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ORIGINAL ARTICLE

Glycosidic bond specificity of glucansucrases: on the role of acceptor substrate binding residues

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Abstract
Many lactic acid bacteria produce extracellular α-glucan polysaccharides using a glucansucrase and sucrose as glucose donor. The structure and the physicochemical properties of the α-glucans produced are determined by the nature of the glucansucrase. Typically, the α-glucans contain two types of α-glycosidic linkages, for example, (α1-2), (α1-3), (α1-4) or (α1-6), which may be randomly or regularly distributed. Usually, the α-glucan chains are also branched, which gives rise to an additional level of complexity. Even though the first crystal structure was reported in 2010, our current understanding of the structure–function relationships of glucansucrases is not advanced enough to predict the α-glucan specificity from the sequence alone. Nevertheless, based on sequence alignments and site-directed mutagenesis, a few amino acid residues have been identified as being important for the glycosidic bond specificity of glucansucrases. A new development in GH70 research was the identification of a cluster of α-glucan disproportionating enzymes. Here, we discuss the current insights into the structure–function relationships of GH70 enzymes in the light of the recently determined crystal structure of glucansucrases.

Keywords: *alternansucrase, dextransucrase, glycoside hydrolase, reuteransucrase, lactic acid bacteria, protein engineering*

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Introduction

Many bacteria form a biofilm matrix to protect them from environmental stress and to keep extracellular digestive enzymes in the vicinity of the cells, thus ensuring that the extracellular enzymes are not providing nutrients to competing organisms (Flemming & Wingender 2010; Walter et al. 2008; Kaditzky et al. 2008). Moreover, biofilms are key to surface colonization, such as tooth enamel colonization by *Streptococcus* species (Nobbs et al. 2009; Freitas et al. 2011). Biofilms are typically composed of polymeric compounds, for example, carbohydrates, proteins and DNA. Several lactic acid bacteria form biofilms that are largely composed of α-glucan polymers, which are synthesized from sucrose by an extracellular glucansucrase (GS) (Schwab et al. 2007; Anwar et al. 2010; Leathers & Bischoff 2011). In this review, we focus on the structure–function relationships of GSs and the structural properties of the α-glucan produced by this class of enzymes.

Glucansucrases

GSs are α-glucan synthesizing enzymes of the glycoside hydrolase family 70 (GH70) (Cantarel et al. 2009), a group of extracellular enzymes produced predominantly by *Lactobacillales* bacteria (van Hijum et al. 2006). These enzymes are also known as glucosyltransferases, abbreviated as 'GTF', because they synthesize α-glucan polymers using the glucose unit of sucrose. GH70 enzymes are evolutionarily closely related to the GH13 and GH77 enzymes and together they form the GH-H clan according to the Carbohydrate-Active Enzymes (CAZy) (http://www.cazy.org) (Cantarel et al. 2009) classification system. The GH-H clan encompasses a large group of enzymes acting on α-glucan carbohydrates such as starch, glycogen, pullulan, sucrose, etc. Well known enzymes of the GH-H clan are α-amylase, branching enzyme, cyclodextrin glucanotransferase, pullulanase and amylomaltase (MacGregor et al. 2001; Kuriki & Imanaka 1999; van der Maarel et al. 2002;

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Kelly et al. 2009b; Palomo et al. 2011). GSs are intensively studied as they are found in many probiotic bacteria, and they are thought to contribute to the probiotic properties via the α-glucan products they make. Moreover, there are indications that the α-glucan products of GSs have prebiotic activities and may act as anti-adhesives hampering colonization by harmful bacteria (Wang et al. 2010).

The catalytic cycle of glucansucrases

Catalysis by GSs is generally believed to be a two-step process, which starts with the binding of the substrate sucrose and the cleavage of the (α1-2)-glycosidic linkage between the glucose and fructose moieties of sucrose yielding a covalent glycosyl-enzyme intermediate. In the catalytic machinery, Asp1025 acts as the nucleophile forming a covalent linkage with the glycosyl moiety, and Glu1063 acts as a general acid/base catalyst (amino acid numbering according to GTF180 of *Lactobacillus reuteri* 180, unless stated otherwise). Following the departure of fructose from the +1 acceptor subsite, an acceptor substrate can enter the acceptor subsites to attack the covalent glycosyl-enzyme intermediate. The resulting product again has an α-glycosidic linkage. The existence of the covalent glycosyl-enzyme intermediate has not yet been demonstrated experimentally for a GH70 enzyme; however, its existence has convincingly been demonstrated by protein crystallography for the GH13 enzymes, cyclodextrin glucanotransferase (Uitdehaag et al. 1999) and amylosucrase (Jensen et al. 2004) and for the GH77 enzyme, amylomaltase (Barends et al. 2007), which also belong to the GH-H clan of glycoside hydrolases.

