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ARTICLE

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Exo-Exo Synergy Between Cel6A and Cel7A From *Hypocrea jecorina*: Role of Carbohydrate Binding Module and the Endo-Lytic Character of the Enzymes

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ABSTRACT: Synergy between cellulolytic enzymes is essential in both natural and industrial breakdown of biomass. In addition to synergy between endo- and exo-lytic enzymes, a lesser known but equally conspicuous synergy occurs among exo-acting, processive cellobiohydrolases (CBHs) such as Cel7A and Cel6A from *Hypocrea jecorina*. We studied this system using microcrystalline cellulose as substrate and found a degree of synergy between 1.3 and 2.2 depending on the experimental conditions. Synergy between enzyme variants without the carbohydrate binding module (CBM) and its linker was strongly reduced compared to the wild types. One plausible interpretation of this is that exo-exo synergy depends on the targeting role of the CBM. Many earlier works have proposed that exo-exo synergy was caused by an auxiliary endo-lytic activity of Cel6A. However, biochemical data from different assays suggested that the endo-lytic activity of both Cel6A and Cel7A were 10^3–10^4 times lower than the common endoglucanase, Cel7B, from the same organism. Moreover, the endo-lytic activity of Cel7A was 2–3-fold higher than for Cel6A, and we suggest that endo-like activity of Cel6A cannot be the main cause for the observed synergy. Rather, we suggest the exo-exo synergy found here depends on different specificities of the enzymes possibly governed by their CBMs.

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KEYWORDS: exo-exo synergy; Cel6A; Cel7A; CBM; cellulose; cellobiohydrolase

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Conflict of interest: KB works at Novozymes A/S, a major manufacturer of industrial enzymes.

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Introduction

Mixtures of different cellulolytic enzymes usually show higher activity than the sum of the constituent enzymes assayed separately. This synergy between cellulases was discovered already in 1950 (Reese et al., 1950), and interest in the phenomenon has greatly increased as it has become clear that it is crucially important for industrial degradation of biomass to soluble sugars. Cellulase synergy has commonly been ascribed to the combined effect of endo-lytic enzymes such as endoglucanases (EG) or lytic polysaccharide monooxygenases (LPMO) on one hand, and processive, exo-lytic cellobiohydrolases (CBH) on the other (Eibinger et al., 2014; Henrissat et al., 1985; Kostylev and Wilson, 2012; Vaaje-Kolstad et al., 2010; Woodward, 1991). This so-called endo-exo synergy may arise as the EG or LPMO attack the chain internally and thus produce new chain ends for CBH attacks. This mechanism, however, does not seem to explain all observations of cellulase synergy, particularly the commonly observed synergy between exo-lytic CBHs such as Cel6A and Cel7A. This so-called exo-exo synergy was first reported by Fägerstam and Pettersson (1980) and has subsequently been observed for a range of systems and conditions (Boisset et al., 2000, 2001; Henrissat et al., 1985; Hoshino et al., 1997; Igarashi et al., 2011; Nidetzky et al., 1994; Tomme et al., 1988; Väljamäe et al., 1998). The extent of the synergistic effect (the so-called degree of synergy defined below) is typically quite similar for both exo-exo and endo-exo synergies (Henrissat et al., 1985; Igarashi et al., 2011; Nidetzky et al., 1994), and this obviously points toward a significant role of both modes.

Current suggestions regarding the molecular underpinnings of exo-exo synergy focus on two main ideas. One interpretation is based on a potential endo-lytic activity of CBHs; particularly Cel6A (Boisset et al., 2000, 2001; Divne et al., 1994; Medve et al., 1994; Poidevin et al., 2013; Ståhlberg, 1993). Thus, if indeed Cel6A conducts frequent internal attacks on the cellulose strand, synergy between Cel7A and Cel6A could simply be a special case of conventional endo-exo synergy, where Cel6A played the role of the

EG. Alternatively, exo-exo synergy could rely on differences in enzyme specificity. In this case, synergy could be envisioned if one enzyme removes certain regions or patches, and hence reveals a new surface that makes up a better substrate for the other enzyme. This idea that one enzyme can remove structures that are problematic to convert for the other, has also been put forward as an alternative explanation for conventional endo-exo synergy (Eriksson et al., 2002; Fox et al., 2012; Jalak et al., 2012; Väljamäe et al., 1998).

