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Processivity of cellulases and chitinases

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Chair of General and Microbial Biochemistry, Institute of Molecular and Cell Biology, Faculty of Science and Technology, University of Tartu, Estonia

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Supervisor:	Senior Research Fellow Priit Väljamäe, PhD Chair of General and Microbial Biochemistry Institute of Molecular and Cell Biology University of Tartu, Tartu, Estonia
Opponent:	Associate Professor Jerry Ståhlberg, PhD Department of Chemistry and Biotechnology, Swedish University of Agricultural Sciences, Uppsala, Sweden
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LIST OF ORIGINAL PUBLICATIONS

Current thesis is based on the following publications, referred to by their Roman numerals.

I Kurašin M., Väljamäe P. (2011) "Processivity of cellobiohydrolases is limited by the substrate" J Biol Chem, 286(1):169–177

II Jalak J., Kurašin M., Teugjas H., Väljamäe P. (2012) "Endo-exo synergism in cellulose hydrolysis revisited" J Biol Chem, 287(34):28802–28815

III Kurašin M.*, Kuusk S.*, Kuusk P., Sørlie M., Väljamäe P. (2015) "Slow off-rates and strong product binding are required for processivity and efficient degradation of recalcitrant chitin by family 18 chitinases" J Biol Chem, 290(48):29074–29085

* Equal contribution

My contribution to the articles is as follows:

Paper I Performed all experiments and participated in data analysis.

Paper II Performed inhibition experiments under single turnover conditions and participated in data analysis.

Paper III Performed all experiments with mutant enzymes and participated in data analysis.

LIST OF ABBREVIATIONS

^{14}CB	^{14}C-labeled cellobiose
^{14C}BC	^{14}C-labeled bacterial cellulose
^{14C}P	^{14}C-labeled product
^{14C}S	^{14}C-labeled substrate
BC	bacterial cellulose
BMCC	bacterial microcrystalline cellulose
CBH	cellobiohydrolase
CBM	carbohydrate binding module
CD	catalytic domain
CNWs	chitin nanowhiskers
DAP	2,6-diaminopyridine
DP	degree of polymerization
E	enzyme
EG	endoglucanase
EL	end label
GH	glycoside hydrolase
HS-AFM	high speed atomic force microscopy
IRG	insoluble reducing groups
k_{cat}	catalytic constant
k_{off}	dissociation rate constant
LPMO	lytic polysaccharide monooxygenase
N^{catal}	the number of catalytic acts
N^{Init}	the number of initiations of processive runs
P^{app}	apparent processivity
P_{Endo}	probability of endo-mode initiations
P^{Intr}	intrinsic processivity
rAC	reduced amorphous cellulose
rBC	reduced bacterial cellulose
S	substrate
SEE	substrate exchange experiments
SmChiA	*Serratia marcescens* chitinase A
SmChiB	*Serratia marcescens* chitinase B
SRG	soluble reducing groups
TrCel6A	*Trichoderma reesei* cellobiohydrolase Cel6A
TrCel7A	*Trichoderma reesei* cellobiohydrolase Cel7A

INTRODUCTION

Increasing living standards and world population necessitate the search for alternatives to petroleum-based products as energy carriers. Thus, cellulose and chitin, the net annual production of which are estimated in billions of tons, have recently become potential candidates as a renewable sources for the production of biofuel for gasoline working vehicles as well as essential chemical compounds for food and pharmaceutical industries. Biosynthesis of cellulose and chitin has been found in almost all kingdoms of life. The main source of cellulose are terrestrial plants, whereas arthropods and fungi are predominant producers of chitin. Both are structural polysaccharides whose main function is to support and protect organisms by increasing cell wall rigidity. The monomers of both cellulose and chitin (i.e. glucose and N- acetylglucosamine respectively) are connected through β-1,4-glycosidic bonds forming long, unbranched chains. These chains are associated into microfibrils with assistance of numerous inter- and intramolecular hydrogen bonds. Microfibrils of cellulose and chitin are embedded into hemicellulose and lignin or protein and calcium carbonate matrix respectively. Such an organization makes these polymers highly resistant to chemical and enzymatic processing. Nevertheless, living organisms have adapted to utilize recalcitrant polysaccharides by secreting a massive battery of enzymes. Although natural enzymes are capable to completely solubilize cellulose and chitin, their application on industrial scale is still not cost-efficient explaining why biofuels cannot compete with fossil transportation fuels to date. Efficient degradation of polysaccharides is achieved by concerted action of different types of enzymes. The main contribution to this process generally comes from processive enzymes that constitute the major part of cellulolytic/ chitinolytic systems. Processivity is defined as the ability of an enzyme to conduct sequential catalytic acts without dissociating from its substrate. It is therefore an important characteristic of cellulose/chitin degrading enzymes, and its investigation is likely to provide a good clue to the improvement of the process of saccharification of recalcitrant polysaccharides.

The first part of the current thesis gives an overview of substrates (cellulose and chitin), enzymes (cellulases and chitinases), and their interactions. Currently available methods on the measurement of processivity, and concomitant pitfalls associated with them are also discussed. In the second part I will focus on the methods for the measurement of cellulase and chitinase processivity that have been developed in the course of my PhD studies. These methods have been tested on the key components of the most studied cellulolytic and chitinolytic systems, namely TrCel7A of the soft-rot fungus *Trichoderma reesei* and SmChiA of the Gram negative bacterium *Serratia marcescens* respectively.

1. REVIEW OF LITERATURE

1.1 Recalcitrant polysaccharides

1.1.1 Cellulose

Cellulose is the most abundant biopolymer on Earth with an estimated annual production between 10^{10} and 10^{12} tons (Hon, 1994; Klemm *et al.*, 2005; Pauly & Keegstra, 2008). The bulk of cellulose is produced by higher plants where it can form up to 50% of the dry mass (Pauly & Keegstra, 2008; Somerville *et al.*, 2004). Biosynthesis of cellulose also exists in bacteria, protists, algae and even animals (McNamara *et al.*, 2015). Cellulose has been used by mankind for centuries in construction and textile industries as well as a fuel. However, discovery of cellulose could be referred to the first half of the 19th century, when the French chemist Anselme Payen isolated it from plants and determined the chemical formula of cellulose, $C_6H_{10}O_5$. Decades later, it was found, that cellulose is a linear polymer of D – glucose residues connected together by β-1,4-glycosidic bonds (Hon, 1994). Similarly to biopolymers like DNA or RNA, cellulose has a specific directionality. The non-reducing end contains a free C4 hydroxyl group and the reducing end contains a free C1 aldehyde group (fig. 1A). C1 of the reducing end is in equilibrium with the cyclic hemiacetal form and the open aldehyde form that makes it a favorable target for specific reactions (Armand *et al.*, 1997; Kipper *et al.*, 2005; Velleste *et al.*, 2010). The length of a cellulose chain or the degree of polymerization (DP) is expressed in the number of glucose units and varies from 100 to 15000, depending on the source and method of preparation (Fleming *et al.*, 2001; Klemm *et al.*, 2005; McNamara *et al.*, 2015). Each glucose unit is rotated by 180^o with respect to its neighbor and thus two consecutive glucose residues, also referred to as cellobiose, may be considered as a repeating unit within a cellulose chain (fig. 1A) (Klemm *et al.*, 2005; McNamara *et al.*, 2015). Structure of a cellulose molecule is stabilized by numerous intra- and intermolecular hydrogen bonds (Nishiyama *et al.*, 2002; Nishiyama *et al.*, 2003).

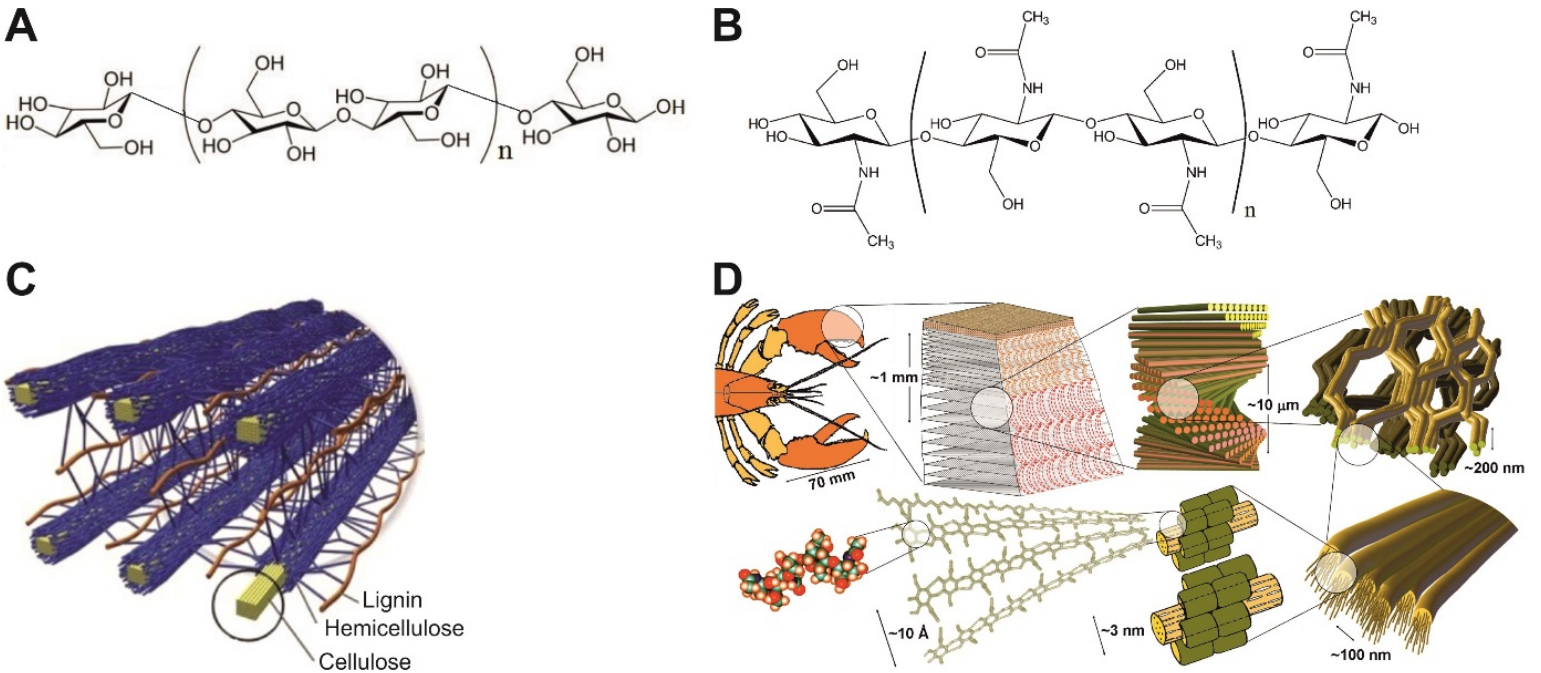

Figure 1. Chemical structure of (**A**) cellulose and (**B**) chitin chains. Reducing end in the form of cyclic hemiacetal on the right and a non-reducing end on the left. The repeating unit, anhydrocellobiose/anhydrochitobiose (n), is enclosed in parentheses. **C.** Higher order structure of cellulose. Cellulose chains are associated via hydrogen bonds into microfibrils. Microfibrils are non-covalently bound to hemicellulose and lignin forming higher order structures such as fibrils. The latter are crosslinked constituting the plant cell wall. Adapted from (Doherty *et al.*, 2011) **D.** Ranking of chitin structures from single chain to exoskeleton of a crustacean. Antiparallel chitin chains are associated through hydrogen bonds into microfibrils. Microfibrils are covalently bound to proteins and form higher order structure chitin-protein fibers. Fibers are embedded into a mineral-protein matrix forming the multilayer cuticle of crustaceans. Reproduced with permission from (Raabe *et al.*, 2005).

Cellulose chains in native cellulose are aggregated into microfibrils which in turn form higher order structures (fig. 1C). Crystallographic studies revealed that within microfibrils cellulose chains are packed in parallel orientation i.e. reducing ends at one side and non-reducing ends at another (Hon, 1994; O'Sullivan, 1997). Fibrillary structure and parallel orientation of chains are determined by biosynthesis of cellulose. Cellulose synthesis is carried out at the plasma membrane by cellulose synthase complexes, rosette-like structures consisting of cellulose synthase subunits (Reiter, 2002; Saxena & Brown Jr, 2005; Y. Watanabe *et al.*, 2015). Each cellulose synthase subunit produces a single cellulose chain from the reducing end toward the non-reducing end. Since all cellulose chains are synthetized in close proximity they are able to form microfibrils by self-assembly at the exit of each single cellulose synthase complex (McNamara *et al.*, 2015; Reiter, 2002). This process, however, is imperfect resulting in the formation of amorphous or disordered regions alternating with crystalline regions within single cellulose microfibrils (Habibi *et al.*, 2010; Nishiyama *et al.*, 2002). Depending on how cellulose chains are packed into crystals, native cellulose can be divided into two polymorphs i.e. Iα and Iβ. Relative amount of Iα and Iβ cellulose is dependent on the origin of cellulose. Algal and bacterial celluloses are rich in Iα, whereas Iβ cellulose is dominant in tunicate, animals and higher plants (Atalla & Vanderhart, 1984; Nishiyama *et al.*, 2002; Sugiyama *et al.*, 1991).

