Determination of the Number-Average Degree of Polymerization of Cellodextrins and Cellulose with Application to Enzymatic Hydrolysis

ARTICLE *in* BIOMACROMOLECULES · MAY 2005

Impact Factor: 5.75 · DOI: 10.1021/bm049235j · Source: PubMed

Determination of the Number-Average Degree of Polymerization of Cellodextrins and Cellulose with Application to Enzymatic Hydrolysis

Y.-H. Percival Zhang† and Lee R. Lynd*,†,‡

Thayer School of Engineering and Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755

Received December 3, 2004; Revised Manuscript Received February 8, 2005

A rapid and accurate method for determining the number-average degree of polymerization (DP_n) was established for insoluble cellulose and soluble cellodextrins as the ratio of glucosyl monomer concentration determined by the phenol−sulfuric acid method divided by the reducing-end concentration determined by a modified 2,2′-bicinchoninate (BCA) method. The modified BCA method, featuring incubation at 75 °C for 30 min, did not result in β-glucosidic bond cleavage, whereas substantial cleavage was observed at higher temperature. Solubilization of insoluble cellulose in cold phosphoric acid prior to measurement of the reducing-end concentration by the BCA method was found not to be necessary for several model celluloses such as microcrystalline cellulose, but such solubilization was required for large fibers of cellulose such as Whatman No. 1 filter paper. The phenol−sulfuric acid method can be used for measuring the glucosyl monomer concentration of soluble cellodextrins, and also for insoluble cellulose if preceded by a liquefaction step. Standard deviations of ≤2% were obtained for both reducing and glucosyl monomer determination and of ≤3% for overall determination of DP. By use of the reported method, hydrolysis of phosphoric acid-swollen cellulose (PASC) by the *Trichoderma reesei* cellulase system was shown to result in a rapid decrease in DP as hydrolysis proceeded. By contrast, the DP of Avicel remained nearly constant during hydrolysis. The specific enzymatic cellulose hydrolysis rate is 100-fold higher for PASC as compared to Avicel.

Introduction

The main product of photosynthesis, cellulose, plays a central role in the global carbon cycle, is an important raw material for industry today, and could play a greatly expanded role as a source of fuels and commodity chemicals in the future.[1−4] Insoluble cellulose is a linear polymer of anhydroglucose units joined by $\beta(1-4)$ linkages with a degree of polymerization (DP) from 100 to 20 000.[4,5] Soluble cellodextrins are short fragments of cellulose with DP from 2 to ∼12, which are soluble for DP ≤ 6 and slightly soluble for 6 < DP < 12.[4] The DP is an important property of cellulosic materials for functionally based models of enzymatic cellulose hydrolysis,[4] as well as for paper-making and other applications.[5−9]

Established methods for DP determination for cellulose involve cellulose dissolution in either metal complex solutions such as Cuam solution,[5,6] organic solvents or inorganic acids (e.g., nitric acid) following derivatization,[6,10] or ionic solutions.[7,8,11] After dissolution, the DP of cellulose may be represented in terms of the number-average, weight-average, or viscosity-average. The number-average DP (DP_n) can be measured by membrane osmometry, cryoscopy, ebullioscopy, vapor pressure oscomometry, size-exclusion chromatography, or determination of reducing-end concentration.[4−6] These established assays require a long time for sample preparation, relatively large sample size, close attention to drying and subsequent dissolution, volatile and/or toxic reagents, and often specialized instruments.

An alternative approach to measuring the number-average DP, although not commonly used at present, is to divide the number of glycosyl residues by the number of chain ends. Utilization of this potentially straightforward approach is at present constrained by shortcomings of available reducing-end sugar assays such as DNS,[12−17] Nelson−Somogyi,[14,18] and 2,2′-bicinchoninate (BCA)[19] methods. In particular, these assays suffer from artifacts due to long glucan chain hydrolysis during the assays and, for many insoluble cellulose samples, incomplete accessibility of chain ends.[17]

In this study, we undertook to develop a simple and rapid protocol for evaluating cellulose DP based on a modified method for measuring reducing ends. This new method offers several advantages relative to established methods, including greater sensitivity, simplified sample preparation, smaller required sample size, less time required, more benign reagents, and simple instrumentation requirements. In addition to circumventing the limitations of standard methods for DP measurement, the proposed protocol has the further advantage that it requires no polymer standards for calibration. Application of this protocol is illustrated for enzymatic

* Corresponding author: e-mail Lee.Lynd@dartmouth.edu.
† Thayer School of Engineering.
‡ Department of Biological Sciences.

10.1021/bm049235j CCC: $30.25
Published on Web 03/18/2005

hydrolysis of Avicel and phosphoric acid-swollen cellulose by the *Trichoderma reesei* cellulase system.

