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Insights into polymer versus oligosaccharide synthesis - Mutagenesis and mechanistic studies of a novel levansucrase from *Bacillus megaterium*

Arne HOMANN^{*,‡}, Rebekka BIEDENDIECK^{†,‡}, Sven GÖTZE^{*}, Dieter JAHN[†] and Jürgen SEIBEL^{*1}

^{*}Department of Technical Chemistry, Technical University Braunschweig, Section of Carbohydrate Technology, Hans-Sommer-Str. 10, D-38106 Braunschweig, [†]Department of Microbiology, Technical University Braunschweig, Spielmannstr. 7, D-38106 Braunschweig, [‡]Arne Homann and Rebekka Biedendieck contributed equally to this work

A novel levansucrase was identified in the supernatant of a cell culture of *Bacillus megaterium* DSM319. In order to test for the contribution of specific amino acid residues to levansucrase catalysis the wild-type enzyme along with 16 variants based on sequence alignments and structural information (see below) were heterologously produced in *Escherichia coli*. The purified enzymes were characterised kinetically and the product spectrum of each variant was determined. Comparison of the X-ray structures of the levansucrases from Gram positive *Bacillus subtilis* and Gram negative *Gluconacetobacter diazotrophicus* in conjunction with the corresponding product spectra identified crucial amino acid residues responsible for product specificity and catalysis. Highly conserved regions like the previously described RDP and DXXER motifs were identified as being important. Two crucial structural differences localised at the amino acid residues Arg³⁷⁰ and Asn²⁵² were of high relevance in polymer versus oligosaccharide synthesis.

Key words: levansucrase, *Bacillus megaterium*, fructo-oligosaccharides, site-directed mutagenesis

Abbreviations used: Ls, levansucrase; Ps, polysaccharides; Os, oligosaccharides

¹To whom correspondence should be addressed (email j.seibel@tu-bs.de)

INTRODUCTION

Global regulatory responses allow *Bacilli* to adapt to changes in environmental conditions [1]. These responses often include changes in the transcription rates of large sets of target genes and thus, the control of a wide variety of cellular processes in these Gram positive bacteria. One of these processes is the synthesis of degradative and polymer building enzymes. The secretion of these enzymes enables the cell to build and utilise highly polymeric nutrients like polysaccharides (Ps), nucleic acids, peptides, and lipids. Ps, e.g. inulin and non digestable oligosaccharides (Os) are known to have human health benefit. They are used by the food industry as nutrition supplements [2; 3] and their potential for immune stimulation is currently under investigation [4].

Levansucrases form Ps by transfer of fructosyl units to Os. This class of enzymes (E.C. 2.4.1.10) is common in eukaryotes and prokaryotes, e.g. plants, fungi (*Aspergillus niger* [5], *Saccharomyces cerevisiae* [6]), and bacteria (*Lactobacilli* sp. [7], *Bacillus subtilis* [8]). In these organisms they build up an extracellular energy reserve of fructans. These exopolysaccharides function as stabilizers of cell membranes protecting them against water stress caused by drought or temperature shift [9]. Furthermore, it is postulated that fructans may be important for survival of bacteria, for phytopathogenesis and symbiosis [10].

Fructosyl transferring enzymes are divided into 2 classes based on the products formed. Inulosucrases (E.C.2.4.1.9) mainly form $\beta(2\rightarrow1)$ bonds producing the polysaccharide inulin while the second class, levansucrases (E.C. 2.4.1.10), form levan which is $\beta(2\rightarrow6)$ linked. The Os and Ps are composed of a head group of α -D-glucopyranoside to which the fructosyl units are coupled.

One of the best characterised members of Gram positive *Bacilli*, *B. subtilis*, secretes the Ls, SacB, into its environment if high amounts of sucrose are available [11]. Ls catalyses the hydrolysis of sucrose as well as transglycosylation reactions with a growing Os chains as acceptor. *B. subtilis* Ls synthesises dominantly high molecular levan with a molecular size of up to 3×10^6 Da. In contrast, the Ls from the Gram-negative *Gluconacetobacter diazotrophicus* mainly synthesises short chain Os and low amounts of levan [12]. Until now, there has been no sufficient explanation for the formation of these different transfructosylation products.

In this study, a novel Ls from *B. megaterium* which is within the same taxonomic group as *B. subtilis* [13; 14] was isolated and its product spectra and reaction kinetics were characterised. Based on amino acid sequence alignments and structural comparisons with enzymes of the glycoside hydrolyse family 68 [15], 16 mutants have been designed by site-directed

mutagenesis. A comparison with the X-ray structures of the levansucrases from *B. subtilis* (74 % identity on amino acid level) and *G. diazotrophicus* (26 %) indicated an overall similar protein structure, carrying identical essential amino acid motifs (RDP, DXXER) but also important differences [16-19]. A detailed biochemical investigation of the 16 Ls variants identified common structure-based functional principles and catalytic specificity modifying determinants.

MATERIALS AND METHODS

Identification of the levansucrase, SacB, from *Bacillus megaterium*

B. megaterium was cultivated aerobically at 37 °C in Luria-Bertani (LB) medium [20] supplemented with 0.5 % (w/v) sucrose. Samples collected at indicated time points after cultivation start were separated by centrifugation into cells and cell-free growth medium (2600 x g, 15 min, 4 °C). For SDS-PAGE [20] analysis of the secreted proteins, 2.5 ml of cell-free growth medium were desalted using PD-10 columns (GE Healthcare, Uppsala, Sweden) prior to concentration by lyophilisation. Proteins were separated by 10 % (w/v) SDS-PAGE and stained with Coomassie Brilliant Blue.

Proteins were identified by peptide mass fingerprinting, as well as by using postsource decay fragmentation data recorded with a Bruker Ultraflex matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer (Ultraflex; Bruker Daltonics, Bremen, Germany). The spectra were evaluated with the Biotoools software (Bruker Daltonics) along with the MASCOT search engine (version 1.9; Matrix Science, London, United Kingdom). The criteria used to accept protein identifications included the extent of sequence coverage (minimum, 30%), the number of peptides matched (minimum number, 5), and the probability score (minimum Mowse score, 70).

Construction of the levansucrase variants by site-directed mutagenesis

Molecular biology methods were outlined previously by Sambrook *et al.* [20]. The 1371 bp coding sequence of *B. megaterium sacB* lacking the signal peptide coding sequence at the 5' end of the gene was amplified by PCR using primers pRBec1_for (5'-TTATTCCATGGCTAAAGGAAACGACAGCAAAG-3'; *Nco* I-site italicised) and pRBec1_rev (5'-TATCAGGATCCGCTATTGCAAAGCGCTCAGTC-3'; *Bam*H I-site itali-

cised). The PCR-product was digested with *Nco* I and *Bam*H I and ligated into the previously *Nco* I/*Bam*H I cut pET11d (Novagen®, San Diego, CA, USA) yielding pRBec1.

