Enzyme-Catalyzed Saccharification of Model Celluloses in the Presence of Lignacious Residues

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Experiments were designed to determine the relevance of enzyme partitioning, between the cellulose and non-cellulose components of pretreated biomass, with respect to rates of cellulose saccharification in a typical biomass-to-ethanol process. The experimental system included three cellulose preparations (differing in physicochemical properties): a representative lignin-rich noncellulosic residue (prepared from dilute acid-pretreated switchgrass), an acid-extracted lignin preparation, and a complete *Trichoderma reesei* cellulase preparation. Enzyme–reactor conditions were typical of those commonly used in biomass-to-ethanol studies. The results were found to be dependent on both the lignin and cellulose preparations used. The noncellulosic lignacious residue, when supplemented at up to 40% (w/w) in cellulose–cellulase reaction mixtures, had little effect on rates and extents of cellulose saccharification. Overall, the results suggest that enzyme partitioning between cellulose and the noncellulosic component of a pretreated feedstock is not likely to have a major impact on cellulose saccharification in typical biomass-to-ethanol processes.

INTRODUCTION

Saccharification of the cellulose and hemicellulose in biomass results in sugar-rich liquid streams useful for the production of a variety of value-added products, including ethanol, furfural, and various functional biopolymers (Fuller et al., 1996). A saccharification process that has received considerable attention involves the dilute acid-catalyzed hydrolysis of the hemicelluloses, followed by the cellulase-catalyzed hydrolysis of the cellulose (McMillan, 1994). The dilute acid operation, referred to as the "pretreatment", is designed to optimize sugar yields from hemicellulose while rendering the cellulose more susceptible to enzymatic hydrolysis (Pinto and Kamden, 1996; Esteghlalian et al., 1997; Converse et al., 1990). The enzymatic susceptibility of the cellulose component of pretreated biomass is affected by many parameters, but one of the more important of these appears to be the lignin fraction of the biomass. Numerous studies have found an inverse correlation between measured lignin contents and cellulose saccharification rate (Yuldashev et al., 1993; Kong et al., 1992; Pinto and Kamden, 1996; Vinzant et al., 1997; Barl et al., 1991), and delignification has been shown to significantly increase rates of cellulose saccharification (Baker, 1973; Schwald et al., 1988). Hence, it is generally accepted that the presence of lignin is associated with reduced rates of biomass cellulose saccharification (Coughlan, 1991).

The presence of lignin in a cellulase–cellulose reaction mixture is often assumed to decrease the quantity of enzyme associated with cellulose due to nonspecific adsorption of enzyme to lignin (Bernandez et al., 1993; Chernoglazov et al., 1988; Excoffier et al., 1991). This assumption is the basis for kinetic models that contain

terms for lignin-bound enzyme (Philippidis et al., 1993b; Ooshima et al., 1990; South et al., 1995). It has also been suggested that the negative effect of lignin is due to steric hindrance (Mooney et al., 1998; Yuldashev et al., 1993). Steric hindrance occurs when lignin encapsulates the cellulose component and makes it less accessible to enzyme catalysts (Fan, 1980).

The objective of this study was to determine the relevance of enzyme partitioning between the cellulose and noncellulose components of pretreated biomass, with respect to rates of cellulose saccharification under traditional saccharification conditions. The noncellulosic component of most interest in this context was lignin. The experimental system included representative cellulose substrates, covering a range of physicochemical properties, and a lignacious residue (LR) prepared by enzyme digestion of the cellulose component of dilute acid-pretreated switchgrass. The experimental design, using physically distinct celluloses and LRs, allowed us to test the relevance of enzyme partitioning in the absence of the confounding steric constraints that are imposed on cellulose accessibility due to the lignin fraction of intact lignocellulosics.

