JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Biomolecular Characterization of the Levansucrase of *Erwinia amylovora*, a Promising Biocatalyst for the Synthesis of Fructooligosaccharides

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Supporting Information

ABSTRACT: *Erwinia amylovora* is a plant pathogen that affects Rosaceae, such as apple and pear. In *E. amylovora* the fructans, produced by the action of a levansucrase (EaLsc), play a role in virulence and biofilm formation. Fructans are bioactive compounds, displaying health-promoting properties in their own right. Their use as food and feed supplements is increasing. In this study, we investigated the biomolecular properties of EaLsc using HPAEC-PAD, MALDI-TOF MS, and spectrophotometric assays. The enzyme, which was heterologously expressed in *Escherichia coli* in high yield, was shown to produce mainly fructooligosaccharides (FOSs) with a degree of polymerization between 3 and 6. The kinetic properties of EaLsc were similar to those of other phylogenetically related Gram-negative bacteria, but the good yield of FOSs, the product spectrum, and the straightforward production of the enzyme suggest that EaLsc is an interesting biocatalyst for future studies aimed at producing tailor-made fructans.

KEYWORDS: *levansucrase, fructans, Erwinia amylovora, infection, HPAEC-PAD*

INTRODUCTION

Erwinia amylovora is a Gram-negative bacterium that causes the devastating disease called fire blight in Rosaceous plants, such as apple, pear, and raspberry. The infection usually occurs in spring, when the temperature increases over 18 °C, and starts when the bacteria present on the flower stigmas infect the plant through the nectaries at the base of the flowers. Within a few days, the infection expands rapidly to the whole blossom and the young shoots and, in a few months, becomes systemic and affects the whole plant. Serious disease outbreaks often lead to significant loss of fruit and trees, with significant negative economic impact.

E. amylovora produces two different types of exopolysaccharides, amylovoran and levan. These cause occlusion of the plant vessels resulting in the characteristic fire blight wilting.[1] Whereas amylovoran is synthesized by several enzymes encoded by the *ams* operon, levan is synthesized by a single enzyme called levansucrase.[2] The enzyme was shown to be involved in the disease,[1,3] and the gene encoding levansucrase was up-regulated during immature pear tissue infection, along with other virulence factors.[4] The levansucrase produced by *Erwinia* was shown to play a role in biofilm formation.[5]

At a mechanistic level, levansucrases (EC 2.4.1.10) are multifunctional enzymes. They perform both the hydrolysis of sucrose, into glucose and fructose, and the synthesis of fructans by direct transfer of a fructosyl moiety to sucrose and/or to a growing glycan polymer chain.[6] On the basis of structural and functional similarity, levansucrases are classified in the glycosyl hydrolase family 68 (GH68),[7] whereas related eukaryotic fructosyltransferases are found in family GH32. Together, the GH68 and GH32 families comprise β-fructofuranosidase clan GH-J. The core structure of bacterial levansucrases, featuring a five-bladed β-propeller topology, the structural determinants for substrate recognition, and the spatial disposition of the key catalytic acidic residues at the active site are highly conserved.[8−11] Despite their conserved active site structure, the kinetic and biochemical properties of levansucrases and their broad spectrum of products differ greatly. Interestingly, some levansucrases, such as SacB from *Bacillus subtilis*, mainly produce long-chain levan, whereas others, such as the enzyme from *Gluconacetobacter diazotrophicus*, synthesize primarily short-chain fructooligosaccharides (FOSs). This difference in product spectrum depends on whether the enzyme is able to catalyze the reaction with a processive or distributive (nonprocessive) mechanism. In a processive mechanism, the product of a catalytic step remains bound to the enzyme, ready for the next catalytic step. Conversely, a distributive mechanism requires that the "intermediate" products dissociate from the enzyme after every step of catalysis. The difference in

Received: May 27, 2013
Revised: November 19, 2013
Accepted: November 25, 2013
Published: November 25, 2013

mechanism seems to be dictated by structural determinants[12,13] that are seemingly localized outside the core active site structure.[14]

The occurrence of fructans, fructose oligomers, and polymers derived from sucrose is widespread in nature, and they can be found in plants and microorganisms.[15] FOSs are fructans with a DP between 2 and 10, whereas longer polymers joined with $\beta(2,6)$ linkages (levan) or $\beta(2,1)$ linkages (inulin) are also common. It has been estimated that at least 15% of flowering plants use $\beta(2,1)$- or $\beta(2,6)$-linked fructans as storage carbohydrates,[16] but the role of fructans in microorganisms is not fully understood yet. Nonetheless, for plant-pathogenic bacteria fructans are produced as a first protection against plant defense mechanisms.[17]

Fructans, in particular FOSs, are emerging as powerful and relatively inexpensive health-promoting food and feed supplements.[18] They are nondigestible in the human upper gastrointestinal tract and act as prebiotics. FOSs selectively stimulate the growth and activity of beneficial colon bacteria that are able to utilize them as a carbon source.[19] Short fructans have a sweet taste and are often used as low-calorie sweeteners, whereas longer chain fructans have a neutral taste and form emulsions with a fat-like texture. These properties make fructans widely used in the food industry. In response to an increasing demand for healthier and calorie-controlled food, the market of prebiotics, and in particular FOSs and inulin, is projected to reach $1.17 billion in Europe and $225.31 million in the United States by 2015 (http://www.strategyr.com). However, it should be noted that the actual market is based on FOSs with inulin-type structure, either extracted from plant sources or produced through biotechnological processes involving the use of microbial enzymes,[20] such as inulinases (EC 3.2.1.7) and fructosyltransferases (EC 2.4.1.9). $\beta(2,6)$-Linked FOSs are present in very low amount in natural sources, so their study, production, and utilization at industrial level are not well developed yet. Nevertheless, their prebiotic and beneficial effects have been demonstrated,[21,22] and the potential of this class of molecules in a still unexploited market is gaining attention. Researchers are currently engaged in the quest for new approaches and enzymes aiming at tailor-made synthesis of a wider repertoire of fructans (DP, linkage type, branching) with new chemical, physical, and biological properties.[23,24]

