Available online at www.sciencedirect.com
ScienceDirect
Enzyme and Microbial Technology 42 (2008) 426–433
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

An improved method to directly estimate cellulase adsorption on biomass solids

Rajeev Kumar [a], Charles E. Wyman [b,*]

[a] *Thayer School of Engineering, Dartmouth College, New Hampshire 03755, USA*
[b] *Chemical and Environmental Engineering Department and Center for Environmental Research and Technology (CE-CERT), University of California, 1084 Columbia Avenue, Riverside, CA 92507, USA*

Received 19 October 2007; received in revised form 20 December 2007; accepted 21 December 2007

Abstract

Based on nitrogen factors (NF) determined for two commercial cellulases produced by the fungus *Trichoderma reesei*, a nitrogen analyzer was used to directly estimate the amount of enzyme adsorbed on biomass solids, rather than relying on the difference between initial and final dissolved protein as typically practiced. For adsorption of cellulase at 4 °C where reactions are minimal, the results for this method agreed closely with those indirectly determined by the usual BCA measurements of dissolved protein. But for hydrolysis at 50 °C, the BCA method indicated that much less protein was adsorbed on the solids compared to the NF method. Because the reagents used in conventional methods are very sensitive to reducing sugars, reagents, chemicals, and especially lignin components released during hydrolysis of lignocellulosic biomass, the result was a high amount of free protein in solution which indirectly affected the amount of protein adsorbed on the solid. Another important advantage of this approach compared to conventional methods is that material balances on protein can be closed. Application of the NF method revealed that sugars and especially cellobiose strongly inhibit cellulase binding, and cellulase binding was completely shut down at cellobiose concentrations greater than 150 g/L. However, this binding inhibition can be reversed using a higher ratio of substrate concentration to inhibitors.

Keywords: Adsorption; Cellulase; Nitrogen; *Trichoderma reesei*; Inhibition; Binding

1. Introduction

The maximum amount of protein that can be adsorbed during enzymatic saccharification of cellulose to glucose is a controlling factor for hydrolysis rates and yields and directly depends on enzyme accessibility to active sites on the solid substrate [1–5]. For low levels of loadings such as with purified cellulase components, a spectrophotometer is typically used to estimate enzyme adsorption based on an extinction coefficient at A_{280}, and FPLC is widely employed to estimate the amount of dissolved protein [6–8]. However, no methods have been reported to directly measure protein adsorption on the surface for higher loadings of either fractionated or complete cellulase systems that are of interest commercially. Instead dissolved protein is measured using colorimetric methods such as BCA, Bradford, and Lowry [9–11] or protein precipitation by acetone [12], with protein adsorption calculated as the difference between the total amount of protein initially added and the amount left in solution at any time. Thus, the values are indirectly determined, and material balances cannot be used to check accuracy. For samples having large amounts of protein, these conventional methods are more prone to flawed results because high protein samples must be diluted to bring the protein concentrations in the range of the standards (1–2 mg/ml) used for calibration. In addition, several studies showed that various substances can interfere with spectrophotometric readings for such procedures [13,14]. Furthermore, the problem may be compounded when working with heterogeneous biomass containing xylan, lignin, glucan, protein, extractives, and ash, because the reagents used in BCA, Bradford, and other assays are very sensitive to dissolved sugars, chemicals, salts, color compounds, and other components that may be released during processing. In this regard, Fig. 1 illustrates the reactivity of BCA reagents with cellobiose, glucose, and xylose. Consequently, estimations of dissolved protein via BCA and Bradford methods may be erroneous for complex cellulosic substrates [15]. Alternatively, components that could

* Corresponding author. Tel.: +1 951 781 5703.
E-mail address: cewyman@engr.ucr.edu (C.E. Wyman).

0141-0229/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2007.12.005

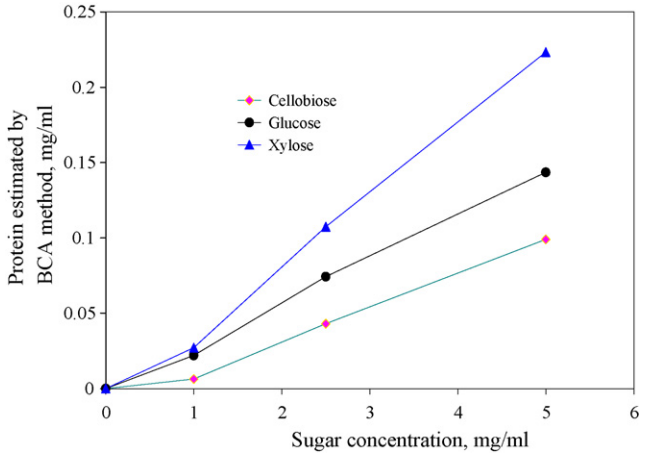

Fig. 1. Amount of protein estimated by the BCA method in response to color developed with different sugars.

interfere with measurements can be removed by ultrafiltration or protein can be precipitated out of solution before quantification [12]. But such approaches further complicate measurements and introduce other potential errors. Although the Kjeldahl method directly measures protein adsorption on solids and is applied to determine the nitrogen and protein content in a range of products such as fertilizers, food, and biomass [16–18], the complex and tedious nature of this prolonged procedure makes it impractical for routine biomass analysis. Thus, a simple and reliable method was needed to directly measure protein adsorption on solids [19], and this study reports a new approach and its efficacy in estimating the amount of adsorbed enzyme at low temperatures and for reaction at hydrolysis conditions.

