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ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 51 (2008) 42–48
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Partition of enzymes between the solvent and insoluble substrate during the hydrolysis of lignocellulose by cellulases

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Received 30 July 2007; received in revised form 8 October 2007; accepted 9 October 2007
Available online 18 October 2007

Abstract

The interfacial and interphasic behavior of enzyme plays an important role in heterologous biocatalysis, such as the enzymatic hydrolysis of lignocellulose. The solid-solution partition of the major cellulases from a highly effective *Trichoderma reesei* cellulolytic system was evaluated during the enzymatic cellulolysis of a pretreated corn-stover substrate. Upon mixing with the insoluble substrate, almost all of the enzymes (including CBH-I, CBH-II, EG-I, EG-II, and BG) were adsorbed, as shown by the protein and activity assay of the solution fraction. No significant desorption was detected during as well as after the cellulolysis, indicating the enzymes' ability to function at the lignocellulose surface. The adsorption was attributed to the specific binding to and activating of cellulose during the cellulolysis, and to the non-specific binding to lignin, particularly after the cellulolysis. The presence of several representative cellulolysis enhancers, substances capable of enhancing the cellulase action on lignocellulosic substrate, led to a significant desorption of the adsorbed cellulases. The effect might be related to the enhancing effect of these substances on the cellulases.

Keywords: Cellulase; Lignocellulose; Adsorption; Hydrolysis

1. Introduction

Creating valuable materials (such as fuel or chemicals) from renewable biomass as well as transforming biomass-derived substances are of strategic importance for our effort in developing alternative energy source, reducing pollution, converting agricultural waste into value-added products, and other sustainable/green industries (for recent review, see [1,2]). Composed with complex carbohydrates, polyphenolics, proteins, and other compounds, biomass materials are highly heterogeneous and resilient towards many conventional processing techniques unless they are under extraordinary conditions (*e.g.* high temperature, pressure, acidity or basicity). However, current research effort in biotechnology has shown promising potentials for economical utilization of biomass feedstocks. In particular, applying enzymes to hydrolyze the polymeric/recalcitrant carbohydrates in biomass into simple/fermentable sugars, under ambient conditions, is of great interest and importance [3–7].

In nature, plant materials can be degraded/transformed by an array of cellulases and other enzymes/accessories produced by microbes (for recent reviews, see [4–6]). An archetypical microbial cellulolysis system is that of *Trichoderma reesei* (*Hypocrea jecorina*), which contains at least two cellobiohydrolases (CBH), six 1,4-β-endoglucanases (EG), and two β-glucosidases (BG) [8]. These cellulases, many of them modularly structured with a catalytic core and one or more carbohydrate-binding modules (CBM), can target different kinds of cellulose and act in synergism [4–6,8,9]. The CBM may, via its hydrophobic affinity, anchor the enzymes onto the surface of cellulose. To effectively hydrolyze cellulose, some cellulases (in particular CBH) apply a "processive" mechanism so that the enzymes may grab and slide along a cellulose chain while hydrolyzing it, to reduce nonproductive detachment/reattachment [9,10]. However, hydrolyzing a biomass substrate with such enzymatic mix is still slow and inefficient in general, due to many factors such as accessibility, instability, and inhibition. How to accelerate the hydrolysis with sufficiently low enzyme dose is one of the major challenges in

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doi:10.1016/j.molcatb.2007.10.004

the commercialization of a viable biotechnology to convert raw biomass feedstock into valuable chemicals [4,7].

The hydrolysis of lignocellulosic substances (the main component of biomass) by cellulases is a heterogeneous catalysis. The insolubility of the substrate requires the soluble enzymes to have affinity and reactivity on interface. The cellulase adsorption onto insoluble cellulose or accompanying non-reactive substances (*e.g.* lignin) plays a major role in the performance of the enzymes. The restriction of the enzymes to two-dimensional surface of cellulose microfibril or one-dimensional cellulose chain may lead to a significant deviation from the conventional kinetics of soluble enzymes [11]. The hydrophobic affinity of CBM, needed for binding a cellulase onto cellulose, may enhance the nonproductive, and often irreversible or inactivating adsorption of the enzymes onto lignin, resulting in decreased efficiency in cellulose hydrolysis [12–18].

Cellulase adsorption by lignocellulose has been the target for active study in the past two decades. Traditionally, the phenomenon has been investigated by Langmuir-type isotherms, measuring the overall protein adsorption under the assumption of uniform adsorbent surface and independent adsorption of individual cellulases, although it is well known that the cellulases can be synergistic partners for biomass hydrolysis. Temporal profiles of the adsorption during hydrolysis have also been semi-quantitatively probed by monitoring selected cellulase activities in the supernatant of hydrolysis suspensions [19–23]. Various interfacial-prone agents, such as surfactants and binding proteins, have been studied for their cellulase-desorbing effect [24–37].

In this study, we investigated the partition of a "complete" *T. reesei* cellulase mix during the hydrolysis of a pretreated corn-stover (PCS) substrate. Using electrophoresis, protein sequencing, protein assay, and cellulase activity measurement, we monitored the temporal adsorption profile of the major cellulases, and correlated it with the effect of two representative cellulase enhancers.