With a view to both gaining insight into its role in plant pathogenesis and exploring its potential as a catalyst for biotechnology applications, we set out to gain insight into the catalytic properties of the *E. amylovora* levansucrase (EaLsc).

MATERIALS AND METHODS

Materials. Sucrose, raffinose, stachyose, lactulose, sucralose, 1-kestose, and nystose as well as potassium acetate and potassium phosphate salts were purchased from Sigma-Aldrich (St. Louis, MO, USA). All buffers and solutions were prepared in Milli-Q water. All other chemicals and solvents were of analytical grade and used as purchased.

Production of Recombinant EaLsc. Cloning, expression, purification, and crystallization of EaLsc were performed as previously described.[25] Briefly, the levansucrase gene *lsc* was amplified from *E. amylovora* strain Ea273 (ATCC 49946), cloned into pETM-30 vector[26] and expressed as recombinant N-terminal GST-tagged protein in *Escherichia coli* BL21 (DE3) cells. The protein was purified by affinity chromatography on a GSTrap 5 mL column (GE Healthcare, Uppsala, Sweden), and the tag was removed using a solubility-enhanced L56 V/S135G TEV protease.[27] The recombinant TEV, the released GST, and residual uncleaved fusion protein were removed by immobilization onto a His-Trap HP 1 mL column (GE Healthcare). Pure EaLsc was obtained after size exclusion chromatography on a Superdex S75 16/60 column (GE Healthcare). Protein purity was confirmed by SDS-PAGE.

EaLsc Activity Assays. The activity of EaLsc was determined using sucrose as substrate. The total activity and the hydrolytic activity were determined by measuring the amount of D-glucose and D-fructose released, whereas the transfructosylation activity was calculated by subtracting the total amount of fructose from that of D-glucose, as previously described.[28] The concentrations of D-glucose and D-fructose were determined either by HPAEC-PAD or by using an enzymatic UV method designed for quantification of these sugars in plant material and food in a microplate format from Megazyme (Bray, Ireland).

The effect of pH on EaLsc activity was evaluated in the range of 5.0−8.0 using 0.1 M potassium acetate buffer (from pH 5.0 to 5.5) and 0.1 M potassium phosphate buffer (from pH 5.5 to 8.0). The reactions were performed at 37 °C for 30 min in the presence of 10 mM sucrose and 21.5 nM (1 μg mL^{-1}) EaLsc. The effect of temperature on EaLsc activity was determined from 25 to 60 °C in 0.1 M potassium phosphate buffer, pH 6.5, with 10 mM sucrose and 21.5 nM EaLsc. Reactions were carried out at 37 °C for 30 min. Reactions were quenched by flash-freezing in liquid nitrogen. The samples were subsequently placed for 10 min in a water bath kept at 80 °C to inactivate the enzyme. All of the reactions were run in triplicate, and each sample was analyzed in duplicate in the D-glucose/D-fructose assay or by high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD).

Determination of Kinetic Parameters. The kinetic parameters for EaLsc total activity were determined at 37 °C. The enzymatic reactions (1 mL) were prepared in 0.1 M potassium phosphate buffer, pH 6.5, containing sucrose (0, 1, 2, 5, 10, 20, 30, 40, 50, 70, and 100 mM) and were started by adding 21.5 nM EaLsc. Aliquots (70 μL) were removed from the reaction mixture every 30 s from time 0 to 5 min and quenched by flash-freezing in liquid nitrogen. The enzyme was then completely inactivated by incubation at 80 °C for 10 min. D-Glucose concentration was measured using the spectrophotometric assay. The initial rate of release of D-glucose, defined as molarity of D-glucose released per second (mM s^{-1}), was calculated at each sucrose concentration by linear regression. Lineweaver−Burk plots were used to determine the apparent Michaelis−Menten constant (K_m) and the maximum velocity (V_{max}). All calculations were performed using Microsoft Office Excel.

Hydrolysis versus Transfer Activity. The hydrolysis versus transfer activity of EaLsc was determined at different concentrations of substrate (from 10 to 1500 mM) and at different temperatures (25−42 °C). Reactions were performed in 0.1 M potassium phosphate buffer pH 6.5 and 37 °C for 24 h using 21.5 nM enzyme. The glucose and fructose contents of the reaction mixture were analyzed by HPAEC-PAD.