Glucansucrase reactions

GSs basically catalyse three types of reaction, namely polymerization (*e.g.* α-glucan formation), hydrolysis and an acceptor reaction. All three reactions start with cleavage of the (α1-2) bond of sucrose and the formation of the glycosyl-enzyme intermediate, and it is the nature of the incoming acceptor substrate, which determines which reaction is catalysed. Of the possible reactions, hydrolysis is the simplest, converting sucrose to glucose and fructose using water as acceptor.

In the polymerization reaction, the glycosyl intermediate is transferred to a growing α-glucan chain. The architecture of the GS active site determines how the incoming glucan chain enters the +1 acceptor subsite and, thus, which glycosidic linkage is synthesized. All four α-glycosidic bond reaction specificities have been observed among GSs, though typically GSs form two types of glycosidic linkages only, for example, (α1-3) and (α1-6), (α1-4) and (α1-6) or (α1-2) and (α1-6). The next level of complexity arises from the fact that the α-glucan products formed are branched, which implies that the acceptor subsites not only can accommodate the non-reducing end of a glucan chain, resulting in chain elongation, but also the glucan chain itself to create a branch. How GSs can specifically recognize and orient acceptor molecules to create highly complex α-glucans is a very intriguing question. Although modelling of acceptor molecules in the active site has provided some clues (Vujicic-Zagar et al. 2010), our current understanding of the glycosidic bond specificity of GSs is only poorly developed.

GSs can also transfer the glycosyl intermediate to a wide variety of hydroxyl-group containing molecules in a so called acceptor reaction, which is useful in (chemo-) enzymatic synthesis, as it prevents all kind of difficulties regarding stereo- and regio-specificity associated with the chemical synthesis of (hybrid) carbohydrate compounds (Hanson et al. 2004; Homann et al. 2009a,b; Leemhuis et al. 2010). The acceptor substrate promiscuity of GSs has been used to elongate short oligosaccharides (Cote & Sheng 2006), to label dextran with ^{13}C-glucose (Mukasa et al. 2001), to glucosylate gentiobiose (Cote 2009a), raffinose (Cote 2009b), nigero-oligosaccharides (Komatsu et al. 2011), flavonoids (Bertrand et al. 2006), hydroquinones (Seo et al. 2009), salicin and phenol (Seo et al. 2005), primary alcohols (Seibel et al. 2006a), methyl, alkyl, butyl and octyl-D-glucopyranosides (de Segura et al. 2006; Richard et al. 2003; Kim et al. 2009) and to synthesize branched thiooligosaccharides (Hellmuth et al. 2007). Another well-known product of the acceptor reaction is the sucrose isomer leucrose (5-*O*-α-D-glucopyranosyl-D-fructose), which is formed as a by-product in α-glucan synthesis, when fructose acts as acceptor with its 5-hydroxyl group. The big advantage of using GSs in chemo-enzymatic synthesis is that they use the cheap compound sucrose as glucose donor, whereas most enzymes used in glycosylation reactions use expensive UDP-glucose or ADP-glucose as glucose donor (Monsan et al. 2010; Seibel et al. 2006b). For the same reason, other glycoside hydrolases have also been applied in carbohydrate synthesis (Desmet & Soetaert 2011), such as the GH13 enzymes amylosucrase (Monsan et al. 2010) and cyclodextrin glucanotransferase (Leemhuis et al. 2010); even though these two enzymes belong to the class of glycoside hydrolases their main activity is transglycosylation (Kelly et al. 2008). The advantage of GSs, and some other glycoside hydrolases,

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is that they do not act on the products they synthesize, in contrast to, for example, cyclodextrin glucanotransferase, which disproportionates its own products.

Glucansucrase products

GSs synthesize α-glucan oligosaccharides and polymers by the stepwise addition of glucose units to a growing α-glucan chain. Theoretically, four types of α-glycosidic bonds can be formed between two glucose moieties, namely (α1-2), (α1-3), (α1-4) or (α1-6). All four α-glycosidic bonds have been observed in α-glucan products made by different GSs, though it must be noted that only a single (α1-2) producing enzyme has been found so far, DSR-E from *Leuconostoc mesenteroides* NRRL B-1299 (Fabre et al. 2005). Moreover, the (α1-2) bonds are formed as single glucose branches of dextrans only, whereas (α1-3), (α1-4) and (α1-6) bonds may connect glucose moieties in the main chain as well as form branches. Common products are dextrans with a backbone of (α1-6) linkages, mutans with mainly (α1-3) linkages, reuteran with randomly distributed (α1-4) or (α1-6) linkages, alternan with alternating (α1-3) and (α1-6) bonds. The α-glucan properties are determined by the types of α-glycosidic linkages, the ratios of α-glycosidic linkage types, degree of branching, or branch length, whether or not the branches are branched as well, and the molecular size of the polymer. Obviously, the structure of the α-glucan is largely determined by the specificity of the GS used to make them, though the reaction conditions also contribute to the final product properties.

