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PURIFICATION OF LEVANSUCRASE BY PRECIPITATION WITH LEVAN

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SUMMARY

Levansucrase (β -2,6-fructan:D-glucose 1-fructosyltransferase, EC 2.4.1.10) forms a complex with levan, which is the product of the enzymic reaction. The specificity of this interaction and the fact that the enzyme-polysaccharide complex precipitates from solution at $100\,000 \times g$, was used as the basis for an efficient procedure for an extensive enzyme purification.

INTRODUCTION

The specific attachment of an enzyme to another macromolecule which is a substrate or the product of its action, was recently used as a basis of an efficient procedure for enzyme purification. Thus, for example, UDPG:glycogen α -4-glucosyltransferase (EC 2.4.1.11)¹, α -amylase (α -1,4-glucan 4-glucanohydrolase, EC 3.2.1.1)^{2,3}, glycogen phosphorylase (α -1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1)^{4,5}, and a fructan hydrolase (fructan 6-fructanohydrolase)⁶, were highly purified by a selective precipitation of the enzyme polysaccharide complex.

Previous observations⁷ have shown that the levan pellets obtained by high speed centrifugation of sucrose-grown *Aerobacter levanicum* homogenates carry down with them a significant amount of levansucrase (β -2,6-fructan:D-glucose 1-fructanohydrolase, EC 2.4.1.10). DEDONDER AND PEAUD-LENOËL⁸ have noted that addition of levan to *Bacillus subtilis* cells affected the rate of appearance of extracellular levansucrase. Formation of an enzyme-polysaccharide complex was suggested as a possible explanation of these phenomena.

An attempt was now made to extend these observations, and to determine whether the formation of a levansucrase-levan complex can be used as the basis for an efficient procedure for the purification of levansucrase.

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EXPERIMENTAL

Materials and methods

Levan was obtained from cultures of sucrose-grown *A. levanicum*^{9,10}. Methods of paper chromatography employed were similar to those used by FEINGOLD *et al.*¹¹. Thin-layer chromatographic separation of sugars was conducted on Kieselguhr G-CaSO₄ (ref. 12) with ethanol-iso-propanol-water (65:23:12, v/v) as the solvent. Electrophoresis was carried out on Sepraphore III cellulose acetate (Gelman Instruments Co., Ann Arbor, Mich.) at 32 V/cm for 40 to 60 min using 2-dimethylamino-propionitrile buffer (pH 7.2) and Brilliant blue FCF as a marker¹³. Overnight immersion in 0.002% nigrosin in 2% acetic acid was employed for protein staining. To detect the levansucrase activity after electrophoretic separation, strips were soaked with 0.02 M sucrose solution in acetate buffer pH 5.4, and, after 15 min incubation dried at 80°. The appearance of a reducing sugar band was detected with the triphenyl-tetrazolium chloride reagent¹⁴ in 50% methanol. Protein was determined colorimetrically¹⁵, using crystalline bovine serum albumin as the standard. Levan was assayed as fructose by the resorcinol method¹⁶.

The standard assay system for the determination of levansucrase activity consisted of 160 mM sucrose, 6 mM (with respect to phosphate) citrate-phosphate buffer (pH 5.4), and enzyme, 0.2–2.0 units/ml, at 37°. Samples were taken for the determination of reducing sugar by the method of SOMOGYI¹⁷ and NELSON¹⁸. A unit of enzyme was defined as that amount which liberates 1 μ mole of reducing hexose per h at standard conditions. The amount of levan produced is equivalent to about 1/3 of this value⁹.

Preparation and purification of levansucrase

A. levanicum was grown for 36 h at 30° on a rotary shaker in the following medium: yeast extract (Difco) 0.5%; NH₄Cl, 0.25%; NaCl, 0.5%; Na₂HPO₄ · 12H₂O, 0.25% and glucose, 1.0%. Cells were collected by centrifugation and washed three times with water. A suspension of cells in water (about 15%, w/w) was disrupted by 10 min sonication in the 10 kcycycle Raytheon oscillator. Debris was removed by centrifugation at 15 000 \times g for 15 min in the cold.

The supernatant was diluted with 4 volumes of cold water to adjust the protein concentration to about 8 mg/ml and 0.1 M acetic acid was carefully added to bring the pH to 4.7–5.0. The heavy precipitate was removed by low-speed centrifugation at room temperature and discarded. The slightly hazy supernatant was centrifuged for 1 h at 100 000 \times g in the Spinco Model L ultracentrifuge.

To the clear supernatant solution, native levan was added at one to two parts (by weight) per 1000 parts of protein. After 10 min in the cold, the solution was centrifuged for 1 h at 100 000 \times g. Most of the enzyme activity was now found in the gummy precipitate tightly adhering to the bottom of the centrifuge tube. This was taken up in water by vigorous stirring in the Vibro-mixer. Native levan was added again as in the previous step and reprecipitated as before. The precipitate was dispersed in a small volume of 5 mM citrate-phosphate (pH 5.4) buffer. Material which caused some cloudiness on standing was removed by low speed centrifugation for 10 min. The purified enzyme remained stable for 2 weeks at 4° or for a longer period when kept frozen.

