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Purification of a novel fructosyltransferase from *Lactobacillus reuteri* strain 121 and characterization of the levan produced

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Abstract

Fructosyltransferase (FTF) enzymes have been characterized from various Gram-positive bacteria, but not from *Lactobacillus* sp. In a screening of 182 lactobacilli for polysaccharide production only one strain, *Lactobacillus reuteri* strain 121, was found to produce a fructan being a levan. Here we report the first-time identification and biochemical characterization of a *Lactobacillus* FTF enzyme. When incubated with sucrose the enzyme produced a levan that is identical to that produced by *Lb. reuteri* strain 121 cells. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

Keywords: Levan; Levansucrase; *Lactobacillus reuteri*

1. Introduction

Lactic acid bacteria (LAB) are a group of Gram-positive, food-grade microorganisms consisting of many genera, e.g. *Lactococcus*, *Streptococcus*, and *Lactobacillus*. Members of these genera possessing the generally regarded as safe (GRAS) status find application in the production of food and feed [1]. LAB are known to produce an abundant variety of exopolysaccharides (EPS) [2] that are of interest for the development of a new generation of food-grade ingredients. LAB EPS may contribute to human health as prebiotics [3], or because of their antitumoral [4], immunomodulating [5], or cholesterol-lowering activity [6].

When screening 182 lactobacilli for EPS production only one strain, *Lactobacillus reuteri* strain 121, was found to produce large amounts of both glucan and fructan homopolymers from sucrose. All other positive strains synthesized polymers consisting of glucose or other sugars [7]. The fructan, a linear polymer consisting of only $\beta(2,6)$

linked fructosyl residues (a levan), had an estimated size of 150 kDa [8].

Enzymes synthesizing levans, fructosyltransferases or levansucrases (sucrose: 2,6- β -D-fructan-6- β -D-fructosyltransferase, E.C. 2.4.1.10), catalyze the following reaction:

Levan polymers are either linear or branched (to varying degrees) at the C-1 position, with molecular masses varying between 20 kDa and several MDa. Levan synthesis has been studied most extensively in *Zymomonas mobilis* [9], *Streptococcus salivarius* [10], and *Bacillus subtilis* [11].

This paper describes the purification and biochemical characterization of a novel FTF enzyme from *Lb. reuteri* strain 121 culture supernatants.

2. Materials and methods

2.1. Strains, media and growth conditions

Lb. reuteri strain 121 (culture collection TNO Nutrition

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and Food Research, Zeist, The Netherlands) was grown at 37°C in MRS medium [12], or in modified MRS medium containing 50 g l⁻¹ raffinose (MRSr) or 50 g l⁻¹ maltose (MRSm), instead of the 20 g l⁻¹ glucose normally present in MRS medium.

2.2. Protein purification

Sucrase activity and protein content were determined in samples from each purification step. One liter of an overnight culture of *Lb. reuteri* strain 121 cells grown on MRSm medium was centrifuged for 15 min at 10 000×g. A saturated ammonium sulfate solution (1.5 l) was added to the supernatant (940 ml) at a rate of 50 ml per min under continuous stirring. The resulting 60% ammonium sulfate solution was centrifuged for 15 min at 10 000×g. The precipitate was resuspended in 10 ml sodium phosphate buffer (10 mM, pH 6.0) and dialyzed overnight against 2 l sodium phosphate buffer (10 mM, pH 6.0). The dialyzed sample (11 ml) was loaded on a hydroxyapatite column (Bio-Rad; 10×6 cm; flow rate 1 ml min⁻¹) equilibrated with sodium phosphate buffer (10 mM, pH 6.0; A). The column was eluted with sodium phosphate buffer (200 mM, pH 6.0; B; flow rate 1 ml min⁻¹) and fractions collected from 0% B to 40% B were screened for sucrase activity. Positive fractions were pooled (7.7 ml), diluted 1:1 (v/v) with 25 mM sodium acetate, 2 M ammonium sulfate pH 5.4, and loaded on a phenyl Superose hydrophobic interaction column (HR 5/5; flow rate 0.5 ml min⁻¹; Pharmacia) equilibrated with a 25 mM sodium acetate, 1 M ammonium sulfate (pH 5.4) buffer. A gradient from 25 mM sodium acetate, 1 M ammonium sulfate pH 5.4 (A) to 25 mM sodium acetate pH 5.4 (B) was applied and fractions were collected from 35% B to 50% B. Fractions with sucrase activity were pooled (3 ml), loaded on a Superdex 200 gel filtration column (XK 16/60; flow rate 1 ml min⁻¹; Pharmacia), and eluted with acetate buffer (25 mM, pH 5.4 containing 0.1 M sodium chloride). Fractions with sucrase activity were pooled (10 ml), dialyzed against 2 l sodium acetate buffer (25 mM, pH 5.4), and stored at -20°C for further studies.