2. Materials and methods

2.1. Enzymes and other proteins

Spezyme-CP cellulase (lot # 301-04075-034; 59 FPU/ml, 123 mg protein/ml), GC-220 cellulase (lot # 301-04232-162; 90 FPU/ml, 184 mg protein/ml), and Multifect xylanase (lot # 301-04021-015; 42 mg protein/ml) enzymes were generously provided by Genencor International (Rochester, NY, USA). Another cellulase in lyophilized form, BioChemika, *Trichoderma reesei*, lot # 1086305, was purchased from Sigma Chemicals (St. Louis, MO), as were bovine serum albumin and chicken egg albumin.

2.2. Substrates

Pure cellulose, Avicel PH-101, was purchased from FMC Corporation (Philadelphia, PA) (cat # 11365, lot # 1094627). Solids prepared by corn stover pretreatment in a Sunds reactor (Metso Paper USA, Inc. Norcross, GA, USA) at 180 °C for 1.5 min with 3% sulfuric acid followed by hot water washing and by pretreatment at 190 °C for 5 min with 3% SO_2 followed by washing were generously provided by the National Renewable Energy Laboratory (NREL, Golden, Colorado) and the University of British Columbia in Canada, respectively. Poplar solids prepared by ammonia recycled percolation (ARP) pretreatment at 185 °C for 27.5 min with a NH_3 to dry biomass ratio of ~3.66 were generously provided by Auburn University. Their compositions as determined by NREL procedures [20] are reported in Table 1.

Table 2
Nitrogen and solids content of Spezyme CP

Drying method	% Nitrogen	Average (standard deviation)	Solids (mg/ml)
Oven @ 105 °C	3.68	3.72 (0.04)	386.0
	3.69		
	3.72		
	3.77		
Oven @ 50 °C	3.93	3.93 (0.05)	382.0
	4.00		
	3.88		
	3.90		
Vacuum dried @ 50 °C	3.89	3.87 (0.03)	374.0
	3.91		
	3.85		
	3.84		
Freeze drying @ −42 °C	3.62	3.74 (0.12)	397.0
	3.74		
	3.87		
	3.73		
Overall average	3.80		384.8

2.3. Nitrogen factor

Lyophilized cellulase and other protein solids were assumed to be pure protein to facilitate estimating the fraction of protein in each. However, because commercial liquid enzyme preparations contain diluents such as sorbitol ($C_6H_{14}O_6$) and sodium chloride (NaCl) and preservatives such as potassium sorbate ($C_6H_7O_2K$) [21], they were dried to determine their total solids content by NREL procedures [22], and the fraction protein was then calculated based on the amount of protein per ml as determined by the standard BCA method [9]. To determine whether protein was lost during drying, it was dried at 105 and 50 °C in a conventional oven (model EW-52501-03, Cole-Parmer Instrument Company, Illinois, USA), at 50 °C in a vacuum oven (model C-05053-10, Cole-Parmer Instrument Company, Illinois, USA), and at −42 °C in a freeze drier (model 10-270B, Labconco Corporation, Kansas City, Missouri). The nitrogen content of the dried and homogenized solids for enzymes and other proteins was then measured using a Flash EATM 112 N/Protein plus CHNS/O Analyzer (CE Elantech, Lakewood, NJ) with aspartic acid/BBOT (2,5-bis-(5-tert-butyl-benzoxazol-2-yl)-thiophen) as a standard (CE Elantech, Lakewood, NJ). Table 2 summarizes the nitrogen and solids content for Spezyme-CP from which nitrogen factor was calculated by the following formula [23]:

$$\text{Nitrogen factor (NF)} = \frac{\%\ \text{protein}}{\%\ \text{nitrogen}}$$

Table 1
Composition of solid substrates

Substrate solid	Pretreatment method and conditions	% Glucan	% Xylan	% Lignin
Avicel-PH 101	None	96.5	1.8	None
Pretreated corn stover	Dilute acid/Sunds reactor 180 °C, 3%H_2SO_4, 1.5 min, 25% solids-W*	59.3	9.3	22.5
Pretreated corn stover	SO_2/190 °C, 3%SO_2, 5 min-W	56.9	11.6	23.8
Pretreated poplar	ARP/185 °C, NH_3:biomass ~3.66, 27.5 min-W	57.5	13.5	24.8

W: washed.

