Synthesis of xyloglucan oligo- and polysaccharides with glycosynthase technology

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ABSTRACT

Xyloglucans are polysaccharides found as storage polymers in seeds and tubers, and as cross-linking glycans in the cell wall of plants. Their structure is complex with intricate branching patterns, which contribute to the physical properties of the polysaccharide including its binding to and interaction with other glycans such as cellulose.

Xyloglucan is widely used in bulk quantities in the food, textile and paper making industries. With an increasing interest in technically more advanced applications of xyloglucan, such as novel biocomposites, there is a need to understand and control the properties and interactions of xyloglucan with other compounds, to decipher the relationship between xyloglucan structure and function, and in particular the effect of different branching patterns. However, due to the structural heterogeneity of the polysaccharide as obtained from natural sources, relevant studies have not been possible to perform in practise. This fact has stimulated an interest in synthetic methods to obtain xyloglucan mimics and analogs with well-defined structure and decoration patterns.

Glycosynthases are hydrolytically inactive mutant glycosidases that catalyse the formation of glycosidic linkages between glycosyl fluoride donors and glycoside acceptors. Since its first conception in 1998, the technology is emerging as a useful tool in the synthesis of large, complex polysaccharides. This thesis presents the generation and characterisation of glycosynthases based on xyloglucanase scaffolds for the synthesis of well-defined homogenous xyloglucan oligo- and polysaccharides with regular substitution patterns.

Keywords: glycosynthase, xyloglucan, xyloglucan endo-transglycosylase, retaining glycoside hydrolase, xyloglucanase, polysaccharide synthesis

SAMMANFATTNING

Xyloglukaner är polysackarider som förekommer som lagringskolhydrater i frön och rotknölar, och som bildar korslänkande glykankedjor i växters cellväggar. Strukturen är komplex och olika förgreningsmönster bidrar till polysackaridens fysikaliska egenskaper såsom bindning och interaktion med andra glykaner, till exempel cellulosa.

Xyloglukan används i stor omfattning i livsmedels-, textil- och pappersindustrin. I och med ett växande intresse för tekniskt mer avancerade användningsområden, såsom nya biokompositer, ökar behovet att förstå och kontrollera xyloglukanets egenskaper och dess interaktion med andra molekyler. Kunskap behövs om förhållandet mellan struktur och funktion hos xyloglukan, och framför allt effekten av de olika förgreningsmönstren. På grund av den heterogena strukturen hos polysackariden såsom den förekommer i naturen har sådana studier inte varit möjliga att genomföra i praktiken. Detta har väckt intresse för att framställa xyloglukananaloger med väldefinierad struktur och regelbundna förgreningsmönster med hjälp av syntetiska metoder.

Glykosyntaser är hydrolytiskt inaktiva muterade glykosidaser som katalyserar bildandet av glykosidbindningar mellan glykosylfluorider och acceptorglykosider. Tekniken uppfanns 1998, och utvecklas för närvarande till ett användbart verktyg för att syntetisera stora, komplexa polysackarider. I denna avhandling beskrivs produktion och karakterisering av xyloglukanasbaserade glykosyntaser för att syntetisera väldefinierade och homogena oligo- och polysackarider av xyloglukan med regelbundna förgreningsmönster.

"Sweets for my sweet,
        sugar for my honey"

- *The Searchers*

For D & G

LIST OF PUBLICATIONS

I   Kathleen Piens,* Maria Henriksson*, **Fredrika Gullfot**, Marie Lopez, Régis Fauré, Farid M. Ibatullin, Tuula T. Teeri, Hugues Driguez and Harry Brumer (2007). Glycosynthase activity of hybrid aspen xyloglucan endo-transglycosylase *Ptt*XET16-34 mutants. *Org. Biomol. Chem.* 5(24): 3971-3978. * *The authors contributed equally to the work*

II   **Fredrika Gullfot**, Farid M. Ibatullin, Gustav Sundqvist, Gideon Davies and Harry Brumer (2009). Functional characterization of xyloglucan glycosynthases from GH7, GH12 and GH16 scaffolds. Manuscript (submitted to *Biomacromolecules*).

The author's contribution:

Publication I: Experimental design and mathematical modelling, pH profiling and kinetic experiments with *Ptt*XET16-34 glycosynthases together with Maria Henriksson.

Publication II: Design, cloning and expression of *Tm*NXG1 glycosynthases, experimental design, characterisation incl. kinetics and product analysis of all presented glycosynthases. Writing of the manuscript incl. figures and tables.

Other publications not included in the thesis:

Pekka B. Mark, Martin Baumann, Jens Eklöf, **Fredrika Gullfot**, Gurvan Michel, Åsa Kallas, Tuula T. Teeri, Harry Brumer and Mirjam Czjzek (2008). Analysis of nasturtium *Tm*NXG1 complexes by crystallography and molecular dynamics provides detailed insight into substrate recognition by family GH16 xyloglucan endo-transglycosylases and endo-hydrolases. *Proteins: Structure, Function and Bioinformatics*, in press.

Laura von Schantz, **Fredrika Gullfot**, Sebastian Scheer, Lada Filonova, Lavinia Cicortas Gunnarsson, James E. Flint, Geoffrey Daniel, Eva Nordberg-Karlsson, Harry Brumer and Mats Ohlin (2009). Affinity maturation generates greatly improved xyloglucan-specific carbohydrate binding modules. Manuscript (submitted to *Biochemical Journal*).

LIST OF ABBREVIATIONS

CNP   Chloro nitrophenyl
DAST   Diethyl amino sulphur trifluoride
DMAP   Dimethyl aminopyridine
FRET   Fluorescence resonance energy transfer
Fuc   Fucose
Gal   Galactose
GDP   Guanosine diphosphate
GH   Glycoside hydrolase
Glc   Glucose
GT   Glycosyl transferase
HF   Hydrogen fluoride
HPAEC   High-pressure anion-exchange chromatography
MEA   Monoethanol amine
PAD   Pulsed amperometric detection
TEMPO   2,2,6,6-tetramethylpiperidine-1-oxy-4-yl
TLC   Thin layer chromatography
UDP   Uridine diphosphate
XET   Xyloglucan endo-transglycosylase
XGO   Xyloglucan-oligosaccharide
Xyl   Xylose

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1 INTRODUCTION

Polysaccharides, the most abundant biomass component on earth, are carbohydrate polymers playing many essential roles in nature. Together with proteins and lipids, they are the key building blocks of all things living. Their diversity and versatility with regards to physical and mechanical properties permit their application for a wide range of purposes. They are essential as components of structural biocomposites such as in the cell wall of plants or the exoskeleton of crustaceans, or as constituents of glycoconjugates such as glycoproteins and glycolipids that play crucial roles in cell signalling and recognition. They serve as energy stores in seeds and tubers, and have immunomodulating or antiviral properties in animals.

The diverse roles and functional properties of polysaccharides in nature carry promises of a vast field of commercial and scientific applications. This inherent potential has been extensively exploited by humans since prehistoric times, such as for cotton- or flax-based textiles, building materials such as wood or reeds, or texture-providing food ingredients. Many of these ancient uses have during history been replaced with modern synthetic compounds and materials, but with diminishing oil supplies and growing environmental concerns, there is a renewed interest for novel carbohydrate-based materials. The potential range is broad, from advanced building components and environmentally friendly consumables to biomedical applications. As a consequence, poly- and oligosaccharides are of interest in a variety of fields, sectors and disciplines, from food technology and cosmetics, over drug development, to materials science and research, both fundamental and applied.

In nature, polysaccharides are synthesised from their respective carbohydrate building blocks by a variety of enzymes such as glycosyl transferases, transglycosidases and phosphorylases. The natural products are often quite heterogeneous with regards to their structure, with e.g. more or less random branching patterns, derivatisations or mixed linkages. For some applications, highly ordered molecular and supramolecular structures are essential, often including requirements for chemical functionalisation. Since these needs are often not fulfilled by purified natural polysaccharides, there is a great interest in synthetic methods for obtaining complex polysaccharides with specific, regular and well-defined structures (Kobayashi et al. 2001; Kobayashi and Ohmae 2006). Such polysaccharides could be homogenous equivalents to their

natural counterparts, but also completely novel carbohydrates with compositions and microstructures not observed in nature (Faijes et al. 2004; Kobayashi and Ohmae 2006).

However, it is difficult and often impossible to build polysaccharides with conventional synthetic approaches based on organic chemistry. The strict demands on regio- and stereoselectivity require complex protection and activation strategies, and the selective formation and hydrolysis of glycosidic bonds is chemically difficult (Daines et al. 2004). As a consequence, interest has turned towards biocatalysis and enzymatic methods for polysaccharide synthesis, in order to meet the demands for readily available, tailor-made poly- and oligosaccharides. The naturally available enzymes of choice for these purposes would be glycoside hydrolases and glycosyl transferases (Thiem 1995), a vast and diverse group of enzymes in terms of specificities, mechanisms, substrates and products. It has been estimated that glycoside hydrolases and glycosyl transferases make up 1–2 % of an organism's genes (Henrissat et al. 2001), indicating that nature knows innumerable ways to handle the advanced synthetic task of polysaccharide polymerisation, thus offering a vast potential repertoire for the biosynthetic chemist in the carbohydrate field.