2.3. Sucrase enzyme activity assay

Sucrose conversion by levansucrase yields glucose (and fructose, which is partly built into the fructan polymer) in a 1:1 molar ratio to the amount of sucrose used. The amount of glucose released allows determination of the overall enzyme activity (total amount of sucrose converted). Glucose was measured with hexokinase (HK; Roche Biochemicals, Switzerland) and glucose-6-phosphate dehydrogenase (G6PDH; Roche Biochemicals) as coupling enzymes. The remaining fructose (as fructose-6-phosphate after HK action) was measured by adding phosphoglucose isomerase (PGI; Roche Biochemicals). Levansucrase activity was assayed at 37°C in a sodium

acetate buffer (25 mM; pH 5.4) with 100 mM sucrose (unless stated otherwise) and 1 mM calcium chloride [10,11,13]. Samples were taken at 3 min time intervals and added to 1/10 volume 1 M sodium hydroxide to stop the reaction. Subsequently, the glucose and fructose contents were determined. One unit of enzyme activity is defined as the release of 1 µmol glucose (total enzyme activity) per min. Enzyme kinetics was studied using standard assay conditions and varying substrate concentrations. Data were fitted with Sigma Plot for Windows 4.0 (Jandell Scientific Software) according to the Michaelis-Menten equation.

2.4. Polymer production and characterization

Polymer was produced by incubating purified levansucrase (1 µg ml⁻¹; final concentration) in a sodium acetate buffer (25 mM, pH 5.4; 1 mM calcium chloride) with 20% sucrose at 37°C for 16 h. Polymeric material was precipitated with 2 volumes of 96% ethanol followed by 10 min centrifugation at 10 000×g. The pellet was resuspended in demineralized water at 4°C during 16 h followed by dialysis overnight against demineralized water. Subsequently, the polymer was precipitated with 2 volumes of 96% ethanol, centrifuged for 10 min at 10 000×g, freeze dried and stored at -20°C for further analysis.

The purified polymer was characterized using nuclear magnetic resonance (NMR; CASS University of Groningen, The Netherlands) for determination of binding types present, and high performance liquid chromatography gel filtration chromatography (HPLC-GFC) analysis for molecular mass determinations. Prior to NMR spectroscopic analysis, the fructan was dissolved in 99.9 atom% D₂O (Isotec Inc.). ¹H and ¹³C NMR spectra were recorded on a Varian UNITY-500 spectrometer (at 500 MHz and 125 MHz, respectively) at a probe temperature of 80°C. The HOD signal was suppressed by applying presaturation during relaxation delay for 2 s. Chemical shifts are reported in Δ units (ppm) relative to the residual deuterated solvent signals for ¹H: Δ4.60 ppm, or for the internal standard methanol for ¹³C: Δ3.35 ppm. For HPLC-GFC analysis fructan was dissolved in elution buffer (20 g l⁻¹ in a 0.15 M sodium sulfate solution) and run on a Polysep GFC-P5000 column (300×7.8 mm; flow rate 1 ml min⁻¹; Phenomenex, Torrance, CA, USA) with an isocratic HPLC pump at 30°C equipped with a Polysep GFC-P5000 guard column (30×7.86 mm; Phenomenex). Fructose dissolved in elution buffer was used as reference. Data integration was done with a Waters Millenium³² (Waters, Milford, MA, USA) software system.

2.5. Sucrose and polymer hydrolysis

Following incubation of MRSr culture supernatants (1/20 diluted) and the purified levansucrase (1 µg ml⁻¹; final concentration) in assay buffer with 50 mM sucrose

for 16 h, the amounts of sucrose consumed, and glucose, fructose, and polymer formed, were determined. Sucrose was hydrolyzed by incubation with β -fructosidase (Sigma-Aldrich, MO, USA). Polymer was isolated as described above. Fructan was hydrolyzed by incubation in a 0.5 M trifluoro acetic acid solution at 50°C for 16 h. Glucan was hydrolyzed by incubation in a 1 M hydrochloride solution at 100°C for 1 h. The acid hydrolyzed samples were neutralized with equimolar amounts sodium hydroxide. The glucose and fructose contents of the samples were determined with the HK/G6PDH and PGI assays (see above).

