Monitoring Cellulase Protein Adsorption and Recovery Using SDS-PAGE

Gang Hu,[†] John A. Heitmann, Jr.,*[,†] Orlando J. Rojas,[†,‡] Joel J. Pawlak,[†] and Dimitris S. Argyropoulos[†]

Department of Forest Biomaterials, North Carolina State University, Campus Box 8005, Raleigh NC 27695-8005, and Department of Forest Products Technology, Faculty of Chemistry and Materials Sciences, Helsinki University of Technology, P.O. Box 3320, FIN-02015 TKK, Espoo, Finland

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was employed to study the sorption behaviors of cellulases on microcrystalline celluloses and hardwood pulp. The adsorption and recovery of cellulases from *Aspergillus niger* and *Trichoderma reesei* were investigated at 25 °C. Cellulase recovery was conducted by rinsing adsorbed enzymes with sodium acetate buffer, Milli-Q water, and sodium hydroxide solution. The initial, equilibrium, and recovered enzymes were analyzed using SDS-PAGE gels. Gels were scanned and analyzed using ImagePro software. The molecular weights of cellulase proteins were determined using a protein marker having seven known proteins. The cellulase system from *Trichoderma reesei* had a higher adsorption on all substrates studied than the cellulase system from *Aspergillius niger*, and higher pH favored desorption from the substrates studied. Experimental results also demonstrated that adsorption and desorption amounts determined by SDS-PAGE were proportional to protein concentrations in their crude mixtures.

Introduction

Cellulase enzymes play a central role in the biological conversion of lignocellulosic materials to fermentable sugars. Cellulase mixtures are composed of three types of isozymes, namely, endoglucanases, cellobiohydrolases, and β-glucosidase.[1−4] These components are believed to work synergistically to convert cellulose to simpler sugars. During the conversion, cellulase adsorption is thought to be a prerequisite for the hydrolysis to occur.[3,5,6] Many efforts have focused on the adsorptive behaviors of cellulases under different conditions using a variety of techniques.[4,7−12] The depletion method is by far the most commonly used method to measure cellulose adsorption. In this method, enzyme adsorption is monitored as the difference in either enzyme activity or protein concentration between the original solution and the equilibrium cellulase solution.[13−15] The enzyme activity is usually measured following the method proposed by the International Union of Pure and Applied Chemistry (IUPAC),[16] and dissolved proteins are measured using colorimetric methods such as that using bicinchoninic acid (BCA), the methods of Bradford and Lowry,[17−19] or protein precipitation by acetone and then calculation of the protein difference to obtain adsorption.[7]

The parameters investigated in cellulase adsorption studies have covered the enzyme systems,[20,21] the substrate characteristics,[22,23] and the medium or operational conditions, such as pH, temperature, ionic strength, etc., in which the enzymatic reaction occurred.[24−26] With the development of new analytical methods and deeper understanding of interfacial phenomena, some new methods have been proposed to measure cellulase adsorption. Quartz crystal microgravimetry (QCM), a technique based on the piezoelectric property of quartz crystals, can provide real time, in situ information on the adsorption behavior of cellulase on cellulose films.[27−29] Staining and labeling techniques have been used to visualize the adsorbed cellulase systems on substrates under a microscope.[4] Most recently, elemental analysis has been proposed to directly measure cellulase adsorption on cellulosic substrates.[6] In this method cellulase adsorption is measured from the nitrogen content on solid substrates. Overall, the approaches presented previously have provided important insights on cellulase adsorption and desorption and have contributed to the development of cellulase systems, substrate treatments, and optimization of reaction conditions. However, distinguishing the adsorption of different isozymes used simultaneously remains a challenging task. Therefore, in this research, we propose sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as an analytical means to measure the adsorption and recovery of different isozymes in cellulase mixtures. Cellulase enzyme mixtures can be obtained from many different sources. *Aspergillus niger* and *Trichoderma reesei* are two of the major sources for commercial cellulases.[3] However, no comparison of adsorption behavior of these two cellulase systems was found in the literature. Most existing research focused on the adsorption of cellulase enzymes from *Trichoderma reesei*. Another objective of this research is to obtain this information.

Compared to native gel electrophoresis, SDS-PAGE is a modified, relatively simple, rapid, and highly sensitive tool to study the properties of proteins, primarily the number of components in protein mixtures and their respective molecular weights.[30] In cellulase research, SDS-PAGE has been used to characterize enzymes produced by different sources. However, SDS-PAGE is less commonly used to study the interaction between cellulases and cellulose substrates. In this work, SDS-PAGE was used as an approach to differentiate the adsorption difference of isozymes on three different cellulosic substrates. Also, cellulase recovery was conducted at three different pH levels using sodium acetate buffer (pH 4.8), Milli-Q water (pH $\sim$ 7.0), and sodium hydroxide solution (pH 10.0), after decanting the equilibrium solution from enzyme and cellulosic substrate mixtures and blotting the substrates. The scanned gel images were obtained and examined using ImagePro software. By comparing the isozyme band positions on SDS-PAGE gels and their relative intensities, an estimation of isozyme molecular weight and the relative adsorption and recovery amounts were

* To whom correspondence should be addressed. Phone: 919-757-8418. Fax: 919-515-6302. E-mail: heitmann@ncsu.edu.
[†] North Carolina State University.
[‡] Helsinki University of Technology.