Previous experiments have demonstrated that the addition of chemically purified lignins to cellulase saccharification systems can significantly reduce rates of cellulose hydrolysis (Excoffier et al., 1991; Chernoglazov et al., 1988; Sewalt et al., 1997). However, the lignins used in those studies may not be representative of the lignin component of pretreated biomass, the chemical and physical properties of which depend on both the pretreatment to which the lignin has been exposed and the method of purification (Glasser et al., 1983; Ooshima et al., 1990). It has been suggested that the lignin component of lignocellulosic materials is best represented by the lignin-rich fraction remaining following extensive enzyme saccharification of the cellulose plus hemicellulose components (Chang et al., 1975;

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Table 1. Composition of Native Switchgrass, Pretreated Switchgrass, LR, and KL

% composition	native switchgrass	pretreated switchgrass[a]	LR[b]	KL
total glycan	56.85	54.0	NA[c]	NA
glucan	31.3	50.0	5.3	ND[d]
xylan	20.56	3.5	NA	NA
galactan	1.86	0.3	NA	NA
arabinan	3.13	0.2	NA	NA
mannan	ND	ND	NA	NA
Klason lignin	21.37	34.55	73	85.4
ASL[e]	3.37	1.5	2.1	ND
ash	7.1	6.8	12.1	14.6

[a] Pretreated at 180 °C, 1.2% H_2SO_4, 1 min. [b] Enzymatically prepared lignacious residue. [c] Not applicable. [d] None detected. [e] Acid soluble lignin.

Crawford, 1981). At a minimum, this residue would reflect the noncellulosic material, the majority of which is lignin, actually present in biomass-to-ethanol reactors. Therefore, enzymatically prepared LR was used in this study. A chemically prepared lignin (analogous to Klason lignin, KL) was also included for comparative purposes. The results of the study suggest that enzyme partitioning between the cellulose and noncellulose components of pretreated switchgrass has but a minimal effect on cellulose saccharification.

MATERIALS AND METHODS

Celluloses. Microcrystalline cellulose (MCC-a, Avicel PH 101) was obtained commercially (FMC, Philadelphia, PA). Amorphous cellulose (AC) was produced from MCC-a according to the method of Isogai and Atalla (1991) using an SO_2–diethylamine–DMSO solvent for cellulose dissolution. Bacterial microcrystalline cellulose (BMCC) was prepared with cultures of *Acetobacter xylinum*. Cultures were incubated in trays (Hestrin, 1963) for 10 days at 30 °C without shaking. Surface layers of cellulose were harvested and purified according to the method of Gilkes et al. (1992). All three cellulose preparations (MCC-a, BMCC, AC) were found to be >96% glucan on the basis of glucose yields following acid hydrolysis (Ehrman, 1992).

Lignins. Lignins were prepared from switchgrass obtained from the National Renewable Energy Laboratory (Golden, CO) (see Table 1 for composition). Switchgrass was dilute acid pretreated in a batch reactor (180 °C, 0.9% H_2SO_4, 5 min) (Esteghlalian et al., 1997) and then water washed to neutral pH. Cellulose was removed from the resulting residue, which was ~61% cellulose, by treatment with *Trichoderma reesei* cellulases (Environmental Biotechnology Inc., Menlo Park, CA) at a concentration of 60 FPU/g of cellulose. Cellulase treatments were done with rotational inversion in a 50 °C incubator for 7 days (Torget, 1993). Following the initial cellulase treatment, the switchgrass residue was washed and again incubated with fresh cellulase (60 FPU/g of original cellulose) for an additional 3 days. Following this enzyme treatment samples were washed with 6 M urea or 3% sodium dodecyl sulfate (SDS) at 40 °C for removal of associated protein (Reese, 1982; Otter et al., 1989). Desorbants were removed through subsequent water washes. The resulting residue (LR) was stored at 4 °C. Compositional analyses were done according to the methods of Magill (1992a,b; KL and acid soluble lignin) and Ehrman (1994; ash and total glucan).

Chemically prepared lignin (essentially the same as KL) was produced by two-stage sulfuric acid treatment (Magill, 1992b) of the pretreated switchgrass. KL was water washed to neutral pH and stored at 4 °C.