1.1 Glycosyl transferases

Glycosyl transferases catalyse the transfer of sugar residues to an acceptor molecule such as a growing polysaccharide chain, or an alternate acceptor such as a lipid moiety or a protein. Non-processive glycosyl transferases condense a single activated sugar residue to its acceptor, while processive glycosyl transferases transfer multiple sugar residues in a serial, one-by-one fashion. Non-processive glycosyl transferases are often involved in the biosynthesis of glycocompounds such as the glycosylation of proteins, while processive glycosyl transferases synthesise larger polysaccharides such as cellulose, starches and mannans (Carpita and McCann 2000).

The substrates of glycosyl transferases are phosphate sugars that are activated by nucleotide groups, or lipid-linked phosphate sugars. The main sugar-activating nucleotide groups in the synthesis of plant cell wall polysaccharides are uridine diphosphate (UDP) or guanosine diphosphate (GDP). In nature, the different nucleotide sugars are synthesised de novo in the cytosol or the Golgi apparatus from glucose nucleotide precursors, see Figure 1 below. Specific pyrophosphorylases catalyse the conversion of glucose 1-phosphate into UDP-D-glucose and GDP-D-glucose respectively, which then may serve as precursors for the synthesis of other nucleotide sugars via interconversion pathways involving enzymes such as epimerases and

dehydrogenases. A second, alternative route for the synthesis of nucleotide sugars from sugar precursors is via salvage pathways, by sugar-specific C-1 kinases and pyrophosphorylase. (Carpita and McCann 2000).
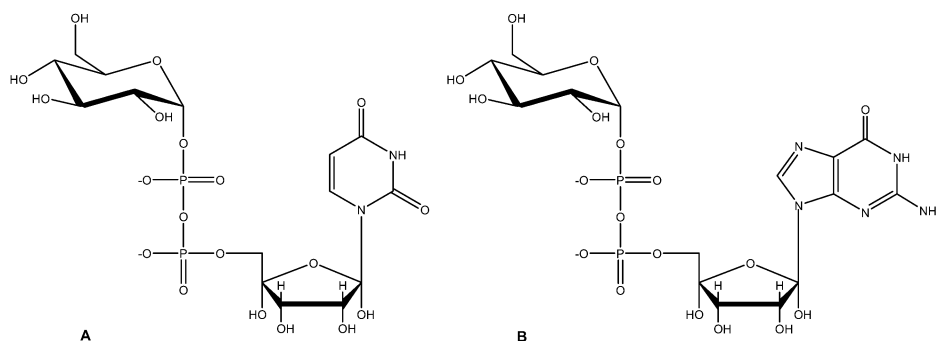

Figure 1: Structures of nucleotide-activated glucoses, the precursors of other nucleotide sugars via interconversion pathways. A: UDP-D-Glucose, B: GDP-D-Glucose.

In the biosynthesis of polysaccharides, the activating nucleotide group is released and the different sugars are polymerised by the relevant glycosyl transferases in a concerted fashion. For example, glucomannans are made by the coordinated transfer of GDP-activated glucose and GDP-activated mannose, and arabinoxylans with UDP-activated xylose and UDP-activated arabinose. With exception for cellulose and callose, the synthesis of these glycans occurs in the Golgi apparatus, but many of the relevant pathways of complex polysaccharide synthesis and polymerisation by the different glycosyl transferases still remain largely unknown (Carpita and McCann 2000).

Glycosyl transferases have been employed with some success in the synthesis of poly- and oligosaccharides in vitro (Crout and Vic 1998; Davies et al. 2001; Hancock et al. 2006; Trincone and Giordano 2006), but are considered somewhat troublesome to work with. The enzymes themselves are often unstable and difficult to express in sufficient amounts, and the complex nucleotide sugar substrates are expensive and hard to obtain. While there are some examples of larger polysaccharides such as hyaluran or β-glucans synthesised by glycosyl transferases (as pure enzymes, in whole cells, or as microsomal fractions, De Luca et al. 1996; Him et al. 2001; Pelosi et al. 2003; Widner et al. 2005), the substrate cost poses serious limits on the method from a practical perspective.

1.2 Glycoside hydrolases

Glycoside hydrolases are enzymes that catalyse the hydrolysis of glycosidic linkages between sugar moieties. In nature, their role is the degradation of poly- and oligosaccharides, for example during cell wall turnover in plants, or to gain access to nutrients by animals and microorganisms. The enzymatic hydrolysis of the glycosidic bond is a reversible equilibrium reaction, in effect meaning that the same enzyme that will hydrolyse the bond between two sugar moieties can in principle also catalyse the formation of the same type of linkage between free sugars. Glycoside hydrolases are abundant, and as proteins that in nature often are secreted by bacteria, they are generally easily manipulated, stable and straightforward to work with, and their glycosyl donor substrates are comparatively inexpensive in contrast to glycosyl transferases that require nucleotide sugars. This makes glycoside hydrolases an interesting alternative for synthetic purposes.

Generally, glycoside hydrolases will follow one of two main mechanistic routes: either the inverting or the retaining mechanism, resulting in the inversion or net retention of the anomeric configuration of the donor saccharide.

The canonical inverting mechanism is a straightforward, one step, single-displacement reaction, shown in Figure 2.
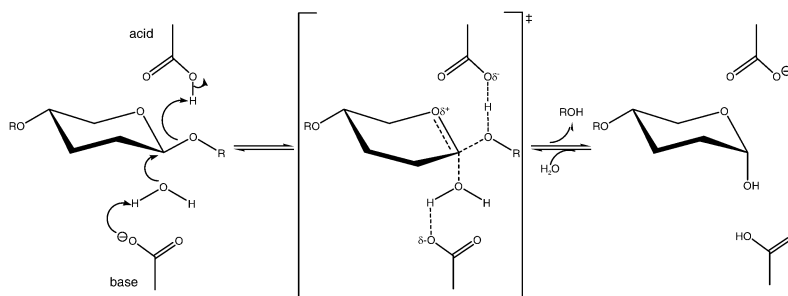
Figure 2: Canonical inverting mechanism of glycoside hydrolysis.

A carboxylic amino acid acts as a general base, and activates a water molecule that will perform a nucleophilic attack on the anomeric carbon at the centre of the glycosidic bond. Simultaneously, a second carboxylic acid residue will act as an acid and permit the breaking of the glycosidic bond and the departure of the leaving group. The overall stereochemistry of the anomeric centre will be reversed in this reaction, yielding an α-sugar from a β-linkage and vice versa.

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Retaining glycoside hydrolases require a more intricate procedure to hydrolyse their substrate while retaining the anomeric configuration. Hydrolysis is performed by a two-step, double displacement reaction according to a mechanism first described by Koshland in 1953 (Davies et al. 1995), see Figure 3.
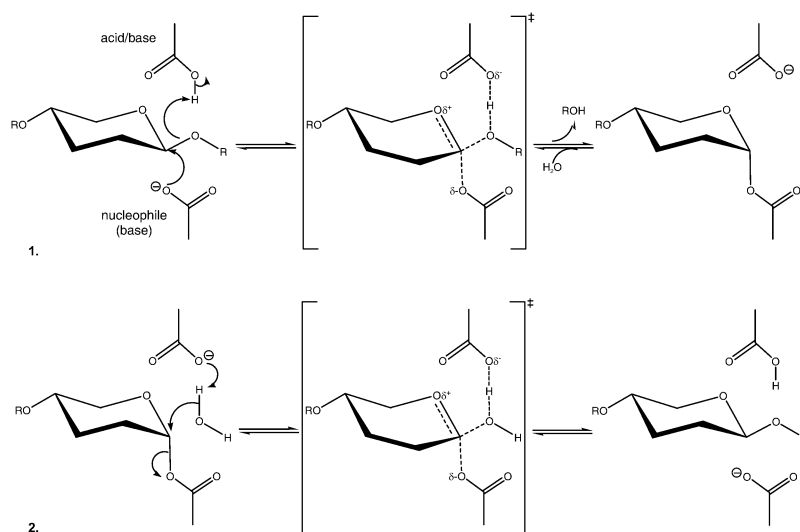

Figure 3: Canonical retaining mechanism of glycoside hydrolysis. 1. Nucleophilic attack and formation of a covalently bound enzyme-glycosyl intermediate. 2. Nucleophilic substitution by an activated water molecule and release of free glycoside.

In the first step, a carboxylic acid residue acts as a nucleophile and attacks the anomeric carbon, while a second carboxylic acid residue will act as an acid and donate a proton. Through this nucleophilic substitution, an enzyme-glycosyl intermediate is formed with the anomeric carbon covalently bound to the nucleophilic residue, in opposite anomeric configuration. In the second step, the incoming water molecule is activated by the unprotonated acid/base, here acting as a base, and in a second nucleophilic substitution the water oxygen attacks the anomeric carbon and releases the glycoside from the covalent bond to the enzyme.

By controlling the reaction conditions to minimise water activity, it is possible to revert the hydrolytic reaction and push the reaction equilibrium to the left. This means both inverting and retaining glycoside hydrolases can be used to form new linkages and add glycosides to the donor instead, by thermodynamic control.

