Cellulose hydrolysis and binding with *Trichoderma reesei* Cel5A and Cel7A and their core domains in ionic liquid solutions

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Abstract

Ionic liquids (ILs) dissolve lignocellulosic biomass and have a high potential as pretreatment prior to total enzymatic hydrolysis. ILs are, however, known to inactivate cellulases. In this article, enzymatic hydrolysis of microcrystalline cellulose (MCC) and enzyme binding onto the cellulosic substrate were studied in the presence of cellulose-dissolving ionic liquids. Two different ionic liquids, 1,3-dimethylimidazolium dimethylphosphate ([DMIM]DMP) and 1-ethyl-3-methylimidazolium acetate ([EMIM]AcO), and two monocomponent cellulases, *Trichoderma reesei* cellobiohydrolase Cel7A and endoglucanase Cel5A, were used in the study. The role and IL sensitivity of the carbohydrate-binding module (CBM) were studied by performing hydrolysis and binding experiments with both the intact cellulases, and their respective core domains (CDs). Based on hydrolysis yields and substrate binding experiments for the intact enzymes and their CDs in the presence of ILs, the function of the CBM appeared to be very IL sensitive. Binding data suggested that the CBM was more important for the substrate binding of endoglucanase Cel5A than for the binding of cellobiohydrolase Cel7A. The CD of Cel7A was able to bind well to cellulose even without a CBM, whereas Cel5A CD had very low binding affinity. Hydrolysis also occurred with Cel5A CD even if this protein had very low binding affinity in all the studied matrices. Binding and hydrolysis were less affected by the studied ILs for Cel7A than for Cel5A. To our knowledge, this is the first systematic study of IL effects on cellulase substrate binding.

Keywords: cellulase, carbohydrate-binding module, hydrolysis, substrate binding, ionic liquid, protein-carbohydrate interaction
1. Introduction

Total hydrolysis of lignocellulosic biomass is currently a very active research area, aiming at the sustainable production of biofuels and chemicals. Enzymatic total hydrolysis of the main polysaccharides in plant biomass produces monosaccharides which can be further converted to products such as ethanol, butanol, lactic acid and fatty acid ethyl esters by microbial fermentations (Bokinsky, et al. 2011; Hofvendahl and Hahn–Hägerdal 2000). The main bottleneck of industrial-scale processing is the recalcitrance of lignocellulosics towards enzymatic hydrolysis. Thus, efficient and economical pretreatment methods are needed.

Many different physical, chemical and biological pretreatment methods have been proposed, though all of them suffer from their own drawbacks (Chandra, et al. 2007; Shill, et al. 2011). Some ionic liquids (ILs), defined as salts with melting points below 100 °C (Sun, et al. 2011), are able to dissolve cellulose and have during the last decade gained significant interest in biomass pretreatment prior to hydrolysis. Swatloski et al. (2002) were the first to demonstrate that cellulose can be dissolved in imidazolium-based ILs, whereafter the dissolution of native lignocellulosic biomass, even wood, has been reported (Kilpeläinen, et al. 2007). Dissolution of microcrystalline cellulose (MCC) in IL followed by regeneration by the addition of an anti-solvent, has been shown to significantly increase the kinetics of enzymatic hydrolysis (Dadi, et al. 2006; Dadi, et al. 2007). To omit the regeneration step, Kamiya et al. (2008) proposed a procedure where enzymatic hydrolysis of cellulose is carried out in the same vessel as its regeneration from IL, without removing the IL between the steps. This one-pot procedure suffers, however, from the fact that enzymes hydrolysing cellulose are inactivated in the presence of high concentrations of IL (Turner, et al. 2003).

For efficient total hydrolysis of cellulose, several cellulase activities are needed. According to the classical hydrolysis theory (Bhat and Bhat 1997), endoglucanases (EC 3.2.1.4) catalyse random hydrolysis along the amorphous regions of the cellulose chains, whereas
celllobiohydrolases (exoglucanases, EC 3.2.1.91 and 3.2.1.176) hydrolyse the crystalline

... cellulose regions either from the reducing or non-reducing end, liberating cellobiose as their

... main product. β-Glucosidases (EC 3.2.1.21) finally hydrolyse the released soluble

cellooligomers to glucose. Cellulases are different in their structure and their mode of action

... but very few studies comparing the IL tolerance of the different cellulase main categories

... have been published. In a recent study, the effect of IL on the hydrolytic action of

... monocomponent *Trichoderma reesei* endoglucanases, celllobiohydrolases and *Aspergillus

... niger* β-glucosidase was evaluated (Engel, et al. 2012). Based on these studies, β-

... glucosidases appear to be particularly sensitive towards ILs. As cellulases are inactivated by

... ILs to different degrees, cellulase cocktails have been optimized specifically for their use in


ILs have been proposed to have harmful effects on cellulase action through their properties

... including: high ionic strength and viscosity of the hydrolysis medium (Engel, et al. 2010) and


In addition to these factors cellulase inactivation probably proceeds through specific

... molecular effects or IL-induced unfolding of proteins (Engel, et al. 2010). ILs with

... hydrophilic anions are more inactivating for enzymes than those with hydrophobic anions

... (Kaar, et al. 2003) which, however, are not generally reported to dissolve cellulose. IL

... induced enzyme inactivation has been shown to be either reversible or irreversible (Kaar, et

... al. 2003).

