Levansucrase of *Bacillus subtilis*

Toshio TANAKA, Susumu OI, Masaru IIZUKA and Takehiko YAMAMOTO

Faculty of Science, Osaka City University, Osaka

Received August 8, 1977

Levansucrase of *Bacillus subtilis* var. *saccharolyticus* was purified from the supernatant of an aerobically incubated suspension of the grown cells in a dilute sucrose solution by chromatography on a column of DEAE-cellulose.

The synthesis of levan by the purified enzyme resulted in most effectively at low temperatures at around 0°C, and the levan synthesized was isolated by passing the enzyme-reaction mixture through a DEAE-cellulose column followed by ethanol precipitation. The levan was isolated in the pure state ($[\alpha]_D^{20}, -47$) and its molecular weight was determined to be 2×10^4 by the gel filtration method.

Levan, a polymer of fructose linked by fructofuranosidic bond, is known to be produced by certain bacteria which grow on sucrose media. Recently Chambert *et al.*¹⁾ published a paper on kinetic studies of levan synthesis by levansucrase (β -2,6-fructan: D-glucose-1-fructosyl-transferase, EC 2.4.1.10) of *Bacillus subtilis* BS 5 by measuring the glucose-releasing velocity from sucrose and isotopic exchange at the reaction.

Formerly, Negoro and Fukumoto isolated saccharase (β -D-fructofuranoside fructohydrolase, EC 3.2.1.26) from culture fluid of *Bacillus subtilis* var. *saccharolyticus*.²⁾ We found that the bacterium also secreted levansucrase actively.

The present paper deals with isolation of levansucrase from the bacterium, levan synthesis by the enzyme under several conditions, especially at several temperatures, and some properties of the levan obtained.

MATERIALS AND METHODS

The microorganism employed and its culture. *Bacillus subtilis* var. *saccharolyticus* preserved as a saccharase producer was employed. The bacterium was grown by the surface culture method using a 5 liters Fernbach flask which contained 700 ml of the medium consisted of 5% soybean cake extract (Soybean cake was gently boiled with twenty volumes of 0.2% NaOH for 1 hr, and the supernatant was neutralized at pH 7.0 with HCl) 5% sucrose, 0.6% $(\text{NH}_4)_2\text{HPO}_4$, 0.02% each of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, KCl and Ca-acetate, pH 7.0, at 37°C

for 48 hr. The grown cells were used for obtaining levansucrase.

Assay of levansucrase. The reaction mixture containing 10% sucrose, 50 mM phosphate buffer, pH 7.0 and enzyme in total 2 ml was incubated at 30°C. After 30 min incubation, the reaction mixture was heated and glucose released was determined by Hatanaka method³⁾ after properly diluting the mixture (usually 10 fold dilution). One unit of levansucrase was defined as the enzyme amount that produced 1 μ mole of glucose per min under the conditions. In the present paper, the reducing power formed in the reaction mixture was also determined as glucose by the method of Somogyi-Nelson,⁴⁾ and one unit of this activity was defined as the enzyme amount that produced reducing sugars equivalent to 1 μ mole of glucose per min under the same conditions as the assay of levansucrase.

Isolation of levan synthesized. The enzyme reaction mixture (25 ml) was applied to a column of DEAE-cellulose (2.4×10 cm) buffered at pH 5.0 with 20 mM acetate buffer. The levan synthesized appeared in the break-through solution while the enzyme remained on the column. The levan in the effluent was precipitated by adding three volumes of ethanol. The precipitate was collected by centrifugation, dissolved into a minimum amount of deionized water, and the solution was dialyzed against water for 24 hr in the cold. The dialyzed solution was lyophilized and weighed to determine the actual yield of levan synthesized to be precipitable with 75% ethanol.

Investigation of levan isolated. The total sugar content of levan isolated was determined by the phenol-sulfuric acid method⁵⁾ using fructose as the standard. The amount of fructose was determined by the method of Kulka.⁶⁾ Identification of constituting sugars was

done by paper chromatography for acid hydrolysate of the sample (hydrolyzed with 0.25 N HCl at 100°C for 2 hr). The paper chromatography was performed by the descending method using Toyo Roshi No. 50 filter paper and the following mixture as the developer: *n*-butanol: pyridine: water, 6:4:3. Reducing sugars on the paper were detected by the ammonia silver nitrate method.

