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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on three original publications, referred to in text by Roman numerals:

I. Jalak J, Väljamäe P. (2010). Mechanism of initial rapid rate retardation in cellobiohydrolase catalyzed cellulose hydrolysis. Biotechnol. Bioeng. 106 871–883.

II. Jalak, J, Kurašin M, Teugjas H, Väljamäe P. (2012). Endo-exo synergism in cellulose hydrolysis revisited. J. Biol. Chem. 287, 28802–28815.

III. Jalak J, Väljamäe P. (2014). Multi-mode binding of cellobiohydrolase Cel7A from *Trichoderma reesei* to cellulose. PLoS ONE 9, e108181.

My contribution to the articles is as follows:

Ref I Performed the experiments with BC and RAC, participated in data analysis.

Ref II Designed and performed the experiments of steady state kinetics, participated in data analysis

Ref III Designed and performed the experiments, analyzed the data

ABBREVIATIONS

$[CBH]_{bound}$	concentration of bound *Tr*Cel7A
$[CBH]_{FA}$	concentration of *Tr*Cel7A with a free active site
$[CBH]_{free}$	concentration of free *Tr*Cel7A
$[CBH]_{OA}$	concentration of *Tr*Cel7A bound on active site level
$[CBH]_{OA\text{-}NP}$	concentration of *Tr*Cel7A nonproductively bound on active site level
$[CBH]_{OA\text{-}prod}$	concentration of *Tr*Cel7A productively bound to cellulose
$[CBH]_{total}$	total concentration of *Tr*Cel7A
BC	bacterial cellulose
BMCC	bacterial microcrystalline cellulose
CBH	cellobiohydrolase
CBM	carbohydrate binding module
CD	catalytic domain
$CD_{TrCel7A}$	catalytic domain of *Trichoderma reesei* cellobiohydrolase Cel7A
DP	degree of polymerization
$DP_{surface}$	degree of polymerization on crystal surface
DS	degree of synergism
EG	endoglucanase
GH	glycoside hydrolase
k_{obs}	observed catalytic rate constant
k_{on}^{obs}	observed association rate constant
LPMO	lytic polysaccharide monooxygenase
MU	4-methylumbelliferone
MUL	4-methylumbelliferyl β-D-lactoside
n_{free}	obstacle-free path
P_{app}	apparent processivity
pNP	para-nitrophenol
pNPL	para-nitrophenyl β-D-lactoside
RAC	regenerated amorphous cellulose
*Tr*Cel5A	*Trichoderma reesei* endoglucanase Cel5A
*Tr*Cel7A	*Trichoderma reesei* cellobiohydrolase Cel7A

1. INTRODUCTION

Cellulose is the main component of plant cell walls and therefore the most abundant biopolymer on Earth. With the annual production of about 4×10^{10} metric tons (Goyal et al., 1991) it has great potential as a renewable energy source. Cellulose consists of linear chains of β-1,4 linked glucose residues. Individual cellulose chains are bound together by hydrogen bonds and *van der Waals* interactions forming microfibrils that are recalcitrant towards both chemical and enzymatic breakdown. Cellulose in plant cell walls is associated with hemicellulose and lignin. Collectively this complex is known as lignocellulose. In nature, cellulose is degraded by microorganisms, mainly bacteria and fungi, which secrete a set of cellulolytic enzymes also called cellulolytic system. The best described cellulolytic system is that of the soft rot fungus *Trichoderma reesei*. The major component of *Trichoderma reesei*'s cellulolytic system is a processive cellobiohydrolase *Tr*Cel7A.

While *Trichoderma reesei* cellulases have been subject of intensive study for decades, the mechanism of cellulase catalyzed cellulose hydrolysis is still not fully understood. One of the biggest shortcomings is the difficulty to measure the rate constant of cellulases acting on cellulose. Problems arise from heterogeneous insoluble substrate as well as from modular structure of the enzyme. It is well known that the rate of enzymatic cellulose hydrolysis drops rapidly in time. The initial burst of activity is followed by a rapid decrease in the hydrolysis rate. Both enzyme- and substrate-related factors have been proposed to explain this phenomenon. Understanding the mechanism and factors that limit the reaction rate are of great importance in enzyme engineering for developing better enzyme cocktails for lignocellulose breakdown.

This work introduces novel methods for determining the rate constants of *Tr*Cel7A catalyzed cellulose hydrolysis (Ref I & II). These methods are used to investigate the mechanism behind the decrease in activity of cellulases during the cellulose hydrolysis (Ref I) and the effect of synergism on this decline in enzyme activity (Ref II). This work also addresses the binding of *Tr*Cel7A to the cellulose by distinguishing between different populations of bound enzyme (Ref III).

2. REVIEW OF THE LITERATURE

2.1. Cellulose

Cellulose is the main component of plant cell walls and, thus, the most abundant biopolymer on Earth. It is an unbranched homopolymer consisting of β-1,4 linked glucose residues (Figure 1, panel A). Since the glucose residues are rotated 180 degrees in relation to each other the shortest repeating unit of cellulose is glucose dimer, cellobiose. This rotation also makes the sides of cellulose chain symmetrical and enables an equal number of hydrogen bonds on both sides. The reducing end glucose residue is distinct from the rest of the chain as it can take both α and β configuration as well as open-chain form. This makes the reducing ends the most reactive part of cellulose. The degree of polymerization (DP) of cellulose chains depends on the source of the cellulose and typically lies between 100 and 15000 glucose units. In a cellulose crystal individual chains are bound to each other through hydrogen bonding and *van der Waals* interactions resulting from pyranose ring stacking. Cellulose can appear in different crystal forms, which determines the number of hydrogen bonds and, thus, the overall recalcitrance of the substrate. In naturally occurring cellulose (cellulose I) parallel cellulose chains form ordered layers in the cellulose crystal. Cellulose I has two distinct crystal forms Iα (triclinic) and Iβ (monoclinic) (Atalla et al., 1993). These two forms coexist with different ratios depending on the source of the cellulose. Cellulose of algae and bacterial cellulose (BC) have higher Iα content whereas Iβ content is higher in plant and tunicin cellulose. Cellulose I is stabilized by interchain (2 per glucose residue) and intrachain (2 – 3 per glucose residue) hydrogen bonds, however, there are no interlayer hydrogen bonds (Zhang and Lynd, 2004). The two crystal forms of cellulose I differ by the conformation of the hydroxymethyl group and hydrogen bonding pattern (Nishiyama et al., 2002, 2003a). The Iα form is considered metastable as it can be irreversibly converted to the Iβ form with hydrothermal treatment (Horii et al., 1987). Despite high crystallinity, cellulose crystals have inherent disorganization in the hydrogen bonding (Nishiyama et al., 2003b) due to intrinsic strain which leads to twisting of the cellulose crystal, estimated to be 1.5° per cellobiose residue (Matthews et al., 2006).

Cellulose II is derived from cellulose I either through mercerization or regeneration. Some mutant strains of *Acetobacter xylinum* also have been reported to be capable of synthesizing it (Kuga et al., 1993; Shibazaki et al., 1998). In Cellulose II the chains are antiparallel to one another. This organization is energetically more favorable and enables the formation of interlayer hydrogen bonds. Other polymorphs of cellulose (III_I, III_{II}, IV_I IV_{II}) are derived either from cellulose I or II through chemical treatment and do not occur naturally (Hon, 1994; O'Sullivan, 1997).

In crystalline form, cellulose chains are packed together into diamond or rectangle shaped microfibrils (Figure 1, panel B). Microfibrils of plant origin

consist of 15–25 chains and are typically about 2.4–3.2 nm in diameter (Kennedy et al., 2007). Algal cellulose microfibrils are larger, about 10 nm in diameter and, thus, have a lower area to mass ratio (Nishiyama et al., 2002, 2003a). In case of cellulose Iα the faces of the pyranose rings in a layer are parallel to the (110) face of the lattice and the hydroxyl groups of the cellulose chain are exposed to ($1\bar{1}0$), (100) and (010) surfaces of the crystal. This arrangement makes the (110) face hydrophobic, while other faces of the lattice are hydrophilic. In cellulose Iβ the orientation of the chains is slightly different and the (100) face is hydrophobic, whereas other faces are hydrophilic. In the perfect crystal the hydrophobic faces are on the narrow edges, essentially the layer consists of a single cellulose chain that is exposed to the surface. Studies with electron microscopy have shown that the corners are often eroded as the chains with fewer interactions with the rest of the crystal dissociate more easily (Sugiyama et al., 1985). Such "worn" edges increase the area of the hydrophobic surface.

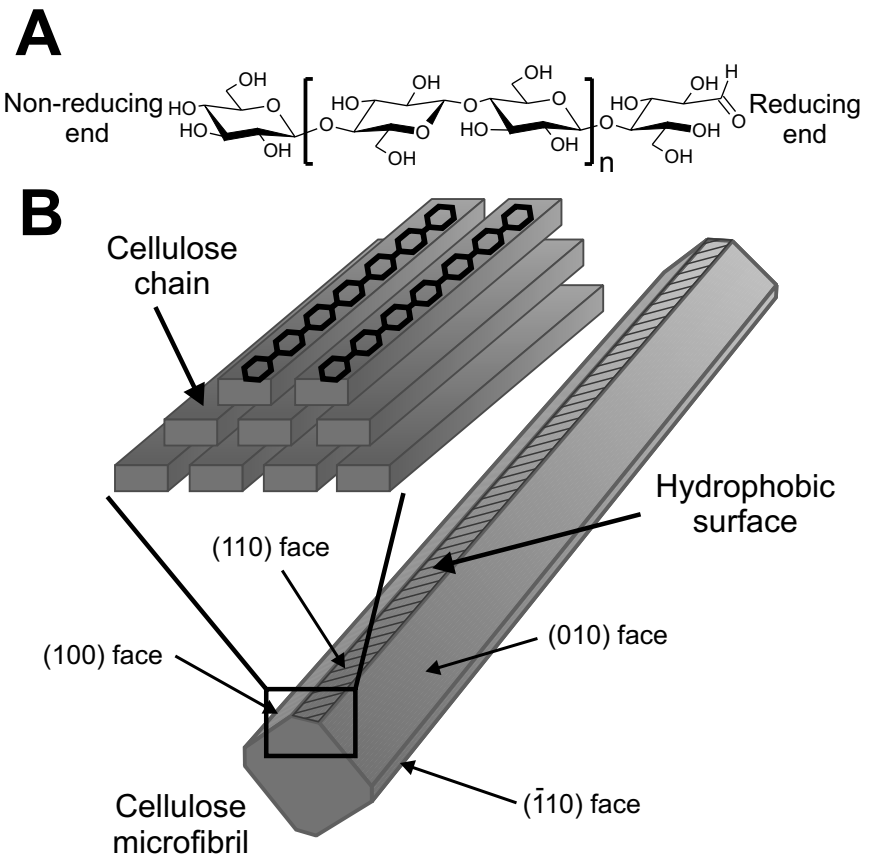

Figure 1. (A) Chemical structure of cellulose chain. The shortest repeating unit, the cellobiosyl moiety is shown in brackets. The reducing end glucose residue is displayed in the open-chain form. For clarity, most hydrogen atoms are omitted from the formula. **(B)** Schematic representation of cellulose Iα microfibril. Pyranose rings of the layer of parallel cellulose chains are exposed to the hydrophobic surface (110) of the microfibril.

Cellulose has also regions where cellulose chains do not form ordered crystal structures. These amorphous parts appear intermittently with crystalline cellulose. The proportion of amorphous cellulose depends on the source and method of preparation of the cellulose. Highly crystalline celluloses such as *Valonia* cellulose can have crystallinity up to 95% (O'Sullivan, 1997), while wood cellulose has crystallinity around 60% (Zhang and Lynd, 2004).

In plant cell walls cellulose is interlinked with hemicellulose, lignin, and pectin. The term hemicellulose incorporates different noncellulose polysaccharides such as xyloglucan, xylan, and mannan (Lynd et al., 2002). Unlike cellulose, hemicelluloses are branched heteropolymers that do not form ordered crystalline structure. Sugar monomers in hemicelluloses can include glucose, xylose, galactose, mannose, arabinose, and rhamnose. While hemicelluloses are generally less recalcitrant than cellulose, its acetylation and complex branching can decrease the efficiency of lignocellulose hydrolysis.

Lignins are a relatively hydrophobic diverse group of heteropolymers consisting of methoxylated aromatic alcohols: paracoumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. The proportions of these monolignols and extent of methoxylation depend on the source of the lignin. Lignin is crosslinked with different carbohydrates in lignocellulose by ester and ether linkages formed through glucuronic acid or arabinose-ferulic acid (Takahashi and Koshijima, 1988). Hemicellulose and lignin typically comprise 20–35% and 5–30% of plant dry weight, respectively.

2.1.1. Cellulosic substrates used in cellulase studies

Celluloses from different sources can have widely different DP, crystallinity and number of free chain ends on the crystal surface. Also, cellulose can be associated with other substances depending on the source. Results of cellulose hydrolysis experiments often depend on the type of cellulose used (Kostylev and Wilson, 2012; Mansfield et al., 1999). In order to obtain comparable results model substrates with defined characteristics are often used in cellulase studies.

Bacterial cellulose (BC), unlike cellulose from plant origin, is pure cellulose i.e. it is not associated with hemicellulose and lignin. BC has DP of about 2000 glucose residues and has a relatively high crystallinity of about 75% (O'Sullivan, 1997; Watanabe et al., 1998). While BC has quite large specific area it has a small number of chain ends available on its surface. Amorphous regions of BC are less recalcitrant and are hydrolyzed first so that partial acid hydrolysis leads to an increase in crystallinity (Väljamäe et al., 1999). The residual cellulose (bacterial microcrystalline cellulose – BMCC) has DP around 100 glucose units (Chanzy and Henrissat, 1985; Väljamäe et al., 1999), which roughly represents the length of crystalline regions in BC. Acid treatment also increases the number of free chain ends available on the cellulose surface.

More commonly cellulosic substrates of plant origin are used in cellulase studies. Avicel is acid treated and ground cellulose from wood pulp and is

considered a blend of crystalline and amorphous forms. It may contain residual hemicellulose but does not contain lignin. The DP of avicel is about 300 glucose residues (Zhang and Lynd, 2004) and its specific area is an order of magnitude smaller than that of the BC and BMCC.

Avicel can be dissolved in phosphoric acid and when regenerated the residual cellulose (regenerated amorphous cellulose – RAC) practically does not contain crystalline regions (Zhang and Lynd, 2006; Zhang et al., 2006). If the treatment is performed at low temperatures the acid hydrolysis will be minimal and the DP will not change significantly (Jeoh et al., 2007; Velleste et al., 2010; Zhang and Lynd, 2005).