2. Experimental

2.1. Materials and instruments

Chemicals used as reagents or buffers were commercial products of reagent or purer grade unless specified otherwise. Low molecular weight (MW) gelatin was obtained from Sigma–Aldrich (FreAlagin M type, MW 0.2–10 kD). Diluted acid-pretreated corn-stover (PCS) preparations (~53% or 59% glycan and 32% or 28% lignin) were kindly provided by US National Renewable Energy Laboratory, and were washed extensively and sieved before use. Carboxymethyl cellulose (CMC) was from Hercules (7L2 type). Phosphoric acid-swollen cellulose (PASC) was prepared from Avicel (FMC, PH101) by a published method [38]. The lignaceous residue of PCS was obtained from 7-day shake-flask PCS hydrolyses (~70%–100% extent) by *T. reesei* cellulases, followed by extensive washing with water and 50 mM Na-acetate of pH 5. Part of the lignaceous residue was heated in boiling water for 5 min to inactivate the adsorbed cellulases.

An experimental "complete" *T. reesei* cellulases preparation from Novozymes was used to hydrolyze the PCS. *T. reesei* culture broths in which the Cel7A (CBH-I) or Cel6A (CBH-II) protein was genetically removed were prepared by Dr. S. Merino and S. Maiyuran of Novozymes. Purified wt *T. reesei* Cel7A and *A. oryzae* Cel3A were prepared at Novozymes [11,39].

Electrophoresis was carried out either on a Bio-Rad SDS-PAGE Criterion device/gels (stained by Bio-Rad BioSafe method) or a Bio-Rad Experion device in accordance to the manufacturer's instruction. Spectrophotometric measurement was carried out on a Molecular Devices SpectraMax 340PC reader with Costar 96-well microplates. In-gel protein tryptic digestion, needed for mass spectrometry-based sequencing/peptide mapping, was made on a Perkin Elmer MultiPROBE II liquid-handling robot. Tandem mass spectrometry was run on a Waters Micromass Technologies Q-ToF micro hybrid orthogonal quadrupole mass spectrometer, coupled with a Dionex Ultimate capillary/nano-flow HPLC system. Sample concentration was made with either Millipore Ultrafree-0.5 filters or a Labconco Freezone 12 L lyophilizer.

2.2. Hydrolysis of pretreated corn stover

PCS hydrolysis was carried out in either 125-mL shake-flasks or 1.7-mL tubes ("mini-scale"), in at least duplicates.

In a typical shake-flask hydrolysis, 10% (w/v) PCS (dry weight) was reacted with 0.5 g/L (corresponding to 5 mg proteins or 3 "filter-paper-unit" (FPU) cellulases per gram of PCS solid, or 9 mg proteins or 6 FPU cellulases per gram of cellulose) cellulases in 50 mM Na-acetate of pH 5, at 50 °C and under 130 rpm shaking. In a typical mini-scale hydrolysis, 5% PCS was reacted with 0.25 g/L (corresponding to 5 mg proteins or 3 FPU cellulases per gram of PCS solid, or 9 mg proteins or 6 FPU cellulases per gram of cellulose) cellulases. Aliquots of the suspension were sampled, and their supernatants were analyzed for soluble reducing sugars by *p*-hydroxybenzoic acid hydrazide (PHBAH), based on a published method [40]. Hydrolysis extent was estimated based on the glycan content of the PCS.

2.3. Cellulase adsorption onto the lignaceous residue of PCS

A washed and re-buffered PCS lignaceous residue preparation (with ~10% initial cellulose remaining), with or without heating in boiling water, was incubated at 3.2% level with a new batch of cellulases (0.5 g/L, corresponding to 16 mg proteins or 10 FPU cellulases per gram of solid, or 140 mg proteins or 95 FPU cellulases per gram of cellulose) for 3 days under the conditions used to hydrolyze the initial PCS. Aliquots of the suspensions were sampled daily, and the supernatants were either directly assayed, or lyophilized and redissolved in smaller volumes of the buffer to make ≥0.3 mg/mL in predicted starting proteins (if all soluble) for SDS-PAGE analysis. Cellulases incubated in the absence of the PCS served as controls.

Another washed and re-buffered PCS lignaceous residue preparation (with ~20% initial cellulose remaining) was incubated at 3.2% level with either cellulases from a Cel7A-deletion *T. reesei* broth (0.2 g/L, corresponding to 6 mg proteins g^{-1} of solid, or 31 mg proteins g^{-1} of cellulose), cellulases from a Cel7A/Cel6A-double-deletion *T. reesei* broth (0.1 g/L, corresponding to 3 mg proteins g^{-1} of solid, or 16 mg proteins g^{-1} of cellulose), or *A. oryzae* BG (0.05 g/L, corresponding to 1.6 mg proteins g^{-1} of solid, or 8 mg proteins g^{-1} of cellulose). The levels of the cellulases corresponded approximately to those in the PCS hydrolysis by the "complete" cellulases.