The suggestion of an (auxiliary) endolytic activity of Cel6A is generally linked to the architecture of its active site region, which is more open and dynamic than the analogous region in Cel7A (Divne et al., 1994; Rouvinen et al., 1990; Varrot et al., 2003; Zou et al., 1999). This is thought to facilitate internal association with the cellulose strand and hence endo-lytic catalysis. This interpretation was used for example by Boisset et al. (2000), who studied Cel7A and Cel6A from *Humicola insolens*. This work used TEM images to elucidate structural changes in cellulose particles during hydrolysis and concluded that Cel6A was an endo-processive CBH. Many subsequent studies have used this classification to rationalize different types of activity data for Cel6A (see Payne et al., 2015 for a review). Direct biochemical evidence for endo-lytic activity of both Cel6A and Cel7A was provided in a ground breaking study by Ståhlberg (1993), but otherwise both qualitative and quantitative measurements of the endo-lytic activity of CBHs remain sparse and it appears that further insights into this is necessary for a better understanding of the catalytic interplay of Cel7A and Cel6A. Turning to substrate specificity, the second plausible cause of exo-exo synergy, some conspicuous disparities in the preference of respectively Cel6A and Cel7A has been identified. Firstly, Cel7A attacks the reducing end of the cellulose strand whereas Cel6A is specific for the non-reducing end (Claeyssens et al., 1990; Davies and Henrissat, 1995). Secondly, Cel6A has been reported to preferentially hydrolyse amorphous cellulose, while Cel7A is superior on crystalline substrates (Bubner et al., 2013; Ganner et al., 2012; Gruno et al., 2004; Ståhlberg, 1993).

To elucidate the importance of these two mechanisms, we have conducted a comprehensive biochemical investigation of mixtures of Cel7A and Cel6A from *Hypocrea jecorina*. The work covered a range of enzyme- and substrate concentrations, and used both wild type enzymes and truncated variants, where the carbohydrate binding module (CBM) and linker had been removed from one or both enzymes. Based on the synergy data and three independent assays for the endo-lytic activity we suggest that substrate specificity, probably governed by the targeting role of the CBM is the main reason for synergy between Cel7A and Cel6A.

Materials and Methods

Enzymes were expressed in *Aspergillus oryzae* and purified as described elsewhere (Borch et al., 2014; Sørensen et al., 2015b) and truncated core enzymes were expressed without linker and CBM. Enzyme concentrations were determined by UV absorption at 280 nm using theoretical extinction coefficients (Gasteiger et al., 2003) of 97,790 $M^{-1}cm^{-1}$ (Cel6A), 82,195 $M^{-1}cm^{-1}$ (Cel6A core), 86,760 $M^{-1}cm^{-1}$ (Cel7A), 80,550 $M^{-1}cm^{-1}$ (Cel7A core), 74,145 $M^{-1}cm^{-1}$ (Cel7B), and 177,880 $M^{-1}cm^{-1}$ (β-glucosidase). Enzyme activity was determined from the end-point concentration of reducing ends in 1 h trials. The substrate was Avicel PH-101 (Sigma–Aldrich St. Louis, MO) and we used loads of either 12 g/L Avicel (low substrate) or 60 g/L Avicel (high substrate). In all experiments with mixtures of Cel6A and Cel7A the total enzyme concentration of CBH was either 0.2 or 2 μM (while the ratio of the two components was varied systematically). In the reference experiments with only one CBH, we used concentrations between either 0 and 0.2 μM or 0 and 2 μM. The concentrations in these mono-component measurements were chosen to match the concentration of the component in the corresponding synergy mixture. All samples contained 10% β-glucosidase (mol βG/mol total enzyme) from *Aspergillus fumigatus*, and all experiments were made in 50 mM sodium acetate pH 5.0 at 25°C. Activity was quantified by the *para*-hydroxybenzoic acid hydrazide (PAHBAH) method (Lever, 1973) and experiments were performed, and quenched as described elsewhere (Sørensen et al., 2015b).

Endo-lytic activity was estimated by real-time measurements with a pyranose dehydrogenase (PDH) biosensor. PDH biosensors were prepared according to a previously published protocol (Cruys-Bagger et al., 2014) except that benzoquinone was used as mediator (instead of 2.6-dichlorophenolindophenol). The substrate used for the PDH measurements was carboxymethylated cellulose (CMC) 90 kDa (9004-32-4 Sigma–Aldrich) with an average molecular mass and degree of substitution of respectively, 90 kDa and 0.7 carboxymethyl substituent per pyranose ring. Sensors were calibrated several times daily in CMC against cellobiose solutions ranging from 0 to 100 μM. Experiments with Cel6A and Cel7A were made with 5 g/L CMC and 1 μM enzyme while for Cel7B, we used 0.5 g/L CMC and 0.2 μM enzyme. These differences in conditions were necessary as the production of cellobiose by Cel7B was otherwise too high and rapid to be captured by the biosensor in real time.

Endo activity was further determined in a simple colorimetric assay using the insoluble substrate azurine crosslinked cellulose; AZCL-HE-Cellulose (Megazyme, Bray, Ireland), which has previously been used to quantify endo-lytic activity (Kračun et al., 2015; Li et al., 2011). For Cel6A and Cel7A we used 10 μM while the enzyme concentration for Cel7B was 0.10 μM. We used 5 g/L AZCL-HE in all measurements and the reaction was allowed to progress for 1 h at pH 5.0 at 25°C in a thermomixer operating at 1100 rpm. Reactions were terminated by centrifugation and the endo-lytic activity was specified as the absorbance in the supernatant at 595 nm per μM enzyme.

