the FEBS Journal

Single amino acid residue changes in subsite −1 of inulosucrase from *Lactobacillus reuteri* 121 strongly influence the size of products synthesized

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Keywords
inulosucrase; *Lactobacillus reuteri*; processivity; site-directed mutagenesis

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(Received 12 June 2006, accepted 10 July 2006)

doi:10.1111/j.1742-4658.2006.05411.x

Bacterial fructansucrase enzymes belong to glycoside hydrolase family 68 and catalyze transglycosylation reactions with sucrose, resulting in the synthesis of fructooligosaccharides and/or a fructan polymer. Significant differences in fructansucrase enzyme product specificities can be observed, i.e. in the type of polymer (levan or inulin) synthesized, and in the ratio of polymer versus fructooligosaccharide synthesis. The *Lactobacillus reuteri* 121 inulosucrase enzyme produces a diverse range of fructooligosaccharide molecules and a minor amount of inulin polymer [with β(2–1) linkages]. The three-dimensional structure of levansucrase (SacB) of *Bacillus subtilis* revealed eight amino acid residues interacting with sucrose. Sequence alignments showed that six of these eight amino acid residues, including the catalytic triad (D272, E523 and D424, inulosucrase numbering), are completely conserved in glycoside hydrolase family 68. The other three completely conserved residues are located at the −1 subsite (W271, W340 and R423). Our aim was to investigate the roles of these conserved amino acid residues in inulosucrase mutant proteins with regard to activity and product profile. Inulosucrase mutants W340N and R423H were virtually inactive, confirming the essential role of these residues in the inulosucrase active site. Inulosucrase mutants R423K and W271N were less strongly affected in activity, and displayed an altered fructooligosaccharide product pattern from sucrose, synthesizing a much lower amount of oligosaccharide and significantly more polymer. Our data show that the −1 subsite is not only important for substrate recognition and catalysis, but also plays an important role in determining the size of the products synthesized.

Glycoside hydrolases (GHs) are a large group of enzymes able to hydrolyze glycosidic bonds, displaying a great variety of protein folds and substrate specificities (http://afmb.cnrs-mrs.fr/CAZY/) [1]. They are divided into 100 different families based on their amino acid sequences. As the amino acid sequence determines the structure of an enzyme, the catalytic mechanism appears to be conserved among the members of a given sequence-based family [2]. GH family 68 (GH68) comprises bacterial fructansucrases (FSs) that are retaining glycosidases. They catalyze transfructosylation reactions involving the formation and subsequent cleavage of a covalent enzyme–substrate intermediate (Ping Pong type of mechanism) [3–5]. FSs catalyze

Abbreviations
DP, degree of polymerization; FOS, fructooligosaccharide; FS, fructansucrase; Inu, inulosucrase; GH, glycoside hydrolase; HPAEC, high-performance anion-exchange chromatography; Lev, levansucrase; SD, Shine–Dalgarno.

hydrolysis when the acceptor that breaks the covalent fructosyl–enzyme bond is water, and/or transglycosylation reactions when the bond is broken by either the growing fructan (polymerization) or oligosaccharide (oligomerization) chain. Evolutionarily, structurally and mechanistically related families are grouped into 'clans'. Enzymes from families GH32 and 68 comprise the β-fructofuranosidase clan GH-J. Members of clan GH-J probably share three identical catalytic residues and use a similar reaction mechanism [6].

Depending on the types of linkage introduced into their products, FSs are divided into inulosucrases (EC 2.4.1.9), synthesizing β(2–1)-linked inulin, and levansucrases (EC 2.4.1.10), synthesizing β(2–6)-linked levan [7]. Family GH68 contains almost 60 members (as of January 2006), which differ significantly in the types of polymer (levan versus inulin) synthesized, and, where studied, in the size of their transglycosylation products [fructooligosaccharide (FOS) versus polymer], and/or their hydrolysis versus transglycosylation activity.

Family GH32 contains more sequences than GH68 and consists of enzymes with broader substrate specificities and activities, e.g. invertases (EC 3.2.1.26), inulinases (EC 3.2.1.7), levanases (EC 3.2.1.65), exoinulinases (EC 3.2.1.80), sucrose:sucrose 1-fructosyl transferases (EC 2.4.1.99), and fructan:fructan 1-fructosyltransferases (EC 2.4.1.100) (http://afmb.cnrs-mrs.fr/CAZY/).

To date, the high-resolution three-dimensional (3D) structures of the GH68 levansucrases from *Bacillus subtilis* (SacB) (with bound sucrose) and *Gluconacetobacter diazotrophicus* (LsdA) have been determined [8,9]. Also, three 3D structures of family GH32 representatives are now available: invertase from *Thermotoga maritima*, fructan 1-exohydrolase from *Cichorum intybus* and exoinulinase from *Aspergillus awamori* [6,10,11]. All five enzymes possess a five-bladed β-propeller fold with a deep, negatively charged, central cavity. Their active sites are positioned at the end of this cavity with a funnel-like opening towards the molecular surface.