*T. reesei* cellulases, also used in commercial cellulase preparations, are the most extensively

... studied cellulolytic enzymes. Of the *T. reesei* celllobiohydrolases, Cel7A hydrolyses the

... cellulose from the reducing chain end and Cel6A from the non-reducing chain end and both

... hydrolyses cellulose in a processive manner (Teeri 1997). Endoglucanases catalyse cellulose

... chain hydrolysis randomly through cycles of adsorption and desorption (Linder and Teeri
1996). *T. reesei* endoglucanases have their catalytically active site in a cleft on the protein surface (Teeri 1997), whereas the active site of *T. reesei* cellobiohydrolases is tunnel-shaped (Divne, et al. 1994, Rouvinen, et al. 1990). Many fungal endoglucanases and cellobiohydrolases have a two-domain structure consisting of a core domain (CD) and a carbohydrate-binding module (CBM), which are linked through a heavily O-glycosylated peptide linker (van Tilbeurgh, et al. 1986). The major endoglucanases (Cel5A, Cel7B) and both cellobiohydrolases (Cel6A, Cel7A) of *T. reesei* all have a two-domain structure with a CD linked to a family 1 CBM (Linder, et al. 1995b). The CBM has been proposed to have multiple roles in the hydrolysis of cellulose: to increase the concentration of cellulase close to the substrate (van Tilbeurgh, et al. 1986), to target the CD to specific sites on the substrate (Carrard, et al. 2000; Fox, et al. 2013) and to disrupt the crystalline structure of the substrate (Arantes and Saddler 2010; Din, et al. 1994). The presence of CBMs has a great impact on the hydrolysis of solid substrates, but does not affect the hydrolysis rates of soluble substrates (van Tilbeurgh, et al. 1986). Recent results also show that the presence of CBM does not affect the actual turnover number of Cel7A on solid cellulose and both the CD and the intact enzyme proceed along a cellulose chain with a similar speed (Igarashi, et al. 2009; Jalak and Välamäe 2010). Recently it was shown, that the CD of *T. reesei* Cel7A is equally efficient in high solid content hydrolysis as the intact cellulase, suggesting that the CBM only improves hydrolysis at low solid contents (Várnai, et al. 2013). Both the CBM and the CD bind to the substrate, but the binding affinity of the CBM is much higher (Palonen, et al. 1999; Ståhlberg, et al. 1991).

Family 1 CBMs are small wedge-shaped domains with a rough and a flat face (Kraulis, et al. 1989). CBM interaction with crystalline cellulose surfaces takes place through three aromatic amino acid residues aligned on the flat face of the CBM (Linder, et al. 1995b). In the CBM of *T. reesei* Cel7A all three aromatic residues are tyrosines, whereas in the CBM of Cel5A one
of the tyrosines is replaced by a tryptophan. Furthermore, there are one asparagine and one glutamine residue on the flat face, which are conserved in the studied family 1 CBMs, and suggested to form hydrogen bonds with cellulose. The CBM of Cel7A binds completely reversibly to cellulose (Linder and Teeri 1996), whereas the binding of the CBM of the other T. reesei cellbiohydrolase, Cel6A, does not show complete reversibility (Carrard and Linder 1999), even though they both belong to family 1 CBMs (Linder, et al. 1995b). Replacement of a single tyrosine with a tryptophan on the flat binding face of Cel7A CBM was shown to significantly increase CBM affinity to cellulose (Linder, et al. 1995a). Thus, large differences in cellulose binding behaviour can be expected even when the CBMs are closely related.