The *sacB* gene was mutated using the QuikChange® site-directed mutagenesis kit (Stratagene). The forward primers used to generate the mutations were as follows (the exchanged amino acids are lower case):

W94A, 5'- GACTTAGATGTAgctGACAGCTGGCC -3';
 D95A, 5'- GACTTAGATGTATGGGctAGCTGGCCATTAC -3';
 V115A, 5'- CCTGCTAGGGCAAAagcAATTTGATATCC -3';
 L118A, 5'- CAAATTGTGTTTGCCgctGCAGGTGATCCAAAAG -3';
 W172A, 5'- CAAACACAAGAAgctTCTGGTTCTGGC -3';
 S173A, 5'- CAAACACAAGAATGGgCTGGTTCTGGCACG -3';
 N252A, 5'- GACACAGGGGATgctCATACGCTAAGAG -3';
 N252D, 5'- GACACAGGGGATgACCATACGCTAAGAG -3';
 N252G, 5'- GACACAGGGGATggtCATACGCTAAGAG -3';
 N252H, 5'- GACACAGGGGATcACCATACGCTAAGAG -3';
 R256A, 5'- GGATAACCATACgctAGCTGATCCTCACTAC -3';
 D257A, 5'- CCATACGCTAAGAGcTCCTCACTACATAG -3';
 E350A, 5'- CACAGTTACAGACGctATTGAACGTGC -3';
 E352A, 5'- GTTACAGACGAAATTGctCGTGCAAACATTTTC -3';
 R370A, 5'- GTTCACCGATTCTgctGGATCTAAAATGACG -3';
 Y421A, 5'- GATAAAACATTTACGgcTTCTCACTTTGCTG -3'

All introduced mutations and the integrity of the residual *sacB* gene were confirmed by DNA sequencing.

Levansucrase production and purification

For cloning and cultivation, *E. coli* cells carrying the corresponding plasmid were grown in Luria–Bertani (LB) medium [20] supplemented with 0.5 mM ampicillin at 37 °C in shaking flasks. Recombinant gene expression was induced with 0.5 mM IPTG after 3 hours and the temperature was lowered to 26 °C for 20 h. For cell disruption, the cell culture was centrifuged at 4 °C for 20 min at 2600 x g and the supernatant discarded. The cells were washed and subsequently dissolved in 50 mM Sorensen's phosphate buffer (pH 6) and disrupted by sonication (sonoplus, Bandelin electronics with sonotrode MS72) for 4 min at 70 % power and 50 % time. After centrifugation at 4 °C and 5200 x g for 20 min, the supernatant contained the recombinantly produced Ls. The enzyme was purified by FPLC

using a 15 ml CM-Sepharose column with a controlled pump mix system (Liquid Chromatography Controller LCC-500 Plus, LKB Pump P-500, Pharmacia) and a UV-detector (Biotech UV-MII, Pharmacia). Loading was done with the crude cell extract in 50 mM Sorensen's phosphate buffer (pH 6.6) with a flow rate of 0.5 ml min⁻¹. For elution, a linear gradient from 50 mM to 1 M of Sorensen's phosphate buffer (pH 6.6) was used. Fractions of 2 ml were collected (autosampler LKB Frac-100, Pharmacia) and analysed by SDS-PAGE (Figure 1). The protein concentrations of the fractions were measured photometrically at $\lambda = 280$ nm (NanoDrop Spectrophotometer ND-100, peqLab Biotechnology).

Characterisation of the wild-type levansucrase

The optimal enzyme concentration was determined in 50 mM Sorensen's phosphate buffer (pH 6.6) at 37 °C and 500 mM sucrose. The tested enzyme concentrations were 73.60, 29.44, 14.72, 7.36, and 4.91 mg l⁻¹ purified Ls, respectively. Reactions were stopped after 1 h by heating (100 °C, 10 min). The release of fructose and glucose from sucrose was analysed by TLC. The optimal substrate concentration for the transfructosylation was determined in 50 mM Sorensen's phosphate buffer (pH 6.6) at 37 °C using 7.36 mg l⁻¹ levansucrase. Sucrose concentrations tested were 1 M, 750, 500, and 250 mM, respectively. Reactions were stopped after 1 h by heating (100 °C, 10 min) and analysed as described above. The pH optimum was determined in 50 mM Sorensen's phosphate buffer at 37 °C, 500 mM sucrose using 7.36 mg l⁻¹ enzyme concentration. The reactions at pH-values of 5, 5.6, 6, 6.6, 7, 7.6, and 8, respectively, were stopped by heating (100 °C, 10 min). Carbohydrate content was analysed by High Performance Anion Exchange Chromatography, (HPAEC, pre column: CarboPac PA1, 4 x 50 mm, main column: CarboPac PA1, 4 x 250 mm Dionex, conductivity detector PAD-2, Dionex at 1 ml min⁻¹). The optimal reaction temperature was determined in 50 mM Sorensen's buffer (pH 6.6) at various temperatures (25, 30, 37, 40, and 50 °C). The optimal reaction conditions for the wild-type and the variants were at an enzyme concentration of 7.36 mg l⁻¹, a substrate concentration of 500 mM sucrose, and a reaction time of 60 min. The release of glucose was measured by HPAEC.

Determination of the kinetic parameters

The kinetic parameters of the wild-type and the variants were determined in 50 mM Sorensen's phosphate buffer (pH 6.6) at 37 °C. Enzyme concentration was 7.36 mg l⁻¹ and the reaction time was 60 min. The substrate concentration varied from 500 to 1 mM sucrose. The release of glucose was measured by HPAEC.

Determination of the product spectrum

The product spectrum was determined for wild-type Ls and each variant using 50 mM Sorenson's phosphate buffer (pH 6.6) and 500 mM sucrose at 37 °C. The enzyme concentration was 100 mg l⁻¹. Samples were taken at indicated times for a period of 9 d. The reaction products were identified using standards (1-kestose, 6-kestose, nystose, all 10 mg l⁻¹) by HPAEC. All unidentified Os were separated by silica gel column chromatography (3.5 x 500 mm, flow rate 500 µl min⁻¹). The structures were analysed by NMR and mass spectroscopy.

Characterisation of the polysaccharide

The formation kinetics of Ps were examined in 50 mM Sorenson's phosphate buffer (pH 6.6) at 37 °C, 500 mM sucrose and 7.36 mg l⁻¹ enzyme concentration. Samples were taken after 30 sec, 30 min, 60 min, 24 h, and 72 h, respectively, and analysed by TLC. The production of Ps was performed in Sorensen's phosphate buffer (50 mM, pH 6.6) at 37 °C with 100 mg l⁻¹ Ls. After 10 min the polymer was precipitated with 1 volume of ethanol (99 % (v/v)). After centrifugation (14,000 x g, 5 min, 4 °C) the supernatant was discarded and the precipitate dried by lyophilisation. Size distribution of formed Ps was characterised by Multi Angle Laser Light Diffraction (MALLD, Dawn DSP, Wyatt Technologies) and RI analysis (RI-101, Shodex).

For the identification of the linkage type, the Ps were produced as described above and dialysed against water at 4 °C over night (Slide-A-Lyzer Dialysis cassettes, Pierce, MWCO 30 kDa). The sample was centrifuged for 5 min at 14,000 x g and the supernatant discarded. The precipitate was washed 4 times with 1 ml of ethanol in water (66 % (v/v)). The Ps were dried by lyophilisation and dissolved in 900 mM NaOD for NMR spectroscopy.

NMR spectroscopy

All samples for NMR analysis were freeze-dried from MilliQ water and dissolved in D₂O (or 900 mM NaOD). Spectra were recorded on a Bruker DRX600 or Bruker DRX400 operating at 600 and 400 MHz for ¹H, respectively. One-dimensional ¹H and ¹³C spectra and phase-sensitive two-dimensional spectra [double-quantum-filtered COSY, NOESY (Nuclear overhauser and exchange spectroscopy), TOCSY (Total correlated spectroscopy), ¹H-¹³C HSQC (Heteronuclear single quantum coherence) and HMBC (Heteronuclear multiple bond

correlation)] were recorded using standard pulse programs at 38.8 °C, and data were analysed using the software packages XWINNMR (Bruker, Germany). Chemical shifts are given in the δ -scale (ppm) and coupling constants J in Hz.

