Effect of Cellulase Mole Fraction and Cellulose Recalcitrance on Synergism in Cellulose Hydrolysis and Binding

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Elucidating the molecular mechanisms that govern synergism is important for the rational engineering of cellulase mixtures. Our goal was to observe how varying the loading molar ratio of cellulases in a binary mixture and the recalcitrance of the cellulose to enzymatic degradation influenced the degree of synergistic effect (DSE) and degree of synergistic binding (DSB). The effect of cellulose recalcitrance was studied using a bacterial microcrystalline cellulose (BMCC), which was exhaustively hydrolyzed by a catalytic domain of Cel5A, an endocellulase. The remaining prehydrolyzed BMCC (PHBMCC) was used to represent a recalcitrant form of cellulose. DSE was observed to be sensitive to loading molar ratio. However, on the more recalcitrant cellulose, synergism decreased. Furthermore, the results from this study reveal that when an exocellulase (Cel6B) is mixed with either an endocellulase (Cel5A) or a processive endocellulase (Cel9A) and reacted with BMCC, synergism is observed in both hydrolysis and binding. This study also revealed that when a "classical" endocellulase (Cel5A) and a processive endocellulase (Cel9A) are mixed and reacted with BMCC, only limited synergism is observed in reducing sugar production; however, binding is clearly increased by the presence of the Cel5A.

Introduction

A critical process in a biomass biorefinery is the enzymatic hydrolysis of cellulose to fermentable sugars. Although cellulases are biocatalysts that can efficiently depolymerize cellulose, developing more effective, thermally stable, low cost cellulase is crucial for successful establishment of biomass biorefineries. There are a number of scientific approaches to "engineer" more effective cellulases, ranging from site-directed mutagenesis to directed evolution. However, rational engineering of cellulases must be based on a comprehensive understanding of the mechanisms governing the depolymerization process. Given that individual cellulases exhibit low activities on microcrystalline cellulose and that the synergistic interaction of multiple cellulases is required to effectively hydrolyze cellulose, elucidating the molecular mechanisms that govern synergism is important for the rational engineering of cellulase mixtures.

Synergistic effects between cellulases have been documented repeatedly by many investigators (*1*−*7*). However, the chemical and mass transport mechanisms that govern synergism are still not well understood. The most popular theory is that cellulases in a mixture increase access to reactive sites on the substrate for each other (*8*). In particular, endocellulases, which have active site configurations conducive to midchain cleavage, are thought to increase the availability of free ends for exocellulases.

A survey of the literature shows that there are at least three key factors that influence the extent to which cellulases synergize: (1) ratio and concentrations of the cellulases in the reaction mixture (*4*, *5*, *9*, *10*); (2) access to binding sites for the mixture cellulases (*11*−*16*); and (3) physical and chemical heterogeneity in the substrate (*17*−*19*).

Studies have shown that lower ratios of the major endocellulases result in higher degree of synergistic effects (DSE) (*4*, *5*, *10*). However, the ratio of bound cellulases has not been extensively documented. Several studies on bound cellulases in mixtures have shown competition for binding sites on the substrate (*14*−*16*). In a previous study (*12*) using equimolar cellulase loading, synergistic cellulase mixtures showed increased substrate binding (*12*), suggesting that the synergistic effect is likely to be partially coupled to synergistic binding.

This study builds on the synergism study of Watson et al. (*5*) and the binding study of Jeoh et al. (*12*) to assess synergism in binding for binary mixtures of *Thermobifida fusca* Cel5A, Cel6B and Cel9A. Native molar ratios of cellulases secreted by *T. fusca* vary with the available carbon source in the growth medium (*20*). *T. fusca* grown on Solka Floc has been observed to secrete the three enzymes in a molar ratio of 0.12:0.72:0.16 Cel5A:Cel6B:Cel9A (*20*). Binary mixtures of these cellulases are synergistic at various molar ratios (*5*, *6*, *12*, *21*). Our specific goal in this study was to observe how varying the loading molar ratios (molar concentration of a cellulase in the mixture divided by the total cellulase molar concentration) of cellulases in binary mixtures determines the bound cellulase fraction. In addition, we wanted to assess the influence of cellulose recalcitrance on bound cellulose fractions.

Experimental Methods

Cellulose and Cellulase Preparation. BMCC (Monsanto Cellulon, Monsanto Company, San Diego, CA) and *T. fusca* Cel5A, Cel6B and Cel9A used in this study were prepared as described previously (*12*). *T. fusca* CD_{Cel5A} was produced and purified by Jung et al. (*17*). The purity of all the cellulases used in this study were better than 99.9% based on observation of a