2.4. Protein assays

Protein profiling was made on either SDS-PAGE or Bio-Rad's Experion electrophoresis system. Diluted samples were concentrated prior to the electrophoresis when needed. Purified *T. reesei* Cel7A of different amount served as controls for estimating the band density on SDS-PAGE. Total protein determination was made with either Pierce BCA kit or Molecular Probes EZQ protein quantification kit, which could avoid interference from reducing sugars. Aliquots of hydrolysis suspensions were either filtered or centrifuged, and the supernatants were subjected to the assays. Protein identification via peptide mapping was done by mass spectrometry.

2.5. Cellulase assays

PASC hydrolysis was carried out with 2 g/L PASC, 0.5 g/L (or 0.25 g/(g PASC)) bovine serum albumin (BSA), and 50 mM Na-acetate, at pH 5 and 50 °C in 96-well plate. CMC hydrolysis was carried out with 5 g/L CMC and 50 mM Na-acetate, at pH 5 and 50 °C. After 30 min, 20 μL supernatant samples were mixed with 180 μL 0.16 M Na-carbonate of pH 10 to stop the hydrolysis, before being assayed for produced sugars with PHBAH. Glucose was used for the calibration, and mixtures omitting either the cellulases or the cellulose served as controls. Hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside (pNPG) was carried out with 4 mM pNPG, 0.01% Tween 20, and 89 mM Na-citrate, at pH 5 and 40 °C in 96-well plate. After 20 min, 100 μL of 1 M Na-carbonate buffer of pH 10 was mixed with 225 μL hydrolysis solution to stop the reaction, and the mixture was measured for its absorption at 405 nm.

2.6. Effect of cellulase "enhancers"

Representing the surfactant/polymer and the protein substances able to enhance cellulases' action on lignocellulose, polyethylene glycol (PEG) 4000 and gelatin (MW of 0.2–1 kD) were tested, at 50 g/L (or 1 g/(g PCS solid)) level, by the mini-scale PCS hydrolysis with cellulases. The gelatin was chosen so that its electrophoretic profile would not interfere with those of the cellulases (MW > 25 kD) on SDS-PAGE. The hydrolysis was run for 5 days, and was sampled/analyzed daily. Mixtures omitting either PCS, PEG4000, or gelatin served as controls. The hydrolysis as well as the soluble proteins was analyzed.

3. Results

3.1. Hydrolysis of pretreated corn stover by cellulases

Under the conditions of this study, the PCS substrate was readily hydrolyzed by the *T. reesei* cellulases. As exemplified in Fig. 1, the hydrolysis proceeded relatively fast at the beginning, reaching ~40%–50% extent in the first day, but then slowed down, reaching ~85% hydrolysis after 6 days. At the mini-scale, the hydrolysis also proceeded relatively fast at the beginning, reaching ~50%–60% extent in day 1, then ~100% extent in day 4.

3.2. Cellulase adsorption onto pretreated corn stover

Upon the mixing, the cellulases were quickly adsorbed onto the PCS substrate. As shown by SDS-PAGE, almost all of the cellulases were absent from the supernatant fraction of the suspension, most likely due to their binding to the cellulose in PCS. The cellulases remained insoluble even when the majority of the cellulose in PCS was hydrolyzed, indicating an adsorption by the lignin. Fig. 2 shows the result obtained for a shake-flask PCS hydrolysis by the cellulases (lanes 2–9). The very faint triple bands of the size ~50–70 kD became more visible when 10 times more supernatants samples were loaded (after 10-fold concentration), although their density was still ~1/5 or less than that of the cellulases incubated in the absence of PCS (lane 2). Similar results were seen with the mini-scale PCS hydrolysis.

In addition to the SDS-PAGE assay, the supernatant of the PCS hydrolysis suspension was also analyzed by the cellulase activity on typical substrates PASC, CMC, and pNPG. When 10% PCS was reacted with 0.25 g/L (corresponding to 2.5 mg proteins or 1.7 FPU cellulases per gram of solid, or 4.5 mg proteins or 3 FPU cellulases per gram of cellulose) cellulases, a ~34%, 51%, 64%, and 68% hydrolysis was observed at days 1, 3, 5, and 7, respectively. Almost all of the PASC-,

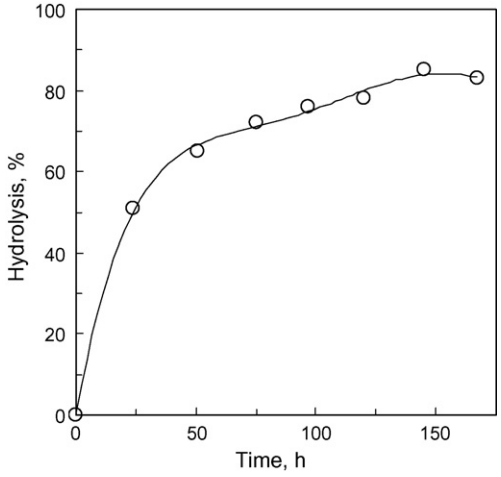

Fig. 1. PCS hydrolysis by *T. reesei* cellulases. Initial concentrations: 10% (w/v) PCS (dry weight), 0.5 g/L (corresponding to 5 mg proteins or 3 FPU cellulases per gram of PCS solid, or 9 mg proteins or 6 FPU cellulases per gram of cellulose) cellulases, 50 mM Na-acetate of pH 5. Incubation conditions: 50 °C, 130 rpm shaking. Detection of released reducing sugar: PHBAH method.