2.6. Amino acid sequences of levansucrase peptide fragments

Purified levansucrase (approximately 12 μ g) was run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels and transferred to PVDF membranes (Millipore Inc.) by Western blotting. After staining with Coomassie brilliant blue without acetic acid and destaining with 50% methanol, the levansucrase protein band was cut out of the PVDF membranes and subjected to N-terminal amino acid sequencing. The protein was digested with chymotrypsin and peptide fragments were separated on an RP-HPLC (Model 120A, Applied Biosystems). Peptide sequencing was performed on an Applied Biosystems model 477A/120A automated gas-phase sequencer equipped with on-line RP-HPLC for detection of the phenylthiohydantoin amino acid derivatives (Eurosequence, Groningen, The Netherlands).

3. Results

3.1. FTF enzyme purification and biochemical characterization

Attempts to purify the FTF enzyme responsible for levan synthesis in *Lb. reuteri* strain 121 from culture supernatants following growth on sucrose or raffinose were unsuccessful, due to the presence of polymer. The strain 121 FTF enzyme binds very tightly to its product and could not be released either by boiling in SDS or by hydrolyzing the levan with hydrochloride acid or trifluoro acetic acid. Sucrase enzymes, producing either glucan or fructan, cannot use maltose as a substrate. *Lb. reuteri* strain 121

grown on maltose was found to possess sucrase activity but no polymers were produced. Sucrase activity in MRSm culture supernatants was approximately 10 times lower (in U mg^{-1}) than in MRSr culture supernatants (results not shown). Sucrase enzyme activity in *Lb. reuteri* strain 121 occurs both in a cell-associated form and as a free, extracellular enzyme [8]. In MRSm cultures sucrase enzyme activity is largely cell-associated, which may explain the lower sucrase activity observed. The FTF enzyme was purified from supernatants of *Lb. reuteri* strain 121 cells grown on MRSm.

Ammonium sulfate precipitation and three column chromatography steps resulted in purification of the FTF enzyme to homogeneity (Table 1), as evaluated by SDS–PAGE and silver staining (results not shown). The yield was 16% and the purification was 350-fold. Purified FTF showed an apparent M_r of 110 000 Da on SDS–PAGE, and 90 000 Da by gel filtration. The gel filtration column was eluted with a non-denaturing buffer preserving quaternary protein structures.

Calcium ions were required for FTF enzyme activity. Activity (glucose release from sucrose; with 1 mM of metal ions and 8 $\mu\text{g ml}^{-1}$ of enzyme; final concentrations) was completely inhibited by Cu^{2+} and Fe^{3+} ions. The following kinetic parameters were determined for FTF (at 8 $\mu\text{g ml}^{-1}$ enzyme concentration): $K_m = 7.0 \pm 0.7$ mM sucrose ($n = 3$); $V_{\max} = 145 \pm 3$ U mg^{-1} ($n = 3$).

Analysis of products formed during incubation of FTF enzyme with 50 mM sucrose revealed that sucrose hydrolysis is the dominant activity of this enzyme, with 14% of sucrose converted into fructan polymer and 86% into fructose.

3.2. In vitro fructan production and fructan analysis

Fructans synthesized from sucrose by the FTF enzyme and by MRSr grown *Lb. reuteri* strain 121 were subjected to NMR and GFC analysis. The one-dimensional (1D) ^{13}C NMR spectrum of the FTF product (Fig. 1) displayed six carbon signals (Table 2). The C-2 resonance ($\delta = 104.0$) indicates the presence of β -fructofuranose. Comparison with the published ^{13}C chemical shifts of fructan produced by *Lb. reuteri* strain 121 grown on MRSr media [8] and *Z. mobilis* levan [14] indicated that the FTF fructan had a $[\rightarrow 6)\text{-}\beta\text{-D-Fruf-(2}\rightarrow)]_n$ structure, a levan. Further confirmation was obtained from the identical ^1H NMR spectra

Table 1
Purification of the levansucrase enzyme from supernatants of *Lb. reuteri* strain 121 cells grown on MRSm medium

Step	Protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Supernatant	163	63	0.4	1	100
AS ^a	46	42	0.9	2.3	68
Hydroxyapatite	2.0	31	16	40	49
Phenyl Superose	0.35	23	65	163	37
Superdex 200	0.072	10	140	350	16

^a60% ammonium sulfate precipitation step.

