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Enzymatic conversion of β-mannans: Analysing, evaluating and modifying transglycosylation properties of glycoside hydrolases

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Abstract

Retaining glycoside hydrolases are enzymes that catalyse the breakdown down of glycans through hydrolysis. Due to the double-replacement mechanism of the retaining glycoside hydrolases (GHs), which form an intermediate with part of the glycan covalently attached to the enzyme, some GHs are able to catalyse synthesis reactions called transglycosylation. In transglycosylation reactions a hydroxyl-containing molecule (acceptor), other than water, acts as a nucleophile which releases the glycan moiety from the covalent intemediate while forming a new glycoside (transglycosylation product). The transglycosylation reaction can be used to transform renewable starting materials such as plant hemicellulose to valuable products, ehich is discussed in the thesis. The work presented in the thesis have explored how GHs interact with glycans and how different aspects of transglycosyaltion reactions affect the final yield of transglycosylations products.

The presented work explores how the open active site structure of two GH26 β-mannanases have made them well adapated to act on heavily galactosylated hemicellulosic β-mannan polysaccharides (**Paper I** and **II**). In addition **Paper I** and **II** explore how substiutions of amino acids in glycan interacting subsites can lead to changes in catalytic properties and how the two GH26 β-mannanases productively interacts with oligosaccharides. The work also examines how variants of GHs can have improved transglycosylation cpacity compared to their wild-type counterparts (**Paper III** and **V**). It investigates how the elimination of saccharide interactions in the +2 subsites can lead to improved transglycosylation capacity in a variant of the GH5 β-mannanase *Tr*Man5A (**Paper III**). The *Tr*Man5A variant displayed greatly improved transglycosylation capacity at the early timepoints. Observed secondary (product) hydrolysis at later times highlighted the importance of analysing prolonged reaction times to determine suitable reaction termination. **Paper III** also demonstrated how enzyme synergy can lead to increased transglycosylation yields, when *Tr*Man5A and a guar α-galactosidase was used in co-incubations where a glactomannan was used as the glycosyl donor. α-Galactosidases were further studied in **Paper IV**, where the transglycosylation capacity of two different α-galactosidases were explored with different glycosyl donors and acceptor molecules. The study showed that the guar α-galactosidase was able to utilise a wide variety of acceptor molecules and glycosyl donors, further expanding potential transglycosylation products that may be produced with the enzyme. **Paper IV** further highlights the negative effects secondary hydrolysis may have on transglycosylation yields. The presented work also shows how targetting highly conserved residues within a glycoside hydrolase family can be used to quickly generate GH variants with improved transglycosylation capacity compared to the wild type GH (**Paper V**). The method relies on protein sequence data and does not require structural knowledge of the target enzyme. Furthermore, the method generates few variants (<20) for screening compared to directed evolution (100s to 1000s) while it appears to be generally applicable as it was succesfully applied to six different GH families covering varying specificities. Improvements was, in part, indicated to be associated with reduced secondary hydrolysis in several of the six GH families in the study.

The results presented in the thesis have expanded the knowledge of different factors that affects and can be manipulated in order to improve the transglycosylation capacity in retaining glycoisde hyroalses. The work presented in the thesis will help further enzymatic synthesis approaches utilising renewable raw-materials.

Key words; β-mannanase, α-galactosidase, transglycosylation, enzyme engineering, MALDI-ToF MS, HPLC

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Coverphoto: “The right tool for the job?”

AI-generated picture by DALL·E 2, using the prompt: “Oil painting of a schematic overview of transglycosylation carried out by a beta-mannanase resulting in the formation of mannoside with a pine in the background”.

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II. Bågenholm, V., **Wiemann, M.**, Reddy, S. K., Bhattacharya, A., Rosengren, A., Logan, D. T., & Stålbrand, H. (2019). A surface-exposed GH26 β-mannanase from Bacteroides ovatus: Structure, role, and phylogenetic analysis of BoMan26B. Journal of Biological Chemistry, 294(23), 9100-9117.

III. Butler, S. J., Birgersson, S., **Wiemann, M.**, Arcos-Hernandez, M., & Stålbrand, H. (2022). Transglycosylation by β-mannanase TrMan5A variants and enzyme synergy for synthesis of allyl glycosides from galactomannan. Process Biochemistry, 112, 154-166.

IV. **Wiemann, M.**, Axell, E., & Stålbrand, H. (2022). A Comparison of the Transglycosylation Capacity between the Guar GH27 Aga27A and Bacteroides GH36 Bo Gal36A α-Galactosidases. Applied Sciences, 12(10), 5123.

V. Teze, D., Zhao, J., **Wiemann, M.**, Kazi, Z. G., Lupo, R., Zeuner, B., Vuillemin, M., Rønne, M.E., Carlström, G., Duus, J. Ø., Sanejouand, Y-H., O'Donohue, M.J., Karlsson, E.N., Fauré, R., Stålbrand, H., & Svensson, B. (2021). Rational Enzyme Design without Structural Knowledge: A Sequence-Based Approach for Efficient Generation of Transglycosylases. Chemistry–A European Journal, 27(40), 10323-10334.

Additional papers not included in the thesis

Bhattacharya, A., **Wiemann, M.**, & Stålbrand, H. (2021). β-Mannanase BoMan26B from Bacteroides ovatus produces mannan-oligosaccharides with prebiotic potential from galactomannan and softwood β-mannans. LWT, 151, 112215.

Ahlgren, E. C., Fekry, M., **Wiemann, M.**, Söderberg, C. A., Bernfur, K., Gakh, O., Rasmussen, M., Højrup, P., Emanuelsson, C., Isaya, G., & Al-Karadaghi, S. (2017). Iron-induced oligomerization of human FXN81-210 and bacterial CyaY frataxin and the effect of iron chelators. PLoS One, 12(12), e0188937.

Contributions to the papers

Paper I	I designed and carried out the experiments on the preferred binding mode of *Ypen*Man26A and the variant W110H. I wrote the first draft of that section of the paper as well as made the figure for the section and gave comments on the whole manuscript.
Paper II	I designed and carried out the preferential binding mode experiments of *Bo*Man26B and the W110H variant and the MST experiments for oligosaccharide-binding of SusD. I wrote the first draft of those sections of the paper and prepared the figures for them. I also took part in revising the manuscript.
Paper III	I took a major part in the design of and carried out the synergy experiments between *Tr*Man5A and Aga27A in cooperation with one of the co-authors. I took a major part in writing the first draft of the synergy section and constructed the MALDI-ToF figure for the section. I took part in data evaluation, data discussion and interpretation and revision of the whole manuscript.
Paper IV	I took part in the design of the project, wrote the first draft of the manuscript and constructed several of the figures. I carried out the experiments evaluating transglycosylation capacity and secondary hydrolysis, some of the screening and stability experiments. I took a major part in data evaluation and analysis, interpretation and revision of the manuscript.
Paper V	I was responsible for the section concerning the GH2 β-mannosidase, except for the NMR analysis. I designed and performed these experiments, expressed and purified *Cf*Man2A and its variant, took a major part in the data analysis, evaluation and interpretation as well as wrote the first draft of this section. I gave comments on the whole manuscript and took part in revising the manuscript (GH2 part).

Populärvetenskaplig sammanfattning

Det moderna samhället har byggts med hjälp av icke-förnyelsebara material såsom kol och olja. De har möjliggjort mycket men vi har blivit mer och mer medvetna om de problem som är kopplade till användandet av dem, till exempel utsläpp av växthusgaser och plasters ansamlande i naturen på grund av nedskräpning och plasters långsamma nedbrytning. Biomassa från växter eller växtbaserade material är ett förnyelsebart alternativ till icke-förnyelsebara material, som skulle kunna möjliggöra övergången till ett potentiellt mer hållbart samhälle. Växtbaserade produkter som papper, bomull och olika typer av byggmaterial har använts under en lång tid av mänsklighetens historia. Under de senaste årtiondena har biomassa från växter även blivit intressant för andra användningsområden, med biobränslen och bioplaster bland de mest välkända.

Växtbiomassa består till stor del av olika typer av sackarider, samma typ av molekyler som finns i vanligt strösocker. Men till skillnad från strösocker som är uppbyggt av två sackaridenheter, består växtbiomassa ofta till stor del av polysackarider som kan bestå av tiotals till flera tusentals sammankopplade sackaridenheter. Polysackarider är en enormt olikartad grupp av molekyler men två typer av polysackarider som de flesta nog känner till är stärkelse och cellulosa. Cellulosa är en av de huvudsakliga beståndsdelarna i växter, men växter innehåller även andra typer av polysackarider inklusive en grupp av rikligt förekommande polysackarider som kallas hemicellulosa. Hemicellulosa är för närvarande oftast endast sedd som en restprodukt, t.ex. i sidoströmmar från skogs- och jordbruksindustrin, men kan potentiellt användas som ett startmaterial för tillverkning av en rad olika produkter.

För att bygga upp och bryta ner olika typer av sackarider har de flesta organismer utvecklat en rad olika enzymer som fungerar som katalysatorer i reaktionerna. En sort av dessa enzymer är glykosid hydrolaser, enzymer som normalt sett bryter ner större sackarider till mindre. Vissa glykosid hydrolaser kan även sätta ihop sackarider med andra typer av sackarider men också andra typer av molekyler genom en reaktion som kallas transglykosylering. Transglykosylering kan potentiellt användas för att tillverka en mängd olika typer av molekyler från hemicellulosa, till exempel biobaserade tensider (ytaktiva ämnen för rengöring) som är milda och biologiskt nedbrytbara.

Arbetet som ligger till grund för avhandlingen har utforskat ett flertal olika glykosid hydrolaser och olika aspekter som kan spela roll i dessa enzymers förmåga att katalysera transglykosylering. Gemensamt för allt arbete som ligger till grund för avhandlingen är att fokus har legat på glykosid hydrolaser som är verksama mot en särskild typ av hemicellulosa, nämligen β-mannan. β-Mannan är den primära hemicellulosan i trädslag som gran och tall som utgör en majoritet av de träd som används i den svenska skogsindustrin. β-Mannan kan vara uppbyggt av en eller flera olika typer av monosackarider men består huvudsakligen av en typ av monosackarid som kallas mannos som bilder en lång polysackarid kedja av sammanlänkade monosackarider. Denna kedja kan ha monosackarider (galaktos) som sidogrupper och glukos finns ofta tillsammans med mannos i huvudkedjan. För att kunna bryta ner denna typ av komplexa polysackarider krävs ofta en kombination av flertalet olika glykosid hydrolaser som klipper isär olika delar av polysackariden. Exempel på olika glykosid hydrolaser är β-mannanaser som klyver bindningar till mannos-enheter och α-galaktosidaser som klyver bindningar till galaktos-enheter.

I **Artikel I** och **II** undersöktes interaktionerna mellan en typ av glykosid hydrolaser, som kallas β-mannanaser, och sackarider och hur strukturen hos ett β-mannanas kan påverka dess förmåga att interagera med olika typer av β-mannan polysackarider. I artiklarna visar vi hur den struktur som de undersökta β-mannanaserna har gör dem väl lämpade för att bryta ner β-mannan som innehåller galaktos-sidogrupper. Dessutom visar vi hur små förändringar i strukturen hos β-mannanaser kan påverka hur de interagerar med sitt substrat.

Artikel III undersökte tranglykosylering för ett β-mannanas, *Tr*Man5A, samt varianter av detta β-mannanas, där små förändringar har gjorts i enzymet med hjälp av genteknologi. Arbetet i artikeln visar hur dessa varianter verkar vara bättre än *Tr*Man5A i vissa typer av transglykosyleringsreaktioner medan *Tr*Man5A är bättre i andra. Utöver det, visar vi i artikeln hur samarbete mellan *Tr*Man5A och ett α-galaktosidas ledde till ökad omsättning av startmaterialet och större mängd transglykosyleringsprodukter.

I **Artikel IV** jämför vi transglykosyleringsförmågan hos två olika α-galaktosidaser. Vi visar hur val av startmaterial kan leda till skillnader i transglykosyleringsförmåga och i vilken omfattning ett glykosid hydrolas bryter ner produkter från transglykosyleringsreaktioner påverkar vilket enzym som är det bästa valet som katalysator i en reaktion.

Artikel V presenterar en generell metod för att generera varianter av glykosid hydrolaser med ökad transglykosyleringsförmåga. Genom att effektivt identifiera viktiga aminosyror inom en familj av besläktade glykosid hydrolaser och generera varianter med förändrade aminosyror kan transglykosyleringsförmågan förbättras. Vi visar att metoden är generell genom att applicera den på ett flertal (sex stycken) olika typer glykosid hydrolaser, där varianter med förbättrad transglykosyleringsförmåga genererades för varje typ av glykosid hydrolas.

Sammantaget bidrar det arbete som ligger till grund för avhandlingen till att öka kunskapen och förståelsen för olika aspekter som kan var viktiga för transglykosyleringsreaktioner. Visionen är att den kunskapsuppbyggnad som presenteras i avhandlingen tillsammans med belyst och framtida forskning och utveckling kan leda till effektiv användning av enzymatisk syntes för omvandling av förnyelsebara råvaror till värdefulla molekyler i storskaliga processer.

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With this thesis a long journey comes to an end. A journey that perhaps has been a bit different than what I expected from the outset due to the onset of a pandemic in the middle of it. Nevertheless, this is a journey that I perhaps never would have been able to finish on my own and there are a great many people I would like to thank for their support and encouragement throughout the years.

First, I would like to thank my supervisor, Henrik Stålbrand, for welcoming me into his research group for my Ph.D. studies and helping me along on this journey into the world of carbohydrate-active enzymes. Thank you for the long, sometimes maybe a bit too long, and interesting discussions we have had and the valuable input you have provided me throughout the years. I would like to think that I have grown and developed at least to some extent during these years, in part due to your guidance.

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Till Julian, min underbara son. Tack för att du sett till att det inte bara blivit jobb de sista månaderna och att jag har fått upptäcka världen tillsammans med dig medans du växt upp. Det här är vad jag har gjort så länge du har levt men nu får vi ge oss ut på nya äventyr tillsammans.

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List of abbreviations

Aga27A	GH27 α-galactosidase A from *Ceratonia siliqua*
*An*Man5A	GH5 β-mannanase A from *Aspergillus nidulans*
*An*Man5B	GH5 β-mannanase ABfrom *Aspergillus nidulans*
*Bo*Gal36A	GH36 α-galactosidase A from *Bacteroides ovatus*
*Bo*Man26A	GH26 β-mannanase A from *Bacteroides ovatus*
*Bo*Man26B	GH26 β-mannanase B from *Bacteroides ovatus*
CAZy	Carbohydrate active enzyme
*Cf*Man2A	GH2 β-mannosidase A from *Cellulomonas fimi*
*Cf*Man26A-50K	Catalytic module and Ig-like domain of GH26 β-mannanase A from *Cellulomonas fimi*
*Cj*Man26C	GH26 β-mannanase C from *Cellvibrio japonicus*
GG	Guar Gum
GH(s)	Glycoside hydrolase(s)
GHX	Glycoside hydrolase family X
GHX_Y	Glycoside hydrolase family X subfamily Y
HPAEC-PAD	High performance anion exchange chromatography with pulsed amperometric detection
HPLC	High performance liquid chromatography
LBG	Locust bean Gum
m/z	Mass over charge ratio
MALDI-ToF MS	Matrix assisted laser desorption/ionisation time of flight mass spectrometry

M*n*	Mannooligosaccharide with *n* mannosyl units
pNP-Gal	*para*-Nitrophenyl galactosidase
PUL	Polysaccharide utilization locus
R171K	Variant of *Tr*Man5A catalytic module
R171K/E205D	Variant of *Tr*Man5A catalytic module
r_S/r_H	Ratio of rate of synthesis over rate of hydrolysis
*Tr*Man5A	GH5 β-mannanase A from *Trichoderma reesei*
WT	Wild type enzyme
*Ypen*Man26A	GH26 β-mannanase A from *Yunnania penicillata*

Introduction

Oil and other non-renewable resources have been an important cornerstone in the development of modern society, being used in a wide variety of products, including fuel and plastics. Energy from fossil fuels is currently the main energy source from a global perspective and makes up a major part of the total global energy production. However, this reliance on non-renewable resources is not without problems. As the name suggests, there is not an endless stream of non-renewable resources available. In addition, problems associated with non-renewable resources such as greenhouse gas emissions and environmental impact have gained much attention. There are also other concerns regarding products such as some plastics with extremely slow degradation in nature.

Plant biomass is a source of renewable starting materials that has gained more and more interest during the 21st century as a feedstock for processes leading to the production of, for example, biofuel and bioplastics. However, early investigations into the usage of plant biomass often used biomass from plants such as corn and sugarcane, so-called first-generation feedstocks. Using these kinds of plants can be problematic as the growth of them for industrial applications may be in direct competition with use of the same land for the cultivation of food [1]. Due to this, plant biomass from second-generation feedstocks such as wood and other non-edible plants have garnered attention as they do not necessarily compete for the same land areas as food and feed production [1].

One example of a second-generation feedstock is the softwood hemicellulose galactoglucomannan. It is the primary hemicellulose in softwoods such as pine and spruce, trees that are important to the Swedish forest industry. Some of the primary products from the Swedish forest industry is wood and pulp, both processes that produces by-products or side-streams that can contain galactoglucomannans [2]. These biproducts can be used for energy in the processing plants, e.g. used for heating, but have also attracted attention as potential targets for further valorisation and production of various types of chemicals and materials.

In nature, a wide variety of enzymes, e.g. retaining glycoside hydrolases, and proteins are involved in the degradation of plant biomass such as galactoglucomannan. The aim of the work that lays the foundation for this thesis has been to investigate different retaining glycoside hydrolases and how they potentially can be utilised and improved as biocatalysts for the valorisation of

mannose-based polysaccharides, such as galactomannan and galactoglucomannan. “Retaining” in retaining glycoside hydrolases refers to the catalytic mechanism utilised by these enzymes which may allow them to catalyse so called “transglycosylation” reactions, which are kinetically controlled and can be utilised for enzymatic synthesis. The interaction between β-mannanases, enzymes that hydrolyses β-mannan polysaccharides, and oligomeric and polymeric substrates was investigated and how these interactions may be influenced by protein engineering. This work also examines various aspects of transglycosylation catalysed by retaining glycoside hydrolases. It explores how enzyme engineering may be used to improve the transglycosylation capacity of retaining glycoside hydrolases and the effects that different types of substrates may have on the transglycosylation capacity of a retaining glycoside hydrolase. The presented work also explores how synergy between different retaining glycoside hydrolases could be used to improve transglycosylation yields.

Taken together, the work presented in this thesis explores various aspects of transglycosylation by retaining glycoside hydrolases and forms a groundwork towards the usage of renewable plant polysaccharides in bio-catalysis with retaining glycoside hydrolases as catalysts to produce well-defined glycoside and saccharide products.

Background

Carbohydrates

Carbohydrates are one of the fundamental building blocks of life and are found performing a wide variety of roles in nature, including energy storage [3], structural integrity [4, 5], and signalling [6]. Carbohydrates can be found as single monosaccharide units, for example glucose, mannose or galactose, or several interconnected monosaccharides units in the form of oligosaccharides and polysaccharides [7]. What length of a glycan where one makes the distinction between oligo- and polysaccharide is somewhat unclear but glycans with less than 10 to 12 monosaccharide units are usually termed oligosaccharides while those with more are referred to as polysaccharides [7, 8]. Oligo- and polysaccharides can consist of the same type of monosaccharide units, e.g. only glucose in starch and cellulose, or different types of monosaccharide units [7]. Furthermore, they can exist as one linear chain, e.g. cellulose, or they can be branched and/or contain substitutions along the mainchain polysaccharide [9, 10], as exemplified by the branched polysaccharide starch [11]. Carbohydrates are one of the most complex structures in nature due to the combination of multiple types of sugars that can be connected in a multitude of ways [12, 13], although all these possible theoretical combinations are unlikely to appear in nature.