Inulosucrase enzymes have been characterized from three bacterial species: *Streptococcus mutans* JC2 [12,13], *Leuconostoc citreum* CW28 [14] and *Lactobacillus reuteri* 121 [15]. Where studied, all other members of family GH68 synthesize levan [7]. *Bacillus subtilis* levansucrase catalyzes the formation of high molecular mass levan without accumulation of FOS, whereas levansucrase of *G. diazotrophicus* synthesizes inulin-type FOS and low amounts of levan [4]. Comparison of the 3D structures of these two levansucrases showed that the structural determinants for synthesis of these different transglycosylation products cannot be identified [9], and no 3D structure is available for an inulosucrase protein at present.

Previously, we have isolated and characterized two FSs from *Lactobacillus reuteri* 121. Levansucrase (Lev) synthesizes a high molecular mass levan [16], and inulosucrase (Inu) synthesizes inulin-type FOS and a small quantity of high molecular mass inulin [17,18]. These two FS enzymes are very similar at the amino acid level (86% similarity and 56% identity, within 768 amino acids), and both depend on Ca^{2+} ions for activity (but to different extents) and display high temperature optima [19]. Using site-directed mutagenesis, we have also identified the catalytic triads in both enzymes [20].

Based on the available 3D structures [8,9] and on alignment of amino acid sequences of family GH68 members (Figs 1 and 2), we have identified three additional fully conserved active site amino acid residues (W271, W340 and R423) as further targets for site-directed mutagenesis in the *Lactobacillus reuteri* 121 Inu enzyme. We propose that the positions of the three conserved residues are identical in the active sites of levansucrase (*B. subtilis* levansucrase PDB: 1PT2) and *Lactobacillus reuteri* Inu at the −1 subsite, interacting with the fructosyl unit of a bound sucrose (Fig. 1; R423 also interacts with the glucose moiety of sucrose at subsite + 1).

In GH68 *Lactobacillus reuteri* Inu, W271 precedes the nucleophile (D272 in Inu and D86 in Bs SacB) and is fully conserved (Fig. 2), whereas in the three

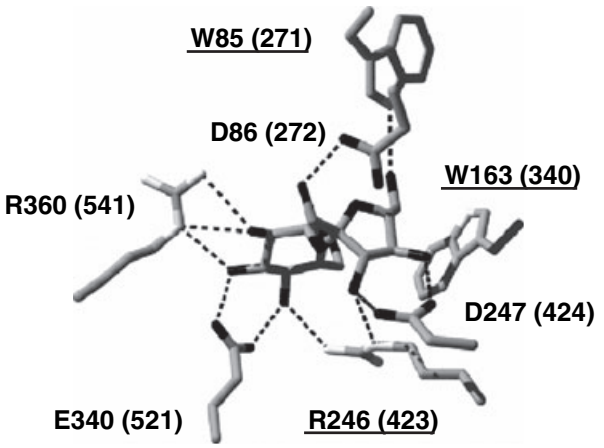

Fig. 1. Close-up view of the active site of mutant E342A of *Bacillus subtilis* SacB levansucrase with a bound sucrose molecule (accession code: 1PT2). The figure was created using the SWISSPDB VIEWER (http://www.expasy.org/spdbv/) [29]. Hydrogen bonds are shown by dashed lines, based on [8]. Numbering of amino acid residues is based on *B. subtilis* SacB, with the numbering of *Lactobacillus reuteri* inulosucrase (Inu) in parentheses. Residues that have been substituted in this study have been underlined.

