Polymerase and hydrolase activities of *Bacillus subtilis* levansucrase can be separately modulated by site-directed mutagenesis

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The levansucrase (sucrose:2,6- β -D-fructan 6- β -D-fructosyltransferase, EC 2.4.1.10) structural gene from a *Bacillus subtilis* mutant strain displaying a low polymerase activity was sequenced. Only one missense mutation changing Arg³³¹ to His was responsible for this modified catalytic property. From this allele we created new mutations by directed mutagenesis, which modified the charge and polarity of site 331. Examination of the kinetics of the purified levansucrase variants revealed that transfructosylation activities are affected differently by the substitution chosen. His³³¹→Arg completely restored the properties of the wild-type enzyme. The most striking feature of the other variants, namely Lys³³¹, Ser³³¹ and Leu³³¹, was that they lost the ability of the wild-type enzyme to synthesize levan from sucrose alone. They were only capable of catalysing the first step of levan chain elongation, which is the formation of the trisaccharide kestose. The variant His³³¹→Lys presented a higher k_{cat} for sucrose hydrolysis than the wild-type, and only this hydrolase activity was preserved in a solvent/water mixture in which the wild-type acted as a true polymerase. The two other substitutions reduced the efficiency of transfructosylation activities of the enzyme via the decrease of the rate of fructosyl-enzyme intermediate formation. For all variants, the sucrose affinity was slightly affected. This strong modulation of the enzyme specificities from a single amino acid substitution led us to postulate the hypothesis that bacterial levansucrases and plant fructosyltransferases involved in fructan synthesis may possess a common ancestral form.

INTRODUCTION

Fructans (insulin or levan type) are soluble polymers of β -D-fructofuranose which are synthesized by several bacterial species [1] and by about 12% of higher plants [2], in which they are stored in high concentration as an alternative to starch. In both Kingdoms, the synthesis occurs from sucrose as the sole substrate. However, plants synthesize fructan by the concerted action of a number of distinct fructosyltransferases [3] exhibiting a high level of fructosyl-donor and fructosyl-acceptor specificities, whereas each species of bacteria produces one exocellular levansucrase (sucrose:2,6- β -D-fructan 6- β -D-fructosyltransferase, EC 2.4.1.10) [1] that is capable of synthesizing branched fructan of high molecular mass from sucrose. For all known levansucrases this polymerase activity is associated with a low level of fructosyl-acceptor specificity. These enzymes catalyse, in fact, the fructosyl transfer from sucrose to numerous acceptors other than levan, such as water (hydrolase activity), short-chain acylalcohols, and various mono- and di-saccharides.

The question arises as to whether such broad enzyme specificity can be restricted by site-directed mutagenesis. We have undertaken such a study with *Bacillus subtilis* levansucrase on the basis of its well-known catalytic properties and mechanism of action [4]. Furthermore, its structural gene cloned into a plasmid vector has been sequenced and may be expressed in *Escherichia coli*, allowing easy construction of protein variants [5,6]. The main difficulty of this approach is to identify, on the wild-type enzyme, the critical amino acids playing key roles in the transfructosylation process. The three-dimensional structure of this protein has not yet been determined at an adequate resolution [7] to permit the precise visualization of the active site. Fortunately, from previous genetic analyses [8], various mutants of *B. subtilis* producing altered levansucrases were isolated. In one of them,

the QB252 strain, the mutation did not affect the thermostability, but modified the catalytic properties of the enzyme in such a way that the polymerase activity was decreased relative to the hydrolase activity.

We have cloned and sequenced this *sacB* allele. As we show in the present study, the mutation involves one substitution, namely Arg³³¹→His. To restore the wild allele and to modify the charge and polarity of this site, we carried out the following amino acid substitutions: His³³¹→Arg, His³³¹→Lys, His³³¹→Ser and His³³¹→Leu.

The catalytic properties of the purified relevant levansucrase variants were analysed. We focus our attention on the ability of these variants to display polymerase activity under conditions where the wild-type acts as an effective polymerase [9]. Furthermore, we attempt to evaluate, for each variant, the kinetic constants of the step of formation and of the step of disappearance of the transient fructosyl-enzyme intermediate which has been shown to play a central role in the transfructosylation processes catalysed by the enzyme [4].