The most relevant substrate for the industry is lignocellulose. In addition to cellulose, these substrates contain hemicelluloses, lignins, and pectins which hamper the action of cellulases. Typically, incubating naturally occurring lignocellulosic biomass with cellulases yields less than 20% of theoretical product (Lynd et al., 2002). To increase the digestibility of the substrate several pretreatment techniques have been used. The most common methods of pretreatment include hydrothermal treatment, acid and alkali treatment, ozonolysis, steam explosion, ammonia fiber explosion, wet oxidation and solubilization with organic solvents or ionic liquids (Alvira et al., 2010).

2.2. Cellulases

Efficient cellulose hydrolysis requires a joint action of different enzymes. Different cellulases have different affinities towards crystalline and amorphous regions of cellulose. Additionally, in the case of lignocellulose, other enzymes are needed for hemicellulose, pectin, and lignin breakdown. The set of enzymes employed by the organism for cellulose utilization is called cellulolytic system.

Different microorganisms have different strategies for cellulose breakdown. Anaerobic cellulolytic bacteria employ complexed cellulase systems, also called cellulosomes (Bégum and Lemaire, 1996; Schwarz, 2001). Cellulosomes are stable enzyme complexes that are either anchored firmly to the cell wall of the bacteria or appear free in the solution. Cellulosomes consist of a noncatalytic glycoprotein scaffoldin and different catalytic modules bound to the scaffoldin through cohesin-dockerin interactions. Attachment to cellulose is mediated by carbohydrate binding module (CBM) that is part of the scaffoldin module. The number and organization of catalytic modules depend on the organism. These catalytic subunits include endo- and exocellulases, hemicellulases, xylanases, chitinases, and lichenases (Bégum and Lemaire, 1996). The cellulosome ensures the proper ratio of different activities, a suitable distance between modules, and the presence of different enzymatic activities all to optimize the synergy between the catalytic components. Additionally, cellulosomes bound to the cell wall ensure efficient oligosaccharide uptake by providing close proximity to the substrate so that the distance over which the products need to diffuse is

relatively short. The best described cellulosomes are from *Clostridium* and *Ruminococcus* families (Schwarz, 2001).

Since the industrial application of complexed cellulase systems would be more complicated, noncomplexed fungal cellulases, most notably those of *Trichoderma reesei*, have received more attention. Filamentous fungi and actinomycetes are capable of penetrating cellulosic substrate through hyphal growth and can secrete cellulases into cavities within the substrate so that high local concentration of the enzyme can be achieved without the formation of high molecular weight complexes. The cellulases of noncomplexed cellulase systems are usually multi-domain enzymes that consist of catalytic domain (CD) and CBM, which are connected by a flexible O-glycosylated linker peptide (Figure 2). While typically there are one of each domain per enzyme molecule, in some cases single enzyme molecule can have multiple CDs and CBMs (Lynd et al., 2002).

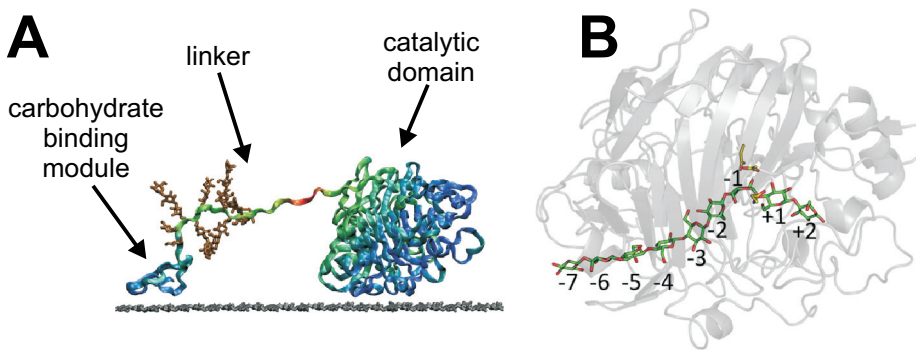

Figure 2. (A) The modular structure of cellobiohydrolase Cel7A from *Trichoderma reesei*. Image adapted from (Zhong et al., 2008) with permission of Springer. **(B)** The catalytic domain of *Trichoderma reesei* cellobiohydrolase Cel7A with highlighted catalytic residues and a cellodextrin chain bound in the substrate binding tunnel. Binding subsites for glucose residues are numbered from –7 to +2; +3 subsite is empty and not marked. Adapted with permission from (Knott et al., 2014) copyright (2014) American Chemical Society.

The active center lies deep in the CD and has more or less closed structure. The substrate binding tunnel or -cleft is lined with aromatic amino acid residues that form binding sites for glucose units. In literature the binding sites for glucose units are numbered (Figure 2) so that the cleavage of the glycosidic bond occurs between sites –1 and +1 with the nonreducing end of the cellulose chain at the "–" side and the reducing end at the "+" side.

The CBM mediates binding to cellulose and plays an important role in the hydrolysis of insoluble substrates while in the case of soluble substrates its effect is negligible. The removal of CBM usually decreases the activity of a given enzyme on insoluble substrates by 50–80% (Gilkes et al., 1992; Tomme et al., 1988) but the effect is strongly dependent on the concentration of

cellulose (Várnai et al., 2013). The binding is mediated through hydrophobic interactions and, to a lesser extent through hydrogen bonding (Linder et al., 1995). Based on binding specificity the CBMs are divided into three groups. Type A binds to hydrophobic surfaces of the cellulose crystal. Type B binds to free single polysaccharide chains. Type C has an affinity towards mono-, di-, and trisaccharides. Boraston *et al* have suggested that type C CBMs also bind to polysaccharide chains in exo- fashion in contrast to the type B CBMs that bind in endo- fashion (Boraston et al., 2004).

CBMs that target crystalline regions of cellulose enable two-dimensional diffusion of the cellulase on the cellulose surface and thereby increase the effective concentration of the enzyme. It has also been speculated that CBMs take a more active role in cellulose hydrolysis by disrupting the substrate and making it more digestible for the CD (Din 1994; Boraston 2004; Arantes 2011), however, several studies contradict this hypothesis (Beckham et al., 2011; Ståhlberg et al., 1991).

CD and CBM are connected by a linker peptide typically 6–59 amino acid residues long. In addition to maintaining a suitable distance between CD and CBM (Srisodsuk et al., 1993) it has been shown that the linker can act in inchworm-like fashion and promote processivity during cellulose hydrolysis (Zhao et al., 2008). It has also been demonstrated that the linker contributes to the binding on cellulose surface (Nakamura et al., 2016; Payne et al., 2013a). While cellulases are typically quite stable enzymes the linker regions are more susceptible to proteolysis (Tomme et al., 1988). The O-glycosylation through Serine and Threonine residues is believed to stabilize the structure of the linker and to increase its resistance towards proteolysis (Beckham et al., 2012).

Most cellulases are glycoside hydrolases (GHs) and catalyze the hydrolysis of the β1,4 glycosidic bond using general acid base catalysis. Based on their primary structure GHs are divided into 133 families (Carbohydrate Active Enzymes database; http://www.cazy.org/) (Henrissat, 1991; Lombard et al., 2014). Enzymes belonging to different GH families have different structures, different amino acids in the catalytic center, and may use different reaction mechanisms. The glycosidic bond is hydrolyzed using either single displacement or double displacement mechanism (Davies and Henrissat, 1995) (Figure 3). In the case of double displacement, the glycosidic oxygen is protonated and a glycosyl enzyme intermediate is formed. With the second nucleophilic substitution at the anomeric carbon, the intermediate is hydrolyzed and the released product has the same configuration as the substrate. In single displacement mechanism, the protonation of the glycosidic oxygen and the nucleophilic attack of the water molecule are simultaneous and yield a product with inverted configuration (Davies and Henrissat, 1995). The mechanism of catalysis is the same within a GH family.

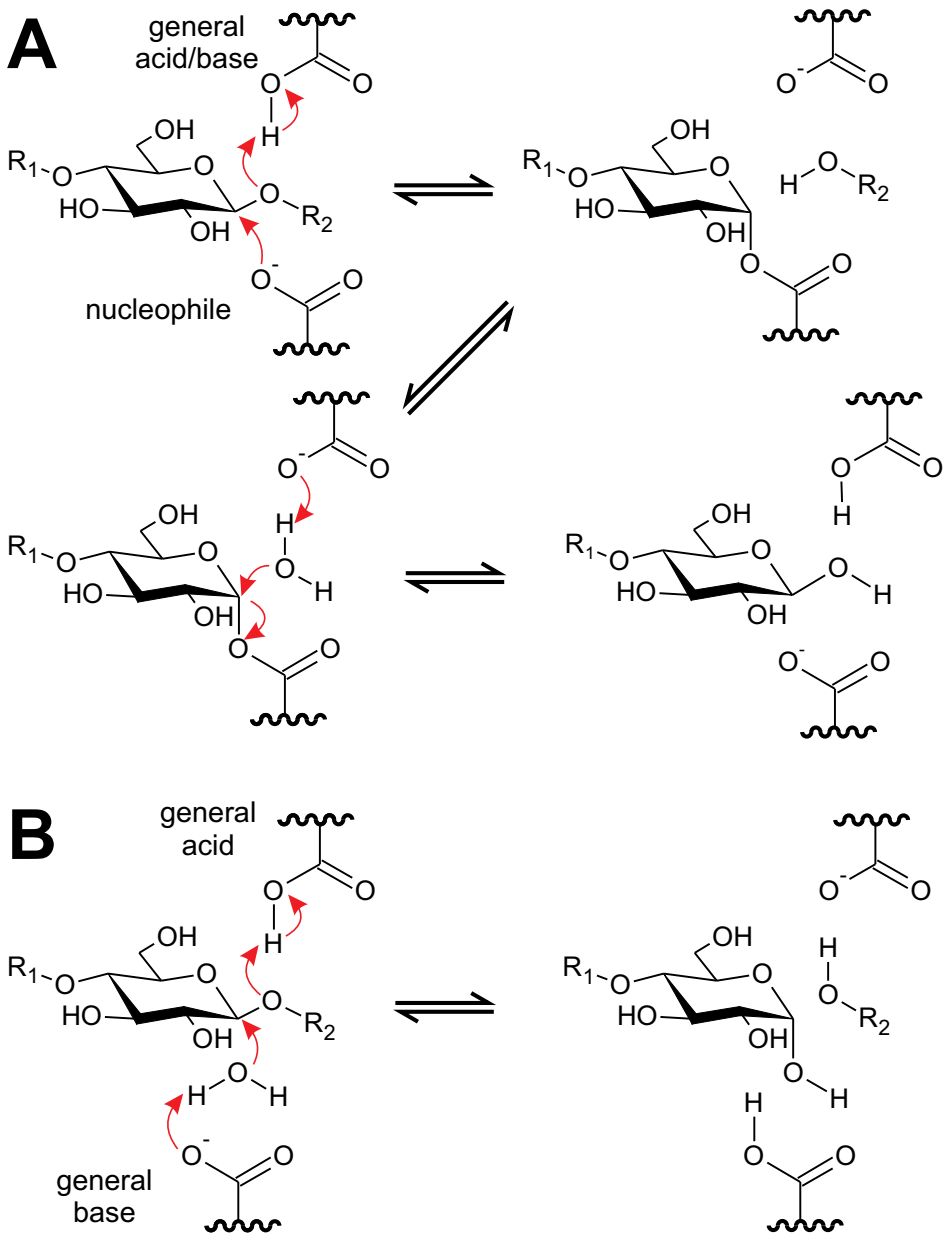

Figure 3. Two major mechanisms of enzymatic hydrolysis of the β-glycosidic bond **(A)** The retaining mechanism with two displacements at the anomeric carbon generates a product with the same configuration as the substrate. **(B)** The inverting mechanism with a single displacement at the anomeric carbon yields a product with inverted configuration.

Based on the mode of action cellulases can be divided into four groups:

- Exo-1,4-β-glucanases that initiate hydrolysis from cellulose chain end. (EC 3.2.1.74). (EC 3.2.1.176) (EC 3.2.1.91).
- Endo-1,4-β-glucanases (EC 3.2.1.4) that randomly cleave the internal bonds of cellulose chain.
- β-glucosidases (EC 3.2.1.21) that convert cellobiose to glucose.
- Lytic polysaccharide monooxygenases (LPMOs) that use oxidative process to cleave cellulose chain.

Exocellulases are enzymes that initiate hydrolysis from cellulose chain ends and can be either processive or nonprocessive. Processivity means that enzyme once bound productively to the substrate performs several consecutive catalytic steps on a single polysaccharide chain. Processive exocellulases – cellobiohydrolases (CBHs) – are the main components of fungal cellulolytic systems. CBHs are specific for either reducing end or non-reducing end and usually both types of CBHs are present in a cellulolytic system. Since the glucose residues in cellulose are rotated 180° in relation to one another, every second β-glycosidic bond is in correct orientation for catalysis. This determines that through processive action CBHs release cellobiose, while the first cleavage of the processive hydrolysis can also yield cellotriose and glucose (Divne et al., 1994; Fox et al., 2012; Kari et al., 2017). CBHs are most effective on crystalline cellulose and hydrolyze cellulose chains from the surface of the crystal layer by layer, so that the specific area and DP of the substrate decrease slowly. CBHs have more or less closed tunnel shaped active site architecture and have multiple binding sites for glucose residues to promote processive action. Futhermore, CBHs display strong interactions with glucose units in product binding sites, which is believed to be the key driver of processive action (Colussi et al., 2015; Knott et al., 2014; Payne et al., 2013b). However, strong binding in product sites also renders CBHs more susceptible to product inhibition (Gruno et al., 2004).

Endoglucanases (EGs) have more open active site (Kleywegt et al., 1997), typically in the shape of a groove. The open architecture of the active site ensures that EGs can freely initiate hydrolysis in any position of the cellulose chain. The action of EGs releases soluble sugars slowly while the DP of the substrate decreases rapidly. EGs acting alone hydrolyze amorphous regions of cellulose effectively, but are unable to hydrolyze highly crystalline portion of the substrate.

To date, it is clear that the endo/exo classification of cellulases is an oversimplification as many enzymes lie somewhere between exo- and endoenzymes. It has been shown that some CBHs can also initiate hydrolysis in endo- fashion (Kurašin and Väljamäe, 2011; Ståhlberg et al., 1993). The contribution of possible endo- activity depends on the architecture of the active site. It has been shown that the loops forming the roof of the active site tunnel of CBHs are flexible and may enable occasional endo attack (Rouvinen et al., 1990). Additionally, although to a lesser extent, some EGs display processivity similarly to

CBHs (Cohen et al., 2005; Kurašin and Väljamäe, 2011). These enzymes can bind to cellulose in endo fashion and proceed with processive action.