Materials and Methods

Materials and Chemicals. All chemicals were reagent-grade purchased from Sigma (St. Louis, MO) unless otherwise noted. Microcrystalline cellulose, Avicel PH105 (20 μm), was obtained from FMC Corp. (Philadelphia, PA). Whatman CC41 and Whatman No. 1 filter paper were purchased from Fisher Scientific (Pittsburgh, PA). Filter paper was cut to 3.5 mm diameter disks with a paper punch or 5 × 20 mm strips with a razor. Soluble cellodextrins including cellotriose (G_3), cellotetraose (G_4), cellopentaose (G_5), cellohexaose (G_6), and longer slightly soluble cellodextrins were produced by mixed acid hydrolysis and separated by chromatography as described previously.[20] Phosphoric acid-swollen cellulose from Avicel PH105, denoted $PSAC_{PH105}$, was prepared by 83.4% phosphoric acid treatment.[21] A cellulase preparation from *T. reesei* [Spezyme, 59 filter paper unit (FPU)/mL] was obtained from Genencor International. Co. (Palo Alto, CA), and β-glucosidase from *Aspergillus niger* (Novozyme 188, 385 IU/mL) was obtained from Sigma.

Reducing-End Determination. BCA working solution was made fresh daily by mixing equal volumes of solution A containing 0.971 g of disodium 2,2′-bicinchoninate, 27.14 g of Na_2CO_3, and 12.1 g of $NaHCO_3$ dissolved in 500 mL of distilled water, with solution B containing 0.624 g of $CuSO_4 \cdot 5H_2O$ and 0.631 g of L-serine dissolved in 500 mL of water.[19] Both reagents were stored separately and were stable for at least a month at 4 °C. One milliliter of BCA working solution was added to 1 mL samples in 13 × 100 mm disposable tubes (Fisher), mixed well, and incubated at 75 °C for 30 min. After tubes were cooled to room temperature, absorbance was measured at 560 nm. Solutions of glucose with concentrations from 0 to 50 μM were run as standards. For samples containing insoluble cellulose, incubation of BCA/sample reaction mixtures was as described above with continuous shaking in a reciprocal shaker and manual shaking every 5 min by reciprocation to keep particles suspended. After cooling, reaction mixtures containing cellulose particles in 13 × 100 mm disposable tubes were transferred into 2 mL microfuge tubes, allowed to settle for several minutes, and centrifuged for 5 min. One milliliter of the supernatant was then transferred to a cuvette, and absorbance was read at 560 nm.

Total Glucosyl Monomer Determination. The glucosyl monomer concentration of cellodextrin-containing samples was measured by the phenol−sulfuric acid method as described elsewhere[22] except that the volume of all reagents and the sample was reduced by 30% to be accommodated in 13 × 100 mm disposable tubes. Absorbance was measured at 490 nm with standard glucose solutions from 0 to 556 μM (0−0.1 g/L). For samples containing insoluble cellulose, 1 mL of concentrated sulfuric acid was added into 1 mL of ~1 g/L cellulose-containing solution and mixed well. After standing for 5 min, the liquefied cellulose solution was diluted 5-fold with distilled water for further assay. Glucose standards were prepared by mixing 1 mL of concentrated sulfuric acid with 1 mL of 1 g/L glucose solution and then diluted to 0.1 g/L with distilled water. The glucosyl monomer concentrations of liquefied cellulose-containing samples were measured by the phenol−sulfuric acid method as described above.

DP Measurement. The number-average DP_n was calculated as the ratio of glucosyl monomer concentration determined by the phenol−sulfuric acid method divided by the reducing-end concentration determined by the modified 2,2′-bicinchoninate (BCA) method. Dissolution in 83.4% phosphoric acid followed by water precipitation was carried out prior to determination of reducing-end concentration by the BCA method as follows. Phosphoric acid treatment involved adding about 100 mg of dried cellulose into 50 mL centrifuge tubes and adding 0.6 mL of distilled water to thoroughly wet samples. Ten milliliters of ice-cold 86.2% H_3PO_4 ($\rho = 1.71$ g/mL) was slowly added to tubes in an ice bath, resulting in a transparent solution after a 30 min incubation with modest stirring. Forty milliliters of ice-cooled distilled water was added gradually to precipitate dissolved cellulose. The supernatant after centrifugation was decanted, and the pellet was washed twice with 40 mL of ice-cold distilled water. One milliliter of 2 M Na_2CO_3 was added to neutralize residual cellulose, and then more ice-cold water was added to wash the residual cellulose three times. Equivalent results were obtained with and without phosphoric acid treatment for fine cellulose particles including Avicel PH105, Sigmacell 20, Sigmacell 101, and Whatman CC41, suggesting that all reducing ends were oxidized by copper ions for these substrates.

