Mutational and biochemical analysis of Lactobacillus reuteri glucansucrase enzymes

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Chapter 8

Summary and Prospects

Lactic acid bacteria have been used to produce fermented food for ages, due to their ability to produce lactic acid and flavor metabolites. The ability of lactic acid bacteria to produce large amounts of exopolysaccharides has drawn strong attention for industrial applications in recent years (15,16,20,30,39,46). α-Glucan homopolysaccharides are synthesized from sucrose by glucansucrase enzymes of lactic acid bacteria and are used as biothickening agents in the food industry, as plasma expander in medicine, and as separation matrix in research (15,16,20,30,39,46). Prebiotic oligosaccharides synthesized by glucansucrases hold great potential as healthy food ingredients (30,46,202). The α-glucans produced by glucansucrases also are important pathogenic factors for dental caries (57-59). They are involved in facilitating the adherence of oral bacteria like *Streptococcus mutans* to tooth surfaces (65). The identification of specific inhibitors of glucansucrase enzymes thus provides a target to prevent dental caries.

Glucansucrases of lactic acid bacteria are classified as glycoside hydrolase family 70 (GH70) enzymes, sharing a catalytic $(\beta/\alpha)_8$ barrel structure with the closely related GH13 and GH77 family enzymes (204), together constituting clan GH-H (http://www.CAZy.org). Using sucrose as donor substrate, glucansucrases catalyze three reactions, namely polysaccharide synthesis, oligosaccharide synthesis or hydrolysis, using a growing glucan chain, oligosaccharides or water as acceptor substrates, respectively (24,28,29). The catalytic residues of glucansucrases (nucleophile D1025, acid/base catalyst E1063 and transition state stabilizer D1136, GTF180 numbering) are identical, but these enzymes catalyze the synthesis of a variety of α-glucans (114). Depending on the glycosidic linkages present, the various α-glucans are divided into five groups: (i) dextran with mainly (α1→6) linkages; (ii) mutan with predominantly (α1→3) linkages; (iii) alternan with alternating (α1→6) and (α1→3) linkages; (iv) reuteran composed of mainly (α1→4) linkages; v) α-glucans with (α1→2) branches. The physico-chemical properties of α-glucans are highly dependent on their structure, especially on linkage types, degree of branching, size (28,29,164). Current studies aim to accomplish the rational synthesis of tailor-made α-glucans with desired properties. All glucansucrases initiate their reaction by cleaving the donor substrate sucrose and first forming a glucosyl-enzyme intermediate. In the second step of the reaction, the glucosyl moiety is transferred to the non-reducing end of

an acceptor substrate with retention of α-anomeric configuration. It has been proposed that the reaction specificity and linkage specificity of glucansucrases are determined by the type of acceptor substrate utilized and by the way in which it binds in the second step of the reaction (24,28,114,151). Prior to the availability of crystal structures of glucansucrases, amino acid residues of several conserved regions have been identified to be important for linkage and reaction specificity by comparing with related GH13 family enzymes (amylosucrase, α-amylase) (88,151). For example, amino acid residues following the transition state stabilizer were identified as putative acceptor binding site residues by sequence alignments and were found to be critical for linkage specificity in several glucansucrases by mutagenesis studies (86,88,109,150,151,165,166). Recently, the elucidation of the crystal structure of GTF180-ΔN revealed an unusual and novel domain organization (100). The polypeptide chain of GTF180-ΔN follows a U-shape course to form five domains (domains A, B, C, IV and V). Except for domain C, the other domains are formed by two discontinuous polypeptide chains from both N- and C-terminal ends. The catalytic core consists of domains A, B and C, which resemble those of family GH13 enzymes. Glucansucrases have two extra and unique domains (IV and V) which are attached to the catalytic core and are not present in family GH13 enzymes. The crystal structure of GTF180-ΔN in complex with the acceptor substrate maltose confirmed the involvement of previously identified amino acid residues in the acceptor substrate binding sites +1 and +2 (100). It also revealed the involvement of many other residues in shaping the acceptor substrate binding site. These residues, especially from domain B, had not been identified in previous mutagenesis studies by sequence alignment due to the novel folding of glucansucrase proteins (U shape). In this PhD project, we structurally characterized the initial oligosaccharides produced by GTFA of *Lactobacillus reuteri* 121 from sucrose, aiming to explore the process of polysaccharide synthesis, and performed semi-rational engineering of glucansucrase GTF180 of *Lactobacillus reuteri* 180 to study structural features that determine its linkage and reaction specificity. These studies provide novel insights into the structure-function relationships of glucansucrase enzymes; novel α-glucans with different structures were produced from sucrose using engineered glucansucrases.