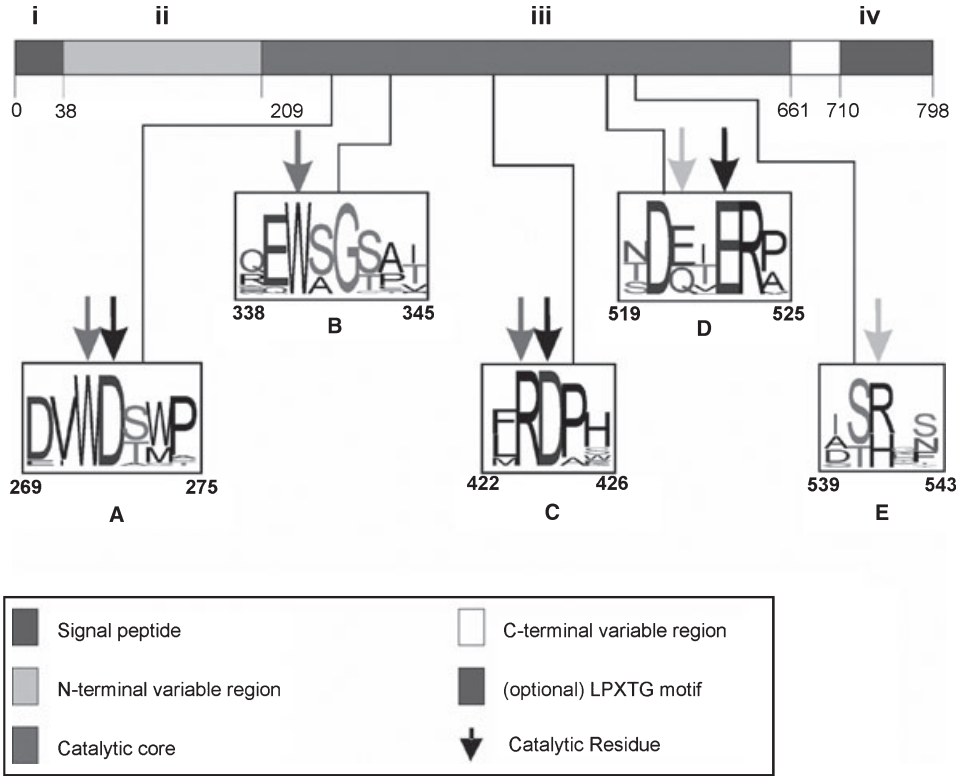

Fig. 2. Schematic representation of family GH68 proteins. The *Lactobacillus reuteri* inulosucrase (Inu) deduced amino acid sequence was used as template (AF459437). The four different regions shown are: (i) N-terminal signal sequence; (ii) N-terminal variable region; (iii) catalytic core; and (iv) C-terminal variable region (which in some cases contains an LPXTG cell wall anchor). Alignments (SEQUENCELOGO, http://weblogo.berkeley.edu/) are shown of short regions in family GH68. Analysis of the 3D structure of levansucrase (SacB) of *Bacillus subtilis* revealed eight amino acid residues involved in sucrose binding (see also Fig. 1). Sequence alignments showed that six of these eight amino acid residues, including the catalytic triad (D272, E523 and D424, Inu numbering), marked with black arrows [20], are completely conserved in GH68. The other three completely conserved residues are located at the −1 subsite (W271, W340 and R423), indicated by dark gray arrows. The other two residues involved in sucrose binding that are not completely conserved are indicated by light gray arrows. The following 20 amino acid sequences were used for the alignment: *Lactobacillus reuteri* Inu (AF459437); *Bacillus subtilis* SacB (X02730); *Gluconacetobacter diazotrophicus* levansucrase (L41732); *Lactobacillus reuteri* levansucrase (AF465251); *Lactobacillus sanfranciscensis* levansucrase (AJ508391); *Streptococcus mutans* SacB (M18954); *Streptococcus salivarius* SacB (L08445); *Geobacillus stearothermophilus* levansucrase (U34874); *Bacillus amyloliquefaciens* SacB (X52988); *Clostridium acetobutylicum* levansucrase (AE007686); *Paenibacillus polymyxa* levansucrase (AJ133737); *Gluconacetobacter xylinus* levansucrase AB034152; *Zymomonas mobilis* levansucrase L33402; *Pseudomonas syringae* levansucrase (AF345638); *Rachnella aquatilis* levansucrase AY027657; *Erwinia amylovora* levansucrase; *Pseudomonas aurantiaca* levansucrase (AF306513); *Leuconostoc citreum* inulosucrases (AY191311); *Lactobacillus johnsonii* levansucrase precursor (AE017202); *Leuconostoc mesenteroides* levansucrase (AY665464).

available GH32 3D structures, an asparagine (N) is present at this position. Although differing largely in size, in both families these residues form hydrogen bonds with the fructose 6-OH at subsite −1 (Fig. 1) [6]. Thus, the Inu mutant W271N was constructed.

W340 has no direct interactions with the substrate, but forms the boundary of the active site at subsite −1 (Fig. 1) [8]. This residue was mutated into an asparagine (W340N) to remove the large aromatic side chain and thereby alter the lining of the sucrose-binding pocket. Family GH32 members possess an aromatic amino acid residue at the equivalent position [6,10].

Positively charged R423, preceding catalytic D424 (transition state stabilizer), interacts with the fructose 3-OH at subsite −1, with the glucose 4-OH at subsite +1 (Fig. 1), and with the carboxylate of catalytic E523 (E342 in Bs SacB; acid–base catalyst) [8]. Mutation R423K would preserve the positive charge at position 423, but the interactions with the glucose moiety and E523 are probably weaker due to the smaller size of the lysine side chain and reduced capability for hydrogen bonding compared to arginine. Introduction

of R423H would remove the charge at that position and probably alter the positions of the substrate and E523.

None of these residues has been mutated before in members of GH68. Provided that there is a high structural similarity between levansucrase and inulosucrase proteins, all three of these residues (W271, R423 and W340; Inu numbering) should be located at the −1 subsite, interacting with the fructosyl unit of a bound sucrose (Fig. 1; R423 also interacts with the glucose moiety of sucrose at the +1 subsite). Information about the functional roles of these conserved FS residues is considered important as a first step to identify structural features responsible for product formation in FS enzymes.

Here we report the characteristic properties of these four mutant *Lactobacillus reuteri* Inu proteins (W271N, W340N, R423H, and R423K). Based on analysis of their total enzyme activities, their transglycosylation activities, and the range of (FOS and polymer) products synthesized, mutations in all three Inu active site residues strongly affected total enzyme activity, whereas mutations in two of these residues changed the amount of polymer and the size distribution of FOS synthesized.