Enzymatic Cellulose Hydrolysis. Enzymatic cellulose hydrolysis was carried out at 50 °C in 50 mM citric acid buffer (pH 4.8) in serum vials with agitation provided by a rotary shaker at 200 rpm. For Avicel, hydrolysis was carried out at 10 g/L Avicel in a total volume of 50 mL with an enzyme loading of 15 FPU of Spezyme cellulase/g of Avicel plus 60 IU of cellobiase/g of Avicel. For PASC, hydrolysis was carried out at 5 g/L PASC in a total volume of 20 mL with an enzyme loading of 0.5 FPU/g of PASC. One milliliter of enzyme hydrolysate sample was withdrawn and transferred to a 2 mL centrifuge tube containing 20 μL of 10 N NaOH at time intervals as indicated. After centrifugation, the supernatant was discarded and the pellet was suspended in 1 mL of 1.1% SDS and boiled for 5 min, centrifuged, and washed in 75% (v/v) ethanol three times followed by 1 mL of distilled water. The pellet was then suspended in distilled water and used for assays of reducing-end concentration as described above.

Results

Reducing-End Determination by the BCA Method. Experiments were undertaken to examine the impact of reaction temperature on sample stability when the concentration of reducing ends was assayed by the 2,2′-bicinchoninate (BCA) method. Solutions containing individual cellodextrins with DP from 2 to 6, denoted G_2 to G_6, at a concentration

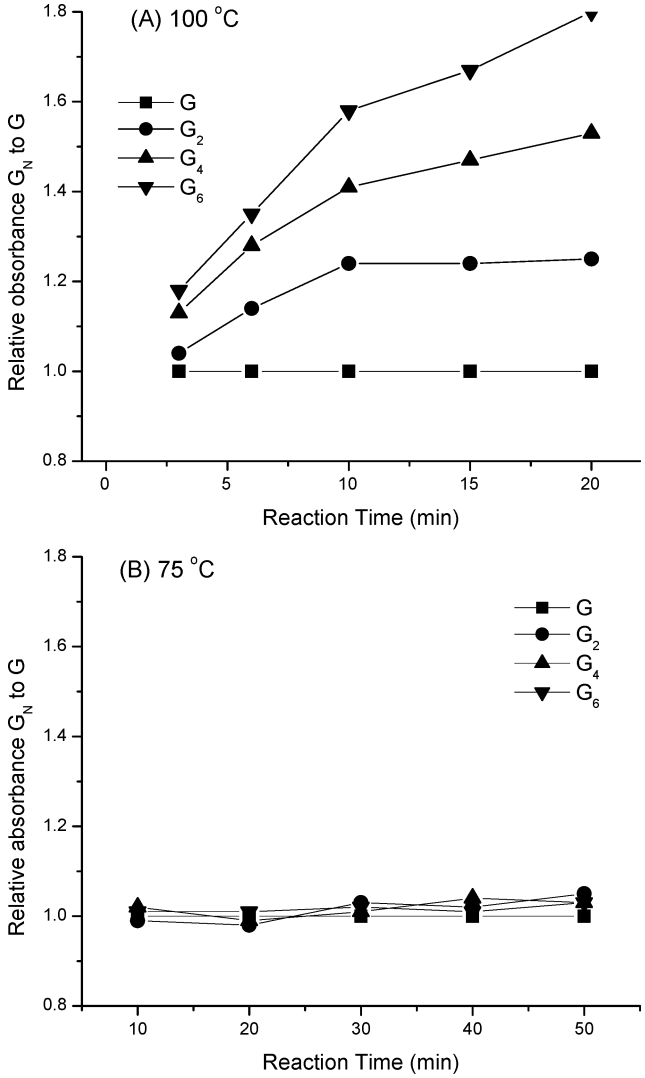

Figure 1. Effect of reaction temperature on the absorbance of cellodextrins relative to equal concentrations of glucose for reducing-end determination by the BCA assay: (A) 100 °C for 15 min; (B) 75 °C for 30 min. Data for cellotriose and cellopentaose are not shown for clarity.