10.1021/ie100731b
Published on Web 08/09/2010

observed. For this work, two cellulases, one from *Asperiglus niger* and another from *Trichoderma reesei*, were used on three different substrates: Avicel PH102, Avicel PH105, and a beaten, bleached hardwood kraft pulp.

Experimental Section

Materials and Instruments. Commercial cellulases were purchased from MP Biomedical Inc. and Sigma Aldrich Co. The cellulase from MP Biomedical Inc. (cat. no. 150583, MPC hereafter) was a yellow dry powder derived from *Aspergillus niger*. The cellulase from Sigma Aldrich (Celluclast, CAS 9012-54-8) was an amber viscous liquid, which was derived from *Trichoderma reesei*. Microcrystalline celluloses (Avicel PH 102 and PH 105) were a gift from FMC BioPolymer Inc. Their nominal particle sizes were 90 and 20 μm, respectively.

Bleached eucalyptus sulfate pulp was purchased from the National Institute of Standards and Technology (NIST, US Department of Commerce, Gaithersburg, MD). This pulp had about 16.7% pentosan content. After it was disintegrated to separate the fiber interlaces, the pulp was beaten to 130 mL CSF (Canadian standard freeness) using a valley beater following TAPPI Method T 200.[31] All pulps were thoroughly washed three times using sodium acetate buffer (pH 4.8, ionic strength 100 mM) and stored in a cold room at 4 °C.

Precast polyacrylamide minigels, 4−20% Precise Protein Gels and 12% Precise Protein Gels, ImmunoPure Lane Marker Reducing Sample Buffer, BupH (tm) Tris-HEPES-SDS Running Buffer, Imperial Protein Stain, SilverSNAP Stain Kit, and Fisher BioReagents EZ-Run Protein Marker were all purchased from Thermo Fisher Scientific Inc. A drying agent was prepared by mixing glycerol, ethanol, and Milli-Q water at concentrations of 3%, 20%, and 77% by volume, respectively.

A Hoefer SE-260 minivertical gel electrophoresis unit coupled with an electrophoresis power supply EPS 301(Amersham Biosciences) was employed for electrophoresis. A 12 × 12 cm Thermo Scientific* Owl* Gel-Dying Kit was used for gel drying using cellophane membranes. A Umax PowerLook III scanner was used to scan the gels. The Image-Pro Plus (Version 4.5.1.22) from Media Cybernetics Inc. was employed to conduct gel band density analysis in transmittance mode (lower transmittance was taken as indicative of higher protein concentration in the gel band). A line profile measurement was selected for image decomposition using a gray scale with 0 and 255 relative units representing black and white, respectively.

Sample Preparation. MP cellulase powders were dissolved in sodium acetate buffer (pH 4.8, ionic strength 100 mM) at a concentration of 20 mg/mL and then diluted to the target concentration using the same buffer solution. Sigma cellulases were handled similarly, but the concentrations were denoted as a volume ratio and the stock solution had a concentration of 2% v/v. All microcrystalline celluloses were hydrated overnight and then centrifugally separated following TAPPI Method UM-256 to remove unbound water.[32]

Cellulase Adsorption and Recovery. Adsorption experiments were performed using the method illustrated in Figure 1. Incubations were performed at a 5% substrate consistency incubated in cellulase solution using a LAB-LINE Incubator-shaker (20 mL of cellulase solution per gram of substrate). After a 1 h incubation, the samples were centrifugally separated using an Eppendorf Centrifuge 5702 following TAPPI Useful Method UM 256[32] and supernatants were collected for SDS-PAGE. The remaining solid substrates were mixed with 20 mL of sodium acetate buffer (pH 4.8), Milli-Q water (pH 7), and caustic water (pH 10), respectively, for enzyme recovery experiments. Recovery experiments were conducted at room temperature. Washing for 30 min was conducted in the LAB-LINE Incubator-shaker, followed by centrifugation to collect the desorbed liquid phase for SDS-PAGE experiments. Before the desorption experiments for the beaten hardwood pulp, ten Kimwipes were used to press the separated pulp to ensure no appreciable residual cellulase solutions were present to interfere with the enzyme recovery.