MATERIALS AND METHODS

Strains and growth conditions

Bacillus subtilis QB252 [*sacA321*, *sacU32*, *sacB*(252)] obtained by Lepesant *et al.* [8], was grown on mineral medium supplemented with glucose as a carbon source. The *B. subtilis* QB112 strain [10] was used as reference for wild-type levansucrase production and sequence.

Escherichia coli XLI B (*recA1*, *gyrA96*, *thi*, *hsdR17*, (*r_k*, *m_k*), *supE44*), *relA1*, λ –, (*lac*), (*F_{proAB}*, *lacI^q*, *lacZ Δ M15*, *Tn10*(*tet^R*)) (Stratagene) grown at 30 °C in Luria–Bertani (LB) or Terrific broth medium [11] supplemented with the appropriate

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antibiotics was used as a host for plasmid pBluescript recombinants. *E. coli* transformation was performed by the calcium-shock procedure, and transformants were selected on LB plates supplemented with 5-bromo-4-chloroindol-3-yl galactoside and isopropyl thio- β -D-galactoside and the appropriate antibiotics [11].

Plasmids and DNA extraction

Chromosomal DNA of *B. subtilis* strains was extracted from culture in exponential phase of growth as described by Saito & Miura [12].

pBluescript was used as cloning vector of the amplified DNA. Plasmid DNA preparations were performed by the alkaline method [11]. When used for sequencing, they were further treated with RNAase, extracted several times with phenol/chloroform (1:1, v/v) and once with chloroform, and finally chromatographed on Ultrogel AcA34. Single-stranded DNA was prepared with M13K07 helper phage according to the protocol proposed by Stratagene.

Cloning and sequencing of the *sacB*(252) allele

Chromosomal DNAs from the QB252 and QB112 strains were used as templates for amplification of the *sacB*(252) allele and the wild-type *sacB* gene by PCR. Before amplification the viscosity of DNA solutions (1 mg·ml⁻¹) was decreased by digestion with *Pst*I; this endonuclease does not possess a hydrolytic site in the wild-type *sacB* gene [5].

Two oligonucleotides, corresponding to nucleotides 169–190 and 1994–1973 of the sequenced DNA fragment [5]:

5'-dCGCGGATCCGGCCCCGTAGTCTGCAAATCCT-3'
5'-dCCGCTCGAGTACGGTTAGCCATTGCTGC-3'

were synthesized (Fig. 1) with the addition of a *Bam*HI site or a *Xho*I site (underlined) to the 5' end of each respectively and used as primers. Approx. 1 μ g of DNA was used for the input, and amplification was carried out with 22 cycles of 1 min denaturation at 94 °C, 3.5 min annealing at 54 °C and 3 min extension at 72 °C. The concentrations of oligonucleotides, dXTP and Mg²⁺ in the reaction mixture were according to the standard procedures suggested by Perkin-Elmer/Cetus. The PCR products (3–5 μ g) were chromatographed on Ultrogel AcA34 (10 ml column) to eliminate excess oligonucleotides. Then the amplified genes (1.82 kb) were digested with *Bam*HI and *Xho*I and ligated into the pBluescript cloning vector.

Fig. 1. Sequencing strategy for the *sacB*(252) allele

The boxed area represents the *sacB* region amplified by PCR, the stippled areas inside indicating the non-coding sequence. The promoter is indicated in the left-hand stippled region by '35' and '10'. *Bam*HI and *Xho*I are the cloning sites into pBluescript introduced into the primers used for PCR. Base numbers refer to the published sequence [5]. Synthetic oligonucleotides used for sequencing the structural gene are the following (numbers in parentheses refer to the published sequence):

- 1 5'-dGGAGGGCTGGAAGAAGCAGACC-3' (407–428)
- 2 5'-dGGCCTGGACGTTTGGGACAGC-3' (705–725)
- 3 5'-dCAGCCACATTTACATCTGACGG-3' (960–981)
- 4 5'-dGGAAGTGAAGATGGCTACCAAG-3' (1259–1280)
- 5 5'-dAACGATATTTACATGCT-3' (1577–1594)
- 6 5'-dCCAAGCATGTAAATATCG-3' (1597–1579)

Single-stranded DNA or plasmid DNA from eight individual transformants in each case was combined and sequenced in parallel using oligonucleotide primers as indicated in Fig. 1. DNA sequencing was conducted by the dideoxy-chain-termination of Sanger *et al.* [13].

