Cellulase Digestibility of Pretreated Biomass Is Limited by Cellulose Accessibility

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ABSTRACT: Attempts to correlate the physical and chemical properties of biomass to its susceptibility to enzyme digestion are often inconclusive or contradictory depending on variables such as the type of substrate, the pretreatment conditions and measurement techniques. In this study, we present a direct method for measuring the key factors governing cellulose digestibility in a biomass sample by directly probing cellulase binding and activity using a purified cellobiohydrolase (Cel7A) from Trichoderma reesei. Fluorescence-labeled T. reesei Cel7A was used to assay pretreated corn stover samples and pure cellulosic substrates to identify barriers to accessibility by this important component of cellulase preparations. The results showed cellulose conversion improved when T. reesei Cel7A bound in higher concentrations, indicating that the enzyme had greater access to the substrate. Factors such as the pretreatment severity, drying after pretreatment, and cellulose crystallinity were found to directly impact enzyme accessibility. This study provides direct evidence to support the notion that the best pretreatment schemes for rendering biomass more digestible to cellobiohydrolase enzymes are those that improve access to the cellulose in biomass cell walls, as well as those able to reduce the crystallinity of cell wall cellulose.

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KEYWORDS: dilute-acid pretreatment; pretreated corn stover; Trichoderma reesei; cellobiohydrolase; cellulase accessibility; cellulose crystallinity

Introduction

A key factor for successful enzymatic conversion of biomass to fermentable sugars is the accessibility of the β(1 → 4) glycosidic bonds in cellulose to cellulase enzymes. Pretreatment regimes must be designed to remove substrate specific barriers to cellulosomes to improve cellulose digestion. The precise nature of the obstacles encountered by the cellulosomes in the complex biomass ultra-structure, however, remains ambiguous. Non-cellulosic polymers in biomass that have been cited as barriers to cellulase activity include primarily the hemicelluloses (Irwin et al., 2003; Yang and Wyman, 2004), lignins (Kim et al., 2003; Yang and Wyman, 2004), and esterification to other polymers (Lam et al., 2003). The physical properties of biomass that may also impact enzyme access to cellulose microfibrils include pore structure (Grethlein, 1985) and substrate surface area (Grethlein and Converse, 1991). Pretreatment effects are typically compared on the basis of improved enzyme digestibility and downstream ethanol production. The link between changes in cell wall chemistry/structure and cellulase digestibility is ultimately dependent on improved access to the cellulose microfibril. Accurate and direct assessment of changes in enzyme accessibility, however, is challenging primarily due to the complexities of both the cellulase system and the biomass.

Plant matter-degrading fungi, such as the Ascomycete T. reesei, produce many glycosyl hydrolases of varying specificities (Coutinho and Henrissat, 1999; Foreman et al., 2003). Furthermore, expression levels of each component can vary depending on media composition and growth conditions (Foreman et al., 2003). Commercial cellulase preparations that are commonly used in biomass conversion contain a broad spectrum of enzyme activities that vary depending upon manufacturing conditions and their intended end use, thus the use of whole cellulase
preparations to study changes in cellulose accessibility is analogous to using a probe of unknown specificity to probe an unknown substrate. This study tests a potentially more effective strategy that utilizes a purified cellulase of known substrate specificity to probe changes in biomass due to pretreatment effects. Cellulobiobohydrolases (also called 1,4-β-D-glucan cellobiohydrolases; exoglucanases; CBH; EC 3.2.1.91) are thought to be processive enzymes and initiate activity from the end of the cellulose chains. T. reesei produces CBH I (Cel7A) and CBH II (Cel6A), which act specifically on the reducing and non-reducing chain ends, respectively (Barr et al., 1996). T. reesei Cel7A comprises nearly 60% of the total protein secreted by the fungus (Nummi et al., 1983). Cellulase enzymes produced by T. reesei have been shown to act synergistically to depolymerize cellulose in biomass with Cel7A playing the primary role of de-crystallizing and hydrolyzing cellulose microfibrils to its monomeric unit, cellobiose (Henrissat et al., 1985). T. reesei Cel7A has been shown to be highly effective on recalcitrant cellulose when acting alone, while also significantly enhancing the overall activity of cellulose mixtures (Irwin et al., 1993). The importance of the Cel7A enzyme in the cellulase system suggests that improvements in overall cellulase activity can be achieved by improving its access to cellulose. T. reesei Cel7A is thus an ideal candidate for use as a probe to assess the impact of the physicochemical changes caused by biomass pretreatment on access to the cellulose fraction.

In previous studies, fluorescence-labeled cellulases from Thermobifida fusca were used to monitor changes in bound cellulase concentrations on bacterial cellulose, a pure cellulose 1α-substrate (Jeoh et al., 2002). The concentration of adsorbed cellulase was determined from the fluorescence intensity of the insoluble fraction of the cellulase–cellulose reactions by correlation to standard curves of known enzyme concentrations. It was determined that cellulase did not interfere with the fluorescence measurements at the target emission wavelengths, allowing direct measurements of the bound enzymes on the cellulose fraction. Biomass, however, is known to auto-fluoresce across the fluorescence spectrum complicating the prospect of conducting similar measurements without correcting for its intrinsic fluorescence.