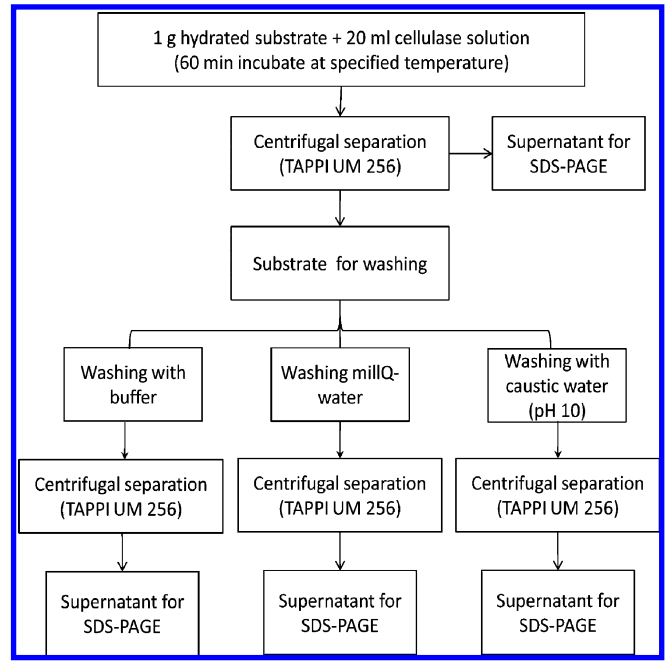

Figure 1. A schematic illustration of cellulase adsorption and recovery experiments to obtain samples for sodium dodecyle sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Electrophoresis. Four parts of sample liquids, including the original cellulase solution, the equilibrium (supernatant) solutions, and the recovered supernatant solutions were mixed with one part of the ImmunoPure Lane Marker Reducing Sample buffer. The sample preparation buffer bearing a red color denatures the cellulase enzymes and serves to indicate where the protein front ends in an electrophoretic run. The prepared mixtures were boiled for 5 min to denature the proteins. After cooling the samples to room temperature, 10 μL samples were placed in gel wells. A control (blank) and a protein marker were each placed into a well when needed. A 5−10 min period was allowed for the proteins to settle down onto the upper surface of the wells, and then electric power was applied to trigger electrophoretic movement. A circulating water bath was employed to prevent overheating of the gel apparatus. Two gels were run simultaneously at 150 V (constant voltage), 240 mA initial current, and 90 min maximum running time. Gel running was discontinued if the red color indicating the protein front reached the bottom of the gels before the end of the set time. Gels were washed in Milli-Q water for 1 h by gently shaking in a shaker bath to remove surfactant. Subsequently, the gels were stained using a SilverSNAP Stain Kit. Stained gels were washed several times to completely remove the staining agent and then dehydrated for at least 30 min by soaking in the drying agent. Thereafter, the gels were air-dried overnight. The dry gels were scanned using the Umax PowerLook III scanner, and images were analyzed using the Image-Pro Plus software.

Results and Discussion

Molecular Weights of Isozymes. The molecular weights of isozymes in MPC and Celluclast were obtained by comparing

Table 1. Proteins, Protein Sources, and Their Molecular Weights in the Protein Marker[a]

protein species	source	molecular weight (kDa)	log (MW)	migration distance[b] (pixels)
β-galactosidase	*E. coli*	116	2.064	184
bovine serum albumin	bovine plasma	66.2	1.821	312
ovalbumin	chicken egg white	45	1.653	424
lactate dehydrogenase	porcine muscle	35	1.544	513
restriction endonuclease Bsp981	*E. coli*	25	1.398	629
β-lactoglobulin	bovine milk	18.4	1.265	735
lysozyme	chicken egg white	14.4	1.158	789

[a] The protein marker was purchased from Fisher Scientific Inc. Information on protein species, sources, and molecular weights were provided by the manufacturer. [b] Migration distance was measured in units of pixels with 400 dpi on a line profile using ImagePro software.

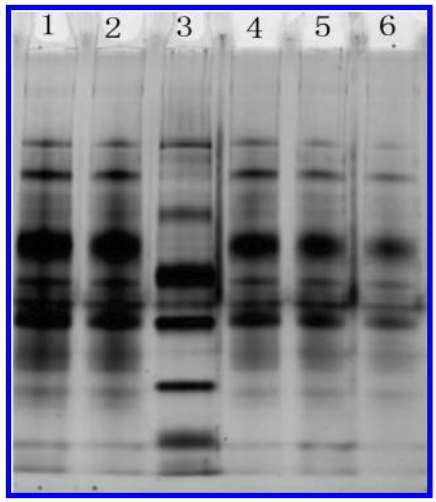

Figure 2. A gel image for the differentiation of isozymes in MPC cellulase. The image also shows the relationship between the band intensities of isozymes and the concentrations of the crude solutions loaded in the wells. MPC cellulase concentrations in well 1 to 2 and 4 to 6 were 0.625 mg/mL, 0.5 mg/mL, 0.4 mg/mL, 0.3 mg/mL and 0.2 mg/mL. The sample in well 3 was the protein marker. The molecular weights of different isozymes were obtained by comparing the migration distances to those of the isozymes in the protein markers (see the text).

their migration distance to a protein marker consisting of seven proteins. Table 1 contains the protein names, the sources for these proteins, and their molecular weights. The fifth column in Table 1 lists the migration distance of different proteins in the protein marker, in units of pixels using a scan mode with 400 dpi resolution. Figure 2 shows the image of a gel that was employed to check the molecular weights of different isozymes in MPC and the relationship between the densities of isozyme bands and the concentrations of sample loadings. The band intensities were analyzed using the scanner line profile analysis and the light transmittance, and the migration distance of the protein marker is recorded in Figure 3.

For isozymes in MPC, a linear correlation was found between the logarithmic value of protein molecular weight MW in the protein marker and the migration distances D_M (pixels): $\log(MW_{MPC}) = -0.00143D_M + 2.28913$, $R^2 = 0.993$, where MW_{MPC} is the isozyme molecular weights of MPC. Similarly, for isozymes in Celluclast, the following fit was obtained: $\log(MW_{Celluclast}) = -0.00171D_M + 2.40717$, $R^2 = 0.993$, where $MW_{Celluclast}$ is the isozyme molecular weights of Celluclast. The molecular weights in both MPC and Celluclast were obtained by applying their respective migration distances (figures not shown) to their respective fit equations as discussed above. Table 2 shows the molecular weights for different isozymes in both MPC and Celluclast.