Table 3
Nitrogen factors determined for selected enzymes and proteins and % nitrogen from literature

Enzyme/protein-commercial name	% Protein	% Avg. nitrogen	Nitrogen factor (NF)
Cellulase-Spezyme CP	32.0 ± 0.8	11.9 ± 0.5	8.40 ± 0.3
Cellulase-GC-220	45.3 ± 0.6	12.4 ± 0.4	7.90 ± .3
Multifect xylanase	12.7 ± 0.4	12.1 ± 0.5	8.28 ± 0.3
Lypolyzed cellulase (BioChemika)	∼100	11.9 ± 0.0	8.39 ± 0.0
Bovine serum albumin (BSA)	∼100	14.0 ± 0.2	7.16 ± 0.1
Chicken egg albumin	∼100	12.8 ± 0.2	7.79 ± 0.1
CBHI of *T. reesei*[a]	–	13.7 ± 0.5	–
CBHII of *T. reesei*[a]	–	13.9 ± 0.5	–
EGI of *T. reesei*[a]	–	13.7 ± 0.5	–
Amino acid residue of CBHI[b]	–	13.8 ± 0.8	–
Amino acids	–	14.2 ± 6.4	–

[a] Nitrogen was determined from amino acid composition of CBHI, CBHII, and EGI of *T. reesei* [50].
[b] CBHI of Trichoderma pseudokiningii S-38, Xu et al. [51].

As an example for Spezyme CP (Table 2),

$$\%\,\text{Protein} = 100 \times (\text{amount of protein per ml/amount of solid per ml})$$
$$= 100 \times (123/384) = 31.96, \qquad \text{NF} = 31.96/3.80 = 8.40.$$

2.4. Calculation of protein adsorption

After drying the substrate, a small fraction (≤6 mg) of each homogenized sample, prepared with pestle and mortar, was weighed in replicate into tin capsules (cat # 240-064-40, CE Elantech, Lakewood, NJ) and sealed. Then the amount of protein adsorbed on the solid substrate was calculated from the nitrogen measurements using the CHNS/O Analyzer. Because lignocellulosic substrates also contain some protein, it was necessary to subtract the amount of nitrogen naturally occurring in each substrate from the total reading to determine the nitrogen content from the adsorbed protein. The nitrogen content measured in this way was converted into the corresponding protein content using the NF established for the enzyme used, and the amount of protein adsorbed in mg per mg of substrate was then calculated.

2.5. Adsorption and hydrolysis experiments

Adsorption experiments were performed in replicate using 0.05N citrate buffer at 4 °C in 50 ml test tubes with a biomass loading containing 0.5–1% glucan (0.5–1% glucan for pure Avicel cellulose) and different cellulase loadings. After equilibration overnight, the tubes were centrifuged and dried at 105 °C for 2 days for further nitrogen analysis. The supernatant was used to determine the amount of protein in solution by the BCA method. Consistent with NREL procedures [24], cellulose hydrolysis was performed in 0.05N citrate buffer (pH 4.8) with tetracycline (200–400 μl of 10 mg/ml in 70%, v/v ethanol) and cyclohexamide (150–300 μl of 10 mg/ml in water) as antibiotics at 50 °C in 125 ml Erlenmeyer flasks with biomass loading containing 1% glucan (1% glucan for pure Avicel) and a total volume of 50 or 100 ml with a given enzyme loading for 4 h. Enzyme action was first stopped by placing the tubes in boiling water for 5 min, and the solids were then centrifuged and dried at 105 °C for further nitrogen analysis. Supernatants were collected to determine the amount of protein in solution and the amount of sugar generated during the course of hydrolysis using a Waters Alliance HPLC system (Model 2695, Waters Corporation, Milford, MA) employing Aminex HPX-87H columns (Bio-Rad laboratories, life science research, Hercules, CA).

3. Results

3.1. Nitrogen factors and protein mass balances

Nitrogen factors (NF) were determined for Spezyme CP cellulase, GC-220 cellulase, Multifect xylanase, lyophilized cellulase (BioChemika), bovine serum albumin (BSA), and chicken egg albumin by the methods described above and found to be very similar, as shown in Table 3. Thus, the NF of these enzymes and protein sources could be used somewhat interchangeably with minimal impact on the results. Based on these values, the nitrogen content of these proteins were determined to be in the range of 11–14%. Furthermore, for Spezyme CP and GC 220 cellulase adsorption on Avicel PH-101 at 4 °C in citrate buffer, adding the measurement of protein on the solids based on the nitrogen factor to that measured in solution by the BCA method

Table 4
Protein mass balances for Spezyme-CP and GC-220 adsorption on Avicel at 4 °C

Enzyme	Loadings	A-Protein adsorbed on Avicel (mg) by NF	F-Free protein in solution by BCA (mg)	A + F - Total protein measured (mg)	Total protein added initially (mg)
Spezyme-CP	A	6.15	10.3	16.5	18.0
	B	13.8	16.1	29.8	30.0
	C	23.8	33.9	57.6	56.0
	D	30.1	51.4	81.5	81.0
GC-220	A	10.4	11.0	21.4	20.5
	B	21.8	25.0	47.0	35.0
	C	25.0	37.8	62.8	60.0
	D	28.0	62.5	90.5	85.0

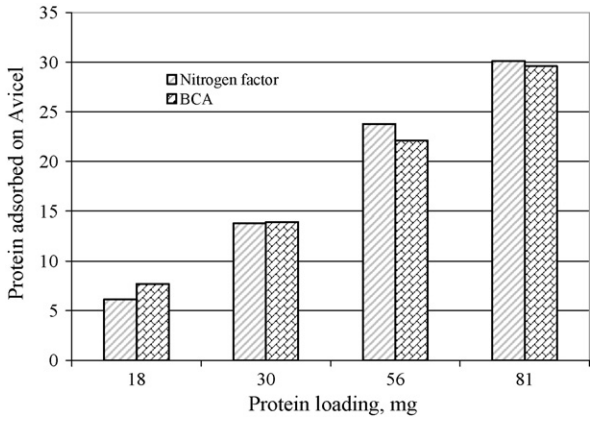

Fig. 2. Amount of protein adsorbed on Avicel at 4 °C for Spezyme CP cellulase as measured by the nitrogen factor and BCA methods at different protein loadings.