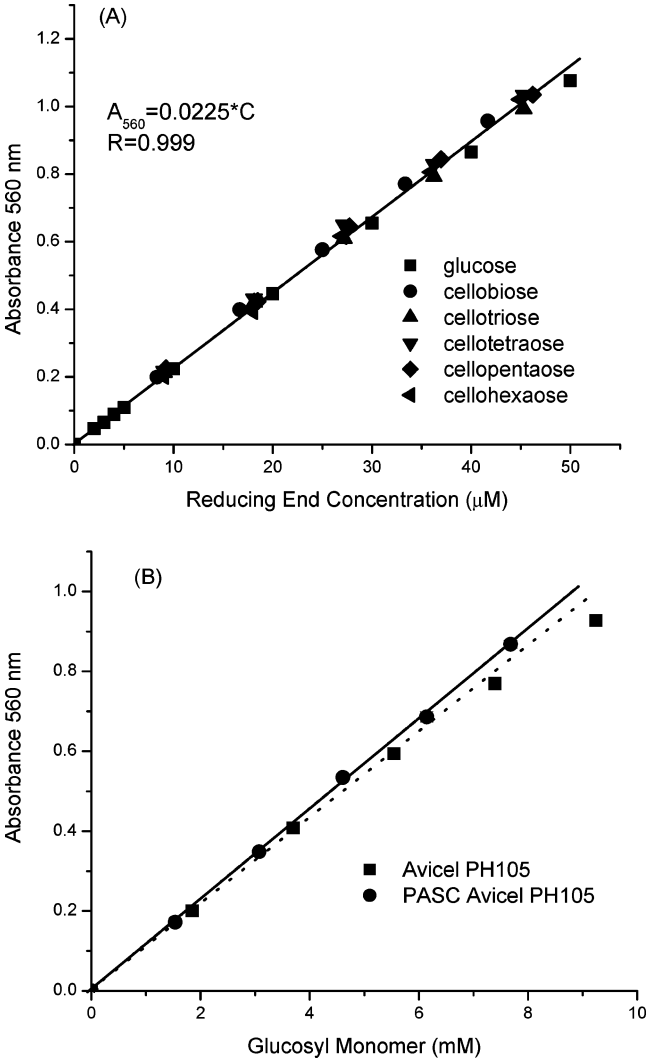

Figure 2. Standard curves for reducing-end determination by the modified BCA assay: (A) soluble cellodextrins; (B) insoluble cellulose.

of 20 μM were mixed with BCA reagent and sampled over time with incubation at either 100, 80, or 75 °C. After cooling, absorbance of cellodextrin-containing solutions was read and expressed as the normalized ratio relative to that of a 20 μM glucose control. At the standard assay temperature of 100 °C, an increase in absorbance, indicative of an increasing concentration of reducing ends and hence hydrolysis, is clearly evident for samples taken over a period of 20 min (Figure 1A). Some degradation of longer cellodextrins (5∼10% for G_3-G_6) occurred at 80 °C after 30 min of reaction (data not shown). However, no increase in absorbance was observed when the incubation temperature was decreased to 75 °C even when the incubation time was extended to 50 min (Figure 1B).

Standard curves of absorbance vs reducing-end concentration were developed for soluble cellodextrins, to determine whether satisfactory results can be obtained with incubation at 75 °C rather than the standard temperature of 100 °C. As may be seen in Figure 2A, a linear relationship is obtained for soluble cellodextrins with DP from 2 to 6 at concentrations from 2 to 50 μM. The standard deviation for replicate samples was excellent over this concentration range at 2%. The linear relation between reducing-end concentrations of known DP values of G8 and G10 and the optical density measured by the BCA method was also obtained (data not shown). For insoluble model cellulose substrates (Avicel FMC PH105 and $PASC_{PH105}$) with unknown DP, a linear relationship between absorbance and glucose monomer concentration was observed for $PASC_{PH105}$ over the range of 0−9 mM and for Avicel at concentrations less than 5 mM glucosyl monomer based on weight (Figure 2B). The deviation from linearity observed at higher Avicel concentrations was found to be sensitive to mixing intensity and is thus attributed to incomplete mixing. For insoluble substrates, stirring during the assay and use of relatively low cellulose concentrations are strongly recommended to obtain reliable and reproducible reducing-end measurements.

Glucosyl Monomer Determination by the Phenol−Sulfuric Acid Method. The phenol−sulfuric acid method[12] was investigated for determination of the glucosyl monomer concentration for both soluble and insoluble β-glucan substrates. A linear relationship was observed between absorbance and glucosyl monomer concentrations for soluble cellodextrins of varying DP over a range of concentrations from 0 to 556 μM (Figure 3A). Such a linear relationship