Cellulase Saccharification. *T. reesei* cellulases (Environmental Biotechnology Inc.) (94.3 FPU/mL, 91.6 ρ-NPGU/mL) were added to each cellulose in concentrations corresponding to 0.5K. K is here defined as the total protein concentration that leads to half-saturation of the cellulose surface, as measured in adsorption isotherm experiments. Total protein additions (0.5K) were 82, 468, and 1120 mg/g for MCC-a, BMCC, and AC, respectively. Reaction conditions were 1% (MCC-a, AC) or 0.5% (BMCC) cellulose in 50 mM sodium citrate (pH 5) at 37 °C; this temperature corresponds to that used in simultaneous saccharification and fermentation protocols (Philippidis et al., 1993a). The lower substrate concentration for BMCC was necessary due to its higher water binding capacity. EL and KL were added to selected reaction mixtures in quantities corresponding to 10 and 40% of total solids. The 40% addition corresponds to the amount of noncellulosic material in a reaction mixture containing 1% (w/v) pretreated switchgrass. Reactions were initiated by the addition of cellulase resulting in total reaction volumes of 1.0 mL. Reactions were terminated at 5, 15, 30, and 60 min and 24 h by centrifugation at 14 000 rpm and removal of supernatant. Initial time points were taken after rapid hand mixing ($\cong$10 s). Reaction mixture supernatants were analyzed for product using a glucose oxidase peroxidase method (Sigma Chemical Co., St. Louis, MO). Early time point samples of AC-based reaction mixtures contained significant amounts of cellobiose and thus were treated with β-glucosidase (Novozym 188, Novo Nordisk, Danbury, CT) before glucose analysis.

Protein Adsorption. Adsorbed protein was calculated as the difference in soluble protein between control (without substrate) and sample (with substrate) reaction mixtures. In reaction mixtures containing cellulose only, soluble protein was measured spectrophotometrically by absorbance at 280 nm using BSA as the reference standard. Protein determination by measurement of absorbance at 280 nm is incompatible with reaction mixtures containing lignin residues. Therefore, soluble protein in reaction mixtures containing lignin was measured by using the Bradford dye binding assay (Bio-Rad, Hercules, CA) using a cellulase color yield of 0.29 $(mg/mL)^{-1}(cm)^{-1}$. Adsorption isotherms were obtained by exposing MCC-a, BMCC, and AC to increasing protein concentrations for 20, 10, and 0.5 min, respectively, which correspond to the shortest time required for maximum adsorption (chosen to minimize the effect of hydrolysis). Adsorbed protein was calculated per gram of residual cellulose. Other reaction conditions were as described for cellulase saccharification.

RESULTS AND DISCUSSION

Saccharification in Model Reaction Mixtures. Reaction time courses show that the addition of LR to reaction mixtures containing AC and BMCC substrates had essentially no effect on the initial phase of hydrolysis (Figure 1b,c). The rate of hydrolysis of MCC-a was only slightly lower in the presence of relatively high concentrations (40%) of LR (Figure 1a). LR had no significant effect on percent converted at 24 h for any of the substrates (Table 2). These results are contrary to those obtained by others using more refined lignin preparations. Sewalt et al. (1997) found a 70% reduction and Excoffier et al. (1991) found a 24% reduction in the extent of hydrolysis after 24 h using 15 and 25% lignin, respectively.

