TWO ACTIVE FORMS OF ZYMOMONAS MOBILIS LEVANSUCRASE: AN ORDERED MICROFIBRIL STRUCTURE OF THE ENZYME PROMOTES LEVAN POLYMERIZATION

Dan Goldman¹, Noa Lavid¹, Alon Schwartz¹, Gil Shoham², Dganit Danino¹, and Yuval Shoham¹
¹Department of Biotechnology and Food Engineering, Technion–Israel Institute of Technology, Haifa, Israel; and ²Department of Inorganic Chemistry and The Laboratory for Structural Chemistry and Biology, The Hebrew University of Jerusalem, Jerusalem, Israel

Running head: Microfibril Structure of Z. mobilis Levansucrase

Address correspondence to: Yuval Shoham, Department of Biotechnology and Food Engineering, Technion, Haifa 32000, Israel. Telephone: 972-4-8293072, Fax: 972-4-8293399; E-mail: yshoham@tx.technion.ac.il

Fructansucrases, members of glycoside hydrolase family 68, catalyze both sucrose hydrolysis, and the polymerization of fructose to β-D-fructofuranose polymers. The resulting fructan polymers are distinguished by the nature of the glycosidic bond – inulin (β-(2-1)-fructofuranose) and levan (β-(2-6)-fructofuranose). In this study we demonstrate that Zymomonas mobilis levansucrase exists in two active forms, depending on the pH and ionic strength. At pHs above 7.0, the enzyme is mainly a dimer, whereas at pHs below 6.0, the protein forms well-ordered microfibrils that precipitate out of the solution. These two forms are readily interchangeable simply by changing the pH. Surprisingly, the manner in which the enzyme is arranged strongly affects its product specificity and kinetic properties. At pH values above 7.0, the activity of the enzyme as a dimer is mainly sucrose hydrolysis and the synthesis of short fructo-saccharides (degree of polymerization 3). At pH values below 6.0, in its microfibril form, the enzyme catalyzes almost exclusively the synthesis of levan (a degree of polymerization greater than 20,000). This difference in product specificity appears to depend on the form of the enzyme, dimer versus microfibril, and not directly on the pH. Images made by negative stain transmission electron microscopy reveal that the enzyme forms a very ordered structure of long fibrils that appear to be composed of repeating rings of 6 to 8 protein units. A single amino acid replacement of H296R abolished the ability of the enzyme to form microfibrils and to synthesize levan at pH 6.0.

Levan and inulin are a group of fructan polymers in which the fructose units are linked via β-(2-1)-linkages (inulin) or β-(2-6)-linkages (levan) (1). Inulin is found mainly in higher plants, whereas levan is primarily synthesized by various bacterial species. In bacteria, levan is synthesized from sucrose by levansucrases (sucrose: 2-6-β-D-fructan 6-β-D-fructosyltransferase, E.C. 2.4.1.10). These enzymes cleave sucrose and use the bond-energy between glucose and fructose to couple a fructosyl unit to a growing fructan (polyfructose) chain that can consist of up to 100,000 fructose units. Levansucrases can also catalyze the fructosyl transfer to a number of acceptors (other than the fructan polymer) such as water (resulting in the hydrolysis of sucrose, an invertase type), sucrose, raffinose, and various mono and disaccharides (2).

The database of carbohydrate active enzymes (CAZY, http://www.cazy.org) classifies bacterial levansucrases (levan synthesis), inulosucrases (inulin synthesis), and invertases (hydrolysis of sucrose) to glycoside hydrolase (GH) family 68 (3). High-resolution crystal structures of the Bacillus subtilis SacB levansucrase complexed with sucrose and of Gluconacetobacter diazotrophicus levansucrase have been described (4-6). These structures reveal a unique five-fold β-propeller topology with a deep, negatively charged binding pocket. The catalytic site contains two acidic residues acting as a general acid/base and a nucleophile, accompanied by a third acidic residue that presumably functions as a "transition state stabilizer". These three catalytic residues are also found in GH families 32, 43, 62, and 68 (7,8). The catalytic mechanism underlying levansucrases occurs via a double displacement reaction in which the first step involves the protonation of the glycosidic oxygen by the acid/base residue (acting as a general acid), which stabilizes the leaving group (i.e., the...
glucose molecule). The nucleophile residue attacks the anomeric carbon of the sucrose scissile bond, forming a covalent glycosyl-enzyme intermediate. In the next step, the acid/base residue, acting this time as a general base, activates either a water molecule (hydrolysis reaction) or a hydroxyl group of the sugar acceptor (transfructosylation reaction), which attacks the anomic center of the glycosyl-enzyme intermediate, resulting in the liberation of the fructose-acceptor product (9).

To date, there is no clear understanding what structural elements govern the outcome of the transfructosylation reaction (hydrolysis versus polymerization) and what elements control the nature of the resulting bond (β-(2-6) versus β-(2-1)).

