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STUDY OF THE EFFECT OF ORGANIC SOLVENTS ON THE SYNTHESIS OF LEVAN AND THE HYDROLYSIS OF SUCROSE BY *Bacillus subtilis* LEVANSUCRASE

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ABSTRACT

The equilibrium between the hydrolase and synthetase activities of levansucrase was determined by progressively substituting water with various organic solvents in the enzymic reaction medium. In the presence of high concentrations of these solvents, the enzyme displayed only synthetase activity. The levan obtained under such conditions had M_r ~10^6 and presented a low molecular dispersity. In the presence of solvent, the K_m values for sucrose and raffinose remained unchanged, but the k_{cat} values were five times higher in comparison to the same constants determined for an aqueous medium.

INTRODUCTION

In aqueous medium, the purified levansucrase of *Bacillus subtilis* catalyzes a transfructosylation reaction from sucrose or raffinose to water (hydrolase activity) or to levan (polymerase activity) according to Eq. *1*. Under the usual conditions of sucrose transformation, the rate of the polymerase activity is low as compared to the hydrolytic activity[1,2].

$$\text{n sucrose} \rightarrow \text{n D-glucose} + \text{levan or (D-fructosyl)}_p + (\text{n} - \text{p})\ \text{D-fructose} \quad (1)$$

In the present work, we investigated the catalytic properties of levansucrase in water-restricted environments. Since we are currently studying the secretion mechanism of this enzyme[3], our initial goal was to study possible modifications of some physical properties, like size and shape of the protein, in media of low dielectric constant which can simulate the apolar medium of the cytoplasmic membrane. We were astonished by the "unconventional" behavior of this enzyme in the presence of organic solvents.

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EXPERIMENTAL

Levansucrase. — Levansucrase was prepared from the culture supernatant of a constitutive strain of *B. subtilis*[4] according to a published method[1].

Substrates. — Uniformly labelled [^{14}C]sucrose was purchased from Amersham Corp. and purified by paper chromatography before use. Raffinose was obtained from Sigma Chemical Co.

Chemicals. — All the solvents used (*i.e.*, acetone, acetonitrile, dimethyl sulfoxide and 1,4-dioxane) were analytical grade from Merck.

Analytical methods. — The pH of water–solvent mixtures was measured with a special electrode for apolar medium (BRV 45, Heito).

Measurements of polymerase activity of levansucrase were carried out according to the following procedure[2]. A solution of 50mM phosphate buffer, pH 6, containing 10mM [^{14}C]sucrose in the chosen solvent at the desired concentration (45 μL) was incubated at 22° in a temperature-controlled cell. The reaction was initiated by addition of an 0.5μM enzyme solution (5 μL). Five aliquots of 10 μL were removed at specified intervals and ^{14}C-labelled sugars were quantitatively analyzed by paper chromatography[2]. The initial velocities were defined as V_g, V_f, and V_l, which are respectively the initial velocity of D-glucose release, D-fructose release, and levan synthesis (expressed as the molarity of D-fructose incorporated in levan). Thus, V_f measures the hydrolase activity and V_l the polymerase activity of the enzyme. Since $V_g = V_f + V_l$, the yield of polymerase activity, Y, is expressed in Eq. 2.

$$Y = 100\ \frac{V_l}{V_g} = 100\left(1 - \frac{V_f}{V_g}\right) \qquad (2)$$

RESULTS

Effects of organic solvents on enzyme behavior. — We studied the variation of the rates of the hydrolase and of the synthetase activity of levansucrase by progressively substituting, in the reaction mixture, water with acetonitrile, acetone, 1,4-dioxane, or dimethyl sulfoxide. These solvents share the same property of being water miscible, but they cause a wide range of dielectric constant and electric dipole moments.

The analytical method used is illustrated in Fig. 1 which shows a comparison of levansucrase catalytic activity in water and in 60% acetonitrile. In this later solvent, the hydrolase activity of the enzyme was completely inhibited as no liberation of free D-fructose was observed. In contrast, V_l had approximately the same value as that of V_g, which means that the yield of levan synthesis under these conditions reached 100%, as compared to only 5–7% in aqueous medium.

The initial rate of sucrose transformation was increased by a factor of three in the presence of 60% solvent. Furthermore, the observation that the rate of sub-