The types and the ratio of the linkages in α-glucans can be simply determined using NMR spectroscopy and/or methylation analysis. The latter method also provides the percentage of terminal residues and branches. However, this type of experiment only provides data on the average composition and not whether the α-glucan contains repeating units or is made up of distinct structural regions. Such insights can be obtained by partial acid hydrolysis and the subsequent purification of all compounds obtained followed by their structural elucidation. This large amount of work has been done for a few α-glucan polymers, yielding structural models for the dextran made by GTF-180 of *L. reuteri* 180 (van Leeuwen 2008b) and the reuteran produced by GTF-A of *L. reuteri* 121 (van Leeuwen 2008c). Once such methodologies are running in a laboratory, it becomes easier to elucidate the fine structure of α-glucans made by other GSs, as has been reported for several GTF-180 mutants (van Leeuwen 2009, 2008a).

The structure of glucansucrases

The CAZy database currently (July 2011) lists 129 GH70 sequences, 47 of which have been biochemically characterised to some extent (Cantarel et al. 2009). GSs are large multi-domain extracellular enzymes. Basically, they consist of an N-terminal signal sequence, an N-terminal region, a catalytic region and a C-terminal region (van Hijum et al. 2006). The size of the N-terminal region is highly variable, it may carry a few sequence repeats, and its function remains unclear. Deletion studies have revealed that large parts of the N-terminal region may be omitted without losing *in vitro* α-glucan synthesizing activity (Kralj et al. 2004; Moulis et al. 2006; Monchois et al. 1999a). The GS catalytic region shows high sequence similarity to the GH13 and GH77 family members, though it is circularly permutated compared to the GH13 and GH77 sequences (Swistowska et al. 2007), and it carries the conserved sequence motifs of GSs. The C-terminal region possesses sequence repeats (Janecek et al. 2000), which provide the C-terminal region with α-glucan binding activity as demonstrated by a number of site-directed mutagenesis and truncation studies (Kingston et al. 2002; Lis et al. 1995; Monchois et al. 1998; Shah et al. 2004). The function of α-glucan binding activity for α-glucan synthesis is, however, not well understood yet.

Recently, our understanding of GSs has made a big leap forward with the elucidation of the first crystal structure of a GS enzyme, GTF-180 from *Lactobacillus reuteri* 180 (Vujicic-Zagar et al. 2010), followed in 2011 by the second crystal structure of a GS protein, GTF-SI from *Streptococcus mutans* (Ito et al. 2011). Both GTF-180 and GTF-SI form α-glucan polymers with (α1-6) and (α1-3) linkages (van Leeuwen et al. 2008b; Ito et al. 2011), but their products are clearly different. GTF-180 makes a water-soluble α-glucan with about 69% (α1-6) linkages, whereas GTF-SI makes an α-glucan with about 90% (α1-3) bonds, which is poorly soluble in water. Notably, in both cases N-terminally truncated proteins were used. The elucidation of a third structure, a truncated form of DSR-E of *Leuconostoc mesenteroides* NRRL B-1299, which makes (α1-2) branches was recently reported at CBM9 in Lisbon by Remaud-Simeon (Remaud-Simeon et al. 2011).

The GS crystal structures confirmed that GH70 enzymes are structurally very similar to GH13 and GH77 enzymes. The catalytic A domain has a $(\beta/\alpha)_8$-barrel fold, which, together with the B- and C-domains (Figure 1), forms a scaffold found in most GH13 and GH77 enzymes. The catalytic residues are located in the A-domain, and also the B-domain provides residues interacting with carbohydrates near the catalytic site. The function of the

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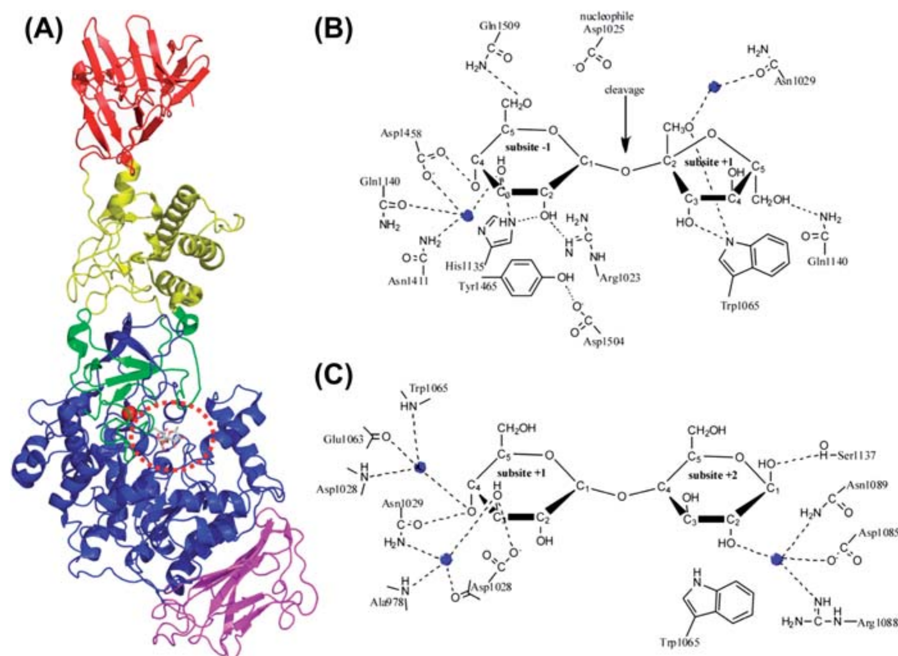