Site-directed mutagenesis

Taking advantage of the unique site *Kpn*I (Fig. 1), 16 nucleotides upstream of the mutation site, oligonucleotides containing a *Kpn*I endonuclease site (underlined) and including mismatches were used as primers in PCR to generate amino acid substitutions in the amplified DNA fragment (1521–1994), using *sacB*(252) as template.

The following (5'-GCGGTACCGTTCAGTACTCCXXX-GGATCAAAAATG-3') oligonucleotides (the site indicated as XXX in bold was substituted by CGC for Arg, AAG for Lys, AGC for Ser, CTC for Leu) were synthesized. The oligonucleotide 1994–1973, described above, was used as a primer for the other strand. The amplified DNA fragments (1521–1994) were, after chromatography on Ultrogel, digested with *Kpn*I and *Xho*I, and purified by electroelution after agarose-gel electrophoresis. Each was ligated to the purified fragment 169–1521 obtained after digestion with *Bam*HI and *Kpn*I of the *sacB*(252) allele and finally ligated into appropriately digested pBluescript vector, generating *sacB* mutants.

Expression and purification of levansucrase variants

E. coli XL1-B recombinants were grown at 30 °C as indicated above. Samples (1 ml) were removed at intervals during growth and frozen. They were then thawed and sonicated briefly (45 s) in 0.1 M-potassium phosphate, pH 6, after addition of chloramphenicol (300 μ g·ml⁻¹) and lysozyme (200 μ g·ml⁻¹). The crude extracts were assayed for levansucrase exchange activity, and aliquots were submitted to electrophoresis and immunoblotting as described below.

Purification of levansucrase variants was achieved from 500 ml of culture. The crude extracts were chromatographed on a hydroxyapatite column (5 ml) which was eluted with a step of 50 ml of 0.5 M-potassium phosphate, pH 6, followed by a linear gradient of 0.5 M–2 M-potassium phosphate, pH 6 [14].

Evaluation of levansucrase amounts by immunoblotting

Proteins were analysed on SDS/10%-(w/v) polyacrylamide gels by electrophoresis and immunoblotting. Incubation with purified rabbit anti-levansucrase antibodies and radioiodinated Protein A was described previously [15]. To determine the amount of levansucrase in the samples, a standard curve was established from immunoblotting of purified levansucrase within the range of 5–50 ng.

Kinetic characterization of the mutant enzymes

Levansucrase of *B. subtilis* catalyses the following general reaction:

Examination of the kinetics of the enzyme activity under steady-state conditions [4] supported unambiguously the conclusion that a fructosyl-enzyme intermediate, EF, occurs in the transfructosylation process. Therefore the mechanism of enzyme action can be described by the two-step model:

where E, S, G and A are symbols for enzyme, sucrose, glucose and fructosyl-acceptors (water, alcohol, fructan etc.) respectively.

A long time ago we proposed [16] an easy operating method to determine the values of the kinetic parameters of this reaction pathway from a minimum of experiments. This method is based upon the measurement of v_F and v_G , which are respectively the initial velocity of fructose and glucose liberation in the presence of various concentrations of a chosen fructosyl acceptor. Such a compound acts both as an inhibitor for free fructose release and as an activator for glucose liberation, since it is generally a better fructosyl acceptor than water.

According to this method, it is possible to estimate with reasonable accuracy the kinetic parameters of eqn. (1) and then to compare the catalytic efficiency and catalytic specificity of the constructed variants.

Enzymes and substrates

Taq polymerase and PCR kit were from Cetus Corp., Norwalk, CT, U.S.A. Sequenase was from USB Corp., Cleveland, OH, U.S.A.

Uniformly labelled [^{14}C]sucrose and [^{14}C]glucose were purchased from Amersham and purified by paper chromatography before use.

Levans of low molecular mass (15 ± 3 kDa) were previously prepared for kinetic studies of levansucrase [4]. Inulin from dahlia tubers (5 kDa) was from Sigma, St. Louis, MO, U.S.A.