Finally, endo-lytic activity was monitored on the basis of changes in the viscosity of CMC semi-dilute solutions, for which the viscosity depends very strongly on molar mass. Steady shear viscosities were measured in a Bohlin VOR rheometer using a C14 couette system with a constant steady shear rate of 14.6 s^{-1} at 25°C (Pedersen et al., 2016). The viscosity changes of 50 g/L CMC were monitored following

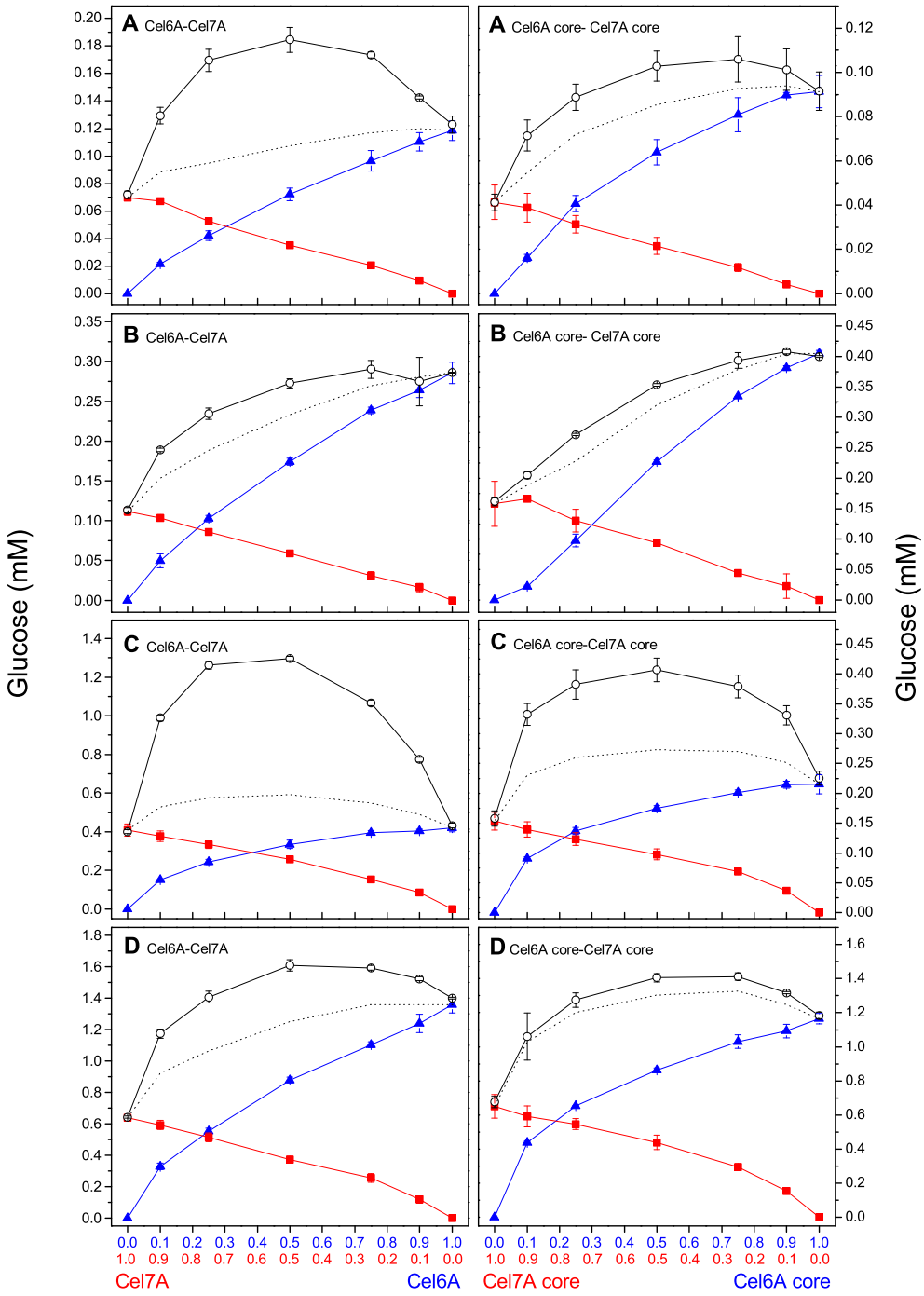

Figure 1. Activity data (formation of glucose) for Cel6A, Cel7A and their mixtures (left column), and for Cel6A core, Cel7A core, and their mixtures (right column). All experiments had 10% β-glucosidase (mol βG/mol CBH). Condition (**A**) 12 g/L Avicel and total [CBH] = 0.2 μM, (**B**) 60g/L Avicel and total [CBH] = 0.2 μM, (**C**) 12 g/L Avicel and total [CBH] = 2 μM, and (**D**) 60 g/L avicel and total [CBH] = 2 μM. Blue triangles: Cel6A (or Cel6A core) in buffer. Red squares: Cel7A (or Cel7A core) in buffer. Dotted line indicates theoretical sum of the mono-components. Open circles: activity of mixtures of Cel6A and Cel7A or Cel6A core, and Cel7A core in different enzyme ratios with a constant enzyme concentration. Symbols are averages of triplicate measurements and error bars represent SD. All activities are plotted as function of fraction of Cel6A/Cel6A core and Cel7A/Cel7A core.

addition of 5 μM enzyme for Cel6A and Cel7A and 1, 0.1, and 0.01 μM for Cel7B. The volume added were 240 μL for all runs. The effect of dilution on the viscosity was determined by addition of 240 μL buffer and subtracted the drop in viscosity caused by the enzymes.