RESULTS AND DISCUSSION

An example of the procedure for enzyme purification outlined above is given in Table I. A purification of nearly two thousand-fold was achieved with a relatively good yield. In several preparations it was noted that a portion of the levansucrase activity tended to precipitate by centrifugation at $100\,000 \times g$ without addition of levan. The dilution of the extracts and acidification usually retarded this precipitation in subsequent steps of purification. This "particulate" nature of the enzyme has yet to be explored.

Native levan precipitates at $100\,000 \times g$ as a transparent gel which carries most of the levansucrase with it. In order to decrease the amount of foreign proteins

TABLE I

PURIFICATION OF LEVANSUCRASE

Cells obtained from 4 l of culture medium (about 10 g) were subjected to the procedure outlined under EXPERIMENTAL.

Fraction	Volume (ml)	Total protein (mg)	Levansucrase		Specific activity
			total (units)	yield (%)	
I. Crude extract	42	1780	25 240	100	14.2
II. pH-5.0 supernatant	195	448	20 400	81	45.5
III. $100\,000 \times g$ supernatant	190	105	19 450	78	185.5
IV. First levan precipitate	60	4.9	10 200	41	2 100
V. Second levan precipitate	11	1.1	8 000	32	7 250
VI. Last supernatant	11	0.28	7 900	31	28 200

trapped in this gel and to secure a high selectivity and specificity of adsorption, the minimum quantity of levan needed for levansucrase precipitation was determined (Table II). Under similar conditions, but with more levan (1 mg/ml), *Trichoderma viride* cellulase (β -1,4-glucan glucanohydrolase, EC 3.2.1.4), *E. coli* β -galactosidase (β -D-galactoside galactohydrolase, EC 3.2.1.23), pancreatic α -amylase (EC 3.2.1.1), *Dactylium dendroides* galactose oxidase (D-galactose:oxygen oxidoreductase, EC 1.1.3.9) and pancreatic ribonuclease (EC 2.7.7.16) were not significantly precipitated from solution.

Impurities were detected even in preparation of high specific activity. In electrophoretic studies on cellulose acetate it was found that the protein with levansucrase activity moved as a single band, with $R_{\text{brilliant blue}}$ 0.67–0.70, which constituted at least 50% of the total protein present. 2 (or 3) additional protein bands devoid of levansucrase activity were found as contaminants. The purest levansucrase preparations catalyzed sucrose cleavage and levan synthesis according to the pattern described for this enzyme in crude cell extracts¹¹. No hydrolytic action on levan was exhibited by these preparations. The enzyme purified by the procedure described still contained levan in different proportions in various preparations obtained. 1 μ g levan per 2–10 μ g protein was detected in the fractions of highest purity prepared. Partial removal of this levan contaminant could be achieved by gel filtration through Sephadex G-100

TABLE II

EFFECT OF LEVAN CONCENTRATION ON LEVANSUCRASE PRECIPITATION

Levan was added to 6 ml of enzyme solution (300 units; 3 mg protein) at pH 5.0 (5 mM acetic acid). The precipitate obtained after 1 h at 100 000 × g was dispersed in 3 ml of 5 mM citrate-phosphate buffer (pH 5.4) and assayed for levansucrase activity.

Weight ratio levan/protein	Levansucrase precipitated (%)
3.3	98
1.7	98
0.067	85
0.013	91
0.0067	95
0.0034	98
0.00067	75
0	14

(Pharmacia, Uppsala, Sweden) or Bio-Gel P-200 (Bio-Rad Labs., Richmond, Calif.) columns.

Levansucrase of *B. subtilis* was shown to have an $s_{20,w}^0$ of 2.7 and a molecular weight of 40 000 (see ref. 19). The enzyme from *A. levanicum* was claimed to have an $s_{20,w}^0$ of 2.28 (see ref. 20) and only an estimation of its molecular weight can be made. Thus assuming a molecular weight of about 30 000 for the enzyme and a molecular weight of $50 \cdot 10^6$ for the branched levan²¹, we estimate from the minimum amount of levan found in purified enzyme fractions that some 2000–3000 protein molecules are found per molecule of polysaccharide, or about one enzyme molecule per every 10–15 terminals of the fructan. These are but preliminary calculations based on isolated experiments. Only further studies based on the preparation of large batches of purified enzyme protein, will allow more accurate evaluations of the levansucrase-levan interaction.

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Act. poss. for purifn: 11/20

Absorb mixed enzymes on ^{good} adsorbent for enzymes.

Try selective elution using substrates; modified substs.,
and/or products.

It might just be possible to achieve a desirable
purification.