Results

Close-up view of the active site of FS enzymes of family GH68

Elucidation of the high-resolution 3D structures of *B. subtilis* levansucrrase and a sucrose-bound inactive mutant protein, and the *G. diazotrophicus* LsdA levansucrase protein, provided clear insights into the reaction mechanism of FS enzymes [8]. Superposition of the available 3D structures revealed that the active sites of these enzymes show extensive overlap with each other [6]. Based on these 3D structures, eight amino acid residues directly involved in binding of sucrose in the active site, and constituting the −1 and +1 sugar-binding subsites (nomenclature according to Davies *et al.* [21]), were identified [8] (Fig. 1). Identical residues were found in the *Lactobacillus reuteri* 121 Inu (Figs 1 and 2) and Lev (Fig. 2) proteins.

Sequence alignment of members of family GH68

Based on the available 3D structures and sequence alignment of family GH68 proteins, residues creating subsite −1 were identified in *B. subtilis* SacB, *G. diazotrophicus* LsdA and *Lactobacillus reuteri* 121 Inu (Figs 1 and 2). This subsite is composed of the catalytic nucleophile (D86 in SacB, D135 in LsdA, and D272 in Inu) [20], the neighboring tryptophan residue (W85 in SacB, W134 in LsdA, and W271 in Inu), two residues located in the 'RDP' motif that are conserved in most members of family GH68 (R246 and D247 in SacB, R308 and D309 in LsdA, and R423 and D424 in Inu), and a tryptophan residue bordering the sucrose-binding pocket and located very close to the fructose moiety at −1 (W163 in SacB, W224 in LsdA, and W340 in Inu). Inu residue D424 acts as transition state stabilizer [20]. Subsite +1 differs among different enzymes from family GH68 and is composed of either an arginine or a histidine residue (R360 in SacB, H419 in LsdA, and R541 in Inu) (see below), either a glutamic acid or a glutamine residue (E340 in SacB, Q399 in LsdA, and E521 in Inu) located two amino acid residues upstream of the acid–base catalyst (E342 in SacB, E401 in LsdA, and E523 in Inu), and the arginine residue present in the 'RDP' motif (also involved in formation of the −1 subsite) (R246 in SacB, R308 in LsdA and R423 in Inu). These differences at the +1 subsite distinguish enzymes from Gram-positive (SacB and Inu) and Gram-negative (LsdA) bacteria, but not polymerizing (SacB), oligomerizing (LsdA and Inu) or inulin/levan-synthesizing (SacB, LsdA) enzymes.

Inu residues W271, W340 and R423, conserved in all members of family GH68, were selected as targets for site-directed mutagenesis. To our knowledge, none of these residues has been mutated and analyzed before in members of family GH68.

Activity of *Lactobacillus reuteri* 121 Inu wild type and active site mutants

Previously, we have constructed and characterized Inu (and Lev) mutants modified in the three catalytic residues E523Q (E503Q) (general acid–base catalyst), D272N (D249N) (catalytic nucleophile), and D424N (D404N) (transition state stabilizer), resulting in at least a 10 000-fold reduction in total activity [20].

The *Lactobacillus reuteri* 121 Inu and Lev wild-type (WT) enzymes possess unusually high temperature optima for their total activities [(V_G), see also Experimental procedures]. The highest total activities were observed at 50 °C [19], whereas the range of synthesized products increased at lower temperatures, and with relatively low activity units [18]. Independent of temperature, the transglycosylation activity ($V_F - V_G$) of Inu increased with sucrose concentrations, reaching 90% of total activity (V_G) at 0.8–1.7 M sucrose. In order to optimize transglycosylation, reactions were performed at 37 °C in a reaction buffer containing 840 mM sucrose.

Table 1. Total (V_G), transglycosylation ($V_G - V_F$) and hydrolytic activities (V_F) (in U·mg^{-1} of protein) of *Lactobacillus reuteri* 121 inulosucrase (Inu) wild type (WT), levansucrase (Lev) WT, and derived Inu mutants. Assays were performed at 37 °C in a buffer supplemented with 840 mM sucrose. Kinetic parameters for total (V_G) activity were determined using a range of seven different sucrose concentrations (1–100 mM). ND, not determined.

	Activities				Kinetic parameters	
Enzyme	Total activity (V_G) (U·mg^{-1})	Transglycosylation ($V_G - V_F$) (U·mg^{-1})	Hydrolysis (V_F) (U·mg^{-1})	Transglycosylation/ hydrolysis	V^G_{max} (u·mg^{-1})	K^G_m (mM)
Inu	147	119	28	4.25	77.7 ± 3.6	12.0 ± 1.8
Lev	101	73	28	2.6	ND	ND
Inu W271N	7.8	6.2	1.6	3.9	2.7 ± 0.2	39.0 ± 2.7
Inu W340N	0.74	0.5	0.24	2	ND	ND
Inu R423H	0.4	ND	ND	–	ND	ND
Inu R423K	2	1.4	0.6	2.3	4.6 ± 0.5[a]	187.0 ± 30.2[a]

[a] The absence of saturation resulted in a high standard error with curve fitting. Data obtained were fitted using the Michaelis–Menten equation.