Results

Synergy Measurements

The activity of Cel6A, Cel7A, and their mixtures (both wild-types and truncated core enzymes) was assessed from 1 h end-point

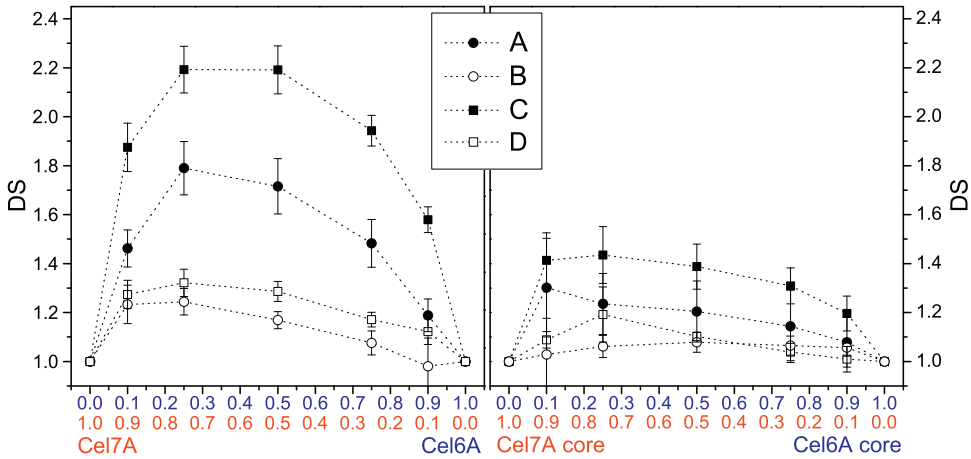

Figure 2. Degree of synergy (DS) calculated according to eq. (1) for pairs of wild-type enzymes (left) and pairs of core variants (right), and plotted as function of enzyme composition. The different curves in each panel refer to the experimental conditions (**A**, **B**, **C,** or **D** specified in Fig. 1). Error bars are SD propagated forward from original SD in Figure 1.

measurements. Four different types of experiments were conducted for each pair of enzymes. These were high enzyme (total concentration of cellulases 2 and 0.2 μM βG), low enzyme (0.2 μM cellulase plus 0.02 μM βG), high substrate (60 g/L Avicel), and low substrate (12 g/L). Results for enzyme mixtures are given by black symbols in Figure 1. To calculate the degree of synergy, the activity of the enzyme mixtures must be compared with the activity of each component in isolation. To this end we measured the glucose concentration in experiments with only one component. These reference experiments were conducted for both enzymes and at all seven mono-component concentrations that occurred in the enzyme mixture measurements. Results for the reference experiments are given in respectively blue (Cel6A) and red (Cel7A) in Figure 1. The apparent activities of the enzyme mixtures