RESULTS AND DISCUSSION

Characterisation of the wild-type levansucrase SacB from *Bacillus megaterium*

When *B. megaterium* was cultivated in LB-medium supplemented with and without 0.5 % (w/v) sucrose, SDS-PAGE comparison of the secretome from both cultivations revealed a dominant protein solely produced in the presence of sucrose (Figure 1). This protein with a relative molecular mass of 52,000 was identified as described in materials and methods (Figure 2). The analysed peptide fragments were homologous to fragments predicted for the Ls SacB, a 473 amino acid residue exoenzyme of *B. subtilis*. BLAST searches of the *B. subtilis* SacB against the translated genome data of *B. megaterium* led to the identification of the SacB protein sequence and the genomic position of the corresponding *sacB* gene [21; 22]. *B. megaterium* SacB consists of 484 amino acids including the 29 amino acids of an *N*-terminal signal peptide. It shows 74 % identity at the amino acid sequence level to SacB from *B. subtilis*. Hence, the *B. megaterium* protein was also named SacB. Nine of the 11 additional amino acids found in *B. megaterium* SacB compared to SacB of *B. subtilis* are adjacent to one another at location 82–90 (considering the numbering of *B. megaterium* SacB). Like *sacB* of *B. subtilis*, *sacB* of *B. megaterium* is the first gene of an operon. It is followed by a gene consisting of 1,563 bp. The deduced protein sequence shows 66 % identity to the levanase LevB from *B. subtilis*. *B. subtilis* *levB* is also the second gene in an operon [22]. The third and last gene in the operon in *B. subtilis* encodes a 520 amino acid protein which might function as a permease [22]. Interestingly, the protein encoded by a gene sequence following the levanase gene *sacB* in *B. megaterium* was annotated as bacterioferritin (Hundertmark, Hiller, Münch, Jahn, 2005, unpublished data). The *sacB* of *B. megaterium* was amplified lacking the coding sequence of the *N*-terminal signal peptide and cloned into an *E. coli* expression vector. After production and purification of the recombinant Ls (Figure 3) the optimal reaction conditions of *B. megaterium* SacB were examined as described in materials and methods. A broad pH optimum between pH 6.0 and 7.0 for the Ls activity was observed. The enzyme activity significantly dropped down at pH values below 5.6 and above 7.6. The temperature optimum of the Ls was found at 45 °C. In standard reactions, long-term temperature treatment revealed a high protein stability at 37 °C for at least 24 h. The optimal reaction conditions for

fructosyl transfer were determined with the purified Ls by measuring the initial velocity of the reaction at 37 °C, pH 6.6, 7.36 mg l⁻¹ purified enzyme and 500 mM sucrose.

Kinetic studies and characterisation of the product spectrum of the wild-type

levansucrase

The kinetic parameters were determined as described in materials and methods (Table 1). The K_m-value 6.6 mM for hydrolysis measured for the wild-type Ls from *B. megaterium* is low compared to other levansucrases, e.g. SacB from *B. subtilis* with 13.5 to 40 mM [23]. This indicates a high affinity to the substrate sucrose supported by a very high k_{cat} (2272 s⁻¹) and a high catalytic efficiency (346 mM⁻¹ s⁻¹, Table 1). The reaction conditions used for the determination of the product spectrum favour hydrolysis (58.4 %) followed by synthesis of polyfructan (22.1 %). Further analysis showed that polyfructan produced by the Ls from *B. megaterium* had a molecular weight (M_w) of 2,711 kDa and consists mainly of β(2→6) linkages, while the polyfructan produced by the Ls from *B. subtilis* had a M_w of 6,295 kDa and was described as β(2→6) linked levan with some β(2→1) branches [8]. Beside the polyfructan formation, the wild-type Ls of *B. megaterium* also synthesised 5 different detectable Os. Three products were identified by HPAEC as 1-kestose (isokestose), 6-kestose, and nystose which are known acceptors for the transfer of fructosyl units [7] (Figure 4).

The two unknown by-products were isolated and analysed by 1- and 2D-NMR and mass spectroscopy (Figure 5, Tables 2 and 3). The 1-D proton spectrum of the first compound showed two anomeric protons (4.6-5.2 ppm), which correspond to an anomeric mixture of two non reducing sugar residues. The 1-H signal the β-configured glucoside-residue resonated at higher field (4.59 ppm) with a large coupling constant (d, 7.96Hz), where the 1-H signal (5.16 ppm) of the α-configured glucose appeared as minor signal of a reducing sugar in a ratio of α:β 1:2. Based on the results of the 2-D COSY spectrum, correlations were observed between the H-1s of the glucose residues and the corresponding H-2s (alpha 3.8-4.1 ppm; beta 3.20-3.16 ppm), which was coupled to H-3s (alpha 3.63-3.68 ppm; beta 3.42-3.40 ppm). The assignments for the anomeric carbon signals (alpha 94.94 ppm; beta 98.79 ppm) and C-2s (alpha 74.27 ppm, beta 76.89 ppm) were achieved by 2-D HSQC spectrum analysis. In addition, ¹H ¹³C HMBC long range ²J and ³J correlations were observed for the H-6 signal of glucose with the ¹³C signals at δ_C 106.52 ppm (alpha, C-2') and 106.55 ppm (beta, C-2') of the fructofuranoside, respectively, which shows correlations for the fructofuranoside H-3' positions (beta 79.78 ppm; alpha 79.68 ppm/4.58 ppm) and H-4' positions (beta 77.45 ppm;

alpha 77.31 ppm/4.07 ppm) (Figure 4). All other carbon and proton signals were assigned from a combination of 2-D COSY, TOCSY, HMBC, and HSQC experiments. All experiments provide strong evidence of an 1:2 α : β mixture of β 6Fru- α , β -Glc (6-*O*- β -D-fructofuranosyl- α , β -D-glucopyranoside, blastose).

The ESI MS of the second compound showed the molecule peak at $m/z = 527$ [M^+Na^+]. This trisaccharide is a non reducing sugar. In the 1-D proton spectrum the anomeric proton resonated at δ_H 5.35 ppm with a small J_{12} value of 3.90 Hz. This proton showed correlation in the 2D-HMBC spectrum with quaternary carbon C-2' (106.45 ppm) of a fructofuranoside residue. In the 2-D HMBC spectrum protons corresponding of the H-6 glucose residue showed correlations to the quaternary carbons C-2'' of a second fructofuranoside residue. Taken together, all signals assigned from the one dimensional and correlation spectroscopy (i.e. COSY, TOCSY, HMBC, DEPT and HSQC), the trisaccharide was identified as β 2,6Fru- α Glc- β 1,2Fru (2,6-*O*- β -D-fructofuranosyl- α -D-glucopyranosyl-1,2-*O*- β -D-fructofuranoside, neokestose).

Functional role of the exchanged amino acids and insights into the reaction mechanism of the levansucrase from *Bacillus megaterium*

Levansucrases belong to glycoside hydrolase family 68 (GH 68) according to CAZy [15] which bind the substrate sucrose in the -1 (fructosyl residue) and +1 site (glucosyl residue). In this family, two X-ray structures of the *B. subtilis* and *G. diazotrophicus* enzymes have been elucidated and both show a five-fold β -propeller topology [18; 19].