Cellulose in plant cell walls forms strong interactions with hemicellulose and lignin (fig. 1 C) (Somerville *et al.*, 2004). Hemicellulose is a heteropolymer of various sugar monomers: xylose, mannose, arabinose etc. (Pauly & Keegstra, 2008). Lignin is a heteropolymer consisting of cross-linked monomers based on phenylpropanoid structures (Doherty *et al.*, 2011). The crystalline structure strengthened by hydrogen bonds and the association with hemicellulose and lignin makes cellulose recalcitrant to chemical and enzymatic degradation (Himmel *et al.*, 2007).

1.1.2 Chitin

Chitin takes the second place in prevalence on Earth after cellulose with an annual production of about 10^{10} tons (Ravi Kumar, 2000; Ravi Kumar *et al.*, 2004). Chitin is naturally found in exoskeletons and cocoons of insects, cell walls of fungi, and crustacean shells where it performs protective and supporting functions (Pillai *et al.*, 2009; Raabe *et al.*, 2005; Ravi Kumar *et al.*, 2004). Chitin is a linear polymer of N-acetyl-D-glucosamine monomers connected by β-1,4-glycosidic bonds. Since each monomer is rotated by 180^{o} relatively to its neighbor, two consecutive N-acetyl-D-glucosamine monomers, also referred to as chitobiose, constitute a minimal repeating unit of the chitin chain (fig. 1B) (Ravi Kumar *et al.*, 2004). Similarly to cellulose, chitin chains have a specific directionality with the reducing end at one side and the non-reducing end at another (fig. 1B). Since there is only one aldehyde group along

the chitin chain the reducing end has a specific chemistry (Horn & Eijsink, 2004). Numerous interchain hydrogen bond interactions favor formation of fibrillar structures such as microfibrils (fig. 1D). Microfibrils are embedded into a polysaccharide/protein matrix (in case of fungi) (Bowman & Fre, 2006) or a protein/calcium carbonate matrix (in case of crustaceans) (Raabe *et al.*, 2005). Such an organization makes chitin a highly crystalline and recalcitrant substrate. Based on chain packing and orientation in microfibrils two polymorphic forms have been proposed for native chitin. In α-chitin chains are packed in anti-parallel orientation (Kameda *et al.*, 2005), whereas β-chitin shows parallel orientation (Gardner & Blackwell, 1975; Nishiyama *et al.*, 2011; Sawada *et al.*, 2012). The most prevalent α-chitin is found in arthropods and fungi (Jang *et al.*, 2004), while β-chitin is a part of the shells of brachiopods and mollusks (Ravi Kumar *et al.*, 2004). α-chitin is thought to be thermodynamically more stable because it is always formed after chitin dissolution and following crystallization (Rinaudo, 2006; Saito *et al.*, 2000).

1.1.3 Polymeric model substrates in cellulase and chitinase research

Because of the presence of additional components such as hemicellulose and lignin, plant cellulose is an inconvenient substrate for detailed studies of enzyme kinetics. Therefore pure celluloses of another origin are often used in cellulase studies, one of which is bacterial cellulose (BC) produced by *Acetobacter xylinum* (McNamara *et al.*, 2015; Saxena & Brown Jr, 2005; K. Watanabe *et al.*, 1998). Unlike plant cellulose BC forms stable suspensions due to adsorption of large amounts of water and it is produced without hemicellulose and lignin (de Oliveira *et al.*, 2011). Heterogeneous acid hydrolysis of bacterial cellulose with HCl removes amorphous regions and results in highly crystalline substrate bacterial microcrystalline cellulose (BMCC) (de Oliveira *et al.*, 2011; Väljamäe *et al.*, 1999). In addition to an increased crystallinity index, BMCC has a reduced degree of polymerization (DP 100–300) (Väljamäe *et al.*, 1999) as compared to BC (DP 1000–3000) (Velleste *et al.*, 2010). Besides BC and BMCC, commercial celluloses like Avicel and Whatman cellulose filter paper are also used as model substrates. Avicel is a chemically extracted wood cellulose with a DP of around 100–300 glucose units. Whatman cellulose filter paper is a cotton linter cellulose with a DP of around 1000–5000 (Terinte *et al.*, 2011). In contrast to crystalline substrates, amorphous cellulose (AC) is produced by dissolving native cellulose in phosphoric acid followed by precipitation in water (Y. H. Zhang *et al.*, 2006). Another substrate of choice is soluble carboxymethyl cellulose (Irwin *et al.*, 1993). It is chemically modified cellulose, where carboxymethyl groups are bound to hydroxyl groups of glucoses. Therefore, carboxymethyl cellulose is thought to be inaccessible to enzymes with closed active sites and is often used to detect endoglucanase activity.

Similar model substrates have also found application in chitinase research. Commercially available raw α- and β-chitins can be purified from protein matrix and calcium carbonate before the use in enzymatic assays. Acid hydrolysis of chitin with HCl results in the formation of chitin nanowhiskers (CNWs) that are analogous to BMCC in cellulase assays (Kuusk *et al.*, 2015). Amorphous chitin is produced by dissolving alkali chitin in crushed ice (Pillai *et al.*, 2009; Rinaudo, 2006). Acetylation of soluble chitosan also results in precipitation of chitin in the amorphous form (Shimojoh *et al.*, 2011). Deacetylation of chitin with alkali, usually concentrated NaOH, result in formation of water soluble chitosan (Eijsink *et al.*, 2008; Pillai *et al.*, 2009). Chitosan with well-defined degree of acetylation has turned out to be valuable substrate in chitinase research.

1.2 Enzymatic degradation of recalcitrant polysaccharides

Biodegradation of cellulose and chitin is an important part of the global carbon cycle. The main degraders of cellulose and chitin are microorganisms whose enzymatic machineries have evolved to efficient utilization of recalcitrant polysaccharides. In nature the main part of cellulose is degraded by fungi (Payne *et al.*, 2015), whereas bacteria are responsible for the turnover of the bulk of chitin (Beier & Bertilsson, 2013).

Efficient degradation of cellulose is achieved by simultaneous action of multiple enzymes that account for the depolymerization of lignin, hemicellulose and cellulose. There are at least four kinds of lignin-degrading enzymes: lignin peroxidases, manganese peroxidases, alkyl aryl etherases, and laccases (Chen *et al.*, 2012). The majority of hemicellulose and cellulose degrading enzymes are glycoside hydrolases (GH) that in turn are divided into families based on amino acid sequence similarities (Lombard *et al.*, 2014). To date 135 GH families have been identified (http://www.CAZy.org/). All known GH-s employ either a double or single displacement catalytic mechanism of glycosidic bond hydrolysis. In the case of double displacement, the hydrolysis product retains its initial β configuration and the enzyme is referred to as a retaining GH. Inverting GH-s use a single displacement mechanism that results in the formation of the product in α configuration (G. Davies & Henrissat, 1995; Rabinovich *et al.*, 2002). In both cases, two carboxylic acid residues (usually Glu) are catalytically essential (Payne *et al.*, 2015). In retaining enzymes one carboxylate is an acid/base catalyst whereas the other is nucleophile. Catalysis by retention involves a covalent glycosyl-enzyme intermediate. In inverting enzymes one carboxylic acid is used to protonate glycosidic bond oxygen whereas the other acts as a base and removes a proton from a water molecule. The mechanism of glycosidic bond hydrolysis is conserved within the GH family.

Enzymes responsible for the decomposition of cellulose are commonly referred to as cellulases (Payne *et al.*, 2015). Being members of the family of

GH, cellulases cleave β-1,4-glycosidic bonds through a single or double displacement catalytic mechanism. At least three types of cellulases are required for efficient cellulose solubilization; i) exocellulases or cellobiohydrolases (CBHs) that hydrolyze cellulose from either reducing or non-reducing end; ii) endoglucanases (EGs) that hydrolyze the cellulose chain from random positions and iii) β-glucosidases that are responsible for the conversion of cellobiose and soluble cello-oligosaccharides to glucose (Lynd *et al.*, 2002). Recently, another type of enzymes, the so called lytic polysaccharide monooxygenases (LPMOs), were found to play a role in cellulose degradation (Eibinger *et al.*, 2014). In contrast to GH-s LPMOs employ oxidative cleavage of glycosidic bonds. Besides cellulases and lignin degrading enzymes the lignocellulose solubilization involves a number of enzymes acting on hemicellulose-substrates. Among these are xylanases, xyloglucanases, mannanases, arabinofuranosidases and different esterases (Benkő *et al.*, 2008; Foreman *et al.*, 2003).

Based on the strategy of cellulose degradation cellulases are also divided into two major classes – complexed cellulases and non-complexed cellulases. Complexed cellulases or cellulosomes are large complexes consisting of multiple enzymes attached to the cell wall via a common protein scaffold (Guimarães *et al.*, 2002). They are typically produced by anaerobic bacteria and represent a minor part of known cellulolytic enzymes (Fontes & Gilbert, 2010; Lynd *et al.*, 2002). In nature the major part of cellulases is represented by non-complexed enzymes (Lynd *et al.*, 2002). Non-complexed cellulases consist of a set of individual enzymes that are secreted mainly by aerobic fungi and bacteria. Recently, another mechanism of cellulose degradation has been reported. The bacterium *Caldicellulosiruptor bescii* secretes an individual enzyme, CelA, that consist of two catalytic domains (CD) and three carbohydrate binding modules (CBM) (Brunecky *et al.*, 2013). Owing to its modular architecture, CelA may be regarded as placing in between cellulosomes and non-complexed cellulases. In the further course of this thesis, I will focus on non-complexed cellulases only.

Degradation of chitin is carried out by chitinases (G. Davies & Henrissat, 1995; Hult *et al.*, 2005). Overall similarity of cellulose and chitin is reflected in the evolvement of similar enzymatic machineries for their degradation. Exochitinases hydrolyze chitin chain from either reducing or non-reducing end whereas endochitinases attack chitin chains at random positions. The major products of chitin hydrolysis are dimers or oligomers of N-acetyl-D-glucosamine which are further degraded to monomers by β-N-acetyl-hexosaminidases (Vaaje-Kolstad *et al.*, 2013). Like in case of cellulose, chitin degradation is also assisted by LPMOs (Hemsworth *et al.*, 2014). In fact, the involvement of LPMOs in the degradation of recalcitrant polysaccharides was first identified in chitin degradation (Vaaje-Kolstad *et al.*, 2010). An alternative pathway of chitin degradation involves its deacetylation to chitosan that can be further hydrolyzed by chitosanases. Chitin decomposition through initial deacetylation was proposed to be more important in soil and sediment compared to water environments (Gooday, 1990).

1.2.1 *Trichoderma reesei* cellulolytic system

The filamentous fungus *Trichoderma reesei* (teleomorph *Hypocrea jecorina*) is the major producer of biomass-degrading enzymes. Industrial strains of the fungus can produce up to 100 g of extracellular protein per liter (Martinez *et al.*, 2008; Payne *et al.*, 2015). Its cellulolytic machinery consists of two cellobiohydrolases, five endoglucanases, two β – glucosidases and one LPMO (Foreman *et al.*, 2003; Martinez *et al.*, 2008). The major components are cellobiohydrolases TrCel7A (formerly CBHI) and TrCel6A (formerly CBHII) that account for 60% and 20% of total cellulases secreted by *T. reesei*, respectively (Chanzy & Henrissat, 1985; Teeri, 1997). Amongst endoglucanases the most abundant is TrCel7B (formerly EGI) followed by TrCel5A (formerly EGII) (Karlsson *et al.*, 2002; Kleywegt *et al.*, 1997). All cellulases, except endoglucanase TrCel12A, have a two-domain structure with a small carbohydrate binding module connected to a catalytic domain through a glycosylated linker peptide (fig. 2A) (Karlsson *et al.*, 2002).

Like all family 7 GH-s TrCel7A is a retaining enzyme (Divne *et al.*, 1998). It is a two-domain enzyme with a molecular weight of 45–65 kDa depending on the extent of its glycosylation (Jeoh *et al.*, 2008; Stals *et al.*, 2004). TrCel7A is an exo-acting cellobiohydrolase that starts hydrolysis from the reducing end of the cellulose chain and produces cellobiose as the main product (Divne *et al.*, 1994; Nutt *et al.*, 1998). Cellobiohydrolase TrCel6A is an inverting enzyme that starts hydrolysis of the cellulose chain from its non-reducing end, releasing cellobiose as the main product (Chanzy & Henrissat, 1985; Rouvinen *et al.*, 1990). Both TrCel7A and TrCel6A are usually thought to be strictly exo-acting processive hydrolases that conduct multiple catalytic acts before leaving from substrate. While their processive mode of action is widely recognized, there is a controversy about the mode of the initial attack. Some studies support strict exo-mode initiation whereas others point to a possible endo-mode initiation (Armand *et al.*, 1997; Ståhlberg *et al.*, 1993; Zou *et al.*, 1999; Varrot *et al.*, 1999).