Plant cell wall polysaccharides

Plant cell wall polysaccharides are some of the most abundant polysaccharides in nature [5, 14]. Polysaccharides are an integral part of the cell walls of plants and are found in the cell walls in the forms of cellulose, hemicellulose and pectin [15]. Besides polysaccharides, various proteins and phenolic compounds, known as lignin, can also be important components of the cell wall of plants [15]. The cell wall of terrestrial plants consists of several layers and can be divided into two types, the primary cell wall and secondary cell wall, with the composition of the cell walls being species dependant [4, 16]. The primary cell wall is formed during the growth of the cell and will expand and reform as the cells grow [16] and generally consists primarily of cellulose, pectin and hemicellulose with some cell wall proteins present as well [17]. Once the cell stops growing, some can also form a thicker and more rigid secondary cell wall that provides structural support [18]. The composition of

the secondary cell wall can vary substantially between different species, but the primary components are generally cellulose, hemicellulose and lignin although all three are not necessarily part of all secondary cell wall [18].

Cellulose

Cellulose is the most abundant biopolymer in nature and consists of linear chains of β-1,4-linked glucosyl units, with the number of monosaccharide units being highly variable and dependant on source of the cellulose [18, 19]. Cellulose is synthesised by a multi-enzyme complex in the cell membrane that forms the glucan chains on the outside of the cell membrane [14]. The chains of linear glucose form microfibrils through association with one another through intermolecular interactions [19] and the microfibrils have both crystalline and non-crystalline, or amorphous, regions [15]. These microfibrils form the structural backbone of the plant cell walls and form larger fibrils interconnected in a matrix of other cell wall components, such as hemicellulose, pectin and lignin [15]. Cellulose has a long history of use in human history in the form of paper and textiles [20] and is currently the target for research for various applications such as biofuels [21, 22] and biomedical applications [23].

Hemicellulose

Hemicellulose is the catch-all name for non-cellulose polysaccharides with equatorial β-1,4-linked backbones and β-1,3-1,4-linked glucans that are present in plant cell walls [4]. Hemicellulose also includes cell wall storage polysaccharides that are found in some plants [4]. Compared to cellulose, hemicelluloses are a much more heterogenous group and the main backbone saccharide can be xylose, glucose or mannose that form the polysaccharides xylan, xyloglucan, glucan, mannan and galactoglucomannan [4]. The backbone polysaccharide can be substituted, i.e. by the attachment of glycosyls [14] or other small molecules such as acetyl or ferulic acids [4, 24], and the degree and what types of substitutions a polysaccharide have vary between species. These substituents can help with the solubility of the polysaccharide, as lack of them often makes the polysaccharides insoluble [14]. Hemicelluloses are synthesised in the Golgi apparatus and then transported to the correct location in vesicles [25]. Just as the substitutions vary between different species, so does what type of hemicellulose that is used [4]. As this thesis has focused on enzymes involved in β-mannan degradation, a more in-depth description of hemicelluloses will be limited to those that are mannose-based.

Mannose-based polysaccharides

β-Mannans are a group of hemicelluloses where β-1,4-linked mannose is the major constituent of the polysaccharide backbone [3, 4]. β-Mannans can be classified into four different subfamilies in the form of linear mannan, glucomannan, galactomannan and galactoglucomannan [3], shown in figure B1. β-Mannans in the form of galactoglucomannan is the primary hemicellulose present in softwoods, e.g.

pine and spruce, [4] while the three other mannan subfamilies often are found as seed storage polysaccharides [3], though they may have other functions, e.g. as the primary structural polysaccharide in some green algae [26].

Linear β-mannans are, as the name suggests, linear chains where the backbone is comprised of β-1,4-linked mannose units but may have a low degree of galactose substitutions (<5%) [3]. Linear mannans have been found to be generally insoluble in water [3, 4] and can be found in various plants [27, 28], such as the the ivory palm nut [29], and various algae [26, 29].

Glucomannans are mannan polysaccharides which have a backbone of β-1,4-linked mannosyl units interspersed with β-1,4-linked glucosyl units, with the ratio between mannose and glucose units being dependant on the source of the glucomannan [30]. Glucomannans are water soluble due to the presence of acetylation along the polysaccharide backbone [30, 31]. Konjac glucomannan, from the tubers of the plant *Amorphophallus konjac*, is one of the most commonly used types of glucomannans and is extracted in the form of flour from the corm of *A. konjac* [32]. Konjac glucomannan can be used as a thickener or emulsifier in food [33] and has been investigated for a wide variety of potential health promoting properties [32].

Galactomannans have a polysaccharide backbone of β-1,4-linked mannosyls with substitutions of α-1,6-linked galactosyls along the backbone [4]. The degree of substitution, or how many galactosyl substituents there are compared to mannosyl units, varies with the source of the galactomannan [4, 9, 34] and the galactosyl substitution makes them water soluble [3, 35]. Two commonly used galactomannans are locust bean gum (LBG) from the seeds of *Ceratonia siliqua* and guar gum (GG) from the beans of *Cyamopsis tetragonoloba* [3]. Guar gum has a higher degree of substitution than locust bean gum, with guar gum having reported galactose to mannose ratios of 1:1.6-2 while locust bean gum has reported ratios of galactose to mannose of 1:3-4 [9, 34]. The higher ratio of galactose to mannose in guar gum compared to locust bean gum makes guar gum more easily dissolvable in water than locust bean gum [36]. Both locust bean gum and guar gum are used in food industry as thickener and emulsifiers, for example in ice cream [9, 34].

The most complex of the characterised β-mannans is galactoglucomannan (GGM). GGM has a polysaccharide backbone of β-1,4-linked mannosyls and glucosyls with substitutions of α-1,6-linked galactosyls and acetyls, which primarily are present on the C-2 and C-3 of the mannosyl units [3]. As the other substituted β-mannans, galactoglucomannan is water soluble [3]. GGM, as mentioned above, is the main hemicellulose in softwoods, such as spruce and pine, and can constitute up to 20 w/w% of the dry weight [37]. GGM has been investigated for various applications such as film formation [38], as a stabiliser in emulsions [39] and as a precursor for production of potential prebiotics [40].

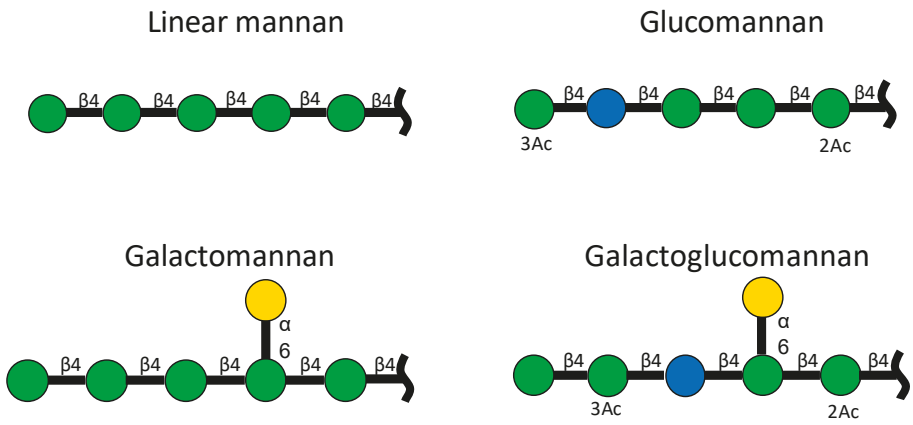

Figure B1. Overview of different type of mannanas.
The figure shows a schematic representation of different types of β-mannans. The mannosyl units are coloured green, the glucosyls are coloured blue and the galactosyls are coloured yellow. The polysaccahride backbones are connected through β-1,4-linkages while the galactosyls substitutions are connected with α-1,6-linkages. The acetylations are attached to either O2 or O3 of the mannosyl units. The figure was made using symbol nomenclature for glycans (SNFG) [41, 42].

Glycoside hydrolases

In order to be able to degrade and reorganise the diverse and complex carbohydrates that exist in nature, a multitude of different glycoside hydrolases (GHs) are required. A GH often primarily targets one specific type of saccharide and linkage, e.g. β-1,4-linked mannose or α-1,6-linked galactose [4, 43, 44], but there are examples of promiscuous glycoside hydrolases (GHs) [45]. GHs can be found in all kingdoms of life, mirroring the importance of carbohydrates as one of the fundamental building blocks of life. Glycoside hydrolases have been divided into different GH families based on their sequences by the so-called Carbohydrate Active Enzyme (CAZy) classification and can be found in the CAZy database (www.cazy.org) [46]. This classification divides glycoside hydrolases into GH families based on sequence similarity of experimentally characterised GHs. Apart from glycoside hydrolases, which primarily hydrolyses or rearranges glycosidic bonds, the CAZy database also contains families of glycosyltransferases (GTs), which catalyse the formation of glycosidic bonds, polysaccharide lyases (PLs), which cleaves glycosidic bonds non-hydrolytically, carbohydrate esterases (CEs), which hydrolyse carbohydrate esters, auxiliary activities (AAs), which are redox-active enzymes, and carbohydrate-binding modules (CBMs), which binds to carbohydrates [46]. While the creation of a new CAZy family requires a characterised protein, putative proteins from GenBank are added daily to the existing families thanks to automation and, if necessary, human curation [46].

Early it was clear that even within GH families there was a large variety in the sequences, e.g. for cellulases [47]. The GH subfamily classification, i.e. subgroupings within the same GH family, allows for a finer phylogenetic analysis of a single GH family as many GH families contains different specificities, i.e. GHs within the family may hydrolyse different sugars and sometimes different linkages. The separation into GH subfamilies has in several cases allowed for the formation of groups with single specificities within a GH family, although this is not guaranteed [48-51].

While the initial classification into glycoside hydrolase families was based on sequence similarity, as more and more 3D structures of glycoside hydrolases became available it became apparent that even when sequences had low similarity, the fold and general tertiary structure could still be highly similar. Moreover, the catalytic amino acids could be conserved between GH families and these GH families may utilise the same catalytic mechanism. Taken together, this led to the now generally accepted suggestion that several GH families could share a common ancestry and prompting the grouping of GH families into clans to highlight this potential common ancestry [52-54].

Glycoside hydrolases are one of two types of enzymes that at the moment make up a majority of the sequences of the CAZy database [46]. As of September 2021, the CAZY database consisted of 171 GH families and almost a million sequences that have been classified as GHs, though only a fraction of these have been characterised [46]. Although the name suggests that these GH families only contain enzymes that perform hydrolysis, this is not the case. Some noteworthy other types of enzymes found in the GH families are transglycosylases [55], enzymes where the glycosidic moiety is attached to another sugar or other type of molecule other than water, and phosphorylases, which forms phosphorylated glycosides [56, 57].

Depending on how a glycoside hydrolase cleaves a saccharide, they can be defined as an *exo*- or *endo*-acting. An *exo*-acting glycoside hydrolase is a glycoside hydrolase that cleaves off saccharides from the end of a saccharide chain [3, 58, 59], most commonly as monosaccharides (**Paper IV and V**) [60-62]. It should however be noted that there are examples of *exo*-acting GHs that cleave off di- or trisaccharides [63, 64]. An *endo*-acting glycoside hydrolase, on the other hand, cleaves internal glycoside bonds in an oligosaccharide or polysaccharide chain (**Paper I-III**) [43, 59].

The main action of a a glycoside hydrolase, i.e. if it is mainly *exo*- or *endo*-acting, may be determined by the topology of its active site. Generally, glycoside hydrolases adopt one of three overarching topologies when it comes to the active sites, pocket (or crater), tunnel or cleft (or groove) (Figure B2) [59]. A pocket topology, as the name suggest, means that the GH has a pocket-formed active site in which the saccharide binds and this topology is common among *exo*-acting GHs [61, 65]. The pocket can either be formed by one enzyme alone or through the

interaction of several monomers when a GH forms oligomeric complexes [61, 65] and is often a fairly restricted topology. The cleft or groove topology on the other hand is more open and may allow for random binding of the saccharide and this type of topology is commonly found in *endo*-acting glycoside hydrolases (**Paper I** and **II**) [59]. The tunnel topology can be seen as an extension of the cleft topology where extended loops around the active site cause it to be more restricted [66].

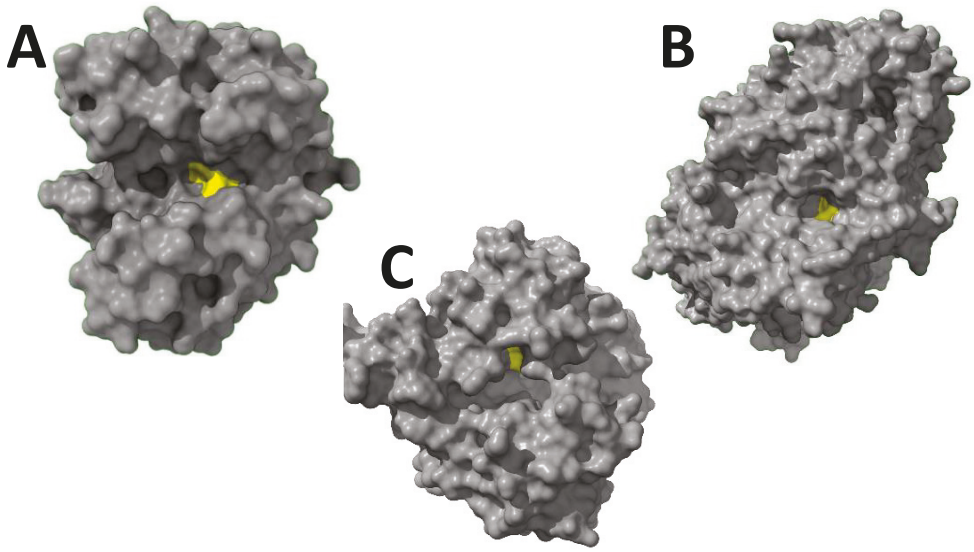

Figure B2. Different topologies of the active site of glycoside hydrolases.
The figure shows a comparison between the three general topologies of the active sites of glycoside hydrolases, with the position of the catalytic residues marked with yellow. The figure was inspired by the representation of topologies presented in Davies and Henrissat [59]. Structure A is a GH26 β-mannanse from *Bacteoides ovatus* with a cleft topology (*Bo*Man26B, PDB ID: 6HF4, **Paper II**), strucutre B is a GH2 β-mannosidase from *Bacteroides thetaiotaomicron* with a pocket topology (*Bt*Man2A, PDB ID: 2JE8, [61]) and structure C is a chitinase from *Serratia marcescens* with a tunnel-like topology (ChiB, PDB ID: 1E6N, [66]). The figure was created with ChimeraX [67, 68].

Reaction mechanisms and subsite nomenclature

Glycoside hydrolases commonly have either an inverting or retaining mechanism, resulting in anomeric configuration of the cleaved sugar either being inverted or maintained compared to that of the substrate [69]. That is, if a GH with a retaining mechanism cleaves a β-linked mannosyl it would produce a β-configured mannose while a GH with an inverting mechanism would produce an α-configured mannose when attacking the same substrate. While many GHs utilise such retaining or inverting mechanisms [70], generally utilising two carboxylates as the catalytic residues [59, 71], there are some examples of other mechanisms among the glycoside hydrolases. For example there are some that utilise other mechanisms

such as substrate-assisted catalysis [59, 72, 73] and NAD-dependant hydrolysis [74]. As most of the enzymes studied in the papers that form the basis of this thesis utilises the retaining mechanism (Figure B3) [69, 70], a more in-depth description will be limited to this particular mechanism.

The classical retaining mechanism is a double-displacement mechanism, with the formation of a covalent glycosyl-enzyme intermediate [69, 70, 75]. In this double-displacement mechanism the two catalytic residues act as a nucleophile and a general acid/base respectively. In the first step of the reaction the catalytic nucleophile performs a nucleophilic attack on the C1, or anomeric carbon, while the general acid/base protonates the leaving group [69]. This leads to the creation of a covalent glycosyl-enzyme intermediate with part of the initial substrate attached to the enzyme while the other part exits the active site. The part of the saccharide leaving the active site after this first step is often referred to as the leaving group. This first step is referred to as the glycosylation step of the reaction [55] and due to the nature of the double-displacement mechanism this glycosyl-enzyme intermediate will have an inverted anomeric configuration compared to the substrate.

With the formation of the glycosyl-enzyme intermediate and exit of the leaving group, the reaction is now ready for the second step. In this second step a water molecule enters the catalytic site and the general acid/base acts as a base, deprotonating the water that then performs a nucleophilic attack against the anomeric carbon [69]. The nucleophilic attack of the water leads to the release of the covalently bound saccharide from the enzyme and this second step is commonly referred to as the deglycosylation step of the reaction [55]. As for the glycosylation step, the deglycosylation step leads to an inversion of the anomeric configuration, resulting in overall retention of the anomeric configuration of the sugar compared to that of the substrate. Both the glycosylation and deglycosylation steps of the reaction occurs through transition states with oxocarbenium ion-like character [69, 76]. One thing to note is that water does not necessarily have to be nucleophile in the deglycosylation step of the reaction. This role can be filed by any molecule with a suitably located hydroxyl group such as saccharides or alcohols (**Paper III–V**) [55, 77-80], and the reaction is then referred to as a transglycosylation reaction. The sugar that is part of the glycosyl-enzyme intermediate is commonly referred to as the glycosyl donor or donor for short, while the molecule that does the nucleophilic attack against the intermediate is referred to as glycosyl acceptor or acceptor for short.

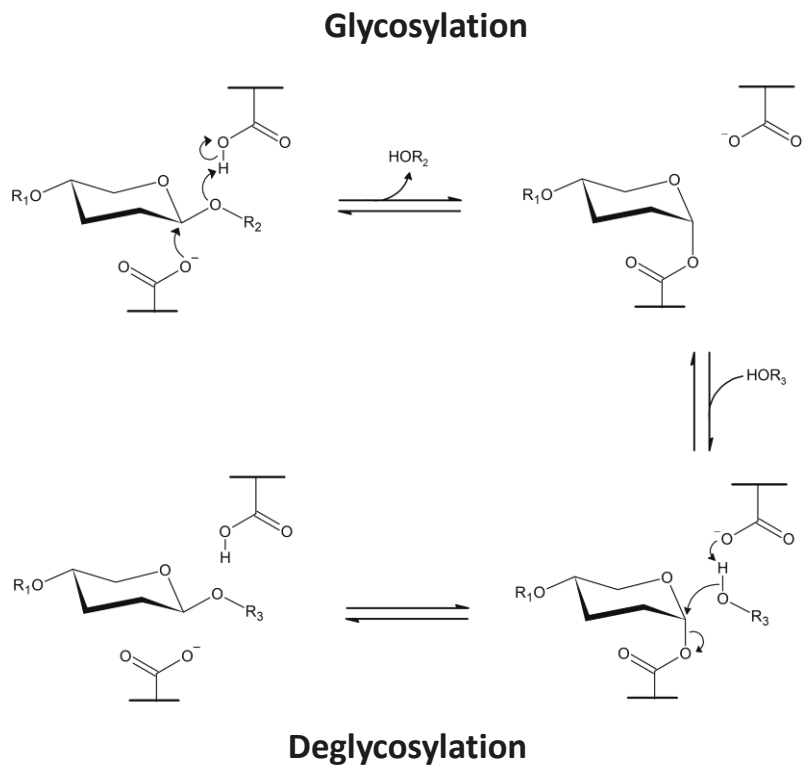

Figure B3. Schematic overview of the retaining mechanism of retaining glycoside hydrolases.
The figure shows an schematic overview of the retaining mechanism [69] with the initial nucleophilic attack by the GH and proton donation by the general acid/base, leading to the formation of the glycosyl-enzyme intermediate, followed by the nucelophilic attack on the resulting glycosyl-enzyme intermediate by the acceptor and finally the deglycosylated enzyme and the released product. R_1 can be either a hydrogen or one or more additional saccharides, R_2 is one or more additonal saccharides and R_3 can be either a hydrogen , i.e. water, or another saccharide or other type of molecule with an hydroxyl group.