Turbidity Assay. Fructan synthesis was monitored in a turbidity assay on microtiter plates. Reactions were performed in 200 μL of 0.1 M potassium phosphate buffer ,pH 6.5, containing sucrose (0, 10, 50, 100, 200, 500, 800, 1000, 1200, and 1500 mM) or raffinose (100 and 150 mM) and started by addition of 25 nM EaLsc. The assays were performed at different temperatures: 21, 25, 37, and 41 °C. The synthesis of fructans was monitored by following the increase in turbidity at 410 nm, reading the absorbance every 5 min for 4 h using a Tecan F200 microplate reader (Männedorf, Switzerland). The amount of products formed was calculated by interpolation to a calibration curve obtained by serial dilution of purified FOSs. FOSs were produced by incubation of 50 nM EaLsc in 0.1 M potassium phosphate buffer, pH 6.5, containing 1 M sucrose, at 37 °C for 4 h and purified according to the method proposed by van Hijum and co-workers.[29]

HPAEC-PAD Analysis. HPAEC was coupled with PAD on a Dionex ICS-3000 system. A Carbo-Pac PA-1 column (2 × 250 mm) (Dionex) connected to a 2 × 50 mm Carbo-Pac PA-1 guard column was used for the separation at room temperature.

For activity assays, isocratic separation of D-glucose, D-fructose, and sucrose was achieved using 100 mM NaOH (prepared from 50% w/w

Table 1. Optimal Reaction Conditions and Kinetic Parameters of EaLsc (Total Activity) in Comparison with Levansucrases from Other Organisms Reported in the Literature

organism	Gram stain	MW	opt pH	opt temp (°C)	K_m (mM)	k_{cat} (1/s)	k_{cat}/K_m (M^{-1} min^{-1})	ref
Erwinia amylovora	−	46520	6.5	37	33.6	502.7	8.97×10^5	
Pseudomonas syringae	−	47603	6	37	160	nd	nd	38
Rahnella aquatilis	−	45939	5	30	50	nd	nd	39
Zymomonas mobilis	−	46762	5	30	18	83	2.76×10^5	33
Gluconacetobacter diazotrophicus	−	63526	5.2	30	11.9	3900	3.27×10^5	11
Bacillus megaterium	+	53933	6.6	45	6.6	2272	2.06×10^7	37
Bacillus subtilis	+	52971	6	37	8	164.6	1.23×10^6	51
Arthrobacter K-1	+	62839	6.5	55	10.4	nd	nd	10
Lactobacillus reuteri 121	+	87621	5	50	21	287	8.20×10^5	28

sodium hydroxide solution) as mobile phase. Flow rate was 0.25 mL min^{-1}, and elution of the sugars was monitored by PAD using the Standard Quad waveform for carbohydrates analysis. Run time was 15 min. Injection volume was 2 μL. Quantification was based on calibration curves generated by serial dilutions of standards between 0.02 and 2 nmol injected (five calibrators). Good linearity was observed in this range of concentrations with coefficient of determination (R^2) values between 0.991 and 0.998. Samples were diluted to give concentrations of the analytes within the linearity range of the detector.

Product profiling was performed using the following mobile phases: solvent A, 0.1 M NaOH; solvent B, 0.1 M NaOH containing 0.6 M NaOAc (prepared by addition of electrochemical grade sodium acetate). These solvents were prepared in Milli-Q water and degassed by sparging with helium for 30 min. Solvent B was filtered through a 0.2 μm nylon filter before sparging. The flow was 0.25 mL min^{-1}, and the gradient profile was 0 min, 0% B; from 0 to 25 min, linear gradient to 60% B; from 25 to 30 min, isocratic 60% B; at 30.1 min back to initial conditions of 0% B and equilibration for 5 min before the next injection. D-Glucose, fructose, sucrose, 1-kestose, and nystose were used as standards.

Gel Permeation Chromatography. Gel permeation chromatography was performed on a TSK-HW40S gel column 15 × 80 cm (Tosoh Bioscience, Japan), using Milli-Q water as a mobile phase and refractive index detector. Flow rate was 0.5 mL min^{-1} and was maintained using an HPLC pump. Four milliliter fractions were collected and analyzed by HPAEC-PAD and MALDI-TOF MS.

Mass Spectrometry. MALDI-TOF MS was performed with a Proflex III, Bruker Analytical Systems Inc. (Billerica, MA, USA), operated in positive ion mode. Samples (1 μL) were mixed with 1 μL of 2,4-dihydrobenzoic acid (10 mg mL^{-1}), and the cocrystallized analytes were ionized by using a laser pulse (337 nm). The laser strength was selected to obtain the best signal-to-noise ratio.

RESULTS AND DISCUSSION

Recombinant EaLsc. *E. amylovora* possesses a single gene encoding a levansucrase (ENA accession no. CBJ48143.1).[30] The amino acid sequence of EaLsc was aligned with the sequences of 40 other functionally characterized levansucrases from different bacteria retrieved from the CAZy database[31] for comparison and phylogeny reconstruction, using Clustal X 2.1 software.[32] The phylogenetic tree is shown in Supplementary Figure S1 in the Supporting Information. As for the majority of levansucrases reported from Gram-negative bacteria, EaLsc does not contain a signal peptide for extracellular secretion, a rather common feature in similar enzymes from Gram-positive microbes. EaLsc shares highest sequence identity with the levansucrases from the genera *Pseudomonas* (71−78%) and *Rahnella* (75−77%), whereas sequence identity drops to about 20% when compared to enzymes from Gram-positive bacteria, such as *B. subtilis* SacB.

