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Transglycosylation by Glycoside Hydrolases - Production and modification of alkyl glycosides

Lundemo, Pontus

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Transglycosylation by glycoside hydrolases

Production and modification of alkyl glycosides

Pontus Lundemo

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To expand the enzymatic toolbox for synthetic chemists it would be favorable to convert glycoside hydrolases into transglycosylases, by limiting their hydrolytic activity. This dissertation investigates the transglycosylation activity of glycoside hydrolases with synthesis and modification of alkyl glycosides, a widely applicable type of surfactants, as model reactions. Reduced hydrolysis for *β*-glycosidases from the thermophilic *Thermotoga neapolitana* was achieved through protein engineering, limiting water content and increasing pH. Complete elimination of the hydrolytic activity with maintained transglycosylation was achieved for synthesis of hexyl-*β*-D-glucoside and the factors resulting in the success are discussed.

In addition, extension of the glycosidic part of alkyl glycosides through the coupling activity of cyclodextrin glucanotransferases is explored. An enzyme kinetics study was undertaken to deduce the optimal reaction conditions to promote coupling for a commercial enzyme. Moreover, a novel cyclodextrin glucanotransferase from *Carboxydocella* species was characterized, shown to have good coupling activity with *γ*-cyclodextrins as donor. This previously poorly studied donor can be used to extend the range of alkyl glycosides that can be produced and thereby the number of applications available.

Key words

Transglycosylation; Glycoside hydrolases; Cyclodextrin glucanotransferases; *β*-glucosidases; Alkyl glycosides; *Thermotoga neapolitana*; Organic reaction media

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Contents

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Populärvetenskaplig sammanfattning

Om vi använder allt tillgängligt fossilt bränsle kommer detta leda till att havsnivån ökar med mer än 50 m, enligt nya rön [1]. Även om detta hot ligger långt in i framtiden, finns ett flertal akuta problem kopplade till den globala uppvärmningen. FNs klimatpanel (IPCC) förutspår i sin senaste rapport en stor ökning av torka, skogsbränder, kraftiga orkaner, svält och massmigreringar år 2100. Dessutom kommer havets ekosystem ha sats ur balans av försurningen som koldioxidutsläppen orsakar, vilket skulle leda till utrotning av tusentals arter. Detta kan dock förhindras, om utsläppen av koldioxid minskar i rask takt för att helt upphöra senast i slutet av århundradet [2].

Stora framsteg görs för att minska utsläppen från fossila bränslen i energi och transportsektorn genom bland annat utveckling av solenergi och alternativa drivmedel. Men fossila bränslen används dessvärre inte enbart i energi och transportsektorn. Faktum är att större delen av alla kemikalier som framställs i världen kommer från en handfull intermediärer producerade i oljeraffinaderier. Det förnyelsebara alternativet, bioraffinaderier, där biomassa används som råvara är på uppgång. Dock ligger verktygslådan som är tillgänglig i bioraffinaderier hundratals år efter i utvecklingen jämfört med den traditionella kemin.

Verktygslådan för bioraffinaderier består i huvudsak av enzymer, proteiner som ansvarar för att alla processer i levande organismer sker. Eftersom biomassa består till största delen av kolhydrater så spelar kolhydrataktiva enzymer en viktig roll. Främst för att bryta ner biomassan till dess sockerbeståndsdelar, tillgängliga för mikroorganismer, men även för uppbyggnad av värdefulla produkter.

En sådan värdefull produkt är alkylglykosider, ytaktiva ämnen som används för att lösa upp feta molekyler i vatten. Dessa används i såväl läkemedelsindustrin som i rengöringsmedel, diskmedel och hudvårdsprodukter. De framställs genom att slå ihop en sockermolekyl med en alkohol.

Glykosylering, att fästa en sockermolekyl på något, är väldigt svårt med traditionell kemi. Det kräver flera steg med hög energiåtgång och metallkatalys. Med enzymer kan det utföras i ett enda steg utan starka reaktionsbetingelser.

Den största mångfalden inom kolhydrataktiva enzymer finns hos glykosid hydrolaser, men dessa kan dessvärre inte användas för glykosylering, då de istället är specialister på att bryta bindningar hos kolhydrater med hjälp av vatten, hydrolys.

Denna avhandling fokuserar på att förstå vad som får glykosidhydrolaser att bryta ner respektive bygga upp föreningar med socker som byggstenar. Genom att designa enzym och reaktionsbetingelser har jag lyckats förbättra utbytet från min modellreaktion från 20 % till nära 100 %. Dessa resultat bidrar till att förstå denna värdefulla enzymklass och för oss närmare målet att kunna använda den stora variationen glykosidhydrolaser för glykosylering och därmed konkurrera ut den smutsigare traditionella kemin.

List of Papers

This dissertation is based on the following papers, provided at the end of the booklet. The papers are referred to in the text by Roman numerals:

I Lundemo P, Adlercreutz P, Karlsson EN. 2013. Improved Transferase/Hydrolase Ratio through Rational Design of a Family 1 *β*-Glucosidase from *Thermotoga neapolitana*. Appl Environ Microb, 79:3400-3405

II Lundemo P, Karlsson EN, Adlercreutz P. 2014. Preparation of two glycoside hydrolases for use in micro-aqueous media. J Mol Catal B-Enzym, 108:1-6.

III Lundemo P, Karlsson EN, Adlercreutz P. Eliminating hydrolytic activity without affecting the transglycosylation of a GH1 *β*-glucosidase. 2015. (Manuscript)

IV Zehentgruber, D.; Lundemo, P.; Svensson, D.; Adlercreutz, P. Substrate complexation and aggregation influence the cyclodextrin glycosyltransferase (CGTase) catalyzed synthesis of alkyl glycosides. J. Biotechnol. 2011, 155 (2), 232−235.

V Ara KZG, Lundemo P, Fridjonsson OH, Hreggvidsson GO, Adlercreutz P, Karlsson EN. A CGTase with high coupling activity using *γ*-cyclodextrin isolated from a novel strain clustering under the genus *Carboxydocella*. Glycobiology. 2014, 25 (5), 514-523.

My contributions to the papers

All work described in this dissertation was performed under the supervision of Professor Patrick Adlercreutz and Professor Eva Nordberg Karlsson.

I I planned and performed the experimental work and wrote the manuscript.

II I planned and performed the experimental work and wrote the manuscript.

III I planned and performed the experimental work and wrote the manuscript.

IV The manuscript was drafted by Professor Patrick Adlercreutz. I performed early characterization work in collaboration with David Svensson and the experimental part with starch as substrate. Daniela Zehentgruber performed most of the kinetics experiments. I partook in data evaluation, making figures and revising the manuscript.

V I planned and performed the characterization of the novel CGTase, isolated by the company Matis Ltd (Reykjavik, Iceland), in cooperation with Kazi Zubaida Gulshan Ara. I wrote major parts of the manuscript, with help of Kazi Zubaida Gulshan Ara.

1 Introduction

Global climate change has stimulated the development of synthetic chemistry from renewable sources. Initially, production of biofuels has been the focus for research efforts. However, crude oil is not only our main source for fuel, but also the raw material for the vast majority of all chemicals and plastics produced, through a limited number of intermediates produced in petroleum refineries. Therefore, to enable the complete transition to a bio-based economy, we must develop competitive biorefineries, where a multitude of chemical products can be produced from renewable feedstock.

The most abundant renewable feedstock available is carbohydrates, comprising around 75 % of the biomass found on earth. Consequently, carbohydrate active enzymes play a key role in the biorefinery concept. Not only are they essential for degradation of biomass, but also have many applications in synthetic processes. The addition of a sugar onto a target molecule, glycosylation, is an important source of structural diversity of natural products. It can alter the properties of compounds in many ways, e.g. improving water solubility [3] or stability [4]. In addition, glycosylation can be used to synthesize alkyl glycosides, a versatile type of surfactant discussed extensively in this dissertation. Considering the wide range of applications, efficient tools for glycosylation are highly desirable.

In nature, glycosylation is mainly performed by Leloir glycosyltransferases. Unfortunately, they are not well suited for glycosylation *in vitro,* as they require expensive nucleotide-activated sugars as glycosyl donors. However, there are three other types of enzymes capable of performing glycosylation: glycoside phosphorylases, transglycosylases and glycoside hydrolases. Among these, glycoside hydrolases is the most widely applicable class, due to their natural abundance, robustness and ability to allow a wide variety of acceptors. Nevertheless, glycoside hydrolases suffer from their dominant hydrolytic activity, diminishing their usefulness for glycosylation.

The work presented in this dissertation has been focused on understanding what governs the propensity for transglycosylation or hydrolysis of glycoside hydrolases. Two types of enzymes have been studied, β-glycosidases and cyclodextrin glucanotransferases, both with the model reaction of synthesis or modification of alkyl glycosides.

1.1 Scope of the dissertation

The dissertation is based on five manuscripts out of which four are published.

Paper I-III deals with β-glycosidases and strategies for overcoming their high hydrolytic activity and favor transglycosylation. The method described in **Paper I** is protein engineering, targeted at altering non-conserved amino acids in the active site. **Paper II** focuses on reducing hydrolysis through limiting the amount of water in the reaction medium. Finally, the approach described in **Paper III** utilizes the different pH optima for transglycosylation and hydrolysis reactions, in combination with further protein engineering.

In **Paper IV** and **Paper V**, transglycosylation of cyclodextrin glucanotransferases is studied, with the aim of increasing the variety of alkyl glycosides that can be produced efficiently. In **Paper IV,** the influence of substrate and product concentrations, and their complexation, on the coupling reaction of a commercial cyclodextrin glucanotransferase is studied. **Paper V** instead focuses on characterization of a novel cyclodextrin glucanotransferase and comparing it to commercial enzymes.

2 Glycosylation

Glycosylation is the process of adding a glycosyl group (e.g. glucose, galactose or fructose) to the functional group of another molecule (glycosyl acceptor). In this dissertation only O-glycosylation will be discussed, where the glycosyl acceptor is the hydroxyl group of another carbohydrate or non-carbohydrate (aglycone) moiety. When the glycosyl group is transferred from one glycoside to form another, the process is called transglycosylation (Figure 1).

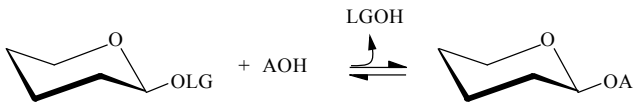

Figure 1 General reaction scheme for transglycosylation of a hexose, where A = Acceptor and LG = Leaving group.

Glycosylation is an important source of structural diversity of natural products. It can alter the properties of compounds in a multitude of ways. Deglycosylating narginine in fruit juices or wine leads to a reduction of bitterness [5, 6]. Addition of a glucose residue to flavonoids greatly increases their water solubility [3]. Glycosylation also play a role in stability, e.g. for the vitamin L-ascorbic acid, were the glycosylated form is significantly more stable [4]. Moreover, glycosylation can have a major effect on skin irritation. Glycosylating the traditional skin lightening agent hydroquinone reduces the cytotoxicity while maintaining the whitening effect [7]. Glycosylation can additionally be used to produce attractive biosurfactants, alkyl glycosides [8], discussed in detail in chapter 5.

2.1 Chemical glycosylation

Understanding the potential of enzymatic glycosylation requires background in conventional carbohydrate chemistry. The earliest known glycosylation method was described by Koenigs and Knorr [9]. This reaction utilizes glycosyl halides, formed through activation with silver or mercury ions, as glycosyl donors. Since 1901, a number of alternative activated glycosyl donors have been described, such as glycosyl trichloroacetimidates [10] or thioglycosides [11]. All these reaction

suffer from the same major limitations, related to the regioselectivity and anomeric configuration of the products.

To enable regioselective glycosylation using conventional chemistry, protection strategies are necessary. A commonly used method is protection with acetic anhydride, removed with sodium methoxide after the glycosylation reaction (Figure 2). Moreover, the anomeric configuration (explained in chapter 3.1.2) of the product is largely governed by the nature of the substituent in position C2. When the C2 oxygen is protected with an alkyl or benzyl group, the α-anomer is preferentially formed. In contrast, a participating group, such as an acetyl group, in the C2 position will result in the anomer opposite to the stereochemistry of the C2 substituent [12].

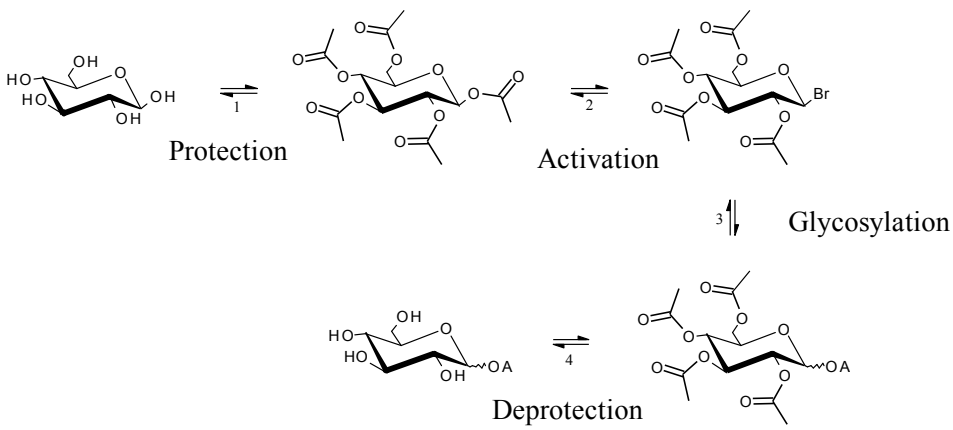

Figure 2 Reaction scheme for regioselective glycosylation using traditional chemistry. 1) protection with acetic anhydride 2) activation of target hydroxyl group with hydrogen bromide 3) glycosylation using silver or mercury salt catalysts 4) deprotection using sodium metoxide. A = Acceptor.

Bearing the above-mentioned complications in mind, enzymatic glycosylation is an attractive alternative. The high stereo and regio-selectivity of enzymes enables single step glycosylation. In addition, enzymatic catalysis avoids the toxic catalysts commonly used in chemical glycosylation and generates less waste, thereby constituting a more environmentally sustainable option [13].

2.2 Enzymatic glycosylation

There are four types of enzymes capable of catalyzing glycosylation: Leloir glycosyl transferases (GT), glycoside phosphorylases (GP), glycoside hydrolases (GH) and transglycosylases (TG). Each group has its own advantages and disadvantages, discussed below and more extensively in a recent review [14].