In this study, fluorescence-labeled, purified T. reesei Cel7A was used as a probe to elucidate factors affecting the digestibility of the cellulose in dilute sulfuric acid pretreated corn stover (PCS) samples. Extents of cellulose hydrolysis and the corresponding concentrations of T. reesei Cel7A bound to the substrate are discussed and related to the physical and chemical properties of the samples.

Materials and Methods

Cellulose Samples

Commercially available pure cellulose samples, Whatman No. 1 filter paper (Whatman, Inc., Florham Park, NJ) and Avicel (PH-102) were used in this study.

Pretreated Corn Stover Sample Preparation

Samples used in this study were produced from corn stover collected from Pioneer 34M95 maize harvested in Colorado in 2002. Corn stover aliquots were subjected to thermo-chemical pretreatment in a pilot-scale vertical reactor using a fixed residence time of approximately 1 min at temperatures ranging from 180 to 200°C; solid loadings between 20% and 35% (g g⁻¹); and acid loadings of 0.03–0.06 acid per dry biomass (g g⁻¹) (Schell et al., 2003).

Raw corn stover was milled to 200 mesh using a Wiley mill (Model 5KH39QN9105, General Electric Motors and Industrial Systems), and hydrated overnight with 5 mM sodium acetate buffer, pH 5.0, under vacuum. Sodium azide (0.02%) was added after re-hydration. PCS samples were washed repeatedly with several exchanges of distilled water. In between exchanges, the samples were centrifuged for 15 min at 4,000 rpm. The supernatant was discarded and the pellet resuspended in fresh distilled water. When the pH of the fibers was above 4.0, the supernatant was exchanged with 5 mM sodium acetate buffer pH 5.0 buffer containing 0.02% sodium azide. The concentrations of the PCS suspensions were established by determination of oven-dry weights on triplicate 1.0 mL samples. The samples were dried overnight in a vacuum oven at 70–80°C and transferred to a conventional drying oven at 95°C for 2–3 h before weighing. The final concentrations of the PCS suspensions were used to calculate experimental loadings.

Amorphous Cellulose Sample Preparation

Amorphous cellulose was prepared from Whatman filter paper and Avicel using the procedure developed by Schroeder et al. (1986). Cellulose was dissolved in a dimethylsulfoxide-paraformaldehyde solution, and then regenerated by slow addition of the cellulose solution to a solution of 0.2 M sodium alkoxide in methanol/i-propanol (1:1). This procedure has been demonstrated to produce amorphous cellulose without altering the degree of polymerization (DP) and reducing end group concentration of the starting cellulose. The amorphous cellulose was characterized by solid state NMR (Schroeder et al., 1986).

Quantitating Reducing-End Groups in Cellulose

The concentration of reducing-end groups in the cellulose samples was quantified using the bicinchoninic acid (BCA) method (Kongruang et al., 2004) against a cellobiose standard curve. Assay volumes of 100 µL containing 0.1–1.0 mg mL⁻¹ cellulose were reacted with 400 µL of the reagent and incubated at 80°C for 30 min. The resulting absorbances of the supernatant were measured at 560 nm, after cooling to room temperature, using a SpectraMAX 190 microtiter plate reader (Molecular Devices, Sunnyvale, CA). Cellulose concentrations were adjusted, such that sample optical density (OD) readings corresponded to
concentrations within 0–0.1 mg mL\(^{-1}\); the linear range of the standard curve. Five replicate measurements were made for each sample.

### Cellulose Crystallinity Measurement by Solid State NMR

Cellulose crystallinity was measured on moist materials prepared by pressing the excess moisture out between two paper towels. Rotors were weighed before and after NMR measurements to ensure that the moisture content did not change during the NMR crystallinity measurement. High-resolution, solid-state \(^{13}\)C NMR spectra were collected at 4.7T with cross-polarization (CP) and magic angle spinning (MAS) in a Bruker Avance 200 MHz spectrometer. Variable amplitude cross-polarization was used to minimize the intensity variations of the non-protonated aromatic carbons that are sensitive to Hartmann–Hahn mismatch at higher MAS rotation rates (Peersen et al., 1993). \(^1\)H and \(^{13}\)C fields were matched at 53.6 kHz and a 1 dB ramp was applied to the proton radio frequency field during the matching period. Acquisition time was 0.051 s and the sweep width was 20 kHz. MAS was performed at 6,500 Hz. Twenty thousand scans were averaged using a 2 ms contact time and a pulse repetition rate of 1.0 s. The cellulose crystallinities shown in Table I were estimated from the solid-state NMR spectra by digitally subtracting the amorphous filter paper cellulose spectrum from the spectra of the other cellulose materials. The subtraction was performed to minimize intensity in the peaks assigned to the C4 and C6 carbons in amorphous regions (~84 and ~62 ppm, respectively) without introducing negative peaks into the NMR difference spectrum. The sample % crystallinity was determined from the integrated intensity of the original and difference spectra.