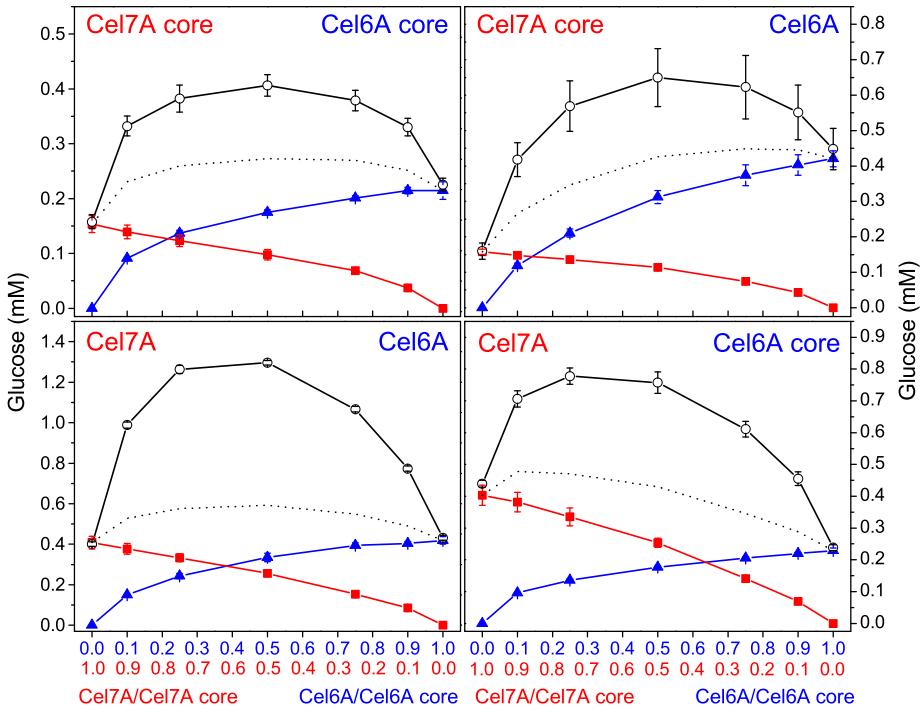

Figure 3. Activity data (formation of glucose) for Cel6A and Cel7A, Cel6A and Cel7A core, Cel6A core and Cel7A, Cel6A core and Cel7A core, and their mixtures at 12 g/L Avicel and [CBH] = 2 μM in the presence of 10% β.-glucosidase. Blue triangles: Cel6A or Cel6A core in buffer. Red squares: Cel7A or Cel7A core in buffer. Dotted line indicates theoretical sum of the mono-components. Open circles represent activity of mixtures at different enzyme ratios. Error bars indicates SD from triplicates.

(black lines) were consistently higher than the sum of the mono-components (dashed lines) and this is a hallmark of exo-exo synergy. The extent of this synergy varied strongly among the tested systems, but it was always higher for pairs of wild-type enzymes compared to pairs of core variants. To assess this quantitatively, we calculated the degree of synergy, DS

$$DS = \frac{A_{Ce16A+Ce17A}}{A_{Ce16A} + A_{Ce17A}} \quad (1)$$

where, $A_{Cel6A+Cel7A}$ is the apparent activity of the enzyme mixture, and A_{Cel6A} and A_{Cel7A} are the apparent activities in the corresponding mono-component experiments (i.e., the two separate experiments with the same mono-component concentrations as in the mixture). Values of DS are plotted as a function of the enzyme composition in Figure 2, and these results underscore that synergy is much stronger for pairs of wild types (both having a CBM) than for pairs of core variants. Regardless of whether the enzymes have a CBM (Fig. 2 left) or not (Fig. 2 right), condition C with low substrate load (12 g/L) and high enzyme concentration (2 μM) gives rise to the strongest synergy. Conversely, synergy for conditions B and D (with high substrate load) consistently showed low DS, and under these conditions synergy between the two core variants could only just be singled out against the experimental scatter.

To further study the role of the CBM for exo-exo synergy, we tested Cel6A-Cel7A enzyme pairs composed of one wild type and one core variant (e.g., Cel6A and Cel7A core). These measurements were all done under condition C (2 μM cellulase and 12 g/L Avicel), where DS had been shown (Fig. 2) to be strongest. Results are presented in Figures 3 and 4, which are designed analogously to Figures 1 and 2. The results for asymmetric pairs of core-wild type enzymes reiterate the general picture for the symmetric enzyme pairs in Figure 2. Thus, the overall trend was that DS decreased when one of the enzymes had no CBM. Closer inspection of Figure 4 suggests that the loss of the CBM from Cel7A had a stronger negative effect on synergy than the loss of the CBM from Cel6A.

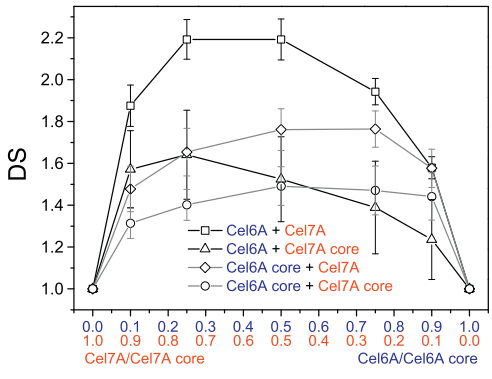

Figure 4. Degree of synergy (DS) calculated according to eq. (1) for the wt-core combinations at condition C (2 μM [CBH] and 12 g/L Avicel). Error bars are SD propagated forward from original SD in Figure 3.

Endo-Lytic Activity

Existing biochemical methods for the distinction of endo- and exo-lytic cellulase activity have different shortcomings, and we therefore conducted three independent assays to assess the endo-lytic activity of the wild-type CBHs. Two of the experimental approaches were based on CMC, which is the standard substrate used to identify endo-lytic cellulase activity (McCleary et al., 2012). In the first of these assays we followed the enzymatic release of soluble sugars in real time by a PDH biosensor (Cruys-Bagger et al., 2014). Results in Figure 5A show an initial phase of rapid hydrolysis followed by a much slower, almost constant reaction rate. The slope in the rapid phase (first 10–20 s in Fig. 5A) is about 10-fold higher than in the slow phase (after 250 s) for both enzymes. The transition between the fast- and slow phase occurs at 5–10 μM cellobiose, which corresponds to the conversion of less than 0.1% of the CMC, and we deduce that this small population of CMC is readily available as substrate for the CBHs, possibly through exo-attack. This interpretation is in accord with both the viscosimetric