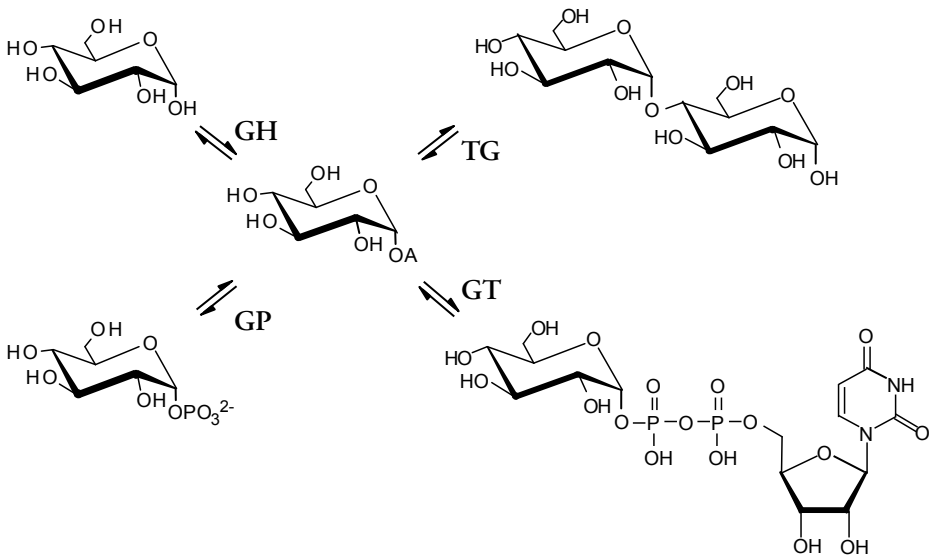

Figure 3 Glycosylation reactions catalyzed by the four types of enzymes; glycoside hydrolases (GH), transglycosylases (TG), glycoside phosphorylases (GP) and Leloir glycosyl transferases (GT). A = Acceptor.

2.2.1 Leloir Glycosyltransferases (GT)

The majority of glycosylation reactions in nature are catalyzed by glycosyl transferases (GT). Most GTs employ nucleotide activated sugars as glycosyl donors and are also called Leloir transferases, after the Nobel laureate who discovered the first sugar nucleotide [15]. They can reach quantitative yields with high selectivity, and exist with a wide range of donor and acceptor specificity. Although very efficient, industrial implementation is limited due to the high price of nucleotide-activated donors. In addition, the catalysts themselves are difficult to work with, as many of them are membrane bound and difficult to express. Enzymes that catalyze mainly transglycosylation and does not use nucleotide activated donors are called non-Leloir glycosyl transferases, and include glycoside phosphorylases (chapter 2.2.2) and transglycosylases (chapter 2.2.4).

2.2.2 Glycoside phosphorylases (GP)

Similar to GT, glycoside phosphorylases (GP) also use activated sugars as glycosyl donors. However, their natural function is not glycosylation, but degradation of the glycosidic linkage in di- and oligosaccharides. They use inorganic phosphate as glycosyl acceptor, resulting in glycosyl phosphate and a saccharide of reduced length [16]. The high energy of the glycosyl phosphate allows the reaction to be reversed and used for synthetic purposes. As for GT, the application of glycoside phosphorylases is limited by the high cost of the glycosyl donor. Although, not only is glycosyl phosphates cheaper than nucleotide activated sugars, but retaining (explained in chapter 3.1.3) GPs can also use sucrose as glycosyl donors, which is significantly cheaper. The acceptor specificity of GP include a wide array of saccharides as well as non-carbohydrate acceptors [17-19] while the donor specificity limits the glycosylation to addition of a glucosyl group, with a few exceptions [20, 21]. Another disadvantage is that GP, in contrast to GT, suffer from a minor hydrolytic side reaction, reducing the yield of the glycosylation.

2.2.3 Glycoside hydrolases (GH)

Glycoside hydrolases (GH) are attractive enzymes because they are readily available, cheap, robust and have a wide range of donor and acceptor specificities [22]. However, analogous to GP, GHs are *in vivo* carbohydrate degrading enzymes. They use water as glycosyl acceptor, resulting in monosaccharides as products. Although monosaccharides are not as activated as glycosyl phosphate, they can be used for glycosylation under certain conditions. This process is called reverse hydrolysis, and work through shifting the thermodynamic equilibrium [8]. An equilibrium beneficial for glycosylation can be achieved through using high substrate concentrations and limiting the amount of water in the reaction, discussed further in chapter 5.3.1. Alternatively, a kinetically controlled transglycosylation reaction can be used, similar to the use of sucrose for GP. This type of reaction is the focus of **Paper I-III**, discussed in chapter 6. Apart from their high hydrolytic activity, glycoside hydrolases often have low regioselectivity, which leads to a mixture of products when the acceptor contains more than one hydroxyl group.

2.2.4 Transglycosylases (TG)

Transglycosylases are retaining glycosidases devoid of their hydrolytic ability, making them ideal for glycosylation. Their major disadvantage is the small number of available specificities, limited to transfer of α-glucosyl or β-fructosyl

groups [23]. One type of TG is cyclomaltodextrin glucanotransferase, which is the focus of **Paper IV-V**, discussed in chapter 7. They belong to GH family 13, highlighting the vague distinction between TG and GH.

2.2.5 Towards efficient tools for glycosylation

The development of efficient tools for glycosylation is attempted from each of the four types of glycosylating enzymes by the research community.

The limitations of GT can be alleviated to a certain extent. The discovery of microbial GTs, efficiently expressed in *E. coli* has improved GT availability [24]. Moreover, the issue of expensive glycosyl donors can be addressed by employing a regeneration system using e.g. sucrose synthase with sucrose as co-substrate [25].

An important development for the use of GH in glycosylation is the development of glycosynthases, first established by Withers [26] and Planas [27] and recently reviewed by Cobucci-Ponzano *et al*. [28]. They are glycosidases where the catalytic nucleophile has been removed by mutagenesis, and instead use activated glycosyl fluoride substrates that allow glycosylation, but not hydrolysis. Although widely applicable, glycosynthases still rely on costly activated glycosyl donors.

Another approach to improve the synthetic use of GH is to limit the hydrolytic reaction and transform them into TG. This approach is extensively discussed in chapter 6, **Papers I-III** and in a recent review by Bissaro *et al.* [29].

For GP, the approach of limiting hydrolysis is also applicable, and has been successfully employed for commercial production of 2-O-(α-D-glucopyranosyl)-sn-glycerol (Glycoin) [30]. The remaining challenge for GP, and for TG, is the extension of substrate specificity, allowing their general use as tools for glycosylation.

3 Glycoside hydrolases

Glycoside hydrolases (or glycosidases) are enzymes that hydrolyze the glycosidic bond that builds up carbohydrates. Since the glycosidic bond is one of the most stable bonds in nature, with a half-live of millions of years [31], glycoside hydrolases are one of the most efficient catalysts in existence, with rate enhancements in the order of 10^{17}-fold [32].

3.1 Classification

Currently there are almost 7000 characterized glycoside hydrolases (http://www.cazy.org/, [33]). Therefore, there is a great need to classify them into different groups. This can be done in a multitude of ways, as detailed below.

3.1.1 Sequence based classification

The most useful classification of glycoside hydrolases was introduced by Bernard Henrissat [34]. It divides all glycoside hydrolases into families, based on amino acid sequence similarity. There are currently 134 glycoside hydrolase (GH) families in the ever-expanding CAZy database (http://www.cazy.org, [33]). In this dissertation, enzymes from GH1 (**Paper I-III**), GH3 (**Paper II**) and GH13 (**Paper IV-V**) will be discussed. Since protein structure is more conserved than amino acid sequence, many of the GH families will have the same fold. Therefore, they have been divided into 14 different clans based on protein fold, discussed in chapter 3.2 [35].

3.1.2 Anomeric configuration

Another way to describe GH is based on the anomeric configuration of their substrates. In carbohydrate chemistry, the anomeric carbon is the hemiacetal (or hemiketal) carbon of a cyclic saccharide. The anomeric configuration is designated according to the configurational relationship between the anomeric centre and the anomeric reference atom (the stereocenter furthest away from the anomeric

centre). If the absolute configuration of the anomeric carbon and the reference atom is the same (R,R or S,S) the saccharide is the α-anomer, and if the absolute configuration is different (R,S or S,R) it is the β-anomer, exemplified in Figure 4 [36]. Glycoside hydrolases act on either α- or β- bonds, rarely both.

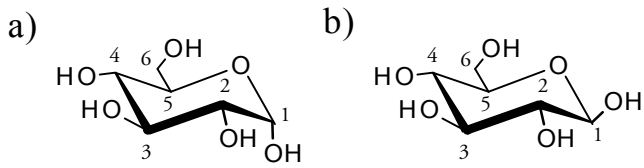

Figure 4 Glucose in two anomeric forms. a) α-D-Glucose, with C1 and C5 both in S configuration. b) β-D-Glucose, with C1 in R configuration and C5 in S configuration.

3.1.3 Mechanism based classification

Glycoside hydrolases can act through a variety of mechanisms, the most common of which are the retaining and inverting mechanism. The names refer to whether the anomeric configuration is retained or inverted during the course of the reaction [37]. The inverting mechanism is carried out in one-step with involvement of two amino acids, a general acid and a general base (Figure 5a). In contrast, the retaining mechanism is carried out in two steps (Figure 5b). First, a nucleophilic attack on the anomeric carbon breaks the glycosidic bond with help of the catalytic acid, which donates a hydrogen atom, enabling the release of the first substrate. Consequently, a covalent glycosyl enzyme intermediate of opposite anomeric configuration is formed. In the second step, an acceptor molecule, deprotonated by the catalytic acid, attacks the covalently bound anomeric carbon and releases the second product. The retaining mechanism is discussed in more detail in chapter 6.1 and more on the mechanism of glycoside hydrolases can be found in several excellent reviews [38-40].

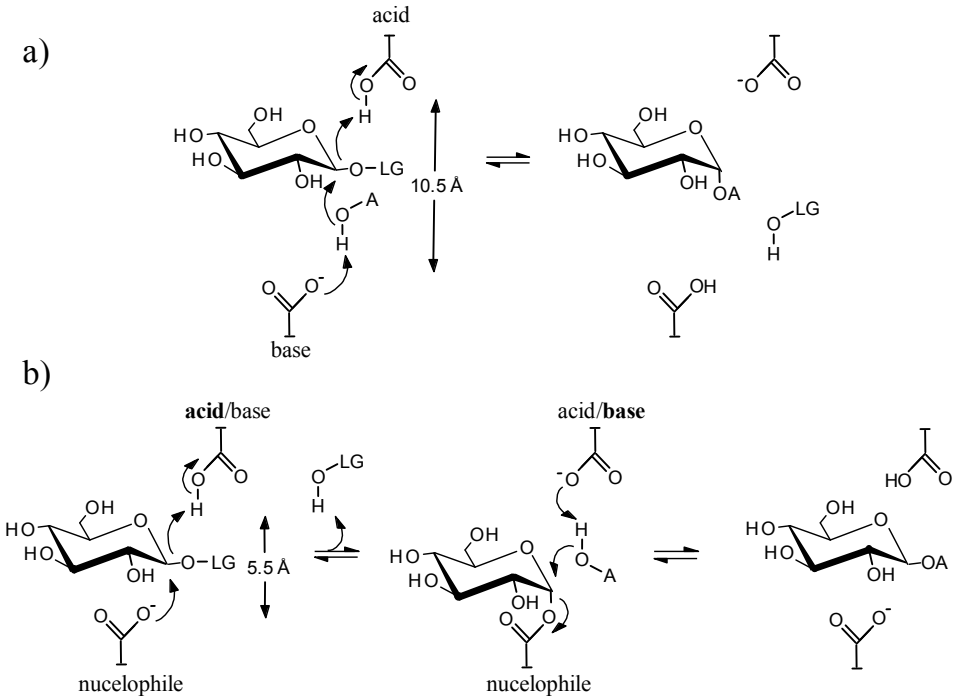

Figure 5 Mechanism of a) an inverting *β*-glucosidase and b) a retaining *β*-glucosidase.

3.1.4 Reaction based classification

Another way to classify GH, common for all enzymes, is the use of Enzyme Commission (EC) numbers [41]. This system is based on the reactions catalyzed. Judging from the name, glycoside hydrolases should all belong to the EC 3.2, hydrolases acting on sugars. However, many GH can also catalyze the transfer of a glycosyl unit, which makes them members of EC 2.4.

3.1.5 Endo or exo acting enzymes

In addition to the classifications above, GHs are either exo- or endo- acting. This refers to their ability to cleave a substrate at the end of the chain or in the middle of it. The structure of the active site cleft will determine which, as discussed in Chapter 3.2.

3.1.6 Enzymes discussed in this dissertation

Throughout this dissertation, the enzymes used will be named according to the convention proposed by Henrissat *et al*. [42]. The first letters, in italic, specificity the organism from which the enzyme originates, followed by three letters signifying the substrate it acts on, what GH family it belongs to and finally a capital letter, to separate multiple occurrences of the same name. Two *β*-glucosidases, *Tn*Bgl1A and *Tn*Bgl3B, along with three cyclodextrin glucanotransferases, *Csp*CGT13, and two commercial enzymes will be treated in this dissertation. The *β*-glucosidases are exo acting and the CGTases are endo acting. All enzymes are retaining, and display both hydrolytic (EC 3.2.1.x) and transglycosylation activity (EC 2.4.x.x).

3.2 Structure

Despite the 134 GH families and 14 structural clans, the overall active site topology can be divided into only three groups; pocket, cleft or tunnel topology. The pocket topology (Figure 6a) is common in enzymes active on small substrates and for exo-acting enzymes, while the cleft and tunnel topologies (Figure 6b and 6c) are found in endo-active enzymes active on polymeric substrates [43]. The tunnel topology (Figure 6c) allows retention of the substrate while releasing the product, called processivity. The *β*-glucosidases in this dissertation have a pocket topology, while the cyclodextrin glucanotransferases have a cleft topology. The structural background relevant for understanding the action of the enzymes in this dissertation is outlined in the following section.

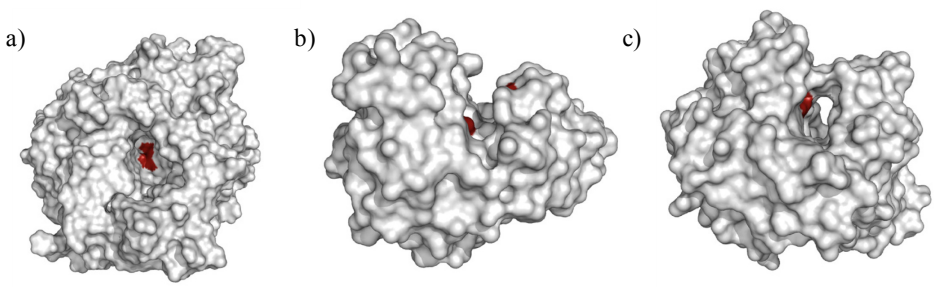

Figure 6 The different active site topology of glycoside hydrolases; a) pocket illustrated by *β*-glucosidase from *Thermotoga neapolitana* (unpublished) b) cleft illustrated by endoglucanase from *Thermonospora fusca* (PDB: 2BOE) c) tunnel illustrated by cellobiohydrolase Cel6A from *Trichoderma reesei* (PDB: 4I5U). The catalytic residues are colored red.