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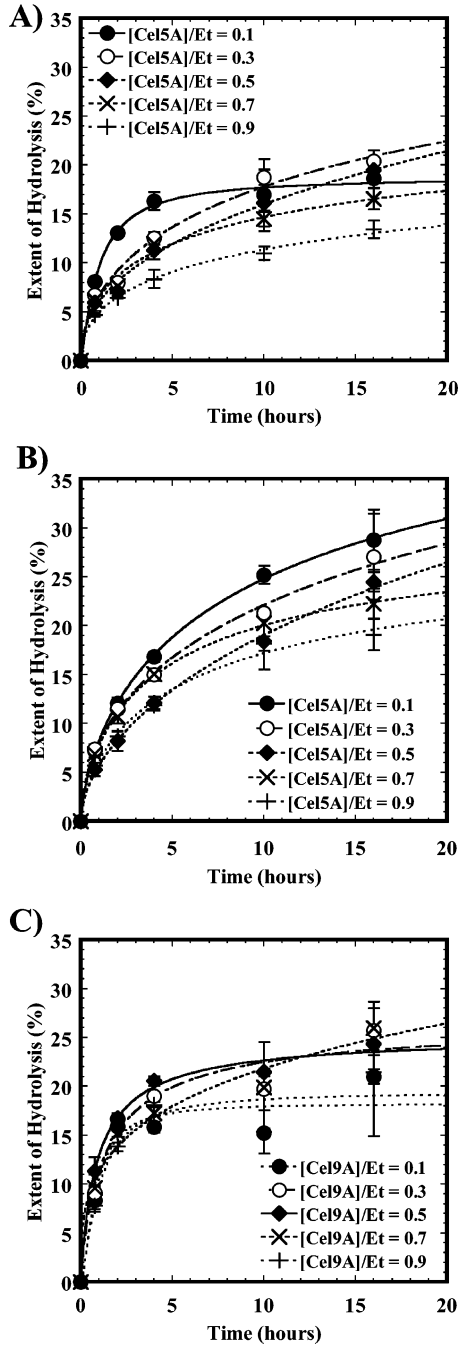

Figure 1. Time courses of BMCC hydrolysis by binary mixtures of *T. fusca* cellulases: (A) Cel5A+Cel6B, (B) Cel5A+Cel9A, and (C) Cel6B+Cel9A. Legend describes the molar ratios of the $E_t = 0.25$ μM loadings.

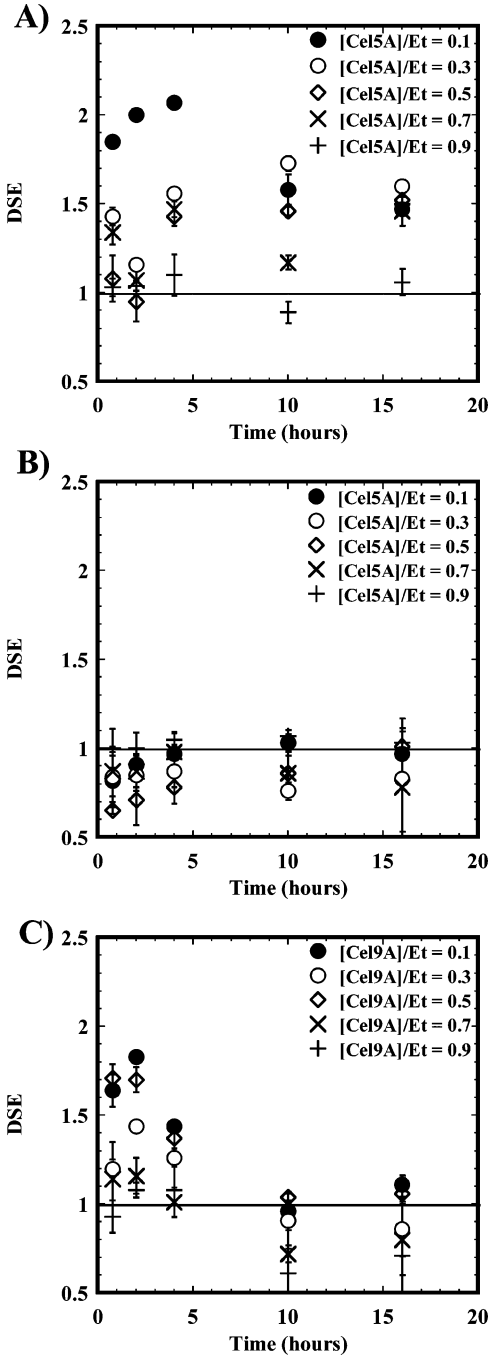

Figure 2. Calculated DSE values throughout the hydrolysis time courses corresponding to data shown in Figure 1: (A) *T. fusca* Cel5A+Cel6B, (B) Cel5A+Cel9A, and (C) Cel6B+Cel9A. Legend describes molar ratios of the $E_t = 0.25$ μM loadings in binary reactions. Line drawn at DSE = 1 as a guide.

single band corresponding to the molecular weight of the target enzyme on SDS−PAGE gels. Cellulases in the binding studies were labeled with either Alexa Fluor 488 (AF488) or Alexa Fluor 594 (AF594) succinimidyl esters (Molecular Probes, Inc., Eugene, OR) as described previously (*12*).

Preparation of Prehydrolyzed BMCC (PHBMCC). Preparation of PHBMCC was carried out as described by Jung et al. (*17*). The reactions, each containing 1 mg/mL BMCC with 5.6 μM CD_{Cel5A} in 5 mM sodium acetate buffer, were carried out in 1.5-mL Costar Spin-x tubes (0.45 μm Nylon, Corning Inc., Corning, NY). The reactions were incubated at 25 °C for 3 h. At the end of the reaction time, the tubes were centrifuged at 13,000 rpm for 1 min. The pretreated BMCC retained in the filters were resuspended with 500 μL of 1% NaOH and then centrifuged again to denature and remove the CD_{Cel5A} adsorbed onto the fiber surface. A previous study by Jung et al. (*17*) showed that the alkaline wash of BMCC did not have a pretreatment effect on the fibers. The fibers were washed three consecutive times by resuspending in 500 μL distilled water, with a centrifugation step between washes. After the final water wash step, the pretreated BMCC was resuspended in 5 mM sodium acetate buffer. The final concentration was determined by triplicate oven-dry weight analysis of 3 mL of the suspension.