Expression and purification of EaLsc in *E. coli* yielded approximately 20 mg of electrophoretically pure enzyme per liter of culture with an apparent molecular weight of 46000 Da (Supporting Information Supplementary Figure S2), in agreement with the predicted value of 46536 Da and with the observations of Gross and co-workers, who first isolated an active form of the enzyme from *E. amylovora* grown in a high concentration of sucrose.[1]

Catalytic Properties of EaLsc. The optimal reaction conditions for EaLsc activity were investigated. Reactions were carried out at different temperatures and pH values, and the effects on total, hydrolytic, and transfructosylation activities were estimated by measuring the amounts of D-glucose and D-fructose released during the reaction. As proposed by several authors,[28,33] the amount of D-glucose released during the reaction can be used to calculate the total activity of the enzyme (V_G), whereas the amount of free D-fructose can be used to evaluate the hydrolytic activity (V_F). The difference between the amount of free D-glucose and the amount of free D-fructose provides a good estimate of the transfructosylation activity of the enzyme ($V_G - V_F$).

EaLsc displayed activity over the pH range from 5.0 to 7.0, but its activity dropped rapidly to 45% at pH 7.5 and to 17% at pH 8.0. The enzyme performed best at pH 6.5 and retained >80% activity in the pH range of 6−7. The total, hydrolytic, and transfer activities all had similar profiles, indicating that the pH at which the reaction occurs does not affect differently these activities. EaLsc was active over a wide range of temperature: 50 °C was the optimal temperature for hydrolytic activity (assayed in the presence of 10 mM sucrose), whereas transfructosylation activity was highest at 37 °C. A similar behavior has been reported for levansucrases from *Rahnella aquatilis*,[34] *B. subtilis*,[35] and *Zymomonas mobilis*.[36] The enzyme activity dropped dramatically at 60 °C, suggesting that the enzyme was heat inactivated.

Despite the multifunctional nature of levansucrases, which makes the determination of kinetic parameters rather complex, we considered it worthwhile to investigate the kinetics of EaLsc total activity (V_G). The initial rates of the reaction were determined at 37 °C over a range of sucrose concentrations (0−100 mM) where the enzyme behavior followed the Michaelis−Menten model, as indicated by the linearity of the corresponding Lineweaver−Burk plots. The values of K_m, k_{cat}, and k_{cat}/K_m were determined by linear regression of the double-reciprocal plot against the substrate concentration (see Table 1). The determined K_m value was 33.6 ± 3.1 mM, k_{cat} was 502.7 ± 43.9 s^{-1}, and k_{cat}/K_m was 8.96×10^5 M^{-1} min^{-1}. The K_m values for other wild-type levansucrases are in the range between 6.6 mM (levansucrase from *Bacillus megaterium*)[37] and

160 mM (levansucrase from *Pseudomonas syringae*).[38] The kinetic parameters determined for EaLsc are very similar to those reported for closely related enzymes. Table 1 shows the kinetic parameters of several characterized levansucrases, including those from Gram-negative bacteria phylogenetically related to EaLsc. The K_m values for the enzymes from *P. syringae*, *R. aquatilis*, and *Z. mobilis* are in the range of 20.6−50 mM, and the k_{cat} and k_{cat}/K_m vary between 6.77×10^5 and 8.64×10^5 M^{-1} min^{-1}. An exception is provided by the levansucrase from *G. diazotrophicus*, which has kinetic properties closer to the enzyme from the Gram-positive *B. megaterium*, having lower K_m and higher k_{cat} values than the other enzymes from Gram-negative organisms. The V_{max} determined for EaLsc was 648 ± 28 U mg^{-1} (where a unit is defined as μmol of substrate hydrolyzed per min), a value very close to 714 U mg^{-1} reported for the enzyme from *R. aquatilis*.[39]

The specific activity of the enzyme varied with both temperature and substrate concentration. The highest observed total activity (V_G) of EaLsc was 883 μmol min^{-1} mg^{-1} (37 °C and 1.5 M sucrose); the highest observed hydrolytic activity (V_F) was 90 μmol min^{-1} mg^{-1} (37 °C and 500 mM sucrose); finally, the maximal transglycosylation activity was 856 μmol min^{-1} mg^{-1} (37 °C and 1.5 M sucrose).

The transglycosylation to hydrolysis ratio (T/H) was determined after 24 h of reaction at 25, 20, 37, and 42 °C at different substrate concentrations (from 10 mM to 1.5 M). The maximum T/H was observed at 37 °C for all of the concentrations tested. Independent of the temperature, the transglycosylation reaction catalyzed by EaLsc increased with substrate concentration in the samples containing >500 mM sucrose, reaching a maximum of 97% at 1500 mM (Figure 1).

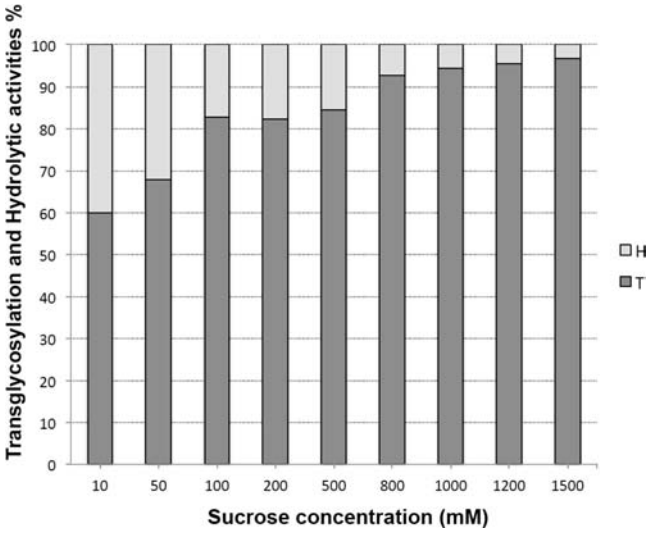

Figure 1. Effect of substrate concentration on transglycosylation and hydrolytic activities expressed as percent of the total activity. The graph refers to reactions performed at 37 °C for 24 h in the presence of different concentrations of substrate (from 10 mM to 1.5 M) and using 21.5 nM EaLsc.