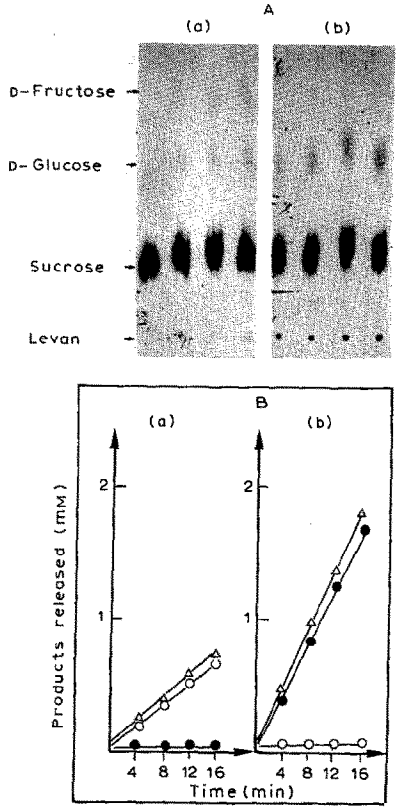

Fig. 1. Kinetics of transformations of [^{14}C]sucrose catalyzed by levansucrase in aqueous medium (a), and in 60% acetonitrile (b). The reaction mixture contained 10mM [U-^{14}C]sucrose and 0.05μM levansucrase in 50mM phosphate buffer, pH 6: A, Paper chromatogram. B, Graphic representation of the concentration of products released: (△—△) D-glucose, (○—○) free D-fructose, and (●—●) D-fructose incorporated in levan.

strate transformation was constant indicated that the enzyme is perfectly stable under such conditions.

The results obtained with the other solvents are listed in Table I. Except for acetonitrile, our investigations were restricted within the range of 0 to 50% (v/v) of each solvent owing to the insolubility of the substrate, sucrose, at higher concentrations.

The data presented in Table I clearly indicate that the yield of levan synthesis is strongly enhanced by the presence of increasing concentrations of organic solvents. Moreover, except for dimethyl sulfoxide, it appears that each solvent acts as an activator of the enzyme. From the data in Table I, it can also be stated that the effect of a particular solvent on the behavior of the enzyme does not merely correlate with the values either of its dielectric constant or of its electric dipole

TABLE I

YIELD OF THE POLYMERASE ACTIVITY OF LEVANSUCRASE WITH RESPECT TO CONCENTRATIONS OF VARIOUS SOLVENTS PRESENT IN THE REACTION MIXTURE

Solvent	ε^b	D^b	*Yield (%)*[a]				
			Solvent–water (v/v)				
			30:70	*40:60*	*50:50*	*60:40*	*70:30*
1,4-Dioxane	2.2	0.4	25 (1.1)	62 (1.4)	100 (2)		
Acetone	20.7	2.9	28 (1.2)	95 (1.6)	100 (2.2)		
Acetonitrile	38.8	3.4	23 (1.1)	86 (1.4)	100 (2)	100 (2.9)	100 (2.3)
Dimethyl sulfoxide	45.0	4.3	40 (1.1)	73 (1.1)	80 (0.7)		

[a]Values indicated in parentheses represent the activation factor measured for the rate of sucrose transformation. [b]The values of the dielectric constant (ε) and of the electric dipole moment (D) of each solvent are given in ref. 13.

moments. Thus, it is difficult to establish, from this limited work, a general law to predict the value for the levansucrase catalysis in water-restricted environments. We have arbitrarily chosen acetonitrile for further studies.

Isolation and analysis of levan synthesized in the presence of 60% acetonitrile. — As shown above, levansucrase in 60% acetonitrile displays only its polymerase

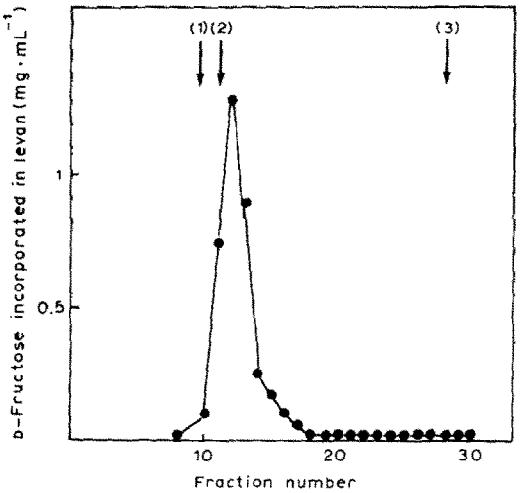

Fig. 2. Chromatography on Bio-Gel A5M column (1.6 × 25 cm) of levan synthesized under initial conditions of sucrose transformation by levansucrase in 60% acetonitrile. The Roé method[12] was performed on each fraction (1.8 mL) collected. Arrows indicate: (1) the void volume of the column, (2) the elution volume of Dextran 2000, and (3) fructose.