All Inu mutants showed strongly reduced V_G compared to Inu WT (20–370-fold). The transglycosylation/hydrolysis ratio remained virtually unchanged for mutant W271N, whereas mutants W340N and R423K showed a two-fold higher hydrolytic activity compared to WT Inu under the conditions tested (Table 1). Mutants W271N, R423K, W340N and R423H displayed only 5.3%, 1.4%, 0.5% and 0.3% of total Inu WT activity, respectively. Kinetic measurements of W271N and R423K showed that affinity for sucrose (K^G_m) had dropped 3–15 times compared to WT Inu.

Polymerization versus oligomerization

Lev WT, Inu WT and Inu mutant W271N were added at equal activity levels to the reaction mixture, allowing proper comparison of the reaction products by TLC (Fig. 3). TLC analysis showed that Inu WT synthesized a range of FOS up to at least degree of polymerization (DP) 9–10 and a small amount of inulin polymer, whereas Lev produced mainly (levan) polymer and smaller-size FOS (mainly kestose and nystose) [18]. Compared to Inu WT, mutant W271N synthesized very similar amounts of FOS up to DP 6, but clearly less of DP 7-9. Interestingly, W271N synthesized more of the larger FOS (> DP 10) molecules and polymer.

With the relatively low remaining activities of mutants W340N, R423K, and R423H, substantially lower activity levels were available for these enzymes for incubation with sucrose in reaction mixtures. No sucrose conversion was detected with mutant R423H when using TLC analysis (24 h of incubation; Fig. 3), although a low activity was clearly detected when a more sensitive assay for glucose release from sucrose was used (initial rates; Table 1). Mutant W340N had lost the ability to synthesize a range of FOS molecules and only synthesized small amounts of kestose and nystose; polymer formation was not detectable (Fig. 3). Mutant R423K still produced a relatively

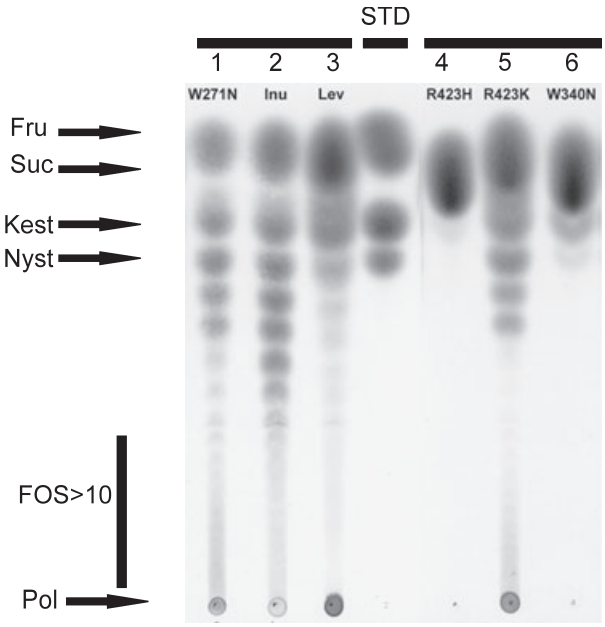

Fig. 3. TLC analysis of the products synthesized by inulosucrase (Inu) wild type (WT), levansucrase (Lev) WT and Inu mutants from sucrose (840 mM) at 37 °C during a 24 h incubation period. The FS enzymes were applied as follows: lane 1, W271N (2 U·mL^{-1}); lane 2, Inu WT (2 U·mL^{-1}); lane 3, Lev (2 U·mL^{-1}); lane 4, R423H (0.016 U·mL^{-1}); lane 5, R423K (0.31 U·mL^{-1}); lane 6, W340N (0.25 U·mL^{-1}). STD, standard containing; Fru, fructose; Suc, sucrose; Kest, 1-kestose (GF2); Nyst, 1,1-nystose (GF3); FOS > 10, fructooligosaccharides with a degree of polymerization larger than 10; Pol, polymer. The plates were run once in butanol/ethanol/water (5 : 5 : 3). Fructose-containing sugars were specifically stained with urea spray [30].

broad range of FOS molecules (in 24 h incubations), despite the lower activity levels of this enzyme used in the reaction mixture (only 0.3 $U \cdot mL^{-1}$ compared to 2 $U \cdot mL^{-1}$ for Inu WT) (Fig. 3). Also, the amounts of FOS synthesized by mutant R423K were comparable (FOS up to DP 6) between mutant and WT enzymes. Mutant R423K failed to produce FOS of DP 6–10, but clearly synthesized more polymer and high-DP FOS (> DP 10) compared to WT Inu. With respect to the range of FOS and the amount of polymer synthesized, mutants W271N and R423K thus resembled each other clearly, strongly differing from Inu WT (Fig. 3).