β-glucosidases hydrolyze cellobiose and soluble cellooligomers into glucose, thereby completing the cellulose breakdown. β-glucosidases act upon soluble substrates and, unlike most CBHs and EGs, these enzymes do not have the modular architecture. β-glucosidases are found in GH families 1, 3, 9, 30, 116 (CAZy) with the majority of fungal β-glucosidases belonging to the family 3. Cellobiose hydrolysis relieves the product inhibition of CBHs, however, β-glucosidases themselves are inhibited by glucose (Singhania et al., 2013; Teugjas and Väljamäe, 2013a).

In 2011 it was shown that cellulases previously classified belonging to families GH61 and CBM33 cleave glycosidic bonds in oxidative manner (Forsberg et al., 2011; Quinlan et al., 2011). In CAZy database these enzymes have been reclassified as auxiliary activities and designated into families AA9 (fungal) and AA10 (bacterial). More families (AA11, AA13) have been added later. These enzymes are copper dependent polysaccharide monooxygenases that oxidize either C1 or C4 in the glucopyranose ring. Type 1 LPMOs produce C1 oxidized compounds (aldonic acids), type 2 LPMOs produce 4-keto sugars through oxidation at C4 carbon, and type 3 LPMOs can oxidize both C1 and C4 of the glucose residue (Phillips et al., 2011). Possible action at C6 has also been suggested (Bey et al., 2013; Quinlan et al., 2011), however, these results are debated (Isaksen et al., 2014). Whether the oxidation occurs at C1 or C4 determines which end of the resulting cut is modified. The position of oxidation may have an effect on synergism with hydrolytic cellulases. For example, the action of reducing end specific CBHs may be hindered if the oxidation occurred at C1 and an aldonic acid is produced. Also, CBHs and EGs have a lower affinity towards cellobionic acid, meaning that cellobionic acid is a weaker inhibitor for cellulases than its non-oxidised counterpart, cellobiose. In contrast, gluconic acid is stronger inhibitor than glucose (Cannella et al., 2012).

LPMOs require external electron donors and molecular oxygen for catalysis. Many different reducing agents, such as gallic acid, ascorbic acid, or reduced glutathione, can provide the electron. In the case of lignocellulose, lignin can act as the electron donor (Kracher et al., 2016). Also, it has been shown that cellobiose dehydrogenase can promote LPMO activity (Langston et al., 2011) which may have physiological significance as its production is upregulated with other cellulases.

Unlike hydrolytic cellulases, most LPMOs consist of a single module (Horn et al., 2012a) and are thought to bind to crystalline cellulose through cellulose binding face that contains the copper binding site (Li et al., 2012), which indicates that LPMOs are active on crystalline cellulose. While the synergism between LPMOs and CBHs can be explained with the cooperation of endo- and exo- activities (see below) the apparent synergistic effect between LPMOs and EGs (two endo- acting enzymes) is less intuitive. The synergism is likely due to their different substrate specificities: LPMOs preferably target crystalline regions of cellulose while EGs have a higher affinity towards amorphous

regions (Nakagawa et al., 2013). No synergy between LPMOs and hydrolytic cellulases has been detected on amorphous substrates (Aachmann et al., 2012).

2.2.1. The cellulolytic system of *Trichoderma reesei*

The white rot fungus *Trichoderma reesei* (anamorph of *Hypocrea jecorina*, the name *Hypocrea* is recommended to be discontinued in favor of *Trichoderma*) (Rossman et al., 2013) was isolated in the South Pacific in the 1940s. *Trichoderma reesei* received attention due to its ability to efficiently degrade cellulose and ever since the cellulases of *Trichoderma reesei* have been subject of intensive research. Most commercial cellulase cocktails consist of enzymes produced by *Trichoderma* species, less often enzymes from *Aspergillus niger* are used as well.

Trichoderma reesei produces two CBHs (Cel7A and Cel6A), that together account for more than 80% of the produced cellulases. Its most prominent cellulase, *Tr*Cel7A (formerly CBHI), is a reducing end specific CBH (Imai et al., 1998) that uses retaining mechanism for catalysis. Two glutamate residues are proposed as catalytic residues: Glu217 as the acid/base and Glu212 as the nucleophile (Divne et al., 1994; Ståhlberg et al., 1996). A third residue, Asp214, is likely involved in the catalysis, however, its role is not yet elucidated (Payne et al., 2015). The substrate binding tunnel of *Tr*Cel7A is formed from four surface loops adjacent to a β-sandwich structure. The 50-Å long tunnel is lined with tryptophan residues and accommodates 10 binding sites for glucose residues (Divne et al., 1998). The subsites are numbered from –7 to +3 with negative numbers for substrate entry and positive numbers for product binding sites.

*Tr*Cel6A (formerly CBHII) is a non-reducing end specific CBH (Barr 1996) that cleaves β-glycosidic bonds with the inverting mechanism. Asp221 has been identified as the catalytic acid and Asp175 is needed to stabilize the transition state (Koivula et al., 2002). In *Tr*Cel6A the active site is covered with two surface loops forming a 20-Å long tunnel adjacent to a distorted β/α -barrel structure. Similarly to *Tr*Cel7A, the tunnel is lined with tryptophan residues. *Tr*Cel6A has altogether at least six binding sites for glucose residues numbered from –2 to +4 (Koivula et al., 1998). Since *Tr*Cel6A is specific to non-reducing end, "+" denotes the "substrate" side of the active site and "–" denotes the "product" side.

The CBHs of *Trichoderma reesei* are not true exoenzymes (Ståhlberg et al., 1993). The surface loops forming the roof of the substrate binding tunnel are flexible and through conformational changes can expose the active site and enable occasional endo attacks (Rouvinen et al., 1990). In contrast, more rigid active site roof leads to increased processivity (Kurašin and Väljamäe, 2011; von Ossowski et al., 2003). *Tr*Cel6A has two surface loops while *Tr*Cel7A has four surface loops forming the roof of the active site. Also, electron microscopy observations of partially hydrolyzed cellulose microfibrils suggested higher

processivity for *Tr*Cel7A (Chanzy and Henrissat, 1985; Imai et al., 1998) and, therefore, it was believed that *Tr*Cel7A has higher processivity than *Tr*Cel6A. Recent findings, however, contradict this hypothesis and show that *Tr*Cel6A displays higher processivity instead (Nakamura et al., 2016). Also, the probability of endo initiation is higher for *Tr*Cel7A than it is for *Tr*Cel6A (Badino et al., 2017).

The cellulolytic system of *Trichoderma reesi* includes at least six EGs: Cel5A, Cel5B, Cel7B, Cel12A, Cel45A, and Cel74A with Cel5A and Cel7B being the most abundant of the set. While the need for two CBHs can be explained by their different chain end specificity, the need for multiple EGs is still poorly understood.

*Tr*Cel7B (formerly EGI) is homologous to the CBH *Tr*Cel7A and shares 45% identity (Penttilä et al., 1986). The most notable difference is the lack of four surface loops that form the roof of the substrate binding tunnel in *Tr*Cel7A (Kleywegt et al., 1997). This makes the active site of *Tr*Cel7B cleft-shaped and promotes endo-activity of the enzyme. Another important difference between *Tr*Cel7A and *Tr*Cel7B lies in the product sites +1 and +2. *Tr*Cel7B lacks three arginine residues present in *Tr*Cel7A that are thought to participate in hydrogen bonding with the product and, thus, boost processive motion. This is in agreement with the paradigm of EGs possessing little processivity. As all family 7 GHs, *Tr*Cel7B uses the double displacement mechanism for catalysis with Glu197 identified as the nucleophile (Mackenzie et al., 1997).

*Tr*Cel5A (formerly EGII) uses the retaining mechanism for cellulose hydrolysis with Glu218 being the catalytic acid and Glu329 the nucleophile. *Tr*Cel5A features $(\beta/\alpha)_8$ barrel topology and a wide and shallow active site groove with five binding sites for glucose residues (Lee et al., 2011). Similarly to most EGs, *Tr*Cel5A does not exhibit a significant degree of processivity and is weakly inhibited by cellobiose. *Tr*Cel5A exhibits higher thermal stability than other major cellulases of *Trichoderma reesei* (namely *Tr*Cel7A, *Tr*Cel7B, and *Tr*Cel6A). Baker *et al* have reported T_m of 75 °C for *Tr*Cel5A that is approximately 10 °C higher than any of the three other major cellulases (Baker et al., 1992). *Tr*Cel5B, another EG belonging to the same GH family, has been predicted by sequence data, but little else is known about this enzyme (Foreman et al., 2003).

*Tr*Cel12A and *Tr*Cel45A (formerly EGIII and EGV, respectively) are minor components of the *Trichoderma reesei* cellulase system. Both enzymes are relatively small in size, which is thought to be advantageous as it gives these enzymes access to small pores in plant cell wall. *Tr*Cel12A consists of a β-sandwich structure with the active site formed by its concave surface (Sandgren 2001). Unlike other *Trichoderma reesei* EGs, *Tr*Cel12A does not have multidomain structure and consist only of CD. *Tr*Cel12A uses the retaining mechanism with Glu116 as the nucleophile and Glu200 as the proton donor (Okada et al., 2000). It has a wider spectrum of substrates than most cellulases and also exhibits activity towards xyloglucan. The crystal structure for *Tr*Cel45A has not been reported yet. Family 45 GHs use the inverting

mechanism for cellulose hydrolysis with aspartic acid residues for catalytic acid and base.

*Tr*Cel74A was first predicted from sequence data (Foreman et al., 2003). EG and xyloglucanase activities have been reported for this enzyme (Benkő et al., 2008). Also, as a family 74 GH, it is known to use the inverting mechanism for catalysis, however, little else is known about this enzyme.

Trichoderma reesei has been shown to produce two β-glucosidases (Cel1A and Cel3A) (Mach et al., 1995; Takashima et al., 1999); additional five β-glucosidases have been predicted by sequence data (Cel1B, Cel3B, Cel3C, Cel3D, and Cel3E) (Foreman et al., 2003). The β-glucosidases of *Trichoderma reesei* appear both as extracellular and cell wall bound enzymes. The enzyme bound to cell wall ensures higher glucose uptake after cellobiose hydrolysis. *Trichoderma reesei* produces β-glucosidases at relatively low concentrations. Moreover, *Trichoderma reesei* β-glucosidases are more sensitive to product inhibition than those of *Aspergillus* species, which is why β-glucosidases from species of *Aspergilli* are more frequently used in enzyme preparations for industrial scale saccharification.

Trichoderma reesei cellulolytic system includes one LPMO, Cel61B, previously classified as GH 61. This enzyme is currently assigned to the auxiliary activity family AA9. The *Tr*Cel61B crystal structure has been solved (Karkehabadi et al., 2008).

2.3. Kinetics of cellulase catalyzed cellulose hydrolysis

In order to improve the performance of cellulases in industrial applications, a better understanding of the cellulase catalyzed cellulose hydrolysis is required. However, there are several limitations that complicate cellulase studies. Cellulose hydrolysis takes place on the solid-liquid interface and the formation of productive enzyme-substrate complex involves several steps. Cellulases have multiple binding modes on cellulose, both productive and nonproductive. Uneven spatial distribution of both substrate and enzyme further complicates the process.

A rapid decrease in rates of cellulase catalyzed cellulose hydrolysis is commonly observed (Lynd et al., 2002; Zhang et al., 1999). The initial hydrolysis rate often decreases more than an order of magnitude within the first minutes of the hydrolysis and cannot be explained by the depletion of the substrate (Bansal et al., 2009; Zhang and Lynd, 2004). This phenomenon has mostly been attributed to CBHs and is often linked to the processive action of these enzymes (Kipper et al., 2005; Praestgaard et al., 2011), but similar behavior at lesser extent has been observed with EGs as well (Murphy et al., 2012). Both substrate- and enzyme-related factors have been proposed to be the cause. The mechanisms proposed to be responsible mainly fall into four categories: (a) product inhibition by cellobiose (Bezerra and Dias, 2004), (b) substrate conversion into more recalcitrant form (Desai and Converse, 1997; Nidetzky and

Steiner, 1993; Zhang et al., 1999), (c) inactivation of the enzyme (Ma et al., 2008), (d) steric hindrance either by other bound enzymes (Bommarius et al., 2008; Igarashi et al., 2011; Väljamäe et al., 1998; Xu and Ding, 2007) or by non-cellulosic polymers of lignocellulose (Eriksson et al., 2002; Várnai et al., 2010).

2.3.1. Processive cycle of CBHs

CBHs are processive enzymes, meaning that CBHs can perform multiple catalytic acts without dissociating from the substrate. In order to elucidate the mechanism of the rapid decrease of rate, it is essential to identify the step in the catalytic cycle that is the bottleneck and limits cellulose hydrolysis. The catalytic cycle of CBHs (with the example of *Tr*Cel7A) can be divided into several distinct steps (Figure 4) (Bansal et al., 2009; Beckham et al., 2011; Payne et al., 2015): (a) binding to insoluble substrate through CBM and/or CD (b) surface diffusion to locate a free reducing end of a cellulose chain (c) threading a cellulose chain into the substrate binding tunnel and formation of productive enzyme-substrate complex (d) hydrolysis of the glycosidic bond (e) product expulsion and formation of another productive enzyme-substrate complex (f) desorption.

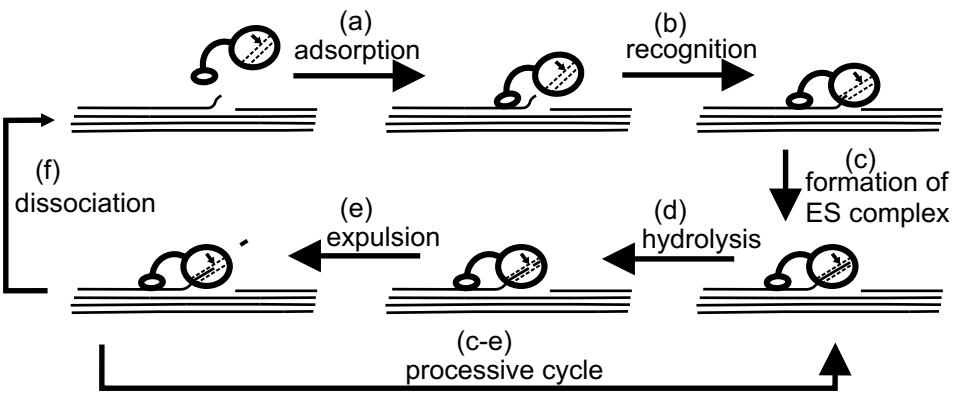

Figure 4. The processive cycle of *Trichoderma reesei* cellobiohydrolase Cel7A acting on cellulose. (a) CBM mediated binding to the cellulose surface. (b) Recognition of a free reducing cellulose chain end on the cellulose surface. (c) Threading of a cellulose chain into the active site of the cellulase and formation of the productive enzyme-substrate complex. (d) Hydrolysis of the β-1,4-glycosidic bond. (e) Product expulsion. Steps (c), (d) and (e) are repeated until the complete degradation of the cellulose chain or enzyme dissociation (f).