While the catalytic site of GHs, where the hydrolysis of the glycosidic bond occurs, is a key part of a glycoside hydrolase, substrate-binding sites in the active site are also important for catalysis as they confer specificity and increased binding (**Paper I**, **Paper II**). These substrate-binding sites are referred to as subsites and each subsite is occupied by one monosaccharide unit. To be able to easily distinguish between different subsites, and to encourage uniform naming within the field, a naming nomenclature has been developed [81], shown in Figure B4. In this nomenclature, subsites are referred to as negative or positive integers, numbered based on their distance from the glycosidic bond that is broken [81]. The glycosidic bond that is broken is located between the monomers bound in the -1 and +1 subsites and as one moves away from it, each additional subsite is one integer higher with

those towards the non-reducing end being assigned negative numbers while those towards the reducing being assigned positive numbers [81]. The positive and negative subsites are often referred to as the glycone and aglycone subsites, respectively (even though saccharide units commonly occupy the positively numbered subsite).

While several types of interactions between protein and carbohydrate contributes to the specificity and binding strength in the subsites, the importance of aromatic amino acids in carbohydrate-binding proteins deserves some extra attention. Aromatic amino acids are in general overrepresented in the proximity of carbohydrates in crystal structures of protein-carbohydrate complexes compared to their general occurrence in a protein sequence [82, 83]. These aromatic residues can contribute to ligand binding (**Paper I** and **II**) [84-86] and as the position of the hydroxyl groups affects the strength of the interaction between the carbohydrate and the aromatic residue it is likely they also play a role in specificity [82, 83, 87].

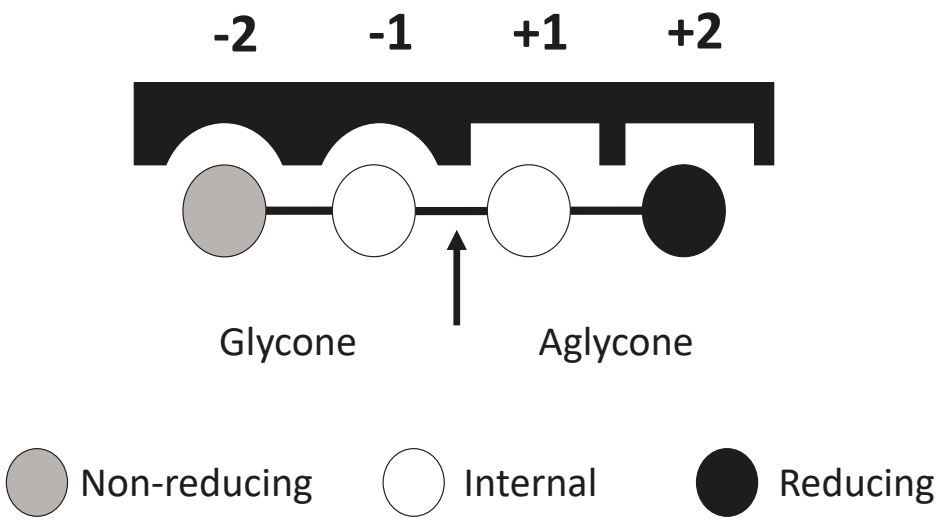

Figure B4. Schematic representation of active site subsites.
The figure shows a schematic representation of a GH with four subsites with bound monosaccahride units and the glycosidic bond that will be cleaved marked with an arrow. The glycosidic bond that is broken is located between subsite -1 and +1 and each subsite that extends beyond -1 and +1 gets an increasing integer, i.e. if two subsites would exists past -1 they would be named -2 and -3 respectively. The negative subsites are often referred to as glycone while the positive subsites often are referred to as aglycone. Both the positively and negatively numbered subsites canbe fewer or extend beyond what is shown in the figure, with the number of subsites depending on the GH.

β-Mannanases

β-Mannanases are glycoside hydrolases that generally cleave β-1,4-linkages in mannan poly- and oligosaccharides in a random *endo*-acting fashion [88-90], generating a varied and often complex product profile when degrading polysaccharides [40, 91]. Besides random *endo*-acting GHs, there are some examples of mannobiohydrolase activity and processivity, i.e. multiple hydrolytic events against the same saccharide without dissociation from the active site, among the β-mannanases [63, 92, 93]. β-Mannanases are found across a number of different types of organisms from differing environments, e.g. fungi (**Paper I** and **III**) [58, 89, 94-96], the human gut microbiota (**Paper II)** [44, 97], extremophiles [98] and plants [99]. The same organism can possess several different β-mannanases that can have different roles in the digestion of complex mannan-based polysaccharides [97, 100, 101]. β-Mannanases can be active towards a large variety of different mannans though their specific activity toward any given mannan polysaccharide can be influenced by the properties of the polysaccharide [94], such as degree of galactose substitutions (**Paper I** and **II**) [84, 94], acetylation [24] and presence of glucose in the main-chain [84]. The degree of influence these factors have will be dependent on the β-mannanase [84, 94, 97]. In addition, depending on the architecture of the active site, different β-mannanases will have differing ability to accommodate substitutions, such galactosyl units, or glucose units in the saccharide backbone in different subsites (**Paper I** and **II**) [102].

The majority of β-mannanases are found in GH families GH5, GH26, GH113 and GH134 (www.cazy.org) [46]. GH5, GH26 and GH113 belongs to clan GH-A, having a TIM-barrel fold, the same catalytic amino acids, located on β-strands 4 and 7, and utilises the retaining mechanism (Figure B3) (**Paper I** and **II**) [103, 104]. GH134 has a lysozyme-like fold and utilises an inverting mechanism [105-107]. This thesis has focused on β-mannanases from GH5 and GH26 (**Paper I-III**) and they will be further presented in the following paragraphs.

GH5 is one of the largest and most diverse GH families in the CAZy database, with 595 characterized members as of September 2022. Besides β-mannanase activity [101, 108, 109], the family also contains, among other, β-glucanases [110], β-xylanases [111], β-mannosidases [112] and lichenases [113] spread over 56 subfamilies [48] (www.cazy.org) [46]. The β-mannanases in GH5 are primarily found in subfamilies GH5_7, GH5_8 and GH5_10, with a majority of those in GH5_7 being of eukaryotic origin, most of the constituents of GH5_8 being of bacterial origin and those characterised in GH5_10 are of eukaryotic origin [100, 102, 108, 109, 114-116]. All of these GH5 subfamilies contain enzymes with determined 3D structure (www.cazy.org) [46] and have seven highly conserved residues located around the -1 subsite in the form of Arg54, Asn168, Glu169 (acid/base), His241, Tyr243, Glu276 (nucleophile) and Trp306, numbered according to *Trichoderma reseei* GH5 β-mannanase [117-119]. The ability to

perform transglycosylation is well documented in GH5 β-mannanases [117-119], including for the GH5_7 *Trichoderma reseei* β-mannanase (*Tr*Man5A) studied in **Paper III** [117-119].

GH26 contains primarily β-mannanases of prokaryotic origin (**Paper II**) [63, 97, 101, 120, 121], though there are some β-mannanases from eukaryotic organisms (**Paper I**) [95, 100], lichenases [122] and xylanases [123] reported in the family as well. As for GH5 β-mannanases, the 3D structure of several GH26 β-mannanases have been determined (**Paper I** and **II**) [63, 97, 100, 124-126]. GH26 β-mannanases have several highly conserved residues in the active site cleft, primarily around the -1 and +1 subsites (**Paper II**) [124, 127]. The highly conserved residues in the active site cleft are D101, H136, R197, H200, E201 (acid/base), G204, W206, F207, D261, Y263, E291 (nucleophile) and W314, using *Bo*Man26B numbering, out of which seven had previously been reported [124, 127] before the publication of **Paper II**. In contrast to GH5, transglycosylation has so far not been reported for native GH26 [100, 128], though indications of transglycosylation have been observed for an engineered variant [129].

β-Mannanases have been investigated for numerous applications. For food and feed processing, investigated applications include clarification of fruit juice to make it less viscous and turbid [128, 130, 131], hydrolysis of mannans from coffee beans for various applications [58, 132, 133] and improving animal feeds [58, 128]. β-mannanases have also been investigated for their ability to produce mono- and oligosaccharides from polysaccharides [2, 94, 134], for production of fermentable saccharides that can be used for bio-based fuel production [135-137], as well as for the potential probiotic applications of mannooligosaccharides (MOS) [40, 138, 139]. In addition, the transglycosylation capacity of GH5 β-mannanases has been explored for the production of different types of mannosides (**Paper III**) [118, 119].

β-Mannosidases

β-Mannosidases are *exo*-acting enzymes where the majority cleave terminal β-1,4-linked mannosyls at the non-reducing end of mannan poly- and oligosaccharides [61, 112, 140-142]. There have been some recent reports of *exo*-acting β-mannosidases that cleave from the reducing end [104, 143]. β-Mannosidases are found in a wide variety of organisms, such as mammals [144], plants [145], fungi [142, 146] and bacteria (**Paper V**) [61, 147]. β-Mannosidases can have different functions in different organisms. Their primary role in bacteria appears to be catabolism of carbohydrates, whereas in mammals they can fill a role in the processing of N-glycans in the lysosomes, as deficiencies in the gene encoding mammalian β-mannosidase may cause lysosomal storage disease [148, 149]. Generally, β-mannosidases can be found in GH1, GH2 [61, 150] and GH5, and the reducing end acting β-mannosidases belong to GH113 [104, 143].

A majority of the β-mannosidases belong to GH2, which just like GH5 is a rather large GH family with several different activities (www.cazy.org) [46]. GH2 belongs to clan GH-A like GH5 and GH26 β-mannanases and the catalytic domain has a TIM-barrel fold. The two catalytic amino acids are glutamates, and they utilise the retaining mechanism [61, 150]. Besides the catalytic domain, several GH2 β-mannosidases also have four additional domains with the catalytic domain situated in the middle of the structure with the four additional domains flanking it [61, 150-152]. In all of the determined GH2 β-mannosidase structure to date it has been shown that one or several of the domains besides the catalytic domain may contribute to the formation of the active site, which has a pocket topology [61, 150-152]. As for GH5 β-mannanases, several β-mannosidases have been shown to have the ability to carry out transglycosylation (**Paper V**) [141, 142, 153, 154].

α-Galactosidases

α-Galactosidases are *exo*-acting GHs that cleaves α-linked galactosyls. They are found in families GH4, GH27, GH31, GH36, GH57, GH95, GH97 and GH110 and carry out the hydrolysis of different α-linked galactosyls, e.g. α-1,6 and α-1,2 (www.cazy.org) [46]. They may target galactooligosaccharides, such as raffinose and stachyose, but some act on polysaccharides with galactosyl substitutions such as locust bean gum and guar gum [35, 62, 155]. α-Galactosidases have been explored for various applications, including removal of the α-gal epitope for xenotransplantation [156], treatment of Fabry Disease [62, 157] and various food and biomass processing applications [62, 158]. As the α-galactosidases studied in **Paper III** and **IV** belong to GH27 and GH36, the scope of this section will be limited to those GH families, which also are the two GH families which contains a majority of the α-galactosidases [159].

Both GH27 and GH36 belong to clan GH-D, with the catalytic domain having a similar $(\beta/\alpha)_8$-fold (or TIM-barrel fold) as clan GH-A enzymes and utilises a retaining mechanism. Instead of two catalytic glutamates, which clan GH-A use, clan GH-D have two aspartates as the catalytic residues [160]. The characterized α-galactosidases in GH27 are primarily of eukaryotic origin while a majority of those in GH36 are of bacterial origin (www.cazy.org) [46]. While activity towards galactosyl substituents of polymeric substrates, such as galactomannan, is well-documented for GH27 enzymes, e.g. for the guar α-galactosidase Aga27A used in **Paper III** [158, 161], GH36 enzymes are in general thought to have limited activity towards polymeric substrates [155, 159]. There are examples of utilisation of galactomannan among the GH36 α-galactosidases, e.g. a tetrameric GH36 α-galactosidase from the gut bacterium *Bacteroides ovatus* (*Bo*Gal36A), studied in **Paper IV**, in which a difference in a loop structure to several other GH36 α-galactosidases is thought to lead to a more open active site cleft, allowing a polysaccharide to enter the active site [35]. The discrepancy in activity towards

polymeric substrates have been suggested to be due to differences in oligomeric states of the enzymes, as many of the characterized GH36 α-galactosidases are tetrameric, causing restriction of the active site, while many GH27 α-galactosidases are monomeric and therefore may have a more open active site [159, 162]. Supporting this suggestion is the observation that tetrameric GH27 enzymes also lacked the ability to degrade internal galactose substituents [159, 163]. Unfortunately, the ability to utilise polymeric substrates does not appear to have been investigated for a monomeric GH36 α-galactosidase from Thermotoga maritima [164]. Several studies have reported the synergistic effects of co-incubations between β-mannanases and α-galactosidases (**Paper II** and **III**) and have shown that while GH36 enzymes may not be able to hydrolyse polymeric substrates, they are able to utilise oligosaccharides from galactomannans that have been pre-hydrolysed by β-mannanases [143, 158, 165, 166].

Just as for GH5 β-mannanases and β-mannosidases, transglycosylation has been reported for α-galactosidases, both for those in family GH27 (**Paper III** and **IV**) [167] and those in family GH36 (**Paper IV**) [79, 168, 169].

Modularity

As in the case of GH2 β-mannosidases, glycoside hydrolases can consist of more than just a catalytic domain [61]. One of the most common and best described of these additional domains for GHs are carbohydrate binding modules (CBMs) [170], which as the name suggests binds to carbohydrates. Among the earliest described CBMs were the cellulose binding domains, associated to cellulases [171]. Currently CBMs are divided into 93 different CBM families in the CAZy database (www.cazy.org) [46]. A function of a CBM can be to help the catalytic module of the enzyme to associate to the polysaccharide that is being degraded [170]. This keeps the enzymes in close proximity to the polysaccharide and increases the enzyme concentration at the polysaccharide [170, 172]. Some CBMs have also been shown to help disrupt the surface of polysaccharide microfibrils in a non-catalytic manner, making the substrate more accessible to the catalytic domain [170, 172]. Furthermore, a study has shown how a CBM may confer specificity to a non-specific glycoside hydrolase [173]. As a CBM is a fully independent domain it is possible to transfer them between glycoside hydrolases, which can be used to improve the catalytic properties of GHs [174].

While it would be natural to expect a CBM to have affinity towards the same glycan as the catalytic domain to which it is attached, this is not always the case. As an example, the β-mannanase from *Trichoderma reesei* studied in **Paper III** has a CBM with affinity for cellulose instead of mannans, thereby providing no beneficial effects when utilizing soluble and insoluble mannans as substrate [175]. However, the presence of the CBM did lead to improved catalytic efficiency when the enzyme was incubated with a mannan/cellulose complex [175], indicating that it might fill

a function in the degradation of complex biological materials such as plant cell walls.

Enzyme synergy

In nature the coordinated effort of several different enzymes, often different glycoside hydrolases but sometimes other types of enzymes as well, is often necessary for the efficient breakdown of complex polysaccharides, as can be seen in Figure B5. This kind of cooperativity where the concerted effort of two or more GHs leads to an increase in product compared to them being used individually is called enzyme synergy. Synergy can either be in sequential manner, where one GH acts after the other, or simultaneous, where the GHs act at the same time [176]. An example of how synergistic systems for polysaccharide degradation has evolved are the polysaccharide utilisation loci (PULs) some bacteria have developed [177, 178]. In PULs, genes encoding proteins for utilization of one type of complex carbohydrate are colocalised and coregulated [177], with the expression of the genes in a PUL producing a set of enzymes apparently well-suited for utilisation of one type of complex carbohydrate. One example of such a PUL is the β-mannan PUL of *Bacteroides ovatus* [97], of which one of the constituting β-mannanases and the α-galactosidase are studied in **Paper II** and **Paper IV**, respectively.

Mirroring nature, enzyme synergy has been applied for saccharification of complex glycans through the utilization of enzyme cocktails [2, 135, 176] and has been applied or investigated for various industrial processes [179, 180]. In **Paper III** we demonstrate how enzyme synergy can be used for improving transglycosylation reactions, an area of enzyme synergy that currently is less well explored.

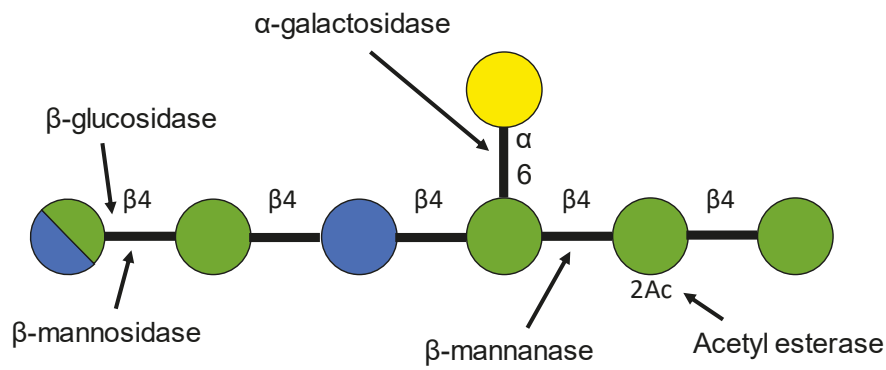

Figure B5. Schematic overview of different enzymes required for degradation of a galactoglucomannan.
The figure represents the cooperative effort of several different types of enzymes that is often required for the efficient degradation of a galactoglucomannan. The enzymes that have been examined in this thesis are β-mannases, β-mannosidases and α-galactosidases.

Transglycosylation

Transglycosylation in nature

As explained in the reaction mechanism section, transglycosylation is the name for retaining mechanism reactions where another molecule than water acts as the nucleophile that attacks the glycosyl-enzyme intermediate. In nature there are several examples of retaining glycoside hydrolases that appear to prefer or almost exclusively perform transglycosylation compared to hydrolysis, so called transglycosylases (TGs) [55]. An interesting example of a transglycosylating retaining glycoside hydrolase is the GH5 β-mannanase from tomato *Le*Man4A, for which different isoforms of the same enzyme displayed differing transglycosylation preferences, indicating the impact enzymatic fine-tuning may have on the transglycosylation capacity of a retaining GH [99]. Enzymes with high native transglycosylation capabilities can have different functions in the cells but have been suggested to fill roles in plant cell wall modification and synthesis of oligo- or polysaccharides [55, 181, 182]. These enzymes are often found in the same families and even subfamilies as enzymes that primarily performs hydrolysis, indicating an evolutionary relationship of TGs and hydrolysing GHs [55, 183]. While TGs often display a high degree of transglycosylation with saccharides as acceptors, this does not necessarily translate to the ability to use other types of molecules, for example alcohols as acceptors [183].

Transglycosylation is also commonly observed for retaining glycoside hydrolases which primarily perform hydrolysis (**Paper III-V**) [79, 182, 184-187]. However, the often low tendency to perform transglycosylation combined with hydrolysis of transglycosylation products, so called secondary hydrolysis [186], makes it more challenging to utilise retaining glycoside hydrolases in transglycosylation reactions. They have therefore been the subject of a wide variety enzyme engineering approaches to try and improve their transglycosylation performance (**Paper III** and **V**) [55, 182].

Engineering retaining GHs to alter transglycosylation capacity

The glycosynthase method

Several different approaches have been tried in order to improve the synthetic capacity of retaining glycoside hydrolases. One of the most successful ones, if looking purely at final yields, is the elegant glycosynthase approach [188]. In a communication by Mackenzie et al, the nucleophile of a retaining β-glucosidase (Abg) was mutated to an alanine (AbgE358A), removing the hydrolytic activity of the enzyme [188]. By incubating the AbgE358A with the activated donors α-

glucosyl fluoride or α-galactosyl fluoride, having the opposite anomeric configuration compared to the natural substrate of the Abg, and various acceptor molecules, products yields between 66-92% were achieved [188]. Part of the explanation presented in the paper for the high yield of synthetic products is the lack of product hydrolysis due to AbgE358A being hydrolytically inept [188]. Product hydrolysis is a common problem that limits the yield of transglycosylation products when it comes to transglycosylation catalysed by retaining GHs that have hydrolytic activity [55, 188] which is essentially abolished or greatly diminished with the glycosynthase approach. The glycosynthase approach has been improved and expanded upon as time has progressed and it has been successfully applied to several glycoside hydrolases from different GH families [189, 190], including β-mannanases [191] and β-mannosidases [192]. The method is not applicable to natural substrates, but requires the use of fluorinated glycosyls, which may be labile depending on reaction conditions [190, 193, 194]. The very low activity of the glycosynthases may lead to either extensive reaction time, up to several days or large enzyme loading, for time efficient synthesis [192-195]. Furthermore, the glycosynthase approach may not always be applicable, as demonstrated by Jahn et al. where one GH26 β-mannanase was turned into a glycosynthase but the approach was unsuccessful for another one [191], though the reason why remains unclear.