Up to date, little data has been published regarding how ILs affect the function of CBMs and cellulase binding to cellulose. Pottkämper et al. (2009) carried out mutations on the CBMs of several bacterial cellulases and based on activity measurements concluded that the CBM may play a key role for cellulase performance in the presence of ILs. We have also previously observed that the presence of low concentrations (20 % w/w) of the IL 1,3-dimethylimidazolium dimethylphosphate [DMIM]DMP did not affect the efficiency of T. reesei Cel5A CD in MCC hydrolysis, whereas the intact Cel5A carrying a CBM suffered a drastic decrease in hydrolysis yield as compared to hydrolysis in buffer (Wahlström, et al. 2012). In this article, we report how the presence of two cellulose-dissolving imidazolium-based ILs, [DMIM]DMP and 1-ethyl-3-methylimidazolium acetate ([EMIM]AcO), affects the hydrolysis of MCC by T. reesei Cel5A and Cel7A (formerly known as endoglucanase II and cellbiohydrolase I, respectively) and their CDs. Furthermore, the effect of ILs on the binding on MCC of these intact T. reesei cellulases and their CDs is elucidated for the first time.
2. Materials and Methods

2.1 Chemicals and enzymes

[DMIM]DMP (> 98 %) and [EMIM]AcO (> 98%) were purchased from Ionic Liquid Technologies (IoLiTec, Heilbronn, Germany). For the preparation of 3,5-dinitrosalicylic acid (DNS) reagent solution according to (Sumner 1924), DNS and potassium sodium tartrate tetrahydrate were acquired from Merck (Darmstadt, Germany). \[^{3}H\]NaBH\(_4\) (100 mCi, 10.9 Ci/mmol) and Ultima Gold™ XR liquid scintillation cocktail were purchased from PerkinElmer (Boston, MA, USA) and 37 % formaldehyde solution with 10 – 15 % methanol added as preservative was from Sigma-Aldrich (Steinheim, Germany). Microcrystalline cellulose (MCC, research grade, particle size 0.020 mm) was purchased from Serva Electrophoresis GmbH (Heidelberg, Germany). The dry weight of MCC was determined as the mass loss for triplicate samples when kept at 105 °C overnight.

\( T. \) reesei Cel5A, Cel5A CD and Cel7A CD were produced, isolated and purified as described in (Suurnäkki, et al. 2000) and \( T. \) reesei Cel7A according to (Rahikainen, et al. 2013).

Endoglucanase activity was determined on carboxymethyl cellulose (CMC) as described in (Wahlström, et al. 2012). Celllobiohydrolase activity was determined on 4-methylumbelliferyl-\(\beta\)-D-lactoside with an assay based on the method in (van Tilbeurgh, et al. 1988). The purity of the enzyme preparations was analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). Samples were run in precast Tris-HCl gradient gels (4–20%, Bio-Rad, Hercules, CA, USA) and visualized with a Criterion stain-free imaging system (Bio-Rad Laboratories Inc., Hercules, CA, USA) in which protein visualization is based on a UV-light driven reaction of tryptophan residues in the presence of trichloro compounds (Kazmin, et al. 2002). The molar concentrations of the monocomponent cellulase preparations were determined based on their absorbance at 280 nm.
using the following molar extinction coefficients (ε): Cel5A 91 000 M⁻¹cm⁻¹; Cel5A CD
77 000 M⁻¹cm⁻¹; Cel7A 83 000 M⁻¹cm⁻¹ and Cel7A CD 80 000 M⁻¹cm⁻¹ (Palonen, et al. 1999).

2.2 Enzymatic hydrolysis of MCC in IL matrices

Enzymatic hydrolysis of MCC (1 % w/w suspension) was carried out by *T. reesei* Cel5A,
Cel5A CD, Cel7A and Cel7A CD in matrices containing [DMIM]DMP or [EMIM]AcO (0 -
50 % w/w) in 0.050 M citrate buffer. MCC (30 mg by dry weight) was weighed in a test tube,
and buffer was added followed by the addition of IL under mixing. The total amount of liquid
was 3 g. The hydrolysis was started by the addition of enzyme (dosage 400 nM,

2.3 Enzyme binding to MCC in IL matrices

2.3.1 Radiolabeling of enzymes through reductive methylation

*T. reesei* Cel5A, Cel5A CD, Cel7A and Cel7A CD were labeled with tritium through
reductive amination with formaldehyde and tritium-enriched \[^3\text{H}]\text{NaBH}_4\) based on a method
by (Means and Feeney 1968) with the following modifications: the reaction buffer had a pH
of 8.5, \[^3\text{H}]\text{NaBH}_4\) was added as solution in 0.01 M NaOH and the reaction time was 60 min.
After the reaction, the reaction mixture was eluted twice in 1 mL fractions through an
Econopac® 10 DG gel column (Bio-Rad Laboratories Inc., Hercules, CA, USA). The protein
containing fractions were pooled, concentrated and the buffer was changed to 0.050 M citrate
buffer (pH 5.0). All buffer exchanges and enzyme concentrations were carried out using 6
mL Vivaspin centrifugal concentrators with polyethersulfone (PES) membranes with a molecular weight cut-off of 5 000 Da (Sartorius Stedim Biotech GmbH, Göttingen, Germany). The final concentration procedure also served as purification of the $^3$H-labeled enzyme preparation, as some residual radioactive material moved to the permeate in the first concentration with very little radioactivity detected in the second permeate.