Amino acid sequence alignments of various Ls enzymes (Figure 6) in combination with structural data of family members pointed towards functional relevant residues. Subsequent site directed mutagenesis of Trp⁹⁴, Asp⁹⁵, Val¹¹⁵, Leu¹¹⁸, Trp¹⁷², Ser¹⁷³, Asn²⁵², Arg²⁵⁶, Asp²⁵⁷, Glu³⁵⁰, Glu³⁵², Arg³⁷⁰, Tyr⁴²¹ was performed in order to explore the structural determinants which are responsible in *B. megaterium* SacB for the transfructosylation activity.

The residues Asp⁹⁵, Glu³⁵² and Asp²⁵⁷ are proposed to form the catalytic triad of the Ls from *B. megaterium* (Figure 7). The substitution of Asp⁹⁵ with alanine inhibited the enzyme activity (Table 4). Sequence alignments with other levansucrases from various bacteria showed that this amino acid residue acts as a nucleophile. It usually attacks the glucopyranosyl residue of the sucrose and forms an enzyme-fructosyl-intermediate with inversion of the glycosidic bond in the intermediate state. The corresponding acid/base catalyst was Glu³⁵². Consequently, an exchange into alanine led to a high decrease in enzyme activity. The variant D257A showed

almost no activity. The residue Asp²⁵⁷ might have been coordinated by hydrogen bonds to the fructofuranoside in positions 3-OH and 4-OH.

Trp⁹⁴, Arg²⁵⁶, Glu³⁵⁰, and Tyr⁴²¹ were selected as their corresponding amino acid residues of the Ls from *B. subtilis* [18; 24] form hydrogen bonds to sucrose and direct it in a productive orientation. Here, the substitution with alanine of each of these amino acid residues in the Ls from *B. megaterium* showed different effects. The variants R256A and E350A were nearly inactive. This agrees with the structural information obtained for the Ls from *B. subtilis* where the corresponding Arg²⁴⁶ formed hydrogen bonds to the 3-OH of the fructofuranoside and also to 4-OH of the glucopyranoside while Glu³⁴⁰ coordinated the 3-OH and 4-OH of the glucopyranoside. The Ls variant W94A still carried 9 % of the catalytic efficiency k_{cat}/K_m while variant Y421A showed 3 % of the wild-type activity. Considering the X-ray structure of the Ls from *B. subtilis*, this Trp formed a hydrogen bond with 6-OH of the fructofuranoside and Tyr of the 2-OH of the glucopyranoside [18]. Due to the low reaction rate hydrolysis was predominantly observed (Tables 6 and 7). The substituted amino acid residues Trp¹⁷² and Ser¹⁷³ were proposed to be part of interactions with aa of the active site. In the Ls of *B. subtilis*, this Trp is situated in the +1 region and functioned to form a pocket for the glucopyranoside of the C-6-OH. Our experiments with Ls variant W172A showed 72-fold increase of the K_m and thus confirmed this function. It was very interesting to notice that substitution of Ser¹⁷³ with alanine effected the catalytic efficiency dramatically, while K_m was comparable to the wild-type Ls. Thus, it is proposed that Ser¹⁷³ does not influence the binding mode of sucrose, but it must somehow participate in subsequent catalysis.

Poly- versus oligosaccharide synthesis

The substitution of Arg³⁷⁰ with alanine of *B. megaterium* SacB resulted in fundamental changes in product formation. The corresponding residue in the Ls from *B. subtilis* in subsite -1 (Arg³⁶⁰), was previously described as a key residue for the formation of 1-kestose [25]. We observed the time dependent accumulation of different Os during Ls variant R370A catalysis. After 60 min reaction time an accumulation of neokestose (2,6- β -Fru- α Glc-1,2- β -Fru, 32.7 mM) was determined while after 19 h blastose (2,6- β -Fru- α , β Glc) was the main reaction product (69.7 mM). A 4-fold K_m -value decrease compared to the wild-type enzyme supported the interactions of Arg³⁷⁰ with the 2-OH and 3-OH of the glucosyl residue in the active site. It is possible that the substitution to Ala favoured an unspecific substrate binding and enabled a fructosylation at C-6 of the glucosyl residue of sucrose yielding neokestose. When the trisaccharide neokestose was accumulating and entering the active site of the *B. megaterium*

Ls variant R370A, the $\beta(2\rightarrow1)$ linkage is cleaved leading to the hydrolysis product blastose. Blastose was supposed to be no donor substrate of the enzyme indicated by its strong accumulation due to an unfavourable $\beta(2\rightarrow6)$ linkage between glucose and fructose.

Residue Asn²⁵² obviously plays an important role for transfructosylation. A substitution to Ala or Gly completely stopped the Ps production at all, while the K_m and k_{cat} -values were not effected (Table 5). Catalysis of this variant was switched from mainly Ps synthesis to hydrolysis (Tables 6 and 7). In contrast mutation of Asn²⁵² to Asp²⁵² effected just downregulation of Ps synthesis (Figure 8), also without changing K_m and k_{cat} values significantly. The X-ray structure of the Ls from *B. subtilis* (74 % identity on amino acid level) enabled insights into the function of this residue. Due to its position in the +2 site, it might stabilise the third fructosyl unit of the growing Os chain and direct it as an acceptor substrate in the optimal position for further transfructosylation. It is located in a channel where the Os chain is able to enter the active site for the transfructosylation reaction. Indeed, based on the Ls structure of *B. subtilis*, it seems likely that no other possibilities are existing for the Os chain to access the catalytic site (Figure 9).

The mutation of Asn²⁵² to Asp²⁵² obviously reduced the coordination of a fructosyl unit in the +2 site. However, it was still possible as indicated by the formation of Ps (Figure 8). The mutations to alanine, glycine and histidine led to a complete knock-out of Ps formation. Very recently, Ozimek *et al.* [26] proposed a model of the sugar-binding subsites in two *Lb. reuteri* 121 fructosyltransferases. For the fructan polymer synthesis the subsites +2, and +3 need a high affinity for binding the growing fructan polymer chain. No structural determinants have been reported so far.

Residues Arg³⁷⁰ and Asn²⁵² which seem to be crucial for the polyfructan synthesis are found to be conserved in Ls from Gram positive bacteria. In contrast, the endophytic Gram negative bacterium *G. diazotrophicus* SRT4 secretes a constitutively expressed Ls (LsdA, EC 2.4.1.10), which mainly converts sucrose into fructo-oligosaccharides. It contains a His⁴¹⁹ instead of Arg³⁷⁰ at the equivalent position. This His residue is found strictly conserved in Gram negative levansucrases. Further, Asn²⁵² is strictly conserved in Gram positive bacteria while in Gram negative this region shows variability. The corresponding part of the three-dimensional structure of LsdA was almost perfectly superimposable with the equivalent residues of the *B. subtilis* SacB in the active site. In contrast the region +2 was not perfectly superimposable (Figure 9).

In conclusion the present study reports the full characterisation of a novel Ls, SacB, from *B. megaterium* and its product spectra. Site-directed mutagenesis clarified the functional roles of

different amino acids which are involved in the formation of different reaction products. There are only few studies existing which concern the Os vs. Ps formation of levansucrases describing a processive or non-processive mechanism [24-26]. So far, no structural insights or amino acids have been identified to give conclusions or clarify differences regarding Ps and Os synthesis among the levansucrases in detail. Here it was shown for the first time that a single amino acid substitution at the +2 subsite switches the enzymatic reaction from Ps to Os formation conserving a high enzyme activity by changing from a processive to a non-processive mechanism. Based on these studies in future more structural details of the +2 and +3 subsites may enable the redesign of this enzyme for the synthesis of tailor-made short-chain fructo-oligosaccharides.

