Carbohydrate Research, 190 (1989) 299–307
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

PURIFICATION AND PROPERTIES OF AN EXTRACELLULAR LEVANSUCRASE FROM *Erwinia herbicola* NRRL B-1678

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(Received October 17th, 1988; accepted for publication, December 22nd, 1988)

ABSTRACT

Levansucrase (EC 2.4.1.10) was purified approximately 40-fold from the extracellular culture fluid of *Erwinia herbicola* NRRL B-1678. The purified enzyme in its native form occurred as an aggregate. Poly(acrylamide) gel electrophoresis in the presence of sodium dodecyl sulfate showed an active monomer having a molecular weight of 48,000. The enzyme had a K_m for sucrose of 28mM and a pH optimum of 6.0, and was stable from −18 to +52°. It was free from α-D-glucosidase, amylase, and glucansucrase activities, but exhibited considerable β-D-fructofuranosidase activity.

INTRODUCTION

Levansucrase (sucrose: 2,6-β-D-fructan 6-β-D-fructosyltransferase, EC 2.4.1.10), which synthesizes the polysaccharide levan from sucrose, is produced by a number of bacteria, including *Bacillus subtilis*[1], *Aerobacter levanicum* (synonym: *Erwinia herbicola*)[2], *Gluconobacter oxydans*[3], *Actinomyces viscosus*[4], and others. Most of the original studies on this enzyme were carried out on that from *Aerobacter levanicum,* which has long been considered to be a constitutive, endocellular enzyme[2,5–7]. More-recent studies on levansucrase have focused on the extracellular enzyme from a constitutive mutant of *B. subtilis* [see, *e.g.*, ref. 8]. However, to date, there have appeared no reports of any studies conducted on the extracellular levansucrase from *E. herbicola.* We recently found that this species secretes appreciable quantities of levansucrase into its growth medium, and describe here a method for production and purification of the enzyme.

MATERIALS AND METHODS

Growth of organism. — Erwinia herbicola B-1678 was obtained from the

*The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

NRRL culture collection of the U.S.D.A. (Peoria, IL). The growth medium consisted of 100 mL of corn-steep liquor (Sigma Chem. Co., St. Louis), 200 g of D-glucitol (sorbitol), and 100 g of KH_2PO_4 in distilled water (10 L). Prior to autoclaving, the pH of the medium was adjusted to 6.9 ±0.1, and the medium was ultrafiltered with a Pellicon apparatus (Millipore, Bedford, MA) provided with a 30,000 nominal M_r cutoff membrane packet. The organism was grown in six Fernbach flasks (1.67 L per flask), and 5 mL of a 3-day-old culture was used to inoculate each flask. Flasks were incubated on a rotary shaker at 100 revolutions per minute for 2.5 d at 29°. Cells were then removed by centrifugation at 15,000*g* for 20 min at 5°.

Preparation and purification of enzyme. — Unless specified otherwise, purification procedures were carried out at 4°. Buffers all contained 0.01% (w/v) of NaN_3 to prevent microbial growth. The cell-free culture-fluid was concentrated by ultrafiltration with the Pellicon apparatus, followed by further concentration to 200 mL in a stirred cell with a regenerated-cellulose membrane filter (Millipore) having a 10,000 nominal M_r cutoff, and dialyzed overnight against 8 L of 20mM potassium phosphate buffer (pH 6.7).

The dialyzed, concentrated culture-fluid was chromatographed by ion-exchange in a column of DEAE-Sepharose Fast Flow (Pharmacia). The salt gradient was monitored conductimetrically, and protein content in the fractions was estimated from their absorbance at 280 nm. Levansucrase was determined as levan-synthase activity by a radiometric assay procedure[9]. The fractions (from the DEAE-Sepharose column) that contained levansucrase activity were pooled, and concentrated to 20 mL by ultrafiltration in a stirred cell as just described. This concentrate was split into two 10-mL portions, each of which was subjected to gel-filtration chromatography on Sephacryl S-300 Superfine (Pharmacia). Fractions were assayed for levansucrase activity by the radiometric method just described. A second method sometimes employed for the determination of levansucrase in column fractions involved the turbidimetric detection of levan formed from raffinose. In this method, 0.1 mL of the column fraction was mixed with 0.9 mL of 0.13M raffinose in 10mM sodium 4-morpholineethanesulfonate (Mes) buffer (pH 6.0). After 30 min at 30°, the absorbance of the reaction mixture was read at 650 nm. Fractions that contained levansucrase activity were pooled, concentrated by ultrafiltration, and chromatographed a second time on Sephacryl S-300 under the same conditions as before. The Sephacryl S-300 column was calibrated with NRRL B-1335-S dextran (M_r $>10^7$), bovine thyroglobulin (M_r 660,000), equine-spleen apoferritin (M_r 443,000), sweet-potato beta amylase (M_r 200,000), yeast alcohol dehydrogenase (M_r 150,000), bovine serum albumin (M_r 66,000), and equine-heart cytochrome c (M_r 12,400).