3.2.1 Catalytic residues

The catalytic site of both inverting and retaining glycosidases is built up of carboxylic amino acids (aspartic or glutamic acid). For inverting glycosidases the general acid and the general base (Figure 5a) are separated by 8 ± 2 Å, while for retaining glycosidases the nucleophile and the acid/base (Figure 5b) are separated by 4.8 ± 0.3 Å or 6.4 ± 0.6 Å [44]. The longer distance for inverting glycosidases allows room for both the substrate and a water molecule that will act as nucleophile, visualized in Figure 5. In addition to a nucleophile and acid/base, CGTases also have a third catalytic residue, an aspartic acid involved in stabilization of the transition state [45].

3.2.2 Enzyme fold

GH1 enzymes, such as *Tn*Bgl1A, belong to clan GH-A and have a classical $(\alpha/\beta)_8$ TIM barrel fold, as depicted in Figure 7a. The catalytic residues are both found in the same domain, but separated in the primary structure by roughly 200 aa [46]. In contrast, enzymes in GH3, such as *Tn*Bgl3B (Figure 7b), are multi-domain enzymes where the two catalytic residues can be in different domains. All GH3 enzymes have an $(\alpha/\beta)_6$ domain in addition to the $(\alpha/\beta)_8$ found in GH1. Apart from the two catalytic domains, *Tn*Bgl3B contains a third domain, which is likely involved in stabilizing the enzyme [47]. CGTases (Figure 7c) are also multi-domain proteins, where one domain has a TIM barrel fold. Commonly, one of the other domains is a C-terminal β-sandwich domain and the other is inserted in the sequence of the barrel and has an irregular structure [48].

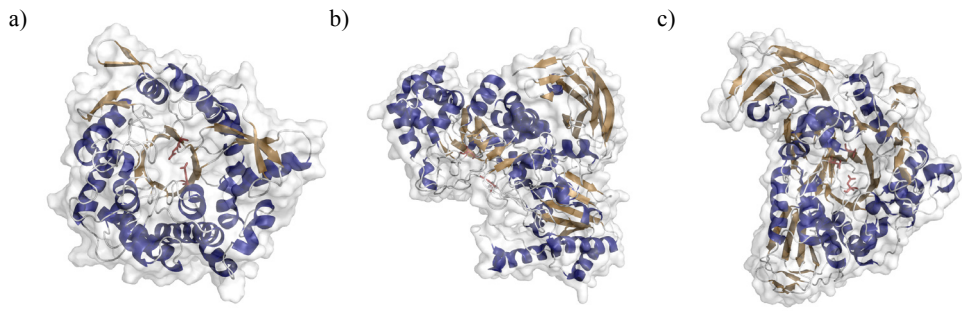

Figure 7 Structure of a) *Tn*Bgl1A b) *Tn*Bgl3B and model of c) *Csp*CGT13 with α-helices in blue and β-sheets in brown and catalytic residues in red.

3.2.3 Active site nomenclature

Given that glycoside hydrolases are active on oligomeric substrates, substrate binding can take place over a large surface. To facilitate the discussion on substrate binding, an active site nomenclature was introduced by Davies *et al.* [49]. Each monomer-binding site is given a number, starting from the bond that is to be cleaved (scissile bond). The subsites towards the reducing end of the substrate are given positive numbers and the subsites towards the non-reducing end are given negative numbers. In this dissertation, the negative subsites will be referred to as the glycone subsites and the positive as aglycone subsites. As seen in Figure 8, the β-glycosidases in this dissertation have three subsites, while the CGTase has nine.

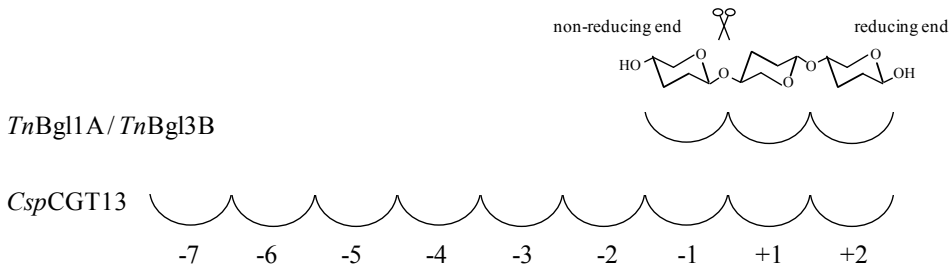

Figure 8 Binding sites for β-glycosidases *Tn*Bgl1A and *Tn*Bgl3B as well as CGTase *Csp*CGT13.

4 Biocatalysis in organic media

An important concept for understanding the work presented in this dissertation is biocatalysis in organic media, which will be discussed in the following chapter. Enzymatic catalysis is normally conducted in aqueous media (water), since that is their natural environment. However, aqueous media has several disadvantages when it comes to industrial bioconversion. It is susceptible to bacterial contamination, many potential substrates and products have poor water solubility, the thermodynamic equilibrium of several processes are unfavorable in water, product recovery is often challenging and water often give rise to unwanted side reactions such as hydrolysis in the case of glycosidase catalyzed transglycosylation.

4.1 Enzyme stability in organic media

A key driving force in protein folding is minimizing the exposure of their hydrophobic core to the hydrophilic solvent, water [50]. Therefore, it was long assumed that enzymes could not function in organic solvents, since they would denature in a hydrophobic solvent. However, this notion was based upon extrapolation from studies in aqueous–organic mixtures, assuming that if enzymes denature in mixtures they would surely denature in neat solvents. This belief was later disproven, first by Price and coworkers [51-53] and later in the pioneering work of Klibanov [54, 55].

The answer to why this is possible lies in the property of water as molecular lubricant, evident by both molecular dynamics simulations and physical measurements [56-58]. In the absence of water, enzymes are very rigid, and thereby kinetically locked in their prior conformation [59]. Not only does this mean enzymes can survive in neat organic solvents, but also leads to increased thermostability in such media [54, 55, 60].

4.2 Enzyme activity in organic media

In general, the catalytic activity displayed by enzymes in neat organic solvents is far lower than in water [61]. The reason for this observation can be one of several factors. Firstly, enzymes are not soluble in organic solvents, and therefore tend to aggregate, which can lead to diffusion limitations or blockage of the active site of the enzymes. This can be alleviated by immobilization on a support with a large surface area [62, 63]. Secondly, a common method to prepare enzymes for use in organic media is lyophilization, which can cause structural perturbations to the enzyme. An effective strategy to limit the damage to the enzyme is to use lyoprotectants such as sugars [64], polyethylene glycol [65] or crown-ethers [66] during dehydration. A third reason for low activity in organic solvents is suboptimal pH, discussed further in section 4.4. Lastly, reduced conformational mobility is a major limitation, which cannot be easily mitigated. The effects can be reduced by 'water mimics' such as glycols [67], formamide [68] and methanol [69] or through choosing a more hydrophobic solvent that is less prone to stripping the enzyme of water [70]. Alternatively, one can circumvent the problem by using higher hydration states.

However, the initial high activity of enzymes in neat solvents is diminished after prolonged exposure. Castillo *et al*. [71, 72] has studied this phenomenon extensively and excluded structural changes, flexibility, hydration and changes to the ionization state as potential causes. Later studies indicate that minor perturbations to active site residues are responsible for the effect [73].

4.3 Thermodynamic water activity (a_w)

The term "neat organic solvent" used in the sections above can be misleading. Most organic solvents used for enzymatic catalysis contains a certain amount of water. Instead of quantifying the amount of water using vol% or mol fraction, the thermodynamic water activity (a_w) is preferred, for several reasons. Firstly, the water activity will be the same in all phases present at equilibrium, which is convenient for two-phase systems and when using solid enzyme support. In addition, enzyme activity is comparable at the same a_w in different solvents, as demonstrated by Valivety and coworkers [74]. The same a_w signify vastly different mol fractions of water, as illustrated in Figure 9, but signifies the same amount of water accessible for the enzyme, affecting the flexibility and thereby the activity.

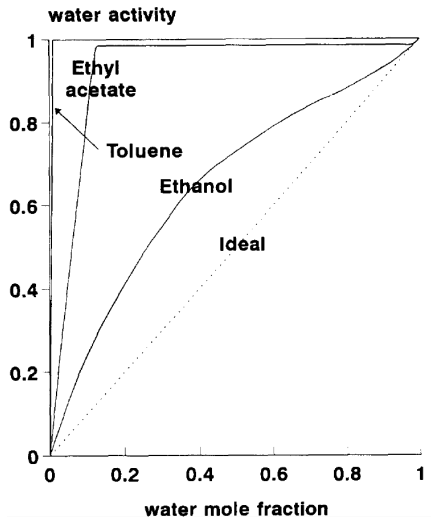

Figure 9 Water activity in aqueous-organic mixtures as a function of composition. Reprinted, with permission, from [75].

Setting the water activity of your solvent is not a trivial task. Several methods can be used to calculate the amount of water to add to reach a certain water activity from dry organic solvents, the most popular of which is the AIOMFAC model (http://www.aiomfac.caltech.edu, [76]). Knowing how much water corresponds to a certain water activity allows setting the water activity through first completely drying the solvent with molecular sieves or through distillation. Another method to set the water activity, first used by Cauwenbergh *et al.*, uses saturated salt solutions for construction of a robust binary system with a precise water activity [77]. A selection of salts and the water activity obtained in saturated solutions of them can be found in Table I.

Table I Water activity of saturated solutions of salts versus temperature [78].

	20°C	25°C
Sodium Hydroxide	0.089 ± 0.024	0.082 ± 0.021
Magnesium Chloride	0.331 ± 0.002	0.328 ± 0.002
Magnesium Nitrate	0.544 ± 0.002	0.529 ± 0.002
Sodium Chloride	0.755 ± 0.001	0.753 ± 0.001
Potassium Chloride	0.851 ± 0.003	0.843 ± 0.003
Potassium Sulfate	0.976 ± 0.005	0.973 ± 0.005

There is a variety of methods to determine the water activity in the gas phase above the binary systems of saturated salt solutions. These include dew point measurements, direct vapor pressure measurements and use of a calibrated

humidity sensor [78]. Conversely, there is no way of directly measuring the water activity in a liquid. Therefore, a common method is to pre-equilibrate solvent in closed chambers containing a series of saturated salt solutions and measure the concentration of water corresponding to each water activity, using e.g. Karl-Fisher titration, as done in **Paper II**.

However, the above-mentioned methods only account for the initial stages of the reaction. Especially for hydrolases, where water is a substrate or product (depending on the direction of the reaction), water activity will change during the reaction. A method to keep a constant a_w was developed by Petersson *et al.* [79]. It uses a flow of air that is either hydrated by water or dried by silica, controlled by a vapor pressure sensor connected to a computer. A simpler method is to use solid salt-hydrates in your reactions, to act as a buffer system [80].

4.4 Hysteresis effects

Not only is enzyme activity dependent on the hydration of the solvent it resides in. It is also dependent on its history of hydration [81]. A catalyst that has been completely dry is generally slower than one that has been carefully equilibrated to the intended water activity from a fully hydrated state. This can be explained by tightly bound waters that will eventually desorb from the enzyme upon extended storage under dry conditions. One gentle drying procedure is to rinse the enzyme preparation repeatedly in a solvent pre-equilibrated to the desired hydration state [82]. This procedure showed promising results, further discussed in **Paper II**.

Another hysteresis effect is that of pH. In aqueous media, pH is one of the major factors influencing enzymatic activity. However, in organic media, pH has no meaning, since there are no Brønsted acids or bases present. Therefore, the ionization state of the enzyme will reflect the pH of the last aqueous solution to which it was exposed [54].

4.5 How much water is needed for activity?

Early work by Careri and coworkers gave the conclusion that a monolayer of water, corresponding to a circa 0.4 g water / g enzyme, is required to fully hydrate proteins [81, 83]. However, a study comparing sorption isotherms of subtilisin Carlsberg in non-polar solvents and air showed that adsorption of loosely bound waters is significantly reduced in nonpolar solvents [84]. This indicates that hydrophobic regions of the protein surface are not covered by water in organic solvents, and that a complete monolayer of water would be thermodynamically

unfavorable in organic media. This result was later supported by molecular dynamics simulations [85, 86]. Instead, at high hydration the thickness of the water network formed around the hydrophilic parts of the protein surface is increased, as depicted in Figure 10.

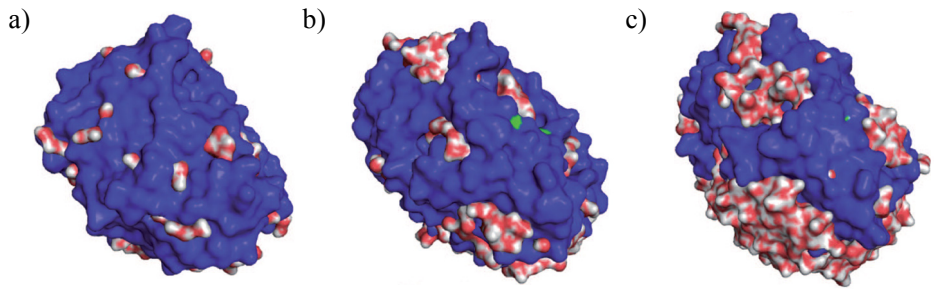

Figure 10 MD simulation snapshot of *Candida antarctica* lipase B at water acitivies a) 0.26 b) 0.49 and c) 0.75. Reprinted, with permission, from [85].

Nevertheless, enzymatic activity can be observed at significantly lower hydration states. The level of hydration required varies between classes of enzymes (Figure 11a). Lipases can handle low hydration conditions, while glycosidases require a large amount of water for their function. Generally, an exponential increase in activity is seen with increased water activity. However, as seen in the comparison of different lipases presented in Figure 11b, some enzymes display maximum activity at hydrations below water saturation. This can be due to water acting as an inhibitor or the interplay between flexibility and stability. Maximum activity is achieved at sufficient hydration for enzyme flexibility while not inducing denaturation. Soares and coworkers hypothesize that unfolding is induced when the water clusters around the hydrophilic parts of the protein are large enough to connect charged groups. Having a medium that allows formation of ion-pairs will make charged groups move to keep themselves neutral and thereby induce structural changes to the protein [58].

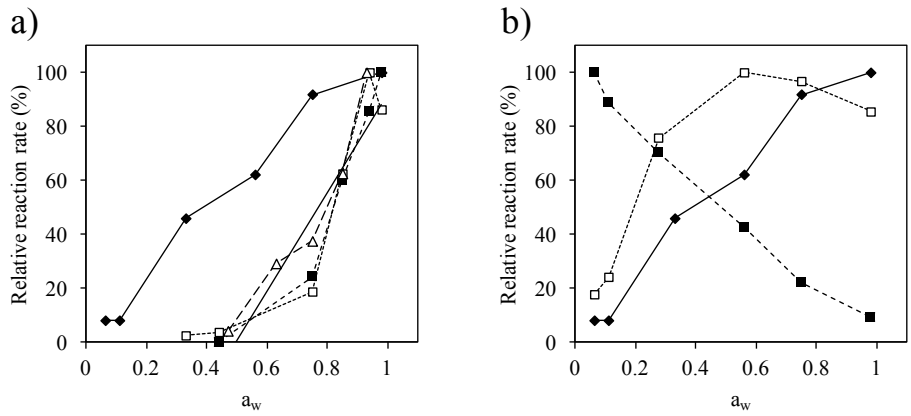

Figure 11 The effect of water activitiy on the catalytic activity of a) lipase from *Pseudomonas sp.* on EP-100 in diisopropyl ether (♦)[87], *α*-chymotrypsin from bovine pancrease on celite in diisopropyl ether (□), alcohol dehydrogenase from horse liver on celite in diisopropyl ether (■) [88] and *β*-glucosidase from almond in hexanol (Δ) [89] b) lipase from *Pseudomonas* sp. on EP-100 in diisopropyl ether (♦)[87], *Candida rugosa* (□) and *Rhizopus oryzae* (■) on EP-100 in diisopropyl ether [90].