Rational and semi-rational engineering

Rational and semi-rational engineering approaches relies on structural and/or mechanistic understanding to guide the design of retaining GH variants with altered transglycosylation capacity. One can target specific amino acids using either structural knowledge or knowledge from homologous glycoside hydrolases as a guide (**Paper III**) [184, 196-199] or a sequence-based approach targeting highly conserved residues of a GH family (**Paper V**) [200, 201]. The rational/semi-rational engineering approach has been successfully applied for altering transglycosylation in a wide variety of different retaining glycoside hydrolases, targeting residues with substrate interactions (**Paper III**) [184, 197, 202, 203], residues involved in water interaction [199] or those interacting with the catalytic residues [204], at or close to the active site.

Random mutagenesis

Another alternative for improving the transglycosylation capacity of retaining glycoside hydrolases is the random mutagenesis approach, and the further developed directed evolution approach. Such methods have been successfully applied for improving both hydrolytic properties [205, 206] and transglycosylation properties of glycoside hydrolases [193, 207, 208]. Compared to a rational engineering approach or the glycosynthase approach, random mutagenesis has its strengths and weaknesses. The perhaps greatest strength of random mutagenesis is that previous structural or mechanistic knowledge is not required and may allow the

discovery of variants with improved properties, through residue substitutions that might not have an obvious rationale. Random mutagenesis often requires extensive screening, as the method depends on the generation of hundreds or thousands of variants [193, 205, 209]. When screening such a large number of variants it is of course ideal if one has access to robust and easy to use methods to screen for the desired activity. While several different screening methodologies that are suitable for screening libraries of retaining glycoside hydrolase variants are available for evaluation of hydrolytic activities [205, 206, 210], library screening for transglycosylation reactions are more challenging and work intensive [193, 207, 208].

Although random mutagenesis was not used in the current work, all of these approaches are possible to combine in different ways and the use of one does not exclude use of another, as demonstrated in several studies [209, 211, 212].

Methods

The following section does not cover all the methods used in the papers that form the basis of the thesis, for that please refer to the individual papers. Instead, it will focus on the key methods that have been used and describes the underlying principle of them in a general way.

Evaluating enzymatic activity of retaining glycoside hydrolases

There are several methods available to determine the activity and specificity of glycoside hydrolases. One commonly used for *exo*-acting glycosidases, e.g. α-galactosidases and β-mannosidases, are use of chromogenic substrates such as *para*-nitrophenyl glycosides (**Paper III-V**) [35, 61, 213]. The hydrolysis of these *para*-nitrophenyl glycosides (pNP-Gly) leads to the release of the chromophore *para*-nitrophenol (pNP) which can be detected spectrophotometrically at 405 nm when it is in its phenolate form. By correlating the absorbance in a sample obtained under known conditions to a standard curve of *para*-nitrophenol, one can calculate the activity of the glycoside hydrolase under the tested conditions. In **Paper III-V,** a discontinuous assay was used where high concentration of sodium carbonate was added to stop the reaction and to ensure that pNP was in its phenolate form [214]. In **Paper V** the release of pNP was also used to monitor the reaction progression by taking samples throughout the reaction time and measuring the amount of released pNP.

When evaluating *endo*-glycosidases, such as β-mannanases, a common assay to determine glycoside hydrolase activity against polymeric substrates, e.g. galactomannans, is the 3,5-dinitrosalicylic acid (DNS) assay (**Paper II and III**) [97, 215]. In the DNS assay, a reducing sugar reacts with DNS reagent causing the sugar to become oxidized while the reagent becomes reduced to 3-amino-5-nitrosalicylic acid [215], which has a high light absorbance at 540 nm [216]. When incubating a glycoside hydrolase with polysaccharides, the hydrolysis of the polysaccharide chains leads to the production of more reducing ends which in turn leads to an increased absorbance at 540 nm as more DNS molecules can be reduced. By correlating the absorbance in the sample with that of a standard curve of an

appropriate saccharide, one can estimate the activity and kinetics parameters towards the polysaccharide substrate. The DNS assay is however often unsuitable for analysis of oligosaccharides as substrate. This is due the often relatively high amounts of free reducing ends at the starting point of the reaction when using oligosaccharides as substrates compared to polysaccharides, which leads to high absorbance in negative controls and thus a limited dynamic range of the assay.

For analysis of glycoside hydrolase activity and kinetic parameters against oligosaccharides and hydrolysis product profiles towards both oligosaccharides and polysaccharides, high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is a commonly used analysis method [217]. In **Paper I and II** HPAEC-PAD was used to determine the product profile of two GH26 β-mannanases against oligosaccharides and in combination with isotope labelling through incubation with ^{18}O-water, analysed with mass spectrometry (MS), (described in more detail below) the preferred productive binding mode could be estimated. In addition, in **Paper II** HPEAC-PAD was used in order to examine the hydrolysis profile towards the galactomannans guar gum and locust bean gum for *Bo*Man26B. The method uses strong alkali as eluent, potentially in combination with other chemicals [40, 217], as sugars are weak anions at high pH and therefore able to interact with the anion exchange column and be separated [217]. The sugars eluting from the column then passes a working electrode where they are oxidized and the electric potential they generate is measured [217]. By correlating elution time and generated electric potential to standard curves of known saccharides it is possible to identify and quantify the saccharides in the analysed samples.

Evaluating transglycosylation in retaining glycoside hydrolases

Initial evaluation of transglycosylation in **Paper III** and **IV** was done with matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-ToF MS). In MALDI-ToF MS the sample one wishes to analyse is deposited onto a stainless steel target plate together with a matrix solution, in **Paper III** and **IV** 10 mg/ml 2,5-dihydroxybenzoic acid (DHB), and allowed to dry, causing the matrix to crystallise with the sample [218, 219]. The deposited sample is analysed with a MALDI-ToF mass spectrometer in which the sample gets ionized through light absorption by the matrix molecule and the ionised analytes are then separated in the mass spectrometer based on their weight and charge [219]. By calculating the theoretical monoisotopic mass of expected transglycosylation products and comparing it to the mass over charge values (m/z) observed in the spectrum from the mass spectrometric analysis it is possible to deduce if the molecules appear to be present in the sample or not. MALDI-ToF MS is a method that allows for rapid

analysis of a large number of samples to detect if transglycosylation products are present but is harder to get quantitative data from [219].

In **Paper III** to **V** high performance liquid chromatography (HPLC) was used to quantify transglycosylation products. HPLC is a common analysis method that can be applied to separate a large variety of different molecules depending on the mobile and stationary phase one use [220, 221]. Dependant on the properties of the analytes, different detectors can be used to detect them. When an analyte has a functional group that has light absorbance, a UV-Vis detector set at an appropriate wavelength, used in **Paper III** and **V**, can be utilised [221]. If the analyte does not have any absorbance a more general detector such as charged aerosol detector (CAD), used in **Paper III** and **Paper IV**, or mass spectrometer can be used instead [221]. Hydrophilic interaction chromatography (HILIC) was used in in order to separate both saccharides and transglycosylation products in the form of glycosides from one another. By using standards of known concentrations, it was possible to identify and quantify the analytes. In the cases where no standards were available, fraction collection combined with analysis of the fractions with MALDI-ToF MS was utilised to confirm the identity of the peaks in the chromatogram from the HPLC separation.

In **Paper III–V** transglycosylation capacity of the examined glycoside hydrolases was evaluated in several different ways. One of the parameters used to evaluate the transglycosylation capacity was yield, looking at how much of the loaded donor substrate had been converted into glycosides (**Paper III** and **V**) or how much of the consumed substrate had been converted into glycosides (**Paper IV**). While yield calculations shows how much of the donor substrate has been converted to transglycosylation products it tells little about what is happening in the reaction, e.g. it does not tell anything about to what extent secondary hydrolysis occurs in the reaction. To address secondary hydrolysis, rate of synthesis over rate of hydrolysis (r_S/r_H) [186, 196] was calculated in **Paper III** and **IV** with a generic formula shown as equation 1. The r_S/r_H-value can also be seen as a value that describes the preference for the retaining GH to catalyse transglycosylation reactions over hydrolysis reactions [186], at least if measured early in the reaction where limited amount of secondary reactions, such as secondary hydrolysis, have had time to occur. A small r_S/r_H ratio indicates that hydrolysis is the reaction that the GH prefer to catalyse while a larger value indicates that the GH may have a preference for transglycosylation.

$$r_S/r_H = \frac{[Transglycosylation\ products]}{[Hydrolysis\ products]} \quad (1)$$

By calculating r_S/r_H at early timepoints in the reactions, one can use this r_S/r_H-value to predict the theoretical yield of transglycosylation products at later stages in the reaction [186]. Deviation from the theoretical yield at later timepoints indicates that unaccounted for reactions, e.g. secondary hydrolysis, has occurred [186].

Preferred substrate binding mode of glycoside hydrolases

An endo-acting glycoside hydrolase can often productively bind a substrate in multiple different ways, generating a range of hydrolytic products [97, 129, 222, 223]. The hydrolytic product profile can be used to determine if the glycoside hydrolase prefers to hydrolyse mannopentaose (M5) into mannotetraose (M4) and mannose (M1) or mannotriose (M3) and mannobiose (M2). The product profile can however not tell how the M5 was positioned in the active site, since, for example, M4 and M1 can be generated from two different productive binding modes, with M5 binding from subsites -4 to +1 or from -1 to +4.

This problem can be addressed in several ways. One method to distinguish between the preferred binding mode of a substrate to a glycoside hydrolase is the use of a sugar alcohol (e.g. mannopenitol or mannohexitol) as substrate [109, 125]. Hydrolysis of the sugar alcohol causes the generation of both a shortened sugar alcohol, e.g. mannitol, and a regular sugar, e.g. mannotetraose, which can be separated by HPAEC-PAD, this allows for analysis of the preferred binding mode of the substrate [109, 125]. Some concerns may be raised due to the use of the sugar alcohol though, as one cannot be sure that the presence of the reduced sugar, i.e. sugar alcohol, interacts with the glycoside hydrolase in the same way as a non-reduced oligosaccharide.

In **Paper I** and **II**, an alternative method for analysis of the preferred substrate binding mode was used where natural, non-reduced oligosaccharides were used as substrates [97, 129, 223]. By combining HPAEC-PAD and MALDI-ToF MS it is possible to determine preferred productive binding mode of a glycoside hydrolase towards a specific oligosaccharide. In this method, the glycoside hydrolase and the oligomeric substrate are incubated in ^{18}O-labelled water, causing the newly formed reducing end to have ^{18}O incorporated (Figure M1B). MALDI-ToF MS analysis can then be used to determine the relative ratio of labelled (^{18}O-incorporated) and unlabelled product based on monoisotopic peak area, which in turn translates to original productive binding preference of the substrate for that particular hydrolysis product. As an example, using the hydrolysis of M5, if labelled M4 (^{18}O-M4) has a five times greater monoisotopic peak area than that of the unlabelled M4 (^{16}O-M4) (Figure M1B), it means that productive binding from subsites -4 to +1 occurred five times more frequently than binding from -1 to +4. Combining this with the

quantitative data from HPAEC-PAD (Figure M1A), i.e. the ratio of hydrolysis into M4 and M1 or M2 and M3, it allows for an overall estimation of the relative preferred binding mode for one specific oligosaccharide, in this case M5. The reactions are carried out at low temperature to avoid spontaneous incorporation of ^{18}O through mutarotation. The method requires compensation for two factors. The first is the presence of any ^{16}O-water present in the reaction, e.g. from enzymes stocks, and the second factor that needs to be adjusted for is the natural occurrence of mannooligosaccharide isotopes, primarily due to the natural occurrence of ^{13}C [129, 223]. Examples of interpreted relative preferred binding modes are presented in **Paper I, Figure 5** and **Paper II, Figure 8**.

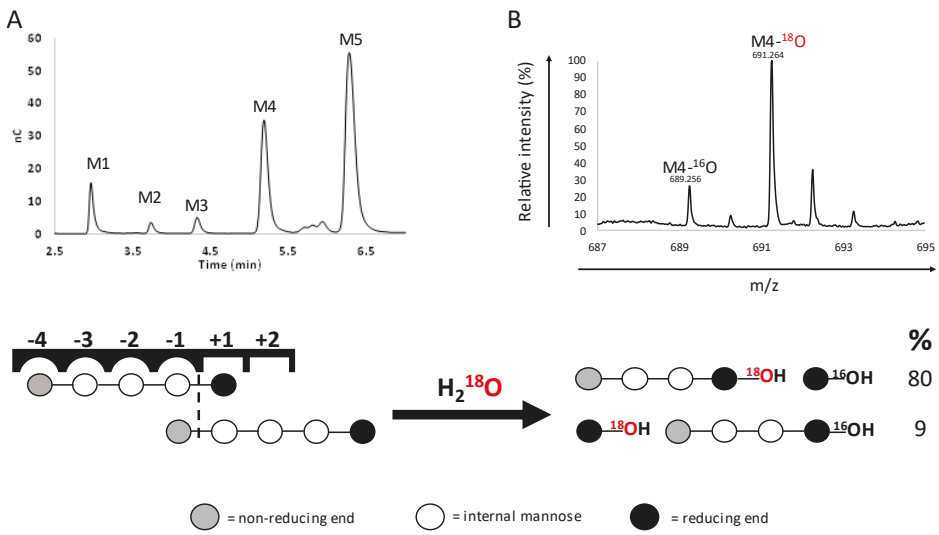

Figure M1. Overview of the analysis steps of the ^{18}O-labelling method.
The figure shows an overview of the different analysis methods that are used in the ^{18}O-labelling method in the form of HPEAC-PAD data (A) and MALDI-ToF MS (B). Below them is a schematic representation of how labelled (^{18}O incorporated) and unlabelled products are formed depending on substrate binding mode. In this example the glycoside hydrolase had a preference for hydrolysing M5 into M4 and M1 (A) and the preferred binding mode of M5 was from -4 to +1 (B), as seen by the greater peak area of the ^{18}O-labelled M4 compare to that of the unlabelled M4. The presented figures are based on data from **Paper I**.

Comparing retaining β-mannanases from GH5 and GH26

Reaction conditions

In earlier studies of the GH26 β-mannanases *Bo*Man26A [97], *Cf*Man26A-50K [129], *Pa*Man26A [100] and *Ypen*Man26A (**Paper I**), no transglycosylation was reported at the tested oligosaccharide concentrations (1-10 mM) for the native catalytic modules. In the work presented in section "Comparing transglycosylation behaviour in GH5 and GH26 β-mannanases", the effect of increased concentration of mannooligosaccharides on the transglycosylation capacity of the aforementioned GH26 β-mannanases was examined. In addition, the ability to utilise methanol as an acceptor was also examined for the four GH26 β-mannanases.

Reactions containing the β-mannanase (2.4 µg/ml *Bo*Man26A, 6.6 µg/ml *Cf*Man26A-50K, 610 µg/ml *Pa*Man26A and 0.36 µg/ml *Ypen*Man26A), 0.4 M mannooligosaccharide (mannotetraose for *Bo*Man26A, mannopentaose for *Cf*Man26A-50K, *Pa*Man26A and *Ypen*Man26A) in 50 mM buffer (potassium phosphate pH 6.5 for *Bo*Man26A, sodium citrate pH 6 for *Cf*Man26A-50K, sodium acetate pH 5.3 for *Ypen*Man26A and sodium acetate pH 5.2 for *Ypen*Man26A) were incubated at 37 °C up to 3 h. The incubations were stopped by heat inactivation (95 °C for 10 minutes). The samples were diluted 100 times with Milli-Q water before depositing 1 µl diluted sample and 1µl 10mg/ml DHB matrix onto a 384 MTP BigAnchor target plate (Bruker). The samples were analysed with MALDI-ToF MS on a AutoFlex Speed mass spectrometer (Bruker) in positive reflector mode and the data was analysed with flexAnalaysis 3.4 (Bruker).

Incubations and with methanol as acceptor were carried out as described above but the concentration of mannooligosaccharide was lowered to 0.35 M and 10 v/v% methanol was added to the reaction mixture and incubation times were prolonged up to 24 hours. Analysis was carried out as described above. In addition, *Tr*Man5A R171K (3.7µg/ml) was incubated with 20 mM M5, 50 mM NaAc pH 5.3 and 10 v/v% methanol at 37 °C up to 1 h. The reaction was terminated and analysed as described above with the exception that the sample was diluted 10 times.

Structural comparison

To investigate the potential +2 subsites of the GH26 β-mannanases *Bo*Man26A (PDB ID: 4ZXO), *Cf*Man26A-50K (PDB ID: 2BVT), *Pa*Man26A (PDB ID: 3ZM8) and *Ypen*Man26A (PDB ID: 6HPF) the crystal structures were superimposed to that of *Cj*Man26C (PDB ID: 2VX6). The structure of *Cj*Man26C has a galactosylated mannotetraose spanning subsites -2 to +2. The superimposition of the crystal

structures could allow for the investigation of potential interactions in a hypothetical +2 subsite for the four studied GH26 β-mannanases, assuming that a bound saccharide is positioned similarly in the active sites of *Bo*Man26A, *Cf*Man26A-50, *Pa*Man26A and *Ypen*Man26A as it does in *Cj*Man26C. The analysis was performed with ChimeraX [67, 68], using the Matchmaker tool to do the superimposition and the Zone select tool to find residues within 5Å of the mannosyl unit bound in the +2 subsite of *Cj*Man26C.

In order to make an initial comparison of highly conserved residues in GH5 and GH26, a structure based sequence alignment of *Bo*Man26B and *Tr*Man5A was done with Expresso [224]. The alignment was further annotated with ESPript 3.0 [225].

Results and discussion

Summary of papers

Paper I, Structure-function relationship in a fungal GH26 β-mannanase

In **Paper I** a GH26 β-mannanase from the fungus *Yunnania penicillata* (*Ypen*Man26A), which had previously been shown to efficiently hydrolyse highly substituted galactomannans [94], was studied. In order to gain a better understanding of enzyme-substrate interactions in β-mannanases, the paper investigated *Ypen*Man26A's kinetics towards the galactomannans locust bean gum and guar gum and how it interacts with and accommodates galactosylated mannans.

Kinetic analysis of *Ypen*Man26A interestingly revealed that the enzyme had a lower k_{cat} on the galactomannan locust bean gum compared to the more heavily galactosyl substituted guar gum galactomannan(**Paper I, Table 4**). This could indicate that the interactions with galactose residues from guar gum could be beneficial for the catalytic rate constant of *Ypen*Man26A. Compared to other characterised GH26 β-mannanases, this appear to be highly unusual as several of them either have a lower or equal turnover number on guar gum (Table R3, **Paper I and II**) [84, 129] .

To study the substrate interaction between *Ypen*Man26A and galactosylated substrates, a catalytically deficient variant of the enzyme was co-crystallised with di-galactosylated mannopentaose. Through a combination of the acquired structure and an overlay with the crystal structure of another GH26 β-mannanase (*Cj*Man26A, PDB ID: 2VX6) with a bound ligand [63], it was shown that *Ypen*Man26A could likely accommodate galactose substitutions in the -3, -2 and -1 subsites (**Paper 1, Figure S2**). The ability to accommodate multiple substitutions along the mannan backbone in the negative subsites is common for several GH26 β-mannanases (**Paper I and II**) [95, 124] possibly making them flexible in how they can bind heterologous substrates, i.e. mannose-based polysaccharides.

