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Immobilisation of levansucrase on calcium phosphate gel strongly increases its polymerase activity

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ABSTRACT

The catalytic properties of levansucrase bound to hydroxyapatite were studied as a possible model for enzyme behaviour when associated in vivo to matrices such as the cell wall of bacteria or tooth surfaces. The activity of the immobilised enzyme was mainly directed towards its polymerase activity. The yield of levan reached 85%. The k_{cat} of the enzyme for sucrose transformation was increased and the K_m for this substrate was unmodified. These properties allow the design of a system for the large-scale production of high-molecular-weight branched-chain levan in vitro in high yield.

INTRODUCTION

Levansucrase (sucrose (2 → 6)-β-D-fructan 6-β-D-fructosyltransferase, EC 2.4.1.10) is produced by a number of bacteria[1]. In vitro, the purified enzyme acts both as sucrose hydrolase and as fructosylpolymerase according to the following reaction:

n sucrose → n D-glucose + levan$p$(D-fructosyl) + $(n-p)$D-fructose

The yield of levan depends on various factors including the presence or the absence of preformed levan[2,3], and the ionic strength[2] and dielectric constant of the reaction medium[4].

However, in vivo bacterial levansucrase is generally found partially bound to the cell wall and partially free in the culture medium. This could indicate that the enzyme displays different catalytic specificities according to its context.

We have attempted to gain insight into this question using the *Bacillus subtilis* levansucrase by comparing the catalytic behaviour of the enzyme in a free state with that when bound to a matrix.

We selected hydroxyapatite as the matrix for the following reasons: (*i*) the enzyme is strongly adsorbed on this phosphate gel[5]; (*ii*) one abundant component

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of the Gram-positive cell wall, teichoic acid[6], is an anionic phosphate polymer displaying some structural similarities with hydroxyapatite and (*iii*) levansucrase of *Streptococcus mutans* which is very similar in activity and sequence to *B. subtilis* levansucrase acts in vivo in close contact with the hydroxyapatite of the tooth surface and is involved in causing cariogenic lesions[7].

EXPERIMENTAL

Levansucrase purification.—Levansucrase was prepared from the culture supernatant of a constitutive strain of *B. subtilis* according to a published method[5].

Substrates.—Uniformly [^{14}C]-labelled sucrose was purchased from Amersham Corp. and purified by paper chromatography before use as described previously. [^{14}C]-Labelled levan was prepared in our laboratory from [^{14}C]sucrose by the action of levansucrase.

Chemicals.—Hydroxyapatite Biogel HTP and Biogel Agarose A-150M were from Bio-Rad Laboratories. The particle size of the hydroxyapatite powder was 10–100 μm.

Analytical methods.—The polymerase activity of levansucrase was measured as follows[3]: a solution of 50 mM phosphate buffer (pH 6) containing [^{14}C]sucrose at the desired concentration was incubated in a temperature-controlled cell. The reaction was initiated by the addition of enzyme. Aliquots were removed at given intervals and [^{14}C]-labelled sugars were quantitatively analyzed by paper chromatography. The trans-fructosylase activity was measured directly from the amount of [^{14}C]glucose released. The yield of the polymerase reaction, Y, can be expressed by the relation:

$$Y = 100\left(1 - \frac{\text{free fructose release}}{\text{free glucose release}}\right).$$

Structural analysis of levan.—Levan was methylated by the method of Hakomori[8]. Methylated levan was hydrolysed and alditol acetates analysed according to the method of Tanaka et al.[9].

RESULTS

Comparison of catalytic activities of free levansucrase and of hydroxyapatite-bound enzyme.—The kinetics of sucrose transformation and the yield of the polymerization reaction were determined (Fig. 1) for the free and the bound enzyme. Immobilisation of the enzyme with hydroxyapatite had two effects on its activity. First, the initial rate of sucrose transformation was increased by a factor of two. Secondly, the polymerisation yield remained constant, around 75% throughout the reaction. In the free state, the enzyme was a poor polymerase during the initial phase of the reaction and polymer yield increased as the reaction progressed.