The yields of FOSs were measured over a period of 4 h at different substrate concentrations and temperatures using a turbidity assay (Figure 2). At this time point, the products of EaLsc were mainly FOSs. Only trace amounts of high molecular weight fructans could be detected, but they could not be properly quantified. FOS production was maximal at 37 °C using 200 mM sucrose as substrate. The quantity of products formed under these conditions with 25 nM EaLsc (1 μg/mL) was around 34 mg/mL. The yield of FOSs was not directly proportional to the concentration of substrate but reached a maximum at 200 mM and then rapidly decreased at higher sucrose concentrations. A similar pattern has been reported for other levansucrases and ascribed to substrate inhibition.[40]

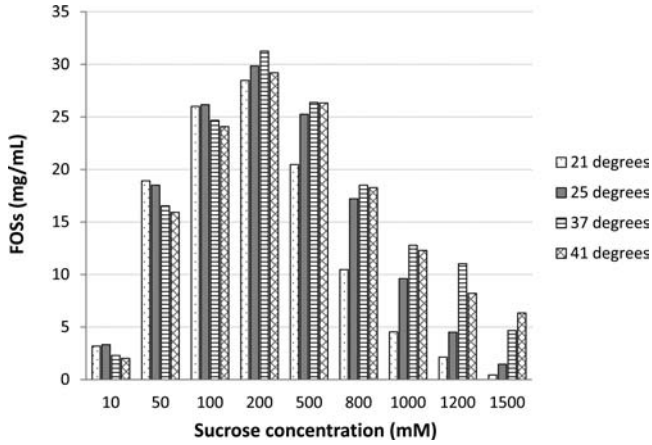

Figure 2. Yields of FOSs measured after 4 h of incubation at different temperatures (25, 30, 37, and 41 °C) and concentrations of substrate (from 10 mM to 1.5 M). Reactions were performed in 0.1 M potassium phosphate buffer, pH 6.5, and started by addition of 21.5 nM EaLsc.

Characterization of the Product Distribution Achieved by EaLsc. The product spectrum of the EaLsc-catalyzed reaction was investigated using sucrose as substrate. HPAEC-PAD analysis revealed that EaLsc forms mainly short-chain FOSs with DP2−6 (Figure 3). Nevertheless, the enzyme is also able to synthesize high molecular weight levan when the reaction is performed at high substrate concentrations (>500 mM). The lack of commercially available standards represents a limitation when trying to identify each observed component of a linear series of FOSs. However, co-injection of pure 1-kestose and nystose allowed the identification of these compounds among the products. Under all conditions used for the activity assays, EaLsc readily produced 1-kestose (retention time = 9.55 min) and at least two other major peaks (retention times = 10.65 and 11.00 min, respectively), which, on the basis of the comparison of their retention times with those reported in the literature,[41] are likely to be 6-kestose and neo-kestose. In contrast, formation of nystose occurred only at high concentrations of sucrose (from 100 mM to 1 M).

The outcome of the reaction was dependent on the initial concentration of sucrose. At low concentration (1 mM), the main products observed were those originating from the hydrolytic activity alone, namely D-glucose and D-fructose, whereas at concentrations of sucrose 10 mM and higher, formation of a considerable amount of FOSs occurred within 1 h of incubation at 37 °C. Longer incubation times did not always lead to an increase of the amount of products. In fact, additional products often appeared and the distribution of products also changed. At low concentrations of substrates some of the product peaks even decreased, as a result of hydrolysis. These results suggest that EaLsc may function using a distributive (nonprocessive) mechanism in which the fructan chain is released from the enzyme after each fructosyl transfer event, as described for some other fructosyltransferases.