Analytical methods. — Total carbohydrate content was estimated by the phenol–H_2SO_4 method[10]. Protein assays were performed according to the method of Bradford[11]. Levan-synthase assays were carried out at 30° by a method, similar to that described for dextransucrase[9], in which the incorporation of D-[U-

^{14}C]fructose from [U-^{14}C]sucrose (New England Nuclear, Boston, MA) into methanol-insoluble polysaccharide was measured. An enzyme unit is defined as the amount of levansucrase that will incorporate one micromole of D-fructose into the polysaccharide in one minute. Routinely, [U-^{14}C]sucrose was used, but [U-(Fru)-^{14}C]sucrose and [U-(Glc)-^{14}C]sucrose were used to differentiate between glucansucrase and levansucrase activities. Raffinose-splitting activity was determined by incubating a volume of enzyme preparation with ten volumes of 0.13M raffinose in Mes buffer (pH 6.0) at 28°, and analyzing 0.1-mL aliquots of the reaction mixture for reducing-sugar content[11] after 10 min.

The purified levansucrase preparation was assayed for contaminating α-D-glucosidase activity by incubating 0.4 mL of enzyme (13.7 U/mL) with 1.6 mL of 33mM *p*-nitrophenyl α-D-glucopyranoside in 10mM Mes buffer (pH 6.0). After 1.5 and 12 h, 0.1-mL aliquots were withdrawn and mixed with 0.9 mL of 0.1M Na_2CO_3. The absorbance was read at 415 nm.

Amylase activity was assayed by incubating 2 mL of levansucrase preparation (13.7 U/mL) with 8 mL of 2% (w/v) soluble starch (Pfanstiehl, Waukegan, IL) in 20mM Mes buffer for 30 min at 28°. The amount of reducing sugar released was measured by the Somogyi–Nelson method[12] on 1-mL aliquots of the reaction mixtures.

The structure of the polysaccharide produced by the purified enzyme was determined by ^{13}C-n.m.r. spectroscopy[13].

For poly(acrylamide)-gel electrophoresis, linear 4–16% acrylamide slab gels with a gradient of 1–8M urea were prepared according to the Jarvik and Rosenbaum modification[14] of the Laemmli procedure[15]. Samples were mixed with concentrated sample buffer to yield a final concentration of 2% sodium dodecyl sulfate (SDS), 100mM 1,4-dithiothreitol, and 10mM Tris buffer (pH 8.0), and then heated in a boiling-water bath for 3 min, loaded onto the gel, and subjected to electrophoresis overnight at 5–7 mA. Gels were fixed and stained with 0.2% (w/v) Brilliant Blue R-250 (Fisher Scientific, NJ) in 10:9:1 (v/v/v) methanol–water–acetic acid, and destained in 17:2:1 (v/v/v) water–ethanol–acetic acid. To detect levansucrase activity within the gel, samples containing enzyme were first mixed with concentrated sample buffer and incubated for 1.5 h at 37°, and subsequently electrophoresed on 4–16% gel containing SDS but no urea. At the end of the electrophoresis, the gel was washed with 1% (w/v) Triton X-100 for 1 h, and then incubated in a solution containing 0.15M sucrose and 1% (w/v) Triton X-100 in 10mM Mes buffer[16] (pH 6.0). Levansucrase activity appeared as white, opaque bands within 1 h.