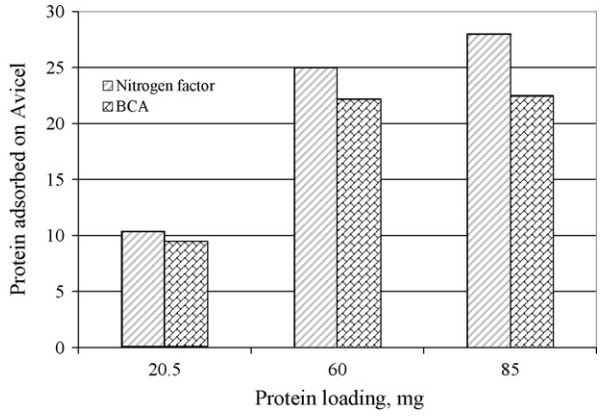

Fig. 3. Amount of protein adsorbed on Avicel at 4 °C for GC220 cellulase as measured by the nitrogen factor and BCA methods at different protein loadings.

agreed very closely with the total added initially for all but one of the protein loadings, as shown in Table 4.

3.2. Protein adsorbed at 4 °C

The adsorption of Spezyme CP and GC 220 cellulases on Avicel was performed in citrate buffer (pH 4.8). The amounts of protein (mg) adsorbed on the solids determined from the direct measurement of nitrogen content with the nitrogen analyzer and indirectly by subtracting the amount of free protein determined by the BCA method from total initially loaded were close. However, the NF measurements tended to be a little higher than those determined for the BCA method, as shown in Figs. 2 and 3 for Spezyme CP and GC220, respectively.

3.3. Protein adsorbed during hydrolysis

Avicel PH-101 and SO_2 pretreated corn stover solids were enzymatically hydrolyzed for 4 h at a loading of 50 FPU/g cellulose (~1.05 mg/ml) as described in Section 2. Almost 58% of the cellulose in Avicel was reacted at these conditions, whereas 95 and 28% of the cellulose and xylan in SO_2 pretreated corn stover were digested, respectively. As shown in Table 5, the difference between the amount of protein in solution as measured by the BCA difference method and that added initially indicated that a negligible amount of protein was adsorbed on Avicel and corn stover solids if the effect of sugars present in the hydrolyzate was neglected. However, for both substrates, the amount of protein adsorbed as measured by the BCA method when interference by the sugars was considered agreed very closely with those for the nitrogen factor method. The BCA method [9] gave a slightly lower value for adsorbed protein for SO_2 pretreated corn stover, apparently due to its reaction with color bodies and lignin components released during hydrolysis.

3.4. Adsorption parameters using NF

Adsorption parameters were estimated by the NF method for Avicel and dilute acid and SO_2 pretreated corn stover at 4 °C and compared with values previously reported in the literature. Each substrate was incubated at a 1–2% glucan content with different loadings of Spezyme-CP protein (20–800 mg/g cellulose) in 50 ml tubes overnight with buffer. Then the tubes were centrifuged repeatedly, the liquid decanted, and solid dried for 2 days. Parameters such as adsorption capacity (mg/g cellulose) and affinity constants ($1/K_d$–L/g) were determined by non-linear regression of the adsorption data to the following Langmuir expression [19] using Polymath software:

$$CE = \frac{\sigma[S_t][E_f]}{K_d + E_f}$$

in which CE is the adsorbed enzyme, E_f the free enzyme in mg/ml, σ the adsorption capacity in mg/mg substrate, S_t the substrate concentration in mg/ml, and K_d is the equilibrium constant = $[C][E]/[CE]$ in mg of enzyme/ml. For Avicel, the maximum adsorption capacity determined by NF agrees closely

Table 5
Amount of protein adsorbed on the solid substrate during hydrolysis as measured by NF and BCA methods and corresponding sugar concentrations in solution

Substrate	Adsorbed protein (mg/gm)			Sugar concentration in solution (mg/ml)	
	NF method	BCA method		Equ. glucose[a]	Xylose
		A	B		
Avicel	18	0.0	22.9	3.22	0.0
SO_2-corn stover	63	0.0	61.2	5.54	0.275

(A) amount of protein adsorbed without excluding the effect of sugars present in the solution; (B) amount of protein adsorbed with excluding the effect of sugars present in the solution as determined by HPLC.

[a] Equivalent glucose = glucose + 1.053*cellobiose.