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In the case of retaining hydrolases, the reaction proceeding through the described enzyme-glycosyl intermediate permits a second attractive synthetic option in the form of kinetic transglycosylation. If the donor substrate is equipped with a good leaving group, the enzyme will be saturated with donor glycoside at all times, and the second step of the reaction mechanism will be rate limiting. Subsequently, the enzyme-bound glycosyl donor can be transferred to a suitable, alternate acceptor that outcompetes water in the reaction, and favours transglycosylation over hydrolysis.

The major downside of using glycoside hydrolases for the synthesis of new glycosidic linkages is that the newly formed products inevitably also become substrates in the regular hydrolytic reaction, thus resulting in low net product yields. To overcome this limitation, several ingenious attempts have been made to use glycoside hydrolases as scaffolds for engineered enzymes that are capable of transglycosylation, but are hydrolytically inactive. The currently most prolific and useful of these novel enzymes are the glycosynthases, which also form the basis of the work presented in this thesis.

1.3 Glycosynthases

In 1998, Stephen Withers and co-workers reported a novel type of enzymes for oligosaccharide synthesis, called glycosynthases (Mackenzie, 1998). Glycosynthases are genetically engineered retaining glycoside hydrolases where the active site nucleophile residue, usually a glutamate, has been replaced by an inactive residue such as alanine, glycine or serine. This essentially prohibits the first step of the wild-type retaining mechanism, and neuters the enzyme in the sense that hydrolysis is no longer possible, neither of substrates, nor of products as in the case of reverse hydrolysis or kinetic transglycosylation, as described above.

The remainder of the active site being intact, the enzyme still possesses the machinery to perform the second step of the retaining mechanism, i.e. the transfer of the enzyme-bound donor glycoside to an incoming acceptor molecule. The stereochemistry of the enzyme-glycosyl intermediate in the wild-type reaction can be mimicked by the provision of glycosyl fluoride donors, in the reverse anomeric configuration of the desired product, i.e. α for a glycosynthase based on a retaining β-glycosidase that will catalyse the formation of a β-linkage between the donor and the acceptor. The α-glycosyl fluoride will fit neatly in the active site donor cleft with the extra cavity created by the mutation, and the reaction required for transglycosylation will

proceed from a stereochemically analogous situation to the second step of the canonical retaining mechanism, see Figure 4.
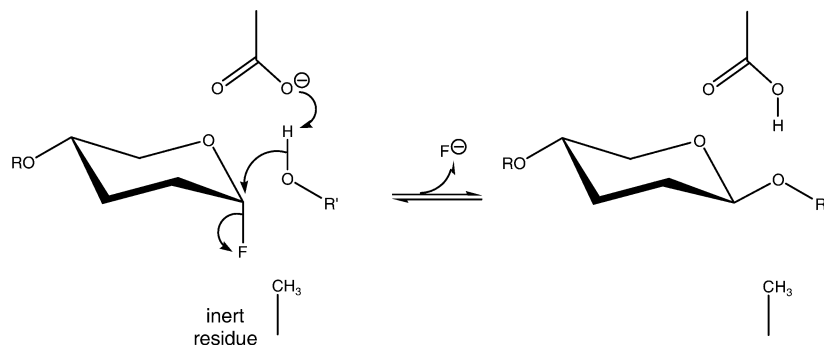

Figure 4: Glycosynthase reaction mechanism. By nucleophilic substitution with fluoride as the leaving group a new glycosidic bond is formed.

The general acid/base residue, here in its function as a base analogous to step two of the canonical retaining mechanism of hydrolysis, activates the acceptor oxygen which in turn attacks the anomeric carbon of the donor. Fluoride departs as the leaving group, and the new glycosidic linkage is formed.

Glycosynthases offer great advantages in the synthesis of desired oligosaccharides or glycocompounds, including fewer synthetic steps versus traditional synthesis, the use of easily manipulated enzymes and inexpensive glycosyl donor substrates as compared to glycosyl transferases and nucleotide sugars, and last but not least, high yields in comparison to glycoside hydrolases employed for kinetically-controlled transglycosylation, due to the incapacitation of the hydrolytic machinery (Crout and Vic 1998; Vocadlo and Withers 2000; Wymer and Toone 2000; Faijes and Planas 2007).

The first reported glycosynthase was based on the *Agrobacterium sp.* exo-β-glucosidase/galactosidase, where the catalytic Glu358 had been replaced by an alanine (Mackenzie et al. 1998). In the same year, the first endo-glycosynthase was reported, the *Bacillus licheniformis* 1,3-1,4-*β*-glucanase E134A (Malet and Planas 1998). A variety of glycosynthases were to follow over the next years, and so far (March 2009), 32 glycosidases from altogether 13 glycoside hydrolase (GH) families have been successfully converted into glycosynthases, summarised in tables 1 and 2 below. In general, endo-glycosynthases are considered highly

regiospecific, catalysing only the type of glycosidic linkages corresponding to their wild-type progenitor activity, while exo-glycosynthases show more promiscuous behaviour, however not necessarily always overlapping with their wild type counterparts (Faijes et al. 2006).

Table 1: Glycosynthases derived from exo-glycoside hydrolases

Origin	Family	Construct(s)	References
Agrobacterium sp. β-glucosidase/galactosidase	GH1	E358A, G, S E358A 1D12 E358A 2F6	(Mackenzie et al. 1998) (Mayer et al. 2000) (Kim et al. 2004) (Kim et al. 2005) (Müllegger et al. 2006) (Scaffidi and Stick 2006) (Scaffidi et al. 2007)
Sulfolobus solfataricus β-glucosidase	GH1	E387G	(Trincone et al. 2000) (Trincone et al. 2003)
Thermosphaera aggregans β-glycosidase	GH1	E386G	(Perugino et al. 2003) (Trincone et al. 2003)
Pyrococcus furiosus β-glucosidase (CelB)	GH1	E372A	(Perugino et al. 2003)
Thermus thermophilus β-glycosidase (TtβGly)	GH1	E338A, G, S	(Drone et al. 2005) (Marton et al. 2008)
Streptomyces sp. β-glucosidase	GH1	E383A	(Faijes et al. 2006)
Pyrococcus horikoshii β-glycosidase	GH1	E324G	(Perugino et al. 2006)
Rice *β-glucosidase* (Bglu1)	GH1	E414A, G, S	(Hommalai et al. 2007)
Escherichia coli β-galactosidase (LacZ)	GH2	E537S E537S/G794D	(Jakeman and Withers 2002)
Cellulomonas fimi β-mannosidase (Man2A)	GH2	E519A, S	(Nashiru O 2001) (Zechel et al. 2003)
Thermotoga maritima β-glucuronidase	GH2	E476A, G, S	(Müllegger et al. 2006)
Escherichia coli β-glucuronidase	GH2	E504A, G, S	(Wilkinson et al. 2008)
Schizosaccharomyces pombe α-glucosidase	GH31	D481G	(Okuyama et al. 2002) (Nakai et al. 2005)
Geobacillus stearothermophilus β-xylosidase (XynB2)	GH52	E335G (library)	(Ben-David et al. 2007) (Ben-David et al. 2008)
Bifidobacterium bifidum α-L-1,2-fucosidase (AfcA)	GH95	N421G N423G D766G	(Wada et al. 2008)

Table 2: Glycosynthases derived from endo-glycoside hydrolases

Origin	Family	Construct(s)	References
Rhodococcus sp. endoglycoceramidase II (EGC II)	GH5	E351A, G, S, D	(Vaughan et al. 2006) (Caines et al. 2007)
Clostridium cellulolyticum cellulase (Cel5A)	GH5	E307G	(Tao et al. 2008)
Humicola insolens cellulase endoglucanase (*Hi*Cel7B)	GH7	E197A, S E197A/H209A E197A/H209G E197A/H209A /A211T	(Fort et al. 2000) (Ducros et al. 2003) (Lin et al. 2004) (Faure et al. 2006) (Saura-Valls et al. 2006) (Faure et al. 2007) (Blanchard et al. 2007) (Piens et al. 2007)[a] (Gullfot et al. 2009)[b]
Trichoderma reesei endoglucanase (*Tr*Cel7B)	GH7	E196A	(Blanchard et al. 2007)
Bacillus halodurans exooligoxylanase	GH8	D263G, A, V, T, L, N, C, P, S	(Honda and Kitaoka 2006)
Cellulomonas fimi endo-1,4-β-xylanase (CFXcd)	GH10	E235G	(Kim et al. 2006)
Thermotoga maritima xylanase (XylB)	GH10	E259A, G, S	(Sugimura et al. 2006)
Clostridium stercorarium xylanase (XynB)	GH10	E293A, G, S	(Sugimura et al. 2006)
Bacillus halodurans xylanase (XynA)	GH10	E301A, G, S	(Sugimura et al. 2006)
Cellulomonas fimi xylanase (Cex)	GH10	E233A, G, S	(Sugimura et al. 2006)
Bacillus licheniformis xyloglucanase (*Bl*XG12)	GH12	E155A	(Gullfot et al. 2009)[b]
Bacillus licheniformis 1,3-1,4-β-glucanase	GH16	E134A	(Malet and Planas 1998) (Fairweather et al. 2002) (Faijes et al. 2003) (Faijes et al. 2004)
Pyrococcus furiosus laminarinase	GH16	E170A	(van Lieshout J 2004)
Populus xyloglucan endotransglycosylase 16-34 (XET16-34)	GH16	E85A, G, S	(Piens et al. 2007)[a]
Tropaeolum majus xyloglucan endotransferase 1/xyloglucanase (NXG1)	GH16	E94A, G, S ΔYNIIG E94A	(Gullfot et al. 2009)[b]
Hordeum vulgare (1,3)-β-D-glucan endohydrolase	GH17	E231A, G, S	(Hrmova et al. 2002) (Fairweather et al. 2003)
Cellvibrio japonicus mannanase (Man26A)	GH26	E320G, S	(Jahn et al. 2003)

[a] Publication I in this thesis
[b] Publication II in this thesis

1.3.1 Applications

Glycosynthases have been applied successfully in the synthesis of various oligosaccharides and glycocompounds, covering a variety of substrate specificities and linkages synthesized (Perugino et al. 2005; Hancock et al. 2006; Faijes and Planas 2007).