The optical rotation of the sample was measured using a quartz cell of 10 cm long on a Rex photoelectric polarimeter. The molecular weight was estimated by the gel filtration method on a column of Sephadex G-200 using several commercial dextrans as a reference.

RESULTS

Isolation and some enzymatic properties of levansucrase

The bacterial cells grown by the method described above were collected by centrifugation, washed with cold water several times by using a centrifuge, and suspended into 30 mM phosphate buffer, pH 7.0, containing 3% sucrose and 1 mM CaCl₂. The suspension was incubated at 36°C overnight with shaking for the enzyme secretion. The suspension was then centrifuged, and to the supernatant an equal volume of cold ethanol (99%) was added to precipitate the enzyme.

The enzyme precipitated was dissolved in a minimum amount of 20 mM acetate buffer, pH 5.0, and the solution was dialyzed against the same buffer. The inner solution was then purified by chromatography on a column of DEAE-cellulose equilibrated with 20 mM acetate buffer, pH 5.0. The enzyme adsorbed was eluted by stepwise increasing the concentration of NaCl containing 20 mM acetate buffer,

pH 5.0. The enzyme activity recovered was about 75%, most of which was eluted between from 0.5 to 1.0 M of NaCl. The rest of the enzyme activity remained on the column, and was not desorbed even with higher concentrations of NaCl (The enzyme remained on DEAE-cellulose was still active as levansucrase when it was used as a suspension.). The purification of the enzyme is summarized in Table I.

General enzymic properties of the enzyme

The optimum pH determined by measuring glucose-releasing activity from sucrose was about pH 6.0. The enzyme was stable in a pH region from 3.5 to 7.5 at 4°C. However, the enzyme was sensitive to heat and lost its activity even on standing at 45°C, pH 7.0 for 15 min.

Levan synthesis under several conditions

1) *Effect of temperature.* There have been very few papers on the effect of temperature on enzymic synthesis of levan. Most of papers so far reported estimated the extent of levan synthesis by measuring glucose liberated, as is done for the assay of levansucrase. However, it has already been made clear that dextran synthesis by dextransucrase does not go parallel with the apparent enzyme activity which increases with increase in temperature to a certain extent.⁽⁷⁾ The effect of temperature on levan synthesis by our enzymes was thus investigated. A 75 ml reaction mixture containing 10% sucrose in 20 mM phosphate buffer, pH 7.0, and 0.37 units of enzyme activity per ml was divided into three equal portions, and they

TABLE I. ISOLATION OF LEVANSUCRASE FROM *Bacillus subtilis*

The bacterial cells obtained by culture using four Fernbach flasks, about 100 g as wet weight, were washed, suspended in 1000 ml of 3% sucrose containing 30 mM phosphate buffer, pH 7.0 and 1.0 mM CaCl₂, and incubated at 36°C for 16 hr. The supernatant was used.

Numbers in parenthesis indicate reducing power formation activity.

Fraction	Total volume, ml	Total levansucrase activity, units	Total protein, mg	Total sugar content, mg	Recovery yield, %
I. Sup. of the incubated cell suspension	940	263 (350)	868	28000	100
II. Ethanol ppt.	80	115 (154)	126	4400	44
III. Eluate from DEAE-cellulose	90	70 (117)	5	10	27

were incubated at 4°C, 20° and 37°C, respectively. Their liberation of reducing sugars and free glucose were determined at certain time of intervals. As shown in Fig. 1, the extent of formation of reducing sugars and free glucose on incubation at 4°C were quite close to each other, indicating that fructose moiety of sucrose was effectively transferred to synthesize levan. Whereas on incubation at 37°C, reducing sugar formed was considerably greater than glucose liberated and the difference increased with time of incubation. The relation between reducing sugar formed and glucose liberated at 20°C was rather close to that at 37°C.

The levan synthesis at the low temperatures was determined by weighing levan by the method described in MATERIALS AND METHODS,

FIG. 1. Progressive Curves of Liberation of Glucose or Formation of Reducing Sugars on Incubation of Levansucrase with Sucrose at Several Temperatures. (10% sucrose, 20 mM phosphate buffer, pH 7.0). ○—○, free glucose; ●—●, reducing sugar.

TABLE II. EFFECT OF TEMPERATURE ON LEVANSUCRASE ACTIVITY AND YIELD OF LEVAN SYNTHESIZED

25 ml of 10% sucrose, pH 7.0 containing 0.37 units (30°C) of levansucrase per ml, 10 days.