Adsorption to the insoluble substrate is a prerequisite of cellulose hydrolysis and it has been shown that *Trichoderma reesei* cellulases preferably bind to the hydrophobic face of the cellulose crystal (Lehtio et al., 2003; Liu et al., 2011). Depending on the substrate and enzyme used achieving the binding equilibrium

can take up to several hours (Maurer et al., 2012), however, the initial binding through CBM is believed to be rapid and not rate limiting for the processive cycle of CBHs (Cruys-Bagger et al., 2014; Kurašin and Väljamäe, 2011). Binding through CBM only is nonproductive. In order to form a productive enzyme-substrate complex, a single cellulose chain needs to be displaced from the crystal and threaded into the catalytic site. CBHs primarily act on chain ends meaning that on substrates with high DP the concentration of free chain ends on cellulose surface could limit the hydrolysis. Endo-initiation is also possible, but this is likely energetically less favorable for processive CBHs (Kurašin and Väljamäe, 2011). The energy required to decrystallize a single cellulose chain depends on the cellulose polymorph and on which face the process occurs (Skopec et al., 2003). Different cellulose polymorphs display different digestibility, indicating that on certain substrates chain displacement could be a rate-limiting step (Gao et al., 2013).

Different methods to determine the k_{cat} of *Tr*Cel7A catalyzed cellulose hydrolysis (for steps c-e in figure 4) have yielded somewhat different results. Using soluble cellooligosaccharides Nidetzky *et al.* determined k_{cat} values of 4.0 s^{-1} and 9.5 s^{-1} for *Tr*Cel7A on cellotetraose and cellohexaose, respectively (Nidetzky et al., 1994a). Gruno *et al.* used short hydrolysis times to assess the initial reaction rates and to determine the k_{cat} of *Tr*Cel7A catalyzed cellulose hydrolysis. The k_{cat} values of 1.5 ± 0.3 s^{-1}, 1.7 s^{-1}, and 2.5 ± 0.3 s^{-1} were found for RAC, BC, and BMCC, respectively (Gruno et al., 2004). Cruys-Bagger *et al.* obtained similar results using transient kinetics. The reported k_{cat} values of *Tr*Cel7A catalyzed cellulose hydrolysis were 5.1 s^{-1}, 4.75 s^{-1}, and 2.4 s^{-1} on RAC, Avicel, and BMCC, respectively (Cruys-Bagger et al., 2012, 2013a). These results are well in line with the results obtained using high-speed atomic force microscopy (Igarashi et al., 2009). The reported velocity of *Tr*Cel7A molecules moving on the cellulose surface was 7.1 ± 3.9 nm/s (Igarashi et al., 2011). Since the length of a cellobiose unit in the cellulose chain is approximately 1 nm (Gardner and Blackwell, 1974) the velocity of *Tr*Cel7A translates into a catalytic constant of 7.1 ± 3.9 s^{-1}. However, β-glucosidases are capable of hydrolyzing β 1–4 glycosidic bond with a rate of more than one order of magnitude greater (Teugjas and Väljamäe, 2013a), indicating that the constraints of the catalysis are evolutionary rather than physical. Under the light of these observations, it seems reasonable to assume that the rate limitation of the processive cycle does not lie in the catalytic step (steps c–e in figure 4).

It has also been proposed that the rate limiting step of the processive cellulose hydrolysis can lie in the dissociation step (Cruys-Bagger et al., 2012, 2013b, 2013a; Kari et al., 2014; Praestgaard et al., 2011). CBHs are processive enzymes and after each catalytic step the enzyme can either continue the processive cycle or dissociate. If the processive movement is hindered, the CBH needs to dissociate first in order to start another processive run. If the dissociation rate is lower than the adsorption rate CBH molecules bound to cellulose nonproductively will accumulate and the rate of cellulose hydrolysis will be governed by the dissociation rate. However, it must be noted that the

adsorption rate depends on the substrate concentration and at low substrate concentrations adsorption can become limiting instead (Cruys-Bagger et al., 2013a; Kari et al., 2014).

2.3.2. Synergism in cellulose hydrolysis

Different cellulases in cellulolytic system display synergism, meaning that the joint activity of the enzymes is greater than the sum of the individual enzyme activities measured separately. Quantitatively it is expressed as degree of synergism (DS) that is the activity of the mixture of the enzymes divided by the sum of the separate activities of the individual components. DS depends on the form of the substrate as well as the ratio of enzymes used.

Several types of synergism between cellulases have been described: a) endo-exo synergism between EGs and CBHs (Eriksson et al., 2002; Henrissat et al., 1985; Väljamäe et al., 1999; Wood and McCrae, 1972) b) exo-exo synergism between CBHs with reducing end and nonreducing end specificity (Badino et al., 2017; Barr et al., 1996) c) synergism between cellulases and β-glucosidases (Singhania et al., 2013) d) synergism between hydrolytic cellulases and LPMOs (Harris et al., 2010). Additionally, intramolecular synergism between CD and CBM is recognized (Din et al., 1994; Kont et al., 2016). However, synergism between different EGs has not been clearly demonstrated (Lynd et al., 2002).

Endo-exo synergism is the most widely studied type of synergy and is believed to be the most significant during cellulose hydrolysis. The canonical model of endo-exo synergism states that EGs generate free chain ends on the cellulose surface for CBHs to act upon (Wood and McCrae, 1972). In agreement with this model, endo-exo synergism is more prominent on substrates with higher DP and on semicrystalline substrates, such as BC and cotton fibers. However, some inconsistencies with this model exist. Different CBHs have different ratios of endo and exo activities for maximum DS. The reported CBH:EG ratio of 95:1 for Cel6A is consistent with the canonical model, while the CBH:EG ratio of 1:1 for Cel7A indicates some other mechanism (Henrissat et al., 1985). However, one must bear in mind that the DS in the case of endo-exo synergism also depends on substrate concentration, which on certain substrates manifests in the form of apparent substrate inhibition (Väljamäe et al., 2001). This means that the optimal enzyme ratios may differ at different cellulose concentrations. Today it is believed that synergism between CBHs and EGs is a more complex process and the model according to which EGs promote CBH activity by generating free chain ends on cellulose surface can only partly explain the synergistic effect (Kostylev and Wilson, 2012). Alternative models propose that EGs promote CBH processivity by removing obstacles on CBH's path (Eriksson et al., 2002; Väljamäe et al., 1999).

2.3.3. Binding

Cellulose hydrolysis takes place on the solid liquid interface for which binding is a prerequisite. Modular cellulases bind to cellulose primarily through CBM using specific noncovalent interactions. CD and linker regions can also adsorb specifically to cellulose independently, but CD, in contrast to CBM, has a greater affinity towards amorphous parts of cellulose (Ståhlberg et al., 1991). Cellulases have been shown to bind preferably to the hydrophobic faces of the cellulose crystal (Liu et al., 2011), which constitutes for a relatively small fraction of the total surface (Nimlos et al., 2012). While cellulases can also bind to hydrophilic surfaces, the affinities for these regions are lower. Additionally, cellulases can bind to lignin, but these interactions are thought to be nonspecific (Berlin et al., 2005; Rahikainen et al., 2013).

Cellulase binding on cellulose is often described with Langmuir isotherm (Equation 1) (Creagh et al., 1996; Kim et al., 1998), which usually gives a reasonably good fit with experimental data.

$$B = \frac{A_{max}[F]}{K_d + [F]} \quad (1)$$

Where B (nmol/g) is bound enzyme per gram of substrate, A_{max} (nmol/g) is the binding capacity of the substrate, K_d (nM) is the dissociation equilibrium constant for enzyme-substrate complex, and [F] (nM) is the concentration of unbound enzyme.

The Langmuir model assumes uniform binding sites, single binding mode, and no interactions between adsorbed molecules. However, modular cellulases can bind to cellulose through different domains independently, so that there are multiple binding modes. Additionally, the higher order structures of cellulose render the binding sites non-uniform as well as enable entrapment of cellulase molecules in cellulose pores. To overcome these limitations Langmuir model with additional binding modes is often used (Jung et al., 2002; Linder et al., 1996; Medve et al., 1997; Ståhlberg et al., 1991). Alternatively, Freundlich isotherm (Jiang et al., 2013; Medve et al., 1997) and Hill's cooperative model (Sugimoto et al., 2012) have been used.

All these models assume equilibrium between bound and free enzyme for which binding reversibility is a critical prerequisite. While many studies have clearly demonstrated fully reversible cellulase binding (Bothwell et al., 1997; Carrard and Linder, 1999; Linder and Teeri, 1996; Palonen et al., 1999), there are several studies that report irreversible or partially reversible binding (Kyriacou et al., 1989; Maurer et al., 2012; Moran-Mirabal et al., 2011; Nidetzky et al., 1994b; Palonen et al., 1999). Nidetzky *et al* have suggested that the inconsistencies stem from the multi-domain structure of the enzyme (Nidetzky et al., 1994b). The adsorption of Cel7A core protein was found to be fully reversible, while the intact enzyme displayed irreversible binding. Studies with purified CBMs have given different results. The adsorption of *Tr*Cel7A

CBM was fully reversible (Linder and Teeri, 1996), while the adsorption of *Tr*Cel6A CBM was irreversible (Carrard and Linder, 1999). Jung et al have suggested that the binding reversibility may depend on the concentration of the cellulase. Experiments with *Thermobifida fusca* cellulases Cel5A, Cel6B, and Cel48A revealed reversible binding at lower enzyme concentrations, while at higher enzyme concentrations the binding was rendered irreversible (Jung et al., 2002).

2.3.4. Michaelis Menten model in enzymatic cellulose hydrolysis

Enzymatic reactions are typically described with Michaelis Menten kinetics. The catalysis is seen as a two step process consisting of a) reversible binding step and formation of productive enzyme-substrate complex b) catalysis and product formation (Equation 2).

$$E + S \rightleftharpoons ES \rightarrow E + P \tag{2}$$

Using the assumption that the total enzyme concentration is much lower than the concentration of the substrate, the rate of the product formation can be given by Michaelis-Menten equation (Equation 3) (Michaelis and Menten, 1913):

$$v = \frac{k_{cat} E_0 [S]}{K_M + [S]} \tag{3}$$

where v is the rate of product formation; k_{cat} is the catalytic rate constant; E_0 is the concentration of the enzyme; [S] is the concentration of the substrate and K_M is the Michaelis constant.

Enzymatic reactions are saturable. Under saturating conditions all enzyme molecules are in productive complex with substrate and increasing the substrate concentration has no further effect on the reaction rate. The rate of the reaction approaches V_{max} and can be given by (Equation 4).

$$v \approx V_{max} = k_{cat} \times E_0 \tag{4}$$

Michaelis Menten kinetics is based on mass action law and, therefore, requires homogeneous reaction conditions. Enzymatic cellulose hydrolysis, however, takes place on cellulose surface and is, therefore, spatially constrained. The excess substrate-to-enzyme ratio used for the quasi-steady state assumption is difficult to achieve, since only a fraction of the cellulose is accessible for cellulases (Hong et al., 2007). Moreover, there are multiple binding modes for cellulases, meaning that while seemingly the saturating conditions can be achieved, all enzyme molecules are not in productive enzyme-substrate complex. For heterogeneous reactions the assumptions used in classical enzyme kinetics do

not hold, the inconsistencies manifest as fractal kinetics with apparent rate orders and time-dependent rate constants (Kopelman, 1988; Väljamäe et al., 2003).

Despite these limitations Michaelis Menten kinetics has been used to describe cellulase catalyzed cellulose hydrolysis (Bansal et al., 2009; Bezerra and Dias, 2004, 2005; Kari et al., 2014). Bezerra and Dias have used integrated Michaelis-Menten equation to determine the kinetic parameters of *Tr*Cel7A catalyzed cellulose (Avicel) hydrolysis and found the k_{cat} to be 2 h^{-1} (Bezerra and Dias, 2004). Cruys-Bagger *et al.* have proposed a kinetic model for processive enzymes to describe the CBH catalysed cellulose hydrolysis. The model is based on the quasi steady-state approximation and the rate of the reaction can be expressed by a hyperbolic function similar to Michaelis-Menten equation (Cruys-Bagger et al., 2013b).

2.3.5. Kinetics of the hydrolysis of low molecular weight model substrates

Because of the complex kinetics observed with polymeric substrates, low molecular weight substrates have often been used in cellulase studies. Amongst these substrates oligosaccharides and chromo- or fluorophore labeled oligosaccharides are most often used. Labeled glucose, cellobiose, and lactose have turned out to be useful in measuring the activity of specific cellulases in complex mixtures.

These low molecular weight substrates consist of mono- or oligosaccharide linked to a chromo- or fluorophore through glycosidic bond (Figure 5). As long as the label is bound to the saccharide, its specific signal cannot be detected. However, when the glycosidic bond between the label and oligosaccharide is hydrolyzed the label is released and it can be quantified by its specific absorbance or fluorescence.

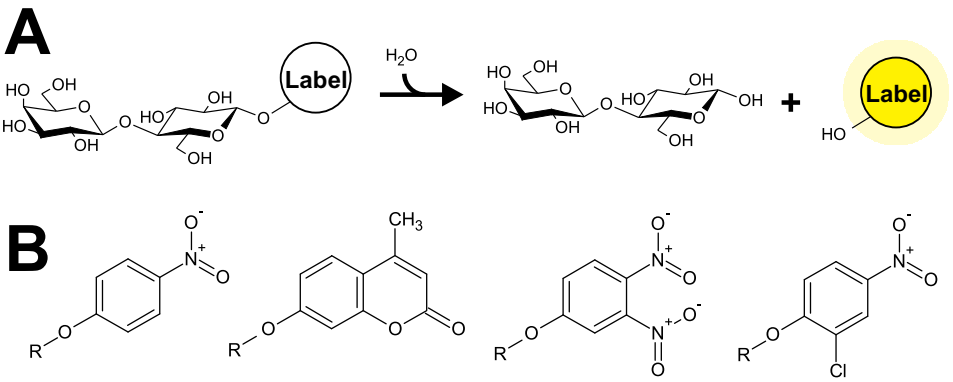

Figure 5. Low molecular weight model substrates used in cellulase studies. **(A)** Hydrolysis of labeled lactose. The specific signal of the label can be quantified once it has been released from the saccharide. **(B)** Different labels used in cellulase studies. From the left to the right: para-nitrophenyl group; 4-methylumbelliferyl group; 1,4-dinitrophenyl group; 2-chloro-4-nitrophenyl group. R in the formulae denotes the sugar moiety.