Synthesis of enzyme substrates and inhibitors

Glycosynthases have traditionally been of particular interest as tools to provide various types of substrates for carbohydrate-active enzymes, for research purposes such as studies of substrate binding and/or specificity or subsite mapping, where suitable compounds are often difficult to obtain by conventional synthetic methods, if at all.

The Cel7B E197A glycosynthase from *Humicola insolens* was successfully employed to synthesise a series of xylogluco-oligosaccharides used for active-site mapping of xyloglucan endo-transglycosylase (XET) (Faure et al. 2006; Saura-Valls et al. 2006; Faure et al. 2007). Stepwise synthesis of hexasaccharide substrates for 1,3/1,4-β-glucanases has been performed in coupled reactions by *Bacillus licheniformis* β-glucanase E134A and *Humicola insolens* Cel7B E197A glycosynthases in a one-pot approach through sequential additions (Faijes et al. 2001). Also, the new glycosynthases based on family GH16 scaffolds presented in this work are thought to find application in the synthesis of galactosylated xylogluco-oligosaccharides for further studies of XET and related enzymes (Piens et al. 2007; Gullfot et al. 2009).

Labelled substrates synthesised so far include cellotriose and –tetraose derivatives with covalently bound 2,2,6,6-tetramethylpiperidine-1-oxy-4-yl (TEMPO) groups, used in experiments to define the orientation of the cellulose chain within binding clefts of cellulose binding domains (Johnson et al. 1999). This was done with the *Agrobacterium sp.* AbgE358A glycosynthase. The same glycosynthase was used in the synthesis of site-specific 13C isotope labelling of cello-oligosaccharides (Brun et al. 2001). Other advanced applications include a cellohexaoside derivative with a naphthalene group at the reducing end and a 4-(4'-dimethylaminobenzeneazo)-benzene derivative at the non-reducing end, used for detection of cellulase activity by fluorescence resonance energy transfer (FRET) (Boyer et al. 2002). Key steps in the synthesis involved the E197A glycosynthase from *Humicola insolens*. Chromophoric 4-methylumbelliferyl disaccharides have been prepared with *Thermosphaera aggregans* β-glycosidase E386G and *Sulfolobus solfataricus* β-glucosidase E387G glycosynthases (Trincone et al. 2003).

Glycosynthases have also proven themselves useful in the synthesis of enzyme inhibitors, such as glycosylated methyl beta-acarviosins as inhibitors of cellulase activity, where the AbgE358S glycosynthase was used to glycosylate the starting molecule (Fairweather et al. 2004). Novel oligosaccharide derivatives as inhibitors of a *Cellulomonas fimi* endo-glucanase were created by coupling N-benzyloxycarbonyl derivatives of isofagomine and tetrahydrooxazine with α-glucosyl fluoride donors by the AbgE358A glycosynthase (Macdonald et al. 2002).

Synthesis of glycocompounds

The synthesis of different glycocompounds and glycoconjugates such as glycoproteins and glycolipids is a task of great and urgent interest. These are usually medically relevant compounds, whose potential cannot be explored due to simple lack of supply (Wong 2005). Several successful pursuits have been made employing glycosynthases to this purpose. For example, glycosylated inositols were produced by treating scyllo-inositol derivatives with α-galactosyl or glucosyl fluoride donors and glycosynthase (Scaffidi and Stick 2006). A series of glucuronides were recently prepared with the *Escherichia coli* β-glucuronidase E504G glycosynthase mutant (Wilkinson et al. 2008). As for more complex glycocompounds, glycosphingolipids have been synthesised in a serial process involving the *Rhodococcus sp.* endoglycoceramidase glycosynthase (Vaughan et al. 2006). A novel glycoprotein was created from *Bacillus circulans* endoxylanase Bcx, using the Agb2F6 glycosynthase for glycosylation, thus proving the feasibility and providing a method for creating neoglycoproteins in vitro (Müllegger et al. 2006). In summary, these are glycosynthase applications considered particularly relevant to the biomedical field, and interest is expected to increase and involve a growing range of products and the development of new methods.

Synthesis of larger polysaccharides

As the glycosynthase methodology matures, interest has turned towards the synthesis of larger polysaccharides, thus comprising a new area of application of the glycosynthase technology (Watts and Withers 2004; Faijes and Planas 2007). The extended active site of endo-glycosynthases may accept larger, complex oligosaccharides, thereby permitting polymerisation by consecutive self-condensation of the glycosyl fluoride substrate. As a first proof of this concept, linear crystalline (1→3,1→4)-β-D-glucan was synthesised with the E134A mutant of a *Bacillus licheniformis* GH16 glucanase using α-laminarobiosyl fluoride donors (Faijes et al. 2004). Since then, similar polymeric products synthesised by other endo-glycosynthases have included β-

(1→4)-glucans (Fort et al. 2000), β-(1→3)-glucans (Hrmova et al. 2002), mixed-linkage β-(1→4)-(1→3)-glucans (Faijes et al. 2001; Fairweather et al. 2002; Faijes et al. 2003) and β-(1→4)-xylans (Kim et al. 2006; Sugimura et al. 2006; Ben-David et al. 2008) including different side-chain substitution patterns, with degrees of polymerisation up to 30 (Faijes and Planas 2007). The work presented in this thesis includes the synthesis of large homogenous xyloglucans with regular ungalactosylated repeats produced by the *Humicola insolens* Cel7B E197S glycosynthase (Gullfot et al. 2009). Altogether, these studies show the potential of glycosynthases in the synthesis of specific polysaccharides with structural homogeneity and/or novel composition not obtainable in their natural counterparts.

1.3.2 Development of the glycosynthase technology

In parallel to the expansion of the synthetic repertoire, the development of the glycosynthase technology is evolving in terms of assays, synthetic processes, but also alternate reaction mechanisms and substrates. In their classical incarnation, glycosynthases are based on retaining β-glycoside hydrolases. There is one reported instance of an α-glycosynthase so far, the *Schizosaccharomyces pombe* α-glucosidase D481G mutant, synthesizing α-glycosidic bonds with β-glycosyl fluoride donors as first proof of concept (Okuyama et al. 2002; Nakai et al. 2005). Also, successful attempts have been made to create glycosynthases from inverting glycoside hydrolases. The *Bacillus halodurans* reducing-end xylose-releasing exo-oligoxylanase works as an inverting glycosynthase by replacement of the general base with an inert residue (Honda and Kitaoka 2006), while the *Bifidobacterium bifidum* α-L-1,2-fucosidase is deprived of a residue normally holding a catalytically necessary water molecule (Wada et al. 2008). There is furthermore an increasing number of reports of glycosynthase-like reactions with other donors than glycosyl fluorides and some following alternative reaction mechanisms, see for example (Michinari et al. 2007) and references therein. A similar approach includes thioglycosynthases and thioglycoligases that catalyse the formation of thioglycosidic linkages, reviewed in (Hancock et al. 2006).

High-throughput glycosynthase screening

The vast majority of all glycosynthases reported so far are the direct result of rational design efforts where a particular glycoside hydrolase is converted to its glycosynthase counterpart by inactivation of the active site nucleophile only. However, the current trend points toward an increase of random mutagenesis and directed evolution based engineering, to expand the glycosynthase repertoire and/or tailor the activity of existing candidates (Hancock et al. 2006).

Such procedures require efficient screening and selection methods, and a few successful assays suitable for high-throughput screening of glycosynthase activity have been developed so far. In the first reported plate-based coupled enzyme screening method, a fluorogenic or chromogenic substance is bound to the acceptor substrate. Upon specific glycosynthase-mediated binding to the α-fluoro donor substrate, the product acts as a substrate to a second enzyme (a cellulase) that releases the fluorophore or chromophore, thus permitting detection of glycosynthase activity by fluorescence (Mayer et al. 2001). Glycosynthase activity can also be linked to the transcription of a reporter gene. This method, denoted chemical complementation, is based on the yeast three hybrid system (Lin et al. 2004; Tao et al. 2006). Upon glycosynthase-mediated coupling of an acceptor with the α-fluoro donor substrate, the reaction product reconstitutes a transcriptional activator, and the transcription of a downstream reporter gene is induced.

A third, more general assay, allegedly applicable to all glycosynthases without restrictions, is based on the release of hydrofluoric acid as a byproduct of the glycosynthase reaction (Ben-David et al. 2008). The benefit of this assay is that it does not require any special chromophoric or modified substrates, but can be used under realistic synthetic conditions.