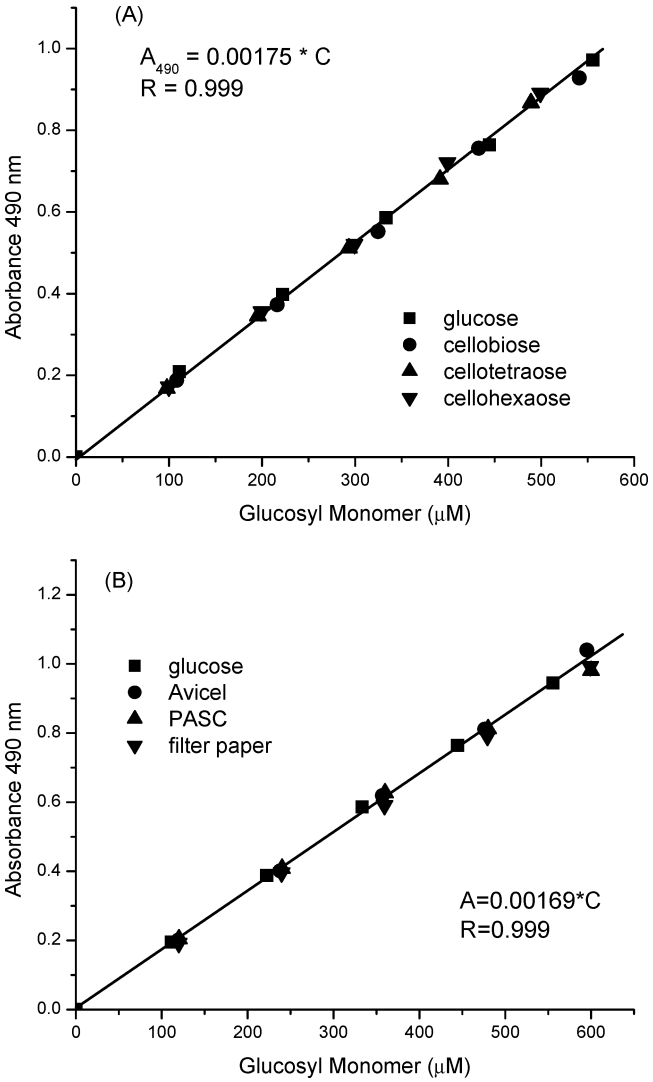

Figure 3. Standard curves for glucosyl monomer determination by the phenol−sulfuric acid method following acid hydrolysis employing the heat of dilution: (A) soluble cellodextrins; (B) insoluble cellulose.

was also obtained for insoluble cellulosic substrates (Avicel, $PASC_{PH105}$, and filter paper) after dissolution with sulfuric acid as shown in Figure 3B. For insoluble cellulose, the reproducibility and robustness of the phenol−sulfuric acid method was improved by adding a dissolution step prior to the assay consisting of mixing sample slurries with equal volumes of concentrated sulfuric acid. By use of dissolution followed by the phenol−sulfuric acid assay, a very good proportionality between absorbance and the amount of cellulose added (standard error ∼2%) was obtained with small (ca. 0.01∼0.07 mg of cellulose) sample sizes.

Degree of Polymerization of Insoluble Cellulose. The DP of cellulose was calculated from the ratio of the molar concentration of glucosyl monomers, measured by the phenol−sulfuric acid method, to the molar concentration of reducing ends, measured by the modified BCA method. Table 1 presents DP values measured for insoluble cellulosic materials with and without treatment with phosphoric acid prior to reducing-end determination. In the absence of phosphoric acid treatment, DP values for Avicel PH105, Sigmacell 20, and Whatman CC41 were 212 ± 7, 209 ± 10, and 212 ± 4, respectively. Very similar DP values were

Table 1. Number-Average Degree of Polymerization of Insoluble Cellulose Measured by the Combined BCA and Phenol−H_2SO_4 Methods

	DP value	
samples	direct	H_3PO_4 dissolution
Microcrystalline Cellulose		
Avicel FMC PH105	212 ± 7	208 ± 6
SigmaCell 20	209 ± 10	215 ± 9
Whatman CC41	212 ± 4	199 ± 9
Fibrous Cellulose		
SigmaCell 101	257 ± 12	263 ± 8
Whatman No. 1 Filter Paper		
0.35 cm disc	2085 ± 54	1850 ± 22
0.5 × 2 cm strip	3420 ± 40	1830 ± 34

obtained with phosphoric acid treatment for these three microcrystalline cellulosic substrates. For Sigmacell 101, a fibrous cellulose, the DP value measured without phosphoric acid (257 ± 12) was also very similar to that obtained with phosphoric acid treatment (263 ± 8). By contrast, the measured DP of Whatman No. 1 filter paper showed a significant dependence upon both whether samples were treated with phosphoric acid and the manner in which samples were cut. For DP calculated from the ratio of individual measurements for monomer and reducing-end concentrations, a standard error of ca. 3% was obtained. For maximum accuracy, we recommend calculating DP from the slopes of absorbance vs glucosyl monomer concentration plots obtained by the phenol sulfuric acid and BCA methods, respectively.