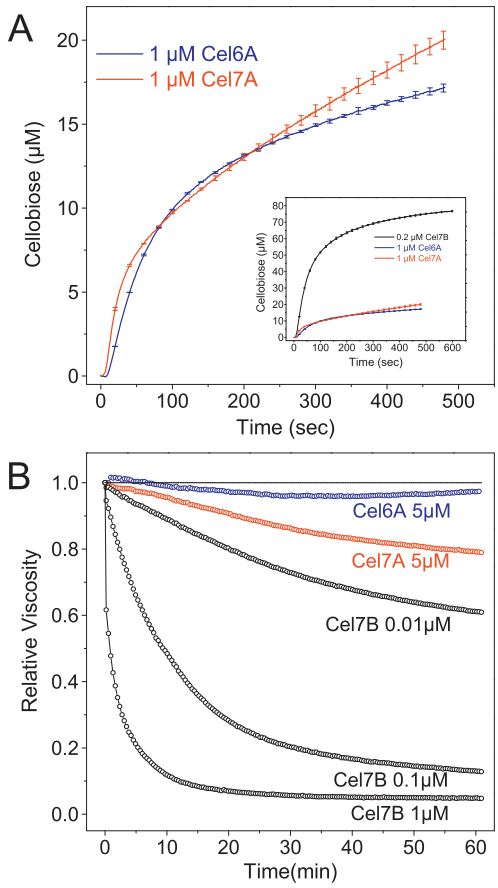

Figure 5. Endolytic activity of Cel6A and Cel7A on carboxymethyl cellulose (CMC) (**A**) Real-time recording of hydrolytic activity. The blue and red trace represent hydrolysis of 5 g/L CMC by respectively, 1 μM Cel6A and 1 μM Cel7A. The black curve in the inset shows the activity of 0.2 μM Cel7B against 0.5 g/L CMC. Error bars (shown at every 20 s) indicate SD from duplicates. (**B**) Relative changes in viscosity of 50 g/L CMC upon enzymatic attack at 25°C, Cel6A (blue), Cel7A (red), and the endoglucanase Cel7B (black).

measurements (see Fig. 5B) and estimates based on the degree of substitution of the CMC. Thus, we estimated the concentration of unlabeled stretches of pyranose rings at the end of the CMC molecule that the CBHs could realistically attack on the basis of the average molecular mass and degree of substitution (see Materials and Methods section). Unlabeled stretched of four pyranose units, for example, statistically occurred at a concentration of about 4 μM for the samples used in Figure 5A, while the analogous number for stretches of six unlabeled pyranose moieties was about 1 μM. These concentrations compare well to the location of the transition in Figure 5A, and we conclude that the degree of substitution of the CMC does not contradict the above interpretation of the transition point. Another possible reason for the transition in Figure 5A is product inhibition, but firstly this would not be expected to show a discrete change as in the figure and secondly the inhibition constant for cellobiose of Cel7A acting on polymeric substrate is hundredths of μM (Gruno et al., 2004; Olsen et al., 2016; Teugjas and Väljamäe, 2013), and product inhibition would therefore only induce insignificant effects on the overall rates in Figure 5A. The inhibition of Cel6A by cellobiose is still lower (Murphy et al., 2013; Teugjas and Väljamäe, 2013), and it appears that product inhibition is an unlikely cause for the sharp change of trace in Figure 5A. After this exo-attack available substrate has been degraded, the biosensor trace reflects endo-lytic activity of the enzyme. Interestingly, this interpretation implies about twice as high endo-lytic activity of Cel7A compared to Cel6A. As a control, the activity against CMC of *H. jecorina* Cel7B, which is traditionally categorized as an endoglucanase, was also measured with the biosensor. Results in the inset of Figure 5A show much higher activity for this enzyme (note that both enzyme and substrate concentrations are strongly reduced compared to the CBH measurements).

We also assessed the endo-lytic activity on the basis of the reduction in the viscosity of CMC solutions. This approach has been used extensively (see McCleary et al., 2012 for a review) and its main advantage is that exo-lytic attacks are essentially mute with respect to viscosity changes. The analysis applied here has been described elsewhere (Pedersen et al., 2016). Results in Figure 5B show that high concentrations (5 μM) of either Cel7A or Cel6A bring about a moderate reduction in the viscosity of a 50 g/L CMC solution over the time scale studied here. Cel7B, on the other hand, reduces viscosity dramatically and normalization of the initial slope in Figure 5B with respect to the enzyme concentration suggests 10^3–10^4 times higher endo-lytic activity of Cel7B compared to the CBHs. These results support the above interpretation of the biosensor measurements inasmuch as the high initial activity of Cel6A and Cel7A on CMC (Fig. 5A) did not lead to detectable viscosity changes in the same systems (Fig. 5B). This behavior is expected if the initial activity burst in Figure 5A reflects exo-lytic hydrolysis of a small population of the CMC, which has accessible strand ends (this reaction would essentially not change the viscosity). More importantly, the results in Figure 5B are also congruent in the sense that Cel7A shows 2–3-fold higher endo-lytic activity than Cel6A.