4.6 Biphasic systems

When using solvents with low water miscibility, one encounters additional complexities such as substrate and product partitioning and surface inactivation due to interfacial tension. However, no inactivation was observed for the *β*-glycosidases from *Thermotoga neapolitana* studied in this dissertation, in agreement with the observation that enzymes from thermophilic sources are more resistant to bi-phasic aqueous-organic media [91]. Partitioning of alkyl glycosides in biphasic systems was studied in detail by Panintrareux and coworkers [92]. They found that the partitioning coefficient, defined as the concentration in the alcohol phase divided by the concentration in aqueous phase at equilibrium, increases with increasing length of the alkyl chain and their corresponding alcohol. As their studies were conducted at 60°C and most experiments in this dissertation are performed at 70°C, experimental apparent partitioning coefficients for alkyl glycoside, p-nitrophenyl-*β*-D-glucoside (pNPG) and p-nitrophenol (pNP) were determined at 70°C, visualized in Figure 12 (**Paper I**).

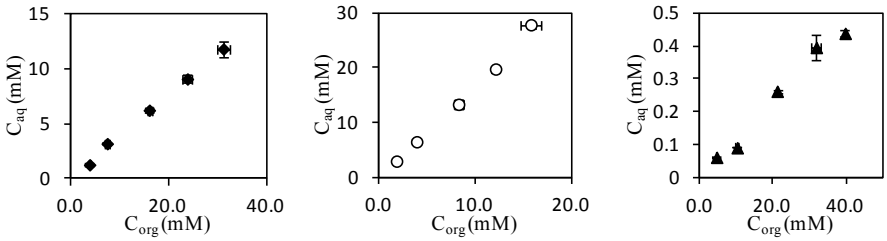

Figure 12 Apparent partitioning of hexyl-*β*-glucoside (♦), p-nitrophenyl-*β*-D-glucoside (○) and p-nitrophenol (▲) plotted as concentration in the aqueous phase (C_{aq}) versus concentration in the hexanol phase (C_{org}). Error bars are 1σ (**Paper I**).

5 Synthesis of alkyl glycosides

Surfactants are among the most commonly produced chemicals today, with a global marked estimated to reach 30 million metric tons in 2030 [93]. In this dissertation, one particular type of surfactant is dealt with, alkyl glycosides.

5.1 Properties and use of alkyl glycosides

Alkyl glycosides are surfactants consisting of a hydrophobic alkyl chain and a hydrophilic glycone group. Varying the length of the alkyl chain as well as the number of glycone residues will affect the properties of the surfactant, making alkyl glycosides a diverse group of surfactants. Important properties for surfactants are critical micelle concentration, surface tension, antimicrobial activity and biodegradability, discussed below along with the current uses of alkyl glycoside surfactants.

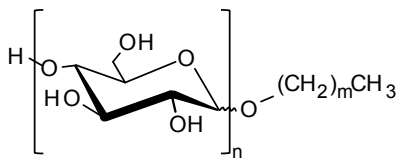

Figure 13 General structure of alkyl glucosides, where n is the number of glucose residues and m+1 is the length of the alkyl chain.

5.1.1 Critical micelle concentration

The critical micelle concentration (CMC) is the concentration at which surfactants aggregate into micelles. Micelles are spherical or cylindrical structures formed through the entropically driven process of minimizing the exposure of the hydrophobic part to a hydrophilic solvent, or *vice versa*. At this concentration, a multitude of other physiochemical properties of the surfactant solution change, such as turbidity, osmotic pressure and surface tension. For surfactants used in washing formulations, a low CMC is desirable, since less surfactant is needed to form surfactants capable of solubilizing the stain or contaminant. For alkyl

glycosides, the CMC decreases with increasing length of the alkyl part, as visualized in Figure 14 [94].

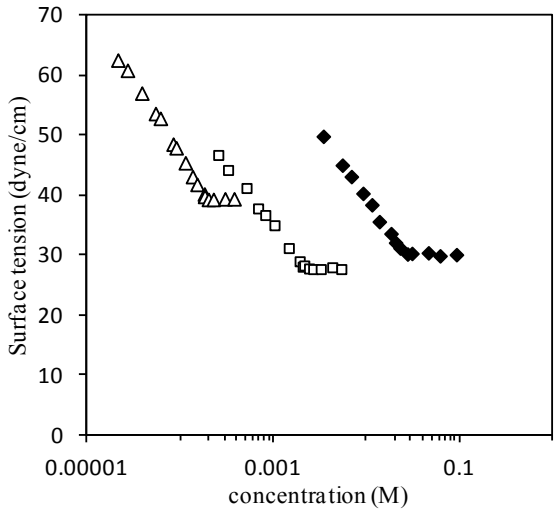

Figure 14 Surface tension versus concentration of alkyl-*β*-D-glucosides at 25 °C; octyl glucoside (♦), decyl glucoside (□) and dodecyl glucoside (Δ) [94].

5.1.2 Alkyl glycosides in detergents and cleaners

Alkyl glycosides have high washing performance over a broad pH range. This makes them applicable both in slightly acidic all-purpose cleaners that are mild for human skin as well as in highly alkaline dishwasher formulations [95]. In all-purpose cleaners, alkyl glycosides also contribute to foam volume and foam stability [96]. Although these properties are not linked to the product performance, they are expected by the consumer and therefore important for purchasing decisions. Moreover, in liquid laundry detergents, alkyl glycosides have been shown to increase the storage stability of the enzymes used therein; cellulases, lipases and proteases [95].

5.1.3 Alkyl glycosides in personal care, biotechnology and pharma

For personal care products, the foaming properties of alkyl glycosides are again important. In addition, surfactant mildness is crucial for personal care products. The mildness of alkyl glycosides is demonstrated e.g. in shampoo, where they reduce the tensile strength of hair less than other surfactants [97]. Another valuable property of alkyl glycosides is their anti-microbial activity, allowing

reduced concentrations of antimicrobial agents in formulations. This property is more pronounced in surfactants with longer alkyl chains [98].

Furthermore, alkyl glycosides are applied in pharmaceutical preparations for improving the bio-availability of a therapeutic agent [99] and in biotechnology research, mainly for solubilizing and stabilizing membrane proteins [100]. Regardless of the intended use, the biodegradability of alkyl glycosides is also beneficial, hindering accumulation and subsequent effects on aquatic life [98].

5.2 Chemical synthesis of alkyl glycosides

Alkyl glycosides were first synthesized by Emil Fischer, more than 120 years ago [101]. He used acid catalysis to react an alcohol with a monosaccharide to form alkyl glycosides and water. Several alternative methods have been developed since then, and has been reviewed by Rybinski and Hill [95].

In industry, Fisher glycosylation is still the preferred route for production of alkyl glycosides, or alkyl polyglycosides (APG) as they are called when produced in technical grade. The production can be done in either a one-step (direct synthesis) or a two-step process (transacetalization). The two-step process uses a shorter alcohol in the first reaction, followed by a transferase reaction with a larger alcohol in the second step. The two-step process is mainly used when polymeric carbohydrates are used as substrates. Both methods are performed at high temperatures and under vacuum, to shift the equilibrium. They are followed by neutralization, distillation of the unreacted alcohol and bleaching before the products are sold as APGs. Although the average degree of polymerization can be adjusted through the ratio of carbohydrate to alcohol, the products will be diverse with regard to number of monosaccharide units and anomeric configuration. Defined alkyl glycosides can be produced using protection chemistry, as discussed in chapter 2.1, or alternatively through enzymatic synthesis.

5.3 Enzymatic synthesis of alkyl glycosides

There are two main routes for enzymatic synthesis of alkyl glycosides, reverse hydrolysis and transglycosylation, presented in detail below. Both are preferably catalyzed by glycosidases, discussed in chapter 3.

5.3.1 Reverse hydrolysis using glycosidases

In aqueous media, glycosidases are hydrolyzing enzymes, catalyzing the breakage of a glycosidic bond (Equation 1). However, glycosidases can also catalyze the reverse reaction, condensation, when the equilibrium is shifted.

$$GlcOH + ROH \rightleftharpoons GlcOR + H_2O \quad (1)$$

$$K = \frac{C_{GlcOR}\, C_{H_2O}}{C_{Glc} C_{ROH}} \quad (2)$$

The yield of alkyl glycosides is determined by the thermodynamic equilibrium. For alkyl glucoside formation with C6 to C12 alcohols, the equilibrium constant, K (Equation 2), has been determined to 1.9, regardless of the length of the alcohol [92]. The equilibrium can be shifted by increasing the concentration of glucose or alcohol, or reducing the concentration of water. However, this is not as trivial as it might seem. First, glycosidases are not very active at low hydration conditions, as discussed in chapter 4.5; hence reducing the water concentration will affect reaction rates. One way to sidestep this hurdle is to start the reaction at water saturation and reduce the hydration as the reaction proceeds towards equilibrium [102]. Secondly, if running in a two-phase system, the solubility of the acceptor alcohol in the enzyme containing aqueous phase is limited. Lastly, the solubility of glucose in monophasic alcohols is low. High temperatures can be used for increasing the solubility of glucose, but may result in oligomeric sideproducts [92]. Another method is to use co-solvents, e.g. 20 % dimethylformamide, shown to increase the solubility of glucose five-fold [103]. A summary of published yields and reactivities for glycosidase-catalyzed formation of linear alkyl glycosides through condensation is presented in Table II. It is clear that the yields and the productivities are lower for longer alkyl glycosides. However, Marana *et al*. used inhibitor constants to show that the affinity for alkyl glycosides is proportional to the length of the alkyl chain for a GH1 *β*-glycosidase [104]. Therefore, it is likely not the enzyme that limits the productivity for longer alkyl glycosides, but rather the low solubility of long alcohols in the aqueous phase. Moreover, the factor influencing the yield of any particular alkyl glycoside the most was found to be the volumetric ratio of alcohol over aqueous phase, where a larger volume of the acceptor alcohol is beneficial [105]. This is logical since partitioning of the product into the alcohol phase is the driving force to shift the equilibrium towards condensation. The vastly higher productivity of *Tn*Bgl1A suggests that the commercial *β*-glucosidase from almond is a crude preparation with low content of active enzyme.

Table II Yield, productivity and experimental conditions for synthesis of alkyl glycosides using reverse hydrolysis with various alcohol acceptors.

Enzyme	Alcohol	T °C	Water content	Yield %	Productivity mmol g^{-1} h^{-1}	Ref
Almond Bgl	Propanol	60	a_w 0.43	69		[106]
	Butanol	60	10%	44	0.34	[107]
	Hexanol	37	5%	10		[108]
	Hexanol	60	5.6%	18	0.56	[92]
	Hexanol	60	a_w 0.71	36		[106]
	Octanol	60	5.6%	10	0.31	[92]
	Octanol	50	10%	15	0.25	[109]
	Octanol	60	a_w 0.81	13		[106]
	Decanol	60	5.6%	3.3	0.07	[92]
Thermotoga neapolitana Bgl1A	Hexanol	70	15%	15	100	**I**

5.3.2 Transglycosylation using glycosidases

Unlike reverse hydrolysis, transglycosylation is a kinetically controlled reaction, and depends more on the properties of the enzyme than on the reaction conditions or thermodynamic equilibrium. The model reaction used to study transglycosylation in this dissertation is depicted in Figure 15. The activated substrate p-nitrophenyl-*β*-D-glucoside (pNPG) is used as glycosyl donor and the medium chain length alcohol, hexanol, as acceptor. In general, much higher reaction rates and transient yields can be obtained through this pathway, as is apparent when comparing Table II and Table III. It is notable that also for transglycosylation the yields are higher for shorter alcohols [110]. Additional factors governing the yield in transglycosylation reactions is discussed in detail in chapter 6, as well as in **Papers I, II** and **III**. Regarding reaction rates, most studies use crude enzyme preparations, which explain the much higher reaction rates for *Tn*Bgl1A. However, it is still significantly faster than the purified enzyme from *Pyrococcus furiosus.*

Table III, Yield, specificity for transglycosylation (r_s/r_h), estimated yield (η), total reaction rates and experimental conditions for synthesis of hexyl glycosides using the transglycosylation activity of catalysts from different sources.

Enzyme	Donor	[Donor] mM	Water content	T °C	pH	yield	r_s/r_h	η	Total reaction rate μmol min^{-1} mg^{-1}	Ref
Aspergillus aculeatus Bgl	pNPG	20	20%	30	5	27%				[111]
Almond Bgl	pNPG	10	a_w 0.96	40	5	71%		73%		[89]
	pentyl glucoside	40	a_w 1.0	50	5		0.82		0.56	[89]
Caldocellum saccharolyticum Bgl	pentyl glucoside	40	a_w 1.0	50	6.5		1.28		0.17	[89]
	pNPG	10	50%	50	6.5	6%		8%		[89]
Escherichia coli Bgal	pNPGal	10	a_w 0.93	50	7	70%		88%		[89]
	pNPGal		a_w 0.92		5		0.27		0.6	[112]
Pyrococcus furiosus Bgl	pentyl glucoside	40	a_w 0.85	95	5	56%		58%		[113]
	pentyl glucoside	100	a_w 0.64	95	5		1.6		2.4	[113]
	pentyl glucoside	100	a_w 0.92	95	5	57%		62%		[113]
	lactose	93	a_w 0.79	75	5	63%+28%			1.71	[114]
F426Y	pentyl glucoside	100	a_w 0.64	95	5		3.7		2	[113]
	pentyl glucoside	100	a_w 0.92	95	5	68%		80%		[113]
Sulfolobus solfataricus Bgal	lactose	93	a_w 0.79	75	6.5	41%+29%			13.04	[89]
	pentyl glucoside	40	a_w 1.0	50	6.5		2.2		1.68	[89]
	pNPGal	10	a_w 0.85	50	6.5	92%		82%		[114]
Thermotoga neapolitana Bgl1A	pNPG	34	15%	70	5.6		0.2	17%	285	**I**
		34	a_w 0.72	70	5.6		1.03	51%	2.3	**II**
		34	15%	70	10		0.38	28%	168.3	**III**
N220F	pNPG	34	15%	70	5.6		1.38	58%	163	**I**
		34	a_w 0.85	70	5.6		3.16	76%	2.4	**II**
		34	15%	70	10		36	97%	72.3	**III**
Thermotoga neapolitana Bgl3B	pNPG	31	16%	60	5.8	80%	5.1		153	[115]
Pichia etchellsii Bgl	methyl glucoside	100	8%	40		48%				[116]

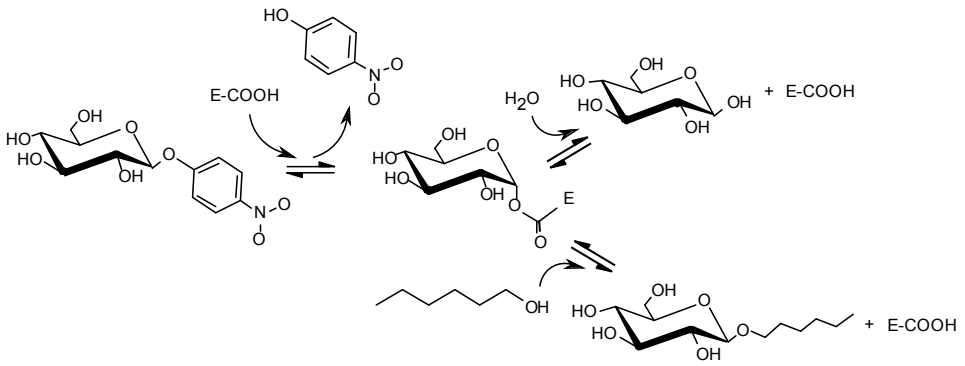

Figure 15 Reaction scheme for the conversion of p-nitrophenyl-*β*-D-glucoside to hexyl-*β*-glucoside (transglycosylation), with conversion to glucose (hydrolysis) as side reaction. When D-glucose is used as the glycosyl donor, formation of hexyl-*β*-glucoside is possible through a condensation reaction (reverse hydrolysis). E-COOH = enzyme.