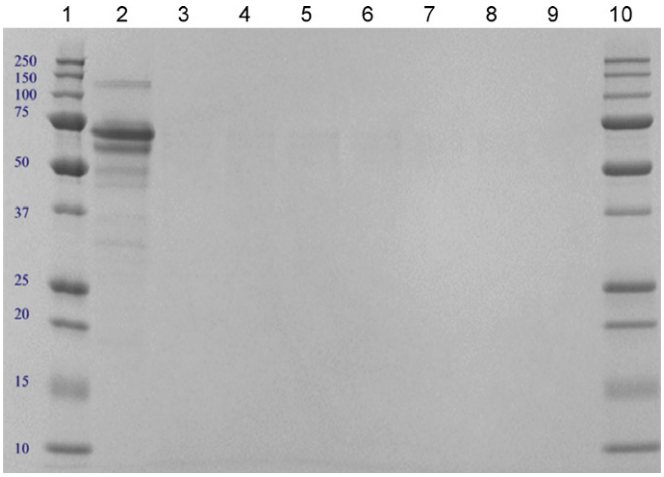

Fig. 2. Cellulase solubilization during PCS hydrolysis. Supernatants of the hydrolysis suspensions sampled at different time: lane 3, day 1; lane 4, day 2; lane 5, day 3; lane 6, day 4; lane 7, day 5; lane 8, day 6; lane 9, day 7. Samples in lanes 2–9 were from the PCS hydrolysis by *T. reesei* cellulases (whose time profile is shown in Fig. 1). Other samples: lanes 1 and 10, MW markers (in kD); lane 2, cellulase solution in the absence of PCS. Major protein bands: CBH-I, ~70 kD; CBH-II and EG-II, ~60 kD; EG-I, ~50 kD; BG, ~130 kD. Sample volume: 30 μL. SDS-PAGE: 8%–16% gel.

CMC-, and pNPG-hydrolysis activities (>90%) were lost in the supernatant in day 1, and the loss maintained through day 7. Based on the BCA assay, the loss of soluble proteins was ~90% at day 1, and ~80% from days 3 to 7. Similar results were also observed for the hydrolysis of 5% PCS with 0.25 g/L (corresponding to 5 mg proteins or 3 FPU cellulases per gram of solid, or 9 mg proteins or 6 FPU cellulases per gram of cellulose) cellulases.

3.3. Cellulase adsorption onto the lignaceous residue of PCS

When two washed and re-buffered PCS lignaceous residue preparation (with ~0% or 10% of the initial cellulose remaining), with or without a heat treatment, were incubated at ~32 g/L level with a new batch of cellulases at 0.5 g/L level (corresponding to 16 mg proteins or 10 FPU cellulases per gram of solid), the remaining cellulose from one lignaceous residue preparation was hydrolyzed. About 90% of the Cel7A and ~100% of the other cellulases were adsorbed, most likely onto the lignin.

When another washed and re-buffered PCS lignaceous residue preparation (with ~20% of the initial cellulose remaining) was incubated at ~32 g/L level with ~0.2 g/L (corresponding to 6 mg proteins g^{-1} of solid, or 31 mg proteins g^{-1} of cellulose) of the cellulases from a Cel7A-deletion *T. reesei* broth, almost all of the proteins, including Cel6A, Cel7B (EG-I), and Cel5A (EG-II), were adsorbed during the 3-day incubation, except a minor band of ~60 kD (~1/20 of the initial proteins based on the band density). When the PCS lignaceous residue preparation was incubated with ~0.1 g/L (corresponding to 3 mg proteins g^{-1} of solid, or 16 mg proteins g^{-1} of cellulose) of the cellulases from a Cel7A/Cel6A double-deletion *T. reesei* broth, almost all of the proteins, including Cel7B and Cel5A, were adsorbed during the 3-day incubation, except the minor band of ~60 kD. When the lignaceous residue preparation was incubated with ~0.05 g/L (corresponding to 1.6 mg proteins g^{-1} of solid, or 8 mg proteins g^{-1} of cellulose) of *A. oryzae* Cel3A, about half of the BG was adsorbed.

The SDS-PAGE bands were identified by mass spectrometry. The result showed that Cel6A, Cel7B, Cel5A, Cel12A EG (EG-IV, ~45 kD), Cel74 xyloglucanase (~100 kD), and swollenin (~70 kD) were among the proteins adsorbed by the liganaceous residue.

When another washed and re-buffered PCS lignaceous residue preparation (with ~30% of the initial cellulose remaining) was incubated at ~4 kg/L level for 24 h with either ~18 g/L (corresponding to 4.4 mg proteins g^{-1} of solid, or 14 mg proteins g^{-1} of cellulose) *T. reesei* Cel7A, 12 g/L (corresponding to 3 mg proteins g^{-1} of solid, or 10 mg proteins g^{-1} of cellulose) cellulases from Cel7A-deletion *T. reesei* broth, 24 g/L (corresponding to 6 mg proteins g^{-1} of solid, or 19 mg proteins g^{-1} of cellulose) cellulases from Cel6A-deletion *T. reesei* broth, 6 g/L (corresponding to 1.5 mg proteins g^{-1} of solid, or 5 mg proteins g^{-1} of cellulose) cellulases from Cel7A/Cel6A double-deletion *T. reesei* broth, or 0.3 g/L (corresponding to 0.07 mg proteins g^{-1} of solid, or 0.2 mg proteins g^{-1} of cellulose) *A. oryzae* Cel3A, results similar to those stated above were observed.