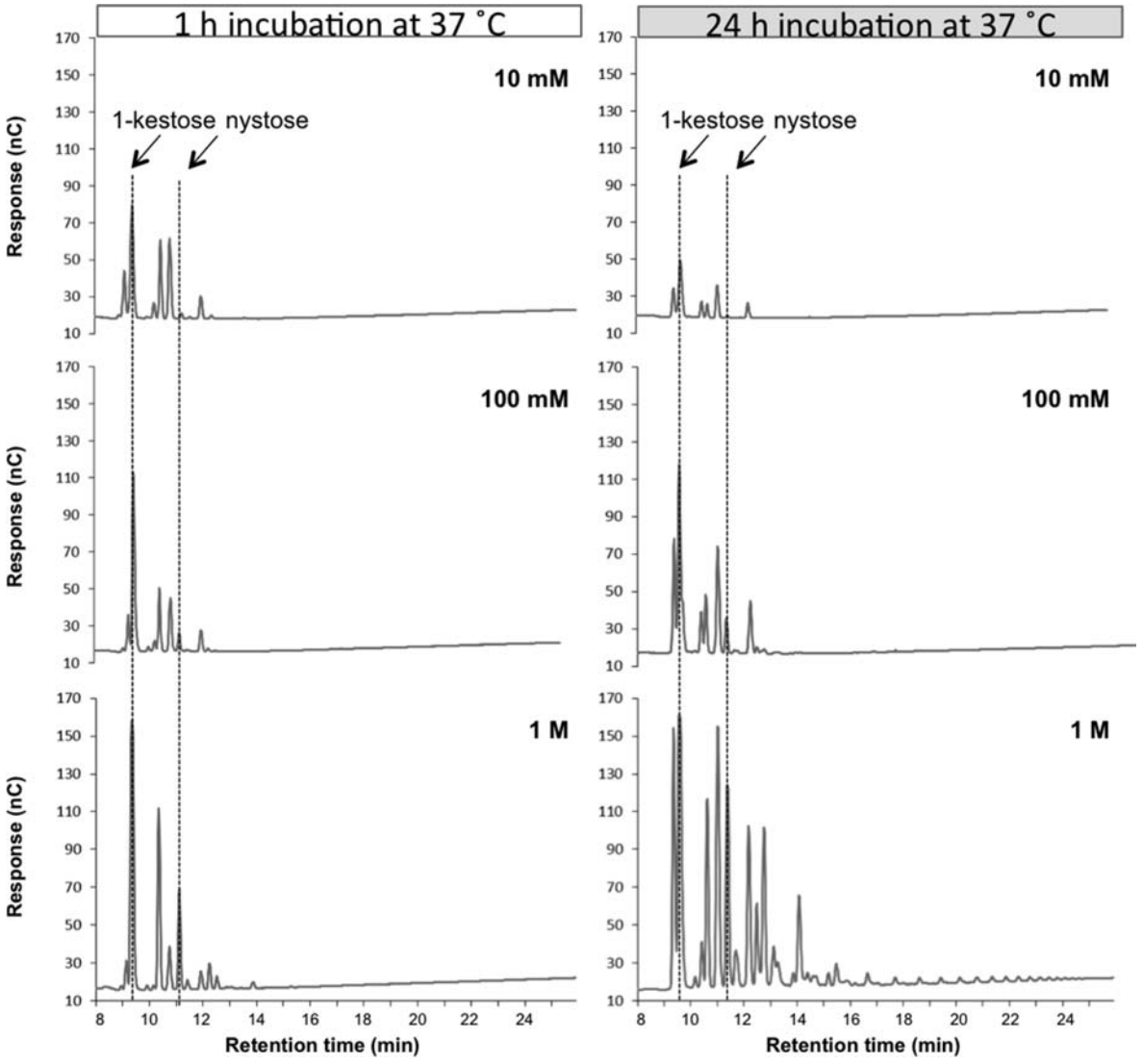

Figure 3. HPAEC-PAD chromatograms of reactions performed at different concentrations of sucrose (10 mM, 100 mM, and 1 M) in 0.1 M potassium phosphate buffer, pH 6.5, and incubated at 37 °C for different lengths of time. Reactions were started by the addition of 21.5 nM EaLsc. Samples containing 10 mM sucrose were injected without dilution; samples containing 100 mM sucrose were diluted 1:10 with water before injection; samples containing 1 M sucrose were diluted 1:100 with water before injection. The retention times of 1-kestose and nystose were determined by injection of standards.

Along with the formation of FOSs, EaLsc catalyzed the formation of levan when the reaction was performed for long incubation times (24 h) and with high concentrations of sucrose (>500 mM). The HPAEC-PAD chromatographic profile of levan showed a linear series of fructooligosaccharides with a DP up to 25−30, and it also displayed the presence of additional small peaks eluting among them, similarly to that previously observed by Ozimek and co-workers[41] (Figure 4). Because bacterial levan is usually a nonlinear polymer with β(2,6)-linked fructosyl chains and β(2,1) branches,[42,43] it is reasonable to speculate that the extra peaks result from different branching of the polymer or from the elongation of β(2,1)-linked products, such as 1-kestose.

To further characterize the product spectrum of EaLsc, fractionation by gel permeation was employed (Supporting Information Supplementary Figure S3). The fractions obtained were analyzed by HPAEC-PAD and MALDI-TOF MS. Figure 5 shows the overlaid MALDI spectra of three main peaks collected during the fractionation. The first peak contained mainly short-chain FOSs and was characterized by the presence of ions at m/z 527 (DP3, e.g., 1-kestose or its isomers 6-kestose and neokestose, see Supplementary Figure S4 in the Supporting Information), 689 (DP4, nystose), 851 (DP5), 1013 (DP6), and 1175 (DP7). The other two peaks contained higher molecular weight FOSs at m/z 1337 (DP8), 1499 (DP9), 1661 (DP10), 1823 (DP11), and 1985 (DP12). The highest DP observed in these experiments was DP18 at m/z 2957, probably due to a decrease in ion-transfer efficiency, because the HPAEC-PAD analysis displayed the presence of fructans with higher degree of polymerization. The spectra displayed a monomodal mass distribution with a mass difference between consecutive ions of 162 Da, corresponding to hexose residues, as expected for fructooligosaccharides.[44,45]

Taken together, the results obtained from HPAEC-PAD and MALDI-TOF MS analysis show that EaLsc produces mainly short-chain FOSs, with a degree of polymerization between DP3 and DP6, whereas levan is synthesized only under