The most common chromogenic labels used in cellulase studies are phenyl group derivates such as para-nitrophenol (pNP) (van Tilbeurgh et al., 1982), 3,4-dinitrophenol (Capon and Thomson, 1979) and 2-chloro-4-nitrophenol (van Tilbeurgh et al., 1988). Under alkaline conditions, these labels can be detected by their specific absorbance. Fluorescence allows more sensitive determination of enzymatic activity. 4-methylumbelliferone (MU) is the most common fluorophore used in cellulase studies (van Tilbeurgh et al., 1982).

Enzymes from different GH families have different hydrolysis patterns on different labeled and non labeled cellooligosaccharides. This specificity allows detection and quantification of different cellulolytic activities from crude culture filtrates, chromatography samples and other complex mixtures (Deshpande et al., 1984).

GH family 7 cellulases (*Trichoderma reesei* cellulases *Tr*Cel7A and *Tr*Cel7B) are capable of releasing the label from reducing end labeled lactose, cellobiose, and cellotetraose (Claeyssens and Henrissat, 1992; Claeyssens et al., 1989; van Tilbeurgh and Claeyssens, 1985). Reactions with labeled cellobiose and cellotetraose, however, are not specific to GH family 7 and these substrates enable multiple reactions. For this reason, labeled lactosides are often preferred (Claeyssens et al., 1990). Within the family, *Tr*Cel7A and *Tr*Cel7B can be further distinguished by their different inhibition by cellobiose as it is a much stronger inhibitor for CBHs than it is for EGs (Gruno et al., 2004; van Tilbeurgh and Claeyssens, 1985).

*Tr*Cel5A can uniquely release the label from reducing end labeled trisaccharides. This reaction, however, is not suitable for detecting *Tr*Cel5A from enzyme mixtures, because the label can also be released from cellotrioside by the sequential activity of GH family 7 cellulases and β-glucosidases.

β-glucosidases can be detected with labeled glucose, this reaction is strongly inhibited by gluconolactone (Reese and Mandels, 1960).

Not all cellulases can be detected with these labeled oligosaccharides. For example, while *Tr*Cel6A is capable of hydrolyzing labeled cellooligomers with three or more glucose residues, the hydrolysis occurs at the glycosidic bond between the second and the third glucose residue from the nonreducing end and the label is not released through its action (Claeyssens et al., 1989; Tilbeurgh et al., 1985).

The kinetic parameters of hydrolysis of low molecular weight substrates correlate poorly with those of cellulose hydrolysis. The label of the model substrates represents a steric hindrance to the cellulase and the binding to the active center may be hampered. This effect varies with different labels (Konstantinidis et al., 1993). In the case of cellooligomers, it has been shown that the kinetic parameters depend on the oligomer length used as a substrate (Koivula et al., 2002; Nidetzky et al., 1994a). The substrate binding tunnels and clefts of cellulases have multiple binding sites for glucose units with different binding affinities. This means that the binding affinities strongly depend on the cellooligomer length with no clear correlation between the cellooligomer length and binding affinity (Koivula et al., 2002). In addition, most cellulases are

capable of catalyzing transglycosylation in parallel to the hydrolysis (Vršanská and Biely, 1992).

While soluble model substrates have been used in inhibition studies, it is important to note that the mechanisms of inhibition can be different for soluble and insoluble substrates (Kuusk et al., 2015; Olsen et al., 2015). In the case of cellulose hydrolysis with CBHs cellobiose acts as a noncompetitive inhibitor. It binds to the product subsites of the substrate binding tunnel and stalls the processive movement of the cellulase. In the case of soluble substrates, cellobiose acts as a competitive inhibitor. For the hydrolysis to occur part of the model substrate needs to bind to the product subsite(s) of the substrate binding tunnel. A cellobiose molecule bound to the product subsites, thus, inhibits the binding of the model substrate.

All in all, these substrates can be used to determine the optimal working conditions for cellulases and their mutants (von Ossowski et al., 2003; Tuohy et al., 2002), but the kinetic parameters obtained using soluble substrates cannot be used to estimate the kinetic parameters on cellulose (Teugjas and Väljamäe, 2013b).

3. AIMS OF THE STUDY

The aim of this study is to investigate enzymatic cellulose hydrolysis and cellulase binding to cellulose on active site level with the intent of determining the mechanism behind the rapid decline in the rate of CBH catalyzed cellulose hydrolysis

The objectives of this thesis can be summarized as follows:

- Develop a method to determine the concentration of *Tr*Cel7A bound to cellulose at active site level
- Determine the k_{cat} of *Tr*Cel7A catalyzed cellulose hydrolysis
- Investigate the effect of endo – exo synergism on the active site mediated binding and k_{cat}
- Elucidate the role of CBM in cellulose hydrolysis
- Study *Tr*Cel7A binding to cellulose in terms of active site mediated binding and non-productive binding with active site free from cellulose chain.

4. RESULTS

4.1. New methods in cellulase studies

The majority of cellulases are multi-domain enzymes consisting of a catalytic domain, linker peptide, and CBM. The multi-domain structure enables active site mediated binding to cellulose as well as solely CBM mediated binding, meaning that both productive and nonproductive binding are possible. Even though cellulases can seemingly be saturated with the substrate, a large fraction of the enzyme is not in the productive enzyme-substrate complex. Enzymatic cellulose hydrolysis does not follow the Michaelis-Menten kinetics and the use of classical methods for determining the kinetic parameters of cellulase catalyzed cellulose hydrolysis is problematic.

To overcome these limitations, we have developed novel methods for determination of kinetic parameters of *Tr*Cel7A catalyzed cellulose hydrolysis. While somewhat different approaches were used in my research the principle of the methods is the same. Cellulose hydrolysis was carried out in the presence of a reporter molecule so that simultaneous hydrolysis of cellulose and reporter molecule occurs. The rate of the reporter molecule hydrolysis correlates to the concentration of the free active sites of the cellulase and enables the determination of the concentration of cellulase-cellulose complexes. The rate of the cellulose hydrolysis and the concentration of cellulase-cellulose complexes, in turn, enables the determination of the turnover rate constant.

4.1.1. Measuring the occupied active centers (Ref I, II, III)

To distinguish the enzyme molecules bound to the substrate at active site level we have developed a method that relies on specific inhibition of low molecular weight reporter molecule hydrolysis by cellulose. In the presence of cellulose, enzyme molecules that are capable of hydrolyzing the reporter molecule are considered as *Tr*Cel7A with a free active site ($[CBH]_{FA}$) and enzyme molecules that do not contribute to the reporter molecule hydrolysis register as *Tr*Cel7A with an occupied active site ($[CBH]_{OA}$). Family 7 GHs are capable of releasing the label from reducing end labeled lactosides and cellobiosides. When labeled lacosides or cellobiosides are used as the reporter molecule the glucose unit binding sites –2, –1, and +1 of the substrate binding tunnel of *Tr*Cel7A must be free from the cellulose chain (Figure 6). Thus, the free *Tr*Cel7A in solution and *Tr*Cel7A-cellulose complexes with the reducing end of the cellulose chain in the glucose unit binding sites up to –3 are capable of releasing the label and are considered $[CBH]_{FA}$. Enzyme substrate complexes with the reducing end of the cellulose chain in the binding sites –2, –1, +1, or +2 of the cellulose binding tunnel are referred to as $[CBH]_{OA}$ (Figure 7).

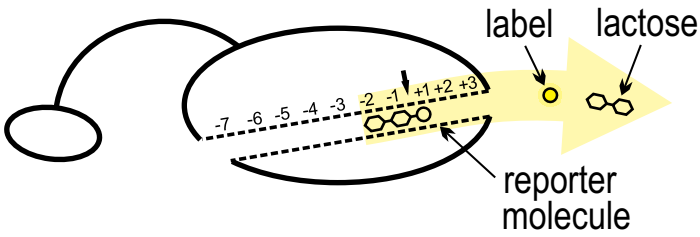

Figure 6. Binding of reporter molecule to the active site of *Tr*Cel7A. The active site of *Tr*Cel7A contains 10 binding sites for glucose residues. The reporter molecule needs to bind to the binding sites –2, –1 and +1 for the hydrolysis to occur.

The rate of the reporter molecule hydrolysis is in correlation with $[CBH]_{FA}$ and by using reference experiments $[CBH]_{FA}$ values can be found. $[CBH]_{OA}$ in turn can be found from the difference between the concentration of total enzyme ($[CBH]_{Total}$) and $[CBH]_{FA}$ (Equation 5).

$$[CBH]_{OA} = [CBH]_{Total} - [CBH]_{FA} \quad (5)$$

The reporter molecule used in this method must meet several criteria. Firstly, the detection of the released label must not be interfered by cellulose hydrolysis. Secondly, the reporter molecule must not interfere with cellulose hydrolysis. Thirdly, the inhibition of reporter molecule hydrolysis must be solely caused by competition with cellulose chain.

In the case of *Tr*Cel7A para-nitrophenyl β-D-lactoside (pNPL) and 4-methylumbelliferyl β-D-lactoside (MUL) were found to be suitable reporter molecules. The hydrolysis results in formation of the detectable chromo- or fluorophore and lactose (Figure 5) (Claeyssens et al., 1990). The released pNP or MU are detectable in alkaline conditions by absorbance or fluorescence, respectively.

The cellulose hydrolysis with CBHs yields cellobiose, which inhibits reporter molecule hydrolysis. An excess of β-glucosidase is required to counter this inhibition, which, in turn, means that control experiments are needed to take into account the β-glucosidase's small activity towards the reporter molecule. If lignocellulose is used in the experiment lignin components released during the hydrolysis can interfere with the detection of pNP and MU. Also, MU, and to a lesser extent, pNP both bind to lignocellulose. This means that the results on lignocellulose are error prone with $[CBH]_{OA}$ being overestimated.

Also, it must be noted that since this method relies on the enzyme's ability to hydrolyze the reporter molecule, it can only be applied to enzymes for which a suitable reporter molecule is available. For example, this method cannot be used to determine the $[CBH]_{OA}$ of *Tr*Cel6A and *Tr*Cel5A if pNPL or MUL is used as the reporter molecule. On the other hand, these enzymes do not interfere with the detection of family 7 GHs and, therefore, this method can be applied to synergistic reaction mixtures as well.

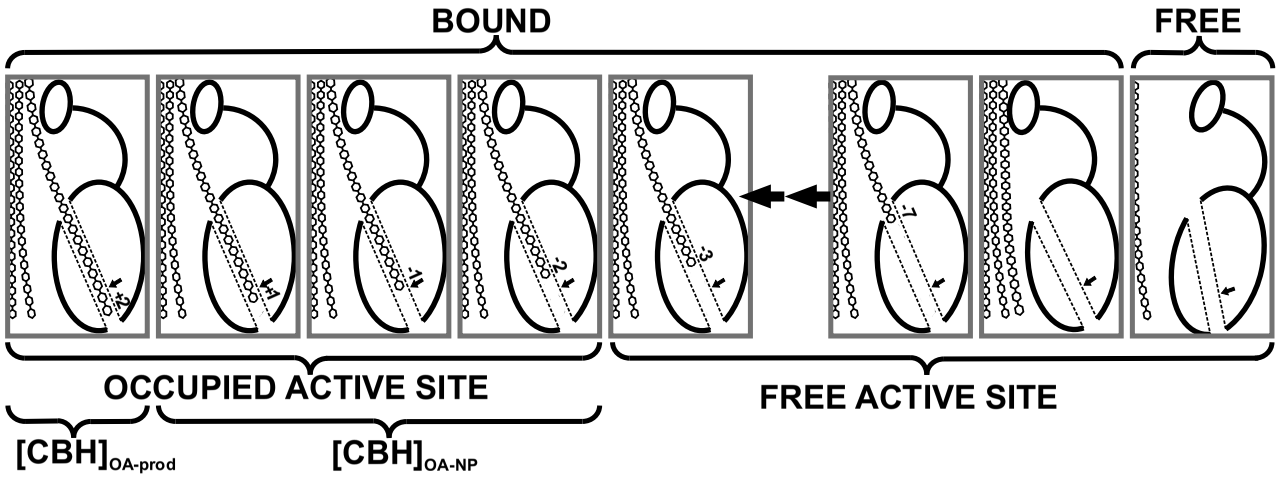

Figure 7 Discrimination between different populations of *Tr*Cel7A molecules. On the left side is the differentiation of bound *Tr*Cel7A ($[CBH]_{bound}$) and free *Tr*Cel7A ($[CBH]_{free}$), on the right side the differentiation between *Tr*Cel7A with active site free for the hydrolysis of reporter molecule ($[CBH]_{FA}$) and *Tr*Cel7A bound on active site level ($[CBH]_{OA}$). $[CBH]_{OA}$ is futher divided into two: nonproductive complexes of *Tr*Cel7A with a cellulose chain in the active site ($[CBH]_{OA-NP}$) and productively bound *Tr*Cel7A ($[CBH]_{OA-prod}$). The glucose unit binding site at which the reducing end of the cellulose chain is situated is numbered. Enzyme-substrate complexes where the reducing end of the cellulose chain is in the glucose binding sites from -6 to -4 are omitted.