Table 6
Enzyme adsorption parameters

Substrate	Adsorption capacity, σ (mg/gm cellulose)	Binding affinity (l/g)	Conditions/method	Source
Avicel PH-101	84	1.84	@ 4 °C/nitrogen factor	This study
	92	1.92	@ 50 °C/—	[19]
	95.2	0.30	@ 4 °C/Bio-Rad	[52]
Dilute acid–corn stover	91[a]	2.49	@ 4 °C/nitrogen factor	This study
	60[a,c]	–	Bradford	[53]
Dilute acid–mixed hardwood	80.6	1.82	@ 40 °C/Bradford	[35]
SO_2–corn stover	124[a]	0.90	@ 4 °C/nitrogen factor	This Study
SO_2–Douglas Fir[b]	342	0.78	@ 4 °C/Bio-Rad	[52]

[a] mg protein/g substrate.
[b] 195 °C, 4.5 min, 4.5% (w/w) SO_2, 52.0% cellulose, 46.1% lignin, and 1.9% hemicellulose.
[c] 180 °C, 1.5 min, 3.0% (w/w) H_2SO_4, 60.0% cellulose.

with values reported in the literature, as shown in Table 6. However, less information has been reported for pretreated biomass, making it difficult to make meaningful comparisons. Nonetheless, adsorption capacities are similar to those reported for other dilute acid pretreatments, although pretreatment conditions, reactor configurations, and substrates differ among studies. On other hand, the adsorption capacities for SO_2 pretreatment are noticeably different from those for Avicel and solids from dilute acid pretreatment. These results infer that the accessibility of β-glycosidic bonds [5] is higher for SO_2 pretreatment, but the very high adsorption capacity for Douglas fir could be attributed to a higher lignin content that is believed to adsorb protein unproductively [6,25–28].

3.5. Impact of sugars on protein adsorption

In enzymatic hydrolysis of cellulose, the inhibition of enzymatic activity by end products (cellobiose, glucose, xylose, and ethanol) and other compounds is well known, with cellobiose being particularly powerful [29–34]. In general, inhibition studies are believed to account for the effect of end products and other compounds on the activity of the catalytic domain of cellulase. However, there are very few studies that show sugars and other compounds affect cellulase adsorption mediated by the cellulose binding domain (CBD), an essential step to heterogeneous reactions like cellulose hydrolysis. Some previous studies showed that dissolved sugars do not affect intact cellulase adsorption [35–37], although, others have found that hydrolysis compounds/sugars may be inhibitory to protein adsorption and may even promote cellulase desorption [38–46]. Stutzenberger and Lintz [38] found that the hydrolyzate of protein-extracted lucerne fibers (PELF), having sugars equivalent to 6% glucose ~175 mM, inhibited binding of endoglucanse and exoglucanase by 27 and 46%, respectively. Wendorf et al. showed that sugar excipients including mono and disaccharides reduced the adsorption of ribonuclease A, bovine serum albumin, and hen egg white lysozyme at the liquid–solid interface [47]. Ooshima et al. reported that the presence of ethanol depressed the adsorption of exoglucanases [42]. However, in another study, Ooshima et al. [35] showed that neither cellobiose nor glucose (up to 20 mg/ml ~58 mM) affected protein adsorption on dilute acid pretreated hardwood using the Bradford method to indirectly estimate protein adsorption [10], although it was not mentioned if interference by sugars with the protein determination was taken into account. In addition, the effect of higher concentrations of sugars and other compounds has seldom been studied. Therefore, it is still not clear if the inhibitory nature of the sugars and other compounds is entirely due to inactivation of the catalytic domain and/or inhibition of the binding domain or a combination of both.

The effect of cellobiose and glucose on cellulase adsorption on cellulose was determined using the method developed in this study. Protein adsorption experiments were performed with Avicel and ARP poplar (cellobiose effect only) at 4 °C, to avoid hydrolysis, in a citrate buffer (pH 4.8) at a protein loading of 40 mg/g cellulose (0.4 mg/ml). Cellobiose and glucose were added to the buffer to bring the concentration from 0 to 80 g/l and from 0 to 100 g/l, respectively, prior to adding protein. Both sugars were found to affect cellulase adsorption negatively, and cellobiose inhibited binding of cellulase more than glucose, as shown in Fig. 4. The binding inhibition for ARP poplar was similar to Avicel at low cellobiose loadings but was a little higher at high loadings. For Avicel, the extent of binding inhibition by 6% glucose for cellulase (about 30%) comprised of about 60%

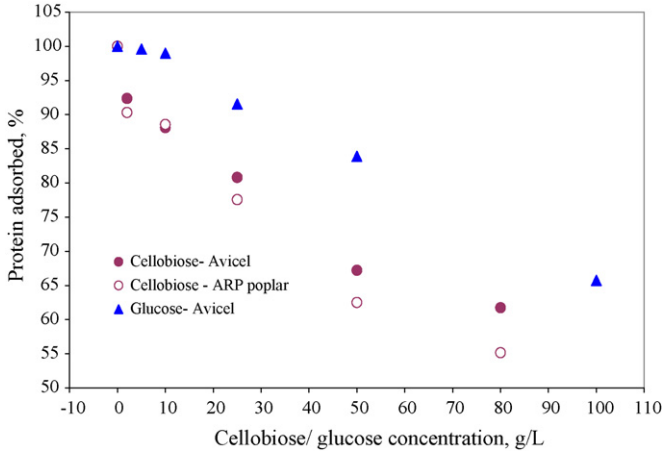

Fig. 4. Effect of cellobiose and glucose on cellulase adsorption as measured by NF method for Avicel and cellobiose only effect for ARP poplar at 4 °C.