In contrast to LR, the addition of KL to reaction mixtures clearly lowered the extent of MCC-a and BMCC saccharification during the early phase of the reaction (Figure 2a,b). AC hydrolysis remained unaltered following KL addition (Figure 2c). The biggest effect was with respect to MCC-a hydrolysis, which decreased by 18 and 67% (after 60 min of hydrolysis) following the addition of 10% KL and 40% KL, respectively. After 24 h, the addition of 10% KL and 40% KL led to 14 and 39% decreases in the extents of MCC-a hydrolysis, respectively. Addition of 10% KL to BMCC-based reaction mixtures had no detectable effect on the extent of saccharification, whereas decreases in the extent of BMCC hydrolysis (~25% after 60 min; ~8%
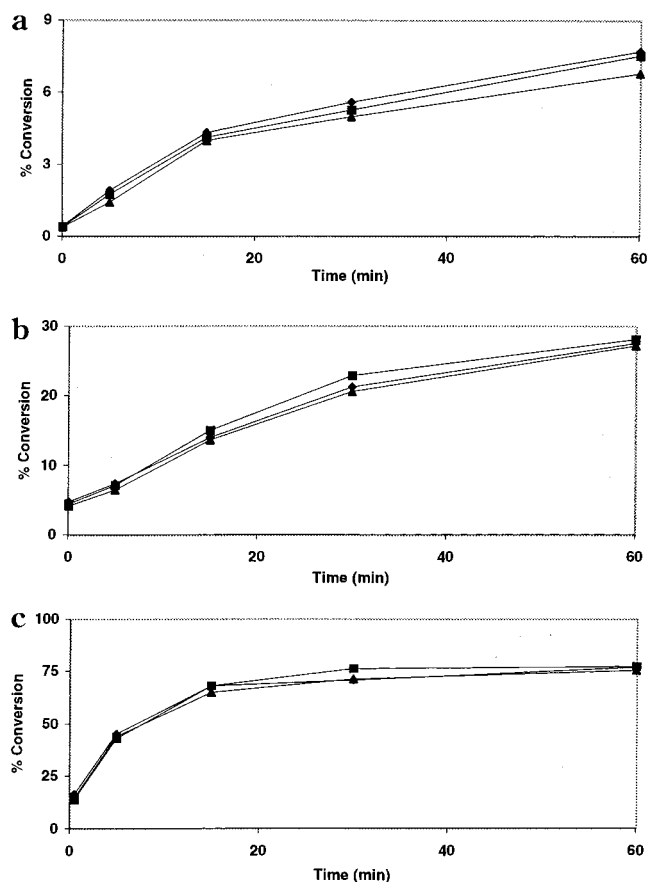
Figure 1. Early stage hydrolysis of three celluloses in the presence of LR: (a) (◆) MCC-a, (■) MCC-a + 10% LR, (▲) MCC-a + 40% LR; (b) (◆) BMCC, (■) BMCC + 10% LR, (▲) BMCC + 40% LR; (c) (◆) AC, (■) AC + 10% LR, (▲) AC + 40% LR. Experiments were performed in 50 mM sodium citrate (pH 5.0) at 37 °C and a protein concentration of $0.5K_A$. Substrate concentrations were 1% for MCC-a and AC and 0.5% for BMCC.

Table 2. Effect of Lignacious Residues on Extent of Cellulose Conversion at 24 h

cellulose	lignacious residue	% conversion	% relative inhibition
MCC-a[a]		36 (0.23)[b]	0
MCC-a	10% LR[c]	35 (0.47)	3
MCC-a	40% LR	36 (0.93)	0
MCC-a	10% KL	31 (0.47)	14
MCC-a	40% KL	22 (0.58)	39
BMCC		78 (1.2)	0
BMCC	10% LR	78 (0.97)	0
BMCC	40% LR	81 (2.2)	0
BMCC	10% KL	78 (2.5)	0
BMCC	40% KL	72 (2.0)	8
AC		99 (5.6)	0
AC	10% LR	101 (0.49)	0
AC	40% LR	98 (2.9)	1
AC	10% KL	97 (0.69)	2
AC	40% KL	99 (1.7)	0

[a] Avicel PH 101. [b] Standard deviation of two replicates. [c] Enzymatically prepared lignacious residue.

after 24 h) were observed when KL addition was increased to 40%. For comparative purposes, other studies of this type (Sewalt et al., 1997) appear to have used cellulose preparations most similar to MCC-a.

Two LR preparations were tested in these experiments. The preparations differed with respect to the method used to desorb residual protein, that is, protein which associated with the noncellulosic components
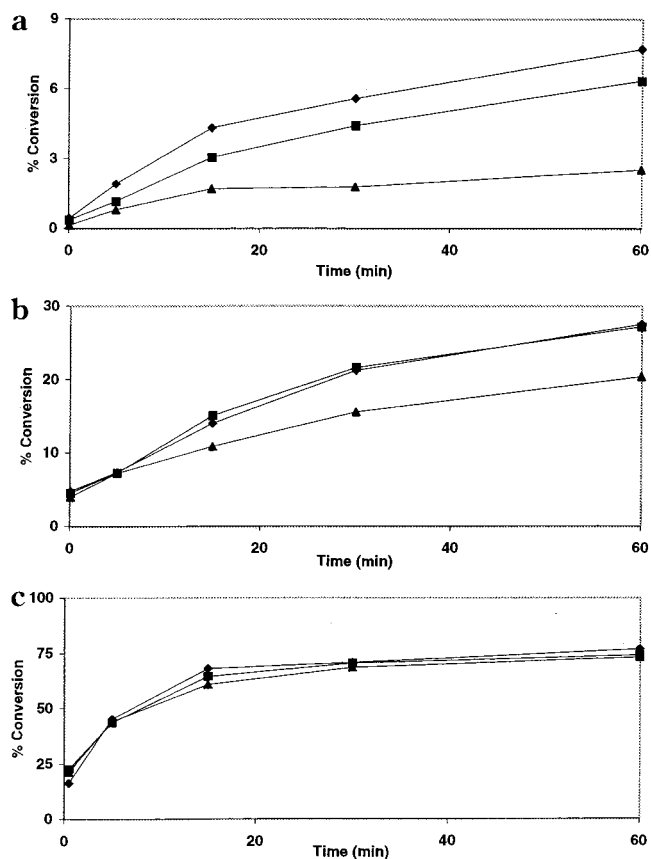