A multiple sequence alignment with several other fungal GH26 β-mannanases revealed that residues in *Ypen*Man26A interacting with mannosyl and galactosyl units overall were highly conserved between these fungal GH26 β-mannanases with the exception for one, *Wsp*Man26A (**Paper II, Figure 3**). *Wsp*Man26A had a

threonine and a histidine predicted to be in the -2 and -4 subsites respectively based on the sequence alignment with *Ypen*Man26A, while the other sequences in the alignment were predicted to have either a glutamate or aspartate and a tryptophan respectively at the equivalent positions (**Paper I, Figure 3**). *Wsp*Man26A had a much lower initial activity towards the more heavily substituted guar gum compared to locust bean gum, a stark discrepancy to the almost equal activity towards both substrates observed for *Ypen*Man26A (**Paper I, Figure 4**) [94]. Based on these findings two variants of *Ypen*Man26A, D37T and W110H, were designed to investigate the role these amino acids played in substrate interactions and/or catalysis.

W110 interacted with a mannose unit in the -4 subsite in *Ypen*Man26A (**Paper I, Figure 4**) and the effect of the substitution of the tryptophan to a histidine appeared to mainly be decreased affinity towards the substrate. The W110H variant had a 17-fold increase in K_M towards locust bean gum accompanied by a minor decrease in k_{cat} (**Paper I, Table 4**) suggesting that the primary role of W110 is substrate binding. This was further supported by analysing the preferential productive binding mode of both W110H and *Ypen*Man26A towards mannopentaose (M5) with the O^{18}-labelling method (described in the Methods section) by analysing the incorporation of ^{18}O into the hydrolysis product when carrying out hydrolysis of M5 in ^{18}O-labelled water. The data showed a shift from primarily productively binding mannopentaose from the -4 to +1 subsites for *Ypen*Man26A to instead productively binding from the -3 to +2 subsites for W110H (**Paper I, Figure 5**). This indicates that the exchange of the tryptophan likely resulted in weakened mannose binding in the -4 subsite.

In *Ypen*Man26A, D37 interacted with a galactose substitution in the -2 subsite (**Paper I, Figure 4**). The effect of the exchange of the aspartate to a threonine appeared to mainly affect the catalytic rate constant as the variant D37T had a lowered k_{cat} towards both locust bean gum and guar gum. The D37T variant also had a slight increase in K_M towards guar gum (**Paper I, Table 4**) as well as reduced k_{cat}/K_M towards di-galactosylated mannopentaose (**Paper I, Table 5**). Decreased k_{cat} towards a di-galactosylated mannopentaose has also been reported for a GH5 β-mannanase variant in which a galactose interacting residue in the -1 subsite was substituted [102]. This indicates that the interactions with galactosyl substitutions of mannose-based oligo- and polysaccharides may not only play a role in substrate binding (K_M) but may also affect the catalytic turnover rate (k_{cat}). Interestingly there is a larger decrease in k_{cat}/K_M of variant D37T with the galactosylated mannopentaose as substrate than with either of the two galactomannans (**Paper I, Table 4** and **5**). A possible explanation for this could be that the longer polysaccharides allow for several alternative binding modes to the enzyme's active site compared to the galactosylated mannopentaose, thereby lessening the effects of the change in a single subsite by additional interactions in other subsites.

The paper highlights the importance of structural knowledge of a GH to understand how the enzyme interacts with the substrate. Single amino acid substitutions caused drastic changes in *Ypen*Man26A's product profile on M5 and its ability to utilise polymeric substrates as seen when comparing the properties of the two variants W110H and D37T to *Ypen*Man26A wild type. Furthermore, it shows that it is possible to engineer GHs towards preferred products, as shown in the shift in product profile in W110H compared to *Ypen*Man26A wild type, a valuable lesson if the end goal is defined hydrolysis or transglycosylation products with specific oligomeric length of the sugar units.

Paper II, Examining structure-function relationship in a bacterial GH26 β-mannanase and conserved residues in GH26

In **Paper II** the structure and properties of a surface-exposed GH26 β-mannanase (*Bo*Man26B) that is encoded as part of a polysaccharide utilization locus (PUL), or gene cluster, for β-mannan degradation in the gut microbe *Bacteroides ovatus* was explored.

*Bo*Man26B had a higher catalytic efficiency towards locust bean gum than guar gum (**Paper II, Table 1**) but compared to some other GH26 β-mannanases, the difference in catalytic efficiency between the two galactomannans was relatively small (Table R3) [84, 129]. This indicates that *Bo*Man26B is tolerant for substitutions along the mannan backbone. Analysis of the product profile of the galactomannans, guar gum and locust bean gum, hydrolysed with *Bo*Man26B further supported that it was tolerant for galactose substitutions as the hydrolysates contained a complex mixture of mannooligosaccharides and galactosylated mannooligosaccharides (**Paper II, Figure 2**). This suggests that *Bo*Man26B is able to interact with and hydrolyse the two galactomannans in a wide variety of ways. Taken together, this supports that *Bo*Man26B is well-equipped to handle galactosyl substitution of galactomannans, well in agreement with the proposed role of the enzyme as the first substrate attacker of the proteins encoded by the *Bacteroides ovatus* mannan-degrading PUL [97].

To examine how *Bo*Man26B interacts with substrates and how it can accommodate galactose substitutions, a crystal structure with a bound di-galactosylated mannopentaose bound was obtained. The crystal structure showed that *Bo*Man26B likely can accommodate galactosyl side groups in all of the negative-numbered subsites except for -2. It also revealed that *Bo*Man26B had a -5 subsite which included a tryptophan (W112) that stacks with the mannosyl unit located in the subsite (**Paper II, Figure 5**). A similar tryptophan providing stacking intertactions in the -5 subsite is present in several GH26 β-mannanases, including those from a protist from *Reticulitermes speratus* (*Rs*Man26A, *Rs*Man26B and *Rs*Man26C) [84,

124] as well as in a GH26 β-mannanase found in the metagenome from cow rumen (CrMan26) [85].

Based on the data obtained from the *Bo*Man26B crystal structure four variants were designed, two targeting W112 (W112F and W112A) and two targeting a lysine interacting with a galactosyl substitution in the -4 subsite (K149S and K149A). Compared to wild-type *Bo*Man26B, the catalytic efficiency for the K149-variants were almost 3-fold lower on both locust bean gum and guar gum, with the largest factor for the decrease with locust bean gum being a 2-fold increase in K_M (**Paper II, Table 1**). The K_M for locust bean gum was more heavily impacted than k_{cat}, whereas the opposite was observed in **Paper I** for the *Ypen*Man26A variant targeting galactose interacting residue in the -2 subsite. This could indicate that interactions with the galactose substitutions at different subsites may have different roles, such that those closer to the targeted glycoside bond mainly modulate the turnover number while those further away might primarily contribute to substrate binding. These single observations are not enough for any general conclusions, yet the subject matter could be of potential interest for further study.

The effect of the W112-variants was much more substantial, resulting in a more than 20-fold decrease in k_{cat}/K_M towards locust bean gum compared to the wild-type *Bo*Man26B (**Paper II, Table 1**). Interestingly, variants targeting a similar tryptophan that provides stacking interactions in the -5 subsite in three other GH26 β-mannanases had varying effects on their kinetic properties towards locust bean gum, as can be seen in Table R1. This shows the complexity of carbohydrate interactions in β-mannanases, as the exchange of seemingly similar amino acids had widely differing results. To further investigate the effects of the two W112 substitutions, the productive binding mode towards mannohexaose, using the ^{18}O-method described in the Methods chapter, was compared to that of *Bo*Man26B. As in the case in **Paper I**, the variants displayed a shift in preferred binding mode with mannohexaose, going from an almost equal preference for binding from -5 to +1 or -4 to +2 for *Bo*Man26B to -4 to +2 being the dominant binding mode for both W112F and W112A (**Paper II, Figure 8**), once more highlighting how enzyme engineering can be used to influence the product profile of β-mannanases.

Table R1. The table shows the effect on the kinetic parameters towards locust bean gum for three different GH26 β-mannanases in which a -5 subsite tryptophan has been exchanged. WT=Native enzyme.

Enzyme (variant)	k_{cat} (s^{-1})		K_M (mg/ml)		k_{cat}/K_M		Reference
	WT	Variant	WT	Variant	WT	Variant	
*Bo*Man26B (W112A)					23.3	0.98	**Paper II**
RsMan26A (W79A)	641	641	5.8	28	111.1	23	[84]
RsMan26B (W79A)	447.4	177.1	3.2	11.5	141.7	15.5	[84]
CrMan26 (W234A)	333.5	453.6	1.62	1.05	205.9	432	[85]

Paper II also covers a phylogenetic and bioinformatic analysis of the GH26 family focusing on the β-mannanases. The analysis identified highly conserved residues in the β-mannanases of the GH26-family (**Paper II, Table 5**), allowing for easier identification of them in the future. Finally, the paper investigated the synergy of *Bo*Man26B and the α-galactosidase *Bo*Gal36A expressed from the same PUL [35, 97]. The synergy experiments showed that the activity of *Bo*Gal36A benefitted more by the presence of *Bo*Man26B than the other way around. The amount of released galactose increased 10-fold in the co-incubation of both enzymes with LBG while the amount of released M2 only increased 2.5-fold compared to *Bo*Gal36A and *Bo*Man26B, respectively, being incubated alone with LBG (**Paper II, Table 2**). Enzyme synergy has previously been demonstrated for GHs expressed from this PUL, as the activity of *Bo*Man26A was shown to be increased towards di-galactoyslated mannopentaose and locust bean gum galactomannan in the presence of *Bo*Gal36A [97].

As in **Paper I**, **Paper II** highlights the importance single amino acid's side chains can have on how an *endo*-acting GH interacts with carbohydrates and the potential of enzyme engineering for changing the product profile. Furthermore, the paper shows how the efficient degradation of complex carbohydrates can benefit from the interplay of different GH families and activities, as is seen in the increased release of saccharides in the co-incubation of *Bo*Man26B and *Bo*Gal36A compared to when they were acting alone on LBG, demonstrating enzyme synergy.

Paper III, How enzyme synergy can improve transglycosylation

In **Paper III** the transglycosylation capacity of a fungal β-mannanase from *Trichoderma reesei* (*Tr*Man5A) was investigated. In addition to the full length enzyme (which has a carbohydrate binding module) the catalytic module of *Tr*Man5A (WTcm) and two variants of WTcm, R171K [117] and R171K/E205D were evaluated.

Initial evaluation of transglycosylation capacity was carried out with mannotetraose (M4) and two acceptors, methanol and allyl alcohol. The initial screening of product formation through MALDI-TOF MS and HPLC showed that all four enzymes produced transglycosylation products with 1 to 3 mannosyl units (**Paper III, Figure 2**), with the R171K/E205D variant having the highest yield (**Paper III, Figure 3**). Surprisingly, allyl mannotrioside (M3-allyl) was the primary product for *Tr*Man5A and WTcm, indicating that the primary productive binding mode was from subsite -3 to +1 for M4, contradicting a previous study showing that WTcm primarily preferred to bind M4 in the -2 to +2 subsites [117]. However, another study had similar observations of hexyl mannotrioside being a major product for *Tr*Man5A when using M4 as donor substrate and hexanol as the acceptor [119].

Transglycosylation where the saccharide acted as acceptor has been established in earlier studies for WTcm with lower M4 concentrations [117]. Due to this, it was hypothesised that *Tr*Man5A and WTcm preferred using M4 as acceptor in the initial reaction conditions, generating elongated saccharides. These elongated saccharides were then proposed to act as the glycosyl donor in subsequent reactions, resulting in the formation of substantial amounts of M3-allyl.

Further comparison of the transglycosylation capacity between *Tr*Man5A and R171K/E205D over prolonged incubation times was carried out, as R171K/E205D had the highest apparent transglycosylation capacity of R171K and R171K/E205D. The comparison was done with allyl alcohol as the acceptor and either M4 or locust bean gum galactomannan as the donor, revealing some interesting differences between the two enzymes. With M4, both *Tr*Man5A and R171K/E205D had continuous production of allyl mannosides during the initial time period. As M4 was depleted, both enzymes displayed secondary hydrolysis, i.e. the hydrolysis of the allyl mannosides [186], although it was limited in *Tr*Man5A and considerably more pronounced for R171K/E205D (**Paper III, Figure 4**). Based on the formation of allyl mannosides and hydrolysis products the ratio of synthesis over ratio of hydrolysis (r_S/r_H) value was calculated, a value describing an enzyme's propensity towards transglycosylation compared to hydrolysis [186, 196]. From the initial r_S/r_H-value, the theoretical yield of transglycosylation products was calculated, with the assumption that r_S/r_H was constant during the reaction, as described in a paper by van Rantwijk [186]. Comparison between the theoretical yield and experimental yield, a way to estimate possible secondary hydrolysis if the former yield is higher than the latter, further supported that there was significant secondary hydrolysis with R171K/E205D. For R171K/E205D the experimental yield of allyl mannosides was 60% lower than the theoretical yield calculated based on the initial r_S/r_H-value. *Tr*Man5A, on the other hand, had an increased experimental yield compared to the theoretical yield. It was proposed that the higher experimental yield was due to transglycosylation where saccharides functioned as both donor and acceptor, a reaction not considered when calculating r_S/r_H. This additional transglycosylation reaction would lead to an apparent r_S/r_H lower than the true value, and subsequently a lower theoretical yield. The elongated saccharides from such reactions could act as substrates in subsequent transglycosylation reactions, leading to a higher experimental yield of allyl mannosides than the theoretical one for *Tr*Man5A. While R171K/E205D had a higher yield of allyl mannosides at the earlier timepoints, the significant amount of secondary hydrolysis with R171K/E205D made it and *Tr*Man5A similar in terms of yield over prolonged incubation times (**Paper III, Section 3.5** and **Table 1**).

When shifting to the polymeric and galactose substituted substrate locust bean gum as the donor substrate, the yield of allyl mannosides decreased for both *Tr*Man5A and R171K/E205D, and *Tr*Man5A had the higher production of allyl mannosides of the two (**Paper III, Figure 7** and **Table 2**). Analysis of the reaction mixture of

*Tr*Man5A with locust bean gum and allyl alcohol with MALDI-TOF MS showed a peak with an m/z corresponding to a heptasaccharide (**Paper III, Figure 8A**), suggesting the accumulation of inaccessible saccharide products. As it has previously been established that higher degree of galactosyl substitutions may sterically hinder β-mannanases [95], it was proposed that the breakdown of locust bean gum lead to the formation of oligosaccharides with galactosyl substitutions in such a way that they became unavailable for further degradation. Efficient hydrolysis of polymeric substrates consisting of several different sugars and/or linkages, such as locust bean gum galactomannan, often requires a mixture of different GHs [158]. Therefore, an α-galactosidase (Aga27A) was added to the reaction mixture and co-incubated with *Tr*Man5A with the aim to remove galactosyl substitutions present on the galactomannan that may hinder *Tr*Man5A. Analysis of the new reaction showed that the peak with a m/z corresponding to a heptasaccharide was drastically reduced (**Paper III, Figure 8C**), supporting that without the galactosidase there was an accumulation of galactosylated oligosaccharides. The addition of Aga27A to the reactions with locust bean gum as glycosyl donor also resulted in an almost 5-fold increase in the yield of allyl glycosides. The increase in yield was in part due to that Aga27A was able to perform transglycosylation, a catalysis not previously described for this enzyme. Moreover, both enzymes had higher yield of transglycosylation products when co-incubated than when incubated alone with the locust bean gum galactomannan (**Paper III, Figure 10 and Table 3**). This increase in yield showed that there was a synergistic effect of the two enzymes, likely since their synergistic actions provided better access to the substrate. Enzyme synergy is well established when it comes to hydrolysis of polysaccharides for *exo*- and *endo*-acting cellulases [226] and has been reported for xylanases and arabinofuranosidases [227] as well as β-mannanases and α-galactosidases [158, 165]. **Paper III** is to the best of our knowledge the first report of enzyme synergy for β-mannanases when it comes to transglycosylation.

The lessons from **Paper III** are that the best catalyst can be dependent on what substrate is used, as *Tr*Man5A had lower yield of allyl mannosides than R171K/E205D with M4 as glycosyl donor but higher with locust bean gum. This was suggested in part be due to the incubations with *Tr*Man5A and M4 had a substantial amount of transglycosylation reactions where saccharides functioned as acceptor, a reaction that likely was limited with the polymeric galactomannan locust bean gum. Furthermore, the choice of glycosyl donor may impose restrictions for the enzyme that are important to understand to be able to circumvent them, e.g. full substrate utilisation for *Tr*Man5A with locust bean gum was limited by galactose substitutions but it was possible to circumvent it through the addition of Aga27A, in the enzyme synergy incubation.

Paper IV, Comparing transglycosylation capacity of two α-galactosidases

In **Paper IV** the transglycosylation capabilities of two α-galactosidases was compared, one from GH family GH27 from the guar plant (Aga27A) and one from GH family GH36 from the gut microbe *Bacteroides ovatus* (*Bo*Gal36A). The two α-galactosidases were compared to see potential differences in transglycosylation capacity between the two GHs and develop tools to evaluate transglycosylation capacity. The enzymes' ability to perform transglycosylation with the glycosyl donors *para*-nitrophenyl-α-galactoside (pNP-Gal), raffinose and locust bean gum galactomannan were tested both in self-condensation reactions, where the same substrate acts both as donor and acceptor, and with the acceptors methanol, propanol, allyl alcohol, propargyl alcohol and glycerol. Initial screening was carried out with MALDI-ToF MS followed by more extensive evaluation with HPLC.

Initial evaluation of the two enzymes' ability to utilise the various acceptors with pNP-Gal as the donor substrate showed that both Aga27A and *Bo*Gal36A were able to perform transglycosylation with all of the tested acceptors (**Paper IV, Table 1**). As both enzymes displayed transglycosylation capabilities with pNP-Gal, the study then continued with evaluating natural saccharides as the donor substrates in the form of raffinose and locust bean gum. With the natural substrate raffinose as glycosyl donor, Aga27A produced transglycosylation products with all acceptors **Paper IV, Table 2)**. With locust bean gum as donor, Aga27A produced transglycosylation products for all acceptors except propargyl alcohol (**Paper IV, Table 3**). BoGal36A on the other hand only produced transglycosylation products with methanol, propanol and glycerol with raffinose as the donor substrate and only with methanol when LBG was used as the glycosyl donor (**Paper IV, Table 2** and **3**), as can be seen in Table R2. The reason for the observed differences in transglycosylation behaviour with the more complex substrates was not fully investigated but a potential contributor is that lowered activity towards the more complex substrates [35] led to an insufficient amount of transglycosylation products to reach the limit of detection.

As both *Bo*Gal36A and Aga27A produced transglycosylation products with methanol as the acceptor with all of the tested substrates, it was used to further compare the transglycosylation capacity of both enzymes. By using two concentrations of raffinose (400 or 40 mM) or locust bean gum as donor substrates, the production of methyl galactoside and galactose was quantified and used to calculate the propensity for transglycosylation, r_S/r_H-value (described in further detail in the Methods chapter), of the two enzymes (**Paper IV, Table 4**). Both enzymes had similar r_S/r_H-value to one another at the endpoint of all incubations although there were differences between substrates. Variation of initial concentrations of raffinose appeared to have limited effect on the r_S/r_H-value while the use of locust bean gum led to lower r_S/r_H-values compared to that of the raffinose

incubations (**Paper IV, Table 4**). The lower r_S/r_H-values with locust bean gum compared to raffinose indicates that either secondary hydrolysis [186] was more prominent with locust bean gum as the donor substrate or that both enzymes preferred hydrolysis over synthesis with the polymeric substrate. It is possible that the secondary hydrolysis is more prominent with locust bean gum as donor substrate due to α-galactosidases in general having lower activity towards polymeric substrates, such as locust bean gum, than oligosaccharides, e.g. raffinose, [35, 166, 228, 229]. It could be speculated that there is a smaller difference in k_{cat}/K_M between methyl galactoside and locust bean gum than it is for methyl galactoside and raffinose, thereby leading to a higher degree of secondary hydrolysis of methyl galactoside when locust bean gum is the donor substrate.