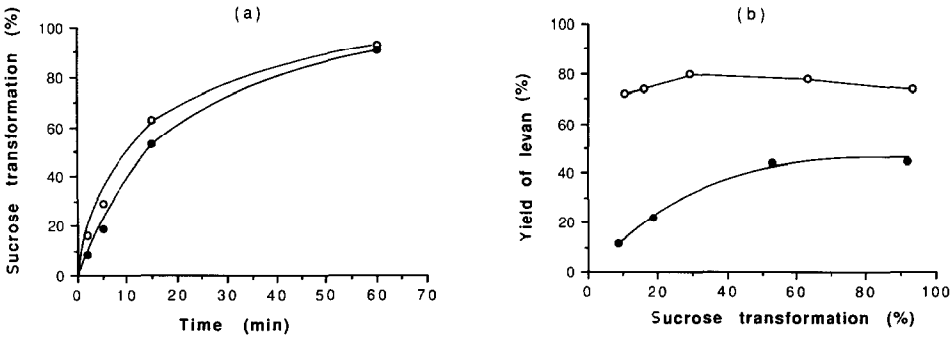

Fig. 1. Catalytic activities of free and of hydroxyapatite-bound levansucrase. (a) Kinetics of sucrose transformation; (b) levan yield plotted against sucrose transformation for free (•) and bound levansucrase (○). Enzyme concentrations were 0.3 μM. The initial sucrose concentration was 0.25 M in 50 mM potassium phosphate buffer (pH 6); the temperature was 25°C. The initial volume of the mixture was 200 μL. Bound enzyme was prepared by mixing 1 mg of hydrated hydroxyapatite with 200 μL of 50 mM potassium phosphate buffer (pH 6), containing 0.3 μM enzyme. After 15 min, the suspension was centrifuged and the reaction initiated by resuspending the pellet in 200 μL of sucrose solution.

These results indicate that the immobilised levansucrase may bind the growing levan chain more strongly than does the free enzyme, thereby enabling a more-efficient polymerase activity.

Size distribution of levan synthesized by immobilised and free levansucrase.—The size distributions of levan synthesised by free and immobilised enzyme were estimated from the chromatographic behaviour of these polymers in agarose A-150M columns (Fig. 2). The degree of polymerisation of levan were quite different. Levan produced by the free enzyme migrated slowly through the column whereas the levan formed by hydroxyapatite-bound levansucrase was completely excluded from Biogel Agarose A-150M. The molecular weight of this levan was therefore higher than 10^7.

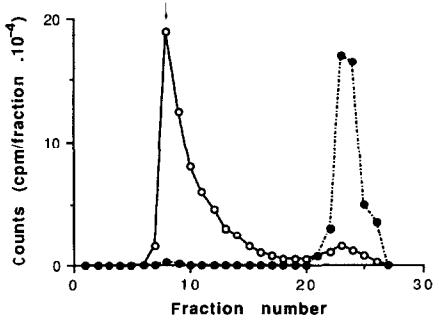

Fig. 2. Chromatography on a Biogel A-150 M column of levan. Levan was produced by the activity of free enzyme (•) or hydroxyapatite-bound enzyme (○). The mixtures at the end of the experiment presented in Fig. 1, were extensively dialysed for 18 h against water to eliminate labelled glucose and free fructose. Approximately the same amount of labelled levan ($6 \cdot 10^5$ cpm) from each preparation was subjected to chromatography, and fractions of 0.5 mL were collected. The volume of the gel in the column was 12 mL. The void volume of the column is indicated by an arrow.

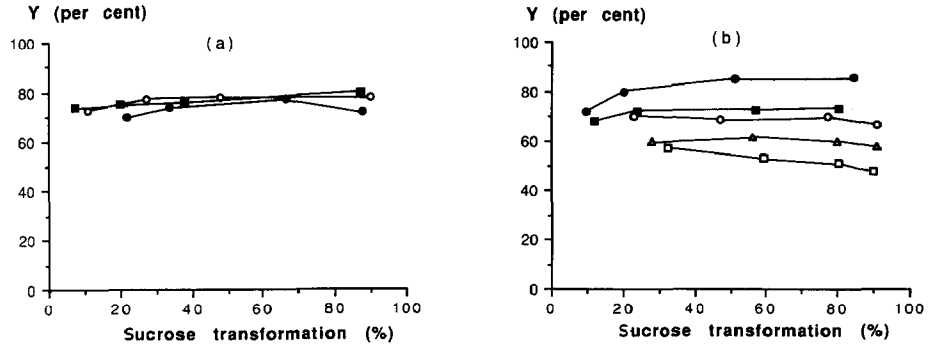

Fig. 3. Yield plotted against sucrose transformation (%) for hydroxyapatite-bound levansucrase. (a) In the presence of various initial sucrose concentrations at 25°C. (●) 0.1 M; (○) 0.5 M; (■) 0.8 M; (b) At various temperatures of reaction, (●) 5°C; (■) 30°C; (○) 40°C; (△) 50°C; (□) 60°C; the initial sucrose concentration in each case was 0.25 M. (Assays were as described in the legend of Fig. 1.)

Modulation of the polymerase activity of the bound enzyme.—We attempted to define the conditions for maximum yield of the polymerase reaction. The density of levansucrase on the hydroxyapatite support had a small effect on polymerase activity since the variation of the hydroxyapatite: levansucrase ratio within the range of 10 to 100 did not modify the yield of levan. We investigated then the effects of initial sucrose concentration and of temperature on the polymerase activity of the bound enzyme. The results obtained are shown in Fig. 3.