Figure 2. Early stage hydrolysis of three celluloses in the presence of KL: (a) (◆) MCC-a, (■) MCC-a + 10% KL, (▲) MCC-a + 40% KL; (b) (◆) BMCC, (■) BMCC + 10% KL, (▲) BMCC + 40% KL; (c) (◆) AC, (■) AC + 10% KL, (▲) AC + 40% KL. Reaction conditions were as described in Figure 1.

Table 3. Adsorption Characteristics of MCC-a, BMCC, AC, LR, and KL

	half-saturating protein concn $(K)^a$ (mg/g of solids)	$0.5K^b$ (mg/g of solids)	max protein adsorption capacity (mg/g of solids)
MCC-a[c]	163	82	92
BMCC	937	468	670
AC	2379	1190	1120
LR[d]	23	NA[e]	35
KL	320	NA	271

[a] Amount of total protein required to obtain half of maximum enzyme adsorption capacity. [b] Concentration of protein used in competition adsorption/hydrolysis experiments. [c] Avicel PH 101. [d] Enzymatically prepared lignacious residue. [e] Not applicable.

during the preparation of LR. In one case, protein was removed with 6 M urea (see Materials and Methods), a treatment known to desorb residual cellulase (Reese, 1982; Otter et al., 1989). Protein was desorbed from the other LR preparation by washing with 3% SDS, a well-established method for the desorption of interfacial proteins (Helenius and Simons, 1975; Rapoza and Horbett, 1990). Results obtained with the two LR preparations were not significantly different, suggesting that the minimal effect of LR on rates of saccharification was not an artifact arising as a consequence of LR preparation.

Protein Adsorption to Reaction Mixture Components. Protein adsorption capacities of reaction mixture components (Table 3) were calculated by fitting a saturation (Langmuir)-type adsorption model to adsorption isotherms. Cellulase adsorption to cellulose does not fulfill the strict requirements for the Langmuir
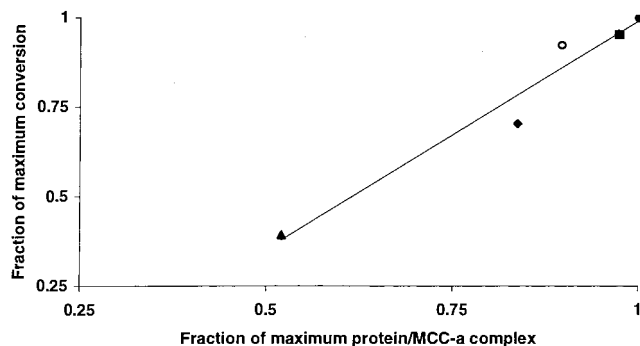
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Figure 3. Fraction of maximum (obtained without lignin addition) MCC-a conversion as a function of the fraction of maximum (obtained without lignin addition) protein/cellulose complex. The five reaction mixtures included are (▲) MCC-a + 40% KL, (◆) MCC-a + 10%KL, (○) MCC-a + 40%LR, (■) MCC-a + 10%LR, and (●) MCC-a. Reaction conditions were as described in Figure 1. Adsorption and conversion data used were obtained after 15 min of hydrolysis.