Table R2. Transglycosylation capacity of *Bo*Gal26A and Aga27A with different acceptors and donor substrates. The table is a summary of **Paper IV** data and shows the reactions for which peaks with m/z corresponding to transglycosylation products were observed with MALDI-ToF MS for *Bo*Gal36A and Aga27A. A + indicates that transglycosylation products were observed while – means that no products could be observed. LBG = locust bean gum.

Acceptor	pNP-Gal		Raffinose		LBG	
	*Bo*Gal36A	Aga27A	*Bo*Gal36A	Aga27A	*Bo*Gal36A	Aga27A
Saccharide	-	+	+	+	-	-
Methanol	+	+	+	+	+	+
Propanol	+	+	+	+	-	+
Glycerol	+	+	+	+	-	+
Allyl alcohol	+	+	-	+	-	+
Propargyl alcohol	+	+	-	+	-	-

The tendency for secondary hydrolysis of the Aga27A and *Bo*Gal36A was examined by monitoring the apparent r_S/r_H over time. This indicated that *Bo*Gal36A had a higher tendency for secondary hydrolysis, as the r_S/r_H-value decreased over time (**Paper IV, Figure 6**), indicating that larger amounts of galactose compared to methyl galactoside formed at later time points compared to earlier ones. For Aga27A, the decrease in r_S/r_H was considerably less pronounced, indicating that secondary hydrolysis was not occurring to the same extent as for *Bo*Gal36A. When it comes to the yields of methyl galactoside (ranging from 27 to 48%) from the reactions with methanol as acceptor and raffinose or locust bean gum as the donors (**Paper IV, Table 4**), the values were comparable to those of other α-galactosidases with various acceptors and glycosyl donors (**Paper III**) [79, 230].

The paper highlights the potential of using simple chromogenic substrates for initial evaluation of transglycosylation potential of α-galactosidases (and potentially other retaining glycoside hydrolases) with the aim to utilise more complex polysaccharides in the end, as the results from pNP-Gal translated well to locust bean gum for Aga27A. The high degree of secondary hydrolysis by *Bo*Gal36A also exemplifies that analysis over prolonged time can give valuable insights, and that timed termination of the transglycosylation reaction may be important, due to the kinetic control of such reactions [186].

Paper V, A quick, general method for improving transglycosylation capacity in retaining glycoside hydrolases

In **Paper V** a method for increasing the transglycosylation capacity of retaining GHs is presented. The method relies on sequence data but otherwise requires little previous knowledge of the glycoside hydrolase or the GH family, except for the specificity of the target enzyme and that it can transglycosylate. The method identifies highly conserved residues within a GH family bioinformatically, and these residues becomes targets for substitution by mutagenesis. Highly conserved residues within a GH family are likely residues that are of importance for the catalytic machinery as these often have a high degree of conservation [231]. The method was shown to be applicable to several different GH families belonging to different GH clans and with different substrate specificities and mode of actions, e.g. *endo*- (GH10) or *exo*-acting (GH2, GH20, GH29, GH5, GH151) GHs as well as those that have a substrate-assisted mechanism.

Through in silico sequence analysis of each GH family, highly conserved residues within each family were identified. These residues were then substituted to similar amino acids in order to retain function but potentially cause perturbations e.g. in the transition state energies in such a way that transglycosylation became more favourable compared to hydrolysis [55]. Changes of the catalytic residues, if known, were avoided as well as substitution of prolines and glycines, as these two residues were assumed to mainly be conserved for structural reasons.

For GH2, the method was tested on a β-mannosidase from *Cellulomonas fimi* (*Cf*Man2A) [213, 232]. The transglycosylation capacity was evaluated with *para*-nitrophenyl mannoside acting as both donor and acceptor in the reaction and *Cf*Man2A was shown to natively have a moderate transglycosylation capacity. For *Cf*Man2A, the bioinformatic analysis generated the suggestion of 12 monosubstituted variants which were expressed, out of which 4 were discarded due to low activity after initial evaluation. Of the remaining 8 variants, 5 were shown to have an increased transglycosylation capacity compared to wild-type *Cf*Man2A while requiring lower or only moderately increased enzyme loading. Compared to the glycosynthase approach for *Cf*Man2A [192], the variants could be added at approximately 100-fold lower enzyme loading (1-2 mg/ml for the glycosynthase variants compared to 2.8-34.3 µg/ml (**Paper V, Table 5**), while still reaching full substrate utilisation within 5 hours compared to the 1 to 5 days that were required in the glycosynthase approach [233]. Furthermore, several of the generated variants showed lowered tendency for secondary hydrolysis, maintaining relatively high amounts of the transglycosylation products over prolonged incubation times while it was rapidly degraded by wild-type *Cf*Man2A (**Paper V, Figure 5**). The variants with improved transglycosylation yield had an improvement of approximately 1.8 to 2.4 times higher yields of transglycosylation products than what was observed for *Cf*Man2A (**Paper V, Table 5**). It should also be highlighted that GH2 is quite a

heterologous family covering several different substrate specificities, further emphasising that the method is a good general method as the selection of targeted residues for *Cf*Man2A were generated based on an initial 20 000 sequences of the GH2-family, of which some were weeded out to lower redundancy.

In the paper GH families 2, 10, 20, 29, 51 and 151 were investigated, representing GHs from clans A, K, R or no clan. Furthermore, the selected enzymes cover a wide variety of substrate sugars such as D/L configuration, α- and β-stereochemistry and pyranose/furanose forms. The method for generating variants with improved transglycosylation capacity was successful for all of the evaluated GH families, even for the glycoside hydrolase from GH151 (**Paper V, Section GH151, α-l-fucosidase *Pt*GH151**) for which very limited information about GH family 151 as a whole was available. The improvement in yield for the reactions ranged between a 1.7- to 9-fold increase when comparing the variant that gave the highest yield to the wildtype for each respective glycoside hydrolase (**Paper V, Table 8**). It was also demonstrated that the generated variants were transposable to equivalent amino acids within the same GH family, as the two best variants for the GH29 enzyme were transposed to another family GH29 enzyme, resulting in higher transglycosylation capacity compared to the wildtype (**Paper V, Table 2**). Taken together, this shows that the method is generally applicable and has several advantages over other methods to generate improved transglycosylation variants. Compared to the previously mentioned powerful glycosynthase approach [188, 190, 192, 195], the variants generated by the presented method in **Paper V** can utilise natural saccharides instead of the expensive and tedious to synthesise activated substrates required for the glycosynthase approach. While the method may not necessarily discover the best glycoside hydrolase variants for improved transglycosylation, it does give a small pool of variants that is easily screened compared to the random mutagenesis approach which requires the generation and analysis of large mutant libraries [234]. And lastly, compared to rational design approaches [235] the presented method does not require any previous structural knowledge of the enzyme to generate variants with improved transglycosylation abilities.

Table R3. Comparison of catalytic properties of GH5 and GH26 enzymes.
The table compares the catalytic properties of a several different GH5 and GH26 β-mannanases on the galactomannan locust bean gum (LBG), guar gum (GG) and the mannooligssacharides mannotetraose (M4), mannopentaose (M5) and mannohexaose (M6).

Enzyme	LBG		GG		M6		M5		M4		References
	k_{cat} (s^{-1})	K_M (g/l)	k_{cat} (s^{-1})	K_M (g/l)	k_{cat} (s^{-1})	K_M (mM)	k_{cat} (s^{-1})	K_M (mM)	k_{cat} (s^{-1})	K_M (mM)	
GH5											
*Tr*Man5Acm (WTcm)	240±5	0.6			20±1	0.05±0.01	13±1	0.08±0.02	6±1	0.31±0.01	[117, 118]
*Tr*Man5A R171K	260±16	1.3			28±1	0.34±0.03	11±1	1.7±0.4	3±<1	10.6±2.4	[117]
*An*Man5A	90	1.4			193±5.8	1.8±0.08	67±2.8	2.9±0.03			[183, 184]
*An*Man5B	15	2.6			61.6±6.7*		36.6±2.6*		40±4.3*		[183]
*An*Man5C	220	3.1			134±5.2	0.6±0.04	112±8.1	1.8±0.13			[183, 184]
*Pa*Man5A	332	0.75									[100]
*Bl*Man5A	1828±87	1.58±0.23					97±2	1.09±0.11			[77]
*Cm*Man5A	300	2.5							1650*		[60]
GH26											
CfMan26A-50k	137±32	1.8±0.7	137±31	16±5	77±1	0.69±0.03	52.42±1	1.13±0.06	11.6±0.6	2.0±0.2	[5, 38]
*Pa*Man26A	55	2.4			40.0*		21.7*		.1*		[100]
*Bo*Man26A	-	-			197*		247*		140*		[97]
*Bo*Man26B	250±12	11±1	122±19.4	12.9±1.7	0.04*						**Paper II** [97]
*Ypen*Man26A	475±5	0.6±0.03	636±19	2.2±0.2							**Paper I**
*Ypen*Man26A W110H	404±18	10±0.8	17±0.6†								**Paper I**
*Rs*Man26A	641	5.8	238	16.8							
*Rs*Man26B	447	3.2	354	26.1							
*Pc*Man26A	2904	8.5									[127]
*Wsp*Man26A	564±26	0.8±0.2	271±31	3.6±1							**Paper I**

*=k_{cat}/K_M (mM^{-1} s^{-1})

†= k_{cat}/K_M (ml mg^{-1} s^{-1})

Comparing transglycosylation behaviour in GH5 and GH26 β-mannanases

Transglycosylation in β-mannanases from family GH26 and GH5

GH5 and GH26 both contain β-mannanases that have similarities to one another. Belonging to clan GH-A, both enzyme families adopts a TIM-barrel fold, utilises a retaining mechanism and the catalytic residues consist of a pair of highly conserved glutamates [236]. Both families contain β-mannanases that span a wide range when it comes to k_{cat} and K_M-values and therefore catalytic efficiency against different mannose-based substrates (Table R1). Comparing kinetic properties of GH5 and GH26 β-mannanases against oligosaccharides and polysaccharides further highlights that it appears difficult to make a clear distinction between β-mannanases from the two GH families on this basis. Although there are many similarities between the two GH families, when it comes to their ability to perform transglycosylation they seem to differ widely. While transglycosylation, both with saccharides and other types of acceptors, is well documented for GH5 enzymes (**Paper III**) [117-119, 141, 183, 184] no such result appear to have been reported for native GH26 enzymes (**Paper I, Paper II**) [97, 129, 237-239]. If the limited data on transglycosylation for GH26 β-mannanases is because it has been less investigated or due to studies have been conducted with negative results and therefore not reported is hard to tell. This possible lack of reporting of negative findings may be a common problem when it comes to sciences [240].

While transglycosylation in native GH26 β-mannanases has not previously been observed, indications of transglycosylation have been observed for a variant of a *Cellulomonas fimi* GH26 β-mannanase [129]. In the study, an alanine in the -2 subsite was changed to an arginine while a phenylalanine in the -3 subsite was changed to an alanine. This resulted in skewed product ratios with a disproportionate amount of longer hydrolysis products when the variant was converting different mannooligosaccharides [129]. Skewed ratios are indicative that transglycosylation occurred in the reaction since if, e.g. M3 is hydrolysed into M2 and M1 it is expected that both products are produced in equimolar amounts when no transglycosylation occurs, assuming M2 is not further hydrolysed. The explanation for the indications of transglycosylation may be that the increased binding strength in the -2 subsite of the *C. fimi* β-mannanase variant [129] could lead to stabilisation of glycosyl-enzyme intermediate. In turn, this may lead to improved transglycosylation, as a more long-lived glycosyl-enzyme intermediate may be beneficial for transglycosylation [201].

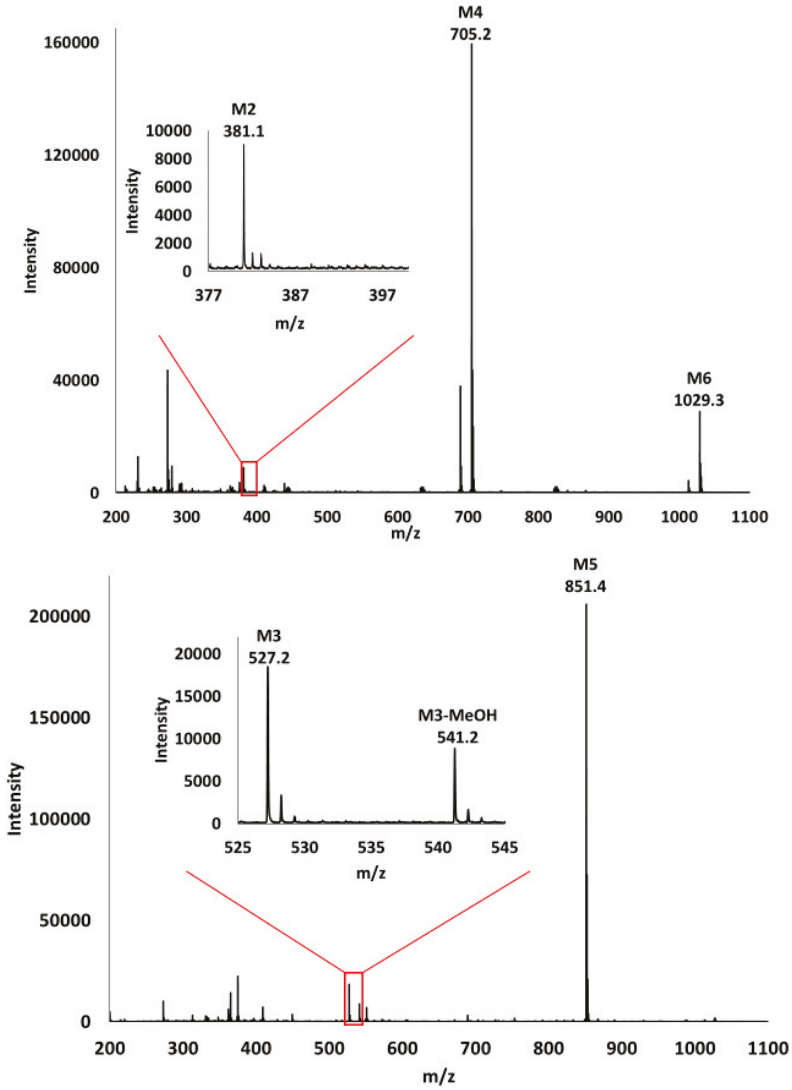

Figure R1. MALDI-ToF MS spectra of transglycosylation reactions with *Bo*Man26A and *Tr*Man5A.
The spectra are from MALDI-ToF MS analysis of reactions with 0.35 M M4, 50 mM KPO_4 pH 6.5, 10 v/v% methanol and 2.4 µg/ml *Bo*Man26A (A) and 20 mM M5, 50 mM NaAc pH 5.3, 10 v/v% methanol and 3.7µg/ml *Tr*ManR171K (B), both reactions incubated for 1h at 37 °C. In the spectrum for the *Bo*Man26B incubation, clear peaks for hydrolysis in the form of M2 (observed m/z: 381.1, expected m/z: 381.1), the M4 susbtrate (observed m/z: 705.2, expected m/z: 705.2) and saccharide transglycosylation products in the form of M6 (observed m/z: 1029.3, expected m/z: 1029.3) was observed but no peak corresponding to transglycosylation with methanol as acceptor (observed m/z: -, expected m/z: 395.1). No mannohexaose was observed in the M4 substrate control for *Bo*Man26A. In the spectrum for the *Tr*ManR171K reactions clear peaks for hydrolysis in the form of M3 (observed m/z: 527.2, expected m/z: 527.2), the M5 susbtrate (observed m/z: 851.4, expected m/z: 851.3) and transglycosylation products where methanol acted as acceptor (observed m/z: 541.2, expected m/z: 541.2) was observed but no saccharide transglycosylation. The expected m/z for *Bo*Man26B are based monoisotopic mass with a potassium adduct while the expected m/z listed for *Tr*Man5A are based on the monoisotopic mass with a sodium adduct.

The preference for several GH26 β-mannanases to productively bind saccharides in the glycone subsites may indicate a lack of or weak interactions with saccharides in the aglycone subsites (**Paper I**, **Paper II**) [100, 124, 129, 239]. Saccharide interactions in the positive numbered subsites has previously been shown to play a crucial role in saccharide transglycosylation by GHs [183, 198, 199, 241], including GH5 enzymes [117, 184]. As an example, the substitution of a single arginine in the +2 subsite of *Tr*Man5A was enough to almost completely abolish transglycosylation activity with saccharide acceptors [117]. Assuming that GH26 enzymes in general have weak interactions with saccharides in the aglycone subsites, i.e. a high K_d for saccharides acting as acceptors, increased acceptor concentration could be beneficial for promoting transglycosylation.

Previous studies on GH26 β-mannanases have in general used low to moderate concentrations of mannooligosaccharides when characterizing the enzymes, e.g. 10 mM M3 for *Cf*Man26A-50K and several other GH26 β-mannanases have been evaluated with mannooligosaccharide concentrations between 1 and 5 mM (**Paper I and Paper II**) [97, 100, 120, 125, 237]. In contrast, transglycosylation has been reported for several GH5 β-mannanases at mannooligosaccharide concentrations of 5 mM (Table R4). In the current work presented in this chapter (not published), indications of saccharide transglycosylation was observed for four different GH26 β-mannanases (*Bo*Man26A, *Cf*Man26A-50K, *Pa*Man26A and *Ypen*Man26A) when they were incubated with 0.4 M of a mannooligosaccharide (Table R4). The presence of transglycosylation products in the reactions was based on observations with MALDI-ToF MS of peaks with m/z corresponding to saccharides of higher mass than the substrate mannooligosaccharide. *Bo*Man26A was further evaluated with lower mannotetraose concentration and transglycosylation products could be observed down to a concentration 20 mM of mannotetraose (Table R4). The observed transglycosylation products for the tested GH26 β-mannanases corresponded with expected results based on preferred productive binding mode (**Paper I, Figure 5** and **Paper II, Figure 8**) [100, 129]. For example, *Bo*Man26A incubated with mannotetraose, which primarily forms a glycosyl-enzyme intermediate with mannobiosyl, appeared to produce mannohexaose (Figure R1) corresponding to a mannotetraose acting as an acceptor of the mannobiosyl.

Superimposition of the crystal structures of the four examined GH26 β-mannanases (Table R4) with that of *Cj*Man26C, crystallised with a mannooligosaccharide in the -2 to +2 subsites (PDB ID: 2VX6) [63], indicated that glycan interactions in the aglycone subsites beyond the +1 subsite may be limited in the studied GH26 enzymes (Figure R2). While *Cj*Man26C had clear interactions with the mannosyl unit in the +2 subsite, only *Bo*Man26A had any residues within 5Å of the mannosyl in the +2 subsite of *Cj*Man26A. Neither of the two residues (E234 and Phe261) in the vicinity of the mannosyl in the +2 subsite of *Bo*Man26A appear to be located so that they can directly interact with a saccharide in the subsite, assuming similar positioning of the saccharide in *Bo*Man26A as *Cj*Man26C (Figure R2 A). The lack

of interactions beyond the +1 subsite could be part of the explanation to the previous lack of observed transglycosylation with saccharides acting as acceptors in GH26 β-mannanases.