Solid-phase synthesis

Another important area of future development and innovation involves synthesis technology. While glycosynthase-mediated reactions conventionally are performed in solution, interesting attempts have been reported using solid-phase methods. Such methods would for example suggest the use of glycosynthases in construction of carbohydrate microarrays, or more generally permit higher synthetic throughput due to improved stability and facilitated recycling. For example, α-galactosyl fluorides have been successfully transferred by Abg E358S and E358G glycosynthases to aminophenyl glucoside and glycopeptide acceptors bound to a PEGA resin solid phase via a backbone amide linker (Tolborg et al. 2002). It is also possible to immobilise the glycosynthase itself, which was done to scale up reactions with the *Streptomyces sp.* β-glucosidase E383A mutant. The glycosynthase was bound to a Ni^{2+}-chelating Sepharose resin, which permitted recycling of the enzyme and increased its stability during both operation and storage (Faijes et al. 2006).

1.4 Xyloglucans

Xyloglucans are an important family of polysaccharides found as cross-linking glycans in the cell walls of plants and as storage polysaccharides in seeds (Carpita and McCann 2000). Cross-linking glycans (often referred to as hemicelluloses) are crucial for cell wall flexibility and cell expansion during growth and differentiation. In the so-called type 1 primary cell walls of dicots and non-commelinoid monocots, xyloglucans bind tightly to the exposed glucan chains of the paracrystalline cellulose microfibrils. They connect these cellulose microfibrils in a kind of network, by spanning the distance between them and binding to either other xyloglucan chains or other cellulose fibrils. This cellulose-xyloglucan framework is further embedded in a pectin matrix (Carpita and McCann 2000). In essence, this architecture provides necessary plasticity by permitting the rigid cellulose microfibrils to move relative each other, without compromising cell wall stability.

1.4.1 Structure and nomenclature

Xyloglucans are based on a linear β-(1→4)-glucan backbone, branched with α-(1→6)-xylose units in regular, repeating patterns. The xylose residues can be further decorated with β-(1→2)-galactoses, which in turn are sometimes extended with α-(1→2)-fucoses. These decorations occur in a tissue- and species dependent manner (Fry et al. 1993; Hoffman et al. 2005; Pena et al. 2008). An overview of the general structure of xyloglucans and the nomenclature of the building blocks is given in Figure 5 below.

Xyloglucans are phylogenetically diverse, and there is a large variety of backbone branching patterns. The most common backbone repeat is the XXXG unit. The widely used xyloglucan from tamarind (*Tamarindus indica*) seeds is comprised of XXXG, XXLG, XLXG, and XLLG motifs, while the presence of fucosylated XXFG and XLFG motifs are characteristic of primary cell wall xyloglucans in dicots.

This elaborate structure is thought to form the basis for xyloglucan solution properties and its interaction with cellulose, and there is a substantial and growing interest in deciphering the relationship between xyloglucan structure and function. Approaches include in muro investigations of the effects of genetically altered xyloglucan composition (Reiter 2002), studies of the association of xyloglucans with bacterial cellulose model systems (Whitney et al. 2006), and biophysical studies of the xyloglucan-cellulose interaction at the molecular level by modelling and
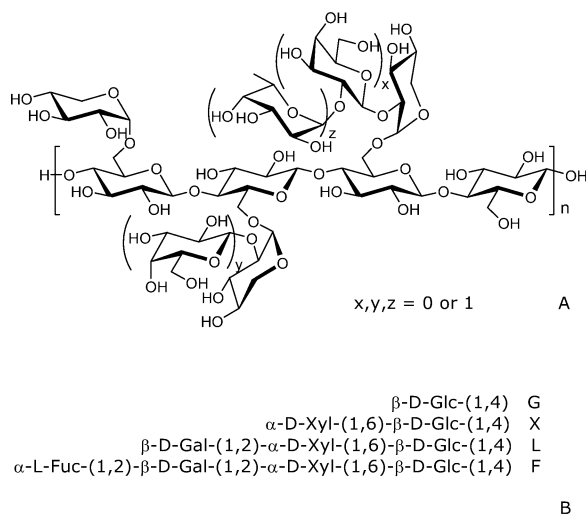
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x,y,z = 0 or 1   A

β-D-Glc-(1,4)   G
α-D-Xyl-(1,6)-β-D-Glc-(1,4)   X
β-D-Gal-(1,2)-α-D-Xyl-(1,6)-β-D-Glc-(1,4)   L
α-L-Fuc-(1,2)-β-D-Gal-(1,2)-α-D-Xyl-(1,6)-β-D-Glc-(1,4)   F

B

Figure 5: General structure (A) and nomenclature (B) of xyloglucan and its building blocks.

experimental work reviewed by (Zhou et al. 2007). While intriguing, the structural complexity poses a serious drawback for relevant in vitro studies, since xyloglucan polymers as found in nature display a large degree of heterogeneity, rendering conclusive investigations on the role of particular structural elements practically impossible. This fact has stimulated an interest in synthetic methods to obtain xyloglucan mimics and analogs with well-defined structure and decoration patterns.

1.4.2 Applications of xyloglucan

Bulk xyloglucan is widely used in the textile industry as a sizing agent, and as a gelling agent in the food industry. Xyloglucan can also be used in papermaking in similar functions as those of starches and galactomannans, to strengthen the sheet and to lower friction. In such applications, the role of xyloglucan is comparatively low-tech, taking advantage of its general gelling or smoothening properties.

As in the case of many industrially promising polysaccharides, the extraordinary properties of xyloglucans have attracted interest in their application in more advanced technologies (Brumer et al. 2004; Gustavsson et al. 2005; Zhou et al. 2005; Bodin et al. 2007; Zhou et al. 2007). Perhaps the most compelling applications of xyloglucan are those reminiscent of its role in the plant cell wall, the binding to and crosslinking of cellulose microfibrils. In such biomimetic approaches,

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xyloglucan has a great and promising potential, not least due to novel, versatile methods for its functionalisation such as the XET technology described below.

The hydroxyl groups of the glucan chains in the paracrystalline cellulose surface form a tight network of hydrogen bonds. Chemical derivatisation of cellulosic surfaces, such as in paper or textile, involves loosening of the crystalline glucan chain structure to get access to free hydroxyl groups. This however compromises the strength of the material to some extent. The XET technology is a novel and innovative method to circumvent this disadvantage, and to permit functionalisation of cellulosic surfaces without compromising material strength.

Small xylogluco-oligosaccharides (XGO) can be derivatised with functional groups one seeks to attach to the cellulosic surface. While such low molecular weight XGOs will not bind to cellulose, the functionalised XGOs can be incorporated into high molecular mass xyloglucan by using xyloglucan endo-transglycosylases (XET). The resulting functionalised xyloglucans will bind readily to cellulose, thus permitting functionalisation of the cellulosic surface without disrupting the crystalline structure and thus compromise material strength. Chemical groups introduced on cellulose surfaces with this appealing method include amino groups, fluorescent dyes, optical brightening agents, protein ligands (biotin), thiol groups, and initiators for polymerisation reactions (Teeri and Brumer 2003; Brumer et al. 2004; Zhou et al. 2005; Zhou et al. 2006). The XET/xyloglucan method has worked with all types of cellulose tested so far, including filter paper, cotton cellulose microcrystals, regenerated cellulose, wood pulp fibres and bacterial cellulose.

Functionalised xyloglucan has a potentially extraordinary role in the field of cutting-edge biocomposites. Cellulose-based biocomposites and nano-technologies often involve controlled polymerisation techniques, for example to produce super-hydrophobic or thermoresponsive surfaces (Lindqvist et al. 2008). Attachment of the polymerisation initiators to cellulose via xyloglucan could circumvent current methodological limitations that occur due to chain degradation or alteration of cellulose morphology (Zhou et al. 2005; Lönnberg et al. 2006). Another intriguing area is in biocomposites, which can be reinforced with cellulose whiskers and nanocrystals. The potential of these promising materials is currently limited by the difficulty to achieve well-dispersed suspensions of the cellulose nanocrystals, but novel synthetic xyloglucan-β-polystyrene block co-polymers can be used as compatibilising agents for the dispersion of the nanocrystals (Zhou et al. 2007 and Q. Zhou, KTH Biotechnology, personal communication).

New materials produced by such innovative methods show great potential for biomedical applications, as high-tech composites, or consumables with extraordinary properties (Bodin et al. 2007). Increased fundamental knowledge about the molecular determinants behind xyloglucan characteristics and behaviour will be essential to harness its full potential for such advanced applications.

2 AIM OF INVESTIGATION

The aim of the work presented in this thesis was the screening, design, production and characterisation of glycosynthases which can polymerise α-xyloglucosyl fluoride substrates.

In a larger perspective, our goal is to develop robust and versatile methods for the preparative synthesis of homogenous xyloglucans with regular substitution patterns not available in nature. Such compounds could pave the way for a scientific breakthrough for structure-function studies of xyloglucan, enabling in vitro experiments regarding the role of particular structural elements for xyloglucan properties and its interaction with cellulose. Also, we seek to synthesise specific oligosaccharides that would be invaluable as ligands for structure-function analyses of xyloglucan-active enzymes, as exemplified by earlier work on *Ptt*XET16-34 characterisation (Baumann et al. 2007; Saura-Valls et al. 2008; Ibatullin et al. 2008), and more recently by a study on *Tm*NXG1 substrate recognition based on crystallography and molecular dynamics (Mark et al. 2008). If well-defined ligands were available that could span the entire binding cleft of the enzyme, findings in this work could be investigated in vitro by performing the relevant binding experiments and biophysical characterisations.