### Enzyme Preparation

*T. reesei* Cel7A was purified from Spezyme CP (Genencor International, Inc., Palo Alto, CA). Spezyme CP was loaded onto a ResourceQ anion exchange column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). After extensive washing with 20 mM Bis-Tris buffer, pH 5.0, bound protein was eluted from the column with a linear gradient of 0–1.0 M sodium acetate buffer containing 1 mM gluconolactone (pH 5.0) until the absorbance reading at 280 nm returned to baseline, indicating the absence of protein in the wash buffer. Bound protein was eluted with 10 mM cellobiose in 100 mM sodium acetate containing 1 mM gluconolactone (pH 5.0). The protein eluted in one peak as indicated by monitoring of absorbance at 280 nm. This peak was pooled, concentrated in the Amicon concentrator with a PM-10 membrane, and examined by SDS–PAGE gel. Gels showed a band corresponding to the target *T. reesei* Cel7A and a faster migrating band corresponding to the apparent molecular weight of the catalytic domain. The pooled sample was spiked with ammonium sulfate to a final concentration of 1.0 M and loaded on to a phenyl-sepharose hydrophobic interaction column (GE Healthcare Bio-Sciences Corp.). The bound protein was eluted with a linearly decreasing ammonium sulfate gradient from 1.0 to 0 M. Fractions with activity on pNP were pooled and concentrated. On SDS–PAGE gel, the concentrated protein solution showed a single band with an apparent molecular weight corresponding to *T. reesei* Cel7A. The final buffer was exchanged with 5 mM sodium acetate pH 5.0 buffer with 100 mM NaCl by size exclusion chromatography (SEC) on a Superose 12 sizing column (GE Healthcare Bio-Sciences Corp.). *T. reesei* Cel7A was labeled with Alexa Fluor 594 succinimidyl esters (Invitrogen Corp., Carlsbad, CA) according to the manufacturer’s recommended protocol. As has been observed with other cellulases (Jeoh et al., 2002), labeling of the *T. reesei* Cel7A did not affect its original activity. Labeled *T. reesei* Cel7A was diluted with unlabeled *T. reesei* Cel7A enzyme for use in the experiments.

### Cellulase Accessibility Experiments

The cellulose accessibility of biomass and the pure cellulose samples was determined using fluorescence-labeled, purified *T. reesei* Cel7A. Triplicate samples (250 μL final volume) containing 1.0 μM *T. reesei* Cel7A with a substrate concentration equivalent to 1.0 mg mL\(^{-1}\) final cellulose on *p*-nitrophenyl lactoside (pNPL) were pooled and concentrated in an Amicon concentrator with a PM-10 membrane (Millipore, Billerica, MA). The protein solution was spiked to a final concentration of 1 mM gluconolactone and loaded on to a *p*-aminophenyl cellulobiose (pAPC) affinity column (Sangeet Hybrid and Penner, 1998). The pAPC column was washed with 100 mM sodium acetate buffer containing 1 mM gluconolactone (pH 5.0) until the absorbance reading at 280 nm returned to baseline, indicating the absence of protein in the wash buffer. Bound protein was eluted with 10 mM cellobiose in 100 mM sodium acetate containing 1 mM gluconolactone (pH 5.0). The protein eluted in one peak as indicated by monitoring of absorbance at 280 nm. This peak was pooled, concentrated in the Amicon concentrator with a PM-10 membrane, and examined by SDS–PAGE gel. Gels showed a band corresponding to the target *T. reesei* Cel7A and a faster migrating band corresponding to the apparent molecular weight of the catalytic domain. The pooled sample was spiked with ammonium sulfate to a final concentration of 1.0 M and loaded on to a phenyl-sepharose hydrophobic interaction column (GE Healthcare Bio-Sciences Corp.). The bound protein was eluted with a linearly decreasing ammonium sulfate gradient from 1.0 to 0 M. Fractions with activity on pNP were pooled and concentrated. On SDS–PAGE gel, the concentrated protein solution showed a single band with an apparent molecular weight corresponding to *T. reesei* Cel7A. The final buffer was exchanged with 5 mM sodium acetate pH 5.0 buffer with 100 mM NaCl by size exclusion chromatography (SEC) on a Superose 12 sizing column (GE Healthcare Bio-Sciences Corp.). *T. reesei* Cel7A was labeled with Alexa Fluor 594 succinimidyl esters (Invitrogen Corp., Billerica, MA) according to the manufacturer’s recommended protocol. As has been observed with other cellulases (Jeoh et al., 2002), labeling of the *T. reesei* Cel7A did not affect its original activity. Labeled *T. reesei* Cel7A was diluted with unlabeled *T. reesei* Cel7A enzyme for use in the experiments.