Zymomonas mobilis, a Gram-negative ethanologenic bacterium, produces levansucrase that was studied extensively (10-14). However, many of the studies of this enzyme resulted in conflicting results regarding its apparent molecular weight and reaction products. Using gel filtration, Crittenden et al. (1994) and Vigants et al. (2003) found that the 46.7 kDa protein of Z. mobilis levansucrase was eluted as an active form in the void volume of the column, indicating a molecular mass of more than 10^6 Da (11,15,16). Crittenden et al. characterized the wild-type levansucrase (rather than the recombinant one) and suggested that its high MW results from the attachment of the enzyme to levan. In that study, the enzyme was also found as a 98,000-Da protein that was only able to hydrolyze sucrose or polymerize short fructo-saccharides. Using purified Z. mobilis levansucrase, Song et al. (1994) reported that the major transfructosylation reaction product was high-molecular weight levan (17), whereas in other studies, the three unit sugar, 1-kestose, was the main reaction product (11).

In this report we demonstrate that Zymomonas mobilis levansucrase can exist in two different active forms, depending on pH and ionic strength. At pH values above 7.0 and low ionic strength, the protein is a dimer, whereas at pH values below 6.0, the protein assembles spontaneously to long, ordered insoluble filaments that are biologically active. Remarkably, the product specificity of the enzyme depends on its quaternary structure and not on the pH per se. A single amino acid replacement of H296R abolished the ability of the enzyme to form microfibrils and to synthesize levan at pH 6.0.

### Experimental Procedures

**Bacterial strains and plasmids-** Zymomonas mobilis (ATCC10988) was obtained from ATCC (ATCC, Manassas, VA, USA). The *Escherichia coli* strains used were XL-1 blue (Stratagene, La Jolla, CA, USA) for general cloning, and BL21(DE3) for expression via the T7 RNA polymerase expression system with pET9d (both from Novagen, Madison, WI, USA).

**DNA manipulation-** DNA was manipulated by standard procedures (18,19). DNA transformation was performed by using the calcium chloride method or electroporation (Gene Zapper, IBI, New Haven, CT, USA). Z. mobilis genomic DNA was isolated using the Marmur's procedure (20), as outlined by Johnson (21). Plasmid DNA was purified using the Qiagen plasmid kit (Qiagen, Inc., Chatsworth, CA, USA). DNA sequencing was performed at the Biological Services Unit at the Weizmann Institute, Rehovot, Israel.

**Cloning of the Z. mobilis sacB gene-** Based on the published DNA sequence of the sacB gene (AAA27695), two PCR primers were designed in a way that enabled the in-frame cloning of the gene into the T7 polymerase expression vector pET9d (Novagen). The N-terminal primer (5'-GACCGACATGTTGAATAAAGCAGGCT T) included an ATG translational start codon inside the PciI restriction site (ACATGT). The C-terminal primer (5'–CCTGTCTTTATTGAATAAATAAGGATCGTGG) included a BamHI restriction site (GGATCC) following the end of the gene. Using these primers, PCR amplification was performed on Z. mobilis chromosomal DNA and the PCR product was digested with PciI and BamHI. The digested product was cloned into a linearized NcoI/BamHI digested pET9d vector resulting in pET9d-sacB and the integrity of the gene was verified by DNA sequencing.

**Site-directed mutagenesis-** Site-directed mutagenesis was performed using the QuickChange Site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The mutagenic primers for the H296R replacement were as follows (the mutated nucleotides are shown in bold): 5'–TTTACGATCAGTCGTCATTGCAG-3' and 5'GTCGAATGACGACTCGATCGTAAA-3'. The mutated gene was sequenced to verify that only the desired mutation was inserted.

2
Purification of Z. mobilis levensucrase

Expression of the Z. mobilis sacB gene was carried out by growing overnight cultures of E. coli BL21(DE3) carrying pET9d-sacB. The cells were grown at 37 °C for 3 hours (OD_{600} of about 0.6), after which the temperature was reduced to 18 °C, and the culture was kept growing overnight to a final turbidity of about 15 OD, at 600 nm. The cells were harvested by centrifugation (10,000xg for 10 minutes), after which the pellet was resuspended in buffer A (50 mM phosphate buffer pH 7.4, 100 mM NaCl and 0.02% sodium azide) and disrupted by two passages through a French® press (Spectronic Instruments, Inc., Rochester, NY, USA) at room temperature. The cell extract was centrifuged (14,000xg for 15 min) and the soluble supernatant was kept at 4 °C. Following the procedure of Vigants et al. (16), the enzyme was selectively precipitated by adding 0.1 M MnCl₂. Next, the solution was centrifuged (14,000xg for 15 min), the pellet was re-suspended in buffer B (50 mM phosphate buffer pH 6.0, 100 mM NaCl and 0.02% sodium azide), and then the soluble protein was passed via a sephacryl S300 26/60 gel filtration column (Pharmacia), running at 1 mL/min with either buffer A at pH 7.4 or with buffer B at pH 6.0.