The specific radioactivity of the $^3$H-labeled enzyme preparation was calculated by determining the radioactivity of the preparation through liquid scintillation counting (LSC, see section 2.3.2) and dividing the value with the molar protein concentration. The specific radioactivities were 1.7, 2.0, 2.2 and 2.4 Ci/mmol for $T$. reesei Cel5A, Cel5A CD, Cel7A and Cel7A CD, respectively. The specific hydrolytic activity of the labeled cellulases was unaffected by the labeling reaction. The labeled cellulase preparations were also analysed by SDS-PAGE, which indicated that no degradation of the cellulase preparations had taken place (Supplementary information 1A and 1B).

2.3.2 Binding experiments to MCC with $^3$H-labeled cellulases and liquid scintillation counting

Binding experiments were carried out in 1 % (w/w) MCC suspension in 0.050 M citrate buffer (pH 5.0) dispersions with added [DMIM]DMP or [EMIM]AcO (0, 20 and 40 % w/w) and initial cellulase concentrations of 0.1 µM – 10 µM. Prior to the experiment, the $^3$H-labeled cellulase preparations were mixed in a 1:50 dilution ratio with unlabeled cellulase, which allowed detection of at least 0.1 µM enzyme concentration in solution. In the binding experiments, the MCC (dosed in 2 % w/w suspension), buffer and IL in a total weight of 0.250 g were mixed in 2 mL polypropene microtubes and cooled to 4 °C followed by addition of the cellulase preparation. An equilibration time of 4 h, which was checked to be enough for equilibration in all the studied matrices at 4 °C, was used. 4 °C was chosen as binding temperature to suppress any enzymatic hydrolysis of the MCC, which would lead to changes
in the cellulose morphology and amount and seriously complicating comparison between
binding results in the different IL solutions. The experiment was stopped by centrifuging the
tubes 8 000 rpm for 4 min, followed by separating the supernatant from the MCC. All
procedures were carried out in environments thermostated to 4 °C. The cellulase binding was
calculated based on the amount of unbound cellulase in the supernatant as quantified by LSC,
by comparing samples with MCC to reference samples without MCC. For LSC, aliquots of
supernatant was mixed with Ultima Gold™ XR liquid scintillation cocktail and the counts
per minute were determined with a liquid scintillation counter (Tri-Carb 2810 TR,
PerkinElmer, Boston, MA, USA) with a 15 min counting time. All binding experiments were
carried out in triplicates. Isotherms were plotted based on the calculated bound enzyme per
gram of MCC against the concentration of free enzyme at equilibrium. The maximal binding
level (B_max, expressed as µmol/g MCC) and the equilibrium dissociation constant (K_d,
expressed as concentration units) were estimated by fitting the data to a hyperbolic, one site
binding model (Equation I) using the GraphPad Prism® 4 software.

\[
\text{Amount of bound enzyme} = \frac{B_{max} \times c_{free}}{c_{free} + K_d}
\]  

where c_{free} denotes the concentration of unbound enzyme in the supernatant.

3 Results and Discussion

3.1 Hydrolysis of MCC with \textit{T. reesei} endoglucanase Cel5A and
cellobiohydrolase Cel7A and their core domains in IL matrices

The enzymatic hydrolysis of microcrystalline cellulose (MCC) with \textit{Trichoderma reesei}
Cel5A and Cel7A and their core domains (CDs) was carried out for 2 or 72 h. The hydrolysis
yield was quantified by analysing the reducing sugars liberated in hydrolysis by the DNS
assay, having a limit of quantification (LOQ) of 1 % hydrolysis yield in the used setup. In the
2 h hydrolysis Cel5A was the only enzyme producing over 1% yields, with yields of 1.5 %, 1.3 % and 1.1 % in buffer, 10 % (w/w) [DMIM]DMP and 10 % (w/w) [EMIM]AcO, respectively (results not shown). The studied ILs do not dissolve MCC at IL concentrations below 80 % under the experimental conditions applied here, as has been shown in (Wahlström, et al. 2012) by light transmission experiments.

Results of the 72 h hydrolyses of MCC in [DMIM]DMP and [EMIM]AcO containing matrices are presented in Figs 1A and 1B. As expected from previous studies (Datta, et al. 2010; Kamiya, et al. 2008; Wahlström, et al. 2012), an increasing concentration of ionic liquid (IL) led to decreasing hydrolysis yields. [EMIM]AcO (Fig 1B) had a stronger inhibiting effect than [DMIM]DMP (Fig 1A) on the studied cellulases in matrices containing over 10 % (w/w) of IL, which is in accordance with our previous studies (Wahlström, et al. 2012). All the studied cellulases had hydrolysis yields above 1 % in up to 40 % (w/w) [DMIM]DMP. Cel5A and Cel5A CD had clearly lower yields than their Cel7A counterparts and Cel5A was the cellulase most affected by the presence of both ILs. In [EMIM]AcO all four cellulases produced over 1 % yields in up to 20 % (w/w) IL, but the yields were under the LOQ for matrices containing 30 % (w/w) [EMIM]AcO or more. By comparing the 2 h and 72 h hydrolysis results it can be concluded that Cel5A was more efficient than the other cellulases in the beginning of the hydrolysis, whereas Cel7A (and Cel7A CD in most matrices) produced higher yields in the longer 72 h hydrolysis.