Individual Cellulase Time Course Reactions. Individual cellulase time course experiments were conducted using 0.025, 0.075, 0.125, 0.175 and 0.225 μmol/g of *T. fusca* Cel5A, Cel6B or Cel9A cellulases. The experiments were conducted with 1

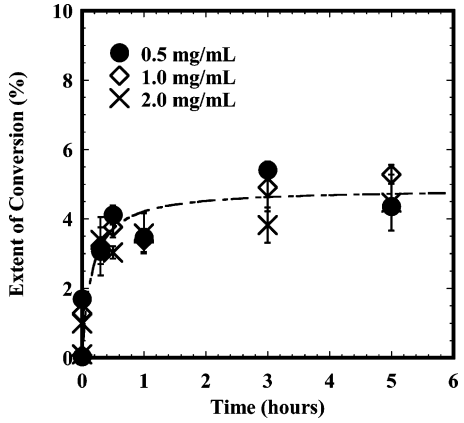

Figure 3. Time course hydrolysis of BMCC by 5.6 μM *T. fusca* CD_{Cel5A}. The legend indicates the initial concentration of BMCC used in the reactions. A line was drawn to guide the eye.

mg/mL BMCC in 250 μL total reaction volumes of 5 mM sodium acetate buffer (pH 5.5). Samples were taken over a 16 h time course at 0.75, 2, 4, 10 and 16 h. The 0.75 h reactions were conducted in 1.5-mL Costar Spin-x tubes (0.45 μm Nylon, Corning Inc., Corning, NY). To prevent excessive evaporative losses, reactions longer than 45 min were conducted in 1.5-mL siliconized microcentrifuge tubes and transferred to Spin-X tubes at the end of the incubation time. The reactions were incubated at 50 °C with end-over-end rotation and stopped by centrifugation in the Spin-X tubes at 13,000 rpm for 1 min. The BMCC remaining in the filter after centrifugation was resuspended in 250 μL of 5 mM sodium acetate (pH 5.5) and analyzed by fluorometry using a Perkin-Elmer luminescence spectrometer (LS50B, Perkin-Elmer Instruments, Oak Ridge, TN) (*12*). The filtered supernatant was analyzed for reducing sugars using the PAHBAH method as described previously (*12*, *22*). Triplicate samples with corresponding triplicate cellulase-only controls were assayed at each reaction time.

Time course experiments on BMCC prehydrolyzed by CD_{Cel5A} (PHBMCC) were conducted under the same conditions as the experiments using untreated BMCC described above. Cellulase loadings of 0.075, 0.125 and 0.175 μM were used in the experiments with PHBMCC.

Varying Ratio, Time Course Reactions. Time course reactions with varying cellulase molar ratios were conducted at a fixed total cellulase loading concentration, E_t, of 0.25 μmol/g using *T. fusca* Cel5A, Cel6B and Cel9A. For the Cel5A+Cel6B, Cel5A+Cel9A, and Cel6B+Cel9A reactions, molar ratios of 0.1, 0.3, 0.5, 0.7 and 0.9 were studied.

The experiments were conducted with 1 mg/mL BMCC with 5 mM sodium acetate buffer (pH 5.5) in 1.5-mL Costar Spin-x tubes (0.45 μm Nylon, Corning Inc., Corning, NY) in 250 μL total reaction volume. The reactions were incubated at 50 °C with end-over-end rotation and stopped by centrifugation at 13,000 rpm for 1 min. The BMCC remaining on the filter after centrifugation was resuspended in 250 μL of 5 mM sodium acetate (pH 5.5) and analyzed by fluorometry (*12*). The filter-flow through was analyzed for reducing sugars using the PAHBAH method (*22*). Triplicate samples with corresponding triplicate cellulase-only controls were taken at 5 s, 0.75, 2, 4, 8 and 16 h reaction times.

Monitoring Cellulose Concentration by Fluorescence. Cellulase concentration was measured by comparing the fluorescence intensity of fluorescence-labeled cellulase samples to a standard curve (*12*). As demonstrated previously, fluorescence labeling of *T. fusca* Cel5A, Cel6B and Cel9A did not affect the specific activities of these cellulases on either CMC or BMCC (*12*). All fluorescence measurements were conducted with a Perkin-Elmer luminescence spectrometer (LS50B, Perkin-Elmer Instruments, Oak Ridge, TN) equipped with a 96-well plate reader and interfaced to a Digital computer (DECpc LPv 433dx, Compaq Computer Corporation, Houston, TX). Measurement conditions and parameters were programmed and controlled through a software package, FL WinLab (Release A, Version 2.0, 1997, The Perkin-Elmer Corporation). Excitation/emission wavelengths for Alexa Fluor 488 (AF488) and Alexa Fluor 594 (AF594) were 488/519 nm and 594/617 nm, respectively. The accuracy of this method has previously been shown to be in the range of 7−9% (*12*).