The glycosynthase technology is a particularly interesting choice for this purpose, and previous work in this area has indeed been successful in the synthesis of certain xylogluco-oligosaccharides (Faure et al. 2006; Saura-Valls et al. 2006; Faure et al. 2007; Saura-Valls et al. 2008). Suitable enzyme scaffolds are usually robust and easy to work with, the required engineering is conceptually straightforward, and the necessary α-xyloglucosyl fluoride substrates are easy to obtain.

3 EXPERIMENTAL METHODS AND MATERIALS

3.1 Measuring glycosynthase activity with a fluoride ion selective electrode

During enzymatic condensation of α-glycosyl fluorides, the anomeric fluoride of the donor glycoside is released upon condensation with the acceptor hydroxyl group. Thus, the reaction can be followed as an increase of fluoride ion concentration in the reaction solution with a fluoride ion selective electrode. While autocondensation is possible, it is assumed to be negligible compared to the enzymatic reaction. Also, some autohydrolysis of the fluoro substrates occurs, and is accounted for by recording the baseline fluoride ion release before adding enzyme to the reaction solution. During final data analysis, this baseline is subtracted from the recorded rate during the enzymatic reaction.

Fluoride ion concentration is measured as an electrical current (mV), and has to be converted to the actual concentration of fluoride ions ($[F^-]$mM) . This is done via a standard curve constructed by recording mV readings at known fluoride ion concentrations. Above a certain concentration level (here: > 0.5 ppm or 26 μM), the relation between mV and fluoride ion concentration is a semilogarithmic function in the general form

$$(1) \quad y(mV) = p \ln\left[F^-\right](mM) + q$$

where p and q are specific experimental constants depending on parameters such as for example pH. For this semilogarithmic region, the conversion function to compute fluoride ion concentration from mV readings will thus be given by

$$(2) \quad \left[F^-\right](mM) = e^{\frac{y(mV)-q}{p}} \quad .$$

3.2 Product analysis by HPAEC-PAD

High-pressure anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is a widely used method in carbohydrate analysis, facilitated by the Dionex ICS-3000 system and carbohydrate-specific CarboPac columns (here: Dionex PA-100).

Carbohydrates are weakly acidic, and will be partially ionised at high pH. This permits selective separation with an anion-exchange stationary phase such as provided by the CarboPac column matrix, which is specifically designed for the separation of mono-, oligo- and polysaccharides. In

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the experiments performed in the present work, the branched oligosaccharides are eluted by a sodium acetate gradient in sodium hydroxide, and the retention time of the different fractions depends on the size of the oligosaccharide. An overview of the relevant chemistry is presented in Figure 6 below.
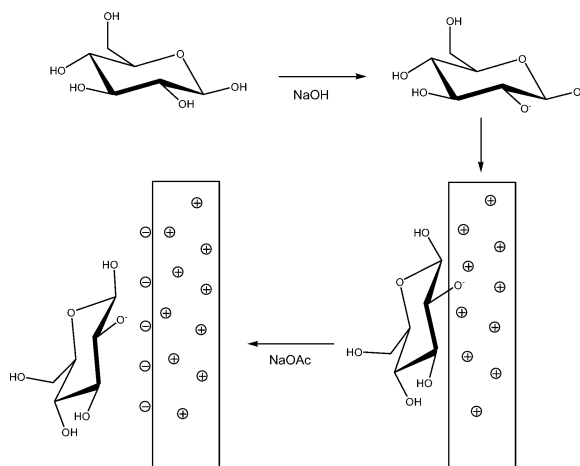

Figure 6: Schematic overview over anion exchange chromatography of carbohydrates. The pH is raised by sodium hydroxide and hydroxyl groups of the sugar are ionised to oxyanions. The ionised sugar binds to the anion exchange stationary phase. A gradient of sodium acetate is added as the eluent; the anionic acetate ions will outcompete the sugar oxyanions on the stationary phase, and release the sugar from the stationary phase. The sugar is eluted.

Detection by pulsed amperometry is performed as a three-step sequence, schematically shown in Figure 7. Upon elution, the carbohydrates are detected by measuring the electrical current at a certain potential as they are oxidised on the surface of a gold electrode. The potential is then raised to oxidise the gold surface, which causes desorption of the oxidised carbohydrates and thus cleans the surface. The potential is lowered again, and the electrode surface is reduced back to gold, completing the pulse sequence, and the next measurement can be performed. The carbohydrate oxidation current in the first step is integrated over time after a certain delay to correct for a charging current due to the change of potential, yielding the detector response in coulomb (C).
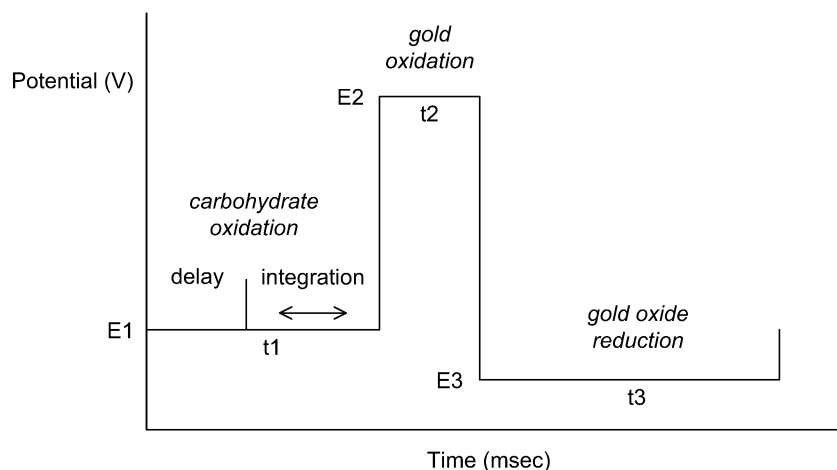
Figure 7: Pulse sequence during pulsed amperometric detection (PAD). The three potentials E1 (oxidation of carbohydrates on gold surface), E2 (cleaning of gold surface by oxidation and desorption of carbohydrate oxidation products) and E3 (restoration of gold surface by reduction) are performed at fixed durations t1, t2 and t3. The electric current resulting from the oxidation of carbohydrates on the gold surface is integrated over time to obtain the output expressed as electric charge.

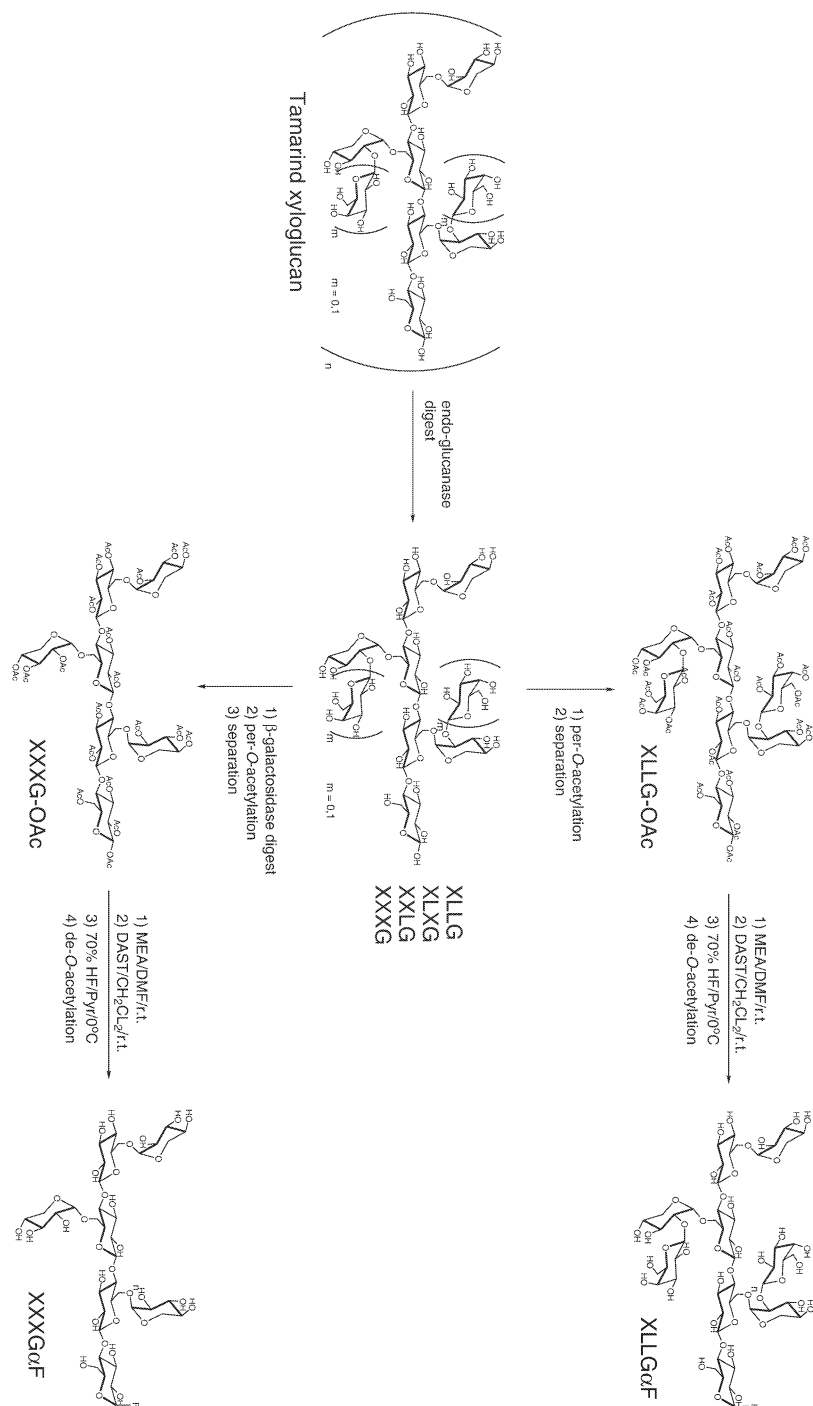## 3.3 Production of xylogluco-oligosaccharides and synthesis of α-xyloglucosyl fluorides