The effect of increasing the [DMIM]DMP concentration on the yield in MCC hydrolysis was different for the intact cellulases and their CDs (Fig 1A). The intact cellulases Cel5A and Cel7A both had higher yields in buffer than their corresponding CDs. This was expected as over 50 % yield losses have been reported in MCC hydrolysis with low substrate concentrations when the carbohydrate-binding module (CBM) has been removed from T. reesei cellobiohydrolases (Tomme, et al. 1988). When changing the matrix from buffer to 10

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% (w/w) [DMIM]DMP, a drastic decrease in hydrolysis yield was observed in the case of the intact cellulases, being 43 % for Cel7A, and 60 % for Cel5A. Thereafter, when further increasing the IL concentration only moderate decreases in the hydrolysis yields were observed. The hydrolysis yields of the Cel5A CD and Cel7A CD were, however, evenly decreased by each increment of [DMIM]DMP in the matrix, being on the same level as for intact cellulases in the [DMIM]DMP matrices. These results suggest that the CBM does not promote the hydrolysis in the presence of [DMIM]DMP. A similar effect of [DMIM]DMP addition on the yield of MCC hydrolysis has previously been reported with T. reesei Cel5A and its CD (Wahlström, et al. 2012).

Surprisingly, MCC hydrolysis yields with Cel7A were differently affected in [EMIM]AcO than in [DMIM]DMP. Cel7A did not suffer a drastic decrease in yield when adding 10 % (w/w) of [EMIM]AcO and the yield decreased quite evenly for each increment of [EMIM]AcO (Fig 1B). The higher hydrolysis yield of 6.8 % with Cel7A in 10 % (w/w) [EMIM]AcO as compared to 5.1 % in 10 % (w/w) [DMIM]DMP was unexpected, as [EMIM]AcO in higher concentrations was more harmful to cellulase performance than [DMIM]DMP. In the case of Cel5A, a 53 % decrease in the hydrolysis yield was observed when going from buffer to 10 % (w/w) [EMIM]AcO (Fig 1B), as was observed with [DMIM]DMP. The addition of [EMIM]AcO decreased the hydrolysis yields of both Cel7A CD and Cel5A CD similarly as those of the intact Cel7A. Unlike in [DMIM]DMP, the hydrolysis yields of intact enzymes were higher than those of the CDs in the presence of [EMIM]AcO (Fig 1B). It appears, that the influence of the CBM on the hydrolysis yield is IL dependent.
3.2 Binding of intact and core domain cellulases to MCC in IL solutions

3.2.1 Cellulase binding to MCC in buffer

The substrate binding of *T. reesei* Cel7A, Cel7A CD, Cel5A and Cel5A CD was followed by determining binding isotherms under standardized conditions. The radiolabeled enzymes were incubated with MCC at 4 °C for 4 h after which the solid cellulose was removed and the concentration of unbound enzyme was measured from the liquid phase by liquid scintillation counting. The four studied cellulases clearly showed different binding behaviour to MCC in buffer solution (0.050 M citrate, pH 5.0) (Fig 2). The two intact cellulases, Cel5A and Cel7A, both exhibited similar binding to MCC. Cel7A CD, lacking CBM, also bound rather well to MCC even though the binding was not as high as for the two intact cellulases. Cel5A CD, on the other hand, had only minor binding to MCC in buffer. Thus, the CBM appeared to be very important for the binding of Cel5A to cellulose, whereas Cel7A was able to bind significantly to cellulose even without CBM. The reason for this difference between the binding of the CDs could be in the different structures of their catalytically active sites, which in cellobiohydrolases (Cel7A) is a tunnel and in endoglucanases (Cel5A) an open-site cleft (Teeri 1997). With the cellulose bound in the active site tunnel of Cel7A the need for a CBM for efficient binding appears not to be so pronounced as with Cel5A. The tunnel-shaped active site of cellobiohydrolases has previously been suggested to cause the tight binding of *T. reesei* Cel7A to cellulose in buffer (Linder and Teeri 1996). Intact cellobiohydrolases have been shown to have the highest binding affinity to cellulose, while the binding affinity is lower for isolated CBMs and even lower for isolated CDs (Palonen, et al. 1999). The binding of Cel7A CD through the active site tunnel has also previously been proposed by (Kotiranta, et al. 1999) who studied *T. reesei* cellulase binding to steam-pretreated willow. Cellulase binding appears, however, to be much dependent on the substrate and applied conditions based on previous studies (Kotiranta, et al. 1999; Palonen, et al. 2004).
3.2.2 Cellulase binding to MCC in aqueous ionic liquid solutions