Figure 1. Crystal structure of the *L. reuteri* 180 GTF-180 glucansucrase with a sucrose molecule bound at the active site (panel A; protein data bank file 3HZ3). Panels B and C give a schematic overview of the interactions between GTF-180 and sucrose (B) and maltose (C), respectively, bound at the active site. The interactions are based on the protein data bank files 3HZ3 (sucrose) and 3KLL (maltose). Note that Tyr1465 makes a stacking interaction with the glycosyl at subsite -1 in the sucrose bound structure, that Tyr1065 makes a stacking interaction with the glycosyl at subsite $+2$ in the maltose bound structure, and that waters are shown as dots. The A, B, C, IV and V domains are shown in blue, green, pink, yellow and red, respectively. The structure contains a Ca^{2+} ion (red sphere) and a sucrose molecule in the active site (sticks). Figure 1A was created using PyMOL (DeLano, 2002).

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C-domain remains to be elucidated; even in GH13 and GH77 enzymes, little is known on the function of this domain. Only for *Bacillus circulans* cyclodextrin glucanotransferase and barley α-amylase 1 (both GH13), there are some experimental evidences showing that the C-domain is involved in glucan binding (Bozonnet et al. 2007; Lawson et al. 1994). In addition to the core structure formed by the A, B and C-domains, GS proteins possess domains IV and V; the structure of domain V has only been elucidated for GTF-180. The function of domain IV is unknown, and no structural homologues are known. Domain V is thought to be a glucan binding domain as it carries the sequence repeats found in the C-terminal region of GS sequences, which were shown to participate in glucan binding (Kingston et al. 2002; Lis et al. 1995; Monchois et al. 1998; Shah et al. 2004). A unique feature of the GS structures is that the polypeptide chain folds in such a way that domains A, B, IV and V are built from both an N- and a C-terminal stretch (Vujicic-Zagar et al. 2010).

Protein engineering of glucansucrase product specificity

Even though GSs have been the subject of mutagenesis studies since the 1990s, the full potential of protein engineering has not been exploited as all mutations reported so far were solely designed on the basis of sequence alignments, due to the absence of a three-dimensional structure. Although a number of residues have been identified as being important for the glycosidic bond specificity of GSs, it was difficult to explain how these mutations altered the reaction specificity. However, by including results of structural and protein engineering studies from evolutionary related GH13 and GH77 enzymes, such as amylosucrase, cyclodextrin glucanotransferase, amylomaltase and α-amylase (Kumar 2010; Kaper et al. 2007; Sarcabal et al. 2000; Leemhuis et al. 2003a,b, 2004), informative attempts to relate structural elements and product specificity in GS were made. Nevertheless, the recent elucidation of the first two crystal structures of GSs (Vujicic-Zagar et al. 2010; Ito et al. 2011) allows a better structural explanation of the effects seen in mutational studies of GSs. Moreover, the three-dimensional structures enable protein engineers to design new mutant GSs in their efforts to expand insights in the structure – function relationship of GH70 enzymes. Ultimately, this is expected to lead to the design of GS variants capable of synthesizing any type of desirable α-glucan regardless of linkage type and degree of branching of the polymer product. In the following paragraphs, we

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will address GS mutations, which affect linkage type specificity.

Because all GS reactions start with the formation of a covalent glycosyl-enzyme intermediate, it is the way in which the acceptor α-glucan is guided towards the reaction centre that determines the type of α-glycosidic linkage formed. Therefore, residues forming acceptor binding subsites (+1, +2, etc.) are likely to be crucial in determining the glycosidic linkage type in the product. In contrast, the main function of donor subsite −1 is the formation of the covalent glycosyl-enzyme intermediate. Although essential for catalysis, it is probably not important for determining reaction specificity; therefore, residues of subsite −1 are not considered in the discussion below.