### Table I. Crystallinities and reducing-end concentrations of pure cellulose samples.

<table>
<thead>
<tr>
<th>Filter paper</th>
<th>Crystallinitya (%)</th>
<th>Reducing-end concentrationb (μmol g(^{-1}))</th>
<th>Regenerated, amorphous form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avicel</td>
<td>61</td>
<td>27.1 (1.5)</td>
<td>28.0 (1.7)</td>
</tr>
<tr>
<td>Filter paper</td>
<td>68</td>
<td>1.3 (0.25)</td>
<td>1.9 (0.50)</td>
</tr>
</tbody>
</table>

aMeasured by solid state NMR.

bMeasured by the BCA method; standard deviation from five replicates in parentheses.
concentration in 5 mM sodium acetate pH 5.0 buffer were prepared for each reaction time assayed throughout a 120 h time course. Reactions were conducted at 38°C, rotating end-over-end and assayed at 1, 4, 24, 48, and 120 h. Each reaction was initiated by the addition of enzyme and terminated by filtration in a 96-well vacuum filter manifold (Innovative Microplate, Chicopee, MA) equipped with a 1.0 μm glass fiber filter. The reaction supernatant was assayed for reducing sugars using the BCA method (Doner and Irwin, 1992) against a cellobiose standard curve. The solid fraction retained in the filter was assayed for bound T. reesei Cel7A concentration.

Bound Enzyme Quantitation

The concentration of bound enzyme on the solids fraction from the accessibility experiments was assayed by fluorometry with adjustments for biomass autofluorescence. Following filtration of the reaction samples, the retained solids (containing PCS + bound T. reesei Cel7A) were re-suspended with 250 μL of distilled water. For each sample, 150 μL of the resuspended solids were transferred to a microtiter plate and read in a FLUOstar optima plate reader (BMG Labtechnologies, Durham, NC) at excitation and emission wavelengths of 584 and 612 nm, respectively. The emission intensities from the samples were converted to concentrations of T. reesei Cel7A using regression parameters from a standard curve of calibration standards that were measured concurrently. To negate the autofluorescence of each of the PCS, a separate calibration was made for each PCS sample digested with Cel7A. The calibration curves contained six levels of standard additions (0–1 μM T. reesei Cel7A) with the same concentration of PCS as used in each of the accessibility experiments. To negate the effects of plate-to-plate or day-to-day variations in the fluorescence measurements, a fresh set of calibration standards (in triplicate, with the appropriate PCS sample) was included with each microtiter plate containing unknown samples from the reactions.

The effect of digestion on the correction of autofluorescence in the calibration standards was examined as follows. Fifteen replicates of a PCS sample were digested to 67 ± 9% by unlabeled T. reesei Cel7A in 5-days, using the conditions described above for the cellulase accessibility experiments. The reactions were terminated by filtration and the solids fractions re-suspended in 125 μL of distilled water. The re-suspended solids were transferred to a microtiter plate, with 75 μL from each replicate pipetted into each well. Standard additions of fluorescence-labeled T. reesei Cel7A including five levels ranging from 0.12 to 2 μM were prepared. Each level was pipetted in triplicates (75 μL per replicate), to the wells containing digested PCS. Final concentrations of T. reesei Cel7A with the digested PCS were 1, 0.5, 0.25, 0.125, and 0.0625 μM. Calibration standards with the same final T. reesei concentrations were then prepared in the same microtiter plate, using undigested PCS. The plate was read in the fluorometer as described in the previous paragraph. The concentrations of T. reesei Cel7A with the digested PCS were determined using regression parameters from the standard curve developed using the undigested PCS. These values were compared to the expected values to determine the effect of extensive digestion on the quantitation method.

Results

Measuring Bound Enzyme Concentrations on Biomass

It has previously been demonstrated that pure cellulose does not contribute to or quench the signal of fluorescence-labeled enzyme allowing for its direct measurement on the solids fraction (Jervis et al., 1997; Jeoh et al., 2002). Applying this method to corn stover was complicated by the intrinsic fluorescence of plant cell wall constituents such as lignin and various extractives, and necessitated modifications in the procedure. We found that the presence of PCS changes the slope of the standard curve used for quantitation of the fluorescence intensity of labeled T. reesei Cel7A (Fig. 1A). Furthermore, we found that PCS samples pretreated to different extents affected the slope of the standard curve to varying degrees (data not shown). To adjust for autofluorescence effects, T. reesei Cel7A concentrations were determined in the presence of PCS (Fig. 1B). The standard curves were prepared using the corresponding PCS sample (Fig. 1A). As shown in Figure 1B, accurate determination of the concentrations was achieved both in the presence of PCS and without PCS, when the correct standard curve was used.