High-performance anion-exchange chromatography (HPAEC) analysis of FS products from sucrose

HPAEC analysis of the reaction products from sucrose allowed a detailed comparison of the product specificities of Inu WT and mutants W271N and R423K. With Inu WT, the amount of FOS synthesized was inversely proportional to the FOS size (Fig. 4A). The largest Inu WT FOS product detectable, with the method used, was DP 18. In the case of the mutant Inu enzymes, this inverted relationship was only seen for oligosaccharides smaller than DP 6. Mutant W271N synthesized FOS of at least DP 20. The larger oligosaccharides were present in small amounts, visible through the whole elution profile (Fig. 4B). Mutant R423K synthesized FOS up to DP 12 (Fig. 4C), but in significantly lower amounts than in WT and mutant W271N samples.

Discussion

Structure–function relationships in bacterial FS enzymes are not yet fully understood. Major questions still to be answered are: what structural features determine the type and size of polymers synthesized, and the ratio of FOS versus polymer produced.

Based on the available structural data, and an alignment of conserved regions in family GH68 enzymes (Figs 1 and 2), eight residues appear to be involved in sucrose binding in the active site of these FS enzymes. Inu residues D272, E523 and D424 were previously identified as catalytic nucleophile, acid–base catalyst and transition state stabilizer, respectively [20]. R541 (R360 in Bs SacB) was identified as a residue forming the acceptor-binding site [22]. Three of the four remaining residues (W271, R423 and W340, but not E521) are completely conserved in family GH68 members and were selected as targets for site-directed mutagenesis.

Inu mutation W271N (exchange of residue present in many GH32 members) resulted in a drastic drop of

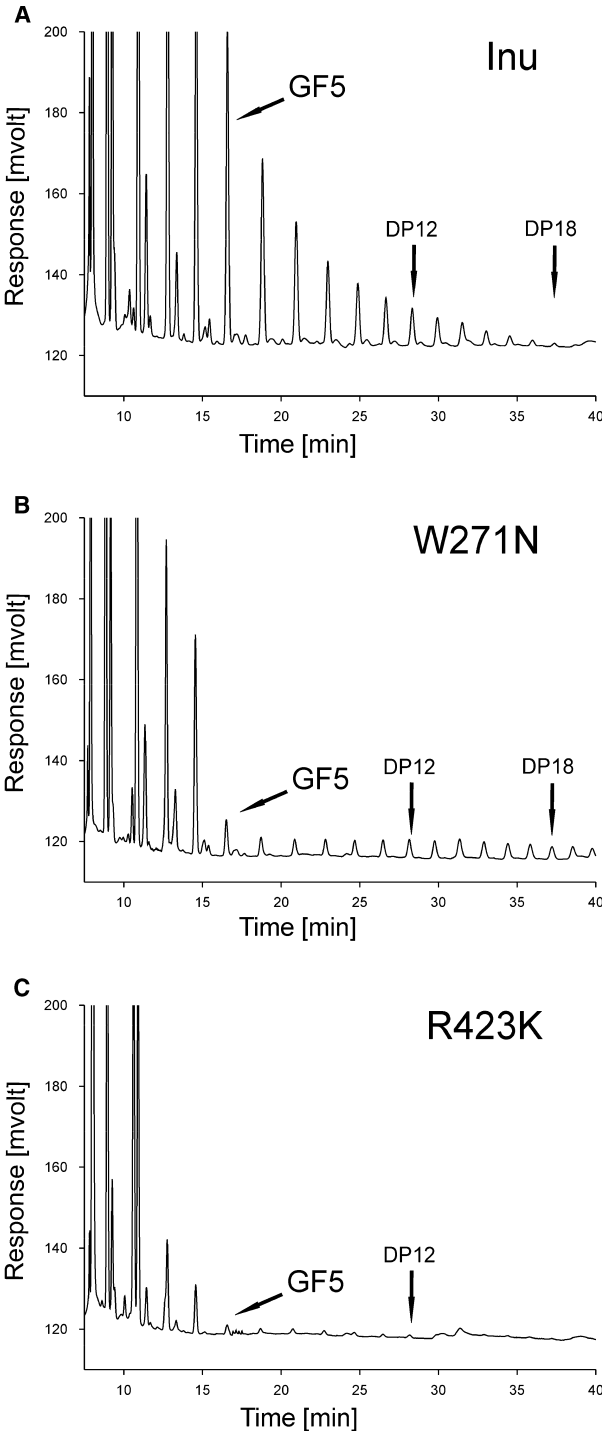

Fig. 4. High-performance anion-exchange chromatography analysis of the products synthesized by inulosucrase (Inu) wild type (WT) (A), and mutants W271N (B) and R423K (C), from sucrose (840 mM) at 37 °C during a 24 h incubation period. The following amounts of enzymes were used: 2.2 $U \cdot mL^{-1}$ of Inu, 2.6 $U \cdot mL^{-1}$ of mutant W271N and 0.2 $U \cdot mL^{-1}$ of mutant R423K. GF5, 1,1,1-kestopentose; DP 12 and DP 18, FOS with a degree of polymerization (DP) of 12 and 18.

enzyme activity and also a three-fold reduction in affinity for sucrose (Table 1). Interestingly, the drop in activity was accompanied by the ability to synthesize larger fructan products compared to the WT. Furthermore, W271N clearly (Figs 1 and 2A) synthesized FOS of DP 10–20, present in equal amounts (Figs 3 and 4B), whereas Inu WT produced gradually lower amounts of FOS upon increasing DP. W271 is located at the bottom of the active site and forms a hydrogen bond with the C6 hydroxyl group of the fructose unit. Introduction of an asparagine at this position might still permit formation of this hydrogen bond. Thus the effects observed on enzyme activity (Table 1) and product specificity (Figs 3 and 4) are probably caused by differences in size between the amino acid side chains. In addition, substitution W271N may also influence the positioning of D272 (nucleophile), partly explaining the observed loss in activity. Changes in the corresponding amino acid residue in *Allium cepa* 6G-FFT showed that also in GH32 this amino acid residue is important in determining activity as well as product specificity [23].