Enzymatic assays- Levansucrase activity was determined by following the release of glucose and fructose and the formation of levan. The release of glucose and fructose was measured by high-performance anion-exchange-chromatography with pulsed amperometric detection (HPAEC-PAD) (Dionex LC30 Instrument, Sunnyvale, CA, USA, equipped with a pulsed amperometric detector (ED40) and a PA1 column). Isocratic elution was performed with 150 mM NaOH as the mobile phase at a flow rate of 1 mL/min. One unit of enzyme (U) releases 1 μmol glucose/fructose per min. Sucrose conversion by levansucrase yields fructose, which can be incorporated into the growing polymer (formation of levan) and glucose in a 1:1 ratio to the amount of sucrose converted. The amount of glucose reflects the total amount of sucrose utilized during the reaction (total activity). The amount of free fructose is a measure of the hydrolytic activity of the enzyme. Thus, subtracting the amount of fructose from glucose provides the transferase activity (22). A typical reaction mixture (1 mL) contained 12.5 μg enzyme and 263 mM sucrose in 150 mM citric acid – Na₂HPO₄, pH 5.0 at 25 °C. Samples (0.1 mL) were removed every 10 min and introduced into 0.9 mL of 15 mM NaOH to stop the reaction. The synthesis of levan was monitored by following the increase in turbidity at 600 nm that occurred due to the insoluble levan. Under typical reaction conditions, more than 90% of the liberated fructose was assimilated to a growing levan polymer. At sucrose concentrations ranging from 5 - 500 mM, the increase in 1 OD_{600} in a 1-cm path cuvette corresponded to the consumption of 120 μmol sucrose per mL. Kinetic parameters were calculated for sucrose-dependent activity. Data analysis was obtained by nonlinear fit reduced chi² with GRAFIT 5.0 software (Erithacus Software Ltd, Staines, UK) using a typical Michaelis-Menten equation with substrate inhibition -

\[ V = \frac{V_{\text{max}} S}{K_m + S + K_i} \]

In this equation: \( V \) – specific activity (U/mg), \( V_{\text{max}} \) – (U/mg), \( S \) – sucrose concentration (mM), \( K_m \) – (mM sucrose), \( K_i \) – substrate inhibition constant (mM sucrose).

Analysis of reaction products- Fructosaccharides were identified by a combination of HPAEC-PAD and TLC. HPAEC-PAD was performed using two buffer eluents, eluent A (150 mM NaOH), and eluent B (150 mM NaOH and 1M sodium acetate). The gradient was programmed as follows: 0-1 min: isocratic elution 100% A, 0% B; 1-30 min: linear gradient to 40% A, 60% B. The elution rate was 1 mL/min. TLC analysis was performed using precoated plates (Silica Gel 60 F 254, 0.25 mm; Merck), and ethyl acetate/methanol/water 7:2:1 as the running solvents.

Negative stain transmission electron microscopy (TEM)- For negative staining-TEM studies, 50 μL of purified enzyme (2 mg/mL) at 50 mM phosphate buffer, pH 7.4 and 100 mM NaCl were added to 450 μL of 50 mM phosphate buffer, pH 5.7 and 100 mM NaCl to a final pH of 6.0 and 0.2 mg/mL enzyme. A carbon-coated grid was placed on a 10-μl sample drop for 2 min, blotted with filter paper, chemically stained with 2% uranyl acetate for 2 min, blotted again, and then air dried. Negatively stained samples were examined in a transmission electron microscope, Tecnai G² 12, operated at 120kV. Images were recorded digitally on a 2K x 2K ultrascan CCD camera with the DigitalMicrograph software package.
RESULTS

Z. mobilis levansucrase exists in two forms. Based on the published genome sequence (23), the Z. mobilis sacB gene was readily cloned via PCR into pET9d and efficiently overexpressed in E. coli using the T7-polymerase expression system. The sacB gene encodes for a 423-amino acid protein with a calculated MW of 46,700. The purification procedure included two steps, MnCl₂ precipitation (15) and gel filtration, resulting in a 98% pure protein based on SDS-PAGE (results not shown). The final gel filtration step was initially performed at pH 7.4 and provided a distinct symmetrical protein peak at Kᵥᵥ = 0.37, corresponding to a MW of 94,000 (Figure 1A). Thus, at pH 7.4 the enzyme appears to be a dimer in solution. Interestingly, the protein taken from this peak did not exhibit appreciable levansucrase activity although the initial sample loaded on the column was active. Combining the various gel filtration fractions or adding various metals to the protein solution did not restore the initial activity. However, when the pH of the purified protein solution was lowered to 6.0 (or below), the solution immediately became highly turbid, resulting in the precipitation of the protein. Unexpectedly, this turbid protein solution exhibited levansucrase activity that was much higher than the initial activity of the sample loaded on the column at pH 7.4. Applying the turbid enzyme solution on the same gel filtration column at pH 6.0 produced the protein peak at the column void volume, suggesting an apparent MW greater than 10⁶ (Figure 1B). These results prompted us to investigate the biochemical properties of the Z. mobilis levansucrase at pH 7.4 and at pHs below 6.0.