5.3.3 Transglycosylation using transglycosylases

A few examples exist of alkyl glycoside synthesis using other enzymes than glycosidases. Sucrose-phosphorylase from *Bifidobacteriumn adolescentis* has been used to produce a wide range of linear and aromatic alky glucosides with sucrose as glycosyl donor, although with low donor yields (<20 %) [117]. Another example is using cyclodextrin glucanotransferase from *Paenibacillus* sp, with *β*-CD as glycosyl donor [118]. A range of alkyl glycosides with short alkyl part was produced, also with low yields.

5.4 Modification of alkyl glycosides

Varying the length of the alkyl chain allows customizing the properties of alkyl glycosides only to a limited extent. Reaching the full potential of this type of surfactant requires the possibility to vary the length of the glycoside part as well. This can be achieved using CGTases, as demonstrated by Yoon *et al*. [119]. A wide range of degrees of polymerization was obtained, through interplay of the coupling and disproportionation reactions of CGTases, further discussed in chapter 7. A significant step towards well-defined alkyl glycosides with oligomeric headgroups was taken by Svensson and coworkers, who added 6 glucose units to dodecyl-maltoside in a single step, using only the coupling reaction of cyclodextrin glucanotransferases [120], further studied in **Papers IV** and **V**.

6 Transglycosylation by β-glycosidases

As mentioned in the introduction, the focus of this dissertation is to understand what governs the propensity for transglycosylation or hydrolysis of glycoside hydrolases. This chapter will discuss exactly that, with the help of the background supplied in previous chapters and with a focus on β-glycosidases, while the next chapter will deal with cyclodextrin glucanotransferases.

6.1 Mechanism

As mentioned in chapter 3.1.3, retaining glycosidases function through a double displacement mechanism. In the first step, the catalytic nucleophile attacks the anomeric carbon of the glycosyl donor and forms a glycosyl enzyme intermediate, with the help of the catalytic acid that protonates the glycosidic oxygen (Figure 16). Upon formation of the glycosyl enzyme, the pKa of the catalytic acid is reduced, due to the removal of the electrostatic repulsion from the charged nucleophile. This allows the same residue to act as a general base in the second reaction step [121]. The intermediate is subsequently deglycosylated by either water (hydrolysis) or another hydroxyl containing acceptor (transglycosylation).

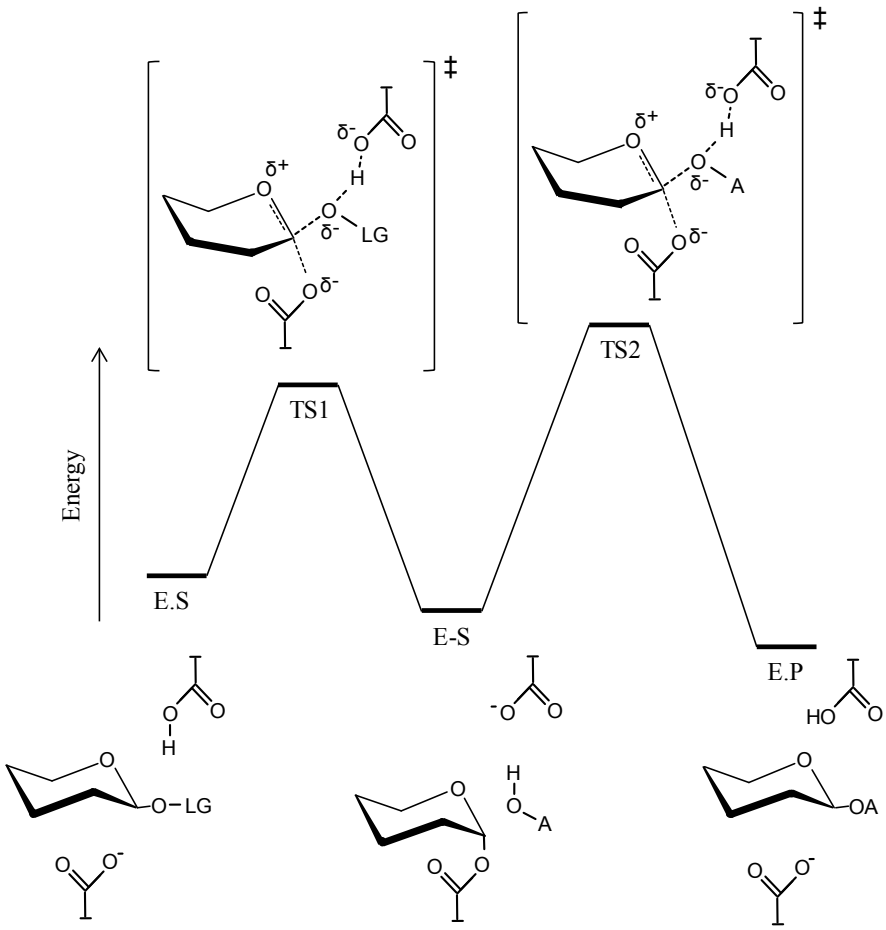

Figure 16 Energy diagram during the double displacement mechanism of retaining glycoside hydrolases.

Both steps go through a oxycarbonium ion-like transition state with a planarity of C5, O5, C1 and C2 in half-chair or boat conformations [122]. Distortion of the pyranose ring is often observed already in the E-S complex, which lowers the activation energy [40, 123]. Although similar, the two transition states are not identical. TS2 is suggested to be more dissociative, that is the glycosyl-enzyme bond is almost broken before the bond to the acceptor is formed [124]. For substrates with good leaving groups (pKa < 8), such as pNPG, deglycosylation is the rate-limiting step while the opposite is true for substrates with poor leaving groups [125].

6.2 Comparison to natural transglycosylases

Transglycosylases are rare in nature. Notable examples include xyloglucan endo-transglycosylases, glucan sucrases and trans-sialidases, discussed in this section, as well as CGTases, which are the focus of the next chapter. Interestingly, they all have homologous hydrolytic enzymes; xyloglucan endo-hydrolases, sucrose hydrolases, hydrolysin sialidases and α-amylases, respectively. Comparing them to their homologous glycosidases could highlight what determines the predominance for hydrolysis or transglycosylation.

A general observation is that transglycosylases are slower than their hydrolytic counterparts. The reason for which is suggested to be a higher energy transition-state, not overcome with water as acceptor [29]. For xyloglucan endo-transglycosylases, structural comparison with their hydrolytic relatives has revealed a difference in loops near the active site [126]. One loop was shown to interfere with the binding in the aglycone subsites of the hydrolytic enzyme, and truncation of the loop increased the ratio of transglycosylation. Further studies revealed that the hydrolytic enzymes have a greater number of hydrogen bonds in the negative subsites, and fewer in the positive subsites aglycone subsite than the transglycosylases [127]. For glucansucrases, there were no structures available until recently, and the first structures have not provided any clear distinction between the hydrolytic and transglycosylating enzymes [128]. More interesting is the comparison between trans-sialidases and their corresponding hydrolases. The active site of a transglycosylase from *Trypanosoma* differs from a hydrolase in the same family by only a few conservative mutations [129]. Molecular dynamics simulations show that the transglycosylase reduces the size of the catalytic cleft upon ligand binding, thereby limiting the exposure to the solvent, while the cleft of the hydrolytic enzyme remains open [130]. There are also transglycosylases in GH1, homologous to β-glucosidases [131]. Unfortunately, no structures are available, making a direct comparison too difficult.

In addition to the above mentioned comparative studies, Larsbrink *et al*. found a potential mechanism for transglycosylases when identifying a novel α-transglucosylase from GH31 [132]. Two water molecules are locked in positions unfavorable for hydrolysis, through a hydrogen-bonding network, depicted in Figure 17.

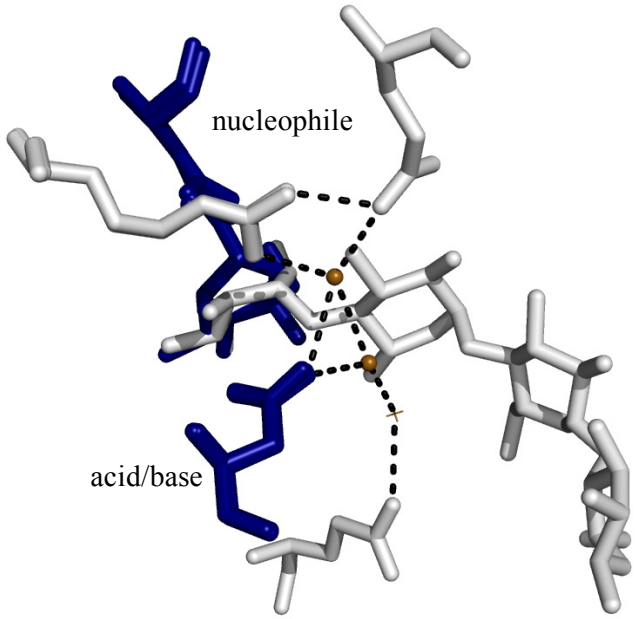

Figure 17 Active site of *α*-transglycosylase from *Cellvibrio japonicus* covalently linked to an inhibitor (PDB: 4B9Z) overlayed with the Michaelis complex with acarbose (PDB: 4BA0). The catalytic residues and the covalent inhibitor are colored blue and the hydrogen bonding network responsible for locking two water molecules (brown) in a position unfavorable for hydrolysis is visualized in black [132].

6.3 Affecting the ratio between transglycosylation and hydrolysis

In order to be useful for glycosylation, the hydrolytic reactivity of *β*-glycosidases needs to be reduced. This can be done in a multitude of ways, presented below.

6.3.1 Mutational studies

Considering that the enzyme used is the main determinant for the rate of transglycosylation and hydrolysis, it is not surprising that protein engineering is the most common approach for increasing the yield in transferase reactions. The following section will summarize the three main hypotheses for improved transglycosylation in *β*-glycosidases; reduced affinity in the glycone subsite, increased affinity in aglycone subsites and affecting the catalytic water.

6.3.1.1 Reduced affinity in negative subsites

Teze *et al.* improved disaccharide yields from 36 % to 82 % through mutations in the -1 subsite [133]. They hypothesized that reducing interactions in the glycone subsite decrease the stabilization of the transition states of the reaction, which affects hydrolysis more than transglycosylation and thereby improves the ratio of transglycosylation. The same effect has been observed in several other mutational studies [134-137].

6.3.1.2 Increased affinity in positive subsites

The second identified mechanism for affecting transglycosylation is modifying the affinity in aglycone subsites. Several studies have highlighted the importance of aromatic residues in the aglycone subsite [138-140]. Moreover, Feng and coworkers reasoned that since deglycosylation is rate limiting, increased affinity of the acceptor would improve transglycosylation, which they also found for a double mutant of a family 1 β-glucosidase [136]. Higher ratios of transglycosylation have also been observed in other mutational studies with improved affinity in aglycone subsites [141-143].

6.3.1.3 Affecting the catalytic water

The third prevalent strategy for promoting transglycosylation in glycoside hydrolases is disrupting the binding of the catalytic water. This has been done by removing a known hydrogen bonding interaction with the catalytic water [144], improving the hydrophobicity of the entrance to the active site [145] or blocking a channel supplying the catalytic water [146], all resulting in reduced hydrolytic activity.

6.3.1.4 Mutational studies of TnBgl1A

The first attempt at improving the synthetic usefulness of *Tn*Bgl1A was aimed at affecting the catalytic water through making the active site more hydrophobic (**Paper I**). Positions in the active site, with previous indications of influence on transglycosylation were targeted. N220 was chosen based on a study on a β-glucosidase from the closely related *Thermus thermophilus*, where the N282T mutation improved transglycosylation, proposedly by affecting N219, corresponding to N220 in *Thermotoga neapolitana* [147]. G222 was targeted due to involvement of the corresponding position, M263, in aglycone specificity for Maize β-glucosidase *Zm*Glu1 [148]. In addition, the main aglycone interacting residue, W322, was studied and one position in the more conserved roof of the aglycone binding site (V168). The kinetic constants of the mutants for hydrolysis and reverse hydrolysis along with the ratio of synthetic activity over hydrolytic activity (r_s/r_h) for transglycosylation are presented in Table IV.

Table IV Kinetic parameters for hydrolysis and reverse hydrolysis along with specificity for transglycosylation of *Tn*Bgl1A and variants thereof (**Paper I**).