Initial gluco-oligosaccharides formed by the reuteransucrase GTFA of *Lactobacillus reuteri* 121

It was largely unclear how glucansucrase enzymes synthesize their polysaccharide products from sucrose, via oligosaccharide intermediates. Does the growing glucan chain remain linked to the enzyme (processive mechanism) or is it frequently released (nonprocessive mechanism)? Therefore we have studied the initial products formed by GTFA of *L. reuteri* 121, also varying sucrose concentration (Chapters 2 and 3). The initial gluco-oligosaccharides produced by reuteransucrase GTFA of *L. reuteri* 121 were characterized by MALDI-TOF-MS and 1D/2D NMR spectroscopy (Chapter 2). Our detailed structural analysis showed that GTFA mainly synthesized linear oligosaccharides starting with the elongation of sucrose with alternating (α1→4) and (α1→6) linkages in the first 6 h of incubation with sucrose. No consecutive (α1→6) linkages were observed in these products. Our data showed that this is the first reported glucansucrase enzyme which synthesizes alternating (α1→4) and (α1→6) linkages. The structural basis for alternating linkage synthesis probably lies in its special acceptor substrate binding sites and remains to be investigated in future studies. Alternansucrase (ASR) of *Leuconostoc mesenteroides* NRRL B-1355 also synthesizes mainly alternating linkages, but with (α1→3) and (α1→6) linkages (52). Malto-oligosaccharides were shown to be relatively poor donor substrates for GTFA, only short oligosaccharides (DP<10) were formed. Instead, malto-oligosaccharides were shown to be effective acceptor substrates, with sucrose as donor substrate. This study provided novel insights into the mechanism of α-glucan synthesis catalyzed by glucansucrases. The novel oligosaccharides produced by GTFA, containing alternating (α1→4) and (α1→6) linkages which are resistant to α-amylase digestion, may find application as prebiotic compounds.

The effects of sucrose concentration on the synthesis of α-glucan were investigated by incubating different concentrations of sucrose with GTFA of *L. reuteri* 121 (Chapter 3). The ratio of oligosaccharide versus polysaccharide synthesized was directly proportional to the concentration of sucrose, while the linkage distributions in the polysaccharides generated at different sucrose concentrations were identical. These results show that size distribution of products (oligosaccharides versus polysaccharides) is kinetically controlled but

the linkage specificity of GTFA-ΔN is not. The oligosaccharides produced at high sucrose concentration (1.0 M sucrose) were isolated and characterized, revealing a more detailed scheme of the growing oligosaccharides synthesized en route to polysaccharides.

Structural basis for polysaccharide synthesis of glucansucrase GTF180 of *Lactobacillus reuteri* 180