Acceptor subsite +1

The +1 subsite has two functions. First, it binds the fructose moiety during covalent glycosyl-enzyme intermediate formation, and as such, it is important for general enzyme activity. Next, an acceptor will approach the catalytic centre by binding in the +1 subsite to attack the covalent intermediate forming the elongated α-glucan product. Obviously, the +1 subsite architecture determines the binding mode(s) of α-glucan acceptors and as such influences the type of linkage formed. In addition, GSs also create branches in the α-glucans, demonstrating that they can hold bulky acceptor substrates. This suggests that specific enzyme–substrate interactions favour the formation of particular linkages. The latter seems especially likely for extending the non-reducing end of glucan chains, as there is ample space allowing for multiple binding modes of acceptor sugars. The involvement of specific enzyme–substrate interactions also fits best with a model in which the +2 and more remote subsites play an important role in reaction specificity. This is supported by the effects observed when residues forming subsite +2 are mutated, as discussed below. For subsite +1, only two residues have been mutated so far, W1065 and Q1140, both of which form H-bonds with the fructosyl moiety of sucrose at subsite +1 (Figure 1). The first residue, W1065, also makes an H-bond with a maltose molecule bound at the +1/+2 subsites (Figure 1). At the corresponding position, a W491G mutation completely inactivated GTF-I of *S. mutans* GS-5 (Table I). Interestingly, DSR-E of *Leuconostoc mesenteroides* NRRL B-1299 is the only GS lacking this tryptophan and possesses a glycine at this position, and it is the only GS known to synthesize (α1-2) bonds. For the second residue, a Q1140E mutation introduced about 3% of (α1-4) forming activity into GTF-180 (van Leeuwen et al. 2009), which naturally forms (α1-6) and (α1-3) bonds only. Two other substitutions (Q1140A and Q1140H) in the same enzyme raised the (α1-6):(α1-3) ratio of the α-glucan products from 2.2 to over 5-fold. Based on the limited amount of experimental data, it appears that W1065 is crucial for catalysis because it forms hydrogen bonds with the O3 and O6 atoms of the fructosyl at subsite + 1 (Figure 1) and that Q1140 influences the glycosidic linkage reaction specificity. However, nothing is known on the role of the other residues at subsite + 1, and more mutation studies are needed to explore this in detail. The best way forward would be to vary the side chain and the ability to interact with carbohydrates of the residues lining the + 1 subsite (Figure 1).

Acceptor subsite +2

Subsite +2 is also important for the type of α-glucan made by GSs. This was revealed by the large impact that some mutations have on the α-glucan structure (Table I), though the complete picture cannot yet be drawn due to lack of data. At subsite +2 Ser1137 makes an H-bond with maltose (Figure 1). Whereas most GSs have a serine at this position, the *L. reuteri* 121 GTF-A that makes α(1-4) and α(1-6) bonds possesses an asparagine at the equivalent position. N1134S and N1134A mutations (position 1137 in GTF-180) increased the fraction of (α1-6) bonds at the expense of (α1-4) bonds (Table I), whereas glutamine, glycine, tyrosine or histidine substitutions hardly affected the glycosidic bond ratio (Kralj et al. 2006). The opposite mutation, S1137N introduced some (α1-4) forming activity in GTF-180, while the wild-type GTF-180 makes (α1-6) and (α1-3) bonds only. The same position has also been targeted by directed evolution in DSR-S of *L. mesenteroides* NRRL B-512F, and the S512C mutation (position 1137 in GTF-180) was found to increase the percentage of (α1-3) bonds from 1.4 to 2.2% at the expense of (α1-6) bonds (Irague 2011). The other subsite +2 residue investigated (Asp1141 in GTF-180) is not conserved among GSs, although a threonine is the most common residue at this position. Mutagenesis data suggests that the nature of the side chain of the amino acid at this position is a key factor in determining the percentage of (α1-3) bonds in the glucan synthesized by GSs (Table I), as a D569T mutation in GTF-I of *S. mutans* strongly increased the fraction of soluble α-glucan, and a T589E mutation drastically lowered the percentage of soluble α-glucan made by GTF-S of *S. mutans* (Table I). Here the assumption is made that glucans high in (α1-3) bonds are insoluble in water. Acceptor

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Table I. Important and mutated residues in glucansucrases. Amino acids are numbered according the *L. reuteri* 180 GTF180 and *S. mutants* GTF-SI structures (Vujicic-Zagar 2010; Ito 2011).