	Hydrolysis			Reverse hydrolysis			Transglycosylation
	k_{cat}	K_M	k_{cat}/K_M	k_{cat}	K_M	k_{cat}/K_M	r_s/r_h
Enzyme	(s^{-1})	(mM)	(s^{-1} mM^{-1})	(s^{-1})	(mM)	(s^{-1} μM^{-1})	
*Tn*Bgl1A	154 ± 3	0.45 ± 0.05	342 ± 39	1.43 ± 0.13	219 ± 61	6.5 ± 1.9	0.20 ± 0.03
V168S	175 ± 6	0.20 ± 0.02	875 ± 80	1.00 ± 0.10	199 ± 73	5.0 ± 1.9	0.20 ± 0.02
N220F	628 ± 25	1.28 ± 0.16	491 ± 64	1.33 ± 0.15	357 ± 104	3.7 ± 1.2	1.38 ± 0.20
G222F	164 ± 5	0.77 ± 0.09	213 ± 26	1.48 ± 0.13	245 ± 66	6.1 ± 1.7	0.22 ± 0.03
W322F	74 ± 3	0.17 ± 0.02	452 ± 58	0.26 ± 0.04	219 ± 122	1.2 ± 0.7	0.12 ± 0.08

It is intriguing that the same mutation can influence the reaction in different ways depending on the media and the substrate used. An enzyme variant that improves the catalytic efficiency for hydrolysis of pNPG, such as V168S, can be detrimental for reverse hydrolysis of glucose and hexanol. Moreover, the enzyme variant with the highest k_{cat} for hydrolysis in aqueous environment, N220F, counter-intuitively has the best ratio of transglycosylation in biphasic media, using the same glycosyl donor. Further, unpublished, studies with cellobiose and lactose as donor showed yet another set of effects, where G222F shows improvements and N220F is severely hampered for transglycosylation (Table V).

Table V Specific initial transglycosylation rate with 1200 mM donor in 15:85 water:hexanol.

	Cellobiose (μmol min^{-1} mg^{-1})	Lactose (μmol min^{-1} mg^{-1})
*Tn*Bgl1A	0.17±0.01	0.15±0.00
V168S	0.16±0.01	0.16±0.01
N220F	0.02±0.00	0.02±0.00
G222F	0.18±0.00	0.17±0.00
W322F	0.10±0.01	0.11±0.00

Notwithstanding the differences observed with varying acceptors, the large shift towards favoring transglycosylation seen for the N220F mutant warranted further investigation. A reduced site saturation library for position N220 was generated, and a few active mutants were characterized (**Paper III**). Two additional mutants showed large improvements in r_s/r_h, N220Y and N220R. Considering arginine is an amino acid with a hydrophilic side group, the reason cannot be simply increasing the hydrophobicity of the active site. Therefore, effects on hydration

behavior, pH profile and pKa of the catalytic residues were studied in search of a common denominator for the three mutations. Studies at decreasing hydration conditions revealed that the three enzyme variants were more reliant on high water activity for favoring transglycosylation (Figure 18).

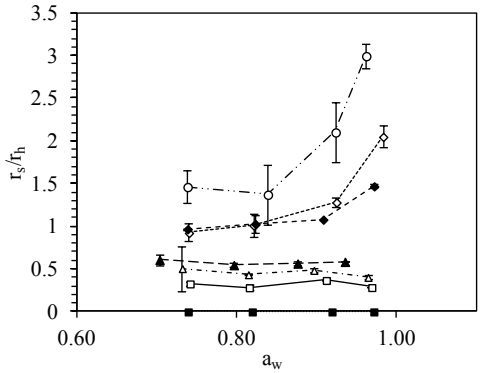

Figure 18 Transglycosylation (r_s) over hydrolysis (r_h) for *Tn*Bgl1A (□) and single mutation N220L (■). N220Y (◊), N220R (♦), N220D (Δ), N220C (▲) and N220F (○), in micro-aqueous hexanol at varying water activity (a_w). Error bars represent 1σ (**Paper III**).

This can be interpreted as rigidity being detrimental for their effect. In this context, it is interesting to note that natural glycosyl transferases are more flexible than glycosidases [149]. However, in what way can increased flexibility lead to a preference for transglycosylation? Molecular dynamics simulations suggest a distortion of the catalytic acid/base for mutants N220Y and N220R after 50 to 500 ns simulation, while no such distortion is seen for wild-type *Tn*Bgl1A (Figure 19).

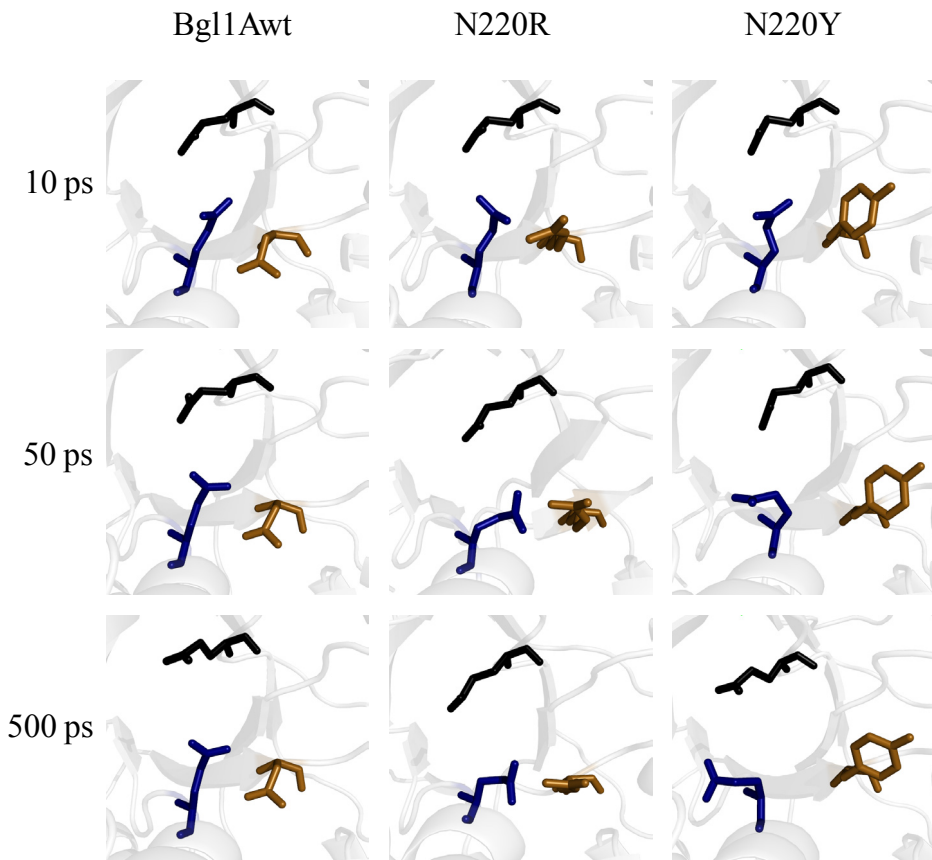

Figure 19 Orientation of the catalytic nucleophile (black), acid/base (dark blue) and position N220 (brown) at different time-points of a molecular dynamics simulation.

Distortion of the catalytic acid/base would hamper the deglycosylation step, and thereby increase the lifetime of the glycosyl-enzyme intermediate. Extending the lifetime of the covalent intermediate would allow more time for the larger acceptor (hexanol) to enter the active site. More speculative, the catalytic conformation could be regained through acceptor binding, as described for a GH13 CGTase by Kelly *et al*. [150]. Such an induced fit mechanism, likely not triggered by the binding of water, would benefit transglycosylation.

Alternatively, deformation of the catalytic acid/base could have an indirect effect on the catalytic water. The only direct interaction to the potential catalytic water in the crystal structure of *Tn*Bgl1A is the acid/base. Repositioning the acid/base could therefore lock the catalytic water in a position unfavorable for hydrolysis, similar to the proposed mechanism of natural transglycosylases, depicted in Figure 17 [132].

6.3.2 Increasing reactant concentration

One of the most influential external parameters in determining the ratio of transglycosylation for *β*-glycosidases is the concentration of substrate. Multiple studies have shown that increased substrate concentrations promote transglycosylation. In most studies, disaccharides are used as both donor and acceptor, making it hard to separate the effects of donor and acceptor concentration [151-154]. However, a study from Richard and coworkers pointed out that the ratio between transglycosylation and hydrolysis should be directly proportional to acceptor concentration, if the reaction ratio is dictated by the competition between water and acceptor [155]. Indeed, the authors show that the ratio of transglycosylation over hydrolysis is directly proportional to the concentration of acceptor alcohol, for a few ethanol derivates.

The specificity for transglycosylation has been shown to be independent of the concentration of glycosyl donor for synthesis of hexyl glycoside from pentyl glycoside [113]. However, several studies aimed at optimizing transglycosylation yields have shown positive effects of increased glycosyl donor [156-158].

6.3.3 Use of organic media

Another important factor intimately linked to the concentration of desired acceptor is the concentration of unwanted acceptor, water. In aqueous environment, the concentration of water is 55 M, much higher than possibly achieved for the desired acceptor. Several studies have focused on reducing this concentration using co-solvents, thereby increasing the yield in transglycosylation reactions. Indeed, improved yields of oligosaccharides have been reported in 30-50 % (v/v) triethyl phosphate [156, 159], 30 % acetonitrile [160], 40 % acetone [161] and non-aqueous lithium chloride in dimethylacetamide [162]. An impressive yield of 97 % was achieved for transglycosylation from pNPGal to GlcNAc using a *β*-galactosidase in a complex glycerol derived solvent [163].

The method of limiting water content is especially suited for synthesis of alkyl glycosides, since the acceptor has high solubility in hydrophobic media, in contrast to saccharides. However, *β*-glucosidases show the counter-intuitively property of reduced selectivity (r_s/r_h) at low a_w [89, 112, 164]. This was also the case for the two *β*-glucosidases from *Thermotoga neapolitana*, *Tn*Bgl1A and *Tn*Bgl3B, when introduced untreated into micro-aqueous hexanol (**Paper II**). Nevertheless, when adsorbed onto porous polypropylene, retained or improved r_s/r_h was observed (Figure 20), allowing an increased yield of alkyl glycosides from 30 % to 50 % using *Tn*Bgl1A. In combination with the N220F mutant, an r_s/r_h of 3.16 was achieved, giving an estimated yield of 76 %. When using the

more gentle drying method, mentioned in chapter 4.4, the enzyme retains more of its activity, although it is still severely hampered compared to at full hydration.

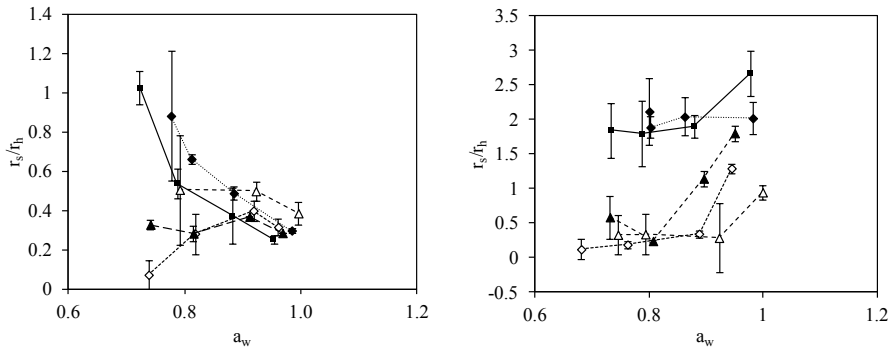

Figure 20 Selectivity for transglycosylation (r_s/r_h) of *Tn*Bgl1A (left) and *Tn*Bgl3B (right) as a function of water activity (a_w). The enzyme was freeze-dried (◊), deposited on Accurel MP1000; vacuum dried (■) or propanol dried (♦), and covalently linked to Eupergit C250L (Δ). Error bars represent 1σ, based on triplicate measurements (**Paper II**).

6.3.4 pH modulation

A most interesting observation is that transglycosylation and hydrolysis have different pH optimum for *β*-glycosidases. This has been utilized to improve the yield in transglycosylation reactions. Bonnin and coworkers showed that, for an exo-galactanase from *Aspergillus niger*, transglycosylation is dominant at pH 7 while hydrolysis surpasses at pH 3.5 [165]. At the lower pH, K_M values decrease with the degree of polymerization of the substrate, while at neutral pH K_M increase with increasing length of the substrate. This suggests that the ionization states of certain residues in the active site are unfavorable for interactions with galactose at neutral pH, and results in improved ratio of transglycosylation. Unfortunately, the authors did not pinpoint if the reduced affinity was in the negative or positive subsites. Another example of higher pH optimum for transglycosylation is seen in a bovine endo-glycosidase [166]. The opposite relation, with a lower pH optimum for transglycosylation was observed for an endo-glucanase from *Rhodotorula glutinis* [161].

A detailed investigation of pH effects on enzyme kinetics was conducted by Seidle and Huber [167]. They found that while the hydrolytic reaction showed the expected hyperbolic profile for V_{max}, the transglycosidic V_{max} was unaffected over the studied pH range (3.0-7.0), for cellobiose as well as oNPG and pNPG as glycosyl donors (Figure 21a). The authors speculate that this could be linked to the protonation state of an active site tyrosine often found with a hydrogen bond to the catalytic nucleophile in structures of *β*-glycosidases and previously shown to be

important for hydrolysis [168]. The hydrogen bond to the tyrosine reduces the energy needed to restore the charged nucleophile in the deglycosylation step. At high pH the tyrosine is deprotonated and can no longer assist in breaking the covalent bond in the glycosyl enzyme intermediate. However, for transglycosylation, with an acceptor that is a better nucleophile than water, this stabilization may not be required.

The same effect is seen for the three beneficial mutations at position 220 of *Tn*Bgl1A (N220F, N220R, N220Y). The transglycosylation activity remains constant over a wide pH range (5-11) while the hydrolytic reactivity is optimal at pH 5.5 (**Paper III**). Thereby, the hydrolytic side reaction can be completely suppressed at high pH. It is noteworthy that this strategy retains the native transglycosylation undiminished, while all other strategies mentioned above have hampered the enzymes catalytic efficiency. However, the gradual decrease in hydrolytic activity over such a wide pH range (Figure 21b) is not consistent with the hypothesis by Seidle and Huber, mentioned above. Moreover, this would not explain why transglycosylation is pH dependent for the wild-type enzyme, and warrants further investigations.

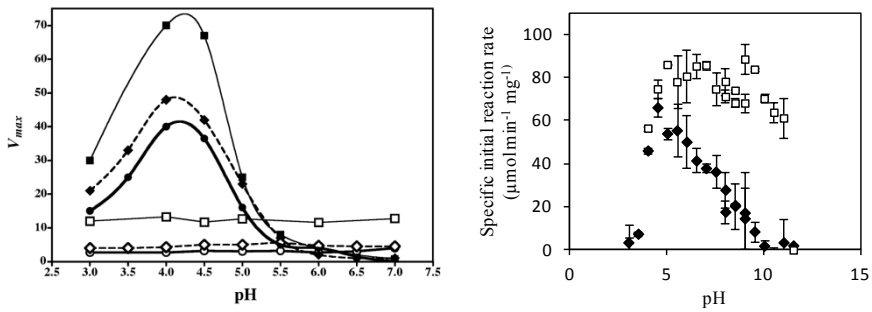

Figure 21 Effects of pH on (left) V_{max} for hydrolysis (filled symbols) and transglycosylation (open symbols) for *Aspergillus niger* Bgl3 with cellobiose (□), pNPG (○) and oNPG (◊) as glycosyl donor, reprinted with permission from [167] (right) specific initial reaction rate of hydrolysis (♦) and transglycosylation (□) of *Tn*Bgl1A mutant N220F (**Paper III**).