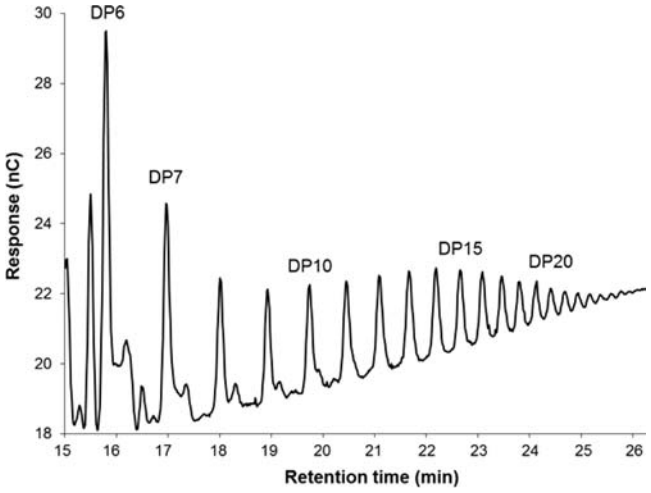

Figure 4. HPAEC-PAD chromatogram of levan produced by EaLsc in 0.1 M potassium phosphate buffer, pH 6.5, containing 1 M sucrose after 24 h at 37 °C. Reactions were started by the addition of 21.5 nM EaLsc.

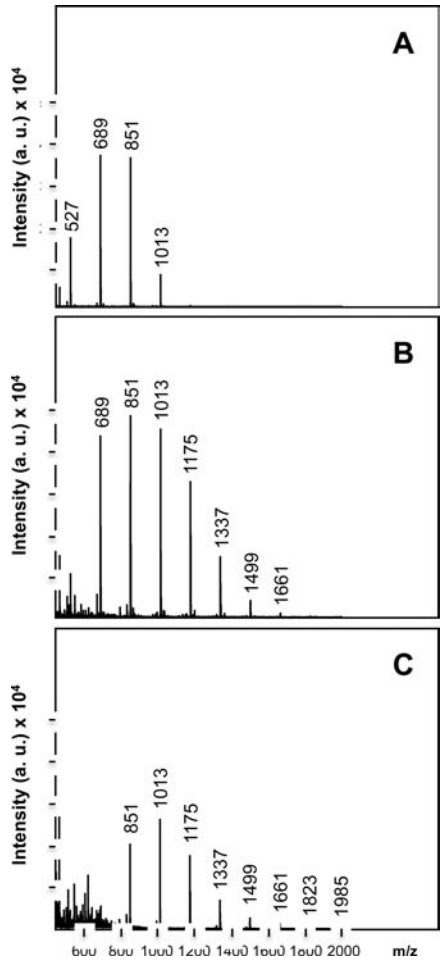

Figure 5. Overlaid MALDI-TOF MS positive ion spectra of three fractions collected during fractionation by gel permeation chromatography (Supporting Information Supplementary Figure S3) of the fructans produced by EaLsc after 24 h of incubation at 37 °C in the presence of 1 M sucrose: (A) fraction containing mainly short-chain FOSs; (B, C) fractions containing high molecular weight fructans.

conditions of high substrate concentration and long reaction times.

Substrate Specificity of EaLsc. Levansucrases that are able to use substrates other than sucrose for the formation of levan and FOSs have been reported. For instance, three levansucrases from *P. syringae*, Lsc1, Lsc2, and Lsc3, and LscA from *P. chlororaphis* displayed activity toward the trisaccharide raffinose (*β*-D-fructofuranosyl-*α*-D-galactopyranosyl-(1→6)-*α*-D-glucopyranoside),[46,47] whereas the *B. subtilis* levansucrase was used for fructosylation of aromatic and aliphatic alcohols.[6] Monosaccharides, such as D-galactose and D-xylose, and disaccharides, such as maltose (4-*O*-*α*-D-glucopyranosyl-D-glucose) and lactose (4-*O*-*β*-D-galactopyranosyl-D-glucose), were shown to act as fructosyl acceptors for *B. subtilis* NCIMB 11871 levansucrase.[48]

In the present study, we investigated the activity of EaLsc toward natural and non-natural substrates (Figure 6). In particular, we focused on raffinose and stachyose (*β*-D-fructofuranosyl-*α*-D-galactopyranosyl-(1→6)-*α*-D-galactopyranosyl-(1→6)-*α*-D-glucopyranoside), which are *α*-galactosides naturally occurring in legumes of the Fabaceae family, such as fava beans and soybeans. These oligosaccharides, which are not digested in the upper intestinal tract due to the absence of the enzyme *α*-galactosidase, have been identified as prebiotics.[49,50] Sucralose (1,6-dichloro-1,6-dideoxy-*β*-D-fructofuranosyl-4-chloro-4-deoxy-*α*-D-galactopyranoside) and lactulose (4-*O*-*β*-D-galactopyranosyl-*β*-D-fructofuranose), which are also non-digestible sugars, were also used as substrates for EaLsc to evaluate its hydrolytic activity toward sucrose analogues in which the D-glucose moiety is replaced by D-galactose and some hydroxyl groups are replaced by chlorine atoms (sucralose). Reactions were performed in 0.1 M potassium phosphate buffer, pH 6.5, at 37 °C in the presence of different concentrations of the sugars, and the amounts of free D-glucose and D-fructose were measured at different time points (0.5, 1, 2, and 24 h). In samples containing lactulose and sucralose no release of D-fructose was observed, indicating that hydrolysis did not occur. However, because the fructosyl moiety of sucralose contains chlorine atoms in positions 1 and 6 that could affect the performance of the enzymes in the assay used for determination of the free sugar, the samples were also analyzed by HPAEC-PAD. These analyses also showed that the enzyme does not hydrolyze sucralose or lactulose (data not shown). On the other hand, small amounts of free D-fructose were detected in the reactions performed using raffinose and stachyose as substrates, indicating that EaLsc was removing fructosyl moieties from the substrates. No free D-glucose could be detected. The yield of FOSs derived by raffinose was measured over a period of 4 h. Using 1 μg of EaLsc, 12.0 mg of FOSs was produced at 37 °C. Analysis of the reaction mixtures by HPAEC-PAD revealed that at 10 mM raffinose, hydrolysis was the dominant reaction; with increasing concentration of the substrate, EaLsc performed substantial transfructosylation, resulting in the formation of FOSs (DP2−5), as shown in Figure 6.