Location	Residue[e]	Mutations (activity%; enzyme)	Function/effect on product	Reference
Subsite −1				
1023/475	**R**[a]			
1025/477	**D**[a]	D551N (dead; DSRS)	Catalytic nucleophile	(Monchois 1997)
1063/515	**E**[a]		Acid/base	
1135/587	**H**[a]	H300F (0.1%; GTF-R)		(Swistowska 2007)
		H561G (dead; GTF-I)		(Tsumori 1997)
		H661R (0.5%; DSRS)		(Monchois 1997)
1136/588	**D**[a]	D301E (0.3%; GTF-R)	Transition state stabilizer	(Swistowska 2007)
1458/909	**D**			
1465/916	**Y**	Y965S (0.9%; GTF-R)		(Wittrock 2008)
1504/955	**D**[a,b]	D1004A (6%; GTF-R)		(Wittrock 2008)
1509/960	**Q**[a,g]	Q937H (1%; GTF-I^{Sd})		(Monchois 2000)
Subsite + 1				
938/380	**L**,D,I		van der Waals	
981/433	**L**		van der Waals	
1028/480	**D**			
1029/481	**N**			
1065/517	**W**,G	W491G (dead; GTF-I)		(Tsumori 1997)
1140/592	**Q**	Q1140E ([c]%; GTF-180)	0→2% (α1-4); 24→16 (α1-3)	(van Leeuwen 2009)
		Q1140A ([c]%; GTF-180)	24→6 (α1-3); 52→69 (α1-6)	(van Leeuwen 2009)
		Q1140H ([c]%; GTF-180)	24→8 (α1-3); 52→76 (α1-6)	(van Leeuwen 2009)
Subsite + 2				
978/430	Y,F,Q,S,**A**			
1028/480	**D**			
1065/517	**W**, G	W491G (dead; GTF-I)		(Tsumori 1997)
1088/540	**R**,H,K,S,T			
1137/589	**S**,N,G	N1134S (193%, GTF-A)	54→18% (α1-4); 46→82% (α1-6)	(Kralj 2006)
		N1134A (263%, GTF-A)	54→36% (α1-4); 46→64% (α1-6)	(Kralj 2006)
		S512C ([c]%; DSRS)	1.4→2.2% (α1-3)	(Irague 2011)
		S1137N ([c]%; GTF-180)	0→3% (α1-4)	(van Leeuwen 2009)
1141/593	T,**D**,S,A,G	D569T ([c]%; GTF-I)	0→24% soluble glucan	(Shimamura 1994)
		T589E ([c]%; GTF-S)	86→2% soluble glucan	(Shimamura 1994)
Subsite + 3				
1088/540	**R**,H,K,S,T			
Other important residues				
827/275	**K**,A,T,R	T350K (116%; DSRS)	70→84% (α1-6); domain IV	(Funane 2005)
926/370	**W**	W344L (4%; GTF-I^{Sd})	No effect	(Monchois 1999b)
931/375	**E**	E349L (41%; GTF-I^{Sd})	No effect	(Monchois 1999b)
934/[d]	N,**D**,K,S,G,T,L,Y	S455K (121%; DSRS)	70→86% (α1-6)	(Funane 2005)
939/381	H,**Q**	H355V (14%; GTF-I^{Sd})	6→9% (α1-6)	(Monchois 1999b)
980/432	**F**,L,Y,M	F353W ([c]%; DSRS)	1.4→3.1% (α1-3)	(Irague 2011)
985/437	**D**	D511N (dead; DSRS)		(Monchois 1997)
987/439	**D**	D513N (4%; DSRS)	No effect on glucan structure	(Monchois 1997)
1031/483	**D**,N,S	D457N ([c]%; GTF-I)	0→37% soluble glucan	(Shimamura 1994)
1145/597	N,R,**Q**,E,D,S	D571K ([c]%; GTF-I)	0→18% soluble glucan	(Shimamura 1994)
1354/[d]	Q,K,**R**,Y,A	K779Q ([c]%; GTF-I)	0→3% soluble glucan	(Shimamura 1994)
1506/957	**V**	V1006A (6%; GTF-R)	Nd	(Wittrock 2008)
1507/958	**P**	P1007A (127%; GTF-R)	Nd	(Wittrock 2008)
1511/962	**Y**	Y1011A (14%; GTF-R)	Nd	(Wittrock 2008)
1589/1041	**K**,T	K1014T ([c]%; GTF-I)	0→14% soluble glucan	(Shimamura 1994)

[a]These residues correspond to the seven conserved GH13 residues (MacGregor 2001).
[b]Asp1504 stabilizes Tyr1465, which has a stacking interaction with the glycosyl moiety at subsite −1.
[c]Only reported to be active.
[d]Region with variable sequence regarding composition and number of amino acids.
[e]Residues in GTF180 are indicated in **bold** font, and in GTF-SI underlined.
[f]DSRS, *L. mesenteroides* NRRL B-512F; GTF-A, *L. reuteri* 121; GTF-180, *L. reuteri* 180; GTF-S, *S. mutans* GS5; GTF-I, *S. mutans* GS-5; GTF-I^{Sd}, *S. downei* MFe28; GTF-R, *S. oralis* ATCC 10557.
[g]Note that nearly all GH13 enzymes have a His at the equivalent position.

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subsite +2 thus plays a crucial role in α-glucan synthesis, with Asn1137 favouring (α1-4) bond formation and a threonine at position 1141 favouring the synthesis of a soluble α-glucan. It remains important to pin-point the roles of the other subsite +2 residues, Ala978, Asp1028 and Arg1088, in α-glucan synthesis, to better understand how GSs work.