Determining the ionization state of individual residues can be done, but requires protein NMR. However, the pKa values of the catalytic residues can be indirectly determined through monitoring the pH dependence of the catalytic efficiency (k_{cat}/K_M), shown to be in good agreement with NMR data [121]. From the mechanism of an enzyme with two dissociable groups (Figure 20), one can derive that the pH dependence of k_{cat}/K_M reflects the ionization of the free enzyme while the pH dependence of V_{max} is influenced by the ionization of the enzyme-substrate complex. As seen in Figure 16, the glycosylation step requires the catalytic nucleophile to be deprotonated while the catalytic acid has to be protonated.

Therefore, the first pKa value of the bell shaped profile of k_{cat}/K_M can be attributed to the nucleophile and the second to the catalytic acid.

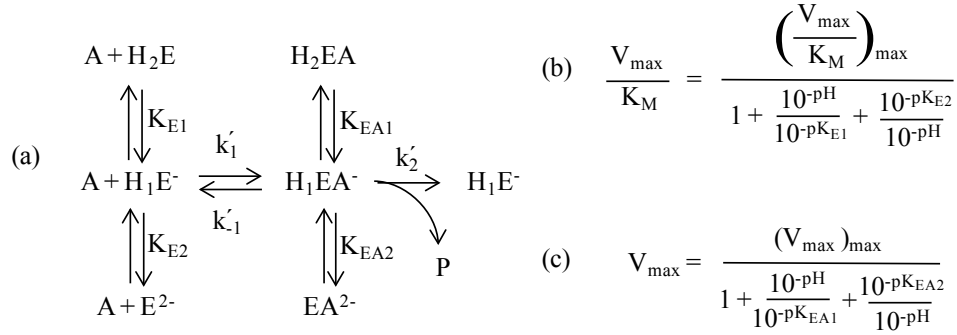

Figure 22 Simplified mechanism of an enzyme with two dissociable groups where only the singly protonated enzyme-substrate complex is catalytically active, and derived equations for apparent V_{max}/K_M (b) and V_{max} (c) [169].

This method was used to evaluate if the pKa values of the catalytic residues were modulated by the N220 mutations. It was found that only N220D and N220Y showed significant perturbations to the pKa of the catalytic nucleophile (+1.1 and +1.0 pH units respectively) and of the catalytic acid/base (+0.5 pH units) for N220Y, while the other mutations left the ionization state unaffected (figure 21). How transglycosylation is retained at higher pH for N220Y/F/R, despite the proposed incorrect ionization state of the catalytic acid, remains to be determined.

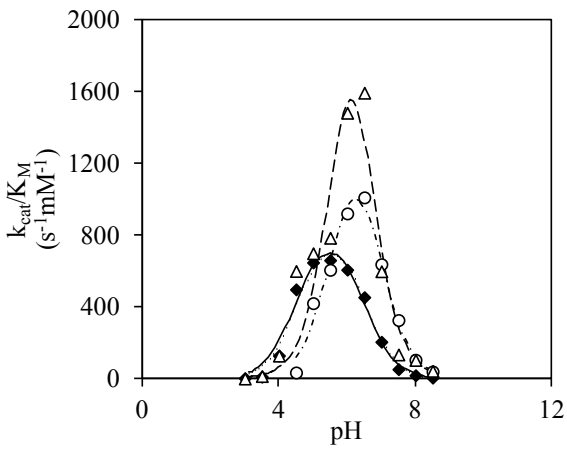

Figure 23 pH dependency of k_{cat}/K_M for *Tn*Bgl1A (♦), N220D (Δ) and N220Y mutant (○) along with their model-fitted curves (**Paper III**).

7 Transglycosylation by cyclodextrin glucanotransferases

Cyclodextrin glucanotransferase (CGTase) is a unique member of GH family 13. Their defining property is the ability to form cyclic oligosaccharides with 6, 7 or 8 glucose units, α, β or γ-cyclodextrins, from starch. This property is presumably used by microorganisms to monopolize on starch substrates by converting them to a form that other microorganisms cannot utilize [170]. Cyclodextrins are widely used in food, cosmetic and pharmaceutical industries due to their ability to form inclusion complexes with hydrophobic molecules [171]. However, what makes CGTases fit into the scope of this dissertation is their ability to, in addition to intramolecular transglycosylation (cyclization), catalyze intermolecular transglycosylation using both linear (disproportionation) and cyclic (coupling) substrates. What dictates the relationship between these reactions and the competing hydrolytic activity is discussed in the following chapter.

7.1 Mechanism

Like β-glycosidases, CGTases use a retaining double-displacement mechanism. However, unlike β-glycosidases, they act exclusively on the α-(1,4)-bond. Another difference is the extra catalytic residue, an aspartic acid critical for transition state stabilization [172]. Four additional residues in the -1 subsite are conserved in both CGTases and their hydrolytic counterparts, α-amylases. They are believed to be involved in substrate binding and distortion, analogous to the mechanism of β-glycosidases [45]. All four reactions catalyzed by CGTases start by substrate binding, formation of a β-linked glycosyl intermediate and dissociation of the first product. This allows binding of an acceptor molecule in the aglycone subsites, which can be either water (hydrolysis), the non-reducing end of the covalently linked glycone (cyclization) or another acceptor with a hydroxyl group (disproportionation/coupling), visualized in Figure 24. Despite acting through a double displacement mechanism, the coupling activity has been shown to follow ternary complex kinetics, where donor and acceptor bind to the enzyme at the same time [173]. However, binding of cyclodextrin would block the acceptor

subsites. This could be explained by acceptor binding near the active site so that it quickly can occupy the aglycone subsites upon formation of the glycosyl enzyme.

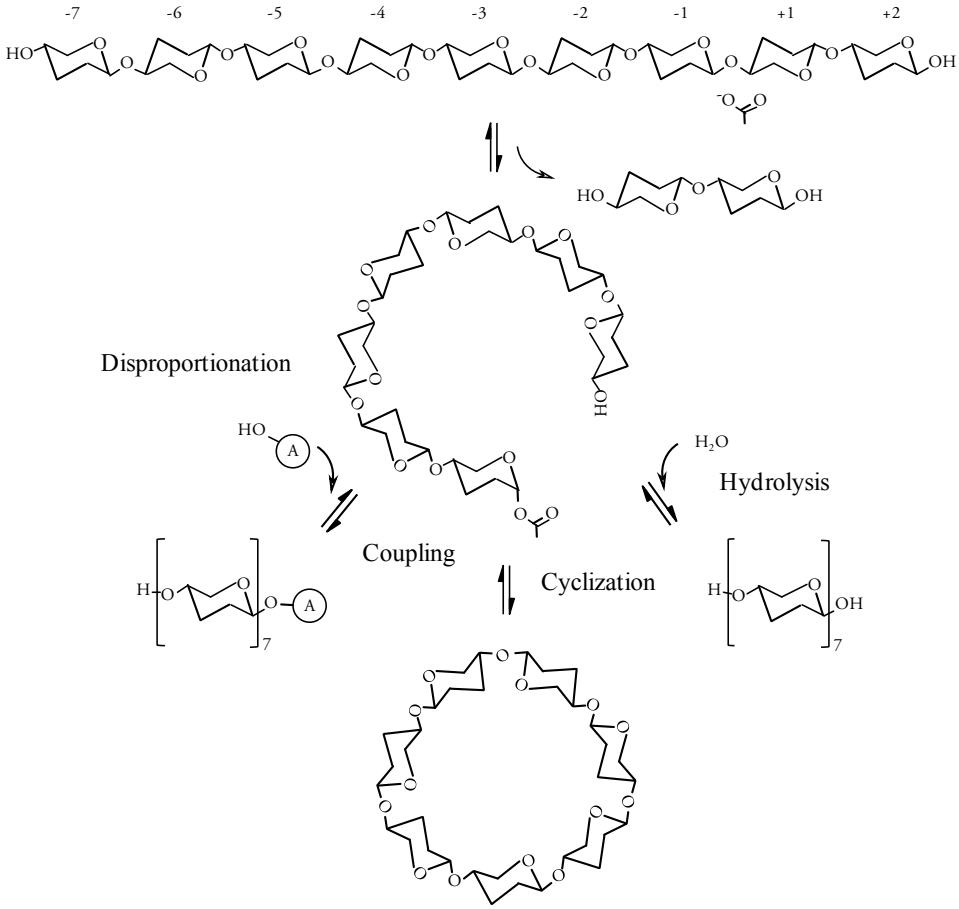

Figure 24 Schematic representation of the reactions catalyzed by CGTases.

7.2 Structural comparison to α-amylases

As mentioned in chapter 6.2, CGTases have their hydrolytic counterparts in α-amylases. Although the overall sequence identity between the two enzymes is low (~30 %), the active sites are more similar. Both have active sites that are accessible for the solvent and hence, restricting the water access to the active site is not the mechanism behind the transglycosylation preference for CGTases. Moreover, no unique binding site for a catalytic water molecule has been identified in the hydrolases [174].

One major difference observed is an extended loop in amylases that block substrate binding in subsite -3/-4, discussed in section 7.3.1 [175]. Another defining difference between the two members is the presence of a tyrosine in the catalytic subsite (Tyr195, according to the numbering of *B. circulans* used consistently in this chapter), where α-amylases have an amino acid with a smaller side chain [176]. Indeed, mutating the tyrosine to leucine or glycine resulted in significant reduction of cyclization and coupling activities while maintaining disproportionation and hydrolysis activity. *In silico* studies suggest that this tyrosine, with the help of other aromatic residues along the pathway, guides the 23 Å movement required for the non-reducing end to reach subsite +1 for cyclization [177].

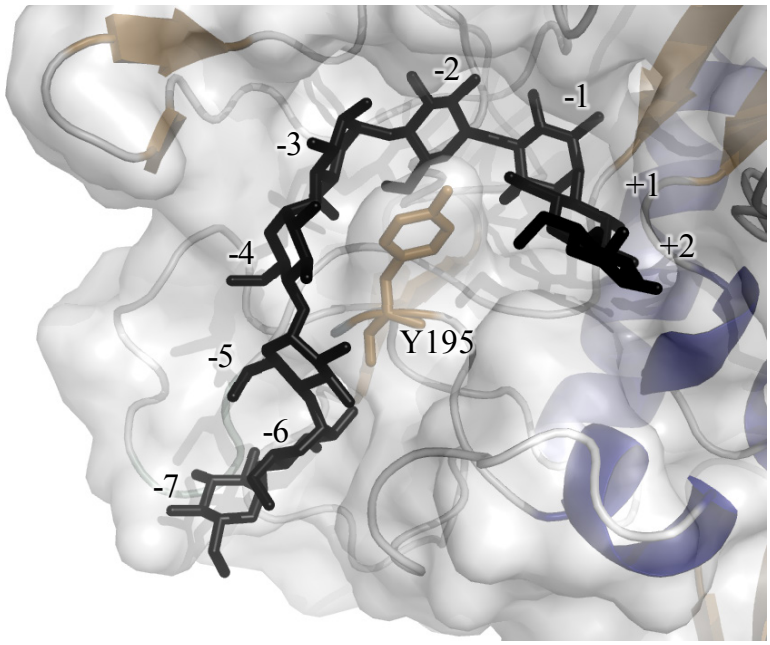

Figure 25 Active site of CGTase from *Bacillus circulans* 251 (PDB: 1CXK), with subsite numbering and Tyr195, different from the smaller residues found in α-amylases, highlighted in brown.

This large movement requires plasticity in the enzyme, observed in crystal structures with bound substrate [45]. Interestingly, when the acceptor subsites are occupied, a conformational change is induced that is likely not induced by water binding. This could partially explain the low hydrolytic activity of CGTases. Further insights are gained from mutational studies, discussed below.

7.3 Affecting the ratio between transglycosylation and hydrolysis

Comparing transglycosylation and hydrolysis for CGTases is not as straightforward as for β-glycosidases, since they catalyze more than one form of transglycosylation. Therefore, the topic is divided below based on which reaction is desired.

7.3.1 Improving the hydrolytic activity

For most uses of CGTases, hydrolysis is an undesired reactivity. However, to understand what governs the propensity for transglycosylation, improving the hydrolytic activity through protein engineering is of interest. This has been extensively studied by the Dijkhuizen group and they have identified three main mechanisms for transforming CGTases into hydrolytic enzymes.

The first is to removing aromatic and hydrophobic interactions in the aglycone subsites [178-180]. The reason this works is analogous to the reason increasing affinity in acceptor subsites favors transglycosylation for β-glycosidases, promoting binding of the desired acceptor molecule.

The second mechanism for converting CGTases into hydrolases is based on structural comparison to amylases, mentioned above. Inserting 5 amino acids, thereby extending a loop aimed at blocking subsites -3 and -4, severely hampered cyclization and disproportionation activities with only minor effects on the hydrolytic activity [181].

Lastly, through directed evolution, the mutation A230V was found to increase the rate of hydrolysis multiple fold while at the same time reducing the cyclization and disproportionation reactions to the same extent [182]. This mutation in the +1 subsite sterically hinders substrate binding in the acceptor subsites. The authors claim that this strongly supports the proposed induced-fit mechanism discussed in the next section, although it could also be for the same reason as removing aromatic and hydrophobic interactions is favorable for hydrolysis.

7.3.2 Favoring the cyclization reaction

Considering the already low hydrolytic activity of CGTases, improving the ratio of cyclization over hydrolysis is not an easy task. However, this was achieved by Kelly and coworkers with a single mutation 10 Å from the active site and 9 Å from the closest subsite, S77P, identified through directed evolution [150]. The authors also attempt to explain the mechanism behind this success with structural

data. A tyrosine residue on the same strand as S77 changes conformation due to a lost hydrogen bond, which in turn affects the hydrogen bond to an arginine. This arginine attains a new conformation that directly affects the orientation of the catalytic acid/base (Figure 26). This distortion may require substrate binding to restore the active orientation of the catalytic acid/base, not achieved with water as acceptor. A similar mechanism has been observed for a glucanotransferase from *Thermus thermophilus* [183, 184].

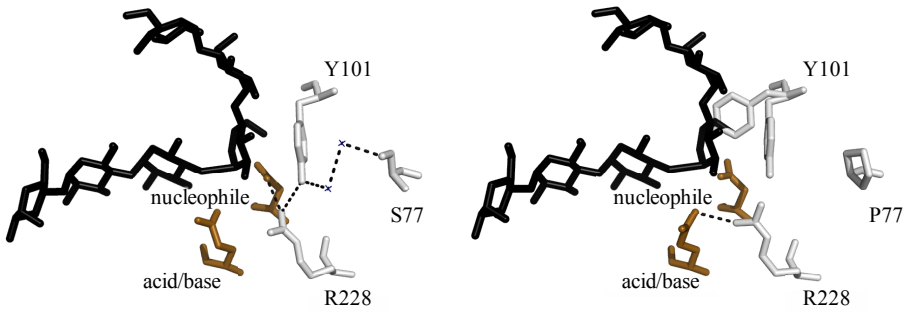

Figure 26 Hydrogen bonding network in CGTase from *Thermoanaerobacterum thermosulfurigenes* disrupted by S77P, allowing hydrogen bonding between R228 and the catalytic acid/base, thereby affecting the orientation of the catalytic acid/base [150].