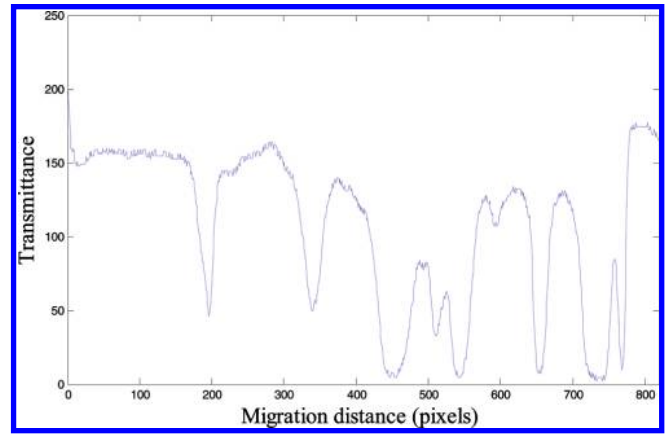

Figure 3. Line profile generated using ImagePro for the protein marker. The scan mode used was in transmittance using a gray scale with 0 corresponding to black and 255 corresponding to white; thus, the lower value indicates higher band intensity. The migration distance was measured from the top of the gel wells where the samples started their migration. Each valley corresponds to a protein. A lower transmittance (higher band intensity) indicates a greater amount of proteins.

Table 2. Molecular Weights of Proteins in Both MPC and Celluclast[a]

	molecular weight of isozyme (kDa)							
cellulase	I	II	III	IV	V	VI	VII	VIII
MPC	110.0	92.2	60.2	47.9	37.6	33.5	29.4	24.6
Celluclast	117.7	98.2	60.0	50.2	37.7	28.8	23.3	18.1

[a] MPC: commercial cellulase from MP Biomedical Inc.

The molecular weights of isozymes in cellulases from both *Trichoderma reesei* (Celluclast) and *Aspergillus niger* (MPC) ranged from about 18 kDa to about 120 kDa. The molecular weights of isozymes IV (see Table 2) are close to those for EGII reported by Goyal et al.,[33] and those of isozymes III are close to the molecular weights of CBHII reported by Kyriacou et al.[34] The number of proteins that can be observed depends on the sensitivity of the staining methods. Eight protein bands were observed in this research from the gels stained by the silver stain kit. The Celluclast contained lower amounts of enzyme proteins with a molecular weight lower than 50 kDa than that of the MPC cellulase. These results are similar to those described by Goyal et al.,[33] who reported molecular weights in the range of 45 to 100 kDa for *Trichoderma reesei* cellulases.

Adsorption on Different Substrates. Cellulase adsorption experiments on three substrates, AvicelPH102, AvicelPH105, and beaten hardwood pulp, were performed at 25 °C. In these experiments, both the initial cellulase solutions (Celluclast and MPC) and the supernatant from the centrifugal separation of the reaction mixtures (1 g substrate with 20 mL enzyme solution, see Figure 1) were loaded into the gels for adsorption comparison. By observing the band intensity differences of those corresponding to the initial solutions versus those for the supernatants separated from different substrates, the adsorption differences can be obtained. The left panel shows the adsorption information for Celluclast and the right panel shows that for MPC (Figure 4). In the gel images, a lighter band (higher transmittance) means lower protein remaining in the equilibrium solution, indicating that more cellulase proteins were adsorbed on the substrates. It is apparent that compared to MPC, Celluclast displayed a higher adsorption on all three substrates,

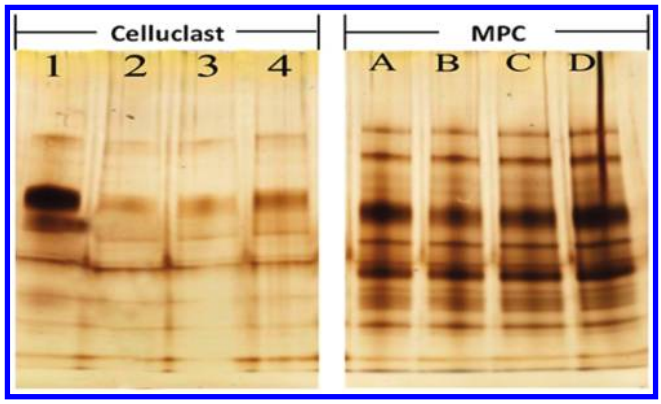

Figure 4. Adsorption of isozymes on three substrates: AvicelPH102, AvicelPH105, and beaten bleached hardwood kraft pulp. The original cellulase concentration for Celluclast was 2% v/v and that for MPC was 5 mg/mL in sodium acetate buffer solutions. All substrates were suspended in aqueous medium at a solid content of 5 wt %. The solutions were diluted by a factor of 10 before loading into gel wells. One and A = original cellulase solution, 2 and B = equilibrium solution from beaten hardwood pulp, 3 and C = equilibrium solution from AvicelPH105, and 4 and D = equilibrium solution from AvicelPH102. Left (1−4) = Celluclast. Right (A−D) = MPC.