Initial-velocity measurements

Transfructosylation reaction from sucrose to water: sucrose hydrolysis. A reaction mixture (45 μl) containing [^{14}C]sucrose at various concentrations (all the values were lower than 50 mM) in 0.05 M-potassium phosphate, pH 6, were incubated at 30 °C. The reaction was initiated by the addition of 5 μl of the enzyme solution. Portions (5 μl each) were removed at intervals and [^{14}C]-labelled sugars were quantitatively analysed by paper chromatography [4]. The initial velocities were defined as v_F and v_G , which are respectively the initial velocity of fructose and glucose release.

Transfructosylation from sucrose to fructose polymer: fructose chain elongation. Levan (or inulin) at various concentrations (1–6 mM) was added to the reaction mixture described above. Initiation of the reaction and analysis of the products were as described.

Transfructosylation from sucrose to glucose: exchange reaction. Glucose at concentrations up to 200 mM was added to the reaction mixture as described above. The conditions of the reaction initiation and product analysis were also as described above. The initial velocity of the exchange reaction is $v_G - v_F$ [16].

RESULTS

Identification of a point mutation on the levansucrase structural gene QB252 mutant as an Arg³³¹ → His substitution

Chromosomal DNAs of *B. subtilis* QB252 and QB112 strains were used as templates to amplify the *sacB* region. The 1.82 kb DNA fragments obtained by PCR as described in the Materials and methods section start 294 bp upstream of the *N*-terminal methionine residue and end 112 nucleotides after the *C*-terminal codon. After cloning them into pBluescript, propagation of the plasmids was done in the host strain *E. coli* XLI-B.

The DNA sequence of the inserted fragments was determined in parallel, on single-stranded DNA, using the synthetic oligonucleotides described in Fig. 1, which allowed the overlapping of the fragments delimited by the primers along the amplified DNA.

A single nucleotide change was identified in *sacB*(252), at position 1542, in reference to the *sacB*(112) sequence, which was found to be the same as the *sacB* published sequence [5],

Fig. 2. DNA nucleotide sequences of the *sacB*(252) allele and the *sacB*(112) wild-type gene

The part of the sequence represented was obtained from single-stranded DNA sequencing of the non-coding strand by using primer 4 as indicated in the legend of Fig. 1. The modified nucleotide is indicated by an arrow. The wild-type codon CGC becomes CAC, changing Arg to His at position 331 of the mature amino acid sequence. (a): *sacB*(252); (b) *sacB*(112).

corresponding to a transition G → A which modifies the Arg³³¹ residue (codon CGC) to a histidine residue (codon CAC), as shown in Fig. 2. The nucleotide sequence of the other strand was done on the double-stranded plasmid DNA using an appropriate oligonucleotide (1605–1597). The change C to T at the same site was obtained, confirming the mutation at this site (result not shown).

Expression, purification and stability of the constructed variants

Levansucrase variants constructed as described in the Materials and methods section were expressed in *E. coli* XL1 B, host of pBluescript recombinants.

Samples from 30 °C culture with a generation time of 80 min were removed at intervals during exponential growth. They were then treated as described in the Materials and methods section for levansucrase assays and quantification after electrophoresis and immunoblotting analysis. The differential rate of levansucrase synthesis by *E. coli* was defined as the slope of the straight line obtained when the amount of levansucrase was plotted against the A_{600} of the *E. coli* culture. The value of this differential rate was approximately the same for each levansucrase variant, namely 1.1 $\mu\text{g}/A_{600}$ unit (about 0.8% of total proteins).

Purifications of levansucrase variants were achieved from chromatography on hydroxyapatite of the crude extracts as described in the Materials and methods section. All variants

displayed the same chromatographic behaviour and were eluted at 1.1 M-potassium phosphate, pH 6. Stock solutions of 40–60 $\mu\text{g}\cdot\text{ml}^{-1}$ in 0.1 M-potassium phosphate, pH 6, were prepared for further kinetic analysis for each purified variant.

The yield of the purification process was 30 %; that is, from 500 ml of *E. coli* culture grown to an A_{600} of 5, we routinely obtained 0.8–1 mg of pure levansucrase variant.

The stability of each variant at 30 °C was deduced from the observed constant value of the differential rate of synthesis in *E. coli* and from the observation that the initial rate of catalysed reactions remained constant for all mutants during the time of the assays.

