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Weak Lignin-Binding Enzymes

A Novel Approach to Improve Activity of Cellulases for Hydrolysis of Lignocellulosics

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Abstract

Economic barriers preventing commercialization of lignocellulose-to-ethanol bioconversion processes include the high cost of hydrolytic enzymes. One strategy for cost reduction is to improve the specific activities of cellulases by genetic engineering. However, screening for improved activity typically uses "ideal" cellulosic substrates, and results are not necessarily applicable to more realistic substrates such as pretreated hardwoods and softwoods. For lignocellulosic substrates, nonproductive binding and inactivation of enzymes by the lignin component appear to be important factors limiting catalytic efficiency. A better understanding of these factors could allow engineering of cellulases with improved activity based on reduced enzyme-lignin interaction ("weak lignin-binding cellulases"). To prove this concept, we have shown that naturally occurring cellulases with similar catalytic activity on a model cellulosic substrate can differ significantly in their affinities for lignin. Moreover, although cellulose-binding domains (CBDs) are hydrophobic and probably participate in lignin binding, we show that cellulases lacking CBDs also have a high affinity for lignin, indicating the presence of lignin-binding sites on the catalytic domain.

Index Entries: Cellulase; lignin; unproductive binding; softwood; hydrolysis.

Introduction

Lignin is the most abundant noncarbohydrate constituent of wood. Its presence in wood represents a major problem for the development of biomass conversion processes. It is widely accepted that lignin interferes

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with cellulose hydrolysis during biomass conversion. Numerous studies have demonstrated an inverse correlation between the rate of biomass hydrolysis and the lignin content *(1–3)*, and an extensive modeling study of 147 lignocelluloses concluded that lignin content was a key factor in determining biomass digestibility *(4)*. Although lignin appears to limit cellulose hydrolysis by forming a physical barrier that restricts enzyme access *(5,6)*, numerous lines of evidence indicate that lignin-enzyme interactions make a significant contribution to the decline in rate observed during hydrolysis of lignocellulose substrates. Improved hydrolysis of lignocellulosic substrates seen in the presence of surfactants *(7–9)* has been attributed to a reduction in nonproductive binding of cellulases to the lignin component *(10)*. A similar mechanism has been invoked to explain the enhancement of lignocellulose hydrolysis seen following the addition of exogenous protein *(11)*. Studies on model lignocellulosic systems have led to the same conclusion: preincubation of a cellulase complex with various isolated lignins reduced activity on filter paper and lowered the level of soluble protein in the reaction mixture *(12)*.

Currently, the high cost of enzymes is a major factor restricting commercialization of biomass conversion processes. One strategy for cost reduction is to improve the specific activities of cellulases by genetic engineering, and significant reductions in enzyme costs have recently been obtained using this approach *(13)*. However, a study of the spontaneous hydrolysis of β-glycosides indicates that enzymes involved in the hydrolysis of β-1,4-glucans are already exceptionally efficient enzymes *(14)*, suggesting that the prospects for further improvement of catalytic rates may be limited. Given the role of lignin in reducing hydrolytic efficiency documented by the examples just cited, an alternative approach, based on protein engineering to reduce lignin-enzyme interactions, appears to offer considerable long-term potential. This approach would be useful even if pretreatment processes could be developed to minimize the steric effects of lignin, because the presence of residual lignin would remain problematic owing to enzyme-binding effects. In addition, it is anticipated that this approach would increase the efficiency of enzyme recycling as well, because binding to residual lignin is also implicated in the low recovery of enzymes following extensive hydrolysis of lignocellulosic substrates. The investigation reported herein provides further evidence to support this strategy.