Enzymatic Cellulose Hydrolysis. Enzymatic hydrolysis experiments with Avicel and PSAC as substrates were undertaken in order to illustrate the utility of the described method for DP measurement. A much lower enzyme loading was used for PASC as compared to Avicel, as described under Materials and Methods, so that hydrolysis occurred over reasonably similar time periods for both substrates. As shown in Figure 4A, the number-average DP of PASC decreased sharply with time during the early stages of hydrolysis and decreased gradually to about 60 thereafter. In the first 20 min of the experiment, at which time 90.5% of the cellulose originally present was not yet hydrolyzed, the DP of PASC decreased from 205 to 118. The reducing-end concentration of the solid phase rose, reached a maximum, and then fell as hydrolysis progressed (inset), suggesting that a larger fraction of bond cleavage occurred due to endoglucanase in the early stages of hydrolysis and due to exoglucanase during the later stages of hydrolysis. In contrast to results obtained with PASC, the DP of residual Avicel remained nearly constant at about 200 over the course of hydrolysis (Figure 4B), with the reducing-end concentration of the solid phase decreasing linearly as hydrolysis proceeded (inset).

Discussion

A method has been developed for determining the number-average DP based on the ratio of the concentration of glucosyl monomers, determined by the phenol−sulfuric acid

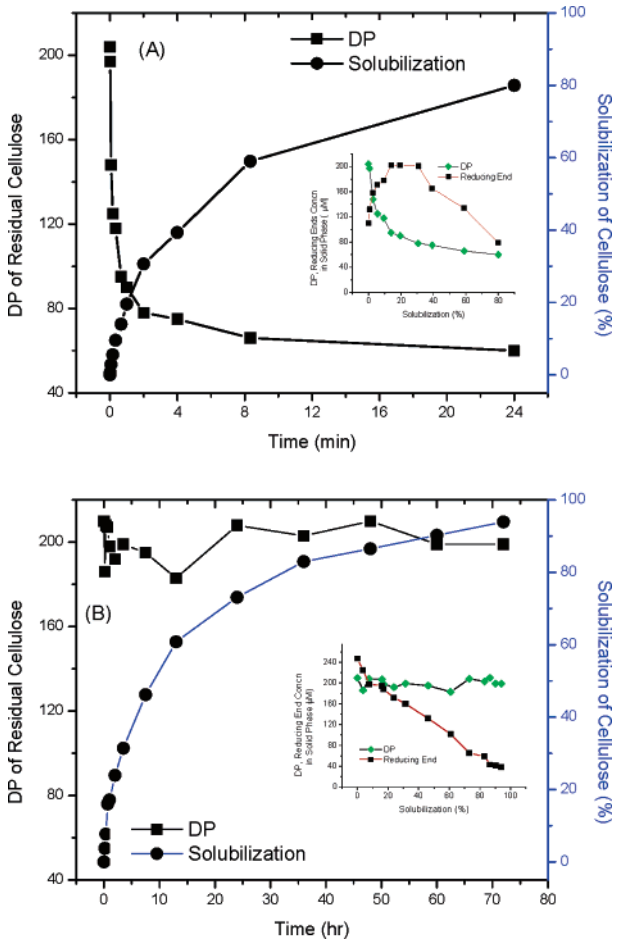

Figure 4. Residual cellulose DP vs time during enzymatic hydrolysis by the *T. reesei* cellulase system: (A) PASC; (B) microcystalline cellulose. Inset: DP and reducing-end concentration as a function of percent solubilization.

method in conjunction with acid hydrolysis, to the concentration of reducing ends, determined by a modified 2,2′-bicinchoninate method (BCA) method. The described method is applicable to insoluble cellulose as well as soluble cellodextrins.

The BCA method has higher sensitivity (2−50 nmol observed here, 1−25 nmol reported elsewhere)[19] as compared to the widely used DNS method (0.1−3.3 μmol for the "micro assay"),[12−17] as well as the Nelson−Somogyi method (12−60 nmol for the "micro assay").[18] Under the modified conditions reported here, 75 °C for 30 min, hydrolysis during the assay was not observed. The fact that measured values are higher for cellobiose and longer cellodextrins than glucose by 20−50% for the DNS method[13−17] and 10−15% for the Nelson−Somogyi method[14] is likely indicative of hydrolysis occurring during the assay, although this has not been investigated directly and the extent of hydrolysis is dependent upon the particular conditions used. Although the BCA method has been used previously to measure reducing ends for soluble polysaccharides at a decreased temperature of 80 °C for maltooligosaccharides, oligogalacturonic acid and chitooligosaccharides,[23] curdlan-like soluble exopolysaccharide,[24] capsular polysaccharide,[25] and (carboxymethyl)-cellulose and cellodextrins,[26] we found that the reaction temperature of 80°C for 30 min resulted in 5−10% higher reading for longer cellodextrins relative to glucose on molar basis.