Biochemical characterization and product specificity. The activity of levansucrase can result in several products. The enzyme can hydrolyze sucrose utilizing a growing chain of fructo-saccharide as the acceptor, resulting in high molecular weight fructan and free glucose. Alternatively, the enzyme can use either water or sucrose as an acceptor; this results in the net hydrolysis of sucrose to fructose and glucose or in the synthesis of short fructo-saccharides. The synthesis of levan by Z. mobilis levansucrase was determined by following the increase in turbidity at 600 nm, resulting from the accumulation of high MW levan. Under standard conditions (25 °C, pH 5.0), the reaction was linear with time for at least 20 minutes and proportional to enzyme concentration. Moreover, the rate at which the turbidity increased was proportional to the rate at which glucose accumulated, indicating that this simple assay reflects the true levan synthesis rate. The highest levan synthesis activity in a 5-minute reaction was at 30 °C and at pH 5.0. The effect of substrate concentration on the initial velocity rate of levan synthesis typifies simple Michaelis-Menten behavior with substrate inhibition. The kinetic constants are summarized in Table 1 and supplemental data S1.

As previously mentioned, at pHs higher than 7.0, Z. mobilis levansucrase did not exhibit levan synthesis activity. To determine whether the enzyme is capable of hydrolyzing sucrose at these pHs, the activity was monitored by determining the sugar content with a high-performance anion-exchange-chromatography with pulsed amperometric detection system (HPAEC-PAD). The rate of glucose release reflects total enzyme activity (transfructosylation together with sucrose hydrolysis), whereas the accumulation of fructose reflects sucrose hydrolysis. Subtraction of fructose accumulation from glucose accumulation yields the transfructosylation activity. The rate release of glucose and fructose were determined at pH 5.0 and 7.2 at different sucrose concentrations. At pH 5.0, the activity was similar to that obtained with the turbidity assay (supplemental data S1) described above and the transfructosylation activity was favored even at very low sucrose concentrations. The reaction fitted the Michaelis-Menten type of kinetics with uncompetitive substrate inhibition (Figure 2A, Table 1). However, at pH 7.2, a completely different activity profile was observed. At this pH, the total activity was somewhat lower (compared to pH 5.0) and favored sucrose hydrolysis rather than transfructosylation. This difference was more pronounced at sucrose concentrations below 250 mM, whereas at substrate concentrations above 250 mM, transfructosylation activity could be measured. At pH 7.0, the dependency of the overall activity on substrate concentrations resembled an S type of behavior (Figure 2B). The data could be best fitted into the proposed ping-pong mechanism of levansucrases (24); however, this model requires the determination of at least nine rate kinetic constants that were not determined independently in this study. To characterize the
reaction products at pH 7.2 and 5.0, the sugar profile was analyzed using HPAEC-PAD and TLC plates (Figure 3). At pH 5.0, a relatively small amount of free fructose was detected and the main product was high molecular weight levan. However, at pH 7.4, the end products were mainly fructose and short fructo-saccharides with a degree of polymerization (DP) 3, with no detectable amounts of levan.

Enzyme quaternary structure. At pH 6.0 and below, Z. mobilis levansucrase solution appears to be turbid and the apparent MW of the protein based on gel filtration is above 10^6 (Figure 1B). To determine whether under the acidic conditions the protein forms a discretely defined structure, we utilized transmission electron microscopy (TEM). To this end, purified enzyme solution at pH 7.4 was acidified to pH 6.0 and samples were taken for TEM analysis at the indicated times (Figure 4). Surprisingly, the enzyme appeared to form well-defined filaments that grew with time. After only a few minutes at pH 6.0, the protein self-assembled into filaments. These filaments elongated separately at first, and after several hours they formed a net-like structure. The fibrils were a few microns long, with a diameter of approximately 12-13 nm. A 3D model of the Z. mobilis levansucrase performed with the Swiss model protein structure homology-modeling server (http://swissmodel.expasy.org) (25) indicated an average monomer diameter of 45 – 65 Å. This value suggests that the fibrils could be constructed from repeating ring units, each containing 6-8 enzyme monomers.

Driving force for enzyme oligomerization. To investigate the driving forces that control the formation of protein fibrils, the oligomerization rate was monitored by measuring the change in protein turbidity with time at different ionic strengths and temperatures. Dimer levansucrase at phosphate buffer (20 mM, pH 7.4) was diluted 6-fold to a final protein concentration of 0.42 mg/ml with 50 mM phosphate buffer pH 5.0, containing different NaCl concentrations, ranging from 150 mM to 1.65 M. The change in protein solution turbidity was measured spectrophotometrically at 600 nm (supplemental data S2A). Increased ionic strength resulted in an increased rate of oligomerization and a shorter initiation time. Similar behavior was observed with increasing temperatures: enzyme solutions incubated at higher temperatures resulted in shorter initiations times and faster oligomerization rates (supplemental data S2B). At the end of these experiments, the enzyme was active and maintained all of its levan synthesis activity. These results strongly suggest that hydrophobic interactions are the dominant driving force for protein self-assembly.