Materials and Methods

Recovery of Enzyme Performance by Addition of Fresh Enzyme

The first experiment was designed to document that the addition of fresh cellulase complex after >75% conversion of a lignocellulosic substrate, when the major part of the substrate surface comprises exposed lignin, results in a significant recovery of the initial hydrolytic rate (Fig. 1). The substrate was Douglas fir, prepared using ethanol organosolv pretreatment

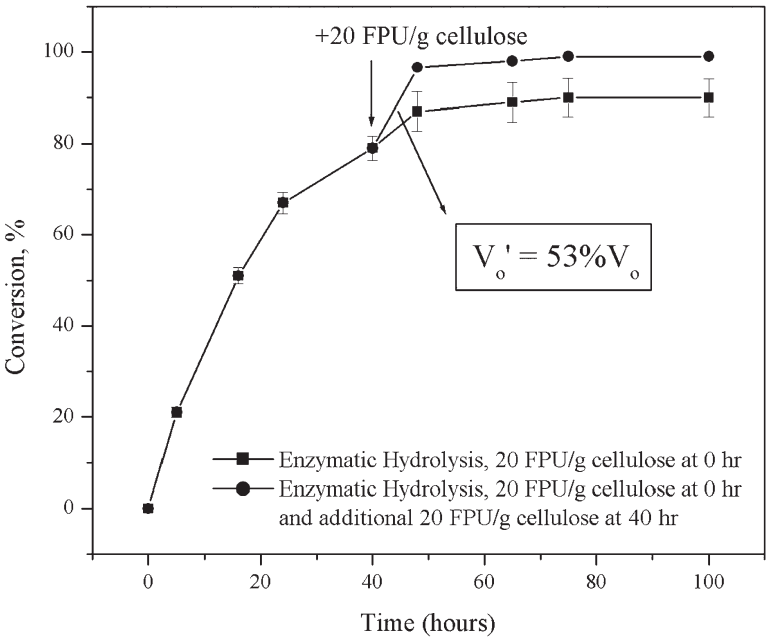

Fig. 1. Recovery of enzyme performance during enzymatic hydrolysis of an organosolv softwood pulp.

(16.4% Klason lignin, 82.6% cellulose, and 2.4% hemicellulose). The enzyme with a filter paper activity of 60.7 filter paper units (FPU)/mL was a commercial *Trichoderma reesei* cellulase preparation, Celluclast® 1.5L (Novozymes), loaded at 20 FPU/g of cellulose and supplemented with an excess of a commercial *Aspergillus niger* β-glucosidase preparation, Novozym® 188 (Novozymes), with a cellobiase activity of 341 cellobiase units (CBU)/mL. The loading of β-glucosidase was 40 CBU/g of cellulose. Hydrolysis (2% substrate consistency) was allowed to proceed for 100 h at 50°C, pH 4.8, and 150 rpm with and without addition of further cellulase (20 CPU/g of cellulose) at 40 h. The experiment was performed in triplicate.

Monitoring of Protein Concentration and Enzyme Activity During Hydrolysis

The second experiment determined the amount of protein and enzyme activity in the supernatant during hydrolysis of ethanol organosolv–pretreated mixed softwood (6% Klason lignin, 91% cellulose, and 3% hemicellulose) from Lignol Innovations (Vancouver, Canada) by *T. reesei* cellulase complex. The conditions of the experiment were as follows: 10 FPU/g of cellulose, 20 CBU/g of cellulose, 100-mL total reaction volume, 24 h, 45°C, 150 rpm, 2% consistency, 250-mL flask volume, pH 4.5. The concentration of protein in solution was corrected for β-glucosidase. The β-glucosidase activity in the supernatant did not change over the hydrolysis;

it showed a constant value of 0.32 U/mL (p-nitrophenyl-β-D-glucopyranoside as a substrate). Furthermore, monitoring of the protein adsorption by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that whereas the intensity of the electrophoresis bands corresponding to cellulases decreased, for β-glucosidase it remained unchanged (data not shown). Protein was measured by the Bradford *(15)* method (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard. Filter paper activity was determined as recommended by International Union of Pure and Applied Chemistry (IUPAC) *(16)*. The glucose concentration was measured by high-performance liquid chromatography (HPLC) with fucose as an internal standard.