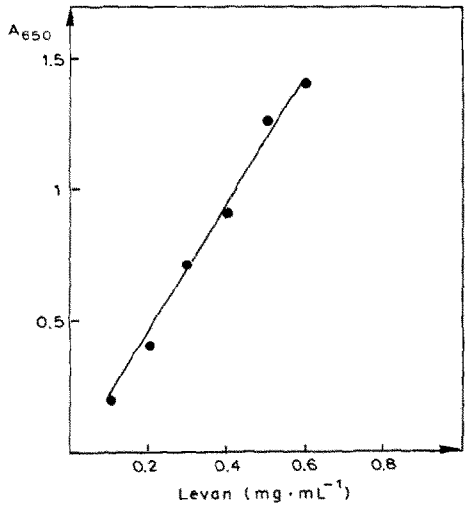

Fig. 3. Absorbance at 650 nm of a 60% acetonitrile solution containing increasing concentration of levan.

activity. Under such a condition, the enzyme activity yielded a cloudy reaction mixture due to the formation of levan, this polymer being insoluble in water and organic solvent mixtures. After centrifugation, the pellet was taken up in water and chromatographed on a Bio-Gel A5M column. Fig. 2 illustrates the size distribution of the levan molecules as a relatively sharp peak indicating mol. wts. ranging from 10^6 to 2.10^6. The composition of the levan synthesized in 60% acetonitrile was

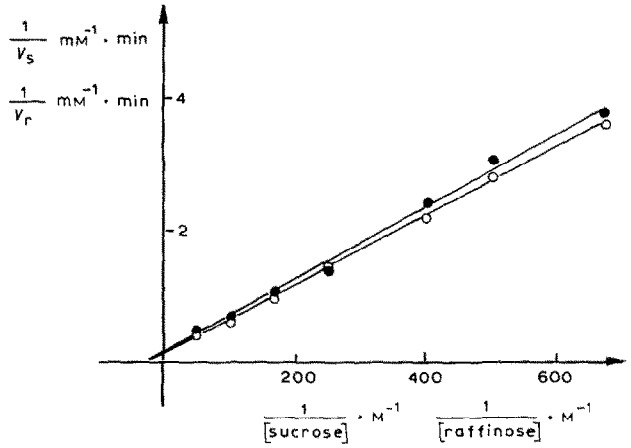

Fig. 4. Kinetics of sucrose and raffinose transformation catalyzed by 0.04μM levansucrase in 60% acetonitrile. Double-reciprocal plots of the initial rate of sucrose transformation V_s (●—●), and of raffinose transformation V_r (○—○) against sucrose and raffinose concentration, respectively.

TABLE II

KINETIC CONSTANTS OF LEVANSUCRASE FOR SUCROSE AND RAFFINOSE

Medium	*Sucrose*		*Raffinose*	
	K_m(M)	$k_{cat} \cdot min^{-1}$	K_m(M)	$k_{cat} \cdot min^{-1}$
Water[a]	2.10^{-2}	3.10^3	$1.5\ 10^{-2}$	3.10^3
60% Acetonitrile[b]	3.2 ± 1.10^{-2}	1.6 ± 0.510^4	$3.3 \pm 1.2\ 10^{-2}$	1.7 ± 0.610^4

[a]Values given for the aqueous medium are from various sources[1,2,7]. [b]Values for 60% acetonitrile are obtained from regression adjustment with the weighted least-square method proposed by Wilkinson[14] of the experimental data presented in Fig. 4.

determined from the complete hydrolysis of a ^{14}C-labelled polymer (heated for 20 min in 0.2M sulfuric acid at 60% ref. 6). [^{14}C]Fructose was the only sugar that could be detected in the hydrolyzate by paper chromatography (results not shown).

Determination of kinetic constants of levansucrase for sucrose and raffinose in 60% acetonitrile. — As indicated above, solutions of levan in the presence of organic solvents are turbid. This effect was used to determine the concentration of the polymer synthesized during the course of enzyme action. Fig. 3 shows a standard curve that relate levan concentration to absorbance at 650 nm. It can be calculated that one absorbance unit corresponds to 0.435 $mg \cdot mL^{-1}$ of levan or 2.7mM fructose incorporated.

The experimental measurements of initial rate of sucrose transformation V_s and of raffinose transformation V_r with respect to their concentration in 60% acetonitrile are shown in Fig. 4. The Lineweaver and Burk double-reciprocal plots were linear. The Michaelis constants for the enzyme with respect to each substrate in water-restricted environments are listed in Table II and compared to the same constants determined for the aqueous medium[1,7]. It is clear that the values of K_m are approximatively the same, but those of k_{cat} are increased for both substrates by a factor of five in the presence of 60% acetonitrile.

DISCUSSION

In concentrated solution of organic solvents, levansucrase displays only its polymerase activity when using sucrose or raffinose as fructosyl donors. The enzyme appears stable and more active toward its substrates than in aqueous medium.

Recently, Klibanov[8] hypothesized that perhaps all enzymes could function in organic solvents. This hypothesis, based on studies of the catalytic behavior of few enzymes in water-restricted environments, appears to be more and more heuristic[9–11] and creates totally new opportunities in the area of applied enzymology. The results presented herein open the way for the synthesis of levan with a high yield. Furthermore, as short chain acyl alcohols may act as D-fructosyl acceptors[1] this allows to extend the transfructosylation reaction of levansucrase to long-chain acyl alcohol solubilized in organic solvent–water mixture.

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