Table 1 Optimal reaction conditions

The optimal reaction conditions for the Ls from *B. megaterium* are shown in comparison to the Ls from *B. subtilis*.

Table 2 NMR data for the identification of blastose

Table 3 NMR data for the identification of neokestose

Table 4 Kinetic parameters of the wild-type levansucrase and its variants.

The reaction was performed with 7.36 mg l⁻¹ enzyme in Sorensen's phosphate buffer (pH 6.6, 50 mM) with 500 mM sucrose. For the variants D95A, R256A, D257A, E350A, and E352A the determination of kinetic parameters was not possible due to missing or too slow release of glucose under these reaction conditions.

Table 5 Kinetic parameters of the wild-type levansucrase and its variants.

The reaction was performed with 7.36 mg l⁻¹ enzyme in Sorensen's phosphate buffer (pH 6.6, 50 mM) with 500 mM sucrose.

Table 6 Product spectrum after 60 min reaction time

All values are given in mM l⁻¹. The sample used for the identification of neokestose is indicated.

Table 7 Product spectrum after 19 h reaction time

All values are mM l⁻¹. The sample used for the identification of blastose is indicated.

Figure 1 Effect of sucrose on the secretome of *Bacillus megaterium* DSM319

B. megaterium DSM319 was cultivated aerobically in 100 ml of LB medium without (lane 1) or with (lane 2) the addition of 0.5 % (w/v) sucrose at 37°C and 250 rpm. Six h after cultivation start, 1.5 ml of cell-free growth medium were desalted using a PD-10 column (GE Healthcare; Uppsala; Sweden) and subsequently lyophilised. The precipitated proteins were analysed via 10 % SDS-PAGE and stained with Coomassie Brilliant Blue G250. Lane M shows Precision Plus Protein Standard (Bio-Rad, Munich, Germany).

Figure 2 Identification of the levansucrase by MALDI/MS

Mass spectrum of the peptide fragment VMKPLITSNTVTDEIER. The levansucrase was identified as described in materials and methods).

Figure 3 SDS-PAGE analysis of the FPLC

Lane 1 is the crude cell extract, 2, flow-through of the FPLC (as described in material and methods), 3, first FPLC peak fraction, 4, protein marker (New England Biolabs, relative molecular masses are indicated in $\times 10^3$), 5-10, FPLC peak fractions. The apparent pure Ls containing fractions are boxed.

Figure 4 Identification of the transfructosylation products

The carbohydrates up to tetrasaccharides were identified by High-Performance Anion Exchange Chromatography (HPAEC). The dotted line is the Os standard (1-kestose, 6-kestose, nystose, all 0.01 g l^{-1}), the solid line is the wild-type Ls reaction (stopped after 19 h at 37°C and 0.5 M sucrose in sorenson phosphate buffer, 50 mM , pH 6.6, enzyme concentration was 0.1 g l^{-1}), the dashed line is the Os standard (concentration as above) mixed with the same wild-type Ls sample.

Figure 5 Correlation spectrum of α,β blastose (HMBC)

Figure 6 Sequence alignment of related levansucrases

Gd is *Gluconacetobacter diazotrophicus* [19], Ps is *Pseudomonas syringae*[28], Ra is *Rahnella aquatilis* [29], Ea is *Erwinia amylovora* [30], Zm is *Zymomonas mobilis* [31], Ss is *Streptococcus salivarius* [32], Pp is *Paenibacillus polymyxa* [33], Ba is *Bacillus amyloliquefaciens* [34], Sm is *Streptococcus mutans* [35], Bs is *Bacillus subtilis* [18], Bm is *Bacillus megaterium*. The amino acids used for the mutagenesis study are indicated and the resulting variant is labeled above the sequence.

Figure 7 The proposed transfructosylation reaction mechanism

The mechanism is based on SacB from *B. megaterium* with sucrose as substrate.

Figure 8 TLC analysis of polymer- vs. oligosaccharide formation activity

The reaction was performed using wild-type levansucrase SacB from *B. megaterium* and indicated variants carrying amino acid exchanges in position Asn²⁵². The reaction conditions were 50 mM Sorensen's buffer, pH 6.6, and 37°C . The enzyme concentration was 7.36 mg l^{-1} each. Std1 and Std2 are carbohydrate standards containing glucose (Glc), fructose (Fru), sucrose (suc) and maltose (not indicated) top down. The sample times are indicated above in [h]. The detectable polymer is encircled.

Figure 9 Local identification of the crucial amino acid residues

This superimposing shows the levansucrases from *Bacillus subtilis* (Bs, purple) and *Gluconacetobacter diazotrophicus* (Gd, cyan) with sucrose in the active site [18; 19; 27; 28]. The analogue amino acid residues of the *Bacillus megaterium* (Bm) Ls are given in parentheses.

Table 1

	Levansucrase, SacB, from <i>B. subtilis</i> [23]	Levansucrase, SacB, from <i>B. megaterium</i>
K _m	13.5 – 40 mM	6.6 mM
TO number (k _{cat})	0.6 – 60 s ⁻¹	2272 s ⁻¹
pH optimum	pH 6 for hydrolysis	pH 6.6 for hydrolysis
T optimum	37 – 50 °C	45 °C
stability	pH 3.5 to 7.5 at 4 °C, no time indicated	> 6 months at pH 6.6 and -20 °C

Table 2

C-atom	α blastose		β blastose	
	δ_C	δ_H	δ_C	δ_H
C-1	94.94	5.16 (d, 3.70)	98.79	4.59-4.57 (d, 7.96)
C-2	74.27	3.49-3.46 (m)	76.89	3.20-3.16 (t, 8.70)
C-3	75.48	3.63-3.68 (m)	78.45	4.42-3.40 (m)
C-4	72.55	3.34-3.40 (m)	72.49	3.88-3.85 (m)
C-5	73.49	3.85-3.88 (m)	77.79	3.84-3.80 (m)
C-6	63.61	3.96-3.89, 3.67-3.62	63.61	3.96-3.89, 3.67-3.62
C-1'	62.99	3.70-3.62 (m)	62.99	3.70-3.62 (m)
C-2'	106.52	-	106.55	-
C-3'	79.68	4.58-4.56 (d, 8.26)	79.78	4.58-4.56 (d, 8.26)
C-4'	77.31	4.07-4.03 (t, 7.60)	77.45	4.07-4.03 (t, 7.60)
C-5'	83.97	3.82-3.76 (m)	84.02	3.82-3.76 (m)
C-6'	65.21	3.77-3.72 (m)	65.32	3.77-3.72 (m)

Table 3

C-atom	neokestose	
	δ_C	δ_H
C-1	62.89	3.91-3.87, 3.76-3.73
C-2	106.45	-
C-3	79.47	4.16-4.13 (d, 8.5)
C-4	77.03	4.11-4.07 (t, 7.4)
C-5	83.88	3.84-3.80 (m)
C-6	65.17	3.83-3.73
C-1'	94.74	5.35 (d, 3.88)
C-2'	73.74	3.53-3.49 (dd, 3.90, 10.00)
C-3'	75.15	3.69-3.66 (m)
C-4'	74.76	3.92-3.88 (m)
C-5'	71.89	3.48-3.45 (m)
C-6'	62.03	3.58 (s)
C-1''	64.14	3.73-3.69, 3.64-3.61
C-2''	106.47	-
C-3''	78.90	4.18-4.16 (d, 8.49)
C-4''	76.64	4.04-4.00 (t, 8.5)
C-5''	84.08	3.87-3.83 (m)
C-6''	65.08	3.80-3.76, 3.68-3.64