Preparation of Organosolv-Pretreated Douglas Fir Pulp

Organosolv-pretreated Douglas fir pulp no. 2 (5.7% Klason lignin, 89.82% cellulose, 2.34% hemicellulose) was provided by Lignol Innovations. Organosolv pretreatment of Douglas fir sample no. 1 was carried out in a 1-L stainless steel pressure reactor (Parr, Moline, IL) using 50% (w/w) ethanol, adjusted to pH 2.4 with 10% (v/v) sulfuric acid, at 195°C and approx 3.2 MPa (460 psi). The solvent:wood ratio was 7:1 (w/w), and the pretreatment time was 40 min. The time required to reach the target cooking temperature was approx 53 min in all cases. After cooking, the reactor was cooled by immersion in ice until the interior temperature was ≤55°C. After pretreatment, the brown liquor was removed by decantation. Following manual removal of any recalcitrant chips, the solids were homogenized for 5 min in 70% (v/v) ethanol at 70°C (pulp:ethanol ≈ 9:1) in a British disintegrator (TMI, Montreal, Canada). Solids were then washed a further three times with warm 70% ethanol and rinsed extensively with water. After washing, the pretreated solids were separated by filtration and stored in sealed plastic bags at 4°C.

Lignin Extraction Procedure

Lignin was prepared by extensive enzymatic digestion of ethanol organosolv-pretreated Douglas fir followed by proteolysis using Pronase (Sigma, St. Louis, MO), a nonspecific proteinase. The conditions of the lignin extraction were as follows: 10 grams of organosolv ethanol pulp was incubated for 72 h in 500 mL of 50 m*M* acetic buffer, pH 4.5. The cellulase loading was 40 FPU/g of cellulose. β-Glucosidase (Novozym 188; Novozymes) was added (80 CBU/g of cellulose) to reduce inhibition of cellobiose. Tetracycline (40 μg/mL) and cycloheximide (30 μg/mL) were added to prevent microbial contamination. After 72 h of hydrolysis, the reaction mixture was centrifuged (2325*g*, 15 min). The supernatant was decanted and 600 mL of distilled water was added to the precipitate. The lignin suspension was sonicated for 60 min. This washing procedure was repeated in triplicate. After washing the residual lignin, it was filtered, air-dried, manually ground, and screened through a 180-mesh screen.

The residual lignin, previously sonicated for 1 h, was incubated overnight with the nonspecific protease, Pronase (1 U/mL; Sigma), at 37°C in 50 m*M* phosphate buffer, pH 7.4, at 150 rpm. After protease treatment and washing with phosphate buffer, the obtained lignin was freeze-dried. The degree of purity of the obtained lignin determined by Klason lignin was >90%. Elemental analysis of the residual lignin before and after protease treatment was performed with a Leco CN-2000 elemental analyzer. The nitrogen content of the lignin before protease treatment was 1.74%, whereas after protease treatment it was 0.86%, which shows that the protease treatment reduced by twofold the protein content.

Adsorption Kinetics of Cellulases

The third experiment examined the adsorption of two recombinant endo-β-1,4-glucanases (EG III [Cel12A] from *Penicillium* sp. and EG I [Cel7B] from *Humicola* sp.) to lignin. The enzymes have similar carboxymethylcellulose (CMCase) activities (49.0 and 42.0 U/mg, respectively), determined as recommended by IUPAC *(16)*. Adsorption kinetics were determined during 90 min of incubation at room temperature, 18 rpm, using 2% lignin and an initial protein concentration of 1 mg/mL in a rotary incubator (FinePCR, Seoul, Korea). The total reaction volume was 1.5 mL in 2.0-mL tubes. Each experiment was performed in triplicate.

Influence of Softwood and Hardwood Lignins on Enzymatic Hydrolysis of Cellulose

The final experiment examined the influence of softwood and hardwood lignin preparations on the enzymatic hydrolysis of α-cellulose by *T. reesei* cellulase preparation (0.91 FPU/mg of protein, 14.1 CMCase U/mg of protein, 2.18 Avicelase U/mg of protein [16]) and a cellulase secreted by *Penicillium* sp. (0.88 FPU/mg of protein, 16.9 CMCase U/mg of protein, 2.03 Avicelase U/mg of protein). Reaction mixtures (containing 2.0% [w/w] α-cellulose, 0.3% lignin [w/w], and 0.7 mg/mL of cellulase in a total volume of 1.2 mL) were incubated at 45°C, pH 4.8, for 30 min and stopped by the addition of 1 *M* Na_2CO_3, and the products were analyzed by HPLC. The high-purity lignin samples (80–95% purity) were prepared from Douglas fir and yellow poplar by ethanol organosolv pretreatment followed by enzymatic hydrolysis and later by the previously mentioned protease treatment.

