme had a specific activity of 90 μmol/min per mg, whereas the cell wall-associated enzyme had a specific activity of 50 μmol/min per mg.

Reaction of the levansucrase preparations with antisera

Several rabbit antisera against live *A. viscosus* cells were assayed for their abilities to inhibit or enhance levansucrase activity, and none of the sera had any effect on enzyme activity. However, in quantitative precipitin tests, an antiserum against *A. viscosus* T14AV precipitated all the levansucrase activity from both enzyme preparations.

Immunodiffusion analysis using the antiserum specific for levansucrase showed that the extracellular and cell wall-associated forms of the enzyme were antigenically identical (Fig. 1, Step A). Addition of sucrose to the center well resulted in the rapid and dramatic formation of a high molecular weight levan by the immunoprecipitated levansucrase (Fig. 1, Step B). The synthesized levan reacted with a mouse myeloma protein (UPC-10), specific for $\beta(2 \rightarrow 6)$ linkages, and with another mouse myeloma protein (UPC-61), which is specific for $\beta(2 \rightarrow 1)$ linkages (Fig. 1, Step C). The fusion of the precipitin arcs formed by the two mouse myeloma proteins and the absence of any spurs indicated that individual polysaccharide molecules contained both $\beta(2 \rightarrow 6)$ and $\beta(2 \rightarrow 1)$ linkages. In its entirety, Fig. 1 demonstrates that a branched levan is formed from sucrose by the activities of a single enzyme or enzyme complex.

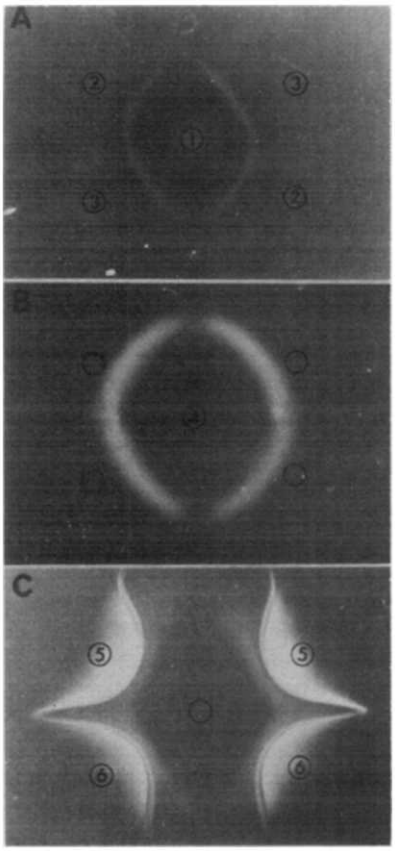
Binding of levansucrase to levan

Levansucrase was incubated with sucrose and the reaction mixture was applied to a column of Bio-Gel Agarose A-50m (exclusion limit $50 \cdot 10^6$ daltons). The enzyme, together with its high molecular weight product levan, eluted at the void volume of the column (Fig. 2A). This confirms the previous finding [7] that the enzyme binds tightly to its product levan. Since the column completely separated sucrose from the enzyme-levan complex, continuous levan synthesis was not required to maintain the association between levansucrase and levan.

Levansucrase was then incubated with preformed levan in the absence of sucrose and the mixture was chromatographed on the same column of Agarose A-50m (Fig. 2B). The levan emerged at the void volume and was not associated with any levansucrase activity. The levansucrase activity appeared in its normal position near the total volume of the column. (A similar experiment using levan which had been boiled and washed by centrifugation gave the same result.) Thus, levansucrase binds tightly to its own product but is unable to bind to preformed levan.

Properties of the levan product

A high purity sample of levan was prepared by incubating sucrose with a catalytic amount of the purified extracellular levansucrase [7]. The ability of this levan to stimulate bone resorption was tested by measuring the release of ^{45}Ca from fetal rat long bones cultured with the substance to be tested [16]. As shown in Table I, levan at various concentrations had no effect on the release of ^{45}Ca. However, the medium from *A. viscosus* culture does contain a