Since cellulose should not contribute to biomass fluorescence, its removal by enzyme digestion should not affect the overall autofluorescence of biomass. This assumption was verified by testing various concentrations of T. reesei Cel7A in the presence of pre-digested PCS samples. Figure 2 shows the experimentally determined enzyme concentrations with respect to the actual concentrations. Below a concentration of 0.5 μM there was only a minimal deviation in the measured concentration from the actual concentration. We did not observe bound T. reesei Cel7A concentrations greater than 0.5 μM on the PCS samples, thus we conclude that this method is valid for the data reported in this study.

The accuracy of the enzyme determination method was verified by measuring T. reesei Cel7A concentrations on the solids fraction and the liquid fraction (reaction supernatant) after terminating a hydrolysis reaction. In these experiments, two lessons were learned; (1) the glass fiber filter used to separate the soluble and insoluble fractions of the reaction mixture can trap free enzyme, thus the supernatant must be pipetted directly from centrifuged samples and (2) PCS extractives in the supernatant of the reaction interfere with the emission of the fluorescence-labeled T. reesei Cel7A enzymes in a manner similar to that of the biomass solids. A T. reesei Cel7A standard curve with an equivalent biomass supernatant concentration must be constructed for accurate determination of T. reesei Cel7A concentrations in the
reaction supernatant. Interestingly, a *T. reesei* Cel7A standard curve in the presence of PCS supernatant overlays a similar standard curve measured in the presence of solid biomass (Fig. 3A). A reaction sample where the solids and the supernatant fractions are determined separately for fluorescence-labeled *T. reesei* Cel7A concentration yields nearly complete mass closure of Cel7A (Fig. 3B). Direct and accurate measurements of *T. reesei* Cel7A bound on biomass can be accomplished with the standard curves for determining bound enzyme concentrations established in the presence of an equivalent concentration of biomass, as in the reaction mixtures.

The Effect of Dilute-Acid Pretreatment on Accessibility of Corn Stover

The effects of pretreatment severity on cellulose digestibility and cellulase binding were studied on a set of PCS samples prepared with a pilot-scale vertical reactor located at the National Renewable Energy Laboratory (Golden, CO) (Schell et al., 2003). The PCS samples were prepared at

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Figure 1. The effect of determining *T. reesei* Cel7A concentrations in the presence of dilute-sulfuric acid pretreated corn stover (PCS). A: Standard curves relating known Alexa Fluor 590 labeled *T. reesei* Cel7A concentrations to fluorescence emission intensities, with and without PCS. B: Determination of “unknown” fluorescence-labeled *T. reesei* Cel7A concentrations with and without PCS using the corresponding standard curve in (A).

Figure 2. Determination of fluorescence-labeled *T. reesei* Cel7A with PCS that was pre-digested for 5-days (87% cellulose conversion).

Figure 3. A: Standard curves of fluorescence-labeled *T. reesei* Cel7A in the presence of PCS and in the presence of PCS supernatant. B: Sum of the enzyme concentrations in a single sample measured in the supernatant and on the solid fraction separately.
various pretreatment severities by changing either temperature or acid concentration. This resulted in samples with residual xylan contents in the range of 2.1–14.1% (Fig. 4). These same samples had a measured average lignin content of 30.8 ± 3.0% (Fig. 4).

The digestibility of the cellulose fraction in the corn stover samples by *T. reesei* Cel7A was improved by dilute-acid pretreatment (Fig. 5A). Up to about 8% xylan content, which corresponds to about 80% xylan removal, decreasing xylan content appeared to improve the extent of cellulose conversion, measured after 5-days of digestion (Fig. 5A). The removal of greater than 80% of the xylan did not significantly improve digestibility by this purified enzyme, indicating that xylan content is not the only factor affecting the digestibility of PCS. Samples with reduced xylan contents showed increased bound concentrations of *T. reesei* Cel7A at 1 h (Fig. 5B). Over a 5-day time course, the bound *T. reesei* Cel7A concentration typically showed maximum binding early in the reaction followed by a gradual decrease (examples shown in Fig. 6A). Bound *T. reesei* Cel7A concentrations at the earliest time provided the clearest indication of the effect of the change in initial xylan concentration on Cel7A accessibility.

**The Effect of Drying on Accessibility**

Two dilute-acid pretreated corn stover samples, one pretreated at high severity, (with a remaining xylan content of 2.1%) and the other pretreated at a significantly lower severity (with a remaining xylan content of 10.4%), were air-dried and re-hydrated, then assayed for accessibility to *T. reesei* Cel7A. The hydrolysis curves show that the digestibilities of the samples substantially decreased as a result of air-drying (Fig. 6B). Importantly, although the digestibilities of the two samples were very different originally, the hydrolysis profiles became very similar after drying.