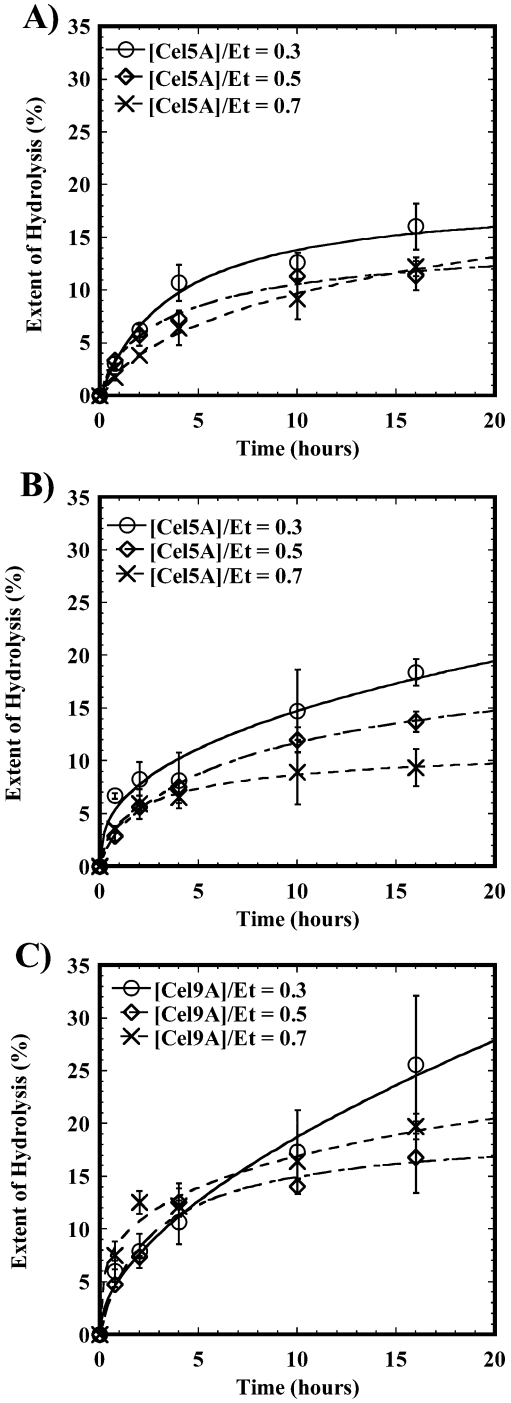

Figure 4. Time courses of PHBMCC hydrolysis by binary mixtures of *T. fusca* cellulases: (A) Cel5A+Cel6B, (B) Cel5A+Cel9A, and (C) Cel6B+Cel9A cellulases. Legend describes molar ratio makeup of the $E_t = 0.25$ μM loadings.

DSE and Degrees of Synergistic Binding (DSB). DSE of the mixtures were calculated using the following equation:

$$\mathrm{DSE}_{\chi} = \frac{\chi_{\mathrm{mix}}}{\sum_{i=1}^{2} \chi_i} \tag{1}$$

where DSE_{χ} is the degree of synergistic effect for extent of hydrolysis (dimensionless), χ_{mix} is the extent of substrate conversion achieved in 0.75 h by a binary mixture of cellulases (%), and χ_i = extent of substrate conversion achieved in 0.75 h by the *i*th component in the binary mixture (%).

The following equation, previously defined by Jeoh et al. (*12*), was used to calculate the degree of synergistic binding in each mixture:

$$\mathrm{DSB} = \frac{[E_{\mathrm{b,mix}}]}{\sum_{i=1}^{2} [E_{\mathrm{b,single}}]_i} \tag{2}$$

where DSB is the degree of synergistic binding (dimensionless), $[E_{\mathrm{b,mix}}]$ is the concentration of bound cellulase in the mixture (μmol/g), and $[E_{\mathrm{b,single}}]_i$ is the concentration of bound cellulase in the *i*th component in the binary mixture (μmol/g).

The DSB values were calculated on the basis of bound cellulase concentrations corrected for the remaining amount of substrate in the reaction (μmol bound cellulase/g remaining substrate).

Results and Discussion

BMCC Hydrolysis. All cellulase mixtures exhibited nonlinear reducing sugar production with time as illustrated in Figure 1. The Cel5A+Cel9A mixture yielded the highest extent of hydrolysis with 30% conversion in 16 h, while Cel6B+Cel9A yielded the second highest extent of hydrolysis with 23% conversion in 16 h. For Cel5A+Cel6B (Figure 1A), a [Cel5A]/E_t ratio of 0.1 was the most effective in hydrolyzing BMCC during the first 4 h of the reaction, followed by [Cel5A]/E_t ratio of 0.3. However, extents obtained for [Cel5A]/E_t ratios of 0.1 and 0.5 converged at 10 h. One important observation is that the two most effective mixtures for hydrolyzing BMCC, Cel5A+Cel9A and Cel6B+Cel9A, are the ones where Cel9A is the dominant component representing up to 90% of the total loading in the mixture.

It is apparent from the results presented in Figure 1A and B that only 10% of the endocellulase Cel5A is needed in a mixture with the exocellulase Cel6B or with the processive endocellulase Cel9A for effective hydrolysis of BMCC. In fact, higher molar ratios of Cel5A negatively impacted the activity of the mixture. This result is consistent with earlier synergism results obtained by Walker et al. (*10*) using Avicel and Watson and Walker (*5*) using BMCC.