The binding isotherms of *T. reesei* Cel7A, Cel7A CD and Cel5A clearly showed that the enzyme binding to MCC was reduced by the presence of ILs (Figs 3A, 3B and 3C). The binding of Cel5A CD to MCC was very low in all matrices and the influence of IL could not be assessed (results not shown). For all the studied cellulases, [EMIM]AcO affected the binding more than [DMIM]DMP and increasing the IL concentration of the binding matrix solution led to lower cellulase binding to MCC, similarly to how the hydrolysis yields were affected by ILs in section 3.1. The binding of Cel5A was clearly more sensitive to the presence of IL than Cel7A and Cel7A CD. In 40 % (w/w) [DMIM]DMP, some binding of Cel5A still occurred, whereas 40 % (w/w) of [EMIM]AcO completely prevented Cel5A from binding to MCC (Fig 3A). Cel7A and Cel7A CD were able to bind to MCC even in the highest IL concentrations in the studied matrices (Fig 3B and 3C). The binding of Cel7A CD was relatively much less affected by the presence of ILs than the binding of the intact cellulases in terms of isotherm shape and $B_{\text{max}}$ value (Fig. 3C and Table 1). The binding isotherm of Cel7A CD was even alike in buffer and in the 20 % (w/w) [DMIM]DMP matrix. Based on the results of the binding studies in IL matrices, the following conclusions could be drawn: The binding ability of *T. reesei* cellulase CBMs is very sensitive to the studied ILs. Cel5A relies to a high extent on its CBM for substrate binding, whereas Cel7A substrate binding takes place efficiently *via* both the CD and the CBM. Interestingly, the binding of Cel7A CD is not very IL sensitive.

The binding of the CBM to cellulose happens mostly through hydrophobic interactions between the three hydrophobic amino acid residues on the flat face of the CBM (Linder, et al. 1995b). It has been shown that the addition of organic solvents to the binding matrix decreases the binding affinity of cellobiohydrolase CBMs (Carrard and Linder 1999), probably due to interference of the solvent with the hydrophobic interactions. The
interference of the hydrophobic interactions by ILs may be one reason for the reduced cellulase binding to MCC observed in this study. In (Carrard and Linder 1999), the effect of matrix pH on CBM (from *T. reesei* Cel6A and Cel7A) binding was found to be less than 10% between pH 2.5 and 11. Both [DMIM]DMP and [EMIM]AcO are basic in buffer solution, but the pH of 40% (w/w) [DMIM]DMP or [EMIM]AcO in citrate buffer does not exceed 6 or 7.5, respectively, reported in (Wahlström, et al. 2012). Thus, it is assumed that the basicity of the ILs as such would not cause large changes to the CBM binding to cellulose. Due to different temperatures used in binding and hydrolysis experiments, binding and hydrolysis results cannot directly be correlated, but it could be expected that the substrate binding of the cellulases is even more interfered by the ILs at higher temperatures than at 4 °C. Finally, ILs have been proposed to induce conformational changes to enzymes leading to inactivation (Sheldon, et al. 2002), and a similar effect of the ILs used in this work on the CBM is possible, which would also lead to lower binding affinity. The observed decrease in the cellulases’ cellulose binding and hydrolysis performance caused by the ILs might be due to both the interference of the ILs with the hydrophobic interactions mediating substrate binding and conformational changes. The conformational changes and protein unfolding caused by ILs should clearly be studied in the future. However, method development may be necessary to carry out such studies in IL environments, as ILs have been reported to cause significant baseline problems in many common methods used for studying enzyme conformation in solution, such as circular dichroism (CD) (Sandoval, et al. 2012).

### 3.2.3 Effect of ILs on B\textsubscript{max} and K\textsubscript{d} of cellulase binding to MCC

The maximal binding level (B\textsubscript{max}) and equilibrium dissociation constant (K\textsubscript{d}) for the binding isotherms were evaluated by fitting the equilibrium points to a hyperbolic function for one site binding using nonlinear regression (Table I). B\textsubscript{max} describes the maximal amount of bound enzyme or alternatively the available binding sites on the sorbent, *i.e.* the isotherms

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plateau value, and $K_d$ is the concentration of free enzyme where the binding corresponds to half the $B_{\text{max}}$. $K_d$ is thus a measure of binding affinity (a small $K_d$ indicates high affinity). For $T. reesei$ Cel5A, Cel7A, and Cel7A CD, the $B_{\text{max}}$ values decreased with increasing IL concentrations. For Cel7A CD, the IL effect on $B_{\text{max}}$ was less pronounced than on the intact enzymes. For Cel5A, the $K_d$ values increased with increasing IL concentration, indicating less binding affinity. Interestingly, the calculated $K_d$ value decreased with increasing IL concentration compared to buffer for Cel7A and Cel7A CD, which was partly in conflict with the general inspection of the binding isotherms. As $K_d$ is directly dependent on $B_{\text{max}}$, $K_d$ values from different experiments should, however, be compared with care.