Contrarily to CBH-s, endoglucanases are distributive enzymes that make only a single or limited number of catalytic acts before leaving the substrate. Endoglucanases TrCel7B, TrCel5A and TrCel12A use the retaining mechanism of hydrolysis of glycosidic bonds, whereas endoglucanases TrCel45A and TrCel74A are inverting enzymes (G. Davies & Henrissat, 1995; Payne *et al.*, 2015). TrCel61 that was initially thought to be an endoglucanase (Saloheimo *et al.*, 1997) was later confirmed to be LPMO (Horn *et al.*, 2012b).

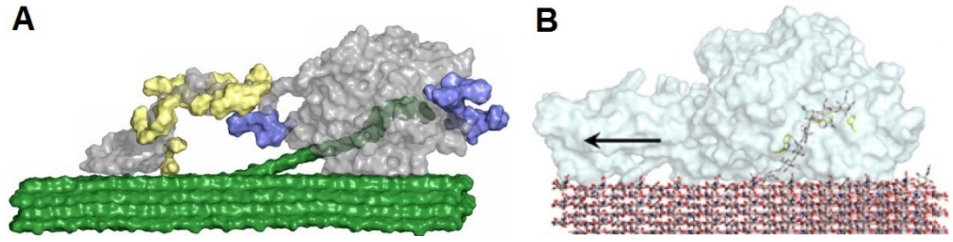

Figure 2. General two-domain structure of *T. reesei* cellulases and *S. marcescens* chitinases. **A.** TrCel7A degrading cellulose (cellulose chain is threaded into catalytic center). Small CBM is connected to a catalytic domain through a 27-residue linker peptide. A cellulose crystal is displayed in green, the enzyme in gray, O- and N- glycans are yellow and blue, respectively. Modified from (Payne *et al.*, 2013b). **B.** SmChiA degrading chitin from the reducing towards the non-reducing end (the moving direction is represented with an arrow). The chitin chain is detached from the crystal and threaded into the catalytic center. CBM is tightly attached to catalytic domain. Adapted from (Jana *et al.*, 2016). Copyright (2016) American Chemical Society.

1.2.2 The chitinolytic system of *Serratia marcescens*

The gram negative bacterium *Serratia marcescens* is one of the most efficient chitin degraders among microorganisms (Beier & Bertilsson, 2013). When grown on chitin it produces three chitinases (ChiA, ChiB and ChiC), one LPMO (CBP21) and a N-acetylhexosaminidase or chitobiase (Vaaje-Kolstad *et al.*, 2013).

S. marcescens chitinases have a two-domain architecture. Different from *T. reesei* cellulases, *S. marcescens* chitinases (except for ChiC) do not have flexible linker peptides and CBM is tightly attached to catalytic domain (fig. 2B) (Payne *et al.*, 2012; Perrakis *et al.*, 1994; van Aalten *et al.*, 2000). All *S. marcescens* chitinases belong to GH family 18. They are retaining enzymes that employ a substrate assisted catalytic mechanism for β-glycosidic bond hydrolysis. In the substrate assisted mechanism the oxygen atom of N-acetamido group of the substrate serves as a nucleophile instead of the carboxylate group of the enzyme which is usually employed by retaining enzymes (Vaaje-Kolstad *et al.*, 2013).

The main component of the chitinolytic machinery of *S. marcescens* is ChiA. It is a processive enzyme moving along the chitin chain from the reducing end toward the non-reducing end releasing chitobiose as the main product (Perrakis *et al.*, 1994). Besides exo-mode initiation from the reducing end, ChiA has been shown to employ also endo-mode initiation (Horn *et al.*, 2006b). Another chitinase produced by *S. marcescens* is ChiB. ChiB is a processive chitinase moving in the opposite direction as compared to ChiA i.e. from the non-reducing end toward the reducing end (van Aalten *et al.*, 2000). The third chitinase ChiC is a non-processive endochitinase. Although initially defined as a separate chitinase ChiC2 was later confirmed to be a catalytic domain derived

by proteolytic cleavage of full length ChiC (Payne *et al.*, 2012). A 21 kDa chitin binding protein acts synergistically with chitinases but its catalytic mechanism remained unknown until the year 2010 when it was shown to be a LPMO (Vaaje-Kolstad *et al.*, 2010).

1.2.3 Synergism between enzyme components

Efficient degradation of cellulose and chitin is achieved only by a concerted synergistic action of different types of enzymes. Synergism means that the total activity of enzymes acting together is higher when compared to the sum of individual activities. The first explanation of synergistic effect between cellulases was proposed by Reese *et al.* in the field defining study more than 50 years ago (Reese *et al.*, 1950). They offered a two component mechanism of cellulose degradation. The C1 component was proposed to swell and expose single cellulose chains and oligomers which are further degraded by the Cx component. Cx was identified as an endoglucanase while the nature of C1 remained unknown. Later on, with discovery of exoglucanase activity in the mixture of cellulolytic enzymes CBHs were ascribed to the C1 component in Reese's C1-Cx system (Kostylev & Wilson, 2012). Further investigations lead to a model of endo-exo synergism which is widely recognized since then. According to this model endo-enzymes hydrolyze polysaccharide chains from random positions (preferably amorphous parts) in a non-processive manner and therefore increase the number of chain ends that in turn are substrates for exo-enzymes. The latter processively degrade crystalline parts of cellulose/chitin exposing previously hidden amorphous parts. Therefore, the activity of one component increases the activity of another and *vice versa* (Wood & McCrae, 1972; Väljamäe *et al.*, 1999). The role of β-glucosidases and chitobiases in synergistic interactions is supposed to be in the degradation of product inhibitors of exoenzymes´ (soluble oligo- and disaccharides) to monosaccharides (Kostylev & Wilson, 2012).

During the past decades a number of alternative models of synergism have emerged (Din *et al.*, 1994; Igarashi *et al.*, 2011; Kostylev & Wilson, 2012; Väljamäe *et al.*, 1999). One of them was aimed to explain the enigmatic synergistic effect between two CBHs (Nidetzky *et al.*, 1994). It was speculated that by hydrolyzing crystalline parts, CBHs uncover new chain ends that have been buried inside the crystal (Barr *et al.*, 1996). Two CBHs with opposite chain end specificity thus create new initiation sites for each other. However, the report on possible endo-activity of some CBHs allowed to adapt the conventional mechanism of endo-exo synergism to explain the cooperation between CBHs (Boisset *et al.*, 2000; Boisset *et al.*, 2001).

An alternative mechanism of endo-exo synergism, whereby EG removes CBH-generated solitary cellulose chains from the crystal surface that otherwise serve as obstacles for CBH, has also been proposed (Väljamäe *et al.*, 1999). This "surface cleaning" mechanism was later adapted to describe the synergistic effect between different enzyme components.

A recently discovered mechanism for the action of LPMO has led to a resurgence of the original C1/Cx model of synergism (Vaaje-Kolstad *et al.*, 2010). By disrupting polysaccharide chains through redox reactions LPMOs increase disorder in the crystalline parts of microfibrils. Since amorphous cellulose/chitin is easier to degrade the overall degradation of polysaccharides is accelerated. Therefore, the action of LPMOs is reminiscent of Reesei´s C1 component whereas GBHs serve as Cx (Kostylev & Wilson, 2012; Morgenstern *et al.*, 2014).

1.3 Processive enzymes

Polymers are widely spread in nature and there is also high abundance of enzymes operating on polymers. A number of these enzymes employ processive catalysis. Once bound to substrate they conduct a multitude of consecutive catalytic acts without dissociating (Breyer & Matthews, 2001). Processive enzymes are dominant components of the cellulolytic and chitinolytic apparatus secreted by fungi and bacteria that play an important role in the global carbon cycle (Martinez *et al.*, 2008).

Processive cellulases and chitinases are efficient in degradation of crystalline cellulose and chitin respectively. Owing to the ability to conduct a number of sequential catalytic acts without dissociation from substrate these enzymes are although slowly, able completely saccharify native substrates without the help of accessory proteins (Igarashi *et al.*, 2011; Teeri, 1997). On the other hand, processivity comes as a penalty in the degradation of amorphous and soluble polymeric substrates (Horn *et al.*, 2006a). The costs and benefits of processivity in degradation of natural substrates remains to be elucidated. To gain better understanding of structure-processivity relationships reliable methods for measuring and quantifying processivity are needed.

1.3.1 Structural determinants of processivity

To date a huge number of the crystal structures of processive and non-processive enzymes is available. Analysis of this information has revealed structural features characteristic to processive enzymes. The most distinctive structural features of processive enzymes are: i) more or less closed active site topology and ii) the presence of multiple consecutive monomer unit binding sites.

Typically, processive glycanases have active sites that rest in a deep cleft that may be partially (fig. 3C) or completely (tunnel shaped) closed (fig. 3A). On the contrary, non-processive glycanases have shallow substrate binding clefts (fig. 3D).

The contribution of active site topology to processivity can be demonstrated by the example of CBH TrCel7A and EG TrCel7B. Both enzymes have about 45% sequence identity and a similar structure of their catalytic domains (fig. 3 A and B). The active site of TrCel7A is tunnel shaped and roofed by four long

surface loops (Divne *et al.*, 1998; von Ossowski *et al.*, 2003). The corresponding surface loops of TrCel7B are shorter resulting in a more open active site cleft (Kleywegt *et al.*, 1997; Taylor *et al.*, 2013). The difference between the processive cellulase of *Trichoderma reesei* Cel6A and its non-processive homolog, the endoglucanase E2 of *Thermomonospora fusca*, lies also in the lack of active site-covering loops in E2. The processive CBH of *Humicola insolens*, Cel6A, has a C-terminal active site loop which is absent in the non-processive EG Cel6B (G. J. Davies *et al.*, 2000). In order to examine the impact of active site topology on processivity Ossowski *et al.* prepared a TrCel7A mutant where the tip of the surface loop was deleted. The mutation caused a twofold reduction in processivity on AC and BMCC (von Ossowski *et al.*, 2003).

In case of partial enclosure of the active site the degree of processivity is thought to be influenced by the depth of the cleft. Thus, the binding sites of processive chitinases ChiA (fig. 3 C) and ChiB of *Serratia marcescens* are in deep clefts whereas non-processive ChiC (fig. 3 D) has a shallow substrate binding cleft. ChiC also lacks several surface loops present in ChiA and ChiB (Horn *et al.*, 2006b; Payne *et al.*, 2012). It has also been shown that flexibility in active site covering loops may stimulate endo activity. Being flexible, the loops covering the active site of TrCel6A may occasionally open and the enzyme may initiate productive association in the middle of cellulose chain. When the enzyme-substrate complex is formed surface loops close and the enzyme proceeds with hydrolysis in processive manner. Enzymes employing this type of mode of action are referred to as endo-processive enzymes (Boisset *et al.*, 2000; Varrot *et al.*, 1999).

Active site topology is not the sole determinant of processivity. Both TrCel7B (fig. 3B) and SmChiA (fig. 3C) have deep clefts that are partially enclosed. However, the first is a distributive endoglucanase while the second is a processive chitinase.

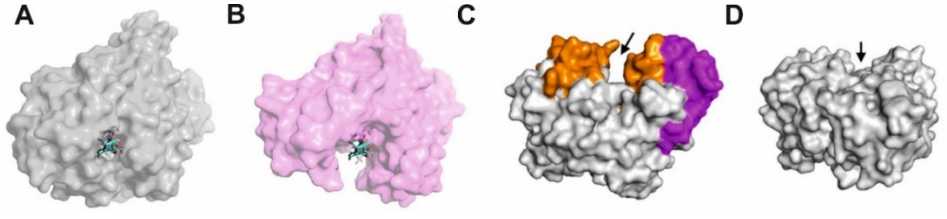

Figure 3. Comparison of active site topology of cellulases and chitinases. **A.** The active site of TrCel7A rests in a long tunnel that is closed by four loops. **B.** The active site of TrCel7B is similar to the one of TrCel7A but some of the loops are absent. **C.** The active site (indicated with arrow) of processive SmChiA rests in a deep cleft. **D.** The substrate binding site (indicated with arrow) of non-processive SmChiC2 has shallow geometry. A and B modified from (Taylor *et al.*, 2013). C and D modified from (Payne *et al.*, 2012)

Another characteristic of processive enzymes is the presence of a number of consecutive glycoside unit binding sites. A multitude of amino acid residues within the catalytic center form hydrogen bonds with the carbohydrate chain and supply the enzyme with sufficient free energy to detach a single polysaccharide chain from the crystal and hold it threaded into the active site after hydrolytic acts (Payne *et al.*, 2013a). Processive cellulases and chitinases involve more such interactions as compared to non-processive enzymes. Thus, the highly processive TrCel7A has a 5 nm long active site with 7 substrate binding sites (-7 to -1) and 3 product binding sites (+1 to +3). Marks "+" and "-" stand for the reducing and non-reducing ends of cellulose chain, respectively, whereas catalysis takes place between -1 and +1 sites (G. J. Davies *et al.*, 1997; Divne *et al.*, 1998). TrCel7A homologs *Trichoderma harzianum* ThCel7A and *Phanerochaete chrysosporium* PcCel7D have also 10 glucose unit binding sites (Muñoz *et al.*, 2001). Although the processive chitinase SmChiA has only 4 substrate binding sites (-4 to -1) and 3 product binding sites (+1 to +3), 6 additional substrate binding sites that rest in CBM may be considered as an extension of the active site cleft, since CBM is tightly attached to the catalytic domain (Aronson *et al.*, 2003). Therefore, ChiA has 13 substrate binding sites in total. With TrCel7A it has been shown that CBM has little or no effect on the rate of processive movement (Cruys-Bagger *et al.*, 2013; Igarashi *et al.*, 2009) and processivity (Brady *et al.*, 2015; Hall *et al.*, 2011; Igarashi *et al.*, 2011). The impact of CBM on processivity of SmChiA and other enzymes with rigidly connected CBM is not known. The impact of the enzyme-substrate hydrogen bonding network in the active site has been also demonstrated experimentally. Substitutions of amino acids that form hydrogen bonds with glucose units in subsites -2 to -4 of the processive endoglucanase Cel9A from *Thermobifida fusca* lead to significantly reduced processivity being accompanied by 85% loss of activity on BC (Li *et al.*, 2007). Substitution of threonine 276 (substrate binding subsite −2) to alanine in SmChiA also results in reduced processivity on chitin (Hamre *et al.*, 2015).