The pH optimum of levansucrase was determined by dialyzing a small portion of the enzyme against distilled water. This enzyme solution was then assayed as already described, at 30°, by mixing 10 μL of enzyme solution with 150 μL of 0.1M buffer and 40 μL of 1.0M [U-^{14}C]sucrose in water, to give a sucrose concentration of 0.2M in the assay mixture. Buffers and the pH ranges at which they were used were as follows: potassium citrate, 2.3–3.7; potassium acetate, 4.0–5.6; potassium

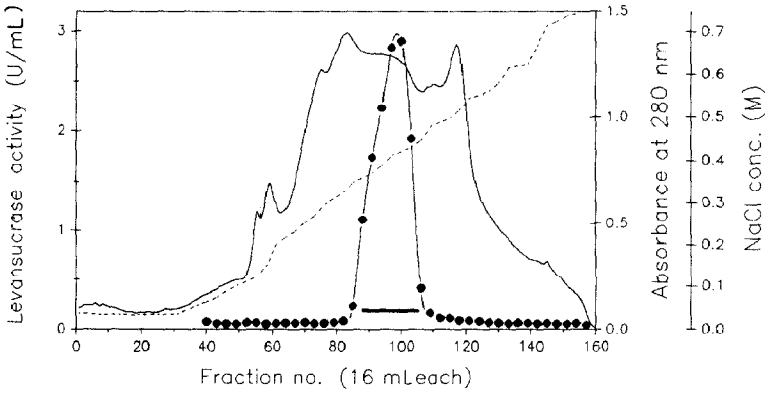

Fig. 1. DEAE-Sepharose Fast Flow ion-exchange chromatography of dialyzed and concentrated B-1678 culture fluid. Levansucrase was assayed radiometrically (see Materials and Methods). Sample size was 200 mL; column size was 5 × 28 cm. Column was eluted at 25° with 20mM potassium phosphate buffer (pH 6.7), followed by a two-liter gradient of zero to 0.8M NaCl. Key: —●—, levansucrase activity; ——, absorbance at 280 nm; ---, NaCl concentration. Bar indicates fractions pooled.

4-morpholineethanesulfonate, 5.7–7.3; and potassium *N,N*-bis-(2-hydroxyethyl)-glycine, 7.4–9.3.

The temperature stability of purified levansucrase was determined by incubating a solution containing 7 μg of purified protein/mL in 10mM Mes buffer (pH 6.0) for 30 min at a chosen temperature. The enzyme solutions were then assayed radiometrically at 30° as already described with a sucrose concentration of 167mM in the assay mixture.

RESULTS AND DISCUSSION

When grown on the medium described, *E. herbicola* B-1678 produced 0.23 unit of extracellular levansucrase per mL of culture fluid within 2.5 d. Little or no cells lysis was observed microscopically prior to centrifugation.

Fig. 1 shows the results of ion-exchange chromatography of the concentrated and dialyzed culture fluid. Levansucrase activity was eluted as a single peak at 0.35–0.45M NaCl. The only raffinose-splitting activity detected was eluted as a single peak corresponding exactly to the levan-synthase peak (data not shown).

Fig. 2 shows an elution profile for the Sephacryl S-300 chromatographic step. The levan-synthase activity was eluted as a single peak, corresponding to a mass of ~400,000–600,000 daltons. Again, levan-synthase activity and raffinose-splitting activity coincided exactly (data not shown). The results of the second gel-filtration chromatography step are shown in Fig. 3. The pooled enzyme fractions from this column were used as the final "purified" enzyme. A summary of the purification of levansucrase appears in Table I.

The apparent molecular weight of the dissociated, monomeric form of the

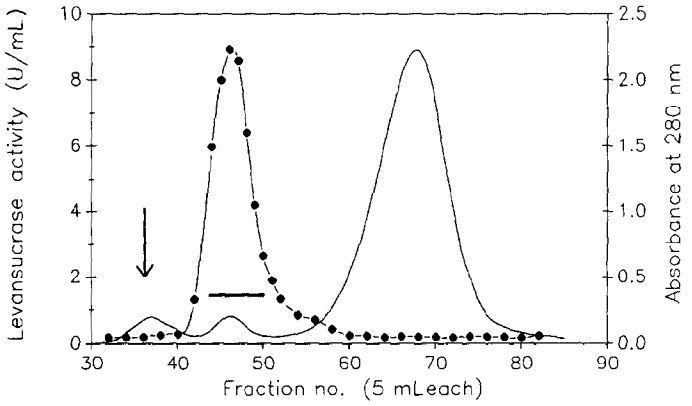

Fig. 2. Sephacryl S-300 Superfine gel-filtration chromatography of pooled and concentrated enzyme fraction from DEAE-Sepharose column. Column size was 2.5 × 98 cm. Sample size was 10 mL. Flow rate was 23 mL.h^{-1}, with 10mM Mes buffer (pH 6.0), at 4°. Levansucrase was assayed as in Fig. 1. Void volume is indicated by arrow. Symbols same as in Fig. 1.