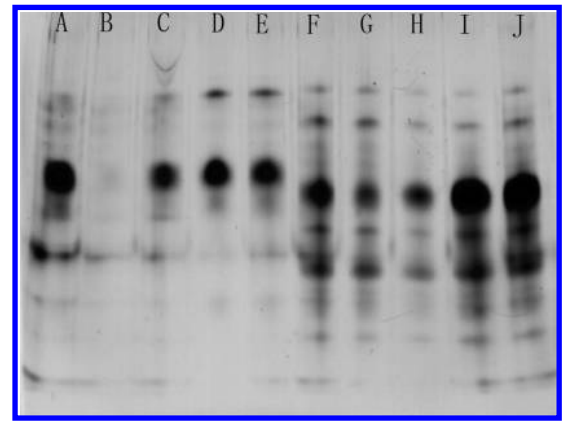

Figure 5. Adsorption on and desorption from AvicelPH105 by cellulases (MPC and Celluclast). Both adsorption and desorption were performed at 25 °C. A−E: Celluclast, F−J: MPC. A and F = original solution with a concentration of 2% v/v and 5 mg/mL, respectively; B and G = equilibrium solution; C and H = recycled at pH 4.8 using sodium acetate buffer; D and I = recycled at pH 7 using Milli-Q water; E and J = recycled at pH 10 using NaOH in water. The volume of recycling agent was half of the original celluase solution used for the adsorption experiments (10 mL vs 20 mL).

which is demonstrated by the different band intensities corresponding to the same substrates (compare the band intensities corresponding to the original cellulase solutions and those corresponding to the residual cellulase solutions after adsorption on the different substrates). For Celluclast, the higher adsorptions occurred with most of the isozymes having a molecular weight greater than 30 kDa. Both cellulase mixtures, the Celluclast and MPC, showed less adsorption on the microcrystalline celluloses than on the beaten hardwood pulp. AvicelPH105 adsorbed more enzymes than AvicelPH102, which is consistent with the respective surface areas (AvicelPH105 has a higher surface area than AvicelPH102). Note that both MCCs have about the same crystallinity of 82−83% from X-ray diffraction.[35] The comparison of cellulase adsorption on hardwood pulp to that on microcrystalline cellulose is more complicated because of the presence of pentosans and amorphous regions in the case of the pulp.[36] It was not clear whether any of the pentosan was removed during beating and washing treatments, and the interaction between cellulase and pentosan was not investigated.

The crystallinity of the hardwood pulp (~63%) was lower than those for the MCCs. The surface areas of AvicelPH102, AvicelPH105, and the bleached beaten hardwood pulp were 0.73, 1.43, and 7.0 m^2/g using the BET nitrogen adsorption technique. As far as the measured parameters were concerned, both the crystallinity and surface areas appeared to affect cellulase adsorption. The results for MPC adsorption on the three substrates were consistent with the results obtained in another investigation using the depletion method where the protein concentrations were determined by UV−vis absorption at a wavelength of 280 nm. The maximum adsorptions found on AvicelPH102, AvicelPH105, and the beaten hardwood pulp were 3.0, 5.0, and 36.8 mg/g-substrate, respectively. It was also observed that the relative ratio of isozymes in the two cellulase mixtures were different. The isozyme amounts in MPC mixtures were more evenly distributed.

A great deal of research has focused on the effect of physical properties on cellulase adsorption and cellulose hydrolysis.[4,37−40] Some researchers have shown that the crystallinity of the substrate plays a more significant role in affecting the extent of enzyme binding and hydrolysis than the respective surface area,[39] whereas some others have attributed more importance to the surface area (or porosity).[38] Our current research supports the opinion that both surface areas and crystallinity affect the cellulase adsorption. By observing the band intensity changes of different isozymes, it can also be concluded that most isozymes in the cellulase mixtures showed adsorption on the substrates studied in this investigation which was in proportion to their concentrations, that is, adsorption nonspecific to the isozyme involved.

Enzyme Recovery. Enzyme recovery was conducted by experiments with the three cellulosic substrates at 25 °C, using the method presented in Figure 1. As explained in the Experimental Section, enzyme recovery was conducted by incubating the recovery solutions (sodium acetate buffer, Milli-Q water, and sodium hydroxide solution) with substrates separated from the adsorption experiments. Figure 5 shows information about the adsorption and subsequent desorption/recovery of all MPC and Celluclast enzymes from AvicelPH105. The original enzyme solution and adsorption equilibrium solutions were both diluted by a factor of 10 to ensure legible band separation, while recovery equilibrium solutions were run without dilution. Since the recycling reagent volumes were half of the amounts used for the adsorptions (10 mL vs 20 mL, see Experimental Section, Figure 1), the same gel band intensity would indicate a concentration of 20 times higher in the original and adsorption equilibrium solutions than in the recovery solutions. The band intensity for equilibrium solutions supported the previous observation that Celluclast adsorbed on the substrate to a larger extent than MPC and that the extent of adsorption was proportional to isozyme solution concentration. The adsorption difference between the two cellulase mixtures can be explained by the existence of different cellulolytic enzymes in the two cellulase systems. Cellulclast is prepared from *Trichoderma reesei*, which produces larger amounts of CBH and EG, while MPC is prepared from *Aspergillus niger*, which produces relatively less CBH and EG and more β-glucanase. These differences can cause the different affinity phenomena described above.[3]