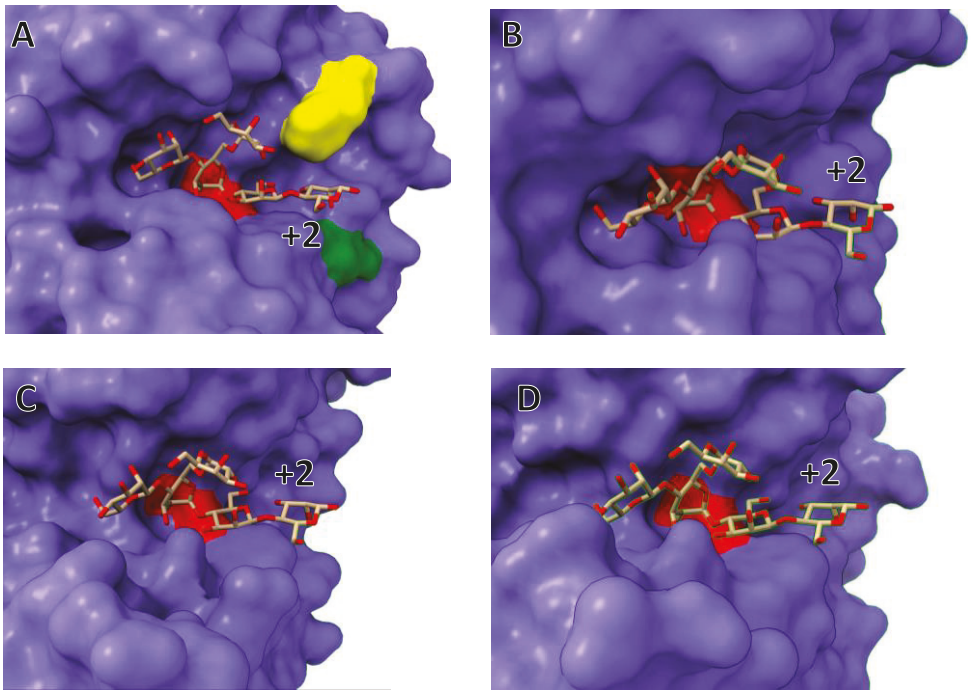

Figure R2. Comaprison of +2 subsites in GH26 β-mannanases.
The figure shows an surface view of BoMan26A (A, PDB ID: 4ZXO), CfMan26A-50K (B, PDB ID: 2BVT), PaMan26A (C, PDB ID: 3ZM8) and YpenMan26A (D, PDB ID: 6HPF) with a galactosylated mannotetraose from *Cj*Man26C (PDB ID: 2VX6) incorporated thorugh superimposition onto the *Cj*Man26C crystal structure. The location of the potential +2 subsite is marked with +2 in the figures. The catalytic residues are shown in red for in all four β-mannnases. Residues which are located within 5Å of +2 mannosyl unit are shown in yellow (F261 in *Bo*Man26A) and green (E234 in *Bo*Man26A).

However, there is another clear difference between the two GH families as we look at transglycosylation with non-saccharide acceptors. As in the case with saccharide acceptors, transglycosylation with non-saccharide acceptors has been observed and published for GH5 β-mannanases (**Paper III**) [119, 183, 223] but no reports appear to be published for GH26 β-mannanases. Even when transglycosylation with saccharides was eliminated in the +2 subsite variant *Tr*Man5A R171K it still maintained the ability to use methanol as an acceptor for transglycosylation (Figure R1 and Table R4) [117]. For the GH26 β-mannanases examined in this section, transglycosylation with methanol as an acceptor could not be detected (Figure R1, Table R4). This indicates that there might be more to the difference in transglycosylation capacity between GH5 and GH26 β-mannanases than limited acceptor interactions for GH26 enzymes.

Table R4. Comparing transglycosylation capacity of GH5 and GH26 β-mannanases.
Transglycosylation capacity of various β-mannanases in family GH5 and GH26. The results for experiments performed in this study are based on observation of peaks with m/z corresponding to transglycosylation products observed in reactions analysed with MALDI-ToF MS. A + indicates observed transglcyosylation products while a – indicates a lack of observed transglycosylation products. For saccharide acceptors, the concentration of saccharide (acting as both donor and acceptor) is listed in parenthesis toegether with which mannoloigosaccharide was used as substrate. For the GH5 β-mannanases (*Tr*man5A, *Tr*man5A R171K, *An*Man5A, *An*Man5B, *An*Man5C), 5 mM mannotetraose was used as the donor substrate in the incubations with methanol. For experiments indicated as "This study" (marked with * in the table) see further desciption in the Methods section.

Enzyme	Acceptor		Reference
	Mannooligossacharide	Methanol	
*Tr*Man5A	+ (5 mM, M4)	+	[117]
*Tr*Man5A R171K	- (5 mM, M4)	+	[117]
*An*Man5A	+ (5 mM, M4)	+	[183]
*An*Man5B	+ (5 mM, M4)	(+)	[183]
*An*Man5C	+ (5 mM, M4)	+	[183]
*Bo*Man26A	- (4 mM, M4), + (20 mM, M4)*	-*	[97], *This study
*Pa*Man26A	- (5 mM, M5), + (400 mM, M5)*	-*	[100], *This study
*Cf*Man26A-50K	- (10 mM, M3), + (400 mM, M5)*	-*	[237], *This study
*Ypen*Man26A	- (1 mM, M5), + (400 mM, M5)*	-*	**Paper I**, *This study

Similar but different

As was shown in **Paper V**, minor changes in the highly conserved residues in and around the -1 subsite may cause radical differences in the transglycosylation capacity of retaining GHs. A comparison of the highly conserved residues in the -1 subsite of GH26 (**Paper II**) and GH5 β-mannanases [108, 242, 243] is therefore interesting in order to try to understand differences that might affect transglycosylation abilities of β-mannanases in the two GH families. A structure-based sequence alignment of *Tr*Man5A and *Bo*Man26B and a superimposition of the crystal structures of *Tr*Man5A (PDB ID: 1QNR) [108] and *Bo*Man26B (PDB ID: 6HF4) (**Paper II**) was made in order to make an initial comparison of the highly conserved residues in family GH5 and GH26 (Figure R3 and R4). Two highly conserved residues in the active site of GH26 β-mannanases, G204 and F207 (*Bo*Man26B numbering), are not displayed in the superimposition of the 3D structures (Figure R4) as they appeared to not be able to interact with a mannosyl unit in the -1 subsite as judged from examination of the structure.

Four residues that are highly conserved in family GH5 and GH26 are potentially conserved between the GH5 and GH26 families as they align in the sequence alignment of *Bo*Man26B and *Tr*Man5A as well as occupy the same space in the superimposition of the 3D structures (Figure R3 and R4). These four residues are the two catalytic glutamates, a tryptophan in the -1 subsite (W314/W306 in *Bo*Man26B/*Tr*Man5A) and a tyrosine (Y263/Y243 in *Bo*Man26B/*Tr*Man5A). The highly conserved tryptophan and tyrosine are thought to interact with the mannose in the -1 subsite and the nucleophile respectively in GH5 [108, 244] and GH26 [124, 127, 244] and have been shown to be critical for catalysis in GH26 [127].

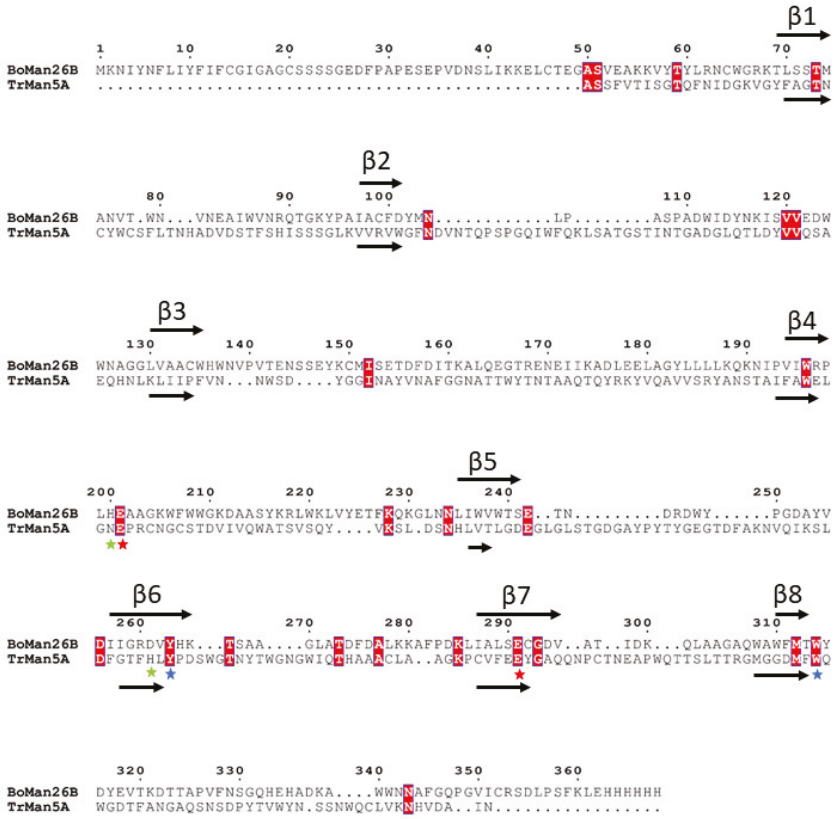

Figure R3. Sequence alignment of *Bo*Man26B and *Tr*Man5A.
A sequence alignment of *Bo*Man26B and *Tr*Man5A with the location of the core β-sheets marked with arrows above (*Bo*Man26A) and below (*Tr*Man5A) the alignment for respective enzyme. The highly conserved catalytic glutamates of clan GH-A is marked with red stars and the tyrosine (Y263/Y243 in *Bo*Man26B/*Tr*Man5A) and tryptophan (W314/W306 in *Bo*Man26B/*Tr*Man5A) that appears to be potentially conserved between the GH5 and GH26 are marked with blue stars. Highly conserved residues in GH5 and GH26, respectively, that align in the sequence alignment but are different residues are marked with a green strar. Identitical residues in the two sequences are highlighted with red background in the alignment.The alignment was done with Expresso [224], which incorporates structural data for the alignment and vizualied with ESPript 3.0 (https://espript.ibcp.fr) [225].

Besides the four residues that may be conserved between GH5 and GH26, there are several other residues in the active site of the two families that have a high degree of conservation in GH5 [108] and GH26 (**Paper II, Table 5**) respectively but do not appear to be conserved between them based on what is observed in the comparison of *Bo*Man26B and *Tr*Man5A (Figure R3 and R4). One of the hallmarks of the GH26 β-mannanases is that the amino acid residue proceeding the acid/base is a histidine (**Paper II**) [127], while most other clan GH-A families have a highly conserved asparagine proceeding the acid/base [245, 246]. In the superimposition of *Bo*Man26B and *Tr*Man5A (Figure R4), one can see potential similarities and

differences in the positioning of conserved residues in the active site of family GH5 and GH26 β-mannanases.

What the effects of these differences in the active site have on the transglycosylation ability of β-mannanases from GH5 and GH26 is an interesting task to study, but is currently unclear. In **Paper V** it was shown how substitutions of highly conserved residues in the -1 subsite of three clan GH-A GHs (a GH2 β-mannosidase, a GH10 β-*endo*-xylanase and a GH51 α-L-arabinofuranosidase) could lead to improved transglycosylation capacity of the produced variant. It is possible that part of the explanation for the apparent weaker transglycosylation capacity observed for GH26 β-mannanases compared to their GH5 counterparts could be attributed to these differences in the -1 subsite. We will return to the discussion of the potential effects of substitution of amino acids in the -1 subsite of retaining glycosidase in terms of transglycosylation in the following section.

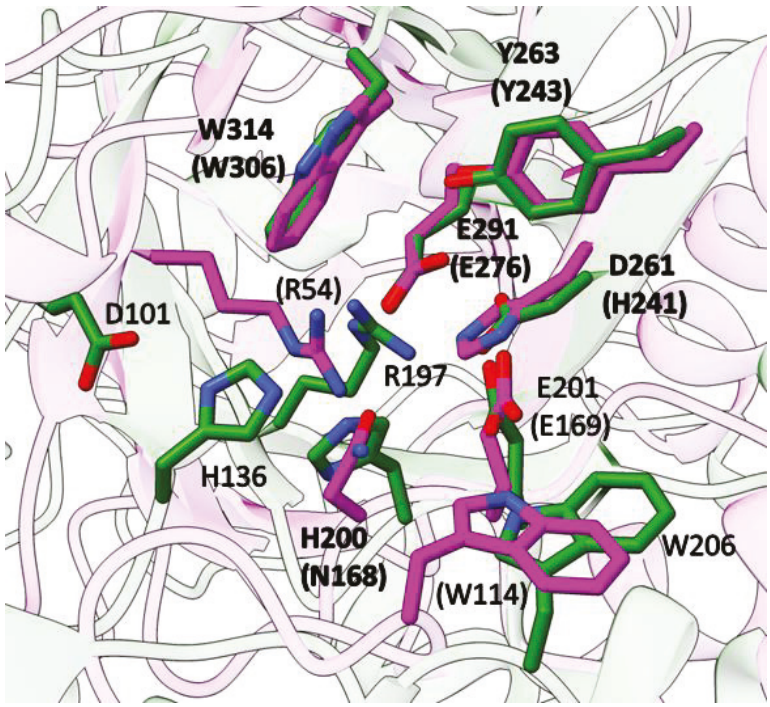

Figure R4. Comparison of the highly conserved residues in the active sites of GH5 and GH26 β-mannanases. The crystal structure of *Tr*Man5A (purple, PDB ID: 1QNR [108]) superimposed on the crystal structure of *Bo*Man26B (green, PDB ID: 6HF4 (**Paper II**)) showing the side-chains of highly conserved residues in the active sites of GH5 and GH26 β-mannanases, respectively. The exception is W114 in *Tr*Man5A, which is not conserved in GH5 but appear to have high stuctural similarity in the active site of GH5, but can be located on different loops in different subfamilies [242]. The numbering of the residues from *Tr*Man5A are shown in parenthesis while those from *Bo*Man26B are without parenthesis in the figure. The numbers for side-chains from residues that align in the sequence alignment and appear to occupy the same space in the superimposition of the 3D structures are shown in bold. The side-chains of the highly conserved residues F207 and G204 (*Bo*Man26B numbering) in GH26 are not displayed in the *Bo*Man26B crystal structure as F207 appeared to be located to far away to interact with a mannose in the -1 subsite while G204 is likely to mainly fill a structural role.

Substrate interactions in glycoside hydrolases

Preferred productive substrate binding mode in glycoside hydrolases and its effect on product profile

The topology of the active site governs how a glycoside hydrolase can productively interact with different substrates and thus the product profile of the GH [59]. The initial binding will likely influence both hydrolysis and transglycosylation product formation both when it comes to hydrolysis and transglycosylation. It will determine if the glycoside hydrolase is endo- or exo-acting [60, 63, 97, 247-249], influence the substrate specificity [48, 122, 244, 248, 250, 251], and the product profile (in term of product length) of a glycoside hydrolase (**Paper I** and **Paper II**) [117, 129, 252].

In **Paper I** and **II** it was demonstrated how the structure of the glycone (negative-numbered) subsites of β-mannanases affect the ability to utilise different polymeric substrates. Both *Ypen*Man26A and *Bo*Man26B were less affected by galactosyl substitutions when hydrolysing the more heavily substituted galactomannan guar gum compared to locust bean gum in contrast to other GH26 β-mannanases, for which a high degree of substitution often had a more adverse effect on the catalytic efficiency (Table R3). This is likely in part due the open active site clefts of both *Ypen*Man26A and *Bo*Man26B, which allows for the accommodation of a galactosyl substituted mannose-based polysaccharide in several of their subsites (-5, -4, -3, -1 and +1 for *Bo*Man26B and -3 to -1 for *Ypen*Man26A) (**Paper I**, **Figure S2** and **Paper II**). Furthermore, the importance of interactions with galactosyl substituents for the catalytic efficiency towards galactomannans was shown for both *Bo*Man26B and *Ypen*Man26A, as variants where galactosyl interactions were weakened or removed led to lowered catalytic efficiency towards galactomannans (**Paper I**, **Table 4** and **Papper II**, **Table 1**). The structure of the active site cleft has previously been shown to play an important role in the kind of substrates that both *endo*-acting β-mannanases and xylanases are able to utilise, as their structure may restrict their ability to accommodate glycosyl substitutions [102, 250]. Substitutions along the polysaccharide backbone is a common feature in many polysaccharides, as we have seen in the form of galactose for locust bean gum and guar gum (Figure B1) [9, 34] and in the form of both acetyl and galactose for the softwood hemicellulose galactoglucomannan (Figure B1) [253, 254]. In other words, if the aim is to utilise saccharides from renewable sources, e.g. the polysaccharides locust bean gum or galactoglucomannan, it is important to understand the limitations that the β-mannanase active site may impose on substrate utilisation. This is exemplified in **Paper III,** where the accumulation of what likely was an indigestible heptasaccharide was observed with MALDI-ToF MS when locust bean gum was incubated with *Tr*Man5A (**Paper III, Figure 8A**). This accumulation was likely due to restrictions caused by one or more galactosyl substituents, as the heptasaccharide

was markedly reduced when the α-galactosidase Aga27A was included in the incubations alongside *Tr*Man5A (**Paper III, Figure 8C**).

Both the glycone and aglycone (positive-numbered) subsites (Figure B4) will influence the preferred binding mode of an oligosaccharide substrate and the chain length of the glycosyl-enzyme intermediate that forms in the double displacement mechanism (**Paper I** and **II**) [117, 223]. However, the structure of the glycone region of a β-mannanase's active site cleft may limit the length and conformation of the glycosyl moiety that forms the glycosyl-enzyme intermediate and subsequently the hydrolysis or transglycosylation products that can be formed. An example of this would be the GH26 mannanases *Bo*Man26A [97] and *Cj*Man26C [63], in which the negative-numbered subsites are partly (*Bo*Man26A) or fully restricted (*Cj*Man26C) beyond the -2 subsite. This leads to the glycosyl-enzyme intermediate exclusively, or almost exclusively for *Bo*Man26A, containing a mannobiosyl unit. This in turn leads to mannobiose being the primary product from hydrolysis [63, 97] and that any potential glycoside generated from either *Bo*Man26A or *Cj*Man26C would in principle be limited to a mannobiosyl as the glycan moiety. Another example of limitations posed by the active site cleft is the pocket topology many retaining exo-glycosidases adopt and where the glycosyl-enzyme intermediate is formed with a monosaccharide unit [59], e.g. the α-galactosidases used in **Paper III** and **Paper IV** or the *C. fimi* β-mannosidase in **Paper V**. If instead a mannanase with a more open active site and a stronger preference for binding in the glycone subsites is used, e.g. *Pa*Man5A [100], *Tr*Man5A (**Paper III**) or *Bo*Man26B (**Paper II**), the hydrolysis product can be longer saccharides and the mannosyl part of potential transglycosylation products can consist of several mannosyl units. Due to these limitations posed by the structure of the enzyme, it is important to understand the enzyme you are working with if the end goal is products of specific characteristics, such as saccharide chain length.

Defined products through modified glycone subsites

One of the goals with using glycoside hydrolase mediated transglycosylation is the formation of well-defined products without the need of extensive protection and deprotection steps often required in organic chemistry [55, 255, 256]. Past research on transglycosylation with retaining GHs and non-saccharide acceptors has included obtaining glycoside hydrolases that functions effectively as catalysts (**Paper III – V**) [79, 118, 196, 200, 257]. The properties of the glycosyl group can affect the properties of a glycoside, i.e. the transglycosylation product, [119, 258] as a glycoside with two saccharide units may behave differently than one with three saccharide units (e.g. different surface activity). Therefore, future work will likely also focus on the production of transglycosylation products with well-defined glycosyl chain-length. In **Paper I** and **II**, it was demonstrated that the hydrolytic product profile can be altered by changing amino acids in *Bo*Man26B and

*Ypen*Man26A which provide key interactions with the substrate. These changes lead to shifts in the preferential binding of mannooligosaccharides from the aglycone to glycone subsites (**Paper I, Figure 5** and **Paper II, Figure 8**). Similar observations have been made in other GHs, where substitution of saccharide-interacting amino acids lead to change in preferential binding mode of the saccharide [129, 259]. This shows that rational engineering of residues in the negative subsites is likely a valid strategy for generating retaining glycoside hydrolases with desired product profiles for transglycosylation, as hydrolytic and transglycosylation products are generated from the same glycosyl-enzyme intermediate (Figure B3). In other words, a change in the hydrolytic profile of a retaining glycoside hydrolase may be expected to give the same shift in the transglycosylation product profile.