In order to determine the rate of reporter molecule hydrolysis and relate it to the $[CBH]_{FA}$ two approaches were used. In (Ref. I) a more complex method was used. The cellulose hydrolysis with *Tr*Cel7A was supplied with pNPL. In order to obtain the rate of pNPL hydrolysis, first, an empirical equation to describe the time course of the pNP formation was found (Equation 6).

$$[pNP] = a(1 - e^{-bt^{1-c}}) \tag{6}$$

Provided with the values of empirical parameters a, b, and c the rate of pNP formation can be found for any time point by using the first order derivative of equation 6 (Equation 7).

$$v_{pNP} = \frac{d[pNP]}{dt} = ab(1-c)t^{-c}e^{-bt^{-c}} \tag{7}$$

In order to relate the v_{pNP} to the $[CBH]_{FA}$ reference curves are needed. In reference samples the cellulose is omitted, so that $[CBH]_{FA} = [CBH]_{Tot}$. While the initial formation of pNP in the reference experiments was linear in time, for longer hydrolysis times pNPL depletion and lactose inhibition must be taken into account. The concentration of the released pNP represents the extent of pNPL hydrolysis as $[pNPL] = [pNPL]_0 - [pNP]$. Also, the concentration of lactose can be found since [pNP] = [Lac]. Since $[CBH]_{FA}$ can change in time when cellulose is present, v_{pNP} was divided with $[CBH]_{FA}$ to take into account the possible changes in $[CBH]_{FA}$. The results of the reference experiments were plotted as $v_{pNP}/[CBH]_{FA}$ *versus* [pNP] and fitted to a rearranged Michaelis-Menten equation including competitive lactose inhibition (Equation 8).

$$\frac{v_{pNP}}{[CBH]_{FA}} = \frac{k_{cat(pNPL)}([pNPL]_0 - [pNP])}{([pNPL]_0 - [pNP]) + K_{M(pNPL)}\left(\frac{1+[pNP]}{K_{i(Lac)}}\right)} \tag{8}$$

In order to obtain the $[CBH]_{FA}$ for a specific time point, we need the v_{pNP} at that time point and the corresponding $v_{pNP}/[CBH]_{FA}$ from the reference curve (designated as $(v_{pNP}/[CBH]_{FA})_{All\text{-}free}$ in Equation 9). The v_{pNP} at the specified time can be calculated using the Equation 7. The corresponding $v_{pNP}/[CBH]_{FA}$ is calculated according to the Equation 8 by using the [pNP] from Equation 6 that corresponds to the same time point. $[CBH]_{FA}$ can then be calculated by using the Equation 9.

$$[CBH]_{FA} = \frac{v_{pNP}}{\left(\frac{v_{pNP}}{[CBH]_{FA}}\right)_{All-free}} \tag{9}$$

A simpler approach to determine the rate of the reporter molecule hydrolysis and the $[CBH]_{FA}$ was used in Ref II and Ref III. In this case the reporter molecule was not present at the initiation of cellulose hydrolysis, instead, it was

added later at a fixed time before the hydrolysis was stopped. In ref II, MUL was used as the reporter molecule and it was added to the reaction 30 seconds before the reaction was quenched. This approach ensures that the times for MUL hydrolysis are the same for each data point. However, it is important that $[CBH]_{FA}$ remains constant throughout the reporter molecule hydrolysis and that the reporter molecule depletion is small enough so it does not have a significant effect on reporter molecule hydrolysis rate. Also, this approach sets a limit to the shortest time interval from the initiation of cellulose hydrolysis that can be used. Since initial rates are used and the times of reporter molecule hydrolysis are the same for all data points, $[CBH]_{FA}$ can be calculated by comparing the concentration of the released label with the results of calibration experiments, where cellulose is omitted.

4.1.2. Determining the observed rate constant of cellulose hydrolysis (Ref I)

The rate of a reaction is the product of the k_{cat} and the concentration of productive enzyme substrate complexes. In order to determine the turnover constant the rates of product formation are needed. We used an empirical equation to describe the time course of glucose formation (Equation 10) and the first order derivative of that equation (Equation 11).

$$[Glc] = a\left(1 - e^{-bt^{1-c}}\right) + gt \tag{10}$$

$$v_{Glc} = \frac{d[Glc]}{dt} = ab(1-c)t^{-c}e^{-bt^{-c}} + g \tag{11}$$

The time courses of glucose formation are fitted to the Equation 10 to obtain the values of empirical parameters a, b, c, and g. Provided with these, the reaction rates for any time point can be found using the Equation 11.

In our experiments, we measured the formation of glucose since an excess of β-glucosidase was included in the reaction mixture. However, the main product of CBH catalyzed cellulose hydrolysis is cellobiose. The rates of glucose formation are divided by two to reflect the formation of cellobiose (v_{CB}) instead of glucose. Using the rate of cellobiose formation and the concentration of enzyme-substrate complex, the observed rate constant for cellulose hydrolysis can be found using the Equation 12 (Figure 8).

$$k_{obs} = \frac{v_{CB}}{[CBH]_{OA}} \tag{12}$$

The found constant is referred to as observed rate constant (k_{obs}) to distinguish it from the true k_{cat}. The $[CBH]_{OA}$ found with the reporter molecule hydrolysis

includes several nonproductive complexes ($[CBH]_{OA\text{-}NP}$) in addition to productive enzyme-substrate complex ($[CBH]_{OA\text{-}prod}$) (Figure 7). The reporter molecules used in the experiments must bind to the glucose binding sites –2, –1, and +1 in the substrate binding tunnel of *Tr*Cel7A for hydrolysis to occur (Figure 6). Nonproductive complexes with cellulose chain end in these subsites also inhibit reporter molecule hydrolysis and are included in the population of $[CBH]_{OA}$ (Equation 13).

$$[CBH]_{OA} = [CBH]_{OA\text{-}prod} + [CBH]_{OA\text{-}NP} \quad (13)$$

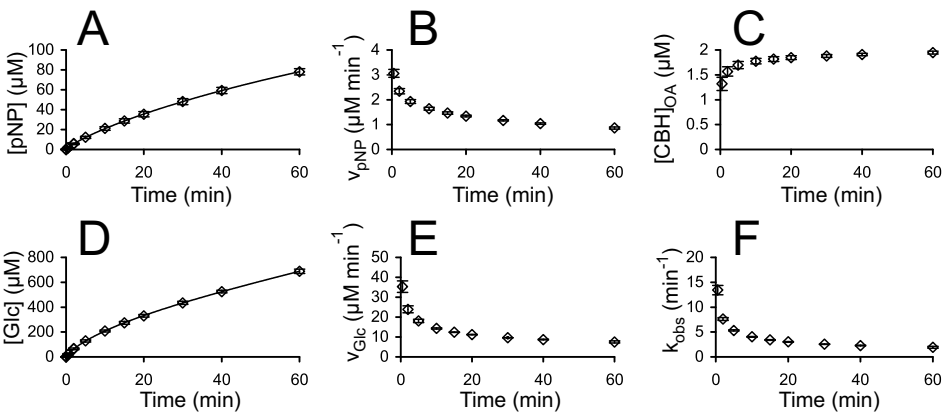

Figure 8. An example of measurement of observed catalytic rate constant for *Tr*Cel7A catalyzed cellulose hydrolysis. Concentrations of *Tr*Cel7A, β-glucosidase, pNPL, and Avicel were 2.5 μM, 0.85 μM, 0.5mM, and 10 mg/ml, respectively. Error bars display standard deviation and are from three independent measurements. **(A)** Time course of pNP released by *Tr*Cel7A. The solid line is according to Equation 6. **(B)** Rate of pNP formation calculated using Equation 7 and parameter values obtained from nonlinear regression analysis of pNP formation using Equation 6. **(C)** Concentration of *Tr*Cel7A with active site occupied by cellulose chain calculated using reference curves, data in panel B, Equation 9, and Equation 5. **(D)** Soluble products released from *Tr*Cel7A catalyzed Avicel hydrolysis in glucose equivalents. The solid line is according to Equation 10 **(E)** Rate of glucose formation calculated using Equation 11 and parameter values obtained from nonlinear regression analysis of glucose formation using Equation 10. **(F)** Values of observed catalytic rate constant calculated according to Equation 12.

Because of the $[CBH]_{OA\text{-}NP}$ the k_{obs} is expected to be lower than the true k_{cat}. The difference between k_{obs} and k_{cat} is determined by the proportion of the $[CBH]_{OA\text{-}prod}$ to the total $[CBH]_{OA}$ as described in the Equation 14.

$$k_{obs} = k_{cat}\left(\frac{[CBH]_{OA-prod}}{[CBH]_{OA-prod}+[CBH]_{OA-NP}}\right) \quad (14)$$

4.1.3. Single turnover method for determining the k_{cat} of cellulose hydrolysis (Ref II)

Enzymatic cellulose hydrolysis displays burst kinetics (Kipper et al., 2005; Praestgaard et al., 2011) and the values of observed rate constants depend on the time used for measurements. The reaction rates need to be measured within the first seconds of the reaction in order to determine the catalytic constant (Cruys-Bagger et al., 2012; Murphy et al., 2012). This limitation can be overcome by using the single turnover method, where each enzyme molecule is allowed to perform only a single processive run on a cellulose chain (Horn et al., 2012b).

In the single turnover method, the enzyme and ^{14}C labeled cellulose are mixed to initiate the hydrolysis. After a short interval, an excess of nonlabeled amorphous cellulose is added to trap all non-bound cellulases (Horn et al., 2012b). Cellulases have a much higher affinity towards amorphous cellulose and under these conditions, each enzyme molecule can perform a single processive cycle on the labeled cellulose and will become trapped on the non-labeled cellulose thereafter. It is important that the trap is added before the enzyme has completed one processive cycle to avoid re-initiations on the labeled cellulose. Formation of the labeled product (expressed in cellobiose equivalents, ^{14}CB) should follow the Equation 15.

$$[^{14}CB] = [^{14}CB]_{max}(1 - e^{-kt}) \quad (15)$$

Where k is the pseudo first order rate constant for passing through one processive run and $[^{14}CB]_{max}$ is the leveling off concentration of the labeled product. $[^{14}CB]_{max}$ is the product of the concentration of productive enzyme-substrate complexes at the trap addition ($[CBH]_{OA}$) and the average number of cellobiose molecules released during one processive run, i.e apparent processivity (P_{app}).

The $[CBH]_{OA}$ can be determined with the use of a reporter molecule hydrolysis as it is described in (3.2.1). Here it is assumed that the initial binding to the cellulose is productive, meaning that all complexes inactive in the hydrolysis of reporter molecule are productive cellulose-cellulase complexes. This assumption is made only for the early stages of the hydrolysis and cannot be generalized. The catalytic constant can be found from the rate constant from Equation 15 and P_{app} as shown in Equation 16.

$$k_{cat} = k \times P_{app} = k \times \frac{[^{14}CB]_{max}}{[CBH]_{OA}} \quad (16)$$

4.2. Mechanism of TrCel7A action on cellulose

4.2.1. Obstacle model (Ref I)

The rate of enzymatic cellulose hydrolysis declines rapidly in time and cannot be explained by the depletion of the bulk substrate (Bansal et al., 2009; Lynd et al., 2002; Zhang and Lynd, 2004). Possible explanations for this decline include

product inhibition (Bezerra et al., 2011), substrate transformation i.e. depletion of "good" substrate so that the ratio of a more recalcitrant form will increase (Nidetzky and Steiner, 1993), depletion of free chain ends on the surface (Desai and Converse, 1997), inactivation of Cel7A through irreversible binding (Ma et al., 2008), and steric hindrance of processive cellulases by hemicellulose, lignin or other bound enzymes (Bommarius et al., 2008; Eriksson et al., 2002; Väljamäe et al., 1998; Várnai et al., 2010; Xu and Ding, 2007).

In our experiments, we also observed a rapid decrease in reaction rate. $[CBH]_{OA}$ did not change significantly throughout the experiments and the decrease in the hydrolysis rate was due to the decrease of k_{obs} in time (Ref I Figure 2). The decrease in k_{obs} did not correlate with the degree of conversion of cellulose nor was there any systematic dependence on the enzyme-to-substrate ratio. The rapid decrease in k_{obs} with hydrolysis time was observed with substrates having different properties such as crystallinity, specific surface area, and the presence of lignin and hemicellulose (Ref I Figure 3).

Since the reactions were supplied with an excess of β-glucosidase, product inhibition cannot be the cause of the decrease in k_{obs}. Depletion of chain ends on the surface should reflect as the decrease of the concentration of active site bound enzyme. As the concentration of the free chain ends on the cellulose surface decreases the population of enzyme bound on active site level should decrease while the population of free enzyme should increase. However, in our experiments $[CBH]_{OA}$ did not undergo significant changes during the hydrolysis (Figure 8 panel C). Steric hindrance by other enzyme molecules bound to the cellulose surface is unlikely to be the main cause of the decline of the reaction rate as the decline was independent of enzyme concentration.

We propose a model, whereby the rapid decline of the reaction rate is caused by obstacles on the path of a processive run. According to this model, the newly formed productive enzyme-substrate complex moves along the cellulose chain at a constant rate. Once the complex encounters an obstacle it is stalled. Since the dissociation rate constant is low (Kurašin and Väljamäe, 2011), the enzyme remains "stuck" on the substrate. The average length of the path the enzyme can pass before becoming stuck is determined by the substrate (Kurašin and Väljamäe, 2011). The contribution of nonproductively bound "stuck" enzyme molecules increases in time, which in turn leads to the decrease of k_{obs}.

The transit times of different steps of the catalytic cycle are in good agreement with this model. Both binding and processive movement are relatively fast, while the transit time for recruitment is more than an order of magnitude longer. The observed association rate constants (k_{on}^{obs}) (Ref II Supplemental Data Table 2) yield transit times for binding between 5 and 10 s. The k_{cat} of $2.2 \pm 0.5\ s^{-1}$ gives a transit time of approximately 0.45 s for passing through steps c-e of a single processive cycle (Figure 9 panel A). Taking into account the processivity of 66 ± 7 CB units predicts a rate constant of $0.033 \pm 0.006\ s^{-1}$ and a transit time of about 30 s for the entire processive run. The dissociation rate constant of *Tr*Cel7A, however, is low ($0.7 \pm 0.1 \times 10^{-3}\ s^{-1}$ on BC) (Kurašin and Väljamäe, 2011) and the predicted transit time for dissociation under these conditions is

around 25 minutes. Since dissociation is much slower than binding and processive run, the k_{obs} of CBH catalyzed cellulose hydrolysis becomes limited by the length of obstacle-free path and k_{off}.