Figure 5 shows that the pH of the solution used to recover the enzymes played an important role in recovery efficiency. It was apparent that higher pH produced higher desorption from AvicelPH 105, for both cellulase enzymes. This was also true for the other two substrates, AvicelPH 102 and beaten hardwood pulp (figures not shown). A hypothesis for the underlying

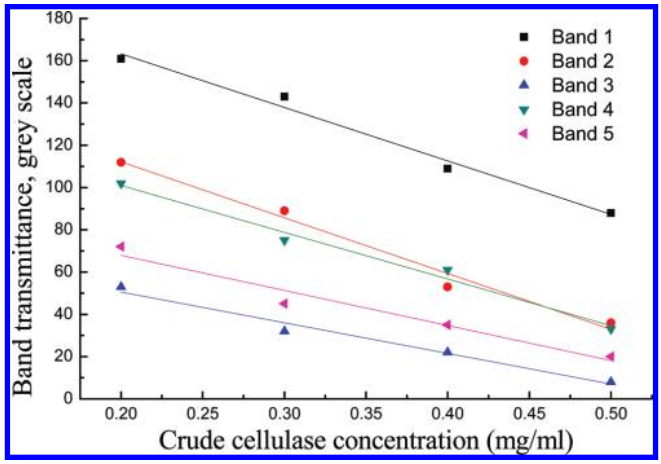

Figure 6. Relationship of isozyme band intensity to cellulase concentration. The four concentrations correspond to wells 2, 4, 5, and 6 in Figure 2 (for MPC cellulase). Correlation coefficients for isozyme 1 to isozyme 5 are 0.98, 0.97, 0.97, 0.98, and 0.94, respectively. Only the most intensive five bands were analyzed in terms of the relationship of band intensity to cellulase concentration.

desorption mechanism is that a low pH (especially pH 4.8, since it has been generally accepted the cellulase enzymes work best at this pH) provides better conditions for the interaction between cellulase isozymes and cellulosic substrates. The washing of substrates therefore could allow a continuation of the hydrolysis process once buffer solutions were added; however, the zero concentration of cellulase in washing with a fresh buffer solutions would favor the dynamic equilibrium to move in the desorption direction. This agrees with the optimum pH range for cellulose hydrolysis by celluase. At pH 7, the buffer conditions for cellulase to present tertiary or quarternary structure may be less favorable. Therefore, the binding domains or even the catalytic domains may not perform at their optimum state, which caused a larger desorption of the adsorbed protein. As a result, this would favor a change of protein structure that may cause the cellulases to have a reduced interaction with the cellulose substrate. At an even higher pH (pH 10 in this research), the hydroxyl ions in the sodium hydroxide solution may have chemical interactions with the side chains of some amino acid residues within the protein chains, especially with those whose side chains are acidic. As a consequence, the hydrogen bond or hydrophobic interactions of cellulase with the substrates is disrupted, which is indicated by the larger recovery at higher pH level. Meanwhile, the swelling of cellulose surfaces at higher pH may have also contributed to increased desorption behavior at pH 10.

Relationship of Band Intensity with Cellulase Concentration. An analysis of the relationship between the band intensity of isozymes with the concentrations of the original cellulase solutions was performed by plotting the transmittance obtained in the line profile as a function of the crude cellulase concentrations (Figure 6), by which a numerical comparison of adsorption and recovery may be obtained. The band intensity of isozymes from gel images was analyzed using the Matlab 2008b software. The intensities of protein bands for four different cellulase concentrations (Figure 6) were determined from their line profiles (figure not shown). The five isozymes or proteins (isozyme fragments) that have the most appreciable bands were analyzed. Figure 6 shows the correlation of the band intensities for the five isozymes that can be easily detected by SDS-PAGE with the concentrations of their original cellulase solutions. The correlation coefficients for the five isozymes (isozyme I−V in Table 2) are 0.98, 0.97, 0.97, 0.98, and 0.94, respectively, which show good linear relationships between the band intensities and the original concentrations of the cellulase solutions in which isozymes existed. The isozymes or proteins with higher concentrations, which can be determined from higher band intensity, gave a better correlation to their original concentrations. Notably, the lines in Figure 6 should be parallel to each other. The pronounced deviation of the line corresponding to band 2 from being parallel to the others may have been caused by a poor scan quality, which subsequently caused the line analysis to be less accurate. The goodness of fit also depends on the staining technique, which can affect the levels of background noise.

The correlation analysis of band intensities versus the concentrations of crude cellulase solutions may serve as calibrations by which a comparison of band intensities of isozymes from gel images for the equilibrium supernatant and recovery solutions with similar migration distances can give residual protein concentrations. This analysis can also work on cellulase recovery by comparing the corresponding band intensities of the recovery solution. It can also be expected that the relative amounts of different isozymes can be determined by using this method. However, development of a comprehensive algorithm is constrained by measurement of the peak areas and overlap of protein bands. Due to these limitations, no further quantitative analyses of cellulase adsorption and recovery ratios were carried out in this work. However, the authors believe that this approach can be developed into a comprehensive method for quick and quantitative analysis of cellulase adsorption and recovery.