Levan synthesis activity depends on the fibril structure of the enzyme. At pHs 7.0 and 5.0, Z. mobilis levansucrase changes both its quaternary structure (from a dimer to long fibrils) and its activity (from sucrose hydrolysis to levan synthesis). These two phenomena could in principle be unrelated. To examine this possibility, we followed the kinetics by which the enzyme changes its product specificity following a step change reduction in pH. If levan synthesis activity depends only on pH (6.0 and below), we expect to see an immediate change in enzyme specificity when the pH drops from 7.0 to 5.0. However, if levan synthesis depends on the fibril's structure, then the change in enzyme specificity will follow the kinetics of fibril formation. Enzyme solutions at pH 7.4 at protein concentrations of 0.22, 0.45, and 0.90 mg/mL were step-changed to pH 5.0 and samples were removed at different time periods, diluted appropriately, and then assayed for levan synthesis under standard conditions (Figure 5). The increase in activity was both time- and protein-concentration dependent. These results indicate that levan synthesis does not depend on the pH per se but requires the fibrillar structure of the enzyme. To further test this conclusion, we determined the activity of an enzyme maintained at pH 7.4 in a pH 5.0 reaction. The reaction was carried out for 3 minutes at 30 °C and the sugar profile was analyzed using HPAEC (Figure 6). Under these conditions, i.e., low protein concentrations and a three-minute reaction, there is not enough time for the enzyme to form fibrils and thus its activity at pH 5.0 is mainly of the dimer form (resembling activity at pH 7.2, Figure 2B). Taken together, these results indicate that levan synthesis depends on the fibril structure of the enzyme and not on the pH per se.

The pH-driven shift in activity from sucrose hydrolysis to levan synthesis is completely reversible. The enzyme exhibited the same intrinsic pH-dependent specific activities (either sucrose hydrolysis at pH 7.4, or levan synthesis at pH 5.0) when shifted repeatedly between the two pHs. To verify that the levan synthesis activity is associated with the protein fibrils (and not, for example, with a
soluble high molecular form of the enzyme) the insoluble fibril protein was washed and its activity was determined. A 0.5 ml enzyme sample (0.5 mg/ml in 50 mM phosphate buffer pH 5.0 and 400 mM NaCl), exhibiting 115 units of levansucrase activity, was centrifuged (14,000xg for 10 min) and 0.45 ml of the supernatant was removed leaving the insoluble pellet. The protein pellet was resuspended in 0.45 ml of the same buffer, and the pellet was washed again in the same procedure. Following the two washing steps, the insoluble fibril suspension retained over 90% of the initial activity, 105 U, and only marginal activity, 3 U, was obtained in the supernatant. These results indicate that the levansucrase activity is indeed associated with the insoluble fibrils.

A single amino acid replacement abolished the ability of the enzyme to form fibrils and to synthesize levan at pH 6.0. Several studies on levansucrases from B. subtilis and Z. mobilis indicated that a single amino acid replacement can either attenuate or abolish the transfructosylation activity but still maintain the hydrolysis activity of these enzymes. Chambert et al. (1991) demonstrated that in B. subtilis levansucrase, Arg360 is essential for the transfructosylation reaction (6,26). Based on these results, His296 in Z. mobilis levansucrase, which corresponds to Arg360 in B. subtilis levansucrase, was replaced by several different amino acids, resulting in the abolishment of levansucrase activity (14,27). Since the pKa of the histidine imidazole ring is 6.0 and the nitrogen may change its charge in the pH range of 7.5 to 6.0, it was tempting to determine whether in Z. mobilis levansucrase, His296 also plays a role in the formation of the fibrils' structure. To this end, H296R replacement was prepared and the mutant protein was over-expressed and purified. At pH 7.4, the protein solution of SacB(H296R) was clear and the enzyme was eluted as a dimer just as the wild-type protein. However, unlike the native enzyme, when the pH was reduced to 6.0, no visible turbidity could be observed (supplemental data S3) and the protein remained as a dimer based on gel filtration (results not shown). When the pH was reduced to 5.0, the protein formed fibril structures, but at a much lower rate (supplemental data S3). The activity of the SacB(H296R) mutant was characterized at pH 7.2 and 5.0 (Figure 7). At both pHs, the activity of the mutant was only about 30% of that of the native enzyme and the activity dependence on substrate concentration exhibited a different pattern than that of the native enzyme. Both the native and the H296R mutant did not reach saturation at sucrose concentrations up to 2 M. With the H296R mutant, at both pHs, the end products were mainly glucose and fructose, indicating sucrose hydrolysis similar to the activity of the native enzyme at pH 7.2. Slight transfructosylation activity was observed at sucrose concentrations greater than 1500 mM and 750 mM at pH 7.2 and pH 5.0, respectively. At both pHs, the formation of high molecular weight levan was not observed by monitoring the increase in turbidity or by observing TLC plates. Short fructo-saccharides (DP 3) were detected only when the mutated enzyme was incubated with high sucrose concentrations. Thus, the H296R mutation appears to affect both the tendency of the enzyme to form microfibril structures at pHs below 6.0, and its ability to synthesize levan.