4 Conclusions

The effect of ionic liquid (IL) on cellulose hydrolysis and substrate binding with $Trichoderma reesei$ monocomponent cellulases and their core domains (CDs) was studied systematically for the first time. Our results show that both the hydrolysis of microcrystalline cellulose (MCC) with $T. reesei$ Cel5A and Cel7A and their binding to MCC are severely interfered by the presence of ILs ([DMIM]DMP and [EMIM]AcO), but to different degrees depending on the structure of the cellulase. Both hydrolysis and binding were found to be more severely interfered by ILs with intact cellulases than with their CDs, suggesting that ILs highly affect the function of the carbohydrate-binding module (CBM). The CBM was clearly more important for Cel5A substrate binding than for the substrate binding of Cel7A and accordingly Cel5A substrate binding was also much more sensitive to the presence of IL. [EMIM]AcO had a stronger detrimental effect on both hydrolysis and substrate binding for all the cellulases as compared to [DMIM]DMP. The decreasing hydrolysis yields in IL matrices cannot be attributed only to lower binding affinities. Substrate binding took place even in matrices with high IL concentrations in which very low hydrolysis was observed. As
was observed for Cel5A CD, high binding affinity is not always necessary for hydrolysis. It is suggested that the different binding affinities and the IL sensitivity of binding of Cel5A and Cel7A is dependent also on the structure of the catalytically active site and its substrate binding zone, which in addition to the CBM is the second site on the enzyme where substrate binding occurs. As ILs appear to affect especially the action of modular cellulases and their CBMs, it may be beneficial to concentrate on cellulases consisting of only a CD for the future screening of IL compatible cellulases.

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## Tables

Table I Estimated values for the maximal binding level ($B_{max}$) and the equilibrium dissociation constant ($K_d$) for *Trichoderma reesei* Cel5A, Cel7A and Cel7A CD in 0.050 M citrate buffer (pH 5.0) containing 0, 20 or 40 % (w/w) of IL. St. Error denotes the estimated standard error for the $B_{max}$ and $K_d$ values.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Cellulase</th>
<th>$B_{max}$</th>
<th>St. Error</th>
<th>$K_d$</th>
<th>St. Error</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>Cel5A</td>
<td>0.5761</td>
<td>0.01773</td>
<td>1.262</td>
<td>0.1116</td>
<td>0.9928</td>
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<tr>
<td></td>
<td>Cel7A</td>
<td>0.5144</td>
<td>0.01552</td>
<td>0.6369</td>
<td>0.05957</td>
<td>0.9846</td>
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<tr>
<td></td>
<td>Cel7A CD</td>
<td>0.3919</td>
<td>0.01903</td>
<td>2.251</td>
<td>0.2587</td>
<td>0.9791</td>
</tr>
<tr>
<td>20 %</td>
<td>[DMIM]DMP</td>
<td>Cel5A</td>
<td>0.4533</td>
<td>0.04968</td>
<td>4.405</td>
<td>0.933</td>
</tr>
<tr>
<td></td>
<td>Cel7A</td>
<td>0.3718</td>
<td>0.01332</td>
<td>0.4691</td>
<td>0.05646</td>
<td>0.9692</td>
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<tr>
<td></td>
<td>Cel7A CD</td>
<td>0.2962</td>
<td>0.0108</td>
<td>0.6692</td>
<td>0.07723</td>
<td>0.969</td>
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<tr>
<td>40 %</td>
<td>[DMIM]DMP</td>
<td>Cel5A</td>
<td>0.1576</td>
<td>0.03551</td>
<td>4.43</td>
<td>2.042</td>
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<td>Cel7A</td>
<td>0.2424</td>
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<td>0.06034</td>
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<td>Cel7A CD</td>
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<td>0.8496</td>
<td>0.1049</td>
<td>0.9746</td>
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<tr>
<td>20 %</td>
<td>[EMIM]AcO</td>
<td>Cel5A</td>
<td>0.3689</td>
<td>0.08776</td>
<td>6.186</td>
<td>2.741</td>
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<tr>
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<td>Cel7A</td>
<td>0.2764</td>
<td>0.0104</td>
<td>0.4915</td>
<td>0.0633</td>
<td>0.9626</td>
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<tr>
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<td>Cel7A CD</td>
<td>0.2052</td>
<td>0.007573</td>
<td>0.4887</td>
<td>0.06277</td>
<td>0.9592</td>
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<tr>
<td>40 %</td>
<td>[EMIM]AcO</td>
<td>Cel5A</td>
<td>Does not converge (very low binding affinity)</td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>Cel7A</td>
<td>0.2244</td>
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<td>0.2074</td>
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<td>Cel7A CD</td>
<td>0.1726</td>
<td>0.01375</td>
<td>0.6492</td>
<td>0.1753</td>
<td>0.8456</td>
</tr>
</tbody>
</table>
List of figures with captions