The yield of the polymerase reaction varied little between different sucrose concentrations within the concentration range explored. The yield remained constant in each case throughout the reaction. The yield was however, temperature-dependent. The maximum yield of 85% was reached at 5°C. The levan formed was partially hydrolyzed at 60°C, causing the apparent yield at high temperatures to decrease as the reaction progressed.

Determination of kinetic constants of immobilised levansucrase.—We measured the initial rate of sucrose transformation catalyzed by bound levansucrase. The Michaelis constant for the enzyme was determined with respect to sucrose (Table I) and was compared to the corresponding constant for the enzyme free in solution. The values of K_m were approximately the same but the k_{cat} of the bound enzyme was about double that of the free enzyme.

TABLE I

Kinetic constants of free and bound levansucrase for sucrose [a]

	K_m (M)	k_{cat} (min^{-1})
Free levansucrase	$1.8 \cdot 10^{-2}$	$3 \cdot 10^3$
Bound levansucrase	$1.2 \pm 0.1 \cdot 10^{-2}$	$5 \pm 0.3 \cdot 10^3$

[a] Values given for free levansucrase are from Chambert et al.[3]; values for bound enzyme were obtained from regression adjustement with the weighted least-squares method of the kinetic results, obtained from measurements of the initial rate of sucrose transformation with respect to this substrate concentration at 25°C.

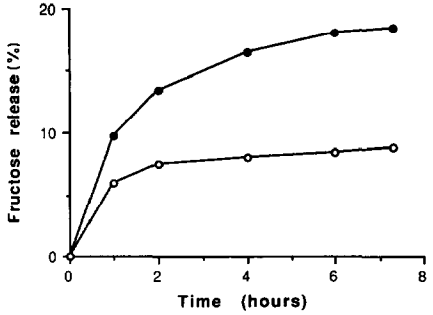

Fig. 4. Kinetics of levan hydrolysis by free enzyme (●) and by hydroxyapatite-bound enzyme (○). The initial concentration of labelled levan was 10 mg mL^{-1} ($9 \cdot 10^6$ cpm mL^{-1}) in 0.05 M potassium phosphate buffer (pH 6). The enzyme concentration was 0.3 μM, initial reaction volume was 100 μL, and temperature was 25°C. The experimental procedure was as described in Fig. 1. Levan hydrolysis was followed from the appearance of free labelled fructose and is expressed as per cent of label released.

Levan hydrolytic activity of the enzyme.—Levansucrase can hydrolyse levan. This hydrolytic activity is stopped at branch points[5]. The catalytic efficiencies of the free and hydroxyapatite-bound enzyme were compared (Fig. 4). The activity was decreased by a factor of two by adsorption onto hydroxyapatite. Thus, either the bound enzyme works in a microenvironnement characterised by water restriction, or the association with hydroxyapatite promotes fructosyl exchange between levan molecules rather than transfructosylation on water.

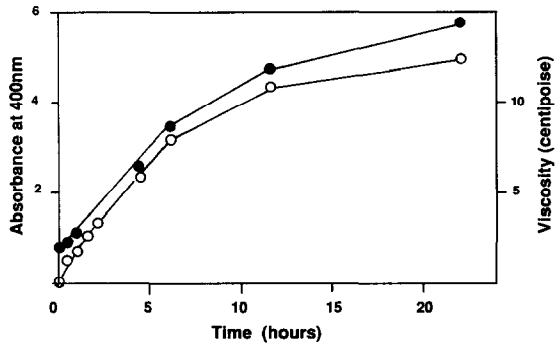

Fig. 5. Kinetics of levan synthesis by hydroxyapatite-bound levansucrase in 100 mL of reaction mixture. Solid hydroxyapatite (150 mg) was resuspended in 2 mL of 0.05 M potassium phosphate buffer (pH 6). Levansucrase (1 mL of 5 mg mL^{-1} solution) was then added and gently stirred for 15 min. The suspension was centrifuged (10 min at 8000*g*), the supernatant was discarded and the pellet resuspended in 100 mL of 10% sucrose (~ 0.3 M) in the same buffer. The reaction was allowed to proceed with gentle stirring at 5°C. Formation of levan was followed on an aliquot after centrifugation by measurements of the absorbance (at 400 nm) resulting from the light scattering by this polymer, and by measurements of the viscosity of the reaction mixture. Viscosity was evaluated using a Baume viscosimetric tube at 25°C. Absorbance at 400 nm (○) (1 absorbance unit corresponds to 8.8 mg mL^{-1} of levan as evaluated from a standard curve); viscosity (●).