**DISCUSSION**

*Z. mobilis* levansucrase exists in two active forms. We have shown that levansucrase from *Z. mobilis* exists in two discrete and active forms. At pHs above 7.0, the enzyme is soluble and is a dimer, whereas at pHs below 6.0 the protein self-assembles into long discrete microfibrils that tend to precipitate out of solution. In both forms the enzyme is active although its substrate and product specificity change. These results clarify previous studies on this enzyme regarding its native molecular weight and specificity. Evidences for the high molecular forms of *Z. mobilis* levansucrase were reported by Yanese et al. (10), Vigants et al. (16), and Crittenden et al. (11). Yanese et al. reported that when the enzyme is precipitated with ammonium sulfate, it forms needle-shaped crystals, as photographed at micronic resolution. Vigants et al. found that the protein appears at the void volume of a gel filtration column, indicating that the enzyme has an apparent MW greater than 10^6. The authors suggested that the high molecular weight of native levansucrase indicates a stable association of several enzyme molecules that are connected together by glycosyl residues, reported to be typical for fructosyltransferases (28). Crittenden et al. found that the levansucrase peak was turbid, and suggested that the enzyme was attached to levan. In that study a second peak of levansucrase was described. That peak exhibited only the activity
of sucrose hydrolysis and the polymerization of fructo-saccharides, and corresponded to a MW of 98,000. These results are consistent with our findings. Z. mobilis levansucrase was shown to be activated towards the synthesis of levan in the presence of 0.1 M NaCl (13). In that work, the initial velocity rate as a function of sucrose concentration provided a sigmoidal-type behavior. Running the same reaction at high salt concentrations (1 M NaCl) reduced the sigmoidal response. These observations are consistent with our findings that high ionic strength promotes the formation of enzyme fibrils, and consequently, the synthesis of levan. The different kinetic constants reported for the enzyme (Km values of 8 mM (29) versus 122 mM (10)) and for product specificity (1-kestose (11) versus high-molecular weight levan (17)) again could be resolved in light of our findings. Z. mobilis levansucrase in its dimer form catalyzes mainly the hydrolysis of sucrose and the synthesis of short-chain fructo-saccharides. In this form of the enzyme, the reaction rate does not reach saturation even at high sucrose concentrations, thus providing high Km values. However, the fibril form of the enzyme that catalyzes levan formation exhibits typical Michaelis Menten behavior with relatively low Km values.

**The driving force for enzyme oligomerization.** The kinetics of fibril formation could be followed by reducing the pH of the solution (from 7.4 to 5.0). TEM images taken at different time points reveal that at early stages the dimer enzyme units first assemble together to form a doughnut-shaped structure composed of six to eight enzyme molecules. This is based on a 3D model structure and the calculated diameter of a monomer. With time, short fibrils appear that progressively become elongated. The kinetics of fibril formation follows typical nucleation-dependent kinetics (30). This phase change depends on the development of nucleation centers that are capable of elongation and steady growth. Typically, the kinetics follows an S-shaped transformation-time curve in which the formation of a critical nucleus is the key rate-determining step, after which fibrillation proceeds rapidly. Both increases in ionic strength and temperature resulted in the acceleration of fibril formation, indicating that hydrophobic interactions dominate a process that is entropy driven. The exact surface sites involved in these interactions are yet to be identified.

The two active forms of Z. mobilis levansucrase exhibit different kinetics and generate different products. We observed that Z. mobilis levansucrase exhibits different kinetic behavior in its two forms. In its fibril form, the enzyme catalyzes almost exclusively high MW levan with no detectable amounts of fructose or short oligofructoses. Thus, in this form the enzyme preferably accommodates the elongated fructo-chain as an acceptor. The initial rate kinetics as a function of substrate concentration exhibits typical Michaelis-Menten behavior of substrate inhibition with kinetic constants of kcct, Km, and Ki of 461 sec⁻¹, 32 mM and 2.1 M, respectively (Figure 2A). Since the effective concentration of levan chain acting as an acceptor is essentially constant during the reaction, the measured Km reflects the binding of the donor to the -1, +1 sites. At least two possible modes of substrate (sucrose) inhibition can be envisioned. In the first, the sequential substrate binding changes and some sucrose molecules occupy the acceptor site +1, +2 before the donor site -1, +1 is occupied, resulting in unproductive binding. Another scenario is that sucrose competes with the levan chain as an acceptor. This should result in the generation of short fructo-saccharides and may not necessarily influence the rate of glucose release (but of course will slow levan synthesis). In our hands, the rates of glucose release and levan synthesis were identical and we did not detect any short fructo-saccharides even at high sucrose concentrations. These results support an inhibition mechanism that involves unproductive binding presumably at the +1, +2 sites. A similar inhibition mechanism was proposed for GH 70 dextran transucrase, which catalyzes a reaction analogous to that of levansucrases, with glucan as the main end product (compare with fructan polymer) (31). The dimer form of Z. mobilis levansucrase catalyzes mainly the hydrolysis of sucrose, and when transfructosylation occurs, it is characterized by the formation of short oligofructoses (mainly DP3). Thus, the acceptor of the fructosyl-enzyme intermediate is either water or sucrose. At sucrose concentrations of up to 250 mM, the rate of fructose release equals that of glucose, indicating that at these sucrose concentrations the favored acceptor is a water molecule. However, at high sucrose concentrations the rate of fructose liberation reaches saturation and transfructosylation occurs, indicating that the sucrose molecule is now the preferred acceptor. The kinetic
constants for the dimer reaction could not be easily obtained since the initial reaction rate as a function of sucrose concentration resulted in a sigmoid type of relation (Figure 2B). This kinetic behavior could be suitable for a ping pong-type mechanism with nine different kinetic constants (results not shown) (24). However, verification of these constants requires extensive equilibrium measurements and is the subject of another study. When the pH of the dimer enzyme solution at pH 7.4 was adjusted to 5.0, a gradual increase in levan synthesis activity was observed. This activity was enzyme concentration-dependent as well as time-dependent, strongly suggesting that the levan synthesis ability is induced by fibril formation.