Catalytic properties of the variants

As proposed in the Materials and methods section the catalytic properties of the variants were studied from an investigation of the various transfructosylation reactions catalysed by levansucrase. Assuming that for all variants these fructosyl transfers were achieved by the same pathway involving the formation of transient fructosyl-enzyme intermediate (reaction 1), we analysed the kinetic experimental data obtained with the help of the kinetic equations derived from this multi-step mechanism.

Transfructosylation from sucrose to water: sucrose hydrolysis. In the presence of low concentrations of sucrose (< 50 mM), the wild-type levansucrase displays only hydrolytic activity [4]. Thus, under such conditions, sucrose does not act as fructosyl acceptor for the initiation of levan chains. Therefore, the kinetic behaviour of the enzyme with respect to the sucrose concentration may be expressed by the Michaelis equation:

$$v_F = v_G = \frac{k_{\text{cat}}[E_T][S]}{K_m + [S]}$$

where v_F , v_G , S , E_T are respectively the initial velocity of fructose and glucose release and concentrations of sucrose and enzyme. Experimental measurements of the initial rate of sucrose hydrolysis were carried out within a concentration range of 1–50 mM-sucrose. The kinetic data obtained enabled us to estimate, from a double-reciprocal plot, the parameters K_m and k_{cat} (V_{max}/E_T) for each variant (Table 1).

Table 1 shows that amino acid substitutions at site 331 strongly modified the sucrose hydrolase efficiency of levansucrase. As expected, the Arg³³¹ variant possessed the same maximum activity as that of the wild-type enzyme [4]. The Ser³³¹ variant displayed only a residual activity (~ 4 % of the wild-type). Surprisingly, it seems that the Lys³³¹ variant was a 2-fold better catalyst than the wild-type. It appeared that the presence of a cationic charge at site 331 was essential for the catalytic activity. However, Michaelis parameters are arrangements of the individual rate

Fig. 3. (a) Plot of the yield (Y) of the fructosyl transfer reaction from sucrose to levan $\{= 100[1 - (v_F/v_G)]\}$ versus levan concentration and (b) plot of the v_G/v_F ratio versus levan concentration

The initial concentration of labelled sucrose was 33 mM. $\circ$, His³³¹ variant; $\blacksquare$, Lys³³¹ variant; $\square$, Leu³³¹ variant; $\bullet$, Arg³³¹ variant; $\blacktriangle$, Ser³³¹ variant. v_G and v_F are the initial rates of glucose and fructose liberation.

constants of the sequential reaction 1, since from the King & Altman procedure [18], assuming $k_2 \gg k_3$, we can establish:

$$k_{\text{cat}} = \frac{k_3 \cdot k_{\text{H}_2\text{O}}}{k_3 + k_{\text{H}_2\text{O}}}$$

and

$$K_m = \frac{k_2}{k_1} \cdot \frac{k_{\text{H}_2\text{O}}}{k_3 + k_{\text{H}_2\text{O}}}$$

This means that additional data are required to evaluate the effects of amino acid substitution upon each step of the transfructosylation process.

Transfructosylation from sucrose to fructose polymer: fructan chain elongation. The yield, Y , of the transfructosylation reaction from sucrose to fructan acceptors was defined [4] as the ratio of the number of fructose residues incorporated in fructan ($v_G - v_F$) to the total number of fructose residues transferred from sucrose (v_G).

It may be expressed as:

$$Y = 100 \left(1 - \frac{v_F}{v_G} \right)$$

This parameter changes with the concentration and the nature of the acceptor [4]. Moreover, the following equation can be derived from mechanism 1, according to the King & Altman procedure [18]:

$$\frac{v_G}{v_F} = 1 + \frac{k_A}{k_{\text{H}_2\text{O}}} [A]$$

Two types of fructan have been tested as acceptor for each protein variant. The first was levans of low molecular mass (15 $\pm$ 3 kDa) previously used for kinetic studies of levansucrase [4], the second was inulin from dahlias (5 kDa). Figs. 3 and 4 show how, for each variant, the increase of each fructan concentration affected both parameters.

Table 1. Values of Michaelis constants for sucrose hydrolysis at 30 °C

These values were evaluated for each variant from the double-reciprocal plot ($1/v_F$ against $1/[S]$). Numerical data were estimated by a weighted regression procedure [17]. The relative weights were assumed to be proportional to the square of the initial velocity. The results are means $\pm$ S.D. ($n = 10$).