Fig. 1. ^{13}C NMR of fructan produced by the levansucrase enzyme purified from *Lb. reuteri* strain 121.

of the FTF fructan (results not shown) and the fructan produced by MRSr grown *Lb. reuteri* strain 121 [8]. The purified FTF enzyme thus is a levansucrase. The molecular masses of the fructans were determined by GFC analysis. The elution profiles of fructan isolated from strain 121 grown on MRSr and of in vitro synthesized fructan by the levansucrase were comparable (results not shown). Both profiles showed two major fractions, representing polymers with an estimated molecular mass of 150 kDa and larger than 2 MDa. The molecular mass of the larger than 2 MDa fraction as well as the molar ratio of both fractions could not be determined because the larger than 2 MDa fraction was in the void volume of the GFC column.

3.3. Amino acid sequencing and sequence comparison

The N-terminal amino acid sequence of the levansucrase protein was determined as (A)QVESNNYNGVAEVN-TERQANGQI(G)(V)(D) (ambiguous residues between parentheses). Amino acid sequences of three internal peptide fragments were determined as 1: (M)(A)HLDVWDS-WPVQDP(V); 2: NAGSIFGT(K); 3: V(E)(E)VYSPKV-STLMASDEVE (ambiguous residues between parentheses). Alignments of the amino acid sequences of the internal peptide fragments of the levansucrase with FTF sequences of *B. subtilis* SacB, *Streptococcus mutans* SacB, and *S. salivarius* FTF revealed clear similarities (Fig. 2). Especially internal peptide fragment 1 appears to be in a highly conserved region of bacterial FTFs. The N-terminal peptide fragment showed no similarity with any known (FTF) proteins (results not shown).

4. Discussion

Screening of a large collection of lactobacilli for EPS production from sucrose identified *Lb. reuteri* strain 121 as the only strain producing a fructan [7]. Methylation analysis of this fructan [8] showed that it contains $\beta(2,6)$ fructosidic bonds, indicating that it is a levan polymer. FTF enzymes have been characterized from *Bacillus* [11,15] and *Streptococcus* [10] species but not yet from *Lactobacillus* species. In view of (a) the beneficial effects that fructans may have on health [3–6], (b) the GRAS status of lactobacilli, (c) the probiotic properties associated with *Lb. reuteri*, and (d) the limited biochemical information available about FTF enzymes, we decided to characterize the levansucrase enzyme responsible for levan production in *Lb. reuteri* strain 121 in more detail.

The *Lb. reuteri* levansucrase affinity for sucrose ($K_m = 7$ mM) is comparable to values reported for FTF of *S. salivarius* ($K_m = 5$ mM; [10]) and SacB of *B. subtilis* ($K_m =$

Table 2

Comparison of ^{13}C NMR chemical shifts of fructan produced by the purified levansucrase and of fructan present in culture supernatants of *Lb. reuteri* strain 121 grown on MRSr [8]

^{13}C NMR	Levan produced by levansucrase	Levan present in strain 121 supernatant
C-1	59.6	61.7
C-2	104.0	105.0
C-3	76.0	78.1
C-4	74.9	76.6
C-5	80.0	81.2
C-6	63.2	64.3

4 mM; [11]). The strong inhibitory effects of Cu^{2+} and Fe^{3+} ions on *Lb. reuteri* levansucrase activity were also observed for the *S. salivarius* [10] and from *B. subtilis* [11] FTF enzymes. Ca^{2+} ions play a role in stabilizing FTF enzymes, e.g. in *S. salivarius* FTF [16]. The precise mechanisms by which heavy metal ions inhibit FTF enzyme activity are currently unknown. The Cu^{2+} -mediated inactivation has been reported to depend on free radical inactivation rather than competition with Ca^{2+} ions [16,17].

The *Lb. reuteri* strain 121 levansucrase (incubated at 37°C with 50 mM sucrose), displays a relatively high sucrose hydrolytic activity (86% of sucrose utilized), compared to both *S. salivarius* [10] and *Bacillus circulans* [15] FTFs (40 and 30% of sucrose utilized, respectively, at 50 mM sucrose). These differences may be based on differences in structural organization of FTF active sites.