	Incubation temperature	
	4°C	37°C
Apparent enzyme activity, units	0.037	0.537
Levan obtained, mg	578	156
Fructose in the hydrolysate, mg	615	161

and the result is shown in Table II. The experiment was carried out using 10% sucrose with 0.37 units of levansucrase per ml, buffered at pH 7.0 with 20 mM phosphate. Table II shows that the lower the temperature of the enzyme reaction, the more synthesis of levan was obtained, though the apparent enzyme activity became significantly small as the temperature was lowered.

2) *Effect of sucrose concentration on levan synthesis at low temperatures.* The effect of sucrose concentration on synthesis of levan by the enzyme was investigated at 4°C for 10 days. As shown in Fig. 2, the synthesis of levan occurred more effectively as the sucrose concentration was increased in the region examined,

FIG. 2. Effect of Sucrose Concentration on Synthesis of Levan.

□, levan recovered, g; ■, fructose in the hydrolysate, g.

and the yield of levan was 60% of fructose moiety of sucrose consumed.

Some properties of levan synthesized

The levan obtained by the method described above was subjected to analysis for constituting sugars and investigation of some properties as levan. The levan showed an optical rotation value of $[\alpha]_D^{20}$, -47, which was almost consistent with that reported by Barker *et al.*⁸⁾ IR spectra showed that the sample gave a typical absorption as levan on the finger print regions; 808, 860 and 925 cm^{-1} . The hydrolysis of the sample with 0.25 N HCl at 100°C for 2 hr gave rise to 103% of fructose. The paper-chromatographic test with the hydrolyzate revealed that fructose was the sole sugar component of the levan obtained. Elementary analysis showed that the sample consisted of 39.9% of C, 6.25% of H, negligible amounts of N (0.04% or less) and no phosphorus.

The elution pattern of the levan at the gel filtration test using a column of Sephadex G-200 is shown in Fig. 3, and the average molecular weight was estimated to be 2×10^4 .

FIG. 3. Gel Filtration of Levan on Sephadex G-200. Using the same column of Sephadex G-200, dextrans of several sizes were applied and eluted as a reference. ○—○, levan; ●—●, dextran.

DISCUSSION

In isolation procedure of levansucrase, almost no change in the ratio of activity of glucose releasing to that of reducing power formation on sucrose as the substrate was

observed. This fact may indicate that the supernatant of the bacterial cell suspension incubated for secretion of levansucrase contains no or negligible amount of the so called invertase. This is most likely from that isolation of invertase from the supernatant of the bacterial suspension has not yet succeeded.

Chambert *et al.*¹⁾ have reported that the levansucrase preparation purified to be absolutely free from levan hydrolyzes sucrose but not synthesize levan. Our enzyme preparation employed in the present paper still contained levan and levan-synthetic activity may have been preserved.

The fact was of interest that the lower the temperature of the enzyme reaction, the more synthesis of levan was obtained. In other word, the higher the temperature of enzyme reaction, hydrolysis of sucrose preferred synthesis of levan. Solving of this phenomenon will be left in the future.

The present paper also presented a purification method of levan enzymatically synthesized. The method was simple and greatly effective, because the levan synthesized by the enzyme was considered as almost pure on the basis of IR spectra, specific rotation value and fructose as the sole constituting sugar. The molecular weight of the levan was estimated to be 2×10^4 in average. This fact was also interesting, because the molecular weight of levan synthesized by unpurified enzyme or the enzyme adsorbed and remained on DEAE-cellulose was much bigger than this. Studies on this problem will be attempted in the future.

REFERENCES

- 1) R. Chambert, G. Treboul and R. Dedonder, *Eur. J. Biochem.*, **41**, 285 (1974).
- 2) H. Negoro and J. Fukumoto, *Kagaku To Kogyo*, **28**, 292 (1954).
- 3) C. Hatanaka, *Nippon Nôgeikagaku Kaishi*, **41**, 448 (1967).
- 4) N. Nelson, *J. Biol. Chem.*, **153**, 375 (1944).
- 5) M. Dubois, K. A. Guilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
- 6) G. R. Kulka, *Biochem. J.*, **63**, 542 (1956).
- 7) K. Itaya and T. Yamamoto, *Agric. Biol. Chem.*, **39**, 1187 (1975).
- 8) S. A. Barker, F. Pautard, I. R. Siddiqui and M. Stacey, *Chem. Ind.*, No. 5, 1450 (1955).