Synergism was observed for only two of the three binary mixtures, Cel5A+Cel6B and Cel6B+Cel9A, (Figures 2) and the Cel5A+Cel6B mixture yielded the highest DSE values. For this mixture, the highest DSE was achieved at the molar ratio of [Cel5A]/E_t = 0.1. Synergism was observed for Cel6B+Cel9A, but only early in the reaction (see Figure 2C). The results from these two mixtures demonstrated that higher ratios of Cel6B are favorable for synergism.

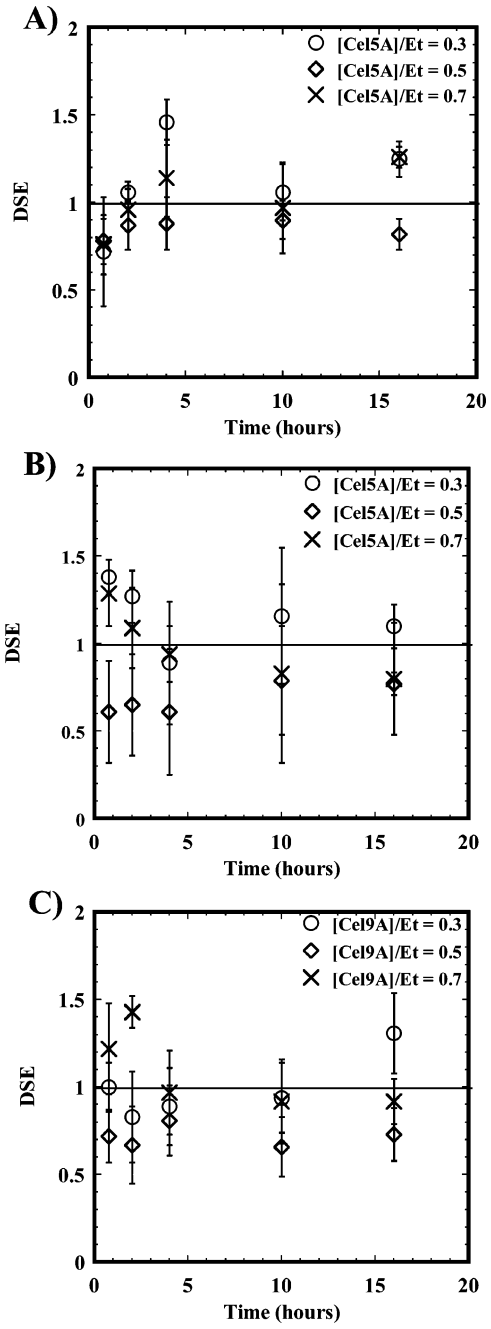

Figure 5. Calculated DSE values throughout the hydrolysis time courses corresponding to data shown in Figure 4: (A) *T. fusca* Cel5A+Cel6B, (B) Cel5A+Cel9A, and (C) Cel6B+Cel9A. Legend describes molar ratio makeup of the E_t = 0.25 μM loadings in binary mixture reactions. Line drawn at DSE = 1 as a guide.

No synergism was observed between the endocellulase Cel5A and the processive endocellulase Cel9A (Figure 2B). DSE values for the Cel5A+Cel9A mixture were consistently near or below 1. However, this mixture yielded the greatest extent of hydrolysis at 16 h (see Figure 1B). Reactions with higher [Cel5A]/E_t values yielded lower hydrolysis extents (see Figure 1B) with DSE values less than 1 (see Figure 2B).

PHBMCC Hydrolysis. BMCC was pretreated by an endocellulase catalytic domain (CD_{Cel5A}) to remove the easily hydrolyzable fraction (EHF) and thus to generate a cellulose that is more resistant to cellulolytic attack. The catalytic domain of Cel5A was found to have limited activity on BMCC, being able to hydrolyze only up to about 5% of the cellulose (Figure 3). On the basis of this observation, we concluded that CD_{Cel5A}