As seen with active site architecture, the presence of multiple binding sites is also not the sole determinant of processivity. For instance, TrCel6A has only 6 glucose unit binding sites (-2 to +4) (Payne *et al.*, 2013b; Teeri, 1997) and is thought to be processive, whereas the non-processive endoglucanase TrCel7B has 10 glucose unit binding sites (-7 to +3) (Kleywegt *et al.*, 1997).

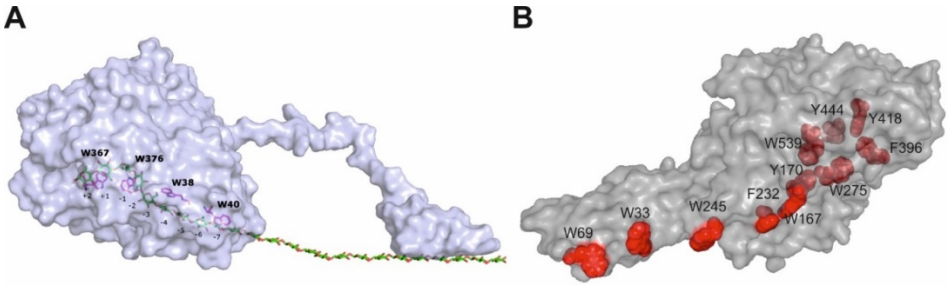

Figure 4. Carbohydrate-aromatic interactions in cellulases and chitinases. **A.** Two-domain structure of TrCel7A with tryptophans responsible for the stacking interactions with the substrate. Adapted from (Kari *et al.*, 2014) **B.** Two-domain structure of SmChiA with amino acids (colored red) responsible for the aromatic-carbohydrate interactions (Zakariassen *et al.*, 2009). The image was made in PyMOL using the crystal structure of the SmChiA (Protein Data Bank code 1EDQ).

Aromatic amino acids that stack with the planar face of the carbohydrate ring via π-interactions also play an important role in determining processivity of cellulases and chitinases (Payne *et al.*, 2011). Proteins generally employ multiple aromatic-carbohydrate interactions in order to achieve a high degree of processivity (fig. 4). Thus, in processive chitinases the conserved sequence motif (SXGG residues 102–105) is followed by a Trp. In non-processive chitinases this Trp is substituted with Ala (Payne *et al.*, 2012). Several Trp point mutations have been shown to turn processive chitinases into non-processive enzymes. These modifications reduce the ability of enzymes to degrade crystalline chitin. In parallel the activity on soluble chitosan increases (Horn *et al.*, 2006a; Payne *et al.*, 2013a). Impact of Trp to Ala point mutations on processivity has been investigated also for cellulases. The substitution of three tryptophans in positions 135, 272 or 367 of TrCel6A to alanine resulted in reduced processivity (Payne *et al.*, 2011). In all cases the loss of processivity was also accompanied by reduced activity on crystalline substrates. W38A and W40A point mutations in TrCel7A also resulted in a lower degree of processivity (Igarashi *et al.*, 2009; Kari *et al.*, 2014; Kont *et al.*, 2016).

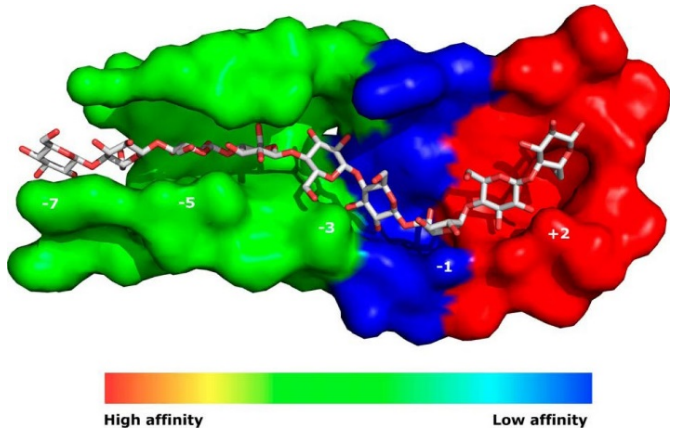

Figure 5. "Heat map" of the active site E212Q mutant of TrCel7A with cellononaose. Numbers correspond to substrate and product binding sites. The strength of binding is represented by coloring according to the legend under the image. Figure is adapted from (Colussi *et al.*, 2015).

In recent piece of work Colussi *et al.* investigated interactions between the catalytically deficient variant of TrCel7A (E212Q) and cellooligosaccharides of different DP by isothermal titration calorimetry (Colussi *et al.*, 2015). They concluded that the binding affinity is not uniformly distributed over the catalytic center (fig. 5). According to the binding affinity the active site of TrCel7A can be dissected into three regions. The first part of the tunnel, an entrance region, has a moderate binding affinity. The binding affinity is lowest near the scissile bond where the cellulose chain is twisted, and strongest in the product expulsion sites at the end of the tunnel (Colussi *et al.*, 2015). This finding is also supported by an *in silico* study on TrCel7A, the main idea of which is that strong interactions in the product binding site are the primary force for the processive movement of the enzyme along the cellulose chain whereas stacking interactions between carbohydrate rings and aromatic amino acids have moderate interaction energies and are obviously required for "guiding" polysaccharide chains through the binding tunnel with subsequent twisting of the cellulose chain (catalytic activation) (Knott *et al.*, 2014).

Taken together, there seems to be no single unequivocal structural determinant of processivity. It rather stems from a sophisticated interplay between active site topology, the number of sugar unit binding sites and pattern of binding energy.

1.3.2 Processivity in quantitative terms

According to its empirical definition the processivity equals to the average number of catalytic events performed by the enzyme per one productive binding event (Lucius *et al.*, 2003). Two different definitions of processivity, intrinsic processivity (P^{Intr}) and apparent processivity (P^{app}) are found in literature (Horn *et al.*, 2012a). P^{Intr} represents the processive potential of an enzyme. It can realize only on an ideal polymer where the length of the processive run is governed solely by the dissociation probability (P_d) of the enzyme-substrate complex (Lucius *et al.*, 2003). It is shown that:

Equation 1. $$P^{Intr} = -\frac{1}{\ln(1-P_d)} \approx \frac{1}{P_d}$$

After association with the polymer chain the enzyme has two possible scenarios: i) dissociate from the substrate or ii) move forward and act catalytically. The probability of first scenario is represented by P_d whereas the probability of second scenario is 1-P_d. P_d can also be expressed by two rate-constants, the dissociation rate constant (k_{off}) and the catalytic constant (k_{cat}).

Equation 2 $$P_d = \frac{k_{off}}{k_{off}+k_{cat}} \approx \frac{k_{off}}{k_{cat}}$$

In the case of highly processive enzymes k_{off} is much smaller than k_{cat}. What follows is that in this case $P_d << 1$ and equations 1 and 2 can be simplified as shown in the rightmost side of the equations, namely that Pd nearly equals k_{off}/k_{cat}. Combining equations 1 and 2 enables to define P^{Intr} in terms of k_{off} and k_{cat} according to equation 3.

Equation 3 $$P^{Intr} \approx \frac{k_{cat}}{k_{off}}$$

On real polymers processivity may be limited by steric obstacles, be it morphological structures of substrate or unproductively bound enzymes (Jalak & Valjamae, 2010; Väljamäe *et al.*, 1998). Therefore, it is more appropriate to use the term apparent processivity (P^{app}). P^{app} is defined as the number of catalytic acts (N^{catal}) divided by the number of initiations of processive runs (N^{Init}).

Equation 4 $$P^{app} = \frac{N^{catal}}{N^{init}}$$

If P^{Intr} shows the processive potential, P^{app} shows the manifestation of this potential on a real polymer (Beckham *et al.*, 2014). On an ideal polymer P_d is independent of the position of the enzyme on the polymer i.e. there is the same P_d value for each consecutive processive step. In this case P^{app} is expected to equal P^{Intr}. On real polymers P^{app} is often far lower than P^{Intr}.

1.3.3 Measuring processivity of cellulases and chitinases

Despite the wealth of structural data on processive cellulases and chitinases there is still a lack of adequate methods to quantitatively assess processivity. Most of the methods, aimed at measuring apparent processivity as discussed below, have certain limitations that are in turn caused by heterogeneity of substrates and complicated analysis of a reaction at the solid/liquid interphase. In measuring P^{app}, the biggest challenge is to determine the number of initiations (eq.4).

The simplest method for measuring the processivity of cellulases and chitinases relies on analyzing the composition of hydrolysis products. Since each consecutive sugar in a polysaccharide chain, be it chitin or cellulose, is rotated by 180° to its neighbor (fig. 1A and 1B), prevalence of even numbered products (mainly disaccharides) is expected for processive enzymes (Horn *et al.*, 2012a). Odd-numbered products (mainly trimers or monomers) can be formed only by initial hydrolytic acts. Therefore, the number of odd-numbered products corresponds to productive initiations of an enzyme on a polymer while the number of even numbered products correspond to the sequential catalytic acts. Herein, processivity is found according to the following equation.

Equation 5 $$P^{app} = \frac{([dimers]-[monomers])}{([trimers]+[monomers])} + 1$$ (Horn *et al.*, 2012a)

In equation 5 it is assumed that enzyme releases only trimers during the initial catalytic act while monomers appear as a result of cleavage of trimers in solution (Horn *et al.*, 2006b). Therefore, corrections with respect to concentration of dimers and trimers has been made (Horn *et al.*, 2012a). Despite the simplicity of the described methodology, it has some disadvantages. Most importantly, the processivity strongly depends on the pattern of the initial-cut products. If the initial hydrolytic acts give rise to dimers, the number of initiations will be underestimated and the processivity will be overestimated. Neither does this approach take into account endo-mode initiations. Cleavage of glycosidic bonds in the middle of a polysaccharide chain does not result in the formation of odd-numbered soluble oligosaccharides and P^{app} will be overestimated. Ambiguity in the initial product pattern is best revealed by the number of equations available in literature (eq. 6–9). Generally, the values of processivity measured with even/odd-numbered products for TrCel7A and SmChiA vary in the range of 5–30 (table 1 and 2). Some of them have been "calibrated" against other methods for P^{app}, like high-speed atomic-force microscopy (HS-AFM), and have been shown to be reliable only for certain enzyme substrate systems (Nakamura *et al.*, 2014). Unfortunately, no general judgement about the applicability of equations 5–9 can be made.

Equation 6 $$P^{app} = \frac{[dimers]}{[monomers]+[trimers]}$$

(Nakamura *et al.*, 2014; von Ossowski *et al.*, 2003)

Equation 7 $$P^{app} = \frac{[dimers]}{[monomers]}$$

(Hamre *et al.*, 2014; Kari *et al.*, 2017)

Equation 8 $$P^{app} = \frac{[dimers]*(1+\alpha)}{2*([trimers]+[tetramers])} + \frac{1+\alpha}{2}$$

where $$\alpha = \frac{[dimers]}{[dimers]+[tetramers]}$$ (X. Z. Zhang *et al.*, 2010)

Equation 9

$$P^{app} = \frac{[processive\ cuts]}{[initial\ cuts]} = \frac{[G2]_{obs} - [G2]_{G3hyd} - [G2]_{EG\ generated} - [G2]_{CBH\ initial\ cuts}}{(1+\alpha+\beta)*([G3]_{obs} + [G3]_{G3\ hyd} - [G3]_{EG\ generated})}$$

detailed derivation of the formula is described in (Fox *et al.*, 2012)

Another approach to find the value of processivity is to quantify the number of soluble (SRG) and insoluble (IRG) reducing groups on native substrates after treatment with an enzyme (Eijsink *et al.*, 2008; Irwin *et al.*, 1993; Vuong & Wilson, 2009). If an enzyme starts hydrolysis using the endo-mode initiation and leaves the substrate before complete hydrolysis of cellulose chain, it forms an insoluble reducing group on cellulose as a result of the first cut. Consecutive processive movement along the polysaccharide chain will be accompanied by the release of soluble products. Therefore, processivity of an enzyme can be calculated as a ratio of the number of soluble sugars to the number of enzyme-generated insoluble reducing ends. Like with the previously described method this approach has several pitfalls. If an enzyme, be it processive or non-processive, starts hydrolysis from the end of the polymer chain, only soluble products will be generated while the number of insoluble reducing ends remains unchanged. As a result, processivity will be overestimated (Horn *et al.*, 2012a). The values of processivity of TrCel7A found by measuring the ratio of SRG and IRG are in the range of 20 (table 1).