3.4. Cellulase desorption from PCS by PEG4000 and gelatin

As tested at the mini-scale, the PCS hydrolysis by *T. reesei* cellulases was enhanced by both PEG4000 and gelatin. The hydrolysis extent at day 1 increased from 64% to ~80% or 90%, respectively, when 50 g/L (corresponding 1 g/(g PASC) or 1.8 g/(g cellulose)) of PEG4000 or gelatin was present. After 2 days, the hydrolysis was essentially complete. Based on SDS-PAGE of the supernatants, almost all the cellulases were adsorbed onto the PCS shortly after the mixing. After day 1, however, significant amounts of the cellulases were resolubilized. With both PEG4000 and gelatin, nearly all of the cellulases were desorbed after the hydrolysis (Fig. 3).

The supernatants from the day 5 hydrolysis were concentrated ~10-fold and then analyzed on Experion (whose microchannel electrophoresis would in general yield electrophoregrams different from those of SDS-PAGE). The electrophoregram of the cellulases showed three major bands of referenced MW of ~68, 112, and 158 kD, with relative density of ~11%, 87%, and 2%, respectively. The "112 kD" band represented Cel7A. When the hydrolysis was made by *T. reesei* cellulases alone, the supernatant sampled after the hydrolysis contained only one major protein band corresponding to that of Cel7A. Comparing with the control (PCS-omitting incubations), ~90% of the cellulases was lost, most likely due to the adsorption on the lignin in PCS. When the hydrolysis was made by the cellulases along with PEG4000 or gelatin, the supernatant contained all three major protein bands. Comparing with the controls (PCS-omitting incubations), essentially all of the cellulases were recovered, most

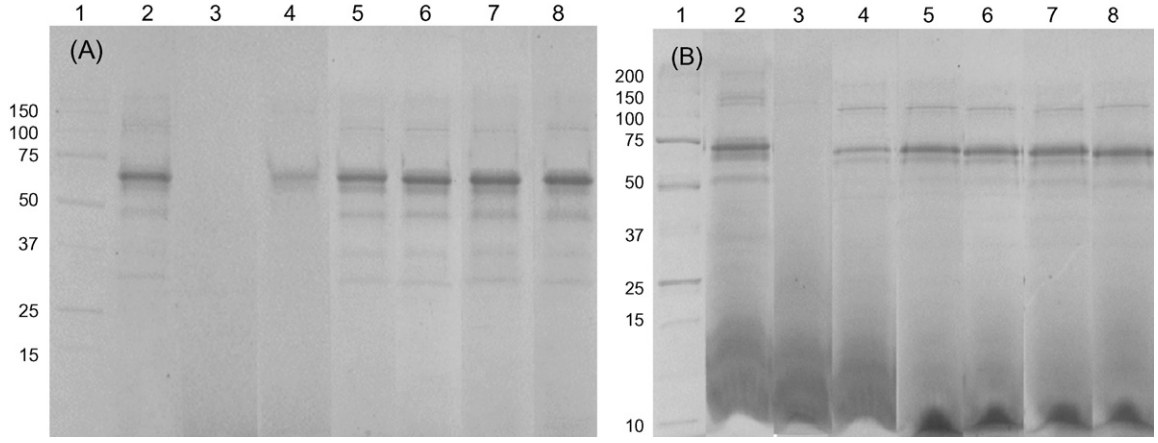

Fig. 3. Cellulase solubilization during PCS hydrolysis. Effect of (A) 50 g/L PEG4000 (corresponding to 1 g PEG/(g PCS solid), or 1.8 g PEG/(g cellulose)) and (B) 50 g/L gelatin (corresponding to 1 g gelatin/(g PCS solid), or 1.8 g gelatin/(g cellulose)). Mini-scale PCS hydrolysis by *T. reesei* cellulases. Supernatants of the hydrolysis suspensions sampled at different time: lane 3, ~0.5 h; lane 4, day 1; lane 5, day 2; lane 6, day 3; lane 7, day 4; lane 8, day 5. Other samples: lane 1, MW markers (in kD); lane 2, cellulase solution in the absence of PCS (the major bands at ~130, 70, 60, and 50 kD corresponded to BG, CBH-I, CBH-II and EG-II, and EG-I, respectively, and the smear between ~10 and 15 kD in (B) corresponded to gelatin). Sample volume: 40 μL. SDS-PAGE: 4%–20% or 8%–16% gels. Composed from different gels.

likely due to the desorption from PCS after the completion of the hydrolysis.