Parameter	Variant ...	His ³³¹	Lys ³³¹	Leu ³³¹	Arg ³³¹	Ser ³³¹
k_{cat} (s^{-1})		22 $\pm$ 2	88 $\pm$ 5	7 $\pm$ 0.6	35 $\pm$ 2	1.6 $\pm$ 0.2
$10^2 \times K_m$ (M)		1.2 $\pm$ 0.1	1.1 $\pm$ 0.1	1.8 $\pm$ 0.2	0.4 $\pm$ 0.04	1.8 $\pm$ 0.2

Fig. 4. (a) Plot of the yield (Y; see Fig. 3) of the transfer reaction from sucrose to inulin versus inulin concentration and (b) plot of the v_G/v_F ratio versus inulin concentration

The initial concentration of labelled sucrose was 33 mM. Symbols are as in Fig. 3.

Fig. 5. Plot of the v_G/v_F ratio versus glucose concentration

The initial concentration of sucrose was 33 mM. Symbols are as in Fig. 3.

Table 2. Values of kinetic parameter k_{lev}/k_{H_2O} and k_{inu}/k_{H_2O} for each variant

These values are estimated from the slopes of the curves presented in Figs. 3(b) and 4(b). k_{H_2O} is considered as a pseudo-first-order rate constant containing the water concentration factor.

Parameter	Variant ...	His ³³¹	Lys ³³¹	Leu ³³¹	Arg ³³¹	Ser ³³¹
$10^{-2} \times (k_{lev}/k_{H_2O})$ (M)		4	1	1	11.1	1.2
$10^{-2} \times (k_{inu}/k_{H_2O})$ (M)		0.3	0	0	2	0

We observed a large discrepancy for the transfer activities of the variants. The reverse variant, His³³¹→Arg, recovered the same transfer activity as the wild-type previously studied [4]; in contrast, the Lys³³¹, Ser³³¹ and Leu³³¹ variants possessed a low transferase activity to levan and no transferase activity to inulin. The straight lines obtained for the variation of the ratio v_G/v_F versus acceptor concentration allowed the evaluation of the ratio k_{lev}/k_{H_2O} and k_{inu}/k_{H_2O} for each variant. Results are presented in Table 2. In the presence of levan the yield of polymerase activity of Lys³³¹, Ser³³¹ and Leu³³¹ variants was one order of magnitude lower than for the wild-type enzyme.

Transfructosylation from sucrose to glucose: exchange reaction. Levansucrase of *B. subtilis* catalyses fructosyl exchange reactions between sucrose donor and glucose acceptor. This exchange activity has already been carefully investigated [16]. It has been shown that, from mechanism 1, mentioned above, and according to the King & Altman procedure [18], we can write the following equations:

$$\frac{[E]}{v_F} = \left(\frac{1}{k_3} + \frac{1}{k_9} \right) + \frac{1}{[S]} \cdot \frac{k_2}{k_1 \cdot k_3} + \frac{k_2 \cdot k_4}{k_1 \cdot k_3 \cdot k_9} \cdot \frac{[G]}{[S]} + \frac{k_4}{k_3 \cdot k_9} [G] \quad (2)$$

$$\frac{v_G}{v_F} = \frac{k_4}{k_9} [G] + 1$$

v_F is the initial rate of fructose release, v_G is the sum of the exchange and the hydrolytic activities of the enzyme.

Fig. 5 depicts the kinetic data obtained for each variant, when the ratios v_G/v_F are measured in the presence of a constant concentration of sucrose, but various concentrations of glucose acceptor. The slope of the straight lines obtained is a direct measurement of the competition between water and glucose to act as a fructosyl acceptor. It is clear that amino acid substitutions at site 331 strongly disturb the efficiency of the exchange reaction. The Ser³³¹ variant has a very weak exchange activity in contrast with the Arg³³¹ variant, which displays the same exchange efficiency as the wild-type previously studied [16]. From the measurements of v_F , either in the presence of various concentrations of glucose and a constant concentration of sucrose or in the presence of various concentrations of sucrose and a constant concentration of glucose, it was possible to evaluate all the kinetic coefficients of eqn. (2) [16].

Then, from the combination of all the data of the levansucrase activities studied, we calculated the individual rate constants for each step of mechanism 1 for all variants. Results are presented in Table 3. We can emphasize the following points.