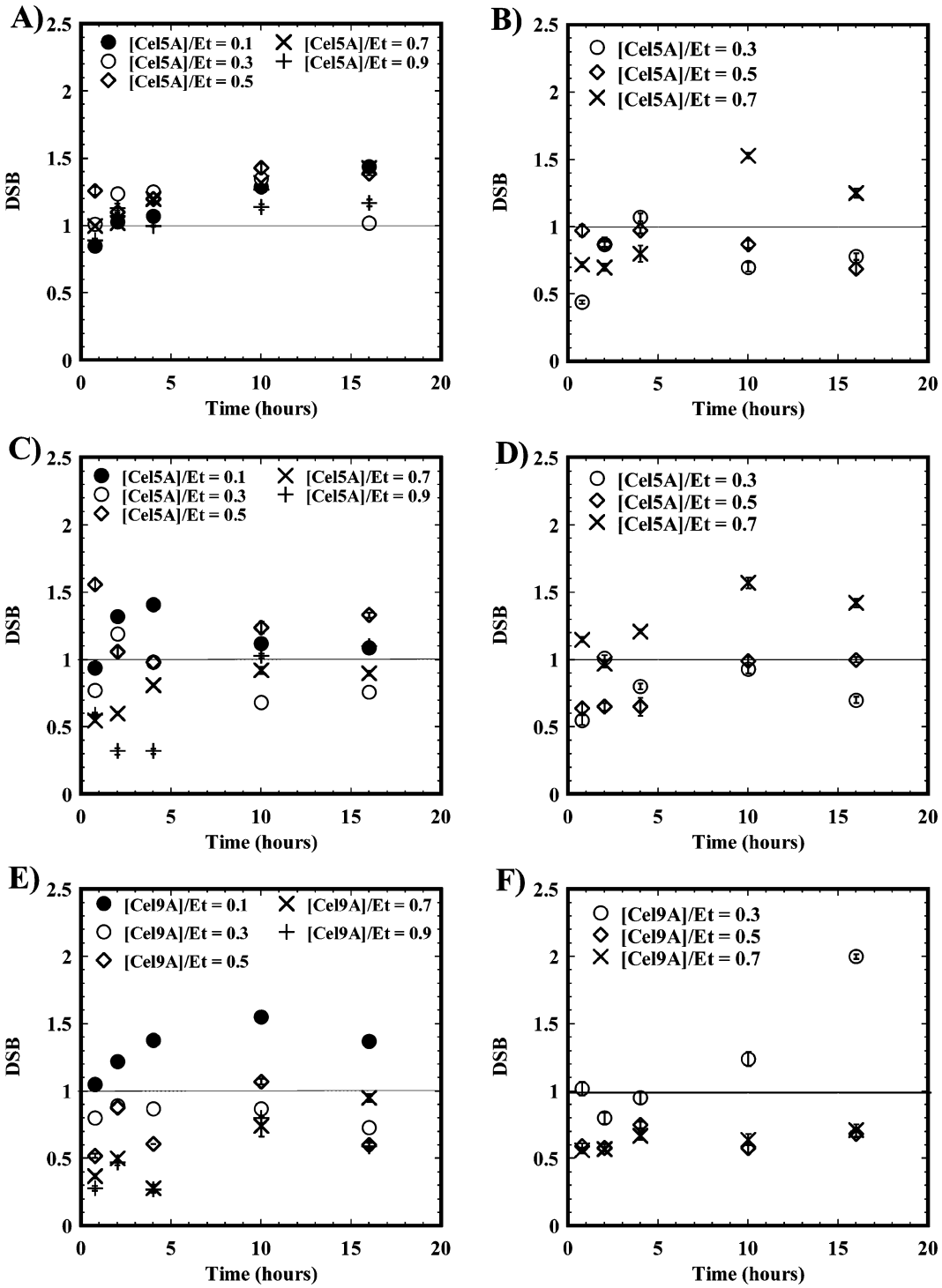

Figure 6. Degree of synergistic binding in (A and B) Cel5A+Cel6B, (C and D) Cel5A+Cel9A, and (E and F) Cel6B+Cel9A on BMCC (left column) and PHBMCC (right column). DSB values were calculated using eq 2. Lines are drawn only to guide the eye.

can only hydrolyze the most easily accessible and hydrolyzable fraction of BMCC. The cellulose remaining after hydrolysis with CD_{Cel5A} (prehydrolyzed BMCC) thus represents the recalcitrant fraction (RF) of BMCC. Beyond the resistance to further attack by an endocellulase catalytic domain, the precise physical nature of PHBMCC was not investigated for this study. It is interesting that the overall extents of hydrolysis on PHBMCC (Figure 4) are effectively 5% less than those of the corresponding mixtures in Figure 1.

The extent of PHBMCC hydrolysis by the binary mixtures followed similar trends as on untreated BMCC. As was the case with untreated BMCC, the lowest level of endocellulase Cel5A yielded the highest hydrolysis extents. Overall, the highest extent of conversion was obtained for the Cel6B+Cel9A mixture followed by the Cel5A+Cel9A mixture. However, synergism in these two mixtures appeared to be relatively insensitive to molar ratio (Figure 5B and C). Although synergism in the Cel5A+Cel6B mixture was more favorable for lower ratios of Cel5A, this dependence was less distinct than on untreated BMCC.

DSE values for all three cellulase mixtures on PHBMCC (Figure 5) were smaller than those observed on BMCC (Figure 2) and tended to diminish to a value of 1.0 or less over the time course of the reaction. Since PHBMCC is a cellulose