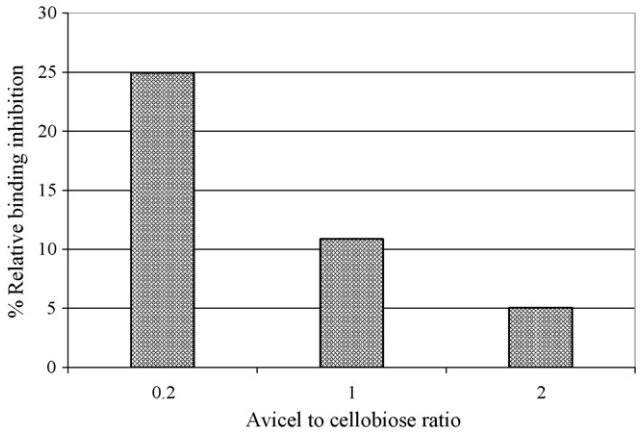

Fig. 5. Effect of cellobiose inhibitor on cellulase adsorption at various Avicel to cellobiose ratio for Avicel at 4 °C with a cellobiose concentration of 50 mg/ml.

CBHI, 20% CBHII, and 12% EGI protein [48], was found to be roughly equal to the inhibition shown by Stutzenberger and Lintz [38].

To understand whether higher substrate concentrations can reduce inhibition of cellulase binding, adsorption experiments were performed with a fixed cellobiose concentration of 50 mg/ml and varying Avicel concentrations from 10 to 100 mg/ml. About 40 mg of cellulase protein/g of cellulose (0.4 mg/ml) was added to the solution having a desired amount of cellobiose and Avicel cellulose. As shown in Fig. 5, the relative binding inhibition (cellulase adsorption on Avicel in the presence of cellobiose relative to adsorption on Avicel alone) was reduced from about 25 to 5% as the ratio of Avicel to cellobiose was increased from 0.2 to 2.

4. Discussion

Measurements of nitrogen content in several protein sources by a nitrogen analyzer gave consistent nitrogen contents in the range of 11–14%, and nitrogen factors (NF) for cellulases, xylanase, and other proteins were found to be very similar and greater than the nitrogen factor (6.25) widely used for estimating the amount of protein in plants [23]. Furthermore, negligible nitrogen losses were found at high temperatures indicating that drying of samples at higher temperatures should not affect the nitrogen content or the NF method applicability. Because nitrogen contents and nitrogen factors were similar for a range of proteins and enzymes, the method should be quite robust for even commercial enzyme preparations that may contain proteins and sources of nitrogen other than cellulases.

Direct nitrogen measurements were then applied to estimate the amount of protein adsorbed at 4 °C on pure cellulose for two cellulases, Spezyme CP and GC220, and the data were compared to indirectly determined protein adsorption measurements based on the conventional BCA method. For both cellulases at all loadings, the amount of protein adsorbed as determined by NF was close to that for the BCA method. However, the NF method generally gave a little higher indication of protein adsorption than the BCA approach, apparently because of the latter's sensitivity to bodies in solution such as the enzyme itself and other proteins. Data for protein adsorption on solids after hydrolysis when sugars and other color developing bodies were in solution along with free protein showed the superiority of the NF to the BCA method. Langmuir adsorption parameters (maximum adsorption capacity, σ) for cellulase at 4 °C were determined using the NF approach for a variety of substrates and generally found to be comparable to values reported in literature.

Application of this method showed that glucose and cellobiose inhibit whole cellulase binding. About 12% inhibition was observed at a 1% cellobiose concentration, a result that is similar to the 10% level reported by Yue et al. [41] for CBHI adsorption. It is also consistent with the small binding inhibition for CBHII reported by Palonen et al. at a very low cellobiose concentration (1.5 mM, ~0.5 g/l) [39]. In addition, our data showed that binding inhibition can be reduced at higher substrate concentrations. Consistent with this, reduced sugars inhibition of cellulase activity by high substrate loadings was reported by Xiao et al. [34]. However, Oh et al. [49] showed cellulase activity was reduced by more than 75% by cellobiose and more than 60% by glucose at loadings of 20 g/l, although binding inhibition was only about 15 and 6% by cellobiose and glucose, respectively, at this loading, as our study showed. This suggests that binding inhibition cannot be solely responsible for a drop in activity during enzymatic hydrolysis of cellulose, and further study is needed.

Acknowledgements

The authors thank the National Institute of Standards and Technology (award 60NANB1D0064) and the US Department of Energy Office of the Biomass Program (Contract DE-FG36-04GO14017) for supporting this research and the Thayer School of Engineering at Dartmouth College for providing the equipment and facilities vital to this research. We also thank Bin Yang and the members of the Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI) among Auburn, Michigan State, Purdue, and Texas A&M Universities, Dartmouth College, the University of British Columbia, the University of California, Riverside (UCR), the National Renewable Energy Laboratory, and Genencor International for their suggestions and other assistance in this research. The corresponding author is very grateful to the Ford Motor Company for funding his Chair at the Center for Environmental Research and Technology for the Bourns College of Engineering at UCR and augmenting support for many projects such as this.