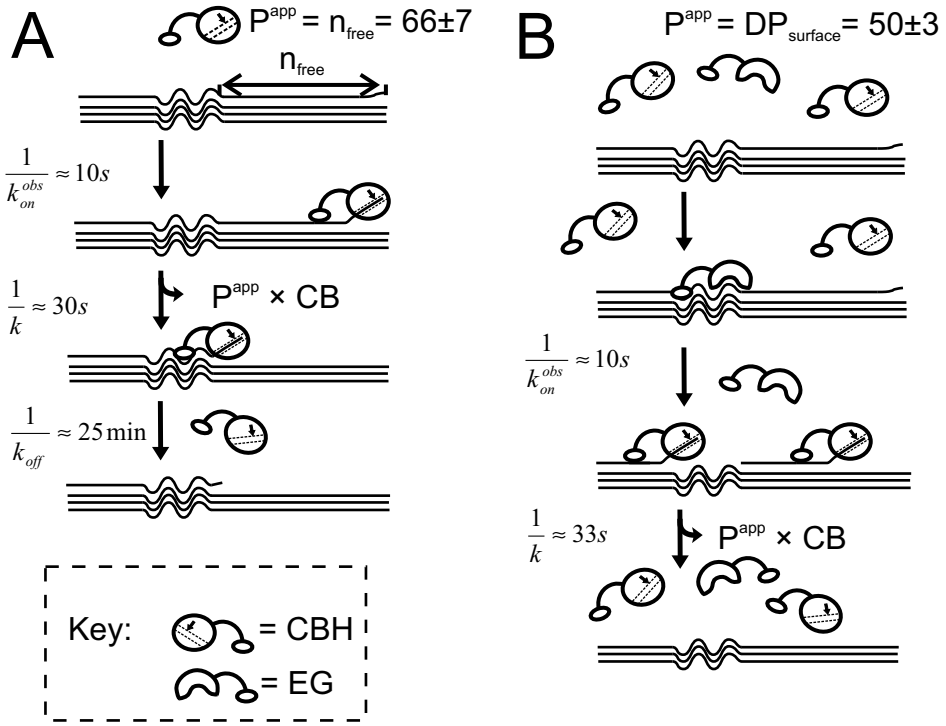

Figure 9. The obstacle model in cellulose hydrolysis with CBH only (A) and with a synergistic mixture of CBH and EG (B). **(A)** Cellulose hydrolysis with an individual CBH. The CBH cannot pass through the amorphous region (wavy lines) and is stalled. The obstacle-free path (n_{free}) is the length of the crystalline region of the cellulose and it limits the apparent processivity of the CBH. Since the transit time for dissociation is much longer than the times for formation of enzyme-substrate complex and processive movement, the steady state rate of CBH catalyzed cellulose hydrolysis is governed by the slow dissociation. The k_{off} value is from (Kurašin and Väljamäe, 2011). **(B)** Synergistic cellulose hydrolysis with CBH and EG. For simplicity, the actions of EG and CBH are depicted as sequential. EGs preferably target amorphous region of cellulose and, thus, generate new chain ends for CBHs to act upon (depicted on the left side of the panel) and also accelerate the recruitment of CBHs by providing ending points (depicted on the right side of the panel). P_{app} for CBHs is determined by the DP of the EG-treated cellulose surface ($DP_{surface}$). The rate of cellulose hydrolysis in steady state approaches the limit set by the velocity of processive movement of CBH.

The nature of the obstacles on the path of processive cellulases can vary with different substrates. BMCC and Avicel are crystalline substrates. In suspension, the microcrystals form aggregates that can interfere with the processive movement of CBH-s. In the case of lignocellulose the noncellulosic polymers can impede the processive movement. In amorphous cellulose the tangled cellulose

chains can form an obstacle on the path of the processive run. BC contains both crystalline regions as well as amorphous and paracrystalline regions. The amorphous regions between crystalline regions could pose an obstacle for processive cellulases.

4.2.2. Alternative mechanism of endo-exo synergism (Ref II)

The conventional view on endo-exo synergism states that the number of free chain ends on cellulose surface limit the hydrolysis. The action of EGs generates additional chain ends for the CBHs to act upon and, thus, increases the ratio of productively bound CBHs (Wood and McCrae, 1972). Indeed, we observed an increase in the concentration of *Tr*Cel7A bound on active site level when reactions were supplemented with EG. However, the increase in $[CBH]_{OA}$ was clearly not sufficient to account for the whole synergistic effect. Largely the synergistic effect was caused by the increase in k_{obs} instead (Figure 10). This means that an additional mechanism contributes to the overall synergism alongside with the conventional mechanism.

In the context of the obstacle model proposed earlier, the rate of the cellulose hydrolysis declines rapidly due to the accumulation of stalled non-productive enzyme-substrate complexes. Our hypothesis is that the added EG increases the k_{obs} and alleviates its decline in time either by removing the obstacles from CBHs path or helping the CBH to overcome them. The k_{cat} 2.2 ± 0.5 s^{-1} found from the single turnover experiment is in agreement with the k_{obs} values of the synergistic cellulose hydrolysis at optimal enzyme-substrate ratios. This indicates that in the presence of EG at the optimal enzyme/substrate ratio the hydrolysis is only limited by the rate of processive movement.

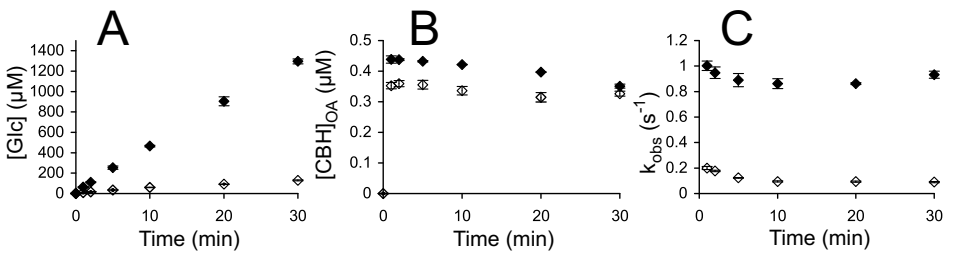

Figure 10. Synergistic effect in BC hydrolysis with CBH *Tr*Cel7A and EG *Tr*Cel5A. *Tr*Cel7A (◇), *Tr*Cel7A + *Tr*Cel5A (◆), error bars display standard deviation and are from three independent measurements. Concentration of *Tr*Cel7A was 0.5 µM, BC concentration was 0.5 mg/ml, and concentration of *Tr*Cel5A, if present, was 0.1µM. All reactions were supplied with 0.85 µM β-glucosidase. **(A)** Glucose formation. **(B)** Concentration of *Tr*Cel7A with cellulose chain in the active site determined by inhibition caused by BC to MUL hydrolysis. **(C)** Observed rate constant values calculated according to the Equation 12.

While the nature of these obstacles is difficult to address experimentally, our experiments suggest that in the case of BC hydrolysis these obstacles can be linked to the amorphous regions of BC. The P_{app} on BC is similar to the length of crystalline regions of the substrate. Our hypothesis is that CBHs cannot pass through the amorphous regions of BC and become stalled upon encountering one. This means that the P_{app} is limited by the length of crystalline parts of BC between amorphous regions. Upon encountering an amorphous part of BC *Tr*Cel7A becomes stalled. The rate of the reaction drops rapidly as more and more enzyme molecules become inactive. EGs, on the other hand, are more active on amorphous substrates. If EGs are present in the reaction mixture, the amorphous regions will be degraded and the recruitment of CBHs is, thus, accelerated (Figure 9 panel B).

The relative contribution of the conventional mechanism of endo-exo synergism and the mechanism described here depends on the enzyme-to-substrate ratio. The contribution of the conventional mechanism of synergism is higher at high enzyme-to-substrate ratios. At low enzyme-to-substrate ratio the majority of *Tr*Cel7A molecules are bound to the substrate at active site level already in the absence of EG. Increasing the number of free chain ends by EG has little effect and the synergistic effect is mostly due to the increase in k_{obs} (Ref II Table 2).

4.2.3. The role of CBM in cellulose hydrolysis (Ref I, II)

It has been suggested, that in addition to facilitating binding to the crystalline cellulose the CBM plays a more direct role in cellulose hydrolysis (Beckham et al., 2010; Mulakala and Reilly, 2005), however, the exact role is not clear (Boraston et al., 2004; Guillén et al., 2010). In order to determine the role of CBM in cellulose hydrolysis, we studied the kinetic parameters of *Tr*Cel7A and its truncated version ($CD_{TrCel7A}$) that consists only of the CD.

The $CD_{TrCel7A}$ had a lower overall activity on cellulose determined by the released soluble sugars when compared to the intact enzyme. The CBM of *Tr*Cel7A, unlike its CD, is known to preferably target crystalline cellulose (Ståhlberg et al., 1991). As expected, the differences were more pronounced on crystalline cellulose, while on the amorphous cellulose the performance was essentially the same. For both, the intact enzyme and truncated enzyme, the rapid $[CBH]_{OA}$ formation was followed with by a plateau, but the plateau level was notably lower for the truncated enzyme. In terms of k_{obs} values the differences between the $CD_{TrCel7A}$ and intact *Tr*Cel7A were smaller. Expectedly, the k_{obs} values were slightly lower for the $CD_{TrCel7A}$ than for the intact enzyme on crystalline substrates and the differences were negligible on amorphous cellulose.

In synergistic reactions, where *Tr*Cel5A was added to the reaction mixture the intact *Tr*Cel7A clearly outperformed $CD_{TrCel7A}$ on low substrate concentrations while at higher BC concentrations the activity was essentially the same (Ref II Figure 3D). This is partly because synergistic hydrolysis of BC

with *Tr*Cel7A and EG displays substrate inhibition (Väljamäe et al., 2001), but at the same time it is known that the CBM is beneficial at low substrate concentrations and has little effect on high substrate concentrations (Kari et al., 2014; Sørensen et al., 2015; Várnai et al., 2013). The effect of added EG on $[CBH]_{OA}$ was different in the case of intact *Tr*Cel7A and $CD_{TrCel7A}$. The addition of *Tr*Cel5A resulted in an increase in the $[CBH]_{OA}$ for the *Tr*Cel7A, while the opposite was seen in the case of $CD_{TrCel7A}$ (Ref II Figure 3B). The k_{obs} values obtained from the synergistic hydrolysis were similar for reactions with intact *Tr*Cel7A and its CD (Ref II figure 3C).

In single turnover experiments the P_{app} and k_{cat} were slightly lower for the $CD_{TrCel7A}$, when compared to the intact enzyme, however, the differences were small and possibly are not significant (Ref II Table 1). This indicates that the proposed mechanism of synergy is the same for both the intact enzyme and CD as well.

Our findings are in agreement with the results published by Igarashi *et al*. that the catalytic domain moves along the cellulose chain at the same velocity as the intact enzyme (Igarashi et al., 2009). The low $[CBH]_{OA}$ of $CD_{TrCel7A}$ suggests that the CBM mainly affects the substrate binding and feeding the cellulose chain into the active site but has little effect on other steps of the processive cycle (Kont et al., 2016).

4.3. Binding of TrCel7A to cellulose

4.3.1. Three binding Modes (Ref III)

The enzymatic hydrolysis of cellulose takes place in the solid-liquid interface and, thus, the adsorption of cellulases to cellulose is a prerequisite for the hydrolysis. *Tr*Cel7A is a multidomain enzyme consisting of CD, linker peptide and CBM that can bind to cellulose independently giving rise to different binding modes.

The CBM and CD of *Tr*Cel7A can bind to cellulose independently so that *Tr*Cel7A bound to cellulose can be divided into two populations: a) *Tr*Cel7A bound to cellulose through its CD and b) *Tr*Cel7A bound to cellulose through its CBM. It must be noted that in this approach we do not distinguish $[CBH]_{FA}$ complexes with a cellulose chain in the substrate binding tunnel (see Figure 7). The complexes where the reducing end of the cellulose chain is in the glucose unit binding sites numbered from –7 to –3 are short-lived and through processive movement the cellulose chain will progress further into the substrate binding tunnel until it forms a $[CBH]_{OA}$ complex. These $[CBH]_{FA}$ complexes with a cellulose chain in the substrate binding tunnel could be stable if there was an obstacle on the path of the processive movement. However, in this case, the obstacle has to be close to the free reducing end of the cellulose chain. We consider this scenario to be unlikely and in the current approach, if $[CBH]_{FA}$ complexes with a cellulose chain in the substrate binding tunnel are present,

these are included in the fraction of *Tr*Cel7A bound to cellulose through its CBM.

To distinguish whether the binding is CD mediated or CBM mediated two approaches were used. In one series the unbound enzyme was separated by filtration so that free enzyme and total bound enzyme can be quantified. In the second series the MUL hydrolysis was carried out in the presence of cellulose so that enzyme molecules with free and occupied active site can be distinguished. These results taken together allow us to divide the enzyme into three populations: a) free enzyme, b) enzyme bound to cellulose on active site level c) enzyme bound to cellulose but with a free active site (Figure 7).

The concentration of the enzyme was varied over four orders of magnitude to reveal possible differences in binding at the active site level and binding through CBM at high and low substrate concentrations. The results were analyzed with nonlinear regression and different models were used for fitting. The models used included Langmuir's single, two and three independent binding site models, Freundlich model, Hill's cooperative binding model and sum of Langmuir's and Hill's model. While several models yielded reasonably good fits with the experimental data, there was a clear systematic deviation between the experiment and fitted mode in the low nanomolar concentration range of free *Tr*Cel7A (Ref III Supporting Information Figure S3).

The Langmuir three independent binding sites model did not provide a better fit over the two sites model, however analyzing the ratios of enzyme bound on active site level and non-productively bound enzyme (with a free active site) indicates at least three distinct binding modes. The high affinity binding mode that dominates in the range of 0–10 nM free *Tr*Cel7A is active site mediated. In the medium affinity binding mode (predominant in the range of 0.1–1 μM free *Tr*Cel7A) the contribution of nonproductively bound enzyme (with free active site) was considerable, while the third, low affinity binding mode, was active site mediated (Figure 11).

In order to determine the binding parameters the dataset was divided into three. We started with a Langmuir's single binding site model and gradually increased the complexity of the model as more data was included in the analysis. Narrowing the dataset to the maximum value of 10 nM free *Tr*Cel7A yielded good fit with the Langmuir single binding site isotherm. Next, we extended the free enzyme range to a maximum of 1 μM *Tr*Cel7A and analyzed the data with Langmuirs two binding sites model. The parameters of the first, high affinity binding mode were fixed and the parameters for the second binding mode were found from the fitting. This approach was extended for the full dataset using Langmuirs three independent binding sites model with the parameters for the first two modes taken from the previous analyses (Ref III Table 1).

Concluding these findings our hypothesis is that the high affinity binding mode corresponds to the productive binding that occurs at the free chain ends at hydrophobic surfaces of the cellulose crystal (Figure 12 panel A). Cellulases are believed to act primarily on the hydrophobic surfaces that constitute a relatively small fraction of the crystal area (Lehtio et al., 2003; Liu et al., 2011; Nimlos et

al., 2012). For the substrate used in our experiments the estimated concentration of free chain ends on the hydrophobic surface is approximately 0.12 µmol per gram of cellulose, which is in good accordance with the A_{max} found for the high affinity binding mode.