The rational design of new glycoside hydrolase variants targeting saccharide interactions requires knowledge of what interactions exist between the GH and the substrate. However, as subsites aside from the -1 subsite can have a low overall degree of conservation within the same family (**Paper I** and **II**), a sequence-based approach like the one used in **Paper V** is likely not an optimal approach. In **Paper I** and **Paper II** the variants were designed based on substrate interactions observed in crystal structures with bound substrate. Since obtaining a 3D structure of a protein through X-ray crystallography often can be a cumbersome task, alternative approaches not directly relying on protein crystallography might be more appealing. Such alternative approaches to examine glycoside hydrolase-substrate interactions could be structure modelling or prediction [184, 260] combined with either overlays with structures that have bound substrate (**Paper I** and **II**) [102, 184] or through docking potential substrates to the obtained models [261-263].

Modifying retaining glycoside hydrolases for improved transglycosylation

As we have seen so far, transglycosylation by retaining glycoside hydrolases is a complex matter and advancements in our understanding of how to improve transglycosylation in retaining GHs have been made in recent years (**Paper V**) [235, 264-266]. We have however, still not reached a general understanding of what gives a retaining GH a high transglycosylation capacity [264]. In part this may be attributed to the great variety in active site structure of retaining glycoside hydrolases, making general conclusions more challenging to make. But another contributing factor is likely that most studies have limited focus to single retaining glycoside hydrolases or GH families [118, 200, 201, 257, 267-269], making conclusions regarding general underlying mechanisms harder in the individual studies. In **Paper V** however, we demonstrated a method for generating retaining GHs with improved transglycosylation that appeared to be generally applicable as

it was successfully implemented for six different GH families. This section will discuss how modifications of glycoside hydrolases can be used to alter their transglycosylation capacity or modifying their acceptor utilization.

Improved transglycosylation through modification of the aglycone subsites

In several retaining GHs, the positive-numbered subsites have been shown to play an important role in transglycosylation, as these subsites may interact with or restrict any molecule that is going to fill the role of acceptor [184, 203, 223, 241, 270]. The role of acceptor can be filled by water (hydrolysis) or, for example, a saccharide or an alcohol (transglycosylation) (Figure B3). In **Paper III** the effect of alterations in the aglycone subsites on transglycosylation capacity of the GH5 β-mannanase *Tr*Man5A was examined. In the paper it was shown how two variants of *Tr*Man5A (R171K and R171K/E205D) had increased transglycosylation capacity with allyl alcohol as acceptor and mannotetraose as donor substrate compared to wild-type *Tr*Man5A (**Paper III, Figure 3 and 4**). The increase in transglycosylation with allyl alcohol was proposed to in part be due to elimination of transglycosylation reactions where M4 acted as acceptor, which has previously been described for the +2 subsite variant R171K [117]. The variants R171K and R171K/E205D would thereby eliminate a competing reaction in the form of transglycosylation where saccharide acts as acceptor while the alterations likely had limited effect on the transglycosylation capacity with allyl alcohol, leading to an overall increased generation of allyl mannosides.

The importance of the aglycone subsites in transglycosylation reactions for certain retaining GHs is well-established in the literature, with several examples of how different modifications in the aglycone region can be used to modify transglycosylation behaviour of retaining glycoside hydrolases [117, 169, 184, 203, 241, 270-273]. These studies highlight how different aspects of potential acceptor interactions influence retaining GHs ability to perform transglycosylation. This can be increased interaction with the acceptor molecule, exemplified by Dilokpimol and co-workers who investigated two homologous GH5 β-mannanases from *Aspergillus nidulans* with differing transglycosylation capacity [184]. The difference in transglycosylation capacity between the two homologues was attributed in part to the presence or lack of a tryptophan in the +1 subsite, revealed through homology modelling of the two GHs [184]. *An*Man5C was shown to have a higher transglycosylation capacity than the homolog *An*Man5A and this appeared in part to be due to an mannose-interacting tryptophan located in the +1 subsite of *An*Man5C [184]. In *An*Man5A the residue in the corresponding location to the tryptophan was instead a serine that appeared to be too far away to have any interactions with the mannose in the +1 subsite. By generating variants with swapped residues at this location in the +1 subsite lead to the generation of an

*An*Man5C variant (*An*Man5C W283S) with reduced transglycosylation capacity compared to wild type *An*Man5C and an *An*Man5A variant (*An*Man5AS289W) that had increased transglycosylation capacity compared to wild type *An*Man5A [184].

Another way to modify the aglycone subsites that has been utilised is modifying the active site to be more open in order to accommodate specific types of acceptors. This is exemplified in a study of a GH13 glucansucrase where screening of a mutant library found variants with improved transglycosylation with the rather bulky flavonoid luteolin [270]. The proposed mechanistic explanation for the increased transglycosylation capacity for several of the variants of the glucansucrase were that the substitution of the amino acids led to better accommodation of luteolin in the aglycone subsites which in turn lead to increased transglycosylation. A similar mechanistic reason is thought to be in part a possible explanation for the apparent increased initial transglycosylation of the R171K/E205D variant compared to the R171K variant in **Paper III** (**Paper III, Figure 3**). The proposed explanation for increased transglycosylation of R171K, compared to *Tr*Man5A wild type, was the suppression of saccharide transglycosylation, likely due to lowered binding in the +2 subsite. The additional improvement in transglycosylation for R171K/E205D was discussed to be due to better accommodation of the allyl alcohol in the active site.

Yet another way to increase the transglycosylation capacity of glycoside hydrolases is to make the catalytic site of the enzyme less accessible to water molecules. Jamek and co-workers showed that transglycosylation of a GH20 β-N-acetylhexosaminidase could be increased by the insertion of a loop close to the active site, with the discussed mechanistic explanation being that the insertion of the loop lead to a more shielded catalytic site that disfavoured water and consequently lead to lowered hydrolysis [271].

Taken together, all of this supports that several aspects of the aglycone subsites affect the transglycosylation capacity of a retaining glycoside hydrolase and how engineering of the aglycone subsites to favour desired interactions, i.e. interactions with the desired acceptor molecule, while disfavouring unwanted interactions, e.g. with water or saccharides, can be a powerful tool to improve transglycosylation. However, as for modifications of the glycone subsites, rational engineering of the aglycone subsites is helped by knowledge of the structure of the glycoside hydrolase that is being modified.

Altering transglycosylation behaviours of retaining GHs through substitutions of amino acids in the glycone region

The various residues in the -1 subsite of a glycoside hydrolase fill several roles, including acting as catalytic residues [59], interacting with and modulating the catalytic residues (**Paper V**) [202, 268, 274] or interacting with the glycosyl providing assistance during the catalysis [202, 275, 276]. In **Paper V** the substitution of highly conserved residues in and around the -1 subsite of retaining GHs was shown to be an efficient way to generate variants with improved transglycosylation compared to the wild-type counterpart. Similar findings have been made in previous studies, where the substitution of residues in the -1 subsite has been shown to increase the transglycosylation capacity of other glycoside hydrolases as well [200, 257, 268].

It has been theorised that changes of highly conserved residues in the -1 subsite may lead to destabilisation of the transition states of retaining glycoside hydrolases [55, 200, 257]. This destabilisation of the transition states can lead to lowered catalytic efficiency of the GHs [55, 200, 257]. It has been put forward that an increased lifetime of the glycosyl-enzyme intermediate may favour transglycosylation over hydrolysis [201]. Furthermore, it has been theorised that the destabilisation of the second transition state may affect the energy barriers for hydrolysis and transglycosylation differently, potentially leading to transglycosylation becoming the favoured reaction in the deglycosylation step [55]. In **Paper V**, reduced catalytic activity was observed for most of the generated GH variants but the effects on transglycosylation capacity was varied. Some variants displayed improved transglycosylation capacities compared to their wild-type GH while other variants had similar or worse transglycosylation capacities compared to their wild type GH (**Paper V, Table 2-7**), highlighting that engineering retaining GHs for improved transglycosylation is not a simple task.

Several studies have investigated the effects of changes in the active site of glycoside hydrolases, either in vitro, in silico or a combination of both [204, 268, 274, 277, 278]. This includes a study from Collet and co-workers where the substitution of a tyrosine interacting with the catalytic nucleophile in a GH5 β-glucanase was proposed to lead to increased lifetime of the glycosyl-enzyme intermediate [274]. Furthermore, the study by Collet et al demonstrated the important role of the base-function of the acid/base in the deglycosylation step and in this case for transglycosylation. In another study conducted by Guo *et al.* it was discovered that a single nucleophile-interacting serine in some GH13 amylosucrases and sucrose hydrolases appeared to play a noticeable role in determining transglycosylation capacity of the investigated GHs [204].

In addition to substitutions in the -1 subsite, alterations of other glycone subsites have also been shown to have potential positive effects on the transglycosylation capacity of retaining glycoside hydrolases [129, 279]. As an example of this, we can

once more look at the study by Hekmat et al. of a variant of the *Cf*Man26A-50K GH26 β-mannanase with weakened interactions to the glycosyl donor in the -3 subsite and strengthened interactions in the -2 subsite [129]. Interestingly, these changes in the glycone subsites resulted in indications of transglycosylation with saccharide acceptors for the *Cf*Man26A-50K variant while no such signs were observed for *Cf*Man26A-50K [129].

Overall, it is clear that disruption of the fine-tuned catalytic machinery of glycoside hydrolases can be an efficient approach to produce enzymes with improved transglycosylation capacity (**Paper V**) [200, 201, 204]. The combination of computational methods and experimental data has led to an increased understanding of how retaining glycoside hydrolases function and can be manipulated [268, 280-282]. The importance of the interactions in the glycone subsites of retaining glycoside hydrolases, and the -1 subsite in particular, for the preferred catalytic route, i.e. hydrolysis vs transglycosylation, has been demonstrated in several studies (**Paper V**) [200, 204, 257, 268] though there is still much to understand.

Proper evaluation of transglycosylation in retaining glycoside hydrolases

As has been described so far in the thesis, several factors go into determining if a retaining glycoside hydrolase is a good candidate to utilise in transglycosylation or not. In **Paper III-V,** secondary hydrolysis negatively affected the amount of transglycosylation products that could be obtained. This is most clear in **Paper III**, where the mannotrioside-product of R171K/E205D was almost fully degraded over prolonged incubation times (**Paper III, Figure 4**). Comparing at what point in the reaction secondary hydrolysis appeared to be more prevalent in the different studies also revealed interesting differences. For *Tr*Man5A (**Paper III, Figure 4**) and for several of the GHs in **Paper V** (**Paper V, Figure 2, 3** and **4**) secondary hydrolysis appeared to primarily occur when most of the donor substrate had been consumed. R171K/E205D (**Paper III, Figure 4**) and *Bo*Gal36A (**Paper IV, Figure 6**) on the other hand appeared to have significant amounts of secondary hydrolysis before a majority of the donor substrate had been consumed. Furthermore, in **Paper III** it is also suggested that unaccounted for reactions, i.e. transglycosylation where the saccharides also acts as acceptor in *Tr*Man5A, led to an apparent lower initial transglycosylation capacity than what the enzyme might actually have. This shows how important proper understanding of the different reactions that occur during the reaction time is to accurately evaluate the transglycosylation capacity of a glycoside hydrolase. As can be seen in **Paper III** and **Paper IV**, estimation of r_S/r_H can be used to indicate the presence of secondary hydrolysis in the reaction but is not sufficient to properly describe the reaction course over the reaction time. A recent

paper by Fagundes et al. present a way to model transglycosylation reactions in retaining glycoside hydrolases [283]. As was done in **Paper III-V**, the authors relate transglycosylation products to the amount of consumed donor substrate. The model presented by Fagundes et al. takes into consideration hydrolysis, transglycosylation and secondary hydrolysis and the approach could influence future studies, leading to better understanding of what happens throughout the reaction and evaluation of different retaining GHs.

Conclusions and future perspectives

The work presented in the thesis has explored various aspects of enzyme-substrate interactions between retaining glycoside hydrolases and different glycans and the transglycosylation capacity of retaining glycoside hydrolases.

In **Paper I** and **II**, two GH26 β-mannanases, *Ypen*Man26A from the fungus *Y. penicillata* and *Bo*Man26B from the bacterium *B. ovatus*, was studied in order to investigate how the structure of their active sites allowed for efficient hydrolysis of the highly galactosyl substituted galactomannan guar gum. It appears as both β-mannanases have adapted a fairly open active site architecture that allows for accommodation galactosyl substituents in several of their subsites. In **Paper III,** the GH5 β-mannanase *Tr*Man5A appeared to be restricted by galactosyl substitution, preventing full utilization of the donor substrate LBG galactomannan. This highlights the importance of the active site architecture of β-mannanases and how it may affect their ability to utilise different type of substrates. In **Paper I** and **II** it was also shown how substitutions of distal tryptophans in the glycone subsites in *Bo*Man26B and *Ypen*Man26A lead to a shift in the preferred productive binding mode, with apparent lowered binding in the subsite containing the substituted tryptophan in both enzymes. The lowered binding in the subsite containing the substituted tryptophan is likely due to lowered affinity for substrate binding in the subsite. The engineering of substrate interactions at specific subsites of retaining, *endo*-acting GHs could potentially be applied to transglycosylation or hydrolysis reactions to steer product formation towards specific saccharide chain-lengths. This approach for generation of specific products is unlikely to be limited to *endo*-acting β-mannanases, but potentially also other *endo*-acting GHs such as *endo*-glucanases or *endo*-xylanases which also utilises natural, renewable substrates.

Paper III investigated transglycosylation capacity of *Tr*Man5A and variants thereof. After initial evaluation, wild-type *Tr*Man5A and the variant R171K/E205D was selected for further investigation of transglycosylation capacity. R171K/E205D appeared to have a higher transglycosylation capacity than wild-type *Tr*Man5A when using M4 as the donor substrate, but also a higher degree of secondary hydrolysis. When using the galactomannan LBG as the donor substrate instead of M4, wild-type *Tr*Man5A instead appeared to be the better enzyme for transglycosylation. It was hypothesised that the higher transglycosylation capacity for R171K/E205D with mannotetraose as the donor substrate was due to the elimination of saccharide transglycosylation with the R171K substitution while the

E205D substitution may lead to an active site that better accommodates the allyl alcohol acceptor. Due to LBG galactomannan being rather large and bulky it is possible that it is a poor acceptor molecule, which could lead to lack of or greatly reduced saccharide transglycosylation in *Tr*Man5A. This lack of transglycosylation where the saccharide acts as acceptor could be part of the explanation to why wild type *Tr*Man5A has higher apparent transglycosylation capacity than R171K/E205D with LBG as the donor substrate but not with M4. **Paper III** emphasizes the importance the aglycone subsites may play in transglycosylation catalysed by retaining GHs, a finding that is supported in previous studies of transglycosylation in other families of retaining glycoside hydrolases [184, 223, 270]. Furthermore, in the section on the previous unpublished data on transglycosylation in GH26 β-mannanases, it was speculated that the apparent lack of subsites past +1 in the examined GH26 β-mannanases could be part of the explanation why transglycosylation with saccharides as acceptors had not previously been observed at low substrate concentrations for these GHs. These results highlight the importance of the aglycone (positive-numbered) subsites in transglycosylation reactions and how they potentially can be engineered to promote desired outcomes in transglycosylation, e.g. disfavour saccharide acting as acceptors, or favouring it if defined length oligosaccharides are the desired product. Further studies of the differences between GH5 and GH26 β-mannanases when it comes to transglycosylation capacity would be of interest. The GH26 β-mannanase *Cj*Man26C could be interesting to include in further investigations due to the presence of a +2 subsite in the enzyme. This may allow investigation of what the effects of a +2 subsite could have on the transglycosylation capacity of GH26 β-mannanases. Furthermore, the application of the method presented in **Paper V** on the GH26 β-mannanases studied in the section "Comparing transglycosylation behaviour in GH5 and GH26 β-mannanases" could be of interest to investigate the effect the residues in their -1 subsites may have on their transglycosylation capacity.

The effect of the donor substrate was investigated in **Paper III** for *Tr*Man5A and in **Paper IV** for the α-galactosidases *Bo*Gal36A and Aga27A. In both papers the shift to a more complex donor substrate, in the form locust bean gum galactomannan, lead to a decrease in transglycosylation yields compared to less complex substrates in the form of oligosaccharides. In **Paper III**, synergy between Aga27A and *Tr*Man5A lead to better utilisation of the polymeric galactomannan locust bean gum and improved yield of transglycosylation products. When moving towards even more complex polysaccharides, such as galactoglucomannan (Figure B1), it is likely that more enzyme activities will need to be included for efficient utilisation of the polysaccharide, e.g. acetyl esterases (Figure B6). Besides enzyme synergy approaches, identification of GHs with transglycosylation capacity that are relatively unhindered by different substitutions and the presence of multiple types of sugar units in the main chain is likely to be valuable when moving towards complex polymeric donor substrates, not only for β-mannans, but also for other renewable heteroglycans, such as xylans. Further investigation on the effects of

choice of donor substrate on transglycosylation capacity could also be of interest, as better understanding of this aspect could help in reaction designs to maximize transglycosylation yields.

In both **Paper III** and **IV**, secondary hydrolysis appeared to be an important factor for the final yield of transglycosylation products as it appeared to be pronounced for both *Tr*Man5A R171K/E205D in **Paper III** and *Bo*Gal36A in **Paper IV** over prolonged incubation times. Interestingly, several of the variants generated in **Paper V** appeared to have lower secondary hydrolysis compared to their wild-type counterpart. This may have been a contributing factor to the apparent higher transglycosylation capacity for some of the variants in **Paper V**. Overall these results shows the complexity of trying to engineer protein with improved transglycosylation capacity as both the choice of substrate and the GH's tendency to catalyse the breakdown of the formed products might have an effect on the final outcome. In future studies it could be interesting to apply the method for generating variants with improved transglycosylation from **Paper V** to *Tr*Man5A R171K/E205D. The presence of secondary hydrolysis observed for R171K/E205D appeared to be one of the main drawbacks of this variant. If variants of *Tr*Man5A R171K/E205D generated through this method displays a similar trend with lowered secondary hydrolysis as was observed in **Paper V**, it could potentially lead to a GH with greatly improved transglycosylation capacity.

In **Paper V** a general method for generating new variants of retaining glycoside hydrolases with potential for improved transglycosylation capacity was presented. The method targets highly conserved residues and relies on sequence data from a GH family without the need of structural knowledge. The method was applied to several different GH families and proved to be successful, leading to the generation of variants with improved transglycosylation compared to the wild-type GH in all the tested GH families. While the method does not necessarily lead to the generation of the variant with the highest possible transglycosylation capacity, it appears to be an efficient way to create initial leads for further enzyme engineering as it generates a relatively small number of variants to evaluate. As the method for improved transglycosylation capacity developed and described in **Paper V** was successfully applied to several GH families, it is likely that its use can be expanded beyond the initial six GH families investigated in the paper. Even if this turns out to not be the case, its successful implementation in family GH2 means that there are a wide variety of different GHs that could potentially be utilised as catalysts in transglycosylation reactions, including β-galactosidases and β-glucosidases (www.cazy.org) [46]. Combining the method presented in **Paper V** with other approaches presented in this thesis and other papers [207, 235] could help the development of retaining glycoside hydrolases with substantially increased transglycosylation capacity.

While the focus of the work presented in the thesis has been on retaining GHs acting on β-mannan, it is clear from the comparison to previous research that has been conducted within the field that some general principles are applicable between different type of GHs. For example, alterations of the of the aglycone subsites appear to be a good way to alter the transglycosylation behaviour in a wide variety of GHs.

Taken together, the findings presented in this thesis and the papers it is based on continue to build up the knowledge on the topic of transglycosylation carried out by retaining glycoside hydrolases. Combining what we have learnt in the presented work with approaches presented by other research groups, e.g. tailoring the microenvironments of a glycoside hydrolase [284] or the reaction conditions in which the transglycosylation is performed [196, 235], can hopefully help bring enzyme catalysed transglycosylation from proof-of-concept and small scale research to large scale reactions that are possible to implement in the valorisation of softwood hemicellulose.

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