It is known that not all reducing ends of insoluble cellulosic samples are accessible by reducing agents such as copper ions.[17] We found that the accessibility of reducing ends was substantially increased by solubilization in ice-cold phosphoric acid followed by water precipitation in the case of Whatman No. 1 filter paper. However, reducing ends appeared to be completely accessible without such swelling in the case of several other laboratory cellulosic substrates (Table 1). We suggest that unknown samples be tested with and without phosphoric acid swelling to determine whether this affects measurable reducing ends of cellulosic samples. The degree of polymerization of cellulose was not impacted significantly by the ice-cold phosphoric acid treatment as shown in Table 1 and reported elsewhere.[28] The DP values measured for various cellulosic samples by the methods described herein are consistent with those reported in the literature for the same or similar materials,[4−6,17] in the case of FMC Avicel PH105, SigmaCell 20 and 101, and Whatman No. 1 filter paper, and also with information provided by the manufacturer in the case of Avicel FMC PH105.

One limitation of the described method is that it cannot be used in the presence of significant protein concentrations. We found that as little as 1 μg of bovine serum albumin results in an absorbance reading large enough to interfere with DP determination (data not shown). In the case of cellulosic samples that contain negligible quantities of protein (i.e., adsorbed cellulase in cellulosic samples), it is desirable to remove protein originating from cellulase enzymes. Treatment in boiling SDS (1.1%) for 5 min followed by thorough washing was found to be sufficient for this purpose, resulting in undetectable protein as measured by the Bradford assay. In the case of cellulosic materials with a significant protein content, for example, many lignocellulosic materials, it will be necessary to remove protein prior to determination of DP by the reported method. It may be noted that very little fibrous protein remains after typical pulping processes used in paper-making,[5,6] so interference with protein would presumably not be a concern for this application. We have not, however, investigated use of the BCA method for reducing-end determination for samples containing appreciable amounts of both cellulose and hemicellulose.

Determination of the glucosyl monomer concentration of cellulose, in general, requires hydrolysis followed by measurement of glucose concentration. By taking advantage of the heat of dilution accompanying the mixing of equal volumes of sample and concentrated sulfuric acid, hydrolysis was conveniently and reproducibly achieved in 5 min. On the basis of accuracy, convenience (reagent stability and preparation), and ease of processing many samples in parallel, we prefer the phenol−H_2SO_4 method. However, a variety of other methods would likely be satisfactory, including HPLC, the hexokinase/glucose-6-phosphate dehydrogenase assay,[28] and the anthrone−sulfuric acid method.[29]

Clear differences in the changes in DP over time during enzymatic hydrolysis of PASC and crystalline cellulose (Figure 4) indicate that the hydrolysis reaction proceeds quite differently on these two substrates. $PASC_{PH105}$ has a large

fraction of β-glucosidic bonds accessible to cellulase. The high accessibility of PASC allows endoglucanase to adsorb and hydrolyze β-glucan bonds randomly, resulting in a rapid reduction of DP within a short time and little solubilization. A sharp decline in the DP of PASC has previously been reported for the cellulase of *Myrothecium* sp.[10] and for endoglucanase from *Ruminococcus albus*,[30] as well as hydrolysis of (carboxymethyl)cellulose by fungal endoglucanase preparations. Avicel has at least 20-fold lower accessibility than PASC.[4] Lower surface area limits endoglucanase adsorption on the surface of cellulose such that hydrolysis of many β-glucosidic bonds cannot occur until they are made accessible by the action of exoglucanases. This kind of peeling or progressive erosion mechanism has been found for hydrolysis of pretreated softwood by the *T. reesei* cellulase[31] and of cotton fibers by *T. reesei* cellobiohydrolase I and endoglucanase I.[32] The specific initial cellulose hydrolysis rate observed here for PASC was more than 100-fold higher than on Avicel [40 vs 0.3 g glucose equivalent h^{-1} (FPU activity)$^{-1}$], which we attribute primarily to the difference in cellulose accessibility for these two substrates. Much higher (1−2 orders of magnitude) hydrolysis rates for homogeneous cellulose as compared to heterogeneous cellulose have also been observed in the case of hydrolysis by mineral acids.[6] The different hydrolysis mechanisms and drastically different hydrolysis rates on two types of celluloses suggest that the hydrolysis step of perfectly pretreated cellulose should not be the rate-limiting step for saccharification.

The described method for determining the DP of insoluble cellulose and soluble cellodextrins can be carried out with samples as small as or even less than 1 mg, does not require sample drying, can be completed in less than a day, involves relatively benign reagents, and has no instrumentation requirements save for a spectrophotometer. By contrast, established methods require dried samples of 100 mg, over a week to complete, involve toxic and/or hazardous reagents, and—in most cases—specialized instruments (for reviews see refs 4−6). Moreover, the described method is sensitive and highly reproducible (ca. 3% standard error), gives a linear response with respect to chain length, and there is no evidence for artifacts associated with substrate hydrolysis during the assay procedure. For many applications involving laboratory cellulosic substrates of the type routinely used for biochemical characterization of cellulase enzymes, the protocol described here would appear to be the preferred method for determination of the number-average DP.