Results and Discussion

The addition of fresh cellulase complex after extensive hydrolysis (>75% conversion) of ethanol organosolv–pretreated Douglas fir resulted in the recovery of >50% of the initial hydrolytic rate (Fig. 1). This result is consistent with the hypothesis that hydrolysis of residual substrate is limited by nonproductive binding of cellulase components to exposed lignin and, presumably, lignin-carbohydrate complex. A similar result was obtained

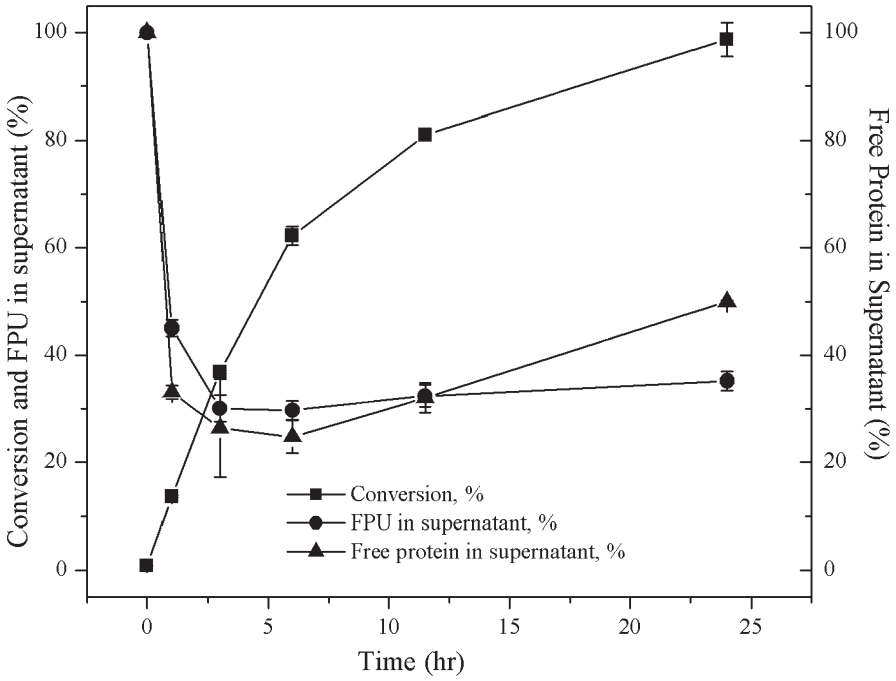

Fig. 2. Monitoring of protein adsorption and filter-paper activity during enzymatic hydrolysis of an organosolv softwood pulp.

using a model reaction system containing steam-exploded spruce and two isolated *T. reesei* cellulases (cellobiohydrolase I and EG I) *(17)*. The result presented here suggests that nonproductive binding also lowers hydrolysis rate of the whole cellulase complex and is an important factor in the hydrolysis of all pretreated biomass with high levels of residual lignin.

The levels of total protein and filter paper activity detected in the reaction supernatant during the hydrolysis of ethanol organosolv–pretreated Douglas fir indicate the capacity for enzyme adsorption by residual lignin (Fig. 2). There was a rapid adsorption of protein during the early stage of hydrolysis, followed by a partial desorption; filter paper activity showed a similar trend. At the end of hydrolysis (~98% conversion), only ~50% of the total protein and 35% of the original filter paper activity were detected in the supernatant.

We further showed that two purified endoglucanases, with similar CMCase activity (EG III from *Penicillium* sp. and EG I from *Humicola* sp.), have significant differences in their affinity for organosolv lignin: at equilibrium, the amounts of these enzymes bound to lignin differed by 12% (Fig. 3). The natural occurrence of endoglucanases with similar activities but different lignin-binding properties provides support for the general concept that enzymes can be engineered for reduced lignin binding without compromising catalytic activity. Moreover, unlike most cellulolytic