Mixtures of Glc_4-based xylogluco-oligosaccharides are obtained by enzymatic digestion of deoiled tamarind kernel powder in buffer solution with crude *Trichoderma reesei* cellulase. The depolymerisation process is followed by thin layer chromatography (TLC), and when completed, the liquid volume is reduced by vacuum and finally freeze-dried to obtain a mixture of xylogluco-oligosaccharides as a white powder. The typical ratio of XXXG, XXLG, XLXG and XLLG obtained is 15:32:7:46, fairly well reflecting the reported ratio in native tamarind xyloglucan (York et al. 1990). To yield pure XXXG, an additional enzymatic step is performed directly after the cellulase digest to remove galactose side groups. This is done by adding *Aspergillus niger* β-galactosidase after approximately 5-6 hours of cellulase digest. After reduction of the liquid volume by vacuum, the product is precipitated from the solution, filtered, redissolved and freeze-dried.

To yield α-xyloglucosyl fluorides from tamarind xyloglucan precursors, the mixture of xyloglucooligosaccharides as obtained by enzymatic hydrolysis is separated into two routes, shown in Figure 8. For XXXGαF, enzymatic removal of galactose side groups is performed, and resulting XXXG oligomers are per-*O*-acetylated as described below. For XLLGαF, the mixture is first per-*O*-acetylated to protect the hydroxyl groups. This is performed by acetic anhydride and pyridine in the presence of catalytic amount of dimethyl aminopyridine (DMAP) at 60°C over night. Pure XLLG-*O*-Ac is then separated from the mixture by flash chromatography on a silica gel with a toluene/acetone gradient.

XXXG-*O*-Ac and XLLG-*O*-Ac are then treated with monoethanol amine (MEA) and acetic acid in dimethyl formamide (DMF) as solvent to remove the protecting acetyl group on the anomeric hydroxyl group. In the next step, this hydroxyl group is replaced by fluoride by treatment with diethyl amino sulphur trifluoride (DAST) in dichloromethane at room temperature. Anomerisation of the resulting β-fluoride into α-fluoride is performed by 70 % hydrogen fluoride (HF) in pyridine. Finally, the compounds are de-*O*-acetylated in a dichloromethane/methanol mixture with catalytic amount of NaOMe, to yield XXXGαF and XLLGαF.

Figure 8: Synthesis of α-xyloglucosyl fluorides

Tamarind xyloglucan

m = 0,1

n

endo-glucanase
digest

XLLG
XLXG
XXLG
XXXG

m = 0,1

1) per-O-acetylation
2) separation

XLLG-OAc

1) MEA/DMF/r.t.
2) DAST/CH₂CL₂/r.t.
3) 70% HF/Pyri/0°C
4) de-O-acetylation

XLLGαF

1) β-galactosidase digest
2) per-O-acetylation
3) separation

XXXG-OAc

1) MEA/DMF/r.t.
2) DAST/CH₂CL₂/r.t.
3) 70% HF/Pyri/0°C
4) de-O-acetylation

XXXGαF

4   RESULTS AND DISCUSSION

4.1   First glycosynthase for galactosylated XGO (Publication I)

The *Humicola insolens* endo-β-glucanase glycosynthase *Hi*Cel7B E197A has been used extensively in the production of xyloglucan oligosaccharides based on XXXGαF donors. However, it is not able to polymerise XLLGαF donors at practically feasible rates. This limitation prompted for the development of new xyloglucan-active glycosynthase variants. The scope of the work presented in publication I was to investigate the potential of nucleophile mutants of a xyloglucan endo-transglycosylase from hybrid aspen (*Populus tremula x tremuloides*, *Ptt*XET16-34) as glycosynthases for the synthesis of xyloglucan oligosaccharides. The results were further compared to the *Hi*CelB E197A glycosynthase, serving as a benchmark for the synthesis of non-galactosylated xylogluco-oligosaccharides and analogs (Faure et al. 2006; Saura-Valls et al. 2006; Faure et al. 2007).

The catalytic nucleophile of *Ptt*XET 16-24, Glu-85, was mutated into glycine, serine, and alanine, which generated glycosynthases with no detectable wild-type activity, but that were capable to oligomerise both XXXGαF and XLLGαF donor substrates. The condensation of XXXGαF by *Ptt*XET16-34 E85A exhibited saturation kinetics with an apparent K_m value comparable to other glycosynthases derived from endo-glycosidases, while the observed k_{cat} value was relatively low. Compared to HiCel7B E197A, the k_{cat} value was approximately five times lower, which was somewhat compensated by a lower K_m value.

Product analysis revealed the time-dependent formation of series of oligomers of the general structure (XXXG)$_n\alpha$F, along with products resulting from the spontaneous hydrolysis of the C1–F bond. The addition of an equimolar amount of XXXG as an alternate acceptor yielded nearly exclusively (XXXG)$_{2-10}$ products in overnight reactions, biased toward lower M_w products compared with reactions with XXXGαF as both the donor and the exclusive acceptor substrate. The addition of alternate acceptors can therefore be used to both fine-tune the glycosynthase product distribution, as well as to control the chemistry at the reducing end of the produced oligosaccharides.

No activity on XLLGαF donors was detected by fluoride ion release or product analysis for *Hi*Cel7B E197A. In contrast, *Ptt*XET16-34 E85A catalysed the condensation of XLLGαF donors with slightly increased k_{cat} and K_m values compared to those found for XXXGαF.

Similar to the results of product analysis observed for XXXGαF, *Ptt*XET16-34 E85A catalysed the homo-condensation of XLLGαF to produce a series of oligomers (XLLG)$_n\alpha$F (n = 2 - 6), which spontaneously hydrolysed to (XLLG)$_n$. Incubation of XLLGαF with the alternate acceptor substrate XLLG led to near-exclusive production of (XLLG)$_n$ in overnight reactions, highly similar to the observations made in the case of XXXGαF with XXXG acceptors.

Interestingly, the degrees of polymerisation of products produced by condensation of XLLGαF were significantly lower than those from XXXGαF. It is likely that the *Ptt*XET16-34 E85A glycosynthase is inhibited by (XLLG)$_n$ products to a greater extent than by (XXXG)$_n$ products, possibly due to additional binding interactions to the pendant Gal residues. Nonetheless, (XLLG)$_n$ oligomers up to n = 6 were observed (M_r 8230), demonstrating that *Ptt*XET16-34 E85A is the first glycosynthase capable of producing homogenously galactosylated xyloglucan fragments.

In summary, the study established that *Hi*Cel7B E197A, wild-type *Ptt*XET16-34, and *Ptt*XET16-34 E85A can all be employed for the synthesis of xyloglucans with regular backbone substitution using appropriate donor substrates. Whereas *Ptt*XET16-34 E85A is able to oligomerise both XXXGαF and XLLGαF, the *Hi*Cel7B E197A glycosynthase cannot be used for the synthesis of galactosylated xyloglucans. The unique ability of *Ptt*XET16-34 E85A to use both XXXGαF and XLLGαF as donor substrates unlocks the possibility to produce a larger variety of xyloglucan oligo- and polysaccharides of defined composition for research and practical applications.

4.2 Xyloglucan synthases from families GH7, GH12 and GH 16 (Publication II)

A growing scientific interest in semi-synthetic homoxyloglucans with controlled and well-defined decoration patterns encouraged our search for novel glycosynthases based on enzymes with wild-type xyloglucanase activity as scaffolds. Xyloglucanase activity (EC 3.2.1.151) has been found amongst glucan hydrolases from six different GH families (Gilbert et al. 2008). Five of these families (GH 5, 7, 12, 16 and 44) employ the canonical double-displacement mechanism of retaining glycosyl transfer (Becker et al. 2001), which forms the basis for the classical glycosynthase reaction. In the study presented in publication II, we screened potential glycosynthase scaffolds from three different glycoside hydrolase families (GH7, GH12, and GH16), based on demonstrable wild-type xyloglucanase activity (reviewed in Gilbert, 2008). The glycosynthases were characterised and compared, including those presented in publication I.