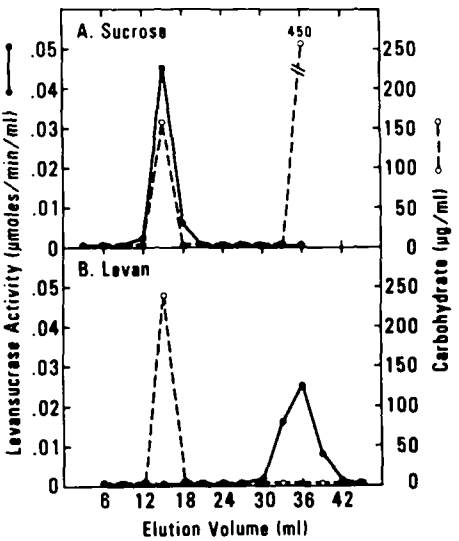


Fig. 1. Formation of a branched levan from sucrose by immunoprecipitated levansucrase. The gel diffusion plate was rinsed in saline between each step. Step A. Reaction of antiserum specific for levansucrase (well 1) with extracellular levansucrase (wells 2) and with cell-bound levansucrase (wells 3) showing antigenic identity between the two forms of the enzyme. The amount of enzyme activity added to wells 2 and 3 was approx. 800 nmol sucrose hydrolyzed/min (approx. 10 μg). Step B. Synthesis of levan by the immunoprecipitated levansucrase. Sucrose (500 μg) was added to the center well (4). Step C. Reaction of fructosan-specific mouse myeloma proteins with levans formed in Step B by the extracellular and cell-bound forms of levansucrase. 375 μg β(2 → 6) specific protein, UPC-10, was added to the upper wells (5). 150 μg β(2 → 1) specific protein, UPC-61 was added to the lower wells (6).

Fig. 2. Agarose A-50m gel filtration chromatography of levansucrase which was incubated either with 3.6 mg (50 mM) sucrose (A) or with 1 mg preformed levan (B) prior to chromatography. The incubations were performed in 0.2 ml 0.1 M potassium phosphate (pH 7.0) for 1 h at 37°C. Levansucrase activity was measured spectrophotometrically. Levan, which appeared in the void volume (elution volume 15 ml), and sucrose and glucose, which appeared in the total volume of the column (elution volume 39 ml), were measured by the phenol-sulfuric acid reaction [28].

potent stimulator of ^{45}Ca release; this material, referred to as Actinomyces Resorption Factor, is currently under study [20].

This high purity levan was also tested for its ability to activate complement by the alternative pathway. Levan was incubated with human serum for 1 h at 37°C; then the amount of alternative complement activity remaining was determined by the kinetic assay. Untreated serum (diluted 1 : 12) lysed 50% of the erythrocytes in 9.3 ± 0.3 min (S.E., n = 5). Serum treated with levan (100 μg/

TABLE I

EFFECT OF LEVAN ON THE RELEASE OF PREVIOUSLY INCORPORATED ^{45}Ca FROM FETAL RAT BONES IN ORGAN CULTURE

Values are means ± S.E. for 6—8 bones cultured for 48 h in the presence or absence (control) of the substance tested.

Substance tested	Percent of total ^{45}Ca released
Control	17 ± 0.3
Levan (100 μg/ml)	16 ± 0.6
Levan (10 μg/ml)	17 ± 0.6
Levan (1 μg/ml)	16 ± 0.8
Levan (0.1 μg/ml)	17 ± 0.5
Parathyroid hormone (0.1 μg/ml)	51 ± 4.0 *
A. viscosus medium (1 : 2 dilution)	41 ± 4.0 *

* Significantly different from control, $P < 0.005$ (Student's one-tailed t test for independent variables).

ml) required 11.7 ± 1.2 min. Serum treated with a more potent activator of complement, lipopolysaccharide at 10 μg/ml, required 24.5 ± 1.8 min. Under these conditions, as a first approximation, the amount of complement activity remaining in the sample is inversely proportional to the time required for 50% lysis [13]. Thus, levan depleted the complement activity of serum by only 20%, while lipopolysaccharide at a 10-fold lower concentration reduced the activity by more than 60%. Lower concentrations of levan were even less effective.

Discussion

As reported earlier by ourselves [7] and others [5,19], the bulk of levansucrase activity produced by *A. viscosus* was found in association with the cell surface. This cell-bound levansucrase activity was located on isolated cell walls. Unlike the levansucrase of *Strep. salivarius* [17], the levansucrase of *A. viscosus* could not be removed from the cell walls with concentrated salt solutions. The cell walls were digested with lysozyme in order to solubilize the enzyme. Whether there is a covalent association between the enzyme and the bacterial cell wall remains to be determined.