Other mutations affecting glucansucrase reaction specificity

In a directed evolution experiment with DSR-S of *L. mesenteroides*, a F353W mutation (position 980 in GTF-180) was found to slightly increase the percentage of (α1-3) bonds (from 1.4 to 3.1%) at the expense of (α1-6) bonds (Irague et al. 2011) (Table I). In GTF-180, the phenylalanine side chain points away from the acceptor subsites, but it is a direct neighbour of L981, which interacts with the substrate at subsite +1 (Vujicic-Zagar et al. 2010). Because the tryptophan is larger than the phenylalanine, this mutation is expected to somewhat change the position of L981, which in turn is likely to influence the reaction specificity.

However, for other mutations reported in the literature it is unclear how they affect the glucan product specificity, as these mutations are very far from the active site and are unlikely to be located at a glucan binding region. For example, a T350K mutation in domain IV (position 827 in GTF-180) of the *L. mesenteroides* DSRS enhanced the fraction of (α1-6) linkages from 70 to 84%. The B-domain mutations S455K in DSRS of *L. mesenteroides* and H355V in GTF-I of *S. downei* (positions 934 and 939 in GTF-180) also raised the percentage of (α1-6) linkages formed in the α-glucan product (Table I). As these two residues are in a loop of the B-domain that forms part of the outer regions of the substrate binding cleft, the mutations may affect the way acceptor substrates are oriented towards the active site and as such explain the effects on glycosidic bond specificity. A D571K mutation in the same GTF-I (position 1145 in GTF-180) also raised the soluble glucan fraction from 0 to 18% (Table I). This residue is located in an α-helix also containing the subsite +1 and +2 residues Q1140 and D1141, which are known to contribute to the glycosidic linkage specificity of GSs (Table I). Then there are three mutations in *L. mesenteroides* GTF-I, D457N, K779Q and K1014T (positions 1031, 1353 and 1589 in GTF-180), which produce more soluble α-glucan (Table I). However, all three mutations are too far away from the catalytic centre to explain the altered reaction specificity.

Double/triple mutations

Several articles report GS variants carrying multiple mutations that produce α-glucans distinct from those formed by the wild-type enzyme. For example, GTF180 of *L. reuteri* 180, synthesizing an α-glucan with about 69% (α1-6) and 31% (α1-3) linkages, started making 12% (α1-4) bonds upon the V1027P/S1137N/A1139S mutations (van Leeuwen et al. 2008a). A follow-up study demonstrated that S1137V is the key mutation for introducing the (α1-4) forming activity (van Leeuwen et al. 2009). From the available structure of GTF180, it is clear that this mutation is located at subsite +2 residues (Figure 1).

The same residues were later targeted in the GS of *L. mesenteroides* B-1299CB4 (DSRBCB4), which makes mainly (α1-6) α-glucan. This enzyme gained 11% (α1-3) and some 2% (α1-4) forming activity upon the S642N/E643V/V644S mutations (Kang et al. 2011). The (α1-4) forming activity was raised to 11% by including V532P/V535I mutations (positions 1027 and 1030 in GTF-180), whereas the double mutant V532P/V535I was unable to change the reaction specificity of the enzyme. Although the individual mutations were not constructed it appears most likely that the S642N substitution (position 1137 in GTF180) is the determining factor, as this residue interacts with carbohydrates at subsite +2 in GS structures (Figure 1). A T350K/S455K mutation (positions 827 and 934 in GTF-180) gave the *L. mesenteroides* DSR-S the ability to create (α1-2) branches on dextrans (Funane et al. 2005), which is quite a rare linkage type for GSs. So far, DSRE of *L. mesenteroides* NRRL B-1299 is the only GS known to make (α1-2) linkages (Fabre et al. 2005). Although both single mutations altered the linkage distribution of the glucan product (Table I), the single mutations on their own are not enough to introduce (α1-2) forming activity. Another combination in DSRS, H463R/T464D/S512T (positions 1088, 1089 and 1137 in GTF-180), increased the fraction of (α1-3) from 1.4 to 6.9% at the expense of (α1-6) linkages (Irague et al. 2011). Because all three residues (indirectly) interact with a maltose molecule bound at the acceptor subsite, at least in GTF-180 (Figure 1), and the individual mutants were not constructed, it is not known whether all three changes are required to obtain this effect. However, from other mutation studies, it is known that the glucan structure can be altered by a substitution at the equivalent position 1137 only (Table I).

Overall, the mutagenesis experiments in GSs have demonstrated that their glycosidic linkage specificity is particularly sensitive to mutations at the acceptor substrate binding subsites +1 and +2, and occasionally also to remote mutations.