Table 4

Variant	K_m [mM]	v_{max} [mM min ⁻¹]	k_{cat} [s ⁻¹]	k_{cat}/K_m [mM ⁻¹ s ⁻¹]
wild-type	6.6 ± 1.1	19.6 ± 0.6	2272	346
W94A	31.9 ± 11.0	8.6 ± 0.9	1000	31
D95A	-	-	-	-
L118A	66.6 ± 22.1	10.6 ± 1.1	1231	19
W172A	480.4 ± 124.8	20.7 ± 3.2	2396	5
S173A	2.3 ± 1.2	3.1 ± 0.3	363	159
N252A	4.1 ± 1.7	12.8 ± 1.1	1480	361
R256A	-	-	-	-
D257A	-	-	-	-
E350A	-	-	-	-
E352A	-	-	-	-
Y421A	51.9 ± 16.3	2.9 ± 0.3	335	6.5

Table 5

Variant	K_m [mM]	v_{max} [mM min ⁻¹]	k_{cat} [s ⁻¹]	k_{cat}/K_m [mM ⁻¹ s ⁻¹]
wild-type	6.6 ± 1.1	19.6 ± 0.6	2272	346
N252A	4.1 ± 1.7	12.8 ± 1.1	1480	361
N252D	8.4 ± 2.3	32.3 ± 1.6	3743	445
N252G	7.5 ± 0.8	19.5 ± 0.4	2256	301
N252H	35.1 ± 30.7	13.2 ± 3.4	1529	43.5
R370A	29.2 ± 11.6	1.5 ± 0.2	179	6.1

Table 6

	wild-type	L118A	W172A	N252A	N252D	N252G	N252H	R370A	Y421A
glc	428.0	85.1	13.0	447.9	463.4	334.9	118.6	138.7	12.2
fru	292.2	71.6	11.3	391.4	362.0	279.9	102.3	103.0	9.6
suc	41.9	407.1	487.0	17.1	0.0	141.8	381.4	321.3	487.5
1-kestose	15.9	3.4	0.0	5.2	3.5	6.9	0.0	3.4	0.0
α,β-blastose	6.4	0.0	0.0	7.3	17.3	9.7	0.0	3.9	0.0
6-kestose	3.5	0.0	0.0	15.0	4.2	6.8	0.0	0.0	0.0
neokestose	2.8	3.5	0.0	5.8	7.4	6.6	0.0	32.7	0.0
nystose	1.5	0.5	0.0	1.7	4.1	0.0	0.0	0.0	0.0

Table 7

	wild-type	L118A	W172A	N252A	N252D	N252G	N252H	R370A	Y421A
glc	449.8	304.1	101.1	459.9	443.8	448.7	431.1	414.4	97.8
fru	359.8	226.7	89.5	399.4	375.5	360.3	360.3	357.2	83.8
suc	0.0	172.2	389.7	0.0	0.0	0.0	27.4	0.0	389.5
1-kestose	7.6	4.5	2.1	4.0	7.7	7.0	6.0	6.0	4.7
α,β-blastose	24.5	7.4	1.1	14.1	21.5	26.3	16.7	69.7	1.1
6-kestose	4.9	4.1	0.6	11.9	14.8	6.7	4.5	1.2	0.0
neokestose	9.5	5.3	5.4	7.0	9.3	15.0	12.3	13.6	6.9
nystose	3.7	2.5	0.0	3.2	3.5	3.4	2.1	2.1	0.0