A single amino acid replacement abolished the ability of the enzyme to form fibrils and to synthesize levan at pH 6.0. Previous studies demonstrated that in Z. mobilis levansucrase, the replacement of His296 attenuated or abolished its transferfructosylation activity (14,27). In light of our findings, it was of interest to examine whether this mutation is related to the ability of the enzyme to form fibrils. The H296R mutation affected the characteristics of Z. mobilis levansucrase in two ways. First, the H296R mutant was unable to form fibrils under mild acidic and ionic strength conditions (pH 6.0 and 300 mM NaCl), and at pH 5.0, the rate of fibril formation was significantly reduced. Second, this mutant was unable to synthesize levan and its activity was mostly towards the hydrolysis of sucrose (up to 1.5 M of substrate), or the synthesis of short oligo-fructoses at higher sucrose concentrations. The total activity (rate of glucose release) of the H296R mutant was about one third of the native enzyme and it increases with a parabolic dependence, with sucrose concentrations up to 2 M. Thus, it appears that His296 plays a dual role both in fibril formation and levan synthesis. Interestingly, it was recently suggested that in B. subtilis levansucrase, Arg360 (homologous to His296) plays a role in binding the acceptor molecule (6). It is possible that the fibril structure promotes the anchoring of lengthened levan polymer to the surface of the enzyme, thus facilitating the high molecular chain to act as an acceptor. Indeed, surface anchoring sites for amyloglucan were proposed for the GH13 amylasucrase from Neisseria polysaccharea (32). Thus, His296 may mediate such anchoring of the acceptors in Z. mobilis levansucrase. In addition, this amino acid might also be involved or positioned in interacting surfaces between enzyme units. Interestingly, levan was not able to prime levan synthesis with the wild type enzyme at pH 7.4 or with the H296R mutant.

In summary, our findings indicate that in its fibril form, Z. mobilis levansucrase prefers levan as the acceptor of the fructosyl unit, whereas in its dimer form the preferred acceptors are water (at sucrose concentrations below 250 mM) or sucrose, at higher substrate concentrations. This change in substrate specificity appears to be unique to Z. mobilis levansucrase because the levansucrases from B. subtilis, Rahnella aquatilis, and Lactobacillus reuteri are capable of synthesizing levan in their soluble form and were not reported to form fibril-like structures.

Biological significance. Z. mobilis is an acidic tolerant bacterium and in nature more than 90% of its strains grow in the pH range of 3.8 - 5.0 (33). All known levansucrases are extracellular proteins and most of the Gram-negative-originated levansucrases are secreted by a signal-peptide-independent pathway (34). It is tempting to speculate that the formation of the enzyme fibrils serves the bacterium in its natural niche. The fibril structure decreases the diffusion of the enzyme away from the bacteria cells, which results in the close association of the cell to the high molecular polymer, levan. Indeed, in Z. mobilis cultures, levan is found attached to cells (35).

ACKNOWLEDGMENTS: This study was supported by the Otto Meyerhof Center for Biotechnology, Technion, established by the Minerva Foundation (Munich, Germany). Y.S. holds the Erwin and Rosl Pollak Chair in Biotechnology at the Technion.
REFERENCES

FIGURE LEGENDS

Fig. 1. Gel filtration chromatograms of Z. mobilis levansucrase at different pH values. Samples were applied on a sephacryl S300 26/60 gel filtration column (AKTA basic, Pharmacia) running at 1 mL/min, at room temperature with 50 mM phosphate buffer, 100 mM NaCl, and 0.02% sodium azide. 280 nm (–), 260 nm(— —) A - Chromatogram at pH 7.4. Levansucrase eluted at 180 mL, corresponding to 94,000 Da. B - Chromatogram at pH 6.0. Levansucrase peaks at the column void volume.

Fig. 2. Effect of sucrose concentration on Z. mobilis levansucrase activity. Activity was assayed at 0.15 M citric acid – Na2HPO4 buffer pH 5.0 (A) or 7.2 (B) containing sucrose at the indicated concentrations at 30 °C. ● - initial velocity rate of glucose release. □ - initial velocity rate of fructose release. ○ - initial velocity rate of polymerization. The inset (B) shows the parabolic relation between total enzyme activity and substrate concentration at low sucrose concentrations.