The molecular mass of FTF enzymes from LAB origin (streptococci only) [10,18] is typically around 90 000–100 000 Da, whereas FTF enzymes of *Bacillus* species [11] or Gram-negative bacteria [9,19] have molecular masses of 50 000–60 000 Da. Gel filtration of the purified strain 121 levansucrase enzyme revealed an apparent molecular mass of 90 000 Da, comparable to that of FTFs from streptococci. Furthermore, internal amino acid sequences of peptides of this strain 121 protein had a higher similarity to FTFs from Gram-positive bacteria (Fig. 2) than to FTFs from Gram-negative bacteria (results not shown). The similarities in FTF molecular masses and the (limited) amino acid sequences of the levansucrase available indicate that the *Lb. reuteri* strain 121 levansucrase is closely related to FTF enzymes characterized from

other LAB, namely *S. salivarius* FTF [10], and *S. mutans* SacB [18]. Both streptococci are well-studied inhabitants of the oral cavity, with fructan synthesized from sucrose most likely contributing to the cariogenicity of dental plaque formation [20]. *Lb. reuteri* strains, in contrast, are residents of the mammalian gut system. It will be interesting to study the in situ functional properties of *Lb. reuteri* strain 121 and the levan produced and their possible roles in the probiotic properties contributed to *Lb. reuteri* strains [4,6].

The GFC profiles of the fructan polymers produced in vitro with *Lb. reuteri* levansucrase showed that there were two dominant size fractions present, one of approximately 150 kDa and one larger than 2 MDa. GFC analyses of the levan produced by *S. mutans* SacB [13] revealed only one size fraction with a molecular mass larger than 2 MDa. Two products with distinct sizes also have been observed for the levan of *B. subtilis* SacB [21]. These authors suggest that the degree of branching is an important factor in determining the molecular mass of the polymer. The levan produced by *Lb. reuteri* strain 121 appears almost unbranched, however (<5% $\beta(2,1)$ linkages; Fig. 1). We are currently searching for alternative explanations for the two very distinct levan size fractions.

This report describes the first-time identification and characterization of a *Lactobacillus* levansucrase. The *Lb. reuteri* strain 121 levansucrase is most closely related to levansucrases of streptococci. However, the *Lb. reuteri* levansucrase displays a relatively high rate of sucrose hydrolysis and the molecular mass distribution of the levan that is produced shows two distinct size fractions. Future work will focus on the identification and characterization of the gene encoding the *Lb. reuteri* strain 121 levansucrase. Expression of the gene in a heterologous host organism and production of larger amounts of levansucrase enzyme and its levan polymer will allow a more in-depth biochemical characterization of the enzyme, the functional properties of its products, and a detailed analysis of the FTF reaction mechanism.

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1.	<i>Lb. reuteri</i> FTF	MAHLDVWDSWPVQDPV
	<i>B. subtilis</i> SacB	---LDVWDSWPQLQAD
	<i>S. mutans</i> SacB	IADLDVWDSWPVQDAK
	<i>S. salivarius</i> FTF	KEEIDVWDSWPVQDAK
		.:*****:.*:
2.	<i>Lb. reuteri</i> FTF	NAGSIFGTK
	<i>B. subtilis</i> SacB	NAGRVFKDS
	<i>S. mutans</i> SacB	NAGSIFGYN
	<i>S. salivarius</i> FTF	NAGPIFGYN
		*** :* .
3.	<i>Lb. reuteri</i> FTF	VEEVYSPLVSTLMASDEVE
	<i>B. subtilis</i> SacB	LKKVMKPLIASNTVTDEIE
	<i>S. mutans</i> SacB	VDQFYTPLLSSTMVSDLE
	<i>S. salivarius</i> FTF	VAELYTPLVTSHMVTDEVE
		: .. .**::: .:***:

Fig. 2. Alignment of the three internal peptide sequences (1–3) obtained from *Lb. reuteri* strain 121 levansucrase protein with *B. subtilis* SacB (X02730), *S. mutans* SacB (M18954), and *S. salivarius* FTF (L08445). (*) Indicates a position with a fully conserved amino acid residue. (:) Indicates a position with a fully conserved 'strong' group: STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW. (.) Indicates a position with a fully conserved 'weaker' group: CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY. Amino acid groups are according to the Pam250 residue weight matrix [22].

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