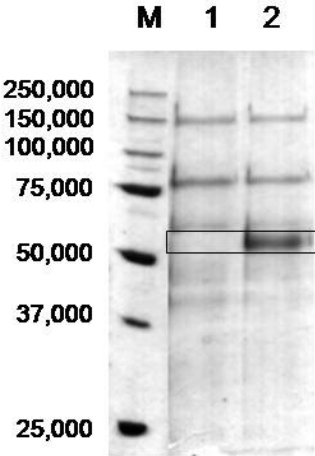


Figure 1

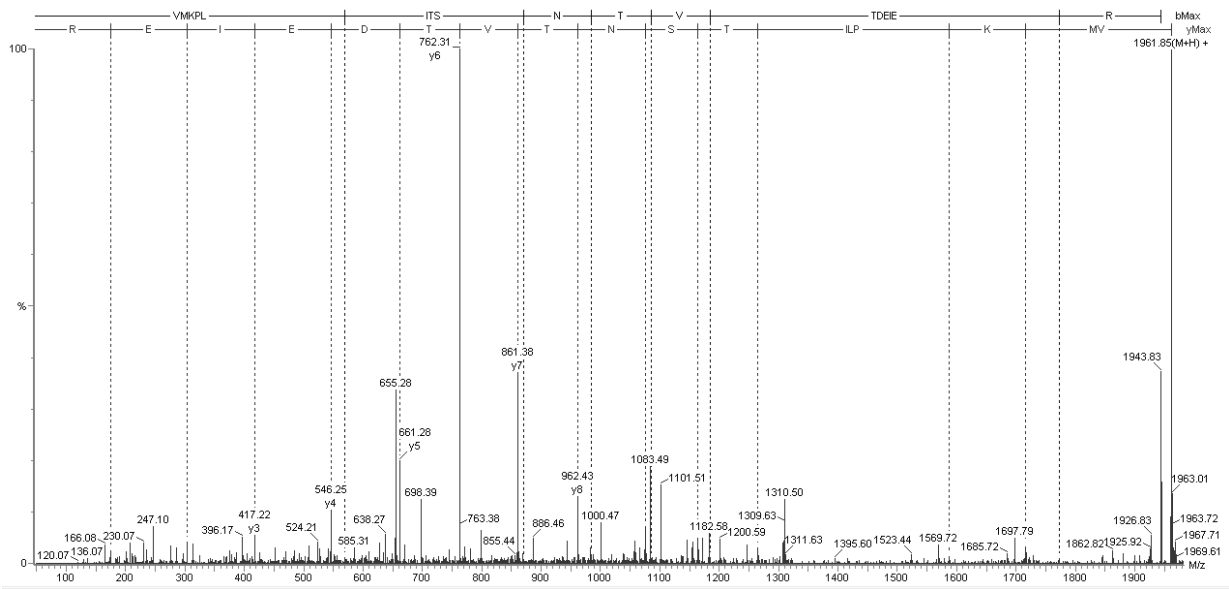


Figure 2

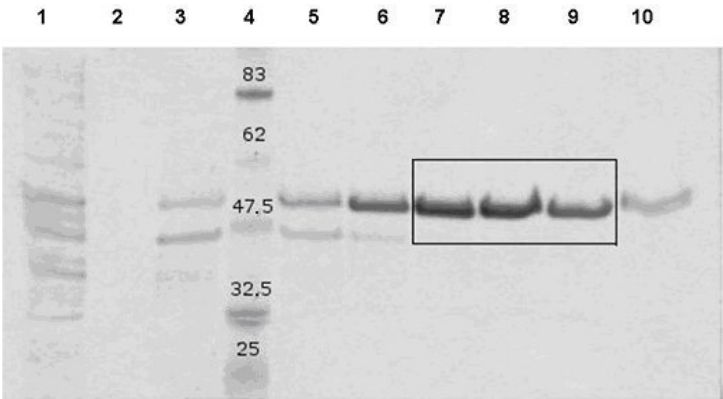


Figure 3

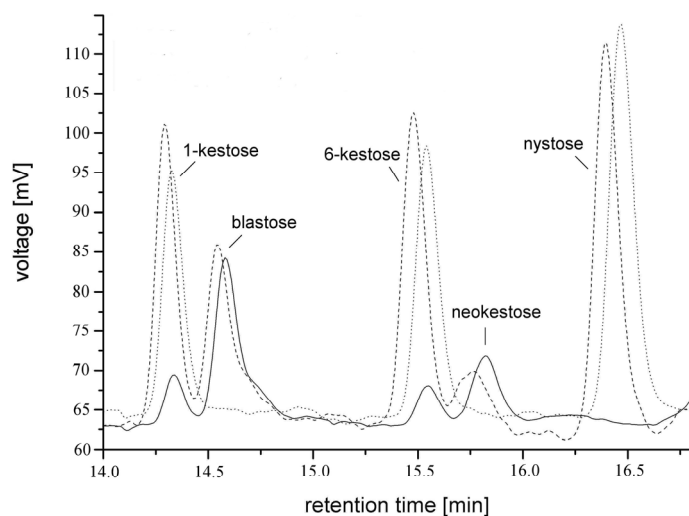


Figure 4

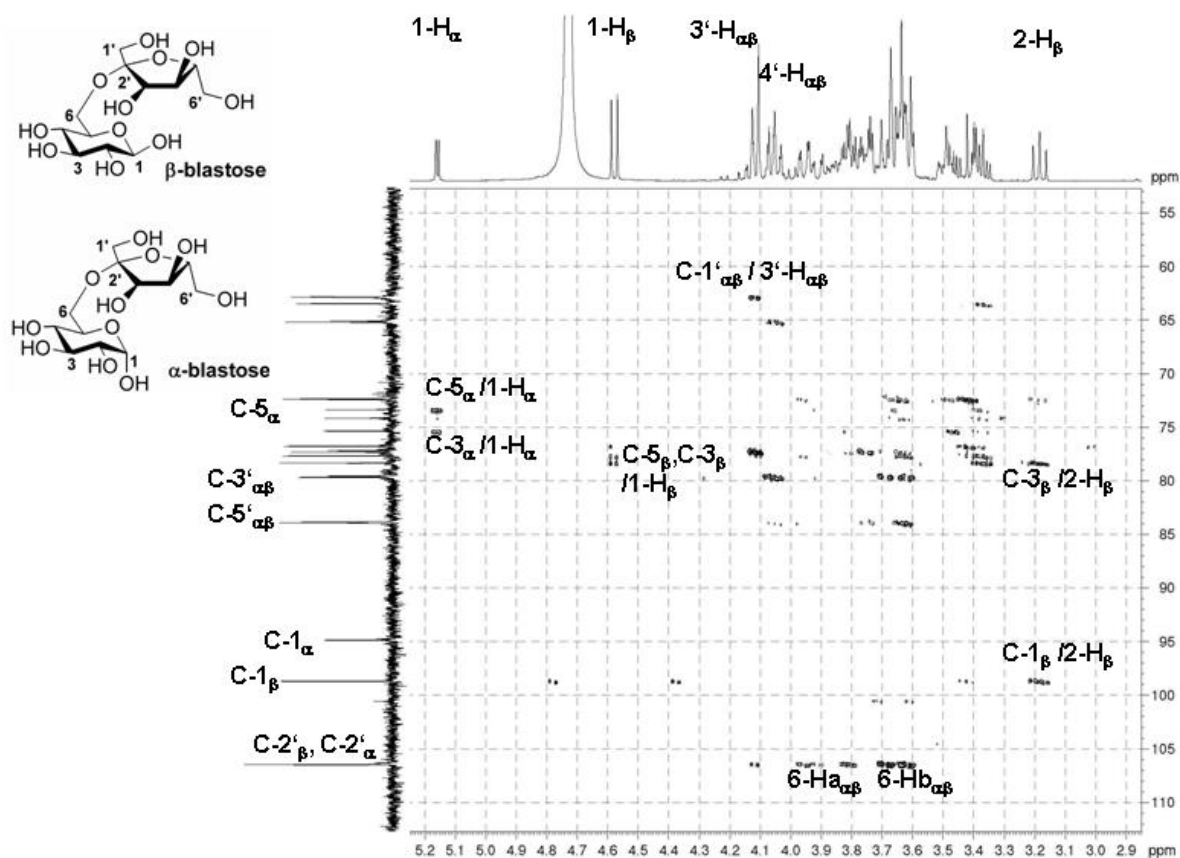


Figure 5

	W94A				W172A				N252A, N252D R256A			
	D95A				S173A				N252G, N252H D257A			
Gd	70	VWVWDTWTLLDKH	82	89	NGWEVIFCLTADP	101	160	QAEWSGSSRLMQ	171	240	QNEF-----FNF	254
Ps	42	VFIWDTMPLRELD	54	61	NGWSVIVTLTADR	73	128	TREWAGTPVLLN	139	194	QNST-----WNFRDPSPFID	210
Ra	42	VFIWDTMPLRSLD	54	61	DGWSVIFTTLTAQR	73	128	SREWAGTPILLN	139	194	QNPY-----WNFRDPSPFID	210
Ea	42	VFIWDTMPLRDFD	54	61	NGWCIIIFTLTADR	73	128	TREWAGTPILLN	139	194	QNAF-----WNFRDPSPFID	210
Zm	40	LWLWDTWPLRDIN	52	59	KGWNVIFSLVADR	71	110	TAEWSGGTIMAP	121	183	ENNL-----WNFRDPHVFIN	199
Ss	284	IDVWDSWPVQDAK	295	303	NGYQLVISMAGAP	315	352	DQQWSGSATVNS	363	424	KFMStf[11]rt	456
Pp	91	FDVWDSWPLQNAD	103	110	KGYNIVFGLAGDP	122	169	SEEWSGSATLTS	180	245	QSVGvdm----dnHTFRDPHYVED	265
Ba	82	LDVWDSWPLQNAD	94	101	NGYHVVFALAGSP	113	160	TQEWSGSATFTS	171	229	QFIDegnyt-sgdnHTLRDPHYVED	253
Sm	243	LDVWDSWPVQDAK	254	262	NGYQLVVAMMGIP	274	311	TQEWSGSATVNE	322	384	QWRStftg---adnIAMRDPHYVED	406
Bs	56	LDVWDSWPLQNAD	68	75	HGYHIVFALAGDP	87	134	TQEWSGSATFTS	145	203	QFIDegnys-sgdnHTLRDPHYVED	227
Bm	91	LDVWDSWPLQNAD	103	110	HGYQIVFALAGDP	122	169	TQEWSGSGTLTK	180	239	QFIDeggyd-tgdnHTLRDPHYIED	263
	E350A				R370A				Y421A			
	E352A				R370A				Y421A			
Gd	333	ANCVNDQTERPQVYLHNGKYYIFTISHRTTFA	363	420	FQSYSHYVMPG	430						
Ps	279	AVGVNDQTERPHYVFQDGKYYLFTISHKFTYA	309	349	FQTYSHCVMPN	359						
Ra	279	AVGVNDQTERPHFVFQDGKYYLFTISHKFTYA	309	349	FQTYSHCVMPN	359						
Ea	279	AVGVNDQTERPHFVFQDGKYYLFTISHKFTYA	309	349	FQTYSHYVMPN	359						
Zm	268	AFGVNDQTERPHVIFQNGLTLYLFTISHDSTYA	298	339	TEAYAHYIMNN	349						
Ss	539	SHMVTDEVERPSVVKMGKYYLFTASRLNKST	569	620	TSTYSYYAVPV	630						
Pp	346	SNLVTDEIERANVFKMNGLWYLFSTRGSKVT	376	420	TWTYAHFAIPQ	430						
Ba	334	SNTVTDEIERANVFKMNGKWLFTDSRGSKMT	364	408	TFTYSHFAVPQ	418						
Sm	491	STMVSDLELRPNVVKLGDKYYLFTASRLNHGS	521	572	TATYSYYAVPV	582						
Bs	304	SNTVTDEIERANVFKMNGKWLFTDSRGSKMT	334	382	TFTYSHFAVPQ	392						
Bm	344	SNTVTDEIERANIFKKDKWLFTDSRGSKMT	374	418	TFTYSHFAVPQ	428						