The medium affinity binding mode includes both enzymes with free and occupied active centers. A considerable fraction of the enzyme is bound only through CBM. The CBM is smaller than the CD of *Tr*Cel7A. It covers 10 cellobiose units of substrate compared to 48 cellobiose units for the CD (Sild et al., 1996). The hydrophobic surface of BC contains approximately 100 cellobiose units per gram of cellulose (Gilkes et al., 1992) meaning that the binding capacity of CBM on the hydrophobic faces is around 10 µmol per gram of cellulose. For CD the binding capacity is 2 µmol per gram of cellulose. The found A_{max} value is within the same order of magnitude with both binding capacities.

We propose that the medium affinity binding mode corresponds to endo mode binding to the hydrophobic surfaces of the cellulose crystal including both productive and nonproductive binding (Figure 12 panel B). The CBM has a higher affinity towards the hydrophobic face of the cellulose crystal, but without a cellulose chain end in the catalytic site, this binding mode is nonproductive. However, CBHs are capable of endo initiation (Kurašin and Väljamäe, 2011; Ståhlberg et al., 1993) and this complex can turn into productive binding when a cellulose chain is displaced from the crystal face and binds to the active site.

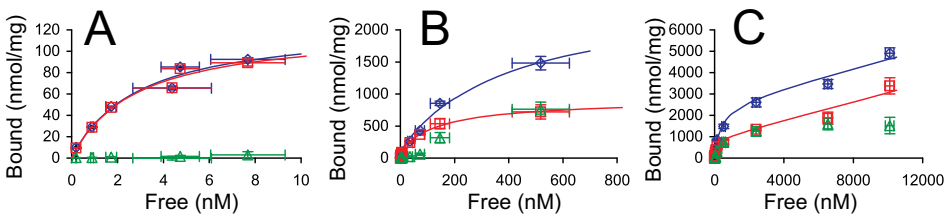

Figure 11. The full binding isotherm is divided into three regions with different dominating binding modes. Total bound enzyme (◇), active site mediated binding (□), bound enzyme with a free active site (△). **(A)** The high affinity binding mode dominates in the range of up to 10 nM free *Tr*Cel7A. This binding mode is active site mediated and the solid lines represent fits to the Langmuir's single binding site model. **(B)** The medium affinity binding mode dominates in the range of 0.1 – 1.0 µM free *Tr*Cel7A. The solid lines represent fits to the Langmuir's two independent binding sites model. **(C)** The low affinity binding mode is dominant at free *Tr*Cel7A concentrations above 1.0 µM. The solid lines represent fits to Langmuir's three independent binding sites model.

The low affinity binding mode that dominates in the range of free enzyme concentrations over 1 µM could represent binding to the hydrophilic surfaces (Figure 12 panel C). The large specific area of the hydrophilic faces of cellulose crystals correlates with the high binding capacity of this binding mode. Cellulases have been shown to bind to the hydrophilic surfaces, but they diffuse to a hydrophobic surface if available (Nimlos et al., 2012). Because of the low

binding affinity and high binding capacity, we were unable to saturate this binding mode and, thus, the exact values for A_{max} and K_d can not be found. The A_{max}/K_d value of total binding and binding on active site level are similar suggesting that the low binding affinity mode is also active site mediated. We propose that the low affinity binding corresponds to the active site mediated binding to the free cellulose chain ends on the hydrophilic surface.

The approach above represents a simplified view of *Tr*Cel7A adsorption. While we did not observe cooperative effects, other authors have reported overlapping binding sites (Sild et al., 1996) and cooperative effects (Sugimoto et al., 2012) in cellulase adsorption. With this additional complexity, the three independent binding sites model can be insufficient to accurately describe the binding of *Tr*Cel7A to BC.

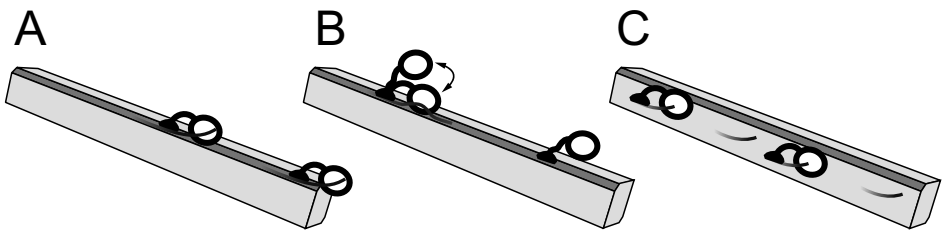

Figure 12. The proposed binding modes of *Tr*Cel7A on cellulose microfibril. The hydrophobic face of the cellulose microfibril is shown in dark gray and free chain ends available for binding through the active site are depicted as protruding lines. **(A)** The high affinity binding mode represents the productive binding to a free cellulose chain end on the hydrophobic face of a cellulose microfibril. This binding mode is both CD and CBM mediated. **(B)** The medium affinity binding mode represents CBM mediated binding to the hydrophobic face of the cellulose microfibril. This complex can become productive through an endo-mode attack. **(C)** The low affinity binding mode may correspond to active site mediated binding to free cellulose chain ends on the hydrophilic faces of the cellulose microfibril.

The determined binding affinity for the high affinity binding mode is among the strongest affinities reported in the literature. K_D values in low nanomolar range have been reported (Creagh et al., 1996; Herner et al., 1999; Moran-Mirabal et al., 2011), however, the majority of the published results fall into the medium binding affinity (Gao et al., 2013; Kamat et al., 2013; Sugimoto et al., 2012; Wahlström et al., 2014). Our results agree with (Shibafuji et al., 2014) that kinetic parameters should be measured at low enzyme concentration.

4.3.2. TrCel7A binding to cellulose is cellulose concentration dependent (Ref III)

The number of binding sites per gram of cellulose and binding affinity are expected not to depend on cellulose concentration. As a rule, cellulase binding studies have been conducted at one substrate concentration.

We measured *Tr*Cel7A binding to BC, varying the BC concentration in the range of 0.01–1 g/L. We focused on the low free *Tr*Cel7A concentration region of the isotherm so that the Langmuir's single binding site model was sufficient to describe the binding. The binding isotherms at different cellulose concentrations did not overlap (Ref III Figure 4 panel A), binding was more efficient at lower BC concentrations (Ref III Figure 4 panel C). Similar results have been obtained using Avicel as substrate (Wang et al., 2011). The decrease in binding affinity can be explained with the association of cellulose microfibrils. Cellulose fibril association is concentration dependent (Kuijk et al., 2013) resulting in lower specific surface per gram of cellulose at higher cellulose concentrations. This reduction of the specific surface has also been linked to the lower association rate constant values at higher cellulose concentrations (Cruys-Bagger et al., 2013a). In this case, the A_{max} value should decrease as the concentration of cellulose increases while K_d remains unaffected. However, our experiments do not enable us to make exact estimations of the A_{max} and K_d values at different cellulose concentrations. The ratio of different binding modes varies in the experiments depending on the highest enzyme concentration included in the measurements which makes the estimation of A_{max} and K_d error prone. A_{max}/K_d, in contrast, depends on the initial slope of the isotherm and is less influenced by the ratio of different binding modes. This decrease in binding efficiency with increased cellulose concentration can contribute to the "solids effect" described in the literature. Increasing the concentration of cellulose (at a constant cellulase-to-cellulose ratio) results in a decrease in cellulose conversion rate (Kristensen et al., 2009; Modenbach and Nokes, 2013; Roberts et al., 2011).

5. CONCLUSIONS

We developed a method to determine the concentration of *Tr*Cel7A bound to cellulose at active site level during cellulose hydrolysis. This method relies on hydrolysis of low molecular weight reporter molecule and allows us to determine the observed catalytic rate constant of *Tr*Cel7A catalyzed cellulose hydrolysis in time. Together with the results from single turnover experiment the k_{cat} can be determined. These methods were applied to both hydrolysis with CBH only and synergistic reactions containing CBH and EG.

A model of CBH action is proposed, where the hydrolysis of cellulose by CBHs is limited by the obstacle-free path on the cellulose surface. EGs avoid stalling of the CBHs by preferably targeting amorphous regions of cellulose that can pose an obstacle.

The key findings of my research can be summarized as follows:

- The decline of cellulose hydrolysis rate is due to accumulation of stalled nonproductive enzyme-substrate complexes
- Amorphous regions of BC can pose an obstacle for CBHs
- EG generated cuts provide both starting and ending points for CBHs during cellulose hydrolysis and enhance the synergistic reaction through accelerated recruitment
- CBM enhances binding to cellulose but has otherwise little effect on cellulose hydrolysis
- Binding to cellulose has at least three distinct binding modes
- Cellulase binding on cellulose is substrate concentration dependent, binding is more efficient at low substrate concentrations

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SUMMARY IN ESTONIAN

Tselluloosi ensümaatilise hüdrolüüsi mehhanismi uurimine madalmolekulaarsete mudelsubstraatide abil

Tselluloos on taimede rakukesta põhikomponent ja seega kõige levinum biopolümeer Maal. Oma laia leviku tõttu on tselluloos potentsiaalne taastuvenergia allikas. Tselluloos koosneb lineaarsetest polüsahhariidiahelatest, milles on glükoosijäägid omavahel seotud β-1,4 glükosiidse sidemega. Tselluloosiahelad on omavahel seotud vesiniksidemete ja *van der Waalsi* jõududega moodustades mikrofibrilli, mis on vastupidav nii keemilisle kui ka ensümaatilisele lagundamisele. Looduses lagundavad tselluloosi peamiselt seened ja bakterid, kes kasutavad selleks komplekti hüdrolüütilisest ja oksüdatiivsetest ensüümidest, mida kokkuvõtvalt nimetatakse tsellulolüütiliseks süsteemiks. Kõige põhjalikumalt uuritud tsellulolüütiline organism on pehmemädanikseen *Trichoderma reesei*.

Trichoderma reesei tsellulolüütilise süsteemi põhikomponent on *Tr*Cel7A. *Tr*Cel7A on tsellobiohüdrolaas – protsessiivne eksoensüüm. *Tr*Cel7A alustab hüdrolüüsi tselluloosi ahela otsast ning mööda ahelat edasi liikudes vabastab glükoosi dimeere, tsellobioosi, seejuures ilma vahepeal ahelalt dissotsieerumata.

Teine klass tsellulaase on endoglükanaasid. Need ensüümid on võimelised hüdrolüüsima glükosiidsidemeid ka tselluloosi ahela keskelt ning ei vaja produktiivse ensüüm-substraat kompleksi moodustamiseks vaba ahelaotsa tselluloosi pinnal. Tselluloosi efektiivseks hüdrolüüsiks on vaja tsellobiohüdrolaaside ja endoglükanaaside sünergistliku koostoimet.

Kuigi tsellulaase on uuritud aastakümneid, ei mõisteta tselluloosi ensümaatilist hüdrolüüsi lõplikult tänini. Üheks puuduseks on universaalsete meetodite puudumine tselluloosi ensümaatilise hüdrolüüsi kineetiliste parameetrite määramiseks. Raskused kineetiliste parameetrite määramisel tulenevad ühelt poolt tselluloosi ehitusest. Ehkki keemiliselt on tselluloos lihtne, moodustavad tselluloosi mikrofibrillid kõrgemat järku struktuure, mis mõjutavad olulisel määral selle reaktiivsust. Teisalt tulenevad raskused tsellulaaside ehitusest – *Trichoderma reesei* tsellulaasid on modulaarse ehitusega ning võivad seostuda tselluloosile nii produktiivselt kui ka mitteproduktiivselt. Tselluloosi hüdrolüüs *Tr*Cel7A-ga hälbib klassikalisest Michaelis-Menteni kineetikast. Hüdrolüüsi kiirus langeb ajas kiiremini, kui see oleks seletatav vaid substraadi ära tarbimisega. Selle nähtuse seletamiseks on välja pakutud mitmeid hüpoteese, mis olemuslikult jagunevad kaheks: reaktsiooni kiiruse langus tuleneb kas substraadi muutumisest raskemini hüdrolüüsitavaks või ensüümi aktiivsuse langusest. Teades mehhanismi, mis põhjustab tselluloosi hüdrolüüsi kiiruse langust, ning missugusel molekulaarsel mehhanismil põhineb sünergism tsellulaaside vahel, oleks võimalik disainida efektiivsemaid ensüümisegusid tselluloosi lagundamiseks.

Minu doktoritöö eesmärgiks on luua meetod *Tr*Cel7A katalüütilise konstandi määramiseks tselluloosi ensümaatilisel hüdrolüüsil ning selle abil tuvastada kiirust piirav etapp tselluloosi lagundamisel. Minu loodud meetod põhineb

madalmolekulaarse reportermolekuli hüdrolüüsil ning võimaldab tuvastada *Tr*Cel7A seostumist tselluloosile aktiivtsentri tasemel. Lahustuva reportermolekuli hüdrolüüsi kiiruse järgi on võimalik määrata vaba aktiivtsentriga *Tr*Cel7A-de kontsentratsioon. Sellest tulenevalt saab leida aktiivtsentri vahendusel tselluloosile seostunud *Tr*Cel7A kontsentratsiooni ning koos reaktsiooni kiirusega omakorda *Tr*Cel7A katalüütilise konstandi tselluloosi hüdrolüüsil. Lisaks rakendasin seda meetodit sünergistlikele reaktsioonisegudele ning määrasin aktiivtsentri vahendatud seostumise osakaalu erinevatel tselluloosi ja *Tr*Cel7A suhetel. Minu uurimustöö olulisemad tulemused saab kokku võtta järgnevalt:

- Tselluloosi ensümaatilise hüdrolüüsi kiiruse järsu languse põhjustab inaktiivsete ensüüm-substraat komplekside akumuleerumine hüdrolüüsi käigus
- Amorfsed piirkonnad bakteriaalse tselluloosi mikrofibrillis takistavad tsellobiohüdrolaaside protsessiivset liikumist
- Endoglükanaaside tekitatud katked tselluloosiahelates kiirendavad tselluloosi hüdrolüüsi võimaldades tsellobiohüdrolaasidel nii seostuda kui ka vabaneda tselluloosi pinnalt
- Tselluloosile seostumisdomeen vahendab seostumist kristallilisele tselluloosile, kuid ei mõjuta olulisel määral hüdrolüüsi kiirust
- Tsellulaaside seostumisel tselluloosile saab eristada vähemalt kolme erinevat seostumisviisi
- Tsellulaaside seostumine tselluloosile sõltub tselluloosi kontsentratsioonist. Madalamatel substraadi kontsentratsioonidel on seostumine efektiivsem

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