Inu W340N showed a very strong reduction in enzyme activity (Table 1), and only 1-kestose was detected on TLC (Fig. 3). Removal of the large tryptophan side chain lining the sucrose-binding pocket will enlarge the size of subsite −1 (Fig. 1). Therefore, a tight fit of the fructose moiety of sucrose in this subsite is clearly favorable for the course of the reaction. Although not interacting directly with the substrate, W340 appears to be important for shaping the active site, vital for positioning of the substrate. Aromatic residues are often involved in the correct orientation of polysaccharides in the acceptor-binding site of different enzymes [24]. Changes in the amino acid residue adjacent to W340 in *Zymomonas mobilis* levansucrase mutant E117Q (E339 in Inu) did not inactivate the enzyme, but rather an increase in polymer formation was observed [25].

Mutation R423H showed the lowest residual activity of all Inu variants (Table 1), and virtually no transglycosylation products were detected on TLC (Fig. 3). Obviously, the histidine side chain interferes with the course of the reaction and exemplifies the importance of arginine at this position.

Substitution R423K introduced a relatively moderate change in the active site (residues of similar size and charge), but caused a very significant reduction in V_G and affinity K_m^G (15-fold) (Table 1). Most striking was the virtually complete absence of FOS synthesis of DP 7–9 in mutant R423K, in combination with a virtually unchanged amount of FOS of DP < 7, and increased synthesis of FOS/polymer of DP > 10 (Fig. 3). HPAEC analysis of the reaction products provided clear evidence for synthesis of FOS up to DP 6 only (Fig. 4C). Apparently, the larger-size material observed on TLC (Fig. 3) represents FOS/polymer that was not visible in the HPAEC analysis.

Inu R423 is part of the highly conserved 'RDP motif' in clan GH-J (Fig. 2C). Clearly, it is involved in a complex network of interactions in the active site of FS enzymes [8]. Nagem *et al*. reported that this R residue (R188 in EI; family GH32) participates in substrate binding, is important for recognition of the sugar ring, and might be responsible for the specificity of the enzyme towards the fructopyranosyl residue [11].

For unknown reasons, GH68 members strongly differ in the range and size of FOS/polymer synthesized. The FS active site mutations analyzed in the present work, targeted completely conserved residues in GH68, and therefore the experimental data presented here do not provide explanations for differences between GH68 members, and between *Lactobacillus reuteri* 121 Inu and Lev. Rather, the mutations introduced may cause further structural changes in the layer of residues surrounding these completely conserved active site amino acids. Further 3D structural information is required to identify such putative changes.

In conclusion, our data show that W271, W340 and R423 (*Lactobacillus reuteri* Inu numbering) fulfill crucial roles in FS enzymes, interacting with the bound sucrose at subsites −1 and +1. Changes in these residues strongly affect overall activity. Mutants W271N and R423K also display changes in their product specificities, especially in polymer versus FOS synthesis. The precise interactions between enzyme and fructose molecules bound to the active site are thus critical for the outcome of the FS transglycosylation reaction. Our data show that the −1 subsite is not only important for substrate recognition and catalysis, but directly or indirectly also plays a role in determination of the size of the products synthesized.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Escherichia coli strain Top10 (Invitrogen, Carlsbad, CA, USA) was used for expression of wild-type (WT) and mutant *Lactobacillus reuteri* 121 Inu (GenBank accession number AF459437) and WT Lev (GenBank accession number AF465251). Plasmid pBAD/myc/his/C (Invitrogen) was used for cloning purposes. Plasmid-carrying *E. coli* cells were grown at 37 °C on LB medium [26] supplemented with 100 $\mu g \cdot mL^{-1}$ ampicillin and 0.02% (w/v) arabinose for *ftf* gene induction. The WT and mutant of Inu were expressed

in *E. coli* as constructs with a C-terminal truncation of 100 amino acid residues, and a C-terminal polyhistidine tag as described [15]. The *bdri* construct was used for optimal Lev expression (see below).