In the third assay for endolytic activity we measured the release of azurine from azurine crosslinked cellulose (AZCL-HE-Cellulose). Results in Table I confirm the interpretation of Figure 5. Thus, we found a 2–3-fold higher endo-lytic activity for Cel7A compared to Cel6A and a 10^3–10^4 times higher activity for Cel7B. In conclusion, we consistently found that the endo-lytic activity of Cel7A was 2–3 times higher than Cel6A, and that these two CBH are at least 1,000 times less endo-active than Cel7B. As the endo-lytic activity of the CBHs is so low, we cannot rule out that the results can be influenced by a slight EG contamination in our samples (a contamination in the order of $1{:}10^4$ by Cel7B, e.g, would influence the results, but be essentially impossible to detect by standard methods). Therefore, our relative endo-lytic activities of the CBHs (10^3–10^4 times less than Cel7B) are upper limits, and the true endo-activity of the CBHs could be even lower. Furthermore we cannot eliminate that differences in EG contamination could influence the relative endo-activity of the two CBHs, but since both CBHs are expressed and purified by exactly the same protocol we find this unlikely.

Table I. Endo-activity of Cel7A, Cel6A, and Cel7B on the endo cellulose-substrate azurine crosslinked cellulose (AZCL-HE-Cellulose) estimated from absorption $A_{595}/\mu M$ enzyme after 1 h hydrolysis. We used 5 g/L AZCL-HE-Cellulose and 10 μM [E] for Cel7A and Cel6A, and 0.1 μM for Cel7B.

	AZCL-HE cellulose activity $A_{595}/\mu M$	Relative activity
Cel7A	0.0133 ± 0.0001	4.23×10^{-4}
Cel6A	0.0058 ± 0.0003	1.84×10^{-4}
Cel7B	31.300 ± 0.2498	1

Discussion

Enzymatic conversion of biomass to fermentable sugars is a key process in emerging industries that produce sustainable fuels and alternatives to petrochemicals from lignocellulosic feedstocks. This conversion (so-called saccharification) requires quite large enzyme doses and minimization of enzyme consumption is therefore vitally important for the economic feasibility of the industry. One important avenue toward lower enzyme consumption is design of enzyme cocktails with a higher degree of synergy. However, the degree of synergy has been shown to depend quite markedly on a range of parameters including surface density of bound enzyme (Medve et al., 1994; Woodward et al., 1988), physical properties of the substrate (Hoshino et al., 1997; Valjamae et al., 1999), hydrolysis time (Boisset et al., 2001; Medve et al., 1998), cellulase mole fraction and substrate conversion (Jeoh et al., 2006; Olsen et al., 2017), and this complex behavior has challenged attempts to elucidate molecular origins of the measured synergy. As a result, discovery of cellulase cocktails with a high degree of synergy remains primarily an empirical endeavor. Clearly, better understanding of the underlying mechanisms would be desirable as it could gradually promote rational elements in the development of enzyme cocktails with more efficient synergy. In the current work we have zoomed in on the origin of the less extensively studied exo-exo synergy.

One central molecular interpretation of exo-exo synergy between Cel7A and Cel6A has been an auxiliary endo-lytic activity of the latter (Boisset et al., 2000; Poidevin et al., 2013), and this understanding has been mainly based on structural evidence (see Introduction section and Payne et al., 2015 for a review). However, this explanation was not supported by the biochemical data presented here. We found

a low endo-lytic activity of both wild types, but Cel7A was more endo-active compared to Cel6A. Our results on the endo-lytic activity of the CBHs were consistent among the three assays types and also congruent to some earlier studies. For example, both Cel7A and Cel6A have been shown to have low activity against CMC (Ståhlberg, 1993), and an earlier work also found that Cel7A was slightly more active than Cel6A on this substrate (Irwin et al., 1993). Our experiments with both CMC and AZCL-HE cellulose indicated a relative endo-lytic activity of the two CBHs, which was 10^3–10^4 times lower than an EG (Cel7B) from the same organism. This minor endo-lytic activity is in line with the observation that Cel7A only produce a small amount of new reducing ends on bacterial cellulose (BC) (Kurasin and Väljamäe, 2011). Overall these results suggest that generation of new chain ends by the CBHs, in particular Cel6A, is of limited importance and hence not the main mechanism behind exo-exo synergy. Finally, we note that in light of the higher endo-lytic activity of Cel7A found here, the assignment of *H. jecorina* Cel6A as an endo-processive enzyme (Boisset et al., 2000) may need further examination.