Table 3. Values of apparent kinetic constants for the reaction pathway of eqn. (1) catalysed by levansucrase variants

Numerical data were estimated by a weighted regression procedure [17]. The results are means ± s.d. (n = 20).

Variant	Parameter ...	k_1/k_2 (M)	k_3 (s ⁻¹)	k_4 (M ⁻¹ ·s ⁻¹)	k_{H_2O} (s ⁻¹)
His ³³¹		35 ± 4	52 ± 5	900 ± 80	27 ± 3
Lys ³³¹		45 ± 5	170 ± 20	4300 ± 300	150 ± 15
Leu ³³¹		50 ± 5	8 ± 1	350 ± 30	33 ± 4
Arg ³³¹		32 ± 4	260 ± 20	4600 ± 300	35 ± 4
Ser ³³¹		44 ± 4	2.0 ± 0.4	26 ± 3	6.6 ± 1.5

(i) The substitution His³³¹→Arg restores the efficiency of the wild-type enzyme, since values of the rate constants of this variant are in agreement with those previously estimated for the wild-type [16].

(ii) Elimination of the cationic charge at site 331 dramatically decreases the constant rate, k_3 , of fructosyl-enzyme formation.

(iii) The efficiency of the transfructosylation reaction from sucrose to glucose, the exchange reaction, is strongly dependent on the presence of a cationic charge at site 331.

(iv) The amino acid substitutions modified slightly, but significantly, the affinity constants of the enzyme for sucrose.

Polymerase activities from sucrose as single substrate. In the presence of a high sucrose concentration [14] or in water/solvent mixture [9] the *B. subtilis* levansucrase catalyses the synthesis of high-molecular-mass levan from the sole sucrose which plays a dual role as fructosyl donor and as first fructosyl acceptor in such a reaction.

We have measured the polymerase activity of each variant under such conditions. Fig. 6 shows how the progressive substitution of water by acetonitrile in the reaction mixture affects the yield of levan synthesized by each variant. As reported for the wild-type [9], this yield is strongly enhanced by increasing concentrations of acetonitrile for the Arg³³¹ variant and to a lesser extent for the His³³¹ variant, in contrast with the Lys³³¹, Ser³³¹ and Leu³³¹ variants, which conserved their hydrolase activity, even in the presence of a high concentration of acetonitrile.

Polymerase activity of the variants in the presence of 0.5 M-sucrose was measured [4] under the initial conditions of transformation of [¹⁴C]sucrose catalysed by each variant.

We observed that Arg³³¹ and His³³¹ variants synthesized ¹⁴C-labelled levans. In contrast, the other variants were unable to

Fig. 6. Yield (Y) of the polymerase activity of the variant $\{= 100[1 - (v_F/v_G)]\}$ versus the concentration of acetonitrile present in the reaction mixture

Symbols are as in Fig. 3. The reaction mixture contained 30 mM-sucrose in 50 mM-potassium phosphate, pH 6.

Table 4. Yield of levan and trioside for each levansucrase variant

This parameter was defined as the ratio of the number of fructose residues incorporated into levans, or into trioside, to the total number of fructose residues transferred.

Product	Variant ...	Yield (%)				
		His ³³¹	Lys ³³¹	Leu ³³¹	Arg ³³¹	Ser ³³¹
Levan		15.1	0	0	38.0	0
Trioside		14.0	17.5	16.5	18.0	18.3

catalyse the fructose polymerization. The only trioside, kestose, identified from its R_F (results not shown), was formed and accumulated. The yield of both levan and kestose formation for all the variants is given in Table 4.

Obviously further characterization is needed to determine the precise nature of kestose in each case, since three different compounds may be synthesized: 1-kestose, 6-kestose or neokestose.

Effect of amino acid substitutions at site 331 on the pH-activity curve of levansucrase

We have limited our experiments to the Arg³³¹ and His³³¹ variants, since they displayed approximately the same catalytic specificities, although their amino acid side chains at site 331 possess a very different ionization constant.

The pH-rate profile for the glucose exchange reaction under conditions of substrates saturation [16] is presented in Fig. 7. As previously published [1], this curve for the wild-type enzyme was consistent with the assumption that two ionizable groups ($pK_1 = 5$; $pK_2 = 7$) take part in the catalytic process. We observed that the pK_1 was unmodified for the His³³¹ variant, whereas its pK_2 was one pH unit lower and approached the value of the imidazole pK .