4. Discussion

4.1. Cellulase adsorption on lignocellulose

Adsorption of soluble cellulases onto insoluble cellulose substrate or non-reactive substances (such as lignin) has been studied in terms of its effect on the hydrolysis rate, enzyme inhibition, or enzyme stability. It has been observed that cellulases would bind to cellulose (often with measurable reversible on/off kinetics), hydrolyze it, and then redissolve in solution when the polymeric substrate is depolymerized/hydrolyzed [21,41]. When lignin is present, however, cellulases may be irreversibly adsorbed by the substance, decreasing their activity on cellulose. The detrimental adsorption may depend on the content, structure, or pretreatment of lignin [12,14–21,42–47].

Among various cellulases, those secreted by *T. reesei* are the mostly studied [8,13,16,43,48,49]. Under conventional hydrolysis conditions for pretreated biomass substrates (*e.g.* corn stover, hard wood, soft wood, Kraft fiber, or reconstituted cellulose–lignin mix), the majority (often > 90%) of the CBHs and EGs could be adsorbed by lignin, likely enhanced by CBM [15,16,50]. The cellulase adsorption might correlate with the lignin content in a lignocellulosic substrate [23]. Lignin could prevent cellulase resolubilization at the completion of cellulose hydrolysis [51]. The nonproductive cellulase adsorption onto lignin was postulated as the main reason that the hydrolysis rate decreases when the hydrolysis progresses, or with elevated substrate levels [23,47]. Adding new cellulases may "restart" the hydrolysis that had been slowed-down due to the lack of available, active enzymes [15].

Cellulase partition in a lignocellulose suspension has been measured with the supernatant for the remaining soluble proteins as well as hydrolytic activities on Avicel, filter paper, CMC, pNPG, or other substrates indicative for CBH, EG, or BG [19–23]. However, the previous investigations were mainly semi-quantitative, often lacking the information on the temporal behavior of individual members in a synergistic enzyme system during the hydrolysis. In this study, we monitored the major *T. reesei* cellulases, *i.e.* CBH-I, CBH-II, EG-I, EG-II, and BG, in a "complete" mixture during the hydrolysis of a PCS substrate. As shown by the electrophoregrams of the supernatants, almost all of the cellulases were adsorbed shortly after being mixed with PCS, likely onto the cellulose in PCS. The enzymes remained apparently adsorbed while hydrolyzing the cellulose into mostly glucose and a small amount of cellobiose, making the hydrolysis a virtual interfacial enzymatic reaction. The adsorption was likely dynamic, allowing the enzymes to move around, or briefly on/off the cellulose, to act on different part of the insoluble substrate. At the completion of the hydrolysis, only a minor amount (<10%) of the initial Cel7A desorbed into solution, indicating that the majority of Cel7A as well as others CBH, EGs, and BG were adsorbed by the lignin in PCS. The activity measurements in the supernatants also showed the adsorption of the CBHs, EGs, and BG during and after the hydrolysis of the cellulose in PCS, consistent with previous studies [19–21,51].

4.2. Desorption of adsorbed cellulases by cellulase enhancers

It has been reported that many surfactants, mostly nonionic ones, could enhance cellulase performance in hydrolyzing lignocellulosic substrates. The effect seemed more profound in the presence of lignin, and has been attributed to the surfactants' ability to reduce the nonproductive cellulase adsorption, widen cellulose accessibility for cellulase, or improve cellulase stability [24–35,52]. Tween 20, Tween 80, and Triton X100 were

among the best enhancers, whose effect might come from their polyalkoxylate structure, because PEG, a group of polyalkoxylate polymers not considered as surfactants, could enhance cellulases equally well [53–55]. It has been postulated that PEG-like polymers and Tween-like surfactants might bind to lignin via hydrogen bonding and hydrophobic interactions, thus reducing the nonproductive binding of cellulases [47,54].

In addition to surfactants and polymers, proteins such as BSA and gelatin have also been reported to enhance cellulase performance in hydrolyzing lignocellulosic substrates [35–37]. The effect has been attributed to a binding of the proteins on lignin, whose competition with cellulase could lead to a reduction of the enzyme's nonproductive adsorption, similar to that of the enhancing surfactants/polymers [37,56]. Indeed, no additive effect was seen when both Tween 20 and BSA was present, although either compound showed an enhancement on cellulase [35].

In this study, PEG4000 and gelatin were chosen as representatives of the cellulase-enhancing polymers/surfactants and proteins, respectively, and were investigated for their effect on the adsorption of cellulases during PCS hydrolysis. Adding PEG4000 or gelatin led to fasted PCS hydrolysis by *T. reesei* cellulases. The electrophoregrams of the supernatants showed that neither compounds prevented the cellulase adsorption during the cellulose hydrolysis. However, after the hydrolysis, almost all of the starting cellulases, including the major CBHs, EGs, and BG, were desorbed from the PCS lignaceous residues in the presence of PEG4000 or gelatin. Thus PEG4000 and gelatin might reduce the nonproductive adsorption of cellulases by lignin, and consequently enhance the cellulases' action on cellulose. The results were consistent with the previous reports on the non-binding of the enhancers on pure cellulose (*e.g.* Avicel) as well as the elevated recovery of cellulase activity in supernatants when the enhancers were present [37,47].