levansucrase from gel electrophoresis in the presence of SDS was ~48,000 (see Fig. 4, lane B). After removal of SDS, this band contained all of the levansucrase activity detected on the gel (see Fig. 4, lane C). Ebert and Stricker[5] found a molecular weight of 22,000 for the cell-associated enzyme. The molecular weight of the extracellular enzyme described here corresponds more closely to those of extracellular levansucrase preparations from *Bacillus subtilis* and *B. amyloliquefaciens*, which are[17] ~52,000. The nature of the large aggregate eluted from the Sephacryl S-300 column is not fully understood, but the conditions necessary to disrupt the aggregate were relatively mild, namely, 2% SDS for 1.5 h at 37°. Levansucrase

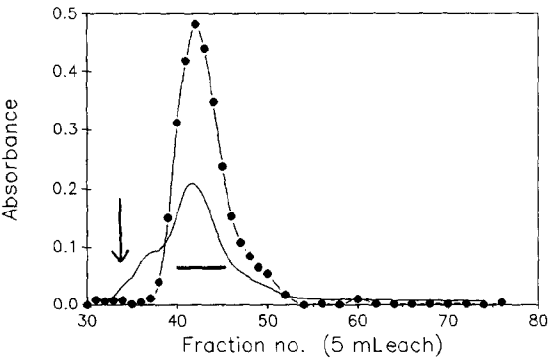

Fig. 3. Sephacryl S-300 Superfine re-chromatography of pooled and concentrated enzyme fraction from Fig. 2. Chromatography was performed as in Fig. 2. Levansucrase was assayed turbidimetrically (see Materials and Methods), and is directly proportional to absorbance at 650 nm. Key: ——, absorbance of fraction at 280 nm; and —●—, absorbance of assay reaction mixture with raffinose at 650 nm after 30 min. Arrow indicates void volume. Bar indicates fractions pooled.

TABLE I

SUMMARY OF PURIFICATION OF EXTRACELLULAR LEVANSUCRASE FROM *Erwinia herbicola* NRRL B-1678

Preparation	*Volume (mL)*	*Units per mL*	*Units per mg of protein*	*Yield (%)*	*Purification factor*
Culture fluid	10,000	0.22	7.3	100	
Concentrated & dialyzed culture fluid	210	7.9	18.4	75	2.5
DEAE-Sepharose fraction, concentrated	20	68	90	62	12.3
1st Sephacryl S-300 column	80	11.5	220	42	30
2nd Sephacryl S-300 column	33	21	300	32	40

activity was detected as a broad peak, even in the presence of 1% (w/v) NaCl, when chromatographed on Sephacryl S-300. This was found for both the purified enzyme and for the enzyme as it occurred in unconcentrated culture fluid. The peak was broad, ranging from <400,000 to >600,000 Da (data not shown). The purified enzyme preparation contained a small proportion of unknown carbohydrate (1 μg/10 μg of protein), which may serve to bind the enzyme molecules together as a large aggregate.

The purified enzyme lacked glucansucrase activity, as evidenced by the fact that no detectable incorporation of D-[U-^{14}C]glucose from [U-(Glc)-^{14}C]sucrose

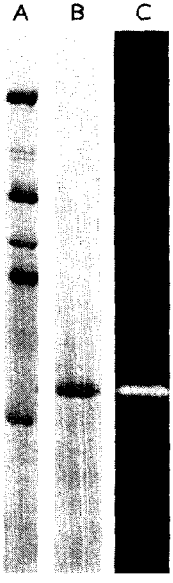

Fig. 4. Analysis of purified levansucrase on 4–16% SDS-poly(acrylamide) gels. Lane A, molecular-weight standards (top to bottom: myosin, M_r 200,000; *E. coli* β-D-galactosidase, M_r 119,000; rabbit-muscle phosphorylase b, M_r 90,000; bovine serum albumin, M_r 63,000; and ovalbumin, M_r 45,000). Lane B, purified levansucrase preparation (1.4 μg of protein containing 0.2 unit of enzyme activity, was loaded), stained for protein with Coomassie Blue. Lane C, corresponding band showing levan-synthase activity within the gel. White band is levan formed from sucrose.

into D-glucan could be detected, while the amount of [U-^{14}C]fructose incorporated from [U-(Fru)-^{14}C]sucrose in a given period was the same as the total ^{14}C incorporated into polysaccharide from [U-^{14}C]sucrose.