Figure 6

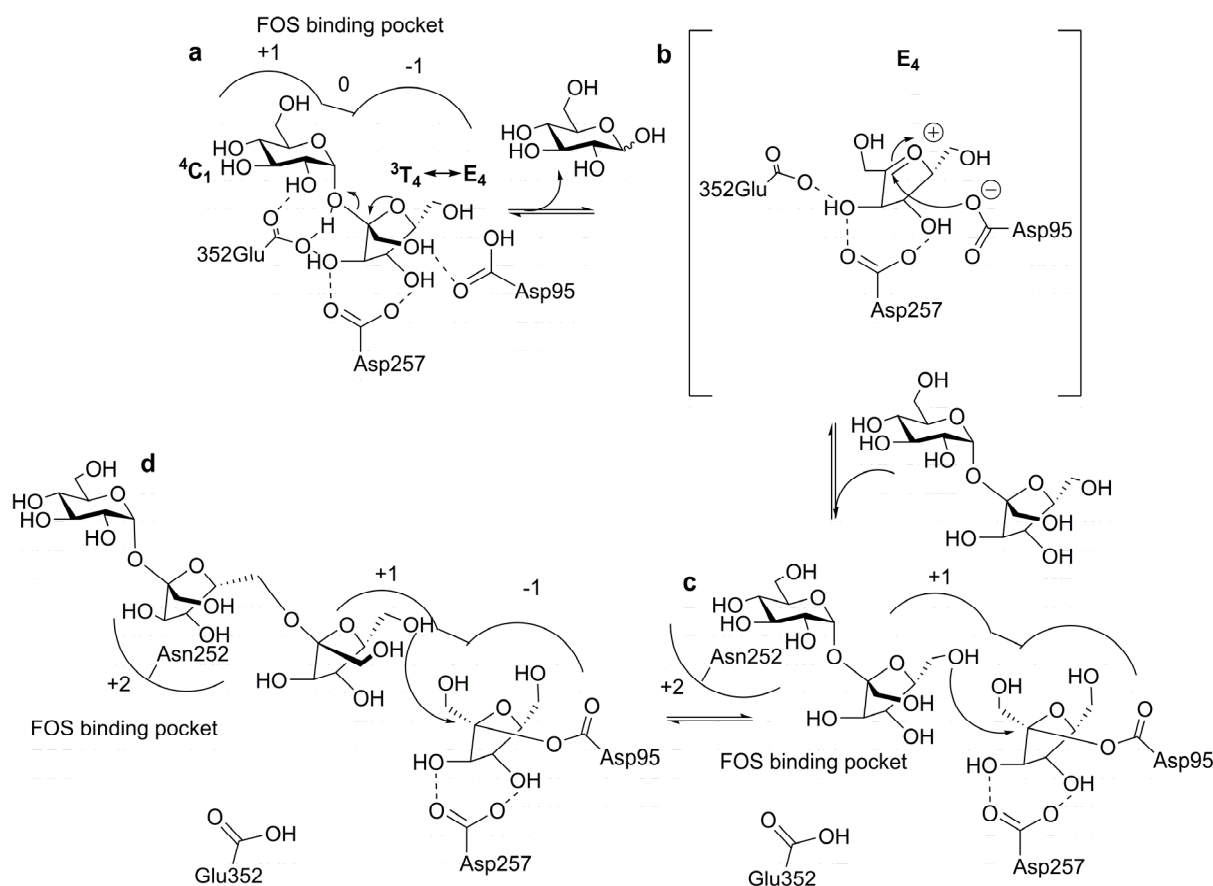


Figure 7

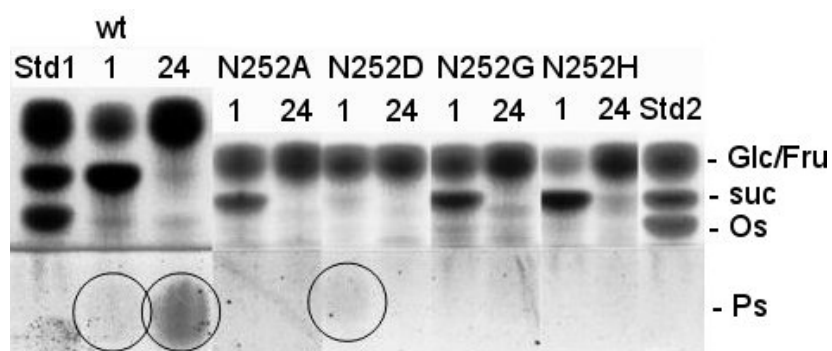


Figure 8

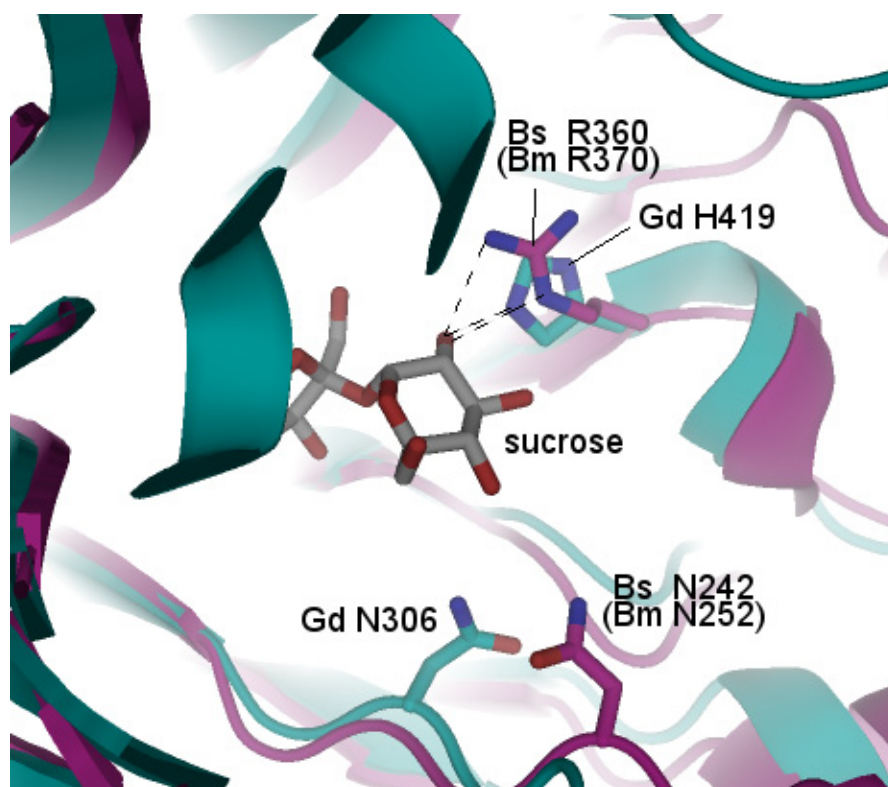


Figure 9

Acknowledgment

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