Although the reason for the decrease in the digestibility of dried biomass is often attributed to decreased accessibility of the enzymes to the cellulose microfibrils, there has been no direct evidence to support or disprove this theory. The binding measurements of *T. reesei* Cel7A during the hydrolysis showed decreased levels of bound Cel7A in the air-dried samples, confirming the notion that cellulase efficacy is limited by reduced access to the cellulose fraction (Fig. 6A). As was observed for the digestibility of these samples, the large differences in bound enzyme concentrations on the original material disappears after drying, with very similar adsorption profiles for the two dried samples.

**The Effect of Cellulose Crystallinity on Accessibility**

The crystallinity of cellulose has long been postulated to play a role in its susceptibility to enzyme digestion (Fan et al., 1980b). To determine the effect of cellulose crystallinity on cellulase accessibility, “amorphous” cellulose...
samples were prepared from two pure cellulose samples with relatively high crystallinities (Table I). The concentrations of reducing-ends were conserved in the amorphous forms, verifying that the celluloses were not hydrolyzed by the regeneration protocol (Table I).

The amorphous forms of the celluloses were found to be significantly more digestible than the original forms (Fig. 7). A close look at the time course data in the first 24 h of the hydrolysis reactions provides some insights that could help to explain the observed improvements in the digestibilities of the cellulose samples (Fig. 7). Specifically, the bound T. reesei Cel7A concentrations on the amorphous forms of both filter paper and Avicel were significantly higher than on the crystalline forms (Fig. 7B and D). The maximum extents of binding on the amorphous forms increased on the order of 15-fold over that of the original forms. The change in crystallinity of the cellulose samples, therefore, may have allowed increased access to T. reesei Cel7A. One could thus infer that the increased access may have contributed to the increased cellulose hydrolysis rates observed in Figure 7.

Discussion

Cellulase-cellulose interactions occur at a liquid–solid interface, requiring a physical adsorption of a cellulase enzyme to its substrate prior to the hydrolysis reaction. The extent to which cellulases can adsorb to cellulose within a lignocellulosic substrate should provide some indication of digestibility. Previous studies have observed correlations between cellulose hydrolysis rates and bound enzyme concentrations (Converse et al., 1990; Ooshima et al., 1991). However, earlier work examining the relationship between bound cellulase and substrate digestibility have been limited by the complexity of the cellulase system (Converse et al., 1990; Ooshima et al., 1990). More recently, advances in cloning technologies and purification procedures have allowed the use of purified enzymes to distinguish between enzyme and substrate effects in the experiments (Eriksson et al., 2002; Palonen et al., 2004).

Additionally, determining bound enzyme fractions in the reactions has become more direct and quantitative, using known extinction coefficients of the well-characterized and carefully purified enzyme components instead of indirect activity based (Meunier-Goddik et al., 1999) or colorimetric methods (Ooshima et al., 1991). However, determining the bound enzyme fraction on biomass is further complicated by the absorbance contributions of various soluble components in biomass (Ucar and Fengel, 1988). One strategy for determining purified enzyme concentrations bound to biomass is to use purified cellulases labeled in vitro with a distinguishing tag (Eriksson et al., 2002; Palonen et al., 2004). The method described in this study employs this strategy, whereby the T. reesei Cel7A enzyme was labeled with a fluorescence tag, and a standard curve was used to correlate measured fluorescence intensities to Cel7A concentrations. This method has been successfully used to determine bound cellulases on a pure cellulose substrate (Jeoh et al., 2002), but becomes problematic with biomass substrates due to the inherent autofluorescence of the plant materials. This problem was solved by developing the standard curves with the addition of equivalent concentrations of biomass to the cellulase standards. Fluorescence-labeled cellulase concentrations bound to biomass can be accurately determined as long as the standard curve is also established in the presence of the same biomass as used in the assay. During the development of this method, one key observation was made: the glass fiber filter used to separate the solid and liquid fractions of the reaction can trap cellulases in the filter. This observation has also been made with nylon membranes. Therefore, measuring cellulase concentration in the filtrate can result in underestimation of the unbound enzyme fraction and consequently, overestimation of the bound enzyme fraction.