The precise role of the domain V in glucansucrases of GH70 family enzymes has remained unclear. In previous studies, different amino acid sequence repeats were identified in the region consisting of domain V and have been found to be involved in glucan binding (28,70,89,90,93,105,117). Comparison of the crystal structures of several glucansucrase proteins revealed a positional variation of domain V reflecting the flexibility of domain V (48,100,103,106). It has been proposed that domain V facilitates polysaccharide synthesis by bringing the glucan chain toward and away from the active site (102,106). In Chapter 4, we report the effects of completely truncating domain V, based on structural information. In view of the identical structures of the polysaccharides synthesized by the GTF180-ΔN and GTF180-ΔNΔV enzymes, we concluded that domain V is not involved in linkage specificity determination. The size distribution of products synthesized by GTF180-ΔNΔV instead was drastically affected, showing virtually no polysaccharide synthesis. Mutagenesis studies in GTF180-ΔN demonstrated that mutations at residues close to the acceptor binding sites in domain B (L940E, L940F) resulted in a significant increase (around 2-fold) in the amount of polysaccharide produced (Chapter 5) (118). The introduction of the same mutations in GTF180-ΔNΔV partially restored polysaccharide synthesis to the level of GTF180-ΔN, but not to that of the same mutants of GTF180-ΔN. These results show that the structural basis for polysaccharide synthesis by GTF180 resides at both these acceptor binding sites and at domain V, representing close and remote binding sites for the (growing) polysaccharide chain, respectively.

Linkage specificity determination in glucansucrase GTF180 of *Lactobacillus reuteri* 180

Amino acid residues shaping the acceptor binding sites +1 and +2 of GTF180-ΔN have been identified by analysis of the available crystal structure of GTF180-ΔN

in complex with the acceptor substrate maltose, and are expected to be critical for the linkage specificity of GTF180-ΔN (100,114). At the +1 subsite, besides conserved catalytic residues, N1029 makes direct and indirect hydrogen bonds with the +1 C4 and C3 hydroxyl group; D1028 forms a water-mediated hydrogen bond with the +1 C4 hydroxyl group, and residues from domain B (L938, L940, A978 and L981) shape the groove near the +1 subsite. At acceptor binding subsite +2, W1065 has a stacking interaction with the glucosyl moiety. Residues S1137, N1138, A1139, Q1140 and D1141, following the transition state stabilizer (D1136), have been shown to be important for linkage specificity (86,88,100,109,114,150,151,165,166), and are located at one side of the +2 glucosyl unit of maltose. Only S1137 has a direct hydrogen bond with the +2 C1 hydroxyl group. At the other side of the +2 glucosyl unit, residues D1085, R1088 and N1089 from α-helix 4 both form an indirect hydrogen bond with the +2 C2 hydroxyl group through the same water molecule. These residues, especially those from domain B, have not been targeted for mutagenesis study prior to the availability of crystal structures. In this PhD thesis, the effects of mutations in amino acid residues close to the +1 and + 2 acceptor binding sites on linkage specificity of products synthesized were investigated, using a semi-rational engineering approach (Chapter 5, 6 and 7).

Mutations in L940 of wild-type GTF180-ΔN all caused an increased percentage of (α1→6) linkages and a decreased percentage of (α1→3) linkages in the products (Chapter 5). Particularly, mutant L940W was unable to form (α1→3) linkages and synthesized a smaller and linear glucan polysaccharide with only (α1→6) linkages. Docking studies demonstrated that the introduction of the large aromatic amino acid residue tryptophan at position 940 partially blocked the acceptor substrate binding groove where L940 locates, likely preventing the isomalto-oligosaccharide acceptor to bind in a favorable orientation for the formation of (α1→3) linkages. L938 mutants (except L938F) showed an altered linkage specificity with mostly an increased (α1→6) linkage synthesis (chapter 6). Residue A978 was found to play an important role in the synthesis of branched linkages. Mutating A978 to larger side chain residues (L, P, F or Y) resulted in a decrease in branched linkage synthesis (~ 50%) and correspondingly an increase in (α1→3) linkage synthesis in the linear sections (Chapter 6). The decreased branched linkage synthesis was probably due to steric hindrance effects of bulky

residues at the groove above the +1 acceptor binding site. All D1028 mutants showed a clear increase in (α1→6) linkage synthesis (Chapter 6). Moreover, D1028Y and D1028W mutants synthesized ~ 50% less branched linkages compared to wild-type GTF180-ΔN, as observed for A978 mutants. Only minor amounts of polysaccharides were synthesized by N1029 mutants (except N1029Y) and they were found to contain mainly (α1→3) linkages (more than 50%) (Chapter 6).