TABLE II

Quantitative results [a] of fractionation of *O*-methyl-D-fructoses obtained from methylation of levan

Levan from	1,3,4,6-tetra-*O*-methyl-D-fructose	1,3,4-tri-*O*-methyl-D-fructose	3,4-di-*O*-methyl D-fructose	Average length of β-(2 → 6)-fructofuranosyl chain
Free levansucrase	8.5	87.0	4.5	12
Bound levansucrase	13.2	75.8	11.0	7–8

[a] Each compound is expressed as per cent of the total amount of methyl-D-fructose. Data from R. Wouters (Raffinerie Tirlemontoise, Belgique).

Preparation of levan on a medium scale from immobilised enzyme.—To test whether immobilised levansucrase could be used for the synthesis and isolation of levan on a large scale, we greatly increased the volume of the reaction mixture. Levan synthesis was followed by measuring the increase of absorbance at 400 nm and the increase of the viscosity of the reaction mixture. The kinetics of levan synthesis, in a 100 mL volume of 10% sucrose in the presence of 5 mg of hydroxyapatite-bound enzyme, at 5°C, is shown in Fig. 5.

The reaction was allowed to proceed for 22 h. Levan concentration reached ~ 40 mg mL^{-1}. The mixture was centrifuged to eliminate the bound enzyme and the supernatant was extensively dialyzed against water and lyophilized.

We obtained 3.4 g of white, fibrous dry levan. This was 68% the maximum theoretical yield. Moreover the levansucrase hydroxyapatite complex pellet retained ~ 70% of initial enzyme activity.

From this pellet, it was possible to perform a second cycle of levan synthesis with a fresh solution of sucrose. The same amount of levan was obtained at the end of the reaction, and thus further cycles appear to be practicable.

Structural analysis of levan synthesised by free and bound levansucrase.—Levan was obtained in the conditions described in Fig. 1 except that unlabelled sucrose was used. The reaction mixtures were extensively dialyzed once no sucrose remained, and then submitted to methylation analysis. Quantitative results of fractionation of the methylated fructose are presented in Table II. The average length of the repeating β-(2 → 6)-linked fructosyl unit of the branched polymers was estimated from the ratio of total *O*-methyl-D-fructose to 1,3,4,6-tetra-*O*-methyl-D-fructose. The average length of β-(2 → 6)-fructofuranosyl chains formed by free levansucrase was ~ 12 fructosyl residues, and that from immobilized levansucrase was 7 to 8 fructosyl residues. Thus, the bound enzyme synthesises a polymer with a more compact structure.

DISCUSSION

Studies of the catalytic activities of enzymes bound to matrices have been used as possible model systems for in vivo[10] enzyme reactions. For bacterial levansucrase, this approach could provide information pertaining to the catalytic activity of the enzyme when bound to cell wall.

Since the envelope structure of Gram-positive bacteria is complex and difficult to obtain in its native state, we studied the properties of *B. subtilis* levansucrase associated with hydroxyapatite. The reasons of this choice are detailed in the introduction.

The most striking feature observed in this work was that such immobilisation of the enzyme directed its activity toward its polymerase action relative to its sucrose-hydrolase activity. The question then arises as to how immobilisation modifies the enzyme specificity.

The first hypothesis which may be proposed is that immobilisation changes the molecular mechanism of the transfructosylation process; new side-chain groups of the enzyme may become involved in catalysis. However this hypothesis is unlikely because a polymerase-defective levansucrase variant we have constructed[11] did not recover this catalytic activity when bound to hydroxyapatite (result not shown).

The second hypothesis is that the activity change results from a microenvironmental effect such as the restriction of water availability. This hypothesis is attractive since we have shown that this enzyme displayed only its polymerase activity in concentrated solution of organic solvents[4]. However we do not know whether the protein is really embedded within the hydroxyapatite gel or only adsorbed on its surface. From the extensive studies of the interaction of proteins with hydroxyapatite, several authors[12,13] concluded that enzymes adsorb onto the surface and postulated that acidic proteins, like levansucrase, bind specifically by complexing the carboxyl groups to the calcium sites on the matrix. This explanation is consistent with the finding that calcium plays a key role in the folding process of *S. mutans*[7] and *B. subtilis* levansucrase[14].

A third possibility is that the diffusion of the growing levan chains is restricted in the microenvironment of the immobilised enzyme.The resulting increase in local concentration of levan could account for the enhancement of the polymerase activity. However, it must be noted that the density of levansucrase on the hydroxyapatite support did not affect the yield of the polymerase reaction. Moreover, covalent immobilisation of the enzyme on other matrices did not change its polymerase efficiency[15].

The biotechnological value of this behaviour should be mentioned, since the methods currently available for the isolation of levan are based on ethanol treatment of growth medium of various bacteria producing levansucrase[1]. Such methods are tedious and result in pure polymers with a final yield lower than those achieved in this work. The performance of hydroxyapatite-bound enzyme could be further improved: for example we have verified that the enzyme acts efficiently on a column under a continuous flow of sucrose solution. The isolation of levan on a large scale would be possible using this type of system.

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