In search for an interpretation that is more consistent with the current observations we note that the CBM promoted exo-exo synergy under all conditions studied here (Figs. 2 and 4). Thus, the highest DS (about 2.2, Fig. 2) was found for mixtures of the two wild type enzymes and removal of one or both CBMs gradually lowered DS. Mixtures of two core variants on high substrate load (60 g/L) showed limited or no synergy ($DS < 1.2$). Results from the asymmetric mixtures (one core and one wild type, Fig. 4) further suggested that the CBM on Cel7A was more important for DS than Cel6A's CBM. This behavior is in line with the interpretation that synergy is connected to the targeting function of the CBM (Carrard et al., 2000; Fox et al., 2013; Herve et al., 2010; Liu et al., 2011; McLean et al., 2002). Different targeting of two enzymes may cause synergy if one enzyme hydrolyzes certain surface structures, crystalline or amorphous regions, and hence expose better substrate for the other. This molecular origin of synergy is independent of whether the enzymes utilize exo- or endo lytic mechanisms, and it has indeed previously been proposed to underlie some cases of endo-exo synergy (Eriksson et al., 2002; Fox et al., 2012; Igarashi et al., 2011; Jalak et al., 2012). In particular, Jalak et al. (2012) suggested that endo-exo synergy reflected a preference of the EG for amorphous sections of BC because sparse amorphous segments make obstacles for the processive movement of Cel7A. In accordance with this, Fox et al. (2012) found that presence of EG increased the processive length of Cel7A. As Cel6A has been suggested to be particularly active on amorphous cellulose (Ganner et al., 2012), an analogous mechanisms could be responsible for the exo-exo synergy observed here. This interpretation is further supported by Igarashi et al. (2011) who showed that presence of Cel6A improved the mobility of Cel7A enzymes and thereby reduced enzyme "traffic jams." This conclusion would also be in line with the reported preference of Cel7A's CBM for the hydrophobic surface of crystalline cellulose (McLean et al., 2002), as well as an earlier observation that sequential exo-exo synergy is observed with pretreatment of Cel6A before action of Cel7A (Väljamäe et al., 1998).

Some studies have suggested that in addition to its role in binding and targeting, the CBM directly assists the catalytic process (Beckham et al., 2010; Din et al., 1991; Guillen et al., 2010; Hall et al., 2011; Lemos et al., 2003; Mulakala and Reilly, 2005; Teeri et al., 1992), and if indeed so, this could also lead to synergy in mixtures of enzymes with different CBMs. We note, however, that a clear positive role of the CBM for activity does not appear from the current results. Looking, for example, at the data in Figure 1B and D, we find that at high substrate loads, the pair of core variants had a comparable or higher activity than the pair of wild types with CBMs. Synergy, on the other hand, was consistently low in these high-solid experiments (see Fig. 2). High activity of CBM-free enzymes in concentrated substrate suspensions, as observed here, has been reported earlier (Le Costaouec et al., 2013; Pakarinen et al., 2014; Várnai et al., 2013), and interpreted as a sign of an off-rate controlled reaction (Sørensen et al., 2015a,b). Thus, if enzyme-substrate dissociation is the rate limiting step, the weaker association of core-variants will speed up the overall reaction at high loads of substrate (increase V_{max}) (Sørensen et al., 2015a,b). More importantly in the current context, comparison of results for wild types and core variants in Figures 1 and 2 shows that high apparent activity and high synergy may occur independently. This observation is consistent with the mechanism of synergy suggested by Jalak et al. (2012) (see the Introduction section). These workers noted that if slow dissociation of enzyme that was stalled in front of obstacles on the cellulose surface was rate limiting, synergy could occur if another enzyme specifically removed such obstacle structures. Jalak et al. (2012) suggested that the obstacles were amorphous regions of cellulose, but the same argument could be valid for other putative structures that obstruct the processive movement of the CBH. For the core variants with higher rates of dissociation, stalling in front of obstacles is likely to be less important, and it follows that removal of such obstacles would not generate the same degree of synergy.

One last aspect of this work concerns the way DS is obtained experimentally. Thus, many earlier studies have measured mono-component activity only at one enzyme concentration, typically corresponding to the total enzyme concentration in the mixtures (see, e.g., Boisset et al., 2000, 2001; Henrissat et al., 1985; Olsen et al., 2017; Tomme et al., 1988). The contribution at other mono-component concentrations that occurred in mixtures was then estimated based on the assumption of a linear dose-activity relationship. However, some of the mono-component dose-activity curves in Figure 1 (red and blue lines) were highly non-linear. This non-linearity is common for cellulases (Bezerra and Dias, 2004; Sattler et al., 1989), and neglect of this will severely influence the calculated value of DS. An extreme example of this can be seen in Figure 1D, where the core enzymes show essentially no synergy (black and dashed curves are almost superimposed). This result, however, is very dependent on the non-linearity of the mono-component activity curves (Fig. 1D), and for these specific results, a linear approximation would give (erroneous) DS values up to 1.6. We strongly suggest that future work includes mono-component activity measurements at several concentrations as it was recently done by Igarashi et al. (2011).

In conclusion we have found that Cel6A and Cel7A from *H. jecorina* show distinct synergy with DS values exceeding two under some conditions. The auxiliary endo-lytic activity of both enzymes was extremely small compared to an endoglucanase from

the same organism, and we suggest that an endo-like activity of the CBH is not the cause of the synergy observed here. The extent of the exo-exo synergy gradually decreased if one or both enzymes did not have a CBM. This observation is consistent with the hypothesis put forward for conventional exo-endo synergy (Eriksson et al., 2002; Jalak et al., 2012) that targeting toward different structures on the cellulose surface can cause synergy. We speculate that the well-known targeting role of the CBM could be the primary cause of exo-exo synergy for Cel7A and Cel6A.

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