The method proposed by Kipper *et al.* relies on hydrolysis of reducing-end fluorescence labeled cellulose (Kipper *et al.*, 2005). In case of reducing-end exo-initiation the initial cleavage of the glycosidic bond will result in the formation of a fluorescent glycoconjugate while further hydrolysis products will be unlabeled. The processivity is found as the ratio of the velocities of the generation of unlabeled to fluorescent products in the steady state. However, if the enzyme starts hydrolysis from the position on the cellulose chain other than the reducing end, no fluorescent label will be released and processivity will be overestimated. Processivity values found for TrCel7A on labeled substrates are much higher as compared to other methods (table 1).

Table 1. Values of apparent processivity of TrCel7A found in literature

Substrate	Method	P^{app}	Reference
Amorphous cellulose BMCC	Even/odd products ratio estimation	14 23	(von Ossowski *et al.*, 2003)
Filter paper	Soluble/insoluble	22	(Irwin *et al.*, 1993)
Amorphous cellulose Cellulose Iα Cellulose III_I	Even/odd products ratio estimation	15.5±0.1 20.3±1.5 34.8±0.9	(Nakamura *et al.*, 2014)
Cellulose III_I	HS-AFM	34	(Nakamura *et al.*, 2014)
Cellulose Iα Cellulose III_I	Single-molecule fluorescence microscopy	42 36.4	(Shibafuji *et al.*, 2014)
Avicel	Even/odd products ratio estimation	5–10	(Medve *et al.*, 1998)
BC BMCC	Fluorescently end-labeled cellulose hydrolysis	88±10 42±10	(Kipper *et al.*, 2005)
Filter paper	Optical tweezers-based single-molecule motility assay	50	(Brady *et al.*, 2015)
Amorphous cellulose Avicel BMCC	Global nonlinear regression analysis	13.7 22.7 22.6	(Cruys-Bagger *et al.*, 2012; Cruys-Bagger *et al.*, 2013)

Contrary to direct measurements of cellulase processivity the method suggested by the group of Peter Westh relies on mathematical model designed to fit experimental data (Cruys-Bagger *et al.*, 2012; Cruys-Bagger *et al.*, 2013). This group employs real-time measurements with amperometric biosensors to conduct experiments in the pre-steady-state regime. This transient stage was chosen because it provides with the basic steps of the catalytic cycle of the enzyme. The advantage of this method is that it also enables to assess important kinetic parameters like the catalytic constant and the dissociation rate constant (table 3). These can be used for the estimation of the intrinsic processivity (eq. 3). On the other hand, the values of kinetic parameters are strongly dependent on the assumptions used for the development of the mathematical model.

Table 2. Apparent processivity of SmChiA found in literature

Substrate	Method	P^{app}	Reference
β chitin	HS-AFM	30.3	(Igarashi *et al.*, 2014)
β chitin α chitin	Even/odd products ratio	7.3 5.9	(Horn *et al.*, 2006b)
chitosan	Chitosan degradation pattern analysis	9.1	(Sikorski *et al.*, 2006)
β chitin α chitin	Even/odd products ratio	30.1 14.2	(Hamre *et al.*, 2014)

Besides the aforementioned, microscopy has also been shown to be a powerful tool in the investigation of enzymes operating on polysaccharides. In 2009, employing TrCel7A, Igarashi *et al*. were able to observe single molecule movement along the polysaccharide chain with HS-AFM which marked an outstanding breakthrough in the field of cellulase research (Igarashi *et al.*, 2009). Later on, an analogous study was conducted on chitinases (Igarashi *et al.*, 2014). The advantage of the method is that it allows for quantification of catalytic (k_{cat}) and dissociation (k_{off}) rate constants (table 3 and 4) besides measuring the value of processivity (table 1 and 2). Combination of HS-AFM with single molecule fluorescence microscopy also enables to determine the degree of processivity of cellulases. For this purpose, the method of HS-AMF is applied for determination of the translation rate (i.e. turnover constant) of enzyme, while single molecule fluorescence microscopy provides with the dissociation rate constant (Shibafuji *et al.*, 2014). In both cases the apparent processivity is found from the ratio of k_{cat} to k_{off}. Recently, another single-molecule motility assay, based on optical tweezers, has been used for the measurement of processivity (tables 1 and 3) (Brady *et al.*, 2015). In spite of many advantages, a disadvantage of these three methods is the need for high-priced specialized equipment which can be limiting for many researchers.

Table 3. Dissociation rate constant of TrCel7A found in literature

Substrate	Method	k_{off} s^{-1}	Reference
Cellulose III_I	HS-AFM	0.2	(Nakamura *et al.*, 2014)
Cellulose Iα Cellulose III_I	Single-molecule fluorescence microscopy (productive/nonproductive)	0.12/0.86 0.14/0.84	(Shibafuji *et al.*, 2014)
Filter paper	Optical tweezers-based single-molecule motility assay	≥0.01	(Brady *et al.*, 2015)
Amorphous cellulose Avicel BMCC	Global nonlinear regression analysis	0.023 0.011 0.010	(Cruys-Bagger *et al.*, 2012; Cruys-Bagger *et al.*, 2013)
BMCC	FRAP	$5x10^{-6}$	(Luterbacher *et al.*, 2013)
Cellulose Iα	Single-molecule fluorescence microscopy	0.033	(Jung *et al.*, 2013)

Table 4. Dissociation rate constant of SmChiA found in literature

Substrate	Method	k_{off} s^{-1}	Reference
β-chitin	HS-AFM	2.36	(Igarashi *et al.*, 2014)

To escape the problems associated with complicated analysis at solid/liquid interphase, one may use highly acetylated water-soluble chitosans. Analysis of the product pattern at a very low extent of substrate hydrolysis by means of size-exclusion chromatography provides with a qualitative description of processivity. Apparently, processive enzymes lead to a slow disappearance of the polymer fraction concomitant with production of dimers and to a lesser extent even-numbered oligomers (DP>2). On the contrary, non-processive endo-enzymes result in a rapid decrease of the polymer fraction and production of equal amounts of even- and odd-numbered long oligomers (Horn *et al.*, 2006b; Horn *et al.*, 2012a; Sikorski *et al.*, 2006). Measuring the change in the DP of substrate at a low degree of scission enables to determine whether an enzyme starts hydrolysis in endo- or exo-manner. Exo-enzymes slowly reduce the average degree of polymerization. On the contrary, DP quickly reduces by the action of endo-enzymes. Based on the data about changes in DP and the assumption that all attacks are endo, one can quantitatively estimate processivity. In this case the change in DP of a polymeric fraction (ΔDP^{pol}) corresponds to the number of initiations of processive runs whereas the average change in DP (ΔDP) corresponds to the number of catalytic acts. Processivity is found as the ratio of ΔDP^{pol} to ΔDP (Sikorski *et al.*, 2006). However, one needs to consider that measured values tend to be lower as compared to those measured on natural substrates. This discrepancy is caused by the particular mechanism of the enzyme. Substrate assisted catalysis requires availability of a N-acetamido group of the sugar in the -1 subsite of the catalytic center of the enzyme, otherwise hydrolysis of glycosidic bond will not occur and the enzyme will slide further resulting in the release of longer (DP > 2) even-numbered products. Therefore, the values of processivity may be underestimated since the number of catalytic acts measured on chitosan, which is deacetylated to a certain extent, does not take into account “sliding” (idling) of the enzyme along the polymer chain. Moreover, exploitation of chitosans is restricted by research on chitinases since there is no analogous substrate for cellulases.

Apart from numerical expression of processivity, examination of morphological changes of crystalline substrates after treatment with enzymes by electron microscopy provides with a qualitative description of the processive mode of action. Observing degradation of Valonia cellulose by TrCel7A and TrCel6A revealed that TrCel6A sharpens the non-reducing end of cellulose microfibrils (Chanzy & Henrissat, 1985) whereas TrCel7A narrows the reducing end of microfibrils (Chanzy *et al.*, 1983). Apparently, sharpening by TrCel6A is caused by lower processivity since the enzyme quickly dissociates from the cellulose chain and starts hydrolysis from the adjacent polymer chain.

TrCel7A, on the contrary, stays on the cellulose chain for longer time intervals resulting in a higher extent of degradation. Qualitative description of the processive mode of action has also been obtained for chitinases on β-chitin (Hult *et al.*, 2005; Uchiyama *et al.*, 2001). Monitoring of how microfibrils are sharpened under electron microscope is a good method in comparative studies e.g. examination of mutational effects or comparing homologous enzymes. Unfortunately, it does not provide with any concrete numerical values.

2. RESULTS AND DISCUSSION

Aim of the study

In recent years, cellulases and chitinases have been of great interest as molecular motors for the production of substances of high value. However, natural enzymes are slow and construction of improved enzymes that can be adapted for industrial uses is required. Therefore, detailed understanding of molecular mechanisms involved in degradation of cellulose and chitin is critical for the rational protein engineering. The current thesis is focused on developing biochemical methods for the measurement of kinetic parameters that promote elucidating elementary reaction steps underlying the hydrolysis of the recalcitrant polysaccharides cellulose and chitin.

2.1 Measuring cellulase processivity under single hit conditions (paper I)

In the first paper, we had set a goal to develop a method that enables reliable measurement of the processivity of cellulases. By the time paper I was published most of the data on processivity had been derived from structural studies, while a small number of biochemical methods, aimed at direct measurements of the degree of processivity, had certain disadvantages (see also chapter 1.3.3).

Apparent processivity (P^{app}) is defined as the ratio of the number of catalytic acts (N^{catal}) to the number of initiation acts (N^{init}) (eq. 3). The most challenging task is the quantification of N^{init}. N^{init} can be measured by following the release of fluorescent glycoconjugate (EL) from reducing-end fluorescence labeled cellulose (Kipper *et al.*, 2005). In that case, however, one can measure the number of initiation acts exclusively from the reducing end while the initiation acts from random position on cellulose are not detectable (fig. 6B) (see also chapter 1.3.3). In order to circumvent this problem, we employed reduced cellulose for conducting hydrolysis experiments. Reduced cellulose has a C1 hydroxyl group at its reducing end instead of a C1 aldehyde group (fig. 6A).The treatment of reduced cellulose with an enzyme therefore results in two major products: i) soluble sugars released by the enzyme (further soluble reducing groups or SRG) and ii) residual cellulose that has C1 aldehyde groups at its reducing ends (further insoluble reducing groups or IRG). Herein the number of IRG corresponds to the number of the initiations of processive runs regardless of whether an enzyme starts hydrolysis from the reducing end or from a random position on the cellulose chain (fig. 6C). Apparent processivity is found by the following equation: (equation 10)

Equation 10 $$P^{app} = \frac{N^{catal}}{N^{init}} = \frac{[IRG]+[SRG]}{[IRG]}.$$

Since the first hydrolytic act will result in the formation of IRG only, this has to be included into the numerator. Despite of the simplicity of the described methodology, it has several pitfalls. If an enzyme starts hydrolysis from the non-reducing end IRG will not be produced and the degree of processivity will be overestimated. Moreover, this methodology cannot be applied to a mixture of cellulases, since there is no possibility to discriminate between IRG generated by different enzymes.