With the purified, fluorescence-labeled T. reesei Cel7A as a probe, we sought to determine the mechanism by which dilute-sulfuric acid pretreatment improves the enzymatic digestibility of biomass. Increases in cellulose digestibilities have been associated with decreased xylan content.
As was also observed in this study, the primary mechanism of dilute-acid pretreatment of corn stover is the hydrolysis and subsequent dissolution of the hemicellulose components (Schell et al., 2003; Yang and Wyman, 2004). In the set of dilute-sulfuric acid pretreated samples studied here, it was shown that the PCS samples had both higher initial T. reesei Cel7A binding capacity and improved digestibilities than the unpretreated corn stover. One interpretation of these data is that removing xylan during pretreatment facilitated cellulase access to the cellulose, which resulted in increased digestibility (Fig. 5). This concept has been discussed in the literature as relating changes in the porosity of the biomass sample to digestibility (Esteghlalian et al., 2001; Grethlein, 1985). NMR measurements conducted on these samples showed that dilute acid pretreatment increased the porosity in corn stover (Ishizawa et al., 2007), suggesting that the observed increase in T. reesei Cel7A access to the cellulose in PCS is due to improved physical access within the biomass structure. However, no measurable differences in porosity were found between these PCS samples (Ishizawa et al., 2007). Sample porosity, therefore, cannot explain the observed differences in T. reesei Cel7A accessibility between differentially pretreated corn stover samples. The DP of cellulose has also been thought to affect digestibility (Knappert et al., 1980; Ucar and Fengel, 1988). The DP of the cellulose in the PCS samples used in this study was not measured; however, acid hydrolysis pretreatment has been shown to decrease cellulose DP (Ucar and Fengel, 1988). One possible explanation for improved digestibilities on severely pretreated samples could be due to a decrease in cellulose DP.

The opposite of “opening up” the structure of biomass to increase cellulase access is to collapse the structure to limit access, which is thought to occur when a biomass sample is dried (Esteghlalian et al., 2001; Grous et al., 1986). The water content in biomass affects the degree of swelling (Browning, 1975), the crystallinity (Fan et al., 1981) and the digestibility (Focher et al., 1981) of cellulose microfibrils. Air-drying of biomass samples is thought to irreversibly collapse the capillary structure (Esteghlalian et al., 2001; Weise, 1998), significantly decreasing internal surface area (Stone and Scallan, 1968; Browning, 1975). Consequently, biomass sample drying is generally understood to negatively affect the enzymatic digestibility of the cellulose fraction in biomass by limiting enzyme access (Esteghlalian et al., 2001; Fan et al., 1980a; Grous et al., 1986). The effect of drying on pulp is described as “hornification,” where the complete removal of moisture allows adjacent cellulose fibers to form hydrogen bonds (Weise, 1998). In this study, drying of the PCS samples was observed to limit the access of T. reesei Cel7A and decrease cellulose digestibility, suggesting that a mechanism similar to hornification may occur even in the presence of other cell wall constituents. We note that two samples, the severely pretreated sample with 2.1% xylan content and the lightly pretreated sample with 10.4% xylan

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**Figure 7.** Twenty-four hour time course data for the extents of hydrolysis and bound T. reesei Cel7A concentrations (plotted as μmol of bound Cel7A per gram of remaining cellulose) on original and amorphous forms of (A and B) filter paper; and (C and D) Avicel.

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Knappert et al., 1980; Yang and Wyman, 2004), as was also observed in this study. The primary mechanism of dilute-acid pretreatment of corn stover is the hydrolysis and subsequent dissolution of the hemicellulose components (Schell et al., 2003; Yang and Wyman, 2004). In the set of dilute-sulfuric acid pretreated samples studied here, it was shown that the PCS samples had both higher initial T. reesei Cel7A binding capacity and improved digestibilities than the unpretreated corn stover. One interpretation of these data is that removing xylan during pretreatment facilitated cellulase access to the cellulose, which resulted in increased digestibility (Fig. 5). This concept has been discussed in the literature as relating changes in the porosity of the biomass sample to digestibility (Esteghlalian et al., 2001; Grethlein, 1985). NMR measurements conducted on these samples showed that dilute acid pretreatment increased the porosity in corn stover (Ishizawa et al., 2007), suggesting that the observed increase in T. reesei Cel7A access to the cellulose in PCS is due to improved physical access within the biomass structure. However, no measurable differences in porosity were found between these PCS samples (Ishizawa et al., 2007). Sample porosity, therefore, cannot explain the observed differences in T. reesei Cel7A accessibility between differentially pretreated corn stover samples. The DP of cellulose has also been thought to affect digestibility (Knappert et al., 1980; Ucar and Fengel, 1988). The DP of the cellulose in the PCS samples used in this study was not measured; however, acid hydrolysis pretreatment has been shown to decrease cellulose DP (Ucar and Fengel, 1988). One possible explanation for improved digestibilities on severely pretreated samples could be due to a decrease in cellulose DP.

The opposite of “opening up” the structure of biomass to increase cellulase access is to collapse the structure to limit access, which is thought to occur when a biomass sample is dried (Esteghlalian et al., 2001; Grous et al., 1986). The water content in biomass affects the degree of swelling (Browning, 1975), the crystallinity (Fan et al., 1981) and the digestibility (Focher et al., 1981) of cellulose microfibrils. Air-drying of biomass samples is thought to irreversibly collapse the capillary structure (Esteghlalian et al., 2001; Weise, 1998), significantly decreasing internal surface area (Stone and Scallan, 1968; Browning, 1975). Consequently, biomass sample drying is generally understood to negatively affect the enzymatic digestibility of the cellulose fraction in biomass by limiting enzyme access (Esteghlalian et al., 2001; Fan et al., 1980a; Grous et al., 1986). The effect of drying on pulp is described as “hornification,” where the complete removal of moisture allows adjacent cellulose fibers to form hydrogen bonds (Weise, 1998). In this study, drying of the PCS samples was observed to limit the access of T. reesei Cel7A and decrease cellulose digestibility, suggesting that a mechanism similar to hornification may occur even in the presence of other cell wall constituents. We note that two samples, the severely pretreated sample with 2.1% xylan content and the lightly pretreated sample with 10.4% xylan
content, became indistinguishable both in terms of digestibility and *T. reesei* Cel7A adsorption profile after drying.