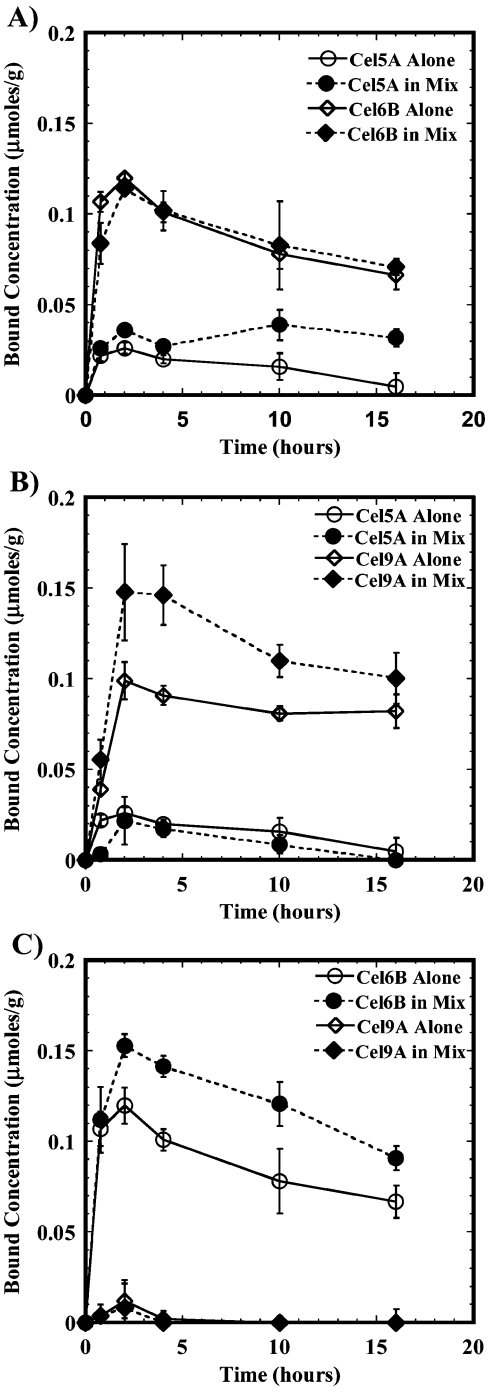

Figure 7. Comparing bound cellulase concentrations in mixture reactions with corresponding individual component reactions for [Cel5A]/E_t = 0.1 of the (A) Cel5A+Cel6B and (B) Cel5A+Cel9A reactions, and (C) [Cel9A]/E_t = 0.1 of the Cel6B+Cel9A reaction on BMCC. Lines are drawn between data points only to guide the eye.

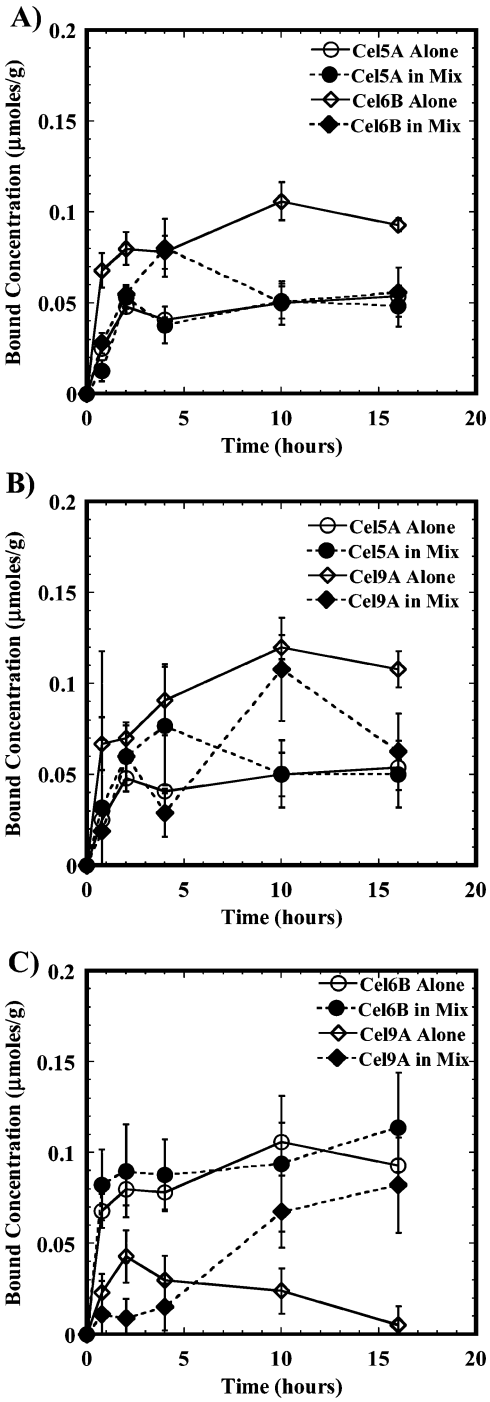

Figure 8. Comparing bound cellulase concentrations in mixture reactions with corresponding individual component reactions for [Cel5A]/E_t = 0.3 of the (A) Cel5A+Cel6B and (B) Cel5A+Cel9A reactions, and (C) [Cel9A]/E_t = 0.3 of the Cel6B+Cel9A reaction on PHBMCC. Lines are drawn between data points only to guide the eye.

resistant to attack by CD_{Cel5A}, these results therefore suggest that cellulose recalcitrance negatively impacts the ability of the cellulases to act synergistically.

Degree of Synergistic Binding. All cellulase mixtures exhibited some synergism in binding to BMCC and to PHB-MCC (Figure 6). For BMCC, this synergism was observed over the entire time course for several ratios (see Figure 6A, C and E). Binding synergism was observed for all of the molar ratios of the Cel5A+Cel6B mixture (Figure 6A). Cel5A+Cel9A exhibited binding synergism for [Cel5A]/E_t values of 0.1−0.5 (Figure 6C). Synergistic binding in the Cel6B+Cel9A mixture on BMCC was only observed for the 0.1 loading molar ratio (Figure 6E). In general, synergism in binding on untreated BMCC tracked synergism in hydrolysis, with a 20−50% increase in total cellulase binding (see Figure 2).

Binding synergism on PHBMCC was observed late into the hydrolysis process, as shown in Figure 6B, D and F, and did not track synergism in hydrolysis. For the Cel5A+Cel6B mixture, a [Cel5A]/E_t of 0.7 yielded the highest DSB (Figure 6B) whereas a [Cel5A]/E_t of 0.3 yielded the highest DSE (Figure 5A) on PHBMCC. This was also true for the Cel5A+Cel9A mixture. For the Cel6B+Cel9A mixture, the highest DSE was observed for [Cel9A]/E_t = 0.7, and the highest DSB were observed for [Cel9A]/E_t = 0.3.