4.2.1 Xyloglucan synthases from family GH7

Enzymatic polymerisation of oligosaccharides into products with high degree of polymerisation is largely dependent on the catalytic turnover (Faijes and Planas 2007). The *Humicola insolens* GH7 cellulase *Hi*Cel7B has served as a scaffold for several useful glycosynthases as described above in this thesis, and since the E197S variant displays a 40-fold higher k_{cat} compared to *Hi*Cel7B E197A for the condensation of other substrates (Ducros et al. 2003), we were interested to see whether *Hi*Cel7B E197S could be used to synthesise xyloglucans with higher degree of polymerisation than those previously obtained with the E197A variant.

The E197S variant was tested on α-xyloglucosyl fluoride substrates under the same conditions as in our previous study (Piens et al. 2007). Using XXXGαF as both donor and acceptor, *Hi*Cel7B E197S exhibited remarkably increased reaction rates compared to its alanine predecessor, making it the fastest (XXXG)$_n$-producing glycosynthase identified thus far. During the condensation of XXXGαF donors a colourless precipitate of (XXXG)$_n$ products is formed, consistent with reported observations that partial enzymatic degalactosylation of native xyloglucan leads to chain aggregation and gelling (Shirakawa et al. 1998; Whitney et al. 2006). The reaction proceeds so quickly that oligosaccharides with low degree of polymerisation are barely discernable by HPAEC-PAD analysis of the products. The reaction is easily scaled up, and XXXG-based homoxyloglucan could be produced in 50 mg batches.

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The partitioning of reaction products into water-soluble and insoluble fractions and subsequent analysis by size exclusion chromatography revealed interesting facts about the aqueous solubility limits of xyloglucan: $(XXXG)_n$ products up to M_w 14 000 mostly remain in the water-soluble fraction, while the insoluble fraction consists of products up to M_w 60 000. Most product is obtained in the M_w range 15 000 - 40 000, corresponding to n ≈ 14 - 36, among the largest products obtained with a glycosynthase so far.

Despite the impressive performance on XXXGαF, the *Hi*Cel7B E197S glycosynthase showed no activity at all on XLLGαF as measured by fluoride ion release or during product profiling by HPAEC-PAD, reflecting the same observations as in our previous study on *Hi*Cel7B E197A. The GH7 *Hi*Cel7B constructs are therefore somewhat limited with regard to their substrate range, since they do not permit condensation of galactosylated α-xylogluco-oligosaccharyl fluoride donors. At the same time, they are superior to all xyloglucan glycosynthases produced thus far for synthetic purposes involving ungalactosylated building blocks. In particular, the *Hi*Cel7B E197S glycosynthase is an outstanding tool for the synthesis of pure, high molecular weight XXXG-based homoxyloglucan.

4.2.2 Xyloglucan synthases from family GH12

The recently characterised *Bacillus licheniformis* XG12 (Gloster et al. 2007) is the first reported instance of xyloglucanase activity within the GH12 family, and no glycosynthase derived from a family GH12 scaffold had been reported so far. Since wild-type *Bl*XG12 hydrolytic activity on xyloglucan is significant (Gloster et al. 2007; Ibatullin et al. 2008), the *Bl*XG12 E155A nucleophile mutant was an appealing candidate in our search for superior xyloglucan synthases. However, only very low activity on xyloglucosyl fluorides was detected.

Some activity on XXXGαF was measured by fluoride ion release, and product analysis by HPAEC-PAD revealed that small amounts of the XXXGαF were consumed, resulting in traces of elongation products. In comparison, activity on XLLGαF was merely detectable, and product analysis did not reveal any elongation products or detectable consumption of XLLGαF substrate.

Interestingly, the results reflect the findings in the kinetic studies previously performed on wild-type *Bl*XG12. Compared to its excellent hydrolytic activity on tamarind xyloglucan, *Bl*XG12 performed only poorly on branched aryl oligosaccharides, such as XXXG-β-2-chloro-4-nitrophenyl and XLLG-β-2-chloro-4-nitrophenyl. With such chloro-nitrophenyl (CNP)

oligosaccharide donors, the rate of hydrolysis is inversely related to the degree of branching of the glucan backbone. Rates observed follow the order GGGG-β-CNP (fastest) > XXXG-β-CNP > XLLG-β-CNP (slowest) (Gloster et al. 2007; Ibatullin et al. 2008).

In conclusion, *Bl*XG12 displays conceptual capability as a glycosynthase, but due to the poor performance in comparison to other available xyloglucan synthases we do not regard *Bl*XG12 E155A an apt choice for the synthesis of xyloglucan-based polymers. However, it is possible that the *Bl*XG12 E155A glycosynthase may find utility in the synthesis of un- or low-substituted glucans, which would have to be investigated in future studies.

4.2.3 Xyloglucan synthases from family GH16

Several GH16 enzymes have served as scaffolds for glycosynthases, including the *Pt*XET16-34 nucleophile mutants reported in publication I. *Pt*XET16-34 follows the retaining mechanism of glycosyl transfer, which is the basis for the glycosynthase reaction, but it is a strict transglycosylase (EC 2.4.1.207) while other glycosynthases are based on hydrolase scaffolds. For this reason, we were interested to derive new xyloglucan synthases from a strict hydrolase within the GH16 family, such as the structurally closely related homolog endo-xyloglucan hydrolase from *Tropaeolum majus* (*Tm*NXG1).

The apparent kinetic parameters for the hydrolysis of Glc$_8$-based xyloglucan oligosaccharides by wild-type *Tm*NXG1 indicated a potentially faster scaffold compared to *Pt*XET16-34, if wild-type rates for hydrolysis were reflected in the glycosynthase reaction. Thus, the catalytic nucleophile Glu-94 of *Tm*NXG1 was replaced by alanine, glycine or serine, yielding hydrolytically inactive enzymes that all displayed activity on both XXXGαF and XLLGαF donors. Also included in this study was the alanine mutant E94A of the engineered XET activity hybrid (Baumann et al. 2007) *Tm*NXG1-ΔYNIIG, which lacks the YNIIG loop adjacent to the acceptor binding site.

The kinetic characterisation revealed saturation kinetics for the polymerisation of both XXXGαF and XLLGαF by *Tm*NXG1 E94A, at faster rates than the previously reported *Pt*XET16-34 E85A glycosynthase. Rates of condensation of the galactosylated XLLGαF donors compared well to *Hi*Cel7B E197A rates on XXXGαF, the benchmark glycosynthase used in the preparative synthesis of xyloglucan oligosaccharides (Faure et al. 2006; Saura-Valls et al. 2006; Faure et al. 2007). The *Tm*NXG1 E94A glycosynthase is therefore well suited for the preparative synthesis of galactosylated xyloglucan oligosaccharides up to certain lengths.

Product analysis revealed time-dependent formation of a series of elongation products with the general structure $(XXXG)_n$, $n \leq 9$, or $(XLLG)_n$, $n \leq 8$ respectively. These products are formed by subsequent condensations catalysed by *Tm*NXG1 E94A and spontaneous hydrolysis of the C-F bond. Along with these products, transient peaks corresponding to $(XXXG)_n\alpha F$ and $(XLLG)_n\alpha F$ products are observed.

An interesting feature is that the product distribution can be controlled by the reaction conditions, to favour the formation of certain products. By including a free xyloglucosyl acceptor, i.e. XLLG, short-length condensation products such as XLLGXLLG accumulates, since reaction products from the condensation of XLLGαF with a fluoride-free acceptor can no longer act as a donor in subsequent reactions. The yield of XLLGXLLG can be enhanced substantially compared to other products in this way, by providing XLLGαF donors and XLLG acceptors at a 1:1 molar ratio. This is useful in the synthesis of specific shorter oligosaccharides, which could serve as ligands for structure-function analyses of xyloglucan-active enzymes, such as the octadecasaccharide XLLGXLLG.

5 CONCLUDING REMARKS AND OUTLOOK

In summary, the xyloglucan glycosynthase activity of *Hi*Cel7B E197S and the new *Tm*NXG1 E94A glycosynthase are key achievements towards our goals as outlined in the Aims of investigation. While the superior catalytic turnover of *Hi*Cel7B E197S sets new benchmarks for the synthesis of large polysaccharides in general and xyloglucan homopolymers in particular, *Tm*NXG1 E94A exhibits a valuable complementarity through its ability to produce XLLG-based xyloglucans, and as such provides a key expansion of the glycosynthase toolbox.

Future steps will include the selective fucosylation of produced xyloglucans, but also the search for a faster glycosynthase capable of condensating XLLGαF into high molecular weight galactosylated homoxyloglucan, analogous to the $(XXXG)_n$ products of *Hi*Cel7B E197S obtained in this work.

Altogether, this study poses a key development in our work on glycosynthases for the production of xyloglucan mimics and homogenous xyloglucans with regular substitution patterns not available in nature. Such compounds permit further characterisation of xyloglucan-active enzymes such as XET, but also relevant in vitro studies of the microenvironment along the xyloglucan polymer as determined by different branching patterns. This latter application is groundbreaking in the sense that such studies have not been possible to perform due to the lack of suitable substrates. The novel homoxyloglucans produced by glycosynthase technology therefore hold promises of a potential scientific breakthrough in the study of cellulose-xyloglucan interactions.

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