However, apart from the attachment to the cell wall, the extracellular soluble levansucrase and the cell wall-associated levansucrase seemed to be identical. The two proteins behaved similarly on ion exchange and gel filtration chromatography. They showed similar values for K_m and specific activity, and they reacted identically with antisera. In addition, the levan products of both enzymes appeared to be branched, based on their reactions with mouse myeloma proteins specific for $\beta(2 \rightarrow 6)$ and $\beta(2 \rightarrow 1)$ linkages in fructosans. The observation that levan produced by the immunoprecipitated levansucrase reacted with both myeloma proteins provides strong support for our earlier suggestion [7] that a single enzyme produces both types of linkage in the levan product. This may reflect the presence of more than one kind of catalytic site or more than one type of subunit within the levansucrase molecule.

The pathogenic potential of levan was evaluated by examining its ability to

activate various host effector mechanisms. Prompted by reports that *A. viscosus* cells activated complement [8,10] and that levan from *A. viscosus* [11] and from another bacterium [9] activated complement, a high purity sample of levan made with purified levansucrase was tested. This preparation of levan did weakly activate complement.

Experiments of our own [20] and by others [21] indicated that *A. viscosus* released into its growth medium a substance capable of stimulating resorption of bone in organ culture. In contrast, the high purity levan had no direct effect on bone resorption. (Crude *A. viscosus* levan, isolated from the growth medium or isolated by incubating cells with sucrose, might be expected to show a positive response because of contaminating Actinomyces Resorption Factor [20].) Of course, in vivo, levan might indirectly cause bone resorption by activating complement [22].

We suggested previously that levan and levansucrase might mediate the aggregation of *A. viscosus* [7]. However, levansucrase did not bind to preformed levan. Therefore, the levan/levansucrase system probably does not act as a bridge between cells, since to bind cells together, a molecule of levan must bind not only the molecule of enzyme which synthesized it, but it must also bind a second molecule of the enzyme on a different cell.

The levansucrase of *A. viscosus* is fundamentally different from the levansucrases which have been extensively studied in *Aerobacter levanicum* and in *Bacillus subtilis*. In contrast to the levansucrase from *B. subtilis* where an enzyme-fructosyl intermediate has been postulated [23], the levansucrase of *A. viscosus* did not reversibly dissociate from its levan product. Nor did the levansucrase of *A. viscosus* require or utilize levan primer. Consequently, each levansucrase molecule from *A. viscosus* probably produced only one molecule of very high molecular weight levan [7]. By comparison, because of the dissociation of enzyme from levan, the molecular weight of *B. subtilis* levan depended on the proportion of levansucrase to low molecular weight levan primer; when excess primer was present, only a few molecules of fructose were transferred onto each primer molecule and the molecular weight of the levan was essentially unchanged. In the absence of primer, very little levan was produced and sucrose hydrolysis predominated, but the levan which was formed reached a molecular weight of 10^7 [24].

The levansucrase of *Ae. levanicum* displayed a mechanism of action which appears to be somewhat similar to that of *A. viscosus* in that no levan primer was needed and a non-dissociating enzyme-levan complex was apparently formed, resulting in the production of high molecular weight levan [18]. However, the levansucrase of *Ae. levanicum* did bind to preformed levan [25]. In fact, it was estimated that the levan product of this enzyme, which has a molecular weight of approx. $5 \cdot 10^7$, can bind 2000—3000 molecules of levansucrase. The binding was essentially irreversible, and has been utilized to purify the enzyme [25].

The physical properties of levansucrase from *A. viscosus* also differentiate it from the levansucrases of these other species. The *A. viscosus* levansucrase is a multi-subunit enzyme [7], having a molecular weight of approx. 220 000 whereas the enzymes of *Ae. levanicum* and *B. subtilis* are monomers of 22 000 and 40 000 daltons, respectively. Other differences between these levansucrases

are that the enzyme from *A. viscosus* is apparently constitutive and extracellular (soluble and cell-wall bound), whereas the levansucrase of *B. subtilis* is sucrose inducible and extracellular, and that of *Ae. levanicum* is constitutive and intracellular.

A constitutive, cell wall-bound levansucrase has also been described from *Strep. salivarius* [17], and a constitutive, extracellular levansucrase has been described from *S. mutans* [26]. The mechanisms of these enzymes are not yet known.

In experimental periodontal disease produced by *A. viscosus*, the severity of the disease is increased by the presence of sucrose in the diet [27]. Unfortunately, except for the effect of levan on complement, the importance of which is questionable, it is not yet clear whether levansucrase plays a significant role in the pathogenic mechanism of *A. viscosus*.

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