In order to study the effects of mutations in residues D1085, R1088 and N1089 on linkage specificity, these three residues were mutated to the corresponding residues present at these positions in various other glucansucrases, identified by sequence alignment (Chapter 7). Secondly, random mutagenesis was performed, targeting each of these three residues separately to investigate their individual roles in linkage specificity (Chapter 7). Our results showed that all combined mutants, and D1085 and R1088 single mutants produced polysaccharides with a higher percentage of branching (from 15% to 22%). They also introduced a small amount of (α1→4) linkages (at the most 5%) in the polysaccharide produced. Single mutation studies showed that residue D1085 and R1088 but not N1089 were responsible for the observed linkage specificity change.

To conclude, our results suggest that linkage specificity of glucansucrase GTF180-ΔN is determined by an interplay of residues from different regions surrounding the acceptor substrate binding groove, not only from domain A but also from domain B. Residues surrounding the +1 and +2 acceptor subsites are critical for linkage specificity of the enzyme and are shown to play different roles. Our studies provide novel insights into the structure-function relationships of glucansucrases and clearly show that production of tailor-made α-glucans by glucansucrase engineering is possible. α-Glucans with potentially different physico-chemical properties [containing 25% to 100% of (α1→6) linkages and 0% to 22% of branching (α1→3) linkages] were produced by GTF180 and its engineered mutants. The novel properties of these α-glucans remain to be determined and may open new prospectives for industrial applications.

Reaction specificity determination of glucansucrase GTF180 of *Lactobacillus reuteri* 180

The relative balance of the three reactions (reaction specificity) catalyzed by glucansucrases can be changed by both enzyme engineering and reaction condition engineering. In this PhD thesis, we studied the effects of specific mutations in GTF180-ΔN on reaction specificity, by determining the percentages of sucrose used for polysaccharide synthesis, oligosaccharide synthesis and hydrolysis. Truncation of domain V, a putative glucan binding domain, resulted in impaired polysaccharide synthesis and a corresponding increase in oligosaccharide synthesis (Chapter 4). Besides the change in linkage specificity, the L940E and L940F mutants also showed a significant increase in polysaccharide synthesis (to about 30% of sucrose) (Chapter 5). The strongest reduction in amount of sucrose used for hydrolysis was observed with L940W (only 4.3%) compared to 23.9% in wild-type GTF180-ΔN. The tryptophan side chain is proposed to increase the hydrophobicity of the active site and/or partially shield off the covalent reaction intermediate for attack by water. With the reduced hydrolysis and polysaccharide synthesis, the L940W mutant is highly efficient in the synthesis of isomalto-oligosaccharides from sucrose and holds great potential for their production. Mutations in N1029 strongly increased the amount of sucrose used for hydrolysis. This revealed that hydrogen bonds of N1029 with the +1 C4 and C3 hydroxyl group are essential for acceptor substrate binding, hence for transglycosylation activity.

To conclude, the reaction specificity of GTF180 can be shifted either to polysaccharide synthesis, oligosaccharide synthesis or hydrolysis by enzyme engineering. Engineering of reaction specificity of glucansucrases may allow efficient production of either polysaccharides or oligosaccharides to meet the demand for different applications.