References

[1] Zhang YH, Lynd LR. A functionally based model for hydrolysis of cellulose by fungal cellulase. Biotechnol Bioeng 2006;94(5):888–98.
[2] Nidetzky B, Steiner W, Claeyssens M. Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: adsorptions of two cellobiohydrolases, two endocellulases and their core proteins on filter paper and their relation to hydrolysis. Biochem J 1994;303(Pt 3):817–23.
[3] Lee Y-H, Fan LT. Kinetic Studies of enzymatic hydrolysis of insoluble cellulose: analysis of the initial rates. Biotechnol Bioeng 1982;XXIV:2383–406.

[4] Hogan CM, Mes-Hartree M, Saddler JN, Kushner DJ. Assessment of methods to determine minimal cellulase concentrations for efficient hydrolysis of cellulose. Appl Microbiol Biotechnol 1990;32(5): 614–20.
[5] Zhang YH, Lynd LR. Determination of the number-average degree of polymerization of cellodextrins and cellulose with application to enzymatic hydrolysis. Biomacromolecules 2005;6(3):1510–5.
[6] Palonen H, Tjerneld F, Zacchi G, Tenkanen M. Adsorption of *Trichoderma reesei* CBH I and EG II and their catalytic domains on steam pretreated softwood and isolated lignin. J Biotechnol 2004;107(1):65–72.
[7] Lemos MA, Mota JATM, Gama FM. A simple method to separate cellulose-binding domains of fungal cellulases after digestion by a protease. Biotechnol Lett 2000;22:703–7.
[8] Valjamae PS, Nutt V, Pettersson A, Johansson G. Acid hydrolysis of bacterial cellulose reveals different modes of synergistic action between cellobiohydrolase I and endoglucanase I. Eur J Biochem 1999;266(2):327–34.
[9] Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, et al. Measurement of protein using bicinchoninic acid. Anal Biochem 1985;150(1):76–85.
[10] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72(1/2):248–54.
[11] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193(1):265–75.
[12] Lee SB, Shin HS, Ryu DDY, Mandels M. Adsorption of cellulase on cellulose: effect of physicochemical properties of cellulose on adsorption and rate of hydrolysis. Biotechnol Bioeng 1982;24(10):2137–53.
[13] Zaia DAM, Zaia CTBV, Lichtig J. Determination of total protein by spectrophotometry: advantages and disadvantages of proposed methods. Quim Nova 1998;21(6):787–93.
[14] Krieg RC, Dong Y, Schwamborn K, Knuechel R. Protein quantification and its tolerance for different interfering reagents using the BCA-method with regard to 2D SDS PAGE. J Biochem Biophys Methods 2005;65(1): 13–9.
[15] Sutcliffe R, Saddler JN. The role of lignin in the adsorption of cellulases during enzymic treatment of lignocellulosic material. Biotech Bioeng Symp 1986;17:749–62.
[16] Helrich K. Official methods of analysis of the association of official analytical chemists. 15th ed. Arlington; 1990. p. 807.
[17] Kamizake NKK, Gonçalves MM, Zaia CTBV, Zaia DAM. Determination of total proteins in cow milk powder samples: a comparative study between the Kjeldahl method and spectrophotometric methods. J Food Comp Anal 2003;16(4):507–16.
[18] Nozawa S, Kenichi Sakaida AH, Suzuki T, Yasui A. Method performance study of the determination of total nitrogen in Soy Sauce by the Kjeldahl Method. Anal Sci 2005;21(9):1129–32.
[19] Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 2002;66(3):506–77.
[20] NREL. Determination of structural carbohydrates and lignin in biomass. Golden, CO: National Renewable Energy Laboratory; 2004, LAP02.
[21] Kanchagar AP. Ph.D. Thesis. Adsorption of purified cellulases on cotton fibers. Athens, Georgia: University of Georgia; 2003.
[22] NREL. Determination of total solids in biomass. Golden, CO: National Renewable Energy Laboratory; 2004, LAP01.
[23] NREL. Determination of protein content in biomass: laboratory analytical procedure. Golden, CO: National Renewable Energy Laboratory; 2004.
[24] NREL. Enzymatic saccharification of lignocellulosic biomass. Golden, CO: National Renewable Energy Laboratory; 1996, LAP009.
[25] Zahedifar M. Ph.D. Thesis. Novel uses of lignin and hemicellulosic sugars from acid hydrolysed lignocellulosic materials: University of Aberdeen; 1996.
[26] Kawamoto H, Nakatsubo F, Murakami K. Protein-adsorbing capacities of lignin samples. Mokuzai Gakkaishi 1992;38(1):81–4.
[27] Monney CA, Mansfield SD, Touhy MG, Saddler JN. The effect of initial pore volume and lignin content on the enzymatic hydrolysis of softwoods. Bioresource Technol 1998;64:113–9.