Understanding the partition of cellulases during their heterogeneous catalysis on the hydrolysis of lignocellulosic substances is of great importance for the development of viable biotechnologies to convert biomass feedstocks into valuable chemicals. The enzymatic reaction is interfacial in nature and requires enzyme adsorption onto its insoluble substrate. Adsorptions that are static, irreversible, nonproductive, inhibitory, or inactivating are of concern. Effective mitigation of the detrimental adsorptions may enhance the hydrolysis or reduce the enzyme dosing/cost [17,23,26,47,51,52]. This may be achieved by genetically or chemically modifying cellulases [48,57,58], modifying lignin or cellulose [12,59], optimizing hydrolysis conditions (pH or ionic strength change), or adding enhancers such as non-ionic polymers. Future studies should focus on systems/conditions more relevant to real biomass hydrolysis, and apply various biophysical, biochemical, and genetic techniques to further elucidate the mechanistic aspects.

Acknowledgements

We thank Dr. S. Merino and S. Maiyuran of Novozymes for providing Cel7A- and Cel6A-deleted *T. reesei* culture media, and Dr. J.R. Cherry for critical suggestions.

References

[1] B. Kamm, P.R. Gruber, M. Kamm, Biorefineries—Industrial Processes and Products Status Quo and Future Directions, Wiley-VCH, Weinheim, 2005.
[2] M.E. Himmel, M.F. Ruth, C.E. Wyman, Curr. Opin. Biotechnol. 10 (1999) 358–364.
[3] J. Houghton, S. Weatherwax, J. Ferrell, Breaking the Biological Barriers to Cellulosic Ethanol: A Joint Research Agenda, US Department of Energy, 2006, http://genomicsgtl.energy.gov/biofuels/.
[4] M.E. Himmel, S.-Y. Ding, D.K. Johnson, W.S. Adney, M.R. Nimlos, J.W. Brady, T.D. Foust, Science 315 (2007) 804–807.
[5] Y.-H.P. Zhang, L.R. Lynd, Biotechnol. Bioeng. 88 (2004) 797–824.
[6] L.R. Lynd, P.J. Weimer, W.H. van Zyl, I.S. Pretorius, Microbiol. Mol. Biol. Rev. 66 (2002) 506–577.
[7] F. Xu, in: K. Ohmiya, K. Sakka, S. Karita, T. Kimura, M. Sakka, Y. Onishi (Eds.), Biotechnology of Lignocellulose Degradation and Biomass Utilization, Uni Publishers, Tokyo, 2004, pp. 793–804.
[8] C.P. Kubicek, D.E. Eveleigh, H. Esterbauer, W. Steiner, E.M. Kubicek-Pranz, *Trichoderma reesei* Cellulases, Royal Society of Chemistry, Cambridge, UK, 1990.
[9] N.S. Mosier, P. Hall, C.M. Ladisch, M.R. Ladisch, Adv. Biochem. Eng. Biotechnol. 65 (1999) 23–40.
[10] T.T. Teeri, A. Koivula, C. Divne, M. Linder, G. Wohlfahrt, L. Ruohonen, J. Lehtiö, T. Reinikainen, M. Srisodsuk, K. Kleman-Leyer, T.K. Kirk, T.A. Jones, in: M. Claeyssens, W. Nerinckx, K. Piens (Eds.), Carbohydrases from *Trichoderma reesei* and Other Microorganisms, The Royal Society of Chemistry, Cambridge, UK, 1998, pp. 3–12.
[11] F. Xu, H. Ding, Appl. Catal. A: Gen. 317 (2007) 70–81.
[12] V.J.H. Sewalt, W.G. Glasser, K.A. Beauchemin, J. Agric. Food Chem. 45 (1997) 1823–1828.
[13] B. Nidetzky, W. Steiner, M. Claeyssens, Biochem. J. 303 (1994) 817–823.
[14] P. Valjamae, V. Sild, A. Nutt, G. Pettersson, G. Johansson, Eur. J. Biochem. 266 (1999) 327–334.
[15] T. Eriksson, J. Karlsson, F. Tjerneld, Appl. Biochem. Biotechnol. 101 (2002) 41–60.
[16] H. Palonen, F. Tjerneld, G. Zacchi, M. Tenkanen, J. Biotechnol. 107 (2004) 65–72.
[17] A. Berlin, N. Gilkes, A. Kurabi, R. Bura, M.B. Tu, D. Kilburn, J. Saddler, Appl. Biochem. Biotechnol. 121–124 (2005) 163–170.
[18] S. Kim, M.T. Holtzapple, Bioresour. Technol. 97 (2006) 583–591.
[19] R. Sutcliffe, J.N. Saddler, Biotechnol. Bioeng. 17 (1986) 749–762.
[20] K. Tatsumoto, J.O. Baker, M.P. Tucker, K.K. Oh, A. Mohagheghi, K. Grohmann, M.E. Himmel, Appl. Biochem. Biotechnol. 18 (1988) 159–174.
[21] H. Ooshima, D.S. Burns, A.O. Converse, Biotechnol. Bioeng. 36 (1990) 446–452.
[22] T.D. Bernardez, K. Lyford, D.A. Hogsett, L.R. Lynd, Biotechnol. Bioeng. 42 (1993) 899–907.
[23] Y.P. Lu, B. Yang, D. Gregg, J.N. Saddler, S.D. Mansfield, Appl. Biochem. Biotechnol. 98 (2002) 641–654.
[24] M. Castanon, C.R. Wilke, Biotechnol. Bioeng. 23 (1981) 1365–1372.
[25] H. Ooshima, M. Sakata, Y. Harano, Biotechnol. Bioeng. 28 (1986) 1727–1734.
[26] D.E. Otter, P.A. Munro, G.K. Scott, R. Geddes, Biotechnol. Bioeng. 34 (1989) 291–298.
[27] J.W. Park, Y. Takahata, T. Kajiuchi, T. Akehata, Biotechnol. Bioeng. 39 (1992) 117–120.
[28] S.S. Helle, S.J.B. Duff, D.G. Cooper, Biotechnol. Bioeng. 42 (1993) 611–617.
[29] M. Kurakake, H. Ooshima, J. Kato, Y. Harano, Bioresour. Technol. 49 (1994) 247–251.
[30] F. Kaya, J.A. Heitmann, T.W. Joyce, Tappi J. 78 (1995) 150–157.
[31] S.J.B. Duff, J.W. Moritz, T.E. Casavant, Biotechnol. Bioeng. 45 (1995) 239–244.
[32] I. Ballesteros, J.M. Oliva, J. Carrasco, A. Cabanas, A.A. Navarro, M. Ballesteros, Appl. Biochem. Biotechnol. 70–72 (1998) 369–381.
[33] J. Wu, L.-K. Ju, Biotechnol. Prog. 14 (1998) 649–652.
[34] W.E. Kaar, M.T. Holtzapple, Biotechnol. Bioeng. 59 (1998) 419–427.