The accessibility of enzymes to cellulose in biomass also appears to be affected by the crystallinity of the cellulose. *T. reesei* Cel7A binds specifically to the reducing-ends of cellulose and must engage a single cellodextrin molecule within its tunnel-shaped active site to initiate hydrolysis (Divne et al., 1998). We observed increased enzyme accessibility from the crystalline cellulose form to the regenerated, amorphous cellulose form as shown by higher levels of bound *T. reesei* Cel7A and higher digestion rates (Fig. 7). We suggest that making the substrate more amorphous improved access to the reducing-ends of cellulose by *T. reesei* Cel7A, thus contributing to the increased hydrolysis rates. Also, action on an amorphous cellulose substrate does not require that the enzyme also decrystallize the targeted cellodextrin from the cellulose microcrystallite. Although a subset of the bound *T. reesei* Cel7A population could be non-productively bound, we cannot distinguish this in our data. A separate experiment using twice the substrate loading (2.0 mg mL$^{-1}$ cellulose) gave the same initial extents of hydrolysis and bound *T. reesei* Cel7A concentrations as shown in Figure 7, demonstrating that at 1.0 mg mL$^{-1}$, the system was below saturating conditions (data not shown). Therefore, despite a 15-fold difference in reducing-end concentrations between the two amorphous cellulose samples, no differences in hydrolysis rates or bound enzyme concentrations were observed. A comparison of 13C CP/MAS NMR spectra of the amorphous cellulose in a dry and hydrated state (Fig. 8) indicates that the amorphous cellulose is slightly more ordered in the hydrated state. The peaks for all the carbons sharpen upon addition of water. Close inspection of the peak carbons shows the formation of two peaks in the region assigned to amorphous C4 carbons (~84 ppm). The appearance of these two peaks suggests that there may be some ordering of the cellulose during the regeneration procedure or a re-ordering of the cellulose during the precipitation/hydration steps. When the amorphous Avicel sample was re-dissolved in paraformaldehyde/DMSO and regenerated for a second time by the same protocol, its digestibility by *T. reesei* Cel7A did not change, thus verifying that the cellulose ordering observed by NMR was not due to incomplete regeneration of the original sample (data not shown).

The cellulose digestion experiments in this study using purified *T. reesei* Cel7A did not yield 100% cellulose conversion. Although there are several factors that may have prevented complete cellulose conversion, one limitation may be the absence of synergistic cellulase enzymes, such as the endoglucanases and β-glucosidases typically co-secreted by the fungus. Additional experiments have shown that adding an endoglucanase to the reactions can result in complete cellulose hydrolysis (Baker et al., 1995; Baker et al., 1998). More detailed investigations using binary cellulase systems are currently underway.

![Image](image-url)

Figure 8. 13C CP/MAS spectra of (A) dry regenerated filter paper cellulose. B: Hydrated regenerated filter paper cellulose.

Conclusions

Probing field dried, dilute acid pretreated corn stover samples with a fluorescence-labeled, purified *T. reesei* Cel7A showed that increased enzymatic digestibility of biomass could be achieved by removing the hemicellulose fraction, which is thought to act as a barrier preventing access to cellulose. The relative importance of sample history was shown by studies of enzyme accessibility to cellulose by digesting dried and corresponding amorphous cellulose samples. Thus, it may be concluded that a pretreatment that removes hemicellulose to allow more cellulase-cellulose interaction, coupled with a means to physically disrupt the crystallinity of the cellulose structure, would greatly improve the enzyme digestibility of the biomass sample.

Measuring changes in physical or chemical properties such as porosity, surface area, and the chemical composition of biomass are indirect means of assessing the impact of pretreatment on the enzymatic digestibility of a biomass sample. Empirical correlations obtained from such measurements often depend on the nature and history of the substrate or the measurement methods, resulting in conflicting conclusions from different research groups. Based on this study, we have identified two important questions to ask in determining the effect of pretreatment on biomass: (1) whether or not the cellulose fraction is made more accessible to the cellulase enzymes and (2) whether or not the cellulose itself has been rendered into a more digestible morphology. Increasing surface area or porosity, or changing the chemistry of biomass, may have little or no
impact on enzyme digestibility unless these criteria are met. We found that these questions can be addressed effectively by directly probing the biomass structure with purified cellulase enzymes.

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References