[28] Yang B, Wyman CE. BSA treatment to enhance enzymatic hydrolysis of cellulose in lignin containing substrates. Biotechnol Bioeng 2006;94(4):611–7.
[29] Mark Holtzapple MC, Shu Y, Hendericson C. Inhibition of *Trichoderma reesei* cellulase by sugars and solvents. Biotechnol Bioeng 1990;36:275–87.
[30] Sinistyn AP, Gusakov AV. A theoritical analysis of cellulase product inhibiton: effect of cellulase binding constant, enzyme/substrate ratio, and beta-glucosidase activity on the inhibition pattern. Biotechnol Bioeng 1992;40:663–71.
[31] Gusakova AV, Sinitsyna AP, Gerasimasb VB, Savitskeneb RY, Steponavichusb YY. A product inhibition study of cellulases from Trichoderma longibrachiatum using dyed cellulose. J Biotechnol 1985;3(3): 167–74.
[32] Gruno M, Valjamae P, Pettersson G, Johansson G. Inhibition of the *Trichoderma reesei* cellulases by cellobiose is strongly dependent on the nature of the substrate. Biotechnol Bioeng 2004;86(5):503–11.
[33] Panagiotou G, Olsson L. Effect of compounds released during pretreatment of wheat straw on microbial growth and enzymatic hydrolysis rates. Biotechnol Bioeng 2007;96(2):250–8.
[34] Xiao Z, Zhang X, Gregg DJ, Saddler JN. Effects of sugar inhibition on cellulases and beta-glucosidase during enzymatic hydrolysis of softwood substrates. Appl Biochem Biotechnol 2004;113–116: 1115–26.
[35] Ooshima H, Burns DS, Converse AO. Adsorption of cellulase from *Trichoderma reesei* on cellulose and lignacious residue in wood pretreated by dilute sulfuric acid with explosive decompression. Biotechnol Bioeng 1990;36:446–52.
[36] Jerry Stahlberg GJ, Pettersson Goran. A New model for enzymatic hydrolysis of cellulose based on the two-domain structure of cellobiohydrolase I. Biotechnology 1991:9.
[37] Girard DJ, Converse AO. Recovery of cellulase from lignaceous hydrolysis residue. Appl Biochem Biotechnol 1993;39/40:521–33.
[38] Stutzenberger F, Lintz G. Hydrolysis products inhibit adsorption of *Trichoderma reesei* C30 cellulases to protein-extracted lucerne fibres. Enzyme Microb Technol 1986;8(6):341–4.
[39] Palonen H, Tenkanen M, Linder M. Dynamic interaction of *Trichoderma reesei* cellobiohydrolases Cel6A and Cel7A and cellulose at equilibrium and during hydrolysis. Appl Environ Microbiol 1999;65(12): 5229–33.
[40] Stutzenberger FJ, Caws MA. Adsorption of *Trichoderma reesei* cellulases on protein-extracted lucerne fibers. J Ind Microbiol 1988;3:273–80.
[41] Yue Z, Bin W, Baixu Y, Peiji G. Mechanism of cellobiose inhibition in cellulose hydrolysis by cellobiohydrolase. Sci China Chem Life Sci 2004;47(1):18–24.
[42] Ooshima H, Ishitani Y, Harano Y. Simultaneous saccharification and fermentation of cellulose: effect of ethanol on enzymatic saccharification of cellulose. Biotechnol Bioeng 1985;27(4):389–97.
[43] Lee Y-H, Fan LT. Some experimental observations on the adsorption of cellulase onto cellulose and on the behavior of adsorbed cellulase. Proc An Bio Eng Symp 9th 1979:13–22.
[44] Matsuno R, Taniguchi M, Tanaka M, Kamikubo T. A model for hydrolysis of microcrystalline cellulose by cellulase. Ann NY Acad Sci 1984;434:158–60. Enzyme Eng.
[45] 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 1986;23(3/4): 263–8.
[46] Estrada P, Acebal C, Castillon MP, Mata I, Romero D. Adsorption of cellulase from *Trichoderma reesei* on wheat straw. Biotechnol Appl Biochem 1988;10(1):49–58.
[47] Wendorf JR, Radke CJ, Blanch HW. Reduced protein adsorption at solid interfaces by sugar excipients. Biotechnol Bioeng 2004;87(5):565–73.
[48] Goyal A, Ghosh B, Eveleigh D. Characterstics of Fungal Cellulases. Bioresour Technol 1991;36:37–50.
[49] Oh KK, Kim SW, Jeong YS, Hong SI. Bioconversion of cellulose into ethanol by nonisothermal simultaneous saccharification and fermentation. Appl Biochem Biotechnol 2000;89(1):15–30.

[50] Banka RR, Mishra S. Adsorption properties of the fibril forming protein from *Trichoderma reesei*. Enzyme Microb Technol 2002;31(6):784–93.
[51] Yan BX, Sun Y. Domain structure and conformation of a cellobiohydrolase from Trichoderma pseudokiningii S-38. J Protein Chem 1997;16(1):59–66.
[52] Yanpin Lu BY, Gregg David, Saddler JohnN, Mansfield Shawn D. Cellulase adsorption and an evaluation of enzyme recycle during hydrolysis of steam-exploded softwood residues. Appl Biochem Biotechnol 2002;98–100:641–54.
[53] Kadam KL, Rydholm EC, McMillan JD. Development and validation of a kinetic model for enzymatic saccharification of lignocellulosic biomass. Biotechnol Prog 2004;20(3):698–705.