The purified levansucrase was also free from α-D-glucosidase and amylase activities. *E. herbicola* is reported to be an amylolytic organism, so these enzymes are likely to be present in spent growth medium[18].

Proton-decoupled ^{13}C-n.m.r. spectroscopy of the product formed from sucrose by the purified levansucrase gave a spectrum virtually identical to that of an *Erwinia* sp. levan published by Seymour *et al.*[13] (not shown), confirming that the polysaccharide produced by the purified enzyme was levan.

The pH optimum of the extracellular levansucrase was 6.0, with little or no activity observed below 3.6 and above 9.0 (see Fig. 5). These values are similar to those found by Ebert and Stricker[5] for the cell-associated enzyme.

The purified enzyme was stable when held for 30 min at temperatures up to ~52°, but was irreversibly denatured when heated to 66°. The purified enzyme was stable to freezing and thawing, and could be lyophilized and subsequently reconstituted with little loss of activity.

When the enzyme was assayed as levan-synthase activity, the apparent K_m of the enzyme for sucrose was found to be 28mM. Ebert and Schenk[19] reported a K_m for sucrose of ~120mM for the cell-associated enzyme. Our K_m value was determined by a radiometric method, since we have found that higher sucrose concentrations (>150mM) interfere with the formation of turbid, high-molecular-weight levan, thus giving the appearance of substrate inhibition when the enzyme is assayed turbidimetrically. Pascal and Dedonder[20] reported a K_m for sucrose of 27mM for a *B. subtilis* levansucrase.

The enzyme preparations contained signficant β-D-fructofuranosidase activity at all stages of purification. This can be attributed to levansucrase activity itself,

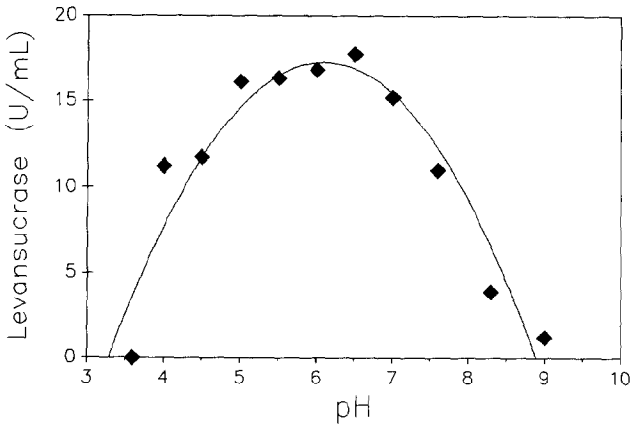

Fig. 5. pH–Activity profile for purified levansucrase. Enzyme was assayed radiometrically at 30°. Sucrose concentration = 0.2M. See Materials and Methods for details.

whereby D-fructosyl units are transferred from sucrose or raffinose to an acceptor—in this case, water. Many compounds containing hydroxyl groups can act as acceptors[21,22]. The ratio of β-D-fructofuranosidase activity to levan-synthase activity was highly variable, and seemed to depend on a number of factors, including temperature, substrate concentration, enzyme concentration, and the concentration of levan and low-molecular-weight acceptors. Some of these effects have also been described for the cell-bound enzyme[19]. It was further noted that, after prolonged incubation (10–100 times the period required to consume all sucrose present), enzyme–sucrose reaction-mixtures were capable of hydrolyzing D-fructose from native levan. This was observed as a diminution in the turbidity of the endogenously produced levan, and a concomitant increase in the amount of D-fructose detected by thin-layer chromatography[23]. Although these activities could be attributed to a contaminating, invertase-like enzyme, a more likely explanation is that *E. herbicola* levansucrase is itself capable of transferring D-fructosyl units from levan to water, in a manner analogous to that described for purified levansucrase from *B. subtilis*[24], and for dextransucrases from *Leuconostoc mesenteroides* and *Streptococcus mutans*[25]. The hydrolysis of levan by purified *B. subtilis* levansucrase has been described as the slow transfer of D-fructosyl units from levan to water through a D-fructosyl–enzyme intermediate[24].

Ebert and Schenk[19] have commented that studies on levansucrase from *E. herbicola* were more difficult than those on similar enzymes from other bacteria, due to the problems inherent in obtaining large amounts of purified enzyme. Our findings should be of great value in facilitating further studies on this enzyme.

ACKNOWLEDGMENTS

We thank Mary Duffy, Jane Philipich, and Holly Dickinson for technical assistance, and Dr. David Weisleder for recording the ^{13}C-n.m.r. spectrum.

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