Conclusion

The molecular weights of isozymes in cellulases from both *Trichoderma reesei* (Celluclast) and *Aspergillus niger* (MPC) ranged from 18 to 120 kDa. This was obtained from image analysis of line profiles from SDS-PAGE gels. The relative ratios of isozymes are different in the two enzyme mixtures. *Trichoderma reesei* (Celluclast) cellulase contained three major components while the other isozymes were present in small amounts. The *Aspergillus niger* (MPC) cellulase contained more evenly distributed isozymes (eight isozymes were detected). All isozymes adsorbed in proportion to their concentration on the three substrates. The isozymes from *Trichoderma reesei* (Celluclast) showed much greater adsorption than the ones from *Aspergillus niger* (MPC). The microcrystalline cellulose with higher surface area adsorbed more isozymes than the one with lower surface area and similar crystallinity. The adsorption results show that both cellulose crystallinity and surface areas affected cellulase adsorption. Both Celluclast and MPC were recovered to different extents at the three pH values used in the experiments. Enzyme recovery increased in the following order: sodium acetate buffer (pH 4.8), (b) Milli-Q water (pH ~7.0), and (c) sodium hydroxide solution (pH 10.0) for both enzyme systems. A linear relationship between the intensities of SDS-PAGE bands corresponding to different isozymes and the concentrations of the crude cellulase solutions was found. Finally, a method to measure protein adsorption and recovery based on the SDS-PAGE technique is suggested for future research.

Literature Cited

(1) Henrissat, B. Cellulases and their interaction with cellulose. *Cellulose* **1994**, *1* (3), 169–196.

(2) Wyman, C. E. Biomass ethanol: Technical progress, opportunities, and commercial challenges. *Annu. Rev. Energy Environ.* **1999**, *24*, 189–226.

(3) Lynd, L. R.; Weimer, P. J.; Zyl, W. H. v.; Pretorius, I. S. Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol. Mol. Biol. Rev.* **2002**, *66* (3), 506–577.

(4) Jackson, L. S.; Heitmann, J. A., Jr.; Joyce, T. W. Visualization of Enzyme Binding to Secondary Fiber Silver-Enhanced Colloidal Gold. *Prog. Pap. Recycl.* **1994**, *3* (2), 32–41.

(5) Hu, G.; Heitmann, J. A.; Rojas, O. J. Feedstock pretreatment strategies for producing ethanol from wood, bark and forest residues. *BioResouces* **2008**, *3* (1), 270–294.

(6) Kumar, R.; Wyman, C. E. An improved method to directly estimate cellulase adsorption on biomass solids. *Enzyme Microb. Technol.* **2008**, *42* (5), 426–433.

(7) Peitersen, N.; Medeiros, J.; Mandels, M. Adsorption of *Trichoderma* cellulase on cellulose. *Biotechnol. Bioeng.* **1977**, *19* (7), 1091–1094.

(8) Ryu, D. D. Y.; Kim, C.; Mandels, M. Competitive adsorption of cellulase components and its significance in a synergistic mechanism. *Biotechnol. Bioeng.* **1984**, *26* (5), 488–496.

(9) Ooshima, H.; Sakata, M.; Harano, Y. Adsorption of cellulase from *Trichoderma viride* on cellulose. *Biotechnol. Bioeng.* **1983**, *25* (12), 3103–3114.

(10) Lee, S. B.; Shin, H. S.; Ryu, D. D. Y.; Mandels, M. Adsorption of cellulase on cellulose: Effect of physicochemical properties of cellulose on adsorption and rate of hydrolysis. *Biotechnol. Bioeng.* **1982**, *24* (10), 2137–2153.

(11) Beltrame, P. L.; Carniti, P.; Focher, B.; Marzetti, A.; Cattaneo, M. Cotton cellulose: enzyme adsorption and enzymic hydrolysis. *J. Appl. Polym. Sci.* **1982**, *27* (9), 3493–3502.

(12) Rabinovich, M. L.; Klesov, A. A.; Grigorash, S. Y.; Kalnina, I. A.; Chernoglazov, V. M. Enzymic hydrolysis of cellulose. VI. Adsorption of cellobiase from a cellulase complex on cellulose. *Bioorg. Khim.* **1982**, *8* (1), 84–95.

(13) Singh, A.; Kumar, P. K. R.; Schugerl, K. Adsorption and Reuse of Cellulases during Saccharification of Cellulosic Materials. *J. Biotechnol.* **1991**, *18* (3), 205–212.

(14) Girard, D. J.; Converse, A. O. Recovery of cellulase from lignaceous hydrolysis residue. *Appl. Biochem. Biotechnol.* **1993**, *39*−*40*, 521–533.

(15) Castanon, M.; Wilke, C. R. Adsorption and recovery of cellulases during hydrolysis of newspaper. *Biotechnol. Bioeng.* **1980**, *22* (5), 1037–1053.

(16) Ghose, T. K. Measurement of cellulase activities. *Pure Appl. Chem.* **1987**, *59* (2), 257–268.

(17) Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **1985**, *150* (1), 76–85.