Binding Time Course. DSB values only reveal whether synergism results in an increase in total bound concentrations, thus providing no insight on whether one or both cellulases in a mixture exhibited increased binding. Such insight is gained by observing the time course of binding for the two cellulases in mixture and individual reactions as shown in Figures 7 and 8 for BMCC and PHBMCC, respectively. On BMCC, binding for all pure cellulases and all mixtures exhibited rapid increases over a period of 2 h, after which they leveled off or decreased (Figure 7). All three cellulase mixtures had one cellulase that exhibited higher binding than was observed with the same amount of that cellulase in single enzyme reactions. For the Cel5A+Cel6B mixture, the increase in binding was observed for Cel5A (Figure 7A), whereas it was Cel9A that exhibited increased binding with the Cel5A+Cel9A mixture and Cel6B for Cel6B+Cel9A. These results are consistent with previous results using binary cellulase mixtures at 1:1 loading molar ratios (*12*).

For PHBMCC, temporal binding trends (Figure 8) were much more varied than for untreated BMCC and there was little evidence of enhanced binding. For the two mixtures containing Cel5A, the observed Cel6B and Cel9A binding concentrations were generally lower than what was observed when these cellulases were reacted alone on PHBMCC (see Figure 8A and B). Furthermore, the [Cel5A]/E_t bound ratio tended to converge to 0.5 despite a loading molar ratio of 0.3. For Cel6B+Cel9A, the observed Cel9A binding was initially lower than Cel9A alone but over time exceeded this level (Figure 8C). This phase of enhanced Cel9A binding also corresponded to increased hydrolysis rates (Figure 4C).

Conclusions

Consistent with previous studies (*4, 5, 10*), DSE was observed to be sensitive to loading molar ratio. In endo-exo mixtures, low ratios of the endocellulase result in the strongest synergistic effect. Although synergism in the endo-processive endo mixture is limited, the data indicate that input molar ratio is still an important factor for obtaining high hydrolysis extents. This effect, favoring high ratios of Cel9A in the mixture, confirms previous observations that Cel9A is more active than Cel5A (*5, 12*). Input molar ratio was a key factor in determining bound enzyme ratio on BMCC. Since all three cellulases have homologous Type II cellulose binding modules (CBM) with similar affinities to BMCC (*19, 23*), one would predict the bound ratio to directly reflect the input ratio. Actual measured bound ratios deviated from this baseline, suggesting that the catalytic domain contributes significantly to the overall binding affinity of the cellulase to the substrate. Bound ratios on PHBMCC showed greater variations, thus further suggesting that the binding influence of the CD is a greater factor on the recalcitrant fraction.

Another major conclusion drawn from this research is that synergism in reducing sugar production decreases as the substrate becomes more recalcitrant. This was evident in the BMCC and the PHBMCC DSE time courses. That the synergistic effect decreases with increasing recalcitrance of the cellulose is counterintuitive. One might expect that cellulose that is more resistant to cellulolytic attack may require more effective cooperation between the cellulase components. The representative recalcitrant cellulose used in this study was created artificially by removing reactive sites to an endocellulase catalytic domain. In reality, through the course of cellulose hydrolysis, the suite of *T. fusca* enzymes may not encounter such a material. However, a decrease in DSE for the binary cellulase mixtures was observed to decrease on untreated BMCC through the time course. Coupled with the lower DSE values observed on PHBMCC, we thus conclude that increasing recalcitrance of the cellulose may in fact contribute to decreasing synergistic effect.

The results of this study reveal that when an exocellulase is mixed with either an endocellulase or a processive endocellulase and reacted with BMCC, synergism is observed in both hydrolysis and binding. In the case of the Cel5A+Cel6B, it was Cel5A that exhibited higher binding, while for Cel6B+Cel9A, it was Cel6B. Thus, the theory that endocellulases increase the availability of free ends for exocellulases to bind is substantiated by the Cel6B +Cel9A results but is negated by the Cel5A+Cel6B results.

Synergistic action on BMCC is coupled to synergistic binding. This was previously reported for equimolar mixtures at higher loadings (*12*) and again confirmed in this study. In binary mixture reactions, overall concentrations on the substrate surface are enhanced. This increase is a result of enhanced binding levels of either one or both of the components in the mixture. Although less prevalent, synergistic binding was also observed on PHBMCC. On the recalcitrant substrate, there appeared to be a competitive binding effect as well. This substrate may have less reactive sites for the catalytic domain, therefore decreasing the overall affinity of the cellulase to the substrate.

This study also revealed that when a "classical" endocellulase and a processive cellulase are mixed and reacted with BMCC, limited synergism is observed in reducing sugar production. However, binding of Cel9A is clearly increased by the presence of Cel5A. For BMCC, DSE values of 1 or less indicated no synergism, whereas for PHBMCC the DSE values are slightly higher. An analysis of the DSB time courses (Figure 6C and D) and the binding time courses (Figures 7B and 8B) revealed that Cel5A created additional sites for Cel9A to bind to BMCC and PHBMCC. This behavior is at odds with the general accepted notion that endo-endo synergism does not occur because endocellulases do not require ends to bind to microcrystalline cellulose; thus, the creation of ends should not enhance the binding of endocellulase. Yet, our results clearly show that the presence of Cel5A enhances the binding of Cel9A, and one must conclude that Cel5A is creating sites for Cel9A binding.

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