1. Fig. 1A Yields of 72 h hydrolysis of MCC at 45 °C with *Trichoderma reesei* Cel5A, Cel5A CD, Cel7A and Cel7A CD, in citrate buffer matrices containing 0 – 50 % [DMIM]DMP. Hydrolysis yields were measured by DNS assay, with a limit of quantification of 1 % yield. Detectable hydrolysis yields below 1 % have been designated as “Traces”. o. d. denotes of dry weight (of the substrate).

2. Fig. 1B Yields of 72 h hydrolysis of MCC at 45 °C with *Trichoderma reesei* Cel5A, Cel5A CD, Cel7A and Cel7A CD, in citrate buffer matrices containing 0 – 50 % [EMIM]AcO. Hydrolysis yields were measured by DNS assay, with a limit of quantification of 1 % yield. Detectable hydrolysis yields below 1 % have been designated as “Traces”. o. d. denotes of dry weight (of the substrate).

3. Fig. 2 Binding isotherms for *Trichoderma reesei* Cel5A, Cel5A CD, Cel7A, and Cel7A CD on MCC in 0.050 M citrate buffer (pH 5.0) at 4 °C.

4. Fig. 3A Binding isotherms for *Trichoderma reesei* Cel5A in 0.050 M citrate buffer (pH 5.0) containing 0 % IL (w/w) (○), 20 % (w/w) [DMIM]DMP (□) or [EMIM]AcO (Δ), or 40 % (w/w) [DMIM]DMP (○) or [EMIM]AcO (●) at 4 °C.

5. Fig. 3B Binding isotherms for *Trichoderma reesei* Cel7A in 0.050 M citrate buffer (pH 5.0) containing 0 % IL (w/w) (○), 20 % (w/w) [DMIM]DMP (□) or [EMIM]AcO (Δ), or 40 % (w/w) [DMIM]DMP (○) or [EMIM]AcO (●) at 4 °C.

6. Fig. 3C Binding isotherms for *Trichoderma reesei* Cel7A CD in 0.050 M citrate buffer (pH 5.0) containing 0 % IL (w/w) (○), 20 % (w/w) [DMIM]DMP (□) or [EMIM]AcO (Δ), or 40 % (w/w) [DMIM]DMP (○) or [EMIM]AcO (●) at 4 °C.
Figures

Fig. 1A
Fig. 1B

![Graph showing hydrolysis yield o. d. (%) vs. Buffer pH and [EMIM]AcO concentration. The x-axis represents Buffer pH 5.0 and various concentrations of [EMIM]AcO (10%, 20%, 30%, 40%, 50%). The y-axis shows the hydrolysis yield o. d. (%) ranging from 0 to 10. The graph includes lines for Cel7A, Cel7A CD, Cel5A, and Cel5A CD, with traces at certain concentrations.](image-url)
Fig. 2
Fig. 3A
Fig. 3B
Fig. 3C
Supplementary material, captions

Supplementary material 1A SDS-PAGE analysis of *Trichoderma reesei* Cel7A (lanes 3 and 4), Cel7A CD (lanes 5 and 6), Cel5A (lanes 7 and 8) and Cel5A CD (lanes 9 and 10) before labelling the proteins with $^3$H through reductive methylation. The protein dosage is 3 µg in the lane to the left and 5 µg in the lane to the right for each cellulase, respectively. Lane 1 and 10 are the molecular weight standards in which the size of each protein is shown in kDa.

Supplementary material 1B SDS-PAGE analysis of *Trichoderma reesei* Cel7A (lanes 3 and 4), Cel7A CD (lanes 5 and 6), Cel5A (lanes 7 and 8) and Cel5A CD (lanes 9 and 10) after labelling the proteins with $^3$H through reductive methylation. The protein dosage is 3 µg in the lane to the left and 5 µg in the lane to the right for each cellulase, respectively. Lane 1 and 10 are the molecular weight standards in which the size of each protein is shown in kDa.
Supplementary material, figures

Supplementary material, Fig 1A
Supplementary material, Fig 1B