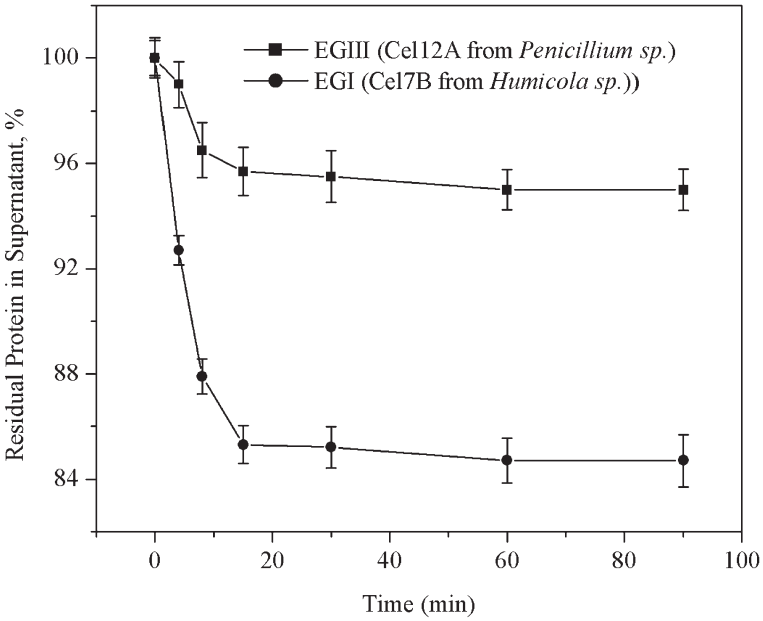

Fig. 3. Adsorption kinetics of two endo-β-1, 4-glucanases on lignin.

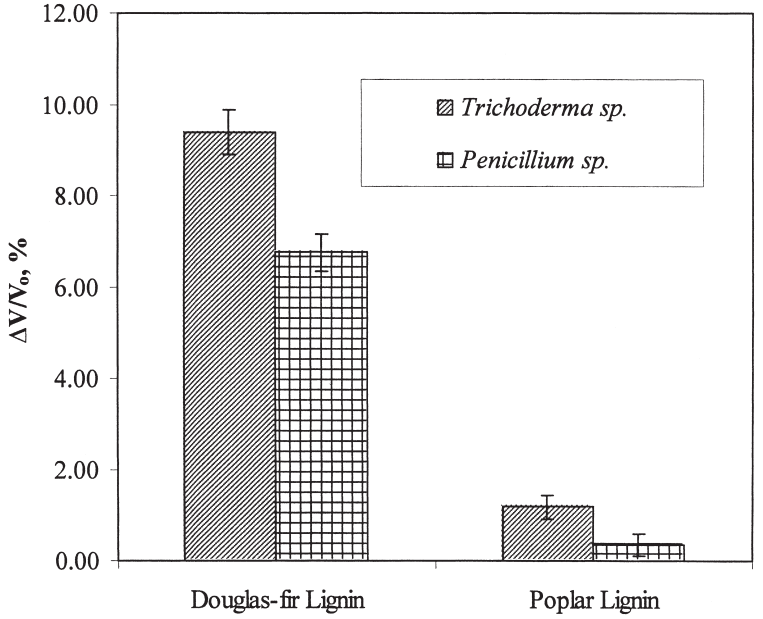

Fig. 4. Influence of a softwood (Douglas fir) and a hardwood (yellow poplar) lignin on cellulose hydrolytic ability of two cellulase complexes. ΔV, difference of initial hydrolytic rates with and without lignin.

enzymes, *Penicillium* EG III and *Humicola* EG I lack CBDs. This is significant because it demonstrates that catalytic domains can make a significant contribution to overall lignin binding and that weak lignin-binding cellulases might be engineered without compromising cellulose binding.

Finally, whole cellulase complexes from *T. reesei* and *Penicillium* sp. were shown to have significant differences in their susceptibility to inhibition by lignin derived from both softwood and hardwood (Fig. 4). Although the commercial *T. reesei* cellulase preparation Celluclast 1.5L is generally used in research studies of lignocellulose enzymatic hydrolysis, it should be noted that it was developed for application in the textile, cereal–processing, and brewing industries. These results provide further support for the notion that the engineering of enzymes with lower lignin affinity could provide a useful strategy for improvement of cellulase activity on lignocellulosic substrates.

Acknowledgments

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