Conclusions and future prospects

In this thesis, a semi-rational mutagenesis approach was applied in the engineering of the glucansucrase GTF180 enzyme, made possible by its recently elucidated crystal structure. This allowed us to identify hot-spot regions in this glucansucrase protein that are critical for its linkage and reaction specificity but had not been investigated in previous studies. The individual roles of these amino

acid residues in enzyme function, including linkage and reaction specificity, and activity, were studied in detail. Several residues from domain B (L938, L940 and A978) and residues from domain A (D1028, N1029, D1085, R1088 and N1089) were found to be important for linkage specificity. Residues A978, D1028, D1085 and R1088 were found to be involved in the branched linkage formation. Residues N1029 and L981 are essential for the transglycosylation reaction. Our studies provide novel insights into the structure-function relationships of glucansucrase GTF180 regarding its linkage specificity, reaction specificity and activity. Considering the similarity of glucansucrases, the importance of these residues can most likely be extended to other glucansucrases. These residues provide new targets for glucansucrase engineering aiming for the synthesis of novel α-glucans. The ultimate goal is to rationally design glucansucrases producing tailor-made α-glucans, i.e. with desired structures and properties. The determination of crystal structures of glucansucrases in complex with longer chain oligosaccharides may identify other protein regions that are important for polysaccharide synthesis and linkage specificity. Molecular dynamic studies represent another potential approach to understand the synthetic process from sucrose to polysaccharides. They may provide us with new insights how to design glucansucrase enzymes for desirable, specific α-glucans, as well as guiding the search for inhibitors of glucansucrases aiming to prevent dental caries. At present it remains unknown what determines the molecular mass of the polysaccharide products of glucansucrases.

Novel α-glucans containing different glyosidic linkages and various amounts of branching were produced by engineered glucansucrases described in this PhD thesis. The physico-chemical properties of these α-glucans remain to be investigated to elucidate their structure-properties relationships. The structures and potential prebiotic activities of the novel oligosaccharides produced by glucansucrases need to be characterized and evaluated systematically. Industrial application processes can be eventually developed based on the information obtained in such studies. In this regard, it is important to note that the (thermo)stability of glucansucrase enzymes remains to be improved; the availability of crystal structures facilitates a computational design approach to achieve this (240).

Glucansucrases present large acceptor substrate promiscuity and have been shown to be capable of using a wide range of compounds like benzenoids, flavonoids and steroids as acceptor substrates (142,201). The acceptor reaction of glucansucrases has been explored to glycosylate organic molecules to improve their solubility, stability, flavour and activity, with the advantage of using the cheap glucose donor sucrose (143,145-147,149). These acceptor reactions need to be further explored and optimized through enzyme engineering and reaction engineering. Such studies are in progress in our laboratory.

In contrast, glucansucrase donor substrate is limited specifically to sucrose, resulting in transfer of only a glucosyl moiety. Various sucrose analogues have been synthesized, in which the glucosyl unit of sucrose is replaced by alternative glycosyl moieties (241-243). These sucrose analogues α-D-xylopyranosyl-β-D-fructofuranoside (Xyl-Fru), α-D-mannopyranosyl-β-D-fructofuranoside (Man-Fru), α-D-galactopyranosyl-β-D-fructofuranoside (Gal-Fru) and α-D-fucosylpyranosyl-β-D-fructofuranoside (Fuc-Fru), have been synthesized by transferring the fructosyl unit of sucrose to other monosaccharides using fructansucrase enzymes (243-246). Use of sucrose analogues as donor substrates by glucansucrase enzymes would allow transfer of a wider range of monosaccharides for oligosaccharide and glycoconjugate synthesis. Glucansucrases will need to be engineered to improve their ability to transfer such different glycosyl moieties. In a recent study, glucansucrase GTFA of *L. reuteri* 121 was reported to use α-D-allopyranosyl-β-D-fructofuranoside as donor substrate and to transfer an allose unit to several acceptor substrates (247). Using sucrose analogues as donor substrate provides a promising opportunity to extend the glycodiversity of glucansucrase products.

In view of all these recent developments, the prospects of producing tailor-made α-glucans by applying enzyme engineering, substrate engineering (sucrose analogues) and reaction engineering are promising. Glucansucrases thus are very interesting enzymes and biocatalysts, and hold strong potential for further industrial application in food, medicine and cosmetic industries.