model, that is, equilibrium process and absence of a saturation effect (Medve et al., 1995; Brash and Horbett, 1995), but the model provides a good fit to the adsorption isotherm data and allows an estimation of adsorption capacities. LR was found to bind relatively little protein, whereas the adsorption capacity of KL was greater than that of MCC-a. This difference in adsorption capacity is likely to explain the contrasting effects the two lignin preparations had on cellulose hydrolysis. Relatively little protein is associated with LR and, thus, it is not expected to appreciably diminish the amount of catalytically productive cellulose–cellulase complex. Much more protein associates with KL and, thus, its addition to a reaction mixture is likely to significantly decrease the amount of cellulase available for cellulose hydrolysis.

The effect of supplemental lignin was most pronounced in experiments using the MCC-a substrate. Figure 3 illustrates the correlation between cellulose-adsorbed protein (calculated using the determined association constant for MCC-a and the measured amount of free protein in reaction mixtures) and the rate of cellulose saccharification (taken as percent of conversion at 15 min). The nearly linear relationship is consistent with supplemental lignin acting as a competing adsorbant. An entirely linear relationship was not expected on the basis of the known synergistic behavior of this system (Nidetzky et al., 1995), the nonlinear substrate–velocity relationships of this system (Penner and Liaw, 1994), and the nonideal enzyme–velocity profiles of individual cellulase components (Medve et al., 1998).

LR was prepared from a herbacious feedstock that had been exposed to high temperatures (180 °C) at low pH during the initial dilute acid pretreatment. The lignin component of LR is thus expected to be condensed, because lignins become highly cross-linked under these conditions (Funaoka et al., 1990). The severity of the pretreatment is expected to affect the degree of condensation, and LRs prepared from less severely pretreated feedstocks are expected to have higher protein binding capacities (Ooshima et al., 1990). Therefore, it is expected that the addition of LRs derived from more mildly pretreated feedstocks would have a more significant (negative) impact on hydrolysis. The pretreatment used in this study was chosen on the basis of its industrial relevance, because it was previously shown to give optimum sugar yields for this feedstock (Esteghlalian et al., 1997).

Application to Biomass Saccharification Studies. Biomass lignin content is well-known to be negatively correlated with rates and extents of biomass saccharification (Vinzant et al., 1997). The experimental design of this study tested whether lignin-rich noncellulosic residues, derived from a representative pretreated herbaceous feedstock, affect cellulose saccharification in the absence of steric hindrance. The affect was found to be minimal, apparently due to the low protein binding capacity of LR. This result suggests that the reported negative impact of the lignin component of dilute acid-pretreated lignocellulosic feedstocks on cellulose saccharification is not principally due to enzyme partitioning (Excoffier et al., 1991; Gregg and Saddler, 1996). The results with this model system are more consistent with the hypothesis that the lignin component of a pretreated feedstock hinders saccharification by physically limiting the enzyme accessibility of the feedstock's cellulose (Yuldashev et al., 1993; Mooney et al., 1998).

The results from this type of study are here shown to be dependent on the cellulose and lignin preparations employed. The optimum preparation(s) for any given study will undoubtedly be dependent on the hypothesis tested. In the present case, the intent was to use preparations that best represent the components present in actual enzyme-based, biomass-to-ethanol, saccharification reactors. Hence, LR derived from an optimally pretreated feedstock was chosen as the primary focus of this study—along with celluloses representing a broad range of physicochemical properties. This is in contrast to those studies that focus on the more refined lignin preparations that are available (Glasser et al., 1983). LR is here envisioned as the lignin-rich noncellulosic residue that actually competes with biomass cellulose as an adsorbent for cellulolytic enzymes—so results based on this preparation are expected to be of direct relevance to industrial biomass-to-ethanol processes. KL was included in this study only as a reference to facilitate comparisons with other research. The results obtained with KL are similar to those from studies using the more refined lignins (Excoffier et al., 1991; Sewaltz et al., 1997).

ABBREVIATIONS USED

AC, amorphous cellulose; BMCC, bacterial microcrystalline cellulose; MCC-a, microcrystalline cellulose; EL, enzymatically prepared lignin; KL, chemically prepared lignin; SDS, sodium dodecyl sulfate.

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