These results may support the hypothesis that the side chain of the Arg³³¹ residue or that of His³³¹ plays its catalytic key role when in its protonated form. However, owing to the multiple

Fig. 7. Variation of the rate of exchange reaction catalysed by the Arg³³¹ variant and the His³³¹ variant as a function of pH

All assays were performed at 30 °C in 0.1 M-potassium phosphate, with 0.1 M-sucrose and 0.2 M-labelled glucose. ●, His³³¹ variant; ○, Arg³³¹ variant. Enzyme concentrations were 1×10^{-6} M for the Arg³³¹ variant and 4.0×10^{-6} M for the His³³¹ variant. Initial exchange rate is expressed as the molarity of labelled glucose exchanged/min.

effects of the pH on the overall state of a protein molecule, and therefore on the stability of the active form, we have to consider such a conclusion with caution.

DISCUSSION

We have shown here that the nature of the amino acid at position 331 dramatically modulates the specificity and the efficiency of the transfructosylation process catalysed by *B. subtilis* levansucrase. Such results may afford a better understanding of the molecular mechanism involved in the catalytic processes of the enzyme and, furthermore, should help us to formulate a hypothesis relevant to the possible existence of a common ancestor for the transfructosyltransferases involved in plant and bacterial fructan synthesis.

With regard to the first point, it is now currently accepted that the mechanism of enzyme catalysing transosylation with retention of the configuration is a two-step mechanism involving a bifunctional catalysis, an acidic group of the protein acting with the assistance of a nucleophile group [19]. For levansucrase, this latter has been identified as the β -carboxy group of an aspartic acid residue [20]. However, no direct evidence has yet been put forward for the nature of the donor of acidic groups. In light of the present results, it is reasonable to postulate that the side chain of the arginine residue at position 331 in the wild-type enzyme should act as a proton donor in the bifunctional catalysis. If we accept this conclusion, it is noteworthy that the change from Arg to Lys or His, the two other possible proton donors, led to very different effects on enzyme activities. Although the side chains of Arg and Lys are approximately the same length, the substitution Arg³³¹→Lys affects the enzyme specificity more strongly than the Arg³³¹→His substitution. However, we have to bear in mind that the guanidinium group and the imidazolium group share the same property of delocalizing the positive charge they carry by resonance upon three atoms organized in plane structure. Lysine does not possess such a property, so its ammonium group is more flexible. We can reasonably assume that steric requirements for the acting groups are more stringent for the polymerase reaction than for the hydrolase activity. The existence of a residual activity for the Ser³³¹ and Leu³³¹ variants should complicate the bifunctional catalytic model proposed above and should indicate that several acidic groups co-operate in the active site during the catalytic act. However, the rate of the step of the transient fructosyl-enzyme formation is strongly dependent on the presence of an acidic donor group at site 331.

The second point we will discuss is relevant to the consequences, at the evolutionary level, of the existence of such a critical amino acid site whose substitution modulates the product specificity of levansucrase so strongly. Unfortunately, few sequence data are available for bacteria and plant fructose polymerases. Comparison of levansucrases from *B. subtilis*, *B. amyloliquefaciens* [21] and *Streptococcus mutans* [22] revealed long stretches of sequence similarities and, as shown below, the site where we demonstrated the implication in enzyme specificity

is well conserved in *B. amyloliquefaciens* levansucrase and partially in the *S. mutans* enzyme:

B. subtilis. ...L F T D S R³³¹ G S K M...
B. amyloliquefaciens ...L F T D S R³³¹ G S K M...
S. mutans ...L F T A S R⁵²¹...

It is noteworthy that the enzyme from *S. mutans* synthesizes a β -1,2-linked fructan, whereas the other enzymes produce mainly a β -2,6-linked polymer. These different structural specificities should, in the future, be correlated with the sequence difference around the critical Arg residue.

Some levansucrase variants studied here partially mimic the catalytic activity of the sucrose:sucrose fructosyltransferase of plants [23]. Such results may mean that enzymes involved in fructan synthesis by bacteria and plants have a common ancestor. This working hypothesis provides a stimulating reason to develop the approach undertaken here.

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