(18) Bradford, M. M. Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing Principle of Protein-Dye Binding. *Anal. Biochem.* **1976**, *72* (1−2), 248–254.

(19) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein Measurement with the Folin Phenol Reagent. *J. Biol. Chem.* **1951**, *193* (1), 265–275.

(20) Ooshima, H.; Burns, D. S.; Converse, A. O. Adsorption of cellulase from *Trichoderma reesei* on cellulose and lignacious residue in wood pretreated by dilute sulfuric acid with explosive decompression. *Biotechnol. Bioeng.* **1990**, *36* (5), 446–452.

(21) Kim, D. W.; Yang, J. H.; Jeong, Y. K. Adsorption of cellulase from *Trichoderma viride* on microcrystalline cellulose. *Appl. Microbiol. Biotechnol.* **1988**, *28* (2), 148–154.

(22) Kyriacou, A.; Neufeld, R. J.; Mackenzie, C. R. Effect of Physical Parameters on the Adsorption Characteristics of Fractionated *Trichoderma reesei* Cellulase Components. *Enzyme Microb. Technol.* **1988**, *10* (11), 675–681.

(23) Converse, A. O.; Girard, D. J. Effect of substrate concentration on multicomponent adsorption. *Biotechnol. Prog.* **1992**, *8* (6), 587–588.

(24) Tanaka, M.; Nakamura, H.; Taniguchi, M.; Morita, T.; Matsuno, R.; Kamikubo, T. Elucidation of adsorption processes of cellulases during hydrolysis of crystalline cellulose. *Appl. Microbiol. Biotechnol. Section Title: Enzymes* **1986**, *23* (3−4), 263–268.

(25) Eriksson, T.; Borjesson, J.; Tjerneld, F. Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. *Enzyme Microb. Technol.* **2002**, *31* (3), 353–364.

(26) Ding, H. Adsorption and synergism of cellulases during hydrolysis of cellulosic materials. Ph.D. Thesis, University of California, Davis, 2000.

(27) Rojas, O. J.; Jeong, C.; Turon, X.; Argyropoulos, D. S. Measurement of cellulase activity with piezoelectric resonators. *ACS Symp. Ser.* **2007**, *954*, 478–494 (Materials, Chemicals, and Energy from Forest Biomass).

(28) Turon, X.; Rojas, O. J.; Deinhammer, R. S. Enzymatic kinetics of cellulose hydrolysis: A QCM-D study. *Langmuir* **2008**, *24* (8), 3880–3887.

(29) Hu, G.; Heitmann, J. A.; Rojas, O. J. In Situ Monitoring of Cellulase Activity by Microgravimetry with a Quartz Crystal Microbalance. *J. Phys. Chem. B* **2009**, *113* (44), 14761–14768.

(30) Hames, B. D.; Rickwood, D. *Gel electrophoresis of proteins: a practical approch*, 2nd ed.; Oxford University Press: Cary, NC, 1990; p 383.

(31) TAPPI. Laboratory Beating of Pulp (Valley Beater Method). In *Test Methods T 200 sp-96*; TAPPI Press: Atlanta, GA, 1998; p 9.

(32) TAPPI. Water Retention Value (WRV). In *TAPPI Useful Methods UM 256*; TAPPI Press: Atlanta, GA, 1991; Vol. 1991, pp 54−56.

(33) Goyal, A.; Ghosh, B.; Eveleigh, D. Characteristics of fungal cellulases. *Bioresour. Technol.* **1991**, *36* (1), 37–50.

(34) Kyriacou, A.; MacKenzie, C. R.; Neufeld, R. J. Detection and characterization of the specific and nonspecific endoglucanases of *Trichoderma reesei*: Evidence demonstrating endoglucanase activity by cellobiohydrolase II. *Enzyme Microb. Technol.* **1987**, *9* (1), 25–32.

(35) Hu, G. Adsorption and Activity of Cellulase Enzymes on Various Cellulose Substrates. Ph.D. Thesis, North Carolina State University, Raleigh, NC, 2009.

(36) Willför, S.; Sundberg, A.; Pranovich, A.; Holmbom, B. Polysaccharides in some industrially important hardwood species. *Wood Sci. Technol.* **2005**, *39* (8), 601–617.

(37) Jackson, L. S. Interaction of Cellulase and Xylanase with Recycled Bleached Kraft Fiber. Ph.D. Thesis, North Carolina State University, Raleigh, NC, 1996.

(38) Grethlein, H. E. The Effect of Pore Size Distribution on the Rate of Enzymatic Hydrolysis of Cellulosic Substrates. *Nat. Biotechnol.* **1985**, *3* (2), 155–160.

(39) Fan, L. T.; Lee, Y.-H.; Beardmore, D. H. Mechanism of the enzymatic hydrolysis of cellulose: Effects of major structural features of cellulose on enzymatic hydrolysis. *Biotechnol. Bioeng.* **1980**, *22* (1), 177–199.

(40) Fan, L.; Lee, Y. H.; Beardmore, D. Major chemical and physical features of cellulosic materials as substrates for enzymatic hydrolysis. *Adv. Biochem. Eng.* **1980**, *14*, 101–117.

Received for review March 26, 2010
Revised manuscript received July 13, 2010
Accepted July 24, 2010

IE100731B