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Recent developments: disproportionating GH70 enzymes

Until recently, it was believed that all GH70 enzymes acted on sucrose. However, recently, it was reported that *L. reuteri* 121 possesses a GH70 enzyme (called GTFB), which disproportionates (α1-4)-oligosaccharides efficiently, but does not utilize sucrose as substrate, even though it shares 46% sequence identity with the regular GS GTF-A of the same *L. reuteri* 121 strain (Kralj et al. 2011). Like the GSs, GTFB transfers single glucose moieties and attaches them to growing α-glucan chains, predominantly via (α1-6) linkages, using (α1-4)-oligosaccharides as glucose donor. It has been known for decades that several GSs have a very low (promiscuous) disproportionation activity (Binder et al. 1983), even though GSs are optimized to cleave the high energy glycosidic linkage in sucrose instead of the stable (α1-4) glycosidic linkage. Therefore, it was surprising to find a GH70 enzyme capable of disproportionating (α1-4)-oligosaccharides at a rate of over 10 times per second. Another remarkable feature of the *L. reuteri* 121 GTFB enzyme is that it creates linear products (Kralj et al. 2011), whereas GSs typically form branched α-glucan polymers. These observations suggest that GTFB may be an evolutionary intermediate between GH70 and GH13 enzymes (Kralj et al. 2011). Phylogenetic analysis suggests that disproportionating-type GH70 enzymes are also present in the *L. reuteri* strains ML4, DSM 20016 and JCM 1112. It is exciting to explore whether these sequences also encode disproportionating-type GH70 enzymes, and to determine their glycosidic linkage type specificity. If the linkage specificity is different from the *L. reuteri* 121 GTFB the α-glucan product range made by GH70 enzymes is even further expanded. The key question is what structural features provide the *L. reuteri* 121 GTFB with its distinct substrate specificity? Sequence alignments show that *L. reuteri* 121 GTFB is somewhat different in the residues forming the acceptor subsites of GSs. Most notable are the replacement of W1065 by a tyrosine, and differences in the 1137-1141 loop, with position 1137, 1140 and 1141 known to be important for glycosidic linkage specificity of GSs (Table I). Mutation of these residues in *L. reuteri* 121 GTFB, and analysis of the products made by mutant enzymes, is currently being performed to find out whether this region is responsible for the unique reaction specificity of the enzyme, or that the key factors are located further away from the active site.

Future perspectives

Matching of the mutagenesis data of the last two decades with the information from the recently elucidated crystal structures of GSs clearly demonstrates the importance of the acceptor subsites + 1 and + 2 for glycosidic linkage specificity. On the other hand, the crystal structures so far do not explain the effects of mutations more distant from the active site. A three dimensional GS structure with a long carbohydrate bound in the substrate binding cleft would be of great help to extend our insights. Until now, all mutations were designed on the basis of sequence alignments and only a few regions of GSs have been targeted by mutagenesis. This limits a deep understanding of the structure-function relationships of GH70 enzymes. We believe that the best way forward to expand the structural insights in this class of enzymes is first to study all acceptor subsite residues by site-directed mutagenesis, and secondly, to identify all residues contributing to glycosidic linkage specificity by using random mutagenesis and high-throughput screening (e.g. directed evolution (Turner 2009; Leemhuis et al. 2009)). Numerous studies have demonstrated that directed evolution is an extremely powerful strategy to identify residues relevant for reaction specificity (Kelly et al. 2009a; Otten et al. 2010; Reetz et al. 2008; Aharoni et al. 2006; Emond et al. 2007), especially for residues that do not directly interact with substrate. Indeed, it has been postulated that substitutions at or near the active site can have too dramatic effects on the reaction specificity and that second shell mutations, or even more distant mutations, can lead to more subtle changes in the active site region, concomitant with subtle or even large changes in reaction specificity of enzymes (Kirby & Hollfelder 2008). The key to success now is to have an effective way of assessing the structure of the α-glucans made by thousands of GS variants, which became feasible with the recently reported high-throughput NMR technology for carbohydrate active enzymes (Irague et al. 2011).

To fully understand the data to be generated from the above experiments, we rely on the elucidation of more three-dimensional structures, preferably of GSs synthesizing (α1-4) bonds, (α1-2) bonds (expected soon (Remaud-Simeon et al. 2011)) and of the disproportionating GH70 enzyme GTFB of *L. reuteri* 121. In addition to these structures, we really require GS structures with long acceptor substrates/products, as such structures will reveal the remote residues interacting with carbohydrates. Also the identification and characterizing of more GH70 enzymes (Fraga et al. 2011; Bounaix et al. 2010; Waldherr et al. 2010; Malik et al. 2009; Kang et al. 2009) contributes to a better understanding of GSs, as it enables protein engineers to relate sequences and reaction specificities.

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The ultimate aim of all these efforts is to obtain the engineering guidelines enabling the creation of GS variants capable of synthesizing any type of desirable α-glucan structure regarding linkage type(s), ratio of linkages within the product, degree of branching, size of the branches, secondary branching and the molecular size of the α-glucan. Such products are expected to be valuable because of their prebiotic properties.

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