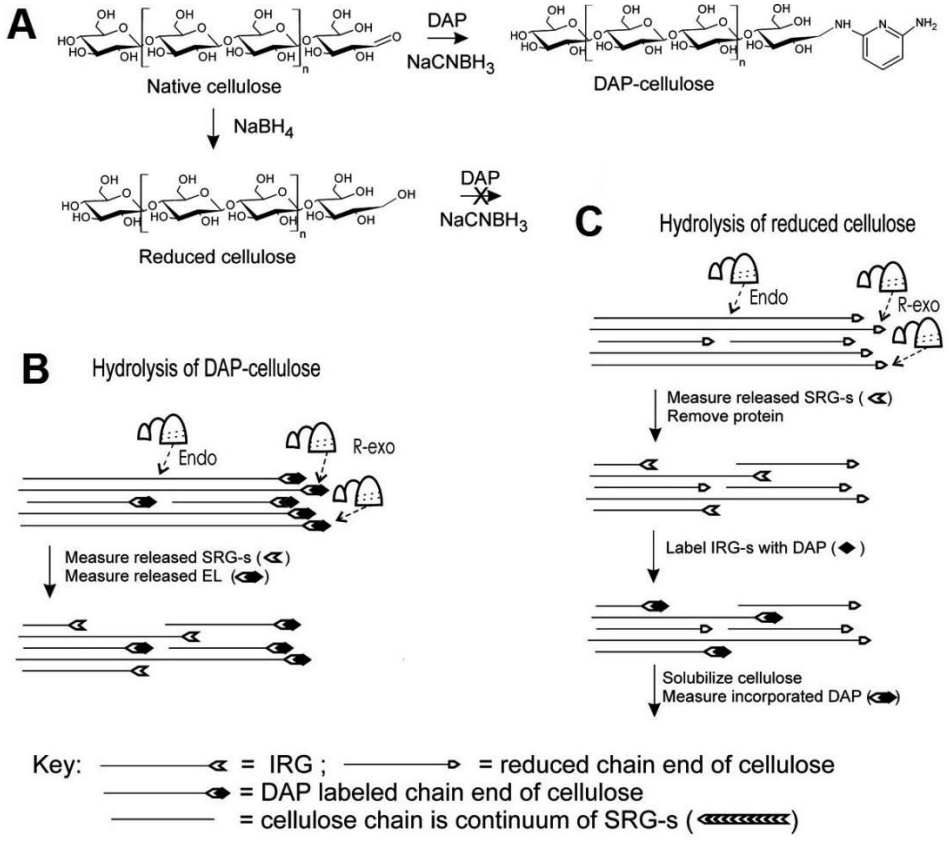

Figure 6. Principle of the measurement of cellulase processivity. **A** Reaction between the reducing end of cellulose and DAP. Owing to the 1:1 stoichiometry, the number of incorporated DAP corresponds to the number of reducing ends **B** The number of released end label (EL) from reducing end labeled cellulose corresponds to the number of exo-mode initiations whereas endo-mode initiations are not followed. **C** Using reduced cellulose as a substrate will result in formation of IRG from reducing-end exo- and endo-mode initiations. Because fluorescent label will react only with the aldehyde group, no labels are incorporated on untreated reduced cellulose. Therefore, only enzyme-generated reducing ends are available for fluorescence labeling.

For precise measurements of the N^{init} one needs to conduct hydrolysis of cellulose under single hit conditions, meaning that IRG generated by an enzyme on a cellulose chain will not be used as a starting point for another processive run. In addition to this, substrates with high DP must be used in order to avoid complete degradation of the cellulose chain, otherwise only SRG will be produced and processivity will be overestimated. We found that reduced bacterial cellulose (rBC) and reduced amorphous cellulose (rAC) prepared from filter paper are appropriate substrates for the processivity measurements. In order to meet the single hit conditions, the rate of formation of IRG should be proportional to the rate of formation of SRG (i.e. in linear dependence) (fig. 7A and fig. 3 in I).

For the quantification of enzyme generated IRG we applied fluorescence labeling of the reducing ends of cellulose with 2,6-diaminopyridine (DAP) (fig. 6A). Fluorescence labeling was chosen since the number of IRG generated by enzyme during hydrolysis is too low for absorbance-based methods.

First of all, we compared two CBHs (TrCel7A and PcCel7D) on reducing-end DAP-labeled and reduced celluloses. If an enzyme initiates the hydrolysis of cellulose from the reducing end only, the number of released end labels (ELs) should be equal to the number of enzyme generated IRG. However, if an enzyme can use endo-mode initiations in parallel with initiations from the reducing end, the number of IRG should exceed the number of EL (fig. 7B). Therefore, the difference between released EL and enzyme generated IRG enables us to estimate the probability of endo-mode initiations (P_{Endo}) according to equation 11.

Equation 11
$$P_{Endo} = \frac{[IRG]-[EL]}{[IRG]}$$

For precise estimation of P_{Endo} the number of EL and IRG should be measured at the same extent of degradation of cellulose (herein, the same amount of released SRG) (fig. 2A and 2C in I). We found, that endo-mode initiations constitute up to 50% for TrCel7A, when measured on BC and up to 90%, when measured on AC. PcCel7D demonstrated even higher P_{Endo} values which is probably due to a more open active site structure in comparison to TrCel7A (table 1 in I) (Divne *et al.*, 1994; von Ossowski *et al.*, 2003). A number of control experiments provided in paper I rule out the possibility that high P_{Endo} values are caused by contamination with EG or modifications at reducing ends of cellulose (supplementary in I, fig. S3 and S4 in I). We also measured P_{Endo} values for EGs (TrCel5A and TrCel12A). As expected, the proportion of endo-mode initiations for EGs was close to 100% (Table 1 in I). Based on our calculated P_{Endo} values, we concluded that cellobiohydrolases TrCel7A and PcCel7D, previously thought to act exclusively from the reducing end, should be placed in between strict exoglucanases and endoglucanases.

Measured numbers of SRG and IRG on reduced amorphous and bacterial celluloses provided us with estimates of the apparent processivity for TrCel7A

and PcCel7D (fig. 7 A and fig. 3 in I). Considering the error limits, P^{app} values were similar for both CBHs when measured on rBC. P^{app} values measured on rAC were also similar between CBHs but almost three times lower as compared to those measured on rBC (table 2 in I). We ascribed this phenomenon to the physicochemical properties of the substrate suggesting that the apparent processivity is defined by the cellulose rather than by the properties of an enzyme. In a previous study by our group we proposed a model where the processive length of CBH is limited by the average length of the "obstacle free path" on a polymer chain (Jalak & Valjamae, 2010). The results obtained in paper I also support this model.

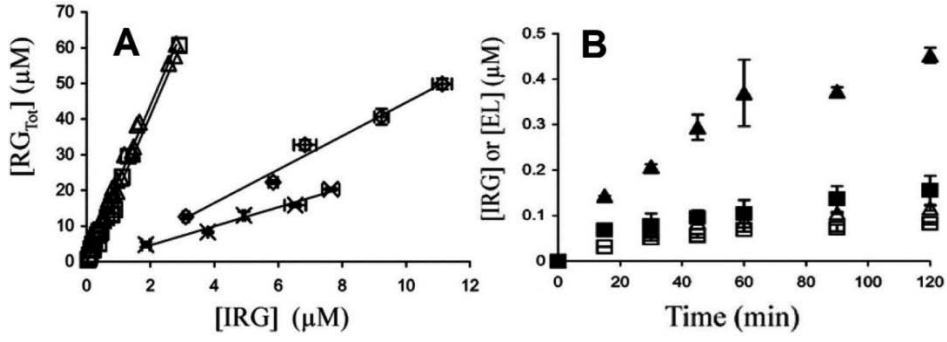

Figure 7. Hydrolysis of reduced and DAP-labeled celluloses. **A.** apparent processivity was measured on rAC for CBHs TrCel7A (□) and PcCel7D (△) and EGs TrCel5A (◊) and TrCel12A (×). RG_{Tot} was found as the sum of SRG and IRG. P^{app} was found as the slope of lines in RG_{Tot} vs IRG coordinates. For details see fig. 3 in I. **B.** Measuring IRG and EL on rBC and DAP-BC respectively. TrCel7A (□ and ■) or PcCel7D (△ and ▲). Open symbols (□ and △) refer to DAP-BC, and filled symbols (■ and ▲) refer to rBC. For details see fig. 2 in I. The slope of generation of IRG provides with estimate of k_{off} according to equation 12.

Since comparison of cellulases on the basis of apparent processivity is not straightforward we decided to estimate the values of intrinsic processivity (P^{Intr}) (see also chapter 1.3.2). According to equation 3, P^{Intr} is determined by two rate constants: the dissociation rate constant (k_{off}) and the catalytic constant (k_{cat}). It has previously been demonstrated by our group that the bottleneck of the catalytic cycle is the enzyme dissociation from the substrate that is governed by k_{off} (Jalak & Valjamae, 2010). The idea that k_{off} is rate-limiting for the recruitment of TrCel7A has been also supported by other studies (Cruys-Bagger *et al.*, 2012; Cruys-Bagger *et al.*, 2013; Igarashi *et al.*, 2011; Kari *et al.*, 2014). If this is the case, the rate of formation of IRG at a "steady state" should be driven solely by the dissociation rate of enzyme from cellulose that in turn enables to find k_{off} according to equation 12 (fig. 7B).

Equation 12 $$k_{off} \approx \frac{\Delta N_{init}}{\Delta t * [ES]} \approx \frac{\Delta [IRG]}{\Delta t * [E]_{Tot}}$$

Because of the low enzyme to substrate ratio used in this study, the approximation that [ES] ≈ $[E]_{Tot}$ is justified, as demonstrated by (Jalak & Valjamae, 2010, 2014).

In paper I we further measured the activity of enzyme in the initial stage of hydrolysis (10 s) to reveal k_{cat} (fig. S5 in I). We assumed that within this time interval enzyme molecules have not passed the "obstacle free path" (Jalak & Valjamae, 2010) and SRG release is governed by k_{cat} of glycosidic bond hydrolysis. We also assumed that all molecules are productively bound to the substrate. k_{cat} values of TrCel7A found in the first study (paper I) are in accordance with those found later (paper II) and by other groups using different methods (Cruys-Bagger *et al.*, 2013; Igarashi *et al.*, 2009; Jung *et al.*, 2013). Provided with the values of catalytic and dissociation rate constants we were able to determine the intrinsic processivity. P^{Intr} values for TrCel7A were almost 4 times higher as compared to PcCel7D both on rBC and rAC. Since the values of k_{cat} for both enzymes were close to each other the difference in P^{Intr} was determined by the difference in k_{off} values (table 2 in I). We also found that P^{Intr} values of CBHs were more than an order of magnitude higher as compared to P^{app} values. With EGs the difference between P^{Intr} and P^{app} values was less obvious (table 2 in I). Furthermore, the k_{cat} values of EGs were within the same order of magnitude with k_{cat} values of CBHs whereas k_{off} values were hugely different. Since the main difference between EGs and CBHs was the value of k_{off}, in paper I we concluded that this parameter may be the primary target for the selection of cellulases.

2.2 Measuring cellulase processivity under single turnover conditions (paper II)

Measurement of the apparent processivity of cellulases under single hit conditions (see also chapter 2.1) has several restrictions. First, it cannot be applied to enzymes that prefer the non-reducing end of a polysaccharide chain as starting point for hydrolysis. Second, only pure preparations of single enzymes can be used. To overcome these limitations, in the second study, we developed a novel method for the measurement of P^{app} of cellulases under single turnover conditions. We employed this method to investigate cellobiohydrolase TrCel7A and its truncated version $CD_{TrCel7A}$ (CBM-less TrCel7A). P^{app} was measured for CBHs acting in isolation or in the presence of EG TrCel5A.

The main idea of the method lies in following the release of the product of hydrolysis during one processive run of the enzyme. CBH and uniformly ^{14}C-labeled bacterial cellulose (^{14C}BC) are mixed within short time intervals after which an excess of non-labeled amorphous cellulose (trap) is added. Amorphous cellulose traps all unbound enzyme as well as enzyme that desorbs

back into solution from ^{14C}BC. Therefore, CBHs that are productively bound to ^{14C}BC by the moment of addition of the trap can realize only a single processive run and the release of radioactively labeled cellobiose (^{14}CB) complies with equation 13 (fig. 8A).

Equation 13 $$\left[^{14}CB\right] = [^{14}CB]_{max}(1 - e^{-kt})$$

In equation 13 the value of [^{14}CB] is limited by the plateau of [^{14}CB]$_{max}$ that in turn is the product of (i) the concentration of productive enzyme-substrate complexes by the moment of addition of the trap ([ES]$_{trap}$) and (ii) the length of processive run (P^{app}) as described by equation 14. The parameter k is the first order rate constant for passing through one processive run (eq. 15).

Equation 14 $$\frac{[^{14}CB]}{[ES]_{trap}} = \frac{[^{14}CB]_{max}}{[ES]_{trap}}\left(1 - e^{-kt}\right) = P^{app}\left(1 - e^{-kt}\right)$$

Equation 15 $$k = \frac{k_{cat}}{P^{app}}$$

First, we assessed the release of [^{14}CB] from ^{14C}BC by adding the trap after 10, 30 and 60 s. We found that the plateau of [^{14}CB]$_{max}$ increases with increasing the time of addition of the trap (fig. 8A). If single turnover conditions are met, the value of [^{14}CB]$_{max}$ is directly proportional to [ES]$_{trap}$, as demonstrated in equation 14. In this case P^{app} must be independent of the time of the trap addition and the value of [^{14}CB]$_{max}$. On the other hand, if CBH molecules can reinitiate processive run on ^{14C}BC before the trap is added, P^{app} will increase with an increasing the time of trap addition. In order to rule out the first or the second scenario one needs to quantify [ES]$_{trap}$. For this purpose, we applied the method for the measurement of the concentration of the enzyme bound to substrate via the active site (Jalak & Valjamae, 2010). When the values of [^{14}CB] from experiments with different times of trap addition were divided by corresponding [ES]$_{trap}$ values ([ES]$_{trap}$ after 10, 30 or 60 s), the plateau values, corresponding to P^{app}, were found similar for all regression lines (fig. 8B). This result points out that single turnover conditions are met and the increased [^{14}CB]$_{max}$ was caused by the increase in [ES]$_{trap}$. The release of [^{14}CB] at different concentrations of the enzyme was also assessed. As in the case of varying the time of the trap addition, the increased values of [^{14}CB]$_{max}$ were caused by the increase in [ES]$_{trap}$ values with increasing enzyme concentration (fig. S1 in II). In the second study we, however, observed a systematic deviation from the single exponential function (eq. 14) when the time of hydrolysis was extended up to 30 minutes. To fit the data second exponent was added. In paper II we proposed that the second exponent corresponds to a smaller population of the enzyme with higher P^{app} and/or lower velocity of processive movement. The idea that TrCel7A molecules form different populations on cellulose has been also reported by other groups in microscopy studies (Igarashi *et al.*, 2011) and

tweezers-based single-molecule motility assay (Brady *et al.*, 2015). Unfortunately, our methodology cannot distinguish between different populations of enzymes and further we focused on the first 180 seconds of hydrolysis where systematic deviations from single exponent are not significant.