In conclusion, the *E. amylovora* levansucrase, EaLsc, was successfully heterologously expressed in *E. coli* in high yield, allowing a thorough characterization of the enzyme's properties. The combination of HPAEC-PAD with MALDI-TOF MS allowed us to shed light on the product profile of the enzyme under different reaction conditions. EaLsc was shown to produce mainly short-chain FOSs (DP3−6); high molecular weight levan was produced only at very high concentrations of

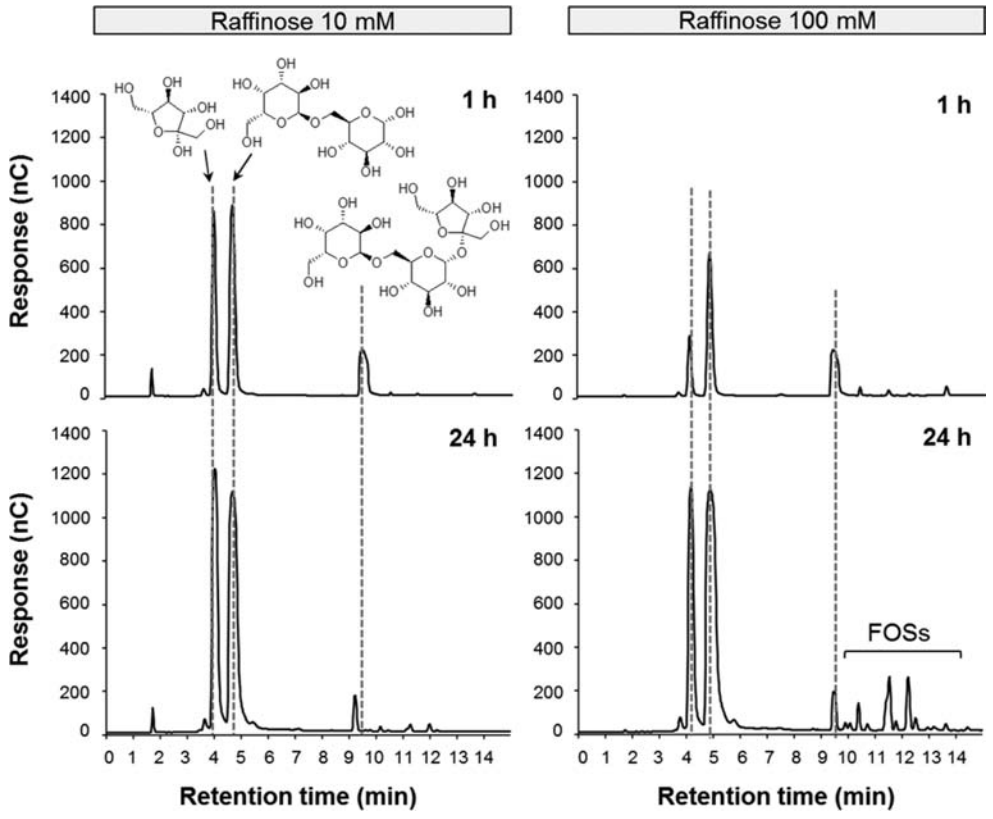

Figure 6. HPAEC-PAD chromatograms of reactions performed at different concentrations of raffinose (10 and 100 mM) in 0.1 M potassium phosphate buffer, pH 6.5, and incubated at 37 °C for different lengths of time. Reactions were started by the addition of 21.5 nM EaLsc. Samples containing 10 mM raffinose were injected without dilution; samples containing 100 mM sucrose were diluted 1:10 with water before injection. The retention time of raffinose was determined by injection of the pure compound.

sucrose (>500 mM) and after long incubation times. These observations are significant on two fronts. First, from a biotechnological point of view, EaLsc is a potential biocatalyst for the production of FOSs. In fact, the high transglycosylation to hydrolysis ratio, the good yield of FOSs, and the ease of enzyme production make it a suitable candidate for further application and engineering studies. Second, under near-physiological conditions (in the Rosaceae, sucrose concentrations in nectar range between 40 and 60% of the total sugars, i. e., around 1 M), EaLsc produces high molecular weight fructans. It has been reported that *E. amylovora* mutants deficient in levan production show reduced virulence,[3] which strongly indicates that EaLsc is involved in the infection process.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was financially supported by the Autonomous Province of Bolzano (project "A structural genomics approach for the study of the virulence and pathogenesis of *Erwinia amylovora*") and by the Free University of Bolzano (project: "*Erwinia amylovora*'s secretome"). Studies conducted at the John Innes Centre were supported by the UK BBSRC Institute Strategic Programme Grant on "Understanding and Exploiting Metabolism (MET)" (BB/J004561/1) and the John Innes Foundation.

Notes

The authors declare no competing financial interest.

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