Acknowledgment. This work was supported by Grant 60NANB1D0064 from the National Institute of Standards and Technology and Grant DE-FG02-02ER15350 from the U.S. Department of Energy.

References and Notes

(1) Lynd, L. R.; Weimer, P. J.; van Zyl, W. H.; Pretorius, I. S. *Microbiol. Mol. Biol. Rev.* **2002**, *66*, 506.
(2) Wahlaud, B.; Yan, J.; Westermark, M. *Biomass Bioenergy* **2004**, *26*, 531.
(3) Wyman, C. E. *Biotechnol. Prog.* **2003**, *19*, 254.
(4) Zhang, Y.-H. P.; Lynd, L. R. *Biotechnol. Bioeng.* **2004**, *88*, 797.
(5) Klemm, D.; Philipp, B.; Heinze, T.; Heinze, U.; Wagenknecht, W. *Comprehensive Cellulose Chemistry*; Wiley−VCH Verlag GmbH: Berlin, Germany, 1998.
(6) Fengel, D.; Wegener, G. *Wood: Chemistry, Ultrastructure, Reactions*; Walter de Gruyter & Co.: Berlin, Germany, 1984.
(7) Striegel, A. M. *Carbohydr. Polym.* **1997**, *34*, 267−274.
(8) Swatloski, R. P.; Spear, S. K.; Holbrey, J. D.; Rogers, R. D. *J. Am. Chem. Soc.* **2002**, *124*, 4974−4975.
(9) Frisoni, G.; Baiardo, M.; Scandola, M. *Biomacromolecules*. **2001**, *2*, 476.
(10) Whitaker, D. R. *Can. J. Biochem. Physiol.* **1957**, *35*, 733−742.
(11) Wu, J.; Zhang, J.; Zhang, H.; He, J.; Ren, Q.; Guo, M. *Biomacromolecules* **2004**, *5*, 266.
(12) Miller, G. L. *Anal. Chem.* **1959**, *31*, 426.
(13) Ghose, T. K. *Pure Appl. Chem* **1987**, *59*, 257.
(14) Wood, T. M.; Bhat, K. M. *Methods Enzymol.* **1988**, *160*, 87.
(15) Coward-Kelly, G.; Aiello-Mazzari, C.; Kim, S.; Granda, C.; Holtzapple, M. *Biotechnol. Bioeng.* **2003**, *82*, 745.
(16) Robyt, J. F.; Whelan, W. J. *Anal. Biochem.* **1972**, *45*, 510.
(17) Kongruang, S.; Han, M. J.; Breton, C. I. G.; Penner, M. H. *Appl. Biochem. Biotechnol.* **2004**, *113/116*, 213.
(18) Somogyi, M. *J. Biol. Chem.* **1952**, *195*, 19−23.
(19) Waffenschmidt, S.; Janeicke, L. *Anal. Biochem.* **1987**, *165*, 337.
(20) Zhang, Y.-H. P.; Lynd, L. R. *Anal. Biochem.* **2003**, *225*, 225.
(21) Zhang, Y. H.; Lynd, L. R. *Anal. Chem.* **2003**, *75*, 219.
(22) Dubois, B.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Anal. Chem.* **1956**, *28*, 350.
(23) Doner, L. W.; Irwin, P. L. *Anal. Biochem.* **1992**, *202*, 50.
(24) Kenyon, W. J.; Buller, C. S. *J. Ind. Microbiol. Biotechnol.* **2002**, *29*, 200.
(25) D'Ambra, A. J.; Baugher, J. E.; Concannon, P. E.; Pon, R. A.; Michon. *Anal. Biochem.* **1997**, *250*, 228.
(26) Carcia, E.; Johnston, D.; Whitaker, J. R.; Shoemaker, S. P. *J. Food. Biochem.* **1993**, *17*, 135.
(27) Lee, S. B.; Shin, H. S.; Ryu, D. D. Y. *Biotechnol. Bioeng.* **1982**, *24*, 2137.
(28) Zhang, Y.-H. P.; Lynd, L. R. *Appl. Environ. Microbiol.* **2004**, *70*, 1563.
(29) Viles, F. J.; Silverman, L. *Anal. Chem.* **1949**, *21*, 950.
(30) Wood, T. M.; Wilson, C. A. *Can. J. Microbiol.* **1984**, *30*, 316.
(31) Ramosb, L. P.; Nazhadb, M. M.; Saddler, J. N. *Enzyme Microb. Technol.* **1994**, *15*, 821.
(32) Kleman-Leyer, K. M.; Siika-Aho, M.; Teeri, T. T.; Kirk, T. K. *Appl. Environ. Microbiol.* **1996**, *62*, 2883.

BM049235J