[35] T. Eriksson, J. Borjesson, F. Tjerneld, Enzyme Microb. Technol. 31 (2002) 353–364.
[36] M. Mandels, E.T. Reese, J. Ind. Microb. Biotechnol. 22 (1999) 225–240.
[37] B. Yang, C.E. Wyman, Biotechnol. Bioeng. 94 (2006) 611–617.
[38] M. Schülein, J. Biotechnol. 57 (1997) 71–81.
[39] J. Langston, N. Sheehy, F. Xu, Biochim. Biophys. Acta Proteins Proteomics 1764 (2006) 972–978.
[40] M. Lever, Anal. Biochem. 47 (1972) 273–279.
[41] G. Carrard, M. Linder, Eur. J. Biochem. 262 (1999) 637–643.
[42] K. Grohmann, D.J. Mitchell, M.E. Himmel, B.E. Dale, H.A. Schroeder, Appl. Biochem. Biotechnol. 20 (1989) 45–61.
[43] P.J. Gerber, T.W. Joyce, J.A. Heitmann, M. Siika-Aho, J. Buchert, Cellulose 4 (1997) 255–268.
[44] H. Palonen, M. Tenkanen, M. Linder, Appl. Environ. Microbiol. 65 (1999) 5229–5233.
[45] L. Meunier-Goddik, M.H. Penner, J. Agric. Food Chem. 47 (1999) 346–351.
[46] J.-G. Shi, G.-M. Zeng, X.-Z. Yuan, F. Dai, J. Liu, X.-H. Wu, World J. Microbiol. Biotechnol. 22 (2006) 1121–1127.
[47] J.B. Kristensen, J. Borjesson, M.H. Bruun, F. Tjerneld, H. Jørgensen, Enzyme Microb. Technol. 40 (2007) 888–895.
[48] T. Reinikainen, O. Teleman, T.T. Teeri, Proteins: Struct., Funct., Gen 22 (1995) 392–403.
[49] H. Ding, F. Xu, in: B.C. Saha (Ed.), Lignocellulose Biodegradation, Symposium Series 889, American Chemical Society, Washington, DC, 2004, pp. 154–169.
[50] V.M. Chernoglazov, O.V. Ermolova, A.A. Klysov, Enzyme Microb. Technol. 10 (1988) 503–507.
[51] D. Lee, A.H.C. Yu, J.N. Saddler, Biotechnol. Bioeng. 45 (1995) 328–336.
[52] M. Alkasrawi, T. Eriksson, J. Börjesson, A. Wingren, M. Galbe, F. Tjerneld, G. Zacchi, Enzyme Microb. Technol. 33 (2003) 71–78.
[53] E. Vlasenko, J. Cherry, F. Xu, PCT Patent WO/2005/067531 (2005).
[54] J. Borjesson, R. Peterson, F. Tjerneld, Enzyme Microb. Technol. 40 (2007) 754–762.
[55] J. Börjesson, M. Engqvist, B. Sipos, F. Tjerneld, Enzyme Microb. Technol. 41 (2007) 186–195.
[56] H. Kawamoto, F. Nakatsubo, K. Murakami, Mokuzai Gakkaishi 38 (1992) 81–84.
[57] J.W. Park, T. Kajiuchi, Biotechnol. Bioeng. 45 (1994) 366–373.
[58] K.N. Park, J.-W. Park, J. Appl. Polym. Sci. 77 (2000) 368–373.
[59] J.P.H. van Wyk, Biotechnol. Technol. 11 (1997) 443–445.