Molecular techniques

An alignment (SEQUENCELOGO, http://weblogo.berkeley.edu/) of 20 members of family GH68 was constructed, showing short regions in FS enzymes with conserved amino acid residues (Fig. 2). Using site-directed mutagenesis, the following *Lactobacillus reuteri* 121 Inu mutants were constructed: W271N, W340N, R423K, and R423H. Single mutations were introduced using the 'megaprimer' method [27] and confirmed by sequencing. For site-directed mutagenesis (synthesis of 'megaprimers'), the following oligonucleotides were used in PCR reactions: i-W271N, 5′-CTGGCCATGA ATC**ATT**TACATCTAAAGG-3′; i-W340N, 5′-CAGCTGA TCCTGA**ATT**TTCTTGTGAAACC-3′; R423H, 5′-CGA TAATATTGCAATGCATGATGCTCATG-3′; r423k, 5′-CGATAATATTGCAATG**AAG**GATGCTCATG-3′. The Lev oligonucleotide BDRI, 5′-GCTAA**A**GAAATCAAG AATATGCC-3′, was used to introduce a silent mutation for improved Lev expression (see below). Additionally, depending on the position of the mutated residue, one of the two flanking primers was used in combination with 'megaprimers': i-pBADRV, 5′-TCTGAGATGAGTTTTTG TTCGG-3′; pBADFD, 5′-TCCTACCTGACGCTTTTTAT CG-3′. The bases in bold indicate mutations introduced; 'i' indicates antisense primer.

Improved expression of Lev

SDS/PAGE analysis of Ni-NTA affinity chromatography-purified Lev revealed copurification of two major protein fractions, 85 kDa and 64 kDa [16]. To eliminate expression of the smaller Lev derivative, the imperfect Shine–Dalgarno (SD) sequence downstream (11 bp) of the alternative start codon (1877 bp from the Lev start codon) was altered (see above). A silent mutation was introduced into the SD sequence at position 1864 (AA**A**GAA) (*bdri* construct). Analysis of purified Lev expressed from *bdri* showed the presence of only a single protein band (data not shown). This improved Lev expression construct was used in all experiments presented in this article.

Purification of FS proteins

All proteins produced were expressed with a C-terminal histidine tag and purified by Ni-NTA affinity chromatography as previously described [15]. Proteins were desalted with 50 mM sodium acetate, pH 5.4, supplemented with 1 mM $CaCl_2$, using a 5 mL Hi-trap desalting column (Amersham, Pharmacia Biotech, Piscataway, NJ, USA). Purity was checked by SDS/PAGE. Enzyme concentrations were determined using Bradford reagent (Bio-Rad, Hercules, CA, USA) with BSA as standard.

FS enzyme activity assay

Activities of the WT and mutant FS enzymes were determined at 37 °C in 50 mM sodium acetate reaction buffer, pH 5.4, supplemented with 840 mM sucrose and 1 mM $CaCl_2$, using purified *Lactobacillus reuteri* 121 enzymes, Lev WT (7.6 $\mu g \cdot mL^{-1}$ protein), Inu WT (1.28 $\mu g \cdot mL^{-1}$) and Inu mutants W271N (32 $\mu g \cdot mL^{-1}$), R423H (25.6 $\mu g \cdot mL^{-1}$), R423K (128 $\mu g \cdot mL^{-1}$), and W340N (32 $\mu g \cdot mL^{-1}$). Kinetic assays were performed using seven different sucrose concentrations ranging from 1 to 100 mM, using Inu WT (6.6 $\mu g \cdot mL^{-1}$), W271N (350 $\mu g \cdot mL^{-1}$) and R423K (490 $\mu g \cdot mL^{-1}$). After preincubation of the reaction mixture at the assay temperature for 5 min, reactions were started by enzyme addition. Samples were taken every 3 min and used to determine the amount of glucose released from sucrose [28]. The amount of glucose formed reflects the total amount of sucrose utilized during the reaction (total activity, V_G). The amount of fructose formed is a measure of the hydrolytic activity (V_F). The amount of glucose minus the amount of fructose reflects the transferase activity (V_{G-F}). One unit of enzyme activity (U) is defined as the release of 1 μmol of monosaccharide per min. Curve fitting of the data was performed with the SIGMAPLOT program (version 9.0) using the Michaelis Menten formula [$y = (a \times x)/(b \times x)$]. In this equation, y is the specific activity ($U \cdot mg^{-1}$), x is the substrate concentration (mM sucrose), a is the maximal reaction rate V_{max}, and b is the affinity constant for the substrate (K_m, mM sucrose).

Detection and quantification of FOS

Products synthesized by the different FS enzymes, from 840 mM sucrose, at 37 °C for 24 h, were analyzed by TLC as described previously [18]. The following enzyme concentrations were used: W271N, Inu WT and Lev WT, 2 $U \cdot mL^{-1}$; R423H, 0.016 $U \cdot mL^{-1}$; R423K, 0.31 $U \cdot mL^{-1}$; W340N, 0.25 $U \cdot mL^{-1}$.

FOS synthesized by Inu WT and mutants W271N and R423K were also analyzed using HPAEC as described previously [18], following incubations of purified FS enzymes in the reaction buffer containing 840 mM sucrose, at 37 °C for 24 h. The following amounts of enzymes were used: 2.2 $U \cdot mL^{-1}$ of Inu WT, 2.6 $U \cdot mL^{-1}$ of mutant W271N, and 0.2 $U \cdot mL^{-1}$ of mutant R423K.

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