Moreover, as for *β*-glycosidases, the pH optimum for transglycosylation (cyclization) and hydrolysis are different [185]. To allow better use of this phenomenon, Wind *et al.* managed to reduce the pH optimum for hydrolysis by more than one pH unit while maintaining the pH optimum for cyclization.

7.3.3 Favoring the coupling activity

For elongation of alkyl glycosides, chapter 5.3.3, the coupling reactivity of CGTases is the desired route, as demonstrated by Svensson and coworkers using a commercial CGTase from Amano Enzyme Inc [120]. However, this activity is not as extensively studied as the cyclization reaction. Therefore, a kinetics study was undertaken to help guide the optimization of the coupling reaction for extending alkyl glycosides (**Paper IV**). The properties of the two substrates in the coupling reaction make it complex to study; cyclodextrin can form inclusion complexes with hydrophobic moieties and the alkyl glycosides form micelles above CMC. Therefore, there are several different reaction species in the mixture, visualized in Figure 27.

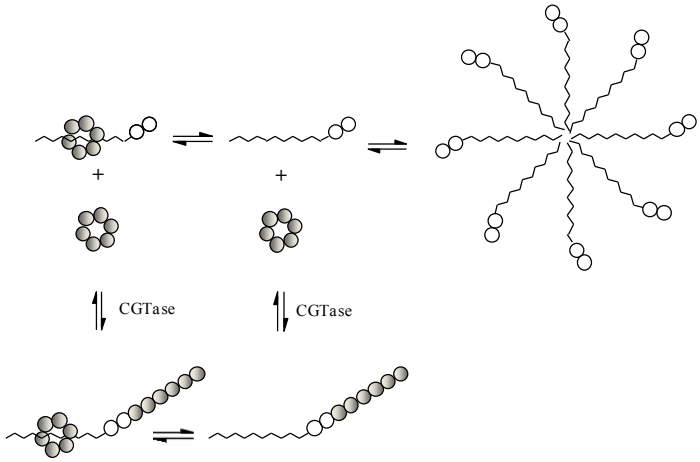

Figure 27 Reaction scheme showing all reaction species involved in CGTase catalyzed coupling between α-cyclodextrin and dodecylmaltoside.

Through following the formation rate of the desired product (dodecyl-β-D-maltooligopyranoside, DDMO) at varying acceptor (dodecyl-β-D-maltopyranoside, DDM) concentrations, simple Michaelis-Menten kinetics was observed when excess of donor (α-cyclodextrin) was used (Figure 28a). Conversely, without excess of donor, the rates decreased with increasing acceptor concentration until equimolar amounts (Figure 28b). This effect can likely be attributed to the complexation of DDM with α-cyclodextrin and subsequent reduction of free cyclodextrin, which apparently is a more potent donor than the complexed form. No rate increase is seen upon further addition of DDM, suggesting that micelles are not good acceptors for the enzyme.

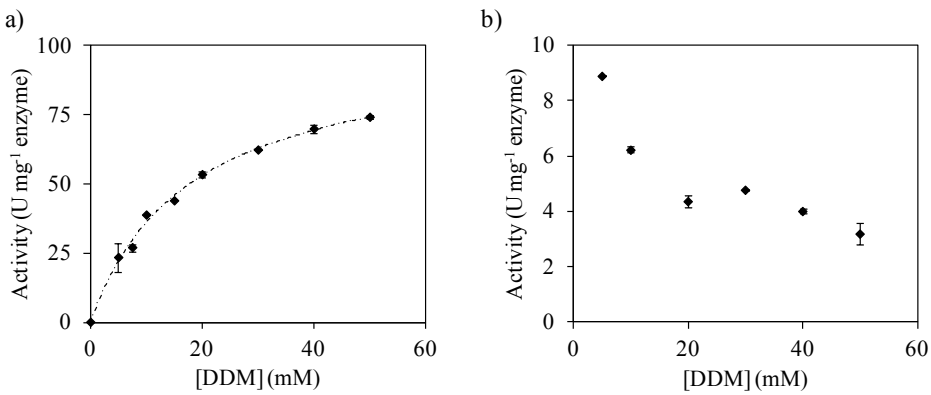

Figure 28 Influence of dodecyl-β-D-maltopyranoside on the initial coupling rate of Amano CGTase in 10 mM sodium citrate buffer, pH 5.2, 60°C containing (a) 300 mM or (b) 25 mM α-cyclodextrin (**Paper IV**).

When instead varying the concentration of the donor, α-cyclodextrin, a sigmoid curve was obtained (Figure 28). A moderate increase in reaction rate was observed until equimolar amounts of donor and acceptor was used, after which the rate increase accelerated. Again, this indicates that free α-cyclodextrin is a better donor than the complexed form. The increased rate above equimolar ratio of donor and acceptor also shows that all DDM must exist in complex form, and that this form is accepted by the enzyme. Due to the low CMC of DDM, it was not possible to distinguish if free alkyl glycosides or complex with α-cyclodextrin is preferred as acceptor. This was studied by Börner and coworkers by using an acceptor alkyl glycoside with a higher CMC value. They found that the monomeric alkyl glycoside is indeed the preferred acceptor, displaying a lower K_M value than the complex [186].

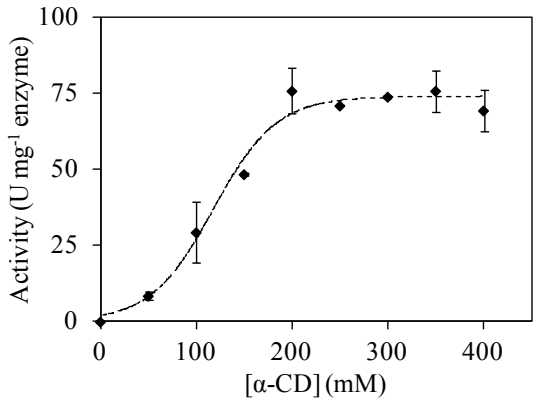

Figure 29 Influence of α-cyclodextrin on the initial coupling rate of Amano CGTase in 10 mM sodium citrate buffer, pH 5.2, 60°C containing 50 mM dodecyl-β-D-maltopyranoside (**Paper IV**).

7.3.4 Donor preference for coupling

To extend the range of alkyl glycosides that can be produced, it is desirable to have CGTases with efficient coupling activity when using larger cyclodextrins than α-cyclodextrin as substrate. Unfortunately, few CGTases are studied with regard to the coupling activity while most are characterized with regard to their specificity in cyclization reactions. It is reasonable to assume that an enzyme preferentially forming a certain type of cyclodextrin would prefer this as donor in the coupling reaction as well. However, this assumption was refuted while characterizing a novel CGTase from the thermophilic organism *Carboxydocella* (**Paper V**). Table VI shows the reaction rates for *Csp*CGT13 and two commercial CGTases for all four reactions catalyzed by the enzymes. As seen in the table, the novel CGTase *Csp*CGT13 preferentially forms α-cyclodextrin, but has a faster coupling and disproportionation activity using γ-cyclodextrin as donor.

Table VI Cyclization, coupling, disproportionation and hydrolysis activities of studied CGTases. Wheat starch was used as substrate for cyclization and hydrolysis and dodecyl-β-maltoside as acceptor for coupling and disproportionation (**Paper V**).

Enzyme		Cyclization (μmol min^{-1} mg^{-1})	Coupling (μmol min^{-1} mg^{-1})	Disproportionation (μmol min^{-1} mg^{-1})	Hydrolysis (μmol min^{-1} mg^{-1})
*Csp*CGT13	α-CD	164.6 ± 4.2	40.5 ± 0.5	66.0 ± 10.7	13.1 ± 0.9
	β-CD	20.6 ± 1.9	25.2 ± 1.9	19.6 ± 4.6	
	γ-CD	7.0 ± 0.5	65.5 ± 0.7	85.1 ± 11.2	
Toruzyme 3.0L	α-CD	355.4 ± 18.3	28.7 ± 0.5	44.8 ± 14.1	23.8 ± 0.2
	β-CD	111.1 ± 0.9	12.6 ± 1.8	12.6 ± 1.2	
	γ-CD	13.9 ± 5.5	21.4 ± 0.2	26.0 ± 3.2	
Amano	α-CD	729.4 ± 13.9	58.9 ± 2.3	0.0 ± 0.0	1.5 ± 3.1
	β-CD	128.0 ± 6.7	11.1 ± 0.0	0.0 ± 0.0	
	γ-CD	30.6 ± 2.2	6.1 ± 0.4	0.0 ± 0.0	

To find clues on what governs the donor preference in cyclization, a comparison was performed between the amino acid sequence of *Csp*CGT13 and sequences of the CGTases that the commercial enzymes are based on (*Thermoanaerobacter* sp. and *Paenibacillus macernas* for Toruzyme 3.0L and Amano enzymes respectively). Variations are seen in subsites -3 and -7 (Table VII). Although subsite -7 plays a large role in cyclodextrin specificity for the cyclization reaction [187, 188], the subsite is too remote to play a role in interactions with a cyclic donor. Therefore, subsite -3 is the more likely candidate in determining specificity in the coupling reaction. The importance of subsite -3 for controlling the coupling reactivity is supported by previous mutational studies. A mutational study of a CGTase from *Bacillus* sp. I-5 showed that Y89S increased the k_{cat} for coupling while reducing it for cyclization [189]. In addition, *Bacillus circulans* CGTase mutant Y89G showed reduced k_{cat} in the coupling reaction, whereas mutant Y89D displayed increased k_{cat} in the same reaction [188]. As seen in Table VII, *Csp*CGT13 has aspartic acid (D) in position 89 and, in accordance with the study by van der Veen *et al*., has high coupling activity. However, the Amano enzyme also displays high coupling activity, despite that *Paenibacillus macerans* CGTase, the origin of the Amano enzyme, has tyrosine (Y) in position 89. Clearly, further mutational studies are required to unveil what governs cyclodextrin specificity and the ratio between coupling and hydrolysis in CGTases.

Table VII Interacting residues in subsite +2 to -7, based on comparison with *B.circulans* 251 (**Paper V**).

Numbering according to CGTase from *B.circulans* 251	Subsite +2 183, 232, 259	Subsite +1 194-195, 230, 233	Subsite -1 100, 140, 227, 229, 257, 327-328	Subsite -2 98	Subsite -3 47, 89, 94, 196, 371	Subsite -6 167, 179-180, 193	Subsite -7 145-147
Thermoanaerobacter sp. ATCC 53627 *(α/β)*	F, K, Y	LF, A, H	Y, H, R, E, HD	H	K, D, S, D, D	Y, GG, N	SET
*Carboxydocella Csp*CGT13 *(α/β)*	F, K, F	LF, A, H	Y, H, R, E, HD	H	K, D, S, D, D	Y, GG, N	NQS
Paenibacillus macernas (α)	F, K, F	LY, A, H	Y, H, R, E, HD	H	K, Y, N, D, D	Y, GG, N	SST

8 Concluding remarks

Unveiling the determinants for transferase and hydrolase activity in glycoside hydrolases would unlock an extensive toolbox for carbohydrate chemists. This dissertation set out to identify such determinants, with production and modification of alkyl glycosides as model reactions.

In the first part of the dissertation, synthesis of alkyl glycosides, glycoside hydrolase catalyzed transglycosylation is the preferred route. A large number of studies have focused on limiting the hydrolytic activity of glycoside hydrolases and thereby improving the yields for transglycosylation, onto a wide range of acceptors. The most successful results have been obtained through directed evolution. However, to allow creation of novel glycosylating tools from any hydrolyzing enzyme, directed evolution is far too labor intensive. In addition, most enzymes variants found suffer from severely reduced activity. Therefore, there is great value in understanding the underlying mechanisms.

Increasing the concentration of the desired acceptor favors transglycosylation. More surprisingly, reducing the concentration of the undesired acceptor does not. I show that it can, if the proper enzyme preparation method is used to avoid detrimental protein-protein interactions (**Paper II**). However, large reductions to catalytic efficiency are observed, linked to the property of water as molecular lubricant.

Another way of favoring transglycosylation is to modulate the affinity of the enzyme for different acceptors. Several studies have shown the importance of aromatic amino acids in the positive subsites. Also on my model enzymes, this is a viable strategy (**Paper I**).

Moreover, destabilizing the second transition state through removing interactions in the negative subsites has been identified as a key step for improving transglycosylation. However, destabilizing a transition state necessarily comes at the cost of reduced catalytic efficiency.

A promising alternative is to utilize the different pH profiles of the transglycosidic and hydrolytic activity of glycoside hydrolases. The transglycosidic activity of a glucosidase from *Apsergillus niger* has been shown to be completely pH independent, while the hydrolytic activity has a distinct optimum. This allows quantitative yields for transglycosylation by simply adjusting the pH of the media. Unfortunately, none of my model enzymes displays this property. However, I

show that single mutant variants of *Tn*Bgl1A do, and that their hydrolytic activity can be completely repressed while maintaining the catalytic efficiency for transglycosylation (**Paper III**). Understanding the molecular determinants for this effect would unlock a novel strategy for converting glycosidases into tools for glycosylation, and warrants further investigation.

Although this first part of the dissertation contributes towards understanding what governs the propensity for glycosylation of glycoside hydrolases, it lacks direct implications for synthetic chemists. That would require using cheaper substrates such as cellobiose or lactose instead of pNPG as glycosyl donor.

The second part of the dissertation, modification of alkyl glycosides, is more applied. It deals with the poorly studied coupling activity of cyclodextrin glucanotransferases for elongation of the glycosidic part of an alkyl glycoside. We show that alkyl glycosides in complex with cyclodextrin are the preferred acceptors and that uncomplexed cyclodextrin is the best glycosyl donor for a commercial cyclodextrin glucanotransferase (**Paper IV**). Furthermore, a novel cyclodextrin glucanotransferase from *Carboxydocella sp*. is characterized (**Paper V**). It shows good coupling activity with γ-cyclodextrin as donor and could, therefore, be useful for extending the diversity of defined alkyl glycosides that can be produced. However, it also displays an unusually high hydrolytic activity to be a cyclodextrin glucanotransferase. Hence, we face the same problem as in the first part of the dissertation. Literature suggest that some of the same mechanisms as in glycosidases are involved in determining the preference for hydrolysis in cyclodextrin glucanotransferases, but no mutational studies were undertaken in this dissertation. Direct comparison to commercial enzymes suggests that subsite -3 is a key position for coupling activity, and call for further investigations.

A major part of this dissertation revolves around enzymes in organic media. A remaining challenge is to explain why certain enzymes are active at very low hydration, while others are not. This would not only be of great value to carbohydrate chemists, but also have implications for other hydrolytic enzymes. Proteases could e.g. be used for building up custom peptides and DNase for building up DNA. Although little progress was made towards using enzymes at low hydration conditions, this dissertation contributes towards that goal through describing other means of limiting the hydrolytic activity of hydrolases, although it is not yet clear how much is generally applicable.

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