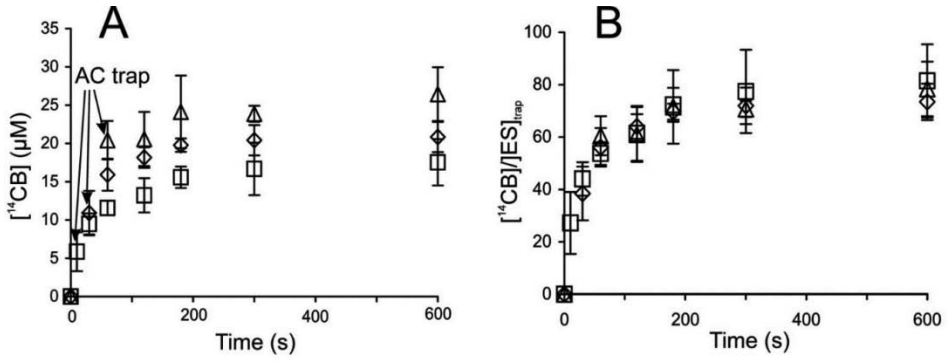

Figure 8. Hydrolysis of ^{14C}BC under single turnover conditions. **A.** After initiation of hydrolysis of ^{14C}BC the AC trap was added after 10 s (□), 30 s (◊), or 60 s (△) and the release of ^{14}CB was followed. **B.** Data from A were converted by dividing the amount of released ^{14}CB by the number of TrCel7A molecules bound to ^{14C}BC at the moment of adding trap (for details see fig. 1 in II)

We compared the data obtained from measurements of P^{app} under single turnover conditions with P^{app} measurements on rBC under single hit conditions (see also chapter 2.1). The resulting values are in good accordance with each other suggesting that both methods are reliable for studies on TrCel7A.

Following the hydrolysis of ^{14C}BC under single turnover conditions also makes it possible to find the catalytic constant as follows from equation 15. We estimated k_{cat} of 2.2 s^{-1} for TrCel7A which is also in accordance with that determined in the course of works presented in paper I (table 2 in I). Unfortunately, we could not measure P^{app} and k_{cat} for $CD_{TrCel7A}$ because of low binding capacity to the substrate and as a result lacking possibility to quantify $[ES]_{trap}$.

An advantage of measuring P^{app} under single turnover conditions over measuring P^{app} under single hit conditions, is that the first can be applied to the mixture of CBH and EG. In case of TrCel7A the presence of EG led to a threefold increase in $[ES]_{trap}$ and a twofold increase of $[^{14}CB]_{max}$ but no changes had been observed in the parameter k. The difference between the increase of $[ES]_{trap}$ and $[^{14}CB]_{max}$ resulted in somewhat reduced values of k_{cat} and P^{app} (table 1 in II). The presence of EG also led to an elevated level of binding of $CD_{TrCel7A}$ to cellulose which, in turn, enabled us to measure values of k_{cat} and P^{app} (table 1 in II). Considering the error limits, we did not find any difference in the catalytic constants and the apparent processivities of TrCel7A and $CD_{TrCel7A}$ in

the presence of EG. Given this background we suggested that CBM is not involved in the processive movement of an enzyme along the polysaccharide chain and its role probably is in increasing the surface density of enzyme molecules on a substrate. The observations that the length and velocity of the processive run of $CD_{TrCel7A}$ is similar to that of the full-length enzyme has been corroborated by microscopy and activity studies on Iα and amorphous celluloses (Igarashi *et al.*, 2009; Nakamura *et al.*, 2013). The study of TrCel7A based on tweezers-based single-molecule motility assay suggests that CBM may even impede the movement of the enzyme along the polysaccharide chain and thus reduce its velocity of the processive run (Brady *et al.*, 2015).

In the course of works as presented in paper II we also studied the endo-exo synergism. According to the conventional interpretation the synergistic mechanism between endoglucanases and cellobiohydrolases is that the first disrupt cellulose chains in random positions (preferentially in amorphous parts) and thus generates starting points for the second (Kostylev & Wilson, 2012). Therefore, an increase in the cellulose degradation rate should be proportional to the increase of the number of enzyme-substrate complexes ([ES]). To test this hypothesis, we investigated the hydrolysis of ^{14C}BC by the mixture of CBH and EG for longer time intervals without the addition of a trap ("steady state" experiments). As expected, the presence of EG under these conditions led to higher levels of the binding of CBH to the substrate (i.e. increased [ES]). However, the increase in [ES] was much less prominent than the increase in the overall speed of saccharification of cellulose (table 2 in II). This result demonstrated that there should be additional mechanisms involved in the interplay between components of synergistic mixtures besides the formation of starting points for CBHs. Previously our group proposed an “obstacle model” postulating that the rapid rate retardation of cellulose hydrolysis by CBHs is determined by slow dissociation of enzymes that are “halted” in front of an obstacle. Herein, we supposed that these obstacles are probably amorphous parts of cellulose. This idea is corroborated by the finding that the value of P^{app} measured in the presence of EG was close to the DP of bacterial microcrystalline cellulose (Väljamäe *et al.*, 1999) where amorphous parts are removed by acidic treatment (see also chapter 1.1.3). In paper II we proposed that the main contribution of EGs to the synergistic effect comes from the removal of obstacles (amorphous parts of cellulose) that, in turn, increases the rate of recruitment of CBHs. Therefore, at optimal enzyme/substrate ratio the observed rate constant for the cellulose hydrolysis approaches the “true” catalytic constant and the overall speed of cellulose digestion becomes limited by the processive movement of CBHs.

2.3 Measuring dissociation rate constant for chitinases (paper III)

In papers I and II we reported that the processive ability of an enzyme is described by its intrinsic processivity that in turn is governed by the dissociation rate constant (k_{off}). By the time paper III was published a number of methods aimed at the measurement of k_{off} of cellulases had appeared in literature (see also table 1) but there was still a lack of methods for the measurement of the k_{off} of chitinases. Chitinases are a class of enzymes involved in the degradation of chitin (see also chapter 1.2). Due to lots of similarities on structural and functional levels with cellulases, research of chitinases promotes elucidating the molecular underpinnings of hydrolysis of cellulose and *vice versa*. As reported in paper III, we developed a novel method for the measurement of k_{off} of chitinases, namely substrate exchange experiments (SEE).

As the name suggests, the principle of SEE is based on measuring the exchange rate of enzyme (E) between the substrate of interest (S) and radioactively labeled substrate (^{14C}S). SEE can be roughly divided into three parts: i) enzyme and substrate are mixed and after certain time intervals ii) 14C-labeled substrate (^{14C}S) is added whereupon iii) the release of radioactively labeled product (^{14C}P) is followed (fig. 9A). The distinctive feature of the release of ^{14C}P is a pronounced lag phase because the enzyme must be released from the substrate before it is available for ^{14C}S (fig. 9C). Concentrations of both S and ^{14C}S are chosen to be saturating for the enzyme. Under these conditions the concentration of free enzyme by the moment of addition of ^{14C}S is negligible and ^{14C}S on-rate ($k_{on}{}^{14C}[{}^{14C}S]$) is higher than both off-rates ($k_{off}{}^{14C}$ and k_{off}) (fig. 9A and 9B). Therefore, the formation of [E^{14C}S] is related to the enzyme dissociation from S as follows from equation 16

Equation 16 $$\left[E^{14C}S\right] \sim 1 - e^{-k_{off}t}$$

In paper III we employed an empirical equation (eq. 17) proposed by Kostylev and Wilson (Kostylev & Wilson, 2013) for the description of the time dependent release of ^{14C}P.

Equation 17 $$\left[^{14C}P\right] = At^{b}$$

Equation 17 has been chosen because of its simplicity and its ability to describe the rate of hydrolysis over the large degree of polysaccharide conversion. Herein, parameter A stands for the total enzymatic activity and is related to the concentration of productively bound enzyme molecules. Parameter b is the hydrolysis power factor. In contrast to parameter A, parameter b reflects the ability of an enzyme to overcome substrate recalcitrance and is not related to enzyme concentration. Since A depends on the concentration of enzyme we combined equations 16 and 17 (eq. 18) for the analysis of the data from SEE.

Equation 18 $$\left[^{14C}P\right] = A\left(1 - e^{-k_{off}t}\right)t^b$$

The sequence of analysis of data was as follows: first, a reference curve (enzyme was added to the mixture of S and ^{14C}S) was analyzed with the help of equation 17 in order to find parameter b, then data from SEE were analyzed using equation 18 where the value of parameter b (calculated from the reference curve) was fixed (fig. 9C).

In paper III we employed SEE to the study chitinase A (ChiA), from *Serratia marcescens,* and its two variants ChiA-W275A and ChiA-W167A. Mutations were done in order to reduce binding energy in the substrate binding site (ChiA-W167A) or in the product binding site (ChiA-W275A). ^{14}C-labeled chitin nanowhiskers (^{14C}CNWs) were used as ^{14C}S in SEE throughout the study. CNWs is an analogue to BMCC in cellulase research, a substrate where amorphous parts are removed by acid hydrolysis (see also chapter 1.1.3). As the substrates of interest CNWs and α-chitin (substrate for the preparation of CNWs) were chosen. We also demonstrated in control experiments that combination of CNWs and ^{14C}CNWs as well as α-chitin and ^{14C}CNWs meet the conditions for conducting SEE.

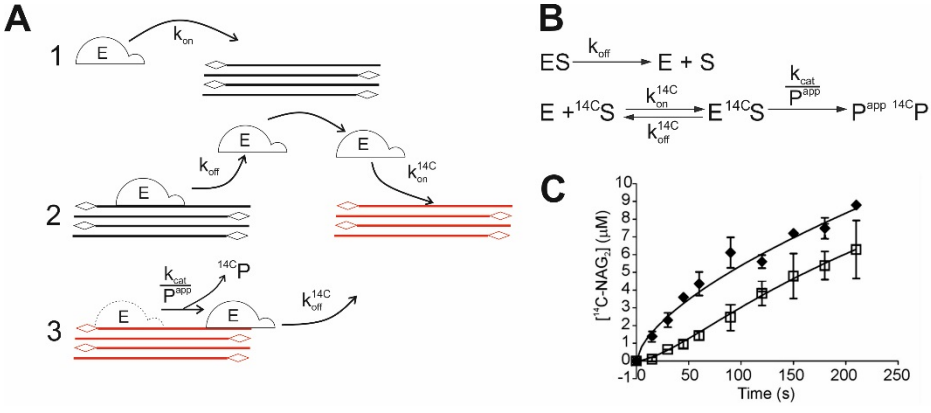

Figure 9. The principle of SEE. **A.** Illustrative representation and reaction sequences in SEE. First, a substrate of interest (S) and an enzyme (E) are incubated together and thereafter an excess of radioactively labeled substrate (^{14C}S) is added. In order to start hydrolysis of ^{14C}S the enzyme must first dissociate from S. Therefore, a lag phase in the formation of radioactively labeled product (^{14C}P) is expected. k_{on} and k_{on}^{14C} are association rate constants (l g^{-1} s^{-1}) for S and ^{14C}S respectively. k_{off} and k_{off}^{14C} are dissociation rate constants (s^{-1}) for S and ^{14C}S respectively. k_{cat} stands for catalytic constant and P^{app} is an apparent processivity. **B.** The same as stages 2 and 3 in A but represented schematically. **C.** ChiA k_{off} measurement on chitin nanowhiskers (CNWs). The reference curve (♦) was analyzed using equation 17. Data obtained from SEE with pre-incubation time 2 h (□) were analyzed using equation 18 (for details see text and fig. 2 in III).

First, we assessed the value of k_{off} on CNWs with a pre-incubation time (i.e. before ^{14C}S was added) of 2 h (fig. 9 and fig. 2 in III). The value of k_{off} for ChiA WT was found to be 0.012 s^{-1}. However, we could not measure the k_{off} value for the mutant enzymes because the lag phase in SEE was absent. Extending the pre-incubation time up to 24 h resulted in the absence of lag phase for all enzymes. This phenomenon might be for two reasons. First, off-rates of enzymes are too high. Second, there is a remarkable amount of free enzyme molecules before the addition of ^{14C}S. We could not discriminate between these two possibilities because of the inability to quantify the amount of free enzyme in the suspension of CNWs.