Fig. 3. Separation of saccharides resulting from levansucrase activity at different pH sucrose solutions. HPAEC-PAD: (A) levansucrase reaction products at 0.15 M citric acid – Na2HPO4 buffer pH 5.0. (B) levansucrase reaction products at 0.15 M citric acid – Na2HPO4 buffer pH 7.2. G- glucose, F- fructose, Suc-sucrose. TLC analysis: (C) Lane 1- fructose/glucose standard. Lane 2 – sucrose standard. Lane 3 – levansucrase reaction products at 0.15 M citric acid – Na2HPO4 buffer pH 5.0. Lane 4 –levansucrase reaction products at 0.15 M citric acid – Na2HPO4 buffer pH 7.2

Fig. 4. Negative stain TEM images of a Z. mobilis levansucrase microfibril structure following the lowering of pH. The pH of 20 mM citric acid – Na2HPO4 buffer pH 7.4 containing 0.2 mg/mL enzyme solution was reduced to 6.0 and images were taken at different time frames. (A) 3 minutes, inset – ring view (B) 10 minutes (C) 1 hour (D) 3 hours (E) 20 hours (F) 20 hours (side view). Notice the ring structure of the cross section. (G) demonstration of short filaments co-existing with the net structure.

Fig. 5. The effect of pH step drop from 7.4 to 5.0 and protein concentration on Z. mobilis levansucrase levan synthesis activity. Levansucrase (at different concentrations as indicated) at 20 mM citric acid – Na2HPO4 buffer pH 7.4 was diluted 6-fold to 50 mM citric acid – Na2HPO4 buffer pH 5.0. Samples were then taken at the indicated times and their levan synthesis activity was assayed at 0.15 M Citric acid – Na2HPO4 buffer pH 5.0 containing sucrose (263 mM) and levansucrase at 25 °C. 0.9 mg/mL (– , ●). 0.45 mg/mL (——— – ○).0.22.5 mg/mL (· · · · · · , □).

Fig. 6. Dimer enzyme activity at pH 5.0. Levansucrase (125μg/mL) kept at pH 7.2 was diluted 10-fold to 0.15 M citric acid – Na2HPO4 buffer pH 5.0 containing sucrose at the indicated concentrations. The activity was assayed with the HPAEC system at 30 °C. ● - initial velocity rate of glucose release. □ - initial velocity rate of fructose release. ○ - initial velocity rate of polymerization.

Fig. 7. Effect of sucrose concentration on Z. mobilis levansucrase H296R mutant activity. Activity was assayed at 0.15 M citric acid – Na2HPO4 buffer pH 5.0 (A) or 7.2 (B) containing sucrose at the indicated concentrations at 30 °C. ● - initial velocity rate of glucose release. □ - initial velocity rate of fructose release. ○ - initial velocity rate of polymerization.
<table>
<thead>
<tr>
<th>Activity</th>
<th>Levan synthesis activity *</th>
<th>Total activity</th>
<th>Hydrolysis activity</th>
<th>Transfructosylation activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (mM)</td>
<td>40</td>
<td>32</td>
<td>18</td>
<td>36</td>
</tr>
<tr>
<td>$K_i$ (M)</td>
<td>2.0</td>
<td>2.1</td>
<td>0.8</td>
<td>2.8</td>
</tr>
<tr>
<td>$V_{max}$ ($\mu$mol/min/mg)</td>
<td>505</td>
<td>572</td>
<td>103</td>
<td>470</td>
</tr>
<tr>
<td>$K_{cat}$ (s$^{-1}$)</td>
<td>411</td>
<td>461</td>
<td>83</td>
<td>379</td>
</tr>
<tr>
<td>$K_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$)</td>
<td>10.3</td>
<td>14.4</td>
<td>4.6</td>
<td>10.5</td>
</tr>
</tbody>
</table>

Levan synthesis activity was monitored by following the increase in turbidity at 600 nm that occurred due to the insoluble levan formation (note that the turbidity contributed by the fibril enzyme is negligible, less than 0.002 OD$_{600}$). The amount of glucose reflects the total amount of sucrose utilized during the reaction (total activity). The amount of free fructose is a measure of the hydrolytic activity of the enzyme. Subtracting the amount of fructose from glucose yields the Transfructosylation activity.

* The increase in 1 OD$_{600}$ in a 1-cm path cuvette corresponded to the consumption of 120 $\mu$mol per 1 mL sucrose (see Methods section).
Figure 1
Figure 3

A  pH 5

B  pH 7.2

C

Levan

GF2  GF3

F

GF2  GF3

Suc

nC

Time (min)

Time (min)
Figure 5
Figure 6
Figure 7

A
pH 5.0

B
pH 7.2

Specific activity (U/mg)

Sucrose (mM)

Specific activity (U/mg)

Sucrose (mM)
Two active forms of Zymomonas mobilis levansucrase: an ordered microfibril structure of the enzyme promotes levan polymerization
Dan Goldman, Noa Lavid, Alon Schwartz, Gil Shoham, Dganit Danino and Yuval Shoham

J. Biol. Chem. published online September 22, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M805985200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2008/09/24/M805985200.DC1

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2008/09/22/jbc.M805985200.citation.full.html#ref-list-1