On α-chitin we first tested pre-incubation times of 10 min and 2 h. In case of ChiA WT and ChiA-W275A the values of k_{off} were higher in the beginning of hydrolysis of α-chitin and decreased with extending pre-incubation time (table 5). However, we did not observe any lag phase for the ChiA-W167A mutant with 10 min of pre-incubation. To figure out whether the absence of a lag phase was caused by the high concentration of free enzyme molecules by the moment of ^{14C}S addition or by the high value of k_{off} we measured enzyme binding to α-chitin. We found that more than 90% of chitinases are bound to chitin via the active site in the first 10 minutes of hydrolysis (fig. 4 in III). We therefore concluded that the value of k_{off} is dependent on the extent of hydrolysis. This hypothesis led us to the question of how k_{off} will change at longer pre-incubation times. To find the answer we conducted a series of SEE with pre-incubation times between 24 and 360 h. We found that the value of k_{off} of ChiA WT was within the standard error over the period of pre-incubation time from 2 h up to 360 h. On the other hand, both mutants demonstrated the absence of lag phase in SEE with longer pre-incubations (table 5 and fig. 3 in III). As in the former case this phenomenon was caused by changes in k_{off} values. Concomitantly with measuring k_{off} in SEE the total activity of enzyme was investigated. The total activity of ChiA WT on α-chitin was similar to that of mutant enzymes within the first 10 minutes but clearly outperformed the mutants with increasing hydrolysis time (fig. 4 in III). In paper III we proposed that similar activity during the initial stage of reaction could be explained by high efficiency of mutants on amorphous parts of α-chitin and differences in activity during later stages are caused by the inability of the mutant enzymes to degrade crystalline parts.

Table 5. Measurements of the value of k_{off} on α-chitin at different incubation time intervals

	k_{off} (10^{-3} s^{-1})		
Enzyme	10 min	2 h	24–360 h
ChiA WT	2.8 ± 0.3	1.5 ± 0.5	1.7 ± 0.3
ChiA-W275A	3.5 ± 1.2	2.2 ± 0.7	fast
ChiA-W167A	fast	6.4 ± 3.7	fast

To tests this hypothesis and to determine the significance of k_{off} we measured initial rates of degradation of CNWs, α-chitin and amorphous chitin employing classical Michaelis-Menten (MM) kinetics for data interpretation. It has been shown that applying of MM-kinetics in the analysis of processive enzymes acting on insoluble polysaccharides is justified with an assumption of excess substrate (Cruys-Bagger *et al.*, 2013). As expected ChiA WT was more efficient on crystalline substrate (CNWs) while mutants clearly outperformed WT on amorphous chitin (table 2 in III). A similar trend has also been demonstrated in the study of Zakariassen *et al.* On soluble chitosan both W167A and W275A clearly outperformed the WT enzyme whereas an opposite result has been obtained on insoluble crystalline β-chitin (Zakariassen *et al.*, 2009). The data obtained from the measurements of initial rates also provided us with the rough estimate of k_{cat} and therefore P^{Intr} (eq. 3). We found that the values of P^{Intr} on α-chitin for all enzymes were in the order of 10^3. These values are in the same order as those found for TrCel7A as presented in paper I (see also chapter 2.1). It is worth mentioning that the active site of TrCel7A has closed topology while the active site of ChiA is groove shaped. Thus the high value of P^{Intr} found for ChiA coincides with the common opinion that the enzyme active site architecture is not the sole determinant of processivity (see also chapter 1.3.1).

It has been previously demonstrated that the loss of processivity is accompanied with substantial increase in the activity on amorphous substrates and the decrease in activity on crystalline substrates. This prompted us to measure P^{app} of chitinases under single hit conditions (see also chapter 2.1). As expected the highest value of P^{app} was measured for ChiA WT. P^{app} values measured for mutants were almost twofold lower (table 3 in III). The measurement of P^{app} under single hit conditions also provided us with the estimates of probability of endo-mode initiations (P^{Endo}). Interestingly, the higher value of k_{off} measured for mutant enzymes was also accompanied with higher values of P^{Endo} (table 3 in III). Another parameter derived from the measurement of P^{app} under single hit condition is k_{IRG} (i.e. the rate constant of formation of IRGs). Depending on what is the rate limiting step in a processive cycle of an enzyme k_{IRG} reflects the rate of association (slow association) or the rate of dissociation (slow dissociation). In paper I we proposed that for cellulases k_{IRG} reflects the rate of dissociation (see also chapter 2.1). Indeed the consistency between k_{off} and k_{IRG} for TrCel7A has recently been confirmed by the study of Kont *et al.* (Kont *et al.*, 2016). In paper III we found that k_{IRG} was an order of magnitude higher than k_{off}. Since ChiA has a modular structure, dissociation from chitin chain is possible in two ways: i) through the catalytic domain only (the enzyme remains attached to substrate via CBM) or ii) the enzyme completely dissociates from the substrate. Herein, we proposed that k_{IRG} corresponds to the first scenario while k_{off} to the second. In other words, after finishing one processive run, the enzyme remains attached to the substrate and initiates a new processive run through lateral diffusion from an adjacent polysaccharide chain of the same substrate.

In summary we propose a model where strong interactions between the enzyme and the substrate and between the enzyme and the product are crucial for the efficient degradation of recalcitrant chitin. According to this hypothesis the enzyme moves along the polymer chain hydrolyzing it until the enzyme encounters an obstacle. Strong interactions in the substrate binding sites do not allow the enzyme to dissociate and switch it into a "standby" regime. At the same time strong interactions in the product binding sites force the enzyme to move forward. If part of an obstacle dissociates from the polymer surface the enzyme starts to move forward and therefore disintegrates the obstacle. A similar behavior for cellulases has previously been shown in a study by Igarashi *et al.* (Igarashi *et al.*, 2011). Using HS-AFM they demonstrated disintegration of a cellulose microfibril by collective "pushing" by multiple cellulases halted in front of an obstacle.

CONCLUSIONS

- The apparent processivity of cellulases is dependent on the nature of the substrate.
- The processive ability of an enzyme is described by its intrinsic processivity.
- Cellobiohydrolases and endoglucanases have similar catalytic constants but largely different dissociation rate constants.
- For single CBHs the limiting step in the catalytic cycle is the dissociation from the substrate.
- For the efficient degradation of recalcitrant polysaccharides strong interactions in the substrate binding site and in the product binding site are required.

SUMMARY IN ESTONIAN

Tsellulaaside ja kitinaaside protsessiivsus

Kahanevate naftavarude ja kasvava inimeste populatsiooni tõttu otsitakse võimalusi kasutada alternatiivseid energiaallikaid. Üheks võimaluseks on kasutada taastuva biokütuse ja tarbekaupade toorainena tselluloosi ja kitiini – enim levinud biopolümeere Maal. Nende peamiseks looduslikuks funktsiooniks on organismi toetamine ning kaitsmine patogeenide ja väliskeskkonna eest. Keemiliselt koosneb tselluloos glükoosi ja kitiin N-atsetüül-D-glükoosamiini monomeeridest. Monomeerid on ühendatud omavahel β-1,4-glükosiidse sidemega ning moodustavad pikad hargnemata ahelad, mis on ühinenud mikrofibrillideks vesiniksidemete abil. Mikrofibrillid moodustavad kõrgemat järku organisatsiooni koos hemitselluloosi ja ligniiniga tselluloosi puhul või kaltsium karbonaadi ja valkudega kitiini puhul. See omakorda muudab tselluloosi ja kitiini kõrge kristallilisusega substraatideks, mis on keemiliselt ja ensümaatiliselt raskesti lagundatavad. Looduses on mikroorganismid kohanenud kasutama tselluloosi ja kitiini toiduallikana. Nad sekreteerivad suurt hulka ensüüme, mis sünergistlikus koostoimes saavutavad polüsahhariidide täieliku degradeerumise. Tselluloosi hüdrolüüsi eest vastutavad ensüümid üldnimetusega tsellulaasid ning kitinaasid lagundavad vastavalt kitiini. Ensümaatilist hüdrolüüsi kasutatakse ka tööstuslikus protsessis, et vältida kontsentreeritud mineraalhapete ja kõrgemate temperatuuride kasutamist. Lootuses saada paremaid ensüüme tööstuslikuks kasutamiseks, on tsellulaasid insenergeneetilise modifitseerimise sihtmärgiks. Ensüümide insenergeneetiliselt muutmiseks on aga vajalik detailne arusaamine tõrksate polüsahhariidide hüdrolüüsi ensüümkineetikast. Vaatamata sellele, et tsellulaase ja kitinaase uuritakse üle poole sajandi, on teadmised ensüümide toimimise molekulaarsetest mehhanismidest veel poolikud. See on tingitud substraadi iseloomust ning tahke/vedel interfaasil toimuvate reaktsioonide analüüsi keerulisusest. Üheks oluliseks tsellulaaside kineetiliseks parameetriks on protsesiivsus, mis väljendub keskmise katalüüsi aktide arvuna, mille ensüüm sooritab enne substraadilt dissotsieerumist. Ensüümide protsessiivsust saab iseloomustada kahe erineva parameetriga: näiline protsessiivsus (P^{app}) ja tõeline protsessiivsus (P^{Intr}). P^{app} on reaalsetel substraatidel mõõdetav parameeter, mis väljendatakse katalüüsi aktide arvu ja protsessiivse tsükli initsiatsioonide arvu suhtena. P^{Intr} on teoreetiline maksimum, mida ensüüm võib realiseerida ideaalsel substraadil. Valdav osa tsellulolüütilistest ja kitinolüütilistest ensüümsegudest, mis sekreteeritatakse mikrokroorganismide poolt, sisaldavad protsessiivseid ensüüme. Seega on protsessiivsuse uurimine oluline biokütuste tootmisprotsessi optimiseerimisel.

Selle töö põhiliseks ülesandeks oli protsessivsete tsellulaaside ja kitinaaside biokeemiline kirjeldus. Selleks töötasime välja uusi meetodeid protsessiivsuse ja teiste kineetiliste parameetrite mõõtmiseks.
Saadud tulemused olid järgmised:

- Ensüümi protsessiivne võime on määratud P^{Intr} poolt ja P^{app} on substraadi iseloomust sõltuv parameeter. P^{Intr} sõltub kahest kiiruskonstandist: katalüütilisest konstandist (k_{cat}) ja dissotsiatsiooni kiiruskonstandist (k_{off}). Tegime kindlaks, et protsessiivsetel ja mitteprotsessiivsetel tsellulaasidel oli sarnane k_{cat}, kuid erinev k_{off}.
- Leidsime, et protsessiivse tsellulaasi hüdrolüüsi kiirust limiteerivaks faktoriks on ensüümi aeglane dissotsieerumine substraadilt.
- Tõrksate polüsahhariidide degradeerimisel on olulised ensüümi tugevad interaktsioonid polümeeriga substraadi sidumiskohtades ja produktiga produkti sidumiskohtades.

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PUBLICATIONS

CURRICULUM VITAE

Name: Mihhail Kurašin
Date of birth: November 10, 1985
Citizenship: Estonian
Phone: +372 5566 8708
E-mail: mihhail.kurashin@gmail.com

Education:

1993–2004	Kohtla-Järve Tammiku Gymnasium
2004–2007	University of Tartu, BSc, gene technology
2007–2010	University of Tartu, MSc, gene technology
2011–	University of Tartu, PhD, gene technology

Language skills:
Russian (native), Estonian, English, Portuguese (basic level)

Research experience:

2005–2017	University of Tartu, Institute of Molecular and Cell Biology, Senior Research P. Väljamäe group
2012–2013	University of São Paulo/USP, São Carlos Institute of Physics, Prof. Igor Polikarpov group, visiting PhD student

Publications:

Kurašin M., Väljamäe P. (2011) "Processivity of cellobiohydrolases is limited by the substrate" J Biol Chem, 286(1):169–177

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ELULOOKIRJELDUS

Nimi: Mihhail Kurašin
Sünniaeg: 10. november, 1985
Kodakondsus: Eesti
Telefon: +372 5566 8708
E-post: mihhail.kurashin@gmail.com

Haridus:

1993–2004	Kohtla-Järve Tammiku Gümnaasium
2004–2007	Tartu Ülikool, BSc, geenitehnoloogia
2007–2010	Tartu Ülikool, MSc, geenitehnoloogia
2011–	Tartu Ülikool, PhD, geenitehnoloogia

Keelteoskus:
Vene (emakeel), Eesti, Inglise, Portugali (algtase)

Teadustöö kogemus:

2005–2017	Tartu Ülikool, Molekulaar- ja Rakubioloogia Instituut, vanemteaduri P. Väljamäe grupp
2012–2013	São Paulo Ülikool, São Carlos Füüsika Instituut, Prof. Igor Polikarpov grupp, külalisdoktorant

Publikatsioonide loetelu:

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